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Expanding the Paradigm for the Outer Membrane: Acinetobacter baumannii in the Absence of Endotoxin

Matthew Joseph Powers and

University of Georgia Infectious disease department, Athens, Georgia, United States

Dr M. Stephen Trent

University of Georgia, Infectious Diseases Department, Athens Georgia United States, strent@uga.edu

Summary

Asymmetry in the outer membrane has long defined the cell envelope of Gram-negative bacteria. This asymmetry, with lipopolysaccharide (LPS) or lipooligosaccharide (LOS) exclusively in the outer leaflet of the membrane, establishes an impermeable barrier that protects the cell from a number of stressors in the environment. Work done over the past 5 years has shown that *Acinetobacter baumannii* has the remarkable capability to survive with inactivated production of lipid A biosynthesis and the absence of LOS in its outer membrane. The implications of LOS-deficient *A. baumannii* are far-reaching – from impacts on cell envelope biogenesis and maintenance, bacterial physiology, antibiotic resistance, and virulence. This review examines recent work that has contributed to our understanding of LOS-deficiency and compares it to studies done on *Neisseria meningitidis* and *Moraxella catarrhalis*; the two other organisms with this capability.

Introduction

A defining characteristic of Gram-negative bacteria is the asymmetric outer membrane, with lipopolysaccharide (LPS) localized exclusively to the outer leaflet (Kamio and Nikaido, 1976; Funahara and Nikaido, 1980). This structure provides an intrinsic barrier against a number of noxious compounds and potential antibiotics in the environment – a barrier that is typically essential for survival and has been reviewed extensively (Nikaido, 2003; Needham and Trent, 2013; Whitfield and Trent, 2014; Henderson *et al.*, 2016). The core sugars and O-antigen of LPS are anchored to the membrane by the glycolipid lipid A, which is essential for cell survival and is a prerequisite for localization of the molecule to the cell surface (Okuda *et al.*, 2016). Until 2012, there were limited exceptions to this rule. Two species, *Neisseria meningitidis* and *Moraxella catarrhalis*, had been shown to survive in the absence of LOS (an analogous molecule lacking the O-antigen polymer) via a directed mutation in a lipid A biosynthetic gene (Steeghs *et al.*, 1998; Peng *et al.*, 2005). The ability for other *Neisseria* and *Moraxella* species to survive in the absence of LOS is most likely not

Corresponding author: Matthew Joseph Powers, powersmj@uga.edu.

conserved, as attempts to make similar mutations in *N. gonorrhoeae* failed to yield viable colonies (Bos and Tommassen, 2005).

In 2012, it was first reported that *Acinetobacter baumannii* could survive in the absence of lipid A as well (Moffatt *et al.*, 2010). This came at a time when *A. baumannii* was an emerging nosocomial epidemic and public health concern across the world; recently being placed as a critical priority pathogen for new antimicrobials by the World Health Organization (WHO | Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics, 2017). While the benefit of an asymmetric outer membrane relative to a standard phospholipid bilayer is apparent due to the impermeable barrier it provides, its lack of essentiality in multiple organisms provides an opportunity to understand mechanisms that are unique to maintenance of asymmetry versus a standard lipid bilayer. *A. baumannii* is the first organism to select for inactivated lipid A biosynthesis in the presence of polymyxins, a class of lipopeptide antibiotics that target LPS. With its clinical relevance combined with its tractability, non-fastidious growth, an expanding repertoire of genetic tools, and animal models, *A. baumannii* represents an important system for studying the outer membrane in the context of antimicrobial resistance and Gram-negative physiology.

The Outer Membrane Barrier

The impermeability of the outer membrane aids in resistance to various antimicrobials including antibiotics, toxic metabolites, antimicrobial peptides, and components of the innate immune response (Delcour, 2009). This impermeability is due in large part to the asymmetry of the outer membrane, with LPS molecules occupying the outer leaflet and glycerophospholipids in the inner leaflet. Intact LPS is a molecule comprised of three distinct moieties – the lipid A anchor, core oligosaccharide, and O-antigen (Raetz, 1990).

Lipid A is a hydrophobic glycolipid, for which the biosynthetic pathway is highly conserved and considered essential for Gram-negative bacteria (Figure 1) (Whitfield and Trent, 2014). Despite this conservation, variations occur downstream of lipid A biosynthesis by way of modifying enzymes that allow for the modification and adaptation of the lipid A species to particular niches increasing bacterial fitness. These modifying enzymes provide a valuable tool for establishing resistance to certain types of antibiotics and altering the permeability of the outer membrane (Raetz *et al.*, 2007).

