Molecular Genetics of Beta-Lactam Sensitivity and Resistance in Mycobacterium Tuberculosis

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Abstract

Mycobacterium tuberculosis threatens global health. Its thick, impermeable cell wall renders it tolerant to high doses of many antibiotics. While we understand the biochemical functions of many cell wall-modifying enzymes, we often do not understand their physiological functions: their spatiotemporal roles in the cell cycle, their substrate preferences, or their individual effects on the macromolecular architecture of the cell wall complex. *Mycobacterium tuberculosis* possesses five peptidoglycan transpeptidases, five lytic transglycosylases, and numerous other peptidoglycan-modifying enzymes that lack precisely-understood roles.

The lytic transglycosylases of *Mycobacterium tuberculosis* are collectively dispensable for *in vitro* growth. We sought to learn what other classes of peptidoglycan-degrading enzyme might substitute for the lytic transglycosylases or become essential in their absence. A high-throughput chemical screen was performed on a strain lacking all five lytic transglycosylases to identify compounds that specifically killed this strain and not wild-type *Mycobacterium tuberculosis*. Among the compounds identified were several members of the cephalosporin class of β-lactam antibiotics. It was shown that the cephalosporins had greater access to the periplasmic β-lactamase of *Mycobacterium tuberculosis* in the lytic transglycosylase-deficient strain and that this strain had enhanced sensitivity to several antibiotics with unrelated mechanisms of action. Together, greater periplasmic access and broadly heightened

susceptibility in the deficient strain suggested a role for the lytic transglycosylases in maintaining the mycolic acid permeability barrier.

To identify the specific penicillin-binding protein target of the cephalosporins, we isolated spontaneously-occurring resistant mutants. These strains contained polymorphisms in *ponA2*, a bifunctional penicillin-binding protein. The polymorphisms conferred sensitivity to heat stress, a phenotype associated with *ponA2* loss of function. To clarify the relation between loss of function and cephalosporin resistance, a *ponA2* deletion strain was created, which exhibited both cephalosporin resistance and sensitivity to carbapenems, another class of β-lactam. Restoration of the wild-type *ponA2* allele suppressed both cephalosporin resistance and carbapenem sensitivity. Inactivation of other transpeptidases did not confer resistance to any β-lactams. The association of penicillin-binding protein inactivation with β-lactam resistance is unusual. One model to explain it is that upon deletion of *ponA2*, *Mycobacterium tuberculosis* compensates for its loss by upregulating a cephalosporin-resistant, meropenem-sensitive transpeptidase.

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Chapter 1

Introduction to the problem of mycobacterial defenses against β-lactams

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Section 1.1: The need for β-lactams in tuberculosis treatment

Tuberculosis causes more than a million human deaths and $50 billion of economic damage each year (1). The disease poses a continuing threat to public health because of the emergence of multidrug-resistant, extensively drug-resistant, and, recently, totally drug-resistant strains of the causative organism, *Mycobacterium tuberculosis* (MTB) (2).

Standard tuberculosis treatment requires the simultaneous use of four drugs: rifampin, an RNA transcription inhibitor; isoniazid, a mycolic acid biosynthesis inhibitor; and ethambutol, an arabinogalactan biosynthesis inhibitor; pyrazinamide, a *trans*-translation inhibitor (3). *Trans*-translation is the process by which stalled ribosomes are recycled (4). The side effects of the tuberculosis treatment regimen can be severe and may discourage many patients suffering tuberculosis from completing the course of treatment. Furthermore, the six-month duration of the treatment makes ensuring its completion difficult in impoverished areas with poor public health systems (5).

Significantly lacking from this list of drugs are any members of the antibiotic classes commonly used to treat respiratory tract infections: β-lactams, fluoroquinolones and macrolides (6), although fluoroquinolones are currently in phase III clinical trials as first-line agents (7, 8). The β-lactams in particular have had the longest use and greatest impact of all antibiotic classes against non-tubercular infections (9). MTB possesses a strong, constitutive β-lactamase activity (10) that renders it highly resistant to β-lactams (11). One potential way to overcome this problem is through concurrent treatment with a β-lactam and a β-lactamase inhibitor. Amoxicillin/clavulanate, one such combination, saw only limited success in several trials (12,

13). Recently, however, the rise of multidrug-resistant tuberculosis has encouraged renewed interest in β-lactam/β-lactamase inhibitor combination therapy. A recent study has shown that meropenem/clavulanate has potent activity against drug-resistant MTB and anaerobic models of persistent MTB infection (14). This finding has led to a clinical trial for meropenem/clavulanate against extensively drug-resistant tuberculosis (15).

The potential clinical use of meropenem/clavulanate, and the search for new antitubercular compounds in general, raise two questions. First, what existing defenses does MTB have against small molecules? Second, what are the means by which it may acquire further resistance? This dissertation shall consider each of these questions in turn, primarily from the perspective of the β-lactams, of which meropenem and other carbapenems are a subclass. Regarding existing defenses, we shall consider the complex architecture of the cell wall, with special emphasis on the lytic transgycosylases of MTB. Regarding the acquisition of further resistance, we shall carefully review existing mechanisms of β-lactam resistance across bacteria and ultimately examine some intriguing mutations we have discovered in MTB.

Section 1.2: Existing defenses: the mycobacterial cell wall

The outer membrane

Two obstacles impede meropenem in its passage through the cell wall to its transpeptidase target in the peptidoglycan of MTB. First, it must traverse the hydrophobic, poorly permeable mycolic acid layer (16). Then, it must escape the hydrolytic activity of MTB's promiscuous, highly active β-lactamase (17). The latter obstacle would not hinder meropenem by much, because meropenem is an inherently poor substrate for the MTB β-lactamase. Furthermore, the β-lactamase is efficiently inhibited by clavulanate in combination therapy (17).

The mycolic acid layer, however, bears consideration. Mycolic acids are $C_{60} - C_{90}$ carboxylic acids covalently linked to arabinogalactan, an underlying polymer of repeating sugars (18). This arabinogalactan layer is itself covalently bound to the peptidoglycan sacculus (19). Together, then, the mycolic acids, arabinogalactan, and peptidoglycan compose one giant macromolecule encasing the cell proper (Figure 1.1) (16).

The mycolic acids extend some 53 to 64 carbons from a carboxylic acid head group, leaving the remaining carbons to form a branch extending from the carboxylic acid α-carbon (21). This 53 to 64 carbon length is substantially longer than the average fatty acid in the plasma membrane, but recent electron microscopy studies indicate that the hydrophobic region of the outer mycolic acid layer is not substantially thicker than the plasma membrane, leaving some doubt as to the exact conformation of mycolic acids in their cellular context (22, 23). What is certain is that they are generally perpendicular to the surface of the cell, and their boundary with the hydrophilic extracellular milieu or capsular area is mediated by a variety of accessory lipids, such as phthiocerol dimycocerosate and the sulfolipids, and glycolipids, such as lipomannan and lipoarabinomannan (24, 25). Together, the mycolic acids on the inside and the free glycolipids on the outside effectively form a mycobacterial outer membrane bilayer (22, 23).

This outer membrane forms a substantial permeability barrier to both nutrients and antibiotics. For the cephalosporin class of β-lactams, by one estimate, the dual limitations of the permeability barrier and β-lactamase activity create a 500 to 5,000-fold concentration differential between the extracellular milieu and the periplasm, compared to a 1.5 to 15-fold differential

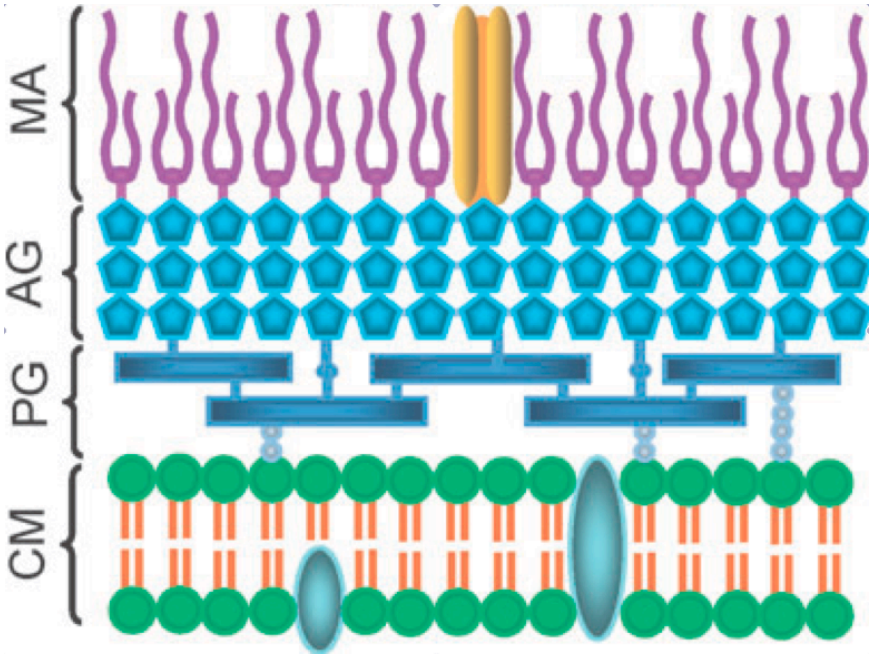
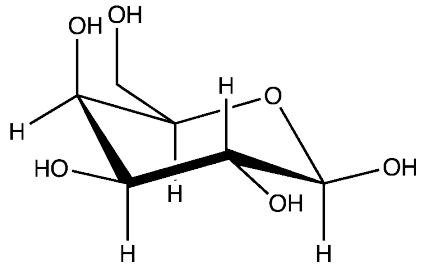


Figure 1.1. The unusual cell wall of *Mycobacterium tuberculosis*. Peptidoglycan (PG), arabinogalactan (AG), and mycolic acids (MA) together compose the mycolyl-arabinogalactan-peptidoglycan complex, a single macromolecule standing between the cell membrane (CM) and the extracellular milieu. The mycolic acids also form the inner leaflet of a membrane bilayer separating the mycolyl-arabinogalactan peptidoglycan complex itself from the extracellular milieu. Adapted from (20).

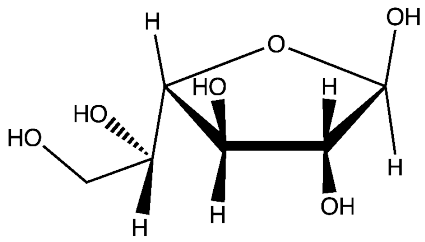
across the outer membrane of *Escherichia coli* K-12 (26). This is true regardless of the hydrophobicity of the cephalosporin.

Beyond the hydrophobicity of the mycolic acids, their unusual length and characteristic unsaturations also contribute to the permeability barrier they form. There are different types of unsaturation, which may vary across species and contribute to the higher or lower permeability of the cell walls of different mycobacteria. For instance, MTB and *Mycobacterium avium* possess a particular *cis*-cyclopropanated α-proximal double bond that other mycobacterial species lack; in direct biophysical comparisons, this modification raised the melting temperature of tubercular mycolic acids by 3 °C (27). In a survey across mycobacterial species, the melting temperature of mycolic acid membranes rose in proportion to the average mycolic acid length for a given species (28). The increases in mycolic acid melting temperature from cyclopropanation and elongation reflect a decrease in fluidity, which is directly related to the ability of particles to diffuse through the membrane (28). These measurements, then, provide a biophysical basis from which to understand the importance of MTB's outer membrane as a permeability barrier.

Two layers of sugar polymers separate the mycolic acid layer from the cell membrane. The first layer, arabinogalactan, comprises a galactan chain some thirty residues in length followed by branching arabinan chains of similar length (29). Notably, all of these sugar residues are furan isomers rather than the more common pyran isomers of other biological glycans (Figure 1.2). Arabinogalactan's higher-order structure is unknown (30). Its biogenesis is not related to that of peptidoglycan, the second sugar polymer layer.



β-D-Galactapyranose



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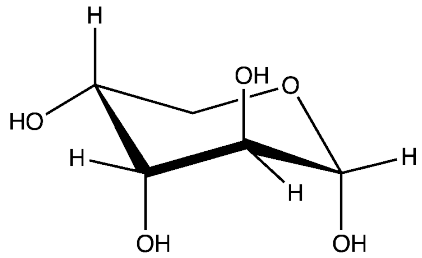
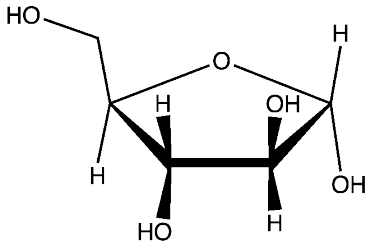


α-D-Arabinopyranose



α-D-Arabinofuranose

Figure 1.2. Comparison of pyran and furan forms of common arabinogalactan sugars.

Peptidoglycan

Mycobacterial peptidoglycan, like all peptidoglycan, is a net-like structure composed of polymers of *N*-acetylglucosamine and muramic acid crosslinked by peptide chains. The peptidoglycan macromolecule is essential, confers bacterial shape, and withstands the turgor pressure resulting from the osmotic differential between the cell and its environment (31). The enzymes responsible for its biogenesis are the targets of numerous antibiotics, including meropenem and other β-lactams (32). Peptidoglycan is also of medical interest because species-specific variations can modulate the host immune reponse to infection (33). The diversity of these functions, and the diversity of the demands across bacterial species in terms of shape, osmotic differential, host environment, and antibiotic exposure mean that the peptidoglycan molecule itself is highly variable from species to species.

The peptidoglycan of MTB must meet some unusual challenges. It is rod-shaped, but elongates from its tips, in contrast to most pathogenic bacteria, which elongate from the middle (Figure 1.3) (34). The peptidoglycan layer is thick, like in other Gram positive organisms, but must coordinate with an outer membrane, like Gram negative ones. It is an intracellular pathogen, and withstands multiple innate immune insults (35, 36), but also tolerates a wide variety of environmental stresses (37).

Peptidoglycan: the glycan chains

The peptidoglycan that meets the diverse requirements for mycobacterial survival necessarily contains some unusual features. For example, the muramic acid residues in the glycan chain contain a mixture of *N*-glycolylation and *N*-acetylation at C1, in contrast to the great majority of bacteria, which have only *N*-acetylation (38). The function of glycolylation is

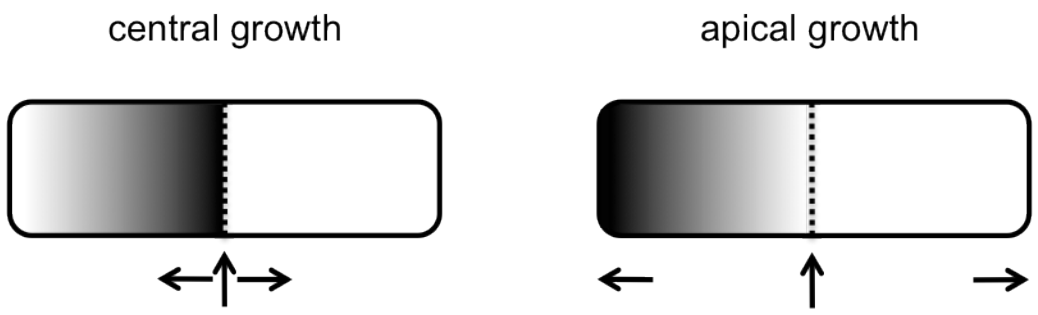


Figure 1.3. Differences in peptidoglycan growth patterns between centrally and apically growing bacteria. In well-studied bacteria such as *E. coli* and *Bacillus subtilis*, peptidoglycan polymerization and crosslinking occurs predominantly at the septum. The age of peptidoglcyan (stronger shading is younger peptidoglycan) increases with distance from the septum (dotted line). In mycobacteria and related genuses, such as corynebacteria and streptomyces, peptidoglycan polymerization and crosslinking occurs predominantly at the apices of the cell. The age of peptidoglycan increases approaching the septum. Adapted with modifications from (34).

unknown, but it may play a role in modulation of the immune response. One mechanism by which it could do this is by making the glycan strands resistant to degradation by lysozyme (39); the glycolyl modification is mere angstroms from the β-1,4-glycosidic bond that lysozyme cleaves, giving it the potential to affect the substrate's interaction with the enzyme active site (40). Glycolylation may have an even more basic biological role in controlling MTB's own peptidoglycan-degrading activity; MTB's genome encodes five enzymes with close homology to lysozyme (40, 41). If glycolylation can alter lysozyme activity, it may alter the activity of these enzymes too.

These lysozyme-like enzymes are lytic transglycosylases, which participate in another aspect of bacterial peptidoglycan's structural variation. This aspect is the modulation of glycan chain length. Glycan chain length is highly variable across species (33), ranging from averages of tens to hundreds of disaccharides in length. Even within individual bacterial cells (strictly, within clonal populations) there is a wide distribution of glycan chain lengths (42). For *Mycobacterium leprae*, the median chain length is thought to be very short (43), but precise calculations do not exist for *M. leprae* or MTB.

There are two ways of controlling the glycan chain length: at the polymerization stage, or through modification of the glycan chain after polymerization.

In most model bacteria, the polymerizing enzyme is a high-molecular weight (HMW) Class A penicillin-binding protein (PBP). Class A HMW PBPs typically contain a glycosyltransferase domain, which links Lipid II, a peptidoglycan precursor, to an existing glycan chain, and a transpeptidase domain, which crosslinks the pentapeptide side chains to those on other glycan chains (Figure 1.4) (44). Beyond their direct peptidoglycan-synthetic functions, Class A HMW PBPs may form homodimers (45) or heterodimers (46) with other

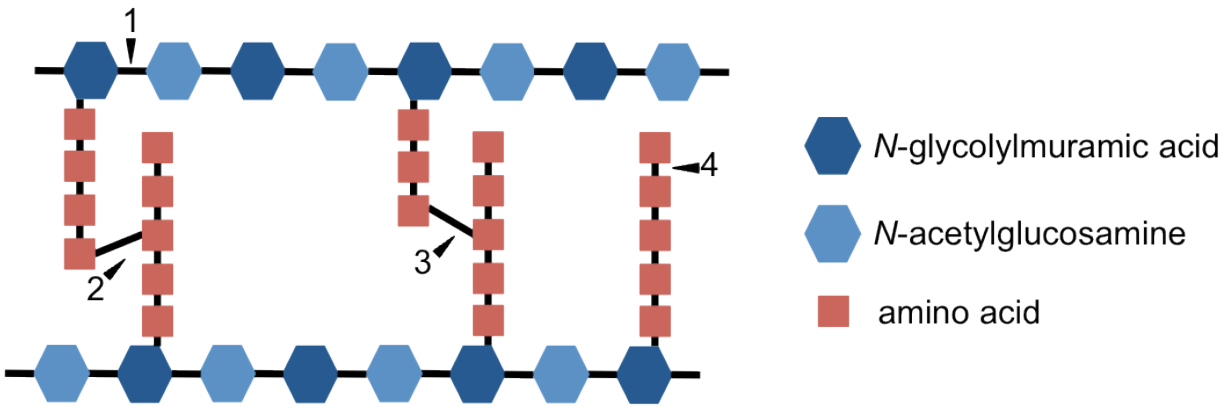


Figure 1.4. Sites of action of selected peptidoglycan-modifying enzymes. (1) is the β-1,4-glycosidic bond, which is cleaved by lysozymes and lytic transglycosylases. (2) is a 4→3 peptide crosslink, which is created by HMW PBP transpeptidases and cleaved by LMW PBP endopeptidases. (3) is a 3→3 peptide crosslink, which is created by L,D-transpeptidases. (4) is a D-alanine-D-alanine bond in an uncrosslinked peptide pentamer, which is cleaved by LMW PBP D,D-carboxypeptidases; there is no theoretical reason this latter reaction cannot occur for the D-alanine-D-alanine bond remaining on a pentamer crosslinked at its third amino acid.

peptidoglycan synthetic enzymes, possibly modulating their activity (47). In addition to PBPs, there exist a few known independent glycosyltransferase-encoding genes with homology to the glycosyltransferase domains of Class A HMW PBPs (48). These independent glycosyl-transferases contribute significantly to the total glycosyltransferase activities of several species, including *E. coli*, *Staphylococcus aureus*, and *Micrococcus luteus* (49, 50). MTB does not possess any known independent glycosyltransferases, but this does not rule out a substantial contribution to glycan chain polymerization from a cryptic glycosyltransferase; at least one Gram-positive organism, *Enterococcus faecium*, possesses such activity without encoding any genes with significant homology to known glycosyltransferase domains (51).

There are several conceivable ways of regulating the chain length during the glycosyltransferase stage of peptidoglycan biosynthesis: through substrate availability, substrate suitability, as by the relative affinity for glycolylated or acetylated muramic acid, or through the processivity of the glycosyltransferase itself. In *E. coli* and *S. aureus*, this last mechanism seems most important, since *in vitro*-reconstituted peptidoglycan biosynthesis produces polymers of similar lengths to those observed *in vivo* (52).

The post-polymerization hydrolysis of existing polymers is important not merely for regulating glycan chain length, but also for peptidoglycan recycling; current *E. coli* models hold that peptidoglycan does not merely insert into laterally expanding cell walls, but moves from inner to outer layers in an aging process (53). Once reaching the outer layers, peptidoglycan hydrolytic enzymes free the glycan chains from the sacculus and digest them before, in a well-understood *E. coli* process, reimporting them into the cytoplasm (54). Recent research has shown that *Bacillus subtilis* also possesses a peptidoglycan recycling system (55).

In *E. coli*, peptidoglycan digestion includes the complete breakdown of a chain to its constituent disaccharide units, requiring an enzymatic lytic transglycosylase function. Many species have multiple peptidoglycan lytic transglycosylases, which may be nonessential both individually and in groups (56, 57). This is also true of other enzymes involved in the peptidoglycan digestion, including muramidases, which release peptide side chains from the glycan strands, and endo- and carboxypeptidases, which degrade the peptide side chains (Figure 1.4) (56, 58).

The lytic transglycosylases of MTB merit special attention because they were the initial focus of our research. There are five of them (Table 1.1), and they are collectively nonessential for *in vitro* growth (60). Since they bear little similarity to the lytic transglycosylases of *E. coli*, the first investigations into their function arose from their similarity to a protein called "resuscitation-promoting factor", encoded by the gene *rpf*, from *Micrococcus luteus* (61). This protein and its mycobacterial homologs were able to stimulate growth of low numbers of cells in broth culture, or cells collected from prolonged stationary phase incubation (61). The finding that MTB had five such factors, and the similarity of the resuscitation phenomenon to the emergence of MTB from the latent phase of human infection, stimulated much excitement (62). The original model postulated that the resuscitation-promoting factors acted by binding a cellular receptor in a manner similar to cytokines (63). The peptidoglycan degradatory function became apparent when the crystal structure of *rpfB* from MTB showed similarity to lysozyme (40). The ability of *M. luteus* Rpf to digest peptidoglycan, and the dependence of both this activity and resuscitation from dormancy on a glutamate corresponding to a catalytic residue in lysozyme, established the peptidoglycan degradatory function beyond doubt (64). Strictly speaking, it is not clear whether Rpf performs a lysozyme-like activity, which hydrolyses the peptidoglycan β-

Table 1.1. Selected peptidoglycan-modifying enzymes of *Mycobacterium tuberculosis*.

Gene	Gene Name	Domains[a]	Essential *in vitro*[b]	Closest *E. coli* Homolog[c]
Rv0050	*ponA1*	Glycosyltransferase Transpeptidase	No	Pbp1b
Rv3682	*ponA2*	Glycosyltransferase Transpeptidase PASTA[d]	No	Pbp1a
Rv2163c	*pbpB*	PBP Dimerisation[e] Transpeptidase	Yes	Pbp3
Rv0016c	*pbpA*	Transpeptidase	No	Pbp2
Rv2864c	-	Transpeptidase N-terminal[f] Transpeptidase	No	Pbp2
Rv3330	*dacB1*	Carboxypeptidase	No	Pbp6b
Rv2911	*dacB2*	Carboxypeptidase	No	Pbp5
Rv0867c	*rpfA*	Lytic Transglycosylase	No	None
Rv1009	*rpfB*	DUF348 x 3[g] G5[h] Lytic Transglycosylase	No	None
Rv1884c	*rpfC*	Lytic Transglycosylase	No	None
Rv2389c	*rpfD*	Lytic Transglycosylase	No	None
Rv2450c	*rpfE*	Lytic Transglycosylase	No	None

[a] Domains contained in the protein are listed from the N-terminus to the C-terminus. Domains were determined using the PFAM server at pfam.janelia.org. Domains without discriptions are discussed at length in the text.

[b] Comprehensive descriptions of essential MTB genes are described in (59).

[c] Homology was determined using the protein NCBI BLAST server against *Escherichia coli* strain K-12. Only homologues with an E-value greater than 0.001 are listed.

[d] PASTA is penicillin-binding protein and serine/threonine kinase-associated: PF03793.

[e] This domain is found in some Class B HMW PBPs: PF03717.

[f] This domain is found at the N-terminus of some Class B HMW PBPs, including MecA of *Staphylococcus aureus*: PF05223.

[g] This is a domain of unknown function: PF03990.

[h] This domain is found in several cell wall-associated proteins: PF07501.

[i] A fold change of 1 indicates that there was no detectable difference between RPF^{null} and RPF^{WT}.

1,4-glycosidic bond and consumes a water molecule, or whether it performs a lytic transglycosylase activity, which cleaves the bond using the muramic acid C6 hydroxyl group in place of water as the nucleophile (65).

Subsequent investigation has shown that deletion of two MTB *rpf* homologs, *rpfA* and *rpfB*, attenuated resuscitation *in vivo* (66), while deletion of all five resulted in a severe survival defect in mice (60). These defects were not associated with filamentation or morphological defects, which often arise from deletion of peptidoglycan-degrading enzymes. The survival defects therefore highlight the importance of regulation of the peptidoglycan macromolecule during infection, while leaving unexplained the precise connection between the biochemical effects of *rpf* homologs and the changes these effects produce in overall cellular physiology.

Peptidoglycan: the peptide crosslinks

While glycolylation and glycan chain length vary within and across species, the most diversity in peptidoglycan structures arises in the peptide side chains. These side chains are diverse, but MTB in particular has a "standard" side chain composition remarkably similar to *E. coli*. Both species possess as their core, most common peptide side chain the sequence L-Ala-γ-D-Glu-*meso*-Dap-D-Ala-D-Ala, where *meso*-Dap is *meso*-diaminopimelic acid and γ-D-Glu is glutamate polymerised from its γ-carboxylic acid group (Figure 1.5) (67, 68). In MTB, the glutamate often forms isoglutamine through amidation of the free α-carboxylic acid. The most common variation on this scheme in MTB is the addition of a single glycine to the ε-amino group on *meso*-Dap (67), similar to the interpeptide bridges in many Gram positive firmicutes (30).

The diversity of peptide side chains cannot match the combinatorial diversity formed by the crosslinking of these side chains. Previously, models assumed that penicillin-binding transpeptidases carried out most or all of this crosslinking (53), and that these transpeptidases were the HMW PBPs. Each HMW PBP contains a transpeptidase domain, which catalyzes the replacement of the peptide bond to the terminal D-Ala of a receiving pentapeptide side chain with the amino group from an invading peptide side chain (Figure 1.5). This amino group is always contributed by the amino acid at P3 (Figure 1.5); in MTB, this is the ε-amino group of *meso*-Dap, but in other species, it may be the side chain amino group of lysine or ornithine instead (69).

As with glycan chain length, the degree of peptide crosslinking modulates the properties of the peptidoglycan macromolecule. More crosslinking results in a finer mesh encircling the cell. It also means that individual crosslinks may be less stressed, since each crosslink bears less of the turgor pressure of the cell contents. This is a consequence of the greater number of crosslinks per unit area in a finer mesh. The diversity of PBP paralogs in most bacterial species suggests transcriptional or translational mechanisms may control the amount of crosslinking that occurs, but other mechanisms exist as well. Carboxypeptidases can remove the terminal D-Ala from a side chain, preventing it from accepting a crosslink from a neighboring peptide strand. Endopeptidases can hydrolyze existing peptide crosslinks, most often at P4 (Figure 1.4, Figure 1.5). While the P4-hydrolyzing endopeptidases are paralogs of HMW PBPs and usually non-essential, as described above, endopeptidases have been described that cleave at nearly every position (70). An endopeptidase cleaving at P2 in MTB is essential (71).

Beyond the degree of crosslinking, the nature of the crosslink also appears to have a major impact on bacterial physiology. While the invading amine group is always a side chain

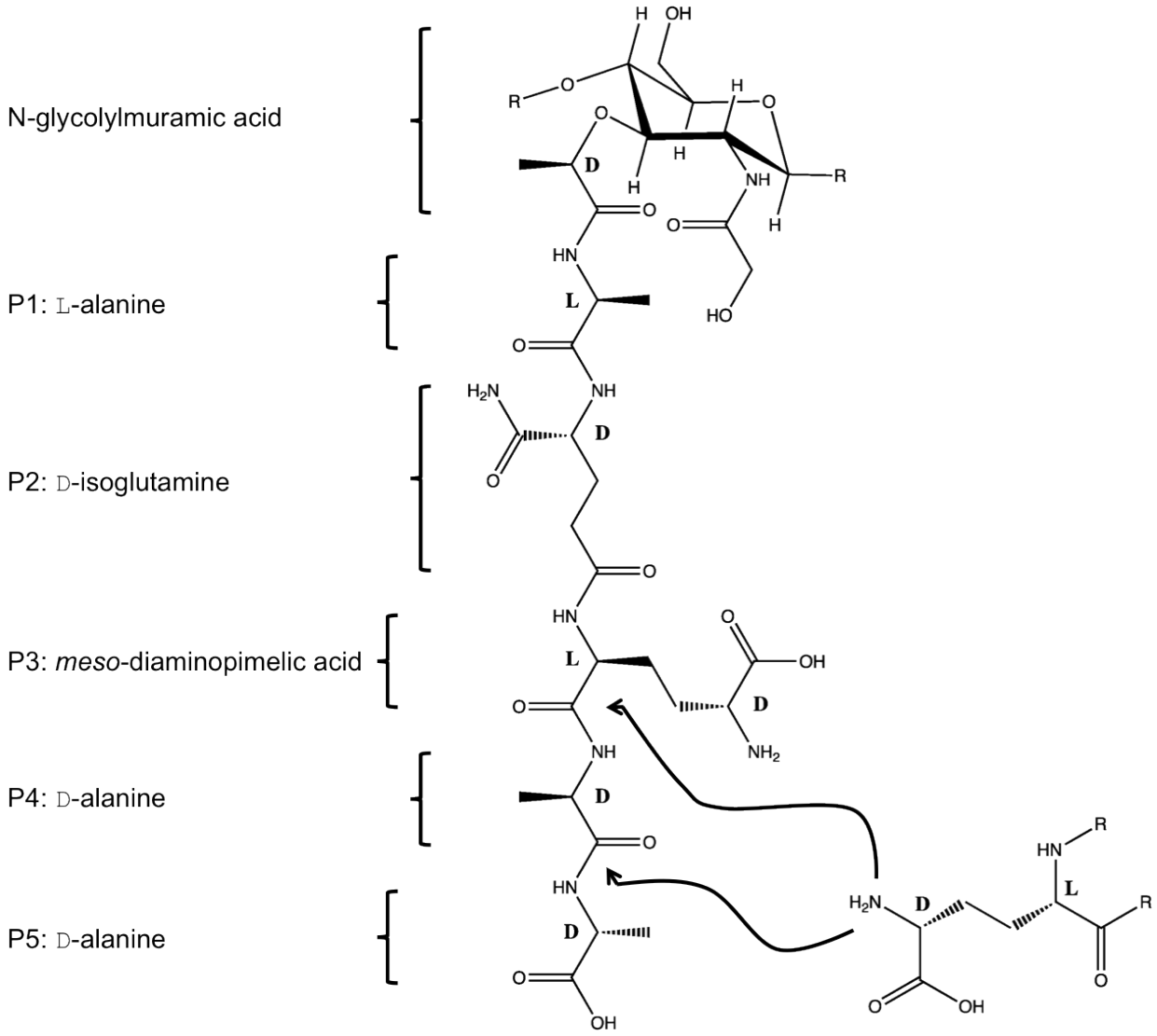


Figure 1.5. Peptidoglycan side chains in MTB contain L-alanine, D-isoglutate, *meso*-diaminopimelic acid, D-alanine, and D-alanine. The stereochemistry of each stereocenter is indicated in bold. The possible reactive carbons to an invading nucleophile are indicated with electron-pushing arrows. D-isoglutamate in MTB is often amidated, as depicted.

from P3, the receiving carboxylic acid can be at P4 or P3 (Figure 1.5) (68). The former crosslink, termed a 4→3 crosslink, predominates in *E. coli* (66). The D,D stereochemistry of the invading and receiving amino acid chiral centers matches the stereochemistry of the D-Ala-D-Ala bond they replace; these bonds are the natural substrates of PBPs (44). In contrast, the 3→3 crosslink, which is formed by an invading amine group from P3 forming a covalent bond with the α-carboxylic acid of P3 *meso*-Dap, predominates in *Mycobacterium abscessus* (72) and stationary phase MTB (67). The L,D stereochemistry of the disrupted link between *meso*-Dap and D-Ala has given the enzymes catalyzing the formation of 3→3 crosslinks the name of L,D-transpeptidases. The investigation of 3→3 crosslinks is relatively new, but it is clear that they play a major role as a determinant of β-lactam sensitivity (73).

