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The Role of MmpL4 in Mycobacterium tuberculosis Cholesterol Metabolism and Virulence

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The Role of MmpL4 in *Mycobacterium tuberculosis*

Cholesterol Metabolism and Virulence

by

Eric Deming Chow

DISSERTATION

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Chapter 2 of this thesis contains previously submitted material for publication.

Abstract

The Role of MmpL4 in *Mycobacterium tuberculosis*

Cholesterol Metabolism and Virulence

Eric Deming Chow

In order to survive in its host, *M. tuberculosis* must be able to survive and thrive in the *in vivo* milieu. *M. tuberculosis* has evolved many virulence factors to deal with the host response to infection. One important element is the MmpL family of proteins. Several of the MmpLs are required for virulence. The *mmpL4*$^{-}$ mutant has the most severe defect of all the *mmpL*$^{-}$ mutants, but the cause of the defect is unknown. Interestingly, in the absence of interferon-γ, the *mmpL4*$^{-}$ mutant has no virulence defect, suggesting that the function of MmpL4 is to counteract a host immune response. Our work has revealed that MmpL4, an RND-like efflux pump, is required for virulence by conferring resistance to 4-cholestene-3-one (4c3), a toxic metabolite of cholesterol degradation. Interestingly, both MmpL4 and cholesterol metabolism are only required when the host interferon-γ signaling pathway is intact.

MmpL4 is a member of a family of related lipid transporters called the MmpLs (Mycobacterial Membrane Protein – Large). This family is homologous to the RND family of transporters found across all kingdoms of life. Several members of the MmpLs have been shown to secrete specific lipids important for virulence. We initially believed this to be the case for MmpL4, but repeated attempts to identify differences in lipid composition in *mmpL4*$^{-}$ mutant cells were fruitless. We discovered that MmpL4 is only

required when host interferon-γ signaling is intact and that this is due to increased toxicity of 4c3 to *mmpL4*$^{-}$ mutants. In addition to 4c3, *mmpL4*$^{-}$ cells are more sensitive to a variety of xenophobic compounds, raising the possibility that MmpL4 secretes other toxic compounds *M. tuberculosis* encounters in the host.

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Chapter 1.

Introduction.

Two thirds of the world's population is infected with *Mycobacterium tuberculosis*, the causative agent of tuberculosis. While most of these individuals are latently infected, approximately 2 million people die each year due to active *M. tuberculosis* infections. In order to cause disease and persist in its host, *M. tuberculosis* needs several virulence factors as well as the ability to adapt to this niche. Lipids play an important role in both aspects. *M. tuberculosis* preferentially metabolizes host lipids during infection and also produces several lipid virulence factors that interact with host cells.

Several studies have identified lipids as the preferred and required carbon source during infection. Research from nearly six decades ago demonstrated that *M. tuberculosis* initially isolated from infected tissues preferentially metabolizes lipids for a source of carbon. In fact, isolates had no activity towards carbohydrates, in contrast to *in vitro* grown *M. tuberculosis* which utilized both carbon sources (Bloch and Segal 1956). Experiments performed much later showed that the utilization of lipids as the sole carbon source requires the isocitrate lyases in the glyoxylate shunt pathway and deletion of the genes encoding them results in severe attenuation of *M. tuberculosis* virulence in mice (McKinney et al. 2000; Muñoz-Elías and McKinney 2005).

In the past several years, more research has shed light on the specific lipids used by *M. tuberculosis* during infection. A gene cluster required for cholesterol degradation in *Rhodococcus* was also found in *M. tuberculosis* (Van der Geize et al. 2007). Further work showed *M. tuberculosis* can import cholesterol and utilize it as an energy source as well as a source of carbon to synthesize lipids. Furthermore, this activity is important in

interferon-γ activated macrophages (Pandey and Sassetti 2008; Chang et al. 2009). Inhibition of this pathway by mutagenesis has shown that intermediates in cholesterol breakdown are toxic (Ouellet et al. 2010; Yam et al. 2009).

While degradation of host-derived lipids is an important carbon source for the bacterium, *M. tuberculosis* also has an amazing capacity for lipid biosynthesis. Its cell wall contains a dense layer of essential mycolic acids which contributes to its low permeability (Brennan and Nikaido 1995). *M. tuberculosis* also synthesizes a number of lipids which are non-essential for *in vitro* growth but required for virulence. Early experiments demonstrated a correlation between production of certain lipids and virulence (Goren, Brokl, and Schaefer 1974b). When the genome of *M. tuberculosis* was sequenced, it revealed a large percentage of the genome is dedicated to lipid synthesis (Tekaia et al. 1999). In addition to synthetic genes, MmpL genes predicted to encode transporters were found in several of these lipid synthetic gene clusters, suggesting a lipid efflux role for the MmpLs (Tekaia et al. 1999).

The importance of lipid virulence factors was confirmed in two signature-tagged mutagenesis studies (Cox et al. 1999; Camacho et al. 1999). In these studies, several mutants defective in the synthesis of pthiocerol dimycolate (PDIM) were identified. PDIM is a lipid critical for virulence. Though its role *in vivo* remains controversial, it is thought to protect the bacilli from reactive oxygen and nitrogen intermediate (Rousseau et al. 2004) and act in concert with the ESX-1 secretion system substrate ESAT-6 to form pores in the phagosomal membrane (Paolo Manzanillo and Eric Chow unpublished results). Additionally, *mmpL7*⁻ mutants were identified in the screen as well. The

mmpL7^{-} mutants were able to synthesize PDIM but unable to secrete it into the cell wall, strongly suggesting that it functions at the interface between pathogen and host (Cox et al. 1999). This study was the first to document a specific function for an MmpL and supported the hypothesis that MmpLs function to secrete lipids synthesized by *M. tuberculosis*. .

The MmpLs belong to the RND family of transporters, which are found across all kingdoms of life and typically contain twelve transmembrane segments and two extramembrane loops (Tseng et al. 1999). Examples include *E. coli* AcrB, which effluxes bile salts, but also a wide variety of drugs and other xenobiotics, and NPC1 which is involved in cholesterol trafficking in vertebrates (Nikaido 2011). Following the study of MmpL7, other MmpLs have also been shown to export mycobacterial lipids. MmpL3 transports mycolic acids, explaining why it is essential (Tahlan et al. 2012; Grzegorzewicz et al. 2012) and MmpL8 transports SL-1278 (Converse et al. 2003; Domenech et al. 2004). Unlike AcrB, these MmpLs have specific substrate specificities. In this work, we report that MmpL4 functions as an efflux pump akin to AcrB. MmpL4 is required for *M. tuberculosis* virulence in the presence of host IFN-γ signaling, and *mmpL4*$^{-}$ mutants are more sensitive to a variety of hydrophobic xenobiotic compounds including 4c3. Interestingly, cholesterol derivatives such as steroid hormones are also important signaling molecules. This raises the possibility that *M. tuberculosis* could secrete cholesterol metabolites to alter the host immune response to increase virulence.

Chapter 2.

M. tuberculosis MmpL4 is required to counteract host interferon-γ defense mechanisms and confers resistance to 4-cholestene-3-one.

Introduction

M. tuberculosis depends on many virulence factors to cause disease. One family of virulence factors is the MmpL family of proteins. Several MmpLs have been shown to be indispensable for virulence, including MmpL4, 7, 8, and 11 (Domenech, Reed, and Barry 2005). Roles have been assigned to MmpL7 and MmpL8 which secrete *M. tuberculosis* lipids. We have been interested in MmpL4 for two reasons. First, it is the most attenuated of all the MmpLs studied. Second, our initial work has demonstrated that the *mmpL4*$^{-}$ mutant is only attenuated when interferon-γ signaling is intact. Understanding the function of MmpL4 could give insight into the host-pathogen interaction that occurs with *M. tuberculosis*.

The MmpLs are predicted to transport *M. tuberculosis* lipids due to previous studies of MmpLs (Tahlan et al. 2012; Grzegorzewicz et al. 2012; Cox et al. 1999; Camacho et al. 2001; Converse et al. 2003) and the proximity of *mmpL* genes to lipid biosynthetic genes in the genome (Tekaia et al. 1999). In this study, we show that MmpL4, unlike other MmpLs, does not secrete an *M. tuberculosis* derived lipid but instead effluxes a toxic host-derived metabolite and behaves similarly to the RND-like efflux pump AcrB. We show that MmpL4 relieves *M. tuberculosis* of 4-cholestene-3-one toxicity, and through this function likely plays a role in facilitating cholesterol metabolism, an activity important for survival in interferon-γ activated macrophages (Pandey and Sassetti 2008). Furthermore, we also show that MmpL4 is required for resistance to clofazimine and verapamil, two clinically approved drugs.

Results

To understand the role of MmpL transporters in *M. tuberculosis* pathogenesis, we created a set of individual mutants with each *mmpL* gene deleted (except for *mmpL3*, which is essential), in the virulent Erdman strain of *M. tuberculosis* (Tahlan et al. 2012; Grzegorzewicz et al. 2012). For *mmpL4, mmpL7,* and *mmpL8,* we used transposon insertion mutants that we had identified in a signature-tagged mutagenesis screen (Cox et al. 1999). For the others, we used specialized transduction to delete each gene from the genome (Glickman, Cox, and Jacobs 2000). To independently determine the importance of each MmpL transporter in *M. tuberculosis* pathogenesis, we infected groups of mice with each of these mutants via tail vein injection and measured their virulence by monitoring host viability (Figure 2.1a). This approach was similar to that of Domenech *et al*., although different strains and infection models were used. In agreement with Domenech *et al.*, strains deficient in *mmpL4, mmpL7, mmpL8,* or *mmpL11* are significantly attenuated. Additionally, we found that the *mmpL9* and *mmpL12* mutant strains were also attenuated in this study, though not as severely as the others.

We focused our attention on MmpL4, as the *mmpL4::tn* strain was the most attenuated during infection (Fig 2.1a) and its substrate(s) have not been determined. To more carefully examine the role of MmpL4 during infection, we performed time course experiments in which we monitored bacterial burdens in tissues, using wild-type, mutant, and *mmpL4*$^{-}$ complementation strains. The *mmpL4*$^{-}$ mutant was severely attenuated for growth during the acute phase of infection, with 1.5 log fewer bacteria in

the lungs and spleen (Fig 2.1b and c). Interestingly, although the *mmpL4* mutant was unable to replicate with normal kinetics *in vivo*, the bacteria were able to persist at these lower levels throughout the course of the infection. Importantly, the virulence of the *mmpL4* strain was restored upon expression of a wild-type copy of the gene, under the control of its native promoter (Fig 2.1b, c).

Since MmpL3, MmpL7, and MmpL8, are involved in the secretion of mycobacterial lipids to the cell surface (Grzegorzewicz et al. 2012; Tahlan et al. 2012; Cox et al. 1999; Converse et al. 2003), we sought to identify a potential substrate of MmpL4 by taking a new lipidomic approach that has been successful in identifying changes between wild type and mutant *M. tuberculosis* (Layre et al. 2011). We harvested bacteria and supernatants from log-phase cultures of wild-type and *mmpL4*$^{-}$ mutant cells, and extracted lipids from both fractions. These crude extracts were separated and analyzed by mass spectrometry. Surprisingly, the profile of the *mmpL4*$^{-}$ mutant was indistinguishable from WT, indicating MmpL4 is not involved in lipid synthesis or secretion (data not shown). Although it is certainly possible that this method is not sensitive enough to identify a minor species that is transported by MmpL4, the remarkable similarity of the lipidome of wild-type and *mmpL4* mutant cells grown in culture prompted us to consider the possibility that the function of MmpL4 is manifest only during infection.

We decided to characterize the *mmpL4* virulence phenotype by examining the importance of various host responses to counteract *M. tuberculosis* infection. Interferon-γ is responsible for inducing a number of antimicrobial factors such as

reactive oxygen and nitrogen species, iron sequestration, antimicrobial peptides, and autophagic targeting. We tested whether MmpL4 has a role in protecting *M. tuberculosis* from these IFN-γ inducible factors by infecting *Ifngr1-/-* mice with wild-type or *mmpL4::tn* strains. In *Ifngr1-/-* mice, the median survival times were almost identical for Erdman and *mmpL4::tn* strains (12 and 13 days respectively) (Fig 2.2, black line), suggesting that MmpL4 functions to counteract a host response downstream of IFN-γ.

While the results from the IFN-γ mouse infection were suggestive of a function of MmpL4 to offset a host response dependent on IFN-γ signaling, the effects of IFN-γ deficiency are profound and pleotropic, and thus the rescue of virulence could have been non-specific to the absence of MmpL4. To address this, we tested whether the MmpL4-dependent growth defect was manifest in infected bone marrow-derived macrophages. Monolayers were infected with *M. tuberculosis* at an MOI of one for two hours and individual wells were lysed at time points following infection and bacterial burdens were determined by plating. In naïve macrophages, *mmpL4::tn* mutant cells grew with kinetics that were indistinguishable from wild-type bacteria (Fig 2.3a). In contrast, *mmpL4::tn* had a one log defect compared to the wild-type and complemented strains in macrophages activated with interferon-γ (Fig 2.3b). Taken together, this data strongly suggests that MmpL4 is required for the survival of *M. tuberculosis* in activated macrophages.

