

# Specific Binding of Nisin to the Peptidoglycan Precursor Lipid II Combines Pore Formation and Inhibition of Cell Wall Biosynthesis for Potent Antibiotic Activity\*

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Unlike numerous pore-forming amphiphilic peptide antibiotics, the lantibiotic nisin is active in nanomolar concentrations, which results from its ability to use the lipid-bound cell wall precursor lipid II as a docking molecule for subsequent pore formation. Here we use genetically engineered nisin variants to identify the structural requirements for the interaction of the peptide with lipid II. Mutations affecting the conformation of the N-terminal part of nisin comprising rings A through C, e.g. [S3T]nisin, led to reduced binding and increased the peptide concentration necessary for pore formation. The binding constant for the S3T mutant was  $0.043 \times 10^7 \text{ M}^{-1}$  compared with  $2 \times 10^7 \text{ M}^{-1}$  for the wild-type peptide, and the minimum concentration for pore formation increased from the 1 nm to the 50 nm range. In contrast, peptides mutated in the flexible hinge region, e.g. [ $\Delta$ N20/ $\Delta$ M21]nisin, were completely inactive in the pore formation assay, but were reduced to some extent in their *in vivo* activity. We found the remaining *in vivo* activity to result from the unaltered capacity of the mutated peptide to bind to lipid II and thus to inhibit its incorporation into the peptidoglycan network. Therefore, through interaction with the membrane-bound cell wall precursor lipid II, nisin inhibits peptidoglycan synthesis and forms highly specific pores. The combination of two killing mechanisms in one molecule potentiates antibiotic activity and results in nanomolar MIC values, a strategy that may well be worth considering for the construction of novel antibiotics.

The antimicrobial peptide nisin is produced by numerous strains of *Lactococcus lactis* and inhibits a broad range of

Gram-positive bacteria (1, 2). It belongs to the lantibiotics, a group of antimicrobial peptides that is characterized by the presence of intramolecular rings formed by the thioether amino acids lanthionine and 3-methylanthionine (3, 4). Nisin has had a long history as a potent and safe food preservative, although recent insight into the molecular mechanism of its bactericidal activity also make it interesting for biomedical applications (5, 6). Generally, the nisin-type subgroup of lantibiotics comprises elongated cationic peptides that have the capacity to adopt amphiphilic structures. Such peptides are assumed to kill microbes by disturbing the integrity of the energy-transducing membrane. Indeed, early experiments demonstrated that nisin or related lantibiotics induced rapid efflux of ions or cytoplasmic solutes such as amino acids and nucleotides. The concomitant depolarization of the cytoplasmic membrane resulted in an instant termination of all biosynthetic processes (7, 8). Structural analysis in the presence of micelles indicated that the hydrophilic groups of the peptide interact with the phospholipid headgroups, and the hydrophobic side chains are immersed in the hydrophobic core of the membrane (9, 10). The wedge model as proposed by Driessen *et al.* (11) takes into account such structural data and proposes that the peptides insert into the membrane without losing contact with the membrane surface, resulting in the formation of a short-lived pore.

Whereas the wedge model may illustrate results obtained with model membranes, a number of effects observed with intact living cells remain unexplained; in particular, the fact that nisin acts on model membranes at micromolar concentrations whereas *in vivo* minimal inhibitory concentration (MIC)<sup>1</sup> values are in the nanomolar range. The discrepancies were explained by the finding that nisin and epidermin use lipid II, the bactoprenol-bound precursor of the bacterial cell wall as a docking molecule for subsequent pore formation (12). The specificity of the nisin-lipid II interaction and the resulting high level activity of nisin was demonstrated in a comparative study with the pore-forming amphiphilic defense peptide magainin 2 (5). We found that, in contrast to magainin, the activity of nisin is enhanced by a factor of  $10^3$  when lipid II is available for targeted pore formation. The fact that nisin specifically binds to lipid II was particularly remarkable, because previously mersacidin and actagardine from the type B class of lantibiotics also had been shown to form a complex with the cell wall

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<sup>1</sup> The abbreviations used are: MIC, minimal inhibitory concentration; DOPC, 1,2 dioleoyl-*sn*-glycero-3-phosphocholine; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol; CF, carboxyfluorescein; OPA, *o*-phthalaldehyde; HPLC, high pressure liquid chromatography; Mes, 4-morpholineethanesulfonic acid.

precursor. In this case however, binding only blocked the precursor from incorporation into polymeric peptidoglycan (13, 14). We therefore assumed that lantibiotics may have defined lipid II binding motifs that could represent interesting lead compounds for the development of novel antibiotics. In particular, these lantibiotics are active against the Gram-positive bacteria that are most prominent with regard to resistance development, e.g. *Staphylococci* and *Enterococci*.

Here we studied the interaction between lipid II and a number of nisin mutant peptides to identify the structural elements of the nisin molecule that are important for the interaction with lipid II and subsequent pore formation. Surprisingly, we identified nisin mutant peptides that were unable to form pores but retained considerable antimicrobial activity through blocking lipid II from incorporation into peptidoglycan. Thus nisin is a dual-function antibiotic with two killing mechanisms combined into one molecule.

#### EXPERIMENTAL PROCEDURES

**Chemicals and Materials**—All chemicals were of analytical grade or better. The phospholipids 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG) were purchased from Avanti Polar Lipids, Inc. and stored at  $-20^{\circ}\text{C}$  in a 9:1 chloroform/methanol solution. Carboxyfluorescein (CF) was purchased from Kodak and purified as described previously (15). Protein concentration was determined using the bicinchoninic acid protein assay reagent (Pierce Chemical Corp.) with bovine serum albumin as a standard.

