THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 269, No. 5, Issue of February 4, pp. 3555–3562, 1994
Printed in U.S.A.

Influence of Amino Acid Substitutions in the Nisin Leader Peptide on Biosynthesis and Secretion of Nisin by *Lactococcus lactis**

(Received for publication, June 14, 1993, and in revised form, October 8, 1993)

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Structural genes for small lanthionine-containing antimicrobial peptides, known as lantibiotics, encode N-terminal leader sequences which are not present in the mature peptide, but are cleaved off at some stage in the maturation process. Leader sequences of the different lantibiotics share a number of identical amino acid residues, but they are clearly different from *sec*-dependent protein export signal sequences. We studied the role of the leader sequence of the lantibiotic nisin, which is produced and secreted by *Lactococcus lactis*, by creating site-directed mutations at various positions in the leader peptide sequence. Mutations at Arg^{-1} and Ala^{-4}, but not at the conserved Pro^{-2}, strongly affected the processing of the leader sequence and resulted in the extracellular accumulation of a biologically inactive precursor peptide. Amino acid analysis and ^1H NMR studies indicated that the precursor peptide with an $Ala^{-4} \rightarrow Asp$ mutation contained a modified nisin structural part with the (mutated) unmodified leader sequence still attached to it. The $Ala^{-4} \rightarrow Asp$ precursor peptide could be activated *in vitro* by enzymatic cleavage with trypsin, liberating nisin. These results confirmed that cleavage of the leader peptide is the last step in nisin maturation and is necessary to generate a biologically active peptide. Several mutations, *i.e.* $Pro^{-2} \rightarrow Gly$, $Pro^{-2} \rightarrow Val$, $Asp^{-7} \rightarrow Ala$, $Lys^{-9} \rightarrow Leu$, $Ser^{-10} \rightarrow Ala$/$Ser^{-12} \rightarrow Ala$ and $Val^{-11} \rightarrow Asp$/$Val^{-13} \rightarrow Glu$ in the leader peptide did not have any detectable effect on nisin production and secretion, although some of them affected highly conserved residues. When mutations were created in the −18 to −15 region of the nisin leader peptide (*i.e.* $Phe^{-18} \rightarrow Leu$, $Leu^{-16} \rightarrow Lys$, $Asp^{-15} \rightarrow Ala$), no secretion or intracellular accumulation could be detected of nisin or its precursors. This suggested that these conserved residues are involved in the maturation process and may interact with lantibiotic-specific modifying enzymes.

The antimicrobial peptide nisin, which is produced and secreted by several strains of *Lactococcus lactis*, is a highly modified peptide with several characteristic features, such as dehydrated Ser and Thr residues (Dha and Dhb, respectively),[1] and five intramolecular thioether ring structures called (β-methyl)-lanthionine residues (Gross and Morell, 1971). Peptides with molecular structures similar to nisin have been found in several other Gram-positive bacteria, and together they have been named lantibiotics (Schnell *et al.*, 1988; for a review see Jung, 1991). Nisin is ribosomally synthesized, and the primary translation product of 57 amino acids is presumed to undergo a number of modifications, catalyzed by specific enzymes, before finally being secreted into the extracellular medium in a bioactive form (Buchmann *et al.*, 1988). The bioactive peptide nisin is composed of the 34 C-terminal amino acid residues of the primary translation product, which are modified in a sequence of events in which specific Ser and Thr residues become dehydrated to form dehydroalanine and dehydrobutyrine, which, in five cases, form intramolecular thioether bridges with the Cys residues present in this part of the precursor peptide (Fig. 1*A*). Intermediate precursor peptides in different stages of dehydration have been isolated from *Staphylococcus epidermidis* strains producing the lantibiotic Pep5, which suggested that sequential dehydration of Ser and Thr residues takes place as a first step in maturation (Weil *et al.*, 1990).

Two natural variants of nisin have been found among lactococcal strains, which have been named nisin A (Gross and Morell, 1971) and nisin Z (Mulders *et al.*, 1991; de Vos *et al.*, 1993). The structural genes for both nisin A (*nisA*) (Buchmann *et al.*, 1988; Kaletta and Entian, 1989; Dodd *et al.*, 1990) and nisin Z (*nisZ*) (Mulders *et al.*, 1991; de Vos *et al.*, 1993) have been cloned and sequenced, and it was shown that the two mature peptides differ in only one amino acid residue (His^{27} in nisin A and Asn^{27} in nisin Z). In *L. lactis* NIZO R5 the production of nisin A is encoded by the 70-kilobase pair conjugative transposon Tn*5276* (Rauch and de Vos, 1992). Recently a 10-kilobase pair DNA fragment of Tn*5276* was cloned from *L. lactis* R5, which contained the gene cluster *nisABTCIPR* (van der Meer *et al.*, 1993, Kuipers *et al.*, 1993a). The genes *nisABTC* have also been found in strain *L. lactis* 6F3 (Engelke *et al.*, 1992), whereas the *nisAB* genes have been reported of *L. lactis* 11454 (Steen *et al.*, 1991). It is assumed that NisT is involved in transport of (precursor) nisin across the cytoplasmic membrane, since it shares significant homology with ATP-dependent translocator proteins (Engelke *et al.*, 1992; Kuipers *et al.*, 1993a). It can be hypothesized that *nisB* and *nisC* encode the enzymes needed for modification of the lantibiotic precursor peptides (Steen *et al.*, 1991; Engelke *et al.*, 1992; Kuipers *et al.*, 1993a). The *nisI* gene encodes a putative lipoprotein, which was shown to be involved in immunity to nisin (Kuipers *et al.*, 1993a). The *nisP* gene was shown to encode a subtilisin-like serine protease, involved in cleavage of the leader peptide sequence from the final precursor peptide (van der Meer *et al.*,

* This work was supported by Contract BIOT-CT91-0265 of the Biotechnology Research For Innovation, Development, and Growth in Europe (BRIDGE) program of the Commission of the European Communities. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: Dha, dehydroalanine; Dhb, dehydrobutyrine; RP-HPLC, reversed phase-high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; Tn, transposon; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

