APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1997, p. 4581–4584 Vol. 63, No. 11
0099-2240/97/$04.00+0

Controlled Gene Expression Systems for Lactic Acid Bacteria: Transferable Nisin-Inducible Expression Cassettes for *Lactococcus*, *Leuconostoc*, and *Lactobacillus* spp.

MICHIEL KLEEREBEZEM,[1]* MARKE M. BEERTHUYZEN,[1] ELAINE E. VAUGHAN,[1]†
WILLEM M. DE VOS,[1,2] AND OSCAR P. KUIPERS[1]

Department of Biophysical Chemistry, NIZO, 6710 BA Ede,[1] and Department of Microbiology, Wageningen Agricultural University, 6703 CT Wageningen,[2] The Netherlands

Received 30 May 1997/Accepted 2 September 1997

A transferable dual-plasmid inducible gene expression system for use in lactic acid bacteria that is based on the autoregulatory properties of the antimicrobial peptide nisin produced by *Lactococcus lactis* was developed. Introduction of the two plasmids allowed nisin-inducible gene expression in *Lactococcus lactis* MG1363, *Leuconostoc lactis* NZ6091, and *Lactobacillus helveticus* CNRZ32. Typically, the β-glucuronidase activity (used as a reporter in this study) remained below the detection limits under noninducing conditions and could be raised to high levels, by addition of subinhibitory amounts of nisin to the growth medium, while exhibiting a linear dose-response relationship. These results demonstrate that the nisin-inducible system can be functionally implemented in lactic acid bacteria other than *Lactococcus lactis*.

Lactic acid bacteria (LAB) are used in a large variety of industrial dairy and other food fermentations. Considerable interest exists in the development of genetic tools that allow production of desired proteins in these LAB, in order to improve the properties of these fermented products. Ideally, these expression systems should allow for the inducible overproduction of the desired proteins at any desired moment during fermentation. *Lactococcus lactis* is one of the best-studied LAB, and most advances in the desired development of genetic tools have been made with this bacterium (for a recent review, see reference 12). One of the most promising controllable expression systems that has been developed is based on the autoregulatory properties of nisin biosynthesis by *Lactococcus lactis* (4, 12). Nisin is a posttranslationally modified antimicrobial peptide that is widely used in the food industry as a natural preservative (6). It has previously been shown that the biosynthesis of nisin by *Lactococcus lactis* is subject to autoregulation, in which nisin functions as a signal molecule that induces the transcription of the nisin biosynthetic gene cluster via a typical two-component regulatory system, consisting of the histidine protein kinase NisK and the response regulator NisR (7, 11, 17). The amounts of nisin required for induction were shown to be far below the MIC of nisin, and induction could also be achieved with supernatants of nisin-producing cultures (3, 11). The nisin biosynthetic gene cluster contains three promoters, of which the *nisA* and *nisF* promoters are subject to nisin-mediated autoregulation, while the *nisR* promoter constitutively drives the expression of the *nisRK* genes (3). Based on this regulated system, several cloning vectors carrying the *nisA* or *nisF* promoter and appropriate *Lactococcus lactis* hosts have been constructed that are specifically suited for controlled, nisin-inducible expression (3, 4). This system for regulated gene expression in *Lactococcus lactis* has been found to show many desirable characteristics: (i) nisin is an ideal molecule to be used as an inducer since it is already widely used in the food industry and can therefore be regarded as a food-grade inducer; (ii) the expression of the desired gene appears to be very tightly controlled, leading to undetectable protein levels in the uninduced state, which even makes it possible to produce lethal proteins (5); (iii) the level of expression is controllable in a dynamic range of >1,000-fold which is directly dependent (linear dose-response relationship) on the concentration of nisin used for induction (3, 11); (iv) very high protein expression levels, which can go up to 60% of the total intracellular protein level, can be reached; and (v) several *nisA* promoter vectors that contain the food-grade selection marker *lacF* instead of an antibiotic resistance gene have been developed (4, 5, 14). Here, we describe the development of a transferable dual-plasmid system that is based on the nisin autoregulatory process. Introduction of these plasmids allowed nisin-inducible gene expression in *Lactococcus lactis* and members of other industrially relevant LAB genera, *Leuconostoc lactis* and *Lactobacillus helveticus*.

