Abstract

*Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) is the only vaccine against tuberculosis (TB). Although in use for nearly a century, it is far from ideal, exhibiting variable protection against pulmonary TB in adulthood and can cause disseminated disease in immunocompromised individuals. Therefore, there is an urgent need to develop more effective and safer TB vaccines than BCG. Understanding molecular factors that influence BCG vaccine properties including safety, immunogenicity, and protective efficacy will help to achieve this goal. This is especially relevant since vaccine candidates designed to boost BCG efficacy have yielded disappointing results in recent clinical trials.

Several hypotheses exist for the variable protection of BCG and one of which concerns BCG strain heterogeneity. While it was widely recognized that different BCG strains exhibited varying degrees of virulence and protection in animal models, it has been attributed to quality control issues during the manufacturing process. However, work from our lab suggests that this variability may reflect true biological differences among BCG strains. Notably, we discovered a correlation between the production of the cell wall lipids, phthiocerol dimycocerosates (PDIMs)
and phenolic glycolipids (PGLs), and BCG vaccine safety record and between the expression of
*phoP*, the transcriptional regulator in the PhoP-PhoR two-component system, and BCG
immunogenicity. Based on these initial observations, the goal of my thesis was to understand the
roles of PDIMs/PGLs and PhoP in mediating BCG vaccine clinical properties, including
virulence, immunogenicity, and protection. Using mouse models of infection and *M. tuberculosis*
challenge, I found that the loss of PDIMs/PGLs reduces virulence and efficacy of BCG without
having an effect on immunogenicity. In examining the role of PhoP, I showed that
overexpression of *phoP* improved protection but also increased virulence of BCG. Taken
together, my work has identified two molecular factors that impact BCG vaccine properties and
suggests that *phoP* overexpression may be a novel strategy to improve BCG efficacy. Further,
my work suggests that there is an intimate link between BCG virulence and protective efficacy,
which provides valuable insight for the selection of BCG strains for current immunization
programs and the design of future TB vaccine candidates.
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# Table of Contents

Acknowledgments ........................................................................................................ iv  
Table of Contents ........................................................................................................ v  
List of Tables ................................................................................................................ ix  
List of Figures ................................................................................................................ x  
List of Abbreviations ................................................................................................... xii

**CHAPTER 1 : INTRODUCTION** .................................................................................. 1  
Chapter 1: Introduction ............................................................................................... 2

1.1 Tuberculosis and *Mycobacterium tuberculosis* .................................................... 2  
   1.1.1 *Mycobacterium tuberculosis* ......................................................................... 2  
   1.1.2 The Global Impact of Tuberculosis ............................................................... 4  
   1.1.3 Pathogenesis and Immunology of TB ........................................................... 5  

1.2 The BCG Vaccine .................................................................................................. 10  

1.3 Current TB Vaccine Development ...................................................................... 11  
   1.3.1 Replacement Candidates: Recombinant BCG and Attenuated *M. tb* Strains .................................................................................................................. 12  
   1.3.2 Booster Candidates: Viral-Vectored and Subunit Vaccines ......................... 14  
   1.3.3 Potential Limitations of Current TB Vaccine Candidates ............................ 16  

1.4 BCG Heterogeneity ............................................................................................. 16  
   1.4.1. Mechanisms of Attenuation of BCG ........................................................ 18  

1.5 Molecular Factors That May Impact BCG Vaccine Properties .......................... 21  
   1.5.1. PDIMs/PGLs and BCG Safety .................................................................. 21  
   1.5.2. PhoP and BCG Immunogenicity ............................................................... 23  

1.6. Thesis Rationale and Outline ............................................................................. 25

**CHAPTER 2 : Both Phthiocerol Dimylocerosates and Phenolic Glycolipids are Required for Virulence of *M. marinum*** ........................................................................ 29
2.1 Introduction ........................................................................................................................................... 30

2.2 Materials and Methods ........................................................................................................................ 33

2.2.1 Bacterial Strains and Culture Conditions ......................................................................................... 33

2.2.2 Generation and Screening of M. marinum φMycobacterium Insertion Library ................................. 34

2.2.3 Molecular Cloning and Complementation ......................................................................................... 34

2.2.4 Quantitative Real-Time PCR (qRT-PCR) ............................................................................................ 35

2.2.5 Thin Layer Chromatography (TLC) Analysis of PDIMs and PGLs .................................................... 35

2.2.6 Zebrafish Infection ............................................................................................................................ 36

2.2.7 Drug Sensitivity Assay ....................................................................................................................... 36

2.2.8 Uptake Assay ...................................................................................................................................... 37

2.3 Results ....................................................................................................................................................... 37

2.3.1 Isolation and Characterization of M. marinum Mutants Defective in Cord Formation ......................... 37

2.3.2 The ppsA::Tn, ppsB::Tn, ppsD::Tn, ppsE::Tn, fadD28::Tn and mas::Tn Mutants of M. marinum Failed to Synthesize PDIMs and PGLs ...................................................... 41

2.3.3 The fadD26 Gene is Involved in the Synthesis of PDIMs but not PGLs in M. marinum ......................... 43

2.3.4 PDIMs and PGLs are Both Required for M. marinum Virulence in Zebrafish ............................... 46

2.3.5 PDIMs and PGLs are Important for Intracellular Growth ............................................................... 50

2.3.6 PDIMs and PGLs Play a Role in Cell Wall Permeability ............................................................... 52

2.4 Discussion .............................................................................................................................................. 55

CHAPTER 3: The Role of PDIMs/PGLs in BCG Virulence and Protective Efficacy ...................................... 61

3.1 Introduction ........................................................................................................................................... 62

3.2 Material and Methods ............................................................................................................................ 65

3.2.1 Bacterial Strains and Culture Conditions ........................................................................................ 65

3.2.2 Generating an Isogenic PDIM/PGL Knockout ................................................................................. 65

3.2.3 Lipid Analysis by Thin Layer Chromatography .............................................................................. 66
3.2.4 Analysis of BCG Virulence in SCID Mice ........................................ 67
3.2.5 Immunogenicity Studies .......................................................... 67
3.2.6 Protection against M. tb Challenge .......................................... 69
3.2.7 Histological Analysis ............................................................ 69

3.3 Results ....................................................................................... 70
3.3.1 Construction of an Isogenic PDIM/PGL-deficient Mutant ............ 70
3.3.2 Loss of PDIMs/PGLS Reduces Virulence of BCG Pasteur ............ 70
3.3.3 Loss of PDIMs/PGLs Does Not Affect Immunogenicity of BCG Pasteur 73
3.3.4 Loss of PDIMs/PGLs affects BCG-mediated Production against M. tb 74

3.4 Discussion .................................................................................. 77

CHAPTER 4 : The Role of PhoP in BCG Immunogenicity and Protection .... 81

4.1 Introduction .............................................................................. 82
4.2 Materials and Methods ............................................................. 83
4.2.1 Bacterial Strains and Culture Conditions ................................ 83
4.2.2 Immunogenicity Assessments .............................................. 84
4.2.3 Analysis of BCG Virulence in SCID Mice ............................... 85
4.2.4 Protection Against M. tb Challenge ...................................... 86

4.3 Results ....................................................................................... 86
4.3.1 Immunogenicity of rBCG-Japan Strains ................................. 86
4.3.2 Comparison of Virulence in SCID Mice ................................. 87
4.3.3 The Overexpression of phoP Improves Efficacy of BCG-Japan .... 90

4.4 Discussion .................................................................................. 93

CHAPTER 5 : DISCUSSION AND FUTURE DIRECTIONS ...................... 98

5.1 Summary of Findings ............................................................... 99
5.2 Major Conclusions ................................................................. 100
5.3 Implications of My Findings ................................................... 103
5.4 Future Directions .................................................................................................................. 106

5.4.1 Increasing the Potency of BCG .......................................................................................... 106

5.4.2 How are PDIMs/PGLs contributing to BCG-mediated protection? ................................. 109

5.4.3 Will restoring PDIMs/PGLs in BCG-Japan improve efficacy? ................................. 111

5.4.4 A Comprehensive Comparison of BCG Strains ............................................................ 112

5.5 Interesting Questions ........................................................................................................... 114

5.5.1 Is it possible to develop a BCG vaccine that prevents reactivation? .......................... 114

5.5.2 Is it possible to develop a sterilizing BCG vaccine? ..................................................... 114

References ................................................................................................................................ 116
List of Tables

Table 2.1. *M. marinum* PDIM/PGL mutants are hypersensitive to hydrophobic and β-lactam antibiotics. ................................................................. 53
List of Figures

Figure 1.1. General characteristics of *M. tb*. ................................................................. 3
Figure 1.2. Overview of the immune response to TB. ............................................................ 6
Figure 1.3. Genealogy of BCG vaccine strains ....................................................................... 19
Figure 2.1. PDIM/PGL biosynthesis in *M. marinum*................................................................. 39
Figure 2.2. Colony morphology and cording phenotype of PDIM/PGL-deficient mutants of *M. marinum*. ........................................................................................................... 40
Figure 2.3. 2D-TLC analysis of PDIMs and PGLs. .................................................................... 42
Figure 2.4. Quantitative RT-PCR analysis .............................................................................. 44
Figure 2.5. Survival of zebrafish infected with *M. marinum* strains. ...................................... 47
Figure 2.6. The PDIM/PGL-deficient mutant did not cause pathological changes in infected zebrafish. .................................................................................................................. 49
Figure 2.7. The loss of PDIMs/PGLs decreases intracellular survival. ...................................... 51
Figure 2.8. Uptake of EtBr and Nile Red by *M. marinum* strains. ......................................... 54
Figure 3.1. Construction of a PDIM/PGL deficient strain of BCG-Pasteur............................... 71
Figure 3.2. The PDIM/PGL deficient mutant of BCG-Pasteur is less virulent in SCID mice ............................................................... 72
Figure 3.3. The loss of PDIMs/PGLs does not affect production of IFNγ. .............................. 75
Figure 3.4. Loss of PDIMs/PGLs affects BCG-mediated protection against *M. tb* .......... 76
Figure 4.1. IFNγ production by rBCG-Japan strains. .............................................................. 88
Figure 4.2. Cytokine production stimulated by rBCG-Japan.*phoP/phoPR* ......................... 89
Figure 4.3. Comparison of rBCG-Japan burden in SCID mice ................................................ 91
Figure 4.4. Efficacy of rBCG-Japan vaccine candidates in a BALB/c mouse model.............. 92
Figure 4.5. BCG-Pasteur strains induce splenomegaly over the course of infection.............. 96
Figure 5.1. PDIMs/PGLs and PhoP contribute to BCG virulence and protection ................. 101
Figure 5.2. Tailored vaccine development strategy ................................................................. 104
Figure 5.3. Testing of rBCG-Japan.*Δlsr2*. ................................................................. 108
Figure 5.4. IFNγ production by rBCG-Japan.glnA1.
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BCG</td>
<td>Bacille Calmette-Guérin</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CFP-10</td>
<td>culture filtrate protein – 10 kDa</td>
</tr>
<tr>
<td>CFU</td>
<td>colony forming unit</td>
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<tr>
<td>DU</td>
<td>duplication</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
<td>ESAT-6</td>
<td>6 kDa early secretory antigenic target</td>
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<td>EtBr</td>
<td>ethidium bromide</td>
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<tr>
<td>HE</td>
<td>hematoxylin-eosin</td>
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<tr>
<td>HIV</td>
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<td>LAM</td>
<td>lipoarabinomannan</td>
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<td>Mbp</td>
<td>mega base pair</td>
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<tr>
<td>MDR</td>
<td>multidrug-resistant</td>
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<td>MHC I</td>
<td>major histocompatibility complex I</td>
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<td>MHC II</td>
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<tr>
<td>MIC</td>
<td>minimum inhibitory concentration</td>
</tr>
<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
</tr>
<tr>
<td>M. tb</td>
<td><em>Mycobacterium tuberculosis</em></td>
</tr>
<tr>
<td>MTBC</td>
<td><em>Mycobacterium tuberculosis</em> complex</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>PDIM</td>
<td>phthiocerol dimycocerosate</td>
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<tr>
<td>PGL</td>
<td>phenolic glycolipid</td>
</tr>
<tr>
<td>PPD</td>
<td>purified protein derivative</td>
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<td>qRT-PCR</td>
<td>quantitative real-time polymerase chain reaction</td>
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<td>single nucleotide polymorphism</td>
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<td>TDM</td>
<td>trehalose dimycolate</td>
</tr>
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<td>Th1</td>
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<tr>
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<td>TLC</td>
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</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
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<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>XDR</td>
<td>extensively drug-resistant</td>
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Chapter 1:

INTRODUCTION
1 Chapter 1: Introduction

1.1 Tuberculosis and *Mycobacterium tuberculosis*

1.1.1 *Mycobacterium tuberculosis*

*Mycobacterium tuberculosis* (*M. tb*) is the most successful bacterial pathogen of man. It is a notorious member of the *Mycobacterium tuberculosis* complex (MTBC), a group that also includes *M. bovis, M. microti* and *M. africanum*. Members of this group share 99.9% nucleotide similarity, are facultative intracellular pathogens, and cause tuberculosis disease in their respective hosts (Boddinghaus, Rogall et al. 1990; Sreevatsan, Pan et al. 1997; Cole, Brosch et al. 1998; Brosch, Gordon et al. 2002). *M. tb* is a Gram-positive rod that belongs to the group of actinobacteria. It is a member of the slow-growing group of mycobacteria, exhibiting a doubling time of 15 – 20 hours. The *M. tb* genome is approximately 4.4 Mbp, which encodes about 4000 genes, and like other mycobacteria, has a high G + C content of ~66% (Cole, Brosch et al. 1998). Interestingly, the *M. tb* genome encodes an abundance of genes (over 250) involved in fatty acid metabolism and a further ~9% of the genome encodes PE/PPE proteins, a family of glycine-rich proteins that are thought to convey antigenic diversity (Cole, Brosch et al. 1998; Karboul, Mazza et al. 2008). The *M. tb* genome also encodes five type VII secretion systems, which are unique from other secretion systems and are used to secrete *M. tb* virulence factors, such as ESAT-6 and CFP-10 (Abdallah, Gey van Pittius et al. 2007). A key feature of *M. tb* and other mycobacteria is its complex lipid-rich cell wall, which is thought to form a hydrophobic barrier that confers impermeability to antibiotics and is responsible for the characteristic acid-fast (or Zhiel-Neelsen) staining (Figure 1.1A). This is largely due to the presence of a group of complex branched-
Figure 1.1. General characteristics of *M. tb*.

(A) High power micrograph of acid-fast *M. tb* from patient sputum identified by Zhiel-Neelsen staining (1000x; image taken from (Lawn and Zumla 2011)). (B) Schematic diagram of mycobacterial cell wall (modified from (Abdallah, Gey van Pittius et al. 2007)). Peptidoglycan, arabinogalactan, and mycolic acids make up the cell wall core. The free lipids including PDIMs, PGLs, TDM, and LAM, are interspersed and exposed on the cell wall surface. PDIM, phthiocerol dimycocerosate; PGL, phenolic glycolipid; TDM, trehalose dimycolate; LAM, lipoarabinomannan.
chained hydroxyl lipids called mycolic acids, which are covalently linked to arabinogalactan in the cell wall (Figure 1.1B). The mycobacterial cell wall also comprises a number of free lipids that have been implicated in virulence, including lipoarabinomannan (LAM) (Chan, Fan et al. 1991), dimycolyltrehalose (TDM/cord factor) (Middlebrook, Dubos et al. 1947; Glickman, Cox et al. 2000), phthiocerol dimycocerosate (PDIM) (Camacho, Ensergueix et al. 1999; Cox, Chen et al. 1999) and phenolic glycolipids (PGL) (Reed, Domenech et al. 2004) (Figure 1.1B). PDIMs and PGLs are of particular interest for this thesis and will be discussed further in subsequent sections.

1.1.2 The Global Impact of Tuberculosis

Tuberculosis (TB), caused by *M. tb*, is an ancient disease that still plagues humanity today. It remains one of the top global health problems with an estimated 8.7 million new cases and 1.4 million deaths from TB in 2011 (WHO 2012). Furthermore, it is estimated that one third of the world’s population (~2 billion people) is latently infected with *M. tb*, representing a large reservoir for reactivation and spread (Corbett, Watt et al. 2003; WHO 2009). Successful eradication of the disease has been hampered by many obstacles including the difficulty of rapid diagnosis, lengthy chemotherapy treatment ranging from 6 to 9 months, and the lack of an effective vaccine. The situation is further complicated by the rise in multi-drug resistant (MDR), defined as resistance to isoniazid and rifampin, and extensively-drug resistant (XTR), defined as resistance to isoniazid, rifampin, and at least one second-line drug, strains of *M. tb*. An additional threat to effective control is *M. tb*/Human Immunodeficiency Virus (HIV) co-infection, which forms a deadly association that claimed 430,000 lives in 2011 (WHO 2012). Although *M. tb* can infect any part of the body, the most common form of the disease is pulmonary TB, which is
the most contagious form and usually manifests in adulthood. Characteristic symptoms of active pulmonary TB include coughing with sputum or blood, chest pains, weakness, and weight loss (WHO 2004). Although treatable, the slow growth of *M. tb* (~15-20 hours doubling time) and its ability to cause persistent infection require a long duration of antibiotic treatment, which fosters patient noncompliance and consequently contributes to the rise of antibiotic resistance and ineffective treatment.

### 1.1.3 Pathogenesis and Immunology of TB

The success of *M. tb* as a pathogen is attributed to its ability to evade the host immune system to establish a latent infection. *M. tb* is primarily transmitted by inhalation of aerosolized droplets generated through coughing by a person with active TB (Figure 1.2). The pathogen enters through the respiratory tract and into the lungs, which is the primary site of infection. In a small fraction of immunocompetent individuals, the infection may be cleared by the immune system (Figure 1.2A) but in an immunocompromised host, primary infection can develop into active TB (Figure 1.2B). In the majority of individuals, however, the bacilli persist in the host to establish a clinically asymptomatic, latent infection that can prevail for decades (Figure 1.2C) (reviewed in (Kaufmann 2001)). In 5 – 10% of latently infected people, the infection can reactivate with a waning of immunity to develop into active TB. Suppression of the immune system dramatically increases susceptibility to *M. tb* infection and is exemplified by the fact that TB is the leading cause of death in HIV-infected individuals (WHO 2012). In these patients, TB progresses rapidly and without proper treatment, patients die from suffocation or respiratory failure resulting from excess pulmonary fluid or tissue damage in the lung, respectively.
Figure 1.2. Overview of the immune response to TB. 
*M. tb* is inhaled through aerosolized droplets and can (A) be cleared (B) develop into active disease or (C) contained within granulomas whereby establishing a latent infection. Upon inhalation, *M. tb* is phagocytosed by macrophage and dendritic cells, their primary niche for replication. Effector T cells, including conventional CD4+ and CD8+ T cells, and macrophages play a primary role in the control of the infection. Activated T cells produce important cytokines, such IFNγ and TNF, which are important for macrophage activation. Activated macrophages induce phagosomal maturation and produce bactericidal molecules, including RNI/ROI, to kill the invading pathogen. Figure modified from (Kaufmann 2001).
*M. tb* enters the lungs and is deposited into the lower airways and alveolar tissues, where they are phagocytosed by resident macrophages and dendritic cells sampling alveolar space (Leemans, Juffermans *et al.* 2001; Wolf, Linas *et al.* 2007). The bacilli reside in a specialized intracellular compartment called the phagosome which would normally undergo maturation and fusion with lysosomes, producing a degradative and microbicidal compartment called the phagolysosome (Armstrong and Hart 1971; Armstrong and Hart 1975). However, *M. tb* is able to subvert phagosome maturation by preventing phagosome acidification and phagosome-lysosome fusion, so that it resides in an early-endosomal compartment, safe from antimicrobial molecules (Russell 2007). Work in mouse models has suggested that infected macrophages and dendritic cells then migrate to the draining lymph node, where they prime naïve T cells (Wolf, Desvignes *et al.* 2008; Cooper 2009). Once activated, effector T cells migrate to the primary site of infection to activate infected macrophages to induce an IFNγ-mediated antimycobacterial response and recruit other immune effectors, including more T cells, B cells, macrophages, and dendritic cells (Cooper 2009). These effector cells form an aggregate to effectively contain *M. tb* in an organized structure called the granuloma, the hallmark of pulmonary TB (Kaufmann 2001). The traditional view suggests that the granuloma effectively “walls off” the infection and establishes a stalemate between the host and pathogen. Interestingly, recent evidence using a zebrafish embryo model of *M. marinum* infection suggests that early granuloma formation may benefit the pathogen. This study showed that granuloma formation during the innate immune response to *M. marinum* infection is a dynamic process, whereby recruitment of uninfected macrophages to the granuloma provides additional cells for expansion and dissemination (Davis and Ramakrishnan 2009). *M. tb* can also adapt to the hypoxic environment encountered in the granuloma and remain in a quiescent state (Roberts, Liao *et al.* 2004; Rustad, Harrell *et al.* 2008), which can be reactivated by host immune suppression. Thus, *M. tb* can establish a latent
infection in the host that can persist for a lifetime. Although the lung is the primary site of infection, without proper immune control *M. tb* can disseminate to other organs and parts of the body. This results from a breakdown of the granuloma, resulting in necrosis, cavitation, liquidation of the cavity, and subsequent spread of the pathogen (Figure 1.2C) (Kaufmann 2001). Disseminated disease, such as miliary TB, is a serious condition and results in 100% mortality if untreated (dos Santos, Deutschendorf *et al.* 2011; von Reyn, Kimambo *et al.* 2011).

Immunological work in humans and mouse models of TB has emphasized the role of cell-mediated immunity in response to *M. tb* infection, particularly through CD4+ T cells and IFNγ. Evidence for this in humans comes from HIV studies that show that a modest reduction in CD4+ T cell counts is sufficient to increase the rate of TB reactivation by 5- to 10-fold (Kwan and Ernst 2011). Several murine studies using antibody depletion or knockout mice have shown that loss of CD4+ T cells results in poor control of *M. tb* infection, culminating in higher bacterial burden and decreased survival (Mogues, Goodrich *et al.* 2001). CD4+ T cells recognize peptide antigens processed through the MHC II endocytic pathway, become activated, proliferate, and differentiate into several subsets, including Th1 and Th2. The Th1 subset is thought to be more important for intracellular pathogens, such as *M. tb*, whereas the Th2 subset is critical against extracellular antigens, such as those produced by allergies or helminth infection (Romagnani 2006). The Th1 subset is characterized by secretion of IFNγ, TNF, and IL-2. Of these, IFNγ has been shown to play an essential role in defense against *M. tb* by stimulating the antimicrobial capacity of macrophages, through production of reactive nitrogen intermediates (RNI) and reactive oxygen intermediates (ROI) (Kaufmann 2001). In addition, IFNγ creates a positive-feedback loop where it stimulates undifferentiated CD4+ T cells to become Th1 cells (Romagnani 2006). The critical role of IFNγ in TB immunity is highlighted by the observation that individuals defective in IFNγ signaling through a mutation in the IFNγ receptor gene are
highly susceptible to mycobacterial infection (Ottenhoff, Kumararatne et al. 1998; de Albuquerque, Rocha et al. 2012). The Th2 subset is characterized by the secretion of IL-4 and IL-10. Production of a Th2 response is thought to be detrimental to protection against TB as IL-10 inhibits the production of IFNγ, resulting in a dampened Th1 response (Kaufmann 2001).

