Lipid II-Based Antimicrobial Activity of the Lantibiotic Plantaricin C

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We analyzed the mode of action of the lantibiotic plantaricin C (PlnC), produced by Lactobacillus plantarum LL441. Compared to the well-characterized type A lantibiotic nisin and type B lantibiotic mersacidin, which are both able to interact with the cell wall precursor lipid II, PlnC displays structural features of both prototypes. In this regard, we found that lipid II plays a key role in the antimicrobial activity of PlnC besides that of pore formation. The pore forming activity of PlnC in whole cells was prevented by shielding lipid II on the cell surface. However, in contrast to nisin, PlnC was not able to permeabilize Lactococcus lactis cells or to form pores in 1,2-dioleoyl-sn-glycero-3-phosphocholine liposomes supplemented with 0.1 mol% purified lipid II. This emphasized the different requirements of these lantibiotics for pore formation. Using cell wall synthesis assays, we identified PlnC as a potent inhibitor of (i) lipid II synthesis and (ii) the FemX reaction, i.e., the addition of the first Gly to the pentapeptide side chain of lipid II. As revealed by thin-layer chromatography, both reactions were clearly blocked by the formation of a PlnC-lipid I and/or PlnC-lipid II complex. On the basis of the in vivo and in vitro activities of PlnC shown in this study and the structural lipid II binding motifs described for other lantibiotics, the specific interaction of PlnC with lipid II is discussed.

Ribosomally synthesized antimicrobial peptides are found to be components of the innate defense systems in almost all forms of life (10, 35). Within the bacterial world, lactic acid bacteria produce a wide range of such antimicrobial peptides, usually referred to as bacteriocins, which display a relatively wide spectrum of activity. The long history of safe use of lactic acid bacteria in food processing has drawn attention to bacteriocins as food biopreservatives, exemplified by the use of nisin in several countries (7). The Lactobacillus plantarum strain LL441 synthesizes the antimicrobial peptide plantaricin C (PlnC), which exerts a potent bactericidal effect on several gram-positive strains, including several food pathogens and spoilage bacteria (11). PlnC was found to dissipate the proton motive force and to induce release of intracellular molecules such as glutamate and ATP (12). Recently, the structure of PlnC has been investigated by nuclear magnetic resonance (28). The highly positive charge of PlnC, concentrated at the N terminus, has been suggested to be needed for electrostatic interaction with the anionic cell surface prior to membrane destabilization, as described for many other pore-forming cationic peptides (27).

PlnC belongs to the large family of lantionine-containing antimicrobial peptides, or lantibiotics. These unique antimicrobial peptides contain lantionine and β-methylthionionine residues which are posttranslationally introduced by specific enzymes encoded by the lantibiotic operons (34). Nisin and mersacidin are the most prominent members of the two lantibiotic groups (types A and B, respectively) proposed by Jung (16) and have been extensively studied (for reviews see references 8, 13, and 24).

The elongated, positively charged type A lantibiotic nisin forms pores in the cytoplasmic membrane of susceptible bacteria and induces loss of vital ion gradients and cytosolic components, depletion of the proton motive force, and the concomitant loss of viability (23). Nisin and other structurally related lantibiotics, e.g., epidermin/gallidermin, use the membrane-bound peptidoglycan precursor lipid II as a docking molecule for pore formation and, thus, combine two modes of action, i.e., pore formation and inhibition of cell wall synthesis, for antibiotic activity in the nanomolar range (4, 6, 32). In contrast, binding of the globular, noncharged type B lantibiotic mersacidin to lipid II leads to inhibition of cell wall biosynthesis at the level of transglycosylation (5).

Considering that cell wall biosynthesis is regarded as a prime antibiotic target, since this pathway is restricted to prokaryotes and low toxicity is expected, it is of relevance to discover novel structures that interfere with it. Recently, two different structural motifs involved in lipid II binding have been suggested for nisin and mersacidin (2). Strikingly, PlnC displays an intermediate structure between nisin and mersacidin, with a highly positive N terminus and a rather compact C terminus with a ring arrangement similar to the mersacidin lipid II binding motif (Fig. 1). Therefore, we investigated the mode of action of PlnC and found that it is a potent inhibitor of in vitro cell wall biosynthesis and forms a complex with the cell wall precursor lipid II. However, we observed that lipid II-mediated pore formation by PlnC occurs only in particular strains. Comparison of the primary structure and in vitro and in vivo activities of PlnC with those of nisin and mersacidin reveal the complexity of lantibiotic-mediated killing and highlight the wide spectrum of interactions displayed by molecules that share the common target lipid II.
Plantaricin C

Nisin

Mersacidin

FIG. 1. Primary structures of PlnC, mersacidin, and nisin. The putative lipid II binding motifs of these peptides are shaded. Dha, dehydroalanine; Dhb, dehydrobutyryl; Ala-S-Ala, lanthionine; Abu-S-Ala, β-methylanthionine.

