Differential antigen expression among strains of Bacille Calmette-Guérin

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Abstract

Mycobacterium bovis Bacille Calmette-Guérin (BCG) strains are live attenuated vaccines. These strains are genetically heterogeneous and the accumulation of deletions and mutations, particularly those affecting regulatory and antigenic proteins, has been proposed to have contributed to overly-attenuated vaccines. To examine this possibility, the effects of the two mutations incurred by BCG vaccines was formally studied through gene-deletion and complementation studies. First, the effect of deletion of the RD2 region was examined by over-expressing the antigenic protein MPB64 in a late strain of BCG and by creating an RD2-knockout strain in *M. tuberculosis.* These two approaches revealed minimal changes attributable to the RD2 region, but stimulated further investigation for other differences contemporaneous with the loss of RD2. By examining differences in gene expression between RD2+ and RD2- strains of BCG, two regions, in addition to the RD2 region itself, that were significantly down-regulated in the latter strains were identified. One region contained genes encoding the highly antigenic proteins MPB70 and MPB83, and the second region contained the gene encoding the extracellular function (ECF) sigma factor, *sigK.* Sequencing of *sigK* across all BCG strains revealed a start codon single nucleotide polymorphism (SNP) in strains with low production of MPB70 and MPB83. Complementation of BCG Pasteur with wild-type *sigK* resulted in up-regulation of *mpb70/mpb83* and increased production of MPB70 and MPB83 proteins. Further studies revealed that the *in vitro* growth rate of BCG Pasteur::*sigK* was slower than that of BCG Pasteur::pMV306 and that cytotoxicity to macrophage-like THP-1 cells was greater following infection with BCG Pasteur::*sigK* than with BCG Pasteur::pMV306. Infection of C57BL/6J mice demonstrated differences between BCG Pasteur::*sigK* and BCG Pasteur::pMV306 in initial deposition, growth rates and persistence. Vaccination of C57BL/6J mice with BCG Pasteur::*sigK* stimulated an MPB70-specific immune response, indicating that restored protein production resulted in increased immunogenicity. Finally, in challenge experiments, mice vaccinated with BCG Pasteur::*sigK* demonstrated equal, or slightly greater, protection than vaccination with BCG Pasteur::pMV306 against subsequent challenge with *M. tuberculosis.* Together, these findings provide an explanation for the loss of MPB70 and MPB83 production by BCG vaccines during prolonged *in vitro* passaging, and provide the basis for further studies aimed at understanding the role of the *sigK-mpb70* regulon in tuberculosis pathogenesis.

Resume

Les souches de *Mycobacterium bovis* Bacille Calmette-Guerin (BCG) sont des vaccins vivants atténués. Ces souches sont génétiquement hétérogènes et il est proposé que l'accumulation des délétions et mutations, particulièrement celles qui affectent les protéines régulatoires et antigéniques ont contribué à cette forte atténuation. Dans ce contexte, nous avons étudié les effets de deux mutations par délétion et complémentation. D'abord, l'effet de la délétion de la région RD2 a été examiné par la sur-expression de la protéine MPT64 dans une souche de BCG tardive, et par la création d'une souche de *M. tuberculosis* dépourvue de RD2. Ces deux approches ont révélé des changements minimes attribuables à la région RD2, mais ont provoqué une investigation approfondie sur d'autres modifications génétiques survenues en même temps que la perte de RD2. En examinant les différences d'expression génétique entre des souches RD2+ et RD2- , nous avons découvert qu'à part la région RD2, deux autres régions étaient fortement sous-régulées dans les souches RD2-. La première région contient les gènes codant pour les protéines antigéniques MPB70 et MPB83, et la deuxième région comprend le gène codant pour le facteur sigma de type ECF (fonction extracytoplasmique), SigK. Le séquençage de *sigK* dans toutes les souches de BCG a révélé l'existence d'une mutation ponctuelle (SNP) dans le codon d'initiation chez les souches à faible production de MPB70 et MPB83. Pour démontrer un lien entre le niveau d'expression et le SNP, BCG Pasteur a été complémenté par *sigK*. Comme prévu, la complémentation s'accompagne d'une sur-régulation des gènes de la région *mpb70/mpb83* ainsi que les protéines correspondantes. Des études additionnelles ont démontré que le taux de croissance de BCG Pasteur::sigK *in vitro* est plus lent que BCG Pasteur::pMV306. De plus, la cytotoxicité des cellules monocytaires THP-1 observée après infection avec BCG Pasteur::sigK est plus importante que celle obtenue après infection avec BCG Pasteur:pMV306. En infectant des souris C57BL/6J, nous avons noté des différences importantes entre BCG Pasteur::*sigK* et BCG Pasteur::pMV306 au niveau de la charge bactérienne initiale, du taux de croissance et de la persistance. La vaccination des souris C57BL/6J avec BCG Pasteur::*sigK* a induit une réponse immunitaire spécifique à MPB70, indiquant que la protéine restaurée a aussi une immunogénicité accrue. Enfin, chez les souris, la protection conférée par BCG Pasteur::*sigK* contre une épreuve par la souche *M. tuberculosis* virulente de type sauvage est comparable ou un peu plus élevée que celle de BCG Pasteur::pMV306. Ensemble, ces résultats élucident la perte de production de MPB70 et MPB83 dans les vaccins BCG durant un passage prolongé *in vitro* et permettent d'étudier de façon approfondie le rôle du régulon de *sigK-mpb70* dans la pathogenèse de la tuberculose.

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Abbreviations

ADC	albumin, dextrose, catalase
BCG	Bacille Calmette-Guérin
CFP	culture filtrate proteins
CFU	colony forming units
DC	dendritic cell
DC-SIGN	DC-specific intercellular adhesion molecule-3 grabbing non-integrin
DNA	deoxyribonucleic acid
DTH	delayed-type hypersensitivity
ECF	extracytoplasmic function
ESAT	early secretory antigenic target
LAM	lipoarabinomannan
LM	lipomannan
LPS	lipopolysaccharide
MOI	multiplicity of infection
MPB	*Mycobacterium bovis* protein
MPT	*Mycobacterium tuberculosis* protein
MVA	modified vaccinia virus Ankara
NOS	nitric oxide synthase
OD_{570}	optical density at 570nm
OD_{600}	optical density at 600nm
ORF	open reading frame
PBMC	peripheral blood mononuclear cells
PDIM	phthiocerol dimycocerosate
PE/PPE	mycobacterial proteins with proline-glutamic acid/ proline-proline-glutamic acid sequence near amino terminus
PIM	phosphatidyl-inositol mannoside
PPD	purified protein derivative
rBCG	recombinant Bacille Calmette-Guérin
RD	region of difference
RNA	ribonucleic acid
RNI	reactive nitrogen intermediate
ROI	reactive oxygen intermediates
SCID	severe combined immunodeficiency
SNP	single nucleotide polymorphism
TB	tuberculosis

Contributions of Authors

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David Sherman and Reiling Liao, at the University of Washington, created the *M. tuberculosis* H37RvΔRD2 strain. Thank you also to David for providing me with the opportunity to work in his lab (prior to the certification of the Level 3 laboratory at the Montreal General Hospital) while characterizing this strain. Deletion of the RD2 region was reconfirmed by Southern blotting by Francois Coulombe.

Serge Mostowy collected the RNA and analyzed the data for tables 3.1 and 3.3. Amplification of *sigK* for sequencing was also performed by Serge. Sequencing was completed at the McGill University and Genome Quebec Innovation Center.

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Complementation of BCG Pasteur with *sigK* was completed by David Alexander, Serge Mostowy and myself.

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1. Literature Review

1.1. Mycobacterium tuberculosis

Tuberculosis (TB) has killed people indiscriminately for centuries and has been well documented both as fact and in fiction for equally as long. Although many people perceive TB as a disease of the past, nearly two million people died of the disease in 2004 and five times as many became newly infected (WHO, 2006a). An estimated two billion people are infected with *Mycobacterium tuberculosis*. Although the majority of these people will not develop active tuberculosis, the minority in whom this does occur is considerable, giving an indication how difficult control and eradication of this disease will be. Acquiring infection with *M. tuberculosis*, like other infectious diseases, is dependent on many factors. Duration of exposure, intensity of exposure and genetic susceptibility are all factors that influence whether someone in contact with a TB case will become infected with *M. tuberculosis*. Once infected, only a fraction of individuals develop active TB. An estimated 5% of infected individuals will develop TB within two years of infection, which is typically termed primary TB. Another 5% will develop active disease at a later time, called reactivation disease. The remaining 90% of infected individuals are not expected to develop disease, unless mitigating factors, such as drugs or other disease, alter the natural history of their infection (Institute of Medicine and Committee on the Elimination of Tuberculosis in the United States, 2000;WHO, 2006b). In the majority of the individuals who do not develop disease, the infection is not cleared, but remains dormant. The ability of *M. tuberculosis* to remain dormant in infected

individuals for decades establishes an enormous reservoir for potential disease outbreaks.

The causative agent of tuberculosis was identified by Robert Koch, a man recognized as a pioneer in bacteriology. He has been credited with the development of numerous techniques that advanced the study of microbiology tremendously, he identified the causative agents of important diseases including anthrax and cholera and he demonstrated a set of criteria, now termed Koch's postulates, used to establish the etiology of a disease. In 1882, Koch first presented his experiments by which he identified *Mycobacterium tuberculosis* as the causative agent of tuberculosis. He meticulously stained the bacteria, isolated bacteria from TB patients and infected animals, cultured bacteria from both sources and re-infected animals with purified cultures. From this, he convincingly demonstrated to his colleagues the etiology of TB and defined the demonstration of the tubercle bacillus as the definite criterion for the diagnosis of TB.

Mycobacterium tuberculosis is a member of the *M. tuberculosis* complex which, in addition to *M. tuberculosis*, includes *M. bovis, M. microti,* and *M. africanum.* Identifying characteristics of *M. tuberculosis* complex organisms include slow growth, dormancy, complex cell envelope, intracellular pathogenesis and genetic homogeneity (Cole *et al.*, 1998). The members of this complex are known for causing TB or TB-like diseases in specific hosts and share greater than 99% sequence homology. The species of the *M. tuberculosis* complex figure among organisms

classified as slow-growing mycobacteria. In contrast to the fast-growing mycobacteria, such as *M. smegmatis* that has replication time of 3-4 hours, slow-growing mycobacteria can have a replication time on the order of hours to days. This slow replication contributes to the difficulty in treating and eradicating TB as drug regimens for treatment are necessarily long. As a means of developing a greater understanding of this organism, the sequenced and annotated genome of *Mycobacterium tuberculosis* was determined, first published in 1998 and subsequently re-annotated in 2002 (Cole *et al.*, 1998;Camus *et al.*, 2002). From this annotation, 4044 predicted genes, known as open reading frames (ORFs) were identified in the 4.4Mbp genome. Conserved hypothetical proteins represent a quarter of the ORFs, while genes encoding intermediary metabolism and respiration represent 22.1% and cell wall and cell processes represent 17.5%. Genes encoding lipid metabolism (5.8%), regulatory proteins (4.7%) and the PE and PPE families of glycine-rich proteins (4.2%) figure less prominently (Camus *et al.*, 2002). Of note, 376 putative proteins show no homology to other known proteins and have been hypothesized to be unique to *M. tuberculosis* (Camus *et al.*, 2002). Identification and characterization of these proteins may provide insight into the mechanism by which *M. tuberculosis* has become such a successful pathogen.

1.1.1. Infection, Host Response and Immune Reaction

1.1.1.1. Cell Envelope

The cell envelope of *Mycobacterium tuberculosis* is a highly complex structure, to which the success of the tubercle bacillus as a pathogen has been partially attributed. The cell envelope is composed of three layers; the plasma membrane, a mycolic acid-arabinogalactan-peptidoglycan complex layer and the outer capsule. The innermost plasma membrane is functionally and structurally similar to other bacterial plasma membranes and is generally not thought to have significant unique properties (Draper and Daffe, 2005). The peptidoglycan layer is highly cross-linked and covalently bound to arabinogalactan, which is further esterified to mycolic acids. This structure makes mycobacteria highly impermeable and contributes to its resilience. Lipoarabinomannan (LAM), phthiocerol dimycocerosate (PDIM) and phosphatidyl-inositol mannosides (PIM) are among the other important components of the cell envelope, and loss of these molecules severely affects the virulence and survival of *M. tuberculosis* (Briken *et al.*, 2004;Onwueme *et al.*, 2005). The capsule of *M. tuberculosis* is largely composed of proteins and polysaccharides, with the main components being the polysaccharides glucan, arabinomannan and mannan. The relatively few lipids found in the capsule tend to be situated towards the inner portion of the capsule. The proposed role of the capsule is to control phagocytosis of the bacterium, such that it confers properties on the bacterium that may permit it to enter macrophages via preferred pathways (Stokes *et al.*, 2004).

1.1.1.2. Uptake Receptors

Upon entry into the lungs, uptake of *M. tuberculosis* is primarily mediated by alveolar macrophages, with monocyte-derived macrophages and dendritic cells playing an increasing role as they are recruited to the site of infection. Uptake of *M. tuberculosis* is mediated through several different receptors. Complement receptor 1 (CR1), CR3 and CR4 play an important role in the uptake of *M. tuberculosis* by alveolar macrophages and monocyte-derived macrophages (Schlesinger *et al.*, 1990;Schlesinger, 1993;Hirsch *et al.*, 1994). Macrophage invasion by mycobacteria can proceed by opsonization with C3b via the alternative complement pathway (Schlesinger *et al.*, 1990) or alternatively, by binding directly with C2a to form a C3 convertase, resulting in the generation of C3b and subsequent opsonization (Schorey, Carroll, and Brown, 1997). In addition, non-opsonized bacteria can bind directly to CR3 and CR4. The mannose receptor provides another significant pathway through which phagocytosis of *M. tuberculosis* occurs (Schlesinger, 1993). Binding of *M. tuberculosis* to the mannose receptor through interactions with the terminal mannose residues of LAM has been described (Schlesinger, Hull, and Kaufman, 1994;Schlesinger *et al.*, 1996). More recently, interactions between the mannose residues of a 19kDa antigen and the mannose receptor have also been identified as a possible route of entry of *M. tuberculosis* (Diaz-Silvestre *et al.*, 2005). Specific inhibition of the individual complement receptors or the mannose receptor significantly reduced *M. tuberculosis* uptake by macrophages and concurrent inhibition of binding to the complement receptors and the mannose receptor inhibits uptake of *M. tuberculosis* to a greater extent than inhibition of the receptors

individually, yet does not fully block phagocytosis. This points to additional mechanisms through which phagocytosis may occur, as suggested by noting that blocking of class A scavenger receptors in addition to blocking the complement and mannose receptors completely abrogates binding (Zimmerli, Edwards, and Ernst, 1996). Uptake of *M. tuberculosis* by dendritic cells (DC) has also been identified as an important pathway of immune stimulation and is mediated primarily through DC-specific intercellular adhesion molecule-3 grabbing nonintegrin (DC-SIGN) (Tailleux *et al.*, 2003). Uptake through DC-SIGN occurs preferentially over either complement receptor or mannose receptor-mediated uptake and induces functional activation of the infected cells (Jiao *et al.*, 2002;Tailleux *et al.*, 2003).

1.1.1.3. Granuloma Formation

Although it is difficult to precisely define its importance, the formation of granulomas is considered essential to the control and containment of *M. tuberculosis* infection. Granulomas in human TB patients are most frequently characterized by a central core of caseating necrosis surrounded by cellular layers composed largely of lymphocytes and macrophages. Examining tissue sections from 20 TB patients, Ulrichs *et al* (Ulrichs *et al.*, 2004) provided a comprehensive description of the organization of the granulomas. They observed that the inner layer of cells surrounding the necrotic core is dominated by macrophages and CD4+ cells, while the outer layer consists predominantly of CD4+ and CD8+ cells. B-cell aggregates, which included both naïve and memory cells, were identified largely in proximity to lymphatics and blood vessels, as well as the central necrotic core. Outside of these

large regions surrounding necrotic centers, smaller peripheral foci of macrophages and lymphocytes were also observed. Mycobacteria were identified in macrophages in the inner circle surrounding the necrotic core and in the peripheral foci, and to a limited extent within the necrotic core.

Consistent with observations from human TB cases, the development of granulomas is also typically observed in animals models of *M. tuberculosis* infection , although the specific characteristics of these granulomas vary between models. In guinea pigs, granulomas typically develop as discrete foci of macrophages mixed with granulocytes and lymphocytes. They progressively develop necrotic cores surrounded by an inner layer of macrophages and neutrophils and an outer layer consisting mainly of lymphocytes. There is further extensive coalescence of granulomas surrounded by macrophages, scattered lymphocytes and neutrophils. CD4+ and CD8+ cells are equally distributed throughout the lymphocyte-rich regions (Turner, Basaraba, and Orme, 2003). The distribution of CD4+ and CD8+ cells is one characteristic of granuloma formation that varies between the guinea pig and mouse models of infection. In mice, the accumulation of lymphocytes surrounding the foci of macrophages (Rhoades, Frank, and Orme, 1997;Cardona *et al.*, 2000) is predominantly composed of CD4+ cells, while CD8+ cells accumulate initially at the periphery and are later found throughout the lymphocyte-rich regions (Gonzalez-Juarrero *et al.*, 2001). Additionally, only small foci of necrosis, if any, are observed and usually only occur late in the disease process. Although neither the guinea pig, nor the mouse is a perfect model of granuloma formation in humans, each has its

similarities to humans. The development of central necrotic cores surrounded by macrophages and lymphocytes in guinea pigs and the early formation of the granuloma and the distribution of lymphocytes in the mouse model closely resemble the characteristics of granulomas in humans. In a number of animal models, susceptibility to *M. tuberculosis* infection or virulence of an *M. tuberculosis* strain, as demonstrated by increased bacterial counts, increased dissemination of bacteria beyond the site of primary infection and/or decreased time of survival, is often associated with poor granuloma formation and organization (Cooper *et al.*, 1993;Bean *et al.*, 1999;Saunders, Frank, and Orme, 1999;Shimono *et al.*, 2003).

1.1.1.4. Innate Immunity

The initial response to *M. tuberculosis* is dependent on three main cell types: macrophages, dendritic cells and natural killer cells. Each of these cell types is important in the initial containment of the bacteria, the innate immune response against *M. tuberculosis* and the initiation of an adaptive immune response. The development of an effective innate immune response against *M. tuberculosis* is thought to require the recognition of mycobacterial components. Toll-like receptors (TLR) represent a family of membrane receptors that respond to microbial products, such as lipopolysaccharide (LPS), lipoproteins and peptidoglycan, and mediate the transcription of genes involved in the innate immune response. TLR2 and TLR4 have been implicated in *M. tuberculosis*-induced cellular activation (Means *et al.*, 1999;Means *et al.*, 2001) and signaling through these TLRs induces secretion of IL-12 and TNFα by macrophages (Jones *et al.*, 2001;Means *et al.*, 2001;Reiling *et al.*,

2002;Drennan *et al.*, 2004). In murine macrophages, expression of a dominant negative form of TLR2 or TLR4 substantially blocks *M. tuberculosis*-induced cellular activation, and co-expression of both dominant negative forms blocked virtually all *M. tuberculosis*-induced activation (Means *et al.*, 2001). Similarly, primary macrophages isolated from TLR2-/- or TLR4-/- mice secrete less IL-12 and TNFα in response to *M. tuberculosis* than wild-type controls (Reiling *et al.*, 2002;Drennan *et al.*, 2004). Although *M. tuberculosis* can activate signaling through both TLR2 and TLR4, most individual ligands that have been identified have been TLR2 ligands. Means *et al.* identified a soluble, heat-stable, protease-resistant factor that mediates TLR2-dependent activation and a heat-sensitive, cell-associated factor that mediates TLR4-dependent activation (Means *et al.*, 1999). The 19kDa lipoprotein secreted antigen (Brightbill *et al.*, 1999), PIM (Jones *et al.*, 2001;Gilleron, Quesniaux, and Puzo, 2003) and lipomannan (LM) (Quesniaux *et al.*, 2004) have also been identified as TLR2-specific ligands, possessing high capacities for induction of IL-12 and TNFα production. Through TLR2 signaling, the 19kDa lipoprotein also induces dendritic cell maturation (Hertz *et al.*, 2001) and activation of alveolar macrophages, enabling killing of intracellular *M. tuberculosis* independent of nitric oxide (NO) release (Thoma-Uszynski *et al.*, 2001).

To further determine the importance of TLRs in protection against *M. tuberculosis*, a number of groups have challenged TLR knock-out mice with *M. tuberculosis* to look for defects in host responses. Contrary to the findings from *in vitro* studies, there has been little evidence indicating the importance of these

pathways from *in vivo* studies. Infections of TLR4-deficient mice with *M. tuberculosis* have revealed at most a very mild phenotype. Two studies using aerosol infections demonstrated no differences in time to death, bacterial counts, recruitment of cells or granuloma formation (Reiling *et al.*, 2002;Shim, Turner, and Orme, 2003). Branger *et al* (Branger *et al.*, 2004) used the intranasal route of infection and demonstrated a slightly increased mortality and higher bacterial counts in the TLR4-defective mice with increased inflammation in the lungs. The deletion of TLR2 may lead to a slightly more pronounced phenotype than that seen in TLR4-deficient mice, although it also remains a relatively modest phenotype. With a low-dose aerosol infection, survival of the TLR2-deficient mice is comparable to wild-type, while the colony forming units (CFU) in the lungs, spleens and livers of infected mice are similar or slightly increased in the TLR2-deficient mice (Reiling *et al.*, 2002;Sugawara *et al.*, 2003a;Drennan *et al.*, 2004). However, infection with a higher infectious dose of *M. tuberculosis* revealed a greater susceptibility of the TLR2 defective mice compared to controls (Reiling *et al.*, 2002;Drennan *et al.*, 2004) and increased inflammation with large coalescing lesions, focal necrosis and few organized granulomas (Drennan *et al.*, 2004). Contrary to TLR4 and TLR2, MyD88 knockout mice have increased susceptibility to *M. tuberculosis* infection demonstrated by decreased survival and increased CFU (Scanga *et al.*, 2004;Fremond *et al.*, 2004). In addition, these mice displayed significant inflammation with increased neutrophilic infiltration, poor granuloma formation and extensive necrosis (Sugawara *et al.*, 2003b;Scanga *et al.*, 2004;Fremond *et al.*, 2004). MyD88, a common downstream signaling molecule of TLRs, is essential for *M. tuberculosis*-induced macrophage

activation (Underhill *et al.*, 1999). Together this indicates that activation of this pathway, regardless of the mechanism through which activation is induced, is important in the protective response against *M. tuberculosis*.

Activation of macrophages through TLRs and phagocytosis of *M. tuberculosis* trigger the induction of both an innate and an adaptive immune response. The early production of TNFα and IL-12 by macrophages plays a crucial role in the development of these responses. TNFα, produced by macrophages, monocytes and dendritic cells stimulated by *M. tuberculosis* or mycobacterial products, can act as an autocrine regulator to stimulate macrophages to produce IL-1, IL-6 and various chemokines, and is important in macrophage activation and granuloma formation. TNFα is also later produced by T lymphocytes to further activate macrophages. Mice lacking TNFα are less capable of containing an *M. tuberculosis* infection, showing increased proliferation and survival of the bacteria, and have a lower mean survival time following infection (Flynn *et al.*, 1995;Bean *et al.*, 1999;Saunders *et al.*, 2005). Granuloma formation in these mice is also impaired, indicated by poorly organized granulomas with extensive necrosis and neutrophilic infiltration (Flynn *et al.*, 1995;Bean *et al.*, 1999). IL-12 is produced mainly by phagocytic cells and exerts its protective effects largely through the induction of IFNγ. IL-12p40 knockout mice are highly susceptible to *M. tuberculosis* infection demonstrated by increased CFU and dramatically decreased time to death compared to wild-type mice. The lack of IL-12 contributes to decreased IFNγ and delayed production of TNFα, to which the authors attributed the delayed macrophage activation signified by the reduced expression of

iNOS in these mice (Cooper *et al.*, 1997). IL-12 is important in the production of IFNγ and to promote the polarization of naïve T lymphocytes to a Th1 phenotype. Early stimulation of NK cells by IL-12, as well as direct stimulation by mycobacterial products, contribute to the early production of IFNγ, which further stimulates macrophages (Trinchieri, 1994;Iho *et al.*, 1999). In response to *M. tuberculosis* infection, dendritic cells produce IL-12 and efficiently present *M. tuberculosis*-derived antigens (Jiao *et al.*, 2002). *M. tuberculosis*-infected DC have been identified in lymph nodes in both TB patients and experimentally infected animals (Jiao *et al.*, 2002;Tailleux *et al.*, 2003) and are thought to be an important contributor to the development of an adaptive immune response against *M. tuberculosis*.

Production of IFNγ is crucial in the control and containment of *M. tuberculosis* infection in mice. IFNγ–knockout mice quickly succumb to infection and are burdened by high numbers of bacteria, which are widely disseminated throughout the mice (Flynn *et al.*, 1993;Cooper *et al.*, 1993). Granuloma formation initially appears similar to wild-type mice, but rapidly progresses to extensive necrosis with neutrophilic infiltration not seen in control mice. In wild-type mice, *M. tuberculosis* infection leads to high serum levels of reactive nitrogen intermediates and increased expression of inducible nitric oxide synthase (iNOS). Neither of these features were present in the *M. tuberculosis*-infected IFNγ–knockout mice, indicating that macrophage activation is severely limited in the absence of IFNγ (Flynn *et al.*, 1993).

1.1.1.5. Adaptive Immunity

1.1.1.5.1. Helper T cells

CD4+ T cells are one of the most important cell types involved in the control of *M. tuberculosis* infection. This has become increasingly evident in part from the high incidence of TB observed in HIV/AIDS patients, in whom the susceptibility to both exogenous infection and reactivation of latent infections is increased (WHO, 2003). Loss of CD4+ cells, shown by murine studies of cell depletion (Muller *et al.*, 1987) and gene knockouts (Caruso *et al.*, 1999;Mogues *et al.*, 2001) impairs the ability to control *M. tuberculosis* infection and substantially decreases time to death. Similarly, depletion of CD4+ cells in chronically infected mice led to reactivation of the low-grade, persistent infection, indicated by increased bacterial proliferation, prominent areas of necrosis in the lungs and decreased survival (Scanga *et al.*, 2000). In addition, bacterial counts can be significantly reduced by adaptive transfer of CD4+ cells into *M. tuberculosis*-infected athymic mice, demonstrating the protective role of these cells (Tascon *et al.*, 1998). A significant Th1 response develops in response to *M. tuberculosis* infection, with high levels of IFNγ and IL-12 being produced, and little IL-4 and IL-10. A lack of either IFNγ or IL-12 leads to severe disease and rapid death. Consistent with this, in *M. tuberculosis*-infected CD4-knockout mice, IFNγ production is initially low, and despite some compensation by CD8+ cells later in infection, these mice succumb to infection significantly earlier than their wild-type counterparts (Caruso *et al.*, 1999) In addition to the importance of IFNγ production, CD4+ cells contribute substantially to infection control by other mechanisms, demonstrated by the depletion of these cells in chronically infected mice

in which infection worsens despite high levels of NOS2 and IFNγ expression being maintained (Scanga *et al.*, 2000).

1.1.1.5.2. Cytotoxic T cells

Although the role of CD8+ T cells in controlling *M. tuberculosis* infection in humans has not been demonstrated through a natural experiment, as has been the case for CD4+ T cells, a number of lines of evidence support the role of cytotoxic T cells as an important component of the adaptive immune response against *M. tuberculosis*. A functional lack of CD8+ cells, through either cell depletion or gene knockouts, leads to an impaired response and increased susceptibility to *M. tuberculosis*, demonstrated by increased bacterial counts, altered histopathology or decreased time to death (Muller *et al.*, 1987;Flynn *et al.*, 1992;Sousa *et al.*, 2000;van Pinxteren *et al.*, 2000;Mogues *et al.*, 2001;Rolph *et al.*, 2001). Additionally, the transfer of CD8+ cells to athymic mice reduces the bacillary burden following infection with *M. tuberculosis*. The ability of these mice to control the infection was dependent on the production of IFNγ (Tascon *et al.*, 1998). In response to mycobacteria, CD8+ T cells are recruited to the sites of infection and are capable of producing IFNγ (Lewinsohn *et al.*, 1998;Serbina and Flynn, 1999;Feng *et al.*, 1999;Smith *et al.*, 1999) and *M. tuberculosis*-infected macrophages and dendritic cells can be directly lysed by antigen-specific CD8+ cells (Tan *et al.*, 1997;Stenger *et al.*, 1997;Lalvani *et al.*, 1998;Serbina *et al.*, 2000). Important aspects of the mechanisms through which CD8+ cells respond to *M. tuberculosis* and help control the infection remain incompletely elucidated, but these cells are now being recognized as significant

contributors to the control of *M. tuberculosis* infection and the loss of these cells cannot be completely compensated for by CD4+ cells.

1.1.1.5.3. Humoral Immunity

Humoral immunity is generally considered to play an insignificant role in protection against *M. tuberculosis*. Johnson *et al.* (Johnson *et al.*, 1997) reported a normal course of infection in B cell deficient mice compared to control mice, as well as similar pathology between the two groups. Vordermeier *et al.* however, reported increased CFU counts in the lungs and spleen of B cell deficient mice (Vordermeier *et al.*, 1996). Despite the increased bacterial load though, the lack of B cells did not confer a lethality phenotype, suggesting humoral immunity may play a minor role in the control of the infection, but not a necessary role for survival. More recently, Bosio *et al* (Bosio, Gardner, and Elkins, 2000) reported reduced immunopathology and delayed bacterial dissemination in B cell knockout mice. Direct comparison of these results is difficult due the differences in strains and route of infection used, but they do illustrate the confusion surrounding the role humoral immunity in *M. tuberculosis* infection. Several mycobacterial antigens that induce a humoral response have been identified and are generally considered for their use as diagnostic agents.

