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A Lactococcal Expression System for Engineered Nisins

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The nisin-producing *Lactococcus lactis* strain FI5876 has been modified and developed for use as an expression system for engineered nisin variants. Insertional inactivation of the resident *nisA* gene had a polar effect on downstream genes, including those involved in nisin immunity. However, subsequent chromosomal rearrangements in this region involving a newly discovered insertion element (IS*905*) generated a strain that was deficient in the *nisA* gene product but expressed those nisin determinants necessary for prenisin maturation, secretion, and immunity. Complementation of the lesion in the *nisA* gene by plasmid-encoded *nisA* genes containing site-specific mutations resulted in the exclusive production of altered nisins containing specific amino acid substitutions.

Nisin is a highly modified peptide antibiotic produced by certain strains of *Lactococcus lactis*. It is of great interest to the food industry because of its efficient antimicrobial activity against a wide range of gram-positive organisms, including many spoilage bacteria and food pathogens such as *Listeria*, *Clostridium*, and *Bacillus* species (12, 25).

Nisin is a member of the family of antibiotics termed lantibiotics. These unusual polycyclic peptides share the structural features of dehydro residues and intrachain sulfide bridges forming lanthionine and β-methyllanthionine rings. The atypical residues are introduced by posttranslational modification of the amino acids serine, threonine, and cysteine in the primary sequence of a precursor peptide (lantibiotics are the subject of a recent extensive review [26]). Biosynthesis of nisin thus involves genes for both the inactive prenisin (*nisA*) and the modifying enzymes responsible for nisin maturation. The *nisA* gene has been cloned and characterized (1, 11, 27) and shown to have a chromosomal location (11, 42). A number of additional, as yet uncharacterized, genes involved in the enzymatic modification of prenisin, translocation, and immunity are encoded by nisin-producing strains (42). These determinants, along with *nisA*, are thought to be clustered together, as has been described recently for the lantibiotics subtilin (28) and epidermin (41). It has been known for some time that nisin determinants can be transferred by conjugation (14), and it has now been established that this ability is due to their carriage on a large conjugative transposon (38).

There is a growing interest in the protein engineering of lantibiotics, both as a means to probe the relationship between their structure and their function and to construct novel variants exhibiting modified properties. The ability to alter, for example, the solubility and stability properties or to broaden the activity spectra of nisin may provide a means of extending the applications of this valuable food-grade antibiotic.

Novel nisins could be constructed by the expression of variant *nisA* genes in a host strain which encodes the necessary maturation machinery and thus can process the modified precursor peptide. The simplest approach is to transform a nisin-producing strain with a recombinant plasmid encoding a variant *nisA* gene. In this background, the host's maturation enzymes are available to process both the resident prenisin and its plasmid-encoded variant. A strategy of this type has been reported for a strain that carries the wild-type nisin transposon (29). However, the disadvantage of this system is that both the host's nisin and the engineered variant are synthesized together, making complex chemical separation procedures necessary prior to analysis of the properties of the novel peptide.

Here we report the derivation of a lactococcal strain that expresses the maturation genes for nisin biosynthesis but is deficient in the *nisA* gene product. The construction involved a number of steps, including recombinant DNA technology, gene replacement, and in vivo chromosomal rearrangements. Transformation of this lactococcal host with recombinant plasmids which express either *nisA* or *nisA* containing a site-directed mutation led to the exclusive expression of nisin or a nisin variant. A number of alterations were made to the primary sequence of prenisin, and the effect of these changes on the biological activity of the mature molecule is described.

MATERIALS AND METHODS

Microbiological techniques and strains used. A number of *L. lactis* subsp. *lactis* strains generated in the course of this work were derived from the nisin-producing strain *L. lactis* FI5876 (11, 22). The construction of the derivative strains and their relevant properties are described below and in Tables 1 and 3. The plasmid-free, non-nisin-producing strain MG1614 (13) was included as a control. *L. lactis* strains were routinely grown at 30°C in M17 medium (43) supplemented with 0.5% (wt/vol) glucose (GM17 medium). The plasmids constructed during this work are listed in Table 2. Chloramphenicol resistance (Cm^r) was selected for at 5 μg/ml; erythromycin resistance (Em^r) was induced at the subinhibitory level of 50 ng/ml and then selected for at 5 μg/ml. Plasmid curing was achieved by growth in GM17 medium supplemented with acriflavine (36).

Escherichia coli MC1022 (3) was the host strain used for construction of recombinant plasmids. Cultures were propagated at 37°C in L broth (32). Selection for ampicillin resistance (Ap^r) was done at 100 μg/ml, and selection for Cm^r was done at 15 μg/ml.

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TABLE 1. Bacterial strains used in this study

Species and strain	Description and derivation	Reference or source
E. coli MC1022		3
L. helveticus CH-1	Culture collection strain	Christian Hansens Laboratories
L. lactis subsp. *lactis*		
MG1614	*L. lactis* subsp. *lactis* 712 cured of plasmids and prophage, resistant to streptomycin and rifampin	13
FI5876	MG1614 carrying nisin transposon Tn*5301*	11
FI7181	FI5876 with Campbell integration of pFI283 in *nisA* (Fig. 1c)	This study
FI6016	MG1614 carrying pFI172	This study
FI7300	FI5876 with Em^r gene from pE194 integrated into *Sac*I site of *nisA* gene (Fig. 1d and 3)	This study
FI7699	FI7300 carrying pFI172	This study
FI7304	Nisin-resistant, Em^s mutant of FI7300 caused by IS*905* integration (Fig. 3)	This study
FI7332	FI7330 cured of pFI172	This study
FI7328	FI7332 retransformed with pFI172	This study
FI7369	FI7332 carrying pFI378	This study
FI7357	FI7332 carrying pFI372	This study
FI7434	FI7332 carrying pFI354	This study
FI7462	FI7332 carrying pFI411	This study
FI7432	FI7332 carrying pFI403	This study
FI7393	FI7332 carrying pFI398	This study

Determination of nisin production by *L. lactis* strains was based on the plate diffusion assay of Tramer and Fowler (44). *Lactobacillus helveticus* CH-1 (Christian Hansens Laboratories A/S, Copenhagen, Denmark) was used as the nisin-sensitive indicator strain. A 0.5-ml sample of an overnight culture, grown in MRS medium (9), was used to seed 50 ml of MRS agar (pH 6.0) containing 1 ml of Tween 20-Ringer's solution (50:50). The wells were loaded with 100 μl of test sample, and the plates were incubated at 4°C for a minimum of 3 h (to allow diffusion) before overnight incubation at 42°C.

Nisin immunity was determined by streaking a loopful of stationary-phase cells on plates containing various amounts of nisin. Nisin (Koch-Lite), dissolved in 0.02 M HCl, was added to GM17 agar up to a maximum concentration of 5×10^3 U/ml, at which growth of all strains was inhibited. *L. lactis* strains were considered to be immune to the highest level of nisin at which growth was evident throughout the streak.

Transformation. Recombinant plasmids were recovered by transformation of *E. coli* by the method of Cohen et al. (8) with the modification of Humphreys et al. (24). *L. lactis* strains were transformed by electroporation as described by Holo and Nes (19) with the following modifications. Cells were grown in GM17 broth supplemented with 2% glycine, and selection was done on GM17 plates containing antibiotic. Sucrose was omitted from the initial growth media and the selection plates. Electroporation was performed with a Gene Pulser apparatus (Bio-Rad) by using a single pulse of 12.5 kV/cm, a capacitance of 25 μF, and a resistance of 200 Ω.

