Development, molecular characterisation and exploitation of the nisin controlled expression system in *Lactococcus lactis*

Pascalle G.G.A. de Ruyter

STELLINGEN

1. Er is meer nodig voor een in voedingsmiddelen aanvaardbaar induceerbaar expressie-systeem dan een lage achtergrondactiviteit en inductie door toevoegen van keukenzout.
(Sanders, J. W., Environmental stress response in *lactococcus lactis*: identification of genes and use of expression signals. PhD Thesis, Groningen)

2. De conclusie dat het vrijkomen van intracellulaire enzymen in de kaasmatrix niet essentieel is voor hun rol in de secundaire proteolyse moet nog bewezen worden.
(Peršic, V. (Auto-)lysis of lactic acid bacteria and proteolysis. Lezing themadag: Baas in eigen kaas, 3 april 1998, Zwolle)

3. De resultaten met betrekking tot de rol van het C-terminale deel van nisine als signaal molecuul zijn nog niet helemaal duidelijk.
(Van Kraaij *et al.* Influence of charge differences in the C-terminal part of nisin on antimicrobial activity and signaling capacity. Eur. J. Biochem. 247:114-120. Dit proefschrift hoofdstuk 2).

4. Versnelde kaasrijping leidt niet per definitie tot oude kaas. (Dit proefschrift, hoofdstuk 6)

5. Als er iets kan bijdragen aan de acceptatie van gebruik van recombinant DNA/ GGO's in voedingsmiddelen dan moeten dat duidelijk aangetoonde gezondheidsclaims zijn.
(Verrips, C. T. *et al.* Barriers to application of genetically modified lactic acid bacteria. Antonie van Leeuwenhoek 70:299-316.)

6. De recombinant DNA discussie gaat tegenwoordig niet zozeer meer over veiligheid maar meer over de vraag of alles, maatschappelijk en ethisch, wat kan, ook mag.

7. De vraag wat de waarde van een individu is als er via de reageerbuis identieke copieën van worden gemaakt, stellen wij ons niet bij de geboorte van een eeneiige tweeling.

8. De mensen leven langer dan ooit, een fenomeen dat ongetwijfeld nodig is geworden door de hypotheken met een dertigjarige looptijd

9. Door het heffen van belasting op de rente van spaartegoeden zal het spreekwoord "wie wat bewaart, heeft wat" niet meer volledig van toepassing zijn.

10. Inkrimping van de varkensstapel met 25% zou de varkenspest in de toekomst tegengaan maar het virus trekt zich waarschijnlijk niets aan van percentages en aantallen varkens.

Stellingen behorende bij het proefschrift
"Development, molecular characterisation and exploitation
of the nisin controlled expression system in *Lactococcus lactis*".

Pascalle de Ruyter, Wageningen, 5 oktober 1998

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PROEFSCHRIFT

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van de Landbouwuniversiteit Wageningen,
dr. C. M. Karssen,
in het openbaar te verdedigen
op maandag 5 oktober 1998
des namiddags te 13.30 uur in de Aula.

Chapter 3 reprinted from: Functional analysis of promoters in the nisin gene cluster of *Lactococcus lactis*. P. G. G. A. de Ruyter, O. P. Kuipers, M. M. Beerthuyzen, I. van Alen-Boerrigter, and W. M. de Vos. J. Bacteriol. 178 (1996) 3434-3439, with kind permission from the American Society for Microbiology.

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Niet als een doode bol vol doode gebergten,
dalen, rivieren en zeeën,
maar als de veelbewogen schouwplaats van menschelijk werken,
leere het kind de aarde kennen.
(woordenboek van opvoeding en onderwijs, 1882)

Aan mijn ouders

Voorwoord

Als allerlaatste dan eindelijk het voorwoord. Heel cliché, maar wat zijn die jaren ontzettend snel verlopen en wat is er veel gebeurd en veranderd in die tijd. Als junior onderzoeker bij het NIZO beginnen leek een zware taak maar door het altijd stimulerende enthousiasme van Willem en Oscar (wat ook nog interessant is..) kon het niet fout gaan. Willem, door jouw altijd kritische houding heb ik geleerd om steeds een stapje dieper te gaan en verder te kijken. Bedankt voor je tijd en begeleiding ook toen ik niet meer zo vaak in de buurt was. Oscar, bedankt dat ik altijd bij je binnen kon vallen en dat je waar ik ook mee bezig was altijd bezorgd was over de vraag: zit fe erbij? Natuurlijk was de altijd gezellige collegiale werksfeer op het NIZO absoluut onvergetelijk. Ik wil iedereen hiervoor graag heel hartelijk bedanken. Als eerste natuurlijk Richard mijn favoriete labgenootje, zorg je nog goed voor de visjes, en natuurlijk Evert, bedankt voor de gezellige etentjes en de leuke uitjes naar Wageningen op woensdag ochtend (toch maar niet op de fiets). Cindy, we begonnen met een kamer te delen in Papendal en het eindigde op een kamer in Groningen. Marke, Ingrid en Ger, bedankt voor de dagelijkse vragen, spulletjes, stammetjes enz. Liesbeth, sorrie dat ik je de eerste weken van je stage met de saaiste man had opgezadeld, maar ik heb het weer goedgemaakt door je op te hemelen in Groningen, toch? Samen ook met Christel en Iris was het een gezellig jaar. Iris wil ik nog speciaal bedanken voor de laatste mutantjes en sequenties. Michiel, bedankt voor alle wijze raad en zinvolle discussies en dat je nog maar vaak als postdoc aan de labtafel mag zitten. Joey, Marja, Jeroen, dat squashen was toch wel heel lekker. Saskia, niet alleen de samenwerking met gus, pepN en zuiveringen maar ook alles buiten het werk om (squashen , tennissen, skien, winkelen) heeft bijgedragen (en zal hopelijk nog niet voorbij zijn) aan een onvergetelijke tijd. Alhoewel ik aan bepaalde NIZO sporten nog wel wat trauma's heb overgehouden. Samen met alle andere collega's op het NIZO, studenten, aio's en postdocs (uit binnen en buitenland) wil ik jullie bedanken voor de hulp, op welke manier dan ook, bij het tot stand komen van dit proefschrift. Tenslotte, wil ik Bakker Lekkerkerk bedanken voor het vertrouwen en de tijd die ik kreeg om dit boekje nog goed af te ronden. Mijn ouders bedanken die mij altijd gestimuleerd hebben mijn eigen weg te kiezen. Leon, bedankt voor de laatste loodjes die je samen met mij gewogen hebt.

Pascalle

Contents

CHAPTER 1

General Introduction

General introduction

Lactic acid bacteria (LAB), including members of the genera *Lactobacillus, Lactococcus, Leuconostoc, Pediococcus* and *Streptococcus*, play an important role in many food and feed fermentations. The ability to preserve various foods by means of fermentation is an ancient and well-appreciated form of biotechnology. Bacteria preserve foods not only as a result of competitive growth (46) but also by their metabolism products and bacteriocin production (96). Moreover, some cultured milk products are supposed to provide certain health and additional nutritional benefits (87). These features explain the major economic importance of the LAB for the food industry, and for the dairy industry in particular. Therefore, considerable efforts are made to increase our knowledge of LAB. Among the LAB, studies on lactococci have advanced most. The genus *Lactococcus* includes five species of which *Lactococcus lactis* represents the best characterized one. In recent years, major advances have been made in unraveling the genetic and molecular basis of important traits of *L. lactis* (19). In addition, *L. lactis* strains can easily been grown and handled in the laboratory serving as models for anaerobic, gram-positive bacteria with a relatively simple metabolism (21). Moreover, there is the perspective that many of the genetic tools developed for *L. lactis* can be applied in other LAB, in many cases with little or no modifications (54, 21a).

L. lactis has a number of desirable properties allowing its use for the production of homologous and heterologous proteins, especially those that are applied in the food industry. Lactococci can be grown at industrial scale on cheap, whey-based media using existing fermentation technology. Moreover, they have the capacity to secrete proteins, allowing surface expression or extracellular production of homologous or heterologous proteins (99,76). Another feature is the possibility of using *L. lactis* as a production host for enzymes and primary and secondary metabolites. This is particularly relevant for the production of compounds that are unique for these bacteria, such as the antimicrobial peptide nisin (28), the flavor compound diacetyl (79), specific exopolysaccharide production (100), or enzymes involved in the generation of cheese flavor (27).

Acceleration of cheese ripening

Cheese is the generic name for a diverse group of fermented milk-based foods produced in at least 500 varieties throughout the world. Cheeses are the most diverse, scientifically interesting, and challenging group of dairy products because of their microbiological and biochemical dynamics and, consequently, inherently unstability. Considering that a basically homogenous raw material is subjected to a more or less common manufacturing protocol, it is fascinating that such a diverse range of products can be produced. The production of rennet-coagulated cheese can be divided into two phases, i. e. manufacture and ripening (Fig.1).

Fig.1: The two phases during cheese production

The manufacturing phase comprises operations, acidification, coagulation, dehydration, shaping and salting, performed during the first 24 hours. The nature and quality of the finished cheese are determined largely by the manufacturing steps. However, it is during ripening that the characteristic flavor and texture of the individual cheese varieties develop (34). Although cheese ripening is a very complex biochemical process, it primarily involves glycolysis, lipolysis and proteolysis, together with numerous secondary changes that are responsible for the characteristic flavor and texture of each cheese variety. These changes are catalyzed by residual rennet, starter bacteria and their enzymes, secondary cultures and their enzymes, non-starter adventitious microflora and their enzymes and indigenous milk enzymes.

Proteolysis appears to be rate limiting in the maturation of many cheese varieties and hence has been the focus of most research on the acceleration of ripening. The proteolytic enzymes involved in the ripening of cheese originate from several sources: milk, coagulant, starter bacteria, non-starter lactic acid bacteria or secondary/adjunct microorganisms. Several methods are used to accelerate ripening (Table 1). These methods aim to accelerate cheese ripening either by increasing the level(s) of putative key enzymes or by providing more favorable conditions for the activity of endogenous enzymes in cheese. One method is based on temperature control because traditionally cheese was ripened in caves or cellars. Since the introduction of mechanical refrigeration for cheese ripening rooms, the use of controlled ripening temperatures has become normal practice. The essential conclusions of these studies were that it is possible to reduce the maturation time by 50 % by ripening at 13-15 °C (63, 33, 32). Since the coagulant is responsible for primary proteolysis in most cheese varieties, it might be expected that ripening may be accelerated by increasing the level or activity of rennet in the cheese curd. However, several studies (95, 11, 42, 48) have shown that increasing the level of rennet in the cheese curd does not accelerate ripening and in fact probably causes bitterness. Addition of exogenous plasmin to cheese milk accelerated the ripening of the cheese without off-flavor development (29, 30, 51). The possibility of accelerated ripening through the use of exogenous (non-rennet) proteinases has attracted considerable attention. The principal problems associated with this approach are ensuring the uniform distribution of the enzyme in the curd and the prohibition of exogenous enzymes in many countries (reviews see references 61, 62 and 33, 34).

Although most lactic acid bacteria are weakly proteolytic, they posses a very complex proteolytic system which is necessary for their fast growth in milk and contains very little free amino acids and small peptides. Since the proteolytic system of the starter bacteria is responsible for the formation of small peptides and amino acids and therefore for flavor development in cheese, it seems obvious to exploit these enzymes for accelerated ripening. Selection of starters can be based on enzyme profiles (10, 12) or based on starter cell lysis, which leads to release of enzymes. Generally *L. lactis* ssp. *cremoris* cultures lyse faster than *L. lactis* ssp. *lactis* strains, although there is considerable interstrain variation within each

subspecies (9, 107). Application of naturally occurring fast-lysing strains is difficult because many of these fast-lysing strains have undesirable cheesemaking properties, e.g., slow acid production or phage sensitivity. Thermoinducible prophage-carrying strains can be useful for accelerated cheese ripening (method 4) through the early release of intracellular enzymes; however extensive loss of these enzymes in the whey has been shown to occur (31). It was also shown that heat-induced lysis of the prophage-carrying strain SK110 is strongly dependent on the growth rate and the pH of the growth medium (67). When strain SK110 was used as a starter culture for the manufacturing of Gouda cheese, a significant increase in the level of free amino acids was measured after six weeks of ripening when a temperature shock was used during the cheese making process. This indicates that lysis of starter bacteria plays an important role in cheese ripening and that temperature-induced lysis of lysogenic strains is a valuable tool to affect the ripening of cheese (67). Phage induced lysis may have potential for accelerated ripening but the technique may be unacceptable to cheese manufacturers due to the possibility of unpredictable acid production (14).

A study on cheddar cheese, manufactured with the bacteriocin-producing strain, *L. lactis* DPC3286 (69), as a starter adjunct, revealed increased levels of starter cell lysis, higher concentrations of free amino acids and a reduction in bitterness (34). This novel method for increasing starter cell lysis in cheddar cheese has many advantages: it requires no special legal approval and the bacteriocin-producing cells are evenly distributed throughout the cheese curd. However, extension of the time for cheese production may be a cause for concern. The considerable knowledge now available on the genetics of cell wall-associated proteinase and many of the intracellular peptidases makes it possible to specifically modify the proteolytic system of starter *Lactococcus*. The availability of *Lactococcus* mutants lacking up to 5 peptidases (68) should facilitate identification of key peptidases and hence the engineering of mutant strains that overproduce these peptidases.

Non-starter lactic acid bacteria which may originate from the milk or the cheese making environment, can also contribute to the ripening of cheese. Traditionally, secondary or adjunct cultures are not used in cheddar-type cheeses but the development and application of such cultures are among the promising approaches towards accelerating ripening. Adjunct cultures of mesophilic lactobacilli do influence proteolysis in cheddar cheese during ripening, mainly at the level of free amino acids formation (34).

The greatest acceleration of ripening has been achieved using a slurry system (57). Ripening of slurries made from chemically acidified curd showed the importance of rennet, lactic acid starter, glutathione and pH. Addition of cheese slurries to cheese milk or cheese curd was reported to accelerate the ripening of cheese (1). In principal cheese slurries are used as a model to study the biochemistry of ripening. The short ripening time, the low cost and the possibility of including numerous parameters in a single study is not possible with cheese making, even on pilot scale.

Since free amino acids are released rather slowly during cheese ripening, a study was undertaken to assess the possibility of accelerating flavor development in cheddar cheese by adding free amino acids to the curd at salting (104). The experiments showed that low concentrations of amino acids activated proteolysis but a very high concentration appeared to be inhibitory.

Table 1: Overview of methods for accelerating cheese ripening

Method	Advantage	Limitations
1. Storage at elevated temperature	Effective, simple, no legal barriers	Non-specific, risk of microbial spoilage, risk of rheological changes and enzyme inactivation
2. Addition of exogeneous enzymes	Natural additive	Not effective (rennet) or expensive (plasmin);Limited commercial use (peptidases)
3. Application of selected starters	Enzyme profile or selection for rapid lysis	None
4. Thermoinducible lysis	Early release of enzymes release of amino acids	Limited strains carrying thermoinducible prophage
5. Bacteriophage assisted lysis	Early release of enzymes release of amino acids	Unpredictable acid production
6. Bacteriocin-induced lysis	More free amino acids Reduction in bitterness	Extension of cheese manufacturing time
7. Application genetically modified starters	Desired enzyme profile	Possible legal barriers
8. Addition of adjunct cultures	Natural microflora	Careful selection
9. Addition of a cheese slurries	Very rapid flavor development	Risk of microbial spoilage Laboratory tool
10. Addition of free amino acids	Diverse selection of flavor	Expensive

In view of the likely significance of autolysis in intracellular enzyme release, flavor development and cheese ripening, the lytic systems of LAB and their bacteriophages are important and several of their salient features are discussed below.

Lytic systems in LAB and their bacteriophages

Biotechnological approaches to accelerate flavor development have focused on supplementation with enzymes or, more recently, the engineering of starter cultures to increase the expression of genes for flavor-generating enzymes such as peptidases. The release of intracellular enzymes from intact starter cells is also a limiting factor in flavor development. An additional approach to flavor acceleration is therefore to effect the lysis of the starter cells at the end of the milk fermentation. Bacteriophages that infect starter strains could achieve this, but such an approach would also contaminate the manufacturing plant with viruses. Modification of the lytic genes offers the prospect of developing a non-infective approach for the exploitation of viruses to effect starter cell lysis without contamination of the dairy environment.

Bacteriophages, lysins and holins

Bacteriophages which can infect and destroy LAB pose a particularly serious threat to dairy fermentations that can result in great economic losses. For temperate as well as for virulent bacteriophages, the release of the native bacteriophage particles implies a hydrolysis step of the bacterial cell wall by a lytic enzyme known as the bacteriophage lysin. This lysin is probably also implicated in the injection of the phage DNA into the cell (108). This key enzyme of the bacteriophage propagation (85) has been extensively studied in *E. coli* (108) and several gram-positive bacteria such as *B. subtilis* (106, 38) and *S. pneumoniae* (37, 83). At least four different kinds of enzymes have been identified that function as endolysin for different bacteriophages: lysozymes, transglycosylases, endopeptidases, and amidases (108). The lysin of lactoccocal bacteriophages, studied until now, is an intracellular protein that lacks an amino-terminal secretory leader (39). This implies that there must be a system for its passage through the cell membrane, so that it can reach its target. A specific class of membrane proteins is required to allow the lysin to access the peptidoglycan, which is after all located outside the cytoplasmic membrane. Because the membrane lesions formed by these proteins are stable and non-specific, these proteins have been termed 'holins' ('hole'-formers). Analysis of their primary sequences suggests a simple membrane topology with two or more membrane spanning helical domains and a highly charged, hydrophilic C-terminus (5, 109) (Fig.2a).

The first analysis of the lytic capacity of lactococcal bacteriophages dates from 1965 when it was reported that the ml3 lysin was a muramidase, like hen egg white lysozyme (73). Almost 25 years later, the lysin from bacteriophage ml3 was partial purified, cloned and sequenced. After that, Shearman et al.(91) reported the expression of the cloned lysin gene of bacteriophage ml3 in dairy starter strains. Lactococcal strains, which were sensitive to lysin, were unaffected by expression of the lysin gene during exponential growth but lysed after reaching stationary phase. However, to fully exploit the lysin, it is necessary to control its production in lactococci. More recently, it was shown that two proteins were expressed from , what at first seemed, a single lysin gene but what later appeared to be two genes (92). Overexpression of the smaller lysin gene or both, overlapping, genes under the inducible T7 promoter in *E. coli* appeared to be lethal. As a consequence, the role of the two gene products in lactococci has to be confirmed. A structural lysin gene that can be controlled by a promoter is of considerable value both for overexpression studies and for exploitation of the lysin gene for lysis of *L. lactis*.

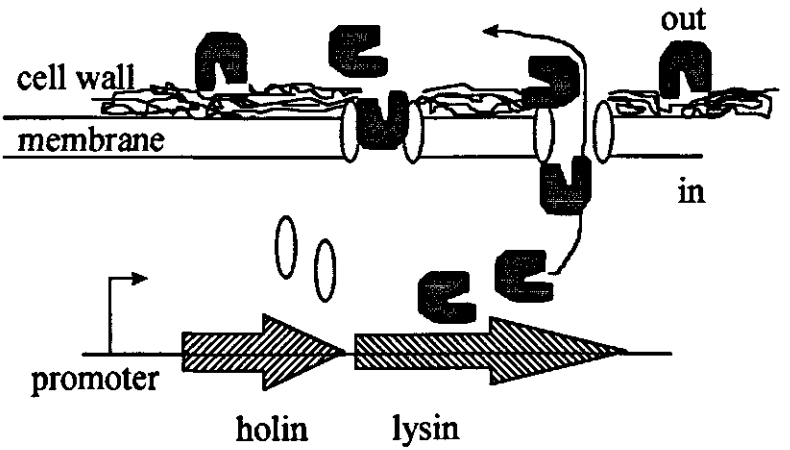

Fig. 2a: Schematic representation of cell wall disruption by the expression of a bacteriophage lysin gene, which relies on the expression of a membrane disruptive holin gene to access its peptidoglycan substrate.