**Nisin Z and Nisin Mutants**—Nisin Z (16) and prepeptide nisin Z with the N-terminal leader sequence of subtilin (17) as well as all mutant peptides (18) were produced in SPYS medium (3% sucrose, 1% peptone (Difco), 1% yeast extract (Difco), 0.2% NaCl, 0.002%  $\text{MgSO}_4$ , and 1%  $\text{KH}_2\text{PO}_4$ , pH 6.8). Peptides were purified from supernatants by hydrophobic-interaction chromatography using Fractogel TSK butyl 650-M (Tosohaas), followed by preparative reversed phase-HPLC using acetonitrile and 0.1% trifluoroacetic acid as eluents. Mutations were verified by mass spectroscopy. Nisin Z is a natural variant of nisin A that only differs in one single residue (Asn instead of His at position 27) and possesses almost identical antimicrobial properties (19, 20). The [ $\Delta\text{N}20/\Delta\text{M}21$ ]nisin A, [S3T]nisin Z, [M17K]nisin Z, and [N20P/M21P]nisin A (21) and the charged mutants [V32E]nisin Z and [V32K]nisin Z were obtained by site-directed mutagenesis. Mutations of the structural genes *nisA* or *nisZ* were performed by two successive polymerase chain reactions, and mutated genes were expressed in *L. lactis* NZ9800 as described previously (18). Nisin stock solutions were stored at  $-80^{\circ}\text{C}$  in 0.05% acetic acid. The MIC was determined by the inhibition of growth of the indicator strains *Micrococcus flavus* DSM 1790 and *Streptococcus thermophilus* Rs in a liquid bioassay (16, 18).

**Lipid II Synthesis**—Lipid II was synthesized *in vitro* using membrane preparations of *Micrococcus luteus* ATCC 4698. Membranes were isolated from lysozyme-treated cells by centrifugation ( $40,000 \times g$ ), washed twice in 50 mM Tris-HCl, 10 mM  $\text{MgCl}_2$ , pH 7.5 and stored under liquid nitrogen. For extraction and purification, the protocol elaborated by Brötz *et al.* (14) for [ $^{14}\text{C}$ ]lipid II was applied. The unlabeled lipid II generated for the present study was detected by exploiting its ability to form a complex with mersacidin. Aliquots of the respective chromatographic fractions were added to a mersacidin activity test (12), and those fractions that were able to antagonize mersacidin activity were collected. The lipid II concentration in stock solutions was determined by quantitative analysis of amino acids and amino sugars using the *o*-phthalaldehyde (OPA) derivatization technique (22). Lipid II samples were hydrolyzed in sealed tubes with 6 N HCl at  $110^{\circ}\text{C}$  for 18 h. After evaporation of HCl, amino acids and sugars were taken up in 200 mM borate buffer, pH 9.5 and derivatized with OPA. The derivatives were separated by reversed-phase  $\text{C}_{18}$  HPLC chromatography using 50 mM phosphate buffer, pH 6.5 (buffer A) and methanol/tetrahydrofuran (97:3) as eluent B. Fluorescence of the derivatives was recorded at 450 nm (excitation at 340 nm) and quantitated using appropriate standards. Purified lipid II was stored in chloroform/methanol (1:1, v/v) at  $-20^{\circ}\text{C}$ .

**Preparation of Vesicles**—Large unilamellar vesicles for the binding, CF efflux, and potassium efflux experiments were prepared by the extrusion technique (23) and treated as described previously (24). Vesicles were made of DOPC with or without 0.065 mol % lipid II (referring to the total amount of lipids). Phospholipid was determined as inorganic phosphate after treatment with perchloric acid (25).

CF-loaded vesicles were prepared with 50 mM CF and then diluted in 1.25 ml of  $\text{K}^+$  buffer (50 mM Mes-KOH, pH 6.0, 100 mM  $\text{K}_2\text{SO}_4$ ) in a final concentration of 25  $\mu\text{M}$  phospholipid (on a phosphorous base). After addition of wild-type or mutant nisin, the induced leakage was monitored for 3 min. The increase of fluorescence intensity was measured at 515 nm (excitation at 492 nm) on a SPF 500C spectrophotometer (SLM instruments Inc.) at  $20^{\circ}\text{C}$ . The nisin-induced CF leakage was expressed relative to the total amount of CF released after lysis of the vesicles by addition of 10  $\mu\text{l}$  of 20% Triton X-100.

Potassium-loaded vesicles were prepared with KCl buffer (150 mM KCl, 30 mM Mes, 20 mM Tris, pH 6.5). The vesicles were added to 5 ml of buffer (30 mM Mes, 20 mM Tris, pH 6.5, 150 mM choline chloride) in a final concentration of 25  $\mu\text{M}$  phospholipid. Nisin or nisin mutant-induced leakage was monitored with a  $\text{K}^+$  selective electrode (15-K, Philips) and a reference electrode (R44/2, Philips). The total amount of  $\text{K}^+$  in the vesicles was determined after addition of 20  $\mu\text{l}$  of a 20% solution of *N,N*-dimethyldodecylamine *N*-oxide (Fluka). Leakage was expressed relative to the total amount of  $\text{K}^+$ . The electrodes were calibrated by titrating known amounts of potassium.

**Radioactive Labeling**—Radioactive labeling of nisin and binding experiments were performed as described previously by Breukink (24). Nisin Z, [S3T]nisin Z and [ $\Delta\text{N}20/\Delta\text{M}21$ ]nisin A were  $^{14}\text{C}$ -labeled by reductive methylation of the  $\epsilon$ -amino group of the lysine residues according to the method of Dottavio-Martin and Ravel (26). The resulting preparations had specific activities of  $4.6 \times 10^4$ ,  $8.2 \times 10^4$ , and  $13.3 \times 10^4$  dpm/nmol, respectively. The radiolabeled peptides were stored in 0.05% (v/v) acetic acid at  $-80^{\circ}\text{C}$ . The binding of the [ $^{14}\text{C}$ ]nisin Z and variants was determined under equilibrium conditions. Vesicles (160  $\mu\text{M}$ , on a phosphorus base) made of DOPC with 4% DOPG or with 0.5 mol % lipid II were incubated at room temperature with  $^{14}\text{C}$ -labeled peptide in 0.5 ml of buffer (25 mM Mes, pH 6.0, 50 mM  $\text{K}_2\text{SO}_4$ ) for 5 min. The mixture was then centrifuged at  $430,000 \times g$  for 45 min to spin down the vesicles with the bound nisin Z. The amount of unbound nisin in the supernatant and of vesicle-bound nisin in the pellet was determined by scintillation counting. No phosphate could be detected in the supernatant after the centrifugation steps, and 90–100% of the applied radioactivity was recovered. The binding isotherms of the nisin-lipid II interaction could be well described by the Langmuir adsorption model assuming that lipid II was the only binding site for nisin in the context of DOPC vesicles.