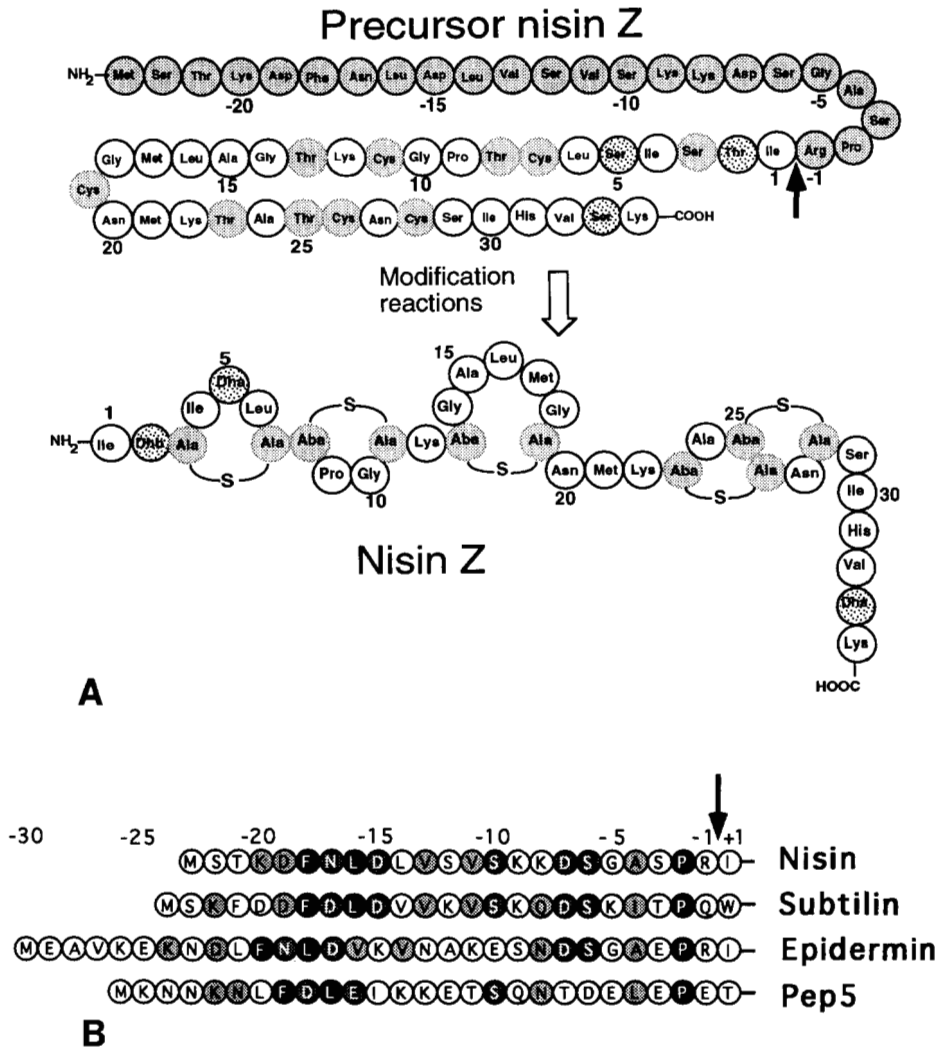

FIG. 1. *A*, primary translation product of the *nisZ* gene and schematic drawing of the primary structure of the mature nisin. The *arrow* in the primary translation product indicates the site for cleavage of the leader peptide from the structural region. Residues in the precursor nisin, which are dehydrated only, are *dotted*, whereas those residues which subsequently form lanthionine bridges are *shaded*. *B*, comparison of leader peptide sequences of nisin, subtilin, epidermin, and Pep5, as deduced from the gene sequences (Banerjee and Hansen, 1988; Buchmann *et al.*, 1988; Kaletta *et al.*, 1989; Schnell *et al.*, 1988). Residues conserved in all four sequences are shown in *black*; residues with a conservative replacement or conserved in three out of four sequences are represented in *dark grey*; other less conserved sites or residues which are conserved but do not occur at identical positions in all sequences are drawn in *light grey shading*. The site of cleavage of the leader peptide from the mature peptide is indicated with an *arrow*.

1993). Finally, *nisR* encodes a positive regulator protein needed for activation of expression of the *nis* genes (van der Meer *et al.*, 1993).

Research on the structure/function relationships and on biosynthesis of nisin has mainly focused on the mature (active) nisin, and several features of the peptide have been proposed to be important for its biological activity. For instance, it was shown that an intact Dha[5] residue is important for antimicrobial activity of nisin (Chan *et al.*, 1989) and that changing the Dha residue at position 5 to a Dhb reduced the antimicrobial properties of nisin 2–10-fold depending on the indicator species (Kuipers *et al.*, 1992). The lanthionine rings are assumed to be of critical importance to maintain the local rigid structure of nisin to allow it to insert into the target membrane and form pores therein. In contrast to the function of nisin and the effect of different residues on the antimicrobial activity of nisin, little is known about the role of the leader peptide. The leader peptide is formed by the 23 N-terminal residues of the deduced primary translation product, but it does not undergo dehydration (van der Meer *et al.*, 1993; Kuipers *et al.*, 1993b), and it is absent in mature nisin (Fig. 1*A*). Leader peptides of different lantibiotics, such as nisin, subtilin, epidermin, and Pep5, exhibit a clear similarity in size and primary structure (Fig. 1*B*). This suggests a critical role of the leader in the process of lantibiotic maturation, which thus may be largely similar in the different lantibiotic producing strains. It has been proposed that the leader peptide is involved in the biosynthetic process (Jung, 1991) for instance (i) by functioning as a recognition or binding part of the molecule for the biosynthetic enzymes, (ii) by interacting with residues of the mature part to keep the molecule in a favorable conformation for the necessary enzymatic reactions and in this way preventing nonspecific modifications, (iii) by keeping the maturating peptide biologically inactive and thus contributing to immunity of the host cell, or (iv) by assisting transport of the precursor peptide across the cytoplasmic membrane.

Here we describe studies to determine the role of the nisin leader peptide sequence in the biosynthetic process of nisin maturation. We created site-directed mutations in conserved residues of the leader sequence to alter the nature of the original residue at that particular position. Some mutations in a region close to the cleavage site prevented the cleavage of the leader peptide from the final precursor. This precursor peptide was secreted into the extracellular medium by the mutant *L. lactis* cells and contained the mutated nisin leader peptide still attached to the fully matured nisin. This result showed that residues critical for leader cleavage and recognition by the leader protease are present in this part of the leader sequence. Another set of mutations, created in a strongly conserved region of different lantibiotic leader peptides, resulted in an almost complete blockade of nisin biosynthesis, without any detectable secretion or intracellular accumulation of precursors. This demonstrates that the leader peptide is indeed involved in nisin biosynthesis.

TABLE I
Primers used in this study

Sites of mutation are underlined in the sequences and the codon changes or the introduced restriction cleavage sites are indicated within parentheses. For primer 4 we used a mixed synthesized oligonucleotide.

Primer	Sequence	Mutation
1	5′-GATTAAATTCTGCAGTTTGTTAG-3′	(*Pst*I)
2	5′-CCCTAAAAAGCTTATAAAAATAGG-3′	(*Hin*dIII)
3	5′-GCATCACCACAAATTACAAGTATTTCGC-3′	(R-1Q)
4	5′-GCATCAG(G/T)ACGCATTACAAGTATTTCGC-3′	(P-2V and P-2G)
5	5′-CAGGTGATTCACCACGCATTACAAGTAT-3′	(A-4D)
6	5′-CGAAGAAAGATTTGGGTGCATCAC-3′	(S-6L)
7	5′-TCGAAGAAAGCGTCAGGTGCATCAC-3′	(D-7A)
8	5′-GTTTCGTTGAAAGATTCAGGTGCATCAC-3′	(K-9L)
9	5′-GGTAGCTGTTGCGAAGAAAGATTCAGGTG-3′	(S-10A/S-12A)
10	5′-TTGGAATCTGATTCGAAGAAAGATTCAG-3′	(V-11D/V-13E)
11	5′-GGCTTTGGTATCTGTTTCGAAG-3′	(D-15A)
12	5′-TTAACAAGGATTTGGTATCTGTTTCG-3′	(L-16K)
13	5′-TTAAACTTGGATTTGGTATCTG-3′	(F-18L)