MATERIALS AND METHODS

Chemicals. All chemicals were of analytical grade or better. Radiolabeled UDP-N-acetylglucosamine ([14C]UDP-GlcNAc) (7.4 GBq mmol⁻¹) was purchased from Amersham Biosciences. 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) was purchased from Avanti Polar Lipids, Inc., and stored at −20°C in chloroform. Protein concentrations of purified PlnC and membrane preparations were determined with the bicinchoninic acid protein assay reagent (Pierce Chemical Corp.), with bovine serum albumin as the standard.

Bacterial strains and culture conditions. Micrococcus flavus DSM 1790 was grown in Trypticase soy broth at 37°C with aeration. Lactococcus lactis subsp. cremoris HP was grown in M17 broth plus 0.5% glucose (Oxoid) at 37°C with aeration. The PlnC producer, L. plantarum LL441, was kindly supplied by J. E. Suárez (University of Oviedo, Spain) and grown in MRS broth (Oxoid) at 32°C.

Purification of PlnC and nisin. PlnC purification was performed as previously described with slight modifications (11). Briefly, supernatants of L. plantarum LL441 cultures were precipitated with ammonium sulfate at 65% (wt/vol). The precipitate was dissolved in 25% acetonitrile in water and eluted through a C8 cartridge (Mega Bond Elut, Variant) with 60% acetonitrile. Active fractions were pooled, evaporated, and applied to a 5-mL cation exchange column (High-S Bio-Rad). PlnC was eluted with an NaCl gradient. Salts were further removed by an additional hydrophobic interaction step. The purity of PlnC was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, as previously described (11). For storage, PlnC samples were freeze-dried. Nisin was obtained and purified from culture supernatants of L. lactis NIZO22186 (18) by chloroform extraction, as described by Bonelli et al. (3).

MIC determinations. MIC determinations were carried out in microtiter plates. M. flavus DSM 1790 was grown in half-concentrated Mueller-Hinton broth (Oxoid), and L. lactis HP was grown in M17 broth plus 0.5% glucose (Oxoid). Serial twofold dilutions of the peptides were made in the appropriate growth medium. Bacteria were added to a final inoculum of 10⁶ CFU/mL in a volume of 0.2 mL. Incubation conditions were 30°C for 24 h for M. flavus DSM 1790 and 30°C for 16 h for L. lactis HP. MIC was read as the lowest peptide concentration causing inhibition of visible growth; determinations were carried out at least in duplicate.

Potassium release from whole cells. Cells were harvested at an optical density at 600 nm (OD600) of 1.0 to 1.5 (3,300 × g, 5°C, 3 min), washed with 50 mL of cold choline buffer (300 mM choline chloride, 30 mM MES [morpholinethanesulfonic acid], 20 mM Tris, pH 6.5), and resuspended in the same buffer to an OD600 of 30. The concentrated cell suspension was kept on ice and used within 30 min. For each measurement, cells were diluted in choline buffer (25°C) to an OD600 of about 3. Peptide-induced potassium efflux was monitored with a microprocessor pH meter (pH 213; Hanna Instruments) with an MI-442 electrode. The assay for synthesis of lipid II-Gly1 was performed in a total volume of 100 μL containing 400 to 800 μg of membrane protein of M. flavus DSM 1790, 10 nmol undecaprenylphosphate, 100 nmol UDP-N-acetylmuramic acid (MurNAc) pentapeptide, and 100 nmol [14C]UDP-GlcNAc in 60 mM Tris-HCl, 5 mM MgCl2 (pH 8), and 0.5% (wt/vol) KCl in buffer, and calculations of percent potassium efflux were performed as described previously (19).

