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Pushing the envelope: LPS modifications and their consequences

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Abstract

The defining feature of the Gram-negative cell envelope is the presence of two cellular membranes with the specialized glycolipid lipopolysaccharide (LPS) exclusively found on the surface of the outer membrane. The surface layer of LPS contributes to the stringent permeability properties of the outer membrane which is particularly resistant to permeation of many toxic compounds, including antibiotics. As a common surface antigen, host immune cells recognize LPS and mount defenses to clear pathogenic organisms. To alter properties of the outer membrane or evade the host immune response, Gram-negative bacteria employ a wide variety of chemical modifications to alter LPS. Here we review key features and physiological consequences of LPS biogenesis and modifications.

Introduction

Molecules at the surface of bacterial cells perform critical roles of interacting with and reacting to the surrounding environment. For Gram-negative bacteria, the surface is composed of an asymmetric outer membrane (OM) with the glycolipid lipopolysaccharide (LPS) exclusively localized to the outer leaflet of the OM[1]. The asymmetric distribution of LPS on the surface produces a potent barrier that is impermeable to many toxic compounds, including antibiotics[2]. To maintain the asymmetry and permeability properties of this unique barrier, most Gram-negative bacteria have dedicated pathways for removing glycerophospholipids that are mislocalized to the outer leaflet. In addition, as a major constituent of the OM, LPS is essential in most Gram-negative bacteria (see box 1) and is critical for virulence[3].

LPS biogenesis begins with synthesis occurring at the cytoplasmic interface of the inner membrane (IM) and then LPS is transported across the IM and to the OM[4,5] (Figure 1a). LPS can be divided into three regions: the conserved lipid A anchor, core oligosaccharide, and O antigen. The conserved lipid A unit is a *bis*-phosphorylated disaccharide of GlcN

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(glucosamine) typically with 4-7 acyl chains (Figure 1b). LPS synthesis begins with the Raetz pathway (Figure 1a), a series of conserved enzymatic steps that produce lipid A with Kdo (3-deoxy-D-*manno*-oct-2-ulosonic acid) sugars, the first units of the core oligosaccharide. Briefly, using the model organism *Escherichia coli* as an example, the Raetz pathway starts with the precursors UDP-GlcNAc (UDP-*N*-acetylglucosamine) and ACP (acyl carrier protein)-bound fatty acids. The successive actions of LpxA (acyltransferase), LpxC (deacetylase), and LpxD (acyltransferase) produce a UDP-2,3-diacylGlcN[3]. The first reaction by LpxA is unfavorable and deacetylation by LpxC is the first committed step of synthesis[3]. A UMP is then cleaved from some of the produced UDP-2,3-diacylGlcN to produce 2,3-diacylGlcN-1-phosphate, also named lipid X[3]. Lipid X production is performed by one of three non-homologous enzymes: *E. coli* and most Gram-negative bacteria use LpxH, α-proteobacteria use LpxI[3] and *Chlamydiae* use LpxG[6]. LpxB then catalyzes formation of a β-1′-6 glycosidic bond between one molecule of lipid X and one molecule of UDP-2,3-diacylGlcN which releases the UDP nucleotide carrier[3]. The tetra-acylated 1-phosphorylated reaction product is then phosphorylated by LpxK at the 4′-position to produce lipid IV_A[3]. This is followed by the addition of the Kdo sugar(s), the number of which varies in different organisms, by the enzyme WaaA (also known as KdtA)[3]. Finally, two acyl transferases, LpxL and LpxM, catalyze the transfer of “secondary” acyl chains completing the Kdo-lipid A domain of LPS[3]. Most bacteria encode homologs of LpxL; however, many bacteria, such as *Francisella*, do not encode an LpxM homolog and others encode distinct acyl-transferases instead of LpxM: LpxJ in many ε-proteobacteria[7] and LpxN in *Vibrio cholerae*[8].

Next, the core oligosaccharide is extended at the cytoplasmic side of the IM, (not covered here) producing lipooligosaccharide (LOS). Some bacteria, including a number of mucosal pathogens, only produce LOS[3]. O-antigen precursors, if produced by the bacteria, are synthesized separately at the cytoplasmic interface of the IM and attached to the lipid carrier undecaprenyl-pyrophosphate[3]. LOS and O-antigen precursors are flipped across the IM by $MsbA_2$ and an O-antigen flippase (vary between organisms), respectively[3]. Depending on the O-antigen biosynthesis pathway utilized, O antigen is either polymerized before flipping or at the periplasmic leaflet of the IM[3]. In both scenarios, O antigen is appended to LOS in the periplasm[3]. LPS or LOS molecules are then transported from the IM to the surface of the OM by the Lpt (LPS transport) machinery[9] (Figure 1a).

Properties of LPS impact OM permeability, resistance to antibiotics, virulence, and recognition by the mammalian host’s immune system. Thus, bacteria have evolved many enzymes that modify LPS to alter its properties and allow them to adapt to the dynamic environments they inhabit. Here we review the current state of the field of LPS biogenesis and its modification. We highlight recent insights into how LPS modifications affect stimulation of host immune response and OM vesiculation. Finally, we discuss how a mechanistic understanding of LPS biogenesis and remodeling has led to development of antibacterial therapeutics.

Altering LPS

Variations to the structure of LPS can be introduced at each step of its biogenesis. For the purpose of discussion, variation in the lipid A structure will be compared to the lipid A anchor produced by *E. coli* K-12 under standard laboratory conditions (Luria-Bertani broth at 37°C, Figure 1b). Under these conditions, the lipid A disaccharide of GlcN has two attached Kdo sugars, is *bis*-phosphorylated and is hexa-acylated. Two primary *N*-linked β-hydroxy-acyl chains are attached at the 2 and 2′ positions and two primary *O*-linked β-hydroxy-acyl chains are attached at the 3 and 3′ positions of the GlcN disaccharide. *E. coli* lipid A then has two secondary acyl chains attached to the β-hydroxyl groups of the 2′ and 3′ acyl chains.

Variations to LPS introduced during the Raetz pathway alter either the sugars or acyl-chains of the Kdo-lipid A domain (depicted in supplementary information S1). Alterations to sugars, GlcN and Kdo, can occur before incorporation into lipid A. Organisms like *Campylobacter jejuni* and *Leptospira interrogans*, utilize the enzymes GnnA and GnnB to oxidize and transaminate UDP-GlcNAc, respectively, converting it into the 3-amino derivative UDP-GlcNAc-3N (2,3-diamino-2,3-dideoxy-D-glucopyranose)[10,11]. These organisms then encode LpxA homologs that selectively utilize the modified UDP-GlcNAc3N sugar[10]. The resulting lipid A in these organisms has 4 amide-linked acyl chains and result in reduced recognition by host immune cells and reduced sensitivity to antimicrobials[11]. Similarly, Kdo is modified by additional enzymes KdnA and KdnB in *Shewanella* species to produce an 8-amino derivative, Kdo8N (8-amino-3,8-dideoxy-D-*manno*-octulosonic acid)[12]. Kdo8N is then activated to its nucleotide-linked form and feeds into lipid A synthesis as a substrate of WaaA. Kdo8N-containing LPS was also found to play a role in protecting cells from antimicrobials[12]. In addition to the possibility of modifications of sugar precursors, variations to Kdo incorporated into lipid A can also occur during or after the Raetz pathway. WaaA of various organisms have variability in the number of Kdo sugars (one, two, three or four) they attach.[13-16] Many organisms will also modify Kdo by hydroxylation (via KdoO)[17] or phosphorylation (via KdkA)[14,18] during lipid A synthesis. Alterations of GlcN and Kdo introduced during the Raetz pathway are constitutive in their respective organism.

Additional diversity arises from the substrate selectivity of the Lpx acyltransferases of different bacteria altering fatty acyl chain characteristics (length, saturation, branching, etc.). Further, the secondary acyltransferases, LpxL and LpxM, can vary in the position of acyl chain attachment and in the number of acyl chains transferred (supplementary information S1). LpxM from *Acinetobacter baumannii*, for example, is bifunctional and transfers a laurate to the 2 and 3′ acyl chains[19]. In addition, many organisms will alter the acyl chains incorporated into lipid A in response to environmental conditions by encoding more than one enzyme that act at the same position (supplementary information S1). This type of variation is well known for organisms like *Salmonella enterica spp*. and *E. coli* that express LpxL, a lauroyl (C12:0) transferase, at higher temperatures and LpxP, a palmitoleoyl (C16:1) transferase, at lower temperatures[3]. In addition, *Klebsiella pneumoniae* encodes two LpxL homologs that are co-expressed under standard growth conditions and compete to add either a lauroyl (C12:0) group, by LpxL1, or myristoyl (C14:0) group, by LpxL2, at the

same position. Finally, organisms like *Leptospira interrogans* and *Francisella tularensis* encode two homologs of LpxD (Figure 1a) that incorporate different length acyl chains[20,21]. This variation in acyl-chain length mediated by *Francisella*[21] LpxD1 and LpxD2 was demonstrated to be in response to temperature, but conditions for which *Leptospira* LpxD2 are expressed have remained elusive[20].

