An antibiotic binds to the ATPase that powers lipopolysaccharide transport

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Accessibility
An antibiotic binds to the ATPase that powers lipopolysaccharide transport

A dissertation presented

by

Janine Margaret May

to

The Department of Chemistry and Chemical Biology

in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the subject of Chemistry

Harvard University Cambridge, Massachusetts June 2016
An antibiotic binds to the ATPase that powers lipopolysaccharide transport

Abstract

The spread of antibiotic resistance has created an urgent need for new antibiotics. The situation is particularly serious for Gram-negative bacteria because they possess an outer membrane (OM) that prevents many antibiotics from entering the cell. The outer leaflet of the OM is composed of lipopolysaccharide (LPS), a complex glycolipid that is critical for creating this permeability barrier. In *Escherichia coli*, seven LPS transport (Lpt) proteins move LPS from its site of synthesis to the cell surface. Compounds that disrupt LPS transport could either kill Gram-negative bacteria directly or sensitize them to other antibiotics. There are currently no antibiotics that target the Lpt pathway.

This work establishes that an antibiotic interacts directly with LptB, the ATPase that powers LPS transport, in addition to its known cellular target. This conclusion is supported by genetic, biochemical, and structural evidence, described below. Mutations in *lptB* that permeabilize the OM to a wide range of antibiotics, including the antibiotic of interest, can be suppressed by compensatory mutations in *lptB*. Most of these suppressor mutations confer resistance to all antibiotics tested, suggesting that they correct the permeability defect. One suppressor mutation, however, selectively confers resistance only to the antibiotic of interest, leading to the hypothesis that this compound binds to LptB in vivo. This compound alters the rate of LptB-dependent LPS transport in in vitro reconstitutions, providing evidence that it affects the activity of LptB. A 2.0-Å crystal structure of the compound bound to LptB was
obtained. The compound binds in the groove region, which contacts coupling helices from transmembrane-domains LptF/G to form a functional ATP-binding cassette (ABC) system. Lethal mutations in the coupling helices can be suppressed by the compound itself or by mutations in \textit{lptB} that change the compound-binding site in the protein, suggesting that the binding observed in the crystal structure is physiologically relevant. If this hypothesis is correct, it may be possible to exploit this interaction to generate a new class of antibiotics effective against Gram-negative bacteria.
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Although I do not discuss my first project in this dissertation, I learned much from that experience. The project was a collaborative effort between the Kahne and Losick labs, and I
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Chapter 1
Targeting outer membrane biogenesis in Gram-negative bacteria

Portions of this chapter are adapted/reproduced from:

1.1 Introduction

The spread of antibiotic resistance is one of the most serious issues facing modern medicine. Antibiotic-resistant Gram-negative bacteria pose a particular challenge, as they possess an outer membrane (OM) that prevents many antibiotics from reaching their targets inside the cell. The OM is an asymmetric bilayer containing phospholipids in the inner leaflet and lipopolysaccharide (LPS) in the outer leaflet, as well as integral membrane proteins and lipoproteins. All OM components are synthesized inside the cell and then transported to the OM via separate pathways. These systems are attractive targets for new antibiotics because defects in OM assembly can lead to cell death or permeabilization to antibiotics that normally cannot penetrate the OM. Small molecules that perturb OM assembly can thus act as antibiotics themselves or potentiate the cell to other antibiotics.

This chapter provides an overview of the antibiotic resistance crisis, particularly as it pertains to Gram-negative bacteria. I describe our current understanding of cell envelope biogenesis in Gram-negative bacteria and highlight recent attempts by the pharmaceutical industry to target LPS biosynthesis and transport.

1.2 A need for new antibiotics, particularly for Gram-negative bacteria

Resistance has arisen to every antibiotic used in the clinic (1). In 2013, the Centers for Disease Control and Prevention estimated that each year antibiotic-resistant infections sicken at least 2 million and kill at least 20,000 patients in the United States, resulting in excess healthcare costs upwards of $20 billion (2). More concerning, however, is the threat to future generations if coordinated action is not taken to combat antibiotic resistance. The Review on Antimicrobial Resistance, commissioned by the United Kingdom Prime Minister, forecasts that in the absence
of a global effort to address antibiotic resistance, global deaths from drug-resistant infections will rise from 700,000 per year today to 10 million per year by 2050, more than currently die from cancer (3). To prevent the onset of a post-antibiotic era, two actions are needed: first, better stewardship of existing antibiotics, including improved monitoring of the spread of resistance, and second, invention of new antibiotics with novel mechanisms of action.

Bacteria can be divided into two classes based on the presence or absence of an outer membrane exterior to the cytoplasmic membrane (4). Monoderm (one membrane) bacteria have a thick layer of peptidoglycan, a mesh-like macromolecule that protects the cell from osmotic stress (5), surrounding the cytoplasmic membrane. In contrast, diderm (two membrane) bacteria possess a thin peptidoglycan layer and an additional outer membrane layer. In the Gram stain protocol, diderms in general do not retain the eponymous stain, earning the label “Gram-negative” (4). Although the Gram-stain classification system imperfectly distinguishes between monoderms and diderms, it will be employed here for simplicity.

Although the spread of antibiotic resistance in both Gram-negative and Gram-positive pathogens is alarming, Gram-negative bacteria pose a particular challenge. Of the six most significant clinical pathogens, termed the “ESKAPE” pathogens, four are Gram-negatives (Klebsiella pneumoniae, Acinetobacter baumanii, Pseudomonas aeruginosa, and Enterobacter spp.) (6, 7). In addition to the ESKAPE pathogens, antibiotic-resistant Neisseria gonorrhoeae, another Gram-negative pathogen, has emerged as a serious threat to public health (8). While drugs effective against Gram-positive bacteria have been developed in recent years, the last class of antibiotics that can kill Gram-negative bacteria, the quinolones, was invented over fifty years ago (9). Gram-negative bacteria are particularly difficult to kill because the outer membrane
prevents many antibiotics from penetrating the cell (10, 11). This impenetrability is due to the unique properties of the Gram-negative cell envelope.

1.3 Overview of the architecture of the Gram-negative cell envelope

The cell envelopes of Gram-negative bacteria have evolved to enable survival in harsh environments, selectively permitting entry of nutrients and discharge of waste. For example, *Escherichia coli*, which lives in the mammalian digestive tract, has a cell envelope that very effectively excludes bile salt detergents (4). The three layers of the Gram-negative cell envelope were first seen clearly by electron microscopy by Kellenberger and Ryter in 1958 (Figure 1.1) (12). The innermost envelope layer visible is the cytoplasmic membrane, also called the inner membrane (IM), which encloses the aqueous cellular contents. Subsequent work established that the middle layer visible is the peptidoglycan, as it can be digested by lysozyme, and the outermost layer is the outer membrane (OM) (13). The inner and outer membranes enclose an aqueous compartment, termed the periplasm, in which the peptidoglycan resides. As our target of interest is involved specifically in OM biogenesis, we will briefly review the properties of the innermost layers of the cell envelope before turning our attention to the structure and assembly of the OM. For simplicity, we will focus primarily on the cell envelope of the model organism *E. coli*. 
Figure 1.1 Double-membrane architecture of the Gram-negative cell envelope. The inner membrane (IM) contains phospholipids (PL) in both leaflets while the outer membrane (OM) contains PL in the inner leaflet and lipopolysaccharide (LPS) in the outer leaflet. The IM and OM enclose the periplasm, an aqueous compartment that contains peptidoglycan. Both the IM and OM contain membrane-anchored lipoproteins. Integral membrane proteins are composed of alpha-helices in the IM and beta-barrels in the OM.

1.4 The inner membrane

As bacteria do not possess intracellular organelles like eukaryotic cells, all essential membrane processes are performed in the IM, including oxidative phosphorylation, protein secretion, and lipid biosynthesis (4). Unlike the OM, the IM contains phospholipids in both leaflets. The IM lipids in *E. coli* are primarily phosphatidyl ethanolamine and phosphatidyl glycerol, although other lipids, like cardiolipin, are present in small amounts (14). Phospholipids are synthesized in the inner leaflet of the IM via the Kennedy pathway (14). The mechanism by which they “flip-flop” from the inner to the outer leaflet of the IM, a thermodynamically unfavorable process, is not completely understood (15). The ATP-binding cassette (ABC)
transporter MsbA is known to flip phospholipids (16), although additional mechanisms are believed to exist. In considering the permeability of the Gram-negative cell envelope, it is important to note that small hydrophobic molecules can freely diffuse across the IM, while hydrophilic molecules cannot. The uptake of hydrophilic nutrients into the cell requires active transport.

The IM contains two types of proteins: membrane-spanning alpha-helical proteins and lipoproteins anchored in the periplasmic leaflet by three acyl chains. As all proteins are synthesized on ribosomes in the cytoplasm, all IM, periplasmic, and OM proteins must be either inserted into or translocated through the IM – a task predominantly conducted by the Sec translocase (17). In addition to the Sec machine, the YidC insertase and the twin arginine translocation (Tat) machine are also involved in translocation and insertion, but they will not be discussed here (18, 19).

The pathway for integral membrane protein insertion differs from that of translocation. Integral IM membrane proteins possess a signal peptide that is recognized by signal recognition particle (SRP), which targets them to the Sec machine for co-translational insertion (20, 21). The core components of the Sec machine, SecYEG, form a protein-conducting channel in the IM (22). As alpha-helices fold on the machine, they are released laterally into the membrane, and the energy for insertion is derived from the ribosome (23). In contrast, the signal peptides of proteins destined for translocation (like lipoproteins, soluble periplasmic proteins, and OM proteins) are recognized by Trigger Factor, which prevents SRP from binding (24). These proteins are kept in an unfolded state by chaperone SecB, which targets them to the Sec machine for post-translational translocation (25). Here the energy for translocation is derived from ATPase SecA (26). The pathways of lipoproteins, periplasmic proteins, and OM proteins
diverge after translocation. For lipoproteins, the signal peptide is cleaved and a series of IM enzymes appends three acyl chains to the N-terminal Cys residue of the mature protein, discussed below (27).

1.5 The periplasm

In comparison to the cytoplasm, the periplasm is a relatively viscous compartment (28). It is also an oxidizing environment, as it contains Dsb proteins that catalyze disulfide bond formation between cysteine residues (29). Other periplasmic proteins include chaperones that facilitate OM protein folding and binding proteins essential for nutrient uptake. Notably, there is no source of energy in this compartment; all biochemical reactions are conducted without ATP.

The periplasm also contains the peptidoglycan layer. In *E. coli*, this layer is approximately six nanometers thick (30). Peptidoglycan is a mesh-like macromolecule that dictates the shape of the cell and stabilizes against osmotic stress (31, 5). It is synthesized from the precursor Lipid II, a disaccharide pentapeptide appended to an undecaprenyl pyrophosphate lipid carrier. The structure of Lipid II is remarkably conserved across bacterial species, with only minor alterations in the peptide sidechain (32). After Lipid II synthesis is completed in the inner leaflet of the IM, Lipid II is flipped to the outer leaflet of the IM by flippase MurJ (33, 34). From there, peptidoglycan glycosyltransferases polymerize the glycan strands, and the penicillin-binding proteins (PBPs) crosslink the peptide sidechains (35). Because peptidoglycan is essential, inhibition of these periplasmic enzymes is lethal. In fact, one of the most successful classes of antibiotics deployed against Gram-negatives, the beta-lactams, inhibits the PBPs (36, 1).
1.6 Key to Gram-negative cell envelope (im)permeability: the outer membrane

The relative impenetrability of Gram-negative bacteria is due primarily to the unique composition of the OM (10). Unlike the IM, which is a typical phospholipid bilayer, the OM is an asymmetric bilayer (37, 38). While the inner leaflet of the OM is composed of phospholipids, the outer leaflet is composed of lipopolysaccharide (LPS), a complex glycolipid. The OM also contains integral beta-barrel proteins and membrane-anchored lipoproteins. In most Gram-negative organisms, LPS consists of a phosphorylated glucosamine disaccharide with six or seven acyl chains (lipid A), a core oligosaccharide, and an extended polysaccharide that can contain hundreds of sugars (O-antigen) (39, 40). LPS molecules pack tightly together in the outer leaflet of the OM, bridged by magnesium ions that neutralize the repulsion between phosphate groups (10). Treatment with EDTA, which chelates the magnesium ions, results in release of LPS to the media (41).

The Gram-negative cell envelope is an excellent barrier to antibiotics in part because the IM and the OM have opposite permeability properties (10, 11). The hydrophilicity of the LPS sugars, coupled with the charges on the phosphate groups and affiliated cations, prevents the diffusion of small hydrophobic molecules through the OM. Small hydrophilic molecules can diffuse through the hydrophilic lumen of beta-barrel porins in the OM, but these compounds cannot easily penetrate the hydrophobic IM. The cell envelope is made more impenetrable by efflux pumps, which expel many hydrophobic molecules that do manage to reach the IM (42).

Because the OM confers intrinsic resistance to many antibiotics, OM biogenesis has emerged as a target in the search for new antibiotics effective against Gram-negative bacteria. Severe genetic defects in OM assembly can lead to cell death, while lesser defects permeabilize the cell to antibiotics that normally cannot enter (10, 11). When LPS is not assembled properly
on the cell surface, patches of phospholipids are thought to form in the outer leaflet of the OM, permitting transmembrane diffusion of hydrophobic molecules (43, 10). All OM components – beta-barrel proteins, lipoproteins, phospholipids, and LPS – are synthesized inside the cell and then must be transported to the OM. Although the mechanism of phospholipid transport remains unknown, the pathways that transport beta-barrel proteins, lipoproteins, and LPS have been identified. The sections that follow review the transport pathways involved in OM biogenesis, with special attention paid to LPS transport.

1.7 Lipoprotein transport to the OM

*E. coli* has over ninety lipoproteins, the majority of which reside in the OM (27). The function of many of these is unknown. There are three essential OM lipoproteins in *E. coli*, all of which play important roles in OM biogenesis: BamD, involved in folding beta-barrels into the OM; LptE, involved in LPS insertion into the OM; and LolB, involved in lipoprotein insertion into the OM (44-46). The most abundant protein in the cell (approximately 500,000 copies per cell) is the OM lipoprotein Lpp, also known as Braun’s lipoprotein (47). Approximately one third of Lpp proteins are covalently linked to peptidoglycan sidechains, effectively stapling the OM to the peptidoglycan layer. Although Lpp is not essential, mutants deficient in Lpp release OM vesicles, indicative of envelope defects (48).

Like all periplasmic and OM proteins, lipoprotein precursors have an N-terminal signal peptide that targets them for post-translational secretion through the Sec or Tat machine (49, 17). Within that signal peptide, prolipoproteins are marked by a consensus sequence, called the lipobox (50). Following translocation to the periplasmic leaflet of the IM, prolipoproteins are processed sequentially by three well-conserved enzymes (Lgt, LspA, and Lnt).
Phosphatidylglycerol/prolipoprotein diacylglyceryl transferase (Lgt) forms a thioether bond between a diacylglycerol molecule and the Cys residue at the end of the lipobox (51). This Cys residue becomes the N-terminal residue of the mature lipoprotein. Lipoprotein signal peptidase (LspA/signal peptidase II) cleaves the signal peptide immediately before the Cys residue (52). The newly freed amino group is then acylated by phospholipid/apolipoprotein transacylase (Lnt) (53). Lgt, LspA, and Lnt are essential in *E. coli*. Notably, the natural product antibiotic globomycin kills Gram-negative bacteria by inhibiting LspA (54, 55).

Because lipoproteins are anchored in the IM by their three acyl chains, lipoproteins destined for the OM cannot cross the hydrophilic periplasm unchaperoned. The localization of lipoproteins (Lol) system, discovered by Tokuda and colleagues, performs this essential function (27). The first step in lipoprotein transport to the OM is extraction of the lipoprotein from the periplasmic leaflet of the IM, executed by ABC system LolCD_{2}E (56). LolD binds and hydrolyzes ATP in the cytoplasm as a dimer, and LolCE are transmembrane domains that facilitate lipoprotein extraction. Lipoprotein binding to LolE stimulates ATP-binding (57). Subsequent hydrolysis of ATP powers the release of the lipoprotein to soluble chaperone LolA, which associates with LolC (58, 59). LolA is a lidded, half-open beta-barrel with a hydrophobic pocket that can accommodate a single acyl chain (60). LolA then transports the lipoprotein across the periplasm to LolB, an OM lipoprotein that shares structural, although not sequence, similarity with LolA (44). LolB has a higher affinity for the lipoprotein than LolA (61). LolA transfers the lipoprotein to LolB in a “mouth-to-mouth” fashion, and the lipoprotein is then inserted into the OM (62).

How are IM lipoproteins differentiated from OM lipoproteins? In *E. coli*, the second residue after the N-terminal Cys usually serves a signal for membrane specificity (63).
presence of an Asp in the second position, especially, causes retention in the IM by preventing recognition by LolCDE (64).

Because the Lol pathway is essential, it has generated interest as an antibiotic target. Recently, a compound has been discovered that appears to inhibit LolA function in vivo (65). This compound has antibacterial activity against *E. coli* and *P. aeruginosa* that can be suppressed by overexpression of LolA (65).

1.8 **Beta-barrel transport to the OM**

While integral membrane proteins in the IM are composed of alpha helices, integral membrane proteins in the OM are folded as beta-barrels. OM beta-barrels are involved in a number of cell processes, including the uptake of nutrients and the release of waste (66). Many small hydrophilic antibiotics, like tetracycline, beta-lactams, and chloramphenicol, likely diffuse through nonspecific beta-barrel channels, called porins (10). Loss of these porins can confer antibiotic resistance (67). In *E. coli*, there are two essential OM beta-barrels, both involved in OM biogenesis: LptD, which inserts LPS into the OM; and BamA, which folds beta-barrels into the OM (68-70).

Beta-barrel proteins are secreted post-translationally in an unfolded state through the Sec machine (17). Following translocation, their signal peptide is cleaved by signal peptidase (71). Because they are prone to aggregation when unfolded, they must be chaperoned in aqueous compartments. In the periplasm, SurA is the primary chaperone of unfolded beta-barrels, although other chaperones, including Skp and DegP, appear to become involved when substrates veer from the SurA pathway (72).
In alpha-helical proteins, hydrogen bonding in the peptide backbone occurs between proximal residues. In beta-barrel proteins, however, hydrogen bonding in the peptide backbone occurs between distal residues. The hydrophobic exterior of a beta-barrel is not assembled until the protein is folded, suggesting that folding is likely coupled to insertion into the membrane (73). Following their journey across the periplasm, beta-barrel proteins are folded and inserted into the OM by the beta-barrel assembly machine (Bam), homologs of which are found in mitochondria and chloroplasts (74-77, 66, 73).

In *E. coli*, the Bam complex consists of five proteins: BamABCDE (70, 78-80). BamA consists of a C-terminal 16-stranded beta-barrel and an N-terminal soluble portion with five polypeptide transport-associated (POTRA) domains (81, 82). BamBCDE are OM lipoproteins that associate with the POTRA domains (83-85). Although only BamA and BamD are essential, loss of any of the components leads to a reduction in folded beta-barrels, increased membrane permeability, and induction of envelope stress responses (86, 79, 45, 80). Beta-barrel folding has been reconstituted in proteoliposomes, and all components are necessary to achieve the maximum rate of folding (87).

The mechanism by which Bam recognizes substrates and facilitates folding and insertion into the OM remains under investigation. As the entire process occurs at the periplasmic leaflet of the OM, it is conducted without an external source of energy. BamA possesses a kink in one of the strands in its beta-barrel domain, suggesting that it may open to insert beta-barrels laterally into the membrane (88). Future mechanistic studies will be assisted by the recent publications of the structure of the entire Bam complex (83-85).
Like the other essential OM biogenesis factors, the Bam components have potential to be exploited as antibiotic targets. Recently, peptides that bind to BamD have been shown to inhibit beta-barrel assembly and confer OM defects (89).

1.9 LPS structure

LPS is essential in *E. coli* and most, but not all, Gram-negative bacteria (90). A few notable pathogens, including *Acinetobacter baumannii* and *Neisseria meningitidis*, normally produce LPS but are viable without it, albeit with diminished virulence (91). In addition, some endosymbiotic organisms, like the causative agent of Lyme disease, *Borrelia burgdorferi*, do not possess the biosynthetic gene clusters necessary for production of LPS (92). Despite these exceptions, the biosynthetic pathway for lipid A is one of the most conserved pathways in Gram-negative bacteria (39, 40).

