Published in final edited form as:
Curr Opin Microbiol. 2013 December ; 16(6): 779–785. doi:10.1016/j.mib.2013.09.007.

On the Essentiality of Lipopolysaccharide to Gram-Negative Bacteria

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Abstract

Lipopolysaccharide is a highly acylated saccharolipid located on the outer leaflet of the outer membrane of Gram-negative bacteria. Lipopolysaccharide is critical to maintaining the barrier function preventing the passive diffusion of hydrophobic solutes such as antibiotics and detergents into the cell. Lipopolysaccharide has been considered an essential component for outer membrane biogenesis and cell viability based on pioneering studies in the model Gram-negative organisms *Escherichia coli* and *Salmonella*. With the isolation of lipopolysaccharide-null mutants in *Neisseria meningitides*, *Moraxella catarrhalis*, and most recently in *Acinetobacter baumannii*, it has become increasingly apparent that lipopolysaccharide is not an essential outer membrane building block in all organisms. We suggest the accumulation of toxic intermediates, misassembly of essential outer membrane porins, and outer membrane stress response pathways that are activated by mislocalized lipopolysaccharide may collectively contribute to the observed strain-dependent essentiality of lipopolysaccharide.

Introduction

Gram-positive bacteria contain a cytoplasmic membrane surrounded by a layer of peptidoglycan; in contrast, Gram-negative bacteria contain a cytoplasm surrounded by what appears to be three layers: an inner membrane, a layer of peptidoglycan and an outer membrane [1,2]. The outer membrane (OM) of Gram-negative bacteria is an asymmetric bilayer with an inner leaflet consisting of phospholipids and an outer leaflet consisting of lipopolysaccharide (LPS). Much of what we know about LPS derives from early work beginning in the 1960s on *Escherichia coli* and *Salmonella typhimurium*. Using a newly developed analytical technique to allow separation of the inner membrane (IM) from the OM [3], Osborn and coworkers established that LPS fractionates to the OM [4]. Remarkably, LPS was subsequently shown to be localized exclusively on the outer leaflet of the OM [5–7]. At the same time, the site of (bio)synthesis of LPS was determined to take place at the inner membrane [8]. Work done by Osborn, Raetz, and others first established

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steps in the biosynthesis of LPS [9–14] and, more recently, the details of LPS transport from its site of synthesis to the cell surface have begun to be uncovered [15]. LPS was shown to be essential and LPS-defective mutants were hypersusceptible to antibiotics [16–18]. The picture that has emerged from the sum of these studies is that individual LPS molecules interact with one another on the cell surface mediated through divalent cations to form a permeability barrier, which prevents entry of small hydrophobic compounds, such as antibiotics, bile salts and detergents, and thus allows Gram-negative bacteria to survive in harsh environments [19–22]. Because the proper assembly of LPS on the cell surface is required to create an effective permeability barrier, genes involved in biosynthesis and assembly (biogenesis) of LPS have become targets for the design of novel classes of antibiotics [23,24].

Historically, interest in developing inhibitors of LPS biosynthesis was predominantly based on the view that LPS was an essential structural component necessary to create an OM. LPS is a large detergent-like molecule comprising three regions; a highly acylated di-GlcNAc backbone (lipid A) connected to a polysaccharide containing repeating sugars (O-antigen) linked through a highly conserved oligosaccharide Kdo/heptose core (Figure 1A). The minimal LPS structure supporting a functional OM and cell viability in enteric bacteria was shown to be Kdo_2-lipid A [14,25]. However, our understanding of the importance of LPS in the physiology of Gram-negative bacteria became clouded by the discovery that certain genera do not require LPS to assemble an OM and survive. Remarkably, certain strains of *Neisseria* can live when their *lpxA* gene encoding the first enzyme in LPS biosynthesis is inactivated, thus depleting these organisms of LPS [26]. One early hypothesis to explain how these strains of *Neisseria* could survive was that capsular polysaccharide was a structural substitute for LPS and became essential in these LPS-deficient strains [27]. However, the ability to construct double mutants lacking both *lpxA* and capsule expression in *N. meningitidis* disproved this theory [28]. Subsequently viable strains of *Moraxella* and *Acinetobacter* completely lacking LPS were isolated and characterized [29,30]. Taken together, these studies called into question the generality of the conclusion drawn from the classic experiments in *E. coli* and *Salmonella*. Clearly the assumption that LPS is simply required as a structural component of the outer leaflet of the OM in all Gram-negative bacteria cannot be correct. The essentiality of LPS varies considerably, depending not only on the genera of Gram-negative bacteria but also on the species, and in some cases, even on the particular strain background. This review will consider alternate explanations to account for strain-dependent LPS essentiality in Gram-negative bacteria and discuss the underlying implications for developing antibiotics targeting LPS.