Although CD4+ T cells play a major role in TB immunology, compelling evidence indicates that CD8+ T cells are also involved in protection. For example, mice deficient in CD8+ T cells or MHC I molecules were more susceptible to M. tb and succumbed earlier to infection (Sousa, Mazzaccaro et al. 2000; Mogues, Goodrich et al. 2001). CD8+ T cells recognize peptide antigens presented by MHC I processed through the cytosolic pathway and have a primary cytotoxic function to lyse infected cells through the release of cytotoxins (perforin, granzymes, and granulysin) (Figure 1.2) (Lewinsohn, Heinzel et al. 2003; Romagnani 2006). In addition, M. tb-specific CD8+ T cells produce IFNγ that contribute to the activation of macrophage-mediated killing (Lewinsohn, Heinzel et al. 2003). The ability of M. tb to present antigens to CD8+ T cells from the phagosome is an interesting phenomenon and has been the focus of considerable investigation. It is thought that M. tb virulence factors, ESAT-6 and CFP-10, create holes in the phagosomal membrane that facilitate phagosomal escape into the cytosol (van der Wel, Hava et al. 2007). Recent studies have illustrated a role of M. tb-infected cell-derived apoptotic vesicles that prime dendritic cells and subsequently activate a robust stimulation of CD8+ T cells (Divangahi, Desjardins et al. 2010). This knowledge has been exploited to develop new vaccine BCG candidates that stimulate both arms of cell-mediated immunity as a strategy to improve protection against TB (Hess, Miko et al. 1998; Grode, Seiler et al. 2005; Sun, Skeiky et al. 2009). Despite the advances in understanding TB immunology and pathogenesis, no correlate of protection in humans has been identified. This highlights the large knowledge gap at present, which is limiting the development of new treatments and vaccines.
1.2 The BCG Vaccine

Bacille Calmette-Guérin (BCG) is the only vaccine licensed for use against TB. BCG was derived from a virulent strain of *M. bovis* that was isolated from the milk of an infected cow. *M. bovis* is a very close relative of *M. tb*, sharing >99.95% sequence identity, and causes TB-like disease in cattle and other mammals (Imaeda 1985; Garnier, Eiglmeier et al. 2003). Around 1901, this strain was brought to the Institute Pasteur and was used by Albert Calmette and Camille Guérin, two former students of Louis Pasteur, to study bovine tuberculosis. During routine cultivation of the isolate, they noticed the emergence of colonies with unusual morphology within a couple of months. Interestingly, infection of guinea pigs with this morphologically distinct isolate showed that the strain exhibited reduced virulence (Behr and Small 1999; Oettinger, Jorgensen et al. 1999). Recognizing the importance of this discovery, they continued the *in vitro* serial passaging of this *M. bovis* isolate for 13 years (total 230 passages) and monitored virulence using numerous animal models until it reached an attenuated state (Oettinger, Jorgensen et al. 1999; Behr 2002). The first human trial occurred in 1921 and was administered to a child whose mother had died of TB shortly after giving birth (Bonah 2005). The vaccinated child did not have adverse reactions to the vaccination and did not develop TB despite his/her mother having contracted the disease. Additional children were tested with the strain with no issues reported (Bonah 2005). Excitingly, this represented the first prophylactic treatment for human TB.

As early as 1924, cultures of BCG were distributed by the Institute Pasteur to laboratories around the world. Since its inclusion in the WHO Expanded Program on Immunization in 1974, BCG has become the most widely used vaccine worldwide with over 100 million doses given annually (WHO 2004). Although BCG is the only vaccine against TB, there are major concerns
regarding its efficacy. While case-control studies have shown that the vaccine is effective at reducing disseminated forms of TB, including miliary TB and tubercular meningitis in children, randomized controlled trials of BCG has shown limited efficacy against adult pulmonary TB (ranging from 0-80%), the most prevalent and contagious form of the disease (Ferguson and Simes 1949; Hart and Sutherland 1977; Miceli, de Kantor et al. 1988; Sharma, Srivastava et al. 1989; Colditz, Brewer et al. 1994; Datta, Vallishayee et al. 1999; Brewer 2000). Another major concern with BCG is its safety in immunocompromised individuals, where disseminated BCG disease has been observed following vaccination in HIV-infected children (Hesseling, Marais et al. 2007). Since the risks of vaccination outweighed the potential benefits, in 2007, the WHO revised its guidelines and recommended that BCG not be given to HIV-infected infants (WHO 2007). Considering that HIV-endemic areas have the highest incidence of TB and HIV-associated deaths accounted for one-third of TB mortality in 2011 (WHO 2012), those populations most in need of vaccination are unable to receive appropriate prophylaxis. In light of this situation, there is an urgent need to develop a TB vaccine that is safer and more effective than BCG.

1.3 Current TB Vaccine Development

The last decade has witnessed a boom in TB vaccine development. Currently, the strategies for developing a new generation of TB vaccines are either to replace BCG with a stronger vaccine that provides a longer duration of protection or to design a vaccine to be used in conjunction with BCG that can be given at a later time to boost existing immunity. Examples from both categories have entered clinical trials and will be discussed below.
1.3.1 Replacement Candidates: Recombinant BCG and Attenuated \textit{M. tb} Strains

The poor efficacy of the current BCG vaccine has prompted the development of several replacement candidates. These include recombinant BCG strains overexpressing immunodominant antigens and attenuated \textit{M. tb} strains. Three recombinant BCG candidates have entered clinical trials. The most advanced is VPM1002 (rBCG\textit{ΔureC-hly}⁺), a BCG-Prague strain overexpressing listeriolysin O from \textit{Listeria monocytogenes}, developed by Stefan Kauffman’s group (Hess, Miko \textit{et al.} 1998; Grode, Seiler \textit{et al.} 2005; Desel, Dorhoi \textit{et al.} 2011). The expression of listeriolysin O mediates phagosomal escape of BCG from the endosome into the cytosol. This promotes apoptosis of the infected cell resulting in the release of BCG-containing apoptotic vesicles (Grode, Seiler \textit{et al.} 2005; Farinacci, Weber \textit{et al.} 2012). These vesicles are subsequently phagocytosed by bystander antigen processing cells and processed via the MHC class I pathway for presentation to CD8⁺ T cells. This mechanism is proposed to induce both arms of cell-mediated immunity through cross-priming. In addition, VPM1002 harbours a urease C deletion (\textit{ΔureC}) in order to maintain the optimal (acidic) pH for listeriolysin O function (Grode, Seiler \textit{et al.} 2005). This candidate showed promising results in preclinical animal trials where vaccination reduced bacterial burden in mice by ~1 log compared to the parental strain and induced potent CD4⁺ and CD8⁺ T cell responses (Grode, Seiler \textit{et al.} 2005; Desel, Dorhoi \textit{et al.} 2011; Farinacci, Weber \textit{et al.} 2012). Phase I clinical trials in adults showed that the vaccine was well tolerated with no major adverse reactions and elicited a moderate increase in BCG immunogenicity in adults (Grode, Ganoza \textit{et al.} 2013). VPM1002 is now in Phase IIa clinical trials in South African infants.
The second recombinant BCG candidate is Aeras-422, a BCG-Danish strain overexpressing perfringolysin O from *Clostridium perfringens*, developed by Aeras Global TB Vaccine Foundation. Similar to VPM1002, this strain was engineered to facilitate phagosomal escape of BCG to cross-prime CD8\(^+\) T cells (Sun, Skeiky *et al.* 2009). Although this candidate showed promising results in pre-clinical animal studies, further characterization was terminated after adverse effects were observed during Phase I trials to assess safety. During the study, two participants developed shingles following vaccination, likely from the reactivation of a latent herpes varicella-zoster viral infection (Kupferschmidt 2011). These adverse effects were attributed to the fact that, unlike listeriolysin O, perfringolysin O does not have a pH restriction and thus can not only function in the endosome but also in the cytosol and extracellularly (Tweten 2005; Bavdek, Kostanjsek *et al.* 2012). Although VPM1002 has shown an acceptable safety profile so far, this does potentially question the use of bacterial toxins in live vaccines.

The third recombinant BCG candidate is rBCG30, a BCG-Tice strain overexpressing the immunodominant Ag85B protein, developed by Marcus Horwitz’s group (Horwitz, Harth *et al.* 2000). Ag85B is the most abundant secreted protein in *M. tb* and BCG and belongs to the mycolyl transferase family comprising Ag85A, B, and C. Ag85A and B have been shown to be highly immunogenic (D'Souza, Rosseels *et al.* 2003). This strain was engineered to amplify the anti-TB CD4\(^+\) T cell response and was the first recombinant candidate to show greater protection (~0.5 to 1 log reduction in *M. tb* CFU) than the conventional BCG in pre-clinical animal studies (Horwitz, Harth *et al.* 2000; Horwitz and Harth 2003). rBCG30 was proven safe in Phase I trials and showed an enhancement of both Ag85B-specific CD4\(^+\) and CD8\(^+\) T cells (Hoft, Blazevic *et al.* 2008). This candidate completed Phase I trials in 2004 but has not advanced to subsequent stages for reasons that are unclear.
BCG has been criticized for becoming over-attenuated to the point of ineffectiveness and for not mimicking the natural immunity occurring after *M. tb* infection (Behr and Small 1997). As such, an alternative to recombinant BCG development is construction of attenuated *M. tb* strains. The leading candidate in this category is *M. tb* ΔphoP (Mtb SO2), developed by Carlos Martin’s group. This strain contains a deletion in *phoP*, which encodes a transcriptional regulator that controls the expression of over 100 genes in the *M. tb* genome and its expression has been linked to virulence (Perez, Samper et al. 2001; Walters, Dubnau et al. 2006). This attenuated *M. tb* strain was shown to be safer than BCG but had comparable levels of protection in mice and rhesus monkeys, showing a ~0.5 to 1 log reduction in *M. tb* burden (Martin, Williams et al. 2006; Verreck, Vervenne et al. 2009). Although approved to enter Phase I clinical trial shortly, there are reservations about the use of live *M. tb* strains for vaccination due to the possibility of reversion to wildtype levels of virulence.

### 1.3.2 Booster Candidates: Viral-Vectored and Subunit Vaccines

Long-term human vaccine studies have shown that the protective efficacy of BCG wanes after 10-20 years (Comstock, Woolpert *et al.* 1976; Hart and Sutherland 1977; Sterne, Rodrigues *et al.* 1998). This time frame coincides with the increased rate of pulmonary TB in adulthood and has been proposed as a hypothesis for the limited efficacy of BCG. As such, the development of booster vaccines is a practical option for improving BCG vaccination since it overcomes the immediate need to replace the current BCG. The boosting strategy has exploited several T cell antigens (eg. Ag85B, PPE18, and ESAT-6 like proteins) that are in the forms of either fusion protein or viral-vectored subunit vaccines (McShane, Pathan *et al.* 2004; Dietrich, Andersen *et*
al. 2006; Radosevic, Wieland et al. 2007; Bertholet, Ireton et al. 2010; Scriba, Tameris et al. 2012). Several of these have entered the clinical pipeline.

The most advanced subunit vaccine is MVA85A, a replication-deficient vaccinia virus expressing Ag85A, developed by Helen McShane’s group. This candidate has shown to boost BCG vaccination in several animal models (McShane, Brookes et al. 2001; Verreck, Vervenne et al. 2009; Vordermeier, Villarreal-Ramos et al. 2009) and was well tolerated in Phase I and Phase IIa clinical trials in the UK and Africa (McShane, Pathan et al. 2004; Sander, Pathan et al. 2009; Scriba, Tameris et al. 2012). These initial studies also showed that the booster enhanced and sustained antigen-specific multifunctional CD4+ and CD8+ T cell production (Beveridge, Price et al. 2007). Other viral-vectored candidates in the clinical pipeline include AERAS-402 and AdAg85A, both of which employ non-replicating adenoviruses. AERAS-402 expresses a fusion protein of immunodominant antigens Ag85A, Ag85B, and TB10.4 and has been shown to enhance cytokine production by both CD4+ and CD8+ T cell in BCG-vaccinated individuals and is now in Phase IIb trials (Radosevic, Wieland et al. 2007; Abel, Tameris et al. 2010; Hoft, Blazevic et al. 2012). Unlike AERAS-402, AdAg85A expresses a single antigen, Ag85A, and was shown to boost cell-mediated immunity in BCG-vaccinated mice and is now in Phase I trials (Santosuosso, McCormick et al. 2006; Santosuosso, McCormick et al. 2007).

Several protein subunit vaccines are also progressing through clinical trials. The furthest among these include M72 and Hybrid 1, a fusion of M. tb proteins Mtb32-Mtb39 (Brandt, Skeiky et al. 2004; Leroux-Roels, Leroux-Roels et al. 2010; Leroux-Roels, Forgus et al. 2013) and Ag85B-ESAT-6 (Dietrich, Andersen et al. 2006), respectively. Both have shown to boost pre-existing BCG-specific cell-mediated immune responses in animal models and adults and are now Phase IIa clinical trials to test for safety.
1.3.3 Potential Limitations of Current TB Vaccine Candidates

While TB vaccine research has gained momentum in recent years, there are still major obstacles. Although a number of candidates have entered the clinical pipeline, it is important to recognize that none of the subunit vaccines have exhibited greater efficacy than BCG when used as stand-alone vaccines and their ability to boost pre-existing BCG-mediated immunity is still being tested. Recently, the most advanced subunit vaccine candidate, MVA85A, exhibited disappointing results in a Phase IIb trial in BCG-vaccinated South African infants, where it induced a modest cell-mediated immune response but failed to protect against TB or *M. tb* infection (Tameris, Hatherill *et al.* 2013). As such, the promise of other booster vaccine candidates is questionable.

Although live TB vaccines including recombinant BCG (e.g. rBCG30, rBCGΔ*ureC-hly*+) and the attenuated *M. tb phoP* mutant showed promising results in preclinical testing, their safety remains in question in HIV-infected individuals. All clinical trials of new live TB vaccines have so far excluded HIV+ individuals. While this cautious approach is logical, there is a need to evaluate new TB vaccines in HIV-infected populations because they are among the most in need of a new and effective vaccine, with an annual TB incidence rate of 5-10%.

1.4 BCG Heterogeneity

Another limitation of the current recombinant BCG approach is that little attention has been paid to the selection of specific BCG strains that are included in the vaccine strategy. As mentioned above, BCG exhibits highly variable efficacy against adult pulmonary TB. The reason for the
variability in protection is not completely understood but several hypotheses have been proposed, including pre-exposure to environmental mycobacteria, nutritional or genetic differences in vaccinee populations, variations among clinical *M. tb* strains, and differences among the BCG strains used for vaccination (Comstock 1994; Fine 1995; Behr 2002; Brandt, Feino Cunha *et al.* 2002; Demangel, Garnier *et al.* 2005; Tsenova, Harbacheuski *et al.* 2007). These hypotheses are not mutually exclusive and may all contribute to the observed variable protection. Of these hypotheses, the heterogeneity of BCG strains is of particular interest. Although colloquially referred to as BCG, there are a number of BCG strains that have been historically used in different vaccination programs (Behr and Small 1999; Oettinger, Jorgensen *et al.* 1999). While early studies revealed that different BCG strains displayed varying degrees of virulence and protection in animals (Dubos and Pierce 1956; Dubos, Pierce *et al.* 1956; Bunch-Christensen, Ladefoged *et al.* 1968; Bunch-Christensen, Ladefoged *et al.* 1970; Ladefoged, Bunch-Christensen *et al.* 1970), these differences were often attributed to quality control issues during the manufacturing process of the strains by different facilities (Milstien and Gibson 1990). The variability in BCG vaccine properties however may reflect true biological differences among BCG strains.

Following the success of the first human trial, cultures of BCG were distributed worldwide. Despite efforts to standardize growth and manufacturing protocols, different passaging conditions were employed, thus continuing the *in vitro* evolution for BCG (Oettinger, Jorgensen *et al.* 1999). As a result, dozens of substrains emerged, including the four that are most commonly used today: BCG-Glaxo (strain 1077), BCG-Danish (strain 1331), BCG-Pasteur (strain 1173 P2), and BCG-Japan (Tokyo strain 172) (WHO 2004). By the 1950s, independent manufacturers began observing the emergence of BCG strains with distinct morphological, biochemical, and immunological phenotypes (Dubos and Pierce 1956; Dubos, Pierce *et al.*
1956). It was not until 1966 that the WHO established the “seed-lot system” to initiate lyophilization of the strains, which halted the further evolution of BCG. However, the dissemination of BCG worldwide fostered the *in vitro* evolution of BCG into different lineages (Figure 1.3). Various genomic comparisons have uncovered extensive genomic divergence, including deletions, duplications and single nucleotide polymorphisms (SNPs), amongst the BCG strains and have revealed a molecular phylogeny that is generally consistent with the historical records of BCG dissemination (Behr and Small 1999; Behr, Wilson *et al.* 1999).

### 1.4.1. Mechanisms of Attenuation of BCG

Two phases of attenuation are likely to have occurred to produce the BCG strains present today. The first phase occurred between 1908 – 1921 during the initial 230 rounds of passaging by Calmette and Guérin that produced the original vaccine, while the second phase occurred with the widespread distribution of BCG starting from 1924 (Behr and Small 1999; Oettinger, Jorgensen *et al.* 1999).

Comparative genomic analyses have identified several chromosomal deletions in BCG, and not surprisingly, these encompass deletions of known virulence factors of *M. tb* and *M. bovis* (Mahairas, Sabo *et al.* 1996; Behr and Small 1999). Among these, only the Region of Difference – 1 (RD1) is absent in all BCG strains (Mahairas, Sabo *et al.* 1996; Pym, Brodin *et al.* 2002). RD1 encodes the ESX-1 secretion system, which is one of five type VII secretion systems found in mycobacteria (Abdallah, Gey van Pittius *et al.* 2007). RD1 encodes nine genes, including two established virulence factors in *M. tb*, the early secretory antigenic 6-kDa (ESAT-6) protein and the culture filtrate protein 10 (CFP-10) (Sorensen, Nagai *et al.* 1995; Berthet, Rasmussen *et al.* 1998). ESAT-6/CFP-10 have been shown to play a role in subverting macrophage killing by
Figure 1.3. **Genealogy of BCG vaccine strains.**
Diagrammatic representation of genetic mutations in BCG strains evolved from the *M. bovis.* DU; duplication. Taken from (Leung, Tran et al. 2008).
preventing phagosomal maturation and facilitating phagosomal escape (Hsu, Hingley-Wilson et al. 2003; Stanley, Raghavan et al. 2003; Guinn, Hickey et al. 2004; Tan, Lee et al. 2006). Interestingly, deletion of RD1 in M. tb does not result in attenuation to BCG levels and reintroduction of ESX-1 in BCG does not restore full virulence, suggesting that additional mutations contribute to the attenuation of BCG (Pym, Brodin et al. 2003; Sherman, Guinn et al. 2004). Indeed, genomic comparisons uncovered several duplications and deletions present in BCG strains (Mahairas, Sabo et al. 1996; Behr, Wilson et al. 1999; Brosch, Gordon et al. 2000; Mostowy, Tsolaki et al. 2003; Brosch, Gordon et al. 2007). Furthermore, over 2000 single nucleotide polymorphisms (SNPs) were discovered by whole genome sequence comparisons between BCG and M. tb and over 700 between BCG and M. bovis (Brosch, Gordon et al. 2007; Leung, Tran et al. 2008; Garcia Pelayo, Uplekar et al. 2009). Most are common to the entire BCG lineage, but some genomic polymorphisms are specific to individual BCG strains (Mostowy, Tsolaki et al. 2003; Brosch, Gordon et al. 2007; Leung, Tran et al. 2008; Garcia Pelayo, Uplekar et al. 2009) (Figure 1.3). For example, the RD2 region is deleted in “late” BCG strains derived after 1927 and the DU2-III duplication is specific to BCG-China, BCG-Prague, BCG-Glaxo, and BCG-Danish derived in the 1940s (Figure. 1.3). This suggests that loss of virulence during the in vitro evolution of BCG involved both the loss of RD1 and the accumulation of attenuating deletions and SNPs. In spite of this plethora of genomic data, how these genetic differences affect vaccine properties, such as safety, immunogenicity, and efficacy, remains largely unknown.
1.5 Molecular Factors That May Impact BCG Vaccine Properties

A major interest in our lab is to uncover the molecular basis for the phenotypic heterogeneity seen amongst BCG strains. The advent of genome sequencing has helped to shift the paradigm of BCG strain heterogeneity to reflect true biological properties, rather than from differences in strain manufacturing. We assert that each BCG strain encompasses its own inherent properties that can be exploited to improve BCG efficacy. To this end, previous work by our lab uncovered a link between established mycobacterial factors, PDIMs/PGLs and PhoP, and BCG safety and immunogenicity.

1.5.1. PDIMs/PGLs and BCG Safety

A previous PhD student in the lab, Jeffrey Chen, uncovered, for the first time, differential expression of established virulence factors amongst BCG strains. Using biochemical analysis by 2D-thin layer chromatography (TLC), he found that three BCG trains, BCG-Japan, -Moreau, and -Glaxo were naturally deficient in the production of phthiocerol dimycocerosates (PDIMs) and phenolic glycolipids (PGLs) (Chen, Islam et al. 2007). PDIMs and PGLs are structurally related, multiple methyl-branched fatty acid-containing lipids in the mycobacterial cell wall and are well established virulence factors (Onwueme, Vos et al. 2005). PDIMs are present only in pathogenic mycobacteria, including those in the \textit{M. tb} complex (\textit{M. tb}, \textit{M. bovis}, \textit{M. microti}, and \textit{M. africanum}). PGLs are also restricted to pathogenic mycobacteria except that in \textit{M. tb} only a subset of clinical isolates produces PGLs (Onwueme, Vos \textit{et al.} 2005). PDIMs were first implicated in virulence using signature-tagged transposon mutagenesis which identified \textit{M. tb}}
PDIM mutants that were attenuated in mice (Camacho, Ensergueix et al. 1999; Cox, Chen et al. 1999). Since then, PDIMs have been shown to mediate receptor-dependent phagocytosis of *M. tb* (Astarie-Dequeker, Le Guyader et al. 2009), and contribute to cell wall permeability (Camacho, Constant et al. 2001) and protection against bactericidal effects of reactive nitrogen intermediates in activated macrophages (Rousseau, Winter et al. 2004). PGLs have been implicated in dampening the immune response by inhibiting the release of proinflammatory cytokines and have been associated with a hypervirulent phenotype of certain *M. tb* clinical isolates (Reed, Domenech et al. 2004; Sinsimer, Huet et al. 2008).

