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Genes Involved in Immunity to the Lantibiotic Nisin Produced by *Lactococcus lactis* 6F3

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The lantibiotic nisin is produced by several strains of *Lactococcus lactis*. The complete gene cluster for nisin biosynthesis in *L. lactis* 6F3 comprises 15 kb of DNA. As described previously, the structural gene *nisA* is followed by the genes *nisB*, *nisT*, *nisC*, *nisI*, *nisP*, *nisR*, and *nisK*. Further analysis revealed three additional open reading frames, *nisF*, *nisE*, and *nisG*, adjacent to *nisK*. Approximately 1 kb downstream of the *nisG* gene, three open reading frames in the opposite orientation have been identified. One of the reading frames, *sacR*, belongs to the sucrose operon, indicating that all genes belonging to the nisin gene cluster of *L. lactis* 6F3 have now been identified. Proteins NisF and NisE show strong homology to members of the family of ATP-binding cassette (ABC) transporters, and *nisG* encodes a hydrophobic protein which might act similarly to the immunity proteins described for several colicins. Gene disruption mutants carrying mutations in the genes *nisF*, *nisE*, and *nisG* were still able to produce nisin. However, in comparison with the wild-type strain, these mutants were more sensitive to nisin. This indicates that besides *nisI* the newly identified genes are also involved in immunity to nisin. The NisF-NisE ABC transporter is homologous to an ABC transporter of *Bacillus subtilis* and the MbcF-MbcE transporter of *Escherichia coli*, which are involved in immunity to subtilin and microcin B17, respectively.

Lantibiotics are lanthionine-containing antimicrobial peptides produced by gram-positive bacteria. Nisin produced by *Lactococcus lactis* 6F3 belongs to the subgroup designated type A lantibiotics, which are linearly shaped (17) and which comprise subtilin (3), epidermin (1), gallidermin (40), and Pep5 (19). Lantibiotics have a high antimicrobial activity against several gram-positive bacteria, such as *Propionibacterium acnes*, staphylococci, streptococci, and clostridia. They contain unusual amino acids, such as dehydroalanine, dehydrobutyrine, lanthionine, and 3-methyl-lanthionine (41).

The biosynthesis of lantibiotics involves posttranslational modifications of ribosomally synthesized precursor peptides (41, 48). For all known lantibiotics, the primary transcript of the nisin structural gene *nisA* is a prepeptide. It consists of a 23-residue leader peptide followed by a C-terminal 34-residue propeptide region from which the lantibiotic is matured. Biosynthesis via posttranslational modifications of ribosomally synthesized prepeptides clearly distinguishes lantibiotics from peptide antibiotics synthesized by multienzyme complexes (gramicidin and valinomycin) and nonpeptide antibiotics, which are products of the secondary metabolism (24).

Nisin is the most prominent lantibiotic. It is used as a food preservative in several countries. It occurs naturally in dairy products (8) and is used as an additive to dairy products or canned food to prevent spoilage by gram-positive bacteria, especially clostridia, listeria, bacilli, and staphylococci (16). Since the realization of the potentially harmful effects of nitrate, which is also used against clostridia in canned food, there has been an increasing interest in nisin and its biosynthesis. Since direct addition of nisin during fermentation is costly and prohibited in many countries, the use of nisin-producing starters is the obvious strategy. A prerequisite for this strategy is the basic understanding of how biosynthesis and immunity are accomplished by nisin-producing strains.

Nisin occurs in two natural variants, nisin A and nisin Z. They are encoded by the genes *nisA* and *nisZ*, which have been cloned several times from different strains. Both variants contain dehydrated amino acids and lanthionine rings, and they differ only in a single amino acid residue at position 27 (histidine in NisA instead of asparagine in NisZ) (9, 29). The genes for nisin biosynthesis are located on a 70-kb conjugative transposon (10, 31) which also contains the genetic information for sucrose metabolism. Several genes encoding proteins which are involved in the biosynthesis of different lantibiotics have been described. Assuming that the genes for biosynthesis, secretion, and immunity are clustered on the same transposon, approximately 12 kb of DNA downstream from the structural gene *nisA* has been cloned and sequenced. The genes *nisB*, *nisT*, *nisC*, *nisI*, *nisP*, *nisR*, and *nisK* have been found to be homologous to the corresponding genes of the subtilin or epidermin gene clusters (2, 23, 38, 39). Gene deletion experiments with the genes *spaB*, *spaC*, *spaT*, *spaR*, and *spaK* in *Bacillus subtilis* have proven that they are essential for subtilin biosynthesis (20, 22, 23). Like *epiP* of *Staphylococcus epidermidis*, *nisP* codes for a subtilisin-like serine protease which is involved in processing of the posttranslationally modified prenisin (12, 39, 47).

