Dissection and Modulation of the Four Distinct Activities of Nisin by Mutagenesis of Rings A and B and by C-Terminal Truncation

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Nisin A is a pentacyclic antibiotic peptide produced by various Lactococcus lactis strains. Nisin displays four different activities: (i) it autoinduces its own synthesis; (ii) it inhibits the growth of target bacteria by membrane pore formation; (iii) it inhibits bacterial growth by interfering with cell wall synthesis; and, in addition, (iv) it inhibits the outgrowth of spores. Here we investigate the structural requirements and relevance of the N-terminal thioether rings of nisin by randomization of the ring A and B positions. The data demonstrate that: (i) mutation of ring A results in variants with enhanced activity and a modulated spectrum of target cells; (ii) for the cell growth-inhibiting activity of nisin, ring A is rather promiscuous with respect to its amino acid composition, whereas the bulky amino acid residues in ring B abolish antimicrobial activity; (iii) C-terminally truncated nisin A mutants lacking rings D and E retain significant antimicrobial activity but are unable to permeabilize the target membrane; (iv) the dehydroalanine in ring A is not essential for the inhibition of the outgrowth of Bacillus cells; (v) some ring A mutants have significant antimicrobial activities but have decreased autoinducing activities; (vi) the opening of ring B eliminates antimicrobial activity while retaining autoinducing activity; and (vii) some ring A mutants escape the nisin immune system(s) and are toxic to the nisin-producing strain NZ9700. These data demonstrate that the various activities of nisin can be engineered independently and provide a basis for the design and synthesis of tailor-made analogs with desired activities.

Lantibiotics are (methyl)lanthionine-containing antibiotics (1, 9) produced by some gram-positive bacteria. The lantionines are posttranslationally formed by enzyme-mediated dehydration of serine and threonine residues followed by enzyme-catalyzed intramolecular coupling to cysteines to form a thioether bridge. The lantibiotic nisin contains one lanthionine and four methyllanthionines (Fig. 1). Nisin autoregulates its own synthesis (19) by binding to the transmembrane protein NisK, which phosphorylates the intracellular response regulator NisR. Phosphorylated NisR activates the nis promoter.

Nisin predominantly acts against gram-positive bacteria. This occurs at nanomolar concentrations as a consequence of the interaction of nisin with the docking molecule lipid II (4, 6). Rings A and B physically interact with lipid II, and this results in membrane permeabilization by hybrid pores of nisin and lipid II (5) and inhibition of cell wall synthesis via lipid II abduction (11). Nisin-producing bacteria are protected against nisin by two self-protection mechanisms: a lipoprotein, NisI, which likely binds and inactivates nisin, and an export system, NisEFG, which presumably extrudes nisin from the cell (20). Another antibiotic activity of nisin is the inhibition of the outgrowth of spores. Nisin’s dehydroalanine in position 5 has been reported to be involved in this inhibitory activity (29). Replacement of the dehydroalanine with an alanine at position 5 of nisin (7) and subtilin (27) strongly reduced the capacity to prevent the outgrowth of spores.

An alignment of lantibiotic sequences revealed a large group of nisin-responding lantibiotics (32) that share the N-terminal rings A and B, ring A being composed of a lanthionine and three other amino acids and ring B being composed of a (methyl)lanthionine and two other amino acids. While the amino acid composition of ring A appears variable, ring B is rather conserved. The opening of ring A strongly reduces antimicrobial activity against Micrococcus luteus NCDO 8166 and leads to a complete loss of antimicrobial activity against Lactococcus lactis MG1614 (8). Only a few conservative ring A mutants (S3T, which has reduced activity [21]; S5A [7]; SST [22]; and SSC [39]) and chemically modified variants (33) have been described, whereas no reports on ring B mutants have appeared. Here, we randomized the amino acids of these functionally important rings to investigate the possibility of engineering nisin mutants with improved or altered characteristics. Surprisingly, a large number of mutants with modulated activities were obtained, providing detailed information about the structural requirements of the different activities of nisin.

MATERIALS AND METHODS

Bacterial strains and plasmids. Strains and plasmids are listed in Table 1.

