Biosynthesis of Lantibiotic Nisin

POSTTRANSLATIONAL MODIFICATION OF ITS PREPEPTIDE OCCURS AT A MULTIMERICAL MEMBRANE-ASSOCIATED LANTHIONINE SYNTHETASE COMPLEX*

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The lantibiotic nisin of Lactococcus lactis is matured from a ribosomally synthesized prepeptide by posttranslational modification. Genetic and biochemical evidence suggests that genes nisB and nisC of the nisin gene cluster encode proteins necessary for prepeptide modification. Inactivation of both genes resulted in complete loss of nisin production. The preparation of membrane vesicles revealed that NisB and NisC are attached to the cellular membrane, and co-immunoprecipitation experiments showed that they are associated with each other. By using the yeast two-hybrid system, which is a highly sensitive method to unravel protein-protein interactions, we could show that the nisin prepeptide physically interacts with the NisC protein, suggesting that NisC contains a binding site for prepeptide. This was also confirmed by co-immunoprecipitation of the NisC protein and the NisA prepeptide by antibodies directed against the leader sequence of the nisin prepeptide. The two-hybrid analysis also confirmed the interaction between NisA and NisC as well as the interaction between NisC and the NisT ABC transporter. A minor interaction was also indicated between prepeptide and the NisB protein. Furthermore, the two-hybrid investigations also revealed that at least two molecules of NisC and two molecules of NisT are part of the modification and transport complex. Our results suggest that lantibiotic maturation and secretion occur at a membrane-associated multimerical lantibiotic synthetase complex consisting of proteins NisB, NisC, and the ABC transporter molecules NisT.

Nisin is a ribosomally synthesized peptide antibiotic containing the unusual amino acids lanthionine, dehydrobutyryne, and dehydroalanine (1, 2). It belongs to a class of peptide antibiotics that is referred to as lantibiotics because of their characteristic thioether bridges consisting of meso-lanthionine and 3-methyl-lanthionine. Nisin occurs naturally in dairy products (3) and is used as a food preservative because it exhibits high levels of antimicrobial activity against several pathogenic Gram-positive bacteria, such as Staphylococcus, streptococci, and clostridia (4). The bactericidal action of nisin and other lantibiotics is mainly caused by pore formation in the cytoplasmic membrane (5–7). Nisin, like the other lantibiotics described so far is ribosomally synthesized. The primary transcript of the nisin structural gene nisA encodes a 57-amino acid prepeptide, which consists of a N-terminal leader sequence followed by the C-terminal prepeptide from which the lantibiotic is matured (1, 2).

Based on the results of Ingram (8) the following model was proposed for the formation of the unusual amino acids. First, a dehydratase reaction occurs at serine and threonine residues, resulting in amino acids dehydroalanine and dehydrobutyryne, respectively. Thereafter, sulfur from neighboring cysteine residues is added to the double bonds, resulting in meso-lanthionine and 3-methyl-lanthionine, respectively. After the isolation of the first lantibiotic structural gene (epiA for epidermin) it was stated that maturation reactions occur at the prepeptide (9). This hypothesis was supported by the isolation of prepeptides containing dehydroalanine (10).

The genes for the biosynthesis of nisin are located on a 70-kilobase pair conjugative transposon (11), which also contains the genetic information for sucrose metabolism. Several genes encoding proteins that are involved in the biosynthesis, secretion, and immunity of different lantibiotics have been characterized (for reviews see Refs. 12, 13, and 51). Proteins encoded by the genes nisB, nisT, nisC, nisI, nisP, nisR, nisK, nisF, nisE, and nisG have been found to be homologous to respective gene products of the subtilin (14–18), epidermin (19–22), gallidermin (23) or Pep5 (24, 25) gene clusters. Gene deletion experiments of the genes spaB, spaC, spaT, spaR, and spaK in Bacillus subtilis have proven that they are essential for subtilin biosynthesis (15, 17, 18). Like epIP, nisP codes for a subtilisin-like serine protease that is involved in processing of the post-translational modified prepeptide (22, 26–28). For nisI, nisF, nisE, and nisG we recently demonstrated an involvement in the self-protection mechanism of the producer against nisin (29). Similar results have also been found for the respective genes of the subtilin-producing strain B. subtilis (16). Many lantibiotic-producing strains have three genes in common considered as lanB, lanC, and lanT. With respect to nisin biosynthesis, the nisT gene encodes a protein of 600 amino acid residues with an predicted molecular mass of 69 kDa. Its gene product shares strong homology with several ATP-dependent transport proteins having two ATP-binding sites and a very hydrophobic region at the N terminus with six potential membrane-spanning domains (26). The NisT protein is expected to be necessary for the secretion of the modified nisin peptide. Proteins encoded by genes nisB and nisC share no homologies with other known proteins in the data bases except similar gene products of other lantibiotic-producing strains. As the functions of all other gene products found in lantibiotic gene clusters became obvious by their similarities to previously described proteins and by biochemical experiments, lanB and lanC most likely encode the proteins catalyzing the modifica-

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Here we report on genetic and biochemical experiments proving the existence of a membrane-bound maturation complex, which we name lanthionine synthetase. These results were obtained by two independent experimental approaches. To prove any interactions between the nisin-prepeptide and its possible maturation proteins we used the yeast two-hybrid system, which is a highly sensitive method to unravel protein-protein interactions (30, 31). This test system detects the functional reconstitution of GAL4, a transcriptional activator from yeast. The interaction of two hybrid proteins, one bearing the GAL4 DNA binding domain and the other fused to the transcriptional activation domain of GAL4, creates a functional activator by bringing the activation domain into close proximity with the DNA binding domain. This results in transcriptional activation of a lacZ reporter gene containing upstream GAL4 binding sites. Independently, co-immunoprecipitation experiments also confirmed the physical interactions indicated by the two-hybrid system. Our results suggest that this multimeric protein complex consists of NisB and at least two NisC subunits for the modification of the prepeptide and in analogy with other bacterial ABC transporters two NisT transporter subunits (32).

