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## **An intimate link between antimicrobial peptide sequence diversity and binding to essential components of bacterial membranes**

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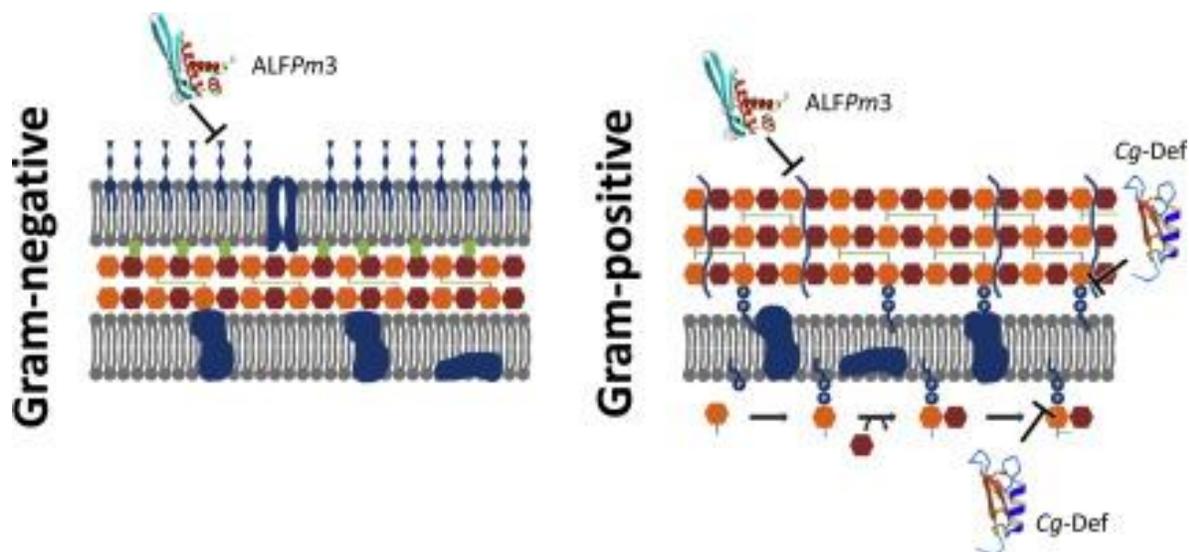
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### **Abstract :**

Antimicrobial peptides and proteins (AMPs) are widespread in the living kingdom. They are key effectors of defense reactions and mediators of competitions between organisms. They are often cationic and amphiphilic, which favors their interactions with the anionic membranes of microorganisms. Several AMP families do not directly alter membrane integrity but rather target conserved components of the bacterial membranes in a process that provides them with potent and specific antimicrobial activities. Thus, lipopolysaccharides (LPS), lipoteichoic acids (LTA) or the peptidoglycan precursor Lipid II are targeted by a broad series of AMPs. Studying the functional diversity of immune effectors tells us about the essential residues involved in AMP mechanism of action. Marine invertebrates have been found to produce a remarkable diversity of AMPs. Molluscan defensins and crustacean anti-LPS factors (ALF) are diverse in terms of amino acid sequence and show contrasted phenotypes in terms of antimicrobial activity. Their activity is directed essentially against Gram-positive or Gram-negative bacteria due their specific interactions with Lipid II or Lipid A, respectively. Through those interesting examples, we discuss here how sequence diversity generated throughout evolution informs us on residues required for essential molecular interaction at the bacterial membranes and subsequent antibacterial activity. Through the analysis of molecular variants having lost antibacterial activity or shaped novel functions, we also discuss the molecular bases of functional divergence in AMPs.

## Graphical abstract



## Highlights

► Specific interaction with Lipid II/Lipid A determines activity of many AMP families ► Diverse amino acid sequences in conserved scaffolds generate functional diversity ► The main mechanisms generating AMP sequence diversity are reviewed ► Mechanisms of resistance are indicative of the essential components targeted by AMPs ► We illustrate this with CS $\alpha$  $\beta$ -defensins and anti-lipopolysaccharide factors families

**Keywords :** Functional diversity, Defensin, Anti-lipopolysaccharide factor, Mechanism of action, Resistance

## 1. Introduction

Antimicrobial peptides and proteins (AMPs) are produced by virtually all species from the living kingdom. Through their antimicrobial properties, they participate in host defense and/or microbial competitions. The established hallmark of a wide range of AMPs is the sharing of amphiphilic and cationic properties, which enable interactions with the negatively-charged bacterial membranes. While AMPs have long been considered as essentially membrane active agents disrupting phospholipid bilayers by diverse means [1], the more recent literature has shown that several AMP families do not directly alter membrane integrity but rather target essential (conserved) components of the bacterial membranes in a process that provides them with potent and specific antimicrobial activities [2]. Thus, antibacterial peptides of diverse origins (both prokaryotic and eukaryotic) have evolved the capacity to bind to given membrane receptors (e.g. microcins that use iron-siderophore receptors to penetrate into enterobacteria) [3] or essential components of the membrane of Gram-positive and Gram-negative bacteria [2]. Thus, lipopolysaccharides (LPS), or their lipid component Lipid A, are targeted by broad series of AMPs of highly diverse structures and origins. These include the well-known polymyxin B from the Gram-positive *Bacillus polymyxa* [4], human  $\beta$ -defensins [5], invertebrate anti-LPS factors [6,7], or Bactericidal/permeability-increasing proteins, which contribute to the defense of both vertebrates and invertebrates [8,9]. Another highly conserved molecular target for AMPs is the peptidoglycan precursor, Lipid II, which is targeted by a broad series of defensins but also well-known antibiotics like vancomycin [10,11]. Similar to the previously mentioned antibacterial peptides, antifungal peptides can also bind essential membrane components such as phospholipids and sphingolipids at the membrane of fungi in a process that leads to fungal cell death [12]. Such interactions that take place at the surface of microorganisms involve highly conserved molecular patterns specific of given phyla of microorganisms and are required for AMP activity. It is now admitted that the co-evolution between immune systems and bacteria has given rise to a rapid diversification of immune effectors and bacterial resistance mechanisms involving modification of essential membrane components (LPS, teichoic acids...). Thus, studying the functional diversity of immune effectors tells us about the essential residues involved in AMP mechanism of action. Similarly, the rapidly increasing knowledge on bacterial membrane modifications conferring resistance to AMPs tells us about those conserved structures targeted by AMPs.

Over the past years, marine invertebrates of different phyla (mollusks, crustaceans) have been found to produce an remarkable diversity of AMPs [13]. Two interesting examples are provided by (i) molluscan defensins, which belong to a broadly conserved family of antibacterial peptides present in phyla as diverse as arthropods, plants, fungi, and (ii) crustacean ALFs, which are restricted to crustaceans and marine chelicerates (horseshoe crabs). These two families of AMPs are remarkably diverse in terms of amino acid sequence and show contrasted phenotypes in terms of antimicrobial activity. While both families are active against multiple types of microorganisms [14,15], molluscan defensins and crustacean ALFs are remarkably more potent against Gram-positive and Gram-negative bacteria, respectively. This specificity is due their specific interactions with Lipid II and Lipid A, respectively. Through those interesting examples, we discuss here how sequence diversity generated throughout evolution informs us on residues required for essential molecular interaction at the bacteria membrane and subsequent antibacterial activity. By comparing to molecular variants having lost antibacterial activity or shaped novel functions through the selection of other residues in a similar structural scaffold, we also discuss the molecular basis of the “neofunctionalization”.

## 2. Sequence diversity of AMPs

Antimicrobial peptides and proteins (AMPs) are recognized as fundamental effectors of the innate immune response. AMPs are found in virtually all organisms, where they exhibit diverse roles in immunity (reviewed in [16]). One of the most recognized role is the microbicidal activity, but they also display immunomodulatory functions such as modulation of cytokine production, chemotactic activity and wound healing, among others (reviewed in ([17]). Over the past years, a high and variable level of sequence diversity has been reported to be a characteristic of several families of AMPs from both vertebrates and invertebrates. There is widespread evidence that all species present a particular arsenal of diversified AMP families [18,19] and it is proposed that the diversity of AMPs in a host may play a role in determining the pathogenicity of a microbe in that species [20] or in shaping its commensal microbiota [21]. Indeed, as beautifully demonstrated in early metazoans (*Hydra*), distinct panels of AMPs determine the diversity of bacterial commensals associated to the host [21]. Similarly, intestinal AMPs shape the structure of the mammalian gut microbiota [22]. It is generally believed that different variants in an AMP family are the result of the functional divergence of isoforms to extend the antimicrobial spectra [23] or acquire novel immune functions. Therefore, great efforts have been done to identify the exclusive arsenal of AMPs that each species can develop and to further recognize the bases of this AMP diversity. Newly identified peptides have been often named after the name of the species they were isolated from rather than after structurally-related AMPs. The lack of a consistent nomenclature for AMPs does not help to identify structural and evolutionary relationships between AMPs isolated from different species. Although some efforts have been made over the past years in the proposal of sequence-based nomenclatures [18,24,25], much remains to be done to define an internationally admitted nomenclature. Nonetheless, different patterns of diversification can be found among AMP families. On the one hand, some AMP families widely conserved throughout evolution, such as defensins, are found in an extensive spectrum of phyla [26]. On the other hand, families including several AMP variants can be restricted to only few species belonging to close phylogenetic groups, such as Anti-lipopolysaccharide factors (ALFs).