LPS synthesis and assembly pathways in *E. coli*

The LPS biosynthesis/transport pathway spans three compartments of Gram-negative bacteria [14]. In *E. coli*, the Kdo_2-lipid A domain is synthesized inside the cytoplasm [11–13]. After sequential addition of sugars to produce the lipid A-oligosaccharide core at the cytoplasmic membrane, this molecule is flipped onto the periplasmic face of the inner membrane by an ABC transporter (MsbA) before the O-antigen is added [31–36]. In the canonical (Wzy-dependent) O-antigen pathway in *E. coli*, O-antigen biosynthesis begins in the cytoplasm with the sequential addition of three to five monosaccharides onto the undecaprenyl monophosphate carrier lipid (Und-P) to make the O-antigen repeat subunit. This oligosaccharide subunit is transported to the periplasmic face of the IM by Wzx and polymerized *en bloc* by Wzy to form a mature O-antigen polysaccharide chain containing as many as 40 to 200 repeat units. This polysaccharide must then be transferred to the lipid A-core acceptor by the O-antigen ligase WaaL [14] prior to transit through the periplasm to the cell surface via the Lpt pathway [36] (Figure 1B). The Lpt pathway consists of seven proteins that form a trans-envelope structure containing an IM complex (LptB/F/G/C)

required to extract LPS from the IM, a bridge (LptA) between the IM and the OM to permit transit across the aqueous periplasmic compartment, and an OM translocon (LptD/E) to allow the large detergent-like LPS molecule to pass through the OM to its final destination on the cell surface [37–54] (Figure 1C).

The loss of LPS biosynthesis from a given organism has deep-seeded consequences for the assembly of other components of the cell envelope. Syntheses of O-antigen, peptidoglycan, secondary cell wall polymers and outer-membrane proteins (OMPs) are impacted by the absence of LPS [14,15]. While LPS itself maybe not be required for viability, the extent to which the essential functions of the cell envelope are compromised by the loss of LPS could ultimately determine whether LPS is essential in any given strain.

Inhibition of LPS biosynthesis could cause accumulation of cell envelope components in inappropriate compartments

Inhibition of LPS biosynthesis (e.g. LpxC deletion, the first committed step of LPS biosynthesis) depletes levels of the oligosaccharide lipid A core within the IM. The lack of oligosaccharide lipid A core acceptor available for O-antigen transfer can potentially cause unligatable Und-PP O-antigen precursors to accumulate. Accumulation of such precursors has been shown to be toxic in *Salmonella*, leading to the suggestion that undecaprenyl sequestration influences essential Und-P dependent pathways [55]. Both O-antigen and peptidoglycan biogenesis utilize this membrane-bound carrier for addition of nucleotide sugars. Because undecaprenyl levels are limited in bacterial membranes, blocking transfer of Und-PP O-antigen to the lipid A core leads to sequestration of Und-P, thereby depleting the Und-P pool available to other essential pathways such as peptidoglycan biosynthesis. This toxicity would be predicted to be highly dependent on the strain background.