Using a NimbleGen tiling array for whole genome comparison, we found a 975 base pair deletion in the PDIM/PGL biosynthetic locus in BCG-Moreau that likely accounts for the loss of PDIMs/PGLs in this strain (Leung, Tran et al. 2008). Similarly, Naka et al. discovered that a single base pair insertion in *ppsA*, a polyketide synthase involved in PDIM/PGL synthesis, in BCG-Japan, which caused a frameshift mutation and thus abolished the production of PDIMs/PGLs (Naka, Maeda et al. 2011). The genetic mutation responsible for the loss of PDIMs/PGLs in BCG-Glaxo has yet to be identified.

Interestingly, the loss of PDIMs/PGLs in BCG-Japan, -Moreau, and -Glaxo correlate with their superior safety records over other BCG strains in clinical studies (Lotte, Wasz-Hockert et al. 1984; Chen, Islam et al. 2007). Analyses of BCG-induced complications in 55 countries revealed that certain strains were correlated with having a higher incidence of adverse reactions, including suppurative lymphadenitis (swelling of the lymph nodes) and osteitis (inflammation of the bone) (Lotte, Wasz-Hockert et al. 1984). For example, BCG-Pasteur, -Danish, and -Russia were associated with higher incidences of suppurative lymphadenitis and osteitis, respectively, whereas BCG-Japan, -Moreau, and -Glaxo, were associated with a lower incidence of adverse
reactions (Lotte, Wasz-Hockert et al. 1984). More convincing observational data comes from changes in national immunization programs. For example, BCG-Sweden was used in Finland until 1978 when concerns over a high incidence of osteitis prompted a switch to BCG-Glaxo. Following this, an immediate decrease in osteitis was observed. However, in 2002, BCG-Glaxo was again replaced by BCG-Danish and an increased in lymphadenitis was observed (Lotte, Wasz-Hockert et al. 1984; Teo, Smeulders et al. 2005). Interestingly, the revised recommendation for BCG vaccination by the WHO in 2007 was based on an increase in observed cases of BCG disseminated disease in South Africa, which coincides with a change in vaccination from BCG-Japan to vaccination with BCG-Danish (Hesseling, Schaaf et al. 2003). Although only a correlation based on clinical data, this was an exciting finding as it may provide the first molecular explanation to account for the differences in BCG virulence and may further support the notion that variability in BCG reflects true biological differences.

1.5.2. PhoP and BCG Immunogenicity

Historically, the potency of BCG was evaluated by its immunogenicity or ability to induce an adequate immune response. Traditionally, this has been measured by the tuberculin skin test, which measures the delayed-type hypersensitivity to a mixture of secreted protein antigens, where the size and induration of the reaction was indicative of the strength of the immune response (Milstien and Gibson 1990). This screening was used as a strain selection method in national immunization programs to choose the most potent and thus possibly more protective BCG strain. Early human and animal studies identified BCG-Prague as being the least immunogenic, where two studies in guinea pigs and children showed that BCG-Prague consistently exhibited significantly lower tuberculin reaction than other BCG strains.
(Vallishayee, Shashidhara et al. 1974; Ladefoged, Bunch-Christensen et al. 1976). Moreover, a comparison of five BCG strains in BALB/c mice also showed that BCG-Prague as one of the lowest immune response inducers (Lagranderie, Balazuc et al. 1996). Because of its low immunogenicity, BCG-Prague was replaced by BCG-Russia in 1981 after 30-years of use (Lotte, Wasz-Hockert et al. 1984), but the underlying mechanism remained unknown.

Recently, we identified a single guanine (G) insertion in the \textit{phoP} of BCG-Prague (Leung, Tran et al. 2008). This insertion caused a frameshift mutation that abrogates the C-terminal DNA binding domain of PhoP. PhoP is the response regulator of the PhoP-PhoR two-component system in \textit{M. tb}, which controls the transcription of over 100 genes, including the positive regulation of some well-established T cell antigens such as Ag85 and PPE18 (Walters, Dubnau et al. 2006). As such, we hypothesized that the \textit{phoP} mutation in BCG-Prague may account for its low immunogenicity. Consistent with this, a previous Masters student in the lab, Andrea Leung, found that complementation of BCG-Prague with a wildtype copy of \textit{phoP} increased expression of the same subset of antigenic proteins (unpublished). Furthermore, she found that mice vaccinated with BCG-Prague complemented with wildtype \textit{phoP} had greater IFN\textsubscript{γ} production from PPD-stimulated splenocytes compared to mice vaccinated with the parental strain (unpublished). This increase in IFN\textsubscript{γ} production was \textasciitilde2-fold, thus partially restoring the immunogenicity of BCG-Prague. Taken together, this supports our hypothesis that the mutation in \textit{phoP} is responsible for the weak immunogenicity exhibited by BCG-Prague.

How the PhoP-PhoR two-component system regulates its target genes has not been fully elucidated. It is believed that PhoR, the membrane-associated sensor histidine kinase, phosphorylates PhoP in response to a currently unknown environmental stimulus, but whether this phosphorylation is required for activity is unknown (Cole, Brosch \textit{et al.} 1998; Walters,
Dubnau et al. 2006). Although there is no evidence to show that PhoP interacts with the promoters of its target genes directly, it has been shown that PhoP autoregulates itself by recognizing three 9-bp direct repeat motifs in the phoP promoter region. Whether this autoregulation functions to activate or repress its own transcription is still controversial (Gupta, Sinha et al. 2006; Gonzalo-Asensio, Soto et al. 2008).

1.6. Thesis Rationale and Outline

BCG is the only licensed vaccine against TB but as discussed previously, it is far from ideal, exhibiting variable protection ranging from 0-80% (Colditz, Brewer et al. 1994; Brewer 2000). In addition, the safety of the vaccine in an immunocompromised population is of a major concern (WHO 2007). With the number of drug-resistant TB cases on the rise (~220 000 – 400 000 cases in 2011, which accounts for approximately 3.7% of new TB cases (WHO 2012)), it is clear that chemotherapy is rapidly becoming ineffective and a potent vaccine is urgently needed. As such a long-term goal of our lab is to develop a recombinant BCG vaccine strain that is both safe and more protective. To this end, my thesis first examines the impact of molecular factors, PDIMs/PGLs and PhoP, on BCG vaccine properties. Identification of molecular factors that influence the clinical properties of BCG, including safety, immunogenicity, and protective efficacy, will shed new insights into novel strategies to improve BCG efficacy.

Previous work by our lab uncovered a link between PDIM/PGL production and BCG safety; however, this is a correlation based on clinical data and does not provide direct evidence (Chen, Islam et al. 2007). In addition, the role of PDIMs and PGLs in mediating other BCG vaccine properties is still unclear. Thus, the first objective of my project was to confirm the role
of PDIMs/PGLs in BCG virulence and to determine their role in BCG immunogenicity and protection. Since the genetic manipulations of BCG and mouse infection studies are arduous and time-consuming, I began my examination of PDIMs/PGLs in *M. marinum*. Although PDIMs and PGLs have been studied extensively in *M. tb*, there are conflicting data on the precise role of PDIMs and PGLs in virulence. For example, early studies examining a PDIM mutant of *M. tb* suggested that PDIMs mediated lung-specific replication of *M. tb* mice (Cox, Chen et al. 1999), however, another study found that a PDIM-deficient strain of *M. tb* replicated poorly in both lungs and spleens (Rousseau, Winter et al. 2004). Additionally, the role of PGLs in virulence is complicated by the fact that these lipids are absent in most clinical strains of *M. tb* and are only produced in a subset of *M. tb* clinical isolates (Reed, Domenech et al. 2004).

*M. marinum* is a close relative of *M. tb* that causes TB-like mycobacterial disease in ectotherms such as frogs and zebrafish. It shares a close genetic relationship with *M. tb*, with >85% nucleotide identity and shares 3000 orthologs with an average amino acid identity of 85% (Stinear, Seemann et al. 2008). Pioneering work by Lalita Ramakrishnan’s group has established a zebrafish-*M. marinum* model of TB that offers several advantages (Prouty, Correa et al. 2003; Pozos and Ramakrishnan 2004). The first is the relative simplicity and ease of using *M. marinum*. Work with *M. tb* requires biosafety level-3 containment, while *M. marinum* can be used in a biosafety level-2 environment. Furthermore, *M. tb* and BCG are notoriously slow growing mycobacteria, with generation times of 15-24 hours, whereas *M. marinum* is a relatively fast growing species, with a generation time of ~7 hours. Thus, experiments using *M. marinum* as a model is relatively fast in comparison to *M. tb* and BCG. Another advantage is that zebrafish infected with *M. marinum* develop granulomatous disease that is similar to human TB, which is characterized by granulomas that contain caseous centres (Prouty, Correa et al. 2003). Zebrafish also have both an innate and adaptive immune system that responds to *M. marinum* infection in a
way that is reminiscent of human infection by *M. tb*. For example, during an infection in zebrafish, *M. marinum* is phagocytosed by macrophages, which then aggregate to form the beginnings of a granuloma-like structure or prime the adaptive arm of the immune system to recruit effector T cells (Davis, Clay *et al.* 2002). Given the rapid growth, pathogenic similarities, and fewer biosafety restrictions compared to *M. tb*, *M. marinum* is increasingly used to study TB pathogenesis. Using the *M. marinum* model, I showed that both PDIMs and PGLs are important for virulence in zebrafish, resistance to antibiotics, and maintaining cell wall permeability (Yu, Tran *et al.* 2012) (Chapter 2).

To elucidate the role of these lipids in BCG vaccine properties, I generated an isogenic PDIM/PGL mutant in BCG-Pasteur, a BCG strain that naturally produces PDIMs/PGLs (Chen, Islam *et al.* 2007). Using these strains, I found that loss of PDIMs and PGLs attenuated the virulence of BCG-Pasteur in mice, but did not affect the immunogenicity. Interestingly, the reduction of virulence concurred with reduced protective efficacy against aerosol challenge with *M. tb* (Chapter 3).

The second part of my thesis is to examine the role of PhoP in BCG immunogenicity and protective efficacy. Previous work by our lab suggested that PhoP may play a role in mediating BCG immunogenicity. As such, we hypothesize that the immunogenicity of BCG may be enhanced by overexpression of *phoP*. Using mice models, I found that overexpression of *phoP* increases IFNγ production and improves the efficacy of BCG-Japan. However, the overexpression of *phoP* also results in the concurrent increase in virulence (Chapter 4).

Overall, I provide experimental evidence that both PDIMs/PGLs and PhoP have a role in mediating BCG virulence and protection, providing direct evidence to illustrate the impact of BCG genetic heterogeneity on vaccine properties. In addition, my work suggests that there is an
intimate relationship between the virulence and efficacy of BCG vaccine strains. Since the loss of PDIMs/PGLs and PhoP occurs naturally in some BCG strains, this finding provides evidence favoring the over-attenuation theory of BCG and provides valuable insights that may impact current immunization programs and future vaccine development (Chapter 5).
Chapter 2:

Both Phthiocerol Dimycocerosates and Phenolic Glycolipids are Required for Virulence of *M. marinum*

A version of this chapter has been published as:


Experiments were performed by Vanessa Tran; with the exception of Figure 2.2, Figure 2.4, Figure 2.5, and Figure 2.6, which were performed by members in Dr. Qian Gao’s lab. Figure 2.3A-C was performed by Ming Li.

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2.1 Introduction

Pathogenic mycobacteria produce two structurally related, methyl-branching fatty acid-containing lipids called phthiocerol dimycocerosates (PDIMs) and phenolic glycolipids (PGLs). PDIMs and PGLs have long chain fatty acid backbones consisting of 3-methoxy (or 3-keto, 3-hydroxy), 4-methyl, 9,11-dihydroxy glycols (phthiocerols) and p-glycosylated phenylglycols (glycosyl phenolphthiocerols), respectively, that are diesterified with di-, tri-, and tetra-methylbranched acyl chains (mycocerosates) (reviewed in (Onwueme, Vos et al. 2005)). PDIMs have been identified in *Mycobacterium tuberculosis* (*M. tb*), *M. africanum*, *M. bovis*, *M. leprae*, *M. marinum*, *M. ulcerans*, *M. kansasii*, *M. haemophilum*, *M. microti*, and *M. gastri*, all of which are pathogenic for humans or animals. PGLs are produced by the same set of pathogenic mycobacterial species except that in *M. tb* only a subset of clinical isolates produces PGLs.

The role of PDIMs in virulence was first suggested by two independent studies using signature-tagged transposon mutagenesis (STM), which identified mutants of *M. tb* that were unable to either produce or properly localize PDIMs to the cell wall, and demonstrated that these mutants were attenuated in animal models of infection (Camacho, Ensergueix et al. 1999; Cox, Chen et al. 1999; Rousseau, Winter et al. 2004). Since then, circumstantial evidence supporting a role for PDIMs in *M. tb* virulence accumulated. The role of PGLs in *M. tb* virulence is less clear and is confounded by the fact that laboratory strains (H37Rv, Erdman) and many clinical isolates, including CDC1551 and MT103, are naturally deficient in PGL production due to a 7-base-pair deletion in *pks15/1*, while some clinical isolates of the East-Asian lineage have an intact *pks15/1* gene and produce PGLs (Reed, Domenech et al. 2004). Mutations of *pks15/1* in *M. tb* HN878, a strain that produces both PDIMs and PGLs and exhibits a hypervirulent
phenotype in infected animals, abolished PGL synthesis and decreased the virulence of the mutant to the level of non-PGL producing strains H37Rv and CDC1551 (Reed, Domenech et al. 2004; Tsenova, Ellison et al. 2005). As such, it was suggested that the production of PGLs in some M. tb strains of the Beijing family is associated with their increased virulence. However, this correlation has been less than perfect (Sinsimer, Huet et al. 2008). Moreover, an H37Rv strain engineered to produce PGLs was not more virulent than the parental strain in the mouse or rabbit model of infection (Sinsimer, Huet et al. 2008). Therefore, the role of PGLs in M. tb virulence remains controversial. In M. bovis (Hotter, Wards et al. 2005) or M. marinum (Alibaud, Rombouts et al. 2011; Chavadi, Edupuganti et al. 2011), simultaneous loss of PDIMs and PGLs has been associated with decreases in virulence. However, the contribution of individual PDIMs and PGLs to virulence in these organisms has not been evaluated.

Significant progress on the biosynthesis of PDIMs and PGLs has been made in recent years. Genes involved in PDIM/PGL synthesis were first identified by Kolattukudy and co-workers (Azad, Sirakova et al. 1996; Azad, Sirakova et al. 1997). They showed that disruption of polyketide synthase (PKS) genes ppsB and ppsC in M. bovis BCG abolished the production of PDIMs and PGLs (Azad, Sirakova et al. 1997). Subsequent genetic studies in M. tb identified other genes involved in PDIM and/or PGL biosynthesis, including fadD26, fadD28, mmpL7, ddrC, and pks15/1, which are in the same genetic region encompassing the ppsA-ppsE genes (Camacho, Ensergueix et al. 1999; Cox, Chen et al. 1999; Reed, Domenech et al. 2004). Together, these studies have uncovered a genetic locus which consists of more than 30 genes believed to be involved in PDIM/PGL biosynthesis and major biochemical steps leading to PDIM/PGL production have been proposed [Figure 2.1 and (Onwueme, Vos et al. 2005)]. Accordingly, phthiocerols and phenolphthiocerols are generated by five PKSs, PpsA-E, which are arranged as a modular PKS system consisting of predicted domains required for the co-linear
assembly of the 3-methoxy, 4-methyl, 9,11-dihydroxy segment. A different PKS, Mas, synthesizes the methyl-branched lipids, mycocerosates. Diesterification of phthiocerols with mycocerosates mediated by an acyltransferase, PapA5, generates PDIMs (Onwueme, Ferreras et al. 2004). Similarly, PapA5 catalyzes the diesterification of phenolphthiocerols with mycocerosates and additional glycosyltransferases are required for the glycosylation to produce the final PGL products (Perez, Constant et al. 2004). While this general scheme is likely to be true, some aspects of this pathway remain unclear. For examples, while fadD28 and mas are thought to be responsible for the synthesis of mycocerosates, a common precursor of both PDIMs and PGLs, disruption of mas or fadD28 in M. bovis BCG abolished PGL but not PDIM production (Azad, Sirakova et al. 1996; Fitzmaurice and Kolattukudy 1998). Genetic disruption of mas in M. tb has not been reported, but inactivation of fadD28 in M. tb Erdman or MT103 strain abrogated the production of PDIMs (Cox, Chen et al. 1999; Camacho, Constant et al. 2001). It is unclear whether this discrepancy reflects differences in the strains studied. For PpsA-E, biochemical evidence is consistent with their role in PDIM/PGL synthesis (Trivedi, Arora et al. 2005), but specific deletion studies have only been described for ppsB and ppsC in M. bovis BCG (Azad, Sirakova et al. 1997), and more recently, ppsD in M. tb H37Rv (Kirksey, Tischler et al. 2011).

In this study, we isolated seven mutants of M. marinum in which ppsA, ppsB, ppsD, ppsE, mas, fadD28 and fadD26 genes were individually disrupted by transposon insertions. We found that ppsA::Tn, ppsB::Tn, ppsD::Tn, and ppsE::Tn mutants were unable to produce PDIMs and PGLs, providing the first genetic evidence for the role of ppsA and ppsE in PDIM/PGL synthesis. Disruption of mas or fadD28 abolished the production of both PDIMs and PGLs, reinforcing their role in mycocerosate synthesis. The fadD26::Tn mutant, in which the transposon inserted in the promoter region, produced a trace amount of PDIMs and a low level of...
PGLs. Zebrafish infection experiments showed that all mutants were severely attenuated and were essentially avirulent under the experimental conditions. Complementation of the \textit{fadD28}:Tn mutant partially restored PDIM/PGL production and virulence in zebrafish, confirming the role of PDIMs/PGLs in \textit{M. marinum} virulence. Complementation of the \textit{fadD26}:Tn mutant resulted in an intermediate level of PDIMs but further reduction of PGLs, and consequently, the recombinant strain remained avirulent in zebrafish. Taken together, our results suggest that PDIMs and PGLs are both required for virulence of \textit{M. marinum}. In addition, we have provided new insights into the biosynthesis of PDIMs and PGLs and their role in mycobacterial virulence.

### 2.2 Materials and Methods

#### 2.2.1 Bacterial Strains and Culture Conditions

\textit{Mycobacterium marinum} M strain (ATCC BAA-535) was used as the parental and wildtype strain for the transposon mutagenesis and subsequent experiments. \textit{M. marinum} cells were grown at 32°C in Middlebrook 7H9 broth (Difco) supplemented with 0.2% glycerol and 10% oleic acid-albumin-dextrose-catalase (OADC) (Difco) or on Middlebrook 7H11 agar (Difco) supplemented with 0.5% glycerol and 10% OADC. \textit{Escherichia coli} strain DH5α was used for routine manipulation and propagation of plasmid DNA. \textit{E. coli} strain DH5α λ \textit{pir}116 (Alexander, Jones et al. 2004) was used for isolation of transposon-containing plasmid. Antibiotics were added as required: kanamycin, 50 µg/ml for \textit{E. coli} and 25 µg/ml for \textit{M. marinum}; hygromycin, 150 µg/ml for \textit{E. coli} and 50 µg/ml for \textit{M. marinum}. 
2.2.2 Generation and Screening of *M. marinum* \(\phi\)MycMar Insertion Library

Propagation of the \(\phi\)MycMar transposon phage and preparation of phage lysates have been described previously (Alexander, Jones et al. 2004). For phage infection, *M. marinum* cells were washed and resuspended in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM MgSO\(_4\), and 2 mM CaCl\(_2\). Phage were added at a multiplicity of infection (MOI) of 10:1 and incubated at 37°C for 3 hours to allow infection to occur. Bacteria were then plated on Middlebrook 7H11 agar supplemented with kanamycin and incubated at 32°C. Kanamycin-resistant (i.e., transposon-containing) *M. marinum* colonies were patched onto Middlebrook 7H11 agar and colonies with unusual morphology were identified by visual inspection.

2.2.3 Molecular Cloning and Complementation

A 4494-bp fragment containing *fadD28* (*MMAR_1765*) gene and *mmpL7* (*MMAR_1764*) was amplified by PCR using chromosomal DNA of *M. marinum* M strain as the template and the forward primer 5’-GAAGATCTAGGAGTGATGCCCATGAGTGTGCCTCCCCTT -3’ and reverse primer 5’-GACTAGTTTACCGGTGCCCAGTCGATTGC -3’, which contains a BglII and a SpeI site, respectively (underlined). The PCR product was cloned into BamHI and SpeI sites of the pSMT3LxEGFP shuttle vector (Humphreys, Stewart et al. 2006), creating pFad28/MmpL7. Similarly, the intact *fadD28* was amplified using the forward primer 5’-GAAGATCTAGGAGTGATGCCCATGAGTGTGCCTCCCCTT -3’ and reverse primer 5’-CCCAGGCTTCTACGTCCAGGCGGCGGAAGTGC-3’. The amplicon was digested with BglII and HindIII and cloned into pSMT3LxEGFP, creating pFadD28. A 1755-bp fragment containing *fadD26* (*MMAR_1777*) was PCR amplified using the forward primer 5’-
CGGGATCCAAGGATGTAGTGCGATGCCGGTGACCGACC -3’ and reverse primer 5’-GACTAGTTCTACCCGTCACGTCCAGCGGATTG -3’. The fragment was cloned into BamHI and SpeI sites of the pSMT3LxEGFP shuttle vector, generating pFadD26. The constructs were confirmed by DNA sequencing. Constructs were electroporated into appropriate mutant strains of *M. marinum* and transformants selected on Middlebrook 7H10 agar plates containing 50 μg/ml of hygromycin.

### 2.2.4 Quantitative Real-Time PCR (qRT-PCR)

Determination of the expression level of *fadD26*, *ppsA* and *ppsE* was accomplished by qRT-PCR using specific primers for each gene: *fadD26*, 5’-CCGATTATCGGGGTCCCACTTTC-3’ (forward) and 5’-CCGTCTTGTGAGCTGGCGTATTTTG-3’ (reverse); *ppsA*, 5’-GACAAGATGGACCCGCAGCAAC-3’ (forward) and 5’-TCAGACATGAGCCGGGAAGAC-3’ (reverse); *ppsE*, 5’-GAACCTCCTCTCCCCAGTGCTAT-3’ (forward) and 5’-GGCTGAACCTGATCGAAGTTGAGC-3’ (reverse). Primers specific for *sigA* (5’-GAAAAACCACCTGCTGGAAG-3’, and 5’-CGCGTAGGTGGAGAATTTGT-3’) were used as an endogenous control to normalize the amount of cDNA template added to each qRT-PCR sample. The cDNA used in these experiments was prepared from RNA samples obtained from three biological replicates. qRT-PCRs were carried out in triplicate using a 7500 Real-Time PCR System (Applied Biosystems) and iQ SYBR green supermixture (Bio-Rad).