The highly conserved lipid A moiety is a glucosamine-based phospholipid that is phosphorylated at the 1 and 4′ positions. While this structure is conserved, the acylation patterns vary in different species. *A. baumannii* produces hepta-acylated lipid A as their major lipid A species, in contrast to hexa-acylated lipid A in *Escherichia coli* (Figure 2) (Boll *et al.*, 2015a). The increased acylation of lipid A provides a higher degree of hydrophobicity relative to standard phospholipids in biological membranes. Lipid A serves as the anchor to two 3-deoxy-D-*manno*-oct-2-ulsonic acid (Kdo) residues, which along with an oligomer of sugars comprises the core region of LPS (Brabetz *et al.*, 1997). Lastly, a repeating O-antigen can be attached to the core oligosaccharide, generating an intact LPS structure. Certain genera, including *Acinetobacter*, lack the lengthy O-antigen

polysaccharide, but display an extended core-oligosaccharide. These endotoxin molecules are termed lipooligosaccharides (LOS) (Preston *et al.*, 1996) (Figure 1). The lipid A (Figure 2) and core moieties in *Acinetobacter* are phosphorylated to varying extents, generating an overall negative charge for the endotoxin molecule (Vinogradov *et al.*, 2002). Furthermore, divalent cation bridging between LPS molecules serves to fortify the membrane through balancing the electrostatic net (Nikaido and Vaara, 1985). This intact outer membrane provides an intrinsic barrier to many potentially toxic compounds, but the unique composition of the outer membrane also makes the cell susceptible to a particular class of antimicrobials – cationic antimicrobial peptides (CAMPS) (Olaitan *et al.*, 2014).

CAMPs, such as polymyxins, are highly effective against Gram-negative bacteria. This class of antibiotics includes two FDA-approved clinical antibiotics; polymyxin B and polymyxin E (colistin). These antibiotics, despite being discovered in 1949, remain the “last-resort” treatment for multi-drug resistant infections due to their high nephrotoxicity in humans (Falagas and Kasiakou, 2006). The amphipathic nature of these compounds with a positively charged cyclic group, makes them highly attracted to the anionic lipid A molecule on the bacterial surface (Velkov *et al.*, 2013). High concentrations of polymyxins displace the divalent cation bridging between LPS/LOS molecules and the acyl chain allows for pore formation and permeation of the outer membrane. Once in the periplasm, this pore formation occurs in the cytoplasmic membrane, causing membrane leakage and disruption of the proton motive force – effectively lethal to the cell (Hancock and Chapple, 1999).

Many bacterial species have modifying enzymes that can reduce the negative charge of LPS/LOS through modification of the lipid A phosphate groups with various amine-containing residues, such as phosphoethanolamine or cationic sugars (e.g. aminoarabinose and galactosamine) (Figure 2) (Trent *et al.*, 2001; Arroyo *et al.*, 2011; Pelletier *et al.*, 2013; Chin *et al.*, 2015). Additional modifications have been demonstrated on the inner core oligosaccharide, which would further reduce the negative charge of LPS/LOS (Kanipes *et al.*, 2001; Tamayo *et al.*, 2005). Remodeling of the bacteria cell surface results in resistance to polymyxins; however, the extent of resistance varies based on the organism and type of LPS/LOS modification.

Despite having modifying enzymes for decreasing polymyxin susceptibility, *A. baumannii* is the only species to date that employs the response of inactivating lipid A biosynthesis as an alternative mechanism of resistance.

Mechanisms of LOS-deficiency in *A. baumannii*

The lipid A biosynthetic pathway, except for certain late acyl-transferases (Fig. 1), is considered essential in Gram-negative bacteria. Mutants that are defective in key processes of lipid A biosynthesis or its transport are inviable with gross morphological changes and disrupted membrane biogenesis (Doerrler *et al.*, 2001; Reynolds and Raetz, 2009; Tomaras *et al.*, 2014; García-Quintanilla *et al.*, 2016; Zhang *et al.*, 2017).

The lipid A biosynthetic pathway consists of nine enzymes, the first seven of which are essential for lipid A biosynthesis and cell survival under standard laboratory conditions.

(Figure 1) (Reynolds and Raetz, 2009). The absence of these genes typically results in lethality. This was recently confirmed to be the case in *A. baumannii* through the inducible expression of two key lipid A biosynthesis genes. It was shown that in the absence of inducer, *lpxH* and *lpxK* mutants were inviable as expected. The use of LpxC inhibitor CHIR-090 (Barb *et al.*, 2007) restored growth, indicating that intermediates from the lipid A biosynthetic pathway, and not the loss of lipid A itself, were responsible for toxicity. This is consistent with the fact that these intermediates are amphipathic, detergent like compounds (Figure 1) (Richie *et al.*, 2016; Wei *et al.*, 2017). This work aligns itself with precedents established in *E. coli* (Garrett *et al.*, 1998; Babinski *et al.*, 2002).