Molecular cloning. Constructs coding for mutant prenisin were made by amplifying plasmid pNZinsA-E3 (18) using a phosphorylated downstream sense primer and an upstream antisense primer with a (nonannealing) peptide-encoding tail. The DNA amplification was carried out by using Phusion DNA polymerase (Finnzymes, Finland). The replacement of ISL of ring A of nisin involved primer 5′-NNNNNNNACTTGTAATGCGTGGTGATGC-3′ and 5′-PO4-T GTACACCCGGTTGTAAAAG-3′, in which N is a randomized nucleotide. The replacement of PG of ring B with XX in nisin involved primers 5′-NNNNNNNT

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The outgrowth of spores. The outgrowth of spores was followed by measuring the OD_{600}. For nisin purified from the supernatant of L. lactis NZ9700, the IC_{50} values were found to be identical to the values obtained from nisin A isolated after production by using L. lactis containing plasmid-encoded enzymes and plasmid-encoded prepropeptide.

**Purification of nisin and nisin mutants.** Wild-type nisin A, the ring A KSI, KFI, and VFG mutants, and ring B PT and PH mutants were purified as follows. Cultures were started from an overnight preculture in GM17 medium with antibiotics by diluting them 100-fold into 250 ml minimal medium (32) buffered with 0.12 M MOPS (pH 7.0) buffer, without antibiotics. The supernatant was diluted with an equal volume of 100 mM lactic acid (pH 4), and subsequently, purification proceeded with a single passage of prenisin over a 5-ml fast protein liquid chromatography HiTrap spheropropion column (Amerham). Elution was performed at pH 4.0 with 1 M NaCl in 50 mM lactic acid. The fraction containing prenisin was desalted on a PD10 column. The collected desalted fractions were thereafter lyophilized. The leader peptide was completely cleaved off by incubating prenisin with NisP-expressing cells for 16 h. Alternatively, 30 min of incubation at 37°C with 0.01 mg/ml trypsin, which readily cleaves after an arginine, was applied. Mass spectrometric measurement of the high-performance liquid chromatography (HPLC)-purified nisin mutants proved that trypsin had not cleaved after lysines. The nisin and nisin mutants were purified on a C_{18} column with reversed-phase HPLC with a gradient of 10 to 50% acetonitrile in 0.1% trifluoroacetic acid. The purified peptide was quantified on the basis of a comparison of the 214-nm HPLC peak area with that of a nisin A standard of known concentration. Analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that the peptides were >95% pure.

**Induction capacity.** The induction capacities of the nisin mutants were determined qualitatively by mass spectrometric measurement of the production of the ITRICK leader peptide by the induced L. lactis NZ9000 ΔacmA containing pTP-ITRICK and pIL3BTC. Quantitative measurement of the induction capacities of the nisin mutants was performed by inducing cells expressing the nisin ring A VFG mutant in the absence of inducing amounts of nisin A. The nisin VFG mutant-expressing cells were incubated for 16 h in GM17 medium containing the appropriate antibiotics with different concentrations of the purified nisin mutant. The trypsin-released activity was determined by measuring growth inhibition, as described above in “Culturing,” with the indicator strain L. lactis LL108 (pOrl 280). The lowest concentration needed to get full expression of the VFG mutant was taken as the minimal induction concentration. This bactericidal...
phenylalanine-containing mutants and the high antimicrobial activity found for the KSI mutant, an additional, KFI, ring A mutant was made. A particular further reason for constructing and also purifying this KFI mutant was its mimicking of ring A of epidermin. The VFG mutant combined potent antimicrobial activity with an absence of induction capacity in liquid media. By contrast, the ring B PH mutant was purified because of its induction capacity combined with an absence of antimicrobial activity. The PT mutant was the ring B mutant with the most potent antimicrobial activity. These purified mutants were accurately characterized with respect to their antimicrobial activities against different gram-positive bacteria, induction capacities, inhibition of the outgrowth of spores, and capacity to permeabilize the membrane.

A strikingly high number of phenylalanine-containing ring A mutants was obtained (see Table S1 in the supplemental material). Only four of the mutants did not contain a phenylalanine at position 4, 5, or 6 (KSI, TKI, VGG, and YQI). Two mutants harbored a charged residue, i.e., a lysine (KSI and TKI). Most of the mutant residues were hydrophobic. The numbers of variants with three (FFY), two (FVW, FSY, FVS, FSF, FAF, FFL, FFY, FFV, and SFF), and one (SFV, VFG, IFS, LFQ, LFA, LSF, and YQI) aromatic residue(s) were 1, 10 and 7, respectively. Interestingly, epidermin and gallidermin contain a phenylalanine at position 5, which in nisin corresponds to a serine that is dehydrated during the posttranslational modification. This serine residue was retained in only 10 transformants, while one mutant (TKI) contained a threonine. The extent of dehydration of all mutants was determined by mass spectrometry (see Table S1 in the supplemental material). Among the mutants that did not lack any dehydrated residue were not only very active ones but also mutants with strongly reduced activity. These data indicate the importance of the amino acid composition for the antimicrobial activity.