**In Vitro Peptidoglycan Synthesis**—A crude membrane fraction was prepared from *Escherichia coli* TB1 by disruption of exponentially growing cells with a Branson Sonifier 250 (Danbury CT) twice for 3–5 min, 50% cooling phase and power level 5. Membranes were collected by centrifugation ( $40,000 \times g$ ), then washed and resuspended in buffer (50 mM Tris HCl, pH 7.5), to give a protein concentration of 8 mg/ml. UDP-*N*-acetylmuramylpentapeptide (UDP-MurNAc-pentapeptide) was isolated from *Staphylococcus simulans* 22. To accumulate the murein precursor, exponentially growing cells were supplemented with 5  $\mu\text{g}/\text{ml}$  vancomycin and incubated for 30 min. After centrifugation, cells were resuspended in distilled water (0.1 g/ml) and extracted by stirring the suspension slowly into 2.5 volumes of boiling water. After 15 min, the suspension was cooled, the cell debris removed ( $48,000 \times g$ ) and the supernatant lyophilized. The lyophilized material was then dissolved in water and brought to pH 2 with 20%  $\text{H}_3\text{PO}_4$ . After acidification, precipitated material was removed by centrifugation, and the supernatant fractionated on reversed-phase  $\text{C}_{18}$  HPLC under isocratic conditions (50 mM  $\text{NaH}_2\text{PO}_4$ , pH 5.2). The UDP-MurNAc-pentapeptide was identified by mass spectrometry. For cell wall biosynthesis assays (35), it was applied in 0.2 mM concentrations together with membranes (160  $\mu\text{g}$  of protein), 0.05 mM [ $^{14}\text{C}$ ]UDP-GlcNAc and 15–60  $\mu\text{M}$  nisin peptides in a total volume of 60  $\mu\text{l}$  of membrane suspension buffer (50 mM Tris-HCl, pH 8, 10 mM  $\text{MgCl}_2$ , 1 mM mercaptoethanol). Incubation proceeded for 45 min at  $32^{\circ}\text{C}$  before the reaction was stopped by heating ( $100^{\circ}\text{C}$ , 30 s). Aliquots were analyzed by paper chromatography (paper GB 002, Schleicher und Schüll, Dassel, Germany) in isobutyric acid/1 M  $\text{NH}_4\text{OH}$  (5:3, v/v). Polymeric peptidoglycan does not migrate from the starting point, and the respective area was cut out and counted in a  $\beta$ -scintillation counter (1900 CA Tri-Carb scintillation counter, Packard, Zürich).

#### RESULTS

**Activity of Wild-type Nisin Z on Lipid II-containing Unilamellar Vesicles**—Previous studies had shown that nisin in low concentrations can induce rapid efflux of CF from multilamellar liposomes when supplemented with 0.1 mol % lipid II. In the absence of lipid II, such liposomes were not affected by nisin concentrations as high as 1.25  $\mu\text{M}$  (12). To characterize in

detail the lipid II effect, we used unilamellar vesicles doped with 0.065 mol % of the cell wall precursor. At 5 nM nisin, significant dye release was observed, and ~60% release was achieved with 50 nM nisin (Fig. 1A). Because dye efflux reached a plateau within less than a minute, we plotted the percentage of dye released after 1 min for a closer inspection of the concentration dependence of the nisin pore formation. Fig. 1B demonstrates that upon the incorporation of lipid II, the sensitivity of DOPC (1,2 dioleoyl-*sn*-glycero-3-phosphocholine) liposomes increased by three orders of magnitude. CF release started at about 1 nM of the lantibiotic, whereas more than 1  $\mu$ M nisin was necessary to affect DOPC liposomes without lipid II. In accordance with previous studies in the absence of lipid II (24), membranes containing the negatively charged lipids DOPG were considerably more sensitive (Fig. 1B) than the DOPC liposomes. However, the difference in sensitivity disappeared after incorporation of 0.065 mol % lipid II (Fig. 1C). Although the overall activity of nisin toward lipid II-containing liposomes was relatively independent from the phospholipid composition, slightly higher release rates were obtained with DOPC as the bulk phase constituent. Under the test conditions applied, the maximum marker release was achieved with nisin concentrations in the range of 0.05 to 0.1  $\mu$ M (Fig. 1C). Application of higher concentrations reduced the dye release efficiency with the DOPC liposomes and with the DOPC/DOPG mixture. With DOPG vesicles, the dye-release rate increased again at 1  $\mu$ M, which agrees well with the results depicted in Fig. 1B, because it coincides with the onset of the marker release activity in the absence of lipid II.

Similar to the dependence of pore formation on peptide concentration, dye release was also dependent on the lipid II concentration in liposomes (Fig. 1D). In a lipid II titration series in which the nisin concentration was kept constant at 10 nM or 100 nM, efflux started at 0.001 mol % lipid II, *i.e.* for an average liposome of 400 nm in diameter and  $7 \times 10^5$  bulk lipid molecules (of 0.70 nm<sup>2</sup>), only seven lipid II molecules per vesicle were sufficient to promote dye release. With increasing peptide concentrations, the release activity reached an optimum, in this case at ~0.1 mol %, and decreased with greater lipid II concentrations. The presence of an optimum in the curves obtained in both titration series suggests that there is an optimal lipid II to peptide ratio, which may be in the range of 1:1, *e.g.* at 0.1 mol % lipid II (optimum in Fig. 1D) the ratio is 1.0:0.4 (lipid II to nisin) for 10 nM nisin, or 1:4 for 100 nM nisin, respectively.

We also replaced the negatively charged CF with K<sup>+</sup> to detect indications for ion selectivity of the pore. Interestingly, the efflux rates did not significantly differ with both markers (*e.g.* Fig. 2; 90% K<sup>+</sup> release after 1 min with 100 nM nisin). The absence of ion selectivity of nisin pores formed in lipid II-doped membranes stands in contrast to data obtained without lipid II (24) and suggests that lipid II not only enhances the pore formation activity of nisin but also has an influence on the pore architecture.