After the Raetz pathway, LPS can be further modified by a diversity of enzymes that generally alter the acyl chains, phosphate groups, the sugar backbone of lipid A, and the core oligosaccharide (Figure 1c-d and supplementary information S2). These modifications have a critical impact on lipid packing, membrane permeability, host recognition, and sensitivity to antimicrobials (discussed later). Alterations to acyl chains of LPS after synthesis typically involve either addition or removal of fatty acids by enzymes in the OM like LpxR, PagL, and PagP[22-24]. These modifications allow organisms to control the membrane characteristics of LPS already transported to the surface. Other alterations to acyl chains of lipid A can occur at the IM including hydroxylation by the enzyme LpxO[25], or, in the unique case by *Vibrio cholerae*, addition of glycine moieties via AlmG[26].

Organisms have evolved a plethora of strategies to alter the charge of lipid A. Briefly, many organisms like *Helicobacter pylori* cleave the phosphates from lipid A to produce a neutral or less negatively-charged version (Figure 1d). The negative charge of lipid A can also be reduced by masking phosphates with positively-charged, phosphoethanolamine (by EptA[27]) and 4-aminoarabinose (by ArnT[27]), or neutral constituents, glucose sugars (by FlmK[28]). In contrast, many bacteria will append additional phosphates to lipid A with the enzyme LpxT[29,30] increasing the negative charge. In addition, Kdo and the core oligosaccharide can be modified by addition of charged moieties, like phosphates or phosphoethanolamine, or addition or removal of sugars[27] (supplementary information S2). Finally, Gram-negative bacteria encode a diversity of biosynthetic and modification enzymes that can alter the structure of O antigen that have been reviewed thoroughly elsewhere[31,32].

Regulation of LPS modifications

LPS modification enzymes can be either constitutively expressed or controlled by regulatory networks. The human-adapted gastric pathogen *H. pylori*, for example, constitutively expresses a highly ordered repertoire of lipid A-modifying enzymes resulting in a tetra-acylated lipid A anchor with reduced phosphorylation[33-36] (Figure 1d). However, in *Salmonella enterica* subsp. *enterica* serovar Typhimurium, a wide range of regulatory mechanisms fine-tune when and to what extent LPS is modified to accommodate a more diverse life style (Figure 2 and supplementary information S3).

Many bacteria utilize two-component systems (TCS) to regulate LPS modification enzymes (supplementary information S3). At their core, these regulation systems utilize a sensor kinase that responds to a signal through autophosphorylation of a histidine residue. The phosphate is then transferred to a cognate response regulatory protein acting as a phosphorylation-mediated switch, turning on and off gene expression. PhoPQ and PmrAB are the most widely-spread TCS that affect lipid A modification genes[37,38]. Here we will briefly touch on the mechanism of these TCS with focus on recent advances.

PhoPQ is best studied in *Salmonella enterica* subsp. *enterica* serovar Typhimurium where it responds to many environmental stimuli including acidic pH, low concentrations of divalent cations, antimicrobial peptides (AMPs), and most recently reported osmotic shock[38-41]. The breadth of stimulating signals varies for PhoPQ of different bacteria[38,42]. However, work on PhoQ of *Salmonella enterica* subsp. *enterica* serovar Typhimurium and *E. coli* has revealed how various signals can feed into one sensor (Figure 2).

PhoQ contains cytoplasmic, transmembrane, and periplasmic domains. The periplasmic sensing domain contains a patch of acidic residues close to the membrane surface that is critical for sensing divalent cation concentrations and cationic AMPs[43]. Mg^{2+} and Ca^{2+} are thought to reduce repulsion between the acidic residues of PhoQ and acidic head group of glycerophospholipids in the IM and favor PhoQ in an off conformation[43]. Repulsion in the absence of divalent cations or disruption of this bridging by binding of cationic AMPs to this site causes a conformational change that favors autophosphorylation[43]. Initially, it was thought that pH changes may also activate PhoQ by affecting the periplasmic sensor domain[44]. However, recently it was suggested that pH changes are sensed by a cytoplasmic domain. When this cytoplasmic domain was altered as a result of mutations in the encoding gene, PhoQ is unable to sense pH changes but can still respond to Mg^{2+} limiting conditions[45]. Additionally, an IM protein in *Salmonella enterica* subsp. *enterica* serovar Typhimurium, UgtL, was proposed to bind PhoQ and amplify the autophosphorylation in response to acidic pH[46]. Finally, osmotic shock was proposed to activate PhoQ by a third mechanism whereby high osmolarity increases lateral pressure exerted by lipids on the transmembrane domains of PhoQ and causes a change in the protein conformation[41]. Altogether, work on *Salmonella enterica* subsp. *enterica* serovar Typhimurium PhoQ has demonstrated the intricacies of how a senor kinase can be adapted for multiple stimuli.

Activated-PhoQ phosphorylates PhoP, which in *Salmonella enterica* subsp. *enterica* serovar Typhimurium and other organisms transcriptionally regulates many virulence factors including lipid A modifications. PhoP directly regulates *pagL* and *pagP* genes, but also indirectly affects other LPS-modification genes through regulation of a small RNA (discussed later) and the protein PmrD[22,47,48]. While not present in all bacteria, *Salmonella enterica spp.* and *E. coli* encode the protein PmrD that mediates coupling between PhoPQ and PmrAB[48,49]. When upregulated by activation of PhoPQ, PmrD binds to phosphorylated PmrA and protects it from being deactivated by phosphatase activity of PmrB[50]. Thus, PhoPQ activation can indirectly feed into PmrAB regulation of genes like *arnT* and eptA[37]. To control the extent of PhoPQ activation, PhoP also upregulates expression of MgrB, an IM protein that provides negative feedback closing the PhoPQ regulatory circuit[51].

PmrB is sensor kinase of the PmrAB TCS and is directly activated by binding of Fe^{3+}, Al^{3+}, and in some organisms Zn^{2+} to a periplasmic, metal-binding, ExxE motif[52]. PmrAB can also be activated by acidic pH, but full activation requires both PmrB and PmrD suggesting part of the activation is through PhoPQ activity[53]. PmrB's proposed pH sensing domain consists of His and Glu residues in the periplasm that may react to altered protonation states[53]. Activation of PmrB causes it to phosphorylate PmrA which directly upregulates aminoarabinose (*arn* operon) and phosphoethanolamine (*eptA*) modifications. Activation also increases transcription of the small protein PmrR, which binds to and inhibits activity of

the lipid A phosphotransferase, LpxT[54]. LpxT and EptA competitively modify the same site on LPS (Figure 1c), so LpxT inhibition enhances phosphoethanolamine modification of lipid A by EptA driving AMP resistance[29].

Further, the combined effects of PmrR, EptA, and ArnT serve as a feedback loop controlling the amount to which PmrAB is activated[54]. Activation of PmrAB by metal ions like Fe^{3+} depends upon the charge of the OM. Upon encountering high levels of Fe^{3+}, bacteria with a highly negatively-charged OM, due to the presence of *bis*- and *tris*-phosphorylated lipid A, experience high entry of Fe^{3+} into the cell where it activates PmrB. High activation of the PmrAB regulon produces the Arn proteins, EptA, and PmrR. PmrR quickly inhibits LpxT reducing the amount of *tris*-phosphorylated lipid A produced and somewhat reducing the negative charge of the OM. Production of aminoarabinose and phosphoethanolamine modified lipid A further reduces the negative charge of the OM. As the charge of the cell surface becomes more positive, less Fe^{3+} reaches PmrB and activation is fine-tuned[54].

In addition to PhoPQ and PmrAB, organisms may have other TCS that allow them to respond to environmental signals (supplementary information S3). Demonstrated in *Salmonella enterica* serovar Enteritidis but likely more wide-spread, the ArcAB TCS is one of the regulators that responds to oxygen availability and regulates the gene encoding LpxO, the oxygenase responsible for hydroxylation of a lipid A acyl chain[55]. *Pseudomonas aeruginosa*, for example, encodes PhoPQ, PmrAB, and three additional TCS that regulate lipid A modification genes. The *Pseudomonas* ColRS system senses Zn^{2+} and specifically upregulates EptA, but not ArnT[56]. Whereas, ParRS and CprRS of *Pseudomonas* sense AMPs and specifically upregulates ArnT[57,58]. While both the sensor kinases of these systems, ParS and CprS, are activated by polymyxins, CprS is also activated by indolicidin and other peptides allowing *Pseudomonas* to respond to many environmental signals[57,58]. *Vibrio cholerae* was also recently shown to have an additional TCS, VprAB (also named CarRS), that senses bile, AMPs, and acidic pH to regulate its *almEFG* operon that modifies lipid A with glycine[59,60]. Finally, certain strains of *Klebsiella pneumoniae* have an additional TCS, CrrAB, that indirectly affects LPS modifications by upregulating the encoding gene for CrrC which in turn up regulates the genes that encode PmrAB[61]. Stimuli that activate CrrAB have yet to be determined. These examples demonstrate the breadth of which TCS are utilized to regulate LPS modifications.