Despite the high level of conservation of ring B, several active mutants were obtained (see Table S2 in the supplemental material). Mass spectrometric analysis demonstrated that a significant fraction of the SS, PS, TS, and PT mutants contained an extra dehydrated residue compared to those in wild-type nisin. The PH, PR, PD, PN, PL, and PP mutants lacked a dehydrated residue and showed partial Cys addition, indicating that one cysteine had not undergone cyclization via coupling to a dehydroresidue.

**Autoinduction and induction.** In an overlay assay with sensitive *L. lactis* cells and trypsin, a variety of halo sizes was observed on the plates when nisin was omitted (see Fig. 2, left plate). This implies that even without the inducer nisin, many of the nisin variants were produced and autoinduced. The presence of inducing amounts of nisin in the plates resulted in an increase in halo size (Fig. 2, right plate). When the assay was performed in the absence of trypsin but using NisP-expressing cells for the overlay, the same halo sizes were observed as in the presence of trypsin. Combining trypsin with NisP-expressing cells led neither to larger nor to smaller halo sizes, which suggests that NisP-mediated leader cleavage was complete and demonstrates that the trypsin-mediated removal of leader peptide did not result in inactivation by cleavage in nisin itself. In the absence of trypsin or NisP, no halos were observed except around the wild-type nisin A-producing strain.
NZ9700, indicating that endogenous leader cleavage is limited. These data indicate that many mutants are equipped with an autoinducing capacity and that all tested mutants could be processed by NisP irrespective of the amino acid composition of ring A and ring B.

In a liquid medium, most leader peptide-containing mutants were unable to self induce. Only prenisin A itself and the ring B PT and PS mutants were able to self induce. Interestingly, after leader cleavage, the ring B PH mutant, which is devoid of antimicrobial activity, also displayed induction capacity (see Table 3). No self-induction was observed when the ring B PH and PR mutants were produced as prenisin. The PR mutant was treated with trypsin and analyzed by mass spectrometry, showing that this nisin mutant is cleaved after the R site (Fig. 3). The N-terminal fragment lacked one dehydrated residue, indicating that Thr at position 8 was not dehydrated. Hence, the observed masses of 1,038, 2,453, and 3,471 Da correspond to IDhbLanIDhaLLanTPR (observed, M/z 1,038 Da), fragment C11-K34, observed, 2,453 Da; theoretical value, 2,454 Da for four-times-dehydrated peptide; whole G10R nisin, observed, 3,471 Da; theoretical value of seven-times-dehydrated peptide, 3,472 Da.

FIG. 3. Trypsin digestion of nisin G10R mutant analyzed by mass spectrometry. The observed average masses correspond to that of IDhbLanIDhaLLanTPR (observed, M + H+, 1,038 Da; theoretical value of three-times-dehydrated peptide, 1,037 Da); fragment C11-K34, observed, 2,453 Da; theoretical value, 2,454 Da for four-times-dehydrated peptide; whole G10R nisin, observed, 3,471 Da; theoretical value of seven-times-dehydrated peptide, 3,472 Da.

Growth inhibiting activity. The levels of activity of nisin mutants against L. lactis and other bacteria were compared
TABLE 2. Susceptibility of cells of different strains to purified nisin and derived ring A and B mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>IC50 (nM) of:</th>
<th>Nisin A</th>
<th>PT mutant</th>
<th>KSI mutant</th>
<th>KFI mutant</th>
<th>VFG mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. lactis NZ9700</td>
<td></td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>18</td>
<td>10</td>
<td>2.7</td>
</tr>
<tr>
<td>L. lactis LL108(pOri 280)</td>
<td>0.4</td>
<td>0.75</td>
<td>0.25</td>
<td>0.4</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>L. lactis LL108(pOri 280)</td>
<td>3.6</td>
<td>5x</td>
<td>67</td>
<td>6.1</td>
<td>125</td>
<td></td>
</tr>
<tr>
<td>S. epidermidis</td>
<td></td>
<td>2.1</td>
<td>5.2</td>
<td>5.6</td>
<td>3.1</td>
<td>11</td>
</tr>
<tr>
<td>S. thermophila</td>
<td></td>
<td>1.2</td>
<td>2.4</td>
<td>0.9</td>
<td>1.5</td>
<td>2.5</td>
</tr>
<tr>
<td>P. pentosaceus</td>
<td></td>
<td>0.5</td>
<td>1.5</td>
<td>1.3</td>
<td>0.4</td>
<td>2.4</td>
</tr>
<tr>
<td>L. johnsonii</td>
<td></td>
<td>0.2</td>
<td>0.30</td>
<td>0.1</td>
<td>0.02</td>
<td>0.2</td>
</tr>
<tr>
<td>L. mesenteroides</td>
<td></td>
<td>0.09</td>
<td>0.19</td>
<td>0.07</td>
<td>0.01</td>
<td>0.12</td>
</tr>
<tr>
<td>M. luteus</td>
<td></td>
<td>0.06</td>
<td>0.20</td>
<td>0.08</td>
<td>0.03</td>
<td>0.39</td>
</tr>
</tbody>
</table>