Despite this, multiple groups have now reported that when grown on high concentrations of colistin (10 μg/mL which is ~20X higher than the average MIC for wild-type *A. baumannii*) colonies appear that are viable (Moffatt *et al.*, 2010; Boll *et al.*, 2016). The colonies lacked lipid A and LOS which is consistent with mutations in early genes of the lipid A biosynthetic pathway as determined by whole-genome sequencing (Boll *et al.*, 2016). These mutations vary by strain and laboratory; however, they all occur in one of the first three genes (*lpxA*, *lpxC*, or *lpxD*) of the lipid A biosynthetic pathway (Figure 1) (Moffatt *et al.*, 2010; Beceiro *et al.*, 2014; Boll *et al.*, 2016). *Acinetobacter* joins *N. meningitidis* and *M. catarrhalis* as the three Gram-negative species viable in the absence of lipid A, excluding the genus Spirochete which have distinctive outer membranes in a class of its own (Haake and Zückert, 2015). *N. meningitidis* was the first organism shown to survive without lipid A and LOS, which was demonstrated in 1998 when a viable *lpxA* mutant was generated *in vitro* (Steeghs *et al.*, 1998). Later, *Moraxella catarrhalis*, a close relative of the genus *Acinetobacter*, was also shown to be viable in the absence of LOS (Peng *et al.*, 2005). An interesting distinction is that both *Neisseria* and *Moraxella* became LOS-deficient through *in vitro* directed mutations. To date, *A. baumannii* is the only organism shown to accumulate lipid A biosynthesis mutants readily under significant outer membrane stress caused by polymyxin antibiotics. That these *lpx* mutations occur in the presence of high colistin suggests the process is determined stochastically rather than through a strictly regulated process. While it is possible these three genes are hot spots for mutations to occur, it is more likely that inactivation of downstream lipid A biosynthetic genes are inviable and drop out under selection.

Concurrent with this work is a study that explores the physiology of *A. baumannii* using a lipooligosaccharide transport mutant (*lptD*) in *A. baumannii* ATCC19606. Remarkably, the *lptD* mutant is viable despite its essentiality in other Gram-negative organisms. LptD is the terminal step in LOS translocation to the outer membrane, and in its absence the entirety of the *lpt* complex is nonfunctional if it forms at all (Okuda *et al.*, 2016). As such, it is interesting that an *lptD* mutant is viable in wild-type *A. baumannii*, as this would lead to an accumulation of intact LOS in the cytoplasm or potentially the periplasm depending on the functionality of the partial Lpt pathway. This work builds on studies that determined the *lpt* pathway is not essential in *Neisseria meningitidis* and the cell can tolerate internal accumulation of LOS (Bos *et al.*, 2004). The accumulation of LOS or lipid A should be lethal regardless of *A. baumannii's* (or any other bacterial species) ability to survive in its absence. Despite this, electron microscopy showed minimal disturbances to the cell. The authors report the *lptD* mutant having intact LOS, suggesting either the cells have a bypass

mechanism to get LOS to the outer membrane or *A. baumannii* is remarkably suited to tolerating internal accumulations of intact LOS. Either outcome would have a significant impact on the field, and warrants further study and examination of these mutants (Bojkovic *et al.*, 2015; Richie *et al.*, 2016; Wei *et al.*, 2017).

Impacts of LOS-deficiency on *A. baumannii* physiology

LOS-deficiency alters the physical and chemical characteristics of the Gram-negative cell envelope leading to effects on membrane biogenesis and physiology. The use of various -omics approaches has worked to decipher these changes *en masse.*

Gene Regulation

A. baumannii has a high degree of strain variation making broad generalizations difficult in the absence of multi-strain studies (Maifiah *et al.*, 2016; Boll *et al.*, 2016). A large-scale transcriptomics study on multiple, commonly used lab strains allowed for the first comparative analysis of transcriptional regulation in the absence of lipid A/LOS. In the four strains studied, 38 distinct gene clusters were up- or down-regulated in *A. baumannii* relative to their LOS-positive counterparts. Strikingly, only five of these were conserved across the strains tested (Boll *et al.*, 2016). These five groups are (1) the *lol* pathway for lipoprotein transport, (2) six lipoproteins, (3) multidrug efflux pumps, (4) the *baeRS* two component-system, and (5) the *mla* pathway for phospholipid transport. All of these are involved in cell envelope maintenance (Raffa and Raivio, 2002; Malinverni and Silhavy, 2009; Lin *et al.*, 2015; Yoon *et al.*, 2016; Grabowicz and Silhavy, 2017; Ekiert *et al.*, 2017).

Outer Membrane Maintenance: the *lol* and *mla* pathways

The *lol* and *mla* pathways are proposed mechanisms for anterograde lipoprotein and retrograde phospholipid transport, respectively (Malinverni and Silhavy, 2009; Konovalova and Silhavy, 2015). LOS-deficient *Acinetobacter* upregulate both of these pathways, although the effects on the outer membrane are unclear. In ATCC strain19606, a common type strain, there are anywhere from 5–25 upregulated lipoproteins upon loss of LOS (Henry *et al.*, 2012; Boll *et al.*, 2016). *Acinetobacter* sp. and *Escherichia coli* have between 80–100 putative lipoproteins in their genomes to provide context (Babu *et al.*, 2006). Some of these lipoproteins localize to the outer leaflet, but their function is unknown (Boll *et al.*, 2016). Increased abundance of lipoproteins in the outer leaflet could simply help occupy space in the outer membrane in addition to glycerophospholipids in the absence of LOS (Figure 3) and this is still under investigation in our laboratory.

