Myung-Dae Seong
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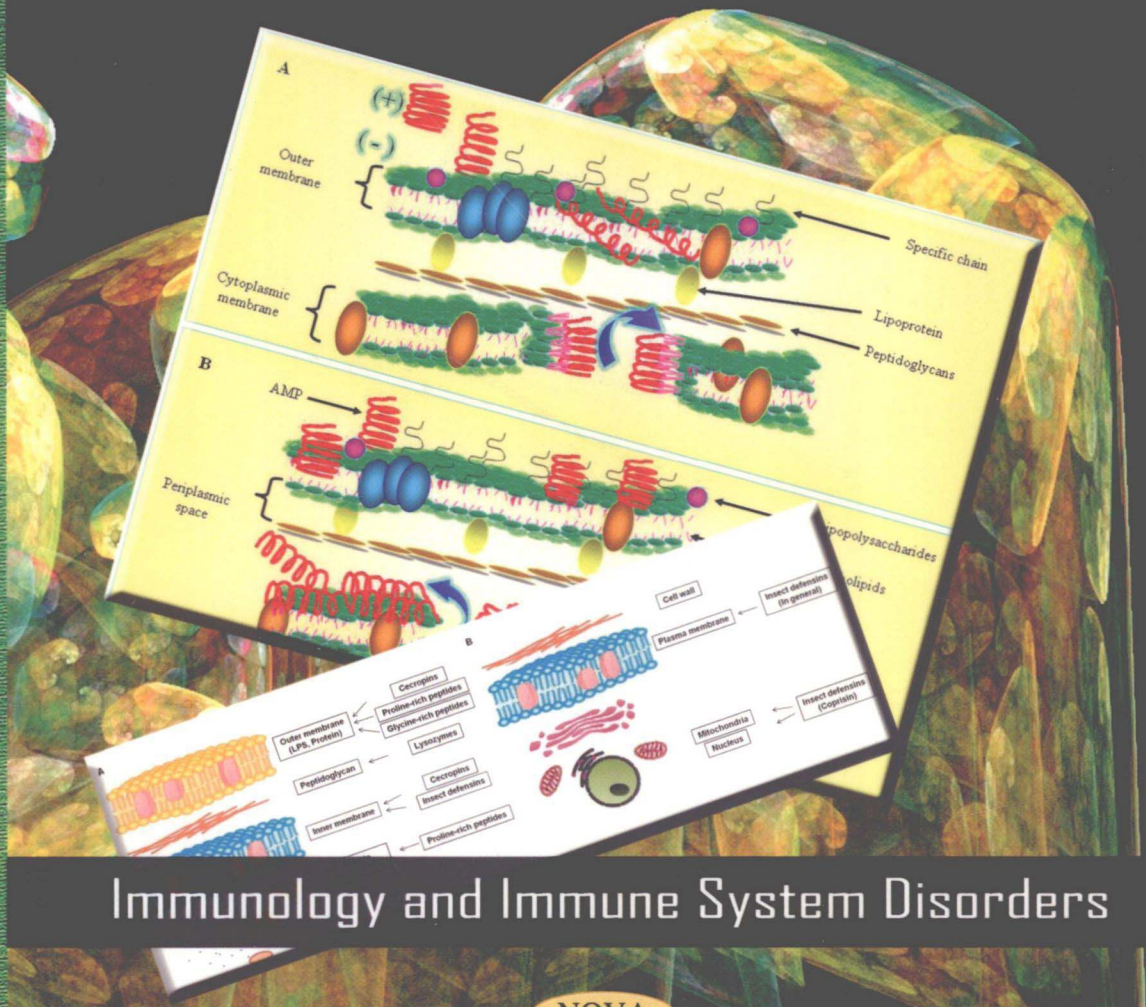
Editors

Antimicrobial Peptides

Properties, Functions and Role in Immune Response



Immunology and Immune System Disorders

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PROPERTIES, FUNCTIONS AND ROLE IN IMMUNE RESPONSE

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PREFACE

Antimicrobial peptides (AMPs) are small peptides which exhibit broad-spectrum antimicrobial activity and often activity against virus. In addition, AMPs exert several functions including endotoxin neutralization, chemotactic and immunomodulating activities, angiogenesis, and wound repair. In this book, the authors discuss the innate immune system and lipopolysaccharides; the biological role of fish antimicrobial peptides; casein-derived peptides; antimicrobial peptides in intestinal inflammation and infection; insect antimicrobial peptides in immunity and mechanisms of action; potential of AMPs as therapeutic tools against infectious disease; and how LC-MS/MS-based quantitative protein profiling can aid mechanistic studies of antimicrobial peptides.

