Cell-Based Discovery Methods Furnish New Compounds Effective Against Gram-Negative Bacteria

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Abstract

Bacterial resistance to antibiotics inevitably results from their clinical use, and we have to continuously develop new antibiotics to stay ahead in this biological arms race. It is particularly important and challenging to develop new antibiotics for Gram-negative bacteria, which possess an outer membrane (OM) with a continuous outward layer of lipopolysaccharide (LPS). This structure forms a permeability barrier that protects bacterial targets from antibiotic engagement, and its assembly requires tens of conserved proteins to cooperate in the biosynthesis and transportation of LPS, many of which are essential for bacterial viability.

The work reported here aims to develop approaches to discover new antibiotics specifically against Gram-negative bacteria. In order to find cell-penetrating compounds and to capture inhibitors of a variety of targets, I focused on cell-based assays instead of *in vitro* methods. Approximately 700,000 compounds were screened for antibacterial activity against *E. coli*, and close to 1,500 compounds were active. Multiple cell-based methods were developed to screen that subset for compounds with activity against promising targets/pathways, including LPS biogenesis.

This thesis discusses in detail one of the cell based assays developed. This particular cell-based filter was based on the assumption that compounds with greater activity against Gram-negative bacteria, which are generally less permeable than Gram-positive bacteria, are likely to

act on targets specific to the former such as those involved in maintaining the outer membrane permeability barrier. We identified a compound with good activity against wt *E. coli* and weak activity against *Acinetobacter baumannii* but no activity against *S. aureus* or *B. subtilis*. Surprisingly, all resistance-conferring mutations in *E. coli* and in *A. baumannii* mapped to the active site of phenylalanine tRNA synthetase. The compound was confirmed to inhibit purified WT enzyme but did not inhibit a purified mutant enzyme. The compound was not active against mammalian cells, but was active against *M. tuberculosis*. In combination, it was shown to prevent resistance to a different tRNA synthetase inhibitor that was demonstrated to be safe in clinical trials, but was withdrawn due to development of resistance. Structure-activity relationship (SAR) studies of the hit compound rapidly led to a five-fold improvement in antibacterial activity and broadened spectrum. Further improvements are expected.

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Acknowledgments

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realized that I was intent on doing my utmost to do all the parts of the work that did not require their participation and would go to great lengths to accommodate their constraints as long as it allowed to screen faster, we were able to establish a productive collaboration.

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faults are more pronounced in some than in others (examples would be inappropriate here), no one is completely free of them.

Prologue: Bacterial infections and antibiotic treatment

The history of humanity is a long record of struggle with many different factors that impede its progress. They include struggle with the ravages of the climate, struggle with the weaknesses of the human body, struggle with the flaws of the human character, to name just a few. The struggles have brought results, as for example replacing the rule of the strong with law and order has restrained many of the flaws of human character. However, the underlying flaws are still there, they still break through occasionally, and constant vigilance is required to maintain what many generations have accomplished.

The human struggle that this thesis is a part of has left its mark across all times, places, and cultures; it is the struggle with bacterial infectious disease. It would not be an overstatement to say that almost any major historical figure had been touched by untimely deaths from bacterial infections. (For example, Abraham Lincoln's fiancée died from Salmonella typhi[1] when he was 26, a death which plunged him into prolonged and profound depression, from which he never completely recovered. That death was not a preventable consequence of his relative poverty at the time: the same happened to Queen Victoria, whose husband died of Salmonella typhi at age 42. Andrew Carnegie's mother and younger brother died from Salmonella typhi[2] when Carnegie was one of the most affluent people in the world, and while his mother was elderly at the time, his brother was not.)

There are many parallels between humanity's struggle with the faults of the human character and its struggle with bacterial infectious disease. First, the triumphs that were accomplished in either are not complete across all places where people live. Just as there are areas of profound lawlessness in this day and age, there also are areas where one's chance of dying from diseases

that are nearly forgotten in some parts of the world is still as great as in remote history. Second, in neither of the areas are accomplishments irreversible: just as crime rates rise in the aftermath of a weakening of law enforcement, so do cases of once-forgotten infectious disease in the aftermath of a lapse in vaccination. Finally, just as the criminal urge constantly explores new possibilities to have its way with impunity even when faced with strong and modern law enforcement apparatus, so do infectious bacteria explore, find, and exploit new ways to ply their trade even in the most advanced healthcare facilities equipped with strong and modern antibacterial treatments. Thus, to retain what we achieved and to make further progress in the field of bacterial infectious disease, we cannot just be content with our achievements and have to keep advancing.

Instances of bacterial infections can be found in recorded history of cultures so remote as Ancient Greece and Ancient Egypt, and later, the Roman Empire[3]. It is pointless to look for references to causal organisms by contemporaries, as the germ theory of disease is only five centuries old, and its admittedly incomplete triumph only took place at the end of the XIX century. However, from the description of symptoms, *M. tuberculosis*, *Y. pestis*, *M. leprae*, and *N. meningitidis* are highly likely to have been the cause of many described cases and epidemics. *T. pallidum* and *V. cholerae* were responsible for more recent epidemics of bacterial infections (XVI and XIX centuries respectively). In addition to these identifiable diseases, many reported cases and epidemics of the past could have been caused by any of a number of bacteria, and the exact causal organism cannot be ascertained.

It is also notable that for most of the recorded history, no effective treatments for infections have been devised, although many superstitions have arisen. There have been several reports of

tetracycline residues in the remains of bones from the first few centuries AD[4,5], but there is no evidence to suggest that they were ingested in response to an infectious disease, or any disease, for that matter. The only effective countermeasure developed has been to quarantine those who were infected. This has been effective to some degree, although in the case of *Y. pestis*, for example, quarantine did not prevent new infections from the fleas of rats.

The last 150 years of progress stand in sharp contrast to the millennia of stagnation in the area of struggle with bacterial infections. A rudimentary form of the germ theory of disease has been formulated by Girolamo Fracastoro in mid-XVI century and bacteria were first seen by Antonie van Leeuwenhoek under the microscope in the late XVII century. Nonetheless, as recently as in 1840s the proposal by Ignaz Semmelweis that puerperal fever was caused by a transmissible agent, a proposal that had been backed by empirical observations, has been met with violent opposition from the medical community, and Semmelweis was committed to an insane asylum, where he died. Only after further work by Louis Pasteur and Robert Koch in the second half of the XIX century was it firmly established that bacterial infections were caused by transmissible particles.

The identification of bacteria as a causal factor in bacterial infections has been tremendously useful in making it possible to develop antibacterial treatments. It has been useful because it allows one to readily assess the efficacy of a large number of compounds as potential treatments either in culture or in simple disease model, as Paul Ehrlich did for syphilis at the turn of the XX century[6]. The compound Arsphenamine, the result of the efforts of Ehrlich, Bertheim, and Sato, was the first compound that could be called an antibiotic. It was commercially available from 1910s and used until the development of penicillin in 1940s.

The time when penicillin was discovered also marked the beginning of discovery and development of multiple other antibiotics. Scientists began to screen soil-dwelling microorganisms for production of antibacterial compounds and to isolate and characterize antibiotics from them. There have also been two major classes of synthetic antibiotics: sulfonamides, that date back to 1930s and fluoroquinolones, which date back to 1960s. However, the approach of screening natural products has yielded most of the antibiotics in clinical use today, and they have been further improved by medicinal chemistry. Since 1970s, however, the rate of discovery of new clinically useful antibiotics has dropped substantially: the antibacterial activity of natural product extracts is commonly found to be due to antibiotics that have already been characterized[7]. In recent years, interest has spiked in looking for new antibacterial producers among organisms that cannot be cultured using normal laboratory techniques[8], and the search has produced one antibiotic candidate, teixobactin[9]. However, we cannot yet conclude with confidence that the antibacterial compounds produced by non-culturable microorganisms are different from the ones that we currently have in either their chemical structures or modes of action. It is so because we do not yet have a large enough sample of the population of antibacterial structures made by non-culturable microorganisms to enable us to draw conclusions. Therefore, it remains to be seen whether that effort will live up to the aspirations of those engaged in it.

Of course, if the set of antibiotics that we already have were sufficient to treat bacterial infectious disease, the diminishing returns of antibiotic discovery, which any human effort inevitably encounters, would be of little consequence. However, in contrast to non-infectious diseases, once a mechanism of resistance to a treatment develops in bacterial disease, it does not limit itself to the patient in whom it arises, does not die with that patient, and does not have to

arise anew in each case. Rather, *because the disease is caused by a transmissible agent, once resistance arises, it can become widespread and firmly established and turn a therapy that once had a miraculous effect into a completely useless procedure*. Antibiotic resistance is made even more of a problem because long times are necessary to determine which antibiotics a particular infection is sensitive and resistant to, which means that treatment often has to start without decent knowledge of how effective it will be in the particular case.

Thus, antibiotic resistance figures prominently in the field of bacterial infectious disease. Studies of how resistance develops and spreads, as well as studies of its costs for bacteria that acquire it, inform us of better ways to use pre-existing antibiotics[10], however, the advance and even the preservation of our position in the field of bacterial infections will ultimately depend on the development of new antibiotics.

This, in turn, raises the question of what these new antibiotics are likely to be like and how we are to discover them. Because any new antibiotic will either employ a new way to target cellular pathways that are already targeted by existing antibiotics, or will target new cellular pathways that no existing antibiotics target, it is worth reviewing the target pathways of antibiotics currently in use.

Essential pathways in a cell can be, with some simplification, divided into those dealing with proteins, lipids, carbohydrates, and nucleic acids. If we examine currently used antibiotics, we find that there are many, perhaps the majority, that target pathways that involve carbohydrate structures (β-lactams and glycopeptides that target peptidoglycan biogenesis), many that target protein-related pathways (tetracyclines, macrolides, aminoglycosides, oxazolidinones, lincosamides, chloramphenicol), and quite a few that target nucleic acids (quinolones,

sulfonamides and antifolates, nitrofurans, rifampicin and its analogues). In contrast, there are very few antibiotics that target lipid-related pathways: daptomycin and polymyxin, triclosan, cerulenin, and platensimycin are about the only ones. Therefore, it would seem that lipid-related pathways are an underutilized class of antibiotic targets.

One can object to the above argument, to the effect that perhaps lipid-related pathways simply are a bad/unpromising class of antibiotic targets. In particular, a common line of reasoning is "if it is such a great target pathway, why did nature not produce many antibiotics to target it". This line of reasoning is misguided: because nature is not a super-organism, nature does not produce any antibiotics. Only some of the organisms that we include in our concept of nature produce antibiotics, and those producer organisms have existed under particular opportunities and constraints, which we know too little about to be able to comprehend[11]. Furthermore, these conditions faced by antibiotic producers in the remote past are sure to be different from ones faced by human antimicrobial therapy today. Perhaps, most obviously, producing antibiotics has surely not been the sole task of the producer organisms; so, it is most likely that any organism that survived and became abundant had limited itself to the production of a few antibiotics that were the sufficient for it under its particular circumstances.

Therefore, it is quite possible that one answer to the antibiotic resistance challenge lies in developing synthetic compounds to target lipid-related pathways for antibacterial treatment, which is the subject of this thesis. In addition to the rather abstract rationale provided in this prologue, there are two specific circumstances that favor the development of lipid-based antibacterial compounds*:* many bacteria have lipid-producing machinery that is not found in

humans, and lipid products produced thereby contribute significantly to bacterial resistance to many antibiotics.

Chapter 1
Role of lipopolysaccharide and the outer membrane in bacterial physiology, pathogenesis, and antibiotic resistance. Its promise as a drug target.

Introduction

Out of the four major chemical components of biological matter (proteins, lipids, carbohydrates, and nucleic acids) lipids play a uniquely definitive role, because the membranes that they comprise are, with some qualifications, the boundary that determines what is part of a given cell, and what is not. This is especially important for organisms like bacteria, which consist of a single cell. Once the membrane ceases to exist in a functional form; so does the organism: while it is possible to propagate bacteria as L-forms lacking a functional peptidoglycan skeleton in highly protective artificial conditions[12], it is impossible to define what propagating bacteria without a membrane could even mean.

Of course, the importance of lipid membranes also applies to higher organisms, including humans, and has implications for antibacterial treatment. The implication is an obvious one, namely, if we are to target lipid membranes in antibacterial treatment, it is important to distinguish between the bacterial and the human membranes in order to kill the bacteria, and not the patient. Therefore, a treatment that simply targets lipid membranes is likely to be harmful for the host.

However, two factors turn the tables in our favor here. Firstly, it is, of course, possible to target pathways of lipid biogenesis, which differ more from bacteria to man than the membranes that they produce. Secondly, and more importantly, bacteria produce a variety of lipid products, which are nowhere to be met with in human cells, and, as this chapter points out the importance of these lipid products in bacterial physiology is far from being merely ornamental.

1.1 Lipopolysaccharide is a prime drug target among lipid products unique to bacteria

Bacteria are the most genetically diverse set of species, and it is therefore not surprising that they produce a wide variety of lipid products that are not found in people. However, in order for a uniquely bacterial lipid product to be a promising drug target, it has to possess two important properties. First, it has to be at least highly important for bacterial growth in a patient if not indispensable for bacterial growth under the vast majority of conditions. Obviously, otherwise, to target it would not be useful for treating infections. Second, it has to be very common among bacteria that cause disease. Because diagnosis of an infection is often slow, and treatment is often preventative, a broad-spectrum drug is preferable to a narrow-spectrum one. Both of these properties are possessed in full by a class of glycolipid molecules known as lipopolysaccharides (LPS). The following sections describe its relevant properties.

1.2 What is LPS and where it is found

Cells of bacterial species possess many different components. To what degree the capacity to produce these cellular components are found in an organism can vary incrementally across bacterial species for some components, while for other components the variation is categorical: a species either has them or does not have them. Secretion systems would be an example of the former: some species have a lot of different ones, some have a moderate number, and some have

none. In contrast, lipopolysaccharide falls squarely in the second category: a given species either possesses the entire apparatus for producing and assembling LPS as well as associated pathways, or it does not. With an allowance being given for exceptions among unknown bacterial species, or species where it has not been studied, bacteria either do or do not have a well-defined apparatus for biogenesis of LPS, although the amounts of LPS produced may vary. Furthermore, bacteria that differ in regard to having LPS, differ profoundly in the overall organization of their cell envelope, and appear to be highly evolutionarily divergent species in general. Therefore, although uniquely bacterial, lipopolysaccharide is widespread and conserved.

Lipopolysaccharide constitutes the outermost layer of the cells that contain it. There, it forms the outer half of a lipid bilayer, known as the outer membrane (OM). As the name suggests, this membrane forms an additional lipid bilayer that encloses the cell, which is itself contained within a cytoplasmic membrane. In the aqueous space between the two membranes, known as the periplasm, resides the peptidoglycan skeleton that is common to most bacteria. This envelope architecture is representative of a class of bacteria known as Gram-negatives (**Figure 1**).

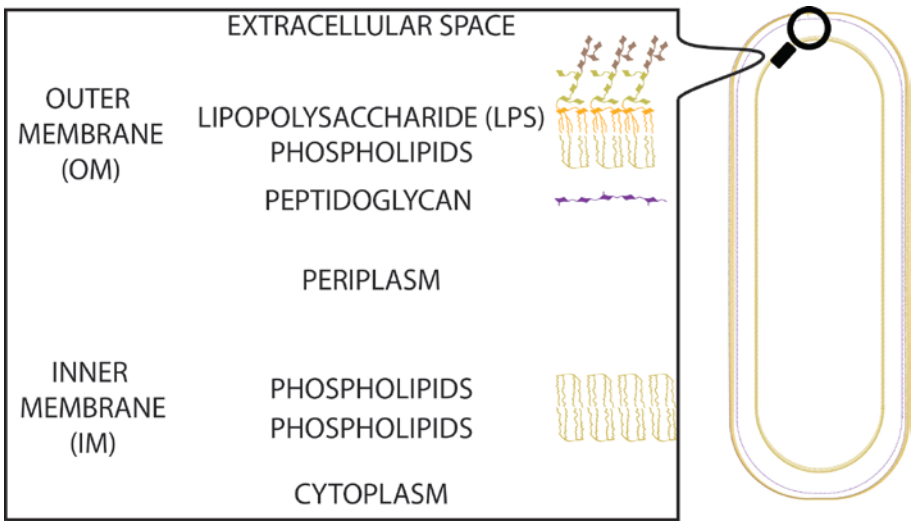

Figure 1 Cell envelope architecture of gram-negative bacteria.

Notably, those bacteria that possess an outer membrane, known as Gram-negatives, tend to possess a far thinner layer of peptidoglycan than those bacteria that have no outer membrane. Also, in bacteria without an OM, peptidoglycan forms the outermost layer of the cell, and correspondingly has a much greater diversity of basic structure and various modifications compared to peptidoglycan of Gram-negatives, in which it is LPS that forms the outermost layer and takes over the function of varying the surface presented to the external environment.

While LPS does vary substantially across and even within species[13], there are certain specific elements of its structure that are highly conserved, and there also are certain structural patterns that tend to be followed[14]. In general, parts of LPS that are closest to the lipid bilayer of the OM tend to be the most conserved, and those parts that are furthest away from it are most varied. For ease of understanding, an LPS molecule is subdivided into three distinct parts on the basis of their structural conservation, namely lipid A, the core region, and the O-antigen (**Figure 2**).

Lipid A is the most conserved part of LPS. It consists of a lipidated disaccharide, and it is those lipid groups that form the outer half of the OM bilayer. The saccharides tend to be glucosamines or glucosamine derivatives, and it is common for the lipid chains to be β-hydroxy lipids, to which secondary acyl chains often attach. The glucosamines are often phosphorylated, and those phosphate groups, in turn, are sometimes modified by addition of phosphoethanolamine or aminoarabinose. This latter type of modification confers protection against polymyxins, cationic antimicrobial peptides that bind to LPS in a phosphate-dependent manner (**Figure 2**). Lipid A is typically highly conserved within a species, with minor variations in its peripheral features, which are typically made in response to environmental stimuli.

The core region is an oligosaccharide that attaches to lipid A and is more varied in its structure. Typically, different strains within a species will produce several cores, although the variation in core structure is still limited. Residues that comprise the core tend to include phosphorylated carbohydrates (**Figure 2**), which chelate soluble cations in the OM. Both lipid A and the core are produced by an incremental elaboration of a pre-existing structure.

O-antigen is the most varied, and the outermost part of lipopolysaccharide. Not all bacteria possess the O-antigen, and those that do not, carry a lipopolysaccharide known as lipooligosaccharide. O-antigen is a polydisperse polymer of an oligosaccharide[15] (with occasional non-saccharide elements) repeat, and the repeating unit varies a lot even within a species. It is common to have hundreds of varieties of the O-antigen within a single species, and over a hundred building blocks are known that can be combined in multiple ways to produce the repeating unit. O-antigen is typically synthesized independently as a polymer, and then attached to the outer core of LPS.

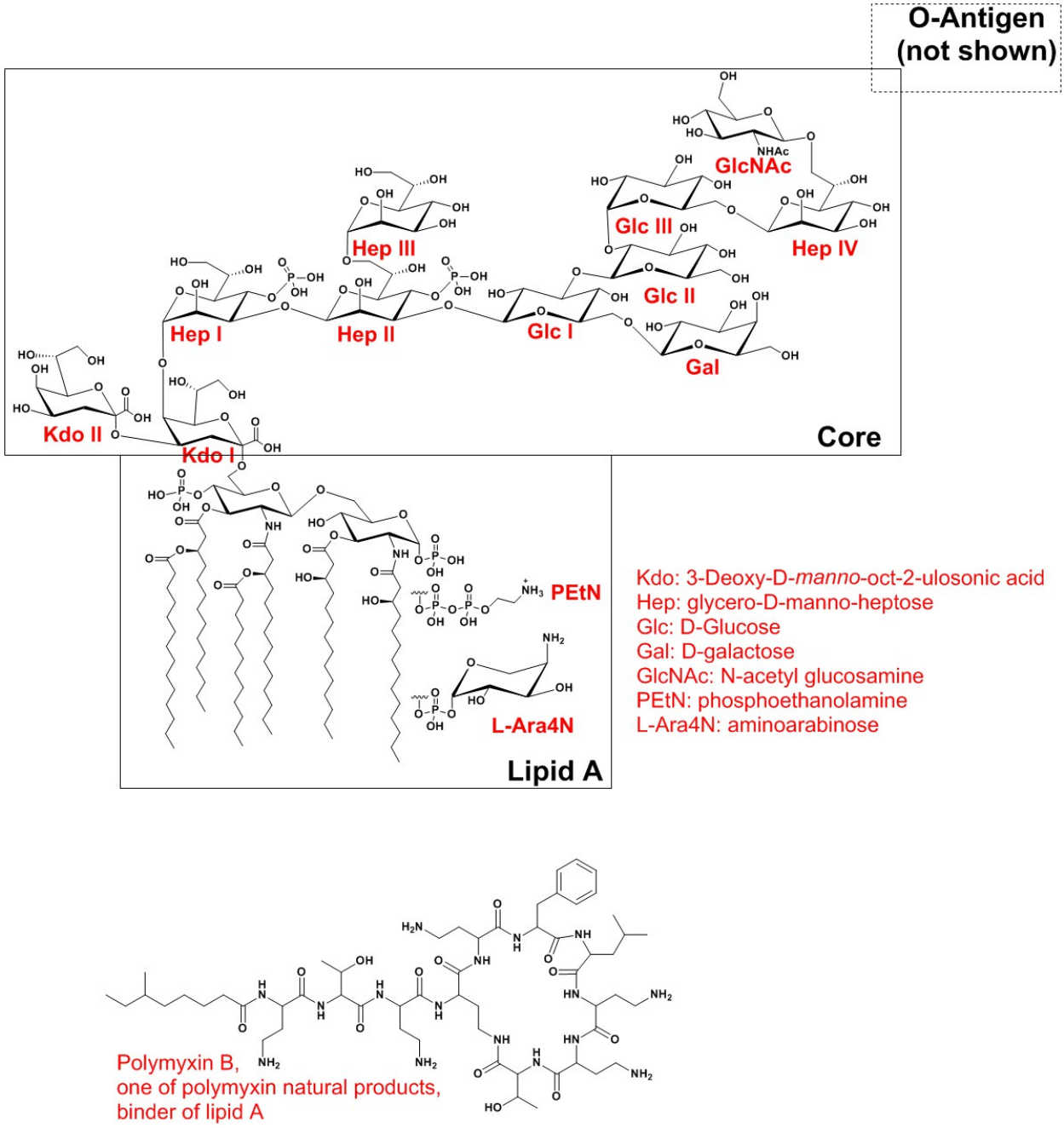

Figure 2 Chemical structure of LPS of *E. coli* and of polymyxin B. LPS is without O-antigen and shows modifications (PEtN and L-Ara4N) that confer resistance to polymyxins, natural products that bind to lipid a and disrupt the membrane. Polymyxin B is a member of that class of antibacterial peptides.

1.3 How LPS is made and how it is transported to its destination

Once we know what the chemical structure of LPS is and where it is located in the cell, an obvious question arises: how is it made and how does it get there? There are two aspects to it: biosynthesis and transport.

1.3.1 LPS Biosynthesis

Biosynthesis of LPS, which has been studied most extensively in *E. coli*, begins with the biosynthesis of Lipid A (**Figure 3**), the most conserved part of the molecule[14]. In the first step, with an unfavorable K_{eq}, LpxA catalyzes the attachment of a β-hydroxy fatty acid (3-hydroxymyristoyl in *E. coli*) to UDP-N-acetylglucosamine, a molecule that is also a precursor in the biosynthesis of the peptidoglycan skeleton (**Figure 39**). In the second step, LpxC catalyzes the deacetylation of the amine in a reaction with a much more favorable Keq, which constitutes the first committed step in the LPS biogenesis pathway. Thus, levels of LpxC are regulated in a cell in order to adjust levels of LPS[16]. LpxC is a metalloenzyme and uses zinc as a cofactor. LpxC is the only enzyme in the LPS biogenesis pathways that has been successfully and thoroughly targeted by chemical inhibitors with Zn-binding hydroxamate groups, although these inhibitors are not used as antibiotics due to human toxicity[17]. In the third step, LpxD attaches a β-hydroxy fatty acid to the amine group that was deacetylated by LpxC. In the fourth step, LpxH hydrolyses the phosphoester bond to cleave UMP and produce Lipid X. In the fifth step, LpxB attaches Lipid X to its precursor via a β,1'-6 linkage. In the sixth step, LpxK phosphorylates the 4' position of the LpxB product to yield Lipid IVa. Thereafter, WaaA attaches two KDO (ketodeoxyoctanoate, aka 3-deoxy-D-manno-octulosonic acid) molecules to lipid IVa, producing

KDO2-LipidIVa. Finally, LpxL and LpxM attach secondary acyl chains to the β-hydroxyl groups of KDO2-LipidIVa and produce KDO2-LipidA.

Once KDO2-LipidA is biosynthesized, a number of glycosyltransferases attach additional, frequently phosphorylated, carbohydrates to it, which constitute the core region of LPS. This is handled by waa(rfa) pathway in *E. coli*, and is not further discussed here[14] because these genes are less conserved across bacteria than early LPS biosynthesis genes and are of lesser interest as antibiotic targets.

