APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1994, p. 814–825
0099-2240/94/$04.00+0

Vol. 60, No. 3

Regulation of Nisin Biosynthesis and Immunity in *Lactococcus lactis* 6F3

G. ENGELKE, Z. GUTOWSKI-ECKEL, P. KIESAU, K. SIEGERS, M. HAMMELMANN, AND K.-D. ENTIAN*

Institut für Mikrobiologie, Johann Wolfgang Goethe-Universität Frankfurt, Marie Curie Straße 9, D-60439 Frankfurt/Main, Federal Republic of Germany

Received 6 October 1993/Accepted 21 December 1993

The biosynthetic genes of the nisin-producing strain *Lactococcus lactis* 6F3 are organized in an operon-like structure starting with the structural gene *nisA* followed by the genes *nisB*, *nisT*, and *nisC*, which are probably involved in chemical modification and secretion of the prepeptide (G. Engelke, Z. Gutowski-Eckel, M. Hammelmann, and K.-D. Entian, Appl. Environ. Microbiol. 58:3730–3743, 1992). Subcloning of an adjacent 5-kb downstream region revealed additional genes involved in nisin biosynthesis. The gene *nisI*, which encodes a lipoprotein, causes increased immunity after its transformation into nisin-sensitive *L. lactis* MG1614. It is followed by the gene *nisP*, coding for a subtilisin-like serine protease possibly involved in processing of the secreted leader peptide. Adjacent to the 3′ end of *nisP* the genes *nisR* and *nisK* were identified, coding for a regulatory protein and a histidine kinase, showing marked similarities to members of the OmpR/EnvZ-like subgroup of two-component regulatory systems. The deduced amino acid sequences of *nisR* and *nisK* exhibit marked similarities to SpaR and SpaK, which were recently identified as the response regulator and the corresponding histidine kinase of subtilin biosynthesis. By using antibodies directed against the nisin prepeptide and the NisB protein, respectively, we could show that nisin biosynthesis is regulated by the expression of its structural and biosynthetic genes. Prenisin expression starts in the exponential growth phase and precedes that of the NisB protein by approximately 30 min. Both proteins are expressed to a maximum in the stationary growth phase.

Many lactic acid bacteria have antimicrobial activity which can be attributed to the production of bacteriocins (28). Bacteriocins are a heterogeneous group of bactericidal proteins produced by gram-negative and gram-positive bacteria. Some bacteriocins are small peptides, whereas others are complex proteins with several distinct subunits having molecular sizes sometimes in excess of 10^6 Da and are often found associated with carbohydrates or lipids.

One subgroup of bacteriocins, the lantibiotics, have received increased interest in the last few years. Lantibiotics belong to the small peptide group of bacteriocins, although they have a broader activity spectrum than most other bacteriocins and in that respect are more similar to antibiotics. Additionally, they differ from the other types of bacteriocins because of their chemical structure. They contain unusual amino acids such as dehydroalanine, dehydrobutyrine, lanthionine, and 3-methyllanthionine. The most prominent lantibiotics are nisin (17), subtilin (16), and epidermin (1). The number of members belonging to this group is steadily growing (for a review, see reference 23). Lantibiotics are ribosomally synthesized as prepeptides and posttranslationally modified (53). A scheme for posttranslational modification has been proposed by Ingram (22). The formation of these unusual amino acids could be explained by posttranslational dehydration of peptide serine and threonine residues, with subsequent addition of neighboring cysteine sulfur to the double bonds of the dehydroamino acids. The primary transcript of lantibiotic structural genes is a prepeptide which consists of an N-terminal leader peptide followed by a C-terminal propeptide from which the lantibiotic is matured. This gene structure was first described for epidermin (53) and was also found for subtilin (5), nisin (6, 11, 24), gallidermin (52), Pep5 (26), and cinnamycin (25).

Principal research interest has focused on nisin, as it is the only lantibiotic that has found practical application. It occurs naturally in dairy products (7) and is occasionally used in food production as an additive to dairy products to prevent spoilage by gram-positive bacteria, especially clostridia, staphylococci, bacilli, and listerias (20). Nisin has two natural variants, nisin A and nisin Z, which differ in a single amino acid residue at position 27 (histidine in NisA is replaced by asparagine in NisZ [9, 41]). The structural genes *nisA* (6, 11, 24) and *nisZ* (41) have been cloned several times from different sources. These were attributed to the 70-kb conjugative transposon Tn*5276* (44) and are genetically linked to the genes for sucrose fermentation (45). On the assumption that the genes for biosynthesis, secretion, and immunity are clustered on the same transposon, several kilobases of DNA downstream of the structural gene *nisA* were cloned and sequenced (12). Three genes, designated *nisB*, *nisT*, and *nisC*, which were homologous to the respective genes of the subtilin (30) and epidermin operons (51) were identified.

Gene deletion experiments proved that the genes *spaB*, *spaC*, and *spaT* are essential for subtilin biosynthesis (30). Adjacent to these genes, two additional open reading frames, *spaR* and *spaK*, were identified recently (29). These genes were also essential for subtilin biosynthesis and encoded a regulatory protein and a histidine kinase, representing a typical two-component regulatory system responsible for the regulation of subtilin biosynthesis.

* Corresponding author. Phone: 69 5800 9525. Fax: 69 5800 9527.

For epidermin, five open reading frames, *epiB*, *epiC*, *epiD*, *epiP*, and *epiQ*, adjacent to the *epiA* structural gene have been identified (51). The genes *epiB* and *epiC* are homologous to *nisB* and *nisC*, and their essential function in epidermin biosynthesis has been proven by mutant complementation (4). Transformation of all *epi* genes into *Staphylococcus carnosus* enabled heterologous epidermin production (51). Gene *epiD* is specific for epidermin biosynthesis. It encodes a flavoprotein involved in the formation of the characteristic C-terminal 2-aminovinylcysteine of the epidermin prepeptide (34). Gene *epiP* codes for a subtilisin-like serine protease which might be involved in cleavage of the leader peptide after secretion of the modified prepeptide.

In this study we report on the identification of four additional open reading frames, *nisI*, *nisP*, *nisR*, and *nisK*, of the nisin operon of *Lactococcus lactis* 6F3 which are involved in immunity against nisin, processing of the posttranslational modified prenisin, and regulation of nisin biosynthesis. Previously genes *nisP* and *nisR* have been also isolated from transconjugant strain *L. lactis* NIZO R5 and the essential function of *nisR* for nisin biosynthesis has been shown (63).

MATERIALS AND METHODS

Strains, media, and plasmids. The nisin-producing strain *L. lactis* 6F3 (kindly provided by T. Hörner, University of Tübingen) and plasmid-free, nisin-nonproducing *L. lactis* strains MG1363 and MG1614 (15) were grown at 30°C in M17 broth (60) supplemented with 0.5% sucrose (M175) or with 0.5% glucose (M17G). *L. lactis* KS100 is a transconjugant obtained from mating the nisin A-producing strain *L. lactis* 6F3 and nisin nonproducer MG1614. For overproduction of nisin and nisin prepeptide detection, CM medium (10), containing 1% sucrose, 1% peptone (Difco), 1% yeast extract (Difco), 0.2% NaCl, 0.002% $MgSO_4 \cdot 7H_2O$, and 1% KH_2PO_4, pH 6.8, was used. *Micrococcus luteus* ATCC 9341 was used as an indicator strain in halo assays.

For subcloning of DNA fragments the *Escherichia coli* strains RRI (F^- *hsdS20* [r_B^- m_B^-] *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*) were used. *E. coli* strains were grown at 37°C in Luria-Bertani broth or agar (GIBCO, Neu Isenburg, Germany). The vectors pUC19 (68) and pBSKRII (Stratagene, Heidelberg, Germany) were used for cloning purposes. If antibiotics were used, the following concentrations were employed: ampicillin, 40 μg/ml; erythromycin, 150 μg/ml; chloramphenicol, 20 μg/ml for *E. coli* and 5 μg/ml for *L. lactis*.

Molecular biology techniques. Established molecular biology protocols were followed (47). 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 (59).

Cloning of the nisin operon. 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 (57) with appropriate DNA fragments as probes. For isolating additional DNA fragments, the 3′-terminal 0.3-kb *Hin*dIII-*Eco*RI segment of the 4.2-kb *Eco*RI fragment (12) was used as a hybridization probe to identify an overlapping adjacent 1.7-kb *Hin*dIII DNA fragment. The downstream 2.9-kb *Eco*RI DNA fragment was isolated similarly by chromosome walking with an appropriate DNA probe.