Chapter 1 – In Gram-negative bacteria, lipopolysaccharide (LPS or endotoxin) is the major component of the outer leaflet of the bacterial cell wall and one of the most potent immunostimulary molecules known. The basic structure of LPS is highly conserved among Gram-negative organisms and consists of a polysaccharide (O-chain) covalently linked to a membrane-bound glycolipid (lipid A) through a core oligosaccharide. The inner sections of LPS are highly anionic due to numerous phosphoryl and carboxyl groups present in its core and lipid A sections.

Even at exceedingly low concentrations, LPS is detected by innate immune system cells bearing the TLR-4/MD-2 receptor-coreceptor, and this recognition induces beneficial responses including moderate fever and local inflammation. However, release of high concentrations of endotoxin by pathogens into the bloodstream triggers acute systemic reactions that may lead to septic shock and eventually to multiorganic failure and death.

Antimicrobial peptides (AMPs) are produced by virtually all types of organisms, and often constitute the first line of defense against microbial pathogens. The highly positive charge of AMPs and their amphiphilic character allow them to bind to anionic residues of the bacterial surface (mainly LPS in Gram-negative bacteria) and to rapidly kill their targets. In addition, AMPs can bind and sequester LPS *in vivo*, thus hampering recognition of this molecule by the immune system. In fact, treatment with AMPs has been shown to prevent sepsis and septic shock in animal models of endotoxemia.

The most important mechanism of resistance to AMPs in Gram-negative bacteria involves the expression of LPS variants with the ability to reduce interaction with AMPs. The LPS modifications include changes in electronegativity and/or hydrohobicity and can affect all the sections of the molecule.

Whereas some bacteria are intrinsically resistant to AMPs, others have sophisticated systems of AMP detection coupled to their LPS modification machinery. In this chapter, the authors will review examples of both types of strategies and will describe how some prominent human pathogens (*Proteus spp*, *Yersinia spp*. *Brucella spp*., *Salmonella spp*., *Bordetella spp*. and *Escherichia coli*) modify their LPS and how these alterations affect the bacterial resistance to AMPs. Interestingly, reduced ability to interact with AMPs correlate in some cases with changes in LPS recognition by cell receptors of the immune system. In addition, bacterial cells expressing these altered LPS display profound changes in virulence and endotoxicity. Examples of these correlations will be discussed in detail throughout the chapter.

Chapter 2 – In this chapter the authors will focus on the functions of known fish antimicrobial peptides (AMPs). Though there is significant information about the gene structure and transcript regulation of several fish AMPs the role of their synthetic or recombinant peptides is scarcely known. Thus, some studies have demonstrated the direct effect of fish AMPs, mainly β-defensins, on fish immunostimulation as well as powerful antibacterial or antiviral peptides.

Chapter 3 – The new advances in protein bioengineering help to explore numerous potentials for releasing biologically functional peptides due to degradation of proteins by specific enzymes. Recently, many researches are aiming to unlock the hidden biological functions of milk proteins/peptides beyond adequate nutritional effects. From the recent research reports, it has become clear that milk proteins are a source of biologically active peptides. These peptides are inactive within the sequence of parent protein and can be released during gastrointestinal digestion or food processing. In the last two decades, a number of bioactive peptides encrypted within the primary structure of casein were described. Several peptides with antibacterial activity and a variety of biological functions were found within the amino acid sequence of this group of milk proteins by employing different enzymatic strategies.