### 2.1. Defensins

Defensins are the most well-known and widely distributed family of AMPs described so far. These host defense peptides were first discovered in the mid 1980's in human neutrophils [27]

and then identified in many groups of multicellular eukaryotes, including fungi, plants and both vertebrate and invertebrate animals. Besides their antimicrobial functions, defensins can also play an important role in many adaptive immune responses, such as neutrophil recruitment, cytokine production and release, phagocytosis enhancement and others [28]. Structurally, they are cationic peptides containing six to eight cysteine residues engaged in three to four intramolecular disulfide bonds. In some rare cases, a fifth disulfide bond has been evidenced. In plant defensins, it is supposed to stabilize further the peptide structure by replacing non-covalent hydrophobic interactions or hydrogen bonds with a covalent bond [29,30]. All defensins contain a  $\gamma$ -core: a unifying structural signature present in virtually all known classes of cysteine-stabilized antimicrobial peptides, which consists in an anti-parallel  $\beta$ -sheet containing the conserved Gly-Xaa-Cys triad [31]. The  $\gamma$ -core structure is proposed to be an archetypal membrane-interaction motif, which highlights the importance of 3D-structures in defensin functions. Based on the spacing and the pairing of their cysteine residues (disulfide bond arrangement), defensins can be classified into different subfamilies [32], which may range from open-ring small peptides ( $\alpha$ - and  $\beta$ -defensins and CS $\alpha\beta$  defensins) to cyclic ( $\theta$ -defensins) or multi-domain polypeptides (big defensins).

Members of a defensin subfamily can have a wide phylogenetic distribution or be restricted to some specific species [33,34]. For instance, while  $\alpha$ -defensins are exclusively found in some mammals (human, mouse, rabbit, hamster, horse) and  $\theta$ -defensins only in Old World primates (rhesus macaque, orangutans, lesser apes),  $\beta$ -defensins are present in virtually all vertebrates (from fish to mammals) [34] and also in some invertebrate species [35,36]. Moreover, pseudogenes for  $\theta$ -defensins are found in humans [37]. In contrast, big defensins are present in some marine invertebrates (horseshoe crabs, mollusks and amphioxus) and CS $\alpha\beta$  defensins in a broad diversity of species including filamentous fungi, vascular plants and different invertebrate groups, such as chelicerates, insects and mollusks [38,39].

**Vertebrate open-ring defensins** share a common conformational structure, consisting of three anti-parallel  $\beta$ -strands stabilized by three disulfide bonds. The cysteine pairing is Cys<sub>1-6</sub> Cys<sub>2-4</sub> Cys<sub>3-5</sub> and Cys<sub>1-5</sub> Cys<sub>2-4</sub> Cys<sub>3-6</sub> for  $\alpha$ -defensins and  $\beta$ -defensins, respectively [40]. **Cyclic  $\theta$ -defensins** from primates are closely related to  $\alpha$ -defensins, which are the result of the cyclization of two small  $\alpha$ -defensin-like precursors of nine amino acids each containing three cysteine residues [41]. Comparatively, **big defensins** are multi-domain polypeptides composed of a hydrophobic N-terminal globular domain and a C-terminal region containing six cysteine residues whose

arrangement is identical to that found in  $\beta$ -defensins [42]. A common origin has been proposed for big defensins from invertebrates and defensins from vertebrates ( $\alpha$ -,  $\beta$ - and  $\theta$ -defensins) [34].

**CS $\alpha\beta$  defensins** (previously referred to as insect or invertebrate defensins) cover a distinct and diverse group of defensins with a no clear phylogenetic relationship with the other defensin subfamilies [38] (**Figure 1**). These molecules contain from six to eight cysteine residues and adopt a characteristic structure composed of an  $\alpha$ -helix connected to an anti-parallel two-stranded  $\beta$ -sheet (the  $\gamma$ -core) by disulfide bonds [26]. This structure is termed as “cysteine-stabilized  $\alpha$ -helix/ $\beta$ -sheet motif” (CS $\alpha\beta$ ) and is also found in other antimicrobial peptides (ASABF from nematodes [43]), scorpion venoms, sweet-tasting proteins and plant trypsin inhibitors [38].

In mollusks, defensins have been initially characterized in species of mussels [44,45]. A diversity of different defensins was then described in oysters, including the CS $\alpha\beta$ -defensin and the big defensin AMP families. Three representative members of the CS $\alpha\beta$ -defensin family (*Cg*-Defs) have been identified from the oyster mantle (*Cg*-Defm) [46] and hemocytes (*Cg*-Defh1 and *Cg*-Defh2) [47], both being constitutively expressed in different tissues. Besides, three representative members from the big defensin family, namely *Cg*-BigDef1, *Cg*-BigDef2 and *Cg*-BigDef3 have been identified and their expression is restricted to hemocytes, both circulating and infiltrating oyster tissues [39].

Oyster CS $\alpha\beta$ -containing defensins comprise a large and diverse family, where each *Cg*-Def is encoded by a separate gene with different genomic organization [48]. Phylogenetic analyses showed that *Cg*-Defs sequences clustered into three separate but constraint groups, in which the three original forms were the most representatives. Furthermore, a highly gene copy number variation (CNV) between individuals has been observed and CNV has been correlated to the variability of *Cg*-Defh1 and *Cg*-Defh2 gene expression [48,49].

The structure of *Cg*-Defm solved by NMR showed that the CS $\alpha\beta$  motif is stabilized by four disulfide bonds (cysteine pattern: Cys<sub>1-5</sub> Cys<sub>2-6</sub> Cys<sub>3-7</sub> Cys<sub>4-8</sub>) [46]. Among antibacterial defensins, only molluscan defensins display a fourth disulfide bridge (**Figure 2A**), which has been proposed to be implicated in the stabilization of the mature peptide [50]. Oyster defensins are expressed as precursors, consisting in a hydrophobic signal peptide followed by the 4.6-4.7 kDa mature peptide positively charged (pI 8.5-8.7). Concerning their functional characterization, antibacterial activities of *Cg*-Defs were studied with recombinant peptides [46,14]. Oyster defensins were shown to be mainly active against Gram-positive bacteria at low minimal inhibitory concentrations (MICs)

(0.01-6  $\mu\text{M}$ ). Conversely, they did not display significant antimicrobial activity against Gram-negative bacteria including oyster pathogens (MICs  $\geq 10 \mu\text{M}$ ).

## 2.2. Anti-lipopolysaccharide factors (ALFs)

Anti-lipopolysaccharide factors (also known as anti-LPS factors or ALFs) are antimicrobial polypeptides exclusively found in marine chelicerates (horseshoe crabs) and crustaceans, which exhibit antimicrobial activity against a large number of Gram-positive and Gram-negative bacteria, fungi and some enveloped virus [15,51–53]. Historically, ALFs were initially purified from the hemolymph (a fluid analogous to the blood in vertebrates) of the horseshoe crabs *Limulus polyphemus* (LALF) and *Tachypleus tridentatus* (TALF) [6]. The term “anti-LPS factor” was introduced due to its potent ability to inhibit the LPS-mediated activation of the horseshoe crab coagulation system [6,7]. In addition to their regulatory anticoagulant activity, horseshoe crab ALFs were also shown to be active against Gram-negative bacteria [51]. In crustaceans, ALFs were identified about ten years after the first report of horseshoe crab ALFs, in two species of penaeid shrimp, *Litopenaeus setiferus* and *Penaeus monodon*, by transcriptomic-based approaches [54,55]. Then, sequences encoding for ALF homologs were described in a wide range of crustaceans, including other penaeid species, lobster, crayfish, freshwater prawns and crabs (reviewed in [56]).

ALFs from both horseshoe crabs and crustaceans are encoded as precursor molecules composed of a signal peptide followed by a mature polypeptide (about 100 residues) containing a hydrophobic N-terminal region and two conserved cysteine residues. The three dimensional structures of the horseshoe crab LALF (solved by X-ray crystallography) and shrimp ALFPm3 (solved by NMR) are extremely similar and consist of three  $\alpha$ -helices (one at the N-terminus and two at the C-terminus) packed against a four-stranded  $\beta$ -sheet [57,58].

In addition to their LPS-binding properties, ALFs can also interact with other microbial surface molecules, such as lipoteichoic acid (LTA) and  $\beta$ -glucan, major cell wall components of Gram-positive bacteria and fungi, respectively [59,60]. Altogether, these findings strongly suggest that the main mechanism of action of ALFs involves their binding to essential microbial cell wall components [18,56].

Different from horseshoe crabs, shrimp ALFs show a high degree of sequence diversity that can be associated to important differences in their biological activities [18]. Based on the primary structure and the range of the theoretical isoelectric point ( $pI$ ) of the mature polypeptides, shrimp ALFs were initially classified into four main groups: ALF-A (anionic and cationic polypeptides of

11.4-11.5 kDa), ALF-B (highly cationic polypeptides of 10.6-11.2 kDa), ALF-C (cationic polypeptides of 11-11.3 kDa) and ALF-D (highly anionic polypeptides of 10.7-10.8 kDa) [18] (**Figure 3**). All four ALF genes are concomitantly expressed at basal levels in an individual shrimp (*Litopenaeus vannamei*) and follow different patterns of gene expression in response to infection. Interestingly, while the ALF-A gene was not modulated, the ALF-B, -C, and -D genes showed to be induced in circulating hemocytes in response to injury and to an infection with the filamentous fungus *Fusarium solani* [18].

