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# Nisin, a Peptide Antibiotic: Cloning and Sequencing of the *nisA* Gene and Posttranslational Processing of Its Peptide Product

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**Nisin produced by *Streptococcus lactis* is used as a food preservative and is the most important member of a group of antibiotics containing lanthionine bridges. To understand the genetic basis of these so-called lantibiotics (Schnell et al., Nature 333:276–278, 1988), we characterized the nisin structural gene, *nisA*, which is located on a plasmid and codes for a 57-amino-acid prepeptide. The prepeptide is processed posttranslationally to the pentacyclic antibiotic. Although nisin and the recently elucidated lantibiotic epidermin from *Staphylococcus epidermidis* are produced by different organisms, their gene organization is identical. As with epidermin, the nisin propeptide corresponds to the C-terminus of the prepeptide. The N-terminus of the prepeptide is cleaved at a characteristic splice site (Pro-−2 Arg-−1 Ile-+1). Remarkably, the N-terminus of prenisin shares 70% similarity with preepidermin, although the propeptide sequences are distinctly different. The structural similarities between these two lantibiotics are consistent with the fact that there is a common mechanism of biosynthesis of these lanthionine-containing antibiotics.**

Several peptide antibiotics have been described as potent inhibitors of bacterial growth. With respect to their biosynthesis, they can be divided into two classes: (i) those that are synthesized by a nonribosomal pathway catalyzed by multienzyme complexes, like gramicidin S (6, 7) and bacitracin (8), and (ii) those that are ribosomally synthesized. Many of these have been shown to undergo posttranslational modification. Whereas multienzyme complexes responsible for nonribosomally synthesized peptide antibiotics have been studied extensively, little is known concerning the biosynthesis of ribosomally formed peptide antibiotics. The most prominent ribosomally synthesized peptide antibiotics are those which are produced by gram-positive bacteria and contain the thioether amino acids lanthionine (Lan) and 3-methyl-lanthionine (MeLan). These antibiotics have been termed lantibiotics because of their characteristic lanthionine bridges (27). The most prominent lantibiotic is nisin (20); others include subtilin (12), ancovenin (32), epidermin (1, 2), gallidermin (15a), Ro09-0198 (16), and Pep5 (25).

Nisin is the most prominent lantibiotic and is used as a food preservative because of its high potency against certain gram-positive bacteria (20, 21, 23). It is produced by *Streptococcus lactis* strains belonging to serological group N. The inhibitory effect of *S. lactis* on lactobacilli was reported as early as 1928 (24). Nisin occurs naturally in dairy products, e.g., milk and farmhouse cheese (5). In several countries, nisin is used to prevent growth of clostridia in cheese and canned food. Since the realization of the potentially harmful effects of nitrite, which is also used against clostridia in canned food, there has been an increasing interest in use of nisin (23). There are two main difficulties in exploiting nisin commercially: (i) the nisin content in commercial preparations is low, and (ii) *S. lactis* produces nisin only slowly. Understanding nisin biosynthesis might overcome these problems.

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The possibility that nisin synthesis might be plasmid controlled was first suggested by Kozak et al. (17). The relationship between plasmids and nisin production was also shown by conjugal transfer of a plasmid gene from donor to negative phenotype recipients (10, 11, 22, 28, 29, 31). However, some investigators have obtained conflicting results which suggested that nisin production is mediated by either chromosomal or plasmid DNA (9).

Recently, the isolation of the gene encoding the prepeptide of the lantibiotic epidermin, *epiA*, clearly showed that lantibiotics are indeed posttranslationally processed from ribosomally synthesized prepeptides (27). Here this result was confirmed for nisin, and remarkably, although nisin and epidermin are produced from different organisms, their gene structure shares several similarities, suggesting a common mechanism of biosynthesis for lantibiotics.

