

## Molecular analysis of the regulation of nisin immunity

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**The genetic determinants controlling immunity to nisin are coordinately regulated, along with biosynthesis genes, in response to an environmental signal, nisin or a nisin analogue. The *nisR* gene product, the putative response regulator of nisin biosynthesis, was found to be a vital component of this induction mechanism. This protein forms part of a two-component regulatory system which controls the expression of genes involved in nisin immunity and biosynthesis. Analysis of the structural requirements of the external signal, using nisin fragments and engineered nisin variants, indicated that the 12 amino-terminal residues of the molecule are a minimum requirement for induction, with an intact ring A being an essential component. Changes throughout the molecule also affected its induction capacity. The production of certain variant nisins by engineered lactococcal strains is reduced in parallel with the strains' immunity to nisin. This can be attributed to inefficient induction by the variant molecule. Treating growing cultures with nisin restored full immunity and maximized the yields of nisin variants by the producer strains.**

**Keywords:** nisin, immunity, regulation, induction, *Lactococcus lactis*

### INTRODUCTION

Biosynthesis of the lantibiotic nisin is a complex mechanism requiring the activity of 11 genes (*nisABTCIPRKFE*) clustered together in the *Lactococcus lactis* genome. It is an inducible system in which the mature nisin molecule provides an external stimulus for expression of nisin-biosynthesis determinants (Kuipers *et al.*, 1995). The ability of nisin-producing bacteria to control the expression of the biosynthesis genes in response to an environmental stimulus involves a two-component regulatory system (Wanner, 1992) comprising a membrane-located 'sensor' protein (NisK) that detects the stimulus (Van der Meer *et al.*, 1993; Engelke *et al.*, 1994) and a cytoplasmic response regulator (NisR) that controls transcription from the *nisA* promoter (Kuipers *et al.*, 1993), thus facilitating an adaptive response.

In order for cells to produce nisin they must necessarily be immune to this antimicrobial peptide. However, at

present, the mechanism by which cells overcome the antagonistic effect of nisin is not fully understood. The *nisI* gene product has been implicated as an immunity protein because expression of the cloned gene in a sensitive *L. lactis* strain confers a degree of immunity on the host cell (Kuipers *et al.*, 1993; Engelke *et al.*, 1994; Qiao *et al.*, 1995). The *nisF*, *nisE* and *nisG* gene cluster is 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 (Siegers & Entian, 1995). It has been established that nisin production is linked to nisin immunity. When nisin-producing strains are rendered Nis<sup>−</sup> by inactivation of the structural gene for pre-nisin (*nisA*), the cells are significantly more sensitive to nisin (Dodd *et al.*, 1992; Kuipers *et al.*, 1993). It was observed that the actual level of immunity in different strains varied, depending on the type of mutation suffered by the *nisA* gene. This prompted an investigation into how induction affects the levels of immunity to nisin in different strains. Evidence is presented which indicates that immunity can be recovered by exposing cells to sub-inhibitory levels of nisin or nisin analogues. The involvement of the sensor histidine kinase (NisK) in the autoregulation of nisin biosynthesis has been reported (Kuipers *et al.*, 1995). Here, we demonstrate that the

**Abbreviations:** Dha, dehydroalanine; Dha5A, dehydroalanine substituted for alanine at position 5; Dha33A, dehydroalanine substituted for alanine at position 33; H27K, lysine substituted for histidine at position 27; I30W, tryptophan substituted for isoleucine at position 30; K12L, leucine substituted for lysine at position 12.

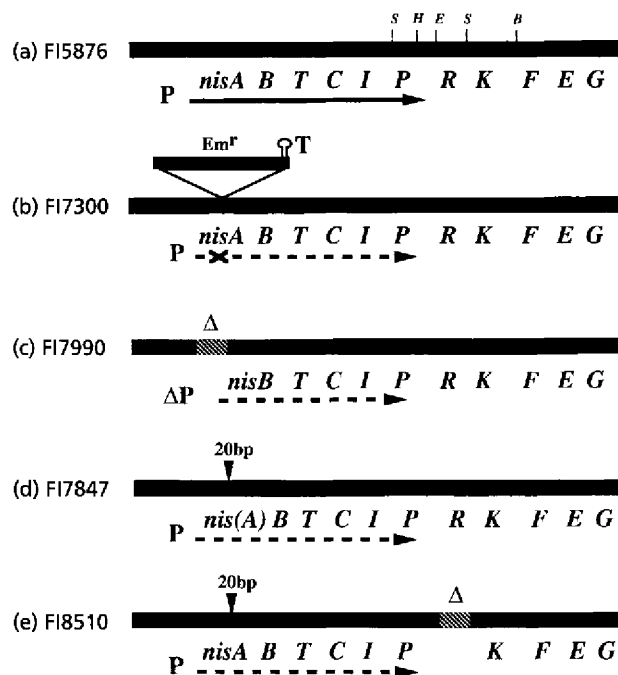
response regulator (NisR protein) is essential for induction of immunity, consistent with the proposal that the two-component regulatory system is integral to the control of expression of the nisin operon.

