

## Insight into Invertebrate Defensin Mechanism of Action

### OYSTER DEFENSINS INHIBIT PEPTIDOGLYCAN BIOSYNTHESIS BY BINDING TO LIPID II

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#### Abstract:

Three oyster defensin variants (*Cg-Defh1*, *Cg-Defh2*, and *Cg-Defm*) were produced as recombinant peptides and characterized in terms of activities and mechanism of action. In agreement with their spectrum of activity almost specifically directed against Gram-positive bacteria, oyster defensins were shown here to be specific inhibitors of a bacterial biosynthesis pathway rather than mere membrane-active agents. Indeed, at lethal concentrations, the three defensins did not compromise *Staphylococcus aureus* membrane integrity but inhibited the cell wall biosynthesis as indicated by the accumulation of the UDP-*N*-acetylmuramyl-pentapeptide cell wall precursor. In addition, a combination of antagonization assays, thin layer chromatography, and surface plasmon resonance measurements showed that oyster defensins bind almost irreversibly to the lipid II peptidoglycan precursor, thereby inhibiting the cell wall biosynthesis. To our knowledge, this is the first detailed analysis of the mechanism of action of antibacterial defensins produced by invertebrates. Interestingly, the three defensins, which were chosen as representative of the oyster defensin molecular diversity, bound differentially to lipid II. This correlated with their differential antibacterial activities. From our experimental data and the analysis of oyster defensin sequence diversity, we propose that oyster defensin activity results from selective forces that have conserved residues involved in lipid II binding and diversified residues at the surface of oyster defensins that could improve electrostatic interactions with the bacterial membranes.

**Keywords:** Antimicrobial Peptides, Bacterial Metabolism, Cell Wall, Defensins, Innate Immunity, Lipid-binding Protein, Mollusk, Plectasin, Pore-forming

## **Introduction**

Antimicrobial peptides (AMPs) are effector molecules of the innate immune system. Present in virtually all living organisms, they provide successful barriers against invading pathogens. Over the past decades, a wide variety of AMPs have been identified, giving evidence of a great diversity in terms of structure, size, and mode of action. Nevertheless, most AMPs are characterized by the prevalence of cationic and hydrophobic amino acids. It is well known that these peptides must display an appropriate balance of hydrophobicity and net positive charge, since their amphiphilic character is essential for their initial interaction with bacterial membranes (for review see (1,2)). Then, AMPs could display a variety of mechanisms of action, killing microbes by membrane disruption (e.g. pore formation) or by altering metabolic processes such as the septum

formation, or the cell-wall, nucleic-acid and protein syntheses (for review see (3-9)).

Defensins are 3–5 kDa AMPs that contain three to four disulfide bridges (4). They are broadly distributed in the animal and plant kingdom (2). Mammalian defensins ( $\alpha$ - and  $\beta$ -defensins) adopt a three stranded antiparallel  $\beta$ -sheet structure while the group of arthropod and plant defensins are composed of an  $\alpha$ -helix linked to an antiparallel two-stranded  $\beta$ -sheet by disulfide bridges, making the so-called cysteine-stabilized  $\alpha$ -helix/ $\beta$ -sheet motif (CS $\alpha\beta$ ). First evidenced in scorpion toxins, this motif is widespread in invertebrate defensins like arthropod and mollusk defensins (5-7), but is also found in plectasin, a defensin from the saprophytic ascomycete *Pseudoplectania nigrella* (8). CS $\alpha\beta$ -containing defensins are mostly active against Gram-positive bacteria (7-9). Conversely, mammalian  $\alpha$ - and  $\beta$ -defensins are usually both active against Gram-positive and Gram-negative bacteria (10).

The diverse spectra of activity of AMPs are believed to be indicative of different modes of action (5). However, the mechanisms of how defensins kill microorganisms are still incompletely understood. It is well established that the amphiphilic structure they adopt is crucial for the first interaction with the microbial surface (11). In addition, several defensins have been reported to damage bacterial and artificial membranes, including mammalian  $\alpha$ - and  $\beta$ -defensins (12,13), as well as arthropod defensins (14,15). However, non membrane-disruptive mechanisms of action have also been proposed, as for the  $\alpha$ -defensin HNP-1, which appears to transit across the cytoplasmic membrane with minimal disruption (13). Thus, over the past years, the debate has increased on how far membrane disruption accounts for the antimicrobial activity of defensins and other AMPs (16-18). Strictly antifungal defensins, which include defensins from plants and from lepidopteran insects, are not only membrane-disrupting agents but also interact with fungal glucosylceramides (19). Similarly, antibacterial defensins, which include mammalian, invertebrate (non lepidopteran) and fungal defensins, can be specific inhibitors of a bacterial biosynthesis pathway. For instance, the antibacterial activity of two mammalian and one

fungal defensins has been recently shown to result from an inhibition of peptidoglycan biosynthesis (20-22).

We have performed here a comparative study of the mechanism of action of antibacterial invertebrate defensins, the cellular targets of which are still unknown. For that, we used as a model three defensin variants characterized in the oyster *Crassostrea gigas*. One was identified from the oyster mantle (*Cg-Defm*) (7), and two others from the immune cells, the hemocytes (*Cg-Defh1* and *Cg-Defh2*) (23). The best studied is by far *Cg-Defm*. Like all oyster defensins, and mussel defensin (6), *Cg-Defm* exhibits four disulfide bonds. Nonetheless, its 3D-structure (7) is very similar to that of plectasin, which contains only three disulfide bonds (8). In a recent study, we showed that the diversity of oyster defensins has arisen through gene duplication and directional selection pressures, and that they cluster in three distinct groups of which *Cg-Defh1*, *Cg-Defh2* and *Cg-Defm* are the representatives (24). As for other antibacterial invertebrate defensins, almost nothing is known at present about oyster defensin mechanism of action.

Here, we asked whether the oyster defensin diversity had a functional relevance in terms of spectrum of activity and mechanism of action. Three representatives of the oyster defensin diversity were therefore expressed as recombinant peptides and studied for their antimicrobial activities and mechanism of action. We found that oyster defensins are mainly active against Gram-positive bacteria, with *Cg-Defh2* and *Cg-Defm* being noticeably more potent than *Cg-Defh1* against several Gram-positive strains. By a combination of *in vivo* and *in vitro* assays, including UDP-MurNAc-pp accumulation assays, thin layer chromatography, surface plasmon resonance, and NMR, we showed that all oyster defensins inhibit peptidoglycan biosynthesis by binding to lipid II. We propose that the residues involved in lipid II-binding have been conserved through evolution, and we show that residues conferring improved antibacterial activity to oyster defensins by modifying their charge distribution are under diversifying selection.