MATERIALS AND METHODS

Strains and Media—The plasmid-free nisin-nonproducing strain L. lactis MG1614 (Ref. 33; kindly provided by M. Gasson, Norwich, United Kingdom) and the nisin-producing strains L. lactis 6F3 (kindly provided by T. Hörner, Tübingen, Germany) and L. lactis K5100 (34) were grown at 30°C in M17 broth supplemented with 0.5% (w/v) sucrose (SM17) or with 0.5% (w/v) glucose (GM17). Micrococcus luteus ATCC 9341 was used as a test strain in nisin assays. M. luteus was also grown at 30°C in GM17 medium. Recombinant plasmids were amplified in Escherichia coli RR1 (F– hsdS20 supE44 ara-14 proA2 lacY1 galK2 rpsL2 20 y-gal5-mtl1) and DH5α (supE44 ΔlacU169 Δ80 lacZM15) hsdR7 recA1 endA1 gyrA96 thi-1 relA1. E. coli strains were grown at 37°C in Luria-Bertani medium (Life Technologies, Inc., 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; chloramphenicol, 20 μg/ml for E. coli and 5 μg/ml for L. lactis. Yeast strain Saccharomyces cerevisiae FY6-52 (MATa, ura3-52, his3-200, ade2-101, lys2-801, trpl-901, leu2-3, 112, can1, gal4-542, gal80-538, URA3::GAL1-1a3c) was used in the two-hybrid assay to monitor protein-protein interactions. The composition of yeast-rich medium and synthetic complete medium has been previously described (35). As a carbon source 4% glucose or 4% galactose was added. Transformants were recovered on synthetic complete medium supplemented with the required amino acids.

Plasmids—The vectors pUC19 (36) and pBSKRII (Stratagene, Heidelberg, Germany) were used for cloning purposes in E. coli. In S. cerevisiae the plasmids pGBT9 and pGAD424 (30, 31) were used to express fusion proteins with the GAL4-binding domain and the GAL4-activation domain, respectively.

Molecular Biology Techniques—Established protocols were followed for molecular biology techniques (37). DNA was cleaved according to the conditions recommended by the commercial supplier of the restriction enzymes (Boehringer, Mannheim, Germany). Restriction endonuclease-digested DNA was eluted from 0.7% agarose gels by the freeze-squeeze method (38).

Plasmid Isolation and PCR—The procedure of Birnboim and Doly (39) was followed 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 1 h. PCR was carried out by following standard procedures (37) in an Eppendorf 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.

Southern Hybridization—Southern blots were carried out according to Southern (40). For Southern hybridization double-stranded DNA fragments were labeled by nick translation using [32P]ATP and DNA polymerase I (Boehringer), as described by Sambrook et al. (37).

Transformation of yeast was carried out as described in Methods (37).

Expression of β-galactosidase activity was determined as described previously (42). Values reported are the average of duplicate assays of four independent transformants. β-Galactosidase activity is expressed as a nanomolar amount of substrate converted per minute (Miller units).

Transformation of L. lactis—L. lactis strains were transformed by electroporation as described by Hidalgo and Nies (44). Cells from a 250-ml overnight culture were harvested by centrifugation (4,000 g, 10 min) and the pellet was resuspended in a Z buffer/5-bromo-4-chloro-3-indoyl-D-galactoside in 1 ml of 50 mM Tris-HCl buffer (pH 7). After the addition of 50 μl of 5-mercaptoethanol, 420 μl of Z buffer/5-bromo-4-chloro-3-indoyl-D-galactopyranoside in 1 ml of N,N-dimethylformamide. The filters were incubated at 30°C and checked periodically for the appearance of blue colonies. For quantitative studies, yeast strains were grown to stationary phase in synthetic medium lacking leucine and tryptophan, diluted to 5 × 105 cells/ml, and then incubated at 30°C for 3–4 h. β-Galactosidase activity was determined as described previously (42). Values reported are the average of duplicate assays of four independent transformants.

Isothermal titration calorimetry was performed with a Gene Pulser apparatus (Bio-Rad) by using a single pulse of 12.5 kV/cm, a capacity of 25 microfarads, and a resistance of 200 Ohm.

β-Galactosidase—Test strain M. luteus ATCC 9341 was grown to an A578 of 0.8, and 0.3 ml was added to 500 ml of molten GM17 agar, mixed, and poured into Petri dishes (20 ml). L. lactis was spread on the agar surface, and the diameter of the zone of M. luteus growth inhibition around the L. lactis cells was determined.

