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Salmonellae PhoPQ regulation of the outer membrane to resist innate immunity

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Abstract

Salmonellae sense host cues to regulate properties important for bacterial survival and replication within host tissues. The PhoPQ two-component regulatory system senses phagosome acidification and cationic antimicrobial peptides to regulate the protein and lipid contents of the bacterial envelope. PhoPQ regulated lipid components of the outer membrane include lipopolysaccharide and glycerolphospholipids. Envelope proteins regulated by PhoPQ, include: components of virulence associated secretion systems, the flagellar apparatus, membrane transport systems, and proteins that are likely structural components of the outer membrane. PhoPQ alteration of the bacterial surface results in increased bacterial resistance to cationic antimicrobial peptides (CAMP) and decreased detection by the innate immune system. This review highlights the complexity of the outer membrane as an environmentally regulated organelle.

Keywords

lipid A; lipopolysaccharide; PagP; PhoPQ; cationic antimicrobial peptide; aminoarabinose; glycerolphospholipid; phosphatidylglycerol; cardiolipin; TLR4/MD2/CD14; Gram-negative bacteria; membrane lipids; Salmonellae; phagosome; molecular pathogenesis

Introduction

During infection, Salmonellae encounter a wide-range of host environments including those found in the lumen of the small intestine and phagosomal vacuoles of epithelial cells and macrophages [1]. Dramatic shifts in nutrient abundance [2–5], pH [6,7], as well as the presence of reactive oxygen and nitrogen species [8,9], membrane damaging cationic antimicrobial peptides (CAMP) [10–12] and bile acids [13–15], are among the

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environmental hazards that bacteria encounter during infection. Here, we review mechanisms of *Salmonellae* PhoPQ40-regulated envelope remodeling and its role in bacterial resistance to innate immune killing.

PhoPQ is activated when *Salmonellae* are within acidifying phagosome vacuoles to promote virulence for animals and humans

The PhoPQ virulence regulators are activated within systemic host environments including the acidified phagolysosome of macrophages [6]. Both activating and null mutations in *phoPQ* severely attenuate *S. Typhimurium* for virulence in murine models of infection indicating regulation mediated by PhoPQ is critical for disease [16,17]. In broth culture, the PhoPQ system can be activated when *Salmonellae* are grown in media of acidic pH [6,18], containing subinhibitory concentrations of CAMP [19], or severely limited in divalent cations [20]. Environmental activation of the inner membrane (IM) PhoQ sensor-kinase increases phosphorylation of PhoP, its cytoplasmic response regulator. Phosphorylated PhoP activates genes encoding OM proteins [21–23], regulators [24,25], components of the intracellular type III secretion system [26], IM transporters that buffer cytosolic pH [27], and enzymes that covalently modify OM barrier components [28,29], while repressing genes encoding the flagellar and invasion associated type III secretion systems [30].

PhoPQ regulates the outer membrane barrier

The outer membrane of Gram-negative bacteria is a complex organelle that provides a barrier protecting bacteria from hazards in their environment (Fig. 1A). The outer leaflet of the OM consists of predominantly lipid A (Fig. 1B), the bioactive component of LPS detected by the TLR4/MD2/CD14 innate immune receptor complex [30]. Lipid A anchors LPS to inner leaflet glycerophospholipids (GPL) through hydrophobic interactions between fatty acyl side chains (Fig. 1C). LPS core oligosaccharide and hypervariable repeated O-antigen extend from the diglucosamine polar head group of the lipid A amphiphile to complete what is often referred to as the OM barrier (Fig. 1A). *Salmonellae* synthesize GPL and lipid A on the inner leaflet of the IM by defined biosynthetic machinery [31]. However, unlike lipid A, which is specifically transported to the OM by an inner and outer membrane spanning protein complex [32] the mechanism of GPL transport to the OM is undefined.