As shown for nisin and Pep5, the mode of action of these amphiphilic cationic peptides is based on the formation of voltage-dependent pores (42). This causes an efflux of ions and molecules of low molecular weight, leading to the breakdown of the membrane potential and depletion of the proton motive force (35). According to this mode of action, the membranes of lantibiotic-producing strains are also sensitive to their own lantibiotics. However, producing strains are much less sensitive than nonproducing strains, and it is of great interest how producer strains accomplish immunity to the synthesized lantibiotic. Immunity seems to be specific for the particular lantibiotic produced, but the molecular basis for immunity is not yet

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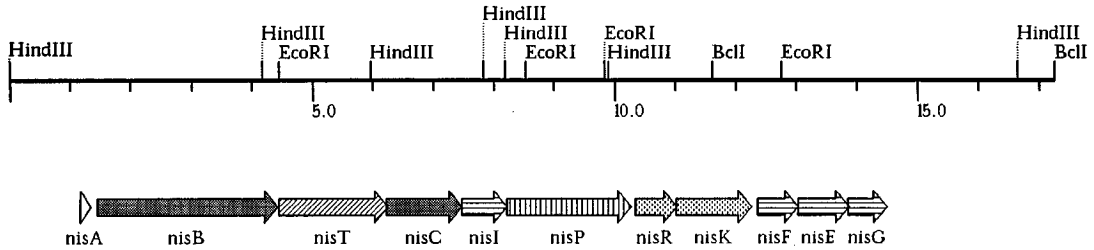

FIG. 1. Restriction map of the *L. lactis* 6F3 nisin gene cluster and open reading frames. Numbers indicate kilobases.

understood. For nisin, involvement of the *nisI* gene in immunity has already been proved (12, 26). Recently three genes were shown to be involved in immunity to subtilin produced by *B. subtilis* (21). In addition to *spaI*, which codes for a putative lipoprotein similar to that encoded by *nisI*, the genes *spaF* and *spaG* seem to be necessary to provide full immunity. For Pep5, a 69-amino-acid gene product of the *pepI* gene provides immunity to the channel-forming action of Pep5 in *S. epidermidis* in the presence of the structural gene *pepA* (33, 34).

In the present study, we report the identification of three further open reading frames belonging to the nisin gene cluster of *L. lactis* 6F3 which may also be involved in immunity to nisin. In a manner similar to that of the subtilin gene cluster, the encoded proteins, NisF, NisE, and NisG, possibly form an ATP-binding cassette (ABC) transporter.

MATERIALS AND METHODS

Strains and media. The plasmid-free non-nisin-producing strain *L. lactis* MG1614 (13a) and the nisin-producing strains *L. lactis* 6F3 (18) and *L. lactis* KS100 (12) were grown at 30°C in M17 broth, supplemented with 0.5% (wt/vol) sucrose (SM17) or with 0.5% (wt/vol) glucose (GM17). *Micrococcus luteus* ATCC 9341 was used as a test strain for nisin activity. *M. luteus* was also grown at 30°C in GM17 medium. Recombinant plasmids were amplified in *Escherichia coli* RR1 (F^- *hsd-520 supE44 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*) and DH5α (*supE44* Δ*lacU169* [φ80 *lacZ*ΔM15] *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*). *E. coli* strains were grown at 37°C in Luria-Bertani medium (GIBCO, Neu-Isenburg, Germany). If antibiotics were used, the following concentrations were employed: ampicillin, 40 μg/ml; erythromycin, 150 μg/ml for *E. coli* and 5 μg/ml for *L. lactis*; and chloramphenicol, 20 μg/ml for *E. coli* and 5 μg/ml for *L. lactis.*

Plasmids. The vectors pUC19 (49) and pBSKRII (Stratagene, Heidelberg, Germany) were used for the purpose of cloning in *E. coli*. The plasmid pSI19 (12) was used for cloning in *L. lactis.*

Molecular biological techniques. Established protocols for molecular biological techniques were followed (36). DNA was cleaved according to the conditions recommended by the commercial supplier of the restriction enzymes (Boehringer GmbH, Mannheim, Germany). Restriction endonuclease-digested DNA was eluted from 0.7% agarose gels by the freeze-squeeze method (45).

Plasmid isolation and PCR. The procedure of Birnboim and Doly (5) was used to isolate the plasmids of *E. coli*. When necessary, these were purified by use of an ultramicrocentrifuge (Beckman TL 100; rotor, TLA 100.2) at 80,000 rpm for 12 h, with a CsCl gradient and ethidium bromide. Plasmid DNA from *L. lactis* was isolated as described previously (12). PCR was carried out according to standard procedures (36) in an Eppendorf Microcycler E apparatus. By using *Taq* DNA polymerase (Boehringer), 35 cycles were performed for 20 s at 94°C followed by 20 s at 55°C and finally by 2.5 min at 72°C.

Southern hybridization. Southern blotting was carried out according to the method of Southern (44). For Southern hybridization, double-stranded DNA fragments were labelled by nick translation with [^{32}P]ATP and DNA polymerase I (Boehringer), as described by Sambrook et al. (36).

Cloning of chromosomal DNA fragments. Total DNA from *L. lactis* 6F3 was isolated as described elsewhere (12). DNA was digested with appropriate restriction enzymes, and restriction sites were mapped according to the method of Southern (44) with appropriate DNA fragments as probes. For isolation of additional DNA fragments, a 3′-terminal fragment of a previously isolated clone containing the genes *nisR* and *nisK* (12) was used as a hybridization probe to identify the adjacent DNA fragment. The isolated 5.6-kb DNA fragment was obtained from a *Bcl*I digestion of chromosomal DNA from *L. lactis* 6F3 and cloned in the plasmid pUC19 to yield pSI30. In a similar way, a 6.7-kb *Hin*dIII DNA fragment was isolated. For DNA sequencing, subclones were constructed in the *E. coli* vector pBSK.