## MATERIALS AND METHODS

**Strains and media.** *S. lactis* 6F3, kindly provided by T. Hörner (University of Tübingen), was grown on medium containing 3.3% Lab lemco powder (Oxoid, Wesel, Federal Republic of Germany [F.R.G.]), 3% malt extract, and 0.38% $Ca(OH)_2$, pH 6.5. *Escherichia coli* RR1 [$F^-$ *hsd-520* ($r^-$ $m^-$) *supE44 ara-14 galK2 lacY1 proA2 rpsL20* $Str^r$ *xyl-5 mtl-1*) was grown on LB medium (1% Bactopeptone [Difco], 0.5% yeast extract, and 0.5% NaCl, pH 7.5) supplemented with ampicillin (40 μg/ml). Incubation for both was at 37°C. Liquid cultures of *E. coli* were grown to late logarithmic phase; *S. lactis* cultures were grown to early logarithmic phase with rotary shaking overnight. Media were purchased from Difco Laboratories (Detroit, Mich.).

**Preparation of *S. lactis* DNA.** DNA was isolated from 1-liter cultures. To inhibit cell wall cross-linking, DL-threonine (final concentration, 20 mM) was added to early-logarithmic-phase cultures and incubated for 1 h at 37°C (4). DNA was released from sensitized cells after treatment with lysozyme (1 h at 37°C) (18) and sodium dodecyl sulfate (SDS; final concentration, 1%). The DNA in the lysate was further purified by ethanol precipitation and CsCl-ethidium bromide density centrifugation in a mini-ultracentrifuge (Beckman

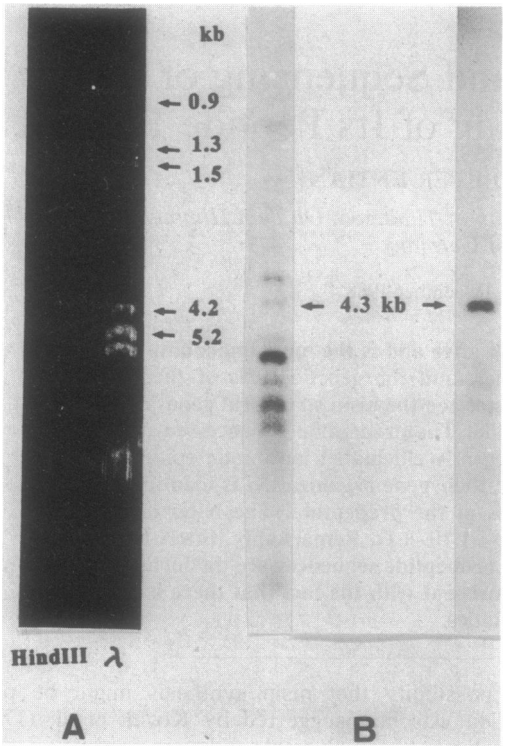


FIG. 1. (A) Agarose gel of *Hin*dIII-digested *Streptococcus lactis* 6F3 plasmid DNA (left lane) and *Eco*RI-*Hin*dIII-digested lambda DNA (right lane). (B) Hybridization of *Hin*dIII-digested plasmid DNA with a wobbled 17-mer oligonucleotide (left lane) and a wobbled 11-mer oligonucleotide (right lane). Both oligonucleotides were derived from the expected propeptide sequence of nisin (13).

model T100 centrifuge, model TLA 100.2 rotor) for 12 h (27). This revealed two ethidium bromide-stained bands (chromosomal and plasmid DNA) which were removed with a syringe needle and extracted four times with an equal volume of 1-butanol saturated with TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), whereas CsCl was removed by dialysis against TE buffer.

**Restriction digestion and electrophoresis.** For restriction digestion, conditions recommended by the commercial supplier (Boehringer, Mannheim, F.R.G.) were followed. Resultant DNA fragments were separated on 0.7% agarose gels. Restriction enzyme-cut DNA was eluted from the gel by the freeze-squeeze method (30).

**Radioactive labeling of DNA.** Oligonucleotides were labeled with [$\gamma$-$^{32}$P]ATP by phage T4 polynucleotide kinase (19). Oligonucleotides were synthesized on a 380B DNA synthesizer (Applied Biosystems, Weiterstadt, F.R.G.) and used without further purification.

**Hybridization with 5′-labeled oligonucleotides.** For hybridization, the method of Southern (28) was used. The hybridization mix, HCl titrated to pH 7, contained sodium citrate (750 mM), sodium chloride (75 mM), sonicated salmon sperm DNA (1 g/liter), Ficoll 400 (final concentration, 0.1%), polyvinylpyrollidone (final concentration, 0.1%), bovine serum albumin (final concentration, 0.1%), and SDS (final concentration, 1%). After hybridization at 23°C, blots were washed thrice with buffer (750 mM sodium citrate, 75 mM sodium chloride).

