Investigations of the early stages of transport by the transenvelope lipopolysaccharide transporter in *E. coli*

Dissertation

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Blake Robert Bertani

Graduate Program in Microbiology

The Ohio State University

2019

Dissertation Committee

Natividad Ruiz, Ph.D., Advisor

Irina Artsimovitch, Ph.D.

Ross Dalbey, Ph.D.

Patrice Hamel, Ph.D.
Abstract

The cell envelope of bacteria mediates their interaction with the outside world and determines what can enter the cell. Gram-negative bacteria have a cell envelope defined by two membranes: an inner membrane, which surrounds the cytoplasm, and an outer membrane, which together with the inner membrane delimits an additional cellular compartment termed the periplasm. The inner membrane of Gram-negative bacteria is primarily composed of a phospholipid bilayer. The outer membrane, in contrast, contains phospholipids in its inner leaflet, and the essential glycolipid lipopolysaccharide (LPS) in the outer leaflet. The presence of LPS in the outer leaflet renders the outer membrane relatively impermeable, and therefore grants the cell resistance to noxious compounds in the environment, such as antibiotics. LPS is synthesized in the cytoplasmic face of the inner membrane, though it can also undergo non-stoichiometric modifications in the periplasmic face, and must thereafter be transported across the rest of the cell envelope. Transversal of the inner membrane by LPS is mediated by the ATP-binding cassette transporter MsbA. LPS extraction from the inner membrane, and subsequent transport across the rest of the cell envelope, is mediated by the LPS transport, or Lpt, complex. The Lpt complex is composed of eight proteins: a dimer of LptB in the cytoplasm that binds and hydrolyzes ATP
to drive LPS transport; two transmembrane domains, LptF and LptG, which form a cavity in the inner membrane that accepts LPS and extracts it; LptC, LptA, and LptD, which form a bridge across the periplasm to allow the hydrophobic portion of LPS to traverse the aqueous periplasm; and LptE, which in conjunction with LptD facilitates the transport of LPS across the outer membrane.

Here, we describe work in which we dissect the molecular mechanisms by which the Lpt system’s inner membrane complex, LptB2FGC, interacts with LPS. In chapter two, we describe the identification of a residue within LptG, K34, which is critical for LPS transport. Through structure-function and suppressor analyses, we show K34 of LptG mediates early contact with unmodified LPS as it enters the cavity formed by LptF and LptG. Further, our results imply that modified LPS interacts with the transporter distinctly from unmodified LPS.

Chapter three describes the structure of the LptB2FGC inner membrane complex, as determined by X-ray crystallography, and defines the path LPS takes to the periplasmic bridge of the Lpt system through site-specific crosslinking. We show that the transmembrane anchor of LptC intercalates between transmembrane domains LptF and LptG and is ergo likely to be functionally involved in transport. Further, the periplasmic bridge is preferentially coupled to LptF, rather than LptG.

In chapter four, we utilized a mutant producing LptG altered at residue K34 to perform suppressor analysis and further elucidate how the LptB2FGC complex interacts with LPS. We found that increased LPS production could
suppress the defects produced by this variant, but not those produced by other altered \textit{lpt} alleles. We therefore postulate that this suppression could be used as a probe of what stages of LPS transport are altered by a given \textit{lpt} allele, or Lpt system inhibitor. Finally, we found that partial-loss-of-function \textit{lpt} alleles suppressed otherwise lethal overproduction of LPS, which we believe supports a model in which LPS is essential to balance the rate at which material is added to the leaflets of the outer membrane.
Acknowledgments

I’ve heard it said that no man is an island, and that’s certainly been true for me during my graduate school career. A large number of people have supported me throughout this endeavor and made it possible. Thus, I’d like to take this opportunity to express to them my gratitude. In no particular order, I’d like to thank my mentor Natividad Ruiz, for her exceptional patience, insight, and when called for, constructive criticism. My advisory committee, for feedback and helpful suggestions, and for bearing with me when I tried to articulate to them my latest ideas. The members of the Ruiz lab, past and present: Emily Butler, for helping me get started in the lab alongside Brent Simpson, who has been both a good co-worker and good friend to me in the last several years; Emily Lundstedt, or “Emily II,” as she has affectionately been dubbed, for being there to bounce ideas off or just listen to me gripe; Sujeet Kumar, for incessant trolling and being trolled in kind (and occasionally actually talking science); Andrew Wilson, for talking shop, and keeping the office stocked with coffee; and finally, Becky Davis, for keeping the ship afloat. I would also like to thank Ao Mei and Giancarlo Casillas-Vargas, undergraduates mentees of mine who both contributed data and strains to my projects, and, I hope, taught me to be a better mentor. My entering class cohort, for helping get me through that first year, and being around to
commiserate in the years after. Last, I would like to thank my friends and family, for helping me cling to sanity during the most stressful portions of my graduate career. I never could have made it this far without the support of everyone mentioned here, and likely at least a few who weren’t (sorry to you neglected folks), and so for helping me get this far, you have my thanks.

-Blake R. Bertani
Vita

2014 ...................................................... B.S. Microbiology, The Ohio State University

2014-2015 ............................................... Graduate Fellow, Department of Microbiology, The Ohio State University

2015-Present .......................................... Graduate Research and Teaching Associate, Department of Microbiology, The Ohio State University

Publications


Fields of Study

Major Field: Microbiology
# Table of Contents

Abstract .................................................................................................................................................. ii  
Acknowledgments ................................................................................................................................ v  
Vita ......................................................................................................................................................... vii  
List of Tables .......................................................................................................................................... xii  
List of Figures .......................................................................................................................................... xiii  
Chapter 1: Background .......................................................................................................................... 1  
  Foreword ............................................................................................................................................... 1  
  1.1 Introduction to the Gram-negative cell envelope and lipopolysaccharides .................................. 1  
  1.2 Structure and function of lipopolysaccharides .............................................................................. 3  
    1.2.1 Main features of the structure of LPS ...................................................................................... 3  
    1.2.2 The function of LPS ................................................................................................................ 5  
  1.3 The lipopolysaccharide biosynthesis pathway ............................................................................. 8  
    1.3.1 Kdo2-lipid A biosynthesis: the Raetz pathway ...................................................................... 8  
    1.3.2 Biosynthesis of the core oligosaccharide ................................................................................. 12  
    1.3.3 Biosynthesis of the O antigen ................................................................................................. 16  
  1.4 Regulation of lipopolysaccharide biosynthesis ........................................................................... 20  
  1.5 Modification of lipopolysaccharide structure .............................................................................. 21  
    1.5.1 Modifications of lipid A .......................................................................................................... 22  
    1.5.2 Modifications to the core oligosaccharide .............................................................................. 26  
    1.5.3 O-antigen modification .......................................................................................................... 28  
  1.6 Lipopolysaccharide transport ........................................................................................................ 29  
    1.6.1 Crossing the IM: MsbA ........................................................................................................... 30  
    1.6.2 From the IM to the OM: the Lpt system ................................................................................. 32  
Chapter 2: A cluster of residues in the lipopolysaccharide exporter that selects substrate variants for transport to the outer membrane ................................................................. 36
List of Tables

Table A.1 Sensitivity to antibiotics of lptG mutants and their basSc derivatives .......................................................... 136
Table A.2 Suppression of lptG(K34D) by basSc is dependent on EptA and ArnT .......................................................................................................................... 138
Table A.3 Specificity of suppression by basSc ............................................................................................................. 139
Table A.4 Strains used in chapter 2 ......................................................................................................................... 140
Table A.5 Primers used in chapter 2 ......................................................................................................................... 142
Table C.1 X-ray data collection and structure refinement ......................................................................................... 178
Table C.2 Primers used to generate plasmids and strains in this study ................................................................. 179
Table C.3 Strains used in this study ......................................................................................................................... 181
Table D.1: Elevated LPS levels suppress lptG(K34D) outer-membrane permeability defects ............................. 183
Table D.2: Suppression byftsH(F37S) is lpt allele specific ......................................................................................... 184
Table D.3 Strains used in Chapter 4 ......................................................................................................................... 185
Table D.4: Primers used in Chapter 4 ......................................................................................................................... 186
List of Figures

Figure 1.1 Architecture of the Gram-negative cell envelope ............................................... 4
Figure 1.2 Lipid A biosynthesis pathway. ............................................................................. 9
Figure 1.3 Structure and biosynthetic enzymes of the *E. coli* K-12 core
oligosaccharide .................................................................................................................. 13
Figure 1.4 Structure of various core oligosaccharides ....................................................... 14
Figure 1.5 Summary of different O-antigen synthesis pathways ....................................... 18
Figure 1.6 Modifications to the structure of lipid A ............................................................ 22
Figure 1.7 Modifications to the structure of the core oligosaccharide ..................................... 26
Figure 1.8 Transport of LPS across the cell envelope .......................................................... 30
Figure 2.1 PEZ model of LPS transport by the Lpt system .................................................. 39
Figure 2.2 Residues in the K34 region of LptG are important for LPS transport. ................. 42
Figure 2.3 Correlation between residue in the cavity of LptG and LPS structure ............... 44
Figure 2.4 Charge requirement in cavity-facing residues in LptG for proper LPS transport .......................................................... 49
Figure 2.5 BasSR-dependent activation of modification of LPS structure by EptA and ArnT suppresses LPS transport defects in *lptG(K34D)* mutants .................................................. 55
Figure 2.6 Activation of LPS modification by *basSc* specifically suppresses defects in the K34-K41 domain of LptG .......................................................... 57
Figure 3.1 Crystal structure of the inner-membrane complex of the LPS transport machine .......................................................................................................................... 73
Figure 3.2 LptC promotes the efficient transport of LPS to LptA ......................................... 76
Figure 3.3 LPS entry into the cavity of the transporter is ATP-independent but extraction out of the membrane and onto the bridge requires ATP .............................................. 79
Figure 3.4 Unidirectional LPS transport is promoted by a gate in the β-jellyroll of LptF ................................................................................................................................. 81
Figure 4.1: Model of the Gram-negative cell envelope and lipopolysaccharide transport system .......................................................................................................................... 88
Figure 4.2: Suppression of *lptG(K34D)* outer membrane permeability defects by *ftsH(F37S)* .......................................................................................................................... 90
Figure 4.3: *ftsH(F37S)* is a partial-loss-of-function allele .................................................... 92
Figure 4.4: Elevated LPS levels suppress *lptG(K34D)* outer-membrane permeability defects .......................................................................................................................... 93
Figure 4.5 Suppression by *ftsH(F37S)* is *lpt* allele specific ............................................. 95
Figure 4.6: Model for LPS essentiality: ................................................................................ 101
Figure A.1: Protein and LPS levels of LptG variants ............................................................ 143
Figure A.2 Activation of BasS changes lipid A profile ......................................................... 144
Figure B.1 Purification and reconstitution of wild-type and inactive complexes of *V. cholerae* and *E. cloacae* LptB\(_2\)FGC. ......................................................... 147
Figure B.2 Structures *V. cholerae* LptB(E163Q)\(_2\)FGC and *E. cloacae* LptB\(_2\)FGC with their electron density maps. ................................................................. 149
Figure B.3 Alignments of *E. coli*, *E. cloacae*, and *V. cholerae* LptB, LptF, LptG, LptC and LptA. ........................................................................ 151
Figure B.4 LptC(ΔTM) stably associates with LptB\(_2\)FG, and facilitates LPS release to LptA as well as full-length LptC without altering the ATPase activity of LptB\(_2\)FG. ................................................................. 153
Figure B.5 Comparison of previously published LptB\(_2\)FG structures to the structure of *V. cholerae* LptB\(_2\)FGC......................................................... 155
Figure B.6 LPS enters the cavity of LptB\(_2\)FGC via LptG TM1, LptF TM5 and the LptC transmembrane helix in a nucleotide-independent manner........................ 157
Figure B.7 *in vivo* photocrosslinking experiments show that LPS passes through the β-jellyroll domains of LptF and LptC, but not LptG. ........................................ 159
Figure B.8 Comparison of the *V. cholerae* and *E. cloacae* structures reveals two states of the gate in LptF. ................................................................. 160
Figure B.9 Complementation and purification of LptF cysteine mutants.......... 162
Figure B.10 Examples of small molecules bound in the crystal structures of *V. cholerae* and *E. cloacae* LptB\(_2\)FGC, and unmodelled electron density in the *V. cholerae* LptB(E163Q)\(_2\)FGC structure......................................................... 164
Figure C.1 Uncropped gels and blots for data shown in main-text and extended data figures................................................................. 166
Chapter 1: Background

Foreword

This section was adapted from the review article *Function and biogenesis of lipopolysaccharides*, for which I was the primary author, published in the journal *EcoSal Plus* in 2018 under Domain 2: Cell Architecture and Growth. Specifically, section 1.1 corresponds to the introduction section, section 1.2 corresponds to the Structure and Function of LPS section, section 1.3 corresponds to the LPS Synthesis Pathway section, section 1.4 corresponds to the Regulation of LPS Biosynthesis section, section 1.5 corresponds to the Modification of LPS Structure section, and section 1.6 corresponds to the LPS Transport section. The original concluding remarks from the publication have been excluded.

1.1 Introduction to the Gram-negative cell envelope and lipopolysaccharides

Gram-negative bacteria are characterized by an envelope that contains two membranes: an inner membrane (IM) that surrounds cytoplasmic components, and an outer membrane (OM) that separates the cell from its environment. These two membranes surround an aqueous cellular compartment termed the periplasm, which contains the peptidoglycan cell wall (Fig. 1.1A) (1). Thus, in Gram-negative bacteria, the OM serves as the first line of defense against environmental threats.
Notably, in contrast to many biological membranes, the OM of most Gram-negative bacteria is not a phospholipid bilayer. Instead, it is a highly asymmetric bilayer that contains phospholipids in the inner leaflet and lipopolysaccharide (LPS) molecules in the outer leaflet (1-3). The glycolipid LPS is the focus of this chapter.

LPS performs several functions in Gram-negative bacteria. The most fundamental function of LPS is to serve as a major structural component of the OM. Perhaps not surprisingly, LPS is an essential component of the cell envelope in most, though interestingly not all, Gram-negative bacteria (4). In addition, LPS molecules transform the OM into an effective permeability barrier against small, hydrophobic molecules that can otherwise cross phospholipid bilayers, making Gram-negative bacteria innately resistant to many antimicrobial compounds (5, 6). LPS can also play a crucial role in bacteria-host interactions by modulating responses by the host immune system.

There are three main areas of LPS biology that are covered in this chapter. We will first discuss the overall structure of LPS and its function from the bacterial and human point of view. We will then review LPS biosynthesis in *Escherichia coli* and *Salmonella*, and discuss how bacteria can regulate LPS synthesis and modify its chemical structure in response to environmental stressors. Lastly, in the 1970s, Mary Jane Osborn and collaborators posed a question that has dominated a great part of LPS biogenesis research in the last two decades: since LPS is synthesized in the IM but displayed at the cell surface, how is it transported across the cell envelope (7, 8)? Here, we will summarize the work that has uncovered a novel
inter-membrane transport system that solves the challenges of shuttling this complex glycolipid across diverse cellular compartments.

1.2 Structure and function of lipopolysaccharides

1.2.1 Main features of the structure of LPS

LPS is a large glycolipid composed of three structural domains: lipid A, the core oligosaccharide, and the O antigen (Fig. 1.1B) (9). Lipid A, the hydrophobic portion of the molecule, is an acylated β-1′-6-linked glucosamine disaccharide that forms the outer leaflet of the OM (9). In *E. coli* and *Salmonella*, the glucosamines are phosphorylated at the 1 and 4′ positions and acylated at the 2, 3, 2′ and 3′ positions (9). Two additional secondary acyl chains are also typically present in the distal glucosamine so that mature lipid A is mostly hexa-acylated (9). The core oligosaccharide is a non-repeating oligosaccharide that is linked to the glucosamines of lipid A (9, 10). The core structure usually contains 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) residues, heptoses, and various hexoses, which can be modified with phosphates and other substituents such as phosphoethanolamine (9-12). The O antigen is an extended polysaccharide that is attached to the core oligosaccharide. It is composed of a repeating oligosaccharide made of two to eight sugars (13-15).

The overall structure of LPS is conserved, but there are many variations that can occur at the species and strain level (9-12, 16, 17). Similarly, the lipid A structure is conserved at the species level; however, as described below, it can
undergo regulated modifications in response to environmental conditions (12, 18-20). The core oligosaccharides vary among species and even between some strains of one species (9-12). However, the most diverse component of LPS is the O antigen (13, 17). Not only can the structure and composition of the O antigen differ within a species at the strain level, but, in addition, some Gram-negative bacteria do not synthesize this component of LPS (13, 21). In such cases, molecules composed of only lipid A and the core oligosaccharide are typically referred to as lipooligosaccharides, or LOS (9). Classically, LOS has been referred to as “rough” LPS, as opposed to “smooth” LPS, which includes the O antigen (9).

Figure 1.1 Architecture of the Gram-negative cell envelope.

A) Depiction of the Gram-negative cell envelope and its components. The inner membrane (IM) contains phospholipids, while the outer membrane (OM) contains
phospholipids in the inner leaflet and lipopolysaccharide (LPS) in the outer leaflet. B) Structure of prototypical LPS produced by *E. coli* (shown is the core structure associated with core type K-12).

1.2.2 The function of LPS

While the structure of LPS (or LOS) may vary among bacteria, in all cases this glycolipid populates much of the cell surface and establishes a permeability barrier that protects the cell from the entry of toxic molecules such as antibiotics and bile salts (5, 22). Additionally, because LPS is the primary bacterial component encountered by the host immune system, LPS often plays a major role in bacterial pathogenicity (20, 23).

The barrier function of LPS stems in part from its strong amphipathic nature. As in other lipid bilayers, the acyl portion of lipid A provides hydrophobic character that inhibits the passage of hydrophilic molecules through the OM. However, in contrast to other bilayers, the core oligosaccharide and O antigen additionally provide extensive hydrophilic character to LPS that makes the OM particularly impermeable to hydrophobic compounds as well (5, 22). The effectiveness of the barrier posed by LPS is also heavily reliant on the ability of LPS to pack densely within the outer leaflet of the OM. This dense packing is mediated in part by hydrophobicity-driven association of the acyl chains of lipid A. As lipid A molecules typically bear a large number (that is, 4-7) of saturated fatty acid moieties, the extensive interactions between these acyl chains result in low
fluidity within the membrane bilayer (5). However, packing of LPS is complicated by the presence of negatively charged phosphate groups throughout its structure. Most salient and conserved are the phosphates of the 1 and 4′ positions of the glucosamines in lipid A, which lie at the exterior surface of the OM, but phosphates can also be found in the core oligosaccharide (9, 12). To prevent repulsion between these negatively charged phosphates, divalent cations such as Mg^{2+} intercalate between LPS molecules, forming polyionic interactions that greatly enhance LPS packing and, consequently, promote the barrier function of the OM (5, 22).

As LPS decorates the surface of many bacterial pathogens, the host immune system has evolved to respond dramatically to its presence, making LPS a PAMP, or pathogen-associated molecular pattern (20, 23, 24). In fact, this response can be so dramatic as to prove toxic to the host. For this reason, LPS has been classically termed “endotoxin” in reference to the cell-associated (endo) toxicity observed for many Gram-negative organisms. Understandably, the immune system has evolved to respond primarily to the most conserved feature of LPS, the lipid A structure, for which host TLR4 (toll-like receptor 4) is the primary receptor (20, 23, 24). However, as mentioned above, there is considerable diversity in LPS structures, even within lipid A. A consequence of this diversity is that different LPS structures have varying ability to trigger the host immune response (20). Thus, while the classical, hexacylated, bisphosphorylated lipid A molecule produced by *E. coli* and *Salmonella* is highly
immunogenic, other forms of lipid A are less so. Some forms of lipid A not only elicit no response themselves, but inhibit the host response to more immunogenic varieties (20, 25-27). In fact, production of less immunogenic lipid A is a strategy used by certain pathogens to evade the host immune response. For example, *Yersinia pestis*, the causative agent of the bubonic plague, modulates the acylation of its lipid A at mammalian body temperature to produce less immunogenic lipid A (28). Alternatively, some organisms evade the host immune response by masking the more conserved aspects of their LPS with a highly variable O antigen (23). Although the O antigen induces the production of antibodies, the length of the O-antigen chain prevents the antibody-mediated deposition of complement at the bacterial cell surface (13, 29, 30). Consequently, the O-antigen structure protects bacteria from lysis by complement. Possession of an O antigen has also been shown to contribute to pathogen evasion of phagocytosis by immune cells (13, 31).

We should also mention that the combination of the antigenicity and great structural diversity of O antigens have been exploited in the clinic to identify and classify pathogens. This application relies on the fact that the immune system produces specific antibodies that recognize one type of O antigen. Collections of O-antigen-specific antisera have been classically utilized to categorize Gram-negative organisms by serotype, that is, antigenicity in serological testing, of their O antigen (17).
1.3 The lipopolysaccharide biosynthesis pathway

1.3.1 Kdo\textsubscript{2}-lipid A biosynthesis: the Raetz pathway

Lipid A was first identified as the lipid component that could be released from the rest of the LPS molecules by mild-acid hydrolysis (32-34). Historically, this degradation product was marked as one of the three structural components of LPS. However, it is worth noting that cells synthesize lipid A together with the Kdo moieties of the core oligosaccharide using a biosynthetic pathway that is the most conserved aspect of LPS synthesis (9). This pathway has been extensively characterized in \textit{E. coli} and \textit{Salmonella} (9, 35) and is referred to as the Raetz pathway because much of the research describing it was led by Christian Raetz and his team (Fig. 1.2).
Figure 1.2 Lipid A biosynthesis pathway.

Modifications to the preceding structure made by each enzyme in the pathway are marked in red, with the exception of the last step, where the modifications made by LpxL and LpxM are colored in red and blue, respectively. Donor molecules are not shown. At low temperatures, LpxP acts instead of LpxL to add a C16:1 palmitoleoyl group instead of a lauroyl group.
The process of lipid A synthesis begins in the cytoplasm with the precursor molecule N-acetyl glucosamine linked to a nucleotide carrier (UDP-GlcNAc). This UDP-GlcNAc precursor is initially acylated by the enzyme LpxA to yield UDP-3-O-(acyl)-GlcNAc (36-38). LpxA is selective for the 14-carbon acyl group β-hydroxymyristate carried by the acyl carrier protein (ACP) (37). This selectivity is based on the LpxA active site functioning as a hydrocarbon ruler that most readily incorporates 14-carbon substrates (39, 40). The acylation of UDP-GlcNAc is unfavorable, however, and thus the first committed step of lipid A synthesis is the second reaction in the pathway, which is the irreversible deacetylation of UDP-3-O-(acyl)-GlcNAc to UDP-3-O-(acyl)-GlcN by the Zn$^{2+}$-dependent metalloenzyme LpxC (41-43). As LpxC catalyzes the first committed step in the synthesis of LPS, much of the regulation of this pathway, which will be discussed below, appears to center around this enzyme. Following the action of LpxC, UDP-3-O-(acyl)-GlcN is subsequently acylated a second time by LpxD to yield UDP-2,3-diacylglucosamine (44, 45). Like its earlier homologous counterpart LpxA, LpxD is selective for β-hydroxymyristate-ACP as a donor (44). In fact, it has been suggested that LpxD could, to some extent, be capable of substituting for LpxA in the first acylation step. However, since both enzymes are essential (44, 46, 47), it appears any cross-specificity between LpxA and LpxD is insufficient to support growth. After the second acylation by LpxD, LpxH removes the sugar nucleotide carrier from UDP-2,3-diacylglucosamine to generate 2,3-diacylglucosamine-1-phosphate, otherwise known as lipid X (48-50). Lipid X is subsequently added by LpxB to a molecule of UDP-2,3-diacylglucosamine (the
product of the LpxD reaction) through a β 1′-6 linkage that releases the UDP nucleotide carrier. The resulting product is a tetraacylated glucosamine disaccharide that is inserted in the inner leaflet of the IM and is sometimes referred to as lipid A disaccharide (51, 52). Following this condensation step, lipid A disaccharide is phosphorylated at the 4′ position by the kinase LpxK, becoming the bisphosphorylated lipid IVₐ (53, 54). As noted above, while not strictly part of lipid A synthesis, the next step is the addition of two Kdo sugar groups of the core oligosaccharide to lipid IVₐ (55-57). This step is mediated by the enzyme WaaA, previously known as KdtA, which sequentially adds Kdo groups to lipid IVₐ from activated Kdo (CMP-Kdo) (56, 57). Finally, two additional acylation events catalyzed by the LpxL and LpxM acyltransferases occur in sequence (58-60). LpxL adds a lauroyl group to the hydroxyl of the 2′-hydroxymyristoyl group and, subsequently, LpxM transfers a myristoyl group to the hydroxyl of the 3′-hydroxymyristoyl group (58-60). Like their earlier counterparts LpxA and LpxD, LpxL and LpxM only utilize substrates carried by ACP (58-60). LpxM functions best after the lauroyl group has already been added by LpxL but it is capable of functioning to some extent in the absence of LpxL activity (60). After the sequential action of LpxL and LpxM, mature, hexacylated lipid A, which also contains the first two Kdo residues of the inner core, is ready to serve as an acceptor for the sugar groups composing the core oligosaccharide.

An important note is the fact that, under normal laboratory growth conditions, about one third of LPS molecules are modified by LpxT, which adds a
second phosphate group to the 1-phosphate of lipid A, utilizing Und-PP as the donor (discussed below in the “Modifications of LPS” section and shown in Fig. 1.6) (61, 62). This reaction is not part of the Raetz pathway and occurs after the core-lipid A molecule is flipped across the IM. In addition, as discussed below, LpxT activity is subject to regulation by environmental conditions.

1.3.2 Biosynthesis of the core oligosaccharide

The core oligosaccharide can be sub-divided into an inner core, which is proximal to lipid A, and an outer core, which becomes the attachment site for the O antigen (9). The inner core is generally well conserved and is composed of Kdo and L-glycero-D-manno-heptose (heptose) groups (9, 63, 64). The outer core constituents are less conserved, and will vary depending on the type of core oligosaccharide, but in general consists of a series of hexoses (9, 63, 64). For the sake of simplicity, we will focus on the synthesis pathway for the K-12 core type from *E. coli* (Fig. 1.3), though various types of core oligosaccharide structures of *E. coli* and *Salmonella* can be found in Fig. 1.4 (9, 11).
Figure 1.3 Structure and biosynthetic enzymes of the E. coli K-12 core oligosaccharide.

Numbers represent bond positions between sugars. Note that nonstoichiometric modifications are not shown. All linkages are α-anomeric unless preceded by the β symbol, which specifies the β-anomeric state. Enzyme names are boxed, with
arrows indicating the linkages they catalyze. It is worth noting that, while the O-antigen ligation site is indicated, *E. coli* K-12 does not typically produce O antigen due to an ancestral mutation that inactivates its synthesis.

**Figure 1.4** Structure of various core oligosaccharides.
Shown are the known core types in *E. coli* (R1-R4, K-12) and *S. enterica* (serovar *typhimurium, Arizonae IIIA*). Numbers represent bond positions between sugars. Note that nonstoichiometric modifications are not shown. All linkages are α-anomeric unless preceded by the β symbol, which specifies the β-anomeric state.

The first step of core oligosaccharide synthesis is the sequential addition by the WaaA enzyme of the first two Kdo groups to the glucosamines of lipid A, which, as discussed above, occurs before the final acylation steps that conclude lipid A synthesis (Fig. 1.2) (56-60). Next, following completion of lipid A synthesis by LpxL and LpxM, the inner core is extended with two heptose residues by the sequential action of WaaC and WaaF (11, 65). ADP-L-glycerod-manno-heptose generally serves as the donor substrate for these inner-core glycosylation reactions (65). After the addition of the heptoses by WaaC and WaaF, the final three steps that complete inner core synthesis must be catalyzed in order by the enzymes WaaP, WaaQ, and WaaY, respectively (66). WaaP is a kinase that phosphorylates the first heptose of the inner core, which was added by WaaC (66, 67). WaaQ then transfers an additional heptose to the second heptose of the inner core, which was added by WaaF. This third heptose added by WaaQ is then phosphorylated by the WaaY kinase (66). Interestingly, while loss of inner core phosphorylation inhibits outer core extension, the loss of the enzymes that extend the outer core also inhibits inner core phosphorylation, implying complexities in the synthesis pathway that have not been fully explored (68).
Synthesis of the outer core begins with the addition of a glucose group to the second heptose, not only in the K-12 core type, but in all *E. coli* and *Salmonella* LPS structures (11, 64). This addition is mediated by WaaG (and its homologs), which utilizes UDP-glucose as its donor substrate (11, 64). The glucose added by WaaG is acted on by the glycosyltransferases WaaO and WaaB, which independently add a glucose and a galactose group, respectively, from UDP-bound donors (11, 64). Additionally, it has been shown that WaaO activity is dependent on divalent cations (64). Next, the penultimate glucose residue is added by the enzyme WaaJ (alternatively known as WaaR), whose activity depends on that of WaaB (11, 69). The final step of core synthesis, the addition of a heptose group to the penultimate glucose, is mediated by the WaaU glycosyltransferase (10, 11). This final heptose group serves as the acceptor of the O antigen after this core-lipid A precursor is translocated to the outer leaflet of the IM.

