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Controlled Gene Expression Systems for *Lactococcus lactis* with the Food-Grade Inducer Nisin

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The kinetics, control, and efficiency of nisin-induced expression directed by the *nisA* promoter region were studied in *Lactococcus lactis* with transcriptional and translational fusions to the *gusA* reporter gene. In the nisin-producing *L. lactis* strain NZ9700, the specific β-glucuronidase activity increased very rapidly after mid-exponential growth until the maximum level at the start of the stationary phase was reached. Expression of the *gusA* gene was also studied in *L. lactis* NZ9800, an NZ9700 derivative carrying a deletion in the structural *nisA* gene that abolishes nisin production, and in *L. lactis* NZ3900, an MG1363 derivative containing the regulatory *nisRK* genes integrated in the chromosome. In both strains, β-glucuronidase activity was linearly dependent on the amount of nisin added to the medium. Without nisin, no β-glucuronidase production was observed. To optimize translation initiation, an expression vector was constructed by fusing the *gusA* gene translationally to the start codon of the *nisA* gene. Use of the translational fusion vector yielded up to six times more β-glucuronidase activity than the transcriptional fusion vector in these strains after induction by nisin. In this way, gene expression can be achieved in a dynamic range of more than 1,000-fold. The β-glucuronidase activity was found to be up to 25-fold higher in extracts of strain NZ3900 than in extracts of strain NZ9800. This translational fusion vector was used for high-level production of aminopeptidase N, up to 47% of the total intracellular protein. These results clearly illustrate the potential of the nisin-inducible expression system for overproduction of desired proteins.

There is considerable interest in the development of food-grade microorganisms for the controlled production of desirable metabolites, enzymes, and other proteins for the food industry. Lactic acid bacteria are used in a variety of industrial dairy and other food fermentations and have potential to be developed as safe production hosts. *Lactococcus lactis* is one of the best-studied lactic acid bacteria for which efficient genetic tools have been developed, including dominant selection markers that are acceptable for use in the food industry (8, 10, 27).

Several strategies have been employed to realize enhanced gene expression in lactococci. High-copy-number plasmids have been developed to increase gene dosage, and various strong constitutive promoters have been characterized (8). Gene expression in *L. lactis* has been the subject of several studies, but only a few regulated promoters have been identified (8, 16, 17, 24, 36, 37). The best-characterized controllable expression system until now is based on the lactose-inducible transcription of the *lac* operon encoding the lactose phosphotransferase system and tagatose-6-phosphate pathway (9, 40). However, application of this system is hampered by the fact that the induction level is less than 10-fold and is mediated by the intermediate tagatose-6-phosphate, the concentration of which cannot be controlled easily, especially not in large-scale fermentations. These drawbacks also apply to the inducible expression system based on the *Escherichia coli* bacteriophage T7 promoter combined with the T7 polymerase gene fused to the *lac* operon promoter, which additionally suffers from the use of a heterologous gene which is not desirable in some food applications (43). Recently, a lactococcal bacteriophage-based system has been developed by combining phage-induced DNA amplification and gene expression (25). This so-called explosive gene expression system allows for an approximately 30-fold increase in protein production, which eventually results in uncontrolled complete lysis, which is not always a desirable feature.

It has been demonstrated previously that the transcription of the lactococcal *nisA* gene is autoregulated (20). The *nisA* gene is the structural gene of the nisin gene cluster encoding the biosynthesis of the antimicrobial peptide nisin (7), which is widely used in the food industry as a natural preservative (4). The fully modified peptide nisin can induce transcription of the *nisA* gene via signal transduction mediated by a two-component regulatory system composed of histidine kinase NisK and response regulator NisR (20, 38). In this report, we describe a series of vectors and strains specifically suited for regulated gene expression, based on transcriptional and translational fusions of the *nisA* promoter region. These vectors and strains allow modulation of expression of any gene in a dynamic range of more than 1,000-fold. They were used to study the kinetics of nisin induction and were applied for high-level expression of the *E. coli gusA* and the *L. lactis pepN* genes, requiring subinhibitory amounts of the food-grade inducer nisin (12).

MATERIALS AND METHODS

Bacterial strains and media. *E. coli* MC1061 (3) was grown in L-broth-based media with aeration at 37°C (31). The lactococcal strains and plasmids used in this study are listed in Table 1. *L. lactis* cells were routinely grown at 30°C in media based on M17 medium (Merck, Darmstadt, Germany) supplemented with 0.5% (wt/vol) glucose (GM17). Chloramphenicol was used at a concentration of 10 μg/ml.