## MATERIALS AND METHODS

*Recombinant expression of Cg-Defs* - Recombinant Cg-Defh1 and Cg-Defh2 peptides were obtained according to the method described for recombinant Cg-Defm production with minor modifications (7). Recombinant Cg-Defh1 and Cg-Defh2 were expressed in *E. coli* Rosetta (DE3) as an N-terminal His6-tagged fusion protein using the pET-28a system (Novagen). By PCR amplification using the forward primer *Cgdefh-F* 5'-GCGCGAATTCATGGGATTTGGGTGTCCG-3', paired with reverse primer *Cgdefh-R* 5'-ATATATGTCGACCTTGAAAGATCTTTACTT C-3', a Met-coding trioxynucleotide was incorporated 5' of each cDNA of Cg-Defh1 and Cg-Defh2 and cloned in-frame with the N-terminal His6 in the EcoRI/SalI sites of pET-28a.

Expression of Cg-Defs was performed as described previously (7). Modifications in purification procedure were introduced to improve the production yield. Affinity chromatography was performed by incubating bacterial cell lysates with TALON® metal affinity resin (Clontech) at a ratio of 25:1 (v/v) in 6M guanidine HCl, 50mM sodium phosphate, 300mM NaCl, 5mM imidazole, (pH 8.5) for 4 h at 4 °C with gently agitation. Then, resin was washed twice by decantation in 6M guanidine HCl, 50mM sodium phosphate, 300mM NaCl (pH 8.5), and fusion proteins were eluted by decantation with two column volumes of 6M guanidine HCl, 50mM sodium phosphate and 1 M imidazole (pH 6.4). Elution was desalted using a reverse phase Sep-pak C-18 cartridge, where the peptide mixture was eluted with a step gradient of 5% and 80% of acetonitrile (ACN) / trifluoroacetic acid (TFA) 0.05%. The 80% ACN fraction containing the peptide mixture was then frozen and lyophilized. The methionine residue introduced at the peptide N-terminus was subjected to CNBr cleavage as described previously (7).

The cleaved peptide mixture was directly folded at pH 8.1 in a refolding solution containing 0.1 M NaHCO<sub>3</sub>, 3 mM reduced glutathione and 0.3 mM oxidized glutathione in the presence of 2 M urea and 25% N,N-dimethylformamide, at room temperature for 72 h. The peptide mixture containing Cg-Defs was then purified as described for Cg-Defm (7). Control of peptide purity was performed by RP-HPLC and mass spectrometry.

*Determination of Minimal Inhibitory Concentrations (MICs)* - Antimicrobial activity of oyster defensins was assayed against several bacteria including *Micrococcus lysodeikticus* CIP5345, *Bacillus megaterium* CIP 6620, *Staphylococcus aureus* CIP 103428, *Staphylococcus aureus* SG511, *Staphylococcus simulans* 22, *S. haemolyticus* (generous gift from P. Bulet), and *E. coli* SBS363. Marine strains were *Brevibacterium stationis* CIP 104228, *Microbacterium maritypicum* CIP 105733, *V. anguillarum* ATCC 19264, *V. nigrispulchritudo* CIP 103195 and the oyster pathogens *V. splendidus* CIP 107715 (also known as LGP32) and *V. aestuarianus* CIP 102971 (also known as LPi 02/41).

Minimum inhibitory concentrations (MICs) were determined in duplicate by the liquid growth inhibition assay based on the procedure described by Hétru and Bulet (25). MIC values are expressed as the lowest concentration tested that causes 100% of growth inhibition (µM). Poor broth (PB: 1% bactotryptone, 0.5% NaCl w/v, pH 7.5) nutrient medium was used for standard bacteria, and artificial sea water (ASW) (26) supplemented with 4 g/l bactopectone and 1 g/l yeast extract (referred to as Zobell medium) at a third strength was used for marine bacteria. Growth was monitored spectrophotometrically at 620 nm on a Multiscan microplate reader (Labsystems).

*Antagonization assays* - Different peptidoglycan precursors, namely undecaprenyl phosphate (C<sub>55</sub>P), UDP-N-acetylmuramyl-pentapeptide (UDP-MurNAc-pp), Lipid II, or UDP-N-acetylglucosamine (UDP-GlcNAc) were tested for antagonization of the oyster defensin antimicrobial activity. Basically, serial dilutions of defensins were performed from 0.25 to 8X MIC, each dilution being incubated in a microtiter plate with the peptidoglycan precursors in a 1:1, 1:2 or 1:5 molar ratio. *S.aureus* SG511 was then added to the microtiter plate as for a conventional MIC determination. Culture medium was half-concentrated Mueller-Hinton broth (Oxoid). After a 18 h-incubation at 37°C, the lowest peptide/peptidoglycan precursor molar ratio that antagonized the antimicrobial activity of the highest defensin concentration (8X MIC) was determined.

*Intracellular accumulation of the final soluble cell wall precursor UDP-N-acetylmuramyl-pentapeptide* - For analysis of the cytoplasmic peptidoglycan precursor pool, *S. aureus* SG511 was grown in half-concentrated Mueller-Hinton broth to an OD<sub>600</sub> of 0.5 and supplemented with 130 µg/ml of chloramphenicol, to prevent *de novo* synthesis of enzymes that may interfere, e.g. through induction of cellular autolysis, with the accumulation of the UDP-linked peptidoglycan precursor in the cytoplasm (27). After 15 min of incubation, each defensin was added at 10X MIC and incubated for another 30 min. Then, cells were extracted with boiling water for 15 min. The suspensions were cooled down and the cell extracts were adjusted to pH 2 with H<sub>3</sub>PO<sub>4</sub>. Insoluble components were removed by centrifugation at 13000 x g for 5 min and the supernatants were filtered through cellulose acetate filters (pore size 0.2 µm) and analyzed by RP-HPLC in 50 mM sodium phosphate buffer pH 5.2, developed in isocratic mode over 30 min at a flow rate 1 ml/min on a Nucleosil 100-C18 column (Schambeck SFD GmbH, Bad Honnef, Germany). UDP-linked cell wall precursors were analyzed and corresponding fractions were confirmed using matrix-assisted laser desorption ionization mass spectrometry (MALDI-TOF MS) in negative mode with 6-aza-2-thiothymine dissolved in 50% (v/v) ethanol/20 mM ammonium citrate as matrix.