a Data are the means of the results of three independent experiments. Values lower than those for nisin A are in bold.

b Data are the IC50 values of the truncated (Δ23–34) versions of the peptides against L. lactis LL108(pOri 280).

Inhibition of the outgrowth of spores. The capacities of the purified nisin A and nisin PT, KSI, KFI, and VFG mutants to inhibit the outgrowth of Bacillus subtilis strain 168 spores were determined. In parallel, the growth inhibition of B. subtilis 168 cells was also measured. The respective IC50 values are shown in Fig. 4. While nisin A inhibited the growth of B. subtilis 168 cells with an IC50 of 7.9 ± 0.6 nM (mean ± standard deviation), much lower concentrations sufficed to inhibit the outgrowth of spores, i.e., an IC50 of 0.3 ± 0.2 nM. The tested mutants showed a similar pattern (Fig. 4). Since the KFI and VFG mutants efficiently inhibit the outgrowth of spores, we conclude that Dha5 is not required for this activity. The KSI and KFI mutants even showed an enhanced capacity to inhibit the outgrowth of spores compared to that of nisin A (Fig. 4).

Truncated nisin mutants. To investigate whether the pore formation and lipid II abdution mechanisms can be separated, C-terminally truncated variants of nisin A and its PT, PH, KSI, KFI, and VFG mutants were constructed. Herein, rings D and E were removed, resulting in nisin A(Δ23–34) mutants. The peptides were purified and subjected to mass spectrometry to determine whether rings A, B, and C were correctly formed. In mutant PH(Δ23–34), two thioether bridges were lacking and a disulfide was present instead (see Table S3 in the supplemental material). For the other truncated mutants, the measured masses agreed with the expected masses for the correct formation of all three thioether rings. All cysteine residues were part of lanthionines, as demonstrated by the lack of CDAP addition (see Table S4 in the supplemental material). To investigate whether the bridging patterns of the truncated mutants were...
TABLE 3. Induction capacities of full-length and truncated (Δ23–34) nisin A and of ring A and B mutants derived therefrom, all without leader peptide

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Minimal induction conc (pM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full-length peptides</td>
<td></td>
</tr>
<tr>
<td>Nisin A</td>
<td>0.3</td>
</tr>
<tr>
<td>PT</td>
<td>0.3</td>
</tr>
<tr>
<td>PH</td>
<td>230</td>
</tr>
<tr>
<td>KSI</td>
<td>30</td>
</tr>
<tr>
<td>KFI</td>
<td>9</td>
</tr>
<tr>
<td>VFG</td>
<td>120</td>
</tr>
<tr>
<td>Δ23–34 truncated peptides</td>
<td></td>
</tr>
<tr>
<td>Nisin A</td>
<td>40</td>
</tr>
<tr>
<td>PT</td>
<td>40</td>
</tr>
<tr>
<td>PH</td>
<td>&gt;4,000</td>
</tr>
<tr>
<td>KSI</td>
<td>&gt;4,000</td>
</tr>
<tr>
<td>KFI</td>
<td>450</td>
</tr>
<tr>
<td>VFG</td>
<td>&gt;4,000</td>
</tr>
</tbody>
</table>

* L. lactis NZ9000 ΔacmA(pIL3BTC pTP-nisVFG) was incubated with different concentrations of inducing nisin mutants. The activity of the VFG nisin produced was determined by measuring growth inhibition with the indicator strain L. lactis 108(pOri 280). The minimal induction capacity was determined as described in Materials and Methods. The experiment was repeated three times; the values shown are from a typical experiment.