Next we sought to identify the condition in interferon-γ activated macrophages that is responsible for the attenuated growth of *mmpL4*$^{-}$ mutants. In murine macrophages, the major killing mechanism activated by interferon-γ is the elicitation of

nitric oxide (NO) via transcriptional induction of the NO synthase, NOS2. Since NOS2 is induced by interferon-γ we determined whether nitric oxide production is responsible for the *mmpL4::tn* defect by infecting *NOS2-/-* mice. If, for example, *mmpL4*$^{-}$ mutant cells are sensitive to NO, we would expect the growth of the *mmpL4*$^{-}$ mutant to be similar to wild-type cells. While removal of NOS2 led to faster kinetics of bacterial growth and animal mortality (Fig 2.2, grey line), there was still a defect of the *mmpL4*$^{-}$ mutant compared to wild-type, and thus this did not phenocopy the interferon-γ knockout mice. In this case, the median survival times were 20 and 27 days for *NOS2-/-* mice infected with wild-type and *mmpL4::tn*, respectively. While *mmpL4::tn* has a virulence defect in *NOS2-/-* mice, this does not eliminate the possibility that *mmpL4::tn* is more sensitive to NO in addition to another factor downstream of interferon-γ. To address this possibility, we treated strains of *M. tuberculosis* in acidified media (pH5.5) in the presence or absence of 3mM sodium nitrite for one week and enumerated CFUs by plating. *mmpL4* and wild-type strains were equally sensitive to NO and low pH (Fig 2.4a), demonstrating that the *mmpL4* virulence defect is not due to NO production by the host or phagosome acidification.

In addition to NO, interferon-γ also provokes superoxide production through the activation of phagosome oxidase (Phox). This pathway appears to be an important mechanism of controlling *M. tuberculosis* infections in humans (Bustamante et al. 2011), though this pathway is dispensable for controlling wild-type infections in mouse models of infection. To test whether *mmpL4* is sensitive to reactive oxygen species (ROS), we performed disc diffusion assays using the superoxide generators plumbagin and

menadione. Discs containing each of the chemicals were placed on 7H10 plates spread with lawns of bacteria and the zones of clearance were measured after 14 days of growth. The experiments demonstrate that *mmpL4* mutant cells are more sensitive to both superoxide generators as indicated by the larger zones of clearance compared to wild type controls (Fig 2.4b). This data leads to the prediction that MmpL4 is required to counteract ROS generated in macrophages. To test this model, we infected wild type and *Cybb-/-* macrophages which lack the ability to generate superoxide. However, *mmpL4::tn* was not rescued in interferon-γ activated *Cybb-/-* macrophages (Fig 2.3c and d) (Pollock et al. 1995). Thus, the *mmpL4* mutant strain is sensitive to superoxide generators in culture, but insensitive to the direct effects of ROS generation in interferon-γ activated macrophages.

Interferon-γ also limits bacterial replication by inducing several mechanisms that function to restrict iron from pathogens (Schaible and Kaufmann 2004). The recent findings that MmpL11 is required for iron-siderophore uptake (Tullius et al. 2011) and that MmpL4 transcription is induced under low-iron conditions (Rodriguez et al. 2002), suggested that MmpL4 may counteract iron sequestration *in vivo*. To test whether *mmpL4*$^{-}$ mutants have a growth defect under low-iron conditions, 10-fold serial dilutions were spotted onto 7H10 plates in the presence or absence of dipyridyl (DPI), an iron chelator. In the absence of DPI, no growth defects were observed (Fig 2.5a). In the presence of 50uM DPI, *mmpL4::tn* and *ΔmmpL4* had considerable growth defects compared to wild-type and complemented strains (Fig 2.5b). However, unlike mycobactin mutants, *mmpL4::tn* and *ΔmmpL4* grew normally in media depleted of iron

(Fig 2.5e and f), demonstrating that *mmpL4* mutants are capable of growth under iron-restricted conditions. Additionally, *mmpL4::tn* secretes similar levels of mycobactins and addition of exogenous iron during macrophage infections did not rescue *mmpL4::tn* (data not shown). Thus, similar to our ROS experiments, *mmpL4* mutant cells are sensitive to chemicals that lead to iron depletion, but not iron depletion *per se*.

In a large transcriptional dataset which subjected *M. tuberculosis* to a variety of xenobiotics (Boshoff et al. 2004), MmpL4 was shown to be induced in response to a variety of chemical treatments. We noticed structural similarity between DPI, plumbagin, and menadione to chemicals that induce MmpL4 such as verapamil and clofazimine, in particular that they are all planar, hydrophobic compounds with aromatic groups (Fig 2.6). Another similarity between the three chemicals is that they are all cell-permeable. Thus, we hypothesized that the reason the *mmpL4*$^{-}$ mutant is not sensitive to low-iron or superoxide *per se* but is more sensitive to the chemicals that we used to generate them is because MmpL4 is required for resistance to these chemicals. MmpL4 could be acting as an efflux pump to eliminate planar hydrophobic compounds from the cell. In the absence of MmpL4, these chemicals build up to toxic concentrations in *M. tuberculosis*. *mmpL4*$^{-}$ mutants should be insensitive to chemicals that do not cross the cell wall. We decided to test this hypothesis with two other iron chelators, the cell permeant 1,10-phenantholine, which is a planar hydrophobic molecule, and DTPA which is cell impermeant. Serial dilutions of strains were spotted on 7H10 plates containing these chelators. The *mmpL4*- mutants had growth defects on 1,10-phenanthroline plates, but not on DPTA plates, as hypothesized (Fig 2.5c and d).

The findings that *mmpL4* mutant cells are sensitive to lipophilic chelators, and MmpL4 is induced by a variety of xenobiotic compounds in culture, led us to test the hypothesis that MmpL4 is required to pump out xenobiotics *in vivo*. We determined the sensitivity of wild type *M. tuberculosis* and *mmpL4*- mutants to several chemicals (Table 2.1). The *mmpL4*$^{-}$ mutants are not more sensitive to standard TB drugs INH, RIF, and EMB, or SDS, a general cell wall stress (Domenech, Reed, and Barry 2005), but are four to eight fold more susceptible to crystal violet, the efflux pump inhibitor verpamil, clofazimine, and plumbagin. The defect of *mmpL4*$^{-}$ mutants is specific as none of the other *mmpL* mutants tested showed increased susceptibility to these chemicals. This data suggests MmpL4 acts as an efflux pump, like AcrB and other RND pumps. Furthermore, sub-inhibitory concentrations of the efflux pump inhibitor verapamil decrease the crystal violet MIC for wild-type and complemented strains, but not *mmpL4*- cells, suggesting that this pump inhibitor also works against MmpL4 (Fig 2.7).

If the function of MmpL4 is to efflux compounds, the sensitivity of the *mmpL4*$^{-}$ mutant should be due to a chemical it encounters in interferon-γ activated macrophages. We tested a battery of compounds that are induced in macrophages, including free fatty acids (arachidonic acid), antimicrobial peptides (LL-37, UB2, RPS-30-2), and tryptophan metabolites (L-kynureneine, cinnabarinic acid) but were unable to identify a compound that specifically inhibited *mmpL4* cells (data not shown).

A screen performed by the Sassetti group for mutants defective in growth on cholesterol gave a clue to the identity of the *in vivo* substrate of MmpL4 (Griffin et al. 2011). *mmpL4* mutants were found to be less competitive in growth on cholesterol.

The mutants' defect was similar in magnitude to that observed in *cyp125* mutants. Cyp125 is a cytochrome P450 that converts the toxic 4-cholesten-3-one (4c3) to a downstream metabolite (Ouellet et al. 2010). Interestingly, cholesterol and its metabolites are planar hydrophobic molecules, similar to the compounds *mmpL4* is more sensitive to (Fig. 2.6), raising the possibility that MmpL4 may also efflux toxic physiologic molecules related to cholesterol such as 4c3. To test this, strains were grown in minimal media containing glycerol (0.1%), cholesterol (0.1mM), or 4c3 (0.1mM) plus glycerol. Strains grew similarly on glycerol and cholesterol but *mmpL4::tn* did not grow in the presence of 4-cholesten-3-one (Fig 2.8). These results indicate that *mmpL4*$^{-}$ is sensitive to exogenously added 4c3, and second, when grown in isolation, *mmpL4* cells do not have a defect when cholesterol is the sole carbon source.

Attempts to rescue *mmpL4* growth in the presence of 4c3 by overexpressing Cyp125 with an integrating complementation construct (Ouellet et al. 2010) failed (Data not shown). This can be explained by the hypothesis 4c3 exerts is toxicity by disrupting membranes (Ouellet, Johnston, and de Montellano 2011). Exogenously added 4c3 will insert into the cell membrane before cytoplasmic Cyp125 is able to act on it, in contrast to 4c3 generated *in vivo* from cholesterol metabolism in the cytosol.

Discussion

In this work, we have identified a novel role for the efflux pump MmpL4. Unlike the other MmpL family members studied, MmpL4 appears to efflux a variety of toxic chemicals in a role similar to *E. coli* AcrB (Nikaido 2011). We have also identified

resistance to 4c3 as an important function for MmpL4. This role is even more interesting given the virulence defect of *mmpL4*$^{-}$ is only manifest in the presence of host interferon-γ signaling, making it a "counter-immune" mutant (Hisert et al. 2004).

We originally hypothesized that MmpL4 exerts its counter-immune function by secreting a lipid that benefits the bacteria since other MmpLs studied have had roles in effluxing mycobacterial lipids (Cox et al. 1999; Converse et al. 2003; Grzegorzewicz et al. 2012; Tahlan et al. 2012) but were unable to determine any differences in the lipid profile of *mmpL4*$^{-}$ cells. Attempts to characterize the *mmpL4*$^{-}$ virulence defect by identifying a host factor downstream of interferon-γ signaling responsible were fruitless. The mutant was not more sensitive to pathways downstream of interferon-γ activation, such as low pH, NO, ROS, iron restriction, autophagy, and antimicrobial peptides.

Attempts to identify an *in vivo* substrate for MmpL4 proved difficult until a study reported a role for MmpL4 in growth on cholesterol (Griffin et al. 2011). In our experiments, *mmpL4*$^{-}$ does not show a growth defect when grown on cholesterol (Fig. 2.8), but does show a defect when 4c3 is present. The magnitude of the *mmpL4*$^{-}$ defect was roughly the same as mutants in *Cyp125*, which encodes the protein that converts 4c3 to downstream metabolites (Griffin et al. 2011).

Our hypothesis for the discrepancy in growth on cholesterol between our results and Griffin *et al.* is that the growth occurred in competition in the screen versus in isolation in our study. During cholesterol metabolism, 4c3 exerts its toxicity in the cell membrane (Ouellet, Johnston, and de Montellano 2011). If the role of MmpL4 is to secrete toxic 4c3 that localizes to the mycobacterial membrane, then in cultures in

which the majority of cells contain functional MmpL4, one would expect 4c3 concentrations to build up in the culture supernatant. This explains why *MmpL4* was identified in the Griffin screen because most of the cells were wild type for *MmpL4*. As 4c3 levels increased, the *mmpL4*$^{-}$ mutants became inhibited, resulting in decreased fitness.

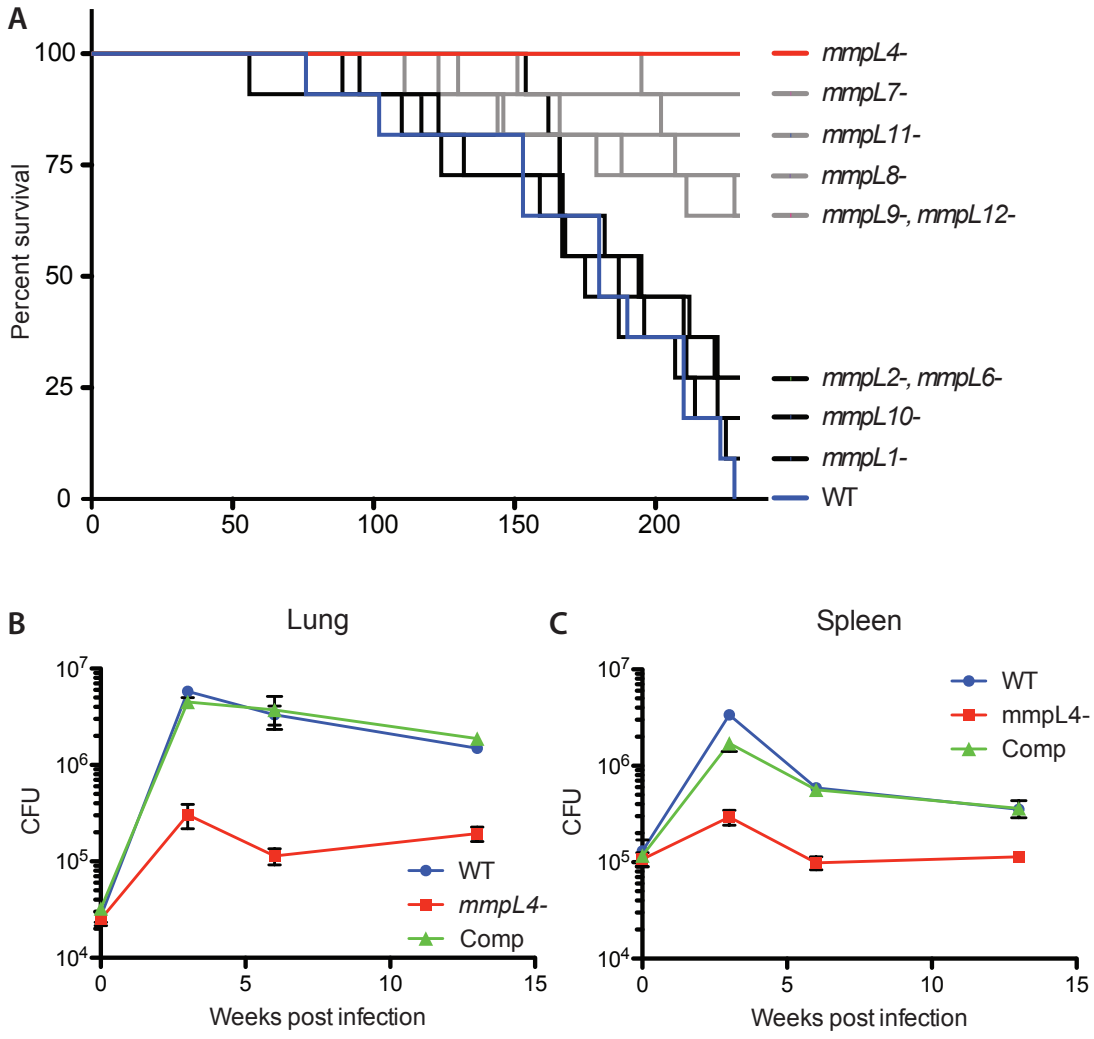
Several pieces of evidence support this model. First, we have shown that MmpL4 is required for resistance to hydrophobic cell-permeant compounds, including 4c3. Second, secretion of cholesterol metabolites has been documented. Androstenedione, androstadienedione, and DHSA have been detected in the culture supernatant of *M. tuberculosis* grown in cholesterol (Nesbitt et al. 2010; Yam et al. 2009). Third, Hsd (Rv1106c), performs the first step in cholesterol degradation and is required for growth on cholesterol (Yang et al. 2011), yet the mutant only showed a moderate growth defect when cholesterol was the sole carbon source in the screen (Griffin et al. 2011). The data suggests that the culture supernatant of the transposon mutant pool contains cholesterol metabolites secreted by *M. tuberculosis* and these metabolites are taken up by *hsd*$^{-}$ cells which have the enzymatic capability to utilize metabolites downstream of cholesterol as a carbon source. When *hsd*$^{-}$ cells are grown in isolation, none of these metabolites are produced, preventing the cells from acquired carbon and halting growth.