1.3.3 Biosynthesis of the O antigen

As might be expected for the outermost, and therefore most exposed, component of LPS, O-antigen structures are highly diverse, with roughly 200 different serogroups identified to date in *E. coli* alone (9, 17). Because of the great diversity of O antigens, the following discussion will focus on the more conserved aspects of O-antigen biosynthesis and its subsequent ligation to the core-lipid A molecule. We should also note that *E. coli* K-12 strains, which are often used in research, do not produce O antigen as the result of an ancestral mutation that inactivates its synthesis in that lineage (21).
The O antigen consists of a variable number of repeating oligosaccharide units, and as such can vary in size from molecule to molecule quite dramatically (9, 70). Additionally, rather than being synthesized directly on the core-lipid A molecule, the O antigen is fully synthesized independently from the rest of the LPS molecule. The O antigen is first built stepwise on a lipid carrier molecule, undecaprenyl phosphate (Und-P), and is then transferred to the core oligosaccharide of the nascent LPS molecule in the periplasmic face of the IM (71, 72). Despite the polymorphic nature of O antigens as a whole, the first step in their synthesis is well conserved, and consists of the transfer of a sugar monophosphate to the carrier molecule Und-P at the inner leaflet of the IM. The resulting sugar-pyrophosphate-undecaprenol (sugar-Und-PP) serves as an acceptor for additional glycosylation reactions (13). Aside from this conserved feature, the routes taken to complete the O antigen vary amongst different organisms and even strains, but generally fall into three categories: the so-called Wzy-dependent pathway, named for the polymerase which founded the group; the ABC-dependent pathway, which, as the name suggests, relies on an ATP-binding cassette (ABC) transporter to translocate the completed O antigen across the IM; and, the synthase-dependent pathway, which is poorly characterized and has only been identified in a single species of Salmonella (9, 13). A summary of these different routes can be found in Fig 1.5.
Figure 1.5 Summary of different O-antigen synthesis pathways.

GT stands for glycosyltransferase, and for the purposes of illustration represents all GTs required to generate the O-antigen. [O] represents a repeating unit of the O-antigen, while the subscript represents the number of repeats present (n being an arbitrary integer). Individual sugar units are represented by “S” inside a hexagon, and are shown bound to an arbitrary nucleotide carrier NDP. The lipid carrier is Und-P.

The Wzy-dependent pathway entails the synthesis of single O units on Und-P, requiring initiation for each O-antigen subunit, and the subsequent flipping of these Und-P-linked O units to the periplasmic face of the IM by the Wzx flippase (9, 13, 73, 74). These O units are then polymerized on a single Und-P carrier molecule through the activity of Wzy. Polymer length is controlled by a partner protein, Wzz, and exhibits a modal distribution of polymer sizes (9, 13, 75-77). Recent structural studies using cryoelectron microscopy have proposed a new model for how Wzz controls polymer length through a synergistic interaction with Wzy (78). Specifically, this model proposes that association between Wzz and Wzy serves to trigger polymerization, with polymer
length being controlled by both a molecular ruler mechanism based on Wzz's polysaccharide-binding capacity, and a molecular stopwatch mechanism based on the time of association between Wzz and Wzy. Finally, the polymerized O antigen is ligated to the core-lipid A acceptor at the outer leaflet of the IM by the ligase WaaL, and the Und-PP carrier is recycled (9). It is worth noting that this biosynthetic strategy of generating O antigen by initiating the synthesis of each subunit on an Und-P molecule places substantial demand on the Und-P carrier pool (9). Indeed, interrupting O-antigen biosynthesis at certain steps in this pathway can lead to the sequestration of Und-P by O-antigen precursors, which causes severe growth defects because Und-P also functions in the synthesis of several envelope glycopolymers, including the essential peptidoglycan cell wall (79, 80).

In contrast to the Wzx-dependent pathway, the ABC-dependent pathway only requires a single initiation event per molecule of polymerized O antigen and carries out the entirety of its polymerization in the cytoplasm (9, 13). Glycosyltransferases first polymerize the completed O antigen on a single Und-P carrier molecule in the inner leaflet of the IM utilizing nucleotide-activated sugar donors. The completely polymerized O-antigen-Und-PP molecule is subsequently flipped to the periplasmic face of the IM by an ABC transporter, where the O-antigen portion is then added to the core-lipid A molecule by the WaaL ligase (13, 81).

The synthase-dependent pathway is peculiar to _S. enterica_ serovar Borreze (rfbO:54) (13, 82). The mechanistic details for this mechanism of O-
antigen synthesis are unclear, but the titular synthase (WbbF) of the pathway is presumed to simultaneously polymerize and translocate the O antigen across the IM (13, 82). As in the other two biosynthetic pathways, the resulting O-antigen-Und-PP molecule is then used as a donor by the WaaL ligase, which transfers the O-antigen polymer to the outer-core oligosaccharide of nascent LPS molecules and releases the lipid carrier, which is then recycled.

1.4 Regulation of lipopolysaccharide biosynthesis

The pathways for the biosynthesis of phospholipids and LPS share a common precursor, β-hydroxymyristate-ACP, the substrate of the FabZ and LpxA enzymes, respectively. As a result, proper balance in lipid biosynthesis, and thereby balanced growth of the IM and OM, requires regulation of LPS synthesis. As stated earlier, LpxC mediates the first committed step in LPS synthesis (9). Consequently, this step becomes a logical control point for the pathway, and indeed, much of our understanding of the regulation of LPS synthesis centers around LpxC. It was noted as early as 1996 that LpxC activity was upregulated when the early steps of LPS synthesis were inhibited, while many other enzymes of the pathway were nonresponsive (83). This response was later shown to be due to regulation of LpxC protein levels by the essential, AAA+ metalloprotease FtsH [for a recent review regarding FtsH, the reader is directed to (84)] (85). Interestingly, FtsH has also been shown to degrade WaaA, which adds Kdo groups to lipid IVA (86). Proteolysis of LpxC by FtsH is dependent on a sequence present at the carboxyl terminus of LpxC, and is controlled by the cellular growth state and levels of the alarmone (p)ppGpp. Specifically, LpxC is stable during
fast growth but degraded by FtsH during slow growth; this relationship is inverted with the loss of (p)ppGpp synthesis (87, 88). The precise signal(s) that directly controls FtsH proteolysis of LpxC has not been elucidated, though feedback through lipid A disaccharide has been proposed as a candidate (46). Nonetheless, a protein that regulates the FtsH-dependent proteolysis of LpxC has also been discovered. The bitopic IM protein LapB (formerly YciM) functions as a negative regulator of LpxC levels in an FtsH-dependent manner (89-91). LapB has also been reported to co-purify with, in addition to FtsH and LPS, several LPS synthesis and transport proteins, including WaaC and the entirety of the LPS transport (Lpt) complex (described below). These co-purification results imply that LapB may serve a larger role in coordinating disparate aspects of LPS biogenesis. However, the precise nature and mechanism of that role and the relevance of some of those interactions are not clear (91). Furthermore, additional proteolytic control of LpxC by an unknown protease has also been proposed (92). Clearly, more research is needed to understand regulation of LPS synthesis.

1.5 Modification of lipopolysaccharide structure

To better adapt to their varying environments, Gram-negative bacteria will often deviate from the LPS biosynthesis pathways described above. The most frequent modifications include: changes in the number and type of acyl chains, as well as the number of phosphates in lipid A; the addition of covalent modifications to lipid A, generally at the 1 and 4’ phosphates, and the core
oligosaccharide; and the conversion of the type of O antigen whenever the genomic locus responsible for O-antigen synthesis is exchanged through horizontal gene transfer (18-20). Additionally, due to the incorporation of nonstoichiometric modifications, LPS synthesized by a single strain is not entirely uniform under any growth condition. The most relevant modifications in *E. coli* and *Salmonella* are discussed in detail below, and those in the lipid A and core oligosaccharide regions are summarized in Figures 1.6 and 1.7.

1.5.1 Modifications of lipid A

![Figure 1.6 Modifications to the structure of lipid A.](image)

Shown are modifications made to lipid A described in the text, alongside the enzymes which mediate them in corresponding colors. **A)** Modifications made to the glucosamine phosphates. While shown in their preferred positions, it is possible for either phosphate to be modified with either substituent. **B)** Modifications made to the acyl groups. The X indicated for LpxO is a hydroxyl (-OH) when LpxO is active, and a hydrogen (-H) when it is not. When acyl chains
are removed, the cleaved bond is shown as a dotted line. It is important to note
that LpxP is part of the conserved Raetz pathway but only active at low
temperatures, at which LpxP substitutes for LpxL to add a C16:1 palmitoleoyl
group instead of a lauroyl group.

Bacteria adapt to different temperatures by modulating the fluidity of their
membranes through alterations of the type of acyl chains of their membrane
lipids (93). LPS is no exception. Under low temperature conditions (~12°C), E.
coli will express an alternate acyltransferase homologous to LpxL, termed LpxP,
that adds a 16-carbon palmitoyl group in place of the 12-carbon lauroyl group
normally added by LpxL (18, 94, 95). Presumably, this modification serves to
offset the decrease in membrane fluidity resultant from lower temperatures (18).
Like its counterpart LpxL, LpxP requires ACP-bound substrates for activity (94).

However, most of the changes to lipid A structure occur in response to the
amount of cations and positively charged antimicrobials in the environment. A
number of enzymes that catalyze those modifications are under control of the
PhoQP two-component regulatory system (96, 97). In Salmonella, the PhoQP
system is primarily implicated in responding to low-levels of divalent cations,
namely Mg^{2+}, the presence of cationic antimicrobial peptides (CAMPs), and host
interactions (97-99). When the kinase PhoQ is activated by these signals, it
phosphorylates its cognate response regulator PhoP. Once phosphorylated,
PhoP activates the transcription of several genes encoding LPS-modifying
enzymes. Among them, PagP is an enzyme that resides in the OM and transfers a palmitoyl group to LPS, resulting in heptacylated LPS species that are implicated in CAMP resistance (18, 100, 101). In this transfer, PagP uses the 16-carbon acyl chains of phospholipids that are mislocalized to the outer leaflet of the OM as donors (18, 100, 101). Additionally controlled by PhoQP are LpxR and PagL (the latter is absent in E. coli), which also reside in the OM and modify the acyl chain content in lipid A. LpxR and PagL, respectively, mediate the removal of the 3′ acyl groups and the 3-hydroxymyristoyl group, which modulate the immunogenicity of lipid A (102, 103).

The PhoQP system also controls modification to the glucosamine disaccharide portion of lipid A, albeit indirectly through another two-component regulatory system. These modifications are directly regulated by the PmrBA (alternatively BasSR for E. coli) two-component system. The PmrBA system is positively regulated by PhoQP through the adapter protein PmrD, which protects the phosphorylated response regulator PmrA (BasR) from dephosphorylation (104-107). In addition, the sensor kinase PmrB (BasS) directly responds to acidic pH and elevated concentrations of certain metals such as Fe$^{3+}$, Al$^{3+}$ and Zn$^{2+}$ (12, 107-109). When phosphorylated, PmrA upregulates the covalent modification of lipid A with 4-amino-4-deoxy-L-arabinose (L-Ara4N) and phosphoethanolamine (PEtN) (18). L-Ara4N modification is mediated by the enzyme ArnT, which transfers L-Ara4N from Und-P to lipid A primarily at the 4′ phosphate, though it can also act on the 1-phosphate (110, 111). PEtN modification is mediated by the enzyme EptA, which transfers PEtN from phosphatidylethanolamine to lipid A
primarily at the 1-phosphate, though it can also act on the 4′-phosphate (112, 113). Activation of PmrBA also inhibits modification of lipid A by LpxT, the kinase that adds a second phosphate group to the 1-phosphate from Und-PP to ~33% of LPS molecules under standard laboratory growth conditions (61, 62). Both the addition of positively charged moieties to lipid A by ArnT and EptA, and the loss of the negatively charged modification catalyzed by LpxT are associated with increased resistance to CAMPs, presumably due to the masking of negative charges (i.e., phosphates) in LPS to which CAMPs bind (62, 107).

In addition, *Salmonella* can alter lipid A through the action of LpxO, which is absent in *E. coli*. LpxO is not regulated by PhoQP and mediates the oxygen dependent hydroxylation of the secondary 3′ acyl group immediately after the carboxyl group in the cytoplasm (114, 115).

It is worth noting that, while these are the primary well-characterized modifications to lipid A carried out by *Salmonella* and *E. coli* species, other species employ similar strategies with alternate modifying groups. For example, certain isolates of *V. cholerae* modify the acyl chains of their lipid A with amino acid moieties (116, 117). Additionally, some *Bordetella* isolates modify the phosphates of their lipid A with glucosamine (118, 119). Both of these modifications promote CAMP resistance, much like many of their *Salmonella* and *E. coli* counterparts.
1.5.2 Modifications to the core oligosaccharide

Figure 1.7 Modifications to the structure of the core oligosaccharide.

Shown is the conserved inner core oligosaccharide (and its linkage to lipid A) in black, with potential modifications being indicated in red (though the alternate rhamnose modification by WaaS when the second Kdo is modified with PEtN by EptB is shown in blue). Numbers represent bond positions between sugars. Enzymes mediating modifications are next to each linkage, and when not associated with *E. coli* K-12, core type associations are listed in parentheses beside the modification.

Given that the outer core is relatively variable, well-characterized modifications tend to be confined to the inner core region. Modifications to the core oligosaccharide include the addition of various sugar groups as well as
other moieties, such as PEtN. What types of modifications occur will also depend to some extent on the core type. The best characterized modifications will be discussed in detail below, and are also summarized in figure 1.7.

Starting from the lipid A-proximal sugars and working up the sugar chain of the core, the second Kdo residue can be modified with additional Kdo, PEtN, rhamnose, or galactose groups (11, 12). Kdo addition is mediated by the WaaZ transferase. Modification of the second Kdo with PEtN is mediated by EptB and confers resistance to polymyxin B and high levels of Ca²⁺ (12, 120-122). Rhamnose addition is mediated by WaaS, and can occur to either the second Kdo or the third Kdo added by WaaZ, but both activities are dependent upon prior addition of the third Kdo (12, 123, 124). When PEtN is present on the second Kdo group, WaaS can only act on the third Kdo (added by WaaZ), whereas it otherwise acts on the second Kdo (12, 124). At least in E. coli K-12, these modifications are associated with induction of the envelope stress response effector σ⁵ [for a review on the function and induction of σ⁵, the reader is directed to (125)], as well as with the truncation of the outer core caused by downregulation of WaaJ (WaaR), which leads to the loss of the terminal glucose and heptose groups (12, 124). In addition, EptB is negatively regulated by PhoQP, but induced by high levels of Ca²⁺ (12, 120, 121). Lastly, addition of galactose to the second Kdo group is mediated by WabA, which is absent in E. coli K-12 and was instead characterized in E. coli with the R2 core type (11).

The first heptose of the inner core may be modified with PEtN, specifically at the phosphate added by WaaP, which confers resistance to CAMPs (126).
This addition is mediated by EptC, which is induced by PhoBR, a two-component system responsive to phosphate-limiting conditions (126-128). The third heptose of the inner core (added by WaaQ) may be modified with several different glucose derivatives, including GlcNAc, GlcN, and glucuronic acid (GlcUA) (11, 126). The former two modifications were characterized in E. coli with the R3 and R1 core types, respectively, whereas the latter is found in E. coli K-12 (11, 126). Modification of the third heptose with GlcUA is mediated by the enzyme WaaH, which like EptC, is induced by the PhoBR system (12, 126). Consistent with its response to phosphate limiting conditions, the GlcUA addition mediated by WaaH proceeds more efficiently in the absence of the phosphate group added by WaaY (12, 126).

1.5.3 O-antigen modification

While modifications to basic O-antigen structures have been reported, they will not be discussed in detail here due to the sheer breadth of O-antigen synthesis (13, 129). More striking than minor additions or replacements of individual sugars is that the O antigen may be replaced entirely. In some cases, a serotype of O antigen is replaced by another through the genetic exchange of part or the entire biosynthetic locus resulting from horizontal gene transfer (16, 130). In addition, the O antigen can be replaced altogether by a different type of polysaccharide. Specifically, in E. coli K-12, it has been shown that induction of capsular colanic acid synthesis results in replacement of the O antigen in LPS with colanic acid repeats in a WaaL-dependent manner (131, 132). A similar phenomenon has been observed with the enterobacterial common antigen (ECA)
Curiously, a missense mutation in \textit{waaL} that broadens the substrate specificity of the ligase led to the discovery that this WaaL variant can modify the LPS core with the peptidoglycan building block by utilizing lipid II as a donor as well (135).

\textbf{1.6 Lipopolysaccharide transport}

In 1972, Mary Jane Osborn and collaborators published seminal studies demonstrating that LPS is synthesized at the IM and, therefore, must be transported across the envelope to the OM. Furthermore, the authors demonstrated that this intermembrane transport is unidirectional (7, 8). That body of work opened a new area of research that eventually led to the discovery of the factors required for LPS transport during the 1990s and 2000s. We now know that, as described in sections above, the core-lipid A and Und-P-linked O-antigen components of the LPS structure are independently synthesized at the cytoplasmic face of the IM. Using different transporters, these two subunits are separately flipped to the periplasmic leaflet of the IM. Once there, the O antigen can be ligated onto the core oligosaccharide moiety by the WaaL ligase. The resulting newly synthesized LPS molecules must then be extracted from the IM, cross the aqueous periplasm, and traverse the OM to ultimately be assembled at the cell surface. We describe in the next sections our current knowledge of these steps in LPS transport.
Figure 1.8 Transport of LPS across the cell envelope.

Shown are representations of MsbA, which mediates the transport of core-lipid A across the IM, and the Lpt complex (LptB<sub>2</sub>FCADE), which mediates LPS extraction from the IM and its transport through the periplasm and OM. As described in the text, the O antigen can be synthesized on Und-P and transported across the IM by different pathways (Fig. 1.5). If made, the O antigen is ligated to core-lipid A in the periplasmic leaflet of the IM by WaaL (not shown).

1.6.1 Crossing the IM: MsbA

Transport of the core-lipid A molecule from the inner leaflet to the outer leaflet of the IM is mediated by a homodimer of MsbA, a flippase that belongs to the ABC transporter superfamily (Fig 1.8) (33, 136-138). MsbA was first
characterized as a multicopy suppressor of the loss of LpxL (previously known as HtrB) activity, and was named accordingly (multicopy suppressor of htrB A) (136). It would not be until years later that the flippase activity of MsbA was demonstrated, both by the accumulation of LPS in the cytoplasmic side of the IM upon MsbA depletion and the in vitro reconstitution of functional MsbA (139, 140). Recent structural studies of MsbA have put forth a model in which LPS directly enters a largely hydrophobic cavity within the MsbA homodimer (141, 142). Interestingly, this cavity is localized in the outer leaflet of the IM, indicating that LPS traverses the IM before being flipped (141, 142). Interactions between this cavity and the lipid A moiety of LPS, together with ATP hydrolysis by MsbA, cause conformational changes in MsbA that lead to the exposure of this cavity to the aqueous periplasmic environment to drive LPS into the outer leaflet of the IM in concert with the closing of the cavity (141). These studies suggest that interaction between positively charged residues within the cavity and the phosphates of lipid A serve as recognition determinants for LPS by MsbA (141, 142). Moreover, the placement of these interactions has been proposed to favor accommodation of shorter acyl chains within the cavity, which may serve to discriminate against flipping of phospholipids (142). It has also been revealed that MsbA’s substrate specificity likely drives the requirement for Kdo2-lipid A as the minimal LPS structure. Flipping of LPS by MsbA is most efficient when the glycolipid contains the late-stage acyl chains, which are added by LpxL and LpxM after the addition of the first two Kdo residues (143-145). The requirement
for Kdo<sub>2</sub>-lipid A as the minimal LPS structure is thus primarily a reflection of the preference of MsbA for hexacylated substrates (142).

1.6.2 From the IM to the OM: the Lpt system

After its translocation across the IM by MsbA, and possibly undergoing the addition of the O antigen and other chemical modifications (e.g. by the addition of L-Ara4N and PEtN) at the outer leaflet of the IM, LPS must be transported across the periplasm and OM. This transport necessitates the extraction of LPS from the IM, the sheltering of its acyl moieties as it traverses the aqueous periplasm, and finally, its translocation across the OM to arrive at its final destination in the outer leaflet of the OM. All of these processes are mediated by a protein complex, termed the Lpt complex (Fig 1.8) (1). The Lpt complex is composed of seven different proteins that span all compartments of the cell: LptB in the cytoplasm; LptF, LptG, and LptC in the IM; LptA in the periplasm, and LptD and LptE in the OM (1, 146). A dimer of LptB proteins together with LptF and LptG form the ABC transporter LptB<sub>2</sub>FG. LptB constitutes the nucleotide-binding domains (NBDs) that bind and hydrolyze cytoplasmic ATP to power the transport of LPS from the IM to the OM (147, 148). Recent structural studies have revealed that LptF and LptG, the transmembrane domains of the LptB<sub>2</sub>FG ABC transporter, form a cavity that is predicted to accommodate LPS during its extraction from the IM (149-151). Whether or not the bitopic IM protein LptC is involved in the extraction process is still somewhat unclear, though it is worth noting that its single transmembrane domain is dispensable for function (152). While the precise mechanism of extraction of LPS from the IM remains to be elucidated, it is known that, eventually,
LPS makes its way to the periplasmic domain of LptC (146, 148). The periplasmic domain of LptC consists of a series of antiparallel β-strands arranged to form a β-jellyroll domain that contains a hydrophobic groove that is thought to shelter the acyl chains of LPS during its transit across the periplasm (153, 154). LptA and the periplasmic domain of LptD, LptF, and LptG possess similar β-jellyroll folds. Moreover, the β-jellyroll domains of LptC, LptA, and LptD have been shown to associate in a head-to-tail manner to extend the hydrophobic groove across the periplasm (154-156). These domains in LptCAD are thus thought to form a bridge extending across the periplasm through which LPS can travel to reach the OM. In support of this model, LPS has been covalently cross-linked to each individual member of this bridge, and the in vitro reconstitution of LPS transport requires the formation of a bridge composed of the Lpt periplasmic components (148, 157, 158). It is worth noting that in addition to its ability to associate with LptC and LptD at either end of the bridge, LptA can also form homo-oligomeric complexes (154, 159). As such, it is possible that more than one LptA protein may be incorporated into the Lpt complex. However, the ability of an LptA variant deficient in homo-oligomerization to complement the deletion of the native lptA gene in E. coli may suggest that one subunit of LptA per complex is sufficient under normal growth conditions (160). Nevertheless, a recent study has demonstrated that, in Salmonella, the width of the periplasm (i.e. the distance between the IM and OM) can be modulated by altering the length of Lpp, the OM lipoprotein that covalently tethers the OM to the peptidoglycan layer (161). It is therefore tempting to suggest
that the number of LptA proteins per Lpt bridge might change depending on the width of the periplasm.

Upon arrival at the OM, LPS must be specifically inserted into the outer leaflet, a process mediated by LptD and LptE (146). The membrane-associated portion of LptD is a large, crenellated β-barrel, in which hydrogen bonding between strands is disrupted along the first and last β strands, creating a crenellation, or small gap (146, 156). LptE is a lipoprotein that resides in the lumen of the barrel portion of LptD (162). Together LptD and LptE are proposed to form the OM translocon responsible for the insertion of LPS into the outer leaflet of the OM. Based on structural studies, it has been proposed that when LPS arrives at the periplasmic β-jellyroll domain of LptD, its acyl chains are directly deposited into the hydrophobic environment of the OM through a gap that exists between this periplasmic domain and the β-barrel domain of LptD; the crenellation of LptD may then provide for a means by which the barrel might open enough to allow lateral passage of the hydrophilic portion of LPS through the lumen of LptD and out into the OM (156). The role of LptE is unclear, but its high affinity for LPS suggests that it is more than simply a plug for the LptD barrel (163, 164). It has also been demonstrated that LptE plays a critical role in the proper assembly of LptD in the OM (162, 165).

Together, these data have led to the so-called “PEZ Model” of Lpt-mediated LPS transport (146). This model likens LPS to the originally Austrian PEZ candies, and the Lpt transport machinery to the mechanical PEZ candy dispenser invented by Oskar Uxa. Such a dispenser works by way of a spring-loaded platform at the
base of the dispenser, which pushes a stack of PEZ candies up through the central channel of the dispenser, so that a candy is always present at the exit of the dispenser and ready to be taken and consumed by the user. Likewise, this model predicts that LPS travels as a stream of molecules, and that the driving force of LPS transport by the Lpt machinery is derived from the LptB2FG transporter at the base of the periplasmic channel formed by LptCAD. Accordingly, the LptB2FG transporter acts much like the spring-loaded platform of a PEZ dispenser by providing constant pressure to the base of the traveling LPS stream by constantly loading new LPS molecules into the channel. Thus, in this model, the channel formed by LptCAD is largely passive in transport, simply providing a compatible route by which LPS molecules might traverse the aqueous periplasm prior to their assembly at the cell surface through the action of the LptDE translocon, which, like the top of the PEZ dispenser, opens up to deliver its cargo. Importantly, the recent \textit{in vitro} reconstitution of LPS transport supports the PEZ model for LPS transport \cite{146, 158}. This great technical achievement has demonstrated that transport of LPS can occur in an ATP-dependent manner between IM-like (i.e. containing LptB2FGC) and OM-like (containing LptDE) proteoliposomes only when they are physically connected by soluble LptA.
Chapter 2: A cluster of residues in the lipopolysaccharide exporter that selects substrate variants for transport to the outer membrane

Foreword

The following chapter adapted from an article of the same title, for which I was the lead author, published in the journal Molecular Microbiology in 2018. Supplementary material from this article is in Appendix A. This work was performed in collaboration with the laboratory of Daniel Kahne. Specifically, the data (and interpretation thereof) shown in Figs. 3D and A.2 were contributed by collaborators from the Kahne Lab (Rebecca Taylor and Emma Nagy, respectively), whereas I, as lead author, contributed to the conception of all other figures.

2.1 Abstract

Most Gram-negative bacteria assemble lipopolysaccharides (LPS) on their surface to form a permeability barrier against many antimicrobials. LPS is synthesized at the inner membrane and then transported to the outer leaflet of the outer membrane. Although the overall LPS structure is conserved, LPS molecules can differ in composition at the species and strain level. Some bacteria also regulate when to modify phosphates on LPS at the inner membrane in order to become resistant to cationic antimicrobial peptides. The multi-protein Lpt trans-envelope machine, which transports LPS from the inner to the outer
membrane, must therefore handle a variety of substrates. The most poorly understood step in LPS transport is how the ATP-binding cassette LptB2FG transporter extracts LPS from the inner membrane. Here, we define residue K34 in LptG as a site within the structural cavity of the *Escherichia coli* LptB2FG transporter that interacts electrostatically with phosphates on unmodified LPS. Alterations to this residue cause transport defects that are suppressed by the activation of the BasSR two-component signaling system, which results in modifications to the LPS phosphates. We also show this residue is part of a larger site in LptG that differentially contributes to the transport of unmodified and modified LPS.

