In vivo cluster formation of nisin and Lipid II is correlated with membrane depolarization

Menno B. Tol, Danae Morales Angeles, Dirk-Jan Scheffers

Department of Molecular Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, The Netherlands

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#Address correspondence to Dirk-Jan Scheffers, d.j.scheffers@rug.nl

a M.B.T. and D.M.A. contributed equally to this work.

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ABSTRACT

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 (Fig. S1). 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, Fig. S1) from flipping the C-terminal tail into and across the membrane. Mutants PP-nisin (N20P, M21P) and ΔΔ-nisin (ΔN20, ΔM21) fail to form pores in liposome-efflux assays (7, 8). Nisin 1-22 (Δ23-34) 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 non-physiological 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.

Nisin, nisin variants and other lantibiotics used in this study all displayed antibacterial activity against *B. subtilis* (Table 1), as determined using the REMA assay, which uses the resazurin/resorufin dye pair to assess metabolic capacity of cells (15)(see supplementary methods). 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 using microscopy. Lipid II was stained with a vancomycin-conjugated BODIPY 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, B), as reported (12, 13, 19, 20), although nisin was more potent at lower concentrations. ΔΔ-nisin was less potent in cluster formation, with only 27% of cells showing clusters at 30 µg/ml and a minimum concentration at which cluster formation was observed of 20 µg/ml. This suggests that the presence of the two amino acids at position 20 and 21 is important for clustering. Nisin 1-22 did not induce cluster formation even at a concentration of 30 µg/ml, which is three times the measured MIC₅₀ (Fig. 1A, Table 1). Mersacidin and mutacin 1140 failed to cluster Lipid II at concentrations far above their MIC₅₀ (Fig. 1B, table 1). This was surprising as all lantibiotics were expected to cluster Lipid II, as
described for PP-nisin in 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 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 hyper-polarized cells with the membrane potential dye DiSC3(5). Addition of nisin leads to depolarization with a concomitant fluorescence increase with an EC50 of 96 nM (Fig. S2A, Table 1). PP-nisin and ΔΔ-nisin were clearly not as active as nisin but caused complete membrane depolarization at higher concentrations (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 (Fig S2B, Table 1). PP-nisin also allows the passage of the probe in vivo, only slightly less efficient than nisin (Fig S2B, Table 1). ΔΔ-nisin and nisin 1-22 are much less efficient in this assay (Fig S2B). ΔΔ-nisin does not quite plateau resulting in very wide confidence bounds 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 sub-fraction 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 nisin1-22 did not, but EC₅₀ values could not be determined for all nisin variants (Fig S3).

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 or similar to MICs for PG containing cells (Table 1), indicating that all 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 (Fig. S4, Table 1). Thus, using L-forms as a way to discern whether nisin variants kill exclusively by inhibiting PG synthesis or also by pore formation,
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 ΔΔ-nisin formed pores in live cells although they are inactive in pore formation in Lipid II-doped 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, mersacidin) or 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 proteins is only possible for those proteins that are not affected by the collapse of the membrane potential that is associated with Lipid II delocalization.
ACKNOWLEDGEMENTS

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FIG 1 *In vivo* clustering of nisin and Lipid II is concentration dependent. (A) Fluorescent 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 in Fig 1A) to cells that lost their edges and have a spotted appearance (arrow in Fig 1A). The percentage of cells with clusters is indicated in parentheses (wt nisin, n = 367 (high), 285 (low); PP-nisin, n = 288 (h), 428 (l); ΔΔ-nisin, n = 297 (h), 303 (l); nisin 1-22, n = 281 (h), 270 (l); see also Table 1). (B) Untreated cells stained with Van-Fl, or treated with mersacidin or mutacin 1140. Percentage of cells with clusters in parenthesis (mersacidin, n = 248; mutacin 1140, n = 215). Scale bar 2 μm (same for all). Fluorescence images were inverted for clarity.
### TABLE 1 Comparison of activity of nisin variants in *B. subtilis* rods and L-forms