Inhibition of in vitro lipid II synthesis. Inhibition of in vitro lipid II formation was analyzed by the lipid II synthesis assay (25) with the addition of radiolabeled [14C]UDP-GlcNAc. Reactions were carried out in a final volume of 150 μL containing 400 to 800 μg of membrane protein of M. flavus DSM 1790, 10 nmol radiolabeled lipid II-Gly1 (25) was performed in a total volume of 100 μL containing 5 nmol lipid II, 10 μg His-tagged glycidyl-tRNA synthetase, 25 μg RNA, and 2.7 μg His-tagged FemX in 100 mM Tris-HCl, 20 mM MgCl2 (pH 7.5), and 0.8% Triton X-100 with 2 mM ATP and 50 nmol [14C]glycine (3.7 GBq/mmol) (Amersham Pharmacia Biotech.). For the inhibition assay, the substrate lipid II and the peptides (at a molar ratio of 1:2) were preincubated for 15 min before addition of the reaction mixture. After incubation for 1 h at 30°C, the reaction mixture (50 μL) was analyzed by TLC using chloroform-methanol-water-ammonia (88:48:10:1) as the solvent (22). Radiolabeled spots were visualized by iodine vapor, excised, and quantified by β-scintillation counting (1900 CA Tri-Carb scintillation counter; Packard).

Inhibition of in vitro lipid II-Gly2 synthesis. The assay for synthesis of lipid II-Gly2 (25) was performed in a total volume of 100 μL containing 5 nmol lipid II, 10 μg His-tagged glycyld-tRNA synthetase, 25 μg RNA, and 2.7 μg His-tagged FemX in 100 mM Tris-HCl, 20 mM MgCl2 (pH 7.5), and 0.8% Triton X-100 with 2 mM ATP and 50 nmol [14C]glycine (3.7 GBq/mmol) (Amersham Pharmacia Biotech.). For the inhibition assay, the substrate lipid II and the peptides (at a molar ratio of 1:2) were preincubated for 15 min before addition of the reaction mixture. After incubation for 1 h at 30°C, the reaction mixture (50 μL) was analyzed by TLC using butanol-acetic acid-water-pyridine (15:3:12:10 vol/vol) (vol/vol) (vol/vol). Radiolabeled spots or lanes were visualized and quantified as described above.
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RESULTS

Pore formation by PlnC in whole cells and liposomes. We analyzed the pore forming activity of PlnC against whole cells of two selected indicator strains, Micrococcus flavus and Lactococcus lactis HP, with high and low susceptibility, respectively (Table 1), using a potassium-sensitive electrode.

Potassium release was detected in M. flavus cells after addition of PlnC at concentrations above 0.1 μM. Addition of PlnC at 0.5 μM (15× MIC) and 1 μM (30× MIC) resulted in release levels of about 73% within 3 min (Fig. 2A). In contrast, no leakage of potassium was observed from L. lactis HP cells after the addition of increasing concentrations of PlnC up to 5 μM (25× MIC). Nisin, however, was as active against this particular strain (Fig. 2B) as against M. flavus and induced potassium release at a concentration of 1 μM, which corresponded to 20× MIC for L. lactis. These results indicate that pore formation may target strain.

Remarkably, when M. flavus cells were preincubated with the non-pore-forming gallidermin mutant peptide A12L gallidermin (3), which binds tightly to lipid II, the cells were no longer affected by PlnC (Fig. 2A). Thus, the pore forming activity of PlnC apparently relies on the availability of the cell wall precursor on the cell membrane. To confirm whether the presence of lipid II could facilitate pore formation, we tested the impact of PlnC on unilamellar liposomes made of DOPC supplemented or not with 0.1 mol% purified lipid II. In the absence of lipid II, PlnC did not induce marker release (data not shown). In the presence of lipid II (Fig. 3), pore formation was not observed even at the highest concentration of PlnC (1 μM), whereas nisin induced marker release up to 80 and 90% after addition at 0.1 μM and 1 μM, respectively (Fig. 3). Overall, the ability of PlnC to form pores was clearly restricted to the most susceptible strain, M. flavus, and did not occur in L. lactis cells or in DOPC liposomes under the experimental conditions used in this study.

Inhibition of lipid II synthesis. Taking into account the very likely involvement of lipid II in the mode of action of PlnC (see above), we hypothesized that PlnC could act mainly as a cell wall synthesis inhibitor. Therefore, we first tested the ability of PlnC to inhibit the formation of lipid II by using an in vitro lipid II synthesis assay on an analytical scale with radiolabeled glycosylation and further cross-linked by transpeptidases (see references 29 and 30 and references therein).

The conversion of the substrate (C₅₅-P) to lipid II was clearly inhibited by addition of PlnC in a concentration-dependent manner (Fig. 4). PlnC, in molar ratios of 1:0.5 and 1:1 (substrate/peptide), reduced the amount of synthesized lipid II from 100% (control, without peptide addition) to 30.4% and 12.4%, respectively. Likewise, when nisin was added at a substrate/peptide ratio of 1:1, the amount of synthesized lipid II was reduced to 20%. Therefore, PlnC is a potent inhibitor of cell wall biosynthesis and very likely, as described for nisin (6), binds to the cell wall precursor lipid I and subsequently blocks lipid II synthesis.