In parallel with the biosynthesis of core-KDO2-LipidA, the precursor to the O-antigen is synthesized independently. There are three pathways that carry out O-antigen synthesis: Wzy-dependent[18], ABC-transporter-dependent[19], and synthase-dependent. For the same reason as with the core, these are not discussed in detail here[20]; it is sufficient to say that all the O-antigen precursors are linked to a polyprenol-phosphate lipid, and the final step of attaching the polymeric O-antigen to the LPS core is performed by WaaL in the periplasm.

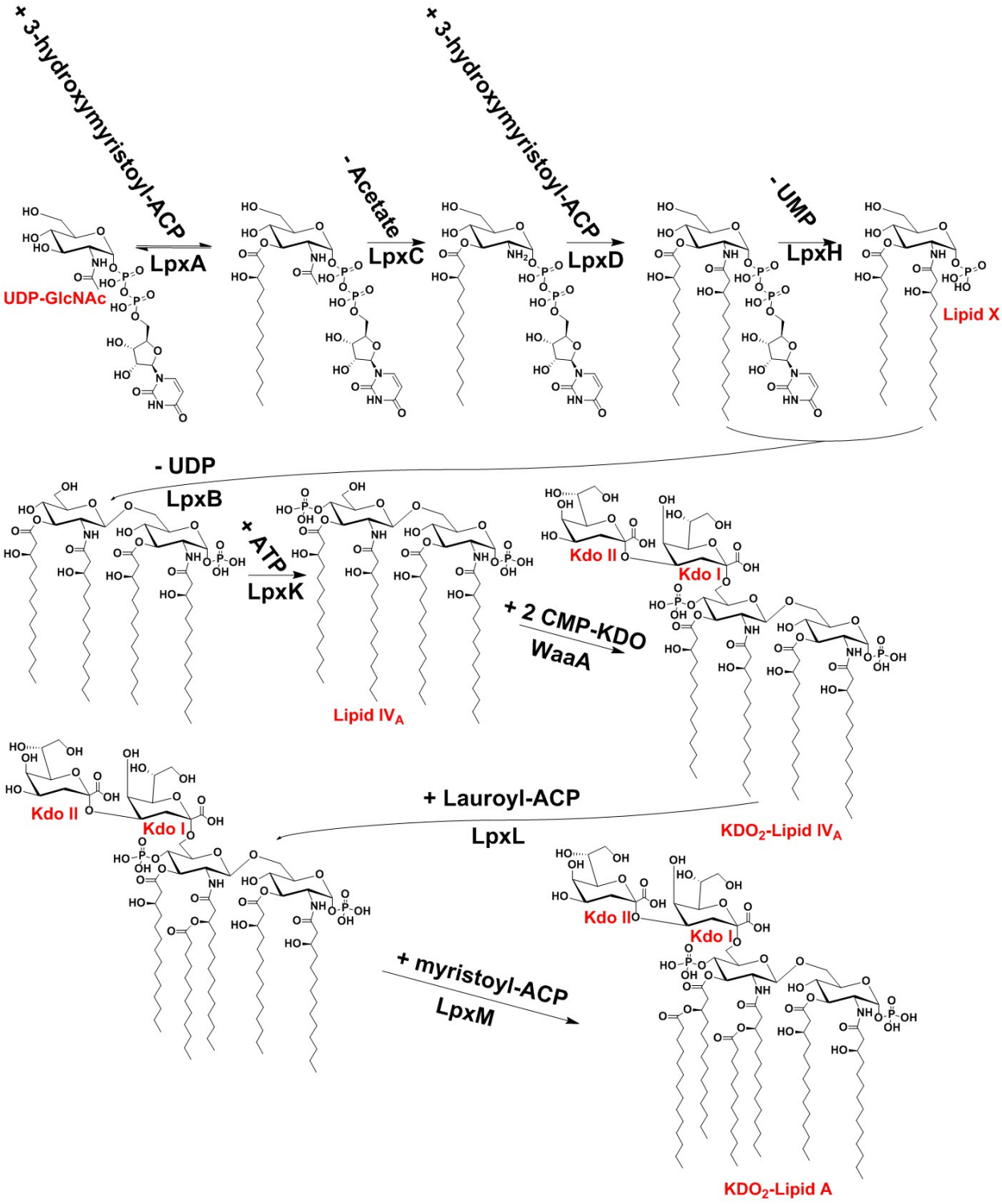

Figure 3 Biosynthesis of Lipid A, the most conserved part of LPS. LpxC catalyzes the first committed step in the pathway, and its proteolysis in *E. coli* controls the amounts of LPS produced.

1.3.2 LPS Transport

In addition to producing the elaborate chemical structure of LPS, which is itself not a trivial task, bacterial cells need to transport it between the two most distant locations of the cell envelope in order for it to serve its role.

The first challenge that needs to be overcome is the flipping of Lipid A-core from the inner face of the inner membrane to its outer face. It is performed by MsbA (**Figure 4**), the best-characterized lipid flippase and an ABC-transporter, which has also been implicated in multidrug efflux[21,22]. It has been crystallized, and its structures are available in complex with various nucleotides[23-25]. However, there is no structure available in complex with LPS, and its mechanism of LPS flipping remains an area of investigation.

Once LPS is flipped to the outer face of the inner membrane, and derivatized with an O-antigen (if the O-antigen pathway is active) this large amphiphilic molecule has to travel to the outer face of the outer membrane across the aqueous layer of the periplasm. This is achieved by the Lpt pathway (**Figure 4**).

The first task of the Lpt pathway is to extract LPS from the inner membrane[26]. This is achieved by an ABC transporter complex LptBBFG in an ATP-hydrolysis-dependent manner. In this process, LptB is the ATPase, and LptFG are integral membrane proteins with which it is associated.

A task that is inseparable from LPS extraction is its transit across the aqueous layer of the periplasm, because LptBBFG does not extract it into this aqueous compartment. Rather, the lipid chains of LPS are believed to be embedded into the groove within a soluble domain of a

membrane-anchored protein LptC. Therefrom, LPS transits the aqueous periplasm with its hydrophobic parts buried in a twisted "lipoduct" of LptA oligomers[27].

In the final step, LPS is directly inserted into the outer face of the outer membrane by the LptDE complex. LptD is a integral membrane β-barrel with a soluble periplasmic domain that is similar to LptA in structure. LptD contains LptE, a lipoprotein, inside of it, which is important for the folding of LptD. The mechanism of LPS insertion by LptDE is not very clear, although it is believed that LPS enters the inside of the barrel and slides out laterally[28]. It is not clear how the sizeable polysaccharide of LPS behaves during the process: because an LPS molecule is probably held by its hydrophobic base on route to the OM, it is unlikely that its tip is easily guided into the opening, and possible that it is dragged through behind the lipidic part of LPS.

It is also notable that the pathway transports its substrate against a concentration gradient, and it is believed that a continuous queue of LPS molecules from its source to its destination is necessary for the transduction of energy from the ABC transporter to the OM[28].

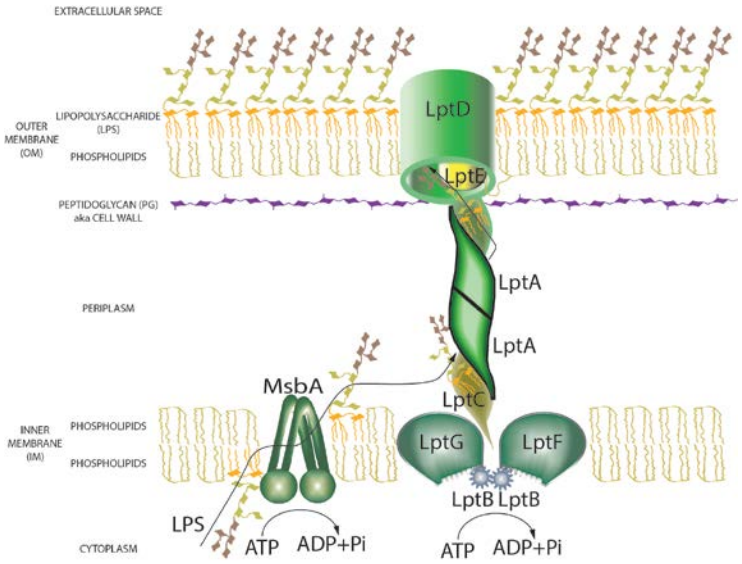

Figure 4 LPS transport pathway

1.3.3 LPS-phospholipid balance in the cell.

An important feature of LPS biogenesis, both at the biosynthesis and transport stages is that it is necessary to maintain the overall quantities of LPS and its distribution across various cellular locations within certain bounds.

For instance, it is known that areas of the OM where LPS is absent (and phospholipid comprises the OM on both sides) exhibit a higher permeability and are often disadvantageous to a cell[29]. While one may then suggest that the way to overcome that problem is to produce an excess of LPS and always have more LPS to put into the OM, that is not a viable approach either. Excess LPS would have to accumulate in the IM[30], and the presence of excess LPS in place of phospholipids in the IM would interfere with normal IM processes.

Therefore, in order to maintain a balance between LPS and phospholipids, a cell has to be able to adjust how much of each is made, and where what is made is located. While we do not possess an all-embracing model of how that occurs, several processes that participate in the task have been characterized, and are important to mention.

Firstly, LPS biosynthesis is regulated by regulating levels of the LpxC protein, which is the first committed step of the pathway. LpxC is subject to proteolytic degradation by FtsH[31], and some further upstream genes in this mechanism of regulation have been identified[32]. However, little is known about how exactly various cellular conditions bring about adjustments in LpxC levels apart from the fact that LpxC levels are kept high in actively growing and dividing cells, and low in stationary phase cells, which is not an adjustment unique to LPS.

Secondly, there is an integral membrane phospholipase, pldA, (**Figure 5**) which resides in the outer membrane, and the location of its active site is such that it can only degrade phospholipids in the outer leaflet of the outer membrane[33,34]. Thus, it seems to be a mechanism for removing areas of phospholipid from the outer leaflet of the OM and restoring the permeability barrier. Interestingly, the enzyme is normally inactive, and its overexpression does not cause damage. Rather, this enzyme becomes active by dimerizing in response to a number of stimuli, which include the presence of phospholipids in the outer leaflet of the OM.

Thirdly, there is an integral membrane protein, pagP, (**Figure 5**) which resides in the outer membrane, and is a palmitoyl transferase, which transfers palmitate residues from phospholipids to LPS molecules[35]. The location of its active site is such that it can only do so by using phospholipids that reside in the outer leaflet of the outer membrane.

Finally, there is a set of proteins that comprise the Mla pathway (**Figure 5**). They are believed to transport excess phospholipids from the outer membrane to the inner membrane[36]. They are homologous to proteins involved in lipid trafficking in other organisms, and permeability effects that are caused by their deletion are suppressed by spontaneous mutants that upregulate pldA, which would suggest that their role is to remove phospholipids from the outer membrane. Mla proteins include an outer-membrane lipoprotein, a soluble periplasmic protein, and a set of inner membrane proteins that form an ABC transporter.

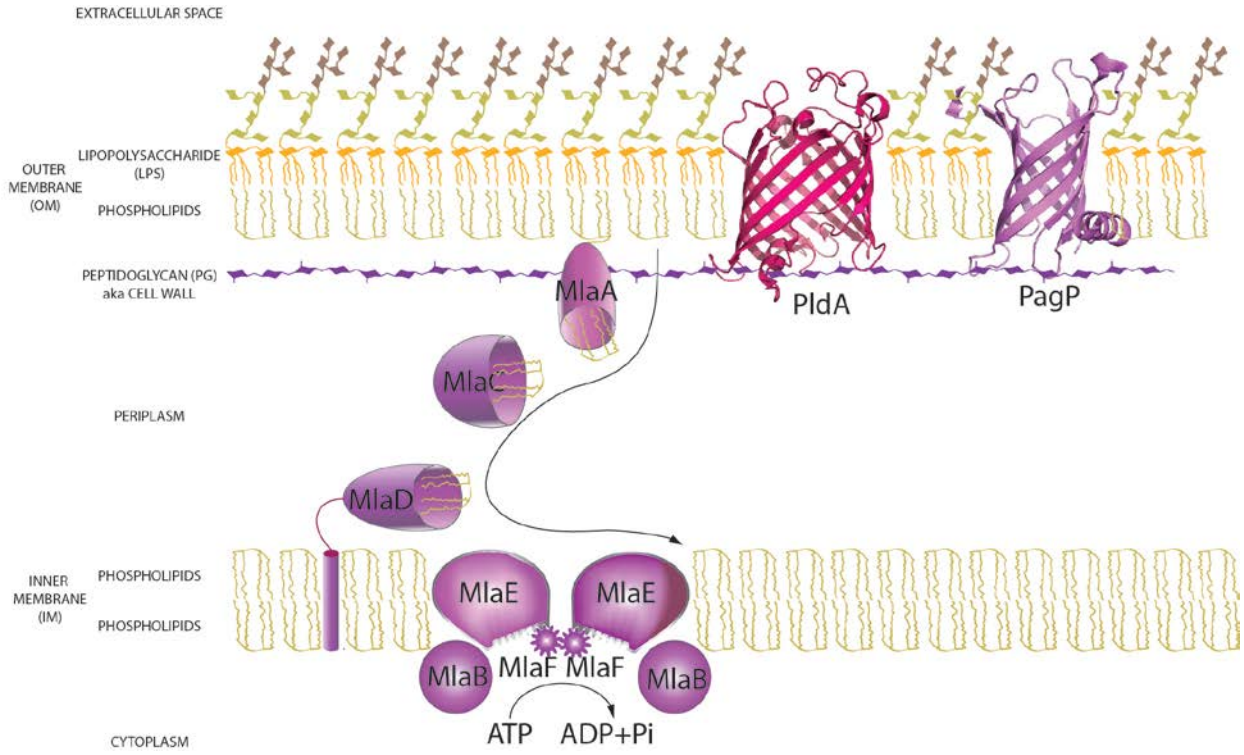

Figure 5 Systems for maintaining LPS-phospholipid balance in the OM. Mla retrograde transporter is believed to transport phospholipids back from the OM to the IM. PldA phospholipase degrades phospholipids present in the outer layer of the OM. PagP palmitoyl transferase transfers palmitoyl chains from phospholipids that end up in the outer layer of the OM to LPS.

In essence, the distribution of phospholipids and LPS in a physiologically normal cell is such that if both were somehow allowed to exchange freely, LPS would flow to the IM, and PL would flow to the OM. While LPS, being restricted to the outer layer of OM probably will not flow back to the IM via a membrane semi-fusion site, this does mean that its transport to the OM requires energy as it moves LPS from a less concentrated compartment to a more concentrated one. Similarly, it appears reasonable that Mla will require energy to add phospholipids to the inner membrane, which is already full of them.

Correspondingly, it would seem that anterograde transport of phospholipids to the OM would not require any energy, but only a conduit for lipids to travel along. Whether it is so or not, and whether such conduit is provided by a protein bridge or by fusion-forming proteins is not known. It is definitely clear that one or very few such conduits will go a long way; so, it would not be reasonable to expect that proteins responsible for their formation would be very highly upregulated under conditions of LPS deficiency.

1.3.4 Accessory pathways that LPS transport depends upon

There are two additional pathways that, while not involved in the transport of LPS directly, have as their main functions, the provision of the machinery that is involved in it directly.

The LOL pathway (**Figure 6**) transports lipoproteins to the outer membrane. OM Lipoproteins are produced on ribosomes inside the cell, translocated across the inner membrane via a dedicated channel, and derivatized with a lipid anchor on the periplasmic side of the IM. Thereafter, lolCDDE, an ABC transporter pulls the lipid anchor from the inner membrane and transfers it to lolA, a periplasmic chaperone, which diffuses to the outer membrane and transfers it to lolB, an OM lipoprotein itself, which inserts the substrate into the outer membrane[37].

The BAM pathway (**Figure 6**) folds integral membrane β-barrels into the outer membrane. These proteins are also made on ribosomes inside the cell and transported across the inner membrane via the same channel as lipoproteins. In the periplasm, they are received by one of a number of chaperones (Skp, SurA, DegP) which transport them to the BAM machine. The BAM machine, which consists of a β-barrel BamA as well as four lipoproteins: BamBCDE, receives the substrate and folds it into the membrane by an unknown mechanism[38].

LptDE, which play a central role in LPS transport, are handled by these two pathways. LptE is a Lol substrate, while LptD is the only substrate of Bam (apart from BamA itself) that is alone indispensable for growth of *E. coli*. (It is possible that multiple combinations of other BAM substrates are indispensable for growth of *E. coli*, but it is possible to delete each independently except LptD and BamA). The folding of LptD by Bam is challenging because it is a particularly large β-barrel that requires LptE in order to fold. Furthermore, the process requires the proper formation of two disulfide bonds in LptD[39]. Thus, this process could be the target for antibiotics that indirectly impact LPS.

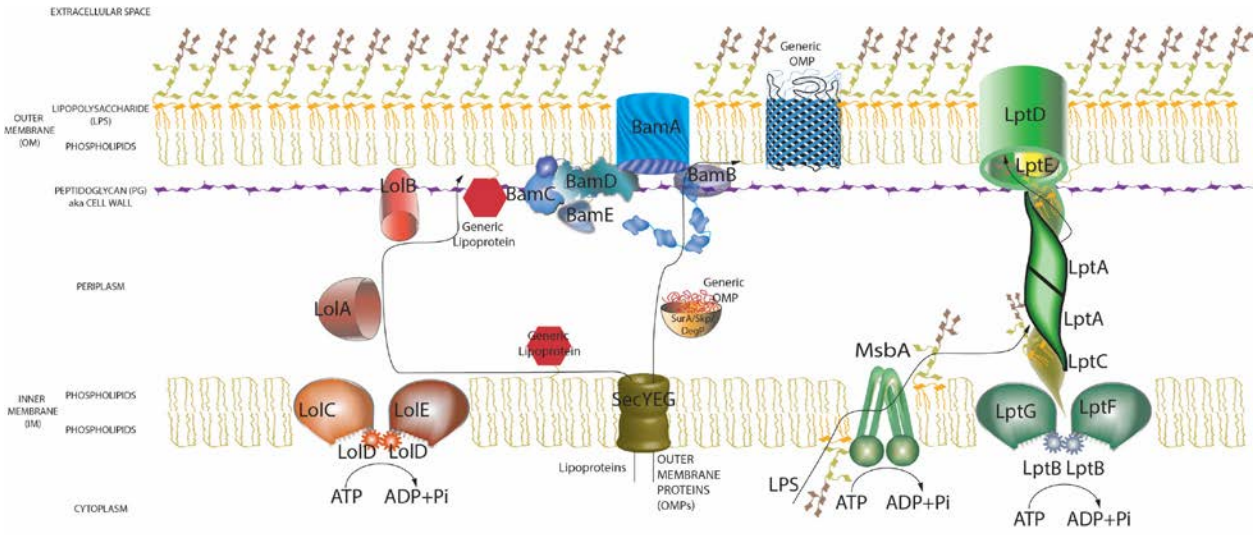

Figure 6 BAM and LOL pathways cooperate with LPS biogenesis to construct the outer membrane. LOL pathway transports lipoproteins, which are a part of both BAM and Lpt machines, to the outer membrane. BAM pathway folds LptD, the terminal part of Lpt pathway.

It is worth mentioning that there has been a report of a synthetic cyclic peptide active against only *P. aeruginosa*, resistance to which arises in LptD[40]. Due to a non-disclosure of the structure and a lack of biochemical and structural tools, it is unclear what the exact mechanism of action is for that peptide. It can be imagined that it binds LPS in a polymyxin-like manner and prevents it from passing through the wild-type LptD channel. It can also be imagined that it binds to LptD

itself in some manner that prevents LPS from passing through it. However, it can also be imagined that it interferes with the folding of LptD by the BAM machine in some way.

1.4 Role of LPS in bacterial physiology

There are elements of biological systems so indispensable and ubiquitous in the terrestrial forms of life (e.g. amino acid glycine) that a discussion of their role in bacterial physiology would be supererogatory. However, such a discussion is by no means superfluous in the case of LPS because LPS-containing bacteria comprise only one of two major classes of bacteria. Thus, it is important to consider the functions that LPS may perform in bacteria that possess it.

Notably, a bacterium does not exist in isolation in nature; so, the physiological functions of a component of it are intimately connected with what it encounters in its environment. However, we will begin by examining the roles of LPS in the life of bacteria that are cultured in the laboratory, and proceed in the next sections to examine the roles of LPS in a bacterium that exists within a patient.

1.4.1 Role of LPS in organisms where we have been unable to remove it

In studying the role of LPS, the approach that most logically suggests itself is to remove or impair LPS function, and compare the bacterial strain that results with its parent strain. Interestingly, it has only proven possible to remove LPS in a subset of bacterial species. In the rest, removal of LPS does not result in viable bacteria.

In the most commonly used laboratory bacterium, *E. coli*, attempts to remove LPS have failed. LpxA, LpxC, LpxD, LpxH, LpxB, LpxK, WaaA, MsbA, and all Lpt genes are indispensable for growth and division in *E. coli*[41]. At the same time, when proteins that are responsible for its

biosynthesis or transport are depleted in genetically engineered strains (**Figure7**), growth slows down and stops; no clear and distinctive phenotypes result.

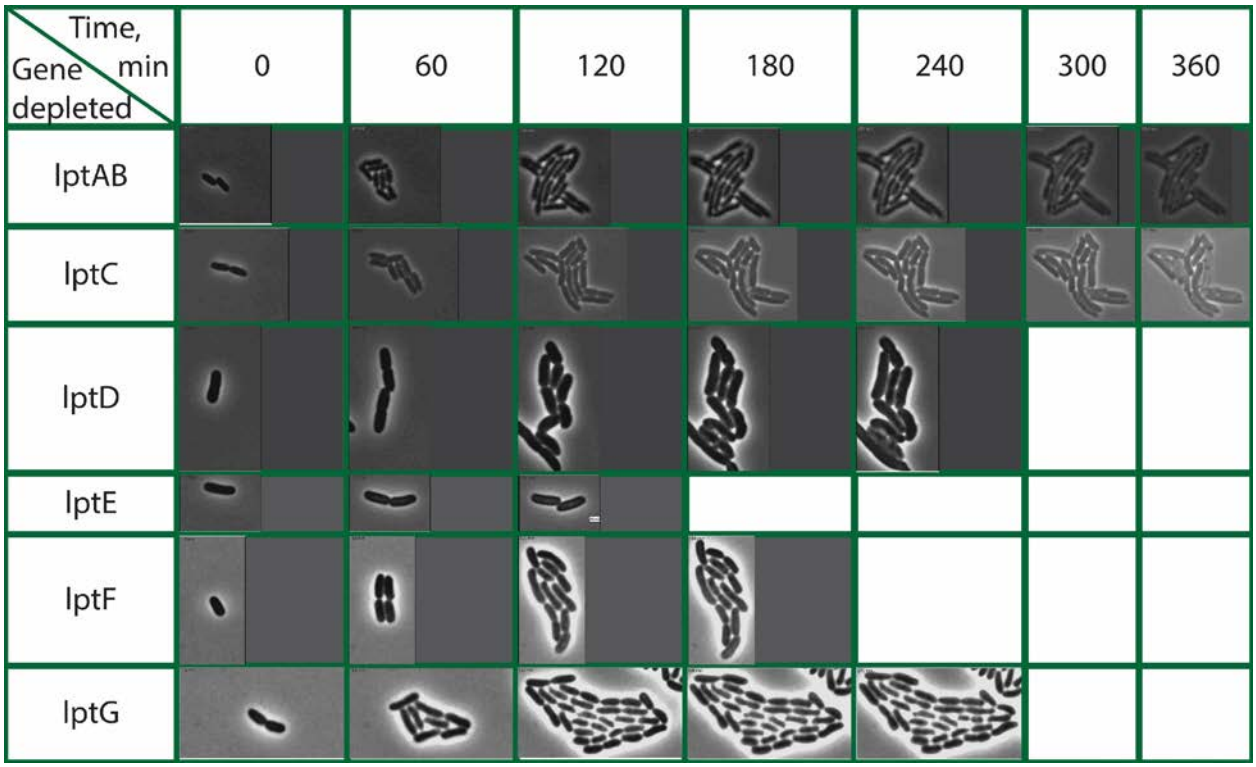

Figure 7 Depletion of LPS biogenesis genes in *E. coli*. Chromosomal copy of each of the above gene is replaced by a copy under the control of arabinose, and the cells are grown under the microscope in the absence of inducer. Stagnation results without any distinctive phenotypes

Nonetheless, many genetic defects that cause a *partial* loss of LPS function result in viable cells, and have been characterized in *E. coli*, and a hallmark feature of those defects has been an increased permeability of the bacterial cell envelope[29]. This is evidenced by the fact that large molecules that are normally unable to penetrate *E. coli* with an intact LPS layer are able to do so once LPS function has been compromised. In addition, soluble components that are entirely confined to the periplasmic space in *E. coli* with an intact LPS layer are found to leak into the growth medium once LPS function has been impaired.

Thus, the major conclusion of studies of the role of LPS in *E. coli* has been that it reinforces the diffusion barrier of the OM. However, that conclusion fails to explain why cells lose viability when LPS is removed: useful though a diffusion barrier may be, unless it prevents something lethal from entering, or prevents something very important from leaving, it should not be necessary for cells to grow and divide. There is currently no indication that either of those two scenarios is true for *E. coli*.

