Genetic Factors Influencing BCG Vaccine Properties

by

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Abstract

Tuberculosis is a re-emerging global health problem. Bacille Calmette-Guerin (BCG), the available vaccine against the disease, is only effective short term and is associated with adverse reactions clinically. The development of new effective vaccines will require an understanding of virulence, immunogenic factors and the beneficial immune responses induced in the human host. My thesis investigates phoP and whiB3, two genes associated with virulence and immunogenicity in *Mycobacterium tuberculosis*. Study of PhoP in a natural phoP mutant, BCG-Prague, and in the clinically safe BCG-Japan, shows that over-expression of PhoP increases the immunogenicity of these vaccine strains. In addition, I found that WhiB3 impacts carbon metabolism in BCG-Birkhaug and BCG-Sweden, although the effect of this on virulence *in vivo* is still unclear. The characterization of genes involved in virulence and immunogenicity allows us to develop novel approaches for improving the efficacy of BCG, which has important implications for future TB vaccine development.
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List of Abbreviations

aa – amino acid
bp – base pair
BCG – Bacille Calmette Guérin
DAT – diacyltrehalose
ELISA – enzyme-linked immunosorbant assay
FACS – fluorescence activated cell sorting
HIV – Human Immunodeficiency Virus
ICS – intracellular cytokine staining
IFNγ – interferon gamma
i.v. – intravenous
M. tb – Mycobacterium tuberculosi
PAT – polyacyltrehalose
PDIM – phthiocerol dimycocerosates
PGL – phenolic glycolipids
PPD – purified protein derivative
RD1 – Region of Difference 1
s.c. – subcutaneous
SL – sulfolipid
TB – Tuberculosis
TLC – thin layer chromatography
WHO – World Health Organization
WT – wildtype
Chapter 1

General Introduction

1.1 The impact of Tuberculosis on human health

One third of the world’s population is infected with *Mycobacterium tuberculosis* (*M. tb*), the causative agent of Tuberculosis (TB) disease. Annually, *M. tb* infects 10 million new people and causes approximately 2 million deaths worldwide (9). These numbers justify its place in the top three global health problems, along with Human Immunodeficiency Virus (HIV) and malaria. Although TB is considered to be an old disease, it has re-emerged since the 1990s due to two main compounding factors: co-infection with HIV and the appearance of drug-resistant strains of *M. tb*. Not only do HIV-positive individuals have an increased susceptibility to *M. tb* infection, the two diseases synergize and exacerbate the effects of each other (61). In addition, the long course of antibiotics required for TB treatment foster patient non-compliance and increases the number of antibiotic-resistant strains. Both multi-drug resistant TB (MDR-TB) and extensively drug resistant TB (XDR-TB) are major challenges in current efforts to control TB since they are unresponsive to drug therapies (10). It is evident that chemotherapy is only a temporary solution and a more effective method of combating TB is through prevention with an effective vaccine.

1.2 Brief introduction of *Mycobacterium bovis* BCG and mechanisms of attenuation

Bacille Calmette-Guérin (BCG) is currently the only available vaccine against TB (9). It is the most extensively used vaccine with over 3 billion people immunized worldwide since its
inception and over 100 million doses administered annually since its inclusion in the World Health Organization (WHO) Expanded Program on Immunization in 1974 (8). This attenuated live vaccine was derived from a virulent strain of *Mycobacterium bovis* (*M. bovis*) in 1921, after 13 years of *in vitro* serial passaging by Albert Calmette and Camille Guérin. From 1924, vaccine strains were distributed to various countries for use in national vaccination programs. Due to lack of refrigeration at that time, BCG stocks were maintained by continued serial culturing every few weeks. However, each production laboratory developed independent propagation techniques for the vaccine, which allowed for a second phase of BCG attenuation. It was not until the WHO introduced the ‘seed lot’ system to initiate lyophilization of the strains in 1966 that the process of *in vitro* evolution stopped (WHO Expert Committee on Biological Standardization, 1966). With over 1000 passages from the original 1921 BCG strain, dozens of different sub-strains emerged that possessed both common and unique mutations (15). This was observed as early as the 1950s, when numerous vaccine producers noted the appearance of the sub-strains, each with distinct morphological, biochemical and immunogenic properties (49-52) (see Section 1.3 for details).

The molecular mechanisms behind BCG attenuation remain unclear. However, it has been shown that the Region of Difference 1 (RD1) genomic region plays a critical role. RD1 is essential for *M. tb* virulence (101) and is present in virulent *M. tb* and *M. bovis* strains but is absent in all BCG strains (15). However, this deletion only partially accounts for the attenuation of BCG since complementation of RD1 alone does not restore virulence to wildtype (WT) *M. tb* levels (15, 101). Furthermore, deletion of RD1 from *M. tb* does not attenuate the strain to the same degree as BCG (110). RD1 encodes a Type VII secretion system, ESX-1 (70, 122), which secretes early secretory antigenic 6 kDa (ESAT-6 (EsxA)) protein and culture filtrate protein 10 kDa (CFP-10 (EsxB)), both well-known antigens belonging to the ESAT-6 family (23, 59).
ESAT-6/CFP10 play a role in virulence by modulating macrophage infection (23, 59) and host immune response (121), highlighting the important roles of these proteins in *M. tb* survival and virulence in the host.

In addition to ESX-1, *M. tb* has four other Type VII secretion systems, one of which, ESX-5, has also shown to be important for virulence in *M. marinum*, a close relative to *M. tb* that causes TB-like disease in fish and amphibians (3, 4). Similar to ESX-1, ESX-5 secretes virulence-associated proteins, including members of the ESAT-6 family.

### 1.3 Variations in BCG clinical properties

#### 1.3.1 Variation in BCG protective efficacy

Clinical and case studies have reported a wide range of efficacy rates for BCG. While it has shown protective efficacy (>80%) against severe disseminated forms of TB in childhood, such as meningitis and miliary TB, it has limited effect against adult pulmonary TB, with efficacy varying from 0-80% (22, 33, 127). Another shortcoming of BCG is its ineffectiveness in those with previous mycobacteria exposure including environmental mycobacteria; established latent *M. tb* infection; or prior BCG vaccination. Previous mycobacteria exposure results in cross-reactive immune responses that prevent BCG from establishing persistence and having a protective effect (34, 45). BCG is therefore given at infancy to minimize the likelihood of cross-immune priming. However, since BCG provides immunity for a maximum of 10 to 20 years, adults lack effective protection (37, 73, 123). This loss of protection coincides with the increase in TB incidences seen in 25 to 35 year olds in Africa (10).
1.3.2 Variation in BCG immunogenicity and safety

BCG sub-strains also exhibit variable immunogenicity. The immunogenicity and potency of TB vaccines has been traditionally measured by tuberculin sensitivity produced by delayed type hypersensitivity to *M. tb* tuberculin, also known as purified protein derivative (PPD) (94). The size of the PPD-induced skin induration was indicative of the strength of a BCG-induced immune response and was used as a method for strain selection in national vaccination programs. The variation in BCG immunogenicity is exemplified in two studies comparing twelve BCG strains in guinea pigs and children, which showed that BCG-Prague consistently exhibited significantly lower tuberculin sensitivity (81, 130). Its weak immunogenicity was also supported by a later study which compared five BCG strains in BALB/C mice and identified BCG-Prague as one of the lowest immune response inducers (82).

Variation in BCG safety has also been demonstrated in vaccination programs worldwide (51, 52, 81, 88). Common adverse reactions to BCG in infants include suppurative lymphadenitis (inflammation of the lymph node) and osteitis (inflammation of the bone). Of the BCG strains that have been widely used, BCG-Pasteur, -Russia, -Sweden, and -Danish are tightly associated with vaccine complications whereas BCG-Japan, -Moreau, and -Glaxo are the least likely to induce adverse reactions (88). Examination of national immunization programs strengthens the link between specific BCG strains and reported complications. For example, the use of BCG-Sweden was associated with high numbers of osteitis cases in Finland. However, in 1978, BCG-Glaxo replaced BCG-Sweden and the cases of osteitis concomitantly dropped. Interestingly, BCG-Danish replaced BCG-Glaxo in 2002, and the incidence of lymphadenitis increased. Although this data is correlative, it suggests that there are true biological differences between the strains that affect safety and immunogenicity.
1.3.3 Genetic polymorphisms in BCG

The heterogeneity of BCG strains has been confirmed by recent molecular and genomic studies (14, 15, 24, 58). Comparative genomic analyses of BCG strains have uncovered extensive genetic polymorphisms including both deletions and duplications. These studies revealed lesions common to all BCG sub-strains and genomic differences exclusive to a particular sub-strain, showing that each BCG is genetically unique (14, 15). A molecular genealogy based on these studies has been established, which is in general agreement with the historical records of BCG dissemination. Some of the biochemical differences among BCG strains observed in early studies have now been confirmed and explained at the molecular level. However, the impact of the genetic and biochemical differences on BCG clinical properties such as safety, immunogenicity, and protective efficacy have not been established until recent studies from our laboratory (see Section 1.4 for details).

1.4 Factors that may impact BCG vaccine properties

Historically, the insufficient protection and variation in BCG safety have been attributed to differences in vaccine manufacturing and administration, discrepancies in trial methods, geographic variations among \textit{M. tb} strains, genetic and nutritional differences in trial populations, and exposure to environmental mycobacteria (13, 19, 36, 45, 54, 128). These hypotheses may not be mutually exclusive and may all contribute to the varying efficacy of BCG. However, recent studies from our laboratory show that these variations may be the result of genetic and molecular differences among the sub-strains. In particular, we found that specific molecular differences among the BCG sub-strains may have an effect on vaccine safety (30, 31).
In addition, our collaborators in China examined 13 different sub-strains of BCG by high resolution comparative genomic analysis. Subsequent data analysis and follow up experiments performed by myself and Vanessa Tran in our lab identified genetic polymorphisms in several well-established virulence genes of *M. tb* (Figure 1) (86), which likely have a major impact on the clinical properties of BCG including immunogenicity and protective efficacy. Understanding the role of these genes and how they contribute to BCG clinical properties is vital to the improvement of BCG.
Figure 1. Updated genealogy of BCG vaccine strains. Diagrammatic representation of the known genetic mutations gathered in BCG sub-strains from the original *M. bovis*. Comparative genomic analysis of 13 different BCG sub-strains by our laboratory revealed the mutations shaded in grey and red. Mutations highlighted in red are investigated further in this work: *phoP* knock out (KO) in BCG-Prague, *whiB3* deletion in BCG-Birkhaug and BCG-Sweden, and the IS6110 insertion in the promoter of *phoP* in BCG-Japan, -Russia, and -Moreau. Genealogy diagram was modified from a previous model (24).
1.4.1 PDIM/PGL and BCG safety

Previous studies from our laboratory established a novel link between the production of phthiocerol dimycocerosates (PDIM) and phenolic glycolipids (PGL) and the safety of BCG (31). PDIM and PGL are important cell wall lipids with established roles in \textit{M. tb} and \textit{M. bovis} virulence (41, 105). They are required for bacterial growth in the host (27, 41) and protection from reactive nitrogen species which are important for macrophage-mediated mycobacterial killing (108). PDIMs may also serve to mask immunogenic components due to their abundance in the cell wall, thereby altering immune recognition by the host (108). PDIMs are found in pathogenic mycobacteria, including \textit{M. tb}, \textit{M. bovis}, \textit{M. africanum}, \textit{M. leprae}, \textit{M. marinum}, \textit{M. ulcerans}, and \textit{M. kansasii} (42, 98). Although less is known about PGLs, their ability to down-regulate pro-inflammatory cytokines and their role in virulence has been characterized \textit{in vitro} and in animal models, respectively (105). Recent studies in our lab show that production of PDIMs and PGLs are abrogated in BCG-Japan, BCG-Moreau and BCG-Glaxo (31). The loss of these lipids may explain the decrease in virulence and low frequency of adverse reactions seen clinically by these strains. While the source of this variation is unclear in BCG-Japan and BCG-Glaxo, our lab identified a genetic deletion (\textit{ΔfadD26-ppsA}) in the PDIM/PGL biosynthetic locus of BCG-Moreau that likely accounts for its inability to produce these lipids (31, 86). Our finding supports the notion that differences among BCG sub-strains reflect genetic heterogeneity of the strains themselves.