DNA sequencing. Oligonucleotides used as primers in sequencing reactions were synthesized with the model 391 DNA synthesizer of Applied Biosystems (Weiterstadt, Germany). Sequencing was carried out on a model 373A DNA sequencer (Applied Biosystems) by using a *Taq* DyeDeoxy Terminator Cycle sequencing kit (Applied Biosystems), according to the protocol of the commercial supplier (37).

Computer analysis of DNA and analysis of amino acid sequences were performed with the DNASIS/PROSIS version 7.0 program (Pharmacia, Freiburg, Germany). For protein homology searches, the databases SWISS-PROT R23.0 and NBRF-PIR R33.0 were used with the FAST A algorithm of Lipman and Pearson (28). Hydrophobicity plots were created according to the method of Kyte and Doolittle (27).

Transformation of *L. lactis*. *L. lactis* strains were transformed by electroporation as described by Holo and Nes (14) with the following modifications. Cells were grown in GM17 broth supplemented with 0.5 M sucrose and 2% glycine. Selection was carried out on GM17 plates containing antibiotic. Sucrose was omitted from the selection plates. Electroporation was performed with a Gene Pulser apparatus (Bio-Rad) by using a single pulse of 12.5 kV/cm, a capacity of 25 μF, and a resistance of 200 Ω.

Nisin bioassay. Test strain *M. luteus* ATCC 9341 was grown to an A_{578} of 0.8, 0.3 ml was added to 500 ml of molten GM17 agar, and the solution was mixed and poured into petri dishes (20 ml). *L. lactis* was spread on the agar surface. The plates were incubated overnight at 30°C, and the diameter of the zone of *M. luteus* growth inhibition around the colonies was determined.

Plate diffusion bioassay for nisin sensitivity. To test nisin immunity, 1 ml of an overnight culture (stationary-phase cells) of a particular *L. lactis* strain grown in GM17 medium (containing the appropriate antibiotic, if required) was added to 250 ml of molten GM17 agar and the solution was mixed and poured into petri dishes (20 ml). Nisin immunity was determined by dissolving purified nisin in 0.02 N HCl and applying various amounts to filter disks on the agar surface. (The stock solution used contained 5 mg of nisin per ml, which is 1.3 M purified nisin.) The agar plates were incubated overnight at 30°C, and the diameter of the inhibition zone was determined.

Nucleotide sequence accession number. The sequence data published here have been submitted to GenBank and are listed under accession number U17255.

RESULTS

Seven genes (*nisB*, *nisC*, *nisT*, *nisI*, *nisP*, *nisR*, and *nisK*) downstream of the nisin structural gene *nisA* of *L. lactis* 6F3 have been identified so far (11, 12, 26). Further cloning and sequencing of an adjacent DNA fragment revealed additional genes belonging to the nisin gene cluster in *L. lactis* 6F3 and further open reading frames in the opposite direction.

Isolation of an adjacent DNA fragment and nucleotide sequence of the identified open reading frames. A 3′-terminal 1.3-kb *Eco*RV fragment of the previously isolated 2.9-kb *Eco*RI fragment containing the genes *nisR* and *nisK* (12) was used as a hybridization probe to determine adjacent restriction sites in the chromosome of *L. lactis* 6F3. A 5.6-kb *Bcl*I fragment was cloned and sequenced. To prove that the isolated DNA really reflects the genomic situation, a 6.7-kb *Hin*dIII fragment was isolated in parallel as a control.

Approximately 12 kb downstream of the nisin structural gene *nisA*, further genes, *nisF*, *nisE*, and *nisG*, were found (Fig. 1). The *nisF* gene encodes a protein of 225 amino acids with a theoretical calculated molecular mass of 24.7 kDa (Fig. 2). The *nisF* reading frame starts at a distance of 102 bp downstream of the end of *nisK*. An inverted repeat consisting of 16 bp within the noncoding region between the two open reading frames