Of course, as with any pathway, there is a possibility that the reason we are unable to remove it and retain viability lies not within the function of its end product, but within the mechanism behind the process that produces that end product.

In particular, the most logical explanation would be that blocking a pathway does not completely remove the pathway from the cell, but deranges it in a way that spreads beyond the pathway itself and has a negative intrusive effect on the rest of the cell[42]. Such scenarios are likely when the pathway is blocked at a downstream stage. In that case, the still active components can either waste cellular resources by consuming them without hope of completion or accumulate an excessive level of precursors in cellular compartments where the precursors interfere with the normal operation of other cellular processes. However, that would not be a problem when the earliest stage is blocked in a pathway as linear as LPS biogenesis in *E. coli*, yet it is impossible to block the earliest stages of LPS biosynthesis in *E. coli* and produce viable cells.

An alternative explanation for our inability to remove LPS in *E. coli* and retain viability may be that while no single important process outside of LPS biogenesis in *E. coli* depends on LPS to a such degree that loss of LPS would cause a drop in its function alone sufficient for a loss cellular

viability, there may be enough of such processes that simultaneous impairments in all of them add up to a loss of viability.

Either way, in order to prove such hypotheses, it is necessary to find a way to remove LPS in *E. coli* while retaining viability and then show what it is contingent upon. Therefore, we don't know what role LPS plays in *E. coli* growth beyond that of a diffusion barrier, and why it is that *E. coli* seems to be unable to survive without it.

1.4.2 Role of LPS in organisms where we have been able to remove it

At the same time, more recently, it has been found that there are a number of bacteria in which LPS can be removed completely without loss of viability. Currently, the list includes *Neisseria meningitidis*[43], *Moraxella catarrhalis*[44], *Acinetobacter* sp[45,46], and *Yersinia ruckeri*[47]. Of these, *Acinetobacter* sp have been studied most widely thus far, and it is the only genus where LPS loss has been observed to occur spontaneously in response to colistin (LPS-binding antimicrobial peptide); therefore, it can provide most additional insights into the roles of LPS.

1.4.2.A Role of LPS in A. baumannii

LPS has been removed through inactivation of the LpxC gene in *Acinetobacter baumannii* 19606, a popular clinical strain, by many groups independently[45,48-50]. The most obvious feature of LPS-deficient strains of *A. baumannii* is that they and are more susceptible to antibiotics (**Figure 11**) than the strains that they are derived from (as may be expected on the basis of the barrier role of LPS), that they are impaired in virulence[51], and that they also grow more slowly.

Overall, the existing knowledge did not reveal any functions for LPS in *A. baumannii* beyond that of a permeability barrier. Therefore, we hypothesized that when *A. baumannii* is propagated

in rich media for multiple generations, genetic variations that enhance fitness under the absence of LPS would become enriched, and would inform us about what pathways suffer most from the lack of LPS in *A. baumannii*. Therefore, we (collaboration between the author and Tim Meredith, Emma Nagy, and Ge Zhang) passaged multiple populations of *Acinetobacter baumannii* 19606 ΔLpxC and of Acinetobacter baumannii 19606 ΔLptD for 2 weeks, characterized the resultant bacteria and performed whole-genome sequencing. Three features emerged from this investigation. Because *A. baumannii* 19606 passaging is not exclusively my work, the following description is brief and is in general terms.

The first feature was that gains in growth rate for *A. baumannii* 19606 ΔLpxC and *A. baumannii* 19606 ΔLptD during serial propagation are gradual. In other words, there is not a single mutational event, but an accumulation of incremental increases in growth rate that restores growth of LPS-deprived *A. baumannii* to wild-type levels. This trend has been observed for many independently passaged populations. Therefore, it appears that there is no single pathway that needs to adjust in order to enable *A. baumannii* to grow faster in the absence of LPS.

The second one was that sequencing of growth-rate-adapted *A. baumannii* 19606 ΔLpxC has not produced a consistent set of genes or pathways that are mutated in order to enable the LPS-less bacteria to grow faster. Many of the mutations found in *A. baumannii* 19606 ΔLpxC were also observed in populations of wild-type *A. baumannii* 19606 that were propagated under the same conditions. In a way, it would seem that observed genetic adaptations have little to do with LPS, and are adjustments to growth in LB in general.

The third feature has to do with genes for removing excess phospholipids from the outer membrane. We found that loss-of-function mutants in genes for pldA and mla components are

commonly found as mutations that arise *late* in the passaging experiments. It appears reasonable that in the absence of LPS, it may be advantageous to disable removal of phospholipids from the outer membrane. However, it is unnecessary to disable these genes in order to recover growth rate in ΔLpxC *A. baumannii*, and it seems that resolving these issues are not an urgent priority of *A. baumannii* ΔLpxC.

Thus, it seems that slowed growth rate of LPS-deficient *A. baumannii* 19606 has little to tell us about the roles of LPS in that organism's physiology. It does appear that absence of LPS renders *A. baumannii* 19606 susceptible to intercompartmental imbalance of phospholipids, which is less of a consequence of the function of LPS than the adaptation of the cell to the presence of LPS.

To probe the same question in a different way, the Boyce lab conducted RNA sequencing of *A. baumannii* 19606 and its ΔLpxA counterpart[52]. They observed an upregulation of genes involved in lipoprotein transport, Mla retrograde transport, BamA, and exopolysaccharide production. However, LPS-related genes were a minority. Of course, it is difficult to interpret which of these adaptations actually reflect the necessities of *A. baumannii* in laboratory culture, and which are a part of an evolved response to the loss of LPS in its natural environment, which may present additional stresses to bacteria without LPS. The relevance of genes identified specifically to the survival of *A. baumannii* 19606 without LPS in the laboratory could be assessed by making knockouts and knockdowns of these genes. However, this was not done, due likely in part to the low efficiency of such experiments in *A. baumannii* 19606. I analyzed the list of 123 upregulated genes in the Boyce study for evolutionary conservation, and it did not reveal any likely candidates for phospholipid transport to the OM on that basis. Of course, as was previously mentioned in this chapter, such genes probably do not need to be upregulated very highly.

All in all, it would seem that *A. baumannii* 19606 has some deficiencies when it comes to growth in LB, but it is necessary to remove LPS to fully observe them. *A. baumannii* without LPS does try to stop mla and pldA from removing PL from the outer membrane, but only as an afterthought, which still does not inform us why LPS is essential in *E. coli.*

1.4.2.B Role of LPS in A. baylyi

Not all *Acinetobacter* species are as difficult to manipulate genetically as *A. baumannii*. While *A. baumannii* is a known human pathogen, which means that its study is of direct relevance for antimicrobial therapy, *A. baylyi* is a non-pathogenic form of *Acinetobacter*, whose natural competence and efficient recombination have made it a model organism as far back as 2004[53]. Of particular interest is the fact that due to the ease of its genetic manipulation, a comprehensive set of single-gene knockouts has been constructed in that organism in 2008[46], which has established the comprehensive pattern of essentiality in LPS-related genes. In effect, it has shown that the only essential genes in the LPS biogenesis pathway of *A. baylyi* are: LpxH, LpxB, LpxK, WaaA, MsbA, LptB, and LptG.

However, because the purpose of the comprehensive knockout study has not been to probe LPS-related pathways, no further tests were performed by de Berardinis et al[46]. Therefore, I used *A. baylyi* to test a simple hypothesis about the mechanism as the one in Swoboda et al.[42] that the late-stage genes in the pathway are essential because their removal irreversibly diverts precursors into a dead-end pathway, and that those late-stage genes could be removed once the pathway was blocked.

At first, I made a simple knockout of LpxC in *A. baylyi*, and its success was both verified by PCR and whole-genome sequencing, as well as by polymyxin resistance (MIC 0.15 μM in WT

A. baylyi and MIC 150 μM in *A. baylyi* ΔLpxC). Notably, LPS-deficient *A. baylyi* does not suffer from a diminished growth rate in LB compared to WT *A. baylyi*, thus seeming to be better adapted to growth in LB than *A. baumannii.*

Thereafter, I went for a knockout of LpxH, LpxB, LpxK, WaaA, MsbA, LptB, and LptG in both WT *A. baylyi* and ΔLpxC *A. baylyi*. While no colonies in WT *A. baylyi* for nulls of LpxH, LpxB, LpxK, WaaA, MsbA, LptB, or LptG were ever obtained (in agreement with de Berardinis et al.[46], all knockouts (of LpxH, LpxB, LpxK, WaaA, MsbA, LptB, and LptG) readily succeeded in ΔLpxC *A. baylyi* (success confirmed both by PCR and by whole-genome sequencing) (see **Figure 8**).

Of course, the inability to knock out the seven above genes in LPS-WT *A. baylyi* does not by itself constitute a proof of impossibility to remove these genes in the strain that is producing LPS. Therefore, an *E. coli* arabinose-inducible expression plasmid, pBAD, has been modified to work in *A. baylyi*, and *A. baylyi* LpxC has been cloned into it. That functional protein is made by this plasmid has been shown by the expression-dependent polymyxin sensitivity of *A. baylyi* ΔLpxC strain that has been transformed with it (**Figure 10**). The vector has a baseline level of leaky expression, as shown by polymyxin sensitivity of the uninduced ΔLpxC pBAD-LpxC. While it was easy to transform an LpxC plasmid into ΔLpxC *A. baylyi*, it has proven impossible to do so in any of the double knockouts with LpxH, LpxB, LpxK, WaaA, MsbA, LptB, and LptG. The only condition under which it has proven to be possible was a combination of severe genetic weakening of upstream sequence and transcriptional repression. Thus, it appears that LPS production is necessary and sufficient for essentiality of the seven genes above (**Figure 8**).

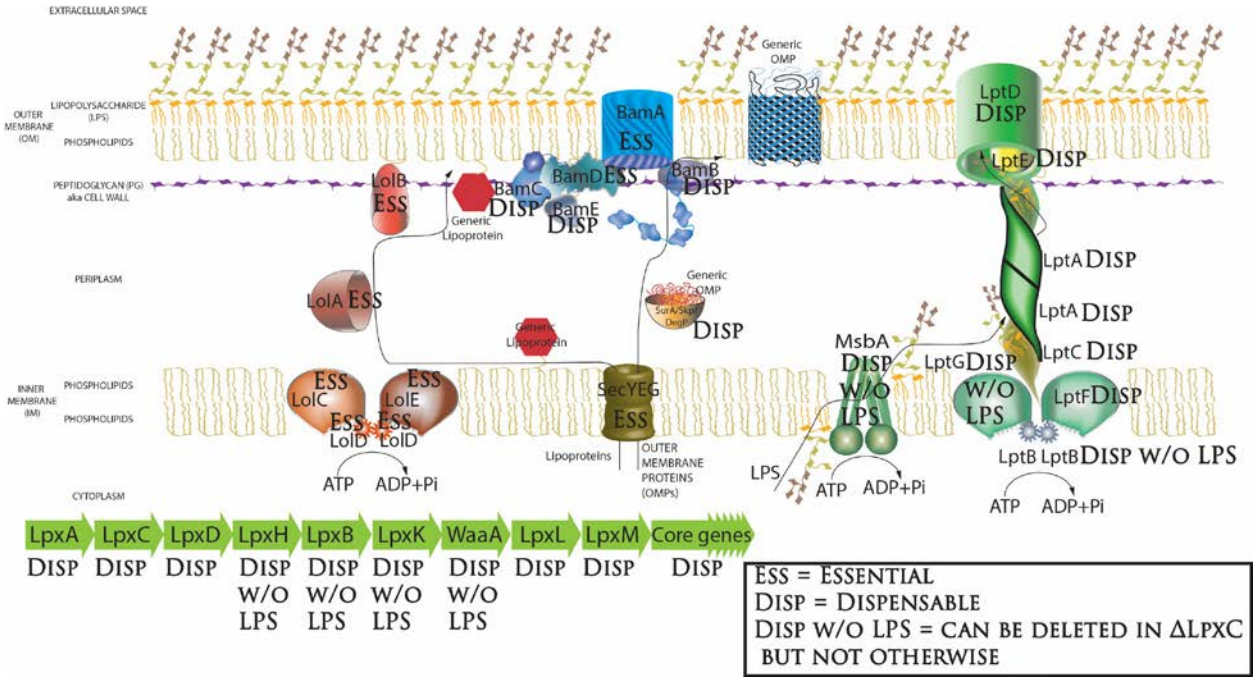

Figure 8 Essentiality and dispensability of OM-related genes in A. *baylyi*. LPS-conditional essentiality of LpxH, LpxB, LpxK, WaaA, MsbA, LptB, and LptG was found by the author. General essentiality of BamA as well as dispensability of LpxC, LptD, and LptE was confirmed by the author.

The other essentialities are assigned on the basis of de Berardinis et al (2008) comprehensive knockout set.

A test of MICs for a panel of known antibiotics (**Figure 9**) had shown identical susceptibilities for the double knockouts and the parent LpxC knockout, implying that these downstream genes play no role in pathways other than LPS biogenesis. This is particularly noteworthy for MsbA because it has a wide spectrum of substrates beyond lipid A[22,54]. If MsbA does perform other essential flippase functions in *A. baylyi*, it certainly cannot be the sole protein that does so.

Strain and MIC, μM / Drug	WT	ΔlpxC	ΔlpxC ΔlpxH	ΔlpxC ΔlpxB	ΔlpxC ΔlpxK	ΔlpxC ΔwaaA	ΔlpxC ΔmsbA	ΔlpxC ΔlptB	ΔlpxC ΔlptG
Aztreonam	70	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Azithromycin	5	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Novobiocin	3	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Coumermycin A1	1.7	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
Cefoperazone	85	15	15	15	15	15	15	15	15
Meropenem	0.144	0.012	0.012	0.012	0.012	0.012	0.012	0.012	0.012
Ampicillin	90	4	4	4	4	4	4	4	4
Ciprofloxacin	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Bacitracin	400	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Nalidixic Acid	>20	14	14	14	14	14	14	14	14
Minocycline	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Furazolidone	40	40	40	40	40	40	40	40	40
Trimethoprim	>150	>150	>150	>150	>150	>150	>150	>150	>150
Rifampicin	1	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014
Linezolid	100	40	40	40	40	40	40	40	40
Chloramphenicol	41	13	13	13	13	13	13	13	13
Moenomycin	2	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0.007
Vancomycin	300	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Ramoplanin	>300	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Geldanamycin	>>150	12	12	12	12	12	12	12	12
Clindamycin	55	7	7	7	7	7	7	7	7
Triclosan	8	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03

Figure 9 After removal of LPS in A. *baylyi*, removal of other genes in lps biogenesis (LpxH, LpxB, LpxK, WaaA, MsbA, LptB, and LptG) has no further effect on susceptibility to a wide range of antibiotics

All in all, work in *A. baylyi* clearly demonstrates that LPS-related genes can only be essential as long as the cell produces LPS. The fact that many late genes can be removed seems to imply that the cell can recognize late-stage blocks and respond to them. That mis-targeted LPS is always the problem is further evidenced by the fact that the fitness of ΔlpxCΔlptD double null in *A. baylyi* is far superior to that of ΔlptD *A. baylyi* alone. It is tempting to hypothesize that LptB and LptG signal the accumulation of LPS to signaling proteins that downregulate LPS biosynthesis. It would be intriguing to identify those proteins and see if they are present in *E. coli.* That may well hold the clue to explaining the difference in LPS gene essentiality between *E. coli* and *Acinetobacter*.

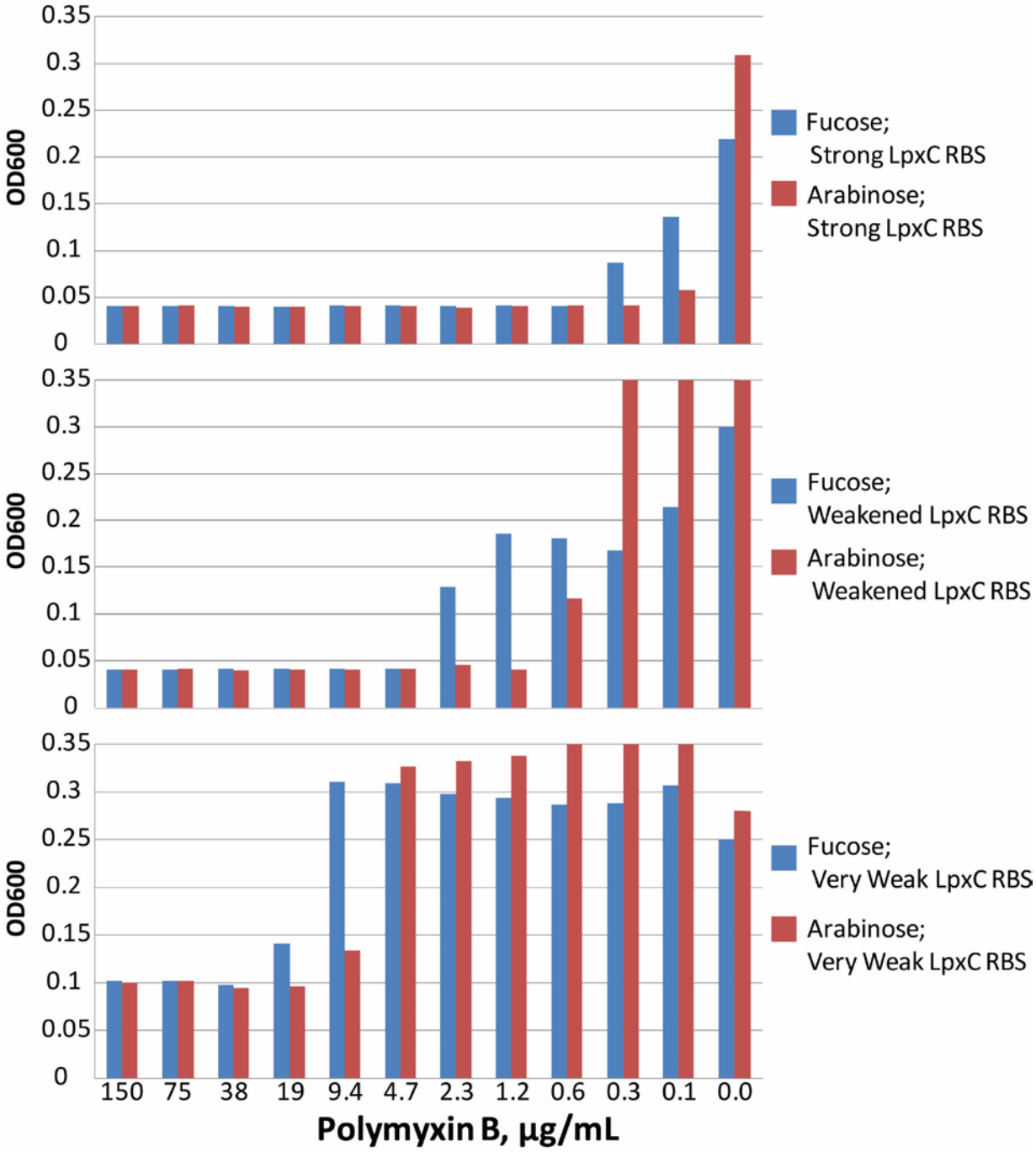

Figure 10 Transcriptional control of LpxC levels controls polymyxin sensitivity in A. *baylyi.* Fucose represses pBAD expression, and arabinose enhances it. Althought induction and repression have an effect, the strength of the Shine-Dalgarno sequence exerts a much stronger influence.

1.5 Role of LPS in bacterial pathogenesis

In fact, the scientific study of LPS began with the discovery of its function in human-pathogen interactions many decades before its role in the cell envelope of bacteria was characterized[55]. LPS was discovered in the laboratory of Robert Koch, who, at the end of the XIX century, was studying the various components of bacteria that elicit inflammation in people. LPS was identified as an inflammatory toxin that retained its toxicity after being heated, while most protein toxins were inactivated by heat. Those observations lead to the term "endotoxin", by which LPS is still referred to, and among even present-day literature that mentions LPS, the vast majority is dedicated to using it for its immunological properties.

Thus, it is worth giving a brief overview of these properties. There are two major roles of LPS that relate to bacterial pathogenesis: its interaction with the innate and with the adaptive immune system.

The interaction of LPS with the innate immune system involves its role as a PAMP (pathogen-associated molecular pattern) – a structural element so common among pathogens that the innate immune system includes a dedicated pathway to recognize it and respond to it. While there are many details known about the recognition process, it is sufficient to say that LPS is recognized by multiple proteins in the organism: LBP (LPS-binding protein, which is soluble), CD14 ("cluster of differentiation 14", which may be soluble or lipid-anchored), and MD2-TLR4 (a soluble protein associated with an integral membrane protein receptor). It is the latter that ultimately transduces the signal inside the cell, which results in the production of proinflammatory cytokines, such as TNFα, IL1β, IFNγ, IL-8, IL-6, IL-1, IL-12. As a result, an inflammatory process ensues that can be overwhelming and cause serious tissue damage. It

appears that TNFα is primarily responsible for the outcome, and its production ultimately causes damage to blood vessel lining in a variety of tissues. Because even picomolar amounts of LPS are sufficient to result in this cascade, the presence of a substantial amount of LPS in the blood during an infection can result in a deadly septic shock.

It is interesting that among the LPS structures that were immunologically studied so far, it is the "default" *E. coli* lipid A that produces the most pronounced inflammatory response, while the inflammatory properties of LPS from other Gram-negative bacteria, including, but not limited to such notorious species as *Yersinia pestis* (plague) and *Francisella tularensis* (tularemia) are far more mild[56]. There is also a flip side to this property: LPS that can thoroughly stimulate the immune response appears to be important for the proper development of the immune system. In particular, a recent prominent study by the Xavier lab[57] investigated the epidemiology of autoimmune diseases in the Baltic region northeastern Europe, and found that the prevalence of such diseases was lower in Russians than in Finns and Estonians. At the same time, the authors found that while *E. coli* was prominent in the intestinal flora of Russians, *Bacteroides* species were prominent in the intestinal flora of Finns and Estonians (all at the stage of infancy). It is known that *E. coli* LPS is immunostimulatory, while *Bacteroides* LPS is immunoinhibitory, and the authors were able to confirm the hypothesis that these differences affect immune development by observing the same difference in susceptibility to autoimmune diseases in mice with the same differences in intestinal microbiota.

The interaction of LPS with the adaptive immune system chiefly concerns the less conserved parts of LPS: O-antigen and the core. In particular, being the most superficial part of LPS, the O-antigen is most frequently targeted by the immune system via antibodies. As a result, its structure

is most often varied by the bacteria to avoid immune recognition; as an example, almost two hundred structural varieties of O-antigen are known for *E. coli*[58].

Finally, even though LPS betrays the presence of bacteria to the immune system of the host, LPS is nonetheless important in producing a successful infection. While molecular mechanisms for the following observation have not been extensively investigated, it is known that *E. coli* with incomplete or insufficient LPS is strongly impaired in its ability to establish an infection, and the same was observed for *A. baumannii* mutants that lack LPS entirely[50]. Thus, the knowledge of the role of LPS in bacterial pathogenesis reinforces the promise of LPS as a drug target.

1.6 Role of LPS in antibiotic resistance

The role of LPS as a diffusion barrier has been alluded to previously, and it assumes a major importance in the context of using antibiotics to treat infections caused by bacteria that possess LPS.

Typically, antibiotic resistance is thought of as arising due to one of the three major acquired mechanisms: either altering the target biological molecule so that it no longer binds the antibiotic, or producing a protein that will excrete that particular antibiotic out of the cell, or producing an enzyme that will chemically inactivate the antibiotic.

While all of these mechanisms are very important for both those bacteria that have LPS, and those that do not, bacteria that have an outer membrane with LPS possess an important advantage when it comes to the last two mechanisms of resistance. First, the uniquely formidable permeability barrier of LPS makes resistance by efflux pumps more effective. Furthermore, the very presence of the outer membrane allows efflux pumps to confer resistance to antibiotics

whose targets would otherwise be exposed outside the cell and indefensible by efflux. Second, bacteria that have an outer membrane with LPS do not have to produce as high a quantity of enzymes that inactivate antibiotics with extracellular targets because these enzymes will not diffuse away when enclosed within the periplasmic space. Finally, it may be argued, although conclusive experimental evidence is lacking on that point, that mutations that confer resistance by changing the target molecule so that an antibiotic no longer binds are more effective when accompanied by a lower intracellular concentration of the drug, which enters the cell in lower quantities. This last point brings to our attention the major role of LPS in antibiotic resistance.

There are many antibiotics which are very effective against the species of bacteria that produce no outer membrane (**Figure 11**). Examples include Vancomycin, Ramoplanin, Bacitracin, Erythromycin, Novobiocin, etc. At the same time, these antibiotics typically have such low activity against bacteria that have an outer membrane with LPS that they are not clinically useful for treating those infections. The targets of these antibiotics are conserved among bacteria; so, something else must account for that difference. For some of them, it is efflux pumps that excrete these compounds beyond the outer membrane. This is evidenced by the fact that Gram-negative bacteria are sensitized to a subset of these drugs once genes for these pumps are inactivated (**Figure 11**). For many of these drugs, however, no amount of tampering with efflux machinery results in sensitization (**Figure 11**). However, when the integrity of the LPS layer of the OM itself is compromised, dramatic sensitization to these drugs is evident, sometimes even to levels below those of organisms that possessed no outer membrane to begin with, and even the complete arsenal of efflux machinery is powerless to restore resistance in that case. This observation holds equally well in *Acinetobacter* species (**Figure 9** and **Figure 11**), which can grow and divide in the total absence of LPS, thus indicating that even if the treatment by

inhibitors of LPS is not antibacterial on its own, it aides many other treatments by compounds that are.