Beyond virulence, our data suggests a clinical role for MmpL4. As shown in table 2.1, *mmpL4* is more sensitive to clofazimine, a drug used to treat leprosy and multi-drug resistant TB. It is not generally used to treat TB infections because of its toxicity and

side-effects. Our results suggest that clofazimine could be a more effective treatment if MmpL4 is inhibited (Fig. 2.7). Clofazimine also transcriptionally upregulates *MmpL4* expression. In addition to clofazimine, several next generation anti-TB compounds including SQ-109, PA-824, and TMC-207 induce *MmpL4* expression as well (Boshoff et al. 2004). While *mmpL4*$^{-}$ mutants didn't show increased sensitivity to these compounds, it will be interesting to see if overexpression of *MmpL4* can lead to resistance, as increased expression of efflux pumps is a known mechanism of antibiotic resistance in bacteria (Nikaido 2011). Lastly, cholesterol metabolites may not be the only physiologic substrate of MmpL4. Biochemical fractionation of macrophage extracts may reveal other antimicrobial compounds the immune system uses to control infections. MmpL4 provide *M. tuberculosis* resistance to these compounds.

Figure 2.1.

***mmpL4*$^{-}$ is less virulent in a mouse model of infection.** Balb/c mice were infected with *M. tuberculosis* strains by tail-vein injections. a) Survival of mice was determined by monitoring weight. b) CFUs from the lung and spleen were enumerated at the time points indicated.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mmpL2-, mmpL6-
mmpL10-
mmpL1-
WT
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Lung
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CFU
10^7
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15
Weeks post infection
WT
mmpL4-
Comp

Figure 2.2.

***mmpL4*$^{-}$ is less virulent in *NOS2*$^{-/-}$ but not *Ifn-γR*$^{-/-}$ mice.** C57bl/6 mice *Ifn-γR1*$^{-/-}$ mice (black lines) or *NOS2*$^{-/-}$ mice (grey lines) were infected with *M. tuberculosis* wild type Erdman (circles) or *mmpL4*$^{-}$ (triangles) via tail vein. Survival time points were determined by weight.

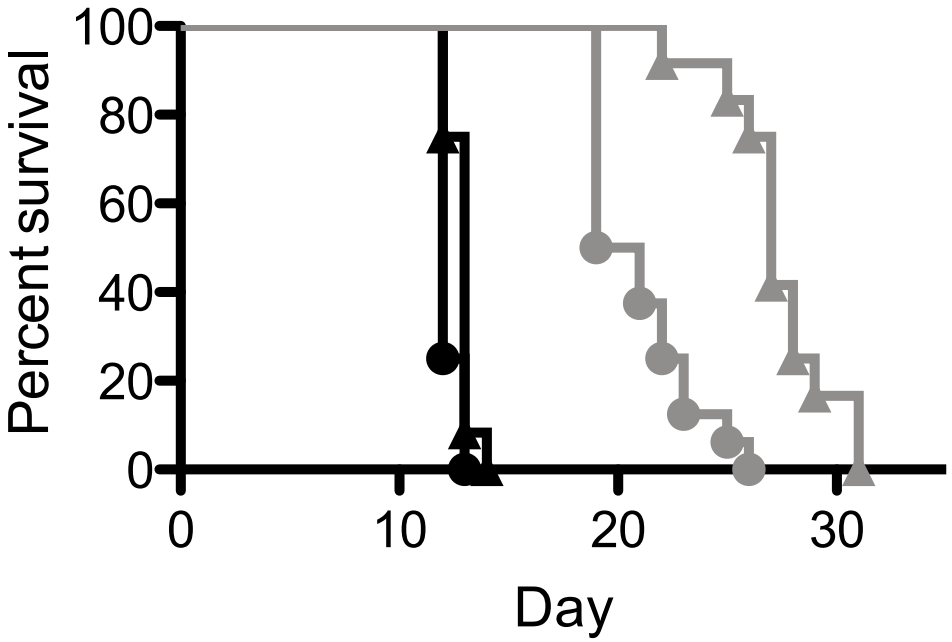

Percent survival
100
80
60
40
20
0
0
10
20
30
Day

Figure 2.3.

***mmpL4*$^{-}$ is more sensitive in WT and *CYBB*$^{-/-}$ interferon-γ activated macrophages.** WT (a and b) or *CYBB*$^{-/-}$ (c and d) BMDMs were infected at an MOI of 1 and CFUs were counted at day 0,1,3,5, and 7. BMDMs were resting (a and c) or activated with interferon-γ (b and d). Each time point was performed in triplicate.

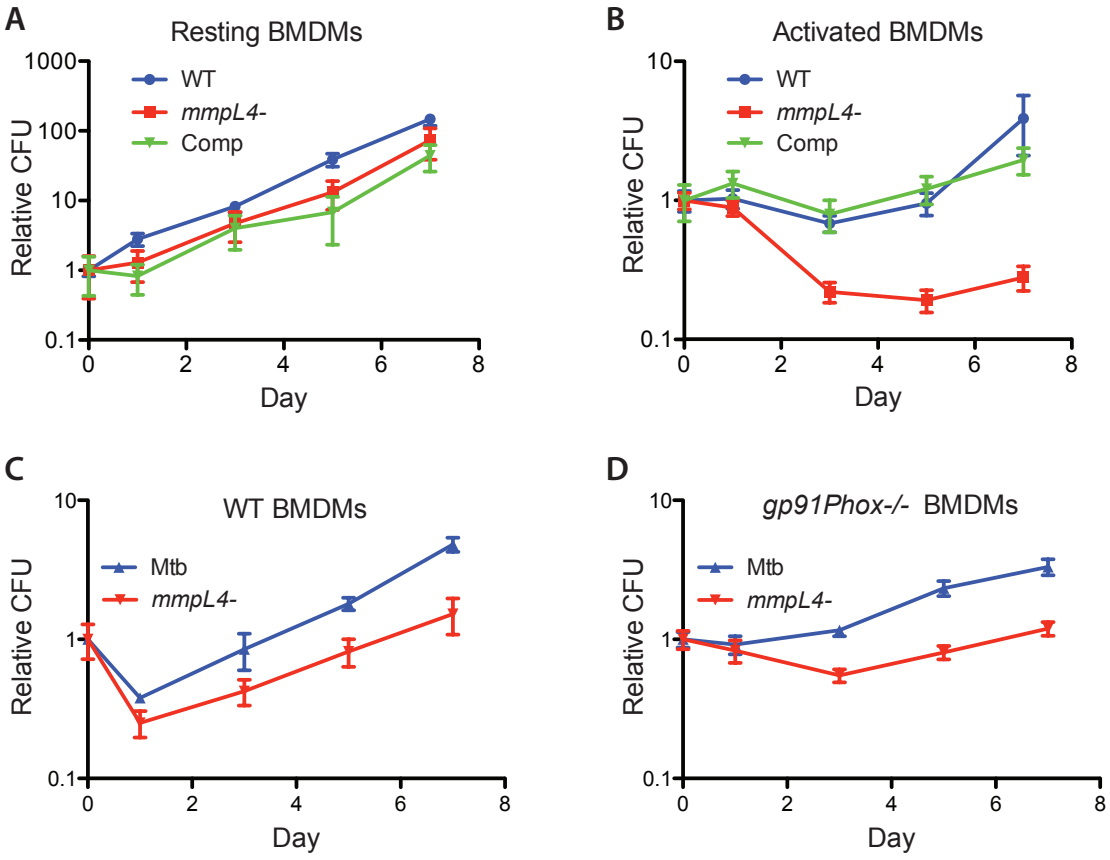

A
Resting BMDMs
1000
100
10
1
0.1
Relative CFU
WT
mmpL4-
Comp
0
2
4
6
8
Day
B
Activated BMDMs
10
1
0.1
Relative CFU
WT
mmpL4-
Comp
0
2
4
6
8
Day
C
WT BMDMs
10
1
0.1
Relative CFU
Mtb
mmpL4-
0
2
4
6
8
Day
D
gp91Phox-/- BMDMs
10
1
0.1
Relative CFU
Mtb
mmpL4-
0
2
4
6
8
Day

Figure 2.4.

***mmpL4*$^{-}$ is more sensitive to superoxide generators plumbagin and menadione.** a) Bacteria were tested against low pH and acidified nitrite. CFUs were plated before and after 6 days of treatment. b) 10ul of 5mM plumbagin in DMSO or 25mM menadione in CHCl3 was tested in a disc diffusion assay. Zones of clearance were measured after 13 days.

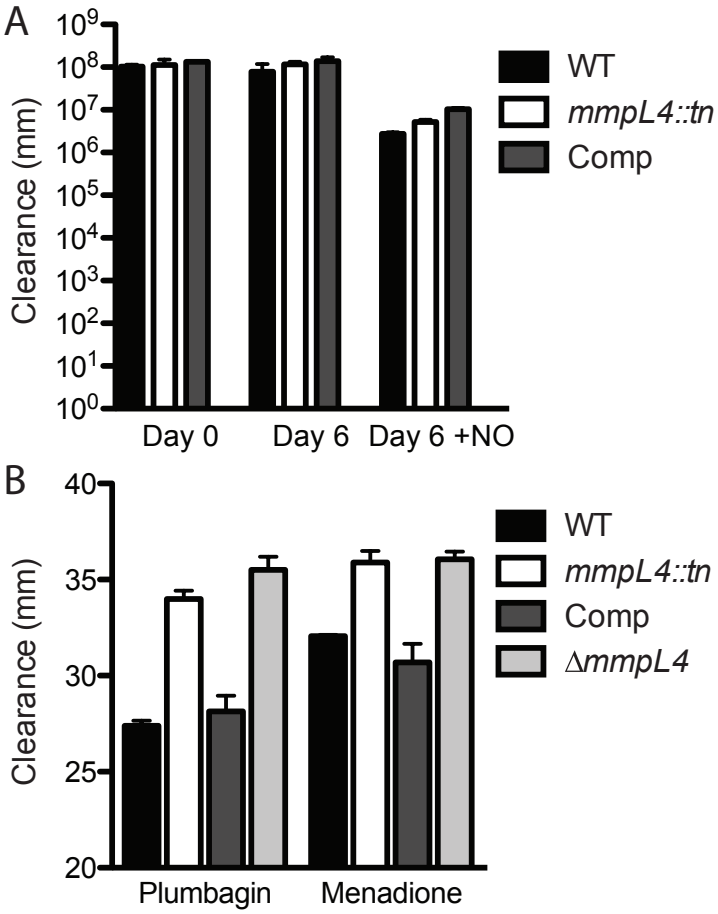

A
Clearance (mm)
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
Day 0
Day 6
Day 6 +NO
WT
mmpL4::tn
Comp
B
Clearance (mm)
40
35
30
25
20
Plumbagin
Menadione
WT
mmpL4::tn
Comp
Δ*mmpL4*

Figure 2.5.

***mmpL4*⁻ is more sensitive to cell-permeable chelators.** 10-fold serial dilutions of bacteria were spotted onto 7H10 plates with a) -, b) 50uM dipyridyl, c) 10uM phenanthroline, and d) 50uM DTPA. Growth in liquid cultures was measured by OD600 in e) iron-replete or f) iron-deficient 7H9 media.