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) using a *Taq* DyeDeoxy Terminator Cycle sequencing kit (Applied Biosystems), according to the protocol of the commercial supplier.

Computer analyses of DNA and analyses of amino acid sequences were performed with the DNASIS/PROSIS version 7.0 program (Pharmacia, Freiburg, Germany). For protein homology searches the data bases SWISS-PROT R23.0 (August 1992) and NBRF-PIR R33.0 (June 1992) were used with the TFASTA algorithm of Lipman and Pearson (37). Hydrophobicity plots were created according to the methods of Kyte and Doolittle (35).

Prenisin detection. *L. lactis* cells were harvested by centrifugation at 5,000 × *g* at 4°C for 10 min and resuspended in 100 μl of loading buffer (5% sodium dodecyl sulfate [SDS], 30% glycerol, 0.01% bromphenol blue, 25 mM Tris-HCl, 0.01% β-mercaptoethanol, pH 6.8). After heating (10 min, 100°C) and short centrifugation (1 min, 14,000 × *g*) the protein content of the supernatant was measured (69). The samples were applied to a discontinuous SDS-polyacrylamide gel electrophoresis system according to established methods (50) using 15% acrylamide tricine gels. Proteins were electroblotted onto Immobilon-P membranes (Millipore; 0.45-μm pore size) by using a discontinuous buffer system (LKB 2117, Multiphor II Elektrophoresis System; Pharmacia-LKB, Freiburg, Germany) as recommended by the commercial supplier. As a reference the lane with standard proteins (Boehringer) was stained with amido black solution (0.5% amido black, 50% methanol, 10% acetate) and washed again with destaining solution (45% methanol, 10% acetate). The sheets were incubated in 20% fetal calf serum (GG-free fetal calf serum; GIBCO BRL, Life Technologies GmbH, Eggenstein, Germany) in phosphate-buffered saline for 20 min. Antibody-containing sera were diluted 1:1,000 with 20% fetal calf serum. Membranes were exposed to antibody solution on a rotary shaker overnight and thereafter washed three times at room temperature for 10 min with TBST (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.05% Triton X-100). Incubation was continued for 2 h with anti-rabbit immunoglobulin G solution (1:5,000 in TBST; alkaline phosphatase conjugate; Sigma, Munich, Germany) followed by three washes with TBST. Thereafter filters were washed for 10 min with AP buffer (100 mM Tris-HCl [pH 9.5], 100 mM NaCl, 5 mM $MgCl_2$) and then incubated in AP buffer containing nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate for the detection of positive bands. Quantification of prenisin was performed by scanning the stained immunoblot with a Personaldensitometer (Molecular Dynamics, Krefeld, Germany).

Detection of NisB protein. *L. lactis* cells were grown overnight in CM medium at 30°C. Cells were harvested by centrifugation and resuspended in 0.2 ml of STET buffer (25 mM Tris-HCl, 10 mM EDTA, 50 mM glucose, pH 8.0) containing 2 mM phenylmethylsulfonyl fluoride. Lysozyme (0.5 mg) was added, and after 2 min of incubation the cell suspension was boiled for 1 min. After the suspension was cooled on ice, 0.5 g of glass beads (0.1-mm diameter) was added, and the suspension was mixed on a laboratory shaker (Heidolph REAX 2000). SDS-polyacrylamide gel loading buffer (0.01% bromphenol blue, 15% sucrose, 6% β-mercaptoethanol, 7.5% SDS) was added, and after centrifugation (1 min, 14,000 × *g*) aliquots were applied to SDS-polyacrylamide gel electrophoresis on 7.5% acrylamide gels. The protein content was determined after chloroform-methanol precipitation according to

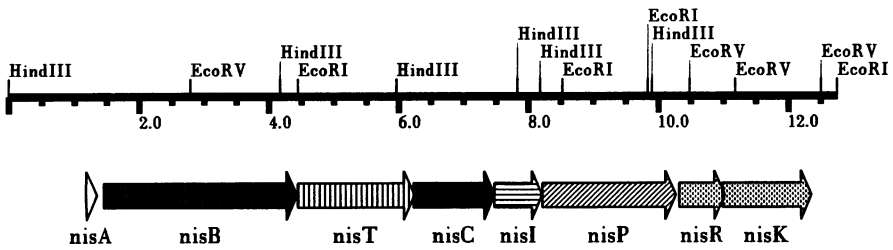


FIG. 1. Map of the nisin operon of *L. lactis* 6F3 with all known genes and restriction sites used for subcloning. Numbers indicate kilobases.

the method in reference 69. Western blot (immunoblot) analysis for detection of NisB was performed as previously described (12).

Nisin bioassay. An overnight culture (0.3 ml) of *M. luteus* ATCC 9341 (adjusted to an optical density at 600 nm of 0.6) was added to 500 ml of molten Luria-Bertani agar, mixed, and poured into petri dishes (10 ml). Filtered 1-ml aliquots of culture fluid, harvested at various times during the growth of the nisin-producing strain, were lyophilized and redissolved in 200 μl of water. Different volumes of this concentrate (reciprocal to the optical density at 600 nm of the culture) were applied with filter disks on the agar surface, and the zone of growth inhibition of *M. luteus* surrounding the filter disk was determined.

Expression of *nisI* in *L. lactis* MG1614. Lactococcal vector pGK12 (32) was used to construct the expression vector pSI19. The unique *Sna*BI site of pGK12 was used to introduce the multiple cloning site of pBluescript SK−. The multiple cloning site of pBluescript SK− was obtained as a 0.173-kb *Bss*HII fragment (blunt ended by Klenow enzyme treatment) and cloned into pBluescript SK−/*Sna*BI. The resulting plasmid, pSI12, was used to generate a gene expression vector by inserting a 0.12-kb DNA fragment of plasmid pMG36e containing the strong constitutive lactococcal promoter P32 (62) into the multiple cloning site. A DNA fragment containing promoter P32 was synthesized via PCR amplification, digested with *Eco*RI-*Bam*HI, and cloned into vector pSI12 to obtain pSI19. A 0.8-kb fragment of plasmid pGEN3 (12) containing the ribosomal binding site and the entire *nisI* gene was isolated by PCR amplification, digested with restriction enzymes *Xba*I-*Bam*HI, and inserted into expression vector pSI19. The resulting plasmid, designated pSI22, was introduced into *L. lactis* MG1614 by electroporation (19).

PCR and plasmid isolation. Plasmid DNA from *L. lactis* was isolated according to a method previously described (36). PCR was carried out by following standard procedures (47) in an Eppendorff Microcycler E apparatus. By using *Taq* DNA polymerase (Boehringer), 35 cycles were performed with 20 s at 94°C followed by 20 s at 55°C and finally by 2.5 min at 72°C.

Plate diffusion bioassay for nisin sensitivity. To examine the effect of *nisI* gene expression on immunity to nisin, 1 ml of an overnight culture (stationary-phase cells) of different *L. lactis* strains grown in M17SG medium (containing the appropriate antibiotic if required) was added to 200 ml of molten M17SG agar, 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 (0.1 to 20 μg) to filter disks on the agar surface. The agar plates were incubated overnight at 30°C, and the diameter of the inhibition zone was determined.