Small RNAs commonly have connections to TCS and can either directly or indirectly regulate lipid A modification genes (supplementary information S3). MicA, a small RNA that represses synthesis of key OM proteins, also negatively regulates PhoPQ expression at the post-transcriptional level thereby impacting LPS structure as well[62]. The sRNA MgrR is upregulated by PhoPQ[63]. In turn, MgrR then inhibits transcription of the *eptB* gene, which encodes a phosphoethanolamine transferase that modifies the second Kdo sugar of the core oligosaccharide[63]. Since activation of PhoPQ typically leads to decoration of lipid A, MgrR inhibition of *eptB* expression is counter-intuitive, but further highlights the complex regulation of LPS modifications. The TCS and small RNAs described in brief here is not a comprehensive overview of all factors that affect transcription or translation of LPS modification genes. Other small RNAs and transcriptional regulators are listed in supplementary information S3.

Regulation of OM enzymes, like PagP, PagL, and LpxR, at the transcriptional level is a relatively slow process as it depends on assembly and turn-over. To more tightly control their activity, these enzymes are also regulated at the level of recognition or availability of their substrates (supplementary information S3). LpxR of *Yersinia entercolitica* and PagL of *Salmonella enterica* subsp. *enterica* serovar Typhimurium both poorly recognize lipid A that has been decorated with aminoarabinose[64,65]. These deacylases can modify lipid A with unmodified phosphates, but their activity is repressed under conditions where aminoarabinose modification at the IM is up-regulated[64,65]. PagP activity is instead regulated by availability of its donor substrate. PagP plays a role in maintaining the asymmetry of the OM by transferring an acyl chain from the glycerophospholipid phosphatidylethanolamine to lipid A[66]. In *Salmonella enterica spp*. and *E. coli*, the activity of PagP is normally low because the outer leaflet of asymmetric OM is lacking glycerophospholipids[2]. Upon OM disruption, such as in Mg^{2+} limiting conditions or exposure to AMPs, glycerophospholipids flip to the outer leaflet resulting in PagP-dependent acylation of lipid A[67]. These examples demonstrate some of the ways LPS modification enzymes are regulated post-translationally.

Consequences of LPS modifications

LPS modifications affect many physiological processes of Gram-negative bacteria. Here effects on permeability of the OM, recognition by immune cells, antimicrobial resistance, and OM vesiculation will be briefly reviewed (Figure 3).

Adaptation of OM permeability

LPS modifications are regulated in response to temperature, metal ion concentrations, pH, AMPs, and other conditions. While these are commonly described for their critical effects on pathogenesis, they also have a role in modulating OM permeability in many environments. Mg^{2+} bridging of the phosphates on lipid A and/or phosphates of the core oligosaccharide of LPS help stabilize the OM. In Mg^{2+} limiting conditions, however, the phosphates of LPS repel each other decreasing OM stability[68]. Through PhoPQ and PmrAB, bacteria like *Salmonella enterica spp*. respond by upregulating PagP, ArnT, and EptA that help to stabilize the membrane by producing more hepta-acylated lipid A (decreasing fluidity) and adding positively-charged moieties (aminoarabinose and phosphoethanolamine) to the 1 and 4′ phosphates[68]. It is not fully clear how having predominantly positively-charged lipid A, which would also charge clash, forms a more stable OM than having predominantly negatively-charged lipid A. Perhaps, because the positively-charged moieties are attached to phosphate groups, the zwitterionic nature of modified lipid A allows cross-bridging to occur between neighboring molecules in the membrane. Alternatively, since PagP activity indicates that some glycerophospholipids are mislocalized to the outer-leaflet, perhaps anionic glycerophospholipids (e.g. phosphatidylglycerol) contribute to cross-bridging positively-charged lipid A species. Still, when Mg^{2+} concentrations are high, constitutive expression of PagP, ArnT, and EptA increases OM permeability[68] indicating that a negatively-charged lipid A domain increases bacterial fitness in the presence of divalent cations. Thus, bacteria employ different lipid A anchors in different environments to regulate OM permeability.

Evading immune system recognition

The human host immune system recognizes and responds to LPS as a common molecular signature of bacteria. LPS or LOS stimulate immune cells by two methods: binding to surface receptors and binding to a non-canonical inflammasome complex if internalized. Surface recognition starts with binding of LPS or LOS to LPS-binding proteins and CD14 (either soluble or membrane-bound) and then transfer of the molecule to toll-like receptor 4 (TLR4)-MD2 complexes on immune cells[69]. Hexa-acylated, *bis*-phosphorylated lipid A is highly recognized by the TLR4-MD2 co-receptor[70,71] (Figure 3a). The signaling pathway stimulated upon TLR4-MD2 binding to lipid A depends on the adaptor proteins that are then recruited (Figure 3a). Recruitment of MYD88 (myeloid differentiation primary response protein 88) results in the MYD88-mediated pathway and production of proinflammatory cytokines. Meanwhile, recruitment of TRIF (TIR domain-containing adaptor inducing IFNβ) results in the MYD88-independent pathway and reduced inflammation[72]. LPS modifications, especially those that remove the phosphates or alter the acylation of lipid A, can reduce either recognition by TLR4-MD2 or alter which signaling pathway is triggered[70,71,73]. As such, many bacteria utilize lipid A modifications to evade recognition by the mammalian immune response. For example, *H. pylori* constitutively produces a dephosphorylated, tetra-acylated species of lipid A that promotes immune evasion and long-term carriage of the organism in the gastric mucosa[74]. Due to differences in modifications and acylation of lipid A, *H. pylori* is 100 to 1000-fold less immuno-stimulatory than *E. coli*[74,75]. In addition, many bacteria upregulate lipid A modifications during infection to evade TLR4-MD2 recognition. Intriguingly, the Bengoechea group[76] detected upregulation of the lipid A modifications in *Klebsiella pneumoniae* directly extracted from infected tissue. This new approach allowed for the direct testing of what lipid A modifications are key for immune evasion in specific tissues. Altered LPS that have desired immune-stimulatory effects have also been explored as therapeutics (Box 2).

LPS that is internalized by host cells, especially from intracellular pathogens, is bound by caspases (caspases 4 and 5 in humans[77], and caspase 11 in mice[78,79]) that then stimulate the inflammasome (Figure 3a). LPS stimulation of the inflammasome triggers an inflammatory cell lysis pathway called pyroptosis[80]. Similar to TLR4 recognition, lipid A is the minimal unit required for inflammasome stimulation[77-79]. Reduced acylation of lipid A reduces recognition by murine caspase 11[78,79] but not by human caspases 4/5[81], indicating that these LPS modifications could be critical for pathogenesis of certain mammalian hosts. Further work is needed to determine if other LPS modifications, especially those employed by intracellular pathogens, allow for evasion of inflammasome recognition in their natural hosts.

Resistance to antimicrobial peptides

AMPs are produced and released by the immune response of the human host during inflammation to non-specifically clear bacteria. In addition, polymyxins are cationic AMPs naturally produced by Gram-positive bacteria and have been adapted for use as antibiotics to treat bacterial infections. AMPs are amphipathic; a typically cationic peptide mediates interaction with negatively-charged LPS with a hydrophobic domain that inserts into membranes forming pores[82] (Figure 3b). Perforation of the OM allows entry and disruption

of the IM resulting in cell lysis[82]. LPS modifications that remove or modify the phosphates of lipid A, modify the phosphates of the core oligosaccharide, and alter the acylation of lipid A can provide protection against AMPs (Figure 3b and supplementary information S2).

Recently it was demonstrated that one factor that contributes to long-term maintenance of commensal bacteria is LPS modifications that provide resistance to AMPs. The gut microbiota is a complex population of bacteria that are maintained long-term, in spite of the mammalian host inflammatory response[83]. Specifically, several *Bacteroidetes thetaiotaomicron* commensal isolates were found to have high resistance to polymyxin B mediated by expression of the lipid A phosphatase LpxF[84]. Further, when comparing the ability of isogenic strains with and without LpxF to colonize the mouse gut and be maintained after inflammation, both *B. thetaiotaomicron* populations could colonize, but the strain without LpxF was displaced by inflammation[84]. This finding indicated that lipid A modifications can contribute to long-term maintenance of commensal gut bacteria. While AMP resistance is a favorable trait for maintaining commensal bacteria, commensals can also become opportunistic pathogens and AMP resistance would make them more difficult to be cleared by the immune response.