Cloning procedures, PCR, and DNA sequencing. *E. coli* MC1061 was used as an intermediate host for cloning and was handled by standard techniques (31). Plasmid DNA was isolated from *E. coli* by using the alkaline lysis method (1) or a Qiagen column purification kit (Diagen GmbH, Hilden, Germany). *L. lactis* was transformed by electroporation (42). Plasmid DNA was isolated from protoplasts of *L. lactis* as described previously (41). Approximately 100 ng of plasmid DNA, unless otherwise specified, was used as a template for amplification by the

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TABLE 1. *L. lactis* strains and plasmids

Strain or plasmid	Relevant properties[a]	Reference(s)
Strains		
MG1363	Plasmid-free and prophage-cured derivative of NCDO 712	13
NZ9700	Nisin-producing transconjugant containing Tn*5276*	21
NZ9800	NZ9700 derivative; Δ*nisA*	21
NZ3000	Δ*lacF*; derived from MG5267 by replacement recombination	34
NZ3900	NZ3000 derivative; *pepN*:*nisRnisK*	5
Plasmids		
pNZ124	Cm^r; 2.8 kb; pSH71 replicon	26
pNZ273	Cm^r; 4.7 kb; pNZ124 carrying the promoterless *gusA* gene from *E. coli*	26
pNZ8008	Cm^r; 5.0 kb; pNZ273 derivative carrying the *gusA* gene transcriptionally fused to the *nisA* promoter	5, 20
pNZ8010	pNZ8008 derivative carrying MCS1	This work
pNZ8020	pNZ8010 derivative without the *gusA* gene carrying MCS2	This work
pNZ8032	pNZ8008 derivative carrying the *gusA* gene translationally fused to the *nisA* promoter	This work
pNZ8035	pNZ8032 derivative containing MCS1	This work
pNZ8037	pNZ8035 derivative without the *gusA* gene	This work
pNZ8040	pNZ8032 derivative containing the *pepN* gene translationally fused to the *nisA* promoter	This work
pNZ8045	pNZ8040 derivative, without *cat-194*, carrying the *lacF* gene	This work

[a] Cm^r, resistance to chloramphenicol.

PCR (29). Routinely, PCR was performed with a total volume of 50 μl containing 1 U of *Taq* polymerase (GIBCO-Bethesda Research Laboratories, Gaithersburg, Md.), 50 mM NaCl, 10 mM Tris HCl (pH 8.8), 2 mM $MgCl_2$, 10 μg of gelatin, 200 μM each deoxynucleoside triphosphate, 10 pmol of each primer, and 2.5 μl of stabilizer (1% W-1; Bethesda Research Laboratories), and the total content was covered with 100 μl of light mineral oil. PCR amplifications were performed in 25 cycles, each cycle consisting of a denaturation step at 95°C for 1 min, a primer-annealing step at 55°C for 1 min, and a primer extension step at 72°C for 2.5 min, with a DNA thermocycler (Perkin-Elmer, Gouda, The Netherlands). Upon agarose gel electrophoresis (31), the amplified DNA yielded a single band that was recovered with a USBioclean kit (U.S. Biochemical Corp., Cleveland, Ohio). Nucleotide sequence analysis of double-stranded plasmid DNA was performed by using an ALF automatic sequencer in combination with autoread kits which include T7 DNA polymerase (Pharmacia Biotech, Roosendaal, The Netherlands). A fluorescent primer (primer 1) with the sequence 5′-GGGTTGGGGTTTCTACAGGACGTA-3′, complementary to positions 325 to 298 of the *gusA* gene (numbering according to reference 18), was used for sequencing. All other DNA manipulations were performed by established procedures (31).

Construction and use of *nisA* transcriptional fusion plasmids. A 0.3-kb *Tth*I-*Sst*I fragment containing the *L. lactis nisA* promoter region, from positions −156 to +156 with respect to the *nisA* transcription site (position 132 according to the numbering of reference 21), was isolated and cloned in pNZ273, a transcriptional fusion vector based on the promoterless *E. coli gusA* gene (26), generating plasmid pNZ8008 (20). The polylinker of pNZ8008 was enlarged by inserting the double-stranded oligonucleotide MCS1, with the sequence 5′-TGGATCCCG GGCTGCAGAATTCTAGACTCGAG/5′-GTCCTCGAGTCTAGAATTCTG CAGCCCGGGATCCATGCA, into plasmid pNZ8008 digested with *Pst*I and *Ava*II. The resulting plasmid, designated pNZ8010, contains eight unique restriction sites (Fig. 1). Plasmid pNZ8010 was digested with *Xho*I, thereby removing the *gusA* gene, and self-ligated, generating pNZ8010ΔG. The polylinker of pNZ8010ΔG was further improved by digesting the plasmid with *Bam*HI and *Xba*I and inserting polylinker MCS2, with the sequence 5′-GATCCGGTACC ACTAGTCCCGGGCTGCAGGAATTCGCATGCGAGCTCGTCGACA GATCTT/5′-CTAGAAGATCTGTCGACGAGCTCGCATGCGAATTCCTG CAGCCCGGGACTAGTGGTACCGGATC, resulting in pNZ8020, which contains 12 convenient cloning sites (Fig. 1).