Inhibition of in vitro lipid II-Gly₁ synthesis by PlnC. The ability of PlnC to bind to lipid II and to inhibit subsequent modification, such as the addition of Gly to the pentapeptide side chain of lipid II catalyzed by FemX, was investigated with an in vitro lipid II-Gly₁ synthesis assay with radiolabeled glycine. The products of the in vitro reaction were analyzed by TLC, and the ratio of glycine incorporation in the presence of each peptide was determined (Fig. 5).

![Graph](http://aem.asm.org/)

**Fig. 2.** Impact of PlnC and nisin on the integrity of the cytoplasmic membrane of Micrococcus flavus (A) and Lactococcus lactis subsp. lactis HP (B). Peptides were added after 30 s, and potassium release was monitored with a potassium-sensitive electrode. PlnC was added at different concentrations: 0.1 μM, white triangles; 0.5 μM, gray triangles; 1 μM, black triangles; 5 μM, diamonds; no peptide addition, asterisks. Cells were preincubated with A12L gallidermin (1 μM) prior addition of PlnC (1 μM) (circles). Potassium leakage is expressed relative to the total amount of potassium (100% value) released after addition of 1 μM nisin (squares).
After incubation of lipid II with FemX in the absence of lantibiotic (control), lipid II-Gly₁ was produced and migrated on the TLC plates with an \( R_f \) value of 0.4 in the solvent system used (Fig. 5A). The molar ratio of radiolabeled glycine incorporated into lipid II was about 0.8, which is close to the theoretical value of one glycine per lipid II molecule (Fig. 5B). As expected, no radiolabel was detected at the application spot.

When lipid II was preincubated with the lantibiotics prior to starting the FemX reaction, different results were observed depending on each peptide. With nisin, no migration was observed on the TLC plate (Fig. 5A), indicating that nisin formed a stable complex with lipid II that remained at the origin. The calculated glycine/lipid II ratio of this complex was strongly reduced to only 0.08 (Fig. 5B). Thus, nisin completely inhibited the FemX reaction. After incubation of lipid II with PlnC, we observed an effect similar to that described for nisin. PlnC also formed a tight complex that remained at the origin in the TLC system (Fig. 5A). However, analysis of the radioactivity revealed that the FemX reaction was less affected by PlnC than by nisin, with a glycine/lipid II ratio of 0.41. Therefore, less than 50% of the substrate was converted to lipid II-Gly₁ (Fig. 5B).

Interestingly, the lipid II binding properties of PlnC and mersacidin appear to differ substantially, as no mersacidin-lipid II complex was retained at the origin and the reaction product migrated in the TLC system (Fig. 5A). Nevertheless, mersacidin also inhibited the FemX reaction. The glycine/lipid II ratio was 0.55 (Fig. 5B), i.e., inhibition of FemX by mersacidin was the least effective among the tested peptides.

**DISCUSSION**

To date, several lantibiotics have been shown to target the peptidoglycan precursor lipid II. Among them, the mersacidin-like peptides inhibit the transglycosylation step of cell wall biosynthesis, thereby blocking the precursor from incorporation into the cell wall (5), whereas nisin-like lantibiotics use lipid II as a docking molecule for high-affinity binding and target-mediated pore formation (4, 6). This study demonstrates that PlnC also falls into the category of lipid II-interacting antimicrobial peptides and acts as a potent cell wall synthesis inhibitor. Moreover, we observed that PlnC shares intermediate features with the prototype lantibiotics nisin and mersacidin concerning both its structure and lipid II interaction, which are also reflected in its biological activity.

PlnC had been previously described as a pore forming lantibiotic that causes dissipation of the proton motive force and an immediate release of preaccumulated solutes in *Lactobacillus fermentum* and *Lactobacillus sake* whole cells (11, 12). However, the requirements for PlnC-mediated pore formation seem to be rather distinctive from those displayed by the prototype type A lantibiotic nisin. In fact, high concentrations of PlnC were needed to achieve cell permeabilization in *M. flavus*. Moreover, in contrast to nisin, there was no marker release from cells of *L. lactis*, indicating that membrane poration by PlnC might be strain specific. Thus, the relative PlnC susceptibility of some strains could be linked to the absence of PlnC-mediated pores.