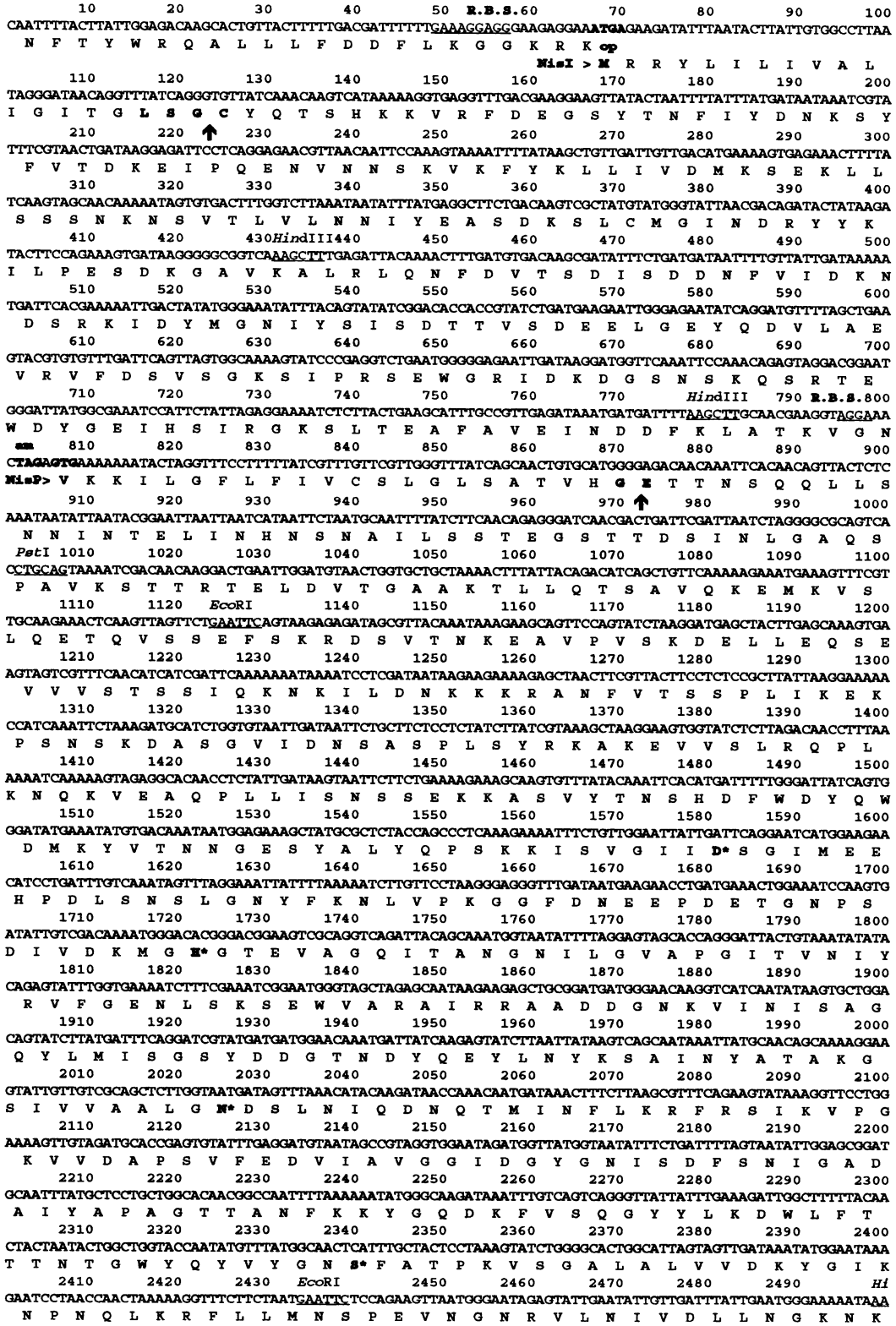
Nucleotide sequence accession number. The sequence data published here were submitted to the EMBL sequence data bank under accession number X76884.

RESULTS

Downstream from the nisin structural gene *nisA* we recently identified three genes (*nisB*, *nisT*, and *nisC*) which are necessary for nisin biosynthesis and transport through the cellular membrane (13) (Fig. 1). Further cloning and sequencing of 5 kb downstream from *nisC* revealed four additional genes involved in nisin biosynthesis of *L. lactis* 6F3.

Nisin immunity gene, *nisI*. The *nisI* gene was identified downstream from *nisC*, and its open reading frame overlaps with the TGA stop codon of the gene *nisC*. The *nisI* gene starts with a ATG codon (position 67, Fig. 2), which is preceded by a potential ribosome-binding site (TGAAAGGAGG) with nearly perfect homology to the consensus ribosome-binding site for *L. lactis* (TGAAAGGAGGT) (61). The *nisI* gene probably encodes a protein of 245 amino acids with a theoretical calculated molecular size of 27.8 kDa. The entire NisI protein showed no significant similarities to other proteins, except that the sequence of its N terminus strongly resembles that of N-terminal signal peptide sequences of lipoproteins from gram-negative *E. coli* as well as from gram-positive *Bacillus* species and *L. lactis*. Bacterial lipoproteins are a group of exported proteins that are anchored to the cellular or outer membrane by lipid moieties. The lipids are covalently linked to the cysteine residue at the distal N terminus of the secreted protein (67). Comparison of the N terminus of NisI with other known lipoprotein signal peptides shows that there is significant similarity to the typical consensus sequence (Leu-X-Gly/Ala ↓ Cys-X) of the cleavage site (arrow) for lipoproteins (Table 1). Additionally, the N-terminal part of NisI has the typical tripartite structure of signal peptides, with a positively charged NH_2 terminus, a hydrophobic core, and a cleavage region, which is also found for signal sequences of bacterial lipoproteins (64). This suggests that the NisI protein is a membrane-bound lipoprotein located on the outside of the cell membrane.

To date no genes similar to *nisI* have been found in the other lantibiotic operons. Because of its singularity and its putative location as a lipoprotein on the outside of the cellular membrane, we thought that it is not directly involved in nisin biosynthesis and tested its possible role in immunity. The *nisI* gene was fused to the constitutive promoter P32 (62) and expressed in the nonimmune nisin-negative strain *L. lactis* MG1614. The same strain was also conjugated with strain *L. lactis* 6F3, resulting in nisin-producing strain *L. lactis* KS100. *L. lactis* MG1614 was highly sensitive to exogenously supplied nisin, showing a weak inhibition zone if 0.1, 0.5, or 1.0 μg of nisin was applied and a strong inhibition zone with 5, 10, or 20 μg of nisin (Fig. 3). In contrast to this, the transconjugant



Continued on following page

FIG. 2. Nucleotide sequence of cloned 5.0-kb region downstream from *nisC* (12) with open reading frames *nisI*, *nisP*, *nisR*, and *nisK*. Possible ribosome-binding sites (R.B.S.), restriction sites, and inverted repeats are underlined. Open reading frames are designated by a one-letter code. Arrows indicate the putative signal peptide cleavage sites of NisI and NisP; the putative membrane anchor sequence (13) of NisP is underlined. Conserved, functional, and active-site amino acids are written in boldface letters and marked by asterisks.