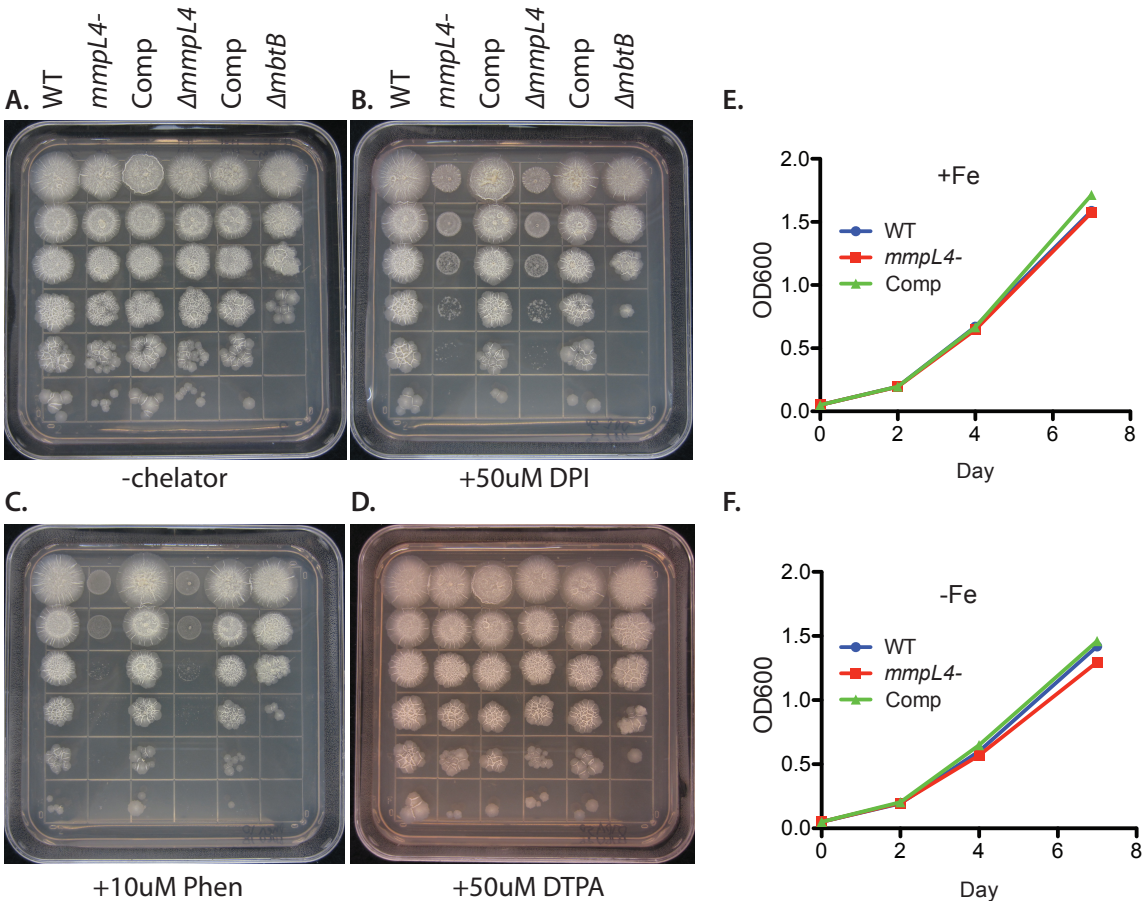
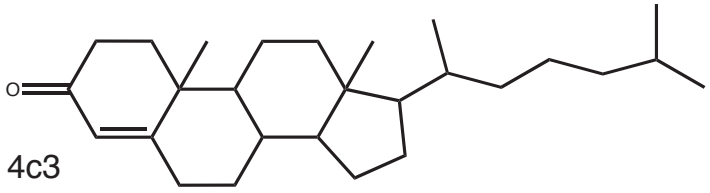

A.
WT
mmpL4-
Comp
ΔmmpL4
Comp
ΔmbtB
B.
WT
mmpL4-
Comp
ΔmmpL4
Comp
ΔmbtB
-chelator
+50uM DPI
C.
D.
+10uM Phen
+50uM DTPA
E.
+Fe
WT
mmpL4-
Comp
OD600
Day
F.
-Fe
WT
mmpL4-
Comp
OD600
Day

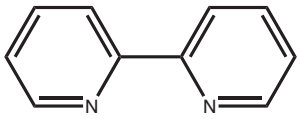
Figure 2.6.

Chemical structures of compounds that show increased inhibition of *mmpL4*⁻ growth.

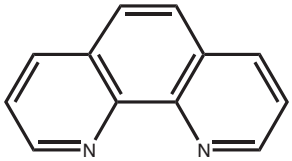
Structures were created in ChemDraw.



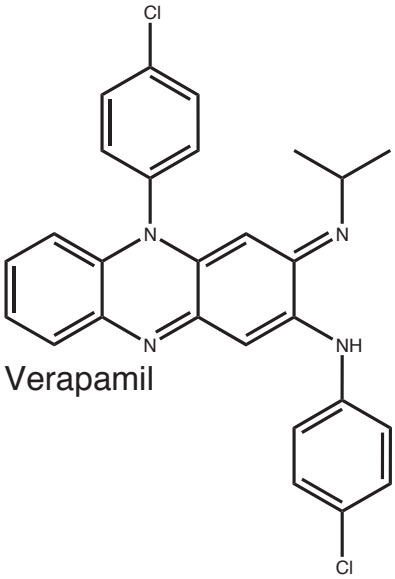
4c3



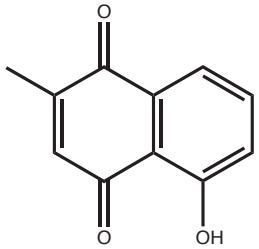
DPI



1,10 Phenanthroline



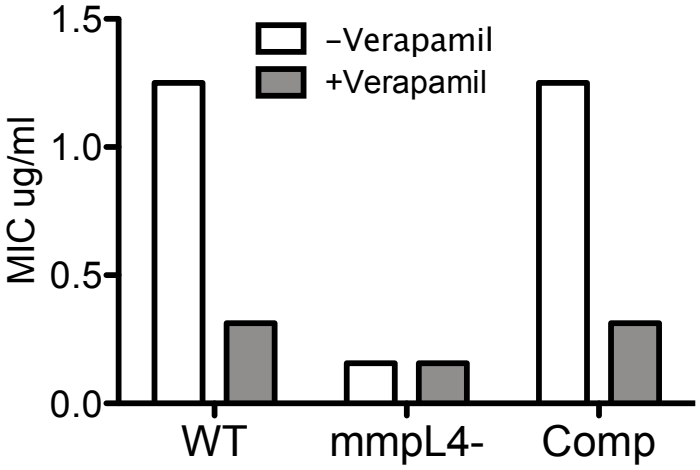
Verapamil



Plumbagin

Figure 2.8.

***mmpL4*⁻ resistance to crystal violet and clofazimine are less sensitive to sub-inhibitory concentrations of verapamil.** Crystal violet (top) and clofazimine (bottom) MICs were determined in the presence or absence of sub-inhibitory concentrations of verapamil (wildtype and complemented, 50ug/ml; *mmpL4::tn* 12.5ug/ml). MICs were performed by serially diluting compounds in 96-well plates and adding 200ul of culture at an OD_{600} of 0.005 and then checking for growth after 6 days. Minimum inhibitory concentrations were selected as the lowest concentration that inhibited visible growth.


1.5
1.0
0.5
0.0
MIC ug/ml
−Verapamil
+Verapamil
WT
mmpL4-
Comp

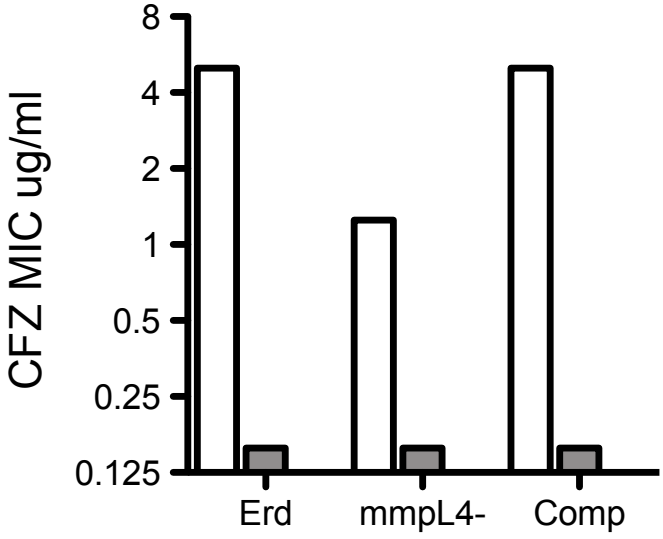

8
4
2
1
0.5
0.25
0.125
CFZ MIC ug/ml
Erd
mmpL4-
Comp

Figure 2.8

***mmpL4*$^{-}$ is more sensitive to 4c3, but has no cholesterol growth defect.** Cultures were grown in minimal media + glycerol to log phase. Cultures were back diluted into minimal media + glycerol, cholesterol, or glycerol + 4c3. Growth was tracked by OD_{600}.

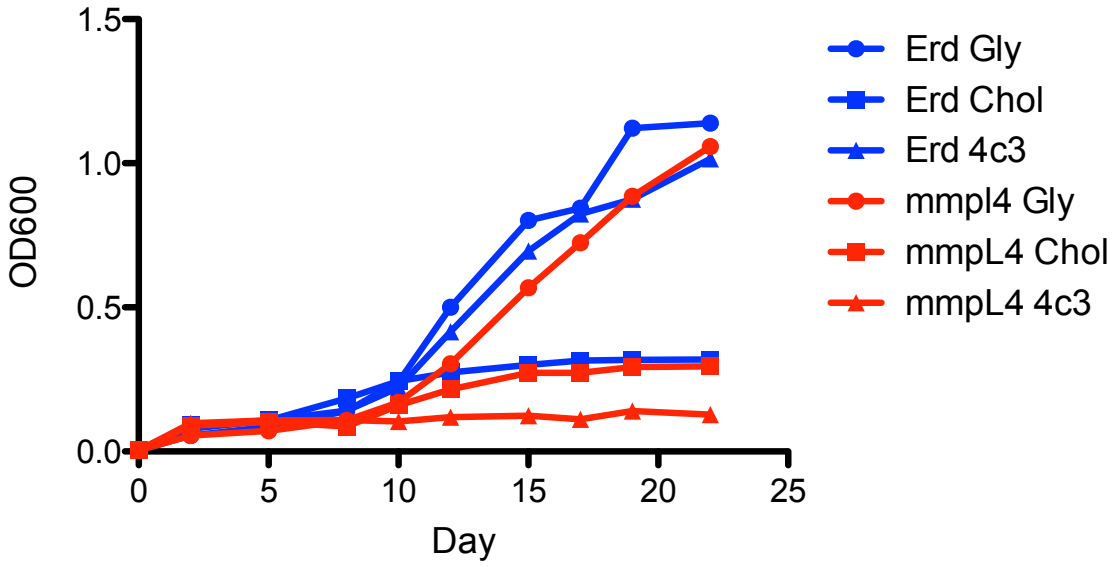

1.5
1.0
0.5
0.0
OD600
0
5
10
15
20
25
Day
Erd Gly
Erd Chol
Erd 4c3
mmpl4 Gly
mmpL4 Chol
mmpL4 4c3

Table 2.1

***mmpL4*$^{-}$ is more sensitive to a variety of chemicals.** MICs were performed by serially diluting compounds in 96-well plates and adding 200ul of culture at an OD_{600} of 0.005 and then checking for growth after 6 days. Minimum inhibitory concentrations were selected as the lowest concentration that inhibited visible growth. MICs are listed relative to the wild-type Erdman strain. The other *mmpL4*$^{-}$ mutants are listed by their respective numbers.

	WT	*mmpL4-*	Comp	1	2	6	7	8	9	10	11	12	13
INH	1	2	1	1	1	1	1	1	1	1	1	1	1
RIF	1	1	2	1	1	1	1	1	1	1	1	1	1
EMB	1	1	1	2	2	1	0.5	1	1	1	1	1	1
SDS (%)	1	1	1	1	1	1	1	1	1	1	1	1	1
Crystal Violet	1	0.125	1	2	1	1	0.5	1	2	1	1	1	1
Verapamil	1	0.125	1	1	1	1	0.5	1	1	1	1	1	1
Clofazimine	1	0.25	1										
Plumbagin	1	0.25	1										
EtBr	1	1	1	1	1	1	0.5	1	2	1	1	1	1
Triclosan	1	1	1	1	1	1	1	1	2	1	1	1	1
Rhodamine	1	1	0.5	1	2	1	0.5	1	1	1	1	1	1
Malachite	1	1	1	1	2	1	1	1	2	2	1	2	1
Acriflavine	1	1	1	1	2	1	0.5	1	1	1	1	1	1

Chapter 3.

Phenolic glycolipid is not sufficient to increase virulence in *M. tuberculosis.*

Introduction

M. tuberculosis devotes a large portion of its genome to polyketide synthases (PKS) that produce various lipids. Although they have comparable genome sizes, *M. tuberculosis* encodes 250 PKSs compared to the 50 found in *E. coli* (Cole et al. 1998). Many of these lipids such as mycolic acids or phthiocerol dimycolate (PDIM) are essential for viability or virulence (Tahlan et al. 2012; Goren, Brokl, and Schaefer 1974a). Differences in virulence of *M. tuberculosis* strains can be accounted for by the ability to produce these virulence lipids.

Our initial work on the lipid transporter MmpL4 suggested that it may be required for the induced production of phenolic glycolipid (PGL) in the Erdman strain of *M. tuberculosis*. Unfortunately, further experiments performed negated this possibility. In the course of these experiments, I generated several tools that allowed further study of PGL itself and decided to address the question of whether PGL is an important virulence factor.

PGL is structurally and synthetically related to PDIM. While PDIM has a phthiocerol backbone synthesized by PpsA-E, PGL has a phenolphthiocerol backbone produced by Pks15/1. In the *w*-Beijing strain of *M. tuberculosis*, PGL has been shown to be required for the hypervirulent nature of the strain (Reed et al. 2004). Our initial data suggested that the Erdman strain of *M. tuberculosis* produces PGL in certain conditions. The was interesting because this strain should be incapable of synthesizing PGL because of a frameshift mutation in *Pks15/1* (Fig 3.1). This raised the question of whether *Pks15/1* in Erdman is functional despite the frameshift mutation and whether PGL

production in this strain is required for virulence.