Although the structure of LPS varies across and within species, the lipid A moiety is relatively well conserved (40). Figure 1.2 depicts the structure of *E. coli* LPS. A clear marker of infection, lipid A (also called endotoxin) is a potent stimulator of the mammalian immune system and the causative agent of septic shock (39). It is often glycosylated with a core oligosaccharide, which exhibits more structural diversity as it extends away from lipid A towards the O-antigen, an extremely variable polysaccharide. The difference in conservation between the lipid A core and the O-antigen is thought to derive from the difference in their functions. While the lipid A portion preserves membrane integrity and the OM permeability barrier, the O-antigen is involved in interactions with the environment and host defenses. In *E. coli* alone, more than 180 different O-antigens have been defined, and some strains, including commonly used lab strains, lack O-antigen entirely (93). LPS lacking O-antigen is called “rough” LPS, as opposed
to “smooth” LPS. At a minimum, most Gram-negative bacteria require lipid A glycosylated with two Kdo (3-deoxy-D-manno-oct-2-ulosonic acid) molecules for growth (40).

**Figure 1.2**  *E. coli* LPS consists of three subunits: lipid A, the core oligosaccharide, and the O-antigen. Kdo = 3-deoxy-D-manno-oct-2-ulosonic acid, Hep = L-glycero-D-manno-heptose, PEtN = phosphoethanolamine, P = phosphate, Glu = D-glucose, Gal = D-galactose.

Under certain conditions, Gram-negative bacteria modify the lipid A structure. These modifications contribute to virulence by enabling evasion of host immune surveillance and conferring resistance to antimicrobial peptides (94). Modification enzymes are found either in the outer leaflet of the IM or in the OM. For example, the *E. coli* IM enzymes ArnT and EptA modify lipid A phosphate groups with 4-amino-4-deoxy-L-arabinose and phosphoethanolamine, respectively (95, 96). These modifications reduce the negative charge of lipid A, rendering cells more resistant to the cyclic lipopeptide antibiotic polymyxin, which binds LPS (97, 98, 96). As another example, the *E. coli* OM enzyme PagP adds an additional acyl chain to lipid A when
phospholipids have entered the outer leaflet of the OM (99, 100). This modification confers resistance to cationic antimicrobial peptides and has been co-opted as a marker for LPS assembly defects (101, 102, 46, 103).

1.10 LPS biosynthesis

The nine-enzyme Kdo₂-lipid A biosynthetic pathway has been well characterized by Raetz and colleagues (39, 40). All steps occur at the interface between the inner leaflet of the IM and the cytoplasm (104). For simplicity, the *E. coli* Kdo₂-lipid A biosynthetic pathway will be discussed here. The first step of *E. coli* lipid A biosynthesis is the acylation of the 3-hydroxy group of UDP-GlcNAc (uridine diphosphate N-acetylglucosamine) by LpxA, which uses an acyl-acyl carrier protein (acyl-ACP) as its donor substrate (105, 106). *E. coli* LpxA accepts only beta-hydroxymyristate, which has a fourteen-carbon chain, but other organisms accept different chain lengths (107). As the equilibrium constant for the first reaction is low (~0.01), the first committed step in the pathway is the deacetylation of the product, UDP-3-O-(acyl)-GlcNAc, by LpxC (108, 109). A zinc metalloamidase, LpxC has been extensively explored as an antibiotic target, discussed below (110, 111). In *E. coli*, LpxC levels are governed by FtsH, a membrane-bound metalloprotease that maintains equilibrium between LPS and phospholipid biosynthesis (112).

After deacetylation, the product is acylated on the free amino group by LpxD, which also utilizes acyl-ACP and is specific for beta-hydroxymyristate (113, 114). The product, UDP-2,3-diacylglucosamine, is cleaved at its pyrophosphate bond by LpxH to give 2,3-diacylglucosamine-1-phosphate, termed Lipid X (115, 116). Disaccharide synthase LpxB generates a beta, 1-6 linkage between Lipid X and another molecule of UDP-2,3-
diacylglucoasmine (117). This disaccharide is phosphorylated by kinase LpxK to form Lipid IVₐ, which possesses some of the immune stimulatory properties of lipid A and enables growth in *E. coli* in the presence of suppressor mutations (118). Kdo₂-lipid A biosynthesis is completed by WaaA, which transfers two Kdo (3-deoxy-D-manno-oct-2-ulosonic acid) residues to lipid IVₐ, and acyltransferases LpxL and LpxM, which add the final two acyl chains (119-121).

Following completion of Kdo₂-lipid A (Re-LPS) biosynthesis, the inner and outer core oligosaccharides are added by the Waa proteins in the inner leaflet of the IM, producing Ra-LPS (39). Ra-LPS is then flipped to the outer leaflet of the IM by ABC transporter MsbA, discovered as a multicopy suppressor of a temperature-sensitive *lpxL* mutant (122-124, 16). In parallel to lipid A-core biosynthesis, O-antigen biosynthesis occurs in one of three pathways – Wzy dependent, ABC transporter dependent, or synthase dependent – all of which require undecaprenyl pyrophosphate carriers (39). LPS biosynthesis is completed in the periplasmic leaflet of the IM, where the O-antigen is ligated to Ra-LPS by WaaL (125, 126).

1.11 **Lipid A biosynthesis as an antibiotic target**

The enzymes in the Kdo₂-lipid A biosynthetic pathway are attractive antibiotic targets because they are essential in most Gram-negative bacteria, well conserved, encoded in single copy, and not present in mammalian cells (39, 40). Acyltransferases LpxA and LpxD have been targeted with peptides that are lethal when expressed intracellularly, work that may facilitate the development of peptidomimetic inhibitors (127, 128). The most well-studied target, however, is LpxC, an excellent target in part because it shares no sequence similarity with other amidases or deacetylases (40).
The Raetz group and colleagues at Merck reported a synthetic hydroxamate-containing compound that inhibited LpxC with a $K_i$ of $\sim 50$ nM (110). This compound had an MIC of 1 $\mu$g/mL against *E. coli*, but was ineffective against *P. aeruginosa* and other Gram-negative organisms (110). Other hydroxamate-containing compounds have been developed since then with improved potency and spectrum (129-131). The structure of inhibitor CHIR-090 bound to LpxC revealed that the hydroxamate moiety coordinates the zinc ion in the active site, while the biphenyl acetyl moiety occupies the hydrophobic groove that normally accommodates the acyl chain of the substrate (132).

The first LpxC inhibitor to enter a human Phase I trial was Achaogen compound ACHN-975 in 2012. When dosed at concentrations predicted to be effective in treating *P. aeruginosa* infections, ACHN-975 performed well in the first Phase I trial (133). A subsequent multiple-dose trial, however, had to be terminated early because subjects developed inflammation at the injection site (133). Despite this setback, Achaogen is continuing to pursue its LpxC inhibitor program.

1.12 LPS transport

As a single *E. coli* cell has approximately $10^6$ lipid A molecules, it is estimated that that LPS molecules must be assembled at the cell surface at a rate as high as 70,000 molecules per minute (134, 135). The surface orientation of LPS was established by Kamio and Nikaido, who found that OM phospholipids are not accessible for labeling at the cell surface, and Muhlradt and Golecki, who labeled LPS on the cell surface by immunoelectron microscopy (37, 38). In 1972, Osborn and colleagues identified the IM as the site of LPS biosynthesis, work that was supported by Muhlradt et al (104, 136). These results were intriguing, as Osborn also found that the
majority of LPS resides in the OM (137). How does LPS travel from its site of synthesis to the cell surface? In eukaryotes, there are three mechanisms of lipid transport: protein-mediated transport, vesicular transport, and diffusion at membrane junctions (138). In the late 1960s, Manfred Bayer observed zones of adhesion between the IM and OM in electron micrographs (139). For many years, it was debated whether these “Bayer bridges” mediate LPS transport, until the discovery of the seven-protein lipopolysaccharide transport (Lpt) system in the early 2000s (140-143).

Within the past few years, a model has emerged to describe how the Lpt system moves its substrate to the cell surface. Although the Lpt system has been studied mainly in E. coli, it appears to be well-conserved across LPS-producing organisms (144). As shown in Figure 1.3, following its synthesis inside the cell, LPS is flipped to the outer leaflet of the inner membrane (IM) by ATPase MsbA and then extracted from the IM by LptB2FGC, an unusual ATP-binding cassette transporter. Following extraction, it traverses a bridge formed by LptA and finally is selectively inserted into the outer leaflet of the OM by the LptDE OM translocon. As there is no ATP in the periplasm, the entire journey following flipping to the outer leaflet of the IM is powered solely by ATP hydrolysis in the cytoplasm (145). The sections that follow review the function and structure of the Lpt system.
Figure 1.3  The LPS transport system in *E. coli*. The ATP-binding cassette (ABC) transporter LptB<sub>2</sub>FGC powers LPS extraction from the IM and transport through a transenvelope bridge composed of LptC, LptA, and the N-terminus of LptD, from which point it is inserted into the OM by the LptDE complex.

1.13 Discovery of the Lpt components, LptABCDE

LptD, an ~87 kDa OM beta-barrel, was the first Lpt protein discovered. It was described first as a determinant of membrane permeability, earning the designation *imp*, for increased outer membrane permeability. In a genetic screen in 1989, Benson and collaborators found suppressors containing mutations in *imp* that enabled *Escherichia coli* cells deficient in *lamB* (the beta-barrel required for maltodextrin import) to survive in media containing maltodextrins as the sole carbon source (68). These *imp* mutants were not only permeabilized to maltodextrins, but also to certain antibiotics and other small molecules that normally cannot cross the OM barrier. Independently, *lptD* was also found to affect organic solvent tolerance (giving rise to its *ostA* designation), again implying a change to membrane structure, although the mechanism at this point was unclear (146).
A more detailed picture of \textit{lptD} emerged following the discovery that it is located immediately upstream of \textit{surA}, a periplasmic chaperone important in the biogenesis of OM beta-barrel proteins (147). Significantly, the operon containing \textit{lptD} and \textit{surA} was found to be upregulated during the $\sigma^E$ stress response, which increases the production of OM biogenesis factors in response to envelope stress (148). Given this suggestion of biogenesis involvement, Braun and Silhavy confirmed the essentiality of \textit{lptD} in \textit{E. coli}, showed that it was co-transcribed with \textit{surA}, and demonstrated that LptD depletion leads to cell chaining followed by lysis (69). Membrane fractionation experiments showed that LptD depletion generates a novel membrane fraction (69), further proving an essential role for LptD in the biogenesis of the cell envelope.

Tommassen and collaborators performed the key experiments that established the function of LptD in LPS assembly. Exploiting the fact that in \textit{Neisseria meningitidis}, unlike in \textit{E. coli}, LPS is not essential (149, 91), they were able to delete \textit{lptD} and consequently observed dramatically reduced LPS levels (102). The small amount of LPS that was produced could not be modified by enzymes present in the OM or enzymes added exogenously, indicating that LptD is essential for LPS assembly in the outer leaflet of the OM. The other component of the OM translocon, essential lipoprotein LptE ($\sim$20 kDa, formerly known as RlpB), was discovered by the Silhavy and Kahne laboratories using LptD as bait (46).

The \textit{lptCAB} (formerly \textit{yrbK} and \textit{yhbNG}) operon, which also contains Kdo biosynthesis genes \textit{kdsDC}, was discovered by the Polissi group in a screen for essential genes (150). Polissi and collaborators found that altered expression of \textit{lptAB} sensitizes cells to compounds that normally cannot penetrate the OM and depletion of LptA or LptB causes LPS to accumulate in the IM (151, 152). Depletion of any of the identified Lpt components – at this time, LptABCDE
– resulted in the same phenotypes, including the build-up of additional membrane material in the periplasm, presumably due to LPS accumulation in the IM (69, 46, 152, 153).

From sequence analysis, it was clear that LptB (~27 kDa) is the cytoplasmic nucleotide-binding domain of an ABC transporter. As ABC transporters are composed of two nucleotide-binding domains and two transmembrane domains (154), Ruiz and colleagues identified the missing transmembrane domains by searching for essential, integral IM proteins of unknown function shared between *E. coli* and another LPS-producing species with a dramatically reduced genome (103). As depletion of the candidate proteins, YjgPQ (each with six transmembrane helices), gave the same phenotypes as depletion of the other Lpt components, they were renamed LptFG (~40 kDa each) (103).

Following discovery of the Lpt components, three questions in particular loomed large. First, how is LPS extracted from the IM? Second, how does LPS travel across the periplasmic space, devoid of ATP? Finally, how does the LptDE complex insert LPS selectively into the outer leaflet of the OM without contaminating the inner leaflet?

### 1.14 Extraction of LPS from the inner membrane

The first step in the transport of LPS across the periplasm is the extraction of LPS from the IM, performed by ABC system LptB2FG. ABC systems couple the energy of ATP hydrolysis with a variety of biological processes, typically transmembrane import and export (154). In a pull-down assay with a histidine tag on LptB, Narita et al. found that the LptB2FG complex associates with LptC (~22 kDa), a bitopic protein with a periplasmic domain that binds LPS (155, 156). The entire complex (LptB2FGC, also called the IM complex) assembles in a 2:1:1:1 ratio, as expected for an ABC transporter (155).
The manner in which ATP hydrolysis is coupled to LPS extraction remains unknown, although mutations in LptB that either prevent ATP hydrolysis or coupling to LptF/G are lethal (157). LptC is known to bind LPS (156), but it is not clear whether LptF/G interact directly or indirectly with LPS. LptC could extract LPS directly, facilitated by the activity of the LptB2FG, or LptF/G could directly extract LPS themselves. While the structure of LptB has been solved by Kahne and collaborators (157), the structure of the entire IM complex has not yet been solved.

1.15 Building a bridge between membranes

Clues to the mechanism of how LPS reaches the OM translocon emerged following the discovery of LptA (152), an LPS-binding, periplasmic protein (~19 kDa) whose depletion in *E. coli* results in accumulation of LPS on the periplasmic side of the IM (158, 153, 159). Two competing models arose to describe the role played by LptA (140). In the first model, inspired by the Lol pathway for lipoprotein transport (160), LptA was proposed to act as a soluble chaperone, shuttling between the IM complex and the OM complex. In the second model, LptA was proposed to form a continuous bridge connecting the IM to the OM.

The first piece of evidence for the bridge model was in place before the discovery of LptA. In a series of experiments with *E. coli* spheroplasts, cells in which the peptidoglycan has been digested and the periplasmic contents released, Tommassen and collaborators observed different requirements for LPS versus lipoprotein transport (161). Addition of periplasmic extract, which contained soluble chaperone LolA, to the spheroplasts released lipoproteins from the IM, but not LPS. Strikingly, the spheroplasts were capable of MsbA-dependent LPS transport to associated OM fragments in the absence of soluble periplasmic components (161). Later, evidence of a trans-envelope complex came from Kahne and collaborators, who found that
all seven Lpt components co-fractionate and co-purify (162). The Polissi laboratory also found LptC to form stable complexes with LptA (163).

As shown in Figure 1.4, crystallographic studies provided a structural model for how the bridge might form. In the presence of LPS, LptA crystallized in long fibrils consisting of two bundles of four LptA proteins stacked N-terminus to C-terminus (164). A previously uncharacterized fold, the LptA structure consists of 16 consecutive, antiparallel beta-strands that resemble a slightly twisted beta-jellyroll. The structure of the periplasmic domain of LptC (~22 kDa), an LPS-binding, bitopic component of the IM complex, was obtained by the Whitfield laboratory (156). The periplasmic domain of LptC is very similar to that of LtpA and consists of 15 consecutive, antiparallel beta-strands, again resembling a beta-jellyroll.

**Figure 1.4** LptC, LptA, and the N-terminus of LptD form a bridge that traverses the periplasm. *left* LptA and the periplasmic domains of LptC and LptD form a filament (164, 156, 165). Residues highlighted are sites that cross-link between the bridge components (166). *right* LptA, LptC and the N-terminal domain of LptD exhibit a beta-jellyroll fold. Highlighted sites in LptA and LptC cross-link to LPS (145). PDB: 2R1A, 3MY2, 4Q35.
With the structural data and the knowledge that the periplasmic N-terminal domain of LptD is also classified in the same structural superfamily as LptA and LptC (designated OstA) (144, 167), the Ruiz and Kahne laboratories further probed the interactions between Lpt components. By introducing a photo-activatable amino acid into various positions in LptA, Freinkman et al. were able to crosslink LptA to both LptC and LptD in vivo and observed an N-to C-terminal orientation of the domains within the bridge (166). Bridge formation apparently does not rely on the transmembrane domain of LptC, as cells lacking this domain are still viable (168). LptA does not interact directly with LptE (169).

These studies, although enabling a consensus about the validity of the bridge model, opened many more questions. For example, it is unknown how many LptA monomers are found in a single bridge. Interestingly, although the structures of LptA and LptC possess many conserved hydrophobic residues in their core, in comparison to mammalian LPS-binding proteins they do not contain an obvious cavity deep enough to fit the lipid A fatty acyl chains of LPS (156). Tran et al. demonstrated in pull-down assays that LptA binds the lipid A core (159), and Okuda et al. demonstrated that residues in the grooves of LptA and LptC can in fact crosslink to LPS (145), but it remains an open question exactly how LPS interacts with the bridge. It is also unclear whether it is transported as stream of single molecules or as aggregates. More work is needed to understand exactly how LPS makes its journey across the periplasm.

1.16 Assembly of the outer membrane translocon: a plug in a barrel

The assembly of the LptDE OM translocon is a feat that requires the coordination of beta-barrel assembly and lipoprotein transport. LptD is a beta-barrel protein that contains two domains: a C-terminal transmembrane beta-barrel domain and a soluble, periplasmic N-terminal
domain (144). Mature LptD possesses two intramolecular disulfide bonds connecting the C-terminal domain to the N-terminal domain; at least one of these bonds must be present for functionality (170). LptE is a lipoprotein anchored in the inner leaflet of the OM (171). As it became apparent that both beta-barrel and lipoprotein biogenesis pathways are involved in the assembly of the LptDE translocon, a number of questions developed. How do LptD and LptE interact? When do the essential disulfide bonds in LptD form? How does the cell regulate assembly to ensure production of functional complexes? In particular, LptE emerged to play a critical role in the folding and proper oxidation of LptD.

Initial studies focused on understanding how LptD and LptE interact. Chng et al. found that LptD and LptE co-purify in a stable 1:1 complex when a polyhistidine-tag is installed on LptE. In fact, LptD cannot be overexpressed without LptE, and the C-terminal domain of LptD strongly protects LptE from proteolytic degradation (172). This finding led to the development of the plug-and-barrel model, in which LptE resides in the lumen of the beta-barrel of LptD. Guided by the structures of three LptE orthologs, Freinkman et al. introduced a photo-activatable amino acid into LptE at various positions and found many to contact LptD (173). For one LptE position, they identified the interaction site in LptD to be part of a putative extracellular loop of the barrel, confirming the plug-and-barrel model (173).

The importance of the plug for barrel biogenesis became quickly apparent. Deletion of nine amino acids in LptD around the identified LptDE interaction site compromises translocon biogenesis and leads to OM defects (173). Seeking to better understand how LptE affects translocon assembly, Chimalakonda et al. introduced random mutations into lptE and screened for mutants with OM permeability defects (174). One such mutant carried the lptE6 allele, which encodes a two-amino-acid deletion that affects LptDE complex formation but not complex
stability. Suppressors to lptE6 that improve LptDE complex formation and restore the barrier function of the OM were found not only in lptD but also in bamA, which encodes the central component of the Bam complex (174). This study suggests that LptD needs to interact with LptE to be folded efficiently on the Bam complex.

1.17 Assembly of the outer membrane translocon: regulation via disulfide bond formation

The critical role played by LptE in the folding of LptD was elucidated further by detailed studies of disulfide bond formation in LptD. LptD contains four cysteine residues, two in the N-terminal domain (Cys31 and Cys173) and two in the C-terminal domain (Cys724 and Cys725). As described above, mature LptD contains two intramolecular disulfide bonds between the N-terminal domain and the C-terminal domain (Cys31—Cys724 and Cys173—Cys725), and at least one is required for LptD to function (170). LptD disulfide bond formation is thought to involve the oxidase DsbA, which introduces disulfide bonds into proteins in the periplasm (175, 176). Deletion of dsbA, presumably possible only when other oxidants are present, leads to defects in LptD oxidation (170).