### 2.2 Introduction

The impermeability of Gram-negative bacteria to small hydrophobic molecules poses a great challenge in the development of antimicrobials (5, 166). This impermeability results from the composition of the Gram-negative cell envelope, which contains two membranes, an aqueous cellular compartment termed the periplasm, and a peptidoglycan cell wall (Fig. 2.1A) (1). The inner membrane (IM) surrounds the cytoplasm and is a typical phospholipid bilayer that is permeable to hydrophobic compounds. The outer membrane (OM), in contrast, contains phospholipids in its inner leaflet and the glycolipid lipopolysaccharide (LPS) in its outer leaflet (3, 167). The structure of LPS drives tight packing of LPS molecules, which decreases the fluidity of the OM and creates a hydrophilic, polyelectrolyte barrier against hydrophobic molecules at the cell surface (1, 5). This barrier-like quality and the fact that LPS is essential in relevant Gram-negative
pathogens make targeting LPS biogenesis a plausible strategy to treat Gram-negative infections (4).

LPS is a large amphipathic molecule composed of up to three regions: lipid A, an acylated glucosamine disaccharide that forms the outer leaflet of the OM; core oligosaccharide, composed of diverse sugars; and O antigen, a polysaccharide made of a repetitive oligosaccharide unit (Fig. 2.1B) (9). Despite these conserved features, LPS structure differs among Gram-negative bacteria with respect to the amount and type of acyl chains on lipid A, the presence of chemical modifications on the glucosamines of lipid A, the identity and arrangement of sugars in the core oligosaccharide and O antigen, and the absence of O antigen (18). In some bacterial species, these structural variations can be regulated by environmental signals. For example, positively charged L-4-aminoarabinose (L-Ara4N) and phospho-ethanolamine (PETN) can be added to phosphates on lipid A (Fig. 2.1B) to increase resistance to antimicrobial cationic peptides by decreasing the binding of these compounds to LPS (18, 110).

Once synthesized and possibly modified at the IM, LPS molecules must be transported across the cell envelope to the OM by the Lpt (Lipo polysaccharide transport) proteins (146, 168). LptAB₂CDEFG form a bridge that spans all compartments of the cell, allowing the coupling of energy, from ATP in the cytoplasm, to the transport of LPS across the envelope (146-148, 151, 158, 169-175). In the PEZ model for LPS transport (Fig. 2.1A), LPS is initially extracted from the IM by the ATP-binding cassette (ABC) transporter LptB₂FG (146, 148-150, 158, 176). Subsequently, LPS traverses the aqueous periplasm with its acyl chains
sheltered inside the hydrophobic groove of the Lpt domains present in LptCAD, with each new molecule of LPS that is extracted from the IM pushing a stream of LPS molecules along this groove (146, 148). Ultimately, LPS molecules in this stream reach the β-barrel of the LptDE translocon, which inserts them in the OM (146, 156, 162, 164, 177). At present, it is unknown how the Lpt machinery transports different LPS variants. Furthermore, it is yet to be determined if post-synthesis, the Lpt system plays a role in regulating the type of LPS displayed at the cell surface in organisms that synthesize various forms of the glycolipid.

Figure 2.1 PEZ model of LPS transport by the Lpt system.
A) LPS is extracted from the IM by the LptB₂FG transporter. LPS transverses the periplasm by sheltering its acyl chains in the periplasmic bridge formed by LptCAD,
followed by translocation to the cell surface through the LptDE translocon. B) Structure of *E. coli* LPS with relevant possible modifications colored in gold (L-Ara4N on the 4′ phosphate) and red (PEtN on the 1 phosphate). Kdo = 3-deoxy-D-manno-oct-2-ulosonic acid, Hep = heptose, Gal = galactose, Glu = glucose.

The most poorly understood step in LPS transport is its energetically unfavorable extraction from the IM by LptB2FG. LptF and LptG, the transmembrane subunits of this ABC transporter, are thought to mediate this extraction and subsequent loading of LPS onto the periplasmic Lpt bridge (146, 148-150, 158, 176). In support of this model, recent crystal structures of the LptB2FG complex have revealed that LptF and LptG form a cavity within the IM that might accommodate LPS during its extraction (149, 150). In this study, we have identified a cluster of residues localized at the membrane-periplasm interface of this cavity that is critical for the transport of LPS. Based on data derived from structure-function and suppressor analyses, and *in vitro* biochemical reconstitution of LPS extraction in liposomes, we propose that a residue within this domain interacts with unmodified LPS through electrostatic interactions. Further, we propose this domain participates in an early step in the recognition of LPS prior to its extraction from the IM by the LptB2FG complex. Our data also suggest that the *E. coli* LptB2FG transporter binds to different LPS substrates in distinct manners.
2.3 Results

2.3.1 Correlation between charges in lipid A and LptG suggests an LPS-interaction site in LptG.

Previous work by Hamad et al. identified a residue in LptG of *Burkholderia cenocepacia* that might interact with LPS (178). In bacteria such as *E. coli* K-12 and *Salmonella*, modification of the phosphates at the C1 and C4’ positions of lipid A with L-Ara4N and PEtN is regulated and usually present only at low levels under standard laboratory growth conditions (18). In contrast, in *B. cenocepacia*, the L-AraN modification is constitutive and essential to viability, and its loss results in phenotypes resembling those reported in *Escherichia coli* cells lacking Lpt function (179). Notably, Hamad et al. showed that substituting aspartate at position 31 (D31) in LptG of *B. cenocepacia* with histidine resulted in an LptG variant (LptGD31H) that suppressed the lethality caused by the absence of L-Ara4N (178). This suggested that in wild-type, residue D31 in LptG might interact with the L-Ara4N moiety in LPS, and that its replacement with histidine might facilitate the transport of unmodified LPS in an L-Ara4N-deficient mutant. In support of this idea, structures of the LptB2FG transporter of *P. aeruginosa* and *K. pneumoniae* show that the equivalent residues in these bacteria are in a cavity formed by LptF and LptG that is thought to accommodate LPS during extraction from the IM (Fig. 2.2A) (149, 150). In fact, the placement of this residue near the periplasmic interface of helix 1 of LptG could allow an interaction between this residue and moieties at the C1 and C4’ positions of lipid A.
Figure 2.2 Residues in the K34 region of LptG are important for LPS transport.
A) Location of relevant LptG residues in the LptB$_2$FG crystal structure from *P. aeruginosa* (PDB = 5x5y). B) Close-up of the relevant residues (labelled by their identities in *E. coli*) shown in panel A. C) Substitutions in the K34 region of LptG cause defects in LPS transport, as determined by enhanced sensitivity to bacitracin (Bacitracin$^S$) using a disc-diffusion assay. Data represents the diameter of the zone of inhibition of growth (in mm); the black dotted line marks the diameter of the antibiotic discs. WT refers to strain NR2761. Substitutions in LptG are
shown; KK/DD refers to the K40D/K41D double substitution. More details in Table S1. D) LPS release activity of LptG<sup>WT</sup>, LptG<sup>K34D</sup>, and LptG<sup>K41D</sup> complexes. LPS release was monitored at various time points by UV-dependent crosslinking to LptAI36Bpa in the presence and absence of ATP. The immunoblot in the top panel was probed with LPS antiserum to detect crosslinked LptA-LPS adducts (marked LptA x LPS), whereas the immunoblot in the bottom panel was probed with LptA antiserum to show uncrosslinked LptA.

Surprisingly, the suppressor <i>B. cenocepacia</i> strain producing LptG<sup>D31H</sup> is viable regardless of whether the phosphates in lipid A are modified with L-Ara4N or not, and residue D31 is not conserved among LptG homologs (178). We wondered whether this lack of conservation is the result of co-evolution with differences in the lipid A structure, given the chemical groups at the C1 and C4′ positions of lipid A vary among bacteria. Indeed, we found a correlation between the charges of moieties at the C1 and C4′ positions of the main type of lipid A produced by a diverse group of Gram negatives and the residue in LptG equivalent to D31 in <i>B. cenocepacia</i> (Fig. 2.3). Often, both groups on lipid A bear the opposite charge of that present in the relevant LptG residue, and the three positions never carry the same charge. This correlation could explain the results obtained by Hamad <i>et al.</i> in <i>B. cenocepacia</i> (178). The lethality caused by the loss of L-Ara4N could result from a charge clash between one of the unmodified phosphates at the C1 and C4′ positions in lipid A and residue D31 in LptG; this clash between negative charges would be relieved in the suppressor strain producing LptG<sup>D31H</sup>
(178). Thus, the correlation between the charges at this residue of LptG and the C1 and C4′ positions of lipid A suggests that these specific positions may interact and, as a result, have co-evolved differently among Gram negatives to facilitate interaction between the LptFG cavity and the different types of LPS they must transport.

Figure 2.3 Correlation between residue in the cavity of LptG and LPS structure.

A) Alignment of LptG proteins from various Gram-negative organisms. Alignment was performed using Clustal Omega (180-182) and the following Uniprot accession numbers: C. jejuni = Q0PB76, H. pylori = E1S717, D. acetophilus = D4H8C5, P. gingivalis = B2RKU5, G. forsetii = A0LYY5, C. crescentus = A0A0H3C7C4, F. tularensis = A0A0B6KiX2, A. baumannii = D0CB66, P.
aeruginosa = Q9HXH5, V. cholerae = A0A0H3Q758, E. coli = P0ADC6, H. influenzae = P45332, B. cenocepacia = U1XMW3, N. meningitidis = A0A0G4BVQ8, S. azorense = C1DTN4, G. uraniireducens = A5G7X0, E. minutum = B2KAY2. Residues examined in this study are colored in the same scheme as figures in the main text. B) Correlation between a specific LptG residue equivalent to D31 in Burkholderia and moieties at the C1 and C4′ positions of lipid A. Residue equivalency was determined by protein alignment (panel A). Moieties on lipid A are based on previously published data and numbers in parentheses denote position on lipid A (C1 or C4′) (183-191). In bacteria where LptG has a negatively (red text) charged amino acid at the relevant site, the C1 and/or C4′ positions in lipid A are frequently modified with positively (blue text) charged groups; in those in which the relevant amino acid is positively (blue text) charged, the C1 and C4′ positions in lipid A contain a negatively (red text) charged phosphate; and, in bacteria with an uncharged (black text) amino acid, the chemical groups at the C1 and C4′ positions in lipid A vary. Furthermore, in bacteria producing lipid A that has the same type of charge at both C1 and C4′ positions, we usually found the opposite, and never the same, charge in the relevant LptG residue. L-Ara4N = L-4-aminoarabinose.

2.3.2 The putative LPS-binding site in LptG is essential for LPS transport.

We next tested in E. coli the idea that the aforementioned sites in LptG and lipid A might interact. Notably, in E. coli, the relevant charges in LptG and lipid A
are reversed with respect to those in *B. cenocepacia*. In *E. coli* K-12, LptG has a positively charged lysine residue (K34) at the position equivalent to D31 in LptG of *B. cenocepacia*, and the phosphates in lipid A are unmodified under normal growth conditions (Fig. 2.3). If charge clashes between these positions in LptG and lipid A were detrimental to LPS extraction from the IM, substituting residue K34 in LptG of *E. coli* with a negatively charged residue should result in phenotypes characteristic of Lpt defects. Because LPS transport to the OM is essential for viability in *E. coli*, and proper transport is required for innate resistance to hydrophobic antibiotics, such defects could result in any of the following phenotypes according to decreasing severity: lethality under all growth conditions; conditional lethality by which mutants can only survive in slow-growing conditions such as minimal medium; and increased sensitivity to hydrophobic antibiotics like bacitracin (192).

Accordingly, we introduced mutations that substituted the positively charged K34 residue in LptG with negatively charged aspartate (K34D) or glutamate (K34E) residues. We generated plasmid-encoded *lptFG* alleles and tested their ability to complement a chromosomal deletion of *lptFG* (193). Haploid strains generated from complementing *lptG* mutant alleles were also tested for defects in LPS transport by assessing their sensitivity to hydrophobic antibiotics. We found that LptG<sup>K34E</sup> was stably produced, but not functional since *lptG(K34E)* did not complement a chromosomal deletion of *lptFG* (Fig A.1). This might explain why an LptG<sup>K34E/R136E</sup> variant had been reported as non-functional (150). In contrast, *lptG(K34D)* complemented, but the resulting *lptG(K34D)* haploid strain
was very sensitive to hydrophobic antibiotics (Figs. 2 and A.1, and Table A.1). These defects suggested that the negative charges on the side chain of the substituted amino acid and LPS might clash, and that this clash might occur more efficiently when position K34 is changed to a glutamate relative to a one-carbon shorter aspartate. To test if these severe defects in LPS transport were limited to changes that reverse the charge, we substituted LptG residue K34 with others that maintain the positive charge (K34R) or neutralize it and are either nonpolar (K34C and K34A) or polar (K34Q). Alleles encoding these substitutions complemented, and while the \( lptG(K34R) \) allele behaved similarly to wild-type \( lptG \), the remaining alleles increased sensitivity to hydrophobic antibiotics, although not as severely as \( lptG(K34D) \) (Fig. 2.2C and Table S1). These results therefore suggest that the charge of residue K34 in LptG is important for LPS transport in \( E. coli \).

We further explored the effect of the charge at position 34 of LptG in \( E. coli \) by exploiting the ability to modify \( LptG^{K34C} \) with cysteine-reactive small molecules that form adducts of different charges since there are no other free periplasmic cysteines in Lpt factors. Compounds 2-sulfonatoethyl methanethiosulfonate (MTSES) and 2-(Trimethylammonium)ethyl methanethiosulfonate (MTSET) cross the OM and react with free sulfhydryl groups in the periplasm to form structurally similar cysteine adducts that are either negatively or positively charged, respectively (Fig. 2.4A) (194, 195). Since substituting K34 in LptG with cysteine conferred mild defects (Fig. 2.2C, Table S1), we thought that we could test the effect of adding differently charged adducts at this position using MTSES and MTSET, provided that the internal cavity formed by LptFG is accessible to the
periplasmic environment. If a positive charge at position 34 is required for proper LptG function, we predicted we might be able to further decrease LPS transport, and thereby increase the OM permeability, in \textit{lptG}(K34C) cells by treating them with negatively charged MTSES but not with positively charged MTSET. To test these predictions, we monitored the growth of wild-type and haploid \textit{lptG}(K34C) cells in the presence of a sub-inhibitory concentration of the lysis-inducing hydrophobic antibiotic bacitracin, which cannot efficiently cross the wild-type OM, but can enter cells with defects in the Lpt system. We then continued to monitor their growth after the addition of either MTSES or MTSET. Under the conditions tested, the growth of wild-type cells was not significantly affected by the addition of either MTSES or MTSET (Fig 4B). However, as predicted, the addition of MTSES, but not that of MTSET, increased the sensitivity of \textit{lptG}(K34C) cells to bacitracin, as reflected by the induction of lysis 1.5 h after treatment (Fig. 2.4B). These results further demonstrated that the positive-charge character of K34 is crucial for its function. \textit{In toto}, the severe phenotypes observed by reversing the charge of K34 in LptG of \textit{E. coli}, the previous data on its equivalent residue in \textit{B. cenocepacia} (178), and the placement of this residue within the internal cavity formed by LptFG support a model in which this site contacts opposite charges in lipid A during its extraction from the IM.
Figure 2.4 Charge requirement in cavity-facing residues in LptG for proper LPS transport.

A) Structures of MTSES, MTSET and their products after they react with free cysteine thiols. B) Shown is sensitivity of various residues in the K34 region of LptG to modification by a negatively charged moiety (MTSES) or positively charged moiety (MTSET) when grown in the presence of sub-inhibitory concentrations of bacitracin. Defects in LPS transport increase sensitivity to bacitracin, which results in a drop in culture turbidity (OD$_{600}$) as a result of lysis. Arrows indicate time of MTSET/MTSES addition. Refer to Experimental Procedures for details.
2.3.3 The periplasmic end of transmembrane domain 1 in LptG constitutes a functional domain in LPS transport.

Having identified the positive charge of K34 in LptG as important for LPS transport, possibly by mediating an interaction with the phosphates of lipid A, we set out to investigate if other charged or polar residues in the same region of LptG are also important for function. The LptB$_2$FG structure (149, 150) revealed two additional positively charged residues (K40 and K41) in helix 1 of LptG that are positioned two turns away from K34, and a negatively charged (D37) and a polar (Q38) residue one turn away from K34 (Fig. 2.2 A-B). We thus generated LptG variants with substitutions at these sites and assessed their function and protein levels.

We found that alleles encoding an aspartate substitution at either K40 or K41 of LptG readily complemented and, unlike $lptG(K34D)$, only caused a mild increase in OM permeability (Fig. 2.2, Table A.1). We then tested for possible functional redundancy between these residues and K34 by constructing double and triple alleles. The $lptG(K40D/K41D)$ allele yielded a haploid strain. This was not surprising, given that an $lptG(K40E/K41E)$ allele has been reported to support growth of an LptFG-depletion strain under depleting conditions (150). However, we observed that the $lptG(K40D/K41D)$ haploid strain was commensurably more sensitive to hydrophobic antibiotics than its parental single mutants (Fig. 2.2C, Table A.1). Further introducing either a K34D or K34A change into the $lptG(K40D/K41D)$ allele resulted in triple mutant alleles that were unable to complement the loss of chromosomal $lptG$, despite causing no notable decrease
in protein levels (Fig. A.1). This synthetic lethality and the proximal location of these residues within LptG suggest these residues function together in LPS transport.

To further evaluate the functional role of the positively charged residues in this domain, we prepared proteoliposomes containing LPS and LptB\textsubscript{2}CFG complexes containing wild-type LptG (LptG\textsuperscript{WT}), LptG\textsuperscript{K34D} or LptG\textsuperscript{K41D}, and examined their ability to extract LPS. To monitor LPS extraction from these liposomes, we added purified LptA-I36\textsubscript{p}BPA-His\textsubscript{6}, an LptA variant containing the UV-crosslinkable amino acid \textit{p}-benzoyl phenylalanine (\textit{p}BPA) (148, 158). In this assay, LPS molecules that are extracted by LptB\textsubscript{2}CFG complexes and transferred to LptA can be covalently trapped in LptA upon UV crosslinking. The resulting LptA-LPS adducts can be detected by a mass shift after immunoblotting for LPS. As previously reported, complexes transferred LPS to LptA in a time- and ATP-dependent manner (Fig. 2.2D) (148, 158). Consistent with the sensitivity data discussed above, the ability to release LPS to LptA was reduced for both variant-containing complexes relative to the LptG\textsuperscript{WT} complexes, with LptG\textsuperscript{K34D} complexes releasing less LPS than those containing LptG\textsuperscript{K41D} (Fig. 2.2D).

We also examined the negative and polar residues (D37 and Q38) of this domain, and found that alleles encoding either alanine or cysteine substitutions at D37 or Q38 also complemented the deletion of chromosomal \textit{lptG}, but the resulting haploid strains exhibited OM-permeability defects (Fig. 2.2C, Table A.1). As before, we also took advantage of the ability to covalently modify cysteine residues with charged sulfhydryl-reactive reagents to investigate the
effect of introducing negative and positive charges at D37 and Q38. Notably, for
lptG(D37C) and lptG(Q38C) cells, we had to use a fourth of the concentration of bacitracin than that used for wild-type and lptG(K34C) strains, and observed effects on growth in a shorter time scale. We do not currently understand if this time difference is related to differences in the accessibility of these residues to the chemical probes or, more likely, to the fact that the lptG(K34C) mutant is less permeable to bacitracin than the lptG(D37C) and lptG(Q38C) mutants (Fig. 2.2C). Nevertheless, our results revealed that reversing the charge of D37 or adding a charge at Q38 conferred sensitivity (Fig. 2.4B), indicating that the negative charge of D37 and the uncharged polar nature of Q38 are important for LptG function. Together, these results show that charged and polar residues at the membrane-periplasm interface of helix 1 in LptG play a crucial role in LPS transport.

2.3.4 Suppressor analysis suggests that residue K34 in LptG interacts with the phosphates of lipid A.

To further investigate the role of K34 in LptG, we selected for suppressors of OM-permeability defects of a haploid lptG(K34D) strain. One suppressor mutation that increased resistance to several hydrophobic antibiotics (Table A.1) generated the basS(L102Q) allele, which changes amino acid 102 in BasS from leucine to glutamine. BasS is the histidine kinase of the BasSR two-component system, which controls chemical modifications of LPS (Fig. 2.5A) (18). Most notably, BasSR upregulates transcription of the arm and eptA operons, which encode the enzymes that modify the phosphates at C1 and C4’ in lipid A with the
positively charged moieties L-Ara4N and PEtN, respectively (Figs. 2.1B, 2.5A) (18, 110, 113).

We wondered if \textit{basS(L102Q)} suppressed \textit{lptG(K34D)} by constitutively activating the BasSR system, as this would lead to increased modification of the lipid A phosphates with positively charged moieties. First, to determine if the \textit{basS(L102Q)} allele activates the BasSR system, we took advantage of the fact that modification of LPS with positively charged moieties results in resistance to the cationic antimicrobial peptide polymyxin B (18). As we predicted, \textit{lptG^+} and \textit{lptG(K34D)} strains carrying \textit{basS(L102Q)} were more resistant to polymyxin B than their respective \textit{basS^+} parents (Fig. 2.5B), indicating that \textit{basS(L102Q)} is a constitutive allele (\textit{basSc}). We next confirmed that this \textit{basSc} allele upregulates modifications in lipid A using MALDI-TOF mass spectrometry (MS) analysis of lipid A isolated from the wild-type strain, and from \textit{lptG(K34D)} mutants carrying either the \textit{basS^+} or \textit{basSc} alleles. MS analysis of the wild-type \textit{basS^+} sample revealed primarily the expected unmodified hexa-acylated 1,4'-bis-phosphate lipid A (Fig. A.2A) (9, 105). In contrast, the \textit{lptG(K34D) basS^+} sample contained equal amounts of unmodified hexa-acylated 1,4'-bis-phosphate lipid A and hepta-acylated 1,4'-bis-phosphate lipid A (Fig. A.2B). The presence of hepta-acylated LPS was expected since deficient LPS transport causes the mislocalization of phospholipids to the outer leaflet of the OM, which induces the enzymatic activity of the OM protein PagP to generate hepta-acylated LPS by transferring an acyl chain from a phospholipid to hexa-acylated LPS (100, 151). In contrast, the \textit{lptG(K34D) basSc} samples contained predominantly hexa-acylated lipid A modified with a single
PETN group and a smaller portion of hexa-acylated lipid A modified with two PETN groups (Fig. A.2C). We also detected a small amount (ca. 1%) of LPS modified with L-Ara4N (Fig. A.2). The increase in PETN modification was expected from the upregulation of \textit{eptA}, while the reduction in the levels of hepta-acylated lipid A was expected from its ability to suppress LPS transport defects conferred by \textit{lptG(K34D)}.

We next tested whether suppression of \textit{lptG(K34D)} by \textit{basSc} is mediated by the modification of the lipid A phosphates. Elimination of the PETN modification by deleting \textit{eptA} in the \textit{lptG(K34D) basSc} strain abolished most of the suppression (Fig. 2.5C, Table A.2). Although we only detected approximately 1% of LPS modified with L-AraN in the MS analysis of the suppressor strain (Fig. A.2), the residual suppression observed in the \textit{lptG(K34D) basSc \Delta eptA} strain was eliminated by deleting \textit{arnT}, which is needed for this modification (Fig. 2.5C, Table A.2) (18, 110). The relative effect of EptA and ArnT on suppression is consistent with the results of the MS analysis of lipid A showing that the \textit{basSc} allele strongly induces the modification of lipid A with PETN (Figs. 2.5A and A.2C).
Figure 2.5 BasSR-dependent activation of modification of LPS structure by EptA and ArnT suppresses LPS transport defects in \textit{lptG(K34D)} mutants.

A) Model of activation of LPS modification by the BasSR system. Activation of the BasS kinase, results in phosphorylation of the BasR response regulator. BasR activates transcription of the \textit{eptA} and \textit{arn} operons, which encode enzymes that modify lipid A with PEtN and L-AraN, respectively. B) Polymyxin resistance conferred by \textit{basSc} as determined by measuring its minimal inhibitory concentration (MIC) for various strains. C) Bacitracin sensitivity (in mm) of the \textit{lptG(K34D)} \textit{basSc} mutant in the presence and absence of \textit{eptA} and \textit{arnT}, as assessed by disc diffusion assay. Black line marks the width of the antibiotic discs. More details in Table A.2.

We next demonstrated that the \textit{basSc} suppressor allele did not change LPS levels (Fig. A.1D) and is not a general suppressor of Lpt defects. Specifically, \textit{basSc} cannot suppress other \textit{lpt} alleles such as \textit{lptD4213}, which alters the OM.
translocon (172), and \textit{lptF}(E84A) and \textit{lptG}(E88A), which alter the helices in LptFG that couple with LptB (193) (Fig. 2.6A, Table A.3). Taken together, our results and those from Hamad \textit{et al.} indicate that electrostatic interactions between residue 34 in LptG of \textit{E. coli} (residue 31 in \textit{Burkholderia}) and moieties at the C1 and C4' positions in lipid A are critical for LPS extraction from the IM.

2.3.5 Differential binding of modified and unmodified lipid A to the LptFG cavity.

Our results support the notion of an interaction between opposing charges at residue 34 of LptG and moieties at the C1 and C4' positions in lipid A in both the wild-type and \textit{lptG}(K34D) strains. However, we observed that the \textit{basSc} allele also suppressed LPS transport defects caused by changes of K34 in LptG to alanine, cysteine, and glutamine (Fig. 2.6B). Furthermore, the \textit{basSc} allele could also suppress defects conferred by changes in LptG residues D37, Q38, K40 and K41 (Fig. 2.6B, Table A.1). Together, these results suggest that \textit{basSc} does not suppress by simply restoring a charge-charge interaction between residue 34 and lipid A. The simplest explanation of the results presented in this study is that residues K34, D37, Q38, K40, and K41 in LptG constitute at least a portion of a binding domain for LPS in the cavity formed by LptFG. Within this domain, residue K34 forms electrostatic interactions with \textit{unmodified} phosphates on lipid A that are important for the extraction of LPS from the IM. In addition, the charges of residues D37, K40 and K41, and the polar character of residue Q38 are also important for the transport of unmodified LPS molecules. Lastly, our results also suggest that LPS molecules with positively charged modifications interact either with a different
domain in the LptFG cavity or with the same domain in helix 1 of LptG but using a different binding mode to that of unmodified LPS.

Figure 2.6 Activation of LPS modification by \textit{basS}^c specifically suppresses defects in the K34-K41 domain of LptG.

A) The \textit{basS}^c allele does not suppress defects in the coupling helix of LptF [\textit{lptF(E84A)} allele] and LptG [\textit{lptG(E88A)} allele], or in the OM translocon LptD (\textit{lptD4213}) as determined by bacitracin sensitivity (in mm) of the wild type and \textit{lpt} mutants carrying either wild-type \textit{basS}^+ (grey/black) or \textit{basS}^c (dark green). The black line represents the width of the antibiotic discs. B) OM-permeability defects of LptG variants with changes in the K34-K41 domain are suppressed by the \textit{basS}^c allele. Shown is bacitracin sensitivity (in mm), as assessed by disc diffusion assay, of WT and LptG variants in \textit{basS}^+/\textit{basS}^c backgrounds. Data for the \textit{basS}^c strains (dark green) are superimposed on their \textit{basS}^+ counterparts. The black line represents the width of the antibiotic discs. More details in Tables A.1 and A.3.
2.4 Discussion

LptF and LptG were identified a decade ago (151), but their role in LPS transport has remained largely obscure even after the recent elucidation of their structure (149, 150). Our results provide the first evidence that in *E. coli*, the cavity formed by LptFG interacts with LPS to extract it from the IM. We have identified an LPS-binding domain in LptG containing a cluster of residues that differentially contributes to the binding of unmodified and modified LPS. This domain is located in the periplasmic end of helix 1 of LptG and contains a positively charged residue (K34) that interacts via electrostatic interactions with unmodified phosphates in LPS. Interestingly, residue K34 is not conserved among LptG homologs, likely because the structure of lipid A varies among bacteria.