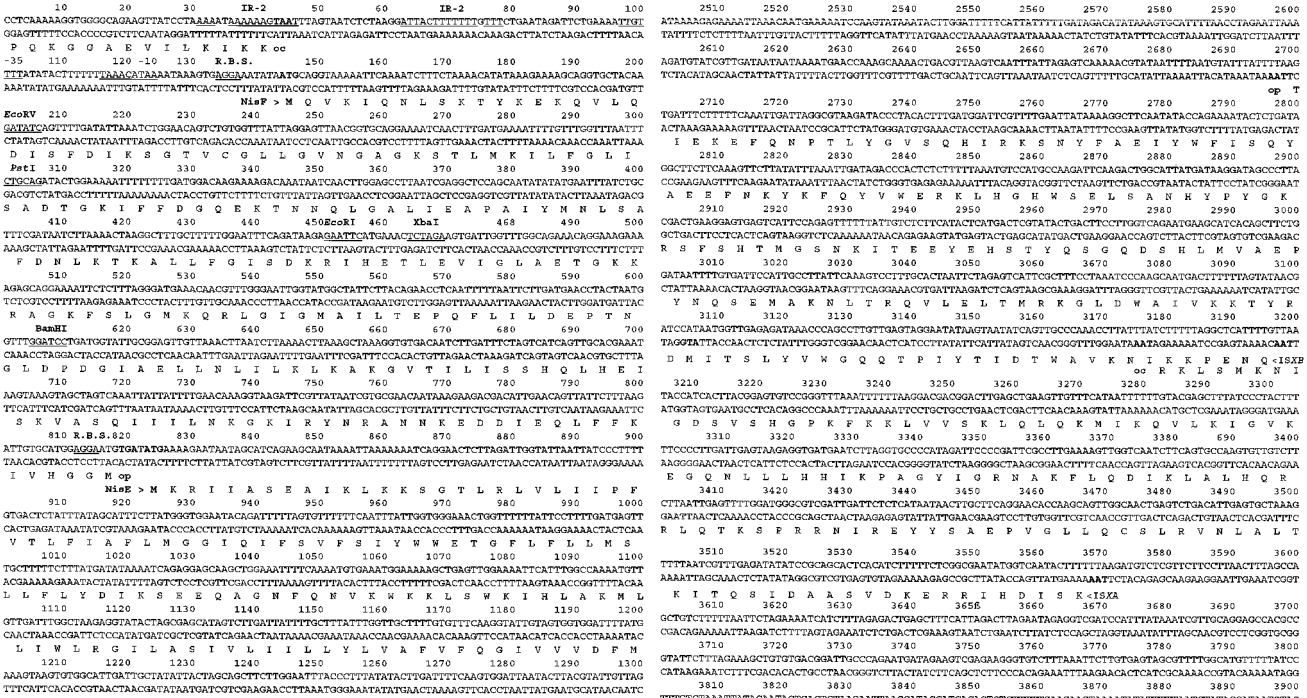

FIG. 2. Nucleotide sequence of cloned 5.0-kb region downstream of *nisK* (12) with open reading frames *nisF*, *nisE*, and *nisG* and with IS*X4*, IS*XB*, and *sacR* in the opposite orientation. Possible ribosome-binding sites (R.B.S.), restriction sites, promoter regions, and inverted repeats (IR) are underlined. Start codons and stop codons are printed in boldface. oc, ochre; op, opal. For protein sequences, the one-letter code is used.

has been identified. This unusual DNA sequence, possibly forming a stem-loop structure, may serve as a terminator of transcription at the end of *nisK*. The left part of the possible stem-loop structure overlapped with four of the C-terminal amino acids of NisK, possibly preventing their translation. The *nisF* gene was preceded by a putative ribosome binding site at an appropriate distance from the ATG start codon. Upstream of *nisF*, a −10 and −35 promoter region similar to others previously described for *L. lactis* was present (46). This indicates that *nisF* has its own promoter.

A further open reading frame, *nisE*, was located downstream of *nisF*. It encodes a protein of 247 amino acids with a deduced molecular mass of 28.2 kDa (Fig. 2). If the second ATG is considered the start codon, the putative gene product would consist of 242 amino acids. The DNA sequence of the fragment was sequenced with eightfold redundancy to ensure that *nisF* and *nisE* are distinct genes and not a result of a sequence frameshift. This was additionally verified by direct genomic PCR sequencing. These results were also confirmed by the fact that a potential ribosome binding site preceded the *nisE* open reading frame. Its correct distance for consensus lactococcal

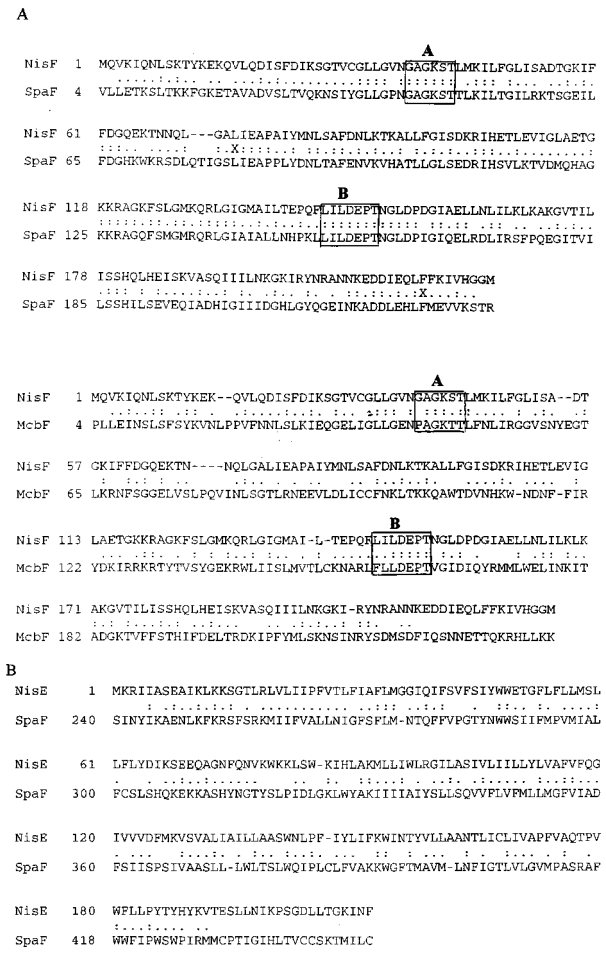

FIG. 3. Analysis of proteins NisF and NisE. (A) Comparison of proteins NisF (*L. lactis*), SpaF (*B. subtilis*), and McbF (*E. coli*). (B) Comparison of proteins NisE (*L. lactis*) and SpaF (*B. subtilis*). Dots indicate similar amino acid residues, and colons indicate identical amino acid residues. The consensus sequences A and B of potential ATP-binding sites are boxed.

