Controlled Gene Expression Systems for Lactococcus lactis with the Food-Grade Inducer Nisin
Ruyter, Pascalle G.G.A. de; Kuipers, Oscar; Vos, Willem M. de

Published in:
Default journal

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
1996

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.
Controlled Gene Expression Systems for *Lactococcus lactis* with the Food-Grade Inducer Nisin

PASCALLE G. G. A. DE RUYTER, OSCAR P. KUIPERS, AND WILLEM M. DE VOS

Department of Biophysical Chemistry, NIZO (The Netherlands Institute for Dairy Research), 6710 BA Ede, and
Department of Microbiology of the Agricultural University Wageningen, 6703 CT Wageningen, The Netherlands

Received 16 May 1996/Accepted 1 August 1996

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 NZ9390 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 (Qiagen 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

---

*Corresponding author. Mailing address: NIZO, Kernhemseweg 2, 6718 ZB Ede, The Netherlands. Phone: 31-318-659511. Fax: 31-318-650400. Electronic mail address: kuipers@nizo.nl.
**TABLE 1. L. lactis strains and plasmids**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant properties*</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MG1363</td>
<td>Plasmid-free and prophage-cured derivative of NCDO 712</td>
<td>13</td>
</tr>
<tr>
<td>NZ9700</td>
<td>Nisin-producing transconjugant containing Cm*</td>
<td>21</td>
</tr>
<tr>
<td>CCGCGGACGCTTGACGTCGGG</td>
<td>Nisin production</td>
<td>21</td>
</tr>
<tr>
<td>NZ9800</td>
<td>NZ9700 derivative; NcoI</td>
<td>21</td>
</tr>
<tr>
<td>NZ3000</td>
<td>ΔlacF; derived from MG5267 by replacement recombination</td>
<td>34</td>
</tr>
<tr>
<td>NZ3900</td>
<td>NZ3000 derivative; pepNnisOmcS</td>
<td>5</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pNZ124</td>
<td>Cm*; 2.8 kb; pSH71 replication</td>
<td>26</td>
</tr>
<tr>
<td>pNZ273</td>
<td>Cm*; 4.7 kb; pNZ124 carrying the promoter- less gusA gene from E. coli</td>
<td>26</td>
</tr>
<tr>
<td>pNZ8008</td>
<td>5.0 kb; pNZ273 derivative carrying the gusA gene transcriptionally fused to the nisA promoter</td>
<td>5, 20</td>
</tr>
<tr>
<td>pNZ8010</td>
<td>pNZ8008 derivative carrying MCS1</td>
<td>This work</td>
</tr>
<tr>
<td>pNZ8020</td>
<td>pNZ8010 derivative without the gusA gene carrying MCS2</td>
<td>This work</td>
</tr>
<tr>
<td>pNZ8032</td>
<td>pNZ8008 derivative carrying the gusA gene translationally fused to the nisA promoter</td>
<td>This work</td>
</tr>
<tr>
<td>pNZ8030</td>
<td>pNZ8032 derivative containing MCS1</td>
<td>This work</td>
</tr>
<tr>
<td>pNZ8037</td>
<td>pNZ8032 derivative without the gusA gene</td>
<td>This work</td>
</tr>
<tr>
<td>pNZ8040</td>
<td>pNZ8032 derivative carrying the pepN gene translationally fused to the nisA promoter</td>
<td>This work</td>
</tr>
<tr>
<td>pNZ8045</td>
<td>pNZ8040 derivative, without cat-194, carrying the laclC gene</td>
<td>This work</td>
</tr>
</tbody>
</table>

* Cm*, 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 MgCl₂, 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 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. DNA sequencing analysis of double-stranded plasmid DNA was performed by using an ALF automatic sequencer in combination with an autocad kit which included T7 DNA polymerase (Pharmacia Biotech, Roosendaal, The Netherlands). A fluorescent primer (primer 1) with the sequence 5′-GGTGTTGCTGGATTGCTTCAATTATTGCGAATTCGAGCTCGCATGCGAATTCCTG-3′ (numbering according to reference 14), carrying a mutated (boldface) NcoI site (underlined). The primers are complementary to positions 1890 to 1901 (14), containing a SmI site (underlined). The amplified PCR product was digested with XmnI and SmI and cloned into pNZ8018 digested with XmnI and SmI, yielding pNZ8030.