1.4.2 \textit{phoP-phoR} polymorphisms

\textit{PhoPR} is one of 11 two-component systems in \textit{M. tb}. Two-component systems are conserved regulatory signal transduction systems often involved in bacterial virulence (46, 92). \textit{PhoR} is a membrane-associated sensor histidine kinase that is thought to phosphorylate \textit{PhoP}, the cytosolic
response regulator, in response to a currently unknown environmental stimulus (35). PhoP (Rv 0757) is a 247 aa, 27.5 kDa protein that transcriptionally controls over 100 genes in M. tb (133). Although there is no evidence yet that PhoP interacts with the promoters of these genes directly, PhoP is able to autoregulate itself by recognizing three 9-bp direct repeat motifs in the phoP promoter region (64) with a head-to-head binding orientation (71). However, whether PhoP requires N-terminal phosphorylation to function and whether it activates or represses its own transcription is still controversial (64, 72).

PhoP is involved in various aspects of mycobacterial virulence and host immune interaction. Without phoP, M. tb is able to survive but cannot grow in macrophages and in mice (100, 133). Furthermore, it was found that a mutation in phoP is partially responsible for the attenuation of avirulent M. tb H37Ra (85). In addition, the upregulation of PhoP was responsible for increasing the virulence of the clinical M. bovis B strain, resulting in a nosocomial outbreak of drug-resistant TB in Spain (107, 120). Interestingly, PhoP mutants of M. tb were found to be more attenuated than BCG-Pasteur, which has an intact phoP gene, highlighting the important role of phoP in virulence (90). PhoP has also been linked to immune modulation by controlling production of trehalose-based lipids (65, 93). This class of lipids include diacyltrehaloses (DAT), implicated in suppressing T cell proliferation in ex vivo studies (109) and sulfolipids (SL), which have a role in host immune modulation by altering cytokine secretion and protecting against reactive oxygen species in the macrophage (78).

Our more recent comparative genomic analysis has revealed extensive polymorphisms in the phoP-phoR locus among BCG strains. Given the important role of PhoP in M. tb, it is likely that polymorphisms in the phoP-phoR locus among BCG sub-strains may contribute to BCG variation. Of interest is a single nucleotide polymorphism in BCG-Prague, where a single
guanine (G) nucleotide insertion between bases 852067 and 852068 (M. tb genome coordinates) results in a frameshift mutation (Figure 2). This mutation abrogates the C-terminal end of PhoP (residues 154-247), affecting the DNA binding domain (71, 86, 114, 134). Other phoP polymorphisms include the insertion (IS) element, IS6110, upstream phoP in BCG-Japan, BCG-Russia and BCG-Moreau. This 1356 bp insertion is situated 18 bp from the phoP start codon at nucleotide 851593 (M. tb genome coordinates) in an inverse orientation to the phoP gene (86) (Figure 3). While this insertion has been described before (58), our study was the first to find the exact insertion site and orientation of IS6110. This insertion may affect the regulation of phoP and impact its important role in virulence. Taken together, the genetic polymorphisms at the phoP-phoR locus, particularly in phoP, will likely have a major impact on BCG clinical properties, which will be the major focus of my thesis study (see Chapter 3).
Figure 2. Schematic diagram of the PhoP frameshift mutation in BCG-Prague. A single guanine insertion (depicted by *) is inserted at nucleotide 852067 and 852068 causing a frameshift mutation, changing residues 154-247 of the DNA-binding domain of PhoP (residues 150-247).
Figure 3. IS6110 insertion into the phoP promoter in BCG-Japan, -Russia, and -Moreau. A schematic representation of the IS6110 insertion in the promoter of phoP (A). IS6110 is inserted 18 bp upstream of the phoP start codon in an inverse orientation. The nucleotide sequence surrounding the IS6110 insertion (B). IS6110 (boxed) is inserted between nucleotides 851592 and 851593 and is flanked by GAA direct repeats. Start codons of phoP and phoR are bolded.
1.4.3 *whiB3* polymorphisms

In addition to polymorphisms in *phoP*, we found that BCG-Sweden and BCG-Birkhaug have a disrupted *whiB3* gene containing a 110 bp deletion (3834822 to 3834932 *M. tb* genome coordinates) in the promoter and start site of *whiB3* (Figure 4). WhiB3 (Rv3416) of *M. tb* is a 102 aa, 11.6 kDa protein that has 52% similarity to the *S. coelicolor* *whiB3* on a DNA level (96). It belongs to a family of seven *whiB*-like genes in *M. tb* which are involved in fatty acid metabolism and pathogenesis (*whiB1*); cell division (*whiB2*); and antibiotic resistance (*whiB7*) (63, 95, 102, 124). WhiB3 is a putative transcription factor that monitors changes in the redox environment of the cell (113). It has been shown to bind to the principal sigma factor, RpoV, of *M. tb*, supporting its role as a transcriptional regulator (124). Like others in the *whiB* family, WhiB3 has four conserved cysteines coordinating an iron-sulfide [4Fe-4S] cluster, which are known to play essential roles in sensing external signals and intracellular redox states (67, 113, 119). This cluster likely signals *M. tb* dormancy in response to stress by interacting with fluctuating NO and O$_2$ levels, resulting in cluster disassembly and modulation of its DNA binding activity (112, 113). WhiB3 has also been shown to trigger dormancy when sensing carbon stress, likely through a similar mechanism since central metabolism affects the intracellular redox environment (17, 126). Interestingly, the WhiB3 ortholog in *S. coelicolor* has been shown to be involved in sporulation, a state reminiscent of *M. tb* dormancy (96, 135).

Although implicated in dormancy, the exact role of WhiB3 in *M. tb* virulence is unclear. For example, the loss of *whiB3* significantly attenuates *M. tb* in a mouse model of infection, but does not have any effect on bacterial replication *in vivo* (124). Although it is clear that WhiB3 is involved in *M. tb* virulence, the role of WhiB3 in BCG remains unknown. Furthermore, the
impact of the \textit{whiB3} polymorphism on the residual virulence of BCG-Birkhaug and BCG-Sweden is undetermined and is investigated in this study (see Chapter 4).

In previous studies comparing the virulence levels of 12 BCG strains, BCG-Sweden, a natural \textit{whiB3} mutant, was found to be more virulent (25, 26, 81) and has often been associated with increased reports of adverse reactions (40, 88). This association is further emphasized when national vaccination programs are evaluated. As described earlier, the cases of osteitis reported in Finland dropped with the switch from BCG-Sweden to BCG-Glaxo, a relatively more attenuated strain missing PDIM/PGLs (88). Although BCG-Birkhaug has been less studied, it is closely related to BCG-Sweden and is expected to have similar levels of virulence (25, 26, 80, 81). Taken together, I hypothesize that the absence of WhiB3-mediated transcriptional regulation may account for the increased virulence and adverse reactions seen in these strains.
Figure 4. Schematic diagram of the \textit{whiB3} deletion in BCG-Birkaug and BCG-Sweden. The promoter and the first 15 aa of the \textit{whiB3} gene are disrupted by a 110 bp deletion of bases 3834822-3834932 (\textit{M. tb} genome coordinates).
1.5 Current Vaccine Development Strategies: rBCG and subunit vaccines

New TB vaccines currently in development are designed to either replace or be used in combination with the existing BCG vaccine. Both approaches attempt to improve the protective efficacy currently afforded by BCG and vaccines from both categories are in various stages of clinical trials (117). The first strategy involves creating a recombinant BCG vaccine that over-expresses an antigenic protein to elicit a better immune response. An example of this is rBCG30, a BCG-Tice strain which over-expresses Antigen 85B (Rv1886c), a highly immunogenic secreted protein. In animal studies rBCG30 provided better protection when compared to its parental BCG strain (75-77). Immunological studies on rBCG30 showed greater induction of interferon gamma (IFNγ), an important surrogate marker often used as a measure of immune activation (91). IFNγ is an essential cytokine required for the activation of the Th1 T cell response that is important for clearing *M. tb* (56, 79). Greater numbers of both CD4+ and CD8+ T cells were also reported for rBCG30 (75), indicative of greater immunological induction. Both CD4+ and CD8+ T cells have been found to be vital in mounting an effective immune response against *M. tb* and are required for immune activation through the production of IFNγ and other cytokines (29, 55, 57). Although there is currently no biological marker for vaccine efficacy, IFNγ production by CD4+ and CD8+ T cells are commonly used as an important correlate of protection (39, 56, 57).

Another recombinant BCG candidate is rBCG::ΔureC-llo+, a urease deficient strain of BCG-Pasteur expressing listeriolysin (*hly*) from *Listeria monocytogenes*. The urease deletion inhibits phagosome de-acidification, allowing optimal pH for listeriolysin function. The expression of
listeriolysin damages the phagosome membrane in which BCG resides, thereby allowing mycobacterial entry into the cytosol (68). This mechanism allows for better CD8+ T cell antigen presentation in the cytosol (68), which aids in cell-mediated lysis of infected cells (55, 139). In addition, more recent studies have shown a correlation between CD8+ T cells with protective efficacy in both humans and mice, although the exact mechanism is unknown (118). In animal studies, rBCG::ΔureC-llo+ protects against M. tb infection better than the parental strain and was even safer in immunocompromised SCID mice (68). This vaccine entered phase I clinical trials in 2008.

Another method to developing a new live vaccine is the strategic attenuation of live M. tb. One such vaccine in development is SO2, a M. tb phoP mutant, which has not yet entered clinical trials (90). While this method retains many immunologically important genes and mimics the immune response induced by a natural infection, there are concerns regarding the safety and possible reversion of the strain to wild type. Studies with SO2 also show that protection in mice is not greater than that already offered by BCG (90).

The second approach to rational vaccine design is to create a subunit “booster” to be used in conjunction with BCG as a “prime”. Subunit vaccines, whether protein or DNA-based, present antigens to the host and boosts immunity through strong IFNγ induction (1). Some currently in development include Hybrid-1 (Ag85B-ESAT-6) and Mtb72f (PPE18-Rv0125), which are fusion proteins of immunogenic M. tb secreted antigens (20, 136). Although each protein subunit vaccine has completed phase I clinical trials as a standalone vaccine, they have not surpassed the protection of BCG (84, 97, 116). However, when used as a booster, Mtb72f has been shown to effectively increase the protection primed by BCG in monkeys (106).
DNA-based subunit vaccines using replication deficient vectors have also been produced in an attempt to stimulate CD8$^+$ T cell recognition. Two of these in development are MVA-85A, a vaccinia virus expressing Antigen 85A (132) and Aeras-402, an adenovirus expressing Antigen 85A, Antigen 85B, and EsxH (a protein of the ESAT-6 family) (89). When tested in monkeys, MVA-85A increased the protection of BCG whereas Aeras-402 boosted T cell responses. Both are currently in Phase I clinical trials. While very different approaches are used in rational vaccine design, they all attempt to compensate for the deficiencies of the current BCG strain.

1.6 Significance and Rationale

Although BCG is still in wide use today, it is not an ideal vaccine. Due to the growing number of *M. tb* infections and increasing incidences of TB disease, development of a new and more effective vaccine is vital. The ideal vaccine must provide both long term efficacy and guaranteed safety. This is of increased importance in countries with high incidences of HIV/AIDS due to the increased risk of adverse reactions. The threat of disseminated BCG disease in immunocompromised HIV+ children has become such a concern that the WHO amended its vaccination policy in 2007 to not immunize HIV+ infants (11), thus subjecting an already vulnerable population to increased risk of TB. However, it has been demonstrated that the risk of disseminated BCG disease and other adverse reactions is associated with only certain strains of BCG. For example, in South Africa, reported cases increased when the vaccine was switched from BCG-Japan (a less virulent strain) to BCG-Danish (a relatively more virulent strain) in 2000 (74). While there are many efforts to repair or replace the current BCG vaccine, our first
concern should be the investigation and selection of a suitable sub-strain for future vaccine development.

1.6.1 The role of PhoP in BCG immunogenicity

BCG-Prague has long been considered a weak vaccine due to its poor immune response in tuberculin tests, which serves as a crude measure for immunogenicity (81, 130). Up until recently, there has been no molecular explanation for this phenotype. Comparative sequence analysis from our lab has shown that unlike the other 12 BCG sub-strains examined, BCG-Prague is the only strain with a phoP mutation (86). PhoP positively regulates 44 genes in *M. tuberculosis* (133), including several well-established T cell antigens that may contribute to tuberculin reactivity. One of these, Antigen 85A is currently in clinical trials as a component of a booster subunit vaccine, highlighting the impact of these antigens on immunogenicity (137). Taken together, this suggests that the PhoP mutation is likely the reason for the loss of immunogenicity seen in BCG-Prague and would also explain the low tuberculin sensitivity seen in previous studies on children and guinea pigs (81, 130).

