Lipid A phosphoethanolamine transferase

Regulation, structure and immune response

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Keywords: antibiotic resistance; lipid A modification; EptA; two-component systems; immune response

Abstract

A wide variety of antibiotics are targeted to the bacterial membrane due to its unique arrangement and composition relative to the host mammalian membranes. By modification of their membranes, some Gram-negative pathogens resist the action of antibiotics. Lipid A phosphoethanolamine transferase (EptA) is an intramembrane enzyme that modifies the lipid A portion of LPS/LOS by addition of phosphoethanolamine. This modification reduces the overall net-negative charge of the outer membrane of some Gram-negative bacteria, conferring resistance to polymyxin. This resistance mechanism has resulted in a global public health issue due to the increased use of polymyxin as last-resort antibiotic treatments against multi drug resistant pathogens. Studies show that, without EptA, pathogenic bacteria become more sensitive to polymyxin and to clearance by the host immune system, suggesting the importance of this target enzyme for the development of novel therapeutic agents. In this review EptA will be discussed comprehensively. Specifically this review will cover the regulation of *eptA* expression by the two component systems PmrA/PmrB and PhoP/PhoQ, the site of modification on lipid A, the structure and catalytic mechanism of EptA in comparison to MCR-1 and *E. coli* alkaline phosphatase, and the host immune system's response to lipid A modification by EptA. The overarching aim of this review is to provide a comprehensive overview of polymyxin resistance mediated by EptA.

Introduction

Antimicrobial therapeutic agents have been developed extensively as treatments against various infectious agents which include bacteria (antibiotics), viruses (antivirals), fungi (antifungals) and parasites (e.g. antimalarials). Infectious agents such as bacteria can develop a variety of mechanisms which allow them to survive drugs exposure that would normally inhibit their growth or kill them [1]. The two major genetic strategies that bacteria have developed to acquire antibiotic resistance are: mutations in chromosomal genes (mutational resistance) and horizontal gene transfer [2,3]. An example of a resistance

mechanism arising from genetic alterations is the modification of the antibiotic's target site, resulting in a decreased affinity of the antibiotic agent. Modification of the target site can be achieved by: point mutations in the genes encoding the target site, enzymatic alterations of the binding site, and/or replacement or bypass of the original target site [2–5].

Due to its unique arrangement and composition relative to host mammalian membranes, the bacterial membrane is a major target of many antibiotics [6]. For example, the outer leaflet of the outer membrane of many Gram-negative bacteria is composed of lipopolysaccharides (LPS), also called endotoxins (Figure 1A). LPS makes up a major component of the outer layer of the outer membrane of most Gram-negative bacteria where it acts as a protective barrier against antibiotics and environmental stresses. LPS is composed of three components: a lipid A moiety, an oligosaccharide core and an O-antigen group (Figure 1B). The hydrophobic lipid A moiety is anchored to the bacterial outer membrane and covalently linked to the hydrophilic oligosaccharide core. The O-antigen domain, made up of oligosaccharide repeating units of varying lengths, is covalently attached to the core oligosaccharide [7]. This O-antigen is commonly found in enteric bacteria, however bacteria that reside on the respiratory and genital mucosal surfaces lack the O-antigen. LPS in which the O-antigen is absent is known as lipooligosaccharide (LOS) [8]. LPS/LOS are important in eliciting immune responses and also play a role in bacterial virulence. Due to their negative charge, they are the target site for cationic antimicrobial peptides (CAMPs) such as polymyxin B and polymyxin E (also known as colistin). CAMPs act by binding to the bacterial membrane and are thought to be involved in the formation of pores resulting in the eventual lysis and killing of the bacteria. However, some Gram-negative pathogens have evolved enzymes that act to modify their membrane structure and biochemical features thereby evading the action of antibiotics directed at the membrane. One example is the modification of the lipid A moiety of the LPS/LOS structure [9].