Results

To verify the nature of *Pks15/1* in the Erdman strain, we amplified the frame shift region by PCR and sequenced the resulting amplicon. We confirmed that the Erdman strain contains a 7 base pair deletion found in Euro-American strains of *M. tuberculosis*. We later determined that the initial data suggesting that the Erdman strain produces PGL was incorrect. We continued to study the role of PGL in virulence in the Erdman strain. We replaced *Pks15/1* with a hygromycin resistance cassette via specialized phage transduction. This strain was complemented with *Pks15/1* cloned under its native promoter into pMV306.kan, an integrating plasmid. A separate plasmid was created which repaired the seven base pair deletion by site directed mutagenesis. This plasmid was transformed into *Δpks15/1* to generate an "intact" strain.

To determine whether PGL was being produced, lipids were extracted and separated by TLC. Glycolipids were visualized by spraying plates with 0.2% anthrone in undiluted sulfuric acid and charred at 140ºC. Glycolipids are stained blue color due to a reaction between carbohydrates and anthrone. Strains containing an intact version of PGL produce PGL (Fig 3.2). To confirm that the blue spots are PGL, two radioactive labeling experiments strains were performed. Strains were grown in the presence of ^{14}C-propionic acid, which labels methyl-branched lipids, or ^{14}C-p-hydroxybenzoic acid, which is incorporated into the phenophthiocerol of PGL. Lipids extracted from these samples contain PGL which migrates in the middle of the TLC plate (Fig 3.3). PDIM,

which runs at the top of the plate, is labeled by propionic acid but not p-hydroxybenzoic acid.

Lastly, unlabeled lipids were extracted, and partially purified by cobalt precipitation of Tween-80. Tween-80 must be removed from samples that are analyzed by mass spectrometry (MS) because Tween-80 ionizes very well and swamps out most other lipids during MS analysis. These lipids were analyzed by Fourier-Transform Ion Cyclotron Resonance MS (Fig. 3.4). The top spectra is derived from ECM13 lipids that were not cobalt precipitated. This spectra shows Tween-80 contamination. The second spectra shows the lipid profile after precipitation. The presence of masses close to PGL is seen in the 1850 m/z range. To confirm that the blue spots identified by TLC are responsible for the ions in this mass range, lipids were purified from that region of the TLC plate. The third spectra shows that these purified lipids run in the expected mass range for PGL. The last row is a control demonstrating the purification protocol with *M. marinum* PGL. The mass of *M. marinum* PGL is lower than *M. tuberculosis* PGL because it has two fewer glycosyl groups. These experiments indicate that complementation with an intact copy of *Pks15/1* confers the ability to produce PGL.

To determine whether the ability to produce PGL confers increased virulence, we infected mice and macrophages to compare the virulence of the various strains generated. In a BALB/c mouse aerosol infection, no differences were observed in CFUs in the lungs livers, and spleen (Fig 3.5 and 3.6). Additionally, complementation with either version of *Pks15/1* did not alter survival times of mice (Fig 3.7).

Discussion

This data is contradictory to the original study implicating PGL in virulence in *M. tuberculosis* virulence (Reed et al. 2004). Subsequently, another group also transformed an intact copy of *Pks15/1* into the non-PGL producing H37Rv strain to make it competent for PGL production (Sinsimer et al. 2008). They found no increase in virulence in the PGL-producing H37Rv strain. Additionally, the Ramakrishnan lab deleted *Pks15/1* from the M strain of *M. marinum,* which normally synthesizes PGL. This PGL-deficient strain, designated KT21 did not have a virulence defect in a zebrafish infection model. If anything, the mutant was hypervirulent compared to the wild type strain (Chris Cosma, personal communication).

There are several possible explanations. First, there may be compensatory mutations in Euro-American strains of *M. tuberculosis* which offset the lack of PGL production in these strains. Second, PGL may not be important for virulence. While the original study complemented the HN878 *Δpks15/1* strain and showed this restored PGL production, they did not test the complemented strain in infection models. This leaves the possibility that the virulence defect is due to a secondary mutation outside of *Pks15/1*.

Lastly, PGL may be required but not sufficient for the increased virulence observed with *w*-Beijing strains such as HN878. For instance, PDIM and ESX secretion are both required for virulence. Mutants in either pathway phenocopy each other (Sridharan Raghavan and Paolo Manzanillo, unpublished results). Work from the Cox lab has shown that the reason for this is both PDIM and ESX secretion are required to

permeabilize the phagosomal membrane (Paolo Manzanillo and Eric Chow unpublished results). It is not out of the question that PGL, which is structurally related to PDIM, also works in concert with another protein. Various toxins are directed to specific membranes by specific glycosyl-protein interactions. An example is the SubAB toxin which is directed by N-glycolylneuraminic acid on targeted cells (Emma Byres et al. 2008). The Erdman strain may not produce this protein co-factor to act in concert with PGL.

Figure 3.1.

Erdman strain of *M. tuberculosis* contains a 7 base pair deletion in *Pks15/1*. The sequence of the ketosynthase domain or *Pks15* is shown compared to the sequence from a *w*-Beijing strain sequence. This mutation results in a premature stop codon after the ketosynthase domain and prevents the Erdman strain from synthesizing PGL.

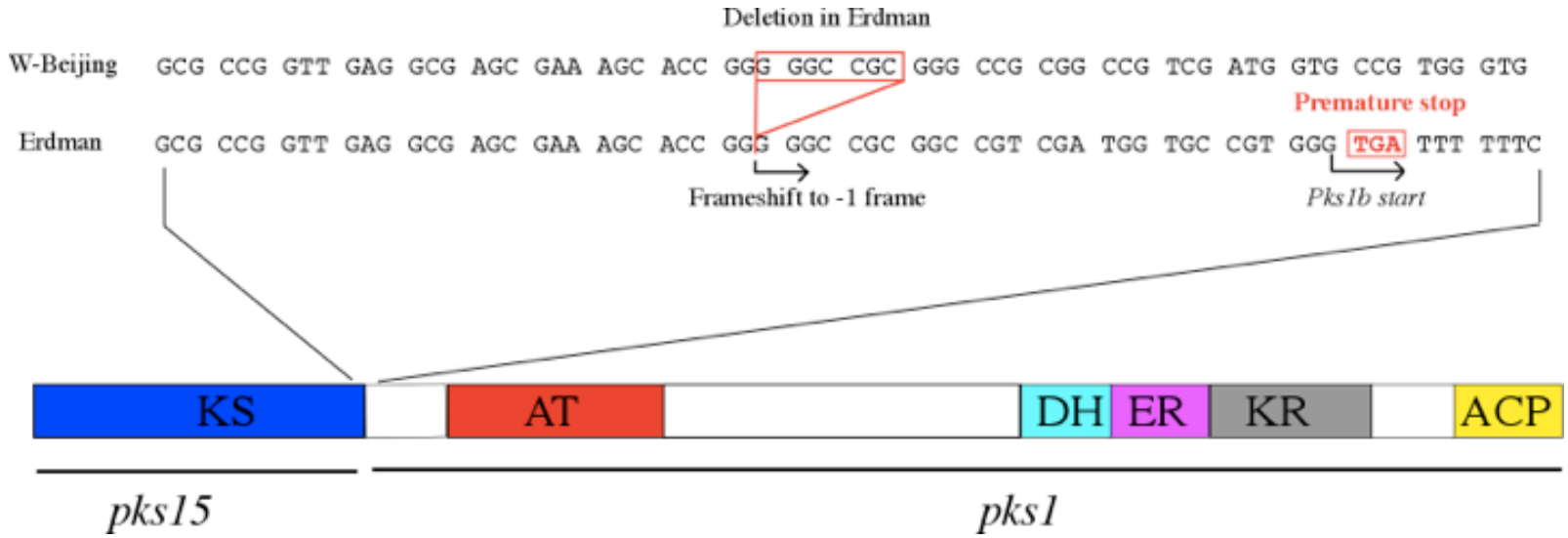

Deletion in Erdman
W-Beijing
GCG CCG GTT GAG GCG AGC GAA AGC ACC GGG GGC CGC GGG CCG CGG CCG TCG ATG GTG CCG TGG GTG
Premature stop
Erdman
GCG CCG GTT GAG GCG AGC GAA AGC ACC GGG GGC CGC GGC CGT CGA TGG TGC CGT GGG TGA TTT TTTC
Frameshift to -1 frame
Pks1b start
KS
AT
DH
ER
KR
ACP
pks15
pks1

Figure 3.2.

Introduction of intact *Pks15/1* confers the ability to synthesize PGL in the Erdman strain of *M. tuberculosis*. Erdman and *Δpks15/1* strains were transformed with plasmids encoding intact *Pks15/1* or *Pks15/1*-KS (H351A catalytic mutations in the KS domain). Total lipids were extracted and separated on silica HPTLC plates in 95:5 $CHCl_3$:MeOH, sprayed with 0.2% anthrone in H_2SO_4, and then charred at 140ºC. Glycosylated lipids react and stain blue in the presence of anthrone.

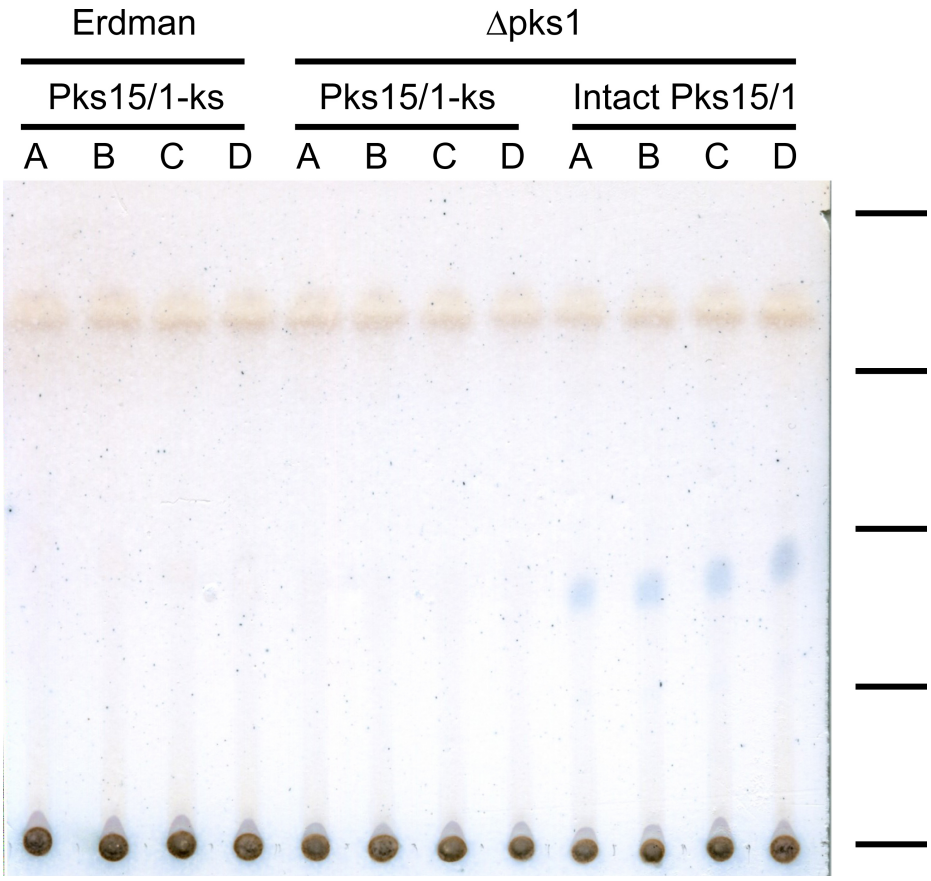
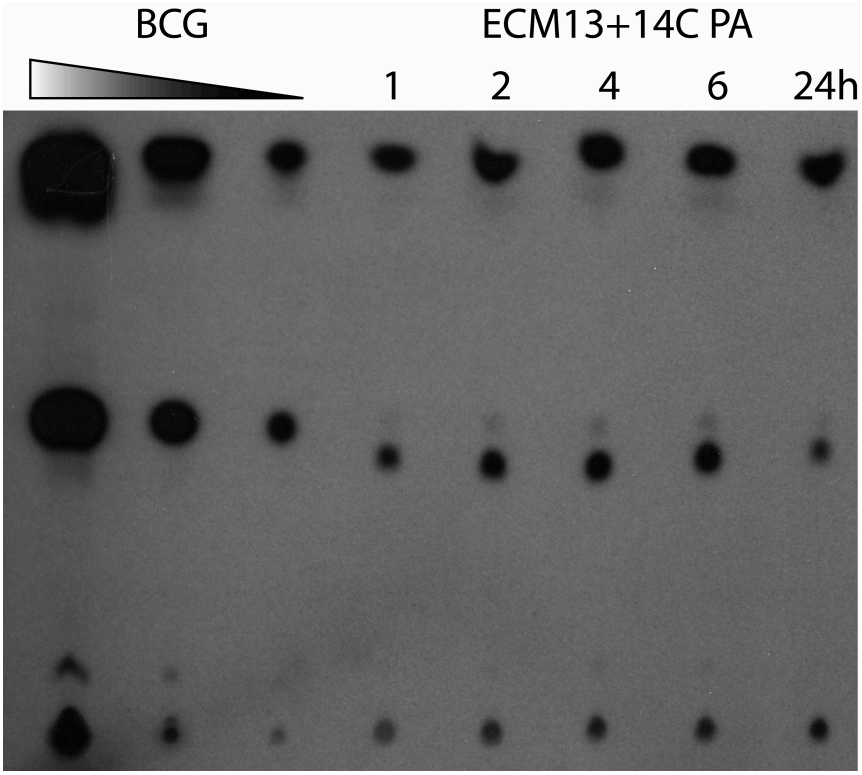

Erdman
Δpks1
Pks15/1-ks
Pks15/1-ks
Intact Pks15/1
A B C D A B C D A B C D

Figure 3.3.