Platteeuw and De Vos (77) reported the cloning and sequence analysis of a lysin gene from the virulent isometric bacteriophage US3. The lysin gene, *lytA*, encoded a 258 amino acid protein. The *lytA* gene was overexpressed in *E. coli* under control of a T7 promoter and SDS PAGE analysis of proteins revealed the *lytA* gene product to be a 29 kDa molecule. Homology searches with the encoded amino acid sequence of LytA revealed a match with the amino terminal region of *S. pneumoniae* autolysin and this led to the suggestion that LytA is an amidase. A second open reading frame was also present on the lysin-expressing clone. This gene was located upstream of *lytA* and encodes a 66 amino acid hydrophobic protein with two putative membrane spanning helices which probably acts as a holin. In several bacteriophage lytic systems a holin gene is linked to the lysin gene (Fig. 2b). In some cases, the lysin gene also expresses a second small protein from within the same coding sequence. This was first observed in bacteriophage ml3 (92). In the other prolate-headed bacteriophages c2 (47) and P001 (44) expression of a similar small protein has been confirmed. Whilst the putative ml3 holin is hydrophobic with a charged carboxy-terminus, it appears to lack the transmembrane domains that are a conserved feature of other holins. For bacteriophage c2, the open reading frame immediately upstream of the lysin gene has been suggested as the holin gene. The third potential holin gene location (93) was located at the end of the bacteriophage bIL67 genome (orf37) (Fig. 2b). Recently, the lysin gene of the small isometric temperate bacteriophage Tuc2009 was identified (3) together with a putative holin gene immediately upstream of the lysin gene. The stop codon of this gene overlaps with the start codon of the lysin gene. The holin gene, *lysB*, of bacteriophage ϕLC3, was located upstream of *lysA*, the lysin gene (4). The open reading frames for the lysin (*lytR*) and the upstream located holin gene (*lytP*) of the temperate lactococcal bacteriophage rlt were also identified (103) (Fig. 2b). Both these genes are required for lysis since expression of the *lytPR* cassette under control of a chloride-inducible promoter resulted in release of intracellular protein upon induction with NaCl (88).

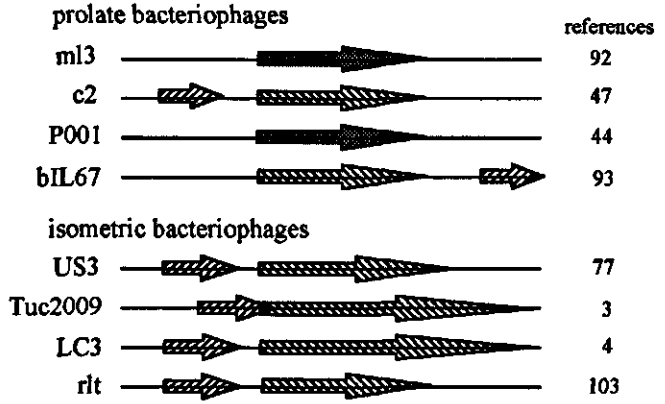

Fig. 2b: Arrangements of lysin (backwards hatched), putative holin genes (forward hatched) and overlapping holin/lysin genes (cross hatched) on the genomes of prolate and isometric lactococcal bacteriophages.

Autolysis

The normal growth and division of bacterial cells involve the activities of cell wall hydrolases that can break down the peptidoglycan. These enzymes probably play a role in cell separation, turn over of cell wall components, sporulation, the development of competence, flagella formation and the activity of some antibiotics (24). In a variety of bacteria, the autolytic machinery has been shown to involve several different hydrolytic activities (82). For *L. lactis* subsp. *cremoris*, autolytic activity has been found to be maximal at neutral pH during exponential growth. The nature of the autolytic enzyme activity was shown to be an *N*-acetylmuramidase. The molecular genetic characterization of the autolytic system of lactococci has recently been initiated with the cloning and DNA sequence analysis of the lactococcal gene for the major peptidoglycan hydrolase, *acmA* (7). An *acmA* deletion mutant was constructed, which was found to grow in long chains, indicating that AcmA is involved in cell separation. In contrast to bacteriophage lysins, AcmA is extracellular, most probably produced as a preprotein with a signal peptide of 57 amino acids which is removed upon secretion (7).

Exploitation of lytic systems

The perceived value of an enhanced autolytic activity is the early release of intracellular enzymes into the cheese curd, providing the potential for enhanced or accelerated flavor development. Several approaches (13) have been taken to isolate new strains that lyse upon reaching stationary phase (7). Autolysis of *L. lactis* by AcmA resulted in the release of intracellular proteins and deletion of the *acmA* gene resulted in complete loss of the autolytic behavior (7). Preliminary results have been reported that AcmA was successfully overproduced with concomitant intracellular enzyme release in *L. lactis* by use of the chloride inducible promoter (88). This chloride controlled expression system which is not completely foodgrade was also used for expression of the *lytPR* genes which showed a low level of control and lysis. As a consequence there is need for highly-controlled expression systems that are food-grade and can be applied in food products.

Gene expression in LAB

Food-grade system

The term food-grade is used to designate systems that are ultimately applicable in the food industry or result in products used in foods (19). The term is used here to qualify gene cloning and expression systems that do not rely on the presence of antibiotic-resistance markers. As a consequence there is a need for food-grade markers by which genetically modified LAB can be selected, the genetic modification can be retained stable, and the desired phenotype can be realized. In general, food-grade markers should be well-defined, acceptable in foods, and applicable in various vectors and hosts. In addition, the marker gene should be derived from the host that is aimed to be genetically modified. The first food-grade system that was described for lactococci, but also can be used in other LAB, was based on the complementation of one or more auxotrophic markers (19).

The detailed characterization of the *L. lactis lac* operon allowed for the identification of the *lacF* gene that was found to complement *L. lactis* strain YP2-5 containing one of the first described mutations in the chromosomal *lac* operon (74). Expression of the *lacF* gene by the vector-located promoter of pNZ305 resulted in complementation of the lactose deficient phenotype of strain YP2-5 and cells containing this plasmid could be selected easily on lactose indicator plates. In addition, the plasmid could be maintained stable by growing its host in media containing lactose. Since lactose is a relatively cheap sugar present in various industrial media based on whey, the use of lactose as a means to maintain selective pressure offers good opportunities for large scale application. Finally, the *lacF* gene has a very small size and could be cloned on a 0.4-kb DNA fragment allowing easy manipulation (17). Various expression vectors have been developed based on the *lacF* fragment (78, 65). The applicability of the food-grade *lacF* complementation system was tested in *L. lactis* (Fig. 2) by replacing the *ery*-β1 of pNZ305 by the lactococcal *pepN* gene encoding a debittering aminopeptidase (98, 19). This is the first example of a industrially useful and completely sequenced plasmid that can be stable maintained in *L. lactis* by food-grade selection. In addition, the resulting strain has been obtained by self-cloning, a special form of genetic modification that has been excluded from the European Directives on the contained use of genetically modified microorganisms (8).

Another food-grade marker system has been based on the ability of some *L. lactis* strains to grow in the presence of nisin. These studies showed (35) that the nisin resistant strains did not produce nisin and the nisin-resistant determinant *nsr* has no relation with the nisin immunity genes *nisI* or *nisFEG*. *L. lactis* cells expressing the *nsr* gene could be selected by specific plating methods and the potential of the *nsr* gene in proving a food-grade marker was illustrated by the cloning of a replication origin (36). Other complementation food-grade systems have been proposed. The lactococcal *thyA* gene for thymidilate synthase was described as a way to select and maintain foreign sequences in *L. lactis* and other bacteria (84). Other systems have been described based on nonsense suppressors and a suppressible purine auxothroph of MG1363 (22), or on the introduction of genes needed for the fermentation of sucrose, which is normally not fermented by *L. lactis*, and selective growth on sucrose containing medium (52). Other examples for food-grade markers could be envisaged in *L. lactis* and other lactic acid bacteria but have not been thoroughly tested.

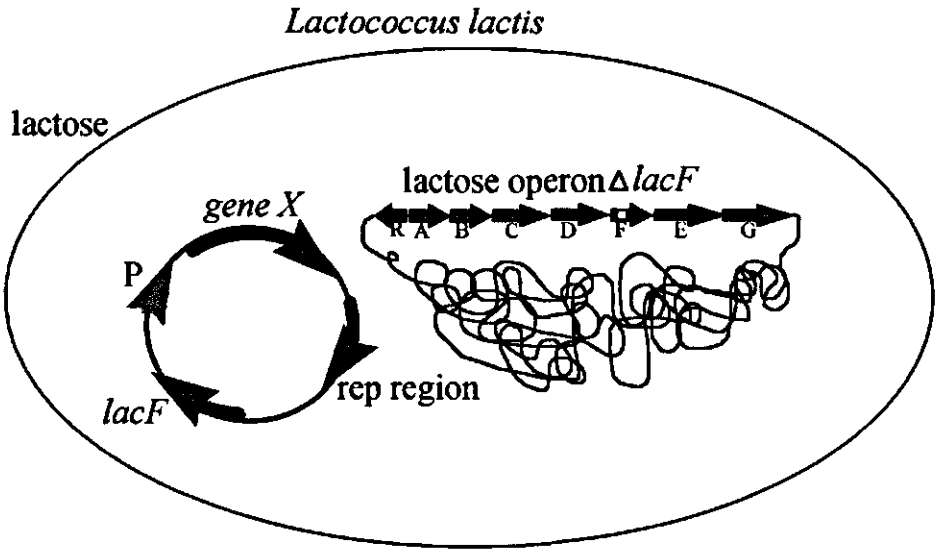

Fig.3: Schematic representation of a gene-expression vector containing the food-grade *lacF* marker which is used for controlled expression of any desirable geneX in a *lacF* deficient strain growing in medium containing lactose for stable maintaining the plasmid.

Control of gene expression

Apart from the traditional applications, lactococci are attractive organisms for the production of commercially valuable products. A lot of research is currently being devoted to the development of strains of LAB with improved characteristics, that may be used to make fermentations more efficient or to overproduce desirable products such as specific enzymes. Because of the current experience in genetics and fermentation technology of *L. lactis*, these bacteria are perfect candidates for production of homologous and heterologous proteins or metabolites. Furthermore, *L. lactis* and its long standing use in the manufacturing of food products could facilitate the acceptance of homologous, heterologous or engineered proteins, produced by these bacteria.

Depending on the nature and application of newly synthesized proteins, several expression strategies are conceivable. One option is to overproduce the new protein intracellularly, after which the cells have to be treated to harvest the protein. Another option is to make *L. lactis* secrete the new protein (99). For cheese making, a combined strategy may be preferred, in which the desired protein or enzyme is produced intracellularly and subsequently released by a system based on autolysis or induced lysis of the host. As it remains still unclear which features of the gene expression signals determine their efficiency in *L .lactis*, the most straightforward approach to heterologous gene expression is based on *L. lactis* -specific gene expression signals. In addition to the generally conserved expression signals (promoter and ribosome binding site), mRNA secondary structure and the nucleotide sequence of the coding region, it may also be advantageous to couple the translation of a heterologous gene to that

of an efficiently expressed homologous gene to accomplish efficient expression of the heterologous gene (19).

A number of genes and operons have been identified in LAB that are inducible and respond to environmental factors. Some of these genes/operons had been isolated and analyzed because of their importance in the fermentation industry and, consequently, their transcription was studied and found to be regulated (for a recent overview see reference 60). Several of these have been developed to use as a controlled expression system including the *lac* promoter (16, 17, 102), the ϕ31 promoter (72) , the chloride inducible *gad* promoter and the nisin inducible *nisA* promoter (discussed below).

Regulated gene expression in L. lactis: Autoregulation of nisin biosynthesis by signal transduction

For a long time it has been known that many LAB can produce antimicrobial compounds. Among these the antimicrobial ribosomally synthesized peptides, generally termed bacteriocins have received special attention, from both scientific sides and the food-industries. Bacteriocins form a heterogeneous group of bactericidal proteins produced by gram-negative and -positive bacteria (53). Some bacteriocins are small peptides, whereas others are complex proteins (for a review see reference 71). One subgroup of bacteriocins, the lantibiotics, have received increased interest in the last few years (56). Lantibiotics belong to the group of bacteriocins, although they have a broader activity spectrum than most other bacteriocins and in that respect are more similar to antibiotics. The most prominent lantibiotics are nisin (40), subtilin (41), Pep5 (86) and epidermin (2). The number of members belonging to this group is steadily growing. Lantibiotics are ribosomally synthesized as prepeptides and posttranslationally modified (20, 49). They contain unusual amino acids such as dehydroalanine, dehydrobutyrine, lanthionine, and 3-methyl-lanthionine. The formation of these unusual amino acids could be explained by posttranslational dehydration of peptide serine and threonine residues, with subsequent addition of neighboring cysteine sulfhydryl to the double bonds of the dehydro-aminoacids. The protein translated from the primary transcript of lantibiotic structural genes is a prepeptide which consists of an N-terminal leader peptide followed by a C-terminal propeptide from which the lantibiotic is matured. Considerable research interest has focused on nisin, as it is the only lantibiotic that has found practical application (15). It occurs in some dairy products and is occasionally used as an additive to dairy products to prevent spoilage by gram-positive bacteria (46). Nisin has two natural variants, nisin A and nisin Z, which differ in a single amino acid residue at position 27 (histidine in nisin A and asparagine in nisin Z (70, 18).

Several strains of *Lactococcus lactis* harbor large conjugative transposons encoding the biosynthetic enzymes of the lantibiotic nisin (Fig. 4a). The structural genes *nisA* (6, 23, 50) and *nisZ* (18, 70) have been cloned several times from different sources. The 11 nisin genes of the 70 kb conjugative transposon Tn*5276*, genetically linked to the genes for sucrose fermentation (80, 81) have been characterized and found to include a region containing the *nisABTCIPRKFEG* genes. Of these nisin genes, *nisA* encodes the nisin A precursor peptide of 57 amino acid residues; *nisB* and *nisC* (25) encode putative enzymes involved in the posttranslational modification reactions (based on homology to genes found exclusively in other lantibiotic gene clusters); *nisT* encodes a putative transport protein of the ABC translocator family that is probably involved in the extrusion of modified precursor nisin (26, 58, 20); *NisI* is the first gene described to be involved in nisin immunity and probably encodes

a lipoprotein (58, 26). The *nisF, nisE* and *nisG* gene cluster are also thought to play a role in self immunity to nisin as strains deficient in any of these functions display a greater sensitivity to nisin (94). *NisP* encodes an extracellular subtilisin-like protease involved in precursor processing (101) and is followed by two open reading frames named *nisR* and *nisK* (Fig. 4a)(94, 20).

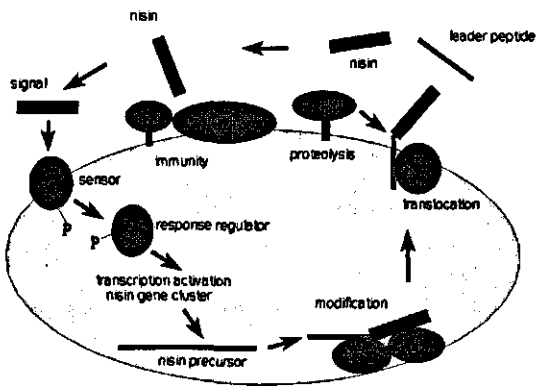

Fig.4a: Model for the autoregulation of the biosynthesis of nisin

The two encoded proteins NisR and NisK, have strong similarities to proteins of two component regulatory systems (97, 75, 105). For NisR the highest similarity was observed with members of the subfamily of the OmpR-like response regulators. The maximum degree of homology (41.3%) was found between NisR and SpaR, the response regulator of subtilin biosynthesis (55). The region with the highest similarity in all response regulators is the N-terminus, where phosphorylation of a conserved Asp residue by the corresponding histidine kinase takes place. NisK shows significant similarity to the histidine kinases of two-component regulatory systems, which act as membrane located sensors of environmental signals. Sequence similarity among histidine kinases is restricted mainly to the C-terminal part, which is located on the inside of the cytoplasmic membrane. This part contains a conserved histidine residue (His-238 in NisK) where autophosphorylation presumably takes place (89), a conserved asparagine residue (Asp-349 in NisK), and a glycine stretch at the C-terminal end (amino acid 380 to the extreme C-terminal end). NisK was similar to SpaK (21.4% identity), necessary for the regulation of the subtilin biosynthesis in *B. subtilis*. Upon disruption of either the *spaR* or *spaK* gene, subtilin production was abolished, indicating the involvement of these gene products in subtilin biosynthesis (55). The regulation was shown to be growth-phase dependent, but an inducing signal was not identified (55, 43). While the structure and function of two-component regulators have been studied in great detail, the nature of the inducing signal has remained unclear in many cases.

Recently, it was demonstrated that fully modified nisin can induce transcription of its

own structural gene as well as of the downstream genes by limited read-through, via signal transduction, by acting as the extracellular signal for the sensor histidine kinase NisK (Fig. 4b) (59). From the *nisA* gene a 260-bp transcript is produced which is initiated at a G residue 42 bp upstream of the AUG start codon (58). At a proper distance from this transcription start a possible promoter sequence was identified. Interestingly, *nisA* transcription was completely abolished when a 4-bp internal deletion was made in the chromosomally located *nisA* gene. When a plasmid carrying the intact *nisA* gene was introduced in the Δ*nisA* strain, a transcript of Δ*nisA* was observed. Apparently, nisin or one of its precursors is required for transcription of its own gene. This was further analyzed (59) by northern blotting using mRNA from the Δ*nisA* strain revealing that indeed Δ*nisA* transcription was restored upon addition of nisin to the culture medium. The amount of transcript was proportional to the amount of nisin added. Moreover, a hampered biosynthesis of nisin interferes with *nisA* transcription: an in-frame deletion of *nisB*, one of the putative modification genes, completely abolished *nisA* transcription, which could be restored by the extracellular addition of nisin. Deletion studies have shown that NisR is also essential for the production of nisin (101). One of the strains used in this study carried the *nisABTCIR* genes on a multicopy plasmid and was shown to secrete fully modified precursor nisin. This result indicates, among other things, that overexpression of *nisR* alone is sufficient for activation of transcription of *nisA*.

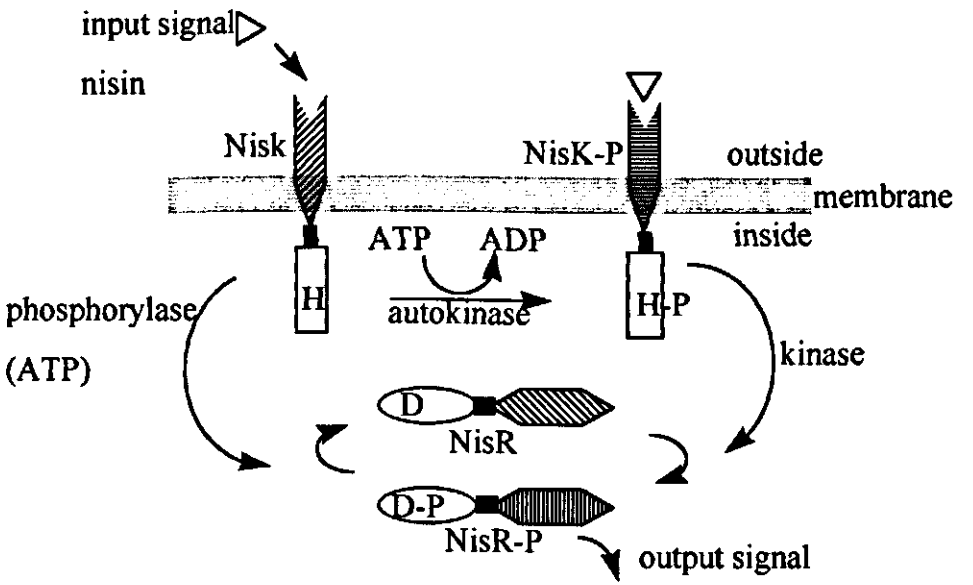

Fig. 4b: Model for nisin signal transduction involving the sensor histidine-kinase NisK and the response regulator NisR.