Modification by incorporation of 4-amino-4-deoxy-L-arabinose (L-Ara4N) [10] and phosphoethanolamine (pEtN) [11] to the lipid A portion of LPS acts to decrease the sensitivity of Gram-negative bacteria to CAMPs. The addition of L-Ara4N and pEtN to the

phosphate groups of the negatively charged lipid A reduces its electrostatic interaction with the α,γ-diaminobutyric acid (Dab) residue of the positively charged polymyxin. This reduced interaction inhibits the disruption of the membrane thus bacterial lysis does not occur [12]. Since polymyxin have been used increasingly as a last-resort antibiotic for treatment of multi drug resistant (MDR) Gram-negative bacteria, especially for *Pseudomonas aeruginosa*, *Acinetobacter baumanii* and Enterobacteriaceae, this resistance mechanism poses a significant global health problem [13,14].

Lipid A phosphoethanolamine transferase (EptA) is the enzyme responsible for modification of lipid A with pEtN and will be the focus of this review. Some examples of pathogenic bacteria that have shown resistance to polymyxin due to the addition of pEtN to lipid A are *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Escherichia coli*, *Shigella flexneri*, *Salmonella enterica* serovar Typhymurium, *Vibrio cholerae* and *Helicobacter pylori* [9,15–20]. Studies on pathogenic *Neisseria* report that, without modification by EptA, the bacterial susceptibility to polymyxin increased more than 100-fold, the killing of the bacteria by normal human serum was enhanced and their sensitivity to the host antimicrobial peptides, LL-37 and protegrin-1, increased by ~10 fold [17,18]. These results suggest that therapeutic agents designed to inhibit the activity of EptA can be used to increase the efficacy of polymyxin and support the clearance of infection by the immune system. Acknowledging the importance of EptA in polymyxin resistance mechanisms and its potential for drug development, a comprehensive understanding of the functional aspects of EptA as well as its regulation and the impact of its enzymatic chemistry will provide a strong foundation to strategically plan further studies aimed at the development of novel inhibitors of the enzyme.

Expression of *eptA* is regulated by PmrA/PmrB and PhoP/PhoQ two-component systems

Expression of *eptA* is induced or repressed by the two-component systems (TCSs) involving PmrA/PmrB and PhoP/PhoQ (Figure 2). PmrA/PmrB is the major regulator of

expression of genes that are involved in modification of LPS in various species of bacteria (e.g. *E. coli*, *Vibrio cholerae*, *Helicobacter pylori*, *Salmonella* Typhymurium, *Shigella flexneri*, *Neisseria gonorrhoeae* and *Neisseria meningitidis*) [9,21]. The PmrA/PmrB regulatory system consists of a cytoplasmic response regulator (PmrA) and a membrane-bound sensor kinase (PmrB). Ferric (Fe^{3+}) iron, aluminium (Al^{3+}), mildly acidic pH (e.g. pH 5.8) and vanadate are the known signals for activation of PmrB in *Salmonella.* In *E. coli*, Zn^{2+} also acts as an activating signal for PmrB [22]. High concentrations of Fe^{3+} or Al^{3+} (i.e. 100 μM) leads to autophosphorylation of PmrB, the phosphoryl group of which is transferred to PmrA, thus activating PmrA. Active PmrA binds to DNA to promote expression of PmrA-activated genes and repress PmrA-repressed genes. *eptA* (also known as *pmrC* or *yjdB*), which is encoded in the *pmrCAB* operon, is one of the genes that is positively regulated by PmrA.

In addition to direct regulation by PmrA/PmrB, *eptA* is indirectly regulated by the PhoP/PhoQ system [21–24]. The PhoP/PhoQ system consists of PhoQ, a membrane-bound sensor kinase, and PhoP, a cytoplasmic based transcriptional regulator. PhoQ senses changes of divalent cations (in particular Mg^{2+} but also Ca^{2+} and Mn^{2+}) in the periplasmic space of the bacterial cell as well as mildly acidic pH. Low (micromolar) concentrations of Mg^{2+} causes PhoQ to dimerize and undergo autophosphorylation. Dimerized PhoQ transfers the phosphate to PhoP which activates its modulation of transcription (increasing the transcription of PhoP-activated genes or repressing the transcription of PhoP-repressed genes). In contrast, high concentrations (millimolar) of Mg^{2+} causes PhoQ to dephosphorylate phospho-PhoP thereby deactivating PhoP [23–26]. PmrD is a small protein activated by PhoP. Active PmrD binds to active phospho-PmrA and prevents its dephosphorylation by PmrB. In short, PmrD maintains PmrA in an active state [21,23,27]. Additionally, the presence of host CAMPs activates PhoQ, by displacement of divalent cations from PhoQ [25,28], thus promoting transcription of certain PhoP and PmrA-dependent genes [29,30].

