Mycobacterial Metabolic Syndrome: Triglyceride Accumulation Decreases Growth Rate and Virulence of Mycobacterium Tuberculosis

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Mycobacterial Metabolic Syndrome: Triglyceride accumulation decreases growth rate and virulence in *Mycobacterium tuberculosis*

A dissertation presented

By

Amanda Jezek Martinot

to

The Committe on Higher Degrees in

Biological Sciences In Public Health

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in the subject of

**Biological Sciences in Public Health**

Harvard University

Cambridge,

Massachusetts

September 2014
Mycobacterial Metabolic Syndrome: Triglyceride accumulation decreases growth rate and virulence in *Mycobacterium tuberculosis*

Abstract

*Mycobacterium tuberculosis* (*Mtb*) mutants lacking the operon *Rv1411c-1410c* encoding a lipoprotein, *Rv1411c* (LprG) and a putative transporter, *Rv1410c* (Rv1410) are dramatically attenuated for growth in mice. Previous work in our lab, using the model organism *Mycobacterium smegmatis*, suggested that this operon regulated the lipid content of the cell wall. Work in other laboratories characterized LprG as a lipid-binding lipoprotein leading us to hypothesize that these bacteria grew poorly due to loss of a key lipid important in the host-pathogen interaction. Based on structural and biochemical studies we hypothesized that this attenuation was due to a lipid transport defect. Using whole cell lipidomic analysis, we found changes in LprG-1410 mutants including accumulation of triacylglyceride (TAG) species in the absence of the transport system. We have identified TAG in outer membrane fractions and supernatants of *Mtb*, have demonstrated the ability of LprG to transport TAG in an *in vitro* vesicle transfer assay, and have co-crystallized LprG with TAG. Moreover, accumulation of intracellular TAG substantially decreases growth under carbon stress *in vitro* and *in vivo* in the mouse model. Our results suggest a far different model – that TAG is ordinarily transported out of the cell and, in the absence of a transporter, limits cell proliferation independent of the host immune response. This suggests that TAG is a key metabolic regulator of cellular growth within the host.
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<tr>
<td>Ac$_2$PIM$_3$</td>
<td>triacylated phosphoinositol mannoside</td>
</tr>
<tr>
<td>Ac$_2$SGL</td>
<td>diacylsulfolipid</td>
</tr>
<tr>
<td>Ac$_3$SGL</td>
<td>triacylated sulfolipid</td>
</tr>
<tr>
<td>Ac$_4$SGL</td>
<td>tetraacylated sulfolipid (a.k.a SL-1)</td>
</tr>
<tr>
<td>C16 PGL</td>
<td>monoglycosyl palmityl phenolphthiotriol dimycocerosates</td>
</tr>
<tr>
<td>CL</td>
<td>Cardiolipin</td>
</tr>
<tr>
<td>CM</td>
<td>carboxymycobactin</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DAT</td>
<td>diacyltrehalose</td>
</tr>
<tr>
<td>DDCM</td>
<td>dideoxycarboxymycobactin</td>
</tr>
<tr>
<td>DDMB</td>
<td>dideoxymycobactin</td>
</tr>
<tr>
<td>FA</td>
<td>fatty acid</td>
</tr>
<tr>
<td>GMM</td>
<td>glucose monomycolate</td>
</tr>
<tr>
<td>GPL</td>
<td>Glycopeptidolipid</td>
</tr>
<tr>
<td>GroMM</td>
<td>Glycerolmonomycolate</td>
</tr>
<tr>
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<tr>
<td>LPE</td>
<td>lysophosphatidylethanolamine</td>
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<tr>
<td>MA</td>
<td>mycolic acid</td>
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<tr>
<td>MB</td>
<td>mycobactin</td>
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<tr>
<td>MDCM</td>
<td>monodeoxycarboxymycobactin</td>
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<td>Abbreviation</td>
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<tr>
<td>MDMB</td>
<td>monodeoxymycobactin</td>
</tr>
<tr>
<td>Mono PGL</td>
<td>monoglycosyl phenylphthiodiolone dimycocerosates</td>
</tr>
<tr>
<td>PA</td>
<td>phosphatidic acid</td>
</tr>
<tr>
<td>PDIM</td>
<td>phthiocerol dimycocerosates</td>
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<tr>
<td>PE</td>
<td>phosphatidylethanolamine</td>
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<tr>
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<td>phosphoinositol mannoside</td>
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<tr>
<td>PM</td>
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<tr>
<td>TAG</td>
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<tr>
<td>TAT</td>
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<tr>
<td>TMM</td>
<td>trehalose monomycolate</td>
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Acknowledgements

First and foremost, I want to thank my husband, Dr. Theodore Martinot, for sacrificing his own academic aspirations so that I could advance my own. He has been, and will always be the center, “the rock,” that keeps me grounded yet continually lifts me up, supporting me in every endeavor I have ever undertaken. I want to thank my father, Dr. Thomas L. Jezek, for opening the world of veterinary medicine up to me and teaching me that with a veterinary degree, I could truly “do anything”. I will always be indebted to Dr. Louis J. Guillette, Jr. who lit my fire for research at the University of Florida during my undergraduate studies and convinced me to pursue a PhD as part of my veterinary medical training. Dr. Jorge Hernandez, at the University of Florida College of Veterinary, opened my eyes to role of veterinarians in global public health and was instrumental in my pursuing a Master’s in Public Health at the University of North Carolina at Chapel Hill where I first became involved in research on *Mycobacterium tuberculosis*. I want to thank the New England Primate Research Center for accepting me into their comparative pathology research training program and supporting me during my PhD studies. Most importantly, I want to thank Dr. Eric J Rubin for being the best mentor possible and for showing me that success in science is just as much about the people you work with and surround your self with, as the science itself. To past and present members of the Rubin lab—I am forever indebted for your advice, support, friendship, and most of all patience. Lastly, I want to thank my mother—for if I had not inherited her drive, passion, and most of all energy, none of this would have been possible.
For my son, Xavier Thomas Martinot—

my best result
Chapter 1

Introduction
SECTION 1.1. TB CONTINUES TO BE A MAJOR GLOBAL HEALTH THREAT. *Mycobacterium tuberculosis* (*Mtb*) is estimated to infect 2 billion people worldwide, or one-third of the world’s population. Among the 8.6 million new cases in 2012, 450,000 people developed multi-drug resistant tuberculosis (MDR-TB), with 170,000 MDR-TB deaths (WHO 2013). The earliest confirmed *Mtb* infection in people dates back 9000 years ago to the Neolithic period (Hershkovitz, Donoghue et al. 2008). The antiquity of this disease reflects the co-evolution of man with microbe. Despite this fact, there are still many aspects of TB biology that we do not understand, namely what predicts whether exposure leads to active versus latent disease. We know from epidemiologic data that only 5% of exposed individuals will go on to develop active disease. The remaining 95% will develop latent disease, which may or may not be diagnosable based on positive PPD skin tests, radiographic evidence of healed tubercles, or positive interferon-γ T lymphocyte stimulation tests (Quantiferon Gold) (Esmail, Barry et al. 2012). Among latent infected individuals, roughly 10% will go on to reactivate with reactivation often linked to immunosuppression, such as that seen in HIV infection. Given the historical success of mycobacteria as a human and animal pathogen, and its inherent resistance to antimicrobials during periods of quiescence, one might argue that mycobacterial fitness and evolutionary “success” lies, at least partly, in its ability to arrest growth, “slow down,” and effectively resist immune clearance and drug treatment. Despite this fact, most research on TB biology focuses on actively growing mycobacteria. We have optimized conditions in order to culture and study this slow-growing organism to test antibiotic susceptibility, to evaluate cytokine secretion profiles during *in vitro* infection of macrophages, and determine immunologic markers of active disease in the human population in our efforts to design an effective vaccine. We do this because it is
difficult to study non-growing, non-culturable organisms. We cannot conduct hypothesis-driven research if we have no phenotypes to test—and meaningful phenotypes require growth. The collective result of these circumstances is that we know very little about how TB slows growth and adapts to a non-growing state. The few models we have, such as the Wayne model of hypoxia, are time-consuming and generally difficult (Wayne and Lin 1982).

Despite these facts, we do know a little about certain aspects of TB growth arrest, mostly based on transcriptome data, leading to the prediction that with down-regulation TB of metabolism, TB simultaneously remolds its cell wall (Kruh, Troudt et al. 2010, Bhamidi, Scherman et al. 2011, Bacon, Alderwick et al. 2014). By studying mycobacterial lipids of the TB cell wall we have a window through which to model the life history of a mycobacterial cell, and hopefully understand important changes in its metabolism that predict a transition to latency. Furthermore, we may gain insight into the regulatory mechanisms that orchestrate these changes, potentially revealing future drug targets. It is likely that TB initiates these metabolic adaptations by sensing its environment. Understanding how TB remolds its cell wall in response to metabolic arrest may therefore provide insight into what host signals, many of which are likely related to host immune pressure, most greatly influence TB growth, growth arrest, and resuscitation from latency.

**SECTION 1.2—SYNOPSIS.** Given the interest in understanding TB virulence factors and the difficulty in studying non-replicating mycobacteria, we asked the question “what genes are required for growth in the mouse?” The results of this initial screen utilizing Himar1 transposon mutagenesis identified two genes, located in an operon, *Rv1410c-1411c* as being individually
conditionally essential for survival in the mouse (Sassetti and Rubin 2003) (Zhang, Reddy et al. 2013). \textit{Rv1411c} encodes for the mycobacterial lipoprotein LprG (Bigi, Gioffré et al. 2004, Gehring, Dobos et al. 2004), additionally identified as important for \textit{in vitro} growth in activated macrophages (Rengarajan, Bloom et al. 2005). LprG is unique among the known mycobacterial lipoproteins in that it exists in an operon with another gene, \textit{Rv1410c}, which encodes for a putative drug efflux pump (Bigi, Alito et al. 2000, Silva, Bigi et al. 2001, Bigi, Gioffré et al. 2004, Ramón-García, Martín et al. 2009, Bianco, Blanco et al. 2011, Bianco, Blanco et al. 2012, Bianco, Clark et al. 2014). This operon has been studied in \textit{Mycobacterium smegmatis}, a fast growing apathogenic mycobacteria, where it was shown that disruption of either gene in the operon results in alterations in the cell wall and the ability of the bacterium to sustain nitrosative stresses and efflux of compounds such as ethidium bromide (Farrow and Rubin 2008).

Mycobacterial lipoproteins within the Lpr family have been shown to have structural homology with proteins in the \textit{E.coli} Lol A-E system (Tanaka, Matsuyama et al. 2001, Takeda, Miyatake et al. 2003, Sander, Rezwan et al. 2004, Sutcliffe and Harrington 2004, Tokuda and Matsuyama 2004, Rezwan, Grau et al. 2007, Okuda and Tokuda 2009, Sanchez, Espinosa et al. 2009, Tschumi, Nai et al. 2009, Tsukahara, Mukaiyama et al. 2009, Kovacs-Simon, Titball et al. 2011, Seshadri, Turner et al. 2012). \textit{E.coli} Lol proteins frequently function as “flippases” moving and positioning important proteins, including other lipoproteins, in the gram-negative outer membrane (Tokuda and Matsuyama 2004). LprG also has homology to the mycobacterial lipoprotein LppX which is predicted to function in conjunction with another mycobacterial pump, \textit{mmpL7}, to localize phthiocerol dimycocerosate (PDIM), an important TB virulence lipid, in the cell envelope (Sulzenbacher, Canaan et al. 2006) (Jain and Cox 2005) (Cox, Chen et al. 2014).
Given these data, we hypothesized that LprG and 1410 were similarly functioning together to position another class of mycobacterial lipids in the cell wall (Fig 1).

Figure 1: Model of LprG-1410 function. (A) LppX crystal as published (Sulzenbacher, Canaan et al. 2006). (B) Model for mmpL7 mediated transport of PDIM through cell membrane, followed by PDIM binding to LppX for positioning on outer cell wall. (C) LprG crystal as published in Drage et al. (Drage, Tsai et al. 2010) (D) Model for Rv1410 mediated transport of triacylated lipid through cell wall, followed by binding to LprG.

A number of studies have identified cell wall associated mycobacterial lipids as key mediators of Mtb pathogenesis. For instance, lipoarabinomannan (LAM) has been predicted to mediate phagolysosome maturation arrest partly through mimicry of host cell lipids (Vergne, Chua et al. 2004, Vergne, Chua et al. 2005, Davis, Vergne et al. 2007). However, little is known about how
mycobacteria process and transport lipids to the mycobacterial outer membrane (OM), where they are known to interact with the host immune system.

The putative lipid cargo of LprG was predicted by Drage et al. to be a tri-acylated phosphatidyl-
myo-inositol mannoside (PIM$_{2Ac3}$) based on elution of acylated PIMs from purified *Mtb* LprG heterologously expressed in *Mycobacterium smegmatis*. In this study they showed that a valine to tryptophan mutation (V91W) in the binding pocket resulted in no LprG-bound PIM$_{2Ac3}$ (Drage, Tsai et al. 2010). However, the binding pocket of LprG is quite large and has been predicted to have the capacity to bind many different di- and triacylated lipids (Jim Sacchettini personal communication). PIMs represent a family of glycolipids believed to be both surface-exposed and anchored to the cytoplasmic membrane (Khoo, Dell et al. 1995). The LprG lipoprotein itself is also thought to be surface exposed due to its ability to activate the innate immune system via TLR 2 (Drage, Pecora et al. 2009). In this work we show that disruption of the LprG-1410 operon results in severe growth attenuation of LprG-1410 *Mycobacterium tuberculosis* mutants in both immune competent and immune deficient mice. We originally hypothesized that loss of *RvLprG-1410c* function resulted in significant cell wall alterations due to mislocalization or absence of a key immunomodulatory PIM. We predicted that loss of a key lipid, potentially important in the host-pathogen interaction, resulted in a significant loss of fitness for the bacterium *in vivo*. We began our studies by using an unbiased lipidomics approach to identify lipidome alterations in LprG-1410 mutants compared to WT cells in an effort to identify the physiologically important lipids that may be the binding partners for LprG. Here we report that the *Rv1410c-1411c* operon, herein referred to as LprG-1410, is involved in regulating intracellular triacylglyceride levels, likely through specificity of Rv1410 for
triglyceride transport. Function of this operon regulates intracellular triacylglyceride levels with downstream effects on global lipid flux that culminate in a dramatically reduced \textit{in vivo} growth rate. This arrested growth state, independent of host immune status, lead us to hypothesize that increased intracellular triglyceride is acting as a metabolic “brake” via negative feedback on phospholipid biosynthesis and fatty acid synthesis accounting for the severe growth defect in mice, that is independent of host immune status. This work represents a significant move forward in our understanding of cell wall biogenesis and growth rate regulation in \textit{Mtb}. Such knowledge provides important information regarding the biology of the mycobacterial cell wall, an understanding of which is crucial to the development of new antimicrobials and vaccines at a time when multidrug resistant strains are increasing in prevalence worldwide.

\textbf{SECTION 1.3 MYCOBACTERIAL LIPOPROTEINS}. Mycobacteria are unique among the Gram-positive bacteria due to the existence of a thick cell envelope that is composed of peptidoglycan, arabinogalactan, and mycolic acids. Functionally, components of the cell envelope of mycobacteria are more similar to the periplasmic space and outer membrane of Gram-negative bacteria than to the cell wall of other Gram-positive bacteria. In particular, mycobacteria have numerous lipoproteins embedded within the cell envelope analogous to the presence of lipoproteins in the outer membrane of Gram-negative bacteria, whereas most lipoproteins of non-mycobacterial Gram-positive bacteria are predominantly anchored to the plasma membrane (Rezwan, Grau et al. 2007). Cell wall associated lipoproteins have been identified as key triggers of the innate immune response to mycobacterial infection due to their known ability to bind TLRs 1,2, and 9 \textit{in vitro} (Bafica 2005, Pecora, Gehring et al. 2006, Pennini, Pai et al. 2006, Pecora 2008, Drage, Pecora et al. 2009). Mice that lack the upstream MyD88 TLR adaptor
protein succumb more rapidly to Mycobacterium tuberculosis (*Mtb*) infection (Feng, Scanga et al. 2003, Sugawara, Yamada et al. 2003, Fremond, Yeremeev et al. 2004, Fremond, Togbe et al. 2007), suggesting that TLR mediated recognition of *Mtb* is an important innate control mechanism. However, despite the highly antigenic nature of the mycobacterial cell wall, *Mtb* is able to evade host defenses, slow its replication, and persist in a state clinically referred to as “latency,” and biologically best described as “dormancy” (Bacon, Alderwick et al. 2014) (Gengenbacher and Kaufmann 2012) (Kim, Lee et al. 2008).

Recent studies have identified mycobacterial lipoproteins as potential players in lipid transport and localization to the outer membrane (OM). The role of mycobacterial lipoproteins in the pathogenesis of *Mtb* infection is presaged by studies that show that mycobacteria with mutations in lipoprotein processing enzymes such as prolipoprotein signal peptidase (LspA) are attenuated in mouse models of *Mtb* infection (Sander, Rezwan et al. 2004, Rezwan, Grau et al. 2007). Over 100 putative lipoproteins have been identified in the *Mtb* genome with predicted functions including signaling, adhesion, and cell wall metabolism. Many mycobacterial lipoproteins have no known homology with other bacteria and the function of most mycobacterial lipoproteins remains elusive (Sutcliffe and Harrington 2004). However, some mycobacterial proteins have been implicated in lipid translocalization into the mycobacterial cell wall. The best characterized of one such lipoprotein is LppX. The LppX lipoprotein is characterized by an α/β fold with a β sheet that forms a U-shaped β-half barrel with a helical twist. Most prominently, it has a large hydrophobic cavity composed of numerous small aliphatic and one phenylalanine (Phe) residue that line the pocket. The β-strand order and number of LppX most closely resembles that of the *E.coli* LolB (Jain and Cox 2005) (Sulzenbacher, Canaan et al. 2006). The cavity of LppX has
been modeled to accommodate PDIM, a known TB virulence lipid (Murry, Pandey et al. 2009). In *E. coli*, LolB is on the receiving end of a vectorial translocation of lipoproteins from LolA, in that its closed, bound form is more stable than that of LolA. This work supported the author’s conclusions that LppX was on the receiving end of PDIM transfer into mycobacterial OM. LppX shares 28% homology with LprG, LprF, and LprA indicating that these mycobacterial lipoproteins likely accommodate mycobacterial lipids. *Mtb* strains lacking PDIM are attenuated for survival in the mouse (Cox, Chen et al. 1999). PDIM is known to be surface exposed and has been shown to be important for uptake of *Mtb* by macrophages (Cambier, Takaki et al. 2014). Interestingly, PDIM is not surface exposed, even with a functional LppX, in the absence of the mmpL7 efflux pump (Camacho, Constant et al. 2001). The class of mmpL (mycobacterial membrane pump Large) pumps falls into the larger RND (resistance, nodulation, and cell division) bacterial pumps of which include the major facilitator superfamily (MFS) pumps of which Rv1410 is classified to belong (Paulsen, Brown et al. 1996). The mmpL pumps in mycobacteria are typically associated with large polyketide synthase gene clusters and members of this class of pump have been linked to proper translocation of not only PDIM (mmpL7), but also sulfolipids (mmpL8), and mycolic acid (mmpL11) (Converse, Mougous et al. 2003, Domenech 2004, Jain and Cox 2005, Pasca, Guglierame et al. 2005, Pacheco, Hsu et al. 2013). Other mycobacterial lipoproteins of interest include LprA, LprQ, LprF, LpqH —some of which have been well characterized as immunomodulatory proteins (Pecora, Gehring et al. 2006, Drage, Pecora et al. 2009), and important for growth resuscitation (Rickman, Scott et al. 2005). Some of these lipoproteins, including LprG, have been shown to modulate cytokine secretion in a TLR2 dependent fashion (Steyn, Joseph et al. 2003, Brülle, Grau et al. 2010, Ciaramella, Cavone et al. 2004, López, Sly et al. 2003, Fortune, Solache et al. 2004, Gehring, Dobos et al.
2004, Pennini, Pai et al. 2006, Pecora 2008). Others such as LpqW have been implicated in PIM and LAM translocation to the mycobacterial cell wall (Crellin, Kovacevic et al. 2008) (Guerin, Kordulakova et al. 2010) and many have been investigated in regards to their potential roles in vaccine development with several groups using over-expression of mycobacterial lipoproteins to increase the antigenicity of vaccine constructs and enhance the immune system recognition of mycobacterial cells (Yeremeev, Lyadova et al. 2000, Zvi, Ariel et al. 2008, Roux, Ray et al. 2011, Seshadri, Turner et al. 2012). Interestingly, this has also been attempted for LprG, our mycobacterial lipoprotein of interest (Turner, Dobos et al. 2004). Of note, overexpressing LprG in a vaccine construct is actually detrimental to mycobacterial control and mice receiving vaccine constructs over expressing the LprG lipoprotein have worse outcomes after Mtb challenge than control mice (Hovav, Davidovitch et al. 2004, Hovav and Bercovier 2006, Hovav, Mullerad et al. 2006). This data is consistent with a model whereby immune recognition of LprG by the immune system is actually pro-inflammatory, although in the context of natural infection it is unclear whether this inflammatory response affords a gain or loss of fitness for mycobacteria. Drage et al. showed that the immunostimulatory function of LprG was dependent on it being bound to its cognate lipid. Work by this group has clearly demonstrated binding of LprG to glycolipids (Drage, Tsai et al. 2010). However, is unclear how loss of this immune recognition via knockout of LprG would be detrimental to the mycobacterium during natural infection.

**SECTION 1.4. HYPOTHESIS.** In this work, I propose an alternate binding partner for the lipoprotein, LprG--mycobacterial triacylglycerides. I propose that the two operonic genes, *Rv1410-Rv1411c*, encode for a lipid transport system unique to mycobacteria that regulates
intracellular triacylglyceride levels, the cell wall lipidome, and growth rate during infection. The following sections outline what is known about triglycerides in prokaryotes and mycobacteria specifically, including synthesis and breakdown. I go on to discuss prokaryotic mechanisms of growth regulation via feedback on fatty acid metabolism. Finally, I propose a model by which triglyceride transport, by LprG-1410, could mediate growth rate regulation in mycobacteria. In chapter 2, I outline how we show that disruption of the \textit{Rv1410-1411c} operon profoundly attenuates \textit{Mtb} infection in both immunocompetent (C57/Bl6) mice and immunosuppressed (Rag-/- and SCID) mice, and that growth attenuation is associated with a dramatically slowed growth rate of our \textit{Mtb} LprG-1410 mutants. In chapter 3, work investigating the lipidome changes with disruption of LprG-1410 is presented. We show that the most significant change to the lipidome of our mutant is a large increase in intracellular triacylglyceride (TAG). TAG are the major component of lipid bodies, a feature associated with a minimally replicating state in \textit{Mtb} (Low, Shui et al. 2010) supporting our conclusion that attenuation is likely due to a slowed growth rate. With an enormous contribution by our collaborators, we have evidence that TAG is a substrate of the LprG-1410 transport system by demonstrating the ability of LprG to transport TAG in an \textit{in vitro} vesicle transfer assay and by showing LprG co-crystallized with TAG. In chapter 4, a mechanism for growth rate regulation under carbon stress, via TAG transport, is examined.

**Section 1.5. Triacylglycerides in Prokaryotes.** Triacylglycerides are an important storage molecule in eukaryotic cells including, yeast, plants, and fungi. However, \textit{Actinomycetes} are unique among prokaryotes in their ability to accumulate large amounts of intracellular triglycerides. Triacylglycerides are fatty acid triesters of glycerol, a glycerol head-group with
three acyl chains attached. Triglyceride annotation describes the number of total carbon species followed by an indication of the number of unsaturations (double bonds) between carbons, i.e. a TAG with a single unsaturation, composed of one 18 carbon, and two 16 carbon acyl chains would be annotated as TAG 50:1. The single unsaturation can be at any position on any of the acyl chains, here shown on the 18-carbon acyl chain (Figure 2).

**Figure 2: Chemical structure of TAG.** The ammoniated TAG 50:1, gives an calculated mass/charge (m/z) ratio of 850.785 and is composed of one 18 carbon acyl chain with a single unsaturation, and two saturated 16 carbon acyl chains.