**Binding Characteristics of Nisin Z to Lipid II-containing Liposomes**—Membrane poration by cationic amphiphilic peptides is considered a sequential process that includes binding to the target membrane, insertion into the membrane, and the formation of a functional pore. Detailed studies with various model peptides (27) and with nisin Z (24) demonstrate that lipids with a net negative charge, such as DOPG, are important for binding via electrostatic interactions (24). Nisin, for example, was bound stoichiometrically to one DOPG molecule per positively charged amino acid residue. In contrast, very little nisin was bound by pure DOPC liposomes, and the above data (Fig. 1C) illustrate that DOPG is not essential when lipid II

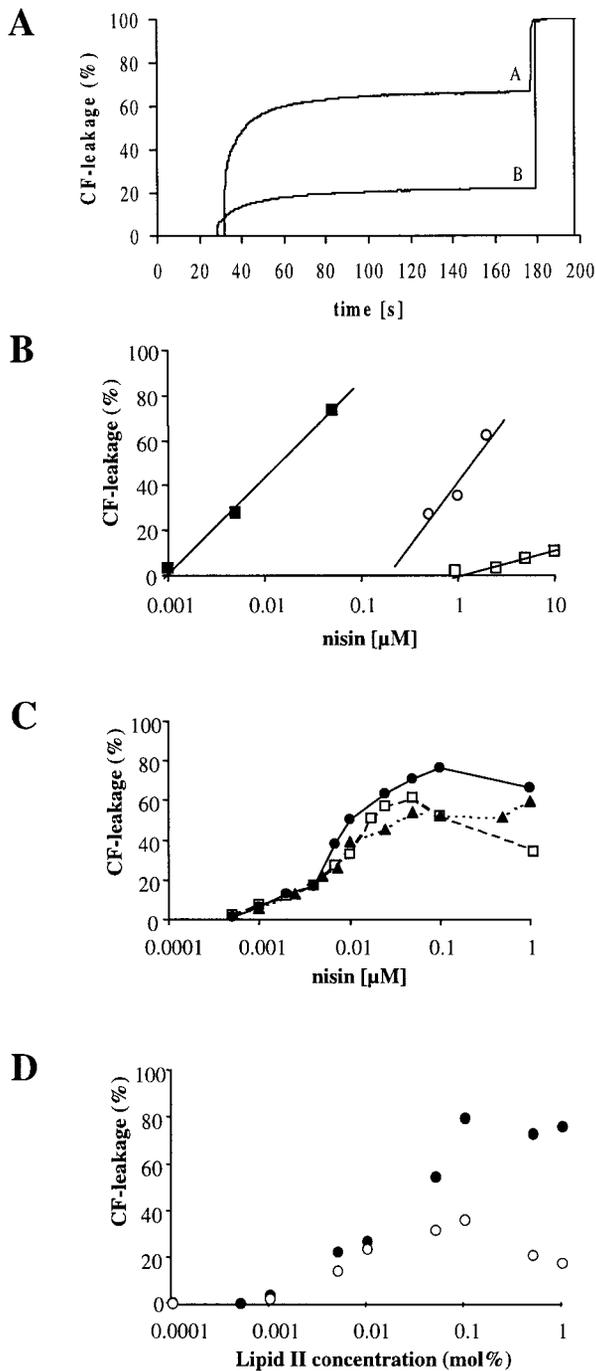
serves as a specific target. We therefore studied the binding behavior of radiolabeled nisin to DOPC liposomes supplemented with lipid II. With lipid II present in 0.5 mol %, nisin showed a binding behavior that was best described by the Langmuir adsorption model with an apparent minimal binding constant of  $2 \times 10^7$  M<sup>-1</sup> (Fig. 3).

The data shown in Fig. 3 indicate that nisin binding saturates when each lipid II molecule is loaded with one nisin Z molecule, indicative of a 1:1 binding stoichiometry, which agrees well with the optimum curves obtained in the peptide and lipid II titration experiments (Fig. 1D). A 1:1 stoichiometry requires that all lipid II is available for binding; however, the procedure for preparation of lipid II-containing liposomes is very likely to result in a symmetric distribution of lipid II headgroups with regard to the bilayer; *i.e.* only 50% of the incorporated lipid II molecules should be outwardly orientated. However, recent experiments with C-terminal His-tagged nisin Z convincingly demonstrate that the lantibiotic is able to translocate across a negatively charged lipid-containing membrane (28). In light of these results it seems likely that in the presence of lipid II, nisin can translocate across the membrane and thus the inwardly orientated lipid II becomes available for interaction with nisin.

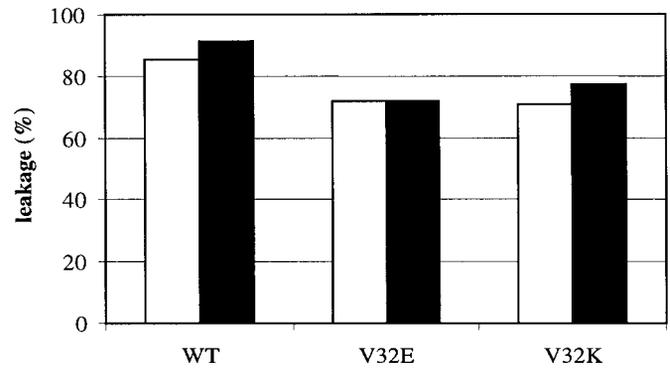
**Structural Requirements for the Lipid II-mediated Pore Formation**—We used the DOPC/0.065 mol % lipid II vesicle system to identify the structural elements in the nisin molecule that are important for the interaction with lipid II and/or subsequent pore formation. Structure-function studies with nisin and other lantibiotics had identified various structural features important for the bactericidal activity *in vivo* or for activities in *in vitro* systems. For instance, the C-terminal part of nisin is important for binding to membranes with a negative surface charge (24) and the two C-terminal amino acids (at positions 33 and 34) are not essential for the activity *in vivo* (29). In contrast, a short flexible region in the central part of the molecules was found to be indispensable not only in nisin and other type A lantibiotics (21) but also of unmodified peptide antibiotics (30). Moreover, strong hints for an important role of the N-terminal double ring system for binding to lipid II came from structural comparison of several type A lantibiotics in a previous study (12). Based on these considerations we selected a number of mutants in the respective regions of nisin (Fig. 4) and compared their activities in the lipid II-containing liposome system to their MIC values for *M. flavus* and *S. thermophilus* (Table I).

**Mutants with Charge Differences**—Introduction of a charged residue in position 32 only slightly reduced K<sup>+</sup> release (Fig. 2) and CF release activities (Fig. 5A) at low peptide concentrations, regardless of whether Val-32 was replaced by a Lys or a Glu residue. Similarly, only minor effects were observed *in vivo*. A strong reduction of the activity of nisin in the liposome assay by almost two orders of magnitude was observed when a Lys residue was introduced into ring C of the peptide (Fig. 5A, M17K), demonstrating that for pore formation a charged residue is not tolerable in the central segment of the molecule. However, *in vivo*, the M17K peptide lost only 50% of its activity against *M. flavus* (Table I), which may be explained by the particular role of anionic cell wall polymers in the overall activity of cationic peptides (see "Discussion").