Modification of lipid A in *Pseudomonas aeruginosa* by phosphoethanolamine transferase termed $EptA_{Pa}$ that adds pEtN moiety exclusively to the 4’ phosphate group was

first reported by Nowicki *et al.* (2015) [31]. Unlike other lipid A pEtN transferases, $EptA_{Pa}$ is regulated by the ColRS two-component system. Upon sensing excess extracellular Zn^{2+}, the ColRS system upregulates $eptA_{Pa}$ transcription and downregulates transcription of *arnT* demonstrating coordinated control over lipid A modifications. Interestingly, this modification has no effect on polymyxin resistance of *P. aeruginosa*.

Specific mutations within the TCS have also been shown to activate the system, thus promoting the expression of genes under its regulation [15]. Several mutations in the PmrA/PmrB system have resulted in increased resistance to polymyxin due to lipid A decoration with pEtN, as well as L-Ara4N. Based on structural studies of PmrA from *Klebsiella pneumoniae* [32], the protein can be divided into two domains: the phosphate receiver domain (REC) and the DNA binding domain (DBD). All PmrA mutations were located in the REC domain [33,34], therefore it is likely that the mutations prevent dephosphorylation of PmrA by PmrB [34]. PmrB is predicted to have seven domains: a cytoplasmic secretion signal domain, two transmembrane domains (TM1 and TM2), a periplasmic domain, a linker domain (HAMP), a histidine-phosphotransfering domain (DHp) and an ATP-binding domain [35,36]. Mutations found in TM1, HAMP, DHp and ATP-binding domains in *Salmonella* sp. resulted in increased resistance to colistin possibly due to the increase of the kinase activity of PmrB compared to its phosphatase activity [34]. Additionally, mutations found in the periplasmic domain of *Pseudomonas aeruginosa* [35] and the cytoplasmic secretion signal domain of *Pseudomonas aeruginosa* [35] and *E. coli* [36] conferred polymyxin resistance in these pathogens. Huang *et al.* have recently published bioinformatic analyses that identify various nonsynonymous mutations on PhoP/PhoQ and PmrA/PmrB in seven pathogenic Gram-negative bacteria. The mutations contribute to resistance to polymyxin through the activation of genes, including *eptA*, responsible for lipid A modifications [37]. Therefore, external stimuli as well as mutations within the systems can indirectly contribute to polymyxin resistance through the upregulation of *eptA* expression.

EptA is able to modify both 1- and 4'- phosphate group of lipid A

eptA (formerly named *lptA*) was first reported in *Neisseria meningitidis* by Cox *et al.* (2003) as a gene that encodes a pEtN transferase specific for lipid A and found to be present in all pathogenic strains of *Neisseria* [11]. The donor substrate in this reaction is phosphatidylethanolamine (PE). The location of pEtN modification by EptA differs slightly among Gram-negative species. In *E. coli* and *Salmonella* Typhymurium, EptA (also known as PmrC) predominantly modifies the 1-phosphate group of lipid A while the 4'-phosphate group is modified by addition of L-Ara4N. However under certain conditions, such as in the absence of L-Ara4N, EptA will modify the 4' position with a second pEtN moiety [38,39]. The addition of pEtN to lipid A in *E. coli* K-12 only takes place when the cells are exposed to mildly acidic growth conditions [38], while in pathogenic *E. coli* O157:H7 this modification is constantly present [40]. In pathogenic *Neisseria* sp. modification of lipid A with pEtN occurs at both the 1 and 4' position due to the absence of Ara4N in the organism, however in gonococci the addition to 4' position is preferred [18,41].

Lipid A modification with L-Ara4N is more common in comparison to pEtN modification due to its efficacy in reducing the overall net negative charge of lipid A (L-Ara4N decreases the net negative charge of lipid A to 0 while pEtN decreases the net negative charge from -1.5 to -1) [15]. It is possible that EptA modifies both phosphate groups of lipid A, when modification with L-Ara4N is not viable, in order to compensate for the differences in enzymes' presence, thus maintaining bacterial resistance against polymyxin as well as maintaining membrane integrity (reducing electrostatic repulsion between LPS/LOS molecules due to the negatively-charged phosphate moieties and acidic sugar). Additionally, variations on the site of modification by EptA may result in different levels of polymyxin resistance across different Gram-negative bacteria. A full understanding of this variation could be useful in optimising the clinical use of polymyxin.