The *mla* ABC-transport system is thought to remove phospholipids from the outer membrane to the inner membrane, actively maintaining asymmetry (Malinverni and Silhavy, 2009). In the absence of LOS, canonical asymmetry is no longer possible. The replacement of lipid A with glycerophospholipids (as no unique lipid species have been detected in LOS-deficient *A. baumannii* to date (Maifiah *et al.*, 2016; Boll *et al.*, 2016) in the outer leaflet most likely provides a constant stress signal to the cell leading to *mla* upregulation. Mislocalization of glycerophospholipids to the outer leaflet of the outer membrane also results in activation of the outer membrane phospholipase PldA and

glycerophosphospholipid degradation (Brok *et al.*, 1996; Langen *et al.*, 2001; Bishop, 2008). While these mechanisms are in place to protect asymmetry, in the absence of lipid A/LOS it is likely their functions are detrimental. Subsequent retrograde removal of phospholipids from the outer membrane would require equivalent anterograde transport of phospholipids to ensure a well-packed membrane. It important to note that the *mla* transport system lacks concrete biochemical evidence for its directionality, a topic under investigation by several laboratories.

Activation of cellular stress response mechanisms

The upregulation of efflux pumps and the *baeRS* two-component system are indicative of a cell under duress (Grabowicz and Silhavy, 2017). The BaeRS system is a conserved response system for cell envelope stress. In *E. coli*, it has a small regulon that includes key efflux pumps. While there is limited data as to the entire regulon of BaeRS in *A. baumannii*, it is reasonable to assume that the subsequent upregulation of efflux pumps suggests a similar function (Lin *et al.*, 2015). Efflux pump activity would be a logical first step at ameliorating the influx of toxic metabolites in the absence of asymmetry (Henry *et al.*, 2012; Boll *et al.*, 2016). Curiously, none of the other major cell envelope stress response systems were upregulated in *A. baumannii* – perhaps due to an expanded role of BaeRS in this species.

Constituting a Fortified Membrane in the Absence of Lipid A

Phospholipid alterations

It remains unclear what makes the outer membrane a functional barrier in the absence of LOS for *A. baumannii.* The overall composition of phospholipids does not appear to drastically change, although the phospholipid phenotype is strain-dependent. While several studies have analyzed multiple strains, the changes seen between LOS-positive and LOS-deficient cells are minor (Maifiah *et al.*, 2016; Boll *et al.*, 2016). In one case, the LOS-deficient strain synthesized phospholipids with shorter acyl-chain lengths, decreasing the overall hydrophobicity of the membrane (Maifiah *et al.*, 2016).

Mimicking LOS through display of surface carbohydrates

Carbohydrate modifications to outer membrane proteins have been shown to be integral to their function (Tytgat and de Vos, 2016). Many Gram-negative bacteria have the capability to glycosylate proteins; however, the mechanisms vary. The two forms of glycosylation, *N*-linked and *O*-linked, utilize completely different methods for generating and attaching glycans to target proteins. Generally, if a bacterial species contains a mechanism for protein glycosylation, it contains either the *N*-linked or *O*-linked system (Nothaft and Szymanski, 2010). A genomics study of *A. baumannii* reveals its capability for *O*-linked glycosylation (Iwashkiw *et al.*, 2012). This hints at the possibility for it to use protein glycosylation as an LOS-mimic in the absence of canonical endotoxin (Figure 3). Furthermore, while strains tend to produce only a single glycan, the glycan is promiscuous in its utility and can be used for glycosylation of proteins and capsule formation (Lees-Miller *et al.*, 2013).

A. baumannii secretes an abundance of the exopolysaccharide poly-N acetylglucosamine (PNAG) (Choi *et al.*, 2009). In certain strains, expression of key PNAG genes is upregulated in the absence of LOS (Henry *et al.*, 2012). The upregulation of exopolysaccharide in stressed cells has been previously documented (Ren *et al.*, 2016), and increased exopolysaccharide could provide a buffer to a compromised outer membrane. Additionally, the generation of outer membrane attached capsule could achieve a similar purpose (Figure 3). This case is best made in *N. meningitidis*, although there are conflicting data. The initial studies of LOS-deficient *N. meningitidis* suggested capsule was essential for survival in this condition (Steeghs *et al.*, 2001). However, the isolation of suppressor mutants in a capsule-deficient, LOS-deficient *N. meningitidis* suggests that capsular polysaccharide is not truly essential (Bos and Tommassen, 2005). The suppressor mutations and subsequent mechanism responsible for this viability remain undetermined.

LOS-deficiency; an in vitro phenomenon?