### 2.2.5 Thin Layer Chromatography (TLC) Analysis of PDIMs and PGLs

The apolar lipids were prepared from *M. marinum* cells (50 mg dry biomass) according to published procedures (Chen, Islam et al. 2007; Ren, Dover et al. 2007). These lipids were
analyzed by two-dimensional thin layer chromatography (2D-TLC) on silica gel 60 plates (EMD Chemicals Inc.). Apolar lipids were developed with petroleum ether/ethyl acetate (98:2, 3×) in the first dimension and petroleum ether/acetone (98:2) in the second dimension. Lipids were visualized by spraying plates with 5% phosphomolybdic acid followed by gentle charring of the plates. For detection of phenolic glycolipids (PGL), the apolar lipid extract was developed with chloroform/methanol (96:4, v/v) in the first direction and toluene/acetone (80:20, v/v) in the second direction, followed by charring with α-naphthol. For quantitative TLC analysis, the *M. marinum* strains were grown to mid-log phase in 7H9-OADC and incubated with 25 µCi of [14C]propionate for 24 hours (specific activity = 54 mCi/mmol; American Radiolabeled Chemicals Inc.). The apolar lipids were prepared from *M. marinum* cells (50 mg dry biomass) and analyzed by 2D-TLC as described above. Lipids were visualized and quantified by phosphorimaging using a storage phosphor screen (GE Healthcare) and Typhoon9400™ imager.

### 2.2.6 Zebrafish Infection

Adult zebra-fish (*Danio rerio*) were infected as described previously (Yu, Niu et al. 2011). To assess the survival, 20 fish per group were infected by intraperitoneal injection with 20 µl of thawed bacterial stocks that were diluted in PBS to reach a dosage of approximately $10^4$ CFU per fish. A Kaplan-Meier curve was calculated for animals infected with each strain including mock-injected (PBS) animals and statistical analysis comparing survival of different groups of animals was calculated by log-rank test.

### 2.2.7 Drug Sensitivity Assay

The drug sensitivity of the *M. marinum* strains was tested using the agar dilution method. Briefly, strains were grown in 7H9 supplemented with ADN (0.5% albumin, 0.2% dextrose,
0.085% NaCl) to OD$_{600}$ ≈1.0. Strains were then adjusted to a concentration $2.5 \times 10^6$ CFU/mL and 4 µL was spotted onto 7H11 agar containing 2-fold dilutions of antibiotic. Plates were visually inspected after 1 week incubation at 32°C to find the minimum inhibitory concentration (MIC).

### 2.2.8 Uptake Assay

The accumulation of Nile Red (Sigma) and ethidium bromide (EtBr) (OmniPur) was measured. Strains of *M. marinum* were grown in 7H9/ADN to an OD$_{600}$ ≈ 1.0, washed twice with 50 mM potassium phosphate buffer (pH 7), and resuspended in 2.5 mL of buffer. The OD$_{600}$ of the resuspended cells was determined, adjusted to OD$_{600}$ = 0.4, and 100 µL of this cell suspension was added in triplicate to a 96-well black fluoroplate (Greiner Bio-One). Nile Red and EtBr were added to a final concentration of 2 µM and 6 µM, respectively. The accumulation of these dyes was measured by fluorescence using a TECAN Infinite® M200 spectrofluorometer with an excitation of 540 nm and emission of 630 nm for Nile Red and an excitation of 545 nm and emission of 600 nm for EtBr.

### 2.3 Results

#### 2.3.1 Isolation and Characterization of *M. marinum* Mutants Defective in Cord Formation

When growing in liquid medium, pathogenic mycobacteria such as *M. tb* and *M. marinum* form serpentine cords, which are large bundles of bacterial cells aggregated in parallel along their long axes (Middlebrook, Dubos et al. 1947; Hall-Stoodley, Brun et al. 2006; Staropoli and Branda
A link between cord formation and virulence was first suggested by the observation that the virulent *M. tb* strain H37Rv formed cords whereas the avirulent strain H37Ra did not (Middlebrook, Dubos et al. 1947). This was later supported by isolation of attenuated strains of *M. tb* and *M. marinum* which were also defective in cording (Glickman, Cox et al. 2000; Gao, Laval et al. 2003). Therefore, as a general strategy to uncover virulence genes, we first visually screened a transposon-insertion library of *M. marinum* for altered colony morphology, followed by microscopic screening for colonies defective in cord formation. The initial screen by colony morphology was done since factors that contribute to cord formation are likely cell surface components. Thus, defects in cording would likely give rise to colonies with altered morphology on agar plates, as demonstrated in our earlier studies (Chen, German et al. 2006; Ren, Dover et al. 2007). We identified 66 mutants after screening approximately 10,000 colonies, which exhibited altered colony morphology compared to the wildtype strain. Of which, 11 mutants were defective in cord formation (see examples in Figure 2.2A and 2.2B).

Genetic characterizations of these mutants revealed that 7 mutants contained transposon insertion site individually within the open reading frames (ORF) of *ppsA, ppsB, ppsD, ppsE, fadD28, mmaA3*, and *PPE38* genes. Three mutants had transposon insertion at different sites within the ORF of *mas* and one mutant had transposon inserted at the promoter region of *fadD26* (Figure 2.1A). Remarkably, 7 of the 9 genes identified (except *mmaA3* and *PPE38*) belong to the presumed PDIM/PGL biosynthetic locus of *M. marinum* (Figure 2.1A), which consists of more than 30 genes spanning ~70 kb in the genome (Onwueme, Vos et al. 2005). These mutants are the focus of this chapter.
Figure 2.1. PDIM/PGL biosynthesis in *M. marinum*.

(A) The PDIM/PGL biosynthetic locus in *M. marinum*. Each arrow represents the open reading frame of predicted genes. The genes disrupted by transposon insertions are highlighted in red and the transposon insertion sites are indicated by filled triangles. (B) Schematic representation of PDIM/PGL synthesis in *M. marinum*. Enzymatic steps catalyzed by disrupted genes (*fadD26, ppsA-E, fadD28, mas*) are emphasized.
Figure 2.2. Colony morphology and cording phenotype of PDIM/PGL-deficient mutants of *M. marinum*.
The PDIM/PGL-deficient mutants exhibited altered colony morphology (A) and were defective in cord formation (B). Results are shown for *M. marinum* wild-type strain (a); representative PDIM/PGL-deficient mutants *fadD28*::Tn (b) and *mas*::Tn (c); and *fadD28*::Tn mutant complemented with the cloning vector (d), intact *fadD28* gene (e), or *fadD28* and *mmpL7* (f). Complementation of *fadD28*::Tn with *fadD28* plus *mmpL7* restored the wild-type colony morphology and partially restored the cording phenotype. Experiments performed by Dr. Qian Gao’s group.
2.3.2 The \textit{ppsA::Tn}, \textit{ppsB::Tn}, \textit{ppsD::Tn}, \textit{ppsE::Tn}, \textit{fadD28::Tn} and \textit{mas::Tn} Mutants of \textit{M. marinum} Failed to Synthesize PDIMs and PGLs

To confirm the role of the identified genes in PDIM/PGL synthesis, Ming Li performed two-dimensional thin layer chromatography (2D-TLC) analyses of PDIMs and PGLs using the apolar lipid fraction isolated from each mutant according to previously published procedures (Chen, Islam et al. 2007). 2D-TLC analysis showed that the synthesis of PDIMs and PGLs were abolished in \textit{ppsA::Tn}, \textit{ppsB::Tn}, \textit{ppsD::Tn}, \textit{ppsE::Tn}, \textit{fadD28::Tn} and \textit{mas::Tn} mutants (Figure 2.3A-B). Unlike previous studies using \textit{M. bovis} BCG, which found that \textit{fadD28::Tn} and \textit{mas::Tn} mutants still produced shorter chain PDIMs (Azad, Sirakova et al. 1996; Fitzmaurice and Kolattukudy 1998), the \textit{mas::Tn} or \textit{fadD28::Tn} mutant of \textit{M. marinum} was devoid of PDIMs. However, our result is consistent with previous studies of the \textit{fadD28::Tn} mutants of \textit{M. tb} (Cox, Chen et al. 1999; Camacho, Constant et al. 2001).

To confirm the role of \textit{fadD28} in PDIM/PGL synthesis, we cloned the intact \textit{fadD28} gene or \textit{fadD28} together with its downstream gene, \textit{mmpL7}, and transformed the constructs into the \textit{fadD28::Tn} mutant. Complementation with each construct partially restored the production of PDIMs and PGLs in the mutant (Figure 2.3B). The identities of PDIMs and PGLs have been confirmed previously by mass spectrometric analysis and/or by radiolabeled 2D-TLC analysis. The MALDI-TOF spectra of purified PDIMs revealed a series of major pseudomolecular ion [M + Na]$^+$ peaks at \textit{m/z} 1238, 1252, 1266, 1280, which corresponds to a C$_{81}$–C$_{84}$ composition of PDIMs and agrees with PDIMs found in \textit{M. marinum} (Chavadi, Edupuganti et al. 2011). To quantify the relative abundance of PDIMs and PGLs, I repeated the 2D-TLC analysis with bacterial cultures labeled with $[^{14}$C$]$propionate, a precursor of PDIMs and PGLs, followed by
Figure 2.3. 2D-TLC analysis of PDIMs and PGLs.

For analysis of PDIMs, equal amount of apolar lipids were developed with petroleum ether/ethyl acetate (98:2, 3×) in the first dimension and petroleum ether/aceto ne (98:2) in the second dimension. Lipids were visualized by spraying plates with 5% phosphomolybdic acid followed by gentle charring of the plates (A-C) or phosphorimaging (D). For analysis of PGLs, the apolar lipid extract was developed with chloroform/methanol (96:4, v/v) in the first direction and toluene/acetone (80:20, v/v) in the second direction, followed by charring with α-naphthol (A-C) or visualized by phosphorimaging (D). (A) PDIM and PGL analysis of pps and mas mutants. All mutants indicated failed to synthesize PDIMs or PGLs. (B) PDIM and PGL analysis of the fadD28::Tn mutant and its complemented strain. The fadD28::Tn mutant failed to synthesize
PDIMs and PGLs. The complemented strain fadD28C, which is the fadD28::Tn mutant with fadD28+mmpL7, partially restored the production of PDIMs and PGLs. (C) **PDIM and PGL analysis of the fadD26::Tn mutant and its complemented strain.** The fadD26::Tn mutant produced residual PDIMs and intermediate levels of PGLs. Complementation with fadD26 (fadD26C) partially restored the production of PDIMs but caused further reduction of PGLs. (D) **Quantitative 2D-TLC analysis.** Bacterial cultures labeled with \(^{14}C\)propionate were subjected to 2D-TLC analysis as described and visualized by phosphorimaging. (E) **Quantitation of the levels of PDIMs and PGLs in different strains.** Data were determined from (D) by phosphorimaging analysis using Typhoon9400™ imager. The data were normalized to the wildtype strain. TLCs in A-C were performed by Ming Li.

quantification by phosphor imaging analysis (Figure 2.3D). This analysis revealed that the levels of PDIMs and PGLs in the fadD28 complemented strain were 25% and 35% of that in the wildtype strain, respectively (Figure 2.3E). The relatively low levels of PDIMs and PGLs in the complemented strains compared to wildtype may be due to sub-optimal expression of fadD28 in the cloning vector. Previously, it was found that complementation of the fadD28::Tn mutant of *M. tb* with intact fadD28 in a plasmid restored only 15% PDIM production, presumably due to the same reason (Camacho, Constant et al. 2001).

### 2.3.3 The fadD26 Gene is Involved in the Synthesis of PDIMs but not PGLs in *M. marinum*

We found that unlike other mutants described above, the fadD26::Tn mutant still produced a trace amount (2%) of PDIMs and a substantial amount (31%) of PGLs, although at a lower level than wildtype (Figure 2.3C-E). In this mutant, the transposon inserted at 313 bp upstream of the predicted start codon of fadD26, which reduces the fadD26 expression to about 9% based on quantitative real time PCR (qRT-PCR) analysis (Figure 2.4). This is consistent with previous findings in *M. tb*, where transposon insertion at the upstream region (113 bp) of fadD26 resulted
Figure 2.4. Quantitative RT-PCR analysis.
The expression levels of *fadD26* and its downstream genes *ppsA* and *ppsE* were determined. Relative expression data (fold change relative to wildtype strain) were obtained using specific primers for each gene and normalized to the level of *sigA*. Results are form three independent experiments. Experiments done by Dr. Qian Gao’s group.
in significantly reduced level of PDIMs, and transposon insertion within the ORF of \textit{fadD26} abolished PDIM production (Camacho, Constant et al. 2001).

The role of \textit{fadD26} in PGL synthesis has not been evaluated in \textit{M. tb} because the strains (Erdman and MT103) from which \textit{fadD26::Tn} mutants were generated are naturally deficient in PGL production due to the \textit{pks15/1} polymorphism (Camacho, Ensergueix et al. 1999; Cox, Chen et al. 1999; Camacho, Constant et al. 2001). We found that in the \textit{fadD26::Tn} mutant of \textit{M. marinum}, the amount of PGLs decreased, which would initially suggest that \textit{fadD26} is also involved in PGL synthesis. However, complementation with the intact \textit{fadD26} gene of \textit{M. marinum} did not increase the level of PGL production in the mutant. Instead, the amount of PGLs was further reduced in the complemented strain (from 31% to 4%) (Figure 2.3C-E). As expected, complementation of the \textit{fadD26::Tn} mutant increased the production of PDIMs, although not to the wildtype levels (57%).

In \textit{M. tb}, \textit{fadD26} and its downstream genes \textit{ppsA} to \textit{papA5} were shown to be transcriptionally coupled (Camacho, Constant et al. 2001). Consistently, we found by qRT-PCR analysis that the transcript levels of \textit{ppsA} and \textit{ppsE} in the \textit{fadD26::Tn} mutant were reduced to 7% and 52% of that in the wildtype strain, and that \textit{trans}-complementation with \textit{fadD26} did not increase their expressions (Figure 2.4). This suggests that like \textit{M. tb}, \textit{fadD26} and its downstream genes (\textit{ppsA-papA5}) in \textit{M. marinum} also form an operon. Accordingly, transposon insertion in the upstream region of \textit{fadD26} causes a polar effect on the expression of \textit{ppsA-papA5} genes, which affects the synthesis of both PDIMs and PGLs. As a consequence, \textit{fadD26} is overexpressed in the complemented strain but the expression of \textit{ppsA-papA5} is still low. Because PpsA-E are PKSs used for both PDIM and PGL synthesis, the elevated FadD26 may outcompete
FadD29, which is specific for the PGL pathway (Figure 2.1), for the limited amount of PpsA-E, resulting in the further reduction of PGL in the complemented strain.

The low expression level of \textit{ppsA-papA5} in the complemented strain may also explain the partial restoration of PDIM production. Previously it was found that complementation of the \textit{fadD26::Tn} mutant of \textit{M. tb} by intact \textit{fadD26} gene only restored 5\% PDIM production (Camacho, Constant et al. 2001). Based on our results, we suggest that \textit{fadD26} is involved in the synthesis of PDIMs but not PGLs in \textit{M. marinum}. Our conclusion is in accordance with a recent study in \textit{M. bovis} BCG, which found that deletion of \textit{fadD26} abolished PDIM production and reduced the level of PGLs, and that complementation only partially restored the PDIM production (Simeone, Leger et al. 2010). Interestingly, in that study, the amount of PGLs in the complemented strain remained unchanged, presumably because \textit{fadD26} was expressed as a single copy gene (i.e., it was cloned in an integrative vector for complementation experiments) and its expression level is not high enough to outcompete FadD29 for the common enzymes (PpsA-E) involved in both pathways (Figure 2.1).

\textbf{2.3.4 PDIMs and PGLs are Both Required for \textit{M. marinum} Virulence in Zebrafish}

To assess the role of PDIMs and PGLs in \textit{M. marinum} virulence, our collaborators performed zebrafish infection experiments. Twenty fish per group were infected with the wildtype \textit{M. marinum} and three PDIM/PGL deficient strains, \textit{mas::Tn}, \textit{fadD28::Tn}, \textit{ppsE::Tn}, and were monitored for survival. All three mutants exhibited attenuated phenotype and were essentially avirulent under the experimental conditions (Figure 2.5). All fish infected with the wildtype strain died by 16 days post infection, whereas none of the fish infected with each of the mutants died at the time the experiment was terminated (30 days post infection) (Figure 2.5). Histological
Figure 2.5. Survival of zebrafish infected with *M. marinum* strains.
Separate tanks, each containing 20 fish infected with indicated strain at $10^4$ CFU bacteria per fish, were monitored for mortalities over a 30-day period. A Kaplan-Meier curve was calculated for animals infected with each strain. All fish infected with PDIM/PGL deficient mutants (*mas::Tn, fadD28::Tn, ppsE::Tn, and fadD26::Tn*) as well as PBS survived. Complementation of *fadD28::Tn* with *fadD28+mmpL7* (*fadD28C2*) partially restored virulence (*p* < 0.001, compared to WT-infected fish) while complementation with *fadD28* alone (*fadD28C1*) did not restore virulence. Complementation of *fadD26::Tn* with *fadD26* (*fadD26C*) did not restore virulence. Infection performed by Dr. Qian Gao’s lab.
analysis revealed no microscopic liver lesions in fish infected with PDIM/PGL-deficient mutants. In contrast, typical necrotizing granulomas were observed in fish infected with the wildtype *M. marinum* (Figure 2.6).

To confirm that the attenuation of mutants was caused by the deficiency of PDIMs/PGLs, we performed the zebrafish infection experiment with the *fadD28::Tn* strain complemented with *fadD28* or with *fadD28* plus *mmpL7*. As expected, the *fadD28::Tn* strain complemented with both *fadD28* and *mmpL7* partially restored virulence, whereas complementation with *fadD28* alone did not restore virulence (Figure 2.5). MmpL7 is a membrane transporter required for transporting PDIMs and possibly PGLs to the cell surface. Previous works in *M. tb* showed that the *mmpL7::Tn* mutant, which still produced PDIMs, was attenuated in virulence because PDIMs were not properly localized (Camacho, Ensergueix et al. 1999; Cox, Chen et al. 1999; Camacho, Constant et al. 2001). The inability to restore virulence in the strain complemented with *fadD28* alone is likely due to the polar effect on *mmpL7* expression caused by the transposon insertion. Consistent with this hypothesis, *fadD28* and *mmpL7* are co-transcribed in *M. tb* (Camacho, Constant et al. 2001).

The median survival time of fish infected with the *fadD28::Tn* strain complemented with *fadD28* and *mmpL7* is 19 days, which is significantly longer than the median survival time of fish (12 days) infected with wildtype *M. marinum* (*P* < 0.001, Log-rank test). This is consistent with the partial restoration of the PDIM/PGL production in the complemented strain (Figure 2.3B).

The *fadD26::Tn* mutant was also avirulent in zebrafish (Figure 2.5). Surprisingly, we found that the *fadD26::Tn* strain complemented with intact *fadD26* remained avirulent in infected zebrafish (Figure 2.5). Since the *fadD26::Tn* mutant produced little PDIMs but
Figure 2.6. The PDIM/PGL-deficient mutant did not cause pathological changes in infected zebrafish.
Zebrafish were infected as conducted in Figure 2.5, with the WT strain of *M. marinum* (E and F), the PDIM/PGL-deficient mutant Δ*mas* (C and D), or PBS as a control (A and B). Histology was assessed at 1 week post infection. A, C, and E: Hematoxylin-Eosin staining, 40×; B, D, and F:Ziehl-Neelson staining, 40×. Progressive necrosis and large numbers of bacteria found within necrotic granulomas of zebrafish infected with the WT strain of *M. marinum* (E and F) but were absent in zebrafish infected with the Δ*mas* mutant strain (C and D). Experiments done by Dr. Qian Gao’s group.
intermediate level of PGLs, we initially thought that the loss of PDIMs was the main cause for its attenuation. We anticipated that complementation with \textit{fadD26}, which partially restored the PDIM production, would at least partially restore virulence. However, two independent zebrafish infection experiments repeatedly showed that the complemented strain remained avirulent. One plausible explanation is that PDIMs and PGLs are both required for \textit{M. marinum} virulence. Thus in the complemented strain, although the level of PDIMs was substantially restored, the PGL level was further reduced, and consequently, the complemented strain still remained avirulent.

### 2.3.5 PDIMs and PGLs are Important for Intracellular Growth

Pathogenic mycobacteria are intracellular pathogens that preferentially replicate inside macrophages during infection. To determine if the zebrafish phenotype discussed above was the result of a defect in intracellular growth in the PDIM/PGL mutants, I performed intracellular growth assay using J774.1 macrophages. Briefly, macrophages were infected with the various \textit{M. marinum} strains at a MOI (multiplicity of infection) of 0.5 and intracellular bacterial growth was assessed at different time points post infection (0, 24, 48, 72, 96 and 168 hour). I found that both the \textit{fadD28::Tn} and \textit{fadD26::Tn} did not replicate as well as wildtype beginning at 72 hours post infection (Figure 2.7). Furthermore, complementation of \textit{fadD28::Tn} partially restored the growth defect while complementation of \textit{fadD26::Tn} did not (Figure 2.7). This is consistent with the zebrafish infection data and suggests that both PDIMs and PGLs are also required for normal intracellular growth.
Figure 2.7. The loss of PDIMs/PGLs decreases intracellular survival.
WT and mutant *M. marinum* strains were grown to mid-log phase and used to infect J774.1 macrophages with a multiplicity of infection (MOI) of ~0.5. To assess intracellular survival, cells were lysed at the indicated time points and bacteria were enumerated to determine CFU/mL (mean ± SD, representative of n=2). Complementation partially restored survival of *fadD28::Tn* but not *fadD26::Tn*. *fadD26 C, fadD26::Tn* plus pFadD26; *fadD28 C, fadD28::Tn* plus pFadD28/MmpL7.
2.3.6 PDIMs and PGLs Play a Role in Cell Wall Permeability

Since PDIMs/PGLs are components of the cell wall, the loss of PDIMs/PGLs could potentially compromise the cell wall integrity. As such, I next examined whether the PDIM/PGL mutants exhibited increased sensitivity to various antibiotics. I chose two mutants, *fadD28::Tn* and *fadD26::Tn*, and their complemented strains for this experiment. Interestingly, I found that both mutants were more sensitive to various hydrophobic drugs including chloramphenicol, rifampicin, tetracycline, erythromycin, ciprofloxacin and ofloxacin, as well as several β-lactams (penicillin G, cephaloridine, and cefazolin) which are hydrophilic (Table 2.1). However, the sensitivity to other hydrophilic drugs, isoniazid, ethambutol, and streptomycin, remained unchanged (Table 2.1).