Polymyxins are cationic AMPs naturally produced by bacteria that have been adapted for use as antibiotics. However, due to toxicity issues polymyxins are considered “last-resort” antibiotics. Still, with the increasing frequency of multi-drug resistant bacteria their use has also become necessary. Many pathogenic bacteria can become resistant to polymyxins through mutations that increase expression of chromosomally-encoded LPS-modifying enzymes like the Arn operon and EptA[37]. Fortuitously, these types of mutations do not easily spread between populations of bacteria. However, *eptA* homologs, named *mcr* genes for *m*obile *c*olistin *r*esistance, have been identified to be encoded in plasmids and phages that can be more easily spread[85]. In the few short years since the first report, mobile homologs of EptA have been identified in many pathogenic bacteria and have been detected worldwide, leading to concerns about spread of resistance to even last-resort antibiotics[85]. These findings only increase the insurmountable evidence that new therapeutics are desperately needed as other LPS modifications that require one enzyme and no special precursors, such as dephosphorylation by LpxF, could also be rapidly spread in a similar manner.

Effects on outer membrane vesicles

Gram-negative bacteria shed OM vesicles (OMVs) containing LPS as they grow. These OMVs are proposed to play roles in cell stress responses, nutrient acquisition, and pathogenesis[86]. Since LPS is a major constituent, altering LPS characteristics impact OMV processes, including recruiting cargo proteins and stimulating vesicle production. Pathogens like enterotoxigenic *E. coli* (ETEC) and *Porphyromonas gingivalis* produce virulence factors that bind to OMVs and are secreted into the environment[87,88]. Recruitment of these factors to OMVs is driven by binding of the cargo proteins to the sugars of LPS at the cell surface (Figure 3c). The pathogen ETEC produces a heat-labile enterotoxin that binds to the Kdo sugars of the core oligosaccharide[88,89]. Binding of labile toxin to LPS may be a mechanism of delivering the toxin on OMVs, but it also partially sequesters the toxin at the OM[89].

Research on *P. gingivalis* has revealed increasingly elaborate interactions between OMV proteins and specific LPS species. Early on, it was noted that LPS glycoforms with a negatively-charged O antigen and a subset of key OM-associated proteins are enriched in OMVs[87]. Mutants that do not produce O antigen had OMVs with altered protein content supporting a role in recruitment[87]. Further, *P. gingivalis* encodes a proposed LPS modification gene, LptO, that affects OMV formation and secretion of gingipains. The activity of LptO is under debate, originally proposed as a lipid A deacylase and recently proposed to instead be a lipid A 1-phosphatase[90,91]. However, mutants with null alleles of *lptO* have 50% reduced OMVs and reduced secretion of proteins recognized by a type IX secretion systems, supporting LptO's role in these processes[90-92]. Finally, a mechanism of recruitment of the disease-associated protein peptidylarginine deiminase (PPAD) to OMV LPS was recently proposed. PPAD citrullinate host proteins[93] and is implicated in the autoimmune disease rheumatoid arthritis[94,95]. PPAD is produced in soluble and LPS-bound forms, but a single amino acid change (Gln 373 to Lys) in PPAD reduces the LPS-bound form of the protein[96,97]. Further, work is needed to clarify how PPAD and other *P. gingivalis* OMV proteins associate with LPS. Another excellent example of how LPS structure influences OMV formation is the interaction of the quorum sensing molecule PQS of *P. aeruginosa* with lipid A[98]. PQS binds to the 4′ phosphate group of LPS, inserts partially into the membrane, and induces curvature that stimulates OMV formation[98,99]. Thus, this signaling molecule stimulates its own secretion into the environment.

Recently chemical alterations to LPS have also been implicated in stimulation of vesicle formation. Specifically, the Feldman group saw that deacylation of LPS by overexpressing PagL increased both the number and size of OMVs released by *Salmonella enterica* subsp. *enterica* serovar Typhimurium[100] (Figure 3d). Interestingly, the Kuehn group[101] also recently found that when they closely monitored OMVs upon shifts between neutral/high Mg^{2+} and acidic/low Mg^{2+} media, PhoPQ and PmrAB activation increases the number and size of OMVs released. Aminoarabinose and phosphoethanolamine modified LPS were found to be under-represented, while hepta-acylated LPS (from PagP activity) was enriched in OMVs[101]. The cooccurrence of increased size and number of OMVs with enrichment of PagP-modified LPS could indicate this modification also stimulates OMV production[101]. The Kuehn group also proposed that exclusion of certain LPS forms from OMVs, like aminoarabinose and phosphoethanolamine-modified LPS upon PhoPQ and PmrAB activation, could be a mechanism of quickly enriching these modified forms in the OM during environmental transitions[101]. These two connections between LPS modifications and OMVs raise many questions for cell envelope biogenesis and bacterial pathogenesis. Under what conditions are PagP and PagL upregulated to stimulate OM vesiculation, to alter fluidity of the OM by altering LPS acylation, or both? Does the selective secretion of PagL- and PagP-modified forms of LPS in OMVs, which are less immune-stimulatory, impact the host immune response to pathogenic bacteria? As discussed earlier, aminoarabinose-modified LPS inhibits the activities of LpxR and PagL; does this serve as a regulatory feed-back to decrease OM vesiculation once the OM LPS content has been appropriately altered?

Inhibitors of LPS biogenesis and modification

Much focus has been put on developing inhibitors of LPS biogenesis and modification as it is an ideal target for antimicrobial treatment due to its uniqueness to bacteria, its essential nature, and its role in virulence. Inhibitors have been explored for many of the enzymes involved in Kdo synthesis and the Raetz pathway (Figure 4)[102,103], focusing on enzymatic steps that are essential in most Gram-negative bacteria. The most promising inhibitors of LPS synthesis have been LpxC inhibitors due to their broad-activity, reviewed thoroughly by Erwin[104]. However, recent findings have suggested that more focus should be put on development of inhibitors of LpxK and LpxH, because, even in bacteria that can grow in the absence of LPS, inhibition of these enzymes results in build-up of toxic intermediates[105,106]. Such inhibitors could be particularly key for treating pathogenic *Acinetobacter baumannii* strains which are commonly multi-drug resistant and can survive in the absence of LOS (see Box 1). Inhibitors of the latter steps in lipid A/LPS synthesis could still prove useful in combination with drugs that normally cannot permeate the asymmetric OM. The most recent studies targeting LPS synthesis have attempted to increase the efficacy of LpxC inhibitors[107], identify compounds that target LpxA through virtual screening methods[108],and develop new high-throughput screening strategies[109,110] for other Lpx enzymes (e.g. LpxH).

Compounds that target transporters of LPS have also been explored as antimicrobials (Figure 4). Two classes of inhibitors were recently reported that target $MsbA_2$. The first are quinolone-like compounds discovered by Genentech that bind to the transmembrane-pocket of $MsbA_2$ and lock it in an LPS-bound, inward-facing conformation[111]. These inhibitors potently inhibit growth of *E. coli* and *Klebsiella*, but are less effective against $MsbA_2$ from *Pseudomonas*, indicating they could pan out to be narrow-range antimicrobials[111]. Kahne and collaborators also recently reported a class of compounds that act as inhibitors of $MsbA_2$ in *Acinetobacter baumannii*[112]. The compounds appear to stimulate wasteful ATP-hydrolysis of $MsbA_2$ that is uncoupled from LPS flipping[112].

A compound that affects coupling of ATP-hydrolysis and LPS transport has also been discovered for the Lpt complex (Figure 4). It was found that the already well-described DNA-gyrase inhibitor, novobiocin, is able to bind to the Lpt machinery[113]. However, novobiocin binding to the Lpt transporter increases LPS transport instead of inhibiting[113]. While novobiocin's effect on LPS transport is not inhibitory, it could be a good starting point for the rationale design of compounds targeting Lpt. Further, novobiocin and novobiocin derivatives, including compounds unable to inhibit DNA gyrase, were found to work synergistically with polymyxins in strains that are naturally sensitive to polymyxins[114]. These findings indicate that increased LPS transport potentiates polymyxins[114] and that combination therapies could be used to reduce the amount of polymyxin needed to treat bacterial infections. However, in strains displaying modified lipid A, novobiocin does not significantly synergize with polymyxin (unpublished, Trent laboratory), indicating that a combination therapy would have limitations in the clinic.

Three additional compounds have been reported that target the periplasmic domains of the Lpt transporter (Figure 4). LptC, LptA, and the N-terminus of LptD contain homologous β jellyroll domains that interact head-to-tail by strand addition to bridge the periplasm[9]. LPS

molecules travel across the Lpt periplasmic bridge with acyl-chains protected in a hydrophobic groove formed by the β-jellyroll domains[9]. A class of macrocyclic peptidomimetics were found to specifically inhibit LPS transport of *Pseudomonas* species by binding to the periplasmic domain of LptD[115,116]. These peptidomimetics are proposed to block LPS as it traverses the periplasm[115,117]. In addition, two previously discovered antibacterial compounds, thanatin and IMB-881, were recently found to act by disrupting assembly of the LPS transporter subunits (Figure 4). Thanatin is capable of binding to the periplasmic domain of LptD and LptA[118]. Binding of thanatin to LptA occurs at the N-terminus β-strand in a way hypothesized to block interactions between LptA and LptC[118]. IMB-881 was also demonstrated to block interactions between LptA and LptC in a screen using a yeast two-hybrid system and subsequent surface plasmon resonance experiments[119]. Thanatin and IMB-881 have antibacterial activity against many Gram-negative bacteria[118,119] and could be developed into useful wide-spectrum antimicrobials.