I hypothesize that PhoP function is directly related to BCG immunogenicity and that over-expression of *phoP* in a BCG strain will likely improve immunogenicity. I will first confirm this hypothesis by testing whether restoring PhoP function in BCG-Prague will improve the immune response elicited by this vaccine strain. I will then determine whether *phoP* over-expression in BCG-Japan increases the immunogenicity of this strain. BCG-Japan was chosen for this study because it is an ‘early’ BCG sub-strain containing fewer genomic deletions, which likely makes it naturally more immunogenic. This strain was also chosen because it has previously been established as a safe vaccine strain due to the lack of PDIM and PGLs. The relative safety of BCG-Japan has been demonstrated in HIV-endemic areas. In 2000, the replacement of BCG-
Japan with BCG-Danish was associated with an increased risk of disseminated BCG disease in HIV-infected children (74, 94).

1.6.2 The impact of WhiB3 on BCG virulence

Although the role of WhiB3 in M. tb remains unclear, it is associated with increased virulence (124) and effective host immunomodulation (112) during infection. WhiB3 also has a role in sensing and responding to NO, O2, and carbon stress, conditions often encountered by M. tb during the infection process. Since BCG-Sweden and BCG-Birkhaug are whiB3 mutants, I will examine the impact of this mutation on carbon metabolism and residual virulence in infected animals, which will help to clarify whether or not the whiB3 mutation has any effect on the clinical properties of these two strains.

Ultimately, the goal of this work is to contribute to the rational design of future TB vaccines that will either complement or replace the current BCG. I propose a novel strategy of repairing an already existing strain of BCG so that immunogenicity and overall efficacy is increased while virulence remains low. This vaccine will aim to be both more effective than the current BCG strains and will also be safe enough to use in HIV-endemic countries.
Chapter 2

Materials and Methods

2.1 Bacterial strains, plasmids and growth conditions

BCG- Prague, -Pasteur, -Japan, -Russia, and -Moreau were grown in Middlebrook 7H9 broth (BD Biosciences) supplemented with 10% ADC (bovine serum album [fraction V], dextrose, and catalase; BD Biosciences) and 0.05% Tween-80 (Sigma) or cultured on Middlebrook 7H11 agar supplemented with 10% OADC (oleic acid, bovine serum album [fraction V], dextrose, and catalase; BD Biosciences). Constructs were made using the Mycobacterium/E. coli shuttle vector pME, a 5.3 kbp plasmid containing a lacZα gene for Blue/White selection with an internal multiple cloning site and a kanamycin resistance cassette for antibiotic selection. Cloned genes were expressed using their native promoter. Mycobacteria strains containing the plasmid pME (Prague pME, Prague pME-phoP, Prague pME-phoP/R, Japan pME, Japan pME-phoP, Japan pME-phoP/R, Birkhaug pME, Birkhaug pME-whiB3, Sweden pME, and Sweden pME-whiB3), were cultured in the same manner as above but with the addition of 25 µg/ml kanamycin.

Plasmid manipulation and propagation was performed using Escherichia coli strain DH5α grown in Luria-Bertani broth with 50 µg/ml kanamycin.

2.2 Cloning of recombinant BCG

In order to complement the phoP mutation in BCG-Prague, a WT phoP gene was amplified from BCG-Pasteur genomic DNA. The 1028 bp product was obtained using forward primer phoP-F (5’- AAAAAAGGTACCGCTTTGTGGCCATGTCAC -3’) and reverse primer phoP-R (5’- AAAAAACTGCAGGCTGCGATCCGATATACTAC -3’) containing a KpnI and a PstI
restriction site (underlined), respectively. Using these restriction sites, the PCR product was cloned into pME, a shuttle vector containing a kanamycin resistance cassette, and was named pME-phoP. The cloned region spans 257 bp upstream of the phoP start site and the phoP gene.

To clone phoP and phoR, a 2501 bp product was amplified from BCG-Pasteur genomic DNA using forward primer phoPR-F (5′- AAAAAAGGTACCGGTCGCAATACCCACGAG-3′) and reverse primer phoPR-R (5′- AAAAACTGCAGCCTCAGTATTTCGGCTTTG-3′) containing a kpnI and pstI restriction site (underlined), respectively. The PCR fragment was then cloned into shuttle vector pME using kpnI and pstI sites and was named pME-phoP/R. The cloned region contains 177 bp upstream of the phoP start codon, both phoP and phoR, the intergenic region between the two genes and 78 bp downstream of the phoR stop codon.

The above constructs as well as the empty vector pME, were propagated in E. coli and then electroporated into BCG-Prague and BCG-Japan strains. Plasmid presence was confirmed by extracting the plasmid from the BCG strain, amplifying in E. coli, and checking for the presence of the intact gene by DNA sequencing (ACGT, Inc., Toronto).

2.3 Microarray analysis of Prague pME and Prague pME-phoP

RNA Isolation

BCG-Prague strains, Prague pME and Prague pME-phoP were grown in triplicate cultures to an OD600 between 0.4 and 0.6. Cells from 40 ml of each culture were collected by centrifugation at 4000 rpm for 10 minutes at room temperature (RT) using a Sorvall Heraeus 75006445 rotor. Cells were resuspended in 1 ml Washing Buffer (0.5% Tween 80, 0.8% NaCl, in RNase free water). The resuspended pellet was then divided into two 2 ml screw top, O-ring bead beating tubes, each containing 1 ml RNAprotect reagent (Qiagen). Tubes were vortexed for 5 seconds,
incubated for 5 minutes at RT, and then centrifuged at 5000 rcf for 10 minutes at RT. Bacterial pellets were stored at -80°C until ready to use.

Thawed pellets were resuspended with 400 µl freshly made Lysis Buffer (20 mM Na acetate, pH 5.5, 0.5% SDS, 1 mM EDTA, in RNase free water). To the tube, 0.8 g of 0.1 mm silica beads (BioSpec Products) and 1 ml acidified phenol:chloroform (5:1, pH 4.5) were added. Cell suspensions were mechanically disrupted in a mini-beadbeater (BioSpec Products) for 1 minute, followed by 1 minute on ice. This was repeated for 3 cycles. Tubes were then incubated at 65°C for 4 minutes and centrifuged at 13K rpm for 5 minutes at 4°C. The resulting aqueous layer was then transferred to a new 2 ml microcentrifuge tube containing 900 µl chloroform:isoamyl alcohol (24:1) placed on ice. To maximize the amount of extracted RNA, 300 µl Lysis Buffer was replaced in each of the bead beating tubes containing the remaining organic phase and mechanical lysis by bead beating, incubation and centrifugation were repeated two more times. Aqueous phases resulting from each round were combined in the same 2 ml tube containing chloroform: isoamyl alcohol mentioned previously. The tubes were then mixed by inversion and centrifuged at 13K rpm for 5 minutes at 4°C. The top aqueous layer was then transferred to a new 2 ml microcentrifuge tube containing 900 µl isopropanol and 90 µl 3M Na acetate, pH 5.5. Tubes were mixed gently by inversion and nucleic acids were allowed to precipitate overnight at -20°C.

The next day, tubes containing precipitated nucleic acids were centrifuged at 13K rpm for 10 minutes at 4°C. The supernatant was decanted and the pellet was washed with 1 ml 70% ethanol. After air drying the pellet for approximately 10 minutes, the pellet was resuspended in 90 µl RNase-free water and heat-dissolved for 10 minutes at 55°C.
Since both DNA and RNA were precipitated, the mixture was then treated with 5 units DNaseI (Fermentas) for 30 minutes at 37°C. DNase inactivation and RNA purification were then performed using an RNeasy purification kit (Qiagen) using the manufacturer’s RNA Cleanup Protocol. The resulting RNA was eluted from the column with 45 µl RNase-free water. A second round of DNA removal was executed using the Turbo DNA-free kit (Ambion) according to the manufacturer’s protocol. The concentration of the RNA was then quantified (NanoDrop ND-1000, Thermo Scientific) and visualized on a 1% agarose gel. Samples were stored at -80°C.

**cDNA Synthesis**

To perform microarray analyses on gene expression, RNA needed to be reverse transcribed into cDNA. Twenty five micrograms of previously extracted RNA was combined with: 25 mM Tris, pH 8.4, 37.5 mM KCl, 25 µg random nonomer primer and RNase-free water to a final volume of 50 µl. The mixture was then incubated for 5 minutes at 65°C and subsequently placed on ice. To this, 50 µl of a mix containing: 25 µM Tris, pH 8.4, 37.5 mM KCl, 3 mM MgCl₂, 20 mM dithiothreitol, 1.2 mM dATP, 1.2 mM dCTP, 1.2 mM dGTP, 0.4 mM dTTP, 0.4 mM 5-(3-Aminoallyl)-2’-Deoxyuridine-5’-Triphosphate (aadUTP), 400 units Superscript II Reverse Transcriptase (Invitrogen), in RNase-free water was added. Tubes were incubated at 42°C for 12 hours to allow reverse transcription into cDNA. Remaining RNA was hydrolyzed by adding 15 µl of 1M NaOH and incubating for 20 minutes at 65°C. The reaction was then neutralized by the addition of the same amount of HCl.

To clean up the reaction and remove excess Tris, the cDNA reaction was purified using QIAquick PCR Purification kit (Qiagen) according to the manufacturer’s enzymatic clean up protocol with minor modifications. Equal volumes of 70% ethanol were substituted for the two rounds of DNA washes. Complementary DNA was eluted by incubating 40 µl of 90°C water on
the column membrane for 1 minute followed by centrifugation at 13K for 1 minute. This was repeated, resulting in 80 µl. The final cDNA concentration was quantified (NanoDrop ND-1000, Thermo Scientific).

**Cy3 and Cy5 Coupling**

Two µg of cDNA was desiccated using a speed vacuum system (Savant DNA 120 SpeedVac Concentrator; Thermo Electron Corporation) and resuspended in 3.5 µl water. The reconstituted sample was then combined with the appropriate fluorescent dye. Cy3 and Cy5 Mono-reactive dye packs (GE Healthcare) were resuspended in 15 µl anhydrous DMSO and 30 µl of 2x Bicarbonate Buffer (230 mM NaHCO₃, 0.5% HCl in water). Subsequently, 3.5µl of the appropriate dye were added to the designated samples. Mixtures were vortexed, spun down briefly in the centrifuge, and then incubated for 60 minutes in the dark. To quench the reaction, 3.5 µl of 4M hydroxylamine was added and incubated for an additional 15 minutes in the dark.

To remove excess dye not conjugated to cDNA, a QIAnuick PCR cleanup kit (Qiagen) was used according to the manufacturer’s protocol with minor modifications. The volume of the cDNA labeling reaction was increased to 50 µl and diluted in double the volume of PB (Qiagen) specified by the manufacturer’s protocol. Resultant purified labeled cDNA was eluted twice in 20 µl EB (10 mM Tris-Cl, pH 8.5) and dried down in a speed vacuum system (Savant DNA 120 SpeedVac Concentrator; Thermo Electron Corporation).

**Hybridization to the Microarray**

Paired samples, one labeled with Cy3 dye and the other with Cy5, were reconstituted and combined with 2X GEx Hybridization Buffer HI-RPM (Agilent) and 10X Blocking Reagent (Agilent) to a final volume of 40 µl. Samples were heated at 95°C for 3 minutes and collected by
centrifugation. Forty microlitres of each sample was placed onto the microarray (Agilent) and hybridized at 65°C for 20 hours.

Before scanning, the microarrays were washed to remove excess cDNA in Wash Buffer 1 (900 mM NaCl, 60 mM NaH$_2$PO$_4$, 6 mM EDTA with 0.005% Sarcosine) by gentle agitation in the dark. Arrays were then washed again in Wash Buffer 2 (90 mM NaCl, 6 mM NaH$_2$PO$_4$, 600 µM EDTA) in the same manner as previous. Microarrays were scanned on a GenePix 4200A Professional microarray scanner (Molecular Devices) using GenePix Pro 6.1 software.

**Microarray Analysis**

Microarray intensities were corrected using Lowess normalization with R software version 1.9. Statistically significant genes were detected using Significance Analysis of Microarrays (SAM) 3.0 (Stanford University). Genes were considered to significantly different if they were within a false discovery rate (FDR) of 5% and showed at least 2-fold change in gene expression.