MATERIALS AND METHODS

Bacterial Strains, Plasmids, Media, and Growth Conditions—*L. lactis* strains used in this study are: NIZO R5, harboring the nisin A transposon Tn*5276*; NZ9700 (Kuipers *et al.*, 1993a), a transconjugant of the plasmid-free strain MG1614 containing a single copy of Tn*5276* (Rauch and de Vos, 1992); and NZ9800, a derivative of NZ9700 in which the *nisA* gene has been exchanged by replacement recombination with a modified *nisA* gene containing a 4-base pair deletion in the pronisin-encoding part and which is therefore no longer able to produce nisin A (Kuipers *et al.*, 1993a). Lactococci were cultivated without aeration at 30 °C in M17 broth (Difco), supplemented with 0.5% (w/v) glucose or sucrose. When analyzed for nisin production, cells were grown in SPYS medium, containing 1% sucrose, 1% bactopepton (Difco), 1% yeast extract (Difco), 0.2% NaCl, 0.002% $MgSO_4 \cdot 7H_2O$ and 1% KH_2PO_4, pH 7.0 (De Vuyst and Vandamme, 1992). For *L. lactis* strains harboring pNZ123-derived plasmids, media were supplemented with 5 μg/ml chloramphenicol. As a host for cloning experiments, we used *Escherichia coli* strain MC1061 (Casadaban *et al.*, 1980), which was cultivated on Luria broth (Sambrook *et al.*, 1989). The wild-type *nisZ* gene was taken from plasmid pNZ9013 (Kuipers *et al.*, 1992), which contains a 280-base pair *Pst*I-*Hin*dIII fragment, carrying the *nisZ* gene of *L. lactis* strain NIZO 22186 (Mulders *et al.*, 1991) under control of the *lac* promoter (van Rooijen and de Vos, 1990) in the vector pNZ123. Mutant *nisZ* genes were cloned in expression plasmid pNZ9019 containing a mutant *nisZ* gene with an additional internal *Pst*I restriction site that is convenient for screening purposes, by replacement of the *nisZ*-containing DNA fragment (Kuipers *et al.*, 1992).

Site-directed Mutagenesis, DNA Techniques, and DNA Sequence Analysis—Site-directed mutagenesis of DNA sequences encoding the nisin leader peptide region involved a two-step polymerase chain reaction amplification and was performed as described before (Kuipers *et al.*, 1991). Mutant polymerase chain reaction fragments were digested with *Pst*I and *Hin*dIII and ligated with plasmid pNZ9019, previously digested with the same enzymes, and subsequently used to transform *E. coli* MC1061. *E. coli* transformants were analyzed for their plasmid DNA content, and the DNA sequence of the *nisZ* region was determined on the double-stranded plasmid DNA with the *Hin*dIII-primer (primer 2, see below) by a modified dideoxy chain termination method (Sanger *et al.*, 1977) as described elsewhere (van der Meer *et al.*, 1993). Plasmids carrying the proper mutation were then introduced by electroporation into *L. lactis* NZ9700 or NZ9800. Plasmid DNA content was subsequently isolated from chloramphenicol-resistant *L. lactis* transformants and analyzed on the restriction enzyme level.

To obtain site-directed mutations in the region of *nisZ* encoding the leader peptide, we used the primers listed in Table I. Restriction enzymes and other DNA modifying enzymes were purchased from Life Technologies, Inc., or United States Biochemical Corp., and used as recommended by the manufacturers. RNA isolation, Northern blotting, and hybridization with a *nisA* gene probe were performed as described previously (Kuipers *et al.*, 1993a).

Production, Purification, and Analysis of Nisin and Precursor Nisin Species—Production and secretion of nisin or nisin precursor species by *L. lactis* wild-type and mutant strains were analyzed in the supernatant of cultures grown to early stationary phase on SPYS medium (A_{620} of 1.0). Cultures (20 ml) were centrifuged for 10 min at 6,000 × *g* to remove the lactococcal cells. The supernatant was subsequently filtrated over a 0.45-μm membrane, 0.10 volume of 100% (w/v) trichloroacetic acid was added, and the mixture was incubated on ice for 16 h to precipitate the protein fraction in the supernatant. The precipitate was then pelleted by centrifugation for 15 min at 6,000 × *g* and the supernatant removed. The pellet was redissolved in 400 μl of 50 mM sodium acetate, pH 5.5, and, when necessary, the pH was adjusted with NaOH to pH 5.5. The protein fraction was then analyzed for the presence of nisin by electrophoresis through 10% Tricine-SDS-PAGE gels (Schägger and Jagow, 1987), which were stained with silver nitrate, and further by examination on RP-HPLC to discriminate between nisin A and nisin Z (see below). Purified 6-kDa precursor of nisin A (van der Meer *et al.*, 1993) was used as a standard on Tricine-SDS-PAGE gels. Large scale isolation of nisin species was performed from the supernatant of 10-liter cultures of *L. lactis* by chromatography on Fractogel TSK butyl 650-S. Preparative RP-HPLC and lyophilization of the $Ala^{-4} \rightarrow Asp$ mutant nisin precursor were performed essentially as described previously (Kuipers *et al.*, 1992), with an altered elution gradient of 5–27% buffer B in buffer A during 35 min at a flow rate of 10 $ml \cdot min^{-1}$. N-terminal sequencing of precursor nisin species was performed at the Stichting Scheikundig Onderzoek in Nederland sequencing facility (Dr. R. Amons, Department of Medical Biochemistry, Sylvius Laboratory, Leiden, The Netherlands) by using an automated gas-phase sequenator (Applied Biosystems Inc, Foster City, Ca). A chemically synthesized nisin leader peptide was kindly provided by Dr. A. Beck-Sickinger of Tübingen University, Tübingen, Germany. Antibodies raised against nisin A and against the synthetic leader peptide were used for Western blotting as described previously (van der Meer *et al.*, 1993).

Analysis of Nisin Species by HPLC—Nisin-containing samples were examined by analytical RP-HPLC, using a Hi Pore RP-318 column (Bio-Rad Laboratories Inc., Brussels, Belgium) at a temperature of 30 °C. Proteins were eluted using a linear gradient from 10 to 23% buffer B (90% aqueous acetonitrile, 0.07% trifluoroacetic acid) in buffer A (10% aqueous acetonitrile, 0.1% trifluoroacetic acid) during 10 min, followed by a 40-min linear gradient from 23 to 28% buffer B in buffer A, at a flow rate of 1.0 $ml \cdot min^{-1}$. The absorbance of the effluent was monitored at 220 nm.

NMR Experiments—^1H NMR spectra were taken at 400.13 MHz on a Bruker AM400 spectrometer interfaced to an Aspect 3000 computer. Experimental conditions were: 1–2 mM protein, dissolved in 10% D_2O, 90% H_2O, pH 3.5, 25 °C. The solvent resonance was suppressed by presaturation. The spectra were referenced to external 3-(trimethylsilyl)-2,2,3,3-tetradeuteropropionic acid sodium salt (British Drug House).