When LptE is limiting, LptD is not oxidized correctly (170), and an LptD species containing a disulfide bond between the first two cysteine residues (Cys31—Cys173) accumulates (177). Mutations in lptD also cause accumulation of the Cys31—Cys173 product (177). To understand whether this species is a dead-end product or an intermediate in the assembly of the LptDE complex, Chng, Xue et al. performed a pulse-chase experiment in which FLAG-tagged LptD was purified from cells pulsed with [35S]-methionine and then chased with cold methionine. As disulfide bond formation affects the migration of LptD on SDS-polyacrylamide...
gels (SDS-PAGE) (170), they were able to determine that Cys31-Cys173 product is indeed on pathway in wild-type cells; it is the predominant form at the beginning of the chase and converted to mature LptD by the end (177). Depletion of LptE thus stalls folding of LptD at an intermediate state.

To develop a more detailed picture of LptD folding, they repeated the pulse-chase experiment and observed six folding intermediates, developing the model depicted in Figure 1.5 (177). LptE is required for disulfide bond rearrangement to form the functional translocon, serving as an important check that both components of the translocon are properly assembled (177). Furthermore, crosslinking studies have indicated that LptA can connect with LptD only when it possesses at least one of the two mature disulfide bonds, ensuring that bridges are not connected to non-functional translocons (166). It is interesting that although other plug-barrel systems have been identified, the LptDE system is the only one in which the plug is a separate polypeptide from the barrel (178-180). Perhaps breaking up the plug and barrel is an elegant solution to ensure that multiple envelope processes are coordinated.
Figure 1.5  Current model for the assembly pathway of the LptDE OM translocon. Several unfolded LptD intermediates exist with varying oxidation states of the four cysteine residues (177). In the non-functional OM translocon, a disulfide bond exists between C31 and C173. In the functional OM translocon, two disulfide bonds are present (Cys31–Cys724 and Cys173–Cys725). Note that LptE is required for disulfide rearrangement.

1.18 The structure of the OM translocon

Once LPS is transported across the periplasm to the OM translocon, it must be selectively inserted into the outer leaflet of the OM. The mechanism of insertion is a matter of ongoing investigation and has been greatly assisted by the recent publications of the structure of the LptDE complex, displayed in Figure 1.6, by the Huang and Dong laboratories (181, 165). Consisting of 26 antiparallel beta-strands arranged in a distinctive kidney shape, the C-terminal domain of LptD is the largest beta-barrel crystallized to date. As predicted, the LptD N-terminal domain consists of two sheets of eleven antiparallel beta-strands exhibiting the beta-jellyroll fold found in LptA and LptC (165). LptE consists of two alpha-helices and four beta-strands (181, 182, 165). Three quarters of LptE is located in the lumen of LptD, and the remainder is positioned on the periplasmic face of the barrel. In addition to directly validating the plug-and-
barrel model, the structures also confirmed the LptDE interaction sites predicted by the crosslinking studies discussed above (173).

**Figure 1.6** Structure of the plug-and-barrel complex of LptD and LptE (165). Disulfide bonds connect the N-terminal domain of LptD to its C-terminal domain. The barrel is thought to open during LPS insertion between beta-strands 1 and 26 (highlighted). PDB: 4Q35.

The structures yield several clues to the mechanism of insertion. Like LptA and LptC, the interior groove of the N-terminal domain of LptD is very hydrophobic, suggesting an interaction with the hydrophobic moiety of LPS (165). In contrast, the interior of the LptDE barrel is fairly hydrophilic, indicating that the barrel is unlikely to interact directly with this same moiety. The prevailing model is that LPS insertion most likely requires the LptD barrel to open between beta-strands 1 and 26, through which LPS can then roll out (173, 181, 165). Several conserved proline residues disrupt these strands, leaving only three main-chain hydrogen bonds between them. Molecular dynamics simulations concur that the interface between beta-strands 1 and 26 is the weakest in the barrel (181). This model is analogous to that proposed by Bishop and van den Berg for beta-barrels PagP and FadL, which are also thought to enable their lipid substrates to diffuse laterally into the membrane via opening of weakly-bonded beta-strands, termed crenels (183-186).
Another important mechanistic question concerns the role played by LptE. Grabowicz et al. have demonstrated that the plugging function of LptE is important (187), but it is unclear how its ability to bind LPS is involved in the insertion mechanism. Malojcic et al. suggest that its ability to disrupt LPS aggregates may be important if LPS is transported across the periplasm in a continuous stream (182). Apparently, in *N. meningitidis*, where LPS is not essential, deletion of *lptE* does not affect LPS levels or localization, suggesting that insertion may occur differently in this organism (188). Additional studies, ideally including structures of the translocon in different conformations, are needed to develop a clearer picture of insertion.

### 1.19 LptD as an antibiotic target

Clinical trials are currently underway for a compound thought to target LptD (POL7080). Discovered by the Robinson group and colleagues at Polyphor, POL7080 is beta-hairpin peptidomimetic that has a nanomolar MIC against *Pseudomonas* species, including important pathogen *P. aeruginosa* (189). It is inactive against other species of Gram-negative bacteria (189).

Because there is no published method for assaying LptD activity directly, the evidence that POL7080 and related compounds interact with LptD is indirect. Resistance to POL7080 can be conferred by a mutation in *lptD* that produces a duplication of six amino acids in the periplasmic domain (189). These residues are not strongly conserved in *E. coli*. An analog of the compound containing a photo-crosslinkable amino acid substituted at one position can be crosslinked in vivo to wild-type LptD, but not to the resistant form (189). Treatment with POL7080-related compounds results in accumulation of abnormal membrane material in the periplasm, similar to Lpt depletions (189, 190). Despite this evidence, in the absence of a direct
biochemical assay of LptD activity and/or a structure of LptD bound to POL7080, it is difficult to conclude definitely that the compound kills cells by targeting LptD. Polyphor’s commitment to continuing clinical trials in the absence of more conclusive data is reflective of the serious need for new agents effective against *P. aeruginosa*.

### 1.20 Conclusion

Therapeutic options for antibiotic-resistant Gram-negative infections are becoming dangerously limited. In some cases, physicians have been forced to return to colistin, a polymyxin antibiotic that was once a drug of last resort due to its toxicity (191). There is a serious unmet need for new Gram-negative antibiotics, but the development pipeline is sparse. The Pew Charitable Trusts estimates that as of September 2015, 39 new antibiotics were in clinical development in the United States (192). Of these 39 antibiotics, only 13 are expected to have activity against Gram-negative pathogens. Of these 13, only one – POL7080 – has a novel mechanism of action, which, as discussed in section 1.19, remains disputed.

There is a clear need for new antibiotic targets in Gram-negative bacteria. The Lpt pathway could be an interesting pathway to exploit because it is conserved across Gram-negative bacteria and essential for both cell viability and maintenance of the OM barrier. Compounds that inhibit this pathway could either kill Gram-negative bacteria directly or permeabilize them to OM-impermeant antibiotics. In principle, any of the seven Lpt proteins could be targets because all are essential. POL7080, for example, may target LptD. Another promising target, however, is the LptB$_2$FGC complex, which possesses the only enzymatic activity in the pathway. The next chapter provides an overview of the mechanism and inhibition of ABC systems, such as LptB$_2$FGC.
1.21 References


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Chapter 2

Mechanism and inhibition of ABC transporters
2.1 Introduction

As discussed in the previous chapter, LptB2FGC is an unusual ATP-binding cassette (ABC) system that may be an attractive target in the development of new antibiotics for Gram-negative bacteria. Comprising one of the largest protein superfamilies, ABC systems couple the energy of ATP binding and hydrolysis to a variety of processes, most notably transmembrane import and export. Although ABC transporters perform diverse functions, they share a common architecture of two nucleotide-binding domains, which catalyze ATP hydrolysis, associated with two transmembrane domains, which facilitate substrate movement. Although no clinically relevant small molecules are known to interact with the nucleotide-binding domains, a number of ABC transporter modulators have been found that target the transmembrane domains. This chapter will provide an overview of the function, structure, mechanism, and inhibition of ABC transporters, with special attention given to LptB2FGC.

2.2 ABC transporters use a common architecture for diverse purposes

ABC systems compose one of the largest protein superfamilies and have been found in all genera of the three kingdoms of life (1). As indicated by their name, all ABC systems possess an ATPase domain, also called the nucleotide-binding domain (NBD). ABC systems utilize the energy of ATP binding and hydrolysis to perform a number of biological processes, most commonly transport across cellular membranes (2-5). ABC systems involved in transmembrane processes contain four subunits: two soluble NBDs paired with two hydrophobic transmembrane domains (TMDs), which enable substrate movement across the membrane (6-8). For simplicity, we will refer to ABC systems containing both NBDs and TMDs as ABC transporters.
ABC systems cluster by sequence into three main groups (1). Class 1 systems are transporters in which the NBDs are fused to the TMDs. Found in both eukaryotes and prokaryotes, Class 1 systems function primarily as exporters of various molecules, including toxins and hydrophobic drugs. A notable example is the human exporter P-glycoprotein, which expels a range of hydrophobic drugs and contributes to multidrug resistance in cancer (9). Class 2 systems consist of fused NBDs with no TMDs. These are involved in non-transport processes, including DNA repair (10), and will not be discussed further here. Class 3 systems are transporters in which the NBDs are not fused to the TMDs. The most well studied Class 3 systems are bacterial binding-protein dependent (BPD) importers, which facilitate the uptake of soluble nutrients such as vitamins, metals, and sugars (11, 12, 4). The ABC system that powers LPS transport, LptB2FG, is a Class 3 system (13, 14).

Across ABC transporters, the NBD is strongly conserved (15, 16). The characteristic sequence motifs of NBDs are discussed below. The TMDs, in contrast, are much more variable, reflective of the diversity of the substrates they handle. Class 1 exporters contain a core of 12 transmembrane helices (six per TMD), while Class 3 importers can contain 10-20 transmembrane helices (5-10 per TMD) (17, 6). Exporters can be heterodimers or homodimers of NBD-TMD fusions, while importers can consist of up to four different proteins (two NBDs and two TMDs). For example, LptB2FG contains two identical copies of the NBD (LptB) and two distinct TMDs (LptF and LptG) (14). The connection between NBDs and TMDs is conserved across ABC transporters. In both Class 1 and Class 3 systems, each TMD possesses a helix, called the “coupling helix,” that fits into a groove of a NBD (18). Although the coupling helix architecture is conserved, the sequence is not, as NBDs form specific side-chain contacts with their TMD partners (6, 7).
2.3 A two-state, alternating access model describes the mechanism of ABC transporters

How do ABC transporters harness the energy of ATP hydrolysis to perform their biological functions? Within the past 14 years, full transporter structures (i.e. including the TMDs) have been published for approximately a dozen ABC systems, including importers and exporters (19-27). These structures, captured in different conformations with and without nucleotides, suggest that ABC transporters couple the energy of ATP hydrolysis to substrate translocation via a conserved two-state mechanism (6-8).

In this model, the transporter is in the “inward-facing” conformation in the absence of nucleotide (Figure 2.1). Here, the NBDs are separated, or open, and the TMDs are open to the cytoplasm. Upon ATP-binding, the NBDs dimerize and pull the coupling helices towards each other, moving the TMDs into an “outward-facing” state open to the extracellular space. In some BPD importers, substrate-loaded binding protein is also required for dimerization (28). In this closed conformation, two molecules of ATP are bound at the interface between the two NBDs. ATP hydrolysis and release of the hydrolysis products ADP and phosphate resets the system to the inward-facing conformation. As the TMDs are alternatively accessible to the intracellular and extracellular compartments, this model is also called the “alternating access” model (29).
The alternating access model is the favored model for the mechanism of ABC transporters. Dimerization of the NBDs accompanied by ATP-binding switches the TMDs from the inward-facing orientation to the outward-facing orientation. ATP hydrolysis and subsequent release of hydrolysis products returns the system to the inward-facing orientation.

The alternating access model is thought to be conserved across ABC transporters, regardless of function or direction of transport. Importers and exporters have been crystallized in both inward- and outward-facing conformations (Figure 2.2). The directionality of transport is likely conferred by the relative binding affinity for the substrate in the two conformations (6, 8). In exporters, the affinity of the TMDs for the substrate is expected to be stronger in the inward-versus the outward-facing conformation. In importers, the opposite is expected.

ABC transporters not involved in import or export are also thought to function via the alternating access model. For example, the Class 1 system MsbA, which flips LPS from the inner to the outer leaflet of the cytoplasmic membrane in Gram-negative bacteria, has also been crystallized in both inward- and outward-facing conformations (30, 31, 23). In the inward-facing conformation, the NBDs are positioned approximately 50 Å apart, a distance also observed in electron microscopy studies (32). Fluorescent resonance energy transfer studies indicate that binding of LPS induces dimerization of the NBDs and ATP hydrolysis (33). These results strongly suggest that MsbA utilizes the conserved two-state mechanism to perform its
novel flippase function. It is likely that the alternating access model is also descriptive of our system of interest, LptB₂FG, although we currently lack structural data for the entire transporter.

Figure 2.2 The alternating access model was derived from crystal structures of Class 1 and Class 3 transporters in both inward- and outward- facing conformations. a) Cartoon representations of Class 3 importers. The molybdate transporter (ModB₂C₂-ModA, from *Archaeoglobulus flugidus*, PDB: 2ONK) contains ligands Mg²⁺, PO₄³⁻, and WO₄²⁻ (21). The maltose transporter (MalFGK₂-MBP, from *E. coli*, PDB: 2R6G) contains maltose and ATP (22). Proteins are colored as separate peptide chains according to the following scheme. Nucleotide-binding proteins (ModC, MalK): gray/black. Transmembrane proteins (ModB, MalFG): blue/navy. Periplasmic binding proteins (ModA, MBP): green. b) Cartoon representations of Class 1 exporters. P-glycoprotein (from *Caenorhabditis elegans*, PDB: 4F4C) does not contain ligands (26). Sav1866 (from *Staphylococcus aureus*, PDB: 2HYD) contains ADP (20). P-glycoprotein is a single peptide chain; Sav1866 is a homodimer.

Within the alternating access framework, the answers to fundamental mechanistic questions appear to vary across transporters. For example, what is the ratio of ATP molecules hydrolyzed to substrate transported? It is thought that most transporters hydrolyze two molecules of ATP for one molecule of substrate transported, but this has only been confirmed in vitro for one transporter (34, 35). Do both ATP-binding sites need to be functional for transport,
or can one suffice? Most transporters appear to require two functional sites, although there exceptions, such as the histidine permease (36). How do ABC transporters prevent futile cycles of ATP hydrolysis in the absence of substrate? In the maltose transporter, dimerization of the NBDs requires binding of the periplasmic binding protein, but this is not universally true (28). Additional studies are needed to understand how different transporter classes carefully coordinate substrate transport with the conformational changes accompanying ATP binding and hydrolysis.

2.4 Nucleotide-binding domains are characterized by conserved ATP binding and hydrolysis motifs

The NBD domain is commonly referred to as the conserved “engine” to which diverse TMDs are coupled. Characterized by three short conserved sequences (the Walker A, Walker B, and signature motifs), all NBDs contain two sub-domains: a RecA-like domain and a helical domain, also called the structurally diverse region (37, 38). The RecA subdomain is well conserved across many ATPases and contains the Walker A (GxxGxGKS/T, where x = any amino acid) and Walker B (ϕϕϕϕD, ϕ = a hydrophobic amino acid) motifs, essential for binding and hydrolyzing ATP (39). The RecA subdomain also contains the switch region, marked by a conserved histidine residue important in hydrolysis (40, 41). The other characteristic ATP binding motif – the signature motif (LSGG[E/Q]) – is found in the helical domain (42, 43). The helical domain is much more variable than the RecA domain and is implicated in interactions with the TMDs (44, 45). Two loops connect the RecA subdomain to the helical subdomain. One of these loops, the Q-loop, is named after a conserved glutamine residue and known to mediate
interactions with the TMDs (19-21). These conserved motifs are highlighted Figure 2.3 in the structure of LptB, obtained by Sherman and collaborators (46).

**Figure 2.3**  ABC transporter NBDs, such as LptB, are characterized by conserved motifs.  a) Cartoon representation of LptB with helices represented as cylinders. All secondary structure assigned by Pymol. PDB: 4P33.  b) Cartoon representation of LptB-ATP with conserved motifs indicated. Coloring chosen to be consistent with ref. (46). PDB: 4P33.

ATP hydrolysis requires dimerization of the NBDs (47, 38). Following dimerization, ATP is bound between the Walker A motif of one monomer and the signature motif of the other, resulting in two ATP molecules bound at the dimer interface (Figure 2.4A). The active site of LptB-ATP will be described here as a representative NBD (46). As shown in Figure 2.4B, the adenosine ring of ATP stacks with a conserved aromatic residue (Y13 in LptB) preceding the Walker A motif (48). The oxygen atoms of the ATP phosphates interact with residues in the Walker A motif, the serine and glycines of the signature motif, and the conserved histidine (H195) from the switch region. ATP hydrolysis requires magnesium (49), which is coordinated by two water molecules, the Walker A motif, and the characteristic glutamine (Q85) from the Q-loop. Although the mechanism of ATP hydrolysis is still controversial (5), a glutamate (E163)
immediately following the Walker B motif is thought to function as the catalytic base that deprotonates the water that attacks the gamma phosphate (47). LptB/E163Q variants are catalytically inactive in vitro and are not viable in vivo (46).

Figure 2.4 ATP hydrolysis requires NBD dimerization. a) Surface representation of LptB-ATP. Two molecules of ATP are bound at the dimer interface (46). PDB: 4P33. b) Active site of LptB-ATP (46). The ATP molecule pictured also makes contacts with the signature motif of the other LptB monomer. Catalytic glutamate was mutated to glutamine (E163Q) to prevent ATP hydrolysis. As LptB was crystallized in the absence of Mg^{2+}, a sodium ion has taken its place (46). Putative hydrolytic water is depicted as a blue sphere; waters coordinating Na^{+} are depicted as gray spheres. PDB: 4P33

Comparisons of apo with ADP- and ATP- bound NBD structures indicate that ATP hydrolysis prompts rearrangement in the active site and movement in the helical domain, which transmits motion to the TMDs (38, 5). In LptB, the most dramatic active site change post-hydrolysis is a sidechain flip in H195, thought to mediate the release of phosphate, which is absent in the LptB-ADP structure (46). LptB/H195 variants show reduced catalytic activity in
vitro and are not viable in vivo (46). As in other NBDs, changes in the LptB active site are accompanied by movement in the helical domain. In this region, Sherman et al. identified the Q-loop residue F90 as particularly important for interactions with TMD partners LptF/G (46). Aromatic residues at this position are relatively well conserved across ABC transporters (46). While LptB/F90A variants are catalytically active in vitro, they are not viable in vivo (46). LptB/F90A cannot pull-down LptF/G, indicative of complex assembly defects (46). This result illustrates the importance of the Q-loop in NBD-TMD interactions.

In addition to the conserved features described above, some NBDs are appended to C-terminal regulatory domains. For example, methionine and molybdate/tungstate transporters have been crystallized with substrates bound to these C-terminal domains (50, 51). Substrate-binding in this region stabilizes an inactive, inward-facing conformation, preventing ATPase activity when import is no longer necessary.

In this section, I have presented LptB as a representative NBD. In ABC transporters, ATP-hydrolysis induces conformational changes in both the conserved and variable regions of the NBD. These changes in turn drive the motion of the TMDs. In the sections that follow, I review attempts to target the NBDs and TMDs of ABC systems.

2.5 Inhibition of human ABC transporters

The human genome encodes 49 ABC transporters (3). Many of these are Class 1 exporters involved in the efflux of sterols, bile salts, and other hydrophobic substrates. To date, 14 ABC transporters have been implicated in Mendelian disorders, including cystic fibrosis and Tangier disease (52, 53, 3). ABC exporters are also implicated in multidrug resistance (MDR) in human cancers (54). Although many ABC transporters are capable of cancer drug efflux in
vitro, only three – P-glycoprotein (Pgp/MDR1/ABCB1), multidrug resistance-associated protein 1 (MRP1/ABCC1), and breast cancer resistance protein (BCRP/MXR/ABCG2) – are thought to contribute significantly to MDR in cancer patients (55). As these are perhaps the most targeted ABC systems, attempts to inhibit these three exporters, especially Pgp, will be discussed below.