An analysis of the structural requirements of the inducing agent was undertaken involving a number of enzymically generated nisin fragments and engineered nisin variants. Nisin immunity was employed as a measure of the effectiveness of induction of the various analogues. The results indicate that the molecular interactions that occur between the external stimuli and NisK require specific parts of the nisin molecule for efficient induction of nisin biosynthesis and immunity.

In the course of studies involving the protein engineering of nisin (Dodd *et al.*, 1992, 1996), it was observed that, in a number of cases, strains producing variant nisin molecules displayed lower levels of immunity than the parent strain FI5876. The genetic linkage of nisin immunity to nisin production is effectively illustrated using engineered strains that produce variant nisins at reduced levels. By inducing full immunity it proved possible to optimize these expression systems, enabling maximum production levels of a variant nisin.

## METHODS

**Bacterial strains.** All strains used in this study are derivatives of the nisin-producing strain *Lactococcus lactis* FI5876 (Dodd *et al.*, 1990). Maps of Nis<sup>-</sup> derivatives are shown in Fig. 1. In each case, a plasmid-encoded wild-type *nisA* gene was converted to a defective gene, which was then incorporated into the chromosome using the technique of gene replacement (Dodd *et al.*, 1996). The construction of FI7300 (Fig. 1b) involved the insertional inactivation of the *nisA* gene by cloning a 1 kb fragment encoding erythromycin resistance (*Em*<sup>r</sup>) into the internal *Sac*I site of the *nisA*-coding region (Dodd *et al.*, 1992). This fragment included a potential rho-independent terminator at the end of the *Em*<sup>r</sup> gene (Horinouchi & Weisblum, 1982). In FI7990, the entire *nisA* gene was deleted as a result of engineering specific restriction enzyme sites on either side of the gene (Dodd *et al.*, 1996). By digesting and re-ligating DNA sequences, a 300 bp sequence including both the coding region and the upstream promoter was deleted (Fig. 1c). FI7847 is derived from FI7300 and carries a 20 bp insertion in *nisA*, resulting in its inactivation (Fig. 1d). In the gene replacement vector used in the construction of FI7300, the *Em*<sup>r</sup> gene and terminator signal were deleted by digestion with *Sma*I, followed by re-ligation. Residual sequences from the multiple cloning site which flanked the *Em*<sup>r</sup> gene leave a 20 bp insertion within the *Sac*I site and cause a frameshift mutation to occur in codon 16 of the *nisA* gene in FI7847. This would effectively truncate the predicted translation product at the carboxy-terminus. In all these Nis<sup>-</sup> strains, the sequences of the downstream genes have been unaffected by the changes introduced into *nisA*. Generating the NisR<sup>-</sup> strain FI8510 (Fig. 1e) required the construction of a gene replacement vector from cloned FI5876 sequences extending from the *Sac*I site in *nisP* to the *Bam*HI site in *nisF*, including the *nisR* and *nisK* genes. Sequences lying between the *Eco*RV site, within *nisR* and the *Sac*I site, 24 bp from the start of *nisK*, were deleted and replaced by a synthetic linker. This was generated by annealing the two complementary primers 5'-CTTGGGTATCAGTGGCATGGGTAAAAA-TATTCAATGCG and 5'-TCGACGCATTGAATATTTT-



**Fig. 1.** Maps of the region of *L. lactis* genome showing the relative location of the nisin genes *nisABTCIPRKFE G* in FI5876 (a) and Nis<sup>-</sup> derivatives FI7300 (b), FI7990 (c), FI7847 (d) and FI8510 (e). Mutations generated by insertion of DNA sequences are shown above the maps. The transcription terminator (T), downstream of the *Em*<sup>r</sup> gene in FI7300 (b), is represented by a hairpin loop. Deletions in chromosomal sequences (Δ) are indicated by a hatched region. The location of the inducible promoter upstream of *nisA* is indicated by P, and transcription of the proposed operon *nisABTCIP* is shown as an arrow below the maps. The broken arrows indicate that transcription may have been affected by the mutation suffered by these strains. Relevant restriction enzyme sites used in the construction of gene replacement vectors are shown above FI5876 (a): S, *Sac*I; H, *Hind*III; E, *Eco*RV; B, *Bam*HI.

TACCCATGCCACTGATACCCAAGAGCT, creating a short *Sac*I-*Sac*I linker containing the start of the *nisK* gene (underlined). As a result of this linkage of sequences flanking *nisR*, an intact *nisK* gene was regenerated including the upstream ribosome-binding site. The final gene replacement vector comprised 1.4 kb of FI5876 sequences containing the 3'-end of the *nisP* gene, a deletion of most of the coding region of *nisR* and a 2 kb downstream region containing a functional *nisK* gene. Using the technique of gene replacement (Dodd *et al.*, 1996), this vector was employed to delete the *nisR* gene from FI7847, generating the NisA<sup>-</sup> NisR<sup>-</sup> strain FI8510 (Fig. 1e).