1.1.2. Mechanisms of Evasion of Host Defenses

1.1.2.1. Reactive Nitrogen Intermediates and Reactive Oxygen Intermediates

The generation of large amounts of reactive nitrogen intermediates (RNI) and reactive oxygen intermediates (ROI) is a major mechanism through which macrophages exert their antimicrobial properties. Mutations in these pathways in humans result in a disease called chronic granulomatous disease, a condition in which afflicted individuals suffer recurrent life-threatening infections due to the inability of phagocytic cells to generate sufficient RNI and ROI to contain the infections. Animal models with mutations in these pathways are highly susceptible to infections from many organisms, including *M. tuberculosis* (Bogdan, Rollinghoff, and Diefenbach, 2000). Several mechanisms have been identified by which *M. tuberculosis* can respond to and protect itself from the bactericidal challenge posed by RNI and ROI. Although no single pathway has been determined to be essential to the ability of *M. tuberculosis* to respond to RNI/ROI, there is evidence identifying key pathways through which *M. tuberculosis* can evade this toxicity.

Alkyl hydroperoxide reductase C (AhpC) is a member of the highly conserved peroxiredoxin family of peroxidases. A compensatory up-regulation of *ahpC* was observed in isoniazid resistant strains of *M. tuberculosis*, whose resistance was due to a mutation in *katG*, the gene encoding the mycobacterial catalase-peroxidase-peroxynitritase. A mutation in *katG* is expected to impair the organism's ability to

metabolize hydrogen peroxide, however, the up-regulation of ahpC was sufficient to compensate for this loss. AhpC has been confirmed to function as both a peroxidase (Hillas *et al.*, 2000) and a peroxynitrite reductase (Bryk, Griffin, and Nathan, 2000). Chen *et al* (Chen, Xie, and Nathan, 1998) demonstrated protection of *Salmonella typhimurium* and of human epithelial cells by *M. tuberculosis* AhpC against hydroperoxides and RNI, as well as protection of the latter from cytotoxicity and apoptosis induced by NOS2. Loss of *ahpC* in *M. tuberculosis* increases susceptibility to cumene hydroperoxide (Springer *et al.*, 2001) and peroxynitrite (Master *et al.*, 2002) and results in decreased survival in resting macrophages (Master *et al.*, 2002).

Deletion of the *katG* gene in *M. tuberculosis* demonstrated the importance of KatG in the response to ROI (Ng *et al.*, 2004). The *katG*-knockout strain was attenuated in C56Bl/6 and $NOS2^{-/-}$ mice, but were indistinguishable from wild-type *M. tuberculosis* in $gp91^{Phox-/-}$ mice that are unable to produce an oxidative burst. Interestingly, KatG was more important for survival of *M. tuberculosis* in mice between two and four weeks of infection suggesting that either the alternative pathways compensate for the deletion of *katG* or that the activity of phagocyte NADPH oxidase wanes during infection.

Several pathways for peroxidase and peroxynitrite reductase activity in *M. tuberculosis* have been proposed, two of which involve AhpC. Bryk *et al* (Bryk *et al.*, 2002) elucidated a pathway composed of dihydrolipoamide succinyltransferase (SucB), dihydrolipoamide dehydrogenase (Lpd), AhpD and AhpC. This pathway efficiently metabolizes hydrogen and alkyl hydroperoxides, as well as peroxynitrite, thereby constituting both peroxidase and peroxynitrite reductase systems. AhpC also shows

peroxidase activity equivalent to that observed in the AhpC/AhpD system when combined with thioredoxin C (TrxC) and thioredoxin reductase (TR). However, a third system constituted of TrxC, TR and thiol peroxidase (Tpx) showed peroxynitrite reductase activity of one order of magnitude greater than that of the AhpC system (Jaeger *et al.*, 2004). AhpC clearly plays a significant role in the protection of *M. tuberculosis* against ROI and RNI, however, it has become clear that it is only one of the several important components comprising the systems fulfilling this function.

Several other pathways have been proposed as protective mechanisms against RNI/ROI, however the relative importance of these pathways and their mechanisms are under investigation. In screening transposon mutants for sensitivity to nitrite, Darwin *et al* (Darwin *et al.*, 2003) identified 12 highly sensitive mutants, five of which had mutations in two genes proposed to be proteasome associated genes. *Rv2115c* has been proposed to help unfold and translocate proteins to the proteolytic core of the proteasome and *Rv2097c* has been proposed to be involved in the recognition of proteins destined for degradation. Although these mutants were highly sensitive to nitrosative stress *in vitro*, the contribution of these proteasomal proteins to *in vivo* adaptation and survival was difficult to assess as the deletion strains were highly attenuated not only in C57Bl/6-derived macrophages and C57Bl/6 mice, but also in iNOS-/- macrophages and mice.

A second pathway involving *M. tuberculosis'* response to protein damage caused by RNI/ROI involves the enzyme methionine sulfoxide reductase (*msrA*), which reduces oxidized methionine residues in proteins. Oxidation of the methionine residues in

proteins may result from exposure to ROI/RNI, such as hydrogen peroxide and peroxynitrite. An *E. coli* strain lacking *msrA* was found to be more sensitive to hydrogen peroxide, GSNO and nitrite, however, this susceptibility could be compensated for by complementation of the strain with *msrA* from either *Escherichia coli* or *M. tuberculosis*, suggesting that MsrA may play a protective role in the response of *M. tuberculosis* to ROI/RNI (St John *et al.*, 2001). To further investigate the role of *msrA*, Douglas *et al* (Douglas *et al.*, 2004) created an *msrA*-disrupted strain in *M. smegmatis*. Contrary to the results in the *msrA* deficient strain of *E. coli*, the Δ*msrA* strain in *M. smegmatis* was not more susceptible to hydrogen peroxide, *S*-nitrosoglutathione (GSNO) or nitrite, but did show higher susceptibility to hydroperoxides. Survival of the Δ*msrA* strain in both unactivated and activated J774A.1 alveolar macrophages was significantly lower than wild-type *M. smegmatis*. Although the role of *msrA* in *M. tuberculosis* has yet to be defined, these early results indicate that *msrA* most likely plays a role not in neutralizing RNI/ROI, but instead in protecting against some of the deleterious effects of the presence of RNI/ROI.

Superoxide dismutase enzymatically converts superoxide to hydrogen peroxide and molecular oxygen, preventing the generation of more toxic molecules such as the hydroxyl radical and peroxynitrite. Although not specific to *M. tuberculosis* , *sodC* has been shown to contribute the resistance of *M. tuberculosis* to ROI (Piddington *et al.*, 2001). A *sodC* knockout strain of *M. tuberculosis* displayed significantly increased sensitivity *in vitro* to killing by superoxide, nitric oxide and a combination of the two compared to wild-type *M. tuberculosis*, as well as lower survival in macrophage

infection. Interestingly, in macrophages isolated from phagocyte oxidase-deficient mice, but not from iNOS-/- mice, this attenuation was abrogated, suggesting that the Δ*sodC* strain was specifically sensitive to killing by ROI, not RNI.

Several other genes have been identified in *M. tuberculosis* as potentially protective against RNI/ROI. The genes *noxR1* (Ehrt *et al.*, 1997) and *noxR3* (Ruan *et al.*, 1999), whose function have not been identified, have been suggested to play a role in protection of *M. tuberculosis* against RNI/ROI. Expression of *noxR1* from *M. tuberculosis* in *E. coli* and *M. smegmatis* and expression of *noxR3* in *S. typhimurium*, conferred resistance *in vitro* to GSNO, nitrite and hydrogen peroxide. Upon deletion of *noxR1* in *M. tuberculosis*, however, there was no observed difference in resistance to RNI between Δ*noxR1* and wild-type *M. tuberculosis*, nor was there a difference in proliferation and survival of these strains following infection of Balb/c mice (Stewart *et al.*, 2000). Further investigation is required to determine the role of these genes in the resistance of *M. tuberculosis* to RNI/ROI.

1.1.2.2.Inhibition of Phagolysosome Fusion

One of the most successful strategies employed by mycobacteria, including *M. tuberculosis,* to evade host defenses is that of phagosome maturation arrest (Armstrong and Hart, 1971;Vergne *et al.*, 2004). Phagosome maturation efficiently enables host cells to destroy phagocytosed bacteria, however, those containing *M. tuberculosis* do not successfully kill the invading pathogen. The hallmark features of a mycobacterial phagosome include decreased vacuolar proton-ATPases, with corresponding limited

acidification (Armstrong and Hart, 1971;Sturgillkoszycki *et al.*, 1994;Clemens and Horwitz, 1995), absence of mature lysosomal hydrolases (Clemens and Horwitz, 1995;Malik, Denning, and Kusner, 2000) and failure to acquire markers of phagosome maturation, including Rab7 and LAMP (Clemens and Horwitz, 1995;Via *et al.*, 1997).

Although the complete mechanism used by *M. tuberculosis* to arrest phagosome maturation has yet to be clearly defined, several strategies have been elucidated. Malik *et al* (Malik, Denning, and Kusner, 2000) demonstrated an alteration in calcium signaling in *M. tuberculosis*-infected phagosomes. Upon uptake of heat-killed *M. tuberculosis* or latex beads, a subsequent increase in calcium concentration, due to both a release from intracellular stores and an influx of extracellular calcium, is consistently observed. However, uptake of live *M. tuberculosis* does not lead to this same increase and this lack of a rise in calcium concentration results in increased survival of the bacteria. A pharmacological increase in calcium concentration resulted in a reversal of the block in phagosomal maturation. How this increase in calcium concentration could play a role in phagosome maturation was further examined by Malik *et al.* (Malik, Iyer, and Kusner, 2001). Phagosomes containing live *M. tuberculosis* exhibit lower levels of calmodulin (CaM), which was linked to the lack of increase in calcium concentration. Furthermore, pharmacological increase in calcium increased the percentage of phagosomes containing activated calmodulin kinase II (CaMKII). An inhibitor of CaMKII, acting by inhibiting the binding of Ca-CaM to CaMKII, blocked the maturation of phagosomes. Two conclusions were drawn from these results: 1) phagolysosome fusion requires Ca-CaM dependent activation of CaMKII and 2) decreased activation of CaMKII contributes to the inhibition of phagosomal maturation.

Early-endosomal autoantigen 1 (EEA1) is recruited to the endosome membrane via Rab5-GTP and phosphotidylinositol 3-phosphate (Patki *et al.*, 1997;Simonsen *et al.*, 1998) and is crucial for the recruitment of syntaxin 6 (Fratti, Chua, and Deretic, 2003). Syntaxin 6 has been demonstrated to be critical for trafficking between the trans-golgi network (TGN) and the endosome, which involves molecules such as vacuolar proton-ATPases and lysosomal hydrolases that are necessary for proper functioning of the phagolysosome (Bock *et al.*, 1997;Mallard *et al.*, 2002). Levels of both EEA1 and syntaxin 6 are lower on phagosomes containing *M. tuberculosis* compared to those containing latex beads, and it is the exclusion of these molecules that has been proposed to be the principal factor in *M. tuberculosis*-induced arrest in phagosome maturation. Two converging pathways involved in the recruitment of EEA1 and ultimately syntaxin 6, are disrupted by *M. tuberculosis* infection, resulting in reduced trafficking between the TGN and the endosome.

The increase in calcium concentration normally induced during phagosome maturation is involved in the recruitment of a phosphotidylinositol 3-kinase, hVPS34, and a serine/threonine kinase that mediates its activity, p150. p150/hVPS34 binds to CaM in a calcium dependent manner and is responsible for the generation of PI3P (Vergne, Chua, and Deretic, 2003). PI3P binds EEA1, and thus disruption of the calcium signaling pathway decreases recruitment of EEA1. *M. tuberculosis* mannose-capped lipoarabinomannan (ManLAM) inhibits the calcium signaling pathway by inhibiting the

initial rise in calcium concentration (Vergne, Chua, and Deretic, 2003), thereby reducing EEA1 and syntaxin 6 recruitment and thus phagosome maturation.

M. tuberculosis inhibits phagosome maturation by interfering with a second overlapping pathway involved in EEA1 recruitment. Infection with *M. tuberculosis* stimulates a MAP kinase, p38, activation of which was previously identified to displace EEA1 associated with endosome membranes (Cavalli *et al.*, 2001). Specific inhibition of p38 increased mycobacterial phagosome co-localization with EEA1, increased the late endosomal marker, LBPA, and increased acidification of the phagosome (Fratti, Chua, and Deretic, 2003), whereas stress-induced activation of p38 decreased EEA1 associated with latex bead phagosomes. Although the pathway requires clarification, it has been proposed that *M. tuberculosis* activation of p38 interferes with the cycling of Rab5, retaining it in its inactive GDP-bound form and thus interfering with both its activation of hVPS34 and its direct binding to EEA1.

Protein kinase G (pknG) is a serine/threonine kinase that is predicted to be localized to the cytoplasm and has been identified by Walburger *et al* (Walburger *et al.*, 2004) to contribute to *M. tuberculosis'* survival in macrophages by blocking lysosomal delivery. Growth of *M. bovis* BCG and *M. tuberculosis* in macrophages was inhibited in a dose-dependent manner by a pknG-specific inhibitor. Immunofluorescent staining indicated that deletion of *pknG* in *M. bovis* BGC resulted in localization of the mutant strain in LAMP-positive vacuoles, whereas the wild-type strain was found in LAMP-negative vacuoles. This was confirmed by cell fractionation of infected macrophages in

which Δ*pknG* BCG was resolved in the lysosomal fractions and wild-type BCG was resolved in the non-lysosomal fractions. Furthermore, growth in macrophages was severely restricted for the Δ*pknG* mutant strains and not for wild-type BCG.

1.1.2.3.MHCII Inhibition

Presentation of antigens is a necessary event in the development of cell-mediated immunity against *M. tuberculosis*, which itself is necessary for the control of *M. tuberculosis* infection. *M. tuberculosis* however, interferes with the proper presentation of antigens, which thereby provides a mechanism by which it can evade immune surveillance. *M. tuberculosis*-infected monocytes have been shown to lack the ability to stimulate T-cell proliferation and IFNγ secretion (Gercken *et al.*, 1994). Noss *et al.* (Noss, Harding, and Boom, 2000) also reported that infection of bone marrow-derived macrophages with *M. tuberculosis* inhibited antigen processing and presentation. Analysis of *M. tuberculosis*-infected monocytes by FACS revealed that surface expression of MHC-II molecules is decreased in infected cells (Gercken *et al.*, 1994;Hmama *et al.*, 1998). Expression of MHCII mRNA is decreased in *M. tuberculosis*-infected cells (Noss, Harding, and Boom, 2000), however, in addition to decreased expression, diminished synthesis and processing of MHCII molecules is the major factor contributing to defective antigen presentation in infected cells (Hmama *et al.*, 1998;Noss, Harding, and Boom, 2000). The *M. tuberculosis* 19kDa lipoprotein has been implicated in the decreased processing and surface expression of MHCII molecules via a TLR2-dependent mechanism and the inhibition of IFNγ-induced gene expression (Noss *et al.*, 2001;Pai *et al.*, 2003;Pai *et al.*, 2004).

1.1.3. Response to External Condition – ECF Sigma Factors

The ability to respond and adapt to changing environmental conditions is crucial for the survival and, in many cases, the virulence of an organism. The specificity of those responses in bacteria is governed by a group of alternate sigma factors, termed extracytoplasmic function (ECF) sigma factors. First described by Lonetto *et al.* (Lonetto *et al.*, 1994), these sigma factors were identified by their similarities to the sigma70 family of sigma factors and by their responsiveness to extracytoplasmic changes. These factors associate with the core RNA polymerase and are required for promoter-specific DNA binding to initiate transcription of the genes required to respond to given extracytoplasmic signals. Three characteristics of ECF sigma factors were described by Lonetto *et al.* (Lonetto *et al.*, 1994): 1) they regulate and respond to extracytoplasmic conditions; 2) many are negatively regulated by genes within their own operons (now termed anti-sigma factors); and 3) they may control only very small regulons.

Two of the earliest described ECF sigma factors are SigE in *Streptomyces coelicolor* and AlgU in *Pseudomonas aeruginosa*. SigE was identified as a sigma factor recognizing one of four promoters from which the *dagA* gene in *S. coelicolor* is transcribed. Upon cloning and sequencing of the *sigE* gene by Lonetto *et al.*, it was found to have a high degree of similarity to sigma70-type sigma factors over most of the conserved regions. Some significant differences were identified however, notably in the region involved in the interaction with the -10 promoter region and in the region involved in preventing the free sigma factor from binding to the promoter. Based on their

similarity to each other and to the sigma70-type sigma factors and their proposed functions, Lonetto *et al.* (Lonetto *et al.*, 1994) proposed seven other sigma factors to be designated as ECF sigma factors, among which they included the *P. aeruginosa* sigma factor, AlgU. *P. aeruginosa* causes chronic infections in cystic fibrosis patients and switching from the nonmucoid to the mucoid form is associated with a worse clinical outcome. This switching has been attributed to increased secretion of alginate, the production of which is under the control of the alginate biosynthetic cluster. Transcription of *algD*, the first gene in this cluster, is initiated by the ECF sigma factor AlgU.

Mycobacterium tuberculosis has 13 sigma factors, ten of which have been classified as ECF sigma factors. Several of these sigma factors have been described and have been found to be involved in response to various stresses.

A *sigC*-knockout strain of *M. tuberculosis* CDC1551 has been described as an immunopathology mutant by Sun *et al* (Sun *et al.*, 2004). Growth in activated alveolar macrophages and in mouse bone marrow derived macrophages was similar between wild-type and the Δ*sigC* mutant. The Δ*sigC* strains also had the capacity to proliferate and persist in mice in both aerosol and intravenous infections. A significant difference between wild-type and the Δ*sigC* mutant was observed in time to death experiments. In both aerosol and intravenous infections, all mice infected with wild-type *M. tuberculosis* died within 235 and 107 days respectively, while no death was observed in mice infected with the Δ*sigC* mutant. Histopathology revealed that infection with wild-type *M.*

tuberculosis led to considerable inflammation with alveolar consolidation and advanced destruction of airspaces. The level of inflammation and destruction of tissue was significantly less in the Δ*sigC*-infected mice and it was concluded that this failure to elicit the same degree of immunopathology as wild-type *M. tuberculosis* was the reason for delayed lethality. Similarly, Karls *et al.* (Karls *et al.*, 2006) suggested that SigC is important for pathogenesis as a Δ*sigC* mutant strain of *M. tuberculosis* H37Rv was highly attenuated in guinea pigs.

Inactivation of SigD also seems to result in an immunopathology mutant, although the phenotype is less well defined. Raman *et al.* (Raman *et al.*, 2004) reported a *sigD*-knockout in *M. tuberculosis* CDC1551 that showed slight attenuation in growth in the lungs of mice, while growth in the spleen was similar to wild-type *M. tuberculosis*, and, as is typical with immunopathology mutants, showed prolonged survival compared to wild-type in a time to death model and little immunopathology. Infection with wild-type *M. tuberculosis* led to extensive lymphocytic infiltration and consolidation of lung tissue, whereas the Δ*sigD* mutant showed less lymphocytic infiltration with significant areas of healthy lung tissue interspersed with smaller granulomas. From these observations, the Δ*sigD* mutant was characterized as an immunopathology mutant. Calamita *et al.* (Calamita *et al.*, 2005), however, reported that although their *sigD*-knockout strain showed comparable growth and survival in activated and resting macrophages as wild-type, similar growth in mice determined by CFU following aerosol infection and prolonged time to death compared to wild-type, they did not observe a decrease in immunopathology. In both the Δ*sigD* mutant and wild-type infected mice,

they observed significant consolidation of lung tissue and granulomatous infection. The differences observed between the two studies could be due to the different strains in which the knockouts were created, as well as to the different mouse strains infected. An additional and significant finding also reported by Calamita *et al.* (Calamita *et al.*, 2005) was TNF production in macrophages in response to *M. tuberculosis* infection. Infection of mouse alveolar macrophage cell line J774A.1 with wild-type *M. tuberculosis* induces TNF production, which was not observed following infection with the Δ*sigD* mutant. The failure to induce cytokine responses similar to wild-type *M. tuberculosis* during infection has been cited as a possible mechanism of the described phenotype of immunopathology mutants (Kaushal *et al.*, 2002), leading the authors to propose that although their mutant did not demonstrate reduced immunopathology in their selected animal model, the Δ*sigD* mutant should be classified as an immunopathology mutant based on the other characteristics observed. Both groups reported approximately 50 genes that were down-regulated in the Δ*sigD* mutant compared to wild-type, however the expression profiles were determined under different growth conditions making a direct comparison difficult.

Disruption of *sigE* in *M. tuberculosis* created a mutant that was more sensitive to exposure to SDS, heat shock and exposure to the oxidative compounds hydrogen peroxide, cumene hydroperoxide and plumbagin (Manganelli *et al.*, 2001). The Δ*sigE* mutant also displayed lower survival than wild-type *M. tuberculosis* in THP-1 macrophage cell line and in both activated and unactivated J774A.1 alveolar macrophages (Manganelli *et al.*, 2001). Growth in mice was dependent on the strains of

mice and of *M. tuberculosis* used. The Δ*sigE* mutant created in *M. tuberculosis* CDC1551 showed similar growth to wild-type in C3H/HeJ mice, while Δ*sigE* created in *M. tuberculosis* H37Rv showed attenuated growth compared to wild-type in Balb/c mice (Ando *et al.*, 2003;Manganelli *et al.*, 2004a). In both cases, however, a delayed time to death was observed in SCID mice, and the Δ*sigE* mutant created in CDC1551 also resulted in a delayed time to death in immunocompetant C3H/HeJ mice.

Upon exposure to SDS, there is an increase in expression of *sigE* and *sigB* in *M. tuberculosis*, however, in the Δ*sigE* mutant, the increase in expression of *sigB* was blunted compared to wild-type, indicating that *sigB* expression in response to SDS is at least in part SigE-dependent. Expression of *sigE* in the Δ*sigE* strain could be detected by the use of a molecular beacon that binds to a region of the *sigE* gene located upstream of the disrupted region and after exposure to SDS, was found to be similar to wild-type, suggesting that SigE is not required for its own expression. Other genes differentially expressed in the Δ*sigE* mutant also include transcriptional regulators, ribosomal proteins, and other factors involved in translation.

Manganelli *et al* (Manganelli *et al.*, 1999) examined the differential expression of the ECF sigma factors in *M. tuberculosis* under various conditions and the most striking response observed was in two conditions: exposure to SDS and heat shock. In both cases, expression of *sigB* and *sigE* was significantly increased and in the latter, a dramatic increase in *sigH* was also observed. Using selective capture of transcribed sequences (SCOTS) and a *sigE*-GFP fusion plasmid, *sigE* and *sigH* were both shown to have increased expression in macrophage infection (Graham and Clark-Curtiss, 1999;Jensen-

Cain and Quinn, 2001). Exposure of *M. tuberculosis* to diamide results in an increase in expression of *sigB*. In both a Δ*sigE* mutant and a Δ*sigH* mutant, diamide-induced expression of *sigB* was severely blunted. In a Δ*sigE*Δ*sigH* double mutant, this response was abolished. Expression of *sigB* in response to exposure to SDS is also dependent on SigE. Expression of *sigE* and *sigB* increase upon exposure to SDS in *M. tuberculosis*, however, in the Δ*sigE* mutant, expression of *sigE* increases as in wild-type and the previously observed increase in *sigB* is significantly lower. This suggests that SigE does not control its own expression, but expression of *sigB* is, at least partially, dependent on *sigE*. Comparison of gene expression in the Δ*sigH* mutant and wild-type *M. tuberculosis* by microarray identified *sigH*, *sigE* and *sigB* all to be significantly down-regulated and similar promoter sites have been identified upstream of all three of these genes. Based on this evidence, a model has been established in which, under conditions of heat stress or oxidative stress, SigH can induce its own expression and in turn, the expression of *sigE* and *sigB*. SigE, along with being inducible by *sigH*, can be induced by heat, surface or oxidative stresses and in turn induce *sigB*. SigB does not appear to be induceable directly by environmental stresses, but instead induces expression of response systems to such stresses upon activation by SigE and SigH.

The loss of SigH in *M. tuberculosis* resulted in increased sensitivity to heat shock and oxidative stress (Raman *et al.*, 2001;Manganelli *et al.*, 2002), however, unlike the Δ*sigE* mutant, no difference was found in survival compared to wild-type *M. tuberculosis* in unactivated or activated macrophages (Manganelli *et al.*, 2002), nor was there a difference between the mutant and the wild-type strains in proliferation and

survival in mice (Kaushal *et al.*, 2002). In immunocompetant mice, a significantly longer time to death was observed for the Δ*sigH* mutant compared to wild-type, but a similar difference was not found in immunocompromised SCID mice. Differences in immunopathology were only detectable in the lungs of infected immunocompetant animals, with the Δ*sigH*-infected mice displaying fewer lesions and smaller granulomas than wild-type infected mice. No differences in immunopathology were observed in the SCID mice (Kaushal *et al.*, 2002). The delay in pathological disease was reported to correlate with a decrease in T-cell recruitment to the lungs in the Δ*sigH* infected mice. Heat shock proteins and components of the thioredoxin-thioredoxin reductase system were reported to be among the genes down-regulated with the loss of SigH.

The ability of *M. tuberculosis* to cope with exposure to hydrogen peroxide has also been partially attributed to SigJ. Loss of this sigma factor increased the susceptibility of cultures to hydrogen peroxide over both long and short exposure times. The Δ*sigJ* mutant was not more susceptible to other stresses such as heat shock, low pH and exposure to SDS, nor was its survival in mice impaired. Proliferation and survival of the Δ*sigJ* mutant and the wild-type *M. tuberculosis* were similar in Balb/c mice and there were no differences in granuloma formation or tissue damage observed, suggesting that other pathways for resistance to oxidative stress may compensate for the loss of SigJ (Hu *et al.*, 2004).

To date, there have been no works published investigating the ECF sigma factor SigK in *M. tuberculosis*. This sigma factor was in fact excluded from a study examining the differential expression of sigma factors in *M. tuberculosis* (Manganelli *et al.*, 1999).

SigL has been proposed by Hahn *et al.* (Hahn *et al.*, 2005) to be involved in the regulation of cell envelope lipid synthesis and the modification of secreted proteins based on analysis of gene expression in *M. tuberculosis* overexpressing *sigL*. Several genes up-regulated in the overexpression mutant are involved in polyketide synthesis, leading to the suggestion that the decreased virulence of the Δ*sigL* strain in mice, seen as an increase in median survival time of mice following intravenous infection, is at least in part due to the disruption of normal expression of these genes. The loss of SigL did not lead to increased susceptibility to SDS or to oxidative or nitrosative stresses. The role of SigL and the SigL-regulon in pathogenesis or response to stress was further examined by Dainese *et al* (Dainese *et al.*, 2006), who confirmed a SigL-dependent promoter of *sigB*, as well as an involvement of SigL in the expression of several polyketide synthases.

The function of the ECF sigma factor SigM was recently investigated by Agarwal *et al.* (Agarwal *et al.*, 2006) and Raman *et al.* (Raman et al., 2006). Both groups observed that expression of *sigM* was low relative to expression of *sigA*. Agarwal *et al.* determined that expression of *sigM* was increased in stationary phase and following heat shock, while Raman *et al.* did not observe any change in expression throughout various growth phases. Up-regulation of expression of a limited number of genes was observed upon overexpression of *sigM,* many of which were identified or putative secreted and

cell-surface associated products and among those commonly identified were four *esat-6* homologues. Raman *et al.* further demonstrated that genes involved in PDIM synthesis and mycolic acid synthesis are negatively regulated by SigM. In animal studies, *ΔsigM*-mutant strains were not observed to be attenuated compared to wild-type *M. tuberculosis* (Agarwal *et al.*, 2006;Karls *et al.*, 2006). Taken together, these experiments suggest that SigM is unlikely involved in virulence of *M. tuberculosis*, but may play a role in survival and adaptation during infection.

Although not all the functions or activating signals of the ECF sigma factors and their downstream effectors in *M. tuberculosis* have been clearly established, it is evident that the ability of the bacteria to adapt to a variety of environmental stresses is dependent on these networks.

1.2. Bacille Calmette-Guérin

1.2.1. Development of BCG

Sponsored by the Institut Pasteur, Albert Calmette and Camille Guérin spent years examining the peculiar characteristics of the tubercle bacillus. Calmette strongly believed that one must understand the "propagation of a disease before even thinking of possible means of treating it effectively" (Guerin, 1978) and thus studied both the clinical manifestations of the disease and the microbiological properties of the disease causing agent in detail. Knowing that many other investigators had been unsuccessful in their attempts to attenuate the bacterium, they began their own studies

using a highly virulent bovine strain of the bacillus, now known to actually have been *M. bovis*. In 1908, they observed that the addition of beef bile to a culture of the bacilli resulted in the disaggregation of the clumps of bacilli normally seen in cultures. Continued passage in the presence of beef bile did not appear to have an effect on the virulence of the bacteria and thus they continued to explore new media on which they could passage the bacteria, finally settling on a medium containing potatoes partially immersed in glycinerated beef bile. It was on this medium that they observed a change in the characteristic appearance of the culture from the typical dry, granular and rough appearance to a moist, waxy and smooth appearance (Guerin, 1978). They continued to passage the culture every three weeks on to new media and in 1921, they described a culture that was observed to be attenuated, yet retained its immunogenic properties in animals. Bacille Calmette-Guérin (BCG) was administered as a vaccine for the first time in July, 1921.

1.2.2. Clinical Trials and Efficacy of BCG

Since the first dose of BCG was administered 85 years ago, numerous studies have been initiated in attempt to define how well BCG protects against tuberculosis, yet the answer to this question remains elusive. One of the earliest studies that showed encouraging results was initiated by Heimbeck, in Norway, in 1927. Upon observation of a high rate of tuberculosis in nursing students who were tuberculin-negative prior to entry to nursing school, he began offering BCG vaccination to these students. A significantly higher number of cases of TB (6 times greater) and of deaths attributable to TB (7 times greater) were observed in the non-vaccinated group

of students compared to the vaccinated group. Also in 1927, Aronson and Dannenberg began a trial in which they vaccinated newborns in Philadelphia. In families with a known case of tuberculosis, they observed 10 deaths out of 84 non-vaccinated children and only one death out of 41 vaccinated children. Similarly, in the families without a known case of TB, two deaths were observed out of the 41 non-vaccinated children and no deaths were observed in the 15 vaccinated children.