**DNA sequencing.** DNA was sequenced bidirectionally by the dideoxy chain termination method (26). Plasmid sequencing was performed as described by Hattori and Sakaki (14). To locate and sequence the *nisA* gene on the cloned DNA fragment, the 17-mer wobbled oligonucleotide probe [ATGGGA(C/G/T)TGC(T)AAC(T)ATGAA] was applied in 30-fold excess as a sequencing primer. This procedure provided a short identifiable region used for reverse priming. For further sequencing of both DNA strands, appropriate oligonucleotides were synthesized as sequencing primers.

**Other abbreviations.** Lan corresponds to *meso*-lanthionine. The *meso*-lanthionine bridges are formed by posttranslational modification at a cysteine and a serine residue. MeLan corresponds to 3-methyllanthionine. The 3-methyllanthionine bridges are formed by posttranslational modification at a cysteine and a threonine residue (15).

## RESULTS

**Isolation of the nisin structural gene, *nisA*.** Chromosomal and plasmid DNA from *S. lactis* 6F3 were isolated. The plasmids obtained were nearly immobile on 0.7% agarose gels, indicating their large sizes. Only after endonucleolytic digestion were distinct bands obtained with the plasmid preparation. Deduced from the postulated propeptide sequence of nisin (Met-Gly-Cys-Asn-Met-Lys, amino acids 17

```
AAAATAAATTATAAGGAGGCACTCAAA ATG AGT ACA AAA GAT TTT AAC TTG GAT
                             M   S   T   K   D   F   N   L   D
                                             -20

TTG GTA TCT GTT TCG AAG AAA GAT TCA GGT GCA TCA CCA CGC ATT ACA
 L   V   S   V   S   K   K   D   S   G   A   S   P   R   I   T
                 -10                                 -1  +1

AGT ATT TCG CTA TGT ACA CCC GGT TGT AAA ACA GGA GCT CTG ATG GGT
 S   I   S   L   C   T   P   G   C   K   T   G   A   L   M   G
                             +10

TGT AAC ATG AAA ACA GCA ACT TGT CAT TGT AGT ATT CAC GTA AGT AAA
 C   N   M   K   T   A   T   C   H   C   S   I   H   V   S   K
    +20                                     +30

TAACCAAATCAAAGGATAGTATTTT
oc
```

FIG. 2. Nucleotide sequence of the nisin structural gene (*nisA*) and deduced amino acid sequence of prenisin. The Shine-Dalgarno sequence 9 bp in front of the ATG codon is boxed, and the proteolytic cleavage site at which the lantibiotic is processed is indicated by an arrow. oc, Ochre stop codon.