S. Typhimurium regulate the structure of LPS through PhoQ sensing and activation, and these structural alterations contribute to resistance to CAMP. Specific changes in OM lipids regulated by PhoPQ include: reducing average O-antigen chain-length [36,37], acylating, deacylating, and hydroxylating lipid A [28,29], derivitizing lipid A [38] and LPS core phosphates with cationic groups (Fig. 1B) [39], palmitoylating OM PG molecules (Fig. 1C) [35], and increasing cardiolipid content. PhoPQ also activates the synthesis of more than two-dozen unique OM proteins including basic proteins of unknown function, such as PagC, which are basic proteins which are likely to complex with negatively charged lipid molecules as part of the OM barrier [23,40,41]. Therefore, upon PhoPQ activation an extensive alteration of lipopolysaccharides, glycerophospholipids, and proteins elaborates an OM barrier more impermeable to CAMP that promotes survival within acidified host phagosomes.

LPS remodeling increases the OM barrier to CAMP while decreasing innate immune recognition to promote bacterial virulence

Cationic amphipathic antimicrobial peptides have diverse membrane-active structures conserved from bacteria to humans [42,43]. Structural diversity and specific bacterial killing mechanisms of CAMP may have led to mammals evolving multiple sub-types, including amphipathic alpha-helical molecules and beta-sheet structures, the latter whose amphipathic nature is maintained by disulfide bonds. Beta-sheet peptides, such as defensins make up 30% of the dry weight of a neutrophil and are secreted into the intestinal lumen by Paneth cells to protect the intestinal crypts. Defensins are critical innate immune effectors likely targeting *Salmonellae* during intestinal colonization and inflammation [44,45].

The activity of CAMP requires their attraction to the anionic phosphate groups that flank lipid A and LPS-core sugar molecule on the surface of Gram-negative bacteria (Fig. 1B). Upon surface interaction, amphipathic CAMP hydrophobic faces insert into the lipid bilayer [43]. On penetration, some alpha-helical antimicrobial peptides may form discrete pore-like structures [46], while others can cause acute membrane blistering near the cell poles [47]. Therefore, CAMP may target specific domains within the OM, rather than inserting randomly or uniformly throughout the bilayer. Regardless of their specific mechanism all antimicrobials must penetrate the Gram-negative OM barrier to kill the microbe.

A variety of studies support that resistance to antimicrobial peptides is important for *Salmonellae* to survive during infection including data demonstrating PhoP-null mutants survive better in macrophages from mice deficient for cathelicidin-related antimicrobial peptide (Cramp), than in macrophages from wild-type mice [48]. Likewise, bacteria with mutations in PhoPQ are attenuated for virulence in a variety of animal and tissue culture models of infection [16] and PhoP-null mutants of *Salmonella enterica* serovar Typhi are attenuated in human volunteers [49,50]. Therefore, CAMP resistance and OM remodeling is likely critical for both human and animal infections caused by *Salmonellae*.

Innate immune surveillance pathways detect invading pathogens using pattern recognition receptors that recognize unique microbe associated molecular patterns [51]. Specific PhoPQ-regulated modifications, such as palmitoylation of lipid A, which generates a molecule with seven acyl side chains, one of carbon length sixteen, can reduce LPS activation of the TLR4-containing receptor complex [30]. The human receptor is optimized to recognize lipid A with six fatty acids of carbon length twelve and fourteen corresponding to lauryl and myristoyl groups, respectively [52,53]. Lipid A molecules and their regulation by PhoPQ are quite diverse among bacterial species. PhoPQ and the enzymes that modify lipid A are highly conserved and implicated in the pathogenesis of a *Legionella pneumophila*, *Bordetella spp.*, *Francisella spp.*, and *Yersinia pestis* [54]. Specific modifications of lipid A have also been associated with adaptation of *Pseudomonas aeruginosa* to the airways of individuals with Cystic Fibrosis [55]. Overall understanding of the enzymes that participate in the de-phosphorylation and the acylation state of lipid A has led to identification of the molecular specificity of the human TLR4 receptor complex and has formed the molecular basis for the use of altered lipid A molecules as important vaccine adjuvants, and therapeutic agents that can activate the receptor without the toxicity of LPS [30, 54].