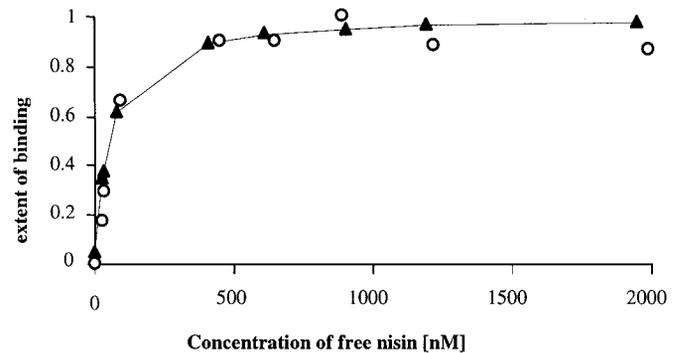
**Hinge Region Mutants**—An even more drastic effect on the activity was found for the hinge region mutants (Fig. 5B). In [ $\Delta$ N20/ $\Delta$ M21]nisin, the flexible segment that connects rings C and D is shortened to just one residue; this peptide did not release more than 20% of the dye even when tested in concentrations higher than 1  $\mu$ M. Replacement of both residues with two prolines [N20P/M21P]nisin reduced the dye release to a



**FIG. 1. Activity of nisin against lipid II-containing liposomes.** A, time course of nisin-induced leakage of CF from DOPC vesicles with 0.065 mol % lipid II. After  $\sim 30$  s,  $0.05 \mu\text{M}$  nisin Z (A) and  $0.005 \mu\text{M}$  nisin Z (B) was added, and the CF efflux was recorded. The 100% leakage level was determined by addition of Triton X-100 after 180 s. The vesicle concentration was  $25 \mu\text{M}$  on a  $P_i$  basis. B, concentration dependence of nisin Z-induced CF leakage from DOPC vesicles with 0.065 mol % lipid II (■), pure DOPG vesicles (○), and pure DOPC vesicles (□). Addition of nisin in a concentration of  $0.015 \mu\text{M}$  to vesicles with lipid II results in 50% leakage. A similar extent of leakage from pure DOPG vesicles was observed when nisin was added in a  $100\times$  higher concentration of  $1.4 \mu\text{M}$ . 50% leakage from pure DOPC vesicles was not achieved even with a  $1000\times$  higher nisin concentration ( $10 \mu\text{M}$ ). The CF leakage was determined after 60 s, and the vesicle concentration was  $25 \mu\text{M}$  on a  $P_i$  basis. C, influence of anionic phospholipids on nisin Z activity. Vesicles were made of DOPC (●), DOPC/DOPG (1:1) (□), and DOPG (▲), each supplemented with 0.065 mol % lipid II. CF leakage was determined after 60 s, and the vesicle concentration was  $25 \mu\text{M}$  on a  $P_i$  basis. D, lipid II concentration dependence of the nisin pore formation activity. DOPC liposomes were supplemented with increasing amounts of lipid II and treated with two concentrations of nisin,  $10 \text{ nM}$  nisin (○) and  $100 \text{ nM}$  nisin (●).



**FIG. 2. Nisin Z and [V32E]- and [V32K]nisin-induced CF leakage (100 nM, open bars) and  $\text{K}^+$  leakage (filled bars) from DOPC vesicles with 0.065 mol % lipid II after 1 min.**



**FIG. 3. Binding behavior of radiolabeled nisin Z to vesicles made of DOPC and complemented with 0.5 mol % lipid II.** Experimental data (○); theoretical data for Langmuir type binding (▲) with  $K = 2 \times 10^7 \text{ M}^{-1}$ . In the theoretical analysis it was assumed that nisin binds only to lipid II.

maximum of  $\sim 10\%$ . [M21G]Nisin was slightly less impaired; however, the 10% efflux obtained at  $0.01 \mu\text{M}$  could not be increased by raising the concentration of the peptide. Although the *in vivo* activities of the hinge region mutants were also reduced, the effect was comparatively less drastic for *M. flavus*. However, for *S. thermophilus*, the MIC values increased by a factor of 25, 40, and 2 for [N20P/M21P]nisin A, [ $\Delta$ N20/ $\Delta$ M21]nisin A, and [M21G]nisin Z, respectively.

**N-terminal Mutants**—Because nisin-(1–12) covering the N-terminal rings A and B had been reported to antagonize binding of wild-type nisin to target cells (31) it was especially interesting to study peptides mutated in this segment of the lantibiotic. However, nonconservative mutations in rings A and B, which could have an impact on the structure of the rings and thus be valuable tools for this study, are apparently incompatible with the biosynthesis machinery and could not be produced (21, 32). We tested three peptides in which the Dhb (*i.e.* the Thr in the prepeptide) in position 2 was replaced by Ser, Ala, and Val, respectively. These mutations had little effect on the MIC and were slightly less active than wild-type nisin Z on lipid II-containing liposomes (Fig. 5C). In contrast, a strong effect was observed with [S3T]nisin. The S3T mutation results in the formation of a 3-methylanthionine ring instead of a lanthionine ring and was found to exert relatively little influence on the activity of the mutant peptide when tested on pure PG-liposomes without lipid II (data not shown). However, with a MIC of 39 nM against *M. flavus*, this peptide was considerably less active *in vivo*.

The apparent discrepancy between the activity of [S3T]nisin *in vivo* and *in vitro* disappeared when lipid II was incorporated into the liposomes. Similar to the activity of the mutant peptide against bacterial cells, its dye releasing activity was strongly

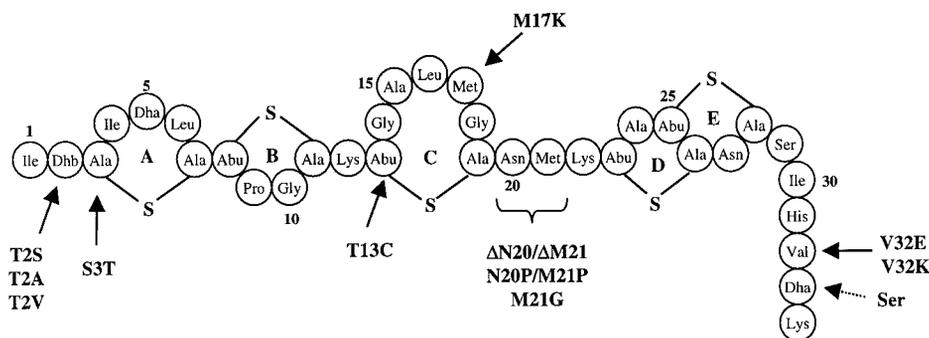


FIG. 4. **Primary structure of nisin Z.** The modifications of the nisin mutants used in this study are indicated by arrows. [V32E]nisin Z and [V32K]nisin Z contain an unmodified Ser residue on position 33 instead of *Dha* in wild-type nisin (dotted arrow).  $\Delta$ N20/ $\Delta$ M21 and N20P/M21P are modifications of nisin A. Nisin A differs from nisin Z at position 27 (His). *Dha*, dehydroalanine; *Dhb*, dehydrobutyrine; *Ala-S-Ala*, lanthionine; *Abu-S-Ala*,  $\beta$ -methylanthionine.