### 3. Molecular Evolution of AMPs

#### 3.1. Host-pathogen interactions and the co-evolutionary theory

Hosts and pathogens live in a strong relationship with each other and their interconnected fight for existence is highly dynamic. At the molecular level, the evolutionary response from this battle is reflected in mutations being under selective pressures [61]. Consequently, natural selection, *i.e.*, the major process by which the evolution of organisms takes place, may act strongly on immune-related genes such as AMPs since hosts adapt to novel, diverse and co-evolving pathogens. In this sense, the general hypothesis of co-evolution proposes that pathogens evolve continuously to escape from the immune response of host and, consequently, the immune system of the host evolves to improve barriers against pathogens. This adaptation of the Red Queen hypothesis [62] predicts that in an “arms race”, both host and pathogen will develop mechanisms that generate diversity and polymorphism of molecules that play key roles in their interplay. Thus, it is not surprising that genes involved in immunity frequently show high levels of genetic diversity and present some of the best examples of positive selection reported to date [63].

At the genomic level, different types of selective forces could shape evolution. On one hand, new alleles that confer a higher fitness to an individual tend to increase in frequency over time until they reach fixation, thus replacing the ancestral allele in the population. This evolutionary process is called positive selection or adaptive evolution (reviewed in [64]). Conversely, new mutations that decrease the fitness tend to disappear from populations through a process known as negative or purifying selection. Also, it may happen that a mutation is advantageous only in heterozygotes but not in homozygotes. Such alleles tend to be maintained at an intermediate frequency in populations by way of the process known as balancing selection. Positive selection favors the fixation of beneficial mutations that lead to evolution of new traits. Therefore, a mutation is said to be adaptive if it performs a function that is in some way advantageous in the population. Negative selection favors the conservation of existing phenotypes or particular amino acid residues functionally constrained, playing an important role in maintaining the long-term stability of biological function of the proteins [65].

### 3.2. Mechanisms of diversification of AMPs

Great efforts have been made to identify the exclusive arsenal of AMPs that each species can develop and to further recognize the bases of the diversity [48,66,67]. The mechanisms involved in the molecular diversification of AMPs have been broadly described in several vertebrate and invertebrate taxa [56,68]. The AMP diversification mechanisms have been studied from an evolutionary point of view, focusing on the positive or negative selective pressure events and genetic mechanisms. The most recognized mechanisms are gene duplication, gene copy number variation, recombination, and allelic polymorphisms. Still, the evolutionary forces that have shaped the diversity of AMPs remain largely unknown. Several studies support the adaptive evolution as the process that drives the diversification of certain AMPs [69–71], while another studies has revealed no evidence of adaptive diversification of AMP sequences despite highly dynamic genomic duplication and allelic polymorphism [61].

Among all AMPs, defensins provide one of the most outstanding examples of a diverse and evolving AMP gene family. Defensins are ancient molecules that are conserved across the eukaryotic kingdom and the diversification patterns of the different families indicate a common evolutionary origin [34]. Phylogenetic analyses on vertebrate and invertebrate defensins revealed a closer relationship between invertebrate defensins and  $\beta$ -defensins than between  $\alpha$ - and  $\beta$ -defensins, suggesting the  $\beta$ -defensins as the first of the vertebrate defensin families [34]. This hypothesis is supported by the presence of  $\beta$ -defensins in phylogenetically distant groups, such as reptiles, birds and fish [72]. Interestingly, plants, fungi and invertebrates display highly homologous CS $\alpha\beta$  defensin peptides, suggesting that they share a common genetic origin [73]. Structural bioinformatics analysis on myxobacterium suggested that CS $\alpha\beta$  defensin-like peptides from myxobacteria might be the potential origin of eukaryotic defensins [74].

Mammalian  $\alpha$ - and  $\beta$ -defensins would share a common ancestor which has evolved by gene duplication and rapid sequence divergence [34,75]. This mechanism of diversification means that each subfamily presents a large degree of sequence variability and that subfamilies display low sequence similarity [76]. Besides,  $\beta$ -defensin diversity has been shaped by birth and dead evolution, positive selection and extensive gene copy number variation [77]. Indeed, both  $\alpha$ - and  $\beta$ -defensins have been subjected to allelic recombination between distinct genomic locations, which generated copy number variation (CNV) between individuals [72]. The variation in the

number of repeated genes among individuals appears to have a role in modulating defensin expression and function [78,79].

Particular patterns of evolution have been evidenced among invertebrate defensins from arthropods and mollusks. A high degree of identity among mature defensins from both groups have been identified, and genetic studies suggests that the exon encoding the mature peptide has experienced exon-shuffling during evolution [80]. Evolutionary analysis on insect defensins suggest a marked diversification and expansion of mature defensin isoforms, and the classic CS $\alpha\beta$  motif has evolved by duplication followed by divergence, producing a diverse cluster of paralogues genes [81]. In the oyster *Crassostrea gigas*, defensin diversity has been produced by recombination events, allelic polymorphism and gene duplication, diverging in three distinct groups which display a highly conserved mature peptide within each group [48]. In addition, several amino acid residues were found to be under positive or negative selection pressures. Like for mammalian defensins, gene copy number variation has been described for oyster defensins, which correlates with the gene expression level of this AMP [49].

The evolutionary history of ALF peptides has been less explored, though phylogenetic analysis revealed a species-specific gene duplication, causing gene orthologues [82]. Molecular analyses on ALF diversity in crustaceans showed no evidence of positive selection but rather is likely to be by purifying selection, determined by models that detect selection on the entire gene or gene region [82]. Still, the employment of site-specific models to examine whether particular amino acid residues were subjected to selective pressures identified positive and negative selection events at certain codons [83]. Thus, it appears that the selective forces operate on specific amino acids of the ALF peptides rather than the complete polypeptide.

Overall, the origin of the diversification of AMPs in both vertebrates and invertebrates appears to display various common patterns, like gene duplication, allelic polymorphisms and, in some cases, evidence of adaptive and negative selection pressures at certain codons. The positive selection in sites encoding specific amino acid residues can be linked to the functional diversification [84]. Therefore, small changes in the primary structure of these molecules can have significant effect on their activities, and positive selection can act at certain sites to increase their potency. On the other side, the sites under purifying selection correspond to residues under strong functional constraints, essential for the function of these peptides. The generation of several variants in AMP families may be indicative of neo- or sub-functionalization processes, due to (i) the high exposure of hosts to potential pathogens, as observed for aquatic organisms, and/or

to (ii) a niche specialization, both phenomena often invoked in the course of evolution of immune genes and in the co-evolutionary arm race between hosts and pathogens.

#### 4. Highlights on the functional consequences of AMP diversity

Adaptive amino acid changes in a conserved structural scaffold are a major force driving new functional emergence. This is well illustrated by the CS $\alpha\beta$ -fold that has developed many diverse biological functions through evolution to participate in both defense and predatory behaviors [38]. As illustrated below, this versatile structural scaffold, which is restricted to fungi, plants, and protostomian animals, indeed supports functions as diverse antimicrobials, toxins, enzyme inhibitors, or tolerance to heavy metals.

##### 4.1. Specialized antimicrobial functions of CS $\alpha\beta$ defensins

Recent studies on CS $\alpha\beta$  defensins have shown that they adopt very diverse and specialized mechanisms of action. Unlike  $\alpha$ - and  $\beta$ -defensins from vertebrates, which are membrane-active peptides with a rather large spectrum of antimicrobial activities (anti-Gram-positive, anti-Gram-negative, antifungal, and occasionally antiviral), CS $\alpha\beta$  defensins have little to no effect on bacterial membranes or model lipid bilayers. Instead, CS $\alpha\beta$  defensins specifically interfere with essential metabolic pathways in Gram-positive bacteria and fungi. Such specific mechanisms of action rely on the capacity of CS $\alpha\beta$  defensins to bind to conserved molecules at the surface of microbes.

##### 4.1.1. *Anti-Gram-positive activity of CS $\alpha\beta$ defensins relies on Lipid II binding.*

While membrane activity was reported for some antibacterial defensins at micromolar concentrations [85], their most potent antibacterial activity was recorded against Gram-positive bacteria at nanomolar concentrations when no membrane damages were observed [86,87]. One highly conserved molecular target that mediates the specific killing of Gram-positive bacteria by CS $\alpha\beta$  defensins is the membrane-anchored cell wall precursor undecaprenyl pyrophosphoryl-MurNAc-pentapeptide-GlcNAc, also referred to as Lipid II. It is believed that the strong affinity of the interaction between Lipid II and CS $\alpha\beta$  defensins explains the potent activity of the peptides. Binding of CS $\alpha\beta$  defensins to Lipid II at the outer leaflet of the bacterial membrane of Gram-positive bacteria results in the inhibition of the cell wall biosynthesis in Gram-positive bacteria. Aside from CS $\alpha\beta$  defensins, Lipid II is a conserved molecular motif that serves as a target for some

human  $\alpha$ - and  $\beta$ -defensins [88,89] as well as many antibiotics (for review see [10,11]). Lipid II is therefore a conserved and essential molecular target at the membrane of Gram-positive bacteria that drives convergent evolution of antimicrobials including cysteine-rich AMPs like defensins.