### 2.4 IFNγ production in Mice

**Inoculation**

Each strain (Prague pME, Prague pME-phoP, Prague pME-phoP/R, Japan pME, Japan pME-phoP, and Japan pME-phoP/R) was grown to an OD$_{600}$ of 0.4-0.7. Cultures were centrifuged at 4000 rpm for 10 minutes at RT and washed once with PBS/0.01% Tween 80. After centrifuging again, the pellet was resuspended in PBS/0.01% Tween 80 and passed through a 25 gauge needle (BD Biosciences) to break up clumps. This was followed by a low speed spin at 1500 rpm for 10 minutes to pellet down larger bacterial clumps. The resulting cell suspension was adjusted to an OD$_{600}$ of 0.4 representing 6.7 x 10$^8$ cells/ml. Cells were plated to confirm this number.
**Spleenocyte Isolation**

Four female C57BL/6 mice (Charles River Laboratories) were inoculated subcutaneously (s.c.) with approximately $1.3 \times 10^8$ cfu in 0.2 ml PBS/0.01% Tween 80 per group. A PBS/0.01% Tween 80 alone group was included as a negative control. Mice were euthanized by CO$_2$ inhalation 8 weeks post-inoculation. Spleens were harvested and spleenocytes were isolated. In brief, spleens were ground between two frosted microscope slides into RPMI media (Invitrogen) and filtered through a 70 µm cell strainer (BD Biosciences). Resultant cell suspensions were then centrifuged at 1000 rpm for 10 minutes at 4°C. The supernatant was decanted and red blood cells were lysed by resuspending the pellet in 1 ml ACK Lysis Buffer (0.15 M NH$_4$Cl, 10 mM KHCO$_3$, 0.1 mM Na$_2$EDTA, pH 7.2). After 2 minutes at RT, the reaction was quenched by adding 30 ml of RPMI and filtered again through a 70 µm cell strainer (BD Biosciences). Samples were then centrifuged at 1000 rpm for 10 minutes at 4°C. Media was again removed and the cell pellet resuspended in 2 ml complete RPMI (cRPMI: RPMI, 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin). Cells were then quantified using a haemocytometer.

**Spleenocyte Activation**

Spleenocytes were seeded in a round-bottomed 96-well plate (Falcon) at a concentration of $5 \times 10^5$ cells/well in 200 µl. To stimulate spleenocytes, 50 µl of purified protein derivative (PPD) (Statens Serum Institute, Denmark) was added to reach a 10 µg/ml final concentration in each well. For non-stimulated wells, 50 µl of cRPMI were added instead. Five wells were seeded per mouse spleen for each condition. As control, media alone was cultured with and without PPD stimulation. Spleenocytes were incubated at 37°C, 5% CO$_2$ for 72 hours.
After 72 hours, cell suspensions for each condition were pooled into a microcentrifuge tube. To obtain cell free supernatant, tubes were spun for 5 minutes at 5K rpm, 10K rpm, and 13K rpm, transferring the media to a new tube after each spin, taking care to avoid the cell pellet. Since extracellular IFNγ release was being measured in this assay, removal of other IFNγ sources that would skew the results was important. Starting with a low centrifuge speed ensured that spleenocytes were not lysed, which would release intracellular IFNγ, and higher speeds ensured all cells were pelleted away from the supernatant. Supernatants were stored at -80°C.

**IFNγ ELISA**

Enzyme-linked immunosorbant assays (ELISAs) for IFNγ were conducted on mouse spleenocytes to determine the extracellular IFNγ released by the activated cells. The OptEIA Mouse IFNγ ELISA set (BD Biosciences) was used to assay IFNγ levels according to the manufacturer’s protocol. In brief, 96-well, flat-bottomed plates (Nunc MaxiSorp) were coated overnight with capture antibody diluted 1:250 in Coating Buffer (0.1 M Sodium carbonate, pH 9.5). Wells were washed 5 times with 300 µl Wash Buffer (PBS with 0.05% Tween 20). Plates were then blocked with 200 µl Assay Diluent (PBS, 10% FBS, pH 7.0) for 1 hour in RT. Following this, 5 rounds of washes were performed with Wash Buffer to remove excess blocking reagent. Then, 100 µl of each sample in Assay Diluent/0.05% Tween 20 were plated in triplicate. Standards ranging from 0 pg/ml to 2000 pg/ml were plated in duplicate. After a 2 hour RT incubation, the plate was washed 5 times with Wash Buffer and 100 µl of Working Detector (detection antibody diluted 1:250 and streptavidin-horseradish peroxidase conjugate diluted 1:250 in Assay diluents) was added. The plate was then again incubated for 1 hour in RT. Wells were washed 10 times with Wash Buffer to rid of any unbound substrates. To develop, 100µl of tetramethylbenzidine (TMB) and hydrogen peroxide was added and incubated
for 30 minutes in the dark. To stop the reaction, 50 ml of Stop Solution (1 M H₃PO₄) was added and the absorbance at 450 nm was measured on a microplate reader (TECAN infinite M200). IFNγ levels were calculated based on a standard curve generated by the standards. Controls including unstimulated media, stimulated media and blank wells were also included to detect background levels and to ensure the absence of any non-specific binding or reactions with the developing substrates. Blank wells containing no capture antibody were blocked, and had either a) detecting antibody, HRP and TMB substrate added, b) HRP and TMB only, or c) detection antibody and TMB only. These combinations were done in order to ensure that detection substrates did not produce a false positive result. T-tests were performed using GraphPad Prism version 5.00 for Windows.

**Intracellular Cytokine Staining (ICS) and Fluorescence Activated Cell Sorting (FACS)**

Spleenocytes were seeded in a round-bottomed 96-well plate (Falcon) at 2 x 10⁶ cells/well in 100 μl. To stimulate cells, 50 ml PPD was added to a final concentration of 12.5 μg/ml. The unstimulated cells received the same volume in media. Extra wells for colour controls were also included and stimulated. Spleenocytes were incubated at 37°C in 5% CO₂. After 19 hours of stimulation, GolgiPlug (BD Biosciences) was added in a 1:1000 final dilution to each well and incubated for an additional 5 hours.

After a total of 24hrs stimulation, plates were gently 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) to remove FBS, which may bind non-specifically to antibodies. After washing, the cell pellet was 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 extracellularly stained with fluorophore conjugated antibodies: FITC-CD4, PE-CD3, PerCy5.5-CD8a (BD Biosciences) diluted in FACS Buffer. The antibody against the CD3 receptor was used as a marker to identify T cells. Antibodies to CD4 and CD8 were used to identify those specific populations among the T cells. The antibodies were incubated with the cells for 30 minutes on ice in the dark. Incubations were then stopped with the addition of 150 μl FACS Buffer and then centrifuged at 1400 rpm for 5 minutes at 4°C.

After removal of the supernatant, cells were permeabilized and fixed in preparation for intracellular cytokine staining by adding 1X CytoFix/CytoPerm (BD Biosciences) to each well. Reactions were incubated for 20 minutes on ice in the dark, after which plates were centrifuged as previous and supernatant was removed. Cells were then washed with 1X PermWash (BD Biosciences) and centrifuged again. Fluorophore conjugated antibody for intracellular IFNγ, APC-IFNγ (BD Biosciences) diluted in PermWash was added and incubated for 30 minutes on ice in the dark. Cells were again centrifuged as above and were resuspended in 200 μl FACS Buffer.

FACS samples were passed through a nylon filter before analysis. FACS analysis was performed on a FACS Calibur (BD) and data analysis was performed using FlowJo V7.6 by Vanessa Tran.

### 2.5 Virulence studies in mice

Virulence studies were conducted using *phoP* strains (Prague pME, Prague pME-phoP/R, Japan pME, and Japan pME-phoP/R) and *whiB3* strains (Birkhaug pME, Birkhaug pME-whiB3, Sweden pME, and Sweden pME-whiB3) grown to an OD_{600} of 0.4-0.7. Cultures were then pelleted by centrifuging at 4000 rpm for 10 minutes at RT and washed once with sterile
PBS/0.01% Tween 80. Once resuspended in PBS/0.01% Tween 80, cells were adjusted until they were at OD$_{600}$ 0.4, which was approximately $6.7 \times 10^8$ cells/ml. Inoculants were each plated to confirm estimated bacterial numbers.

Six week old, male C57BL/6 mice (Charles River Laboratories Inc.) were inoculated intravenously (i.v.) with approximately $1.3 \times 10^8$ cfu in 0.2 ml PBS/0.01% Tween 80 of the appropriate BCG strain. Four mice were included in each group. PBS/0.01% Tween 80 alone was also included as a negative control group. At days 1, 14, 21, and 42 after injection (Day 21 timepoint was omitted in WhiB3 studies), mice were sacrificed by CO$_2$ asphyxiation. The lungs, liver, and spleen were harvested and homogenized. Homogenates were then serially diluted and plated onto Middlebrook 7H11 plates containing 10% OADC and 25 µg/ml kanamycin. Colony forming units (CFUs) were counted 3 weeks later. Bacterial counts from organ homogenates harvested 24 hours after inoculation (Day 1 time point) were used as an indicator of initial infection titre.

### 2.6 2-D Lipid Analysis of PDIMs and PGLs

**Extraction of Apolar Lipids**

BCG strains used for phoP related investigations (Prague, Prague pME, Prague pME-phoP, Prague pME-phoP/R, Japan, Japan pME, Japan pME-phoP, Japan pME-phoP/R) were grown to mid-log phase. Each culture was pelleted by centrifuging at 3500 rpm for 20 minutes at RT and was stored at -20ºC. Pellets were then lyophilized (Lyph-Lock 10; Labconco) for 24 hours and dried cells were stored at -20ºC until ready to use.

Fifty mg of dried pellet from each strain was extracted with 2 ml methanol-0.3% NaCl (100:10 v/v) and 1 ml petroleum ether for 15 minutes on a rotator at RT. Samples were centrifuged at
2600 rpm for 8 minutes at RT to separate the aqueous and organic phases. After separation, the top petroleum ether phase was transferred to a new 3.7 ml glass tube (Fisher Scientific). The remaining cell pellet was re-extracted with the addition of 1 ml petroleum ether in the same manner as described above. Both petroleum ether layers were collected in the same tube and evaporated using a Reacti-Therm Heat/Stirring Module (Pierce).

2-D Apolar Lipid Analysis

Ten mg of apolar lipids resuspended in dichloromethane were spotted onto a Silicon 60 TLC plate (Whatman). For PDIM analysis, plates were separated in the first dimension with petroleum ether: ethyl acetate (98:2 v/v) three times. Petroleum ether: acetone (98:2 v/v) was used to develop in the second dimension. Plates were visualized with 5% molybdophosphoric acid (5% phosphomolybdic acid in ethanol), followed by charring. For PGL analysis, plates were developed in the first dimension with chloroform: methanol (96:4 v/v) and in the second dimension with toluene: acetone (80:20 v/v). Plates were visualized with α-naphthol (9.75% α-naphthol, 10.5% sulphuric acid, 83% ethanol) followed by charring.

2.7 Lipid analysis for DATs, PATs, and SLs

Extraction of Lipids

*phoP* strains (BCG Pasteur pME, Prague, Prague pME, Prague pME-phoP, and Prague pME-phoP/R) were each grown to mid-log phase. Cultures were then pelleted by centrifuging at 3500 rpm for 20 minutes at RT and stored at -20°C. Pellets were lyophilized (Lyph-Lock 10; Labconco) for 24 hours and stored at -20°C.
Lipids from 50 mg dried bacteria were extracted with 2 ml chloroform:methanol (1:2 v/v) for 20 minutes. Tubes were then centrifuged at 2600 rpm for 8 minutes at RT and the liquid was transferred to a separate tube. Lipids were re-extracted using 2 ml chloroform:methanol (2:1 v/v) following the same procedure. Liquid containing the extracted lipids were combined and evaporated at 37°C using a Reacti-Therm Heat/Stirring Module (Pierce).

_Lipid Analysis_

Ten mg of lipids resuspended in dichloromethane were spotted onto Silica Gel 60 TLC plates (Whatman). To develop DATs, PATs, and SLs, chloroform: methanol: water (60:16:2 v/v) was used. To develop PATs alone, petroleum ether: acetone (92:8) was used. Lipids in both cases were visualized by dipping in 5% molydophosphoric acid (5% phosphomolydic acid in 95% ethanol).

_2.8 qRT-PCR of phoP in IS6110 strains_

_RNA Isolation_

BCG-Pasteur, -Japan, -Russia, and –Moreau were grown to an OD$_{600}$ of 0.4-0.6. Subsequent RNA isolation followed the same protocol as stated above for the microarrays.