Several other formal trials were initiated in the 1930s, two of which took place within First Nations communities in Canada and the United States and a third trial of interest took place in Chicago. In these trials, vaccination occurred either at birth or in childhood and all children were considered to be living in a high incidence environment. Significant protection against both TB cases and death due to TB was observed in all three populations, with protection ranging from 75-83% (Guerin, 1978).

After World War II, both the US Public Health Service and the Medical Research Council in the UK recommended large-scale trials. Four such trials took place beginning in 1949-1950, one of which showed a high level of protection, two a moderate/low level and one an insignificant level of protection. The trial demonstrating the highest efficacy took place in England, enrolling 54,239 individuals between 1950-52, who were aged 14 or 15 at the time of entry, and following these individuals for 20 years. Over this time period, the overall protective efficacy was determined to be 77% and although the efficacy was high over the entire

time period, when divided into five-year intervals, the efficacy decreased in each subsequent interval. However, because the countrywide incidence also decreased dramatically in England during this time period and so few cases were observed within the last interval (15-20 years after vaccination), the study concluded that the estimate of low protective efficacy during this interval was not precise and the overall protection provided by BCG vaccination against tuberculosis in this population was high (Hart and Sutherland, 1977).

A similar trend of decreasing efficacy over time was observed in Madanapalle in southern India (Frimodt-Moller, Acharyulu, and Pillai, 1973). The difference in outcome with this trial, however, was the overall protective efficacy for the 21-year observation period was determined to be only 20%. The highest efficacy was observed in the first nine years, during which the protective efficacy was estimated to be 83%, however, over the remainder of the follow-up period, several cases arose in the vaccinated group, as well as the control group, resulting in a low overall estimate of efficacy.

The US Public Health Service initiated a large-scale trial in Puerto Rico (Comstock, Livesay, and Woolpert, 1974), selecting this location because of the high mortality rate from tuberculosis in the country during the 1930s and 1940s. Vaccinations took place between 1949 and 1951, although enrolment was significantly lower than expected. An overall reduction of tuberculosis cases of 28.7% was observed over the 19-year follow-up. In contrast to the studies in England

and India, the protection attributable to vaccination varied insignificantly over the five-year intervals of observation.

A large-scale trial from which questions regarding the use of BCG in the United State arose, took place in Georgia and Alabama beginning in 1950. Over a 20-year follow-up, very little protection due to BCG vaccination was observed. However, it was noted by the authors that although protection was seemingly low, the numbers of cases that had arisen in both the vaccinated and the control groups was considerably lower than expected and thus "...there was little opportunity for *any* vaccine to contribute much to the control of tuberculosis." (Comstock and Palmer, 1966).

Further questions arose from the release of two studies, one in Chingleput, India (Baily, 1980) and the other in the Karonga district in Malawi (Ponnighaus *et al.*, 1992), which demonstrated no benefit from BCG vaccination. The Chingleput region was chosen because of its prior lack of BCG vaccination in the population and the moderate rate of TB in the region. Vaccinations took place from July, 1968 to March, 1971. 366,625 people were registered, of whom 77.8% were vaccinated (all ages). In the 15-year-followup, there was no indication of protection in BCG vaccinated individuals compared to placebo group. In Malawi, routine BCG vaccination of school-aged children began in 1974. Between 1979 and 1989, 180 cases of tuberculosis were identified in 83,455 individuals in whom BCG scar status could be

determined. As in Chingleput, the rate of tuberculosis did not differ between the BCG scar negative and the BCG scar positive groups.

Despite the extensive use of BCG worldwide and the numerous clinical trials in which BCG has been evaluated, a subset of which is presented here, its efficacy as a vaccine against tuberculosis still remains in question. A meta-analysis by Colditz *et al* (Colditz *et al.*, 1994) reported an overall protective effect of BCG vaccination of 50%, however, when extrapolating to any given population, this value is considered to be misleading since it does not address two important factors: the risk of exposure and the environment of that population. From a summary of the estimates of BCG efficacy by Fine (Fine, 1995), the overall trend suggests that, albeit low in some cases, BCG does afford some degree of protection against tuberculosis in most populations. A recent meta-analysis of the effect of BCG vaccination on tuberculous meningitis and miliary tuberculosis in children concluded that BCG vaccination is a highly cost-effective measure against childhood tuberculosis and supported the continued use of BCG, particularly in high-incidence environments (Trunz, Fine, and Dye, 2006).

1.2.3. Failure of BCG

The variable performance of BCG in clinical trials has raised several questions concerning its overall efficacy and what factors contribute to the observed discrepancies. The significant protection provided by BCG against TB in children demonstrates that BCG can effectively, and consistently, generate a protective

immune response. However, since this protection is not seen as consistently in adults or over the long-term, attention has turned to identifying the factors that contribute to either the loss or the maintenance of a protective immune response.

1.2.3.1. Failure of BCG – Environmental Mycobacteria

In comparing the different levels of protection against *M. tuberculosis* provided by vaccination with BCG, it has been suggested that exposure to environmental mycobacteria may influence the immune response that BCG is capable of inducing. Crossreactivity of many species of mycobacteria, specifically with BCG, has been demonstrated in both animal models and in humans (Lozes *et al.*, 1997;Oftung *et al.*, 1998), suggesting that exposure to other mycobacteria may influence the immune response to BCG. Generally, higher levels of protection have been observed in temperate environments and lower levels in more tropical environments. Although there are many factors that may influence this trend, exposure to non-tuberculous mycobacteria has been proposed to be a major factor. Comparison of immune responsiveness to various mycobacterial antigens in similar non-vaccinated populations in the UK and Malawi suggested, from positive IFNγ and DTH responses, that both populations had been exposed to a variety of mycobacteria. However, the greater prevalence of responsiveness to PPDs from environmental mycobacteria in the Malawian population suggested much higher exposure in this population (Weir *et al.*, 2003). Following vaccination, the increase in IFNγ and DTH responses observed in the UK population were much greater than those observed in the Malawian population (Black *et al.*, 2002). Within the Malawian population,

individuals with low responsiveness to environmental mycobacteria prior to vaccination demonstrated a greater increase in IFNγ response to *M. tuberculosis* PPD following vaccination than individuals with initial high responsiveness (Black *et al.*, 2001). Two hypotheses, regarding the exposure to environmental mycobacteria, explaining the apparent failure of BCG have been proposed: the masking hypothesis and the blocking hypothesis.

1.2.3.1.1. Environmental Mycobacteria: Masking Hypothesis

The masking hypothesis suggests that environmental mycobacteria stimulate a baseline level of immunity to *M. tuberculosis* and any additional protective immunity generated by BCG vaccination is limited. Palmer and Long (Palmer and Long, 1966) originally suggested this hypothesis based on their observations that vaccinating guinea pigs with environmental mycobacteria could protect against an *M. tuberculosis* challenge to a varying degree, but also reduced the protective effects of subsequent BCG vaccination. Similar results were observed by Orme and Collins (Orme and Collins, 1984) with *M. avium* infection in mice. Mice pre-sensitized with *M. avium* were protected against an *M. tuberculosis* challenge, but no further protection was observed in mice that were both pre-sensitized and BCG vaccinated. The level of protection induced by prior sensitization depended on the species of mycobacteria, with *M. avium* demonstrating the highest level of induced protection (greater than BCG), *M. kansasii* and *M. simiae* inducing a moderate level and no protection being induced by *M. scrofulaceum*. Regardless of the level of protection afforded by sensitization, all groups of mice (unsensitized/vaccinated or sensitized/vaccinated)

demonstrated similar levels of protection. The protection induced by the pre-sensitization with environmental mycobacteria masked the protection that would otherwise be induced by BCG, making the protection attributable to BCG apparently lower.

The measured effect will be the difference between the protection afforded by sensitization and that provided by BCG, effectively changing the non-vaccinated control group from a mycobacteria naïve group to a previously exposed group. This hypothesis suggests that the baseline to which vaccination is compared will depend upon the degree of sensitization in the population, with a higher degree of sensitization providing a higher degree of masking and therefore a lesser apparent effect. De Lisle *et al.* (de Lisle *et al.*, 2005) compared the protective efficacy of BCG in guinea pigs, both sensitized and unsensitized with *M. avium*, against an *M. bovis* challenge. Sensitization alone afforded a low level of protection. BCG vaccination in sensitized animals showed equal or slightly lower protection than BCG vaccination in unsensitized animals when each group was compared to control animals that were both unsensitized and unvaccinated. The reduction in CFU counts in vaccinated-sensitized animals when compared to unvaccinated-sensitized animals, however, is less than the reduction seen when compared to unvaccinated-unsensitized animals since sensitization alone provided a base level of protection. The apparent reduction in CFU is dependent on the control group to which the vaccinated group is compared, while the actual CFU counts were similar in all vaccinated groups regardless of sensitization.

Based on this hypothesis, BCG vaccination in a previously sensitized population will indeed provide less protection than in a population with relatively low exposure to mycobacteria because, to some extent, a protective immune response has already been generated in this population. This apparent failure of BCG in widely exposed populations will then reflect a redundancy in immune responses, rather than an inability to stimulate a protective response. It does suggest that a vaccine that can further boost the immune response above that stimulated by environmental mycobacteria would be advantageous.

1.2.3.1.2. Environmental Mycobacteria: Blocking Hypothesis

The blocking hypothesis suggests that the immune response generated by environmental mycobacteria to common mycobacterial antigens is sufficient to block the replication of BCG, which is crucial for the induction of protective immunity. This hypothesis, contrary to the masking hypothesis, suggests that BCG cannot induce a strong protective immune response in highly sensitized populations. Prior sensitization with certain mycobacteria can inhibit BCG growth in animal models (Lozes *et al.*, 1997;Brandt *et al.*, 2002;Demangel *et al.*, 2005). Brandt *et al.* (Brandt *et al.*, 2002) originally proposed the blocking hypothesis based on observations that BCG vaccinated mice were significantly protected against *M. tuberculosis*, while mice that were first sensitized and subsequently vaccinated were not protected. Demangel *et al.* (Demangel *et al.*, 2005) found that mice pre-sensitized with *M. avium* inhibited BCG growth to a greater extent than *M. vaccae* or *M. scrofulaceum* and

correspondingly, BCG vaccination in mice pre-sensitized with *M. vaccae* and *M. scrofulaceum* induced greater protection against *M. tuberculosis* than in *M. avium* sensitized mice. As a comparison, a recombinant strain of BCG (BCG::RD1), which has been shown to persist longer than BCG in mouse infections, was tested in the same model. Growth of BCG::RD1 was not as restricted in pre-sensitized mice as BCG and, most interestingly, it induced greater protection in the lungs and similar protection in the spleen of pre-sensitized mice compared to unsensitized mice. It was concluded that if BCG replication is not inhibited, then a protective immune response can be generated in addition to that provided by exposure to environmental mycobacteria. Therefore, use of a strain of BCG whose replication is not inhibited by prior exposure of the host to environmental mycobacteria or by a vaccine that does not depend on replication to induce a protective response may be most beneficial.

1.2.3.2. Failure of BCG – Attenuation of BCG

Among the variables that must be considered when analyzing the protective efficacy of BCG, the vaccine itself must be examined. Behr and Small (Behr and Small, 1997) demonstrated by plotting vaccine efficacy versus passage number for the strains of BCG that had been used in multiple trials, that increased passage number correlated with decreased efficacy in four of the five strains analyzed. Behr further described the strains used, the passage numbers and the trials in which they were used and although acknowledging that all the variables between the trials could not be controlled for, the analysis revealed a trend of decreasing efficacy to be associated with increased passage number (Behr, 2001). Analysis of some the genetic changes

that have accumulated in the BCG strains over time may elucidate phenotypic changes affecting vaccine efficacy and is a focus of this thesis.

1.2.4. Current Use

BCG strains are currently the only vaccines used for tuberculosis prevention and are widely used throughout the world, with an estimated 2 million doses given per week. WHO vaccination policies vary by country, however, the most common recommendation is for vaccination at birth (Fine P.E. *et al.*, 1999). Currently, there are five substrains (Russia, Tokyo, Danish, Moreau and Pasteur) that account for greater than 90% of production (WHO, 2005).

1.3. Evolution of BCG

1.3.1. Phylogeny

As BCG started to be used around the world, aliquots were disseminated at various times to several locations and continued passaging occurred at many sites worldwide, in addition to the Pasteur Institute. As these aliquots were cultured in various laboratories, there were reports of varying morphological characteristics, growth characteristics and reactions in animal and human hosts.

In 1996, Mahairas *et al.* (Mahairas *et al.*, 1996) analyzed the genetic differences between *M. bovis* and strains of BCG and, through the use of subtractive genomic hybridization, three distinct regions of difference (RD) were identified. The

10 ORFs comprising RD3 were identified as missing in all BCG strains analyzed, but present in virulent laboratory strains of *M. tuberculosis* and *M. bovis*. Upon comparison with clinical strains of *M. tuberculosis*, this region was only found in a minority of these strains and thus determined to not likely be of high importance in the virulence of *M. tuberculosis*. This predicted prophage was possibly absent in the *M. bovis* from which BCG was derived or may have occurred during the multiple passages leading to the attenuation of BCG. RD2 was identified as a 10.8kB, 11 ORF region, missing in a subset of BCG strains. Two imperfect direct repeats are located at the deletion breakpoints, suggesting a mechanism by which this region may have been deleted. The RD1 region was found to be present in all clinical strains of *M. tuberculosis*, as well as the laboratory strains H37Rv and Erdman and in *M. bovis*, while it is lacking in all strains of BCG. Based on this distribution and the prediction that at least one gene in the RD1 region is a virulence factor or involved in the regulation of other virulence factors, RD1 was predicted to be the attenuating mutation of BCG.

By constructing a genealogy based on written records of BCG dissemination and using IS*6110* and *mpt64* molecular typing, Behr and Small (Behr and Small, 1999) established a preliminary phylogeny of BCG, which was further elaborated upon by DNA microarray analysis comparing *M. bovis* and BCG strains to *M. tuberculosis* H37Rv (Behr *et al.*, 1999). From this, 16 deletions were identified, five of which are BCG-specific deletions (one missing in all BCG – RD1, four missing from various substrains – RD2, RD8, RD14, RD16). In addition to confirming

previously identified deletions, Mostowy *et al.* (Mostowy *et al.*, 2003) identified three further deletions, two of which are specific to one strain each (RDRussia, RDFrappier) and one that is deleted from both BCG Denmark and BCG Glaxo. Figure 1.1 shows the complete phylogeny of BCG.

1.3.2. RD1

The attenuation of *M. bovis* can at least in part be attributed to the loss of the RD1 region. Pym *et al* (Pym *et al.*, 2002) complemented RD1, RD3, RD4, RD5 and RD7 into BCG Pasteur to examine what, if any, role these regions played in the attenuation of BCG. Only complementation of RD1 led to a significant phenotypic change. Colony morphology of BCG Pasteur::RD1 appeared more similar to *M. tuberculosis* or *M. bovis* than typical BCG, developing a spreading, less rugose morphology. Virulence in mice was partially restored, with modest differences in persistence also observed. In SCID mice, BCG::RD1 proliferated to a 100-1000-fold higher level than BCG, as well as inducing severe inflammation and the formation of granuloma-like structures not seen in BCG-infected mice. The level of virulence, however, was not fully restored to the levels of *M. tuberculosis* H37Rv.

Deletion of the RD1 region severely attenuated *M. tuberculosis* H37Rv (Lewis *et al.*, 2003). While *M. tuberculosis* H37Rv infected and multiplied rapidly in THP-1 cells, H37RvΔRD1 increased only 3-fold over 7 days, appearing much more similar to BCG, which did not replicate intracellularly. Similarly, H37Rv infection led to more than 80% destruction of the cell monolayer over 7 days, while very little cell death

was observed in BCG- or H37RvΔRD1-infected cells. Murine infections with the RD1 knockout strain also revealed a BCG-like phenotype. Contrasted to the rapid proliferation of H37Rv in the lungs and dissemination to the spleen following aerosol infection, growth of H37RvΔRD1 and BCG Russia was slow, with delayed dissemination to the spleen. Infection with H37RvΔRD1 and BCG Russia was indistinguishable by histopathology, revealing very little inflammation and no granuloma formation at 6 weeks, a time after which H37Rv infection led to severe inflammation and granuloma formation. Differences in growth and histopathology were apparent in a long-term infection model (57-68 weeks) (Sherman *et al.*, 2004), indicating that deletion of RD1 did not fully attenuate *M. tuberculosis* to the levels of BCG. Survival of mice infected with H37RvΔRD1 was significantly prolonged compared to H37Rv, yet led to more rapid death than BCG, again indicating that loss of RD1 did not fully attenuate *M. tuberculosis* to BCG levels (Hsu *et al.*, 2003).

Attenuation of virulence of H37RvΔRD1 strains in murine infection was confirmed by Hsu *et al.* (Hsu *et al.*, 2003), who additionally demonstrated that disruption the ESAT-6/CFP10 operon was sufficient to attain similar levels of attenuation as those observed with the loss of RD1. ESAT-6 and CFP10 are secreted proteins, which are encoded for by genes in the RD1 region. Lung histopathology of Balb/c mice infected with this strain revealed the bacilli are largely contained in intact macrophages located in the alveolar spaces, whereas evidence of macrophage lysis and the presence of *M. tuberculosis* in macrophages located in interalveolar walls was apparent in wild-type *M. tuberculosis*-infected mice. Disruption of other genes in the

RD1 region (*Rv3871, Rv3876/3877*) resulted in strains defective in the secretion of ESAT-6 and CFP10 (both proteins were produced and detected intracellularly) and attenuated in SCID mice. It was proposed that ESAT-6 is an effector molecule responsible for cytolysis, inducing cell-to-cell spreading and tissue invasiveness.

Further evidence that other genes in the RD1 region are required for secretion of ESAT-6 and CFP was provided by Gao *et al.* (Gao *et al.*, 2004) and Guinn *et al.* (Guinn *et al.*, 2004). Disruption of individual genes in the RD1 region, specifically *Rv3870*, *Rv3871* and *Rv3876*, led to the loss of secretion of ESAT-6 and CFP, even though these proteins were detectable within the bacilli. Decreased cytolysis and cell-to-cell spreading have been attributed to the lack of secretion of these proteins. During infection of THP-1 cells with *M. tuberculosis*, the percentage of cells infected increased over time and the majority of cells were infected with fewer than 10 bacilli. With secretion-deficient mutants, the percentage of cells infected remained steady, while the proportion infected with greater than 10 bacilli increased, indicating the ability of the bacteria to replicate but not to spread to new cells (Guinn *et al.*, 2004). Individual mutants were comparably attenuated in animal models to H37RvΔRD1. Early suggestions proposed that ESAT-6 and CFP10 activity is mediated through the formation of a pore, however recent structural data contradicts this idea suggesting a signaling and receptor binding function of the complex (Renshaw *et al.*, 2005). The proteins form a stable 1:1 dimer (Renshaw *et al.*, 2002) and the formation of this dimer appears to be important for virulence (Brodin *et al.*, 2005).

ESAT-6 and CFP10 are both immunogenic proteins, stimulating strong cellular immune responses during *M. tuberculosis* infection. This has been demonstrated both in TB patients (Johnson *et al.*, 1999;Dillon *et al.*, 2000), as well as in several animal models (Brandt *et al.*, 1996;Vordermeier *et al.*, 2001;Brusasca *et al.*, 2001;Kamath *et al.*, 2004), suggesting the importance of these proteins in early infection. The strongly induced immune response to these proteins has prompted their inclusion in various vaccine formulations (to be discussed in section 1.4). Additional gene products encoded for in the RD1 region have been examined for immunogenic properties. Although ESAT-6 and CFP10 induce the strongest responses, cellular responses to PPE68 (*Rv3873*), *Rv3878* and *Rv3879c* have also been detected (Okkels *et al.*, 2003;Agger *et al.*, 2003;Liu *et al.*, 2004). There is significant interest in identifying immunogenic proteins in the RD1 region for their potential use in diagnostic testing for *M. tuberculosis* infection.

1.3.3. RD2

Several years after the development and continued passaging of BCG, reports of an additional loss of virulence of BCG were published (Oettinger *et al.*, 1999). From the established timeline of deletions in BCG, it has been noted that this secondary loss of virulence coincided with the loss of the RD2 region and it has been speculated that a function link exists between the observed change in virulence and the loss of this region. The genes in the RD2 region include a ribonucleotide reductase subunit, *lysE*-family and *lysR*-regulatory proteins, a methyltransferase and

the antigenic protein MPT64, the most widely described of the RD2 gene products (Figure 1.2).

MPT64 was identified as a culture filtrate protein, abundantly produced and secreted, and detectable as early as day 2 in *in vitro* cultures (Harboe *et al.*, 1986;Fifis *et al.*, 1991;Andersen *et al.*, 1991). The highly immunogenic protein induces a prominent cellular immune response in animal models, including guinea pigs and cattle (Haga *et al.*, 1995;Oettinger *et al.*, 1995;Lightbody *et al.*, 1998). Similarly, a strong response to MPT64 is detectable in TB patients (Roche *et al.*, 1994;Roche, Feng, and Britton, 1996). From the strength of these responses, the potential importance of MPT64 in inducing a protective immune response and its inclusion in DNA and subunit vaccines has been supported. The absence of *mpt64* in several substrains of BCG was first noted by Li *et al.* (Li *et al.*, 1993) and it was subsequently confirmed as a constituent of the deleted RD2 region (Mahairas *et al.*, 1996), raising the question of the possible effect this loss has had on the protective immunity BCG is capable of inducing.

Several other RD2 products have been identified as inducing immune responses in animals and TB patients, however, as the levels of induction have been relatively low, none have been classified as major antigens (Cockle *et al.*, 2002;Liu *et al.*, 2004).

M. tuberculosis is known to be intrinsically resistance to macrolides, although sensitivity of certain strains of BCG to macrolides has been reported. This phenotype has recently been attributed to the loss of the RD2 gene *Rv1988* (Buriankova *et al.*, 2004), which encodes a product showing strong similarity to the Erm family of methyltransferases. Methylation of the 23S rRNA by these methyltransferases reduces binding of macrolides to the ribosome, inhibiting the macrolide-stimulated dissociation of the peptidyl-tRNA from the ribosome, thus conferring resistance.

The role of the ribonucleotide reductase subunit R2-1 encoded by *nrdF1* (*Rv1981c*) appears to be secondary to that of *nrdF2* (*Rv3048c*). The R2-2 subunit, encoded by *nrdF2*, preferentially associates with the R1 subunit to form a functional holoenzyme. The R2-1 subunit can form an active holoenzyme with R1 only when present in high concentrations (Yang *et al.*, 1997). In addition, a knockout of R2-2 in *M. tuberculosis* could only be successfully created when the strain was first complemented with a second copy of R2-2, indicating that R2-1 could not compensate for the loss of R2-2 and may serve a different, unidentified function (Dawes *et al.*, 2003).

1.3.4. Other Deletions

Several other deletions, some of which affect multiple strains and some of which are strain specific, have been identified. They range from 21 to 9073 bp in size and include genes annotated as transcriptional regulators, a magnesium transporter (*Rv1811c*), a sigma factor (*sigI–Rv1189*), *mce4* family proteins (*Rv3495-3497*), and a

PE_PGRS family protein (*Rv1768*). Three genes annotated as transcriptional regulators have been deleted from BCG (*Rv1773c, Rv1985c, Rv3405c*), making this category significantly over-represented in the deletions in BCG compared to their frequency in the genome (Behr *et al.*, 1999). In addition to these deletions, several significant SNPs that have resulted in functional changes have also been identified. In the *mmaA3* gene, a G to A mutation was found at position 293 and this point mutation resulted in impaired production of methoxymycolic acid in all strains derived after 1931 (Belley *et al.*, 2004). Two point mutations have also been identified in *Rv3676*, a gene encoding the transcriptional regulator cAMP receptor protein (CRP). The first mutation is present in all strains of BCG, while the second affects those derived after 1931, however, both mutations decrease the DNA binding ability of the protein and prevent CRP from binding to the promoter with which it normally associates (Spreadbury *et al.*, 2005).

1.4. Vaccine Development

1.4.1. Live Vaccines

The development of a new live vaccine against tuberculosis has been actively pursued in recent years and this work has focused on two approaches: modifying BCG to enhance immunogenicity and developing attenuated strains of *M. tuberculosis*.

1.4.1.1. Recombinant BCG

Several strains of recombinant BCG have shown promising results in their ability to protect against tuberculosis in animals models and have been moved forward into clinical trials. Modifying BCG for use as a new vaccine against *M. tuberculosis* has the advantages of the good safety record of BCG and the relatively low cost of production of the vaccine.

Although the RD1 proteins ESAT-6 and CFP10 are often associated with the virulence of *M. tuberculosis*, they induce significant cellular immune responses and are thought to be highly important during infection, making them potential candidates for improving vaccine-induced protective immunity. A recombinant strain of BCG complemented with the RD1 region (BCG::RD1) successfully secretes these proteins and induces ESAT-6 and CFP10-specific response in splenocytes of vaccinated mice (Pym *et al.*, 2003). In vaccinated mice and guinea pigs challenged with *M. tuberculosis*, BCG::RD1 protects as well or better than the wild-type BCG. CFU in the lungs were similar between the two strains, but fewer CFU were present in the spleens of BCG::RD1-vaccinated animals at most time points. In addition, gross pathology of the spleens revealed fewer and smaller tubercles on the surface of the spleens from BCG::RD1-vaccinated mice compared to wild-type BCG-vaccinated mice. However, a safety concern has been raised regarding this potential vaccine. This strain is significantly more virulent in SCID mice (time to death) compared to the parental strain (Brodin *et al.*, 2004). Pre-clinical studies of this vaccine are ongoing (Kamath *et al.*, 2005).

A recombinant strain of BCG expressing the *M. tuberculosis* 30kDa major secretory protein Ag85B is currently being evaluated in a Phase I clinical trial. This protein is expressed and secreted in large amounts by *M. tuberculosis* both *in vitro* and *in vivo* and is considered to be an important protective antigen. Over-expressing major antigens in BCG to induce protective immunity is based on the theory that extracellular proteins are potent immunoprotective molecules and the demonstration that vaccinating with such proteins induces a protective immune response. Recombinant BCG strains expressing this protein (rBCG30) induce a strong DTH response in guinea pigs. Vaccination with rBCG30 protected guinea pigs extremely well against aerosol infection with *M. tuberculosis* Erdman (Horwitz *et al.*, 2000). *M. tuberculosis* CFU counts were significantly reduced in the lungs and spleens, although to a lesser extent in the lungs (significant in only 1 of 2 experiments), compared to both unvaccinated and, more interestingly, BCG (parental strains) vaccinated animals (Horwitz *et al.*, 2000). The median survival of rBCG30 vaccinated guinea pigs was also significantly increased from 24 to 30 weeks, compared to BCG-vaccinated guinea pigs (unvaccinated median survival was 15.5 weeks) (Horwitz and Harth, 2003).

A new recombinant BCG showing greater protection than the parental BCG has recently been reported (Grode *et al.*, 2005). This strain is urease deficient and expresses listeriolysin (Hly) from *Listeria monocytogenes* ($\Delta ureC hly^{+}$ rBCG). Expression of Hly serves two functions: first, the mycobacterial phagosomal membrane is perforated by Hly resulting in translocation of mycobacterial antigens to

the cytosol and subsequent presentation on MHCI molecules and second, perforation of the phagosomal membrane induces apoptosis which promotes cross-priming of mycobacterial antigens. The loss of urease, which is suggested to be involved in the *M. tuberculosis*-induced blocking of phagosomal acidification, allows the phagosome to acidify normally and reach a pH at which Hly functions most efficiently (Grode *et al.*, 2005). Significant protection against low (30 CFU) and medium (200CFU) dose *M. tuberculosis* H37Rv infections in Balb/c mice was induced by the $\Delta ureChly^+$ rBCG strain compared to unvaccinated mice at all time points examined and compared to parental BCG at some time points. Interestingly, $\Delta ureChly^+$ rBCG also protected against a challenge with the highly virulent Beijing strain, against which parental BCG did not protect. To address the question of safety raised by the introduction of a known virulence factor into BCG, SCID mice were infected with a very high dose (10^8), at which mice infected with the parental BCG strain had a mean survival time of 25 days. The mice infected with the $\Delta ureChly^+$ rBCG strain were clearly attenuated, surviving for a mean of 80 days.

1.4.1.2. Attenuated Mycobacterium tuberculosis

Several strains of attenuated *M. tuberculosis* are being considered as potential vaccine candidates, although for one of these strains to be put into use the question about safety would have to be thoroughly addressed. At a meeting in Geneva, in November, 2004, on the development of TB vaccines, three attenuated strains were presented. Deletion of PhoP, which is part of a two component system thought to regulate the expression of virulence genes, severely attenuates *M. tuberculosis* in a

mouse model, however, even though growth of the knockout is limited, it does persist as long as wild-type *M. tuberculosis* in a short-term model. As persistence of BCG is important for developing a protective immune response, this may be regarded as an advantageous phenotype for a potential vaccine derived from an attenuated *M. tuberculosis* strain. The two other strains discussed used a *panCpanD*-deleted strain as a backbone, upon which either *lysA* or RD1 was additionally deleted. Both strains were highly attenuated and showed protective efficacy similar to that induced by BCG vaccination (Sambandamurthy *et al.*, 2002;Sambandamurthy *et al.*, 2005).

1.4.2. DNA Vaccines

There have been a multitude of DNA vaccines tested for their ability to induce a protective response against *M. tuberculosis* infection. The most successful of these vaccines have demonstrated protection in animal models similar to that of BCG, but used alone they have yet to surpass the protective efficacy of BCG. Several have shown success, however, in their ability to act in a prime-boost strategy to enhance the protection induced by BCG. Some of the genes used in DNA vaccines include *Rv3875* (ESAT-6), *Rv3804c* (Ag85A), *Rv1886c* (Ag85B), *hspX*, *sodA*, *hsp65*, *hsp70*, *mpt83*, *mpt64*.