Molecular techniques. Total genomic DNA from *L. lactis* strains was prepared according to the method of Lewington et al. (33). Plasmid DNA was isolated by the sodium dodecyl sulfate alkaline lysis method. Covalently closed circular DNA was purified by CsCl-ethidium bromide gradient centrifugation (35). Restriction enzymes and other DNA-modifying enzymes from various sources were used according to the suppliers' recommendations. Conditions used for polymerase chain reaction (PCR) analysis were as described previously (22). The following primers were used in this study: primer 1, 5′-AAGAATCTCTCATGAGT; primer 1a, 5′-GGAAATAAGAGGCAATTT; primer 2, 5′-CCATGTCT GAACTAACA; primer 3, 5′-GTGGAATACGGGTTTG; primer 4, 5′-TAAATAATTTATAGCTATTG; primer 5, 5′-CAGAGCTCTGATGGGTTG (*Sac*I site underlined); primer 6, 5′-GTAGAATTCCGTTTATCGTTTGGAG (*Eco*RI site underlined); primer 7, 5′-GCAACTTGTCAGTGTAGTATT CAC; and primer 8, 5′-GTGAATACTACACTGACAAGT TGC. Determination of nucleotide sequences of plasmid

TABLE 2. Lactococcal plasmids used in this study

Plasmid	Description and derivation	Reference
pFI172	Plasmid pTG262 carrying a 5.5-kb *Sal*I fragment of nisin transposon Tn*5301* including the *nisA* gene	11
pFI283	Gene replacement vector based on *E. coli* vector pMTL23p with a Cm^r gene from pC194 and a *nisA* gene from pFI172 inactivated by insertion of an Em^r gene from pE194 (Fig. 1a)	This study
pFI354	pFI172 carrying a 1.2-kb *Sac*I-*Eco*RI deletion including C terminus of *nisA* (Fig. 5b)	This study
pFI378	pFI354 carrying a PCR fragment restoring the *nisA* gene (Fig. 5c)	This study
pFI411	As pFI378 but encoding *nisA*/H27Q	This study
pFI403	As pFI378 but encoding *nisA*/H27Q,V32I	This study
pFI398	As pFI378 but encoding *nisA*/H27Q,T23S	This study
pFI372	pFI354 carrying a *Sac*I-*Eco*RI oligonucleotide causing the *nisA*/Δ21–34 mutation	This study

DNA was performed by the dideoxy-chain termination method (40). Sequenase version 2.0 (United States Biochemical Corp.) was used according to the supplier's recommendations.

Construction of the gene replacement vector pFI283. The *nisA* gene of FI5876, cloned into the shuttle vector pTG262 to generate pFI172, has been described previously (11, 22). A 2-kb *Acc*I-*Sal*I fragment from this construct, containing *nisA* and the start of *nisB*, was subcloned into the pBR322-based vector pMTL23P (4). A 1-kb fragment encoding the Em^r gene of the staphylococcal plasmid pE194 (20) was inserted into the unique *Sac*I site in the cloned *nisA* gene. This insertion resulted in disruption of the *nisA* gene. The Em^r gene in this construction was transcribed in the same direction as the *nisA* gene and was flanked on either side by approximately 1 kb of lactococcal DNA sequences. A unique *Eco*RV site in the adjacent polylinker of the vector sequences was used to insert a 2-kb fragment carrying the Cm^r gene originating from the staphylococcal plasmid pC194 (21). A map of the resulting recombinant plasmid, pFI283, is shown in Fig. 1a.

Site-directed mutagenesis. Site-directed mutagenesis of the *nisA* gene was carried out by PCR-mediated overlap extension (18) with plasmid pFI172 DNA as a template. Primers 5 and 6, which contain a *Sac*I site and an *Eco*RI site, respectively, defined the ends of a 254-bp fragment encoding the C-terminal 20 amino acids of prenisin (see Fig. 6). A pair of overlapping complementary primers (7 and 8) were designed from sequences within this region of *nisA* which included a single base change from the original *nisA* sequence (Fig. 6). These were used in PCR amplifications in conjunction with one of the terminal primers (Fig. 5d) to create two partially complementary fragments with the specific mutation located in the overlapping region. The fragments were annealed to provide a template for a subsequent PCR involving the same primers that determine the two ends of the *Sac*I-*Eco*RI fragment (primers 5 and 6 [Fig. 5d]). The final PCR-generated fragment containing the specific mutation was purified by isolation from an agarose gel with a DEAE-NA 45 membrane (Schleicher & Schuell). Ragged ends were modified with T4 DNA polymerase and polynucleotide kinase. The blunt-ended fragment was cloned into the *Sma*I site of pUC18 (46), and the nucleotide sequence of the manipulated region was determined to confirm that the selected mutation was present.

Deletion of a 1.26-kb *Sac*I fragment from pFI172 generated plasmid pFI354, which encodes only the N-terminal part of *nisA* (Fig. 5b). A *Sac*I-*Eco*RI fragment from the pUC18 derivatives was then subcloned into pFI354 to recover an uninterrupted *nisA* reading frame (Fig. 5d). The various pFI354 derivatives are listed, together with their site-specific *nisA* mutations, in Table 4.

Construction of a truncated *nisA* gene. The complementary oligonucleotides 5′-CTGATGGGTTGTAACTAAG-3′ and (*Eco*RI) 5′-AATTCTTAGTTACAACCCATCAGAGCT-3′ (*Sac*I) were designed so that, as a result of annealing, overhangs which were complementary to a *Sac*I and an *Eco*RI site were created at each end. The synthetic fragment contained sequences from the *Sac*I site within *nisA* to the asparagine codon at position 20 (Fig. 6), followed by a TAA stop codon and a partial *Eco*RI site. The double-stranded fragment was cloned directly into pFI354 digested with *Sac*I and *Eco*RI (Fig. 5b), and the nucleotide sequence of the truncated *nisA* gene was confirmed.

Nucleotide sequence accession number. The GenBank/EMBL accession number of the nucleotide sequence in Fig. 6 is M27277.

RESULTS

Insertional inactivation of *nisA*. The gene replacement vector pFI283 was constructed to insertionally inactivate the chromosomally encoded *nisA* gene. It carries a cloned *nisA* gene which is disrupted by the insertion of an Em^r gene (Fig. 1a and Materials and Methods). The nisin-producing strain *L. lactis* FI5876 was transformed with pFI283, and six Em^r transformants were obtained. The plasmid does not encode a replication origin functional in *L. lactis*, and hence the recovery of the Em^r gene in these transformants required integration of this marker into the recipient chromosome.

Reciprocal recombination between homologous sequences on pFI283 (Fig. 1a) and the chromosome of FI5876 (Fig. 1b) could generate two types of transformants. A single crossover event would result in the entire plasmid integrating into the chromosome (Campbell integration). A double crossover event, one crossover on each side of the Em^r gene in pFI283, would exchange the wild-type chromosomal *nisA* for the insertionally inactivated copy, with subsequent loss of Cm^r encoded by the nonreplicating plasmid (gene replacement). Both recombination mechanisms have been shown to operate in *L. lactis* (7, 30, 31). The two alternative types of recombination could be distinguished phenotypically in the transformants obtained by screening for Cm^r. Five of the Em^r transformants were found to be Cm^r, suggesting that in these strains Campbell-type integration had occurred (Fig. 1c). One transformant, designated FI7300, had recovered only Em^r, suggesting that this strain had undergone gene replacement (Fig. 1d).

These proposed chromosomal rearrangements were investigated at the molecular level by PCR analysis with primers 1 and 2, which specifically amplify a 0.9-kb fragment from FI5876 chromosomal sequences containing the *nisA* gene and flanking regions (Fig. 1b and 2, track 3). When DNA from the Em^r Cm^s transformant FI7300 was used as a template, a 1.9-kb fragment was amplified by primers 1 and 2 (Fig. 2, track 5). A 1-kb increase in the size of this fragment would be expected if the Em^r gene were integrated in this part of the chromosome (Fig. 1d) and is consistent with the proposal that gene replacement had replaced the parental wild-type *nisA* gene with the insertionally inactivated copy.

The five Em^r Cm^r transformants (represented by strain FI7181 [Fig. 1c]) gave identical results with preliminary PCR analysis. By using template DNA from these transformants, two fragments were amplified by primers 1 and 2 (Fig. 2, track 4); the sizes of these fragments were equivalent to those of the fragments generated in FI5876 (Fig. 2, track 3) and FI7300 (Fig. 2, track 5). Campbell integration in these strains would result in two copies of *nisA* (the parental wild-type copy and the insertionally inactivated copy [Fig. 1c]), and hence amplification of both genes would generate the two different fragments observed.