_cdDNA Synthesis_

RNA was reverse transcribed into cDNA in the same manner as stated above for the microarrays. However, reactions contained only 15 µg RNA and dNTPs used in the reaction were as follows: 0.6 mM dATP, 0.6 mM dCTP, 0.6 mM dGTP, and 0.6 mM dTTP (Fermentas). Negative control reactions were performed in parallel without the addition of enzyme. Resultant cDNA was quantified (NanoDrop ND-1000, Thermo Scientific) and visualized on a 1% agarose gel.
Quantitive PCR (qPCR)

Total cDNA for each BCG strain was diluted to a concentration of 20 ng/µl. A standard curve was generated by designating the 20 ng/µl aliquot as the 100 copy sample and doing 10-fold dilutions. The 20 ng/µl solution was then diluted 1:10 and used as the unknown sample to be quantified. The 15 µl qPCR reactions were done on a 96-well plate (twintec PCR plate, Eppendorf) with each well containing 2X SYBR Green Jumpstart Taq Ready Mix (Sigma), 0.5uM of respective forward primer, 0.5uM of respective reverse primer, and 4.5 µl of the appropriate cDNA sample. Primers used for the detection of phoP were forward primer phoP-F (5’- TATCGGCGAACGTCAGTCGAACAT -3’) and reverse primer phoP-R (5’- TATCGGCGAACGTCAGTCGAACAT -3’). Sigma factor A (sigA), a housekeeping gene, was used for normalization and was detected using forward primer sigA-F (5’- TCGAGGTGATCAACAAGCTG -3’) and reverse primer sigA-R (5’- TGGATCTCCAGCACCTTCTC -3’). Cycling conditions were as follows: 95ºC for 2 min, and then 40 cycles of 95ºC for 15 seconds, 55ºC for 15 seconds, and 68ºC for 20 seconds. After cycling was completed, a melting curve was performed where PCR products were heated with increasing temperature over 20 minutes from 60ºC to 95ºC. This process detected when double stranded PCR products melted to confirm that each reaction contained only one type of PCR product.

Standards and unknown samples were done in triplicate. A negative control for each set of primers was included for each BCG strain tested. The negative control sample consisted of the product from the negative cDNA synthesis reaction previously done without reverse transcriptase. Quantitive PCRs was performed in a Mastercycler ep realplex real-time
thermocycler (Eppendorf) using Realplex 4.4 software. T-tests were performed using GraphPad Prism version 5.00 for Windows.

2.9 **WhiB3 plate assays**

WhiB3 plate assays were conducted according to Singh et. al. (113). Briefly, Birkhaug pME, Birkhaug pME-whiB3, Sweden pME, and Sweden pME-whiB3 were grown to mid-log phase in 7H9 broth and normalized to an OD$_{600}$~1.0. Twenty µl of each strain were spot-plated onto each plate and kept at 37ºC for 3 weeks. Plates were comprised of Dubos media (glycerol omitted) with 1.5% agar and an added carbon source. Dubos agar was supplemented with no additional carbon source (basal) or 50 mM glucose, 50 mM succinate, 50 mM fumarate, 50 mM pyruvate, 50 mM citrate, or 25 mM acetate.
Chapter 3

Role of PhoP in BCG Immunogenicity

Introduction

Compared to other BCG sub-strains, BCG-Prague is considered a weak vaccine due to its low PPD reactivity, which is used as a measure of immunogenicity (81, 130). However, the mechanism behind this was unclear until recent analysis by our lab revealed that BCG-Prague contains a mutation within the phoP gene, a defect not present in any of the other 12 strains examined (86). This single guanine (G) insertion caused a frame shift mutation affecting the majority of the C-terminal DNA-binding domain, likely abrogating PhoP function. PhoP is responsible for transcriptionally upregulating 44 genes in M. tb, some of which are well established immunogenic antigens (133). phoP mutants of M. tb were previously shown to be attenuated, including the avirulent H37Ra strain which also has a mutation in the DNA binding domain of the protein (85). Taken together, I hypothesize that the non-functional PhoP in BCG-Prague is unable to induce immunogenic T cell antigens and thus is responsible for the weak immune response elicited by this strain.

To test this hypothesis, I complemented the natural phoP defect in BCG-Prague with either the WT phoP gene alone or WT phoP and phoR on an external plasmid. Using these strains I determined by microarray whether PhoP regulated known immunogenic proteins and confirmed that the upregulation of these antigenic proteins induced a stronger immunogenic reaction in a mouse model of infection. I also tested the residual virulence of the strains to ensure that the addition of PhoP did not inadvertently increase virulence. In addition, the importance of PhoP
was demonstrated by assessing the effect of an IS6110 insertion on phoP expression in BCG-Russia, BCG-Japan, and BCG-Moreau and associating this to their clinical vaccine properties.

Results

3.1 PhoP regulates genes associated with BCG Immunogenicity

As the first step to confirm my hypothesis, I constructed a recombinant BCG-Prague containing a WT phoP gene on a plasmid (named Prague pME-phoP) and performed microarray analysis. The WT phoP gene was cloned from BCG-Pasteur, which previous NimbleGen analysis had shown to contain no mutations in the phoP-phoR locus (86).

My microarray analysis reveals that out of approximately 4000 genes in the BCG genome, 67 were upregulated by PhoP by at least 2-fold, some of which encode proteins associated with host immune induction. In addition, six genes were downregulated by PhoP. These genes along with their genomic loci and functions are listed in Table 1. One particular gene of interest is fbpA, which encodes Antigen 85A, a highly antigenic secreted protein (16, 99). Antigen 85A belongs to a complex of proteins including Antigen 85B and 85C. Together they are the main constituents of the proteins secreted by mycobacteria (60) and are also bound to the cell surface (104). While the exact role of the Antigen 85 complex is still unknown, it may be involved in attachment and uptake of the bacilli into host cells (138). Antigen 85A is also a well established T cell antigen. Host antibodies against Antigen 85A block bacterial attachment and prevent mycobacterial infection of host cells. Consistent with my finding, Antigen 85A was also upregulated by PhoP in an expression profile study done by Walters, et al., which compared M. tb H37Rv and an isogenic phoP mutant (133). These results suggest that Antigen 85A is an important immunogenic factor across mycobacterial species.
Another group of upregulated genes encode ESAT-6-like proteins: EsxJ (Rv1038c), EsxK (Rv1197), EsxL (Rv1198), EsxM (Rv 1792), EsxN (Rv1793), EsxO (Rv 2346c), EsxP (Rv2347c), EsxV (Rv3619c), and EsxW (Rv 3620c). These are nine out of ten known ESAT-6-like proteins secreted by the ESX-5 secretion system, which has been linked to virulence and immunomodulation in *M. marinum* (3, 5, 111). The remaining ESX-5 secreted protein, EsxI (Rv1037c), was upregulated but did not pass the 2-fold cut off (data not shown). Furthermore, other ESAT-6-like proteins secreted by other ESX systems were not present, indicating that this upregulation was specific for the ESX-5 secretion system. ESX-5 is one of five type VII secretion systems in *M. tb*, which are specialized secretion systems found in actinomycetes. The most well characterized member of this family is ESX-1, which secretes the important virulence factors ESAT-6 (EsxA) and CFP-10 (EsxB) in *M. tb*. Interestingly, other than ESX-1, ESX-5 is the only other ESX system with an associated role in virulence (2). It also facilitates cell-to-cell spread of *M. marinum* in infected macrophages, a function shared by ESX-1 (4). However, ESX-5 does not complement ESX-1 deletion, which suggests that they have distinct roles in virulence (2). The parallels between the ESX-1 and ESX-5 secretion systems, namely the secretion of the ESAT-6-like and PE/PPE proteins, suggest that ESX-5 may have a similar role in virulence and immunogenicity as ESX-1.

In addition to the ESAT-6-like proteins, expression of genes encoding PE25, PE_PGRS35, PPE19, PPE41, and PPE 60 were increased. These proteins belong to the PE/PPE family of proteins, which are characterized by N-terminal proline-glutamic acid (PE) and proline-proline-glutamic acid (PPE) motifs. Although the exact role of these proteins is unknown, they have been implicated in virulence (87, 103), immune evasion (21, 44), and are a source of mycobacterial antigenic variation (12, 21). PE/PPE proteins are unique to mycobacteria and are highly expanded in pathogenic strains, accounting for about 9% of the coding genome of *M. tb*
Consistent with my results, PPE19 and PPE60 were also significantly upregulated by PhoP in the aforementioned *M. tb* study by Walters et al. (133). Interestingly, PPE41 is secreted by ESX-5 (4), further supporting the link between PhoP and ESX-5 secretion.

Taken together, I have shown that PhoP positively regulates the expression of known T cell antigens in BCG-Prague. Although the majority of the PhoP-regulated genes from my microarray analysis were distinct from the results obtained in *M. tb* by Walters et al. (133), this large discrepancy was not unexpected. The study of BCG is often complicated by the fact that the strains harbour many unidentified and uncharacterized mutations that may indirectly affect the expression of genes. This genetic heterogeneity emphasizes the importance of conducting studies in specific BCG strains since studies done in other mycobacteria and even other BCG strains are not always representative.
Table 1. Comparison of Prague pME and Prague pMe-phoP by microarray.*

**Genes upregulated by PhoP in BCG-Prague**

<table>
<thead>
<tr>
<th>Gene #</th>
<th>Gene</th>
<th>Function</th>
<th>Mean Fold</th>
<th>q-value (%)</th>
<th>Classification</th>
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<td>ppiA</td>
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<td>4.783</td>
<td>information pathways</td>
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### Genes repressed by PhoP in BCG-Prague

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The transcription profiles of Prague pME (WT) and the complemented strain Prague pME-phoP were compared to determine genes under the control of PhoP. Strains were grown in Middlebrook 7H9 media (supplemented with 10% ADC, 0.05% Tween 80) and were harvested at an OD600 nm between 0.4-0.6. Three independent microarrays (Agilent) were performed using different biological RNA samples for each. Microarrays were normalized using Lowess normalization. Genes with 2-fold or greater changes compared to WT and with a q-value ≤ 5% (% chance of being a false positive), as determined by SAM statistical analysis, were included in this list. Genes are listed in the order they occur in the BCG genome along with the average fold change, q-value, and their annotation according to the *M. tb* genome on TUBERCULIST (Pasteur Institute). There are 67 genes upregulated and 6 downregulated by PhoP. In blue: genes that are ESAT-6-like proteins belonging to ESX-5 secretion system (9 of 10 known). In green: genes that are part of the PDIM/PGL synthesis locus.
3.2 Recombinant BCG-Prague expressing PhoP induces an elevated immune response

Since PhoP regulates antigenic proteins in BCG-Prague (Antigen 85A, ESAT-6-like and PE/PPE proteins), I wanted to determine whether PhoP-dependent regulation of these proteins affect levels of immune induction. IFNγ is a surrogate marker of immune response and its production by immune cells is correlated with protective BCG vaccination (18, 54). To test the role PhoP plays in immunogenicity, I compared IFNγ induction in Prague pME to two PhoP-complemented strains, Prague pME-phoP, and Prague pME-phoP/R. As described in Materials and Methods (Chapter 2), four mice per group were subcutaneously (s.c.) inoculated with each strain and spleenocytes were isolated eight weeks post-injection. Secreted IFNγ was then measured by ELISA from spleenocytes that were either stimulated with 10 µg/ml PPD, or left unstimulated.

My results show that restoration of \textit{phoP} in BCG-Prague increases IFNγ production (Figure 5). IFNγ levels rose significantly from $5,327 \pm 3,096$ pg/ml in Prague pME to $12,510 \pm 9,393$ pg/ml in Prague pME-phoP (p< 0.05, n=4). Surprisingly, a decrease in IFNγ was reported in Prague pME-phoP/R ($3,872 \pm 2,272$ pg/ml). These results show that PhoP-regulated proteins affect the immunogenicity of BCG-Prague since complementation with PhoP increased IFNγ production. This further supports the loss of \textit{phoP} as the mechanism behind the loss of immunogenicity in BCG-Prague.
Figure 5. The effect of phoP on BCG-Prague IFN\(\gamma\) production in stimulated mouse spleenocytes. BCG-Prague strains (Prague pME, Prague pME-phoP, Prague pME-phoP/R) were inoculated subcutaneously into C57BL/6 mice. Eight weeks post-inoculation, spleenocytes were harvested and stimulated with 10\(\mu\)g/ml PPD. Extracellular IFN\(\gamma\) production was determined by ELISA. Results reported are IFN\(\gamma\) levels produced by PPD-stimulated spleenocytes adjusted by subtracting IFN\(\gamma\) released in corresponding unstimulated spleenocytes. Statistical analysis using a T-test show significant difference between Prague pME and Prague pME-phoP (p<0.05, n=4)(*).
3.3 Recombinant BCG-Japan over-expressing PhoP induces an increased immune response

Since my ultimate goal is to make a more effective vaccine, I wanted to see whether the over-expression of PhoP in BCG-Japan would increase the immunogenicity of the strain. BCG-Japan has a consistent safety record in clinical studies mainly due to the loss of PDIM/PGLs, cell wall lipids tightly associated with virulence. In addition, BCG-Japan is an ‘early strain’ and retains more characteristics of the original BCG strain compared to the later evolved BCG-Prague (14). I hypothesized that the over-expression of phoP would make BCG-Japan a more immunogenic vaccine while still maintaining its safety due to the absence of PDIM/PGLs. To test this, IFNγ induction was measured in the same manner as before using Japan pME, Japan pME-phoP, and Japan pME-phoP/R (Figure 6). Both Japan pME-phoP (26,364 ± 13,057 pg/ml IFNγ) and Japan pME-phoP/R (31,772 ± 4,260 pg/ml IFNγ) elicited higher IFNγ production in mice spleenocytes compared to Japan-pME alone (11,550 ± 2,759 pg/ml IFNγ). The IFNγ levels induced by Japan pME-phoP (p< 0.01, n=4) and Japan pME-phoP/R (p< 0.001, n=4) were also both significantly different from the amount produced by Japan pME.