Outline of the thesis

The aim of this thesis is the development and molecular characterization of the nisin controlled expression (NICE) system and its exploitation for the controlled lysis of LAB. This starts in Chapter 2 which describes the fusion of the *nisA* promoter to the promoterless β-glucuronidase gene (*gusA*) of *E. coli* which resulted in expression of this heterologous gene in *L. lactis* NZ9800 (Δ*nisA*), only upon induction with nisin species. Subinhibitory amounts of nisin, nisin mutants or nisin analogues can acts as inducers. Moreover, there is no direct relationship between the antimicrobial activity and the signaling capacity of nisin. The promoters in the nisin gene cluster were characterized in Chapter 3 by primer extension and transcriptional fusions to the *gusA* gene. Three promoters, upstream of *nisA*, *nisR* and *nisF*, which all gave rise to *gusA* expression in the nisin producing strain NZ9700, were identified. The *nisR* promoter was shown to direct nisin-independent *gusA* expression in *L. lactis* MG1363 while the *nisF* promoter, like the *nisA* promoter is nisin-inducible. Chapter 4 describes the kinetics, control, and efficiency of nisin-induced expression directed by the *nisA* promoter region in *L. lactis* with transcriptional and translational fusions to the *gusA* reporter gene. This allowed for the construction of food-grade inducible and density-dependent gene expression cassettes. These were exploited for the controlled overexpression of a variety of useful genes in *L. lactis*. In Chapter 5 the partially purified regulator protein NisR was shown to bind to the *nisA* promoter region by gel mobility shift assays. Two pentanucleotide sequences required for effective transcription activation and probably for NisR binding were identified by mutational analysis of this promoter region. Controlled expression of the lytic genes *lytA* and *lytH*, which encode the lysin and the holin proteins of the lactococcal bacteriophage ϕUS3, respectively, was accomplished in Chapter 6 by application of the food-grade nisin-inducible expression system. Simultaneous production of the lysin and the holin was essential to obtain efficient lysis and concomitant release of intracellular enzymes. These features provide a flexible system suitable for a wide range of applications in starter cultures. Finally, in Chapter 7 an overview is presented with concluding remarks.

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CHAPTER 2

Determination of the induction capacity of nisin, nisin-mutants and nisin analogs

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SUMMARY

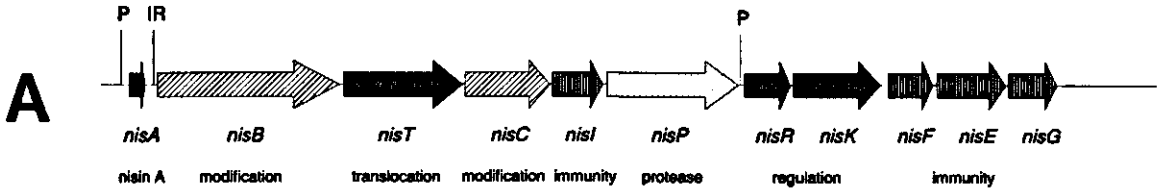
The posttranslationally modified, antimicrobial peptide nisin is secreted by strains of *Lactococcus lactis* that contain the chromosomally located nisin biosynthetic gene cluster, *nisABTCIPRKFEG*. Fusion of a *nisA* promoter fragment to the promoterless reporter gene *gusA*, resulted in expression of *gusA* in *Lactococcus lactis* NZ9800 (Δ*nisA*), only upon induction with nisin species. The expression level of *gusA* was directly related to the amount of inducer that was added extracellularly. Subinhibitory amounts of nisin, nisin mutants or nisin analogues were found to act as inducers, whereas other antimicrobial peptides or the unmodified precursor peptide are incapable of induction. There was no relationship between antimicrobial activity and signaling capacity of nisin mutants. The presence of modified residues in the mature nisin molecule is of crucial importance for induction capacity; in particular, residues 1-11 are minimally required for the putative interaction with the NisK sensor protein. Taken together, these data indicate that besides its function as an antimicrobial peptide, nisin also acts as a secreted signal molecule that induces the genes involved in its own biosynthesis. This cell-density dependent regulation of gene expression is an example of a quorum sensing mode in gram-positive bacteria.

INTRODUCTION

Nisin is an antimicrobial peptide (1-3), widely used in the food industry as a safe and natural preservative. The ribosomally synthesized precursor peptide undergoes extensive posttranslational modification, which includes dehydration of serine and threonine residues and the formation of thio-ether bridges named (ß-methyl)lanthionines, resulting in five ring structures named A, B, C, D and E (Fig. 1B). Peptides containing these characteristic modified residues are named lantibiotics (4). Eleven genes organized in a cluster have been implicated in the complex biosynthesis of nisin, *i.e. nisABTCIPRKFEG* (5-11). Both nisin A and nisin Z producing strains are common in nature and both structural genes, *nisA* and *nisZ*, have been cloned from various strains (5,6,12,13).

The proteins encoded by *nisR* (8) and *nisK* (10) were shown to be involved in the regulation of nisin biosynthesis (8,10,44). NisR is a response regulator and NisK is a sensor histidine kinase, both belonging to the class of two-component regulatory systems (14-16). Two gene products were found to be involved in the regulation of the biosynthesis of the related lantibiotic subtilin (19), and these also belong to the class of two-component regulators, *i.e.* SpaR, the response regulator, and SpaK, the sensor histidine kinase (20,21). Upon disruption of either of these genes, subtilin production was abolished, indicating the involvement of these gene products in subtilin biosynthesis (20). The regulation of subtilin production was shown to be growth-phase dependent, but an inducing signal was not identified (20,21).

While the structure and function of two-component regulators have been studied in great detail (14-16), the nature of the inducing signal has remained unclear in many cases. It is demonstrated here that fully modified nisin, mutant nisin species and nisin analogues can induce the transcription of the *nisA* structural gene, as well as the downstream genes by limited read-through. Furthermore, it is demonstrated that this induction process is mediated via signal transduction via NisR and NisK. Al together, the nisin induction system resembles a quorum sensing phenomenon in which nisin acts as a peptide pheromone (45).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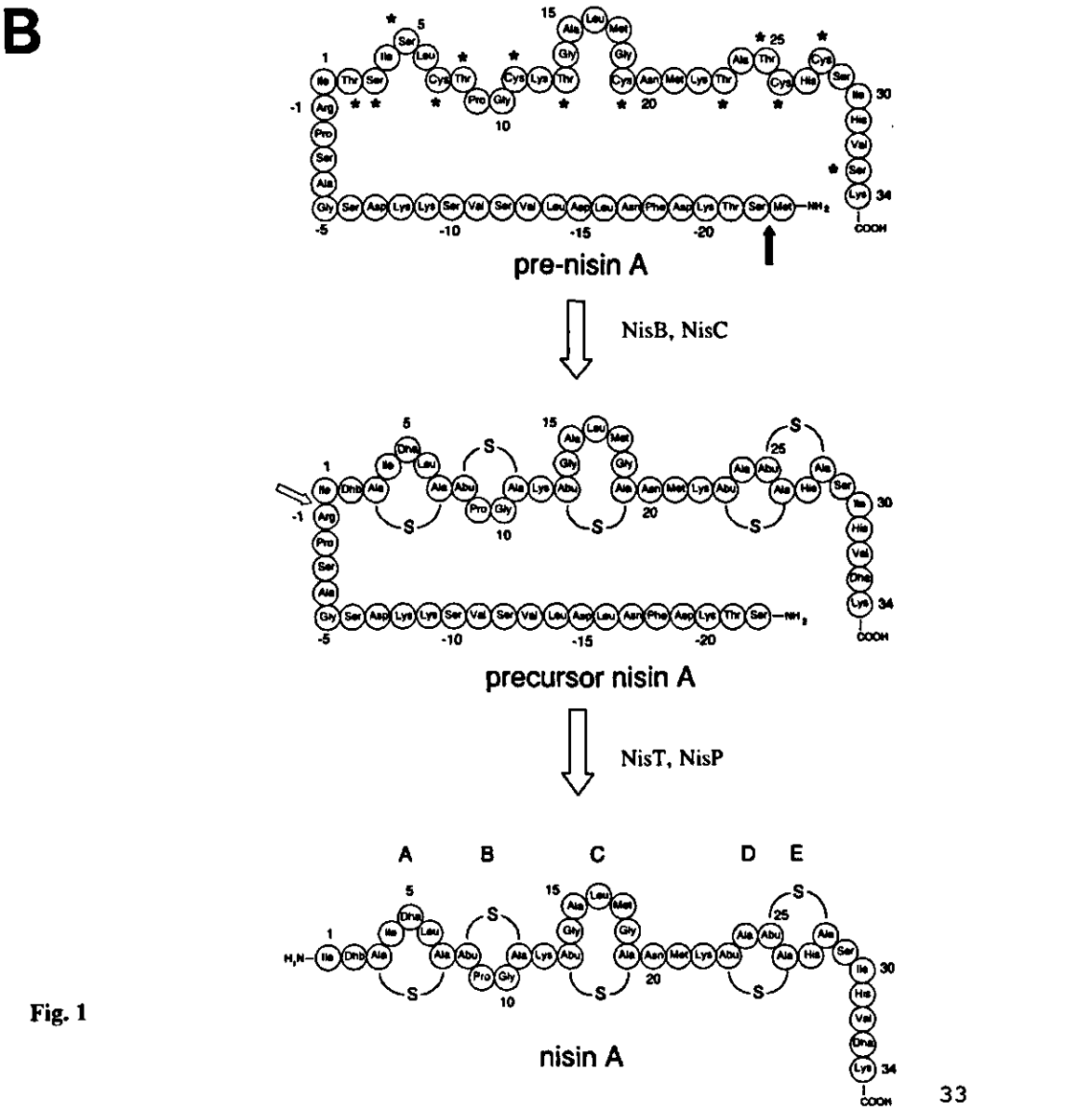
B
pre-nisin A
NisB, NisC
precursor nisin A
NisT, NisP
A
B
C
D
E
nisin A

Fig. 1

Fig. 1. Panel A: Organization of the nisin gene cluster. Established (*nisAIPRKFEG*) and putative (*nisBCT*) functions of the gene products are indicated. P denotes mapped promoters and IR denotes an extensive inverted repeat sequence that could act as a rho-independent terminator (7). Panel B: Schematic outline of the biosynthesis of nisin A. Rings are labelled A,B,C,D,E. Asterisks indicate residues that will be modified during the posttranslational modification reactions. The black arrow indicates processing of the N-terminal Met residue, while the white arrow indicates processing of the leader peptide by the action of NisP(8).

MATERIALS AND METHODS

Strains and plasmids. Strains *L. lactis* MG1614 (22), NZ9700, a nisin producing transconjugant containing Tn*5276* (23), and NZ9800, a derivative of NZ9700, in which the *nisA* gene has been exchanged by replacement recombination with a modified *nisA* gene containing a 4-bp deletion in the pronisin-encoding part (Δ*nisA*) and which is therefore no longer able to produce nisin A, have been described previously (9). *L. lactis* strains were cultivated without aeration at 30°C in M17 broth (Difco), supplemented with 0.5% (w/v) glucose or sucrose. For *L. lactis* strains harboring pNZ273-derived plasmids (24), media were supplemented with 10 mg/ml chloramphenicol. Strain NZ9800Δ*nisI*Δ*nisP* (39) was used as a leader peptidase deficient strain. As a host strain for cloning experiments, *Escherichia coli* strain MC1061 (26) was used.

The *nisA* promoter region including part of the *nisA* gene was isolated as a 1442 bp *Bgl*II-*Ecl*136II fragment from plasmid pNZ9000 (8). This fragment was cloned in pNZ273 containing the promoterless *gusA* gene (24), which had been digested with *Bgl*II and *Sca*I, generating plasmid pNZ8003. Part of the upstream promoter region was deleted by digesting pNZ8003 with *Bgl*II and *Tth*111I. These sites were made blunt by Klenow polymerase and ligated, generating plasmid pNZ8008, which eventually contained a 312 bp *nisA* promoter fragment in front of the *gusA* gene. Another part of the *nisA*-promoter region, including the complete *nisA* gene and the first part of the *nisB* gene, was isolated as a 1904-bp *Bgl*II-*Mun*I fragment from plasmid pNZ9000. This fragment was cloned in pNZ273 (24), which had been digested with *Bgl*II and *Eco*RI, generating plasmid pNZ8002. A 1442-bp *Bgl*II-*Ecl*136II promoter fragment was deleted in pNZ8002 generating pNZΔ8002 by making the *Bgl*II site blunt with Klenow polymerase and subsequent ligation to the *Ecl*136II site. All constructs were initially made in *E. coli* MC1061 (26). Plasmids pNZ8008, pNZ8002 and pNZΔ8002 were used to transform *L. lactis* NZ9700 and *L. lactis* NZ9800 (9) and transformants were obtained by selecting for resistance to chloramphenicol.

DNA techniques. Restriction enzymes and other DNA modifying enzymes were purchased from Gibco/BRL Life Technologies (Gaithersburg, Md.), or United States Biochemicals (Cleveland, Ohio), and used as recommended by the manufacturers.

Inactivation of chromosomal *nisK* by gene replacement. The chromosomal copy of the *nisK* gene was inactivated by introduction of an erythromycin resistance gene (28) into the open reading frame of *nisK*. For this purpose, a 2.8-kb *Hin*dIII-*Eco*RI chromosomal DNA fragment from strain NZ9700 containing the 3'-part of the *nisP* gene and the intact *nisR* and *nisK* genes was cloned into pUC19 (29). This construct was designated pNZ9150 and the final deletion strain NZ9850 (44).

Production, purification and characterization of mutant nisins. Production and purification of mutant species of nisin Z have been described previously (25,44). The mutant nisin Z species S-33, V32W/S-33, V32K/S-33, V32E/S-33 and V32E/nisZ(1-32) have been isolated and confirmed in the same way (27). Nisin A(1-32) was isolated and prepared as described (31). The precursor nisin Z, containing the subtilin leader peptide (sl-nisin Z) has been obtained as described before (30). Purified lacticin 481 has been isolated previously (32). Purified Pep5 (33) was obtained from Prof. Dr. H.-G. Sahl, Bonn, Germany. Unmodified precursor nisin A was obtained from the laboratory of Prof. Dr. G. Jung, Tübingen, Germany. Preparations of subtilin (19) and lactococcin A (34) consisted of culture supernatants of producing strains, which were confirmed to possess substantial antimicrobial activity in agar-diffusion assays. Purified nisin fragments (35-38) were obtained from Prof. T. Shiba, Protein Research Foundation, Osaka, Japan. Antimicrobial activities against *L. lactis* MG1614 were determined essentially as described before for *Micrococcus flavus* (25). *L. lactis* was cultured in GM17 broth at 30 °C with an initial A_{600nm} of 0.025 and outgrowth was measured when the culture without nisin had reached an A_{600nm} of 0.8.

ß-Glucuronidase assays. Lactococcal cells (1 ml) were harvested at 1.5 h after induction with nisin (or nisin mutants, fragments or other antimicrobial species) and adjusted in NaPi-buffer (50 mM $NaHPO_4$, pH 7.0) to a final A_{600nm} of 2.0. The cells were permeabilized by adding 50 ml acetone/toluene (9:1) per ml of cells followed by 10 min incubation at 37°C. The extracts were used immediately in the assay. For the determination of ß-glucuronidase activity 40 ml of extract was added to 950 ml of gus-buffer (50 mM $NaHPO_4$, pH 7.0, 10 mM ß-mercaptoethanol, 1 mM EDTA, 0.1% Triton X-100) and 10 ml 100 mM para-nitro-ß-D-glucuronic acid (Clonetech Lab. Inc., Palo Alto, California). The mixture was incubated and the increase in A_{405nm} was measured at 37°C.

RESULTS

In order to investigate whether the *nisA* promoter could be used to regulate the expression of heterologous genes in *L. lactis*, and to obtain a more quantitative assay of induction capacity, a nisin promoter fragment of 312 bp containing part of the *nisA* structural gene was fused to the promoterless reporter gene *gusA* of *E. coli* on plasmid pNZ273 (24). This construct, named pNZ8008, was used to transform *L. lactis* strain NZ9800, which contains the nisin transposon Tn*5276* (9) with a 4-bp deletion in the middle of the *nisA* gene (*ΔnisA)*. The resulting strain was assayed for ß-glucuronidase activity, with and without induction by nisin. Without induction, ß-glucuronidase activity could not be demonstrated, whereas wild-type nisin A and nisin Z effectively induced ß-glucuronidase activity (Fig. 2).

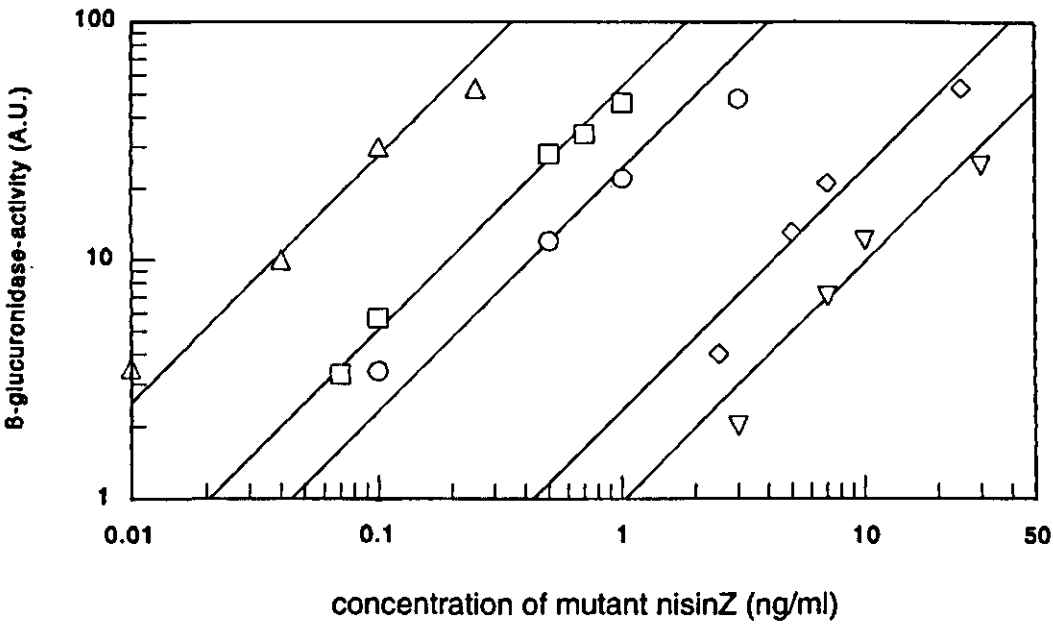

Fig. 2. Dose-response of purified (mutant) nisins as inducers of *gusA* expression in strain *L. lactis* NZ9800 harboring pNZ8008.
Nisin species: Δ, T2S nisin Z; □, M17W nisin Z; 0, wild-type nisin Z; ◊, S3T nisin Z;∇, I1W nisin Z. Standard errors were less than 20% for each given value.