The adaptation of *L. lactis* FI5876 to express engineered *nisA* genes has been described previously (Dodd *et al.*, 1996). In strains FI8327, FI8256, FI8278 and FI8290, the genes *nisA*/H27K, *nisA*/K12L, *nisA*/130W and *nisA*/Δ21M, respectively, have been incorporated into the genome of FI5876 in place of the wild-type *nisA* gene.

**Construction of pFI1002.** The *nisR* gene of FI5876 was isolated from a λEMBL3 genomic library (Dodd *et al.*, 1990) on a *Hind*III-*Sac*I fragment and cloned into the lactococcal shuttle vector pTG262 (Transgene), generating plasmid pFI1002. The cloned 1.13 kb fragment extends from the end of the *nisP* gene to the start of the *nisK* gene and includes the transcription

signals upstream of *nisR*. The molecular techniques used in these constructions were performed as described previously (Dodd *et al.*, 1996).

**Microbiological techniques.** Cultures were grown in M17 medium (Terzaghi & Sandine, 1975) supplemented with 0.5% (w/v) glucose (GM17 medium). Incubations were at 30 °C, unless carrying out the gene replacement protocol, in which case cultures were grown at either 28 °C or 37 °C (Dodd *et al.*, 1996). Screening strains for resistance to antibiotics was carried out at the following levels: erythromycin (Em<sup>r</sup>), 5 µg ml<sup>-1</sup>; streptomycin (Sm<sup>r</sup>), 200 µg ml<sup>-1</sup>.

*Escherichia coli* MC1022 (Casadaban & Cohen, 1980) was the host strain for construction of gene replacement vectors derived from the temperature-sensitive replicon pG<sup>+</sup>host6 (Appligene). Cultures were propagated at 37 °C in L broth (Lennox, 1955). Selection for pG<sup>+</sup>host6-encoded Em<sup>r</sup> and ampicillin resistance (Ap<sup>r</sup>) was carried out at 400 µg ml<sup>-1</sup> and at 200 µg ml<sup>-1</sup>, respectively.

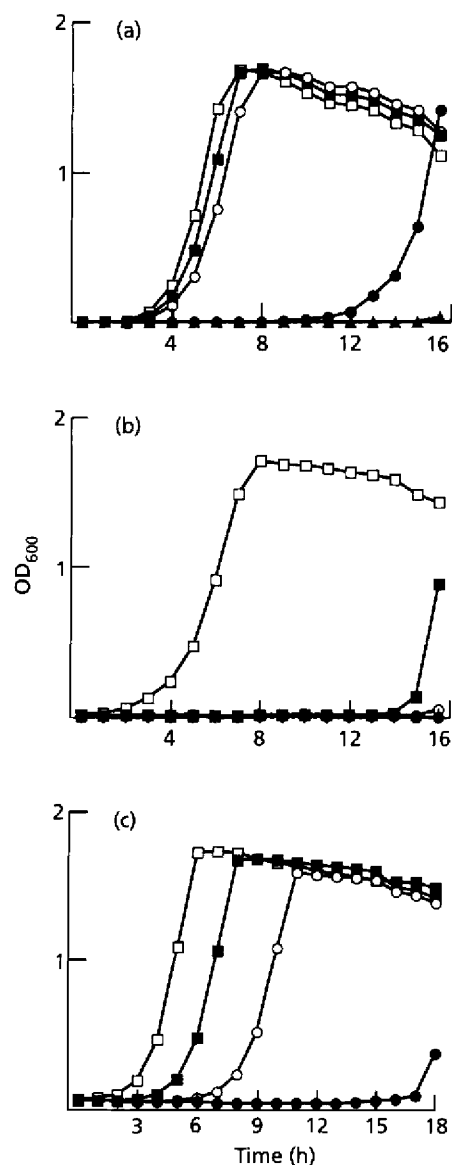
**Nisin assays.** Bioassays for production of nisin, or nisin variants, were based on the plate diffusion assay of Tramer & Fowler (1964) and were performed as described previously (Dodd *et al.*, 1996). An indication of the degree of immunity to nisin was obtained from streaking or spotting (3 µl aliquots) cultures onto the surface of a series of GM17 agar plates containing nisin at increasing concentrations. Immunity was taken as the lowest concentration of nisin at which growth was unaffected after 48 h incubation. Immunity assays in broth were carried out in sterile cuvettes containing GM17 broth inoculated with stationary phase cultures (1 in 500 dilution). Nisin, or nisin variant, at various concentrations, was added to the cuvettes and growth at 30 °C was monitored hourly (OD<sub>600</sub>; UVIKON 860 spectrophotometer). In microtitre plate assays, the wells (in triplicate), containing 200 µl GM17 broth, were inoculated with stationary phase cultures (1 in 200 dilution). Nisin, or nisin variant, was added and growth at 30 °C was monitored (OD<sub>630</sub>) on an automated microtitre plate reader (Dynotech). The amount of growth in each sample was determined at the time when the parent strain, in the absence of added nisin, had reached maximum cell density, usually at *t* = 6–7 h.