Isolation of Membrane Vesicles of L. lactis—Membrane vesicles preparation of L. lactis were based on a method described for Streptococcus cremoris (44). Cells from a 250-ml overnight culture were harvested by centrifugation (4,000 × g, 10 min, 4°C), washed with 30 ml of 100 mM Tris-HCl buffer (pH 8), and suspended in 2 ml of 100 mM Tris-HCl buffer (pH 8) containing 2 mM phenylmethylsulfonyl fluoride. The cell suspension was diluted with 4 ml of 100 mM Tris-HCl buffer (pH 8) containing 10 mM MgSO4, and 30 mg of egg lysozyme (E. Merck AG, Darmstadt, Germany). After incubation at 37°C for 40 min, 3 ml of NaCl (final concentration, 0.5 M) was added for cell lysis and the solution was diluted immediately with 14 ml of 100 mM Tris-HCl buffer (pH 8) containing 50 μg of DNase I and 50 μg of RNase A (Merck) per ml and incubated at 37°C for a further 20 min. After centrifugation (1,000 × g, 60 min, 4°C) the supernatant was aspirated carefully with a Pasteur pipette, the sediment, which contained remaining whole cells and cell debris, sedimentation of membrane vesicles from the supernatant was achieved by centrifugation at 48,000 × g at 4°C for 30 min. Membrane vesicles were carefully resuspended with a Potter-Elvehjem homogenizer in 50 mM Tris-HCl buffer (pH 8) containing 10 mM MgSO4. Vesicles were washed once in the same centrifugation parameters, and finally aliquots of 0.1–0.5 ml of suspended membrane vesicles were rapidly frozen and stored in liquid nitrogen.

Co-immunoprecipitation—Membrane vesicles were solubilized with 1% dodecyl-β-D-maltoside for 1 h at 4°C. After 60 min of centrifugation at 100,000 × g (4°C) the supernatant was divided into two fractions, and 200 μl of the supernatant was diluted with 200 μl of TENT buffer (25 mM Tris-HCl, pH 7.5, 5 mM EDTA, 250 mM NaCl, 0.1% Triton X-100). After the addition of 50 μl of washed 10% Pansorbin (Staphylococcus aureus cells; Calbiochem-Novabiochem GmbH, Bad Soden, Germany), the mixture was incubated for 2 h at 4°C. After centrifugation (100,000 × g, 30 min) the supernatant was collected. The pellet was then treated and the supernatant was collected. The pellet was then treated and the supernatant was collected.

1 The abbreviations used are: PCR, polymerase chain reaction; bp, base pair; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
Germany) the samples were incubated at 22 °C for 1 h. After centrifugation (10,000 × g, 1 min, 4 °C) the supernatant was collected and incubated overnight at 4 °C with 5 μL of the respective antisera. To the suspension 100 μL of prewashed 10% Pansorbin were added, and the sample was incubated for 15 min at 22 °C. After centrifugation of the StaphA (Pansorbin) antibody-antigen complex (2 min, 10,000 × g, 4 °C) through a sucrose cushion (1 M sucrose in TENT buffer), the supernatant was removed. The pellet was washed twice and loaded onto a SDS-polyacrylamide gel electrophoresis followed by Western blot analysis.

Antibody Isolation—A 1.3-kilobase pair Sau3A/HindIII fragment of chromosomal DNA from L. lactis 6F3 containing 99 base pairs of the nisC gene was cloned into vector pATH3 (45). The hybrid gene trpE/nisC was expressed in E. coli RR1, and the hybrid protein was isolated as described in Ref. 45. The hybrid protein TrpE/NisC was further purified by preparative SDS-gel electrophoresis and isolated by electroelution. The purified fusion proteins were used for rabbit immunization as described previously (26).

SDS-polyacrylamide Gel Electrophoresis and Western Blot Analysis—SDS-polyacrylamide gel electrophoresis and Western blot analysis were performed as described previously (26, 34). Molecular weight standards for SDS-polyacylamide gel electrophoresis were obtained from Sigma-Aldrich (Deisenhofen, Germany).

RESULTS

Genetic Analysis of Genes nisB, nisT, and nisC—The nisin-producing, plasmid-free strain L. lactis KS100 obtained from conjugation of L. lactis 6F3 with L. lactis MG1614 (34) was used to investigate the physiological significance of the genes for biosynthesis of nisin. The respective genes were interrupted by insertion of the erythromycin resistance marker (Table II). The flanking regions that allowed homologous recombination on either side of the insertion were approximately 1 kilobase pair each. In order to inactivate the nisB gene in the chromo-

some of L. lactis KS100 the cells were transformed with plasmid pGEN5, which is not able to replicate in L. lactis cells. In the case of nisT pSi127 was used for inactivation of the gene encoding the ABC transporter in the chromosome of L. lactis KS100. Plasmid pSi140 was used for transformation of L. lactis KS100 in order to inactivate the chromosomally located nisC gene. The phenotypes of mutants within the genes nisB, nisT, and nisC were investigated by means of the nisin bioassay with M. luteus as test organism. As shown in Fig. 1A all mutants were no longer able to produce nisin. Western blot analysis with antibodies directed against NisB and NisC revealed that the respective gene products were no longer detectable in the mutants (Fig. 1, B and C).