The mechanism of cancer drug efflux is not well understood. Pgp, MRP1, and BCRP can export a range of hydrophobic compounds. Unlike bacterial importers, which transport soluble substrates across membranes, exporters must expel compounds that can diffuse across membranes. Two hydrophobic drug efflux models have been proposed: the “hydrophobic vacuum cleaner model” and the “flippase” model (56). In both models, the exporters take up their substrates directly from the membrane. In the vacuum cleaner model, the compound is expelled to the aqueous extracellular space. In the flippase model, the exporter flips compounds from the inner to the outer leaflet of the membrane, rather than into the aqueous phase. These models are not mutually exclusive and are difficult to differentiate experimentally because hydrophobic compounds rapidly repartition into the membrane (55).

Many diverse compounds, including calcium channel blockers, steroids, and tyrosine kinase inhibitors, have been shown to modulate cancer efflux pump activity in vitro and in vivo (57, 58). These compounds, called modulators, do not kill the cancer cells directly, but instead facilitate the uptake of cytotoxic compounds previously expelled (59). Modulators can act via several mechanisms. Classical modulators, such as verapamil and cyclosporin A, are transporter substrates thought to have high rates of spontaneous flip-flop within the membrane bilayer (60, 61). Treatment with a classical modulator thus locks the exporter in a futile cycle of ATP hydrolysis and transport that cannot overcome the intrinsic flip-flop rate. Other compounds,
such as tariquidar, are not transported, but display very high affinity for the drug-binding pocket in the TMDs (62-64).

Three generations of modulators have been taken into clinical trials (65). The first, which included verapamil and cyclosporine A, demonstrated high toxicity but low effectiveness. Second generation modulators were designed with higher binding affinity but still exhibited prohibitive toxicity. The third generation, which includes tariquidar, is still in clinical trials. Treatment of MDR with modulators is compounded not only by their toxicity, but also by their ability to increase the expression of efflux pumps, potentially making the cancer even more refractory to treatment (55).

It is important to note that the modulators taken into the clinic are thought to interact with the TMDs of the transporters, specifically the drug-binding sites. Some steroids and flavonoid compounds, found in fruits, vegetables and other plants, are thought to bind to the NBD of Pgp (66-68). When flavonoids are incubated with purified Pgp NBD, they can quench the intrinsic fluorescence of tryptophan residue positioned in the RecA subdomain at the back of the NBD, away from the ATP-binding site interface (67, 26). The exact NBD binding sites of these compounds, however, remain unknown, and they also likely interact with the TMD binding sites as well (69, 70). Flavonoids have been shown to stimulate and inhibit Pgp activity in cell culture, depending on the assay conditions used, and studies conducted to examine how flavonoid compounds affect drug uptake in animals have not been conclusive (70). The clinical utility of flavonoids as efflux pump modulators has yet to be established.

2.6 Inhibition of ABC transporters in Gram-negative bacteria
The *E. coli* genome encodes 79 ABC systems, the majority of which are binding-protein dependent importers (71). Several of these systems have the potential to be exploited as new antibiotic targets. There are three essential ABC transporters, all involved in cell envelope biogenesis (discussed in Chapter 1): MsbA, a Class 1 homodimer that flips the LPS Lipid A-core moiety from the inner leaflet to the outer leaflet of the IM; LolCDE, a Class 3 system that extracts lipoproteins from the IM; and LptB$_2$FG, a Class 3 system that extracts LPS from the IM (72, 31, 13, 14). Other systems have been found to be essential under certain conditions. For example, the transporter FtsEX, thought to regulate peptidoglycan hydrolysis, is essential for cell division in low-salt media (73, 74).

In addition to these essential systems, there are other transporters implicated in virulence. Although the major Gram-negative multidrug efflux pumps (*e.g.* AcrAB-TolC) are powered by the proton motive force and not ATP (75), ABC exporters are involved in the secretion of virulence factors. Class 1 ABC exporters are found in Type I secretion systems, which discharge protein toxins like haemolysin (76, 77). It has been proposed that targeting virulence factors rather than essential processes may provide a means to combat infection with a lower risk of resistance (78), although molecules with this mechanism of action have not yet come to fruition in the clinic.

To date, no antibiotics have been reported that target these essential and virulence-associated ABC systems in Gram-negative bacteria. The Kahne lab has conducted in vitro screens for small molecules that inhibit the ATPase activity of LptB (79, 80), although no small molecules have been demonstrated to interact directly with LptB$_2$FGC. MsbA is perhaps the most promising target for traditional TMD-binding inhibitors, as there is evidence that MsbA
may function as a multidrug exporter as well as a flippase (81, 82). Perhaps it will be possible to target these drug-binding sites as was done for P-glycoprotein.

2.7 Conclusion

It is important to note that there is precedent for targeting ABC systems for antibiotic development. The antibiotic targocil binds to the TMDs of TarGH, an essential ABC system that exports teichoic acids, components of the Gram-positive cell wall, to the cell surface (83). Given the severity of the antibiotic resistance crisis for Gram-negative bacteria, the three essential, conserved Gram-negative ABC systems – all involved in envelope biogenesis – deserve careful consideration in the search for new antibiotic targets. In this vein, the next two chapters describe the discovery of the first small molecule demonstrated to interact directly with LptB₂FGC.

2.8 References


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Chapter 3

Genetic and biochemical evidence that novobiocin interacts with LptB₂FGC
3.1 Introduction

In this chapter, I present genetic and biochemical evidence that the natural product novobiocin interacts with LptB2FGC, the ABC transporter that powers lipopolysaccharide (LPS) transport. This is the first small molecule demonstrated to interact with LptB2FGC. In the sections that follow, I show that novobiocin can suppress defects in LptB in vivo, generating the hypothesis that novobiocin can bind LptB. I also show that novobiocin affects LPS transport in in vitro reconstitutions – further evidence that novobiocin interacts with LptB2FGC.

All of the genetic experiments were conducted by Professor Natividad Ruiz with the assistance of her technician Rebecca Davis, and her student Brent Simpson at The Ohio State University. The biochemical experiments were performed by me in collaboration with Dr. David Sherman and Dr. Suguru Okuda in the Kahne lab.

3.2 Partial-loss-of-function mutant lptB1 is permeabilized to antibiotics that normally cannot penetrate the outer membrane

The discovery that novobiocin interacts with LptB2FGC originated in the characterization of *E. coli* mutants with defects in LptB, the ATPase that powers LPS transport (Figure 3.1A). To facilitate the discovery of LptB inhibitors, Sherman and collaborators published structures of *E. coli* LptB bound to ATP and ADP (1). As LptB was purified and crystallized with a C-terminal histidine tag, Professor Ruiz attempted to install the tag on chromosomal *lptB* to assess whether it affects LPS transport. Remarkably, she was not able to isolate any colonies with the tag, suggesting that it confers LPS transport defects too severe to support growth.

While attempting to add the histidine tag, she isolated a mutant, *lptB1*, that contains a frameshift mutation in the tag that appends 34 amino acids onto the C-terminus of LptB (Figure
To characterize \textit{lptB1}, she performed a disk diffusion assay with antibiotics that can kill \textit{E. coli} strains with membrane defects, but not wild-type \textit{E. coli}. These antibiotics, which include bacitracin, erythromycin, novobiocin, rifampicin, and vancomycin, cannot reach their targets within wild-type cells because they cannot penetrate the OM or are efficiently expelled by efflux pumps. For example, the minimum inhibitory concentration (MIC) of novobiocin against \textit{E. coli} drops 100x upon deletion of the major efflux pump component \textit{acrA} (2). As shown in Figure 3.1C, \textit{lptB1} is sensitive to bacitracin, novobiocin, rifampicin, and erythromycin, while wild type is not. This result indicated that \textit{lptB1} is a partial-loss-of-function mutant: it is viable, but has a defective OM.

\textbf{Figure 3.1} Partial-loss-of-function mutant \textit{lptB1} is permeabilized to antibiotics that normally do not penetrate the OM. a) Schematic of LPS transport in \textit{E. coli}. LptB binds and hydrolyzes ATP in the cytoplasm, powering LPS transport from the IM to the OM. b) \textit{lptB1} has a frameshift mutation that appends a 34-amino acid tag onto the C-terminus of chromosomal \textit{lptB}. c) Zones of growth inhibition observed in disk diffusion assay for wild type (wt) and \textit{lptB1}. Cells were plated on Luria Broth (LB) plates containing 6-mm disks soaked with designated antibiotic. Nov: novobiocin. Bac: bacitracin. Em: erythromycin. Rif: rifampicin. (Data from N. Ruiz.)

3.3 Most intragenic \textit{lptB1} suppressor mutations correct the permeability defect, conferring resistance to multiple antibiotics
To further characterize \( lptB1 \), Professor Ruiz plated \( lptB1 \) on novobiocin and vancomycin to obtain resistant mutants (Figure 3.2). She obtained mutations not within the known target pathways of these antibiotics, but within \( lptB1 \). The simplest explanation for how these intragenic suppressor mutations confer resistance in the \( lptB1 \) background is that they correct the defect produced by the 34-amino acid tag, restoring the OM permeability barrier. Professor Ruiz reasoned that if this were the case, then these mutations should confer resistance not only to the antibiotic used during the selection, but also to other antibiotics that cannot penetrate an intact OM.

![Figure 3.2](image)

**Figure 3.2** The permeability defects of \( lptB1 \) can be suppressed by intragenic mutations, one of which (\( lptB1/R144H \)) confers resistance only to novobiocin. *left* Zones of growth inhibition measured in disk diffusion assay for wild type, \( lptB1 \), and \( lptB1 \) suppressors as described in Figure 3.1C. *right* Representative disk-diffusion plates for novobiocin-specific suppressor \( lptB1/R144H \) and representative general suppressor \( lptB1/T45A \). (Data from N. Ruiz.)

Indeed, as shown in Figure 3.2, all but one of the suppressor mutations conferred resistance to all of the antibiotics tested (novobiocin, bacitracin, erythromycin, and rifampicin). The exception, \( lptB1/R144H \) (highlighted in yellow), confers resistance only to novobiocin.
*lptB1/R144H* provided the first indication that novobiocin may affect the Lpt pathway and will be discussed in section 3.5 below.

### 3.4 How do the general suppressor mutations confer resistance to multiple antibiotics?

With the exception of *lptB1/S243stop*, which truncates the C-terminal 34-amino acid tag, the mechanism by which the suppressor mutations correct the *lptB1* defect for multiple antibiotics is unknown. Professor Ruiz observed that protein levels in *lptB1* are reduced relative to wild type, but none of the suppressor mutations were found to improve protein levels (data not shown). Our current hypothesis – informed by the structure of the C-terminus of LptB, ATPase activity assays, and the location of the suppressor mutations – is that LptB1 has reduced ATPase activity, and the suppressor mutations improve this activity.

Although the C-terminus of *E. coli* LptB is not visible in the structures published by Sherman and collaborators, it is visible in the structure of *Burkholderia phymatum* LptB, crystallized with an N-terminal histidine tag in the absence of nucleotide (PDB: 4WBS). As shown in Figure 3.3A, the C-terminus of *B. phymatum* LptB appears to reach between the Walker A and switch motifs, both important in ATP-binding and hydrolysis (discussed in Chapter 2) (3). The sequence of LptB is well conserved between *E. coli* and *B. phymatum*, so we expect a similar configuration for *E. coli* LptB (Figure 3.3B). David Sherman found that LptB2FGC complexes purified with a C-terminal histidine tag exhibit significantly reduced ATPase activity compared to complexes purified with an N-terminal histidine tag (Figure 3.3C), suggesting that C-terminal tags disrupt key catalytic interactions. It is likely that LptB1 has impaired ATPase activity similar to LptB-his.
The C-terminus of LptB is important for ATPase activity. a) The C-terminus of\textit{B. phymatum} LptB contacts the Walker A and switch regions, important for ATP binding and hydrolysis. Conserved motifs are colored as follows: yellow – Walker A, orange – Walker B, red – switch region, blue – signature motif, and teal – Q-loop. C-terminus colored purple with sidechains shown as sticks for M274, F272, L268, and Y267. PDB: 4WBS. b) Alignment of the C-terminal regions of \textit{E. coli} and \textit{B. phymatum} LptB. Sequence alignment performed with Clustal Omega (4, 5); coloring by conservation added in Jalview (6). c) ATPase activity in detergent of \textit{E. coli} LptB\textsubscript{2}FGC complexes purified with a C-terminal versus an N-terminal histidine tag on LptB. Free phosphate release was measured with a molybdate-based assay (7). (Data from D. Sherman.)

Significantly, the suppressor mutations cluster near the Walker A, signature motif, and switch regions (Figure 3.4). Again, these motifs are known to be important in the binding and hydrolysis of ATP (3). Perhaps mutations in these regions change the affinity of LptB for ATP and/or the rate of hydrolysis to compensate for the defects in the C-terminus. Additional work is needed to understand both the role that the C-terminus plays in catalytic turnover and how the suppressor mutations compensate for changes to the C-terminus.
Figure 3.4  Mutations that suppress the lptB1 permeability defect cluster near regions important for ATPase activity. Suppressors (from Figure 3.3) are represented as purple spheres, and conserved motifs are colored as follows: yellow – Walker A, orange – Walker B, red – switch region, blue – signature motif, and teal – Q-loop. PDB: 4P33. (Data from N. Ruiz.)

3.5  lptB1/R144H: a novobiocin-specific suppressor that grows better in the presence of novobiocin

In addition to the general suppressors discussed above, Professor Ruiz isolated one suppressor – lptB1/R144H – that clearly conferred resistance only to novobiocin (Figure 3.2). Presumably, it does not correct the lptB1 defect in a manner analogous to the general suppressors discussed above. This curious finding was made even more remarkable by the observation that lptB1/R144H grows much better in the presence of novobiocin than in its absence. As shown in Figure 3.5, lptB1/R144H displays growth defects on LB plates unless novobiocin is present. This result prompted two questions: How does lptB1/R144H confer resistance to novobiocin, and novobiocin alone? And, why does lptB1/R144H grow better in the presence of novobiocin?
Figure 3.5  lptB1/R144H grows better in the presence of novobiocin. Efficiency of plating wild type, lptB1, and lptB1/R144H on LB plates with and without novobiocin (33 μg/mL). Spots range from undiluted overnight culture to 10^-6 dilution. (Data from N. Ruiz.)

3.6  Novobiocin can suppress lethal mutations in lptB

To characterize the R144H mutation in the absence of the lptB1 tag, Professor Ruiz constructed lptB/R144H, a haploid strain in which tagless lptB/R144H is expressed from a plasmid. Strikingly, lptB/R144H is even more compromised than lptB1/R144H: it dies on Luria Broth (LB) plates unless novobiocin is present (Figure 3.6A).

Since the surprising discovery of the novobiocin-dependence of lptB/R144H, the Ruiz lab has found that novobiocin can suppress other lethal mutations in lptB. This property is allelespecific. For example, novobiocin can suppress the lethality of lptB/R144H and lptB/R144Q, but not lptB/R144A or lptB/R144F, as assessed by plating on LB media in haploid cells. Novobiocin also cannot rescue catalytically inactive mutants, such as lptB/E163Q, K42A, or H195A (data not shown). In total, novobiocin has been found to suppress the lethality of six mutations: R144H, R144Q, R145A, I148S, G33C/L72R, and L93F. These mutations cluster near the signature motif and the groove region that interacts with transmembrane domains LptF/G (Figure 3.6B).
Figure 3.6  Novobiocin can suppress the lethality of *lptB* mutations.  a) Efficiency of plating on LB plates with and without novobiocin (5 µg/mL).  Strains are haploid with tagless LptB expressed from a plasmid (NR754 *tet2 A*lptB (pET23/42-LptB)).  Cultures were grown overnight in minimal media.  Spots range from undiluted overnight culture to 10⁶ dilution.  b) *lptB* mutations suppressed by novobiocin (R144H, R144Q, R145A, I148S, G33C/L72R, and L93F, highlighted in magenta) cluster near the signature motif and groove regions.  PDB: 4P33.  (Data from N. Ruiz and B. Simpson.)

It is worth noting that of the six lethal mutations that can be suppressed by novobiocin, two – R144H and G33C/L72R – can also be suppressed by a C-terminal histidine tag (data from the Ruiz lab, not shown).  In the case of R144H, the *lptB*1 34-amino acid tag also enables growth, as seen in Figure 3.5.  Although it is unknown how a C-terminal tag can suppress defects arising from mutations in the signature motif and groove region, these results further point to the importance of the C-terminus in the activity of LptB.

3.7  Two potential explanations for how novobiocin suppresses defects in LptB

How does novobiocin suppress deleterious mutations in *lptB*?  The first explanation we considered is that novobiocin’s ability to suppress Lpt defects is an indirect consequence of inhibition of its known target: DNA gyrase (8).  To test this hypothesis, the Ruiz lab examined whether nalidixic acid, another gyrase inhibitor (9), also enables *lptB*/R144H to grow.  It does
not (Figure 3.7). Although novobiocin and nalidixic acid target different subunits of gyrase, this result weakens the argument that gyrase inhibition is responsible for the suppression of Lpt defects.

![Figure 3.7](image)

**Figure 3.7** Nalidixic acid, another DNA gyrase inhibitor, cannot suppress the lethality of lptB mutations. Disk diffusion assay for lptB/R144H, a haploid strain in which tagless lptB/R144H is expressed from a plasmid. The growth wings, highlighted in blue ink, are thought to arise nonspecifically whenever novobiocin is present with a bacteriostatic antibiotic. NB: novobiocin. NA: nalidixic acid. (Data from B. Simpson.)

An alternative explanation is that novobiocin interacts with another, hitherto unknown protein in the cell. We reasoned that it could interact directly with a component of the Lpt pathway or with a component in a connected pathway. For example, deletion of fabH, a gene involved in fatty acid biosynthesis, can also suppress Lpt defects (10). The Ruiz lab has also found that minimal media can suppress Lpt defects, including those conferred by lptB/R144H, presumably by slowing growth and thus reducing pressure on the Lpt pathway (data not shown). To test whether novobiocin interacts directly with a component of the Lpt pathway, versus interacting with a connected pathway, we employed biochemical tools developed in the Kahne lab to study LPS transport.

### 3.8 Novobiocin accelerates LPS release to LptA in vitro
To test whether novobiocin affects the first step of LPS transport – the transfer of LPS from the IM to LptA – we adopted an in vitro assay developed by Okuda et al (11). Using technology developed in the Schultz lab (12), Okuda incorporated the unnatural amino acid ρBpa, which crosslinks to adjacent molecules following UV irradiation, into various sites in LptA to find one that crosslinks to LPS (LptA*). Using right-side-out (RSO) vesicles prepared from E. coli over-expressing tagless IM complex (LptB2FGC), he found that in the presence of ATP, these vesicles release LPS to purified LptA* in a time-dependent manner (Figure 3.8A). As expected, LptA-LPS crosslinks are observed only when the IM complex is overexpressed, ATP is present, and the sample is UV irradiated (11).

![Diagram](image_url)

**Figure 3.8** Novobiocin accelerates LPS release from membrane vesicles to LptA. a) In the presence of ATP, RSO vesicles prepared from cells overexpressing tagless LptB2FGC transfer LPS to soluble LptA*, which contains a photo-crosslinkable amino acid at a position that binds LPS. b) Western blot from the vesicle assay showing the amount of LPS crosslinked to LptA* after a 1-hr incubation period with varying concentrations of novobiocin. The exposure time refers to the amount of time the film was placed on the blot. Long exposure included here to show that LPS is released to LptA* even in the absence of novobiocin.

Using the RSO vesicle assay, Okuda and I added varying concentrations of novobiocin to the RSO vesicles prepared from cells overexpressing tagless wild-type complex and assessed LPS release to LptA* after 1 hour. Remarkably, novobiocin was found to increase LPS release to LptA* from the RSO vesicles (Figure 3.8B). This is the first small molecule demonstrated to have this effect.
Because RSO vesicles are prepared from membranes isolated from whole cells, they contain other proteins in addition to LptB$_2$FGC. To assess whether novobiocin interacts with LptB$_2$FGC versus another component of the vesicles, we utilized a pure reconstitution developed by Dr. Suguru Okuda and David Sherman. In the pure reconstitution, the IM complex is purified in detergent and then rapidly diluted into liposomes composed of polar lipid extract and Ra-LPS. Okuda and Sherman have found that these proteoliposomes transfer LPS to LptA* in a time-dependent manner (Figure 3.9A). As in the RSO vesicle system, LptA-LPS crosslinks are observed in the pure reconstitution only when the IM complex is present, ATP is added, and the sample is UV irradiated.