Section 1.3: The β-lactam mechanism of action

The molecular targets of the β-lactams

The β-lactams were the first antibiotics discovered, and have to date been among the most successful, both in use and diversity, having spawned hundreds of semi-synthetic derivatives (9). Although scientists have discovered a diverse range of bacterial resistance mechanisms, this dissertation will show that MTB can acquire resistance through a hitherto undescribed mechanism, the loss of PBP function. To understand the details of how this mechanism may work, a detailed consideration of the means by which β-lactams may act is necessary.

Exposure to β-lactams may induce several different effects. The observable outcomes range from bacteriostasis and morphological alterations to rapid bacteriolysis (74). Although effects vary for particular species (75), antibiotics (74), and exposure conditions (76), all known

effects result from the inhibition of peptidoglycan-modifying peptidases outside of the cell membrane. These peptidases are the (D,D-) PBPs and the L,D-transpeptidases (77).

The most important effects of exposure to β-lactams are cell death and bacteriolysis. Current models hold that cell death arises only from the inhibition of peptidoglycan-synthesizing HMW PBPs (67) and L,D-transpeptidases (73), as distinct from the low-molecular weight (LMW) endo- and carboxypeptidase PBPs, which hydrolyze peptidoglycan. While the phenotypic alterations arising from deletion of the LMW PBPs indicate that they may be responsible for some of the morphological effects of β-lactam treatment, they are not usually essential (78).

For the two most diverse classes of β-lactams, the penicillins and cephalosporins, bacteriolysis and cell death probably occur through inhibition of the HMW PBPs (74, 77). Although cephalosporin binding to the L,D-transpeptidases can occur (79), the failure to identify these proteins in most early assays for β-lactam binding proteins suggests the phenomenon is not common.

The third major class of β-lactams, the carbapenems, also display high affinity for certain Class A HMW PBPs (80), and so it has been assumed that the carbapenems target these PBPs too (81). Recently, the finding that carbapenems form adducts with the L,D-transpeptidases that form 3→3 crosslinks (82), and that they potently kill organisms rich in these crosslinks (14), has raised the possibility that these enzymes may present an additional or alternate molecular target for the carbapenems (73).

Whatever the relevant protein targets are in the cell, the β-lactams mimic the natural D-Ala-D-Ala substrate of the PBPs and inhibit them by forming a slowly-hydrolyzing adduct at the active site (83). The hypothesis that carbapenems may target the L,D-transpeptidases has force

because a distinctive feature of these carbapenems is the opposite chirality of the α-carbon corresponding to P4 of the peptide substrate (Figure 1.5, Figure 1.6) (81, 83). This corresponds to the difference between D,D- and L,D-transpeptidation.

Molecular pathways to cell death

The potential targets of β-lactams, then, are clear, but the means by which the inhibition of these targets leads to the observed effects of β-lactam exposure is less clear. In fact, the precise mechanism by which β-lactams kill bacteria is an enduring mystery (77, 85, 86, 87). The functional diversification of PBPs may have complicated efforts to solve the mystery, as may have the multiplicity of PBP paralogs in the typical bacterial genome.

In *E. coli*, the best-studied system, the effects of exposure to several β-lactams have received comprehensive study. β-lactams that specifically inhibit PBP3 lead to filamentation. β-lactams that specifically inhibit PBP2, the function of which is poorly described, induce the formation of ovoid cells. β-lactams that specifically inhibit the collective PBP1 lead to rapid lysis (74). The collective PBP1 comprises the PBP1a, PBP1b, and PBP1c proteins of *E. coli*, which were not separable in initial observations of the *E. coli* PBP complement. PBP1a, PBP1b, and PBP1c are all of the Class A HMW PBPs of *E. coli*.

The bacteriolytic effect of PBP1 inhibition is consistent with a number of data indicating that the Class A HMW PBPs are collectively essential in many species. This has been genetically demonstrated in *E. coli* (88) and *B. subtilis* (89). In *Corynebacterium glutamicum*, a close relative of MTB with a closely corresponding set of PBPs, the deletion of its full complement of Class A HMW PBPs leads to severe morphological defects (90). Why precisely these phenotypes should arise is not clear; both glycosyltransferase and transpeptidase activities

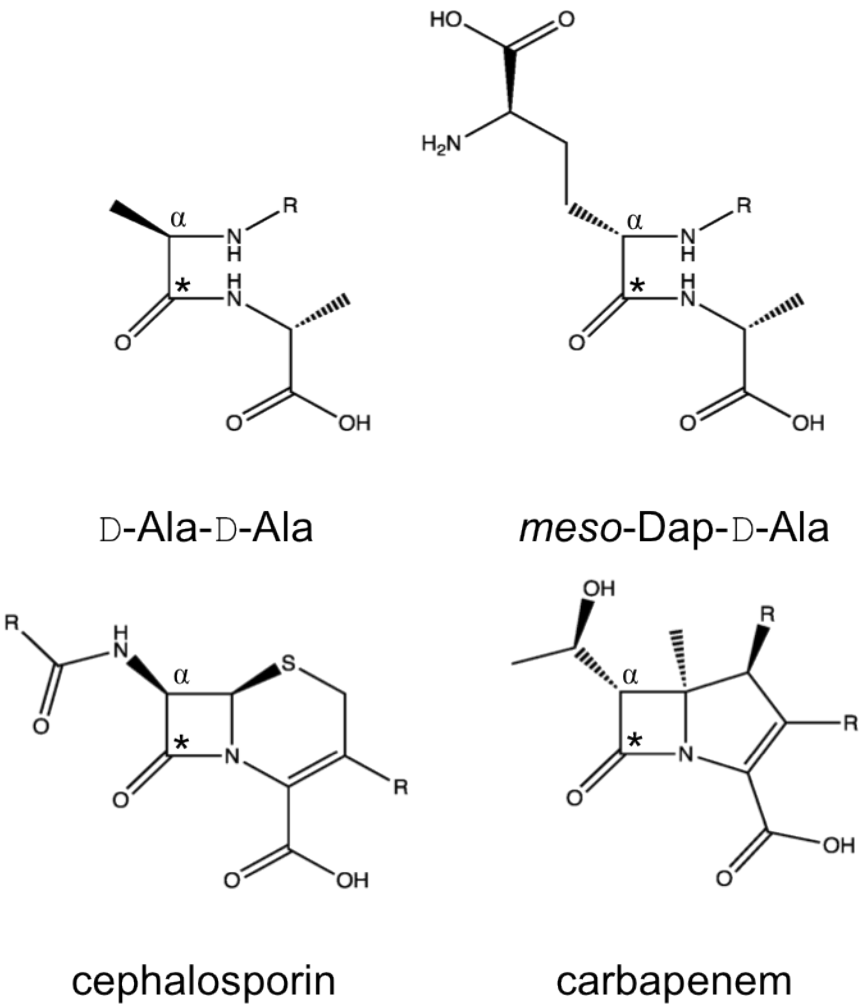


Figure 1.6. The cephalosporin and carbapenem cores have opposite chirality at the carbon α to the electrophilic carbonyl carbon (*). In peptidoglycan, the corresponding α-carbon (α) has opposite chirality at P4 and P3, where crosslinks are formed for D,D and L,D-transpeptidases, respectively. The strained conformation of peptidoglycan depicted is intended to illustrate the correspondence; the actual conformation at a transpeptidase active site is nonetheless likely to force β-lactam and peptide chains into a similar correspondence, since the β-lactams are substrate mimics for the peptide chains.

are biochemically redundant, with monofunctional glycosyltransferases and Class B PBPs, respectively. One hypothesis is that some Class A HMW PBPs may serve an essential scaffolding function (91), though whether β-lactam binding would disrupt such a function bears further investigation.

The effects of PBP3 inhibition are also consistent across species. PBP3, also called FtsI, has a more distinct and well-defined function than other PBPs, as a catalyst of crosslinking during septal peptidoglycan synthesis. It is recognizable across species and essential for septation and viability in *E. coli*, *B. subtilis*, and *C. glutamicum* (92, 93, 94, 95).

Killing by inhibition of crosslinking

The simplest model for killing by β-lactams, then, is that whatever the profile of inhibition by low concentrations of a given β-lactam, killing effects at bactericidal concentrations ultimately arise from the inhibition of the well-conserved and essential functions of either FtsI or the collective Class A HMW PBPs (74). The inhibition of either of these crosslinking functions could result in a cell wall with gradually weakening mechanical stability, which would ultimately succumb to the turgor pressure within the cell, rupturing violently (96, 97).

Killing by autolysis

A finding in *Streptococcus pneumoniae* ultimately showed that this simple model did not suffice to explain the β-lactam mechanism of action. *S. pneumoniae* has an autolytic system that causes it to undergo massive loss of viability upon reaching stationary phase (98). The chemical inactivation of the major component of this autolytic system, *lytA*, conferred tolerance to penicillin (99). The *lytA* gene encodes a peptidoglycan amidase (99). Genetic inactivation of

amidases in *E. coli* (100) and endopeptidases in *B. subtilis* (101) confirmed that the requirement of β-lactam-mediated bacteriolysis for autolysins was widespread. Notably, in all of these species, β-lactam tolerance was not complete; bacteriostatic, but not bacteriolytic, effects still occurred, and in some cases there was still a gradual loss of viability in the presence of β-lactams (98, 101, 102); these observations imply that either additional autolysins or another pathway of cell death must exist.

The requirement for autolysins led to the balanced growth model of killing by β-lactams (103). In this model, the proper maintenance of peptidoglycan requires a balance of synthetic activities, primarily from PBPs, and lytic activities from autolytic peptidoglycan hydrolases; inhibition of PBPs by β-lactams tips the balance in favor of the lytic activities, and the cell wall gradually degrades until it is too weak to withstand the turgor pressure of the cell, resulting in release of cytoplasmic contents and death (103).

Killing by induced autolysis

This model has endured to the present as a partial explanation of β-lactam killing (104). The most significant refinement to the model has been the realization that autolysins are under close control and depend on cellular signals to perform β-lactam-mediated bacteriolysis (103). This refinement sprang from two lines of evidence. First, in *E. coli*, *B. subtilis*, *S. pneumoniae*, *S. aureus*, and *E. faecium*, manipulation of culture conditions could uncouple PBP inhibition and bacteriolysis in time (105, 106), demonstrating that the autolysins require a particular signal or condition to be active. Second, even in autolysis-defective bacteria, growth and sometimes even division continue normally (56, 99, 100, 101), which shows that other, redundant peptidoglycan-hydrolyzing enzymes must exist, insofar as peptidoglycan hydrolysis is necessary for normal

growth. The necessity of some form of peptidoglycan degradation is a feature of current models of peptidoglycan maintenance (53, 107). Therefore, even with autolysins deleted, there must be other peptidoglycan degradatory machinery active. These other, redundant peptidoglycan-hydrolyzing enzymes must act under different conditions than the penicillin-induced autolysins, again implying that the cell restricts or activates at least one of the two groups – the autolysins, or the other enzymes.

Both of these observations show that the cell can, under some conditions, control the activity of autolysins (103). For the Gram positive organisms, an elegant model exists wherein the local acidity of cell wall regions controls hydrolase activity (108). Several factors in turn modulate this local acidity: the buffering capacity and pH of the extracellular milieu, the proton motive force, and the D-alanylation of teichoic acids. The balance of these forces can cause fine-tuned local variations across the cell wall (108). This model is more problematic for *E. coli*, which lacks teichoic acids and the thick peptidoglycan of Gram positive organisms, which are critical features of the model (108).

Killing by holins

The balanced growth model does not explain cell death that occurs without bacteriolysis, which occurs in some streptococci (109, 110). In these systems, loss of colony-forming ability and protein and RNA synthesis occur without any detectable change in optical density. In the initial report of this phenomenon, an uncharacterized mutation reduced this cell death by four orders of magnitude (109).

The identity of this mutation did not become clear until years later, when other researchers found a mutation in the *cidAB* operon of *S. aureus* conferring a similar amount of β-

lactam resistance. CidA bears homology to holin proteins, and its deletion lowered the extracellular peptidoglycan hydrolase activity of *S. aureus* mutants (111). Holins are small proteins that tend to disrupt the cell membrane; their effect here on extracellular peptidoglycan hydrolase activity is consistent with their usual function in phage infection. In phage infection, peptidoglycan hydrolases remain intracellular, where they cannot act, until the appropriate point in the infection cycle. At the appropriate point, the balance of holins and antiholins changes to permeabilize the cell membrane; the released hydrolases digest the peptidoglycan, and packaged phage can diffuse freely through the permeabilized membrane and digested peptidoglycan to infect other cells (112).

Notably, while endogenous holins evidently perform the same biophysical function in *S. aureus* during β-lactam-induced death as phage-encoded holins do during phage escape, the method of killing to which endogenous holins contribute is non-lytic, indicating that it does not require the release of peptidoglycan hydrolases. Instead, cell death may occur simply as a result of the collapse of the cell membrane permeability barrier, followed by loss of the proton motive force and dissipation of the cellular machinery (108, 110). Consistent with this model, the antiholin regulator of *cidAB*, *lrgAB*, activates in response to decreases in the proton motive force, allowing it to prevent inappropriate killing by *cidAB*-mediated permeabilization (113).

Homologs of *cidAB* occur in *E. coli* and many Gram positive bacteria, but there are no obvious homologues in MTB or other actinomycetes. This is not surprising, because the prevailing explanation for the existence of *cidAB* is that it has a role in biofilm development (108); the holins facilitate the release of DNA from dying staphylocci, which increases biofilm integrity (114). The exploration of biofilm roles in MTB pathogenesis is still in its infancy, but biofilms do not play a role in the classical model of granulomatous infection. Although, MTB

lacks obvious *cidAB* homologs, the possibility that some such system may contribute to β-lactam-mediated cell death cannot be excluded, since the holins are merely small, amphiphilic helical proteins, and proteins meeting this description are abundant in MTB's genome (41).

Killing by oxidative damage

Another recent proposal is that killing by β-lactams may occur through the disregulation of the tricarboxylic acid cycle and the Fenton reaction, leading to widespread, lethal hydroxyl radical damage to all cellular macromolecules (115). The precise contributions of this pathway to killing, and the causal role of hydroxyl radical damage in this killing, require additional work (115, 116), but the very existence of widespread internal damage during exposure to β-lactams reinforces a crucial point: the existence of pathways to cell death involves sensing of β-lactams.

Cellular sensing of β-lactams

The oxidative damage pathway, the holin:antiholin pathway, and the induced autolysis pathway to bacterial cell death all require some means by which the cell can sense the state of its peptidoglycan, or sense the presence of β-lactams directly. Without signal transduction, the active processes in these pathways would never be initiated.

There are several possible mechanisms the MTB cell could use to monitor the state of its peptidoglycan synthetic machinery. One mechanism is through a sensor protein. *S. aureus* uses such a mechanism, the BlaR1 protein, to monitor β-lactam concentrations so that it can avoid unnecessary expression of its supplementary, highly β-lactam-resistant PBP, PBP2a. PBP2a is the principle effector of methicillin resistance in methicillin-resistant *S. aureus* infection. To respond to treatment with methicillin, a sensor domain resembling a transpeptidase domain

resides outside the cell, connected by a transmembrane helix to an internal peptidase domain that controls the expression of PBP2a, upregulating it when β-lactams are present (117). MTB has a close homolog of BlaR1. This gene, Rv1845c, is thought to respond to β-lactams, but has only a BlaR1-like response domain, with no sensor domain (118). MTB nonetheless possesses many candidate domains that may bind to β-lactams, including a number of β-lactamases and undescribed transpeptidases not thought to contribute directly to β-lactamase activity. It also possesses two genes that encode the PBP-associated serine and threonine kinase-associated domain, a specialized domain that may bind peptidoglycan-like substrates and play a role in sensing (119). It is possible that any of these proteins may interact with BlaR1 or another transmembrane regulator to convey direct information about the presence of β-lactams.

Another mechanism through which MTB may sense the presence of β-lactams is indirectly, by monitoring the state of its peptidoglycan. One way to do this would be through the peptidoglycan recycling pathway, by which various peptidoglycan fragments are transported directly to the cytosol. In *E. coli*, this pathway provides information through the proportion of tri-, tetra-, and pentapeptides imported by AmpD, a disaccharide-peptide transporter. An increased ratio of pentapeptides to tripeptides indicates a lack of PBP activity, implying the presence of β-lactams and leading to expression of the AmpC β-lactamase (120). While MTB does not possess clear homologs to the proteins of the Amp pathway, it and other Gram positive bacteria evidently do have a peptidoglycan recycling system, as indicated by the description and characterization of the NagZ protein in *B. subtilis* (55). Investigation into this system is only beginning. MTB possesses a NagZ ortholog (41).

Finally, bacteria may possess additional ways of sensing β-lactams by somehow directly monitoring the activity or inhibition of their β-lactam sensitive transpeptidases. In *C.*

glutamicum, which is closely related to MTB, depletion of FtsI (a Class B HMW PBP), but not its inhibition by cephalexin, resulted in overexpression of the structural protein DivIVA (95). The overexpression of this protein implies that cephalexin-bound FtsI activates some signaling pathway distinct from the one activated by absence of FtsI activity, which does not lead to DivIVA overexpression. Since FtsI activity is absent in both cases, there should be no difference that can be detected from the state of the peptidoglycan itself. *C. glutamicum*, which falls in the same actinomycete family as MTB, may possess a sensor molecule like BlaR1 or may have some entirely novel way of monitoring FtsI activity.

FtsI may represent a special case; unlike most PBPs, it is individually essential in most species and well-conserved across all species (92). There is more evidence that there may be distinct cellular signaling pathways to monitor FtsI. In *E. coli*, cells appear to be able to react to FtsI inhibition by turning on the SOS response and inducing the *dpiBA* operon (121); as with *C. glutamicum*, the specific mechanism of signaling is not known (121).

Section 1.4: Potential defenses: β-lactam resistance across bacteria and its implications for mycobacteria

Given the uncertainty surrounding the precise series of steps by which exposure to β-lactams leads to bacterial cell death, it would be remarkable if there existed a complete catalog of the mechanisms by which bacteria might acquire resistance to the β-lactams. Nonetheless, two broad classes of resistance mechanisms have occurred over and over again (Figure 1.7). The first broad class contains those mechanisms that decrease the effective concentration of β-lactam at the general site of action, the periplasm or cell wall zone. This class comprises changes in outer membrane permeability, changes in β-lactamase activity, and changes in efflux pump

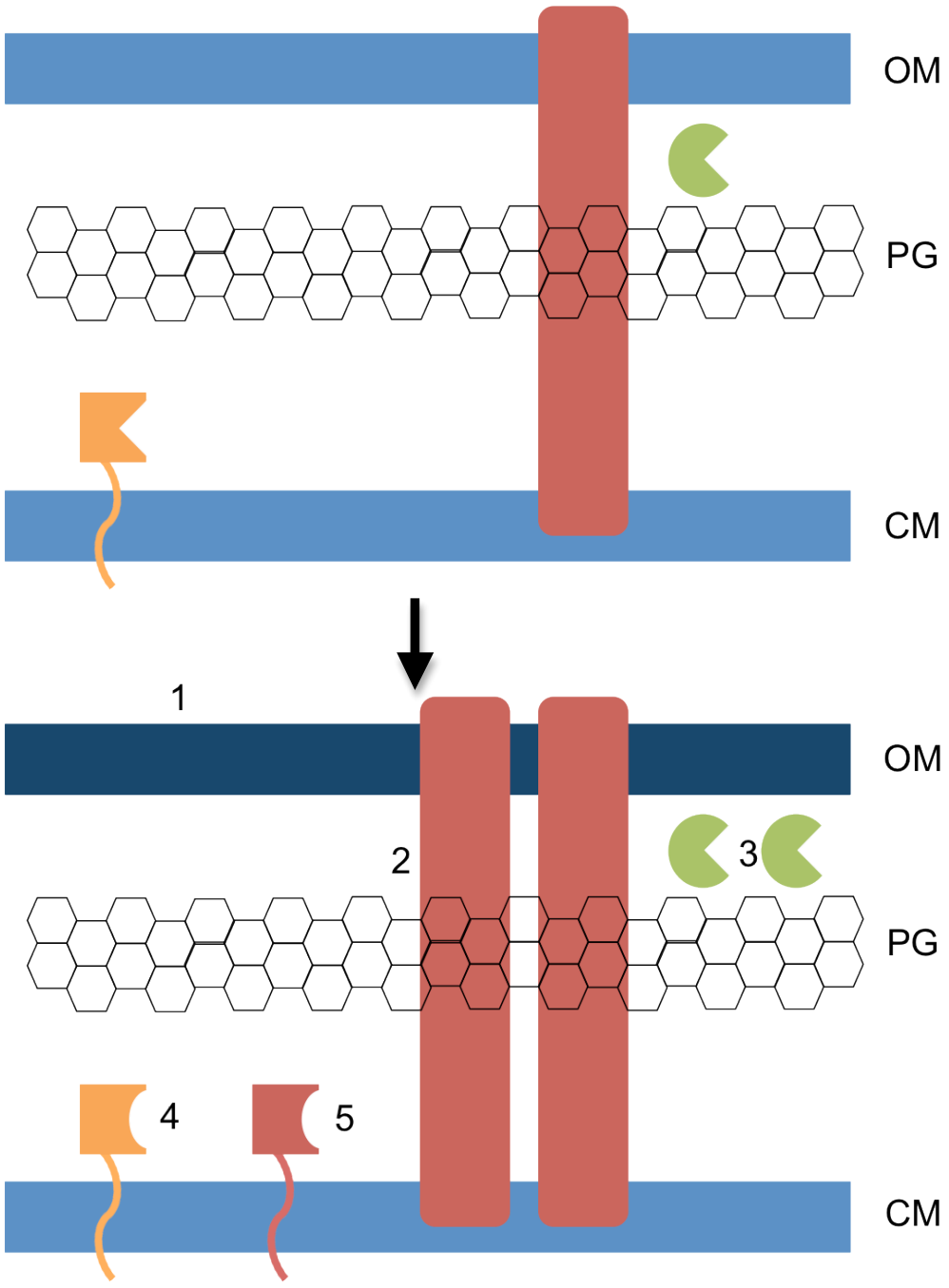


Figure 1.7. Overview of β-lactam resistance mechanisms. A simplified mycobacterial cell wall is depicted from the outer membrane (OM) through the peptidoglycan (PG) to the cell membrane (CM). In the bottom panel, the same cell wall is depicted after having utilized the full complement of resistance mechanisms discussed here: [1] alterations in permeability of the outer membrane; [2] increase in efflux pump expression; [3] increase in β-lactamase expression; [4] target site modification of the constitutive PBP; [5] and expression of a new, β-lactam refractory PBP.

activity. The second broad class of β-lactam resistance mechanisms is the modification of the PBP profile of the cell in such a way that sufficient transpeptidase activity exists for survival at a given β-lactam concentration, where before it had not. This may occur through target site modification, in which an individual PBP acquires a mutation altering its affinity for β-lactam substrates, or through acquisition or expression of previously unused PBPs with low β-lactam affinity.

Permeability

Changes in outer membrane permeability could occur by diffusion through the hydrophobic bilayer itself or through alterations in the population of porins it contains (122). In *Pseudomonas aeruginosa*, which like MTB has a combination of permeability and efflux defenses (123), β-lactam diffusion through porins is much more important than direct diffusion across the lipid bilayer: increasing β-lactam hydrophobicity leads to decreasing effectiveness, since the increased bilayer diffusion does not compensate for loss of mobility through porins. Therefore, although both alterations in porin content (124) and outer membrane composition (125) correlate with acquisition of β-lactam resistance, outer membrane changes probably act through the populations of porins that are permitted in the outer membrane (122, 125).

MTB is similar to Gram negative organisms, in that it possesses a well-defined outer membrane with porins for nutrient diffusion. Also like Gram negatives, diffusion of β-lactams into MTB probably occurs through hydrophilic pathways rather than across its lipid bilayer (26). Since few model Gram positive organisms possess similar outer membranes to MTB, and against these few, β-lactams are generally ineffective, little literature exists on the possibility of MTB modifying its porin or outer membrane characteristics to resist β-lactam diffusion through its

hydrophilic entry points. Expression of a porin from a closely related organism, *Mycobacterium smegmatis*, in the MTB model *Mycobacterium bovis* BCG, increased growth rate and sensitivity to several antibiotics, implying that low hydrophilic diffusion is a limiting factor in growth (126, 127), and that further diminution of these pathways could not increase β-lactam resistance without deleteriously affecting growth. Notably, however, some research has identified loss of function mutations in sulfolipid biogenesis genes that appear to confer β-lactam resistance in MTB. Sulfolipids are components of the outer membrane. This research only showed genetic association, and did not identify changes in the sulfolipid population, or show a causal link between gene loss and resistance (128); nonetheless, the possibility exists that diffusion across the lipidic components of the outer membrane does play an unappreciated role in β-lactam access to the periplasm.

β-lactamase activity

The second method of altering effective β-lactam concentration is through alteration of the β-lactamase profile. MTB possesses a strong, promiscuous β-lactamase, BlaC (129), and numerous apparent β-lactamase paralogs (41) with undefined activity; not all are necessarily β-lactamases. The *blaC* gene itself is under control of at least two stress-responsive transcription factors (118). Together, these factors suggest a broad potential for MTB to alter its existing β-lactamase transcription levels and substrate specificities through spontaneous mutation. MTB has not yet realized this potential in the clinic; a broad survey of clinical strains found little variation in the β-lactamase activities present (130).

Despite this potential, MTB lacks one of the most important resistance determinants for the carbapenem class of β-lactams. Metallo-β-lactamases, which tend to have extremely broad

substrate specificity, activity against carbapenems, and resistance to standard β-lactamase inhibitors (131), are not present in the MTB genome. MTB is unlikely to acquire one of these genes, because despite their recent spread in other organisms, MTB has very low potential for horizontal gene transfer (132, 133). This removes one of the most serious potential weaknesses of the tuberculosis therapy highlighted by this review, treatment with meropenem/clavulanate.

Multidrug efflux pumps

The third method of altering effective β-lactam concentration is through multidrug efflux pumps. Multidrug efflux pumps are a widespread mechanism of antibiotic resistance. In the case of MTB and other organisms possessing an outer membrane, all of the multidrug efflux pump families require adaptor proteins to facilitate transport from the cytoplasm or periplasm across the outer membrane. Although MTB does not possess homologs to the known adaptor proteins (41, 134), it does possess numerous multidrug efflux pumps (128). One of these exports β-lactams, and its loss confers β-lactam sensitivity (128). Together, these findings suggest that transcriptional alteration of efflux pump profiles has great potential to confer additional β-lactam resistance.

Target site modification

The second broad class of mechanisms of β-lactam resistance is modifications of the transpeptidase activity that allow it to evade β-lactam binding. The most prominent method for doing this is through target site modification, which can make a PBP more refractory to a given β-lactam. Target site modification may work through two methods. First, point mutations in the target site may alter the deacylation rate, allowing the PBP to eject the suicide substrate and

regain its functionality on a short timescale. Such mutations confer modest resistance in *S. pneumoniae* (135). Second, point mutations can alter the affinity – the quotient of the acylation rate constant for the β-lactam divided by its dissociation constant for the PBP in question – of the PBP, resulting in an improved ratio of peptidoglycan to β-lactam binding (77). While target site modification often occurs through genetic exchange and the formation of mosaic alleles differing by as much as 15% from the original parent alleles (136), target site modification by genetic exchange would likely be unavailable to MTB because of its low potential for horizontal gene transfer. Instead, it would have to acquire resistance through individual point mutations. Such mutations can confer modest resistance to β-lactams. Like all target site modification of PBPs, point mutations are usually dominant (77, 137).

Alteration of the penicillin-binding protein profile

Instead of altering an existing transpeptidase through target site modification, the cell may acquire or express a new PBP. The most prominent example of this sort of resistance is PBP2a in *S. aureus*. PBP2a has very low affinity for many β-lactams (138), but is sufficient to carry on transpeptidation when all other PBP transpeptidase functions are inhibited (139). PBP2a is inducible by a β-lactam-sensing system (117), and its expression does not alter the remainder of the PBP profile of the cell (140), which is intuitively consistent with its dominant mode of action (137); expression of nonfunctional homologs does not interfere with the action of the dominant PBP.

S. aureus acquired PBP2a from a mobile genetic element (141); the possibilities for MTB acquiring PBP2a or a similar resistance mechanism are therefore limited by MTB's low potential for horizontal gene transfer. Nonetheless, the fact that PBP2a is repressed under normal

conditions illustrates an important point: MTB may not express its entire complement of PBPs except under stressed conditions. Some have hypothesized that *ponA2*, a Class A HMW PBP, may have special sterically obstructive elements that render it refractory to β-lactam binding (44), and experimentally, PonA2 does bind to some β-lactams poorly (142). Our laboratory has noted that PonA2 seems to express poorly at the translational level, perhaps because it is not part of the housekeeping peptidoglycan biosynthesis machinery under β-lactam-diminished conditions (unpublished data). PonA2 is therefore a speculative example of the potential for a PBP2a-like resistance mechanism in MTB.

A relatively unexplored mechanism of resistance that fits broadly into alteration of the PBP profile is the replacement of normal crosslinking functions with L,D-transpeptidation in *E. faecium*. In this mechanism, under inhibition by β-lactams, PBP crosslinking stops, while L,D-transpeptidation continues. An unusual feature of this system is that the L,D-transpeptidase does not take the usual pentapeptide as a substrate; instead, it exclusively uses tetrapeptide, which must be created from pentapeptide by a β-lactam-insensitive D,D-carboxypeptidase (143, 144). There is no energetic or biophysical reason why tetrapeptide should be the required substrate; this requirement appears to be a result of the substrate preference of the *E. faecium* L,D-transpeptidase. Therefore, although MTB does not possess any orthologs of the *E. faecium* carboxypeptidase (41), it does possess several L,D-transpeptidases, and so it is conceivable that it could acquire resistance through L,D-transpeptidation.