*Determination of the membrane potential using TPP+* - *S. aureus* SG511 was grown in 50% Mueller-Hinton broth to an OD<sub>600</sub> of 0.5-0.6. To monitor the membrane potential, 1 µCi/ml of [<sup>3</sup>H]tetraphenylphosphonium bromide (TPP<sup>+</sup>; 26 Ci/mMol) was added (the lipophilic TPP<sup>+</sup> diffuses across the bacterial membrane in response to a trans-negative membrane potential). The culture was then treated with each defensin at 10X MIC (1.2, 2.5 and 5 µM *Cg*-Defh2, *Cg*-Defm and *Cg*-Defh1 respectively), and sample aliquots of 100 µl were filtered through cellulose acetate filters (pore size 0.2 µm) and washed twice with 5 ml of 50 mM potassium phosphate buffer. The filters were dried and placed into scintillation fluid, and the radioactivity was measured with a liquid scintillation counter. Non specific TPP<sup>+</sup> binding was determined by measuring the TPP<sup>+</sup> incorporation in cells pre-treated with butanol. The

pore-forming lantibiotic nisin (3.6 µM corresponds to 10X the MIC) was used as control. For calculation of the membrane potential ( $\Delta\psi$ ), the TPP<sup>+</sup> concentrations were applied in the Nernst equation ( $\Delta\psi$ ) = (2.3 X R X T/F) X log(TPP<sup>+</sup> inside/TPP<sup>+</sup> outside), where *T* is absolute temperature, *R* is the universal gas constant and *F* is the Faraday constant. Mean membrane potential values were calculated from the results of three independent experiments.

*In vitro synthesis and purification of peptidoglycan precursors* - Lipid II was prepared as described previously (28). Briefly, lipid II was synthesized using membrane preparations of *Micrococcus flavus* DSM 1790. Membranes were isolated from lysozyme-treated cells by centrifugation (40.000 x g), washed twice in 50 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, pH 7.5, and stored under liquid nitrogen until use. Membrane proteins (100 to 200 mg) were incubated with 2.5 µmol C<sub>55</sub>P, 25 µmol UDP-MurNAc-pp, and 25 µmol UDP-GlcNAc in 60 mM Tris-HCl, 5 mM MgCl<sub>2</sub>, pH 8, and 0.5% (w/v) Triton X-100. UDP-MurNAc-pp was purified as described previously (29). Bactoprenol-containing products were extracted with butanol/pyridine acetate (2:1; vol/vol; pH 4.2), purified as described and analyzed by TLC, using phosphomolybdic acid (PMA) staining (28). Reaction mixtures were incubated for 1 h at 30°C, and lipids were extracted with the same volume of *n*-butanol-6 M pyridineacetate (2:1, v/v), pH 4.2. Purification of lipid II was performed on a DEAE cellulose column (0.9 x 25 cm, DEAE SS type; Serva, Heidelberg, Germany) and eluted with a linear gradient of chloroform-methanol-water (2:3:1, v/v/v) to chloroform-methanol-300 mM ammonium bicarbonate (2:3:1, v/v/v).

*Thin Layer Chromatography (TLC)* - Lipid II (2 nmol) was vacuum-dried for 5 min at room temperature and *Cg*-Defh2, in aqueous solution, was added in molar ratios ranging from 0.1 to 1 with respect to an amount of lipid II. Reaction mixtures were vortexed and incubated at 30°C for 30 min and then applied onto TLC plates (TLC Silica Gel 60 F<sub>254</sub>, Merck). Plates were developed in butanol-acetic acid-water-pyridine (15:3:12:10, vol/vol/vol/vol), and stained with phosphomolybdic acid.

*Surface plasmon resonance (SPR)* - SPR experiments were carried out at 25°C on a BIACORE 2000 instrument (GEHealthcare, Biacore AB, Uppsala, Sweden). The binding studies were performed on L1 sensor chips (GEHealthcare) coated with lipids prepared as follows. Two kinds of small unilamellar vesicles (SUV) were prepared by sonication from 100% pure DOPC and from a mixture composed of DOPC and lipid II in a 99.2 : 0.8 molar ratio. SUVs were coated on Fc1 and Fc2 respectively. Lipid layers on L1 sensor chips were made according to the protocol previously described (30). The coating of the lipid layers gave a response in the range of 6000 to 6500 RU.

For kinetics, peptides were simultaneously injected at 250 mM on the two flow-cells at a flow rate of 30 µL/min. Dissociation was monitored over 400s in HBS running buffer (10 mM HEPES pH 7.4, 300 mM NaCl). New lipid layers were prepared for every injection to avoid the need for a regeneration step. The response of peptide binding on DOPC layers was taken as a negative control and subtracted from the response on Lipid II-containing DOPC layers. To determine the binding ratio, RU levels were measured at the plateau, just after the end of injection for the three defensins, nisin Z and tachyplesin. Two separate experiments were performed for each peptide injected. To compare the peptides in terms of binding, the response of Defh2 was given a 100 % value.

*NMR spectroscopy* - NMR experiments were performed on a Bruker Avance 700 spectrometer equipped with a triple resonance cryoprobe and pulse field gradients. The 500 µl sample containing 1 mg of *Cg-Defh2* was prepared in 95:5 H<sub>2</sub>O:D<sub>2</sub>O in a 10 mM phosphate buffer at pH 5.5. The carrier frequency was set at the water frequency. The resonance of water was suppressed by the WATERGATE method (31) and the <sup>1</sup>H reference spectrum of *Cg-Defh2* was recorded at 17°C and typically obtained with 128 scans. In a second experiment, the *Cg-Defh2* sample (350 µg in 500 µL, 10 mM phosphate buffer, pH 5.5) was poured into a vial containing a 1:1 stoichiometric amount of lipid II (75 nmol) initially solubilized in the chloroform/methanol 1:1 mixture. The <sup>1</sup>H spectrum of the *Cg-Defh2*-lipid II complex was recorded under the conditions used for *Cg-Defh2*

alone, so that both spectra could be compared. Data were processed using the XWINNMR programs.

*Sequence analysis and three-dimensional structure modelisation* - The molecular weight and isoelectric point of three defensins variants were calculated by the protein calculator program (<http://www.scripps.edu/~cdputnam/protcalc.html>) in which pI values are calculated assuming that all residues have pKa values equivalent to the isolated residues. Amino acid sequences were aligned with ClustalW2 ([www.ebi.ac.uk/clustalw/](http://www.ebi.ac.uk/clustalw/)).