*Rhodococcus* are model organisms for studying bacterial TAG given that up to 87% of the bacterial dry mass is made up of triglyceride (Alvarez and Steinbüchel 2002). The biosynthetic machinery for TAG biosynthesis in this organism is well characterized and much of what we know about TAG synthesis and hydrolysis in prokaryotes has been derived from work on this organism. TAG are generally thought of as a reserve carbon source in prokaryotes and organisms such as *Rhodococcus jostii (RHA_1)* and *Rhodococcus opacus* (PD360) have been exploited for biofuel research due to their ability to accumulate TAG (Janssen, Ibrahim et al. 2013). The few bacterial species for which TAG synthesis has been described, namely *Streptomyces, Nocardia, Rhodococcus, Mycobacterium, Dietzia, Gordonia,* and *Acinetobacter* typically generate TAG during conditions of excess carbon but nitrogen limitation (Olukoshi and
Packter 1994) (Alvarez, Kalscheuer et al. 2000). As it pertains to *Mycobacterium tuberculosis*, TAG accumulation is associated with early log phase (O.D.~0.2-0.3) or late stationary phase growth (O.D. >2) (Sartain, Dick et al. 2011). TAG biosynthesis is linked to phospholipid biosynthesis via a common precursor molecule, diacylglycerol (DAG). For this reason, during logarithmic growth, it is thought that most fatty acid primers are diverted into phospholipid synthesis. In *Streptomyces*, there is a strong correlation between diacylglycerol transferase (DGAT) activity and TAG content (Olukoshi and Packter 1994) indicating that this enzyme is possibly responsible for the switch from phospholipid metabolism to TAG biosynthesis as cells transition from exponential growth to stationary phase growth. In fact, DGATs serve as the sole class of enzymes that function exclusively in the biosynthesis of triglycerides, as the other enzymes in TAG anabolism are shared with phospholipid synthesis as described below. This feature is important to our work given that we have operonic level transcriptional data linking transcriptional regulation of *Rv1411c-Rv1410c* (LprG-1410) operon with the operon *Rv3814-3816c*, encoding the mycobacterial DGAT, *Rv3816* (Baranowski and Rubin *unpublished*).

**SECTION 1.6. TRIACYLGLYCERIDE METABOLISM IN ACTINOMYCETES.** Recent work in *Rhodococcus* has comprehensively identified the key enzymes in the biosynthesis and degradation of TAG in this organism (Chen, Ding et al. 2014). We know from studies in *Rhodococcus* that TAG biosynthesis begins with the incorporation of acetyl-CoA typically generated via β-oxidation of fatty acids, into malonyl-CoA. This carboxylation is carried out by acetyl-CoA carboxylase (ACC), the best-known homolog of which is accA1 *Rv2501c* in mycobacteria. Malonyl-CoA then functions as extender units onto which a series of iterative reactions catalyzed by malonyl-coA transacylase (FabD, *Rv2243*) result in the addition of two-carbon units to the acyl chains resulting in the elongation of the short, medium, or long-chain
acyl units that will be incorporated into triglycerides. Acyl carrier proteins (ACP) then facilitate
the transfer of acyl chains in conjunction with the function of condensing FabF-FabB enzymes,
after completion of each condensing cycle, and the elongated acyl chain is transferred back to the
ketosynthase to start a new cycle. Prokaryotic FASI is well recognized as a holoenzyme with all
necessary enzymatic modules translated as a single polypeptide chain, collectively known as the
fatty acid synthase (FAS). Mycobacteria are unique in that they possess both the mammalian-like
FasI and the prokaryotic-like FasII. In mycobacteria FasI results in the bimodal product
formation of either C16 units or C24 (Mycobacterium smegmatis) and C26 (Mycobacterium
tuberculosis) units. These intermediate or long-chain fatty acids can then be incorporated into
phospholipid or triacylglyceride synthesis or be transferred to FasII for further elongation to
form the complex mycolic acids that are the structural basis for the mycobacterial cell wall
(Mondino, Gago et al. 2013).

As it pertains to triacylglyceride synthesis, intermediate (C16) or long-chain (C24-C26) acyl
primers are joined to an acyl-carrier protein to form acyl-ACP. In the Kennedy scheme of
triacylglyceride synthesis, the glycerol backbone of the triglyceride is added in the form of
glycerol-3-phosphate (G3P) by glycerol-3-phosphate acyltransferase then incorporated step-wise
into lyso-phosphatidic acid and phosphatidic acid. Potential candidates for these two enzymes in
Mycobacterium tuberculosis include Rv2482c and Rv2483c (PlsB2 and PlsC). Phosphatidic acid
(1,2 diacylglycerol-3-phosphate) is a key precursor in both phospholipid biosynthesis and
triacylglyceride biosynthesis as this intermediate may be converted into either the liponucleotide
intermediate CDP-DAG via the action Rv2881c (cdsA) (Nigou and Besra 2002) or into DAG via
the action of a phosphatidic acid phosphatase enzyme (PAP) (Comba, Menendez-Bravo et al.
2013). The gene Rv3816c of Mycobacterium tuberculosis is predicted to encode a 1-acyl-sn-
glycerol-3-phosphate acyltransferase and is located in an operon with two other genes predicted to encode acyltransferase enzymes of similar function. This class of enzymes allows the direct acylation of Acyl-ACP to form phosphatidic acid, therefore potentially bypassing the formation of lyso-phosphatidic acid. Long-chain acyl-CoA is then transferred to DAG to form triacylglycerides (TAG) via the action of diacylglycerol transferases (DGAT), commonly referred to as tag synthases or *tgs* (Kennedy 1961) (Vigeolas and Geigenberger 2004). Mycobacteria have 15 annotated *tgs* genes or which the best characterized is *tgs1* (*Rv3130c*) (Daniel, Deb et al. 2004). A simplified TAG synthetic scheme is shown here (Fig 3).
Figure 3: Model for Coupling of Fatty Acid, Phospholipid, and Triacylglyceride Synthesis in *Mtb*. Acetyl CoA primers enter into Fasl (mycobacterial fatty acid synthase I). Monoacylglycerol-3-phosphate (Acyl-G3P) is converted to lyso-phosphatidic acid (Lyso-PA) then phosphatidic acid (PA) by the genes *PlsB2* and *PlsC*, respectively. Phosphatidic acid precursors destined for phospholipid synthesis will have the addition of a cytidine di-phosphate nucleotide (CDP-DAG) that then enters into the phospholipid (PL) synthetic pathway. Phosphatidate phosphatases will remove the phosphate group to form diacylglycerol (DAG). The combined actions of acyltransferases *Rv3816c* and diacylglycerol transferase, *tgs1*, are predicted to result in the formation of mycobacterial triacylglyceride (TAG). In this work, we propose that TAG is transported across the inner membrane by the combined action of *Rv1410c* and *Rv1411c* (*LprG*). In *E.coli*, FadR is a transcriptional activator of the FASII enzymes; in mycobacteria, FasR is the transcriptional activator of FASI (black inset). In *E.coli*, acyl-ACP primers act as negative feedback inhibitors on phospholipid metabolism via repression of transcription of both β-oxidation and FasII induction by FadR and FabR (red inset). *Rv3208* encodes the *Mycobacterium tuberculosis* FasR homolog of this gene.

This synthetic scheme represents an oversimplified view of TAG biosynthesis in actinomycetes. A comprehensive study in Rhodococcus identified 22 different reactions involving 457 candidate enzymes attributed to fatty acid biosynthesis, TAG biosynthesis, TAG storage and degradation,
and fatty acid degradation in this organism. In addition to 16 tgs genes, 45 lipases/esterases and 40 long-chain fatty acid CoA ligases were predicted (Chen, Ding et al. 2014). This suggests that many of the enzymes involved in mycobacterial triglyceride metabolism and regulation are uncharacterized and unannotated as such. Furthermore, free fatty acids (FFA) bound to acyl carrier proteins (acyl-ACP) are known to be key regulators in fatty acid metabolism in model organisms such as *Escherichia coli* (*E. coli*) and *Bacillus subtilis* (*B. subtilis*) (Zhang and Rock 2009). As mentioned previously, mycobacteria are unique among prokaryotes in that they encode both the eukaryotic like FasI and the prokaryote FasII. In *E. coli* and *B. subtilis*, FasII activity is highly regulated by free fatty acids via negative feedback loops involving transcriptional repressors of fatty acid synthesis, such as FadR. In this system, acyl-ACP binding to transcription factors such as *FadR* impact transcription of FasII. It has recently been determined that mycobacteria possess a TetR like transcription factor that is predicted to be a FadR homolog. In *Mycobacterium smegmatis*, this transcription factor was shown to be a FasI activator and therefore termed FasR (Nickel, Irzik et al. 2010, Zhang and Rock 2010, Mondino, Gago et al. 2013). Given the complexity of the mycobacterial lipidome and the large number of genes devoted to lipid synthesis in *Mtb*, the existence of such a factor supports the hypothesis that fatty acid synthesis is regulated by feedback loops in mycobacteria as well. This is important for the work presented here in that we have identified a suppressor mutation for one of our LprG-1410 phenotypes (malachite green susceptibility) that maps back to a single nucleotide polymorphism resulting in a F123C mutation in the proposed *Mycobacterium tuberculosis* homolog to the newly characterized *Mycobacterium smegmatis*, FasR. In the case of *Mycobacterium smegmatis* FasR, binding to the promoter region of the *fas-acpS* operon results in *fas* transcription (Mondino, Gago et al. 2013). The authors go on to show that long-chain fatty
acids are inhibitory to FasR binding to the promoter region of the *fas-acpS* operon in that decreased *fas* transcription was demonstrated in the presence of intermediate and long chain fatty acids (C16-C22). These data suggest a model by which levels of FFA contribute to negative feedback on *fas* expression via binding to the FasR protein and preventing its ability to bind DNA (Mondino, Gago et al. 2013). In *E.coli*, β-oxidation and FasII activation are tightly co-regulated in that unsaturated fatty-acid synthesis is regulated by the tetR family transcription factor, FabR. E.coli only possess the FasII system and binding of the FadR transcription factor results in down-regulation of β-oxidation genes and upregulation of *fabA* and *fabB*, the FasII genes involved in anaerobic formation of unsaturated fatty acids (Fujita, Matsuoka et al. 2007). In *E.coli*, FabR acts as a transcriptional repressor of *fabA* and *fabB* (Feng and Cronan 2011).

The coordinated function of the FadR and FabR therefore mediate growth rate by regulating fatty acid synthesis and incorporation into phospholipids for membrane biogenesis (Zhang and Rock 2010). FFA negative feedback on FasI in the mycobacterial model involves decreased binding of a transcriptional activator (FasR) to shut down FasI expression. Although this model is mechanistically different, the principles of FFA negative feedback regulation are the same. It is becoming increasingly evident that FasI and FasII activity is co-regulated in mycobacteria, but currently there is no research looking into the role of TAG biosynthesis and degradation in these potential feedback loops. In addition it is unclear whether there are factors regulating mycobacterial β-oxidation as is seen in other model bacterial systems. In order for mycobacteria to utilize the fatty acids (FA) stored in TAG, TAGs must first be hydrolyzed to liberate the long-chain fatty acids. Liberated long-chain FA then must undergo β-oxidation to provide acetyl-coA units that may feed into either the TCA cycle or back into FasI for lipid synthesis (Wheeler 2009) (Venkatesan and Wierenga 2013). Increased transcription of genes involved in β-
oxidation during macrophage infection supports the notion that Mtb utilize lipids during infection (Schnappinger, Ehrt et al. 2003). For our purposes, it is interesting to speculate how TAG metabolism and TAG transport may affect the ability of Mtb to access fatty acids for β-oxidation. Given that in other model organisms, β-oxidation occurs across membranes, it is tempting to speculate that TAG transport across mycobacterial cell membranes, may impact access to these enzymes and/or contribute to currently uncharacterized FFA feedback loops on fatty acid synthesis as seen in E. coli.

SECTION 1.7. HYPOXIA MODEL OF GROWTH ARREST—IMPLICATIONS FOR THE IN VIVO TRANSITION TO LATENCY. Most of what we know about the importance of TAG in mycobacterial growth comes from research utilizing the hypoxia model of non-replicating persistence in Mycobacteria (Wayne and Lin 1982, Wayne 1994). This system is thought to be the best approximation of mycobacterial metabolism during dormancy, a non-replicating state of persistence that is predicted to represent what is clinically referred to as latency in the human population. Approximately one third of the world’s population is infected with latent tuberculosis (LTBI). It has been proposed that “latent” mycobacteria evade host immune cell destruction by either minimizing or altering the quality of immune cell recognition. This ability to evade immune detection is likely related to residence in protective host cell niches, growth rate changes, and subsequent alterations to the cell wall (Seiler, Ulrichs et al. 2003, Neyrolles, Hernández-Pando et al. 2006, Daniel, Maamar et al. 2011). The presence of lipid bodies and high triacylglyceride (TAG) content is reported to be a feature of latent or dormant mycobacteria (Fang, Wallqvist et al. 2012, Low, Rao et al. 2009, Daniel, Maamar et al. 2011, Daniel, Deb et al. 2004). TAG accumulation and latency are both associated with decreased drug susceptibility (Deb, Lee et al. 2009, Baek, Li et al. 2011). An antibiotic course of nine months duration is
necessary to clear and prevent reactivation of latent mycobacteria (IUATCOP 1982). The need for this prolonged treatment course is attributed to the inability of drugs to target cells that are not actively growing (Zhang, Sala et al. 2012, Solapure, Dinesh et al. 2013). Although it is impossible to know the true features of latent mycobacteria due to small numbers and poor detection methods, high TAG content and intermittent arrested growth or decreased growth rate are well-accepted characteristics of these cells (Beste, Laing et al. 2007).

Mycobacteria likely experience low oxygen tension in the human lung as a result of the antimycobacterial immune response (Via, Lin et al. 2008). Control of mycobacterial replication is associated with the formation of the “granuloma.” (Saunders, Frank et al. 1999) The granuloma that develops secondary to mycobacterial infection is unique in the human—currently no animal model entirely recapitulates all the histopathologic and epidemiologic features of human TB disease. The mycobacterial granuloma consists of a necrotic core, filled with caseum, hence termed “caseous.” Small numbers of mycobacteria are thought to persist in the caseum for an extended period of time in a non-replicating state. This necrotic core is surrounded by a rim of activated macrophages and multinucleated giant cells, further surrounded by layers of infiltrating B and T lymphocytes as the adaptive immune response progresses. A mature granuloma where TB replication is controlled is further characterized by infiltration of fibroblasts, calcification of necrotic material, and contraction (Russell, Cardona et al. 2009, Russell 2011, Lin, Ford et al. 2014). It is important to note, that although many animals develop caseous granulomas in response to mycobacterial infection, only primates and occasionally rabbits are reported to control mycobacterial replication upon the development of granulomas. Rabbits have been reported to have spontaneous latency in that 100% of experimentally infected animals achieve
culture negative status at 24 weeks post infection, yet reactivate after immunosuppressive glucocorticoid therapy. This model may become useful for studying certain aspects, of non-culturable mycobacterial persistence, but the lack of reagents and the lack of disease spectrum make them a non-ideal animal model for studying TB latency due a lack of predictive power (Subbian, O’Brien et al. 2013). The cynomologous macaque model is the best approximation for the development of latent *Mtb* disease (Capuano, Croix et al. 2003, Flynn, Capuano et al. 2003, Lin, Pawar et al. 2006, Lin, Rodgers et al. 2009). Even then, only 55% will develop latent disease, and latency in this setting is a six-month course typically. An important distinguishing feature of the macaque model is the development of a spectrum of lesions with differing natural histories in which individual populations of mycobacteria are predicted to undergo a concomitant spectrum of changes that is more typical of the human disease condition (Lin, Ford et al. 2014). Unfortunately, the macaque model is laborious, expensive, management intensive, and ethically and often politically challenging, not to mention, far from representative of a condition that occurs in 95% of exposed humans that lasts on the order of decades, not months.

Given these difficulties in studying mycobacterial latency, the Wayne model was developed in order to study the characteristics of these mycobacteria in order to better understand the mechanisms by which these mycobacteria not only persist, but also “awaken” to go on to cause disease decades after the initial infection. In this model, gradual oxygen depletion leads to a non-replicating persistent state, where mycobacteria are resistant to antimicrobials and are lipid-laden, consistent with features described of latent mycobacteria (Wayne and Hayes 1996, Seiler, Ulrichs et al. 2003). Mycobacteria within granulomas are considered to be in a hypoxic environment for the long duration of latency, of which may last over 25 years or more. In fact
only 10% of exposed individuals will ever experience reactivation of disease, much of which is
dependent on host immune status. Early work utilizing this model identified that a main
transcriptional feature of these cells was the upregulation of genes known to be strongly
regulated by hypoxia, and induced by the transcription factor DosR (Rv3133c), commonly
referred to as the DosR regulon (Park, Guinn et al. 2003). The Dos reguon is composed of some
fifty genes of which transcription is upregulated via binding of the DosR transcription factor
during hypoxia (Pethe, Sequeira et al. 2010). It is well known that mycobacteria accumulate lipid
bodies under hypoxic conditions and can be visualized by cryoelectron microscopy, and special
stains such as Nile Red (Greenspan, Mayer et al. 1985).

It has been determined that the main constituent of mycobacterial lipid bodies is TAG
(Wältermann, Hinz et al. 2004). TAG synthase genes, such as Rv3130c (tgs1) and TAG lipases
(LipY) are both upregulated under hypoxic conditions and in lipid-loaded macrophages (Rustad,
Sherrid et al. 2009, Daniel, Maamar et al. 2011, Park, Guinn et al. 2003). In the Wayne model of
hypoxia, Mtb upregulates DosR genes and by doing so, preferentially metabolizes glucose over
glycerol, and shuttles glucose precursors into the reductive side of the TCA cycle in order to
maintain a favorable redox status (Fang, Wallqvist et al. 2012). However, metabolism of glucose
via the glycolytic pathway is thought to result in the production of toxic metabolites and it was
recently shown that Mtb can survive longer during hypoxia in a non-replicating state if glucose is
removed from the media (Phong, Lin et al. 2013). It has been consistently predicted that the main
carbon source for mycobacterial during chronic infection is lipids (Russell 2003). This is based
on the attenuation of mycobacteria with mutations in the glyoxylate shunt (McKinney, Höner zu
Bentrup et al. 2000, Muñoz-Elías and McKinney 2005). The glyoxylate shunt is the pathway
canonically used by lipid metabolizing organisms whereby acetate entering the TCA cycle is “shunted” into glyoxylate→malate→oxaloacetate allowing the conversion of simple carbon sources into carbohydrates when complex carbons such as glucose are not available. Both malate and oxaloacetate then enter the gluconeogenic pathway allowing the formation of glucose from fat (Eoh and Rhee 2014, Eoh and Rhee 2013). However, it is unclear what contribution host-derived lipids versus intracellular stores of mycobacterial lipids in the form of triglycerides, account for the lipid utilization via the glyoxylate shunt during hypoxia and latency (Fig 4).
Given that mycobacteria under hypoxic conditions develop characteristic large lipid bodies composed of TAG, that TAG synthase (tgs) genes are upregulated as part of the DosR response, and during nitrosative acid stress—all of which are considered stressors during infection of the human host, it is likely that \textit{Mtb} utilizes intracellular TAG as a carbon source during infection and latency. \textit{Tgs1} preferentially incorporates C26 acyl chains into TAG and the mycobacterial FasI system has been reported to be the fatty acid synthase responsible for generating C26 acyl
chains in mycobacteria (Sirakova 2001). Others have shown that FasI is responsible in large part for TAG synthesis in that known FasI targeting drugs, such as cerulenin (Boshoff, Mizrahi et al. 2002, Parrish, Kuhajda et al. 1999, Kremer, De Chastellier et al. 2005), abolish TAG synthesis in mycobacteria (Kremer, De Chastellier et al. 2005). The potential role for TAG metabolism in mycobacterial growth regulation during latency and TB reactivation has been highlighted by research showing that TAG hydrolysis is necessary for regrowth of mycobacteria after reaeration of hypoxic cultures (Low, Rao et al. 2009). *Mycobacterium bovis* BCG cells grown under hypoxic conditions were unable to be recovered after treatment with the lipase inhibitor, tetrahydrolipostatin (THL). Others have shown that *LipY* is an important TAG lipase in *Mtb* (Deb, Daniel et al. 2006). Both structural predictions and functional modification of secretion signals of *LipY* indicate that this lipase is a membrane-bound lipase (Singh, Singh et al. 2010). *LipY* is interesting in that it is a PE/PPE protein, a class of proteins with unknown function, unique to mycobacteria. *LipY* is one of the few proteins in this class for which the function has been defined and recent work by Daleke et al. show that this protein is likely secreted by the *esx5* secretion system, a Type VI secretion system, unique to slow-growing mycobacteria (Daleke, Cascioferro et al. 2011) (Bottai, Di Luca et al. 2012). This is interesting in the context of this work, given that we have evidence linking co-operonic transcriptional regulation of LprG-1410 with the *esx1*, *esx2*, and *esx5* operons (Baranowksi and Rubin unpublished).

**Section 1.8 Triacylglyceride accumulation is a feature of *Mtb* growth in vivo.** As mentioned above, TAG synthesis and hydrolysis have been shown to be important features of growth resuscitation in the hypoxia model of mycobacterial dormancy. This model is the best approximation to the metabolic state of latency TB *in vivo*. TAGs have been shown to be a
feature of mycobacteria recovered from in vitro macrophage cultures, particularly in foamy macrophage cultures induced with the addition of oleic acid to culture media (Daniel, Maamar et al. 2011). TAGs have been shown to be a feature of mycobacteria isolated from human sputum samples (Garton, Christensen et al. 2012, Garton, Waddell et al. 2008), and TAGs are increased in mycobacteria seen in foamy macrophages from human granulomas (Caire-Brändli, Papadopoulos et al. 2014).

In summary, we have identified a genetic operon Rv1410-1411c, that encodes a transporter and lipoprotein that we believe is a triacylglyceride transporter. In the following chapters, I will outline evidence to support a model by which intracellular TAG levels are a function of LprG-1410 function. Loss of LprG-1410 function coincides not only with TAG accumulation, but also growth arrest in immune competent mice and grow rate reduction in immune compromised mice. TAG transport may be necessary for proper lipolysis by LipY in order to liberate FFA for β-oxidation. Growth rate regulation is predicted to be a function of increased TAG levels potentially contributing to negative feedback on FasI via increased binding of either TAG or FFA to transcriptional activator FasR. Decreased FasR binding of FasI will likely decrease rates of cell biogenesis, contributing to growth arrest under conditions of carbon stress, explaining the in vivo growth defect.
SECTION 1.9. REFERENCES.


carbon flow is necessary to limit the accumulation of toxic metabolic intermediates under hypoxia. *PLoS ONE.* **8:** e56037.


Chapter 2

LprG-1410 function regulates growth rate \textit{in vivo} independent of immune function.
SECTION 2.1 ATTRIBUTIONS. Competition infections of Nos2-/-, Phox -/-, Phox -/- with Aminoguanidine, and IFN-γ -/- Mice, and the Rag -/- survival experiment were performed by Mary Farrow, former graduate student in the Rubin lab. A version of these results was previously reported in her dissertation entitled “Characterization of the lprG-Rv1410c operon in Mycobacterium tuberculosis” (Farrow M. 2007). All histopathology presented from these experiments, as shown, was performed by the author.