This experiment in *E. faecium* is distinctive in that it shows a mechanism of β-lactam resistance that relies on loss of PBP function, and loss of dependence on this function, rather than gain of it in the face of increasingly harsh exposures to β-lactams. The authors obtained this distinctive resistance phenotype by five serial passages (144). In this dissertation, we hope to

show the generation, in a single step, of a mutant that may rely on a similar mechanism, and that, in any case, confounds all of the above explanations for β-lactam resistance.

Section 1.5: Dissertation overview

Our interest in the cell wall began with the resuscitation-promoting factors of MTB; we thought the essential role during infection of a class of peptidoglycan-modifying enzymes that otherwise lacked obvious phenotypes intriguing. In Chapter 2, we use a high-throughput chemical screen to identify compounds that specifically kill MTB lacking resuscitation-promoting factors. One of the most prominent classes of compounds that does so is the cephalosporin class of β-lactams. In Chapter 3, we further investigate the cephalosporins in detail. We show that cephalosporins have increased potency against the resuscitation-promoting factor-deficient strain because of a permeability defect in its mycolic acid layer; we propose that this permeability defect may explain the importance of the resuscitation-promoting factors during infection.

In Chapter 4, we investigate the physiologically relevant target of the cephalosporins. We show that spontaneous resistance arises from mutations in the *ponA2* Class A HMW PBP. These mutations confer partial loss of function in the transpeptidase domain and to lead to meropenem sensitivity as well as cephalosporin resistance. We propose a model of *ponA2* loss of function-mediated resistance wherein PonA2 activity is replaced by the activity of a cephalosporin-resistant, meropenem-sensitive transpeptidase.

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Chapter 2

Chemical genetics reveals a broad vulnerability in resuscitation-promoting factor-deleted Mycobacterium tuberculosis

Attributions

The high-throughput chemical screen against wild-type *Mycobacterium tuberculosis* H37Rv was designed and performed by Sarah Stanley and John Aquadro. The screening design was modified, and the high-throughput chemical screen against *Mycobacterium tuberculosis* H37RvΔ*rpfACDEB* was performed by James Gomez and Carl Wivagg. Screening compound preparation and plate mapping were handled by the Broad Institute Chemical Biology Platform. All further molecular biological work and analysis was performed by Carl Wivagg.

Marked portions of this work were published as a manuscript by Carl Wivagg and Deborah Hung in *Antimicrobial Agents and Chemotherapy* 2012, volume 56, pages 1591-1594.

Chapter 2: Chemical genetics reveals a broad vulnerability in resuscitation-promoting factor-deleted Mycobacterium tuberculosis

Section 2.1: Abstract

The bacterial cell wall has provided many of the most successful targets for antimicrobial compounds, while remaining a fertile area for investigations into bacterial physiology. One such investigation identified the resuscitation-promoting factors of *Mycobacterium tuberculosis* as collectively dispensable for *in vitro* growth but essential for survival during infection of a host. The resuscitation-promoting factors are predicted to encode lytic transglycosylases. We sought to probe the function of these genes using a chemical synthetic lethal screen. A diverse library of known bioactive, commercial, natural isolate, and diversity-oriented synthesis compounds was screened for the ability to selectively inhibit growth of a strain lacking the resuscitation-promoting factors but not wild-type *M. tuberculosis*. Several diversity-oriented synthesis scaffolds showed activity across multiple compounds, but the inhibition of wild-type *M. tuberculosis* by similar compounds, the lack of stereospecificity in the scaffolds, and the activity of some compounds in an erythrocyte lysis assay led to the conclusion that these compounds did not act through inhibition of a synthetically essential enzymatic process. Among the known bioactive compounds, 9 out of 31 β-lactams screened showed resuscitation-promoting factor specific activity. Slight sensitization to known bioactive compounds with unrelated mechanisms of action, such as rifampin and the macrolides, suggested that the increased activity was the result of a broad permeability defect caused by the deletion of the resuscitation-promoting factors, rather than synthetic lethal interactions with specific genes.

Section 2.2: Introduction

Chemical biology provides a number of useful advantages that complement classical approaches to understanding bacterial physiology. Among these are the ability to rapidly and reversibly inhibit a protein target, to perturb the function of essential genes, and to modulate enzymatic activity in biologically relevant niches, such as during host infection (1). We thought these tools would be advantageous in studying the resuscitation-promoting factors of *Mycobacterium tuberculosis*, which are a recently identified family of cell wall-acting enzymes (2, 3).

Chemical genetics is uniquely appropriate for molecular biological examinations of the cell wall. Each class of cell wall-modifying enzymes – transpeptidases, carboxypeptidases, lytic transglycosylases, endopeptidases, and amidases – typically has multiple paralogs in any given species (4, 5, 6). This redundancy makes classical genetic approaches to understanding gene function difficult and time-consuming. The difficulty arises not merely from the large number of potential paralogs for a given function, but also from the fact that these paralogs may not be identifiable in advance. For example, endopeptidases and carboxypeptidases may both have annotations as low-molecular weight penicillin-binding proteins, and in fact some of these proteins may perform both enzymatic activities (7). This lack of clarity necessitates a laborious combinatorial approach to generate mutants with informative phenotypes (8).

Chemical genetics, in contrast, can sometimes provide a single small molecule, like β-lactams for the transpeptidases, or 2-nitrophenylthiocyanates for the lytic transglycosylases of MTB (9), that can reversibly or irreversibly inactivate a whole class of proteins without genetic manipulation. With attention to dosing, single small molecule inhibitors can behave either as specific inhibitors of individual paralogs, or as general inhibitors of a class (10). Chemical

genetics also provides a facile approach to study the components of the extracellular peptidoglycan maintenance machinery that are essential, such as FtsI; cephalexin and piperacillin are specific inhibitors of this essential protein in *E. coli* (11).

Mycobacterium tuberculosis (MTB) in particular benefits from a chemical genetic approach, because classical genetic studies remain difficult, since even a modest amount of laboratory growth can lead to loss of mycobacterial virulence factors (12). Such losses can alter mycobacterial physiology in unpredictable ways. Moreover, MTB's 18-24 h doubling time can make the generation of a mutant strain fully deleted for a family of paralogs take years (13, 14, 15).

**MTB is the causative agent of tuberculosis and resists killing by many antibiotics. This resistance arises partly from the unique mycobacterial long-chain mycolic acids that are covalently bound to the cell wall peptidoglycan by intermediate arabinogalactan polymers, encasing the bacterium in a poorly permeable, hydrophobic shell. Bacteria regulate growth and division using many classes of peptidoglycan-modifying enzymes to remodel the cell wall, including, in MTB, the five resuscitation-promoting factors (*rpfA-E*), which are thought to correspond to peptidoglycan lytic transglycosylases in Gram-negative organisms. Like lytic transglycosylases, RPFs are not essential for *in vitro* growth. Thus, deletion of all five RPF homologues (H37RvΔ*rpfACDEB*, hereafter RPF^{null}) in MTB has no effect on bacterial viability in broth culture, although one growth model on an agar support shows delayed colony formation (15). In contrast, the RPFs are essential for growth and persistence in a mouse model of MTB infection (15). Since cell wall growth and division can apparently proceed *in vitro* in the absence of RPFs, it is unclear what their essential role is *in vivo*.

Homology studies, demonstrated enzymatic activity (16), and interactions with other cell-wall modifying enzymes (17) all suggest that RPFs have a function modifying peptidoglycan. Because of the covalent interaction between the peptidoglycan and the mycolic acids, alterations to peptidoglycan may affect the outer membrane, the integrity of which contributes to hallmark mycobacterial characteristics, including clumping of cells in culture, impermeability, and broad antibiotic tolerance. RPFs may therefore contribute to these characteristics.

In order to elucidate further roles of the RPFs in *M. tuberculosis*, we performed a high-throughput chemical screen of 26,000 compounds to identify molecules that preferentially inhibit growth of RPF^{null} but not wild-type *M. tuberculosis* (RPF^{WT}), thus providing a synthetic lethal phenotype with the RPFs.**

Section 2.3: Results and discussion

Chemical screen and selection of hits

We designed a high-throughput chemical screen for logarithmically growing RPF^{null} in 384-well format. Growth of RPF^{null} in the presence of diverse small molecules was measured after four days, using constitutively-expressed red-fluorescent protein. A total of 26,000 compounds were screened.

**The results from the screen against RPF^{null} were compared to results from a parallel screen against RPF^{WT} (18). We selected for follow-up 168 compounds that were hits against RPF^{null} but not RPF^{WT}; hits were defined as those compounds whose mean activity exceeded the activity of rifampin at a concentration at which the Z' of rifampin, equaled zero (19). Rifampin is a tuberculocidal antibiotic that provided clear killing and was used as a control in the screen; Z' is a measure of separation between negative and positive controls. An additional 42

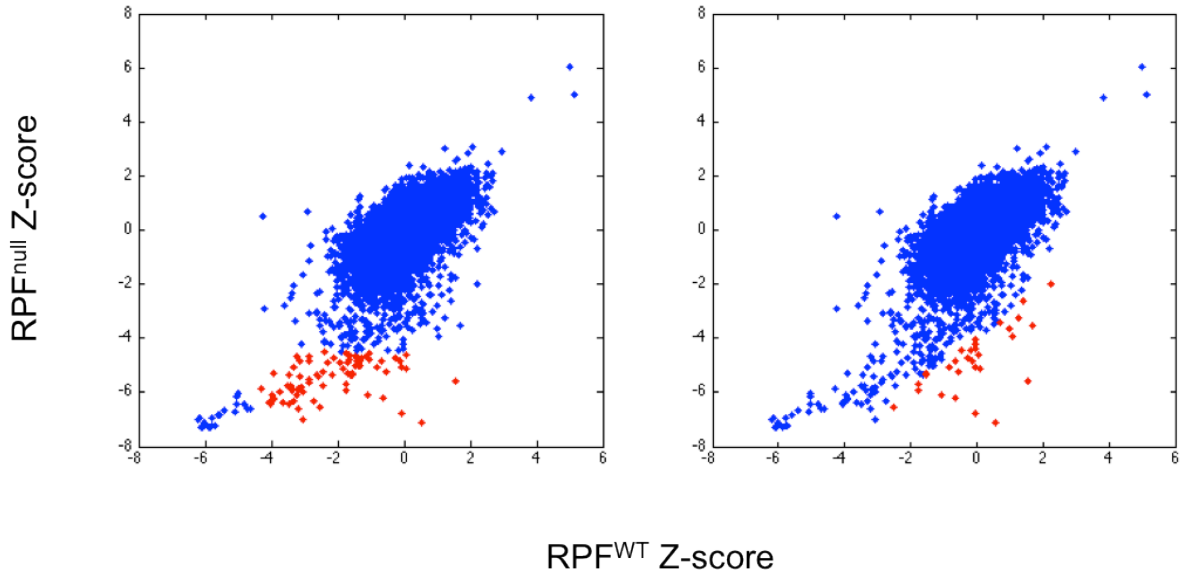


Figure 2.1. Selection of compounds from an RPF synthetic lethal screen. Selection of compounds that were designated hits (red) from the entire population of screened compounds (blue) was performed using two methods. First (left), hits were selected for which activity exceeded a predefined threshold (see text) in the strain of interest, RPF^{null}, but not in RPF^{WT}. Second (right), hits were selected for which the difference in growth between RPF^{WT} and RPF^{null} exceeded an arbitrary threshold. There was overlap between the selection methods.

compounds were selected for which the pairwise difference in growth for each molecule between RPF^{WT} and RPF^{null} exceeded an arbitrary threshold (Figure 2.1). Some compounds with significant differences in activity between the two strains may not have been identified because the screening concentration exceeded the inhibitory concentration of both strains.**

Each of the selected compounds was retested against RPF^{null} and against H37RvΔ*rpfACBED*, an alternatively-derived RPF knockout strain (Table 2.1). This second strain was used as a biological replicate, to eliminate concerns that secondary mutations arising from the lengthy passaging of RPF^{null} during its creation might be contributing to vulnerability to the RPF^{null}-inhibitory compounds. The strains were also tested against the parent strain, RPF^{WT}; this strain differs from H37Rv because it lacks phthiocerol dimycocerosate (PDIM) (Table 2.1) (20), a virulence factor known to influence the mycobacterial permeability barrier to some small molecules (21).

The retest was performed using the screening methodology, at full or half screening concentration, depending on compound availability. Compounds were also retested at five twofold dilutions below the maximum dose to evaluate their potency. Of the retested compounds, 76 were eliminated because they had similar activity against RPF^{WT} and RPF^{null}, indicating that either they had undetected activity in the original screen against H37Rv, or that their activity against RPF^{null} was a result of PDIM deficiency, not the loss of RPFs. The cutoff for elimination corresponded to about 33% inhibition of RPF^{WT} growth.

Culling the remaining 134 compounds to an interpretable list presented a significant challenge, with 36 data points (2 duplicates $\times$ 3 strains $\times$ 6 doses) per compound. Three criteria were of importance: potency, reproducibility, and specificity. The former two factors were evaluated by mean effect size and the tendency of graded inhibition to occur at lower doses,

Table 2.1. Strains used in this work.

Name	Description	Source
Mycobacterium tuberculosis H37Rv	Laboratory strain	E Rubin
H37Rv PDIM$^-$ (RPF^{WT})	Alternate lineage of H37Rv containing a frameshift mutation in *mas*, which is required for biosynthesis of phtiocerol dimycocerosate.	V Mizrahi
H37RvΔ*rpfA*	H37Rv PDIM$^-$ containing an unmarked deletion of the gene *rpfA*	(13)
H37RvΔ*rpfB*	H37Rv PDIM$^-$ containing an unmarked deletion of the gene *rpfB*	(13)
H37RvΔ*rpfC*	H37Rv PDIM$^-$ containing an unmarked deletion of the gene *rpfC*	(13)
H37RvΔ*rpfD*	H37Rv PDIM$^-$ containing an unmarked deletion of the gene *rpfD*	(13)
H37RvΔ*rpfE*	H37Rv PDIM$^-$ containing an unmarked deletion of the gene *rpfE*	(13)
H37RvΔ*rpfAB*	H37RvΔ*rpfA* containing an unmarked deletion of the gene *rpfB*	(13)
H37RvΔ*rpfACD*	H37RvΔ*rpfA* containing an unmarked deletion of the genes *rpfC* and *rpfD*	(14)
H37RvΔ*rpfACBE*	H37RvΔ*rpfA* containing an unmarked deletion of the genes *rpfA*, *rpfB*, and *rpfE*	(15)
H37RvΔ*rpfACDE*	H37RvΔ*rpfACD* containing an unmarked deletion of the gene *rpfE*	(15)
H37RvΔ*rpfACDB*	H37RvΔ*rpfACD* containing an unmarked deletion of the gene *rpfB*	(15)
H37RvΔ*rpfACDEB* (RPF^{null})	H37RvΔ*rpfACDE* containing an unmarked deletion of the gene *rpfB*	(15)
H37RvΔ*rpfACBED*	H37RvΔ*rpfACBE* containing an unmarked deletion of the gene *rpfD*	(15)
H37RvΔ*rpfACDEB*:: pH*rpfCDE*	H37RvΔ*rpfACDEB* with *rpfC*, *rpfD,* and *rpfE* under the control of their native promoters on the integrating plasmid pHINT	this work

while specificity was evaluated by inhibition of RPF^{WT}. These criteria were transformed into a set of selective rules.

1. The compound must have an effect size of at least 33% inhibition of growth at the maximum dose.

2. The compound must have a non-zero effect size at the second-highest dose.

3. The compound must have more inhibition of the RPF^{null} than of RPF^{WT} at the maximum and second-highest doses.

In all, 47 compounds remained after this selection process (Table 2.2).

Follow-up with H37RvΔ*rpfE* and H37RvΔ*rpfAB*

We wished to understand whether all of the RPFs shared one collective function, or whether they had specific, individual functional roles. In particular, we wondered whether one or two RPFs might play a dominant role in sensitization to various small molecules, with the rest being irrelevant. To answer this question, we decided to investigate whether RPF-dependent resistance to the small molecules described above was typically dependent on the presence of individual RPFs or on the class of genes as a whole. If RPF-dependent resistance was dependent on individual RPFs, we would expect some single RPF knockouts to display the same increases in sensitivity to certain small molecules that RPF^{null} displayed. If, on the other hand, RPF-dependent resistance could be conferred by any RPF, regardless of its genetic origin, we would expect single knockouts to be broadly resistant, with most compounds being only effective against RPF^{null}.

Due to limitations on the amount of many compounds available, only two additional RPF strains could be tested. Strains H37RvΔ*rpfAB* and H37RvΔ*rpfE* were selected because it is

Table 2.2. Selection of hits from high-throughput chemical screening of RPF^{null}.

Type of Selection	Number of Compounds
Compounds screened	26000
Cherry-picked for further analysis	210
Inactive against H37Rv PDIM^{null}	134
Selected by for high potency	47
Of which	
Active against H37RvΔ*rpfAB* and H37RvΔ*rpfE*	16
Active against H37RvΔ*rpfAB* and not H37RvΔ*rpfE*	2
Active against H37RvΔ*rpfE* and not H37RvΔ*rpfAB*	1
Active against H37Rv RPF^{null} only	28

believed that *rpfA*, *rpfB*, and *rpfE* are more likely to have singular functional roles than *rpfC* and *rpfD*. This belief is predicated on the greater size of the former genes and their greater importance in several phenotypes (15). For example, *rpfB* contains several accessory domains that appear to help it achieve localization or substrate specificity, and an *rpfB* knockout has a delayed-growth phenotype in a mouse model of latent infection (22).

Strains H37RvΔ*rpfAB* and H37RvΔ*rpfE* were tested as above for RPF^{WT} and RPF^{null}: compounds were tested against strains over a six-point dose-response curve for each compound, beginning at the concentration used for the original high-throughput screen, which was between 5 and 10 μM for most compounds.

The majority of the compounds, 28 out of 47 (Table 2.2), were ineffective against any strain studied except RPF^{null}. This result was consistent with the hypothesis of broad functional redundancy among the RPFs.

More surprisingly, 16 out of the 47 were effective against all three strains: RPF^{null}, H37RvΔ*rpfE* and H37RvΔ*rpfAB*. This finding was not strictly consistent with either the hypothesis of broad functional redundancy or of specific roles for specific RPFs. Rather, it implies that *rpfE* shares a broad functional role with either *rpfA* or *rpfB*, but cannot replace their individual activities. The conclusion that the genes share a broad functional role follows from the fact that loss of *rpfA*/*rpfB* and loss of *rpfE* lead to the same phenotype: sensitization to the 16 small molecules. The conclusion that sharing a broad functional role, they do not act in the same specific functional niches follows from the fact that *rpfA*/*rpfB* cannot completely compensate for *rpfE* or vice versa; instead, each of these 16 compounds can inhibit a strain that has lost either *rpfA*/*rpfB* or *rpfE*.

Discussion of chemical properties of hits

β-lactams

Out of 31 β-lactams screened, 9 had specific activity against RPF^{null}. Two others had specific activities that were not detected in the screen because the screening concentration was not high enough. There was no obvious molecular profile of physical properties that distinguished those compounds with activity against RPF^{null} from those without; among the physical properties considered were hydrophobicity, polarity, molecular weight, charge state, and the presence of acidic or basic sites (Figure 2.2). All of the β-lactams contained the cephalosporin core, except cloxacillin. This β-lactam had a bulky residual group in common with the cephalosporin hits, but such residual groups are common across β-lactams (Figure 2.3).

Tuberculocidal diversity-oriented synthesis compounds

Out of the 47 compounds, 24 were members of three classes of DOS (diversity-oriented synthesis) compounds. DOS compounds are designed to probe areas of chemical space not represented by small molecules prepared for standard "drug-like" commercial libraries; in particular, they tend to have more chiral centers and more three-dimensional shape than compounds designed by other approaches (23, 24).

Each of the three classes of DOS compounds represented among the 47 hits had a separate core structure upon which several growth-inhibitory compounds were based. These compounds were not pursued further because other variations of the same core structure inhibited the growth of RPF^{WT} (Figure 2.4). The small differences between compounds that were specific to RPF^{null} and those that were not led us to hypothesize that slight differences in

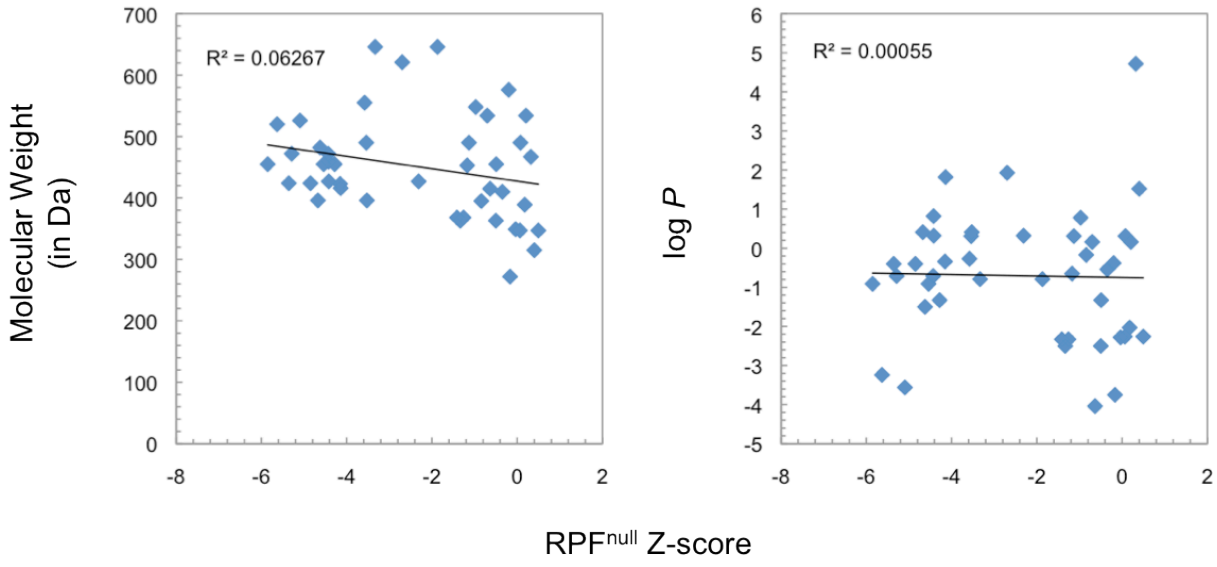


Figure 2.2. Correlation of β-lactam activity from the chemical synthetic lethal screen with molecular properties. β-lactams with activity against RPF^{null} did not have distinct molecular weights (left) or octanol/water partition ratios(right) from those with no activity. All β-lactams screened are plotted; trendlines were determined by least squares linear regression.

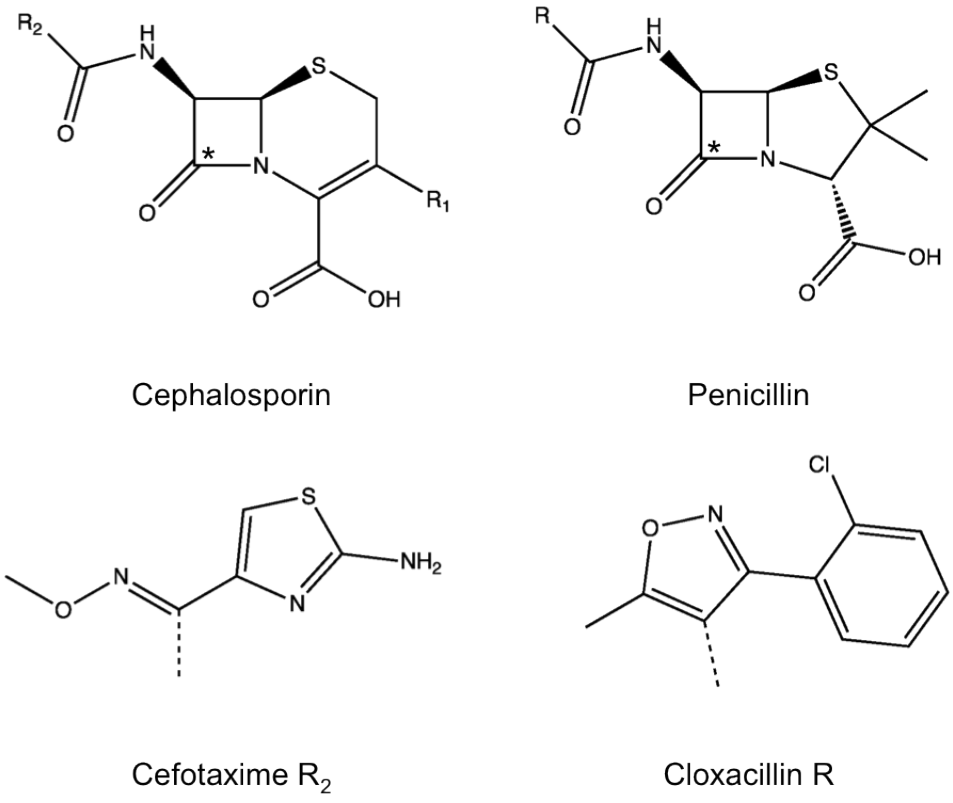
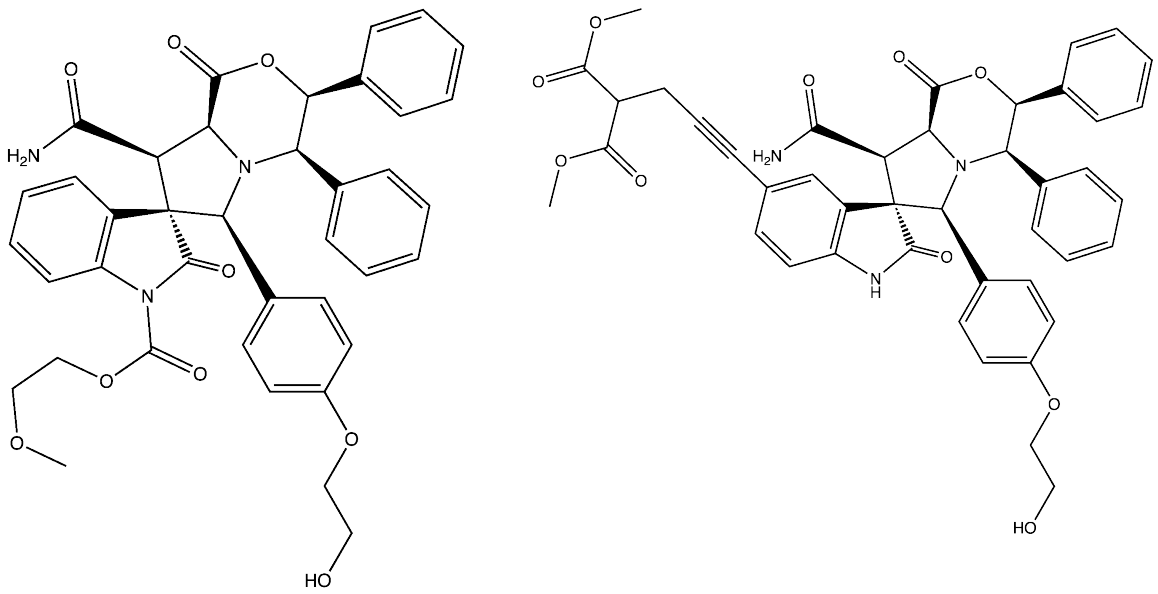
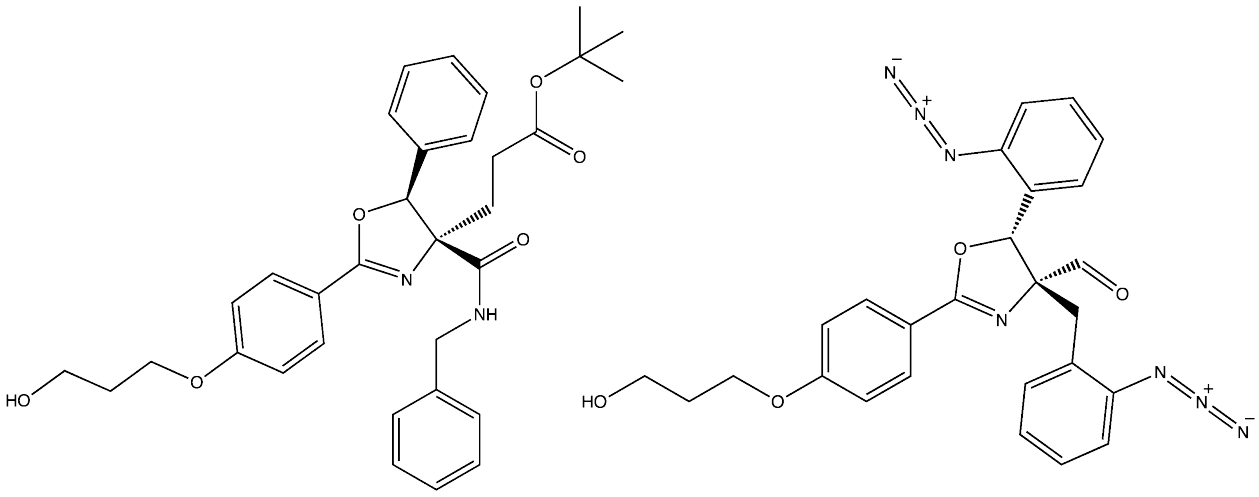


Figure 2.3. Comparison of cephalosporin and penicillin hits from the chemical synthetic lethal screen. The cephalosporin core structure often contains residual groups at R_1 and R_2 of up to 800 Da. The cloxacillin modification of the penicillin core contains a similarly bulky group.

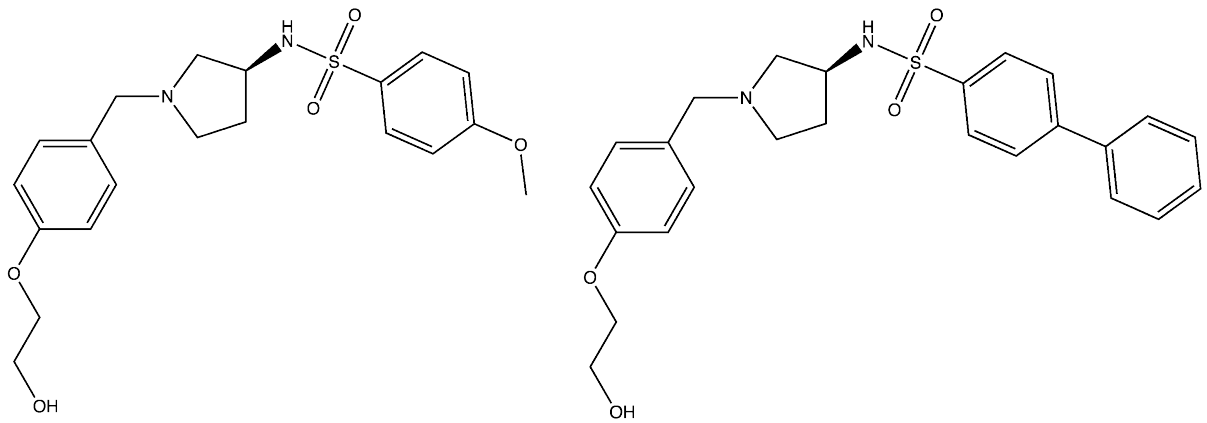


DOS Class 1



DOS Class 2

Figure 2.4 continued on next page



DOS Class 3

Figure 2.4 (Continued). Representative chemical structures for synthetic lethal DOS compounds. Chemical structures are displayed for each of the three DOS scaffolds showing activity against both specific activity against RPF^{null} and general activity against RPF^{WT} and RPF^{null}. The left compound for each class is an RPF^{null}-specific example compound, while the right compound is an example compound with activity against RPF^{WT}.

chemical characteristics from side group variation might affect the relative ability of each compound to traverse the cell walls of RPF^{WT} and RPF^{null}.