Closer inspection of these results shows that BCG-Japan strains cause stronger IFNγ production than BCG-Prague strains. The lowest inducer of IFNγ among the Japan strains, Japan pME, produced higher readings than the highest inducer of IFNγ in the Prague strains, Prague pME-phoP. This phenomenon may be due to the over-expression of PhoP in BCG-Japan strains, which already have a functional phoP gene in addition to the one introduced on the external plasmid. Evidence presented later in this work also shows that PhoP expression in BCG-Japan is naturally high and not properly regulated, thereby compounding the level of PhoP over-expression. It is clear from these results that while PhoP affects the immunogenicity of both
BCG sub-strains, more work is needed to characterize the type of immune response elicited by both strains.
Figure 6. The effect of *phoP* on BCG-Japan IFNγ production in stimulated mouse spleenocytes. Japan pME, Japan pME- phoP, and Japan pME-phoP/R strains were injected subcutaneously into C57BL/6 mice. Eight weeks post-inoculation, spleenocytes were harvested and stimulated with 10µg/ml PPD. Extracellular IFNγ production was determined by ELISA. Reported numbers are IFNγ levels produced by PPD-stimulated spleenocytes adjusted for background IFNγ released in corresponding unstimulated spleenocytes. Statistical analysis using a T-test show significant difference between Japan pME and Japan pME-phoP (p<0.01, n=4) (***) and between Japan pME and Japan pME-phoP/R (p<0.001, n=4) (****).
3.4 Over-expression of PhoP induces higher levels of IFNγ-producing CD4+ T cells in recombinant BCG-Japan

I have shown that PhoP is involved in host immune induction through its control of secreted antigens. These antigens are recognized by T cells, which play an essential role in immunological memory. In order to be effective, the vaccine strain must elicit the proper immune response, including the induction of the proper T cell subtypes. Of particular interest are changes in CD4+ and CD8+ T cells because both these populations are required for a protective immune response against *M. tb* (55, 57, 131). While my ELISA data provided overall IFNγ levels released by immune cells, it is still unclear what T cell subsets are activated specifically. It is expected that strains with increased *phoP* expression would also show increased numbers of T cells. To determine the relative amount of each T cell subpopulation induced by PhoP, Vanessa Tran from my lab performed ICS (Intracellular Cytokine Staining) and FACS (Fluorescence Activated Cell Sorting) analysis on mouse spleenocytes isolated for previous ELISA experiments. This allowed the identification and enumeration of T cell subpopulations producing specific cytokines of interest. Spleenocytes were either stimulated with PPD or left unstimulated and were stained for extracellular T cell markers CD3+, CD4+ and CD8+ using fluorophore conjugated antibodies. A CD3+ antibody was used to identify T cells by recognizing a T cell receptor. Antibodies for CD4+ and CD8+ were specific for their respective T cell subtypes. Cells were also stained for intracellular expression of IFNγ. Only CD3+ CD4+ IFNγ+ or CD3+ CD8+ IFNγ+ T cell populations were examined for this study. Cell counts in each population of interest derived from unstimulated spleen cells were considered background and were subtracted from the counts obtained from stimulated spleenocytes.
Results from BCG-Prague strains show that Prague pME (122,425 ± 93,371 cells) have higher counts of IFNγ producing CD4⁺ T cells compared to Prague pME-phoP (87,375 ± 59,485 cells) and Prague pME-phoP/R (51,400 ± 45,183 cells) (Figure 7a). The same is seen in CD8⁺ populations with Prague pME (97,250 ± 103,284 cells) producing higher cell counts than Prague pME-phoP (48,075 ± 27,137 cells) and Prague pME-phoP/R (26,575 ± 23,566 cells) (Figure 7b). The high levels of both CD4⁺ and CD8⁺ T cell induction observed in Prague pME was surprising since they contradict earlier IFNγ ELISA results and cannot be explained at this time.

IFNγ producing T cells were also examined in Japan pME, Japan pME-phoP, and Japan pME-phoP/R to determine whether over-expressing phoP would have an effect on T cell populations. Both Japan pME-phoP (70,980 ± 41,778 cells) and Japan pME-phoP/R (128,575 ± 39,951 cells) showed an increase of CD4⁺ IFNγ producing T cells when compared to Japan pME (46,825 ± 56,710 cells) (Figure 7c). However, no statistically significant differences were found between the strains. The number of IFNγ producing CD8⁺ T cells induced by Japan pME (52,100 ± 36,118 cells), Japan pME-phoP (46,760 ± 35,217 cells), and Japan pME-phoP/R (47,525 ± 44,644 cells) were similar among the three strains (Figure 7d). Although not statistically significant, the increased CD4⁺ T cells in recombinant BCG-Japan strains is a promising result that further demonstrates the role of PhoP in immunogenicity.
Figure 7. The effect of *phoP* on IFNγ-producing CD4+ and CD8+ T cells in the spleen. FACS analysis was performed on activated spleenocytes stained for intracellular IFNγ and the extracellular T cell markers CD4, CD8, and CD3. Specific T cell populations induced by recombinant BCG-Prague strains were identified and enumerated: CD3+CD4+ IFNγ+ T cells (A) and CD3+CD8+ IFNγ+ (B). The same was done for T cell populations reported for recombinant BCG-Japan constructs: CD3+CD4+ IFNγ+ T cells (C) and CD3+CD8+ IFNγ+ (D). Numbers from activated spleenocytes were background subtracted using counts from unstimulated spleenocytes stained in parallel. Four mice per BCG strain were tested. No significant differences were found using a T-test.
3.5 The over-expression of PhoP/R does not increase BCG-Prague and BCG-Japan virulence

As mentioned previously, PhoP is a virulence factor in M. tb. One potential concern is that over-expression of phoP in BCG may increase its virulence and have a negative impact on safety. My microarray analysis found seven genes in the PDIM/PGL biosynthetic locus that were also upregulated with PhoP expression. PDIMs and PGLs are structurally related cell wall lipids that have been linked to virulence (66, 78, 108).

To assess whether virulence in Prague pME-phoP/R was increased compared to WT BCG-Prague, I intravenously (i.v.) injected each strain into immunocompetent mice and enumerated the bacterial burdens in the lung, spleen and liver. My results show that virulence in BCG-Prague is generally unaffected by the addition of a functional PhoP (Figure 8). Although Day 21 counts in the liver indicate a slight increase in virulence, this may not be significant enough to produce a noticeable clinical difference.

The same experiments were performed in BCG-Japan, using Japan pME and Japan pME-phoP/R, to ensure that over-expression of PhoP does not result in increased virulence. The results also show that PhoP does not increase virulence of BCG-Japan strains in mice (Figure 8).
Figure 8. The effect of \textit{phop} on BCG-Prague and BCG-Japan virulence. To determine the effect of \textit{phop} on virulence, strains containing \textit{phop} on an external plasmid (Prague pME-phop/R and Japan pME-phop/R) were compared to their WT equivalents (Prague pME and Japan pME, respectively). Four mice per group were inoculated with each strain. Lungs, spleen and livers were harvested at days 1, 14, 21, and 42 post-inoculation. Homogenized organs were plated and CFUs were enumerated after 3 weeks. Some time points had no data (ND) due to inappropriate selection of dilution ranges for plating organ homogenates.
3.6 PhoP has no effect on PDIM and PGL synthesis

Since my microarray results indicated an upregulation of seven genes in the PDIM/PGL biosynthesis locus due to the over-expression of *phoP*, I wanted to verify that their production was affected. To do this, I used TLC (thin layer chromatography) to separate the different lipid classes based on their relative polarity. In order to investigate the possible link between *phoP* expression and PDIM/PGL synthesis in BCG-Prague, I performed 2D-TLC (two-dimensional thin layer chromatography) analysis on the strains compared previously by microarray, Prague pME and Prague pME-phoP. In addition, I also performed 2D-TLC analysis on the other BCG-Prague strains used in this work, Prague pME-phoP/R, as well as a WT strain without a vector. To do this, apolar lipids were extracted from each of the recombinant strains and separated chromatographically using the appropriate solvents as described in Materials and Methods (Chapter 2).

TLC analysis revealed an unexpected loss of PDIM and PGL production in some BCG-Prague strains, which may account for the upregulation of the seven PDIM/PGL biosynthetic genes seen in my microarray expression study. As expected from previous studies in our lab comparing lipid production among BCG strains (31), BCG-Prague showed both PDIM and PGL production (Figure 9A-1 and 9B-1). Surprisingly, there was a loss of both lipids in Prague-pME, which is considered WT (Figure 9A-2 and 9B-2). While Prague pME-phoP produced PDIM and PGLs (Figure 9A-3 and 9B-3) as anticipated, these lipids were also absent in the Prague pME-phoP/R strain (Figure 9A-4 and 9B-4). Active PDIM/PGL production in the PhoP-complemented strain (Prague pME-phoP) combined with the loss of PDIM/PGL production in the WT strain (Prague pME) resulted in an artificial increase in expression of PDIM/PGL biosynthesis genes in my microarray since the WT strain was not producing these lipids. These results indicate that
PDIM/PGL upregulation in my microarray analysis was affected by inconsistencies in the lipid production in the BCG-Prague strains themselves and not by PhoP-dependent regulation.

PDIMs and PGLs are complex lipids associated with virulence and are sometimes selected against in vitro, a phenomenon seen in other labs (47). It is hypothesized that random lipid loss occurs because their production requires a large expenditure of energy that is wasted when grown in culture. Unfortunately, this finding has complicated the results of not only the microarray, but also of mouse studies done in this work that would be affected by the drop in virulence in the Prague pME strain. Nonetheless, this serves as confirmation that PDIM/PGL production is not regulated by PhoP. This result is consistent with previously published studies in M. tb H37Rv, where PDIM production and PhoP regulation were completely independent from one another (32).

In addition to BCG-Prague strains, BCG-Japan strains (Japan pME, Japan pME-phoP, and Japan pME-phoP/R) constructed for mice experiments were also analyzed by TLC. Since BCG-Japan is naturally deficient in PDIM/PGL production (31), they are not expected to produce either lipid. As predicted, 2-D TLC analysis showed no production of PDIMs or PGLs in any of the strains (Figure 9C and 9D).
Figure 9. PDIM and PGL lipids in BCG-Prague and BCG-Japan bacterial strains by two-dimensional thin layer chromatography. Apolar lipids from BCG-Prague strains (Prague, Prague pME, Prague pME-phoP, Prague pME-phoP/R) and BCG-Japan strains (Japan, Japan pME, Japan pME-phoP, Japan pME-phoP/R) were analyzed by TLC. PDIMs for BCG-Prague strains (A) and BCG-Japan strains (C) were separated using the solvents petroleum ether/ether acetate (98:2 v/v) three times in the first dimension and petroleum ether/acetone (98:2 v/v) in the second dimension. Plates were then charred with 5% molydophosphoric acid. PGLs for BCG-Prague strains (B) and BCG-Japan strains (D) were developed using chloroform/methanol (96:4 v/v) in the first dimension and toluene/acetone (80:20 v/v) in the second dimension. Lipid spots were visualized using α-naphthol/sulphuric acid, followed by charring of the plate. PDIMs and PGLs are shown as indicated.
3.7 PhoP has no effect on DAT, PAT and SL production in BCG

The cell wall of mycobacteria is comprised of many complex lipids that serve both protective and pathogenic roles. More than half of the 44 genes upregulated by PhoP in *M. tb* are associated with cell wall components including lipid biosynthesis and secretion (133). Included in this list are *pk*2 and *msl*3, which encode enzymes involved in the biosynthesis of sulfolipids (SLs), diacyltrehalose (DATs), and polyacyltrehaloses (PATs) (48, 115). These lipids are only found in pathogenic mycobacteria closely related to *M. tb* and are crucial to the bacteria’s ability to survive in its host (42, 78). Previous studies with an *M. tb H37Rv* phoP mutant demonstrated that PhoP regulates the biosynthesis of these trehalose-containing lipids (32, 65). Although the expression of these biosynthetic genes was not significantly upregulated in my microarray analysis, lipid production could still be affected. In order to determine whether PhoP in BCG-Prague controls production of these lipids as well, a one-dimensional TLC was performed to separate and visualize DATs, PATs, and SLs. In addition, the recapitulation of these lipids will also provide evidence that *phoP* has functionally complemented the defective PhoP in BCG-Prague. To do this, lipids were extracted using chloroform/methanol and developed in chloroform/methanol/water (60:16:2 v/v) for DATs and PATs, or petroleum ether/acetone (98:2 v/v) for SLs as described in Material and Methods (Chapter 2).