Although capable of inducing a modest protective response, single antigen vaccines have not induced a response as protective as BCG. To improve upon this, multi-gene vaccines have been tested. Delogu *et al* (Delogu *et al.*, 2002) screened a series of single antigen vaccines for their ability to induce a protective response in a

short-term, low dose aerosol challenge and from these, selected 10 that demonstrated significant protection (eight of which encode CFP – including *mpt83*, one PE and one PPE). The antigens were fused with the tissue plasminogen activator signal sequence to ensure efficient secretion. Vaccination with either the combination DNA vaccine or BCG induced significant protection following aerosol infection demonstrated by a decrease in CFU in the lungs and spleen, improved histopathology and increased mean survival period. The combination vaccine administered a dose of each individual component that was ineffective as a single vaccine. In combination, however, this dose induced protection similar to that induced by BCG. This lead the authors to suggest that multi-cistronic plasmids, from which several antigens could be expressed, may permit an increase in dosage that can be administered and perhaps enhance protective immunity.

This approach was used with two immunodominant proteins, ESAT-6 and Ag85B, to create a plasmid from which a fusion protein was expressed and secreted (Derrick, Yang, and Morris, 2004). Vaccination with the plasmid DNA induced significant protection from an *M. tuberculosis* challenge, indicated by reduced CFU in the lungs and spleen and an increased mean survival time that were comparable to BCG and that far exceeded the efficacy of either single antigen. By using a fusion gene plasmid, this effectively allowed for the amount of each antigen per vaccination to be increased for the same total amount of DNA.

Another approach taken to address the limitation on the quantity of DNA that can used per vaccination is the use of cationic PLGA (DL-lactide-co-glycolide) microspheres (Cai *et al.*, 2005). The microspheres serve three functions: to act as mediators of DNA transfection targeted to phagocytic cells, to protect against biological degradation by nucleases and to enhance gene expression. A combination of DNA vaccines encoding Ag85B, MPT64 and MPT83 was tested in mice as both non-encapsulated and encapsulated DNA. The non-encapsulated DNA was injected three times at three-week intervals for a total amount of 900ug (300ug each plasmid), while a total dose of only 90ug of encapsulated DNA was administered in one injection. Both vaccination strategies resulted in a reduction of CFU in the lungs and spleen of *M. tuberculosis*-infected mice to levels comparable with BCG. The high efficacy of the low-dose, single injection vaccination with encapsulated DNA makes this formulation an enticing prospect for further studies.

1.4.3. Subunit Vaccines

Many individual antigens have been identified for their immunogenic properties and assayed for their ability to induce a protective response to *M. tuberculosis* in animal models. Although some degree of protection has been demonstrated in mouse models following vaccination with subunit vaccines, the level of protection has not shown any improvement over that induced by BCG. As with DNA vaccines, however, the importance of multiple components in subunit vaccines is being raised.

Two immunodominant proteins, Ag85B and ESAT-6, have been used to create a multi-component subunit vaccine based on a fusion protein of the two antigens. PBMCs from mice vaccinated with the fusion protein can be stimulated to produce IFNγ in response to both Ag85B and ESAT-6 and vaccination effectively reduced CFU in the lungs and spleen following an aerosol challenge with *M. tuberculosis* to levels comparable with BCG (Olsen *et al.*, 2001). Additionally, vaccination of guinea pigs with the fusion protein, protected the animals against weight loss as well as BCG, whereas vaccination with the individual components or a mixture of the components did not increase the mean survival time (Olsen *et al.*, 2004). This is the first reported subunit vaccine to have shown significant protection in the guinea pig model.

ESAT-6 is a valuable diagnostic reagent and as such the use of a vaccine containing ESAT-6 is not necessarily a favourable approach to a new vaccine. Based on this and the promising results of the Ag85B-ESAT6 fusion protein, Dietrich *et al* (Dietrich *et al.*, 2005) pursued candidates to replace ESAT-6 in the fusion protein and maintain the effectiveness of the vaccine. TB10.4 is a member of an ESAT-6 related subfamily that is recognized by a high proportion of TB patients (Skjot *et al.*, 2002). Vaccination with the Ag85B-TB10.4 fusion protein induced a strong IFNγ response in PBMCs stimulated with either antigen, and the response the TB10.4 was stronger in mice vaccinated with the fusion protein than in those vaccinated with TB10.4 alone. Vaccination of mice with the Ag85B-TB10.4 fusion protein significantly reduced CFUs in the lungs following an aerosol challenge with *M. tuberculosis* to levels

comparable to those found in mice vaccinated with either BCG or the Ag85B-ESAT6 fusion protein. As both proteins are present in BCG and recognized in BCG-vaccinated individuals, this vaccine is being tested as a BCG-booster vaccine.

The first subunit vaccine to be tested in humans is a recombinant polyprotein Mtb72F, developed as a fusion of two antigens, Mtb32 (*Rv0125*) and Mtb39 (*Rv1196*), that were identified in PPD-positive individuals and in animal models as potential vaccine candidates. Vaccination with rMtb72F in either of two adjuvants tested reduced CFU by ~0.6 logs in the lungs of aerosol-challenged mice, regardless of the qualitative and quantitatively different immune responses generated by the different vaccine formulations. More importantly, vaccination extended survival of guinea pigs following aerosol challenge with *M. tuberculosis* to greater than a year, which is comparable to BCG and significantly longer than the 30-week survival of the control animals (Skeiky *et al.*, 2004).

1.4.4. Prime-Boost Strategies

As BCG has been and continues to be used so extensively throughout the world, developing a new vaccination strategy that incorporates BCG can take advantage of the established vaccination programs, as well as benefit individuals who have been previously vaccinated. Heterologous prime-boost strategies can effectively generate CD4+ and CD8+ T cell responses and have been proposed as an effective strategy to boost BCG-induced immunity.

Boosting BCG with the Mtb72F subunit vaccine, which was shown to be highly effective as a subunit vaccine, modestly increased protection. Survival of guinea pigs vaccinated with BCG and boosted with Mtb72F was significantly longer than those vaccinated with BCG alone, with three animals surviving the length of the experiment compared to one BCG-vaccinated animal. Histopathology of all surviving animals, including the non-boosted BCG-vaccinated guinea pig, revealed small granulomas associated with large airways and blood vessels, whereas all animals that died before the end of the experiment showed large coalescing lesions, consolidating most of the lung tissue. Although the surviving animals showed similar pathology regardless of the vaccine used, boosting with Mtb72F resulted in a longer mean survival time, with more animals showing favourable histopathology results. Boosting with Mtb72F DNA vaccine, in contrast to the subunit vaccine, did not significantly prolong survival time in *M. tuberculosis*-challenged guinea pigs compared to animals vaccinated with BCG alone. Non-boosted BCG-vaccinated animals, however, displayed large coalescing lesions with central regions of necrosis, whereas animals receiving the booster showed less inflammation and signs of healing (Brandt *et al.*, 2004).

Boosting BCG vaccination with modified vaccinia virus Ankara expressing Ag85A (MVA85A) has been proposed as an effective combination to enhance BCG-induced immunity. In BCG-vaccinated Balb/c mice, MVA85A boosting induces strong antigen specific CD4+ and CD8+ responses, in spleen, lungs and lung lymph nodes, which were significantly greater than those observed in mice vaccinated with

BCG alone. CFU in the lungs and spleen of *M. tuberculosis*-challenged mice were significantly reduced in both BCG-vaccinated and the BCG-vaccinated, MVA85A-boosted mice relative to control mice, with the latter vaccination group also showing significantly lower CFU relative to BCG-vaccinated mice. Boosting with BCG however, also decreased the CFU to similar levels as the MVA85A boosted levels (Goonetilleke *et al.*, 2003). In further studies using the guinea pig model of infection, boosting with MVA85A induced significant protection compared to control animals demonstrated by prolonged survival, decreased CFU and decreased consolidation of lung tissue, however, the protection observed was similar to that induced by BCG alone (Williams *et al.*, 2005). This vaccination model has been moved forward into clinical trials and to date MVA85A has shown good tolerance and immunogenicity in vaccinated individuals (McShane *et al.*, 2004;McShane *et al.*, 2005). Interestingly, boosting with MVA85A stimulated a strong response in both BCG-vaccinated individuals, as well as individuals determined to have been previously exposed to environmental mycobacteria, suggesting MVA85A, in addition to being a component of a new vaccination strategy, may have value as a booster of previously vaccinated and exposed individuals.

1.5. Research Objectives

As we now recognize that there are significant genetic differences between strains of BCG and as strains of BCG are continuing to be used to vaccinate children worldwide against tuberculosis, it could prove to be highly beneficial to understand the phenotypic differences between strains arising from the various genetic changes that have occurred. To this end, the work in this thesis has been completed in an endeavour to understand what impact some of these genetic changes have had on the affected strains of BCG. More specifically, the first objective of this thesis is to examine if the loss of the RD2 region contributed to ongoing attenuation of BCG strains. The deletion of this region resulted in the loss of both antigenic and regulatory proteins, both of which could affect the ability of BCG to induce a protective immune response against *M. tuberculosis*. To further address differences in antigenic proteins between strains of BCG, the second objective focuses on identifying the cause of the differential expression of two antigenic proteins, MPB70 and MPB83, in various strains of BCG. The third objective of this thesis was to evaluate the impact of expression of these proteins in BCG by restoring their expression in a low-producing strain of BCG. Taken together, these three objectives will hopefully provide some insight into how BCG strains have evolved through *in vitro* culturing and what impact these changes have had on the ability of these vaccines to protect against tuberculosis.

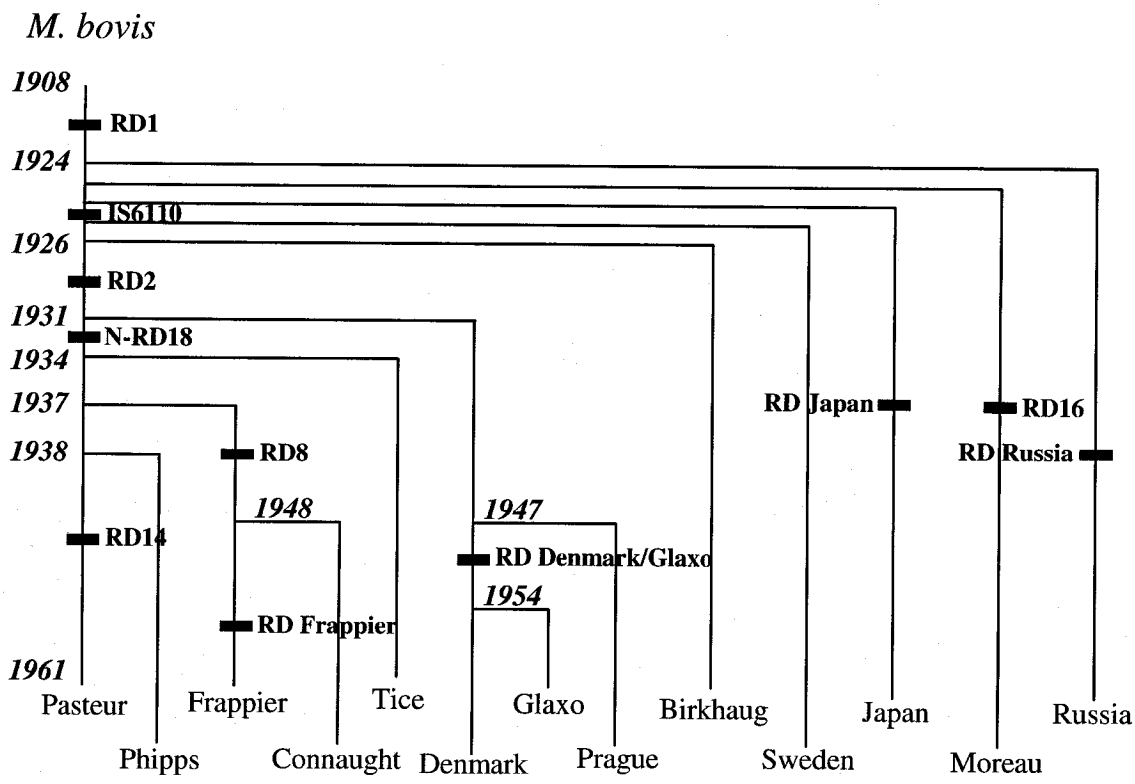


FIGURE 1.1 – Phylogeny of BCG strains. Vertical axis represents the timeline from the derivation of BCG from *M. bovis* in 1908 to the lyophilization of BCG in 1961. Horizontal axis represents the various strains of BCG. Genetic deletions have been labeled.

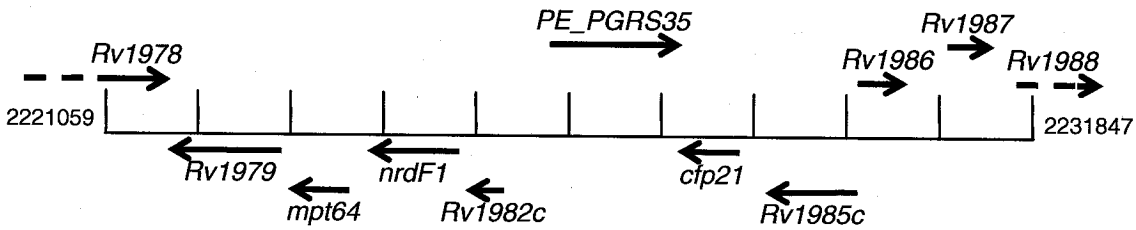


FIGURE 1.2 – RD2 region. Arrows represent genes in the RD2 region. Location of the region in the genome is indicated in basepairs.

2. *Materials and Methods*

Bacterial cultures. Unless otherwise stated, BCG strains and *M. tuberculosis* H37Rv were grown at 37°C in Middlebrook 7H9 medium (Difco Laboratories, Detroit, Mich.) containing 0.05% Tween 80 (Sigma-Aldrich, St. Louis, Mo.) and 10% albumin-dextrose-catalase supplement (Becton Dickinson and Co., Sparks, Md.) on a roller platform (Wheaton). Cultures were grown to mid-log phase and stored as 1 ml aliquots in 7H9 medium containing 15% glycerol, at -80°C. Frozen aliquots were thawed, as needed, and diluted in fresh 7H9 medium supplemented with Tween 80 and ADC and grown with rotation at 37°C.

THP-1 cell culture. The human monocytic cell line THP-1 was acquired from ATCC (American Type Culture Collection, #TIB-202). Cells were maintained in RPMI 1640 media (Wisent) containing 2mM L-glutamine and supplemented with 10% heat-inactivated fetal bovine serum (Wisent) and 20 mM HEPES (Wisent), at 37°C with 5% CO_2 in vented tissue culture treated flasks (Falcon). Cell density was maintained between 0.5 – $8.0x10^5$ cells/ml. Aliquots were frozen in complete RPMI medium with 5% DMSO and stored in liquid nitrogen. As needed, aliquots were quick thawed, washed with complete RPMI and re-suspended in new media.

Animals. C57BL/6J mice were ordered from Jackson Laboratory (Bar Harbor, Maine). Mice were housed in the Conventional Animal Facility in the Montreal General Hospital for the BCG experiments and in the Biosafety Level 3 Animal Facility for the *M.*

tuberculosis infections. All procedures were approved by the Facility Animal Care Committee.

Deletion of RD2. To knockout the RD2 region, the genomic regions flanking the RD2 region were amplified by PCR using the primers listed in Table 2.1. PCR products for the regions upstream and downstream of RD2 were cloned into the plasmid pKO (provided by D.R. Sherman) to flank the kanamycin resistance cassette, creating the constructs pKO-RD2. The same PCR products were also cloned into the multiple cloning site of p2NIL (Parish and Stoker, 2000). The plasmid pGOAL19 (Parish and Stoker, 2000) was digested with PacI and the fragment containing the marker genes of interest was ligated into the p2NIL vector containing the cloned fragments digested with the same enzyme, creating the construct pNIL/pGOAL-RD2. All constructs were verified by restriction analysis and purified before use. Bacteria for transformation were grown to mid-log phase and pelleted by centrifugation. Bacteria were washed three times in 10% glycerol (20ml, 10ml, 5ml) and re-suspended at 1/50 of the initial volume in 10% glycerol (200µl per 10ml culture). BCG strains were mixed with 500-1000ng of plasmid DNA (either pKO-RD2 or p2NIL/pGOAL-RD2) and electroporated (BTX Harvard Apparatus, Holliston, MA or Bio-Rad Laboratories, Hercules, CA) using the settings 2500V, 1000Ω and 25µF in 2-mm gap cuvettes (VWR). 800µl of warm 7H9 media was added immediately after electroporation and bacteria were incubated at 37°C overnight. Bacteria were then plated on 7H10 agar with hygromycin (50µg/ml) or kanamycin (25µg/ml) and incubated at 37°C until colonies appeared, after approximately 3 weeks. Individual colonies were re-suspended in 7H9 and incubated at 37°C for approximately

one week. For PCR screening, 100µl aliquots were centrifuged and bacteria were re-suspended in water. Lysates were prepared by boiling samples for 20 minutes and used as templates for PCR screening. For detection of site-specific integration, primer pairs consisted of a vector-specific primer and a genomic DNA-specific primer (see table 1), allowing for amplification only from DNA in which the plasmid had integrated at either the upstream or the downstream homologous regions. Colonies in which site-specific integration was detected were plated on 7H10 agar with 2% sucrose and kanamycin (no kanamycin used for pNIL/pGOAL-RD2) and incubated at 37°C for approximately 4 weeks. When colonies were detected, individual colonies were selected and grown in 7H9 media. Colonies were screened for gene deletions by PCR as described above.

Complementation of *mpb64* and *sigK*. To complement *mpb64,* the *mpb64* region was amplified from BCG Russia using the primers MPT64compF and MPT64compR (see Table 1). The amplified product was digested with *Hind*III and *Kpn*I (Fermentas) and cloned into the plasmid pKZ4 digested with the same restriction endonucleases. To complement *sigK*, the *sigK* region (including the complete gene and 288bp upstream), was amplified by PCR from *M. tuberculosis* H37Rv, BCG Russia, BCG Birkhaug and BCG Pasteur, using the primers Rv0445cL and sigKR (see Table 2.1). The amplified products were cloned into the T-vector, pDRIVE (Qiagen). The *sigK* region was then removed by digestion with *Hind*III and *Kpn*I and ligated to the integrative mycobacterial vector pMV306 (Stover *et al.*, 1991) cut with the same restriction endonucleases. Integrity of the cloned genes was confirmed by DNA sequencing. The resulting plasmids and the empty vectors were electroporated into BCG Denmark (*mpb64*) or BCG Pasteur

(*sigK*), as described above for gene deletions. Transformants were grown at 37°C on Middlebrook 7H10 plates with hygromycin (50μg/ml) for *mpb64* transformants or kanamycin (25 μg/mL) for *sigK* transformants. Complementation was PCR-confirmed by amplifying the cloned gene with vector-specific primers for the regions flanking the insert and these amplicons were sequence-confirmed for all transformants.

DNA Extraction. *Genomic DNA Extraction:* Bacteria was grown to an OD_{600} of 0.3 to 0.5, pelleted by centrifugation (3000rpm, 10 minutes), transferred to 2ml screw-cap tubes and re-pelleted. The pellet was re-suspended in 400μl fresh lysis buffer (Na-acetate 20 mM, SDS 0.5%, EDTA 1mM), after which 1ml of acidified phenol:chloroform (5:1, pH 4.5; Ambion) and 0.8g of silica beads (Sigma) were added to the tube. Bacteria were lysed by agitation using a reciprocal shaker (FastPrep FP120, Savant Bio 101) set at a speed of 6.5 for 45s. The tubes were incubated on ice for 10 min and then centrifuged at 4°C. The aqueous phase was collected and mixed with an equal volume of chloroform-isoamylalcohol (24:1), vortexed for 1 minute and centrifuged at maximum speed for 5 minutes. The aqueous phase was collected, mixed with an equal volume of isopropanol and 1/10 volume of sodium acetate, and stored overnight at –70°C. DNA was pelleted by centrifugation, air dried and resuspended in nuclease-free water.

High Molecular Weight DNA Extraction for Southern blotting: Bacteria were transferred to 2ml microcentrifuge tubes and heated at 80°C for 20 minutes. Tubes were centrifuged and the bacteria were resuspended in 500μl of TE buffer (100mM Tris/HCl - pH 8.0, 10mM EDTA). Lysozyme (10mg/ml) was added and tubes were incubated at 37°C overnight. 70μl of 10% SDS and 10μl of proteinase K (10mg/ml) were added and tubes

were incubated at 65°C for 20 minutes. 100µl of NaCl (5M) and 100µl of CTAB/NaCl were added, tubes were vortexed and incubated at 65°C for 10 minutes. 750µl of chloroform-isoamylalcohol was added to each tube, tubes were vortexed and centrifuged. Aqueous phase was transferred to new tubes and mixed with 550µl of isopropanol. Tubes were then incubated overnight at -20°C. DNA was pelleted by centrifugation, washed with cold 70% ethanol and air-dried. The pellet was re-suspended in TE buffer.

RNA extraction. Bacteria was grown to an OD_{600} of 0.3 to 0.5, pelleted by centrifugation (3000rpm, 10 minutes), resuspended in 1 ml of washing buffer (0.5% Tween 80, 0.8% sodium chloride) and transferred to 2ml screw-cap tubes. This suspension was centrifuged and the pellet was resuspended in 400µl fresh lysis buffer (Na-acetate 20 mM, SDS 0.5%, EDTA 1mM), after which 1ml of acidified phenol:chloroform (5:1, pH 4.5; Ambion) and 0.8g of silica beads (Sigma) were added to the tube. Bacteria were lysed by agitation using a reciprocal shaker (FastPrep FP120, Savant Bio 101) set at a speed of 6.5 for 15s, followed by 4.5 for 15s. The tubes were incubated on ice for 10 min and then centrifuged at 4°C. The aqueous phase was transferred into a new tube and was replaced with 300µl of lysis buffer. Cell lysis and collection of the aqueous phase was repeated three time. An equal volume of chloroform-isoamylalcohol (24:1) was added to the pooled aqueous phase (~800µl), tubes were mixed by inversion and centrifuged at 4°C. RNA was precipitated by adding an equal volume of isopropanol and 1/10 volume of sodium acetate (Ambion) to the aqueous phase and stored overnight at –20°C. RNA was pelleted by centrifugation at 4°C, washed with 70% ethanol and air dried for 10 minutes. The pellet was re-suspended

in RNase-free water (Ambion). Genomic DNA contamination was digested with DNase-I (Fermentas) and RNA was purified with RNeasy Purification Kit (Qiagen), following the manufacturer's protocol. The quality of RNA was confirmed by denaturing gel electrophoresis (formaldehyde).

Protein preparation and immunoblot analysis.

Protein preparation BCG Complemented Strains: Cultures were grown in 7H9 media on a rolling platform for 7 days. The cultures were then centrifuged and the supernatant was filtered with a 0.22um membrane filter and concentrated with an Amicon Ultra-15 Centrifugal Filter Unit, 10 000 MWCO. Cell pellets were frozen and whole cell lysates were prepared by resuspending the cell pellet in 100ul PBS and boiling for 20 minutes. If necessary, the culture filtrate proteins (CFP) were precipitated by the following protocol: 1 volume of sample was mixed with 3 volumes of methanol, 1 volume of chloroform, 4 volumes of water. Samples were centrifuged at max speed (~13200rpm) for 1 minute. The upper phase was removed and replaced with 4 volumes of methanol and mixed briefly. Protein was pelleted at max speed for 15 minutes (Wessel and Flugge, 1984). Protein was re-suspended in 100ul PBS and the final concentration was determined using Coomassie Plus Protein Determination Kit (Pierce) following standard protocol.

Immunoblotting – MPB64: 5µl of the concentrated CFP for each strain was added to an equal volume of SDS-loading buffer, heated at 80°C for 5 minutes, and loaded into each lane. SDS-PAGE was performed under reducing conditions using the Mini-PROTEAN 3 electrophoresis system (Biorad) with 10% polyacrylamide gels. Proteins were transferred

to a polyvinylidene difluoride membrane. Membranes were blocked in PBS containing 2% BSA and 0.05% Tween 20, and then probed with primary antibodies for 1 hour at room temperature. Bound antibodies were recognized by horseradish peroxidase-labelled anti-mouse Ig. All antibodies were diluted in PBST with 1% BSA. Mouse monoclonal antibody L24b4 (Oettinger and Andersen, 1994) was used at a dilution of 1/50 and the HRP-conjugated anti-mouse antibody was used at a dilution of 1/10000. Protein bands were detected using ECL Plus™ Western Blotting Detection Reagents (Amersham).

Immunoblotting – MPB70 and MPB83: A total of 10µg of CFP for each sample or 2ul of cell lysate was loaded in each lane. Samples were added to SDS-loading buffer and heated to 80°C for 5 minutes. SDS-PAGE was performed under reducing conditions using the Mini-PROTEAN 3 electrophoresis system (Biorad) with 12% polyacrylamide gels. Proteins were transferred to a polyvinylidene difluoride membrane. Membranes were blocked in PBS containing 2% BSA and 0.05% Tween 20, and then probed with primary antibodies for 1 hour at room temperature. Bound antibodies were recognized by horseradish peroxidase-labelled anti-mouse Ig. All antibodies were diluted in PBST with 1% BSA. Mouse monoclonal antibodies 1-5C (anti-MPB70) and MBS43 (anti-MPB83) (Wiker *et al.*, 1998) were used at a dilution of 1/500 and the HRP-conjugated anti-mouse antibody was used at a dilution of 1/10000. Protein bands were detected using ECL Plus™ Western Blotting Detection Reagents (Amersham).

BCG Strains Protein Preparation and Immunoblotting – MPB70 and MPB83: BCG strains were cultivated as surface pellicles on liquid synthetic Sauton medium for 3 weeks

at 37°C. The bacteria were washed and disrupted by a bead beater to yield a cellular extract and the culture medium was filtered to remove residual bacteria and concentrated by ammonium sulphate precipitation at 80% saturation. The antigens were separated under reducing conditions by horizontal sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in precast 8-18% gradient Excel gel using a Multiphor II unit 2117 (Amersham Pharmacia). After separation, the proteins were transferred to a nitrocellulose membrane (pore size, 0.2 μm) by diffusion blotting (Olsen and Wiker, 1998) and the gel was stained with CBB. The membranes were blocked with PBS containing 2% bovine serum albumin (BSA) and 1% gelatin and incubated with antibodies overnight. Bound antibodies were recognized by horseradish peroxidase-labelled anti-rabbit or anti-mouse Ig. As substrate, 3,3-diaminobenzidine was added to visualize the bound antibodies.

Southern blotting. 2μg of DNA was digested with *Pvu*II at 37°C for 1 hour. Digested DNA was run on a 0.8% agarose gel at 60V overnight. The gel was exposed to UV for 10 minutes and then incubated in HCl (0.25M) for 10 minutes and twice with NaOH (0.4M) for 20 minutes. DNA was then transferred to a nylon membrane (Amersham) and the membrane was incubated with 4x SSC (0.6M NaCl, 60mM sodium citrate, pH7.0) for 10 minutes. Probes were amplified by PCR using primers for the *Rv1986* gene of RD2 and for the IS6110 sequence and labeled with an ECL-labeling kit (Amersham), following manufacturer's protocol. Membranes were pre-hybridized at 42°C for 1 hour and hybridized with the ECL-labeled probes at 42°C overnight. Membranes were washed once with 5x SSC (0.75M NaCl, 75mM sodium citrate, pH7.0) for 5 minutes,

twice with wash buffer (0.3x SSC, 0.4% SDS) for 15 minutes and twice with 2x SSC (0.3M NaCl, 30mM sodium citrate, pH7.0) for 5 minutes. Probes were detected using ECL Direct Nucleic Acid Detection System (Amersham).

PCR amplification and sequencing across *Rv0445c* (*sigK*). The sequence of *Rv0445c* (*sigK*) was determined by amplifying the gene and flanking regions from isolates of *M. canettii*, *M. tuberculosis* H37Rv, *M. tuberculosis* H37Ra, *M. africanum* (n = 2), *M. microti* (n = 2), *M. caprae* (n = 2), *M. bovis* (n = 2) and 13 members of the BCG family – BCG Russia (ATCC 35740), BCG-Moreau, BCG-Japan, BCG-Sweden, BCG-Birkhaug (ATCC 35731), BCG-Prague, BCG-Glaxo (ATCC 35741), BCG-Denmark 1331 (ATCC 35733), BCG-Tice (ATCC 35743), BCG-Frappier (ATCC 35735), BCG-Connaught, BCG-Phipps (ATCC 35744) and BCG-Pasteur 1173. The sequence was amplified using the primers sigKL (5'- agctcgagcagctcaaaatc -3') and sigKR (5'- acgcgtcaccccaactact -3') and amplicons were sequenced by di-deoxy terminal sequencing at the McGill University and Genome Quebec Innovation Center. To look for differences between the amplified sequence and the prototype genome sequences, results were compared by BLAST analysis to *M. tuberculosis* H37Rv using Tuberculist (http://genolist.pasteur.fr/TubercuList/), *M. tuberculosis* 210 and CDC1551 using the sequences provided at NCBI (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi), *M. bovis* AF2122/97 using Bovilist (http://genolist.pasteur.fr/BoviList/), and the assembly sequence of BCG Pasteur (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/m_bovis).

Real-time quantitative RT-PCR. Targeted gene expression levels were determined using RT-PCR with molecular beacons or SYBR green, according to established

protocols (Manganelli *et al.*, 1999;Mostowy *et al.*, 2004). In brief, RNA was extracted from log-phase cultures and converted to cDNA using reverse transcriptase (Fermentas) and random hexamer primers, following the manufacturer's protocol. cDNA was amplified using a Roche LightCycler with the Roche LightCycler-FastStart DNA kit using fluorescent molecular beacons specifically designed for the genes of interest or with the LightCycler-FastStart DNA SYBR Green Kit. To provide a normalization standard for mRNA expression, expression of *sigA* was also determined, and the level of expression of the gene of interest was divided by that of *sigA* to normalize for differences in total mRNA extracted (Manganelli *et al.*, 1999). Sequences of the primers used for molecular beacon and SYBR green analysis and the sequences of the molecular beacons used are as listed in Table 2.1.