In light of evolution, an outer membrane complete with lipid A and LOS/LPS must be the most fit version of the Gram-negative cell envelope. With the existence of both Gram-negative and Gram-positive bacteria, there is the underlying question of which came first. A majority of bacterial phyla are diderms, containing an inner and outer membrane (Hug *et al.*, 2016). The monoderm bacteria are restricted largely to the *Firmicutes* and *Actinobacteria*, although key exceptions of diderm *Firmicutes* allow for an interesting comparative study on the evolution of the cell envelope. Antunes and co-workers (Antunes *et al.*, 2016) examined the diderms in the *Firmicutes* and found intact genes for lipid A biosynthesis, LPS transport, lipoprotein transport, and unique diderm flagellar genes. That these genes exist in the *Firmicutes* suggests that monoderm bacteria arose multiple times in evolutionary history through several independent events. It is curious to look at the loss of LOS in the context of a step towards transitioning from being a diderm to a monoderm. While mimicking millions of years of evolution in a test tube is entirely infeasible, the ability to inactivate lipid A biosynthesis could be a critical first step in eventually shedding an outer membrane. Remarkably, the fact that LOS-deficient *A. baumannii* maintains an outer membrane is a testament to the mechanisms in place to safeguard asymmetry and an outer membrane as a barrier.

The ability to survive in the absence of lipid A and LOS is not necessarily conserved among a given genus or even species. When a number of *A. baumannii* isolates were tested for LOS-deficiency, only about 50% were capable of surviving in the absence of LOS: a fact that highlights the immense lack of knowledge in terms of signals and physiological prerequisites for inactivating lipid A biosynthesis (Boll *et al.*, 2016). In one particular case, this inability was linked to the expression of PBP1a, which is a penicillin-binding protein involved in peptidoglycan synthesis and maintenance (Boll *et al.*, 2016). There is no clear evidence based on sequence similarity of *A. baumannii* genomes for why particular strains have high or low levels of PBP1A (unpublished results, Trent Lab).

A. baumannii contains two bifunctional penicillin-binding proteins, PBP1a and PBP1b, that are capable of both transglycosylase and transpeptidase activity (Cayô *et al.*, 2011). In *E. coli*, these proteins function in different capacities despite performing identical chemistry

and loss of both is synthetically lethal (Typas *et al.*, 2010). The expression and localization has not been explored in *A. baumannii.* The current dogma in *E. coli* suggests that outer membrane lipoproteins regulate the activity and localization of PBP1a and PBP1b, yet a lack of any obvious homologs for regulatory lipoproteins suggests their mechanisms of maintenance and activity are unique from *E. coli* (Lupoli *et al.*, 2014). Potentially, PBP1a-mediated cell elongation is fatal in LOS-deficient cells due to a compromised membrane and an increase in overall osmotic and turgor stress that results from it. This is supported in part by a small group of studies that examined cell morphology of LOS-deficient cells; however, more in-depth analysis of cell shape morphology with high-resolution techniques will bolster these claims (Soon *et al.*, 2011; Soon *et al.*, 2012). This link between cell shape and LOS-deficiency warrants further exploration and study.

LOS-deficiency in the Clinic

That *A. baumannii* can survive in the absence of LOS *in vitro* is an interesting phenotype in and of itself; however, its impact on physiology suggests it is largely deleterious to its virulence. LOS-deficient *A. baumannii* fail to activate toll-like receptor 4 (TLR4), the main TLR that detects endotoxin in eukaryotic hosts (Kim *et al.*, 2013; Moffatt *et al.*, 2013; Boll *et al.*, 2016). LOS-deficient *N. meningitidis* and *A. baumannii* both have significant *in vitro* growth defects (Bos and Tommassen, 2005; Beceiro *et al.*, 2014). Although a direct comparison is inappropriate due to difficulty in monitoring growth rate *in vivo*, LOS-deficient *A. baumannii* exhibit reduced fitness in both *C. elegans* and a mouse infection model (Beceiro *et al.*, 2014). Furthermore, inhibition of lipid A biosynthesis through a large concentration of LpxC inhibitors *in vivo* inhibited lethal infection from *A. baumannii* due to its inability to shed LPS molecules and activate TLR4. The inhibitor used, LpxC-1, had no *in vitro* activity against *A. baumannii*, although it is undetermined whether LpxC-1 treated *A. baumannii* were truly LOS-deficient in this study or if the compound is simply ineffective against *A. baumannii* LpxC. (Lin *et al.*, 2012; Tomaras *et al.*, 2014). Additionally, inhibition of canonical virulence factors including type-VI secretion factors and surface-based pili likely contribute to reduced virulence *in vivo* (Henry *et al.*, 2012).

While LOS-deficient *A. baumannii* can be readily isolated *in vitro*, its isolation in the clinic is overwhelmingly rare. The lack of published clinical cases of LOS-deficient *A. baumannii* is presumably due to lipid A modification mechanisms (Fig. 2) that allow for survival in the presence of clinical levels of colistin and polymyxin B without a significant growth defect (Lesho *et al.*, 2013). A transcriptomic study of eight patients infected with *A. baumannii* yielded a number of clinical isolates with single nucleotide polymorphisms increasing transcription of *pmrAB* that encodes for a two-component regulatory system controlling lipid A modifications (Kröger *et al.*, 2016). The same study identified nonsynonymous mutations in a lipopolysaccharide transport gene (*lptF*) and lipid A transport gene (*msbA*). The *in vitro* resistance to colistin of these clinical isolates was only 0.5 μg/mL. Thus, these strains would be considered highly susceptible to the antibiotic and would lack lipid A modifications consistent with colistin resistance (> 2 μg/mL). This makes it likely that host-induced factors lead to active remodeling of the outer membrane and this phenotype was not stable *in vitro* (Wright *et al.*, 2017). That the expected phenotype was not detected *in vitro* suggests these mutations might be an artifact of next-generation sequencing, and is