Membrane Localization of NisC—In order to detect the NisC protein in cells of L. lactis, polyclonal antibodies directed against NisC were used. The antibodies cross-reacted with a protein with a molecular mass of approximately 47 kDa, which

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**Table I**

| Plasmid | Description | PCR primer used for amplification |
|---------|-------------|----------------------------------|
| pSi87   | 202-bp PCR fragment containing the entire nisA gene cloned into pGAD424 Smal/PstI | 5'-GGAGCCATCCAACTCCTACGAGAAATACCC-3' 5'-CTTATAAGGATCCGAGGAGAAGA-3' |
| pSi88   | 202-bp PCR fragment containing entire nisA gene cloned into pGAD424 Smal/PstI | 5'-GGAGCCATCCAACTCCTACGAGAAATACCC-3' 5'-CTTATAAGGATCCGAGGAGAAGA-3' |
| pSi89   | 1255-bp PCR fragment containing nisC (410 aa) cloned into pGAD424 BamHI/PstI | 5'-TCAGAGCAATATTAGGATGTCCGATG-3' 5'-TTAAATACTGATTTCTCTCC-3' |
| pSi90   | 1255-bp PCR fragment containing nisC (410 aa) cloned into pGAD424 BamHI/PstI | 5'-TCAGAGCAATATTAGGATGTCCGATG-3' 5'-TTAAATACTGATTTCTCTCC-3' |
| pSi117  | 1830-bp PCR fragment containing nisT (595 aa) cloned into pGAD424 EcoRI/BamHI | 5'-GGACTATCCATGATGAAAGTCC-3' 5'-CTTATAAGGATCCGAGGAGAAGA-3' |
| pSi118  | 1830-bp PCR fragment containing nisT (595 aa) cloned into pGAD424 EcoRI/BamHI | 5'-GGACTATCCATGATGAAAGTCC-3' 5'-CTTATAAGGATCCGAGGAGAAGA-3' |
| pHZ1    | 1482-bp PCR fragment containing the 5' end of nisB (494 aa) cloned into pGAD424 BamHI/SalI | 5'-GAGTAGAACAAAACTGAAAGTTCC-3' 5'-GAAATACATGACAGGATGAATTC-3' |
| pHZ2    | 1482-bp PCR fragment containing the 5' end of nisB (494 aa) cloned into pGAD424 BamHI/SalI | 5'-GAGTAGAACAAAACTGAAAGTTCC-3' 5'-GAAATACATGACAGGATGAATTC-3' |
| pHZ3    | 1513-bp PCR fragment containing the 3' end of nisB (502 aa) cloned into pGAD424 EcoRI/SalI | 5'-TGAGAATAAAGAATCTACTACGTGTG-3' 5'-ATCCATCTAGTCTCTTATCCATG-3' |
| pHZ4    | 1513-bp PCR fragment containing the 3' end of nisB (502 aa) cloned into pGAD424 EcoRI/SalI | 5'-TGAGAATAAAGAATCTACTACGTGTG-3' 5'-ATCCATCTAGTCTCTTATCCATG-3' |
| pHZ5    | 948-bp PCR fragment containing the 3' end of nisT (301 aa) cloned into pGAD424 EcoRI/BamHI | 5'-AGGACTATTTCTGAATCATTATTTATCC-3' 5'-GTATCGATCCGATCAGGAGCC-3' |
| pHZ6    | 948-bp PCR fragment containing the 3' end of nisT (301 aa) cloned into pGAD424 EcoRI/BamHI | 5'-AGGACTATTTCTGAATCATTATTTATCC-3' 5'-GTATCGATCCGATCAGGAGCC-3' |

**Table II**

| Plasmid | Genetic marker and/or description | Reference(s) |
|---------|----------------------------------|--------------|
| pGEN4   | 2.7-kb Dral/HindIII (blunt ended) fragment of chromosomal DNA from L. lactis 6F3 in pBSK EcoRV | This work |
| pGEN5   | Em' gene of pE194 in pGEN4 EcoRV | This work, 49 |
| pSi140  | Em' gene of pE194 in pGEN3 Ncol (blunt ended) | This work, 49 |
| pSi122  | 1.8-kb EcoRI fragment of chromosomal DNA from L. lactis 6F3 in pDia504 | This work, 49 |
| pSi127  | Em' gene of pE194 in pSi122 Ndel | This work, 49 |
is in accordance with the predicted molecular mass of NisC deduced from the DNA sequence (Fig. 1C). After centrifugation of cell extracts from nisin-producing L. lactis cells followed by Western blot analysis, most of the cross-reacting activity was present in the sediment, indicating that the NisC protein was associated with the membrane fraction of the cell extracts. In order to test this possibility, vesicles of L. lactis cells were prepared, and proteins attached to the membrane vesicles were analyzed by immunoblot analysis. The 47-kDa NisC protein was associated with the membrane fraction. After resuspension followed by repeated centrifugation, the NisC protein was still found in the vesicle fraction and only slight depletion was observed, indicating that NisC is attached to the membrane (Fig. 2). The same result has already been observed for the NisB protein (26). The finding that both proteins are associated with the vesicle fraction strongly supports our hypothesis that the biosynthesis of nisin occurs at the cellular membrane of L. lactis cells.

Construction of Fusion Proteins with the Yeast GAL4 Binding and Activation Domains—The yeast two-hybrid system was used to investigate proteins encoded by the nisin gene cluster for their possible physical interactions. In-frame fusions of the respective genes with the parts of the GAL4 gene encoding the DNA-binding and transcriptional activation domain of GAL4 (Fig. 3A) were constructed as described in Table I. The results of β-galactosidase assays monitoring the interactions of proteins encoded by genes from the nisin gene cluster fused to the GAL4 DNA-binding and activation domains, respectively, are shown in Tables III and IV. In order to identify the protein probably catalyzing the first modification reaction, we tested for possible interactions with the NisA prepeptide. A blue coloration of yeast cells, which is indicative of an interaction, was observed for NisC and the NisA prepeptide. When we investigated whether the NisB protein binds the prepeptide, we additionally observed a weak interaction of the nisin prepeptide with N-terminal part of NisB. The C-terminal half of NisB did not interact with NisA within the yeast cells, suggesting that the prepeptide is attached to the N terminus of NisB. Furthermore, we could observe interactions between the NisC protein and both the N-terminal and C-terminal part of NisB. In addition to this, the NisC protein also interacted with the C-terminal domain of the NisT protein.