Further PCR analysis involved primer 1a, which is specific for sequences upstream of primer 1 (Fig. 1b) and is derived from sequences which lie outside the region of homology present on the gene replacement vector pFI283 (Fig. 1a). Primers 1a and 2 amplify a 2.6-kb fragment of FI5876 chromosomal DNA (Fig. 2, track 9), and as would be expected, this fragment increased in size to 3.6 kb as a result of insertion of the Em^r marker in *nisA* when FI7300 DNA was used as a template (Fig. 2, track 11). The fact that

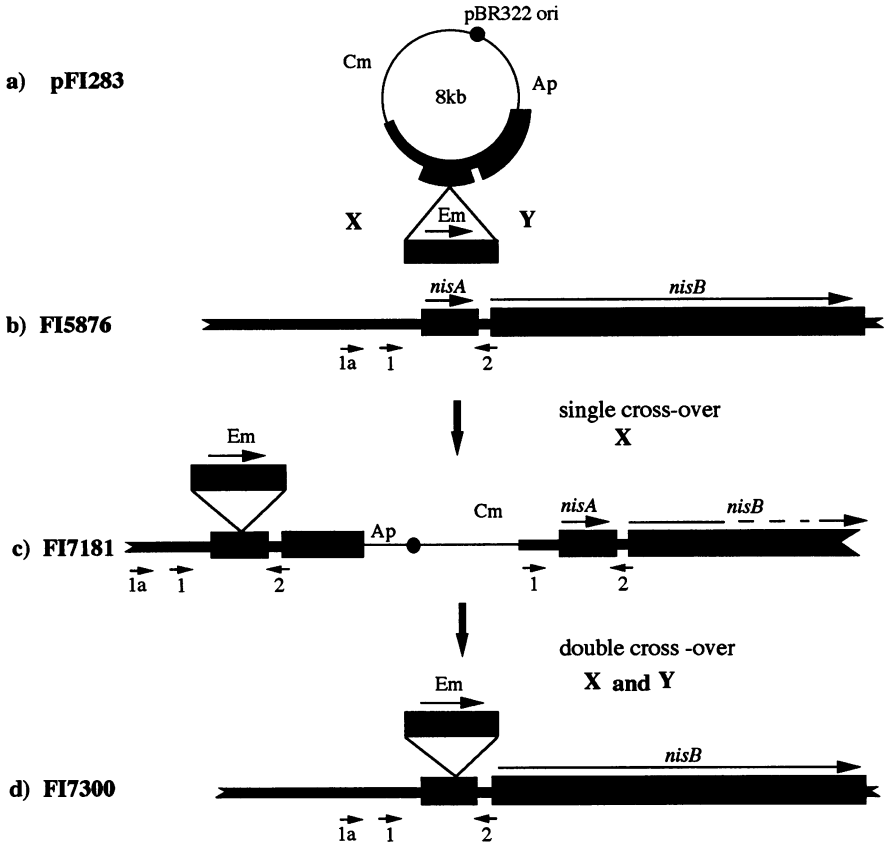

FIG. 1. Strategy for *nisA* gene replacement. The maps of plasmid pFI283, the gene replacement vector (a), and FI5876 (b), FI7181 (c), and FI7300 (d) show equivalent regions of the chromosome encoding *nisA* and flanking sequences. Thin lines represent plasmid DNA, and thick lines represent lactococcal chromosomal DNA. The *nisA* and *nisB* genes are indicated by black boxes, and DNA sequences containing the Em^r determinant are shown as shaded boxes. The direction of transcription of the genes is indicated by arrows above the maps. The small numbered arrows below the maps represent primers used in PCR analysis. A single recombination event between lactococcal sequences to the left of the Em^r determinant on pFI283 and homologous sequences on FI5876 (X) results in Campbell integration of the plasmid with the organization of sequences as shown for FI7181. Recombination between pFI283 sequences on both sides of the Em^r determinant and homologous FI5876 sequences (X and Y) leads to gene replacement, as found with FI7300. ori, origin of replication.

FI7181 DNA also generated a band of 3.6 kb (Fig. 2, track 10) and not 2.6 kb (as in the parent strain, FI5876 [cf. Fig. 2, track 9]) indicated that the organization of chromosomal sequences resulting from Campbell integration was as shown in Fig. 1c, with the Em^r gene integrated in the upstream copy of *nisA*.

The expression of nisin determinants in FI7181 appeared to be unaffected by the chromosomal rearrangements, and both nisin production levels and immunity were indistinguishable from those of FI5876 (Table 3). FI7300, having lost the parental *nisA* gene as a consequence of gene replacement, no longer produced nisin. Furthermore, the insertion in the *nisA* gene in this strain appeared to have affected nisin immunity levels (reduced to <500 U/ml [Table 3]).

In an attempt to recover nisin production in the *nisA*-deficient host FI7300, the strain was transformed with pFI172, which encodes *nisA*, and six transformants were tested for nisin production. The bioassays yielded negative results, indicating that the chromosomal mutation in this host could not be complemented by provision of the *nisA* gene product in *trans* (Table 4).

Activation of genes for immunity to nisin and modification. Reduction in nisin immunity due to the insertional inactivation of *nisA* in FI7300 may be caused by a polar effect on downstream genes. This may also result in reduced expression of genes required for modification, thus preventing complementation of the *nisA* mutation in this host. Derivatives of FI7300 which had spontaneously reverted to wild-type levels of nisin immunity were selected by growth in medium containing nisin at inhibitory levels. Colonies which grew on agar plates containing 10^3 U of nisin per ml were picked, and the cells were grown in medium containing the same level of nisin. One such mutant, designated FI7304, expressed wild-type levels of nisin immunity, did not produce nisin, and furthermore was no longer Em^r (Table 3).

PCR analysis of FI7304 DNA using primers 1 and 2 resulted in amplification of a 3.2-kb fragment (Fig. 2, track 6), which was 1.3 kb larger than the equivalent FI7300 fragment generated by the same primers (Fig. 2, track 5). This suggested that loss of Em^r was not caused by a deletion in this region of the genome. The retention of the Em^r gene sequences was confirmed by PCR with primers 3 and 4, which are specific for a region at the 3′ end of the Em^r gene and amplify a 0.4-kb fragment (Fig. 3b). As these primers generated an FI7304 fragment of 1.7 kb (data not shown), it was concluded that an additional 1.3 kb of DNA was inserted in this region of the Em^r gene (Fig. 3c). This results in loss of Em^r gene function with concurrent recovery of nisin immu-

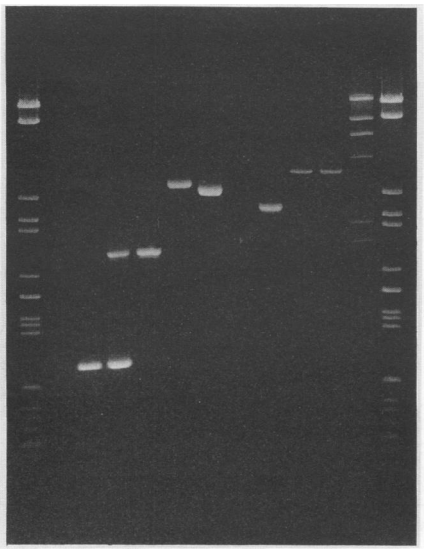

FIG. 2. Agarose gel electrophoresis of PCR fragments generated with primers 1 and 2 (tracks 2 to 7) and primers 1a and 2 (tracks 8 to 11). Tracks 1 and 13, lambda DNA digested with *BglI*. Tracks 2 to 11 show template chromosomal DNA from strains MG1614 (track 2), FI5876 (track 3), FI7181 (track 4), FI7300 (track 5), FI7304 (track 6), FI7332 (track 7), MG1614 (track 8), FI5876 (track 9), FI7181 (track 10), and FI7300 (track 11). Track 12, DNA digested with *Hin*dIII. The 1.2% agarose gel was electrophoresed for 2.5 h at 100 V.

nity (see below). A comparison of fragments from FI7300 and FI7304 generated by amplification with primers 3 and 2 and with primers 3 and 4 was consistent with this interpretation (Fig. 3b and c).

The extra DNA sequences gained by FI7304 were amplified with primers 3 and 4 (Fig. 3c), and this PCR fragment was used to probe a Southern blot of restriction enzyme-digested genomic DNA from the parent strain, FI5876. A number of fragments hybridized to the probe (data not shown), indicating that the additional DNA in FI7304 is present in multiple copies in the genome of this strain. Further investigation has revealed that these repeated sequences represent a new lactococcal insertion sequence designated IS*905* (unpublished data). As has been demonstrated for other insertion elements (2, 48), transcriptional read-through from a potential promoter within IS*905* may lead to turn-on of downstream genes. Such promoter activity could account for the observed increase in nisin immunity exhibited by FI7304 (equivalent to that of the parent strain [Table 3]) and may also have restored the expression of genes required for processing of prenisin to a level sufficient to facilitate *nisA* complementation.