There are many different factors which may contribute to whether a bacterial strain is susceptible to accumulation of the Und-PP O-antigen. For instance, some O-antigen serotypes utilize a second distinct biosynthetic pathway, the ABC transporter-dependent pathway [14]. The O-antigen homopolymer is assembled on a single Und-P carrier in the cytoplasm prior to transport and ligation to the oligosaccharide-lipid A acceptor on the periplasmic face of the IM [14,56]. Those strains that utilize this ABC pathway would clearly be less susceptible to the accumulation of O-antigen because the demand for the carrier lipid is far less. Secondly, different strains of bacteria modify their lipid A core with O-antigen to varying extents [57]. Some bacteria tend to cap a larger portion of lipid A-oligosaccharides with O-antigen, producing smooth LPS, whereas other organisms tend to have predominantly underivatized lipid A-oligosaccharide core present on the surface (rough LPS) [14]. The extent to which lipid A-oligosaccharides are end-capped with O-antigen (smooth to rough ratio) reflects the flux through the pathway, which in turn is related to the usage of Und-P carrier. A final factor contributing to the sensitivity of a given strain to O-antigen accumulation and the resulting Und-P sequestration is whether cellular mechanisms exist to process the stalled intermediates. Ordinarily, after the O-antigen polysaccharide is transferred to the lipid A-core via WaaL, the newly released Und-PP is recycled back to its active monophosphoryl form Und-P via pyrophosphatases, which liberates carriers enabling the next round of O-antigen and peptidoglycan biosynthesis [58]. It is possible that certain pyrophosphatases can also cleave the O-antigen precursors when its biosynthesis or ligation stalls and allow the Und-P lipid carrier to be recycled. Indeed, in certain *E. coli* strains with group 4 capsules, a fraction of O-antigen is normally released by hydrolysis to form an extracellular capsule polysaccharide layer [59,60]. This discussion is simply meant to illustrate that there might be many strain-specific mechanisms to relieve the buildup of O-antigen intermediates that would otherwise result in toxicity due to sequestration of the lipid carrier.

Inhibition of LPS biosynthesis could affect the assembly and function of membrane proteins

In addition to LPS, the outer membrane of Gram-negative bacteria contains two major classes of proteins: lipoproteins and integral membrane proteins of β-barrel structure. The exact function of most membrane β-barrel proteins is not known, but many are believed to form pores (porins) in the membrane to provide nonspecific channels across the OM to allow entry of nutrients, which are generally small and hydrophilic [22,61]. It is believed that LPS facilitates porin assembly and function by acting as a molecular chaperone [35]. For example, the porins OmpC and OmpF depend on LPS for trimerization [62–64] and for maintaining proper channel gating function [65], while the protease OmpT requires LPS for its proteolytic activity [66]. While the complement of essential OMPs has only been defined in a limited number of species, there are two outer-membrane β-barrel proteins known to be essential in *E. coli*. One, LptD is a component of the heterodimeric OM translocon responsible for LPS transport and assembly on the cell surface [38,39,41,49–51]. In those strains of *Neisseria* where LPS is not essential, LptD becomes non-essential as well [39]. The other BamA is an essential component of the five-protein complex responsible for assembling all OMPs [67–70]. In fact, there are some endosymbionts that have evolved minimal genomes and do not contain genes involved in LPS biogenesis pathway (either Lpx or Lpt) [71,72]. However BamA is generally found to be essential even in minimal genomes, suggesting some β-barrel proteins must be present to permit passage of metabolites across the outer membrane. Clearly, different strains of bacteria have unique nutrient requirements and hence may depend on a specific repertoire of porins for essential nutrient uptake. In the case where these porins depend on LPS for folding/function, LPS would become indispensable.