The lipid profiles of BCG-Pasteur, BCG-Prague, Prague pME, Prague pME-phoP, and Prague pME-phoP/R showed no discernable differences in banding patterns (Figure 10) meaning that DAT, PAT, and SL production are not affected by PhoP in BCG-Prague. As a consequence, the restoration of the trehalose-containing lipids, signifying functional complementation of the defective *phoP* gene in BCG-Prague, also could not be confirmed. To further complicate the analysis, the banding patterns were distinct from other TLC analyses done in *M. tb* and *M. tb*
phoP mutants (32, 65, 133). Therefore, lipid bands cannot currently be identified and would require further analysis by mass spectrophotometry, which was not performed in this study. Due to numerous genetic lesions in BCG that have yet to be characterized, any number of varying factors could have an effect on the biosynthesis of these lipids. Lipid profiles of DATs, PATs, and SLs have never been performed in BCG strains and remain unknown. Since banding patterns between BCG-Prague and BCG-Pasteur (a strain containing no known phoP-phoR mutations) are similar, pre-existing aberrations in trehalose-containing lipids may be common among all or at least multiple BCG sub-strains. Additionally, because the stimulus for the phoP-phoR system is not known, it is possible that PhoP activation has not reached the threshold needed to drive up lipid regulation.
Figure 10. Comparison of DATs, PATs, and SLs in BCG-Prague bacterial strains by thin layer chromatography. Lipids from Pasteur pME, Prague pME, Prague pME-phoP, and Prague pME-phoP/R were extracted from lyophilized cells using chloroform/methanol (2:1 then 1:2 v/v). Lipids were developed in chloroform/methanol/water (60:16:2 v/v) (A), or petroleum ether/acetone (98:2 v/v) (B). Bands were visualized by charring with 5% molydophosphoric acid. Specific lipids could not be identified at this time since banding patterns were unique from previous lipid analyses done in M. tb and could not be directly compared.
3.8  *phoP* expression is upregulated in BCG strains containing IS6110

PhoP has a role in virulence in *M. tb* (85, 100) and its upregulation is connected to a hypervirulent strain of *M. bovis* (107, 120). The PhoP protein autoregulates its own transcription by using recognition sites in the promoter of its gene. However it is currently debated whether this is a mechanism for activation (64) or repression (72) of *phoP* expression. Comparative sequencing of 13 BCG sub-strains using the NimbleGen technique revealed three BCG sub-strains with IS6110 present in the promoter region upstream of *phoP*. These strains include BCG-Japan, BCG-Russia, and BCG-Moreau. To determine the effect of IS6110 on *phoP* expression and the possible correlation with variations in clinical virulence, I performed qRT-PCR on the strains and compared them to BCG-Pasteur, which has no mutations in the *phoP*-pho*R* locus (86). I predicted that differing levels of *phoP* expression may account for the differences in virulence seen in these BCG sub-strains clinically. To do this, RNA from the strains were extracted and reverse transcribed into cDNA as described in Materials and Methods (Chapter 2) and quantified by real time PCR. Levels of *phoP* transcripts were normalized with sigma factor A (*sigA*), a commonly used house-keeping gene so that the different strains could be compared. Three biological samples were examined for each strain.

Results show that all three IS6110-containing strains have increased *phoP* expression when compared to BCG-Pasteur (Figure 11). The differences were statistically significant in each case when strains were compared using an unpaired, two-tailed T-test. The most pronounced increase in *phoP* expression was in BCG-Japan with 1.039 ± 0.079 copies *phoP/sigA* in contrast to BCG-Pasteur with 0.366 ± 0.045 copies *phoP/sigA* (almost 3-fold increase). BCG-Moreau showed a smaller increase of 0.741 ± 0.062 (2-fold increase) while BCG-Russia displayed a much more
modest increase of 0.496 ± 0.046 copies $phoP/sigA$ (1.4 times that found in BCG-Pasteur).

Consistent with my result, $phoP$ expression in BCG-Japan was also higher than BCG-Pasteur in a study by Brosch et al. (24).
Figure 11. Expression levels of phoP in BCG strains containing IS6110. Quantitative RT-PCR was used to determine the relative expression levels of phoP among the IS6110 containing strains, BCG-Japan, -Russia, and -Moreau. BCG-Pasteur was used as a comparison strain since no mutations were detected in the phoP-phoR locus. Copies of phoP were normalized to copies of the sigma factor A gene (sigA) present in each tested sample. Results are expressed as a relative ratio of phoP to sigA. All three BCG strains had increased phoP expression compared to BCG-Pasteur as determined by a T-test, as indicated. BCG-Japan showed the highest upregulation of phoP (p<0.001, n=3) (**); BCG-Russia had the smallest difference (p<0.05, n=3) (*); and BCG-Moreau had an intermediate level of phoP upregulation among the strains (p<0.01, n=3) (**).
Discussion

Evidence from this chapter demonstrated the important role of PhoP in BCG immunogenicity. Complementation of phoP in BCG-Prague and the over-expression of phoP in BCG-Japan resulted in the upregulation of genes encoding T cell antigens and other potentially immunogenic proteins. The restoration of these antigens by phoP complementation successfully increased the immunogenicity of BCG-Prague. In addition, phoP over-expression in BCG-Japan achieved higher levels of IFNγ induction to PPD, a mixture of M. tb secreted antigens that likely mimics a natural infection, than any other single vaccine candidate to date. This feat was also accomplished without a concomitant increase in virulence. Since both parental BCG strains were relatively safe strains clinically, the new re-engineered strain will likely have low incidence of adverse reactions as well.

Since the PhoPR system of M. tb is largely uncharacterized, it is not known whether phoR is affected by the natural phoP mutation in BCG-Prague. Studies in DosRST, another mycobacterial two-component system, showed that deletion of the response regulator (DosR) led to abrogated expression of the sensor kinase (DosS), which was not repaired even with the reintroduction of DosR (38). To address this concern I had included both phoP and phoP/R constructs in my immunogenicity studies. If PhoR has no effect on PhoP expression or function, then the two strains were expected to behave similarly in experiments.

Immune induction studies showed inconsistencies in IFNγ production between phoP and phoP/R expressing strains. The most striking example was the large difference in IFNγ production between Prague pME-phoP and Prague pME-phoP/R. However, the weak IFNγ induction by Prague pME-phoP/R may be due to the loss of virulent PDIM/PGL lipids (Figure 9). In addition, despite having similar overall IFNγ release (Figure 6), Japan pME-phoP and Japan pME-phoP/R
exhibited differential activation of IFNγ-producing CD4+ T cells (Figure 7). This unexplained discrepancy suggests that the effects caused by the presence of the signal transducer, PhoR, cannot be entirely discounted. Since the over-expression of PhoR is the only discernable difference in these strains, the enhanced expression of the sensor kinase may alter the induction of specific immune cells. This will likely be resolved with further study comparing the behaviour of the two constructs in BCG-Pasteur, which is WT in the \textit{phoP-phoR} locus, and also by ensuring that PDIM/PGL production is maintained.

Since PhoP is associated with virulence and immunogenicity, I tried to establish a link between the increased \textit{phoP} expression seen in the three IS6110-containing BCG sub-strains (BCG-Russia, -Japan, -Moreau) and their clinical behaviour. BCG-Russia is known for its high virulence and adverse reactions as a vaccine (25, 26, 88) and the upregulated expression of \textit{phoP} may be partially responsible for this phenotype. Conversely, BCG-Japan and BCG-Moreau are both consistently low in reactogenicity (88, 94) but showed the highest \textit{phoP} expression among the strains tested here (Figure 11). This discrepancy can be explained by the absence of the virulent PDIM and PGL lipids (31), which no doubt also has an effect on their reactogenicity. Due to the extensive role of these lipids in virulence, it would not be surprising if their loss masked the increased effects brought on by \textit{phoP} upregulation.

The upregulation of \textit{phoP} in all three IS6110-containing strains support the autorepression model of \textit{phoP} regulation. The 1356 bp IS6110 fragment contains no identifiable transcriptional start site and its insertion 18 bp from the \textit{phoP} start site likely interferes with the three known PhoP binding sites: DR1 (-61 to -69), DR2 (-47 to -55), and DR3 (-3 to -11) (72). In agreement with the repression model, this presumably abrogates PhoP-dependent repression of the promoter and
drives up \textit{phoP} expression as a consequence. Supporting this is the higher \textit{phoP} expression in \textit{M. tb} H37Ra, a \textit{phoP} mutant with abrogated DNA binding ability (59, 62).
Chapter 4

Impact of WhiB3 on BCG Metabolism and Residual Virulence

Introduction

WhiB3 has been associated with virulence in mycobacteria. WhiB3 has a vital role in *M. bovis* since a *whiB3* mutant was deficient in growth by approximately five logs in mice compared to WT *M. bovis*. This same mutant was even further attenuated when examined in a gerbil animal model (124). Since gerbils develop necrotic lung granulomas that are rich in fatty acids but low in other carbon sources, WhiB3 has been implicated in metabolic switchover because mutants are unable to adapt (129). Further supporting its role in regulatory switchover is the maximal induction of WhiB3 during the early phase of infection, coinciding with granuloma formation (129).

Both BCG-Birkhaug and BCG-Sweden are natural *whiB3* mutants due to a mutation disrupting the promoter and start site of the gene (86). Although the role of WhiB3 in *M. tb* virulence is still being uncovered, it has been shown to function as a transcriptional regulator responsible for metabolic switchover in response to NO, O₂, or carbon stress (113). Due to its similarity to the *B. subtilis* *whiB3* gene, it has been implicated in mycobacterial dormancy. WhiB3 may also have a role in immunomodulation through its regulation of trehalose-containing lipids, which may also serve a secondary role as a byproduct of redox regulation. I hypothesize that as seen in other mycobacteria, the *whiB3* mutation affects BCG metabolism and residual virulence and its loss in BCG-Birkhaug and BCG-Sweden contributes to the attenuation of these strains. To test
this, I complemented each strain with a WT \textit{whiB3} gene on an external plasmid and then compared the \textit{in vitro} growth of each strain on plates supplemented with different carbon sources. After observing a difference in growth, I tested whether WhiB3 also has an effect \textit{in vivo} in a mouse model of infection.

**Results**

### 4.1 Carbon metabolism is affected in BCG \textit{whiB3} mutants

Recent studies showed that in addition to NO and O$_2$, nutrient depletion signals persistence in \textit{M. tb} and influences expression of respiratory and metabolic enzymes that modulate the intracellular redox state (17). When compared to WT, an \textit{M. tb whiB3} mutant grew poorly on carbohydrate-based carbon sources, but grew more effectively on acetate, a short chain fatty acid molecule (113). This suggested that the \textit{whiB3} mutant had a diminished ability to sense and/or adapt to the various carbohydrate sources and also reflected the bacteria’s preference for fatty acids during infection. Taken together, this implicated WhiB3 in transcriptionally regulating the switchover to fatty acid metabolism, a vital adaptation in the host macrophage.

To test whether this was also the case in BCG, I examined BCG-Birkhaug and BCG-Sweden on different carbon sources in the same manner as the study in \textit{M. tb} (113). WT or \textit{whiB3}-complemented strains were compared for growth and colony morphology on various carbon sources (glucose, succinate, fumarate, pyruvate, citrate and acetate). In each case, the \textit{whiB3}-complemented strain grew to an equal or higher density than the WT strain (Figure 12). Growth differences were more pronounced when comparing Birkhaug pME and Birkhaug pME-whiB3, especially on plates supplemented with glucose, acetate and fumarate. Differences between
Sweden pME and Sweden pME-whiB3 were minimal. Plate assays were repeated twice with similar results.

