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In Vivo Cluster Formation of Nisin and Lipid II Is Correlated with Membrane Depolarization

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Nisin and related lantibiotics kill bacteria by pore formation or by sequestering lipid II. Some lantibiotics sequester lipid II into clusters, which were suggested to kill cells through delocalized peptidoglycan synthesis. Here, we show that cluster formation is always concomitant with (i) membrane pore formation and (ii) membrane depolarization. Nisin variants that cluster lipid II kill L-form bacteria with similar efficiency, suggesting that delocalization of peptidoglycan synthesis is not the primary killing mechanism of these lantibiotics.

Lantibiotics form a class of antimicrobial peptides that contain thioether rings formed by lanthionine residues. Nisin, the most studied lantibiotic, is a 34-residue peptide produced by *Lactococcus* species with antimicrobial activity against a wide range of Gram-positive bacteria (see Fig. S1 in the supplemental material). Nisin targets lipid II, the precursor molecule for peptidoglycan (PG) synthesis (1), and kills via two modes of action: (i) formation of large membrane pores and (ii) interference with PG synthesis.

Two lanthionine rings in nisin (A and B) form a pyrophosphate-binding cage that binds lipid II and is highly conserved among lipid II-binding lantibiotics (2). The C terminus of nisin is important for membrane integration (3, 4). Nisin-lipid II complexes (8:4 stoichiometry) form pores in the membrane (5–7) that result in the efflux of small molecules and influx of sodium ions, which will lead to cell death. Mutations in the hinge region of nisin either block or severely inhibit pore formation activity, presumably by preventing the hinge region (residues N20, M21, and K22) (see Fig. S1 in the supplemental material) from flipping the C-terminal tail into and across the membrane. Mutants PP-nisin (N20P M21P) and $\Delta\Delta$ -nisin (Δ N20 Δ M21) fail to form pores in liposome efflux assays (7, 8). Nisin 1-22 (Δ 23-34) cannot dissipate the membrane potential of sensitive *Lactococcus* species (9). Similar to nisin 1-22, mutacin 1140 and mersacidin bind lipid II but are too short to span the membrane (6, 10). Mutants that do not efficiently form pores are thought to act by affecting cell wall synthesis only.

Two mechanisms for lantibiotic interference with PG synthesis are proposed: “occlusion” and “clustering.” Occlusion is the binding to the pyrophosphate moiety of lipid II, which blocks incorporation of lipid II into glycan strands (11). Clustering is the formation of nonphysiological domains containing lipid II and nisin in the membrane, which results in delocalized PG synthesis (12).

Recently, we used PP-nisin as a tool to cluster lipid II into domains to determine the effect of delocalized lipid II on the localization of proteins involved in PG synthesis (13). PP-nisin was expected not to affect the membrane potential of live cells (7); however, we found that PP-nisin induced membrane potential loss (13). This compromised the localization of many membrane-associated proteins, including MreB (14). Here, we further investigated the effects of various nisin mutants on lipid II cluster formation and pore formation using live *Bacillus subtilis* cells.

The nisin, nisin variants, and other lantibiotics used in this study all displayed antibacterial activity against *B. subtilis* (Table 1), as determined using the resazurin microplate assay