The loss of LPS could also disrupt the structure and function of the inner membrane. It is possible that inhibition of LPS biosynthesis leads to accumulation of glycolipid (e.g., Und-PP O-antigen, Und-PP enterobacterial common antigen [73,74], or other secondary cell envelope polymers [75]) intermediates. One could imagine many scenarios through which these accumulated glycolipid intermediates could compromise IM functions. The accumulation of glycolipid intermediates could influence the functions of IM proteins. For example, various essential IM proteins are involved in peptidoglycan biosynthesis [76]. Since both the O-antigen and Lipid II contain an Und-PP activating group, accumulation of Und-PP O-antigen could compete with Lipid II inhibiting cell wall synthesis. Additional glycolipids could also influence the proper assembly of inner-membrane proteins by affecting the lipid environment in the IM. Simply by affecting IM bilayer packing, these accumulated glycolipids could also create physical defects in the IM and cause problems by dissipation of membrane potential. Here again, the presence of strain-specific mechanisms to relieve the buildup of O-antigen intermediates (or other secondary cell envelope polymers) would determine whether a given Gram-negative organism would be susceptible to LPS deletion.

Inhibition of LPS biosynthesis could trigger stress response pathways causing inhibition of growth

Inhibition of growth could result directly from the loss of LPS or from the cellular response to the loss – activation of an alternative genetic program in response to the stress of LPS deletion. It has been shown that the accumulation of mistargeted and/or misfolded outer membrane proteins in the periplasm is detected by a sensor protein, DegS, initiating a proteolytic cascade that results in activation of the σ^E -dependent envelope stress response system [77,78] (Figure 1D). The σ^E -transcription factor up-regulates both the expression of

genes involved in the targeting and assembly of OMPs, as well as for genes encoding proteases in order to clear misfolded substrates [79]. Literally hundreds of genes are turned on in order to restore the intracellular trafficking of OMPs. Because various OMPs require LPS to fold, inhibition of LPS biosynthesis could also cause misfolding of porins initiating the envelope stress response resulting in growth stasis until the intracellular levels of LPS can be restored [80–82]. A recent report has suggested that there are surveillance systems that directly detect mistargeted LPS in the periplasm of *E. coli*, and triggers the envelope stress response by activating σ^E (Figure 1D) [83]. The fact that bacteria respond to LPS and OMP defects or loss by activating a quality control mechanism further emphasizes the essentiality of LPS in maintaining OM integrity. In *E. coli* and *Salmonella*, a particularly stringent OM stress response has presumably evolved in order to allow these organisms to colonize the gut, where a high concentration of detergent-like molecules (*e.g.* bile salts) must be tolerated [84]. Hence, LPS may, in part, be required to prevent growth stasis triggered by stress response systems. Although relatively less is known about stress response systems in other Gram-negative organisms, it is conceivable that such OM quality control surveillance systems may not be as prominent as their counterparts in *E. coli* and *Salmonella* [35]. These bacteria may continue to grow and divide in an OM compromised state, whereas other strains would cease growing due to stress response signaling.

Conclusion

For several decades LPS was thought to be an essential structural component of the OM of Gram-negative bacteria just as amino acids are essential to the structures of proteins. However, with the discovery of LPS-deficient organisms it is now clear that the essentiality of LPS to Gram-negative bacteria is more complex. It seems reasonable that LPS was selected in Nature because when combined in an asymmetric bilayer with phospholipids it produces an unusual membrane that prevents the passage of toxic hydrophobic molecules into the cell. However, at this point, maintaining a proper LPS permeability barrier is tangential to its essentiality. Having become so heavily integrated into the cell envelope physiology, its removal may affect other metabolic processes through indirect means. Whether it is possible to delete LPS from a given organism will depend on the cellular context and perhaps even how it is removed. While LPS may be non-essential in some organisms, its loss is not inconsequential. Strains lacking LPS are less virulent [85] and much more susceptible to antibiotics that normally do not penetrate the OM [29,30,86]. It is still believed that compounds which interfere with the functions of LPS synthesis, transport, or assembly, will have the potential to function on their own as antibiotics as well as to potentiate the entry of other existing antibiotics normally excluded by the OM. In fact, a better understanding of the importance of LPS biogenesis on bacterial physiology could provide clues as to the specific vulnerabilities of a given Gram-negative pathogen to inhibition of LPS at different steps in synthesis and assembly.

Acknowledgments

This work was supported by the Blavatnik Biomedical Accelerator Fund and the National Institutes of Health (NIH) grant AI081059 to D.K.