Construction and use of *nisA* translational fusion vectors. To introduce an *Nco*I restriction site at the ATG start codon of the *nisA* gene, three nucleotide substitutions were made by PCR with approximately 10 ng of pNZ8008 used as template DNA. This procedure required one mutagenic primer, 5′-GTTAAAA TCTGCAGTACCCATGGTGAGTGCC, containing a *Pst*I site (underlined) and three substitutions (boldface), generating a new *Nco*I site (underlined), and an antiparallel primer, 5′-CCAAGATCTAGTCTTATAACTATACTG, containing a *Bgl*II site (underlined). The primers are complementary to the regions from positions +62 to +31 and −164 to −137 with respect to the *nisA* transcription start (21). The amplified fragment was digested with *Bgl*II and *Pst*I and cloned into pNZ8008 which had been digested with *Bgl*II and *Pst*I, generating pNZ8018. Plasmid pNZ8018 was sequenced with primer 1 to confirm the integrity of the *nisA* promoter and the presence of the *Nco*I site.

The *Nco*I site in the chloramphenicol resistance gene of pNZ8008 was removed by PCR-mediated megaprimer mutagenesis, as described previously (22), using the mutagenic primer 5′-AAATGAAGTCCAAGGAATAATAGAAAG, complementary to positions 1680 to 1706 of the *cat-194* sequence (numbering according to reference 14), carrying a mutated (boldface) *Nco*I site (underlined). Two amplification rounds were used, the first one with the mutagenic primer and the antiparallel primer 5′-CCTGTAAAGAATGACTTCAAAGAG, complementary to positions 1566 to 1589 (14), containing an *Xmn*I site (underlined). The second round was performed with the purified first fragment as a primer, together with the second primer 5′-CCAGTCATTAGGCCTATCTGAC, complementary to positions 1880 to 1901 (14), containing a *Stu*I site (underlined). The amplified PCR product was digested with *Xmn*I and *Stu*I and cloned into pNZ8018 digested with *Xmn*I and *Stu*I, yielding pNZ8030.

An *Nco*I restriction site was introduced at the ATG start codon of the *gusA* gene by use of PCR mutagenesis. The gene was amplified by using pNZ8008 as a template and two primers, 5′-GGAGTCCCCCATGGTACGTCC (containing three substitutions [boldface] generating the new *Nco*I site [underlined]) and 5′-GCACTCGAGAAGCTTTCATTG (containing an *Xho*I and a *Hin*dIII site [underlined]). Each PCR cycle consisted of a primer-annealing step at 50°C. The PCR-amplified *gusA* gene was cloned as an *Nco*I-*Hin*dIII fragment in pNZ8030 digested with *Nco*I-*Hin*dIII, generating pNZ8032 (Fig. 1). Subsequently, the double-stranded oligonucleotide (MCS1) was inserted in pNZ8030, digested with *Pst*I and *Ava*II. The resulting plasmid pNZ8035 was finally digested with *Xho*I, thereby removing the *gusA* gene, and self-ligated, generating pNZ8037 (Fig. 1).

The *pepN* gene (34) was cloned as a 2.5-kb *Nco*I-*Xho*I fragment in pNZ8032 digested with *Nco*I-*Xho*I, generating pNZ8040 (see Fig. 5). The *pepN* gene was obtained by Expand Long Template PCR (Boehringer, Mannheim, Germany) performed as recommended by the manufacturer, using 2 ng of pNZ1120 (34) as a template and as primers the oligonucleotides 5′-GCAACTGCAGGAGAA GCCATGGCTGTAAAACG, containing two substitutions (boldface) generating a new *Nco*I site (underlined) at the ATG start codon of the *pepN* gene, and 5′-CCTTATTCTCGAGTTGATTGTTCTATCG, containing an *Xho*I site (underlined).

For construction of a fully food-grade vector, the chloramphenicol acetyltransferase gene of pNZ8040 was deleted by a restriction digestion with *Sal*I and *Bgl*II and replaced by the food-grade marker gene *lacF* (27), isolated as a 0.4-kb *Sal*I-*Bam*HI fragment from plasmid pNZ307, generating pNZ8045. pNZ307 is a pUC18 derivative (44) harboring a 405-bp *Nco*I-*Xmn*I fragment containing the lactococcal *lacF* gene (6).