SECTION 2.2 PREFACE. The attenuation seen with LprG-1410 loss is dramatic (Sassetti and Rubin 2003). Both Rv1411c and Rv1410c were listed as two of the most underrepresented genes recovered after passage of a library of transposon mutants in the mouse. Bigi et al. report that loss of LprG-1410 function results in 2.5 logs lower colony-forming units (cfu) in the spleen compared to wild-type and complement infected mice after 35 days of infection (Bianco, Blanco et al. 2011). A range of disparate phenotypes have been reported with this mutation in both Mycobacterium tuberculosis and Mycobacterium bovis, ranging from increased susceptibility to malachite green, an antifungal agent that is non-toxic to mycobacteria and used in standard mycobacterial growth media (Bianco, Blanco et al. 2012), increased sensitivity to oxidative stress and growth defects in low iron media (Farrow unpublished), and inconsistent reports of increased antibiotic susceptibility (Ramón-García, Martín et al. 2009, Bianco, Blanco et al. 2012). Some groups have focused on potential phospho-inositol-mannoside (PIM) binding partners of LprG and TLR2 activation, in an attempt to explain the growth defect due to a defect during the host-pathogen response (Drage, Tsai et al. 2010). Overall, there is little continuity and no consensus in the field as to the mechanism for growth attenuation. Using forward genetics has been difficult in assigning function to these genes. In most cases, potential substrates for
mycobacterial substrates can be divined by annotation of nearby genes on the chromosome, as was the case for PDIM and mmpL7 (Jain and Cox 2005). However it is unusual for a lipoprotein and transporter to be in an operon, there is no precedent for such an organization in model organisms such as E.coli, and nearby genes offer no functional insight into the potential substrates for the LprG-1410 system. In this chapter, I will highlight experimental results that show that a defect in the host-pathogen interaction is unlikely. Given the severe attenuation seen in both immune competent and immune deficient mice, we believe the growth defect is not host-induced but intrinsic to the bacterium. More importantly we show that loss of gene function is not catastrophic for the cells in the absence of adaptive immunity. In fact, these mycobacterial mutants can cause death in SCID mice. The dramatically slowed growth rate of the LprG-1410 mutant that we report in SCID mice is only consistent with a pre-existing metabolic defect leading to slow growth. Typically, during the course of TB infection in immune competent mice, exponential growth of mycobacteria is seen during the first three weeks of infection despite pressure exerted by the innate immune system. This implies that innate mechanisms of control are inadequate to slow growth. Only until after the onset of adaptive immunity at three weeks, does the growth of Mtb in the mouse plateau, remaining slow and stable for months in the mouse during chronic infection. We first show that LprG-1410 mutants are attenuated in mice lacking key components of the innate immune system, namely oxidative burst by both neutrophils and macrophages. We then go on to show that in two genetic mouse backgrounds with defective adaptive immunity (RAG -/-, SCID), but completely intact innate immunity, that growth rate is dramatically slowed. In fact, we were unable to rescue growth rate in any of the immune deficient mouse backgrounds we tested. Therefore, we conclude that the attenuation seen with loss of LprG-1410 is secondary to a metabolic defect of the bacterium, independent of
**host immune response.** The fact that this phenotype is only apparent during *in vivo* growth suggests that the phenotype is “activated” due to growth conditions within the host. Given that we believe that immune pressure is not the initiating factor for the expression of this slow growth phenotype, we hypothesize that the cause is carbon source stress *in vivo* due to catabolism of odd-chain fatty acids derived from cholesterol.

**SECTION 2.3 LPRG-1410 FUNCTION DOES NOT AFFECT IN VITRO GROWTH RATE IN RICH MEDIA OR MACROPHAGES.** LprG-1410 function together or independently does not affect growth of *Mycobacterium tuberculosis* (*Mtb*) under standard, rich conditions culture conditions. No growth defect of RvΔLprG-1410, Rv::tn1410, RvΔLprG, and RvΔ1410 was noted in 7H9/OADC (Fig 5A,B).
**Figure 5: Growth of LprG-1410 mutants in vitro.** A) Mycobacterium tuberculosis PDIM+ H37Rv (black), RvΔLprG-1410 (red), RvΔLprG-1410+pLprG (aqua), RvΔLprG-1410+p1410 (orange), and RvΔLprG-1410+pLprG-1410 (dark blue) B) PDIM “low” H37Rv (black), Rv::tn1410 (red), Rv::tn1410+p1410 (blue). Cells were cultured in 7H9+OADC to log phase (Middlebrook) and diluted back to an OD 0.005. Growth was monitored by increase of OD600 over time. Error bars, mean +/- SD.

LprG and Rv1410 were both predicted to be required for growth in macrophages (Rengarajan, Bloom et al. 2005). RvΔLprG-1410 grew comparable to wild-type (Rv) in J774 with and without interferon-gamma (IFN-γ) stimulation (Fig 6 A,B), THP1 (Fig 6C), and bone marrow derived macrophages (Fig 6D). Colony-forming units (cfu) were enumerated on 7H10 plates that contain low level (0.25%) malachite green. Given reports of decreased recovery of LprG-1410
mutants off plates containing malachite green, cfu reported here are likely underestimates for the growth of the LprG-1410 mutant strains (Bianco, Blanco et al. 2012, Gelman, McKinney et al. 2012).

**Figure 6: Growth of LprG1410 mutants in macrophages.** (A) J774 (B) J774 with INF-γ (C) THP1 human monocytic line (D) murine bone-marrow derived macrophages (BMM) were cultured in DMEM (A,B) with (A) or without (B) IFN-γ in 96 well plates or DMEM (C) or R10 (D) in 24 well plates, and infected with H37Rv, Rv::tn1410, or Rv::tn1410+p1410 at MOI 1:1 (A,B,C) or MOI 10:1 (D). Cells were lysed with Triton-X at 24, 48, and 72 hours. Lysates were titered and plated onto Middlebrook 7H10. Bacterial colony-forming units were counted at 3-4 weeks. Error bars, mean +/-SD.
SECTION 2.4 LprG-1410 function is necessary for survival of Mycobacterium tuberculosis in mice. Independently, both LprG and Rv1410, have been predicted to be conditionally essential for survival in the mouse (Sassetti and Rubin 2003). Using transposon site mutagenesis and looking for transposon site insertion mutants recovered from mouse spleens at 4 weeks and 8 weeks post-infection, Sassetti determined that LprG and Rv1410 were two of the most under-recovered transposon mutants. Zhang et al. confirmed the conditional essentiality of LprG and Rv1410 using TnSeq (Zhang, Reddy et al. 2013). This methodology is more sensitive in detecting genomes from recovered mouse libraries and places LprG and Rv1410 among the top 50% of essential genes based on genome count ratios recovered from in vitro vs. in vivo libraries. Bigi et al. reported that LprG (P27) mutants were attenuated for growth in Balb/c mice (Bigi, Gioffré et al. 2004). It is generally thought that disruption of LprG will have polar effects on the entire operon and therefore result in loss of expression and translation of Rv1410. Indeed, LprG-1410 has only one transcriptional start site upstream of LprG (Shell personal communication). In order to confirm the essentiality of both these genes, the transposon insertional mutant Rv::tn1410 recovered from the initial Sassetti TraSH library was isolated and complemented. Upon infection C57Bl6 mice, only wild type (Rv) and the complemented strain Rv::tn1410+p1410 could be recovered from lungs and spleens (Fig 7).
**Figure 7:** Growth of LprG-1410 mutant in mice. C57Bl6 mice were infected by intravenous tail vein infection with 1x 10^6 cfu of WT (black, Rv), mutant (red, Rv::tn1410), or complement (blue, Rv::tn1410+p1410). Mice were harvested at 1, 3, and 3 months post-infection and lungs and spleen homogenate plated for cfu. Error bars, mean +/- SD.

**SECTION 2.5 GROWTH OF LPRG-1410 MUTANTS CANNOT BE RESCUED IN MICE WITH DEFICIENCIES IN KEY COMPONENTS OF INNATE IMMUNITY IMPORTANT FOR MTB CONTROL.**

Many gene products and proposed virulence factors of *Mtbc* are thought to assist TB in subverting or evading the innate responses to initial infection. The importance of *Mtbc* control by oxidative killing via inducible nitric oxide (*iNOS*) in macrophages or via superoxide in neutrophils is well described (Hernandez-Pando, Schön et al. 2001, Lee, Yang et al. 2009, Barrera, Kramnik et al. 1994, Gotoh and Mori 1999, Bekker, Freeman et al. 2001, Davis, Vergne et al. 2007). Mycobacterial lipids have been implicated in protecting *Mtbc* from some of these oxidative stressors, therefore we predicted that growth of LprG-1410 mutants would be rescued in immune deficient mouse backgrounds lacking oxidative killing capacity. We performed competition experiments in mice with defects in in the ability to produce nitric oxide via cytokine inducible nitric oxide synthase (*nos2^-/-*). Nitric oxide mediated killing is an important host anti-
mycobacterial immune response and mice deficient in iNOS rapidly succumb to TB mediated disease (MacMicking, North et al. 1997). NO is also an important mediator of TNF-alpha antimicrobial activity and therefore mice deficient in NOS2 are expected to be a good proxy for the loss of the effect of TNF-alpha activation (Nacy, Meierovics et al. 1991). Importantly, the presence of lipids in the mycobacterial cell wall namely, PGL-1, LAM, and DIM have been implicated in protecting TB from host inflicted NO damage by acting as oxygen free radical scavengers, down-regulating phagolysosomal fusion, and modulating IFN-γ expression (Chan, Fan et al. 1991). Therefore we hypothesized that growth of our lipid altered LprG-1410 mutants may experience growth rescue in the nos2/- immune compromised host background. This was not the case. We failed to rescue growth of the LprG-1410 mutant in mice lacking inducible nitric oxide synthase (Fig 8A,B).

Similarly, neutrophil innate function is also important for host-mediated antimicrobial defense. The NADPH oxidase enzyme is a multicomponent enzyme of which phox is a component. Phox-/- mice thereby have defective NADPH oxidase and abnormal neutrophil mediated killing of Mtb. (Yang, Shin et al. 2009) (Scanga, Mohan et al. 2001). We infected Phox-/- mice (Jackson, Gallin et al. 1995), which cannot produce superoxide, with both wild type and mutant strains. In both the lung and spleen the mutant appeared attenuated relative to wild type M. tuberculosis to an extent similar to or greater than that seen in C57Bl/6 mice (Fig 8C). Given the synergistic effects of reactive oxygen and reactive nitrogen species (Nathan and Shiloh 2000), we also examined the contribution of LprG and Rv1410 in vivo in the absence of both reactive oxygen and nitrogen. Phox-/- mice were fed the NOS inhibitor aminoguanidine (2.5%, ad lib.) to mimic the phenotype of a Phox/NOS2 double knockout mouse model (Flynn, Goldstein et al. 1995,
Scanga, Mohan et al. 2001, Flynn, Scanga et al. 1998, Chan, Tanaka et al. 1995). The mutant was attenuated in both organs in these mice as well (Fig 8D).
Figure 8. LprG-1410 function is necessary for growth of *Mtb* in mice lacking innate effector functions. Lung and spleen colony forming units from mice infected with H37Rv (black) and Rv::tn1410 (red) in competition. (A) C57Bl/6 mice (B) nos2/-/- mice (C) Phox/-/- mice (D) Phox/-/- mice fed aminoguanidine (NOS2 inhibitor). Numbers represent mean and standard error of three to five mice per time point.
Section 2.6. Rv1410 is required for M. tuberculosis virulence in the absence of an adaptive immune response. The macrophage is equipped with a variety of antimicrobial effectors in addition to reactive oxygen and nitrogen radicals. Many of these are induced by the pro-inflammatory cytokine IFN-γ (Flesch and Kaufmann 1987, Ding, Nathan et al. 1988, Flesch and Kaufmann 1991, Rengarajan, Bloom et al. 2005), which is produced mainly by activated T cells after induction of the adaptive immune response. Host ability to secrete IFN-γ is one of the most critical immunologic mechanism of TB control in both mice and humans (Cooper, Dalton et al. 1993, Flynn, Chan et al. 1993). IFN-γ secretion is also down-stream of innate control mechanisms initiated by pathogen associated molecular patterns (PAMPs), including TLR2. The surprising and dramatic attenuation of the Rv1410 mutant in mice unable to mount oxidative and nitrosative responses led us to question if LprG-1410 was required to resist IFN-γ stimulated effector functions during the adaptive immune response. We infected IFN-γ −/− mice (Dalton, Pitts-Meek et al. 1993) with H37Rv and the tn1410 mutant and measured the organ burden in lung and spleen. As in all other mouse models, we found that the mutant was attenuated relative to wild type M. tuberculosis in both organs (Fig 9A,B).
**Figure 9**: LprG-1410 function is necessary for growth of *Mtb* in mice lacking IFN-γ. IFNγ-/- mice were infected with a 3:1 ratio of mutant (Rv::tn1410, red) to WT (H37Rv, black) via tail-vein injection. Cfu was measured in lung (A) and spleen (B) at 1, 7, and 30 days post infection. Error bars, mean +/- SD of three to five animals per time point.

**SECTION 2.7 MUTATIONS IN RvLPRG-1410 RESULT IN ATTENUATION DUE TO DECREASED GROWTH RATE.** It was surprising to us that a mutation that caused such profound *in vivo* attenuation, even in the absence of key elements of the innate and adaptive immune response would not result in a more obvious growth defect in macrophages. Despite being identified as important for growth in macrophages (Rengarajan, Bloom et al. 2005), we were unable to uncover a reproducible macrophage growth defect with single strain infections. Furthermore, newer *in vivo* TraSH data with more sensitive detection of mutant outgrowth by measurement of mycobacterial genomes, although still calling *LprG* and *Rv1410* conditionally essential, clearly showed recovery of mutants with transposon insertions (Zhang, Reddy et al. 2013). Therefore we asked the question whether mutants were being killed and cleared or whether the attenuation phenotype was secondary to an *in vivo* growth rate defect. Mycobacterial cells where genetic disruption resulting in a non-growing “persistent” or slow-growing state would still be detectable using newer TnSeq genome sequencing methodology for evaluating transposon mutagenized libraries, but may be underrepresented since outgrowth (on plates) would be proportional to
growth rate in vivo. In this scenario mutants with slowed growth rate will represent a smaller proportion of sampled cells, the effect of which will be amplified by recovery and scraping of plate grown cells for genomic DNA preparation and TnSeq. To test this hypothesis we first performed a survival experiment with the Rv::tn1410 mutant and wild type H37Rv. All of the Rv::tn1410 mutant infected mice survived past the pre-determined endpoint of 5 months (Fig 10A), however when three of these animals were sacrificed for end of study cfu and histopathologic evaluation it was surprising to note that animals had significant numbers of mycobacteria in the lung (Fig 10B,C).
Figure 10. LprG-1410 function is necessary for growth and virulence of *Mtb* in *Rag* -/- mice lacking an adaptive immune system. Two groups of 14 *Rag* -/- mice were infected with 1x10^6 cfu of either H37Rv (black) or Rv::tn1410 (red) by tail vein injection. Mice were followed for 5 months. H37Rv infected *Rag* -/- mice died within 40 days of infection. Mutant infected mice lived out to five months post-infection (A). At end of study, all mice were sacrificed and lungs harvested from three mice for cfu and histopathology. Numerous acid-fast mycobacteria were covered from the lung (B) acid-fast staining of Rv::tn1410 in lung, 400x magnification. (C) Multibacillary growth of Rv::tn1410 in macrophages of lung, 1000x oil magnification.

Given that these animals were intravenously infected and that approximately 1% of bacteria administered via tail vein injection reach the lungs, this suggested that the mutant was viable and replicating over time after seeding in the lung. There was significant lung pathology associated with the multibacillary expansion of the mutant. The interstitium of the pulmonary parenchyma was expanded by large infiltrates of viable and apoptotic macrophages filled with numerous acid-fast bacilli. Alveoli were collapsed and filled with histiocytes undergoing varying degrees
of cell death. Numerous apoptotic bodies were noted and macrophages diffusely stained for cleaved-caspase 3 by immunohistochemistry, a marker for apoptosis (data not shown). There was significantly less necrotic cell death and minimal neutrophil infiltrate compared to what was seen at time of death in \textit{Rag-1-} mice infected with wild-type H37Rv. Without earlier time points with which to compare WT infected animals and mutant infected animals at similar bacillary loads, it was impossible to determine if and how disease progression differed in mice infected with the WT versus mutant strains. Furthermore, given that only three mice were sacrificed at the end of study, it was impossible to conclude whether the progressive disease was uniform across the entire survival cohort of mice infected with the mutant. For this reason, we generated a clean double knockout of \textit{LprG-1410} on the background of a confirmed PDIM positive parent strain and repeated the survival experiment, this time with SCID mice utilizing aerosol infection to better monitor growth kinetics in the lungs of the mutant infected mice. \textit{Rag-/-} mice lack the capacity for VJD recombination and therefore have no functional B and T lymphocytes. They are considered the gold standard for evaluating the contribution of adaptive immunity to infection control in immunological studies. SCID mice have a similar defect as a part of a greater spectrum of severe combined immunodeficiency, including the absence of functional B and T lymphocytes. They are considered slightly “leakier” in terms of B and T lymphocyte function, but overall should recapitulate the same phenotype—leakiness in this case could have resulted in even less growth of the mutant, given that the presence of any functional B or T cells could contribute to the control of the mycobacterial infection. However, this was not the case. For the first five months, the Rv\textit{Δ}Lpr\textit{G-1410} mice recapitulated the survival curve seen with \textit{Rag-/-} mice infected with Rv::\textit{tn1410}. However, all mutant infected mice died at six months post-infection (Fig 11A). Mutant infected mice had disseminated mycobacterial disease with numerous
aggregates of viable and necrotic macrophages filled with acid-fast bacilli in the lung, spleen, heart and kidney indicating that at least in the absence of adaptive immunity, these mycobacteria did replicate and were able to cause death, albeit at a significantly slowed rate. WT and complement infected mice died by 48 days with $5 \times 10^7$ cfu and 60 days with $5.8 \times 10^7$ cfu in the spleen respectively at time of death (TOD). Mutant infected mice died by 185 days with $2.3 \times 10^6$ cfu in the spleen at time of death (Fig 11B). An additional cohort of SCID mice were aerosol infected with the $\text{Rv} \Delta \text{LprG-1410}$ mutant in parallel with those assigned to the survival group for the purpose of following bacterial cfu and histopathology in the mutant longitudinally during the survival experiment. Bacterial cfu from lung at time of death compared to the 24-hour inoculum approximates the in vivo doubling rate of the mutant in mice lacking adaptive immunity to be between 4-5 days compared to 2.4 days for the wild type and complimented strain (Fig 11C).

**Figure 11.** LprG-1410 function is necessary for normal growth rate and virulence of $\text{Mtb}$ in SCID mice lacking an adaptive immune system: SCID mice were infected via aerosol with 50-100 cfu of $\text{H37Rv}$ (black), $\text{Rv} \Delta \text{LprG-1410}$ (red), and $\text{Rv} \Delta \text{LprG-1410::LprG-1410}$ (blue) and followed for six months. WT and complement infected mice died by 60 days post-infection. Mutant infected mice died at 6 months post-infection (A). Colony-forming units in spleen of WT (black), mutant (red) and complement (blue) at time of death (TOD). Cfù in lung of mutant infected mice increases over time (C).
If the mutant were replicating at the same rate as wild-type *Mtb* but a greater proportion were being killed, we would anticipate differences in the type of pulmonary pathology, in particular, the degree of macrophage infiltrate, necrosis, and apoptosis, in the WT, complement, and mutant infected mice. These changes may have been probed to evaluate the type and quality of innate effector functions that contributed to infection control. However, this was not the case. There was minimal macrophage infiltrate (**Fig 12E**), scarce histopathologic evidence of bacterial replication or host cell necrosis compared to wild-type and complement infected mice (**Fig 12A,B,C**), and no gross pathology at matching time-points at 60 days post-infection (**Fig 12D**).
Figure 12. Gross and microscopic pathology of SCID mice infected with *Mtb* LprG-1410 mutants compared to wild type and complement infected SCID mice: Hematoxylin and eosin (H&E) of lung from mice infected with (A) Rv and (C) RvΔLprG-1410::LprG-1410 at time of death, 200x magnification. (B) Representative lung section from time sacrifices of mice infected with RvΔLprG-1410, 60 days post-infection, 200x magnification. Cfu recovered from remaining lung was (A) 1x10⁸ for WT (B) 5x10⁴ for mutant and (C) 1x10⁸ for complement-infected mice. (D) Fixed lung from WT (*Rv—right*), mutant (middle), and complement (left). (E) Inset of (B) showing small foci of macrophage infiltrate in mutant, 600x magnification.

We determined that the attenuation of the LprG-1410 mutant was directly correlated with the slowed growth rate due to the fact that the pathology associated with outgrowth of the mutant is
comparable at six months post-infection to that seen in WT and complement infected mice at 60 days post-infection. Large foci of viable and degenerative macrophages and acid-fast bacteria are noted in numerous organs including heart, kidneys, lung, and spleen at time of death (Fig 13). The fact that large numbers of bacteria were culturable from lung and spleen and that infection with the mutant uniformly killed the mice, indicated that mice infected with the mutant succumbed to disease in a parallel fashion as WT and complemented infected mice, only at a dramatically reduced rate.

**Figure 13. Ziehl-Neelson Acid Fast staining of SCID mice infected with *Mtb* LprG-1410 mutant at time of death.** Lung from representative SCID mouse infected with RvΔLprG-1410 at time of death (6 months post-infection). (A) Ziehl-Neelson acid-fast stain of lung section from lesion seen grossly in (C) showing large numbers of acid-fast mycobacteria within large aggregate of macrophages, 200x magnification. Cfu from lung was 1x10^6 at time of death. (B) Inset of (A), 600x magnification.

**SECTION 2.8. DISCUSSION.** Given the comparable histopathology of the mutant and large numbers of bacteria cultured from lung and spleen of the mutant infected SCID mice at time of death, we concluded that attenuation of the mutant and delayed time to death was a function of the growth rate *in vivo* being twice as long in mice with a dysfunctional LprG-1410 operon. We hypothesized, based on our inability to rescue growth in various immune deficient mouse backgrounds, that “immune” pressure, per say, was unlikely to be the cause of the slower
doubling time in vivo compared to in vitro. It is unknown whether the mutant would show the
same “slow growth” phenotype in the other immune deficient mouse strains such as the IFN-γ-/-,
Nos2-/-, Phox-/-, and AG Phox-/- mice using single strain infections. The experiments reported
for the above-mentioned immune deficient mice were mixed strain infections (competition
experiments) and the WT bacteria would have uniformly killed the mice, precluding any
determination of recoverability of the mutant strain at later time points. One interpretation of our
result is that loss of adaptive immune pressure did partially rescue growth, but it is clear that
neither oxidative killing by innate effector cells, loss of interferon-gamma mediated killing
during the innate immune response or down-stream of the adaptive immune response, nor B and
T cell mediated immunity could restore normal growth and virulence of the LprG-1410 mutant.

We predict that loss of LprG-1410 function results in an inherent metabolic defect of the
bacterium.

We hypothesize that growth conditions in the host that are independent of immunity likely
contribute to the conditional “expression” of this slow-growth phenotype. The fact that we
couldn’t recapitulate the growth arrest under standard culture conditions or in macrophages, led
us to hypothesize that stresses, such as carbon-source, oxygen tension, and pH, independently, or
collectively, render growth conditions in the mouse unfavorable for our mutant. Two
mycobacterial mutants described as having a similar phenotype, namely, normal growth under
standard culture conditions and in macrophages but lack growth in the mouse, are the isocitrate
lyase (icl) mutant and the PhoPR two-component regulator mutant. Isocitrate lyase is a key
enzyme in the glyoxylate shunt, a carbon utilization pathway used by mycobacteria persisting on
fatty acids (Wayne and Lin 1982). The icl mutant, however, is attenuated in activated
macrophages and grows in the mouse during the acute phase of infection, but cannot persist during chronic infection (McKinney, Höner zu Bentrup et al. 2000). In addition, growth of the icl mutant is rescued in IFN-γ -/- mice, explained by the finding that icl is upregulated in activated but not non-activated macrophages (McKinney, Höner zu Bentrup et al. 2000). Isocitrate lyase is preferentially required during chronic infection supporting the conclusion that mycobacteria subsist on fatty acids during chronic infection. The LprG-1410 mutant can still grow in activated macrophages, is attenuated in both acute and chronic infection of the mouse, and is not rescued in the IFN-γ -/- mouse background. Therefore, it is unlikely that LprG-1410 function falls within the pathway of the glyoxylate shunt. However, propionate is considered a toxic by product of odd-chain fatty acid catabolism and the glyoxylate shunt has been shown to be important for propionate detoxification (Gould, van de Langemheen et al. 2006, Eoh and Rhee 2014). This is important in light of the work to be presented in Chapter 4 where we investigate the susceptibility of our mutant to growth in propionate.