How LPS is extracted from the IM remains unknown, but it has been proposed that it enters into a central cavity formed by LptFG and that, once inside, movement of the ATPase LptB in the cytoplasm is transduced to LptFG so that they undergo conformational changes that lead to LPS extraction towards the periplasmic Lpt domains of LptF and/or LptG, and, eventually, LptC (Fig. 1A) (146, 148-150, 193). The domain we have identified to be critical for LPS transport is localized inside the LptFG cavity, right at the periplasmic interface of transmembrane 1 of LptG, where the hydrophilic portions of lipid A might be at the initial stages of extraction from the IM (Fig. 2.2A). Changing the positive charge of position K34 to a negative charge in this domain of LptG in *E. coli*, which normally produces bisphosphorylated lipid A, causes defects in LPS transport that can be ameliorated by modifying the phosphates on lipid A with positively charged
moieties. These findings are in line with results previously described in *Burkholderia* (178), and support a model stating that the charged side chain in this position of LptG interacts with the opposite charges at positions C1 and/or C4′ of lipid A molecules. Indeed, in *Burkholderia*, lipid A is constitutively modified with positively charged L-Ara4N and LptG has a negatively charged aspartate at the relevant position 31; inversely, in *E. coli* the phosphates in lipid A are normally unmodified and the relevant position in LptG, K34, is positively charged. However, this model alone cannot explain how, in wild-type *E. coli*, Lpt can transport both negatively and positively charged lipid A-containing LPS, since this bacterium can modify LPS with L-Ara4N and PEtN under certain conditions (18, 110, 113). The aforementioned model predicts that such modifications would result in a clash between positive charges in lipid A and K34 in LptG that would be expected to result in LPS transport defects. Yet, this is not the case, even in strains that constitutively activate the BasSR-regulated modifications of lipid A (Fig. 2.6A). Moreover, altering the charge and polar nature of residues near K34 in LptG (D37, Q38, K40, and K41) also causes defects in Lpt function that can be suppressed by constitutively activating BasSR in the presence of native K34 in LptG (Fig.6B).

To reconcile all these data, we propose that in wild-type *E. coli*: 1) residues K34, D37, Q38, K40 and K41 of LptG form at least a portion of a domain that mediates interactions between LPS and the cavity of LptFG; 2) this cluster of residues exhibits differential binding to unmodified LPS, with residue K34 mediating electrostatic interactions with a phosphate in lipid A that are crucial for the extraction of unmodified LPS from the IM; and, 3) modified LPS binds either to
this domain in LptG in a different way to that of unmodified LPS, or contacts a yet-to-be-identified site in the LptFG cavity. According to this model, wild-type *E. coli* can transport both types of LPS because the LptFG cavity can interact with both forms of LPS, albeit differently. In contrast, in the *lptG(K34D)* mutant, unmodified LPS, which is the predominant form synthesized under normal growth conditions, clashes with the negatively charged aspartate, thereby causing severe defects in LPS transport. These defects can then be suppressed by constitutively activating the BasSR system because this activation shifts the type of LPS the mutant produces from negatively charged to mostly positively charged, PEtN-modified LPS. This shift in the type of LPS molecules that are synthesized favors a different mode of interaction of LPS with the LptFG cavity, thereby increasing LPS transport and suppressing OM permeability defects in the *lptG(K34D)* mutant.

Our findings, and the fact that these LptG residues are localized at the membrane-periplasm interface of the LptFG cavity, lead us to propose that this cluster of residues mediate interactions with LPS at an early step in the extraction process. As such, the identity of these residues could serve to dictate how different LPS variants interact with the LptFG cavity prior to extraction. Since modification of lipid A occurs before LPS engages with LptB2FG, it is also possible that this cluster of residues in LptG could ultimately control the type and proportion of LPS being displayed at the cell surface. In fact, in *Burkholderia*, in which L-AraN modification of lipid A is essential and constitutive, LptFG may have evolved to only handle modified substrate (178). This could imply that residue D31 of LptG in *Burkholderia* may only be able to directly interact with L-AraN-modified LPS.
through electrostatic interactions, much like residue K34 in LptG of *E. coli* interacts with the unmodified phosphates. Residue D31 of LptG in *Burkholderia* would also prevent transport of unmodified LPS by clashing electrostatically with the phosphates. Alternatively, D31 might only serve to discriminate against unmodified LPS while L-AraN-modified LPS interacts differently with the transporter, much like we propose modified LPS does in *E. coli*. Altogether, we propose that the cavity of LptFG and the structure of LPS have co-evolved in different bacteria to ensure efficient transport and possibly modulate the diversity of LPS at the OM.

### 2.5 Experimental Procedures

#### 2.5.1 Strains and growth conditions.

Strains (Table A.4) were typically grown at 37°C in either lysogeny broth (LB) or M63 supplemented with 0.2% (wt/vol) glucose with aeration for liquid cultures and on 1.5% agar plates for solid media. When appropriate, media was supplemented with ampicillin (125 µg/mL), bacitracin (100 µg/mL), chloramphenicol (20 µg/mL), isopropyl-β-D-1-thiogalactopyranoside (0.16 mM), kanamycin (30 µg/mL), novobiocin (33 µg/mL), polymyxin B (2 µg/mL, 10 ug/mL), tetracycline (25 µg/mL), vancomycin (25, 75 µg/mL), and X-Gal (33 µg/mL).

#### 2.5.2 Mutant construction

*lptG* alleles were generated through site-directed mutagenesis PCR using the pBAD18LptFG3 plasmid (193), or pCDFDuet.LptBFG (148) as templates, PfuTurbo polymerase (Agilent Technologies, Inc.) or KOD Hot Start Polymerase (Novagen), and primers listed on Table A.5. Complementation tests and construction of haploid strains bearing mutant *lptG* alleles was carried out using
the segregation-defective pRC7LptFG plasmid system as described (193).

Mutant alleles of *eptA*, *arnT*, and *basS* were introduced into strains by P1<sub>vir</sub> transduction either through linkage to nearby marker *zjd2211::Tn10* (for *basSc*) or by selecting for kanamycin resistance (for Δ*eptA::kan* and Δ*arnT::kan*). The *kan* marker was removed by pCP20-encoded FLP recombinase (196). Allele replacement was verified by PCR and the presence of the *basS(L102Q)* allele was confirmed by DNA sequencing.

2.5.3 Selection and mapping of suppressors

A 100-µL aliquot of a culture of strain NR3673 [haploid *lptG(K34D)* strain] grown overnight in M63 with glucose was plated on LB solid medium supplemented with bacitracin (100 µg/mL). A single suppressor isolate was selected for mapping based on its robustly restored resistance to multiple antibiotics. The suppressor was mapped via P1<sub>vir</sub> co-transduction using as donor a randomly generated mini-Tn10 library (197). Loss of suppression was monitored by the inability to grow in the presence of vancomycin (25 µg/mL). A mini-Tn10 transposon marker, referred to as tet2-2, ~25% linked to the suppressor allele was inserted at minute 94 of the *E. coli* chromosome. Sequencing of this region of the chromosome revealed a change in nucleotide 305 of *basS* from T to A, and thereby altering residue 102 of the BasS protein from L to Q. This allele was therefore named *basS(L102Q)*.

2.5.4 Antibiotic sensitivity

To assay OM permeability to bacitracin, novobiocin, rifampin, and erythromycin, 100 µL of overnight cultures of haploid strains were grown in M63
with glucose and subjected to disc diffusion assays as described previously (147). MICs were determined by presence or absence of growth in liquid media in the presence of the compound at various concentrations in 96-well plates as described previously (197).

2.5.5 MTSES/MTSET assays

Strains were grown overnight in 5-mL cultures either in LB or M63 with glucose, and then diluted 1:500 or 1:250, respectively, in fresh LB containing sub-inhibitory concentrations of bacitracin (4 μg/mL for NR2761 and NR4077, 1 μg/mL for NR4518 and NR4232). These cultures were incubated at 37°C and their growth was monitored by measuring their optical density at 600 nm (OD_{600}) every 30 min for 2-3 h until an OD_{600} of ~0.2 was reached, upon which 0.4 mM of MTSES or MTSET was added. After adding these cysteine-reactive reagents, we continued to monitor OD_{600} every 30 min until a total elapsed time of 5 h.

2.5.6 Overexpression and purification of the Lpt inner membrane complex

To overexpress His_{6}-LptBFGC, His_{6}-LptBFG-K34D-C, and His_{6}-LptBFG-K41D-C, KRX cells were transformed with pET22/42.LptC and either pCDFDuetHis_{6}LptBFG, pCDFDuet.LptBFG(K34D) or pCDFDuet.LptBFG(K41D). Overexpression and purification of each inner membrane complex variant was done as previously reported (147). Cultures were grown at 37°C after diluting overnight cultures 1 to 100 into fresh LB broth supplemented with 50 μg/mL spectinomycin (Sigma) and 50 μg/mL carbenicillin (Teknova). Expression was induced with 0.02% L-rhamnose at OD_{600} ~ 1, and cultures were grown for 16 h at 18°C. Cells were harvested by centrifugation at 4,200 × g for 20 min and
resuspended in 50 mM Tris-HCl (pH 7.4), 300 mM NaCl, 2 mM MgCl₂, supplemented with 1 mM phenylmethanesulfonyl fluoride (PMSF, Sigma), 100 μg/mL lysozyme (Sigma), and 100 μg/mL DNase I (Sigma). The resuspended cells were passaged through an EmulsiFlex-C3 high-pressure cell disruptor three times. The cell lysate was centrifuged at 10,000 × g for 10 min to remove unbroken cells. Membranes were isolated by centrifugation at 100,000 × g for 1 h. Membranes were resuspended and solubilized in 20 mM Tris-HCl (pH 7.4), 300 mM NaCl, 5 mM MgCl₂, 10% (vol/vol) glycerol, 1% n-dodecyl-β-D-maltopyranoside (DDM, Anatrace), 2 mM ATP at 4 °C for 1 h, followed by centrifugation at 100,000 × g for 30 min. The supernatant was applied to Ni-NTA Superflow resin (Qiagen), and eluted with 20 mM Tris-HCl (pH 7.4), 300 mM NaCl, 10% (vol/vol) glycerol, 0.05% DDM, 100 mM imidazole. The eluate was concentrated with an Amicon centrifugation filter, 100-kDa molecular weight cutoff (MWCO, Amicon Ultra; Millipore), and then subjected to size exclusion chromatography on a Superdex 200 10/300 GL column (GE Healthcare) in 20 mM Tris-HCl (pH 7.4), 300 mM NaCl, 10% (vol/vol) glycerol, 0.05% DDM. Fractions were pooled and concentrated to ~5 mg/mL. All complexes were analyzed by SDS-PAGE to assess purity. Protein was flash frozen in liquid nitrogen and stored at -80°C until use.

2.5.7 Overexpression and purification of LptA

LptA-I36pBPA-His₆ was overexpressed in the periplasm and purified by making spheroplasts, as previously reported (148). BL21(λDE3) cells with pSup-BpaRS-6TRN and pET22b-LptA-I36pBPA-His6 were grown to OD600 ~ 0.5 in
500 mL LB broth supplemented with 50 μg/mL carbenicillin, 30 μg/mL chloramphenicol, and 0.8 mM pBPA. Protein expression was induced with 50 μM IPTG (Sigma) for 2 h at 37°C. The cells were harvested and converted to spheroplasts. The periplasmic fraction was incubated with Ni-NTA Superflow resin (Qiagen), washed with 20 mM Tris-HCl (pH 8.0), 300 mM NaCl, 20 mM imidazole, and then eluted with 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 200 mM imidazole. The purified LptA-I36pBPA-His6 was concentrated with 10 kDa cut-off Amicon centrifugal concentrators (Millipore) to ~2.5 mg/mL, flash frozen with liquid nitrogen and kept at -80°C in 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 200 mM imidazole containing 10% glycerol.

2.5.8 IM complex proteoliposome preparation

Inner membrane proteoliposomes were prepared as previously described (158). Aliquots of *E. coli* polar lipid extract stock solution and LPS were thawed and sonicated briefly to homogenize. Proteoliposomes containing the Lpt inner membrane complex variants were prepared by a detergent dilution method (198). Prior to dilution, a mixture with the following final concentrations was prepared in 20 mM Tris-HCl (pH 8.0), 150 mM NaCl buffer: 7.5 mg/mL lipid stock, 0.5 mg/mL LPS, 0.25% DDM, and 0.86 μM purified inner membrane complex. To make this mixture, the DDM was first added to the lipid stock solution to make detergent-destabilized liposomes. LPS was added to this mixture, which was kept on ice for 10 min to allow for mixed detergent-phospholipid-LPS micelles to form. The protein complex was added, and the mixture was incubated on ice for 20 min. The mixture was then transferred to an ultracentrifuge tube and diluted 100× with cold
20 mM Tris-HCl (pH 8.0), 150 mM NaCl. After letting the diluted mixture sit on ice for 30 min, the proteoliposomes were pelleted by ultracentrifugation at 300,000 × g for 2 h at 4°C. The proteoliposomes were resuspended in 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, diluted 100× again, then pelleted by ultracentrifugation at 300,000 × g for 2 h at 4°C. For every 100 μL original mixture (prior to the first dilution step), 250 μL cold 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10% glycerol was added. If the resuspended proteoliposomes were not used immediately, they were flash frozen in liquid nitrogen and stored at -80°C.

2.5.9 Reconstitution of LPS release to LptA

The reconstitution of LPS release from proteoliposomes to LptA was conducted similarly to the ATPase assay described above. All assays were done in 50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 10% glycerol (final concentrations). Reactions contained 60% inner membrane proteoliposomes by volume (prepared as described above, thawed on ice). The remaining volume was composed of Tris-HCl, NaCl, and glycerol such that the final concentrations would be the above values. All assays were done in 50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 10% glycerol (final concentrations). Reactions contained 60% IM proteoliposomes by volume (prepared as described above, thawed on ice). The remaining volume was composed of Tris-HCl, NaCl, and glycerol such that the final concentrations were the above values. LptA-I36pBPA-His₆ was added to a final concentration of 2 μM prior to starting the assay. Assays were initiated at 30°C with the addition of ATP/MgCl₂ (final concentrations were 5 mM ATP, 2 mM MgCl₂). At specified time points, 25 μL aliquots were removed from the reactions and added to a microtiter
plate, which was subsequently irradiated with UV light (365 nm) on ice for 3 min using a B-100AP lamp. Following UV-irradiation, samples were added to 225 μL cold 20 mM Tris-HCl (pH 8.0), 150 mM NaCl supplemented with 0.2% DDM. To each sample, 250 μL 20% TCA was added. The proteins were precipitated and gel samples prepared as described above. Samples were boiled and subject to SDS-PAGE followed by immunoblotting.

2.5.10 Preparation of lipid and LPS stock solutions.

For proteoliposome preparation, *E. coli* polar lipid extract (Avanti Polar Lipids, Inc.) was dissolved in water and sonicated for 30 minutes to make a 30 mg/ml aqueous suspension stock. The lipid stock solution was flash frozen in liquid nitrogen and stored at -80°C. A 2 mg/mL aqueous suspension stock of LPS from *E. coli* EH100 (Ra mutant, Sigma) was dissolved in water and sonicated for 30 minutes. The LPS stock solution was flash frozen in liquid nitrogen, and stored in aliquots at -80°C.

2.5.11 Isolation of lipid A from *E. coli* cells.

Cultures of each strain were grown in LB medium (200 mL) at 37°C. Cells were harvested when they reached OD ~ 1.0 and then washed with 25 mL phosphate buffered saline, pH 7.4. Lipid A was isolated from each sample using the Bligh-Dyer extraction method and acid hydrolysis (199). Briefly, pellets were resuspended in 95 mL of a single-phase Bligh-Dyer mixture (chloroform:methanol:PBS (pH 7.4); 1.0:2.0:0.8 v/v). The mixture was allowed to sit at room temperature for 30 minutes for lysis. The sample was centrifuged at 2,000 x g for 20 minutes and then the pellet was washed with an additional 25
mL of single phase Bligh-Dyer mixture. After centrifugation, the pellet was suspended in 2.4 mL hydrolysis buffer (50 mL sodium acetate, pH 4.5, 1% SDS) and boiled for 30 minutes. Lipid A was harvested by extractions in two-phase Bligh-Dyer mixture (chloroform/methanol/water, 2:2:1.8 v/v). After extraction, the combined chloroform layers were dried by rotary evaporation and the sample was transferred using a 4:1 chloroform:methanol mixture. The dried sample was washed once with acidified ethanol (2% HCl in EtOH) and 2x with ethanol to ensure complete removal of SDS (200). Samples were then lyophilized and stored at -20°C.

2.5.12 Mass spectrometry of lipid A species

Samples were analyzed using a Bruker Ultraflextreme MALDI-TOF/TOF mass spectrometer. An ATT matrix was used and it was prepared as follows. A saturated solution of 6-aza-2-thiothymine in 50% acetonitrile and a saturated solution of tribasic ammonium citrate were combined (20:1, v/v). Lipid A samples were dissolved in chloroform:methanol (4:1). The matrix (0.5 uL) was deposited on the sample plate and allowed to dry and then an equal volume of sample was added on top of the matrix. Each spectra represents the average of ~50 laser shots. Data were acquired in negative-ion mode with a reflectron analyzer and delayed extraction.

2.5.13 Immunoblotting

Levels of LptG variants were assessed by immunoblotting with rabbit anti-LptG antisera and horseradish peroxidase-conjugated anti-rabbit antibody from goat (GE Healthcare Life Sciences) using whole cell lysates from overnight
cultures that were normalized for cell density based on OD$_{600}$ readings, as described previously (193). LPS levels were determined similarly, substituting anti-LPS antibody (Bio-Rad 4329-5004) for the primary antibody and horseradish peroxidase-conjugated anti-mouse antibody from sheep (GE Healthcare Life Sciences) for the secondary antibody. Quantitation of LPS levels was carried out with Imagelab 5.2.1 (Bio-Rad).
Chapter 3: Structural basis of unidirectional export of lipopolysaccharide to the cell surface

Foreword

The following chapter was adapted from an article of the same title, for which I was a contributing author, published as a letter in the journal Nature in 2019. Specifically, I contributed to the design and data collection for cysteine disulfide-crosslinking experiments, which are represented in figures 4D and B.9 (data I collected is in fig. B.9a-d). Extended data is in appendix B, and supplementary information is in appendix C. This article was published alongside a related article by Li et al. (201).

3.1 Abstract

Gram-negative bacteria are surrounded by an inner cytoplasmic membrane and by an outer membrane that serves as a protective barrier to limit entry of many antibiotics. The distinctive properties of the outer membrane are due to the presence of lipopolysaccharide (202). This large glycolipid, which contains numerous sugars, is made in the cytoplasm and a complex of proteins forms a membrane-to-membrane bridge that mediates transport of lipopolysaccharide from the inner membrane to the cell surface (202). The inner-membrane components of the protein bridge comprise an ATP-binding cassette (ABC) transporter that powers transport, but how this transporter ensures
unidirectional lipopolysaccharide movement across the bridge to the outer
membrane is unknown (146). Here we describe two crystal structures of a five-
component inner-membrane complex that contains all the proteins required to
extract lipopolysaccharide from the membrane and pass it to the protein bridge.
Analysis of these structures, combined with biochemical and genetic
experiments, identifies the path of lipopolysaccharide entry into the cavity of the
transporter and up to the bridge. We also identify a protein gate that must open
to allow movement of substrate from the cavity onto the bridge.
Lipopolysaccharide entry into the cavity is ATP-independent, but ATP is required
for lipopolysaccharide movement past the gate and onto the bridge. Our findings
explain how the inner-membrane transport complex controls efficient
unidirectional transport of lipopolysaccharide against its concentration gradient.

3.2 Main Text

Lipopolysaccharide (LPS) biosynthesis is completed in the outer leaflet of
the inner membrane. LPS is then transported to the outer membrane by a protein
bridge that comprises seven conserved lipopolysaccharide transport proteins
(LptB2FGCADE, Fig. 3.1a) (158, 203). The inner membrane components,
LptB2FG, comprise an ATP-binding cassette (ABC) transporter (151, 169, 176), a
family of proteins that is conserved in all domains of life (204, 205). ATP binding
and hydrolysis by the cytoplasmic ATPase LptB provides the energy to move
LPS across the periplasmic bridge (147, 148). LptB2FG forms a stable sub-
complex with another component, LptC, which is anchored in the membrane by a
single transmembrane (TM) helix (170, 176). LptC receives LPS from LptFG and
transfers it to LptA (148, 158), which connects the inner membrane complex to LptDE, the outer-membrane translocon (158, 162, 164, 173, 174, 206). The bridge model for LPS transport has been likened to a Pez candy dispenser in which candies are pushed up the stack and out of the dispenser by a spring at the base (146, 158). LptB2FG serves as the spring, but how the complex functions to achieve unidirectional LPS transport is not understood at a molecular level.

LptC, which contains a periplasmic domain that binds LPS (148, 153), is the key to understanding unidirectional movement of LPS. Recent structures of LptB2FG (149, 150) did not define the path taken by LPS during movement into the cavity and out of the membrane. We screened homologs of LptB2FGC from multiple Gram-negative bacteria for functional protein expression (Fig. B.1), and obtained crystals of *Vibrio cholerae* and *Enterobacter cloacae* LptB2FGC complexes that were refined to 2.85 Å and 3.2 Å resolution, respectively (Fig. 3.1b, Fig. B.2, and Table B.1).

LptF and LptC form a continuous β-jellyroll via an edge-to-edge interaction between their C- and N-terminal β-strands (Fig. 3.1b), and there are numerous contacts between side chains on the convex surfaces of the LptF and LptG β-jellyroll sheets. The transmembrane anchor of LptC interdigitates between LptG TM1 and LptF TM5 (Figs. 3.1b and 3.1c). To our knowledge, no other ABC-system contains a transmembrane helix from another protein incorporated directly into the transporter (205, 207). Below we show that this helix regulates transport activity.
Figure 3.1 Crystal structure of the inner-membrane complex of the LPS transport machine.

a, A schematic showing the seven protein components of the LPS transport machinery and movement of LPS from the inner membrane to the outer leaflet of the outer membrane. The soluble protein LptA and the periplasmic domains of LptC and LptD form a protein bridge across the aqueous periplasm. b, Ribbon diagram of *V. cholerae* LptB2FGC, with LptC in pink, the two copies of the ATPase LptB in brown and the transmembrane components LptF and LptG in green and blue, respectively. The membrane is shown in grey. c, Ribbon diagram depicting the view from the periplasm into the cavity between the transmembrane helices.

To probe the *in vivo* relevance of the crystal structures, we incorporated a photocrosslinkable unnatural amino acid (*p*-benzoylphenylalanine, *p*BPA) at
multiple sites in LptFGC (208) and tested for protein-protein contacts. We replaced several residues at the interface between LptC and LptF, and at the terminal β-strand of the LptG β-jellyroll (Fig. 3.2a). We found extensive crosslinks between LptC and LptF, but the only crosslink between LptC and LptG was from a residue on the convex side of LptC that faces LptG (Fig. 3.2b, Fig. B.4a). We conclude that the marked asymmetry observed in the crystal structure is physiologically relevant.

LptC creates a single pathway for entry and movement of LPS onto the bridge. Although LptC mutants lacking the TM helix can survive in the laboratory, only full length LptC is found in the wild, implying a strong selection for the TM helix (35, 152). To probe the role of the LptC TM helix, we compared the functions of LptB₂FG complexes containing either no LptC, wild-type LptC or LptC with the TM helix removed (hereafter LptC(ΔTM)) in proteoliposomes. LPS transport to LptA was greatly diminished in the absence of LptC, but LptC(ΔTM), which stably associated with LptB₂FG (Fig. B.4b), supported transport comparably to full-length LptC (Fig. 3.2c). However, even when a large excess (10X) of LptC(ΔTM) was used, the ATPase activity of the LptC(ΔTM) complex was substantially higher than for LptB₂FGC (158) (Fig. 3.2d; Fig. B.4c). Because LptB₂FGC containing full length LptC uses less ATP to transport LPS than LptB₂FG with LptC(ΔTM), we conclude that the TM domain of LptC modulates ATP hydrolysis to achieve more efficient coupling of ATP hydrolysis and LPS movement.
Previous structures of LptB2FG suggested an alternating-access model with two possible gates on opposite sides of the transporter for entry of LPS into the cavity (Fig. 3.2e, left panel, and Fig. B.5) (149, 150). In the absence of LptC, these entry paths are structurally similar and related by two-fold rotational pseudosymmetry. However, the presence of the LptC TM helix breaks the symmetry and differentiates the potential points of entry. Our structures define a single path for LPS entry into the cavity between LptG TM1 and LptF TM5 where the TM segment of LptC is located; this path is directly aligned with the concave surface of the continuous β-jellyroll formed by the interaction LptF and LptC. Entry between LptG TM5 and LptF TM1 is prevented by the steric barrier created by the convex surface of the continuous LptC-LptF β-jellyroll (Fig. 3.2e, right panel).
Figure 3.2 LptC promotes the efficient transport of LPS to LptA.

a, View of the junction between the β-jellyrolls of LptG (blue), LptF (green) and LptC (pink) in the *V. cholerae* LptB2FGC structure. Amino acids shown as red sticks were substituted with *p*BPA for experiments in b; corresponding *Escherichia coli* residues are shown in parentheses (see Fig. B.3). b, *E. coli* LptB2FGC complexes containing *p*BPA were photocrosslinked in vivo and adducts were detected by pulling down His-tagged LptC and immunoblotting with antibodies against LptC, LptF and LptG. Corresponding LptC immunoblots are shown in Fig. B.4a. c, *E. coli* LptB2FG or LptB2FGC complexes were reconstituted into LPS-containing proteoliposomes and their ability to transport LPS to LptA was assessed (see Fig. B.1b). d, ATP hydrolysis by LptB2FG or LptB2FGC in proteoliposomes was monitored by measuring levels of inorganic phosphate. Bars represent the averages of three biological replicates, with error bars showing the s.d. e, The *Pseudomonas aeruginosa* LptB2FG structure (left, Protein Data Bank (PDB) ID 5X5Y) (149) and *V. cholerae* LptB2FGC structure (right). The *Klebsiella*
*pneumoniae* LptB2FG structure (PDB ID 5l75) (150) is shown in Fig. B.5a. Blots shown in b and c are representative of data from three biological replicates.

To probe how LPS moves into the cavity and up to LptC, we used *in vivo* photocrosslinking to determine where LPS binds. (148) We trapped LPS in two distinct regions of the transporter (Fig. 3.3 and Figs. B.6 and B.7): at the base of the transport machine between the interdigitated LptC helix and LptG TM1 and LptF TM5 (Fig. 3.3a and 3.3b, and Fig B.6c and B.6d) and at the interface of the β-jellyroll between LptF and LptC (Fig. 3.3c, Fig. B.7). We did not observe crosslinks to LPS at the alternate entry point between LptG TM5 and LptF TM1 (Fig. B.6e). The crosslinks at the base of the transporter indicate how LPS enters the cavity; we trapped LPS at two sites, one located just outside the cavity as defined by crosslinks to LptC(M19pBPA) and LptF(S315pBPA), and one located inside the cavity as defined by LptC(G21pBPA) and LptG(S30pBPA). The crosslinks at the interface between the β-jellyrolls of LptF and LptC (LptC(F78pBPA) and LptF(R223pBPA), Fig. 3.3b,c and Fig. B.6a) indicate how LPS might move from the cavity onto the bridge, and are also consistent with the single path for movement defined by the structures.