(REMA), which uses the resazurin-resorufin dye pair to assess the metabolic capacity of cells (15) (see the methods in the supplemental material). The MIC₅₀s determined correspond well with MIC values reported in the literature for the various compounds (8, 16–18). The capacity of these compounds to cluster lipid II was tested by microscopy. Lipid II was stained with a vancomycin-conjugated BODIPY (boron-dipyrromethene) fluorophore, and cells were imaged. Control cells show a large amount of lipid II in the septum and additional lipid II on the cell edges, whereas nisin and PP-nisin induce the formation of spotty clusters with the loss of defined fluorescent cell edges (Fig. 1A and B), as reported previously (12, 13, 19, 20), although nisin was more potent at lower concentrations. $\Delta\Delta$ -Nisin was less potent in cluster formation, with only 27% of cells showing clusters at 30 μ g/ml and a minimum concentration at which cluster formation was observed of 20 μ g/ml. This suggests that the presence of the two amino acids at positions 20 and 21 is important for clustering. Nisin 1-22 did not induce cluster formation even at a concentration of 30 μ g/ml, which is 3 times the measured MIC₅₀ (Fig. 1A and Table 1). Mersacidin and mutacin 1140 failed to cluster lipid II at concentrations far above their MIC₅₀ (Fig. 1B and Table 1). This was surprising as all lantibiotics were expected to cluster lipid II, as described for PP-nisin in giant unilamellar vesicles (GUVs) and live cells and for mutacin 1140 in GUVs only (12). The hinge region mutants, nisin 1-22, and mutacin 1140 all have the ring A/B cage, yet mutacin 1140 and nisin 1-22 were not effective in clustering, suggesting that the cage itself is insufficient for clustering.

As not all lantibiotics tested clustered lipid II, we decided to

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TABLE 1 Comparison of activities of nisin variants in *B. subtilis* rods and L-forms

Lantibiotic	Rods				L-forms					
	MIC ₅₀ ^a , μg/ml (nM) ^a	Clustering, yes/no (%) ^b	Concn for clustering, μg/ml (nM) ^c	Δψ dissipation, μg/ml (nM) ^d	95% confidence interval, μg/ml (nM)	Pore formation, μg/ml (nM) ^d	95% confidence interval, μg/ml (nM)	MIC ₅₀ ^e , μg/ml (nM) ^a	Δψ dissipation, μg/ml (nM) ^e	95% confidence interval, μg/ml (nM)
WT nisin	2.5 (753)	Yes (99.6)	0.5 (151)	0.32 (96)	0.29–0.35 (87–105)	0.03 (9.0)	0.029–0.032 (8.7–9.6)	0.2 (60)	0.47 (142)	0.42–0.53 (127–160)
PP-nisin	5 (1,530)	Yes (99.0)	1.5 (459)	3.7 (1,133)	3.4–4.0 (1,041–1,224)	0.10 (30.6)	0.091–0.101 (27.9–30.9)	6.25 (1,913)	0.83 (254)	0.75–0.91 (229–278)
ΔΔ-Nisin	12.5 (8,136)	Yes (27.0)	20 (6,509)	4.0 (1,302)	3.9–4.2 (1,269–1,367)	2.8 (911)	0.22–5.45 (72–1,773)	25 (8,136)	1.87 (609)	1.46–2.29 (475–745)
Nisin 1-22	10 (4,691)	No (0.0)	>30 (>14,073)	ND ^f	ND	0.68 (319)	0.49–0.87 (230–408)	50 (23,455)	ND	ND
Mersacidin	25 (13,706)	No (0.0)	>60 (>32,895)							
Mutacin 1140	0.47 (207)	No (0.0)	>10 (>4,416)							

^a MIC₅₀ obtained from REMA.

^b Determined by visual inspection of microscopy images (Fig. 1). Percentages of cells with lipid II clusters for WT nisin (*n* = 285), PP-nisin (*n* = 428), ΔΔ-nisin (*n* = 303), nisin 1-22 (*n* = 270), and mersacidin (*n* = 248) are shown, all at 30 μg/ml. The concentration of mutacin 1140 (*n* = 215) was 10 μg/ml.

^c Lowest concentration at which clustering was observed or highest concentration tested (without observing clustering).

^d EC₅₀ obtained from fitting the dose-response curve per Fig. S2 in the supplemental material.

^e EC₅₀ obtained from fitting the dose-response curve per Fig. S4 in the supplemental material.