TABLE 3. Nisin production and immunity

Strain	Em^r	Nisin production (NisA)[a]	Immunity to nisin (U/ml)[b]	Transformation frequency[c]	
				pTG262	pFI172
MG1614	−	−	<10	2.0×10^5	4.0×10^4
FI5876	−	+	$(1–3) \times 10^3$	2.0×10^4	70
FI7181	+	+	$(1–3) \times 10^3$	NT[d]	NT
FI7300	+	−	$(2–5) \times 10^2$	3.0×10^5	3.0×10^3
FI7304	−	−	$(1–3) \times 10^3$	1.5×10^4	24
FI7332	−	−	$(1–3) \times 10^3$	4.0×10^5	3.0×10^5

[a] Nisin production was determined by the plate diffusion assay.
[b] Levels at which strains were immune to nisin ranged between two values. At the lower level growth was unaffected, but at the higher level inhibition of growth was evident.
[c] Transformation was done by electroporation with 1 μg of plasmid DNA. Frequencies are given as transformants per microgram of DNA.
[d] NT, not tested.

Expression and maturation of plasmid-encoded *nisA*. FI7304 was transformed with the *nisA*-encoding plasmid pFI172. Transformants were obtained at a low frequency (Table 3), and the majority did not produce nisin in bioassays. One transformant, designated FI7330, was found to yield nisin at levels of approximately 50% of that of the parent strain FI5876 (Fig. 4), and this strain was analyzed further. Quantitation of nisin production in GM17 batch cultures of the parent strain FI5876 and the complementing strain FI7330 gave yields of 994 and 240 U/ml, respectively. It was presumed that FI7330 had undergone a spontaneous mutation, within either the plasmid or the chromosome sequences, which resulted in nisin production. Isolation of plasmid DNA from FI7330 yielded a molecule indistinguishable from pFI172 on the basis of restriction enzyme analysis (data not shown). Curing FI7330 of plasmid DNA to generate the plasmid-free strain FI7332 resulted in a loss of nisin production (Fig. 4). However, when plasmid pFI172 was introduced back into the latter strain, high transformation frequencies were obtained (Table 3), and all transformants produced nisin in bioassays.

When DNA from the plasmid-free strain FI7332 was analysed by PCR using primers 1 and 2, a small size reduction (200 bp) in the amplified fragment was observed. The deletion was in the vicinity of the IS*905* insertion in FI7304 (Fig. 2, cf. tracks 5 and 6). Primers 3 and 4 (derived from the Em^r gene sequences [Fig. 5b]) did not generate a fragment with this template, indicating that the deleted sequences in FI7332 included a region at the 3′ end of the Em^r gene in which IS*905* had been inserted. The deletion does not extend beyond the *nisA* gene, as primer 2 (specific for sequences at the end of *nisA*), together with either primer 1 or 3, resulted in fragment amplification (Fig. 3d). The small chromosomal rearrangement in FI7332 did not affect nisin immunity, which is conferred at a level indistinguishable from that of the parent strain (Table 3).

Expression of variant *nisA* genes. The expression vector pFI354 was constructed for incorporation of specific mutations into the *nisA* gene. It was derived from pFI172 by deletion of a 1.26-kb fragment containing the 20 C-terminal codons of the *nisA* gene (Fig. 5a and b). PCR amplification of pFI172 DNA using primers 5 and 6 (Fig. 5d and 6) generated a fragment which contained the deleted C-terminal end of the *nisA* gene, extending from the internal *Sac*I site to an *Eco*RI site (introduced by primer 6) in the sequence 249 bp downstream (Fig. 6). This fragment, which included the intercistronic region and the first 25 codons of *nisB*, was cloned as a *Sac*I-*Eco*RI fragment into pFI354 to generate pFI378 (Fig. 5c), in which an intact *nisA* gene was created. The functionality of this reconstructed gene was tested by introducing it into the FI7332 expression system and demonstrating in bioassays that nisin production occurred (Table 4 and Fig. 4).

To produce engineered nisin molecules, site-directed mutagenesis was carried out with the nucleotide sequence of the *nisA* gene which is contained within the 249-bp *Sac*I-*Eco*RI fragment (Fig. 6). Initially, the *nisA* codon selected for alteration was the codon for His-27, which lies within ring E of the mature nisin A molecule (Fig. 7). Primers 7 and 8 were

TABLE 4. Nisin activity

Strain	Host strain	Plasmid	Plasmid nisin gene	Nisin activity	
				Plate diffusion[a]	Colony overlay[b]
FI5876			*nisA*	++	++
MG1614				−	−
FI6016	MG1614	pFI172	*nisA*	−	−
FI7300				−	−
FI7699		pFI172	*nisA*	+	+
FI7304				−	−
FI7330[c]	FI7332	pFI172	*nisA*	+	+
FI7332				−	−
FI7328[c]	FI7332	pFI172	*nisA*	+	+
FI7434	FI7332	pFI354		−	−
FI7369	FI7332	pFI378	*nisA*	+	+
FI7462	FI7332	pFI411	*nisA*/H27Q	+	+
FI7432	FI7332	pFI403	*nisA*/H27Q,V32I	+	+
FI7393	FI7332	pFI398	*nisA*/H27Q,T23S	−	+
FI7357	FI7332	pFI372	*nisA*/Δ21–34	−	−

[a] Zones of growth inhibition were as follows: ++, 24 mm; +, 18 to 21 mm (including the 8-mm bore of the well).
[b] Zones of growth inhibition were as follows: ++, 23 mm; +, 15 to 18 mm.
[c] FI7330 was generated by transforming FI7304 with pFI172 and involved a spontaneous chromosomal deletion (see text). As the host strain is no longer FI7304, a new strain designation was allocated. After curing of pFI172, this strain, FI7332, was retransformed with pFI172 to generate FI7328. Strains FI7330 and FI7328 are thus probably identical, although their constructions were independent.

designed to exchange this histidine residue for a glutamine residue by PCR-mediated site-directed mutagenesis (Fig. 5d). A naturally occurring variant of nisin A called nisin Z, which contains an asparagine residue in place of His-27, has been identified (15, 37). Incorporation of glutamine, whose charge is similar to that of asparagine, in the same position would thus represent a conservative substitution in the nisin Z amino acid sequence. The mutation involved a single-base-pair change that resulted in the histidine codon CAT being changed to the glutamine codon CAG (Fig. 6). The variant gene was designated *nisA*/H27Q in accordance with accepted nomenclature (10).

In many cases, determination of the nucleotide sequences of the PCR-generated fragments revealed that nonselected base changes were also present in different clones. As a consequence of this lack of complete fidelity of the *Taq*I polymerase, it was possible to identify and construct genes containing, in addition to the mutation at position 27, an isoleucine in place of valine 32 (termed *nisA*/H27Q,V32I), and in an independent recombinant, a serine in place of threonine 23 (termed *nisA*/H27Q,T23S). Both of these secondary mutations involved alteration of a single base pair and resulted in a conservative amino acid change in the prenisin sequence. The structure of mature nisin A, together

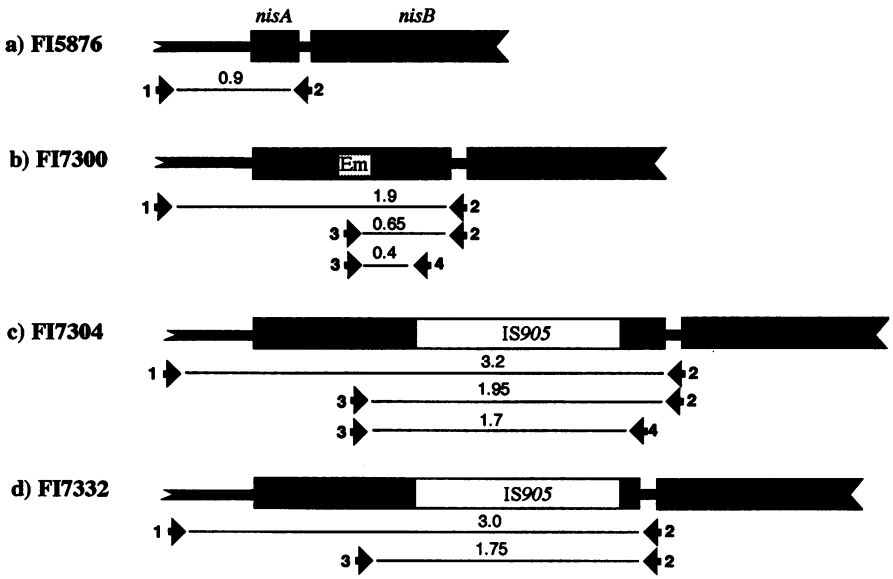

FIG. 3. Maps of equivalent chromosomal regions of the nisin-producing strain FI5876 (a) and derivatives FI7300 (b), FI7304 (c), and FI7332 (d). The *nisA* gene and the start of the *nisB* gene are indicated by black boxes. The insertions in *nisA* are signified by a shaded box (Em^r) and an open box (IS*905*). Primers used for analysis of *nisA* insertional inactivation are shown as numbered arrows below the maps. Lines connecting primers represent the amplified fragments, with sizes given in kilobases.