To examine whether the increased drug sensitivity of the above mutants is caused by a general increase in cell permeability, I used fluorescence spectroscopy to measure whole cell accumulations of ethidium bromide (EtBr) and Nile Red, representatives of the hydrophilic and hydrophobic compounds, respectively. The results showed that both compounds accumulated more rapidly and to higher levels in mutant cells compared to wildtype cells, indicating an increase in cell wall permeability (Figure 2.8). The two complemented strains were not complemented in the drug sensitivity and permeability assays, although there is partial complementation in their ability to exclude ethidium bromide, which is consistent with the partial restoration of PDIM/PGL production in these strains (Figure 2.3).
Table 2.1. *M. marinum* PDIM/PGL mutants are hypersensitive to hydrophobic and β-lactam antibiotics. Strains were grown to mid-log phase and $10^4$ CFU were spotted on 7H11/ADN plates containing 2-fold dilutions of antibiotic. The minimum inhibitory concentration (MIC) (µg/mL) was determined after incubation at 30°C for 1 week (representative of n=3).

<table>
<thead>
<tr>
<th>Strain*</th>
<th>CEP</th>
<th>CEF</th>
<th>PEN</th>
<th>AMP</th>
<th>CAM</th>
<th>ERY</th>
<th>TET</th>
<th>RIF</th>
<th>OFL</th>
<th>CIP</th>
<th>STREP</th>
<th>INH</th>
<th>EMB</th>
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<tbody>
<tr>
<td>WT</td>
<td>5</td>
<td>20</td>
<td>64</td>
<td>512</td>
<td>20</td>
<td>32</td>
<td>4</td>
<td>0.96</td>
<td>4</td>
<td>0.5</td>
<td>4</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>fasD28::Tn</td>
<td>2.5</td>
<td>10</td>
<td>32</td>
<td>256</td>
<td>10</td>
<td>8</td>
<td>2</td>
<td>0.48</td>
<td>2</td>
<td>0.25</td>
<td>4</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>fasD28::C</td>
<td>2.5</td>
<td>10</td>
<td>32</td>
<td>256</td>
<td>10</td>
<td>8</td>
<td>2</td>
<td>0.48</td>
<td>2</td>
<td>0.25</td>
<td>4</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>fasD26::Tn</td>
<td>2.5</td>
<td>10</td>
<td>32</td>
<td>256</td>
<td>10</td>
<td>8</td>
<td>2</td>
<td>0.48</td>
<td>2</td>
<td>0.25</td>
<td>4</td>
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<td>1</td>
</tr>
<tr>
<td>fasD26::C</td>
<td>2.5</td>
<td>10</td>
<td>32</td>
<td>256</td>
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<td>2</td>
<td>0.25</td>
<td>4</td>
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<td>1</td>
</tr>
</tbody>
</table>

* fasD28::fasD28::Tn plus pFasD28/MmpL2; fasD28::C, fasD26::Tn plus pFasD26.

* CEP, cephaloridine; CEF, cefazolin; PEN, penicillin; AMP, ampicillin; CAM, chloramphenicol; ERY, erythromycin; TET, tetracycline; RIF, rifampicin; OFL, ofloxacin; CIP, ciprofloxacin; STREP, streptomycin; INH, isoniazid; EMB, ethambutol.
Figure 2.8. Uptake of EtBr and Nile Red by *M. marinum* strains.
Whole-cell accumulations of EtBr and Nile Red by *M. marinum* were measured by fluorescence spectroscopy. Results are from three independent experiments (mean ± SEM). The *fadD26::Tn* and *fadD28::Tn* strains exhibited more rapid and higher levels of accumulation for EtBr and Nile Red than the WT strain. Complementation partially restored the accumulation of EtBr but had no effect on Nile Red uptake. *fadD26 C, fadD26::Tn* plus pFadD26; *fadD28 C, fadD28::Tn* plus pFadD28/MmpL7.
2.4 Discussion

Despite intensive studies in recent years, the biosynthesis of PDIMs and PGLs and their role in mycobacterial pathogenesis remain incompletely understood. For example, while there is substantial evidence supporting the involvement of PDIMs in *M. tb* virulence, a recent study performed whole genome sequencing of multiple lab stocks of two representative strains of H37Rv (ATCC 25618 and ATCC 27294), which were isolated from the same patient in different years, and found that strains of ATCC 25618 maintained in different laboratories contained a frameshift mutation in *mas* (Ioerger, Feng et al. 2010), and therefore cannot produce PDIMs (Kana, Gordhan et al. 2008). However, these strains of ATCC 25618 are fully virulent in mice (Parish, Smith et al. 2003; Kana, Gordhan et al. 2008). It is not clear whether the ATCC 25618 strains contain compensating mutations that retain virulence despite the loss of PDIMs. Similarly, the role of PGLs in *M. tb* virulence remains controversial (Cox, Chen et al. 1999; Sinsimer, Huet et al. 2008) and is complicated by the fact that many laboratory and clinical strains of *M. tb* are naturally deficient in PGLs. Because of this, we believe it is important to study the biosynthesis and biological function of PDIMs and PGLs in other pathogenic mycobacteria, which may help to clarify some of the issues associated with *M. tb* studies. In this study, we examined the biosynthesis and function of PDIMs and PGLs in *M. marinum*, which is the closest genetic relative of the *M. tb* complex. Pioneering works by Ramakrishnan and co-workers have established *M. marinum* as an excellent model to understand various aspects of host-pathogen interactions in *M. tb* pathogenesis [reviewed in (Tobin and Ramakrishnan 2008)]. *M. marinum* and *M. tb* share many virulence determinants, such as the ESX-1 secretion system (Tobin and Ramakrishnan 2008). As such, knowledge we gain on PDIMs/PGLs in *M. marinum* may be applicable to *M. tb*. 
We found that disruption of multiple genes in the presumed PDIM/PGL biosynthetic locus in *M. marinum* abolished the production of PDIMs and PGLs, and complementation of one of the mutants (fadD28::Tn) restored PDIM/PGL synthesis, thus providing direct genetic evidence for the involvement of this locus in PDIM/PGL synthesis. This is consistent with two recent independent studies which showed that disruption of *tesA*, the gene upstream of *fadD26*, also abolished the PDIM/PGL synthesis in *M. marinum* (Alibaud, Rombouts et al. 2011; Chavadi, Edupuganti et al. 2011). Together, these studies demonstrate that although there are some differences in the chemical structures of PDIMs and PGLs between *M. marinum* and *M. tb* (Onwueme, Vos et al. 2005), these two organisms employ similar biosynthetic machinery to produce PDIMs and PGLs. In addition, our study provides evidence for the roles of *ppsA* and *ppsE* in PDIM/PGL synthesis.

One intriguing difference between structures of PDIMs/PGLs in *M. marinum* and *M. tb* is that in *M. marinum* (and closely related *M. ulcerans*), the mycocerosates are deoxtrorotary as opposed to levotrorotary found in *M. tb* and all other mycobacterial species studied (Onwueme, Vos et al. 2005). We found that the deletion of *mas* in *M. marinum* abolished the production of PDIMs and PGLs, confirming its role in mycocerosate synthesis. Sequence alignment revealed that Mas homologs from *M. marinum* and *M. ulcerans* are nearly identical (99.5% sequence identity) and they are more distantly related to Mas from other mycobacteria such as *M. tb* (78.14%), *M. bovis* including BCG (78.09%), and *M. leprae* (75.9%). This is consistent with the notion that Mas in *M. marinum* and *M. ulcerans* synthesizes the *R* enantiomer of mycocerosates, while its homologs in *M. tb* and other mycobacteria are involved in synthesizing the *S* enantiomer of mycocerosates. Future mutagenesis study of these Mas homologs will help to identify residues important for the stereochemistry of mycocerosates.
Deletion of \textit{fadD28} and \textit{mas} in \textit{M. marinum} abolished the production of both PDIMs and PGLs, which is consistent with previous studies of the \textit{fadD28}:Tn mutant of \textit{M. tb} (Cox, Chen et al. 1999; Camacho, Constant et al. 2001). However, in \textit{M. bovis} BCG, \textit{fadD28}:Tn and \textit{mas}:Tn mutants still produced PDIMs but with shorter chain mycocerosates (Azad, Sirakova et al. 1996; Fitzmaurice and Kolattukudy 1998). It was suggested that in \textit{M. bovis} BCG, another PKS catalyzed the synthesis of the shorter chain mycocerosates (Fernandes and Kolattukudy 1998). However, the identity of this enzyme has not been described and the identity of these ‘short chain mycocerosates’ has been questioned by others (Onwueme, Vos et al. 2005). Our results suggest that in \textit{M. marinum}, like in \textit{M. tb}, Mas is the only PKS involved in mycocerosate synthesis, and that \textit{fadD28} is also involved in this pathway (Figure 2.1).

In addition to \textit{fadD28}, there are three other \textit{fadD} genes in the PDIM/PGL locus (\textit{fadD26}, \textit{fadD22}, and \textit{fadD29}, Fig. 1). These genes encode fatty acyl-AMP ligases that convert long-chain fatty acids to acyl-adenylates (Trivedi, Arora et al. 2004). Recent studies in \textit{M. bovis} BCG found that \textit{fadD22} and \textit{fadD29} are required for PGL synthesis only, and \textit{fadD26} is specifically involved in PDIM synthesis (Ferreras, Stirrett et al. 2008; Simeone, Leger et al. 2010). Consistently, we found that \textit{fadD26} is involved in the synthesis of PDIMs but not PGLs in \textit{M. marinum}. FadD26 and FadD29 are thought to transfer different fatty acyl substrates to the same set of PKSs, PpsA-E, for reiterative elongation, leading to phthiocerols and phenolphthiocerols, respectively (Figure 2.1). We found that the expression level of \textit{fadD26} affects the synthesis of PDIMs and indirectly the production of PGLs, suggesting that the production of PDIMs and PGLs is connected and that the ratio of these two lipids may be maintained by the relative abundance of enzymes specific for each pathway (FadD26 and FadD29) and enzymes common to both pathways (PpsA-E). In wildtype cells, since \textit{fadD26} and \textit{ppsA-E} are transcriptionally coupled, the induction of \textit{fadD26} expression will also increase the levels of PpsA-E, which may lead to higher productions.
of both PDIMs and PGLs. Conversely, the repression of fadD26 will lead to lower levels of both PDIMs and PGLs because of the decreased ppsA-E expression. As such, the bacteria can efficiently regulate the amounts of PDIMs and PGLs produced by controlling the expression of fadD26. This also implies that the production of PGLs is dictated by the level of PDIMs, such that wildtype cells cannot produce exceedingly higher level of PGLs without a concurrent increase in PDIM production. It is tempting to suggest that bacteria may employ this mechanism to maintain a constant ratio of PDIMs/PGLs to maximize their effect in virulence.

Our results suggest that in M. marinum, not only are both PDIMs and PGLs essential for virulence, they need to be expressed at appropriate levels such that insufficient production of either lipid will compromise virulence and result in attenuation. For example, the low levels of either PDIMs in the fadD26::Tn mutant or PGLs in the complemented strain resulted in complete attenuation. Furthermore, the amounts of PDIMs and PGLs appear to correlate with the level of virulence, as demonstrated by the partial recovery of virulence in the fadD28 complemented strain that only had partial restoration of PDIMs and PGLs.

In other mycobacteria, the relative role of PDIMs and PGLs in virulence is less clear. It has been demonstrated that loss of PGLs in M. tb or M. bovis strains, which still produce PDIMs, compromise their virulence (Reed, Domenech et al. 2004; Hotter, Wards et al. 2005). Conversely, Sinsimer et al. showed that a recombinant H37Rv strain that expresses intact pks15/1 thus producing PGLs was not more virulent than the parental strain (Sinsimer, Huet et al. 2008; Ioerger, Feng et al. 2010). The effect of removing PDIMs from a M. tb strain that retains PGLs has yet to be evaluated. Previous studies by Sinsimer et al. (Sinsimer, Huet et al. 2008) and Ioerger et al. (Ioerger, Feng et al. 2010) did not find a good correlation between the production of PDIMs or PGLs with virulence among M. tb strains. However, it should be noted
that these comparative studies were performed on strains that are not isogenic and alternative explanations cannot be excluded. Together, these studies suggest that PDIMs may play a more critical role than PGLs in *M. tb* virulence. PGLs are probably not required for but will augment virulence presumably in combination with PDIMs.

Previous studies of PDIM/PGL biosynthesis and their role in virulence have been complicated by recent findings that laboratory H37Rv strains including the ATCC 27294 strain frequently undergo spontaneous mutations leading to loss of PDIMs, which has caused errors in identifying genes involved in PDIM synthesis and virulence (Andreu and Gibert 2008; Domenech and Reed 2009). This stresses the importance of performing genetic complementation experiments to validate that a specific phenotype is actually caused by a given mutation, as well as performing analysis of PDIM production in all strains used in studies. Unfortunately, except for some genes in the PDIM/PGL biosynthetic locus, complementation experiments have not been carried out for a number of other genes in *M. tb* reported to be involved in PDIM synthesis (Onwueme, Vos et al. 2005), and their involvements in PDIM synthesis are uncertain.

The mechanisms by which PDIMs and PGLs mediate virulence remain unclear. PGLs are thought to inhibit the proinflammatory cytokine responses (Reed, Domenech et al. 2004). PGLs of *M. leprae* are involved in bacterial attachment and invasion of Schwann cells (Ng, Zanazzi et al. 2000). A recent study found that PDIMs may mediate a receptor-dependent phagocytosis of *M. tb*, allowing it to create a protective niche by preventing phagosomal maturation (Astarie-Dequeker, Le Guyader et al. 2009). Elevated cell wall permeability and increased antibiotic sensitivity have also been previously described in PDIM deficient mutant of *M. tb* (Camacho, Constant et al. 2001; Rousseau, Winter et al. 2004), and recently, in a PDIM/PGL-deficient mutant of *M. marinum* (Alibaud, Rombouts et al. 2011; Chavadi, Edupuganti et al. 2011).
Consistently, we found that the PDIM/PGL deficient mutants of *M. marinum* exhibited increased cell wall permeability and antibiotic sensitivity. I also found that the PDIM/PGL mutants were impaired for growth in macrophages. The weakening of the permeability barrier of mycobacterial cell wall may also compromise the intracellular survival of the PDIM/PGL deficient mutants, contributing to attenuation in virulence.
Chapter 3:
The Role of PDIMs/PGLs in BCG Virulence and Protective Efficacy

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Tran,V., Li, M., and Jun Liu. 2013. The protective efficacy of BCG is related to its level of virulence. Manuscript in prep.

All experiments were performed by Vanessa Tran. Ming Li assisted in the mouse work.
3

3.1 Introduction

Tuberculosis (TB) remains one of the leading causes of death worldwide. The World Health Organization (WHO) estimated that in 2011 there were ~1.4 million deaths and ~8.7 million new cases of TB (WHO 2012). Global efforts to eradicate *Mycobacterium tuberculosis* (*M. tb*), the causative agent of TB, has been thwarted by the HIV pandemic and the evolution of multi-drug- (MDR-) and extensively-drug-resistant (XDR-) strains of *M. tb*. Bacille Calmette-Guérin (BCG), an attenuated strain of *Mycobacterium bovis*, remains to be the only licensed vaccine against TB. Although in use for nearly a century, the BCG vaccine is far from ideal. While clinical studies have shown that the vaccine is effective at reducing disseminated forms of TB, including miliary TB and tubercular meningitis in children (Colditz, Berkey *et al.* 1995; Trunz, Fine *et al.* 2006), BCG has limited efficacy against adult pulmonary TB (Colditz, Brewer *et al.* 1994; Brewer 2000), the most contagious form of the disease. Another major concern with BCG is its safety in immunocompromised individuals, where disseminated BCG disease has been observed following vaccination in HIV-infected children (Hesseling, Marais *et al.* 2007; Azzopardi, Bennett *et al.* 2009). Since the risks of vaccination outweighed the potential benefits in 2007, the WHO revised its recommendation and declared that HIV infection was a contraindication for giving BCG (WHO 2007). Considering that HIV-endemic areas have the highest rate of TB and HIV-associated deaths accounted for one-third of TB mortality in 2011, those populations most in need of vaccination against TB are unable to receive appropriate prophylaxis (WHO 2012). Therefore, the development of a more effective and safe TB vaccine is urgently needed.
Although commonly referred to as BCG, there are a number of BCG strains that have been historically used in different vaccination programs (Behr and Small 1999; Oettinger, Jorgensen et al. 1999). Further, these different strains exhibit genetic heterogeneity and therefore are not isogenic (Mahairas, Sabo et al. 1996; Behr and Small 1999; Brosch, Gordon et al. 2007; Leung, Tran et al. 2008; Garcia Pelayo, Uplekar et al. 2009). While early studies revealed that different BCG strains displayed varying degrees of virulence and protection in animals (Dubos and Pierce 1956; Dubos, Pierce et al. 1956; Bunch-Christensen, Ladefoged et al. 1968; Bunch-Christensen, Ladefoged et al. 1970; Ladefoged, Bunch-Christensen et al. 1970), these differences were often attributed to quality control issues during the manufacturing process of the strains by different facilities (Milstien and Gibson 1990). Variability in BCG vaccine properties however may reflect true biological differences among BCG strains. For example, biochemical analysis of phthiocerol dimycocerosates (PDIMs) and phenolic glycolipids (PGLs) in 12 BCG strains, found that BCG-Japan, -Moreau, and -Glaxo were naturally deficient in PDIM/PGL production (Chen, Islam et al. 2007). Intriguingly, these BCG strains exhibit superior safety records in clinical studies (Lotte, Wasz-Hockert et al. 1984; Liu, Tran et al. 2009).

PDIMs and PGLs are structurally related, multiple methyl-branched fatty acid-containing lipids in the mycobacterial cell wall and are well established virulence factors (Onwueme, Vos et al. 2005). PDIMs and PGLs are also only found in pathogenic mycobacteria except that in \( M. tb \) only a subset of clinical isolates produces PGLs (Onwueme, Vos et al. 2005). PDIMs were first suggested to be important for \( M. tb \) virulence using signature-tagged transposon mutagenesis which identified \( M. tb \) PDIM mutants that were attenuated in mice (Camacho, Ensergueix et al. 1999; Cox, Chen et al. 1999). Since then, PDIMs have been shown to mediate receptor-dependent phagocytosis of \( M. tb \) (Astarie-Dequeker, Le Guyader et al. 2009), and contribute to
cell wall permeability (Camacho, Constant et al. 2001) and protection against bactericidal effects of reactive nitrogen intermediates in activated macrophages (Rousseau, Winter et al. 2004). The role for PGLs in virulence has been suggested to be in dampening the immune response by inhibiting the release of proinflammatory cytokines and the presence of PGLs has been associated with a hypervirulent phenotype of certain M. tb clinical isolates (Reed, Domenech et al. 2004; Sinsimer, Huet et al. 2008). In Chapter 2, I also establish that production of both PDIMs and PGLs are required for virulence of M. marinum in zebrafish (Yu, Tran et al. 2012).

Given the critical role of PDIMs/PGLs in mycobacterial virulence and immunogenicity, we hypothesized that the differential expression of PDIMs/PGLs may influence the biology of BCG strains, which in turn could affect vaccine efficacy and virulence. To assess this, I generated a PDIM/PGL-deficient strain of BCG-Pasteur, a BCG strain that naturally produces PDIMs/PGLs. I found that the PDIM/PGL-deficient strain of BCG-Pasteur was less virulent in SCID mice and was less protective than the parental strain against M. tb infection in BALB/c mice. My results demonstrate that the protective efficacy of a BCG vaccine strain is related to its level of virulence, and provide direct evidence to support the notion that over-attenuation of a BCG strain leads to decreased efficacy. Our finding has important implications for future vaccine development.
3.2 Material and Methods

3.2.1 Bacterial Strains and Culture Conditions

*Mycobacterium bovis* BCG strains, BCG-Pasteur and BCG-Japan, were grown at 37°C in Middlebrook 7H9 broth (Difco™) supplemented with 0.2% glycerol, 10% albumin-dextrose-catalase (ADC; BD BBL™), and 0.05% Tween 80 or on Middlebrook 7H11 agar (Difco™) supplemented with 0.5% glycerol and 10% oleic acid-albumin-dextrose-catalase (OADC; BD BBL™). *Escherichia coli* strain DH5α was used for routine manipulation and propagation of plasmid DNA. *E. coli* DH5α was grown in LB broth (BioShop) or on LB agar (BioShop). Antibiotics were added as required: kanamycin, 50 µg/ml for *E. coli* and 25 µg/ml for BCG; hygromycin, 150 µg/ml for *E. coli* and 75 µg/ml for BCG.

3.2.2 Generating an Isogenic PDIM/PGL Knockout

Specialized phage transduction was used to generate a PDIM/PGL-deficient mutant of BCG-Pasteur as described previously (Bardarov, Bardarov Jr *et al.* 2002). Briefly, the allelic exchange construct was made by amplifying upstream and downstream regions flanking the *fadD28* gene from BCG-Pasteur genomic DNA using the primer sets 5' - ACTAGTGATTTTCGACACTCGGTAA - 3' (*SpeI*) / 5'- AAGCTTGTCTTCTTTGAAGGT -3' (*HindIII*) and 5'- TCTAGAGATTTTCACGCCTTT - 3' (*XbaI*) / 5' - GGTACCAGTTTCGATA ATG G - 3' (*KpnI*), respectively (restriction sites are underlined). The upstream amplicon was digested and ligated into a *SpeI/HindIII*-digested pJSC284 cosmid, containing a hygromycin resistance marker (hyg<sup>R</sup>). The resulting vector was then digested with *XbaI* and *KpnI* and ligated to the downstream amplicon, creating the complete allelic exchange construct. Correct insertion of both amplicons was confirmed by PCR using locus-specific primers. The recombinant
construct was cloned into a conditionally replicating TM4 shuttle phasmid, pHLR, and specialized transducing mycobacteriophage were generated by electroporating *M. smegmatis* mc^²^155 at the permissive temperature (30°C). Putative knockout mutants were obtained by transducing BCG-Pasteur at the non-permissive temperature (37°C) and selecting hygromycin-resistant colonies. Deletion of *fadD28* was confirmed by Southern blot (Amersham) analysis using a 500 bp probe against the upstream region of *fadD28*, generated with primers 5’-TCCAACCTCGTCTCAGCT - 3’ and 5’- CGCCAT GGGTCCACCA -3’, following the manufacturer’s protocol.