Microarray Analysis. Microarray hybridization and analysis were done as previously described (Mostowy *et al.*, 2004). In brief, mRNA from BCG strains and complemented strains was extracted during log-phase *in vitro* growth and labeled with Cy3 or Cy5 dUTP by reverse-transcriptase (Amersham Biosciences). Labeled cDNA was hybridized to microarrays composed of oligonucleotide probes from the TB Array-Ready Oligo Set™ (Operon) that had been printed onto Sigmascreen™ microarray slides (Sigma). Comparisons were performed between *M. tuberculosis* H37Rv and *M. tuberculosis* H37RvΔRD2, between BCG Russia and BCG Pasteur, between BCG Birkhaug and BCG Denmark, and between Pasteur::*sigK* and Pasteur::pMV306 (empty vector). In each case, duplicate hybridizations were performed for each dye combination (Cy3/Cy5 and Cy5/Cy3), resulting in four hybridizations per comparison. Hybridized arrays were

scanned with Scanarray 5000XL (PerkinElmer, Freemont, Calif.) and hybridization results were quantified with Scanalyze software (http://rana.Stanford.EDU/software/). Array analysis was performed as previously described (Mostowy *et al.*, 2004) in order to determine a Z-score, indicative of how many standard deviations a data point lies from the population mean, for each gene. Z-scores for each gene were averaged across replicates within each experiment to minimize the probability of observing such variation by chance alone and genes with average Z-scores of 2 or greater are presented.

Growth Curves. Stationary or rolling cultures of the selected strains were passaged with a 25-gauge needle to establish a single-cell suspension. The optical density at 600nm (OD_{600}) was measured and the cultures were diluted to the desired initial density in 7H9 media in triplicate. Cell density of each culture was measured daily by removing 0.8ml aliquot from each culture, passaging each aliquot through a 25-gauge needle to remove clumps and measuring the OD_{600}.

THP-1 Infections. THP-1 cells were seeded in 24-well tissue culture-treated plates (Falcon) at a density of 1.0×10^5 cells per well in complete RPMI media with 100nM phorbol 12-myristate 13-acid (PMA; Sigma). Cells were incubated at 37°C with 5% CO_2 for 48 hours, at which time cells were washed and incubated in RPMI media with 100U/ml recombinant human IFNγ (BioDesign International) for 24 hours. For each infection, bacterial cultures were grown to mid-log phase, quantified by measuring the OD_{600}, diluted in warm RPMI and added to each well at an MOI of 1, 10, 25 or 50. Cells were incubated at 37°C with 5% CO_2 for 4 hours, after which the cells were washed three

times in warm RPMI. Complete RPMI was added to each well and cells were incubated at 37°C with 5% CO_2 for 20 hours (total infection time 24 hours). Cytotoxicity was determined by measuring the metabolism of an oxidation-reduction dye (AlamarBlue; Biosource International). AlamarBlue was diluted in warm RPMI and added to each well for 6 hours, at which time the OD_{600} and OD_{570} of each sample were measured. Percentage of reduction of AlamarBlue was calculated according to the manufacturer's instructions.

Vaccination of mice. Bacterial cultures were grown to mid-log phase and re-suspended in PBS at a concentration of 10^7/ml. Inoculum doses were confirmed by serial dilution plating on 7H10 agar containing hygromycin (*mpb64*) or kanamycin (*sigK*). Mice were immunized by subcutaneous infection at the base of the tail with 10^6 bacteria in 100µl of PBS using a 1.0-ml insulin syringe.

Mouse Splenocytes and IFNγ quantification. Four weeks after vaccination, mice (5 per group) were sacrificed by CO_2 inhalation and the spleens were removed and placed in HBSS with 2% FBS. Spleens were passed through a nylon mesh filter and rinsed with HBSS with 2% FBS. Cells were pelleted and re-suspended in ACK buffer for 5 minutes to lyse red blood cells. Cells were washed three times with complete RPMI. Cells were plated in 96-well plates at a density of 5.0×10^5 cells per well in complete RPMI with various concentrations of antigens (RPMI alone, concanavalin A, MPB70 – MPB70 was provided by Harald Wiker). After 72 hours of culture, IFNγ was quantified in serial

dilutions of supernatant by ELISA (BD Biosciences Pharmingen; San Jose, Ca) according to the manufacturer's protocol.

Intravenous infection of mice. For growth studies, naïve mice were infected with 10^6 bacteria in 100µl of PBS intravenously. For challenge studies, mice were infected with 10^6 bacteria in 100µl of PBS intravenously 10 weeks after vaccination. Bacterial cultures were grown to mid-log phase and re-suspended in PBS at a concentration of 10^7/ml. Inoculum doses were confirmed by serial dilution plating on 7H10 agar. Mice were placed in a restraining cone and infected by injecting 100 µl in the caudal tail vein with a 1.0-ml insulin syringe. On the indicated days, mice were sacrificed by CO_2 inhalation. The lungs and spleen were removed and placed in 0.025% saponin–PBS on ice. Tissue homogenates were prepared with a Polytron PT 2100 bench-top homogenizer (Kinematica AG, Lucerne, Switzerland). The Polytron was washed sequentially in distilled H_2O–0.05% SDS–70% ethanol–PBS before the next organ was processed. Serial dilutions of the organ homogenates were plated on 7H10 agar containing hygromycin or kanamycin. For histopathologic evaluations, the renal vein was cut, and 20 ml of PBS followed by 20 ml of 10% formalin was perfused via the left atrium with a 30-ml syringe. Organs were embedded in paraffin, mounted, and stained with hematoxylin and eosin and for AFB.

Statistical Methods. Data was analyzed by ANOVA and Kruskal-Wallis methods. For the ANOVA method, differences between means were assessed by Tukey's test. A p value of <0.05 was considered significant.

TABLE 2.1 Sequences of Primers

NAME OF PRIMER	SEQUENCE
RD2 Deletion	
RD2R5	5' – caatgtcaccgatcgtcg – 3'
RD2L5	5' – ccgatgatcttctgtttgacc – 3'
RD2R3	5' – ggtggtcgccgtggagttg – 3'
RD2L3	5' – tacaactgcgacaaagcgtc – 3'
New RD2R5	5' – ataaaagcttaacaaaatacatcatcgcgcc – 3'
New RD2L5 pKO	5' – ataagcatgcccgatgatcttctgtttgacc – 3'
New RD2L5 p2NIL	5' – ataacacgtgccgatgatcttctgtttgacc – 3'
New RD2F3	5' – ataaggatccggtggtcgccgtggagttg – 3'
New RD2R3	5' – ataaggtacctggcggagggagtgttcgt – 3'
mpt64 Complementation	
MPT64compF	5' – ataaaagcttcggtgctgcaaactactcc – 3'
MPT64compR	5' – ataaggtaccgtctaggccagcatcgagtc – 3'
sigK Complementation	
Rv0445cL	5' – agctcgagcagctcaaaatc – 3'
sigKR	5' – acgcgtcaccccaactact – 3'
Molecular Beacons	
sigA	5' – cctcgcgtcgaagttgcgccatccgagcgagg – 3'
sigK	5' – gcagcctgtcgaccgagtccgttggctgc – 3'
mpb70	5' – gcagccagctcaatccgcaagtaaacctgggctgc – 3'
mpb83	5' – gcagccagcatcctgacctaccacgtgaggctgc – 3'

Beacon and SYBR green Primers

	Left Primer	*Right Primer*
sigA	5' – tgcagtcggtgctggaca – 3'	5' – cgcgcaggacctgtgagc – 3'
sigK	5' – agtttgactccgccaaaggt -3'	5' – gcaccatagcgcacttcc – 3'
mpb70	5' – ctcgaacaatccggagttgaca – 3'	5' – acaccgtgtactgaccgctgtt – 3'
mpb83	5' – atcaactcaagactgacgccaaac – 3'	5' – caccttgcagggtctgatgg – 3'
dxr	5' – gggacttgaggtcatcgaaa – 3'	5' – tatggaaatcacaggcagca – 3'
Rv2871	5' – gtatcgatgacgagctgtacc – 3'	5' – ttggacgatagatcgacacc – 3'
Rv2872	5' – gactatcggggtttgcttga – 3'	5' – tcaacatcggacgctaactg – 3'
dipZ	5' – tcggttggtatcaggcctac – 3'	5' – ggtccaagtggcgtagttgt – 3'
Rv2876	5' – agtgggagttcgacgtcagt – 3'	5' – acgtgatcaggaaccagtcc – 3'
Rv2877c	5' – ggttccatgtatggctacgg – 3'	5' – agccagatagatcgctacgc – 3'
mpb53	5' – gttcggtctggccaatacac – 3'	5' – cgtccagaaccacaacacc – 3'
Rv2879c	5' – gcgacgggtgtctattgagt – 3'	5' – taccgtgcaggaaactcctt – 3'
Rv2880c	5' – aacacggtctgcatttcctc – 3'	5' – cccatacccatgaacaccac – 3'
cdsA	5' – tggtcgttgtctgcatgatt – 3'	5' – gagccattttccgggtagac – 3'
Rv0441c	5' – tgggtgccaaaaaggtagac – 3'	5' – ccacaacagggtgacttcg – 3'
PPE10	5' – caattcggcactgatgtttg – 3'	5' – caggctaggtactgggttgc – 3'
Rv0443	5' – cgggtgcaggatatacaggt – 3'	5' – tagtaccccgacagcaggtc – 3'
Rv0444c	5' – ggccgagcaagttctgac – 3'	5' – gcagccacatctgatacacg – 3'
Rv0446c	5' – tggcaactgtgggtattcaa – 3'	5' – cagcaggtaggtcatcagca – 3'
ufaA1 L	5' – ccgacctttcgacctagttg – 3'	5' – tgaacattgcgcacgaatac – 3'
Rv0448c	5' – cttctacgtttcgccgtttc – 3'	5' – tcatcgcgatctgtcttgtc – 3'
Rv0449c	5' – cccacacccactatctggac – 3'	5' – acatcgacatttccgactcc – 3'
mmpL4	5' – ctaaattcgcgaacgactcc – 3'	5' – cttccagtgacgggacaaat – 3'
mmpS4	5' – cctggaaaacagcaaaccat – 3'	5' – ccacgatatttcccatcacc – 3'

3. *Results*

3.1. Characterization of the RD2 region

BCG was derived from *Mycobacterium bovis* by continuous *in vitro* passaging during the years 1908-1921. Through genomic studies, it is now known that the RD1 region, which is present in all virulent *M. bovis* isolates, is deleted from all strains of BCG and has been identified as the attenuating mutation leading to the derivation of BCG. From 1921 to 1961, BCG were continually propagated *in vitro*, in numerous laboratories worldwide under a variety of conditions, until BCG Pasteur 1173 was lyophilized for storage and preservation after 1173 bi-weekly passages. Given that BCG arose from a mutation that occurred during *in vitro* growth, it has been speculated that subsequent mutations may have had an additional impact on characteristics such as growth, virulence and antigenicity – all of which may affect the ultimate phenotype upon which BCG is evaluated, its protective efficacy. RD2, encompassing 11 ORFs and missing from BCG strains obtained from the Pasteur Institute after 1931, represents such a mutation. The significance of RD2, in terms of the ability of BCG strains to grow, persist, stimulate an immune response and protect against tuberculosis is presently unknown. The genes in the RD2 region include major antigenic proteins, a PE family protein and a probable transcriptional regulator, all of which can be proposed to play a significant role in the ability the of bacteria to interact with or adapt to the host environment, and thus upon their deletion, result in further attenuation.

To evaluate any potential phenotype arising from the deletion of RD2, three approaches were pursued: 1) A late strain of BCG was complemented with the gene

mpb64 to evaluate the possibility that expressing MPB64 may increase its protective efficacy in a mouse challenge experiment; 2) a *M. tuberculosis* RD2-knockout strain, H37RvΔRD2, was created in the laboratory of David Sherman, at the University of Washington (Seattle, Washington), and evaluated for *in vitro* growth, cytotoxicity in a monocytic cell line and overall changes in gene expression; and 3) early and late strains of BCG were compared for differences in global gene expression, with an intent of revealing any potential phenotypes for subsequent targeted study.

3.1.1. MPB64 Over-expression

The loss of antigenic proteins from the later strains of BCG has been proposed to contribute to the lack of efficacy in some clinical trials (Behr, 2002) and the importance of extracellular proteins in developing a protective immune response has been demonstrated by successfully protecting guinea pigs against a *M. tuberculosis* challenge by vaccinating with extracellular antigens (Pal and Horwitz, 1992;Horwitz *et al.*, 1995). In the RD2 region, the gene *mpt64* encodes for the highly antigenic protein, MPT64, that has been previously identified as a candidate for new vaccines. To determine if the loss of this antigenic protein has impacted on the degree of protective immunity generated by BCG, BCG Denmark was complemented with *mpb64*, expressed with the MOP promoter on an integrating plasmid, creating the strain BCG Denmark::*mpb64*. The mycobacterial optimal promoter, MOP, is an artificial promoter region derived from promoter sequences from the BCG heat shock protein gene, *hsp70*, and an *Escherichia coli tac* promoter and is engineered to induce high gene expression from the plasmid.

First, expression of *mpb64* in the complemented strains was assessed. Figure 3.1a shows the expression of *mpb64* in three complemented strains, as well as BCG Russia (an early strain with an intact an RD2 region) and BCG Denmark (a late strain with deletion of the RD2 region). As expected, expression was detected in BCG Russia and no expression was found in BCG Denmark. Expression was detected in all three complemented strains examined and each strain showed greater expression than BCG Russia. To verify that gene expression correlated with protein production, the culture filtrate proteins from the complemented strains were analyzed qualitatively by immunoblotting for MPB64. As shown in Figure 3.1b, MPB64 could be detected in the culture filtrate proteins of the complemented strains, indicating that, similar to BCG Russia, these strains can produce and secrete the protein. Protein samples for immunoblotting were not prepared from the same cultures that were used to determine gene expression and thus the RNA expression values do not correlate with the quantity of protein detected in cultures. In related work, Horwitz and colleagues created recombinant strains of BCG that overexpress Ag85B. Although this antigen is present in all strains in BCG, vaccination with the recombinant strains displaying increased Ag85B production conferred enhanced protection against an *M. tuberculosis* challenge in guinea pigs and greater expression of the antigen correlated with better protection (Horwitz *et al.*, 2000). Based on these results, the strain of BCG Denmark that expresses the highest level of *mpb64* was selected for further studies as it presents the greatest potential to highlight any improvement in vaccine efficacy attributable to MPB64.

The ability of the BCG Denmark::*mpb64* strain to induce a protective response was evaluated in C57BL/6 mice. Mice were vaccinated subcutaneously with either BCG Denmark::pKZ4 (empty vector control) or BCG Denmark::*mpb64* and subsequently challenged by intravenous infection with BCG Russia. The latter strain was selected as a surrogate of a virulent strain in which the RD2 region is intact, because at the time of the experiment, construction of the level 3 laboratory was incomplete and experiments with *M. tuberculosis* were not permitted. Protection was determined by enumeration of colony forming units (CFU) of BCG Russia in the spleen and lungs of infected mice at four and eight weeks following the challenge. At both time points, protection induced by vaccination with either strain was greater in the lungs than in the spleen. The reduction of CFU in the spleens of mice vaccinated with either strain, compared to unvaccinated mice (sham-vaccinated with PBS), was 1 log at four weeks and 0.5 log at eight weeks. In the lungs, at four weeks, vaccination with BCG Denmark::pKZ4 induced a 1.3 log reduction in CFU compared to unvaccinated mice. In mice vaccinated with BCG Denmark::*mpb64*, however, CFU in the lungs were reduced an additional 0.3 log compared to BCG Denmark::pKZ4 vaccinated mice (figure 3.2). This difference was no longer observed after eight weeks, at which time an equal 1 log reduction in CFU was seen in mice vaccinated with either vaccine.

3.1.2. Attempted deletion of RD2 in BCG and subsequent RD2 deletion in M. tuberculosis H37Rv

In attempt to delete the RD2 region from early strains of BCG, three constructs were made using two plasmids, pKO and p2NIL/pGOAL19. The first construct, pKO-RD2, contained a downstream homologous region of 953 bp and an upstream homologous region of 730 bp flanking a kanamycin resistance cassette, which would allow for the knockout strain to be marked with kanamycin resistance. In addition to the kanamycin resistance cassette, both plasmids also contained a hygromycin resistance cassette. BCG Russia, BCG Sweden and BCG Birkhaug were electroporated and plated on 7H10 with hygromycin for selection of integration of the plasmid. Site-specific integration at either the downstream or the upstream homologous region was screened for by PCR. Approximately 20% of the colonies screened had downstream site-specific integration of the plasmid and no colonies with upstream site-specific integration were identified. Colonies with site-specific integration were re-plated on 7H10 medium containing sucrose as a counter-selection method conferred by the presence of the *sacB* gene on the plasmid. Two outcomes arose upon plating of the site-specific integrants on sucrose-containing plates, either very few colonies grew on the selective medium over the course of 4-6 weeks, or very high growth was seen with colonies forming within 1-2 weeks. The colonies appearing on the plates at 4-6 weeks were screened for each of the three expected outcomes: 1) deletion of the RD2 region; 2) reversion to wildtype by loss of the plasmid; or 3) growth on sucrose with maintenance of the plasmid by inactivation of the *sacB* gene.

Although the *sacB* gene may allow for a high degree of selection, reduction of selective power, presumably by inactivation of *sacB*, has been reported (Pavelka, Jr. and Jacobs, Jr., 1999). All colonies appearing on these plates were screened for the loss of the RD2 region, as well as the presence of the plasmid. No strains having lost the RD2 region were identified and greater than 90% of the colonies screened had maintained the plasmid, as detected by PCR.

The majority of the plates on which colonies appeared at 1-2 weeks could not be screened due to the high density of colonies. When isolated colonies could be selected, screening by PCR indicated maintenance of the plasmid. This suggested that a mutation in the *sacB* gene was acquired in the parent colony prior to plating on sucrose. For further experiments, such plates were not analyzed.

A two-plasmid system was developed by Parish and Stoker (Parish and Stoker, 2000), which consisted of a manipulation vector and a second vector containing various marker genes, in our case selected to include *sacB* for counter-selection. This allowed for the addition of the selection markers at a single restriction site in the manipulation vector subsequent to the cloning of the homologous regions. The construct pNIL/pGOAL-RD2 was created using the same homologous regions as the pKO-RD2 construct. Approximately 70% of colonies screened from hygromycin selection were single cross-overs with downstream site-specific integration of the plasmid. No upstream site-specific integration was detected. All colonies that were positive for site-specific integration were plated on sucrose for selection of double cross-over transformants. Approximately 50% of colonies screened from sucrose

selection had maintained the plasmid while retaining the RD2 region. The vast majority of the remaining colonies screened were positive for detection of the RD2 region and negative for the plasmid, suggesting a reversion to wild-type.

Upstream site-specific integration of the plasmid was detected in five colonies selected off sucrose plates, all of which arose from one parent colony. Since initial site-specific integration in the parent colony was at the downstream region, this was suggestive of a double recombination event leading to the elimination of the RD2 region. Both upstream and downstream site-specific integration were confirmed by PCR in each of the five colonies. Further characterization of these colonies, however, also revealed the continued presence of *mpt64*, a gene in the RD2 region, and both the *hyg* and *kan* genes present on the plasmid. Examination of these cultures by PCR and Southern blotting for the possibility of these being mixed cultures containing an RD2-knockout were inconclusive and additional attempts to isolate the knockout were unsuccessful.

A second pKO-based construct was designed with longer regions of homology flanking the kanamycin resistance cassette. Parish *et al.* demonstrated that in six of seven constructs used to knockout various genes involved in amino acid synthesis, recombination occurs preferentially on the side with the longer flanking region and although elimination of the gene of interest requires recombination at both flanking regions, a size greater than 1kb was suggested to be beneficial (Parish *et al.*, 1999). The upstream region in the second pKO-RD2 construct was 1290bp in length and the downstream region was 1472bp. Similar to the first pKO construct, all site-specific integration of the plasmid in the colonies screened from the hygromycin selection was

downstream site-specific integration. No RD2-knockouts were identified from the colonies screened from the sucrose selection.

In parallel, efforts were expended to also attempt to directly delete the RD2 region from virulent *M. tuberculosis* H37Rv. Because the level 3 containment laboratory was not yet completed at McGill, this work, using the methodology outlined above, was performed in the laboratory of David Sherman, at the University of Washington. Deletion of the RD2 region was obtained in both *M. tuberculosis* H37Rv and *M. tuberculosis* H37RvΔRD1, and confirmed by Southern blot (Figure 3.3).

3.1.3. H37RvΔRD2 Growth Characterization

To assess the impact of the loss of the RD2 region, we first examined the *in vitro* growth characteristics of the *M. tuberculosis* H37RvΔRD2 strain. The *in vitro* growth curve of H37RvΔRD2 was indistinguishable from that of H37Rv, as shown in Figure 3.4. The growth rates of the two strains in lag and log phases of growth were similar and the strains reached similar maximum densities.

Cytotoxicity to THP-1 cells caused by infection with *M. tuberculosis* can be observed as a decrease in cellular metabolism of an indicator dye, alamar blue. Infection of THP-1 cells with *M. tuberculosis* H37Rv results in destruction of the cell monolayer over the course of the infection and a decrease in cell metabolism, resulting from cell death (Lewis *et al.*, 2003). Contrary to H37Rv, infection with an attenuated strain of *M. tuberculosis,* as demonstrated by infection with *M.*

tuberculosis H37RvΔRD1 (Lewis *et al.*, 2003), does not result in either a decrease in metabolism or destruction of the monolayer. To assess what degree of attenuation, if any, resulted from the deletion of the RD2 region, THP-1 cells were infected with H37Rv or H37RvΔRD2 and cell metabolism was determined four and seven days following infection. H37Rv and H37RvΔRD2 both induced a high degree of cell death, as determined by a significant decrease in metabolism of alamar blue (figure 3.5) and destruction of the cell monolayer (data not shown). There was no difference between these strains suggesting that the loss of RD2 may not lead to any loss of virulence of *M. tuberculosis,* as measured by cytotoxicity to host macrophage-like cells.

3.1.4. Microarray of Early BCG vs. Late BCG and Identification of mpb70/mpb83

In an attempt to identify potential phenotypes arising from the loss of the RD2 region, global gene expression of selected early strains of BCG was compared to that of late strains of BCG by whole genome microarray. The results summarizing the direct comparison of BCG Russia with BCG Pasteur and BCG Birkhaug with BCG Denmark are presented in Table 3.1. The genes that were consistently found to be differentially expressed between early BCG and late BCG were limited, many of which were annotated as conserved hypothetical proteins (open reading frames encoding conserved elements of unknown function). Of particular note, however, were the genes *mpb70* and *mpb83*. MPB70 and MPB83 have been previously

identified as antigenic proteins that are highly produced in some strains of BCG, but almost undetectable in others.

To confirm the microarray results, quantitative RT-PCR was used to compare the expression of these genes in the same strains of BCG that were used for the microarray comparisons. Expression of *mpb70* and *mpb83* was significantly higher in the early strains (BCG Russia and BCG Birkhaug) compared to the late strains (BCG Denmark and BCG Pasteur), with a calculated difference of greater than 50-fold (Figure 3.6).

This difference was further confirmed by immunoblotting of culture filtrate proteins and whole-cell extracts from all BCG strains (Figure 3.7). MPB70 is a secreted protein, thus detectable in the CFP, and MPB83 is a membrane-bound protein and most often detectable in the cellular extracts, although can also be found in the CFP. MPB70 and MPB83 were both clearly detected in all early strains, while in late strains of BCG, MPB70 was undetectable and MPB83 was only faintly detectable. These results correlate with the subset of strains previously identified as being either high- or low-producers of MPB70 and MPB83.

3.1.5. Microarray H37Rv vs. H37RvΔRD2 and Expression of mpt70/mpt83

From the previous results indicating a concurrence of the high-producing strains with the presence of the RD2 region and the low-producing strains with its absence, we hypothesized that the reduction in expression of *mpb70* and *mpb83* could be

consequential to the deletion of the RD2 region. To examine this possibility, whole genome microarray comparison of gene expression between H37Rv and H37RvΔRD2 was used. The only genes consistently observed to be down-regulated in the deletion mutant were genes of the RD2 region itself, suggesting that transcription of genes in the RD2 region does not significantly influence expression of genes outside of this region during standard laboratory *in vitro* growth (data not shown). Notably, neither *mpt70*, nor *mpt83* were identified as differentially expressed as a function of RD2 disruption.

3.2. Reduced expression of mpb70 and mpb83 is due to a start codon mutation in sigK

The identification of antigenic proteins that induce a strong cellular immune response during *M. tuberculosis* infection has been a major focus in TB vaccine development. MPB70 was isolated from the culture filtrate of BCG Tokyo and was found to account for more than 10% of the secreted proteins (Nagai, Matsumoto, and Nagasuga, 1981). The abundance of this protein in the culture filtrate and the high reactivity of BCG-sensitized animals to MPB70 instigated further characterization of this protein. Sensitization of guinea pigs with various strains of BCG and subsequently testing for DTH responsiveness to MPB70 revealed that not all strains of BCG induced reactivity to the protein (Miura *et al.*, 1983;Haslov, Andersen, and Bentzon, 1987). A highly homologous protein, showing significant cross-reactivity with MPB70 and similar induction of DTH response in guinea pigs, was later identified and called MPB83 (Harboe *et al.*, 1995). Further characterization of BCG strains resulted in the division of strains into high- and low-producers of these proteins (Abou-Zeid *et al.*, 1986;Wiker *et al.*, 1996). The sequence of *mpb70* in the high- and low-producers is identical, but analysis of mRNA and Northern blots revealed significantly different levels of expression between high- and low-producers (Matsuo *et al.*, 1995). Further studies provided evidence that *mpt83* (*Rv2873*) and *mpt70* (*Rv2875*) form part of the same transcriptional unit (Juarez, Torres, and Espitia, 2001). Taken together, this information suggests that the difference in expression between high- and low-producing BCG strains could be due to a shared but unknown defect in transcription.

3.2.1. *Identification and sequencing of sigK*

As the deletion of RD2 did not impact upon the expression of *mpt70* and *mpt83* as initially hypothesized, the initial microarray data examining the differences in gene expression between early and late strains of BCG were revisited. A second region, separate from the *mpt70/mpt83* region, was also significantly down-regulated in late (low-producing) strains of BCG. This region includes a gene encoding a predicted regulatory protein, annotated as a probable alternate RNA polymerase sigma factor classified in the extracytoplasmic function (ECF) sigma factor subfamily (See Table 3.1). To explore the possibility that a mutation in *sigK* might be linked to the expression phenotype, we first compared the sequence of *sigK* from *M. tuberculosis* H37Rv to sequences generated by the genome sequencing projects for *M. bovis* 2122 and *M. bovis* BCG Pasteur. Alignment searches predicted that BCG Pasteur had a mutation at the third basepair of *sigK*, prompting formal sequencing and comparison across members of the *M. tuberculosis* complex, including different strains of BCG.

Sequencing of *sigK* revealed a guanine to adenine mutation at the third nucleotide of the coding region in low-producing strains of BCG compared to high-producing strains, resulting in a change from the typical AUG start codon to an AUA start codon in the corresponding strains. The AUG start codon was observed in all members of the *M. tuberculosis* complex except for the eight low-producing strains of BCG obtained after 1927 (table 3.2). While the codon AUA has been identified as a functional start codon in *Escherichia coli*, *Bacillus subtilis* and *Salmonella spp*, levels

of translation are substantially reduced with this codon compared to the conventional AUG start codon (Romero and Garcia, 1991;Sussman, Simons, and Simons, 1996).

3.2.2. *Complementation of sigK*

As the start codon mutation corresponded precisely with the low-producing strains, the functional consequences of this mutation were evaluated in a low-producing strain, BCG Pasteur, by complementing this strain with *sigK* with an AUG start codon. Using an integrating vector, pMV306, BCG Pasteur was complemented with wild-type *sigK* from *M. tuberculosis* H37Rv, BCG Russia or BCG Birkhaug. In addition, this strain was complemented with a second copy of *sigK* from BCG Pasteur (mutant *sigK*) and with the empty vector to serve as controls. Complementation was confirmed by PCR using vector-specific primers (data not shown).

3.2.3. *Expression of sigK in complemented strains*

A common characteristic of ECF sigma factors, which has been confirmed for other *M. tuberculosis* ECF sigma factors (Helmann, 2002;Manganelli *et al.*, 2004b), is that their expression is auto-regulated and as such, we first determined the effect of *sigK* complementation on its own expression using quantitative RT-PCR. As negative controls, complementation with the empty vector or with the mutant *sigK* resulted in no change of *sigK* expression, shown by BCG Pasteur::pMV306 and BCG Pasteur::*sigK*Pasteur. The latter indicates that introducing a second copy of the mutant gene did not alter levels of transcription. In contrast, complementation with wild-type *sigK*, demonstrated with BCG Pasteur::*sigK*H37Rv, BCG

Pasteur::*sigK*Russia and BCG Pasteur::*sigK*Birkhaug, showed a marked increase in transcription of *sigK* (figure 3.8). Similar results were obtained with a second clone of each of the same strains (data not shown). Since the mutation in *sigK* is predicted to impair translation, not transcription, and complementation of wild-type, but not mutant-type, *sigK* served to markedly increase *sigK* expression, these results were consistent with auto-regulation of this gene, as is typically observed with ECF sigma factors.

3.2.4. Transcription of mpb70/mpb83 and MPB70/MPB83 production in complemented strains

Expression levels of *mpb70* and *mpb83* in the complemented strains were analyzed by quantitative RT-PCR. In BCG Pasteur::*sigK*Pasteur and BCG Pasteur::pMV306, levels of mRNA were comparable to those previously demonstrated in low-producing strains of BCG, indicating the introduction of the empty vector, or a second mutant copy of the gene, had no effect on *mpb70/mpb83* expression. In contrast, the same strains in which an increase in *sigK* expression was observed (BCG Pasteur::*sigK*H37Rv, BCG Pasteur::*sigK*Russia and BCG Pasteur::*sigK*Birkhaug), manifested highly increased levels of transcription of *mpb70* and *mpb83*, comparable to the levels observed with high-producing strains of BCG (figure 3.9). The same results were obtained with a second clone of the same strains (data not shown).