Complementation with intact *Pks15/1* confers the ability of Erdman strain to synthesize PGL. ECM13, which contains an intact copy of *Pks15/1,* was incubated with ^{14}C propionic acid (PA), top, or ^{14}C-p-hydroxybenzoic acid (pHBA), bottom, for 1, 2, 4, 6, or 24 hours. Lipids were extracted and then separated by TLC. BCG samples containing lipids labeled with ^{14}C-PA were included as positive controls on both plates.


BCG
ECM13+14C PA
1
2
4
6
24h

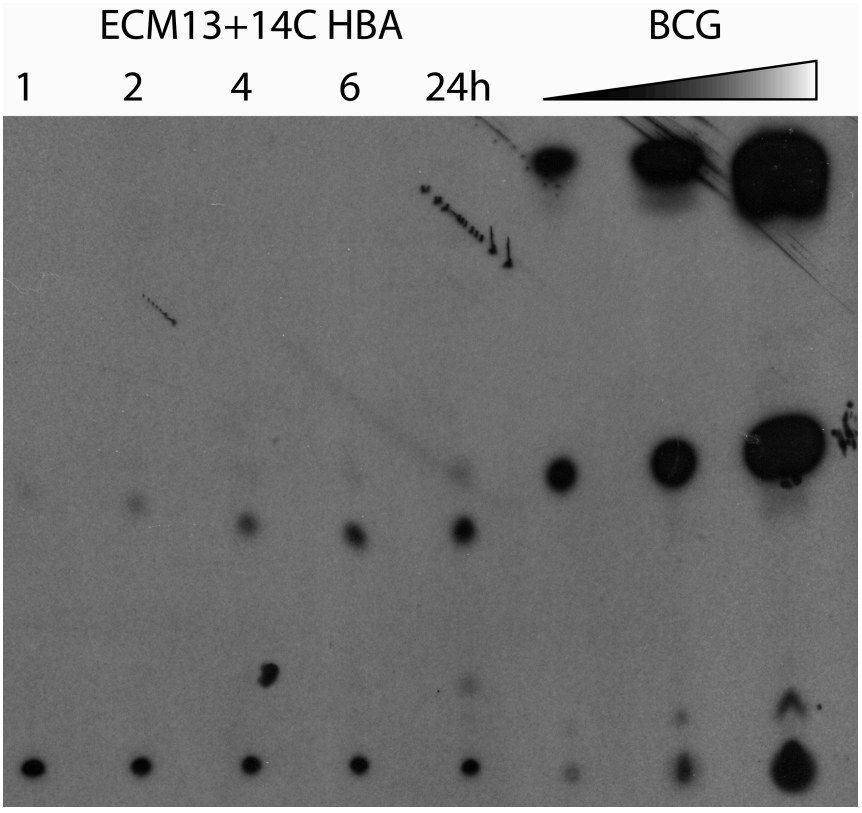

ECM13+14C HBA
BCG
1
2
4
6
24h

Figure 3.4.

Mass spectrometry confirms ECM13 produces PGL. ECM13 and purified PGL were run on an FT-ICR MS. Top, lipids from ECM13 resuspended in hexanes were back extracted against water show Tween contamination. Second row, cobalt-precipitated ECM13 lipids show PDIM and PGL as $NH4^+$ adducts. Third row, ECM13 lipids extracted from the PGL-region on TLC show the correct mass for PGL. Last row, PGL purified from *M. marinum* shows the mass of mono-glycosyl PGL.

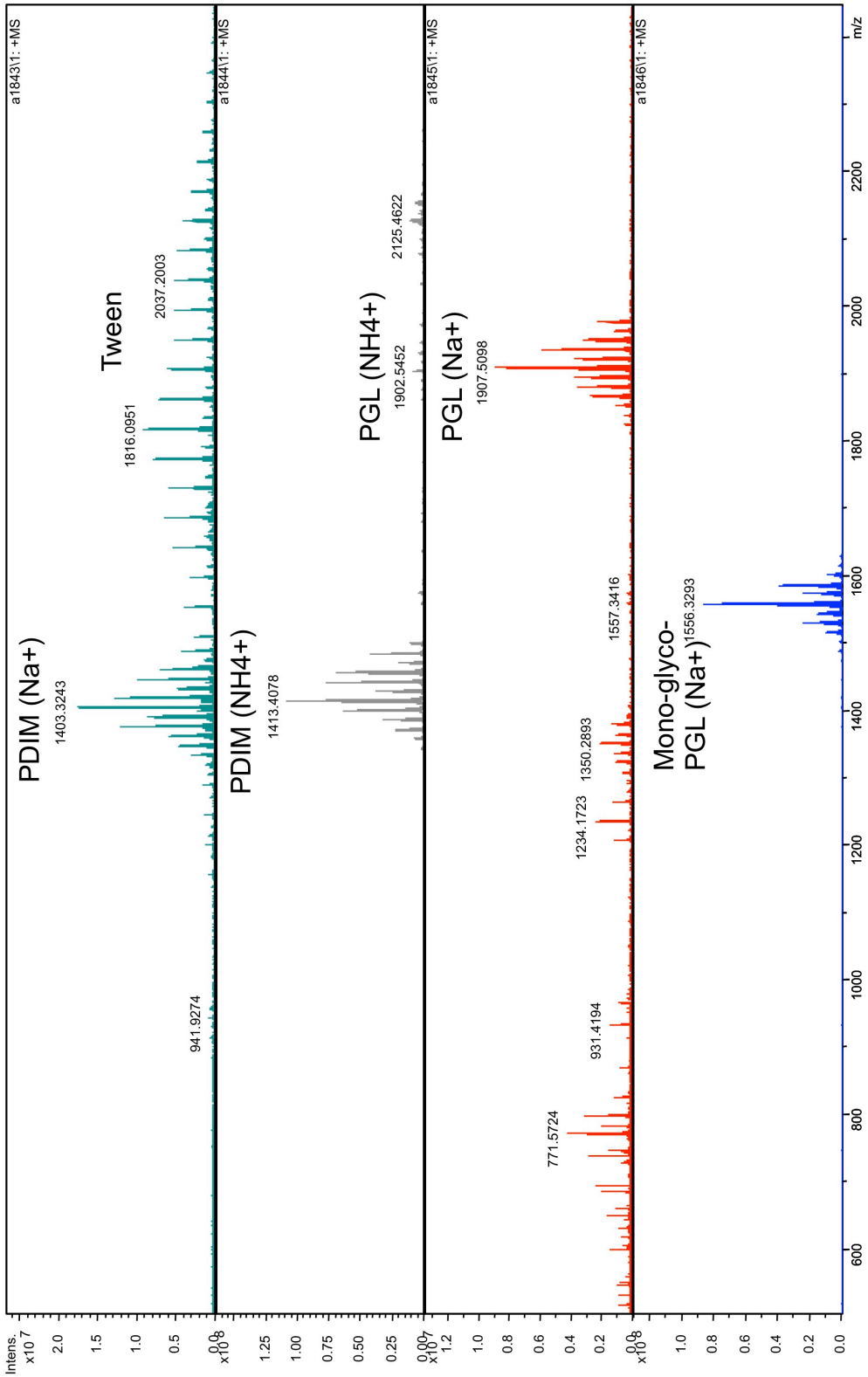

a18431\1: +MS
PDIM (Na+)
1403.3243
941.9274
Tween
1816.0951
2037.2003
a18441\1: +MS
PDIM (NH4+)
1413.4078
PGL (NH4+)
1902.5452
2125.4622
a18451\1: +MS
PGL (Na+)
1907.5098
771.5724
931.4194
1234.1723
1350.2893
1557.3416
a18461\1: +MS
Mono-glyco-
PGL (Na+)
1556.3293
Intens.
m/z

Figure 3.5.

PGL production does not affect lung CFUs. Balb/C mice were infected via aerosol with approximately 250 CFU/mouse. CFUs were enumerated from homogenized lungs of infected mice at time points indicated.

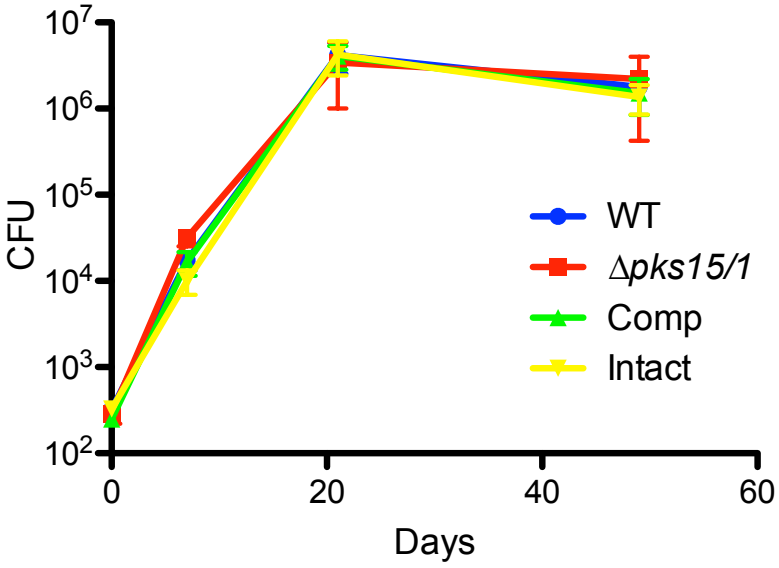
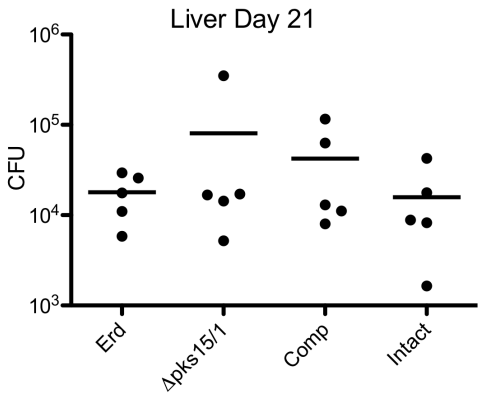
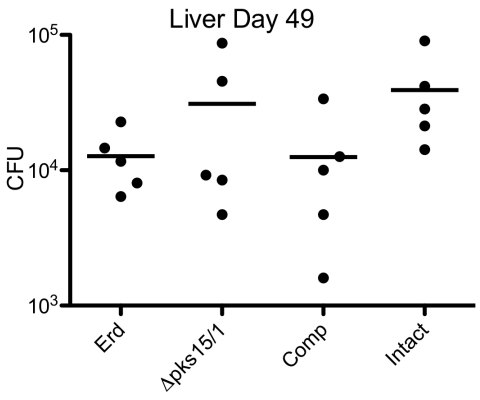
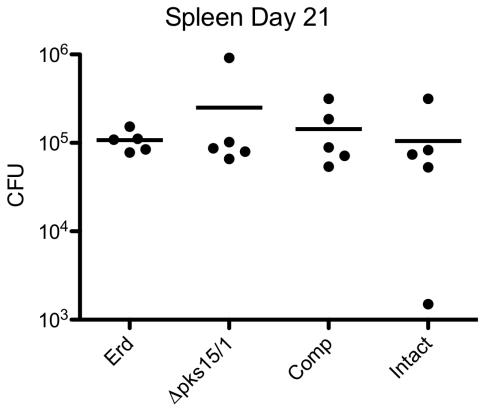

10^7
10^6
10^5
10^4
10^3
10^2
CFU
0
20
40
60
Days
WT
Δpks15/1
Comp
Intact

Figure 3.6.

PGL production does not affect Liver and Spleen CFU. Balb/C mice were infected via aerosol with approximately 250 CFU/mouse. CFUs were enumerated from the livers and spleen of infected mice 21 and 49 days post infection.