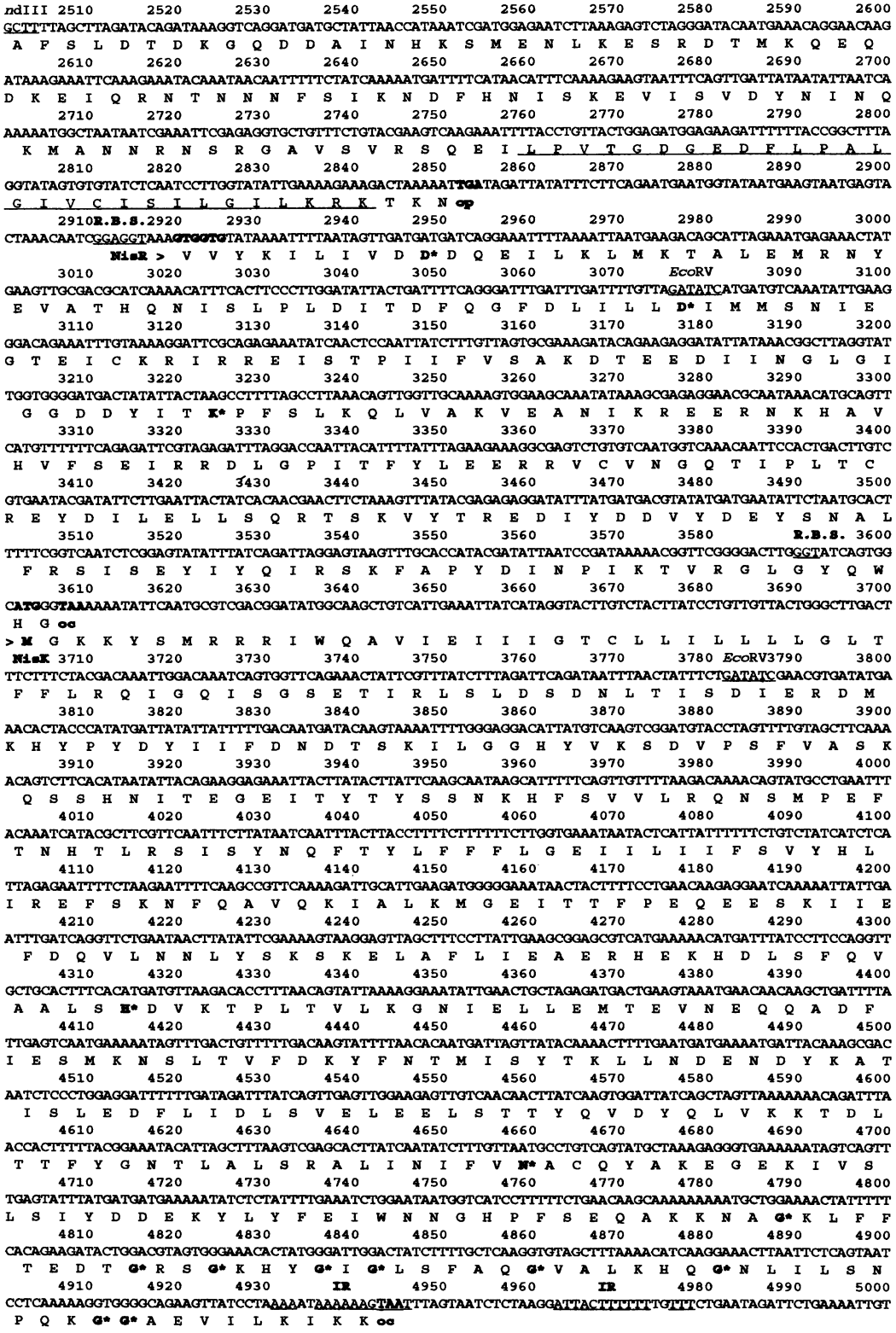


FIG. 2—*Continued.*

strain *L. lactis* KS100 and transformant strain *L. lactis* MG1614/22 (harboring the constitutively expressed *nisI* gene) were not sensitive to 0.1, 0.5, or 1.0 μg of nisin and showed only weak inhibition of growth with 5, 10, or 20 μg of nisin. Although the transconjugant strain KS100 was slightly less sensitive to nisin compared with the *nisI* transformant strain MG1614/22, *nisI* conferred increased immunity to the nisin-sensitive, nonproducing strain MG1614.

Nisin processing. The gene *nisI* is followed by the *nisP* gene within 1 bp of the end of the *nisI* open reading frame. The *nisP* gene possibly starts with a GTG codon (position 806, Fig. 2) preceded by a potential ribosome-binding site (AGGA) within 6 bp of the GTG start codon and ends with a TGA stop codon (position 2852, Fig. 2). It encodes a putative protein of 682 amino acid residues, corresponding to a calculated molecular size of 74 kDa. Examination of data bases for homologous proteins showed that NisP has characteristic similarities to serine proteases, especially with the group of subtilisin-like serine proteases. Members of this group are, for example, the major intracellular serine protease ISP-I from *Bacillus subtilis* (31) and thermitase from *Thermoactinomyces vulgaris* (38). The characteristic conserved amino acid motifs of the active and catalytic sites of this group are also strongly conserved in NisP. The three active-site residues Asp, His, and Ser and the oxyanion hole residue Asn are essential for the function of these proteins (56); in NisP these catalytic residues are predicted to be Asp-259, His-306, Ser-512, and Asn-407, respectively (Fig. 2). The *nisP* gene from *L. lactis* 6F3 is almost identical to the *nisP* gene from *L. lactis* NIZO R5 which was recently described and shown to cleave synthetic prenisin when expressed in *E. coli* (63). The only difference is a threonine residue at position 500 in *L. lactis* 6F3 instead of the reported alanine residue.

NisP is also very similar to EpiP, a serine protease from the epidermin operon which may be involved in processing the posttranslationally modified, secreted prepeptide of the lantibiotic epidermin (51). The catalytic domain of NisP (amino acid residues 220 to 570) shows great similarity in sequence and length to the corresponding segment of EpiP (41%) (amino acid residues 220 to 462). In contrast to EpiP, the N-terminal pre-pro sequence of NisP differs considerably in size (220 versus 110 residues), and the C-terminal extension with the possible membrane anchor (Fig. 2) found in NisP is completely absent in EpiP.

Regulation of nisin biosynthesis. The *nisP* gene is followed by two additional open reading frames, *nisR* and *nisK*. The *nisR* open reading frame starts 49 bp distant from the end of the *nisP* stop codon with a GTG codon (position 2923, Fig. 2), which is not unusual for *L. lactis*. It is preceded by another GTG codon; it is thus unclear which of these codons functions as the original start codon. However, the second GTG codon is the correct distance (7 bp) from a consensus lactococcal ribosome-binding site (GGAGGT). Assuming that the second GTG functions as the start codon, *nisR* encodes a protein of 228 amino acids with a calculated molecular size of 27 kDa. The *nisR* open reading frame overlaps with a further open reading frame, *nisK*. It starts with an ATG codon (position 3702, Fig. 2), ends with TAA as a stop codon (position 4943, Fig. 2), and probably encodes a protein of 447 amino acids with a deduced molecular size of 51.3 kDa.

The two proteins, NisR and NisK, have strong similarities to proteins of two-component regulatory systems. For NisR the highest similarity was observed with members of the subfamily of the OmpR-like response regulators, e.g., PhoP (39) from *Salmonella typhimurium* (25.0% identity), PhoB (3) from *Pseudomonas aeruginosa* (32% identity), PhoP (55) from *B. subtilis* (31.2% identity), and OmpR (8) from *E. coli* (31.4% identity). The maximum degree of homology (41.3%) was found between NisR and SpaR (29), the response regulator of subtilin biosynthesis (Fig. 4). The region with the highest similarity in all response regulators is the N terminus, where phosphorylation of a conserved Asp residue by the corresponding histidine kinase takes place (48, 49). It is the location of three active-site residues, the site of phosphorylation included, which are conserved in all members of the family and, by sequence identity, in NisR are predicted to be Asp-10, Asp-53 (site of phosphorylation), and Lys-102.

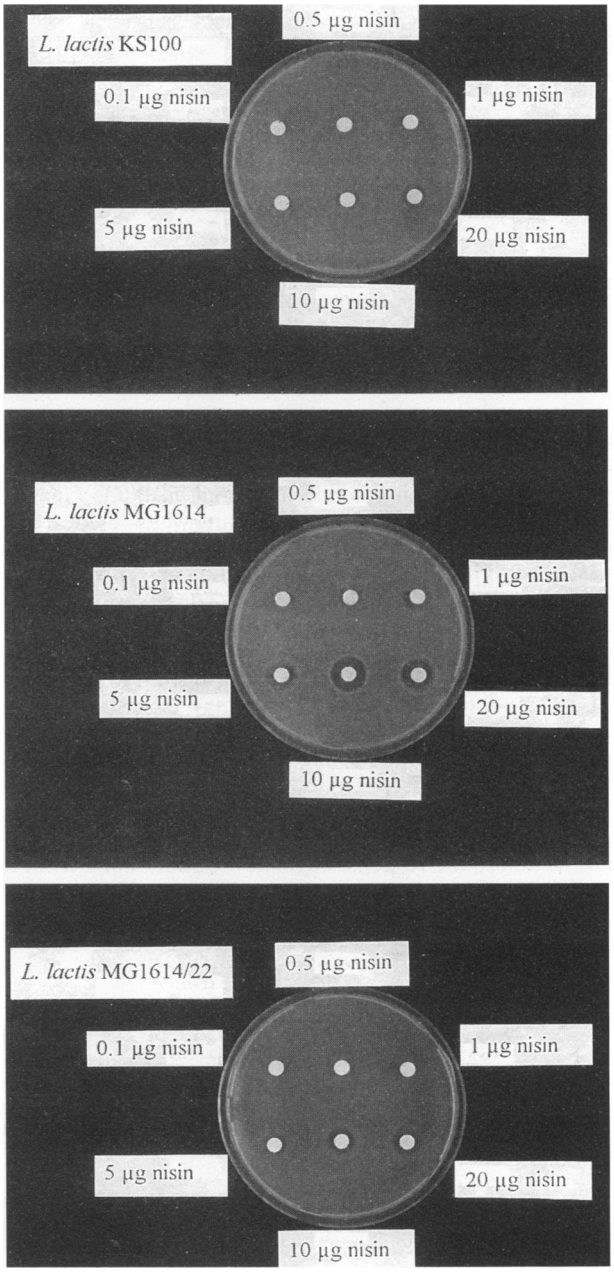


FIG. 3. Bioassay for detection of immunity against nisin: effect of different amounts of nisin on growth of the nisin-producing transconjugant *L. lactis* KS100, the nisin nonproducing, nisin-sensitive strain *L. lactis* MG1614, and *L. lactis* MG1614/22 transformed with the *nisI* gene (bottom).