As it pertains to the phenotype of severe Mtb attenuation in the absence of adaptive immunity, the phoP mutation is equally attenuating in the SCID mouse as our LprG-1410 mutant in terms of survival, and likely more so, as Mtb with phoP mutations are being developed as a live vaccine strains and there are no reports of any pathologic change in SCID mice infected with phoP mutants out to six months (Martin, Williams et al. 2006). Incidentally, it is interesting that PhoP is part of a two-component regulatory system that mediates lipid metabolism in Mtb, including sulfolipid metabolism. The phoP mutation results in loss of sulfolipids (SL-1), diacyltrehaloses (DAT), and polyacyltrehaloses (PAT) (Walters, Dubnau et al. 2006). Furthermore, the phoP mutant has lower levels of the virulence lipid phthyciol-dimycoloserase
(PDIM) (Chesne-Seck, Barilone et al. 2008) (Lee, Krause et al. 2008). The lab-adapted apathogenic H37Ra strain has also been shown to lack production of SL-1, PAT, and DAT, and PDIM. The lack of production of SL-1, DAT, and PAT in H37Ra has been definitively linked to a point mutation resulting in a leucine to serine amino acid alteration in the PhoP protein in this strain. However, restoration of these lipids by expression of the wild-type version of phoP does not restore virulence of this strain in the murine model. Furthermore, triple knock-out of the biosynthetic genes for these three lipids classes ($\Delta mls3\Delta pks2$) does not result in a growth defect in vivo in mice, leading the authors to conclude that other PhoP regulatory targets alone or combination with loss of PDIM likely account for the in vivo attenuation of the H37Ra strain (Chesne-Seck, Barilone et al. 2008). Incidentally, one such operon controlled by phoP regulation is aprABC. The aprABC locus is linked to intraphagosomal pH sensing and phagosomal growth of Mtb in macrophages. Of interest to us is the fact that the H37Rv$\Delta aprABC$ mutant was shown to have increased levels of triacylglycerides (TAG) that is aprA dependent (Abramovitch, Rohde et al. 2011). Our lipidomics analyses of the Rv$\Delta LprG-1410$ and Rv::tn1410 outlined in Chapter 3, show that accumulation of triglyceride is a major lipidome alteration, which led us to question whether triglyceride accumulation could explain the immune pressure independent growth arrest of our strains in vivo.

As it pertains to in vivo growth phenotypes associated with alterations in TAG levels, the H37Rv$\Delta whiB3$ strain has also been reported to accumulate PDIM and TAG upon genetic disruption. The whiB3 has been shown to be a iron-responsive transcription factor, that regulates anabolism of the complex lipids, PAT, SL-1, DAT, PDIM, and TAG in response to oxidative/redox stress in Mtb (Steyn, Collins et al. 2002, Singh, Guidry et al. 2007, Singh,
Crossman et al. 2009). Mycobacterial lipids have been implicated in countering the redox stress that *Mtb* experiences upon exposure to the acidified phagosome and due to propionate metabolism when it persists on fatty acids *in vivo* as a carbon source during chronic infection. It was hypothesized that greater channeling of lipid precursors into PDIM and TAG with loss of this regulatory mechanism may offer enhanced protection during propionate stress. Indeed, WhiB3 mutants had a survival advantage under toxic levels of propionate (20mM) *in vitro* and RvΔwhiB3 mutants grew normally in macrophages and *in vivo* in the mouse model (Steyn, Collins et al. 2002). Given that both ΔaprABC and ΔwhiB3 mutants are reported to have dramatic lipidome alterations characterized not only by the loss of complex lipids, SL-1, DAT, PAT, and accumulation of TAG, yet no attenuation phenotype was noted in the mouse, we hypothesized that loss of key lipids and increases in TAG levels alone were unlikely to account for the severe attenuation we see with disruption of LprG-1410. Furthermore, the hypervirulent, *Mtb* Beijing strain is also reported to produce high levels of TAG (Reed, Gagneux et al. 2007), supporting a more complex linkage between the increased TAG in our LprG-1410 mutant and attenuation.

In summary, we originally hypothesized that attenuation was due to altered (enhanced) recognition by innate immune effector cells such as macrophages and neutrophils, or due to enhanced T cell priming of the adaptive immune response due to an altered lipid profile of our LprG-1410 mutant. It is thought that many TB surface lipids influence which monocyte and dendritic cell surface receptors are engaged by the bacterium during infection, leading to more “favorable” downstream events, and even thwarting phagolysosome fusion thereby evading host immune control of infection (Kang, Azad et al. 2005). However, these hypotheses have been
difficult to prove mechanistically due to surface receptor redundancy. We originally set out to test the ability of our mutant to alter the cytokine secretion profile of macrophages \textit{in vitro}, predicting that different lipid composition may skew the immune response to a Th1 response instead of the more “tolerant” Th2 response (Manca, Reed et al. 2004). We considered whether a different lipid profile could influence macrophage differentiation and therefore increase macrophage activation towards an M1 profile—also associated with enhanced mycobacterial killing (Kahnert, Seiler et al. 2006, Bouhlel, Derudas et al. 2007). We also hypothesized that perhaps enhanced immune recognition by innate or adaptive effectors would enhance clearing of the cells. All of these hypotheses were based on the idea that loss of a key immunomodulatory lipid would result in enhanced immune recognition and killing by the host leading to attenuation.

If this were the case, we would predict that there would be histopathologic evidence of innate effector (monocytic and neutrophilic) mediated killing and/or altered recruitment of cytotoxic T lymphocytes and NK cells to the site of infection. We began testing these hypotheses by evaluating recruitment of effector cells to lung by flow cytometry and immunohistochemistry (IHC) and by looking at expression of important cytokines such as iNOS and arginase by IHC. In all cases the mutant recruitment profile of lymphocytes (B and T), macrophages, and neutrophils was comparable in the relative proportions of cells arriving in lung in response to infection (data not shown). The main difference was that there were far fewer numbers of all these cells by histopathology (data not shown). Furthermore, monocytes did induce expression of both iNOS and arginase in response to infection with the mutant as seen by IHC (data not shown). Collectively, these data did not support the hypothesis of altered immune recognition, trafficking, or response to LprG-1410 mutants. The data support the hypothesis that fewer numbers of the LprG-1410 mutants, due to a slowed growth rate, resulted in the attenuation
phenotype. Small numbers of LprG-1410 mutant bacilli rendered their presence benign during the first months of infection, resulting in little pathology and minimal cellular recruitment. As numbers of these cells increased slowly over time, pathology and tissue damage followed, eventually leading to death.

**SECTION 2.9. METHODS.**

**SECTION 2.9.1 CULTURE OF MYCOBACTERIA.** Mycobacterial strains were grown in 7H9 with OADC (Middlebrook), 0.2% glycerol and Tween 80. For intravenous mouse infections, titered stocks were prepared, frozen, and thawed on the day of infection. For aerosol infections, titers were determined at designated O.D. 0.75- 0.80, and actively growing cells were used to prepare the aerosol inoculum to eliminate stochastic differential viability between WT and mutant strains during the freeze-thaw process.

**SECTION 2.9.2 CELL CULTURE AND MACROPHAGE INFECTIONS.** J774 murine macrophages and THP1 human monocytic macrophages were maintained in T75 flasks in DMEM. THP1 macrophages were activated overnight with 50 ng/ml phorbol myristate acetate (PMA) (Sigma) scraped and seeded into 24 well plates at 5x10^5 cells per well, 24 hours prior to infection. Murine bone marrow derived macrophages were isolated from 8 week-old C57/Bl6 mice as previously described (Weischenfeldt and Porse 2008) and differentiated in L929 conditioned, then maintained in R10 media. Mycobacteria were grown to an optical density (OD) 0.3. The day of infection, bacterial cultures where diluted in PBS, sonicated, and filtered with a 5um filter (Millipore) to achieve a single cell suspension. Filtered cells were left to settle for 15 minutes
and the upper 80% of the filtrate was removed and measured spectrophotometrically. An OD of 1.0 was calculated as equivalent to 3x10^8 bacterial cells/ml. Bacterial filtrate was then diluted into warm cell culture media to the desired concentration to achieve a multiplicity of infection (MOI) of either 1:1 (bacterial cells to cultured cells) or 10:1. Culture media was removed from 24 well plates and cells were washed once with 1xPBS. Cell culture media containing bacteria was then added to wells and incubated for 4 hours to allow for phagocytosis. After 4 hours media was removed and cells were washed with 1xPBS and fresh media added to cells. The bacterial inoculum was titered to confirm equal viability of the inoculum between strains. Macrophages were lysed with 0.1% TritonX100 after 4 hours, 24 hours, 3 days, and 5 days. For interferon-gamma (IFNγ) stimulation, recombinant murine IFNγ (Life Technologies) was added to culture media at concentration of 100U/ml at 48 hours post-infection of macrophages. Cells were maintained in IFNγ for 12-15 hours then washed.

**Section 2.9.3 Mouse strains.** Six- to eight-week-old mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The various mouse strains and their relevant immunological characteristics are shown in Table 1.

### Table 1. Mouse strains used for all experiments.

<table>
<thead>
<tr>
<th>Defect</th>
<th>Strain</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bl6</td>
<td>C57Bl/6J</td>
<td>Jackson</td>
</tr>
<tr>
<td>NOS2−/−</td>
<td>B6.129P2−Nos2tm1Lau/J</td>
<td>Jackson</td>
</tr>
<tr>
<td>Phox−/−</td>
<td>B6.129S6−Cjbltm1Dmy/J</td>
<td>Jackson</td>
</tr>
<tr>
<td>IFNγ−/−</td>
<td>B6.129S7−Ifngtm1Dy/J</td>
<td>Jackson</td>
</tr>
<tr>
<td>Rag1−/−</td>
<td>B6.129S7−Rag1tm1Dy/J</td>
<td>Jackson</td>
</tr>
<tr>
<td>SCID</td>
<td>B6.CB17−Prkdc−/−/Sz3</td>
<td>Jackson</td>
</tr>
</tbody>
</table>
Mice were housed under sterile conditions in a BSL3 facility, and all animal experiments were performed under an animal protocol approved by Harvard University.

**SECTION 2.9.4 Mouse infections.** Six-eight week old, female mice were acquired for all experiments. *Nos2-/-, Phox-/-, IFNγ-/-* were infected intravenously (i.v.) by tail vein injection with $10^6$ *M. tuberculosis* cfu. Infecting doses were confirmed by plating, and three to five mice per group were harvested at the given time points. Spleens and lungs were placed in 4 ml PBS, and homogenized with a tissue homogenizer. Homogenates were diluted and plated onto either Middlebrook 7H10 agar or Middlebrook 7H9+1.5% Bacto Agar (Difco) containing OADC enrichment (Middlebrook) and 0.2% glycerol. Plates were incubated at 37°C, and colonies were counted at three to four weeks. A subset of *Nos2-/-* received the reagent aminoguanidine *ad lib*. For survival of *Rag-/-* mice infected with *Rv* and *Rv::tn1410*, 13-14 mice per group were singly infected with the indicated strains and followed out to five months. For survival of SCID mice infected with *Rv, RvΔLprG-1410, and RvΔLprG-1410::LprG-1410*, five mice per group were infected via aerosol of 50-100 cfu and followed for 6 months. An additional 10 SCID mice were infected with *RvΔLprG-1410* and followed longitudinally. Groups of five mice were sacrificed at time of death of *Rv* and *RvΔLprG-1410::LprG-1410* in order to measure cfu and evaluate histopathology over time. For survival of SCID mice infected with *RvΔtgs1*, *Rv::tn1410*, *Rv::tn1410Δtgs1*, and *Rv::tn1410::LprG-1410* mice were infected via aerosol infection with 100-200 cfu and followed for 6 months.

For the Bl/6 and *NOS2-/-* competition experiments, the indicated strains were mixed at a ratio of 3:1, unmarked: marked. H37Rv is unmarked, *Rv::tn1410* is kanamycin-resistant, and *Rv::tn1410*
+ LprG-1410 is kanamycin and hygromycin resistant. To determine the burden of each strain, spleens and lungs were harvested and plated for colony-forming units on 7H10 agar with and without kanamycin (25 µg/mL), or with and without kanamycin (25 µg/mL) plus hygromycin (50µg/mL). For IFNγ-/-, Phox-/-, and aminoguanidine experiments, the indicated strains were mixed at a ratio of 3:1, marked: unmarked. Bacterial burdens were determined by plating in the presence or absence of kanamycin.

**SECTION 2.9.5 Histopathological analysis of mouse lungs.** Spleen, liver, kidney, heart and lung were collected in 10% formalin, fixed for 24 hours in 10% formalin and stored at 4°C in 70% ethanol. Lungs were inflated with 10% formalin prior to fixation. Fixed organs were embedded in paraffin, and lung sections were stained with hematoxylin-eosin, or Ziehl Neelsen Method acid-fast staining, and observed by light microscopy.
SECTION 2.10. REFERENCES


Chapter 3

LprG-1410 function is an important mediator of global lipid flux in *Mycobacterium tuberculosis*
**SECTION 3.1 ATTRIBUTIONS.** Cloning of protein expression vectors and LprG protein purification was performed by Lu Bai in the Seeliger Lab at Stonybrook University. Jahangir Iqbal of the Hussain Lab at SUNY Downstate Medical Center performed triglyceride transfer assays. Jennifer Han-Chun Tsai of the Sacchettini Lab at Texas A&M University performed LprG co-crystallization. Tan-Yun Cheng of the Moody Lab at Brigham and Women’s Hospital, assisted with collisional mass spectrometry and interpretation. All lipidomics analyses were performed in the Moody Lab whose members, especially Dr. Emilie Layre, provided the technical training, technical support, and reagents for the work presented here. Methods as written were provided by the authors attributed with slight modification. I want to thank Daniel Rubin for his tremendous help cloning the complementation vectors. I also want to acknowledge Zuri Sullivan’s efforts and support working with the BCG versions of the LprG-1410 mutation not presented here and for her general dedication and friendship during the two years I mentored her.

**SECTION 3.2. PREFACE.** Previous work in our laboratory showed the function of LprG -1410 in *Mycobacterium smegmatis* affected key features of the mycobacterial cell wall. Disruption of the operon resulted in increased uptake of the lipophilic dye, Congo Red, and decreased sliding motility, a characteristic attributed to the hydrophobicity of the cell wall and associated with glycopeptidolipid (GPL) in the apathogenic soil saprophyte, *Mycobacterium smegmatis* (Martinez, Torello et al. 1999, Recht, Martinez et al. 2000, Deshayes, Laval et al. 2005, Farrow and Rubin 2008). It is well known that *Mycobacterium tuberculosis* does not produce GPL (Schorey and Sweet 2008), furthermore, lipidomics analysis of *Mycobacterium smegmatis* LprG-1410 knock-out strains showed no major alterations in GPL levels (data not shown). Therefore,
we hypothesized that LprG-1410 function was important for translocation of another class of mycobacterial lipids, potentially important for virulence. A co-crystal of LprG bound to triacylated phosphoinositol mannoside (Ac$_2$PIM$_3$) had previously been published (Drage, Tsai et al. 2010), and we set out to identify potential LprG phospholipid substrates by whole cell lipidomic analysis. We anticipated that lack of a surface exposed phosphoinositol mannoside (PIM) was affecting trafficking of LprG-1410 in the phagosome and we predicted that the mutant would be more readily transferred to the phagolysosomal compartment, an outcome that would enhance killing of our LprG-1410 mutants and explain the attenuation seen in the mouse model.

However, as reported in Chapter 2, we were unable to show a macrophage growth defect, and initial lipidomics analyses using negative mode ionization (best for detecting phospholipids) failed to show any major alterations in phospholipids between the mutant and wild-type (WT) strains (data not show). For this reason, we questioned whether phospholipids represented the biologically important lipid substrate of the LprG-1410 transport system. We opted to take an unbiased approach to identifying potential lipid substrates using lipidomics. Here we report major alterations in lipid flux between WT and LprG-1410 mutants, with triacylglyceride accumulation representing a key lipid alteration that has profound implications for the observed \textit{in vivo} growth defect and growth rate alterations reported in Chapter 2.

\textbf{SECTION 3.3 LOSS OF LPRG-1410 FUNCTION RESULTS IN GLOBAL INCREASE IN LIPIDS COMPARED TO WT MYCOBACTERIUM TUBERCULOSIS}. Comparative lipidomic analysis was performed on lipids extracted from whole cells using chloroform and methanol before or after washes with the detergent dioctyl sulfosuccinate sodium salt (AOT) using normal phase, positive
mode high performance liquid chromatography/mass spectrometry (HPLC/MS). Disruption of LprG-1410 function results in global lipidome changes in RvΔLprG-1410 compared to wild-type cells. During logarithmic growth, approximately 1500-2000 of detectable lipid ions can be mapped using the MycoMass database (Layre, Sweet et al. 2011). Amongst mappable lipids with validated retention times, major classes of lipids include triacylglycerides (9%), cardiolipins (8%), diacyltrehaloses (8%), phosphatidylethanolamine (7%), and triacyltrehaloses (6%) (Fig 14).
Figure 14. Distribution of ions mapping to *Mtb* lipids from logarithmically growing cells: Percent condensed, automatically annotated ions identified across all strains analyzed. Raw mzData files were converted using MassHunter, processed in R using XCMS, and peaks deconvoluted and aligned across samples. The aligned output consisted of an accurate mass, retention time (RT), and intensity for each peak. Data was imported into excel and uploaded into the MycoMass database for automated ion annotation. Ion annotations were condensed as described above. Ions mapping to triacylglycerides (TAG) noted in red. Ions mapping to phospholipids in grey: Cardiolipin (CL), phosphoinositol (PI), phosphatidyglycerol (PG), phosphatidylethanolamine (PE), phosphatidic acid (PA). Ions with 2 or less species mapping to a class or intermediates (such as Lyso-PI) were included in “Other.” Diacylated sulfolipid (Ac₂SGL) was <0.01%. *n*=1448.

Lipid alterations with disruption of the LprG-1410 operon were evaluated using GenePattern (v2.0, Broad Institute, Cambridge MA) (Reich, Liefeld et al. 2006), utilizing a filter of two-fold increase in ion counts over wild type levels at *p*<0.01. Analysis was limited to known TB lipids as annotated in the MycoMass database (Layre, Sweet et al. 2011). Unknown ions represent a
combination of ions generated from contaminants, ions from media, and uncharacterized TB lipids, and were excluded from the analysis. The decision to focus on changes in characterized TB lipids was based upon preliminary lipidomics evaluation of the WT and mutant TB strains including a search for increase or decrease in uncharacterized TB lipids (no MycoMass ID assigned). No overlap between replicates was identifiable for fold change analysis of unmappable ions (data not shown). Following MycoMass annotation of lipids with two-fold or greater increase in the mutant strain, manual verification of lipid class was performed based on known retention times of TB lipids, chemical nature of the lipid species, and predicted separation behavior using normal phase liquid chromatography. For example, ions that mapped to phospholipid species (hydrophilic and predicted to elute late on the column) were eliminated from analysis if the corresponding retention time overlapped with hydrophobic lipids (known to elute first on the column). As a general rule, ions that mapped to phosphatidylethanolamine (PE), phosphatidic acid (PA), phosphatidylinositol (PI), lyso-phosphatidic acid (LPA), phosphatidylglycerol (PG), lysophosphoinositol (LPI), and other phospholipid species were eliminated from analysis if the retention time was less than 8 minutes. In addition, ions that mapped to lipids known to not be synthesized by Mycobacterium tuberculosis (Mtb), such as glycopeptidolipids (GPL), were also excluded from analysis (Schorey and Sweet 2008). The Mycomass database provides up to five lipid matches based on the mass-to-charge (m/z) ratio. Matches are assigned based on the measured m/z to predicted m/z by structure with the first match providing an assignment within 10 ppm for the calculated m/z. In cases where the first match did not fit with the biological behavior on the column, but the second match did, the second “best” match was used for lipid annotation. For the purposes of this unbiased screen we felt that this methodology, while potentially prematurely discarding useful data about lipid flux,
would decrease the false positive hit rate, and provide a good first pass screen for major changes in lipid perturbations by disruption of the LprG-1410 operon. On average, this screening and mapping method allows for consistent identification of roughly 1500-2000 mappable TB lipids. This method was used repeatedly over a number of growth conditions and lipid loading and the breadth of percent ions mapping to the various lipids in the TB lipidome remained constant confirming that this work-flow allowed for adequate detection of all major classes of TB lipids (Fig 15).

**Figure 15: Volcano plot comparing lipidome of LprG-1410 mutant compared to wild type *Mtb*.** H37Rv (WT) and ΔLprG-1410 were grown on plates in triplicate and lipids extracted from cell pellets by successive chloroform: methanol washes. Lipid was normalized by weight and 5μg of lipid from each strain was evaluated by LC/MS. Data was integrated into GenePattern (Broad Institute). Lipidomes of ΔLprG-1410 and WT were compared. Lipid ions present at two-fold greater in mutant compared to WT at a p-value <0.01 are highlighted in red. Ions were mapped to lipid species using the MycoMass database. IDs were manually cross-referenced by retention time. Ions with inaccurate RT were eliminated from analysis.
SECTION 3.4 OVEREXPRESSION OF LprG-1410 DECREASES TRIACYLGLYCERIDE LEVELS IN Mtb DURING LOG PHASE GROWTH. Lipidomes were initially screened looking for decrease in lipids in the mutant compared to WT using both positive mode and negative mode HPLC/MS. It became evident early in the investigation that there were no major gain or loss of lipids in the mutant compared to WT during log phase growth in 7H9 media with oleic acid supplementation (data not shown). However, large increases in lipids were noted in the mutant compared to the complement and overexpression strains. TAG represented 61% of lipid ions increased in the mutant compared to the LprG-1410 overexpression strain and 40% of the lipid ions increased in the mutant compared to the LprG-1410 complemented strain. Of interest, PDIMs, phosphatidylglycerols (PG) and diacylglycerol lipid ions were also predicted to be increased in the mutant compared to the LprG-1410 overexpressor and the LprG-1410 complement, but these changes represented a much smaller proportion of the total ions changing with loss of the LprG-1410 operon. It is important to note that ions were assigned to lipid classes based on automated annotation using the MycoMass database and represent both confirmed and unconfirmed matches. Collectively, this first pass screen supports the hypothesis that overexpression of LprG-1410 decreases triacylglyceride levels in Mtb during the logarithmic growth phase (Figure 16).
Figure 16: Triacylglycerides are decreased with overexpression of LprG-1410. Cells were collected during log phase growth at OD 0.6 +/- 0.1. Lipids were extracted, centrifuged, and dried. LC/MS was performed on 5ug of lipid from WT (H37Rv), Mutant (ΔLprG-1410), Complement (ΔLprG-1410::pLprG-1410), and Overexpression (Rv::LprG-1410). Ion counts were integrated in GenePattern (Broad Institute, Cambridge, MA) and percent ions representing two fold change or greater with A) Overexpression (n= 54) or B) Complementation (n= 94) compared to mutant are shown.

Overexpression was confirmed by western blot for native LprG (data not shown). Given that triacylglyceride accumulation is typically associated with stationary phase growth or TB dormancy (Deb, Daniel et al. 2006, Fang, Wallqvist et al. 2012, Sartain, Dick et al. 2011) we predicted that physiologically LprG-1410 may not normally be expressed at high levels during log phase growth explaining the few lipidomic differences between the WT and mutant strains under these conditions. For this reason, all future analyses focused on cells isolated from plates or from stationary phase cultures.

SECTION 3.5. DISRUPTION OF THE LPRG-1410 OPERON RESULTS IN TRIACYLGLYCERIDE ACCUMULATION DURING STATIONARY PHASE GROWTH. Triacylglyceride increases were most prominent during stationary phase growth and from plates consistent with what has been
previously reported (Sartain, Dick et al. 2011). Lipidomics analyses on plate grown cells collected four weeks post-plating showed that 28% of ions increased in the mutant compared to WT mapped to triacylglycerides, followed by phosphatidylethanolamine (PE) at 19%, and diacyltrehalose (DAT) at 12% (Fig 17).