Induction of strains with *nisA* promoter-containing plasmids, enzyme activity,

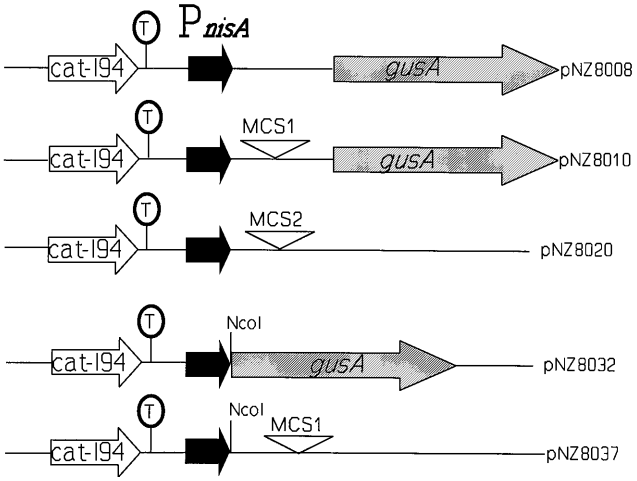

FIG. 1. Schematic representation of the organization of the constructed expression vectors containing the inducible *nisA* promoter. In constructs pNZ8032 and pNZ8037, the *Nco*I restriction site is shown to indicate the possibility of making translational fusions with the *nisA* start codon. T, terminator of the chloramphenicol acetyltransferase gene *cat-194*. Multiple cloning sites for MCS1, *Bam*HI, *Sma*I, *Xma*I, *Pst*I, *Eco*RI, *Xba*I, *Xho*I, and *Ava*II; sites for MCS2, *Bam*HI, *Kpn*I, *Spe*I, *Sma*I, *Pst*I, *Eco*RI, *Sph*I, *Sst*I, *Sal*I, *Bgl*II, *Xba*I, and *Xho*I.

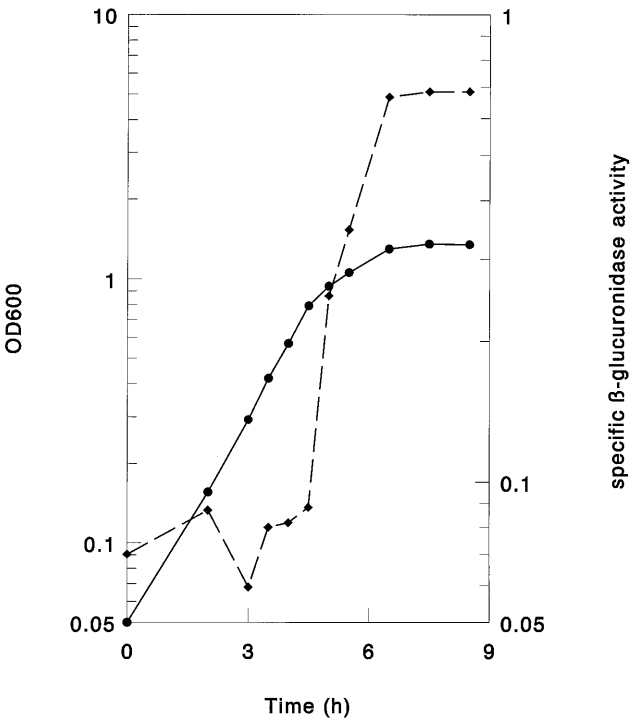

FIG. 2. β-Glucuronidase activity (broken line) determined in cell extracts during growth (optical density at 600 nm [OD600]; solid line) of the nisin-producing strain NZ9700 harboring pNZ8008. The activities are expressed as specific activity (increase in A_{405} per minute) per optical density unit.

and protein analysis. Several *L. lactis* strains were used as hosts for induction studies of *nisA* promoter-containing plasmids. Histochemical screening for *gusA*-positive clones was performed with 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) (Research Organics Inc., Cleveland, Ohio) at a final concentration of 0.5 mM (26). The *L. lactis* strains harboring plasmids with *gusA* or *pepN* under control of the *nisA* promoter were grown until an A_{600} of 0.5 was reached and induced with different concentrations of nisin or not treated. Growth was continued for 90 min (unless stated otherwise), cells were harvested, and cell extracts were prepared as described previously (5). These extracts were used for quantitative determination of β-glucuronidase or aminopeptidase N activity, using para-nitro-β-D-glucuronic acid (Clonetech Lab, Inc., Palo Alto, Calif.) or lysyl-*p*-nitroanilide (Fa. Bachem, Bubendorf, Switzerland), respectively, as described previously (11, 26, 34). Protein concentrations were determined as described previously (2), using bovine serum albumin as a standard. A sample of 100 μl of the extracts was mixed with equal amounts of sample buffer, and 20 μl was applied to a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel (23). The protein fractions were quantified as a percentage of the total intracellular protein by scanning and digitizing the gel, using an image-analyzing system and the computer programs Iris Video Digitizer and Image Quant (Molecular Dynamics, Zoetermeer, The Netherlands).