TABLE 1. Comparison of the signal peptide sequence of NisI with other known lipoprotein signal peptide sequences

Protein	Source	Signal peptide[a]	Reference
Braun's lipoprotein	*E. coli*	MKATKLVGAVILGSTL**LAG**↓CS	42
β-Lactamase	*Bacillus licheniformis*	MKLWFSTLKLKKAAAVLLFSCVA**LAG**↓CA	43
β-Lactamase	*Bacillus cereus*	MFVLNKFFTNSHYKKIVPVVLLSCAT**LIG**↓CS	21
β-Lactamase	*Bacillus* sp. (alkalophilic)	MIVPKKFFHISHYKKMLPVVLLSCVT**LIG**↓CS	27
PrsA	*B. subtilis*	MKKIAIAAITATSILA**LSA**↓CS	33
PAL-related protein[b]	*B. subtilis*	MRYRAVFPMLIIVFA**LSG**↓CT	18
PrtM[b]	*L. lactis*	MKKKMRLKVLLASTATALLL**LSG**↓CQ	65
NisI[b]	*L. lactis*	MRRYLILIVALIGITG**LSG**↓CY	This paper

[a] ↓, cleavage site for signal peptidase II (the consensus cleavage site has been written in boldface).
[b] The lipoprotein character of the protein is deduced only from similarity.

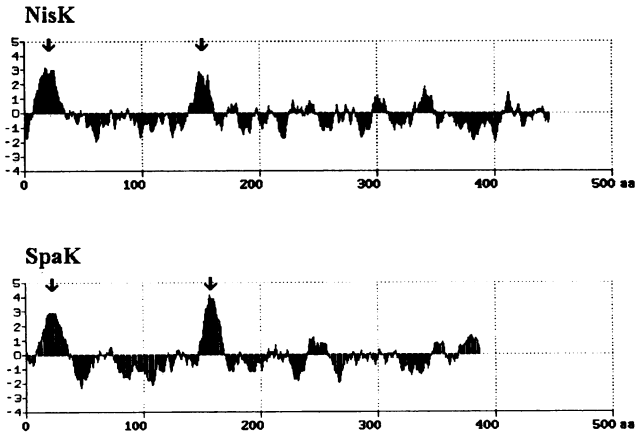
NisK shows significant similarity to the histidine kinases of two-component regulatory systems, which act as membrane-located sensors of environmental signals (for a review, see reference 58). Sequence similarity among histidine kinases is restricted mainly to the C-terminal part, which is located on the inside of the cytoplasmic membrane. This section contains a conserved histidine residue (His-238 in NisK) where autophosphorylation takes place (48), a conserved asparagine residue (Asp-349 in NisK), and a glycine-rich stretch at the C-terminal end (amino acid 380 to the extreme C-terminal end, Fig. 2). NisK was similar to EnvZ (8) from *E. coli* (19.2% identity) and to SpaK (21.4% identity), necessary for the regulation of subtilin biosynthesis in *B. subtilis* (Fig. 5). The similarity to other histidine kinases, such as PhoR (54) from *B. subtilis* (18.4% identity) or PhoM (2) of *E. coli* (19.3% identity), is limited to the C-terminal region of the protein containing the conserved histidine residue, which plays a role in signal transduction to the response regulator. Beside similarity at the amino acid level, a characteristic histidine kinase hydrophobicity plot was determined for both NisK and SpaK (Fig. 6). Two hydrophobic regions at the N terminus are interrupted by a hydrophilic stretch, and the C-terminal part is also hydrophilic. The two hydrophobic regions are possibly transmembrane domains, and the hydrophilic region in between may correspond to the extracellularly located sensor domain. The hydrophilic C-terminal region might be the signal-transducing domain with kinase activity.

To confirm the regulation of nisin biosynthesis, we monitored the growth-dependent expression of both prenisin and NisB, which is probably involved in the modification reactions (12). Prenisin expression was monitored by using polyclonal antibodies directed against the leader sequence (Fig. 7a). Its expression strongly increased when cells reached the mid-logarithmic growth phase (Fig. 7c), reaching a maximum in the early stationary growth phase. In the stationary phase, prenisin expression decreased slowly, with approximately 60% residual activity remaining after 26 h in the stationary phase. The NisB protein was very poorly expressed in the first 4 h after inoculation and thereafter strongly increased in the late logarithmic growth phase, reaching its maximum in the stationary

FIG. 4. Similarity of *L. lactis* regulatory protein NisR to SpaR, the regulator of subtilin biosynthesis from *B. subtilis* (29), and response regulator OmpR from *E. coli* (8). Identical amino acid residues relative to NisR are indicated by colons; similar amino acid residues relative to NisR are indicated by dots. Functional or conserved amino acid residues (Asp-10, Asp-53, and Lys-102) are marked by asterisks. The arrow indicates a phosphorylation site.

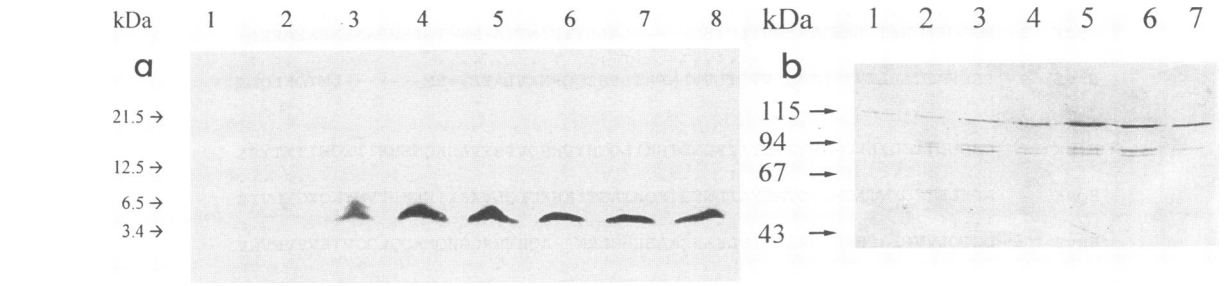
```
NisK  1- MGKKYSMRRRIWQAVIEIIIGTCLLIL---LLLGLTFFLRQIG-QI-SG-------SETIRLSL-
SpaK  1- MGIGFKGRKTLLRELVKYMVTLCISLV---VLALLYIFINTIA-MN-TGFSHPANYNEREAEKL-
EnvZ  9- RSSFARTLLLIVTLLFASLVTTYLVVLNFAILPSLQQFNKVLAYEV-RM-------LMTDKLQLE

NisK      DSDNLTISDIERDMKHYPYDYIIFDNDTSKILGGHYVKSDVPSFVASKQSSHNITEGEITYTYSS
SpaK      APKLETIDKVTADMIPDTMSYAILNKETKQKTAGTIKEKDLQLVKKKIEKKPYVNYKQKGYLVIE
EnvZ      DGTQLVVPPAFRREIYRELGISLYSNEAAEEAGLRW--AQHYEFLSHQMAQQLGGPTEVRVEVNK

NisK      NKHFSVVLRQNSMPEF-TNHTLRS--ISYN-QFTYLF-FFLGEIILIIFSVYHLIREFSKNFQAV
SpaK      RNNEYCVLQYSLRADF-SSPLLRKYLPNYELTSICIL-IILLIIVISIITTY-FANRLRKHFETL
EnvZ      SSP-VVWLKTWLSPNIWVRVPLTE--IHQG-DFSPLFRYTLAIMLLAIGGAWLFIRIQNRPLVDL

                                                                        *
NisK      QKIALKMGEITTFPEQEESKIIEFDQVLNNLYSKSKELAFLIEAERHEKHDLSFQVAALSHDVKT
SpaK      NVITRYIKEQNLQFTPEFTHIKEFDDVIDSLIEMRDALQSSLEALWRLEKNKKEQIGALAHEIKI
EnvZ      EHAALQVGK-GIIP----PPLREYG--ASEVRSVTRAFNHMAAGVKQLADDRTLLMAGVSHDLRT

NisK      PLTVLKGNIELLEMTEVNEQQADFIESMKNSLTVFDKYFNTMISYTKLLNDENDYKATISLEDFL
SpaK      PITIIKGNAELLSLSMQNEEQAEYTKYILGAGNQIEQYIYQLIHLSKTEDALTIHLEKASVDELT
EnvZ      PLTRIRLATEMM-----SEQDGYLAESINKDIEECNAIIEQFIDY---LRTGQE--MPMEMADLN

                                                  *
NisK      IDLSVELEELSTTYQVDYQLVKKTDLTTFYGNTLALSRALINIFVNACQYAKEGEKIVSLSIYDD
SpaK      ETLVKDISAYKGNKNINISFKKENLMKEAKIDWQLLHRALLNILTNAVDYTPEGG-TVSVHAESI
EnvZ      AVLG-EVIAAESGYEREIETALYPGSIEVKMHPLSIKRAVANMVVMAARY---GNGWIKVSSGTE

NisK      EKYLYFEIWNNGHPFSEQAKKNAGKLFFTEDTGRSGKHYGIGLSFAQGVALKHQGNLILSNPQKG
SpaK      LRYFIFL -387
EnvZ      PNRAWFQVEDDGPGIAPEQRKHLFQPFVRGDSARTISGTGLGLAIVQRIVDNHNGMLELGTSERG

NisK      GAEVILKIKK -447
EnvZ      GLSIRAWLPVPVTRAQGTTKEG -450
```