The complementation plasmid was generated by amplifying a 2094 bp fragment containing a wildtype (WT) copy of *fadD28*, using the forward primer 5’ - GGTACCAAGCCAGTTAGGGGC -3’ (KpnI) and reverse primer 5’ - AAGCTTCAGTCCG GGGAGGAC -3’ (HindIII), and cloned into a KpnI/HindIII-digested pME shuttle vector to generate pFADD28.

### 3.2.3 Lipid Analysis by Thin Layer Chromatography

Production of PDIMs/PGLs was examined using 2D-thin layer chromatography (TLC), according to published procedures (Chen, Islam *et al.* 2007; Ren, Dover *et al.* 2007). Briefly, the apolar lipid fraction was extracted from 50 mg (dry weight) of BCG and analyzed on silica gel 60 plates (EMD Chemicals Inc.). For detection of PDIMs, apolar lipids were developed with petroleum ether/ethyl acetate (98:2, 3×) in the first dimension and petroleum ether/acetone (98:2) in the second dimension. Lipids were visualized by staining plates with 5% phosphomolybdic acid followed by gentle charring. For detection of PGLs, the apolar lipid extract was developed with chloroform/methanol (96:4, v/v) in the first direction and toluene/acetone (80:20, v/v) in the second direction, followed by charring with α-naphthol.
3.2.4 Analysis of BCG Virulence in SCID Mice

All of the animal procedures were approved by the University of Toronto Animal Care Committee. Female Fox Chase CB17<sup>®</sup> SCID mice were purchased from Charles River Laboratories and the mice were age-matched (7-8 weeks) within each experiment. Mice (4-6 per group per time point) were infected intravenously via the tail vein with $10^4$ or $10^5$ CFU of the different BCG strains in 0.2 mL PBS/0.01% Tween 80. At 1, 7, 21, 42, 52, and 79 days post infection, the lungs were harvested, homogenized in PBS, and plated on 7H11 agar to enumerate bacterial burden. CFU was counted after incubation at 37°C for 3 weeks. Bacterial counts from lung homogenates harvested at day 1 post infection were used as an indicator of initial infection dose.

3.2.5 Immunogenicity Studies

Female C57BL/6 mice were purchased from Charles River Laboratories and were age-matched (6 weeks) within each experiment. Four to nine mice per group were inoculated subcutaneously on the scruff of the neck with approximately ~$10^4$ CFU in 0.2 ml PBS/0.01% Tween 80 of wildtype BCG-Pasteur or the knockout strain. Control mice were given 0.2 mL of PBS/0.01% Tween 80. After 9 weeks, mice were euthanized, splenocytes were isolated, and intracellular IFNγ was measured. Briefly, splenocytes were seeded at $2 \times 10^6$ cells/well in 100 μl in triplicate and stimulated with 2.5 μg/well of purified protein derivative (PPD) (Statens Serum Institute, Denmark) or complete RPMI (cRPMI; RPMI/10% FBS/1% L-glutamine/1% penicillin/streptomycin) as a control and incubated at 37°C and 5% CO₂. After 19 hours of stimulation, GolgiPlug (BD Biosciences) was added in a 1:1000 final dilution and incubated for an additional 5 hours. After a total of 24 hours stimulation, plates were centrifuged at 1400 rpm
for 5 minutes at 4°C. The supernatant was removed and the cell pellet was washed in 200 μl FACS Buffer (0.5% BSA/PBS), and resuspended in Fc Block (eBiosciences) diluted in FACS Buffer (1:400) and incubated for 15 minutes on ice in the dark. An additional 150 μl of FACS Buffer was added, mixed, and plates were centrifuged at 1400 rpm for 5 minutes at 4ºC. Supernatant was removed and cells were stained for extracellular T cell surface markers: CD3-PE, CD4-FITC, and CD8a-PercyPCy5.5 (BD Biosciences) diluted in FACS Buffer, and incubated for 30 minutes on ice in the dark. Following extracellular marker staining, the cells were washed with 150 μl FACS Buffer and permeabilized and fixed with 1X CytoFix/CytoPerm (BD Biosciences) for 20 minutes. Cells were then washed with 1X PermWash (BD Biosciences) and incubated with IFNγ-APC (BD Biosciences) for 30 minutes to stain for intracellular IFNγ. Cells were centrifuged as above, resuspended in 200 μl FACS Buffer, and analyzed on a BD FACSCalibur™ flow cytometer (BD Biosciences). A total of 300 000 events per sample were collected in the lymphocyte gate and analyzed using FlowJo V7.6. Gates for analysis were set based on isotype controls.

Quantitative measurements of IFNγ production was also determined using an enzyme-linked immunosorbant assay (ELISA) using the OptEIA Mouse IFNγ ELISA set (BD Biosciences). Samples used for ELISAs were supernatants from splenocytes stimulated for 72 hours. Briefly, samples were added in triplicate to 96-well, flat-bottomed plates (Nunc MaxiSorp) pre-coated with capture antibody and measurements were carried out following the manufacturer’s protocol. The absorbance was read at 450 nm on a microplate reader (TECAN infinite M200). IFNγ levels were calculated based on a standard curve generated using an IFNγ standard.
3.2.6 Protection against *M. tb* Challenge

Groups of 13-15 female BALB/c mice (Charles River Laboratories) were vaccinated subcutaneously on the scruff of the neck with ~$10^5$ CFU of the BCG strains in 0.2mL PBS/0.01% Tween 80 or PBS/0.01% Tween 80 alone as a control. At 8-weeks post vaccination, mice were aerogenically challenged with 400-600 CFU of *M. tb* H37Rv using a GlasCol nebulizer. Mice were euthanized at 5 and 9 weeks post challenge (6-7 mice per group per time point) to harvest the lung and spleen. A portion of the organs were fixed in 10% formalin for histological analysis. The remaining portion was homogenized and plated on 7H11 agar to enumerate burden of *M. tb* in the lung and spleen. Plates were incubated at 37°C and counted after 2.5-3 weeks.

3.2.7 Histological Analysis

Fixed tissues were embedded at the Centre for Modeling Human Disease (Toronto Centre for Phenogenomics) into paraffin blocks. Serial 4 μm thick sections prepared and kept at 37°C for more than 12 hours. The sections were deparaffinnized in three changes of xylene for 3 minutes each and rehydrated in four consecutive washes of alcohol (100%, 100%, 95%, and 70%) for 3 minutes each. Sections were stained with hematoxylin and eosin (EMD Chemicals) or Acid Fast stain kit (Surgipath) according to standard procedures and were examined using a Leica microscope.
3.3 Results

3.3.1 Construction of an Isogenic PDIM/PGL-deficient Mutant

A BCG-Pasteur strain deficient in PDIMs/PGLs was generated by target deletion of \( fadD28 \), which encodes a fatty acyl-AMP ligase involved in PDIM/PGL biosynthesis (Figure 3.1A-B) (Onwueme, Vos et al. 2005). Deletion of \( fadD28 \) was confirmed by Southern blot using a 500 bp probe against the upstream region of \( fadD28 \) (Figure 3.1A-B). The abrogation of PDIM/PGL synthesis in the knockout strain was confirmed by 2D-TLC analysis (Figure 3.1D). The \( fadD28 \) knockout strain grew equally well as the parental strain in 7H9 medium (Figure 3.1C). Transformation of plasmid pFADD28, which contains WT \( fadD28 \), into the knockout strain restored the production of both PDIMs and PGLs (Figure 3.1D).

3.3.2 Loss of PDIMs/PGLS Reduces Virulence of BCG-Pasteur

To determine the impact of PDIM/PGL expression on the degree of BCG virulence, I compared the virulence of the WT, \( \Delta fadD28 \), and complemented strains in severely immunocompromised SCID mice, a mouse model that has been commonly used to assess the virulence of recombinant BCG or attenuated \( M. \) \textit{tb} vaccine strains (Pym, Brodin \textit{et al.} 2002; Grode, Seiler \textit{et al.} 2005; Martin, Williams \textit{et al.} 2006). Groups of 30 SCID mice were infected intravenously via the tail vein with \( \sim 10^4 \) colony forming units (CFU) (low dose) of each strain. An additional cohort was infected with \( \sim 10^5 \) CFU (high dose) of the WT and \( \Delta fadD28 \) strains to determine if there was a dosage effect. I found that bacterial count of the \( \Delta fadD28 \) strain in the lung of infected mice was significantly less compared to WT or the complemented strain (Figure 3.2A-B), suggesting that the \( \Delta fadD28 \) strain is less virulent than WT. This is further supported by the observation that in
Figure 3.1. Construction of a PDIM/PGL deficient strain of BCG-Pasteur.

(A) Genomic organization of WT (Pasteur) and ΔfadD28 strains. Dashed lines indicate products of restriction digestion with ClaI and BoxI. (B) Southern blot analysis. Chromosomal DNAs isolated from WT and three randomly picked ΔfadD28 clones were digested with ClaI and BoxI and blotted with a 500 bp probe of fadD28, which yielded a 3.5 kb and 1.8 kb fragment, respectively, and agreed with prediction (A). (C) In vitro growth curve of WT (Pasteur) and ΔfadD28 strains. Strains were grown in 7H9/ADC/Tween 80 at 37°C. Growth was measured at OD₆₀₀. (D) 2D-TLC analysis of PDIMs and PGLs. For PDIM analysis, apolar lipids were developed with petroleum ether/ethyl acetate (98:2 v/v, 3 times) in the first dimension (1st) and petroleum ether/acetone (98:2, v/v) in the second dimension (2nd). Lipids were visualized by charring with 5% phosphomolybdic acid. For PGL analysis, the apolar lipid extract was developed with chloroform/methanol (96:4, v/v) and toluene-acetone (80:20, v/v), followed by charring with α-naphthol. PDIMs, phthiocerol dimycocerosates; PGLs, phenolic glycolipids.
Figure 3.2. The PDIM/PGL deficient mutant of BCG-Pasteur is less virulent in SCID mice.

(A-B) BCG burden in SCID mice. SCID mice were infected intravenously with ~10^4 (A) or ~10^5 (B) CFU of BCG-Pasteur (Pasteur), the ΔfadD28, or complemented strain (ΔfadD28 + pFADD28). Bacterial burden in lungs were determined at various time points over 11 weeks. *, p<0.05; **, p<0.01; Two-Way ANOVA. (C) Scruffiness and dehydration of SCID mice in the high dose (10^5 CFU) infection group. At 52 days post-infection (dpi), mice infected with WT BCG exhibited severe dehydration while the ΔfadD28-infected mice appeared normal. (D) Body weight of SCID mice infected with BCG. The weight of SCID mice infected with ~10^5 CFU of BCG-Pasteur at 52 dpi compared to ΔfadD28-infected mice and BCG-Pasteur-infected mice in the low dose group (each dot represents one mouse, mean ± SD, **, p<0.01; ***, p<0.001; One-Way ANOVA). (E) Lung pathology of BCG-Pasteur- and ΔfadD28-infected mice from the high dose group at 52 dpi. (F) Lung histology of BCG-Pasteur- and ΔfadD28-infected mice from the high dose group at 52 dpi; arrow denotes magnified granuloma-like lesion. HE, hematoxylin-eosin stain; AF, acid fast stain. Ming Li assisted with the organ harvest.
the high dose cohort, mice infected with the WT strain exhibited observable disease phenotypes, including severe dehydration (Figure 3.2C), and significant weight loss (Figure 3.2D), which were not seen in mice infected with the ΔfadD28 strain at the same time points post infection (Figure 3.2C-D). Because of the severe disease phenotype, by day 52, WT-infected mice had to be euthanized and later time points could not be collected. The lungs of mice infected with the high dose of WT BCG-Pasteur also exhibited gross pathological evidence of disease, with numerous surface nodules observed, whereas few were seen in the ΔfadD28-infected mice (Figure 3.2E). Consistently, histological analysis of lung tissues from WT-infected mice had numerous acid-fast staining (AF) positive granulomatous lesions, whereas lungs from ΔfadD28-infected mice showed few scattered lesions (Figure 3.2F). Taken together, my results demonstrate that loss of PDIMs/PGLs reduces the virulence of BCG-Pasteur.

### 3.3.3 Loss of PDIMs/PGLs Does Not Affect Immunogenicity of BCG-Pasteur

To assess if the loss of PDIMs/PGLs affects the immunogenicity of BCG-Pasteur, I determined the production of IFNγ in vaccinated C57BL/6 mice. Currently, there is no proven immunological correlate of protection or “biomarker” for efficacy (Mittrucker, Steinhoff et al. 2007; Soares, Scriba et al. 2008). However, a critical role of IFNγ in the control of TB has been demonstrated in mice (Cooper, Dalton et al. 1993; Flynn, Chan et al. 1993) and humans (Jouanguy, Altare et al. 1996; Newport, Huxley et al. 1996). As such, antigen specific IFNγ production, mostly by CD4⁺ T cells has been used most widely as measure of protective immunity and vaccine efficacy, despite the fact that IFNγ alone is insufficient for protection against TB (Hanekom, Dockrell et al. 2008). Thus, to examine the role of PDIMs/PGLs in immunogenicity of BCG, I used a C57BL/6 immunocompetent mouse model and measured
antigen (PPD) specific IFNγ production from both CD4\(^+\) and CD8\(^+\) T cells by intracellular cytokine staining. Interestingly, I found that the loss of PDIMs/PGLs in BCG-Pasteur did not significantly alter the amount of IFNγ production from both CD4\(^+\) and CD8\(^+\) T cells, where comparable levels were observed between the WT- and ∆fadD28-infected groups (Figure 3.3A). Detection of IFNγ production by ELISA also yielded similar results (Figure 3.3B). Taken together, my results indicate that loss of PDIMs/PGLs does not affect BCG immunogenicity.

### 3.3.4 Loss of PDIMs/PGLs affects BCG-mediated Production against *M. tb*

To determine if the PDIM/PGL-deficient mutant of BCG-Pasteur retained the same capacity to protect against *M. tb*, I used an aerosol challenge model in BALB/c mice. Groups of 13-15 mice were vaccinated subcutaneously with \(\sim 10^5\) CFU of BCG-Pasteur, ∆fadD28, ∆fadD28 + pFADD28, or PBS as a control. At 8-weeks post-vaccination, the mice were aerogenically challenged with 400-600 CFU of *M. tb* H37Rv and bacterial burden in the lung and spleen was determined at 5 and 9 weeks post-challenge. Interestingly, I found that the PDIM/PGL-deficient strain did not protect as well as the WT against *M. tb* infection (Figure 3.4). At 5 weeks post-challenge, WT-vaccinated mice had significantly lower *M. tb* burden in the lungs compared to the PBS control group, with a \(~4\)-fold reduction in CFU (Figure 3.4A, p<0.001). However, vaccination with ∆fadD28 caused only a \(~1.5\)-fold reduction in CFU compared to the PBS group and this difference was statistically not significant. The *M. tb* burden in ∆fadD28-vaccinated mice was also significantly higher than that in WT-vaccinated mice (\(p<0.05\)) and importantly, the protection was restored to WT levels in the complemented strain (∆fadD28+PFADD28-vaccinated group) (Figure 3.4A). Similar results were observed in the spleen, although the
Figure 3.3. The loss of PDIMs/PGLs does not affect production of IFNγ.

(A) Intracellular cytokine staining analysis of IFNγ production by CD4+ and CD8+ T cells. C57BL/6 mice were immunized subcutaneously with the WT BCG-Pasteur, ΔfadD28, or PBS/0.01% Tween 80. At 9 weeks post-vaccination, mice were sacrificed and splenocytes were harvested. Splenocytes were incubated with or without PPD for 24 hours followed by staining for T cell surface markers (CD3-PE, CD4-FITC, CD8a-PercyPCy5.5) and intracellular IFNγ (IFNγ-APC). Samples were analyzed by BD FACSCalibur™ and FlowJo® Software. Pooled results from two independent experiments; each data point represents one mouse. (B) ELISA analysis of IFNγ production. The supernatant from unstimulated and PPD-stimulated splenocytes was assayed for IFNγ using the OptEIA Mouse IFNγ ELISA set (BD Biosciences) following the manufacturer’s protocol. Representative results from one mouse experiment (mean ± SD; One-Way ANOVA; ns=not significant).
Figure 3.4. **Loss of PDIMs/PGLs affects BCG-mediated protection against *M. tb***.

BALB/c mice were vaccinated subcutaneously with ~10^5 CFU of the BCG strains or PBS as a control. At 8-weeks post vaccination, mice were aerogenically challenged with ~400-600 CFU of *M. tb* using a GlasCol nebulizer. Mice were sacrificed at 5 and 9 weeks post challenge and organs were examined for bacterial burden and pathology. Bacterial burden of *M. tb* in the (A) lung and (B) spleen were determined by plating tissue homogenate on 7H11 agar (6 mice per group per time point; mean ± SEM; *, *p* < 0.05; **, *p* < 0.01; ***, *p* < 0.001; ns=not significant; one-way ANOVA, Bonferroni post-test). (C) **Histological analysis of lung sections from mice in each group at 9 weeks post-challenge**. Samples are stained with hematoxylin-eosin (HE). Arrows indicate regions of granuloma-like lesions. Top row is 50× magnification; bottom row is 200× magnification. Ming Li assisted with tissue collection.
differences were not statistically significant (Figure 3.4B). At 9 weeks post-challenge, a similar trend was observed but the differences were not as drastic, which may be due to the fact that BALB/c mice have begun to control the M. tb infection at this time point.

In this challenge experiment, I also included a BCG-Japan-vaccinated group for comparison. As mentioned above, BCG-Japan is a natural PDIM/PGL-deficient mutant. Consistently, vaccination with BCG-Japan achieved levels of protection that were similar to ΔfadD28-vaccinated mice in the lung and spleen at both 5 and 9 weeks post-challenge (Figure 3.4A-B).

Histological analysis of M. tb challenged mice showed consistent differences in lung pathology between the different BCG vaccinated cohorts. Vaccination with WT BCG-Pasteur or the complemented strain reduced the number of granuloma-like lesions in the lung, whereas mice vaccinated with the BCG-Pasteur ΔfadD28 strain or BCG-Japan had lung pathology similar to the PBS control group (Figure 3.4C). The extent of the granulomatous lesions therefore appears proportional to the degree of bacterial burden in the lung. Taken together, my results suggest that the loss of PDIMs/PGLs compromises the ability of the BCG vaccine to protect against M. tb infection.

3.4 Discussion

A major drawback of the BCG vaccine is its limited effect against pulmonary TB in adults with efficacy estimates from clinical studies ranging from 0 to 80% (Colditz, Brewer et al. 1994; Brewer 2000). Several hypotheses have been proposed to explain the variable efficacy (reviewed in (Fine PEM 1999; Andersen and Doherty 2005)), including one that concerns the in vitro
evolution of BCG strains. All BCG vaccine strains are descendants of the original BCG strain isolated in 1921, which was 80% efficacious in humans but no longer exists. Each substrain has undergone additional in vitro passaging until seed lots were established in the 1960s (Behr 2002; Liu, Tran et al. 2009). Different passage numbers at multiple sites (for example, 172 passages for BCG-Japan and >1000 passages for BCG-Pasteur) caused divergent evolution, resulting in a number of BCG substrains that differ from each other and from the original vaccine. It was hypothesized that current BCG strains might have been over-attenuated by excessive in vitro passaging and consequently lost efficacy (Behr and Small 1997). Consistent with this hypothesis, early animal studies by Dubos and Pierce in the 1950s (Dubos and Pierce 1956; Dubos, Pierce et al. 1956) and Bunch-Christensen et al. in the 1970s (Bunch-Christensen, Ladefoged et al. 1968; Bunch-Christensen, Ladefoged et al. 1970; Ladefoged, Bunch-Christensen et al. 1970) found that BCG strains differed in virulence and that there appeared to be a correlation between the level of virulence and protective efficacy. In addition, comparative genome analysis in the 1990s established that BCG strains are not isogenic and that their genetic changes are generally consistent with the historical record of BCG dissemination (Behr and Small 1999). It is now clear that deletion of chromosome region RD1, which is common to all BCG strains and likely occurred between 1908-1921, contributes to the attenuation of BCG (Mahairas, Sabo et al. 1996; Behr and Small 1999; Pym, Brodin et al. 2002). Additional genetic mutations specific to individual BCG strains are required to account for the differential virulence of BCG strains observed in animal studies. However, such evidence has been lacking. In 2007, we observed differential production of PDIMs and PGLs among BCG strains and found that BCG-Moreau, -Japan, and -Glaxo are naturally deficient in PDIM/PGL production (Chen, Islam et al. 2007). Consistently, the genetic mutations responsible for the loss of PDIMs/PGLs in BCG-Moreau and -Japan were subsequently uncovered (Leung, Tran et al. 2008; Naka, Maeda et al. 2011).
Interestingly, the loss of PDIMs/PGLs in these three BCG strains correlates with their superior safety records in clinical studies (Lotte, Wasz-Hockert et al. 1984; Liu, Tran et al. 2009). However, since this correlation was made among BCG strains that are not isogenic, direct evidence for the impact of PDIMs/PGLs on BCG vaccine properties was needed. In this study, I addressed this question by constructing a PDIM/PGL-deficient strain from BCG-Pasteur, which naturally produces PDIMs/PGLs, and performed comparative studies of the isogenic strains. I found that loss of PDIMs/PGLs from BCG-Pasteur attenuates its virulence but also compromises its protective efficacy, indicating that there is an intimate relationship between BCG efficacy and virulence. Since the loss of PDIMs/PGLs occurs naturally in at least three BCG strains, my study provides the first piece of experimental evidence for the over-attenuation hypothesis. Thus BCG-Japan, -Moreau, and -Glaxo have evolved independently and acquired the same phenotype (loss of PDIMs/PGLs) by different genetic mutations, resulting in over-attenuation. Other genetic mutations including a number of single nucleotide polymorphisms (SNPs) have been detected in each BCG strain (Mostowy, Tsolaki et al. 2003; Brosch, Gordon et al. 2007; Leung, Tran et al. 2008; Garcia Pelayo, Uplekar et al. 2009; Liu, Tran et al. 2009), but it remains to be determined if these mutations affect the virulence of a given strain and consequently its efficacy.