identical to those of full-length mutant peptides, postsource decay was applied and the fragments were analyzed (see Table S5 in the supplemental material). Since only a few fragments were observed, conclusions about the bridging patterns could be drawn. The observation of fragments y10 and y11, which correspond to ring C (Fig. 1) indicates that this ring is correctly formed. The y14 ion is also found for all mutants, indicating that Cys11 of ring B is not connected to the alternative dehydroamino acid Dha5. Thus, rings B and C are correctly formed. With the exception of the PH mutant, fragments that confirm normal bridging patterns were obtained, indicating that ring A is also properly formed (see Table S5 in the supplemental material).

The truncated nisin A and derived mutants showed strongly decreased induction capacities, but truncated nisin A and mutant PT remained more-effective inducers than the other mutants (Table 3). The truncated mutants had retained some antimicrobial activity against L. lactis LL108(pOri 280) (Table 2), but this varied among the mutants. Truncated nisin A (IC$_{50}$, 3.6 nM) and the truncated KFI mutant (IC$_{50}$, 6.1 nM) showed significant levels of activity: their IC$_{50}$ values were only 9- and 15-fold higher than that of full-length nisin. Truncation led to larger increases for the PT (77-fold increase in IC$_{50}$). VFG (96-fold increase in IC$_{50}$), and KSI (268-fold increase in IC$_{50}$) mutants (Table 2). This relatively larger decrease in activity for the latter mutants might indicate that pore formation by the corresponding full-length mutants might contribute more to the antimicrobial activity than the inhibition of cell wall synthesis. Next, we determined whether the truncated mutants were still capable of permeabilizing the membrane. Therefore, L. lactis LL108(pOri 280) cells were suspended in a sodium buffer and a Δψ was induced by adding the potassium ion ionophore valinomycin. The resulting Δψ caused by the electrogenic efflux of potassium ions was monitored by the decrease in the fluorescence of the Δψ-sensitive probe DiSC$_3$(5). The addition of full-length nisin A (Fig. 5, dotted line) or the KSI, PT, KFI, or VFG mutant (not shown) caused a rapid dissipation of the Δψ, as shown by the recovery of the DiSC$_3$(5) fluorescence. In contrast, neither truncated nisin A (Fig. 5, solid line) nor any of the other truncated mutants affected the Δψ. Hence, the removal of the C-terminal region containing rings D and E resulted in a complete loss of the membrane permeabilization activity. This implies that the antimicrobial activity observed for the truncated peptides likely results from the remaining capacity to inhibit cell wall synthesis. To validate this suggestion, the ability to inhibit the outgrowth of B. subtilis spores was also monitored. This activity was diminished to an extent similar to that of the ability to inhibit the growth of B. subtilis cells (not shown), although the outgrowth of spores was inhibited at lower concentrations than those resulting in the inhibition of cells, as discussed before for inhibition by full-length peptides. These data indicate that the inhibition of cell wall synthesis contributes significantly to the antimicrobial effect of nisin A and the KFI mutant but less to the antimicrobial effects of the KSI, VFG, and PT mutants.

DISCUSSION

Following binding to the peptidoglycan precursor lipid II, nisin exhibits two activities that ultimately result in cell death: (i) nisin permeabilizes the membrane by forming transmembrane hybrid pores composed of lipid II and nisin (12, 38) and (ii) it inhibits cell wall synthesis (4, 5, 6) by displacing lipid II.
from the septa (11). The binding of nisin to lipid II involves a pyrophosphate cage, formed by rings A and B of nisin (15). Differences at positions 4, 5, and 6 in natural homologs of nisin reside at the circumference of the pyrophosphate cage. This suggested that mutations would be allowed in positions 4 to 6. Nisin also autoinduces its own synthesis by a mechanism involving the interaction with NisK, inhibits the outgrowth of spores, and is recognized by the self-protection mechanisms that provide immunity to the producing cells. The latter involves NisI and the ABC transporter NisEFG.