Modelisation of *Cg-Defh1* and *Cg-Defh2* was performed by SWISS-MODEL (<http://swissmodel.expasy.org/>) by using the *Cg-Defm* structure as template (Protein Data Bank identifier 2B68). Modelisation was chosen over NMR determination since *Cg-Defh1* and *Cg-Defh2* share 84%, and 79% sequence identity with *Cg-Defm*. In addition, the 8 cysteines involved in four conserved disulfide bridges and the proline are found at conserved positions between the three variants (Figure 1). Model quality was evaluated by the assessment tools including in the software to estimate the reliability of the resulting model. Structure visualization was performed in the 3D molecule viewer, a component of vector NTI Advance 10 (Invitrogen). The structures obtained for *Cg-Defh1* and *Cg-Defh2* are very close to the *Cg-Defm* structure. From the superimposition of the backbone atoms, root mean square values were measured at 0.11 and 0.10 Å for *Cg-Defh1* and *Cg-Defh2*, respectively.

## RESULTS

*Oyster defensins are mainly active against Gram-positive bacteria.* Three variants of oyster defensins representative of the 3 phylogenetically distant groups previously described (24) were expressed as recombinant peptides in *E. coli*. Models of the *Cg-Defh1* and *Cg-Defh2* structures were built by using the *Cg-Defm* (PDB, 2B68) as a template (Fig. 1). The expression and purification of the three defensin variants was optimized as described in materials and methods. After folding and final purification, peptide purity was estimated above 95% by both RP-HPLC and mass spectrometry. The defensin variants were

then tested for antimicrobial activity. In the liquid growth inhibition assay, all recombinant oyster defensins were active at very low concentrations (10 nM range) against most of the Gram-positive bacteria tested but did not show significant antimicrobial activity (20  $\mu$ M and above) against Gram-negative bacteria, including oyster pathogens (Table 1). *Cg-Defh2* was the most potent, showing MIC values 2 to 4-fold lower than *Cg-Defh1* and *Cg-Defm* against most of the Gram-positive strains. The higher difference was observed between *Cg-Defh2* and *Cg-Defh1*, which displayed a 40-fold difference in MIC values against the marine bacterium *B. stationis* CIP 101282. Thus, while the three defensin variants displayed a similar spectrum of activity, *Cg-Defh2* and *Cg-Defm* were noticeably more potent than *Cg-Defh1* against the Gram-positive strains.

*Oyster defensins do not compromise membrane integrity of S. aureus.* We asked whether oyster defensins had deleterious effects on the bacterial membrane of Gram-positive bacteria by monitoring the membrane potential of *S. aureus* SG511 exposed to oyster defensins. The membrane potential was monitored for a period of 20 min after peptide addition from the distribution of the lipophilic cation TPP<sup>+</sup> inside and outside the bacterial cells after treatment. The pore-forming peptide nisin Z was used as a positive control. Oyster defensins did not induce any significant change in membrane potential when used at 10X MIC (*i.e.* 6, 12 and 24  $\mu$ g/ml for *Cg-Defh2*, *Cg-Defm* and *Cg-Defh1*, respectively) (Fig 2). However, at those concentrations, all three defensins induced a total loss of cultivability of *S. aureus* SG511 (data not shown). In contrast to oyster defensins, addition of nisin Z at 10X MIC (40  $\mu$ g/ml), induced a significant decrease of the membrane potential of *S. aureus* SG511 (Fig 2). Altogether, this indicates that at lethal concentrations (10X MIC), oyster defensins do not compromise the membrane integrity of *S. aureus* SG511.

*Oyster defensins are inhibitors of the peptidoglycan biosynthesis.* The antibacterial activity of oyster defensins being mostly directed against Gram-positive bacteria (table 1), we investigated their potential interference with the biosynthesis of peptidoglycan, which is readily

accessible in Gram-positives. This biosynthesis consists in a series of cytoplasmic reactions yielding soluble precursors of peptidoglycan followed by membrane-bound steps, which start with the anchoring of the last soluble precursor, UDP-N-acetylmuramyl-pentapeptide (UDP-MurNAc-pp) to the lipid carrier undecaprenylphosphate (C<sub>55</sub>P). Consequently, to analyze the interference of oyster defensins with peptidoglycan biosynthesis, we monitored the accumulation of UDP-MurNAc-pp. For that, we analyzed the cytoplasmic pool of soluble peptidoglycan precursors in cell extracts of defensin-treated *S. aureus* SG511.

RP-HPLC and mass spectrometry analysis of bacterial cell extracts showed that the three oyster defensins caused the intracellular accumulation of UDP-MurNAc-pp, in a similar manner to vancomycin (Fig 3). The continuous biosynthesis and subsequent defensin-induced accumulation of UDP-MurNAc-pp in the cytoplasm is indicative of the absence of leakage and therefore consistent with the absence of membrane damage observed above. Moreover, it strongly suggests that oyster defensins are inhibitors of peptidoglycan biosynthesis. Since the synthesis of UDPMurNAc-pp itself is not impaired, the inhibition by oyster defensins should occur at the membrane-bound steps of the biosynthesis.

*Lipid II has antagonist effect on oyster defensin antibacterial activity.* Antagonization assays were performed to identify the putative targets of oyster defensins in peptidoglycan biosynthesis. Briefly, after the anchoring of UDP-MurNAc-pp to C<sub>55</sub>P, the resulting lipid I is converted into lipid II by addition of N-acetylglucosamine (GlcNAc). Lipid II is subsequently translocated across the cytoplasmic membrane, polymerized by transglycosylation, and finally cross-linked by transpeptidases, which form the peptidoglycan network (32). Therefore, several intermediates of the peptidoglycan biosynthesis, namely Lipid II, C<sub>55</sub>P, UDP-MurNAc-pp and UDP-GlcNAc, were used as potential antagonists in the liquid growth inhibition assay. Lipid II was the only compound that prevented the oyster defensin antimicrobial activity (table 2). Indeed, when used at a 1:1 molar ratio, oyster defensins became unable to inhibit the growth of *S. aureus* SG511 at concentrations as

high as 8X MIC (table 2). Neither activated cell wall sugars (UDP-MurNAc-pp and UDP-GlcNAc) nor the lipid carrier undecaprenylphosphate, (C<sub>55</sub>P) had similar antagonist effects (table 2).

