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. KUIPERS1

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 postranslationally 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-inducing LAB (3, 11). The amount of nisin required 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 HindIII-PstI 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 to *Escherichia coli* MC1061 [2], selected on an L broth-based medium [15] containing ampicillin [100 μg/ml]). For cloning purposes, the 2.3-kb ScaI-PstI fragment from pNZ9500 containing the nisR and nisK genes without the nisR promoter (but with the nisR ribosome binding site [3]) was subcloned into EcoRV-PstI-digested pBluescriptII (Stratagene, La Jolla, Calif.). From the resulting plasmid (pNZ9540) the nisRK fragment was reisolated as an XhoI-XbaI fragment and cloned into SalI-XbaI-di-
mants that showed resistance to chloramphenicol (5 μg/ml) were readily obtained. 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-galactosidase measurements (3). When MG1363 harboring pNZ8008 and pNZ9530 was not induced with nisin, the β-galactosidase 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-galactosidase activity (Fig. 1). In contrast, uninduced MG1363 cells harboring pNZ8008 and pNZ9531 showed a basal β-galactosidase activity level (not shown) that had remained unobserved in the histochemical β-galactosidase 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-galactosidase 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-galactosidase 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

<table>
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<tr>
<th>Plasmid</th>
<th>Relevant characteristic(s)</th>
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<tr>
<td>pUC19ery</td>
<td>Amp’, Ery’</td>
<td>10</td>
</tr>
<tr>
<td>pBluescript II</td>
<td>Amp’</td>
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</tr>
<tr>
<td>pIL252</td>
<td>Amp’, Ery’, low-copy-number, broad-host-range cloning vector</td>
<td>16</td>
</tr>
<tr>
<td>pIL253</td>
<td>Ery’, high-copy-number, broad-host-range cloning vector</td>
<td>16</td>
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<tr>
<td>pNZ9500</td>
<td>Amp’, Ery’, pUC19ery derivative containing a 2.7-kb chromosomal DNA fragment from strain NZ9700 that contains 3’ ends of nisR and nisRK</td>
<td>This work</td>
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<tr>
<td>pNZ9540</td>
<td>Amp’, nisR promoter followed by nisRK cloned into pBluescript II</td>
<td>This work</td>
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<td>pNZ9520</td>
<td>Ery’, nisR RK cloned in pIL253, expression of nisRK driven by rep read-through</td>
<td>This work</td>
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<tr>
<td>pNZ9521</td>
<td>Ery’, nisR RK cloned in pIL253, expression of nisRK driven by nisR promoter and rep read-through</td>
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<tr>
<td>pNZ9530</td>
<td>Ery’, nisR RK cloned in pIL253, expression of nisRK driven by rep read-through</td>
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<tr>
<td>pNZ9531</td>
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<td>This work</td>
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<tr>
<td>pNZ8008</td>
<td>Cam’, promoterless gusA reporter gene, cloned behind the nisA promoter</td>
<td>11</td>
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**FIG. 1.** Nisin-dependent gusA expression in Lactococcus lactis cells harboring pNZ8008. The different strains tested are NZ9800 (closed squares), NZ9390 (open squares), and the double transformants of MG1363 harboring, besides pNZ8008, the plasmid pNZ9530 (open circles) or pNZ9531 (open triangles).
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 (nisA [10, 11]) and NZ93900 (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 NZ93900) 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 specific 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-
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.

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