Chapter 4 – Antimicrobial peptides (AMPs) constitute an important part of innate immunity. AMP expression is often increased in response to colonic infection and inflammation. They also demonstrate a broad range of antimicrobial effects.

Over the last two decades, the roles of antimicrobial peptides have been discovered and explored. Certain AMPs such as alpha defensin HD 5-6 and beta defensin HBD1 are constitutively expressed while others including defensin HBD2-4 and bactericidal/permeability increasing protein (BPI) are associated with Inflammatory Bowel Disease (IBD). Gene expression of several AMPs (beta defensin HBD2-4 and cathelicidin) is induced in response to invasion of gut microbes. Cathelicidin can directly modulate colitis while other AMPS such as lactoferrin and hepcidin are utilized as biomarkers of IBD disease activity.

The application of AMPs for therapeutic purposes is still at an early stage of development. A few endogenous host-based AMPs (cathelicidin, elafin and SLPI) were shown to alter colitis when delivered intravenously or intracolonically in mouse colitis models. Novel AMPs (synthetic or artificial non-human peptides) with potent antimicrobial or anti-inflammatory property have been developed and may represent an alternative therapeutic approach against colitis and intestinal infection in the future. This report details the latest development of AMP-related research with emphasis in innate immunity and pathophysiology of colitis and intestinal infection.

Chapter 5 – Antimicrobial peptides (AMPs) are small peptides which exhibit broad-spectrum antimicrobial activity and often activity against virus. In addition AMPs exert several functions including endotoxin neutralization, chemotactic and immunomodulating activities, angiogenesis, and wound repair. In recent years, the AMPs have been implicated in several diseases including psoriasis, atopic dermatitis, rosacea, and Crohn's ileal disease among others. Because of their therapeutic effects, some of these peptides have been used successfully in the treatment of several pathologies such acne vulgaris, periodontal disease, and cancer. This chapter provides an overview of the main AMPs in humans, and discuss their properties, functions, and role in immune response.

Chapter 6 – Every living organism from prokaryotes to humans produces antimicrobial peptides (AMPs) as a component of innate immunity. AMPs less than 100 amino acid residues display broad-spectrum activity against pathogenic bacteria, fungi, and viruses. Particularly, insects that are the largest class within the animal kingdom due to a remarkable evolutionary success, have been continuously exposed to pathogenic microorganisms. Therefore, insects possess the potent antibacterial defense reactions and mainly rely on innate immunity rather than adaptive immunity because of energy efficiency. As a result, a single insect produces approximately 10-15 peptide antibiotics upon detection of invading the pathogens and the peptides are secreted into hemolymph. Insect AMPs are divided into five main classes: cecropins, insect defensins, glycine-rich/proline-rich peptides, and lysozymes. Although their groups and structures are diverse, insect AMPs are typically cationic and amphipathic structures, allowing them to interact with the anionic microbial surface and to insert into cytoplasmic membrane consisting of phospholipids. This action results in disruption of membrane integrity, like depolarization and pore formation. In addition to the membrane-active property, some insect AMPs have been recently reported to exert antimicrobial activity via different mechanisms including deactivation of bacterial protein and induction of yeast apoptosis. In case of targeting the cytosolic components by entering the cell without membrane perturbation, AMPs inhibit the synthesis of bacterial protein such as molecular chaperone DnaK and outer membrane protein or induce apoptotic death. During apoptosis induced by AMPs, intracellular reactive oxygen species accumulation and mitochondrial dysfunction play major roles in the pathway. Thereafter, various apoptotic phenotypes such as phosphatidylserine externalization, DNA and nuclei damage are shown in yeast cells. Insects have a highly successful immune system and the accurate understanding on the mechanisms can be used to design more potent AMPs.