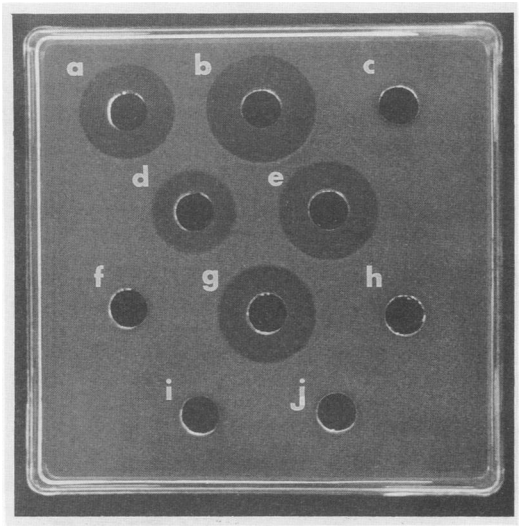

FIG. 4. Plate diffusion bioassay. The wells contain samples from the following strains (*nisA* gene in parentheses if present): FI7330 (a), FI5876 (*nisA* on Tn*5301*) (b), MG1614 (c), FI7332/pFI411 (*nisA*/H27Q) (d), FI7332/pFI378 (*nisA*) (e), FI7332/pFI372 (*nisA*/Δ21–34) (f), FI7332/pFI403 (*nisA*/H27Q,V32I) (g), FI7332/pFI398 (*nisA*/H27Q,T23S) (h), FI7332 (plasmid free) (i), and FI7332/pFI354 (*nisA*) (j). See Table 2 for plasmid-encoded *nisA* or *nisA* variant genes.

with the locations of the PCR-generated mutations in the processed molecule, is shown in Fig. 7.

The Nis$^-$ strain FI7332 was transformed with pFI354 and with the various derivative plasmids containing specific *nisA* mutations. The transformants were tested for antimicrobial activity in plate diffusion assays and colony overlays (Table 4). Strains containing the *nisA*, *nisA*/H27Q, and *nisA*/H27Q,V32I genes exhibited nisin activity in the plate diffusion assays. The levels of activity of the nisin variants were lower than that of a wild-type nisin-producing strain (FI5876) but comparable to that of a strain that produced nisin A by complementation (FI7332/pFI378 [Table 4 and Fig. 4]).

The biological activity exhibited by the strain containing *nisA*/H27Q,T23S was reduced to below the level of detection in the plate diffusion assay. However, zones of inhibition of a size similar to that of the *nisA*/H27Q strain were evident in colony overlays, suggesting that this assay is more sensitive than the plate diffusion assay method. The preparation of cell extracts for testing in the plate diffusion assay involves both heat treatment and reduction of the pH to 2.0 (44). One possibility was that the mutations in the *nisA*/H27Q,T23S gene had rendered the processed nisin A molecule unstable at high temperature or low pH. However, when these treatments were omitted from the extraction procedure, activity was not regained.

Plasmid pFI372, derived from pFI354, was engineered to express the truncated gene *nisA*/Δ21–34, which encodes the N-terminal 20 residues of nisin A (see Materials and Methods and Fig. 6 and 7). Transformation of FI7332 with pFI372

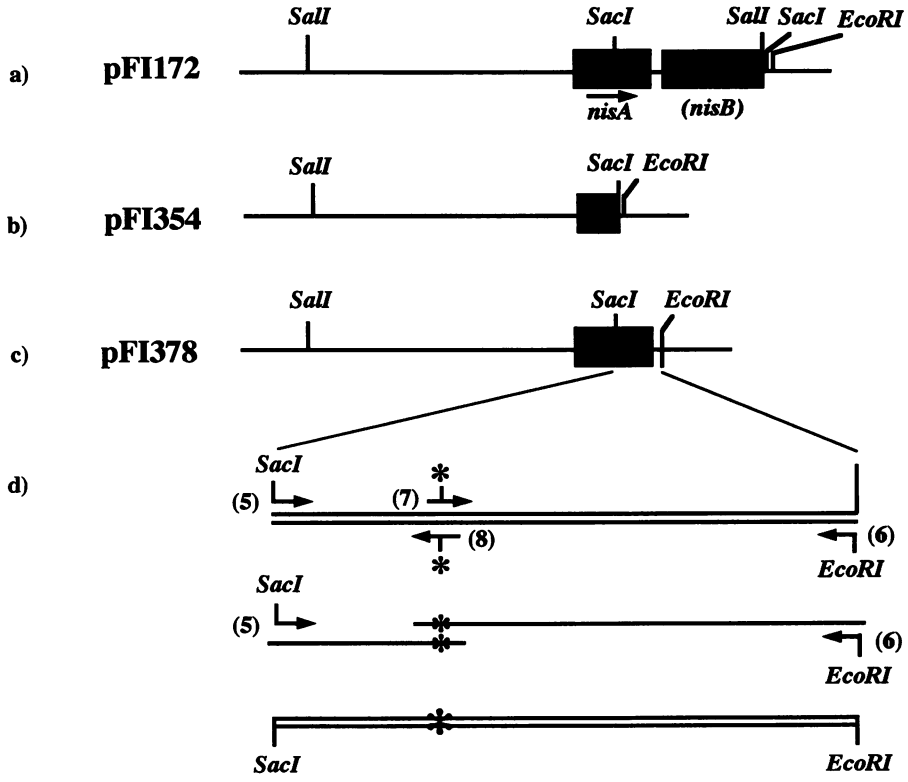

FIG. 5. Linear maps of plasmids pFI172 (a), pFI354 (b), and pFI378 (c). Genes within the cloned sequences are boxed, and relevant restriction sites are given above the maps. (d) Strategy for site-directed mutagenesis of plasmid-encoded *nisA*. The double-stranded nucleotide sequence between the *Sac*I and *Eco*RI sites of pFI378 is represented by a double line. The sites at which primers anneal are shown as arrows above and below the lines. The mismatches incorporated in primers 7 and 8 (Fig. 6) and the mutations they generate in the amplified fragment are indicated with asterisks.