Among  $CS\alpha\beta$  defensins, targeting of Lipid II is best illustrated by plectasin from the fungus *Pseudoplectanina nigrella* [90]. The plectasin-mediated sequestration of Lipid II from penicillin-binding proteins prevents its incorporation in peptidoglycan [10]. The same mechanism of Lipid II sequestration was observed with oyster defensins [87], which are highly similar to plectasin in terms of three dimensional structure. By comparing the sequences of the highly diverse oyster defensins with plectasin, we found that residues under purifying selection were involved in plectasin binding to Lipid II [48]. Indeed, among oyster defensin highly conserved residues [48], Phe2, Gly3, Cys4, and Cys25 are involved in plectasin-Lipid II interaction (**Figure 2A**). Such a purifying selection is characteristic of strong functional constraints (e.g. residues essential for the peptide activity) [65]. Aside from those conserved positions, sites of diversification were observed in oyster defensins, in particular at position 16 were the presence of positively charged residues improved antibacterial activity, probably by increasing electrostatic interactions between defensins and the negatively charged membranes of bacteria [87].

#### **4.1.2. Antifungal activity of $CS\alpha\beta$ defensins relies on interaction with membrane lipids at the fungal membrane.**

While primarily active against Gram-positive bacteria,  $CS\alpha\beta$  defensins of invertebrates have often been shown to display antifungal activities against filamentous fungi [91]. However, among  $CS\alpha\beta$  defensins, a substantial number of peptides are strictly antifungal. In particular, this is the hallmark of plant defensins [12]. But such a strictly antifungal activity has also been found in some insect defensins like the  $CS\alpha\beta$ -containing drosomycin. Although a similar antifungal activity is observed for those defensins, their mode of action can be extremely diverse [92]. Many of them interact with sphingolipids of different classes at the fungal membrane: the radish Rs-AFP2 (DEF-RASAT) interacts with GlcCer, whereas the dahlia DmAMP1 interacts with M(IP)2C. On the contrary, the tobacco NaD1 interacts with a variety of phospholipids, but not with sphingolipids (for review see [12]).

#### 4.1.3. Structural determinant of antibacterial and antifungal defensins.

To understand the functional consequences of defensin sequence diversity, we aligned amino acid sequences of antibacterial and antifungal defensins whose spectrum of activity and/or mechanism of action had been characterized (**Figure 2**). All peptides contain the minimal 6 cysteines of CS $\alpha\beta$  defensins as well as a conserved Gly-Xaa-Cys sequence that belongs to the  $\gamma$ -core motif conserved among all cysteine-stabilized antimicrobial peptides [31]. Both antibacterial and antifungal defensins are most often cationic ( $7.7 < pI < 9.7$ ), with some rare exceptions like the molluscan defensin McDef and the fungal Eurocin ( $pI=6.8$ ), both antibacterial (**Figure 2A**). However, the charged amino acids are distributed differently in antifungal and antibacterial defensins. In particular, patches of basic amino acids present in antibacterial are lacking in antifungal defensins (**Figure 2B**). The local concentration of positive charges in antibacterial defensins might play an important role in initiating electrostatic interactions with bacterial membranes. As fungal membranes are more zwitterionic than the negatively-charged bacterial membranes [93], this property may not have been selected throughout evolution for antifungal defensins.

When comparing amino acid sequences of CS $\alpha\beta$  defensins, they cluster according to their antibacterial/antifungal mechanisms of action rather than to their phylogenetic origin (**Figure 1**). For instance, insect drosomycin clusters with antifungal defensins from plants and fungal plectasin and eurocin cluster with antibacterial defensins from invertebrates. This indicates that both the antibacterial and antifungal functions have been preserved in diverse phyla of life. Interestingly, while the number of cysteines varies from 6 to 8 in antibacterial defensins, there is to date no indication that the number of disulfide bonds modifies the biological properties of the peptides and in particular their spectrum of activity. Consistently, clustering of defensin amino acid sequences does not follow the number of cysteines neither (**Figures 1 and 2A**). It is still largely unknown why a fourth disulfide bond is found in certain variants of molluscan defensins like oyster Cg-Defs, clam MCDef or mussel MGD-1. It has to be noticed that the position of this fourth disulfide bond, which is conserved among species of mollusks, slightly differs in antifungal and antibacterial defensins, suggesting its apparition could have occurred at different times during evolution.

Finally, the N-terminal sequence of CS $\alpha\beta$  defensins, which contains the first cysteine of the sequence, is an important region that differentiates antibacterial and antifungal peptides (**Figure**

2). In antifungal defensins, although a certain degree of amino acid sequence variation is observed, all peptides contain a N-terminal  $\beta$  strand lacking in antibacterial defensins (**Figure 2B**). This  $\beta$  strand is stabilized onto the whole structure by the first conserved disulfide bond of the  $CS\alpha\beta$  motif. In antibacterial defensins, the N-terminal sequence is well conserved. The first amino acids form a conserved sequence of two types, either Gly-Phe-Gly-Cys or Ala-Thr-Cys-Asp-Leu (**Figure 2A**). We discuss below how this may play an important role in their interactions with essential components of the bacterial membranes and subsequent antibacterial activity.

#### 4.1.3.1. Antibacterial $CS\alpha\beta$ defensins.

Antibacterial defensins from fungi and invertebrates including mollusks (oyster, mussel, clam), insects (dragonfly, mosquitoes, flies...) and arachnids (tick, spider, scorpion) share a three dimensional structure consisting in two  $\beta$  strands and a  $\alpha$  helix stabilized by three to four disulfide bridges (**Figure 2A**). Plectasin and oyster defensins have been characterized in details for their interaction with Lipid II [86,87]. In addition, some peptides like the fungal eurocin and the insect lucifensin have been studied for their mechanism of action in the frame of the plectasin study. Similar to plectasin and oyster defensins, both peptides were shown to directly interact with Lipid II [86]. While other possibilities such as convergent evolution can also account for the similarities observed between these AMPs, a common genetic origin for the antibacterial defensins of fungi, mollusks and arthropods seems highly probable. This theory is supported by the conservation among these AMPs of not only the  $CS\alpha\beta$  motif but also conserved amino acid sequences.

Two types of consensus sequence are observed among antibacterial defensins that differ by their N-terminal sequence and other conserved positions (**Figure 2A**). The first consensus sequence  $GFGC X_{(5-10)} C X_{(2)} H C X_{(6-8)} GYC X_{(6-8)} CXC X_{(1-12)}$  starts with four residue Gly-Phe-Gly-Cys (GFGC) present in the Lipid II-binding peptides plectasin, *Cg*-Defens and eurocin. They were shown to be involved in the plectasin/Lipid II interaction [86] (**Figure 2A**). This sequence is widespread among antibacterial defensins from diverse kingdoms of life (fungi, mollusks, arachnids, insects...) and could therefore be an important determinant of Lipid II binding in antibacterial defensins. The cysteine residue from the Gly-Tyr-Cys (GYC) sequence conserved in all most known antibacterial defensins is also involved in Lipid II binding [86]. Therefore, both the N-terminal sequence and the  $\gamma$ -core of antibacterial defensins would be involved in the interaction with Lipid II. However, other unidentified residues are likely implicated in this interaction. Indeed, the three oyster *Cg*-Defens,

which all contain the GFGC and GYC sequences, revealed differential binding affinities for Lipid II that correlated with the variation in their antimicrobial potency [87]. Moreover, another clade of antibacterial defensins lacks the GFGC sequence and contains instead a conserved Ala-Thr-Cys-Asp-Leu (ATCDL) sequence at their N-terminus (**Figures 1 and 2A**). The consensus sequence is the following: ATCDL  $X_{(10)}$  CAXHC  $X_{(6)}$  GGYC  $X_{(5)}$  CVCRN. In particular, the ATCDL sequence is found in lucifensin which was also reported to bind to Lipid II [86]. The determinants of this molecular interaction remain to be determined. To our knowledge, the affinity for Lipid II of GFGC- and ATCDL-containing antibacterial defensins has not been compared to date. The lucifensin example still strongly suggests that potentially all antibacterial defensins can bind to Lipid II as part of their mechanism of action.

#### **4.1.3.2. Antifungal $CS\alpha\beta$ defensins.**

Most of the current knowledge on antifungal defensins comes from plant defensins (for review see [12]). Antifungal defensins share a very similar three dimensional structures, consisting in three  $\beta$  strands and a  $\alpha$  helix. The N-terminal  $\beta$  strand, which is not part of the canonical  $CS\alpha\beta$  motif, is specific of antifungal defensins. Whether this  $\beta$  strand contributes to the specific antifungal activity of those  $CS\alpha\beta$  defensins remains to be established. An important amino acid sequence diversity is observed among antifungal defensins and to date, there is no known consensus sequence that identifies antifungal defensins beyond their conserved cysteine array and one to two conserved Gly-Xaa-Cys motifs (**Figure 2B**). The overall consensus sequence of antifungal defensins is the following:  $X_{(1-8)}$  C  $X_{(8-10)}$  C  $X_{(3-7)}$  C  $X_{(3)}$  C  $X_{(7-9)}$  GXC  $X_{(4-8)}$  CXC  $X_{(3)}$  C  $X_{(0-15)}$ . Structure/activity studies on antifungal defensins have evidenced an essential role of the  $\gamma$ -core motif for the antifungal activity of most of the plant defensins (for review see [12]). For instance, in Rs-AFP2 and MsDef1 and MsDef4, which differ by both their primary sequence and mechanism of action, sites important for antifungal activity include the  $\gamma$ -core region GXC  $X_{(4-8)}$  C (**Figure 2B**) [94,95]. Consistently, this  $\gamma$ -core is known as an important determinant for peptide interactions with microbial membranes [31].