Chapter 7 – With the rise of bacterial resistance to conventional antibiotic, there is a growing interest in anti-infective agents with fundamentally different modes of action than that of traditional antibiotics. Antimicrobial peptides (AMPs) are emerging as particularly innovative candidates in the antimicrobial drug research area to counteract bacterial mechanisms of antibiotic resistance. The properties that make AMPs promising alternatives to antibiotics include: a cidal activity on a broad range of infectious agents, a rapid and potent antibacterial activity including against multidrug resistant strains, a synergy with some classical antibiotics, an activity against biofilms, a lipopolysaccharide endotoxin neutralization activity, and an effectiveness in animal models. Some impediments, however, have hampered the attractiveness of these promising molecules for pharmaceutical use; i) AMPs are less efficient than current treatments on some antibiotic susceptible bacterial strains, ii) such molecules have a limited stability inside the host and the *in vivo* toxicology is unknown, iii) the high cost of manufacturing, iiii) and concerns about the acquisition of

resistance to both therapeutic and endogenous AMPs. In this short communication, these impediments and strategies that may overcome or are currently overcoming them will be discussed.

Chapter 8 – Novel antimicrobial drugs are in urgent need to overcome the continuous growth in the emergence of microbial resistance to current drugs. Antimicrobial peptides, a group of relative short (less than 100 amino acid residues), positively charged and amphiphilic peptides produced by a wide range of organisms as part of their first line of defense, are excellent candidates for the new drugs. Systematic and comprehensive understanding their mechanisms of action was thus urgently required. The microbial proteome adjusts rapidly in response to antimicrobial-agent challenge. These responses are highly specific for the physiological impairment encountered and usually directed at either compensating the loss of a particular function or counteracting the inflicted damage. For this reason, proteomic analysis may aid mechanistic studies of antimicrobial agents such as antimicrobial peptides. This chapter begins with an introduction of antimicrobial peptides, their structure classification, mode of action and potential role as novel antimicrobial-drug candidates are also discussed. A brief introduction of proteomic analysis is then presented, the role of quantitative protein profiling in mechanistic studies of antimicrobial peptides is discussed. Finally the quantitative protein profiling platform established by our lab for aiding the mechanistic studies of antimicrobial peptides will be presented.

CONTENTS

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Chapter 1

MODIFICATIONS IN LIPOPOLYSACCHARIDE THAT REDUCE INTERACTION OF BACTERIAL PATHOGENS WITH THE INNATE IMMUNE SYSTEM AND CAUSE RESISTANCE TO ANTIMICROBIAL PEPTIDES

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ABSTRACT

In Gram-negative bacteria, lipopolysaccharide (LPS or endotoxin) is the major component of the outer leaflet of the bacterial cell wall and one of the most potent immunostimulary molecules known. The basic structure of LPS is highly conserved among Gram-negative organisms and consists of a polysaccharide (O-chain) covalently linked to a membrane-bound glycolipid (lipid A) through a core oligosaccharide. The inner sections of LPS are highly anionic due to numerous phosphoryl and carboxyl groups present in its core and lipid A sections.

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targets. In addition, AMPs can bind and sequester LPS *in vivo*, thus hampering recognition of this molecule by the immune system. In fact, treatment with AMPs has been shown to prevent sepsis and septic shock in animal models of endotoxemia.

The most important mechanism of resistance to AMPs in Gram-negative bacteria involves the expression of LPS variants with the ability to reduce interaction with AMPs. The LPS modifications include changes in electronegativity and/or hydrohobicity and can affect all the sections of the molecule.

Whereas some bacteria are intrinsically resistant to AMPs, others have sophisticated systems of AMP detection coupled to their LPS modification machinery. In this chapter, we will review examples of both types of strategies and will describe how some prominent human pathogens (*Proteus spp*, *Yersinia spp. Brucella spp., Salmonella spp., Bordetella spp.* and *Escherichia coli*) modify their LPS and how these alterations affect the bacterial resistance to AMPs. Interestingly, reduced ability to interact with AMPs correlate in some cases with changes in LPS recognition by cell receptors of the immune system. In addition, bacterial cells expressing these altered LPS display profound changes in virulence and endotoxicity. Examples of these correlations will be discussed in detail throughout the chapter.