To determine the effect of expression of wild-type *sigK* in BCG Pasteur on protein synthesis, culture filtrate proteins and whole cell lysates from the *sigK*-complemented strains of BCG Pasteur were analyzed by SDS-PAGE and immunoblotting. MPB70 was detected in the culture filtrate proteins from BCG Pasteur::*sigK*H37Rv, BCG Pasteur::*sigK*Russia and BCG Pasteur::*sigK*Birkhaug, but could not be detected in Pasteur::*sigK*Pasteur or BCG Pasteur::pMV306 (figure 3.10a). Similarly, MPB83 was detected in the whole cell lysates of the same clones in which MPB70 was abundantly detected in the culture filtrates (figure 3.10b). Upon complementation of wild-type *sigK,* BCG Pasteur was able to produce MPB70 and MPB83 in a pattern consistent with high-producing strains.

3.2.5. Microarray of complemented strains, qRT-PCR confirmation

Based on observed differences in expression of the genes neighbouring *sigK* and *mpb70/mpb83* by microarray-based analysis of four BCG strains, we used quantitative RT-PCR to determine the effect of wild-type *sigK* complementation on transcription in these regions. As shown in figure 3.11a, expression of the genes *Rv0443c-Rv0449c* was increased over 2-fold with *sigK* complementation. Transcription of *Rv2874, Rv2876* and *Rv2877c* was increased over 10-fold in the *sigK*-complemented strains compared to the control strain. *Rv2878c* (*mpt53*) was also increased, although to a lesser extent, with 4-fold increase seen in the *sigK*-complemented strains. Contrary to expectations from a previous study indicating that *Rv2871-Rv2874* are co-transcribed (Juarez, Torres, and Espitia, 2001), we did not detect any increased expression of either *Rv2871* or *Rv2872*, suggesting that these two

genes are under separate transcriptional control. Based on the differences in transcription, this *sigK*-regulated gene region includes *Rv2873* through *Rv2878*, but not *Rv2872* or *Rv2879*. (figure 3.11b).

To further examine the role that *sigK* plays in global gene expression, we analyzed global transcription in BCG Pasteur::*sigK*H37Rv, BCG Pasteur::*sigK*Russia and BCG Pasteur::*sigK*Birkhaug compared to BCG Pasteur::pMV306 by DNA microarray (table 3.3). Results revealed increased expression of the majority of genes presented in figure 3.11, with significant changes in expression as measured by both induction ratios and Z-scores. There were no genes outside of either the *mpb70/mpb83* or the *sigK* regions that were found to be significantly up-regulated, suggesting a high degree of specificity of this system. Because of the stringency of the analysis performed, genes whose expression was induced in 3 of 4 arrays, but whose expression could not be optimally quantified on the fourth, were not included in the table (e.g. *mpb83*). Only four genes were repressed with introduction of wild-type *sigK*.

*3.3. Characterization of BCG Pasteur::*sigK

The importance of MPB70 and MPB83 emerged as their immunological properties were studied. Both proteins can induce cellular and humoral responses in a variety of animals including mice, rabbits, guinea pigs and cows (Miura *et al.*, 1983;Haslov, Andersen, and Bentzon, 1987;Hewinson *et al.*, 1996;Wiker *et al.*, 1998). Although MPB70 and MPB83 are classically considered to be antigens of *M. bovis* rather than *M. tuberculosis*, the ability of MPB70 to stimulate lymphocyte proliferation in a significantly greater proportion of TB patients than BCG vaccinees points to production of this protein during natural infection by *M. tuberculosis* (Roche *et al.*, 1994). In fact, while the *in vitro* production of MPT70 in *M. tuberculosis* is comparable to the low-producing BCG strains, a strong increase in expression of both *mpt70* and *mpt83* has been observed during intracellular infection by *M. tuberculosis* (Schnappinger *et al.*, 2003). MBP70 has also been shown to be recognized by T-cells from donors with a wide variety of HLA backgrounds and was suggested as a good candidate for subunit vaccines due to its recognizability by a high proportion of the population (Al Attiyah *et al.*, 2003). To determine the consequence of high production of these proteins, we compared our isogenic strains of BCG Pasteur that either fail to produce these proteins (BCG Pasteur::pMV306 - empty vector control) or produce them in high quantities (BCG Pasteur::*sigK*) in *in vitro* and *in vivo* models.

3.3.1. In vitro Growth

To characterize the impact of expression of the *sigK* regulon on growth, the *in vitro* growth rates of BCG Pasteur::pMV306 and BCG Pasteur::*sigK*Russia were compared (Figure 3.12). BCG Pasteur::*sigK*Russia consistently displayed a longer lag phase, but recovered to reach a similar maximum growth rate and maximum density as BCG Pasteur::pMV306. Similarly, when grown on solid media, Pasteur::pMV306 colonies consistently appeared 2-5 days earlier than Pasteur::*sigK*Russia colonies.

3.3.2. THP-1 Virulence Assay

As previously described, the level of cytotoxicity induced by an infecting strain can be assessed using a THP-1 cell infection model. THP-1 cells were infected with BCG Pasteur::pMV306 and BCG Pasteur::*sigK*Russia with an MOI of 50:1, 25:1, 10:1 and 1:1 for 24 hours and metabolism of the indicator dye alamar blue was measured as an indication of cytotoxicity (Figure 3.13). At an MOI of 1:1, the cells infected with either strain appeared healthy and metabolism was comparable to uninfected cells. No difference in metabolism between infected cells was observed. Metabolism of the cells infected with an MOI of 10:1 was lower than that of uninfected cells and those infected with BCG Pasteur::*sigK*Russia consistently displayed significantly lower metabolism than those infected with BCG Pasteur::pMV306. At both 25:1 and 50:1, BCG Pasteur::*sigK*Russia displayed significantly greater cytotoxicity than BCG Pasteur::pMV306. Metabolism of cells infected with either strain at these MOIs was significantly lower than that of uninfected cells and destruction of the cell monolayer was evident.

3.3.3. Infection of C57Bl/6 mice

Characterization of the growth of potential vaccine strains in animal models highlights two important characteristics of live vaccines against tuberculosis: the persistence of the strain and the pathogenicity of the strain. To determine if the expression of *mpb70* and *mpb83* impacts on the growth and survival of BCG Pasteur in an animal model, C57BL/6 mice were infected with BCG Pasteur::pMV306 and BCG Pasteur::*sigK*Russia. The pattern of growth of each strain was similar in the two infections performed. Mice were infected with an average of 2.5 x 10^6 CFU in the first experiment and 5.7 x 10^6 CFU in the second experiment and the infection doses of the strains were not significantly different within each experiment. Despite the same inocula, BCG Pasteur::*sigK*Russia consistently showed higher CFUs in the lungs and spleen on day 1 (Figure 3.14), although this was not statistically significant in either infection. By day 21 in both experiments, the CFU in the lungs and spleen increased to the maximum levels observed and were almost identical between BCG Pasteur::*sigK*Russia and BCG Pasteur::pMV306. In the first experiment, the BCG Pasteur::*sigK*Russia CFU increased 3.6 and 4.6 times, in the spleen and lungs respectively, from day 1 to day 21, whereas the BCG Pasteur::pMV306 CFU increased 8.3 and 8.4 times, in the spleen and lungs respectively. Similarly, in the second experiment, BCG Pasteur::*sigK*Russia CFU increased 3.6 and 2.4 times from day 1 compared to 6.9 and 3.8 times, in the spleen and lungs respectively, for BCG Pasteur::pMV306. On days 42 and 84, the CFU declined for both strains in the spleen and lungs, however, in the two experiments, BCG Pasteur::pMV306 declined to lower levels than BCG Pasteur::*sigK*Russia.

3.3.4. *Splenocytes – IFNγELISA*

To determine if increased expression of *mpb70* in BCG Pasteur::*sigK*Russia induced an antigen-specific immune response, splenocytes were isolated from vaccinated animals four weeks following vaccination and cultured with MPB70. Splenocytes from animals vaccinated with either BCG Pasteur::pMV306 or BCG Pasteur::*sigK*Russia responded strongly when cultured with an unspecific mitogen concanavalin A, but only those cultured from animals vaccinated with BCG Pasteur::*sigK*Russia produced IFNγ in response to stimulation with MPB70. As shown in figure 3.15, splenocytes from BCG Pasteur::*sigK*Russia vaccinated animals produced high levels of IFNγ in response to purified MPB70, while levels of IFNγ from splenocytes isolated from BCG Pasteur::pMV306 vaccinated animals were almost undetectable. Both cultures responded to CFP purified from cultures of either strain (data not shown).

3.3.5. *Intravenous Challenge with BCG Russia in BCG Pasteur::pMV306 and BCG Pasteur::*sigK*Russia vaccinated mice*

To evaluate the impact of the presentation of the antigenic proteins MPB70 and MPB83 to the immune system on the protective response induced by vaccination with BCG, a mouse challenge model was used. C57Bl/6 mice were vaccinated with BCG Pasteur::*sigK*Russia or BCG Pasteur::pMV306 (subcutaneous injection with 10^6 CFU) and challenged intravenously with BCG Russia ten weeks after vaccination. As construction of the level 3 laboratory for the use of *M. tuberculosis* was incomplete, BCG Russia was used as a surrogate of a virulent strain expressing both MPB70 and

MPB83. Vaccination with either strain reduced the bacterial load in the lungs and spleen of challenged mice compared to unvaccinated mice (Figure 3.16). At week 4, vaccination with either strain resulted in a significant reduction in CFU as compared to unvaccinated mice, with a 1.5 log reduction in CFU observed in the lungs and a 1.0 log reduction in the spleen. At week 8, the reduction in bacterial load in the Pasteur::*sigK*Russia vaccinated mice compared to unvaccinated mice was 0.7 log greater in the lungs and 0.4 log greater in the spleen than the reduction in the Pasteur::pMV306 vaccinated mice. The reduction in CFU in the Pasteur::pMV306 vaccinated mice, however, was less than anticipated and the bacterial load in the spleen was not significantly different from that of the unvaccinated mice.

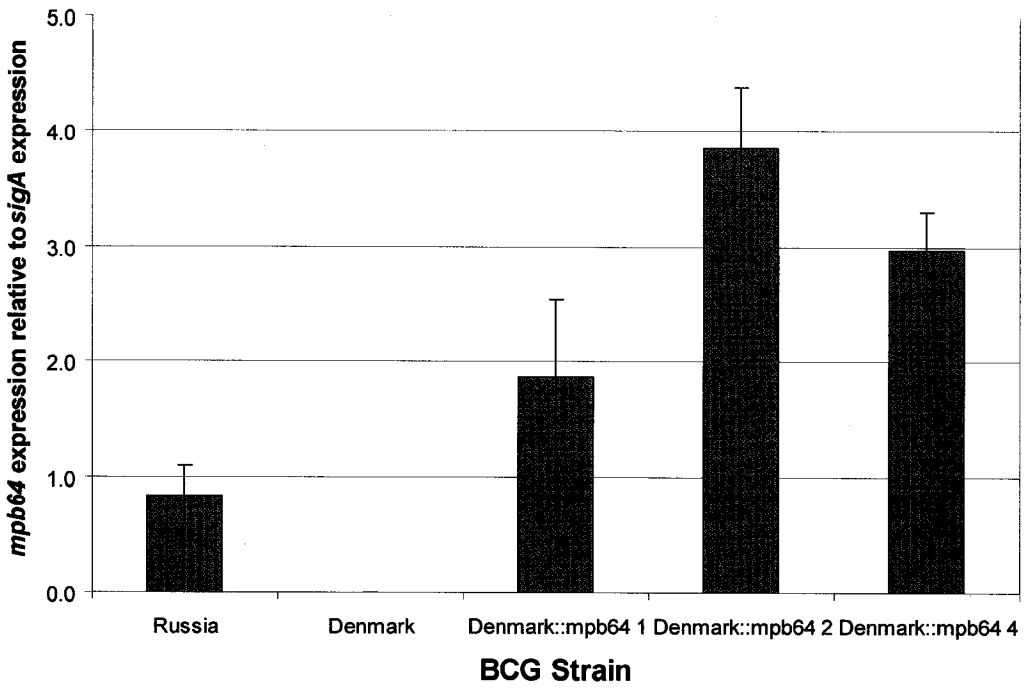
3.3.6. Intravenous Challenge with M. tuberculosis H37Rv in BCG Pasteur::pMV306 and BCG Pasteur::sigKRussia vaccinated mice

Upon completion of the level 3 laboratory, further challenge studies were pursued to better characterize the ability of BCG Pasteur::*sigK*Russia to induce a protective immune response compared to BCG Pasteur::pMV306. As before, C57BL/6 mice were vaccinated subcutaneously with BCG Pasteur::*sigK*Russia or BCG Pasteur::pMV306, but were instead challenged with *M. tuberculosis* H37Rv by intravenous infection ten weeks after vaccination. The bacterial load in the lungs and spleen were determined at four and eight weeks after the *M. tuberculosis* challenge. In the first experiment (Figure 3.17), the average vaccination dose was 6.3×10^5 CFU of the designated strain of BCG and mice were challenged with 4.6×10^6 CFU of *M. tuberculosis* H37Rv. In the second experiment (Figure 3.18), mice were vaccinated

with an average of 2.7 x 10^6 CFU of BCG and challenged with 1.2 x 10^6 CFU of *M. tuberculosis* H37Rv.

At four weeks post-challenge, significant levels of protection were observed with both vaccination strains compared to unvaccinated mice (figures 3.17a, 3.18a). The greatest levels of protection observed were in the lungs of the Pasteur::*sigK*Russia vaccinated mice in both experiments, in which a 1.3-1.4 log reduction in CFU compared to unvaccinated mice was observed. At eight weeks post-challenge, a significant reduction in *M. tuberculosis* CFU was again observed in both vaccination groups compared to unvaccinated controls. In the first experiment, BCG Pasteur::pMV306 and BCG Pasteur::*sigK*Russia induced similar levels of protection. No difference in CFU reduction was observed between the groups vaccinated with BCG Pasteur::pMV306 or BCG Pasteur::*sigK*Russia (figure 3.17b). In the second experiment, both strains induced a significant level of protection compared to unvaccinated mice and there was no difference between the two vaccinated groups in the reduction of *M. tuberculosis* CFU in the lungs. Reduction of CFU in the spleen in BCG Pasteur::*sigK*Russia vaccinated mice, however, was almost 0.5 log greater than the reduction observed in the BCG Pasteur::pMV306 vaccinated group (figure 3.18b).

A.



B.

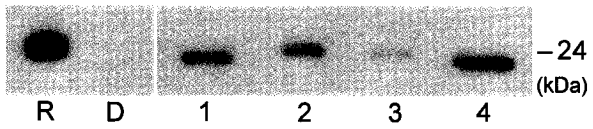
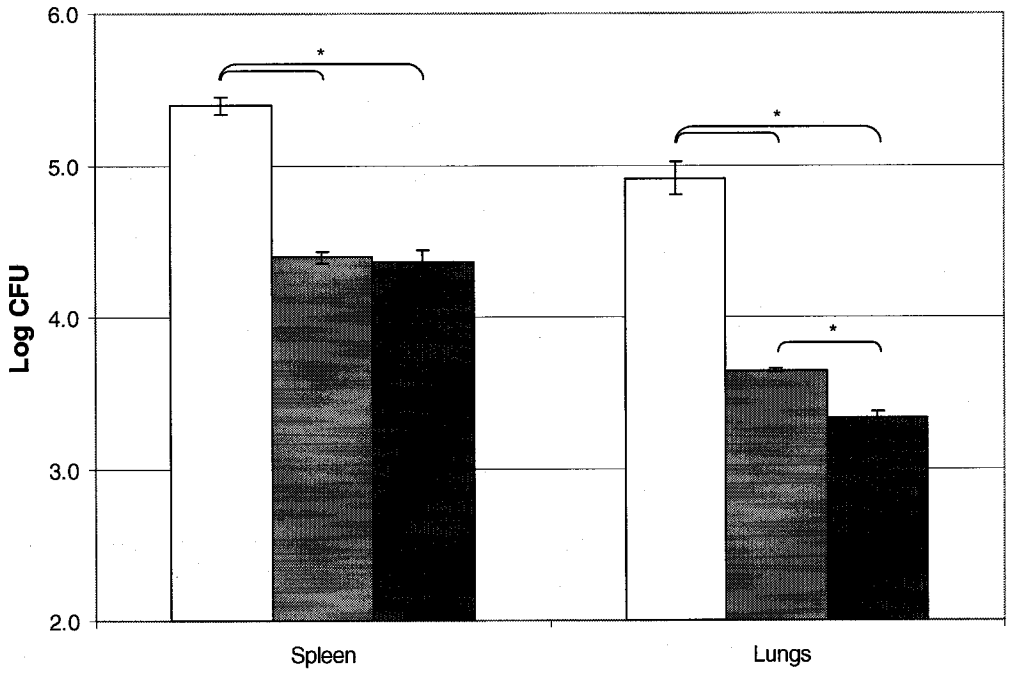


FIGURE 3.1 – A. Expression of *mpb64* upon complementation of BCG Denmark with *mpb64* from BCG Russia. Values are expressed as a ratio of gene expression in complemented strains compared to BCG Denmark::pKZ4. All values were normalized to the levels of *sigA* mRNA and error bars represent the standard error of the mean. B. SDS-PAGE and immunoblotting of culture filtrate proteins from BCG Russia, BCG Denmark and complemented strains of BCG Denmark, using Mab L24b4. The strains used were as follows: R – BCG Russia, D – BCG Denmark, 1-4 – BCG Denmark::*mpb64* 1-4

WEEK 4



WEEK 8

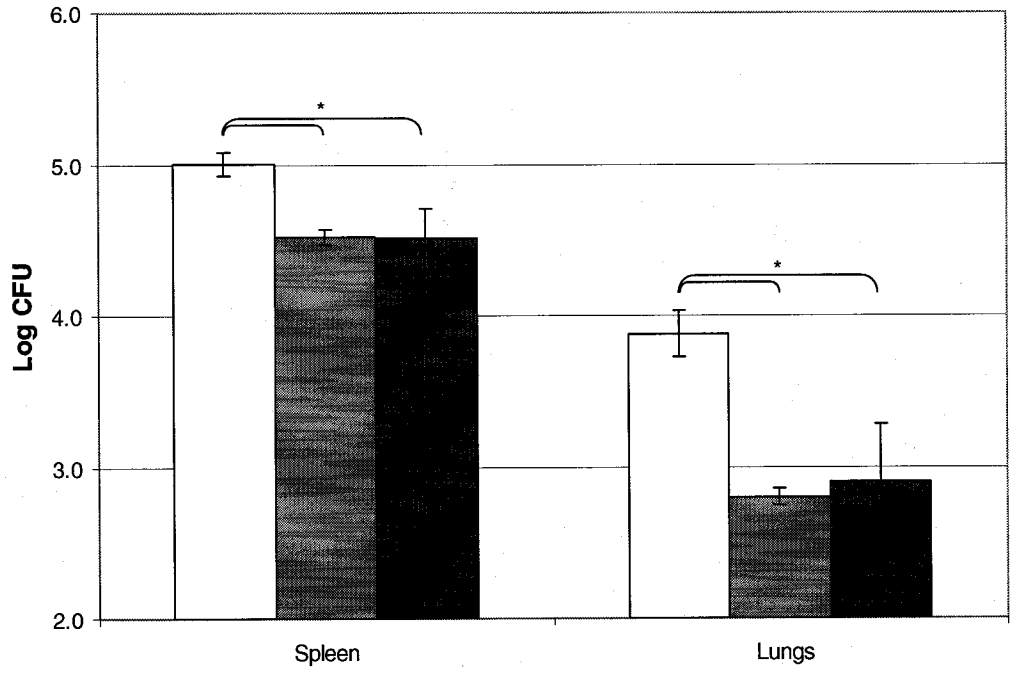


FIGURE 3.2 – Bacterial counts in the spleen and lungs of BCG Russia challenged animals, at 4 and 8 weeks, following vaccination with PBS (□), BCG Denmark::pKZ4 (▨) or BCG Denmark::*mpb64* (■). Results are expressed as the mean of 3-5 animals per group and error bars represent the standard error of the mean. (*, $p < 0.05$)

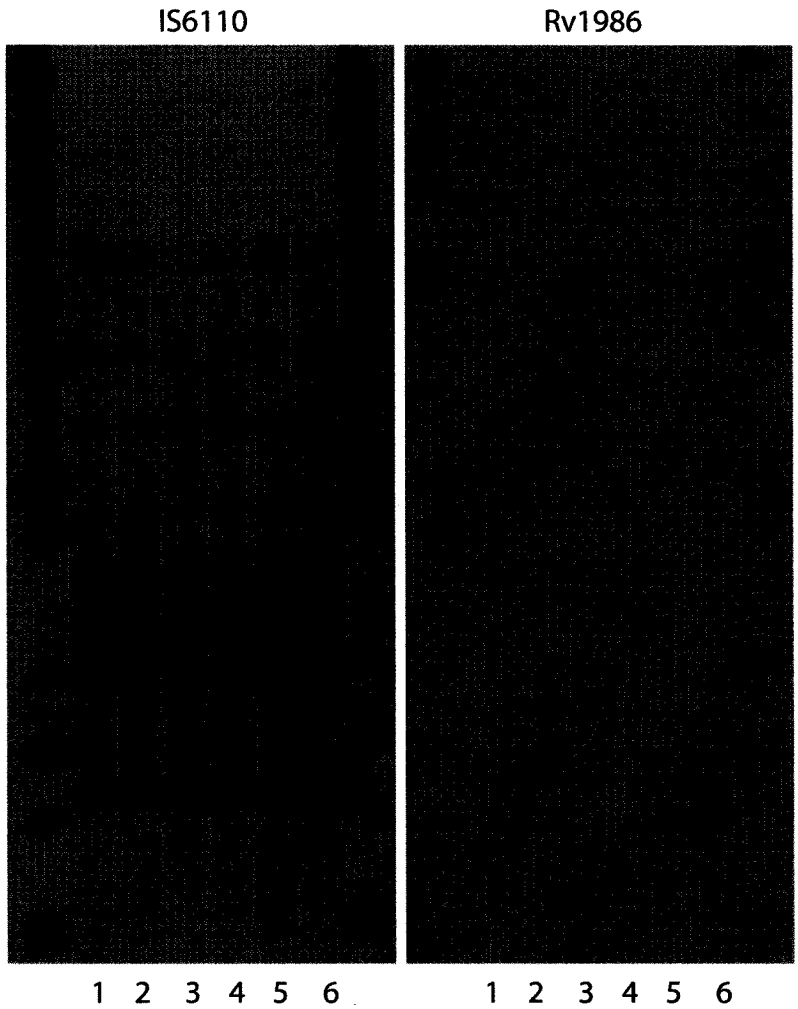


FIGURE 3.3 - Southern blot analysis of *Pvu*II-digested chromosomal DNA from *M. tuberculosis* H37Rv (lanes 5 and 6), *M. tuberculosis* H37RvΔRD2 (lanes 3 and 4) and *M.tuberculosis* H37RvΔRD1ΔRD2 (lanes 1 and 2), using probes for IS6110 and *Rv1986*.

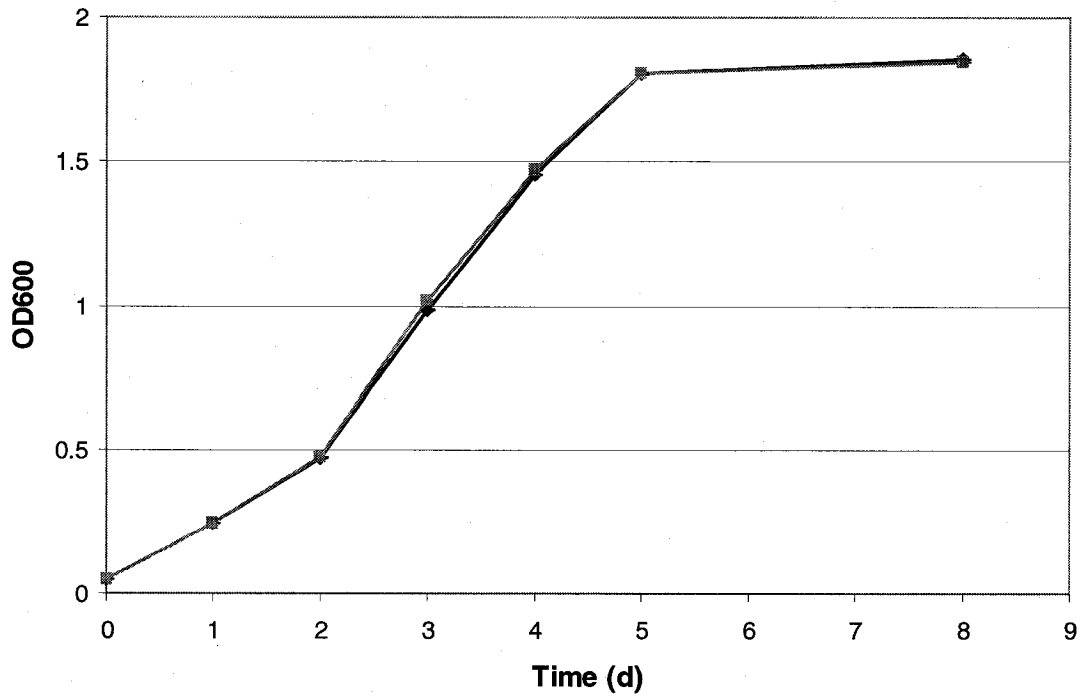


FIGURE 3.4 – *In vitro* growth of *M. tuberculosis* H37Rv and H37RvΔRD2. Growth was followed by measuring OD_{600} of cultures of *M. tuberculosis* H37Rv (◆) and *M. tuberculosis* H37RvΔRD2 (▩). Data for each time point is the mean and SEM of three cultures.

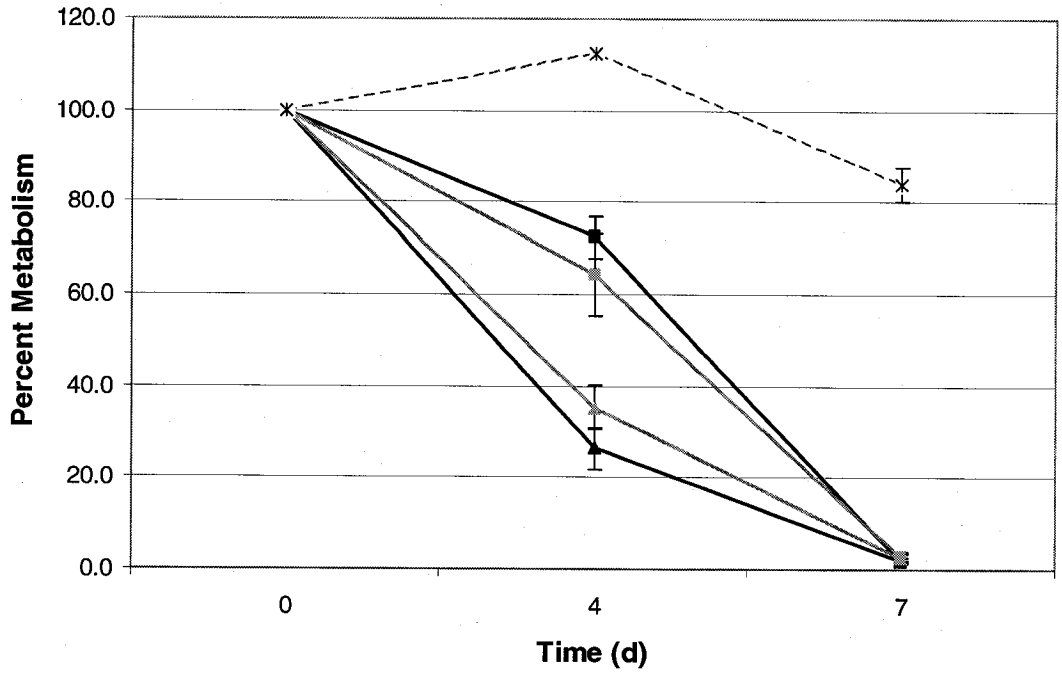


FIGURE 3.5 – Infection of human macrophage THP-1 cell line with *M. tuberculosis* H37Rv (black) and H37RvΔRD2 (grey), with an MOI of 0.5 (□) or 1.0 (△). Cytotoxicity was determined as a function of cell metabolism and indicated as a percent of metabolism compared to day 0. Metabolism by uninfected cells is represented by the dashed line.

ORF	Gene name	Russia vs Pasteur		Birkhaug vs Denmark		Gene Product
		Fold change	Z-score	Fold change	Z-score	
Rv0445c	*sigK*	-5.29	3.56	-7.14	3.04	Sigma factor
Rv0446c		-5.79	3.82	-8.34	3.36	Conserved transmembrane protein
Rv0447c	*ufaA1*	-6.91	3.79	-8.14	3.17	Cyclopropane-fatty-acyl-phospholipid synthase
Rv0448c		-15.82	4.22	-11.09	4.68	Conserved hypothetical protein
Rv0449c		-14.46	5.35	-19.16	5.07	Conserved hypothetical protein
Rv2627c		3.10	2.93	5.02	2.18	Conserved hypothetical protein
Rv2707		-3.53	2.42	-3.93	2.28	Conserved transmembrane protein
Rv2873	*mpb83*	-13.71	6.47	-36.87	5.00	Cell surface lipoprotein
Rv2875	*mpb70*	-17.51	6.31	-33.99	5.45	Major secreted immunogenic protein
Rv2876		-10.04	4.64	-12.81	4.21	Conserved transmembrane protein
Rv2878c	*mpb53*	-3.55	3.92	-26.41	2.25	Soluble secreted antigen
Rv3681c	*whiB4*	2.73	2.08	3.06	2.22	Transcriptional regulatory protein

TABLE 3.1 – Predicted genes whose *in vitro* expression was significantly dysregulated between early versus late BCG. The Z-score, indicative of how many standard deviations a data point lies from the population mean, is the average for that gene across replicate (n=2) microarrays of Russia (early BCG) versus Pasteur (late BCG) and Birkhaug (early BCG) versus Denmark (late BCG). Genes with average Z-scores of 2 or greater for both early versus late BCG comparisons are presented and the fold change is calculated from a normalized log-ratio of that gene. The fold change in late BCG expression is expressed as a ratio, with early BCG expression for that gene providing the reference value of 1. Genes for which expression was decreased in late BCG are indicated by a negative sign, genes with increased expression in late BCG are reported by a positive sign. Open reading frame (ORF) and gene name are listed by Rv number as annotated for *M. tuberculosis* H37Rv (http://genolist.pasteur.fr/TubercuList/). Genes from the same region of the genome are grouped and presented in a darker shade.