Structural requirements of the inducer molecule tested by use of nisin mutants and other related peptides. The induction capacity of several mutant nisin species was also determined (Table 1) (25, 18). Purified T2S and M17W nisin Z were found to induce higher expression of *gusA* than wild-type nisin A or Z, whereas S3T and I1W nisin Z were found to have lower induction capacity (Fig. 2). It was calculated that less than 5 molecules of the best inducer (T2S nisin Z) per cell were sufficient to activate transcription, which illustrates the high efficiency of the signal-transduction processes. Some of the antimicrobial peptides tested, including the homologous (56%) lantibiotic subtilin, the lantibiotic lacticin 481 and the antimicrobial peptide lactococcin A, did not elicit induction of *gusA* expression (results not shown). These results are concordant with the transcription analyses in which the peptides evoked no restoration of transcription (44). More detailed insight into the structural requirements of the inducer molecule was obtained by using more mutant nisins and nisin fragments in the *gusA* reporter assay (Table 2). The antimicrobial activity and the induction capacity were mainly affected without mutual relationship by modifications in the first three rings. In addition, the chemically modified nisin variant nisin A(1-32)-peptide, in which the two C-terminal residues are absent (31), was found to exhibit similar activity and signaling capacity as wild type nisin A. Hinge regions appear to be present in each lantibiotic and seem to be of importance for antimicrobial activity and to a variable extent also for induction capacity (Table 2: N20P/M21P and ΔN20M21). Whereas introduction of removal of dehydrated residues can either stimulate or reduce activity (Tables 1 and 2: T2S, S3T, S5T, M17Q/G18Dhb).

Table 1. Comparison of induction capacity with antimicrobial activity against strain *L. lactis* MG1614 of nisin Z and nisin Z mutants.

Nisin Z (mutant)	Induction capacity[a] (%)	Activity against *L. lactis*[b] (%)
M17W nisin Z	220	12
nisin Z	100	100
S3T nisin Z	11	2
I1W nisin Z	3	47

[a] The induction capacity of nisin Z was taken as 100%; values were calculated by measuring the distances between the dose-response curves of nisin Z and each of the nisin species.

[b] The minimal inhibitory concentration of nisin Z against *L. lactis* MG1614 (14 ng/ml) was taken as 100 % activity.

Standard errors were less than 20% for each given value.

To obtain more insight into the contribution of C-terminal residues, mutant nisin species have been constructed in which a negatively charged Glu or a positively charged Lys residue has been introduced. Previously, it was shown that V32E/nisinZ(1-32) has an extra net negative charge in the C-terminal part compared to the V32E/S-33 nisin Z. The antimicrobial activity of V32E/nisinZ(1-32) is reduced but the induction capacity seems to be even stronger affected by the negative charge (27). Since an unmodified residue at position 33 did not impair nisin induction or antimicrobial activity, the loss of activity and induction capacity of the Val substitutions was probably due to charge differences. Nisin mutants with an extra or altered positive charge, such as those with the following alteration, V32K/S-33, N27K, H31K and

K12P, had no influence on antimicrobial activity but strongly reduced the signaling capacity, except for M17K. There was no relationship between antimicrobial activity of the mutant nisin species against strain *L. lactis* MG1614 and their induction capacity (Tables 1 and 2).

Table 2: Properties of engineered nisin species compared to properties of nisin A and nisin Z

Alteration	Characteristic	Antimicrobial activity	induction capacity
	N-terminal mutations		
I1W	fluorescent label	similar	strongly reduced
I1W/T-2	unmodified Thr-2	reduced	reduced
T2S	Dhb to Dha	increased	increased
S3T	Ala-S-Ala to Abu-S-Ala	strongly reduced	reduced
S5T	Dha to Dhb	reduced	similar
	Hinge region mutations		
K12P	positive charge reduction	similar	strongly reduced
N20P/M21P	altered flexibility	reduced	similar
ΔN20M21	partial removal hinge	strongly reduced	strongly reduced
	Third ring mutations		
M17Q/G18Dhb	novel Dhb	similar	reduced
M17W	fluorescent label	reduced	increased
M17K	lysin in ring 3	similar	similar
	C-terminal mutations		
NisA(1-32)	absence last two residues	similar	similar
H31K	altered positive charge	similar	reduced
N27K	extra positive charge	similar	strongly reduced
S33	unmodified ser33	similar	similar
V32W/S-33	fluorescent label	slightly reduced	strongly reduced
V32K/S-33	positive charge	similar	reduced
V32E/S-33	negative charge	reduced	reduced
V32E/*nisZ*(1-32)	extra negative charge	reduced	strongly reduced

Activities were measured by determination of MIC values of purified mutant nisins against *Micrococcus flavus*, as has been described previously (25,17,27).
Signaling capacities of wild-type nisin was taken as 100% value. Standard errors were less than 20% for each given value.
Activity or capacity:

Increased	> 100%
Similar	80-100%
Reduced	20-80%
Strongly reduced	< 20%

The influence of the leader peptide on signaling properties was also studied. Sl-nisin Z, which consists of the subtilin leader fused to the pronisin Z, and its tryptic fragment [ITPQ]-nisin Z exhibit at least 200-fold lower antimicrobial activity than nisin Z (30). These data suggest that the leader sequence has a function in keeping the fully matured lantibiotic part inactive prior to secretion (30). Similarly, no antimicrobial activity was observed with the synthetically made, and unmodified nisin precursor or the biologically processed and fully modified precursor peptides (8,30). The signaling capacities of sl-nisin Z and [IPTQ]-nisin Z were compared to those

precursor peptides and the wild type nisin (Table 3). Both N-terminally extended peptides showed a low induction activity in strain NZ9800 harboring pNZ8008, suggesting the need for a free N-terminus for induction. Similarly, the unmodified synthetic precursor peptide did not elicit induction of *gusA* expression in strain NZ9800 carrying pNZ8008, while the expression after induction with the modified precursor was reduced (Table 3). This clearly shows the importance of the modifications of the nisin molecule for induction. However, when strain NZ9800Δ*nisI*,Δ*nisP* (39), which can not produce an active leader peptidase, harboring pNZ8008 was induced with the modified precursor, b-glucuronidase activity was not detectable any more (Table 3). This can be explained by the fact that the leader peptide of the modified precursor was cleaved off by NisP in strain NZ9800, which was not possible in the leader peptidase-deficient strain.

Table 3: Induction capacities of nisin-leader mutants compared to the wild type nisin Z in two different strains.

Mutants	Strain NZ9800 harboring pNZ8008	Strain NZ9800Δ*nisI*,Δ*nisP* harboring pNZ8008
[ITPQ]-nisin Z	< 20%	nd
sl-nisin Z	< 20%	nd
synthetic precursor	undetectable	nd
modified precursor	80%	<1%
Nisin Z	100%	100%

nd= not determined

In order to determine the critical parts of nisin that are required for induction, a set of synthetic nisin A fragments (35-38) (Table 4) was used. The minimal requirement (2% induction of that of nisin A) for retaining induction capacity was found to reside in residues 1-11 of nisin A, comprising the first two rings. Addition of the third ring enhanced induction (8-30% induction of that of wild-type nisin A), whereas a severe decrease in induction was caused by deleting the N-terminal residues Ile-1 and Dhb-2 (0-1% induction, Table 4). Fragments that contained rings B and C, or rings D and E (Fig. 1B), were not capable of acting as a signal effector.

Table 4: The minimal requirement for retaining induction capacity obtained by using synthetic nisin A fragments (36).

Nisin A fragments (residues)	Induction capacity (%)	Rings present
nisin A	100	A,B,C,D,E
1-21	30	A,B,C
1-19	8	A,B,C
1-11	2	A,B
3-19 (L-Ala-5)	1	A,B,C
3-19 (D-Ala-5)	undetectable	A,B,C
3-19	undetectable	A,B,C
8-19	undetectable	B,C
22-34	undetectable	D,E

The minimal inhibitory concentration of nisin Z against *Micrococcus flavus* (11 ng/ml) was taken as 100 % activity.
All nisin fragments contained the modified residues as they are present in wild-type nisin A (Fig. 1B), unless indicated otherwise at position 5. Standard errors were less than 20% for each given value.

Influence of the intergenic region between *nisA* and *nisB* on expression of genes downstream of *nisA*. To determine the influence of a large inverted repeat sequence located in the intergenic region between *nisA* and *nisB* on expression of genes downstream of *nisA* (Fig. 1A), plasmid pNZ8002 was constructed, in which the nisin promoter fragment including *nisA* as well as the intergenic region and the first part of *nisB* was fused to the *gusA* gene. This plasmid was able to direct expression of *gusA* in strain NZ9800 only after induction with nisin species, albeit to an approximately 50-fold reduced level relative to *gusA* expression in pNZ8008 in strain NZ9800. When the nisin promoter fragment was removed from pNZ8002, yielding pNZΔ8002, ß-glucuronidase activity was completely abolished, even in the presence of an inducer. These results indicate that expression of at least one downstream gene, *i.e. nisB*, is coregulated and dependent on the presence of the *nisA* promoter. Most likely, expression of the other downstream genes *nisTCIP* is also dependent on the *nisA* promoter by limited read-through, since a significant increase in immunity levels, for which NisI is partially responsible (9), was found in the induced state relative to the uninduced state of strain NZ9800.

In further experiments, the nisin-producing strain NZ9700, which contains the intact Tn*5276*, with either plasmid pNZ273, containing the promoterless *gusA* gene, or pNZ8008, containing the *nisA* promoter fragment followed by the *gusA* gene, was used in an agar-diffusion assay (8) to determine the amount of nisin produced. Fifty times less nisin production and severely reduced immunity was observed when plasmid pNZ8008 was present, compared to the situation where pNZ273 was present. This can be explained by titration of the response regulator NisR by the multi-copy presence of the *nisA* promoter region containing a putative NisR binding site.

Requirement of *nisK* expression for signal transduction. The chromosomal *nisK* gene was insertionally inactivated by introduction of an erythromycin-resistance gene (28) in strain NZ9800, which yielded strain NZ9850 (44). Strain NZ9850 was also transformed with pNZ8008 but expression of the *gusA* gene could not be induced by the addition of nisin A. While induction with nisin A (0.0005 to 0.0025 mg/ml) resulted in ß-glucuronidase activity in strain NZ9800, no activation could be measured in strain NZ9850 harboring pNZ8008, with the same inducer concentrations (Fig. 2). No polar effects of the *nisK* disruption on the expression of the *nisFEG* genes downstream of *nisK* are expected, since a promoter has found to be present in front of *nisFEG* (43). These results indicate that an intact *nisK* gene is required for the signaling cascade that leads to induction of the *nisA* promoter

DISCUSSION

We have demonstrated that transcription of *nisA* is autoregulated *via* signal transduction by a two-component regulatory system, and depends on the extracellular presence of the secreted and fully modified peptide. Subinhibitory amounts of nisin, mutant nisin species or nisin analogues can also act as inducers, whereas other antimicrobial peptides or the modified and unmodified precursor peptides are incapable of induction. There is no relationship between antimicrobial activity and signaling capacities of mutant nisin species. This effect can be attributed to differences in the mode of action, while the antimicrobial activity of nisin is dependent on its pore-forming activity in membranes (40-42), the induction capacity is likely to be dependent on the interaction with NisK. The latter interaction could be either direct with the surface-exposed domain of nisin or indirect, following membrane insertion, with the transmembrane part of NisK (45,46).

The experiments using engineered nisin species and the nisin fragments indicate that the N-terminal region (residues 1-11) of the fully modified nisin molecule plays a crucial role in the induction process whereas the more C-terminally located residues have only an enhancing effect. The C-terminal region was shown to be responsible for the initial interaction of nisin with the target membrane (47). Partial removal of the hinge region (ΔN20M21) showed the influence on activity and induction capacity of the distance between or the orientation of rings ABC with respect to rings DE. Analysis of enzymatically generated nisin fragments, to function as inducers of immunity, have demonstrated that the 12 amino-terminal residues were sufficient (48). Thus, the most probable site of molecular interaction with the sensor protein NisK will be the N-terminal part, in particular residues 1-11 of the nisin molecule, although additional interactions with the rest of the molecule cannot be excluded.

The results with the constructs pNZ8002 and pNZΔ8002 showed that the expression of at least *nisB* is dependent on the *nisA* promoter. Recently, it was also shown that the number of genes that are subject to the same autoregulation can be extended to include those involved in self-immunity, the most likely candidates being *nisI* and *nisFEG* (48). This suggests that coexpression of *nisI* and *nisA*, along with the intervening *nisB*, *nisT* and *nisC*, occurs in response to induction with nisin. The absence of a transcription terminator at the end of *nisI* and lack of a convincing promoter in front of *nisP* indicates that the complete polycistronic transcriptional unit, controlled by the inducible *nisA* promoter, includes six genes and extends from *nisA* to *nisP*.

There may be several evolutionary reasons for the autoregulation of nisin gene transcription *via* signal transduction, *e.g.* i.) to save energy by control of the integrity of the gene cluster, since any disfunctional biosynthetic gene will abolish inducer formation, and thus expression of biosynthetic genes, ii.) to raise immunity levels in response to high nisin production by neighboring cells, in other words, amplifying the response to environmental signals, or iii.) to promote cell to cell communication that allows the production of antimicrobial peptides in high quantities in a concerted action, thereby decreasing the chance of resistance development in target organisms.

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CHAPTER 3

Functional analysis of promoters in the nisin gene cluster of *Lactococcus lactis*

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

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

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

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

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

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

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TABLE 1. *L. lactis* strains and plasmids

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

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

MATERIALS AND METHODS

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

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

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

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

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

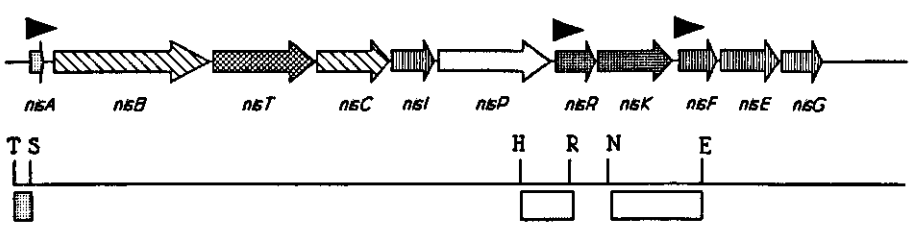

FIG. 1. Schematic representation of the organization of the nisin gene cluster, location of the nisin promoters, and fragments inserted in the *gusA* reporter plasmid pNZ273 (20). Relevant restriction sites are indicated: T, *Tth*1; S, *Ssr*I; H, *Hin*dIII; R, *Eco*RV; N, *Nde*I; E, *Eco*RI.

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

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

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

[b] ND, not determined.

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

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

RESULTS

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

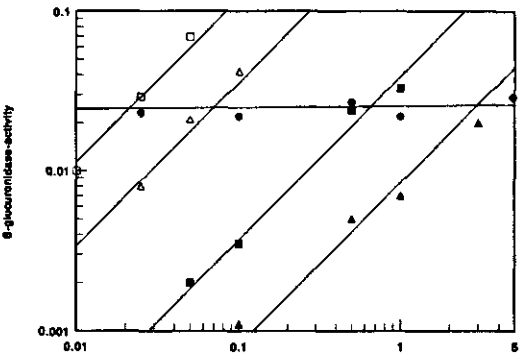

FIG. 2. Dose-response curves of *gusA* expression in cell extracts of *L. lactis* NZ9800 or *L. lactis* NZ3900 directed by the *nisA* promoter, *nisR* promoter, or *nisF* promoter induced with several concentrations of nisin. The β-glucuronidase activity is shown as specific activity per optical density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

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

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

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

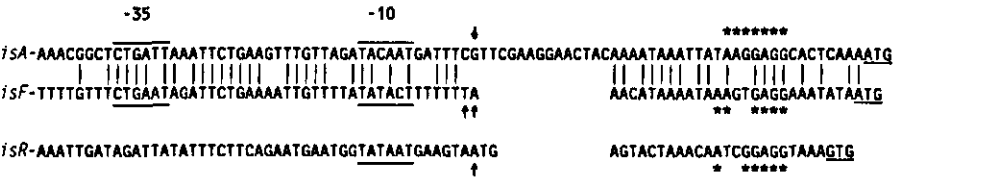

FIG. 3. Comparison of the *nisA*, *nisR*, and *nisF* promoter sequences. Arrows indicate the transcription initiation sites mapped by primer extension. The main start site of the *nisF* promoter was mapped on nucleotide 116 (numbering according to the work of Siegers and Entian [25]) with primer 1. The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

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

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

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

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

DISCUSSION

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

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

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

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

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

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

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

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

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

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CHAPTER 4

Controlled gene expression systems for *Lactococcus lactis* with the food-grade inducer nisin

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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,

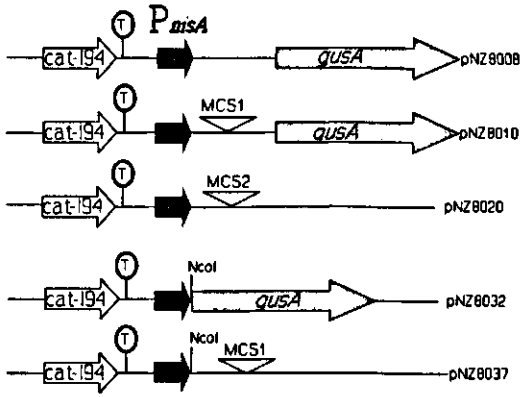

FIG. 1. Schematic representation of the organization of the constructed expression vectors containing the inducible *nisA* promoter. In constructs pNZ8032 and pNZ8037, the *Nco*I restriction site is shown to indicate the possibility of making translational fusions with the *nisA* start codon. T, terminator 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.

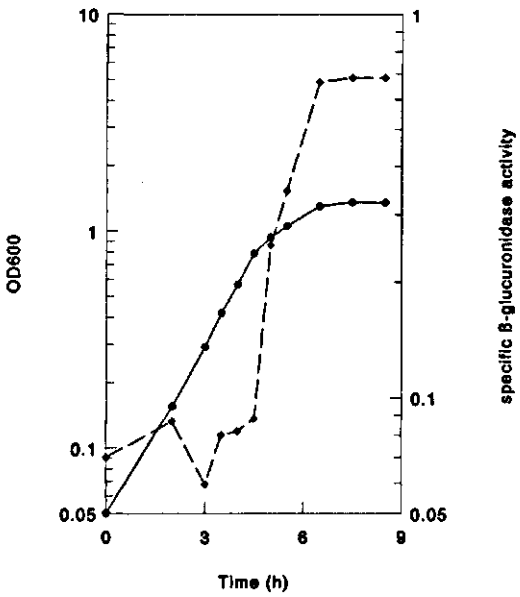

FIG. 2. β-Glucuronidase activity (broken line) determined in cell extracts during growth (optical density at 600 nm [OD600]; solid line) of the nisin-producing strain NZ9700 harboring pNZ8008. The activities are expressed as specific activity (increase in A_{405} per minute) per optical density unit.

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 *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 (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).