THE INNATE IMMUNE SYSTEM AND THE LIPOPOLYSACCHARIDE

Despite continuous medical advances, bacterial infections still remain among the world leading causes of death and bring about incalculable economic losses year after year. This is particularly evident for sepsis and septic shock, which claim more than 210,000 deaths in the United States annually (Skrupky et al., 2011). In the majority of the cases, these conditions arise subsequent to an infection and are the result of a disproportionate response of the immune system to microbial factors (Cinel and Opal, 2009).

In sepsis caused by Gram-negative organisms, endotoxin is the major immunostimulatory factor. This molecule can be released into the extracellular milieu by cell division or by the action of immune system effectors (Holzheimer, 1998). In some cases, therapy with some antibiotics can even enhance LPS liberation from the bacterial cells thus worsening the symptoms of sepsis (Kirikae et al., 1997).

The first event in the interaction of the innate immune system with endotoxin involves binding of LPS to a serum protein called LPS binding protein (LBP). The LPS-LBP complex is then recognized by specific receptors such as CD14. Receptor binding triggers intracellular signalling through the Toll Like Receptor-4(TLR-4) /Myeloid differentiation factor 2 (MD-2) leading to the synthesis and release of potent pro-inflammatory mediators (Lopez-Bojorquez et al., 2004; Alexander and Rietschel, 2001). These include IL-1, IL-6 and TNF-α, which are then followed by IFN-γ, IL-1, IL-2, IL-8, IL-12, MIP-2, prostaglandin E2 (PGE2) and reactive oxygen species (ROS). Local secretion of these factors is a key event to controlling infection but becomes detrimental if mediators are produced systemically and/or in excessive amounts. Under the latter conditions pro-inflammatory mediators cause massive cell migration into tissues, coagulation and other profound physiological alterations that, when combined, lead to tissue hypoperfusion and organic failure (Annane et al., 2005).

The innate immune system can recognize endotoxins from a wide variety of Gram-negative organisms. This capacity relies on the fact that some sections of LPS display little

structural variability. In particular, lipid A is the most structurally conserved part of the molecule and the motif recognized by TLR-4/MD-2 (Jerala 2007). Not surprisingly, the lipid A has been called the "endotoxic principle", since it causes all the symptoms associated with endotoxemia when administered systemically (Zähringer et al., 1999).

The structural conservation of LPS is exploited by the innate immune system not only passively (i.e. for recognition) but also in an active way, namely via the production of bactericidal compounds targeting the bacterial cell surface. Thus, as part of its arsenal, the human immune system secretes a wide variety of AMPs that bind to LPS and disorganize the bacterial envelope. Typically, AMPs are relatively short (12 to 100 amino acids), positively charged (net charge of +2 to +9) and amphiphilic (Jenssen et al., 2006). The initial interaction between AMPs and the bacterial surface is mainly electrostatic and involves the cationic residues of the peptides and some conserved LPS regions with high concentrations of negatively charge groups. This mechanism of defense is very conserved evolutionary and AMPs have been found in the vast majority of organisms including microorganisms, plants, invertebrates, amphibians, birds, fishes and mammals. AMPs have been shown to efficiently bind and neutralize LPS *in vivo* and to protect animals against lethal endotoxic shock (Gutsmann et al., 2010; Brandenburg et al., 2010). The most extensively studied AMP is the antibiotic polymyxin B, whereas prominent examples of human AMPs include defensins, cathelicidins, and lactoferricin.

This chapter summarizes both the structural features common to all types of endotoxins and the modifications that some relevant bacterial pathogens introduce in their LPS to avoid recognition by the innate immune system. These structural changes affect interaction of LPS both with receptors of the innate immune such as TLR-4/MD-2 and with innate immune effectors (AMPs, among others). Interestingly, LPS variants with reduced ability to interact with AMP correlate in some cases with changes in LPS recognition by the immune system.

SALMONELLA ENDOTOXIN, THE "CANONICAL" LPS

The genus *Salmonella* comprises a group of Gram-negative bacterial pathogens of humans and animals that cause diseases such as enteric fever and some types of severe gastroenteritis. Like many other enterobacteria, this pathogen uses the oral fecal route to access to its ecological niche, the gut, and for its transmission between hosts. *Salmonella* and *Escherichia coli* are two groups of closely related microorganisms and their LPSs are sometimes chemically indistinguishable. The LPS of *Salmonella-Escherichia* has been by far the most extensively studied of all and is considered as the archetypical or "canonical" endotoxin molecule.