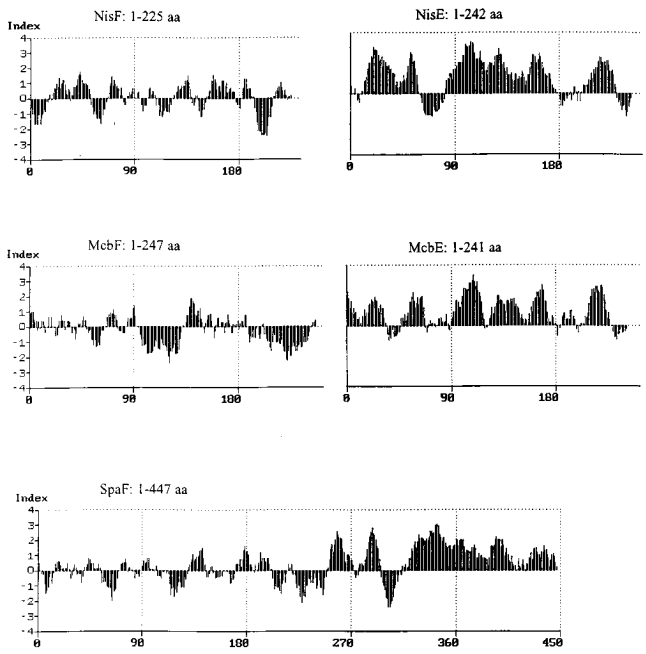

FIG. 4. Comparison of predicted hydrophobicity profiles for NisF, NisE, McbF, McbE, and SpaF. Positive numbers indicate hydrophobicity. aa, amino acids. Hydrophobic domains were estimated according to the method described by Kyte and Doolittle (27).

ribosome binding sites (46) strongly favors the second ATG as the start codon.

Another open reading frame, *nisG*, overlapped with the 3′ end of *nisE*. The *nisG* gene starts with an ATG codon which is preceded by a potential ribosome binding site (AGGAGT). The *nisG* gene probably encodes a protein of 214 amino acids with a deduced molecular mass of 24.2 kDa (Fig. 2).

Homology of NisF and NisE to ABC transporters. The entire NisF protein is 45.5% similar to the N-terminal domain of the recently reported SpaF protein, which shares strong homologies with ABC transporters of the HisP subfamily (25). As a common feature, the ATP-binding sites of these transporters are located in the N-terminal part of the protein. NisF shares two potential ATP-binding sites with proteins of this group (Fig. 3A). The second open reading frame, *nisE*, encodes a predominantly hydrophobic protein similar to the C-terminal part of SpaF (20.2% over the entire protein) (Fig. 3B). The hydrophobicity analysis of NisF and NisE together resembles that of the entire SpaF protein, and this also favors a similar function as an ABC transporter (Fig. 4). Furthermore, there is an additional similarity of NisF and NisE to the McbF-McbE transporter of *E. coli* (13) (Fig. 3 and 4). McbE appears to be an integral membrane protein consisting of 241 amino acids with a predicted molecular mass of 27.8 kDa. The *mcbF* gene encodes a protein consisting of 247 amino acids with a molecular mass of 28.8 kDa. McbF contains two ATP-binding sites in a manner similar to that of NisF and other known ABC transporters (6). McbF and McbE are thought to form a membrane complex which is involved in the specific transport of microcin B17 and in immunity to this bacteriocin. NisF is 28.6% similar to McbF over the entire protein. The similarities of NisF-NisE to SpaF of *B. subtilis* and to McbF-McbE of *E. coli*, which are involved in immunity to subtilin and microcin B17, respectively, suggest that NisF, NisE, and NisG may have similar functions.

Similarities of NisG to colicin immunity proteins. The *nisG* gene encodes a predominantly hydrophobic protein. Similarly, immunity proteins described for different colicins are also predominantly hydrophobic, with three or four potential mem-

TABLE 1. Descriptions of plasmids used

Plasmid	Genetic marker and/or description	Reference(s)
pSI16	2.0-kb *Pvu*II fragment of pGEN3 in pUC19 *Hin*dII	This work
pSI20	Em^r gene of pE194 joined to pSI16 *Hin*dII	This work, 15
pSI29	6.7-kb *Hin*dIII fragment of chromosomal DNA of *L. lactis* 6F3 in pUC19 *Hin*dIII	This work
pSI30	5.6-kb *Bcl*I fragment of chromosomal DNA of *L. lactis* 6F3 in pUC19 *Bam*HI	This work
pSI32	2.5-kb *Xba*I fragment of pSI29 in pBSK *Xba*I	This work
pGEN3	4.2-kb *Eco*RI fragment of chromosomal DNA of *L. lactis* 6F3 in pUC19 *Eco*RI	12
pSI41	5.8-kb *Cla*I-*Bst*XI fragment of pSI29 in pBSK *Cla*I-*Bst*XI	This work, 15
pSI48	Cm^r gene of pC194 in pSI41 *Eco*RI-*Pst*I	This work, 15
pSI53	Cm^r gene of pC194 in pSI32 *Sna*BI	This work, 15
pSI62	Cm^r gene of pC194 in pSI32 *Nru*I	This work, 15