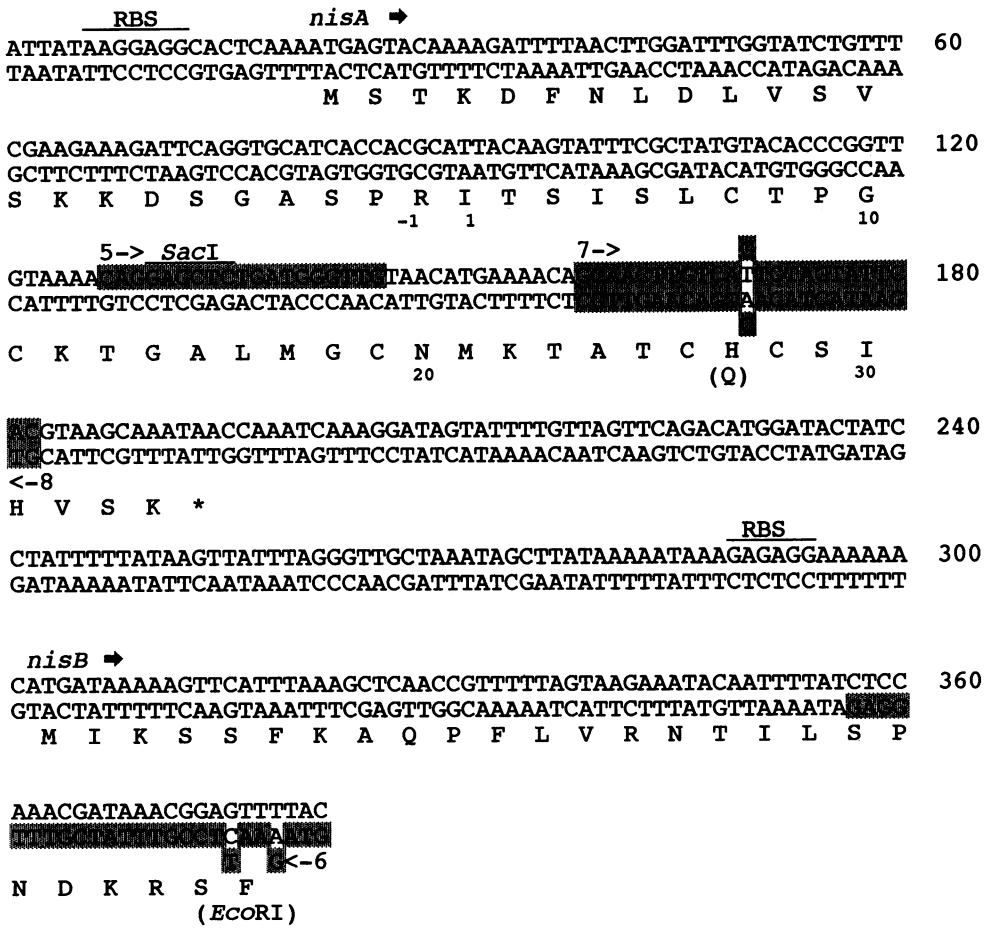

FIG. 6. Double-stranded nucleotide sequence of the *nisA* gene and flanking regions. Coding regions preceded by ribosome binding sites (RBS) are indicated, and the primary translation products are given below the sequence. The point of cleavage of the N-terminal leader sequence of prenisin is located between the arginine (R) at position −1 and the isoleucine (I) at position 1. Shaded regions represent the nucleotide sequences of synthetic oligomers used as primers for PCR-mediated site-directed mutagenesis. The 5′ ends of the primers and the directions in which the PCR proceeds are indicated by numbered arrows. Specific mismatches included in the primers are represented by shaded nucleotides above or below the sequence. Primers 7 and 8 are designed to substitute a glutamine (Q) residue for His-27 (H), and primer 6 has an *Eco*RI site incorporated into the sequence downstream of *nisA*.

generated transformants that did not exhibit antimicrobial activity in either assay (Table 4 and Fig. 4).

DISCUSSION

We have developed an expression system for nisin production that is based on complementation of a deficiency in the *nisA* gene product of *L. lactis* FI5876. The strategy employed for construction of the *nisA*-deficient host strain FI7332 initially involved integration of an Em^r gene in the chromosomally encoded *nisA* gene. The effect of this insertion was not only loss of nisin production but also a reduction in immunity to nisin (FI7300 [Table 3]). From analysis of this region of the nisin transposon, Tn*5301*, it appears that *nisB* and genes further downstream are transcribed by read-through from the *nisA* gene (10a, 42). In this case, insertional inactivation of *nisA* in FI7300 could have a polar effect on other genes in the nisin operon.

The Em^r gene, followed by a potential rho-independent terminator (20), was inserted in the chromosome of FI7300 such that the coding region was transcribed in the same direction as the *nisA* gene. It is not known whether this

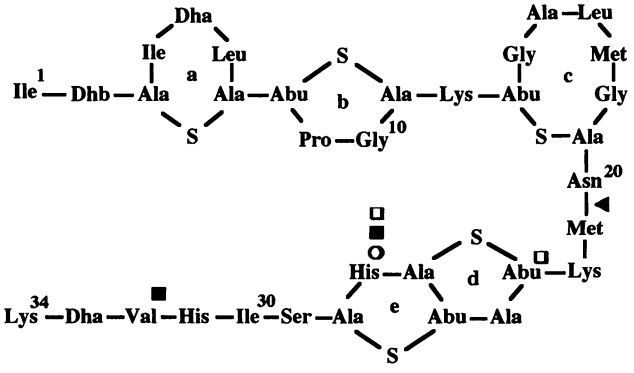

FIG. 7. Structure of nisin A (from reference 17). The modified residues are dehydroalanine (Dha), dehydrobutyrine (Dhb), aminobutyrate (Abu), lanthionine (Ala-S-Ala), and β-methyllanthionine (Abu-S-Ala). The amino acids which would undergo substitution as a result of site-directed mutagenesis are indicated by symbols above the relevant residue. Predicted molecular alterations to nisin A/H27Q, nisin A/H27Q,T23S, and nisin A/H27Q,V32I are indicated by ●, □, and ■, respectively. The arrowhead between residues 20 and 21 marks the C terminus of nisin A/Δ21–34.

sequence acts as a terminator of transcription of the nisin operon, but the effect of the Em^r insertion on nisin immunity in this strain is consistent with a reduction in the expression of downstream genes. The mutation caused by this insertion in FI7300 cannot be complemented in *trans* by provision of pFI172-encoded *nisA*. This is presumed to be due to a deficiency in expression of the modifying enzymes brought about by the polar mutation in *nisA*.

The fact that this apparent loss of expression was concurrent with a reduction in nisin immunity prompted a search for FI7300 derivatives which had spontaneously reverted to wild-type levels of immunity. FI7304 is one such isolate that had lost Em^r as a result of insertional inactivation by a newly identified insertion element, IS*905* (unpublished data). Transformation of FI7304 with the shuttle vector pTG262 occurred at a frequency of approximately 10^4 transformants per μg of DNA. This frequency dropped by about 3 orders of magnitude when the transforming plasmid encoded the *nisA* gene (pFI172 [Table 3]). A similarly low frequency was obtained when the wild-type nisin-producing strain FI5876 was transformed with pFI172. However, the same plasmid transformed the Em^r insertionally inactivated strain FI7300 at elevated frequencies (Table 3). The variation in transformation frequencies may thus reflect the levels of expression of genes responsible for prenisin maturation in these different hosts. The introduction of pFI172 (a multicopy plasmid encoding *nisA*) might result in a much higher prenisin level than that determined by a single chromosomal copy on Tn*5301*. Hence, in those host strains expressing the maturation enzymes at wild-type levels, subsequent processing may lead to overproduction of active nisin. Under these conditions, the nisin immunity system may not be adequate, and the viability of any transformed cells would thus be limited.

Of those rare FI7304/pFI172 transformants that were obtained, all were Nis$^-$ except for one, namely, FI7330. In order for these transformants to be viable, nisin production has been lost or expression has been depressed to levels permissible for cell viability. In the case of FI7330, the detrimental effect of overexpression of *nisA* is thought to be compensated for by a reduction in the levels of expression of the modifying enzymes. This presumed decrease in expression of genes downstream of *nisA* did not detectably reduce the level of immunity to nisin in FI7330 (Table 3). Curing FI7330 generated the plasmid-free strain FI7332, which could then accept pFI172 or derivatives in transformations at a frequency 4 orders of magnitude higher than that of FI5876 or FI7304 (Table 3). The plasmid-encoded *nisA* complements the mutation in FI7332, resulting in nisin production at a level approaching that of the nisin-producing parent (Fig. 4).

The conversion of FI7300 to FI7322 was brought about by a series of independent in vivo DNA rearrangements involving the newly identified IS*905*. The initial insertion of this element in the Em^r gene of FI7300 resulted in an apparent increase in the levels of both nisin immunity and nisin-modifying enzymes, as proposed above. Preliminary analysis has revealed that IS*905* exhibits homology with the staphylococcal element IS*256*, two copies of which flank the aminoglycoside resistance determinant of the compound transposon Tn*4001* (39). Interestingly, putative promoters which could initiate turn-on of genes downstream of the site of insertion have been identified in the termini of IS*256* (2). The internal drug resistance determinants of Tn*4001* are thought to be under the control of a promoter encoded within one terminus of the upstream copy of IS*256*. Similar transcriptional read-through from an IS*905*-encoded promoter could account for the polar effects associated with insertion in the Em^r gene in FI7304. Indeed, sequences at the extreme right end of IS*905* conform precisely to the consensus −35 region of promoters in gram-positive bacteria (16), which may provide a partial promoter sequence for the expression of downstream nisin determinants. The efficiency of expression of such genes would therefore depend on sequences adjacent to the insertion which would provide the −10 region and transcription initiation site of a hybrid promoter.