In further experiments the NisA protein was divided in the leader peptide and the prepeptide region, and both parts were fused to the respective GAL4 domains. These constructs were also assayed for interaction with NisC, but no interaction of NisC with either part of NisA could be observed, indicating that the complete NisA protein is necessary for an interaction with NisC. The two-hybrid analysis system also allowed us to test for self-interactions of proteins involved in nisin maturation and transport by fusing the gene of interest to the DNA binding

**Fig. 1. Inactivation of genes nisB, nisC, and nisT.** A, bioassay for nisin production. Strains were streaked onto plates containing M. luteus as a test organism. The zone of growth inhibition around the cells of the wild-type strain L. lactis KS100 indicates nisin production, whereas no growth inhibition can be observed around cells of the nonproducing strain MG1614 and mutants of KS100 where the genes nisB, nisC, or nisT has been inactivated, respectively. B, immunoblot analysis of NisB. Lane 1, molecular mass standards (kDa); lanes 2, 3, and 4 (from left to right), protein extracts of L. lactis wild-type cells (KS100), nonproducing cells (MG1614), and nisB disruption mutant. C, immunoblot analysis of NisC. Lane 1, molecular mass standards (kDa); lanes 2, 3, and 4 (from left to right), protein extracts of L. lactis wild-type cells (KS100), nonproducing cells (MG1614), and nisC disruption mutant.

![Proteins Involved in Nisin Biosynthesis](image-url)
Proteins Involved in Nisin Biosynthesis

A

![Diagram](image)

**Fig. 3.** A, organization of the genes nisA, nisB, nisT, nisC, and corresponding protein fragments used in the two-hybrid assays. The genes encoding proteins NisA, NisB, NisT, and NisC are indicated by arrows, and the lengths of protein fragments encoded by plasmids derived from pGBT9 and pGAD424 are shown below. B, composition of the suggested lanthionine synthetase complex derived from the data obtained in the two-hybrid assays. Lengths of bars correspond to the sizes of interacting fragments indicated by the number of amino acids.

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**Table III**

| Protein Fragments | NisA | NisB (aa 1–494) | NisB (aa 491–993) | NisC | NisT (aa 299–600) |
|-------------------|-----|----------------|------------------|-----|------------------|
| White             | Blue | White          | White            | Blue| White            |
| Blue              | White| White          | Blue             | Blue| Blue             |
| White             | White| White          | White            | White| White            |
| Blue              | Blue | Blue           | Blue             | Blue| Blue             |

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**Table IV**

| Plasmids | Proteins fused to the GAL4 domains | β-Galactosidase activity (units/mg⁻¹) |
|----------|-----------------------------------|-------------------------------------|
| pGBT9 × pGAD424 | No | 0.8 |
| pSI87 × pSI90 | NisA and NisC | 8.1 |
| pSI88 × pSI89 | NisA and NisC | 7.8 |
| pSI87 × pSI89 | NisA and NisC | 3.2 |
| pSI88 × pSI87 | NisA and NisC | 3.5 |
| pSI89 × pSI87 | NisA and NisC | 8.0 |
| pSI89 × pSI87 | NisA and NisC | 8.2 |
| pSI90 × pSI87 | NisA and NisC | 7.8 |
| pSI90 × pSI87 | NisA and NisC | 8.1 |
| pSI89 × pSI87 | NisA and NisC | 7.9 |
| pSI90 × pSI87 | NisA and NisC | 8.4 |
| pSI89 × pSI87 | NisA and NisC | 8.2 |
| pSI90 × pSI87 | NisA and NisC | 7.7 |
| pSI117 × pSI118 | NisA and NisC | 8.3 |
| pSI117 × pSI118 | NisA and NisC | 3.8 |

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and activation domain as well. When yeast cells were cotransformed with plasmids carrying fusions of the nisC gene with the GAL4 activation domain and the DNA-binding domain we observed β-galactosidase activity, which suggests that the protein complex that mediates the maturation of prenisin contains more than one NisC protein. The same result (blue coloration of the yeast cells) was also observed when plasmids carrying fusions of the entire nisT gene with both parts of GAL4 were cotransformed. This finding suggests that at least two NisT molecules are part of the supposed complex, which is in accordance with the general view that bacterial ABC transporters occur as dimers (46). The results of the two-hybrid investigations suggested that all proteins involved in nisin maturation are associated in a multimeric complex consisting of proteins NisB, NisC, and NisT (Fig. 3B).