We compared *in vivo* crosslinking intensities at the different LPS-binding sites (Fig. 3.3b) in wild-type and catalytically dead complexes to probe which steps in LPS transport are ATP-dependent. Crosslinking was negligible to the β-jellyroll of LptC and LptF in the complex that contained the inactive LptB(E163Q) (147) ATPase, but was much stronger to binding sites at the base of the cavity.
By contrast, the LPS crosslinks to the β-jellyroll of LptC and LptF were much stronger in complexes that contain wild-type LptB. These results suggested that entry into the cavity of the LptB2FGC transporter does not require ATP hydrolysis, but accumulation of LPS in the β-jellyroll does. Increased crosslinking intensities at the base of the cavity for the inactive transporter is likely to reflect the build-up of LPS in the inner membrane owing to impaired transport.(170)

We performed in vitro crosslinking experiments to clarify whether entry into the cavity or extraction from the membrane requires ATP. Crosslinking to LPS at residue LptC(G21pBPA), at the base facing the interior of the cavity, was observed in the absence of ATP (resting state) and in a pre-hydrolysis state in which ATP is present but hydrolysis is not possible owing to a lack of Mg2+ (+EDTA, Fig. 3.3d). Crosslinking to LptC(F78pBPA) in the β-jellyroll was not observed under these conditions. Similar results were obtained for the complex containing the LptB(E163Q) mutant or when the non-hydrolyzable ATP analog AMPPNP was used (Fig. B.6c). Weak crosslinking to LptC(F78pBPA) was observed in the presence of ATP and vanadate (Fig. 3.3d), consistent with a transporter that can extract but cannot turn over continually owing to inhibition by ADP-vanadate. Taken together, these results show that entry into the transporter cavity does not require ATP; however, ATP is required to extract LPS out of the membrane.
Figure 3.3  LPS entry into the cavity of the transporter is ATP-independent but extraction out of the membrane and onto the bridge requires ATP.

a, Ribbon diagrams of LptB2FGC structures showing functionally relevant sites, with LptG in cyan, LptC in pink and LptF in green. Left, the gate through which LPS enters the cavity. Right, the junction of the β-jellyrolls between \textit{V. cholerae} LptC and LptF. Residues shown as red sticks were substituted with \textit{p}BPA for photocrosslinking experiments shown in b–d; the corresponding \textit{E. coli} residues are shown in parentheses. b, \textit{in vivo} photocrosslinking experiments comparing LptC–LPS crosslink strength at three positions in LptC in LptB2FGC complexes that contain either wildtype LptB or LptB(E163Q), which cannot hydrolyze ATP. Crosslinks to LPS were detected with antibodies against LPS. c, In wild-type complexes, residues at the LptC–LptF interface were substituted with \textit{p}BPA and photocrosslinked to LPS in \textit{vivo}. d, \textit{E. coli} LptB2FGC variants with \textit{p}BPA substitutions at positions M19, G21 or F78 were reconstituted into proteoliposomes that contain LPS. The ability of these LptC variants to crosslink LPS was tested by exposure to UV light after 30-min incubation with either buffer only or buffer with
ATP–Mg2+, ATP–VO4 – or ATP– EDTA. See Fig. B.6 for additional crosslinking experiments that support an external and an internal binding site for LPS at the entry gate, as well as experiments showing undetectable LPS binding to sites in the LptG β-jellyroll. Data shown in b–d are representative of results from three biological replicates.

The LPS transporter presents a problem not found in other ABC transporter systems, because LPS in the bridge can potentially flow backward when the cavity reopens. Unless the rate of entry of LPS into the cavity is much faster than backward flow, there must be a mechanism to prevent the latter or transport would be inefficient. A notable feature of our LptB2FGC crystal structures is that the β-jellyroll of LptF exists in two structurally distinct states: in the *E. cloacaec* complex it is open (Fig. 3.4a and 3.4b), whereas in the *V. cholerae* complex it is closed (Fig. 3.4b, Fig. B.8). To determine if transport requires access to the open state, we introduced a pair of cysteines capable of forming a disulfide bond into LptF to trap it in the closed state (Fig 3.4c). Cells that express LptF containing the single cysteine mutations were viable, but those that produce the double cysteine variant — LptF(Cys2) — were nonviable, even when cellular levels were similar to those of wild-type LptF (Fig. B.9a-c). LptF(Cys2) was dominant negative, which implies that the mutant LptF formed a non-functional complex with the other inner-membrane Lpt components (Fig. B.9d). We successfully purified LptB2F(Cys2)GC complexes (Fig. B.9e), which shows that the loss of viability was not due to an assembly defect. Using an *in*
vitro LPS transport assay (Fig. 3.4d), we observed transport by the LptB_{2}F(Cys2)GC mutant complex only upon the addition of DTT (Fig. 3.4e), which reduced the disulfide and allowed LptF to access an open state. In contrast, ATP hydrolysis occurred regardless of whether the gate in LptF was open or closed (Fig. B.9f), which indicates that gate-opening is not directly coupled to ATP hydrolysis. We infer that movement of the substrate, driven by ATP binding or hydrolysis, pushes the gate open. Spontaneous gate closure behind the substrate provides a mechanism to reduce the rate of backward flow when the cavity reopens.

Figure 3.4 Unidirectional LPS transport is promoted by a gate in the β-jellyroll of LptF.

a, Surface representation of *E. cloacae* LptB_{2}FGC in a gate-open conformation. Unresolved residues between the transmembrane helix and β-jellyroll of LptC are denoted with a dashed magenta line. The edges of the open surface of the LptF
and LptC β-jellyroll domains are highlighted with a dashed blue line. b, The distinct gate-closed and gate-open conformations of the *V. cholerae* complex (top) and the *E. cloacae* complex (bottom) at the LptF–LptC interface. c, Two spatially proximal amino acids in the *V. cholerae* LptF β-jellyroll, S157 and I234, were replaced with cysteines in the corresponding *E. coli* LptF to enforce a closed conformation by spontaneous formation of a disulfide bond. *V. cholerae* LptF residues 154–157 and 233–236 are shown as semitransparent spheres to emphasize the close packing of side chains. d, LPS release to LptA I36pBPA from proteoliposomes containing LptB2FGC with LptF cysteine variants; UV-dependent photocrosslinking to LPS was detected with an LPS antibody, and His-tag blots show the amount of LptA(I36pBPA)–His6 in each sample. See also Figs.B.8, B.9. The blots are representative of experiments performed in triplicate. e, Model depicting LPS movement through LptB2FGC onto the bridge. The opening and closing of the gate in LptF is denoted by the symbols defined in b.

On the basis of the structural and functional studies reported here, we propose the following model for LPS transport. First, newly synthesized LPS present in the inner membrane binds at the entry point defined by the LptC TM segment intercalated between LptG TM1 and LptF TM5 (Fig 3.4f), and ATP-independent entry of LPS into the cavity past the LptC TM segment occurs (209). ATP-dependent cavity constriction then provides the force to push LPS out of the membrane. The LptC TM helix coordinates ATP hydrolysis with extraction from...
the membrane, and this role in transport efficiency apparently provides such an
important fitness advantage that it is conserved in the wild even though it can be
bypassed in the laboratory under certain conditions. (152, 210) The gate in the
β-jellyroll of LptF also improves transport efficiency by preventing backward
movement of LPS into the membrane as the cavity reopens, ensuring
unidirectional traffic from LptC to LptA and through LptDE to the outer
membrane.

As correct LPS transport is critical for survival, the structures and
functional information reported here may lead to new strategies for the
development of antibiotics. Such strategies may include binding tightly to LPS in
the cavity of the machinery to jam it or disrupting interfaces between components
of the machinery to reduce efficiency of transport. Compounds that impair outer-
membrane assembly would make Gram-negative bacteria sensitive to many
antibiotics that cannot normally penetrate into the bacteria.
Chapter 4: Suppression of lipopolysaccharide transport defects by increased synthesis and its implications for lipopolysaccharide essentiality

Foreword

The following chapter is based on on-going research in the laboratory of Natividad Ruiz. I am the primary author of the following section and contributed to the ideas described, as did other members of the Ruiz lab. I generated all the data shown.

4.1 Abstract

Gram-negative bacteria possess two membranes, an inner membrane, enclosing cytoplasmic components, and an outer membrane, which delineates the cell. This outer membrane is highly asymmetric in lipid content, containing phospholipids in the inner leaflet, and tightly packed lipopolysaccharides in the outer leaflet. As a result, the outer membrane poses a barrier to the entry of toxic molecules, such as antibiotics, into the cell. How lipopolysaccharide is transported from its site of synthesis in the cytoplasm to its assembly point in the outer membrane is not understood. Previously, we reported on a critical residue within the lipopolysaccharide transport complex, which we found to be important in early interactions with the substrate. Here, we exploited a defective variant of the transport complex in which this residue was altered to discern how this problem might be overcome, thereby giving us insight into the transport
mechanism. We found that defects caused by this variant could be suppressed by increased lipopolysaccharide synthesis, but interestingly, defects in lipopolysaccharide transport caused by other alleles could not. The suppression conferred by increased lipopolysaccharide synthesis could therefore be a potential tool for distinguishing if early substrate binding, or some other downstream stage of lipopolysaccharide transport, has been affected by other alleles or inhibitors. Additionally, we found that lipopolysaccharide overproduction was better tolerated by strains defective in lipopolysaccharide transport than by wild-type strains, which supports a model in which lipopolysaccharide essentiality is tied to balanced membrane synthesis.

4.2 Introduction

Gram-negative bacteria are characterized by having a dual membrane system: An inner membrane, enclosing cytoplasmic components, and an outer membrane, which defines the boundaries of the cell and together with the inner membrane delineates an additional cellular compartment termed the periplasm (1). Anchored to the outer membrane is the peptidoglycan cell wall, which gives the cell its shape and protects it from osmotic lysis (1). With respect to lipid composition, the inner membrane is a relatively typical biological membrane, composed of a bilayer of phospholipids (1). The outer membrane, in contrast, is a highly asymmetric structure, containing phospholipids in the inner leaflet of its bilayer, and the complex glycolipid lipopolysaccharide, or LPS, in the outer leaflet (1, 211). LPS is an amphipathic molecule, with a highly acylated, and ergo hydrophobic, bisphosphorylated di-glucosamine membrane anchor that is
attached to a series of hydrophilic sugar polymers (211). Due to LPS’s amphipathic nature and tight packing, the outer membrane of Gram-negative bacteria presents a potent barrier to the entry of toxic molecules, such as antibiotics, into the cell (5, 211). Additionally, LPS is essential in most, but interestingly not all, Gram-negative bacteria, though the exact nature of this essentiality has not been fully clarified (4, 211).

LPS is synthesized in the cytoplasmic leaflet of the inner membrane, from which it must be transported to be assembled in the outer leaflet of the outer membrane (211). LPS must therefore traverse the inner membrane, the aqueous periplasm, and the outer membrane. Transport across the inner membrane is driven by an ATP-binding cassette (ABC) transporter, MsbA (139, 141, 211). Once in the outer leaflet of the inner membrane, LPS must still pass through the periplasm and outer membrane. This poses extra difficulty, in that there is no ready source of energy in the periplasm, such as ATP, to drive this process. This problem is solved by the Lpt (lipopolysaccharide transport) system, which is composed of seven unique proteins, and forms a proteinaceous bridge that spans the cell envelope (Fig. 4.1) (211). This architecture allows the coupling of cytoplasmic ATP hydrolysis to the transport of LPS across the periplasm and outer membrane (211). Specifically, a dimer of LptB proteins in the cytoplasm binds and hydrolyzes ATP to drive continuous binding and extraction of LPS from the inner membrane by the transmembrane domains LptF and LptG (147, 151, 211). LptF and LptG each possess six transmembrane segments, which form a cavity that accepts LPS from the inner membrane and mediates its extraction
LptF and LptG also each contain a so-called “β-jellyroll” domain, a hydrophobic, concave groove composed of β-strands that shelters LPS as it traverses the periplasm, though at this time it seems only the β-jellyroll of LptF serves this function (201, 211, 212). The β-jellyroll domain of LptF is linked to another β-jellyroll domain from LptC, which additionally has a transmembrane anchor domain that interdigitates between the transmembrane domains of LptF and LptG, though the significance of this is still under investigation (201, 211, 212). The β-jellyroll domain of LptC further links to the β-jellyroll domain of LptA, which is wholly periplasmic and connects to the β-jellyroll domain of LptD at its opposite end, forming a continuous trans-envelope bridge (155, 203, 211). LptD is an outer membrane β-barrel protein, whose N-terminus also includes a β-jellyroll domain (211). LptD is assembled in complex with an outer membrane lipoprotein, LptE, which forms a plug-in-barrel structure with LptD (162, 211). The hydrophilic portion of LPS passes through the lumen of the LptD β-barrel to allow direct insertion of LPS into the outer leaflet of the outer membrane (156, 211).
We recently reported on a critical residue within the LptF/G cavity responsible for making early contacts with LPS as it enters the cavity (213). Here, we utilized a variant at this residue to further elucidate how the Lpt system functions by selecting for suppressor mutations that could suppress the defects associated with this variant. We found that the defects conferred by altering this residue could be suppressed by increasing LPS synthesis. Interestingly however, increased LPS synthesis was not a general suppressor of Lpt defects, making
this suppression a potential tool for evaluating the effects of different \textit{lpt} alleles, or inhibitors of the Lpt system, on different stages of transport. Moreover, we found that certain instances of increased LPS synthesis were tolerated by our Lpt partial loss-of-function strains, but not by Lpt wild-type strains. We believe this finding supports a model in which LPS is essential for balancing the rates of assembly of the leaflets of the outer membrane, rather than any intrinsic property of LPS itself.

\textbf{4.3 Results}

As we reported previously, residue K34 of LptG, located within the cavity formed by LptF and LptG at the inner-membrane/periplasm interface, interacts with LPS in the early stages of extraction from the inner membrane (213). We sought to further interrogate how the LptB$_2$FG complex interacts with LPS by selecting for suppressor mutations of the \textit{lptG(K34D)} allele, which causes defects in LPS transport, and thereby confers increased outer membrane permeability that was used as the basis for selection (see Materials and Methods for details). We isolated several \textit{ftsH} alleles capable of suppressing \textit{lptG(K34D)}. Specifically, we isolated several point mutations, substituting phenylalanine 37 to serine [\textit{ftsH(F37S)}], phenylalanine 221 to a leucine [\textit{ftsH(F221L)}], valine 280 to a glycine [\textit{ftsH(V280G)}], and valine 302 to a phenylalanine [\textit{ftsH(V302F)}]. These mutations all proved capable of suppressing the outer membrane permeability defects associated with \textit{lptG(K34D)}.

\textit{FtsH} is an essential inner membrane protease and has an established role in regulating LPS synthesis by degrading the rate-limiting biosynthetic enzyme,
To gain some insight into how these alleles might be working, we attempted to move the *ftsH*(F37S) and *ftsH*(V302F) alleles into a wild-type *E. coli* strain and interestingly, found that while *ftsH*(F37S) was viable in this background, *ftsH*(V302F) was not. Moreover, the *ftsH*(V302F) suppressor strain ultimately proved to be unstable, meaning it readily acquires additional suppressor mutations. We therefore proceeded to characterize the *ftsH*(F37S) as our primary representative of *ftsH* allele suppression of *lptG*(K34D) membrane-permeability defects (Fig. 4.2), but took this to imply that these alleles were, to some extent, loss-of-function alleles, based on the essentiality of *ftsH* (47, 84, 214) and the lethality of these suppressor *ftsH* alleles in the wild-type background.

![Figure 4.2: Suppression of *lptG*(K34D) outer membrane permeability defects by *ftsH*(F37S).](image)

Shown is disc diffusion assay data, in which the *ftsH*⁺ parent mutant *lptG*(K34D) is colored orange, and the corresponding *ftsH*(F37S) derivative strain is colored
Based on this deduction, increased LPS synthesis seemed a reasonable avenue through which suppression might take place. To investigate this possibility, we assayed steady-state cellular levels of LPS and a known FtsH substrate for proteolysis, the heat-shock sigma factor RpoH (84, 215). We found that the levels of both LPS and RpoH were elevated in strains carrying the \textit{ftsH}(F37S) allele relative to those with wild-type \textit{ftsH} (Fig. 4.3), further supporting that \textit{ftsH}(F37S) is a partial-loss-of-function allele.
Figure 4.3: \( ftsH(F37S) \) is a partial-loss-of-function allele.

Left: Western blot assaying steady-state levels of RpoH during exponential growth.
Right: ProQ Emerald 300 stain for LPS levels. Strains: \( lpt^+ fts^+ \) = NR4492, \( lpt^+ ftsH(F37S) \) = NR5380, \( lptG(K34D) fts^+ \) = NR5232, and \( lptG(K34) ftsH(F37S) \) = NR6107. The data shown is representative of three independent experiments.

These results supported the notion that suppression of the \( lptG(K34D) \) outer-membrane permeability defects by \( ftsH(F37S) \) occurred through elevated LPS levels. To ascertain if this is indeed the mechanism of suppression, we sought to elevate LPS levels in \( lptG(K34D) \) strains independently of the \( ftsH(F37S) \) allele by providing additional copies of the \( lpxC \) gene on a plasmid. We found that introducing \( lpxC \) on a low-copy number plasmid into a \( lptG(K34D) \) strain was sufficient to suppress the outer-membrane permeability defects associated with \( lptG(K34D) \) in a manner similar to the \( ftsH(F37S) \) allele (Fig. 4.4). Moreover, we found that introducing the \( lpxC \)-encoding plasmid into \( ftsH(F37S) \) strains resulted in ready acquisition of suppressor mutations, presumably due to
the toxic overproduction of LPS by excessive LpxC levels resulting from reduced degradation by FtsH\(^{F37S}\) (85), consistent with the notion that \(ftsH(F37S)\) is a partial-loss-of-function allele. As the above results indicated that RpoH was also elevated in the context of the \(ftsH(F37S)\) allele, we performed the same experiment with a plasmid containing \(rpoH\) but found this did not confer suppression. These results further support the notion that \(ftsH(F37S)\) suppresses outer-membrane permeability defects in the \(lptG(K34D)\) mutant by increasing the levels of LPS in the cell, presumably thereby facilitating transport.

Figure 4.4: Elevated LPS levels suppress \(lptG(K34D)\) outer-membrane permeability defects.

Shown is disc-diffusion assay data for either WT (NR4492) or \(lptG(K34D)\) (NR5232) strains carrying either the empty pACYC184 vector, or derivatives expressing \(lpxC\) or \(rpoH\). Shown on the Y-axis is the diameter (in mm) of the zone of inhibition for each strain for the antibiotic bacitracin. Full data is shown in Table
D.1. The dotted black line represents the width of the disc. The data shown is representative of two independent experiments.

If increasing LPS levels is sufficient to drive transport, such a mechanism of suppression could be expected to be general, and therefore capable of suppressing a variety of lpt alleles. To assess this possibility, we combined ftsH(F37S) with lptD4213, as well as lptF(E84A) and lptG(E88A), which have been established to confer LPS transport defects, specifically at the level of outer-membrane translocon biogenesis and coupling of the transmembrane domains LptF and LptG to the nucleotide-binding-domain LptB (172, 193). To our surprise, ftsH(F37S) proved lethal in the context of lptD4213 and did not suppress the outer-membrane permeability defects of either lptF(E84A) or lptG(E88A), indicating that suppression is, at least to some extent, allele specific (Fig. 4.5).
Figure 4.5 Suppression by \textit{ftsH(F37S)} is \textit{lpt} allele specific.

Shown is disc diffusion assay data, in which the \textit{ftsH}+ parent mutant is colored magenta, and the corresponding \textit{ftsH(F37S)} derivative strain is colored green. The wild-type control is shown in gray for reference. Shown on the Y-axis is the zone of inhibition (in mm) for the antibiotic bacitracin. Full data is shown in Table D.2. The dotted black line represents the width of the disc. The data shown is representative of at least three independent experiments.

\textbf{4.4 Discussion}

Taken together, our data supports a model in which \textit{ftsH(F37S)} suppresses the outer-membrane permeability defects of \textit{lptG(K34D)} by reducing proteolysis of LpxC, elevating its levels in the cell, which results in increased LPS biosynthesis that ultimately helps to drive LPS transport. Remarkably however, the \textit{ftsH(F37S)} allele is not capable of suppressing all defective \textit{lpt} alleles. This implies that increased cellular levels of LPS are not a general driver of all steps of LPS transport, but rather affect them differentially. This stands to reason, as increased concentrations of LPS in the inner membrane should presumably facilitate
interaction of the substrate with its transporter, thus helping to drive the early steps of transport, but should have less influence on steps that occur following LPS binding to the transporter. As residue K34 of LptG has been established to be involved in early interaction of LPS with the transporter (201, 213), it seems reasonable that \textit{lptG(K34D)} can be suppressed by increasing LPS levels. In contrast, \textit{lptF(E84A)} and \textit{lptG(E88A)} were not suppressed, which we believe implies they facilitate step(s) of transport following substrate interaction (e.g.: collapse of the cavity and resultant extraction of LPS). Supporting this notion, these alleles alter the coupling helices of LptF and LptG, which connect these transmembrane domains to the nucleotide binding domains, and ergo couple their movement to ATP binding and hydrolysis by the nucleotide binding domains (193, 207). Moreover, it has been established that entry of LPS into the cavity of LptF and LptG is ATP-independent (212). However, additional \textit{lpt} alleles will need to be tested for suppression by increased LPS biogenesis before firm conclusions can be reached. Should this line of reasoning hold true, suppression by increased LPS biogenesis, and therefore by the \textit{ftsH(F37S)} allele, may prove to be a useful diagnostic tool for determining what steps of LPS transport have been affected by either further \textit{lpt} alleles, or potentially, inhibitors of the Lpt system.

We also observed that certain \textit{ftsH} alleles, which our data suggest are partial-loss-of-function alleles, could suppress the defects of \textit{lptG(K34D)}, but were lethal in an otherwise wild-type \textit{E. coli} strain. That sufficient loss of FtsH function fails to complement in wild-type \textit{E. coli} is not surprising, given that \textit{ftsH}
encodes an essential inner-membrane protease (47, 84, 214). Perhaps more surprising is that these alleles (here, we used \textit{ftsH(V302F)} as a representative) complement in the context of the reduced Lpt system function conferred by \textit{lptG(K34D)}. This implies that loss of significant FtsH function, and concomitant increased LPS production, can be suppressed by partial loss of Lpt system function. We believe this finding has interesting ramifications with respect to LPS essentiality.

While LPS biogenesis has been established for some time as essential in many Gram-negative bacteria, the precise basis for this essentiality is not well understood (4). As a major component of the cell envelope, one could imagine that the requirement for LPS biogenesis is structural, and that without LPS, it is simply not possible to form a complete cell envelope. The viability of certain species of Gram-negative bacteria in the absence of LPS, namely \textit{Neisseria Meningitidis}, \textit{Moraxella catarrhalis}, and \textit{Acinetobacter baumannii}, argues against this hypothesis (216-218). In fact, the viability of these species in the absence of LPS has been used as a means of probing the nature of LPS essentiality. A publication by Boll \textit{et al.} linked LPS essentiality to the function of a peptidoglycan polymerase, PBP1a, the absence of which coincided with concomitant overproduction of surface-exposed lipoproteins that may be compensating for the absence of LPS in the outer-membrane (219). Furthermore, Powers and Trent found that, while \textit{Acinetobacter baumannii} is viable without LPS, fitness of an LPS-free strain is greatly improved by the loss of PldA, a phospholipase responsible for removing phospholipids from the outer leaflet of the outer
membrane, and the Mla (maintenance of lipid asymmetry) system, which traffics phospholipids back to the inner membrane from the outer leaflet of the outer membrane (Fig. 4.6A) (220, 221). Together with our data, we believe this evidence supports a model in which LPS is essential not because of any intrinsic structural property, but because LPS must be present to balance the rate of material addition to the outer membrane leaflets. In the absence of LPS, which normally constitutes most of the outer leaflet of the outer membrane, increased production of surface-exposed lipoproteins, or ceasing removal of phospholipids from the outer leaflet of the outer membrane, increases the rate of material addition to the outer leaflet, thereby rebalancing it with growth of the inner leaflet (Fig. 4.6B, D). In the event LPS is overproduced, increasing the rate material is added to the outer leaflet of the outer membrane, balance can be restored by reducing the rate of transport of this material to the outer leaflet of the outer membrane, which can be accomplished by Lpt system defects. In further support of this idea, it has been found that suppressor mutations allowing the loss of FtsH or its positive regulator LapB (which would increase LPS biosynthesis, due to elevated levels of LpxC, which would no longer be degraded by FtsH) were isolated in $lpxC$. These suppressor mutations decrease LpxC function to compensate for its increase in levels. In addition, suppressors of $ftsH$ lethality were found in $fabZ$, which encodes a fatty acid synthase protein which directs a common acyl precursor of both LPS and phospholipids into phospholipid synthesis (85, 89, 91). Redistributing this precursor to favor phospholipid synthesis over LPS production would again serve to rebalance the rate at which
material is added to the outer membrane leaflets. This redistribution would both reduce the amount of the common precursor available for LPS synthesis, which was too rapid owing to the loss of FtsH or LapB, while simultaneously increasing the rate of synthesis of its counterpart in the inner leaflet: phospholipids. Also in support of this model, May and Sutterlin et al. identified a conditionally lethal, gain-of-function allele of *mlaA*, a gene encoding the outer-membrane component of the Mla system, which they named *mlaA*<sup>*</sup> (222, 223). The *mlaA*<sup>*</sup> allele produces aberrant MlaA that allows translocation of phospholipids from the inner leaflet of the outer membrane to the outer leaflet. The resulting increase in phospholipids in the outer leaflet of the outer membrane activates PldA, an outer membrane phospholipase that cleaves a fatty acid from outer leaflet phospholipids. Ultimately, free fatty acids produced from phospholipids by PldA are trafficked back to the cytoplasm by FadD and added to the cell’s acyl-ACP pools. This increase in acyl-ACP pools in turn activates LPS synthesis (Fig. 4.6A). Therefore, the *mlaA*<sup>*</sup> allele results in both the depletion of phospholipids in the inner leaflet of the outer membrane and the increase in growth of the outer leaflet: the MlaA<sup>*</sup> variant moves phospholipids from the inner leaflet to the outer leaflet and indirectly increases LPS synthesis through the action of the phospholipase PldA and the recycling of fatty acids by FadD. Notably, the lethality of the *mlaA*<sup>*</sup> allele could be suppressed by reducing Lpt function (223), akin to the scenario we observed with *ftsH*(V302F) and *lptG*(K34D). We support a model for this suppression in which when LPS is overproduced, either as a result of the *ftsH*(F37S) or *mlaA*<sup>*</sup> alleles, Lpt defects decrease its traffic to the
outer-leaflet of the outer membrane. This decreased traffic to the outer leaflet restores balanced synthesis of the outer membrane leaflets, despite the overproduction of LPS, which would otherwise cause the outer leaflet to grow too quickly relative to the inner leaflet (Fig. 4.6B, C). Whether or not the lethality of imbalanced outer membrane leaflet synthesis stems ultimately from disruption of the outer membrane alone, or due to the cell’s attempts to compensate by trafficking lipid content from the inner membrane to the outer membrane, as suggested by the observations of Sutterlin and May et al., is unclear.
Figure 4.6: Model for LPS essentiality:

LPS is required for balancing outer membrane leaflet biogenesis. We propose a model in which LPS is essential not for any intrinsic structural property, but because it serves to balance the rates at which the inner and outer leaflets of the outer membrane are constructed. A) Schematic for regulators of outer membrane biogenesis B) Model for normal outer membrane assembly. The inner leaflet is represented with phospholipids, while the outer leaflet is represented by LPS (as in Fig. 4.1). The rate at which material is added to the inner leaflet is represented by the width of the grey arrow, whereas the rate at which material is added to the outer leaflet is represented by the dark blue arrow. In wild-type strains, these rates should be balanced, as indicated by the equals sign between them. C) Model for the basis of the lethality of LPS overproduction and means of suppressing it. Color coding is as in panel A, with the addition of a dark blue arrow between the leaflets to represent the mlaA\* directed transfer of phospholipids from the inner leaflet to the outer leaflet. On the left, LPS overproduction causes the outer leaflet to grow more rapidly than the inner leaflet, causing the cell to lyse. On the right, this rapid growth can be suppressed by reducing the rate of transport to the outer leaflet through defects in the Lpt system (denoted as lpt\text{down} in the figure). D) Model for survival of LPS-free organisms. Color coding is the same as in panels B-C. On the right, LPS-free organisms lack a major component of the outer leaflet, and therefore have greatly reduced transport to this leaflet. This
problem can be addressed by substituting lipoproteins for LPS on the cell surface, and by getting rid of systems that remove phospholipids from the outer leaflet of the outer membrane, such as PldA and Mla.
4.5 Materials and Methods

Materials and Methods:

Strains and growth conditions. Strains were grown at 37°C in either lysogeny broth (LB) or M63 supplemented with 0.2% (wt/vol) glucose with aeration for liquid cultures and on 1.5% agar plates for solid media. Media was supplemented with ampicillin (125 µg/mL), bacitracin (100 µg/mL), chloramphenicol (10 or 20 µg/mL), isopropyl-β-D-1-thiogalactopyranoside (IPTG - 0.16 mM), kanamycin (30 µg/mL), tetracycline (25 µg/mL), vancomycin (25 µg/mL), and X-Gal (33 µg/mL) when appropriate. A full list of strains can be found in table D.3.