TABLE I  
Antimicrobial activities of nisin and nisin variants determined as MIC against *Micrococcus flavus* and *Streptococcus thermophilus*

Peptide	<i>Micrococcus flavus</i>		<i>Streptococcus thermophilus</i>		Activity on liposomes
	nM	$\mu$ g/liter	nM	$\mu$ g/liter	
Nisin Z (wild type)	3.3	11	1.8	6	+++
V32E	13.5	45	30.0	100	++
V32K	3.3	11	15.0	50	++
M17K	6.6	22		ND <sup>a</sup>	+
T13C	>60	>20	>60	>200	—
$\Delta$ N20/ $\Delta$ M21 <sup>b</sup>	26.4	88	74.6	250	—
N20P/M21P <sup>b</sup>	9.8	33	44.7	150	—
M21G	9.9	33	3.6	12	—
T2S	1.5	5	0.9	3	++
T2A	4.5	15	3.0	10	++
T2V	3.3	11	1.8	6	++
S3T	39	130	14.1	47	+
Nisin with subtilin leader	>60	(>200)		ND	—

<sup>a</sup> ND, not determined.

<sup>b</sup> Both hinge region mutants were constructed in the *nisA* genetic background.

reduced in the presence of lipid II (Fig. 5C); the concentration necessary to release 50% of CF dropped about 75-fold from 0.015  $\mu$ M (Fig. 1B) to 1.15  $\mu$ M for the mutant peptide. The concentration dependence of the pore formation by the S3T mutant was unchanged as compared with the wild-type peptide (Fig. 5C, parallel lines), indicating that the affinity of the peptide for lipid II was impaired rather than its pore formation activity. In fact, the binding constant of this mutant peptide dropped about 50-fold to  $0.043 \times 10^7$  M<sup>-1</sup>. The strong effect of the incorporation of an additional methyl group points out a crucial role of the conformation of the N-terminal ring of nisin in the specific binding of lipid II.

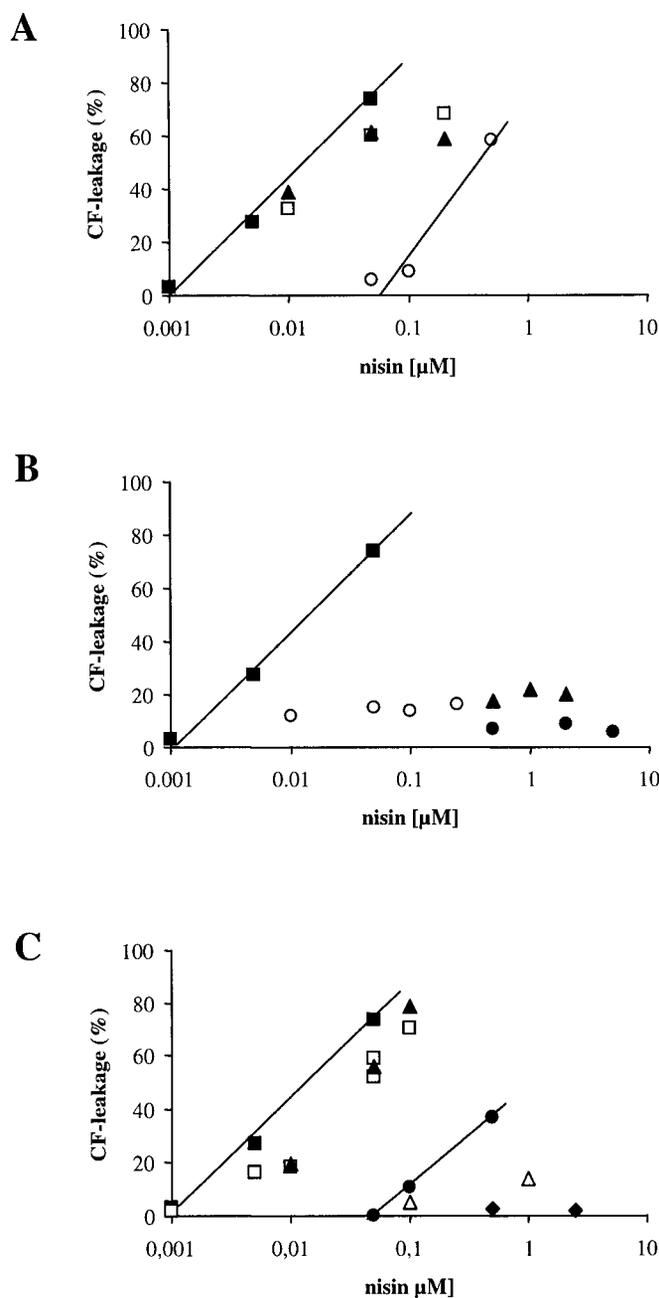
Another interesting mutant that demonstrated the importance of a correct structure of the N-terminal part of nisin is the T13C peptide. This mutation results in the formation of a disulfide bond instead of a thioether, which influences considerably the conformation of ring C (33). Accordingly, this peptide was almost inactive *in vivo* (Table I) and in the liposome assay in the presence of lipid II (Fig. 5C). N-terminal extensions of nisin had also been found to be detrimental to the activity of the lantibiotic; e.g. the modified but uncleaved nisin Z precursor peptide was completely inactive against *M. flavus* (34). A related precursor in which the leader peptide of nisin was exchanged for the subtilin leader segment (17) did not show any activity against lipid II-containing liposomes as well (Fig. 5C).