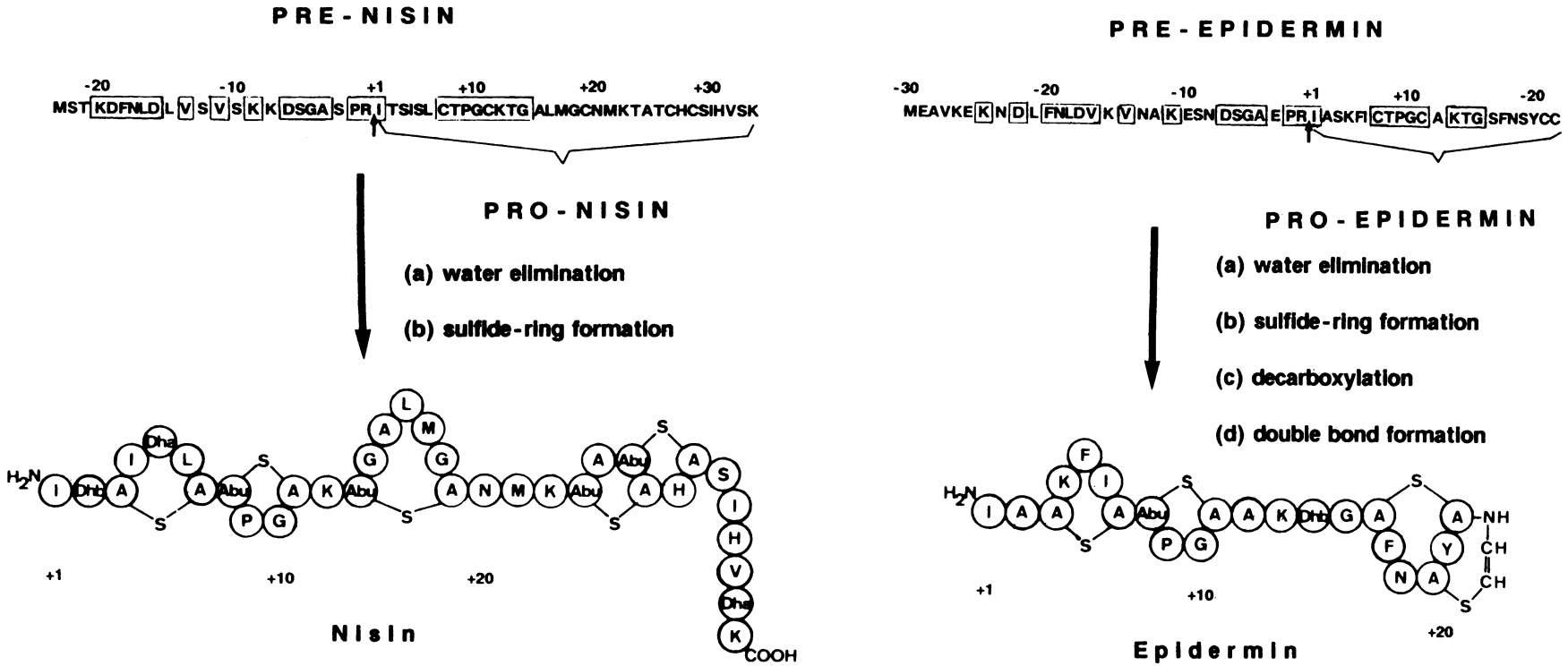


FIG. 3. Comparison between the nisin (left) and the epidermin (right) prepeptide and possible maturation steps (see text). Corresponding amino acids are boxed. The characteristic cleavage site at which both lantibiotics are processed is indicated by a small arrow. Abbreviations: Abu, aminobutyric acid; Dha, dehydroalanine; Dhb, dehydrobutyrine.

to 22), a wobbled 17-mer DNA probe [5′-ATGGGA(C/G/T)TGC(T)AAC(T)ATGAA-3′] was synthesized and used as a hybridization probe. There were at least eight signals after hybridization of the plasmid digest with the 17-mer DNA probe (Fig. 1). Accordingly, an additional 11-mer wobbled oligonucleotide [5′-TGC(T)ACA(C/G/T)CCA(C/G/T)GG-3′] corresponding to Cys-Thr-Pro-Gly, amino acids 7 to 10, was used to identify the nisin signal (Fig. 1). A 4.3-kilobase (kb) *Hin*dIII fragment gave a strong signal with the 11-mer oligonucleotide and a minor signal with the 17-mer oligonucleotide. No further signals identical in size were obtained. The digested chromosomal DNA did not give similar hybridization signals. This indicated that the nisin structural gene was located on a plasmid, in agreement with successful curing experiments (9).

**Structure of the *nisA* gene.** The 4.3-kilobase-pair (kbp) *Hin*dIII fragment was subcloned into pUC19, and the 17-mer wobbled oligonucleotide was used as a sequencing primer in 30-fold excess. The expected propeptide sequence of nisin (13) identified the nisin structural gene, which was designated *nisA*. The pronisin sequence derived from the DNA sequence was in complete agreement with that proposed by Gross and Morell (13). An open reading frame and a methionine codon were found to be at an appropriate distance from a Shine-Dalgarno sequence. Hence, the prepeptide of nisin consists of 57 amino acid residues (Fig. 2). The propeptide, corresponding to the amino acid residues from which nisin is matured, is located at the C-terminus of the prepeptide. It is processed from the prepeptide between $Arg^{-1}$ and $Ile^{+1}$, and a proline residue is present at position −2. Thus, a structure is maintained that is characteristic of many proteolytic splice sites.