In particular, compounds of DOS Class 1 (Figure 2.4) were hypothesized not to have an RPF-specific mechanism of action because several active compounds had stereocenters with inverted chirality relative to the rest; since enzymes are chiral molecules, a notional enzyme target of DOS Class 1 would be expected to interact with only scaffolds containing one chirality. Furthermore, 7 out of 17 DOS Class 1 compounds displayed modest activity in an erythrocyte lysis assay. Erythrocytes are eukaryotic cells lacking DNA, and consequently much enzymatic machinery; any compound that lyses both erythrocytes and bacteria is unlikely to act through an enzyme-specific mechanism. Out of all the compounds tested in this assay, DOS Class 1 were the only compounds that showed any activity against erythrocytes (Figure 2.5).

Macrocycles

Another class of compounds displaying similar behavior to DOS Class 1 were a series of large peptide-mimetic macrocycles (Figure 2.6). Only two of these molecules passed the potency and specificity filters above, but upon inspection, it appeared that the others had failed because, although displaying much greater inhibition of RPF^{null}, they also modestly inhibited growth of RPF^{WT} (Figure 2.6). The cyclic shape and numerous hydrogen bonding opportunities afforded by these molecules, as well as the fact that they comprised three sets of enantiomeric pairs, led us to hypothesize that they acted as ionophores.

The macrocycles were tested in an erythrocyte lysis assay, but did not display any activity at screening doses, in comparison to DOS Class 1 compounds, which displayed potent,

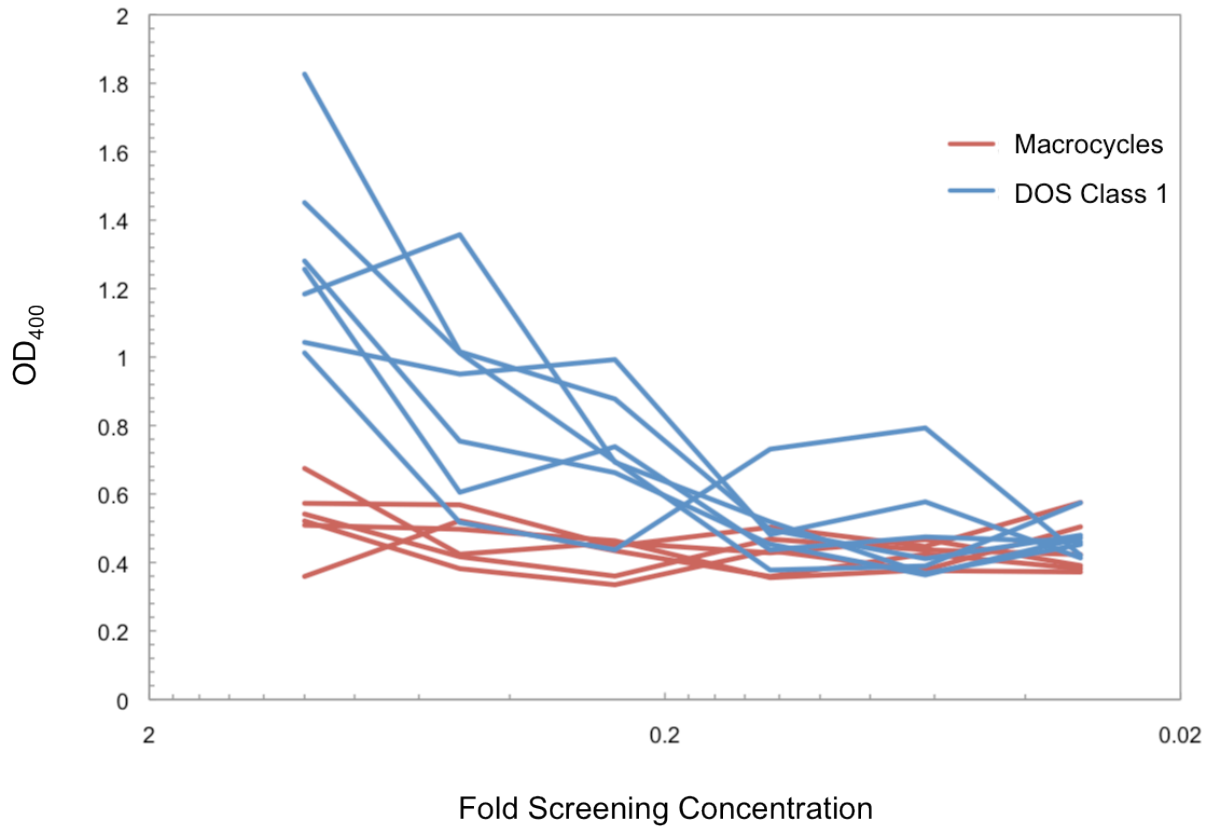


Figure 2.5. Dose-response relationships for small molecules in an erythrocyte lysis assay. Small molecules were subjected to an erythrocyte lysis assay over a range of concentrations. Erythrocyte lysis after 24 h was determined by measuring the optical density at 400 nm, which in this assay corresponds to the amount of hemoglobin released. Individual compounds are not marked here for clarity. Members of DOS Class 1 cause clearly elevated erythrocyte lysis at high concentrations.

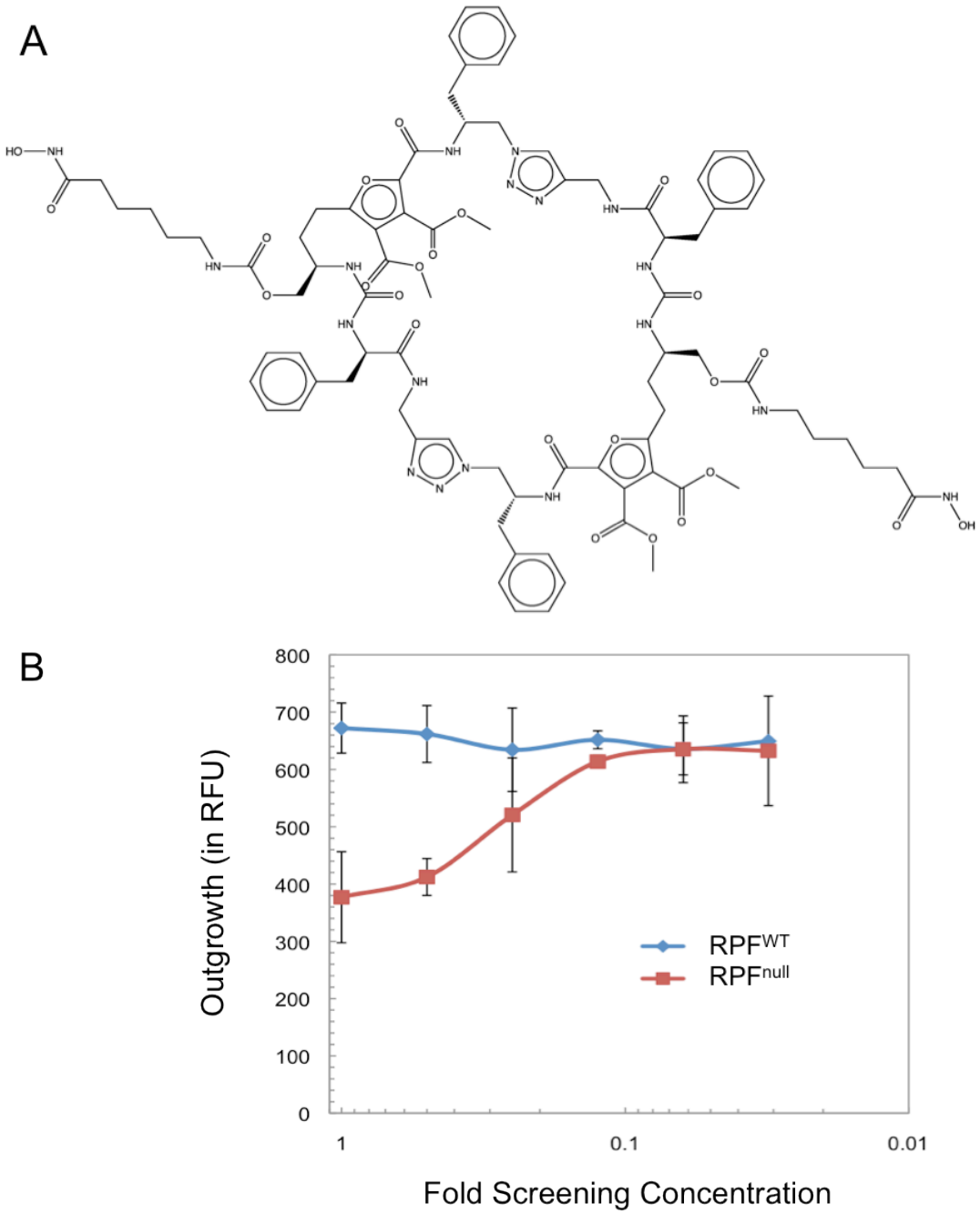


Figure 2.6. Peptide-like macrocycles, a class of RPF^{null}-specific growth-inhibitory molecules. XW_SM2_11 (A), typical of the peptide-like macrocycles, displays modest RPF^{null}-specific inhibitory activity over at least one logarithm of concentrations (B). Inhibitory activity was determined by measuring outgrowth by relative fluorescent units, as described in Materials and methods.

dose-dependent activity at multiple dilutions of screening concentrations (Figure 2.5). This suggests that they may be acting in an MTB-specific manner rather than as ionophores.

Eight-membered rings

Four hits were azocine derivatives (Figure 2.7) that shared an *N*-linked benzyl methyltrifluoride ether. Eight other azocine compounds sharing the benzyl substitution did not show activity in either screen.

Unclassified compounds

Several compounds showed RPF-specific activity but no structural similarities (Figure 2.8) to other RPF-specific compounds. Like the eight-membered rings, these compounds were deemed insufficiently-represented to merit the same level of interest as the other classes of hits.

Slight vulnerability of RPF^{null} to other antibiotics

Finally, a parallel investigation of RPF^{null} noted a modest sensitization of RPF^{null} to the macrolide class of antibiotics on agar (25); our dose-response assay noted a similar phenotype in liquid culture (Figure 2.9). Dose-response assays with other common antibiotics noted very moderate increases in RPF^{null} sensitivity to vancomycin and rifampin, as well as sensitivity to all macrolides (Table 2.3). These changes in sensitivity were not noted in the chemical screen because, for RPF^{null}-specific activity to be detected, the screening concentration must fall within a very small window of specificity; many compounds, such as rifampin, were screened at concentrations that exceeded both the RPF^{null} IC_{90} (inhibitory concentration of 90 % of growth) and the higher RPF^{WT} IC_{90}.

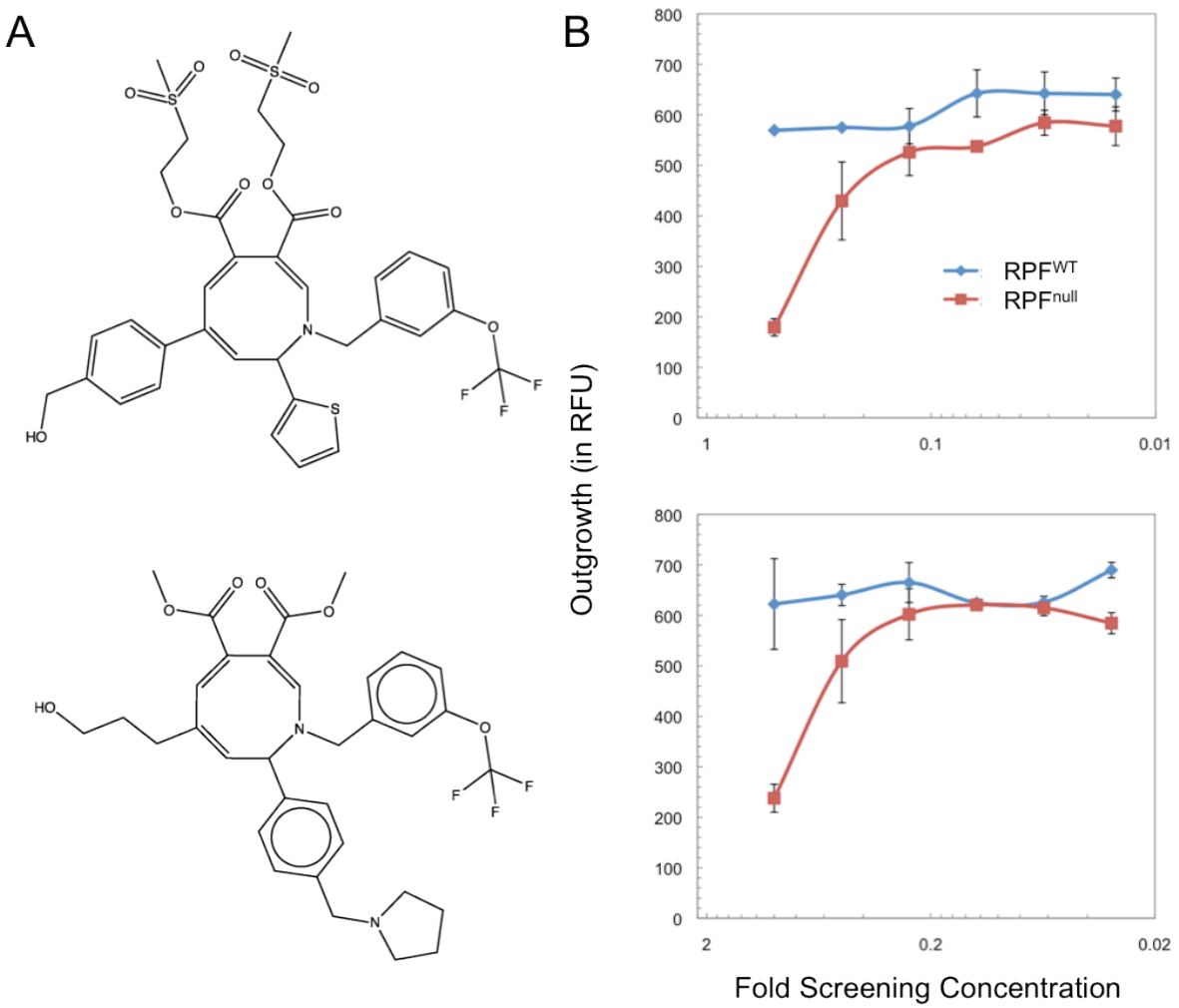
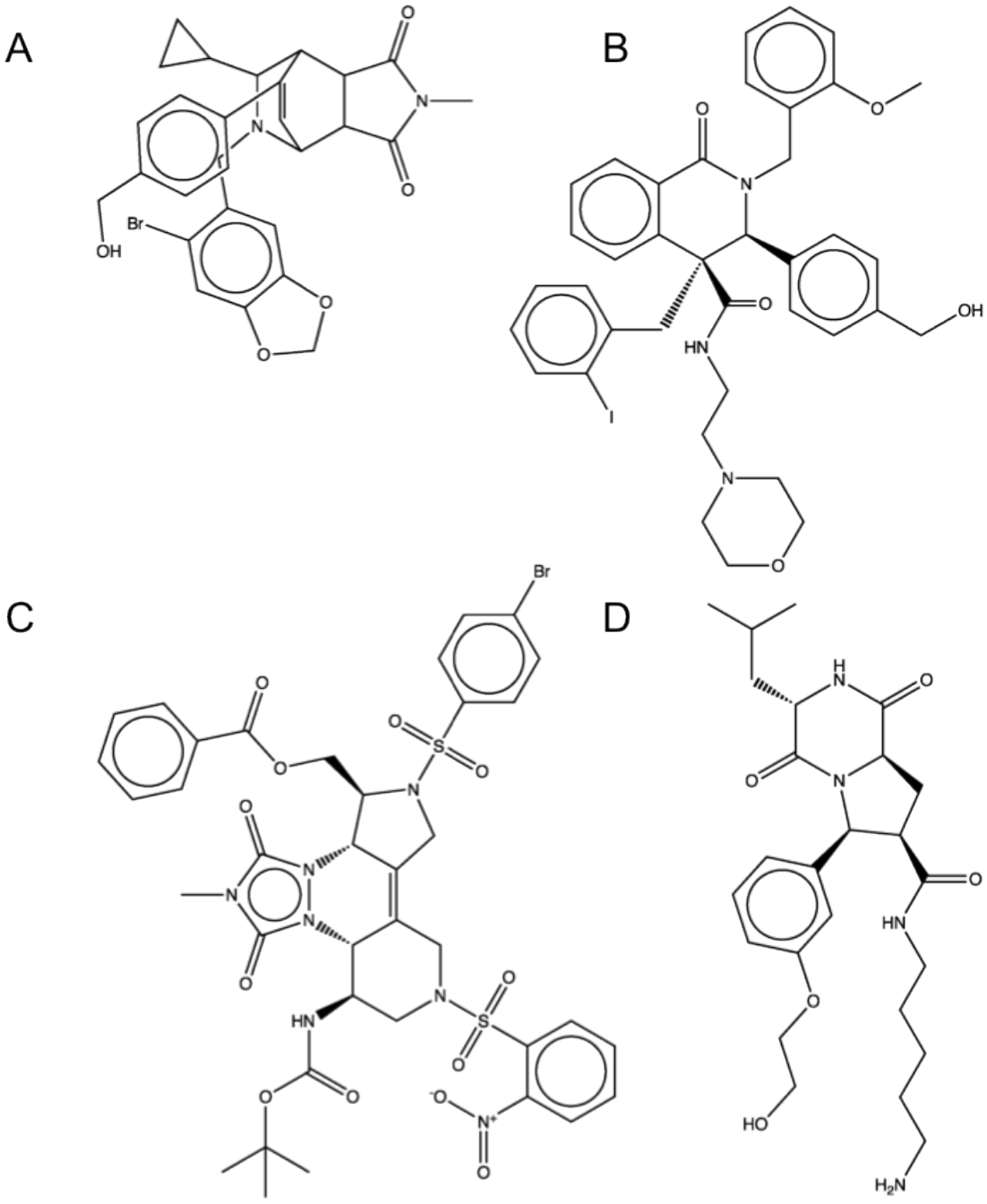


Figure 2.7. 1-(4-trifluoromethoxy)-benzyl azocines, a class of RPF^{null}-specific growth inhibitory small molecules. These 1-(4-trifluoromethoxy)-benzyl azocines (A), showed moderate RPF^{null}-specific activity in dose-response assays (B). Inhibitory activity was determined by measuring outgrowth by relative fluorescent units, as described in Materials and methods. Two other azocine derivatives, not pictured, showed similar activity.



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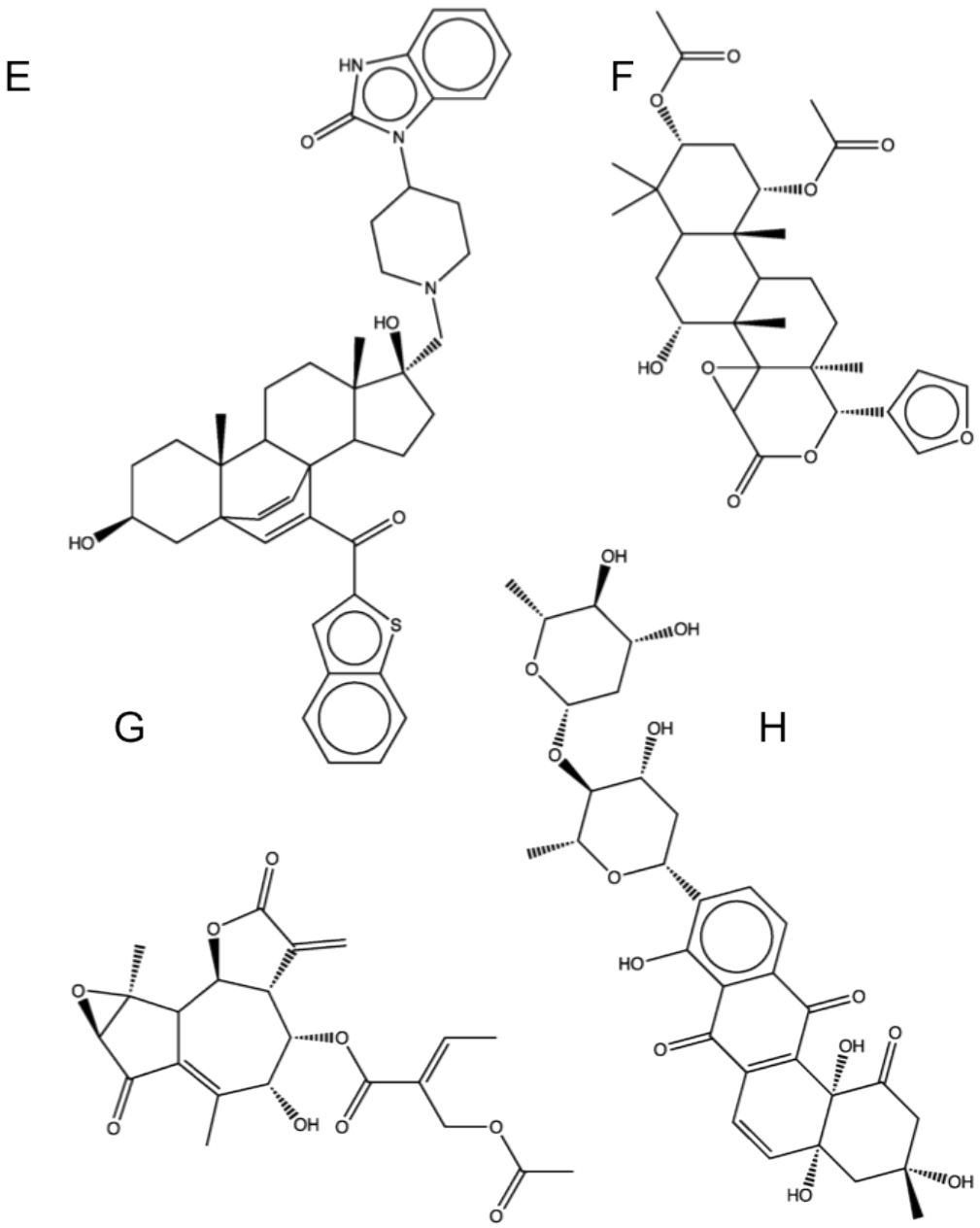

Figure 2.8 (Continued). Singly-represented small molecules scaffolds with RPF^{null}-specific growth inhibitory activity. All of these compounds (A-H) displayed RPF^{null}-specific activity, but did not have obvious structural features in common with the other hits from the screen.

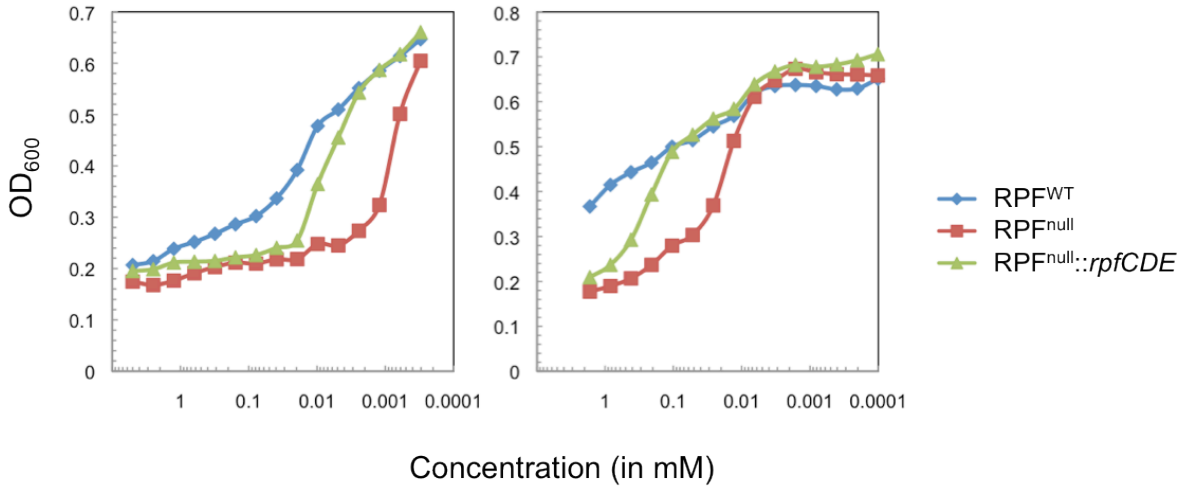

Figure 2.9. Inhibition of RPF^{null} by the macrolide class of antibiotics. The macrolides clarithromycin (left) and azithromycin (right) showed substantial RPF^{null}-specific activity, which could be complemented by the addition of *rpfC*, *rpfD*, and *rpfE* in single copy under the control of their native promoters. Erythromycin, not shown, displayed similar activity, but degraded rapidly. See Materials and methods, Chapter 3.

Table 2.3. Increases in sensitivity of RPF^{null} to common antibiotics.

Antibiotic	Fold Sensitization [a]
Amikacin	1
Cefotaxime	8
Ciprofloxacin	1
Clarithromycin	128
Clofazimine	1
Isoniazid	1
Rifampin	2
Vancomycin	8

[a] A fold change of 1 indicates that there was no detectable difference between RPF^{null} and RPF^{WT}.

Section 2.4: Conclusion

The most parsimonious explanation for the sensitization of RPF^{null} to a wide variety of compounds is that these compounds all preferentially inhibit RPF^{null} through a single vulnerability introduced by the deletion of the RPFs. Two well-described classes of antibiotics, the cephalosporins and the macrolides, showed broad RPF^{null} specificity among many compounds within the classes, but the known mechanisms of these two classes of compounds are quite distinct: cephalosporins target the cell wall (26), while macrolides affect the ribosome (27). Furthermore, none of the compounds described in the screen for specific inhibitors of RPF^{null} show structural similarity to either the macrolides or the cephalosporins, suggesting that these compounds have distinct modes of inflicting cell death or bacteriostasis.

One candidate vulnerability through which all of these compounds may gain potency is the permeability of the RPF^{null} outer membrane. This possibility is supported by the sensitization of RPF^{null} to sodium dodecyl sulfate (SDS) (15); in Gram negative bacteria, disruption of the outer membrane results in increased SDS sensitivity (28). It is also suggested naturally by the covalent linkage of the RPF-regulated peptidoglycan to the inner mycolic acid leaflet of the outer membrane (29); from such a linkage, it follows that disruptions in the peptidoglycan may have effects on the mycolic acids.

We pursued the hypothesis that permeability was a mechanism of RPF^{null} sensitization to small molecules in the specific context of cephalosporin sensitivity in Chapter 2.

Section 2.5: Materials and methods

High-throughput chemical screening

Mycobacterium tuberculosis strain H37RvΔ*rpfACDEB* (a generous gift of V. Mizrahi) was marked with a hygromycin resistance cassette-expressing vector, pUV15, containing red fluorescent protein under the control of the promoter for Rv3583c, a strongly, constitutively expressed mycobacterial gene.

The strains were grown at 37 °C and 2 RPM to mid-log phase (OD_{600} 0.6 – 0.8) in Middlebrook 7H9 medium containing 0.2% glycerol, 0.05% Tween 80, and Middlebrook OADC Enrichment (Becton Dickenson #212351). Cell clumps were removed by centrifugation at 58 x*g*, and the OD_{600} was adjusted to 0.05. Cells were then aliquoted into 384-well plates containing compounds, 20 μL per well. The average compound concentration was ~25 μM in 7H9 medium with an additional 0.5% dimethyl sulfoxide.

Cells were grown for four days at 37 °C, and then growth was assessed by measuring fluorescence (λ_{ex} 444 nm λ_{em} 484 nm). Compounds were designated as inhibitory if the mean growth in duplicate compound wells was less than the mean growth of control wells containing rifampin at a concentration at which the Z factor (15) equaled zero. Compounds were designated as specifically inhibitory to RPF^{null} if the difference in growth between H37Rv (22) and RPF^{null} was less than the difference between DMSO controls for H37Rv and rifampin controls for RPF^{null}.

Secondary assays

Compounds selected for follow-up were placed in 7H9 medium in 384-well plates as above, but at six separate two-fold dilutions below the screening concentration. Measurement of growth was carried out as described for high-throughput screening. The growth of strains H37Rv RPF^{WT}, H37RvΔ*rpfAB*, and H37RvΔ*rpfE* was assessed by measuring the fluorescence resulting from pUV3583c-GFP, a constitutively active GFP reporter maintained by hygromycin, with λ_{ex} 485 nm and λ_{em} 538 nm.

Erythrocyte lysis assay

Compounds selected for follow-up were placed in 384-well plates in 20 µL phosphate-buffered saline.

Recently drawn whole human blood with acid-citrate-dextrose anticoagulant mixture was mixed with RPMI medium supplemented with fetal bovine serum. Whole cells were collected by centrifugation and washed twice in the same mixture; they were finally resuspended at twice their original blood concentration.

Aliquots of 20 µL whole blood cells were added to the 384-well compound plates and incubated at 37 °C for 24 h. Cells were sedimented and hemoglobin release in the isolated supernatant was measured. This measurement was performed by adding an equal volume of DIHB-250 reagent (Gentaur Molecular Products) to supernatants, incubating for 5 min, and measuring OD_{400}.

Dose-response curves

Compounds were stored at -20 °C in dimtheyl sulfoxide (DMSO). The potency of compounds was determined as follows: 384-well plates were filled with 20 µL 7H9 medium and

100 nL DMSO per well. Compounds were arranged in the plates in twofold dilutions from the highest compound concentration to the lowest.

MTB grown to mid-logarithmic phase (OD_{600} 0.5 – 1.0) was centrifuged at 58 x*g* to remove clumps and then diluted in fresh 7H9 medium to OD_{600} 0.1. Cells were added in 20 µL aliquots to each well, with individual strains being measured in quadruplicate, sextuplicate, or octuplicate.

Cells were grown for 100 – 200 hr at 37 °C in sealed containers. After incubation, growth was measured by OD_{600}. The IC_{90} was computationally identified for each singlicate dilution series as the lowest concentration at which OD_{600} was less than 10 % of the growth in the absence of drug; for combinations of strain and drug for which singlicate measurements yielded different results, a range was reported. In additional to these technical duplicates, all strain and drug combinations were tested in at least two independent experiments. Although absolute numbers differed between experiments due to variation in drug stocks, differences between strains remained constant.

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Chapter 3:

The cephalosporin susceptibility of a strain of *Mycobacterium tuberculosis* lacking the resuscitation-promoting factors results from a change in permeability

Attributions

The scientific work in this chapter was performed by Carl Wivagg. Toshiro Ohsumi assisted with whole-genome sequencing analysis.

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Chapter 3: The cephalosporin susceptibility of a strain of *Mycobacterium tuberculosis* lacking the resuscitation-promoting factors results from a change in permeability

Section 3.1: Abstract

The resuscitation-promoting factors of *Mycobacterium tuberculosis* are essential for survival in a mouse model of tuberculosis infection, but are dispensable for *in vitro* growth. Previously, we showed that resuscitation-promoting factors are required for wild-type tolerance to several antibiotics, including cephalosporins, macrolides, and vancomycin. Here, we sought to investigate the mechanism by which the resuscitation-promoting factors maintain this tolerance. Focusing on the case of the cephalosporins, we investigated the primary determinants of cephalosporin sensitivity and resistance: permeability, β-lactamase activity, and target site modification. The permeability of the mycolic acid outer membrane of *Mycobacterium tuberculosis* was directly evaluated by comparing the access of extracellular substrates to the periplasmic β-lactamase activity; in resuscitation-promoting factor-deficient strains, this access was greatly increased. The contribution of specific resuscitation-promoting factors to cephalosporin sensitivity was evaluated, and the results broadly supported the hypothesis that there exists a "functional hierarchy" of importance for these genes, in which *rpfA* and *rpfB* contribute to specific cellular functions, while the other resuscitation-promoting factors show some level of redundancy. Together, these results indicate that the resuscitation-promoting factors are important for the coordination of the mycolic acid and peptidoglycan layers of the *Mycobacterium tuberculosis* cell wall. Their function in maintaining the impermeability of the cell wall may contribute to their *in vivo* survival defect.