Suppressor selection and mapping. A 100-µL aliquot of overnight culture of either strain NR4063 \([lptG(K34D) \Delta eptA]\) or strain NR5232 \([lptG(K34D) \text{tet}2]\) was plated on LB agar with bacitracin. For NR4063 derived suppressors, a single suppressor isolate was selected for mapping based on its restored resistance to multiple antibiotics. Mapping was conducted via P1\text{vir} co-transduction, using a randomly generated mini-Tn\text{cam} library as the donor (224). Loss of suppression in transduced isolated was monitored by their ability to grow on LB agar with bacitracin. A mini-Tn\text{cam} marker disrupting the \(yhcA\) gene (and therefore named \(yhcA::\text{cam}\)) was found to be ~30% linked to the suppressor mutation. Subsequent sequencing of this region of the chromosome identified a point
mutation in \textit{ftsH}, resulting in the alteration of valine 302 to a phenylalanine. This suppressor allele was therefore named \textit{ftsH(V302F)}. For suppressors of \textit{NR5232}, these were selected in the context of \textit{tet2}, a tetracycline resistance marker integrated at approximately minute 72 of the chromosome. Suppressor isolates were evaluated for linkage to this marker, resulting in three additional suppressors with \(~30\%\) linkage to this marker, which when this region of the chromosome was sequenced (a list of primers can be found in table D.4), yielded point mutations in \textit{ftsH} corresponding to \textit{ftsH(F37S)}, \textit{ftsH(F221L)} and \textit{ftsH(V280G)}.

\textbf{Antibiotic sensitivity.} To assay OM permeability to bacitracin, novobiocin, rifampin, and erythromycin, 100 µL of overnight cultures of haploid strains were grown in LB and subjected to disc diffusion assays as described previously (147).

\textbf{Plasmid construction.} pACYC184 derived plasmids encoding FtsH, LpxC, and RpoH, were produced by digesting pACYC184 plasmid DNA with XbaI and HindIII restriction enzymes to prepare the recipient vector. Inserts were prepared from \textit{E. coli} \textit{NR754} chromosomal DNA via colony PCR with the 5XbaI-ftsH and 3HindIII-ftsH, 5XbaI-LpxC and 3HindIII-LpxC, and 5XbaI-RpoH and 3HindIII-RpoH primers respectively (a list of primers can be found in table D.4). These PCRs were digested with DpnI overnight, followed by XbaI and HindIII restriction enzymes. The digested recipient vector was treated with Antarctic phosphatase
prior to undergoing ligation with the amplified insert in a ~5:1 molar reaction (insert:vector). This ligation reaction was electroporated into DH5α competent cells, and successful transformants were selected for on LB agar with chloramphenicol (20 mg/ml). Plasmid was prepared from successful transformants and screened by restriction enzyme digest and sequencing of the insert.

**Determination of RpoH levels.** RpoH Levels were determined by immunoblotting of whole-cell lysates from mid-log cultures with rabbit anti-RpoH antisera horseradish peroxidase-conjugated anti-rabbit antibody from goat. Samples were prepared by growing cells to an OD$_{600}$ ~0.5, followed by pelleting cells and resuspending in a volume of sample buffer normalized by OD$_{600}$ readings, resolved on SDS-PAGE gels, and imaged as described previously (193).

**Determination of LPS levels.** LPS levels were determined by ProQ Emerald 300 LPS staining (ThermoFisher Scientific) of mid-log cultures. Samples were prepared by growing cells to an OD$_{600}$ ~0.5, pelleting an amount of culture based on their OD$_{600}$ value (2 mL for OD$_{600}$ = 0.5), and resuspending the pellets in 50 ul of LPS sample buffer (0.66 M Tris pH 7.6, 2% [w/v] SDS, 10% [v/v] glycerol, 4% [v/v] β-mercaptoethanol, 0.1% [w/v] bromophenol blue). Samples were then subjected to proteinase K digestion at 56°C overnight (adding 10 ul of 2.5 mg/ml proteinase K to each sample). 15 ul of each sample was resolved on a 15% SDS-PAGE gel and imaged as described in the ProQ Emerald 300 staining
protocol (Molecular Probes) via UV transillumination on a Chemidoc XRS+ system with ImageLab software (Bio-Rad).

**Determination of allele lethality by disruption of linkage.** Lethality of \( ftsH \) alleles was determined by disruption of linkage in co-transduction. \( ftsH \) alleles, linked to either \( tet2 \) or \( yhcA::cam \), (~25% linked), were transduced into recipient strains with a kanamycin resistance marker, \( yhbX::kan \), adjacent to \( ftsH \), but on the opposite side relative to \( tet2\!/yhcA::cam \) (linkage ~ 23%). Recipients were then screened for kanamycin resistance, and when linkage between \( tet2\!/yhcA::cam \), was disrupted (0% rather than 23%, i.e.: all transductants remain kanamycin resistant), the \( ftsH \) allele in question was deemed lethal in that background, as this region of the chromosome was not being replaced.
Chapter 5: Concluding Remarks

The work presented in this document has focused on elucidating how the LPS transport complex, or Lpt system, interacts with its substrate in the early stages of transport. Chapter one presents a review of our knowledge of LPS function, synthesis, modification, and, prior to the works presented in this document, transport. In reality, however, the work presented in chapter two began before the structure of LptB_{2}FG was published, and as such, while the general architecture and mechanism of the transporter were established, we had comparatively little to go on in identifying how the transmembrane components of the transporter, LptF and LptG, might interact with LPS.

As described in chapter two, our first real clue came from a publication from Hamad et al., who noted that the essentiality of LPS modification with aminoarabinose could be suppressed by an alteration to LptG in Burkholderia (179, 225). This ultimately led us to discover that residue K34 of LptG in E. coli, the equivalent to the residue altered to suppress aminoarabinose essentiality in Burkholderia, was critical to the proper transport of unmodified LPS (213). That residue K34 of E. coli LptG does not appear critical for the export of phosphoethanolamine-modified LPS implies that different LPS species are transported in distinct manners, and further, that the Lpt system may play some
role in which of these species ultimately populate the outer membrane. Neither of these implications have been thoroughly explored, but present interesting questions for further exploration. Does the mechanism of transport vary substantially among LPS species, or are there just minor adjustments? Given their generally similar structure, one would suspect the latter, but there is no data available at this time to support one hypothesis over the other. Does Lpt contribute to the selection of LPS species for display at the cell surface? It seems apparent that in *Burkholderia*, this is the case, as cells simply are not viable without aminoarabinose available with which to modify their LPS, at least without a compensating modification to LptG. However, it is at present unclear if this extends to other organisms, particularly those that can produce multiple LPS species, such as *E. coli*. Perhaps Lpt therefore plays a major role in dictating the barrier characteristics of the outer membrane, rather than this being dictated strictly by which LPS molecules are synthesized. While these questions remain, this work is among the first to start elucidating how LPS interacts with the cavity formed by LptF and LptG during its extraction from the inner membrane by Lpt.

Chapter three describes the LptB2FGC structure, and experiments based on this structure that illuminate the path LPS takes through the transporter. We show that the LptF β-jellyroll is preferentially coupled to the β-jellyroll of LptC (212). The function of the LptG β-jellyroll is therefore unclear, though based on its conservation in nature, it seems reasonable to presume it has some function. We hypothesize that the LptG β-jellyroll serves as a structural scaffold for the β-
jellyroll of LptF, but the function of the LptG β-jellyroll, if it has one, remains to be clarified. Another remarkable feature of the structure is the transmembrane helix of LptC, which is intercalated between the LptF and LptG transmembrane domains (212). This renders the LptB2FGC transporter asymmetric, and is, as far as we know, a structural feature unique to this transporter. While remarkable, the precise mechanistic import of this feature remains unclear, particularly given that removal of the LptC transmembrane helix confers no observed LPS transport defects (152). Again, however, the conservation of the LptC transmembrane helix in nature suggests it serves some function, and reduced ATP hydrolysis rates by wild-type LptB2FGC complexes, relative to complexes lacking LptC, suggests that it may serve to curtail inappropriate ATP hydrolysis. Nonetheless, more work is needed to clarify the role of the transmembrane helix of LptC in LPS transport.

This work was published alongside similar work done by Li et al. which in general supported our conclusions (201). Notably, Li et al. were able to observe LPS in the cavity formed by LptF and LptG, and their findings support those reported in chapter two describing the importance of residue K34 of LptG in contacting LPS in the early stages of extraction. They proposed a model in which LPS, upon entering the cavity, forms a handful of interactions with LptFG, resulting in loose association. Subsequent exit of the LptC transmembrane helix causes the cavity to collapse around LPS, resulting in tighter binding. Finally, binding of ATP by LptB brings the LptB dimer together, collapsing the cavity and expelling LPS from the cavity, ultimately resulting in it being loaded onto the
bridge formed by the β-jellyroll domains of LptF, LptC, LptA, and LptD. Insofar as it is described here, we agree with their model. However, it is unclear what drives the exit of the transmembrane helix of LptC, and the mechanistic details of LPS extraction remain to be elucidated.

In chapter four, we describe the isolation of partial-loss-of-function alleles of \textit{ftsH} that suppress the outer membrane permeability defects of \textit{lptG(K34D)} by causing increased LPS production. Notably, suppression by these alleles was specific, and thus it is our hope that this mechanism of suppression may be useful as a diagnostic tool for determining if early or late stages of LPS transport have been affected by a given \textit{lpt} allele, or inhibitor of the Lpt system. However, how specific this suppression is remains to be clarified, which will dictate what level of resolution one can achieve by this method.

Additionally, we found that the increased LPS production caused by some of our \textit{ftsH} alleles was lethal in \textit{lpt} wild-type strains. However, these alleles complemented in a \textit{lpt} partial-loss-of-function, \textit{lptG(K34D)}, strain. In conjunction with previous published work (219, 220, 222, 223), we feel this supports a model in which LPS essentiality is due to the need to balance the rate at which material is added to the leaflets of the outer membrane.

In summary, the works presented here shed new light on how the LPS transport system interacts with its substrate in the early stages of transport, how LPS is directed onto the Lpt bridge, and finally, into the nature of LPS essentiality. However, we still lack mechanistic details on how LPS transport
occurs, and further work needs to be done to verify if balancing leaflet synthesis is the sole reason for LPS essentiality.
References


22. Carpenter TS, Parkin J, Khalid S. The Free Energy of Small Solute Permeation through the Escherichia coli Outer Membrane Has a Distinctly


44. Kelly TM, Stachula SA, Raetz CR, Anderson MS. The firA gene of Escherichia coli encodes UDP-3-O-(R-3-hydroxyacyl)-glucosamine N-


67. Yethon JA, Whitfield C. Purification and characterization of WaaP from Escherichia coli, a lipopolysaccharide kinase essential for outer membrane


110. Trent MS, Ribeiro AA, Lin S, Cotter RJ, Raetz CR. An inner membrane enzyme in Salmonella and Escherichia coli that transfers 4-amino-4-deoxy-L-arabinose to lipid A: induction on polymyxin-resistant mutants and role of a novel


Table A.1 Sensitivity to antibiotics of *lptG* mutants and their *basS<sup>c</sup>* derivatives

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Bacitracin</th>
<th>Novobiocin</th>
<th>Rifampin</th>
<th>Erythromycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR2761</td>
<td><em>lptFG&lt;sup&gt;+&lt;/sup&gt;</em></td>
<td>7.2 (9) ± 0.3 (0)</td>
<td>7 (9.3) ± 0 (0.6)</td>
<td>7 (14) ± 0 (0)</td>
<td>9 (11.7) ± 0 (0.6)</td>
</tr>
<tr>
<td>NR4027</td>
<td><em>lptG(K34R)</em></td>
<td>8.7 (9.5) ± 0.6 (0.5)</td>
<td>7 (12.2) ± 0 (0.3)</td>
<td>7 (14.2) ± 0 (0.3)</td>
<td>9.8 (12.5) ± 0.8 (1.3)</td>
</tr>
<tr>
<td>NR4803</td>
<td><em>lptG(K34R) basS&lt;sup&gt;c&lt;/sup&gt;</em></td>
<td>7 (7.8) ± 0 (0.3)</td>
<td>7 (9.7) ± 0 (0.3)</td>
<td>7 (13.7) ± 0 (0.8)</td>
<td>9.5 (11.8) ± 0.5 (0.3)</td>
</tr>
<tr>
<td>NR4077</td>
<td><em>lptG(K34C)</em></td>
<td>12 (12) ± 0.5 (0.5)</td>
<td>8.7 (15.5) ± 2.9 (3)</td>
<td>9.2 (17.2) ± 1.9 (0.8)</td>
<td>11.5 (16.3) ± 0.5 (0.6)</td>
</tr>
<tr>
<td>NR4358</td>
<td><em>lptG(K34C) basS&lt;sup&gt;c&lt;/sup&gt;</em></td>
<td>8.2 ± 0.3</td>
<td>7 (9.8) ± 0 (1)</td>
<td>7 (14) ± 0 (1)</td>
<td>9.8 (12.3) ± 0.3 (0.6)</td>
</tr>
<tr>
<td>NR3740</td>
<td><em>lptG(K34A)</em></td>
<td>14 (12) ± 0 (3.5)</td>
<td>9 (14.3) ± 3.5 (6.1)</td>
<td>9.2 (17.5) ± 3.8 (4)</td>
<td>11.8 (14) ± 1 (2)</td>
</tr>
<tr>
<td>NR4362</td>
<td><em>lptG(K34A) basS&lt;sup&gt;c&lt;/sup&gt;</em></td>
<td>7.3 (7.8) ± 0.3 (0.3)</td>
<td>7 (9.5) ± 0 (0.5)</td>
<td>7 (13.2) ± 0 (0.3)</td>
<td>10.2 (12.7) ± 0.8 (0.8)</td>
</tr>
<tr>
<td>NR4084</td>
<td><em>lptG(K34Q)</em></td>
<td>16 ± 1.5</td>
<td>13 (23.7) ± 1.7 (1)</td>
<td>8.5 (19.3) ± 2.6 (1.2)</td>
<td>13.2 (18.8) ± 0.8 (1.6)</td>
</tr>
<tr>
<td>NR4357</td>
<td><em>lptG(K34Q) basS&lt;sup&gt;c&lt;/sup&gt;</em></td>
<td>9 ± 0.5</td>
<td>11.2 (18.2) ± 0.6 (1.6)</td>
<td>7 (14) ± 0 (0)</td>
<td>10.5 (13.7) ± 1.3 (1)</td>
</tr>
<tr>
<td>NR3673</td>
<td><em>lptG(K34D)</em></td>
<td>21.7 (21.7) ± 1.3 (1.3)</td>
<td>13.7 (28.3) ± 0.6 (2.1)</td>
<td>24 (24) ± 1.7 (1.7)</td>
<td>15.2 (21) ± 0.8 (0.9)</td>
</tr>
<tr>
<td>NR3951</td>
<td><em>lptG(K34D) basS&lt;sup&gt;c&lt;/sup&gt;</em></td>
<td>10.7 (12.3) ± 0.6 (1.2)</td>
<td>13.7 (18.5) ± 0.6 (1.3)</td>
<td>7 (15) ± 0 (0)</td>
<td>11 (15.2) ± 0.9 (0.3)</td>
</tr>
<tr>
<td>Strain</td>
<td>Mutation</td>
<td>Value 1</td>
<td>Value 2</td>
<td>Value 3</td>
<td>Value 4</td>
</tr>
<tr>
<td>-----------</td>
<td>-------------------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
</tr>
<tr>
<td>NR433</td>
<td>lptG(D37A)</td>
<td>13.5 ± 0.9</td>
<td>9.8 (16) ± 2.8 (1.3)</td>
<td>10.5 (17.8) ± 3 (0.8)</td>
<td>11.8 (17.7) ± 1.3 (1.2)</td>
</tr>
<tr>
<td>NR4904</td>
<td>lptG(D37A) basSc</td>
<td>9.7 ± 0.3</td>
<td>7 (14.3) ± 0 (0.3)</td>
<td>7 (16) ± 0 (1)</td>
<td>10.5 (14.2) ± 0.5 (0.8)</td>
</tr>
<tr>
<td>NR5118</td>
<td>lptG(D37C)</td>
<td>19.2 ± 1.3</td>
<td>13.5 (24.7) ± 0.5 (1.5)</td>
<td>22.5 ± 0.5</td>
<td>14.7 (20.3) ± 0.3 (1.5)</td>
</tr>
<tr>
<td>NR4401</td>
<td>lptG(D37C) basSc</td>
<td>13.7 ± 0.6</td>
<td>13 (21) ± 1 (0.5)</td>
<td>12 (17.8) ± 1 (0.8)</td>
<td>13.7 (18.7) ± 0.6 (1.5)</td>
</tr>
<tr>
<td>NR4319</td>
<td>lptG(Q38A)</td>
<td>14.2 ± 0.3</td>
<td>7 (18) ± 0 (2)</td>
<td>11.3 (19.3) ± 3.8 (0.6)</td>
<td>13.2 (17.3) ± 0.8 (1.5)</td>
</tr>
<tr>
<td>NR4402</td>
<td>lptG(Q38A) basSc</td>
<td>8.8 ± 0.3</td>
<td>7 (11.7) ± 0 (0.8)</td>
<td>7 (14) ± 0 (0.5)</td>
<td>10 (14) ± 0.9 (1)</td>
</tr>
<tr>
<td>NR4322</td>
<td>lptG(Q38C)</td>
<td>19.5 ± 1.3</td>
<td>15.7 (26.8) ± 2.1 (0.3)</td>
<td>23.2 ± 0.3</td>
<td>14.2 (21.7) ± 1.6 (2.1)</td>
</tr>
<tr>
<td>NR4403</td>
<td>lptG(Q38C) basSc</td>
<td>11.3 ± 0.6</td>
<td>10.2 (17.7) ± 3 (3.2)</td>
<td>8.7 (16.2) ± 2.9 (1.8)</td>
<td>11.7 (15) ± 0.6 (1)</td>
</tr>
<tr>
<td>NR3674</td>
<td>lptG(K40D)</td>
<td>10.7 ± 0.6</td>
<td>7 (20.2) ± 0 (1)</td>
<td>8.2 (15) ± 2 (3)</td>
<td>11.5 (15.5) ± 0.5 (0.5)</td>
</tr>
<tr>
<td>NR4363</td>
<td>lptG(K40D) basSc</td>
<td>7 (8) ± 0 (0.5)</td>
<td>7.3 (12.5) ± 0.6 (4)</td>
<td>7 (13.8) ± 0 (0.3)</td>
<td>9.8 (13) ± 0.3 (0)</td>
</tr>
<tr>
<td>NR3675</td>
<td>lptG(K41D)</td>
<td>10.7 ± 0.6</td>
<td>7 (14.3) ± 0 (0.6)</td>
<td>7 (16) ± 0 (1)</td>
<td>11 (14.7) ± 0 (0.6)</td>
</tr>
<tr>
<td>NR4356</td>
<td>lptG(K41D) basSc</td>
<td>7 (8.2) ± 0 (0.8)</td>
<td>7 (9.8) ± 0 (0.8)</td>
<td>7 (14) ± 0 (1)</td>
<td>9.7 (12.5) ± 0.6 (0.9)</td>
</tr>
<tr>
<td>NR3741</td>
<td>lptG(K40D/K41D)</td>
<td>14.8 ± 0.8</td>
<td>9.7 (22) ± 4.6 (1)</td>
<td>11.8 (19.7) ± 4.3 (1.2)</td>
<td>10.8 (18) ± 3.3 (1)</td>
</tr>
</tbody>
</table>
Table A.2 Suppression of \textit{lptG(K34D)} by \textit{basS\textsuperscript{c}} is dependent on EptA and ArnT

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Zone of inhibition (mm)\textsuperscript{a}</th>
<th>Bacitracin (mm)</th>
<th>Novobiocin (mm)</th>
<th>Rifampin (mm)</th>
<th>Erythromycin (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR2761</td>
<td>\textit{lptG\textsuperscript{+}}</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NR3952</td>
<td>\textit{lptG(K34D)}</td>
<td></td>
<td>20.3 ± 0.6</td>
<td>15.7 (28.2) ± 0.6 (2.3)</td>
<td>15 (17) ± 0 (1)</td>
<td>20.3 (24.2) ± 5.5 (1.3)</td>
</tr>
<tr>
<td>NR4063</td>
<td>\textit{lptG(K34D) ΔeptA}</td>
<td></td>
<td>22 ± 1.3</td>
<td>16.5 (31.3) ± 4.3 (3.2)</td>
<td>16.3 (21) ± 0.8 (1.7)</td>
<td>21 (25.3) ± 5.2 (2.3)</td>
</tr>
<tr>
<td>NR4117</td>
<td>\textit{lptG(K34D) ΔeptA/arnT}</td>
<td></td>
<td>22.3 ± 1.5</td>
<td>15 (32) ± 1 (3.6)</td>
<td>16.8 (23) ± 1.3 (1.7)</td>
<td>26 ± 2.6</td>
</tr>
<tr>
<td>NR3951</td>
<td>\textit{lptG(K34D) basS\textsuperscript{c}}</td>
<td></td>
<td>10.8 ± 0.3</td>
<td>13.2 (15.3) ± 0.8 (1.5)</td>
<td>12.3 (15) ± 0.6 (1)</td>
<td>11 (16.3) ± 0 (0.6)</td>
</tr>
<tr>
<td>NR4062</td>
<td>\textit{lptG(K34D) basS\textsuperscript{c} ΔeptA}</td>
<td></td>
<td>17.3 ± 0.6</td>
<td>14 (24.7) ± 1 (0.6)</td>
<td>14 (20.7) ± 0 (1.2)</td>
<td>16.2 (20.8) ± 3.8 (0.3)</td>
</tr>
<tr>
<td>NR4116</td>
<td>\textit{lptG(K34D) basS\textsuperscript{c} ΔeptA/arnT}</td>
<td></td>
<td>21.5 ± 1.3</td>
<td>14.7 (30.7) ± 0.6 (3.1)</td>
<td>16 (22) ± 0 (1)</td>
<td>25 ± 2.6</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Sensitivity was assessed by disc diffusion assays performed in three independent experiments. Values denote the diameter (in mm) of zones in which no growth was observed, whereas values in parentheses denote the diameter of areas of partially inhibited growth. The same format was used to denote standard deviation values. Discs were 6 mm in diameter.
Specificity of suppression by bas$^c$

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Bacitracin</th>
<th>Novobiocin</th>
<th>Rifampin</th>
<th>Erythromycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR2761</td>
<td>lptFG$^+$</td>
<td>7 (8.2) ± 0 (0.3)</td>
<td>7 (9) ± 0 (0)</td>
<td>7 (14.3) ± 0 (0.8)</td>
<td>9.3 (12.2) ± 0.6 (0.8)</td>
</tr>
<tr>
<td>NR3954</td>
<td>lptFG$^+$ bas$^c$</td>
<td>7 (9) ± 0 (1)</td>
<td>7 (9.8) ± 0 (0.3)</td>
<td>7 (14.2) ± 0 (0.6)</td>
<td>9.8 (12.2) ± 0.8 (0.8)</td>
</tr>
<tr>
<td>NR3265</td>
<td>lptG(E84A)</td>
<td>14.2 ± 0.3</td>
<td>7 (10.3) ± 0 (0.3)</td>
<td>7 (19.5) ± 0 (0.5)</td>
<td>14 (20.2) ± 0.5 (0.3)</td>
</tr>
<tr>
<td>NR4382</td>
<td>lptG(E84A) bas$^c$</td>
<td>15.8 ± 0.8</td>
<td>7 (11) ± 0 (0)</td>
<td>7 (20.7) ± 0 (1.2)</td>
<td>16.2 (22) ± 0.3 (0.9)</td>
</tr>
<tr>
<td>NR2762</td>
<td>lptG(E88A)</td>
<td>13.3 (29.5) ± 1 (1.5)</td>
<td>7 (15.2) ± 0 (0.3)</td>
<td>13.2 (30.7) ± 0.3 (5.8)</td>
<td>13.3 (33.7) ± 0.6 (3.1)</td>
</tr>
<tr>
<td>NR4383</td>
<td>lptG(E88A) bas$^c$</td>
<td>14.7 (28.3) ± 0.6 (2.3)</td>
<td>7 (15.2) ± 0 (1)</td>
<td>14.7 (36) ± 1.5 (3.6)</td>
<td>12.5 (37.2) ± 0.5 (0.3)</td>
</tr>
<tr>
<td>NR760</td>
<td>lptD4213</td>
<td>22.8 (26) ± 0.3 (0.5)</td>
<td>19.8 (27.5) ± 0.8 (1.3)</td>
<td>19.3 (26.8) ± 0.3 (0.3)</td>
<td>25 (29.8) ± 0.9 (0.8)</td>
</tr>
<tr>
<td>NR4372</td>
<td>lptD4213 bas$^c$</td>
<td>22.2 (25.5) ± 0.3 (0.5)</td>
<td>20.7 (30.2) ± 0.8 (0.3)</td>
<td>21.3 (33.2) ± 0.6 (0.3)</td>
<td>24.8 (32.5) ± 0.6 (0.5)</td>
</tr>
</tbody>
</table>

*Sensitivity was assessed by disc diffusion assays performed in three independent experiments. Values denote the diameter (in mm) of zones in which no growth was observed, whereas values in parentheses denote the diameter of areas of partially inhibited growth. The same format was used to denote standard deviation values. Discs were 6 mm in diameter.*
Table A.4 Strains used in chapter 2