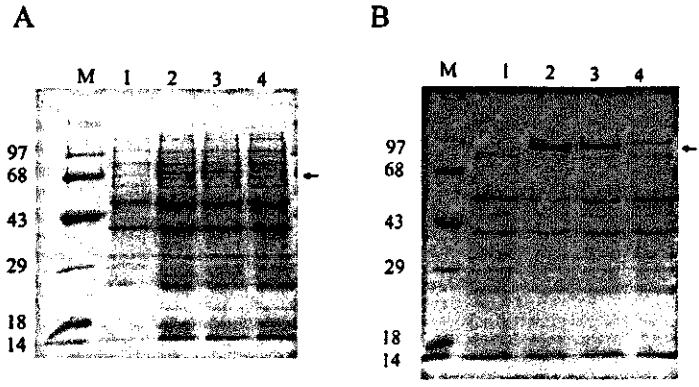

FIG. 3. Coomassie blue-stained gels after SDS-PAGE of extracts of strain NZ3900 containing pNZ8032 or pNZ8040, producing β-glucuronidase (A) or aminopeptidase N (B). (A) Lane 1, uninduced cells; lanes 2 to 4, induction with 2.5, 0.5, and 0.05 ng of nisin A per ml, respectively; lane M, molecular weight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

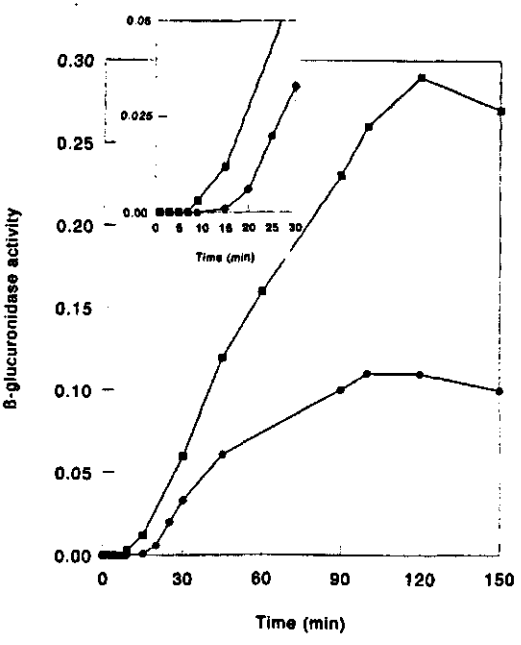

FIG. 4. Kinetics of induction: β-glucuronidase activities (specific activity per optical density [at 600 nm] unit) determined in cell extracts of strains NZ9800 (●) and NZ3900 (■), both harboring pNZ8032, during the time after induction with 1 ng of nisin A per ml.

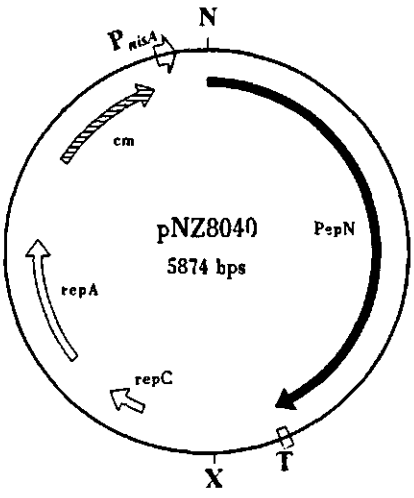

FIG. 5. Physical map of the PepN-overproducing construct pNZ8040. The lactococcal *pepN* gene was cloned behind the inducible *nisA* promoter. Relevant cloning sites: N, *Nco*I; X, *Xho*I. T, transcriptional terminator.

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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CHAPTER 5

Mutational analysis of the *nisA* promoter and characterisation of the binding site of the response regulator protein NisR of *Lactococcus lactis*

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SUMMARY

The antimicrobial peptide nisin induces its own biosynthesis probably by acting as a peptide pheromone for a quorum-sensing module involving the sensor protein NisK and the response regulator NisR. Mutational analysis of the promoter fragment upstream of the structural *nisA* gene was performed and its effect on the transcription initiation efficiency in *Lactococcus lactis* was studied using the *Escherichia coli* reporter gene *gusA*. A fragment including 39 bp upstream from the transcriptional start was found to be sufficient for promoter activity and inducibility by nisin. Two pentanucleotide sequences centered at -26 and -37 were shown to be required for transcriptional activation of the *nisA* promoter, probably functioning as a binding site for NisR. Further mutational analysis of the direct repeat and scanning deletion analysis indicated that these pentanucleotide sequences, the canonical -10 sequence, and their spacing are required for inducible transcriptional activation of the *nisA* promoter. The *nisR* gene was fused to histidine residues and overexpressed in *E. coli* after which the His-tagged NisR was partially purified using nickel-NTA agarose binding. Gel mobility shift assays showed that overproduced His-tagged NisR binds directly to the *nisA* promoter region.

INTRODUCTION

Cell-density-dependent gene expression appears to be widely spread in bacteria. An example of such a quorum-sensing mode in gram-positive bacteria is the production of antimicrobial peptides by lactic acid bacteria. A specific class of these peptides is formed by the lantibiotics, peptides that undergo extensive posttranslational modifications, which result in formation of lanthionine and β-methyllanthionine residues forming thioether ring structures. The genes required for lantibiotic biosynthesis, export and immunity are generally clustered (8, 28). Interestingly, both the nisin and the subtilin biosynthetic gene clusters include genes encoding a sensor protein (*nisK* and *spaK*, respectively) and a response regulator (*nisR* and *spaR* respectively) that have been shown to be essential for lantibiotic production (6, 14, 16, 18, 29). Moreover, nisin has been shown to be the inducing signal that is likely to be sensed by NisK, in this way autoregulating its own biosynthesis (18).

The nisin biosynthesis pathway is encoded by the *nisABTCIPRKEFG* gene cluster, in which the regulatory genes *nisR* and *nisK* are under control of a constitutive promoter while the expression of the other genes is controlled by the nisin-inducible *nisA* and *nisF* promoters (5). The *nisA* and *nisF* promoter sequences have significant similarities. These include a canonical -10 sequence which is spaced by 20 bp from a less conserved -35 sequence, and two conserved pentanucleotides centered at -26 and -37 that could be important for transcriptional control. The encoded proteins NisR and NisK show strong sequence similarities to proteins of two-component regulatory systems (23, 14). Since expression of *nisRK* in other lactic acid bacteria than *L. lactis* allows nisin-inducible *nisA* promoter activity in these bacteria, NisR and NisK are the only components required for the signal-transduction pathway involved in nisin autoregulation (15). It has also been shown that NisR, when overproduced, promotes nisin production in the absence of an intact *nisK* gene, probably due to NisR autophosphorylation and lack of NisK mediated dephosphorylation (29).

Remarkable high sequence identity is found between NisR and the response regulators from the gram-positive bacteria, e.g., SpaR (16) from *Bacillus subtilis* (42.2%) and VanR (9) from *Enterococcus faecalis* (36.1%). The region with the highest similarity in all response regulators is the N-terminus, where phosphorylation of a conserved Asp residue by the corresponding histidine kinase takes place (27). Three essential residues, the site of phosphorylation included, are conserved in all members of the family and, by sequence comparison, are predicted to be in NisR Asp-10, Asp-53 (site of phosphorylation), and Lys-102. The deduced NisR amino acid sequence shows highest similarity with members of the subfamily of the OmpR-like response regulators, e.g. OmpR (4) from *E. coli* (31.4% identity). It has been found that the OmpR protein activates the *ompF* and *ompC* promoters by binding to a region upstream of the -35 region of those promoters (3, 13).

In this study, we provide evidence for the fact that NisR has a direct role in the regulation of the nisin biosynthesis by showing that partially purified His-tagged NisR protein binds to the *nisA* promoter by gel mobility shift assays. Moreover, mutational analysis including deletions and base-pair substitutions of the *nisA* promoter fragment provided insight on the minimal requirements for nisin inducibility and promoter strength.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Escherichia coli* MC1061 (2) was grown in L-broth-based media with aeration at 37°C (26). *E. coli* JM109 (DE3)LysS (Promega Corporation, Madison, Wis.) was grown in L-broth-based media containing 1% (wt/vol) glucose and chloramphenicol (10 μg/ml) with aeration at 37°C. *Lactococcus lactis* strains were grown at 30°C in M17 (Merck, Darmstadt GmbH) broth containing 0.5 % (wt/vol) glucose (GM17). When appropriate, the media contained chloramphenicol (10 μg/ml) or ampicilline (50 μg/ml). For gene expression by use of the T7 expression system in *E. coli* JM109(DE3)LysS, cells were induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to the growth medium at a concentration of 0.5 mM.

DNA methodology, reagents and enzymes. Isolation of plasmid DNA from *E. coli* and standard recombinant DNA techniques were performed according to Sambrook et al. (26). Isolation of plasmid DNA from *L. lactis* was performed as described previously (30). DNA fragments were isolated from agarose gels by using the GlassMAX DNA Isolation Matrix System (BRL, Life Technologies, Inc). Transformation of *L. lactis* strains was performed according to Wells et al. (32). Nucleotide sequence analysis of double-stranded plasmid DNA was performed using an ALF automatic sequencer in combination with Autoread kits (Pharmacia Biotech) which includes T7 DNA polymerase. A fluorescently labeled primer with the sequence 5' - GGGTTGGGGTTTCTACAGGACGTA-3', complementary to positions 325 to 298 of the *gusA* gene (numbering according to reference 24), was used for sequencing. Restriction enzymes and other DNA modifying enzymes were purchased from Gibco/BRL Life technologies (Gaithersburg, Md.), and used as recommended by the manufacturers. Oligonucleotides were purchased from Pharmacia.

Construction of plasmids. The *nisA* promoter region was cloned as an approximately 200 bp *Bgl*II-*Eco*RI PCR amplified fragment with different mutations, in pNZ273 containing the promoterless *gusA* gene (24). Approximately 100 ng of template pNZ8008 DNA (18) was used for constructing the plasmids in Table 1, except that for the constructs pNZ8069, pNZ8074, and pNZ8075, the plasmids pNZ8059, pNZ8058 and pNZ8057 were used as templates, respectively, and were subjected to amplification by the polymerase chain reaction (25). Routinely, PCR was performed in a total volume of 50 μl containing 1 U of *Taq* polymerase (GIBCO-Bethesda Research Laboratories, Gaithersburg, Md.) as described before (6). PCR amplifications were performed by use of the following primers (Table 1, restriction sites are underlined) combined in all cases with primer 0: 5'-GGTCCGGAATTCCTGCAG, except that for plasmid pNZ8070 primer I and II were used. PCR yielded single bands upon agarose gel electrophoresis (26) that were recovered with the USBioclean Kit (U.S. Biochemical Corp., Cleveland, Ohio), digested with *Bgl*II (or *Bam*HI in primer 3) and *Eco*RI (in primer 0) and cloned into pNZ273, which had been digested with *Bgl*II and *Eco*RI, generating the constructs of Table 1. For the construction of pNZ8070, the PCR product and pNZ273 were digested with *Bgl*II and *Ava*II and ligated.

Table 1: primers and annealing temperatures used in a PCR reaction for construction of plasmids.

Primer	plasmid	temp. (°C)
5'-GCGAAGATCTGATTAAATTCTG	pNZ8051	50
5'-GCGAAGATCTCGAGCATAATAAACGG	pNZ8052	50
5'-CGCGGATCCATGTGATTAAATTCTGAAG	pNZ8053	60
5'-GCGAAGATCTGATTAAATTTTGAAGTTTG	pNZ8054	60
5'-GCGAAGATCTGATTAAATTCTAAAGTTTGTTAG	pNZ8055	60
5'-GCGAAGATCTGATTAAATTCTGAAGTTT(CAT)TTAGA-TACAATG	pNZ8056	60
5'-GCGAGATCTAATAAACGGCTTTGACAAAATTCTG	pNZ8057	50
5'-GCGAAGATCTGATTAAATTCTGAATGTTAGATAC	pNZ8058	50
5'-GCGAGATCTAATAAACGGCTTTGACAAAATTCTGAATGT-TAGATAC	pNZ8059	50
5'-GCGAAGATCTGATTAAATTCGAAGTTTG	pNZ8060	55
5'-GCGAAGATCTGATTAATTTCTGAAGTTTG	pNZ8061	55
5'-GCGAAGATCTGATTAAAATCTGAAGTTTG	pNZ8062	55
5'-GCGAAGATCTGATTAAATACTGAAGTTTG	pNZ8063	55
5'-GCGAAGATCTGATTAAATTCAGAAGTTTG	pNZ8064	55
5'-GCGAAGATCTGATTAAATTCTGTAGTTTGTTAG	pNZ8065	55
5'-GCGAAGATCTGATTAAATTATTAAGTTTGTTAG	pNZ8068	50
5'-GCGAAGATCTTTGACAAAATTATTAATGTTAG	pNZ8069	50
I: 5'-GTAGGTCCTTCGAACGAAATC	pNZ8070	50
II: 5'-CAGCTCCAAGATCTAGTC	pNZ8070	50
5'-GCGAAGATCTGATTTTCTGAAGTTTGTTAG	pNZ8072	50
5'-CTCCAGATCTGATTAAATTATTAATGTTAG	pNZ8074	50
5'-GCGAGATCTTTGACAAAATTATTAAGTTTG	pNZ8075	50
5'-GCGAGATCTAATAAACGGCTATTATTAAATTCTG	pNZ8076	50
5'-ATAAACAGATCTGATTAAATTAAGTTTGTTAGATACAATG	pNZ8077	50
5'-CAGGAGATCTCGGCTATTAAATTCTGAAGTTTG	pNZ8078	50
5'-CTCCAGATCTGATTAAATTCTGAAGTTTGTTAGCAATGATT-TCGTTCGAAG	pNZ8079	50
5'-GCGAGATCTAATAAACGGCTTTGACAAAATTAAGTTTGT-TAGATAC	pNZ8080	50

For the construction of pNZ8073, two oligonucleotide fragments, fragment 1: 5'-GATCTGAT TTTTATCTGATCAAACAATCATACAATGATTTCG and fragment 2: 5'-AATTCGAAAT CATTGTATGATTGTTTGATCAGATAAAAATCA were mixed, heated to 90°C and cooled down slowly for the annealing. This fragment was ligated with pNZ273, digested with *Bgl*II and *Eco*RI.

All constructs were initially made in *E. coli* and the mutations were confirmed by sequence analysis of the promoter region. The constructs were used to transform the plasmid free strains *L. lactis* MG1363 (11) and *L. lactis* NZ3900, an MG1363 derivative which contains the *nisRK* genes integrated in the chromosome (6).

ß-glucuronidase assays. For determination of β-glucuronidase activity, *L. lactis* cells were grown to an A_{600nm} of 0.5, induced with nisin A (1 ng ml^{-1}) or not induced, and grown for another 90 min. Cells were harvested and resuspended in NaPi-buffer (50 mM $NaHPO_4$, pH 7.0) to a final A_{600nm} of 2.0. The cells were permeabilized by adding 50 µl acetone/toluene (9:1 v/v) per ml of cells followed by 10 min incubation at 37°C. Forty µl of the extracts was used immediately in the ß-glucuronidase assay by adding 950 µl of GUS-buffer (50 mM $NaHPO_4$, pH 7.0, 10 mM ß-mercaptoethanol, 1mM EDTA, 0.1% Triton X-100) and 10 µl 100 mM para-nitro-ß-D-glucuronic acid (Clonetech Lab. Inc., Palo Alto, California). The mixture was incubated at 37°C and the increase in A_{405nm} (ΔA min^{-1}) was used to calculate the specific ß-glucuronidase activity per OD_{600nm}-unit.

Overproduction and partial purification of NisR. The *nisR* gene (29) was obtained by PCR (Using Pwo polymerase, Boehringer, Mannheim, Germany) performed as recommended by the manufacturer, using 100 ng of pNZ9510 as a template, which contains a *ScaI-SalI* fragment of pNZ9500 (15) cloned into *SmaI-SalI* digested pUC19. As primers the oligonucleotides 5'-GAATTCGAGCTCGGTACCCAC, containing an *Eco*RI site (underlined) and 5'-CGCTGGATCCTTA*GTGATGATGGTGATGGTG*CCCATGCCACTGATACCCAAG, containing a *Bam*HI site (underlined), a new stopcodon (bold) and the nucleotide sequence for six histidine residues (Histag, in italics) were used. The PCR product was isolated as a 0.7-kb *Eco*RI-*Bam*HI *nisR*-Histag fragment and cloned into pNZ9540 (15), digested with *Eco*RI and *Bam*HI, behind the T7 promoter generating pNZ8090. This plasmid was used to transform *E. coli* JM109(DE3)LysS, which contains a chromosomal copy of the T7 RNA polymerase gene under control of the *lacUV5* promoter and plasmid pLysS that contains the T7 lysozyme gene. T7 lysozyme represses the basal activity of T7 RNA polymerase. For NisR overproduction 100 ml cells were grown at 37°C and induced at an optical density at 600 nm of 0.4 - 0.5 by adding IPTG to an final concentration of 0.5 mM. Incubation was continued for 2 hours and cells were harvested and resuspended in 1 ml NaPi-buffer (50 mM $NaHPO_4$, pH 7.0). A sample of 100 µl of these cells was mixed with equal amounts of sample buffer, and 5 µl was applied to a sodium dodecyl sulfate (SDS)-12.5% polyacrylamide gel (19) to confirm the production of the His-tagged NisR protein as an approximately 29 kDa band after Coomassie brilliant blue staining of the gel.

For the enrichment of the His-tagged NisR protein under native conditions, Ni-NTA agarose (Qiagen) was used in a batch procedure. An overnight culture of *E. coli* JM109(DE3)LysS harboring pNZ8090 was used to inoculate 250 ml of medium (4%), cells were grown, induced with IPTG, harvested and resuspended in 2.5 ml NaPi-buffer. Cells were lysed by adding triton X-100 (2%) and lysozyme (1mg/ml) and incubated on ice for 20 minutes. The cells were subjected to three subsequent freeze (N_2(l)) thaw (RT) cycles to improve cell lysis. DNAse and RNAse were added to a concentration of 1µg/ml, incubated on ice for 15 minutes, centrifuged for 20 minutes at 4°C, and the supernatant (cell-free extract) was decanted and saved on ice. The 50% Ni-NTA agarose slurry was thoroughly resuspended and 250 µl was added to 1 ml of supernatant (containing the His-tagged NisR protein) in an eppendorf reaction tube. Also 10 mM imidazole was added to inhibit the binding of non-tagged contaminating proteins. This suspension was mixed and incubated, rotating, at room temperature for 45 minutes. The suspension was centrifuged in an eppendorf centrifuge for 10 seconds, washed with 500 µl buffer I (50 mM NaH_2PO_4, 300 mM NaCl, 25 mM imidazole, pH 8.0) and the protein was eluted with 250 µl of buffer II (50 mM NaH_2PO_4, 300 mM NaCl, 300 mM imidazole, pH 8.0). A prepacked, disposable PD-10 column containing sephadex G-25 M (Pharmacia, Sweden) was used for rapid desalting and buffer exchange. An SDS-polyacrylamide gel (12.5%) system was used to examine the purity of the His-

tagged NisR preparations and to confirm its expected size. Protein concentrations were determined by the method of Bradford (1) using bovine serum albumin (Sigma) as standard.

Determination of the oligomerization state of His-tagged NisR by gel filtration. The desalted His-tagged NisR fraction was loaded onto an HR 10/30 Superdex 75 column (Pharmacia), preequilibrated with 50 mM NaPi buffer, 300 mM NaCl pH 8.0, and the protein was eluted at 0.44 ml/min and collected in 500 µl fractions. The protein molecular weight standards (Mw of 1.4, 6.5, 20.0, 36.0 and 67.0 kDa, respectively) were applied to the column under the same conditions, and the elution volume and molecular weight of each standard were used to generate a standard curve to determine the molecular weight of the NisR protein.