^f ND, not detected.

further study the pore-forming activity of nisin (variants) in live cells, using fluorescent dyes to monitor membrane depolarization and pore formation in 96-well plate assays. Membrane depolarization was measured in hyperpolarized cells with the membrane potential dye DiSC₃ (5). Addition of nisin leads to depolarization with a concomitant fluorescence increase with a 50% effective concentration (EC₅₀) of 96 nM (Table 1; see Fig. S2A in the supplemental material). PP-nisin and ΔΔ-nisin were clearly not as active as nisin but caused complete membrane depolarization at higher concentrations (see Fig. S2A), which was unexpected as they were reported to be deficient in pore formation (8, 12). Nisin 1-22 was inactive in our depolarization assay, as reported earlier (9).

The membrane depolarization observed with PP-nisin and ΔΔ-nisin was surprising; therefore, the pore formation capacity of these nisin variants was determined. The quenching of the membrane-permeable DNA stain SYTO9 by membrane-impermeable propidium iodide (PI) influx was used as a proxy for pore formation in live *B. subtilis* cells by nisin (variants). Efficient influx of the propidium probe was detected with nisin, with an EC₅₀ of 9.0 nM (Table 1; see Fig. S2B in the supplemental material). PP-nisin also allows the passage of the probe *in vivo* only slightly less efficiently than nisin (Table 1; see Fig. S2B). ΔΔ-Nisin and nisin 1-22 are much less efficient in this assay (see Fig. S2B). ΔΔ-Nisin does not quite plateau, resulting in very wide confidence intervals for the EC₅₀ (Table 1). SYTO9 quenching by nisin 1-22 reaches a plateau at half the level of quenching caused by nisin, indicating that nisin 1-22 can induce pore formation but not to the extent that membrane potential is altered. This assay does not resolve whether or not a subfraction of cells is responsible for the observed probe influx. It is possible that live cells can counteract the depolarization effects of pore formation to a certain extent—e.g., by resealing unstable pores. The protonophore carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) did not cause propidium influx (not shown), indicating that depolarization of the membrane alone does not cause propidium influx. A potassium efflux assay using the potassium indicator PBFI confirmed that nisin, PP-nisin, and ΔΔ-nisin cause potassium efflux, whereas nisin 1-22 did not, but EC₅₀s could not be determined for all nisin variants (see Fig. S3 in the supplemental material).

All nisin variants that cluster lipid II induced membrane depolarization. ΔΔ-Nisin induced both clustering and pore formation at much higher concentrations than nisin and PP-nisin, suggesting that both events are linked. To establish whether pore formation is the main killing mechanism for these nisin variants, we used L-forms that grow and proliferate in the absence of a cell wall (21). L-forms will be killed by nisin variants that form pores, while nisin variants that kill by inhibition of PG synthesis alone will be ineffective. Although lipid II synthesis is blocked or reduced in L-forms, nisin is still effective against L-forms due to the presence of other precursor lipids similar to lipid II (lipids III and IV [22]). MIC values for nisin, PP-nisin, and ΔΔ-nisin in L-forms were either lower than or similar to the MIC values for PG-containing cells (Table 1), indicating that all of these variants kill with a similar efficiency irrespective of the presence of a cell wall. The MIC₅₀ for nisin 1-22 increased 5-fold. Membrane depolarization of L-forms was also found to be similar to depolarization of cells containing PG (Table 1; see Fig. 4 in the supplemental material). Thus, by using L-forms as a way to discern whether nisin variants kill exclusively by inhibiting PG synthesis or also by pore formation,