Development of a two-plasmid nisin-inducible system. Previous studies indicate that the *nisRK* genes are the only *nis* genes required for nisin-mediated signal transduction and *nisA* or *nisF* promoter activation in *Lactococcus lactis* (4, 11). In order to evaluate the possibility of functional implementation of this strictly controlled expression system in other LAB, various *nisRK* plasmids were constructed (plasmids used in this study are listed in Table 1). A 2.7-kb *Hin*dIII-*Pst*I chromosomal DNA fragment from strain NZ9700, containing the 3′ part of the *nisP* gene and the complete *nisR* and *nisK* genes, was cloned into similarly digested pUC19ery (10), resulting in pNZ9500 (transformed [15] to *Escherichia coli* MC1061 [2], selected on L broth-based medium [15] containing ampicilin [100 μg/ml]). For cloning purposes, the 2.3-kb *Sca*I-*Pst*I fragment from pNZ9500 containing the *nisR* and *nisK* genes without the *nisR* promoter (but with the *nisR* ribosome binding site [3]) was subcloned into *Eco*RV-*Pst*I-digested pBluescriptII (Stratagene, La Jolla, Calif.). From the resulting plasmid (pNZ9540) the *nisRK* fragment was reisolated as an *Xho*I-*Xba*I fragment and cloned into *Sal*I-*Xba*I-di-

* Corresponding author. Mailing address: Department of Biophysical Chemistry, NIZO, P.O. Box 20, 6710 Ede, The Netherlands. Phone: 31-(0)318-659629. Fax: 31-(0)318-650400. E-mail: kleerebe@nizo.nl.

† Present address: Nestlé Research Center, Nestec Ltd., Vers-chez-les-Blanc, Lausanne, Switzerland.

TABLE 1. Plasmids used in this study

Plasmid	Relevant characteristic(s)	Source or reference
pUC19ery	Amp^r, Ery^r	10
pBluescript II	Amp^r	
pIL252	Ery^r, low-copy-number, broad-host-range cloning vector	16
pIL253	Ery^r, high-copy-number, broad-host-range cloning vector	16
pNZ9500	Amp^r, Ery^r, pUC19ery derivative containing a 2.7-kb chromosomal DNA fragment from strain NZ9700 that contains 3′ ends of *nisP* and *nisRK*	This work
pNZ9540	Amp^r, *nisR* promoter followed by *nisRK* cloned into pBluescript II	This work
pNZ9520	Ery^r, *nisRK* cloned in pIL253, expression of *nisRK* driven by *rep* read-through	This work
pNZ9521	Ery^r, *nisRK* cloned in pIL253, expression of *nisRK* driven by *nisR* promoter and *rep* read-through	This work
pNZ9530	Ery^r, *nisRK* cloned in pIL252, expression of *nisRK* driven by *rep* read-through	This work
pNZ9531	Ery^r, *nisRK* cloned in pIL252, expression of *nisRK* driven by *nisR* promoter and *rep* read-through	This work
pNZ8008	Cam^r, promoterless *gusA* reporter gene, cloned behind the *nisA* promoter	11

gested pIL252 or pIL253 (16), resulting in plasmids pNZ9530 and pNZ9520, respectively. A 2.4-kb *Hpa*II-*Pst*I fragment from pNZ9500, containing the *nisR* promoter sequence followed by the *nisR* and *nisK* genes was cloned into similarly digested pIL252 or pIL253, resulting in plasmids pNZ9531 and pNZ9521, respectively. The plasmids pNZ9520, pNZ9521, pNZ9530, and pNZ9531 all contain the *nisR* and *nisK* genes in the same transcriptional orientation as the replication genes present on pIL252 or pIL253. Since the *repG* gene (the last gene within the *rep* operon) is not followed by a transcription termination sequence, it is expected that for pNZ9520 and pNZ9530 the transcription of *nisRK* will depend entirely on the transcription read-through from the *rep* promoter. The *rep* promoter activity is repressed by the product of the *repF* gene, which is present in pIL252 but is disrupted in pIL253, leading to the respective low and high copy numbers of these vectors (16). Transcription of the *nisRK* genes in the pNZ9521 and pNZ9531 constructs is driven by the *nisR* promoter and is probably enhanced by read-through activity of the *rep* promoter. Therefore, these plasmids (Table 1) are expected to result in different expression levels of the NisR and NisK proteins and for functional analysis they were introduced in *Lactococcus lactis* MG1363 (8; procedure for electroporation described in reference 19) harboring pNZ8008, a pNZ273 derivative that contains a transcriptional fusion of the promoterless *gusA* reporter gene to the *nisA* promoter (11). Previously, we established that the replicons of these plasmids were compatible (18) and transformants that showed resistance to chloramphenicol (5 μg/ml) and erythromycin (3 μg/ml) were readily obtained. Transformants harboring pNZ8008 in combination with either pNZ9530 or pNZ9531 gave rise to white colonies on GM17-X-Gluc (M17 [Merck, Darmstadt, Germany] supplemented with 0.5% glucose and 0.5 mM 5-bromo-4-chloro-3-indolyl glucuronide [X-Gluc; Research Organics Inc., Cleveland, Ohio]) plates when no nisin was added to the plates. In contrast, blue colonies were obtained when these transformants were plated on GM17-X-Gluc plates in which nisin (2.5 ng/ml) was included. Transformants harboring pNZ8008 in combination with pNZ9520 or pNZ9521 gave rise to blue colonies on GM17-X-Gluc plates without nisin induction, indicating transcriptional activity of the *nisA* promoter under noninducing conditions. These results suggest that the plasmids pNZ9530 and pNZ9531 (in contrast to pNZ9520 and pNZ9521) generate functional expression of the *nisRK* genes in *Lactococcus lactis* at a level that allows regulatory characteristics similar to those when single copies of these genes are chromosomally encoded.