Co-immunoprecipitation of NisB and NisC—Although the two-hybrid results were conclusive and indicated a physical interaction of proteins NisB and NisC, further support by alternative experiments was necessary to exclude possible artifacts. To prove that NisB and NisC are attached to each other co-immunoprecipitation experiments were carried out by using antibodies directed against NisB and NisC, respectively. Membrane vesicles of L. lactis cells were incubated with 1% dodecyl-β-D-maltoside in order to solubilize membrane-associated proteins. The resulting protein solution was thereafter incubated with antibodies directed against the NisC protein, and the protein-antibody complexes were precipitated with protein A from S. aureus (see "Materials and Methods"). After SDS-polyacrylamide gel electrophoresis, the proteins precipitated by the NisC-specific antibody were further analyzed by NisB-specific antibodies. As shown in Fig. 4A, our results clearly revealed that NisB was co-precipitated by the NisC-specific antibody. The same result was obtained when the proteins were first precipitated with the NisB-specific antibody and further analyzed for the presence of NisC (Fig. 4B). A number of unspecific bands were also observed due to protein A cross-reactions. To distinguish the unspecific signals from specific ones we used strain L. lactis MG1614, which is identical to L. lactis KS100 except for the fact that this strain lacks the genes for sucrase metabolism and nisin production, for co-immunoprecipitations. By using strain MG1614, all unspecific signals, but no signals corresponding to the size of NisB or NisC, were observed. In preparations of L. lactis cells, which were not treated with protein A of S. aureus the unspecific signals were not present, which also confirms that the unspecific signals are due to unspecific reactions of protein A. The finding that proteins NisB and NisC can be co-immunoprecipitated by antisera specific for the other protein supports the results obtained from the two-hybrid experiments and justifies the conclusion that the two proteins NisB and NisC are subunits of a common protein complex.

Co-immunoprecipitation of NisA and NisC—We also investigated the possible interaction of the nisin prepeptide NisA with the NisC protein, by co-immunoprecipitation with antibodies directed against the NisC protein and against the nisin prepeptide (34). Cell extracts of nisin-producing L. lactis cells were incubated with antibodies directed against the prepeptide, and the protein-antibody complexes were precipitated with protein A of S. aureus (see "Materials and Methods"). After SDS-polyacrylamide gel electrophoresis the proteins precipitated by the NisA antibody were further analyzed by antibodies directed against NisC. Again vesicles of the strain MG1614, which does not contain the nisin gene cluster, were used to prove the specificity of the signals. As shown in Fig. 4C our experiment clearly revealed that NisC was co-precipitated by prepeptide-specific antibodies, which confirms biochemically the finding of the two-hybrid assay that the nisin prepeptide is bound to the NisC protein.

Co-immunoprecipitation of NisA and NisB—When cell extracts of L. lactis KS100 were treated with antibodies directed against the prepeptide and the immunoprecipitate was subsequently analyzed with NisB-specific antibodies, we were not able to detect the NisB protein. On the other hand, when cell extracts were first incubated with NisB-specific antibodies and the samples were subsequently analyzed for the presence of the prepeptide after precipitation, a weak signal was detectable (Fig. 4D), indicating an interaction of both proteins. The fact
that a co-immunoprecipitation only occurred by using NisB-specific antibodies, but not when NisA antibodies were used for precipitation, possibly indicates that the NisB protein covers the leader sequence of the prepeptide and makes it inaccessible to the NisA antibodies.

**DISCUSSION**

Since the isolation of lantibiotic structural genes, which proved that lantibiotics are encoded by distinct genes, several genes involved in lantibiotic biosynthesis have been identified flanking the structural genes. The genes found near the structural genes of different producers show strong similarities indicating their similar function in lantibiotic maturation, secretion, processing, immunity, and the regulation of biosynthesis. The lantibiotics are considered to be formed by posttranslational modifications that convert the ribosomally synthesized prepeptides into peptides that contain the characteristic ring structure of lantibiotics. Two reactions have been proposed for the maturation of lantibiotics, dehydration of serine and threonine residues in the prepeptide region and the addition of sulfur from neighboring cysteine residues to the resulting double bonds.

Our experiments revealed that the genes nisB and nisC encode two proteins of 117.5 and 47 kDa that are both associated with the cellular membrane. Inactivation of the genes by insertion of antibiotic resistance markers completely abolished nisin production in both cases, demonstrating their involvement in the biosynthesis of nisin. Furthermore, the results of the two-hybrid assay and co-immunoprecipitation experiments indicated that these proteins are attached to each other. In addition to this we could also demonstrate that the NisB protein as well as NisC interact with NisA. Therefore, we assume that proteins NisB and NisC form a complex that mediates the maturation of the nisin prepeptide. Since the proteins encoded by genes nisB and nisC share no homologies with other known proteins in the data bases except products of similar genes found in the gene clusters of different lantibiotic producers, we suppose that they might catalyze reactions that are specific for lantibiotic maturation.

The NisA antibody we used in the co-immunoprecipitation experiments is directed against the leader sequence of the prepeptide. Co-immunoprecipitation of NisB with NisC as well as NisB and NisC interact with NisA. Therefore, we assume that proteins NisB and NisC form a complex that mediates the maturation of the nisin prepeptide. Since the proteins encoded by genes nisB and nisC share no homologies with other known proteins in the data bases except products of similar genes found in the gene clusters of different lantibiotic producers, we suppose that they might catalyze reactions that are specific for lantibiotic maturation.

The NisA antibody we used in the co-immunoprecipitation experiments is directed against the leader sequence of the prepeptide. Co-immunoprecipitation of NisB was possible with the prepeptide antibody, which is directed against the leader sequence of NisA. However, when the cell extracts were first incubated with antibodies directed against NisB, the prepeptide could be co-immunoprecipitated. Co-immunoprecipitation in only one direction indicated that the nisin-prepeptide is not accessible to its leader-directed antibody in the NisB-NisA complex, suggesting that the leader peptide region of the prepeptide is involved in NisB binding. The finding that the complex consisting of NisA and NisC could be precipitated by the antibody directed against the NisA leader sequence indicates that in the complex the leader peptide is still acces-