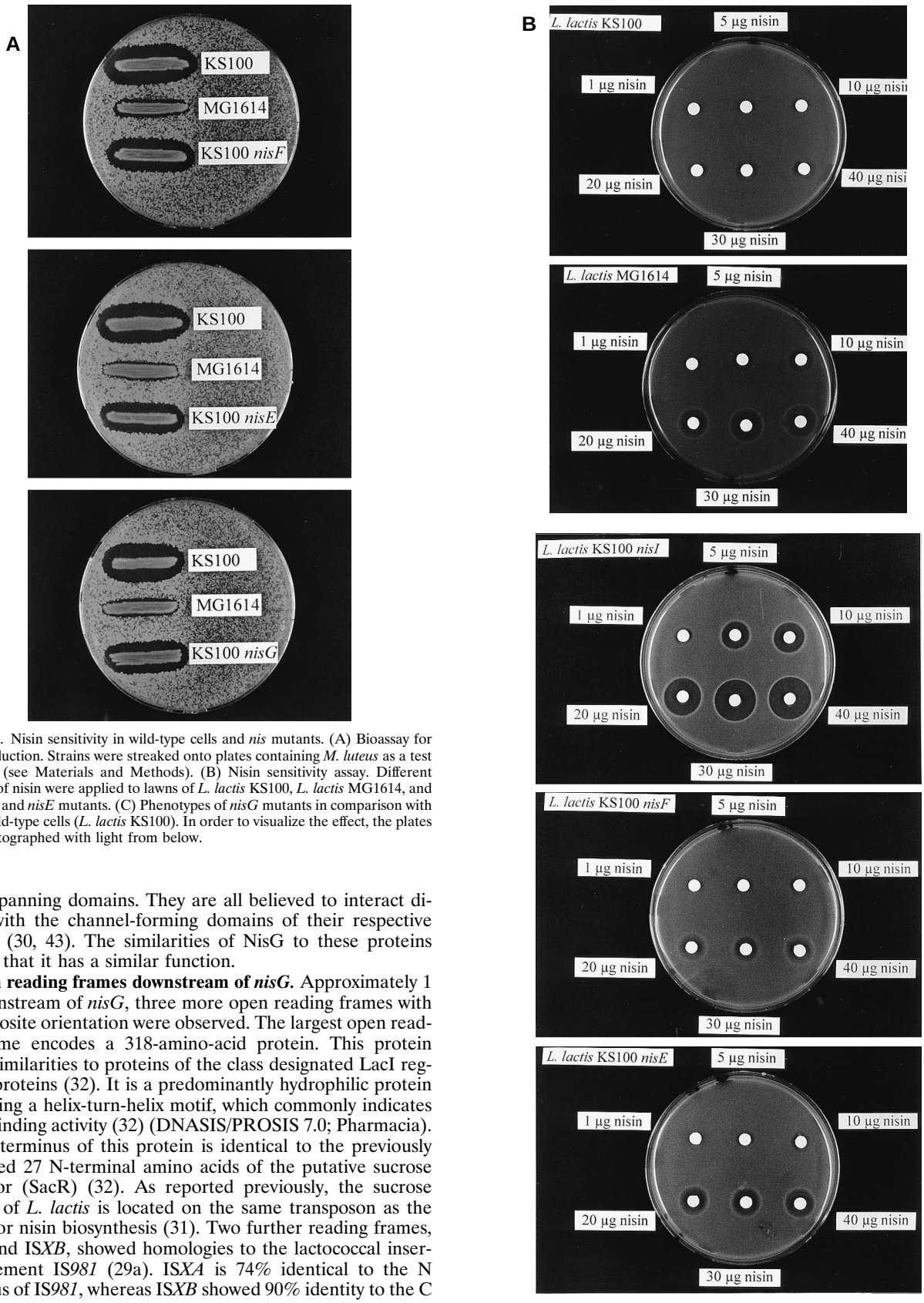

FIG. 5. Nisin sensitivity in wild-type cells and *nis* mutants. (A) Bioassay for nisin production. Strains were streaked onto plates containing *M. luteus* as a test organism (see Materials and Methods). (B) Nisin sensitivity assay. Different amounts of nisin were applied to lawns of *L. lactis* KS100, *L. lactis* MG1614, and *nisI*, *nisF*, and *nisE* mutants. (C) Phenotypes of *nisG* mutants in comparison with that of wild-type cells (*L. lactis* KS100). In order to visualize the effect, the plates were photographed with light from below.

brane-spanning domains. They are all believed to interact directly with the channel-forming domains of their respective colicins (30, 43). The similarities of NisG to these proteins suggest that it has a similar function.