**Construction of nisin variants.** Protein-engineered nisins were generated and purified using the expression system described previously (Dodd *et al.*, 1996). Enzymically generated nisin fragments were produced by digesting nisin with trypsin (nisin<sub>1-12</sub>) or α-chymotrypsin (nisin<sub>1-20</sub>) (Chan, *et al.*, 1996). Treatment of nisin with HCl in glacial acetic acid generated (*des*-ΔAla5)nisin (Chan *et al.*, 1989). The 23 amino acid pre-nisin leader sequence was synthesized on an automated peptide synthesizer (Pepsynthesizer 9050; Millipore).

## RESULTS

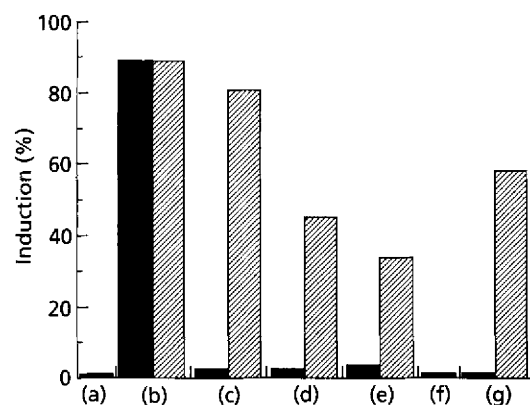
### *L. lactis* FI7847 – induction of immunity to nisin

Insertional inactivation of the *nisA* gene in strain FI7847 not only rendered this strain Nis<sup>-</sup> but also resulted in a significant reduction in its level of self-immunity to nisin. In plate assays, FI7847 displayed approximately 50% of the immunity levels of the Nis<sup>+</sup> parent strain FI5876 (no growth detectable above nisin concentrations of 20 µg ml<sup>-1</sup>). The capacity to recover wild-type levels of immunity in FI7847 was investigated by exposing growing cells to low levels of nisin prior to assaying for immunity in broth. When cultures were incubated overnight in the presence of subinhibitory levels of nisin (100 ng ml<sup>-1</sup>), immunity was significantly increased and,



**Fig. 2.** Immunity to nisin. Cultures were assayed for nisin immunity by determining the effect of nisin on growth in broth containing nisin at the following concentrations: 0 (□), 2 µg ml<sup>-1</sup> (■), 5 µg ml<sup>-1</sup> (○), 10 µg ml<sup>-1</sup> (●) and 20 µg ml<sup>-1</sup> (▲). OD<sub>600</sub> readings were taken hourly for cultures of (a) FI5876, (b) FI7847 and (c) FI7847 that had previously been grown overnight in the presence of 100 ng nisin ml<sup>-1</sup>.

in contrast to untreated FI7847 (Fig. 2b), cells challenged with up to 5 µg nisin ml<sup>-1</sup> grew well (Fig. 2c). The addition of nisin to the growth media had the effect of slightly increasing the lag phase when compared to that of FI5876 (Fig. 2a). Therefore, preincubating FI7847 with subinhibitory levels of nisin induces immunity to a level approaching that of the Nis<sup>+</sup> parent strain FI5876. This response was found to be dependent on the amount of nisin that the cells were exposed to, prior to assaying for immunity. Treating growing cells with 1 ng ml<sup>-1</sup> did not induce immunity. A concentration of 10 ng ml<sup>-1</sup> was sufficient for immunity to be restored, although growth



**Fig. 3.** Induction of immunity in FI5876 and Nis<sup>-</sup> derivatives. (a) MG1614; (b) FI5876; (c) FI7847; (d) FI7300; (e) FI7990; (f) FI8510; (g) FI8540. Cultures that had been preincubated with 100 ng nisin ml<sup>-1</sup> (hatched boxes) and the equivalent untreated cultures (filled boxes) were assayed in microtitre plates for nisin immunity. OD<sub>600</sub> values were taken when cultures grown in GM17 broth in the absence of nisin had reached maximum cell density. Induction, expressed as a percentage, is a measure of growth in the presence of nisin (2 µg ml<sup>-1</sup>), relative to growth in the absence of nisin. Values represent the mean of three experiments with standard errors less than 20%.

in 2 µg nisin ml<sup>-1</sup> was significantly slower (growth peaked approximately 2 h later) than that of cultures induced with 100 ng ml<sup>-1</sup> (Fig. 2c).

To test whether this induction phenomenon was reversible, FI7847 cells that had been pretreated with nisin were washed and used to inoculate fresh GM17 broth. After approximately 10 generations growth in the absence of nisin the cells were assayed and immunity was found to be comparable to that of cells which were not pre-exposed to nisin (Fig. 2b).