Strain and MIC, μM / Drug	*E. coli*	*E. coli* ΔTolC	*E. coli* LptD-Imp4213	*A. baumannii*	*A. baumannii* ΔlpxC	*P. aeruginosa*	*S. aureus*	*B. subtilis*
Meropenem	0.16	0.16	0.16	11.77	0.35	40.00	1.61	1.22
Aztreonam	0.20	0.16	0.01	50.00	3.84	8.00	>300	20.03
Ciprofloxacin	0.05	0.01	0.01	2.50	0.96	0.27	1.00	0.29
Nalidixic Acid	19.00	2.52	1.22	10.00	10.98	300.00	63.11	25.58
Novobiocin	125.00	0.93	0.93	1.60	0.10	>700	0.15	2.04
Coumermycin A1	20.00	15.16	0.17	12.00	0.72	14.00	0.08	11.20
Azithromycin	12.00	0.87	0.16	25.00	0.50	299.99	8.66	5.82
Minocycline	0.89	0.25	0.40	0.09	0.10	7.20	0.27	0.12
Furazolidone	5.20	1.45	0.78	63.00	50.00	148.18	19.98	0.96
Trimethoprim	0.54	0.46	0.21	136.50	43.84	364.00	19.89	0.95
Rifampicin	8.00	8.00	0.01	1.20	0.08	23.00	0.02	0.32
Chloramphenicol	14.00	7.75	8.00	400.00	76.00	76.00	15.86	10.70
Moenomycin	27.66	27.66	0.02	1.44	0.09	40.00	0.04	0.30
Vancomycin	200.00	200.00	0.60	117.00	0.12	>300	1.41	1.41
Ramoplanin	200.00	200.00	3.29	100.00	0.40	>200	0.40	0.11
Linezolid	>300	42.69	99.96	150.00	99.96	>150	5.00	2.61
Polymyxin E	0.15	0.15	0.15	0.60	158.00	0.31	79.00	4.90
Polymyxin B	0.14	0.14	0.14	0.14	72.00	0.28	18.00	2.26

Figure 11 Comparative drug susceptibility of *E. coli* and of its permeability mutants as well as of clinically important Gram-positive and Gram-negative pathogens. It is evident that ΔTolC *E. coli* is more sensitive to some drugs than its WT parent, but *E. coli* LptD-Imp4213 is considerably more sensitive to almost all antibiotics than its WT parent. Similarly, LPS-deprived *A. baumannii* is considerably more sentive than its WT parent strain to all antibiotics except LPS-binding polymyxins. *P. aeruginosa* is notably insensitive to a wide range of antibiotics. Gram-positive bacteria *S. aureus* and *B. subtilis* are, on the whole, more sentive to antibiotics than WT Gram-negative bacteria.

To summarize, LPS is a very promising drug target for the following reasons. It is very common among infectious bacteria (E. coli, A. baumannii, P. aeruginosa, K. pneumoniae, N. gonorrhoeae, V. cholera, Y. pestis), and the fundamental proteins that underlie its biogenesis are highly conserved. At the same time, there is no similar pathway in humans; so, there human toxicity is less of a concern than with more conserved targets. Most infectious bacteria require LPS for growth and division; so, its inhibition is expected to be a broad-spectrum standalone bacterial treatment. Furthermore, direct inhibition of LPS biogenesis has the potential to diminish the risk of septic shock. At the same time, incomplete inhibition of LPS (or its inhibition in bacteria that

can survive without it) makes the bacteria much more susceptible to the defenses of the immune system and to the action of other drugs.

1.7 Methods for Chapter 1

1.7.1 Methods for depletion strain imaging

Depletion strains for this experiment were from Natividad Ruiz and Alessandra Polissi (FL907 transduced into NR754 is the LptAB depletion strain[59], FL905 transduced into NR754 is the LptC depletion strain[59], NR1134 is the LptD depletion strain, AM689 is the LptE depletion he strains grown at 37 ˚C with shaking in LB supplemented with 0.2% arabinose were diluted 1:100 into LB supplemented with 0.2% arabinose and grown at 37 ˚C with shaking for 2 hours. The cells were harvested by centrifugation and washed 3 times with LB without arabinose. Then, 1 µL of cell culture was placed on a coverslip, covered by an LB-agar pad without arabinose, sealed with a silicone ring and another coverslip and imaged on an inverted microscope at 37 ˚C using a phase-contrast 100x oil immersion objective.

1.7.2 Methods for *A. baylyi* strain construction

A. baylyi ADP1 (purchased from ATCC) was used in these experiments. Knockouts of *A. baylyi* were constructed according to the methods of de Berardinis et al paper of 2008[46] and were verified by PCR as well as by whole-genome sequencing. An *A. baylyi* -compatible version of pBAD was constructed by inserting the origin of replication of pWH1266 (purchased from ATCC) into pBAD-HisA using Gibson assembly and transformed into *A. baylyi* according to de Berardinis et al paper of 2008[46].

1.7.3 Methods for *A. baylyi* MIC measurements

Overnight cultures of appropriate *A. baylyi* strains were diluted 1:100 and grown for 2 hours. They were normalized to OD600 of 0.0001 and used to inoculate microplates with concentration gradients of appropriate antibiotics. Plates were incubated without shaking overnight and were read for OD600 after 24 hours. MICs were determined as the lowest concentrations at which OD readings reached the lowest values that no longer decreased with increasing concentration.

1.7.4 Methods for measuring dependence of polymyxin susceptibility on induction of LpxC expression in *A. baylyi*

The *A. baylyi* LpxC gene was cloned into pBAD both with the native strong Shine-Dalgarno sequence of pBAD (CAGGAGG) and with a weakened Shine-Dalgarno sequence (CACACAGG) and a severely weakened Shine-Dalgarno sequence (CGGGCGG). The plasmids were transformed into *A. baylyi* ΔLpxC. These strains were used to inoculate a concentration gradient of polymyxin B in a microplate supplemented with either arabinose (1.5%) or fucose (0.75%) for induction or suppression. OD600 was measured after 24 hours of incubation.

1.7.5 Methods for measuring MICs of *E. coli* and its mutants as well as of *A. baumannii*, *P. aeruginosa*, *S. aureus*, and *B. subtilis*.

A derivative of *E. coli* K-12 MC4100, its ara+ revertant, NR754 was used (obtained from Natividad Ruiz). *A. baumannii* strain 19606 was used (purchased from ATCC); *A. baumannii* strain 19606 ΔlpxC::kan was obtained from Tim Meredith. *P. aeruginosa* strain PAO1 was used (purchased from ATCC). *S. aureus* strain Newman was used (obtained from the Walker lab). *B. subtilis* strain 3610 was used (obtained from the Losick lab). All bacteria were grown for 2 hours in a shaker after 1:100 dilution of an overnight culture, and were normalized to OD600 of 0.0001 and used to inoculate microplates with concentration gradients of appropriate antibiotics. Plates

were incubated overnight and were read for OD600 after 24 hours. MICs were determined as the lowest concentrations at which OD readings reached the lowest values that no longer decreased with increasing concentration.

1.7.6 Methods for whole-genome sequencing for all projects described in this work

For all strains and species described in this work, genomic DNA was purified from 1 mL of overnight culture using Invitrogen PureLink kit according to manufacturer's instructions. DNA was prepared for sequencing using Illumina's Nextera kit, and its quality was checked using Agilent BioAnalyzer and qPCR QC kit by KAPA Biosystems according to manufacturer's instructions. Samples were sequenced on Illumina's HiSeq 2500 in high-output mode 2x 125-bp reads.

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Chapter 2
Screening and target ID of novel small molecule antibiotics for Gram-negatives: FAB-based suppression of LPS defects and large-scale screening

2.1 The choice of approach to screen for inhibitors of OM assembly

As was shown in detail in Chapter 1, LPS is a very promising target for developing novel antibacterial compounds. Compounds that would inhibit its biogenesis pathways would be very useful; however, to actually develop new compounds for that purpose is a lot more challenging than just to realize their importance.

The first point of consideration is the type of assay to be employed in screening for inhibitors of outer membrane assembly.

There are two major classes of screening approaches: the target-based and cell (or organism)-based methods. Target-based methods are performed in cell-free systems and usually rely on biochemical assays that contain a minimum of components necessary to reconstitute the biological process of interest and to measure its inhibition. Cell-based or organism-based screening methods rely on cells that may be tissue-cultured or constitute whole organisms, such as bacteria, fungi, *C. elegans*, *D. melanogaster*, and even sometimes *D. rerio* larvae.

In a world with perfect experimental methods and with a treasure trove of great chemical compounds, the choice between the two approaches would not matter. It would not matter

because both approaches would return the same compounds. However, in the world that we have to deal with, all methods return some compounds that we do not want fail to return some compounds that we do want. Likewise, there are both nuisance compounds that appear promising at first, and disappoint later, as well as compounds that appear unpromising at first, but can be chemically improved to become quite valuable. These facts mean that both the target-based and cell-based methods have advantages and disadvantages that apply differently to different biological systems.

2.1.1 Features of target-based screening

Target-based methods have the advantage that the target of a small molecule discovered by their use is known immediately. However, in order to employ a target-based method, one usually has to reconstitute the activity of the target *in vitro*, or to develop some surrogate assay. To do so in whatever way is often difficult, and to do so in a way that is robust and scalable is more difficult still. Moreover, the results from such effort may easily turn out to be a poor model of what actually happens in a cell, and a model that cannot be scaled up to boot. In addition, small molecule inhibitors that are active and are detected *in vitro* may be unable to reach their target *in vivo*, may be degraded by the cell, or may have been detected because they are disrupting the assay itself rather than the process that it seeks to model.

A wide variety of biochemical tools have been developed to reconstitute activities of many different cellular processes, and to survey them is beyond the scope of this work. The important consideration from the standpoint of high-throughput screening is that these methods span a spectrum of applicability to high-throughput use.

The easiest to adapt to high-throughput screening are assays that monitor enzymatic activity, the making and breaking of covalent bonds. A variety of methods, based on measurements of absorbance, fluorescence, and luminescence are available to report on a vast range of enzyme activities, and all of them scale very well. Of these, fluorescence and absorbance suffer from the fact that candidate compounds found in screening libraries can occasionally affect these signals leading to false-positive and false-negative results, but that can be dealt with. Luminescence is more resistant to these effects, but requires more expensive reagents. In the same category are assays that monitor the status of an interaction of a small molecule with a macromolecule. The small molecule can be fluorescently labeled, and the polarization of its fluorescence signal will indicate whether it is bound to a large molecule. An example of that approach is the assay that looks for molecules that can displace Moenomycin pharmacophore from the transglycosylase binding site[1].

Slightly more difficult to measure in high throughput are protein-protein interactions that cannot be reduced to monitoring enzymatic activity upon the joining or dissociation of the two proteins. This could include protein interactions in cell-cell recognition, or innumerable intracellular protein-protein interactions. These can be assayed in high throughput using AlphaScreen, a methodology that relies on singlet oxygen diffusion from a bead that is linked to one of the two proteins to the bead that is linked to the other. This approach is reliable and scalable, but quite expensive[2].

Finally, the most difficult to evaluate in high-throughput target-based screens are processes that involve membrane protein folding and intercompartmental transport of cellular components. This is so because, on the one hand, the successful outcome of such processes often cannot be directly

deduced from formation or breaking of covalent bonds, and, on the other hand, the target proteins involved in such processes are often generally difficult to purify and work with biochemically.

Common to all these approaches is the fact that molecules that work on a target *in vitro*, can fail to work on it *in vivo*, a factor that is of special importance in bacteria, where it led to multiple failures of drug development efforts[3]. Molecules found in target-based screens have a very high chance of not being able to engage that target in a cell due to its permeability barriers and efflux machinery.

It is also an important consideration that, for the same reason that one always knows the target of molecules picked up in target-based screens, one can never serendipitously discover an inhibitor of a different target in the pathway of interest or in a pathway related to it by using a target-based screen.

Moreover, because reconstituting a cellular process biochemically often requires its thorough understanding (Indeed, the ability to reconstitute a process *in vitro* is often adduced as a proof of such understanding), it follows that inhibitors of new and poorly understood cellular processes, inhibitors the discovery and characterization of which would be most scientifically valuable, usually cannot be discovered in reconstitution-based screens.

2.1.2 Features of cell- or organism-based screening

As was mentioned before, using cells or organisms for high-throughput screening of small molecules has the distinct advantage that we immediately know that the compounds we find work *in vivo*, whatever our definition of "working *in vivo*" may be. However, there are two

principal challenges in this approach: first, to find moderate and weak compounds that may be improved by chemical modifications later and, second, to understand exactly what the compounds that we found are doing in the cell.

These two problems loom especially large for screening in bacteria as opposed to screening in TC cells and small eukaryotes.

First, bacteria, and especially bacteria with an LPS-containing outer membrane, have a very formidable arsenal to keep out and pump out small molecules in general[4]. This means that most small molecules in screening libraries will have no effect on them at all. As a result, it is often necessary to partially undermine these permeability barriers by mutations. While this is possible to do, it is necessary to be cautious about how it is done, if the molecules are eventually supposed to work in a cell with intact permeability barriers.

Second, when it comes to bacteria, the main purpose of small molecule screening is the discovery of antibacterial compounds, and, as a result, the compounds of interest will frequently kill cells or arrest growth before a phenotype that could be detected in a screen will arise. Thus in contrast to screening in systems which we seek to alter, but not destroy, when screening in bacteria, we face major difficulties in understanding what compounds are doing to cells, and, therefore, in discriminating between compounds that act on our pathways of interest and those that do not.

Nonetheless, several approaches exist that attempt to deal with these difficulties with varying degrees of success. The main types of such screening approaches are summarized below

The major class of high-throughput assays for antibacterial compounds relies on viability- and growth-based approaches that attempt to go beyond the obvious. Of course, the obvious fact is that antibacterial compounds are expected to reduce viability and inhibit growth, but that fact on its own does not provide any information about how a compound is doing it. However, on the basis of what may be known about a particular class of targets, more sophisticated approaches can be devised that still rely on viability and growth inhibition.

In particular, the knowledge of the target or class of targets may inform us that compounds that inhibit them would be (1) less effective against certain mutants or in the presence of another compound, or (2) more effective against certain mutants or in the presence of another compound, or (3) boost the antibacterial effect of another compound, or (4) suppress the antibacterial effect of another compound. Such screening assays have the advantage that the setup and readout is cheap and easy. However, they suffer from the fact that screening is typically done at a single concentration. This is a problem because the observation of all of the above effects is only possible within a certain window of the concentration range, and the differential effect may be subtle. Thus, what is gained at the primary screen stage has to be paid for in extensive dose-response follow-up to eliminate false-positives.

A variation that is sometimes possible on the viability and growth inhibition approach is to bypass the target or pathway entirely. If the result towards the attainment of which the target pathway works is supplied externally in a way that is not susceptible to the same mode of inhibition, it is possible to develop a rather robust way to screen for inhibitors, which would not be effective in the engineered system. This can be achieved by transplanting machinery for the same function from a very different species, or externally supplying an essential metabolite. An

example of that would be to screen for inhibitors of MurJ in *E. coli* by looking for compounds that are not effective against *E. coli* cells in which a copy of Amj (ydaH) from B. subtilis is present – a different protein that is capable of performing the same function[5].

A different kind of approach relies on transcriptional reporter assays. In such assays, the inhibition of the target evokes a specific stress response that deploys a transcriptional program. If the promoters involved are known, they can be put upstream of reporter genes, the products of which can be detected by assays based on absorbance, fluorescence, or luminescence. Such assays can be very useful for high throughput when we seek to affect, but not destroy cells with compounds[6]. Unfortunately, such assays are difficult to implement in high throughput for the discovery of antibacterial compounds because of the concentration dependence in combination with the antibacterial effect. Too much antibacterial compound will result in cell death or growth arrest before the transcriptional response can take place, whereas too little will not evoke a strong response, and it is not realistic to be spot-on in terms of concentration in high throughput.

Finally, another powerful approach used in cell-based screening is high-content imaging[7]. Here, cells or organisms are imaged after being subjected to small molecule treatment, and compounds of interest are picked on the basis of a desired phenotype. However, this approach is quite poorly suited to bacteria for a number of reasons. First, bacteria are quite small in spatial extent and that limits the variety of phenotypes that can be observed in them, especially limited is sub-cellular localization. The near complete list of phenotypes is: gross morphological changes, localization of fluorescence to the poles, localization of fluorescence to the septum, localization of fluorescence to the membrane, and diffuse or punctuate fluorescence throughput the cell. As always, these effects are concentration-dependent, and are rarely specific enough for target

determination. For example, cell filamentation is known to occur in response to a wide variety of stresses. Second, because bacteria are small, it is technically challenging to image them, and most of the above phenotypes are impossible to observe clearly on existing high-content microscopes – they are only visible with high-power oil-immersion optics not suited to high throughput. Third, bacteria are generally not adherent to microplate surfaces and are harmed by polycationic adherent coatings. This complicates the multiple washing and staining steps necessary for high-content imaging. Finally, many of the answers that imaging of bacteria would provide can be obtained equally well by relying on assays that are easier to implement and which produce far fewer gigabytes of data, which in turn are also easier to analyze than images.

While it is often very informative for the understanding of the mode of action of a particular compound to see under a microscope how bacteria react to treatment by small molecule over time, this is not at all suitable to high-throughput screening.

For outer membrane biogenesis, there are multiple considerations that favor the choice of cell-based assays. First, there is a rather large number of potential targets in the pathway, and it is not clear at this stage which of them are better to aim for. Thus, it is unrealistic to reconstitute the entire LPS biogenesis pathway, even in a dedicated experiment at this stage, let alone for screening in high throughput. At the same time, in a cell, it is possible to target any of the proteins of the pathway. Second, even the subsets of targets in the biogenesis pathway, the underlying sub-processes involve complexes of multiple membrane proteins, which are difficult to work with and are poorly suited to high-throughput assays. Finally, because the desired inhibitors are expected to undermine existing permeability barriers, it is advantageous to use as

starting points molecules that have some *in vivo* activity in spite of those permeability barriers. All in all, cell-based methods appear to be the most suited to this class of inhibitors.

2.1.3 Choosing a background

However, the common shortcomings of cell-based methods apply to this class of targets as well, and it is important to address them.

The main of those challenges is to weaken the penetration barriers in order to achieve higher concentrations that would allow to pick up hits with lower on-target activity while at the same time not picking up molecules that do not cross the intact permeability barrier at all because it is notoriously difficult to engineer bacterial permeability into an inhibitor that lacks it.

One of the oldest ways of increasing the permeability of the bacterial outer membrane relies on the mutant Imp4213[8], which contains a deletion of 23 amino acids in LptD. It was initially discovered as the mutant that allowed *E. coli* without LamB maltoporin to grow on minimal media with maltodextrins as the sole carbon source, and it was found that it increased the permeability of the outer membrane dramatically. There are several hypotheses to explain why the mutation leads to a more permeabilized outer membrane. It is known that LptD protein with the 4213 mutation is poorly folded by the Bam machine[9], leading to a decreased number of its copies in the cell. This would naturally be expected to result in an incomplete coverage of the outer membrane by LPS, which would make it easier for molecules to diffuse across. There is, however, an additional factor that may be a contributor: LptD-Imp4213 is stalled on Bam, and the resultant complex may itself form a channel through which molecules would diffuse. Imp4213 is not a particularly good mutation to rely on for increased permeability in a high-throughput screen: it permeabilizes cells very strongly **(Figure 12**), letting in molecules that

would otherwise never stand a chance to enter and have an effect, but, more importantly, it causes a variety of other defects in the cell, which is evidenced by the fact that some normally non-essential genes in other pathways become essential in Imp4213 cells (Natividad Ruiz; unpublished data).

A different approach to lower the permeability barrier is to use a strain that is missing the TolC protein. It is an outer-membrane protein involved in many efflux systems[4], and removing it does not allow molecules that never got in to enter, but its removal does protect molecules that enter and are pumped out from this fate (**Figure 12**). The efflux machinery of *E. coli* K-12 is so arranged that multiple pumps that reside in the inner membrane and consume energy to pump out small molecules, connect to TolC, which resides in the outer membrane and use it as a conduit for their ejection flow. Thus, removing TolC renders all of these pumps ineffectual. Mutants that lack TolC show a dramatic sensitization to drugs that are efflux pump substrates, but are just as resistant as TolC-wt strains to drugs that never get in to begin with. In this sense, ΔTolC is a more appropriate way to sensitize cells to experimental small molecules for high-throughput screening because it excludes molecules that never cross the OM. However, TolC knockouts are not very suitable for cell-based assays that go beyond the testing of simple antibacterial effect. It appears that TolC plays a role not only in drug efflux, but also in detoxification through unknown partners, and removing all efflux is harmful to the cell – some genes that are not normally essential become essential once TolC is removed (Natividad Ruiz; unpublished data).

Yet, a third way to achieve increased permeability is neither to alter a gene's coding sequence, nor to remove it altogether, but to decrease its levels. This is the approach taken in this chapter, and the genes targeted for decreased expression are lptF and lptG, which are cotransctibed. The

mutation was constructed by the Ruiz lab, and consists of a pARA promoter inserted upstream of LptFG. The expression levels of the two proteins are reduced in the absence of arabinose, but not to zero, and addition of arabinose restores them to WT. Resulting cells can grow and divide, but are more susceptible to antibiotics due to decreased levels of LPS in the OM. However, that increased susceptibility is lower than either in Imp4213 or in ΔTolC (**Figure 12**). Thus, this background allows a moderate increase of susceptibility. Furthermore, its effects on other genes do not spread beyond the pathway, and it is more suitable as a background for diverse cell-based assays.

Strain and MIC, µM / Drug	*E. coli*	*E. coli* LptD-Imp4213	*E. coli* ΔTolC	*E. coli* LptFG↓
Meropenem	0.16	0.16	0.16	0.09
Aztreonam	0.20	0.01	0.16	0.10
Ciprofloxacin	0.05	0.01	0.01	0.02
Nalidixic Acid	19.00	1.22	2.52	9.50
Novobiocin	125.00	0.93	0.93	47.00
Coumermycin A1	20.00	0.17	15.16	16.00
Azithromycin	12.00	0.16	0.87	11.00
Minocycline	0.89	0.40	0.25	0.36
Furazolidone	5.20	0.78	1.45	5.20
Trimethoprim	0.54	0.21	0.46	0.30
Rifampicin	8.00	0.01	8.00	8.00
Chloramphenicol	14.00	8.00	7.75	14.00
Moenomycin	27.66	0.02	27.66	10.00
Vancomycin	200.00	0.60	200.00	157.48
Ramoplanin	200.00	3.29	200.00	200.00
Linezolid	>300	99.96	42.69	>300

Figure 12 MICs (µm) of known antibiotics for the three sensitized screening backgrounds recapitulate trends discussed in the text. Imp4213 is highly sensitized to all classes of compounds; ΔTolC is highly sensitized only to pump substrates, but not to others. LptFG↓ is moderately and uniformly sensitized to antibiotics. Most importantly, LptFG↓ is not known to be synthetic lethal with anything outside of lptFG.

2.1.4 Detecting the on-pathway hits

The other challenge common challenge of cell-based assays to be addressed here is the pathway specificity. Obviously, OM assembly is essential for growth and division in *E. coli*; thus, molecules that inhibit it will be antibacterial, but so will be molecules that affect any of the other essential pathways.

A number of approaches discussed earlier are unsuited to high-throughput screening in this case. While there are transcriptional reporter strains that will respond to interference with OM assembly[10-12], they in fact respond to membrane disruptions that result from such interference, and will also respond to nonspecific disruptors such as detergents and chelators. At the same time, OM assembly pathways are quite conserved; so, it is not possible to bypass them with a different pathway. Finally, high-content imaging is not suited to the task either because bacteria are too small to monitor intracellular membrane trafficking in them by microscopy, and, as above, membrane disruptions caused by detergents and other non-specific agents would constitute a major source of off-pathway hits with this class of methods in high-throughput screening even if the sub-microscopic structures could be resolved.

Thus, comparative susceptibility appears to be the method of choice for high-throughput screening in this case. Such methods require the knowledge of the pathway and what the best method is depends on the specific pathway. Furthermore, development of such a method is particularly challenging in this case because such methods are typically validated with a pre-existing inhibitor of the pathway, which is not available for any aspect of OM assembly except for LpxC inhibitors. This means that genetic defects had to be used as a substitute for inhibitors in assay development.

As was mentioned before, in relative susceptibility assays, bacteria can be rendered either more or less susceptible to on-pathway compounds by the use of either a mutation or a small molecule. It is important to first address the first distinction for this class of inhibitors.

It is generally much harder to devise something that will improve the ability of bacteria to withstand an adverse influence than to devise something that will impair their ability to survive. In general, it is much harder to better something than to wreck it. Thus, there is no shortage of partial loss-of-function mutations in OM assembly that render cells more vulnerable to further inhibition of those pathways. However, this approach suffers from an outrageous lack of specificity in general, and especially so in this particular case. Because the many pathways that comprise cellular physiology are highly interconnected, it is difficult to be specific in impairing one of them without impairing at least some of the others. The extent to which a cell is generally weakened to small molecules by weakening it towards a subset of them varies depending on the pathways involved, but this is particularly high for weakening OM assembly pathways. This is the case because the OM normally serves as a permeability barrier that excludes harmful small molecules; thus, undermining it will cause all molecules to be more effective and fail as an on-pathway filter.