**Figure 3.9** Novobiocin accelerates LPS release from proteoliposomes containing LptB$_2$FGC to LptA. a) In the presence of ATP, proteoliposomes prepared with purified (LptB-his)$_2$FGC transfer LPS to soluble LptA*. b) Western blot from the proteoliposome assay showing the amount of LPS crosslinked to LptA* after a 1-hr incubation period with varying concentrations of novobiocin.

Using the pure reconstitution, Okuda, Sherman, and I found that novobiocin again increases LPS release to LptA*, albeit at higher concentrations (Figure 3.9B). This result indicates that novobiocin interacts with and affects the activity of LptB$_2$FGC directly.

There are two issues raised by the pure reconstitution that warrant additional attention. The first is that I observed novobiocin activation of LPS release in the pure reconstitution only when I purified the IM complex with a C-terminal 6-histidine tag on LptB. Dr. Okuda also independently observed this effect. When I purified the IM complex with an N-terminal 6-
histidine tag on LptB, however, novobiocin did not appear to activate LPS release. As discussed above, the C-terminal tag on LptB impairs activity of the complex. Consequently, proteoliposomes prepared with (LptB-his)$_2$FGC are much less active than proteoliposomes prepared with (his-LptB)$_2$FGC. The most likely explanation for our failure to see novobiocin-induced activation in the N-tagged pure reconstitution is that we have not yet found the optimal conditions for this system, which already has high activity relative to the C-tagged system. The fact that novobiocin strongly activates LPS release in the RSO vesicle assay, which utilizes tagless LptB$_2$FGC, indicates that the effect is not an artifact of the C-terminal tag.

The second issue concerns the manner in which novobiocin activates LPS release. Does novobiocin affect the rate of ATP hydrolysis, the coupling between hydrolysis and extraction, or both? David Sherman found that novobiocin does not strongly affect the ATPase activity of LptB-his alone (data not shown), although this data may not be reflective of the entire complex. While performing the proteoliposome assay with the C-tagged system, I measured the amount of free phosphate released at each novobiocin concentration. It appears that novobiocin may slightly increase ATPase activity as well as LPS release, but more data, collected in replicates, are needed. This experiment would ideally be performed with the N-tagged system, which does not suffer from impaired ATPase activity, once the conditions are optimized.

Although additional work is needed to better probe the mechanism of how novobiocin affects LPS release, the data presented here show that novobiocin can indeed interact, at least in vitro, with another system in addition to gyrase: LptB$_2$FGC.

3.9 Does novobiocin affect the activity of LptB/R144H in vitro?
The discovery that novobiocin activates LPS release in vitro provided impetus for returning to the question posed above: how does novobiocin suppress defects in LptB caused by R144H and other mutations? To address this question, we reasoned it would be instructive to compare the activity of wild-type complexes to mutant complexes in the presence and absence of novobiocin. For this experiment, we chose the vesicle assay, as novobiocin exerts the strongest effect on wild-type complexes using this method.

The cells used to prepare the vesicles are merodiploid; they possess the wild-type chromosomal copies of the lptBFGC genes in addition to plasmid copies for overexpression. In order to adapt the vesicle assay to study mutant complexes, it was important to verify that (1) the LPS release signal detected is due to overexpression of the mutant complexes, not background expression of the wild-type complex from the chromosome, and (2) the mutant complexes are expressed to the same level. To address issue (1), I prepared vesicles from cells that did not contain plasmid (n/p). As shown in Figure 3.10, these vesicles released almost no LPS, indicating that LPS release from background expression of the wild-type complex is negligible. To address issue (2), I assayed the levels of LPS and LptBFC in the vesicles by Western blot. There were no differences in protein or LPS levels, even for catalytically-inactive mutants (data not shown).

As discussed above, the Ruiz lab found that novobiocin suppresses the lethality on LB plates of lptB/R144H and lptB/R144Q, but not lptB/R144A. I wondered whether we would also observe differences between these mutants in vitro (Figure 3.10). Interestingly, LptB/R144A vesicles are not capable of LPS release, even in the presence of novobiocin. They appear to be as inactive as the vesicles prepared with LptB/E163Q, which cannot hydrolyze ATP. In contrast, vesicles prepared with the LptB/R144H or LptB/R144Q variants appear to be more active than
vesicles prepared with the wild-type complex. While this result is surprising, the observation that LptB/R144H and LptB/R144Q vesicles behave differently than LptB/R144A vesicles mirrors the allele specificity observed in vivo.

![Figure 3.10](image)

**Figure 3.10** Vesicles prepared with LptB/R144H release more LPS than those prepared with wild-type LptB. Vesicles were prepared from merodiploid cells overexpressing untagged complex with the indicated mutation on the overexpressed copy of \(lptB\). Western blot shows the amount of LPS crosslinked to LptA* after a 1-hr incubation period with indicated concentration of novobiocin. The no plasmid (n/p) sample indicates that LPS release due to chromosomal expression of the wild-type complex is negligible.

Surprisingly, novobiocin does not appear to strongly affect the activity of LptB/R144H complexes in the vesicle assay (Figure 3.10, Figure 3.11). It is possible that additional optimization is needed to observe novobiocin’s effect here. I have observed that vesicles prepared with LptB/R144H have a higher background signal in the absence of ATP compared to vesicles prepared with wild-type LptB (data not shown). This signal is most likely due to contamination from cellular ATP during the preparation of the vesicles. As LptB/R144H vesicles are very active, even low concentrations ATP could generate a strong LPS release signal. It may not be possible to draw conclusions about novobiocin’s effect on LptB/R144H complexes until this background level is reduced.
Figure 3.11  LPS release from vesicles prepared with LptB/R144H does not appear to be affected by novobiocin. Western blot shows the amount of LPS crosslinked to LptA* after a 1-hr incubation period with indicated concentration of novobiocin.

It will also be necessary to examine these mutants in the pure reconstitution. In my hands, the LptB/R144H complex displayed dramatically less ATPase activity than wild-type complex when purified with an N-terminal histidine tag, even though it did not display any obvious complex formation defects during purification. Tristan Owens and Becca Taylor in the Kahne lab, however, have observed equal, or slightly greater, activity of the R144H complex over the wild-type complex when purified with a tag on LptC, a result that is more consistent with my observations in the vesicle assay.

The observation that the R144H variant may be more active than wild type is surprising, as R144 contacts residues important for catalytic activity and coupling with the transmembrane domains. The R144 side chain hydrogen bonds with D162 of the Walker B motif and Q85 of the Q-loop in the LptB-ATP and LptB-ADP structures, respectively (PDB: 4P33 and 4P32). Interestingly, in the apo LptB structure from *Burkholderia*, the side chain of the analogous residue (R177) does not participate in hydrogen bonding (PDB: 4WBS), suggesting the Walker B and Q-loop interactions are mediated by ATP-binding. Mutation of the analogous residue (Q140) in MalK, the nucleotide-binding domain of the maltose transporter, has been found only
3.10 **Coumermycin A1 also suppresses lethal mutations in \textit{lptB}**

Novobiocin is a member of the aminocoumarin family of antibiotics, discussed in Chapter 5. Another member of this family is coumermycin A1, which structurally resembles a dimer of novobiocin and also is a potent inhibitor of DNA gyrase (Figure 3.12) (15). Unlike novobiocin, coumermycin A1 has antibacterial activity against wild-type \textit{E. coli}. It is thought not to be an efflux pump substrate, as the MIC against wild-type \textit{E. coli} is comparable to the MIC against \textit{E. coli} lacking the major pump component \textit{tolC} (Vadim Baidin – personal communication). Can coumermycin A1 also suppress defects in LptB, or is this property specific to novobiocin?

Brent Simpson tested coumermycin A1’s ability to suppress the lethality of \textit{lptB/R144H} on LB plates. The results were striking. As shown in Figure 3.12, a disk of novobiocin enables \textit{lptB/R144H} to grow from a large radius extending around the disk. Coumermycin A1, on the other hand, enables \textit{lptB/R144H} growth only within a tight ring. Presumably cells too close to the coumermycin A1 disk cannot grow due to coumermycin’s inhibition of gyrase, and cells too far from the disk cannot grow due to the defect in LptB. This result suggests that coumermycin A1 can suppress Lpt defects in a manner similar to novobiocin, albeit in a narrow concentration range.
I examined the effect of coumermycin A1 on LPS release from vesicles prepared from cells overexpressing tagless, wild-type complex. At low concentrations, coumermycin A1, like novobiocin, stimulates LPS release (Figure 3.13). At higher concentrations, coumermycin A1 appears to inhibit LPS release, although it is possible that it may be disrupting the vesicles due to its hydrophobicity. It is important to note that unlike novobiocin and coumermycin A1, the small molecule 6,7-dihydroxycoumarin does not affect LPS release (Figure 3.13). This suggests that the coumarin moiety alone is not sufficient to activate LPS release in vitro.

### 3.11 Conclusion

Figure 3.12  Coumermycin A1 also suppresses defects caused by lptB/R144H. Disk diffusion assay for lptB/R144H, a haploid strain in which tagless lptB/R144H is expressed from a plasmid. NB: novobiocin. C: coumermycin A1. (Data from B. Simpson.)

Figure 3.13  At low concentrations, coumermycin A1 accelerates LPS release from membrane vesicles to LptA. Western blot shows the amount of LPS crosslinked to LptA* after a 1-hr incubation period with indicated concentration of coumermycin A1 or 6,7-dihydroxycoumarin.
In this chapter, I present genetic and biochemical evidence that the gyrase-inhibitor novobiocin interacts with an additional system in E. coli: LptB2FGC. The Ruiz lab has demonstrated that novobiocin can suppress lethality caused by mutations in lptB. We believe this suppression to be mediated through a direct interaction with LptB2FGC because novobiocin affects the LPS release activity of LptB2FGC in a pure reconstitution. The data presented in this chapter provided impetus for the structural studies pursued in Chapter 4, which disclose where novobiocin interacts with the complex.

3.12 Materials and Methods

3.12.1 Strains and Materials

E. coli strain Nova Blue [endA1 hsdR17 (rK12− mK12+) supE44 thi-1 recA1 gyrA96 relA1 lac F′[proA+B+ lacIqZΔM15::Tn10] (TetR)] from Novagen was used for plasmid manipulations. E. coli strain BL-21(λDE3) [F− ompT gal dcm lon hsdS8(rB− mB−) λ(DE3)] from Novagen was used for right-side-out vesicle preparation. E. coli strain KRX [F′, traD36, ΔompP, proA+B+, lacIΔ(lacZ)M15ΔompT, endA1, recA1, gyrA96 (Nal’), thi-1, hsdR17 (rK− mK+), e14− (McrA’), relA1, supE44, Δ(lac-proAB), Δ(rhaBAD)::T7 RNA polymerase] from Promega was used for protein purification.

The plasmids are used in this study are listed in Table 3.1. To study LptB variants in the right-side-out vesicle assay, lptB mutations were installed in pCDFDuet-LptB-LptFG by site-directed mutagenesis with primers purchased from Integrated DNA technologies (Table 3.2). Mutagenesis was confirmed with DNA sequencing by Beckman Coulter. PCR amplification was performed with KOD Hot Start DNA polymerase from Novagen. Restriction enzyme DpnI was
purchased from New England Biolabs. All other materials were purchased from Sigma Aldrich unless otherwise noted.

### Table 3.1 Plasmids used in this study

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<th>Description</th>
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<tr>
<td>pSup-BpaRS-6TRN</td>
<td>encodes tRNA and aminoacyl-tRNA synthetase to incorporate pBpa at amber stop codons (TAG)</td>
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<td>pCDFDuet-LptB-LptFG</td>
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<td>(11)</td>
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<tr>
<td>pBAD/HisA-LptC</td>
<td>encodes untagged LptC</td>
<td>(11)</td>
</tr>
<tr>
<td>pCDFDuet-LptB-his8-LptFG</td>
<td>encodes LptB with a C-terminal his8 tag in site 1 and untagged LptFG in site 2</td>
<td>D. Sherman</td>
</tr>
<tr>
<td>pET22/42-LptC</td>
<td>encodes untagged LptC</td>
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### Table 3.2 Site-directed mutagenesis primers (for pCDFDuet-LptB-LptFG)

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<td>Ecoli_lptB E163Q-r</td>
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<td>Ecoli_lptB R144Q-r</td>
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#### 3.12.2 Purification of LptA/I36pBpa-his for photocrosslinking

For LPS release assays, LptA/I36pBpa-his was purified as described in ref. (11).

Overnight cultures of BL-21(λDE3) containing plasmids pSup-BpaRS-6TRN and pET22b-LptA/I36Am-his were diluted 100x into LB Miller media containing 50 μg/mL carbenicillin, 30 μg/mL chloramphenicol, and 0.8 mM pBpa (Bachem). Cultures were grown at 37 °C, 220 rpm to OD 0.6, at which point overexpression was induced with 50 μM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cultures were incubated at 37 °C, 220 rpm for 2 h.

Cells were harvested by centrifugation at 5250 xg, 4 °C for 20 min. Pellet was resuspended on ice in 50 mM Tris-HCl (pH 8.0), 0.5 M sucrose supplemented with 300 μg/mL lysozyme and 100 μg/mL DNAsae. To promote spheroplast formation, an equal volume of 50
mM Tris-HCl (pH 8.0) with 3 mM ethylenediaminetetraacetic acid (EDTA) was added slowly, and the suspension was incubated on ice for 30 min.

Spheroplasts were pelleted by centrifugation at 6000 xg, 4 °C for 15 min and discarded. The supernatant was centrifuged at 100,000 xg, 4 °C, for 30 min. In preparation for nickel affinity chromatography, Ni-NTA Superflow resin (Qiagen) was washed with water and equilibrated with column buffer [20 mM Tris-HCl (pH 8.0), 300 mM NaCl, 20 mM imidazole]. After the supernatant was applied to the column twice, the resin was washed twice with 20 column volumes of column buffer. Protein was eluted in two batches of 3 column volumes of Tris-buffered saline (TBS; 20 mM Tris [pH 8.0], 150 mM NaCl) with 200 mM imidazole.

Eluate was concentrated in a 10-kDa molecular weight cut-off (MWCO) centrifugation filter (Amicon; Millipore) to ~3 mg/mL. Glycerol was added to a final concentration of 10% (vol/vol), and protein aliquots were flash frozen and stored at -80 °C. Protein concentration was measured using the Biorad DC protein assay.

3.12.3 Preparation of right-side-out (RSO) membrane vesicles

Right-side-out (RSO) vesicles were prepared as described in ref. (11) with minor modifications. To prepare the vesicles used in Figures 3.8, 3.11, and 3.13, overnight cultures of BL-21(λDE3) cells containing plasmids pCDFDuet-LptB-LptFG (encoding wild-type LptB or LptB/R144H) and pBAD/HisA-LptC were diluted 100x into LB Miller media containing 50 µg/mL carbenicillin and 50 µg/mL spectinomycin. Cultures were grown at 37 °C, 220 rpm to OD 1.0, at which point overexpression was induced with 0.02% arabinose and 10 µM IPTG. Cultures were incubated at 37 °C, 220 rpm for 2 h.
Cell culture (25 mL) was pelleted by centrifugation at 6000 xg, 4 °C for 10 min. Cells were resuspended in 2.5 mL 50 mM Tris-HCl (pH 7.4), 0.25 M sucrose supplemented with 150 μg/mL lysozyme and 50 μg/mL DNAse. Cell suspension was vortexed briefly and incubated on ice for 1 min. To promote spheroplast formation, 2.5 mL 50 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 3 mM EDTA was added slowly, and the suspension was incubated on ice for 30 min.

Spheroplasts were harvested by centrifugation at 6000 xg, 4 °C for 15 min and lysed in 1-2 mL lysis buffer (TBS, 5 mM MgCl₂, 0.1 mM EDTA, 10 μg/mL DNAse I, 5 mM ATP). Samples were centrifuged at 16,000 xg, 4 °C for 15 min and resuspended in the supernatant to complete the lysis. Vesicles were collected by centrifugation at 200,000 xg, 4 °C for 30 min. Vesicles were resuspended in 1 mL storage buffer (TBS, 5 mM MgCl₂, 10% glycerol, 5 mM ATP), flash-frozen, and stored at -80 °C. The protein concentration of the vesicles was measured using the Biorad DC protein assay.

Different growth conditions were used to prepare the vesicles used in Figure 3.10, as cells containing plasmids encoding LptB variants E163Q and R144A grew considerably more slowly than cells containing plasmids encoding wild-type LptB. The pCDFDuet-LptB-LptFG plasmid was mutagenized as shown Table 3.2. Overnight cultures of BL-21(λDE3) cells containing plasmids pCDFDuet-LptB-LptFG (encoding wild-type LptB or a variant) and pBAD/HisA-LptC were diluted 100x into LB Miller media containing 50 μg/mL carbenicillin and 50 μg/mL spectinomycin. Cultures were grown at 37 °C, 220 rpm to ~ OD 0.7, at which point the temperature was reduced to 16 °C. Fifteen minutes later, overexpression was induced with 0.02% arabinose and 10 μM IPTG. Cultures were incubated at 16 °C, 220 rpm for 16 h. Plasmids were harvested from cells post-overexpression, and lptB was sequenced. No
suppressor mutations were detected. Vesicles were prepared from the cultures as described above. In parallel, vesicles were prepared from plasmidless BL-21 as a control.

3.12.4  **LPS release assay from RSO vesicles**

LPS release from RSO vesicles to LptA* (LptA/I36pBpa-his) was assayed as described in ref. (11) with minor modifications. RSO vesicles (50 µg protein) were diluted into reaction buffer (TBS, 5 mM MgCl₂, 10% glycerol) with 5 mM ATP in a total reaction volume of 100 µL. Novobiocin sodium salt, coumermycin A1, and 6,7-dihydroxycoumarin stock solutions (50x) were prepared in dimethyl sulfoxide (DMSO). Vesicles were pre-incubated with designated compound for 15 min on ice. Samples not treated with compound were supplemented with DMSO to 2%. The reactions were initiated by addition of 3 µg of LptA* (LptA/I36pBpa-his) and incubated at 30 °C for 1 h. Following the incubation period, samples were transferred to a 96-well plate and irradiated with UV light (365 nm) for 3 min on ice.

Following UV irradiation, samples were solubilized on ice for ~1 h in 1 mL Buffer A: 20 mM Tris-HCl (pH 8.0), 300 mM NaCl, 5 mM MgCl₂, 20 mM imidazole, 100 µg/mL lysozyme, 50 µg/mL DNase I, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% (wt/vol) Anzergent 3-14 (Anatrace). Debris was removed by centrifugation at 18,500 xg, 4 °C for 30 min. In preparation for nickel affinity chromatography to isolate LptA*, 250 µL Ni-NTA Superflow slurry was washed with water and equilibrated with 0.5 mL Buffer B (20 mM Tris-HCl (pH 8.0), 300 mM NaCl, 20 mM imidazole, 0.02% Anzergent 3-14). After sample supernatants were applied to columns 3x, resins were washed with 1 mL Buffer B. Proteins were eluted in one batch with 0.5 mL elution buffer (TBS, 200 mM imidazole, 0.02% Anzergent 3-14) and precipitated with 10% (wt/vol) trichloroacetic acid (TCA), followed by a cold acetone wash. Precipitates were
resuspended in 50 µL Laemmli sample buffer (2x) containing 5% (vol/vol) β-mercaptoethanol and subjected to immunoblotting as described below.