*Oyster defensins bind irreversibly to the bacterial cell wall precursor lipid II.* The binding of oyster defensins to lipid II was first assayed by thin layer chromatography (TLC) by incubating the oyster defensins with lipid II at molar ratios ranging from 1:0.1 to 1:2. With the addition of increasing amounts of oyster defensins, the free lipid II band disappeared from the TLC plate and became undetectable at a 1:1 molar ratio, as shown in Fig. 4A for Cg-Defh2. This strongly suggested that lipid II and oyster defensins bind to each other in a 1:1 stoichiometry. Interestingly, the lipid II:Cg-Defh2 complex could not be recovered after a 1:1 butanol / pyridine acetate extraction suggesting the formation of an insoluble complex in aqueous solution (data not shown).

Surface plasmon resonance (SPR) experiments were then performed to fully demonstrate the interaction and obtain quantitative data on the binding of the three oyster defensin variants. We used small unilamellar vesicle (SUV) in our experimental design to avoid the formation of lipid II micelles in aqueous solution. SUV of DOPC / 0.2 mol % lipid II, and DOPC / 0.8 mol % lipid II were prepared and immobilized on a SPR chip for interaction assays. We compared the binding of oyster defensins to that of nisin Z (positive control), and of tachyplesin, an invertebrate LPS-binding antimicrobial peptide (negative control).

While little to no binding of oyster defensins was observed on DOPC vesicles (data not shown), a significant binding of the three defensin variants was observed on DOPC / 0.8 mol % lipid II vesicles (Fig 4B). Overall, interaction kinetics were similar for defensins and nisin Z, although they differed by their association phase (Fig 4B). The binding of oyster defensins appeared rather irreversible with no decay in the RU values over time. Similar results were obtained on DOPC / 0.2 mol % lipid II vesicles although signals were less intense (data not shown). As expected, tachyplesin did not bind to the DOPC / 0.8 mol % lipid II vesicles, and even gave a negative signal most likely due to the membrane disruption properties of tachyplesin (33). In two independent experiments, the three oyster defensins displayed higher binding to lipid II than nisin Z used as a

positive control (2- to 4-fold higher RU values) (Fig 4C). Interestingly, differential binding was also observed among the oyster defensin variants. Indeed, Cg-Defh2 and Cg-Defm, which are also the most active (table 1), gave similar association levels to DOPC / 0.8 mol % lipid II vesicles, with a 100% and 92% mean response, respectively (Fig 4C). By comparison, the mean response of Cg-Defh1, which is the less active variant (table 1), was calculated at 48%. Altogether, this shows that oyster defensins are strong ligands of lipid II and that differential binding occurs between variants.

In order to further explain the molecular basis of the differential binding of the defensin variants to lipid II, we performed an NMR study in which the Cg-Defh2 <sup>1</sup>H spectrum was recorded in the absence and in the presence of lipid II (1:1 molar ratio). While both lipid II and Cg-Defh2 were soluble in phosphate buffer, they precipitated as soon as they were mixed together. Indeed, the Cg-Defh2 signals disappeared uniformly with the addition of lipid II, preventing us from identifying the amino acids that specifically bind lipid II (data not shown). The defensin was found associated with lipid II giving rise to an insoluble complex.

## DISCUSSION

Results showed that oyster defensins kill *S. aureus* through binding to lipid II, which results in inhibition of peptidoglycan biosynthesis. To our knowledge, this is the first detailed description of the mechanism of action of an antibacterial defensin from invertebrates. Indeed, until now, only invertebrate defensins with antifungal activity (lepidopteran defensins) had been characterized in terms of cellular targets (19). The trapping of peptidoglycan precursors is common to many antimicrobials including glycopeptides, lipopeptides, lipodepsipeptides, lantibiotics, and other AMPs (34). However, such a mechanism of action was only very recently identified for one fungal defensin named plectasin (20) and two mammalian defensins representative of the  $\alpha$ -defensins, namely HNP-1 (21), and  $\beta$ -defensins, namely HBD-3 (22). The best-documented AMP in this respect is probably Nisin Z which combines a pore-forming activity and the trapping of lipid II (35). It was used as a positive control throughout this study.



We showed here that oyster defensins inhibit the peptidoglycan biosynthesis by trapping its membrane-bound precursor, lipid II. Indeed, incubation of *S. aureus* with oyster defensins resulted in the accumulation of the last soluble precursor of peptidoglycan, UDP-MurNAc (Fig. 2). Moreover, lipid II antagonized oyster defensin activity against *S. aureus* whereas undecaprenylphosphate (C<sub>55</sub>P), which serves as a lipid carrier in Lipid II, as well as UDP-MurNAc and UDP-GlcNAc, the cell wall sugars involved in the disaccharide moiety of lipid II, did not (table 2). Antagonization by lipid II occurred at a 1:1 molar ratio (table 2), as also observed for nisin Z (36) and for mammalian  $\beta$ -defensin HBD-3, although the latter was also antagonized by C<sub>55</sub>P and negatively charged phospholipids when added in up to a 5 fold molar excess (22).

Altogether this suggests that targeting of lipid II by oyster defensins requires lipid II-specific molecular determinants such as the pentapeptide side chain, which is targeted by the glycopeptide antibiotics (*e.g.* vancomycin), or the disaccharide-pyrophosphate moiety, which is targeted by the lantibiotics (34). Based on the recent study of plectasin (20), a peptide highly similar to oyster defensins, we tend to favor the latter hypothesis. Unfortunately, we were unable to determine those molecular determinants by NMR due to the insolubility of the Cg-Defh2/lipid II complex in aqueous buffer. Such an insolubility was previously reported for another invertebrate AMP referred to as anti-lipoplysaccharide factor, which precipitated when incubated in presence of lipid A, used as a docking molecule at the surface of Gram-negative bacteria (37). Such precipitations can result from the hiding of the peptide charges at the peptide-lipid interface of the complex giving rise to an unfavorable pI value for the solubility of the complex. The disappearance of the defensin and lipid II signals from the NMR spectra occurred at a 1:1 molar ratio, in agreement with the molar ratio required for antagonization of oyster defensin antibacterial activity (table 2). Both experiments support the formation of a 1:1 stoichiometric insoluble complex in aqueous buffer. That stoichiometry, which was also observed for plectasin:lipid II complex formation (20), was also confirmed here by TLC, as indicated by the absence of free lipid II on the plate at this molar ratio (Fig 4A).