### Induction of immunity in Nis<sup>-</sup> strains

Cultures of the nisin-producing strain FI5876 and its Nis<sup>-</sup> derivatives (Fig. 1) were assayed for immunity to nisin and their ability to have immunity restored by induction was investigated. Strains FI7300 and FI7990 have suffered mutations to the *nisA* gene that respectively introduced a transcription terminator or deletion of the upstream promoter (Fig. 1b, c). Both mutations are likely to affect expression of genes downstream of *nisA*. Plate assays indicated that these mutations had rendered the cells more sensitive than FI7847 to nisin (immunity to nisin 10% wild-type level) and induction of immunity to nisin was also found to be less effective. Under induction conditions which restored complete immunity to FI7847 (Fig. 3c), both FI7300 and FI7990 displayed sensitivity to nisin (Fig. 3d, e).

### Role of NisR in induction of immunity

To test the role of the two-component regulatory system in immunity to nisin, a derivative of FI7847 (Fig. 1d) was generated that lacked the *nisR* gene. The effect of this

**Table 1.** Induction of immunity by nisin fragments

Cultures were preincubated with the inducing agent at either 30 or 300 nM and assayed for nisin immunity in microtitre plates. Molar concentrations were used to ensure that, when dealing with small fragments of nisin, a comparison of induction capacity could be made between the equivalent number of molecules (30 nM nisin is approximately 100 ng ml<sup>-1</sup>). Immunity was determined by growth of cultures in the presence of 2 µg nisin ml<sup>-1</sup> relative to growth in the absence of nisin, and is expressed as a percentage.

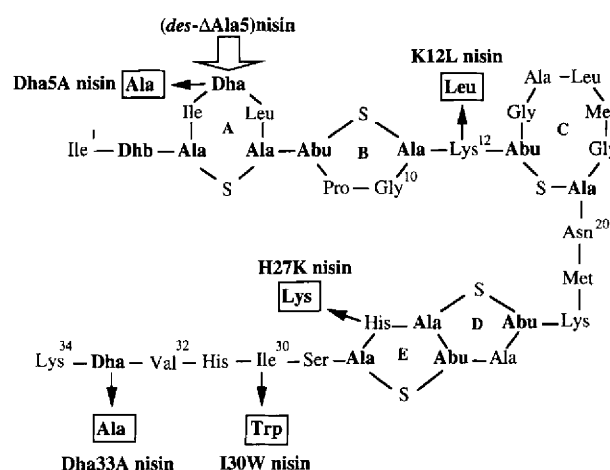
Inducing agent (nisin fragment)	Immunity with inducing agent at:		Lanthionine rings
	30 nM	300 nM	
Nisin <sub>1-34</sub> (wild-type)	100	98	ABCDE
Nisin <sub>1-32</sub>	98	97	ABCDE
Nisin <sub>1-20</sub>	3	108	ABC
Nisin <sub>1-12</sub>	1	9	AB
( <i>des</i> -Dha5)Nisin <sub>1-32</sub>	1	5	BCDE
Pre-nisin leader <sub>(1-23)</sub>	1	3	—

mutation, in strain FI8510 (Fig. 1e), was tested by assaying for immunity to nisin. FI8510 (NisA<sup>-</sup> NisR<sup>-</sup>) displayed a negligible level of immunity (Fig. 3f) comparable to that of the non-nisin-producing strain MG1614 (Fig. 3a). As with MG1614, the sensitivity of FI8510 was such that it did not grow in the presence of inducing levels of nisin. Hence, the ability to induce immunity was lost as a result of the deletion of *nisR*.

The *nisR* gene and upstream promoter region of FI5876 were cloned into the lactococcal vector pTG262, generating pFI1002. The ability of the plasmid-encoded gene to complement the NisR<sup>-</sup> deficiency in FI8510 was tested by transforming this strain with pFI1002. The resulting strain, designated FI8540, was able to respond to induction with nisin. The induced strain had recovered immunity, although not to the same level as that of the parental strain FI7847 (Fig. 3g).

### Structural requirements of inducing agents

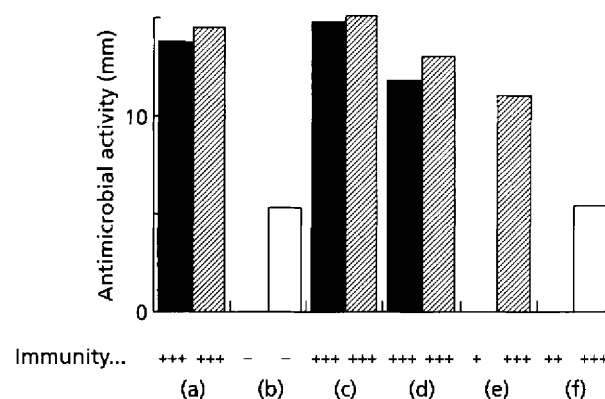
Using nisin immunity as an indicator of induction, the effectiveness of various derivatives of nisin as inducing agents was investigated. The nisin fragments tested were all able to induce immunity in FI7847, but with the smaller fragments the level of immunity was significantly lower (Table 1). When equimolar concentrations of nisin fragments were used it was evident that, at 30 nM (= 100 ng nisin A ml<sup>-1</sup>), the minimum requirement for induction of immunity was nisin<sub>1-32</sub> (Table 1). At the higher concentration of 300 nM, the nisin fragment nisin<sub>1-20</sub> was as effective at restoring immunity as the complete molecule (Table 1). Nisin<sub>1-12</sub> did not function as an inducing agent until a concentration of 600 nM was used. The only difference between nisin<sub>1-32</sub> and (*des*-ΔAla5)nisin<sub>1-32</sub> is cleavage of ring A (Chan *et al.*, 1989) in