Outer membrane remodeling mechanisms promoted by the PhoPQ system

Derivatizing LPS phosphates with aminoarabinose and ethanolamine

Upon sensing pH and CAMP, PhoPQ increases transcription of *pmrD* and the *pmrCAB* operon to activate the PmrA response regulator [24–26]. Activated PmrA induces transcription of genes encoding enzymes that covalently modify lipid A and core sugar phosphates with aminoarabinose (L-Ara4N) and phosphoethanolamine (pEtN) (Fig. 2) [56]. The initial step for L-Ara4N modification is catalyzed by the PhoP and PmrA-regulated UDP-glucose dehydrogenase (PagA, or Ugd) which oxidizes UDP-glucose within the cytosol for amine addition [38]. Remaining components of the L-Ara4N-LPS modification pathway are encoded within an operon, *pmrHFIJKLM*, which is activated by PhoPQ through PmrA [56]. When L-Ara4N synthesis is mostly complete, the UDP-nucleotide is exchanged for a undecaprenyl-phosphate carrier lipid on the inner leaflet of the IM (Fig. 2) [57]. The carrier and L-Ara4N are then flipped into the outer leaflet of the IM where L-Ara4N is transferred to nascent lipid A phosphates by ArnT, also known as PmrK. Finally, O-antigen is loaded onto the core structure and assembled LPS molecules are transported across the periplasm and inserted into the OM by the Lpt system [32].

Decreased negative charge conferred by cationic L-Ara4N groups on lipid A molecules diminishes CAMP binding sites for drugs like colistins, which are bacterial-derived polymyxins increasingly used as last resort antibiotics against multidrug resistant Gram-negative bacteria, such as *P. aeruginosa*, *Acinetobacter baumannii* and *Klebsiella pneumoniae*. Aminoarabinose modification is required for *S. Typhimurium* to fully colonize the murine small intestine [12,24] and PhoPQ likely senses CAMP within the intestinal lumen to activate the PmrA regulon [12]. Further understanding of the role of L-Ara4N in intestinal colonization and survival may reveal roles for OM remodeling during *S. Typhimurium* competition with the intestinal microbiota.

Decreasing O-antigen chain length

O-antigens form a discrete heterogeneous layer outside the bacterial cell that is in direct contact with host environments [58]. These immunodominant polysaccharides are under extreme selective pressure and therefore highly variable, likely as a result of selective pressure by bacteriophages. O-antigens are involved in immune evasion [59–61], as well as resistance to killing by host complement [62] and bile acids [15,63,64]. PhoPQ activation reduces the overall number of O-antigen repeats through the action of the PmrA-regulated membrane protein complex, Wzz_{st}. A more uniform and shorter O-antigen, or reduced synthesis of more rare forms of LPS with very long-type O-antigens with greater than one hundred subunits may contribute to O-antigen-related phenotypes [36,37]. Recent structure-function analysis suggests the Wzz_{st} IM protein complex selectively measures shorter long-type O-antigens with between sixteen and thirty-five subunits (Fig. 2) [65]. Mechanistically, it remains unclear how average reductions in O-antigen chain length contribute to the PhoPQ-regulated barrier, but it is plausible that a more uniform shorter O-antigen somehow promotes barrier function while decreasing recognition by the adaptive immune system, analogous to the role of lipid A remodeling in innate immune evasion.

Hydroxylating a lipid A myristoyl group

Initial chemical characterization of PhoPQ-regulated LPS structures measured an abundance of uniquely hydroxylated myristoyl groups [36]. Likewise, lipid A species with 2-hydroxymyristate substitutions are produced by *S. Typhimurium* during infection of macrophages [66]. Regulated hydroxylation of myristoyl groups is catalyzed by the IM dioxygenase, LpxO [67]. PhoPQ does not regulate transcription of *lpxO* nor does 2-hydroxymyristate play a defined role in OM barrier function making its physiologic role and mechanism of activation by PhoPQ unknown. The function of LpxO may be linked to VisP. VisP is a periplasmic peptidoglycan binding protein that may physically interact with LpxO [68] and exhibits a genetic relationship with *lpxO* during infection. Mutations in the *S. Typhimurium visP* locus result in bacteria that are defective for systemic infection of mice [68]. The *visP* phenotype is partially synthetic, since systemic defects can be relieved by second site mutations in *lpxO*. However, since *lpxO* single mutants show minimal defects in causing systemic disease, it seems likely that the activities of VisP and LpxO are incompatible with growth in the absence of the other protein. It is interesting to speculate that the function of the periplasmic protein VisP is in some way related to specific hydroxylated LPS.