The binding of oyster defensin to lipid II was shown to be very strong, as determined here by SPR. First, oyster defensins bound to DOPC vesicles containing 0.8 mol% lipid II in an almost irreversible manner, as indicated by the absence of decay in the RU values during the dissociation step (Fig. 4B & C). This prevented us from calculating dissociation constants. Since the binding of oyster defensins was dependent on the lipid II molar concentration in the DOPC vesicles, and was undetectable in the absence lipid II-free vesicles (data not shown), it was assessed that the binding is due to lipid II only. Second, all defensin variants (Cg-Defm, Cg-Defh1, and Cg-Defh2) gave binding responses 2 to 4 times higher than nisin Z, used here as a positive control (Fig. 4B & C). As in the present study, the binding of HNP-1 to lipid II was also recently evidenced by SPR (21). However, we cannot compare both studies since in the HNP-1 study, the peptide was immobilized on the SPR sensor chip to monitor the binding of soluble lipid II, which most likely forms micelles in such an aqueous solution.

From our results, the binding of oyster defensins to lipid II likely occurs at the outer leaflet of the cytoplasmic membrane, *i.e.* extracellularly when referring to Gram-positive bacteria. Indeed, at lethal concentrations (0.1  $\mu$ M, *i.e.* 10 x MIC), oyster defensins did not damage the cytoplasmic membrane of *S. aureus* (Fig. 3). For this reason, they should have no access to the inner leaflet of the cytoplasmic membrane where lipid II is synthesized and decorated with a pentaglycine interpeptide bridge, before it is translocated across the cytoplasmic membrane. From this result, we think that the mechanism of action of oyster defensins involves trapping and sequestration of extracellular lipid II, thus blocking its polymerization into mature peptidoglycan.

Consistent with the use of lipid II as a molecular target, oyster defensins have been shown here to be highly active against Gram-positive bacteria (10 nmolar range) and barely active (above 10  $\mu$ M) against Gram-negative bacteria (table 1). Indeed, in Gram-negative bacteria the peptidoglycan is protected by the outer membrane preventing access of oyster defensins to the periplasmic space in the absence of membrane damages. As a consequence, we think that the different susceptibility of Gram-

positive and Gram-negative bacteria to oyster defensins results from a differential access to lipid II, which is readily accessible (extracellular) in Gram-positive bacteria, and which requires outer membrane damages to become accessible in Gram-negative bacteria. Such membrane damages can be created by high concentrations of oyster defensins, as observed above 10  $\mu$ M against the Gram-negative oyster pathogen, *V. splendidus* LGP32 (38).

Interestingly, the mammalian  $\alpha$ -defensin HNP-1 (21) and human  $\beta$ -defensin HBD3 (22), were recently found to bind lipid II. However, unlike invertebrate defensins, mammalian defensins are both active against Gram-positive and Gram-negative bacteria (12,13,39). If both mammalian and invertebrate antibacterial defensins have a similar target, what makes the invertebrate defensins so selective towards Gram-positive bacteria? One hypothesis is that unlike invertebrate antibacterial defensins, which require lipid II to be readily accessible, mammalian defensins could use their membrane-disrupting properties (40,41) to gain access to Lipid II. Indeed, the membrane activity of invertebrate defensins appears much weaker than that of mammalian defensins. This was shown here for oyster defensins (Fig 3), and earlier for the *Drosophila melanogaster* defensin A, which required very high defensin / bacterial cell ratio ( $10^5$ :1) to induce potassium efflux in *Micrococcus luteus* (15).

As shown here for oyster defensins, evidences are increasing that invertebrate defensins, and more generally  $Cs\alpha\beta$ -containing defensins are specific inhibitors of a bacterial biosynthesis pathway rather than mere membrane active agents. This was shown previously for purely antifungal defensins from plants and from lepidopteran insects (heliomicin) (42), all of which interact with fungal glucosylceramides (19). From the present study and the recent plectasin study (20), antibacterial defensins carrying a  $Cs\alpha\beta$  motif could have a similar mechanism of action, which involves Lipid II-trapping and subsequent inhibition of peptidoglycan biosynthesis. This is consistent with their preferential activity against Gram-positive bacteria (5,8,43,44). The example of heliomicin, which does not bind to lipid II (20) and lacks antibacterial activity (42), reinforces our

thesis that lipid II-binding is an essential determinant of invertebrate defensin antibacterial activity. The binding of plectasin to lipid II was proposed to involve two cysteines engaged in one disulfide bond and two additional amino acids at the N-terminus (20). Although sharing only 54 to 58% of sequence identity with plectasin (data not shown), the four residues are conserved in the oyster defensin sequences (Fig 1). Therefore, as in plectasin, Phe2, Gly3, Cys4 and Cys25 could be essential determinants of oyster defensin binding to lipid II. Other (yet unidentified) residues from the oyster defensin sequence could also be involved in lipid II-binding, thus explaining the differential affinity of the three variants for this peptidoglycan precursor.

The present study supports the hypothesis that strong selective pressures have directed oyster defensins towards the design of new variants displaying higher potency. Indeed, some amino acids of the oyster defensin sequences have been shown to be the subject of diversifying selection (24). Interestingly, the most active defensins, *Cg-Defm* and *Cg-Defh2* (Table 1), display a charged residue at one such position (Lys16 and Arg16, respectively), instead of Gly16 in *Cg-Defh1*. Those residues are highly exposed at the surface of oyster defensins (Fig 1). Similarly, an additional lysine on the surface of the plectasin improved its antibacterial activity, probably by promoting a better binding to the cell wall and membrane of target bacteria (20). Therefore, variations in oyster defensin potency could depend on charge distribution driven by sites under diversifying selection. This is supported by the finding that the difference in potency among oyster defensins are much higher against the marine strain *B. stationis* (40 fold) than against all other bacteria (less than 8 fold), which unlikely belong to the oyster alochtone microflora and have unlikely co-evolved with the oyster immune system.

Aside from those sites of diversification, oyster defensin variants have highly conserved residues (24), three of which (Gly3, Cys4 and Cys25) (Fig. 1) are involved in the plectasin-lipid II interaction. Such a purifying selection is characteristic of strong functional constraints (*e.g.* residues essential for the peptide activity) (45). In conclusion, from this and our previous evolutionary study (24), we propose that oyster defensin activity results from selective forces that

have, on the one hand, conserved the residues involved in lipid II-binding (positions 3, 4 and 25), and on the other hand, diversified residues (position 16) that could improve electrostatic interactions with the negatively-charged membranes of bacteria.

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## FOOTNOTES

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**Table 1.** Antimicrobial activities of recombinant oyster defensins. MIC values are expressed as the lowest concentration tested that causes 100% of growth inhibition ( $\mu\text{M}$ ).