FI7304 was found to have undergone a small deletion (approximately 200 bp) in the vicinity of the right end of IS*905* to generate FI7332 (Fig. 5); this deletion enabled the latter strain to express plasmid-encoded *nisA* and process the gene product to active nisin. A putative hybrid promoter which spanned the terminus of IS*905* would thus be lost as a result of such a deletion event. The creation of a new, possibly weaker, promoter may have led to expression of the downstream genes in FI7332 at levels low enough to permit complementation of the chromosomal lesion in this host. In these various strains, the activity of the terminator that follows the Em^r gene needs to be considered. Experiments are in progress to elucidate the precise nature of the various rearrangements and the effects these mutations have on the expression of other genes involved in nisin biosynthesis.

The ability of the *L. lactis* FI7322 expression system to produce variant nisins was tested by introducing plasmids encoding *nisA* genes which had been subjected to site-directed mutagenesis. The mutated *nisA*/H27Q gene encoded a prenisin molecule in which His-27 was substituted for a glutamine residue (Fig. 7). This represents a conservative change in the amino acid sequence of the natural nisin variant nisin Z (15, 37), in which an asparagine residue is found at position 27. Subtle differences in the solubility properties (23, 29) and growth inhibition spectra (15) of nisin A and nisin Z have been reported. These may be due to the altered charge distribution in the nisin Z molecule brought about by the substitution of a histidine (protonated at pH 6.0) with an uncharged asparagine residue. The engineered variant nisin A/H27Q was found to have biological activity in bioassays (Table 2). It might be expected that this nisin derivative would exhibit properties closer to those of nisin Z. However, preliminary bioassays suggested that the diffusion properties of nisin A/H27Q were not improved and may even be lower than those of nisin A (Fig. 4). The differentiation of diffusion and antimicrobial activity is not possible in these bioassays, and accurate assessment of variant nisins such as nisin A/H27Q will require more detailed investigation.

Nisin A/H27Q,V32I contains a secondary mutation three residues from the COOH terminus of the mature molecule. Substitution of Val-32 for Ile-32 represents a conservative amino acid change in the nisin A sequence (Fig. 7). The C-terminal Dha-33 (dehydroalanine) and Lys-34 of nisin A can be deleted without loss of activity (5), suggesting that this region of the molecule does not play a vital functional role. The substitution at position 33 would be expected to have a minimal affect on the structure of the molecule. Bioassays indicated that biological activity was retained at a level comparable to that of nisin A expressed under the same conditions (Fig. 4).

At present, the expression of plasmid-encoded mutated *nisA* genes by the complementation system of FI7332 can be detected only if the primary translation product is modified and the subsequently processed nisin variant retains biological activity. Failure to detect zones of inhibition in assays may thus be due to either (i) lack of expression of the mutated gene, (ii) failure of the primary translation product to mature, or (iii) correct processing of the primary transla-

tion product but loss of activity due to the changed amino acid sequence. Antibodies have been raised against mature nisin and leader sequence (33a), and immunoassays will be used to distinguish between these alternatives.

The Thr-23 to Ser-23 substitution in the engineered variant nisin A/H27Q,T23S represents a conservative amino acid change and as such might be expected to exhibit levels of antimicrobial activity comparable to those of nisin A. This particular threonine residue is involved in posttranslational modification to form β-methyllanthionine and generate ring D of nisin A (Fig. 7). Theoretically, dehydration of the hydroxyl group in the substituted Ser-23 side chain could be followed by lanthionine ring formation, as occurs in ring A of nisin A (Fig. 7). However, the biological activity of this nisin variant appears to have been adversely affected by the secondary mutation. Low levels of activity were detectable in colony overlays (Table 4), suggesting that gene expression was occurring, but it is not yet known whether maturation (i.e., formation of ring D) was partially inhibited by the Thr-to-Ser substitution or whether the fully processed molecule has reduced activity as a consequence of the amino acid change.

Introduction of pFI372, which encodes the truncated *nisA* gene (*nisA*/Δ21–34), into the FI7332 expression system did not lead to any detectable nisin activity in either plate diffusion or colony overlay assays (Table 4). Posttranslational processing of this truncated prenisin molecule would generate a structure composed of only rings A, B, and C of mature nisin A (Fig. 7). On the basis of biological tests of synthetic nisin fragments, it has been reported that the minimum structure required for activity is residues 1 to 19 (47). Our failure to demonstrate activity with a potentially larger molecule may thus indicate either that expression of the truncated gene has been affected or that the missing C-terminal region of the molecule prevents correct processing, hence blocking biosynthesis of nisin A/Δ21–34 at the maturation stage.

A protein engineering strategy has been developed to generate specific nisin variants. The expression system, involving a non-nisin-producing lactococcal host strain (i.e., FI7332) and plasmid-encoded or mutated *nisA* genes, has advantages over other expression systems which produce a mixture of normal and variant nisins. The strain described here allows the exclusive production of nisin variants, and hence the effect of a particular mutation on biological properties and antimicrobial activity can be readily assessed prior to its purification. The extraction and purification of variant nisins are in progress, and two-dimensional nuclear magnetic resonance analysis will be performed on these molecules to confirm the predicted structures and to compare the conformations with that established for nisin A (6, 34, 45). The system described here opens the way to produce a wide range of nisin variants which can be used to probe structure-activity relationships within the nisin molecule.

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REFERENCES

1. **Buchman, W. B., S. Banerjee, and J. N. Hansen.** 1988. Structure, expression, and evolution of a gene encoding the precursor of nisin, a small protein antibiotic. J. Biol. Chem. **263**:16260–16266.
2. **Byrne, M. E., D. A. Rouch, and R. A. Skurray.** 1989. Nucleotide sequence analysis of IS*256* from the *Staphylococcus aureus* gentamicin-tobramycin-kanamycin-resistance transposon Tn*4001*. Gene **81**:361–367.
3. **Casadaban, M. J., and S. N. Cohen.** 1980. Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. J. Mol. Biol. **138**:179–207.
4. **Chambers, S. P., S. E. Prior, D. A. Barstow, and N. P. Minton.** 1988. The pMTL *nic*$^-$ cloning vectors. I. Improved pUC polylinker regions to facilitate the use of sonicated DNA for nucleotide sequencing. Gene **68**:139–149.
5. **Chan, W. C., B. W. Bycroft, L.-Y. Lian, and G. Roberts.** 1989. Isolation and characterisation of two degradation products derived from the peptide antibiotic nisin. FEBS Lett. **252**:29–36.
6. **Chan, W. C., L.-Y. Lian, B. W. Bycroft, and G. C. K. Roberts.** 1989. Confirmation of the structure of nisin by complete ^{1}H N.m.r. resonance assignment in aqueous and dimethyl sulphoxide solution. J. Chem. Soc. Perkin Trans. I **1**:2359–2367.
7. **Chopin, M.-C., A. Chopin, A. Rouault, and N. Galleron.** 1989. Insertion and amplification of foreign genes in the *Lactococcus lactis* subsp. *lactis* chromosome. Appl. Environ. Microbiol. **55**:1769–1774.
8. **Cohen, S. N., A. C. Y. Chang, and L. Hsu.** 1972. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. Proc. Natl. Acad. Sci. USA **69**:2110–2114.
9. **De Man, J. C., M. Rogosa, and M. E. Sharp.** 1960. A medium for cultivation of lactobacilli. J. Appl. Bacteriol. **23**:130–135.
10. **De Vos, W. M., G. Jung, and H.-G. Sahl.** 1991. Definitions and nomenclature of lantibiotics, p. 457–463. *In* G. Jung and H.-G. Sahl (ed.), Nisin and novel lantibiotics. ESCOM, Leiden, The Netherlands.

10a. **Dodd, H. M., et al.** Unpublished data.