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH5α</td>
<td>F' φ80lacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17 (rK-, mK+) phoA supE44 thi-1</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>NovaBlue</td>
<td>endA1 hsdR17 (rK12-mK12+) supE44 thi-1 recA1 gyrA96 relA1 lac F'[proA+B+lacIqZΔM15::Tn10] (TetR)</td>
<td>Novagen</td>
</tr>
<tr>
<td>KRX</td>
<td>F', traD36, ΔompP, proA+B+, lacIq, Δ(lacZ)M15 ΔompT, endA1, recA1, gyrA96 (Nalr), thi-1, hsdR17 (rK-, mK+), e14- (McrA-)</td>
<td>Promega</td>
</tr>
<tr>
<td>WD102</td>
<td>W3110 zjd2211::Tn10 basR^+</td>
<td></td>
</tr>
<tr>
<td>NR4921</td>
<td>NR754 ΔlptFG let2-2 basS(L102Q) (pBAD18LptFG3/LptG/K34D)</td>
<td>This study</td>
</tr>
<tr>
<td>NR754</td>
<td>MC4100 ara^+</td>
<td>(151)</td>
</tr>
<tr>
<td>NR2759</td>
<td>NR754 ΔlptFG (pRC7KanLptFG)</td>
<td>(193)</td>
</tr>
<tr>
<td>NR4340</td>
<td>NR754 ΔlptFG basS(L102Q) zjd2211::Tn10 (pRC7KanLptFG)</td>
<td>This study</td>
</tr>
<tr>
<td>NR2761</td>
<td>NR754 ΔlptFG (pBAD18LptFG3)</td>
<td>(193)</td>
</tr>
<tr>
<td>NR3955</td>
<td>NR754 ΔlptFG zjd2211::Tn10 (pBAD18LptFG3)</td>
<td>This study</td>
</tr>
<tr>
<td>NR4027</td>
<td>NR754 ΔlptFG (pBAD18LptFG3/LptG/K34R)</td>
<td>This study</td>
</tr>
<tr>
<td>NR4803</td>
<td>NR754 ΔlptFG basS(L102Q) zjd2211::Tn10 (pBAD18LptFG3/LptG/K34R)</td>
<td>This study</td>
</tr>
<tr>
<td>NR4077</td>
<td>NR754 ΔlptFG (pBAD18LptFG3/LptG/K34C)</td>
<td>This study</td>
</tr>
<tr>
<td>NR4358</td>
<td>NR754 ΔlptFG basS(L102Q) zjd2211::Tn10 (pBAD18LptFG3/LptG/K34C)</td>
<td>This study</td>
</tr>
<tr>
<td>NR3740</td>
<td>NR754 ΔlptFG (pBAD18LptFG3/LptG/K34A)</td>
<td>This study</td>
</tr>
<tr>
<td>NR4362</td>
<td>NR754 ΔlptFG basS(L102Q) zjd2211::Tn10 (pBAD18LptFG3/LptG/K34A)</td>
<td>This study</td>
</tr>
<tr>
<td>NR4084</td>
<td>NR754 ΔlptFG (pBAD18LptFG3/LptG/K34Q)</td>
<td>This study</td>
</tr>
<tr>
<td>NR4357</td>
<td>NR754 ΔlptFG basS(L102Q) zjd2211::Tn10 (pBAD18LptFG3/LptG/K43Q)</td>
<td>This study</td>
</tr>
<tr>
<td>NR3673</td>
<td>NR754 ΔlptFG (pBAD18LptFG3/LptG/K34D)</td>
<td>This study</td>
</tr>
<tr>
<td>NR3951</td>
<td>NR754 ΔlptFG zjd2211::Tn10 basS(L102Q) (pBAD18LptFG3/LptG/K34D)</td>
<td>This study</td>
</tr>
<tr>
<td>NR4433</td>
<td>NR754 ΔlptFG (pBAD18LptFG3/LptG/D37A)</td>
<td>This study</td>
</tr>
<tr>
<td>NR4804</td>
<td>NR754 ΔlptFG zjd2211::Tn10 basS(L102Q) (pBAD18LptFG3/LptG/D37A)</td>
<td>This study</td>
</tr>
<tr>
<td>NR4518</td>
<td>NR754 ΔlptFG (pBAD18LptFG3/LptG/D37C)</td>
<td>This study</td>
</tr>
<tr>
<td>NR4401</td>
<td>NR754 ΔlptFG zjd2211::Tn10 basS(L102Q) (pBAD18LptFG3/LptG/D37C)</td>
<td>This study</td>
</tr>
<tr>
<td>NR4319</td>
<td>NR754 ΔlptFG (pBAD18LptFG3/LptG/Q38A)</td>
<td>This study</td>
</tr>
<tr>
<td>NR4402</td>
<td>NR754 ΔlptFG zjd2211::Tn10 basS(L102Q) (pBAD18LptFG3/LptG/Q38A)</td>
<td>This study</td>
</tr>
<tr>
<td>NR4232</td>
<td>NR754 ΔlptFG (pBAD18LptFG3/LptG/Q38C)</td>
<td>This study</td>
</tr>
<tr>
<td>NR3674</td>
<td>NR754 ΔlptFG (pBAD18LptFG3/LptG/K40D)</td>
<td>This study</td>
</tr>
<tr>
<td>NR4363</td>
<td>NR754 ΔlptFG basS(L102Q) zjd2211::Tn10 (pBAD18LptFG3/LptG/K40D)</td>
<td>This study</td>
</tr>
<tr>
<td>NR3675</td>
<td>NR754 ΔlptFG (pBAD18LptFG3/LptG/K41D)</td>
<td>This study</td>
</tr>
<tr>
<td>NR4356</td>
<td>NR754 ΔlptFG basS(L102Q) zjd2211::Tn10 (pBAD18LptFG3/LptG/K41D)</td>
<td>This study</td>
</tr>
<tr>
<td>NR3741</td>
<td>NR754 ΔlptFG (pBAD18LptFG3/LptG/K40D/K41D)</td>
<td>This study</td>
</tr>
<tr>
<td>NR4359</td>
<td>NR754 ΔlptFG basS(L102Q) zjd2211::Tn10 (pBAD18LptFG3/LptG/K40D/K41D)</td>
<td>This study</td>
</tr>
<tr>
<td>NR3079</td>
<td>NR754 (pBAD18LptFG3)</td>
<td>(193)</td>
</tr>
<tr>
<td>NR4840</td>
<td>NR754 (pBAD18LptFG3/LptG/K34E)</td>
<td>This study</td>
</tr>
<tr>
<td>NR4841</td>
<td>NR754 (pBAD18LptFG3/LptG/K34A/K40D/K41D)</td>
<td>This study</td>
</tr>
<tr>
<td>NR4842</td>
<td>NR754 (pBAD18LptFG3/LptG/K34D/K40D/K41D)</td>
<td>This study</td>
</tr>
<tr>
<td>NR3952</td>
<td>NR754 ΔlptFG zjd2211::Tn10 (pBAD18LptFG3/LptG/K34D)</td>
<td>This study</td>
</tr>
<tr>
<td>JW4082</td>
<td>BW25113 yjdF::kan</td>
<td>(47)</td>
</tr>
<tr>
<td>JW5729</td>
<td>BW25113 yjcZ::kan</td>
<td>(47)</td>
</tr>
<tr>
<td>JW5730</td>
<td>BW25113 ΔeptA::kan</td>
<td>(47)</td>
</tr>
<tr>
<td>JW2251</td>
<td>BW25113 ΔarnT::kan</td>
<td>(47)</td>
</tr>
<tr>
<td>NR4047</td>
<td>NR754 ΔlptFG zjd2211::Tn10 basS/L102Q ΔeptA::kan (pBAD18LptFG3/LptG/K34D)</td>
<td>This study</td>
</tr>
<tr>
<td>NR4062</td>
<td>NR754 ΔlptFG zjd2211::Tn10 basS/L102Q ΔeptA::frt (pBAD18LptFG3/LptG/K34D)</td>
<td>This study</td>
</tr>
<tr>
<td>NR3996</td>
<td>NR754 ΔlptFG zjd2211::Tn10 basS(L102Q) ΔarnT::kan (pBAD18LptFG3/LptG/K34D)</td>
<td>This study</td>
</tr>
<tr>
<td>NR4048</td>
<td>NR754 ΔlptFG zjd2211::Tn10 ΔeptA::kan (pBAD18LptFG3/LptG/K34D)</td>
<td>This study</td>
</tr>
<tr>
<td>NR4063</td>
<td>NR754 ΔlptFG ΔeptA zjd2211::Tn10 (pBAD18LptFG3/LptG/K34D)</td>
<td>This study</td>
</tr>
<tr>
<td>NR4063</td>
<td>NR754 ΔlptFG ΔeptA::frt zjd2211::Tn10 (pBAD18LptFG3/LptG/K34D)</td>
<td>This study</td>
</tr>
<tr>
<td>NR4117</td>
<td>NR754 ΔlptFG ΔeptA::frt ΔarnT::kan zjd2211::Tn10 (pBAD18LptFG3/LptG/K34D)</td>
<td>This study</td>
</tr>
<tr>
<td>NR4062</td>
<td>NR754 ΔlptFG ΔeptA::frt ΔarnT::kan zjd2211::Tn10 basS/L102Q (pBAD18LptFG3/LptG/K34D)</td>
<td>This study</td>
</tr>
<tr>
<td>NR4116</td>
<td>NR754 ΔlptFG ΔeptA::frt ΔarnT::kan zjd2211::Tn10 basS/L102Q (pBAD18LptFG3/LptG/K34D)</td>
<td>This study</td>
</tr>
<tr>
<td>NR3954</td>
<td>NR754 ΔlptFG zjd2211::Tn10 basS(L102Q) (pBAD18LptFG3)</td>
<td>This study</td>
</tr>
<tr>
<td>NR3265</td>
<td>NR754 ΔlptFG (pBAD18LptFG3/LptF/E84A)</td>
<td>(193)</td>
</tr>
<tr>
<td>NR4382</td>
<td>NR754 ΔlptFG zjd2211::Tn10 basS(L102Q) (pBAD18LptFG3/LptF/E84A)</td>
<td>This study</td>
</tr>
<tr>
<td>NR2762</td>
<td>NR754 ΔlptFG (pBAD18LptFLptG/E88A)</td>
<td>(193)</td>
</tr>
<tr>
<td>NR4383</td>
<td>NR754 ΔlptFG zjd2211::Tn10 basS(L102Q) (pBAD18LptFG3/LptG/E88A)</td>
<td>This study</td>
</tr>
<tr>
<td>NR760</td>
<td>MC4100 lptD4213</td>
<td>(197)</td>
</tr>
<tr>
<td>NR4372</td>
<td>NR754 lptD4213 zjd2211::Tn10 basS(L102Q)</td>
<td>This study</td>
</tr>
</tbody>
</table>
Table A.5 Primers used in chapter 2

<table>
<thead>
<tr>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>5LptG-K34R</td>
</tr>
<tr>
<td>3LptG-K34R</td>
</tr>
<tr>
<td>5LptG-K34C</td>
</tr>
<tr>
<td>3LptG-K34C</td>
</tr>
<tr>
<td>5LptG-K34A</td>
</tr>
<tr>
<td>3LptG-K34A</td>
</tr>
<tr>
<td>5LptG-K34Q</td>
</tr>
<tr>
<td>3LptG-K34Q</td>
</tr>
<tr>
<td>5LptG-K34D</td>
</tr>
<tr>
<td>3LptG-K34D</td>
</tr>
<tr>
<td>K34D-f</td>
</tr>
<tr>
<td>K34D-r</td>
</tr>
<tr>
<td>K41D-f</td>
</tr>
<tr>
<td>K41D-r</td>
</tr>
<tr>
<td>5LptG-D37A</td>
</tr>
<tr>
<td>3LptG-D37A</td>
</tr>
<tr>
<td>LptG-D37C Sense</td>
</tr>
<tr>
<td>LptG-D37C Anti</td>
</tr>
<tr>
<td>LptG-Q38A Sense</td>
</tr>
<tr>
<td>LptG-Q38A Anti</td>
</tr>
<tr>
<td>5LptG-K40D</td>
</tr>
<tr>
<td>3LptG-K40D</td>
</tr>
<tr>
<td>5LptG-K41D</td>
</tr>
<tr>
<td>3LptG-K41D</td>
</tr>
<tr>
<td>5LptG-K40D/K41D</td>
</tr>
<tr>
<td>3LptG-K40D/K41D</td>
</tr>
<tr>
<td>5LptG-K34A/40/41D</td>
</tr>
<tr>
<td>3LptG-K34A/40/41D</td>
</tr>
<tr>
<td>5LptG-K34A/40/41D</td>
</tr>
<tr>
<td>3LptG-K34A/40/41D</td>
</tr>
<tr>
<td>5LptG-K34E</td>
</tr>
<tr>
<td>3LptG-K34E</td>
</tr>
<tr>
<td>LptBY47Asense</td>
</tr>
<tr>
<td>LptBY47Aanti</td>
</tr>
<tr>
<td>5BasS</td>
</tr>
<tr>
<td>3BasS</td>
</tr>
<tr>
<td>5EptA</td>
</tr>
<tr>
<td>5EptA471</td>
</tr>
<tr>
<td>3EptA</td>
</tr>
<tr>
<td>5arnT</td>
</tr>
<tr>
<td>3arnT</td>
</tr>
</tbody>
</table>
Figure A.1: Protein and LPS levels of LptG variants.

A-C) Immunoblot analyses of whole cell lysates, normalized based on the OD\textsubscript{600} values of overnight cultures, from strains encoding LptG variants were performed using anti-LptG antisera. Molecular weight markers are shown on the left in KDa. Labels correspond to the following strains: WT = NR2761 (panels A and C) and NR3079 (panel B), K34R = NR4027, K34C = NR4077, K34A = NR3740, K34Q = NR4084, K34D = NR3673, K40D = NR3674, K41D = NR3675, K40D/K41D = NR3741, K34E = NR4840, K34A/K40D/K41D = NR4841, K34D/K40D/K41D = NR4842, D37A = NR4433, D37C = NR4518, Q38A = NR4319, Q38C = NR4232.
D) LPS levels do not change significantly among mutant and suppressor strains. LPS immunoblotting of whole cell samples normalized by OD$_{600}$ was performed and analyzed as described in Experimental Procedures. Average and standard deviation values derived from three independent experiments are shown. In each experiment, LPS levels were normalized to those of the wild-type (WT) strain (NR2761). From left to right, strains used were NR2761, NR3954, NR3673, NR3951, and NR4116.

Figure A.2 Activation of BasS changes lipid A profile.

A-C) MALDI-TOF spectrum for lipid A isolated from the wild-type strain NR3955 (panel A), $lptG(K34D)$ strain NR3952 (panel B), $lptG(K34D)$ bas$S^c$ strain NR3951
(panel C). D) Lipid A structures of species corresponding to the observed m/z peaks labeled in panels A-C. The additions of PEtN and L-Ara4N are shown on a single LPS molecule in panel D for illustrative purposes, but the m/z values provided correspond to LPS modified with either a single PEtN (orange) or L-Ara4N (green) group. In the analysis of the \textit{lptG(K34D) basS} strain NR3951, we could detect about 1% of LPS being modified with L-Ara4N (peak with m/z value of 1927.4). This modification was absent in the wild-type (NR3955) and \textit{lptG(K34D)} (NR3952) strains. In some biological repeats of the unsuppressed \textit{lptG(K34D)} strain, we observed the presence of some PEtN-modified lipid A and a concomitant reduction in hepta-acylated 1,4'-bis-phosphate lipid A. This phenotype, which is intermediate between that of the \textit{lptG(K34D)} and \textit{lptG(K34D) basS} strains, was likely caused by the appearance of spontaneous suppressor mutants that arose in the cultures of the \textit{lptG(K34D)} strain from which lipid A was purified.
Appendix B: Extended Data for Chapter 3
Figure B.1 Purification and reconstitution of wild-type and inactive complexes of *V. cholerae* and *E. cloacae* LptB\(_2\)FGC.

a, To assess the protein quality in purified LptB\(_2\)FGC complexes, samples were boiled in SDS loading buffer, separated by PAGE using a 4–20% gradient polyacrylamide gel, and then stained with Coomassie blue dye. (1) Wild-type, *E. cloacae*; (2) catalytically inactive LptB(E163Q), *E. cloacae*; (3) wild-type, *V. cholerae*.
cholerae; and (4) catalytically inactive LptB(E163Q), V. cholerae. Note that V. cholerae LptF and LptG co-migrate in this gel, but can be separated under other conditions. Protein samples are each representative of ten or more replicates. b, Schematic of a reconstitution of LPS transport in which purified LptB2FGC and LPS have been incorporated into liposomes by the detergent-dilution method. Addition of LptA modified with a photocrosslinkable amino acid (I36pBPA for E. cloacae, V34pBPA for V. cholerae), along with ATP and Mg2+ leads to time-dependent accumulation of LPS in LptA. LPS transport is monitored by exposing the samples to UV light at the time point shown, quenching transport by addition of SDS loading buffer, PAGE to separate LPS-LptA adducts from LPS and, finally, western blotting for LPS. c, Results of the experiment described in b. Blotting for LPS shows time-dependent accumulation of LPS in LptA when the proteoliposomes contain wild-type LptB2FGC complexes, but accumulation of LPS is not observed when EDTA is substituted for Mg2+, or when the complexes contain LptB(E163Q) rather than wild-type LptB. His6 blots were used to assess the amount of LptA(XpBPA)–His in each sample. E. coli and E. cloacae LptA(I36pBPA)–His6 run as a doublet whereas V. cholerae LptA(I36pBPA)-His6 does not. Blots are representative of data from three biological replicates.
Figure B.2 Structures *V. cholerae* LptB(E163Q)2FGC and *E. cloacae* LptB2FGC with their electron density maps.

a, Ribbon diagram of *V. cholera* LptB(E163Q)2FGC (left) and corresponding $2F_o - F_c$ simulated-annealing composite omit map (right) contoured at 1.0σ. and carved 2.5 Å from the model. b, Ribbon diagram of *E. cloacae* LptB2FGC (left) and corresponding $2F_o - F_c$ simulated-annealing composite omit map (right) contoured at 1.0σ. and carved at 2.5 Å from the model. c, Electrostatic surface
potential of *V. cholerae* LptB(E163Q)\(_2\)FGC depicted with the most basic residues in blue and most acidic residues in red. The approximate edges of the inner membrane are denoted with grey dashed lines. The central panel is in the same orientation as in a. The left panel is a 180° rotation around the axis perpendicular to the membrane. In the right panel, the view in the central panel has been rotated 180° around an axis parallel to the membrane in the plane of the page, and the periplasmic domains are clipped away to give a view of the central cavity between the transmembrane helices. For reference, a ribbon diagram (analogous to Fig. 3.1c) is shown above the surface diagram.
Figure B.3 Alignments of *E. coli*, *E. cloacae*, and *V. cholerae* LptB, LptF, LptG, LptC and LptA.

a, Sequences of Lpt proteins from *E. coli* K12, *E. cloacae* subsp. *cloacae* (ATCC 13047) and *V. cholerae* C6706. Percent identity of the *E. cloacae* and *V. cholerae* sequences to the *E. coli* sequence are listed after each alignment. Note that public databases (including Uniprot and NCBI) contain a misannotated LptF *cholerae* sequences to the *E. coli* sequence are listed after each alignment. Note that public databases (including Uniprot and NCBI) contain a misannotated LptF.
sequence for *E. cloacae* subsp. *cloacae* (ATCC 13047), with bases
GTGATAATCATAAGATATCTG missing from the beginning of LptF; LptF
homologues from other *E. cloacae* strains do not appear to contain this error.
Alignments were made using ClustalO and then coloured in MView
(https://www.ebi.ac.uk/Tools/msa/clustalo/).
Figure B.4 LptC(ΔTM) stably associates with LptB2FG, and facilitates LPS release to LptA as well as full-length LptC without altering the ATPase activity of LptB2FG. 

a, in vivo photocrosslinking experiment from Fig. 3.2b, shown with the corresponding anti-LptC western blot. b, in vivo photocrosslinking experiments (Fig. 3.2) showed that a ρBPA substitution at position R223 of LptF could form LptF–LptC adducts after exposure to UV. In this experiment, proteoliposomes containing LptF, LptB2F(R223ρBPA)G were incubated with soluble ΔTM-
LptC(ΔTM) at 1×, 2×, 5× or 10× molar excess of LptC and exposed to UV light to test whether LptC(ΔTM) associated with the LptB\textsubscript{2}F(R223pBPA)\textsubscript{G} complexes. Western blots for LptC (left) and LptF (right) were used to assess the amount of LptC–LptF adduct formed (top panels) as well as the amounts of LptC and LptF in each sample (bottom panel). The LptF and LptC blots were performed on the same set of samples. The intensity of the LptC–LptF adduct band does not increase with LptC(ΔTM) beyond 2× to 5× molar excess. This result suggests that at those concentrations of LptC(ΔTM), the binding site for LptC(ΔTM) at LptF(R223pBPA) is saturated. The intensity of the LptF band (bottom right panel) does not decrease further because residues R223pBPA can also form intramolecular crosslinks to other parts of the LptF β-jellyroll, and these may be favored over intermolecular crosslinks to LptC(ΔTM). c, d, Fig. 3.2c, d shown with 5× and 10× molar excess of LptC(ΔTM). Blots shown in a, b and c are representative of data from three biological replicates.
Figure B.5 Comparison of previously published LptB₂FG structures to the structure of *V. cholerae* LptB₂FGC.

a, Top-down views of the *P. aeruginosa* LptB₂FG structure (left, PDB ID 5X5Y) (149), *K. pneumoniae* LptB₂FG structure (center, PDB ID 5I75) (150) and our *V. cholera* LptB₂FGC structure (right). b, Alignments of *P. aeruginosa* LptB₂FG (left,
dark blue) and *K. pneumoniae* LptB₂FG (right, dark blue) with *V. cholerae* LptB₂FGC (LptC, pink; LptF, green; LptG, teal; and LptB, grey).
Figure B.6 LPS enters the cavity of LptB$_2$FGC via LptG TM1, LptF TM5 and the LptC transmembrane helix in a nucleotide-independent manner.

Western blots from Fig. 3.3b are shown alongside blots from additional in vivo photocrosslinking experiments comparing LPS crosslinking in wild-type and LptB(E163Q) backgrounds. b, In vitro reconstitution (as in Fig. B.1b) of
LptB$_2$FGC, LptB$_2$FGC(M19pBPA), LptB$_2$FGC(G21pBPA) and LptB$_2$FGC(F78pBPA) shows that the variants with amber codons incorporated in the LptC transmembrane helix release LPS to LptA(I36pBPA) as well as wild type. c, In vitro photocrosslinking of LptB$_2$FGC pBPA variants reconstituted into proteoliposomes with LPS, as in Fig. 3.3d, comparing complexes containing LptB(E163Q) to those containing wild-type LptB, and the effects of AMPPNP relative to ATP. d–f, Additional crosslinking experiments with pBPA incorporated at other positions in the transmembrane helix of LptC, LptF TM1 and TM5, and LptG TM1 and TM5. g, h, Ribbon diagrams of *E. cloacae* LptB$_2$FGC, with views of the two potential gates in the LptFGC transmembrane helices; LptG (teal), LptC (pink), and LptF (green). Residues of interest are shown as sticks. Blots for LptG(S30pBPA) are shown in Extended Data Fig. 3.7b. Blots shown in a–f are representative of data from three biological replicates.
Figure B.7  *in vivo* photocrosslinking experiments show that LPS passes through the β-jellyroll domains of LptF and LptC, but not LptG.

a. Blots from Fig. 3.3c are shown for reference.  
b. *In vivo* photocrosslinking experiments performed analogously to those in Fig. 3.3b, c. The UV-photocrosslinkable amino acid pBPA was substituted at positions in the periplasmic β-jellyroll domains of LptC, LptF and LptG. Positions in the interior of the LptF and LptC can crosslink to LPS, whereas those facing outside the β-jellyroll do not. No crosslinks to LPS were observed at ten positions in the LptG
β-jellyroll; by contrast, LptG(S30pBPA), in TM1 and facing the cavity of the transmembrane domain, can crosslink LPS. c, Ribbon diagram of the structure of the \textit{V. cholerae} complex, showing the β-jellyroll domains of LptC, LptF, and LptG. Side chains of residues that crosslink LPS are shown in red, whereas residues that do not crosslink LPS are shown in blue. Blots shown in a and b are representative of data from three biological replicates.

Figure B.8 Comparison of the \textit{V. cholerae} and \textit{E. cloacae} structures reveals two states of the gate in LptF.

a, An overlay of the \textit{V. cholerae} (LptG, teal; LptF, green; and LptC, magenta) and \textit{E. cloacae} (LptG, grey; LptF, orange; and LptC, pink) structures shows two conformations of the loop between the third and fourth β-strands of the LptF periplasmic domain. b, Magnified view of this loop and an adjacent loop from
LptC in the *V. cholerae* structure. LptF residues 179–183 and LptC residues 71–74 are depicted as sticks on top of the cartoon. c, Magnified view of the same region in *E. cloacae*. The $2F_\text{o} - F_\text{c}$ omit maps in b and c are contoured at $1.0\sigma$ and carved 2.5 Å from the protein.
Figure B.9 Complementation and purification of LptF cysteine mutants.

a, Table of results from assays that tested the ability of plasmids encoding *E. coli* LptF variants to complement a Δ*lptF* strain. b, To assess whether the LptF double-cysteine mutant was being expressed and translated properly, we measured LptF–Flag levels by anti-Flag western blot in whole-cell lysates from merodiploid *E. coli* strains containing either pBAD18-LptF-Flag or pBAD18-
LptF(S157C/I234C)-Flag. c, Disc diffusion assays were carried out to assess the outer-membrane permeability (relative to wild type) of *E. coli* strains expressing only plasmid-encoded LptF variants. Values denote the diameter (in mm) of regions with no growth, and values in parentheses denote regions of inhibited growth. Discs were 6 mm in diameter. d, Disc diffusion assays were performed as in c, except that experiments were performed in merodiploid strains with wild-type chromosomal copies of *lptFG*. Alleles are listed as follows: plasmid-encoded *lptFG* alleles, a solidus and then the chromosome-encoded *lptFG* alleles. e, Coomassie-stained samples of purified LptB2FGC used in the LPS release assay: (1) LptF–Flag; (2) LptF(S157C)–Flag; (3) LptF(I234C)–Flag; and (4) LptF(S157C/I234C)–Flag. LptC contained a C-terminal thrombin-cleavable His7 tag used for nickel-affinity chromatography, which was cleaved before reconstitution into liposomes. f, The ATPase activity of *E. coli* LptB2FGC reconstituted into liposomes was assessed by measuring the rate of phosphate release over time. Data are mean ± s.d. of results from three technical replicates, each of two biological replicates, except two replicates that were performed with one LptB(E163Q)2FGC sample. Data in b and e are representative of data from three biological replicates. Data in c and d are mean ± s.d. calculated from three independent experiments.
Figure B.10 Examples of small molecules bound in the crystal structures of *V. cholerae* and *E. cloacae* LptB<sub>2</sub>FGC, and unmodelled electron density in the *V. cholerae* LptB(E163Q)<sub>2</sub>FGC structure.
a, Two views of the one molecule of novobiocin bound at the interface between two non-crystallographically symmetric copies of *E. cloaca* LptB, with $2F_o - F_c$ simulated-annealing composite omit map contoured at 1.5$\sigma$ and carved at 2.0 Å.
b, Electron density for several ordered detergent molecules is present in the structure of *V. cholerae* LptB$\_2$FGC; here, a DDM molecule is shown bound to LptG with an $F_o - F_c$ Polder map contoured at 2.5$\sigma$. c, Ordered PEG400 at the interface of *V. cholerae* LptB and LptG, with the $2F_o - F_c$ map contoured at 1.0$\sigma$ and carved at 2.0 Å. d, View of the lumen between the transmembrane helices of LptC (purple), LptF (green) and LptG (teal), looking towards the inner membrane from the periplasm. The $2F_o - F_c$ map is contoured at 1.0$\sigma$ (blue mesh) and the $F_o - F_c$ map is contoured at 3.0$\sigma$ (green and red meshes). e, View of the same region from within the membrane, with LptF helices in the foreground clipped away; the perspective is approximated by the teal arrow at the top right of d.
Appendix C: Supplementary Data for Chapter 3

Figure C.1 Uncropped gels and blots for data shown in main-text and extended data figures.