**FIG. 4. Co-immunoprecipitation experiments.** A, co-immunoprecipitation of NisB and NisC analyzed with NisB-specific antibodies. After precipitation, protein complexes were separated on 7.5% SDS-polyacrylamide gels. The NisB cross-reacting band is marked. Lane 1, molecular mass standards (kDa). Lane 2, NisB antibody-protein complex precipitated with protein A of S. aureus. Lane 3, NisC antibody-protein complex precipitated with protein A of S. aureus. Lanes 2 and 3 show the result of the co-immunoprecipitation experiment carried out with nisin-producing L. lactis KS100 cells. Lanes 4 and 5, control experiments with L. lactis MG1614 lacking the genes for nisin biosynthesis. Lane 6, vesicle fraction of L. lactis KS100 as positive control. Additional bands in lanes 2, 3, 4, and 5 are due to unspecific cross-reactions of the used antibodies with protein A of S. aureus. B, co-immunoprecipitation of NisB and NisC analyzed with NisC-specific antibodies. Lanes 1–6 are identical to lanes 1–6 in Fig. 4A. The NisC protein is marked. C, co-immunoprecipitation of NisC and NisA analyzed with NisC-specific antibodies. Lane 1, molecular mass standards (kDa). Lane 2, NisC antibody-protein complex precipitated with protein A of S. aureus. Lane 3, NisA antibody-protein complex precipitated with protein A of S. aureus. Lanes 2 and 3 show the result of the co-immunoprecipitation experiment carried out with L. lactis KS100 cells. Lanes 4 and 5 show control experiments with vesicles of L. lactis MG1614 with NisC-specific antibodies (lane 4) and NisA-specific antibodies (lane 5). Lane 6, vesicle fraction of L. lactis KS100 as positive control. Additional bands in lanes 2, 3, 4, and 5 are due to unspecific cross-reactions of the used antibodies with protein A of S. aureus. NisC protein is marked by an asterisk. D, co-immunoprecipitation of NisB and NisA analyzed with NisA-specific antibodies. After precipitation protein complexes were separated on 15% Tricine-polyacrylamide gels. Lane 1, molecular mass standards (kDa). Lane 2, NisB antibody-protein complex precipitated with protein A of S. aureus. Lane 3, NisA antibody-protein complex precipitated with protein A of S. aureus. Lanes 4 and 5 show control experiments with vesicles of L. lactis MG1614 with NisC-specific antibodies (lane 4) and NisA-specific antibodies (lane 5). Additional bands in lanes 2, 3, 4, and 5 are due to unspecific cross-reactions of the used antibodies with protein A of S. aureus.
lacticin 481 production contain genes that encode proteins CylM and LctM (47, 48), whose C-terminal domains exhibit strong similarities with LanC proteins, whereas no similarity with LanB proteins was observed (12). Interestingly the lack of LanB-homologous proteins is correlated with differences in the leader peptides. The structural genes for these lantibiotics encode prepeptides whose leader peptides differ from those encoded by the nisin, epidermin, subtilin, and Pep5 gene clusters (12). The fact that organisms that produce lantibiotics with class two leader sequences like lacticin 481 and cytolysin contain only proteins with similarities to LanC (12) is in accordance with our results suggesting that LanB may mainly interact with the lantibiotic leader and that the catalytic subunit of the lanthionine synthetase complex for modification of the prepeptides is located within the LanC proteins. LanB proteins might be necessary for the recognition of the prepeptides, stabilization of the complex, and maintenance of a conformation of the prepeptides that allows the modification reactions to proceed. In cytolysin- or lacticin 481-producing cells, the function of the LanB proteins might be provided by the N-terminal domain of the CylM and LctM proteins, respectively.

We propose the existence of a lanthionine synthetase complex of at least 350 kDa consisting of NisB, at least two molecules of NisC, and a NisT dimer (Fig. 5). Due to unsuccessful attempts to isolate the complex by the native blue gel method (50) we propose that NisC is only loosely attached to the NisT transporter molecules. However, the genetic and biochemical data gave convincing evidence that lantibiotic prepeptides are matured at a multimeric lanthionine synthetase complex that catalyzes the dehydration of amino acid residues and the subsequent formation between the dehydrated residues and neighboring cysteine residues within the lantibiotic prepeptides.