Figure 17: Triacylglycerides are increased with disruption of the LprG-1410 operon during stationary phase growth. A, Percent of total ions annotated by class on plate grown cells harvested at 4 weeks using Mycomass identification and retention time verification, \( n = 1470 \). B, Percent ions annotated by class with two fold or greater increase in RvΔLprG-1410 compared to H37Rv, \( p < 0.01, \ n = 91 \). Triacylglyceride (TAG) highlighted in red; phospholipids including phosphatidylethanolamine (PE), phosphatidylinositol (PI), cardiolipin (CL), phosphatidylglycerol (PG), and phosphatidic acid (PA) shown in purple.

TAG identity was confirmed using tandem mass spectrometry (MS/MS) for selected ions that were increased in the mutant compared to wild type. Figure 17 (A) shows the predicted fragmentation pattern for TAG 52:1. This TAG species was predicted to contain two 18 carbon
acyl chains and one 16 carbon acyl chain with one unsaturation resulting in the mass-to-charge ratio (m/z) 876.79 present in the lipid sample as seen in the extracted ion chromatogram (EIC) (Fig 18A). The measured m/z of 876.79 is consistent with the addition of an ammonium charge (NH3+) to the predicted uncharged m/z of 859.77. The parent ion was identified and collided. Collision induced disassociation (CID) yielded the two major predicted product ions 577.519 and 603.535 (Fig 18B,C).

**Figure 18: Collisional mass spectrometry on triacylglyceride accumulated in LprG-1410 mutant.** (A) Calculated mass and fragmentation pattern predicted for TAG 52:1 based on MS/MS and extracted ion chromatogram (BPC product) from HPLC/MS performed to isolate m/z 876.80 from *RvΔLprG1410* derived lipid. (B,C) Collision-induced disassociation of the parent ion resulted in the formation of two predicted product ions 577.51 m/z (B) and 603.52 m/z (C).
Section 3.6 Disruption of \textit{Rv1410} is sufficient for triglyceride accumulation. In order to increase confidence that TAG accumulation was specific to disruption of the LprG-1410 operon, additional lipidomics analyses on \textit{Rv::tn1410} was performed in parallel with \textit{RvΔLprG-1410} to look for overlap in the lipid accumulation phenotype. Increased levels of triacylglycerides ranging from small (TAG 36:0) to large (TAG 60:2) was noted in both \textit{RvΔLprG1410} and \textit{Rv::tn1410} compared to WT. Fold change increases ranged from 2-100 fold depending on the strain evaluated and the TAG species (Fig 19).

\textbf{Figure 19.} Bar graph of ion counts mapping to triacylglyceride from comparative lipidomics. WT (H37Rv) and two mutant strains (H37RvΔLprG-1410 and Rv::tn1410) were grown in triplicate on 7H9 plates. Cells were scrapped and lipids extracted in successive rounds of chloroform: methanol (2:1, 1:1 1:2). Solubilized lipids were separated from the remaining cell pellet, dried, normalized by weight and 5 ug loaded for LC/MS analysis. Ions were aligned using R (v2.15.2, R Foundation for Statistical Computing, Vienna, Austria) and run through the MycoMass database. Bar graph of ion counts mapping to triglyceride that eluted from column at retention time 0-5 minutes. Red=\textit{RvΔLprG-1410}; Orange=\textit{Rv::tn1410}; Black=\textit{Rv}. Error bars, mean +/-SD.
Overlap of lipid species with mappable ions representing nine fold or higher change in both mutant strains compared to WT is shown below (Fig 20).

Figure 20. Venn diagram of lipid classes with increases in LprG-1410 mutants. Overlap of lipid ions annotated by Mycomass ID that were increased 9 fold or greater (p<0.01) in mutant compared to WT. Ions were mapped with MycoMass and condensed after retention time verification. Ions were grouped into lipid classes based on automated annotation. Shown, lipid classes in which ions were 9 ≥ fold increased in the mutant (Rv::tn1410 or RvΔLprG-1410) compared to WT. n= 99, lipid ions in Rv::tn1410; n=39 lipid ions in RvΔLprG-1410; Total number of ions representing class overlap, n=19. Number of individual ion species mapping to class in ()

The more stringent cut-off of nine fold was necessary in order to separate out the more numerous lipid fluctuations (in the two-fold to ten fold range) in Rv::tn1410 compared to WT. Rv::tn1410 had 99 mappable ions that were nine fold or greater increased at p<0.01, RvΔLprG-1410 had 39 respectively. This increased variation in the lipidome between H37Rv and Rv::tn1410 reflects the increased variability in lipid profiles between non-isogenic parent strains. Importantly the TAG accumulation phenotype was present in both the Rv::tn1410 and RvΔLprG-1410 mutant
strains. By evaluating the three strains (H37Rv, Rv::tn1410, RvΔLprG-1410) we felt we could increase confidence that overlap in lipid perturbations between the strains reflected the contribution of the LprG-1410 operon, specifically that of Rv1410 to the resultant lipid profile, which shows that loss of Rv1410 is sufficient for the TAG accumulation phenotype. This is consistent with our model predicting the function of Rv1410 to be epistatic to that of LprG. Automated annotation suggests that both mutant strains had increases in carboxymycobactins (CM, n=2), monodioxycarboxymycobactins (MDCM, n=3), and triacylglycerides (TAG, n=7). However, given the secreted nature of CM and the low abundance of MDCM, there is a high probability that these are false positive hits due to misannotation. Automated annotation is only used as a screening mechanism to prioritize lipid hits for further validation and characterization. TAG species represented the area of greatest overlap between the two mutant strains with increased levels of seven TAG species between the two strains indicating that TAG increases represented 28% of the overlapping lipid accumulation profile. The fact that this overlap was present at the more stringent cut-off of 9 fold increase over wild-type at a p-value of <0.01 reflects the magnitude of the lipid perturbation in regards to the TAG accumulation phenotype compared to other lipid alterations in the strains. In addition, TAG represented the lipid class with the greatest proportion of ions increased greater than two-fold compared to Rv at p<0.01 in RvΔLprG-1410 (25%) and Rv::tn1410 (19%) (data not shown). For this reason, TAG was chosen for further investigation and validation.

TAGs composed of 50-60 carbons were consistently increased greater than five fold and with dominant ion (990.94 m/z) in the TAG 60 series representing TAG 60:1, increased 43 fold on average across the Rv::tn1410 replicates (p=2.76^{-05}) and 31 fold on average in the RvΔLprG-
$1410 \ (p=0.002) \ (\text{Fig 21A})$. Complete overlap with the triglyceride accumulation phenotype was noted for the 60-62 carbon series between the two mutant strains (Fig 21B).

**Figure 21:** Extracted ion chromatogram and mass spectra of elevated triacylglyceride in LprG-1410 mutants. (A) Extracted ion chromatogram (EIC) for m/z 990.94, predicted ion for TAG 60:1 by MycoMass. Overlay of three replicates each of RvΔLprG-1410 and Rv::tn1410 in red (Mut); three replicates of H37Rv in black (WT). (B) Predicted structure of TAG 60:1. (C) Mass spectra extracted at 3 minutes showing 60 carbon TAG series: TAG 60:2 (m/z 988.93), TAG 60:1 (m/z 990.94), and TAG 60:0 (m/z 992.95).
TAG accumulation was reversible with complementation of the LprG-1410 operon. Large TAGs ranging from 54-63 carbon TAG species consistently showed three fold or greater ion counts in mutant compared to wild type. A trend towards reversible accumulation was seen across most TAG species, with statistically significant complementation present for TAG 59:1 (3 fold), TAG 59:2 (2 fold), TAG 61:2 (3 fold) and TAG 62:2 (2 fold) as shown below (Fig 22).

**Figure 22: Complementation with LprG-1410 reverses TAG accumulation during stationary phase growth.** Bar graph of ion events mapping to 56-62 carbon TAG species. Error bars, mean +/- SD. * p<0.05, ** p<0.01, ***p<0.001, 2-way ANOVA with Bonferroni post-test. * Mut vs. WT, † Mut vs. Comp. Lipids were extracted from plate grown WT (H37Rv), Mutant (RvΔLprG-1410), and Compliment (RvΔLprG-1410::LprG-1410) after 4 weeks of growth. HPLC/MS was performed and comparative lipidomics performed.

Based on automated annotation, other complementable changes in the lipidome included increased levels of triacyltrehalose (TAT), diacyltrehalose (DAT), and diacylglycerol (DAG). These lipid changes may reflect other possible substrates for LprG-1410 transport but the magnitude and significance levels for these changes were inconsistent across biological
replicates and for this reason were not evaluated further. It is interesting to note that within more than one run, alterations in phospholipids such as phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and phosphatidylinositol (PI) were noted in the mutant strain as indicated (Fig 23, left). However, this lipid flux was never complementable by automated annotation under any condition tested (Fig 23, right). Modest alterations in diacylglycerol (DAG) levels were also noted in the mutant, typically ranging 2-3 fold for a smaller number of DAG species compared to fluctuations in TAG. Further validation of the identity of the events mapping to DAG is necessary, but these changes were complementable. DAG is a common precursor to both TAG and phospholipid, and perturbations in this lipid may have been difficult to detect using comparative lipidomics methodology if precursors are rapidly converted as substrates into other lipids. However, if DAG levels are also affected by the function of LprG-1410, this may contribute to some of the differences in the PE levels predicted in the mutant by automated annotation.
Figure 23. Percentage of events mapping to lipid classes by automated annotation among those increased in LprG-1410 mutant compared to wild type and complemented cells. Lipids were extracted from plate grown WT (H37Rv), Mutant (RvΔLprG-1410), and Compliment (RvΔLprG-1410::LprG-1410). Ions increased greater than two-fold at p<0.05 in the mutant compared to WT (n=160) and mutant compared to complement (n=86) were mapped with MycoMass and condensed after retention time verification. Pie graphs representing percentage of events mapping to lipid classes by automated MycoMass annotation. Total mapped ions, n = 1342.

**SECTION 3.7. LPRG-1410 FUNCTION AFFECTS INTRACELLULAR LEVELS OF TRIACYLGLYCERIDES.** Accumulation of intracellular triacylglycerides with disruption of the LprG-1410 operon was also seen using a combination of reverse micellar extraction of outer membrane lipids (OM) and traditional chloroform: methanol extraction of inner membrane lipids (Bansal-Mutalik and Nikaido 2011, Bansal-Mutalik and Nikaido 2014). This methodology allows for stripping of lipids from the outermost portion of the cell wall using a combination of hexane and dioctyl sulfoisuccinate salt (AOT) to allow for evaluation of intracellular and inner membrane bound lipids post-AOT extraction. This methodology improved detection of some less abundant lipids, presumably due to increased extractability consistent with what was previously reported by Nikaido et al. (Fig 24A). No significant differences in the major phospholipid classes were detected (Fig 24B). This experiment was only performed once due to technical difficulties and the results need to be validated.
Figure 24: Inner membrane lipids from stationary phase liquid cultures mapped using automated annotation. H37Rv (black, WT), RvΔLprG-1410 (red, Mut), RvΔLprG-1410::pLprG-1410 (dark blue, Comp), and Rv::LprG-1410 (aqua, OE) were grown in 7H9 +OADC without Tween for 4 weeks. Cells were centrifuged and supernatant removed. Outer membrane (OM) lipids from the cell pellet were extracted twice in Heptane/10mM AOT. Inner membrane lipids (IM) were extracted with 2:1 chloroform: methanol, separated, dried and normalized. Comparative lipidomics was performed with HPLC/MS and ions mapped with Mycomass and validated based on retention time. (A) Percent ions of total ions annotated by lipid classes across all strains. (B) Bar graph of ion event counts based on automated annotation for representative lipid classes.
Using this methodology, a more distinct separation in classes of lipids that accumulate in the mutant compared to WT were noted with DAT representing 26%, PDIM representing 21%, and triglycerides representing 17% of events annotated and increased two fold or greater in the mutant compared to WT. “Other” represents lipid classes where 5% or less of ions mapped (Fig 25 A,B).
Figure 25: Comparative lipidomics on inner membrane lipids from WT and LprG-1410 mutant *Mtb* using automated annotation. Cells were grown for 4 weeks in liquid culture to OD 3. Cells were washed twice with heptane 10mM AOT and pelleted. The pellet represents inner membrane (IM) fraction. The IM was then extracted with chloroform: methanol 2:1, solubilized lipids dried and normalized. LC/MS was performed on 2.5 ug of lipid from H37Rv (WT), ΔLprG-1410 (MUT), ΔLprG-1410::LprG-1410 (Comp), and Rv::LprG-1410 (OE). Data was integrated in to GenePattern and lipids increased in Mutant compared to WT in a volcano plot (A). Percent ions mapping to TAG (red), DAT (blue), and PDIM (green) are shown in (A,B). Bar graph of ion counts mapped to TAG species by MycoMass (C).
Sulfolipids and PDIM are thought to predominantly be surfaced exposed and therefore large amounts of these lipids were likely removed during the heptane/AOT wash. Fluctuations in the presence of these lipids intracellularly could also shed insight into the lipid specificity for LprG-1410. It is interesting to note that both PDIM and DAT are predicted to have intracellular increases based on automated annotation in addition to TAG using this methodology. PDIM has many structural similarities to TAG and is thought to bind to LppX, another bacterial lipoprotein with structural similarity to LprG (Sulzenbacher, Canaan et al. 2006). LppX is reported to have a smaller lipid-binding pocket than LprG and therefore the larger binding pocket of LprG could likely also accommodate PDIM. Unfortunately, given the nature of spontaneous PDIM loss with continuous passage, we did not feel confident evaluating the potential contribution of LprG on PDIM transport in these studies.

TAG accumulation in the ΔLprG-1410 mutant was consistent compared to WT and overexpression strains (OE) and represented the only phenotype that was complementable using this methodology. The accumulation phenotype was demonstrated for TAG containing 59-68 carbons (TAG 59:0-TAG 68:0) with the most prominent effect being evident for TAG (60-62) (Fig 25C) consistent with evaluation of whole cell lipid extracts. Comparison between the AOT (outer membrane) fractions of these strains was attempted but could not be quantitatively compared between strains due to the inability to eliminate AOT from the lipid preps. AOT is a large molecule with a molecular weight of FW 444.56. The presence of the molecule precluded normalization of lipid loading for mass spectrometry. Ongoing studies using other normalization methods to a known standard or internal standard are currently being developed to quantitatively assess the presence of TAG in outer membrane fractions of the mutant.
SECTION 3.8. TRIACYLGLYCERIDES ARE FEATURE OF THE MYCOBACTERIUM TUBERCULOSIS OUTER MEMBRANE. Heptane/AOT derived lipid extracts representing the outer membrane fraction of *Mtb* show enrichment of PDIM and sulfolipids consistent with previous reports indicating the presence of these lipids in the outer portions of the mycobacterial cell wall. Heptane/AOT extracts show the presence of triacylglycerides in the outer membrane of *Mtb* (Fig 26).

**Figure 26. Levels of outer membrane and inner membrane triacylglycerides from WT *Mtb*.** H37Rv was grown in 7H9 +OADC without Tween in triplicate for 4 weeks. Cells were pelleted by centrifugation. Supernatant was removed and outer membrane (OM) lipids were extracted twice with heptane/10mM AOT and combined. Lipids and AOT were dried. The remaining pellet was extracted with 2:1 chloroform methanol. An equal amount of AOT/lipid (OM) and inner membrane (IM) lipid preparation was evaluated by HPLC/MS. Bar graph of 59-68 carbon TAG species as mapped by MycoMass and retention time validated.
Hexane only extraction of broth-grown and plate grown \textit{Mtb} was also performed. This extraction protocol is considered to be a “gentle” extraction method that selectively extracts the outer-most lipids of the \textit{Mtb} cell wall. Reverse micellar extraction was more effective at retrieving triacylglycerides than hexanes alone (data not shown) suggesting that triacylglycerides are located intermediately within the outer membrane of \textit{Mtb}.

\textbf{SECTION 3.9. OVEREXPRESSION OF LPRG-1410 INCREASES PRESENCE OF TRIACYLGlycerIDES IN SUPERNATANTS OF MYCOBACTERIUM TUBERCULOSIS.} Given that we predicted LprG-1410 was a TAG transporter, we predicted we might be able to detect differences in TAG in supernatants from WT versus mutant cells. Here we show that TAG are normally present in low levels in supernatants in wild type, ΔLprG-1410, and complemented strains consistent with our prediction that TAG are intermediately located within the cell wall. However, overexpression of LprG-1410 on a wild type background increases the presence of TAGs (Fig 27A). In order to normalize for potential unequal shedding of cell wall lipid based on the effect of the LprG-1410 mutation, we normalized internally to total cell wall lipids and show that the ratio of TAG in supernatant lipid compared to an equal amount of lipid prepped from the whole cells via chloroform: methanol extraction is still higher for the overexpression strain (Fig 27B).
Figure 27: Triacylglycerides in supernatants from log phase growth of *Mtb*. (A) Extracted ion chromatogram for TAG 60:1 (m/z 990.94) for H37Rv (WT), RvΔLprG1410 (Mut), RvΔLprG1410::LprG1410 (compliment), and Rv::LprG1410 (OE). (B) Bar graph of ratio of ion counts for TAG 60:1 based on 10ug of lipid loaded for HPLC/MS. Ratio is expressed as # counts for m/z 990.94 from supernatant (Lipid Sup) to # ion counts for m/z 990.94 from chloroform:methanol extraction (CMW).

**SECTION 3.10. LPRG TRANSFERS TRIACYLGLYCERIDE ACROSS VESICLES.** Purified HIS-tagged mycobacterial lipoproteins LprG, LprGV91W, LprA, and LppX were generated by expression in *Escherichia coli* and purified. Lipoproteins were added to equimolar mixtures of donor vesicles containing fluorescently labeled yet quenched TAG and acceptor vesicles. Percent transfer between vesicles was measured as an increase in fluorescence in the acceptor vesicle fraction over time. The specificity of LprG for binding and transfer of TAG in this assay but not LprGV91W, LprA, or LppX suggests that although the large binding pocket of LprG is structurally predicted to accommodate a number of small to large lipid species with a range of
two-four acyl chains, there is specificity in the function of this lipoprotein in triglyceride transfer between membranes (Fig 28).

![Graph](image)

**Figure 28. Mtb LprG transports triacylglyceride (TAG) between lipid bilayers.** The movement of NBD-labeled TAG from donor vesicles with high TAG content to acceptor vesicles without TAG is detected as an increase in NBD fluorescence due to dequenching. A truncated soluble construct of LprG (lacking the N-terminal secretion signal and lipidated cysteine) can mediate this transfer. The V91W mutation (LprG-VW) abrogates activity. Analogous constructs of LprA and LppX have no activity. Error bars show standard deviation of three technical replicates. Only one replicate each was performed for LprA and LppX.

**SECTION 3.11. LPRG CO-CRYSTALLIZES WITH TRIACYLGLYCERIDE.** It has been previously published that the binding cavity of LprG can accommodate triacylated lipid species. Specifically, it has previously reported that LprG co-crystallizes with triacylated phosphoinositol mannoside (Ac₂PIM₃) by Drage et al. (Drage, Tsai et al. 2010). This study primarily focused on
potential phospholipid binding substrates of LprG and the analysis was limited to negative mode ionization which is not ideal for detecting triglycerides. Given our data showing transfer activity of LprG for TAG, we wanted to confirm structurally that TAG can bind in the pocket of LprG. Here we show that LprG does indeed bind triacylglyceride and co-crystallizes with LprG (Fig 29). Interestingly, in this crystal, only two acyl chains fit inside the pocket of LprG with the third acyl chain interacting with a hydrophobic groove on the surface of LprG. This may permit LprG binding of TAG with longer acyl chains (Han Chun-Tsai personal communication).

**Figure 29:** *Mtb* LprG co-crystallized with tripalmitate TAG 48:0.

**SECTION 3.12 DISCUSSION.** Previous work on LprG-1410 structure and function has highlighted the importance of gene function for survival in the mouse (Bigi, Gioffré et al. 2004), resistance to oxidative stress (Ramón-García, Martín et al. 2009), TLR2 activation in the presence of bound phospholipid ligands (Drage, Pecora et al. 2009), and susceptibility to malachite green stress
(Bianco, Blanco et al. 2012). Most of the existing research on the lipoprotein LprG has focused on its potential role in phospholipid binding and its potential role in the host-pathogen interaction as it pertains to activation of the innate immune response via TLR2 activation. The arguments as presented suggest that when LprG is bound to its cognate lipid, hypothesized to be an immunomodulatory PIM, activation of TLR2 would initiate a cascade of events leading to an innate immune response. This model suggests that LprG binding of lipid should effectively enhance immune recognition. Given this model, we would anticipate that loss of LprG would actually benefit the bacterium by allowing it to evade recognition by the innate immune response and therefore enhance survival in immune competent mice, the opposite of what is reported. Numerous studies have called into question the specific role of TLR2 activation on Mtb pathogenesis in that TLR2-/- mice are not hypersusceptible to infection, pathology, or general disease progression (Hölscher, Reiling et al. 2008).

For this reason, we predicted that LprG’s lipid binding role was likely important for the structural integrity of the cell wall. We hypothesized that perhaps LprG and 1410 worked together to position a structurally important phospholipid in the cell wall explaining it’s severe attenuation in the mouse model. However, our initial in vitro studies showing normal growth under standard culture conditions and minimal growth defect in macrophages placed this hypothesis lower on the list. Regardless, we pursued an unbiased comparative lipidomic analysis looking for alterations in phospholipid synthesis given the structural and binding data reported (Drage, Tsai et al. 2010). Using negative mode ionization (reported to be more effective for ionizing phospholipid species) we found minimal differences in phospholipid levels compared to WT Mtb. The most prominent lipidomic alteration across both positive mode and negative mode
comparative lipidomic analysis pointed us to an accumulation phenotype of triacylglycerides. In fact we initially disregarded these findings attributing the alteration to a generalized stress response of the mutant. After repeated efforts to identify “another” lipid alteration that could explain the growth attenuation in the mice we began to seriously consider triacylglyceride as the substrate for our hypothesized lipid transport system. In retrospect, this was not far-fetched. LprG had been structurally predicted to preferentially bind triacylated lipids. Given the hypothesized importance of LprG in binding phospholipids, previously work had used negative mode ionization methodologies thereby potentially “missing” this important lipid. Although we feel confident that LprG does have the capacity to bind phospholipid species, here we report that the biologically important binding partner as it pertains to the growth defect seen LprG-1410 mutants in mice, is triacylglyceride.

This work shows that the main lipidomic alteration in both single (Rv::tn1410) and double (RvΔLprG-1410) LprG-1410 mutants is accumulation of triacylglyceride species. It is important to note that LprG is positioned upstream to Rv1410c in the operon, therefore lipidomics analysis of the Rv::tn1410 mutants should represent a strain with normally expressed levels of LprG. This predicts that Rv1410 functions epistatically to that of LprG as it pertains to the growth phenotype observed in mice. This only makes sense if Rv1410 is restricting LprG access to an important lipid-binding partner. Given that Bigi et al. report a continued growth defect in mice with single LprG mutants, and the fact that single transposon mutants in LprG, as reported by Sassetti et al. (Sassetti and Rubin 2003, are also highly underrepresented in vivo, it follows that there must be a lipid species or class of lipids that are important for TB growth in the mouse that
are transported by the combined function of LprG-1410 even if LprG is potentially promiscuous in its lipid binding partners (phospholipids and triacylglycerides).

Here we show that disruption of the LprG-1410 operon disrupts global lipid flux in *Mycobacterium tuberculosis* with the predominant, complementable lipid alteration being an increase in total (cell wall and intracellular) triacylglyceride, and more specifically intracellular levels of TAG. We show that not only can LprG co-crystallize with TAG but it can also transfer TAG in a vesicle transfer assay. We show that, indeed, TAG is a component of the outer cell wall of mycobacteria—a fact that has intermittently been reported for *Mycobacterium smegmatis* (Bansal-Mutalik and Nikaido 2014) and proposed for *Mycobacterium tuberculosis* (Ortalo-Magné, Lemassu et al. 1996), but never before reported or confirmed in *Mycobacterium tuberculosis*. Most importantly, we describe a completely novel feature of prokaryotic triacylglyceride biology, in reporting that triacylglyceride transport may be an important feature of cell homeostasis and metabolic regulation akin to what is described for eukaryotic cells.