11. **Dodd, H. M., N. Horn, and M. J. Gasson.** 1990. Analysis of the genetic determinant for production of the peptide antibiotic nisin. J. Gen. Microbiol. **136**:555–566.
12. **Fowler, G. G., and M. J. Gasson.** 1990. Antibiotics—nisin, p. 135–152. *In* N. J. Russel and G. W. Goulds (ed.), Food preservatives. Blackie and Sons, Glasgow, United Kingdom.
13. **Gasson, M. J.** 1983. Plasmid components of *Streptococcus lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. J. Bacteriol. **154**:1–9.
14. **Gasson, M. J.** 1984. Transfer of sucrose fermenting ability, nisin resistance and nisin production into *Streptococcus lactis* 712. FEMS Microbiol. Lett. **21**:7–10.
15. **Graeffe, T., H. Rinalta, L. Paulin, and P. Saris.** 1991. A natural nisin variant, p. 260–268. *In* G. Jung and H.-G. Sahl (ed.), Nisin and novel lantibiotics. ESCOM, Leiden, The Netherlands.
16. **Graves, M. C., and J. C. Rabinowitz.** 1986. In vivo and in vitro transcription of the *Clostridium pasteurianum* ferredoxin gene. J. Biol. Chem. **261**:13744–13753.
17. **Gross, E., and J. Morell.** 1971. The structure of nisin. J. Am. Chem. Soc. **93**:4634–4635.
18. **Ho, S. N., N. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease.** 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene **77**:51–59.
19. **Holo, H., and I. F. Nes.** 1989. High-frequency transformation, by electroporation, of *Lactococcus lactis* subsp. *cremoris* grown with glycine in osmotically stabilized media. Appl. Environ. Microbiol. **55**:3119–3123.
20. **Horinouchi, S., and B. Weisblum.** 1982. Nucleotide sequence and functional map of pE194, a plasmid that specifies inducible resistance to macrolide, lincosamide, and streptogramin type B antibiotics. J. Bacteriol. **150**:804–814.
21. **Horinouchi, S., and B. Weisblum.** 1982. Nucleotide sequence and functional map of pC194, a plasmid that specifies inducible chloramphenicol resistance. J. Bacteriol. **150**:815–825.
22. **Horn, N., S. Swindell, H. M. Dodd, and M. J. Gasson.** 1991. Nisin biosynthesis genes are encoded by a novel conjugative transposon. Mol. Gen. Genet. **228**:129–135.
23. **Hugenholtz, J., and J. C. M. De Veer.** 1991. Application of nisin A and nisin Z in dairy technology, p. 440–447. *In* G. Jung and H.-G. Sahl (ed.), Nisin and novel lantibiotics. ESCOM, Leiden, The Netherlands.

24. **Humphreys, G. O., A. Weston, M. G. M. Brown, and J. T. Saunders.** 1979. *In* S. W. Glover and L. O. Butler (ed.), Transformation—1978. Cotswold Press, Oxford.
25. **Hurst, A.** 1981. Nisin. Adv. Appl. Microbiol. **27:**85–123.
26. **Jung, G.** 1991. Lantiobiotics: a survey, p. 1–34. *In* G. Jung and H.-G. Sahl (ed.), Nisin and novel lantibiotics. ESCOM, Leiden, The Netherlands.
27. **Kaletta, C., and K.-D. Entian.** 1989. Nisin, a peptide antibiotic: cloning and sequencing of the *nisA* gene and posttranslational processing of its peptide product. J. Bacteriol. **171:**1597–1601.
28. **Klein, C., C. Kaletta, N. Schnell, and K.-D. Entian.** 1992. Analysis of genes involved in biosynthesis of the lantibiotic subtilin. Appl. Environ. Microbiol. **58:**132–142.
29. **Kuipers, O. P., W. M. G. J. Yap, H. S. Rollema, M. M. Beerthuyzen, R. J. Siezen, and W. M. De Vos.** 1991. Expression of wild-type and mutant nisin genes in *Lactococcus lactis*, p. 250–259. *In* G. Jung and H.-G. Sahl (ed.), Nisin and novel lantibiotics. ESCOM, Leiden, The Netherlands.
30. **Leenhouts, K. J., J. Kok, and G. Venema.** 1989. Campbell-like integration of heterologous plasmid DNA into the chromosome of *Lactococcus lactis* subsp. *lactis*. Appl. Environ. Microbiol. **55:**394–400.
31. **Leenhouts, K. J., J. Kok, and G. Venema.** 1990. Stability of integrated plasmids in the chromosome of *Lactococcus lactis*. Appl. Environ. Microbiol. **56:**2726–2735.
32. **Lennox, E. S.** 1955. Transduction of linked genetic characters of the host bacteriophage P1. Virology **1:**190–206.
33. **Lewington, J., S. D. Greenaway, and B. J. Spillane.** 1987. Rapid small scale preparation of bacterial genome DNA suitable for cloning and hybridization analysis. Lett. Appl. Microbiol. **5:**51–53.
33a. **Leyland, M.** Personal communication.
34. **Lian, L. Y., W. C. Chan, S. D. Morley, G. C. K. Roberts, B. W. Bycroft, and D. Jackson.** 1992. Solution structures of nisin A and its two major degradation products determined by NMR. Biochem. J. **283:**413–420.
35. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
36. **McKay, L. L., K. A. Baldwin, and E. A. Zottola.** 1972. Loss of lactose metabolism in lactic streptococci. Appl. Microbiol. **23:**1090–1096.
37. **Mulders, J. W. M., I. J. Boerrigter, H. S. Rollema, R. J. Siezen, and W. M. De Vos.** 1991. Identification and characterization of the lantibiotic nisin Z, a natural nisin variant. Eur. J. Biochem. **201:**581–584.
38. **Rauch, P. J. G., M. M. Beerthuyzen, and W. M. De Vos.** 1991. Molecular analysis and evolution of conjugative transposons encoding nisin production and sucrose fermentation in *Lactococcus lactis*, p. 243–249. *In* G. Jung and H.-G. Sahl (ed.), Nisin and novel lantibiotics. ESCOM, Leiden, The Netherlands.
39. **Rouch, D. A., M. E. Byrne, Y. C. Kong, and R. A. Skurray.** 1987. The *aacA-aphD* gentamicin and kanamycin resistance determinant of Tn*4001* from *Staphylococcus aureus*: expression and nucleotide sequence analysis. J. Gen. Microbiol. **133:**3039–3052.
40. **Sanger, F., A. R. Coulson, B. G. Barrell, A. J. H. Smith, and B. A. Roe.** 1980. Cloning in single-stranded bacteriophage as an aid to rapid DNA sequencing. J. Mol. Biol. **143:**161–178.
41. **Schnell, N., G. Engelke, J. Augustin, R. Rosenstein, V. Ungermann, F. Gotz, and K.-D. Entian.** 1992. Analysis of genes involved in the biosynthesis of lantibiotic epidermin. Eur. J. Biochem. **204:**57–68.
42. **Steen, M. T., Y. Chung, and J. N. Hansen.** 1991. Characterization of the nisin gene as part of a polycistronic operon in the chromosome of *Lactococcus lactis* ATCC 11454. Appl. Environ. Microbiol. **57:**1181–1188.
43. **Terzaghi, B. E., and W. E. Sandine.** 1975. Improved medium for lactic streptococci and their bacteriophages. Appl. Microbiol. **29:**807–813.
44. **Tramer, J., and G. G. Fowler.** 1964. Estimation of nisin in foods. J. Sci. Food Agric. **15:**522–528.
45. **Van De Ven, F. J. M., H. W. Van Den Hooven, R. Konings, and C. W. Hilbers.** 1992. NMR studies of lantibiotics. The structure of nisin in aqueous solution. Eur. J. Biochem. **202:**1181–1188.
46. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13amp7 derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19:**259–268.
47. **Wakamiya, T., K. Fukase, M. Kitazawa, H. Fujita, A. Kubo, Y. Maeshiro, and T. Shiba.** 1990. Lanthionine peptide nisin: study of a structure-activity relationship, p. 60–64. *In* J. E. River and G. R. Marshall (ed.), Peptides—chemistry, structure and biology. ESCOM, Leiden, The Netherlands.
48. **Zafarullah, M., D. Charlier, and N. Glansdorff.** 1981. Insertion of IS3 can "turn-on" a silent gene in *Escherichia coli*. J. Bacteriol. **146:**415–417.