RESULTS

Development of cloning vectors based on a transcriptional fusion with the *nisA* promoter. To allow for the development of *nisA* promoter-based vectors, a series of plasmids based on the vector pNZ8008 was constructed (Fig. 1). Plasmid pNZ8008 is an expression vector containing the reporter gene *gusA* which is preceded by the *nisA* promoter region ranging from positions −156 to +156 with respect to the *nisA* transcription start (21), including −35 and −10 sequences, the putative NisR binding site (5), and the ribosome binding site as well as part of the *nisA* coding region including translational stop signals (20). Nisin-induced expression of the *nisA* promoter region was studied by introducing pNZ8008 in the nisin-producing *L. lactis* strain NZ9700, a derivative of the plasmid-free strain MG1614, carrying the nisin-sucrose conjugative transposon Tn*5276* (28). Plasmid pNZ8008 was stably maintained in the resulting Cm^r transformants, in spite of its homology with the 312-bp *nisA* promoter region of the nisin gene cluster present in the chromosome of NZ9700. All transformants generated blue colonies on plates containing X-Gluc, suggesting constitutive β-glucuronidase expression. To determine the kinetics of *gusA* expression mediated by the *nisA* promoter region in the nisin-producing strain NZ9700, the specific β-glucuronidase activity was assayed during growth of the culture and found to increase very rapidly after mid-exponentional growth, until the maximum level at the stationary phase was reached (Fig. 2).

To determine the different expression levels in other strains, expression of the *gusA* gene was also studied in *L. lactis* NZ9800, an NZ9700 derivative carrying a deletion in the *nisA* gene that abolishes nisin production, and in *L. lactis* NZ3900, an MG1363 derivative which contains the *nisRK* genes integrated in the chromosome. Since no nisin is produced by those strains, *gusA* expression was studied in the absence and presence of extracellular nisin A (0.075 ng ml^{-1}). The β-glucuronidase activity specified by pNZ8008 was determined and found to be 25 times higher in extracts of strain NZ3900 than in those of strain NZ9800 (Table 2). In addition, no detectable β-glucuronidase activities were found in strains NZ9800 and NZ3900, harboring pNZ8008, without induction with nisin (Table 2). On the basis of the detection limit of the β-glucuronidase assay, it can be concluded that the promoter is switched off to an undetectable background level and the induction factor exceeds 1,000 (Table 2).

To exploit further pNZ8008, its polylinker was enlarged by inserting a double-stranded oligonucleotide containing the multiple cloning site MCS1, generating pNZ8010. To develop an even more convenient expression vector with other unique restriction sites, the *gusA* gene was removed and another multiple cloning site (MCS2) was inserted, resulting in pNZ8020 (Fig. 1). Plasmids pNZ8010 and pNZ8020 have been used successfully for cloning genes which are transcriptionally fused to the controlled *nisA* promoter (19, 35, 39).

Translational fusion of the *gusA* gene to the *nisA* promoter. To optimize translation initiation, an expression vector based on the *nisA* transcription and translation signals was constructed by introducing an *Nco*I site at the ATG start codon of the *nisA* gene which can be used for translational fusions of other genes with the efficient *nisA* ribosome binding site on the promoter region. This approach was tested by the simultaneous introduction of an *Nco*I restriction site at the ATG start codon of the *nisA* and the *gusA* genes and a fusion of the *nisA* promoter to the *gusA* gene, generating pNZ8032. To show the applicability of this translational fusion vector, β-glucuronidase activities were determined in the *L. lactis* strains NZ9800 and NZ3900 (Table 2). A sixfold-higher level of nisin-inducible

TABLE 2. Expression of the *gusA* gene either transcriptionally (pNZ8008) or translationally (pNZ8032) fused to the *nisA* promoter, after induction with nisin A (0.075 ng/ml), in several lactococcal strains

L. lactis strain	Characteristics	β-Glucuronidase activity[a]	
		pNZ8008	pNZ8032
MG1363	No Tn*5276*[b]	<0.1	<0.1
NZ9800	Tn*5276*; Δ*nisA*	3	20
NZ3900	No Tn*5276*; *nisRK*[c]	80	130

[a] β-glucuronidase activity is shown as specific activity (10^3) per optical density (at 600 nm) unit.
[b] Tn*5276* denotes the conjugative nisin-sucrose transposon.
[c] The *nisRK* genes are integrated on the chromosome (5).