It is well known that in yeast, mammalian cells, and plants that triglyceride levels and transport are important metabolic regulators of cell function. In yeast, the transition from log phase growth to stationary phase growth is characterized by a switch from phospholipid metabolism to triglyceride metabolism based on the specificity of growth phase dependent levels of TAG and phospholipid precursor molecules and efficiency of the various diacylglycerol transferases (DGATs) in incorporating precursor molecules into these down-stream lipids (Horvath, Wagner et al.). In Arabidopsis triglyceride levels regulate seed formation and germination based on the availability of TAG as a storage lipid. Furthermore, a role for TAG in membrane lipid
remodeling of the thylakoid of senescent leaves has also been proposed (Li-Beisson, Shorrosh et al. 2013). In mammals, transfer of triglyceride is systemic with movement of lipoprotein-bound triglyceride in plasma to distant parts of the organism by the blood-stream. Triglycerides are then imported into eukaryotic cells by transfer lipoproteins, which upon uptake, lead to subsequent intracellular signaling and cellular function. It is already well accepted that the mycobacterial metabolic state and response to stresses such as acid and pH (Baker, Johnson et al. 2014), hypoxia, and antibiotic susceptibility (Baek, Li et al. 2011) are correlated with increases in intracellular triglyceride levels, presumably as a function of increased triglyceride synthesis as mycobacteria transition to a non-growing state. Therefore it is not unreasonable to expand our concept of mycobacterial growth regulation as a function of triacylglyceride transport across the cell wall in addition to synthesis as a means to 1) quickly adapt growth rate to environmental stresses 2) store lipid precursors in a cellular compartment that may also be home to important lipases and 3) interact with the host macrophage.

Given these data, we present a model whereby LprG-1410 function is consistently “ON” during normal growth of mycobacteria under favorable conditions. We would predict that LprG-1410 function would be down regulated under conditions of stress, such as hypoxia, acid, antibiotic stress, and starvation thereby allowing intracellular accumulation of TAG precipitating growth arrest and potentially transition to a “dormant” like state. Existing expression data profiling of LprG-1410 has typically shown that increased expression is associated with antibiotic stress with is consistent with Rv1410’s known function in toxic compound efflux such as shown by Farrow and Rubin (Farrow and Rubin 2008). In fact, it has been typically difficult to identify other stress conditions under which LprG-1410 expression is induced such as cholesterol, acid, and hypoxia.
This supports the hypothesis that down regulation of LrpG-1410 function is more important to mycobacterial physiology under these conditions and is consistent with our model that down regulation of LprG-1410 function mediates increases in intracellular TAG during stress. Our lab has begun assessing operonic level co-regulation of genes based on the well-characterized expression data sets for mycobacteria (Boshoff 2004), which are well-known to be preferentially biased towards conditions of antibiotic stress, acid stress, and metabolic stress. Combining these data with the Mtb operon data acquired from comprehensive mapping of Mtb transcription start sites (Shell manuscript in preparation), we can confidently use operonic co-regulation to probe TB biology. Using this methodology we find that LprG-1410 operonic level expression is highly co-regulated with genes important for virulence, such as the esx1 locus, Rv3868-3871, (p<2.80E-14) which is consistent with it being important for host survival. More importantly, the modeling predicts co-expression of LprG-1410 with operons containing individual genes involved in triacylglyceride synthesis. LprG-1410 is significantly co-expressed with the Rv3813c-3816c operon (p<1.71E-13), the second most significant hit behind esx-1 (Baranowski and Rubin unpublished). This operon contains three annotated acylglycerol transferases, one of which is Rv3816c, predicted to be the acyltransferase responsible for acylation of glycerol-3-phosphate to monoacylglycerol that is the precursor to triacylglyceride in the TAG synthetic scheme (Baek, Li et al. 2011). Given that in other model organisms growth rate is a function of the balance between phospholipid and TAG synthesis via the availability of phosphatidic acid (PA) and diacylglycerol (DAG) precursor molecules (Figure 2, Chapter 1), it is possible that LprG binds both phospholipids and triacylglycerides and that regulation of intracellular TAG flux is partly regulated by Rv1410 function. This places LprG-1410 function at a key regulatory
step for cell wall biogenesis, potentially regulating the flux of diacylglycerol precursors into either phospholipid or TAG synthesis and thereby regulating cell growth in vivo.

**SECTION 3.13 METHODS.**

**SECTION 3.13.1 CULTURE OF MYCOBACTERIA.** The H37Rv lab strain was used for all experiments unless otherwise noted. Cells were routinely cultured in Middlebrook 7H9 broth and supplemented with 10\% (vol/vol) OADC (Middlebrook), 0.2\% glycerol, and 0.05\% Tween80. For cells destined for lipidomics analysis, Tween-free media was used. Cells were maintained at 37°C with shaking.

**SECTION 3.13.2. CONSTRUCTION OF THE ΔLPRG-1410 MUTANT BY RECOMBINEERING.** Mycobacterial phage-mediated recombineering was employed to create the double knockout of the *Rv1411c-Rv1410c* (LprG-1410) operon based on methodology previously described (Murphy 2011, Wei, Krishnamoorthy et al. 2011). A cosmid library (generated by Dr. Chris Sassetti) of mycobacterial genes was used to isolate the genomic template for the LprG-1410 gene. Cosmid 2_33 contains roughly 30kb of genomic DNA from *Mtb* and was confirmed to contain the genes *Rv1411c-Rv1410c* (LprG-1410) by primers (AJM1F and AJM1R). The cosmid was amplified, purified, and transformed into the *E.coli DH5α* previously transformed with the temperature sensitive (replication at 30°C), arabinose inducible (pAraBAD), tetracycline-marked recombineering plasmid *pkd119*. Recombination was suppressed in the presence of 1\% glucose therefore culture and maintenance of the pkD119 plasmid required maintenance in media supplemented with glucose and tetracycline. Primers with 50 bp of homology to genomic
sequence upstream to LprG and downstream of 1410 were designed to amplify a chloramphenicol/hygromycin resistance cassette. The resultant linear PCR product was transformed into the temperature-sensitive, lambda-red recombinase containing *E.coli* strain, pkd119 (Murphy 2007). Allelic exchange resulted in the creation of a recombinant cosmid containing the resistance cassette flanked by genomic sequence upstream and downstream of LprG-1410.

Recombinant cosmids were amplified and purified and used as a template to generate the *Mtb* recombineering construct containing 500bp of genomic homology upstream and downstream of the LprG-1410 operon on either side of the chloramphenicol/hygromycin resistance cassette. For selection in *E.coli* chloramphenicol (15ug/ml) was used; hygromycin (50ug/ml) in *Mtb*. The linear recombineering PCR construct was transformed into a confirmed PDIM positive mycobacterial *H37Rv* strain that had previously been transformed with mycobacterial Che9c phage recombinase-expressing pNIT::recET-sacB-kan (Wei, Krishnamoorthy et al. 2011) that is inducible with isovaleronitrile (IVN). Competent, recombinase-expressing mycobacteria are generated as follows: Cells are grown to OD 0.8. Cells are then incubated for 8 hours with 1mM isovaleronitrile (Sigma-Aldrich 308528) after which 2M glycine is added and cells incubated overnight. Cells are washed with 10% glycerol and the 3kb linear PCR product containing 500 bp of chromosomal homology upstream and downstream of the LprG-1410 genetic locus flanking the chloramphenicol/hygromycin resistance cassette is electroporated into mycobacterial cells as previously described (Hatfull and Jacobs 2014). Recombinants were selected on hygromycin (50ug/ml) and PCR confirmed (AJM15F,R; AJM16F,R). The
The recombinase plasmid was cured by counterselection on 10% sucrose in the absence of kanamycin and confirmed by patching (Fig 30).

Figure 30: Genetic manipulation of *Mtb* to generate LprG-1410 double knockout. The cosmid 2_33 containing the LprG-1410 genes was used as a template to amplify a linear PCR product (ajm2F, ajm2R) with homology 50bp upstream and down stream of the operon flanking a hygromycin/chloramphenicol (Chlor/Hyg) resistance cassette. Step (1) Recombineering was performed in *E.coli* using the pkD119 to exchange the Chlor/Hyg cassette with the genetic locus on the 2_33 cosmid. Step (2) the recombinant cosmid is used as a template to generate a linear PCR product (ajm3F, ajm3R) with 500 bp chromosomal homology upstream and downstream of the LprG-1410 operon (replaced by Chlor/Hyg). Step (3) the 3kb linear PCR product is transformed into *Mtb* previously transformed with pRecET, expressing the mycobacterial che9 phage recombinase under control of an isovaleronitrile inducible promoter (pNIT). Step (4), successful recombination results in a chromosomal deletion of LprG-1410 with replacement by the chloramphenicol/hygromycin cassette. The recombinase plasmid is cured by counterselection on 10% sucrose.

The knockout was confirmed by diagnostic PCR (Fig 31) using primers listed in Table 2, and Southern blot (Fig 32).
Figure 31. Diagnostic PCR to confirm knockout of the LprG-1410 operon. Template DNA was genomic from boiled cell lysates unless otherwise indicated. Lane 1, \( Rv \) (ajm11F,R). Lane 2, \( \Delta LprG-1410 \) (clone 24.4) (ajm11F,R). Lane 3, \( Rv \) (ajm12F,R). Lane 4, \( \Delta LprG-1410 \) (ajm12F,R). Lane 5, \( Rv \) (ajm8F,R). Lane 6, \( \Delta LprG-1410 \) (ajm8F,R). Lane 7, pOriK6 positive control plasmid for Chloramphenicol/Hygroycin cassette (ajm8F,R). Lane 8, \( Rv \) (ajm13F,R). Lane 9, \( \Delta LprG-1410 \) (ajm13F,R).
Figure 32: Southern Blot confirming knockout of LprG-1410 operon. Genomic DNA was purified from Lane (1) Rv::tn1410+pLprG1410, Lane (2) RvΔLprG-1410+pLprG-1410, Lane (3) Rv::tn1410, Lane (4) RvΔLprG-1410, Lane (5) H37Rv. Digoxigenin labeled DNA probes were made with PCR DIG Probe Synthesis Kit (Roche) using primer set (ajm13F, ajm13R). Genomic DNA was digested with BAMHI. Chromosomal LprG-1410 will be present on a 2.1 kb DNA fragment. DNA was transferred, cross-linked, hybridized with probe, and visualized with Anti-Digoxigenin-AP Fab Fragment (Roche). (A) DNA loading gel (B) membrane showing (1) probe binding to 6.1KB fragment consistent with LprG-1410 present on complementing plasmid (positive control) and 4.1 kb fragment consistent with band shift due to transposon insertion in Rv::tn1410, (2) probe binding to 6.6 kb fragment on complementing plasmid (positive control) and loss of binding to LprG -1410 chromosomally in KO, (3) probe binding to 4.1 kb fragment consistent with band shift due to transposon insertion (4) no probe binding in RvΔLprG1410, and (5) probe binding to 2.1 kb fragment in H37Rv. A list of plasmids and primers used for this work can be found below (Table 2).
<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Use</th>
<th>5’-3’ sequence</th>
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</thead>
<tbody>
<tr>
<td><strong>ajm1F</strong></td>
<td>Recombineering Step 1: confirm gene presence on cosmid 2_33</td>
<td>ATGCAGGCCACCCAGACGCCA</td>
</tr>
<tr>
<td><strong>ajm1R</strong></td>
<td>Recombineering Step 1: confirm gene presence on cosmid 2_33</td>
<td>TTAGAGCGGCCTCCACTTGGG</td>
</tr>
<tr>
<td><strong>ajm2F</strong></td>
<td>E.coli Recombineering for LprG1410 KO Step 2: 50bp homology flanking resistance cassette</td>
<td>ACCCCCATCCTCGGCAGCGCCACGCAACTCACAG</td>
</tr>
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<tr>
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<tr>
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<tr>
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<td>Hyg F</td>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
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<tr>
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<tr>
<td><strong>ajm12R</strong></td>
<td>500 flank 1410 R (500 bp product)</td>
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</tr>
<tr>
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</tr>
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SECTION 3.13.3 CONSTRUCTION OF COMPLEMENTATION CONSTRUCTS. *Rv1411c-Rv1410c* was cloned from genomic DNA with homology to Gateway *attB* cloning sites using the Gateway BP Clonase II system (Life Technologies) as previously described (Katzen 2007). Briefly, gateway destination vector pDEAJM1_LprG1410 was designed containing genomic homology to the 5’UTR and 3’UTR of the L5 site (*glyV*) flanking *Rv1411c-Rv1410c*, the tetracycline repressor and TetOFF promoter. Plasmids were transformed into *H37Rv, RvΔLprG-1410, and Rv::tn1410*. Successful integration of the desired sequences was confirmed using binding upstream to the L5 site, outside region of homology included on gateway plasmid (AJM66) and inside the cloned gene on destination vector (AJM49). Genes were constitutively expressed in the absence of tetracycline. Expression was confirmed via Western Blot (data not shown) for native LprG (US Biological).

SECTION 3.13.4 LIPID EXTRACTION, NORMALIZATION, AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY MASS SPECTROMETRY. *Mtb* was grown in 25 ml volume in inkwells in triplicate in tween-free 7H9. For OD analysis an additional replicate containing tween was included. Cells were harvested at OD 0.6 +/- 0.1 as described in Layre et al. for logarithmic phase analysis (Layre, Sweet et al. 2011). For stationary phase analysis, cells were cultured as above in 25 ml volume and were collected when all cultures had no further increased in OD, typically at 4 weeks post-inoculation. Stationary phase OD was typically 2-2.5 for *Mtb*. For plate grown lipid cultures, strains were grown in tween free or tween containing media until late logarithmic phase grown (OD 0.75-1.0). Cells were transferred in triplicate (100ul per plate) to reconstituted 7H9 plates (1.5% Bacto agar) that did not contain malachite green. Plates were stored at 37°C for four weeks at which time plates were scraped and cells treated with 30 ml
chloroform: methanol (C:M) 2:1 and left overnight in the BSL3 prior to removal. Further successive extractions were performed in C:M 1:1 and 1:2 each with one hour long extraction. Extractable lipids were separated by centrifugation, collected, and dried under nitrogen evaporation. Dried lipids were resuspended in C:M 1:1 to a concentration of 1mg/ml. For high performance liquid chromatography mass spectrometry (HPLC-MS) analysis, 20ug of dry lipid was resuspended in 100% solvent B (isopropanol: methanol, 70:30 [v/v], 0.02% [m/v] formic acid, 0.01% [m/v] ammonium hydroxide). MS analysis was performed with an Agilent 6520 Accurate Mass QtoF mass spectrometer as previously described (Layre, Sweet et al. 2011).

**SECTION 3.13.5 REVERSE MICELLAR EXTRACTION OF OUTER MEMBRANE LIPIDS.** Extraction of outer membrane lipids in *Mtb* was performed using methods based on those reported by Nikaido *et al.* (Bansal-Mutalik and Nikaido 2014). Briefly, 10 ml volumes of *Mtb*, were cultured in tween-free 7H9 in triplicate in small inkwells. An additional tween-containing replicate was used to measure OD. After 4 weeks of culture, 1 ml of *Mtb* was collected and centrifuged to remove supernatant. Equal volume of heptane/10mM AOT (Sigma) was added to the cell pellet and transferred to glass conicals. Cells were extracted overnight and supernatant collected after sedimentation. An additional, 30 min with gentle rocking, heptane/AOT extraction was performed and supernatants combined. Supernatants were filtered using polypropylene hydrophobic, 1 um filters (Cole Palmer). Remaining cell pellets were extracted with 3 ml C:M 2:1 overnight. For evaluation of inner membrane and cytosolic lipids (IM), lipids were collected by centrifugation and dried. Lipidomic analysis was performed as described above.
SECTION 3.13.6 COLLISIONAL MASS SPECTROMETRY. As described in (Madigan, Cheng et al. 2012).

SECTION 3.13.7 EXTRACTION OF SUPERNATANT DERIVED LIPIDS. Approximately 30 ml of culture filtrate was removed from mycobacterial cells grown in tween-free media after centrifugation. Supernatants were filtered twice using 1 um filters (Millipore). Lipids were extracted by addition of 6N HCl (0.3% v/v) of supernatant followed by addition of 140% ethyl acetate (v/v) for 30 minutes with gentle rocking. Supernatants were centrifuged and the upper organic phase collected and pooled for evaporation to dryness using Genevac (SP Scientific). Lipid weights measured and dried lipids resuspended to 1mg/ml in chloroform: methanol 1:1.

SECTION 3.13.8 R, GENE PATTERN, STATISTICAL ANALYSIS. Raw data files were converted to mzData files using MassHunter (Agilent Technologies) and processed in R using XCMS conversion (v1.24) (Smith, Want et al. 2006) allowing peak deconvolution and alignment across samples. Output data provided 1) accurate mass (m/z) 2) retention time 3) peak intensity. The dataset was exported into GenePattern (v2.0, Broad Institute). Using the “Multiplot Preprocess” and “Multiplot” functions, triplicate lipidomic data was used to generate “volcano” plots comparing the lipidomes of two Mtb strains, in this case “mutant” versus “wild type,” or “mutant” versus “complement.” Log fold change intensity is plotted on the y-axis and Log fold p-value on the x-axis using a student’s paired t-test and Hochberg multiple comparison correction (Acharya 2014). A filter for changes two fold or greater at p-value <0.05 between strains was used to identify variations in lipid features. For evaluation of ratios between supernatant-derived and whole cell lipid extracts, ion counts generated post-deconvolution and
overlay using R script were used to generate a supernatant: cell-associated ratio for each set of triplicates. Average ratios were compared between strains for each lipid species as identified via the automated MycoMass annotation. Student’s *t*-test was used to compare ratios.

SECTION 3.13.9. CLONING OF LIPOPROTEIN EXPRESSION VECTORS. A non-acylated (NA) version of MtbLprG (NA-MtbLprG) that excludes the signal sequence and changes the acylated cysteine to a methionine was cloned from *M. tb* H37Rv genomic DNA. Site-directed mutagenesis of NA-MtbLprG was performed to achieve a V91W amino acid mutation. For expression in *E. coli*, the target gene was cloned into pET24b behind the IPTG-inducible T7-lac promoter and in frame with a TEV protease recognition site and a C-terminal His$_{6}$-tag.

SECTION 3.13.10. OVEREXPRESSON OF HIS$_{6}$-TAGGED RECOMBINANT PROTEINS. For expression in *E. coli*, BL21(DE3) competent cells were transformed with the pET24b constructs and cultured in Luria-Bertani broth (LB); 50 µg/mL kanamycin was used for selection. When the OD$_{600}$ was approximately 1, 0.1 mM IPTG was added to induce protein expression for 4 h at 37 °C. Bacteria were isolated by centrifugation at 5000 xg for 20 min at 4 °C. The pellet was stored at -20 °C or lysed immediately.

SECTION 3.13.11. PURIFICATION OF HIS$_{6}$-TAGGED PROTEINS. All steps were performed at 4 °C unless otherwise noted. To purify proteins from *E. coli*, cell pellets were resuspended in lysis buffer (20 mM Tris, 200 mM NaCl, 1 mM DTT, 0.2 mM EDTA, 10 mM imidazole, 10% glycerol, pH 7.4) and sonicated 5s on/off for 10 min total processing time. Nickel affinity chromatography (HisTrap FF 5mL, GE Healthcare) was used for initial purification. After injection, protein samples were washed with 5 column volume (CV) binding buffer (Buffer A, 50 mM Tris, 1 mM DTT, 10% glycerol, pH 7.4), and bound proteins were eluted with a 0-50%
gradient of elution buffer (Buffer A with 1M imidazole) over 20 CV. All target proteins eluted at an imidazole concentration of approximately 100 mM and were analyzed for purity by SDS-PAGE. If contaminating proteins co-eluted, a second purification step by anion exchange chromatography (HiTrap Q HP 5 mL, GE Healthcare) was performed. The protein was eluted with a gradient of 0-100% high salt buffer (Buffer A with 1M NaCl) over 20 CV. The pH of the buffer was adjusted to at least 1 unit higher than the theoretical isoelectric point (pI) of the target protein. All of the proteins were purified in a final step by size exclusion (HiPrep 16/60 120 mL, GE Healthcare). The mobile phase was buffer A without glycerol. Purified proteins were verified by tryptic digest and mass spectrometry (MS), flash frozen in liquid nitrogen and stored at -80 °C in Buffer A. Using the theoretical extinction coefficient, proteins’ concentrations were estimated by A280. All of the non-acylated proteins (NA-MtbLprG, V91W NA-MtbLprG, NA-MtbLprA, NA-MsmegLprG and A91W NA-MsmegLprG) were stably expressed in *E. coli* and/or in *M. smegmatis*; purified and verified by MS as described above.

**SECTION 3.13.12 TRIGLYCERIDE TRANSFER ASSAY.** Briefly, transfer assay was performed in 96 well “U” Bottom Microtiter plates (Thermo Labsystems, Franklin, MA). Donor vesicles containing 450nmol of phosphatidylcholine (PC) and 14nmol of fluorescently labeled NBD-TAG/ml were mixed 1:1 with acceptor vesicles containing 2400 nmol of PC/ml in the presence of either LprG, LprA, LppX, LprGV91W, and MPT (control—microsomal transfer protein) and buffer for a final volume of 100ul. Plates were incubated at 37°C or at room temperature and fluorescence monitored at different time periods using a 7620 Microplate Fluorimeter (Cambridge Technology, Watertown, MA) using 460nm excitation and 530 nm emission wavelengths. Blank values were obtained by eliminating transfer protein and adding isopropanol
to donor vesicles. Percent transfer: (Arbitrary fluorescence units in assay well-blank values)/(total fluorescence units-blank value) x100 (Hathar et al. 2004).

SECTION 3.13.13 CO-CRYSTALLIZATION WITH TRIGLYCERIDE. The gene LprG was amplified from Mtb H37Rv genomic DNA and ligated to the expression vector (pET30b) such that the protein construct of LprG included residue 35-231 without the N-terminal signal peptide. The protein expressed in the *E. coli* strain BL21 (DE3) and purified by nickel-affinity chromatography was concentrated to 10mg/mL. Prior to the crystallization, powder tri-palmitic glyceride (TAG), weighed to make the concentration three times higher than the protein concentration, was directly added to LprG. The mixture of LprG and TAG was incubated on ice for 1 hour. Crystals were obtained in the condition containing 0.1M sodium acetate buffer pH 4.5 and 25% (w/v) PEG3350. Crystals were mounted in the cryo-protectant (30% (v/v) of ethylene glycol and 70% (v/v) of crystallization condition). Data were collected to resolution of 1.8 Å at the Advance Photon Synchrotron (beamline 23-ID, the Argonne National Lab). Processed with HKL2000, the space group of the LprG-TAG complex crystals was C2 and cell dimension were a = 95.7 Å, b = 71.6 Å, c = 61.8 Å, α = 90°, β = 106.6°, γ = 90°. Structural solution was obtained by molecular replacement (MOLREP) using apo-LprG structure as an initial model. The structure of the complex structure was built and refined with coot, CCP4 and PHENIX. The final R-work and R-free were 20.4% and 23.4%, respectively.
SECTION 3.15 REFERENCES


Horvath, S. E., A. Wagner, E. Steyrer and G. Daum Metabolic link between phosphatidylethanolamine and triacylglycerol metabolism in the yeast Saccharomyces cerevisiae. BBA - Molecular and Cell Biology of Lipids. 1811: 1030-1037.