3.12.5  Purification of LptB-his-LptFGC

For the pure reconstitution, LptB-his-LptFGC was purified as described in ref. (1), with minor modifications. Overnight cultures of KRX cells transformed with plasmids pCDFDuet-LptB-his8-LptFG and pET22/42-LptC were diluted 200x into LB Miller media containing 50 µg/mL carbenicillin and 50 µg/mL spectinomycin. Cultures were grown at 37 ºC, 220 rpm to OD 0.9, at which point overexpression was induced with 0.02% (wt/vol) L-rhamnose. Cultures were incubated at 37 ºC, 220 rpm for 3 h. Cells were harvested by centrifugation at 4250 xg, 4 ºC for 20 min. Pellet was resuspended in 50 mM Tris-HCl (pH 7.4). Cells were re-pelleted by centrifugation at 8000 xg, 4 ºC for 20 min and flash frozen.

Pellet was thawed and resuspended in 50 mM Tris-HCl (pH 7.4) supplemented with 1 mM PMSF, 100 µg/mL lysozyme, and 50 µg/mL DNAse I. Cells were lysed 3x through a high-pressure cell disruptor. Unbroken cells were removed by centrifugation at 5000 xg, 4 ºC for 10 min. To pellet membranes, cell lysate was centrifuged at 100,000 xg, 4 ºC for 60 min. Membranes were resuspended in 20 mM Tris-HCl (pH 7.4), 300 mM NaCl, 5 mM MgCl₂, and 10% glycerol. Following two rounds of homogenization, membranes were flash frozen.

Membranes were thawed, supplemented with 1% (wt/vol) n-dodecyl-β-D-maltopyranoside (DDM, Anatrace) and 2 mM ATP, and solubilized at 4 ºC for 2 h with gentle rocking. In preparation for cobalt affinity chromatography, Talon metal affinity resin (Clontech) was washed with water and equilibrated with Buffer C: 20 mM Tris-HCl (pH 7.4), 300 mM NaCl, 10% glycerol, and 0.05% DDM. Membrane suspension was centrifuged at 100,000 xg, 4
°C for 90 min, and supernatant was applied to Talon column 3x. Resin was washed with 20 column volumes of Buffer C and 10 column volumes of Buffer C with 5 mM imidazole. The protein complex was eluted in 3 batches of 3 column volumes of Buffer C with 25 mM imidazole. Eluate was concentrated in a 100-kDa MWCO centrifugation filter to ~2 mg/mL and flash frozen.

Protein complex was further purified by size exclusion chromatography on Superdex 200 10/30 GL column in Buffer C. Fractions containing protein complex were pooled and concentrated in a 100-kDa MWCO centrifugation filter to ~10 mg/mL. Aliquots were flash frozen and stored at -80 °C. Protein concentration was measured using the DC protein assay.

3.12.6 Proteoliposome preparation

Proteoliposomes were prepared by a detergent dilution method according to the procedure developed by Dr. David Sherman and Dr. Suguru Okuda. Aqueous stock suspensions of *E. coli* polar lipid extract (20 mg/mL, Avanti Polar Lipids, Inc.) and LPS from *E. coli* EH100 (2 mg/mL, Ra mutant, Sigma) were prepared, sonicated for 20 min, and flash frozen for storage at -80 °C. Thawed aliquots were sonicated briefly before use.

Before dilution, a mixture containing liposomes (7.5 mg/mL), DDM (0.25%), LPS (0.5 mg/mL), and protein complex (0.135 mg/mL) was prepared in 1x TBS as follows. After the polar lipid extract stock suspension was diluted into water, DDM was added to destabilize the liposomes, and LPS was added to form LPS-phospholipid-DDM micelles. Following the addition of TBS stock, the mixture was incubated on ice for 10 min. Protein was then added to the mixed micelles, and the mixture was incubated on ice for 20 min.
The mixture was rapidly diluted 100x with cold TBS and incubated on ice for 30 min. Proteoliposomes were harvested by centrifugation at 300,000 xg, 4 °C for 2 h. The supernatant was discarded, and the proteoliposomes were diluted 100x into cold TBS again. Proteoliposomes were again harvested by centrifugation at 300,000 xg, 4 °C for 2 h. The supernatant was discarded, and the proteoliposomes were resuspended in cold TBS with 10% glycerol (250 µL for every 100 µL of original mixture prior to dilution). Proteoliposomes were flash frozen and stored at -80 °C.

3.12.7 LPS release assay from proteoliposomes

LPS release from proteoliposomes to LptA* (LptA/I36pBpa-his) was assayed using the procedure developed by Dr. David Sherman and Dr. Suguru Okuda. Reaction mixture was composed of 60% (vol/vol) thawed proteoliposomes in 50 mM Tris-HCl (pH 8.0), 500 mM NaCl, and 10% glycerol (final concentrations). LptA* was added to a final concentration of 0.04 mg/mL. A novobiocin sodium salt stock solution (50x) was prepared in DMSO. Proteoliposomes were pre-incubated with novobiocin for 15 min on ice. Samples not treated with drug were supplemented with DMSO to 2%. The reactions were initiated by addition of 5 mM ATP and 2 mM MgCl₂ (final concentrations) and incubated at 30 °C for 1 h. Following the incubation period, 30 µL of each sample were transferred to a 384-well plate and irradiated with UV light (365 nm) for 3 min on ice.

Following UV irradiation, 25 µL of each sample were added to 225 µL cold TBS with 0.22% DDM. Proteins were precipitated by adding 250 µL 20% TCA and washed with cold acetone. Precipitates were resuspended in 50 µL Laemmli sample buffer (2x) containing 5% (vol/vol) β-mercaptoethanol and subjected to immunoblotting as described below.
3.12.8  **SDS-PAGE and immunoblotting**

Protein samples in Laemmli sample buffer were loaded onto homemade Tris-HCl 4-20% polyacrylamide gradient gels (SDS-PAGE). After electrophoresis, proteins were transferred onto Immun-Blot PVDF membranes (Biorad) and subjected to immunoblotting with LptA antiserum raised in rabbit (16) and LPS antiserum raised in mouse (HyCult Biotechnology). Blots were probed with donkey anti-rabbit and sheep anti-mouse secondary antibodies conjugated to horseradish peroxidase (GE Amersham). Bands were visualized with ECL Prime Western Blotting Detection Reagent (GE Amersham) on Biomax Light Film (Kodak).

3.12.9  **Efficiency of plating and disk diffusion assays (Ruiz lab)**

All efficiency of plating (described in text) and disk diffusion assays (1) were conducted in the Ruiz lab. Strains included in the figures are listed in Tables 3.3 and 3.4. Plasmid pET23/42-LptB was constructed in ref. (1).

### Table 3.3  **lptB1 and suppressor strains (N. Ruiz)**

<table>
<thead>
<tr>
<th>Strain name</th>
<th>Genotype</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR754</td>
<td>MC4100 ara+</td>
<td>wild-type strain</td>
</tr>
<tr>
<td>NR1768</td>
<td>lptB1</td>
<td>lptB1 allele encodes protein sequence depicted in Fig. 3.1b</td>
</tr>
<tr>
<td>NR1963</td>
<td>lptB1/R144H</td>
<td>selected on novobiocin (33 µg/mL) plates</td>
</tr>
<tr>
<td>NR3025</td>
<td>lptB1/T45A</td>
<td>selected on vancomycin (75 µg/mL) plates</td>
</tr>
</tbody>
</table>

### Table 3.4  **LptB variant strains (B. Simpson)**

<table>
<thead>
<tr>
<th>Strain name</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>lptB (wt)</td>
<td>NR754 tet2 ΔlptB (pET23/42-LptB)</td>
</tr>
<tr>
<td>lptB/R144H</td>
<td>NR754 tet2 ΔlptB (pET23/42-LptB/R144H)</td>
</tr>
<tr>
<td>lptB/L93F</td>
<td>NR754 tet2 ΔlptB (pET23/42-LptB/L93F)</td>
</tr>
</tbody>
</table>

3.12.10  **ATPase assay of the complex (D. Sherman)***
The ATPase activity of LptB2FGC with C- and N-terminal histidine tags on LptB was assessed by Dr. David Sherman using the protocol described in ref. (7). Complex was purified using plasmids pCDFDuet-his6-LptB-LptFG and pCDFDuet-LptB-his8-LptB-LptFG with pET22/42-LptC according to the protocol described in ref. (1).

3.13 References


Chapter 4

Structure of novobiocin bound to LptB
4.1 Introduction

In the previous chapter, I introduced genetic and biochemical evidence that the gyrase inhibitor novobiocin interacts with LptB2FGC, the ABC transporter that powers LPS transport. Although at that point we did not know how novobiocin interacts with the complex, we hypothesized that it may be interacting with LptB, the nucleotide-binding domain of the transporter. Here, we report the co-crystal structure of novobiocin bound to LptB. This is the first demonstration of a direct interaction between a small molecule and a component of the Lpt pathway.

In the sections that follow, I present the novobiocin-binding site, as well as genetic evidence that this site is physiologically relevant. I discuss the conservation of this site across ABC transporters and put forth three models for considering how novobiocin might affect the activity of LptB2FGC. The structural work was done by me in collaboration with Dr. David Sherman and Dr. Michael Lazarus in the Kahne lab. The genetic experiments were conducted by Professor Natividad Ruiz and her student, Brent Simpson.

4.2 Novobiocin binds at the crystallographic dimer interface of LptB

We soaked novobiocin into LptB-ADP crystals obtained under conditions reported by Sherman and collaborators (1). Under these conditions, LptB crystallizes as a dimer with ADP bound at each active site. The dimer interface is formed by each monomer’s hydrophobic groove region, which interacts with transmembrane domains LptF/G. We obtained a 2.0-Å structure containing unambiguous density for two novobiocin molecules bound symmetrically at the dimer interface (Figure 4.1A). The ADP was not displaced, consistent with the observation that novobiocin does not inhibit the activity of the complex.
As will be discussed further in the Appendix, the novobiocin-binding site in LptB bears no similarity to the novobiocin-binding site in the GyrB, its known target (2, 3). LptB and GyrB are not homologous, and the novobiocin-binding site in GyrB partially overlaps the ATP-binding site, which is not the case in LptB. The LptB-ADP-NOV crystal structure presents a novel protein-ligand interaction.

Figure 4.1  Novobiocin binds to LptB. a) Crystals of LptB-ADP were grown as reported previously and soaked with novobiocin. Two molecules of novobiocin were found in a 2.0-Å structure (LptB-ADP-NOV) bound symmetrically at the dimer interface. b) As each LptB monomer contacts both novobiocin molecules, there are two potential novobiocin-binding sites (nov1 and nov2).

4.3 The nov1 site is the relevant binding site

Because each LptB monomer contacts both novobiocin molecules, there are two novobiocin-binding sites in the crystal structure, both located near the groove region (Figure 4.1B). Both sites make important contacts with the LptB monomer. To determine which site is the correct site, we consulted crystal structures of intact ABC systems, such as the maltose transporter (MalK2FG) solved by the Chen lab (Figure 4.2A) (4). In this structure, the groove is
almost completely filled with helices, termed coupling helices, from the transmembrane domains (Figure 4.2B) (5). In the LptB-ADP-NOV structure, one binding site (nov1, Figure 4.2B) is located to the side of the hydrophobic groove, while the other (nov2, Figure 4.2B) fills the groove. As complete disruption of the interaction between LptB and the coupling helices of LptF/G would most likely inhibit, rather than activate, LPS release activity, we favor the nov1 binding site as the correct site. Additional evidence supporting the choice of the nov1 binding site is presented in section 4.5.

Figure 4.2 Comparisons with other ABC systems indicate that the nov1 site is the relevant binding site. a) Structure of an intact ABC system: the maltose transporter (4). (PDB: 3PV0) b) Comparison of the groove region of the maltose transporter with that of LptB-ADP-NOV. Tyr87 of MalK structurally aligns with Phe90 of LptB. The nov1 site is positioned to the side of the groove that normally accommodates the transmembrane domain coupling helices. The nov2 site occludes the groove.

4.4 The nov1 binding site is positioned in a region important for mediating interactions with LptF/G

The nov1 binding site is located in the Q-loop near the helical subdomain, a region involved in interactions with the transmembrane domains (6, 7). Remarkably, novobiocin interacts with a residue in this region that has been identified as essential for proper complex
assembly: F90. As shown in Figure 4.3A, F90 participates in hydrophobic interactions with the coumarin ring of novobiocin.

Sherman and collaborators found that the aromatic side chain of F90 is essential for LPS transport. The F90A variant is nonfunctional in vivo, as it cannot complement the loss of the wild-type \textit{lptB} allele (1). A more conservative substitution, F90Y, is functional in vivo, but haploid strains with this variant display permeability defects (1). F90 variants are compromised not in catalytic activity, but in complex formation. Although LptB/F90A-his possesses wild-type levels of ATPase activity, LptB/F90A-his does not pull down the other components of the complex in pull-down assays (1).

Figure 4.3 Novobiocin binds in a region that mediates interactions with LptF/G. a) The coumarin moiety of novobiocin is involved in hydrophobic interactions with F90, previously shown to be important for function in the groove region (1). Density contouring depicted at 1.9 sigma. b) LptB/F90pBpa crosslinks to LptF in vitro. IM complex was purified with photocrosslinkable amino acid \textit{p}Bpa substituted at position 90 (his-LptB/F90pBpa-LptFGC) and irradiated with UV light for 10 min. Western blots with both \(\alpha\)-LptB and \(\alpha\)-LptF antibodies show a UV-dependent, higher molecular weight band, indicating crosslinking occurred. Crosslinking may also have occurred between LptB and LptG, but was difficult to assess due to the lack of a specific LptG antibody.

Recently, we have discovered that F90 interacts directly with the transmembrane domains LptF/G. As depicted in Figure 4.2B, F90 faces into the coupling helix groove. Upon
substituting the photo-crosslinkable amino acid \( \rho \text{Bpa} \) into various positions in LptB (8), Brent Simpson detected crosslinking between LptB/F90\( \rho \text{Bpa} \) and both LptF and LptG in vivo (data not shown). To enable identification of the LptF/G residues crosslinked to LptB by mass spectrometry (a project continued by Tristan Owens), I attempted crosslinking at the F90 position in vitro. UV irradiation of purified his-LptB/F90\( \rho \text{Bpa} \)-LptFG generated a high molecular weight band visible on both anti-LptB and anti-LptF western blots, indicating crosslinking occurred between LptB and LptF (Figure 4.3B). These in vivo and in vitro crosslinking studies indicate that F90 interacts directly with LptF/G.

Given that F90 forms a direct, essential interaction with LptF/G, it is remarkable that novobiocin binds directly above it. It is likely that novobiocin interacts directly with LptF and/or LptG in addition to LptB.

4.5 The nov1 binding site accounts for novobiocin’s ability to suppress defects in LptF/G

Additional evidence for the validity of the nov1 binding site at the LptB-LptF/G interface has emerged from studies of the coupling between LptB and LptF/G. The Ruiz lab identified the LptF/G coupling helices by searching for residues predicted to be in cytoplasmic helices conserved across species in both LptF and LptG. They have since substituted \( \rho \text{Bpa} \) into these putative coupling helices and demonstrated that they crosslink back to LptB. These coupling helices each contain an absolutely conserved glutamate: E84 in LptF and E88 in LptG. As shown in Figure 4.4A, simultaneous mutation of these glutamates to alanines is lethal (\( \text{lptFG}^* = \text{lptF/E84A-lptG/E88A} \)). This lethality can be suppressed by the allele \( \text{lptB/R91S} \) (Figure 4.4A), as well as the R91C and R91H variants (data not shown).
Novobiocin also interacts with R91. As shown in Figure 4.4B, the benzamide ring of novobiocin participates in cation-pi interactions with R91 (Figure 4.4B) (9). As this interaction significantly alters the orientation of the R91 sidechain, we wondered whether novobiocin also could suppress the lethality of \textit{lptFG*}. Indeed, it can (Figure 4.4A). Novobiocin’s ability to phenocopy \textit{lptB/R91S} is significant for two reasons. First, it demonstrates that novobiocin can suppress mutations in LptF/G as well as in LptB. Second, it indicates that the binding we see in the crystal structure is relevant to the activity observed in vivo. I consider each point below.

\textbf{Figure 4.4}  Novobiocin affects interactions between LptB and LptF/G. a) Efficiency of plating \textit{lptFG (wt)}, \textit{lptB-R91S}, coupling helix mutant \textit{lptFG*}, and \textit{lptFG*}/\textit{lptB-R91S} on LB media with and without novobiocin (5 µg/mL). Spots range from undiluted overnight culture to $10^{-6}$ dilution. (Data from B. Simpson.) b) The benzamide ring of novobiocin participates in cation-pi interactions (dashed lines) with Arg91. Density contouring depicted at 1.9 sigma.

As before with defects in LptB, novobiocin’s ability to suppress defects in LptF/G is allele-specific. The Ruiz lab found that novobiocin is capable of suppressing \textit{lptF/E84D-lptG/E88D} in a narrow concentration range but can never suppress \textit{lptF/E84R-lptG/E88R} (data not shown). Similarly \textit{lptB/R91S} can suppress \textit{lptF/E84D-lptG/E88D} only in minimal media and can never suppress \textit{lptF/E84R-lptG/E88R} (data not shown).
It is unknown how novobiocin and mutations in R91 suppress coupling helix defects. R91 is well conserved across species, although the Ruiz lab has found that substitution with A, E, K, or S confers no defects (data not shown) (1). Simpson also found that LptB/R91pBpa can crosslink to LptF and/or LptG in vivo, although the identities of the crosslinked residues are unknown. In the LptB-ATP and LptB-ADP structures, R91 can participate in a hydrogen bond with Q136, while in the LptB-ADP-NOV structure it cannot. To assess whether this hydrogen bond is important, the Ruiz lab is currently investigating whether mutations in Q136 can also suppress lptFG* in vivo. It may also be informative to study the coupling helix mutant with the LPS release assays described in Chapter 3.

The discovery that novobiocin can phenocopy a mutation in its binding site speaks to an important question posed in Chapter 3: is novobiocin’s ability to suppress Lpt defects in vivo an indirect consequence of inhibition of its known target, DNA gyrase? In Chapter 3, we show that nalidixic acid, another gyase inhibitor, cannot suppress Lpt defects, suggesting that Lpt suppression is not mediated through gyrase. This experiment is complicated, though, by the fact that nalidixic acid and novobiocin target different subunits of DNA gyrase (10). The observation that novobiocin mimics mutations at residue R91 suggests that novobiocin suppresses Lpt defects via a direct interaction with LptB. Taken together, the genetic, biochemical, and structural data presented so far strongly support a model in which novobiocin binds to LptB in vivo. Ultimately, however, to prove this point it will be desirable to obtain novobiocin derivatives that act on LptB but not gyrase.

4.6 How conserved is the nov1 binding site across ABC transporters?
As discussed in Chapter 2, both human and bacterial ABC transporters are important targets in drug development. The pharmaceutical industry has worked extensively to inhibit the major human efflux pumps that contribute to multidrug resistance in cancer (11). More recently, the essential ABC systems in Gram-negative bacteria have emerged as targets in the search for new antibiotics (12, 13). As nucleotide-binding domains are relatively well conserved (14), we wondered whether novobiocin could also interact with these important systems. Is the novobiocin-binding site present in these transporters?

It should be noted that because novobiocin binds at the interface between LptB and LptF/G, we currently only have a picture for half of the binding site. Novobiocin presumably makes other important contacts with LptF and/or LptG. We also do not yet know which residues in LptB are essential for the interaction. We are currently testing point mutations in the vicinity of the nov1 binding site for the ability to diminish novobiocin’s in vitro or in vivo activity. With these caveats in mind, I employed residues F90, R91, and R92 as a proxy for the nov1 binding site. In LptB, F90 and R91 are almost universally conserved across species, although R92 is more variable (1).

Figure 4.7 excerpts an alignment of diverse nucleotide-binding domains, including those from the essential Gram-negative transporters (LptB2FGC, LolCDE, MsbA, and FtsE), crystallized Gram-negative importers (MalK, BtuD, MetN, ModC), and the major human multidrug efflux pumps (Pgp, MRP1, and BCRP). This sequence alignment differs slightly from structural alignments; for example, F427 from Sav1866 aligns structurally with F90 from LptB, not R91 as predicted by sequence (1). It is clear, however, that the region around the nov1 binding site, unlike the Walker A motif, is not conserved in the other essential Gram-negative transporters or in the human multidrug efflux systems.
Novobiocin binds to LptB in a region with relatively low sequence conservation across ABC systems. Novobiocin-binding site highlighted with a black box. Sequence alignment performed with Clustal Omega (15, 16); coloring added in Jalview (17). Accession numbers: LptB (E. coli, NP_417668.1), LoID (E. coli, NP_415635.4), MsbA (E. coli, NP_415434.1), MalK (E. coli, NP_418459.1), BtuD (E. coli, NP_416224.1), MetN (E. coli, NP_414741.1), ModC (E. coli, NP_415286.1), CysA (E. coli, NP_416917.1), LivG (E. coli, NP_417912.1), FtsE (E. coli, NP_417920.1), Sav1866 (S. aureus, Q99T13.1), Pgp (H. sapiens, NP_000918.2), MRP1 (H. sapiens, NP_004987.2), BCRP (H. sapiens, NP_004818.2), CFTR (H. sapiens, NP_000483.3).