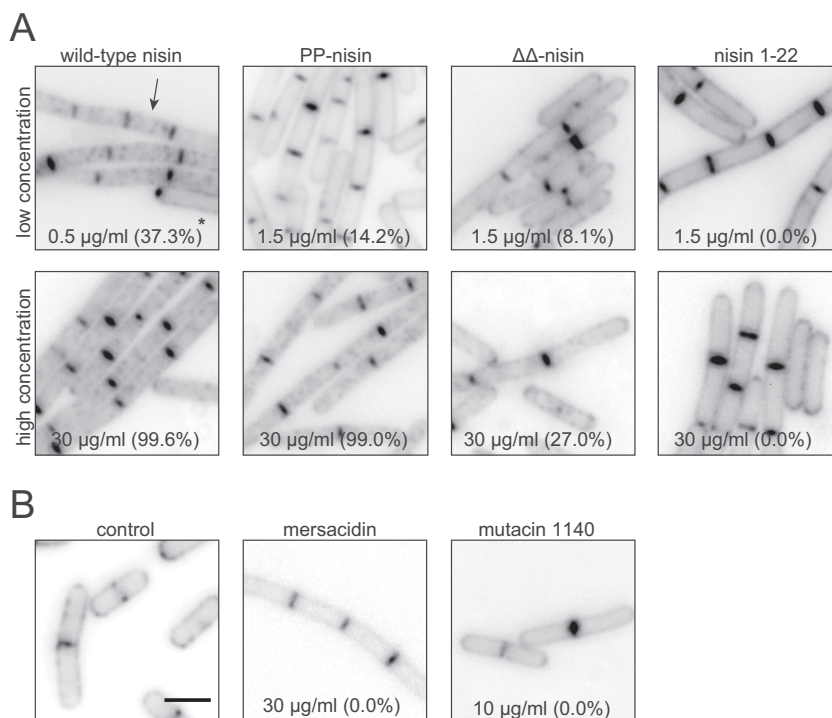


FIG 1 *In vivo* clustering of nisin and lipid II is concentration dependent. (A) Fluorescence microscopy of *B. subtilis* 168 after incubation with nisin and staining of lipid II with fluorescent vancomycin (Van-Fl). Concentration-dependent clustering of lipid II can be observed by a change in phenotype from cells with defined edges and without spots (asterisk) to cells that lost their edges and have a spotted appearance (arrow). The percentages of cells with clusters are indicated in parentheses for each group: wild-type nisin, $n = 367$ for high concentration and 285 for low; PP-nisin, $n = 288$ for high and 428 for low; $\Delta\Delta$ -nisin, $n = 297$ for high and 303 for low; and nisin 1-22, $n = 281$ for high and 270 for low (Table 1). (B) Untreated cells stained with fluorescent vancomycin or treated with mersacidin or mutacin 1140. Percentages of cells with clusters are shown in parentheses: for mersacidin, $n = 248$, and for mutacin 1140, $n = 215$. Scale bar, 2 μm (same for all panels). Fluorescence images were inverted for clarity.

we conclude that only nisin 1-22—which kills cells much more efficiently when PG synthesis is required—predominantly targets PG synthesis.

The results presented here suggest that lantibiotic-induced cluster formation of lipid II coincides with membrane depolarization. Surprisingly, mutacin 1140 clusters lipid II in GUVs (12) but fails to do so in live cells (Fig. 1), and PP-nisin and $\Delta\Delta$ -nisin formed pores in live cells, although they are inactive in pore formation in lipid II-doped 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) liposomes (8). This suggests that lantibiotics have a stronger pore-forming activity on live cell membranes, which could be caused by either differences in lipid composition, the presence of protein in the membranes, or the presence of a membrane potential. Neither lipid II binding (by nisin 1-22 or mersacidin) nor membrane depolarization (e.g., by CCCP [13]) alone is sufficient to form lipid II clusters. This strongly suggests that nisin-lipid II cluster formation results in depolarization, although we cannot formally exclude that depolarization results in clustering.

These findings have implications for the proposed killing modes of nisin-like lantibiotics: nisin variants capable of membrane depolarization may inhibit PG synthesis as well, but our results suggest that this is not important for killing as cell-wall-less L-forms are killed by these compounds with similar or higher efficiency. Nisin 1-22, the only nisin variant that exclusively targets PG synthesis, should work through occlusion not clustering, as we never observed clusters formed by lipid II and nisin 1-22.

Similarly, occlusion is the mode of action of mersacidin and mutacin. An implication of our finding is that monitoring the effects of lantibiotic-mediated lipid II delocalization on cell wall synthesis is only possible for those proteins that are not affected by the collapse of the membrane potential that is associated with lipid II delocalization.

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