LPS Composition

Like other "smooth" LPSs, *Salmonella* LPS consists of long O-polysaccharide or O-antigen (most often made of repeating units of a short oligosaccharide) linked to the core-lipid A. In almost all O-serotypes, the O-polysaccharide bears no significant charge and several studies have shown that it hinders the action of AMPs. On the other hand, the core

oligosaccharide of *Salmonella* LPS carries several negatively charged groups (phosphates and 3-Deoxy-D-manno-octulosonic acid residues (KDO)) and the two phosphoryl groups of lipid A. *Salmonella* lipid A is typically composed of a phosphorylated glucosamine disaccharide carrying six acyl chains of 12 to 14 carbon residues (Figure 1). The core acid and phosphorylated sugars lay close to the phosphorylated disaccharide backbone whose hydrophobic acyl chains anchor the LPS to the bacterial outer membrane. This peculiar structure is the target of AMPs which first electrostatically bind to the corresponding LPS sections and then disrupt the membrane causing structural damage and dramatic permeability changes (Powers and Hancock, 2003; Yeaman, 2003). Similarly, the minimal structure of *Salmonella* LPS carrying full endotoxic activity consists of the lipid A disaccharide backbone with the two phosphoryl groups, and six fatty acids (saturated, and in part, 3-hydroxylated) including 3-acyloxyacyl groups (Rietschel et al., 1994). For *E. coli* LPS, it has been described that five of the six acyl chains interact with the hydrophobic pocket in MD-2 whereas the other one is partially exposed on the surface of MD-2 facilitating the interaction with TLR-4 through their hydrophobic residues (Park et al., 2009).

LPS Modification and Effect in the Immune Response

Modifications altering interaction of LPS with the innate immune system have been described in several *Salmonella* serovars. From a biochemical point of view, LPS remodeling includes changes in its hydrophobicity (increase or reduction of the acylation in lipid A) and in its net charge, the latter associated normally with the removal of phosphate groups or their substitution with aminocompounds. All these changes, summarized in Figure 1, are regulated by complex regulatory systems like those based on a membrane bound sensor and its corresponding response regulator, called two-component sensory/regulatory systems. In *Salmonella*, the most important systems of this type involved in LPS modification are PhoP-PhoQ and PmrA-PmrB.

The PhoP-PhoQ sensory/regulatory system was first described in *Salmonella* (Miller, et al., 1989). PhoQ is a sensor kinase bound to the bacterial membrane that can be activated by several stimuli such as acidic pH, the presence of certain AMPs and low levels of magnesium and calcium. (Alpuche Aranda, et al., 1992; Bader et al., 2003; Prost, et al., 2008). All these signals are thought to be characteristic of acidified phagosomes. The sensor kinase acts by phosphorylating the cytoplasmatic component, PhoP, a regulator that controls the transcription of a large number of genes involved in the remodeling of the Gram-negative envelope. Those modifications include the addition of palmitate groups into lipid A moiety (*pagP*), deacylation of lipid A (*pagL, lpxR*) or hydroxylation (*lpxO*) (Bishop et al., 2000; Raetz and Whitfield, 2002; Reynolds et al., 2006). Since LPS hydrophobicity is one of the key players of the interaction with the immune system, changes in the acylation or deacylation of the LPS have a great influence in the immune response (Kawasaki, et al., 2005; Rietschel et al., 1994).

Interestingly, the PhoP-PhoQ system was reported not only to be necessary for *Salmonella* resistance to AMPs and for its intracellular survival within phagocytes but also for *Salmonella* virulence in animal models and in humans (Alpuche Aranda et al., 1992; Ernst et al., 2001; Kawano et al., 2010; Miller et al., 1989; Prost et al., 2008). At the molecular level, it has been described that the *pagL*-dependent removal of the β-hydroxymyristoyl

residue at position 3´ might reduce the cytokine inducing potential of bacterial cells (Raetz and Whitfield, 2002) and that palmytoylation of lipid A mediated by *pagP* antagonizes signal transduction in human cells lines (Tanamoto and Azumi, 2000).