My finding has important implications for current BCG immunization programs and for the development of the next generation of TB vaccines. My data suggests that using a “safe” BCG strain for vaccination can have negative consequences on the protective efficacy. Despite its limitations, BCG is unlikely to be replaced in the near future and will continue to play a major role in future TB vaccine development, either as an integral component (e.g. recombinant BCG) or as a prime immunization to be boosted by subunit vaccines. The lack of protective efficacy of MVA85A, the most advanced subunit vaccine candidate thus far, in a recent clinical trial study (Tameris, Hatherill et al. 2013) further underlines the critical role of BCG in future vaccine development.
compositions and the importance of live vaccine research. The positive correlation between virulence and efficacy that we observed suggests that when developing recombinant BCG or attenuated *M. tb* strains as future vaccines, there needs to be a fine balance between these two factors in order to achieve optimal protection while maintaining an acceptable level of safety.
Chapter 4:
The Role of PhoP in BCG Immunogenicity and Protection

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All experiments were performed by Vanessa Tran. Ming Li assisted with the mouse experiments.
4

4.1 Introduction

Previous work by our lab revealed a novel link between PhoP expression and BCG immunogenicity. Specifically, we found that BCG-Prague, a BCG strain that consistently induced a weak tuberculin reaction (Vallishayee, Shashidhara et al. 1974), is a natural *phoP* mutant (Leung, Tran et al. 2008). PhoP is a response regulator of the PhoP-PhoR two-component system in mycobacteria and positively regulates >40 genes, including several well-established T cell antigens, such as Ag85 and PPE18, that have been exploited in TB vaccine development (Walters, Dubnau et al. 2006; Sander, Pathan et al. 2009). Due to its poor immunogenicity, BCG-Prague was considered a “weak” vaccine but the reason for this was unknown. However, we recently found a single G insertion in the C-terminal DNA-binding domain of PhoP, which likely abrogates its function and accounts for the low immunogenicity of BCG-Prague (Leung, Tran et al. 2008). To test this hypothesis, a previous Masters student in our lab, Andrea Leung, found that reintroduction of wildtype *phoP* into BCG-Prague increased the expression of both these T cell antigens and IFNγ production in mice, thus supporting the notion that the mutation in *phoP* is responsible for the weak immunogenicity. Based on this work, I wanted to determine if PhoP also had a role in mediating other BCG vaccine properties including virulence and protection. Further, I hypothesized that the overexpression of *phoP* could be used to enhance immunogenicity and thus protective efficacy of BCG and can be used to generate a new vaccine candidate.

An ideal TB vaccine should be both protective against TB and safe. To satisfy both requirements, I examined the overexpression of *phoP* in BCG-Japan. Based on previous work, I chose BCG-Japan, a natural PDIM/PGL mutant, as the parental strain to generate recombinant
BCG-Japan strains (rBCG-Japan). BCG-Japan was chosen over the other natural PDIM/PGL mutants, BCG-Moreau and BCG-Glaxo, because it also lacks triacylglycerols (TAGs), which are important for virulence (Chen, Islam et al. 2007), and contains less genomic deletions, making it more likely to be broadly immunogenic (Behr, Wilson et al. 1999; Leung, Tran et al. 2008). Furthermore, a number of studies have shown that among BCG strains tested, BCG-Japan is generally the most attenuated (Bunch-Christensen, Ladefoged et al. 1968; Ladefoged, Bunch-Christensen et al. 1970; Lagranderie, Balazuc et al. 1996). Since the regulation of phoP is still unclear, two rBCG-Japan strains were made: one overexpressing phoP (rBCG-Japan.phoP) and one overexpressing phoP and its cognate sensor kinase, phoR (rBCG-Japan.phoPR). These strains were made by Andrea Leung.

To assess the effect of phoP overexpression on BCG vaccine properties, I tested the recombinant BCG-Japan strains in mice and found that overexpression of phoP improved protection against an aerosol challenge of *M. tb* but also increased the virulence of BCG-Japan. These results suggest that the more virulent BCG-Japan strain is more protective against *M. tb* and further supports the link between BCG virulence and efficacy I established previously. In addition, these results highlight a novel strategy to increase the potency of BCG vaccine strains.

### 4.2 Materials and Methods

#### 4.2.1 Bacterial Strains and Culture Conditions

*Mycobacterium bovis* BCG-Japan strains were grown at 37°C in Middlebrook 7H9 broth (Difco™) supplemented with 0.2% glycerol, 10% albumin-dextrose-catalase (ADC; BD BBL™),
and 0.05% Tween 80 or on Middlebrook 7H11 agar (Difco™) supplemented with 0.5% glycerol and 10% oleic acid-albumin-dextrose-catalase (OADC; BD BBL™). *Escherichia coli* strain DH5α was used for routine manipulation and propagation of plasmid DNA. *E. coli* DH5α was grown in LB broth (BioShop) or on LB agar (BioShop). Antibiotics were added as required: kanamycin, 50 µg/ml for *E. coli* and 25 µg/ml for BCG; hygromycin, 150 µg/ml for *E. coli* and 75 µg/ml for BCG.

### 4.2.2 Immunogenicity Assessments

Female C57BL/6 mice were purchased from Charles River Laboratories and were age-matched (6 weeks) within each experiment. Four to five mice per group were inoculated subcutaneously on the scruff of the neck with approximately ~10⁴ CFU in 0.2 ml PBS/0.01% Tween 80 of wildtype BCG-Japan or the recombinant BCG-Japan strains. Control mice were given 0.2 mL of PBS/0.01% Tween 80. After 9 weeks, mice were euthanized, splenocytes were isolated, and intracellular IFNγ was measured. Briefly, splenocytes were seeded at 2x10⁶ cells/well in 100 µl in triplicate and stimulated with 2.5 µg/well of purified protein derivative (PPD) (Statens Serum Institute, Denmark) or complete RPMI (cRPMI; RPMI/10% FBS/1% L-glutamine/1% penicillin/streptomycin) as a control and incubated at 37°C and 5% CO₂. After 19 hours of stimulation, GolgiPlug (BD Biosciences) was added in a 1:1000 final dilution and incubated for an additional 5 hours. After a total of 24 hours stimulation, plates were centrifuged at 1400 rpm for 5 minutes at 4°C. The supernatant was removed and the cell pellet was washed in 200 µl FACS Buffer (0.5% BSA/PBS), and resuspended in Fc Block (eBiosciences) diluted in FACS Buffer (1:400) and incubated for 15 minutes on ice in the dark. An additional 150 µl of FACS Buffer was added, mixed, and plates were centrifuged at 1400 rpm for 5 minutes at 4°C. Supernatant was removed and cells were stained for extracellular T cell surface markers: CD3-
PE, CD4-FITC, and CD8a-PercyPCy5.5 (BD Biosciences) diluted in FACS Buffer, and incubated for 30 minutes on ice in the dark. Following extracellular marker staining, the cells were washed with 150 μl FACS Buffer and permeabilized and fixed with 1X CytoFix/CytoPerm (BD Biosciences) for 20 minutes. Cells were then washed with 1X PermWash (BD Biosciences) and incubated with IFNγ-APC (BD Biosciences) for 30 minutes to stain for intracellular IFNγ. Cells were centrifuged as above, resuspended in 200 μl FACS Buffer, and analyzed on a BD FACSCalibur™ flow cytometer (BD Biosciences). A total of 300 000 events per sample were collected in the lymphocyte gate and analyzed using FlowJo V7.6. Gates for analysis were set based on isotype controls.

Quantitative measurements of IFNγ production was also determined using an enzyme-linked immunosorbant assay (ELISA) using the OptEIA Mouse IFNγ ELISA set (BD Biosciences). Samples used for ELISAs were supernatants from splenocytes stimulated for 72 hours. Briefly, samples were added in triplicate to 96-well, flat-bottomed plates (Nunc MaxiSorp) pre-coated with capture antibody and measurements were carried out following the manufacturer’s protocol. The absorbance was read at 450 nm on a microplate reader (TECAN infinite M200). IFNγ levels were calculated based on a standard curve generated using an IFNγ standard.

4.2.3 Analysis of BCG Virulence in SCID Mice

All of the animal procedures were approved by the University of Toronto Animal Care Committee. Female Fox Chase CB17® SCID mice were purchased from Charles River Laboratories and the mice were age-matched (7 weeks) within each experiment. Mice (4 per group per time point) were infected intravenously via the tail vein with $10^5$ CFU of the different BCG strains in 0.2 mL PBS/0.01% Tween 80. At 1, 7, 21, and 43 days post infection, the lungs
were harvested, homogenized in PBS, and plated on 7H11 agar to enumerate bacterial burden. CFU was counted after incubation at 37°C for 3 weeks. Bacterial counts from lung homogenates harvested at day 1 post infection were used as an indicator of initial infection dose.

4.2.4 Protection Against M. tb Challenge

Groups of 10-12 female BALB/c mice (Charles River Laboratories) were vaccinated subcutaneously on the scruff of the neck with \( \sim 10^5 \) CFU of the BCG strains in 0.2 mL PBS/0.01% Tween 80 or PBS/0.01% Tween 80 alone as a control. At 8-weeks post vaccination, mice were aerogenically challenged with \( \sim 300 \) CFU of M. tb H37Rv using a GlasCol nebulizer. Mice were euthanized at 5 and 9 weeks post challenge (6-7 mice per group per time point) to harvest the lung and spleen. Samples were homogenized and plated on 7H11 agar to enumerate burden of M. tb in the lung and spleen. Plates were incubated at 37°C and counted after 2.5-3 weeks.

4.3 Results

4.3.1 Immunogenicity of rBCG-Japan Strains

Protection against TB has been shown to be dominated by T cell mediated immunity (Kaufmann 2001). The production of proinflammatory cytokines (e.g., IFNγ and TNF) and cytokines of the Th1 T cell response (e.g., IL-2) have been widely used to measure protective immunity of BCG vaccines (Lagranderie, Balazuc et al. 1996; Davids, Hanekom et al. 2006; Meya and McAdam 2007). To evaluate the immunogenicity of the rBCG-Japan strains, C57BL/6 mice were immunized subcutaneously with the parental strain, the rBCG-Japan stains, or PBS/0.01%
Tween 80 as a control. At 9 weeks post-vaccination, splenocytes were harvested, stimulated with PPD, and cytokine production was measured by intracellular cytokine staining and flow cytometry. I observed that \textit{phoP} overexpression increased the immunogenicity of BCG-Japan (Figure 4.1). Specifically, overexpression of \textit{phoP} alone induced a modest increase of PPD-stimulated IFN\textgamma\textgreek{ of 1.25-fold compared to the parental strain, but this difference was not statistically significant. However, overexpression of the entire two-component system, containing both \textit{phoP} and \textit{phoR}, significantly increased IFN\textgamma\textgreek production by \textasciitilde{}2-fold (Figure 4.1). This suggests that \textit{phoR} might be important for the correct regulation of \textit{phoP} and subsequent induction of antigenic targets in this overexpression system. To examine more comprehensively the immunogenicity profile of the \textit{phoP} overexpression strains, I tested an array of cytokines. Since it is known that the Th1 arm of the cell-mediated immune response plays a key role in protection against \textit{M. tb}, I used a multiplex ELISA to determine if the rBCG-Japan strains elicited a Th1 polarized response. Surprisingly, I found that vaccination with either the parental or recombinant BCG-Japan strains induced similar levels of both Th1 and Th2 cytokines after PPD stimulation (Figure 4.2). This suggests the overexpression of \textit{phoP} in BCG-Japan enhances IFN\textgamma\textgreek production but does not broadly increase immunogenicity.

\textbf{4.3.2 Comparison of Virulence in SCID Mice}

PhoP has been shown to contribute to virulence in \textit{M. tb} (Perez, Samper \textit{et al.} 2001; Martin, Williams \textit{et al.} 2006; Walters, Dubnau \textit{et al.} 2006; Lee, Krause \textit{et al.} 2008) and \textit{M. bovis} (Rivero, Marquez \textit{et al.} 2001; Soto, Menendez \textit{et al.} 2004). Therefore, to determine if the overexpression of \textit{phoP} and \textit{phoR} increased the virulence of the recombinant strains, I performed an infection experiment in a SCID mouse model. Similar to before, 8-week old female mice were injected intravenously with rBCG-Japan.pMe, rBCG-Japan.\textit{phoP}, rBCG-Japan.\textit{phoPR}, or
Figure 4.1. IFNγ production by rBCG-Japan strains.
C57BL/6 mice were immunized subcutaneously with the parental control strain, the rBCG-Japan stains, or PBS/0.01% Tween 80 as a control. At 9 weeks post-vaccination, mice were sacrificed and splenocytes were harvested. Splenocytes were incubated with or without PPD for 24 hours followed by staining for T cell surface markers (CD3-Pacific Blue, CD4-PECy7, CD8-FITC) and intracellular IFNγ (IFNγ-PE). Samples were analyzed by BD FACSCalibur™ and FlowJo® Software (4-5 mice per group; mean; representative of n=2; *p<0.05, ns=not significant, t-test).
Figure 4.2.  Cytokine production stimulated by rBCG-Japan.phoP/phoPR.
C57BL/6 mice were immunized subcutaneously with the parental control strain, the rBCG-Japan strains, or PBS/0.01% Tween 80 as a control. At 9 weeks post-vaccination, mice were sacrificed and splenocytes were harvested. Splenocytes were incubated with or without PPD (10 µg/mL) for 72 hours and the supernatant was harvested and cytokine production was measure by multiplex ELISA (Mouse Th1/Th2 9-plex Multi-Spot® ELISA (MSD)) (mean ± SEM). No significant differences in cytokine levels were observed between the parental and recombinant BCG-Japan strains (1-way ANOVA, Bonferroni post-test).
PBS/0.01% Tween 80 as a control. I found that overexpression of \textit{phoP/phoR} did not increase bacterial burden in the lungs (Figure 4.3). Interestingly, overexpression of \textit{phoP} alone increased BCG replication in SCID mice, beginning at 21 days post infection and becoming significantly different by 43 days post infection with a \~1.2 log difference in CFU compared to rBCG-Japan.pMe (Figure 4.3). Again, this suggests that the presence of \textit{phoR} is important for the proper regulation of \textit{phoP} function.

### 4.3.3 The Overexpression of \textit{phoP} Improves Efficacy of BCG-Japan

To determine if the overexpression of \textit{phoP} or \textit{phoP/phoR} increased the protective efficacy of BCG-Japan, I used an aerosol \textit{M. tb} challenge model in BALB/c mice. Briefly, groups of 10-12 mice were vaccinated subcutaneously with the parental BCG-Japan, the recombinant strains, or PBS/0.01% Tween 80 as a control. At 8 weeks post-challenge, mice were challenged aerogenically with virulent \textit{M. tb} H37Rv and target organs were harvested over time to determine bacterial burden. At 5 weeks post-challenge, BCG vaccination caused a significant reduction of \textit{M. tb} burden in lung, with a 2.4-fold, 3.8-fold, and 2.3-fold reduction for rBCG-Japan.pMe, rBCG-Japan.\textit{phoP}, and rBCG-Japan.\textit{phoPR}, respectively (Figure 4.4). Further, vaccination with rBCG-Japan.\textit{phoP} resulted in an additional 1.6-fold reduction compared to vaccination with the parental strain. By 9 weeks post-challenge, only mice vaccinated with rBCG-Japan.\textit{phoP} had significantly reduced lung burden compared to the PBS control group and protection was greater than that achieved with the parental strain (Figure 4.4). A similar trend was observed in the spleen, where vaccination with either the recombinant strains significantly reduced the bacterial burden compared to the PBS control group, with a 3.9-fold and 3.2-fold reduction for rBCG-Japan.\textit{phoP} and rBCG-Japan.\textit{phoPR}, respectively, whereas the parental strain did not significantly reduce splenic burden (Figure. 4.4). This trend extended to 9 weeks
Figure 4.3. Comparison of rBCG-Japan burden in SCID mice.
8-week old female SCID mice were infected intravenously with ~$10^5$ CFU of parental or rBCG-Japan strains. Lungs were harvested at 1, 7, 21, and 43 days post infection, homogenized in 5 mL PBS/0.01% Tween 80, and plated on 7H11 agar. Colonies were counted after ~3 weeks incubation at 37°C (4 mice per group per time point; mean ± SD; ***p<0.001; ns=not significant, ANOVA, Bonferroni post-test). Ming Li assisted with tissue collection.
Figure 4.4. Efficacy of rBCG-Japan vaccine candidates in a BALB/c mouse model.
Groups of mice were vaccinated with the recombinant BCG-Japan strains or PBS as a control. At 8-weeks post vaccination, mice were aerogenically challenged with ~350 CFU of \textit{M. tb} using a GlasCol nebulizer. Mice were sacrificed at the various time points to collect organs for tissue homogenization. Bacterial burden of \textit{M. tb} in the lung and spleen were determined by plating tissue homogenate on 7H11 agar (5 mice per group per time point; mean ± SEM; *p<0.05; **p<0.01; ***p<0.001; ANOVA, Bonferroni post-test). Ming Li assisted with tissue collection.
post-challenge but did not reach statistical significance due to larger variations. Taken together, these results suggest that overexpression of phoP during vaccination can reduce the growth of *M. tb* in the lungs and provide exciting evidence that this strategy could be used to improve the efficacy of BCG.

4.4 Discussion

The link between *phoP* and BCG immunogenicity was first proposed by our lab with the discovery that BCG-Prague, a BCG strain that is traditionally thought to be weakly immunogenic, is a natural *phoP* mutant. We proposed that the natural *phoP* mutation was responsible for the weak immunogenicity of BCG-Prague. Evidence to support this notion was provided by studies that showed increased transcription of known PhoP targets and enhanced IFNγ production with reintroduction of wildtype *phoP* into BCG-Prague (unpublished data).

Thus we hypothesized that *phoP* overexpression could be used as a strategy to improve BCG efficacy by boosting immunogenicity. Here I showed that the overexpression of *phoP* in BCG-Japan did indeed boost immunogenicity and improve protection against *M. tb*. Specifically, I found that overexpression of *phoP* increased the recall IFNγ immune response following PPD stimulation compared to the parental BCG-Japan strain, where a significant increase was seen with the overexpression of both *phoP* and *phoR*. However, the overexpression did not enhance the induction of other cytokines tested. Although rBCG-Japan-*phoP* induced less IFNγ than rBCG-Japan-*phoPR*, this strain was more protective against an *M. tb* challenge, suggesting that immunogenicity does not correlate with protection. This is consistent with the fact that IFNγ is necessary but not sufficient for protection against TB, and as such is an imperfect correlate of
In Chapter 3, I provided evidence to support the correlation between virulence and protection, where I showed that an isogenic PDIM/PGL mutant of BCG-Pasteur was less virulent in SCID mice and also less protective against *M. tb* infection (Figure 3.2 and Figure 3.4). Conversely, in this chapter, I found that overexpression of *phoP* in BCG-Japan increased virulence in SCID mice but also improved protection. This provides further empirical evidence to support the notion that BCG efficacy is related to its level of virulence. Thus, protection appears to be correlated with virulence and not immunogenicity.

Considering that the link between PhoP and virulence has been established by other groups, it is not surprising that overexpression of *phoP* in BCG-Japan increased virulence compared to the parental strain. I found that rBCG-Japan*phoP* was able to replicate more effectively in immunocompromised SCID mice. This is consistent with a previous study that showed that a *phoP* mutant of *M. tb* replicated poorly in bone-marrow-derived macrophages and was severely attenuated in mice (Perez, Samper *et al.* 2001). In addition, it was found that overexpression of *phoP* by an IS6110 insertion in the promoter region, was responsible for the hypervirulence of an *M. bovis* B strain that caused a nosocomial outbreak of multi-drug resistant TB in Spain (Rivero, Marquez *et al.* 2001; Soto, Menendez *et al.* 2004). Finally, whole genome sequencing of an attenuated strain of *M. tb*, H37Ra, identified a mutation in the DNA-binding region of *phoP* that partially contributes to the decreased virulence of this strain (Lee, Krause *et al.* 2008). Considering the superior safety profile of BCG-Japan in clinical studies, it is unlikely that the overexpression of *phoP* increased virulence to levels equal to or surpassing those of more virulent BCG strains, such as BCG-Pasteur. Indeed, I observed that SCID mice infected
with the BCG-Pasteur strains developed splenomegaly, an indication of infection, over the course of experiment, whereas mice infected with the BCG-Japan strains including rBCG-Japan\textit{phoP} did not (Figure 4.5). Furthermore, mice infected with rBCG-Japan\textit{phoP} still had much lower bacterial burden compared to mice infected with a similar dose of the BCG-Pasteur strains. For example, by day 43 post infection, the lung burden of rBCG-Japan\textit{phoP} (~$10^4$ CFU/lung) (Figure 4.4) was 1-3 logs lower than the burden of the BCG-Pasteur PDIM/PGL knockout (~$10^5$ CFU/lung) or the wildtype BCG-Pasteur (~$10^7$ CFU/lung) (Figure 3.2B). A comparison of growth between the recombinant BCG-Japan and more virulent BCG strains, such as BCG-Pasteur, would directly test the relative safety of these strains. Considering the link between BCG virulence and efficacy, the fact that the \textit{phoP} overexpression strains were still relatively attenuated may have influenced its potency.

Although promising, the increased protection with rBCG-Japan\textit{phoP} was modest showing only ~2-fold reduction in \textit{M. tb} burden compared to the parental BCG strain. This small improvement may be due to the fact that BCG-Japan was used as the parental backbone. BCG-Japan was chosen as the backbone for the recombinant BCG construction based on its superior safety record. However, the consequence of this is that it is inherently less protective than other, more virulent BCG strains. Indeed, in Chapter 3, I showed that BCG-Japan did not protect as well against \textit{M. tb} infection compared to BCG-Pasteur (Figure 3.4). Since the recombinant BCG-Japan work was done concurrently with the work described in Chapter 3, our choice of BCG was based on safety which we realize now is not necessarily appropriate. How this improved efficacy compares to other BCG strains, such as BCG-Pasteur is still in question. A direct comparison of these strains in the aerosol challenge model would clarify this. Nonetheless, the data described in this chapter provides additional evidence to support the notion that BCG virulence and protection are intimately related and suggests that \textit{phoP} overexpression is a promising novel strategy to
Figure 4.5. BCG-Pasteur strains induce splenomegaly over the course of infection. Spleen weight was measured prior to tissue homogenization from SCID mice intravenously infected with (A) BCG-Pasteur strains or (B) recombinant BCG-Japan strains. Four mice per group per time point (mean ± SEM).
improve efficacy. Future work aimed to improve BCG efficacy could use more inherently virulent BCG strains, such as BCG-Pasteur, to increase protection.
Chapter 5:

DISCUSSION AND FUTURE DIRECTIONS
5

5.1 Summary of Findings

The last decade has witnessed a boom in TB vaccine development with an unprecedented number of candidates entering clinical trials. However, this optimism has been met with disappointing results thus far with the leading candidate showing no protection against TB or *M. tb* infection (Tameris, Hatherill *et al.* 2013). Further, a criticism of the current recombinant BCG strategies is that the rational decision on choosing certain BCG strains for development has been overlooked. Prior to this work, our lab discovered a link between PDIM/PGL production and *phoP* expression with BCG safety and immunogenicity, respectively. Here I demonstrate that PDIMs/PGLs and PhoP play a role in virulence and potency of BCG and provides the first direct evidence to link these molecular factors to BCG vaccine properties.