That said, the nov1 site may be present in some transporters. In addition to the well-studied ABC systems cherry-picked above, I also included two nucleotide-binding domains found in a BLAST search for E. coli proteins similar to LptB: CysA and LivG, involved in sulfate and branched-chain amino acid uptake, respectively (18). These are the only E. coli nucleotide-binding domains I found to contain analogs of both F90 and R91. Interestingly, mutations in cysteine biosynthesis and RNA synthetase genes have been found to confer resistance to novobiocin in E. coli through an unknown mechanism (19, 20). One of the
implicated genes, \textit{cysB}, is a transcriptional regulator of \textit{cysA} (21). It is tempting to speculate that novobiocin may also interact directly with CysA and/or LivG.

Because novobiocin binds in the helical subdomain, also called the variable region, it is unsurprising that its binding site may be present in closely- but not distantly- related transporters. In the past, nucleotide-binding domains have been deemed challenging to target with specificity due to their high degree of conservation. The fact that novobiocin binds in the variable helical subdomain, and not the conserved RecA subdomain, may be advantageous in future attempts to optimize its antibiotic activity.

4.7 Novobiocin binding induces conformational changes in the helical subdomain

The data presented above indicate that novobiocin binds directly to LptB at the LptF/G interface. This result raises many additional questions, perhaps most importantly: how does novobiocin affect the activity of the entire transporter (LptB$_2$FGC)? In vivo, novobiocin suppresses the lethality of mutations near the groove region of LptB as well as in the coupling helices of LptF/G. In biochemical reconstitutions, novobiocin accelerates LPS transfer to LptA from wild-type LptB$_2$FGC. Does the LptB-ADP-NOV structure shed light on the mechanism behind these observations?

It is important to note that the LptB-ADP-NOV structure – which lacks the transmembrane domains – gives an incomplete picture of the interaction between novobiocin and the IM complex. LptB was also crystallized with a C-terminal tag, which confers ATPase defects and might alter some of the changes caused by novobiocin binding. That being said, comparison of the LptB-ADP structures with and without novobiocin is instructive. In Figure 4.5, LptB-ADP-NOV (red) is aligned with LptB-ADP (gray) by the Walker A motif. While the
RecA subdomain is relatively constant between the two structures, in the novobiocin structure the helical subdomain shifts up, in the direction of the transmembrane domains. Sherman and collaborators, by comparing LptB-ATP structures with the LptB-ADP structures, have found that helical subdomain shifts down upon ATP hydrolysis (1). This motion is thought to drive the movement of the transmembrane domains. Perturbation of the interaction between the RecA and helical subdomains could affect the ABC system in multiple ways, discussed below.

**Figure 4.6** Novobiocin binding shifts the helical subdomain relative to the RecA-like subdomain. a) Cartoon representation of LptB-ADP, with helices depicted as cylinders. Secondary structure assigned by Pymol (22). PDB: 4P32. b) Ribbon representation of LptB-ADP (gray) aligned with LptB-ADP-NOV (red) by the Walker A region (residues G36 – T43).

4.8 Three models for how novobiocin might affect LptB2FGC activity

How might novobiocin-mediated changes in the helical subdomain affect LPS release? Figure 4.6 presents a theoretical framework based on the alternating access model for considering how novobiocin could affect the Lpt transporter (6, 7). Here, I have deconstructed LPS release into three connected but distinct processes: complex formation, ATP binding and
hydrolysis, and transmembrane domain movement. Each process could be affected by changes in the helical subdomain, which not only interacts with transmembrane domains, but also contains the ATP-binding signature motif. Although novobiocin may influence multiple processes simultaneously, I discuss each separately below:

    a) Complex formation. Although the mechanism by which the LptB₂FGC complex assembles is not completely understood, it is known that mutations in lptB (i.e. F90A) can prevent proper interactions with LptF/G. While wild-type complexes are fairly stable, it is conceivable that some of the groove mutations described in Chapter 3, or the coupling helix mutant described above, could behave similarly to the F90 variant. Perhaps novobiocin facilitates complex formation for these mutants. It may be possible to test this hypothesis using a pull-down assay with a tag on LptB, as developed by Sherman and collaborators (1).

    b) ATP binding and hydrolysis. Novobiocin’s ability to accelerate LPS release from wild-type complexes could be caused by an increase in the rate at which ATP is bound and hydrolyzed. To test this hypothesis, it will be necessary to measure how novobiocin affects the ATPase activity of the complex. Ideally this experiment would be conducted in proteoliposomes with N-terminally tagged complex, as the C-terminal tag confers ATPase defects (discussed in Chapter 3).

    c) Transmembrane domain movement. Conversely, novobiocin could accelerate LPS release by increasing the number of LPS molecules that are extracted per round of ATP hydrolysis. Without changing the rate of ATP hydrolysis, novobiocin could alter the conformational changes in the transmembrane domains that accompany hydrolysis. Again, it will be necessary to measure the rate of ATP hydrolysis to test this hypothesis.
Figure 4.7  Framework for considering how novobiocin could affect LPS release, based on the alternating access model described in Chapter 2 (6, 7). Novobiocin could affect a) complex formation, b) the rate of ATP binding and/or hydrolysis, or c) transmembrane domain movement, which dictates the number of LPS molecules transported per hydrolysis cycle. Novobiocin could affect some or all of these aspects of transport.

To summarize, although the in vitro data discussed in Chapter 3 indicates that novobiocin activates LPS release from wild-type complexes, we have not yet been able to tease apart its effect on ATP hydrolysis versus transmembrane domain movement. Even less is understood
about the groove region and coupling helix variants suppressed by novobiocin (e.g. LptB/R144H and LptF/E84A-LptG/E88A) in vivo, as we have yet to observe novobiocin affect the activity of these variants in vitro. How can novobiocin, which accelerates LPS release for wild-type complexes, suppress the R144H variant, which appears to have increased basal levels of LPS release? Are these results not representative of the systems in vivo, or does novobiocin exert different effects on wild-type versus mutant complexes? Additional biochemical work is needed to establish how novobiocin suppresses groove region and coupling helix variants.

4.9 Conclusion

In this chapter, we present the crystal structure of novobiocin bound to LptB. This result strongly suggests, if not proves, that novobiocin’s ability to activate LPS release in vitro and suppress Lpt defects in vivo is due to its direct interaction with LptB. Although the genetic and biochemical evidence presented in Chapter 3 suggested that novobiocin affects LPS transport, the crystal structure was key in establishing that novobiocin interacts directly with LptB.

Our current understanding of the novobiocin-LptB interaction is depicted in Figure 4.8. Novobiocin binds in the groove region at the interface between LptB and LptF and/or LptG. For wild-type complexes, novobiocin-binding accelerates LPS release. The mechanism by which it affects LPS release is not known and could involve changes in complex formation, ATP hydrolysis, and/or transmembrane domain movement.

Although we have no evidence that novobiocin’s interaction with LptB is detrimental to wild-type cells, it may be possible to exploit this interaction to disrupt LPS transport. Because genetic disruption of the interaction between LptB and LptF/G is lethal, Sherman and collaborators proposed in 2014 that targeting this interface may be a sound strategy for
developing new antibiotics. In this vein, novobiocin may serve as springboard for the design of new antibiotics, discussed in the appendix.

Figure 4.8 Model: novobiocin binds at the LptB-LptF and/or LptB-LptG interface and accelerates hand-off to LptA.

4.10 Dissertation summary

There is a critical need for new antibiotics effective against Gram-negative bacteria, which are characterized by an outer membrane that prevents the entry of many antibiotics. Proper assembly of the outer membrane requires transport of lipopolysaccharide (LPS) from the inner membrane, where its synthesis is completed, to the outer membrane. LPS transport is powered by the essential complex LptB$_2$FGC, a member of the ATP-binding cassette transporter superfamily, which couples the energy of ATP hydrolysis to transmembrane processes. In this dissertation, I present biochemical and structural evidence that the natural product antibiotic novobiocin interacts with LptB, the nucleotide-binding domain of the transporter. I summarize this work below.

This project began with the observation that novobiocin, an antibiotic which normally cannot penetrate the outer membrane, can suppress lethal defects in LPS transport in *E. coli* –
work performed in the Ruiz lab. This activity does not appear to be mediated via novobiocin’s interaction with its known target, DNA gyrase, because other gyrase-inhibiting antibiotics do not have a similar effect. Intrigued by this result, I worked with other Kahne lab members to assess whether novobiocin affects LPS-transport in in vitro reconstitutions of LptB2FGC activity. Indeed, we found that novobiocin surprisingly accelerates, rather than inhibits, LPS-transport from wild-type complexes. This result indicated that novobiocin interacts directly with LptB2FGC.

Although the nature of this interaction was unclear, we hypothesized that novobiocin may be interacting with LptB. I soaked novobiocin into LptB-ADP crystals and obtained a co-crystal structure. The crystal structure strongly suggests, if not proves, that novobiocin interacts directly with LptB. Novobiocin binds to LptB at the interface with transmembrane domains LptF/G. Because novobiocin can phenocopy mutations in this region, we are reasonably confident that novobiocin’s ability to suppress LPS-transport defects in vivo is derived from its interaction with LptB. While novobiocin itself does not have antibacterial activity against Gram-negative bacteria, we may be able to design analogs that do. Because novobiocin’s binding interaction with LptB is significantly different from its interaction with gyrase, it could be possible to modify novobiocin to maintain activity on both targets.

This work will be of interest to the outer membrane, ABC transporter, and novobiocin communities. Novobiocin is the first small molecule demonstrated to interact directly with a component of the Lpt pathway, as well as the first non-substrate small molecule crystallized with the nucleotide-binding domain of an ABC transporter. Going forward, this work raises a number of questions: How does novobiocin affect LPS release for wild-type and defective complexes? Does it alter complex formation, ATP binding and hydrolysis, transmembrane domain
movement, or some other aspect of the catalytic cycle? Can we design novobiocin analogs that sufficiently disrupt LPS transport to facilitate their own uptake or the uptake of other antibiotics?

The majority of antibiotics in clinical use today are natural products or their derivatives (23). It is tempting to speculate that the LptB-novobiocin interaction is not a coincidence, but a product of nature’s attempt to break down the outer membrane barrier. Regardless, the work presented here validates LptB as a target in the search for new antibiotics effective against Gram-negative bacteria.

4.11 Materials and Methods

4.10.1 Strains and materials

*E. coli* strain Nova Blue [endA1 hsdR17 (rK12− mK12+) supE44 thi-1 recA1 gyrA96 relA1 lac F’[proA+ B+ lacI(qZ)ΔM15::Tn10] (TetR)] from Novagen was used for plasmid manipulations. *E. coli* strain KRX [F’, traD36, ΔompP, proA+B+, lacI(q), Δ(lacZ)M15] ΔompT, endA1, recA1, gyrA96 (Nalr), thi-1, hsdR17 (rK−, mK+), e14− (McrA), relA1, supE44, Δ(lac-proAB), Δ(rhaBAD)::T7 RNA polymerase] from Promega was used for protein purification.

The plasmids are used in this study are listed in Table 4.1. For photocrosslinking experiments, an lptB/F90Am mutation was installed into pCDFDuet-his6-LptB-LptFG by site-directed mutagenesis with primers purchased from Integrated DNA technologies (Table 4.2). Mutagenesis was confirmed with DNA sequencing by Beckman Coulter. PCR amplification was performed with KOD Hot Start DNA polymerase from Novagen. Restriction enzyme DpnI was purchased from New England Biolabs. All other materials were purchased from Sigma Aldrich unless otherwise noted.
Table 4.1  **Plasmids used in this study**

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<tr>
<th>Name</th>
<th>Description</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>pET22/42-LptB-his8</td>
<td>encodes LptB with a C-terminal his8 tag</td>
<td>(12)</td>
</tr>
<tr>
<td>pCDFDuet-his6-LptB-LptFG</td>
<td>encodes LptB with an N-terminal his6 tag in site 1 and untagged LptFG in site 2</td>
<td>(1)</td>
</tr>
<tr>
<td>pET22/42-LptC</td>
<td>encodes untagged LptC</td>
<td>(1)</td>
</tr>
<tr>
<td>pSup-BpaRS-6TRN</td>
<td>encodes tRNA and aminoacyl-tRNA synthetase to incorporate pBpa at amber stop codons (TAG)</td>
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Table 4.2  **Site-directed mutagenesis primers**

<table>
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<tr>
<th>AA change</th>
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<th>Primer sequence (5’ to 3’)</th>
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</thead>
<tbody>
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<td>F90Am*</td>
<td>LptB-F90Am-f</td>
<td>CAG GAA GCC TCC ATT TAG CGT CGC CTC</td>
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<tr>
<td></td>
<td>LptB-F90Am-r</td>
<td>AAC GCT GAG GCG ACG CTA AAT GGA GG</td>
</tr>
</tbody>
</table>

*Primers designed by David Sherman

4.10.2  **LptB-his overexpression and purification for crystallography**

LptB-his (full-length LptB with a C-terminal his8 tag) was purified as described in ref. (1) with minor modifications. Overnight cultures of KRX cells (Promega) transformed with plasmid pET22/42-LptB-his8 were diluted 100x into LB Miller media containing 50 µg/mL carbenicillin. Cultures were grown at 37 °C, 220 rpm to OD 0.8, at which point the temperature was reduced to 16 °C. Following 30 min of shaking at 16 °C, overexpression was induced with 0.2% L-rhamnose. Cultures were grown at 16 °C, 220 rpm for 14 h.

Cells were harvested by centrifugation at 5000 xg, 4 °C for 20 min. Pellet was resuspended in Buffer A: Tris-buffered saline (TBS; 20 mM Tris [pH 8.0], 150 mM NaCl), 20% (vol/vol) glycerol, and 0.5 mM Tris(3-hydroxypropyl)phosphine (THP: EMD Milipore). To facilitate lysis, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 50 µg/mL lysozyme, and 50 µg/mL DNAse I were added to the cell suspension. Cells were lysed 3x through a high-pressure cell disruptor. Unbroken cells were removed by centrifugation at 6000 xg, 4 °C for 10 min. To pellet membranes, cell lysate was centrifuged at 100,000 xg, 4 °C for 30 min. Membranes were discarded and 10 mM imidazole was added to the supernatant.
In preparation for nickel affinity chromatography, Ni-NTA Superflow resin (Qiagen) was washed with water and equilibrated with Buffer A supplemented with 10 mM imidazole. Cell lysate supernatant was incubated with equilibrated Ni-NTA resin at 4 °C for 1 h with gentle rocking. Following incubation, flow-through was removed and resin was washed with 20 column volumes of Buffer A with 20 mM imidazole. Protein was eluted in one batch with 2.7 column volumes of Buffer A with 200 mM imidazole. Eluate was concentrated in a 10-kDa molecular weight cut-off (MWCO) centrifugation filter (Amicon; Millipore) to ~50 mg/mL and flash frozen.

Protein was further purified by size exclusion chromatography on Superdex 200 10/30 GL column in Buffer A. Fractions containing protein were pooled and concentrated in a 10-kDa MWCO centrifugation filter to ~50 mg/mL. Protein aliquots were flash-frozen and stored at -80 °C. Protein concentration was measured using the Biorad DC protein assay.

4.10.3  LptB-his crystallization and novobiocin soak

LptB-his was crystallized using conditions described in ref. (1). Purified LptB-his was diluted into Buffer A to a concentration of 20 mg/mL. The 20 mg/mL stock was diluted 2x into TBS, yielding a final protein concentration of 10 mg/mL and glycerol concentration of 10%. This solution was incubated with 2.5 mM ATP and 2.5 mM MgCl₂ for 1 h on ice before setting up drops.

Crystals were grown by vapor diffusion in hanging drops at room temperature. 1 μL protein solution was mixed with 1 μL reservoir solution consisting of 100 mM MES (pH 6.5) and 30% (wt/vol) PEG 4000. As observed in ref. (1), flat, triangular crystals appeared after several days.
For the novobiocin soaks, crystals were transferred to 2-µL drops of reservoir solution (100 mM MES (pH 6.5), 30% PEG 4000) containing 2.5 mM novobiocin sodium salt. Crystals were soaked for ~90 min at room temperature and then flash-frozen in cryoprotectant containing 100 mM MES (pH 6.5), 33% PEG 4000, 24% glycerol, and 2.5 mM novobiocin.

4.10.4 Crystallography data collection

The X-ray diffraction data for the LptB-ADP-NOV crystals were collected at 0.97918 Å at 24-ID-E of the Advanced Photon Source at Argonne National Laboratory. LptB-ADP-NOV belongs to the space group \( \text{C}_{121} \).

4.10.5 Crystallography data processing and structure determination

The dataset was indexed and integrated using iMosflm (24) and scaled using the CCP4 (25) program AIMLESS (26). The structure was solved by molecular replacement with Phaser (27) using the complete LptB-ADP structure from ref. (1) as a search model (PDB: 4P32). Initial rounds of refinement in Phenix (28-30) were performed with rigid body refinement, simulated annealing, and ADP (atomic displacement parameter or \( B \)-factor) refinement, yielding a model with \( R_{\text{free}} \) and \( R_{\text{work}} \) values of 29.9% and 25.0%, respectively. This model contained clear unassigned density at the LptB dimer interface.

Following manual placement of novobiocin into the unassigned density in COOT (31), the model was further refined in Phenix with cycles of minimization, simulated annealing, and ADP refinement, interspersed with manual editing in COOT. Waters and magnesium ions were placed, and the refinement was completed using cycles of minimization, ADP refinement, and translation/libration/screw (TLS) refinement with TLS parameters from the TLS motion.
determination server (32). Ligand restraints were generated using the Mogul geometry optimization in eLBOW (33), and coordination sphere restraints were generated with ReadySet (30). The $R_{\text{free}}$ and $R_{\text{work}}$ values for the final LptB-ADP-NOV structure are 22.0% and 17.9%, respectively.

Much of the software used in this project was installed and configured by SBGrid (34). Figures were prepared using Pymol (22).

Table 4.3 Data collection and refinement statistics

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<th>Data Set</th>
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</tr>
<tr>
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<tr>
<td>Angles ($\alpha$, $\beta$, $\gamma$), °</td>
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<tr>
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<tr>
<td>Resolution range, Å</td>
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<tr>
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<td>Completeness, %</td>
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<tr>
<td>Multiplicity</td>
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<td>Refinement*</td>
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</tr>
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<tr>
<td>No. of ions</td>
<td>2</td>
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<tr>
<td>Average $B$-factor, Å$^2$</td>
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<tr>
<td>Protein</td>
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<td>Ligands</td>
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<td>Solvent</td>
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<td>Disallowed, %</td>
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<td>Bond lengths, Å</td>
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<td>Bond angles, °</td>
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*Values in parentheses are for the shell with the highest resolution.
For crosslinking studies, his-LptB/F90pBpa-LptFG was purified as described in ref. (1), with minor modifications. Overnight cultures of KRX cells transformed with plasmids pSupBpaRS-6TRN, pCDFDuet-his6-LptB/F90Am-LptFG, and pET22/42-LptC were diluted 100x into LB Miller media containing 30 µg/mL chloramphenicol, 50 µg/mL carbenicillin, 50 µg/mL spectinomycin and 0.5 mM ρBpa (Bachem). Cultures were grown at 37 °C, 220 rpm to OD 0.9, at which point overexpression was induced with 0.02% (wt/vol) L-rhamnose. Cultures were incubated at 37 °C, 220 rpm for 3 h. Cells were harvested by centrifugation at 5000 xg, 4 °C for 20 min. Pellet was resuspended in TBS. Cells were re-pelleted by centrifugation at 8000 xg, 4 °C for 10 min and flash frozen.