The activity of the PmrA-PmrB system also leads to the production of LPS variants with altered pattern of cytokine induction. These modifications affect the ability of monocytes/macrophages to recognize *Salmonella* cells (Rietschel et al., 1994) and the virulence in mice (Gunn et al., 2000). This two- component sensory/regulatory system is activated not only in acidic environments such as the phagosome of macrophages (Faucher et al., 2006), but also in the presence of cations (ferric, aluminum) and AMPs (Nishino et al., 2006; Gunn and Miller, 1996; Raetz and Whitfield, 2002).

The PmrA-PmrB system activates genes involved in the attachment of 4-amino-4-deoxy-L-arabinose (Ara4N) (*pmrE* and the *pmrHFIJKLM* operon also called *pmrF* locus) and phosphoethanolamine (*pmrC o eptA*) to the *Salmonella* lipid A diglucosamine backbone. Since these chemical groups are positively charged, their addition to the lipid A makes the LPS less anionic thereby reducing the affinity of AMPs for the bacterial surface and mediating AMP resistance (Gunn, 2001; 2008; Yeaman, 2003).

A different strategy that *Salmonella* uses to lower the negative charge of its LPS involves the removal of phosphate groups. Touzé *et al* identified a phosphotransferase, LpxT, that adds a second phosphate group to monophosphorylated lipid A, increasing the negative charge of the bacterial surface (Touzé et al., 2007). Recently, it has been reported that PmrA interferes with this modification by downregulating *lpxT*. Furthermore, the same authors described that phosphoethanolamine addition to lipid A requires the simultaneous inhibition of *lpxT* by PmrA (Herrera et al., 2010).

Besides lipid A, other portions of the LPS are also implicated in the interaction of *Salmonella* endotoxin with the host immune system. Thus, modifications in the LPS core and O-antigen (Figure 1), have been shown to influence to a large extent the susceptibility of *Salmonella* to both serum and AMPs (Tamayo et al., 2005). These structural changes were also described to mediate resistance to complement (Murray et al., 2006) and to enhance bacterial survival in intestinal cells (Duerr et al., 2009; Zenk et al., 2009) dendritic cells (Zenk et al., 2009) and macrophages. This mechanism of resistance is likely dependent on the ability of the O-chain to act as a physical barrier preventing the recognition of those conserved molecular patterns that are located more internally in the LPS.

Finally, the RcsC/YojN/RcsB phosphorelay system can also mediate LPS modification in *Salmonella*. This system was firstly identified in *E. coli* where it controls capsule synthesis and exopolysaccharide production thereby playing an important role in biofilm formation (Latasa et al., 2012; Majdalani and Gottesman, 2005). The sensor component of this phosphorelay is activated by envelope stresses such as high osmolarity, desiccation, low temperature and exposure to AMPs (Farris et al., 2010; Hagiwara et al., 2003; Sledjeski and Gottesman, 1996). This system promotes the activation of the wzz_{st}(*cld*) gene product which in turn causes O-antigen elongation and mediates serum resistance (Delgado et al., 2006). The wzz_{st} gene is also activated by the PmrAB system.

In addition *to E. coli* and *Salmonella*, other Gram-negative bacteria share the above-mentioned mechanisms for LPS modification. Thus, orthologous of PmrAB and PhoPQ have been detected both in *Yersinia spp* and *Pseudomonas aeruginosa*. However, although these systems mediate changes of LPS similar to those described for *Salmonella*, they appear to

respond to distinct regulatory stimuli (McPhee et al., 2006; Moskowitz et al., 2003; Oyston et al., 2000; Raetz, 2001; Reines et al., 2012).