DNA binding assay. A 200 bp *BglII-Eco*RI fragment was excised from pNZ8051 and labeled by filling in the 3'recessed ends of both sites with the Klenow fragment of DNA polymerase in the presence of [α-^{32}P]dATP (3000 Ci/mmol, Amersham Corp.), dCTP, dGTP, and dTTP. Binding of His-tagged NisR with the end-labeled probe was performed in 20 µl assay mixture which contained: 20 mM Tris-HCl pH 8.0, 100 mM KCl, 10 mM $MgCl_2$, 2mM dithiothreitol, 2 mM EDTA, 100 µg/ml poly d(I-C), 20% glycerol, approximately 5 ng end-labeled fragment, and His-tagged NisR at varying concentrations (0 to 9 µg). After 30 min. of incubation at room temperature, 1 µl of 20x sample buffer (200 mM Tris pH 8.0, 0.8% bromophenol blue) was added and reaction mixtures were loaded on a polyacrylamide gel (acrylamide:bisacrylamide, 30:0.5) in 50 mM Tris-borate, 0.5 M EDTA (pH 8.0). The gel was pre-run for 30 min. at 15 V/cm at room temperature. Electrophoresis was performed under the same conditions. After drying the gel was autoradiographed.

RESULTS

Characterization of the minimal *nisA* promoter region required for controlled *gusA* expression Plasmid pNZ8008 (18) is an expression vector containing the *gusA* reporter gene preceded by the *nisA* promoter region including positions -156 to + 156 with respect to the *nisA* transcription start (17). Several derivatives of pNZ8008 were constructed that contain deletions in this promoter region (Fig. 1). Plasmids containing the promoter fragments from position -56 to +156 (pNZ8052), position -39 to +156 (pNZ8051) and position -156 to +7 (pNZ8070) were used to transform *L. lactis* NZ3900, an MG1363 derivative which contains the *nisRK* genes integrated in the chromosome, in order to compare the mode of control of the shortened promoter regions with that of the nisin-inducible *nisA* promoter that drives expression of the *gusA* gene in pNZ8008. The induction by nisin and the promoter efficiency were determined as a function of the external nisin concentration. The β-glucuronidase (GusA) activities were determined and found to be similar in all extracts. In addition, no detectable GusA activities were found without induction with nisin (data not shown). Thus, the *nisA* promoter region including 39 bases upstream of the transcription start is sufficient for promoter activity and inducibility by nisin. This region contains a -10 sequence and two directly repeated pentanucleotide sequences (Fig. 2).

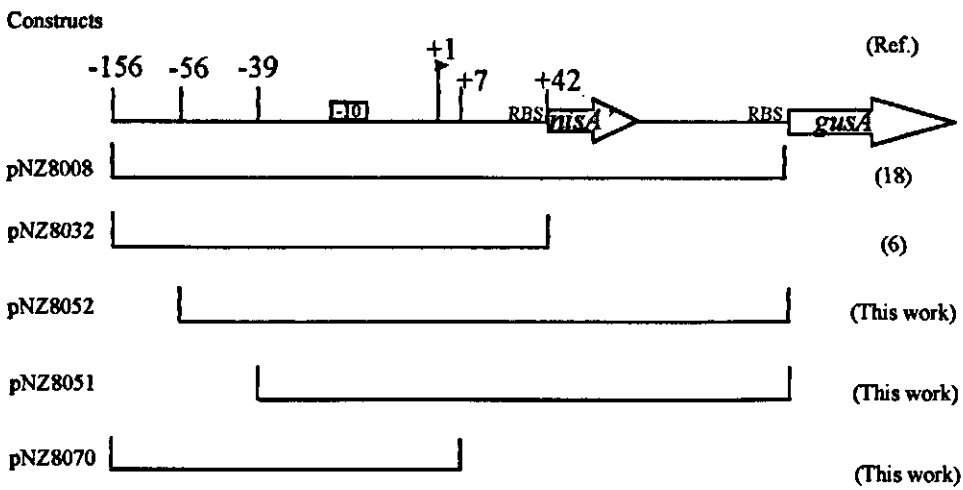

Fig. 1. Schematic representation of the *nisA* promoter regions cloned in front of the *gusA* reporter gene.

Location of the DNA binding sites for NisR by site-directed mutagenesis. To study the role of the conserved region consisting of a pentanucleotide direct repeat in more detail, a set of different *nisA*-promoter fragments was generated by PCR, containing single or multiple basepair substitutions and/or deletions, and cloned in front of the *gusA* reporter gene (Fig. 2). To determine the promoter strength and the inducibility by nisin, all the resulting plasmids were introduced in both strain *L. lactis* NZ3900 and in strain *L. lactis* MG1363, lacking the signal transduction machinery for nisin. Substitutions outside the two pentanucleotide repeats or substitutions of the T or A inside the repeat did not lead to differences in inducibility or promoter strength (pNZ8056, pNZ8061-8065) (Fig. 2). Substitution of the C or the G in the repeat to a T or an A (pNZ8054, pNZ8055) led to controlled but decreased *gusA* expression in NZ3900 after induction with nisin A (1 ng/ml) compared to the wild type expression. No GusA activity was detected in MG1363 harboring these plasmids. Substitutions of both the C and the G in either one of the pentanucleotide repeats (pNZ8068, pNZ8076) or the deletion of the middle T in the repeat centered at -26 (pNZ8060) abolished inducibility and promoter activity (Fig. 2). A small decrease in GusA activity but no difference in inducibility was observed between *L. lactis* NZ3900 cells harboring pNZ8008 and cells harboring the constructed plasmid containing the inversion mutant (pNZ8073), which shows that only the two pentanucleotide sequences and the -10 region are required for inducibility.

Most promoters in *L. lactis* are characterized by the presence of canonical TTGACA and TATAAT sequences that are spaced by an average of 17 bp and located at -35 and -10, respectively (7). However, the *nisA* promoter shows an aberrant -35 sequence (CTGATT) and a spacing of 20 bp between this sequence and the canonical -10 sequence. In order to generate a more consensus promoter, mutations were made in the *nisA* minimal promoter fragment. Substitution of the C at -38 by a T (pNZ8057), resulting in the -35 sequence TTGATT, did not result in a difference in *gusA* expression in both the *L. lactis* strains (Fig. 2). In addition, when this mutation was introduced in combination with a double CG substitution at positions 25 and 27 (pNZ8075), or in combination with a 3-bp deletion outside the boxes (pNZ8059), leading to a spacing of 17, no GusA activity was found. However, when these mutations were combined with a -35 region that was changed into a more consensus one (TTGACA; pNZ8069), constitutive *gusA* expression was found in *L. lactis* NZ3900 and MG1363. This constitutive expression was also found in case of a consensus -35 region and a deletion of the middle CTG basepairs at position -26 (pNZ8080).

The 3-bp deletions, which either changed one of the pentanucleotide repeats (pNZ8078, pNZ8077) or the spacing between the repeats (pNZ8072) abolished GusA activities, probably because NisR was prevented from binding. The middle CTG deletion at position -26 (pNZ8077) did not show constitutive expression (compared to pNZ8080) probably because of the lack of a consensus -35 sequence. The deletions downstream the repeats were assumed to prevent RNA polymerase from binding because of the changed spacing between the repeats and the -10 region.

Fig. 2. Sequence of the wild-type (-35 and -10 sequences in bold) and the mutated *nisA* promoter fragments (mutations in bold) and a summary of the *gusA* expression assays in *L. lactis* strain NZ3900, with or without induction by nisin (1 ng/ml), or in *L. lactis* strain MG1363. The direct repeat is underlined. The specific β-glucuronidase activity per optical density (at 600 nm) unit is 0,13 ± 0,2 (100%). + = 100% ; +/- = 3% - 6% ; +* = 10% - 30% ; - = < 0,1%.

Construct	-37 -26 -10 +1	NZ3900 no Nisin	NZ3900 with Nisin	MG1363
8051	TCTGATTAAATTCTGAAGTTTGTTAGATACAATGATTTCG	-	+	-
1 or 2-basepair substitutions/deletions				
8053	TGTGATTAAATTCTGAAGTTTGTTAGATACAATGATTTCG	-	+	-
8076	TATTATTAAATTCTGAAGTTTGTTAGATACAATGATTTCG	-	-	-
8068	TCTGATTAAATTATTAAGTTTGTTAGATACAATGATTTCG	-	-	-
8060	TCTGATTAAATTC-GAAGTTTGTTAGATACAATGATTTCG	-	-	-
8054	TCTGATTAAATTTTGAAGTTTGTTAGATACAATGATTTCG	-	+/-	-
8055	TCTGATTAAATTCTAAAGTTTGTTAGATACAATGATTTCG	-	+/-	-
8061	TCTGATTAAATTCTGAAGTTTGTTAGATACAATGATTTCG	-	+	-
8062	TCTGATTAAAATCTGAAGTTTGTTAGATACAATGATTTCG	-	+	-
8063	TCTGATTAAATACTGAAGTTTGTTAGATACAATGATTTCG	-	+	-
8064	TCTGATTAAATTCAGAAGTTTGTTAGATACAATGATTTCG	-	+	-
8065	TCTGATTAAATTCTGTAGTTTGTTAGATACAATGATTTCG	-	+	-
8056	TCTGATTAAATTCTGAAGTTTATTAGATACAATGATTTCG	-	+	-
Inversion mutant				
8073	TCTGATTTTTATCTGATCAAACAATCATACAATGATTTCG	-	+/-	-
Consensus -35 and spacing				
8057	TTTGACAAAATTCTGAAGTTTGTTAGATACAATGATTTCG	-	+	-
8075	TTTGACAAAATTATTAAGTTTGTTAGATACAATGATTTCG	-	-	-
8059	TTTGACAAAATTCTGAA---TGTTAGATACAATGATTTCG	-	-	-
8074	TCTGATTAAATTATTAA---TGTTAGATACAATGATTTCG	-	-	-
8069	TTTGACAAAATTATTAA---TGTTAGATACAATGATTTCG	+*	+*	+*
8080	TTTGACAAAATT---AAGTTTGTTAGATACAATGATTTCG	+*	+*	+*
3-Basepair deletions				
8078	T---ATTAAATTCTGAAGTTTGTTAGATACAATGATTTCG	-	-	-
8072	TCTGATT---TTCTGAAGTTTGTTAGATACAATGATTTCG	-	-	-
8077	TCTGATTAAATT---AAGTTTGTTAGATACAATGATTTCG	-	-	-
8058	TCTGATTAAATTCTGAA---TGTTAGATACAATGATTTCG	-	-	-
8079	TCTGATTAAATTCTGAAGTTTGTTAG---CAATGATTTCG	-	-	-

Construction and overproduction of a His-tagged NisR protein. A fusion of the *nisR* gene was made with six codons for histidine residues at the 3'end, under control of the T7 promoter generating pNZ8090. Production of His-tagged NisR in *E. coli* JM109(DE3)LysS under control of the inducible T7 promoter system was analyzed. Upon induction of *E. coli* JM109(DE3)LysS carrying plasmid pNZ8090, a clear additional protein band appeared on the Coomassie brilliant blue-stained SDS-polyacrylamide gel (Fig. 3, lane 1). This band was not visible in protein extracts from induced cells of *E. coli* JM109(DE3)LysS carrying pBluescript II (data not shown). The apparent molecular weight of the His-tagged protein was estimated to be about 29 kDa. This is in good agreement with the predicted size for the Histag-NisR product of 27,381 Da. Therefore, we conclude that the overproduced protein is the His-tagged NisR protein.

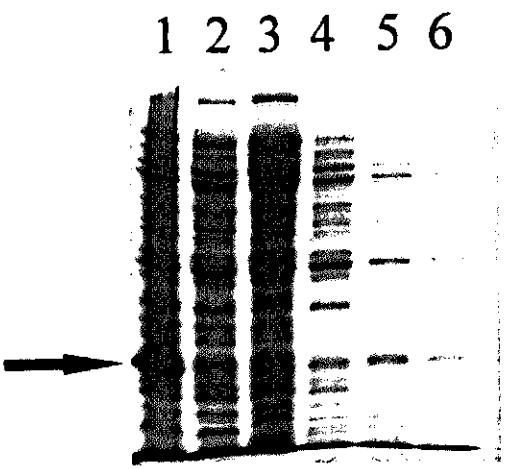

Fig. 3. Overproduction and preliminary purification of His-tagged NisR. Coomassie brilliant blue-stained gel after SDS-PAGE of extracts of strain *E. coli* JM109(DE3)LysS containing pNZ8090, producing His-tagged NisR. The fractions from each purification step were analyzed: Lanes: (1) 5 µl whole cell lysate; (2) 5 µl cell-free extract; (3) 15 µl of the wash-fraction eluted with 10 mM imidazole; (4) 15 µl of the wash-fraction eluted with 25 mM imidazole; (5) 15µl of the fraction eluted with 200 mM imidazole; (6) 15µl of the fraction eluted with 300 mM imidazole. The location of the overproduced NisR is indicated (arrow).

Partial purification and binding of the His-tagged NisR protein to the promoter region of the *nisA* promoter. In order to study its interaction with the promoter region of the *nisA* gene *in vitro*, the NisR regulator protein was partially purified from the overproducing *E. coli* strain harboring pNZ8090. The purification of His-tagged NisR was performed using a batch-wise Ni-NTA agarose binding procedure followed by desalting column chromotography.

Gel filtration was used to determine the oligomerization state of His-tagged NisR. When the sample containing the partially purified His-tagged NisR, was loaded on the column, proteins eluted at several peaks at different molecular weights (data not shown). However, the protein peak of about 30 kDa was identified as His-tagged NisR by SDS-PAGE, indicating that His-tagged NisR behaves predominantly as a monomer in solution under these conditions.

To determine whether the His-tagged NisR could bind to the *nisA* promoter, the partially purified fraction (fig. 3 lane 6) was incubated with the ^{32}P labeled 200-bp *nisA* promoter fragment of pNZ8051 for a gel mobility shift assay. With increasing His-tagged NisR concentrations (estimated to be 0, 3, 6, and 9 μg), we observed that the promoter fragment mobility decreased, suggesting that His-tagged NisR binds to the *nisA* promoter (Fig.4). When the labeled fragment was incubated with extracts that did not express *nisR*, or when a labeled 200-bp promoter fragment from pNZ8080 with His-tagged NisR concentrations (<10 μg) was used, no shift at all was observed (results not shown).

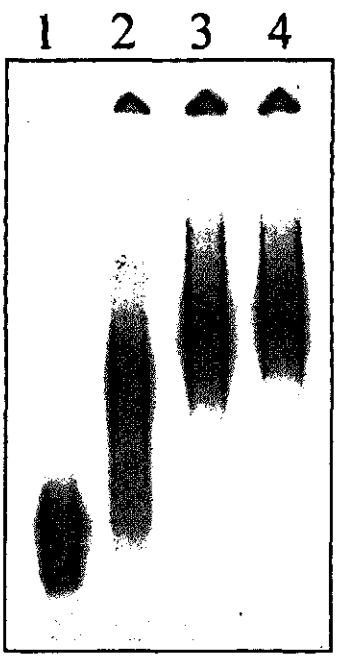

Fig. 4. Autoradiograph of a gel mobility shift analysis with or without protein extracts with His-tagged NisR. The DNA fragments containing the *nisA* promoter region of pNZ8051 were incubated with (1) no protein extract, (2) 3 μg, (3) 6μg, (4) 9 μg of His-tagged NisR extract.

DISCUSSION

Expression of *gusA* under control of several *nisA* promoter fragments, differing in length, was determined in order to assess the minimal promoter region involved in transcriptional control. This was found to be a fragment containing 39 bp upstream from the transcriptional start. The *nisA* promoter fragment of pNZ8008 was further analyzed for inducibility by nisin and transcription efficiency by use of site-directed mutagenesis. A direct repeat harboring two pentanucleotide sequences, centered at -26 and -37 upstream of the -10 region, was found to be present in both the *nisA* and *nisF* promoters (Fig. 2). Both pentanucleotides appear to contain critical residues involved in transcriptional regulation. Notably, substition of the C or the G residues at positions -25 and -27 decreased β-glucuronidase activity after induction by nisin while substitution of both the C and the G in either one of the pentanucleotide repeats abolished inducibility and activity. A deletion of 3-bp at position -25 to -27 together with a consensus -35 region resulted in constitutive *gusA* expression. Further mutational analysis of the direct repeat and scanning deletion analysis indicated that these pentanucleotide sequences and the spacing between the repeats and the -10 are required for inducible transcriptional activation of the *nisA* promoter. The importance of the spacers between the consensus sequences has also been reported in a recent study of synthetic constitutive *L. lactis* promoters (12).

Inspection of the *nisA* promoter reveals a remarkable lack of a consensus -35 region This leads to the suggestion that NisR, by binding to the repeat centered at -37, serves RNA polymerase by recognizing the *nisA* promoter. The process of transcription initiation consists of several events. First, recognition and binding of the σ factor-RNA polymerase complex to the promoter region takes place. This could suggest that NisR acts as a σ factor and binds together with RNA polymerase to the promoter region. However, there is no homology of NisR with known σ-factors from *L. lactis* or other bacteria and, moreover, this can not explain the functionality of NisR in other lactic acid bacteria (15). Therefore, we assume that NisR binds as a dimer to the pentanucleotide repeats and thereby triggers RNA polymerase, including σ-factor, to form a closed complex formation. This also explains the importance of the spacing between the pentanucleotide repeats and the -10 region. When NisR can not bind the DNA, such as in case of mutations within the pentanucleotide repeats, conserved -35 and -10 sequences spaced by 17 bp are necessary for RNA polymerase binding.

Many regulatory proteins that bind to DNA have been shown to occur as multimers. In addition, it has been demonstrated that unphosphorylated regulator proteins can be monomers in solution but dimerize upon phosphorylation (10, 22). For this reason, we examined the oligomerization state of the native NisR protein. Experiments, including molecular weight determinations on a Superdex 75 column, demonstrated that the His-tagged NisR was a monomer in solution. A possible explanation could be that NisR is predominantly in the unphosphorylated form. Another possibility is that the NisR protein is able to form multimers only during or after binding to the DNA molecule. In this regard it is interesting that the *nisA* and *nisF* promoter regions contains two tandem copies of the pentanucleotide sequence.

Phosphorylation has been found to increase the binding activity of regulator proteins to the target gene promoters. This has been shown by binding of the *phoB* promoter by the *B. subtilis* PhoP regulator protein, that also belongs to the OmpR subfamily of response regulators (20). Less protein was needed to observe retardation of the *phoB* promoter when PhoP was phosphorylated, indicating that PhoP-P binds more efficiently (20). However, it should be considered that it is not certain at present whether the His-tagged NisR protein we purified from *E. coli* represents the nonphosphorylated form of NisR, because there is *in vitro* and *in vivo* evidence that some response regulators can be phosphorylated rather nonspecifically by a low-molecular weight phosphodonors

(e.g. acetyl phosphate) in *E. coli* (21, 31). Therefore, it would be interesting to determine whether NisR can be phosphorylated *in vitro* and, if phosphorylated, whether it will bind more efficient to the *nisA* promoter fragment.

Presently, we favor the possibility that one or more NisR binds upstream of the -10 region to the pentanucleotide repeats. NisR would then assist RNA polymerase to trigger *nisA* transcription efficiently. However, to substantiate this model the NisR binding sites in the *nisA* promoter should be located by footprinting and other DNA binding studies with purified NisR in conjunction with wild-type and mutated promoter fragments.