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REFERENCES
1. Buchmann, G. W., Banerjee, S. and Hansen, J. N. (1988) J. Biol. Chem. 263, 16260–16266.
2. Kaletta, C. and Entian, K.-D. (1989) J. Bacteriol. 171, 1597–1601.
3. Chevallier, R. J. F., Fournaud, J., Levebre, E. and Moquet, G. (1957) Ann. Technol. Agric. 2, 117–137.
4. Hurst, A. (1981) Adv. Appl. Microbiol. 27, 263–265.
5. Benz, R., Jung, G. and Sahl, H.-G. (1991) Nisin and Novel Lantibiotics, ESCOM Scientific Publishers, Leiden, Germany.
6. Sahl, H.-G. (1985) J. Bacteriol. 162, 833–836.
7. Schübler, F., Benz, R. and Sahl, H.-G. (1989) Eur. J. Biochem. 182, 181–186.
8. Ingram, L. (1969) Biochim. Biophys. Acta 184, 216–219.
9. Schnell, N., Entian, K.-D., Schneider, U., Götz, F., Zähner, H., Kellner, R., and FEMS Microbiol. Lett. 45, 225–230.
10. Weil, H.-P., Beck-Sickinger, A. G., Metzger, J., Stevanovic, S., Jung, G., Josten, M., and Sahl, H.-G. (1990) Eur. J. Biochem. 194, 217–223.
11. Dodd, H. M., Horn, N. and Gasson, M. J. (1990) J. Gen. Microbiol. 136, 555–566.
12. de Vos, W. M., Kuipers, O. P., van der Meer, J. R., and Siezen, R. (1995) Mol. Microbiol. 17(3), 427–437.
13. Sahl, H.-G., Jack, R. W., and Bierbaum, G. (1995) J. Biol. Chem. 270, 827–835.
14. Banerjee, S. and Hansen, J. N. (1988) J. Biol. Chem. 263, 9508–9514.
15. Kaletta, C., Klein, C., Schnell, N., and Entian, K.-D. (1991) Nisin and Novel Lantibiotics, ESCOM Scientific Publishers, Leiden, Germany.
16. Klein, C. and Entian, K.-D. (1994) Appl. Environ. Microbiol. 60, 2793–2801.
17. Klein, C., Kaletta, C. and Entian, K.-D. (1993) Appl. Environ. Microbiol. 59, 296–303.
18. Klein, C., Kaletta, C., Schnell, N. and Entian, K.-D. (1992) Appl. Environ. Microbiol. 58, 132–142.
19. Allgaier, H., Jung, G., Werner, R. G., Schneider, U. and Zähner, H. (1986) Eur. J. Biochem. 160, 9–22.
20. Augustin, J., Rohde, R., Wieland, B., Schneider, U., Engelske, G., Entian, K.-D. and Götz, F. (1992) Eur. J. Biochem. 204, 1149–1154.
21. Schnell, N., Engelske, G., Augustin, J., Rosenthal, B., Ungermann, V., Götz, F. and Entian, K.-D. (1992) Eur. J. Biochem. 204, 57–68.
22. Schnell, N., Engelske, G., Augustin, J., Rosenthal, B., Götz, F. and Entian, K.-D. (1991) Nisin and Novel Lantibiotics, ESCOM Scientific Publishers, Leiden, Germany.
23. Schnell, N., Entian, K.-D., Götz, F., Horner, T., Kellner, R. and Sahl, H.-G. (1989) FEMS Microbiol. Lett. 58, 263–268.
25. Meyer, C., Bierbaum, G., Heldrich, C., Reis, M., Suling, J., Iglesias-Wind, M. I., Kempter, C., Molitor E., and Sahl H.-G. (1995) Eur. J. Biochem. 232, 478–489
26. Engelke, G., Gutowski-Eckel, Z., Hammelmann, M., and Entian, K.-D. (1992) Appl. Environ. Microbiol. 58, 3730–3743
27. Kuipers, O. P., Beethuyzen, M. M., Siezen, R. J., and de Vos, W. M. (1993) Eur. J. Biochem. 216, 281–291
28. van der Meer, J. R., Polman, J., Beethuyzen, M. M., Siezen, R. J., Kuipers, O. P., and de Vos, W. M. (1993) J. Bacteriol. 175, 2578–2588
29. Siegers, K., and Entian, K.-D. (1995) Appl. Environ. Microbiol. 61, 1082–1089
30. Chien, C. T., Bartel, P. L., Sternglanz, R., and Fields, S. (1991) Proc. Natl. Sci. U. S. A. 88, 9578–9582
31. Fields, S., and Song, O. (1989) Nature 340, 245–246
32. Blight, M. A., and Holland, I. B. (1990) Mol. Microbiol. 4, 873–880
33. Gasson, M. J. (1983) J. Bacteriol. 154, 1–9
34. Engelke, G., Gutowski-Eckel, Z., Kiesau, P., Siegers, K., Hammelmann, M., and Entian, K.-D. (1994) Appl. Environ. Microbiol. 60, 816–825
35. Niederacher, D., and Entian, K.-D. (1987) Mol. Gen. Genet. 206, 505–509
36. Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) Gene (Amst.) 33, 103–119
37. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
38. Tautz, D., and Renz, N. (1983) Anal. Biochem. 132, 503–517
39. Birnboim, H. C., and Doly, J. (1979) Nucleic Acids Res. 7, 1513–1518
40. Southern, E. M. (1975) J. Mol. Biol. 98, 503–517
41. Klebe, R. J., Harriss, J. V., Sharp, D., and Douglas, M. G. (1983) Gene (Amst.) 25, 333–341
42. Guarente, L. (1983) Methods Enzymol. 101, 181–191
43. Holc, H., and Nes, I. F. (1989) Appl. Environ. Microbiol. 55, 3119–3123
44. Otto, R., Lageveen, R. G., Veldkamp, H., and Konings W. N. (1982) J. Bacteriol. 149, 733–738
45. Koerner, T. J., Hill, J. E., Myers, A. M., and Tzagoloff, A. (1991) Methods Enzymol. 194, 477–490
46. Higgins, C. F. (1995) Cell 82, 693–696
47. Gilmore, M. S., Segarra, R. A., Booth, M. C., Bogie, C. P., Hall, L. R., and Clewell, D. B. (1994) J. Bacteriol. 176, 7335–7344
48. Rinco, A., Dufour A., Le Pogam, S., Thuault, D., Bourgeois, C. M., and Le Pennec, J. P. (1994) Appl. Environ. Microbiol. 60, 1652–1657
49. Horinouchi, S., and Weisblum, W. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 7079–7083
50. Schagger, H., Cramer, W. A., and von Jagow, G. (1994) Anal. Biochem. 217, 220–230
51. Entian, K.-D., and de Vos, W. M. (1996) Antonie Leeuwenhoek 69, 109–117