At the same time, because there are hardly any molecules known that do anything specific to OM assembly pathways, making it impossible to use one that will specifically render them more robust, we focused on genetic alterations that will do so.

In counterpoint to the strategy of sensitization, where sensitizing to one class of molecules is likely to sensitize to many additional classes of molecules, protecting from one class of molecules is UNLIKELY to protect from additional classes of molecules. The low likelihood is

due to the fact that no evolutionary tradeoff would be involved in such an improvement, and the resulting genotype would more likely be found as WT than as an improved one. On the contrary, usually such engineered protection comes at a cost, and the resultant strains are more sensitive to other adverse influences. This plays into the hands of the scientist because while the activity of on-target molecules drops toward the engineered strain, the activity of off-target molecules rises, making the two easier to distinguish (**Figure 13**).

Direction of influence \ Type of influence	Gene mutation	Small molecule
Sensitization	- Any sensitization is rarely specific to a single target - Sensitization of OM is very nonspecific due to accompanying permeabilization to a wide range of molecules	- Sensitization is rarely specific to a single target - Sensitization of OM is very dose-dependent for small molecules in *E. coli* (because LPS is essential) and it is also non-specific
Protection	- Protection is likely to be specific to the target; otherwise, it would have been in WT genotype - If the cost of protection is expressed in sensitivity to off-target molecules, the signal-to-noise of the assay is considerably improved	- No small molecule is known to protect *E. coli* OM pathways from inhibition

Figure 13 Sensitization and protection in high-throughput screening assays. Protecting against inhibitors of the target of interest at the cost of sensitizing the cell to inhibitors of other targets is the optimal strategy.

The protective mutations that form the basis of screening in this project were discovered fortuitously in the lab of Natividad Ruiz, our collaborator at Ohio State[13]. The basal strain for the investigation was a previously mentioned strain of *E. coli* K-12 with a mutation in the promoter of LptFG, which resulted in lower levels of these proteins in the cell in the absence of arabinose

induction. That strain was used in a chemical mutagenesis screen for partial loss-of-function mutations in LptFG that would be suppressed by overexpression of the proteins. Chemically-induced mutants were tested for viability with and without arabinose, and LptG P282S was found and confirmed as a mutant that required arabinose to grow.

Thereafter, a selection was carried out for suppressors of LptG P282S lethality at low levels (**Figure 14**). After promoter mutants that restored high expression levels were eliminated, the class of mutations that emerged and was confirmed consisted of various impairments in fatty acid biosynthesis (FAB). Of these, we focused on loss-of-function mutants in FabH for future work, because this gene can be deleted entirely, and revertants to wt FabH cannot arise, which is not the case for point mutations in essential FAB genes.

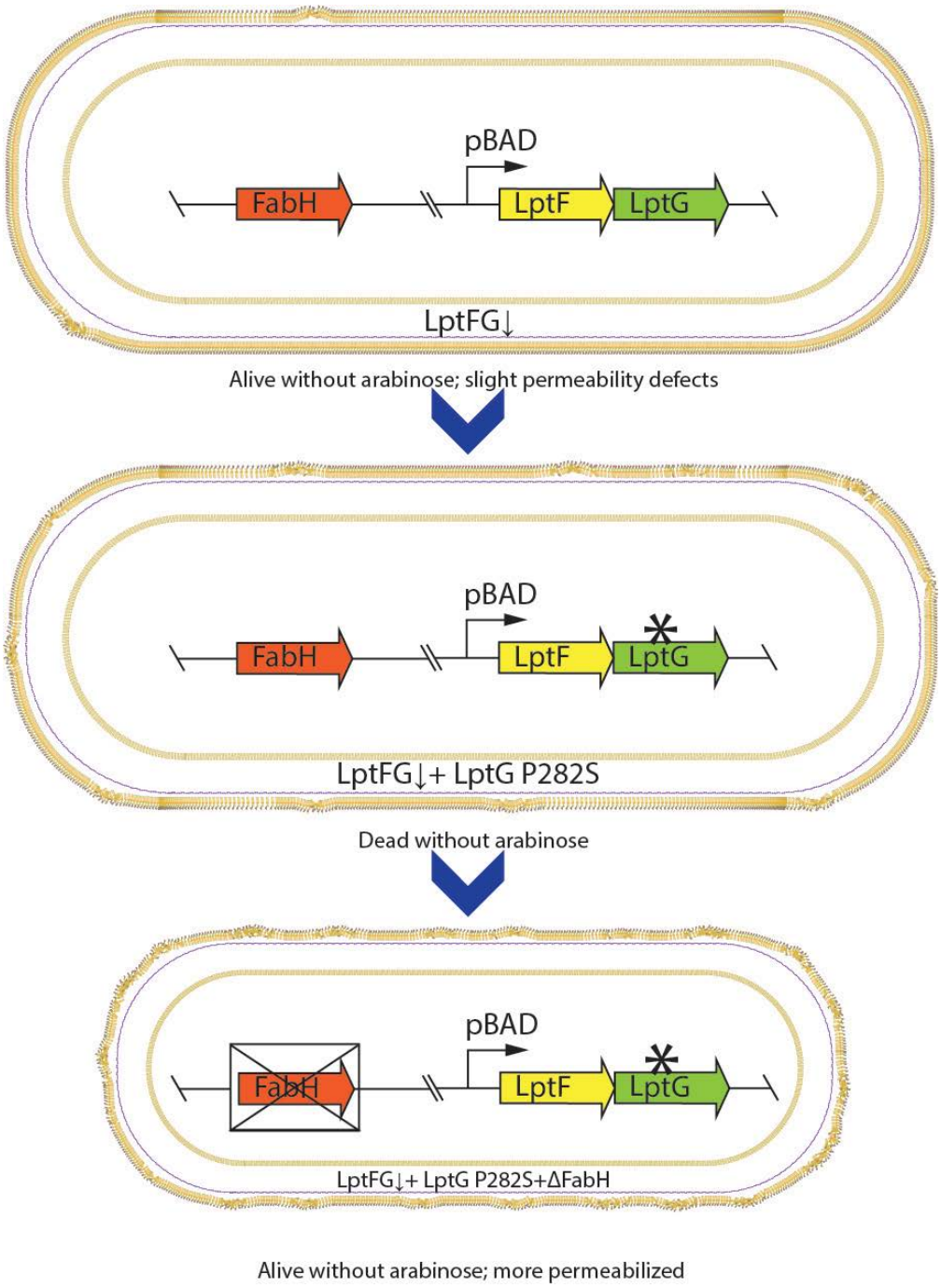

Figure 14 Chronology of discovery of suppression of Lpt defects by FabH. Cells without FabH are physically smaller and grow at a slower rate, which likely enables them to cope with impaired LPS biogenesis. Because FabH removal alters the chemical composition of cellular lipids, cells lacking FabH are permeabilized.

The protective effect is clear and was also confirmed for other genes in LPS transport and biosynthesis. For instance ΔfabH::kan allele suppresses the conditional synthetic lethality that lptD4213 and lptFV48S exhibit (Natividad Ruiz; unpublished data). The lptD4213 lptFV48S double mutant cannot grow in LB but it grows in minimal media. The triple mutant lptD4213 lptFV48S ΔfabH::kan can grow in LB and minimal media. Also, ΔfabH suppresses lethality in LB at 42 degrees of an LpxL temperature-sensitive mutant(Natividad Ruiz; unpublished data). In addition, resistant mutants to LpxC inhibitors map to FAB genes[14].

However, it is not known for certain why fatty acid biosynthesis impairments confer a protective effect against interference with LPS biogenesis. Nonetheless, a plausible explanation exists, and it is of some importance for the work described below.

It has been found that cells with impairments in fatty-acid biosynthesis are much smaller in size and grow slower than their WT counterparts, a trend that is independent of nutrient availability. Thus, their growth requires a substantially smaller amount of membranes and, consequently places lower demands on processes that produce those membranes. Thus, they would seem to be able to cope with impairments in OM assembly that would kill a cell that was going full throttle. Notably, this explanation would seem to suggest that not only LPS biogenesis, but also Bam and Lol pathways may be more resistant to inhibition in cells with impaired fatty acid biosynthesis. However, there are no validated small molecule inhibitors of those pathways to test that quickly, and genetic experiments, which are lengthier, were not attempted.

However, lowering susceptibility to compounds by slowed growth carries the risk of low specificity for a given pathway. In fact, in order for the binding of an antibiotic to its target to result in cell death, it is often necessary that a cell be actively growing and dividing so that the

lack of the target's activity may be felt. This is evidenced by the fact that, in the extreme case of lowered growth rate, it seems that it is dormant cells that are responsible for the phenomenon of persistence: a higher level of antibiotic tolerance by a subset of cells that are genetically identical to the rest of the population[15]. In any case, it is established quite firmly that β-lactams, antibiotics that inhibit the crosslinking of the peptidoglycan skeleton have less of an effect, the slower a cell grows[16]. Thus, a probability of a similar non-specificity existed for impairments of fatty acid biosynthesis.

However, fortunately, impairing fatty acid biosynthesis does not only slow growth, but also alters the lipid composition of membranes, resulting in greater permeability of cells to small molecules in general (**Figure 15**). Thus, most classes of known antibiotics are actually more effective against cells with impaired fatty acid biosynthesis than against cells with intact fatty acid biosynthesis. This is a substantial advantage because hits obtained by this method would have to be more active against cells with fortified membranes than against cells with permeabilized membranes. Thus, hit molecules must not only be ahead of off-pathway compounds, but also must overcome a handicap.

Strain and MIC, µM / Drug	*E. coli*	*E. coli* ΔFabH
Meropenem	0.16	0.09
Aztreonam	0.20	0.10
Ciprofloxacin	0.05	0.05
Nalidixic Acid	19.00	9.60
Novobiocin	125.00	75.00
Coumermycin A1	20.00	5.00
Azithromycin	12.00	5.00
Minocycline	0.89	0.89
Furazolidone	5.20	2.30
Rifampicin	8.00	8.00
Chloramphenicol	14.00	14.00
Moenomycin	27.66	3.80
Vancomycin	150.00	18.00
Ramoplanin	200.00	29.39
Linezolid	>300	300.00

Figure 15 Removal of FabH sensitizes E. *coli* to a variety of antibiotics, and does NOT confer a broad "slow grower" protection.

However, an important difficulty arises with any approach of this type that was alluded to in earlier, namely concentration dependence. As the dose-response shows, FabH deletion rescues cells from the effects of LpxC inhibitor at a certain range of concentrations (**Figure 16**). At a low level of compound, neither strain is affected, while at a high level of compound, both are inhibited. There is no way to fully resolve that problem when screening at a single dose in high throughput.

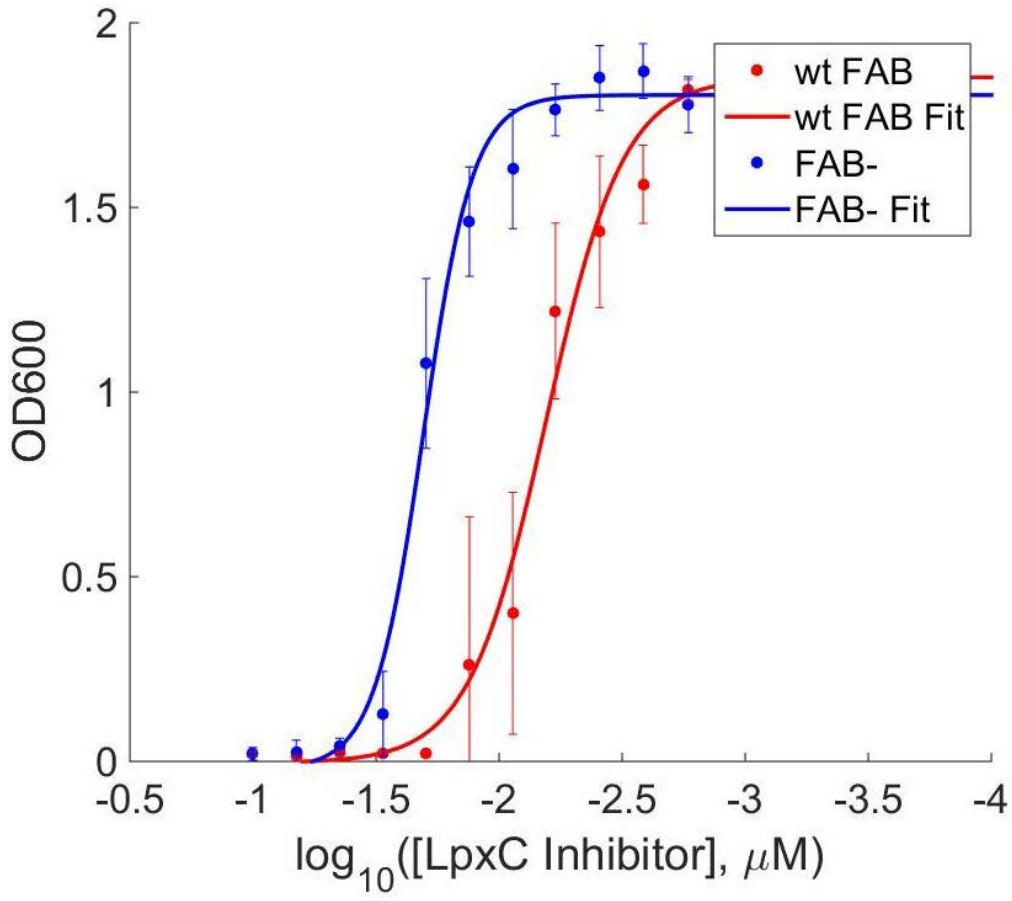

Figure 16 Removal of FabH renders cells less sensitive to LpxC inhibitor

Pushing the concentration to the high end will return more false-positives that will inhibit both strains at high concentrations, but only ΔFabH at lower values. Pushing the concentrations toward lower values would return more false-negatives which would have had the desired behavior at higher concentrations. The first approach is more inclusive, and the second approach is more selective. Because, as the following section will illustrate, most small molecules in screening libraries will not inhibit bacterial growth, the first approach made more sense, and we proceeded to screen ~700,000 compounds using it.

2.2 High-throughput antibacterial screening

Even though our assay relied on comparative susceptibility of two strains, and we began the screen by testing two strains in parallel against library compounds, we quickly switched to screening only the FabH-WT strain in the primary assay. The main reason for doing so has been that there were just not very many anti-*E. coli* compounds in screening libraries. Therefore, there was no point in exposing both kinds of strains to tens of thousands of inactive compounds and thereby wasting both time and supplies.

Thus, we screened ~700,000 compounds at the highest possible concentration, which was either 50 μM or 25 μg/ml, depending on the specific library. After defining primary hits as compounds that produced a final OD600 lower than three standard deviations under the mean, we ended up with about 1300 primary hits, about 0.18% hit rate (**Figure 17**). This is not a high hit rate, and this is the hit rate for antibacterial activity only – the hit rate for compounds that are less active against ΔFabH is much lower as what follows will show.

All in all, the lesson taught by this experience is that when screening for novel antibacterial compounds, it is best not to deploy intricate and costly assays for pathway specificity at the primary high-throughput stage. Intricate assays will suffer from the general sloppiness that is an inalienable attribute of high-throughput screening. At the same time, costly assays will be wasteful at this stage due to a low hit rate. Rather, as the following sections will illustrate, it is better to initially select the antibacterial compounds by using a simple, cheap, and easily scalable assay, and to look for pathway specificity later within that subset.

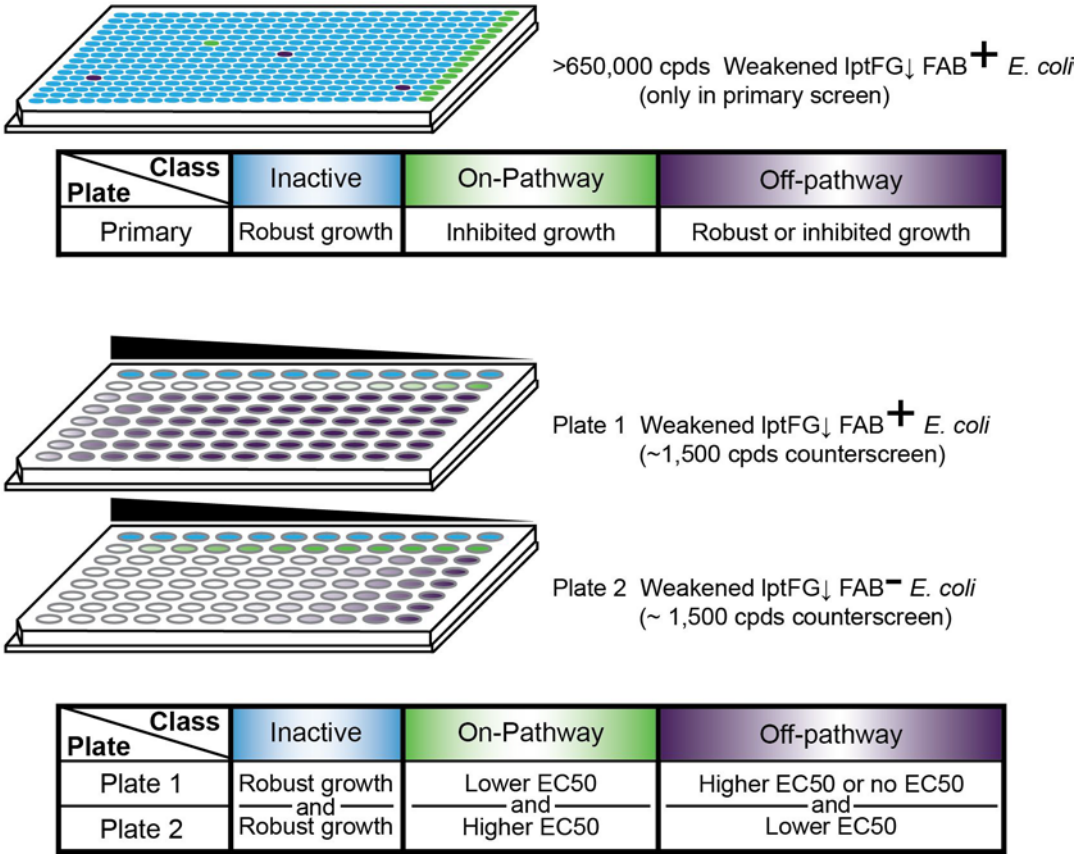

Plate \ Class	Inactive	On-Pathway	Off-pathway
Primary	Robust growth	Inhibited growth	Robust or inhibited growth

Plate \ Class	Inactive	On-Pathway	Off-pathway
Plate 1 and Plate 2	Robust growth and Robust growth	Lower EC50 and Higher EC50	Higher EC50 or no EC50 and Lower EC50

Figure 17 High-throughput screening workflow for *E. coli.* Primary screen was carried out against LptFG↓ FabH-WT *E. coli* only in order to save time and supplies. Approximately 1,500 candidate compounds produced by that effort were counterscreened in dose-response against LptFG↓ FabH-WT *E. coli and* LptFG↓ ΔFabH *E. coli*.

2.3 Primary follow-up

As the primary screening progressed, we have been confirming antibacterial activity of primary hits, ordering additional quantities of confirmed hits and testing them in dose-response against FabH-WT and ΔFabH strains. We also ordered analogues of hits that could not themselves be ordered due to depleted supplies. In addition, we ordered commercially available analogs of confirmed hits, which were tested against FabH-WT and ΔFabH strains to explore SAR.

These efforts yielded ~50 cores that had a greater activity against WT-FabH than against ΔFabH. Thus, the overall hit rate from screening was about 70 ppm, which is quite low. However, having obtained additional quantities of commercially available antibacterial hit compounds, we produced our own screening library of ~1200 compounds from them, which has already been useful in many ways.

2.4 Methods for Chapter 3A

2.4.1 Methods for the determination of MICs of various screening backgrounds and ΔFabH

All bacteria were grown for 2 hours in a shaker after 1:100 dilution of an overnight culture, and were normalized to OD600 of 0.0001 and used to inoculate microplates with concentration gradients of appropriate antibiotics. Plates were incubated overnight without shaking and were read for OD600 after 24 hours. MICs were determined as the lowest concentrations at which OD readings reached the lowest values that no longer decreased with increasing concentration.

2.4.2 Methods for measuring susceptibility of FabH+/- strain pair to the LpxC inhibitor

The strains (NR1099 = NR754 LptFG↓ and NR2015 = NR754 LptFG↓ ΔFabH) were grown for 2 hours after 1:100 dilution of overnight cultures, and were normalized to OD600 of 0.0001 and used to inoculate microplates with concentration gradients of LpxC inhibitor (PF-5081090). Plates were incubated overnight and were read for OD600 after 24 hours.

2.4.3 Methods for the high-throughput screening procedure and confirmation/counter-screen

Screening relied on using 1,000x frozen aliquots of log-phase NR754 LptFG↓ in order to save time. These aliquots were prepared as follows. A dilution (1:100) of an overnight culture is grown for two hours. The cells are harvested and washed once in fresh LB. Then, the cells are

resuspended in a volume of LB with 15% glycerol that will accomplish an OD600 of 0.2 and frozen at -80 degrees. For screening, these aliquots are thawed and diluted 1:1,000 into fresh LB.

Assay microplates are filled with 30 µL of LB, and 300 nL of compound stocks is transferred into them by a robotically manipulated pin tool. 30 µL of culture is added to them to achieve a starting OD600 of 0.0001 as well as positive control in the form of LpxC inhibitor, and the plates are incubated for 22.5 hours at 37 ˚C without shaking and read for OD600.

Whenever possible, hits were confirmed for antibacterial activity using small volumes provided as cherrypicks by the ICCB-L screening facility

Powders of confirmed hits were reordered from suppliers whenever available, and tested against NR754 LptFG↓ and NR754 LptFG↓ ΔFabH in dose-response between 150 µM and 1 µM in a log2 gradient according to the MIC protocol above.

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Chapter 3
A novel inhibitor of Phenylalanine tRNA synthetase in *E. coli* and *A. baumannii*

3.1 Using spectrum of activity to profile screening hits

The most experimentally straightforward and cheap way to characterize primary hits for an antibacterial candidate is to measure their antibacterial activities in a variety of species and mutants. Little of this information is known directly from the primary screen because a weakened strain usually serves as the basis for cell-based assays, and, obviously, there are no cells in *in vitro* based assays.

As far as the selection of strains for such testing is concerned, it is generally informative to include a variety of bacteria that have an outer membrane with LPS (Gram-negatives) and of those that do not (Gram-positive and mycobacteria), as well as mutants impaired in efflux and mutants with compromised permeability barriers.

Intuitively, it may appear that the best antibiotic candidates are the compounds that have the highest activity against the most strains: after all, the more bacteria a compound can kill the better. However, that is only true if the compound does not kill the patient as well. Unfortunately, compounds that emerge from screening libraries and have high broad-spectrum antibacterial activity are most often nonspecific in their action and broadly toxic to all life forms.

This consideration may also suggest testing primary hits against toxicity to mammalian cells to rule out toxic compounds. However, that is usually inadvisable at an early stage before the target

is known. This is so because a hit compound may often have more than one activity, and it may be possible to eliminate mammalian toxicity while preserving antibacterial activity. Furthermore, when looking for inhibitors of targets with no existing inhibitors, even compounds that have mammalian toxicity are valuable because they still allow the study of the novel target in bacteria and may thus suggest new ways to look for inhibitors of the novel target. Thus, testing for mammalian toxicity at an early stage is likely to lead to discarding of potentially valuable compounds.

A comparison of antibacterial activities of a compound against bacteria that have an outer membrane with LPS (Gram-negative) and those that do not (Gram-negative mutants lacking LPS, Gram-positives, and mycobacteria) is likely to be informative in a number of ways.

On the one hand, compounds that are more effective against bacteria without an LPS-containing outer membrane are typically in the majority. That is the case primarily because an outer membrane with LPS is a formidable permeability barrier, and compounds have a hard time crossing it no matter what their target is. Thus, the set of compounds that are less active against bacteria with LPS-containing OM than against the rest is not highly enriched in compounds of interest. Although inhibitors of characteristically Gram-positive targets (for example, wall teichoic acids) would be in this set, they would be lost among many generally toxic compounds.

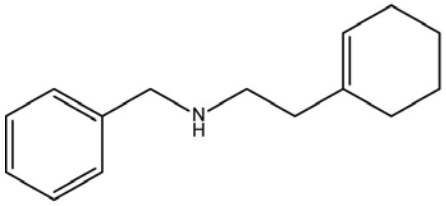
On the other hand, compounds that are more effective against bacteria that have an outer membrane with LPS than against those that do not will be far fewer in number, and, therefore, far more interesting. That is the case because there are not many mechanisms that enable compounds to have a stronger antibacterial effect on a strain with a formidable permeability barrier than on a strain without one. Such compounds either act directly on the permeability

barrier or pathways that assemble (more likely for LPS-deprived vs. LPS-containing Gram-negatives of the same species, **Figure 10**), or act on targets that are evolutionarily divergent (more likely for different species). Either way, this susceptibility pattern implies that the action of these compounds has to be clean and cannot be due to non-specific toxicity; therefore, such compounds deserve further study.