consistent with similar findings in the Trent Lab (unpublished results, Trent Lab). In contrast, virulent LOS-deficient *N. meningitidis* have been isolated, albeit infrequently, in clinical settings. Interestingly, this isolate contained a mutation in *lpxH*, which is further downstream than any viable isolate generated *in vitro* (Piet *et al.*, 2014).

The clinical threshold for colistin and polymyxin B concentrations *in vivo* is low due to its nephrotoxicity in humans. The delivery of colistin via a convertible pro-drug means that its accumulation to target concentrations in the body is slow in contrast to polymyxin B which is delivered as the active drug (Nation *et al.*, 2014). While the dosing regimen in the clinic appears to have no effect on the efficacy of the antibiotic, *A. baumannii* was capable of developing resistance in a stable (no reversion of resistance) and unstable (reversion of resistance) fashion (Cheah *et al.*, 2016). Whether *A. baumannii* inactivates LOS/lipid A in the host remains to be determined. The low isolation frequency of LOS-deficient cells from the host environment suggests that this phenomenon has an unknown impact on virulence that needs to be further explored. However, with limited knowledge of the natural reservoirs of *A. baumannii*, it is possible this endotoxin-free, non-immunogenic state could be important for bacterial persistence.

Conclusion

Over the past five years, significant progress has elucidated a number of physiological changes that occur in Gram-negative cells that lack lipid A and LOS in their outer membrane. This organism represents a new frontier for understanding membrane biology, and represents a continued shift in the paradigm of LPS-essentiality for bacterial membranes. Despite this progress, there are serious gaps in our knowledge of LOS-deficient membranes, its regulation, and mechanisms for membrane fortification in *A. baumannii.*

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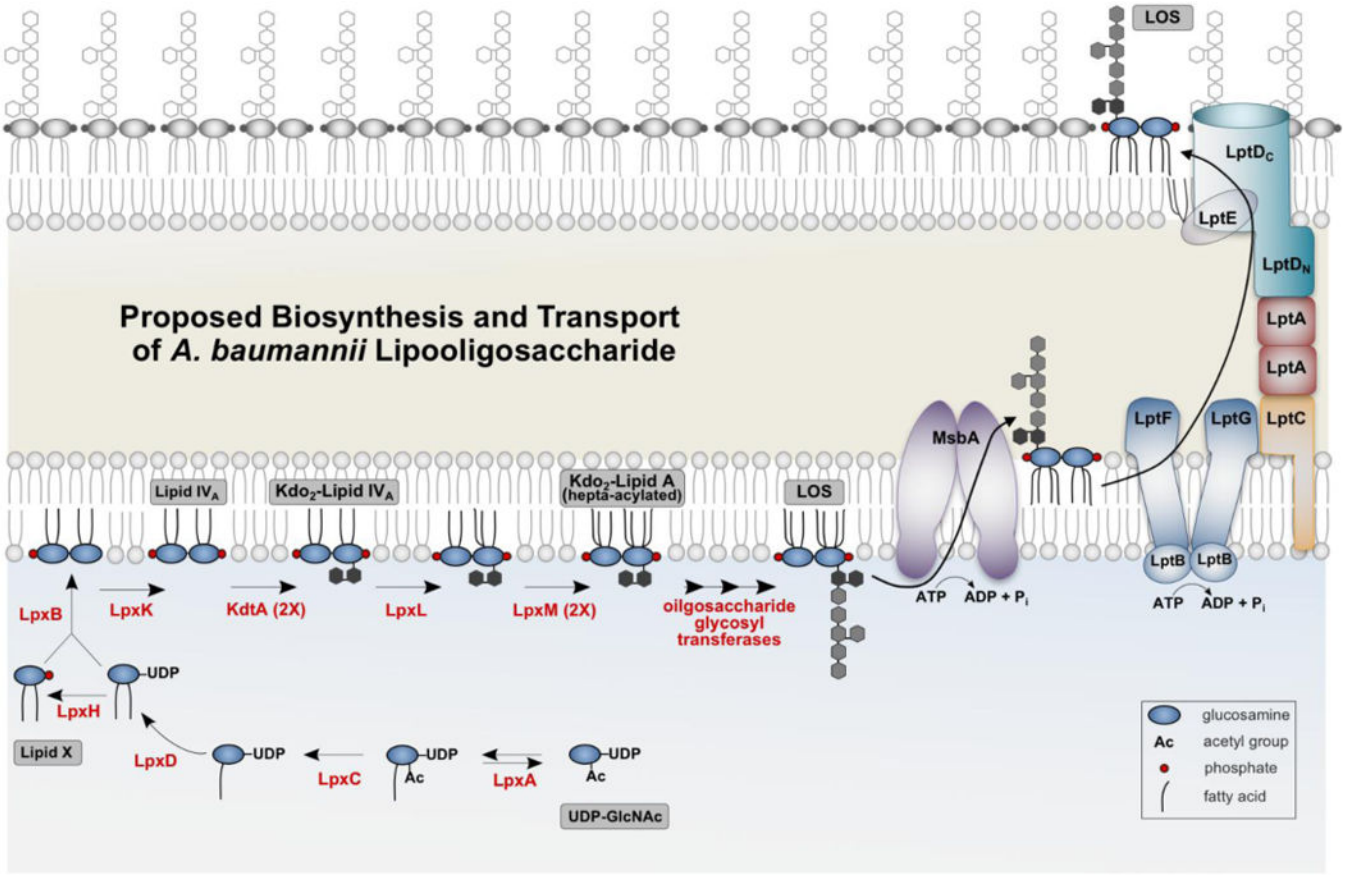