Finally, inhibitors that block the expression or directly inhibit the enzymatic activity of lipid A modifying enzymes have been reported (Figure 4). Since LPS modifications are critical for virulence of many pathogens and antibiotic resistance, these compounds could be used to directly treat many bacterial infections or in combination therapy to potentiate polymyxins. Inhibitors were discovered that caused down regulation of PmrAB and reduced lipid A modifications in *Acinetobacter baumannii* and *Klebsiella pneumoniae*[120]. Indeed, these compounds potentiated polymyxins[120]. Substrate mimics have also been reported that can directly inhibit ArnT which would be expected to have a similar effect[121]. These compounds could prove to be useful for treating bacteria with PmrAB-mediated resistance to polymyxins. However, they would be unable to reverse polymyxin resistance arising from the plasmid-encoded homologs of *eptA* (*mcr* genes) whose expression is PmrA-independent. No compounds have yet been identified that target EptA, but the recent full-length structure of the enzyme could serve as a starting point for rational design of inhibitors[122]. Altogether, much work has been devoted to designing or identifying compounds that inhibit LPS biogenesis and modification. More work is needed to explore which of these has potential as a clinical antimicrobial or to identify new compounds with promise.

Concluding remarks and future directions

Here we have touched on the striking diversity of the chemical alteration of LPS employed by pathogenic bacteria and the associated physiological consequences. Yet, it is clear that we do not have a complete story of all biological processes that LPS is involved in and many open questions remain. It is still unclear why LPS is essential in almost all Gram-negative bacteria. Further work on bacteria able to survive in the absence of LPS, like *A. baumannii*, will provide clues to what additional roles LPS holds as a major constituent of the OM. Also, from a bacterial physiology standpoint, LPS biogenesis is a complex process; LPS is synthesized on the cytoplasmic side of the IM, transported across multiple membranes, and modified in all compartments of the cell. While the major synthetic enzymes and transporters have been identified in many bacteria, how synthesis and transport are coordinated to ensure efficient assembly of the bacterial OM is unknown. Do LPS synthetic enzymes form large synthome complexes? In organisms that constitutively modify LPS, what are the checkpoints to make sure these have occurred? (Box 3) Further, LPS biogenesis

and its modification likely have connections to other cellular processes in bacteria that have yet to be discovered. For example, the LPS modification enzyme LpxT utilizes undecaprenyl-pyrophosphate as a phosphate donor and contributes to recycling of this carrier molecule[123]. Conceptually, this also opens up the possibility that undecaprenyl-pyrophosphate could serve as phosphate donor for other processes in the periplasm, where no ATP available. In addition, EptC in *Campylobacter jejuni* is a phosphoethanolamine transferase that remarkably modifies lipid A, a heptose sugar of the core oligosaccharide, N-linked glycans of proteins and the rod of the *C. jejuni* flagellum[124,125]. Many bacteria contain multiple homologs of phosphoethanolamine transferases; how many of these are involved in LPS modifications, protein modifications or both? LPS modification enzymes could have a larger role in regulating activity of cell envelope proteins.

It has also become apparent that LPS has critical roles in interactions between bacteria and higher organisms. We have briefly reviewed how LPS can stimulate the human immune system, and both commensal and pathogenic bacteria have been implicated in immune system development impacting the incidence of asthma, allergies, and autoimmune diseases. Many studies have shown that the rate of autoimmune diseases is heightened in regions with improved sanitation and reduced childhood exposure to infectious agents[126]. Recently, in a study that followed gut microbiota development in infants from various regions, high abundance of *Bacteroidetes* in the gut during infancy was correlated with high incidence of autoimmune diseases[127]. *Bacteroidetes* species produce modified LPS that is less immuno-stimulatory, suggesting that LPS stimulation of the immune system is critical during development[127]. Finally, it is important to note that changes in lipid A/LPS structure also impact interaction between bacteria and plants with a number of LPS modifying enzymes characterized in *Rhizobium* species[128]. Our concepts of how bacteria contribute to the development of higher organisms are evolving and LPS is likely to have a major role in many more aspects of these processes.

Supplementary Material

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Suggested Glossary terms

Lipooligosaccharide
A form of LPS with an extended core oligosaccharide, but lacking O antigen

TLR4/MD-2 (Toll-like receptor 4/myeloid differentiation factor 2)
A pattern-recognition receptor of the innate immune system that recognizes LPS/LOS initiating a robust signal cascade and inflammatory response in mammals

Stringent response
A stress response of bacteria in reaction to nutrient limitation resulting in extreme physiological changes

Small RNAs

Typically short, non-coding RNA molecules that interact with mRNAs to regulate gene expression or interact with proteins to regulate activity

Outer membrane vesicles (OMVs)
Small, spherical outer-membrane blebs that are released from Gram-negative bacterial cells and contain membrane and periplasmic components

Inflammasome
A intracellular, multiprotein complex in mammalian cells that recognizes microbial molecules and activates inflammatory response include pyroptosis and pro-inflammatory cytokines

Pyroptosis
An inflammatory, programmed cell death that typically associated with infection of intracellular pathogens

Capsule
A thick layer of polysaccharides that surrounds a bacterial cell, also referred to as capsular polysaccharide

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Box 1:

Abandoning Lipid A to become resistant

Neisseria meningitidis, Moraxella catarrhalis, and *Acinetobacter baumannii*, have been demonstrated to be viable when lipid A synthesis is disrupted[129-131]. However, only *A. baumannii* strains were found to inactivate lipid A synthesis in a selectable manner to provide resistance to cationic AMPs[129]. Loss of LOS in *A. baumannii* comes with fitness costs: reduced growth rate, reduced virulence, and sensitivity to many antibiotics[132]. However, clinical isolates are capable of inactivating lipid A synthesis supporting that this extreme mechanism of resistance could occur in hospitals or patients[129]. Further, screening for this type of colistin resistance during treatment is needed to determine the clinical relevance. Work on LOS-deficient *A. baumannii*, have started to unravel how a bacterium can survive when lipid A synthesis is inactivated. Comparing the ability of multiple strains of *A. baumannii* to inactivate LOS synthesis, it was found that peptidoglycan synthesis is a critical factor; LOS-deficient mutants could be isolated from strains with low protein levels of penicillin binding protein 1A (PBP1A) or with disruption of the encoding gene for PBP1A, *ponA*, but not from strains expressing higher levels of PBP1A[133]. Further, LOS-deficient *A. baumannii* respond by increasing transcription of genes encoding for lipoprotein transport and surface-exposed lipoproteins[133,134]. These results indicate peptidoglycan and lipoprotein synthesis affect the ability for *A. baumannii* to survive with an OM consisting of a symmetric glycerophospholipid bilayer. Furthermore, a short-term evolution experiment demonstrated that LOS-deficient bacteria can increase overall fitness through inactivation of genes required for the removal of glycerophospholipids from the outer leaflet of the OM[135]. Since LOS-deficient *A. baumannii* need to fill the outer leaflet of the OM with glycerophospholipids, it is logical that enzymes that remove or degrade these lipids in the outer leaflet would be disadvantageous. Specifically, mutations that result in disruption of the Mla transporter, transports mislocalized OM glycerophospholipids back to the IM; disruption of PldA, phospholipase that degrades mislocalized glycerophospholipids; or disruption of both increase growth rate of LOS-deficient *A. baumannii* strains[135]. Disruption of Mla and PldA are expected to allow for higher accumulation of glycerophospholipids in the outer leaflet of the OM replacing LOS. Work in *A. baumannii* has demonstrated that LOS biogenesis is interconnected with biogenesis of other cell envelope components (peptidoglycan, OM lipoproteins, and OM glycerophospholipids) and rewiring these connections is necessary to grow in the absence of LOS. It remains unclear why lipid A synthesis is essential in some organisms and not in others; however, LOS-deficiency in *Acinetobacter* has provided unique insights into cell envelope biology.

Box 2:

Altered LPS as adjuvants and vaccines

LPS can be utilized to stimulate the immune response, but high stimulation causes severe damage to tissues and organs, as occurs during sepsis[72]. LPS variants that stimulate the immune response without toxic effects have been explored as immunotherapeutics. In 1982, Ribi and colleagues[136] described a method for chemically-altering LPS species from *Salmonella minnesota* to produce a mixture of mono-phosphorylated lipid A species (MPL) with reduced toxicity. After further development by the Corxia Corporation and GlaxoSmithKline Biologicals, this led to the FDA approval in 2009 of MPL, primarily 3-O-deacyl-4′-monophosphoryl lipid A, as an adjuvant in vaccines called MPL adjuvant™ [72]. The MPL adjuvant™ shows reduced activation of the MYD88-dependent response and thus induces less toxic inflammation[72], while providing key adjuvant properties. To further fine-tune the lipid A induced immune-response, our group[137] and others[138,139] have bioengineered non-pathogenic bacteria to produce various LPS glycoforms by combinations of overexpressing and knocking out of genes involved in lipid A synthesis and modification. In our work with *E. coli*[137], altering the phosphates, number of acyl chains, position of acyl chains, and acyl chain hydroxylation of lipid A individually or in combination can give a range of TLR4-MD2 recognition and cytokine response. A similar range of immune stimulation was observed by the Van der ley[139] and Ernst[138] groups for LPS with various modifications produced by *Neisseria meningitidis* and *Yersinia pestis*, respectively. Interestingly, not all trends observed for TLR4-M2 recognition of lipid A species produced by *E. coli* held true for *N. meningitidis*, highlighting the importance of studying LPS-dependent immune modulation by various organisms.