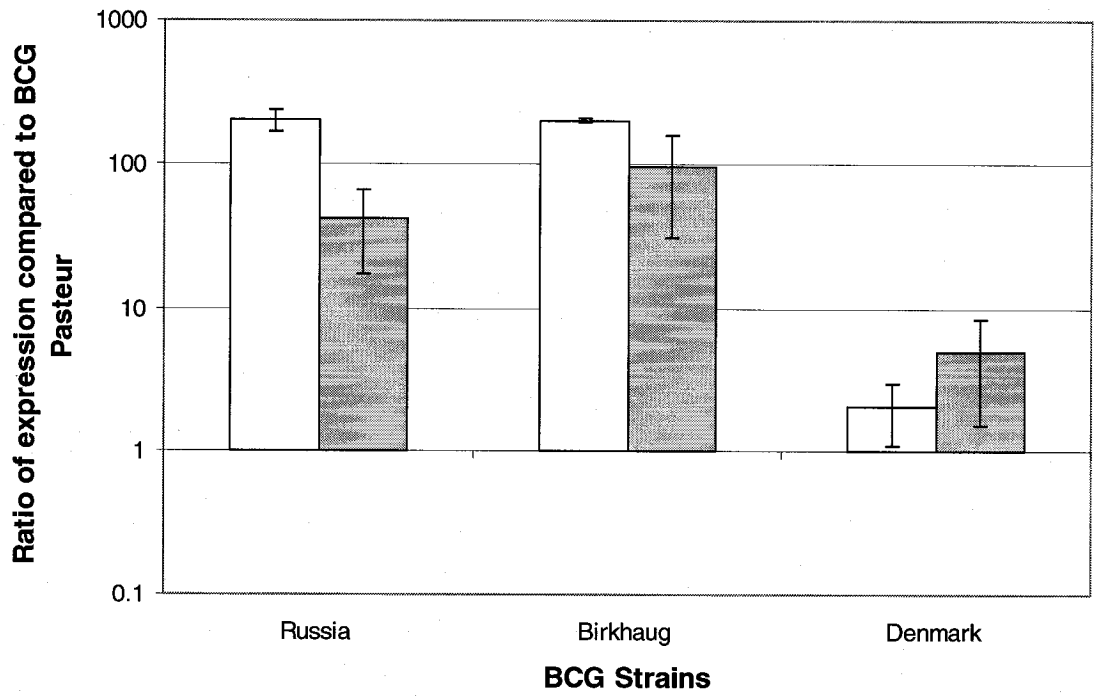


FIGURE 3.6 – Expression of *mpb70* (white) and *mpb83* (grey) in *M. bovis* BCG strains. Values are expressed as a ratio of gene expression compared to that of BCG Pasteur. All values were normalized to the levels of *sigA* mRNA and error bars represent the standard error of the mean.

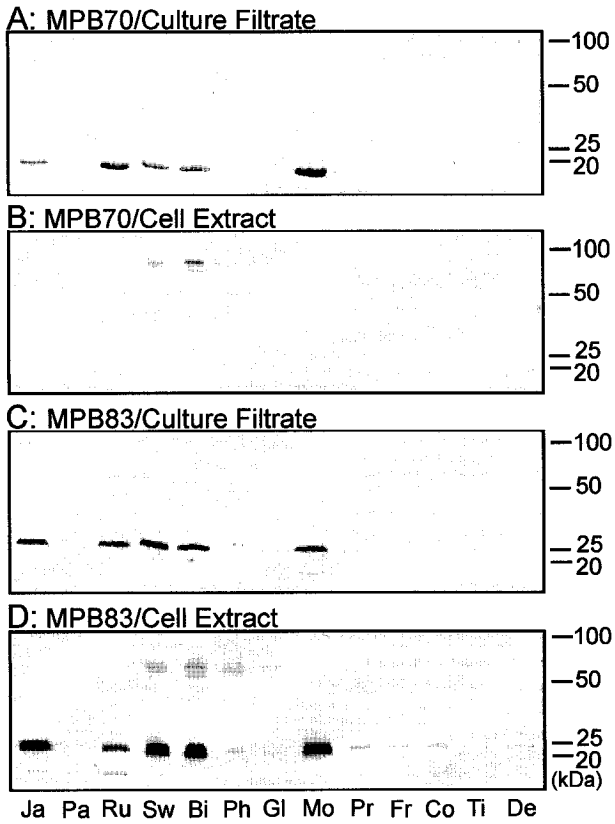


FIGURE 3.7 – SDS-PAGE and immunoblotting of culture filtrate proteins (A, C) and cell extracts (B, D) from BCG strains, using MAbs 1-5c for MPB70 (A, B) and MBS43 for MPB83 (C,D). The strains used were as follows; Ja-BCG Japan, Pa-BCG Pasteur, Ru-BCG Russia, Sw-BCG Sweden, Bi-BCG Birkhaug, Ph-BCG Phipps, Gl-BCG Glaxo, Mo-BCG Moreau, Pr-BCG Prague, Fr-BCG Frappier, Co-BCG Connaught, Ti-BCG Tice, De-BCG Denmark.

Strains	3bp of start codon
M. canettii	AUG
M. tuberculosis H37Rv	AUG
M. tuberculosis H37Ra	AUG
M. africanum	AUG
M. microti	AUG
M. caprae	AUG
M. bovis	AUG
BCG Russia	AUG
BCG Moreau	AUG
BCG Japan	AUG
BCG Sweden	AUG
BCG Birkhaug	AUG
BCG Prague	***AUA***
BCG Glaxo	***AUA***
BCG Denmark	***AUA***
BCG Tice	***AUA***
BCG Connaught	***AUA***
BCG Frappier	***AUA***
BCG Phipps	***AUA***
BCG Pasteur	***AUA***

TABLE 3.2 – Sequence analysis of *sigK* for two representative isolates of different *M. tuberculosis* complex members, with the exception of *M. canettii* where only one isolate was sequenced.

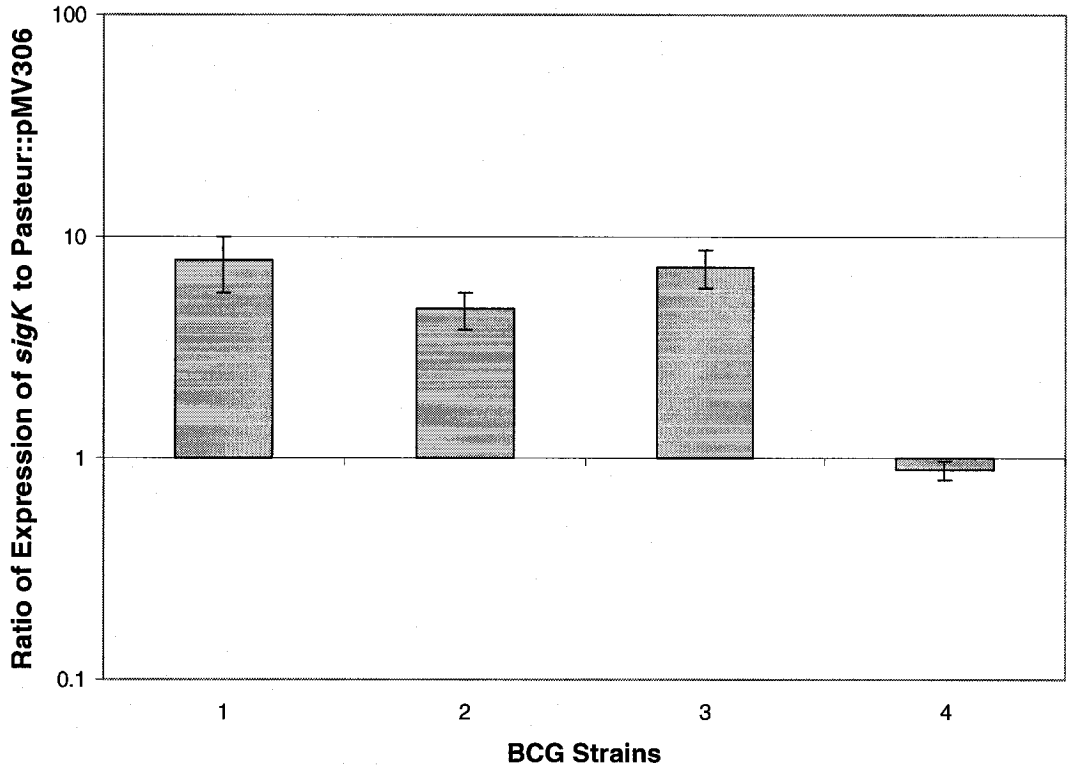


FIGURE 3.8 – Expression of *sigK* upon complementation of BCG Pasteur with *sigK* from 1 - *M. tuberculosis* H37Rv, 2 - BCG Russia, 3 - BCG Birkhaug and 4 - BCG Pasteur. Values are expressed as a ratio of *sigK* expression in complemented strains compared to BCG Pasteur::pMV306. All values were normalized to the levels of *sigA* mRNA and error bars represent the standard error of the mean.

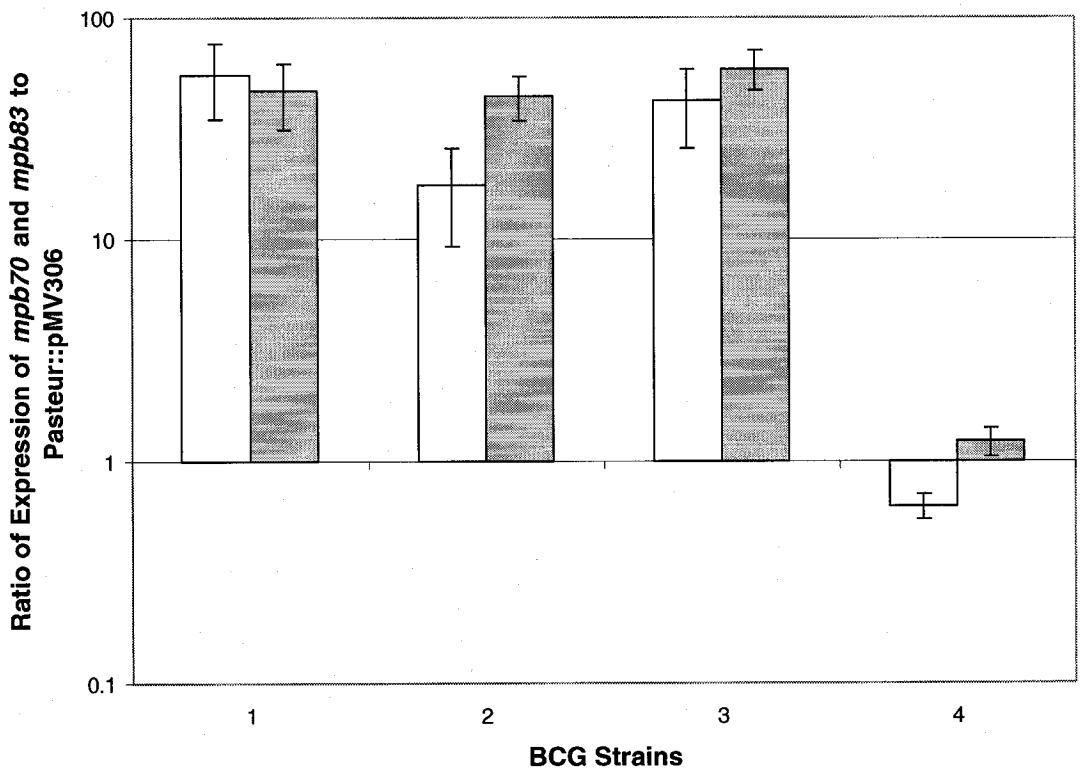


FIGURE 3.9 – Expression of *mpb70* (white) and *mpb83* (grey) upon complementation of BCG Pasteur with *sigK* from 1 - *M. tuberculosis* H37Rv, 2 - BCG Russia, 3 - BCG Birkhaug and 4 - BCG Pasteur. Values are expressed as a ratio of gene expression in complemented strains compared to BCG Pasteur::pMV306. All values were normalized to the levels of *sigA* mRNA and error bars represent the standard error of the mean.

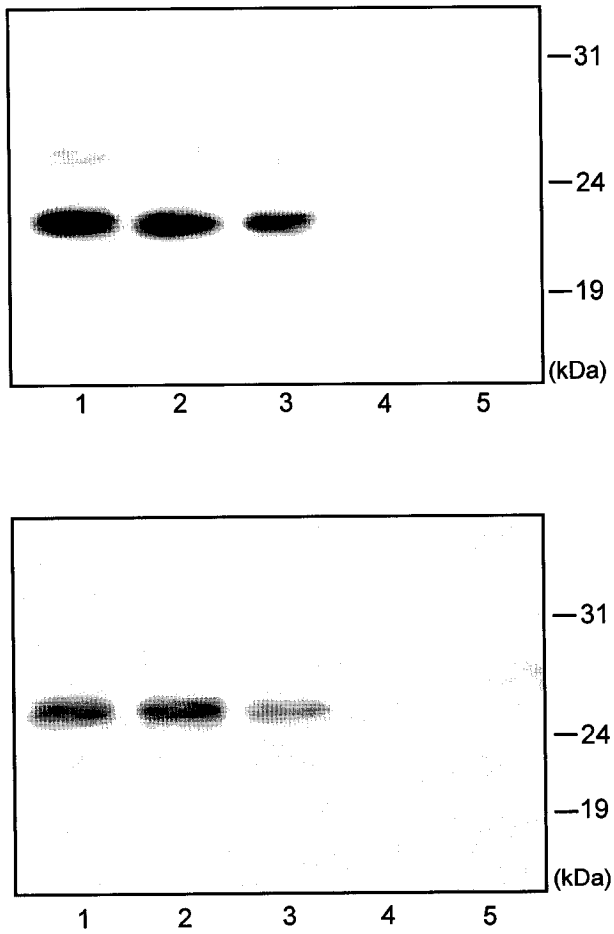


FIGURE 3.10 – SDS-PAGE and immunoblotting of culture filtrate proteins (top) and cell extracts (bottom) from complemented strains of BCG Pasteur, using MAbs 1-5c for MPB70 and MBS43 for MPB83. The strains used were as follows:
1 - BCG Pasteur::*sigK*H37Rv, 2 - BCG Pasteur::*sigK*Russia,
3 - BCG Pasteur::*sigK*Birkhaug, 4 - BCG Pasteur::*sigK*Pasteur,
5 - BCG Pasteur::pMV306.

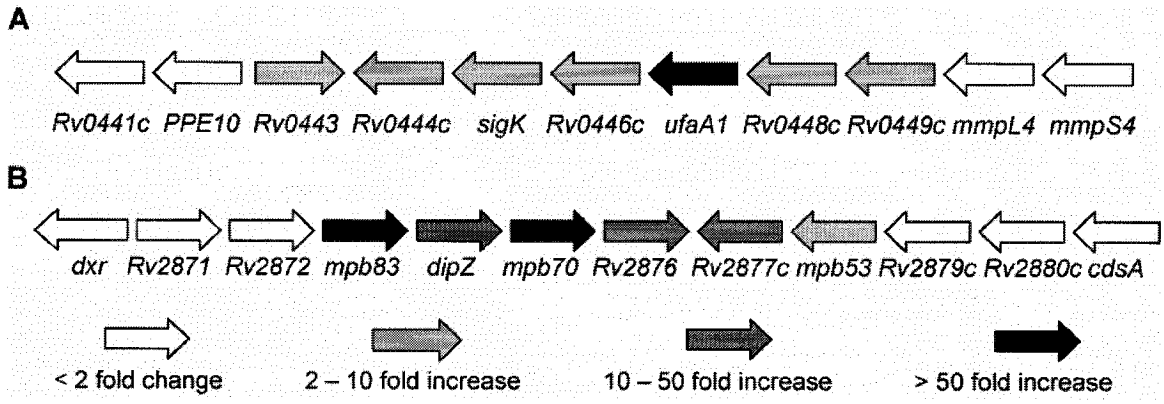


FIGURE 3.11 – A. Expression analysis of the genes *Rv0441c* to *Rv0450c*. B. Expression analysis for the genes *Rv2870c – Rv2881c*. Levels presented represent the ratio of gene expression in *sigK* complemented strains compared to the control strain BCG Pasteur::pMV306. All values were normalized to the levels of *sigA* mRNA and the ratio presented represents the mean of results from different clones, specifically BCG Pasteur::*sigK*H37Rv, BCG Pasteur::*sigK*Russia and BCG Pasteur::*sigK*Birkhaug.

ORF	Gene name	BCG Pasteur::*sigK*Birkhaug vs. BCG Pasteur::pMV306		BCG Pasteur:: *sigK*H37Rv vs. BCG Pasteur::pMV306		Gene product
		Fold change	Z-score	Fold change	Z-score	
Rv0445c	*sigK*	2.7	2.6	2.6	2.4	Sigma factor
Rv0446c		6.1	5.3	7.9	5.2	Conserved transmembrane protein
Rv0447c	*ufaA1*	5.0	4.5	5.0	4.0	Cyclopropane-fatty-acyl-phospholipid synthase
Rv0448c		15.1	7.8	9.4	5.6	Conserved hypothetical protein
Rv0449c		11.8	6.5	8.3	5.2	Conserved hypothetical protein
Rv1884c	*rpfC*	-3.0	3.2	-3.6	3.3	Probable resuscitation-promoting factor C
Rv1886c	*fbpB*	-2.1	2.2	-3.2	2.9	Secreted antigen 85-B
Rv2031c	*hspX*	-2.1	2.2	-2.6	2.4	Heat shock protein X
Rv2874	*dipZ*	836.6	15.0	88.8	9.5	Possible integral membrane protein
Rv2875	*mpb70*	62.2	11.7	17.7	7.2	Major secreted immunogenic protein
Rv2876		12.5	7.4	13.1	6.2	Conserved transmembrane protein
Rv2878c	*mpb53*	37.6	8.1	3.7	3.3	Soluble secreted protein
Rv3681c	*whiB4*	-2.2	2.1	-2.2	2.0	Transcriptional regulator protein

TABLE 3.3 – Predicted genes whose in vitro expression was significantly dysregulated between BCG Pasteur::pMV306 (empty vector) and BCG Pasteur::*sigK* (wild type). The Z-score, indicative of how many standard deviations a data point lies from the population mean, is the average for that gene across replicate (n=2) microarrays of BCG Pasteur::*sigK*Birkhaug versus BCG Pasteur::pMV306 and BCG Pasteur::*sigK*H37Rv versus BCG Pasteur::pMV306. Genes with average Z-scores of 2 or greater for both comparisons are presented and the fold change is calculated from a normalized log-ratio of that gene, with BCG Pasteur expression for that gene providing the reference value of 1. Open reading frame (ORF) and gene name are listed by Rv number as annotated for M. tuberculosis H37Rv (http://genolist.pasteur.fr/TubercuList/). Genes from the same region of the genome are grouped and presented in a darker shade.

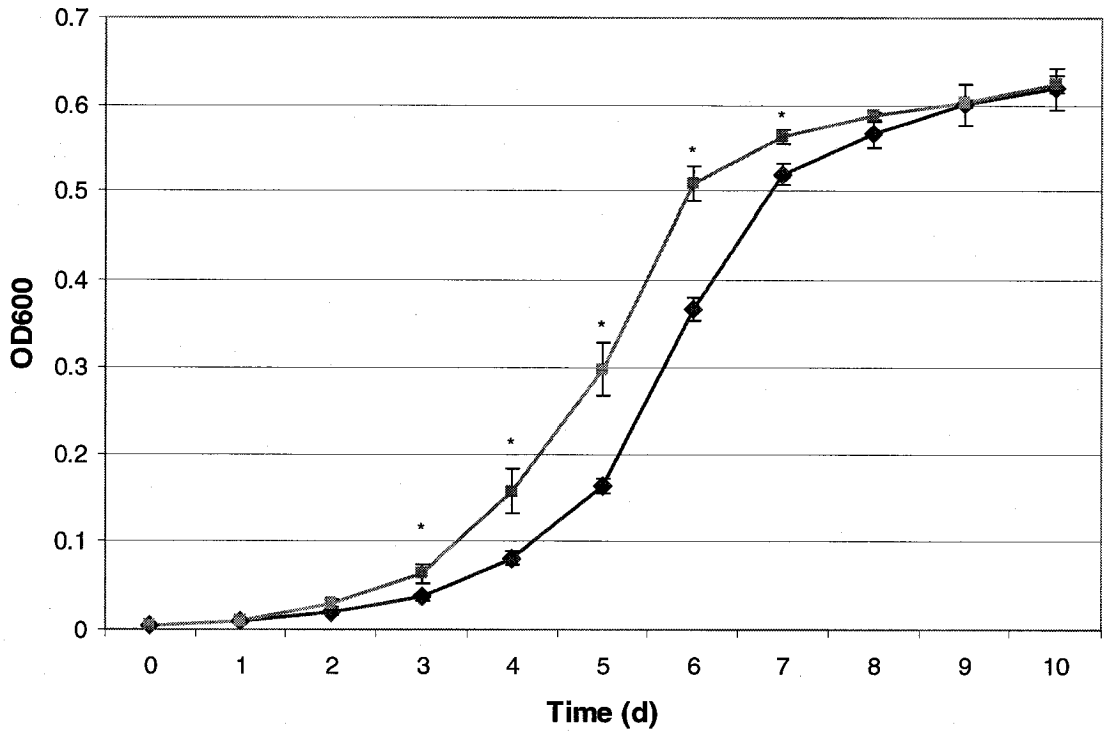


FIGURE 3.12 – In vitro growth of BCG Pasteur::*sigK*Russia and BCG Pasteur::pMV306. Growth was followed by measuring OD_{600} of cultures of BCG Pasteur::*sigK*Russia (◆) and BCG Pasteur::pMV306 (■). Data for each time point represents the mean of three experiments, each measured in duplicate and error bars represent the standard error of the mean. (*, $p < 0.05$)

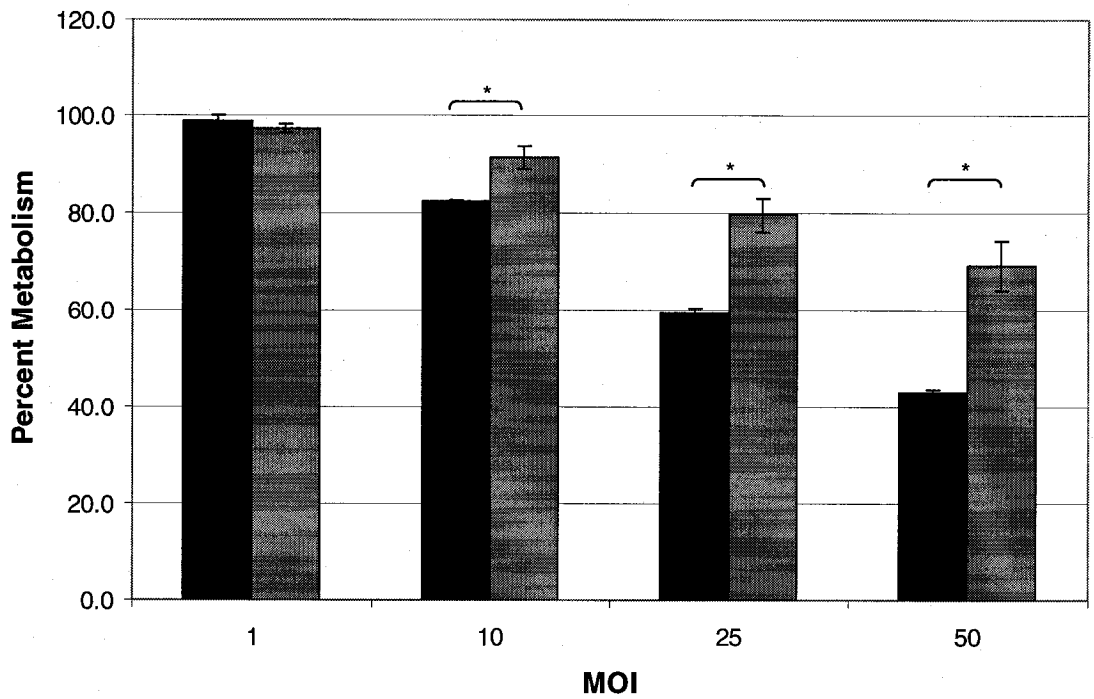


FIGURE 3.13 – Infection of human macrophage THP-1 cell line with BCG Pasteur::*sigK*Russia (■) and BCG Pasteur::pMV306 (▩). Cytotoxicity was determined as a function of cell metabolism and indicated as a percentage of metabolism compared to uninfected cells at 24hrs. Data is a representative sample of five experiments and is the mean of triplicate samples. Error bars represent the standard error of the mean. (*, $p < 0.05$)

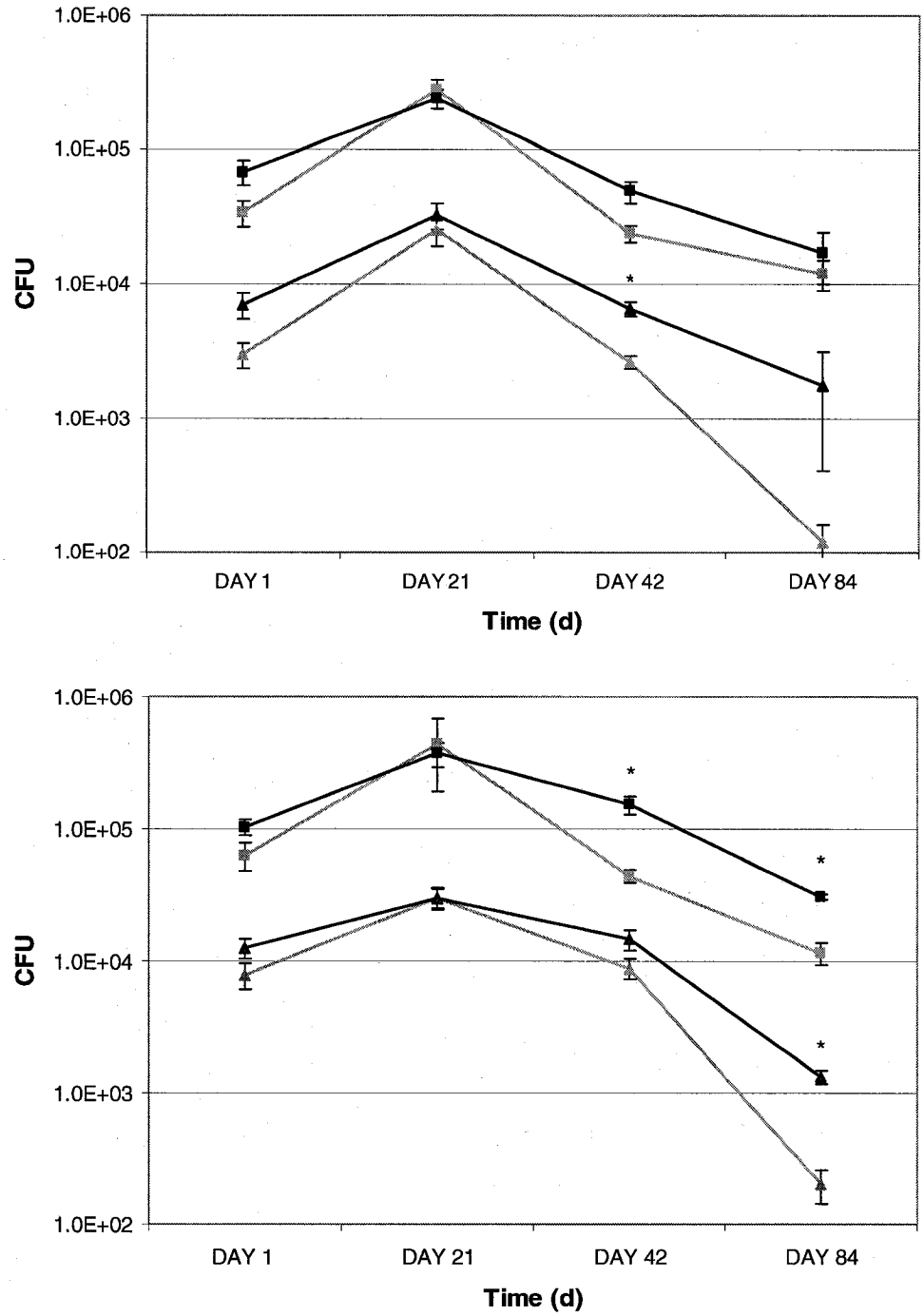


FIGURE 3.14 – Bacterial growth in lungs (△) and spleens (□) after an intravenous infection of C57Bl/6 mice with BCG Pasteur::*sigK*Russia (black) and BCG Pasteur::pMV306 (grey). Upper and lower panels represent two separate infections, data points are the mean of 4-5 mice per group and the error bars represent the standard error of the mean. (*, $p < 0.05$)

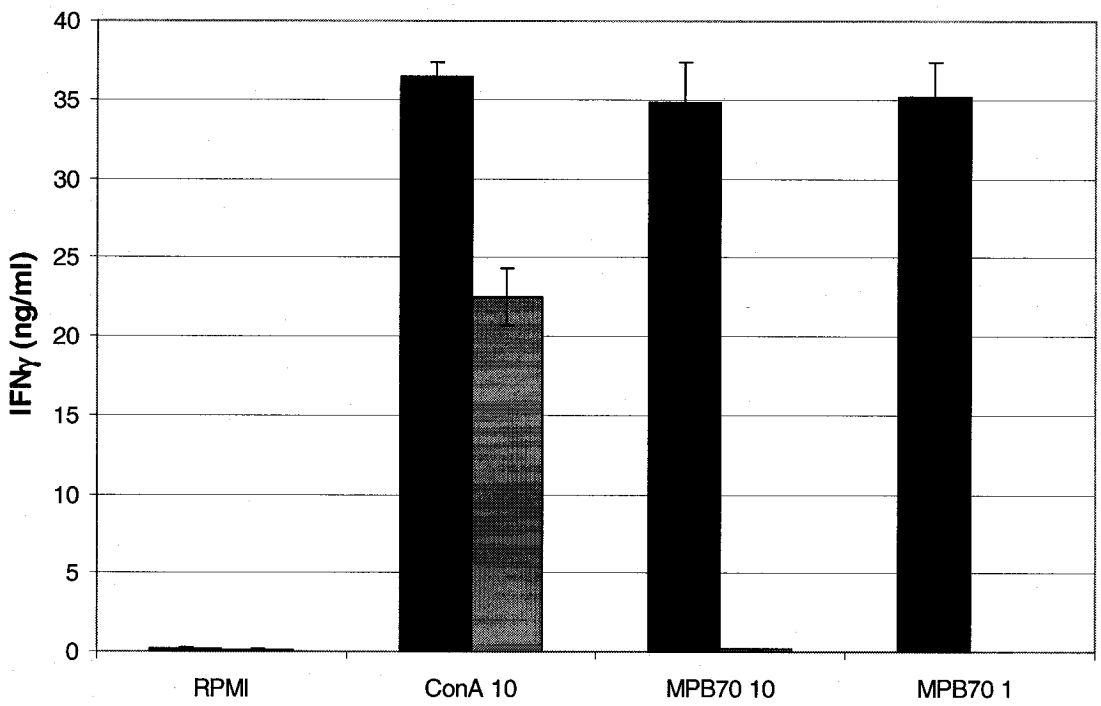
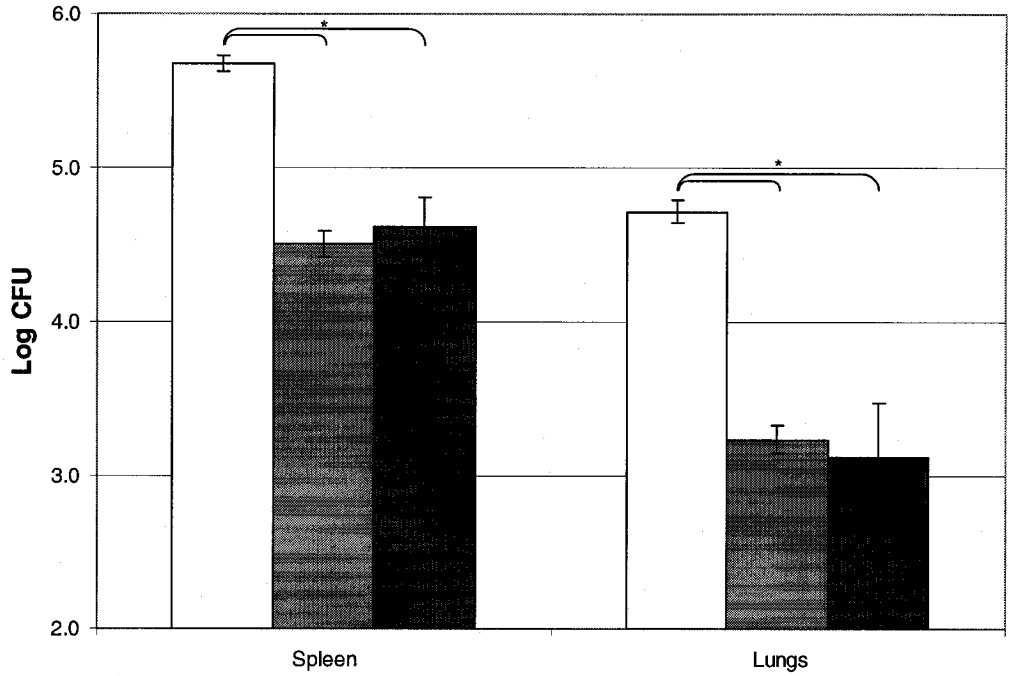


FIGURE 3.15 – IFNγ production by splenocytes isolated from BCG Pasteur::pMV306 (▩) and BCG Pasteur::*sigK*Russia (■) vaccinated mice and cultured with RPMI, conconavalin A or MPB70 for 72hrs. IFNγ was measured by ELISA. Data represents the mean of triplicate samples and the error bars represent the standard error of the mean.