Structure and Catalytic Mechanism of EptA

EptA is a membrane protein that catalyses the transfer of phosphoethanolamine (pEtN) from phosphatidylethanolamine (PE) to the 1- and 4'- phosphate headgroups of the lipid A (Figure 3) [9,15–20]. Multiple sequence analysis (MSA) of EptA across ten clinically relevant Gram-negative bacteria species as well as *E. coli* MCR-1/2 show that the soluble C-terminal catalytic domain of the protein is more conserved than the N-terminal transmembrane domain (mean pairwise sequence identity of 50% and 31% respectively) [42]. Mobilised colisitin resistance (MCR) is a new member of the phosphoethanolamine transferase enzyme family that is highly homologous to EptA [43–45]. Like EptA, it also catalyses the addition of pEtN to the phosphate headgroup of lipid A. Phylogenetic analyses placed the two enzymes into two neighbouring sub-clades which suggest that the enzymes have parallel evolutionary paths. However unlike EptA, whose gene is chromosome encoded, the *mcr* gene is plasmid-mediated and therefore the resistance determinant is readily mobilised/transferrable to other bacteria [43,46], accelerating the development of antibiotic resistance in Gram-negative bacteria.

The high diversity of the membrane composition across different bacterial species may contribute to the higher sequence variability of the transmembrane domain. The atomic structure of the catalytic domain of EptA from *N. meningitidis* (*Nm*EptA) has been initially solved [47] and shows that the enzyme exhibits a similar fold and significant homology of active-site residues to phosphatases such as *E. coli* alkaline phosphatase [48]. Following this, the crystal structure of full-length *Nm*EptA [49] was determined, revealing two discretely folded domains: a hydrophobic transmembrane domain embedded in the cytoplasmic membrane and a C-terminal soluble periplasmic-facing domain. The two domains are connected by a bridging helix and an extended loop (Figure 4A). While a number of structures of homologues have been determined for the catalytic domain [50–54] only *Nm*EptA has been structurally characterised in its full-length form.

The N-terminal domain of *Nm*EptA is helical with 5 helices arranged approximately parallel to each other extending into the membrane however only one of these helices is sufficiently long enough to span the full width of the inner membrane. Loops between the

membrane helices are short on the cytoplasmic facing surface while they are more extended on the periplasmic facing surface of the domain. A long loop between transmembrane helices 3 and 4 contain two periplasmic facing helices termed PH2 and PH2' (Figure 4B). These small helices are positioned close to the periplasmic facing soluble domain and have been proposed to form the walls to the substrate binding site where the pEtN donor lipid is thought to bind. The conserved residue that is proposed to interact with the amine group of pEtN, Glu114, is located in PH2 helix. A pEtN molecule that would form a covalent enzyme intermediate can be accommodated between this residue and the catalytic nucleophile Thr280 [49]. Further mutational studies showed that mutation of this residue into alanine (E114A) inactivates the enzyme, further supporting its role in substrate binding [55].

The C-terminal periplasmic facing domain of *Nm*EptA exhibits a hydrolase fold composed of a seven-stranded mixed beta pleated sheet sandwiched by alpha helices. Ten cysteine residues are present in this domain and are involved in 5 intramolecular disulphide bonds as expected given the oxidising environment of the periplasm where this domain is localised (Figure 4B). These disulphide bonds are proposed to be important for the structural integrity and stability of the protein. The overall fold of the domain exhibits distinct structural similarity to alkaline phosphatase from *E. coli*, a metal binding enzyme that catalyses the hydrolysis and transphosphorylation of phosphate monoesters through a covalent serine-phosphate intermediate. Here we compare the metal binding sites between *Nm*EptA, MCR-1 and alkaline phosphatase in order to provide some insight regarding the catalytic reaction mechanism of the enzyme. The broad family of lipid A phosphoethanolamine transferases exhibit conserved metal-binding residues and a conserved catalytic threonine (Thr280 in *Nm*EptA) that acts as the nucleophile for substrate attack and formation of an enzyme intermediate. A Zn^{2+} ion is located at the carboxyl end of the beta pleated sheet and is tetrahedrally coordinated by 4 residues: Asp452, His453, Glu240 and the catalytic Thr280 (Figure 4B). This Zn^{2+} ion is identically positioned in both the soluble domain and the full-length *Nm*EptA structures (PDB 4KAV and 5FGN respectively). In MCR-1 the identical metal binding residues are Asp465, His466, Glu246 and Thr285 (Figure 5A) [50–54].