To examine the role of PDIMs/PGLs, I generated isogenic PDIM/PGL mutant strains of BCG-Pasteur to directly examine the role of these lipids in BCG vaccine properties. Given the evidence to support the role of PDIMs/PGLs as virulence factors in *M. tb* (Camacho, Ensergueix *et al.* 1999; Cox, Chen *et al.* 1999; Camacho, Constant *et al.* 2001; Reed, Domenech *et al.* 2004; Rousseau, Winter *et al.* 2004), *M. bovis* (Hotter, Wards *et al.* 2005), and *M. marinum* described above (Yu, Tran *et al.* 2012), it was not surprising that I found that the loss of PDIMs/PGLs reduced the virulence of BCG-Pasteur in SCID mice. Although BCG virulence was affected, the loss of PDIMs/PGLs did not alter BCG immunogenicity as both parental and knockout strains produced similar levels if PPD-stimulated IFNγ from both CD4+ and CD8+ T cells. Interestingly, I found that vaccination with the PDIM/PGL-deficient BCG-Pasteur was less effective compared to the parental strain at reducing *M. tb* burden following an aerosol challenge.
To determine if PhoP affected BCG vaccine properties, I examined the overexpression of phoP in BCG-Japan. I found that overexpression of phoP or phoP/phoR enhanced the production of BCG-mediated IFNγ. Further, I found that rBCG-Japan,phoP replicated more rapidly in SCID mice compared to rBCG-Japan,phoPR and was more effective at reducing M. tb burden following an aerosol challenge.

5.2 Major Conclusions

Results from both my projects provide strong evidence that the virulence level of BCG is intimately related to its protective efficacy. Dubos and Pierce in 1956 hypothesized that a not-too-low residual virulence is an important attribute of BCG: “…there is no doubt that immunity is an expression of the ability of the BCG organisms to multiply in the body of the vaccinated individual. The immunity is dependable and lasting if the BCG culture is endowed with great invasiveness, whereas it is at best weak and transient if the BCG culture has become very attenuated” (Dubos and Pierce 1956). This led to the speculation that current available BCG strains might have been over-attenuated due to excessive passage in vitro and, consequently, lost efficacy. However, definitive evidence such as the direct implication of established virulence factors and/or virulence genes has been lacking. My work establishes that both PDIMs/PGLs and PhoP can mediate BCG virulence and protection (Figure 5.1). Based on previous work, PDIMs/PGLs may contribute to BCG virulence by providing cell wall integrity, resistance to RNI, and preventing phagosomal maturation. Like PDIMs/PGLs, PhoP is an established virulence factor for M. tb and may contribute to BCG virulence through the secretion of
Figure 5.1. PDIMs/PGLs and PhoP contribute to BCG virulence and protection. The presence of PDIMs/PGLs and the overexpression of \textit{phoP} in BCG increases virulence and protection against \textit{M. tb} challenge. The mechanisms by which these molecular factors exert their effects are proposed.
ESAT-6-like proteins. How these proteins modulate BCG virulence merits further investigation. Although PhoP controls the expression of antigenic proteins, my results suggest that the increase in IFNγ production with phoP overexpression does not correlate with improved protection. Thus, how PDIMs/PGLs and PhoP contribute to BCG-mediated protection is unclear but may be related to the increased persistence in the host and thus prolonged immune stimulation.

Taken together, results from both my projects provide evidence to support the over-attenuation theory and suggests that there needs to be a fine balance between these two factors in order to achieve the optimal protection while maintaining an acceptable level of safety. The only randomized trial study directly comparing two different BCG strains was carried out by comparing BCG-Pasteur and BCG-Glaxo, and found that BCG-Pasteur, administered at a lower dosage, provided a small (40%) but statistically significant greater protection against childhood forms of TB than BCG-Glaxo (Milstien and Gibson 1990). This is consistent with my finding, that is, BCG-Pasteur is more virulent than BCG-Glaxo, which is a natural PDIM/PGL-deficient mutant, and therefore more protective.

Consistent with my finding, one recent study examined the effect of the RD2 deletion, which is common to the “late” BCG strains, on BCG efficacy and found that the loss of RD2 did not affect protection against pulmonary M. tb infection, but was associated with increased M. tb dissemination (Kozak and Behr 2011). This coincided with the fact that the RD2-containing BCG strains disseminated more readily to the spleen. Additionally, deletion of RD2 from M. tb H37Rv attenuated virulence in mice (Kozak, Alexander et al. 2011). Together, this suggests that the loss of RD2 in BCG may also contribute to its attenuation and efficacy.
5.3 Implications of My Findings

My findings have important implications for national immunization programs and future recombinant BCG vaccine development. It is our belief that no one strain is superior and that a blanket strategy to cover all populations is unfitting. We propose that BCG vaccine administration and development needs to be tailored for the targeted population (Figure 5.2). For example, in HIV-endemic areas where HIV infection is a contraindication to BCG vaccination, safety is of primary concern. However, in a more immunocompetent population, a more virulent and thus more protective BCG strain could be well tolerated. TB is a leading cause of death in HIV-infected individuals living in Africa (WHO 2012). However, prophylactic treatment for this vulnerable group has been lacking since the WHO declared that HIV infection in infants was a contraindication for BCG vaccination (WHO 2007). This decision was based on an increase incidence of BCG disease that coincides with a change in vaccine strain in South Africa from BCG-Japan to BCG-Danish (Hesseling, Schaaf et al. 2003). Given the evidence I provide in this thesis to support the fact that different BCG strains are genetically and phenotypically distinct, this might prompt the WHO to reconsider using less virulent BCG strains for immunization. That is, instead of leaving this vulnerable population completely unprotected, return to vaccinating with BCG-Japan, a strain that is naturally deficient in PDIMs/PGLs and has a good history of being safe. Furthermore, novel vaccine candidates designed specifically for an immunocompromised population can use less virulent BCG strains as the backbone. Our initial strategy of choosing BCG-Japan as the parental strain to construct the phoP overexpression strain was based on its superior safety record. Although our rBCG-Japan,phoP strain showed a modest increase in protection, it provides promising results for a vaccine that may be appropriate for an immunocompromised population. Conversely, to accommodate an immunocompetent
Figure 5.2. Tailored vaccine development strategy.
Our proposed strategy is to develop new recombinant BCG vaccines that are suitable for the target population, such that less virulent BCG strains can be used in immunocompromised populations and more virulent BCG strains can be used in immunocompetent populations.
population that can tolerate more virulent BCG strain, future TB vaccine development targeted for this group may consider using more virulent BCG strains such as BCG-Pasteur as the parental backbone in order to maximize efficacy. Traditionally, BCG strains have been thought to possess ‘equivalent’ clinical vaccine properties. Thus, it is gratifying to see that the WHO recently recognized that the biological differences among BCG strains could indeed have an impact on their vaccine properties and recommended the rational selection of specific BCG strains for future TB vaccine development (Walker, Brennan et al. 2010). My findings provide additional insight into which BCG strain could be best selected for specific immunization programs.

My work also has important implications for current TB vaccine development using the attenuated *M. tb* strategy. For example, the leading attenuated *M. tb* candidate, *M. tb* SO2, was shown to be safer than BCG in preclinical testing (Martin, Williams et al. 2006). In order to satisfy the requirement for attenuated *M. tb* candidates to harbour at least two non-reverting independent mutations before entering clinical trials, an additional deletion in *fadD28*, which encodes a fatty acyl-AMP ligase involved in PDIM production, was generated (MTBVAC01) (Kamath, Fruth et al. 2005). Given that PDIMs are another established virulence factor in *M. tb*, it is possible that the deletion of both *phoP* and *fadD28* could greatly reduce virulence, and as my findings suggest, this could adversely affect efficacy, possibly to the point of impotence. The efficacy of the *phoP/fadD28* double mutant of *M. tb* is unknown as no preclinical protection data has been published.


5.4 Future Directions

My initial recombinant vaccine studies revealed that rBCG-Japan, *phoP* is a promising new candidate; however, the potency of this vaccine is still relatively weak exhibiting a ~2-fold reduction in *M. tb* burden compared to the parental strain. As such, future work should focus on strategies to improve efficacy and build upon the work presented in this thesis.

5.4.1 Increasing the Potency of BCG

In Chapter 3, I directly compared the efficacy of two BCG strains, BCG-Pasteur and BCG-Japan, to reduce *M. tb* burden following an aerosol challenge and found that vaccination with BCG-Pasteur was inherently more protective than BCG-Japan. As mentioned, BCG-Japan is naturally deficient in PDIMs/PGLs and has shown a good safety record compared to BCG-Pasteur in clinical use. This is consistent with our notion that virulence and protection are related properties of BCG. Given this observation, one way to increase the potency of new recombinant BCG candidates is to use more naturally protective (ie. virulent) BCG strains as the parental backbone. Based on the recombinant BCG data presented in Chapter 4, I predict that overexpression of *phoP* in a BCG-Pasteur background will further enhance BCG-mediated protection.

The potency of the recombinant BCG could also be enhanced by further boosting the overexpression of *phoP*. The recombinant strains tested in this work contained overexpression systems under the native promoter of *phoP*. Quantitative RT-PCR showed that overexpression of *phoP* was only ~2-3-fold compared to the parental strain. Thus, it is reasonable to speculate that greater protection can be achieved by further increasing the expression of *phoP*. This can be
done by expressing \textit{phoP} under a high-expression promoter, such as \textit{P}_{smyc}. This promoter has been shown to increase expression of target genes by 100-fold (Kaps, Ehrt \textit{et al.} 2001; Ehrt, Guo \textit{et al.} 2005). I have constructed this new high-expression \textit{phoP} plasmid and the generation of new recombinant BCG-Pasteur strains is currently underway by other members of the lab. The heightened overexpression of \textit{phoP} can be determined by qRT-PCR.

Similar to the \textit{phoP} overexpression strategy, another way to increase immunogenicity is by deletion of the global repressor, \textit{lsr2}. A previous PhD student in the lab, Blair Gordon, discovered that Lsr2 is a global regulator in mycobacteria that negatively regulates a number of genes, including the PE/PPE family of proteins in \textit{M. tuberculosis} (Gordon, Li \textit{et al.}). PE/PPE proteins are the major source of antigen variation and have been shown to be highly immunogenic, inducing strong T cell and B cell responses (Choudhary, Mukhopadhyay \textit{et al.} 2003; Chaitra, Shaila \textit{et al.} 2007; Narayana, Joshi \textit{et al.} 2007; Chaitra, Shaila \textit{et al.} 2008). Therefore, another strategy to increase the immunogenicity of BCG is to knockout \textit{lsr2} to increase the expression of multiple PE/PPE proteins. Although initial immunogenicity tests of an \textit{lsr2} knockout in BCG-Japan did not show a significant enhancement of IFN\textsubscript{\gamma} (Figure 5.3A), subsequent preliminary protection experiments by the technician in our lab, Ming Li, shows that the \textit{lsr2} deletion mutant of BCG-Japan exhibited improved protection compared to the parental strain (Figure 5.3B). This suggests that this is a promising strategy that can be exploited to improve BCG and should be examined in detail.

The potency of BCG is suggested to be related to its ability to persist in the host. Our lab previously identified a link between BCG growth and \textit{glnA1} expression that can potentially be used to improve the fitness of BCG. \textit{glnA1} encodes a glutamine synthetase (GS) that is involved in nitrogen metabolism, catalyzing the synthesis of glutamine and glutamate, which is important
Figure 5.3. **Testing of rBCG-Japan.Δlsr2.**

(A) **Deletion of lsr2 does not affect IFNγ production.** C57BL/6 mice were immunized subcutaneously with the parental control strain, the rBCG-Japan strains, or PBS/0.01% Tween 80 as a control. At 9 weeks post-vaccination, mice were sacrificed and splenocytes were harvested. Splenocytes were incubated with or without PPD for 24 hours followed by staining for T cell surface markers (CD3-Pacific Blue, CD4-PECy7, CD8-FITC) and intracellular IFNγ (IFNγ-PE). Samples were analyzed by BD FACSCalibur™ and FlowJo© Software (4-5 mice per group; mean).

(B) **Efficacy of rBCG-Japan.Δlsr2 in a BALB/c challenge model.** Groups of mice were vaccinated with the recombinant BCG-Japan strains or PBS as a control. At 8-weeks post vaccination, mice were aerogenically challenged with ~350 CFU of *M. tb* using a GlasCol nebulizer. Mice were sacrificed at 5 weeks post-challenge to collect organs for tissue homogenization. Bacterial burden of *M. tb* in the lung was determined by plating tissue homogenate on 7H11 agar (5 mice per group per time point; mean ± SEM). Data and graph courtesy of Ming Li.
for bacterial growth. A former PhD student in the lab, Jeffrey Chen, showed that BCG strains lack functional enzymes to degrade alanine and serine and the accumulation of these undegraded amino acids blocks GS activity and inhibits growth of BCG (Chen, Alexander et al. 2003). As such, we hypothesized that overexpression of *glnA1* would overcome this growth inhibition and lead to increased survival in the host and thus improve BCG efficacy. Initial immunogenicity studies of *glnA1* overexpression in BCG-Japan did not show a significant increase in IFNγ production compared to wildtype (Figure 5.4). However, given that studies including my own show that BCG immunogenicity does not always correlate with protection, this strategy merits further investigation in the aerosol challenge model.

These strategies are not necessarily mutually exclusive and can be combined to maximize protection. The construction of a BCG-Pasteur.*Δlsr2* mutant supplemented with the high-expression vector containing *phoP* is currently being made by other members of the lab. It will be interesting to see the outcome of these new recombinant BCG strains. Obviously, increasing the potency could result in the concurrent increase in virulence and this will need to be tested in SCID mice. Even if deemed “too virulent” for an immunocompromised population, these candidates could still have utility in a more tolerant, immunocompetent population, as discussed above.

### 5.4.2 How are PDIMs/PGLs contributing to BCG-mediated protection?

In Chapter 3, I showed that PDIMs/PGLs contribute to virulence and that this affects BCG efficacy. PDIMs/PGLs are established virulence factors that have been investigated in other pathogenic mycobacteria, such as *M. tb* and *M. marinum*. Although some molecular mechanisms
Figure 5.4. IFNγ production by rBCG-Japan.glnA1.
C57BL/6 mice were immunized subcutaneously with the parental control strain, the rBCG-Japan stains, or PBS/0.01% Tween 80 as a control. At 9 weeks post-vaccination, mice were sacrificed and splenocytes were harvested. Splenocytes were incubated with or without PPD for 24 hours followed by staining for T cell surface markers (CD3-Pacific Blue, CD4-PECy7, CD8-FITC) and intracellular IFNγ (IFNγ-PE). Samples were analyzed by BD FACSCalibur™ and FlowJo® Software (4-5 mice per group; mean).
of PDIM/PGL-mediated virulence have been elucidated for these organisms, little work has been done to characterize a PDIM/PGL mutant of BCG. I showed that the PDIM/PGL mutant of BCG-Pasteur did not replicate as well as wildtype in SCID mice. Given that the mutant displayed similar *in vitro* growth to wildtype (Figure 3.1C); it is likely that the growth defect is the consequence of a host-pathogen interaction. For example, PDIMs have been shown to facilitate phagocytosis by macrophages for *M. tb* (Astarie-Dequeker, Le Guyader *et al.* 2009). To determine if this is also true for BCG, uptake and replication in macrophages could be assessed using a bone-marrow-derived macrophage infection experiment. This would determine if the growth defect of the PDIM/PGL mutant I observed in SCID mice was due to rapid clearance by macrophages. Considering SCID mice are immunocompromised and thus lack an adaptive immune response (ie. T cell response), it is likely that the innate immune response is restricting growth. Further, macrophage killing is mediated through the production of reactive nitrogen intermediates (RNI) and PDIMs have been shown to protect *M. tb* against this cidal activity (Rousseau, Winter *et al.* 2004). To determine if PDIMs/PGLs play a similar role in BCG, intracellular growth of the wildtype and knockout mutant could be compared in macrophages in the presence of L-NAME, which inhibits the production of RNI. A more thorough characterization of the PDIM/PGL mutant of BCG may help us to better understand how these lipids affect BCG virulence and efficacy.

### 5.4.3 Will restoring PDIMs/PGLs in BCG-Japan improve efficacy?

In Chapter 3, I showed that the loss of PDIMs/PGLs in BCG-Pasteur decreases efficacy to levels similar to BCG-Japan, a BCG strain naturally deficient in these lipids (Figure 3.4). Thus, it stands to reason that reintroduction of PDIM/PGL production in BCG-Japan should increase its
efficacy to BCG-Pasteur levels. It was recently shown that the PDIM/PGL deficiency in BCG-Japan is caused by single base pair insertion in \textit{ppsA}, a polyketide synthase involved in PDIM/PGL biosynthesis, rendering it non-functional (Naka, Maeda et al. 2011). Further, this study showed that reintroduction of wildtype \textit{ppsA} restored PDIM/PGL production (Naka, Maeda et al. 2011). To determine if the re-introduction of these lipids restores efficacy, protection can be assessed using the BALB/c \textit{M. tb} aerosol challenge model we established. If the re-introduction of PDIMs/PGLs does not restore efficacy, this suggests that other mutations are present in BCG-Japan that also affect its effectiveness as a vaccine. This would be interesting considering that BCG-Japan is an “early” strain compared to BCG-Pasteur and should contain less genomic deletions.

5.4.4 A Comprehensive Comparison of BCG Strains

While it is recognized that the various BCG strains differ in their ability to induce a potent immune response and protect against \textit{M. tb}, conflicting data from animal and human studies has hindered the recommendation or favour of one BCG strain over another (as reviewed in (Ritz, Hanekom et al. 2008). Further, the BCG strains chosen in these studies was generally based on lineage or usage (Castillo-Rodal, Castanon-Arreola \textit{et al.} 2006; Horwitz, Harth \textit{et al.} 2009) and thus failed to correlate these differences in immunogenicity and efficacy with genetic differences between strains. Given the wealth of genomic data now available and the relative ease of whole-genome sequencing, it is possible to perform a comprehensive analysis of the BCG strains that exist to gain insight into the extent by which genetic differences influence BCG-specific immune response, virulence, and efficacy. To do this, I propose to first compare their ability to protect against \textit{M. tb} using the aerosol challenge model in BALB/c mice. Using a mouse model for this comprehensive analysis has the advantage of being relatively fast, simple, and inexpensive
compared to human studies. A comparison of virulence can be done in SCID mice to determine the ability of the BCG strains to replicate in target organs and overall survival. Although I did not find a correlation between protection and production of Th1 cytokines, it is possible that other immune effectors are involved that were not assessed in my work, such as the Th17 response. Thus, differences in immunogenicity can be determined initially using a whole blood assay coupled with a multiplex ELISA to examine a larger repertoire of cytokines. Alternatively, this could be done using cytokine staining and multi-parameter flow cytometry, which has the advantage of identifying specific cell populations. Using the genomic and phenotypic data obtained, BCG strains can potentially be grouped into various categories based on virulence, immunogenicity, and protection, which can then be linked to specific genetic traits (ie. production of PDIMs/PGLs). Furthermore, it would also be interesting to compare the immune response in the mouse model pre- and post-\textit{M. tb} challenge to examine the time and strength of the recall immune response, specifically examining the memory T cell response. This could possibly shed light to understand why a certain BCG strain confers greater protection than another.

A drawback to this study is that the mouse model is not necessarily reflective of human infection. Nevertheless, gaining more insight into the mechanisms of BCG-mediated protection will provide invaluable information in not only how to design more effective vaccines, but also may uncover biomarkers for protection, which is an urgent need in the field. This comprehensive analysis will also allow us to delineate BCG strains into useful categories and build a consensus on which strain is optimal for use.
5.5 Interesting Questions

5.5.1 Is it possible to develop a vaccine that prevents reactivation?

It is estimated that one third (~2 billion people) of the world’s population is latently infected with *M. tb*. This represents a huge reservoir for reactivation to active disease and transmission. An interesting phenomenon also occurs with immigrants latently infected with TB who reactivate after living in Canada for several years. This presents a major public and global health risk. One vaccine candidate, H56, has shown promising results in a post-exposure and latent model of TB in mice and macaques. This subunit vaccine candidate employs a multistage vaccination strategy and expresses early antigens, ESAT-6 and Ag85B, and latency-associated protein, Rv2660c (Aagaard, Hoang *et al.* 2011). Vaccination with H56 was shown to control reactivation post-*M. tb* infection and reduce bacterial burden in mice and macaques (Aagaard, Hoang *et al.* 2011; Lin, Dietrich *et al.* 2012). Interestingly, a previous PhD student in the lab, Blair Gordon, found that the global gene regulator, Lsr2, represses genes in *M. tb* that are involved in persistence. As such, the secretome of *M. tb Δlsr2* represents a potential reservoir for latency-associated antigens. To uncover these targets, the supernatant of the *M. tb Δlsr2* can be screened for proteins that protect mice against an *M. tb* aerosol challenge. These novel latency-associated antigens can then be developed into subunit vaccine candidates and tested for control of reactivation in a mouse model of latent TB.

5.5.2 Is it possible to develop a sterilizing BCG vaccine?

Unlike BCG, vaccines against other infectious diseases in current use are generally thought to induce sterilizing immunity that is antibody-mediated. A truly effective BCG vaccine would not only protect against TB but prevent *M. tb* from establishing an infection. Although cell-mediated
immunity has been proclaimed to be the primary mechanism of protection, there is mounting evidence to challenge the paradigm that humoral immunity plays little role in the protection against *M. tb* (Maglione and Chan 2009; Achkar and Casadevall 2013). Only one vaccine candidate, IKEPLUS, an *M. smegmatis* strain expressing the esx-3 locus from *M. tb*, has shown promising sterilizing immunity (Sweeney, Dao *et al.* 2011). This study found that vaccination with IKEPLUS led to a marked reduction in *M. tb* burden, which fell below the limit of detection in the liver of mice surviving for over 200 days. Although promising, this candidate again solely triggers the cell-mediated immune response. I propose that a BCG vaccine strain that carries antigens to trigger both cell-mediated and humoral immunity could work synergistically to prevent disease and clear infection. One such candidate could combine the recombinant work described above (e.g. rBCG-Pasteur, *phoP*) with over-production of lipoarabinomannan (LAM), a glycolipid that has shown to elicit a potent antibody response (Brown, Cruz *et al.* 2003; de Valliere, Abate *et al.* 2005). The overexpression of *manB* from *M. tb*, a phosphomannomutase involved in LAM biosynthesis, was shown to increase levels of LAM in *M. smegmatis* (McCarthy, Torrelles *et al.* 2005).

Although numerous TB vaccine candidates are in the pre-clinical and clinical pipeline, it is imperative to continue the search for new antigens and develop novel strategies to improve the BCG vaccine. In spite of the disappointing results from clinical trials thus far, even modest advances are valuable in the fight against TB. In fact, WHO surveillance data suggests that a 1% improvement in protective efficacy could save 18 000 lives and prevent 83 000 new *M. tb* infections annually (Corbett, Watt *et al.* 2003).
References


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