Thus, one of the follow-up assays performed on the antibacterial compounds found in the screen has been to compare their activities against Gram-negative and Gram-positive bacteria to try to find inhibitors of OM assembly.

3.2 The identification of the primary hit

As a result, a compound was found (**Figure 18**) that was active against *E. coli*, *A. baumannii*, and *Enterobacter*, but inactive against *S. aureus* and *B. subtilis*. Furthermore, the structure of the compound was a rather simple secondary amine, which, if it acted through general toxicity, would have been far more effective against the more permeable Gram-positives.



Bacterial Strain	MIC, μM
E. coli	50
A. baumannii	100
M. tuberculosis	150
S. aureus COL	>>150
S. aureus Newman ΔdltA	>>150

Figure 18 Hit compound with higher Gram-negative than Gram-positive activity

3.3 Target identification using resistant mutants

Perhaps one of the most elegant and unambiguous indicators of the cellular target of an antibacterial compound is the observation that it is sufficient to mutate one gene that codes for a target in order to confer resistance to the antibacterial effect. In particular, if the level of resistance thus conferred is high, the molecule is likely to be highly selective for its target.

Of course, there is also a downside to such observation, namely, if it is possible to generate high-level resistance to a molecule, its utility as an antibiotic may be limited. However, that conclusion may be premature because there is often a fitness cost to resistance[1], and resistant mutants obtained in the laboratory have often been known to be impaired in virulence[2]. Furthermore, when looking for an inhibitor of a target for which no inhibitors exist, this is a secondary consideration.

However, the approach for raising resistant mutants is not without its pitfalls. There are multiple reasons why it may not yield the results that are as clear as one may hope. First, it is possible to get resistant mutants that acquire resistance not through a mutation in the target, but through a mutation that upregulates efflux pumps or alters the specificity of efflux pumps. Of course, such mutants are not very informative. Second, it is often possible to get mutants in genes tangential to the target, which provides indirect information, and so is more useful than resistance through generic detoxification, but it does not reveal the actual target. Of course, such outcomes are not surprising because the cells are under a selective pressure to alleviate the antibacterial effect of a compound in the most efficient way possible, not to inform the investigator about the

compound's mode of action. Third, it is possible that no resistance at all develops to a compound. That, in turn, could be caused by a number of mechanisms.

First, it is possible that the compound has no specific target in which resistance could develop, but is generally toxic. That is an unattractive scenario, but a common one nonetheless. Second, the compound could have two essential targets, in which case it would be necessary to acquire resistance in both at the same time. If the frequency of resistant mutations is 10^{-8} for each of them, it is 10^{-16} for both at the same time – a very low likelihood event. Third, it may be that the compound is so well suited to the target that no straightforward resistance acquisition is possible, or that the target is such that it loses its function once it is altered. This seems to be the case with Moenomycin A in Gram-positives[3].

Thus, instead of attempting to characterize our secondary amine using specialized methods, we chose to perform an unbiased genetic selection for resistance and sequence the resulting mutants; something that we did for other compounds as well, but it is for this one that the approach has been particularly effective. Instead of returning mutations that affect general detoxification and efflux, it returned mutants in Phenylalanine tRNA synthetase in both *E. coli* and *A. baumannii* (**Figure 19**). In addition, all of the resistant mutants were insensitive to the compound up to 0.5 mM, which implies that its activity is entirely on target.

Species	Mutation in PheS	MIC, μM
E. coli	A294S (GCC->TCC)	>>500
E. coli	F250I (TTT->ATT)	>>500
E. coli	F250L (TTT->TTA)	>>500
E. coli	G130R (GGG->AGG)	>>500
E. coli	G172D (GGC->GAC)	>>500
E. coli	I175N (ATC->AAC)	>>500
E. coli	I175S (ATC->AGC)	>>500
E. coli	V173L (GTA->TTA)	>>500
E. coli	V275E (GTG->GAG)	>>500
E. coli	V279E (GTG->GAG)	>>500
A. baumannii	G171C (GGT->TGT)	>>500

Figure 19 Resistant mutants to the compound converge on the same target in two species and confer a high level of resistance, which implies that the compound is not generally toxic

3.4 Location of mutations within the protein's crystal structure in E. coli

Furthermore, it produced a variety of different mutations in the *E. coli* protein, for which a crystal structure has been solved[4], which allowed us to map them (**Figure 20**).

Phenylalanine tRNA synthetase is one of the largest and most complex tRNA synthetases[5], and it has a dimer-of-dimers $(\alpha\beta)_2$ organization, wherein PheS is the α subunit, and PheT is the β-subunit. PheS is responsible for the first step of the reaction, which is the activation of the amino acid, and PheT joins in the second step of recognizing $tRNA^{Phe}$ and transferring the amino acid onto it (**Figure 20**). Notably, a tRNA molecule interacts with both heterodimers: the acceptor region interacts with the PheS active site in one heterodimer, while the anticodon loop is recognized by PheT of the other heterodimer in the dimer of dimers[6].

Phenylalanine-binding pocket contains some hydrophobic and some hydrophilic areas that properly orient the $–NH_2$ and –COOH groups of phenylalanine and pheylalanine-AMP for the reactions (**Figure 21**). Two phenylalanine residues in the enzyme (F248 and F250) interact with the phenylalanine substrate in an edge-to-face manner[7].

As **Figure 22** and **Figure 23** show, all of the mutations map around the phenylalanine recognition pocket, suggesting that it is where the drug binds. Interestingly, one of the mutations found (A294S) has been previously described as altering substrate specificity of the enzyme[8], which further indicates that the drug binds in the phenylalanine-binding pocket. Another pair of mutations F250I and F250L maps to one of the two phenylalanine phenylalanine-recognizing residues.

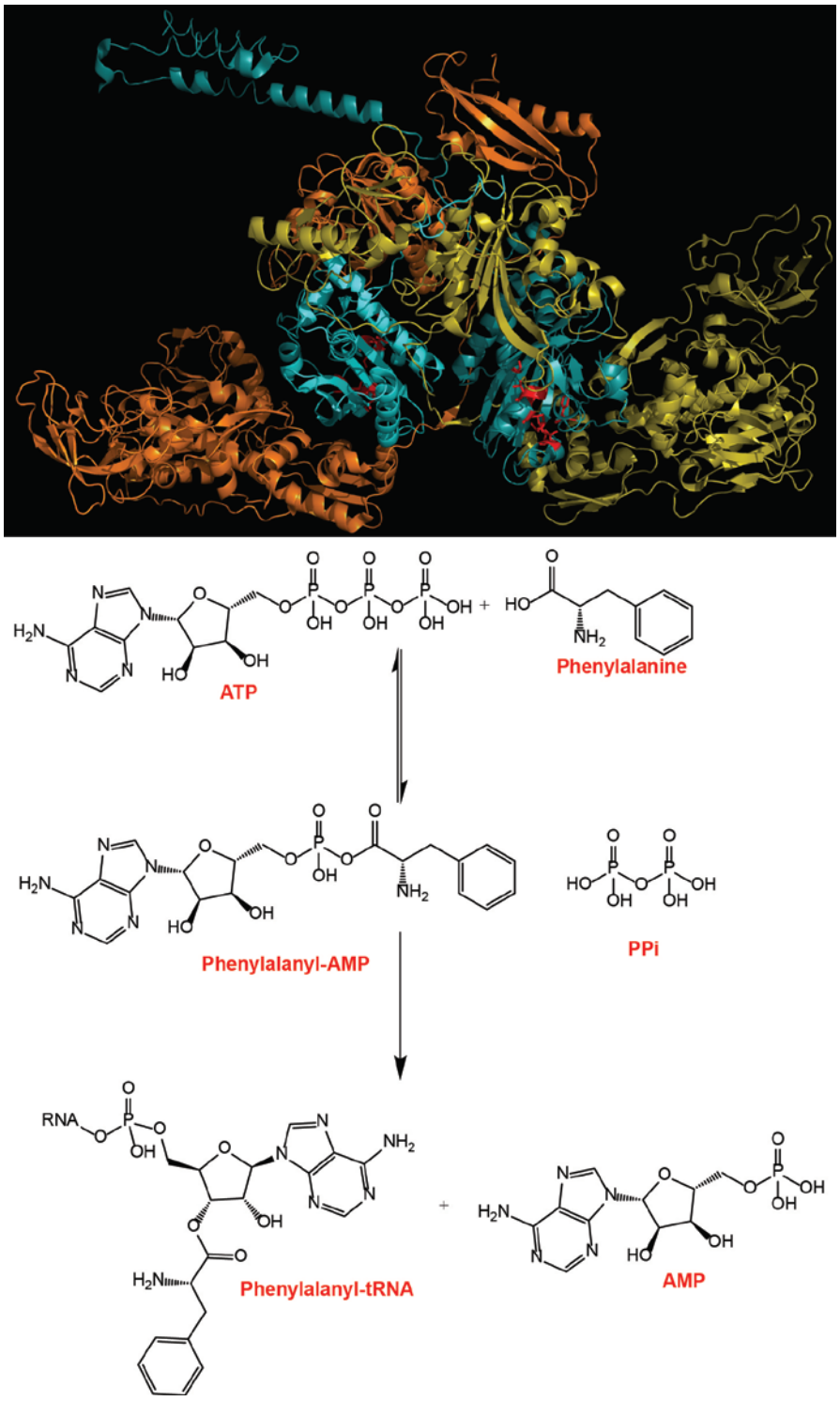


FIGURE 20 Structure of the *E. coli* PheRS in its $(\alpha\beta)_2$ architecture and the reaction performed by it. Residues in positions that correspond to mutations that confer resistance are highlighted in red.

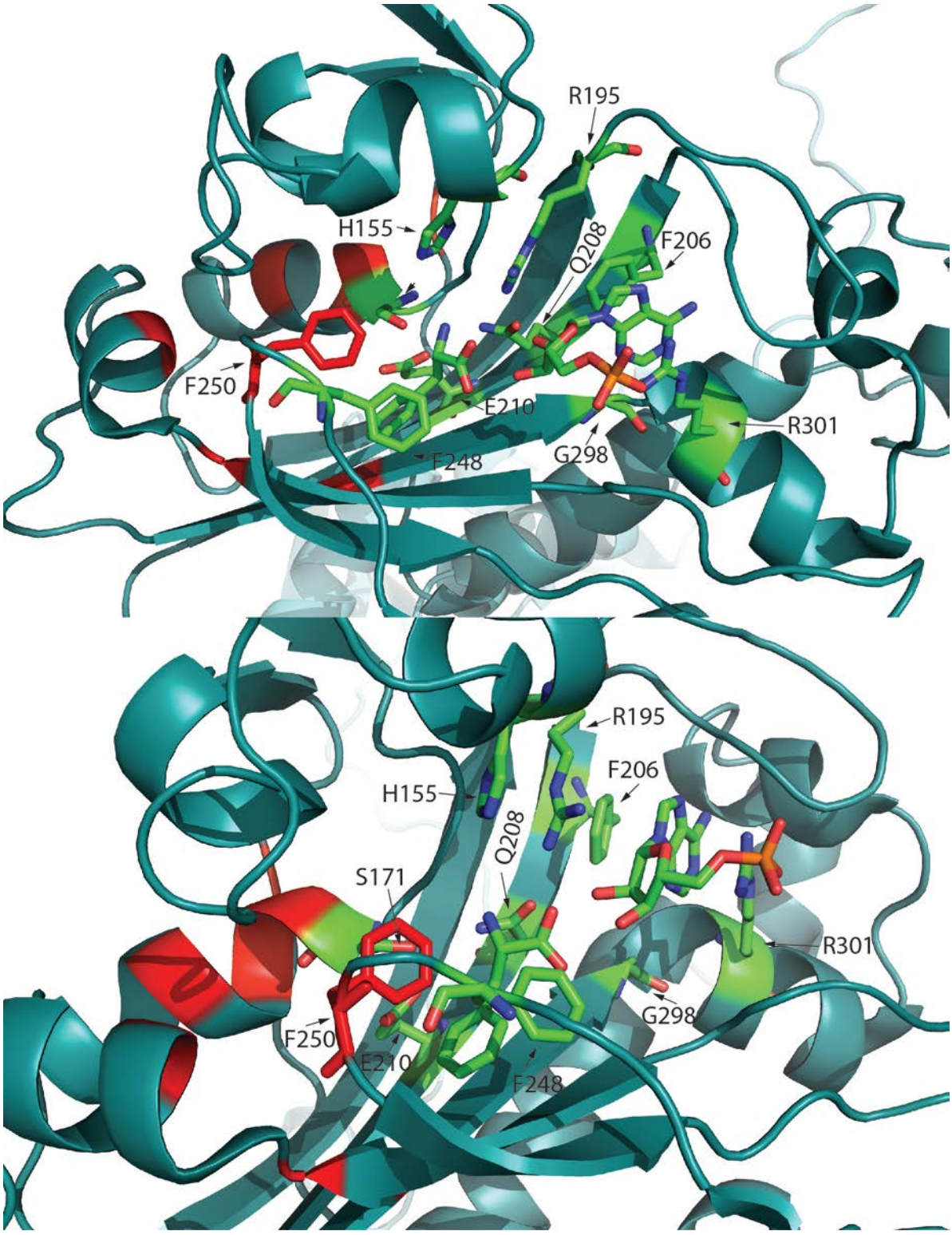


Figure 21 Active site residues of E. coli PheRS responsible for proper positioning of phenylalanine and ATP.

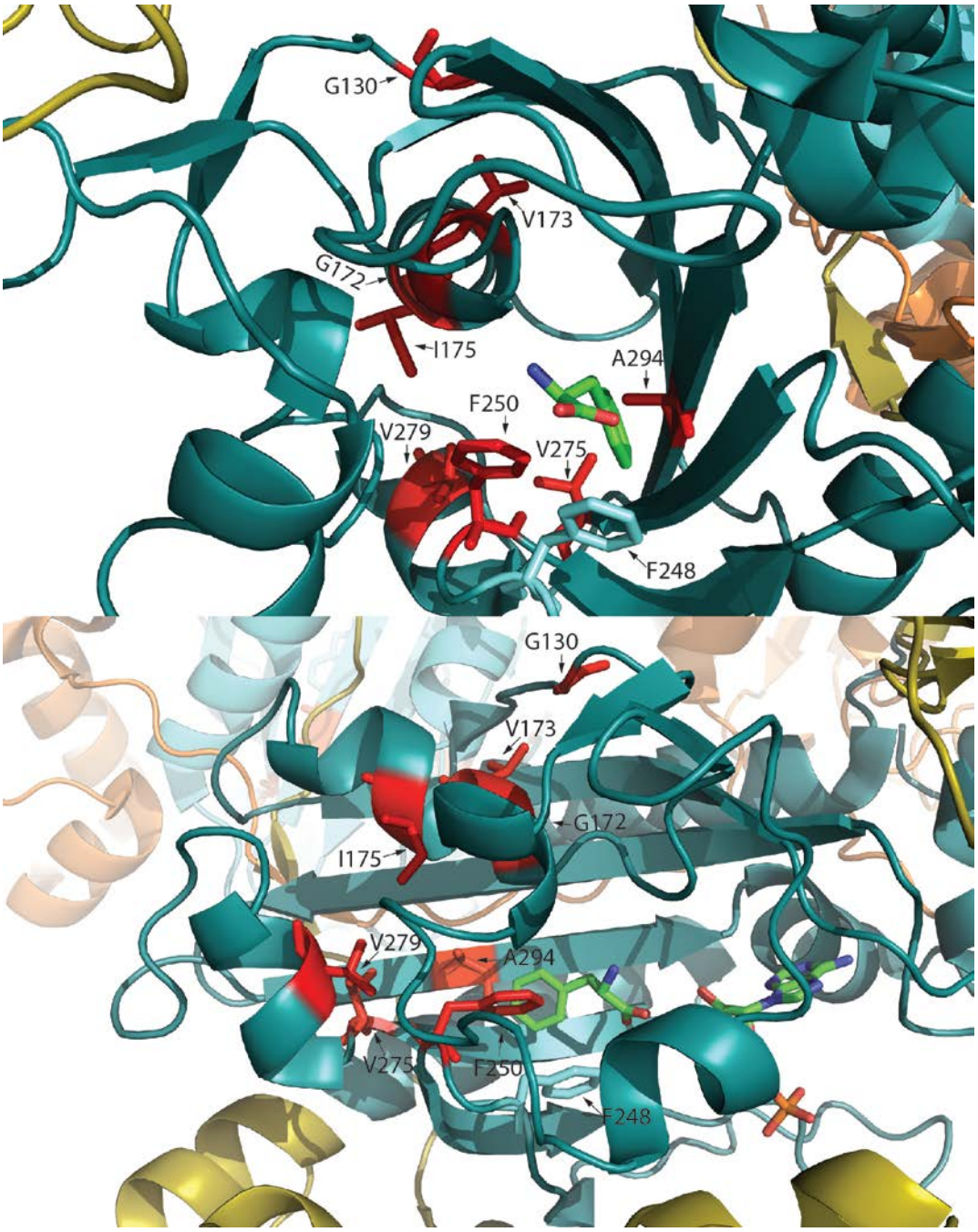


Figure 22 Phenylalanine binding pocket of *E. coli* PheRS. Residues in positions that correspond to resistance mutations are highlighted in red. One of them is a phenylalanine residue that is a part of a pair of phenylalanines that recognize the incoming phenylalanine (F250). The other residue of the pair is F248. Bound phenylalanine is colored according to its constituent atoms. Bound AMP is omitted from the top image for clarity.

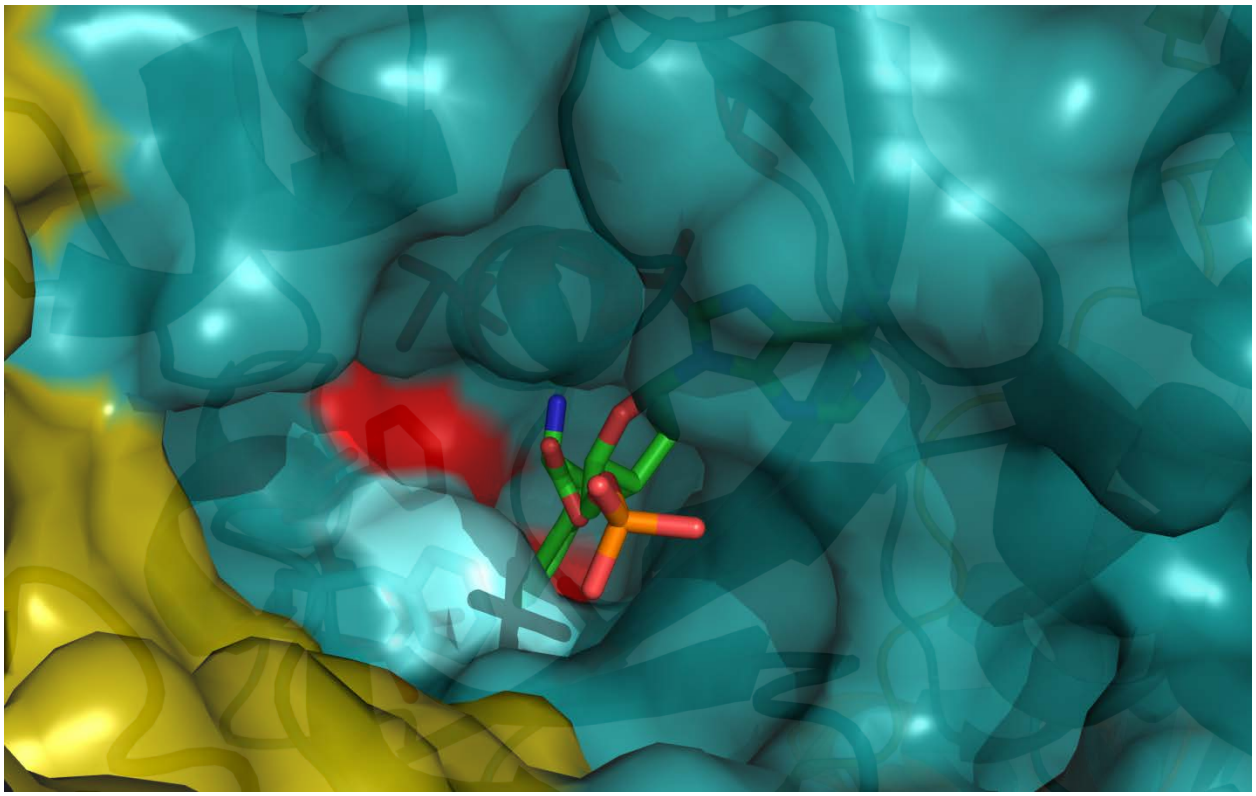
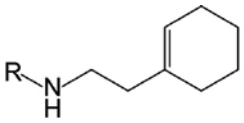


Figure 23 Protein surface with phenylalanine and AMP bound. Residues that correspond to resistance-conferring mutations are highlighted in red. Only two of them F250 and A284 form the lining of the pocket. The rest form a part of the scaffold for the pocket.

3.5 SAR studies of the hit scaffold in vivo

Due to simplicity of the structure, we were able to perform SAR at an early stage and found that activity of the molecule is completely lost if any variation is introduced into the benzylamine portion (**Figure 24**). In particular, only one ortho-methyl substitution is tolerated, and even it diminishes activity. Having two ortho methyls abolishes it altogether; presumably, the single o-methyl group has to face away from the binding pocket. All tested substitutions on the benzyl ring in the meta and para positions abolish activity; so does introduction of heteroatoms into the benzyl ring, addition of extra carbons between the nitrogen and the benzyl ring, and methylation of the benzylic carbon. This indicates that SAR is very tight around that area and that it mimics phenylalanine in the binding pocket.

Variations on the benzylamine side negatively affect activity



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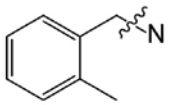
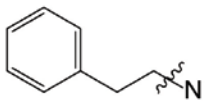
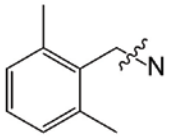
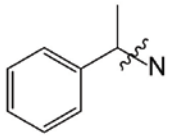
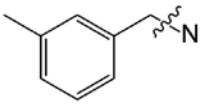
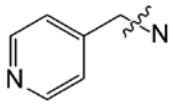
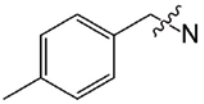
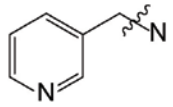
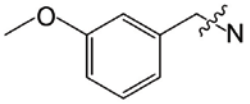
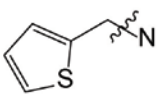
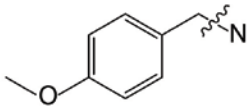
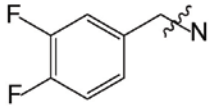
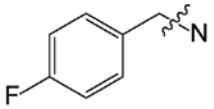
	90		>>150
	>>150		>>150
	>>150		>>150
	>>150		>>150
	>>150		>>150
	>>150		>>150
	>>150		

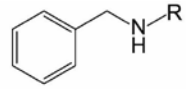
Figure 24 Structure-activity relationship indicates that preservation of the benzylamine side is necessary for activity

Amino-acyl tRNA synthetases (AaRSs) have long been considered an attractive class of antibiotic targets[9]. Their ubiquitous nature, high degree of conservation within a broad spectrum of bacterial species and considerable divergence between prokaryotic and eukaryotic enzymes have made AaRS's the subject of numerous antibacterial programs. (However, the only tRNA synthetase inhibitor to reach clinical use is the natural product mupirocin[10,11], which is effective only against Gram-positive bacteria.) Thus, we sought to investigate if compounds similar to

ours have been previously characterized for this purpose. A variety of small molecules have been tested as candidate inhibitors of the prokaryotic enzyme. However, the only scaffold close to our class has been a set of analogs of benzphetamine (studies in mid-1970s), and the strongest of those has been norbenzphetamine[12,13]. Thus, we sought to compare the activity of our compounds to that of norbenzphetamine. Initially, we saw no antibacterial activity for norbenzphetamine using our standard protocol; however, when we tested it in minimal media as the authors did, we observed weak antibacterial activity with an MIC of 150 μM. It is likely that phenylalanine in rich media suppresses the antibacterial effect of norbenzphetamine. Thus, it is advantageous for our class of inhibitors that they are not susceptible to such influences of rich media.

Notably, because similar types of molecules have been used clinically, i.e. benzphetamine, prospects are good for safety of this class of drugs as well. In fact, we tested the original hit against Vero cells, and no toxicity was observed up to 150 μM.

We have also explored SAR on the cyclohexene-yl side of the amine (**Figure 25**). There is SAR there, but it appears to be a lot less clear-cut: it is possible to remove the double bond and still retain activity. It is also possible to replace the six-membered ring with a seven-membered one and retain a diminished level of activity, but replacing it with a five-membered ring abolishes it altogether. If one, rather than two, methylene groups are between the amine and the cyclohexene, the activity is abolished. It is possible to replace cyclohexene with norbornane and retain a diminished level of activity, but not with norbornene. All in all, it was unclear what the SAR is exactly for that part of the molecule.