Removing R-3-hydroxy-myristate from lipid A

The outer membrane of Gram-negative bacteria contains few proteins with catalytic activity [69,70]. *S. Typhimurium* encodes two specialized OM phospholipases whose role in barrier remodeling is fairly well understood. PagL is found in select Gram-negative bacteria including *Salmonellae* and *Pseudomonads* [71]. The enzyme hydrolyzes myristate of carbon length fourteen from lipid A to generate pentaacylated structures in *S. Typhimurium* (Fig. 1B) [28]. Although PagL levels are increased on PhoPQ activation, demyristoylated lipid A is not observed in broth culture [28], or within macrophages [66]. Inhibition of PagL activity by L-Ara4N modified lipid A phosphates may explain why PagL activity is not observed in PhoPQ-activated bacteria. Demyristoylated lipid A structures are produced by *S. Typhimurium* when genes necessary for L-Ara4N modification are disrupted [72], or when specific residues within the outer leaflet surface loops of PagL are mutated [73,74]. This additional post-translational regulation may suggest that L-Ara4N-modified lipid A molecules bind PagL and render it inactive until membrane damage occurs. Such regulation could be important within host tissues, since demyristoylated lipid A structures have reduced TLR4-activating properties [30].

Transferring palmitoyl groups to lipid A and phosphatidylglycerol within the OM

Most OM phospholipases share an integral membrane beta-barrel structure with an active site exposed to the outer leaflet [73,75,76]. In response to OM damage [77,78], or PhoPQ activation [29,78], the conserved PagP enzyme transfers palmitoyl groups from the *sn-1* position of GPL to lipid A using an outer leaflet active site (Fig. 1A) [75,77]. Increased palmitoylation increases the hydrophobicity of the OM and prevents penetration of amphipathic alpha-helical CAMP like C18G and LL-37 [54]. Heptaacylated lipid A structures with one palmitoyl group demonstrate reduced activation of TLR4 [52], indicating the effects of lipid A palmitoylation are two-fold. In *E. coli* and *S. Typhimurium*, damage to

the OM bilayer activates the PagP enzyme [29,77,79]. Penetration of CAMP, depletion of divalent cations, or treatment with detergents can cause LPS molecules to become displaced from the outer leaflet [34,83]. To maintain bilayer integrity, GPL from the inner leaflet flip into the outer leaflet forming GPL microdomains within the OM, which activate PagP palmitoyltransferase activity [77,80–83]. In contrast, when bacteria are not stressed the OM must be predominantly asymmetric [82]. Highly specialized and broadly conserved enzymes like OMPLA specifically degrade GPL that have reached the outer leaflet. Therefore, GPL must aberrantly flip into the outer leaflet at some slow rate during cell division [80,81,83]. Hydrolysis of GPL molecules yields lyso-GPL structures that can act as detergents and disrupt barrier stability. Genetic analysis supports that Gram-negative bacteria have a retrograde transport system that recycles GPL products back to the IM [83]. Recent work demonstrates that PagP has dual substrate specificity within the OM and in addition to lipid A, PagP transfers palmitoyl groups to phosphatidylglycerol on the bacterial surface (Fig. 1A) [35]. Therefore, when the PhoPQ system is activated GPL may be increasingly translocated to the outer leaflet to increase barrier hydrophobicity.

Regulating OM glycerolphospholipids

Recent work from our laboratory indicates that the PhoPQ system regulates the GPL component of the OM. Like for lipid A, the PagP enzyme also catalyzes the transfer of palmitoyl groups to PG within the OM (Fig. 1A) [35]. The synthesis of these unique *sn*-3' palmitoylated acylphosphatidylglycerols, referred to as palmitoyl-PG, may contribute to the function of PagP in promoting resistance to antimicrobial peptides. Our group also noted an increase in cardiolipin (CL) content of the OM on activation of PhoPQ (Fig. 1C) [35], though no specific mechanism has been defined to explain this observation. It is interesting to speculate that CL may facilitate specific formation of microdomains within the OM that promote cell division and OM lipid remodeling when PhoPQ is activated [84–86].