Altering LPS has also been utilized to increase the efficacy of OMV vaccines. OMVs have been used as successful vaccine platforms, particularly for *Neisseria meningitidis*[140], because they display molecular signatures of the pathogen, but are unable to replicate. LPS modifications that stimulate OMV formation or that alter the adjuvanticity have been combined with these platforms to have desired immune-stimulatory effects. In addition, the Feldman[141] and DeLisa[142,143] groups have developed methods to produce OMVs that contain LPS whose core oligosaccharide is modified with pathogen-associated glycans (capsule, heterologous O antigens, and other surface-displayed glycans). These approaches capitalize on the substrate promiscuity of the O-antigen ligase, WaaL, which can transfer a variety of sugar repeats from the undecaprenyl-pyrophosphate carrier to LPS[3]. The genes for producing a desired pathogen-associated glycan are heterologously expressed in non-pathogenic *E. coli* K-12 strains that do not produce O antigen[141-143]. The pathogen associated glycan is appended to LPS by WaaL and modified LPS is released in OMVs. Since LPS in OMVs is still endotoxic, the *E. coli* strains that produce these OMV vaccines can be further engineered to have modified lipid A with reduced endotoxicity, while retaining adjuvant properties[142]. These vaccine platforms have been used to produce OMVs displaying a variety of disease-associated glycans that upon immunization provide protection against the associated pathogen in disease models (e.g. *Streptococcus pneumoniae*,

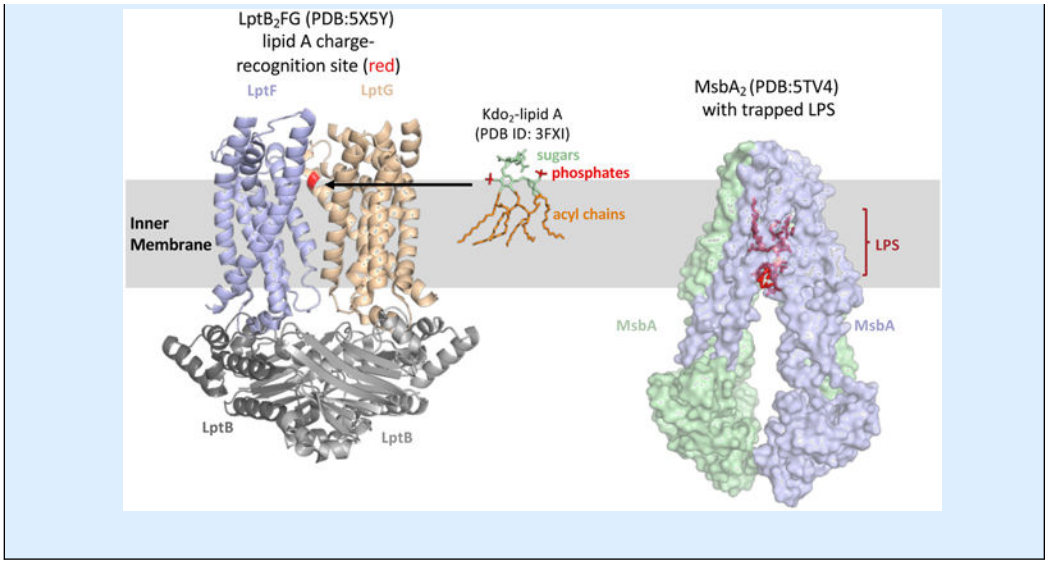
Campylobacter jejuni, *Francisella tularensis subsp. Tularensis*, and *Neisseria meningitidis*)[141-143]. Further, displaying glyco-engineered LPS on reduced-endotoxic and commensal strains of *E. coli* instead of OMVs could be promising as oral-administrable, cost-effective, whole-cell vaccines.

Box 3:

Selecting which LPS to transport

To assure LPS synthesis is complete before transport begins, the IM flippase, $MsbA_2$, transports hexa-acylated LPS more efficiently than LPS with a tetra-acylated lipid A domain[144]. The molecular mechanism for selectively is still not understood, but mutations that alter a proline either in transmembrane 1 or 2 of MsbA resulted in increased transport of lipid IV_A in strains with disrupted Kdo synthesis[145]. Recent cryo-EM structures of $MsbA_2$ prior to and after flipping suggests a trap and flip model where lipid A binds in a deep cavity of $MsbA_2$ (see figure) that allows it to translocate to the outer leaflet of the IM prior to flipping[146]. ATP hydrolysis then causes transmembrane rearrangement to flip and release lipid A[146]. Selectivity for hexa-acylated lipid A likely occurs prior to the "trap" state as it would be energetically unfavorable to release lipid A back into the inner leaflet once this step is reached.

Many modifications of LPS and attachment of O antigen, if present, to the coreoligosaccharide occur at the periplasmic leaflet of the IM[3]. Presumably, it would be advantageous to assure that desired core-lipid A modifications and O-antigen addition occur before transport by the Lpt machinery. Work in *Burkholderia cenocepacia* and *E. coli* suggests that the Lpt machinery recognizes charges at the 1 and 4′ positions of lipid A in order to control which molecules are transported. In *B. cenocepacia*, LPS is predominantly aminoarabinose-modified and this modification is essential for viability[147]. *B. cenocepacia* that produce unmodified, negatively-charged lipid A are only viable with a compensatory mutation that alters an Asp residue in the substrate cavity of LptFG (colored red on LptG in figure) to neutral His suggesting a charge interaction between transporter and substrate[148]. Further analysis of this residue in LptG suggested it may be coevolved with the charge of the 1 and 4′ position of lipid A produced by various Gram-negative bacteria[149]. In *E. coli*, LptG contained a positively-charged residue at this site and lipid A is predominantly unmodified at the 1 and 4′ phosphates. Further, mutations in *E. coli* that alter this residue in LptG to have a negative charge conferred defects that could be suppressed by mutations that increased the amount of lipid A modified with positively-charged phosphoethanolamine[149]. These findings suggest that charge-charge interactions between the GlcN disaccharide backbone of lipid A and LptG are one way that a bacterium selects which form of LPS is transported to the OM. For *Burkholderia cenocepacia* gating which LPS is transported to the OM is a convenient way to assure that a constitutive modification of lipid A occurs. However, it is clear that not all bacteria with constitutive modifications to LPS utilize similar regulation mechanisms because constitutive modifications are not essential in many organisms like *H. pylori*.