Section 3.2: Introduction

Previously, our laboratory performed a chemical screen for small molecules that specifically inhibited growth of a strain of *Mycobacterium tuberculosis* (MTB) lacking all five resuscitation-promoting factors (RPFs), H37RvΔ*rpfACDEB* (RPF^{null}), but did not inhibit growth of wild-type MTB (RPF^{WT}). One of the most significant classes of hits was the cephalosporin class of antibiotics.

The cephalosporins were interesting for several reasons. First, although they are not currently popular in tuberculosis chemotherapy, recent work has shown that some β-lactams, such as meropenem, have potent sterilizing activity against MTB. This work focused explicitly on meropenem because of its low rate of hydrolysis by the MTB β-lactamase (1). Another compound with a low rate of MTB β-lactamase hydrolysis is cefotaxime; its K_D is in the millimolar range (2). Cefotaxime is also one of the cephalosporins that acts against RPF^{null} with enhanced potency, which could potentially make it a viable candidate for therapeutic use in combination with an RPF inhibitor (1).

Beyond clinical reasons, understanding the mechanism by which RPF^{null} is sensitized to the cephalosporins presented an opportunity to learn about RPF biology. The RPFs are thought to be peptidoglycan lytic transglycosylases, which cleave the β-1,4-glycosidic bond between *N*-acetylglucosamine and muramic acid (3). But little is known about the specific cellular spatial or temporal contexts in which individual RPFs function, and little is known about what other pathways may be affected by peptidoglycan β-1,4-glycosidic bond hydrolysis. For instance, the peptidoglycan region in which RPFs function is covalently bound to the surrounding arabinogalactan and mycolic acid layers (4), which may be affected by peptidoglycan conformation. The multiplicity of RPF paralogs in MTB raises further biological questions; it is

not clear whether they are functionally redundant or have diversified to perform separate functions.

Some characteristics appear to be specific to individual RPFs. For example, only RpfB and RpfE interact with RipA, a peptidoglycan endopeptidase (5). RpfA is the only RPF under regulatory control by the cAMP receptor protein encoded by Rv3676 (6). Deletion of *rpfA* also results in a notably different transcriptional response in comparison to the other RPFs (7).

Many characteristics, however, are common to all of the RPFs. They display a uniform expression pattern during growth in broth culture, with high expression in early logarithmic phase, gradually decreasing to undetectability in stationary phase (8). Deletions of several separate RPF subsets leads to decreased survival during host infection (9, 10, 11). Finally, our work in Chapter 2 showed that deletion of both *rpfE* and *rpfA*/*rpfB* resulted in heightened sensitivity to multiple small molecules.

Among these small molecules, extensive research has been done concerning the cephalosporins subgroup. There are three broad ways a two-membrane organism like MTB commonly acquires β-lactam sensitivity (12): [1] through alteration of its permeability; [2] through alteration of its β-lactamase activity; [3] through modification of its penicillin-binding proteins.

In this chapter, we investigate the possible contribution of each of these factors to the altered sensitivity of RPF^{null} to cephalosporins; in Chapter 4, we investigate in more detail the ability of modification of penicillin-binding proteins to contribute to changes in β-lactam sensitivity.

Section 3.3: Results and discussion

**In Chapter 2, we identified 18 β-lactams with RPF^{null}-specific inhibitory activity; nine were selected among the 47 most potent compounds from a screen for RPF^{null}-specific growth inhibitory compounds. These were the penicillin dicloxacillin and the cephalosporins cefazolin, cefotaxime, cefditoren, ceftriaxone, cefmetazole, cefoperazone, cephaloridine, and cephapirin. Cefotaxime was selected for more intensive investigation. The IC_{90} (the concentration at which 90 % of growth was inhibited) for RPF^{null} against cefotaxime was ~8-fold lower than in RPF^{WT} (Table 3.1), consistent with our screening results. We observed similar sensitization of the mutant for ceftriaxone and cefamandole. Colony-forming unit (CFU) measurement for RPF^{WT} and RPF^{null} verified that observed effects on OD_{600} arose from bactericidal activity (Figure 3.1).

To determine which RPFs contributed to RPF^{null}'s increased β-lactam sensitivity, we measured the cefotaxime IC_{90} against a range of mutants lacking one or more RPFs (Table 3.1). No individual RPF gene was singlehandedly responsible for RPF^{WT}-level cefotaxime resistance. The biggest increases in sensitivity came with the loss of *rpfA* and *rpfB*. In general, with the progressive loss of RPFs, cefotaxime sensitivity gradually increased. The additive effects observed suggest functional redundancy of the RPFs. Restoration of *rpfC*, *rpfD*, and *rpfE* by integration of a plasmid carrying these genes at the mycobacteriophage L5 attB site suppressed cefotaxime sensitivity in RPF^{null}.**

This complementation of RPF^{null} strongly suggested that cefotaxime sensitivity was a result of the RPF deletions, not any secondary genomic mutations acquired during the generation of RPF^{null}. Target site modification, which is the alteration of the β-lactam binding site on a penicillin-binding protein to change its affinity for β-lactams, most often occurs through genetic mutation of the penicillin-binding protein gene (12). In addition to our complementation of

Table 3.1. IC_{90}s of RPF^{null} and related strains to cephalosporins.

β-Lactam	Strain[a]	IC_{90} (μM)[b]
Cefotaxime	H37Rv (RPF^{WT})	26
	H37RvΔ*rpfA*	13-26
	H37RvΔ*rpfB*	13-26
	H37RvΔ*rpfC*	26
	H37RvΔ*rpfD*	26
	H37RvΔ*rpfE*	26-52
	H37RvΔ*rpfAB*	26
	H37RvΔ*rpfACBED*::pH*rpfCDE*	6.5-26
	H37RvΔ*rpfACBE*	6.5
	H37RvΔ*rpfACBED* (RPF^{null})	1.6-3.3
	H37RvΔ*rpfACDEB*	0.78
Cefamandole	H37Rv	390-780
	H37RvΔ*rpfACDEB*	98
With potassium clavulanate (1 μM)	H37Rv	24
	H37RvΔ*rpfACDEB*	1.5
Ceftriaxone	H37Rv	3.2-6.3
	H37RvΔ*rpfACDEB*	1.6

[a] From (7, 10, 11).

[b] Differences in IC_{90}s are representative of at least two independent experiments containing at least four duplicates each; for cefamandole, amounts of clavulanate were varied across duplicates.

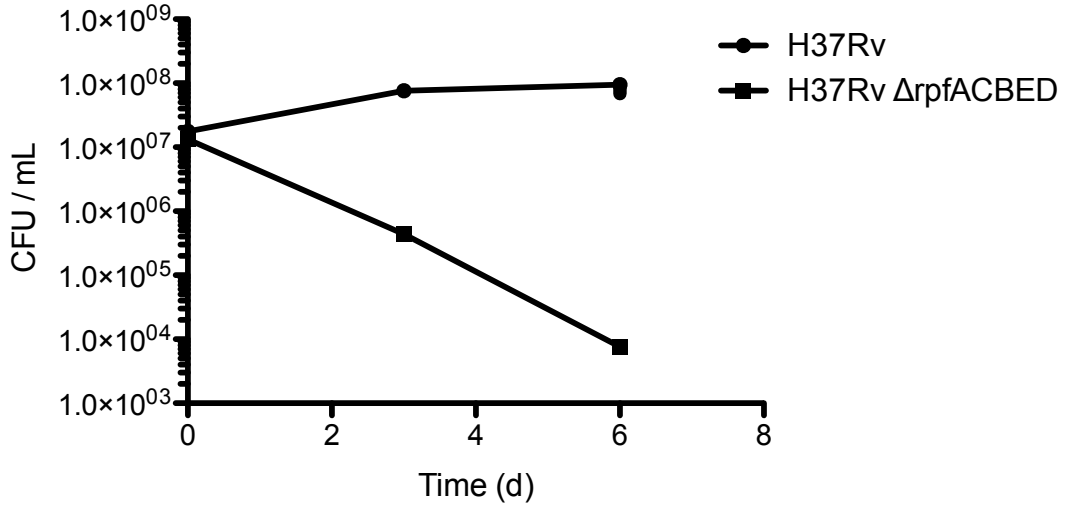

Figure 3.1. RPF^{WT} and RPF^{null} were grown in 3 μM cefotaxime and surviving bacteria were enumerated at the indicated time points by plating for colony-forming units on 7H10 agar. The result shown is representative of the experiment performed in triplicate. Error bars for duplicates are drawn, but may not be apparent due to precision.

RPF^{null}, we performed whole-genome sequencing to identify any possible confounding secondary mutations. We detected no mutations in any of MTB's penicillin-binding proteins. This strongly suggested that target site modification was not the means by which RPF^{null} had become sensitized to cefotaxime, so we turned our attention to changes in the MTB β-lactamase.

**To test whether increased β-lactam sensitivity in RPF^{null} could be due to loss of β-lactamase activity with sequential deletion of the RPFs, we measured the cefamandole IC_{90} of RPF^{WT} and RPF^{null} in the presence of 1 μM clavulanate, a β-lactamase inhibitor. If differences in β-lactamase activity account for the differences in cephalosporin sensitivity in the two strains, its inhibition by clavulanate should cause the cefamandole IC_{90} values for RPF^{WT} and RPF^{null} to converge. In fact, treatment with clavulanate did not cause this convergence; instead, a small divergence was observed (Table 3.1), thus demonstrating that loss of β-lactamase activity does not account for the increased β-lactam sensitivity in RPF^{null}.

Since RPF^{null} is also hypersensitive to SDS and macrolide antibiotics relative to RPF^{WT}, we hypothesized that it may have a general permeability defect underlying both these phenotypes as well as cephalosporin sensitivity. To test this, using previously reported methods (13), we measured the relative rates of cefamandole hydrolysis by intact RPF^{WT} and RPF^{null} cells (Figure 3.2). When these rates are normalized to their respective rate of hydrolysis in lysates, they provide a comparison of the relative rates of diffusion across the intact outer membrane; in effect, this provides a measure of relative permeabilities, provided that the concentration of cefamandole internal to the permeability barrier is below the order of the β-lactamase K_m. In highly permeable cells, intracellular hydrolysis of cefamandole is assumed to be rate-determining while in poorly permeable cells, diffusion through the permeability barrier is assumed to be rate-limiting.

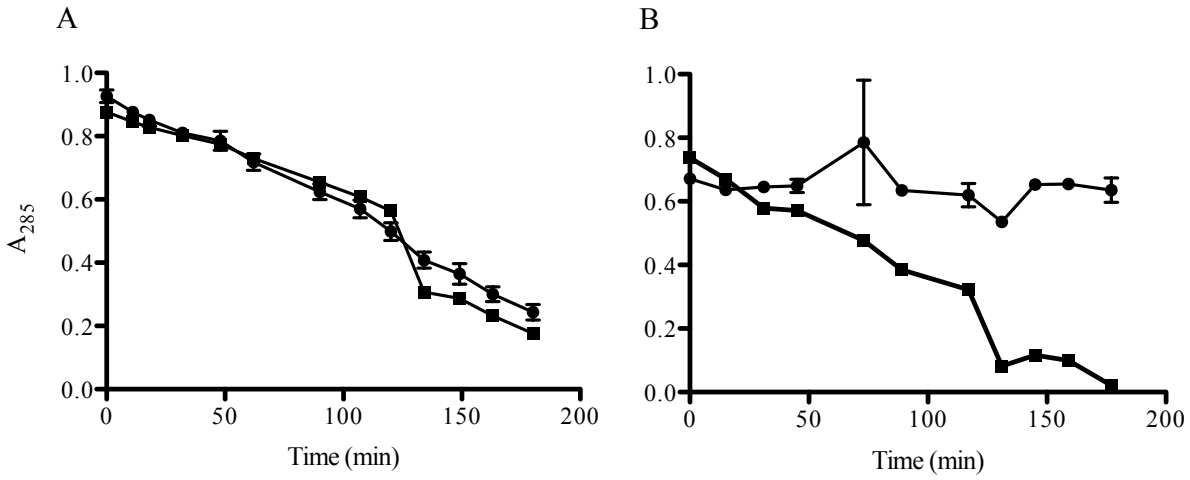

Figure 3.2. Kinetics of cefamandole hydrolysis. Absorption at 285 nm of H37Rv (●) or H37RvΔ*rpfACDEB* (■). Samples were mechanically lysed (A) or resuspended in phosphate-buffered saline intact (B) and exposed to 20 mM cefamandole nafate. Subsequently, absorption measurements were taken at the indicated times. The illustrated values are averages of two technical replicates for each strain. Parameters v_{lysed} and v_{intact} are the slope and error calculated from the aggregated data for each strain.

We calculated a v_{intact}/v_{lysed} ratio of 1.09 +/- 0.04 for RPF^{null}, compared to an RPF^{WT} v_{intact}/v_{lysed} ratio of 0.07 +/- 0.09. We also found that the loss of cefamandole sensitivity observed with the restoration of *rpfC*, *rpfD*, and *rpfE* in RPF^{null} was reflected in a correspondingly lower v_{intact}/v_{lysed} ratio of 0.19 +/- 0.04, indicative of decreased permeability. These results show that RPF^{null} has increased permeability to cefamandole.

We have found that the deletion of RPFs in *M. tuberculosis* H37Rv strain results in increased sensitivity to cephalosporins because of a resulting increased permeability in its outer membrane. While this work provides a clear demonstration of the change in permeability with respect to cephalosporins, it is possible that the reported sensitivity of RPF^{null} to other small molecules, including SDS (11), the macrolide class of antibiotics, vancomycin, and rifampin (14) may also be due to this same permeability defect. This finding suggests that changes in mycobacterial cell physiology at the peptidoglycan level may have far-reaching effects on the essential mycolic acid permeability barrier and that such alterations may affect the ability of the mutant bacilli to survive *in vivo*. Specifically, loss of particular cell wall-modifying enzymes may decouple the rates of peptidoglycan and outer membrane synthesis, as suggested in a model by Heidrich *et al*. (15) thus causing defects in the outer membrane that permit a wide range of molecules to diffuse in and out of the cell.

Our work shows that genetic ablation of RPF activity can potentiate a number of small molecules against mycobacteria, including known antibiotics presently unused in therapy. The use of RPF inhibitors should have similar effects. Recently, Demina *et al.* (16) have identified several small molecules with promising RPF inhibitory activity, while more generally, bulgecin is a known inhibitor of lytic transglycosylases. β-lactams are historically one of the most successful antibiotic classes, and the use of the β-lactamase inhibitor clavulanate to potentiate

them against mycobacteria has recently been proposed (1). This work provides a mechanistically orthogonal approach to potentiating β-lactam antibiotics, and other antibiotics.**

Section 3.4: Materials and methods

Strains and growth conditions

Mycobacterium tuberculosis H37Rv and its derivatives were grown to mid-logarithmic phase at 37 °C in 30 mL sterile square containers; culture aeration was maintained by shaking at 100 RPM with a constant head space of 20 mL.

The growth medium was Middlebrook 7H9 Broth supplemented with Middlebrook OADC Enrichment (Becton Dickenson #212351), 0.2 % glycerol, and 0.05 % Tween 80.

Complementation

The genes *rpfC*, *rpfD*, and *rpfE* were introduced to RPF^{null} on the plasmid pHINT under the control of their native promoters (construct a generous gift of V. Mizrahi): RPF^{null} was grown to logarithmic phase (OD_{600} 0.6 – 1.0) and then pelleted at 2,850 x*g* for 10 min. Cells were washed three times in 10 % glycerol, 0.05 % Tween 80 at 37 °C and then resuspended in the same solution at 1/20 the original volume. The pHINT construct, 1 μg, was transformed into 400 μL cells in a 1 mm electroporation cuvette. This mixture was electroporated at 2,500 V, 1,000 Ω, 4 μF. The cells were allowed to recover for 24 h in 5 mL 7H9 medium in the growth conditions described above. Colonies were isolated after growth on 7H10 agar medium supplemented with Middlebrook OADC Enrichment, 0.5 % glycerol, and 0.05 % Tween 80.

Permeability assay

Cells were grown to mid-logarithmic phase, OD_{600} 0.6 – 1.0. To measure v_{intact}, cells were pelleted by centrifugation at 2,850 x *g* and resuspended at 20x their original concentration. Cells were mixed in a 9:1 ratio with a prepared stock of 10 mg/mL cefamandole nafate in phosphate-buffered saline. Cefamandole concentration was monitored by the absorbance at 285 nm, which formed a characteristic peak centered at this wavelength in 7H9 medium or phosphate-buffered saline. For data collection, cell-drug mixtures were vigorously mixed, and then 100 μL aliquots were withdrawn at 5 – 15 min intervals, centrifuged at 16,100 x *g* for 2 min to remove debris, transferred to 96-well plates and read by a SpectraMax plate reader (Molecular Devices). A rate was determined from the linear least squares regression, calculated by Microsoft Excel.

The v_{lysed} parameter was calculated like v_{intact}, except cells were resuspended at 10x instead of 20x their original concentration in 7H9 medium, not phosphate-buffered saline. Mechanical lysis was performed using a beadbeater (BioSpec): 0.5 mL 0.1 mm zirconia/silica beads and 1 mL cells were beaten at maximum speed in a 1.5 mL conical microfuge tube in 6x 30 s pulses.

Dose-response curves

Compounds were stored at -20 °C in dimtheyl sulfoxide (DMSO) or -80 °C in 7H9 medium. The potency of compounds was determined as follows: 384-well or 96-well plates were filled with 20 μL 7H9 medium per well; if a compound in a DMSO stock was being tested 384-well (96-well) plates were filled with 20 μL (90 μL) 7H9 medium and 1 % or 2 % DMSO. Compounds were arranged in the plates in twofold dilutions from the highest compound concentration to the lowest.

MTB grown to mid-logarithmic phase (OD_{600} 0.5 – 1.0) was centrifuged at 58 x *g* to remove clumps and then diluted in fresh 7H9 medium to OD_{600} 0.1. Cells were added in 20 µL (10 µL) aliquots to each well, with individual strains being measured in quadruplicate, sextuplicate, or octuplicate.

Cells were grown for 100 – 200 hr at 37 °C in sealed containers. After incubation, growth was measured by OD_{600}. The IC_{90} was computationally identified for each singlicate dilution series as the lowest concentration at which OD_{600} was less than 10 % of the growth in the absence of drug; for combinations of strain and drug for which singlicate measurements yielded different results, a range was reported. In additional to these technical duplicates, all strain and drug combinations were tested in at least two independent experiments. Although absolute numbers differed between experiments due to variation in drug stocks, differences between strains remained constant.

Whole-genome sequencing

Genomic DNA was prepared from RPF^{null} as follows: cells were spun at 2,850 x *g* and resuspended in 1 mL of the supernatant. They were heated to 100 °C for 15 min. The cells were spun at 2,100 x *g* 20 min and resuspended in 0.95 mL bug lysis solution: 25 mM Tris pH 7.9, 10 mM EDTA, 0.9 % dextrose. The cells were spun at 16,100 x *g* 10 min, then resuspended in 0.5 mL bug lysis solution containing 1 mg/mL lysozyme. Cells were incubated at 37 °C 20 h. 100 µL 10% SDS was used to terminate the reaction and 50 µL Proteinase K (New England BioLabs P8102) was added. This mixture was heated to 55 °C for 20 min. 200 µL 5 M NaCl was added. To this mixture, 160 µL cetrimide solution was added: 4.1 % NaCl, 10 % cetrimide. This mixture was heated to 65 °C 10 min. DNA was extracted with 1 mL 24:1 chloroform:isoamyl

alochol. Phase separation was achieved with centrifugation at 16,100 x *g* 5 min; the extraction was repeated. 800 µL aqueous phase was extracted and mixed with 560 µL 2-propanol. This was incubated at room temperature for 5 min and then spun at 16,100 x *g* 5 min. The supernatant was aspirated and 1 mL 70 % ethanol added, followed by mixing by inversion. This mixture was spun at 16,100 x *g* 5 min and then air-dried. DNA was resuspended in 50 µL TE pH 7.8. Whole-genome sequencing was performed by the Broad Institute using Illumina technology with 36 bp reads. Alignment and screening for mutations were performed using the VAAL algorithm (17).

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Chapter 4

PonA2 is required for mycobacterial cephalosporin sensitivity and carbapenem resistance

Attributions

Carl Wivagg performed all of the molecular biological work except for the transposon screen. Paul Montgomery, Matthew Thompson, Roger Smith, and Michael FitzGerald performed the transposon screen. James Sacchettini and Thomas Ioerger sequenced two of the *ponA2* mutants. The manuscript was written by Carl Wivagg.

Chapter 4: PonA2 is required for mycobacterial cephalosporin sensitivity and carbapenem resistance

Section 4.1: Abstract

Recent studies have renewed interest in the β-lactam class of antibiotics as a potential treatment for *Mycobacterium tuberculosis* infection. To understand the potential opportunities and limitations of this approach, we sought to identify the specific transpeptidase targets of the β-lactams in *M. tuberculosis*. We isolated spontaneous β-lactam-resistant mutants and identified causative mutations in the penicillin-binding protein *ponA2*. In contrast to many penicillin-binding protein mutations, these mutations were recessive and occurred in both the transpeptidase target domain of the gene and the separate glycosyltransferase domain. To examine *ponA2* function more closely, we created a *ponA2*^{null} strain. This strain was also cephalosporin-resistant, but it was sensitized to carbapenems and had no phenotype with respect to penicillins. Since loss of function is an unusual mechanism of β-lactam resistance, we wondered whether other *M. tuberculosis* penicillin-binding proteins could also confer resistance through loss of function. Null mutations in other penicillin-binding proteins did not confer resistance to cephalosporins or carbapenems. We propose to explain the unusual behavior of the *ponA2* mutants with a model wherein *ponA2* transpeptidation is replaced by the activity of an alternate transpeptidase in a *ponA2*^{null} mutant. The different affinities of PonA2 and the alternate transpeptidase would account for the altered susceptibilities to the various classes of β-lactam.

Section 4.2: Introduction

Tuberculosis is a global threat that causes 1.4 million deaths per year (1). The causative organism, *Mycobacterium tuberculosis* (MTB), has developed concurrent resistance to every

first- and second-line antibiotic used against it (2); more common multidrug resistance to a subset of these antibiotics is spreading and probably exists in nearly every country (1). The scientific community's ongoing interest in applying antibiotics currently not used in tuberculosis treatment, such as β-lactams, has therefore acquired a new urgency (3, 4). β-lactams are currently used only rarely in the clinic because of MTB's high intrinsic tolerance to them (5), but recent work has shown that combination therapy with the β-lactamase inhibitor clavulanate may make several β-lactams viable treatment options (6).

Of particular importance in developing effective therapeutic regimens for any drug is an understanding of how the drug acts and by what means the target pathogen may become resistant to treatment. In the case of the β-lactams, it has long been understood that the antibiotic acts by forming a long-lived acyl-enzyme intermediate at the catalytic serine of its target, a penicillin-binding protein (PBP), thus obstructing its activity and the resultant impact on bacterial viability (7).

Bacteria usually possess several paralogous PBPs. These PBPs are thought to be collectively essential enzymes responsible for synthesizing the peptidoglycan sacculus that encases the bacterium and opposes the turgor pressure resulting from the osmotic differential between the bacterial cytoplasm and the extracellular milieu (8). The PBPs have two primary synthetic functions. The first is a glycosyltransferase function, wherein they processively polymerize the individual subunits of peptidoglycan, called disaccharide pentapeptide, into long glycan strands. The second is a transpeptidase function, wherein they crosslink pentapeptide from neighboring strands to form the final, netlike peptidoglycan sacculus. The β-lactams mimic the substrate of the transpeptidase crosslinking domain. The inhibition of the transpeptidase crosslinking function leads to a weakened sacculus. There is some debate about the exact

process by which the weakened sacculus resulting from β-lactam treatment leads to bacterial cell death, but in the simplest model, the weakened sacculus cannot withstand the turgor pressure resulting from the osmotic differential between the bacterial cytoplasm and the extracellular milieu, and the result is an explosive release of the cytoplasmic contents (8, 9, 10).

A precise understanding of the mechanism of bacterial cell death requires the identification of the particular PBPs that, when inhibited, lead to bacterial cell death. Since not all PBPs are essential, β-lactams probably need inhibit a subset of them to be effective. Similarly, resistance can arise from any mutation that alters the affinity of some member of this subset of PBPs, regardless of the inhibition of the other PBPs; therefore, resistance is dominant.

The PBP paralogs of a given species divide into several conserved functional classes. These include [1] bifunctional Class A high-molecular weight (HMW) PBPs, which possess both glycosyltransferase and transpeptidase activities; [2] Class B HMW PBPs, possessing only transpeptidase activities; and [3] both low-molecular weight (LMW) carboxypeptidases and endopeptidases, which hydrolyze rather than form peptide bonds (11). [4] Recently, researchers have identified a fourth class of proteins that act on a different catalytic site in the disaccharide pentapeptide; the different stereochemistry at this site has led them to be termed L,D-transpeptidases, while typical PBPs are D,D-transpeptidases (12).

The simplest models of β-lactam killing suppose that PBP activities are inhibited, and so the subset of PBPs that are bound by a particular β-lactam, must be essential. In MTB and related species, transposon mutagenesis experiments indicate that LMW PBPs and L,D-transpeptidases are non-essential (13). One Class B PBP is essential, while deletion of pairs of the other class A and B HMW PBPs results in severe growth and morphological defects. This genetic evidence therefore suggests that these PBPs may be the β-lactam targets in MTB.

Indeed, Class A and B HMW PBPs are generally hypothesized to be the critical β-lactam targets in most bacterial species. Nonetheless, even within the Class A and B HMW PBPs, it is not clear whether all of the transpeptidase domains must be inhibited by β-lactams for cell death to ensue, or whether only a subset of them form the molecular target.

β-lactam resistance in MTB is not well-studied because of the current lack of β-lactam use in the clinic. In other species, β-lactam resistance sometimes occurs by target site modification; target site modification is an alteration in the binding site of the target protein that lowers its affinity for the antibiotic that inhibits it. Resistance to β-lactams by target site modification typically occurs in the Class A and B HMW PBPs (8), providing further evidence that these classes contain the targets of the β-lactams.

This picture is complicated by the fact that in MTB in particular, there is increasing appreciation of the importance of the L,D-transpeptidases. These enzymes catalyze the crosslinking of amino acids at the third amino acid of nearby peptidoglycan monomers, creating 3→3 crosslinks; in contrast, PBPs catalyze the crosslinking of the fourth amino acid of one monomer to the third amino acid of another, creating a 4→3 crosslinks (11). MTB and *Myocbacterium abscessus*, a closely related strain, have both recently been shown to possess primarily 3→3 rather than 4→3 crosslinks (14, 15). The relative importance of D,D- and L,D-transpeptidases in these species may therefore differ from their relative importance in other bacterial species.

Meropenem, a member of the carbapenem subclass of β-lactams, has recently been shown to form *in vitro* adducts with an MTB L,D-transpeptidase, encoded by the gene Rv0116c (14). In *Enterococcus faecium*, the PBP 4→3 crosslinking pathway can be completely bypassed by 3→3 crosslinking (16). Even in this situation, meropenem retains activity, suggesting it can

act by inhibition of L,D-transpeptidases. In MTB, meropenem is effective against even cells that are not actively growing; stationary phase MTB is rich in 3→3 crosslinks (14). Meropenem's effectiveness against nongrowing MTB may therefore be a result if its ability to inhibit these L,D-transpeptidation as well as or instead of PBPs (17).

In the work that proposed meropenem as a promising antitubercular therapeutic agent, it was initially selected for study because of its low affinity for the MTB β-lactamase (6). The same researchers identified several cephalosporins that showed similarly low affinity but were not pursued (18). We set out to investigate the β-lactam targets in MTB using the simpler case of these cephalosporins. The cephalosporins are simpler because they show much lower affinity for L,D-transpeptidases, and so probably act by inhibition of Class A and B HMW PBPs. We sought to identify HMW PBPs important for cephalosporin resistance; we hoped that these PBPs might be the targets of cephalosporins leading to β-lactam mediated killing.

Section 4.3: Results

Isolation of Cefotaxime-Resistant Mutants

We wished to identify the cephalosporin resistance-determining PBP in MTB. The strategy we selected was to isolate spontaneously cefotaxime-resistant mutants; in previous experiments in our lab (19), cefotaxime had worked well as a model cephalosporin, providing changes in antibiotic resistance that correlated with changes in other cephalosporins, including ceftriaxone, cefamandole, and cefmetazole. We hypothesized that some spontaneously cefotaxime-resistant mutants would contain target site modifications in the PBP most capable of functioning in the presence of cefotaxime. We made multiple attempts to generate spontaneous mutations in wild-type H37Rv, but no resulting strain showed reproducible resistance in liquid

culture. This difficulty may result from the fact H37Rv was able to grow – with significant delays – at concentrations of cefotaxime very close to the solubility limit. It may also result from antibiotic degradation over the long timespan required to form colonies (3-4 weeks).

Instead of generating resistant clones from H37Rv, we generated cefotaxime-resistant clones by plating logarithmically growing H37RvΔ*rpfACDEB* on 7H10 plates containing cefotaxime. H37Rv*ΔrpfACDEB* is a strain of *M. tuberculosis* in which the five resuscitation-promoting factors, *rpfA-E*, were sequentially deleted. In a previous study, it was shown to have a tenfold increased sensitivity to several β-lactam antibiotics (19).

We isolated seven independent cefotaxime-resistant colonies from H37Rv*ΔrpfACDEB*. We performed whole-genome sequencing to identify mutations in two of them. Both strains contained single mutations in the Rv3682 gene, designated *ponA2* (Figure 4.1, Table 4.1, Table 4.2), which encodes one of two bifunctional PBPs in MTB. No other polymorphisms were identified that were not also present in the parent H37Rv*ΔrpfACDEB* strain. We sequenced the *ponA2* gene in the remaining, unsequenced cefotaxime-resistant strains and identified single-nucleotide *ponA2* polymorphisms in each strain (Figure 4.1). Unexpectedly, the range of mutations spanned both the glycosyltransferase and transpeptidase domains of PonA2; in other species, mutations linked to resistance typically occur only in the transpeptidase domain, which is the site of β-lactam binding and inhibition (8, 11). All of the mutations were non-synonymous single-nucleotide polymorphisms, and many caused changes within amino acid families, such as alanine to valine (Figure 4.1).

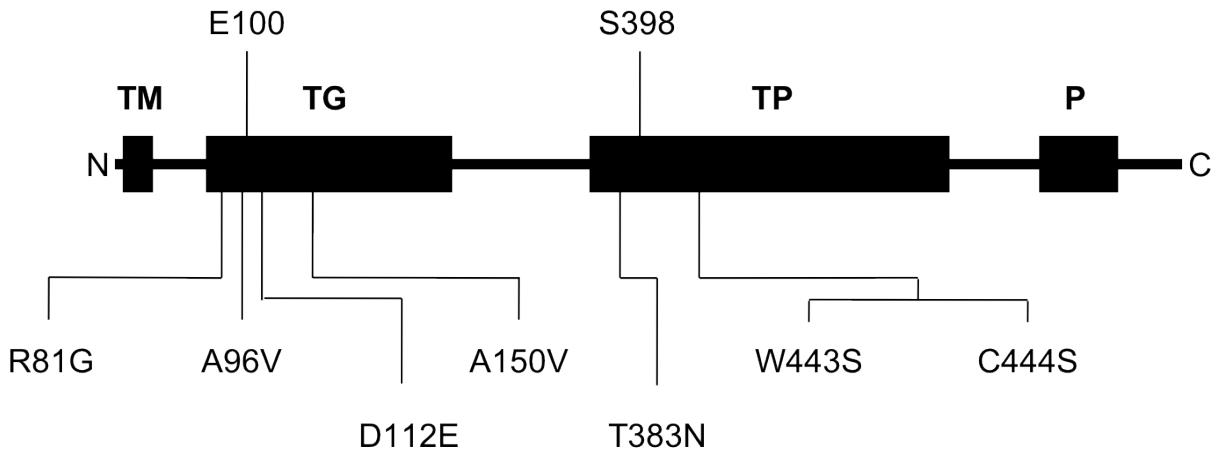

Figure 4.1. PonA2 is an 810 amino acid protein containing a transmembrane helix (TM), a glycosyltransferase domain (TG, PF00912), a transpeptidase domain (TP, PF00905), and a penicillin-binding protein and serine/threonine kinase-associated domain (P, PF03793). Above the diagram are indicated the catalytic residues associated with the TG and TP domains. Below the diagram are indicated the amino acid changes resulting from spontaneous mutations conferring cefotaxime resistance.