In alkaline phosphatase two Zn^{2+} ions and a Mg^{2+} ion are bound to the structure and have been proposed to play important roles in substrate binding and catalysis. The structurally identical Zn^{2+} ion to that seen in *Nm*EptA and MCR-1 is tetrahedrally coordinated by conserved residues Asp369, His370, Asp51 and the catalytic nucleophile Ser102 (Figure 5B) and is proposed to play a role in stabilising the catalytic nucleophile, Ser102, the hydroxyl group of which undergoes deprotonation by a Mg^{2+}-coordinated hydroxide ion [48]. The second Zn^{2+} ion in alkaline phosphatase is coordinated by His412, Asp327 and His 331. This Zn^{2+} ion functions to position the substrate and enables the release of the alcohol leaving group after cleavage from the phosphoester. Additionally it acts to coordinate the nucleophilic hydroxide ion for the second step of the reaction involving hydroxide ion attack on the phosphorus atom and hydrolysis of the serine-phosphate intermediate [48]. Given the importance of the second Zn^{2+} ion in the reaction mechanism of alkaline phosphatase, it is possible that the second Zn^{2+} ion binding site is also required for the catalytic mechanism of EptA. The first Zn^{2+} ion is sufficient for the removal of pEtN from PE (as shown by an *Nm*EptA enzymatic assay using a fluorescently labelled substrate [49]) however the second Zn^{2+} ion might be needed for the transfer of pEtN to the lipid A substrate for the completion of the reaction. The above-mentioned enzymatic assay does not assess the second half of the enzymatic reaction, that is the transfer step.

This second Zn^{2+} ion is found in the structure of the soluble domain of *Nm*EptA only when crystals are soaked in a solution containing 1 mM $ZnSO_4$ [47]. In the case of MCR-1 it is observed in the structures as the protein was purified in buffer containing $ZnCl_2$ [54] or as the cell culture medium contains $ZnCl_2$ [50]. However in both *Nm*EptA and MCR-1 only two metal coordinating ligands (His465/His478 and His383/His395 for *Nm*EptA/MCR-1 respectively) are observed [47,54]. In the soluble domain structure of MCR-1 the second Zn^{2+} ion is also coordinated by a water molecule and by the side chain of Glu300 from a symmetry related molecule [50,54]. Soaking of crystals of the soluble domain of *Nm*EptA with magnesium salts failed to show binding [47] suggesting that the mechanism for activating the Thr280 nucleophile may differ from that proposed for alkaline phosphatase.

However, attempts to probe Mg^{2+} and additional Zn^{2+} binding sites in the full-length structure have not yet been carried out and full metal coordination may require portions of the transmembrane domain. In alkaline phosphatase, the residues that coordinate the bounding Mg^{2+} ion are Asp51, Thr155 and Glu322 [48]. The importance of the Zn^{2+} ion to the structural integrity of the protein is further supported by the fact that attempts to remove the metal ion from crystals of the enzyme through treatment with 10 mM ethylenediaminetetraacetic acid (EDTA), were unsuccessful suggesting it is very tightly bound to the protein [47]. Furthermore, incorporation of 0.85 mM EDTA to MCR-1's colistin MIC experiments reduced the MIC of colistin to vector-only controls suggesting that the metal is critical to the catalytic activity of the enzyme [54].