**Inhibition of *In Vitro* Peptidoglycan Biosynthesis by Nisin Peptides**—For [S3T]nisin Z, we observed comparable reductions in its affinity for lipid II, its pore formation activity on liposomes, and its *in vivo* activity. In contrast, the hinge region mutants were completely inactive in pore formation but retained comparatively high *in vivo* activity (Table I). Because

the residual activity e.g. of  $\Delta$ [N20/ $\Delta$ M21]nisin against *Micrococcus* strains or *S. thermophilus* was in the same range of concentrations as the activity of mersacidin and vancomycin (30–70 nM, Ref. 14) we speculated that the nisin hinge region mutants could exert their antibiotic activity primarily via inhibition of cell wall biosynthesis. Both mersacidin and vancomycin form a complex with lipid II and thereby block *de novo* synthesis of polymeric peptidoglycan (14). We therefore compared the inhibitory activity of nisin and of the relevant mutant peptides in an established *in vitro* cell wall biosynthesis assay (35) based on isolated *E. coli* membranes. The wild-type peptide clearly inhibited the formation of polymeric peptidoglycan in a concentration-dependent manner (Fig. 6). As expected, the  $\Delta$ N20/ $\Delta$ M21 deletion mutant was fully inhibitory and may even have a somewhat stronger effect in this assay, explaining its relatively strong *in vivo* activity. In contrast, the S3T mutant had almost no inhibitory effect, which correlates well with its reduced affinity for lipid II and with its pore formation activity as depicted in Fig. 5C.

## DISCUSSION

In this report we have analyzed the first known case of a pore formation mechanism by an amphiphilic antibiotic peptide that is based on specific interaction with a defined integral component of bacterial cytoplasmic membranes. We demonstrate that the lantibiotic nisin binds with high affinity to the membrane-bound bacterial cell wall precursor lipid II, which consists of the bactoprenol-carrier lipid and the complete monomeric disaccharide-pentapeptide peptidoglycan subunit. The high potency that derives from such a target-mediated poration may well explain why nisin is so much more effective against bacteria in which the docking molecule is available for binding as



**FIG. 5. Activity of nisin mutant peptides on the lipid II-doped liposomes.** The CF leakage from DOPC vesicles containing 0.065 mol % lipid II was determined after 60 s; the vesicle concentration was kept at 25  $\mu\text{M}$  on a  $\text{P}_i$  basis. *A*, activity of charge mutants: wild-type nisin (■), V32E (□), V32K (▲), M17K (○). *B*, activity of hinge region mutants: wild-type nisin (■),  $\Delta\text{N20}/\Delta\text{M21}$  (▲), N20P/M21P (●), M21G (○). *C*, activity of N-terminal mutants: wild-type nisin (■), T2S (□), T2A (○), T2V (▲), S3T (●), T13C (△), and nisin Z prepeptide with subtilin leader (◆).

compared with a peptide that apparently acts in a nontargeted fashion such as magainin 2 (5). The results reported here and in previous papers on the molecular mechanisms of activity of nisin in the absence of lipid II (7, 8, 18, 24, 28, 36) show that nisin can permeabilize membranes via two different mechanisms. At high concentrations ( $\mu\text{M}$  range) pores can be formed without lipid II, *i.e.* in a target-independent fashion. For optimal activity in the absence of lipid II, membranes should contain 50–60% negatively charged phospholipids, and the positively charged C terminus of nisin is important for initial binding (24) and for antimicrobial activity (18). Pores formed under such conditions are significantly anion-selective (24); the

membrane potential supports pore formation, and the wedge model (37) may possibly describe mechanistic details. In contrast, when lipid II is available, nisin can disturb the barrier function of a bilayer already at nM concentrations and a model taking into account features elaborated in this study is presented in Fig. 7.

Throughout this study we observed a striking correlation between the activity of nisin *in vivo* and its activity against lipid II-doped DOPC vesicles in contrast to pure DOPG or DOPC vesicles (Fig. 1*B*). The most important observation in this regard is that MIC values and the concentrations necessary for activity against lipid II-containing liposomes are in the same range, *i.e.* nanomolar instead of micromolar against lipid II-free liposomes. Also, high affinity binding to a docking molecule as an initial step of the activity can explain the discrepancy between the activity of some mutants on model membranes and against intact bacterial cells. In this regard, the S3T mutation as described earlier is the most clear example. For the hinge region mutants the correlation between the dye release activity (Fig. 5*B*) and the *in vivo* activity (Table I) was less obvious. However, what appears to be a discrepancy at first sight, rather highlights a unique feature of nisin that was not identified in other antibiotics so far. The N terminus seems to be involved in the binding of nisin to lipid II, whereas the hinge region between ring clusters A, B, C and D, E is most important for pore formation (as depicted in Fig. 7). Whereas peptides mutated in this segment lose the pore formation activity, their capacity for binding lipid II is less affected. Therefore, nisin retains the activity to prevent lipid II incorporation into polymeric peptidoglycan, thus acting in a way that is analogous to the antibiotics mersacidin and vancomycin (14). In contrast, the S3T mutation affects initial binding to lipid II and thus equally affects cell wall biosynthesis and the pore forming activity.

For the charged mutants, comparison of the *in vivo* activity and *in vitro* liposome results is complicated by the fact that the Gram-positive cell wall is a highly charged anionic polymer that acts as a binding matrix for polycationic peptides. Several studies (*e.g.* Ref. 38) have demonstrated the importance of the accumulation of cationic peptide antibiotics in the cell wall, which results in highly increased local concentrations of the peptides as compared with the surrounding media. Thus, although pore forming activity of the M17K peptide is affected *in vitro*, the increase in its net positive charge should lead to higher accumulation. Increased local concentration in addition to effective inhibition of peptidoglycan biosynthesis could explain the relatively small impact on the M17K mutant *in vivo* activity. In line with this interpretation is the relatively small effect on dye release of the introduction of either a positive or a negative charge in position 32, which *in vivo* resulted in wild-type activity when a positive charge is introduced (V32K) and a quite significant reduction when the net positive charge is further reduced (V32E).

The identification of a specific target enables for the first time rational design strategies, and a clear picture for structural and functional relationships in nisin is emerging. Although specific target-mediated poration may be detected more often in the future, nisin seems to be a particular case in that two different killing mechanisms are combined in one molecule. For both mechanisms, structural features of the peptide and defined segments in the molecule can now be identified. The results convincingly demonstrate that the interaction of nisin with lipid II is relevant *in vivo* at least for those bacterial strains, which are of high or intermediate sensitivity toward the lantibiotic. Low level sensitivity, which may start in the

FIG. 6. Inhibition of *in vitro* peptidoglycan synthesis by nisin Z and mutant peptides. The amount of polymeric peptidoglycan produced in the presence of the peptides is given relative to an untreated control. The experiment shown is representative of three independent experiments.