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CHAPTER 6

Food-grade controlled lysis of *Lactococcus lactis* for accelerated cheese ripening

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Food-grade controlled lysis of *Lactococcus lactis* for accelerated cheese ripening

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An attractive approach to accelerate cheese ripening is to induce lysis of *Lactococcus lactis* starter strains for facilitated release of intracellular enzymes involved in flavor formation. Controlled expression of the lytic genes *lytA* and *lytH*, which encode the lysin and the holin proteins of the lactococcal bacteriophage ΦUS3, respectively, was accomplished by application of a food-grade nisin-inducible expression system. Simultaneous production of lysin and holin is essential to obtain efficient lysis and concomitant release of intracellular enzymes as exemplified by complete release of the debittering intracellular aminopeptidase N. Production of holin alone leads to partial lysis of the host cells, whereas production of lysin alone does not cause significant lysis. Model cheese experiments in which the inducible holin-lysin overproducing strain was used showed a fourfold increase in release of L-Lactate dehydrogenase activity into the curd relative to the control strain and the holin-overproducing strain, demonstrating the suitability of the system for cheese applications.

Keywords: cheese ripening, lysin, holin, *Lactococcus lactis*

Lactic acid bacteria are gram-positive bacteria that are widely used in a variety of dairy fermentation processes. They contribute to flavor formation and texture development while protecting the product from spoilage organisms. Notably, strains of the lactic acid starter bacterium *Lactococcus lactis* are of great economic importance because of their world-wide use in cheese making. It is assumed that the lysis of lactococci during cheese ripening results in the release of intracellular proteolytic and esterolytic enzymes, which contribute to flavor development. This process is relatively slow and enhancement of the rate of lysis could result in accelerated cheese ripening. In addition, lysis of starter bacteria is an attractive possibility for secretion of overproduced intracellular enzymes from lactococci in a food-grade manner[1,2]. Lysis can be achieved by host-encoded autolysis or through the action of lytic bacteriophages[3]. Both processes require hydrolytic enzymes, e.g., amidases or muramidases, that gain access to the cell wall either via direct secretion[4] or via cell-membrane disruption by a holin[5]. Holin genes are generally found immediately adjacent to lysin genes and code for small peptides with high structural similarity[6–8]. Lysis induced by bacteriophage infection has been combined with enhanced gene expression in *L. lactis*, yielding a system that can be exploited for accelerated cheese ripening[9]. However, controlled production of only the lytic proteins offers a noninfective approach for developing lactococcal strains that lyse upon demand. Initial attempts to exploit the lysin of ΦML3 in *L. lactis* were unsuccessful because a tightly controlled gene expression system was not available[10,11].

Recently, an inducible expression system has been developed for *L. lactis* that is based on the food-grade antimicrobial peptide nisin[2,12,13]. Subinhibitory amounts of nisin can induce transcription of genes cloned under control of the *nisA* promoter, in a linear dose-response. This is accomplished via signal transduction mediated by a two-component regulatory system comprising the histidine kinase NisK and the response regulator NisR[2,12,13]. This tightly controlled system allows for the construction of improved and specialized cheese starter cultures that will release their intracellular enzymes efficiently at an early stage in the cheese curd. We report the construction of lactococcal strains that express the holin (*lytH*) and the lysin (*lytA*) genes of bacteriophage ΦUS3 (ref. 14) in a controlled manner, permitting induced lysis. Furthermore, we show that the *lytH* gene encodes a protein that itself is capable of disrupting the cellular membrane, thereby facilitating release of intracellular enzymes without disturbing other starter strains.

Results

Nisin induced lysis: Effect of holin and lysin production on growth and viability of *L. lactis*. The adjacent genes *lytA*, encoding a lysin, and *lytH* (previously designated orf2) encoding a putative holin, from the *L. lactis* bacteriophage ΦUS3, have the gene order *lytHA*, which are separated by 150 bp[14]. Both genes were cloned either individually or as the lytic cassette *lytH-lytA* into the expression vector pNZ8010 (ref. 2) under control of the nisin-inducible *nisA* promoter, resulting in plasmids pNZ8011 (*lytHA*), pNZ8012 (*lytA*), and pNZ8013 (*lytH*), respectively. These plasmids were introduced in strain *L. lactis* NZ3900, an MG1363 derivative containing the *nisRK* signal transduction genes, integrated in the *pepN* locus on the chromosome[12,15]. The addition of the inducer peptide nisin (0.01 μg/ml) to growing cells of strain NZ3900 harboring the expression plasmid without the lytic genes did not cause any effect on growth (data not shown). In contrast, the addition of the same amount of nisin to strain NZ3900 harboring pNZ8011 containing the lytic cassette *lytH-lytA* resulted in a decrease in optical density (Fig. 1A). The number of colony forming units dropped more than 10,000-fold, indicative of cell lysis (data not shown). The extent of lysis was dependent on the amount of nisin added (Fig. 1A), and lysis could be induced throughout the exponential growth phase although the extent of lysis varied (Fig. 1B).

As expected, induction of only *lytA* expression using 0.01 μg nisin/ml did not cause any significant lysis of *L. lactis* NZ3900 cells harboring pNZ8012 (Fig. 2A). However, the induction of *lytH* expression using 0.01 μg nisin/ml almost immediately halted the growth of cells harboring pNZ8013 (Fig. 2A). Because the ribosome binding site of

release their intracellular contents. The latter will not happen if partial lysis by the holin-producing adjunct culture is permitted to occur, because these cells will not damage the surrounding bacteria. This can be advantageous when only release of enzymes of the adjunct starter culture is desired. Model cheeses were made that exhibited a fourfold faster release of LDH into the curd when the holin-lysin–producing strain was used relative to the other two strains, which is indicative of facilitated release of relevant flavor-forming enzymes in the curd. Instead of adding purified nisin, it is also possible to add small amounts of a fermentation broth from a nisin-producing *L. lactis* strain or even more simply to include a small amount of nisin-producing bacteria in the starter culture, which enables in situ inducer production. It might also be worthwhile to implement nisin-inducible holin-lysin systems in other adjunct starter cultures such as lactobacilli, streptococci, enterococci, and leuconostocs.

Cheese ripening costs, which amount to $13,000,000 per week for the annual production of Gouda cheese in the Netherlands, may be reduced significantly by application of the adjunct starter cultures described here.

Experimental protocol

Bacterial strains and culture conditions. *E. coli* MC1061 was grown in L-broth based medium with aeration at 37°C[6,19]. *L. lactis* strains were routinely grown at 30°C in media based on M17 (Merck, Darmstadt, Germany) supplemented with 0.5% (wt/vol) glucose (GM17). Chloramphenicol and erythromycin were used if appropriate at a concentration of 10 μg/ml). For model cheese making, cells of strain *L. lactis* ssp. *cremoris* SK110 were used, which were precultivated for 20 h at 20°C in milk.

Molecular cloning procedures. *E. coli* MC1061 was used as an intermediate host for cloning and was handled using standard techniques[19]. Plasmid DNA was introduced into *L. lactis* by electroporation[20]. Plasmid DNA was isolated from protoplasts of *L. lactis*[21]. Restriction fragments and plasmids were analyzed by agarose gel electrophoresis and purified by use of the USBioclean kit (*U.S. Biochemical Corp., Cleveland, OH*).

Construction of plasmids. A 1300-bp SspI-EcoRI fragment containing the *lytH* and *lytA* genes was isolated from pNZ1308 (ref. 14), treated with Klenow polymerase and cloned in pUC19, which was digested with HincII, to construct pUC19I. A 990-bp EcoRV fragment containing the lytA gene from pNZ1308 was also cloned in pUC19 digested with HincII, generating pUC19II. A 500-bp Sau3AI-SspI fragment containing the lytH gene was isolated from pNZ1308 and cloned in pUC19, digested with HincII, generating pUC19III. Subsequently, the lytH-lytA cassette and the lytH gene were isolated respectively as 1300- and 500-bp BamHI-EcoRI fragments from pUC19I and pUC19III and cloned under control of the nisA promoter in the expression vector pNZ8010 (ref. 2), which had been digested with BamHI and EcoRI, to construct plasmid pNZ8011 (lytH-lytA) and pNZ8013 (lytH). Plasmid pNZ8012 was constructed by cloning a 990-bp XbaI-PstI fragment from pUC19II in pNZ8010 which had been digested with XbaI and PstI. For construction of the translational fusion vectors, an NcoI site was introduced at the ATG start codon of the lytH gene by use of PCR mutagenesis. The gene was amplified by using approximately 100 ng pNZ8011 as a template and two primers: 5′-GCAAACAACCATGGTAACTAGAATG (containing two substitutions [boldface] generating the new NcoI site [underlined]) and 5′-CGAAGTA-CAACAAGCTTACGTCAATC (containing a HindIII site [underlined]). The primers are complementary to positions 353 to 378 and position 588 to 562 (according to the numbering of ref. 14). The amplified lytH gene was cloned as an NcoI-HindIII fragment into pNZ8032 (ref. 2), which was digested with NcoI and HindIII generating pNZ8036. For the construction of pNZ8038, the 1.2-kb BbsI-XhoI fragment of pNZ8011 containing part of lytH and complete lytA was cloned into the 3.1-kb vector fragment of pNZ8036, digested with BbsI and XhoI.

Nisin induction and monitoring of lysis. An overnight culture of *L. lactis* containing an expression plasmid was inoculated into fresh medium (GM17 with chloramphenicol 10 μg/ml) and grown until an A_{600nm} of 0.5 was reached. The cells were induced with different concentrations (0.02, 0.01, 0.005, or 0.001 μg/ml) of nisin A (referred to as nisin) or not treated. At several time intervals, cells were harvested and cell extracts were prepared[7]. These extracts or the culture supernatants were used for quantitative determination of aminopeptidase N activity using lysyl-*p*-nitroanilide (Fa. Bachem, Bubendorf, Switzerland) as a chromogenic substrate for PepN[16]. To exclude possible strain dominance in coculture experiments, strains were grown separately and mixed when an A_{600} of 0.5 was reached. In the mixed culture experiments with strains NZ3900 carrying pNZ8038 and MG1363 carrying pNZ1120, the individual strains were grown until an A_{600} of 0.5 was reached, cells were spun down and suspended together in fresh GM17 prewarmed at 30°C without antibiotics in the absence or presence of nisin.

Model cheese manufacturing. Model cheese was manufactured from pasteurized (30 min, 63°C) Nilac (a highly standardized low heat spray powder; Netherlands Institute for Dairy Research [NIZO], Ede, The Netherlands) skim milk supplemented with 0.1% yeast-extract. Cheese milk was inoculated with 2% of a full-grown culture of *L. lactis* ssp. *cremoris* SK110 complemented with either 0.4% NZ3900(pNZ8020), 0.4% NZ3900(pNZ8036), or 0.4% NZ3900(pNZ8038) of full grown cultures and incubated at 30°C. Sixty minutes after inoculation 0.023% rennet was added, and 75 min after incubation 1 μg/L nisin A was added for induction of the lytic genes. After 25-min setting of the milk, the curd was subsequently cut, drained, pressed, vacuumized, and stored at 13°C. LDH activity was assayed[22]. Released enzyme activity was determined in the whey, which was separated during ripening, and in the curd. The curd sample was diluted fivefold in 2% sodium citrate buffer (wt/vol) and subsequently homogenized for 5 min in a stomacher (Lab-Blender 400, Seward London). The sample was centrifuged for 10 min at 10,000 G to remove whole cells.

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CHAPTER 7

Summary and concluding remarks

SUMMARY AND CONCLUDING REMARKS

Lactic acid bacteria are gram-positive bacteria that are widely used in a variety of dairy fermentation processes. Notably, strains of the lactic acid starter bacterium *Lactococcus lactis* are of great economic importance because of their world-wide use in cheese making. The characteristic aroma, flavor and texture of cheese develops during ripening of the cheese curd through the action of numerous enzymes derived from the cheese milk, the coagulant, and the starter and non-starter bacteria. Ripening is a slow and consequently an expensive process that is not fully predictable or controllable. Principal methods by which accelerated ripening may be achieved include: an elevated ripening temperature, use of modified or adjunct starters, addition of exogenous enzymes, and use of cheese slurries. The advantages, limitations, technical feasibility and commercial potential of these methods are discussed in Chapter 1 of this thesis.

Since the growth of lactococci ceases at or shortly after the end of curd manufacture, their intracellular enzymes are ineffective until the cells die and lyse. It would be expected that the sooner starter enzymes are released through lysis, the sooner they can participate in flavor forming reactions and hence the faster the rate of cheese ripening could be. There is not a single compound or class of compounds which appears to be responsible for the full flavor of cheese. Several volatile components contribute to the flavor of cheese (8). In hard-type cheeses, such as Gouda and Cheddar, proteolytic enzymes from mesophilic lactococci play a crucial role in the formation of free amino acids during ripening (19). The enzymes from lactococci are also very important for the formation of flavor components from amino acids (27). However, to promote an adequate interaction between substrates and enzymes, lysis of cells leading to the release of intracellular enzymes into the cheese matrix, is considered to be essential.

In order to improve the properties of fermented products, in particular cheese, considerable interest exists in the development of genetic tools that allow production of desired proteins in lactic acid bacteria. Recently, the nature of the environmental stimulus that activates the regulatory pathway involved in nisin biosynthesis by *L. lactis* has been elucidated (13). Nisin is a ribosomally synthesized antimicrobial peptide which is widely used in the food industry as a natural preservative. Introduction of a 4 bp deletion in the structural *nisA* gene (*ΔnisA*) of a *L. lactis* strain that normally produces nisin, resulted not only in loss of nisin production but also in abolition of *ΔnisA* transcription. Transcription could be restored by the addition of subinhibitory amounts of nisin to the culture medium, which is an important finding leading to the insight that nisin may have both antimicrobial and signaling activity (13). The auto-regulatory process involved in nisin biosynthesis can be considered as a special form of quorum sensing in *L. lactis* (10)

Deletion, complementation and sequence comparison studies showed that the unusual *nisA* promoter is controlled in a signaling pathway that depends on the presence of intact *nisR* and *nisK* genes and requires fully mature nisin as the inducer. To further characterize this novel communication system at the molecular level, the unique interaction that is expected between nisin and the receiver part of the NisK sensor protein has been analyzed (Chapter 2). This was done by studying the response of the signal transduction machinery to nisin analogues produced by either protein engineering or organic synthesis. Nisin Z and several of its mutants were able to induce transcription. The N-terminal domain of

nisin was found to be essential for efficient communication and nisin mutants with improved and decreased signaling efficiency were identified. Transcriptional activation varied several hundred-fold depending on the actual mutation, with the T2S and M17W mutants of nisin Z being more potent inducers than nisin Z itself. Related peptides like the lantibiotics subtilin, lacticin 481, and Pep5, as well as the unmodified synthetic precursor of nisin A did not induce transcription. By fusing a *nisA* promoter fragment to the promoterless *E. coli* reporter gene *gusA*, induction capacities could be quantified and it was established that less than 5 molecules per cell of the best inducer (nisin Z T2S) are sufficient to activate Δ*nisA* transcription. Induction capacity and antimicrobial potency are clearly two different, independent characteristics of the nisin molecule. Synthetic nisin A fragments were used to show that the minimal requirement for induction capacity resided in the first 11 residues, comprising the first two ring structures (A and B) of nisin A (Fig. 1).

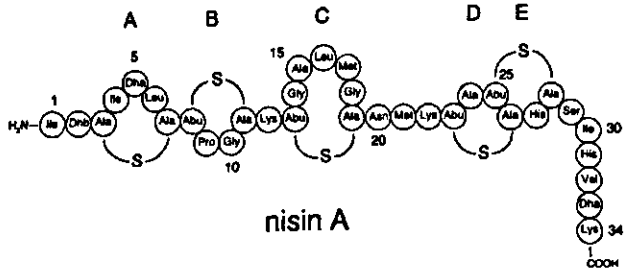

Fig. 1: Schematic outline of nisin A. Rings are labeled A, B, C, D, E.

Chapter 3 describes the characterization of the promoters in the nisin gene cluster *nisABTCIPRKFEG* of *L. lactis* by primer extension and transcriptional fusions to the *E. coli* promoterless β-glucuronidase gene (*gusA*) (3). Three promoters preceding the *nisA*, *nisR*, and *nisF* genes, all gave rise to *gusA* expression in the nisin-producing strain. The *nisR* promoter was shown to direct nisin-independent *gusA* expression in *L. lactis* MG1363. In the *L. lactis* strains, which contain the *nisRK* genes and the *nisF-gusA* fusion plasmid, a similar regulation by nisin was found as with the *nisA* promoter fragment. When the *nisK* gene was disrupted, no β-glucuronidase activity directed by the *nisF* promoter could be detected even after induction with nisin. These results show that, like the *nisA* promoter, the *nisF* promoter is nisin-inducible. The *nisF* and *nisA* promoter sequences share significant similarities and contain a conserved region that could be important for transcriptional control (see also Chapter 5).

Based on this regulated *nisA* promoter several cloning vectors were developed carrying the *nisA* promoter (Chapter 4). These vectors were tested in appropriate *L. lactis*

hosts that were specifically suited for controlled, nisin-inducible expression (4). These vectors and strains allow modulation of expression of several genes in a dynamic range of more than thousand-fold. They were used to study the kinetics of nisin induction and were applied for high level production of the *L. lactis* aminopeptidase N requiring subinhibitory amounts of the food-grade inducer nisin.

Controlled gene expression systems also provide the opportunity to study the effects of varying intracellular levels of proteins. Because of the growing interest in gene expression and organization, studies on regulation of gene expression in *L. lactis* have obtained increasing attention. Various expression systems using stable, broad host-range vectors and regulated promoters have been described for lactic acid bacteria (Table 1) (14).

Table 1: Characteristics of inducible expression systems for lactic acid bacteria

LAB	Inducible element	Inducing factor	Expressed gene(s)	Ratio induced: uninduced	Ref
L. lactis	*lacA* or *lacR* promoter	Lactose	*cat-86, luxAB*	< 10	6,25
L. lactis	*lacA*/T7 promoter	Lactose	gene for TTFC	< 20	26
L. lactis	*dnaJ* promoter	Temperature	*amyS*	< 4	24
L. lactis	*sodA* promoter	Aeration	*lacZ*	2	23
L. lactis	*prtP* or *prtM* promoter	Absence of peptides	*gusA*	< 8	17,18
L. lactis	repressor/operator ϕr1t	Mitomycin C	*lacZ*	70	20
Lb. pentosus	*xylA* promoter	Xylose	*cat-86*	60-80	16
L. lactis	Pal70 promoter	pH, Temperature	*lacZ*	50-100	8
L. lactis	*trpE* promoter	Absence of tryptophan	*LacZ*	100	2
L. lactis	ϕ31 promoter and ori	ϕ31 infection	*LacZ*	>1000	21
L. lactis	*gad* promoter, regulator *gadR*	Chloride	*lacZ, lytPR, acmA*	>1000	23
L. lactis	*nisA/nisF*	nisin	*gusA,pepN, lytHA*	> 1000	this work

These systems are based on transformation of lactic acid bacteria with plasmids containing effective expression signals, which can be regulated by inducers, repressors or environmental factors. Ideally, they will allow the controllable overproduction of proteins at high levels at any desired moment during industrial fermentation. To be able to use inducible gene expression systems in food production, the inducing signal should be either a safe food additive or a change in a physical parameter that can be easily applied in an industrial process. Considering this, the nisin controlled expression (NICE) system offers the best possibilities for regulating gene expression in lactic acid bacteria and has several advantages for application. First of all, nisin has a long history of safe use in the food industry and the NICE system is easy to use at low-costs because induction of cultures can take place by simply adding small subinhibitory amounts of nisin or a culture containing a nisin-producing *L. lactis* strain. In addition, the NICE system is versatile and flexible because several different expression strains and plasmids are available. Moreover, expression can be tightly controlled in a dynamic range, enabling production of lethal proteins and a controllable level of expression. Finally, a fully food-grade NICE system has been developed based on *lacF*-deficient lactococcal strains and the *lacF* gene as selective marker (15). Recently, it was established that the NICE system can also be functionally implemented in other lactic acid bacteria than *L. lactis* i. e. in *Lactobacillus helveticus* and *Leuconostoc lactis*. For this purpose transferable dual plasmid systems were developed, consisting of one plasmid expressing *nisRK* to a specific desired level and the other one containing the nisin-inducible promoter (11).