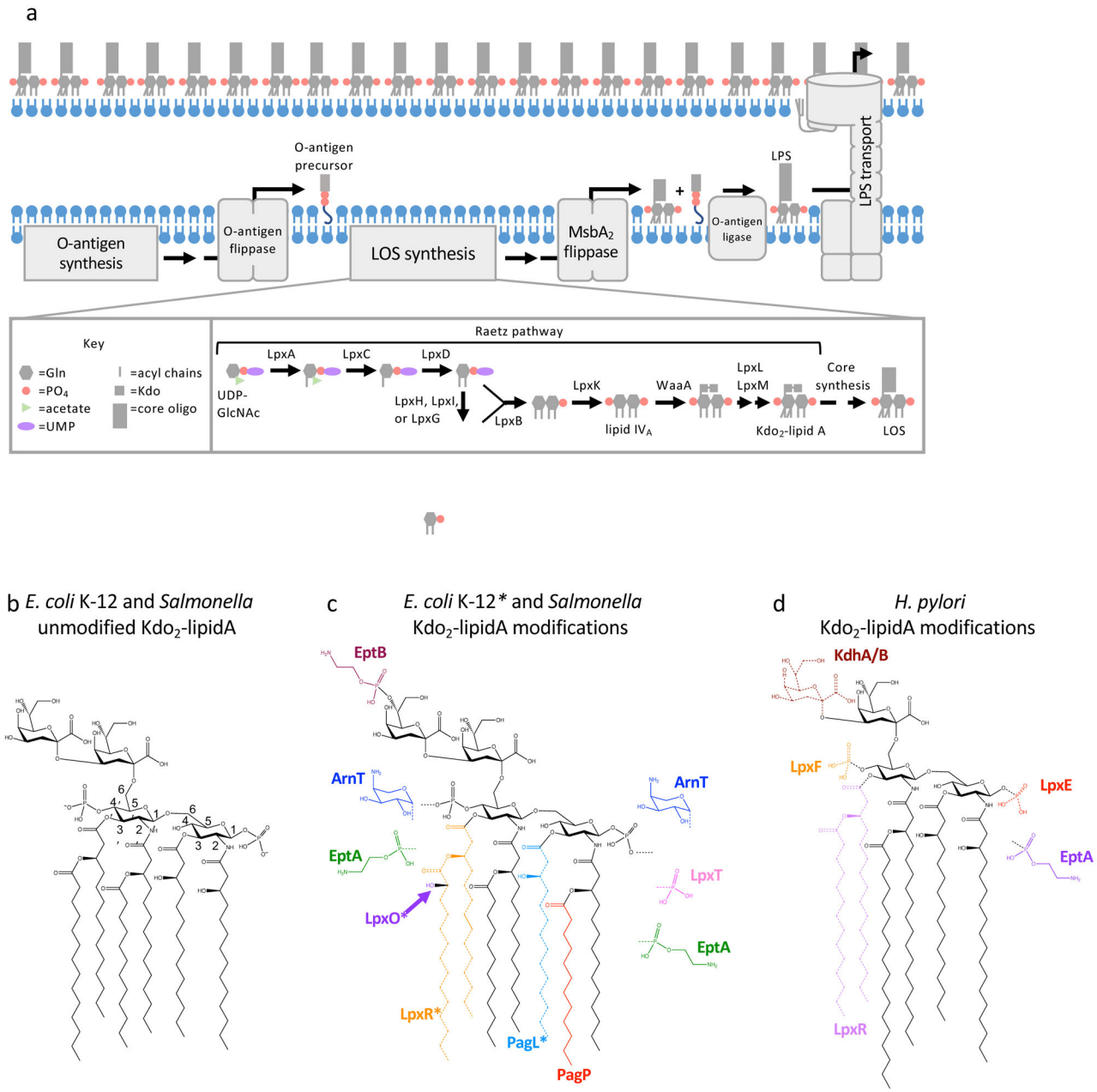


Figure 1. Lipopolysaccharide biogenesis, structure, and modifications.

(a) Overview of LPS biogenesis in *E. coli* and *Salmonella*. Briefly, synthesis of the lipid A and core domains of LPS occurs in the cytoplasm and at the cytoplasmic interface of the IM. O antigen, if present, is synthesized separately attached to the carrier lipid, undecaprenyl-pyrophosphate. LOS and O-antigen precursors are flipped across the IM separately by $MsbA_2$ and O-antigen flippases, respectively. O antigen is attached to LOS on the periplasmic side of the IM. Finally, LPS is transported from the IM to the surface of the OM by the Lpt complex. (b) Unmodified Kdo_2-lipid A synthesized by *E. coli* K-12 and *Salmonella enterica spp.* strains (c and d) Summary of chemical modifications of Kdo_2-lipid A that can occur after synthesis in *E. coli K12* and *Salmonella enterica spp.* (c) and

Helicobacter pylori (d). Enzymes that catalyze the modification are color coded along with the chemical group. Chemical groups drawn with dotted lines indicate the enzyme catalyzes hydrolysis to remove the group. Asterisks indicate that LpxO, LpxR, and PagL are present in *Salmonella enterica spp*. strains but not present in *E. coli K12* strains.

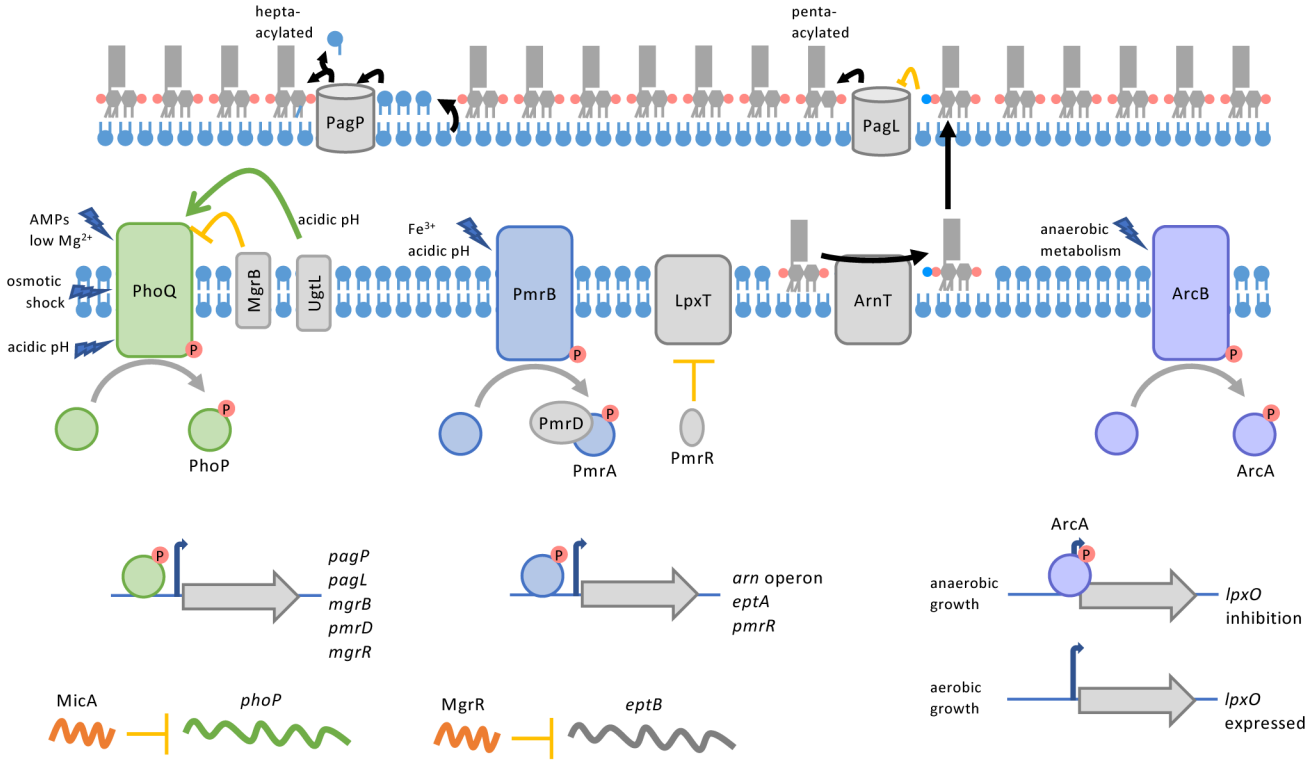


Figure 2: Regulation of LPS modifications

(a) Regulation of LPS modification enzymes in *Salmonella enterica* subsp. *enterica* serovar Typhimurium. TCS PhoPQ, PmrAB, and ArcAB regulate genes that encode enzymes that alter the acylation (PagP, PagL), modify phosphates (aminoarabinose by ArnT and phosphoethanolamine by EptA), and hydroxylate an acyl chain (LpxO) of lipid A, respectively. PhoPQ also upregulates the protein PmrD which binds to and protects phosphorylated-PmrA, connecting these TCS. Small RNAs are connected to PhoPQ regulation; MicA inhibits translation of PhoP, and MgrR, when upregulated by PhoPQ, inhibits the gene *eptB*, encoding a core-oligosaccharide modifying phosphoethanolamine transferase. In addition, PhoPQ and PmrAB upregulate genes involved in negative feed-back loops for the respective TCS. PhoPQ upregulates MgrB that binds to and inhibits PhoQ. PmrAB upregulates the small protein PmrR and genes that for modifying lipid A with aminoarabinose (*arn* operon) and phosphoethanolamine (*eptA*). PmrR post-translationally inhibits the lipid A phosphotransferase LpxT. Decrease in LpxT activity and increased lipid A modification by ArnT and EptA alter the charge of the OM so that metal ions that activate PmrB are blocked from entering the cell. Finally, the activity of OM enzymes PagP and PagL are regulated by availability and modification of their substrates, respectively. When glycerophospholipids are mislocalized to the outer leaflet of the OM, PagP catalyzes the transfer of an acyl chain from donor glycerophospholipids to acceptor lipid A molecules. PagL deacylation of lipid A is inhibited by aminoarabinose modification of lipid A.