The structure of full-length *Nm*EptA revealed extra electron density near to the bound Zn^{2+} ion and between the PH2 and PH2' helices which was compatible with a single molecule of dodecylmaltoside (DDM) detergent molecule (Figure 4C) in which the enzyme was solubilised and purified [49]. The presence of the DDM molecule helped to identify the substrate binding region of the enzyme. Furthermore, this led to a proposal that the protein is flexible and that the crystal structure may represent a pEtN bound complex. A large conformational change in the relative orientation of the two domains would likely be required to accommodate a large lipid A (receiver) substrate for transfer of the ethanolamine from the enzyme intermediate. Molecular dynamic simulations of *Nm*EptA in a modelled lipid bilayer showed that the protein is conformationally flexible [49]. It is possible that binding of the acceptor lipid substrate might reveal a second Zn^{2+} binding site in the enzyme.

A Mg^{2+} ion binding site is not present in *Nm*EptA despite attempts to obtain a complex by soaking crystals of the soluble domain *Nm*EptA in magnesium salts [47]. Additionally a Mg^{2+} ion binding site has not been found in the soluble domain of MCR- 1 [50,54]. The role of Mg^{2+} ion in alkaline phosphatase is to coordinate the water molecule that acts as the general base to deprotonate the catalytic nucleophile Ser102 [48]. However, it is worth noting that there are variations in terms of the number (from one up to three) and the identity (Ca^{2+}, Mn^{2+}, Zn^{2+} and/or Mg^{2+}) of the divalent metal ions involved in the reaction

mechanism of enzymes that belong to the alkaline phosphatase superfamily, with the metal ion that coordinates and activates the catalytic nucleophile common to all superfamily members. Additionally, in arylsulfatase (AS) and phosphonate monoester hydrolase (PMH), both of which have only one active-site metal ion, a basic residue takes the role of the second Zn^{2+} ion of alkaline phosphatase [56]. Thus it is also possible for EptA to have only one active-site metal ion.

The crystal structure of full length *Nm*EptA provides a strong basis for structure-guided drug design, an approach that makes use of the known 3D structure of a target bound to its natural ligand or a drug to optimise inhibitor potency and specificity [57]. The active site region of *Nm*EptA is known through identification of the catalytic nucleophile, Thr285, and the conserved residues that tetrahedrally coordinate the bound Zn^{2+} ion. Additionally, the bound DDM molecule, observed in the structure, has been used to hypothesise the conformation of the pEtN-bound state of the enzyme. However, conformational changes to the enzyme that could take place upon lipid A binding may reveal other catalytically important residues which have not yet been identified. Therefore, elucidation of the molecular details for the complete catalytic cycle of the enzyme including the number and the role of metal ions in the mechanism will greatly aid the development of inhibitors of EptA.

Lipid A modification by EptA alter the response of the immune system

Upon infection by Gram-negative bacteria, LPS molecules are released from the bacterial outer membrane in the form of outer membrane vesicles (OMVs) [58]. Due to its conserved structure among various bacterial species, LPS serve as pathogen-associated molecular patterns (PAMPs) that bind to pattern-recognition receptors (PRRs) in the host. The PRR complex that recognises LPS is comprised of LPS-binding protein (LBP), cluster of differentiation 14 (CD14), Toll-like receptor 4 (TLR4) and lymphocyte antigen 96 (MD2) [59–62]. LBP is a glycoprotein which forms a high-affinity complex with LPS micelles (K_D = ~1 nM) [59]. LBP, bound to LPS micelles, forms a tertiary complex with a molecule of CD14 and

within this complex the transfer of a single LPS molecule to CD14 takes place. CD14 binds to the LBP-LPS complex with high affinity and dissociates rapidly after the transfer of a single LPS molecule from LBP to CD14 [60]. CD14 then transfers the LPS molecule to the TLR4/MD2 complex which forms a stable 1:1 complex and dimerizes with a second TLR4/MD2 complex upon binding to LPS (Figure 6A) [58]. This interaction triggers a signalling cascade [62] which leads to inflammatory responses and clearance of the infection. However, systemic inflammatory responses that are caused by the sudden release of high concentrations of LPS into the bloodstream could have deleterious effects. The lipid A portion of the biologically most potent LPS molecules consists of a β-1,6-linked D-glucosamine disaccharide with six saturated fatty acid chains and is phosphorylated at 1- and 4'- positions of the glucosamine disaccharide. Structural variants of this lipid A are less recognisable by the host's PRRs, thus eliciting weaker immune responses [63,64]. In the context of this review, unmodified lipid A refers to the bis-phosphorylated lipid A carrying six saturated fatty acid tails while modified lipid A refers to other lipid A variants (e.g. mono-phosphorylated, tri-phosphorylated, hypo-acylated or over-acylated lipid A as well as lipid A decorated with various moieties).