#### 4.1.4. Novel functions of CS $\alpha\beta$ defensins.

It is now understood that the CS $\alpha\beta$  scaffold supports a broad series of functions in peptides. In peptides from invertebrates, this motif is shared by toxins and antimicrobial peptides (defensins). “Neofunctionalization” has been proposed to explain the origin of scorpion toxins from CS $\alpha\beta$  defensins [96,97]. Not surprisingly, many toxins are recognized as capable of exerting direct antimicrobial effects, and reciprocally both antibacterial and antifungal defensins like plectasin and Psd1, respectively, can exert toxin activities by blocking ion channels [98,99]. Recently, experimental conversion of a defensin into a toxin enabled to establish an evolutionary relationship between two distantly related protein families [100]. This could be achieved by shortening the loop separating the first two cysteines in a defensin in which the GYCXX conserved motif was replaced by the “scorpion toxin signature” GKCXN.

Beyond the ancestral and intimate link between toxins and defensins, some CS $\alpha\beta$  defensins have also evolved very specific and poorly explored functions. For instance, some plant defensins with antifungal activity are essential to zinc detoxification in plants [101], some others are insecticidal [102] or pollen tube attractants [103]. Since all antifungal defensins whose three dimensional structures have been determined have a similar backbone, functional specificities are likely to arise primarily from differences in the amino acid composition. In some cases, the determinants of such a specialization have been explored. For example, it was recently shown that four residues (Val-Phe-Phe-Ala) in the  $\gamma$ -core of plant defensins are conserved among plants tolerant to zinc [104]. However, most of the time, the molecular determinants of such a neofunctionalisation remain to be determined. Another striking and still non understood example of neofunctionalisation in CS $\alpha\beta$  defensins is Xtox from *Spodoptera frugiperda* [105]. This protein encoding 11 repeats of defensins is devoid of antibacterial and antifungal activity, even after *in vitro* proteolytic maturation. Still, the corresponding gene is highly induced in response to infection, suggesting an essential but undermined role in the insect immune response.

#### 4.2. Neofunctionalization in anti-lipopolysaccharide factors

As in defensins, antimicrobial activity in ALFs is dependent on an antiparallel  $\beta$ -hairpin structure. This scaffold, which encompasses a conserved charged sequence, is delimited by the two paired cysteines (**Figure 3**). This structure defined as the LPS-binding domain [57] is generally composed of one negatively and six positively-charged residues [Glu<sub>21</sub>, Lys<sub>22</sub>, (Lys/Arg)<sub>31</sub>, Lys<sub>35</sub>, (Lys/Arg)<sub>46</sub>, (Lys/Arg)<sub>48</sub>, (Lys/Arg)<sub>58</sub>] that interact with the negative charges of the Lipid A moiety of

LPS [57,106] (**Figure 3**). A broad range of studies have shown that synthetic  $\beta$ -hairpins corresponding to this cationic region had the ability to mimic the antibacterial and anti-LPS activity of the whole molecule [106–110].

#### **4.2.1. LPS-binding domain and specificity of ALF antimicrobial spectrum.**

Shrimp ALFs are diverse not only in terms of sequences, but also biochemical properties and gene expression (see chapter 2.2.). In particular, several residues important for LPS-binding are not conserved among the four ALF groups A-D. Not surprisingly, this translates into important variations in terms of biological activities [18]. Cationic polypeptides from Group B, such as ALFPm3 (or *Penmon* ALF-B1), are the most active members of shrimp ALFs and display an LPS-binding domain [15,58] (**Figure 3**) that involves residues conserved in large transmembrane proteins establishing strong interactions with LPS [58]. Among ALFs, group D gathers peptides with a negative net charge, which have lost most of their antimicrobial activity [18]. In this group of anionic ALFs, most of the residues involved in LPS-binding of cationic ALFs are lacking (**Figure 3**). As consequence, they have impaired LPS-binding properties and display very low antimicrobial activity [18]. These evidences strongly support the role of the charged residues carried by LPS-binding domain in the interaction of ALFs with LPS. Moreover, it strongly suggests that ALFs are evolving towards novel functions beyond anti-Gram negative. While *in vitro* antimicrobial activity of ALFs has been extensively studied in crustacean species (reviewed in [56,111]), most of the biochemical information available to date is related to cationic ALFs (mainly group B), preventing us from deciphering the molecular basis of the ALF specificity of action.

#### **4.2.2. Multiple antimicrobial functions of ALFs and interactions with other essential membrane components.**

Recent data were obtained on the activity of shrimp ALFs using RNA interference (RNAi) *in vivo*. They showed that ALFs from the different groups differ in their spectrum of activity, which nonetheless covers a diversity of microbial agents. Thus, the silencing of LvALF1 (Group A) from *L. vannamei* resulted in increased susceptibility of shrimp to bacterial and fungal infections [112]. The silencing of ALFPm6 (Group C) from *P. monodon* led to a significant increase in susceptibility of shrimp to both bacterial and viral infections [113]. Thus, sequence diversity among ALF groups translates into different spectra of activity.

The molecular interactions responsible for those multiple activities include ALF binding to lipoteichoic acid (LTA) and  $\beta$ -glucans, major cell wall components of Gram-positive bacteria and fungi, respectively [59,60]. Recent studies suggested that ALFs are also able to interact with and inhibit viral shrimp pathogens, such as the White spot syndrome virus (WSSV) [114]. In particular, it has been shown that shrimp ALF $Pm3$  binds to the WSSV189 envelope protein of WSSV [115]. However, the amino acids involved in the interaction with those viral, fungal or bacterial essential membrane components are still unknown, preventing us from interpreting most of the functional consequences of sequence diversity in ALFs.

While most of the previous studies have considered the role of ALFs in the defense against pathogens, Group B ALFs were recently shown to control the commensal microbiota in shrimp hemolymph (blood stream). Thus, silencing of ALF $Pm3$  (Group B) resulted in a rapid and lethal propagation of bacteria in the hemolymph of healthy shrimp [113]. Supporting the essential role of ALFs in controlling the hemolymph microbiota, silencing of a soluble C-type lectin (MjHeCL) in the kuruma prawn *Marsupenaeus japonicus* led to the proliferation of bacteria in the shrimp hemolymph, resulting in shrimp death [116]. Altogether, those recent data support the hypothesis of multifunctional ALFs, with a spectrum of activity that goes far beyond their ability to bind LPS and subsequently kill Gram-negative bacteria. The sequence variations responsible for ALF antimicrobial specificities still remain to be determined.

## 5. Resistance mechanism and molecular targets at microbial membranes

As illustrated in this review, in diverse families of AMPs, the specificity of the molecular interactions occurring at the surface of microorganisms determines the AMP spectrum of activity (**Figure 4**). AMPs such as defensins or ALFs, which target Lipid II, LTA or Lipid A at the bacterial membrane, are often active against a broad series of Gram-positive or Gram-negative bacteria. On the contrary, some other families of AMPs have a narrow spectrum of activity. For instance, microcins from enterobacteria are only active against a limited number of phylogenetically-related strains (for review see [3]). Indeed, their activity depends on their interaction with specific receptors - in that case iron-siderophore receptors - at the bacterial membrane, which are only found in susceptible strains [3].

Mechanisms of resistance against AMPs are increasingly described and they are highly informative on the essential components of bacterial membranes targeted by AMPs. In some cases, resistance is conferred by the simple loss of a given receptor. This has been easily

evidenced for resistance to microcins [117]. In that case, functional redundancy between diverse families of iron-siderophore receptors, enables the loss of one receptor required microcin-binding activity without consequences on bacterial viability. However, sometimes, the molecular targets are not dispensable. As largely described in this review, often the AMP docking molecules are essential component of the membrane structure. Still, diverse mechanisms of resistance compatible with bacterial survival have been evidenced that directly modify the structure and as a direct consequence, often the charge of the AMP molecular target, without altering membrane integrity. Such modifications are common to Gram-positive and Gram-negative bacteria as modification of anionic cell surface constituents repels cationic AMPs, preventing them from reaching the cytoplasmic membrane and eventually disrupt its integrity.

The best described mechanism of resistance is certainly that affecting the Lipid A structure. Indeed, as a target for a broad variety of AMPs, this essential component of the Gram-negative outer membrane can be chemically modified by the addition of free amine groups which lower its negative net charge, and subsequent electrostatic interactions between bacteria and cationic AMPs. Studies on diverse Gram-negative bacteria have shown that addition of free amine groups at various positions on Lipid A structure is a major mechanism conferring resistance to polymixin B and other cationic AMPs that directly interact with Lipid A (for review see [118]). Those chemical modifications are observed on both the acyl chains and the disaccharide moiety of Lipid A (**Figure 4**). For example, addition of chemical groups like phosphoethanolamine and aminoarabinose increases resistance to AMPs. The enzymes responsible for such modifications are EptB and ArnT, respectively [118]. In addition, acylation or deacylation of Lipid A by LpxO/LpxR and PagL/PagP, respectively contribute to AMP resistance. Those chemical modifications are regulated by two component systems among which the best described is the PhoPQ system [119].