SAR is not clear on the non-benzyl side 

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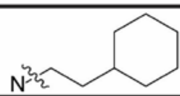
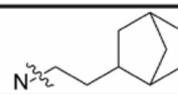
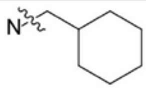
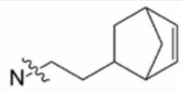
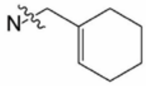
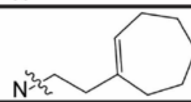
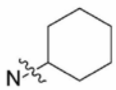
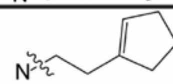
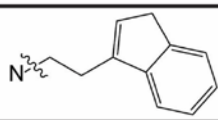
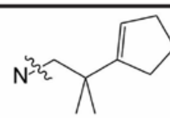
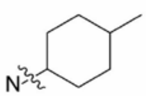
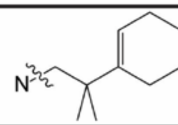
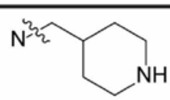
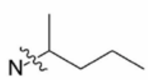
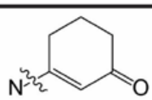
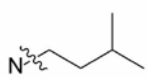
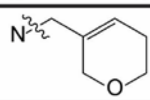
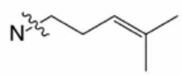
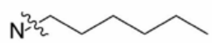
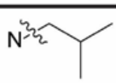
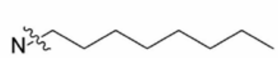
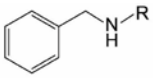
	91		150
	>>150		>>150
	>>150		150
	>>150		>>150
	>>150		>>150
	>>150		>>150
	>>150		>>150
	>>150		>>150
	>>150		>>150
	>>150		>>150
	>>150		>>150

Figure 25 Structure-activity relationship for the non-benzylamine side of the inhibitor: there is some flexibility, but only within a range of structures.

However, we knew that α-methylation improved activity significantly for the benzphetamine-derived compounds[12], and we explored the effects of methylation on our class of compounds. Interestingly, α-methylation improved its potency dramatically for one enantiomer, and

abolished it completely for the other (**Figure 26**). Furthermore, α-methylation bestowed activity upon several structures that were inactive otherwise.

SAR for the non-benzyl side 

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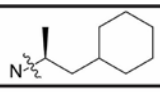
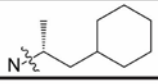
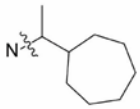
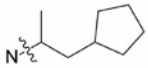
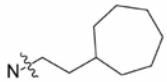
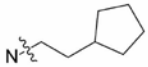
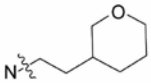
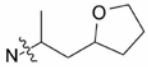
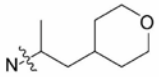
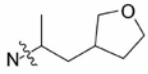
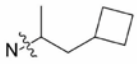
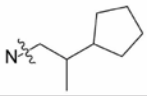
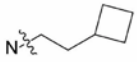
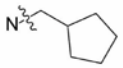
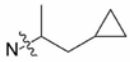
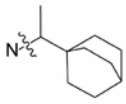
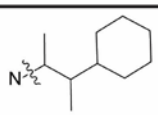
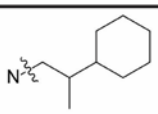
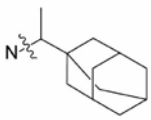
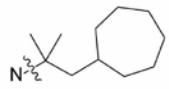
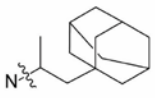
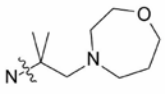
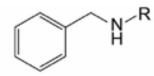
	15		>>150
	90-50		91
	150-91		>>150
	>>150		>>150
	>>150		>>150
	>>150		>>150
	>>150		>>150
	>>150		~300
	~300		150
	~250		>>150
	~250		>>150

Figure 26 α-methylation improves activity in a sterospecific manner

At the same time, multiple aromatic and heterocyclic analogs were inactive (**Figure 27**):

SAR for the non-benzyl side 

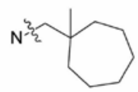
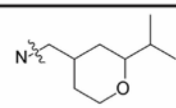
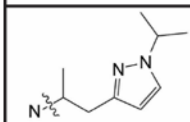
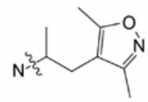
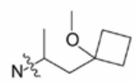
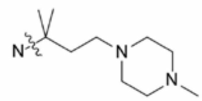
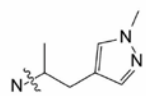
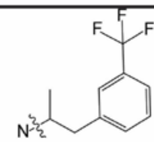
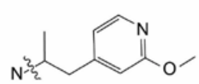
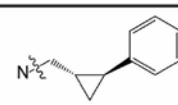
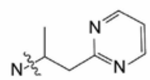
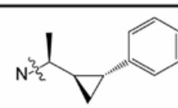
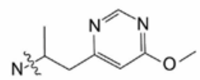
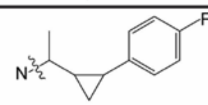
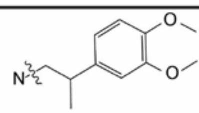
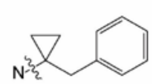
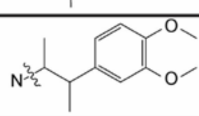
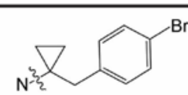
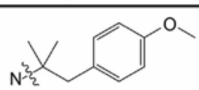
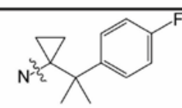
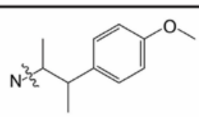
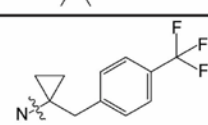
R group structure	WT *E. coli* MIC, μM	R group structure	WT *E. coli* MIC, μM
	>>150		>>150
	>>150		>>150
	>>150		>>150
	>>150		>>150
	>>150		>>150
	>>150		>>150
	>>150		>>150
	>>150		>>150
	>>150		>>150
	>>150		>>150
	>>150		>>150

Figure 27 Structure-activity relationship indicates multiple inactive scaffolds to avoid

These improvements indicate that it would be possible to improve the antibacterial properties of the molecule by focusing on the non-benzylamine side. We also aim to solve a crystal structure

of the molecule in complex with the protein in order to guide our medicinal chemistry efforts. From the structure in **Figure 23**, without performing any computational docking, it is possible to see that, assuming that the benzylamine binds in the same ways as phenylalanine, the other side of the amine cannot reach the adenosine-binding pocket. Moreover, it is much too different from adenosine to bind there and making it more like adenosine (introducing heteroatoms) abolishes activity, which indicates that it is more likely to bind the pocket next to the phenylalanine binding pocket and opposite the AMP binding site that is formed by the opposite sides of F248 and F250 as well as by P247 and I145 (**Figure 28**). If that is indeed the case, it may be possible to identify a branching point where the compound may be modified to extend into the ATP binding site. However, it is important to keep in mind that, while increasing enzyme binding, it is equally important to maintain the ability of compounds to penetrate cells.

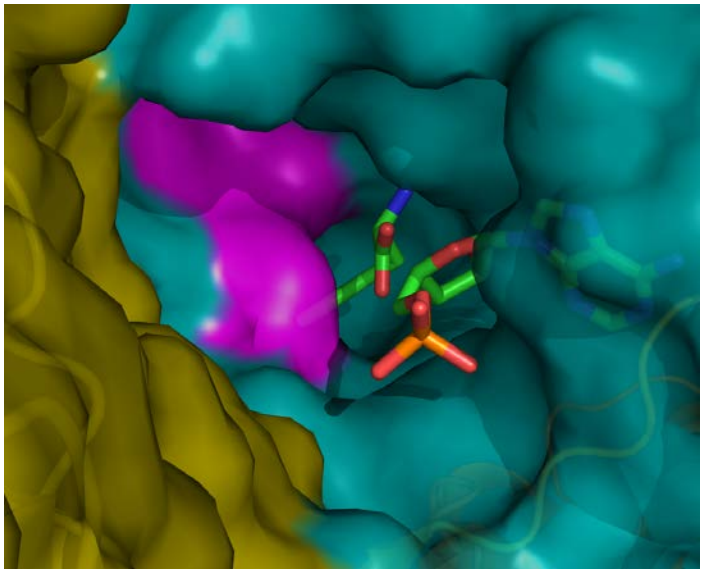


Figure 28 A hydrophobic patch (pink) outside of the phenylalanine binding pocket which may bind the non-benzylamine part of the inhibitor

3.6 Biochemical characterization with the target in vitro

In order to evaluate the ability of these compounds to penetrate cells, we correlated whole-cell activity with on-target activity against the enzyme. We purified the E coli PheST enzyme in both WT and F250L resistant forms according to a previously published protocol [14]and reconstituted its activity *in vitro* using a method described by the lab of Paul Schimmel[15]. We found that resistant enzyme was not inhibited by the compound up to 400 μM, whereas the WT enzyme was inhibited at 12 μM. Because MIC against WT E coli is between 30 and 70 μM, it appears that incomplete penetration limits the effectiveness of the compound (**Figure 29**). We also tested the improved analog, and found that it inhibits the WT enzyme at below 0.5 μM (compared to 15 μM MIC against E coli). This indicates that gain in antibacterial activity is due to increased activity against the target as well as that penetration limits the effectiveness of this compound as well.

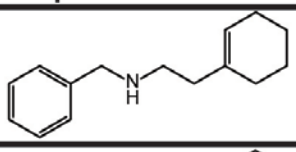
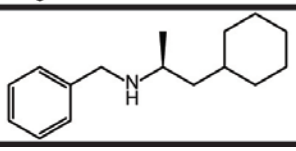
Enzyme / Compound	*E. coli* WT PheRS	*E. coli* PheRS F250L
	IC50 6 μM	IC50 >>400 μM
	IC50 0.0625 μM	IC50 >> 270 μM

Figure 29 Biochemical reconstitution reproduces *in vivo* observations

3.7 Resistance to this class of inhibitors

A valid objection to this class of compounds is that resistance may develop rapidly in the clinic. In addition to the aforementioned possibilities of different fitness of resistant mutants in the laboratory and in a patient, it is definitely possible to use a combinatorial strategy to avoid the

development of resistance, and we have obtained preliminary proof of it. Recently, Glaxo Smith Kline tried to introduce a leucyl tRNA synthetase inhibitor for use against Gram-negatives, AN3365[16,17]. This compound had good potency but failed in Phase II due to a high frequency of resistance. There are good indications that we can overcome the high frequency of resistance by employing two compounds that target different tRNA synthetases. In fact, the validity of this combination strategy is evidenced by the fact that we could not obtain resistant mutants by plating bacteria on combination (adjusted for dose equivalence) of our dialkyl amine and a commercially available analog (Tavaborole) of the GSK compound series, whereas either of the drugs could be overcome by resistance when used alone (**Figure 30**).

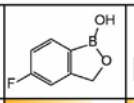
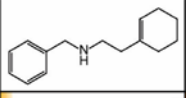
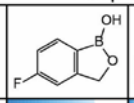
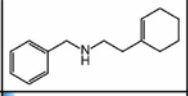
Resistant colonies from 10^8 cells after 36 h				Frequency of resistance			
Population #			Both (adjusted for dose equivalence)	Population #			Both (adjusted for dose equivalence)
1	64	2	0	1	6.E-07	2.E-08	0.E+00
2	50	2	0	2	5.E-07	2.E-08	0.E+00
3	77	3	0	3	8.E-07	3.E-08	0.E+00
4	56	3	0	4	6.E-07	3.E-08	0.E+00
5	55	4	0	5	6.E-07	4.E-08	0.E+00
6	53	3	0	6	5.E-07	3.E-08	0.E+00
7	54	4	0	7	5.E-07	4.E-08	0.E+00
8	80	21	0	8	8.E-07	2.E-07	0.E+00
Average	61	5	0	Average	6.1E-07	5.3E-08	0

Figure 30 Combination of Tavaborole and hit compound abolishes resistance in *E. coli*

3.8 AaRS-OM connections

It is interesting to consider why these phenylalanine tRNA synthetase inhibitors are more effective against the less permeable Gram-negative bacteria. It could well be that the difference is just due to on-target activity, and we will investigate that possibility by purifying and testing PheRS from *S. aureus* and *B. subtilis*. It is also possible that the greater effect of these

compounds on Gram-negative bacteria is due to some consequence to the LPS biogenesis pathways. It is known that binding of uncharged tRNAs to the ribosome results in stringent response, which is mediated by ppGpp[18]. Stringent response regulation encompasses a wide range of cellular processes[19], which include FtsH-mediated degradation of LpxC[20] mentioned in Chapter 1; so, perhaps inhibition of PheRS is particularly harmful if accompanied with a mis-regulation of LPS biogenesis. Also, tRNA synthetases have been implicated in regulatory functions beyond tRNA-related ones[21,22]; in particular, T. Thermophilus PheRS has been shown to specifically bind DNA[23]. Perhaps differences in such regulatory functions account for differences in susceptibility. Interestingly, a recent report[24] mentioned that a mutation in PheS in *A. baumannii* appears to confer some protection against LPS binder polymyxin E (described in Chapter1).

3.9 Methods for Chapter 3

MICs were measured as described in previous chapters except that the MIC for M. tuberculosis was measured by the Rubin lab in microplates under a gradient of concentrations.

All resistant mutants were selected from individual overnight cultures started from single colonies. Agar plates with drugs were monitored for up to 72 hours for the appearance of new colonies.

For measurement of rates of resistance, concentrations were 3x MIC for individual compounds or 1.5x MIC for each when used in combination (the drug-drug interaction for these was found to be additive)

Genome sequencing was performed as described in Chapter 1.

Protein was cloned and purified according to a previously published procedure[14] and biochemical assay used ATP-PPi exchange according to the protocol published by the Schimmel lab.[15]

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Chapter 4
Compounds that rescue cells from LptB defects and exhibit activity with purified Lpt inner-membrane complex

4.1 Phenyloxazolepiperidine series

In contrast to the outcome of the previous section, it is possible for no resistant mutants to arise under selection by a screening hit. Such an outcome resulted with one of the hit compounds; it was found as being less effective against ΔFabH than against WT-FabH *E. coli* (**Figure 31**). However, no resistance to it resulted from a selection.

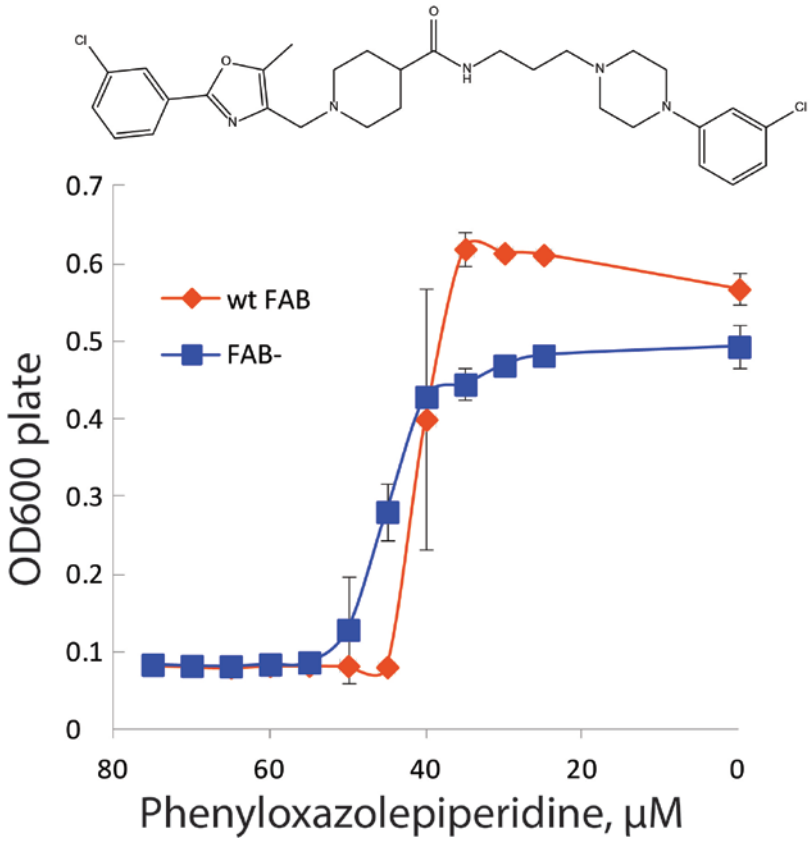

Figure 31 Phenyloxazolepiperidine that is suppressed by FabH

Normally, such an outcome may imply that a compound has non-specific toxicity, and, having no target, cannot become less effective by cell's acquiring a mutation. However, in this case, such an outcome is unlikely because the molecule was more effective against a more fortified cell than against a more permeabilized one, which would contradict non-specific toxicity. Therefore, the presence of two targets for this compound is likely. Nonetheless, spontaneous selections failed to reveal anything about what the targets may be, and alternative approached had to be attempted.

A complementary approach to screening for spontaneous mutants is to screen collections of single gene overexpression strains for decreased susceptibility. It is, of course, also possible to screen collection of gene knockouts; however, that is less promising for two reasons. First, it is easier to get a spontaneous null mutation in a selection, than it is to get a spontaneous mutation that results in overexpression. Second, knockout mutants can only involve non-essential genes, whereas overexpression collections include both types of genes. Using an overexpression screen has some downsides as well. First, overexpression of many essential genes is toxic in itself above a certain level; so, getting the combination of concentration of drug and of the level of gene overexpression right can be crucial to observing the effect. Second, in the case of multi-protein machines, it may be necessary to overexpress all of the components at the same time: even if it is hoped that the extra copies of a subunit-target are to just bind the drug and not to be associated with the rest, it may be possible that the target can bind the drug only in complex with other components of the machine that it is a part of.

Thus, the next experiment attempted was the screen of the ASKA collection[1] for decreased susceptibility to the compound. ASKA is a library of *E. coli* strains, each of which contains a

plasmid overexpressing a gene. Each cloned gene contains an N-terminal His tag, and a C-terminal GFP tag, which are not optimal for the purpose of screening for drug targets because these modification can affect the function of a gene, but it is the most widely available overexpression library.

Microplates with supra-MIC concentration of the compound were inoculated with ASKA clones. Following incubation, wells with visible growth were sampled, and the plasmids from them were isolated and sequenced. There were multiple wells of growth, but only two that contained plasmids coding for essential genes: FabI and LptB. It may be suspected that overexpressing these genes would simply fortify the permeability barriers to all compounds in a non-specific manner. However, those two gene hits were specific because multiple other hit compounds were tested at the same time and in the same manner and none returned either LptB or FabI from wells of growth.

As can be imagined, modulating drug efficacy by overexpression has multiple pitfalls for LPS transport: there are many proteins that make up a functional machine, and pronounced overexpression of membrane components among them is toxic. Furthermore, even a successful delicately engineered overexpression experiment would not be entirely conclusive in the absence of a positive control compound. Therefore, alternative sources of evidence of LptB engagement were sought.

One such test arose on the basis of genetic experiments in the lab of our close collaborator, Prof. Natividad Ruiz. The initial experiment utilized LptB1, strain with a form of LptB that contains an artificial C-terminal extension, which impairs its function (Natividad Ruiz; unpublished data). This results in outer membrane defects and renders cells sensitive to drugs that normally fail to

reach their intracellular targets (Novobiocin, Bacitracin, etc…). Thus, N. Ruiz carried out a selection for mutants that would restore novobiocin resistance. Among these mutants, LptB-R144H was identified as intriguing because it allowed LptB1 to grow on antibiotic plates with novobiocin only, leaving the cells unable to grow on plates with other drugs like bacitracin, etc. A reconstruction of this mutation by N. Ruiz in WT-LptB background (as opposed to LptB1) revealed that it possessed a plating defect on LB agar, which could be rectified by low amounts of Novobiocin (5 μg/mL).

This suggested that Novobiocin, which is a well-characterized, clinically used inhibitor of a DNA topo-isomerising enzyme GyrB also interacts with LptB; more biochemical and structural evidence for this has been found in our lab (S. Okuda, J. May, D. Sherman unpublished data).

Thus, it was worthwhile to test the effects of the screening hit on R144H mutant. It was tested, and it was found that the screening hit rescued R144H mutant from plating defect in a concentration-dependent manner (**Figure 32**).

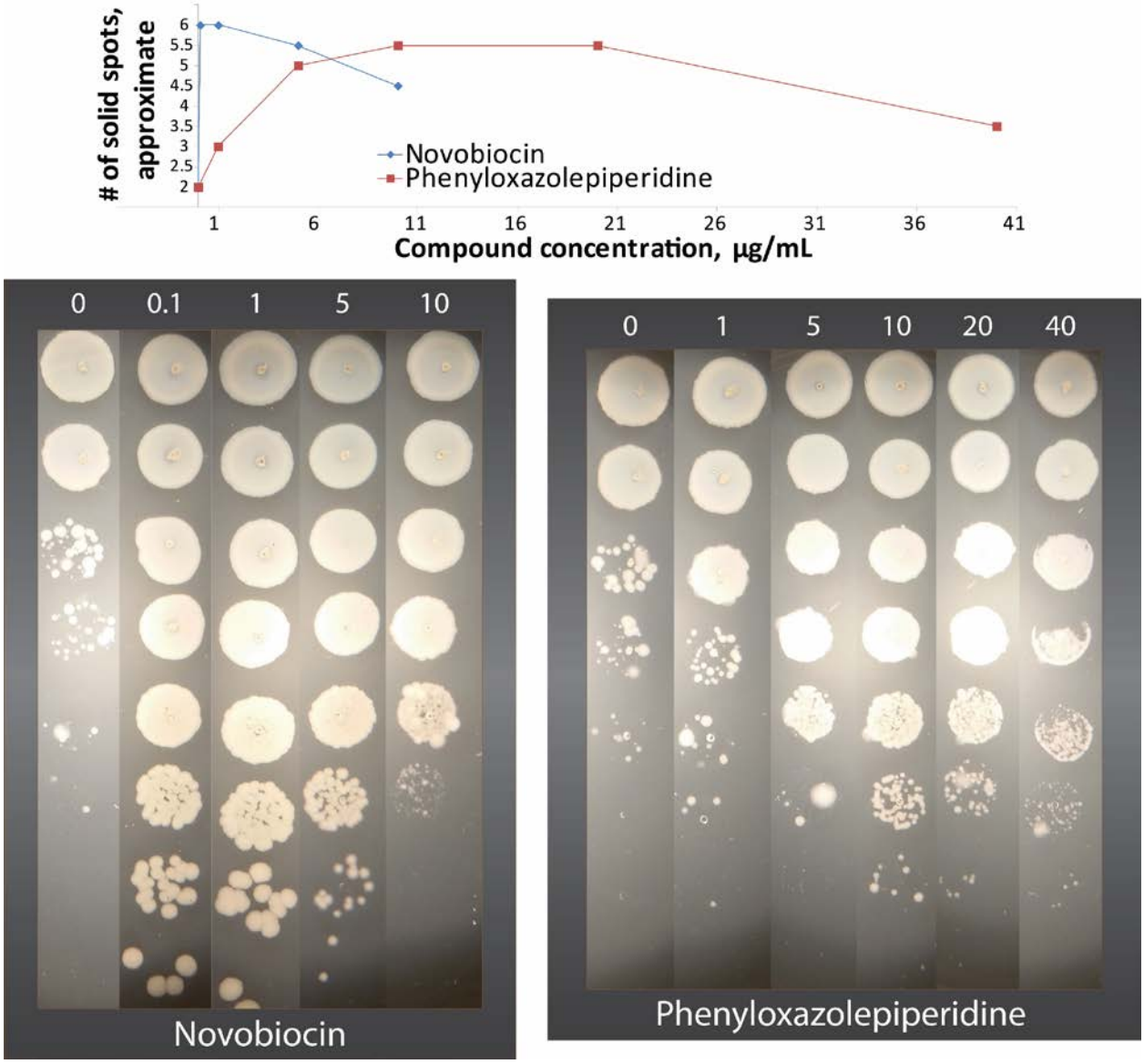

Figure 32 Novobiocin and phenyloxazolepiperidine screening hit rescue growth of R144H on solid media.

This result was promising and, because multiple analogues of the compound were on hand from SAR by catalogue efforts, SAR of R144H plate growth rescue was evaluated, and several additional active compounds were found (**Figure 33**).