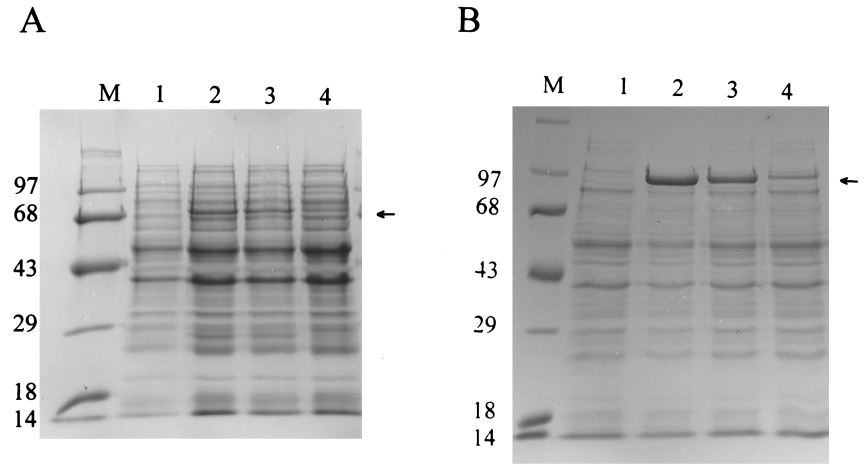

FIG. 3. Coomassie blue-stained gels after SDS-PAGE of extracts of strain NZ3900 containing pNZ8032 or pNZ8040, producing β-glucuronidase (A) or aminopeptidase N (B). (A) Lane 1, uninduced cells; lanes 2 to 4, induction with 2.5, 0.5, and 0.05 ng of nisin A per ml, respectively; lane M, molecular weight marker (in kilodaltons). (B) Lane 1, uninduced cells; lanes 2 to 4, induction with 0.5, 0.1, and 0.05 ng of nisin A per ml, respectively; lane M, molecular weight marker (in kilodaltons). The locations of the overproduced proteins are indicated (arrows).

gusA expression was obtained in strain NZ9800 harboring pNZ8032 than in strain NZ9800 harboring pNZ8008. The activity in strain NZ3900 with pNZ8008 is about 25 times higher than that in NZ9800, but the increase with pNZ8032 is only twofold (Table 2). We observed growth problems with this strain in the induced state, so very high levels of β-glucuronidase production might be lethal to the cells. It was not possible to determine β-glucuronidase activity in strain NZ9700 because of the structural instability of pNZ8032 in this nisin-producing strain.

A cell extract of strain NZ3900 harboring pNZ8032 was used to visualize β-glucuronidase production in SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The 68-kDa protein band of β-glucuronidase was clearly visible after induction with low concentrations of nisin A (Fig. 3A), concomitant with an increase in β-glucuronidase activity (data not shown). These results demonstrate that the heterologous enzyme β-glucuronidase can be overproduced in *L. lactis* to high levels in a strictly controlled way when the gene encoding it is translationally fused to the *nisA* promoter.

Kinetics of induction. To study the regulation of the *nisA* promoter in the translational *nisA-gusA* promoter fusion plasmid in strains NZ9800 and NZ3900, the kinetics of β-glucuronidase activities were determined at several times after induction with nisin A. After 90 to 120 min, the maximum level of activity is reached in both NZ9800 and NZ3900 (Fig. 4). After addition of 1 ng of nisin A ml^{-1} to the non-nisin-producing strain NZ3900 harboring pNZ8032, β-glucuronidase activity could be measured after a lag phase of approximately 10 min, whereas in strain NZ9800 harboring pNZ8032 this lag phase is approximately 15 min. With a lower induction concentration of nisin (0.1 ng ml^{-1}), the lag phase before β-glucuronidase activity is increased in both strains to 20 to 25 min (data not shown). The greatest increase of activity upon nisin induction is observed when strain NZ3900 is used (Fig. 4).