Here we demonstrate that by mutating ring A or B at one or more positions (i.e., positions 4, 5, and 6, as well as 9 and 10) one or more of nisin's functional characteristics can be modulated. We obtained a series of mutants with entirely new combinations or selections of the activities known for nisin. Mutants with enhanced or reduced bactericidal activity, with strongly reduced antimicrobial activity but significant maintenance of induction capacity, without significant autoinduction capacity but with antimicrobial activity, and with the ability to circumvent the self-protection mechanism of the producing strain were obtained. In addition, mutants that lacked Dha5 but that retained or even showed an enhancement in the capacity to inhibit the outgrowth of spores were obtained. These data clearly indicate that the various activities of nisin can be modulated by altering the composition of ring A or B. The nisin fragment comprising residues 1 to 12 [nisin(1–12)] containing rings A and B does not have antimicrobial activity and has only low induction capacity (10), but as far as antimicrobial activity is concerned, it retains nisin-antagonizing activity (8). Membrane binding by this fragment is strongly reduced (28), possibly because the peptide bears only one positive charge, a lysine. Our data on the ring A VFG mutant indicate that the composition of ring A affects its (auto)inducing capacity. With leader peptide, the VFG mutant had no inducing capacity, and without leader peptide, only poor inducing capacity. However, the VFG mutant retained strong antimicrobial activity. Apparently there is no or no strong correlation between lipid II binding and induction capacity. Changes throughout the nisin molecule affect the signaling capacity, the A ring being essential (10). The C-terminal truncation of residues 23 to 34 described in this report reduced the induction capacity. Charge alterations in the C-terminal part of nisin also resulted in a reduction of induction capacity (40). These data indicate that several regions of the nisin molecule contribute to its induction capacity.

A nisin variant with an open ring C has been reported to have lost essentially all antimicrobial activity (8). Here we report on variants with mutations of rings A, B, and C that are still equipped with antimicrobial activity. Therefore, it seems that ring C is needed for activity, possibly by binding other (truncated) nisin peptides and inducing the segregation of lipid II (11). Nisin A(Δ23–34) and mutants derived therefrom are unable to permeabilize the target membrane but retain antimicrobial activity. This activity of truncated nisin A is about 10-fold higher than that reported for nisin(1–20) (8), which could be the result of the additional positive charge provided by Lys22. The remaining activity of our truncated variants likely results from the inhibition of cell wall synthesis following binding to lipid II. This opens the possibility of selectively optimizing truncated nisin mutants with respect to cell wall synthesis inhibition. The relevance of the mechanism of inhibition of cell wall synthesis has been reinforced by the activity of several highly bactericidal lantibiotics that do not form pores, such as short lantibiotics (2) and a hinge-region mutant of nisin (N20P M21P) (38, 41). Bonelli et al. (2) demonstrated that, in model membranes, lipid II-mediated pore formation by gallidermin, which shares the ring pattern of rings A and B of nisin but consists of only 22 amino acids, depends on the membrane thickness. With intact cells, pore formation was less pronounced than that observed with nisin and occurred only in some strains. Moreover, gallidermin is approximately 10-fold more effective in cell killing than nisin, which might be related to a more-efficient inhibition of cell wall biosynthesis. Ring C, the hinge region, and the C terminus of nisin have been subjected to a limited mutational analysis (8, 22, 39). Interestingly, the N20K and M21K mutants displayed antimicrobial activities against gram-negative Shigella, Pseudomonas, and Salmonella species (43). Further mutational analysis of these regions may result in additional mutants with interesting characteristics.

Nisin has been reported to inhibit the outgrowth of spores via a mechanism that involves Dha5, which is thought to react with protein thiol groups in the spore wall. Dha5 is indeed a reactive residue and likely the least-stable residue of ring A that is of functional importance. Here we obtained nisin mutants without a Dha residue in ring A that efficiently inhibit the outgrowth of spores. In addition, C-terminal truncation of nisin A, while the Dha5 was retained, resulted in a major loss in the spore outgrowth inhibitory activity. Therefore, our data demonstrate that this residue is not essential for spore outgrowth inhibition. This opens the possibility of generating new nisin variants, devoid of any unstable dehydroresidue, which still effectively inhibit the outgrowth of spores.

Previously, we have shown that the lantibiotic enzymes have a broad substrate specificity (16, 18, 32). Now we demonstrate that the activities of nisin can be modulated differentially by mutagenesis of rings A and B. Already, when selecting only against L. lactis, a large number of interesting ring A and B mutants were obtained. These rings show a large mutational freedom and can be used as targets for improving lantibiotics. Therefore, it will be of interest to apply the same method for randomization of rings A and B and select for activity against pathogenic strains. In combination with the possibility of engineering new lanthionine rings, tremendously large possibilities exist for the design and synthesis of new lantibiotics and bioactive peptides with desired properties.

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