In agreement with \textit{M. tb}, the presence of \textit{whiB3} allowed the strains to grow better on all carbohydrate-based carbon sources. However, the differences are not as pronounced as those seen in the \textit{M. tb} study and in some cases, are indiscernible (i.e. BCG-Sweden on citrate and fumarate supplemented plates). This trend is also evident when strains were grown on acetate, which opposed the result seen in \textit{M. tb}. The variation from \textit{M. tb} results cannot currently be accounted for and is likely due to other accumulated mutations in the BCG strains.
Figure 1. The effect of \textit{whiB3} on carbon metabolism in BCG-Birkhaug and BCG-Sweden. The ability to utilize different sources of carbon was compared between \textit{whiB3} mutants (Birkhaug pME and Sweden pME) and \textit{whiB3}-complemented equivalents (Birkhaug pME-\textit{whiB3} and Sweden pME-\textit{whiB3}). Birkhaug pME-\textit{whiB3} colonies have denser growth on all carbon sources compared to Birkhaug pME. The same is seen to a lesser degree in Sweden pM-\textit{whiB3} verses Sweden pME. Plates were made of Dubos agar supplemented with the indicated carbon course. Colonies shown are at 3 weeks growth.
4.2 Virulence is not affected in BCG whiB3 mutants

Both BCG-Birkhaug and BCG-Sweden metabolize various carbon sources more effectively when complemented with a functional whiB3 gene. I wanted to next determine whether this phenotype seen in vitro would affect their growth and virulence in vivo. To do this, I took the same strains (Birkhaug-pME, Birkhaug pME-whiB3, Sweden pME, Sweden pME-whiB3) and inoculated mice through i.v. injections and measured bacterial load in the lung, spleen and liver at different time points as described in Materials and Methods (Chapter 2).

My results indicated that complementation with WhiB3 did not increase virulence of these BCG strains (Figure 13). However, CFU counts in some organs reflect a marginal increase in virulence in WhiB3-containing strains. For example, the livers of mice infected with BCG-Birkhaug strains show that Birkhaug-whiB3 grew better than Birkhaug pME by Day 42 despite having lower starting inoculant counts at Day 1. In addition, CFUs in BCG-Sweden infected livers also show a WhiB3-associated increase in virulence. Although the CFUs at these described time points are statistically significantly different (not shown), it is still unclear if the changes are substantial enough for a clinical effect. Unfortunately, more accurate comparisons of the strains are hampered by the unequal inoculations of the strains as indicated by the Day 1 time point.
Figure 12. Effect of whiB3 on virulence in BCG-Birkhaug and BCG-Sweden. To determine whether whiB3 has an in vivo effect on BCG virulence, whiB3 mutant strains (Birkhaug pME and Sweden pME) and their complemented counterparts (Birkhaug pME-whiB3 and Sweden pME-whiB3) were compared. Four mice per group were inoculated and sacrificed on days 1, 14 and 42 post inoculation. Lungs, spleen, and liver were homogenized and plated. CFU counts were enumerated after 3 weeks incubation at 37ºC.
Discussion

Results from this chapter showed that the impact of WhiB3 on BCG is minimal. Unlike in \textit{M. tb} and \textit{M. bovis}, WhiB3 expression in BCG-Birkhaug and BCG-Sweden did not lead to a substantial increase in virulence. This suggested that unlike ESAT-6 and PDIM/PGLs, WhiB3 is not a major virulence factor. Since BCG is already deficient in ESAT-6 secretion and other virulence mechanisms, the effects of the \textit{whiB3} deletion are over shadowed and do not result in a significant change in virulence. However, since \textit{M. tb} contains many virulence factors the consequence of \textit{whiB3} loss was not masked by other attenuations. This result is consistent with previous clinical findings. When comparing the virulence of 12 BCG sub-strains, BCG-Sweden was identified as one of the more virulent strains (25, 26, 81). BCG-Sweden has also often been associated with increased reports of adverse reactions compared to many other BCG sub-strains (40, 88). While the risk of BCG-induced osteitis was 0.01 cases per million for BCG-Japan, reported cases for BCG-Sweden were 32.5 and 43.4 cases per million in Sweden and Finland, respectively (88). This association was further emphasized when national vaccination programs were evaluated. As described earlier, the cases of osteitis reported in Finland dropped when BCG-Sweden was replaced by BCG-Glaxo, a strain missing PDIM/PGLs (88). Although BCG-Birkhaug has been less studied, the two strains are closely related and are expected to have consistent levels of virulence (25, 26, 80, 81).

Another unexpected result was the altered ability for the \textit{whiB3} mutants to grow on acetate. While the presence of \textit{whiB3} in \textit{M. tb} led to decreased growth, BCG strains showed the opposite effect of increased growth with \textit{whiB3} complementation. Since WhiB3 may be involved in the sense and switchover from carbohydrates to fatty acid metabolism, this growth difference could
indicate that WhiB3 function in BCG is somehow dysregulated. This could also explain why the addition of a functional WhiB3 had no significant effect on the virulence of the BCG strains.
Chapter 5

General Discussion and Future Directions

This work investigates the effect of two virulence factors, PhoP and WhiB3 on the attenuation and immunogenicity of BCG. The dramatic effect of PhoP on immunogenicity has been established several times in this study. Microarray expression analysis demonstrates that PhoP positively regulates genes encoding proteins or protein family members associated with mycobacterial virulence and immunogenicity. These include the upregulation of Antigen 85A, a dominant T cell antigen that is exploited in several subunit vaccines (89, 132); and members of the ESAT-6-like and PE/PPE family of proteins, which have roles in virulence and possibly immune recognition (23, 44, 87, 121). Interestingly, all nine upregulated esx genes as well as PE41 are secreted by the ESX-5 secretion system (2, 4). Although the role of ESX-5 is unclear in BCG, preliminary studies done in M. marinum and its similarity to ESX-1 of M. tb suggests it is an important contributor to virulence and immunogenicity.

The crucial role of PhoP in BCG immunogenicity is further validated by my in vivo mouse work. The re-introduction of phoP in the natural phoP mutant, BCG-Prague, greatly improved the immunogenicity of the strain as measured by IFNγ production (Figure 5), showing that the loss of phoP is at least partially responsible for its reduced vaccine potency. The immunogenicity of BCG-Japan, a strain with naturally high levels of PhoP expression due to the presence of IS6110 (Figure 11), was further enhanced with the over-expression of PhoP (Figure 6). In fact, the level of IFNγ induction surpassed vaccine candidates that are currently in pre-clinical and clinical trials. More importantly, the over-expression of PhoP in both strains did not result in a concomitant increase in virulence compared to the parental BCG.
Although the vaccine candidates currently in development have shown promising results, they have major limitations. DNA and protein subunit vaccines have shown potential as boosters in conjunction with BCG but have shown limited success as standalone vaccines (53, 89, 97, 106, 132). The success of this strategy is debatable considering the poor efficacy of BCG. Attempts at using other avirulent or attenuated strains of mycobacteria have also been unsuccessful, including *M. vaccae* (43), recombinant *M. vaccae* expressing *M. tb* antigens (6), and the *M. tb phoP* mutant (7, 28). In addition, the safety of these live vaccines is a primary concern. Although some strains of BCG can cause adverse reactions, the general safety of BCG has been proven in humans over decades of vaccination. Thus, it is favourable to build upon existing strains of BCG with proven safety records rather than engineering a completely new strain. However, the unique characteristics of each BCG sub-strain should be taken into consideration when choosing a parental strain, which was not done for the current BCG vaccine candidates.

Gene expression profiling of immune responses after BCG vaccination show that BCG strains activate distinct immune pathways (140). This is supported by their unique behaviour clinically, especially in terms of residual virulence and adverse reactions (51, 52, 81, 88). It is evident that BCG sub-strains have unique vaccine properties. Identifying and understanding these differences is crucial for vaccine development and evaluation since the benefits and drawbacks of each strain will be known.

BCG will undoubtedly remain an important component of TB vaccine development, whether as recombinant vaccine or the ‘prime’ in a prime-boost regimen. However, the development of a new vaccine against TB has many obstacles. Not only must it be more effective than the existent BCG, it should also be safe enough to use in both the general population and in immunocompromised individuals, such as those affected by HIV. Attempts at creating a new
vaccine, whether a recombinant BCG or a subunit vaccine, have so far fallen short. While they are promising, none has considerably surpassed the performance of the current BCG and have not properly addressed the need for increased safety in immunocompromised individuals who are at the most risk of TB infection. My work proposes a novel vaccine strategy that exploits the natural properties of BCG. Most importantly, safety was a primary factor in choosing both BCG-Prague and BCG-Japan as parental strains. Although preliminary, the over-expression of PhoP in these strains increased the immunogenicity to very promising levels that surpassed current vaccine strategies. As such, these are excellent candidates for future TB vaccine development. Future work will need to be done to completely characterize these strains and assess their protection efficacy.

**Evaluate the protection efficacy of the vaccine strains**

In order to determine the vaccine potential of these strains, the protection efficacy will be examined in a mouse model of infection. To do this, C57BL/6 mice will be vaccinated with BCG-Prague and BCG-Japan over-expressing PhoP and then challenged with aerosolized *M. tb*, mimicking the natural infection route of TB. The ability of these strains to protect mice from *M. tb* infection will be determined by *M. tb* burden in target organs and compared to parental BCG as well as other sub-strains commonly considered potent vaccines such as BCG-Pasteur or BCG-Russia. This will help determine whether the increased immune response is significant enough to enhance protection. The safety of these recombinant BCG strains will be assessed in an immunocompromised SCID mouse model of infection. This will ensure the safety of these strains for use in HIV-positive individuals, who are at high risk. More in depth investigation into the effects of increased PhoP expression, protective efficacy, and safety will demonstrate that these strains are superior candidate TB vaccines.
Investigate the PhoP-PhoR System

The PhoPR two-component system of *M. tb* is largely uncharacterized. It belongs to the diverse OmpR/PhoB subfamily of response regulators, which have important roles in stress adaptation and virulence (125). PhoP of *Salmonella enterica* serovar Typhimurium is positively autoregulated and responds to Mg$^{2+}$ starvation, resulting in the regulation of over 40 genes, some of which are virulence factors (69). PhoB in *Bacillus subtilis* however regulates itself through repression mechanisms and senses phosphate starvation (83). Currently, the activating signal for the *M. tb* PhoPR system remains unknown and its method of regulation is also debated. Both the association of two-component systems with virulence and adaptation mechanisms and the PhoP-dependent regulation of secreted antigenic genes found in this work suggest that PhoPR is activated by an environmental cue during host infection. My work also shows that PhoP likely represses its own transcription since disruption of the promoter with IS6110 leads to upregulated expression (Figure 11) in various BCG strains. To confirm phoP autorepression, the three DR sites used for PhoP recognition will be re-introduced into the IS6110-containing strains, BCG-Japan, BCG-Russia, and BCG-Moreau in the proper location and phoP expression will be examined. Lowered phoP expression in these ‘repaired’ strains will support the autorepression model. Alternatively, IS6110 will be inserted into BCG-Pasteur in an attempt to increase phoP expression. Once characterized, these sites can be exploited to control phoP expression levels in future recombinant vaccine strains.

Another necessary experiment would be to repeat the microarray expression analysis with a Prague pME-phoP strain positive for PDIM and PGLs. While unlikely, PDIM/PGLs may have unexpected secondary effects on the microarray results due to their role in virulence. However, the increased IFNγ production also seen in Prague pME-phoP/R, which produce PDIM/PGLs
indicate the loss of these lipids may have no significant effect. Additional expression analyses should also be done to determine whether the PhoP regulon is similar in other BCG sub-strains. Due to the many other uncharacterized mutations in BCG sub-strains, PhoP may not be the only mutation responsible for regulation of ESX-5 genes in BCG-Prague.

Investigate the role of ESX-5 in immunogenicity

The ESX-5 secretion system likely plays an important role in BCG virulence due to its similarity to ESX-1 and its connection to virulence in *M. marinum*. The microarray results reveal that nine out of ten ESAT-6-like proteins as well as PE41, known to be secreted by ESX-5, were upregulated with PhoP function. To specifically examine the role of ESX-5 on BCG virulence and immunogenicity, isogenic ESX-5 BCG mutants will be constructed and immunological studies in mice will be performed as described in this work. To avoid any effects that irregular PhoP expression may have on the system, BCG-Pasteur will be used to generate the mutants since it has normal *phoP* expression. This allows more direct examination of ESX-5 secretion and ascertains any differences in the ESX-5-specific immune response induced by these strains.

According to the *M. marinum* model, ESX-5 secretion actively decreases pro-inflammatory host cytokines, alters macrophage expressed surface antigens, and is involved in phagosome escape and spread (3, 4). This is predicted to hinder bacterial recognition and killing by the host to increase bacterial load. If this is true, the loss of ESX-5 proteins in BCG-Prague could contribute to early clearance of the strain and thus not allowing adequate time for the host to develop an immunological response. To investigate this proposed mechanism, the ESX-5 mutant and WT BCG strains will be used to infect mice and the levels of pro-inflammatory cytokines, such as TNFα, will be determined and compared.
My work has established molecular factors that partially account for the differences seen in BCG virulence and immunogenicity. Using this knowledge, I have developed two promising vaccine candidates, BCG-Prague expressing PhoP and BCG-Japan over-expressing PhoP, which will contribute to the development of new TB vaccines that are more safe and effective. This also validates the novel approach to vaccine development taken by our laboratory, in which BCG strain selection based on unique vaccine properties is a key step in rational vaccine design.
References