Open reading frames downstream of *nisG*. Approximately 1 kb downstream of *nisG*, three more open reading frames with the opposite orientation were observed. The largest open reading frame encodes a 318-amino-acid protein. This protein shows similarities to proteins of the class designated LacI regulator proteins (32). It is a predominantly hydrophilic protein containing a helix-turn-helix motif, which commonly indicates DNA binding activity (32) (DNASIS/PROSIS 7.0; Pharmacia). The N terminus of this protein is identical to the previously published 27 N-terminal amino acids of the putative sucrose regulator (SacR) (32). As reported previously, the sucrose operon of *L. lactis* is located on the same transposon as the genes for nisin biosynthesis (31). Two further reading frames, IS*XA* and IS*XB*, showed homologies to the lactococcal insertion element IS*981* (29a). IS*XA* is 74% identical to the N terminus of IS*981*, whereas IS*XB* showed 90% identity to the C terminus of IS*981*. This indicates that both reading frames were derived from an ancestorial insertion element. However,

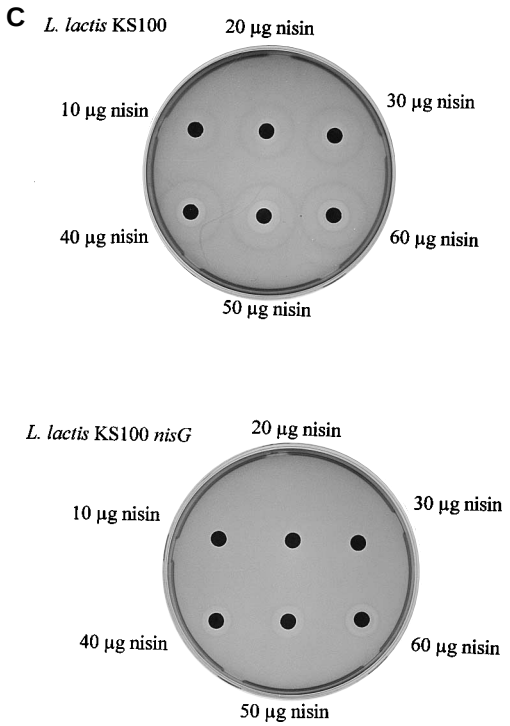

FIG. 5—*Continued.*

no suitable Shine-Dalgarno sequence could be found for IS*XA*, suggesting that this insertion element is not functional.

Genetic analysis of the open reading frames *nisI*, *nisF*, *nisE*, and *nisG*. The nisin-producing, plasmid-free strain *L. lactis* KS100 obtained by conjugation of *L. lactis* 6F3 with *L. lactis* MG1614 was used to investigate the physiological importance of the identified reading frames for nisin biosynthesis. The respective genes were interrupted by insertion of chloramphenicol or erythromycin resistance markers (Table 1). The flanking regions which allowed homologous recombination at either side of the insertion were approximately 1 kb each. For disruption of *nisF*, part of the gene was substituted by the *cat* gene from plasmid pC194 (15). The resistance marker was inserted into a *Pst*I-*Eco*RI site within *nisF*. Digestion of plasmid pSI41 (a subclone of pSI29 containing the genes *nisF*, *nisE*, and *nisG*) with *Pst*I-*Eco*RI resulted in a deletion of 150 bp of *nisF*. Plasmid pSI48, obtained by insertion of the *cat* gene into *nisF*, was used for transformation of *L. lactis* KS100. This plasmid does not replicate in *L. lactis*. In the case of *nisE*, plasmid pSI32, a 2.5-kb *Xba*I fragment of pSI29 cloned into pBSK, was used. The *cat* gene was inserted into a single *Sna*BI site within the coding region of *nisE*, yielding plasmid pSI53. For disruption of *nisG*, the same plasmid, pSI32, was used, but in this case the *cat* gene was inserted into a single *Nru*I site within the *nisG* gene, leading to plasmid pSI62. Another gene within the nisin gene cluster of *L. lactis* 6F3, *nisI*, which has already been shown to be involved in immunity to nisin (12), was also inactivated by insertion of a resistance marker. In this case plasmid pSI16, a subclone of pGEN3 (12), was used for *Hin*dIII digestion and insertion of the gene for erythromycin resistance. The resulting plasmid, pSI20, was used for disruption of the *nisI* gene in the chromosome of *L. lactis* KS100.