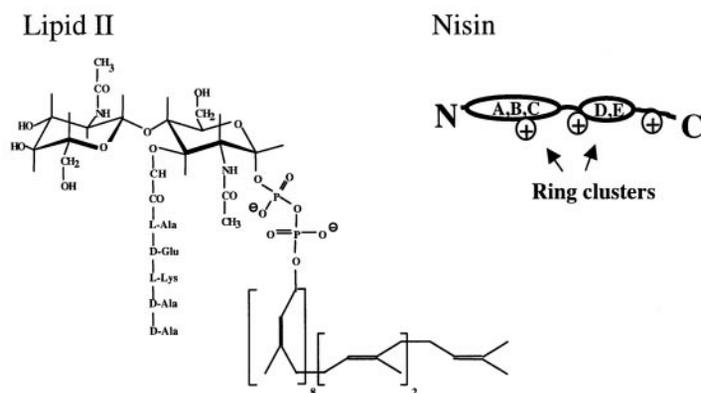
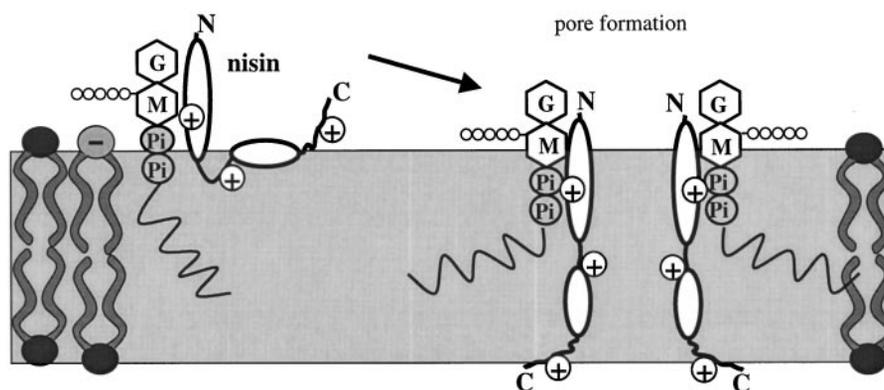
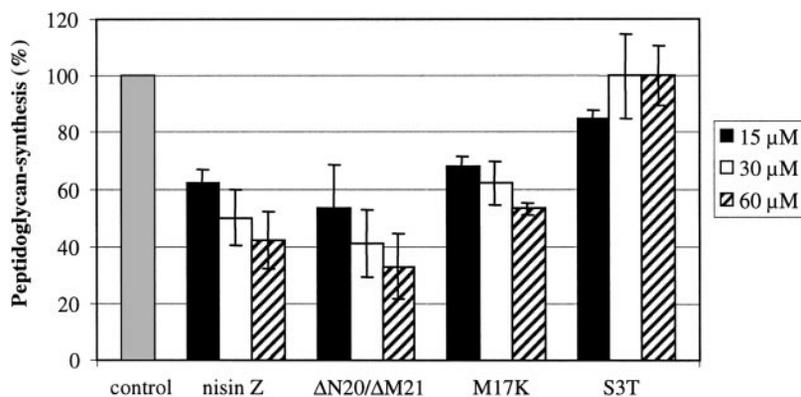


FIG. 7. Model for the lipid II-mediated nisin pore formation. Based on our results, we propose that nisin first binds to the outwardly orientated carbohydrate moiety of lipid II in a 1:1 stoichiometry. The N-terminal segment of the lantibiotic is essential for binding, and a negative surface charge is not necessary. The C-terminal part of nisin is then assumed to translocate across the membrane, in accordance with the translocation found in the absence of lipid II (28). For this step the flexible hinge region between ring clusters A, B, C and D, E is important. Recent preliminary results with lipid II-doped black lipid bilayers indicated that the pore stability increases from the millisecond range to several seconds and that the pore formation process becomes voltage-independent.<sup>2</sup> This suggests that interactions with the lipid II moiety stabilize a conducting transmembrane orientation of the peptides. Interactions of  $\epsilon$ -amino groups of lysine side chains with pyrophosphate groups of lipid II may be helpful in this regard and could also explain the reduced anion selectivity of the pore. Several nisin/lipid II complexes are presumed to assemble for a functional pore thus explaining the rapid efflux from liposomes and living cells of molecules of the size of carboxyfluorescein, amino acids, and ATP (7). The position of the lipid moiety of lipid II in the bilayer is unknown but may be of ultimate importance for nisin pore formation; structural details of the molecule are given in the *bottom* of the figure. The *cartoon* of nisin points out functionally important segments of the molecule; for the complete structure of nisin see Fig. 6.

micromolar concentration range (corresponding to  $\sim 3 \mu\text{g/ml}$ ) could result from lipid II-independent activity as observed with pure DOPG/DOPC liposomes and could explain the low activity of nisin against microorganisms that do not contain lipid II in their membranes, such as yeasts and fungi, as well as its low toxicity in animal model studies (2).

Thus, the accessibility of the docking molecule may be ultimately relevant for the activity of the lantibiotic. Because cell wall biosynthesis is a tightly controlled process, and the bacitroprenol-bound central carriers in that process are limited in number, one can assume that these molecules are in intimate contact with proteins involved in the membrane-associated steps of cell wall formation. Depending on the type of interactions with such proteins, the access to lipid II may be restricted

<sup>2</sup> I. Wiedemann, R. Benz, and H.-G. Sahl, unpublished observations.

to such an extent that even Gram-positive bacteria could become comparatively insensitive toward nisin as observed for the genus *Listeria* (39). There is in fact very little knowledge on how cell wall synthesis is functionally organized, and how bactoprenol carriers interact with the enzymes; it is not even clear how lipid II, in particular the bactoprenol C<sub>55</sub> lipid tail (Fig. 7), is embedded within the membrane, e.g. in a trans-bilayer fashion or in between the monolayers. We consider it realistic that the results reported here not only will form the basis for new strategies to rationally improve nisin and related lantibiotics, but also will enable us to address fundamental questions about the structural organization of bacterial cell wall biosynthesis and the underlying mechanisms by which bacteria can become resistant to antimicrobial peptides.

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