Liver Day 21
CFU
10^6
10^5
10^4
10^3
Erd
Δpks15/1
Comp
Intact


Liver Day 49
CFU
10^5
10^4
10^3
Erd
Δpks15/1
Comp
Intact


Spleen Day 21
CFU
10^6
10^5
10^4
10^3
Erd
Δpks15/1
Comp
Intact

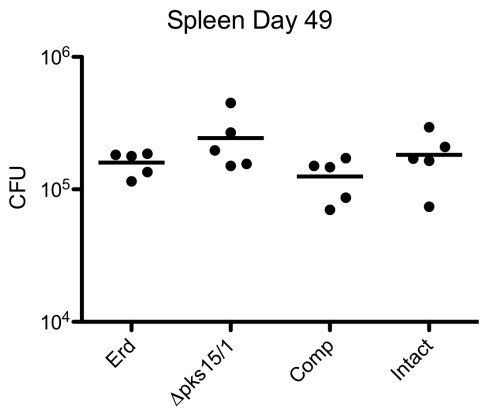
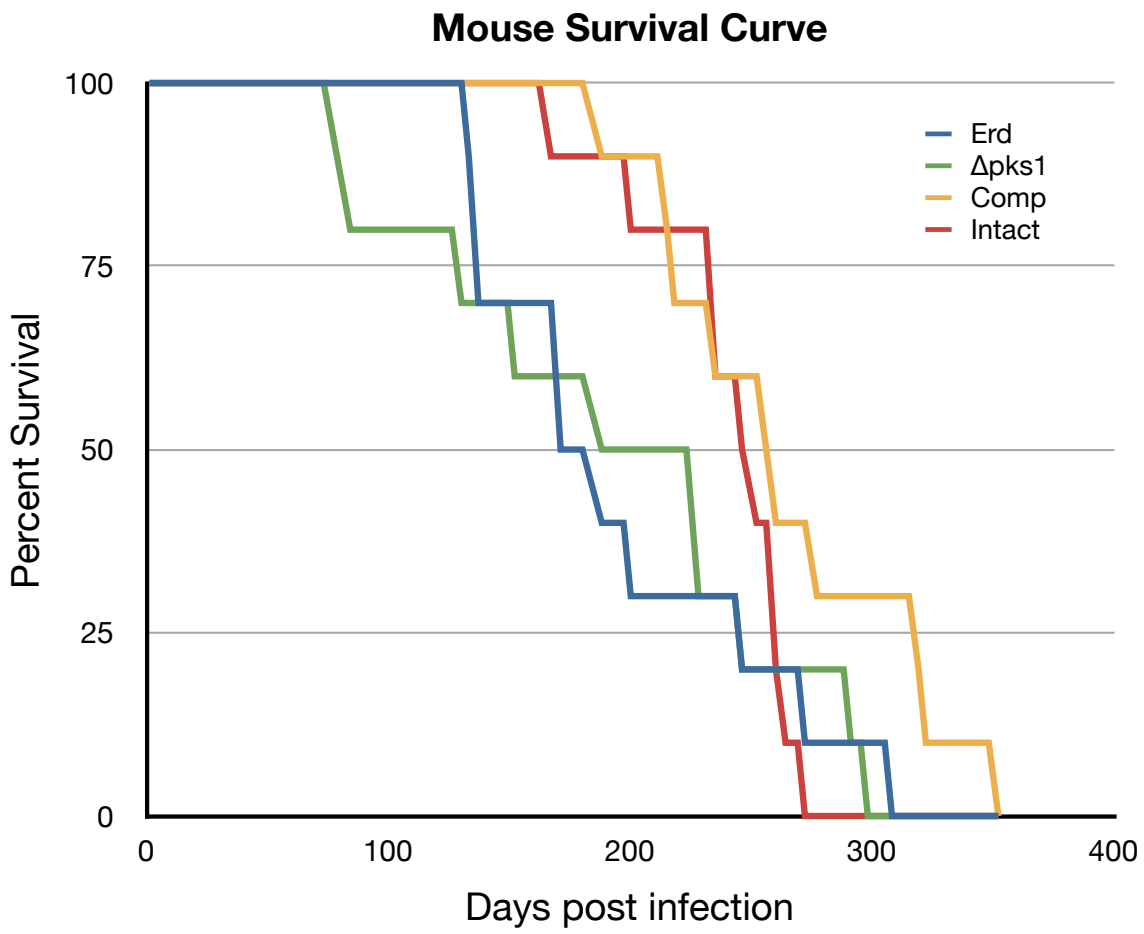

Spleen Day 49
CFU
10^6
10^5
10^4
Erd
Δpks15/1
Comp
Intact

Figure 3.7.

PGL production does not increase virulence in Balb/C mice. Balb/C mice were infected via aerosol with approximately 250 CFU of wild type Erdman, *Δpks15/1*, or *Δpks15/1* complemented with wild type or intact *Pks15/1* under the control of the native promoter. Endpoints were determined by loss of weight.


Mouse Survival Curve
100
75
50
25
0
Percent Survival
0
100
200
300
400
Days post infection
Erd
Δpks1
Comp
Intact

Chapter 4.

Conclusions and perspectives.

We have identified a new role in virulence for the *M. tuberculosis* transporter MmpL4. The *mmpL4*⁻ mutant is of great interest due to its severe virulence phenotype, which depends on the presence of host interferon-γ, a cytokine critical for the control of mycobacterial infections (Kamijo et al. 1993). Identification of *M. tuberculosis* pathways to combat this host response would improve our understanding of this important host-pathogen interaction. During the course of this work, we have discovered that in contrast to other MmpLs studied thus far, MmpL4 effluxes a wide range of substrates and at least one physiologically relevant one, 4c3. In addition to counteracting 4c3 toxicity, MmpL4 is able to protect *M. tuberculosis* from several other hydrophobic xenobiotic compounds similar to other RND-like transporters such as AcrB and MexB. This function for MmpL4 may reveal other important functions for future study such as drug resistance or the secretion of biologically active metabolites by *M. tuberculosis*.

While 4c3 was the only physiologically relevant substrate identified, the fact that MmpL4 has a wide variety of substrates opens up the potential of other *in vivo* substrates. Indeed, several papers have shown that when *M. tuberculosis* is grown in media containing cholesterol, it secretes catecholic cholesterol metabolites, androst-4-ene-3,17-dione (AD), and androsta-1,4diene-3,17-dione (ADD) (Yam et al. 2009; Nesbitt et al. 2010). It will be interesting to see if MmpL4 is required for the secretion of these chemicals since they are structurally similar to 4c3. Additionally, activated macrophages may produce other antimicrobial compounds that MmpL4 can detoxify through its efflux activity.

The secretion of AD and ADD is interesting because these cholesterol metabolites are steroid hormones and could have effects on the host. Indeed, oxysterols - cholesterol derivatives - are important signaling molecules that have roles in immune system regulation (Liu et al. 2011; Hannedouche et al. 2011), as well as *M. tuberculosis* pathogenesis (P. Manzanillo, unpublished results). Future experiments will include testing cell wall and culture supernatant extracts from *M. tuberculosis* grown in cholesterol in various bioassays, including immune cell migration and cytokine production. If these *M. tuberculosis* extracts contain activity important for virulence, identification of the efflux pumps (if MmpL4 is not responsible) should reveal other proteins important for virulence.

As this work and others (Domenech, Reed, and Barry 2005) have shown, the other *mmpL* mutants do not appear to be anymore sensitive to compounds even though they all share strong homology with MmpL4. Interestingly, in the environmental pathogen *M. marinum*, there has been a large expansion in the number of *MmpL4* genes (Jain, Chow, and Cox 2008). The increased exposure of *M. marinum* to environmental chemicals and a wider host range may have provided the selective pressure to expand its efflux pump repertoire to combat a wider range of chemical insults. Structural studies comparing MmpL4 with the other MmpLs may reveal why MmpL4 has a wider substrate specificity.

There are still other MmpLs with unknown substrates. These include MmpL9, 11, and 12 which have virulence defects (Fig 2.1). MmpL11 has been shown to be involved in iron acquisition but the mechanism is unknown (Tullius et al. 2011).

Lipidomic experiments have not revealed differences in the lipid composition of these mutants (M. Jain, unpublished results). One explanation is that the substrates of these MmpLs are not encountered by *M. tuberculosis in vitro*. These substrates could be lipids or toxic compounds that are simply not produced or encountered during standard culturing conditions. Studies of these uncharacterized MmpLs should start with lipidomic experiments with various types of treatments that induce the expression of these MmpLs.

The increased sensitivity of *mmpL4*$^{-}$ mutants to clofazimine and verapamil and the ability of verapamil to sensitive *M. tuberculosis* to clofazimine (Fig 2.8) are interesting from a therapeutic research aspect. Both clofazimine and verapamil are FDA-approved drugs that have been used for several decades. Clofazimine is used to treat *M. ulcerans* and *M. avium* infections and verapamil is used to treat angina and high blood pressure. Clofazimine is not a standard tuberculosis treatment due to its toxicity at therapeutic doses, but is used as a last resort for multidrug resistant tuberculosis infections. This research raises the possibility of using lower concentrations of clofazimine if it is administered concurrently with verapamil. Similar experiments have shown positive results in mice with rifampin (Louw et al. 2011). The addition of efflux pump inhibitors to *M. tuberculosis* drug regimens could decrease both the time and dose of treatments as well as directly decrease the virulence of the bacterium.

Chapter 5.

Materials and Methods.

Culture growth. Cultures were grown in 7H9 Media supplemented with 10% OADC, 0.05% Tween-80, and 0.5% glycerol. Cultures were harvested at 0.6-0.8 OD600 and synced several days before.

Mouse infection. Mouse infections were described previously (Ohol et al. 2010). Briefly, strains were grown in 7H9 media supplemented with 10% OADC, 0.5% glycerol, and 0.05% Tween-80. Cultures were synced and harvested in log phase by centrifugation. Pellets were washed twice in PBS, resuspended in PBS, and sonicated in a cup sonicator for 15 seconds at 90% power. The innocula was loaded into a nebulizer. Mice were exposed for a duration of time that resulted in roughly 250 CFU per mouse. CFUs were enumerated from the lung, spleen, and liver at times indicated. In the time to death experiments, mice were sacrificed when they lost 15% of their maximum body weight.

Macrophage CFU infections. First, check to make sure CO_2 tanks in the closet next to the BSL3 anteroom entrance are full. If not order more and hook them up to the system. A day or two before the infection, plate macrophages in 12-well dishes at $2x10^5$ cells/well one day before infection and infected at an MOI of 1. To prepare the innocula, 13ml of each strain was spun at 500 RPM for 5 minutes to remove clumps. 11.5ml were transferred to a new 15 ml conical, being careful to avoid the pellet. These cells were then pelleted at 3000 rpm for 5 minutes and washed twice with 10ml PBS without Ca^{2+}/Mg^{2+}. After the second wash, the pellet was resuspended in 6ml and then sonicated twice for 7 seconds at 90% with a Branson sonicator. The tubes were inverted between each sonication. The appropriate amount of bacteria was diluted in 10% horse

serum in DMEM to get $2x10^5$ bacteria/600ul and applied to macrophages. Horse serum is used because its complement is not sensitive to heat inactivation. The plates were spun down for 10 minutes at 1000 RPM. Innocula was aspirated and two washes with warm PBS + Ca^{2+}/Mg^{2+} were performed. 2ml of warm BMMO media was added to each well.

To enumerate CFUs, wells were aspirated and lysed in triplicate. 500ul of 0.5% TritonX-100 in PBS was added to wells. 250ul of the lysed cells were added to 1.75ml of PBS + 0.05% Tween-80. Dilutions were performed and 100ul of each dilution was plated on 7H10+10% OADC + cyclohexamide (100ug/ml). Colonies were counted 3 weeks later.

Macrophage infections with *M. tuberculosis* expressing LuxABCDE. Erdman and *mmpL4::tn* were transformed with a plasmid (a kind gift from P. Manzanillo) containing a constitutive promoter driving the *P. luminescens* LuxABCDE operon to generate autoluminescent mycobacteria. Macrophages were plated at $5x10^4$ cells/well in white 96-well plates. Infections were performed as described earlier for normal macrophage infections, but an MOI of 4 was used. Luminescence readings were taken on a Tecan M200 plate reader before media was added back to aspirated wells.

Harvesting lipids from *M. tuberculosis*. First, for experiments that will be put on a mass spectrometer, it is critical that plastics are not used. $CHCl_3$ and other organic solvents will extract phthalates and other compounds from plastics and contaminate the samples. Use glass vials with PTFE-coated caps and use glass pipettes when using $CHCl_3$. The two exceptions are MeOH and ethyl acetate. You can use polypropylene micropipette tips (avoid serological pipettes with ethyl acetate). There are plastic-

coated glass bottles in the BSL3 containing solvents. These bottles are plastic-coated as a safety mechanism against breakage of glass in the BSL3. The bottles also have red PTFE-lined caps. To transfer more solvent to the BSL3, fill a glass bottle with solvent and cap it with a red PTFE-coated cap and bring it down to the BSL3. In a BSC, pour the solvent into the appropriate plastic-coated bottle. Sterilize the empty bottle and cap from the BSC and the BSL3 with Vesphene IIse.

To harvest lipids from *M. tuberculosis*, it is important that strains being compared are grown similarly and harvested at the same density. After centrifugation, decant the supernatant. Save it in a conical if you will be performing extractions on the supernatant (described below). Resuspend the pellet in MeOH and transfer it into a glass vial. Shake the vial to ensure the interior surfaces are covered with MeOH, ensuring the sterility of the vial. Wipe the vial out of the biosafety cabinet and again to get it out of the BSL3. Add an equal volume of $CHCl_3$ and shake the vial. Allow extractions to proceed overnight and spin down at 250 x g in a Beckman clinical centrifuge. Remove the organic layer and dry it down under a nitrogen stream and store at -20ºC. If detergents need to be removed for subsequent experiments such as mass spectrometry, a cobalt precipitation can be performed.

To extract lipids from culture supernatants, transfer 45ml of supernatant to a 50ml polypropylene conical tube. Add 6ml of ethyl acetate with a glass pipette. Shake the samples to mix and centrifuge at 3000 RPM for 5 minutes. Transfer as much of ethyl acetate layer with a P1000 into a glass vial, being careful not to disturb the interface. Add another 5ml ethyl acetate to the conical and shake, and repeat. Dispose of glass

pipettes in the sharps container. Add an equal volume of MeOH to the ethyl acetate extractions to sterilize the sample and wipe out of the BSL3. Dry down the sample under a nitrogen stream and store at -20ºC.

To harvest lipids from the cell wall of *M. tuberculosis*, resuspend the cell pellet with 3ml hexanes and transfer to a 15ml conical. Notice how the cells sink rapidly in hexanes. Sonicate for 30s at 20%. The cells should still sink rapidly. If they do not, they have likely been disrupted and the integrity of the surface extraction cannot be trusted. If the cells sink, centrifuge and transfer the hexanes layer to a glass vial, being careful to avoid the interface. Add an equal volume of MeOH to sterilize the samples and wipe the vial out of the BSL3. Dry the samples under a nitrogen stream and store at -20ºC.