Various lipid A modification enzymes have been found to alter lipid A by the addition or removal of acyl chains and phosphate groups, as well as the transfer of various moieties, such as sugars, phosphoryl groups or amino acids which alter the normal host-pathogen interactions. As mentioned above, EptA modifies the 1- and 4'-phosphate groups of lipid A by the addition of pEtN [9]. Mutational studies of LBP shows that basic residues located in the N-terminal domain are important for the LPS-LBP interaction [62,65]. These residues are thought to interact with the negatively-charged phosphate groups of lipid A. Thus, interaction between LPS and LBP might be disrupted by masking the negative-charge through addition of pEtN by EptA.

Unmodified lipid A binds strongly to the TLR4/MD2 complex (Figure 6B) and promotes dimerization of the complex, while modified lipid A may exhibit silent or antagonistic characteristics towards TLR4/MD2. Modified lipid A with silent characteristics

are not sensed by the human TLR4-mediated innate immune system, while modified lipid A with antagonistic characteristics can bind to TLR4/MD2 but does not promote dimerization with the second TLR4/MD2 complex [64]. Although the exact effects of lipid A modification on signalling through the TLR4/MD2 complex have not been fully determined, the interaction between the negatively-charged phosphate group of lipid A and the charged residues at the opening of the MD2 pocket might be obstructed by the presence of a positively-charged pEtN moiety. The hydrophilic interactions between the 1-phosphate of lipid A and positively-charged residues in TLR4 on the dimerization interface might also be altered by the presence of pEtN [66].

Interestingly, even though structural and mutational studies indicate that the addition of pEtN to lipid A might disrupt the interaction between LPS and the PRR complex, bioactivity studies of *Neisseria* show that lipid A with pEtN decoration stimulates stronger pro-inflammatory responses in comparison to lipid A without pEtN [67,68]. Jarvis and colleagues observed that the LOS of pathogenic *Neisseria* exhibit some structural heterogeneity. Lipid A with a higher degree of phosphorylation induced a higher level of TLR4 signalling [67,69]. Additionally, mass spectrometry analysis of *N. meningitidis* strain 89I also showed the presence of natural variations in the degree of phosphorylation and acylation within a single strain [70]. Therefore, in pathogenic *Neisseria,* stronger pro-inflammatory responses due to the addition of the pEtN by EptA may cause an increase in the degree of overall lipid A phosphorylation which may compensate for the decreased electrostatic interaction between Lipid A and the PRR complex, due to the presence of the positively charged ethanolamine group. Further studies on the interaction between pEtN-decorated lipid A and the PRR complex might provide a deeper understanding of the effect of pEtN decoration on the innate immune response.

In pathogenic *Neisseria* lipid A modification by EptA has been shown to not only stimulate pro-inflammatory responses but also support the colonisation of mucosal surfaces [71,72] and increase *Neisseria gonorrhoeae* resistance to complement-mediated killing [18,73] as reviewed in detail by Kahler *et al.* [74]. While the *eptA* gene is conserved among

the pathogenic *Neisseria*, almost all of the commensal *Neisseria* strains, with the exception *N. lactamica* and one of two strains of *N. polysaccharea*, do not have *eptA*. Interestingly, despite similarities in phosphorylation and acylation of lipid A between *N. lactamica* and *N. meningitidis*, *N. lactamica* does not induce inflammation. However the LOS from *N. lactamica* has a similar potential to that of the LOS from *N. meningitidis* in inducing the transcription of Tumor Necrosis Factor-α (TNF- α) in an *in vitro* cell assay [68]. This may suggest that *in vivo N. lactamica* has mechanisms to decrease the inflammatory response caused by EptA modification of lipid A. While there are reports of the role of EptA in pathogenicity of *Neisseria*, there is currently a lack of detail regarding its role in other pathogenic organisms.