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Highlights

- We discuss the cellular role of lipopolysaccharide within Gram-negative bacteria.
- We propose explanations for why lipopolysaccharide is essential in certain organisms.
- We consider implications for developing lipopolysaccharide-targeting antibiotics.

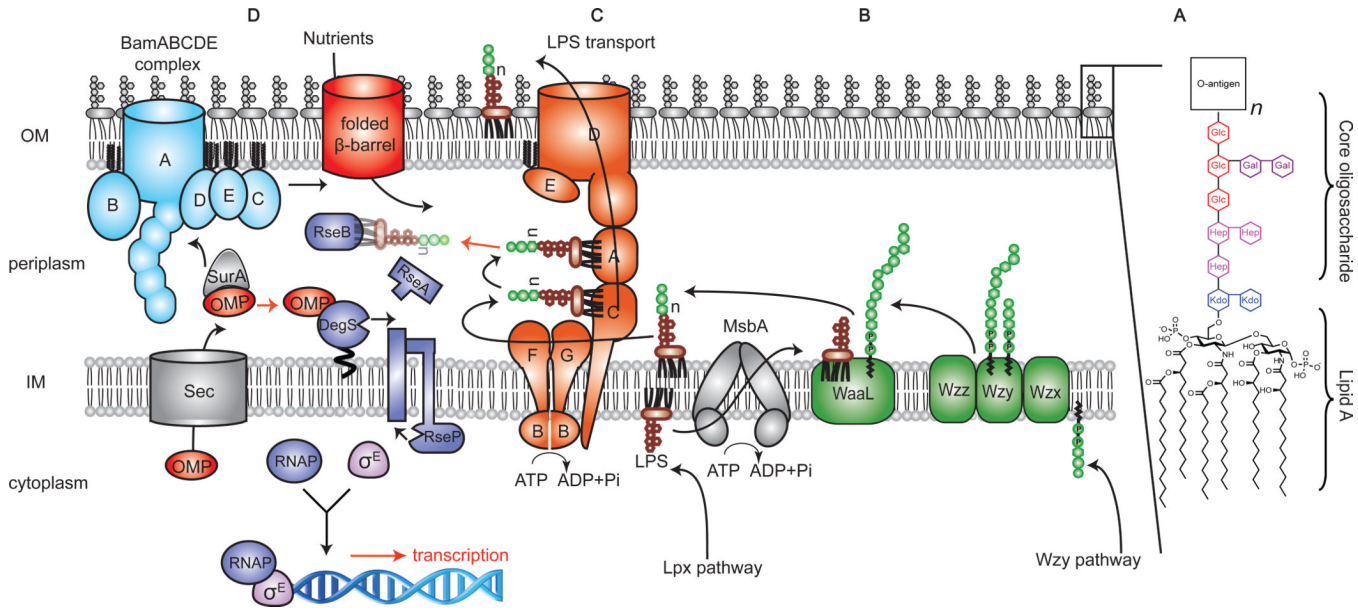

Figure 1.

Pathways that mediate outer membrane (OM) biogenesis and maintain OM integrity. (A) LPS is a complex glycolipid that consists of three regions: lipid A, a core oligosaccharide and an O-antigen polysaccharide. (B) O-antigen is assembled through the Wzy-dependent pathway. The O-antigen repeat unit is assembled in the cytoplasm and flipped to the periplasmic face of the IM by Wzx; O-antigen is polymerized by Wzy and the chain length is modulated by Wzz. (C) LPS is biosynthesized in the cytoplasm, flipped to the periplasmic face of the inner membrane (IM) and transported through the periplasm to the outer leaflet of the OM via the Lpt pathway. Ligation to O-antigen depends on the particular strain background. (D) Mislocalized or misfolded OMPs and LPS loss or defects initiate the σ^E envelope stress response pathway. LPS loss or defects affect porin assembly; misassembled porin binds to DegS, degrading RseA and initiating the σ^E stress response. LPS signal could directly bind to RseB, subjecting RseA to proteolysis by DegS to activate the σ^E stress response.