<table>
<thead>
<tr>
<th>Lantibiotic</th>
<th>MIC&lt;sub&gt;50&lt;/sub&gt; (µg/ml) (nM)</th>
<th>Clustering (yes/no) (%)</th>
<th>Concentration for clustering (µg/ml)</th>
<th>ΔΨ dissipation (µg/ml) (nM)</th>
<th>95% conf. bounds</th>
<th>Pore formation (µg/ml) (nM)</th>
<th>95% conf. bounds</th>
<th>α MIC&lt;sub&gt;50&lt;/sub&gt; (µg/ml) (nM)</th>
<th>ΔΨ dissipation (µg/ml) (nM)</th>
<th>95% conf. bounds</th>
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<tbody>
<tr>
<td>WT nisin</td>
<td>2.5</td>
<td>yes</td>
<td>0.5</td>
<td>0.32</td>
<td>0.29-0.35</td>
<td>0.03</td>
<td>0.029-0.032</td>
<td>0.2</td>
<td>0.47</td>
<td>0.42-0.53</td>
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<td>PP-nisin</td>
<td>5</td>
<td>yes</td>
<td>1.5</td>
<td>3.7</td>
<td>3.4-4.0</td>
<td>0.10</td>
<td>0.091-0.101</td>
<td>6.25</td>
<td>0.83</td>
<td>0.75-0.91</td>
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<tr>
<td>ΔΔ-nisin</td>
<td>12.5</td>
<td>yes</td>
<td>29</td>
<td>4.0</td>
<td>3.9-4.2</td>
<td>2.8</td>
<td>0.22-5.45</td>
<td>25</td>
<td>1.87</td>
<td>1.46-2.29</td>
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<tr>
<td>Nisin 1-22</td>
<td>10</td>
<td>no</td>
<td>&gt; 30</td>
<td>ND</td>
<td>ND</td>
<td>0.68</td>
<td>0.49-0.87</td>
<td>50</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Mersacidin</td>
<td>25</td>
<td>no</td>
<td>&gt; 60</td>
<td>ND</td>
<td>ND</td>
<td>319</td>
<td>230-408</td>
<td>23455</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>Mutacin</td>
<td>13706</td>
<td>0.0%</td>
<td>&gt; 32895</td>
<td>ND</td>
<td>ND</td>
<td>319</td>
<td>230-408</td>
<td>23455</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

<sup>a</sup> MIC<sub>50</sub> obtained from REMA assay.

<sup>b</sup> Visual inspection of microscopy images (Fig 1), % indicates percentage of cells with Lipid II clusters for WT nisin (*n* = 285), PP-nisin (*n* = 428), ΔΔ-nisin (*n* = 303) and nisin 1-22 (*n* = 270) and for mersacidin (*n* = 248), all at 30 µg/ml. Mutacin 1140 was at 10 µg/ml (*n* = 215).

<sup>c</sup> Lowest concentration at which clustering was observed, or highest concentration tested (without observing clustering).

<sup>d</sup> EC<sub>50</sub> obtained from fitting dose-response curve, (Fig S2)

<sup>e</sup> EC<sub>50</sub> obtained from fitting dose-response curve, (Fig S4)

<sup>f</sup> ND, not detected.
A

<table>
<thead>
<tr>
<th>Low Concentration</th>
<th>High Concentration</th>
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<tbody>
<tr>
<td><strong>wild-type nisin</strong></td>
<td>0.5 μg/ml (37.3%) *</td>
</tr>
<tr>
<td><strong>PP-nisin</strong></td>
<td>1.5 μg/ml (14.2%)</td>
</tr>
<tr>
<td><strong>ΔΔ-nisin</strong></td>
<td>1.5 μg/ml (8.1%)</td>
</tr>
<tr>
<td><strong>nisin 1-22</strong></td>
<td>1.5 μg/ml (0.0%)</td>
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</tbody>
</table>

B

<table>
<thead>
<tr>
<th>Low Concentration</th>
<th>High Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>control</strong></td>
<td>30 μg/ml (0.0%)</td>
</tr>
<tr>
<td><strong>mersacidin</strong></td>
<td>30 μg/ml (0.0%)</td>
</tr>
<tr>
<td><strong>mutacin 1140</strong></td>
<td>10 μg/ml (0.0%)</td>
</tr>
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