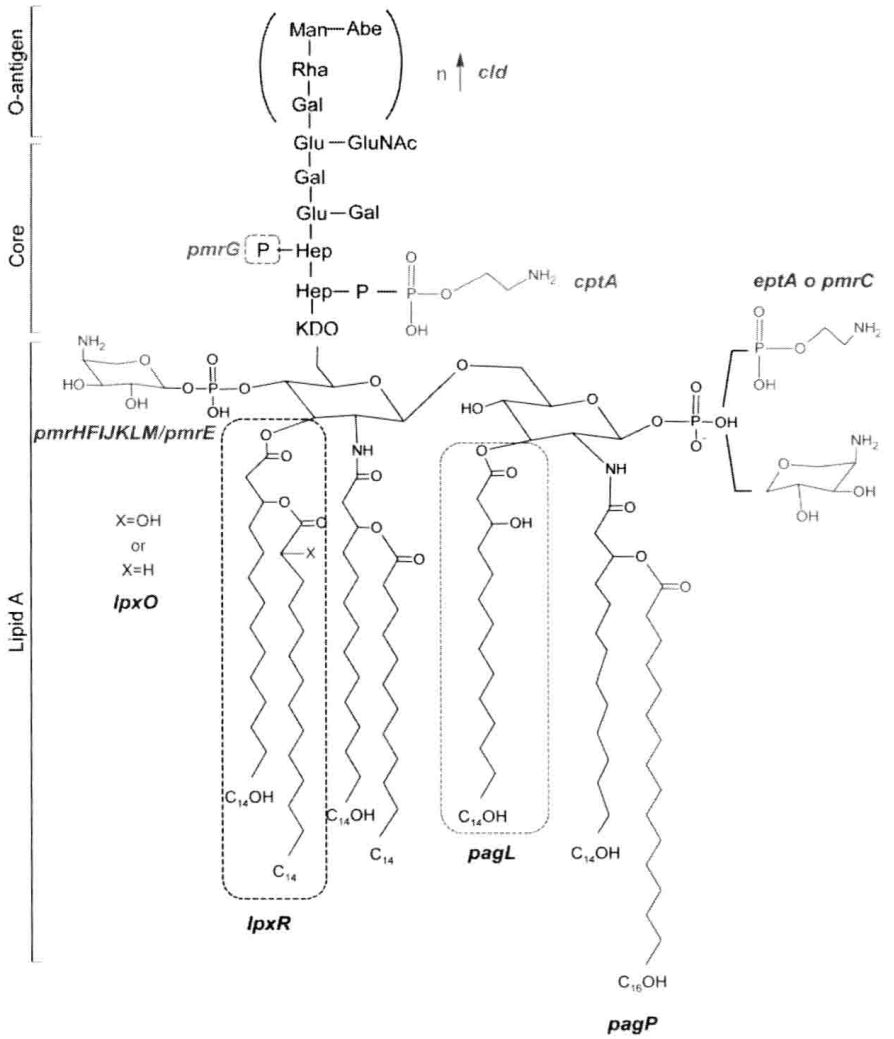

Figure 1. Chemical structure of *Salmonella enterica* Typhimurium LPS. Some LPS groups can be modified by the two component systems PhoP-PhoQ (in blue) and PmrA-PmrB (in red), which regulate the expression of genes involved in the resistance of *Salmonella* to bactericidal mechanism of the innate immune system including AMPs. Abbreviations: *pagP*, palmitoyl transferase; *pagL*, 3-O-deacylase; *lpxR*, 3′-*O*-deacylase (regulation unknown); *lpxO*, production of 2-hydroxy myristate; *eptA* o *pmrC*, lipid A phosphoethanolamine phosphotransferase; pmrHFIJKL/pmrE, involved in 4-aminoarabinose addition; *cptA*, heptose I phosphoethanolamine phosphotransferase; *pmrG*, heptose II phosphatase; *cld*, O-antigen chain length determinant.

Supramolecular Structure

Apart from the nature of the components that form the LPS, the supramolecular structure greatly influences its activity. Like other amphiphiles, monomeric LPS molecules can form aggregates. This organization in lamellar, cubic and hexagonal structures affects the endotoxicity of the LPS being the former state the least biologically active and the latter the