Overexpression of *pepN* in *L. lactis* NZ3900. To demonstrate the applicability of the *nisA* promoter for overproduction of endogenous lactococcal enzymes, pNZ8040, carrying the *pepN* gene translationally fused to the *nisA* promoter, was introduced into strain NZ3900 (Fig. 5). Cell extracts of strain NZ3900 harboring pNZ8040 were used to determine the specific activity of aminopeptidase N, after induction with nisin A and in the absence of nisin A (Table 3). With 0.5 ng of nisin A ml^{-1} used as an inducer, the aminopeptidase N activity amounted to 25 mmol mg^{-1} min^{-1}. SDS-PAGE of cell extracts of NZ3900 harboring pNZ8040 showed the overproduction of the expected 95-kDa aminopeptidase N (34) after induction with nisin A at concentrations of 0.5, 0.1, and 0.05 ng ml^{-1} (Fig. 3B). The protein bands on the gel were quantified, and

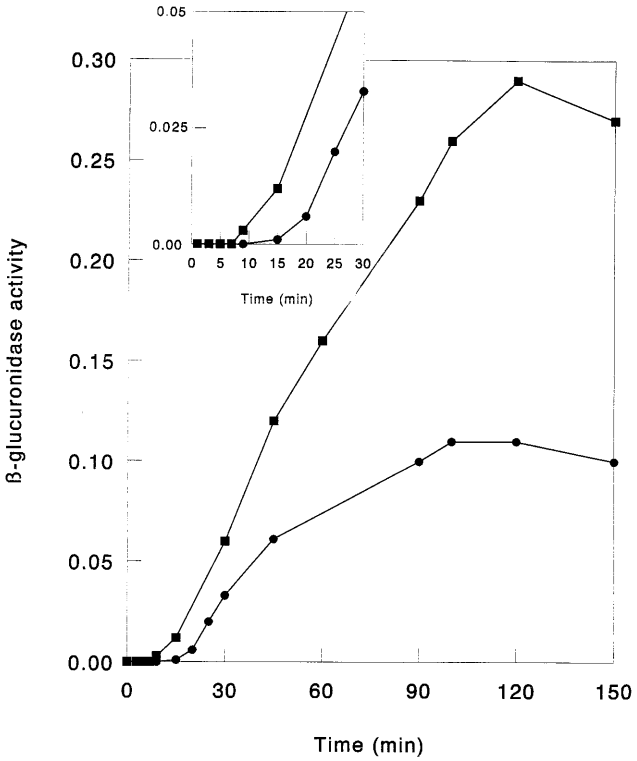

FIG. 4. Kinetics of induction: β-glucuronidase activities (specific activity per optical density [at 600 nm] unit) determined in cell extracts of strains NZ9800 (●) and NZ3900 (■), both harboring pNZ8032, during the time after induction with 1 ng of nisin A per ml.

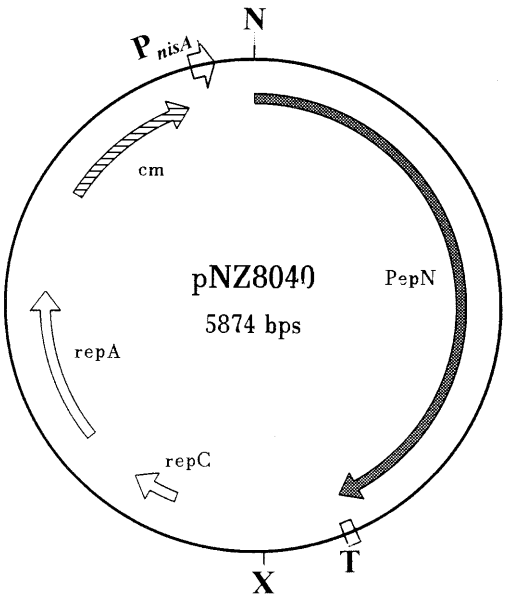

FIG. 5. Physical map of the PepN-overproducing construct pNZ8040. The lactococcal *pepN* gene was cloned behind the inducible *nisA* promoter. Relevant cloning sites: N, *Nco*I; X, *Xho*I. T, transcriptional terminator.

the results showed that after induction with 0.5 ng of nisin ml^{-1}, approximately 47% of the intracellular protein is formed by the overproduced PepN protein (Table 3) and that there is a linear dependency on inducer concentration, as has been shown before by use of enzymatic assays (5, 20).

The genetic marker that is used in the vectors described here is based on the transferable chloramphenicol resistance gene (*cat-194*), which can easily be replaced by the *lacF* marker gene (6, 27). In combination with the *lacF*-deficient strain *L. lactis* NZ3900, this *lacF* marker provides a system perfectly suitable for food application (27). To test this approach, the *cat-194* marker of pNZ8040 was replaced by the *lacF* gene and the resulting plasmid, pNZ8045, was introduced into *L. lactis* NZ3900. Lactose-utilizing transformants were used to determine PepN activity in the presence and absence of nisin. Induced cells showed PepN activity similar to that of cells of NZ3900 harboring pNZ8040 (Table 3).