An NcoI 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′-GGAGTCTCCCATGCATTGCTACGTCGCC (containing three substitutions [boldface]) generating the new NcoI site (underlined) and 5′-GACTCTTAGAAGACCTTTGAATTG (containing an XhoI and an HindIII site [underlined]). Each PCR cycle consisted of a primer-annealing step at 50°C. The PCR-amplified gusA gene was cloned as an NcoI-HindIII fragment in pNZ8030 digested with NcoI-HindIII, generating pNZ8032 (Fig. 1). Subsequently, the double-stranded oligonucleotide (MCS1) was inserted in pNZ8030, digested with PstI and AvaI. The resulting plasmid pNZ8035 was finally digested with XhoI, thereby removing the gusA gene, and self-ligated, generating pNZ8037 (Fig. 1).

The pepN gene (34) was cloned as a 2.5-kb NcoI-XhoI fragment in pNZ8032 digested with NcoI-XhoI, 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′-GCATGGCTGTCGTTAAAAACG-3′ and containing two substitutions (boldface) generating a new NcoI site (underlined) at the ATG start codon of the pepN gene, and 5′-GACTTCTTAGAAGACCTTTGAATTG-3′, generating a HindIII site (underlined).

For construction of a fully food-grade vector, the chloramphenicol acetyltransferase gene of pNZ8040 was deleted by a restriction digestion with SaI and BglII and replaced by the food-grade marker gene lacI (27), isolated as a 0.4-kb Sall-BamHI fragment from plasmid pNZ207, generating pNZ8045. pNZ8045 is a pUC18 derivative (44) harboring a 405-bp pUC18 lacI fragment containing the lactococcal lacI 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 NcoI 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, BamHI, SmI, XhoI, PstI, EcoRI, XbaI, NcoI, and AvaII; sites for MCS2, BamHI, KpnI, SpeI, SmI, PstI, EcoRI, SpeI, Srl, SalI, BglII, XhoI, and Xhol.](image-url)
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-o-glucuronide (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 Tn5276 (28).

Plasmid pNZ8008 was stably maintained in the resulting Cm' 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-exponential 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 NcoI 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 NcoI 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**

<table>
<thead>
<tr>
<th>L. lactis strain</th>
<th>Characteristics</th>
<th>β-glucuronidase activity&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pNZ8008</td>
</tr>
<tr>
<td>MG1363&lt;sup&gt;b&lt;/sup&gt;</td>
<td>No Tn5276&lt;sup&gt;c&lt;/sup&gt;</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>NZ9800</td>
<td>Tn5276; ΔnisA&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3</td>
</tr>
<tr>
<td>NZ3900</td>
<td>Tn5276; nisRK&lt;sup&gt;c&lt;/sup&gt;</td>
<td>80</td>
</tr>
</tbody>
</table>

<sup>a</sup> β-glucuronidase activity is shown as specific activity (10<sup>4</sup>) per optical density (at 600 nm) unit.

<sup>b</sup> Tn5276 denotes the conjugative nisin-sucrose transposon.

<sup>c</sup> The nisRK genes are integrated on the chromosome (5).
**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 lacticoccal 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. 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).

**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.
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 NeoI 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 SDSPAGE.

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 absence or presence of different amounts of nisin A**

<table>
<thead>
<tr>
<th>Nisin A concen (ng/ml)</th>
<th>Sp act(^a)</th>
<th>% of total protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.05</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>0.07</td>
<td>10</td>
<td>ND(^b)</td>
</tr>
<tr>
<td>0.1</td>
<td>14</td>
<td>26</td>
</tr>
<tr>
<td>0.25</td>
<td>21</td>
<td>ND</td>
</tr>
<tr>
<td>0.5</td>
<td>25</td>
<td>47</td>
</tr>
</tbody>
</table>

\(^a\) 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).

ACKNOWLEDGMENTS

We are grateful to Michel Kleerebezem, Jeroen Hugenholtz, and Roland Siezen for critically reading the manuscript and to Liesbeth Bijl for technical assistance.

This work was partly financed by the Mesdag Foundation (Leeuwarden, the Netherlands).

REFERENCES