RESULTS AND DISCUSSION

Effect of Site-specific Mutations in the Nisin Z Leader Peptide on Production and Secretion of Nisin—To analyze the role of specific residues in the leader peptide sequence on nisin biosynthesis, we created a number of site-specific mutations in the leader-encoding region of the *nisZ* gene, resulting in altered amino acid residues in the nisin Z leader peptide (Fig. 2). The mutations were chosen primarily at residues or motifs conserved among the four related lantibiotic leader peptides (Fig. 1*B*) and generally applied in such a way that the nature of that particular residue would be altered drastically. Mutated *nisZ* genes were cloned in pNZ9019 and expression was established

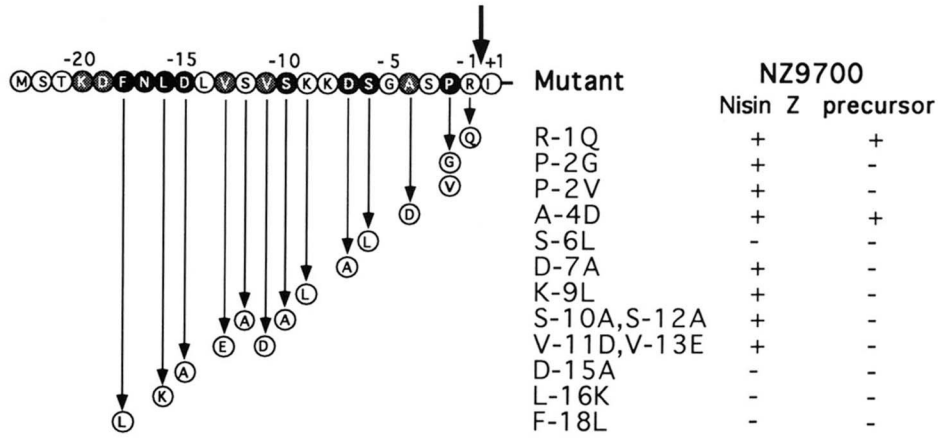

FIG. 2. **Overview of the generated site-directed mutations in the nisin Z leader peptide.** Amino acid alterations are given in one letter code. The *right part* of the figure gives an interpretation of the results of SDS-PAGE and HPLC analysis of the different mutants in *L. lactis* strain NZ9700 with respect to the presence of nisin Z or precursor nisin Z in the extracellular medium.

in *L. lactis* strain NZ9700. This strain is a nisin A producer, but will simultaneously produce nisin Z when the *nisZ* gene is present on the expression plasmid pNZ9013 (Kuipers *et al.*, 1992).

Production of nisin and its secretion into the extracellular medium by the different mutant strains was characterized first by analyzing the supernatant of late-log cultures on SDS-PAGE gels (Fig. 3, *A* and *B*). All mutants were found to produce and secrete peptides of approximately 3.5 and/or 6.0 kDa. Using SDS-PAGE it cannot be distinguished whether nisin A, nisin Z, or both are present in the 3.5-kDa band. By using HPLC we could detect the presence of nisin A and nisin Z separately in the culture supernatants of mutant strains, and we compared this to the production of nisin A and nisin Z by strain NZ9700 harboring pNZ9013 (*nisZ*). Changing the leader peptide sequence of nisin Z at several unique positions showed strong effects on the production and secretion of nisin Z (Fig. 2). Two mutant strains (carrying the leader mutations $Arg^{-1} \rightarrow Gln$ and $Ala^{-4} \rightarrow Asp$) produced and secreted an extra peptide with an apparent molecular mass of 6 kDa on SDS-PAGE (Fig. 3*A*, *lanes 1* and *4*). On RP-HPLC the 6-kDa peptide appeared as an additional peak with a shorter retention time than nisin A and Z; a peak eluting at exactly the same position as nisin Z was observed in both cases, suggesting a slow processing of the mutant precursors (Fig. 4*A*). The 6-kDa peptide that was produced by the $Ala^{-4} \rightarrow Asp$ mutant strain was purified by HPLC and analyzed further by ^1H NMR (see below). Several other mutations, *i.e.*, $Pro^{-2} \rightarrow Gly$, $Pro^{-2} \rightarrow Val$ (Fig. 4*B*), $Asp^{-7} \rightarrow Ala$ and $Val^{-11} \rightarrow Asp/Val^{-13} \rightarrow Glu$, did not affect production and secretion of nisin A and nisin Z by the lactococcal cells, although differences in production levels of mutants were found in some cases, *e.g.* higher production of the $Ser^{-10} \rightarrow Ala/Ser^{-12} \rightarrow Ala$ mutant (Fig. 4*B*) and lower production of $Lys^{-9} \rightarrow Leu$. It was unexpected that changing the highly conserved Pro residue at position −2 to a Gly or Val had no obvious effect on nisin Z biosynthesis or processing. The only effect we found was that strains with these mutations ($Pro^{-2} \rightarrow Gly$ and $Pro^{-2} \rightarrow Val$), and with the $Asp^{-7} \rightarrow Ala$ mutation in the leader peptide, showed a 2-fold slower growth on SPYS medium than did strain NZ9700 harboring pNZ9013. However, the final production and secretion levels of nisin A and of nisin Z in cells harvested at an A_{620} of 1.0 were not different (results not shown). In strains containing one of the following mutations, $Ser^{-6} \rightarrow Leu$, $Asp^{-15} \rightarrow Ala$, $Leu^{-16} \rightarrow Lys$ and $Phe^{-18} \rightarrow Leu$ in the nisin leader peptide, the production of mutant nisin Z, but not of nisin A, was drastically reduced or not detectable at all on HPLC. An example of the elution pattern of such a mutant ($Phe^{-18} \rightarrow Leu$), deficient in nisin Z production in strain NZ9700, is shown in Fig. 4*B*. When we screened the cytoplasmic protein fraction of these mutant strains (*i.e.* mutations at positions −18, −16, −15, or −6) for intermediate (mutant) precursor nisin peptides by Western blotting and immunological staining techniques with antibodies raised against the nisin leader peptide, we did not find any intracellular accumulation of precursor nisins, whereas in the case of wild-type *nisZ* expression in strain NZ9700, a 6-kDa precursor band was visible. Therefore, we