Table 4.1. Strains used in this work.

Name	Description	Source
H37Rv	Laboratory strain virulent *Mycobacterium tuberculosis*	E Rubin
H37Rv pREC	H37Rv with an acetonitrile-inducible plasmid for assisted homologous recombination	this work, (20)
H37Rv WT	H37Rv pREC after undergoing sucrose selection to lose pREC	this work
H37RvΔ*ponA2*	H37Rv pREC marked with a hygromycin resistance cassette and after undergoing sucrose selection to lose pREC	this work
H37Rv *ponA2*::Tn	H37Rv containing a transposon at amino acid 737 in *ponA2* (coordinate 4124127)	E Rubin
H37RvΔ*rpfACDEB*	H37Rv deleted for the genes *rpfA*, *rpfB*, *rpfC*, *rpfD*, and *rpfE*	(21)
ponA2^{R81G}	Spontaneous mutation of H37RvΔ*rpfACDEB* isolated on cefotaxime.	this work
ponA2^{A96V}	Spontaneous mutation of H37RvΔ*rpfACDEB* isolated on cefotaxime.	this work
ponA2^{D112E}	Spontaneous mutation of H37RvΔ*rpfACDEB* isolated on cefotaxime.	this work
ponA2^{A150V}	Spontaneous mutation of H37RvΔ*rpfACDEB* isolated on cefotaxime.	this work
ponA2^{T383N}	Spontaneous mutation of H37RvΔ*rpfACDEB* isolated on cefotaxime.	this work
ponA2^{W443S}	Spontaneous mutation of H37RvΔ*rpfACDEB* isolated on cefotaxime.	this work
ponA2^{C444S}	Spontaneous mutation of H37RvΔ*rpfACDEB* isolated on cefotaxime.	this work
ponA2^{R81G}:: *ponA2*^{WT}	*ponA2*^{R81G} with *ponA2* under the control of its native promoter on pMV306-G2, a single-copy integrating plasmid	this work
ponA2^{A96V}:: *ponA2*^{WT}	*ponA2*^{A96V} with *ponA2* under the control of its native promoter on pMV306-G2, a single-copy integrating plasmid	this work
ponA2^{W443S}:: *ponA2*^{WT}	*ponA2*^{W443S} with *ponA2* under the control of its native promoter on pMV306-G2, a single-copy integrating plasmid	this work
ponA2^{C444S}:: *ponA2*^{WT}	*ponA2*^{C444S} with *ponA2* under the control of its native promoter on pMV306-G2, a single-copy integrating plasmid	this work
H37RvΔ*ponA2*:: *ponA2*^{WT}	H37RvΔ*ponA2* with *ponA2* under the control of its native promoter on pMV306-G2, a single-copy integrating plasmid	this work
H37RvΔ*ponA2*:: *ponA2*^{A96V}	H37RvΔ*ponA2* with the *ponA2*^{A96V} allele under the control of its native promoter on pMV306-G2, a single-copy integrating plasmid	this work
H37RvΔ*ponA2*:: *ponA2*^{C444S}	H37RvΔ*ponA2* with the *ponA2*^{C444S} allele under the control of its native promoter on pMV306-G2, a single-copy integrating plasmid	this work
H37RvΔ*ponA2*:: *ponA2*^{E100Q}	H37RvΔ*ponA2* with the *ponA2*^{E100Q} allele under the control of its native promoter on pMV306-G2, a single-copy integrating plasmid	this work
H37RvΔ*ponA2*:: *ponA2*^{S398A}	H37RvΔ*ponA2* with the *ponA2*^{S398A} allele under the control of its native promoter on pMV306-G2, a single-copy integrating plasmid	this work
H37Rv pUVi-*ponA2*	H37Rv with an additional copy of *ponA2* under the control of a strong inducible promoter on pUV15tet, a multicopy episomal plasmid	this work
H37Rv pUVi-*ponA2*^{W443S}	H37Rv with the *ponA2*^{W443S} allele under the control of a strong inducible promoter on pUV15tet, a multicopy episomal plasmid	this work
14:C9 pUVi-*ponA2*	14:C9 with *ponA2* under the control of a strong inducible promoter on pUV15tet, a multicopy episomal plasmid	this work
14:C9 pUVi-*ponA2*^{W443S}	H37Rv *ponA2*::Tn with the *ponA2*^{W443S} allele under the control of a strong inducible promoter on pUV15tet, a multicopy episomal plasmid	this work

Table 4.2. IC_{90}s of H37Rv and daughter strains to cephalosporins.

Parent	Strain	IC_{90}[a]
H37Rv∆*rpfACDEB*	parent	1
	ponA2 R81G	2
	ponA2 A96V	2
	ponA2 D112E	4
	ponA2 A150V	4
	ponA2 T383N	n.d.
	ponA2 W443S	8
	ponA2 C444S	8
	ponA2 C444S + pMV306 *ponA2* WT	1
H37Rv wild-type	pUV15tet GFP[c]	1
	pUV15tet *ponA2* wild-type	4
	pUV15tet *ponA2* W443S	2
H37Rv *ponA2*::Tn[d]	pUV15tet GFP	1
	pUV15tet *ponA2* wild-type	4
	pUV15tet *ponA2* W443S	2
H37Rv∆*ponA2*	parent	1
	pMV306 *ponA2* WT	0.125
	pMV306 *ponA2* A96V	0.25
	pMV306 *ponA2* C444S	0.5
	pMV306 *ponA2* E100Q	1
	pMV306 *ponA2* S398A	1

[a] IC_{90}s are expressed in multiples of the parent strain resistance.
[b] The IC_{90} for this strain was not determined.
[c] The plasmid pUV15tet is a multicopy plasmid containing *ponA2* under the control of an inducible promoter. See Materials and methods.
[d] This strain contains a transposon insertion near the C-terminus of *ponA2*. These assays were performed in the presence of 96 µg/mL potassium clavulanate.

Complementation of *ponA2* mutations

Dose-response curves showed that the mutations in *ponA2* were associated with varying modest levels of resistance; most strains were able to grow at cefotaxime concentrations from 2- to 10-fold greater than the parent strain (Table 4.2). To confirm that these mutations caused the observed cefotaxime resistance, the W443S allele was cloned and expressed episomally in H37Rv under the control of several promoters. Under heterologous promoters of various strengths, PonA2^{W443S} failed to confer resistance to cefotaxime in dose-response assays (data not shown).

Resistance arising from target site modification is usually dominant (8), so the inability of *ponA2* mutant alleles to confer resistance in a merodiploid context was puzzling. Together, the numerous experiments failing to show resistance arising from the mutant allele in the presence of the wild-type allele implied either that the mutant allele was not being expressed or that it was recessive. To test the hypothesis that the *ponA2* mutations were recessive, the wild-type allele was introduced into one of the originally isolated mutants, PonA2^{C444S}, in single copy under the control of its native promoter. The resulting strain lost all resistance associated with the PonA2^{C444S} genotype (Figure 4.2, Table 4.2), confirming that the PonA2^{C444S} mutation caused resistance and that it was recessive.

Deletion of *ponA2* leads to cefotaxime resistance

We wished to test whether these unusual recessive β-lactam resistance mutations could confer resistance in MTB H37Rv, or whether their resistance depended on the H37RvΔ*rpfACDEB* background in which they had originally been identified. Since the mutations were recessive, simply expressing them in H37Rv *in trans* and testing resistance

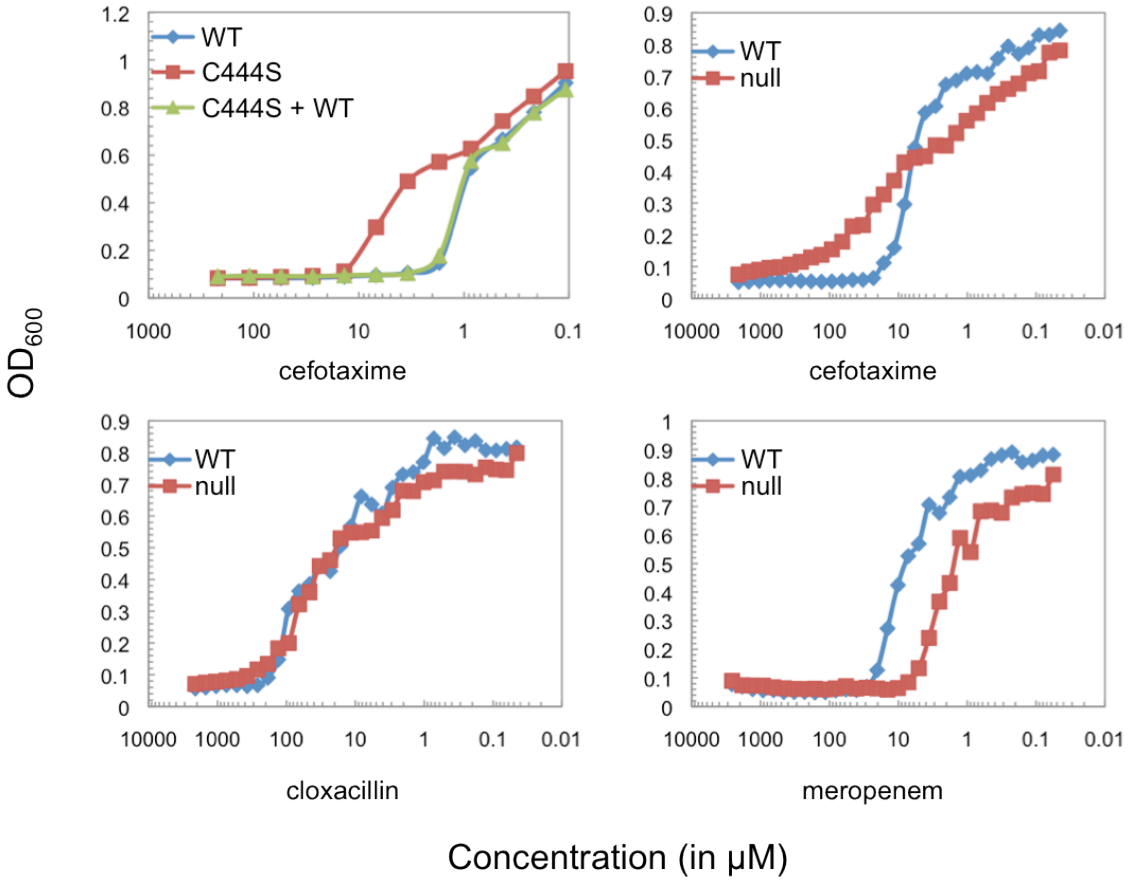

Figure 4.2. Sensitivity conferred by *ponA2* alleles of *Mycobacterium tuberculosis*. In the background H37RvΔ*rpfACDEB* (upper left), resistance conferred by the C444S allele can be suppressed by addition of the wild-type allele (WT) under the control of its native promoter on a single-copy integrating plasmid. The null allele confers resistance to cefotaxime (upper right), sensitivity to meropenem (lower right), and has no effect on cloxacillin susceptibility (lower left).

would not work. Instead, H37RvΔ*ponA2*, containing a clean deletion of *ponA2*, was created. The complete loss of *ponA2* from H37Rv actually conferred an even higher level of resistance than the *ponA2*^{C444S} allele had conferred in H37RvΔ*rpfACDEB* (Figure 4.2, Table 4.2). Previously, decreased ceftriaxone susceptibility has been observed in *Mycobacterium smegmatis* deleted for *ponA2* (22), but the same laboratory showed that *ponA2* disruption in MTB actually increased susceptibility to β-lactams (23). These results may be explained by the unusual dose-response relationship for H37RvΔ*ponA2* (Figure 4.2). Although the concentrations of cefotaxime at which H37RvΔ*ponA2* can grow are substantially higher than its parent, H37RvΔ*ponA2*'s growth rate at these high levels was very slow. Furthermore, at levels of cefotaxime well below the wild-type inhibitory concentration, H37RvΔ*ponA2* continued to display slower growth than in the absence of drug (Figure 4.2). The effect at low levels of drug, while modest, was reproducible in many independent assays.

Complementation of H37RvΔ*ponA2* with *ponA2*^{WT} under the control of its native promoter suppressed the new resistance phenotype completely, restoring the wild-type growth pattern (Table 4.2). In contrast, the *ponA2*^{A96V} allele, which had had one of the lowest susceptibilities among the resistant mutants, only partially suppressed the resistance of H37RvΔ*ponA2*; this was consistent with the low level of resistance conferred by this allele in H37RvΔ*rpfACDEB* (Table 4.2). The intermediate phenotype of *ponA2*^{A96V} between *ponA2*^{null} and *ponA2*^{WT} led us to the hypothesis that the *ponA2* SNPs may provide resistance through partial loss-of-function, in contrast to the greater resistance phenotype displayed by the *ponA2*^{null} complete loss of function allele. This would be consistent with the recessive behavior of the SNPs, which is otherwise inexplicable by the well-described β-lactam resistance mechanisms.

Mutations in PonA2 confer cefotaxime resistance by partial loss of function

Previously, it has been shown that disruption of *ponA2* by transposon mutagenesis confers hypersensitivity to heat stress, lysozyme, and sodium dodecyl sulfate (24). These phenotypes were only investigated in a transposon mutant, and so it is not certain which PonA2 domains they depend on; rather, they provide a functional test for the presence of PonA2. We were curious whether our *ponA2* SNPs, which appear to confer PonA2 loss of function vis-à-vis cefotaxime resistance, also cause loss of function in whichever PonA2 domain is responsible for these other phenotypes.

We expressed the *ponA2*^{WT}, *ponA2*^{A96V}, and *ponA2*^{C444S} alleles under the control of their native promoter in H37RvΔ*ponA2*. The *ponA2*^{A96V} and *ponA2*^{C444S} alleles represent mutations from the glycosyltransferase and transpeptidase domains, respectively. The mutant alleles conferred significantly increased sensitivity to 24 h of incubation at 45 °C compared to *ponA2*^{WT} (Figure 4.3). The relative sensitization conferred by the *ponA2*^{A96V} and *ponA2*^{C444S} alleles in comparison to *ponA2*^{WT} was comparable to that seen for PonA2 loss of function in previous assays (24). This result provides functional evidence that the *ponA2* SNPs and *ponA2* loss of function-mediated cefotaxime resistance arise from the same biological role of PonA2 as its heat stress phenotype.

PonA2 transpeptidase activity is essential for cefotaxime sensitivity and heat stress tolerance

PonA2 has two catalytic activities, a glycosyltransferase activity for glycan chain polymerization, and a transpeptidase activity for peptide crosslinking (11). Its paralog PonA1 has both of these catalytic activities (11) and plays a structural role, physically interacting with

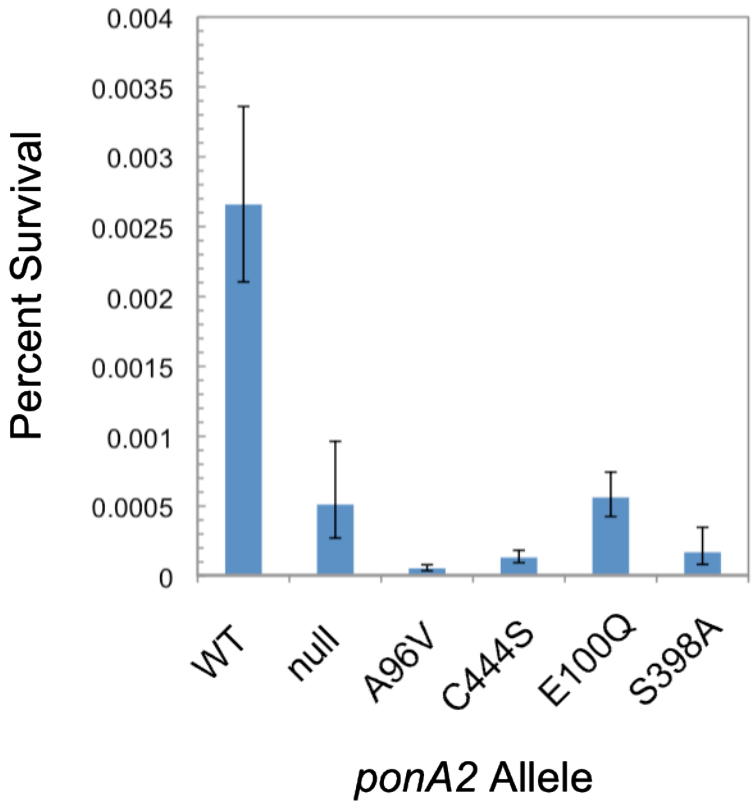

Figure 4.3. Surival of *Mycobacterium tuberculosis ponA2* mutants following heat stress. H37RvΔ*ponA2* was subjected to incubation at 45 C for 24 h. Colony-forming units in suspension were enumerated before and after incubation, and a percent survival was calculated. A96V is a mutation in the *ponA2* glycosyltransferase domain, while C444S is a mutation in the transpeptidase domain. E100Q and S398A are mutations at the active sites in the glycosyltransferase and transpeptidase domains, respectively.

and contributing to the activity of the endopeptidase RipA (25). Therefore, beyond its catalytic roles, PonA2 may also play a structural role or contribute to an additional function with its PASTA domain (Figure 4.1), about which relatively little is known.

To understand better which domain or feature of PonA2 is responsible for making H37Rv sensitive to cefotaxime, we constructed by site-directed mutagenesis a *ponA2*^{S398A} allele. S398 contains the active nucleophilic hydroxyl moiety responsible for forming an acyl-enzyme intermediate in PonA2-mediated transpeptidation, or peptidoglycan crosslink formation (11). This mutant, expressed under the control of the native promoter, failed to complement the cefotaxime hypersensitivity phenotype of H37RvΔ*ponA2*, while the wild-type allele expressed on the same vector completely restored sensitivity. Furthermore, the transpeptidation mutant showed the same heat stress hypersensitivity phenotype that the *ponA2*^{null} and *ponA2* single-nucleotide polymorphic alleles showed, indicating that PonA2 transpeptidation is essential for heat stress tolerance as well (Table 4.2, Table 4.3, Figure 4.3).

The catalytically inactive glycosyltransferase mutation, *ponA2*^{E100Q}, showed the same heat stress and cefotaxime susceptibility phenotypes as *ponA2*^{S398A}, but this result was expected, because the transpeptidase activity of bifunctional PBPs is believed to be glycosyltransferase-dependent (26). In other words, the inactivation of glycosyltransferase activity also inactivates transpeptidase activity.

All together, then, these results show that *ponA2* plays a key role in the molecular events following exposure of MTB to β-lactams. Its presence renders MTB vulnerable to β-lactam exposure, and this vulnerability depends on the catalytic transpeptidase activity of PonA2. The probable reason for vulnerability, then is the covalent inactivation of the PonA2 transpeptidase

Table 4.3. Susceptibility of PBP loss of function mutants to β-lactams.

PBP Disrupted	cloxacillin[a]	cefotaxime[a]	cephalexin[a]	meropenem[a]
none	-	-	-	-
PbpA	S	S	S	S
Rv2864c product	-	-	-	-
PonA1	n.d.	-	n.d.	-
PonA2	-	R	R	S
PonA2 + PonA2^{WT} [b]	n.d.	-	n.d	-
PonA2 + PonA2^{S398A} [b]	n.d.	R	n.d.	S

[a] - no change relative to wild-type S sensitized relative to wild-type
R resistant relative to wild-type n.d. not determined
[b] "+" indicates that the second allele was expressed under the control of its native promoter from the attB locus after integration on the vector pMV306-G2.

activity by β-lactams. These findings also illustrate the importance of H37Rv*ΔrpfACDEB* as a tool for the investigation of spontaneous resistance.

Disruption of other PBPs confers only sensitivity to β-lactams

The uniqueness of β-lactam resistance arising from PonA2 loss of function led us to wonder whether other PBP loss-of-function mutations could confer similar phenotypes. We selected mutants containing insertions in *ponA1*, *pbpA*, and Rv2864c, which are the three other non-essential PBPs in MTB, from an arrayed transposon library. Rv2864c and *pbpA* encode Class B HMW PBPs, while *ponA1* encodes MTB's remaining bifunctional glycosyltransferase/transpeptidase Class A HMW PBP. We assayed these mutants for sensitivity to cefotaxime. The *pbpA* mutant showed greatly increased susceptibility in its dose-response curve, while the *ponA1* and Rv2864c mutants did not reproducibly differ from wild-type (Table 4.3). These phenotypes are not surprising; deletions of orthologous PBPs in the closely related organism *Corynebacterium glutamicum* produced similar changes, with very minor sensitization to some β-lactams and modest sensitization to others (27). In no case in *C. glutamicum* did deletion of a PBP result in resistance (27).

To evaluate the spectrum of β-lactams that showed similar behavior to the cephalosporin cefotaxime, we compared the responses of the PBP mutants to this cephalosporin with the responses to meropenem, a β-lactam of the carbapenem family, cloxacillin, a β-lactam of the penicillin family, and cephalexin, another cephalosporin (Table 4.3). The *pbpA* mutant displayed increased sensitivity to all β-lactams tested, while *ponA1* and Rv2864c did not show any reproducible phenotypes. For *ponA2*, the null mutation was verified as cefotaxime-resistant as described above. The *ponA2* mutant was also resistant to cephalexin, but displayed increased

sensitivity to meropenem and no phenotype at all for cloxacillin (Figure 4.2, Table 4.3). All of these *ponA2* phenotypes could be suppressed by complementation with the wild-type *ponA2* allele but not the *ponA2*^{S398A} catalytic null mutant.

Screen for other factors affecting ceftriaxone sensitivity

In *Escherichia coli*, protein interaction studies have shown interactions between Class A HMW PBPs, Class B HMW PBPs, lytic transglycosylases, and endopeptidases (28, 29). These findings have led to the proposal that peptidoglycan synthesis proceeds through multienzyme complexes. Related work in MTB has shown that PonA1, a Class A HMW PBP, interacts with an endopeptidase, RipA (25), which itself interacts with the lytic transglycosylase RpfB (30); this interaction indicates that although phylogenetically distant, MTB, like *E. coli*, may use multienzyme complexes to synthesize its own peptidoglycan (25).

We wondered whether PonA2 might be acting in such a complex. If so, loss of other genes encoding members of the complex might phenocopy loss of PonA2. Alternatively, if loss of PonA2 was conferring cephalosporin resistance through some other functional pathway, perhaps by acting as a scaffolding protein or through its PASTA domain, it seemed possible that other genes might be involved in this functional pathway too.

To address these possibilities, we screened an arrayed transposon library containing insertions in 2,500 MTB genes in 96-well plates in 31 μg/mL ceftriaxone. Ceftriaxone is a cephalosporin that showed similar resistance profiles to cefotaxime for our panel of PBP mutants (data not shown). The 31 μg/mL ceftriaxone concentration represents a concentration at which growth would be expected of H37RvΔ*ponA2*, but not of wild-type H37Rv.

We grew the transposon mutants in duplicate 96-well plates in the presence of drug for 14 days and then measured their growth by optical density. To select hits, we calculated the Z score of each strain's growth, for the high-drug condition, for the low-drug condition, relative to the population mean and standard deviation for its plate:

$$Z = \frac{x - \mu}{\sigma}$$

where x is the sample value, μ is the plate mean, and σ is the plate standard deviation. We set a hit cutoff of $Z > 3$ to consider only transposon mutants that behaved significantly different from other mutants on the same plate. We calculated Z scores across plates rather than across the screen to account for plate-to-plate variation.

Seven genes showed significant growth at 31 μg/mL (Table 4.4). These hits were resistant to ceftriaxone. One of these six mutants carried an insertion in *dacB2*, a low-molecular weight PBP, which hydrolyzes rather than forms D,D peptide bonds in peptidoglycan. The D,D carboxypeptidases, although they bind β-lactams, are not considered likely to be the targets whose inhibition leads to killing (8, 11); for this reason, we did not include them in our panel of PBPs in Table 4.3. If DacB2 confers resistance through the same pathway as PonA2, it must act downstream of PonA2. This follows from energetic considerations: transpeptidases like PonA2 cannot form a new peptide bond without cleaving a preexisting one. The substrate specificity of DacB2 is well-understood; it cleaves the D,D peptide bond that would act as the preexisting bond in PonA2-mediated transpeptidation. Without that peptide bond, transpeptidation cannot proceed.

Another strain contained a transposon insertion in Rv3680. Since this gene is the first gene upstream of *ponA2* on the 5′ strand and encodes an ATPase anion exporter (31), we reasoned that it was unlikely to confer cefotaxime resistance by itself and was probably acting

Table 4.4. Transposon insertion locations conferring ceftriaxone resistance.

Gene	Name[a]	Function
Rv0169	*mce1A*	unknown transmembrane protein
Rv0626		antitoxin
Rv1373		glycolipid sulfotransferase
Rv2577		unknown
Rv2911	*dacB2*	carboxypeptidase
Rv3493c		unknown transmembrane protein
Rv3680		probable anion transporter ATPase

[a] Where blank, no gene name exists.

through polar effects on *ponA2*. This result needs to be confirmed by complementation. No transposon insertions in *ponA2* itself were evaluated in the screen.

A functional connection between the remaining five genes and *ponA2* is less apparent. Three have undefined functions (Table 4.4). One is a glycolipid sulfotransferase of undefined substrate specificity (31), and so it is difficult to speculate how its activity may contribute to ceftriaxone sensitivity. The remaining gene is an antitoxin. Antitoxins typically repress the activity of cognate toxins; in the case of Rv0626, the toxin is the downstream gene Rv0627, a ribonuclease that presumably affects mRNA stability of specific transcripts (32). Typically, toxin-antitoxin systems represent a way for bacteria to respond to stressful conditions (32); the Rv0626 antitoxin is known to be downregulated during macrophage infection (33), which suggests that its cognate toxin actively affects the transcriptome in these conditions. The genes affected by the Rv0627-Rv0626 system are not known.

Screen for other factors affecting β-lactam resistance

We had previously observed that loss of *pbpA* caused cefotaxime and meropenem sensitization. We concluded that *pbpA* was necessary for intrinsic resistance to both β-lactams (Table 4.3). As with PonA2, we sought to identify other genes that might act in the *pbpA* transpeptidation pathway. We repeated the screen of transposon insertion mutants, this time screening at subinhibitory antibiotic concentrations to identify sensitized genes. We screened against both meropenem and ceftriaxone. Screening conditions were identical to those used above, but with 2 μg/mL meropenem and 4 μg/mL ceftriaxone.

Using the same significance cutoff of $Z > 3$, and averaging the results of the ceftriaxone and meropenem screens, we identified 18 genes that conferred sensitivity to both ceftriaxone and

meropenem (Table 4.5). Six of these genes were undescribed conserved hypothetical coding sequences, while two encoded transcription factors of unknown specificity. Several hits were in known cellular pathways with unclear relationships to peptidoglycan metabolism or β-lactam-mediated killing (Table 4.5).

One hit stood out as being of particular significance. This was Rv3811, which encodes a secreted amidase. In fact, this amidase is the only MTB member of the amidase family PF01510 (31), of which *Streptococcus pneumoniae lytA* is another member. In *S. pneumoniae*, *lytA* is an autolysin essential for bacteriolysis during penicillin-induced cell death (34). LytA and other members of PF01510 cleave the bond between glycan strands and peptides in peptidoglycan, which can be useful during peptidoglycan recycling or to remove crosslinks in peptidoglycan (35). The reason this amidase might confer resistance to β-lactams in MTB is unclear.

Meropenem resistance in Mycobacterium smegmatis arises through a different mechanism

These experiments have shown that loss of both *ponA2* and *pbpA* confers meropenem sensitivity in MTB. We therefore hoped to pinpoint *ponA2* or *pbpA* as the meropenem resistance-determining mycobacterial PBP. It is difficult to perform experiments with meropenem and MTB because of the antibiotic's short half-life in MTB growth medium, in comparison to the ~24 h doubling time of MTB. Consequently, we switched to the model organism *Mycobacterium smegmatis*, a close relative of MTB with a ~3 h doubling time.

As we did previously with cefotaxime, we hoped to identify mutations that conferred resistance by target site modification in the resistance determining PBP. We isolated spontaneously meropenem-resistant mutants of *M. smegmatis*. In contrast to the MTB cefotaxime resistance by loss of *ponA2*, which conferred resistance to cefotaxime but sensitivity

Table 4.5. Transposon insertion locations conferring β-lactam sensitivity.

Gene	Name[a]	Function
Rv0049		unknown
Rv0204c		unknown transmembrane protein
Rv0820	*phoT*	inorganic phosphate transport
Rv0821c	*phoY2*	regulator of inorganic phosphate transport
Rv1049		transcription factor
Rv1124	*ephC*	possible oxidative damage response
Rv2349c	*plcC*	transmembrane phosphoesterase
Rv2418c		unknown
Rv2502c	*accD1*	fatty acid metabolism
Rv2645		unknown
Rv3005c		possible sulfur oxidation
Rv3183		transcription factor
Rv3196A		unknown
Rv3207c		unknown transmembrane protein
Rv3406		possible small molecule catabolism
Rv3524		transmembrane sensor
Rv3787c		possible phosphatase regulator
Rv3811		secreted amidase

[a] Where blank, no gene name exists.

to meropenem, these mutants were resistant to both cefotaxime and meropenem (Figure 4.4, cf Figure 4.2). No mutations were identified in the *ponA2* or *pbpA* orthologs, although a poorly-expressed *ponA2* paralog was not checked. The resistance was not due to changes in β-lactamase expression, since high doses of clavulanate did not eliminate the difference. This work shows that MTB may develop β-lactam resistance through other means than *ponA2* loss of function. Work is still being performed to identify the causative mutations.

Discussion

We have shown that mutation of *ponA2* confers cephalosporin resistance in MTB and that this resistance is recessive. We have shown that these mutations function by loss or impairment of *ponA2* function, and that loss of *ponA2* function confers not only cephalosporin resistance, but also meropenem sensitivity.