Conclusions and future directions

Work over the last 20 years has demonstrated that the OM is not a static organelle but rather a highly regulated and mosaic surface barrier. The regulated lipid and protein components of the OM likely create microdomains for specific barrier functions while allowing multicomponent protein complexes to assemble. In this regard, perhaps the need for a second type III secretion system for *S. Typhimurium*'s intracellular lifestyle reflects the ability of the remodeled membrane to support the specific assembly and secretion of the phagosome-active, but not the surface-active secretion system. The recent observation that PhoPQ regulate OM GPL suggests that like for lipid A, OM GPL regulation may be important for many Gram-negative pathogens. Future work will define whether mechanisms of OM GPL regulation are conserved in other species and whether specific GPL structures are recognized by innate immune receptors. Lastly, suppression of the host innate immune response is a general principle of Gram-negative bacterial pathogenesis. Recently, it was shown that LPS activates the Nlr4/Acs inflammasome and Caspase-11 through an unidentified receptor that detects distinct lipid A acylation patterns within the cytoplasm of macrophages [87–91]. Therefore, it is conceivable that regulation of lipid A allows *S.*

Typhimurium to reduce activation of Caspase-11, or alternative cytosolic host-cell death pathways encountered by bacteria during infection of animals and humans.

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peptides with bacterial outer membranes could aid design of peptide antibiotics to treat multidrug resistant Gram-negative pathogens.

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HIGHLIGHTS

- The Salmonellae PhoPQ virulence system is a major regulator of outer membrane (OM) lipids and proteins.
- Lipopolysaccharide (LPS) regulation increases bacterial resistance to host killing.
- PhoPQ regulates modification to LPS phosphates and controls acylation of lipid A.
- PhoPQ promotes palmitoylation of phosphatidylglycerol (PG) and increases cardiolipin content of the OM.
- The PhoPQ-regulated OM palmitoyltransferase, PagP, transfers palmitoyl groups to PG within the OM of *S. Typhimurium*.

Figure 1. PhoQ-regulated remodeling of outer membrane structures

(A) Structural organization of the *S. Typhimurium* outer membrane (OM). PagP hydrolyzes palmitoyl groups of carbon length sixteen (C16:0) from glycerophospholipid (GPL) donor substrates and transfers them to lipid A molecules with six acyl substituents of carbon length twelve (C12:0) and fourteen (C14:0), or to the polar head group of phosphatidylglycerol (PG) molecules that have flipped (dashed arrow) into the OM outer leaflet on account of PhoPQ activation, or OM damage inflicted by antimicrobial peptides. The PagP palmitoyltransferase produces lipid A molecules with seven acyl chains with one palmitoyl group, as well as triacylated palmitoyl-PG molecules with a palmitoyl group extending from the *sn*-3' head-group position [35]. (B) PhoPQ regulates the chemical structure of *S. Typhimurium* lipopolysaccharides (LPS). Modifications in red are directly regulated by the PhoPQ system. Those in blue are indirectly regulated by the PhoPQ system through the PmrA response regulator. Dashed lines indicate acyl groups cleaved by PagL and PagP. (C) Chemical structures of specific *S. Typhimurium* GPL. The PagP enzyme transfers palmitoyl groups from the *sn*-1 position of GPL donor substrates to the *sn*-3' position of PG acceptor substrates by transesterification. The two palmitoyl-PG species depicted are generated by the PagP enzyme within the OM of *S. Typhimurium* [35]. CL = cardiolipin, or diphosphatidylglycerol, PE = phosphatidylethanolamine, acyl-PG = acylphosphatidylglycerols.

Figure 2. Bacterial adaptation to host antimicrobial peptides requires coordinated synthesis of diverse OM structures

Cationic antimicrobial peptides (CAMP) like C18G, an amphipathic α -helical human-derived cathelicidin, damage acute outer membrane (OM) surfaces to expose a thin layer of peptidoglycan, the periplasmic space, and eventually the inner membrane (IM). PhoQ binds CAMP on the outer leaflet of the IM to activate phosphorylation of PhoP, which induces PmrA and its regulon. PmrA regulates: ArnT, an aminoarabinose (L-Ara4N) transferase, EptA, a lipid A phosphoethanolamine (pEtN) transferase, Wzz_{st}, a long-type O-antigen translocase, and CptA, a LPS core pEtN transferase.