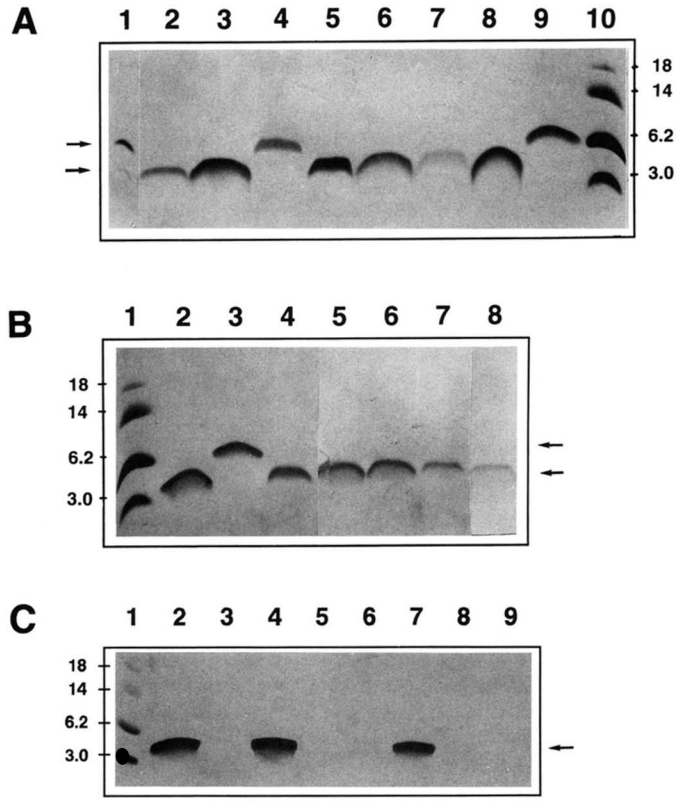

FIG. 3. **Silver-stained Tricine-SDS polyacrylamide gels of trichloroacetic acid-precipitated supernatants of late log cultures of *L. lactis* NZ9700 with the different mutant *nisZ* plasmids.** *A*, *lanes*: *1*, $Arg^{-1} \rightarrow Gln$ (run on another but fully comparable gel); *2*, $Pro^{-2} \rightarrow Gly$; *3*, $Pro^{-2} \rightarrow Val$; *4*, purified $Ala^{-4} \rightarrow Asp$; *5*, $Ser^{-6} \rightarrow Leu$, *6*, $Ser^{-10} \rightarrow Ala/Ser^{-12} \rightarrow Ala$; *7*, $Asp^{-7} \rightarrow Ala$; *8*, purified nisin A; *9*, purified precursor nisin A; *10*, molecular mass markers. *B*, *lanes*: *1*, molecular mass markers; *2*, purified nisin A; *3*, purified precursor nisin A; *4*, $Val^{-11} \rightarrow Asp/Val^{-13} \rightarrow Glu$; *5*, $Asp^{-15} \rightarrow Ala$; *6*, $Leu^{-16} \rightarrow Lys$; *7*, $Phe^{-18} \rightarrow Leu$; *8*, $Lys^{-9} \rightarrow Leu$ (run on another gel). *Arrows* point to the positions of nisin A or/and nisin Z (*lower*) and precursor nisin Z (*upper*). *C*, trichloroacetic acid-precipitated supernatants of late log cultures of *L. lactis* NZ9800 with or without mutant *nisZ* plasmids. *Lanes*: *1*, molecular mass marker; *2*, NZ9700; *3*, NZ9800; *4*, NZ9800 containing pNZ9013 (*nisZ*); *5*, NZ9800 + $Leu^{-16} \rightarrow Lys$; *6*, NZ9800 + $Phe^{-18} \rightarrow Leu$; *7*, NZ9800 + $Asp^{-7} \rightarrow Ala$; *8*, NZ9800 + $Arg^{-1} \rightarrow Gln$ and 9) NZ9800 + $Ala^{-4} \rightarrow Asp$.

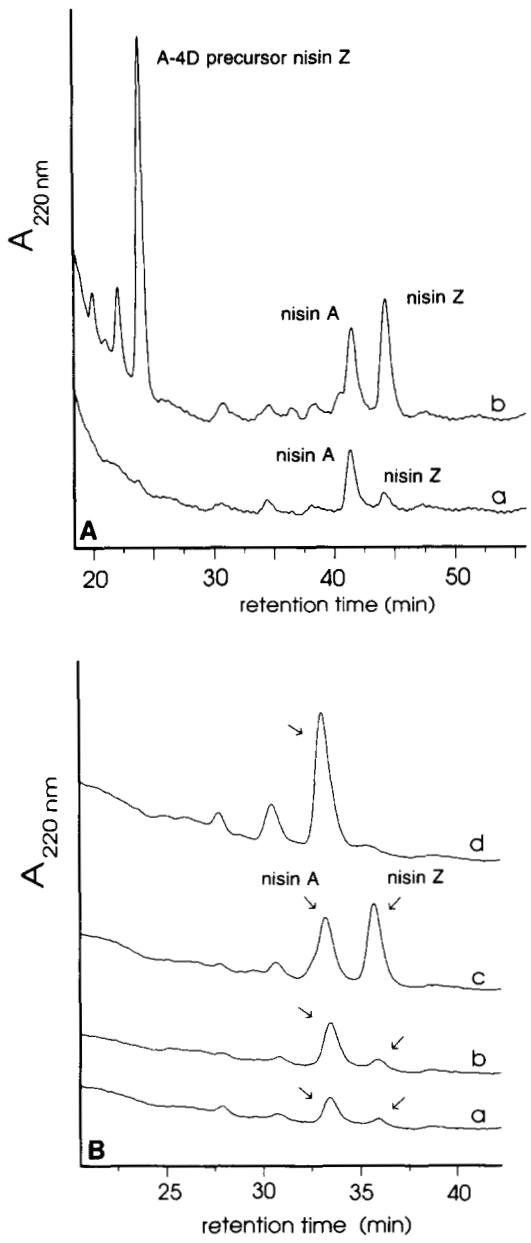

FIG. 4. **RP-HPLC analysis of culture supernatants of the different nisin Z mutant-producing strains.** *A*: *trace a*, NZ9700 harboring pNZ9013, producing wild-type nisin A and nisin Z; *trace b*, Ala^{-4} → Asp precursor nisin Z produced by NZ9700. Positions of the precursor nisin Z, nisin A, and nisin Z are indicated. *B*, profiles of nisin A and nisin Z (pNZ9013 in strain NZ9700) (*trace a*), Pro^{-2} → Val nisin Z produced by NZ9700 (*trace b*), Ser^{-10} → Ala/Ser^{-12} → Ala nisin Z produced by NZ9700 (*trace c*), and Phe^{-18} → Leu nisin Z produced by NZ9700 (*trace d*). *Arrows* point to the positions at which nisin A and Z elute. Peaks eluting just in front of the species described are degradation products of the intact nisin species that we always find after trichloroacetic acid precipitation.

assume that these mutations blocked the biosynthetic pathway of nisin Z. Most of these mutations occurred in the region −18 to −15, which is strongly conserved among the different leader peptides (Fig. 1*B*), demonstrating the importance of this region for a proper completion of the biosynthetic process. The sensitivity of residues in this region even for relatively conservative changes was demonstrated by the Phe^{-18} → Leu mutation, which did not result in any detectable nisin Z production (Fig. 4*B*).