Pellet was thawed and resuspended in 50 mM Tris-HCl (pH 7.4) supplemented with 1 mM PMSF, 100 µg/mL lysozyme, and 50 µg/mL DNAse I. Cells were lysed 3x through a high-pressure cell disruptor. Unbroken cells were removed by centrifugation at 5000 xg, 4 °C for 10 min. To pellet membranes, cell lysate was centrifuged at 100,000 xg, 4 °C for 45 min. Membranes were resuspended in 20 mM Tris-HCl (pH 7.4), 300 mM NaCl, 5 mM MgCl₂, and 10% glycerol. Following two rounds of homogenization, membranes were supplemented with 1% (wt/vol) n-dodecyl-β-D-maltopyranoside (DDM, Anatrace) and 2 mM ATP and solubilized at 4 °C for 1 h with gentle rocking.

In preparation for cobalt affinity chromatography, Talon metal affinity resin (Clontech) was washed with water and equilibrated with Buffer B: 20 mM Tris-HCl (pH 7.4), 300 mM NaCl, 10% glycerol, and 0.05% DDM. Membrane suspension was centrifuged at 100,000 xg, 4 °C for 30 min, and supernatant was applied to Talon column 3x. Resin was washed with 20
column volumes of Buffer B and 10 column volumes of Buffer B with 5 mM imidazole. The protein complex was eluted in 3 batches of 3 column volumes of Buffer B with 25 mM imidazole. Eluate was concentrated in a 100-kDa MWCO centrifugation filter to <1 mg/mL and flash frozen.

Protein complex was further purified by size exclusion chromatography on Superdex 200 10/30 GL column in Buffer B. Fractions containing protein complex were pooled and concentrated in a 100-kDa MWCO centrifugation filter to ~1 mg/mL. Aliquots were flash-frozen and stored at -80 °C. Protein concentration was measured using the DC protein assay.

4.10.7  In vitro crosslinking with his-LptB/F90pBpa-LptFG

Purified his-LptB/F90pBpa-LptFG was irradiated with UV light (365 nm) for 10 min on ice. Protein samples with and without UV treatment were diluted 1:1 in Laemmli sample buffer and loaded onto homemade Tris-HCl 4-20% polyacrylamide gradient gels (SDS-PAGE). After electrophoresis, proteins were transferred onto Immun-Blot PVDF membranes (Biorad) and subjected to immunoblotting with LptB (1) and LptF (35) polyclonal antisera raised in rabbit. Following immunoblotting with anti-rabbit-horseradish peroxidase antibodies raised in donkey (GE Amersham), bands were visualized with ECL Prime Western Blotting Detection Reagent (GE Amersham) on Biomax Light Film (Kodak).

4.10.8  Efficiency of plating assays (Ruiz lab)

All of efficiency of plating assays (described in text) were conducted in the Ruiz lab. Strains included in the figures are listed in Table 4.4. The genotype of NR754 is MC4100 ara+. 
Table 4.4 *lptFG* and suppressor strains (B. Simpson)

<table>
<thead>
<tr>
<th>Strain name</th>
<th>Genotype</th>
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<td>lptFG (wt)</td>
<td>NR754 Δ<em>lptFG</em> (pBAD18-LptFG3)</td>
</tr>
<tr>
<td>lptB/R91S</td>
<td>NR754 tet2 lptB*R91S</td>
</tr>
<tr>
<td>lptFG*</td>
<td>NR754 Δ<em>lptFG</em> (pBAD18-LptF/E84A-LptG/E88A)</td>
</tr>
<tr>
<td>lptFG*-lptB/R91S</td>
<td>NR754 tet2 lptB<em>R91S Δ</em>lptFG* (pBAD18-LptF/E84A-LptG/E88A)</td>
</tr>
</tbody>
</table>

4.12 References


22. The Pymol Molecular Graphics System, Version 1.7 Schrodinger, LLC.


Appendix

Novobiocin as a starting point for the development of antibiotics effective against Gram-negative bacteria
A.1 Introduction

In Chapters 3 and 4, I presented genetic, biochemical, and structural evidence that the natural product antibiotic novobiocin interacts with LptB, the nucleotide-binding domain of the ABC transporter that powers lipopolysaccharide (LPS) transport in Gram-negative bacteria. The known target of novobiocin is DNA gyrase. At this point, we have no evidence that LptB is a secondary target of novobiocin. In vitro, novobiocin activates, rather than inhibits, LPS transport. In vivo, novobiocin suppresses defects in LPS transport in *Escherichia coli*. Currently, it is unknown whether mutations in lptB could raise the minimum inhibitory concentration of novobiocin, which would unequivocally demonstrate that novobiocin’s interaction with LptB is detrimental. It may, however, be possible to generate novobiocin analogs that do have significant, deleterious interactions with LptB.

This appendix considers novobiocin’s potential to serve as a starting point for the development of new antibiotics effective against Gram-negative bacteria. As discussed in Chapters 1 and 3, LptB is an excellent target because it is essential and involved in cell envelope biogenesis. Genetic data suggest that even partial inhibition of LPS transport could compromise the integrity of the outer membrane. In this way, LptB-targeting antibiotics could potentiate their own uptake. Furthermore, it may be possible to modify novobiocin to improve its activity against LptB while maintaining activity against DNA gyrase: a dual target strategy. In this chapter, I review our understanding of novobiocin’s interaction with DNA gyrase and discuss the possibility of targeting both gyrase and LptB.

A.2 Clinical history of novobiocin and other aminocoumarins
Novobiocin is an excellent starting scaffold in part because it has already been used in the clinic. A product of the golden era of antibiotics, novobiocin is a natural product effective against Gram-positive, although not Gram-negative, bacteria (1). It was simultaneously discovered by several pharmaceutical companies (Lepetit, Merck, Pfizer, and Upjohn) in 1955-56 and ultimately commercialized by Upjohn primarily for the treatment of penicillin-resistant Staphylococcus aureus, which emerged as a threat in the late 1940s (2-7). It was also employed to a lesser extent against pneumococcal pneumonia in cases in which penicillin could not be administered due to patient allergies (8-10). As a monotherapy, it was approved for both oral and intravenous administration with a typical daily dose of 1-2 grams (11, 12). Novobiocin’s clinical use was hampered by its side effects and the occasional development of resistance during therapy (13, 7, 14, 15). The most common side effect was rash, followed by a low incidence of gastrointestinal intolerance and hematological disorders, although it was proposed later that these side effects might have been caused by impurities (8, 16, 14, 17, 18). During the 1960s-70s, the use of novobiocin declined rapidly following the introduction of other antistaphylococcal antibiotics, including the cephalosporins and beta-lactamase stable penicillins, such as methicillin and oxacillin (10). Upjohn ceased production of novobiocin in 1999, and it was finally withdrawn from the Food and Drug Administration Orange Book in 2011 (19).

Novobiocin is a member of the aminocoumarin family of antibiotics, synthesized by various Streptomyces strains. It consists of a coumarin moiety attached to an unusual sugar (L-noviose) via a glycosidic bond and an aromatic acid via an amide bond (Figure 5.1). Characterization of the biosynthetic pathway revealed that the noviose moiety is derived from D-glucose, while the coumarin and benzamide moieties are derived from tyrosine (20). During the 1960s, two other aminocoumarin antibiotics were discovered: coumermycin A1 (Roche and
Bristol-Myers, 1965) and clorobiocin (Rhone-Poulenc, 1969) (21-23). Coumermycin A1 and clorobiocin have improved potency against both Gram-positive and Gram-negative bacteria (24). Coumermycin A1 is composed of two noviose-coumarin “monomers” asymmetrically bridged by a pyrrole group. It also possesses a pyrrole substituent on the 3-hydroxy group of the noviose instead of a carbamate. Clorobiocin is identical to novobiocin, except it possesses the pyrrole group on the 3-hydroxy group of the noviose and a chlorine atom instead of a methyl group on the coumarin ring.

![Structure of the aminocoumarin antibiotics novobiocin, clorobiocin, and coumermycin A1](image)

**Figure A.1** Structure of the aminocoumarin antibiotics novobiocin, clorobiocin, and coumermycin A1 (1).

Although coumermycin A1 has more antibacterial potency than novobiocin, it has extremely poor aqueous solubility (10). Both Roche and Bristol-Myers terminated clinical development of coumermycin A1 due to poor oral bioavailability and toxicity issues (25-29). Bristol-Myers ran two programs varying the amide group attached to the pyrrole-noviose-coumarin core from coumermycin A1, but were unable to find a compound with properties better than those of novobiocin (30-34). While clorobiocin has good aqueous solubility, clinical work was never initiated, presumably due in part to the disappointing performance of coumermycin
A1 and the declining sales of novobiocin (10, 1). Novobiocin remains the only aminocoumarin antibiotic to progress past Phase I clinical trials (1).

A.3 Type IIA topoisomerases as antibiotic targets

Although it was known in the 1950s that the aminocoumarins inhibit DNA synthesis, their target was unknown until 1976, when Gellert et al. discovered that they inhibit *Escherichia coli* DNA gyrase, a Type IIA topoisomerase (35, 36). As the bacterial chromosome is a covalently closed circular system, DNA processing events, like replication and transcription, produce different topological configurations (*e.g.*, relaxed, positively-supercoiled, negatively-supercoiled, knotted, catenanes). DNA replication, for example, produces positive supercoiling ahead of the replication fork and pre-catenanes behind it (37). These configurations must be interconverted, a task that is performed by the topoisomerases, an essential family of enzymes that acts primarily via supercoiling and decatenation (38). Type II topoisomerases hydrolyze ATP to introduce double-strand breaks in the DNA, pass another DNA strand through the opening, and then re-ligate the broken strand (39). Most bacteria have two essential Type IIA topoisomerases: DNA gyrase, which primarily introduces negative supercoiling during replication, and topoisomerase IV (topo IV), which primarily decatenates DNA (35, 40, 38). In *E. coli*, DNA gyrase and topo IV are ~40% sequence identical (41). Type IIA topoisomerases are tetramers consisting of two DNA-binding proteins (GyrA in DNA gyrase; ParC in topo IV) and two ATP-binding proteins (GyrB and ParE) (42-45).

Essential in all bacteria, Type IIA topoisomerases are excellent antibiotic targets because they have no close homologs in humans (46). They are targeted by two clinically-used classes of antibiotics: the aminocoumarins, which bind to GyrB/ParE, and the quinolones, which bind to
GyrA/ParC (36, 47). When quinolones bind to the GyrA/ParC subunits, they poison the cell by stabilizing the covalent topoisomerase-DNA complex, thereby introducing DNA double-strand breaks (48). In contrast, the aminocoumarins act as competitive inhibitors at the ATP-binding site in GyrB/ParE (46). For the quinolones, in general gyrase is the primary target in Gram-negative bacteria, while topo IV is the primary target in Gram-positive bacteria (49). For the aminocoumarins, gyrase is the primary target for both Gram-negative and Gram-positive bacteria, although topo IV inhibition is also significant, discussed below (50).

Multiple biochemical assays have been developed to assess topoisomerase inhibition, including ATPase, supercoiling, decatenation, and relaxation assays. IC₅₀ values vary significantly depending on the method and conditions used (50). In general, supercoiling assays are considered most appropriate for DNA gyrase, and decatentation assays are preferred for topo IV. Both of these assays are based on the observation that different topological isomers of DNA (i.e. supercoiled versus relaxed and concatenated versus decatenated) run differently on agarose gels. Although a range of values have been observed using these assays, in general novobiocin inhibits gyrase at nanomolar levels and topo IV at micromolar levels, consistent with the assertion that gyrase is the primary target (Table 5.1, data from (24)). Clorobiocin and coumermycin are even more potent inhibitors in vitro (24).

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<th>E. coli</th>
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<tr>
<td></td>
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<td>coumermycin A1</td>
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</tbody>
</table>

| Table A.1 | In vitro and in vivo activity of the aminocoumarins, as measured by Alt and collaborators (24). Gyrase inhibition assessed with supercoiling assay; topo IV inhibition assessed with decatenation assay. MICs measured against S. aureus ATCC 29213 and E. coli ATCC 25922. |
It is worth noting that DNA gyrase is a member of the GHKL superfamily of ATPases, named after DNA gyrase, molecular chaperone heat-shock protein 90 (Hsp90), certain protein kinases, and DNA repair enzyme MutL (51). Novobiocin has been shown to weakly inhibit Hsp90, an important target for cancer therapeutics (52, 53). Analogs have since been developed with more potent activity (54). In developing novobiocin analogs to target LptB, it may be important to consider their effects on other GHKL family members.

A.4 Resistance to aminocoumarins

As discussed above, the clinical use of novobiocin to combat *S. aureus* infections was limited in part by the emergence of resistance. The frequency of spontaneous resistance in *S. aureus* grown with novobiocin at a concentration four-fold higher than the MIC is estimated to be in the range of $10^{-7}$ to $10^{-8}$, a level consistent with the observed clinical resistance (55, 56, 1). Aminocoumarin resistance can arise through multiple routes, most commonly through mutations in the target, *gyrB* (57). As expected, these mutations cluster around the aminocoumarin-binding site (58-60). When coumermycin-resistant mutants are generated spontaneously in *E. coli*, the most frequently mutated residue is R136 (often to His, Cys, or Ser), a site that is also frequently mutated in novobiocin-resistant strains of other species, including *S. aureus* (61-63, 55). The fitness cost of this mutation is significant: purified *E. coli* GyrB bearing an R136 mutation has only 10% of the supercoiling activity (20-50% of the ATPase activity) of the wild-type protein (63). Target-based resistance appears to be limited by the proximity of the novobiocin-binding site to the active site (64, 46).

While GyrB is the primary target of the aminocoumarins in all species, the importance of the second target, ParE, varies. In *S. aureus*, resistance can be generated sequentially in *gyrB*,
then in *parE*, and then in *gyrB* again with increasing novobiocin concentrations (55). This is not the case in *E. coli*. While a cysteine substitution at R132 in *parE* (the homologous site to R136 in *gyrB*) confers resistance to novobiocin in in vitro supercoiling assays, it does not raise the minimum inhibitory concentration for permeabilized *E. coli* strains already containing resistant gyrase (65). Presumably the concentration of novobiocin required to inhibit topo IV in vivo also inhibits the resistant form of gyrase.

Some bacteria become resistant to the aminocoumarins by expressing enzymes that modify the compounds themselves. While the aminocoumarin biosynthetic gene clusters encode for a resistant form of GyrB (20), novobiocin-producing organism *Streptomyces niveus* also can inactivate novobiocin by carbamylating the 2-hydroxy group of the noviose (66). Other bacteria inactivate novobiocin by cleaving the amide bond between the coumarin ring and the benzamide moiety or by hydroxylating the prenyl group (67, 68).

A novobiocin derivative that potently targets both GyrB and LptB may be less susceptible to the development of resistance. Although this dual target strategy would not protect against some forms of resistance, such as drug-modification, the probability of target-based resistance may decrease if mutations are required in both GyrB and LptB.

### A.5 Intrinsic aminocoumarin resistance in Gram-negative bacteria

As discussed in previous chapters, Gram-negative bacteria are intrinsically resistant to moderate concentrations of novobiocin because novobiocin is an efflux pump substrate (69). For example, in *Pseudomonas aeruginosa*, novobiocin facilitates its own efflux by binding a transcriptional repressor of the of the MexAB-OprM multidrug efflux pump (70). Novobiocin-binding promotes dissociation of the repressor from the DNA promoter, thus increasing
expression of the pump. When these pumps are disabled, or the outer membrane is compromised, the MIC of novobiocin drops dramatically. Deletion of the major \textit{E. coli} efflux pump component \textit{acrA} increases the sensitivity to novobiocin 100-fold (71). In Chapter 3, we observed that mutations in \textit{lptB} also sensitize \textit{E. coli} to novobiocin, validating our dual-target approach.

Clorobiocin and coumermycin A1 are much more effective against Gram-negative bacteria (24). The reasons for this are complex, as antibacterial activity depends both on ability to penetrate the cell and affinity for gyrase. The methylpyrrole substituent on noviose enables clorobiocin and coumermycin A1 to bind GyrB more tightly, leading to more potent inhibition in vitro (discussed below). Clorobiocin, however, does appear to be a pump substrate, as permeabilized \textit{E. coli} and \textit{P. aeruginosa} strains show increased sensitivity to clorobiocin compared to wild type (72). At least part of clorobiocin’s improved antibacterial activity against Gram-negative bacteria must then stem from its higher affinity for gyrase.

Interestingly, removal of the major pump component \textit{tolC} does not appreciably alter the minimum inhibitory concentration for coumermycin (Vadim Baidin, personal communication). In Chapter 3, we observed that coumermycin also suppresses Lpt defects in vivo and affects LPS release in vitro. Is coumermycin not a pump substrate, or is it disrupting LPS transport sufficiently to facilitate its own uptake? To address this question, Brent Simpson in the Ruiz lab is currently assessing whether mutations in the novobiocin-binding site of LptB raise the MIC of coumermycin.

A.6 Structure of novobiocin bound to GyrB
The first crystal structure of GyrB, which was of the *E. coli* protein complexed with nonhydrolyzable ATP analog ADPNP, was obtained in 1991 (73). Complexes with novobiocin and clorobiocin were then published in 1996-1997 (74, 75). As expected for a competitive inhibitor, the aminocoumarin binding site partially overlaps the ATP-binding site (Figure 5.2). This binding interaction is clearly different from that observed with LptB in Chapter 4. The most important novobiocin binding interaction is a set of hydrogen bonds formed between the noviose carbamate and D73 (*E. coli* GyrB numbering) with an affiliated water molecule (1). The adenine of ATP also participates in hydrogen bonding interactions with D73; this is the only interaction that is shared between ATP and the aminocoumarins. The coumarin ring hydrogen bonds with R136, a frequently mutated residue in resistant strains. In clorobiocin and coumermycin A1, the noviose carbamate is replaced by a methylpyrrole that not only forms hydrogen bonds with D73, but also engages a hydrophobic pocket formed by V43, A47, V71, and V167. This interaction is thought to be primarily responsible for clorobiocin and coumermycin’s increased potency in vitro (1).

![Figure A.2](image)

**Figure A.2** The aminocoumarin-binding site partially overlaps the ATP-binding site in GyrB. a) Structure of *E. coli* GyrB bound to ANP, a non-hydrolyzable ATP analog (76). PDB: 4WUC. b) Structure *E. coli* GyrB bound to clorobiocin (CBN) (77). PDB: 1KZN. c) Close-up of the aminocoumarin-binding site. D73 interacts with both ATP and the aminocoumarins. R136 hydrogen bonds with the coumarin ring and is commonly mutated in aminocoumarin-resistant strains (1).
Notably, the benzamide moiety of novobiocin and clorobiocin does not appear to make any key contacts with GyrB. In vitro, the benzamide ring is not required for inhibition of _S. aureus_ gyrase (78). It is, however, required for in vivo potency against _S. aureus_, suggesting that it may play a role in penetrating the cytoplasmic membrane (1). The observation that the benzamide ring appears to be unimportant for binding GyrB is central to our dual target strategy, discussed below.

A.7 Strategies for targeting LptB alone and with GyrB

The key to the dual target approach is that novobiocin binds to GyrB and LptB differently. The benzamide moiety, in particular, appears to play a key role in the LptB but not the GyrB interaction. As shown in Chapter 4, this ring forms a cation-pi interaction with R91 and R92 of LptB. It may be possible to alter the benzamide ring to improve activity against LptB without losing activity against GyrB. Michael Mandler in the Kahne lab is currently synthesizing novobiocin derivatives with variations in the benzamide moiety and testing them for antibacterial activity. Compounds with improved antibacterial activity can be characterized with DNA gyrase assays discussed above and LPS release assays described in Chapter 3. We are also developing an assay to assess binding to LptB, and potentially the entire complex, using isothermal titration calorimetry.

Even if activity against GyrB is lost, LptB can be exploited as a target in its own right. It is likely that compounds could be lethal by either inhibiting or activating LPS release. That is, too much flux through the Lpt pathway appears to be as detrimental as too little. For example, the LptB/R144H variant, which is nonfunctional in vivo, is more active than wild type in vitro.
The mechanism behind this lethality is unclear. Perhaps accelerating LPS release wastes ATP and other cellular resources, or disrupts the Lpt bridges. As novobiocin activates LPS release from wild-type complexes in vitro, it may be more straightforward to find analogs that act as activators rather than inhibitors.

A.8 References


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