**Fig. 4.** Molecular structure of nisin and nisin variants. Amino acid substitutions, in different engineered molecules, are indicated by arrows to boxes. The large arrow in ring A indicates the position at which cleavage occurs to generate (des- $\Delta$ Ala5)nisin.

the latter nisin derivative (Fig. 4). However, while nisin<sub>1-32</sub> functions effectively as an inducing agent, the ability of (des- $\Delta$ Ala5)nisin<sub>1-32</sub> to restore immunity in strain FI7847 has been abolished (Table 1). The leader peptide was also unable to induce immunity at any of the concentrations tested.

All the engineered peptides displayed the ability to induce nisin immunity in FI7847, although to varying degrees (Table 2). The alteration to ring A in Dha5A nisin A (Fig. 4) did not appear to adversely affect induction. In contrast, the substitution of a Dha for an alanine at position 33 in Dha33A nisin A and Dha5,33A nisin A had the effect of



**Fig. 5.** Effect of induction on immunity and production in strains producing engineered nisins. (a) FI5876; (b) FI7990; (c) FI8327; (d) FI8256; (e) FI8278; (f) FI8290. Antimicrobial activity is expressed as the diameter (mm) of the zone of inhibition (after subtracting the size of the well) of the indicator organism in plate bioassays. Samples from untreated cultures are shown as solid bars and the hatched bars represent samples from cultures that have been preincubated with nisin (100 ng ml<sup>-1</sup>). The open boxes indicate where a small zone of inhibition was generated due to the nisin in the broth (100 ng ml<sup>-1</sup>) included in the incubation. These samples, from strains FI7990 (b) and FI8290 (f), do not produce any additional antimicrobial activity as a result of the induction. Immunity was determined in plate assays on GM17 agar containing 20 µg nisin ml<sup>-1</sup>. Immunity was expressed in terms of the amount of growth relative to the wild-type strain FI5876. + + +, Confluent growth; + +, >10 colonies; +, <10 colonies; -, no growth.

significantly lowering the induction capacity of the molecules. This was restored by increasing the concentration of the molecules 10-fold. K12L nisin A, H27K nisin A and I30W nisin A (Fig. 5) were able to induce

**Table 2.** Induction of immunity by nisin and engineered nisin variants

Strain	Inducing agent (nisin variant)	Immunity* with inducing agent at:		Activity (%)†
		100 ng ml <sup>-1</sup>	1 µg ml <sup>-1</sup>	
MG1614	-	2	4	-
FI5876	-	94	100	-
FI7847	-	3	3	-
FI7847	A (wild-type)	104	104	100
FI7847	Dha5A	114	107	52
FI7847	Dha33A	17	101	52
FI7847	Dha5,33A	34	106	13
FI7847	H27K	86	ND	100
FI7847	K12L	81	ND	100
FI7847	I30W	41	ND	81

ND, Not determined.

\* Cultures were preincubated with the inducing agent at a concentration of either 100 ng ml<sup>-1</sup> or 1 µg ml<sup>-1</sup> and assayed for nisin immunity in microtitre plates (see legend to Table 1).

† Activity against *L. lactis* MG1614 (Gasson, 1983), relative to nisin A, was determined from minimum inhibitory concentrations. The value for nisin A (0.13 µg ml<sup>-1</sup>) was taken as 100% (Dodd *et al.*, 1996).

immunity, although to a lesser degree than the wild-type molecule (Table 2).

### Induction of immunity in strains producing nisin variants

The effect of induction in strains producing variant nisins was investigated. Immunity of cells that had been preincubated with nisin was determined and compared to that of the equivalent untreated cultures in plate assays. The NisA<sup>-</sup> strain FI7990 had significantly reduced immunity (Dodd *et al.*, 1996) and this could not be restored by induction (Fig. 5b). With derivative strains encoding a variant *nisA* gene, immunity had been affected to varying extents; however, in all cases, full immunity was restored in response to preincubation with nisin (Fig. 5c, d). A negligible increase in the production of antimicrobial activity, as a result of nisin induction, was evident in the parent strain FI5876 and the derivatives FI8327 and FI8256 (Fig. 5a, c, d). In the case of the nisin variant produced by FI8278, this yield increase was more striking. With no nisin induction, inhibition was not detected in bioassays. However, after preincubating the cells with nisin, antimicrobial activity in the supernatant sample had significantly increased (Fig. 5e). Pretreatment of FI8290 cells with nisin did not result in the production of antimicrobial activity. This engineered strain, encoding the variant gene *nisA*/Δ21M, did, however, display intermediate levels of immunity and recovered full immunity in response to induction.