DISCUSSION

As which features determine the efficiency of gene expression in *L. lactis* have not been determined completely, the most straightforward approach to developing expression vectors is the use of cognate lactococcal signals. A regulated expression system was developed by using the *L. lactis nisA* promoter cloned in a promoter-probe vector harboring the *gusA* reporter gene. The *nisA* gene contains a promoter sequence which can efficiently control transcription initiation depending on the extracellular concentration of the antimicrobial peptide nisin. Recently, it has been shown that induction of the *nisA* promoter relies on the products of the genes *nisR*, encoding the response regulator, and *nisK*, encoding the histidine kinase sensor (20, 38).

It was shown that the *nisA* promoter-based expression vector pNZ8008 can be used to express the *gusA* gene in several lactococcal strains, containing the chromosomal *nisRK* genes necessary for signal transduction. In the nisin-producing strain NZ9700, nisin induces its own production as well as the expression of the gene of interest cloned behind the *nisA* promoter. The expression "quorum sensing" can be used to describe this regulatory system which couples cell density to expression of a particular trait (30). It has long been known that the nisin production rate is maximal towards the end of the logarithmic growth phase (15). In some cases, it can be an advantage to use a nisin-producing strain for continuous overexpression of proteins, since active induction by adding nisin is no longer needed.

Other strains that are very useful for overexpression of genes using the nisin-inducible expression system are the non-nisin-producing strains NZ9800 and NZ3900. The response in strain NZ3900 harboring a *nisA-gusA* fusion plasmid is 25 times higher and with the same inducer concentration is detectable earlier than the response in strain NZ9800. This property may be due to the fact that this strain does not contain the *nisI* (21) and *nisFEG* genes, which are involved in nisin immunity (32), in this way preventing the putative interaction of extracellular nisin with the immunity proteins and leading to a higher available nisin concentration for induction. Strain NZ3900 has shown to be extremely useful for the overproduction of proteins of interest (35). The nisin concentration necessary for induction is far below the MIC of 14 ng/ml (20). This offers the possibility of using the system in dairy applications in combination with conventional starters that will not be inhibited by the inducing nisin concentrations.

To determine effects of translation initiation, coding sequences can be fused directly to the *nisA* initiation codon at a unique *Nco*I site that includes the initiating ATG codon. The translational fusion of the *gusA* gene to the *nisA* promoter in pNZ8032 showed considerably higher activity than the transcriptional fusion of *gusA* to the *nisA* promoter (pNZ8008). Therefore, it was possible to produce the heterologous protein β-glucuronidase, using the translational fusion vector, in sufficient amounts to allow visualization of the product by SDS-PAGE.

The controlled overproduction of *pepN*, by use of a translational fusion in *L. lactis* NZ3900, allows rapid extraction and purification of great amounts of aminopeptidase N that can be used for biochemical studies. Direct application of this strain in dairy and other food fermentations is also feasible. The results indicate that approximately half of the total intracellular protein constitutes aminopeptidase N, a level of overproduction that, to our knowledge, has never been described before for lactic acid bacteria. Considering the specific activity of 25 mmol mg^{-1} min^{-1} in cell extracts of NZ3900 harboring pNZ8040, compared with the specific activity of purified aminopeptidase N (33), it is likely that all of the overproduced protein is active.

TABLE 3. Specific aminopeptidase N activity and quantified PepN protein fractions as a percentage of the total intracellular protein in cell extracts of NZ3900 harboring pNZ8040 grown in the absence or presence of different amounts of nisin A

Nisin A concn (ng/ml)	Sp act[a]	% of total protein
0	0	0
0.05	8	9
0.07	10	ND[b]
0.1	14	26
0.25	21	ND
0.5	25	47

[a] Shown as millimoles per minute per milligram of protein.
[b] ND, not determined.

In view of the measured β-glucuronidase activities of strain NZ3900 harboring pNZ8032 (Table 2), an induction factor of at least 1,000-fold can be calculated. Furthermore, no detectable *gusA* expression is observed without induction of the *nisA* promoter, which offers the advantage of overexpression of lethal genes in *L. lactis*. Recently, *nisF* expression also was found to be controlled by nisin, albeit the expression levels were lower than those obtained with the *nisA* promoter (5). This offers the possibility of using also the *nisF* promoter as an alternative for the *nisA* promoter for a highly controllable expression system. Thus, the series of vectors and strains described here are ideally suited for high-level, food-grade, controlled overproduction of desired proteins. Up to now, this has been achieved with a number of homologous and heterologous proteins that could be produced in large quantities (2 to 60% of total intracellular protein) in a strictly controlled and food-grade manner, with great potential for practical application (19, 35).

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