Similarly, in Gram-positive bacteria, chemical modifications of the polyanionic lipoteichoic acid structure, which change the overall charge of the bacterial surface, confer a major resistance to cationic AMPs. D-alanylation of teichoic acids, which is catalyzed by the *dlt* operon is probably the best described mechanism to date for its role in resistance to AMPs and virulence *in vivo* [120–122]. Those structural modifications of major membrane components are performed by enzymatic machineries whose transcription is induced upon exposition of bacteria to sub-lethal concentrations of AMPs [123,124]. The GraRS system has been well described for its role in sensing AMPs and controlling the *dlt* operon [123,124]. Finally, modifications of the Lipid II structure have been reported to confer resistance to AMPs and antibiotics (for recent review see

[125]). Unlike in the previous examples, the modifications of the Lipid II structure tend to be constitutive. They confer a high degree of resistance to antimicrobials, showing further the essential role of Lipid II as a major target for AMPs. Resistant strains are also highly informative on the Lipid II structural motifs targeted by antibiotics and AMPs. Thus, Gram-positive bacteria highly resistant to vancomycin have a modified structure of Lipid II in which the D-Ala-D-Ala dipeptide to which vancomycin binds (**Figure 4**) is replaced by a D-Ala-D-Lac depsipeptide [126]. Not surprisingly, those vancomycin-resistant strains do not show cross-resistance with plectasin, which was proposed to essentially bind to the pyrophosphate moiety instead of the D-Ala-D-Ala of Lipid II [86]. On the contrary, amidation of D-Glu in Lipid II structure results in a less negatively charged peptidoglycan and an increased resistance to cationic AMPs including plectasin. This gain of resistance is consistent with the interaction of plectasin N-terminal amine with the carboxyl group of the D-Glu residue [86].

A few studies have addressed the *in vivo* significance of bacterial resistance to AMPs in animal models of infections, in particular in mice, but also in two instances, in a human model of infection (for recent review see [127]). They have highlighted the importance of surface charge modification in the outcome of several infectious diseases caused by Gram-positive or Gram-negative bacteria.

## 6. Conclusion

With the emergence of next generation sequencing, an exponential number of AMPs have been discovered in genomes of species from all branches of the kingdom of life. These data reveal how diverse and polymorphic AMP sequences are, not only at the inter-specific level but also at intra-specific and individual levels. This questions us even further on the functional meaning of sequence diversity. As illustrated in this review article, it is still rather difficult to predict the function of a given peptide on the basis of its primary sequence or structural scaffold. Much more functional studies will be needed to address this question, revealing a major technical gap between high throughput sequencing techniques and classical biochemical characterizations. As functional studies are a prerequisite to study functional divergence, the development of innovative and rapid biochemical tools will be an important issue to address. In parallel, microbiological tools are developing, informing us on the mechanisms of resistance. By sequencing genomes and/or transcriptomes of resistant bacteria, we will have access to the enzymatic machineries conferring resistance and as a consequence to the molecular targets of AMP variants.

Besides its evolutionary interest, functional divergence of AMPs should also be a major source of inspiration for the rational design of new drugs with specific mechanism of action, with a limited risk of toxicity. A clearer understanding of these relationships and the immunological roles of AMPs should also enable the design of shorter active peptides based on molecular signatures like the  $\gamma$ -core of defensins as a structural basis for the design of more effective anti-infective and immunotherapeutic agents and strategies.

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### Figure captions

**Figure 1.** Phylogenetic tree of different defensin groups including antibacterial and antifungal CS $\alpha\beta$  defensins and vertebrate defensins from human. **Antibacterial CS $\alpha\beta$  defensins** from: the mollusks (M) *Crassostrea gigas* (Cg-Defm: AEO45023, Cg-Defh1: ACQ76280, Cg-Defh2: ACQ73009), *C. virginica* (AOD: P85008), *Mytilus edulis* (DEFA\_MYTED: P81610, DEFB\_MYTED: P81611), *M. galloprovincialis* (MGD-1: P80571) and *Ruditapes philippinarum* (MCdef: AFP50008), the chelicerates (C) *Ixodes scapularis* (Scapularisin: EEC08934), *Androctonus australis* (DEF4\_ANDAU: P56686) and *Leiurus quinquestriatus* (DEF4\_LEIQH: P41965), the insects (I) *Aeschna cyanea* (DEFI\_AESCY: P80154), *Lucilia sericata* (Lucifensin: P86471), *Drosophila melanogaster* (DEFI\_DROME: P36192), *Anopheles gambiae* (DEFI\_ANOGA: Q17027) and *Aedes aegypti* (DEFC\_AEDAE: P81603) and the fungus (F) *Pseudoplectania nigrella* (Plectasin: Q53106), *Trichophyton interdigitale* (DEF\_TRINT: EZF32930) and *Eurotium amstelodami* (Eurocin: 2LT8). **Antifungal CS $\alpha\beta$  defensins** from: the plants (P) *Nicotiana glauca* (NaD1: Q8GTM0), *Raphanus sativus* (Rs-AFP1: P69241, Rs-AFP2: P30230), *Medicago sativa* (MsDef1: Q9FPM3), *M. truncatula* (MtDef4: G7L736), *Pisum sativum* (Psd1: P81929), *Pachyrrhizus erosus* (SPE10: 3PSM), *Petunia hybrida* (PhD1: Q8H6Q1) and *Saccharum officinarum* (Sd5: 2KSK) and the insect (I) *D.*

*melanogaster* (Drosomycin: AAF47767). **Vertebrate defensins** from human (H): alpha-defensins (HNP1: NP\_004075, HNP3: AAA35753) and beta-defensins (HBD2: NP\_004933, HBD3: NP\_061131).

**Figure 2.** Alignment of amino acid sequences of (A) antibacterial and (B) antifungal CS $\alpha\beta$  defensins with characterized antimicrobial activity/mechanism of action. Positively and negatively charged amino acids are displayed in blue and red, respectively. Cysteines are highlighted in black. Highly conserved residues are highlighted in grey. The conserved N-terminal motifs of antibacterial defensins GFGC and ATCDL are highlighted in blue. Residues of plectasin involved in interaction with Lipid II and conserved in other sequences are indicated with a + sign. The position of  $\alpha$ -helices (grey boxes),  $\beta$ -sheets (grey arrows) and disulfide bond (brackets) is based on the 3D structure of *Cg*-Defm (PDB ID: 2B68) and Rs-AFP1 (PDB ID: 1AYJ) for antibacterial and antifungal defensins, respectively. Disulfide bonds conserved in all sequences are in black lines. Disulfide bonds found in molluscan defensins only are in dashed line. **Antibacterial CS $\alpha\beta$  defensins:** *Crassostrea gigas* (*Cg*-Defm: AEO45023, *Cg*-Defh1: ACQ76280, *Cg*-Defh2: ACQ73009), *C. virginica* (AOD: P85008), *Mytilus edulis* (DEFA\_MYTED: P81610, DEFB\_MYTED: P81611), *M. galloprovincialis* (MGD-1: P80571), *Ruditapes philippinarum* (MCdef: AFP50008), *Ixodes scapularis* (Scapularisin: EEC08934), *Androctonus australis* (DEF4\_ANDAU: P56686), *Leiurus quinquestriatus* (DEF4\_LEIQH: P41965), *Aeschna cyanea* (DEFI\_AESCY: P80154), *Lucilia sericata* (Lucifensin: P86471), *Drosophila melanogaster* (DEFI\_DROME: P36192), *Anopheles gambiae* (DEFI\_ANOGA: Q17027), *Aedes aegypti* (DEFC\_AEDAE: P81603), *Pseudoplectania nigrella* (Plectasin: Q53106), *Trichophyton interdigitale* (DEF\_TRINT: EZF32930) and *Eurotium amstelodami* (Eurocin: 2LT8). **Antifungal CS $\alpha\beta$  defensins:** *Nicotiana glauca* (NaD1: Q8GTM0), *Raphanus sativus* (Rs-AFP1: P69241, Rs-AFP2: P30230), *Medicago sativa* (MsDef1: Q9FPM3), *M. truncatula* MtDef4: G7L736), *Pisum sativum* (Psd1: P81929), *Pachyrrhizus erosus* (SPE10: 3PSM), *Petunia hybrida* (PhD1: Q8H6Q1), *Saccharum officinarum* (Sd5: 2KSK) and *D. melanogaster* (Drosomycin: AAF47767).

**Figure 3.** (A) Phylogenetic tree of Anti-lipopolysaccharide factors (ALFs). **Shrimp ALFs:** *Litopenaeus vannamei* (LvALF1 or *Litvan* ALF-A: EW713395, ALFLv3 or *Litvan* ALF-B: ABB22833, LvALF2 or *Litvan* ALF-C: EW713396, *Litvan* ALF-D: FE152534), *L. setiferus* (*Litset* ALF-D: BE846661), *L. stylirostris* (*Litsy* ALF-D: AAY33769), *L. schmitti* (ALFLsch or *Litsch* ALF-B: ABJ90465), *Farfantepenaeus paulensis* (ALFFpau or *Farpau* ALF-B: ABQ96193), *Penaeus monodon* (ALFPm2 or *Penmon* ALF-A:

ABP73291, ALFPm3 or *Penmon* ALF-B: ABP73289, ALFPm6 or *Penmon* ALF-C: ADM21460), *Marsupenaeus japonicus* (MjALF2 or *Marjap* ALF-A: BAH22585, M-ALF or *Marjap* ALF-C: BAE92940) and *Fenneropenaeus chinensis* (ALFc or *Fenchi* ALF-B: AAX63831, FcALF2 or *Fenchi* ALF-C: AFU61125, FcALF6 or *Fenchi* ALF-D: AFU61129). **Horseshoe crab ALFs:** *Tachypleus tridentatus* (TALF: P07087) and *Limulus polyphemus* (LALF: P07086). (B) Alignment of amino acid sequences representative of shrimp ALFs (Group A, B, C and D) with LALF. Positively and negatively charged amino acids are displayed in blue and red, respectively. Cysteines are highlighted in black. Highly conserved residues are highlighted in grey. Residues involved in LPS-binding of ALFPm3 [58] are indicated with a + sign. The position of  $\alpha$ -helices (grey boxes),  $\beta$ -sheets (grey arrows) and disulfide bond (brackets) is based on the 3D structure of ALFPm3 (PDB: 2JOB). Disulfide bonds conserved in all sequences are in black lines.