### DISCUSSION

Autoregulation of nisin biosynthesis has recently been described with the demonstration of inducible *nisA* transcription in response to extracellular nisin (Kuipers *et al.*, 1995). Here we have shown that self-immunity of the nisin-producing strain FI5876 is also subject to nisin induction. This phenomenon would not normally be apparent as nisin-producing cells would be expected to be in a perpetual state of induction with continual production and immunity to nisin. Using the NisA<sup>-</sup> strain FI7847, it was possible to investigate the effect of nisin induction on the immunity function in the absence of nisin production. The nisin promoter of FI7847 is unaffected by the frameshift mutation suffered by the *nisA* gene (Fig. 1d) and expression of downstream genes would be expected to occur as a result of transcriptional readthrough. With this strain, it was possible to demonstrate that immunity to nisin could be restored to the level of the Nis<sup>+</sup> parent strain, as a result of induction with subinhibitory levels of nisin. Induction is a reversible phenomenon and the elevated immunity level was lost if the inducing agent was removed from the external environment. Cells reverted to the untreated state and were as sensitive as cells that have not been pre-exposed to nisin.

Kuipers *et al.* (1995) demonstrated that expression of the adjacent *nisB* gene is under the same autoregulation as *nisA*, with both being dependent on the presence of the upstream *nisA* promoter. From the work presented here, the number of genes in the cluster that are subject to the

same autoregulation can be extended to include those involved in self-immunity, the most likely candidate being *nisI* (Kuipers *et al.*, 1993; Engelke *et al.*, 1994; Qiao *et al.*, 1995). This suggests that coexpression of this gene and *nisA*, along with the intervening *nisB*, *nisT* and *nisC* genes (Fig. 1), occurs in response to induction with nisin. The absence of a transcription terminator at the end of *nisI* and lack of a convincing promoter for *nisP* argues that the complete polycistronic transcriptional unit, controlled by the inducible *nisA* promoter, includes six genes and extends from *nisA* to *nisP*. If transcription is prevented, due to deletion of the promoter (in the case of FI7990; Fig. 1c) or by introduction of a transcriptional terminator in *nisA* (in the case of FI7300; Fig. 1b), induction is impeded and immunity levels are reduced (Fig. 3). This is consistent with the proposal that genes within this transcriptional unit are involved in immunity. It was interesting to note that induction in these latter two strains is not completely abolished. The level of immunity displayed by these strains, approximately 40% of the parental strain (Fig. 3d, e), may be explained by limited transcriptional readthrough to the downstream genes including *nisI*. An alternative interpretation is that another gene(s), outside the putative *nisA*-*nisP* operon, is involved in immunity, as proposed by Qiao *et al.* (1995). It has been suggested that the *nisF*, *nisE* and *nisG* genes fulfil this role (Siegers & Entian, 1995). Furthermore, it has recently been reported that the expression of these three genes is under the control of an inducible promoter that responds to nisin in the external environment (O. P. Kuipers and others, personal communication). While further transcriptional analysis of the different cistrons is required, it can be speculated that, in the absence of a NisI function, an alternative immunity mechanism may exist that supplies a lower level of protection to the host cell.

The amount of inducing agent present in the external environment affects the level of immunity. It was only possible to detect induction of immunity with nisin at 10 ng ml<sup>-1</sup> (3 nM) or above. This concentration is 100-fold higher than that reported by Kuipers *et al.* (1995) for induction of transcription of the *nisA* gene. They found a reduction (~50-fold) in the level of transcription of sequences downstream of *nisA* and this was attributed to the presence of a large inverted repeat in the intergenic region between *nisA* and *nisB*. The function of this putative hairpin-loop structure may be to limit transcriptional readthrough to the genes downstream of *nisA*, which could account for the observed requirement for higher levels of the inducing agent to bring about full immunity.

An investigation of the role of the two-component regulatory system in nisin induction involved the construction of a strain that lacked the *nisR* gene, as well as containing a defective *nisA* gene. Nisin immunity in this strain, FI8510 (Fig. 1e), had effectively been abolished as a result of the chromosomal mutations. Its sensitivity was significantly higher than that of strains with reduced immunity due to impaired transcription, i.e. FI7847, FI7300 and FI7990 (Fig. 4). Unlike in these latter strains, it did not prove possible to elevate the levels of immunity

in FI8510 by induction with nisin (Fig. 3). Complementation of the deficiency by provision of a plasmid-encoded *nisR* gene restored an inducible immunity phenotype in strain FI8540 (Fig. 3). This is consistent with NisR acting as a response regulator necessary for activating transcription of the immunity determinants. The lower immunity level displayed by FI8540 may be due to the fact that the *nisR* gene is encoded by a multicopy plasmid rather than being cotranscribed with the chromosomal *nisK* gene. It has been demonstrated that NisK is an essential component of the nisin regulatory mechanism. Kuipers *et al.* (1995) created a NisK<sup>-</sup> strain in which transcription was not induced by external nisin. The results presented here complement these findings and underline the vital role of the two-component regulatory system in the autoregulation of nisin biosynthesis and immunity.