FIG. 5. Similarities between NisK and the histidine kinases SpaK from *B. subtilis* (29) and EnvZ (8) from *E. coli*. Identical amino acid residues relative to NisK are indicated by colons; similar amino acid residues relative to NisK are indicated by dots. Functional histidine residue His-238 (site of phosphorylation) and conserved Asn-349 are marked by asterisks.



phase, decreasing by approximately 50% after overnight incubation (Fig. 7b). No nisin activity was observed by bioassay within the first 5 h after inoculation. Thereafter nisin activity strongly increased, reaching a maximum in the stationary growth phase. After overnight incubation nisin activity decreased to about 30% of the maximal value. Comparing the kinetics of NisA, NisB, and nisin activity revealed that prenisin expression clearly preceded that of NisB and nisin activity, which on the other hand is coordinately expressed with NisB.

FIG. 6. Comparison of the predicted hydrophobicity profiles for NisK and SpaK. Hydrophobic putative membrane-spanning segments are indicated by arrows.



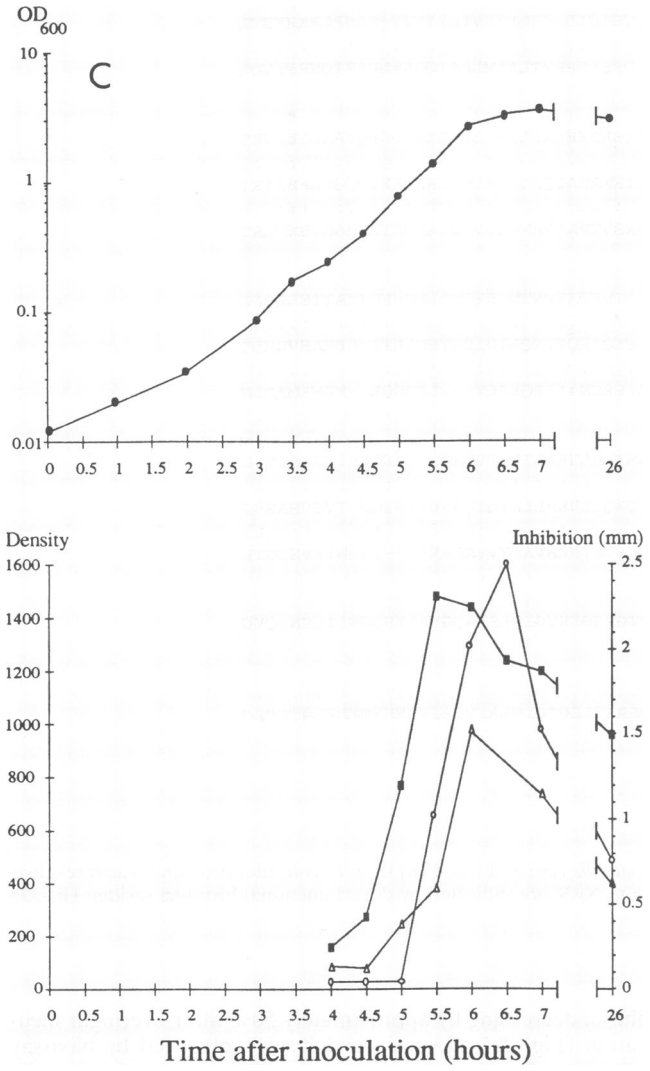


FIG. 7. Growth phase-dependent kinetics of prenisin and NisB expression and nisin production. (a) Immunoblot analysis of prenisin expression (lane 1, 4 h; lane 2, 4.5 h; lane 3, 5 h; lane 4, 5.5 h; lane 5, 6 h; lane 6, 6.5 h; lane 7, 7 h; lane 8, 26 h). Times correspond to hours after batch culture inoculation. Molecular size standards are given on the left. (b) Immunoblot analysis of NisB expression (lane 1, 4 h; lane 2, 4.5 h; lane 3, 5 h; lane 4, 5.5 h; lane 5, 6 h; lane 6, 7 h; lane 7, 26 h). Times correspond to hours after batch culture inoculation. (c) Densitometric analysis of prenisin expression (squares), NisB expression (triangles), and nisin activity (diameter of inhibition zones; circles). The corresponding growth curve is given in the upper panel. OD_{600}, optical density at 600 nm.

These results show that the expression of prenisin and that of NisB are both regulated, probably by the NisK-NisR two-component regulatory system.

DISCUSSION

Nisin is the most important member of the lanthionine-containing peptide antibiotics now commonly referred to as lantibiotics. Genes involved in nisin biosynthesis are organized into an operon-like structure starting with the structural gene *nisA* (6, 24) followed by genes *nisB*, *nisC*, and *nisT*, which we described previously (12). According to the present hypothesis, modification reactions occur on the intact prepeptide (53) by dehydration of serine and threonine residues followed by subsequent sulfur addition from neighboring cysteine residues. This has been proven by the isolation of modified Pep5 prepeptides from *Staphylococcus epidermidis* 5 (66). NisB and NisC could correspond to the enzymes necessary for the modification reactions. In addition, the membrane localization of NisB was recently shown (12). The NisT protein seems to be involved in transport through the cellular membrane (12). Isolation of adjacent DNA fragments downstream of *nisC* revealed four further open reading frames, the first of which was *nisI*. The NisI protein derived from the DNA sequence has a leader sequence characteristic of lipoproteins, and expression of the *nisI* gene in a nisin-sensitive nonproducer conferred increased nisin resistance to this strain. This clearly shows that NisI is involved in immunity against exogenously supplied nisin. No other genes similar to *nisI* have been found in other lantibiotic operons investigated thus far. There is little information on immunity or resistance determinants against lantibiotics in the literature. In the case of Pep5 from *S. epidermidis* 5, a gene, *pepI*, which conferred immunity against Pep5 to its producer was identified (46). The *pepI* gene encodes a small peptide of 69 amino acid residues which provides immunity when expressed in conjunction with the structural gene *pepA*. NisI and PepI have no sequence similarities. A gene, *nsr*, conferring resistance against nisin has been isolated from *L. lactis* subsp. *lactis* biovar diacetylactis DRC3, which is a nisin nonproducer (14). The 318-amino-acid Nsr protein was predicted to be membrane associated as a result of its hydrophobic N terminus. However, the level of resistance was only 10% of that of a nisin-producing strain. Hybridization of an *nsr* gene probe with chromosomal DNA of the nisin producer *L. lactis* 11454 revealed that no homologous gene was present in the genome of this strain. These results show that the Nsr protein led to an acquired resistance, thus differing considerably from the immunity afforded to nisin-producing strains.

The mechanism of immunity remains speculative. Considering the lipoprotein-like character of NisI, attached to the exterior of the cellular membrane by lipid moieties, this protein might confer resistance by floating in the membrane and interacting directly with nisin or by disturbing the association of nisin aggregates, thus preventing channel formation.