**Comparison between prenisin and preepidermin.** Epidermin, from *S. epidermidis*, was the first lantibiotic for which the prepeptide sequence has been reported (27); nisin, from *S. lactis*, is the second. Comparison of these prepeptides, synthesized by different organisms, should reveal common characteristics of lantibiotics (Fig. 3). Both lantibiotics are encoded by distinct genes, and the propeptides corresponding to the matured lantibiotics are located at the C-termini of the prepeptides. Lantibiotics are processed at a characteristic proteolytic splice site, $Pro^{-2}X^{-1}\downarrow Y^{+1}$, where X corresponds to a basic amino acid residue and Y corresponds to a hydrophobic residue. The N-terminus of the prepeptides left of the splice site contains 30 (preepidermin) and 23 (prenisin) amino acid residues, of which 16 are identical. The cysteine residues from which the sulfide bridges are formed are not found within the N-terminal part of the prepeptides.

Most remarkable is the N-terminal conservation of the prepeptides. Although the producing strains *S. lactis* and *S. epidermidis* have diverged, these basic structures have been preserved, indicating an essential role during lantibiotic biosynthesis. Similar conservation is not observed within the propeptide sequences. Only those amino acid residues forming the N-terminal *meso*-lanthionine and 3-methyl-lanthionine bridges are similar, whereas no similarities are observed within the C-terminus. These observations give some support to the previously stated hypothesis that the N-terminus of the prepeptide may play an important role in the synthesis of lantibiotics.

## DISCUSSION

From the similarities in prepeptide structure of the two lantibiotic prepeptides described, the question arises how the modification and processing steps occur. Our recently presented hypothesis (27) favors the occurrence of enzymatic modifications at the prepeptide before cleavage, possibly supported by the α-helical N-terminus. This hypothesis was based on the observation that lantibiotics are also toxic for most producer strains and that processing of the mature lantibiotic during secretion may solve the problem of high intracellular concentrations. The lack of cysteine residues and the strong conservation at the N-terminal sequences may also support this view (Fig. 3). Alternatively, the N-terminal sequence might serve as a signal sequence for secretion. However, this seems less likely, as the N-terminal sequences of nisin and epidermin do not share common characteristics with either cotranslational or posttranslational signal sequences (3). Unlike epidermin, for which four steps of maturation have been proposed (27) (water elimination, sulfide ring formation, decarboxylation, and double bond formation), nisin requires only the first two reactions. Whereas in the epidermin molecule water elimination occurs at all serine and threonine residues, $Ser^{29}$ of nisin is not modified. This serine residue is directly adjacent to a 3-methyllanthionine bridge, which may prevent water elimination from $Ser^{29}$. Water elimination of $Thr^{25}$ may be followed immediately by sulfide addition from $Cys^{28}$, both reactions being catalyzed by a single enzyme.

In the last 5 years, nisin has become much more important as a food preservative because carcinogenic *N*-nitrosamine is formed from nitrite, which is an alternative for preserving canned food. Nisin has several advantages: it occurs naturally in dairy products; its toxicity is negligible; it is only active under conditions used to store dairy products and canned food, namely at low pH and after preheating; as a peptide, it is rapidly degraded proteolytically; and it does not inhibit growth of gram-negative intestinal flora. The lantibiotic nisin is uniquely specific as a food preservative, and disadvantages generally known for other antibiotics, such as resistance and toxicity, do not occur.

The prenisin structure reported here proves that lantibiotics are similarly organized and hence demonstrates common biosynthetic pathways. Our results may make it possible to obtain nisin-overproducing strains. Furthermore, combined with chemical structural analysis, future design and expression of new lantibiotics may yield new possible applications.

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## ADDENDUM IN PROOF

After this paper was submitted, Buchman et al. (G. W. Buchman, S. Banerjee, and J. N. Hansen, J. Biol. Chem. **263**:16260–16266, 1988) reported the isolation of the nisin structural gene from genomic DNA. We found the *nisA* gene to be located on purified plasmid DNA. Strain divergency or the different DNA purification methods used may explain this; the *nisA* sequences reported are absolutely identical.

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