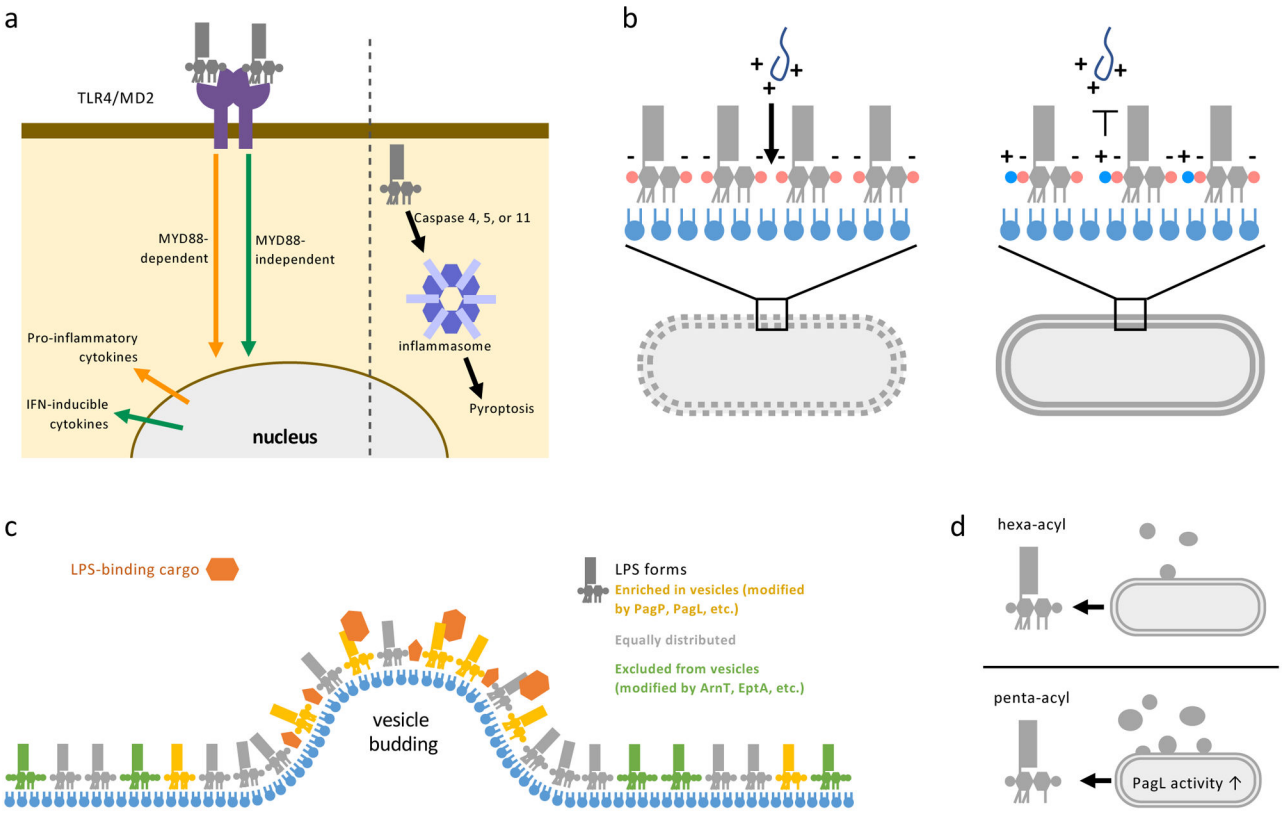


Figure 3: Consequences of LPS modifications

(a) LPS can stimulate immune cell responses through recognition by surface receptors (left) or binding to the cytoplasmic inflammasome (right). TLR4/MD2 receptors on the surface of mammalian immune cells recognize lipid A and can activate two signaling pathways. The myeloid differentiation primary response protein 88 (MYD88)-dependent pathway upregulates proinflammatory cytokines that lead to inflammation and bacterial clearance. Alternatively, signaling through TIR domain-containing adaptor inducing IFNβ (TRIF), known as the MYD88-independent pathway, produces interferon (IFN) inducible cytokines that result in less inflammation, but are critical for adjuvanticity. Modifications of the phosphates and acyl chains of lipid A affect how well LPS is recognized by the TLR4/MD2 receptor and which signaling pathway is induced. Inflammasome recognition is mediates by caspases and lead to an inflammatory cell death pathway called pyroptosis. Modifications to acyl chains of LPS reduce stimulation of murine inflammasome response but does not affect stimulation of human cell-lines inflammasomes. (b) Cationic antimicrobial peptides (AMPs) produced by host immune cells or used as antibiotics (such as Polymyxin B and Colistin) to treat infectious bacteria act by first forming charge-charge interactions with the highly negatively-charged OM. AMPs then perforate the OM followed by the IM, leading to lysis of bacterial cells. LPS modification that reduce the negative charge or alter the acyl chains of lipid A provide resistance against AMPs by charge repulsion or decreasing the fluidity of the OM. (c) Gram-negative bacteria release vesicles that bud from the OM called outer membrane vesicle (OMVs). When the LPS in the OM and OMVs released are compared, certain chemical forms of LPS are enriched, equally distributed, or excluded (colored gold, grey, and green respectively) in OMVs. Some cargo proteins (orange) associated with OMVs

such as heat-labile enterotoxin in enterotoxigenic *E. coli*, are recruited through specific interactions with LPS. Allowing the selective recruiting and secretion of certain proteins in OMVs. (d) Certain chemical forms of LPS, such as penta-acylated LPS produced by PagL activity, increase the size and number of OMVs released by bacteria, indicating that LPS modification can stimulate OMV formation.

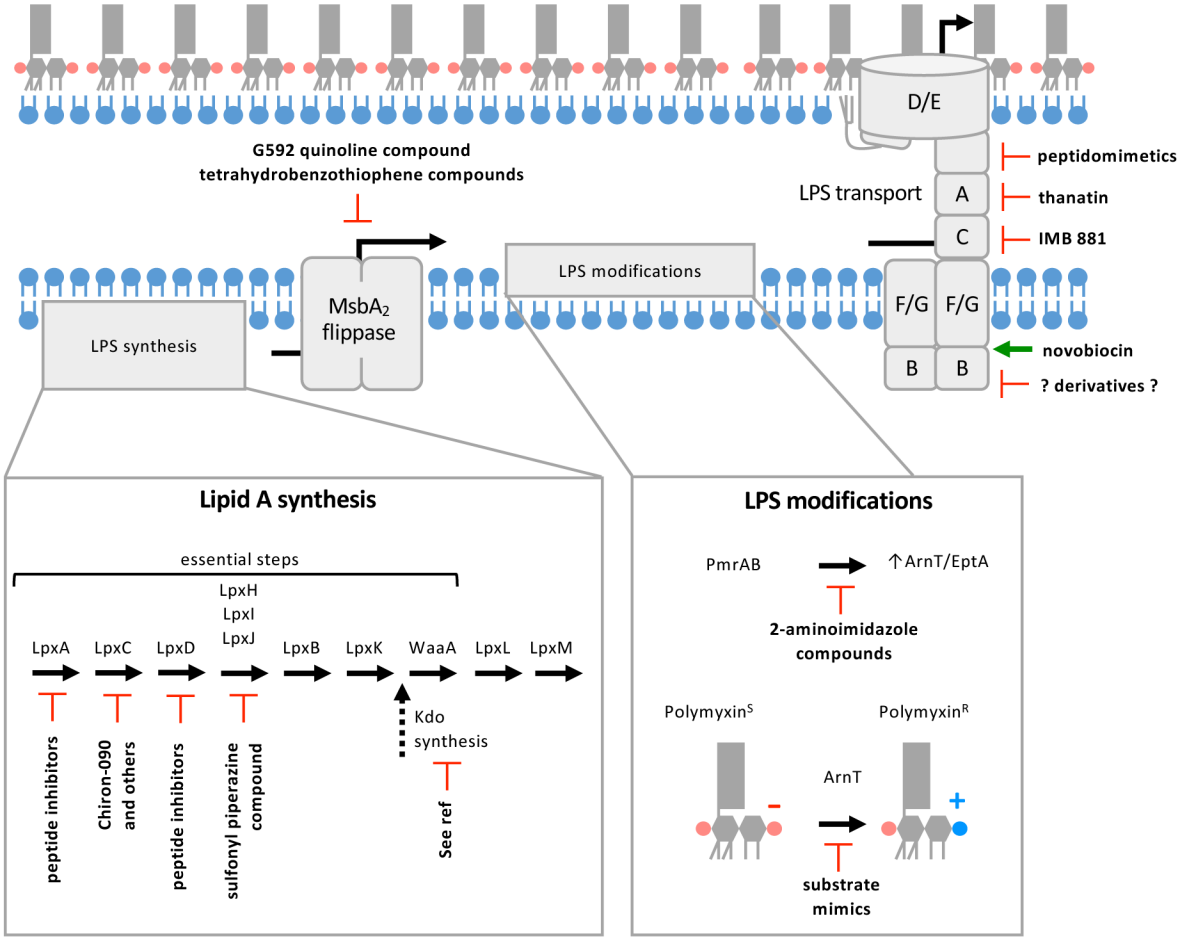


Figure 4: Summary of therapeutic strategies to target LPS biogenesis.

Compounds have been identified that inhibit (indicated by red blocked arrows) enzymes involved in lipid A synthesis, LPS flipping by $MsbA_2$, regulators of LPS modifications, enzymes that modify LPS, and transport of LPS to the OM. The DNA gyrase novobiocin has also been demonstrated to bind the Lpt machinery and activate LPS transport (indicated by green arrow). While this activity does not inhibit growth, it does synergistically increase the efficacy of polymyxins.