Our findings have implications for molecular models of β-lactam action. Broadly, they show that each class of β-lactams has specific genetic determinants of sensitivity and resistance. This implies that the specific subset of transpeptidases through which each class induces cell death may differ. Of particular interest is cephalexin: this cephalosporin, like piperacillin, is usually considered to be an FtsI-specific inhibitor, while other cephalosporins are not (36, 37); our findings clearly show that cephalexin, like the other cephalosporins, is rendered ineffective by *ponA2* loss of function. Cephalexin, ceftriaxone, and cefotaxime have greatly differing side chains, but the same core site mimicking the D-alanine-D-alanine substrate in pentapeptide side chains. Since their susceptibility profiles against the various PBPs are the same, it seems likely that the core is responsible for their binding specificity. The PonA2 active site has several

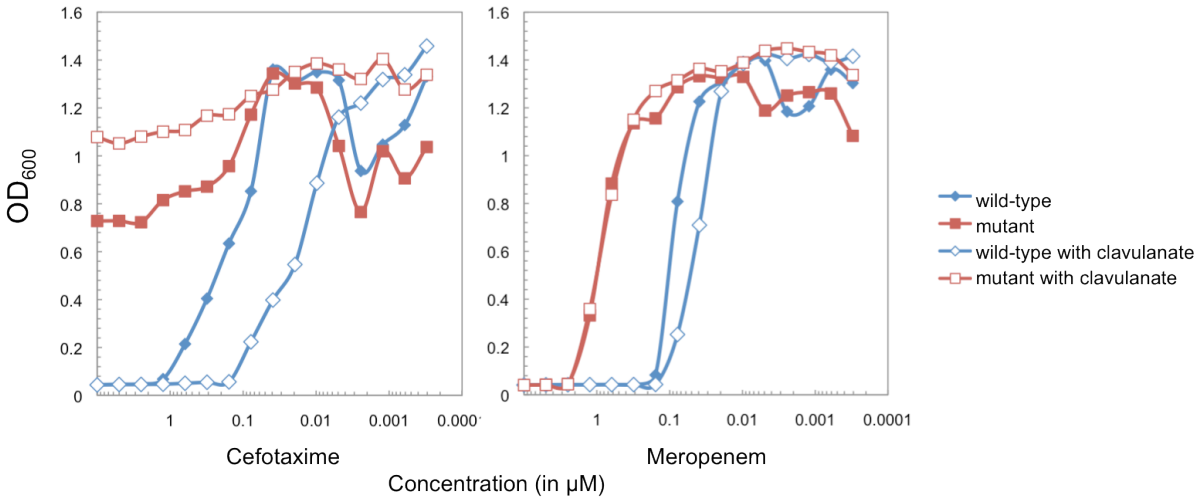

Figure 4.4. Susceptibilities of meropenem-resistant *Mycobacterium smegmatis*. Clavulanate (open circles) inhibits the mycobacterial β-lactamase; any differences in dose-response relationships that persist in the presence of clavulanate are therefore not a result of differences in β-lactamase activity between strains.

conserved structural motifs across its mycobacterial orthologs (11), which may determine its greater affinity for the cephalosporin core or lesser affinity for the carbapenem core.

The ability of *ponA2* loss of function mutations to confer recessive resistance show that *ponA2* plays a unique role in β-lactam resistance. To our knowledge, there is no precedent for the loss of a PBP conferring any level of β-lactam resistance in any organism. We believe that loss of *ponA2* must result in some sort of cellular adaptation under normal conditions that renders MTB more resistant to cephalosporins when it encounters them. One such adaptation could be that loss of *ponA2* leads to regulatory changes that result in its role being filled by a previously inactive cefotaxime-resistant, meropenem-sensitive transpeptidase (Figure 4.5). We are currently trying to identify an upregulated transpeptidase in H37RvΔ*ponA2* by global transcriptional analysis. Candidates include the L,D-transpeptidases, which are believed to bind carbapenems but not other classes of β-lactams, or one of the known PBPs. Although we evaluated the known PBPs *ponA1*, *pbpA*, and Rv2864c for contributions to β-lactam susceptibility, only *pbpA* loss of function caused a change. Loss of *pbpA* function resulted in sensitivity to both cefotaxime and meropenem, this gene does not seem to be a reasonable candidate for the cefotaxime-resistant, meropenem-sensitive transpeptidase; its presence contributes only to resistance, not to sensitivity. We cannot eliminate the possibility that the replacement transpeptidase is encoded by *ponA1* or Rv2864c; these mutants showed no shift in β-lactam susceptibility, which could result from as simple an explanation as a lack of expression under normal conditions.

The discovery of mutations in the glycosyltransferase domain of PonA2 was initially surprising, but since glycosyltransferase activity is believed to be required for transpeptidase activity (11), and since loss of transpeptidase activity is sufficient to confer all of the observed

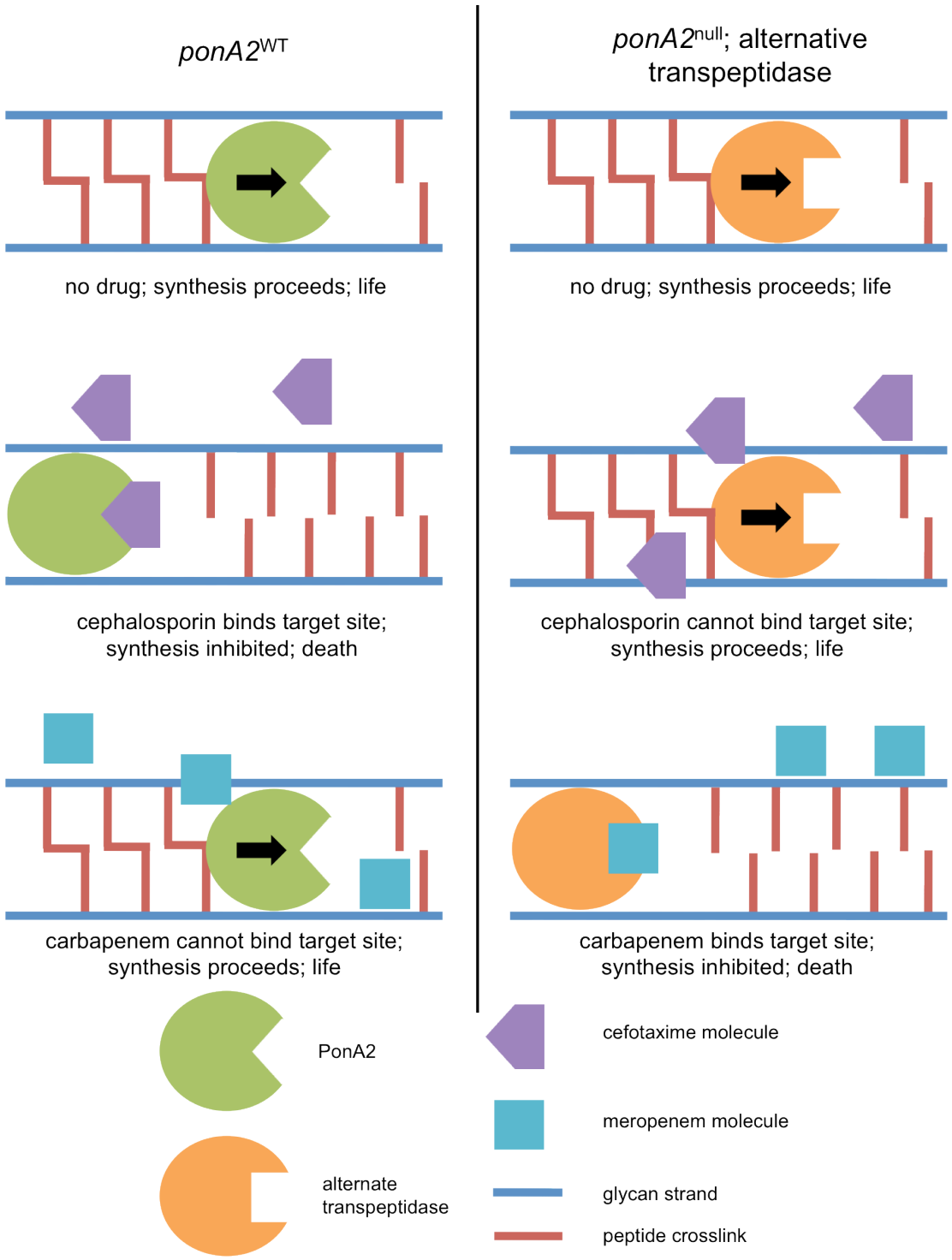

Figure 4.5. A model for responses to different β-lactams. When PonA2 is present (left panel) crosslinking of peptide strands proceeds, unless cephalosporins are present. When cephalosporins are present, PonA2 is inhibited and death ensues. When PonA2 is absent (right panel), its function is replaced by an alternate transpeptidase. This peptidase crosslinks peptide strands normally unless carbapenems are present. When carbapenems are present, this transpeptidase is inhibited, and without PonA2, death ensues.

phenotypes, it seems possible that the glycosyltransferase mutations acted by modulating the output of the transpeptidation function. The dependence of the β-lactam sensitivity phenotypes on the activity of the transpeptidase domain was expected, since this activity is the target of the β-lactams. The contribution that transpeptidase activity may give to heat stress tolerance is less clear. In their original paper noting heat stress sensitivity in a *ponA2*-transposon-disrupted strain, Vandal et al. speculated that stronger peptidoglycan might be required to withstand heat (24); the transpeptidase activity certainly contributes to the netlike properties that impart structural integrity to the peptidoglycan macromolecule.

The findings have important implications for the development of resistance in a clinical context. Transposon insertions in *ponA2* cause attenuation in mouse lungs and spleens (24). Therefore, resistance through loss of *ponA2* function would result in attenuation. But these results suggest that complete loss of PonA2 function is not necessary for resistance; partial resistance can be acquired by very minor substitutions, such as the one-carbon change from aspartate to glutamate. Although these mutations confer only very small changes in cephalosporin susceptibility, a series of such mutations can create an opportunity for clinically relevant resistance (38). Furthermore, the finding that changes leading to cephalosporin resistance can engender meropenem sensitivity strongly suggests that β-lactams are not all equivalent in treatment, and that there are potential gains from treatment with multiple β-lactams. This is consistent with emerging data that carbapenems may act against L,D-transpeptidases (17). The great differences among MTB mutants in susceptibility to different β-lactams underline the diversity within this antibiotic family and the vast potential it contains for future tuberculosis treatment.

Section 4.3: Materials and methods

Strains and growth conditions

Mycobacterium tuberculosis H37Rv and its derivatives were grown to mid-logarithmic phase at 37 °C in 30 mL sterile square containers; culture aeration was maintained by shaking at 100 RPM with a constant head space of 20 mL. Alternatively, cultures were grown in 1 L cylindrical containers with aeration maintained by rotation at 2 RPM with a head space of greater than 900 mL. For *Mycobacterium smegmatis*, cultures were grown in 250 mL Erlenmeyer flasks; culture aeration was maintained by shaking at 250 RPM with a constant head space of 200 mL.

The growth medium was Middlebrook 7H9 Broth supplemented with Middlebrook OADC Enrichment (Becton Dickenson #212351), 0.2 % glycerol, and 0.05 % Tween 80. For *Mycobacterium smegmatis*, Middlebrook OADC Enrichment was omitted and replaced with equivalent concentrations of albumin, dextrose, and sodium chloride.

Genetic manipulation of *Mycobacterium tuberculosis*

Cells in the logarithmic phase of growth – OD_{600} 0.5 – 1.0 – were centrifuged at 2,850 x *g* 10 min 37 °C, washed twice in 10 % glycerol 0.05 % Tween 80, and resuspended in 1/10 volume of the same solution.

The inducible multicopy plasmid pUV15tetORm (39), the integrating single-copy plasmid pMV306-G2, and the PCR product Δ*ponA2* (Table 4.1) were introduced to *M. tuberculosis* H37Rv by electroporation of 1 μg DNA in a 1 mm electroporation cuvette at 2.5 kV, 8 μF, 1 MΩ.

The integrating single-copy plasmid pMV306-G2 was derived from pMV306 (40) as follows: the small fragment from pNEB193 (New England BioLabs) released by EcoRI and XbaI was cloned into the PacI site followed by the small fragment from SalI and HindIII digestion cloned into the PmeI site. The resulting construct was digested with SgfI and BspHI to introduce the zeocin resistance cassette an unpublished plasmid (a generous gift of E Rubin).

Cells were recovered from electroporation in 5 mL 7H9 in otherwise normal growth conditions for 24 h. They were then plated on 7H10 agar medium supplemented with Middlebrook OADC Enrichment, 0.5 % glycerol, and 0.05 % Tween 80. After 18 – 24 d, single colonies were cultured. For Δ*ponA2*, a further selection was performed on these isolates, using 7H10 agar medium as described above, with 5 % sucrose, to eliminate the recombineering plasmid.

Isolation of spontaneous resistant mutants

Cultures in the logarithmic phase of growth were plated onto 7H10 agar medium supplemented with Middlebrook OADC Enrichment, 0.5 % glycerol, and 0.05 % Tween 80. For cefotaxime (Sigma-Aldrich C7912), the concentration used to select resistant mutants was 25 μg/mL. For meropenem (LKT Laboratories M1770), the concentration used was 11 μg/mL.

Heat stress assay

Cells were pelleted at 2,850 x *g* and washed in 7H9 medium twice. They were then resuspended to OD_{600} 0.05. Cell densities at time zero were assessed by colony-forming unit (CFU) plating. Cells were diluted 1/10 into fresh 7H9 medium and incubated at 45 °C for 24 h. Cell densities were again assessed by CFU plating, with a limit of detection of 10 CFU/mL. All

CFU measurements were performed in triplicate, and results are representative of two independent experiments. Heat stress survival was calculated as a percentage of CFU in the culture at time zero.

Dose-response curves

Compounds were stored at -20 °C in dimtheyl sulfoxide (DMSO) or -80 °C in 7H9 medium. The potency of compounds was determined as follows: 384-well plates were filled with 20 μL 7H9 medium per well; if a compound in a DMSO stock was being tested 384-well plates were filled with 20 μL 7H9 medium and 1 % or 2 % DMSO. Compounds were arranged in the plates in twofold dilutions from the highest compound concentration to the lowest.

MTB grown to mid-logarithmic phase (OD_{600} 0.5 – 1.0) was centrifuged at 58 x *g* to remove clumps and then diluted in fresh 7H9 medium to OD_{600} 0.1. Cells were added in 20 μL aliquots to each well, with individual strains being measured in quadruplicate, sextuplicate, or octuplicate.

Cells were grown for 100 – 200 hr at 37 °C in sealed containers. After incubation, growth was measured by OD_{600}. The IC_{90} was identified for each singlicate dilution series as the lowest concentration at which OD_{600} was less than 10 % of the growth in the absence of drug; for combinations of strain and drug for which singlicate measurements yielded different results, a range was reported. In additional to these technical duplicates, all strain and drug combinations were tested in at least two independent experiments. Although absolute numbers differed between experiments due to variation in drug stocks, differences between strains remained constant.

Where dose-response curves were measured with an inducible promoter, strains were first grown to OD_{600} 0.5 – 1.0, diluted to OD_{600} 0.025 in fresh medium in the presence of anhydrotetracycline, and incubated for 7 d using normal growth conditions. Afterward, dose-response curves were prepared normally, but medium contained anhydrotetracycline. Anhydrotetracycline was added from a 100 ng/μL 95 % ethanol stock, to a final concentration of 0.125 ng/μL, a concentration empirically judged to be noninhibitory.

Transposon library screens

Screen – Transposon mutants were incubated in 7H9 medium in 96-well paltes for 7 days at 37 °C to an OD_{600} of 0.05 – 0.20. The cells were mixed, and aliquots of 5 μL were transferred to 96-well screening plates containing 95 μL 7H9 medium and the following drug concentrations: [1] no drug; [2] 31 μg/mL ceftriaxone (Sigma-Aldrich C5793); [3] 4 μg/mL ceftriaxone; [4] 42 μg/mL meropenem; [5] 2 μg/mL meropenem. These plates were incubated for 14 days at 37 °C, after which growth was measured by OD_{600}.

Analysis – Duplicates were averaged. Z scores were calculated for each plate using the plate mean and standard deviation of bacterial growth as assessed by OD_{600}. To calculate sensitization, Z scores were calculated from the difference between the growth of each strain in the presence and absence of low drug.

Whole-genome sequencing

Genomic DNA was prepared from RPF^{null} as follows: cells were spun at 2,850 x *g* and resuspended in 1 mL of the supernatant. They were heated to 100 °C for 15 min. The cells were spun at 2,100 x *g* 20 min and resuspended in 0.95 mL bug lysis solution: 25 mM Tris pH 7.9, 10

mM EDTA, 0.9 % dextrose. The cells were spun at 16,100 x *g* 10 min, then resuspended in 0.5 mL bug lysis solution containing 1 mg/mL lysozyme. Cells were incubated at 37 °C 20 h. 100 µL 10% SDS was used to terminate the reaction and 50 µL Proteinase K (New England BioLabs P8102) was added. This mixture was heated to 55 °C for 20 min. 200 µL 5 M NaCl was added. To this mixture, 160 µL cetrimide solution was added: 4.1 % NaCl, 10 % cetrimide. This mixture was heated to 65 °C 10 min. DNA was extracted with 1 mL 24:1 chloroform:isoamyl alochol. Phase separation was achieved with centrifugation at 16,100 x *g* 5 min; the extraction was repeated. 800 µL aqueous phase was extracted and mixed with 560 µL 2-propanol. This was incubated at room temperature for 5 min and then spun at 16,100 x *g* 5 min. The supernatant was aspirated and 1 mL 70 % ethanol added, followed by mixing by inversion. This mixture was spun at 16,100 x *g* 5 min and then air-dried. DNA was resuspended in 50 µL TE pH 7.8. Whole-genome sequencing was performed by Texas A & M University using Illumina technology.

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Chapter 5

Discussion

Chapter 5: Discussion

Section 5.1: Summary of results

When this research began, the peptidoglycan-degrading enzymatic function of the resuscitation-promoting factors (RPFs) had just come to light (1). It was also known that deletion of multiple RPFs could deleteriously affect survival in mouse models of tuberculosis infection (2). In fact, several separate RPF deletion mutants showed attenuated survival in macrophages (2, 3), in persistence in mice, and in reactivation from a mouse model of latent infection (4).

The connection between all of these infection-related phenotypes and the specific enzymatic function of RPFs was unclear, since they are collectively dispensable for *in vitro* growth (2). One hypothesis was that RPFs might act to resuscitate cells from dormancy by releasing the cell from a tightly restrictive peptidoglycan shell, similar to the emergence from the spore state in *Bacillus subtilis* (5). Another hypothesis was that RPFs might release peptidoglycan fragments as signaling molecules, which would then stimulate emergence from the dormant state (5).

These models explained how peptidoglycan degradation might affect resuscitation, but they did little to explain why a *Mycobacterium tuberculosis* (MTB) strain lacking four RPFs could not establish infection, let alone escape from the latent phase. The connection between the biochemical peptidoglycan degradatory function and survival during infection intrigued us.

The results of our chemical screen suggested a new physiological role for the RPFs linking their biochemical function to their *in vivo* phenotypes. Specifically, it showed that the RPFs are essential for maintaining the low permeability of the MTB mycolic acid layer. The chemical screen showed that the RPFs were required for tolerance to a wide array of small

molecules. Some of these molecules retained activity against RPF-deficient strains even when their chirality was inverted, which indicated that they acted through means other than enzyme inhibition. Others were active against both RPF-deficient strains and human erythrocytes, which also strongly suggested a common mechanism of potentiation against the RPF-deficient strains.

We focused on the cephalosporin class of β-lactam antibiotics, which contained many members with activity against RPF-deficient strains. We showed that the cephalosporins have specific bactericidal activity against RPF^{null}, a strain lacking all five MTB RPFs. We showed that this activity can be partially suppressed by complementation of RPF^{null} with *rpfC*, *rpfD*, and *rpfE*. This finding supports growing evidence that the RPFs have some degree of functional redundancy (6). To elaborate the mechanism by which these RPFs affected sensitivity to cephalosporins, we then showed that one cephalosporin, cefamandole, has enhanced access to the protected periplasmic compartment of MTB in the RPF^{null} strain; we showed this by demonstrating that cefamandole can be depleted from growth medium more rapidly by the periplasmic β-lactamase activity of the RPF^{null} strain than the wild-type strain, even though the β-lactamase activity itself is constant. To verify that this phenotype is RPF-related, we showed that it can be suppressed by addition of *rpfC*, *rpfD*, and *rpfE*.

Vulnerability to multiple antibiotics and unclassified small molecules suggests that the RPF^{null} strain might have some general, common mechanism that enhances the activity of each of these molecules. The enhanced access of cefamandole to the periplasmic compartment indicates that this general mechanism of enhanced activity might be a change in the permeability of the outer mycolic acid layer of the MTB cell wall. This change in permeability could explain the *in vivo* survival defects of RPF^{null} and other RPF-deleted strains. Specifically, macrophages control MTB infection with soluble small molecules such as reactive oxygen and nitrogen

species (7, 8), and the intact outer membrane permeability barrier is thought to be essential for low permeability against these types of small, hydrophilic solutes (9). Its disruption or impairment would be devastating for the progress of infection, which is consistent with the poor survival of RPF^{null} *in vivo* (2).

The investigation of the cephalosporins led us naturally to the question of how these molecules might act. The precise links between the inhibition of the peptidoglycan synthetic machinery by β-lactams and subsequent β-lactam-induced cell death and bacteriolysis are an area of ongoing research (10), and especially so in mycobacteria, for which even less is known about peptidoglycan growth, maintenance, and degradation than in better-studied model organisms (11). We used RPF^{null} to study β-lactam resistance in MTB, since the high intrinsic resistance of MTB to β-lactams makes study with the wild-type organism challenging in laboratory conditions.

Spontaneous mutants of RPF^{null} acquired cephalosporin resistance by mutation of the gene *ponA2*, a penicillin-binding protein (PBP). We showed a causal link between mutations spanning both the glycosyltransferase and transpeptidase domains of *ponA2*, a range of mutations that has not been shown to cause β-lactam resistance in other organisms. We showed that these mutations were recessive and that they conferred heat stress sensitization, a phenotype associated with *ponA2* loss of function. These findings suggested that the *ponA2* mutations might be conferring resistance through *ponA2* loss of function. We created a *ponA2* deletion ($ponA2^{null}$) mutant and found that this mutant displayed even more cephalosporin resistance than the spontaneous mutants. All together, these findings establish loss of PBP function as a mechanism of β-lactam resistance.

Since β-lactam resistance by PBP loss of function is unusual, we wondered whether loss of any other MTB PBPs could also confer resistance. We tested transposon insertion mutants in *ponA1*, *pbpA*, and Rv2864c, the remaining nonessential PBP genes in MTB. The results of these tests showed that intact *pbpA* was required for full resistance to all β-lactams tested, while *ponA1* and Rv2864c disruption did not result in any phenotypes. Only *ponA2* conferred a resistance phenotype upon disruption. Interestingly, *ponA2* deletion conferred not only cephalosporin resistance, but meropenem sensitivity. This finding allows us to conclude that the different β-lactams have highly different binding profiles in MTB.

To expand upon these observations, we performed a transposon screen to identify other genes that might play a role in β-lactam resistance. We found a low-molecular weight PBP, *dacB2*, whose disruption also conferred resistance to ceftriaxone. A number of other genes whose disruption conferred resistance encoded conserved hypothetical proteins.

To find genes that, like *pbpA*, were required for cephalosporin and carbapenem resistance, we screened the transposon library for mutants that could not grow in subinhibitory concentrations of either of these two antibiotics. Among the genes that were sensitized to both antibiotics was Rv3811, a secreted amidase that is a distant ortholog of *lytA*, the *Streptococcus pneumoniae* autolysin essential for β-lactam-induced bacteriolysis.

Section 5.2: Current conclusions and future questions

The demonstration that *ponA2* and *dacB2* can confer β-lactam resistance by loss of function necessitates a new model for a mechanism of β-lactam resistance. Some organisms, such as *Staphylococcus aureus*, can resist β-lactam-induced cell death so long as they have a single β-lactam-resistant transpeptidase activity. In *S. aureus*, this is the inducible, β-lactam-

refractory PBP2a; so long as PBP2a is functional, the presence of other transpeptidases is irrelevant (12). In contrast, in MTB, the presence of *ponA2* transpeptidase activity confers sensitivity rather than resistance.

Despite this apparent contrast, the methicillin resistance in *S. aureus* provides a hint of the simplest explanation for *ponA2*-mediated cephalosporin resistance in MTB. PBP2a is not normally expressed in *S. aureus*; instead, it is induced by the β-lactam sensor protein BlaR1 (13). This happens quickly enough to rescue the cell from death during β-lactam treatment. From the perspective of peptidoglycan, the sequence of events is that first, peptidoglycan crosslinking stops as the β-lactam-sensitive PBPs are inhibited. Then, PBP2a is induced and crosslinking resumes, while the normal PBPs become irrelevant because β-lactam binding has rendered them catalytically inactive and hindered.

We propose that like *S. aureus*, MTB has two states. The first is a vulnerable state where β-lactams bind the available PBPs and, without some response by the cell, ultimately lead to death. The second is a resistant state where a β-lactam insensitive PBP, like PBP2a, carries on peptidoglycan crosslinking and the cell is safe. The difference between *S. aureus* and MTB is that in MTB, the resistant state can only be achieved by the loss of *ponA2*; after deletion of *ponA2*, the β-lactam-refractory PBP is expressed and the bacterium is safe. When *ponA2* is present, no transpeptidase is induced upon exposure to β-lactams, *ponA2* and other extant PBPs are inhibited, and the cell dies.

This theory makes testable predictions. Genetically, it predicts that there exists another transpeptidase gene that, if deleted, will suppress cephalosporin resistance in *ponA2*. Biochemically, it predicts the appearance of a new carbapenem-binding activity in a *ponA2*

knockout compared to wild-type MTB. We are searching for the transpeptidase by trying to identify upregulated RNA transcripts in the *ponA2* knockout.

One provocative possibility is that the upregulated transpeptidase is an L,D-transpeptidase. MTB possesses numerous genes encoding L,D-transpeptidases (14), and current research indicates that they are generally cephalosporin-resistant and meropenem-sensitive (15). If this possibility were true, it would be highly significant, because it would show the replacement of 4→3 crosslinking by PBPs with 3→3 crosslinking by L,D-transpeptidases. This has only been demonstrated once before, in *Enterococcus faecium* (16). In that species, this replacement conferred resistance to ampicillin (16).

The resistant state need not involve the upregulation of an L,D-transpeptidase, or even a transpeptidase at all; there are many ways that MTB could acquire cephalosporin resistance, of which we have provided a detailed overview in Chapter 1.4. For example, the loss of *ponA2* could somehow lead to the upregulation of a cephalosporin-resistant efflux pump or, through altered protein-protein interactions, to a decrease in the cephalosporin affinity of another existing PBP; the ability of protein-protein interactions to affect PBP β-lactam affinity has also been observed in *E. faecium* (17). We prefer the L,D-transpeptidase hypothesis because the upregulation of one transpeptidase is an intuitively appealing response to the loss of another.

Regardless of the specific mechanism of resistance, one fact is clear: loss of *ponA2* leads to some adaptation in the molecular physiology of the cell that renders it cephalosporin-resistant. In order for loss of *ponA2* to lead to such an adaptation, the cell must have some way of sensing the loss of *ponA2*. This method could be indirect, through sensing of the downstream molecular products of PonA2 activity, direct, through protein-protein interactions with PonA2, or a combination of the two.

An example of an indirect mechanism would be the *Escherichia coli* Amp system. PBP transpeptidation leads to the removal of the terminal D-Ala in peptidoglycan peptide stems, so the Amp system reimports a sampling of peptidoglycan degradation products and detects the ratio of D-alanylated peptide stems to non-D-alanylated peptide stems (18). Changes in this ratio indicate a change in PBP activity. MTB does not possess orthologs to the genes of this particular system (14), but it is an example of an indirect PBP sensing system.

A direct mechanism of sensing PonA2 would be the physical interaction of PonA2 with a transmembrane sensor. We are not aware of any specific examples of an interaction of a PBP with a transmembrane sensor, but no theoretical considerations preclude such a possibility.

If direct interaction is involved, existing knowledge better supports a combination mechanism of direct and indirect sensing. For example, direct interaction of the PonA2 paralog PonA1 with a partner endopeptidase has been clearly demonstrated; PonA1 not only interacts with this partner endopeptidase, RipA, but modulates its activity (19). In a combination mechanism, the PBP-partner interaction is the direct component, while the indirect component would be the effect that this interaction has on the downstream products of the partner's activity. The partner could be an endopeptidase, like RipA, or any of a number of other peptidoglycan-modifying enzymes.

The sensing of and subsequent cellular response to the loss of *ponA2* raise a fundamental question: what is the difference between chemical inhibition of function and genetic loss of function (Figure 5.1)? Why does the cell respond to total loss of the *ponA2* gene, but when it is faced with the much simpler problem of the inhibition of PonA2 by cephalosporins, it fails to respond adequately and dies?

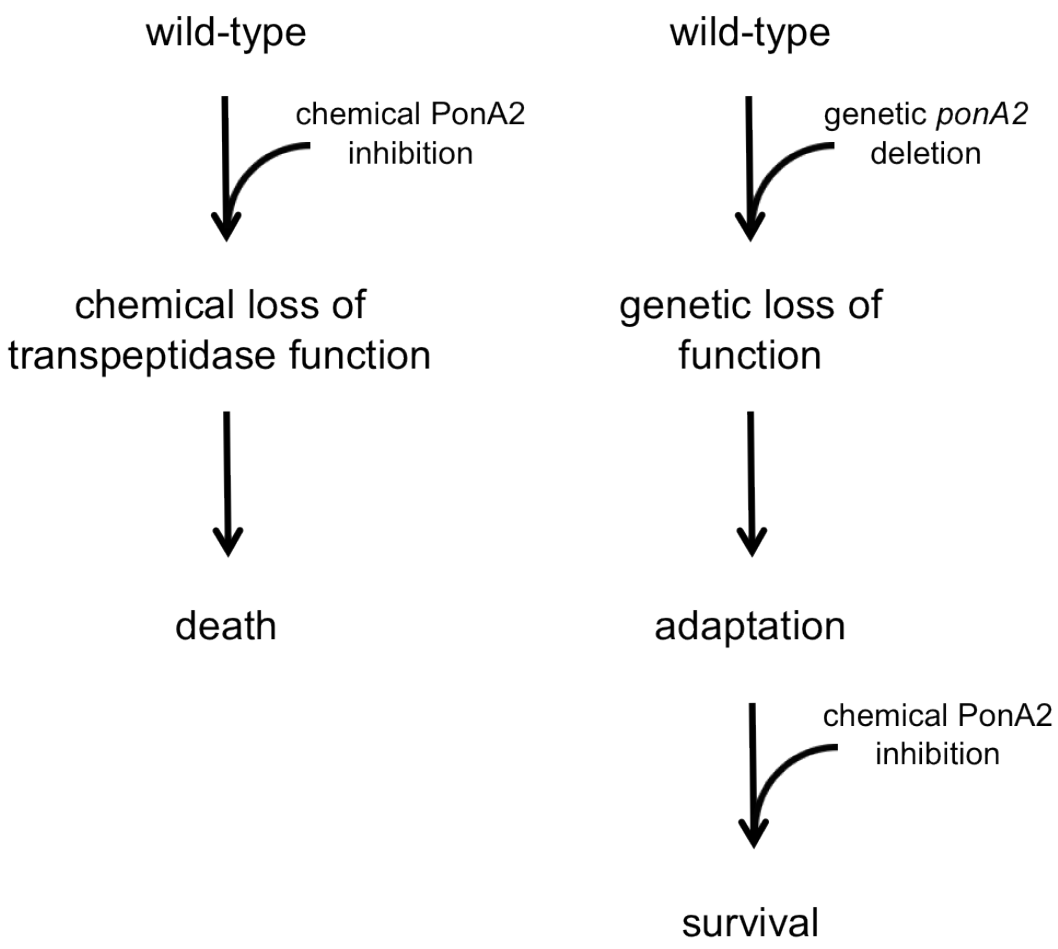

Figure 5.1. Schematization of the differing responses of wild-type and *ponA2* deletion strains to cephalosporin treatment. In the wild-type, chemical inhibition of PonA2 by cephalosporin treatment leads to cell death. Genetic deletion of *ponA2*, rather than leading to cell death also, leads to some sort of adaptation. Not only does this adaptation allow the cell to survive, it also renders it resistant to future cephalosporin treatment.

There are two ways to account for the difference. One is that the difference is timing. The other is that cellular adaptation to *ponA2* loss of function is not related to its transpeptidase activity.