Radioactive lipid labeling and extraction. Special precautions must be taken when labeling *M. tuberculosis* with radioactive compounds. Swipes must performed after use to ensure no radioactive contamination has occurred in the BSL3. To perform swipes, add one volume working concentration Vesphene IIse with one volume scintillation fluid. Swipe surfaces of the biosafety cabinet, incubator, centrifuge, and any other space used. All waste is dumped into a 1 liter roller bottle with concentrated Vesphene IIse and then wiped out of the BSL3 to be disposed of properly as radioactive waste. Currently at UCSF radioactive waste takes precedence over biological waste. Only use micropipettes when manipulating radioactive material because they fit inside the waste roller bottle.

Synced log-phase cultures were transferred to 15ml conical tubes containing ^{14}C-propionic acid or p-hydroxybenzoic acid. After one day of labeling, pellets were

collected. If larger volumes for time-course or pulse-chase experiments are needed, 50ml conical tubes can be used. The conicals are placed in secondary containment and then placed in the incubator. For endpoint analysis, overnight to 24 hours of labeling is sufficient. Cells are collected by centrifugation. Pellets are resuspended with MeOH, transferred to glass vials, and wiped out of the BSL3. Lipids isolated from the supernatant and cell wall are performed as described earlier.

Thin layer chromatography analysis of lipids. Lipids were resuspended in $CHCl_3$ or petroleum ether and spotted on to silica HPTLC plates and developed in 95:5 $CHCl_3$:MeOH to detect PGL or 98:2 petroleum ether:acetone to resolve PDIM. After running the plates and drying, plates were sprayed with H_2SO_4 or H_2SO_4 + 0.2% anthrone and heated at 140ºC in a Fisher Isotemp oven. Anthrone staining causes glycolipids to turn blue. It is not required when detecting unglycosylated lipids.

To detect radioactive lipid TLCs, clear a phosphorimager plate by illuminating with intense white light for 10 minutes. After running the TLC in the appropriate solvent system, lay a sheet of thin Mylar over the TLC plate and then place the phosphorimager plate on top. The mylar protects the phosphorimager plate from being contaminated with radioactive material.

Cobalt precipitation of detergents. Tween and tyloxapol are used to grow *M. tuberculosis* in suspension. These detergents are extracted along with lipids and can cause problems with downstream analysis. To remove these detergents, a cobalt precipitation can be employed. The dried sample is resuspended in 4 ml hexanes, 3 ml MeOH, and 1ml cobalt precipitation solution (3 g cobalt nitrate, 20 g ammonium

thiocyanate, 100ml Milli-Q water) (Shen, Hawari, and Kamen 2004). After mixing and centrifugation, the hexanes layer is removed and another hexanes extraction is performed and then dried down. The cobalt solution causes the detergent to partition into the water/MeOH bottom phase while lipids are found in the hexanes phase. To increase yields, the water/MeOH phase can be back extracted four more times with hexanes.

The sample is resuspended in 4 ml hexanes, 3 ml MeOH, and 1ml cobalt precipitation solution (3 g cobalt nitrate, 20 g ammonium thiocyanate, 100ml Milli-Q water) (Shen, Hawari, and Kamen 2004). After mixing and centrifugation, the hexanes layer is removed and another hexanes extraction is performed. The two fractions are dried down and resuspended in petroleum ether.

PDIM purification. To purify PDIM, large cultures were grown in 1 liter roller bottles with vented caps and kept in suspension with a stir bar and grown to stationary phase. Pellets were resuspended in MeOH and transferred into glass vials and wiped out of the BSL3. Pellets were extracted 5 times with MeOH. All spins are performed at 250xg in a Beckman tabletop clinical centrifuge. MeOH extracts a contaminating band that runs close to PDIM on TLCs without extracting PDIM. The identity of this band is unknown but appears as a purple band after spraying with H_2SO_4. Next, the pellet is extracted twice with petroleum ether. Tween or tyloxapol is removed as described earlier. The samples are resuspended in petroleum ether. A preparative TLC plate is scored and the extract is loaded onto the plate. The plate is developed in 98:2 petroleum ether:acetone. The scored portion of the plate is broken off and sprayed

with H_2SO_4 and heated at 140º C in a Fisher Isotemp oven. Areas corresponding to PDIM are scraped off into a glass vial and extracted with $CHCl_3$ and dried down.

FT-ICR MS analysis. For FT-ICR experiments, dried lipids were cleaned up with a cobalt precipitation (Shen, Hawari, and Kamen 2004) to remove detergents and then resuspended in 2:1 $CHCl_3$:MeOH. Samples were run in both positive and negative ion mode on a Bruker Daltonics ApexII FT-ICR mass spectrometer as described previously (Jain et al. 2007).

Minimum inhibitory concentration experiments. MICs were performed by serially diluting compounds in 96-well plates and adding 200ul of culture at an OD_{600} of 0.005. Plates were incubated at 37ºC and wells were scored for growth after 6 days. Compounds were diluted in water or DMSO. Concentrations of solvent did not exceed 2.5%. Minimum inhibitory concentrations were selected as the lowest concentration that inhibited visible growth.

Plasmids. I've cloned a series of plasmids that may be of use for Cox lab members. They are based on pEC076 and pEC077. Both of these plasmids are derivative of the integrating pMV306.K plasmid. They have a MOP promoter followed by a PacI site and multiple cloning site. pEC076-based plasmids will have a slightly lower expression level (~75% of pEC077). I have also cloned several c-terminal epitope tags including 1x and 3x FLAG, HA, etc. The utility of these plasmids is the PacI site. PacI is a robust cutter that only cuts once or twice in the *M. tuberculosis* genome meaning it can be used for almost any cloning experiment from *M. tuberculosis*. The same cloning strategy can be used to clone a gene into any of these plasmids.

The LucABCDE strain that Paolo Manzanillo has been an extremely useful too as an alternative to plating for CFUs. However, there were times when competition experiments are required and would benefit from a similar system. I have cloned two click beetle luciferases from the Promega Chroma-Glo system, CBR*luc* (red-emitting) and CBG68*luc* (green-emitting) into pEC077. These plasmids are pEC170 and pEC171. CBR*luc* is not as bright as CBG68*luc*, so you expect one strain to give a lower signal, you should take this into account. An alternative use for this system is as a reporter. Cells can be transformed with a plasmid that contains the MOP promoter driving CBR*luc* and your promoter of interest driving CBG68*luc*. In this case, readouts can be normalized without having to take OD_{600} measurements, which can be difficult to do accurately in a plate format.

Table 5.1 Plasmids and Strains

Strain or Plasmid	Genotype/Description	Source/ref.
M. tuberculosis		
Erdman	wild-type	W. R. Jacobs, Jr.
JCM80	Erdman *mmpL4::tnHygR*	
JCM84	JCM80 + pJSC282	
JCM118	*pks15/1::HygR*	This study
phJSC706	Erdman *ΔmmpL4*	This study
phJSC707	Erdman *Δpks15/1*	This study
ECM11	JCM118 + pEC11	This study
ECM13	JCM118 + pEC31	This study
ECM129	phJSC706 + pJSC282	This study
ECM150	JCM80 + pLUC plasmid (from P. Manzanillo)	This study
E. coli		
BEC001	AG100+pSPORTI	This study
BEC002	AG100+pAcrB	This study
BEC003	AG100+pEC173	This study
BEC004	AG100+pEC174	This study
BEC005	AG100A(ΔacrAB)+pSPORTI	This study
BEC006	AG100A+pAcrB	This study
BEC007	AG100A+pEC173	This study
BEC008	AG100A+pEC174	This study
Plasmids		
pMV306.Kan	Integrating shuttle vector	W. R. Jacobs, Jr.
pJSC604	Erdman *PKS15/*1 genomic region cloned into EcoRV ClaI of pMV306.kan	This study
pEC11	The rest of the operon from pJSC604 was removed	This study
pEC27	*Pks15* KS region amplified with oligos 146/147 Topo-cloned into pCR2.1	This study
pEC28	7bp deletion introduced by SDM into pEC27	This study
pEC31	Intact *Pks15/1* created by sub-cloning pEC28 insert into pEC11	This study
pEC41	*M. marinum MmpSL4* knockout construct cloned into pJSC232	This study
pEC48	*M. marinum Pks1* knockout construct cloned into pJSC232	This study
pEC49	mCherry from pROW1 cloned behind *MmpS4* promoter in HindIII-NcoI of pMV306.K	This study
pEC68	MOP→*MmpS4* in pMV306.K Xba/HindIII	This study
pEC69	pEC68 without stop codon	This study
pEC70	pEC69+eGFP HpaI/ClaI	This study
pEC71	MOP→mCherry Xba/ClaI pMV306.K	This study
pEC72	pEC71 without stop codon	This study
pEC73	pEC69+mChery ClaI/HpaI	This study
pEC74	MOP→FLN ESAT6 306.K Xba/HindIII	This study
pEC75	MOP→FLN CFP10 306.K Xba/HindIII	This study
pEC76	MOP-SD-PacI-NcoI→306.K Xba/NcoI	This study
pEC77	MOP-SD1-PacI-NcoI→306.K Xba/NcoI	This study
pEC78	pEC76+eGFP PacI/ClaI	This study
pEC79	pEC76+mChery PacI/ClaI	This study
pEC80	pEC77+eGFP PacI/ClaI	This study
pEC81	pEC77+mCherry PacI/ClaI	This study

pEC84	Domain 1 MmpL4→pEC76 PacI/ClaI	This study
pEC85	pEC84 without stop codon	This study
pEC87	Domain 2 MmpL4 without a stop codon →pEC76 PacI/ClaI	This study
pEC92	pEC76 + C-term HA after HpaI	This study
pEC97	pEC77+C-terminal Myc after HpaI	This study
pEC98	pUC19 + PacI-MmpS4-NS-HindIII into XbaI/HindIII	This study
pEC99	pUC19 + PacI-MmpL4-NS-HindIII into XbaI/HindIII	This study
pEC100	pEC76 + C-terminal FLAG after HpaI	This study
pEC101	pEC100 3xFLAG	This study
pEC102	pEC77 + C-terminal FLAG after HpaI	This study
pEC104	pUC19 + PacI-MmpSL4-NS-HindIII into XbaI/HindIII	This study
pEC105	pUC19+ PacI before SmaI	This study
pEC106	MmpL4 D1 → pUC19 XbaI-PacI-D1-HindIII	This study
pEC107	MmpL4 D2 "	This study
pEC108	MmpL7 D2 "	This study
pEC109	MmpS2 → pEC92 PacI/HindIII (HA)	This study
pEC110	MmpL4→pEC92 PacI/HindIII (HA)	This study
pEC113	MmpS4→pEC97 Pac/HindIII (Myc)	This study
pEC115	MmpSL4→ pEC97 Pac/HindIII (Myc)	This study
pEC116	MmpL4-D4→ pEC97 Pac/HindIII (Myc)	This study
pEC117	MmpS4→pEC102 PacI/HindIII (FLAG)	This study
pEC118	MmpL4→pEC102 PacI/HindIII (FLAG)	This study
pEC119	MmpL4→pEC102 PacI/HindIII (FLAG)	This study
pEC121	SacB→306.Hyg AvrII/BstBI	This study
pEC122	*M. marinum* MmpSL4 knockout construct into BamHI pEC121	This study
pEC123	*M. marinum* Pks1 knockout construct into BstBI pEC121	This study
pEC124	MOP→ from pEC77 into SacII pMV261.K	This study
pEC125	MOP→PacI from pEC76 into SacII pMV261.K	This study
pEC139	MmpL4-D1→pEC102 PacI/HindIII (FLAG)	This study
pEC140	MmpL4-D2→pEC102 PacI/HindIII (FLAG)	This study
pEC141	MmpL7-D2→pEC102 PacI/HindIII (FLAG)	This study
pEC142	PacI-MmpL4-STOP → pUC19 XbaI/HindIII	This study
pEC143	PacI-MmpSL4-STOP→pUC19 XbaI/HindIII	This study
pEC144	MmpL4→pEC77 PacI/HindIII	This study
pEC146	MmpL4-D1→pLIC-HMK	This study
pEC147	MmpL4-D2→pLIC-HMK	This study
pEC148	MmpSL4+STOP → pEC77 PacI/HindIII	This study
pEC149	MmpSL4+STOP → pEC124 PacI/HindIII	This study
pEC153	SodA→pEC077 PacI/ClaI	This study
pEC154	SodA→pEC124 PacI/ClaI	This study
pEC155	SodC→pEC077 PacI/ClaI	This study
pEC156	SodC→pEC124 PacI/ClaI	This study
pEC157	MmpL4-D1 →SLIC MOP C-tag 6xHis 3xFLAG	This study
pEC158	MmpL4-D2 →SLIC MOP C-tag 6xHis 3xFLAG	This study
pEC159	MmpL7-D2 →SLIC MOP C-tag 6xHis 3xFLAG	This study
pEC169	HyPer GFP→pEC77 PacI/ClaI	This study
pEC170	CBRLuc→pEC77 PacI/ClaI	This study
pEC171	CBG68Luc→pEC77 PacI/ClaI	This study
pEC173	MmpSL4→pSPORTI XbaI/HindIII	This study
pEC174	MmpL4→pSPORTI XbaI/HindIII	This study
pEC175	*M. marinum* Mmar1877 KO construct →pEC121	This study
pEC176	*M. marinum* Mmar0255 KO construct →pEC121	This study

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