Chapter 4

Intracellular Triacylglyceride Levels Regulate Growth Rate *in vitro* during Carbon Source Restriction
SECTION 4.1 PREFACE. In chapter 2 we outline results showing that LprG-1410 mutants have a growth defect that is present irrespective of host immune pressure, leading us to hypothesize that LprG-1410 results in a metabolic defect that slows growth rate. In chapter 3 we show that a major lipidome feature of LprG-1410 disruption is accumulation of triacylglycerides. We show that this accumulation phenotype is specific for intracellular lipids, and that the transporter function of Rv1410 is likely epistatic to LprG in that single Rv1410 mutants (Rv::tn1410) have the same TAG accumulation phenotype as the LprG-1410 double knockout strain. Furthermore in chapter 2 we show that the single Rv1410 mutant also has a decreased growth rate even when a functional copy of the LprG gene is present. We go on to show that LprG can not only bind TAG via co-crystallization, but also transport TAG via a vesicle transfer assay. The fact that TAG is known to be important for growth down regulation during hypoxia and likely during acid and antibiotic stress, led us to hypothesize that the accumulation of TAG in our mutants was the cause for growth rate attenuation in the mouse. Given that it is well known that mycobacteria use host-derived lipids as a carbon source, we predicted that the reason we couldn’t recapitulate a slow growth phenotype under standard, rich media culture conditions or in macrophage culture was secondary to the fact that these cells were not under sufficient carbon source stress in these conditions. We opted to test growth phenotypes of our mutants during carbon restriction, utilizing propionate or cholesterol as single carbon sources, with the hope that we could phenocopy growth rate alterations between the strains as we predicted to occur during host infection.

Mycobacterial catabolism of host-derived fatty acids during infection is supported by the conditional essentiality of the isocitrate lyase gene in vivo (McKinney, Höner zu Bentrup et al.
2000, Muñoz-Elías and McKinney 2005). It is known that mycobacteria utilize cholesterol during host infection (Pandey and Sassetti 2008, Griffin, Gawronski et al. 2011). It is also known that cholesterol catabolism results in the formation of propionate (Yang, Nesbitt et al. 2009), a odd-chained three carbon fatty acid that is toxic to mycobacteria at high concentrations (Eoh and Rhee 2014, Gould, van de Langemheen et al. 2006, Lee, VanderVen et al. 2013). The following work highlights our investigations using cholesterol and propionate containing media to probe the role of LprG-1410 in \( Mtb \) growth rate regulation under carbon source stress. We show that we can induce a growth defect in our LprG-1410 mutants, compared to WT strains, using carbon stress. We attempt to reverse this growth defect by modulating intracellular levels of triacylglyceride, without complementation of the LprG-1410 genetic operon, in order to link TAG levels to growth arrest of our mutant strains. We are unable to successfully modify TAG levels sufficiently to reverse growth arrest via further genetic alterations. However, we are able to rescue growth with acetate and glycerol supplementation in the LprG-1410 mutant. We predict this result is a function of modifying acetyl Co-A levels and inducing the tricarboxylic acid (TCA) cycle. The fact that TCA cycle induction cannot be achieved by overexpression of \( citA \), encoding for the mycobacterial citrate synthase gene, suggests that acetyl-coA levels are low in our mutant. These results contribute to the proposed model whereby triacylglyceride transport by LprG-1410 impacts acetyl-coA levels intracellularly, limiting bacterial growth and suggest that TAG transport may be required for 1) TAG lipolysis and/or 2) \( \beta \)-oxidation of free fatty acids.

**SECTION 4.2. CARBON SOURCE RESTRICTION LIMITS GROWTH OF LPRG-1410 MUTANTS.**

We hypothesized that we could slow the growth rate, and therefore recapitulate the *in vivo*
growth defect, of LprG-1410 mutants in vitro with carbon source restriction. *Mtb* is thought to predominantly catabolize host-derived lipids during chronic infection, particularly cholesterol. For this reason we first tested growth in Sauton’s minimal media with 0.01% cholesterol. Our LprG-1410 mutants displayed slower growth rates in cholesterol than wild type Mtb (Fig 33).

![Graph showing growth of Mtb in cholesterol](image)

**Figure 33: Growth of Mtb in cholesterol.** H37Rv (WT), Rv::tn1410 (red triangles, solid red), RvΔLprG-1410 (black triangles, dashed, red), RvΔLprG-1410::LprG-1410 (black circles, dashed blue), Rv::tn1410::LprG-1410 (solid blue, blue inverted triangles), were cultured in Sauton’s minimal media with cholesterol 0.01%, with 0.05% tyloxapol. Growth was measured as OD600 at the indicated times. LprG-1410 mutants have moderately slower growth rate than wild type and complement strains and reach lower peak ODs compared to wild type and complement strains. Error bars indicate means +/- standard deviations.

Growth characteristics of *Mtb* with cholesterol as the sole carbon source is interesting in that there is normal doubling time (18-24 hours) of WT cells during the first five days of growth, but growth abruptly levels off after cells reach an approximate O.D. of 0.5. This suggests that metabolites associated with cholesterol metabolism effectively slow growth rate after reaching a critical point. Given that cholesterol is known to be catabolized into two acetate molecules,
one propionate molecule, and pyruvate (Yang, Nesbitt et al. 2009), we predicted that this premature “leveling” off of growth was secondary to the accumulation of propionate in the cells. Propionate is known to be toxic to *Mycobacterium tuberculosis*, as is reported for other bacteria, at high molar ratios, typically at concentrations exceeding 10mM. Propionate is the simplest form of a 3-carbon straight-chain lipid; therefore catabolism of any odd-chain fatty acids will result in the formation of propionate. Propionate toxicity is proposed to occur secondary to accumulation of 2-methylisocitrate (2-MIC). In *Salmonella*, propionate metabolism via the methyl-citrate cycle results in the accumulation of the intermediate 2-methylcitrate (2-MIC) which has been shown to inhibit 1,6 fructose bisphosphatase, a key enzyme in gluconeogenesis (Rocco and Escalante-Semerena 2010). Work by Rhee *et al.* goes on to show that a functioning methylcitrate cycle is necessary for *Mtb* survival *in vivo* in order to prevent toxic accumulation of propionate and the intermediate 2-MIC in the host when utilizing fatty acids (Eoh and Rhee 2014). A key enzyme linking the methylcitrate cycle and glyoxylate shunt in *Mtb* is isocitrate lyase (*icl*) due to the fact that, in *Mtb*, this enzyme dually functions as a methyisocitrate lyase (MCL) in addition to its isocitrate lyase (ICL) function (Gould, van de Langemheen et al. 2006). In addition, *Mtb* has been shown to assimilate propionate by channeling propionate into branched chain lipid synthesis, such as PDIM and triacylglyceride (Lee, VanderVen et al. 2013). Given our hypothesis that LprG-1410 is a TAG transporter, mediating intracellular levels of this important storage molecule, we asked whether LprG-1410 mutants would have a growth phenotype with propionate as the sole carbon source.
SECTION 4.3. **LprG-1410 mutants are hypersusceptible to propionate as sole carbon source compared to WT.** We predicted that LprG-1410 mutants would be either 1) hypersusceptible or 2) resistant to the effects of propionate. If the presence of propionate increases intracellular TAG levels and intracellular TAG levels are important in regulating growth rate, then we would predict our mutant to be hypersusceptible to propionate. However, if TAG functions as a sink for toxic propionate, then we would predict that the mutant would be resistant to propionate toxicity given that it is a known TAG accumulator. In fact, it has been shown that mutations in *aprABC*, also known to result in increases in PDIM and TAG, confer resistance to toxic levels of propionate, typically described as 20mM (Abramovitch, Rohde et al. 2011). Here, we show that both RvΔ*LprG-1410* and Rv::tn*1410* are hypersusceptible to propionate at 10mM, a concentration typically considered permissible for growth of wildtype *Mtb (Fig 34).*
Figure 34: Growth of *Mtb* with propionate as sole carbon source. H37Rv (black), RvΔLprG-1410 (red), Rv::tn1410 (red-hashed), and RvΔLprG-1410::LprG-1410 (blue) were grown in triplicate in Sauton’s with 10mM propionate and 0.05% tyloxapol, at 37° with shaking. Growth measured at OD600. Error bars, mean, +/- standard deviation.

This finding is consistent with the first hypothesis, that propionate metabolism increases TAG synthesis, and that intracellular TAG levels function in regulating growth rate, likely via a negative feedback mechanism.

Given the hypersusceptibility phenotype, we wanted to determine if LprG-1410 mutants had defects in the methylcitrate cycle, therefore resulting in the enhanced propionate toxicity. One common way to test this hypothesis is to provide an alternate pathway for propionate assimilation into the TCA cycle via the methlymalonyl-CoA (MMP) pathway (Savvi, Warner et al. 2008). This pathway requires a VitB12 co-factor to activate the methyl-malonyl CoA mutase (MCM) responsible for converting methylmalonyl-CoA to succinate. The MMP has not
only been shown to be active in Mtb but important for TB’s ability to grow in the host. Given that Mtb does not synthesis VitB12, at least to any appreciable extent in vitro, this pathway is thought to be exclusively active in the host. TB is thought to acquire VitB12 from the host given that Mtb lacking genes important for the methlycitrate cycle cannot survive on propionate in vitro but have normal growth in the mouse (Muñoz-Elías, Upton et al. 2006). By culturing TB in the presence of VitB12 in vitro, the MMP pathway can be activated and has been shown to relieve propionate toxicity in vitro in WT Mtb and in strains with a defective methylecitrate cycle (Savvi, Warner et al. 2008). We therefore predicted that if LprG-1410 mutants had some unknown defect in its ability to assimilate propionate via the MCC that induction of the MMP pathway would relieve the toxicity. Here we show that this is not the case. VitB12 does not rescue growth of the LprG-1410 mutants (Fig 35).

![Figure 35: Growth of Mtb in propionate-restricted media with vitamin B12 supplementation.](image)

H37Rv (black) and RvΔLprG-1410 (red) were grown in 7H9 without OADC supplementation, 10mM propionate, +/- 10 ug/ml VitB12 (dashed lines) at 37°C with shaking. Growth measured by OD 600, mean +/- standard deviation.
SECTION 4.4. LPRG-1410 MUTANTS HAVE A GENERALIZED GROWTH DEFECT ON FATTY ACIDS. We then went on to test whether the growth defect we observed in cholesterol was specific to cholesterol or whether the LprG-1410 mutant would show defects when forced to catabolize even-chain lipids as well as odd-chained lipids. To test this hypothesis we grew our strains in short chain fatty acid (FA) acetate (C2), intermediate length FA, palmitic acid (C16), and long-chain FA, lignoceric acid (C24). Growth rate is extremely slow for both WT and mutant strains under these restrictive conditions, however LprG-1410 mutants seem to be at a disadvantage in palmitic acid (C16). These are preliminary results and further experiments are planned to determine the growth phenotype of the mutant on restricted odd and even chain FA (Fig 36).

Figure 36: Growth of Mtb with single fatty acid carbon sources. RvΔLprG-1410::LprG-1410 (Comp, black) and RvΔLprG-1410 (mutant, red) were grown in Sauton’s media supplemented with either 0.01% (A) acetate (C2) (B) palmitic acid (C16) or (C) Lignoceric acid (C24). Growth measured by OD600. Error bars, mean +/- SD.

SECTION 4.5. ACETATE AND GLYCEROL RESCUE LPRG-1410 MUTANT GROWTH DEFECT ON PROPIONATE. Given the technical difficulty and long incubation times on restrictive fatty
acids, we focused on utilizing the propionate growth defect of the mutant to test mechanisms by which we could rescue the slow growth phenotype of our LprG-1410 mutants. Specifically, we wanted to test whether we could rescue growth by attempting to modulate intracellular levels of triacylglyceride. We first attempted to decrease TAG levels by diverting acetyl Co-A precursors away from TAG synthesis and into the TCA cycle by overexpressing the citric acid synthase gene (**citA**). This had been shown by **Baek** et al. to modulate TAG levels during hypoxia (Baek, Li et al. 2011). Indeed, the WT growth defect in 10mM propionate was partially rescued by overexpression of **citA** in H37Rv, however no effect was seen on growth of our LprG-1410 mutants (**Fig 37**).

**Figure 37:** Effect of **citA** overexpression on growth of **Mtb** in propionate as sole carbon source. **H37Rv** (black), **H37Rv+pcitA** (black dashed), **RvΔLprG-1410** (red), **RvΔLprG-1410 +pcitA** (red dashed) were grown in Sauton’s minimal media with 10mM propionate, at 37°C with shaking. Growth measured by OD 600, mean +/- standard deviation. Overexpression of citrate synthase (**citA**) in **Rv** rescues growth of **Rv** in 10mM propionate. Growth rate of LprG-1410 mutant is not rescued.
We then tried adding back various concentrations of acetate (C2) given that Lee et. al. recently showed that acetate supplementation could rescue the growth of *icl* mutants in propionate (Lee, VanderVen et al. 2013). Indeed supplementation of 5mM acetate improved growth of both WT and LprG-1410 mutants in the presence of 10mM propionate, but acetate alone was unable to fully restore LprG-1410 growth to wild-type levels in propionate. Given that we had already determined that it was unlikely that a defect in the methlycitrate cycle and glyxoylate shunt was contributing to the propionate phenotype based on our VitB12 experiments, and given the conflicting results that acetate but not *citA* could rescue growth—we suspected a defect in anapleurosis due to inadequate acetyl Co-A levels in our mutant. The primary source of acetyl-CoA under conditions where the cell is forced to catabolism lipids is β-oxidation of fatty acids. Under highly restrictive growth on selective FA, LprG-1410 function may drive anapleurosis by promoting β-oxidation of FA derived from intracellular TAG. In support of the hypothesis that our LprG-1410 mutants lack sufficient anapleurosis of the TCA cycle, we go on to show that the addition of 0.2% glycerol to Sauton’s media containing 5mM acetate and 10mM propionate fully restores growth of the LprG-1410 mutant to wild-type levels. (Fig 38).
Figure 38: Rescue of growth of *Mtb* with addition of acetate and glycerol in presence of propionate. H37Rv (black) or RvΔLprG-1410 (red). Sauton’s minimal media with addition of (A) 10mM propionate (dashed lines), 10mM propionate, 5mM acetate (solid lines), (B) 10mM propionate, 5mM acetate, 0.2% glycerol at 37° C with shaking WT. Growth measured by OD600, mean +/- standard deviation. (A) Growth of RvΔLprG-1410 is partially rescued in 5mM acetate. (B) Growth of RvΔLprG-1410 is fully restored to WT levels in 5mM acetate with 0.2% glycerol in presence 10mM propionate.

Glycerol is metabolized to pyruvate and likely contributes to a secondary source of acetyl-CoA (Fig 4, Chapter 1). It should be noted that increasing acetate to 10mM in the presence 10mM propionate, did not offer any additional benefit (data not shown), and actually slightly slowed the growth kinetics of the LprG-1410 mutant. This experiment was only conducted once, but reflects the importance of the ratio of acetate and propionate in the cell (C2:C3 balance) as previously described (Lee, VanderVen et al. 2013).

**SECTION 4.6. BLOCKING LIPASE ACTIVITY EXACERBATES LPRG-1410 GROWTH DEFECT ON CHOLESTEROL AS SOLE CARBON SOURCE.** We hypothesized that intracellular TAG levels were affecting growth rate. Fifteen TAG synthases (*tgs*) genes are encoded in the TB genome (Deb, Daniel et al. 2006) therefore genetically attempting to decrease TAGs by knocking-out
TAG synthesis and looking for growth rescue would be difficult. In fact, we created a TAG synthase 1 knock-out ($\Delta$tgs1) using the $Rv::tn1410$ mutant strain as parent and we were unable to detect any alterations in TAG levels by lipidomics in standard media, no growth rescue in propionate or cholesterol, and no return of virulence in SCID mice (data not shown). Given that some level of TAG is thought to be protective in the presence of propionate, we were worried that eliminating TAG synthesis might actually decrease growth of both WT and LprG-1410 mutants making interpretation of the independent role of TAG levels difficult to interpret. For this reason, we attempted to exacerbate the growth defect of LprG-1410 mutants by preventing TAG breakdown by lipases. Tetrahydrolipostatin (THL) has been shown to decrease TAG lipolysis in the hypoxia model of $Mtb$ growth (Ravindran, Rao et al. 2014). Therefore we asked whether LprG-1410 mutants would be hypersusceptible to THL during growth on cholesterol. We first determined the minimum inhibitory concentration (MIC) of THL for WT cells grown in Sauton’s minimal media supplemented with 0.8% glycerol and determined that the MIC for wild type H37Rv was between 2-4 ug/ml. Reported MICs for THL vary depending on the species tested. Both $Mycobacterium smegmatis$ and $Mycobacterium bovis, BCG$ have remarkably increased resistance to THL compared to $Mtb$ (Kremer, De Chastellier et al. 2005). For our first experiment we used a concentration of 5 ug/ml, which in retrospect was too high, as seen by the complete inhibition of growth during the first 5 days of culture, during which, exponential growth of $Mtb$ is typically noted. However, this concentration was only bacteriostatic for strains with a functional LprG-1410 in cholesterol. The growth of WT and LprG-1410 complemented strains recovered during the following 10 days in culture although never reaching the expected OD of 0.5 in the absence of drug. However, the $\Delta$LprG-1410 mutant never recovered growth during exposure to THL (Fig
We did not plate for cfu, therefore we cannot determine if the THL was bacteriocidal for our mutant, although we would predict, based on our hypothesis that high TAG levels precipitated growth arrest of the mutant, that recovery on plates would be possible. We repeated the experiment at half the concentration of THL (2.5ug/ml) and again show that the LprG-1410 mutant is hypersusceptible to growth arrest (Fig 39B).

Figure 39: Effect of tetrahydrolipstatin on growth of \textit{Mtb} when cholesterol is sole carbon source. WT (Rv, black), mutant (\textit{ΔLprG-1410} clone 24.4, red), or complement (Rv\textunderscore{ΔLprG-1410}::LprG-1410, 24.4+86, blue) were cultured with (dashed) or without (solid) tetrahydrolipstatin (THL) at (A) 2.5 ug/ml (B) 5ug/ml, in Sauton’s with 0.01% Cholesterol. Growth measured by OD600, error bars, mean +/- standard deviation. The LprG-1410 mutant is hypersusceptible to THL at 2.5 ug/ml (A) THL completely abolishes growth of LprG-1410 mutant at 5 ug/ml (B).

THL is predicted to target thioesterase (TE) domains and potentially targets numerous lipases and possibly polyketide synthases, such as those encoded by pks13, which has a TE domain and is involved in mycolic acid synthesis, (Parker, Barkley et al. 2009, Ravindran, Rao et al. 2014). Therefore, is likely to have off-target effects. However, these data do support TAG levels as being key modulators of growth rate during catabolism of exogenous fatty acids. We
have shown that the MIC for THL for *Mtb* is >10 fold higher (62.5ug/ml) in 7H9 supplemented with OADC ([Fig 40B](#)) compared to what was determined in carbon restricted Sauton’s with 0.8% glycerol ([Figure 40A](#)).

![Sauton’s 0.8% Glycerol](#)

**Figure 40: Effect of carbon source on minimum inhibitory concentration of tetrahydrolipostatin.** The strains indicated, WT= *Rv*, Δ=ΔLprG-1410, Comp= ΔLprG-1410::LprG-1410, tn1410=Rv::tn1410, tnComp= Rv::tn1410::LprG-1410, and Δtgs1=RvΔtgs1 (tag synthase 1) were grown in 96 well plates in either (A) Sauton’s with 0.8% glycerol (B) Sauton’s 0.8% glycerol for 6 days at 37°C with shaking. Alamar blue was added to control (no drug wells), when color change indicated alamar blue was added to remaining well on Day 7. MIC determined based on color change to dark blue (indicated in black boxes).
The reported MIC for THL is <30ug/ml (Kremer, De Chastellier et al. 2005). These differences in reported MIC reflect the importance of growth conditions, more specifically carbon sources, when testing drugs that may alter the levels of TAG.

**SECTION 4.7. SUPPRESSOR MUTATION LINKS LPRG-1410 FUNCTION TO REGULATION OF FASI VIA THE FASI ACTIVATOR, FASR.** LprG-1410 mutants are known to be hypersusceptible to malachite green via an unknown mechanism (Bianco, Blanco et al. 2012). It has also been shown that cells treated with cell-wall acting antibiotics have decreased recovery on malachite green after the removal of antibiotic from the media suggesting that malachite green resistance in *Mtb* is a function of cell wall integrity (Gelman, McKinney et al. 2012). Malachite green is present in 7H10 and 7H11 (Middlebrook) agar used for routine culture of *Mtb* at a concentration of 0.25ug/ml. Although we routinely recovered our mutants, RvΔLprG-1410 and Rv::tn1410, without difficulty on 7H10, we became concerned about the presence of malachite green in the standard agar after encountering spurious results in colony recovery after passage of our LprG-1410 mutants in macrophages. We performed a targeted experiment using ten fold the malachite green concentration present in standard 7H10 media (2.5ug/ml) and compared cfu recovery on 7H9 + 1.5% Bacto agar containing no malachite green. This was based on preliminary experiments showing increased susceptibility of *Mycobacterium smegmatis* LprG-1410 mutants to malachite green at a similar concentration (data not shown). We confirmed that this concentration of malachite green had no effect on recovery of wild type cells, but was completely cidal for our ΔLprG-1410 mutant (Fig 41B).
Figure 41: Effect of malachite green on plate recovery of *Mtb*. H37Rv (WT) and RvΔLprG-1410 (KO) were grown to O.D. 0.8-0.9 and serial dilutions of 100ul culture was plated in triplicate on reconstituted 7H9 salts + 1.5% Bacto agar with 2.5ug/ml malachite green (MG) and no malachite green (no MG). After 4 weeks of growth on plates, colonies were counted and a recovery ratio (# colonies on no MG/ # colonies on MG) was determined and reported as log-fold change (A). Plates containing malachite green with no growth of KO were left to incubate for another 2-3 weeks. A single malachite green resistant (MGR) suppressor colony was recovered and regrown. Malachite susceptibility was retested. (A) top, no growth defect of WT; middle (no growth of KO); bottom, small but numerous colonies of the MGR KO. (C) Colony counts from (A) at 4 weeks post-incubation. The recovery ratio of the WT and KO is consistent with previous experiments. The MGR KO has intermediate recovery on malachite green confirming a stable, and likely genetic, phenotype.
Colony-forming units were counted at 4 weeks and RvΔLprG-1410 plates (containing no colonies) were left at 37°C for an additional three weeks to see if malachite green suppressors would arise. Given that the target of malachite green is unknown, we reasoned that a malachite green suppressor mutation on a ΔLprG-1410 background would potentially provide insight into the function of our genes of interest. After an additional three weeks of growth, only two colonies were recovered from plates containing 2.5ug/ml malachite green. Only one of these colonies was recoverable/expandable after sub-culturing in rich media (7H9+OADC). This suppressor strain, henceforth referred to as “malachite green resistant” (MGR), was retested for resistance to malachite green and confirmed to be moderately resistant. Colonies were able to be counted at 4 weeks, not seven weeks, suggesting to us that this was a stable genetically determined resistance, not phenotypic resistance (Fig 41 A,C). We then performed whole genome sequencing on the ΔLprG-1410 (24.4) parent strain and the suppressor strain ΔLprG-1410 MGR to identify a genetic determinant that could confer malachite green resistance. Compared to the wild type H37Rv strain, RvΔLprG-1410 (clone 24.4) was confirmed to have a deletion of Rv1410c-Rv1411c/LprG with ~2kb antibiotic marker insertion with no secondary mutations. The malachite resistant mutant, Rv24.4_MGR had two additional unique mutations: a SNP in Rv3208:F123C and an indel -G, 140 bp upstream of Rv3849/EspR (Thomas Ioerger, personal communication). Given that the deletion (indel –G) was in an intergenic non-coding region of an in vitro non-essential gene, it was unlikely to be responsible for the malachite resistance phenotype. Rv3208 is predicted to encode the Mycobacterium tuberculosis homolog to FadR, the fatty acid synthase (FasI) activator well characterized in Mycobacterium smegmatis (Mondino, Gago et al. 2013). Rv3208 is predicted
to be essential in vitro by Himar1-based transposon mutagenesis (Griffin, Gawronski et al. 2011). Protein structural predictions placed the F123C phenylalanine to cysteine mutation in the core α-helical bundle, likely affecting DNA binding of this transcription factor (Jessica Seeliger, personal communication). Given that our mutant is viable in vitro, we reasoned that this mutation likely modified DNA binding but did not abolish it, and most likely would result in decreased transcription of the FasI gene (Fig 42).

**Figure 42:** Predicted protein structure of FasR with F123C mutation. *Rv3208*, predicted FadR of *Mtb*, with F123C, phenylalanine to cysteine, single point mutation. Structure based on TetR from *Rhodococcus jostii*, RHA1_ro02733.