For each figure, the cropped regions are denoted by boxes.
From figure 3.2C – blots shown overlain marker images taken concurrently with Azure c600 imaging system
Figure 3.2c, Figure B.4

α-LptA

α-LPS

kDa

25

20

15

LptF(R223pBPA)

α-LptC

15

20

25

37

50

75

kDa

ΔTM-LptC

UV

- + + + + + + + + + +


- - 1x 2x 5x 10x

o-LptA

o-LptF

168
Figure 3.3B – Anti-His5-HRP blot shown overlain marker images taken concurrently with Azure c600 imaging system

Extended data figure 3.6a – Anti-His5-HRP blot shown overlain marker images taken concurrently with Azure c600 imaging system
**Figure 3.3d and Figure B.6b**

<table>
<thead>
<tr>
<th>WT E. coli LptB,F,G</th>
<th>LptC(XpBPA)</th>
<th>ATP/Mg²⁺ ± EDTA</th>
<th>± VO₄⁻</th>
<th>t (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>0 30 30</td>
</tr>
<tr>
<td>M19</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0 30 30</td>
</tr>
<tr>
<td>G21</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0 30 30</td>
</tr>
<tr>
<td>F78</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0 30 30</td>
</tr>
</tbody>
</table>

**Figure 3.3d**

![Image](https://example.com/image1.png)

Anti-LPS

**Figure B.6b**

![Image](https://example.com/image2.png)

Anti-LptA

<table>
<thead>
<tr>
<th>WT E. coli LptB,F,G</th>
<th>LptC(XpBPA)</th>
<th>ATP/Mg²⁺ ± EDTA</th>
<th>± VO₄⁻</th>
<th>t (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>0 30 30</td>
</tr>
<tr>
<td>M19</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0 30 30</td>
</tr>
<tr>
<td>G21</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0 30 30</td>
</tr>
<tr>
<td>F78</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0 30 30</td>
</tr>
</tbody>
</table>

**Figure 3.3d**

![Image](https://example.com/image3.png)

Anti-LPS

**Figure B.6b**

![Image](https://example.com/image4.png)

Anti-LptA

<table>
<thead>
<tr>
<th>WT E. coli LptB,F,G</th>
<th>LptC(XpBPA)</th>
<th>ATP/Mg²⁺ ± EDTA</th>
<th>± VO₄⁻</th>
<th>t (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>0 30 30</td>
</tr>
<tr>
<td>M19</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0 30 30</td>
</tr>
<tr>
<td>G21</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0 30 30</td>
</tr>
<tr>
<td>F78</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0 30 30</td>
</tr>
</tbody>
</table>

**Figure 3.3d**

![Image](https://example.com/image5.png)

Anti-LPS

**Figure B.6b**

![Image](https://example.com/image6.png)

Anti-LptA
Figure 3.3C –

<table>
<thead>
<tr>
<th>Lpt</th>
<th>LptC</th>
<th>LptF</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBPA</td>
<td>UV</td>
<td></td>
</tr>
<tr>
<td>Y42</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>T47</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>R212</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>R223</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Y230</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>R220</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

- Anti-LPS
- Anti-His
- Anti-LptF
Figure 3.4E

<table>
<thead>
<tr>
<th>LptF</th>
<th>WT</th>
<th>S157C</th>
<th>S157C + I234C</th>
</tr>
</thead>
<tbody>
<tr>
<td>t (min)</td>
<td>0</td>
<td>15</td>
<td>30</td>
</tr>
<tr>
<td>DTT</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

kDa

50
37
25
20
15
10

anti-LPS

<table>
<thead>
<tr>
<th>LptA</th>
<th>anti-His (LptA36pBP)-His</th>
</tr>
</thead>
<tbody>
<tr>
<td>t (min)</td>
<td>0</td>
</tr>
<tr>
<td>DTT</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure B.1– panel (c),

<table>
<thead>
<tr>
<th></th>
<th><strong>E. cloacae LptB,FGC</strong></th>
<th></th>
<th><strong>V. cholerae LptB,FGC</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>t (min)</strong></td>
<td>0</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>LptB</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ATP</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>UV</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EDTA</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**kDa**

75 | 50 | 37 | 25 | 20 | 15

**kDa**

75 | 50 | 37 | 25 | 20 | 15
Figure B.6 – panel (b), (c), (e)

**Table B.6.1**

<table>
<thead>
<tr>
<th></th>
<th>WT E. coli LptB GFP</th>
<th>M19 G21</th>
<th>E78</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LptC</strong>&lt;sup&gt;(α)BPA&lt;/sup&gt;</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td><strong>ATP/Mg&lt;sup&gt;2+&lt;/sup&gt;</strong>&lt;sup&gt;±&lt;/sup&gt;</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td><strong>t (min)</strong>&lt;sup&gt;−&lt;/sup&gt;</td>
<td>0</td>
<td>30</td>
<td>30</td>
</tr>
</tbody>
</table>

**Panel B**

- **LptC**<sup>(α)BPA</sup>:
  - WT E. coli LptB GFP:
    - ATP/Mg<sup>2+</sup>:
      - UV:
        - 0 min: +, −, −, −
        - 30 min: +, −, −, −
  - M19 G21:
    - ATP/Mg<sup>2+</sup>:
      - UV:
        - 0 min: +, −, −, −
        - 30 min: +, −, −, −
  - E78:
    - ATP/Mg<sup>2+</sup>:
      - UV:
        - 0 min: +, −, −, −
        - 30 min: +, −, −, −

**Panel C**

- **LptC**<sup>(α)BPA</sup>:
  - WT E. coli LptB GFP:
    - ATP/Mg<sup>2+</sup>:
      - UV:
        - 0 min: +, −, −, −
        - 30 min: +, −, −, −
    - AMPPNP/Mg<sup>2+</sup>:
      - UV:
        - 0 min: +, −, −, −
        - 30 min: +, −, −, −
  - M19 G21:
    - ATP/Mg<sup>2+</sup>:
      - UV:
        - 0 min: +, −, −, −
        - 30 min: +, −, −, −
    - AMPPNP/Mg<sup>2+</sup>:
      - UV:
        - 0 min: +, −, −, −
        - 30 min: +, −, −, −
  - E78:
    - ATP/Mg<sup>2+</sup>:
      - UV:
        - 0 min: +, −, −, −
        - 30 min: +, −, −, −
    - AMPPNP/Mg<sup>2+</sup>:
      - UV:
        - 0 min: +, −, −, −
        - 30 min: +, −, −, −

**Figure B.6 (cont.)**

- **Panel B**
  - (α-<em>His</em>):
    - kDa:
      - 75
      - 50
      - 37
      - 25
      - 20
      - 15
- **Panel C**
  - (α-LPS):
    - kDa:
      - 75
      - 50
      - 37
      - 25
      - 20
  - (α-LptC):
    - kDa:
      - 75
      - 50
      - 37
      - 25
      - 20

**Legend**

- **LptC**<sup>(α)BPA</sup>
- **ATP/Mg<sup>2+</sup>**
- **AMPPNP/Mg<sup>2+</sup>**
- **UV**
- **t (min)**
- **kDa**

**Note**: The table and figures represent data from experiments involving the LptC protein expressed in E. coli with different treatments, including ATP/Mg<sup>2+</sup> and AMPPNP/Mg<sup>2+</sup> additions, UV treatment, and time points of 0 and 30 minutes. The kDa values indicate the molecular weight markers used in the gel electrophoresis experiments.
Figure B.6—panels (f)
Figure B.7– panel (b), (c),
Figure B.5—panels (b), and (d)
Table C.1 X-ray data collection and structure refinement.

A table of statistics from X-ray data collection alongside the statistics generated during structure refinement.

<table>
<thead>
<tr>
<th>Data collection</th>
<th>( V. ) cholerae LptB(E163Q)2FGC</th>
<th>( E. ) cloacae LptB2FGC†</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Space group</strong></td>
<td>C 2</td>
<td>P2_12_1</td>
</tr>
<tr>
<td><strong>Cell dimensions</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( a, b, c ) (Å)</td>
<td>167.35, 80.72, 202.99</td>
<td>116.72, 157.21, 297.25</td>
</tr>
<tr>
<td>( \alpha, \beta, \gamma ) (°)</td>
<td>90.00, 112.18, 90.00</td>
<td>90.00, 90.00, 90.00</td>
</tr>
<tr>
<td><strong>Resolution (Å)</strong></td>
<td>49.27-2.85 (2.93-2.85) *</td>
<td>49.33-3.20 (3.25-3.20) *</td>
</tr>
<tr>
<td><strong>( R_{\text{sym}} ) or ( R_{\text{merge}} )</strong></td>
<td>0.183 (2.82)</td>
<td>0.355 (18.02)</td>
</tr>
<tr>
<td><strong>( I/\sigma I )</strong></td>
<td>7.4 (0.8)</td>
<td>6.7 (0.6)</td>
</tr>
<tr>
<td><strong>Completeness (%)</strong></td>
<td>99.6 (100.0)</td>
<td>99.9 (99.2)</td>
</tr>
<tr>
<td><strong>Redundancy</strong></td>
<td>6.4 (6.7)</td>
<td>16.7 (15.7)</td>
</tr>
<tr>
<td><strong>CC(_{1/2})</strong></td>
<td>0.995 (0.305)</td>
<td>0.995 (0.295)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Refinement</th>
<th>( V. ) cholerae LptB(E163Q)2FGC</th>
<th>( E. ) cloacae LptB2FGC†</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Resolution (Å)</strong></td>
<td>49.27 – 2.85</td>
<td>49.33-3.20</td>
</tr>
<tr>
<td><strong>No. reflections</strong></td>
<td>58672</td>
<td>89469</td>
</tr>
<tr>
<td><strong>( R_{\text{work}} ) / ( R_{\text{free}} )</strong></td>
<td>24.21 / 29.17</td>
<td>27.90 / 32.01</td>
</tr>
<tr>
<td><strong>No. atoms</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>10212</td>
<td>19625</td>
</tr>
<tr>
<td>Ligand/ion</td>
<td>233</td>
<td>100</td>
</tr>
<tr>
<td>Water</td>
<td>53</td>
<td>8</td>
</tr>
<tr>
<td><strong>B-factors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>110.5</td>
<td>136.5</td>
</tr>
<tr>
<td>Ligand/ion</td>
<td>119</td>
<td>142.7</td>
</tr>
<tr>
<td>Water</td>
<td>81.8</td>
<td>103.6</td>
</tr>
<tr>
<td><strong>R.m.s. deviations</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bond lengths (Å)</td>
<td>0.0073</td>
<td>0.0072</td>
</tr>
<tr>
<td>Bond angles (°)</td>
<td>0.93</td>
<td>0.94</td>
</tr>
<tr>
<td><strong>PDB ID</strong></td>
<td>6MJP</td>
<td>6MIT</td>
</tr>
</tbody>
</table>

†Number of crystals: 3

*Values in parentheses are for highest-resolution shell.
Table C.2 Primers used to generate plasmids and strains in this study.

Primers used to generate new plasmids for protein expression or complementation assays, including primers for amplifying genes from genomic DNA.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>General</th>
<th>Sequence 5' to 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>site_1_upstrm_rev</td>
<td>AT GGT ATA TCT CCT TAT TAA AGT TAA ACA AAA TTA TTT CTA CAG GGG</td>
<td></td>
</tr>
<tr>
<td>site_1_dwnstrm_fw</td>
<td>GGA TTC GAG CTC GGC GCG CCT GCA GGT C</td>
<td></td>
</tr>
<tr>
<td>site_2_upstrm_rev</td>
<td>CAT ATG TAT ATC TCC TTA TAC TTA ACT AAT ATA CTA AGA TGG GG</td>
<td></td>
</tr>
<tr>
<td>site_2_dwnstrm_fw</td>
<td>GGT ACC CTC GAG TCT GTG AAA GAA ACC G</td>
<td></td>
</tr>
<tr>
<td>2242_upstrm_rev</td>
<td>CAT ATG TAT ATC TCC TTA AAG TTA AAC AAA ATT TCT AGA GGG G</td>
<td></td>
</tr>
<tr>
<td>2242_dwnstrm_fwd</td>
<td>CTG GTA CCG CGC GGC AGC CAC CAC C</td>
<td></td>
</tr>
<tr>
<td>pBADHisA_up_r</td>
<td>GGT TAA TCT CTC TTA GGC CAC CAA AAA ACG</td>
<td></td>
</tr>
<tr>
<td>pBADHisA_dwn_f</td>
<td>AAG CTT GGC TGT TTT GGC GGA TG</td>
<td></td>
</tr>
<tr>
<td>thrombin-HisHis-f</td>
<td>CTG GTA CCG CGC GGC AGC CAC CAC C</td>
<td></td>
</tr>
<tr>
<td>pelB-rev</td>
<td>GGC CAT CGC CGG CTG GGC AGC GAG GAG GAG CAC CAC</td>
<td></td>
</tr>
<tr>
<td>pet22b_3'_rev</td>
<td>CACCACCACCAACACCACACTGAGATCCGGC</td>
<td></td>
</tr>
<tr>
<td><strong>Enterobacter cloacae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ecloa_B_fwd</td>
<td>AAT AAT TTT GTT TAA CTT TAA TAA GGA GAT ATA CCA TGG CAA CAT TAA CTG CAA AAA ATC</td>
<td></td>
</tr>
<tr>
<td>Ecloa_B_rev</td>
<td>TCG ACC TGC AGG CGC GCC GAG CTC GAA TCC TCA GAG TCT GAC TCC CCC</td>
<td></td>
</tr>
<tr>
<td>Ecloa_F_fwd</td>
<td>ATT AGT TAA GTA TAA GGA GAT ATA CAT ATG ATA ATC ATA AGA TAT CTG GTG CCG G</td>
<td></td>
</tr>
<tr>
<td>Ecloa_G_rev</td>
<td>CGG TTT CTT TAC CAG ACT CGA GGG TAC CTC AGG AGC GGT TCA GCA GCA</td>
<td></td>
</tr>
<tr>
<td>Ecloa_C_fwd</td>
<td>TTT GTT TAA CTT TAA GGA GAA GAT ATA CAT ATG AGT AAA ACC AGA CGT TGG G</td>
<td></td>
</tr>
<tr>
<td>Ecloa_C_rev</td>
<td>GGT GTT GCC TGC CGC CGC GTA CCA GAG GCT GAG TTT GTG TGT TTT GAA TTT</td>
<td></td>
</tr>
<tr>
<td>pelB-</td>
<td>ACT GGC GAT ACC GAA CAG CCG</td>
<td></td>
</tr>
</tbody>
</table>
| Ecloacae_LptA_fwd| GCCGGATCTCAGTGGTGTTGATTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTG
Ec_LptA_I36Am_r
v
GAG ACT GCG TAT CGG ACT CGA TAT GCT ACG GCT GTT CCG TAT CGC CAG TAA C
EcLoa_LptB_E163
Q_fwd
CCATTTCGAGGCGTTGACCCC
EcLoa_LptB_E163
Q_rev
TTGGT
pBADHisA_Ecloa_LptC_fwd
CGTTTTTTGGGTAAGGAGGAATTAACC ATG AGT AAA ACC AGA CGT TGG GTT ATC
pBADHisA_Ecloa_LptC_rev
GGGGTCAACGCCAAGCAGCAGGACGGATGAAAC TTCGG
pBADHisA_Ecloa_LptC_fwd
CTCTCATCCGCCAAAACCAGCAGCAGT
pBADHisA_Ecloa_LptC_rev
CTCTCATCCGCCAAAACCAGCAGCAGT
his7-tbn-LptC_r
GTGTG TGT GTG GTG GTG GTT GCT GCC GGC CGG TAC CAG AGT TTG TTT TTG TTG

**Vibrio cholerae**

C6706_B_fwd
AAA TAA TTT TGT TTA ACT TTA ATA AGG AGA TAT ACC
ATG GCC ATC TTA AAA GCA CAG C
C6706_B_rev
TCG ACC TGC AGG CGC GCC GAG CTC GAA TTC TCA TAG
ACG GAA TGG TTC GCC GAG
C6706_F_fwd
TTA GTT AAG TAT AAG AAG GAG ATA TAC ATA TGA TTA
TTG TTA GAT ATT TGA TCC GAG AAC
C6706_G_rev
CGG TTT CTT TAC CAG ACT CGA GGG TAC CTT ATA GCT
TTG TAC CCA ATA GCC
C6706_C_fwd
TTT GTT TAA CTT TAA GAA GGA GAT ATA CAT ATG AGT
TTG TCG CGC ATT GTT TAC
C6706_C_rev:
GGT GGT GGC TGC CGC GCG GTA CCA GAG GTG TGA
GCG TTT CAT ATC TAC C
pelB_vibrio_lptA_fwd
CTGCTCCTCGCCACGCCGCATGCCAC
pet22b_vibrio_LptA_rev
GCGGATCTCTAGTGCGCGGATGCC

C6706-LptA-V34Am_fwd
CTCTCTACCGACAGAGAACAAC
C6706-LptA-V34Am_rev
CTCTCTACCGACAGAGAACAAC
C6706-LptB-E163Q_fwd
GCTAACCCACAATTTATCTTGCTGGAT CAG
C6706-LptB-E163Q_rev
GGGGTCAACGCCCAAAACCAGCAGCAGT
his7-thrombin_clean
GTGGTGGCTGCCGCGCGGTACCAGAGGTGTGAGCGTTTCA
his7-thrombin_fwd
CTCTCTACCGACAGAGAACAAC
pBAD-c6706-C_fwd
CGTTTTTTGGGTAAGGAGGAATTAACC ATG AGT AAA ACC AGA CGT TGG GTT ATC
pBAD-c6706-C_rev
CGTTTTTTGGGTAAGGAGGAATTAACC ATG AGT AAA ACC AGA CGT TGG GTT ATC

---

180
LptF cysteine variants

LptFTAGsense GACTACAAGGACGACGACGACAAAGGTATGCACGTCCTTCTGTGGTGG
LptFTAGanti CTTGTCGTCGTCGTCCTTGTAGTCTTTTGGTCGAATTTTCGCGAGGAAC
5LptF-S157C GACTAATGCGACCTGCTGCTGCTTCAATCAGAAAAGCGTTG
3LptF-S157C GATGAAACCGACCGCTGCCATTAGTCTCGCTTGGCTGGAATTG
5LptF-I234C TCAGGGGATCTGCGTCACCAGGCGGTGTCGCTC
3LptF-I234C CCTGGTGACCGCGATCGCTGCTGGAAGTC

Table C.3 Strains used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH5α</td>
<td>F’ ϕ80lacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17 (rK-, mK+) phoA supE44 λ thi-1</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>NR2759</td>
<td>NR754 ΔlptFG (pRC7KanLptFG)</td>
<td>(193)</td>
</tr>
<tr>
<td>NR5593</td>
<td>NR754 ΔlptFG (pBAD18LptFG3/LptF/180FLAG)</td>
<td>This study</td>
</tr>
<tr>
<td>NR5686</td>
<td>NR754 ΔlptFG (pBAD18LptFG3/LptF/180FLAG/S157C)</td>
<td>This study</td>
</tr>
<tr>
<td>NR5687</td>
<td>NR754 ΔlptFG (pBAD18LptFG3/LptF/180FLAG/I234C)</td>
<td>This study</td>
</tr>
<tr>
<td>NR5710</td>
<td>NR754 (pBAD18LptFG3/LptF/180FLAG)</td>
<td>This study</td>
</tr>
<tr>
<td>NR5712</td>
<td>NR754 (pBAD18LptFG3/LptF/180FLAG/S157C)</td>
<td>This study</td>
</tr>
<tr>
<td>NR5713</td>
<td>NR754 (pBAD18LptFG3/LptF/180FLAG/I234C)</td>
<td>This study</td>
</tr>
<tr>
<td>NR5714</td>
<td>NR754 (pBAD18LptFG3/LptF/180FLAG/S157C/I234C)</td>
<td>This study</td>
</tr>
</tbody>
</table>

Genotypes of *E. coli* stains used in this study.
Table D.1: Elevated LPS levels suppress \( lptG(K34D) \) outer-membrane permeability defects

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Zone of inhibition (mm)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Bacitracin</td>
</tr>
<tr>
<td>NR5951</td>
<td>( lptG^+ ) (pACYC-empty)</td>
<td>7.3 (10.3) ± 0.4 (1.1)</td>
</tr>
<tr>
<td>NR5952</td>
<td>( lptG^+ ) (pACYC-lpxC)</td>
<td>7.8 (10.8) ± 1.1 (0.4)</td>
</tr>
<tr>
<td>NR5953</td>
<td>( lptG^+ ) (pACYC-rpoH)</td>
<td>7.3 (10.5) ± 0.4 (1.4)</td>
</tr>
<tr>
<td>NR5954</td>
<td>( lptG(K34D) ) (pACYC-empty)</td>
<td>19.8 (22) ± 1.1 (2.1)</td>
</tr>
<tr>
<td>NR5955</td>
<td>( lptG(K34D) ) (pACYC-lpxC)</td>
<td>15.5 ± 0.7</td>
</tr>
<tr>
<td>NR5956</td>
<td>( lptG(K34D) ) (pACYC-rpoH)</td>
<td>20 (22) ± 2.8 (0)</td>
</tr>
</tbody>
</table>

*aSensitivity was assessed by disc diffusion assays performed in two independent experiments. Values denote the diameter (in mm) of zones in which no growth was observed, whereas values in parentheses denote the diameter of areas of partially inhibited growth. The same format was used to denote standard deviation values. Discs were 6 mm in diameter.
Table D.2: Suppression by *ftsH*(F37S) is *lpt* allele specific

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Zone of inhibition (mm)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Bacitracin</th>
<th>Novobiocin</th>
<th>Erythromycin</th>
<th>Rifampin</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR2761</td>
<td><em>lptG</em>&lt;sup&gt;+&lt;/sup&gt;</td>
<td></td>
<td>7.8 (10) ± 0.8 (1.0)</td>
<td>7 (10.7) ± 0 (0.6)</td>
<td>7 (15.3) ± 0 (0.6)</td>
<td>9.5 (13) ± 0.5 (0)</td>
</tr>
<tr>
<td>NR5232</td>
<td><em>lptG(K34D)</em></td>
<td></td>
<td>18.8 ± 0.8</td>
<td>15 (27.2) ± 1 (0.8)</td>
<td>22.7 ± 0.6</td>
<td>15 (22.8) ± 0 (0.8)</td>
</tr>
<tr>
<td>NR6107</td>
<td><em>lptG(K34D) ftsH</em>(F37S)</td>
<td></td>
<td>13.0 (16.2) ± 0.7 (2.3)</td>
<td>10.3 (21.9) ± 0.4 (0.9)</td>
<td>13.4 (20.6) ± 1 (1.9)</td>
<td>14.4 (20) ± 0.5 (0.7)</td>
</tr>
<tr>
<td>NR2761</td>
<td><em>lptG</em>&lt;sup&gt;+&lt;/sup&gt;</td>
<td></td>
<td>7.4 (9.9) ± 0.7 (0.7)</td>
<td>7 (9.9) ± 0 (0.7)</td>
<td>7 (14.8) ± 0 (1)</td>
<td>9.8 (13.1) ± 0.7 (0.5)</td>
</tr>
<tr>
<td>NR3265</td>
<td><em>lptF(E84A)</em></td>
<td></td>
<td>14.8 ± 1.1</td>
<td>7.6 (11.8) ± 1.3 (1.3)</td>
<td>8.3 (20.4) ± 2.9 (1.3)</td>
<td>15 (21.8) ± 0.7 (1.5)</td>
</tr>
<tr>
<td>NR5528</td>
<td><em>lptF(E84A) ftsH</em>(F37S)</td>
<td></td>
<td>12.8 (14.5) ± 2.2 (1.6)</td>
<td>7 (11.6) ± 0 (1.1)</td>
<td>7.8 (21.4) ± 2.3 (0.8)</td>
<td>13.8 (21.2) ± 0.4 (1)</td>
</tr>
<tr>
<td>NR2762</td>
<td><em>lptG(E88A)</em></td>
<td></td>
<td>11.5 (21.7) ± 0.9 (8.4)</td>
<td>7.7 (17.8) ± 1.6 (2.6)</td>
<td>10.7 (20.5) ± 2.3 (3.2)</td>
<td>13.1 (19.2) ± 0.5 (2.9)</td>
</tr>
<tr>
<td>NR5529</td>
<td><em>lptG(E88A) ftsH</em>(F37S)</td>
<td></td>
<td>10.4 (21.4) ± 2.4 (8.7)</td>
<td>7 (13.6) ± 0 (4.0)</td>
<td>8.1 (19.2) ± 2.1 (3.4)</td>
<td>12.3 (18.4) ± 0.8 (2.4)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Sensitivity was assessed by disc diffusion assays performed in at least three independent experiments. Values denote the diameter (in mm) of zones in which no growth was observed, whereas values in parentheses denote the diameter of areas of partially inhibited growth. The same format was used to denote standard deviation values. Discs were 6 mm in diameter.
Table D.3 Strains used in Chapter 4

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH5α</td>
<td>F⁻ φ80lacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17 (rK⁺, mK⁺) phoA supE44 λ thi-1</td>
<td>Life technologies</td>
</tr>
<tr>
<td>NR4492</td>
<td>NR754 tet2 ΔlptFG (pBAD18LptFG3)</td>
<td>This study</td>
</tr>
<tr>
<td>NR5380</td>
<td>NR754 ftsH(F37S) ΔlptFG::frt tet2 (pBAD18LptFG3)</td>
<td>This study</td>
</tr>
<tr>
<td>NR5232</td>
<td>NR754 ΔlptFG::frt tet2 (pBAD18LptFG3/LptG/K34D)</td>
<td>This study</td>
</tr>
<tr>
<td>NR6107</td>
<td>NR754 ftsH(F37S) ΔlptFG::frt tet2 (pBAD18LptFG3/LptG/K34D)</td>
<td>This study</td>
</tr>
<tr>
<td>NR5951</td>
<td>NR754 ΔlptFG::frt tet2 (pBAD18LptFG3; pACYC184)</td>
<td>This study</td>
</tr>
<tr>
<td>NR5952</td>
<td>NR754 ΔlptFG::frt tet2 (pBAD18LptFG3; pACYC184-lpxC)</td>
<td>This study</td>
</tr>
<tr>
<td>NR5953</td>
<td>NR754 ΔlptFG::frt tet2 (pBAD18LptFG3; pACYC184-rpoH)</td>
<td>This study</td>
</tr>
<tr>
<td>NR5954</td>
<td>NR754 ΔlptFG::frt tet2 (pBAD18LptFG3/LptG/K34D; pACYC184)</td>
<td>This study</td>
</tr>
<tr>
<td>NR5955</td>
<td>NR754 ΔlptFG::frt tet2 (pBAD18LptFG3/LptG/K34D; pACYC184-lpxC)</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>NR5956</td>
<td>NR754 (\Delta lptFG::frt) tet2 (pBAD18LptFG3/LptG/K34D; pACYC184-rpoH)</td>
<td>This study</td>
</tr>
<tr>
<td>NR5957</td>
<td>NR754 (\Delta lptFG::frt) tet2 ftsH/F37S (pBAD18LptFG3/LptG/K34D; pACYC184)</td>
<td>This study</td>
</tr>
<tr>
<td>NR5958</td>
<td>NR754 (\Delta lptFG::frt) tet2 ftsH/F37S (pBAD18LptFG3/LptG/K34D; pACYC184-lpxC)</td>
<td>This study</td>
</tr>
<tr>
<td>NR5959</td>
<td>NR754 (\Delta lptFG::frt) tet2 ftsH/F37S (pBAD18LptFG3/LptG/K34D; pACYC184-rpoH)</td>
<td>This study</td>
</tr>
<tr>
<td>NR2761</td>
<td>NR754 (\Delta lptFG::frt) (pBAD18LptFG3)</td>
<td>(193)</td>
</tr>
<tr>
<td>NR3265</td>
<td>NR754 (\Delta lptFG::frt) (pBAD18LptFG3/LptF/E84A)</td>
<td>(193)</td>
</tr>
<tr>
<td>NR2762</td>
<td>NR754 (\Delta lptFG::frt) (pBAD18LptFG3/LptG/E88A)</td>
<td>(193)</td>
</tr>
<tr>
<td>NR5528</td>
<td>NR754 ftsH(F37S) (\Delta lptFG::frt) tet2 (pBAD18LptFG3/LptF/E84A)</td>
<td>This study</td>
</tr>
<tr>
<td>NR5529</td>
<td>NR754 ftsH(F37S) (\Delta lptFG::frt) tet2 (pBAD18LptFG3/LptG/E88A)</td>
<td>This study</td>
</tr>
<tr>
<td>NR4943</td>
<td>NR754 (\Delta lptFG::frt) (\Delta yhbX::kan) (pBAD18LptFG3)</td>
<td>This study</td>
</tr>
<tr>
<td>NR5349</td>
<td>NR754 lpd4213 yhbX::kan</td>
<td>This study</td>
</tr>
</tbody>
</table>

Table D.4: Primers used in Chapter 4

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ → 3’)</th>
<th>Usage</th>
</tr>
</thead>
<tbody>
<tr>
<td>5ftsH</td>
<td>TTCGCTCCCTGTTACGAAG</td>
<td>ftsH sequencing</td>
</tr>
<tr>
<td>5ftsH455</td>
<td>CAGATCAAACGACCTTTGC</td>
<td>ftsH sequencing</td>
</tr>
<tr>
<td>5ftsH116 3</td>
<td>ATGGTTGAGTTCGAGAAAGC</td>
<td>ftsH sequencing</td>
</tr>
<tr>
<td>-------------</td>
<td>----------------------</td>
<td>----------------</td>
</tr>
<tr>
<td>3ftsH</td>
<td>TACCCCTGGGCAAAGAGTTTC</td>
<td>ftsH sequencing</td>
</tr>
<tr>
<td>5XbaI-ftsH</td>
<td>TATATCTAGACCTGACGCTGTTTTTTAAACAC</td>
<td>Plasmid construction</td>
</tr>
<tr>
<td>3HindIII-ftsH</td>
<td>TCCAAAGGCTTTACCTGGGCAAAGAGTTTC</td>
<td>Plasmid construction</td>
</tr>
<tr>
<td>5XbaI-lpxC</td>
<td>AAACCTAGAGCGGAATGTATAGTACACTTC</td>
<td>Plasmid construction</td>
</tr>
<tr>
<td>3HindIII-lpxC</td>
<td>TTCAAAAGGCCTGGAGAGAGTGCCAGATTTG</td>
<td>Plasmid construction</td>
</tr>
<tr>
<td>5XbaI-rpoH</td>
<td>AGCTTCTAGACAGTTGTTGCTACCACCTGAAG</td>
<td>Plasmid construction</td>
</tr>
<tr>
<td>3HindIII-rpoH</td>
<td>AACCAAGCTTTTCATCCAGGGTTCTCTGC</td>
<td>Plasmid construction</td>
</tr>
</tbody>
</table>