The regulation of nisin biosynthesis appears to be highly complex. The first of the biosynthetic proteins to be expressed is prenisin. Its expression precedes, by about 30 min, that of NisB, which probably encodes one of the maturation enzymes. Nisin activity coincides with NisB expression, which also implicates NisB in the maturation of the prepeptide. Surprisingly, even after overnight incubation in the stationary phase, prenisin and NisB were still present at approximately 60% of the maximum level. NisB expression in the stationary phase indicates that modification reactions still occur, which in turn indicates that prenisin is continuously synthesized in the stationary phase. This finding may open new production possibilities by using immobilized nisin-producing cells.

The expression of the biosynthetic genes is clearly regulated. The nisin regulatory system seems to be very similar to that recently identified for the regulation of biosynthesis of the lantibiotic subtilin from *B. subtilis* (17a, 30). For *B. subtilis* it was shown that gene disruption of either of the proteins SpaR and SpaK led to a failure in subtilin production. On the basis of the striking similarities between nisin and subtilin biosynthesis, we assume that the regulatory proteins NisR and NisK are also essential for nisin biosynthesis. Recently the NisR part of the two-component regulatory system has also been identified in *L. lactis* NIZO R5 (63). However, downstream from the cloned *nisR* gene from *L. lactis* NIZO R5 no coding information for NisK was present. Three possibilities may explain the lack of *nisK* in *L. lactis* NIZO R5: (i) the *nisK* gene was lost during preparation of the genomic library, (ii) the *nisK* gene was lost during transconjugation, or (iii) the *nisK* gene was not present in the nisin producer used as a donor for transconjugation. According to the last two cases, another histidine kinase could substitute the *nisK* function.

The NisK histidine kinase shows strong similarities to the C terminus of all histidine kinases belonging to two-component regulatory systems. The C terminus of histidine kinases is intracellularly located and necessary for response regulator phosphorylation. The N-terminal part is different for histidine kinases. It is extracellularly located and acts as either a sensor or a receptor. Interestingly, NisK shows homology over the entire protein with SpaK of *B. subtilis* as well as with EnvZ of *E. coli*. SpaK regulates the biosynthesis of the lantibiotic subtilin (29), and EnvZ is involved in osmoregulation (for a review, see reference 40). All three regulatory systems have a common feature in that they respond to environmental stimuli and could have evolved from a common ancestral protein.

ACKNOWLEDGMENTS

We thank C. Klein for stimulating discussion.

This work was supported by Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 169) and Fonds der Chemischen Industrie. G. Engelke and K. Siegers received grants from the federal state Hessen and the Graduiertenkolleg "Proteinstrukturen, Dynamik und Funktion," respectively. Z. Gutowski-Eckel was supported by a Liebig-grant from the Fonds der Chemischen Industrie.

REFERENCES

1. **Allgaier, H., G. Jung, R. G. Werner, U. Schneider, and H. Zähner.** 1986. Epidermin: sequencing of a heterodet tetracyclic 21-peptide amide antibiotic. Eur. J. Biochem. **160:**9–22.
2. **Amemura, M., K. Makino, H. Shinagawa, and A. Nakata.** 1986. Nucleotide sequence of the *phoM* region of *Escherichia coli*: four open reading frames that may constitute an operon. J. Bacteriol. **168:**294–302.
3. **Anba, J., M. Bidaud, M. L. Vasil, and A. Lazdunski.** 1990. Nucleotide sequence of the *Pseudomonas aeroginosa phoB* gene, the regulatory gene for the phosphate regulon. J. Bacteriol. **172:**4685–4689.
4. **Augustin, J., R. Rosenstein, B. Wieland, U. Schneider, N. Schnell, G. Engelke, K.-D. Entian, and F. Götz.** 1992. Genetic analysis of epidermin biosynthetic genes and epidermin-negative mutants of *Staphylococcus epidermidis*. Eur. J. Biochem. **204:**1149–1154.
5. **Banerjee, S., and J. N. Hansen.** 1988. Structure and expression of a gene encoding the precursor of subtilin, a small protein antibiotic. J. Biol. Chem. **263:**9508–9514.
6. **Buchman, G. W., 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.
7. **Chevalier, R. J., J. Fournaud, E. Levebre, and G. Mocquot.** 1957. Mise en evidence des streptocoques lactiques inhibiteurs et stimulants dans le lait et les fromages. Ann. Technol. Agric. **2:**117–137.
8. **Comeau, D. E., K. Ikenaka, K. Tsung, and M. Inouye.** 1985. Primary characterization of the protein products of the *Escherichia coli ompB* locus: structure and regulation of synthesis of the OmpR and EnvZ proteins. J. Bacteriol. **164:**578–584.
9. **de Vos, W. M., J. W. M. Mulders, R. J. Siezen, J. Hugenholtz, and O. P. Kuipers.** 1993. Properties of nisin Z and distribution of its gene, *nisZ*, in *Lactococcus lactis*. Appl. Environ. Microbiol. **59:**213–218.
10. **De Vuyst, L., and E. Vandamme.** 1992. Influence of the carbon source on nisin production in *Lactococcus lactis* batch fermentations. J. Gen. Microbiol. **138:**517–578.
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. **Engelke, G., Z. Gutowski-Eckel, M. Hammelmann, and K.-D. Entian.** 1992. Biosynthesis of the lantibiotic nisin: genomic organization and membrane localization of the NisB protein. Appl. Environ. Microbiol. **58:**3730–3743.
13. **Fischetti, V. A., V. Pancholi, and O. Schneewind.** 1990. Conservation of a hexapeptide sequence in the anchor region of surface proteins from Gram-positive cocci. Mol. Microbiol. **4:**1603–1605.
14. **Froseth, B. R., and L. L. McKay.** 1991. Molecular characterization of the nisin resistance region of *Lactococcus lactis* subsp. *lactis* biovar diacetylactis DRC3. Appl. Environ. Microbiol. **57:**804–811.
15. **Gasson, M. J.** 1983. Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. J. Bacteriol. **154:**1–9.
16. **Gross, E., and H. Kiltz.** 1973. The number and nature of α,β-unsaturated amino acids in subtilin. Biochem. Biophys. Res. Commun. **50:**559–565.
17. **Gross, E., and J. L. Morell.** 1971. The structure of nisin. J. Am. Chem. Soc. **93:**4634–4635.

17a. **Gutowski-Eckel, Z., C. Klein, K. Siegers, K. Bohm, M. Hammelmann, and K.-D. Entian.** 1994. Growth phase-dependent regulation and membrane localization of SpaB, a protein involved in biosynthesis of the lantibiotic subtilin. Appl. Environ. Microbiol. **60:**1–11.

18. **Hemilä, H.** 1991. Sequence of a PAL-related lipoprotein from *Bacillus subtilis*. FEMS Microbiol. Lett. **82:**37–42.
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. **Hurst, A.** 1981. Nisin. Adv. Appl. Microbiol. **27:**85–123.
21. **Hussain, M., F. I. J. Pastor, and J. O. Lampen.** 1987. Cloning and sequencing of the *blaZ* gene encoding β-lactamase III, a lipoprotein of *Bacillus cereus* 569/H in *Escherichia coli*. J. Bacteriol. **169:**579–586.
22. **Ingram, L.** 1970. A ribosomal mechanism for synthesis of peptides related to nisin. Biochim. Biophys. Acta **224:**263–265.
23. **Jung, G., and H.-G. Sahl (ed.).** 1991. Nisin and novel lantibiotics.