To study the nisin response of the transformants harboring pNZ8008 in combination with either pNZ9530 or pNZ9531 in more detail, the *nisA* promoter activity was determined as a function of the concentration of externally added nisin by quantitative β-glucuronidase measurements (3). When MG1363 harboring pNZ8008 and pNZ9530 was not induced with nisin, the β-glucuronidase activity remained below the detection limits (<0.0001 U of activity per optical density unit [AU]), but after induction with nisin at increasing concentrations, these cells showed a typical linear dose-response relationship between nisin concentration and β-glucuronidase activity (Fig. 1). In contrast, uninduced MG1363 cells harboring pNZ8008 and pNZ9531 showed a basal β-glucuronidase activity level (not shown) that had remained unobserved in the histochemical β-glucuronidase plate assay, which can probably be explained by the relatively poor sensitivity of the latter enzyme assay. Furthermore, when these cells were induced with nisin at increasing concentrations, it appeared that a nisin-inducible increase of β-glucuronidase activity could be obtained only at relatively high nisin concentrations. These studies showed that the absolute activity level at a certain nisin concentration was only slightly higher than the level that was obtained in the double transformant harboring pNZ9530 and pNZ8008. MG1363 cells harboring pNZ8008 and either pNZ9520 or pNZ9521 showed very high basal levels of β-glucuronidase activity (0.07 to 0.11 AU) and could hardly be induced with nisin (data not shown).

The dose-response relationships obtained with pNZ8008 and pNZ9530 or pNZ9531 containing double transformants were compared with those obtained with previously developed

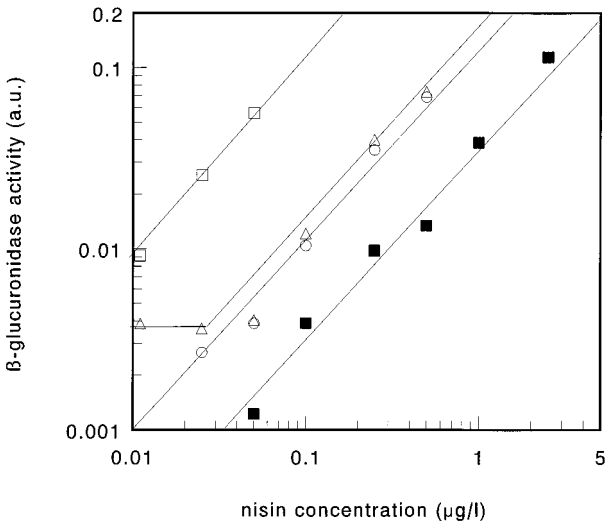

FIG. 1. Nisin-dependent *gusA* expression in *Lactococcus lactis* cells harboring pNZ8008. The different strains tested are NZ9800 (closed squares), NZ3900 (open squares), and the double transformants of MG1363 harboring, besides pNZ8008, the plasmid pNZ9530 (open circles) or pNZ9531 (open triangles).