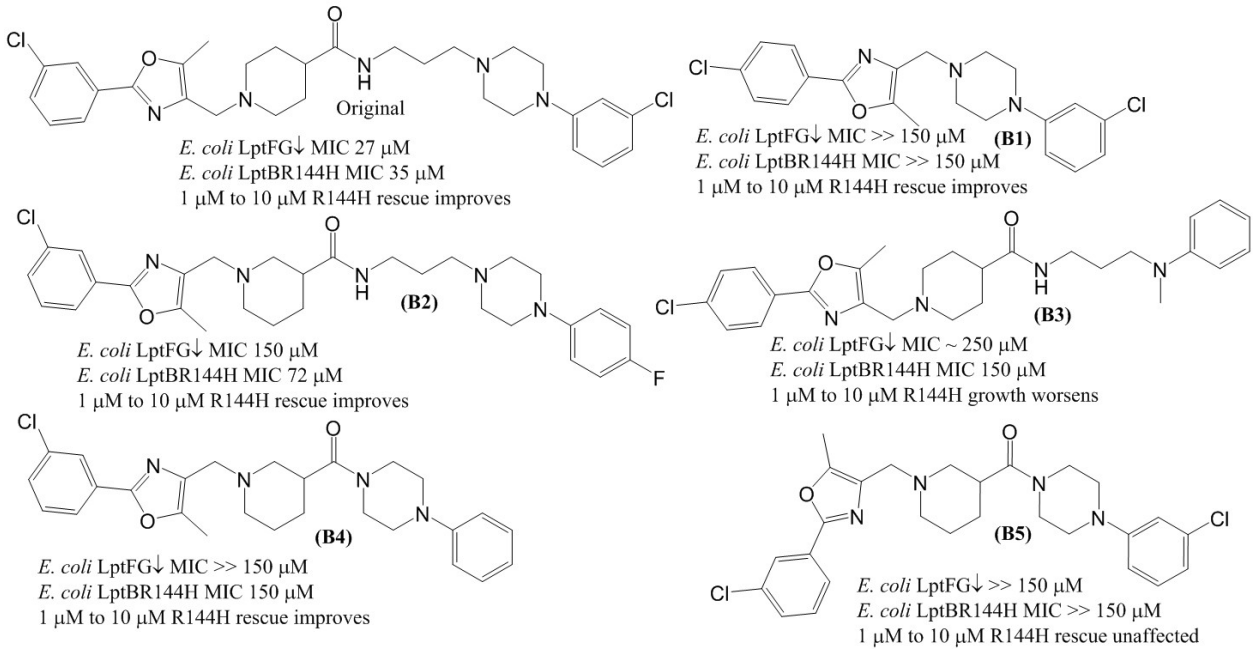

Figure 33 Additional compounds that rescue the solid growth defect of LptB-R144H

One trend was immediately obvious from these experiments, namely that the target responsible for the stronger antibacterial action and target responsible for R144H rescue are not the same target: some of the more potent compounds exacerbate rather than ameliorate the R144H plating defect at 5 μM (**Figure 36**). However, this is not merely a matter of how close the concentration tested is to the MIC because there are both compounds with MIC of 35 μM that rescue well at 10 μM (Original) and compounds with MIC of 35 μM **(D1)**, **(D3)**, 72 μM **(D4)**, **(W4)** or no MIC **(W10)**, **(W12)** that exacerbate the plating defect at 5 μM. Moreover, testing for rescue at 1 μM and 10 μM showed that dose dependence of the effect can go both ways: for some compounds rescue of R144H improves with an increase in concentration; for others, it worsens. This dose dependence is also quite separate from the MICs of the compounds: going from 1 μM to 10 μM, extent of rescue increases for both the original hit (MIC 35 μM) and **(B1)**, a compound with no

observable antibacterial effect. Furthermore, the extent of rescue drops going from 1 μM to 10 μM for both compound **(B3)** (MIC 150 μM) and compound **(D2)** (MIC 17 μM (**Figure 34**).

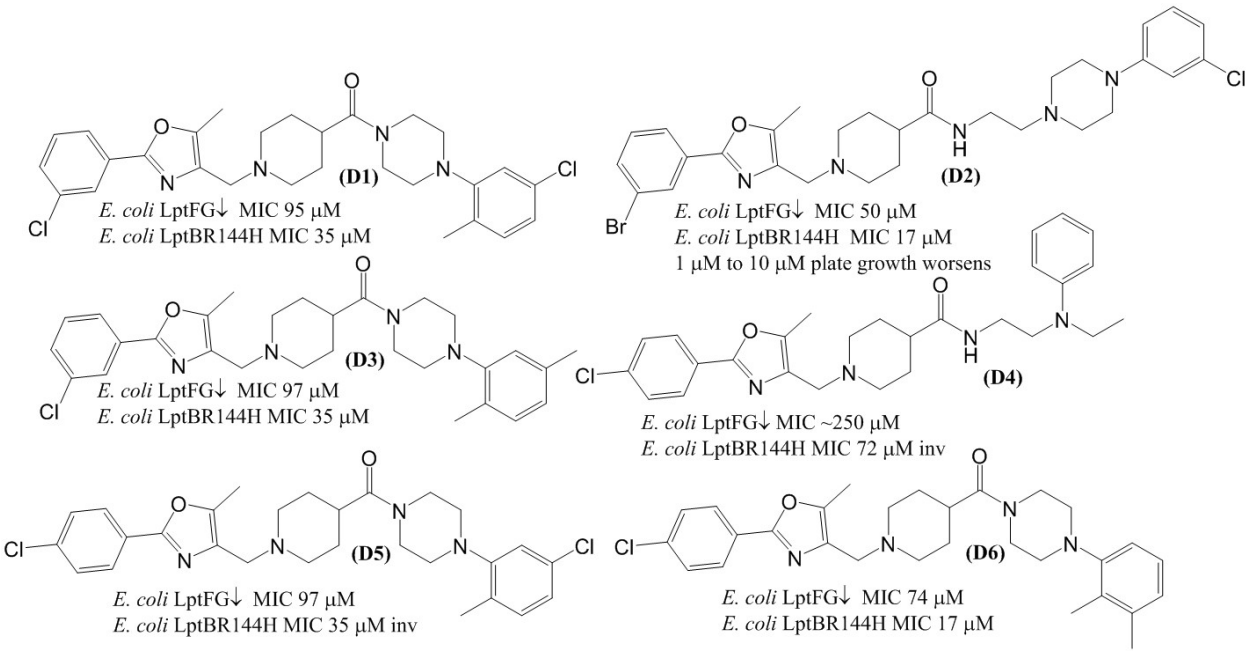

Figure 34 Analogs that are detrimental to growth of LptB R144H on agar

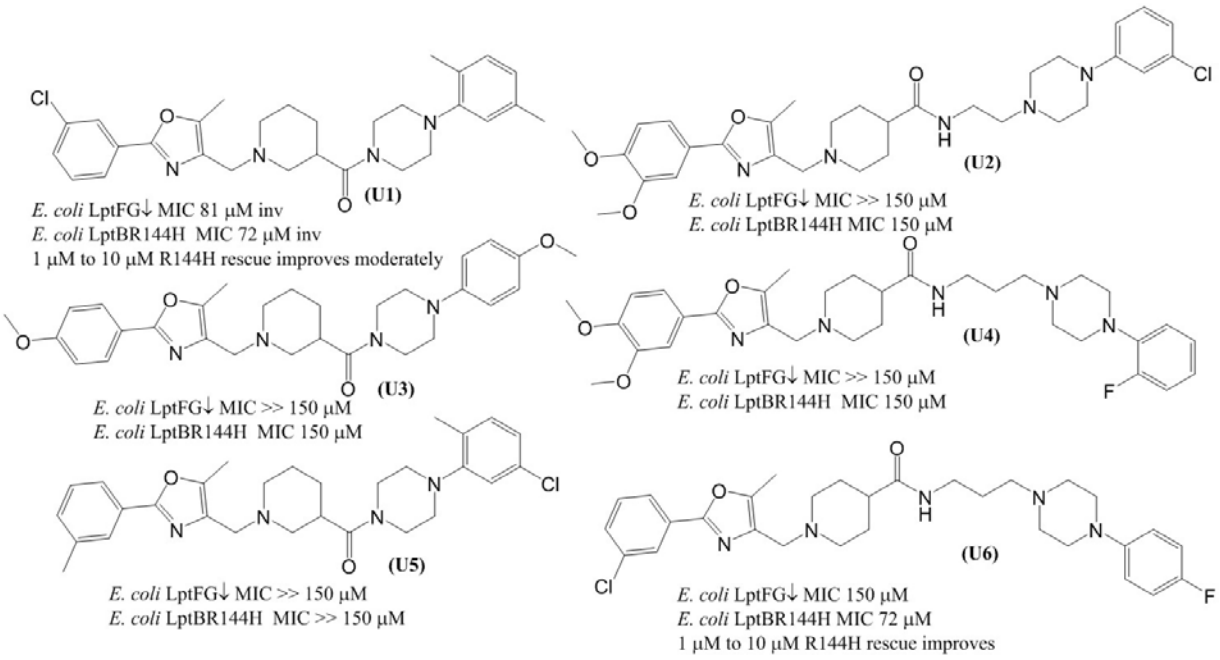

Figure 35 Structures that effect a moderate rescue of R144H or do not change its growth on agar

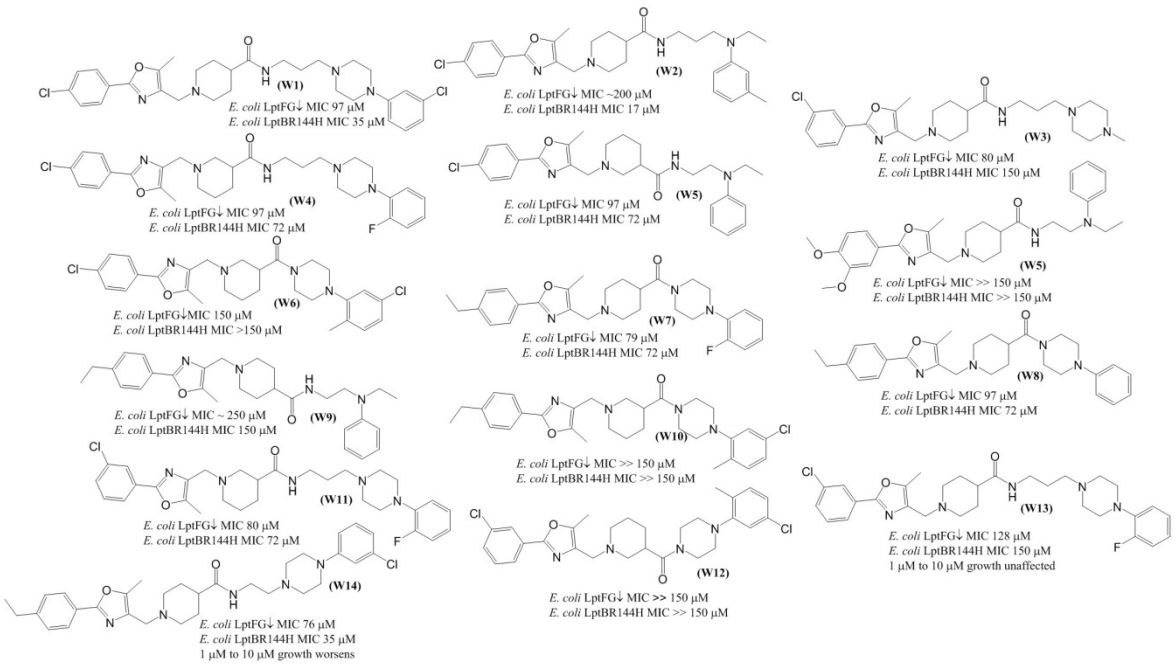

Figure 36 Structures that worsen growth of LptB R144H on agar

Because multiple structural aspects differ in these analogues, it is difficult to come up with a clear SAR. Nonetheless, it seems at least clear that anything other than phenyl-piperazine at the right end of the molecule fails to improve solid growth with an increase in concentration. Structural studies and further SAR is necessary to probe the activity.

Notably, biochemical characterization of this compound in the reconstitution[2] of LPS release from LptBBFGC in proteoliposomes to LptA shows dose-dependent increased release to LptA and indicates target engagement by this compound. (**Figure 37**)

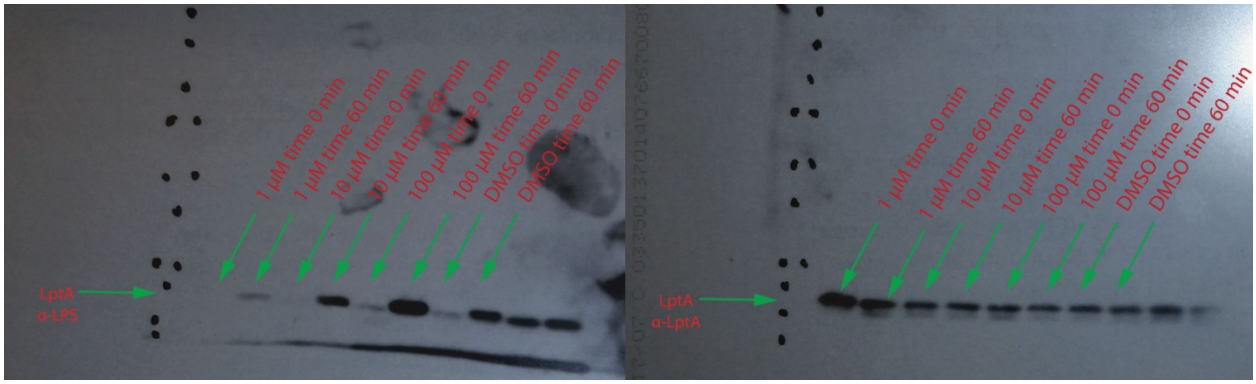

Figure 37 Original compound activates LPS release in proteoliposomes (experiment performed by David Sherman and Becca Taylor) in a manner similar to that of novobiocin.

4.2 Assay optimization for screening at Calibr

It is clear that the behavior of the phenyloxazolepiperidine class just described is somewhat strange, and while we seek to understand it, we also are looking for more inhibitors: the effort to find an inhibitor of OM biogenesis in *E. coli* does not stop at 700,000 molecules. As it was mentioned in the previous chapter, the near absence of active and promising chemical structures in screening libraries when it comes to novel antibacterial compounds is a major hurdle in

looking for them. Thus, as we follow up on hits that we did obtain, we are also looking for additional chemical structures.

One such effort involves collaboration with California Institute for Biomedical Research (Calibr), which has a collection comparable in size to the number that we screened at Harvard. However, the Calibr compound structures are different, and their screening workflow is a lot more automated, which allows for a large number of compounds to be screened in a far shorter time.

However, Calibr is not primarily geared towards antibacterial screening, which means that the concentrations used in their screening protocols are considerably lower than what we have used at ICCB-L at Harvard. Consequently, the approach of screening at 50 μM was not suitable, and we had to adjust our assay to be able to detect hits at 5 μM.

The initial screening, as at ICCB, has to simply pre-select antibacterial compounds, and the strategy of using ΔTolC for that purpose is quite adequate because it sufficiently raises the hit rate as was described earlier in Chapter 2. However, ΔTolC does not work for comparison of FabH-WT and ΔFabH strains because it is not possible to produce a viable strain that lacks both TolC and FabH – as was alluded to earlier in this chapter, TolC likely has unknown partners, and disabling all efflux compromises the ability of the cell to deal with various metabolic disruptions.

Strain and MIC, µM / Drug	*E coli* ΔacrF, ΔmacA, ΔmdtF, ΔemrY, ΔmdtC, ΔemrA, WT FabH	*E coli* ΔacrF, ΔmacA, ΔmdtF, ΔemrY, ΔmdtC, ΔemrA, ΔfabH
Aztreonam	0.26	0.08
Azithromycin	0.9	0.44
Novobiocin	6	0.95
Coumermycin A1	9.36	4.38
Cefoperazone	0.014	0.014
Meropenem	0.12	0.046
Ciprofloxacin	0.015	0.015
Bacitracin	2000	347
Minocycline	1	0.63
Trimethoprim	2	1.18
Rifampicin	4.88	4.88
Linezolid	42	18
Chloramphenicol	5	2.5
Moenomycin	40	7.6
Vancomycin	200	27.8
Ramoplanin	200	200
Triclosan	0.043	0.043
LpxC Inhibitor	0.0215	0.0516

Figure 38 MICs for FabH +/- strain pair in multi-pump null background

Thus, in collaboration with the lab of Natividad Ruiz, we proceeded to knock out all of the known inner membrane partners of TolC (**Figure 38**) in order to reconstruct most of its phenotype of efflux deficiency. That was successful, and it was still possible to remove FabH in the resultant background. This suggests that there are unknown partners/functions of TolC; however, more importantly, this allowed us to retest hits from a pilot batch obtained by screening at Calibr against ΔTolC, and we found that a number of them appeared to be less effective against ΔFabH than to WT-FabH, which is promising. Currently, the follow-up of those compounds and preparation for full deck screening are underway.

3.4 Methods for Chapter 4

Dose-response testing of phenyloxazolepiperidine compound against lptFG↓ and LptFG↓ΔFabH was carried out as described in Chapter 2.

For spot-titer testing of the phenyloxazolepiperidine compounds for rescue of LptB-R144H, a log10 dilution series of an overnight culture of NR3174 = NR754 ΔlptB::tet2 (pET23/42-lptB/R144H) was prepared, and 5 μL per dilution were transferred onto agar plates with compounds and incubated for 24 hours.

Reconstitution was carried out as described by Okuda et al[2]

Testing of Calibr compounds was carried out in 1536-well plates. Appropriate strains at starting OD of 0.001 were added to the plates, and the plates were incubated with humidification for 7.5 hours. The plates were developed with BacTiterGlo (Promega) according to manufacturer's instructions and luminescent signal was measured on a plate reader.

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Chapter 5: Anti-*E. coli* sublibrary and MurJ inhibition

As was mentioned earlier, purchased hits from antibacterial screening in *E. coli* were formatted into a sublibrary of ~1200 compounds. This allows screening for inhibitors of essential targets much more efficiently because such inhibitors would be expected to have an antibacterial effect, and, thus, it is possible to achieve the same results with 60-fold less effort than when screening 700,000 compounds.

One use that this library has been put to is the screening for inhibitors of MurJ, an integral inner-membrane protein that flips the precursors for peptidoglycan skeleton of the cell across the inner membrane.

Peptidoglycan is located between the inner and outer membranes in Gram-negative bacteria, and it consists of linear polymers of a disaccharide repeat (N-acetylglucosamine, N-acetylmuramic acid). Adjacent chains are crosslinked to each other via oligopeptides that are attached to N-acetylmuramic acid.

The monomer precursor for this polymer is the molecule known as lipid II (**Figure 39**). It contains the disaccharide with the oligopeptide as well as a C55 bactoprenol-pyrophosphate lipid that anchors it in the membrane. This precursor is biosynthesized inside the cell and is flipped across the inner membrane by MurJ in order to be incorporated into peptidoglycan.

MurJ is an essential protein and a promising antibiotic target because peptidoglycan biogenesis is perhaps the best validated process for antibiotic treatments; thus, inhibitors of this protein would be valuable candidates for novel antibiotics.

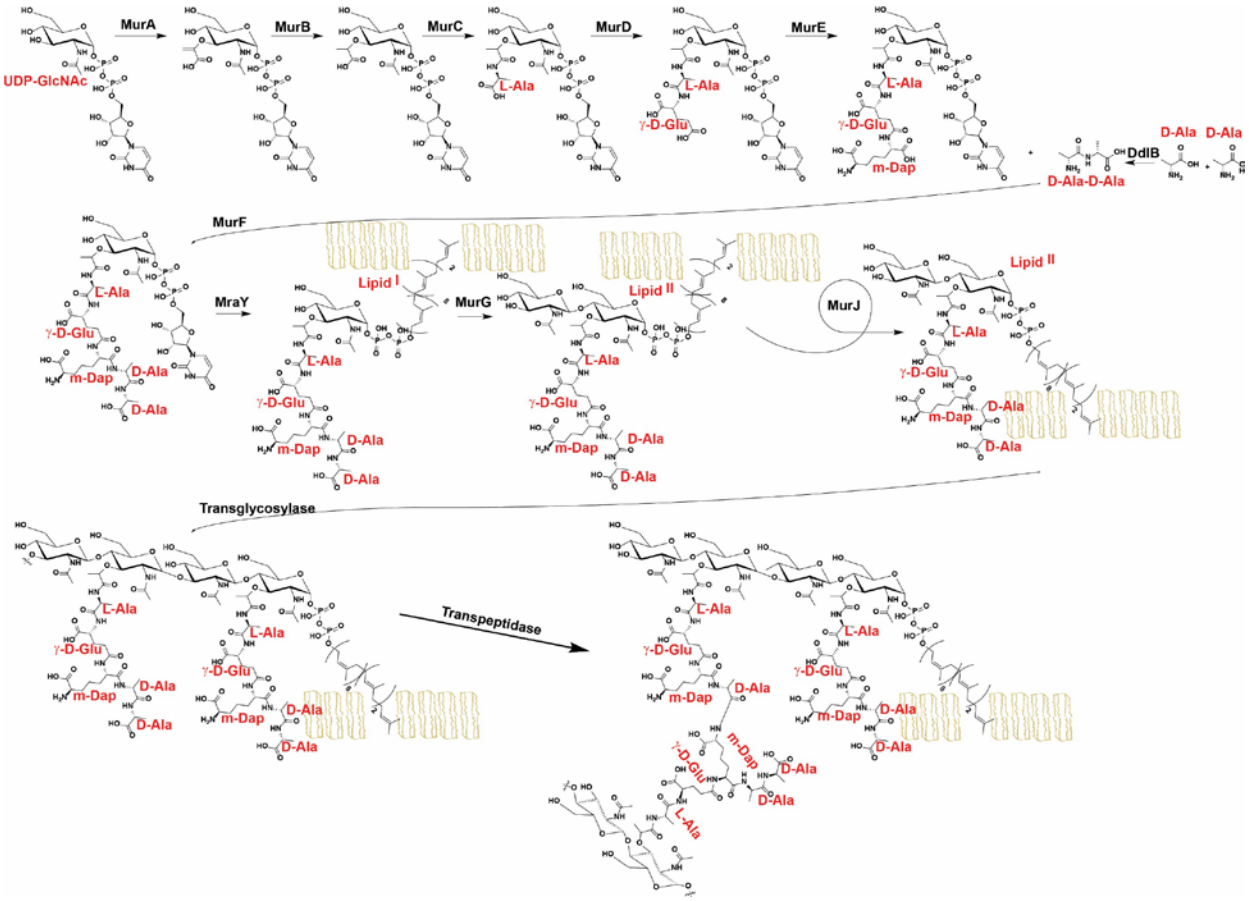

Figure 39 Biosynthesis, flipping, and polymerization of peptidoglycan

Because MurJ has not been extensively characterized, and was only established[1] as lipid II flippase in 2014, there are not many ways to screen for its inhibitors. It is possible to look for cytoplasmic accumulation of lipid II in *E. coli*, in an assay developed by F. Rubino in our lab, but that is a 2-day assay that relies on western blotting for readout, and, therefore, is unsuited for high throughput. Thus, we decided to employ the most obvious approach, namely overexpressing MurJ and looking for compounds that became less effective with overexpression.

The first attempt to do so was a very straightforward one: cloning the flippase gene onto the same arabinose-inducible plasmid as in Chapter 1, pBAD, transforming it into *E. coli*, and exposing the strain to compounds with and without inducer. This approach produced several

compounds, which were tested for lipid II buildup, and among which a number of weak hits were found (data not shown).

However, there were several problems associated with the approach used in this first attempt. First, overexpression of membrane proteins is generally toxic, and the high level of induction used in this assay could cause additional adverse effects that distorted the readout. Second, because the assay was done with both the chromosomal MurJ intact AND the flippase-overexpressing plasmid, which has a leaky level of expression, there was no way to weaken the non-overexpressing strain by lowering MurJ copy numbers in order to increase the window of detection for weak inhibitors. Third, because there are currently no antibodies to native forms of the protein, it is not possible to detect lipid II flippase levels without a tag on all of its copies in the cell.

Thus, we decided to refine our approach to screening for MurJ inhibitors by overexpression. In order to both achieve a firmer control over expression levels and in order to be able to measure levels of MurJ, we made haploid strains where a single copy of FLAG-tagged MurJ was under an inducible promoter on a plasmid. This allowed us to monitor levels of MurJ under different induction conditions. Furthermore, we also made a version of this construct with all native cysteins deleted, and an A29C mutation. This construct was previously reported in Sham et al[1] and it allows to abolish MurJ function by treatment with a cysteine-reactive small molecule MTSES in a manner that mimics small molecule inhibition, which is helpful for optimizing screening assays. Finally, we placed those constructs in a ΔTolC background in order to make the target more accessible to small molecules.

Importantly, establishing this finer control of MurJ levels has proven advantageous for the screening assay. First, the induction of a haploid MurJ-FLAG enabled a fine level of control over observable MurJ levels. It was found that quite low levels of MurJ are sufficient to sustain viability and that toxicity is observed at moderate levels of overexpression. Furthermore, it was found that moderate overexpression of MurJ slightly increases the susceptibility of *E. coli* to pre-existing antibiotics, implying that the overexpression assay is likely to have the specificity of overcoming a handicap by hits that was previously described for FabH. Second, the use of ΔCys A29C allele allowed us to validate the assay with a hit-like small molecule. This has in fact revealed that moderate MurJ inhibition suppresses the toxicity of moderate MurJ overexpression, further extending the detection window in the screening assay.

Currently, the assay is being both deployed to screen the sublibrary and further attempts at improving it are made by lowering the levels of MurJ under no induction conditions. It is hoped that that will pre-sensitize the strain to MurJ inhibitors, further widening the window for their detection.

Methods for Chapter 5:

MurJ was cloned from *E. coli* NR754 and into pBAD with the strong RBS of the plasmid. The resultant plasmid was transformed into NR754. The sublibrary was screened against NR754 with empty pBAD and NR754 with pBAD-MurJ at 0.2% arabinose at 1800x, 600x and 200x dilution of the compound stocks. Hits were scored on the basis of OD differential after 24 hours.

The above plasmid was then modified to add N- and C-terminal FLAG tags and to mutagenize it to ΔCys-A29C by us. It was then transformed into NR754 and NR754ΔTolC by the lab of Natividad Ruiz and the chromosomal copy was removed.

References for Chapter 5:

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(1) Sham, L. T.; Butler, E. K.; Lebar, M. D.; Kahne, D.; Bernhardt, T. G.; Ruiz, N. *Science* **2014**, *345*, 220.