Fig. 1. Proposed biosynthesis and transport of *A. baumannii* lipooligosaccharide (LOS)

Typically, the Kdo_2-lipid A domain of LPS or LOS is required for growth of gram-negative bacteria. The *A. baumannii* genome contains homologs of the necessary Lpx enzymes to synthesize the Kdo_2-lipid A substructure similar to that of *E. coli*, although the *Acinetobacter* enzymes have not been fully characterized. Each step of Kdo_2-lipid synthesis requires a single enzyme (red) with the early steps of the pathway catalyzed by soluble enzymes and the latter steps catalyzed by membrane associated proteins. Each acyl transferase has a preferred acyl chain specificity that is dictated by an active site hydrocarbon ruler and acyl-ACP (acyl carrier protein) serves as the acyl donor. Although the Kdo sugars are part of the inner core-oligosaccharide, in some organisms Kdo addition is required for lipid A biosynthesis as the final two steps, catalyzed by LpxL and LpxM, require the presence of covalently attached Kdo. In *E. coli*, both LpxL and LpxM catalyze the addition of a single acyl chain producing the final hexa-acylated lipid A species. However, *A. baumannii* LpxM catalyzes the addition of two acyl chains resulting in an hepta-acylated lipid A structure (Boll *et al.*, 2015). The remaining sugars of the oligosaccharide of LOS are extended on the cytoplasmic leaflet of the inner membrane prior to transport by the ABC transporter MsbA that flips *A. baumannii* LOS to the periplasmic face of the inner membrane. Although absent in *Acinetobacter*, for organisms with a complete LPS structure, the O-antigen domain is added on the periplasmic face of the inner membrane. Finally, the intermembrane transport of LOS is performed by the Lpt (LPS transport) proteins that comprise an envelope-spanning translocation machine.