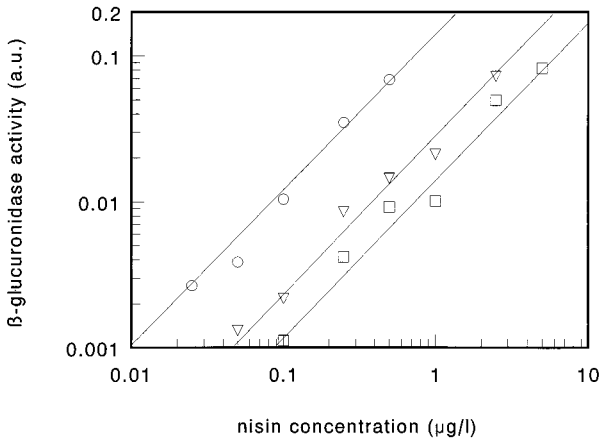

FIG. 2. Dose-response relationships of *gusA* expression in pNZ8008 and pNZ9530 harboring double transformants of *Lactococcus lactis* MG1363 (circles), *Leuconostoc lactis* NZ6091 (triangles), and *Lactobacillus helveticus* CNRZ32 (squares) with regard to the concentration of extracellularly added nisin.

nisin-inducible systems in which the *nisRK* genes were expressed from the chromosome. Therefore, β-glucuronidase activities were determined in pNZ8008-carrying derivatives of *Lactococcus lactis* strains NZ9800 (Δ*nisA* [10, 11]) and NZ3900 (carrying a single chromosomal copy of the *nisRK* genes [4]) after induction with various concentrations of nisin (Fig. 1). The dose-response relationships obtained for these strains corresponded very well with the previously described measurements (3, 4), including undetectable β-glucuronidase activity levels under uninduced conditions (not shown for NZ3900) and the typical linear dose-response characteristics upon induction with nisin. Remarkably, these typical dose-response characteristics are completely shared by the *Lactococcus lactis* MG1363 double-transformant derivatives carrying the two plasmids pNZ8008 and pNZ9530. A disturbance of this dose-response relationship is observed in the double transformants carrying pNZ9531, pNZ9520, or pNZ9521 in combination with pNZ8008, which is characterized by a basal level of *nisA* promoter activity in the absence of the inducer nisin that appears to reflect the expected *nisRK* expression level. Furthermore, in the case of the pNZ9520 or pNZ9521 double transformants a reduced or abolished inducibility of the *nisA* promoter activity by nisin is observed (data not shown). In previous experiments it has been shown that high expression levels of the *nisR* gene lead to constitutive activity of the *nisA* promoter (17), which might result from autophosphorylation activity of NisR or aspecific cross talk, leading to phosphorylation of NisR. These results clearly indicate that the level of *nisRK* expression generated by pNZ9530 and pNZ9531 allows strictly controlled, nisin-inducible overexpression of a gene cloned under control of the *nisA* promoter in *Lactococcus lactis* and emphasize the importance of the expression level of the two components involved in the nisin-mediated signal transduction, NisR and NisK.

Functional analysis of the two-plasmid system in heterologous LAB. The plasmids used in the dual-plasmid system described above are based on broad-host-range cloning vectors. This allowed us to study the functionality of the nisin-inducible system in other LAB. On the basis of the results obtained with *Lactococcus lactis* MG1363 as a host, pNZ9530 was chosen for transfer of *nisRK* to other species of LAB. The plasmids pNZ9530 and pNZ8008 were transformed (by procedures described in references 13 and 1, respectively), to *Leuconostoc lactis* NZ6091 (13) and *Lactobacillus helveticus* CNRZ32 (9), and both single and double transformants were selected. The observed transformation frequencies for both strains were between 10^5 and 10^6 transformants per μg of pNZ8008 DNA but were typically around 100-fold lower for pNZ9530 (or other pIL252 or pIL253 derivatives). Remarkably, all single-transformant derivatives grew as quickly as their plasmid-free parental strain, but the double-transformant derivatives grew only approximately half as fast. This effect on the physiology was not observed in *Lactococcus lactis* and could be caused by plasmid load and/or possible host-dependent partial incompatibility of the two plasmids.

The β-glucuronidase activity and its response to nisin induction was analyzed for both *Leuconostoc lactis* and *Lactobacillus helveticus* transformants and compared to the data obtained with *Lactococcus lactis* as a host. In qualitative X-Gluc–MRS (Merck) plate assays, none of the single transformants gave blue colonies. However, the double transformants of both *Leuconostoc lactis* and *Lactobacillus helveticus* gave rise to blue colonies when the plates contained nisin. This indicated that the nisin-inducible gene expression system can be functionally implemented in these bacteria by transformation with these plasmids.