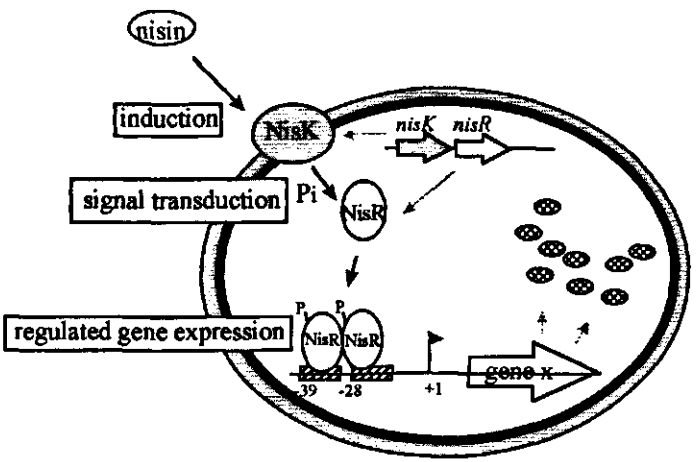

Fig. 2: Schematic model of the NICE system. The two pentanucleotide repeats, located upstream of the *nisA* transcription initiation site (arrow), are indicated by forward hatching. (See text for further explanation).

After establishing the mechanism of induction and controlled expression, the *nisA* promoter element, an essential component of the NICE system (Fig. 2), was studied in more detail (Chapter 5). In the nisin autoregulation process the NisR protein is expected to act as the response regulator, activating transcription of target genes. The *cis*-acting elements for NisR were identified as the *nisA* and *nisF* promoter fragments and these were further analyzed for inducibility. Expression of *gusA* under control of several *nisA* promoter fragments was monitored in order to determine the minimal promoter region. This analysis showed that transcriptional control is determined by a fragment containing 39 bp upstream of the *nisA* transcription start. A direct repeat consisting of two pentanucleotides, centered at -37 and -26, was shown to be present in both the *nisA* and *nisF* promoters. Mutational analysis of this direct repeat indicated it is required for transcriptional activation of the *nisA* promoter probably as a binding site for NisR (Fig. 2). Moreover, several 3 bp deletions showed that inducibility by nisin was also dependent on the spacing between these repeated pentanucleotides and the transcription initiation site. This also resembles a recent study on constitutive promoters of *L. lactis* which demonstrates that the context in which the consensus sequences are embedded, the spacing, clearly is important for promoter strength (9).

Based on the substantial similarity between the nisin and subtilin biosynthesis pathways, one can proposes a signaling peptide function for the mature subtilin molecule produced by *B. subtilis*. This assumption is supported by the finding that the production of subtilin is dependent of the presence of intact *spaR* and *spaK* genes (12). Furthermore, the *cis*-acting elements for SpaR could be the *spaI* and the *spaB* promoter fragments. In this regard it is interesting to note that the *spaI* and *spaB* putative promoter regions, like the *nisA* and *nisF* promoter regions, contain two tandem copies of a unique pentanucleotide sequence (Fig. 3).

```
nisA   TCTGATTAAATTCTGAAGTTTGTTAGATACAATGATTTCGTTCG
nisF   TCTGAATAGATTCTGAAAATTGTTTTATATACTTTTTTTAAACA
spaI   TTTGATTAAATTTTGATAAAAGTATTCTAGAATGGTCTGCATCC
spaB   CTTGATATTTTTTTTGATTTTTAGAATGTATAGTAAAAATAGAGT
```

Fig.3: Comparison of the *nisA* and *nisF* promoters (transcription start site (+ 1) indicated in bold italics) and the putative *spaI* and *spaB* promoter sequences. The -35 and -10 sites are underlined, the pentanucleotide sequences are indicated in bold.

Preliminary results described in Chapter 5 showed the direct binding of His-tagged NisR to the *nisA* promoter region. The symmetry in the two recognition motifs may support the possibility that NisR binds as a dimer (Fig. 2). This has to be further substantiated because the purified His-tagged NisR was identified as a monomer in solution in absence of DNA. However, the regulator protein, which is probably unphosphorylated, may dimerize upon phosphorylation or form a multimer only when bound to the DNA molecule. In addition, a number of transcriptional regulators have been reported that direct bending of the DNA helix at the contacted sequences (21). Therefore, a central question, which remains unanswered, is whether protein-induced bending plays an active role in transcription initiation or is just a consequence of protein binding. Protein-induced bending may act in transcriptional activation independently of (but in addition to) protein-protein interaction between the activator and RNA polymerase.

Chapter 6 describes the use and the possibilities for applications of the NICE system for the production of lytic enzymes (5). In view of the general importance of bacteriophages as an industrial problem in the dairy industry and the likely significance of autolysis in

intracellular enzyme release and flavor development in food products, lytic systems of lactic acid bacteria and their bacteriophages receive increasing attention. For the release of progeny from the host cell, the bacteriophages appear to encode a set of enzymes that degrade the host cell-envelope. This consists of several structural components, including peptidoglycan layer and cytoplasmic membrane. In coliphages, such as lambda, cell lysis has been assumed to depend upon bacteriophage-encoded proteins: e.g. holin and endolysin (1). Holins have thought to form a hole in the cytoplasmic membrane, through which the endolysin can attack the peptidoglycan layer.

Expression of the holin/lysin cassette (*lytPR)* of the lactococcal bacteriophage r1t and the autolysin gene (*acmA*) of *L. lactis* mediated by the chloride-inducible promoter in *L. lactis* resulted in cell lysis (23). This system has the disadvantage that it is not food-grade and that lysis is relatively slow in the presence of maximal induction. Controlled expression of the lytic genes *lytA* and *lytH*, which encode the lysin and the holin proteins of the lactococcal bacteriophage ϕUS3, respectively, was accomplished by application of the food-grade NICE system. Simultaneous production of lysin and holin is essential to obtain efficient lysis and concomitant release of intracellular enzymes as exemplified by complete release of debittering intracellular aminopeptidase N. Production of holin alone resulted in partial lysis of the host cells, whereas, production of lysin alone did not cause significant lysis. Model cheese experiments in which the inducible holin-lysin overproducing strain was used, showed a four-fold increase in release of L-lactate dehydrogenase activity into the curd relative to the control strain and the holin-overproducing strain, demonstrating the suitability of the system for cheese applications. This may eventually result in faster flavor formation and to new flavor balances in cheese, which are attractive features for both producers and consumers.

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SAMENVATTING

SAMENVATTING

De primaire functie van de melkzuurbacterie *Lactococcus lactis* als startercultuur voor de produktie van gefermenteerde melkprodukten is de goed onderzochte omzetting van melksuiker in melkzuur. De omzettingen die leiden tot de vorming van smaak zijn echter minder goed gekarakteriseerd. De afbraak van melkeiwit door proteases en peptidases speelt een belangrijke rol bij de vorming van smaak in gefermenteerde melkprodukten. Er is relatief weinig bekend over het belang en aandeel van andere intracellulaire enzymen bij smaakvorming. Wel is vastgesteld dat door het openbarsten, het zogenaamde lyseren, van melkzuurbacteriën, vrijkomende intracellulaire enzymen in belangrijke mate bijdragen aan de smaakvorming. Een aantrekkelijke manier om de kaasrijping te versnellen is dan ook om de beschikbaarheid van deze intracellulaire enzymen te vergroten door gecontroleerde lysis van de startercultuur te realiseren. Naast het gebruik als startercultuur bij de bereiding van gefermenteerde melkprodukten kunnen cultures met voorspelbare lytische eigenschappen toegepast worden als gastheer voor de overproduktie en secretie van intracellulaire enzymen in zowel gefermenteerde voedingsmiddelen als ook in industriële fermentaties. Tenslotte kan gecontroleerde lysis de voorspelbaarheid van de overleving van gemodificeerde starterculturen in commerciële produkten vergroten.

Hoofdstuk 1 beschrijft de voor- en nadelen alsmede de technische en commerciële mogelijkheden van een aantal methoden voor versnelde kaasrijping, zoals een verhoogde rijpingstemperatuur, gemodificeerde starters en het gebruik van enzymen. Daarnaast worden in dit hoofdstuk lytische systemen en gecontroleerde expressie-systemen in melkzuurbacteriën besproken. Recentelijk is een gecontroleerd expressie-systeem ontwikkeld dat gebruik maakt van het toevoegen van de voedselveilige, food-grade inducer nisine. Het principe van een expressie systeem is een gen, onder controle van geschikte en efficiënte transcriptie- en/of translatiesignalen, tot expressie kan worden gebracht in een geschikte gastheer, hetgeen leidt tot de produktie van het gewenste eiwit. In een gecontroleerd expressie-systeem wordt het eiwit van interesse geproduceerd als reactie op een signaal van buitenaf, bijvoorbeeld door een bepaalde toevoeging aan het groeimedium, een verandering in pH, osmotische waarde of temperatuur. Nisine is een antimicrobieel peptide dat wordt geproduceerd door verschillende stammen van *Lactococcus lactis* , en wordt gebruikt in de voedingsmiddelenindustrie als conserveermiddel, omdat het de groei van ongewenste bacteriën tegengaat.

Hoofdstuk 2 beschrijft de inductie-capaciteit van nisine, gemuteerde nisine soorten en nisine analogen. Het posttranslationele gemodificeerde nisine-molecuul wordt herkend aan de buitenkant van de cel door het sensor eiwit NisK. NisK geeft dit signaal door via signaaltransductie aan het regulator eiwit NisR. Het *nisA* promoter fragment, uit het nisine gencluster, werd gefuseerd met het promoterloze *gusA* reporter gen uit *Escherichia coli*, coderend voor β-glucuronidase. Deze fusie leidt, alléén na toevoeging van nisine, tot expressie van *gusA* in *L. lactis* NZ9800, een stam die geen nisine produceert door een deletie in het *nisA* gen (*ΔnisA*). Het expressie niveau van *gusA* is direct afhankelijk van de hoeveelheid nisine die wordt toegevoegd. Hele lage hoeveelheden nisine maar ook bepaalde gemuteerde nisine soorten of nisine analogen kunnen als inducer fungeren, terwijl andere peptiden of het ongemodificeerde precursor nisine niet in staat zijn te induceren. Er is geen direct verband tussen de antimicrobiële activiteit en de inductie capaciteit van gemuteerde nisine soorten aangetoond. De gemodificeerde aminozuur-residuen in het mature nisine-molecuul blijken noodzakelijk voor de inductie-capaciteit, waarvan vooral de eerste 11 residuen van belang zijn voor de vermoedelijke interactie met het sensor eiwit NisK. Dit betekent dat nisine niet alleen een antimicrobieel eiwit is maar ook ook een gesecreteerd signaal molecuul dat de expressie van

zijn eigen biosynthese genen induceert. Deze celdichtheid-gerelateerde vorm van regulatie is een voorbeeld van "quorum sensing" in Gram-positieve bacteriën.

In hoofdstuk 3 worden de promoters in het nisine gencluster verder gekarakteriseerd met behulp van primer extensie en transcriptionele fusies met het promoterloze *gusA* gen. Voor de genen *nisA*, *nisR* en *nisF* zijn drie promoters geïdentificeerd die leiden tot *gusA* expressie in de nisine-producerende stam *L. lactis* NZ9700. Omdat de transcriptionele autoregulatie van *nisA* door signaaltransductie via de sensor NisK en het regulator eiwit NisR is aangetoond, werd ook de mogelijke nisine-afhankelijke expressie van *gusA* onder de controle van de *nisR* en *nisF* promoters onderzocht. De *nisR* promoter gaf een nisine-onafhankelijke expressie van *gusA* te zien in de stam *L. lactis* MG1363, die het nisine gencluster niet bevat. De stam *L. lactis* NZ9800 (*ΔnisA)* met het *nisF-gusA* fusie plasmide, produceerde alleen β-glucuronidase na inductie met nisine. Dezelfde regulatie werd gevonden in *L. lactis* NZ3900, een stam met alleen de *nisR* en *nisK* genen op het chromosoom. Op het moment dat het *nisK* gen werd onderbroken, werd geen *nisF* promoter afhankelijke β-glucuronidase activiteit gevonden, zelfs niet na inductie met nisine. Deze resultaten tonen aan dat net als de *nisA* promoter, de *nisF* promoter nisine induceerbaar is. De *nisF* en de *nisA* promoter sequenties hebben significante overeenkomsten en bezitten een geconserveerde regio van 29 bp die van belang zou kunnen zijn bij de transcriptionele regulatie.

In hoofdstuk 4 wordt de gereguleerde *nisA* promoter gebruikt voor de ontwikkeling van gecontroleerde expressie-systemen voor *L. lactis*. De kinetiek, controle en efficiëntie van de nisine-geïnduceerde expressie zijn bestudeerd in *L. lactis* met behulp van transcriptionele en translationele fusies met het *gusA* reporter gen. In de nisine-producerende stam NZ9700 nam de specifieke β-glucuronidase activiteit erg snel toe na de mid-exponentiële groei tot aan een maximum bij het bereiken van de stationaire groeifase. In de stammen NZ9800 en NZ3900 was de β-glucuronidase activiteit afhankelijk van de hoeveelheid nisine die werd toegevoegd aan het medium. Zonder nisine werd geen β-glucuronidase geproduceerd. Om de translatie-initiatie te optimaliseren, werd een expressie-vector gemaakt door middel van een translationele fusie van het *gusA* gen met het start codon van het *nisA* gen. Het gebruik van deze vector leidt tot zes keer meer β-glucuronidase activiteit dan met de transcriptioneel gefuseerde vector in deze stammen na inductie met nisine. Op deze manier kan de expressie van een gen tot meer dan 1000-keer verhoogd worden. De β-glucuronidase activiteit bleek 25 keer hoger in extracten van stam NZ3900 dan in extracten van stam NZ9800. Door het gebruik van de translationeel gefuseerde vector werd het enzym aminopeptidase N overgeproduceerd tot 47% van het totaal intracellulair eiwit. Deze resultaten laten duidelijk de mogelijkheden zien van de toepassing van het nisine-induceerbare expressie-systeem (NICE-systeem) voor de overproduktie van elk gewenst eiwit.

Na het bestuderen van het inductie-mechanisme en de gecontroleerde expressie wordt het *nisA* promoter gebied nader bestudeerd in hoofdstuk 5. In de nisine-autoregulatie is het NisR eiwit het regulator eiwit dat de transcriptie van de betreffende genen activeert. De *cis*-werkende elementen voor NisR zijn de *nisA* en *nisF* promoter fragmenten en deze worden dan ook verder bestudeerd op induceerbaarheid. Expressie van *gusA* gereguleerd door verschillende *nisA* promoter-fragmenten, leidt tot de bepaling van een minimaal promoter fragment van 39 basen stroomopwaarts van de *nisA* transcriptiestart, dat betrokken is bij gecontroleerde transcriptie. Kleine veranderingen, substituties of deleties, in één of twee baseparen in het -35 tot -10 gebied van de *nisA* promoter, leiden tot het opheffen van induceerbaarheid. Een stukje sequentie bestaande uit twee penta-nucleotiden (TCTGA) in het -10 tot -35 gebied, is aanwezig in zowel de *nisA* als de *nisF* promoter. Mutagenese van dit penta-nucleotide toonde dat dit betrokken is bij de transcriptionele activatie van de *nisA* promoter, en waarschijnlijk dient als bindingsplaats voor NisR. NisR werd met behulp van een histidine-staart overgeproduceerd en gedeeltelijk gezuiverd

met affiniteits-chromatografie. Voorlopige resultaten met gel retardatie studies tonen aan dat het NisR eiwit bindt aan de *nisA* promoter. Vervolg onderzoek zal dit echter nog moeten bevestigen.

In hoofdstuk 6 wordt het nisine inductie-systeem toegepast voor het uiteindelijke doel van dit proefschrift, namelijk gecontroleerde lysis van melkzuurbacteriën voor versnelde kaasrijping. Bij verschillende bacteriofagen van melkzuurbacteriën zijn op het DNA genen gevonden die coderen voor zowel lysines, lytische enzymen die een celwandafbrekende functie hebben zoals lysozyme, amidases en muramidases, als ook voor holines, eiwitten die poriën in de bacteriële membraan kunnen maken. Lysines en holines zij beide nodig om bacteriële cellen te lyseren. Het holine is waarschijnlijk nodig voor de translocatie van het lysine door de celmembraan zodat deze de celwand bereikt en afbreekt. Een eerste aanzet tot de constructie van gemodificeerde *L. lactis* stammen die onder gecontroleerde omstandigheden kunnen lyseren is gegeven door de moleculaire analyse van een tweetal lysis genen van de *L. lactis* bacteriofaag US3, het lysine-coderende gen *lytA* en het holine-coderende gen *lytH*. In dit hoofdstuk wordt beschreven hoe deze lysis genen gekoppeld kunnen worden aan de nisine-induceerbare *nisA* promoter. Produktie van LytA en LytH in *L. lactis* NZ3900 na toevoeging van nisine leidt tot lysis. Het feit dat intracellulaire enzymen hierbij vrijkomen werd aangetoond door de activiteit te bepalen van het vrijkomend intracellulair aminopeptidase N. Produktie van alleen het holine leidt tot gedeeltelijke lysis van de *L. lactis* cellen, terwijl produktie van alleen het lysine niet resulteerde in lysis. Experimenten met model-kaas waarin de holine- en lysine-overproducerende stam werd gebruikt tonen een viervoudige toename van de L-lactaat dehydrogenase activiteit in de kaas ten opzichte van de holine-overproducerende stam. Deze resultaten laten duidelijk de toepassingsmogelijkheden zien van dit systeem voor het gebruik in kaas en de mogelijkheden voor versnelling van de rijping van dit en andere gefermenteerde zuivelprodukten.

CURRICULUM VITAE

De schrijfster van dit proefschrift werd op 28 juni 1968 geboren te Maastricht. Na het behalen van het VWO diploma aan het Stedelijk Lyceum te Maastricht in 1986 werd de studie gezondheidswetenschappen aan de Limburgse Universiteit begonnen. Deze studie was niet volgens verwachting en daardoor werd in 1987 begonnen aan de studie biotechnologie aan het HLO in Delft. Het afstuderen, de stage bij het SSDZ in Delft en de ingenieurs titel gaven niet genoeg voldoening en hetzelfde jaar, 1991, werd begonnen met de studie biologie aan de Universiteit van Utrecht. Door deze studie in voltijd en deeltijd te volgen kon binnen twee jaar het doctoraalexamen worden behaald. Onderzoek aan probiotica als afstudeervak bij de vakgroep voor voedingsmiddelen van dierlijke oorsprong (VVDO) in Utrecht wekte de interesse in melkzuurbacteriën. De afstudeerscriptie bij de vakgroep moleculaire microbiologie onder leiding van Prof. Hoekstra gaf meer inzicht in de algemene genetica. Met deze inzichten werd zij in 1993 als junior onderzoeker aangesteld bij het Nederlands Instituut voor Zuivelonderzoek (NIZO). Binnen de genetica groep van de afdeling biofysische chemie werd het in dit proefschrift beschreven onderzoek verricht onder leiding van Prof. Willem de Vos en Dr. Oscar Kuipers. Momenteel is zij werkzaam als hoofd Quality & Development bij Bakker Lekkerkerk Holland bv.

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