Purification and Characterization of Ala^{-4} → Asp Nisin Precursor Peptide—The 6-kDa peptide secreted by the strain with the Ala^{-4} → Asp mutation was purified from the supernatant of 10-liter culture by hydrophobic interaction chromatography and RP-HPLC. The different peaks in the chromatogram of the Ala^{-4} → Asp supernatant (Fig. 4*A*) were collected, lyophilized, and analyzed by SDS-PAGE (not shown). This confirmed that the major peak with a retention time of 23 min on analytical RP-HPLC (Fig. 4*A*) and 20 min on preparative RP-HPLC (Fig. 6) was a 6-kDa peptide species. About 10 mg of lyophilized peptide was obtained and further analyzed by N-terminal sequencing, ^1H NMR, and by an *in vitro* enzymatic cleavage assay to determine the possible biological activity of this peptide. N-terminal sequencing gave the following sequence for the purified peptide: STKDFNLDLVSVSKKDSGDSPRI. This is identical to the primary sequence of the leader peptide deduced from the mutated *nisZ* gene, except for the absence of the N-terminal methionine, which is usually cleaved off in bacterial cells. This suggests that this peptide is a precursor form of nisin in which the leader sequence is still attached to the nisin part (in some stage of modification). The apparent molecular mass of 6 kDa is in good agreement with such a precursor nisin peptide of 56 amino acids. The N-terminal sequencing stopped after Ile^1, indicating that Thr^2 is converted to Dhb, which blocks further Edman degradation. The N-terminal analysis further showed that Ser and Thr residues in the leader peptide itself are not modified, as was found likewise for the leader peptides of Pep5 (Weil *et al.*, 1990), of nisin A (van der Meer *et al.*, 1993), and of subtilin, which had been fused to the pronisin part (Kuipers *et al.*, 1993b).

Comparison of the one-dimensional ^1H NMR spectrum of the Ala^{-4} → Asp precursor peptide of nisin Z with that of wild-type nisin Z (Kuipers *et al.*, 1992) showed that the typical vinyl-proton resonances of the dehydrated residues of the mature nisin Z peptide (*i.e.* Dhb^2, Dha^5, and Dha^{33}) were also present in the Ala^{-4} → Asp precursor peptide (Fig. 5). This suggested that the nisin moiety of this peptide was at least partially modified in a proper way. Further preliminary results of two-dimensional ^1H NMR experiments indicated that it is likely that the nisin part is completely matured with respect to dehydration and thioether bridge formation. A complete validation of the structure of the Ala^{-4} → Asp precursor peptide by two-dimensional ^1H-NMR is in preparation. The Ala^{-4} → Asp precursor peptide, which itself showed no biological activity, could be activated to its bioactive form *in vitro* by incubation with trypsin. This should result in cleavage of the precursor at the same position after Arg^{-1} as occurs during normal maturation *in vivo* by the protease NisP (van der Meer *et al.*, 1993). No cleavage by trypsin occurs in the nisin mature part under these conditions (van der Meer *et al.*, 1993). Tryptic digestion resulted in the release of a peptide from the Ala^{-4} → Asp precursor with the same retention time on RP-HPLC as nisin Z (Fig. 6). When tested in a bioassay with *Micrococcus flavus*, this peptide had an antimicrobial activity comparable with nisin A and nisin Z, *i.e.* at least 200 times higher than that of the uncleaved Ala^{-4} → Asp precursor nisin Z. These results indicated that the 6-kDa peptide secreted by the Ala^{-4} → Asp mutant strain was very likely a precursor peptide of nisin Z, which had the leader peptide sequence still attached to the completely matured nisin Z moiety. Although no attempts were made in this study to isolate and purify the 6-kDa peptide from the strain producing the Arg^{-1} → Gln mutant, it seems likely that this peptide is also an uncleaved precursor form of nisin Z similar to that of the Ala^{-4} → Asp mutant. Interestingly, the mutation at the −1 position in the nisin leader peptide results in a Gln residue as also found at that position in the subtilin leader (Fig. 1*B*). The finding that the Arg^{-1} → Gln precursor peptide is not efficiently cleaved by the nisin leader protease shows that a clear difference in specificity exists between the

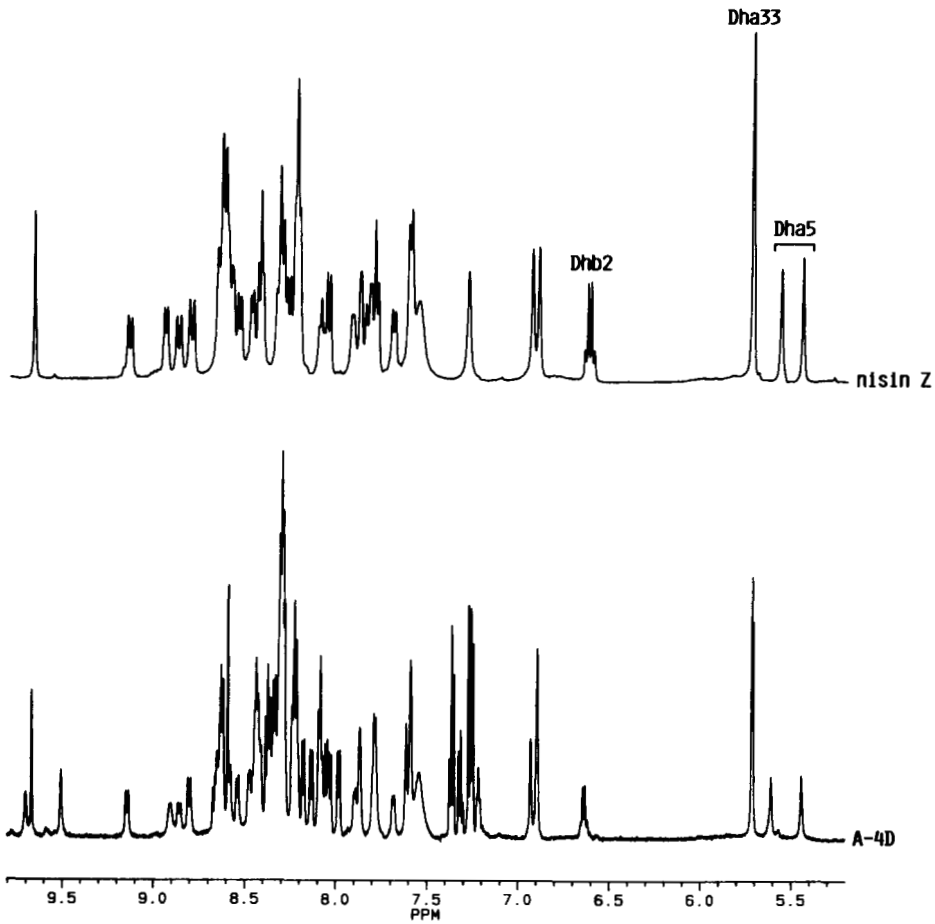

FIG. 5. **Low field region of the 400-MHz ^{1}H NMR spectra of wild-type nisin Z (*top*) and Ala^{-4} → Asp precursor nisin Z (*bottom*).** Resonances of the relevant dehydrated residues are indicated in the nisin Z spectrum. Experimental conditions: 10% D_2O, 90% H_2O, pH 3.5, 25 °C.