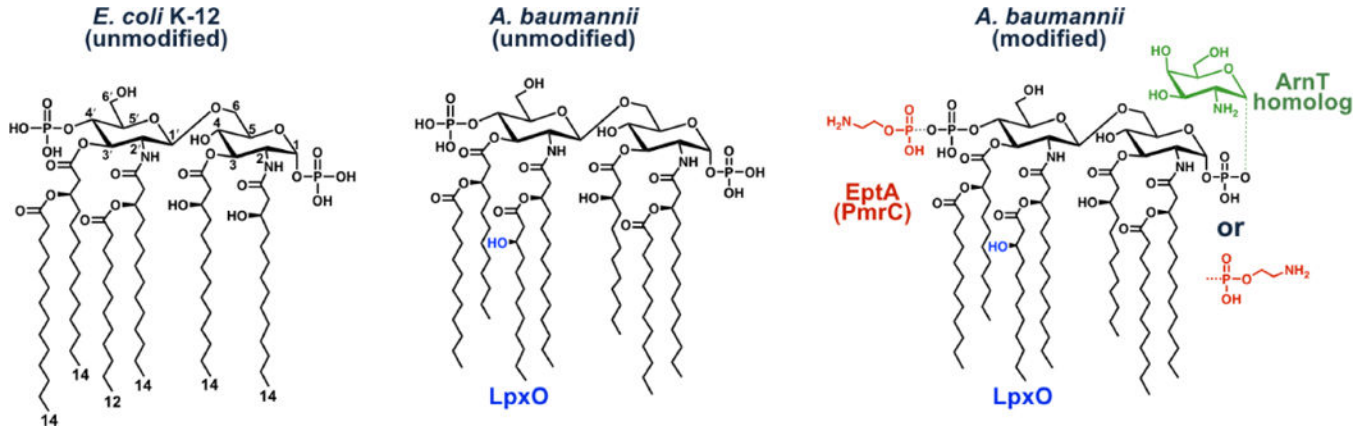

Fig. 2. Comparison of lipid A structures of *E. coli* and *A. baumannii*

The predominant lipid A species of *E. coli* K-12 is hexa-acylated and phosphorylated at the 1- and 4′-positions. *A. baumannii* is similar to that of *E. coli* except an additional acyl chain is found in an acyloxyacyl-linkage at the 2-position arising from a second acylation event catalyzed by LpxM (Figure 1). Wildtype *A. baumannii* lipid A also contains an additional hydroxyl group on the 2′-linked secondary acyl chain that likely arises from the action of an LpxO homolog found in the A. *baumannii* genome (Boll *et al.*, 2015). In colistin resistant *A. baumannii* that maintain LOS, the lipid A phosphate groups can be modified with galactosamine and with phosphoethanolamine residues. Phosphoethanolamine modification can occur at both phosphates (Arroyo *et al.*, 2011), whereas only single modified species of galactosamine have been reported (Pelletier *et al.*, 2013) thus far. EptA (PmrC) catalyzes the addition of phosphoethanolamine in *A. baumannii* (Arroyo *et al.*, 2011) and galactosamine addition is likely catalyzed by an *A. baumannii* ArnT homolog. In *Salmonella* and *E. coli*, ArnT transfers the sugar L-4-aminoarabinose to lipid A phosphate groups (Needham and Trent, 2013).

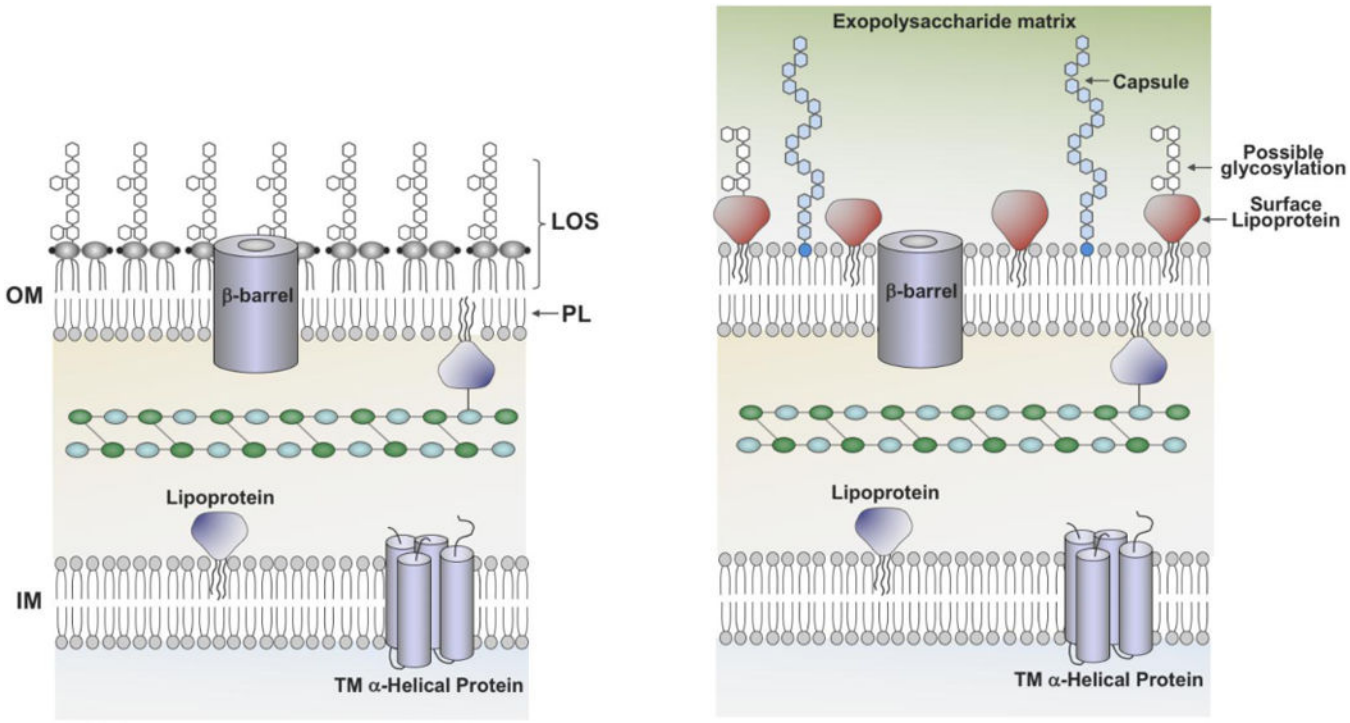

Fig. 3. Potential changes in cell-surface architecture during LOS/lipid A deficiency

Left: A wild-type Gram-negative cell envelope with typical asymmetry in the outer membrane. *Right*: A depiction of putative mechanisms for altering the cell envelope and fortifying the membrane in the absence of asymmetry. Lipoproteins exposed to the surface (Boll *et al.*, 2016) could play a critical role in filling space in a more porous outer leaflet. The capacity for *A. baumannii* to glycosylate these lipoproteins hints at a potential mechanism for mimicking LOS. The generation of capsular polysaccharide and exopolysaccharide provide an additional level of protection that could impede the influx of noxious compounds and buffer the LOS-deficient cell from the environment.