To analyze the induction characteristics of the nisin-inducible expression system in the pNZ8008- and pNZ9530-harboring derivatives of *Leuconostoc lactis* and *Lactobacillus helveticus*, β-glucuronidase activities were quantitatively determined (3) after induction with various nisin concentrations (Fig. 2). The dose-response relationships obtained for both bacteria showed essentially the same characteristics as those obtained for *Lactococcus lactis*, i.e., no detectable β-glucuronidase activity level when not induced with nisin and a linear dose-response curve for β-glucuronidase activity that is directly dependent on the concentration of externally added nisin (Fig. 2). These findings indicate that no aspecific cross talk between NisR and endogenous sensor proteins or other kinases occurs. The main difference observed is that the absolute levels of β-glucuronidase activity per unit of optical density at 600 nm (AU) at a given nisin concentration were lower in both *Leu-*

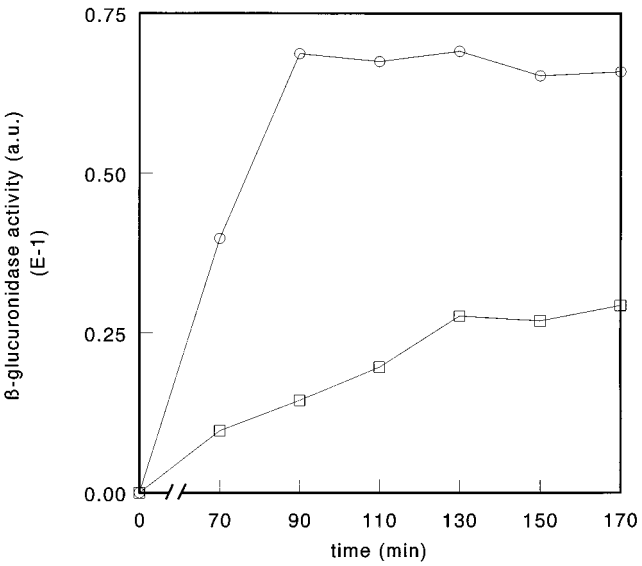

FIG. 3. β-Glucuronidase activity measured in cell extracts of pNZ8008 and pNZ9530 double-transformant derivatives of *Lactococcus lactis* MG1363 (circles) and *Leuconostoc lactis* CNRZ32 (squares) after various periods of induction with nisin (0.5 μg/liter).

conostoc (approximately 5-fold) and *Lactobacillus helveticus* (approximately 10-fold) than in *Lactococcus lactis*. To investigate whether this was caused by slower induction kinetics as a result of the slower growth of the double-transformant derivatives of these bacteria, growth was continued for periods longer than the standard 90 min after nisin induction, shown to give maximal response in *Lactococcus lactis* (3), and, subsequently, cells were assayed for β-glucuronidase activity (Fig. 3). Although these experiments indeed showed that the maximal level of β-glucuronidase activity was reached after an induction period of approximately 130 min (Fig. 3), absolute activity levels were never as high as those obtained for *Lactococcus lactis* (Fig. 3). A possible explanation for this observation is that, although the system is induced, the *nisA* promoter is not recognized by the transcription initiation machinery of these heterologous hosts as efficiently as it is in *Lactococcus lactis*. Alternatively, it could be that the *nisA* promoter-derived transcript is recognized with reduced efficiency by the translation machinery of these hosts compared to that of *Lactococcus lactis*. Nevertheless, our results clearly indicate that with the dual-plasmid system nisin-controlled gene expression can be transferred to other LAB. The *nisRK* plasmids described in this work allow variation of the *nisRK* expression level in order to reach the level required for functional use of the induction system. This level was obtained with the same construct in the bacteria tested (pNZ9530) but might require one of the other constructs (Table 1) in other LAB or in other gram-positive bacteria.

The nisin-inducible gene expression system has proven to have very useful characteristics in *Lactococcus lactis*, in which it has been used to overproduce a number of homologous and heterologous proteins (12), and the results presented in this work prove the feasibility of constructing controlled gene expression systems that are functionally transferable to phylogenetically unrelated LAB.

This work was supported by the European Community Biotechnology program (contracts BIOT-CT94-3055 and BIOT-CT96-0498).

We are grateful to Roland Siezen, Jan Wouters, Jeroen Hugenholtz, and Evert Luesink for critically reading the manuscript.