ESCOM Science Publishers, Leiden, The Netherlands.
24. **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.
25. **Kaletta, C., K.-D. Entian, and G. Jung.** 1991. Prepeptide sequence of Ro 09-0198: the first structural gene of a duramycin type lantibiotic. Eur. J. Biochem. **199**:411–415.
26. **Kaletta, C., K.-D. Entian, R. Kellner, G. Jung, M. Reis, and H.-G. Sahl.** 1989. Pep5, a new lantibiotic: structural gene isolation and prepeptide sequence. Arch. Microbiol. **152**:16–19.
27. **Kato, C., Y. Nakano, and K. Horikoshi.** 1989. The nucleotide sequence of the lipo-penicillinase gene of alkalophilic *Bacillus* sp. strain 170. Arch. Microbiol. **151**:91–94.
28. **Klaenhammer, T. R.** 1988. Bacteriocins of lactic acid bacteria. Biochimie **70**:337–349.
29. **Klein, C., C. Kaletta, and K.-D. Entian.** 1993. Biosynthesis of the lantibiotic subtilin is regulated by a histidine kinase/response regulator system. Appl. Environ. Microbiol. **59**:296–303.
30. **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.
31. **Koide, Y., A. Nakamura, T. Uozomi, and T. Beppu.** 1986. Cloning and sequencing of the major intracellular serine protease gene of *Bacillus subtilis*. J. Bacteriol. **167**:110–116.
32. **Kok, J., K. J. Leenhouts, A. J. Haandrikman, A. M. Ledeboer, and G. Venema.** 1988. Construction of plasmid cloning vectors for lactic streptococci which also replicate in *Bacillus subtilis* and *Escherichia coli*. Appl. Environ. Microbiol. **48**:726–731.
33. **Kontinen, V. P., P. Saris, and M. Sarvas.** 1991. A gene (*prsA*) of *Bacillus subtilis* involved in a novel, late stage of protein export. Mol. Microbiol. **5**:1273–1283.
34. **Kupke, T., S. Stevanovic, H.-G. Sahl, and F. Götz.** 1992. Purification and characterization of EpiD, a flavoprotein involved in the biosynthesis of the lantibiotic epidermin. J. Bacteriol. **174**:5354–5361.
35. **Kyte, J., and R. F. Doolittle.** 1982. A simple method for displaying the hydropathic character of proteins. J. Mol. Biol. **157**:105–132.
36. **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.
37. **Lipman, D. J., and W. R. Pearson.** 1985. Rapid and sensitive protein similarity searches. Science **227**:1435–1441.
38. **Meloun, B., M. Baudys, V. Kostka, G. Hausdorf, C. Frommel, and W. E. Hohne.** 1985. Complete primary structure of thermitase from *Thermoactinomyces vulgaris* and its structural features related to the subtilisin-like proteinases. FEBS Lett. **183**:195–200.
39. **Miller, S. I., A. M. Kukral, and J. J. Mekalanos.** 1989. A two-component regulatory system (*phoP*, *phoQ*) controls *Salmonella typhimurium* virulence. Proc. Natl. Acad. Sci. USA **86**:5054–5058.
40. **Mizuno, T., and S. Mizushima.** 1990. Signal transduction and gene regulation through the phosphorylation of two regulatory components. The molecular basis for the osmotic regulation of the porin genes. Mol. Microbiol. **4**:1077–1082.
41. **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.
42. **Nakamura, K., R. M. Pirtle, I. L. Pirtle, K. Takeishi, and M. Inouye.** 1980. Messenger ribonucleic acid of the lipoprotein of the *Escherichia coli* outer membrane. II. The complete nucleotide sequence. J. Biol. Chem. **255**:210–216.
43. **Nielsen, J. B. K., and J. O. Lampen.** 1982. Membrane-bound penicillinases in Gram-positive bacteria. J. Biol. Chem. **257**:4490–4495.
44. **Rauch, P. J. G., and W. M. de Vos.** 1992. Characterization of the novel nisin-sucrose conjugative transposon Tn*5276* and its insertion in *Lactococcus lactis*. J. Bacteriol. **174**:1280–1287.
45. **Rauch, P. J. G., and W. M. de Vos.** 1992. Transcriptional regulation of the Tn5276-located *Lactococcus lactis* sucrose operon and characterization of the *sacA* gene encoding sucrose-6-phosphate hydrolase. Gene **121**:55–61.
46. **Reis, M., and H.-G. Sahl.** 1991. Genetic analysis of the producer self-protection mechanism ("immunity") against Pep5, p. 320–331. *In* G. Jung and H.-G. Sahl (ed.), Nisin and novel lantibiotics. ESCOM Science Publishers, Leiden, The Netherlands.
47. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
48. **Sanders, D. A., B. L. Gillece-Castro, A. M. Stock, A. L. Burlingame, and D. E. Koshland.** 1989. Identification of the site of phosphorylation of the chemotaxis response regulator protein, CheY. J. Biol. Chem. **264**:21770–21778.
49. **Sanders, D. A., and D. E. Koshland, Jr.** 1988. Receptor interactions through phosphorylation and methylation pathways in bacterial chemotaxis. Proc. Natl. Acad. Sci. USA **85**:8425–8429.
50. **Schägger, H., and G. von Jagow.** 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1–100-kDa. Anal. Biochem. **166**:368–379.
51. **Schnell, N., G. Engelke, J. Augustin, R. Rosenstein, V. Ungermann, F. Götz, and K.-D. Entian.** 1992. Analysis of genes involved in the biosynthesis of lantibiotic epidermin. Eur. J. Biochem. **204**:57–68.
52. **Schnell, N., K.-D. Entian, F. Götz, T. Hörner, R. Kellner, and G. Jung.** 1989. Structural gene isolation and prepeptide sequence of gallidermin, a new lanthionine containing antibiotic. FEMS Microbiol. Lett. **58**:263–268.
53. **Schnell, N., K.-D. Entian, U. Schneider, F. Götz, H. Zähner, R. Kellner, and G. Jung.** 1988. Prepeptide sequence of epidermin, a ribosomally synthesized antibiotic with four sulphide-rings. Nature (London) **333**:276–278.
54. **Seki, T., H. Yoshikawa, H. Takahashi, and H. Saito.** 1988. Nucleotide sequence of the *Bacillus subtilis phoR* gene. J. Bacteriol. **170**:5935–5938.
55. **Seki, T., H. Yoshikawa, H. Takahashi, and H. Saito.** 1991. Cloning and nucleotide sequence of *phoP*, the regulatory gene for alkaline phosphatase and phosphodiesterase in *Bacillus subtilis*. J. Bacteriol. **169**:2913–2916.
56. **Siezen, R. J., W. M. de Vos, J. A. M. Leunissen, and B. W. Dijkstra.** 1991. Homology modelling and protein engineering strategy of subtilases, the family of subtilisin-like serine proteinases. Protein Eng. **4**:719–737.
57. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
58. **Stock, J. B., A. J. Ninfa, and A. M. Stock.** 1989. Protein phosphorylation and regulation of adaptive responses in bacteria. Microbiol. Rev. **53**:450–490.
59. **Tautz, D., and N. Renz.** 1983. An optimized freeze squeeze method for the recovery of DNA fragments from agarose gels. Anal. Biochem. **132**:503–517.
60. **Terzaghi, P. E., and W. E. Sandine.** 1975. Improved medium for lactic streptococci and their bacteriophages. Appl. Microbiol. **29**:807–813.
61. **van de Guchte, M., J. Kok, and G. Venema.** 1992. Gene expression in *Lactococcus lactis*. FEMS Microbiol. Rev. **88**:73–92.
62. **van de Guchte, M., J. M. B. M. van der Vossen, J. Kok, and G. Venema.** 1989. Construction of a lactococcal expression vector: expression of hen egg white lysozyme in *Lactococcus lactis* subsp. *lactis*. Appl. Environ. Microbiol. **55**:224–228.
63. **van der Meer, J. R., J. Polman, M. M. Beerthuyzen, R. J. Siezen, O. P. Kuipers, and W. M. de Vos.** 1993. Characterization of the *Lactococcus lactis* nisin A operon genes *nisP*, encoding a subtilisin-like serine protease involved in precursor processing, and *nisR*, encoding a regulatory protein involved in nisin biosynthesis. J. Bacteriol. **175**:2578–2588.
64. **von Heijne, G.** 1989. The structure of signal peptides from bacterial lipoproteins. Protein Eng. **2**:531–534.
65. **Vos, P., M. van Asseldonk, F. van Jeveren, R. Siezen, G. Simons, and W. M. de Vos.** 1989. A maturation protein is essential for production of active forms of *Lactococcus lactis* SK11 serine proteinase located in or secreted from the cell envelope. J.

Bacteriol. **171:**2795–2802.
66. **Weil, H.-P., A. G. Beck-Sickinger, J. Metzger, S. Stevanovic, G. Jung, M. Josten, and H.-G. Sahl.** 1990. Biosynthesis of the lantibiotic pep5. Eur. J. Biochem. **194:**217–223.
67. **Wu, H. C., and S. Hayashi.** 1986. Lipoprotein secretion in bacteria, p. 260–265. *In* L. Leive (ed.), Microbiology—1986. American Society for Microbiology, Washington, D.C.
68. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.
69. **Zamenhoff, S.** 1957. Preparation and assay of desoxyribonucleic acids from animal tissue. Methods Enzymol. **3:**696–704.