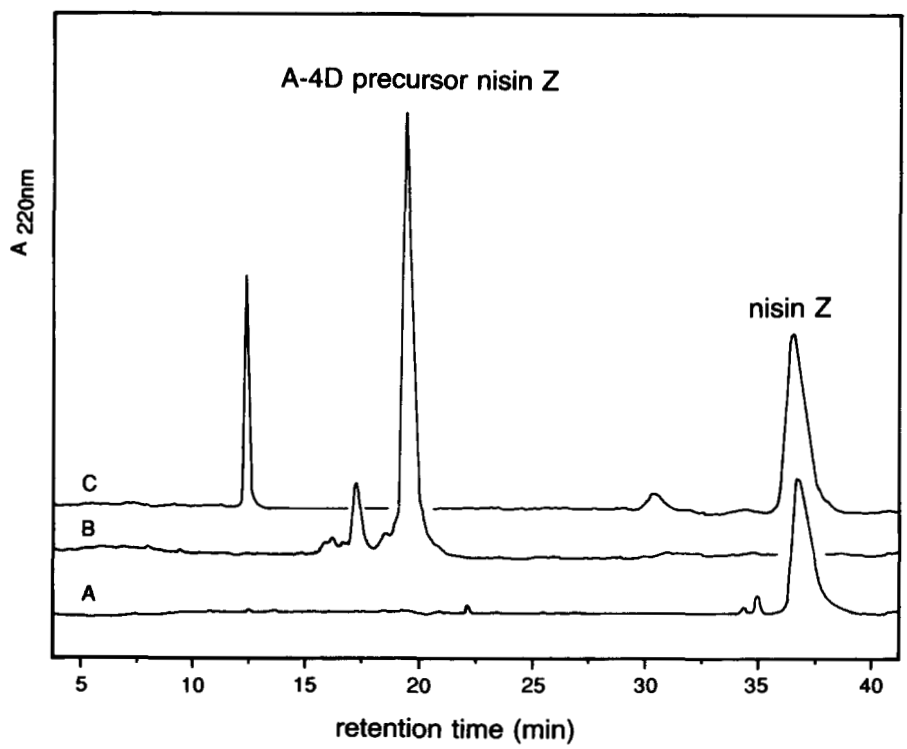

FIG. 6. **Preparative RP-HPLC elution profiles of the purified Ala^{-4} → Asp nisin Z precursor peptide, before (*trace B*) and after (*trace C*) incubation with trypsin *in vitro*.** The elution pattern of wild-type nisin Z is shown by *trace A*. The positions of the precursor peptide, the resulting nisin Z peptide, and wild-type nisin Z are indicated. The fraction eluting at 12.5 min probably represents a tryptic fragment of the Ala^{-4} → Asp leader peptide and was not further analyzed.

leader proteases of the nisin and subtilin biosynthetic pathways. This is in accordance with the finding that a gene fusion between DNA encoding the leader of subtilin and the pronisin part also results in the production of a 6-kDa peptide in *L. lactis* NZ9700 (Kuipers *et al.*, 1993b). Our results demonstrate that residues in this region of the leader peptide close to the cleavage site are important for recognition by the leader protease (NisP) which removes the leader peptide from the mature nisin

(see also below).

Production of Mature Nisin Is Essential for Nisin Biosynthesis—All the experiments to screen the different leader mutations described so far were carried out in *L. lactis* strain NZ9700, which is a nisin A-producing strain that will simultaneously produce nisin Z when the *nisZ* gene is introduced on a plasmid. The production of nisin A by this strain, however, makes analysis of the effect of mutations in the *nisZ* gene more laborious and may perhaps lead in some cases to competition between precursor peptides of nisin A and nisin Z for available sites on the modifying enzymes. Therefore, we analyzed a number of the mutated *nisZ* genes in *L. lactis* strain NZ9800, a derivative of NZ9700 which carries a 4-base pair deletion in the chromosomal *nisA* gene, but which is otherwise not affected in any of the other known *nis* biosynthetic genes (Kuipers *et al.*, 1993a). When plasmids containing the wild-type *nisZ* gene (such as pNZ9013) were introduced into this strain, production of nisin Z was established without any nisin A present in the extracellular medium (Kuipers *et al.*, 1993a). However, when plasmids carrying *nisZ* mutations were transformed into strain NZ9800, we found remarkable differences in production and secretion of nisin Z or precursor nisin Z when compared with strain NZ9700. Only those mutants of *nisZ* that gave rise to production and secretion of mature nisin Z in strain NZ9700 also did so in strain NZ9800 (*e.g.* $Asp^{-7} \rightarrow Ala$, Fig. 3*C*, *lane 7*), whereas those that blocked nisin Z production in strain NZ9700 (*e.g.* $Phe^{-18} \rightarrow Leu$) likewise did so in strain NZ9800 (Fig. 3*C*, *lanes 5* and *6*). In contrast, the two mutants of *nisZ* which led to the production and secretion of precursor nisin Z (*e.g.* $Arg^{-1} \rightarrow Gln$ and $Ala^{-4} \rightarrow Asp$) in strain NZ9700 did not do so in strain NZ9800 (Fig. 3*C*, *lanes 8* and *9*). In these cases no intracellular accumulation of precursor peptides could be detected by Western blotting using anti-leader-peptide antibodies. To see whether the reason for the lack of production of some of the mutants was a blockade at the level of transcription of the mutant genes, RNA was isolated and Northern blotting was performed, followed by hybridization with a radiolabeled *nisA* gene fragment. The results revealed that all mutant genes were transcribed, since in every case a typical transcript of about 340 nucleotides was visible. Taken together, these data suggest that a defined structural characteristic of the precursor peptide is required for completion of the biosynthetic process, possibly by activating transcription of biosynthetic genes downstream of *nisA*. In strain NZ9700 mature nisin A and/or its wild-type leader peptide could fulfill this function of autoregulating its own biosynthesis and (mutant) nisin Z biosynthesis. Whether this possible autoregulatory effect takes place on the level of transcription or at any later stage is not known yet, but we found recently that in strain NZ9800 transcription of the *nis* operon is blocked in the absence of a functional *nisA* gene *in trans* (Kuipers *et al.*, 1993a).

Cleavage of the Leader Peptide Sequence from the Nisin Precursor Peptide Is Not Necessary for Transport across the Cell Envelope—The different enzymatic steps that lead to full maturation of nisin and other lantibiotics are not known in detail, although the final structural characteristics of the molecule (*i.e.* dehydrated residues, lanthionine rings) and the partial description of intermediates in the biosynthesis of Pep5 (Weil *et al.*, 1990) have provided support for the different steps in the biosynthetic process that have been postulated (Schnell *et al.*, 1988). The present study shows that mutation of 2 residues in the leader peptide of nisin Z, $Arg^{-1} \rightarrow Gln$ and $Ala^{-4} \rightarrow Asp$, leads to secretion of a precursor peptide that most likely contains a fully matured nisin structural part into the extracellular medium. This indicates that cleavage of the leader sequence from the precursor is the last step in nisin biosynthesis and that cleavage itself is not a prerequisite for transport of this peptide across the cytoplasmic membrane.