**Figure 4.** Tentative model of interaction of oyster defensin *Cg-Defm* (PDB ID: 2B68) with Lipid II and shrimp ALFPm3 (PDB ID: 2JOB) with lipopolysaccharide (LPS) and lipoteichoic acid (LTA). A schematic view of the interactions is displayed on the left panel. Presumed interactions of defensins with Lipid II involves the pyrophosphate moiety and the D-glutamate of Lipid II. At low peptide concentration, the binding of oyster defensins to Lipid II likely occurs at the outer leaflet of the plasma membrane after Lipid II is translocated, blocking its polymerization into mature peptidoglycan of Gram-positive bacteria. At higher peptide concentration, some membrane permeabilization may occur and the binding of Lipid II could also take place at the inner leaflet of the membrane. Shrimp ALFs can bind to both LPS from Gram-negative bacteria and LTA from Gram-positive bacteria. LPS-binding probably involves seven charged residues located in the cysteine-stabilized  $\beta$ -hairpin of ALF structure. LTA-binding sites are still unknown. On the right panel, chemical structures of *Salmonella* Lipid A and *Staphylococcus aureus* Lipid II are provided. The Lipid II structure is presented with the Glycine pentapeptide substitution specific of *S. aureus*. OM: Outer Membrane, PP: Periplasm, PM: Plasma Membrane, PG: Peptidoglycan, MurNAc : N-Acetylmuramic acid, GlcNAc: N-acetyl-D-glucosamine.

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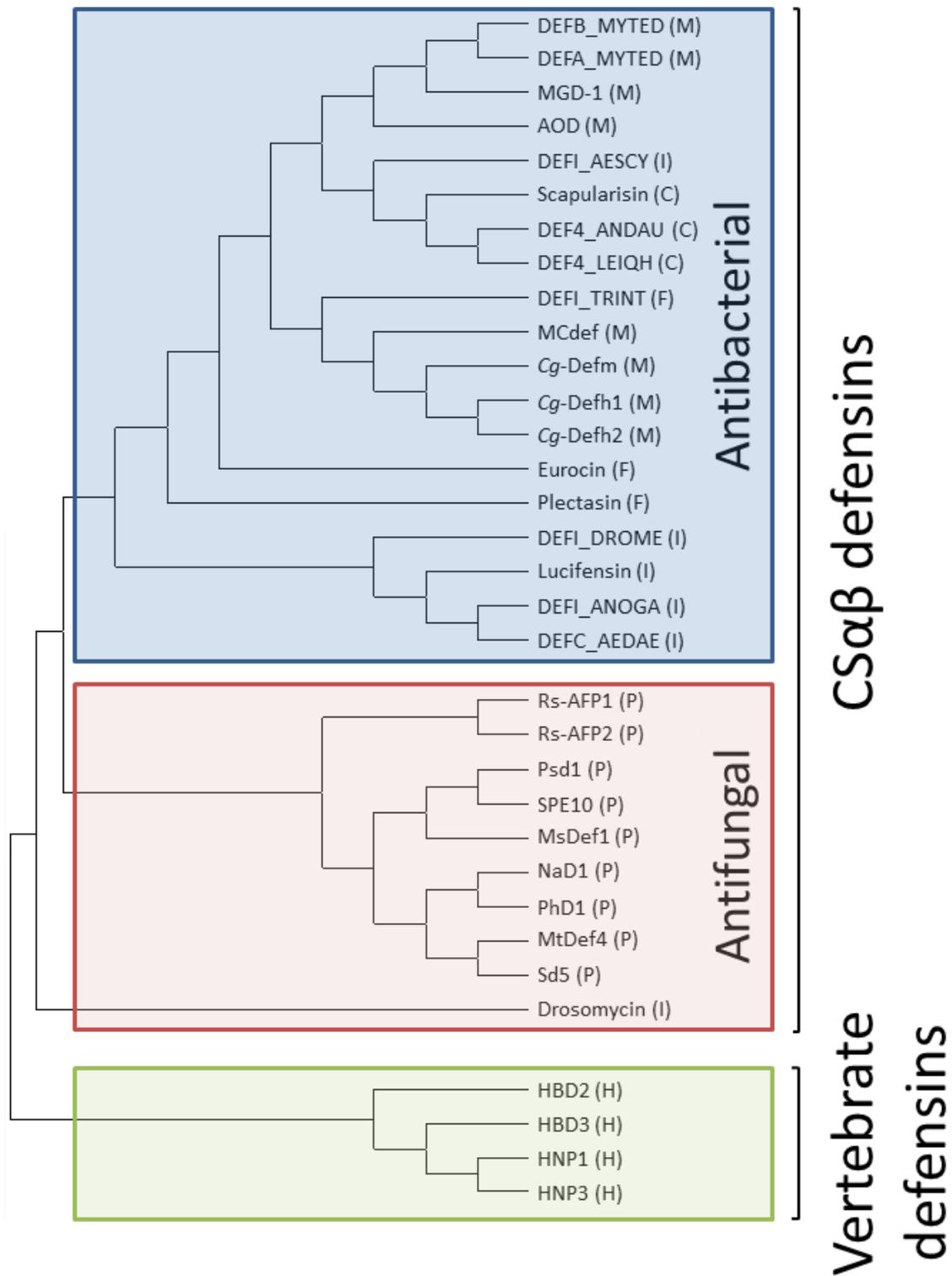