Another example that highlighted the differences between the abilities of nisin and PlnC to form pores stemmed from liposome studies. Lipid II-mediated pore formation enables nisin to kill in vivo in a nanomolar range (6, 32), and the presence of lipid II in artificial membranes stabilizes the pores and increases the pore lifetime (33). The data obtained here, by the competition assay with the lipid II binding gallidermin peptide mutant, also strongly suggest that PlnC targets the cell wall precursor. However, the lipid II-containing liposomes did not release entrapped CF, whereas nisin did so efficiently. The lack of pore formation by PlnC may be due to a different lipid requirement in membrane model systems compared to nisin (9, 31, 32). Indeed, CF efflux has been reported for *Escherichia coli*-derived liposomes (12) whose net charge is highly negative, in contrast to the more zwitterionic lipid, DOPC, used in this study. Furthermore, considering the possible globular and compact structure of PlnC, as suggested by the published thioether bridging pattern (28), the peptide might be too short to form a functional, membrane-spanning pore in C₁₈:₁ liposomes, as described for the type-A lantibiotics epidermin/galidermin (3).
The significance of lipid II for the biological activity of PlnC was clearly highlighted by the in vitro cell wall biosynthesis assays. PlnC strongly interfered with lipid II synthesis and bound tightly to lipid II. Binding to lipid II may be tentatively assigned to the same lipid II binding motif found in mersacidin (2). These peptides share an identical ring structure in the central part of the molecule (amino acids 12 to 18) and amino acid Glu17 (Fig. 1), which has been shown to be indispensable for the in vivo activity of mersacidin (26). However, the molecular interaction between lipid II and PlnC differs from that between lipid II and mersacidin, as indicated by the different behaviors of the peptide-lipid II complexes. PlnC formed a tight complex with lipid II that did not dissociate in TLC plates. Nuclear magnetic resonance studies of the mersacidin structure in different environments revealed remarkable conformational changes in which the junction between Ala12 and Abu13 serves as a hinge region for the opening and closure of the ring structures, and it has been suggested that electrostatic interactions play a central role in the mersacidin-lipid II interaction (14). This flexible hinge region is absent in PlnC, because of a thioether bridge comprising the amino acids at positions 12 and 15.

In spite of a lipid II binding motif that resembles that of mersacidin, the overall inhibitory features of PlnC are more similar to those of nisin. In contrast to nisin, mersacidin had been shown to not inhibit in vitro lipid II synthesis (5), whereas nisin forms a complex with both lipid I and lipid II (21). Here, we found that PlnC also interacts with both cell wall precursors, i.e., it strongly inhibits in vitro lipid II synthesis and forms a stable complex with lipid II, indicating that both lantibiotics may target the same structures on lipid II.

The interaction of nisin and lipid II is well characterized, as the solution structure of the complex has recently been disclosed. The N-terminal double-ring system of nisin was found to form a binding cage for the pyrophosphate linkage group of lipid II, and six hydrogen bonds between the backbone amides of rings A and B of nisin (amino acids 3 to 11 [Fig. 1]) and the pyrophosphate moiety were identified (15). However, nisin possesses a completely different structure, and the lipid II binding motif that enables nisin to interact with lipid II/lipid I is not present in PlnC (Fig. 1). Therefore, we suggest that additional linking sites must be displayed by PlnC, besides the lipid II binding motif also present in mersacidin, that enable the strong interaction with lipid I. As PlnC contains a highly positively charged N terminus, which is missing in mersacidin, the additional charge may contribute to lipid II binding. Interestingly, after tryptic cleavage of this N-terminal stretch, the in vivo activity of PlnC is lost (11).

It has become evident that the in vivo activity of lantibiotics cannot be assigned to a single factor, e.g., interaction with isolated cell wall precursors. Rather, it turns out that cell wall synthesis inhibition and pore formation may contribute differently to the antimicrobial activity depending on the target strain. Additionally, intrinsic factors of the target strains which have been shown to have a strong impact on the in vivo activity of antimicrobial peptides, e.g., surface net charge, phospholipid composition, and autolytic activity, among others, must be considered (1, 17, 20).

Remarkably, PlnC antimicrobial activities, which result in MICs in the nanomolar range against susceptible bacteria, are consistent with the structurally intermediate position of this peptide between the well-characterized type A and type B lantibiotics nisin and mersacidin, respectively. Therefore, PlnC appears to be a good model for in-depth structure-function studies aimed toward better understanding of the multiple biological activities of lantibiotics and, thereby, for designing novel food preservatives and powerful drugs against antibiotic-resistant pathogens.
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