MIC ( $\mu\text{M}$ )	<i>Cg-Defh2</i>	<i>Cg-Defm</i>	<i>Cg-Defh1</i>
<b>Gram-positive bacteria</b>			
<i>M. lysodeikticus</i> CIP 5345	0.01	0.01	0.03
<i>B. megaterium</i> CIP 6620	0.03	0.03	0.06
<i>S. aureus</i> CIP 103428	0.25	2	2
<i>S. aureus</i> SG511	0.12	0.25	0.5
<i>S. simulans</i> 22	0.5	1	2
<i>S. haemolyticus</i>	2	2	6
<i>M. maritopicum</i> CIP 105733T	1	1	2
<i>B. stationis</i> CIP 101282	0.1	0.2	4
<b>Gram-negative bacteria</b>			
<i>V. splendidus</i> CIP 107715	>40	>40	>40
<i>V. aestuarianus</i> CIP 102971	>40	>40	>40
<i>V. anguillarum</i> ATCC 19264	>40	>40	>40
<i>E. coli</i> SBS 363	20	20	40
<i>V. nigripulchritudo</i> CIP103195	>40	>40	>40

**Table 2.** Antagonization of antimicrobial activity of recombinant oyster defensins against *S. aureus* SG511 by putative target molecules of cell wall biosynthesis. +: antagonization, -: normal antimicrobial activity. Antagonists were added at diverse molar ratio with respect to each peptide. The lower molar ratio at which the antagonization occurred is given.

Antagonist	<i>Cg-Defh2</i>	<i>Cg-Defm</i>	<i>Cg-Defh1</i>	molar ratio antagonist/ <i>Cg-Defh</i>
$\text{C}_{55}\text{P}$	-	-	-	-
Lipid II	+	+	+	1:1
UDP-MurNAc-pp	-	-	-	-
UDP-GlcNAc	-	-	-	-

## FIGURE LEGENDS

**Fig. 1.** Three dimensional structure and amino acid sequences of *Cg*-Defs. Comparison of the *Cg*-Defh2 and *Cg*-Defh1 structures obtained by molecular modelling from the *Cg*-Defm structure (PDB 2B68). **A.** 3D-structure showing the distribution of the cationic (blue), and anionic (red) residues for the oyster defensins. Cysteine residues are in yellow. **B.** Amino acid alignment of three oyster defensin variants showing cationic (blue) and anionic (red) amino acids. The calculated molecular weight and estimated isoelectric point (pI) of every defensin is given on the right. Arrows point out at charged amino acids under diversifying selection (24). Asterisks indicate amino acids conserved in plectasin that are involved in the plectasin-lipid II interaction (20).

**Fig. 2.** Lack of membrane depolarisation in oyster defensin-treated *S. aureus* SG511. *S. aureus* loaded with [<sup>3</sup>H]-tetraphenylphosphonium bromide were incubated with 10X MIC of *Cg*-Defm *Cg*-Defh2, *Cg*-Defh1, or the pore-forming nisin Z. The intracellular [<sup>3</sup>H]-tetraphenylphosphonium bromide concentration was determined 1, 5 and 20 min after peptide addition, and the membrane potential was calculated according to the Nernst equation. Histograms indicate *S. aureus* membrane potential before (Ctrl, white bars) and two times after the addition of each peptide (light and dark grey bars).

**Fig. 3.** Accumulation of the UDP-MurNAc-pp peptidoglycan precursor in *S. aureus* SG511 treated with oyster defensins. *S. aureus* SG511 were exposed to oyster defensins (A) or vancomycin (positive control, B) at 10X MIC. The negative control (B) corresponds to untreated *S. aureus* (i.e. w/o inhibitor). The cytoplasmic pool was analysed by RP-HPLC on a Nucleosil 100-C18 column under isocratic conditions. Intracellular accumulation of the final soluble cell wall precursor UDP-MurNAc-pp (arrow) was confirmed by mass spectrometry (data not shown).

**Fig. 4.** Binding of oyster defensin to lipid II. The oyster defensin / lipid II complex formation was studied by TLC (A) and SPR (B and C). In the TLC experiment, *Cg*-Defh2 was added to lipid II in molar ratios ranging from 1:0.1 to 1:1. After migration, the TLC plate was stained with phosphomolybdic acid. Whereas free lipid II migrates in the TLC plate, *Cg*-Defh2 and the *Cg*-Defh2/lipid II complex remain at the application spot. In the SPR experiment, the sensorgrams (B) depict the interaction of oyster defensins, nisin Z (positive control) and tachyplesin (negative control) with immobilized DOPC vesicles containing 0.8 mol % lipid II. The control sensorgrams (peptide interaction with immobilized DOPC vesicles) were subtracted from the data presented. The data from two independent experiments (two different liposome preparations) are summarized in (C), where the binding of each peptide to the lipid II-containing vesicles is expressed as a percentage of *Cg*-Defh2 binding (100 %).