Regarding timing, the *ponA2*^{null} mutant had a chance to adapt to conditions of gradually diminishing PonA2, as the protein was diluted over many generations after the removal of the source gene from the parent cell. In contrast, inhibition of PonA2 by β-lactam exposure in a culture, except at very low concentrations, occurs very rapidly in comparison to the doubling time of MTB. The physiological consequences of β-lactam exposure may become irreversible before the cell has a chance to respond to the inhibition of its peptidoglycan synthetic machinery. The biological difference that accounts for the different concentrations at which the wild-type and mutant die, in this scenario, is that death in the mutant occurs in a bacterium that had the chance to adapt to diminished PonA2 over generations, while in the wild-type, death occurs before such adaptation.

The other way to account for the difference is that the stimulus for cellular adaptation to *ponA2* loss of function could be loss of some other function than transpeptidation. One candidate function could involve PonA2's PBP and serine/threonine kinase-associated (PASTA) domain; this domain is also found in an MTB transmembrane protein kinase, where it is believed to act as a periplasmic sensor (20). Another candidate function could involve the glycosyl-transferase activity of PonA2, which is not inhibited by β-lactams. However, if loss of the glycosyltransferase or PASTA functions were responsible for adaptation, restoration of these activities without transpeptidation should suppress cephalosporin resistance. Our experiments showed a transpeptidase catalytic null *ponA2* allele did not suppress cephalosporin resistance.

Finally, the loss of function that stimulates adaptation to *ponA2* deletion could involve a scaffolding role for PonA2, as with PonA1 and RipA above. In such a scenario, loss of interaction with a partner protein would occur in the *ponA2*^{null} mutant, but not upon chemical inhibition of PonA2.

Regardless of the particular differences between genetic and chemical loss of *ponA2* function, there are several significant implications of our research. (1) The acquisition of cephalosporin resistance implies some cellular response to the loss of *ponA2*, possibly the upregulation of an alternate transpeptidase. (2) The existence of such a response implies a mechanism of sensing active PonA2, which may involve detection of the protein itself or its synthetic products. (3) This mechanism of sensing cannot be completely explained by existing concepts because it involves the ability to distinguish inhibition of transpeptidation from its genetic ablation.

The nature of the cellular response to loss of *ponA2* and the mechanism by which it is sensed are open scientific questions. Their answers can only lead to an enhanced understanding of the complicated field of peptidoglycan metabolism.

Section 5.3: Concluding remarks

This project began with an inquiry into the cellular function of the resuscitation-promoting factors. During the course of our work, some researchers have developed small molecule inhibitors of the RPFs. Our work, which showed that one cellular function of the RPFs is to protect against a broad array of small molecules, indicates that inhibition of RPFs may potentiate many of these small molecules. Indeed, since RPFs are required for *in vivo* survival, the RPF inhibitors have the potential to be useful therapeutic agents on their own.

Among the agents that RPFs potentiate against, cephalosporins and other β-lactams are a critical element of the current pharmaceutical armamentarium against non-tuberculosis respiratory infections. This work shows that the primary *in vitro* route to cephalosporin resistance is *ponA2* loss of function. Since *ponA2* loss of function attenuates *M. tuberculosis* (20), cephalosporins may have potential to overcome the problem of drug resistance in a pathogen that has total drug resistance to first-line therapeutic agents (21).

The unexploited potential of the cephalosporins and other β-lactams against tuberculosis, and the desperate need for new therapeutic agents against this disease, bespeak possibilities of a future where the hyperbolic language of "magic bullets" once used to describe penicillin and streptomycin might again become relevant.

Section 5.4: References

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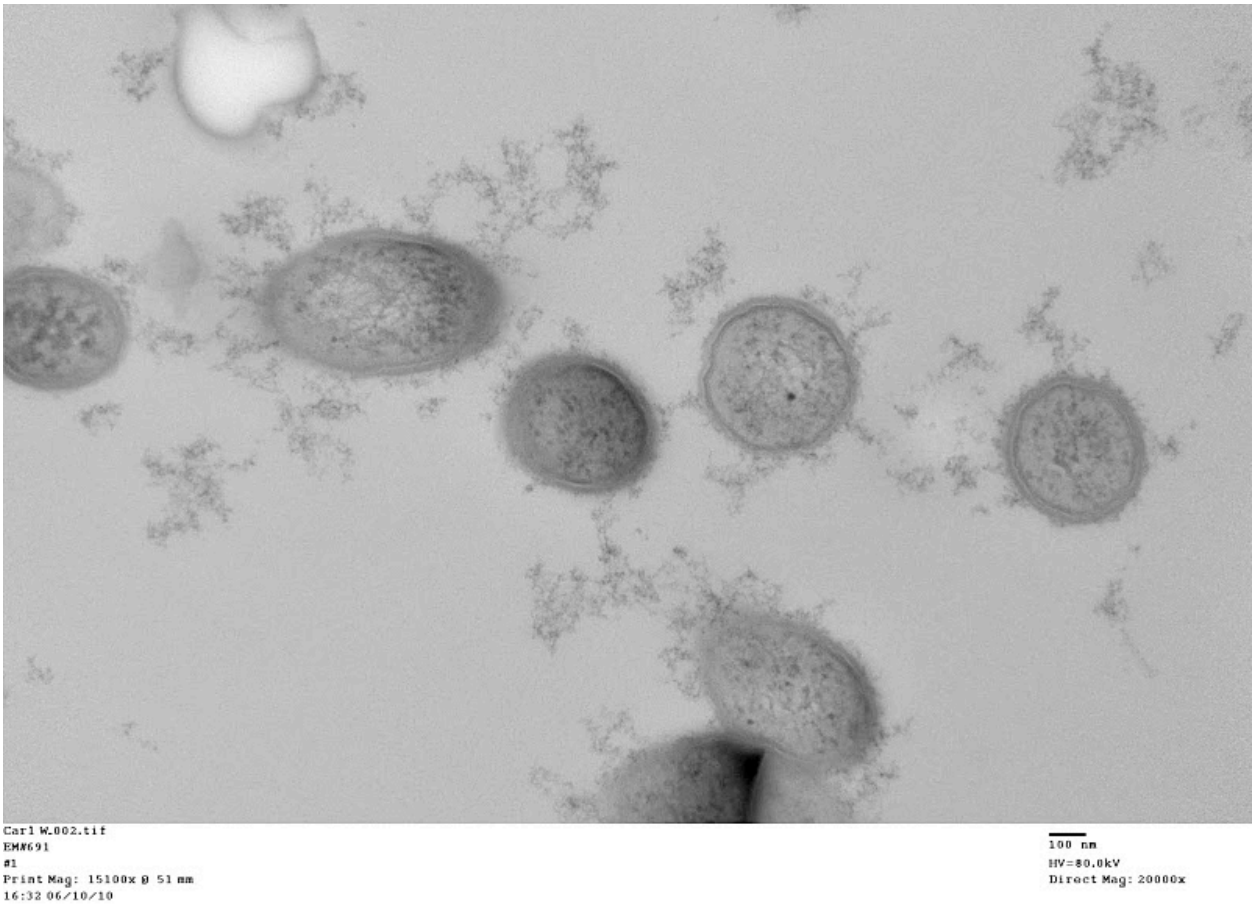
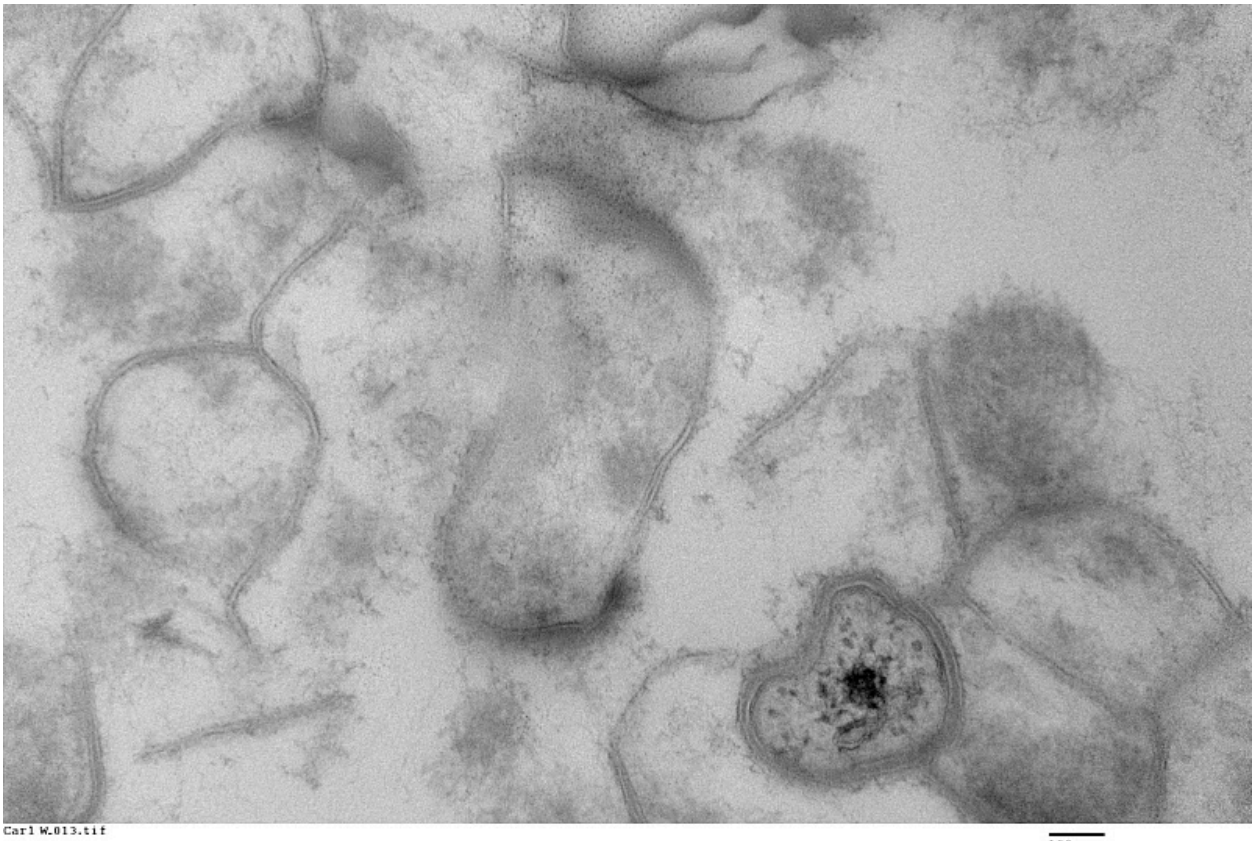
Appendices to the dissertation

Appendix 1: Electron microscopic studies of H37RvΔ*rpfACDEB* and related strains

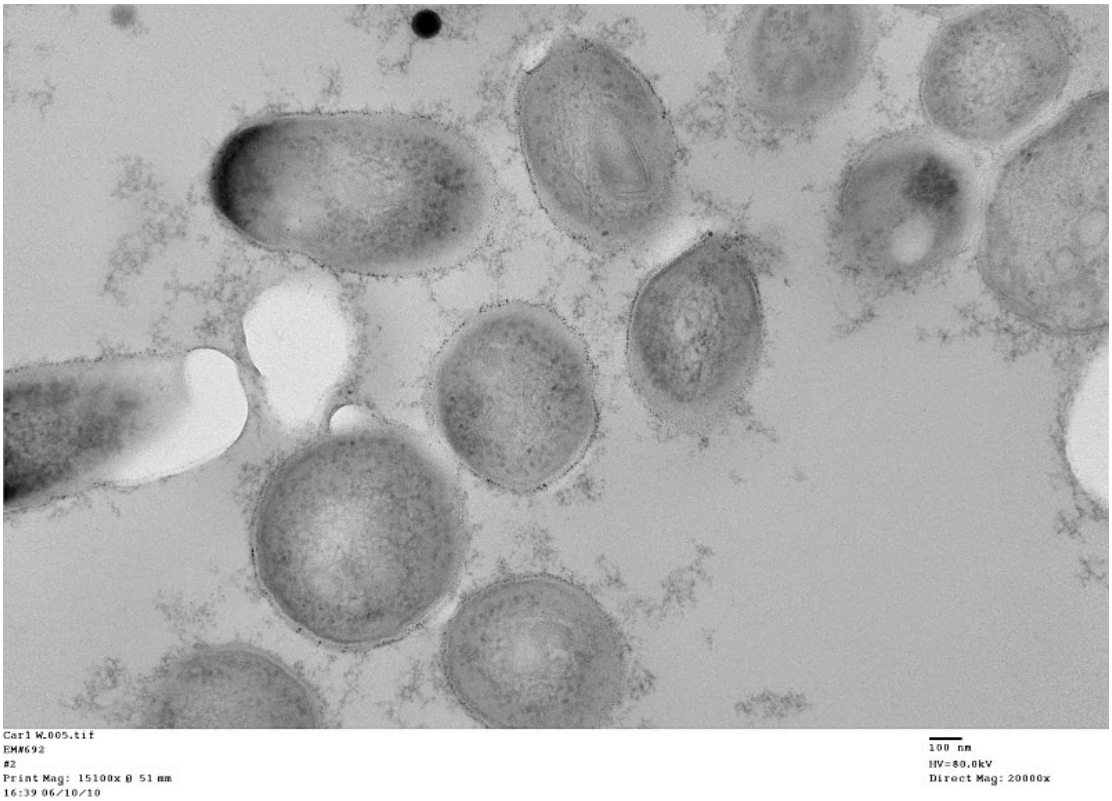
A (H37Rv, logarithmic phase)



B (H37Rv, carbon-starved)

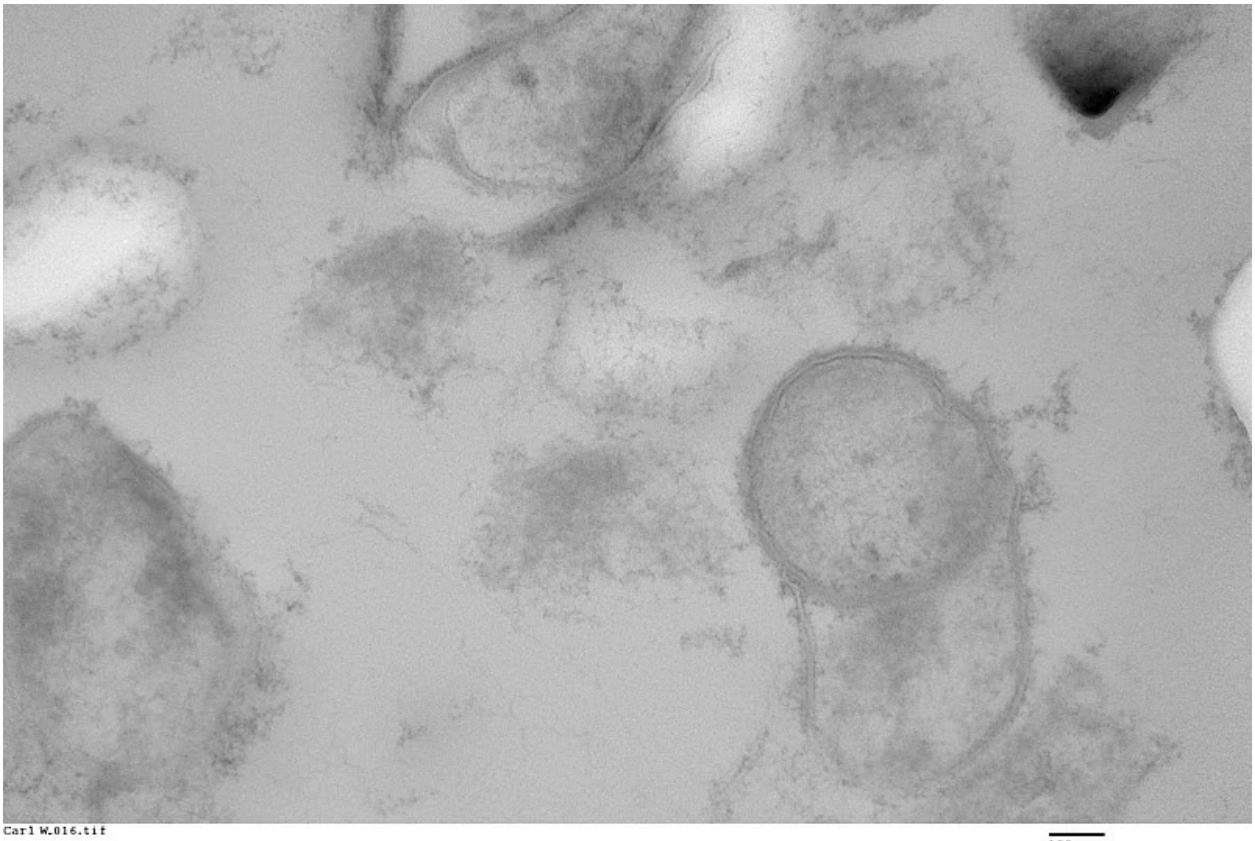


C (H37Rv∆*rpfACDEB*, logarithmic phase)

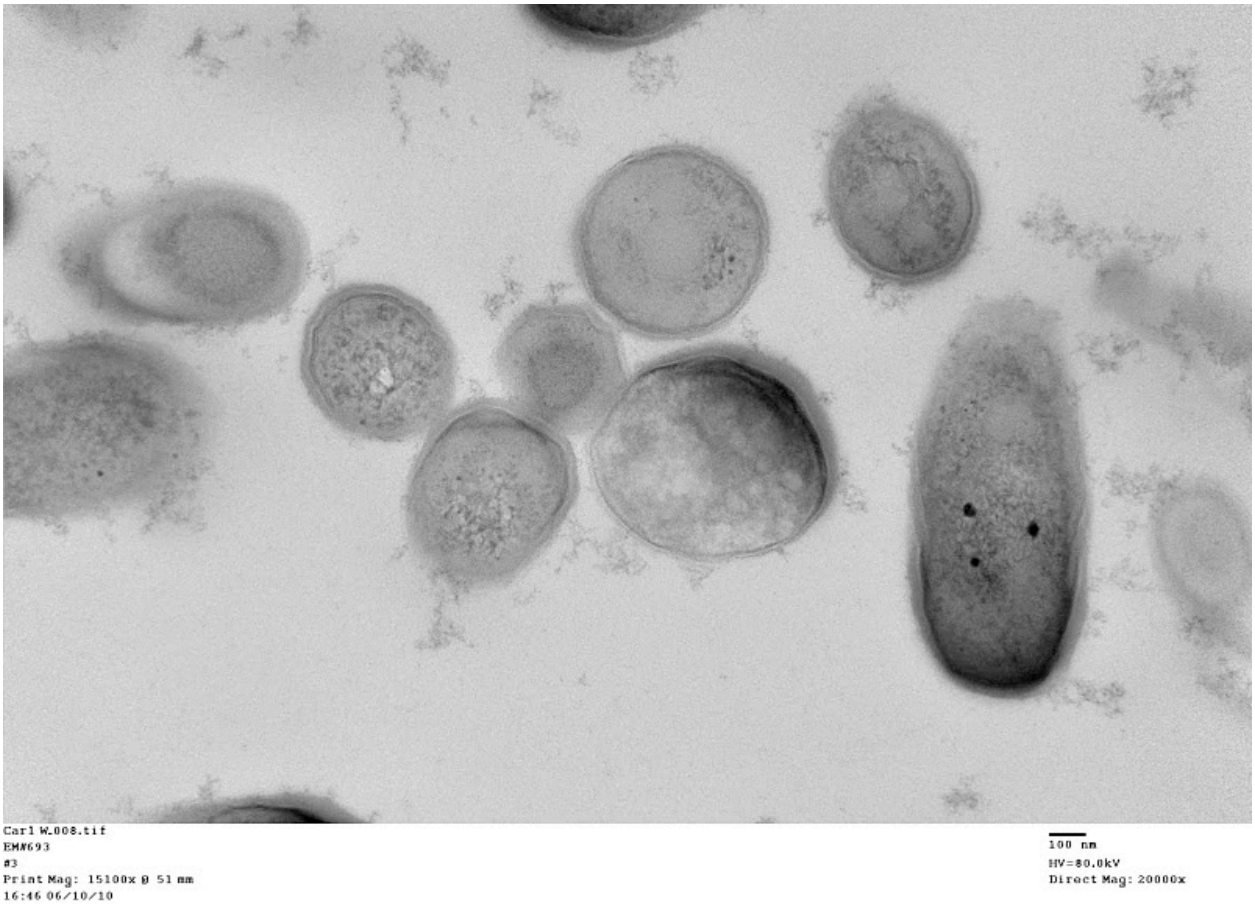



Carl W005
Carl W. Sample B
Cal: 0.627 nm/pix
4:23:07 p 04/09/10
100 nm
HV=80.0kV
Direct Mag: 30000x
X:909.8115 Y: 336.4342

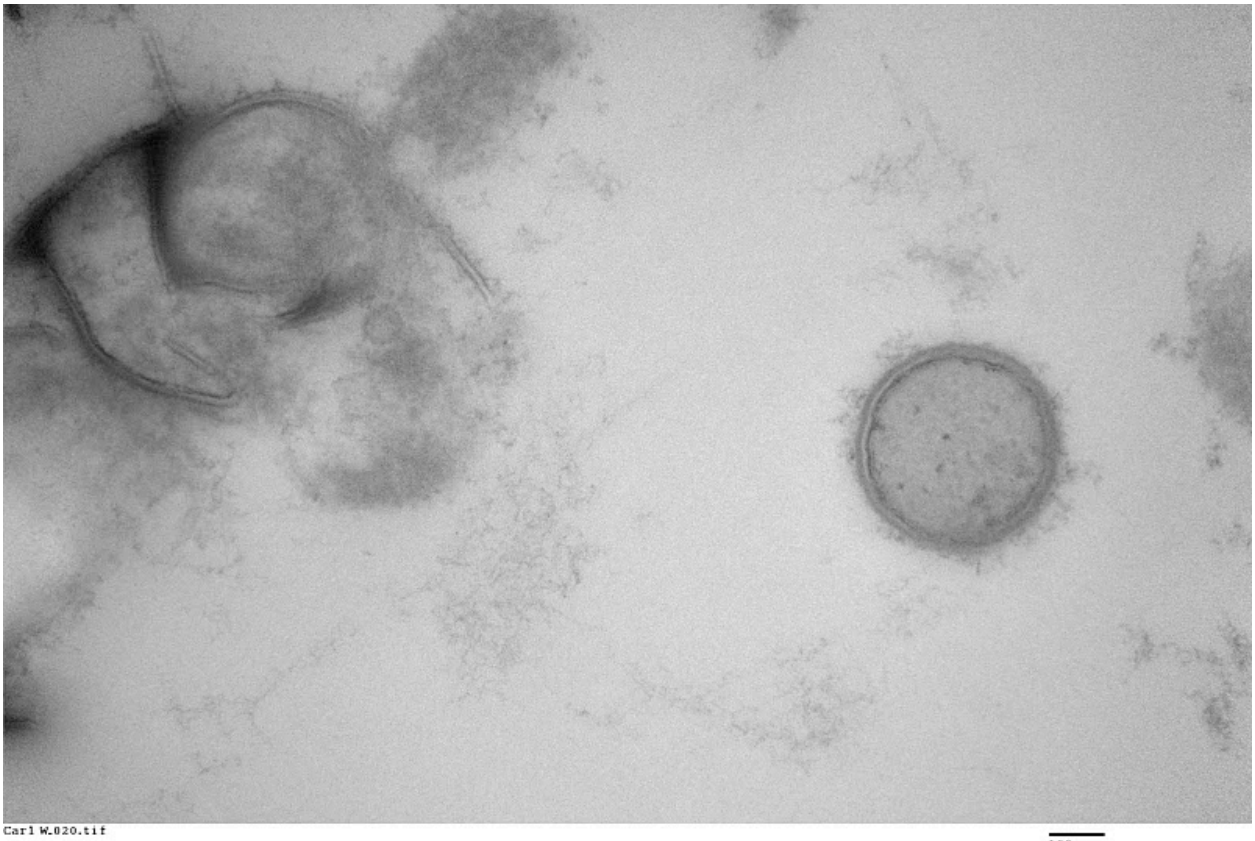
D (H37RvΔ*rpfACDEB*, carbon-starved)



E (H37RvΔ*rpfACDEB ponA2*^{G1328T}, logarithmic phase)



F (H37RvΔ*rpfACDEB ponA2*^{G1328T})



We separately collected clonal populations of H37RvΔ*rpfACDEB*, its parent H37Rv strain, and H37RvΔ*rpfACDEB ponA2*^{G1328T}, a cefotaxime-resistant spontaneous mutant daughter strain of H37RvΔ*rpfACDEB*, under two conditions:

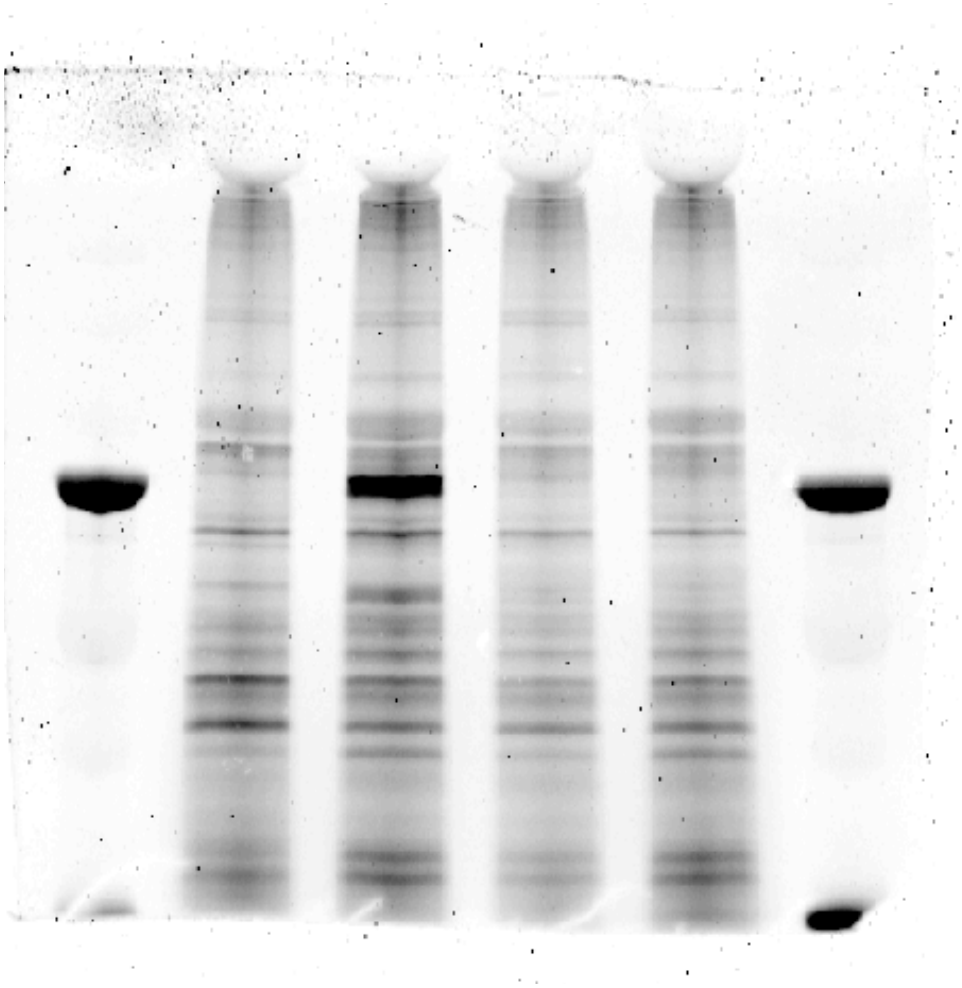
1. exponential growth – OD_{600} 0.6 – 0.8

2. carbon starvation – grown to OD_{600} 0.6 – 0.8, washed and resuspended in PBS with 0.05 % tyloxapol at OD_{600} 0.2, incubated at 37 °C for 4 weeks

We collected the cells by centrifugation, washed in PBS with 0.05 % Tween 80, and then resuspended in a fixative containing 2 % paraformaldehyde, 2.5 % glutaraldehyde, and 0.1 M sodium cacodylate at pH 7.4. Fixed cells were subjected to transmission electron microscopy by the Harvard Medical School Department of Cell Biology core facility.

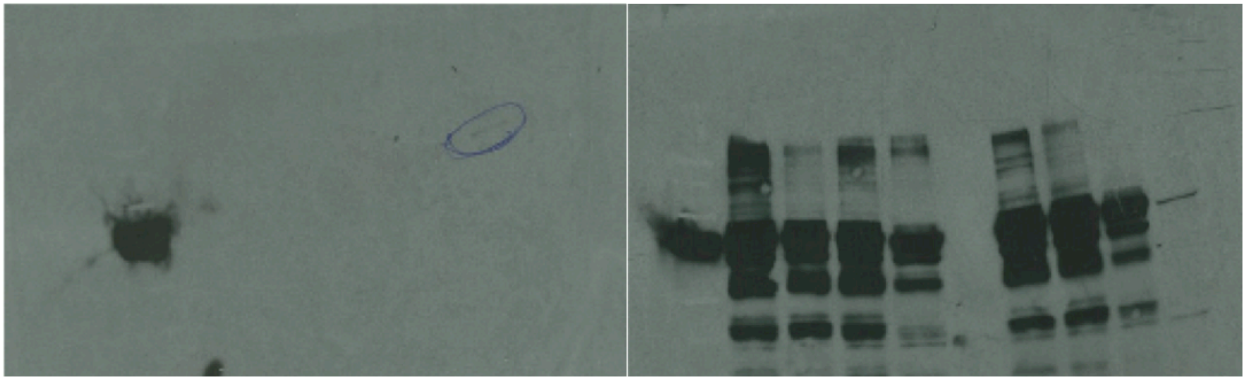
The results do not present obvious differences between H37Rv and its daughter RPF-depleted strains. The slightly granular appearance of the H37RvΔ*rpfACDEB* strain in this image does not appear consistently across samples (see second image in (C)).

Appendix 2: Biochemical studies of PonA2 activity



We expressed PonA2 (lane 2) and the PonA2^{R81G} (lane 3) and PonA2^{W443S} (lane 4) isoforms from the pET-28a vector in *Escherichia coli* BL21-DE3 cells. We induced constructs expressing each isoform and the parent construct (lane 1) for 1 hr in cells growing at 37 °C 250 RPM with 1 mM IPTG. We lysed the cells by beadbeating, pelleted inclusion bodies, and then collected membranes by centrifugation at 100,000 x *g* 1 hr; we stained the collected membranes with 10 μM bocillin, for 90 min. The flanking dark bands of the gel are from a protein ladder; they occur at 75 kDa and result from the fluorescence of the marker. These results indicate that

the wild-type PonA2 isoform expresses in a catalytically active form at these conditions to a much greater extent than either of the mutant isoforms.



This α-His Western blot, tagging the PonA2 protein using the pET-28a endogenous histidine tag, shows the induction of PonA2^{W443S} in soluble form under a variety of conditions. From left to right: ladder, 37 °C 1 mM IPTG, 300 μM IPTG, 100 μM IPTG, 30 μM IPTG, no IPTG, and 30 °C 1 mM IPTG, 300 μM IPTG, 100 μM IPTG, 30 μM IPTG. The left blot shows cell lysates after the removal of inclusion bodies by centrifugation; the circled band corresponds to the 75 kDa position for 30 °C 30 μM IPTG. The right blot shows the same lysates before the removal of inclusion bodies.

These results indicate that although PonA2^{W443S} is abundantly expressed, it has a tendency to form inclusion bodies. Together, these blot results and the results of the bocillin staining experiment above show that mutant PonA2 isoforms may be less catalytically active and have fundamentally different solubility characteristics from the wild-type isoform, supporting our hypothesis that these alleles may lead to PonA2 that is impaired in function.