As stated above, inflammatory responses are useful for clearance of bacterial infection however, hyper-inflammatory responses can lead to systemic inflammatory response syndrome (SIRS) which can be fatal for the host. Since addition of pEtN by EptA trigger stronger pro-inflammatory responses, inhibitors of EptA could potentially prevent or minimise SIRS. Also mentioned above, early studies show that, in the absence of EptA, the bacteria become more sensitive towards killing by normal human serum and host antimicrobial peptides. All of these findings suggest that EptA is an excellent target for the development of therapeutic agents to combat polymyxin resistance.

Conclusion

Gram-negative pathogens are becoming increasingly resistant to various classes of antibiotics, resulting in the need to use polymyxin as last-resort drugs. However, some Gram-negative pathogens have evolved resistance mechanisms against polymyxin, incurring serious threats to global public health. One such resistance mechanism is enzymatic modification of the outer membrane by the enzyme EptA. EptA reduces the net-negative charge of the membrane through addition of pEtN to the lipid A moiety which prevents the binding of polymyxin to the membrane. This modification also acts as a key virulence factor enhancing the pathogenicity of the bacteria [74].

One strategy to counter this problem is through development of novel antivirulence agents that inhibit lipid A modification by EptA. Inhibition of EptA will, hopefully, restore the efficacy of polymyxin, support the clearance of infection by the immune system and minimise the proliferation of polymyxin resistance. In this review, we discuss the biology of EptA, including its transcriptional regulation pathway by the bacteria, its catalytic chemistry, as well as the response to modifications of the LPS/LOS substrate by the host. This overview indicates that EptA may be an excellent target for drug-development to combat bacterial resistance to polymyxin. While much of this work has been undertaken for the enzyme from pathogenic *Neisseria*, further investigations are required for other pathogenic Gram-negative bacteria. A better understanding of the catalytic mechanism of EptA including the involvement of active-site metal ions and different enzyme conformational states will provide important biochemical information to assist in further inhibitor development.

Acknowledgement

This work is supported by the National Health and Medical Research Council of Australia Grants APP1003697 and APP1078642. A.S. acknowledges the University of Western Australia for support through an International Research Training Program Fees Offset Scholarship and a University Postgraduate Award.

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Figure Captions

Figure 1. The Gram-negative bacteria cell envelope. (A) Schematic representation of the location and the structure of the Gram-negative cell envelope. (B) General structure of Gram-negative LPS/LOS. Created with BioRender.com.

Figure 2. Schematic representation for activation and interaction of the PhoP/PhoQ and PmrA/PmrB two-component systems by various signals, which regulates the expression of *eptA.* P denotes the phosphate.

Figure 3. Reaction catalysed by EptA.

Figure 4. Molecular structure of *Nm*EptA. (A) Ribbon representation of *Nm*EptA structure with the N-terminal transmembrane domain represented in blue and the C-terminal soluble domain represented in red. (B) Secondary structure of the C-terminal soluble domain. The Zn^{2+} ion is represented as an orange sphere, the coordinating residues are shown as green-coloured sticks and the intramolecular disulphide bonds is shown as yellow-coloured sticks. (C) Secondary structure of the N-terminal transmembrane domain with the helical numbering labelled. The conserved glutamate residue (E114) proposed to interact with the amine group of pEtN is shown as green-coloured sticks and the DDM molecule is shown in ball-and-stick representation.

Figure 5. Metal coordination residues of *Nm*EptA, MCR-1 and *E. coli*'s alkaline phosphatase. (A) Metal ion binding site of *Nm*EptA superimposed to MCR-1 in comparison to (B) *E. coli*'s alkaline phosphatase. The Zn^{2+} ion is represented as orange sphere and the Mg^{2+} ion is represented as a green sphere. *Nm*EptA residues are shown as green-coloured sticks, MCR-1 residues are shown as magenta-coloured sticks. *E. coli* alkaline phosphatase

residues are shown as pink-coloured sticks. The yellow dashed lines indicate hydrogen bond interaction between the amino side chains and the metal ions.

Figure 6. The structure of TLR-4/MD-2 in complex with LPS [60]. (A) The overall structure of the TLR-4/MD-2/LPS complex. (B) Unmodified lipid A bound to the TLR-4/MD-2 complex. TLR-4/MD-2 complex is shown in ribbon representation. The lipid A component of LPS is shown in ball-and-stick representation, and the core-oligosaccharide is shown as white-coloured sticks. PDB 3FXI.