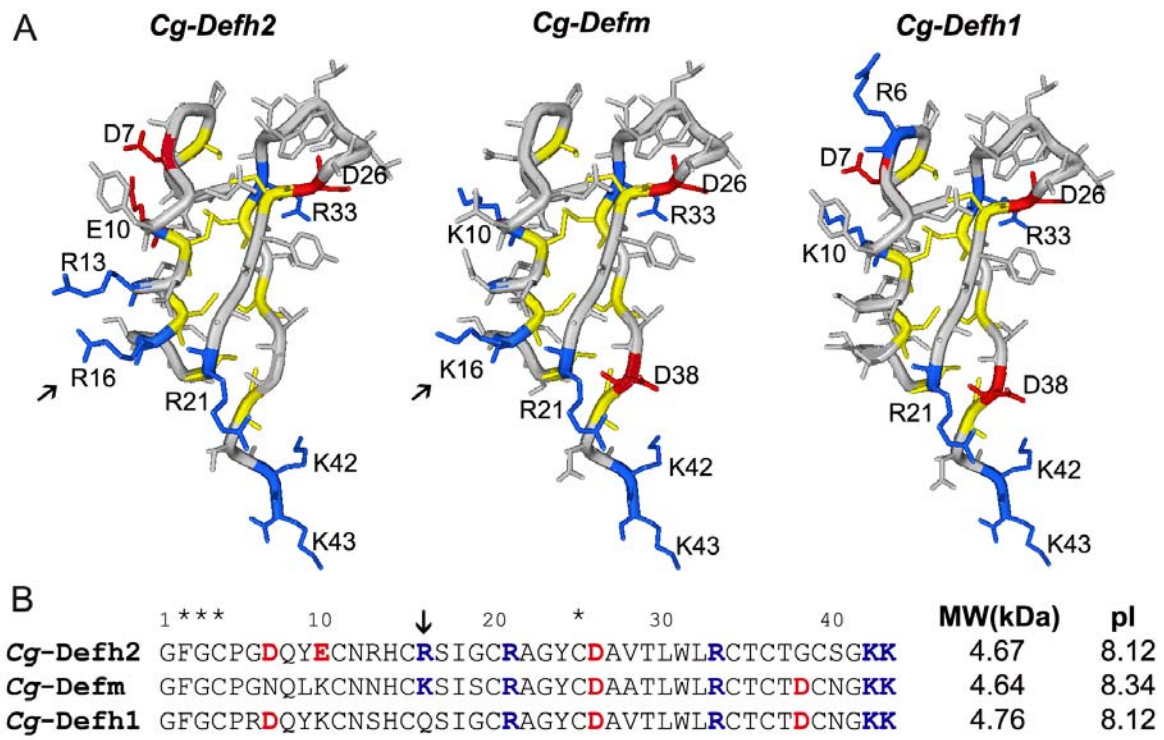


Figure 1

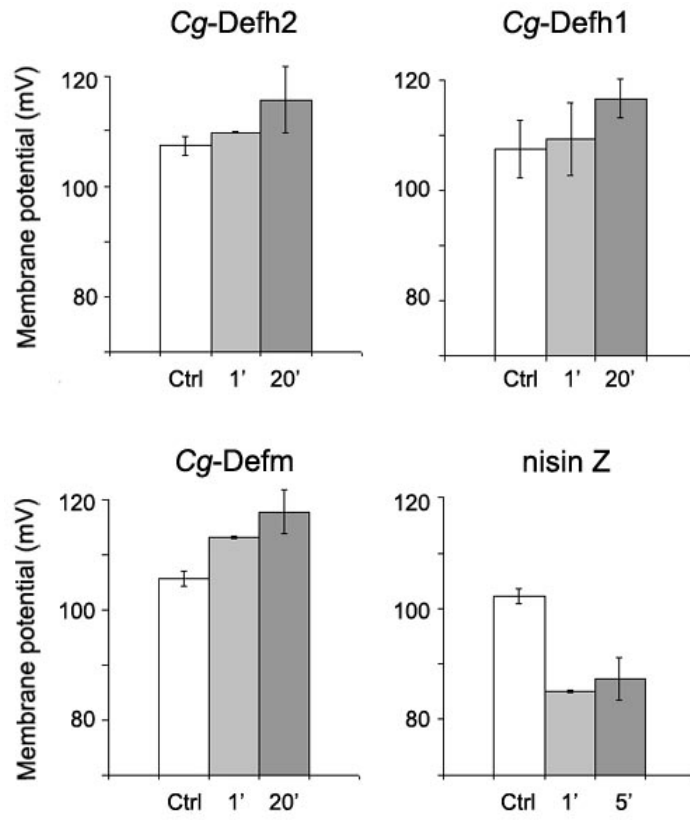


Figure 2



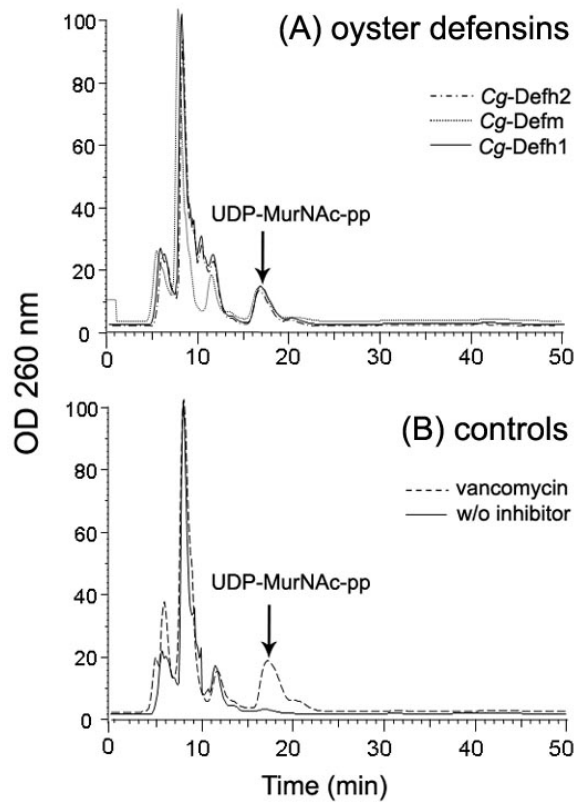


Figure 3

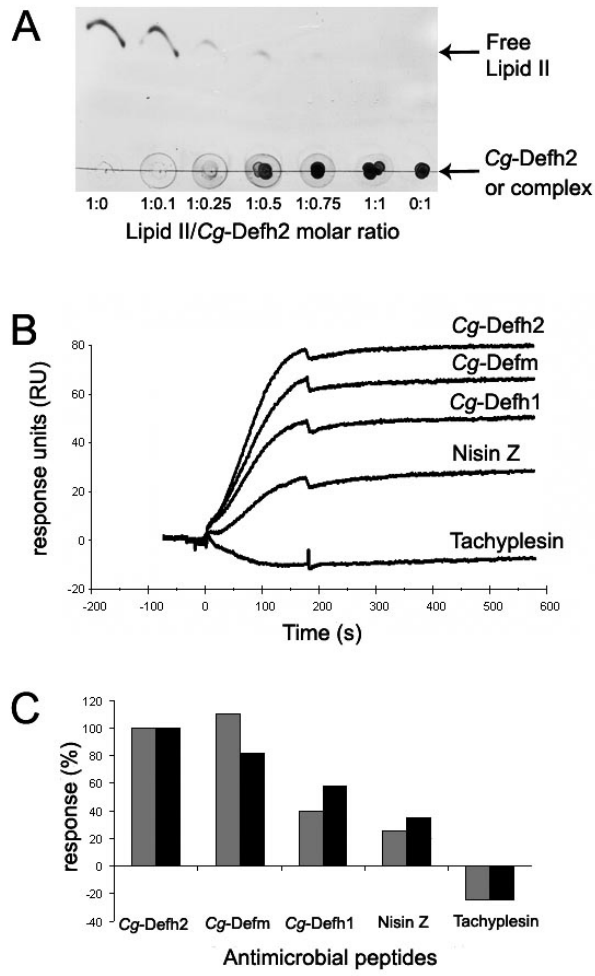


Figure 4