To identify which regions of nisin are required for induction, the capacity of a range of structural variants to function as inducers of immunity was assessed. Analysis of enzymically generated nisin fragments demonstrated that all five rings of the processed nisin molecule were necessary for efficient induction of immunity, although the 12 amino-terminal residues (rings A and B) were sufficient if the concentration was increased 20-fold (Table 1). A significant finding was that when ring A was broken in [*des*-Dha5]nisin<sub>1-32</sub>, induction was abolished (Table 1). The opening of this ring (Fig. 4) would cause an increase in the flexibility of the molecule and radically change the conformation of this region of nisin (Chan *et al.*, 1989). This suggests that ring A plays a major role in the molecular interactions between the inducing agent (nisin) and the membrane-located sensor protein NisK. An alteration to the constituent amino acids in ring A is not likely to have such an extreme effect on its conformation. The substitution of Dha for an Ala in the variant Dha5A nisin A simply results in loss of a double bond and an additional hydrogen at position 5 (Chan *et al.*, 1989). The biological activity of this engineered nisin was only slightly lower than that of nisin A (Dodd *et al.*, 1996) and its ability to function as an inducer of immunity was unaffected by the introduced changes (Table 2). Interestingly, amino acid changes made in other regions of nisin, including the carboxy-terminal tail, resulted in a reduction in the capacity of the molecule to induce immunity. The induction capacity of both Dha33A nisin and I30W nisin was less than 50% of that of the wild-type molecule (Table 2). It may be that overall conformational changes brought about by these mutations influence the binding properties of the molecule. The reduced efficiency of interaction between the inducing agent and the receptor molecule could be overcome by supplying the signal peptide at a higher concentration (Table 2). In similar work reported by Kuipers *et al.* (1995), it was also found that the amino-terminal region of the molecule (nisin<sub>1-11</sub>) was important for induction. From both studies, it was apparent that the ability of a nisin variant to act as an inducer of biosynthesis and immunity was not directly related to its specific activity against *L. lactis* MG1614 (Table 2). This suggests that antimicrobial interactions

with the membrane of sensitive cells resulting in pore formation (Giffard *et al.*, 1996; Abee, 1995; Gao *et al.*, 1991; Sahl, 1991) are distinct from the signal peptide/membrane sensor interactions that lead to induction of biosynthesis and immunity.

Increased knowledge of the role of induction in biosynthesis and immunity is of value in the exploitation of lactococcal expression systems based on genetic adaptation of the nisin operon (Dodd *et al.*, 1992, 1995, 1996). Bioassays of cell-free extracts demonstrate that peptide yields can vary considerably between different nisin variants, regardless of their specific activity. This can be attributed to variation in the induction capacity of the different engineered nisins. The biosynthesis of I30W nisin A by strain FI8278 is a good example of how poor induction by the engineered molecule (Table 2) results in very low levels of production. However, by adding subinhibitory levels of nisin to the growth media, immunity increased and, with it, the ability to produce significantly higher quantities of the modified peptide (Fig. 5e). The specific activity of the purified peptide was not much lower than that of the wild-type molecule, but its capacity to function as an inducer was considerably reduced (Table 2). Those strains which produce a nisin variant that functions efficiently as an inducer (e.g. FI8327 producing H27K nisin A) behave in a similar manner to FI5876. The strains are fully immune to nisin and production of antimicrobial activity is effectively at its maximum level (Fig. 5a, c). It was interesting to note that, while FI8290 (encoding *nisA*/Δ21M) did not generate any detectable antimicrobial activity, its immunity to nisin was above background level and, significantly, was found to be higher than that of FI8278 (Fig. 5e, f). This suggests that the nisin operon in FI8290 is expressed, resulting in the production and secretion of a processed Δ21M nisin A molecule. Furthermore, this shortened peptide, although not as efficient as nisin, retains its ability to act as an inducing agent. Such a molecule, lacking biological activity, may be of use in the development of lactococcal production systems in which the inducible *nisA* promoter is employed for the expression of heterologous peptides and proteins. The absence of biological activity by the inducing agent means that such an expression system need not involve any of the nisin immunity functions. A closely related line of work involves protein engineering the nisin molecule using lactococcal expression systems (Dodd *et al.*, 1995, 1996). The results reported here demonstrate that production levels of variant nisins can also benefit from induction, ensuring that the potential of the expression systems extends to the generation of those variant molecules that do not efficiently autoregulate their own biosynthesis.

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Received 6 March 1996; revised 7 May 1996; accepted 13 May 1996.