Phenotypes of *nisI*, *nisF*, *nisE*, and *nisG* mutants. The effects of mutations within the genes *nisF*, *nisE*, and *nisG* were first assessed by means of the nisin bioassay with *M. luteus* as the test organism (see Materials and Methods). All mutants were still able to produce nisin (Fig. 5A). This indicated that the respective genes were not directly involved in the biosynthesis of nisin. To investigate their possible role in immunity, the sensitivity to increasing concentrations of nisin was tested. As described previously, constitutive expression of the *nisI* gene in *L. lactis* MG1614 resulted in increased immunity to nisin, even if the cells were still more sensitive to nisin than those of the wild-type strain *L. lactis* KS100 (12). To confirm these results, the sensitivity of mutants with mutations in the *nisI* gene to increasing concentrations of exogenously applied nisin was investigated. In comparison with the wild-type strain *L. lactis* KS100, the *nisI* mutants exhibited a marked increase of sensitivity to exogenously applied nisin (Fig. 5B). The *nisF* and *nisE* mutants were also markedly more sensitive to exogenously applied nisin than the wild-type cells, although the increase in nisin sensitivity was less pronounced than that for the *nisI* mutants (Fig. 5B). Interestingly, mutants with mutations in the structural gene were also slightly more sensitive to nisin than the wild-type strain. Inactivation of *nisG* did not result in markedly increased sensitivity to exogenously applied nisin, but in comparison with the wild-type strain, the mutant exhibited a different phenotype. Whereas inhibition zones were clear with *nisG* mutants, they were more turbid with wild-type cells. Additionally, in the nisin diffusion assay, a ring of cells with reduced growth was observed with wild-type cells but not with mutants (Fig. 5C). Possibly the growth of wild-type cells is slowed down after application of nisin but the cells can overcome its toxicity by adaptation. These results suggest that the *nisG* mutant also became more sensitive to nisin.

DISCUSSION

Since the isolation of lantibiotic structural genes, which proved that lantibiotics are encoded by distinct genes, several genes involved in lantibiotic biosynthesis near the structural genes have been identified. The genes found near the structural genes of different producers show strong similarities, indicating their similar functions in lantibiotic maturation, secretion, and processing and in regulation of the biosynthesis. The genes for the biosynthesis of nisin are clustered on a 70-kb transposon. The gene cluster starts with the structural gene *nisA* (7, 18), which is followed by the genes *nisB*, *nisT*, *nisC* (11), *nisI*, *nisP*, *nisR*, and *nisK* (12, 47). The proteins NisB and NisC are assumed to catalyze the modification reactions during maturation of the prepeptide (11). The NisT protein seems to be involved in transport (11). NisI has already been reported to be involved in immunity to nisin (12, 26). NisP has been shown to be necessary for processing of the secreted prepeptide (47). NisR and NisK correspond to a two-component regulatory system consisting of a response regulator (NisR) and a histidine kinase (NisK), which regulate the biosynthesis of nisin (12). The identification of three open reading frames 1 kb downstream of *nisG* and in the opposite direction, of which two show strong similarities to an insertion element and one belongs to the sucrose operon (32), indicates that all the genes belonging to the nisin gene cluster of *L. lactis* 6F3 have been identified.

In order to make nisin acceptible for application in the food industry, the understanding of nisin immunity is of major importance. Although the nisin produced by some strains of *L. lactis* is the most prominent bacteriocin of lactic acid bacteria, little is known about nisin immunity. The self-protection necessary to overcome the action of their own products by a specific mechanism is a common feature of bacteriocin-producing cells. For channel-forming colicins, immunity proteins

which are predominantly hydrophobic with three or four potential membrane-spanning domains have been described (43). In the case of colicin E1 and colicin A of *E. coli*, specific integral membrane proteins are presumed to interact directly with the channel-forming domains of the respective colicins (43). Recently, three proteins, SpaI, SpaF, and SpaG, involved in the self-protection mechanism against the lantibiotic subtilin have been identified (21). The SpaI protein is a lipoprotein which is similar to the NisI protein, which increases immunity to nisin after its expression in a nonproducing *L. lactis* strain (12). Here we report on the identification of three genes, *nisF*, *nisE*, and *nisG*, which are involved in immunity to nisin. NisF shows strong similarity to McbF of *E. coli*. McbF, which also contains two ATP-binding sites, is thought to form a membrane complex together with McbE, a predominantly hydrophobic protein. This complex is expected to be involved in the specific transport of microcin B17 and in immunity to this peptide antibiotic. In the case of *L. lactis*, the hydrophobic partner which forms a complex with the ATP-binding domain (NisF) of the ABC transporter might be NisE or NisG.

Like several colicins and other linear lantibiotics, both nisin and subtilin have a common mode of action, depolarizing the energy-transducing cytoplasmic membrane via formation of aqueous channels (35). As in the case of subtilin and microcin B17, the ABC transporter could play a role in the self-protection mechanism against nisin by transport of the lantibiotic. Since the channel-forming activity of nisin has been reported to be dependent on the orientation of the transmembrane potential (4), it can act only from the outside of the cells. The active transport of mature nisin to the inside of the cells and subsequent degradation may be a means to protect the cells from damage caused by their own products. Another more specific mechanism may be provided by the action of NisI, which might interact directly with the channel-forming molecules (12).

In the case of several channel-forming colicins, immunity is conferred by specific membrane-spanning proteins, which are believed to interact directly with the channel-forming domains of their respective colicins (43). For example, the hydrophobicity plot of the colicin N immunity protein and that of NisG are very similar in configuration. As an alternative to the function as a hydrophobic domain in a putative ABC transporter, NisG might act in a manner similar to that of the colicin immunity proteins by interaction with pore-forming nisin molecules.

Our results suggest that there are different mechanisms by which nisin-producing cells protect themselves. These mechanisms involve at least the products of the genes *nisI*, *nisF*, *nisE*, and *nisG*.

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