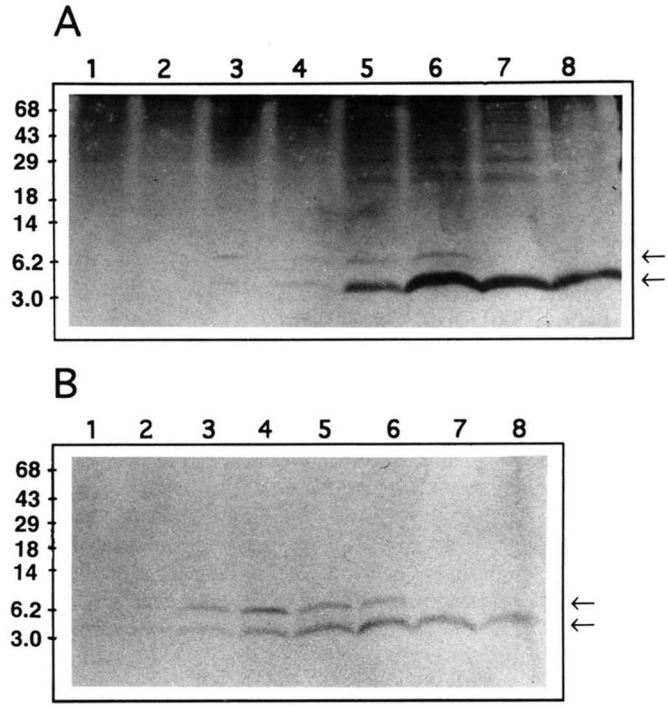

FIG. 7. *A*, Tricine-SDS-PAGE analysis of trichloroacetic acid-precipitated culture supernatant of *L. lactis* NIZO R5 (nisin A wild-type strain) during batch growth on SPYS medium. *B*, Western blot of SDS-polyacrylamide gel of trichloroacetic acid-precipitated culture supernatants of *L. lactis* NIZO R5, incubated with anti-nisin A antibodies, and stained using peroxidase-labeled mouse anti-rabbit antibodies. The analysis was performed with culture supernatants of cells harvested at A_{620} of 0.05 (*lane 1*), 0.11 (*lane 2*), 0.16 (*lane 3*), 0.21 (*lane 4*), 0.31 (*lane 5*), 0.49 (*lane 6*), 0.71 (*lane 7*), and 1.55 (10 times diluted; *lane 8*). *Arrows* point to the positions of nisin A (3.5 kDa) and precursor nisin A (6.0 kDa).

We have obtained further evidence that cleavage of the leader peptide sequence is actually taking place on the outside of the cell envelope. During exponential growth of strain NIZO R5, which produces wild-type nisin A, we detected increasing amounts of nisin A in the supernatant, but during a short period of growth, a 6-kDa peptide was also detectable (Fig. 7*A*). This 6-kDa peptide reacted both with antibodies raised against nisin A (Fig. 7*B*) and with antibodies against a chemically synthesized leader peptide of nisin A (results not shown). Furthermore, when analyzed by RP-HPLC this 6-kDa peptide eluted with a retention time similar to the $Ala^{-4} \rightarrow Asp$ precursor peptide, suggesting that it represents precursor nisin A that has escaped proteolytic cleavage and activation. This wild-type nisin A precursor peptide could be isolated in large amounts from the supernatant of *L. lactis* cells which contained the cloned *nis* biosynthetic gene cluster *nisABTCIR*, but which did not carry a functional *nisP* gene for the leader protease (van der Meer *et al.*, 1993). The nisin A precursor peptide was also found to be biologically inactive, similar to the $Ala^{-4} \rightarrow Asp$ precursor peptide of nisin Z, and could be activated by *in vitro* enzymatic cleavage of the leader peptide by either trypsin or NisP (van der Meer *et al.*, 1993). *In vivo*, the cleavage is supposed to be carried out by the gene product of *nisP*, which is located distal from *nisI* on Tn*5276* (van der Meer *et al.*, 1993). The amino acid sequence of the encoded protease suggested it to be a secreted subtilisin-like serine protease that contains a C-terminal membrane anchor (van der Meer *et al.*, 1993). Modeling studies of the active site and the substrate binding region of the NisP protease, with a precursor nisin as substrate, which were based on homologies with subtilisin and thermitase com-

plexed to inhibitors, suggested that the two mutations chosen at position −1 and −4 (*i.e.* $Arg^{-1} \rightarrow Gln$ and $Ala^{-4} \rightarrow Asp$) indeed reduced the possibility of the substrate to fit and interact in the substrate binding cleft (Siezen, 1993). Interestingly, these modeling studies also predicted that substitutions of the conserved Pro^{-2} residue to a Gly or Val would not affect cleavage of the leader sequence by the protease.

What Is the Role of the Leader Peptide Sequence in the Biosynthetic Process?—Our results show that 2 residues in the region close to the cleavage site between the leader peptide and mature nisin are essential for recognition by the leader protease, *i.e.* Ala^{-4} and Arg^{-1}, whereas the conserved Pro^{-2} can be exchanged by a Gly or Val without apparent effects on processing. A further critical region, which is strongly conserved among leader peptides from different lantibiotics (Fig. 1*B*), is located at positions −18 to −15. Substitution of residues at positions −18, −16, and −15 in this region leads to a blockade of nisin Z production, even when the changes introduced are relatively conservative (*e.g.* Phe to Leu), by an unknown mechanism. It could be that region −18 to −15 is important for recognition by modifying enzymes, such as the possible candidates NisB and NisC (Engelke *et al.*, 1992; Kuipers *et al.*, 1993a) and/or by the transport system. Although we did not detect substantial amounts of precursor nisin Z species intracellularly in mutants which were impaired in nisin Z biosynthesis, we cannot rule out the possibility that residues in the −18 to −15 region of the nisin leader are involved in transport. Except for one other mutation generated in the leader peptide ($Ser^{-6} \rightarrow$ Leu), the rest of the leader mutations did not have a detectable effect on nisin Z biosynthesis. This means that the nature of the residues at these positions (−13 to −9, −7, and −2) is not critical for the biosynthetic process. It is obvious that the pro-region of nisin also contains elements (*e.g.* Ser, Thr, and Cys residues) which must be recognized by the modifying enzymes and, after modification, by the transport system. Indications for such elements were obtained when site-specific mutants were constructed in the section of the *nisZ* gene encoding the mature part of nisin Z (Kuipers *et al.*, 1992). Some of these mutants (*e.g.* $Ser^{5} \rightarrow$ Thr) also failed to be produced in strain NZ9800.

It remains to be solved how the primary translation product of the *nisA* or *nisZ* gene is directed toward the putative modifying enzymes, what the exact sequence of events is in the modification process of the nisin structural region, and what the trigger is for translocation of the matured precursor peptide. The mutation strategy employed here appears to be useful for the elucidation of the molecular interactions during these processes.

Acknowledgments—We are grateful to Dr. A. G. Beck-Sickinger and Prof. Dr. G. Jung for providing the synthetic leader peptide of nisin. We thank Joey Marugg and Jeroen Hugenholtz for critically reading the manuscript; Paula Both, Charles Slangen, and Patrick van den Bogaard for technical assistance; and Henk van Brakel, Joop Mondria, and Simon van der Laan for preparation of the figures.

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