REFERENCES

1. **Bhowmik, T., and J. L. Steele.** 1993. Development of an electroporation procedure for gene disruption in *Lactobacillus helveticus* CNRZ 32. J. Gen. Microbiol. **139:**1433–1439.
2. **Casabadan, M. J., and S. N. Cohen.** 1980. Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. J. Mol. Biol. **138:**179–207.
3. **de Ruyter, P. G. G. A., O. P. Kuipers, M. M. Beerthuyzen, I. van Alen-Boerrigter, and W. M. de Vos.** 1996. Functional analysis of promoters in the nisin gene cluster of *Lactococcus lactis*. J. Bacteriol. **178:**3434–3439.
4. **de Ruyter, P. G. G. A., O. P. Kuipers, and W. M. de Vos.** 1996. Controlled gene expression systems for *Lactococcus lactis* with the food-grade inducer nisin. Appl. Environ. Microbiol. **62:**3662–3667.
5. **de Ruyter, P. G. G. A., O. P. Kuipers, and W. M. de Vos.** Food-grade controlled lysis of *Lactococcus lactis* for accelerated cheese ripening. Submitted for publication.
6. **de Vos, W. M., O. P. Kuipers, J. R. van der Meer, and R. J. Siezen.** 1995. Maturation pathway of nisin and other lantibiotics: post-translationally modified antimicrobial peptides exported by gram-positive bacteria. Mol. Microbiol. **17:**427–437.
7. **Engelke, G., Z. Gutowski-Eckel, P. Kiesau, K. Siegers, M. Hammelman, and K.-D. Entian.** 1994. Regulation of nisin biosynthesis and immunity in *Lactococcus lactis* 6F3. Appl. Environ. Microbiol. **60:**814–825.
8. **Gasson, M. J.** 1983. Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. J. Bacteriol. **154:**1–9.
9. **Khalid, M. N., and E. H. Marth.** 1990. Purification and partial characterization of a prolyl-dipeptidyl aminopeptidase from *Lactobacillus helveticus* CNRZ 32. Appl. Environ. Microbiol. **56:**381–388.
10. **Kuipers, O. P., M. M. Beerthuyzen, R. J. Siezen, and W. M. de Vos.** 1993. Characterization of the nisin gene cluster *nisABTCIPR* of *Lactococcus lactis*, requirement of expression of the *nisA* and *nisI* genes for development of immunity. Eur. J. Biochem. **216:**281–291.
11. **Kuipers, O. P., M. M. Beerthuyzen, P. G. G. A. de Ruyter, E. J. Luesink, and W. M. de Vos.** 1995. Autoregulation of nisin biosynthesis in *Lactococcus lactis* by signal transduction. J. Biol. Chem. **270:**27299–27304.
12. **Kuipers, O. P., P. G. G. A. de Ruyter, M. Kleerebezem, and W. M. de Vos.** 1997. Controlled overproduction of proteins by lactic acid bacteria. Trends Biotechnol. **15:**135–140.
13. **Platteeuw, C., G. Simons, and W. M. de Vos.** 1994. Use of the *Escherichia coli* β-glucuronidase (*gusA*) gene as a reporter gene for analyzing promoters in lactic acid bacteria. Appl. Environ. Microbiol. **60:**587–593.
14. **Platteeuw, C., I. van Alen-Boerrigter, S. van Schalkwijk, and W. M. de Vos.** 1996. Food-grade cloning and expression system for *Lactococcus lactis*. Appl. Environ. Microbiol. **62:**1008–1013.
15. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
16. **Simon, D., and A. Chopin.** 1988. Construction of a plasmid family and its use for molecular cloning in *Streptococcus lactis*. Biochimie **70:**559–566.
17. **van der Meer, J. R., J. Polman, M. M. Beerthuyzen, R. J. Siezen, O. P. Kuipers, and W. M. de Vos.** 1993. Characterization of the *Lactococcus lactis* nisin A operon genes *nisP*, encoding a subtilisin-like serine protease involved in precursor processing, and *nisR*, encoding a regulatory protein involved in nisin biosynthesis. J. Bacteriol. **175:**2578–2588.
18. **Vos, P., M. van Asseldonk, F. van Jeveren, R. Siezen, G. Simons, and W. M. de Vos.** 1989. A maturation protein is essential for production of active forms of *Lactococcus lactis* SK11 serine proteinase located in or secreted from the cell envelope. J. Bacteriol. **171:**2795–2802.
19. **Wells, J. M., P. W. Wilson, and R. W. F. Le Page.** 1993. Improved cloning vectors and transformation procedure for *Lactococcus lactis*. J. Appl. Bacteriol. **74:**629–636.