Figure 1

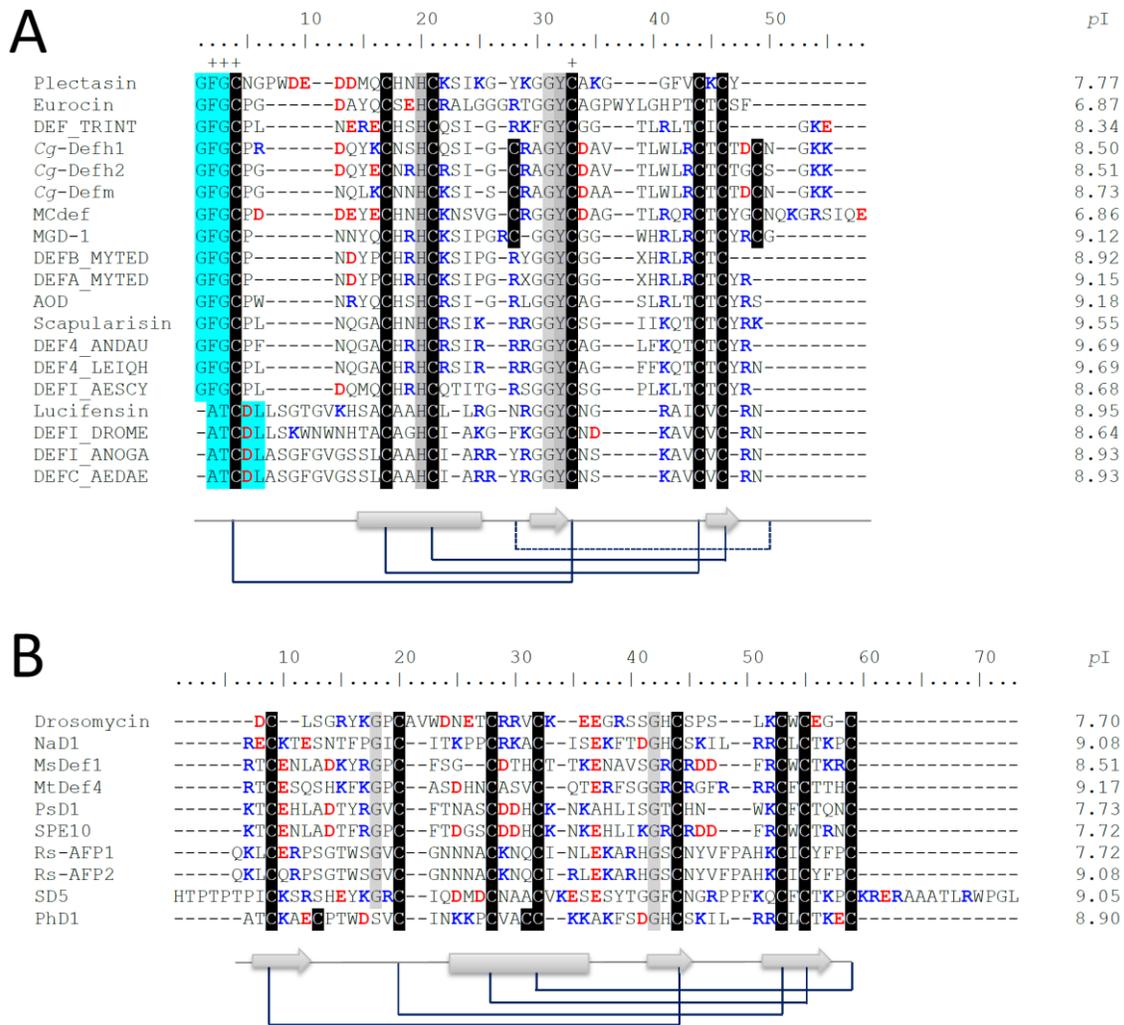


Figure 2

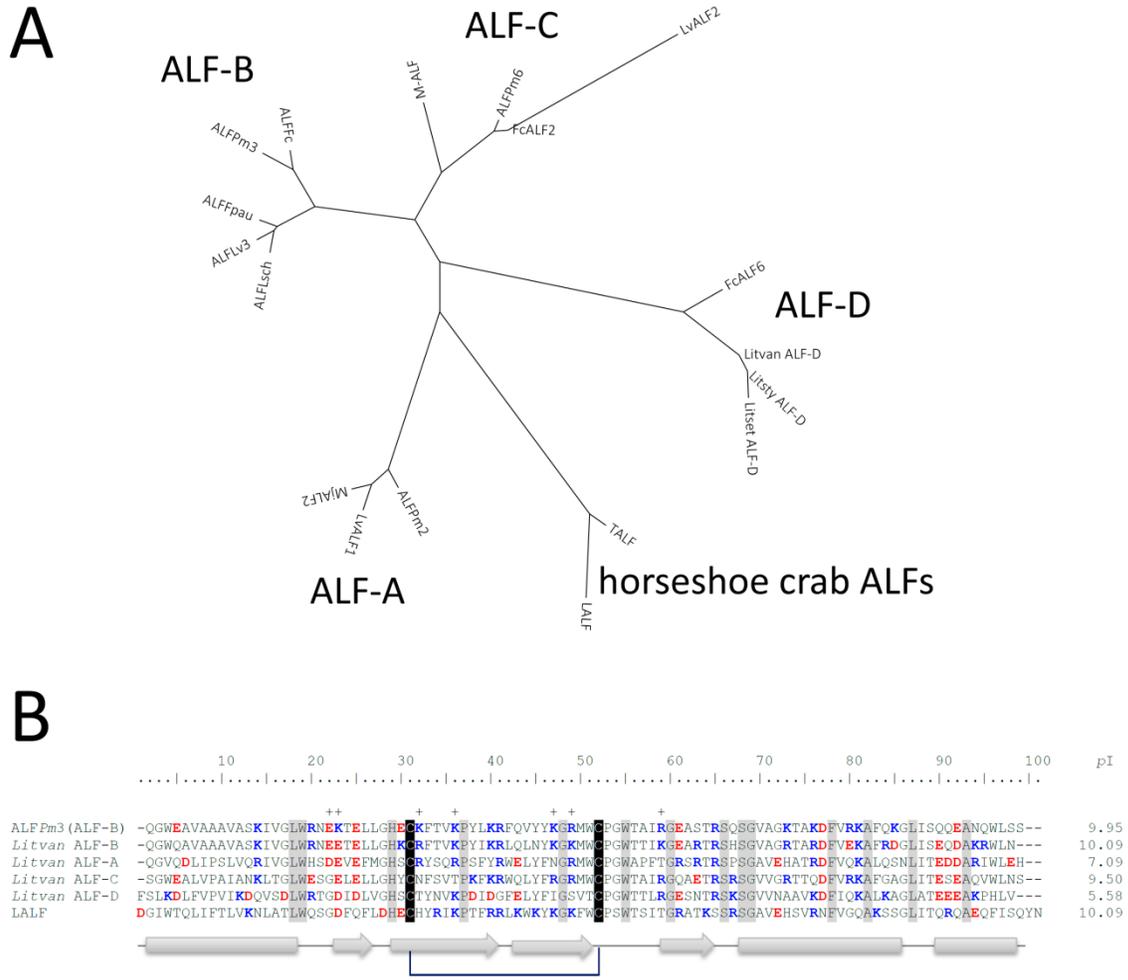


Figure 3

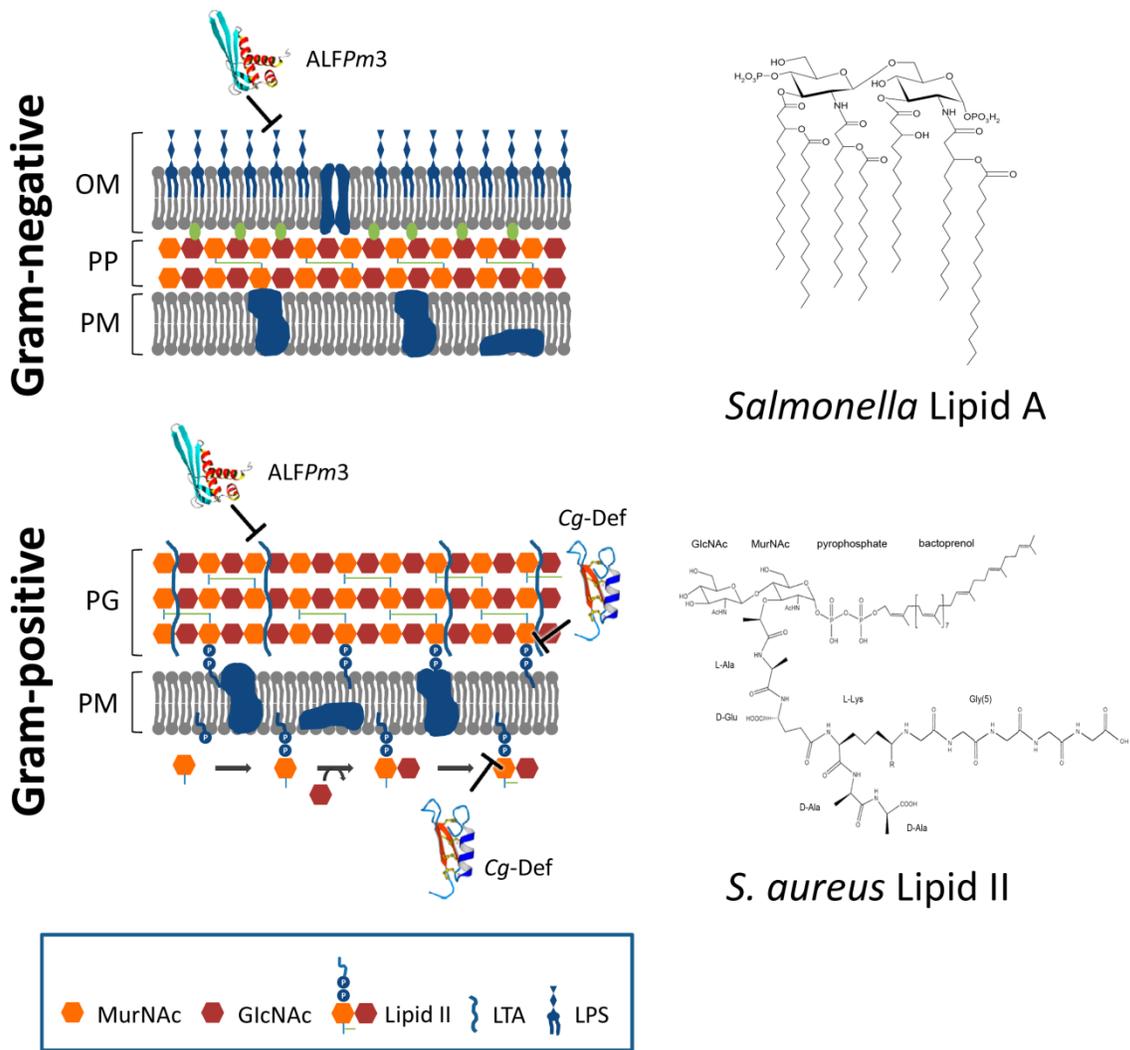
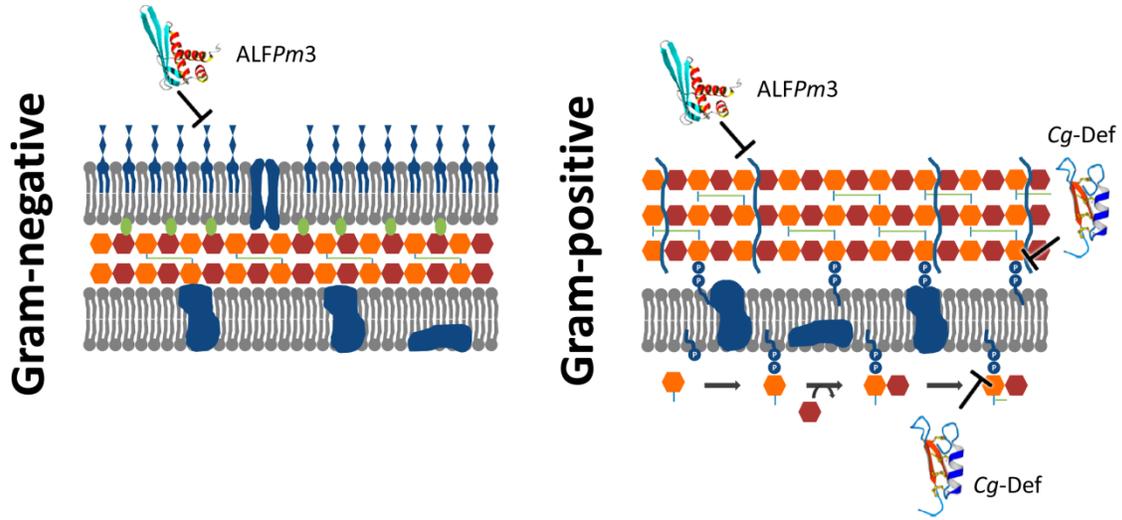


Figure 4



Graphical abstract

ACCEPTED MANUSCRIPT