**Section 4.8** *Rv3208::F123C exacerbates the growth defect of ΔLprG-1410 in propionate and cholesterol*. We wanted to confirm that the *Rv3208::F123C* mutation had a phenotypic effect other malachite green resistance to place this gene in our pathway of interest in determining the growth effects of LprG-1410 function in the mouse. So, we tested
growth of the MGR mutant in propionate and cholesterol. Indeed, \textit{Rv}\Delta LprG-1410-\textit{Rv3208::F123C} had an exacerbated growth defect compared to \textit{Rv}\Delta LprG-1410 with loss of LprG-1410 alone with both these carbon sources (Fig 43).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure43.png}
\caption{Effect of FasR F123C mutation of growth of LprG-1410 mutants in propionate and cholesterol. \textit{Rv} (WT, black), \textit{Rv}\Delta LprG-1410 (red-solid), \textit{Rv}\Delta LprG-1410::LprG-1410 (blue), and \textit{Rv}\Delta LprG-1410-\textit{Rv3208::F123C} (24.4 MGR, red-dashed) were cultured in Sauton’s minimal media supplemented with (A) 10mM propionate or (B) 0.01% cholesterol. Growth measured by OD600; error bars, mean+/− standard deviation. The MGR suppressor mutant is more susceptible than with loss of LprG-1410 alone.}
\end{figure}

\textbf{Section 4.9. Fatty Acids do not Rescue Growth of \textit{LprG-1410} Mutant with \textit{Rv3208::F128C} in Propionate.} Given that Fasl activation by the FasR transcription factor in \textit{Mycobacterium smegmatis} was titratable based on the presence of free fatty acids, we envisioned a model whereby the presence of high TAG due to the LprG-1410 mutation was contributing to negative feedback on Fasl via FasR binding. In this model successive rounds of β-oxidation of free fatty acid (FFA) liberated from TAG by the action of lipases would generate short (C2, acetate), mid (C16), and long chain (C24) FFA that therefore could
modulate FasR activation of FasI. In this scenario, normal TAG hydrolysis would generate FFA that could result in positive or negative feedback on FasI depending on the relative amounts of short and long chain FFA liberated from TAG (Fig 44A). One possible scenario would be that decreased TAG transport leads to increased levels of free long-chain fatty acids (FFA) leading to decreased FasR binding → decreased FasI transcription → decreased C16 and C24 FA to feed into FasII for cell wall biogenesis and growth (Fig 44B).

Figure 44: Model for role of LprG-1410 and FasR in regulating growth rate of Mtb. (A) In presence of a functional TAG transport system (LprG-1410), carbon stress leads to generation of TAG → TAG lipolysis results in liberation of free fatty acids (FFA) that undergo β-oxidation to form acetate/acetyl-CoA units relieving negative feedback on FasI via binding of the FasR transcription factor. FasR binding activates FasI leading to increased growth rate via generation of cell wall lipids via downstream action of FasII. (B) With disruption of LprG-1410 function, TAG levels increase intracellularly. High intracellular TAG leads to increased levels of intermediate or long-chain FFA resulting in negative feedback on FasI via decreased binding of FasR.
In this model, the addition of short-chain FA (such as acetate) would relieve negative feedback via FasR → allow FasI activation → FasII activation → cell biogenesis → increasing growth rate accounting for the rescue of our LprG-1410 mutant. In the presence of an additional F123C mutation in Rv3208, perhaps the transcription factor has decreased binding to either short or long-chain fatty acids impeding its ability to regulate FasI activity under carbon stress. This would lead to enhanced down-regulation of growth rate and potentially loss of viability in our carbon stress assay.

Given this model, we would predict that the F123C mutation could result in either 1) increased or 2) decreased binding of FasR upstream of FasI. Increased binding may result in FasI being constitutively active resulting in cidal effects due to high growth-rate under carbon source stress. Alternatively, the mutation could result in decreased binding, effectively shutting off FasI and leading to complete growth arrest regardless of the type and quantity of intracellular FFA. Although, we have just begun to characterize the growth of the F123C mutant under carbon stress and have yet to begin investigating the effects of this point mutation on transcription of FasI, we do have preliminary data suggesting decreased responsiveness of the ΔLprG-1410 MGR strain to the presence of FFA under propionate stress. We have previously demonstrated that acetate partially rescued the growth of our LprG-1410 mutant in propionate. Here we show that both acetate and the intermediate chain FA, palmitic acid (C16) rescue growth of the LprG-1410 mutant in propionate (Fig 45A). However, growth of ΔLprG-1410 MGR is unable to be rescued in the presence of propionate by either acetate (C2) or palmitic acid (C16) (Fig 45B).
Figure 45: Effect of FasR F123C on rescue of propionate stress by addition of fatty acids. All strains were grown in Sauton’s minimal media with 10mM propionate. (A) H37Rv (Black), RvΔLprG-1410 (red), RvΔLprG-1410 with 0.01% acetate (blue) or with 0.01% palmitate (green). Loss of LprG-1410 function results in increased lag phase as previously shown. This growth defect is rescued by addition either acetate or palmitic acid. (B) H37Rv (Black—curve reproduced from A), RvΔLprG-1410-Rv3208::F123C (MGR, red-hashed), RvΔLprG-1410-Rv3208::F123C with 0.01% acetate (blue-hashed) or with 0.01% palmitate (green-hashed). The MGR LprG-1410 mutant has a more prominent growth defect in propionate. The growth defect is not rescued by addition of either acetate or palmitic acid.

SECTION 4.10. DISCUSSION. One alternative hypothesis is that LprG-1410 mutants have a defective MMP pathway leading to increased propionate toxicity in vivo. It has been shown that the MMP pathway is the preferred pathway for propionate assimilation into the TCA cycle in vivo, therefore even in the presence of an active MMC pathway, the strain may experience increased propionate toxicity in vivo since this predicted to be the predominant pathway used during growth in the host (Muñoz-Elías, Upton et al. 2006). This hypothesis was raised due to the fact that Rv1411c-1410c is spatially upstream of three putative genes in
this pathway. *Rv1492-1493*, encodes the MutAB gene for the methylymalonyl-CoA mutase (MCM) and *Rv1496*, although annotated as a putative arginine transporter, is predicted to be the mycobacterial MeaB, a GTPase necessary for the assembly of the MCM in *Methylobacterium extorquens* (Savvi, Warner et al. 2008) (Korotkova and Lidstrom 2004). However, we felt this was unlikely given that the MMP pathway is completely dispensable *in vivo* based on transposon site mutagenesis studies looking conditional essentiality of these genes *in vivo* (Sassetti and Rubin 2003, Zhang, Reddy et al. 2013). Furthermore, the MMP pathway has been implicated as potentially useful to *Mtb* for propionate detoxification during chronic infection, explaining the dispensability of MCC genes *in vivo*. Given that LprG-1410 mutants fail to grow during acute infection even in immune deficient murine genetic backgrounds, with only partial rescue during chronic infection of severe combined immunodefficient mice, this was even more unlikely.

β-oxidation in mammalian, yeast, and plant cells occurs across the membranes of mitochondria or peroxisomes. In most prokaryotes, β-oxidation is important for catabolizing lipids acquired extracellularly from the environment. In E.coli, fatty acyl CoA synthase, the first enzyme in the β-oxidation cascade, is thought to be involved in translocation of long-chain FA, derived extracellularly, across the inner membrane (Schmelter, Trigatti et al. 2004). Given that TB is thought to utilize host-derived lipids for survival during chronic stages of infection in the host, it is plausible that this model holds true for mycobacteria as well, placing enzymes responsible for β-oxidation in the periplasm of mycobacteria. However, mycobacteria and *Actinomycetes* in general are unique, not only in their capacity to synthesize and store large quantities of triacylglyceride, but also catabolize triacylglyceride as an energy
source during starvation. A number of \textit{Mtb} lipases are predicted to be located within the cytoplasm (Singh, Singh et al. 2010), but importantly, it is becoming well recognized that mycobacteria possess lipases located within the cell wall, some of which are secreted and implicating in breaking down host-derived lipids (Cotes, Dhouib et al. 2007) (Dhouib, Laval et al. 2010). Therefore it is possible that β-oxidation of TAG requires transport of TAG from the cytosol into the cell wall for access to lipases and that this process is disrupted in LprG-1410 mutants. In this model loss of TAG transport results in decreased TAG hydrolysis and decreased FFA for β-oxidation and generation of acetyl-CoA, limiting growth in the host.

In this work, we show that acetate alone is insufficient for restoring growth of LprG-1410 mutants in the presence of propionate, while the addition of both acetate and glycerol restores the growth rate of LprG-1410 mutants to that of WT cells. Importantly, \textit{Mtb} growth rate in media containing acetate, glycerol, and propionate is comparable to growth rate in full 7H9 broth media, essentially negating any potential propionate toxicity in WT and mutant cells alike. Under acidic conditions, where TAG levels are known to be high, and growth slowed, it has been shown that carbon supplementation at the anapleurotic node of oxaloacetate, pyruvate, acetate, and PEP rescues growth (Baker, Johnson et al. 2014). For this reason, we believe that rescue of our LprG-1410 mutants with acetate and glycerol in the presence of propionate is also likely occurring at the anapleurotic node. In fact, we would anticipate that our LprG-1410 mutants would also have a growth defect under acidic conditions, although this is currently untested. Two potential explanations for growth rescue of glycerol at neutral pH include 1) glycerol can contribute to anapleurosis by conversion to pyruvate from glyceraldehyde-3-phosphate and 2) glycerol feeds into phospholipid biosynthesis byway of
DAG under favorable growth conditions contributing to phospholipid anabolism. These effects may promote growth even in the presence of high levels of TAG. In the simplest model, hydrolysis of TAG generates FFA and glycerol leading to growth of wild-type cells. It is possible that LprG-1410 function may be needed for normal TAG hydrolysis.

It is becoming increasingly clear that stresses such as acid, carbon source, and hypoxia are resulting in shifts in central carbon metabolism for *Mtb* resulting in triacylglyceride increases and slowed growth. Currently it is unclear whether triacylglyceride accumulation is the consequence of such a shift in central carbon metabolism or whether TAG levels per se are contributing to this shift. Given the importance of cell wall biogenesis in the replication rate of *Mtb* it is likely that TAG levels contribute to negative feedback mechanisms known to regulate lipid metabolism in other bacterial species. Specifically, it is well described that acyl-ACP is a known regulatory molecule impacting both acetyl-CoA carboxylase and FadR mediated regulation of β-oxidation in *E.coli* (Fujita, Matsuoka et al. 2007). *Rv3208* has recently been characterized as the mycobacterium tuberculosis homolog of FasR, a tetracycline repressor family activator of fatty acid synthase 1 (Fasl) in *Mtb*. Fasl is known to be the enzyme responsible for the generation of long C16-C24 fatty acid chains that are incorporated into TAG. We have new data linking LprG-1410 function to FasR in that we isolated a suppressor mutation of LprG-1410 that maps back to FasR. LprG-1410 mutants are known to be hypersusceptible to high levels of malachite green by a currently unknown mechanism. While testing this phenotype in *Mtb*, we isolated a suppressor mutation that allows partial rescue of growth of LprG-1410 mutants in the presence of high levels of malachite green. The F128C point mutation in the FasR gene was the only mutation identified
by whole genome sequencing of the suppressor mutant compared to the LprG-1410 double knockout. This mutation has been predicted to affect function of this transcription factor, but we currently have not tested whether the mutation enhances or inhibits binding of FasR. It is unlikely that the mutation completely prevents binding in that FasR has been predicted to be essential in vitro for Mtb (Griffin, Gawronski et al. 2011).

Collectively, we have built a model in which high triacylglyceride levels due to lack of transport, promote growth arrest of Mtb under carbon source stress. This phenotype is reversible with the addition of acetate and glycerol and is exacerbated in the presence of a lipase inhibitor. These data combined suggest that TAG hydrolysis may be impaired in the absence of LprG-1410 function. The isolation of a suppressor mutation mapping back to FasR suggests that TAG levels may feedback into a fatty acid synthesis regulatory loop based on FasI expression. In summary, we propose a model by which TAG transport critically regulates intracellular TAG levels during growth in the host. Not only is this a completely novel mechanism of growth rate regulation in Mtb, it sheds light into the important metabolic role of TAG in overall cell homeostasis—information that greatly improves our understanding of how TB may down regulate its growth rate during a transition to latency.

**SECTION 4.11. METHODS**

**SECTION 4.11.1 CARBON RESTRICTION.** Mycobacteria were cultured as previously described. For carbon restriction Sauton’s minimal media was used. Sauton’s media contains: Asparagine 0.5 g/L, KH2PO4 1.0 g/L, Na2HPO4 2.5 g/L, Ferricammonium citrate 50 mg/L,
MgSO4*7H20 0.05g/L, CaCl2 0.05 g/L, ZnSO4 0.01 mg/L and 10% Tyloxapol (vol/vol) 5ml/L. Propionate and acetate (Sigma) were supplemented as sodium salts at 5mM, 10, and 20mM concentration where indicated. Cholesterol, palmitic acid, oleic acid, and lignoceric acid (Sigma) were supplemented at 0.01%. Fatty acids and cholesterol 500x stocks were made with 50:50 ethanol/tyloxapol. Stocks were warmed to 95°C. Fatty acids were quickly added to pre-warmed Sauton’s media while stirring. Growth curves were all performed in small ink wells and growth measured by OD600. Mycobacteria were grown to late log phase OD 0.8-1.0 then diluted back 1:100 to OD 0.01. Fatty acids and cholesterol precipitate in the media over time. Therefore, media blanks for the spectrophotometer need to be maintained at 37 degrees during the length of the growth experiment. Where indicated, Vitamin B12 at 10ug/ml was added to culture media. Vitamin B12 causes red discoloration to media, and media containing only Vitamin B12 was used as a blank for spectrophotometric studies. Antibiotics used to maintain genetrically manipulated strains included kanamycin (25 ug/ml), hygromycin (50ug/ml), and zeocin (20 ug/ml). Where indicated, tetrahydrolipostatin was added at either 2.5 ug or 5ug/ml in propionate restricted media (data not shown) and cholesterol restricted media. Glycerol was supplemented at 0.2% where indicated. Growth curves were followed for 10-21 days depending on carbon source.

**SECTION 4.11.2 GENETIC MANIPULATION OF Mtb.** *H37Rv* (WT), *RvΔLprG-1410*, and *Rv::tn1410* were transformed with the episomal plasmid, pUV15tetO, to constitutively express *citA* (*Rv0889c*). The *RvΔtgs1* mutant, the *RvΔtgs1::pMV306::tgs1* complementation strain, the plasmid, pUV15tetO:*citA* and the suicide plasmid, pJM1::hygromycin were generously provided by Dr. Chris Sassetti at the University of Massachusetts Medical School.
SECTION 4.11.3 DRUG TESTING. Mycobacteria were grown to log phase and diluted back to OD 0.006. Serial dilutions of tetrahydrolipstatin (Sigma) were made in 96 well plates in either 7H9 with OADC or with Sauton’s with 0.8% glycerol. Mycobacteria were added to wells (total volume 200ul) and allowed to grow for 6 days at 37°C with shaking. Resazurin 0.02% was added on the 6th day at 10ul per well and cells were incubated overnight as above. Color change from pink to purple/blue indicated growth inhibition (pink positive control).

SECTION 4.11.4 MALACHITE GREEN SUSCEPTIBILITY ASSAY. Malachite green (oxalate salt) was purchased from Sigma. Agar (7H9 + 1.5% Bacto agar) was prepared and autoclaved. Sterile-filtered malachite green was added to agar at concentration of 2.5ug/ml. Plates poured and saved. Strains were grown to O.D.0.8-0.9 (late log phase) and ten-fold dilutions prepared. Three dilutions were prepared in triplicate and plated in duplicate with 100ul per plate. Dilutions for WT and complement Rv were $10^{-4}$, $10^{-3}$, and $10^{-2}$. For cells grown on malachite green, lower dilutions were used, $10^{0}$, $10^{-1}$, and $10^{-2}$. Cfu were counted at 4 weeks (or 7 weeks for suppressor).

SECTION 4.11.5 SEQUENCING. Genomic DNA was isolated from H37Rv, RvΔLprG-1410, and malachite green resistant (MGR) RvΔLprG-1410 as previously described (Parish and Brown 2011). Whole genome sequencing was performed by Dr. Tom Ioeger at Texas A&M University.
SECTION 4.12 REFERENCES


Chapter 5

Summary and Future Directions
**SECTION 5.1 SUMMARY.** In summary, we have determined that a major lipidomic alteration of triacylglyceride accumulation is seen with disruption of the LprG-1410 operon. We believe that *Rv1411c* (LprG) and *Rv1410c* (1410) function together to form a triglyceride transporter. Although LprG may function to bind other cell wall lipids such as triacylated phospholipids, the function of Rv1410 likely offers the specificity of this transport system for triacylglycerides. This work compliments the reported findings of others who have investigated the function of Rv1410 in *Mycobacterium bovis*, where it is commonly referred to as P55, a nomenclature denoting the size of the protein. Work by Ramon-Garcia determined that P55 is important in redox balance in *M. bovis*, likely through a lipid transport mechanism (Ramón-García, Martín et al. 2009). Interestingly, it was shown that P55 expression was induced in the presence of triclosan, a drug targeting fatty acid synthesis. Furthermore, P55 mutants were resistant to menadione, the Vitamin K analog known to promote lipid oxidation. This finding is consistent with the presence of increased triacylglyceride in our *M.tb* LprG-1410 mutant, given that TAG is a proposed sink for lipids damaged by peroxidation and functions to prevent the cell from reactive oxygen species. The work of Ramon-Garcia on the function of P55 in *M.bovis* also highlighted an increased lag phase during growth *in vitro*. We have started investigating the growth of the *Mycobacterium smegmatis* LprG-1410 mutants looking at growth phenotypes under carbon source stress where we also see a delay in lag phase growth and decreased O.D. at stationary phase. Furthermore, they report a difference in colony-size, consistent with what we observe in LprG-1410 mutants in *Mtb* (data not shown). We have also generated a knockout of LprG-1410 in *Mycobacterium bovis*, BCG
that was the basis of Zuri Sullivan’s undergraduate thesis work. She showed a similar clumping phenotype, increased transducibility by the Himar1 transposon carrying mycobacteriophage, and a growth defect in macrophages (data not show)—phenotypes that we are currently following up on. Mary Farrow’s thesis work on LprG-1410 in *Mycobacterium smegmatis* provided important insights into the work presented here. She determined that in some oxidative stress assays that the LprG-1410 *Mycobacterium smegmatis* mutants were hypersusceptible. This phenotype was reproduced in the Rv::tn1410 mutant *Mtb* strain. However, growth of this strain was not rescued in mice defective in oxidative killing mechanisms employed during infection *in vivo*, such as neutrophil mediated RNI production and macrophage production of ROS. Furthermore, IFN-γ is known to mediate macrophage killing via production of ROS as well and our mutant remained attenuated in IFN-γ -/- mice, one of the most immunosuppressed models of *Mtb* infection possible. The role of IFN-γ in controlling TB replication in the mouse model is well described. The pronounced attenuation of our LprG-1410 mutants despite loss of this important effector-molecule further reinforces a pre-existing metabolic defect of this bacterium.

A model whereby LprG-1410 mediates triacylglyceride transport explains many of seemingly disparate phenotypes linked to these genes’ function. Triglycerides are known mediators of redox balance in many eukaryotic (animal and plant) and prokaryotic organisms. Given the rapidity that mycobacterial cells can modify their TAG levels during growth suggests alternative mechanisms at play in addition to synthesis and hydrolysis (Sartain, Dick et al. 2011). LprG-1410 function may act as a “triacylglyceride
dump” when cells want to maintain exponential growth; translational down-regulation of the protein pump (1410) may be more efficient in mediating intracellular TAG levels compared to hydrolytic mechanisms involving lipases such as LipY. Alternatively, TAG synthesis may be too energy inefficient and slow-- by decreasing pump expression, mycobacteria may be able to counter oxidative stress simply by decreasing TAG transport. Furthermore, important lipases may be sequestered in the cell wall to prevent access to lipid bodies during storage intracellularly. TAG transport may be another regulatory mechanism by which lipolysis is controlled and therefore β-oxidation when TB is subsisting on intracellular or extracellularly derived host lipids.

We have provided three independent lines of evidence supporting the conclusion that triacylglycerides are a substrate for LprG-1410 mediated transport: 1) TAG is present in supernatants of Mtb cell cultures and increased expression of the operon increases the presence of TAG in supernatants 2) LprG co-crystallizes with TAG 3) LprG uniquely transfers TAG in a functional assay compared with other mycobacterial lipoproteins, and transfer is abrogated with a mutation in the binding pocket of LprG. Furthermore, we demonstrate by selective lipid extraction of the outer membrane that TAGs are a component of the mycobacterial cell wall, a feature previously suggested by work in Mycobacterium smegmatis (Bansal-Mutalik and Nikaido 2014) but not confirmed in Mycobacterium tuberculosis until now. This work therefore expands our understanding of the lipid composition of the mycobacterial cell wall and functional importance of cell wall lipids in lipid homeostasis and growth rate regulation.
It is likely that LprG-1410 functions to transport other lipids in \textit{Mtb} as well. Given the large size of the LprG binding pocket, and co-crystallization studies with other triacylated lipids, LprG-1410 may be important for transporting many lipids including free fatty acids (FFA).

\textbf{SECTION 5.2 FUTURE DIRECTIONS.} We are currently working on generating tag synthase knock-outs on the background of LprG-1410 knock-outs to determine the contribution of TAG transport for some of the observed phenotypes such as malachite green susceptibility and colony-size previously reported. We are generating mutants with the SNP F123C in the mycobacterial FasR homolog to determine its effect on Fas I transcription. We hope to not only fully characterize negative feedback mechanisms of FFA regulation of FasI transcription by FasR in \textit{Mtb}, but more specifically, determine if, and to what degree, TAG synthesis and TAG levels have on this potential negative feedback mechanism. We are also very interested in understanding the contribution of FasR function in the background of LprG-1410 mutants as it pertains to lipid homeostasis. We have lipid extracts from these strains ready to be evaluated.

Lastly, we are working on cloning lipase overexpression strains to evaluate the effect of lipolysis on LprG-1410 mutant growth during carbon restriction. We are also generating tag synthase mutant strains in the background of LprG-1410 loss that will allow us to determine more specifically the role of 1410 in TAG transport and more importantly effects on growth rate in the host. SCID mice were infected with \textit{Rv::tn1410}, \textit{Rv::tn1410\_Atgs1}, and \textit{Rv::tn1410::LprG-1410}. This survival experiment is ongoing, but at
7 months both the *Rv::tn1410* and *Rv::tn1410Δtgs1* mutants are still alive. We have yet to evaluate the histopathology and cfu from these animals. If *tgs1* produces the TAG species that are the substrates for LprG-1410 transport, then we would expect that loss of *tgs1* function should decrease intracellular TAG levels and potentially reverse the growth rate phenotype that we have seen with loss of LprG-1410 function. As far as we can tell based on initial results, loss of *tgs1* function does not rescue growth of the LprG-1410 mutant *in vivo*. Possible explanations for the lack of rescue include: 1) *tgs1* does not produce the TAG substrates for LprG-1410 mediated transport 2) *tgs1* is not active during growth in the mouse or 3) transport of TAG drives some important physiological function independent of overall of TAG levels. We know that loss of TAG synthesis has no effect on survival in immune competent or immune deficient mice therefore TAG synthesis or TAG levels must not be the sole mechanism by which TB counters *in vivo* host pressures, including, but not limited to, propionate toxicity, redox stress, and lipid oxidation. Given that *tgs1* function is best described in the context of hypoxia, it is clear that these mutant strains need to be tested for growth resuscitation in hypoxia. The mouse model is not recognized as a hypoxic model—therefore, the choice of *tgs1* out of all the annotated tag synthases may not have been ideal. It is also possible that loss of one tag synthase results in upregulation of other tag synthases. Future work will need to focus on further elucidating the upstream and downstream effects of TAG transport in order to determine the mechanism by which TAG levels and TAG transport may regulate growth rate in mycobacteria.
SECTION 5.3 REFERENCES.