WEEK 4



WEEK 8

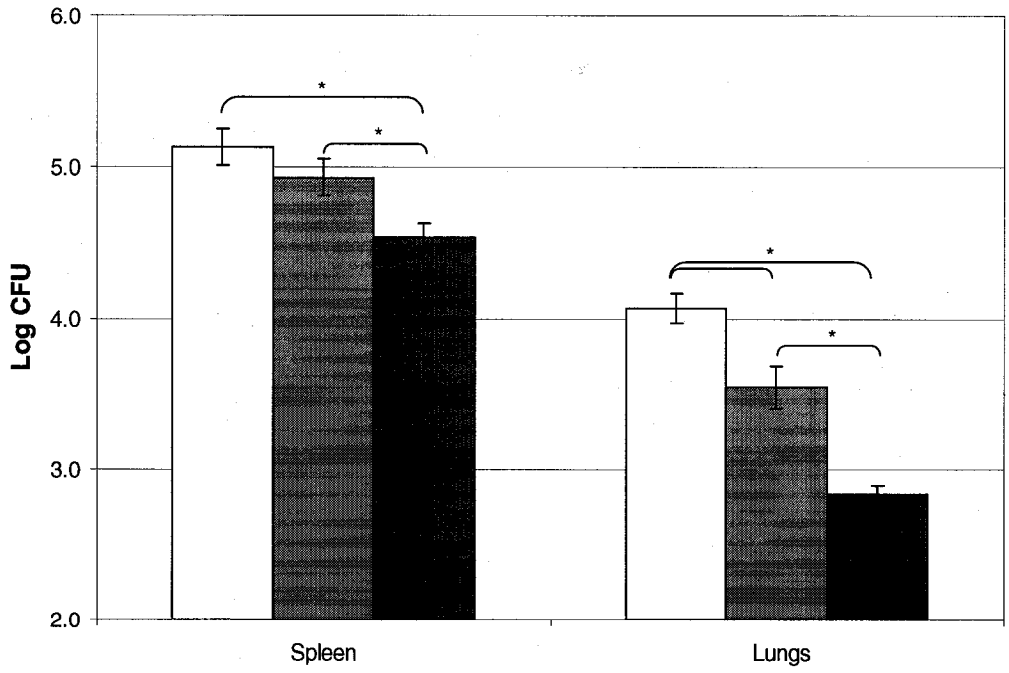
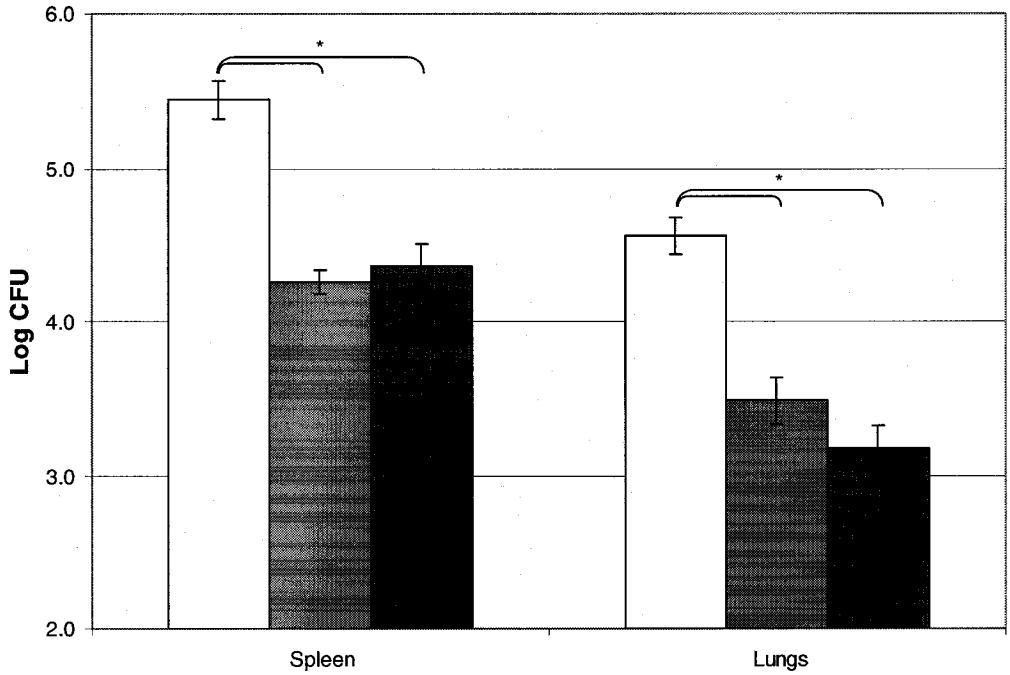


FIGURE 3.16 – Bacterial counts in the spleen and lungs of BCG Russia challenged animals, at 4 and 8 weeks, following vaccination with PBS (□), BCG Pasteur::pMV306 (▨) or BCG Pasteur::*sigK*Russia (■). Results are expressed as the mean of 3-4 animals per group and error bars represent the standard error of the mean. (*, $p < 0.05$)

WEEK 4



WEEK 8

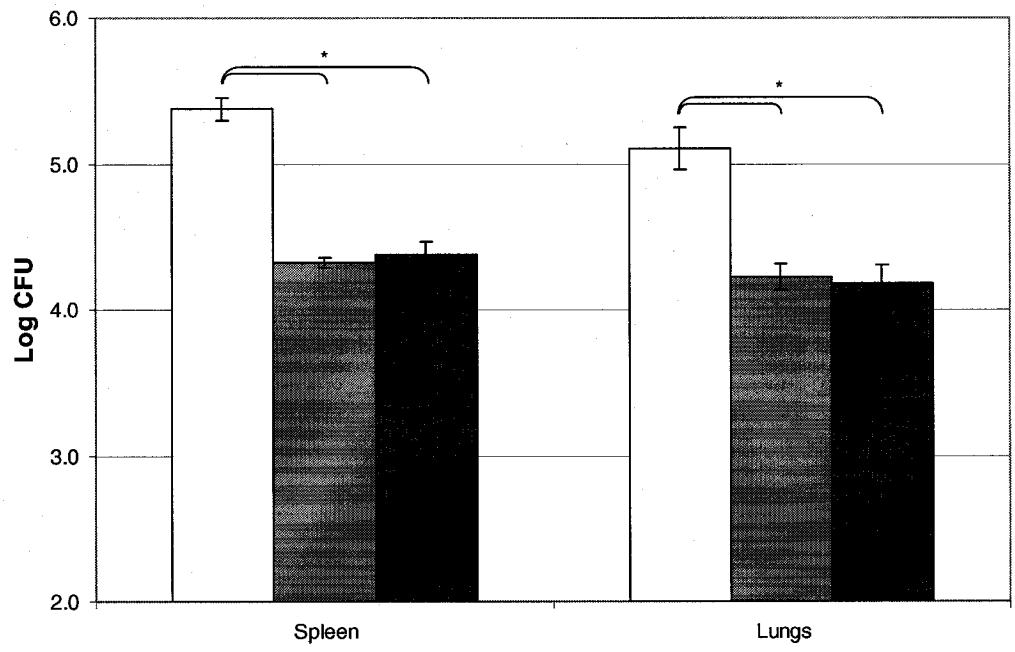
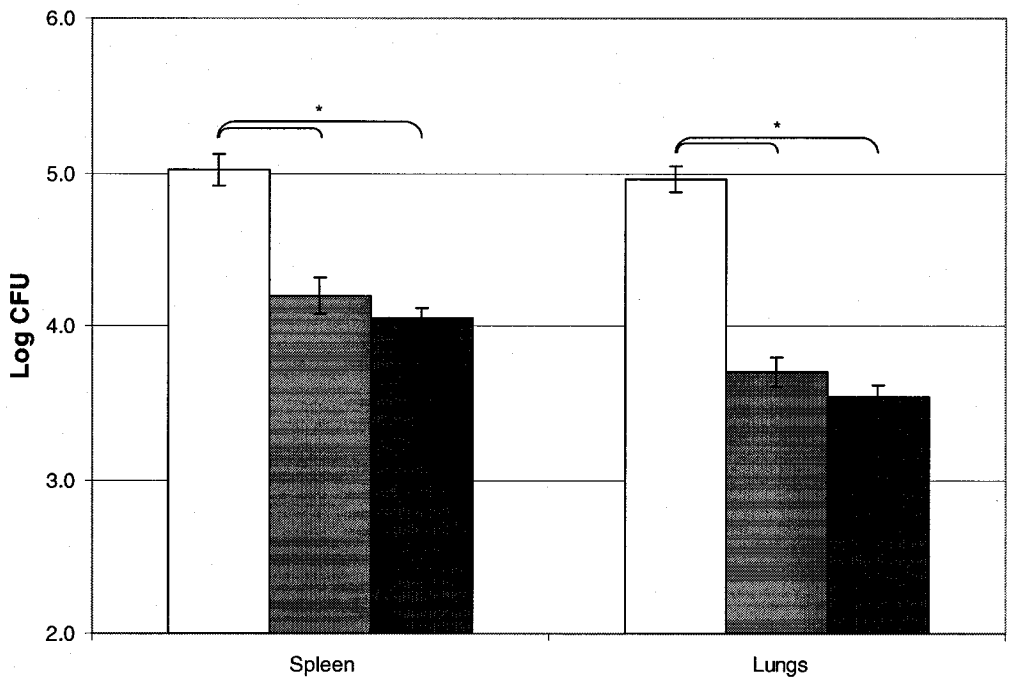


FIGURE 3.17 *– M. tuberculosis* H37Rv Challenge 1. Bacterial counts in the spleen and lungs of *M. tuberculosis* H37Rv challenged animals, at 4 and 8 weeks, following vaccination with PBS (□), BCG Pasteur::pMV306 (▩) or BCG Pasteur::*sigK*Russia (■). Results are expressed as the mean of 5 animals per group and error bars represent the standard error of the mean. (*, $p < 0.05$)

WEEK 4



WEEK 8

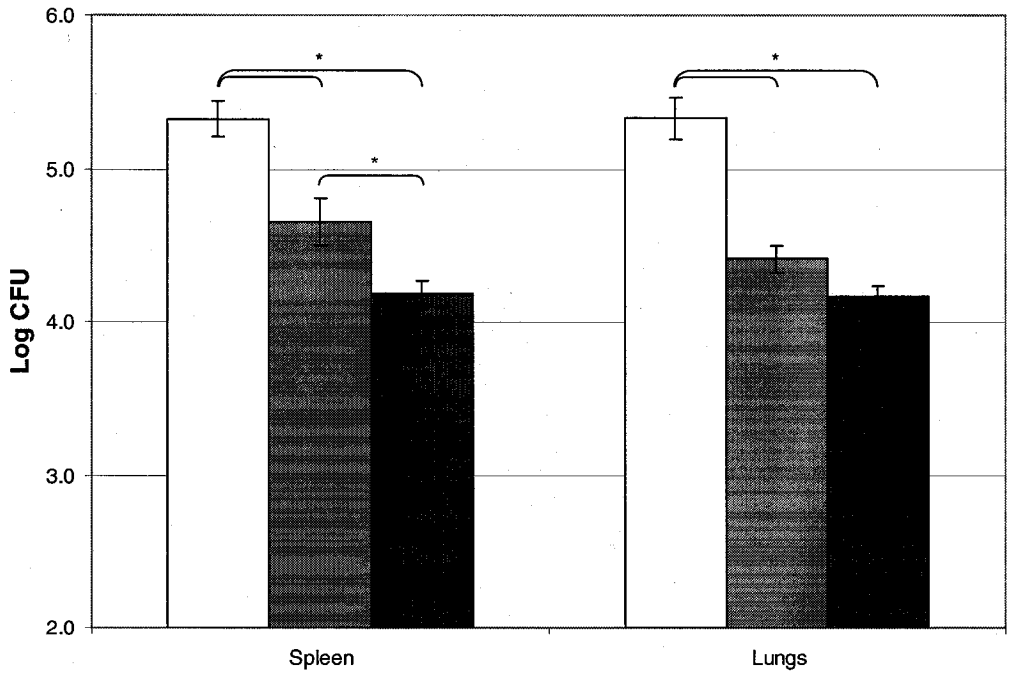


FIGURE 3.18 – *M. tuberculosis* H37Rv Challenge 2. Bacterial counts in the spleen and lungs of *M. tuberculosis* H37Rv challenged animals, at 4 and 8 weeks, following vaccination with PBS (□), BCG Pasteur::pMV306 (▩) or BCG Pasteur::*sigK*Russia (■). Results are expressed as the mean of 5-6 animals per group and error bars represent the standard error of the mean. (*, $p < 0.05$)

4. Discussion

4.1. RD2

Through the use of comparative genomics, it has been revealed that BCG has undergone significant *in vitro* evolution (Mostowy *et al.*, 2003). Compared to virulent *M. bovis*, the average BCG strain has lost 18 890 bp, affecting 19.3 ORFs. Notably, the prolonged propagation of BCG *in vitro* has resulted in as much loss of genomic content as has been sustained by clinical isolates of *M. tuberculosis.* The selective pressures experienced in laboratory-controlled *in vitro* conditions will be different than those faced by clinical isolates, and consequently, the loss of genomic content in laboratory-adapted strains likely indicates the selection of phenotypes that are better adapted for *in vitro* growth. A reduction in the diversity of response mechanisms, although potentially deleterious *in vivo*, may represent an adaptation to improve *in vitro* growth. Having become attenuated by a mutation acquired during *in vitro* growth and having subsequently sustained further substantial deletions, characterization of the resulting phenotypic changes in BCG strains is expected to provide insight into how these strains, in becoming adjusted to *in vitro* conditions, have changed in their ability to adapt to *in vivo* growth and to stimulate a protective immune response.

After the time period between 1927 and 1931, there were reports describing a decrease in virulence in BCG (Oettinger *et al.*, 1999). This time period coincides with the deletion of RD2, a region spanning 10.8 kB and 11 ORFs, deleting among others a possible transcriptional regulator, a major antigenic protein (MPB64) and a

surface protein in the PE_PGRS family. The RD2 region was therefore hypothesized to have played a role in the decrease in virulence reported in BCG, however, direct comparisons between early and late strains of BCG to identify the effects of the RD2 deletion would be confounded by the numerous other deletions accumulated in the various strains of BCG. The RD2-knockout strain of *M. tuberculosis*, created in the laboratory of David Sherman, provided us with the opportunity of studying the impacts of the loss of RD2 in isolation from other deletions. The loss of this region, however, did not obviously impact upon *in vitro* growth or virulence in THP-1 cells. Growth curves measuring *in vitro* growth for wild-type *M. tuberculosis* H37Rv and *M. tuberculosis* H37RvΔRD2 were identical through lag, log and stationary phases, suggesting that the loss of RD2 did not contribute to an overt *in vitro* growth advantage. Infection of THP-1 cells has previously been used as a model of virulence, in which an RD1-knockout strain was shown to be severely attenuated compared to wild-type *M. tuberculosis* (Lewis *et al.*, 2003). This same model was used to compare virulence of the RD2-knockout strain to that of *M. tuberculosis* H37Rv. Both strains were found to cause a similar reduction in cell metabolism, indicating that any difference in virulence between these strains is, at most, minimal. Kato-Maeda *et al.* (Kato-Maeda *et al.*, 2001) proposed that the accumulation of deletions among clinical isolates generally diminishes their virulence and similarly Behr *et al.* (Behr *et al.*, 1999) speculated that further genomic deletion has led to the overattenuation of current BCG vaccines. The lack of a virulence phenotype in the RD2-knockout strain could be proposed to be a result of the strain in which the deletion was recreated. The effects of the deletion could in fact be negligible, as seen,

in a strain that has not suffered previous attenuating mutations, while the same deletion in BCG, an already attenuated strain relative to *M. tuberculosis*, could prove more debilitating as the ability of BCG strains to compensate for the deletion of RD2 may be more limited than that of *M. tuberculosis* H37Rv. This is supported by the observation that although the deletion of RD1 in *M. tuberculosis* H37Rv severely attenuated this strain compared to wild-type *M. tuberculosis*, long-term animal studies revealed that *M. tuberculosis* H37RvΔRD1 remains more virulent than BCG Russia (Sherman *et al.*, 2004).

To characterize the potential impact of the loss of the antigenic protein MPB64 through the loss of RD2 in late strains of BCG, BCG Denmark was complemented with *mpb64*. The complemented strains expressed *mpb64* at a level 3-4 times greater than BCG Russia, and the secreted protein was detectable by Western blotting of the CFP. Vaccination with extracellular proteins was demonstrated to effectively induce a protective immune response against *M. tuberculosis* in mice and guinea pigs (Pal and Horwitz, 1992;Andersen, 1994). Horwitz *et al* (Horwitz *et al.*, 1995) proposed that extracellular proteins should play a key role in generating an immunoprotective response since they are released into intracellular compartments making them available for proteolytic processing and MHC presentation, and vaccination with such proteins should thus induce a population of lymphocytes that is capable of recognizing and targeting infected cells. Since, however, vaccination with extracellular proteins induced an immune response that was less protective than that induced by BCG, Horwitz *et al.* (Horwitz *et al.*, 2000) attempted to enhance this

response by creating a strain of BCG that overexpresses a major secretory protein, Ag85B. This strain of BCG effectively protected against an *M. tuberculosis* challenge in guinea pigs, with levels of protection greater than that induce by BCG. Although not as abundant as Ag85B, MPT64 is an effectively secreted protein and does induce a significant immune response in animal models and in TB patients (Haga *et al.*, 1995;Oettinger *et al.*, 1995;Roche, Feng, and Britton, 1996;Mustafa *et al.*, 1998;Lightbody *et al.*, 1998). From the consistency of the responses induced and given that MPT64 is an *M. tuberculosis* complex-specific antigen (Harboe *et al.*, 1986;Abe, Hirano, and Tomiyama, 1999), MPT64 has been included in diagnostic tests for tuberculosis, which have shown a high degree of specificity and sensitivity (Nakamura *et al.*, 1998;Hirano *et al.*, 2004). The Ag85 complex, however, has homologues in many mycobacterial species (Wiker and Harboe, 1992) and Ag85-specific responses are detected in non-tuberculosis patients more frequently than are MPT64-specific responses. This suggests that protection dependent on a strong response to Ag85-complex antigens may be limited in the degree of protective response that can be generated above the immunity stimulated by previous exposure to mycobacteria. However, as MPT64 is a strong immunostimulatory secreted protein with limited distribution in other mycobacterial species, inclusion of this protein in a vaccine may have greater potential to induce a protective response than Ag85-complex antigens in populations determined to have high levels of exposure to environmental mycobacteria. Also of interest, Ag85B is produced by all strains of BCG and increasing the expression by 2-6 fold significantly increased the protection afforded by vaccination with the recombinant strain. Of the rBCG30 strains tested,

the strain expressing higher levels of Ag85B was found to induce the greatest protective response (Horwitz and Harth, 2003). MPB64 is not produced in eight BCG strains, therefore it was hypothesized that restoring the expression of *mpb64* in a strain that otherwise lacks this gene would increase the protective immunity over that generated by vaccination with the parental strain. In addition, the expression level in BCG Denmark::*mpb64* was higher than that in BCG Russia, which, similar to overexpressing Ag85B, would in theory emphasize any benefit derived from expressing this protein. At four weeks post-challenge, expression of *mpb64* appeared to be beneficial, as vaccination with BCG Denmark::*mpb64* induced a 1.6 log reduction in CFU in the lungs, while BCG Denmark reduced CFU in the lungs by 1.3 log compared to unvaccinated animals. Although this difference was no longer observed at eight weeks, nor was it observed in the spleen, further testing in an aerosol *M. tuberculosis* challenge model or in a model with environmental mycobacteria pre-sensitization may be informative. In unpublished data, a recombinant strain of BCG expressing *mpt64* was found to be more protective against an *M. tuberculosis* challenge than the parental strain of BCG, and although the level of protection was stated to be less than that induced by rBCG30, no details regarding the recombinant strain expressing *mpt64* were provided (Horwitz, 2005).

Although no phenotypic changes have yet been identified in *M. tuberculosis* H37RvΔRD2, changes in gene expression may reveal potential phenotypes. To this end, we compared the global gene expression of two early strains versus two late strains of BCG and found limited, but striking differences. Only two regions were

significantly dysregulated, however, one of these regions includes genes encoding two highly antigenic proteins, MPB70 and MPB83. These proteins are of particular interest because of their high production in some strains of BCG and because previous attempts to identify the cause of the differential expression were unsuccessful. As the RD2 region encompasses a potential transcriptional regulator and the difference in expression and protein production is clearly a division between early and late strains of BCG, the deletion of this region was proposed as a likely cause of the loss of expression of these genes in late strains of BCG. Further investigations into the second region in which expression differs between early and late strains of BCG, however, revealed that a mutation in this region, and not the deletion of RD2, was responsible for the decreased expression of *mpb70* and *mpb83* in late strains of BCG. This will be explored further in section 4.2.

To date, no clear phenotype has been associated with the loss of the RD2 region in *M. tuberculosis* H37Rv. However, this does not preclude the possibility of any detrimental or beneficial effects arising from the loss of this region in BCG. Although the region itself does not cause significant attenuation in a virulent strain of *M. tuberculosis*, the possibility that the loss of this region may have contributed to a further attenuation of BCG must still be examined. The accumulation of deletions has been proposed to lead to the attenuation of clinical isolates of *M. tuberculosis*, and thus the accumulation of deletions in BCG may also be a pathway by which BCG has become more attenuated with continued passaging. To fully understand the effects of

subsequent deletions in BCG, isogenic strains with accumulated mutations continue to be required.

4.2. Expression of mpb70 and mpb83 as a function of sigK and the potential role of sigK

Comparative genomic studies have demonstrated the substantial genome decay experienced by BCG strains during a half-century of *in vitro* passage (Mostowy *et al.*, 2003). While DNA microarray-based comparisons have efficiently uncovered genomic deletions as an evident form of evolution, these tools overlook other forms of genomic variability, such as duplications and single nucleotide polymorphisms (SNPs). With the targeted study of specific genes, there has come the recognition of numerous loss-of-function SNPs during BCG evolution, including a SNP that impairs synthesis of methoxymycolic acids (Behr *et al.*, 2000), a SNP predicted to decrease the DNA-binding ability of a CRP homologue (Spreadbury *et al.*, 2005) and the start codon mutation described here. Remarkably, along with the loss of the RD2 region (Mahairas *et al.*, 1996), these three SNPs all coincide with the interval of 1927-1931, suggesting either a period of considerable *in vitro* evolution or the replacement of one BCG stock with another at the Pasteur Institute in the late 1920's.

Our data indicate that a SNP in the start codon of the ECF sigma factor, *sigK*, occurred between 1927-1931 at the Pasteur Institute and resulted, either directly or indirectly, in a major drop in production of the antigenic proteins MPB70 and MPB83. Previous attempts to identify the cause of the differential production of these

proteins between high- and low-producing strains were inconclusive and attributed the differences in gene expression to differential transcriptional efficiencies (Matsuo *et al.*, 1995), suggesting that a difference in transcription initiation may be the cause. The mutation in the start codon of *sigK* effectively results in a difference in transcriptional efficiencies between high- and low-producing strains as transcription in the low-producing strains can only be initiated by the low levels of SigK that are produced from the AUA start codon, whereas the greater efficiency of translation of *sigK* mRNA in high-producing strains increases the transcription of the *sigK*-regulated genes. The most common naturally occurring initiation codons are AUG and GUG (61% and 35% respectively in *M. tuberculosis*), however AUA has been documented to be a functional start codon in bacteria (Romero and Garcia, 1991;Sussman, Simons, and Simons, 1996;Schoenhals, Kihara, and Macnab, 1998), as well as in *S. cerevisiae*, *Caenorhabditis elegens* and humans cells (Zitomer *et al.*, 1984;Peabody, 1989;Hernandez *et al.*, 2002;Ko and Chow, 2003). The efficiency of AUA as a start codon is significantly lower than that of the predominant AUG start codon and has been demonstrated to have less than 10% of the activity of the AUG initiation codon in *E. coli* (Romero and Garcia, 1991;Sussman, Simons, and Simons, 1996). The AUG to AUA mutation in *sigK* may have been selected for by conferring a growth advantage *in vitro*, as shown by the faster growth of BCG Pasteur::pMV306 compared to BCG Pasteur::*sigK*Russia. Although the role of *sigK* and the *sigK*-regulon in infection are unknown, the growth advantage conferred by losing the expression of this regulon may have been of greater benefit *in vitro* to a continually

passaged laboratory culture than the potential benefit of retaining the normal *in vivo* function of this regulon.

SigK is one of ten extracytoplasmic function (ECF) sigma factors annotated in the *M. tuberculosis* H37Rv genome. As the name suggests, these regulatory elements mediate responses to changing external conditions (Manganelli *et al.*, 2001;Ando *et al.*, 2003;Hu *et al.*, 2004;Manganelli *et al.*, 2004a). As an indicator of the possible role the *sigK*-regulon may play in adapting to extracellular conditions, *mpb70* and *mpb83* are among the most highly up-regulated genes at 4, 24 and 48 hours in *in vivo* macrophage infection as determined by Schnappinger *et al* (Schnappinger *et al.*, 2003). Infection of unactivated and activated bone marrow- derived macrophages from both $NOS2^{+/+}$ and $NOS2^{-/-}$ mice induced similar up-regulation of *mpb70* and *mpb83*, suggesting that induction of expression of these genes, and likely their function, is independent of the activation state of the macrophages and of the generation of RNI. A common feature of ECF sigma factors is their control over relatively small regulons (Bashyam and Hasnain, 2004). For instance, by microarray analysis, the regulon of *sigC* has been estimated to contain 13, 14, and 18 genes, in exponential, early stationary and late stationary growth phases respectively (Sun *et al.*, 2004). This is consistent with our observations of 13 genes being consistently up-regulated in wild-type *sigK*-complemented strains. Four genes were observed to be consistently down-regulated in the *sigK*-complemented strains, however, the role of *sigK* in the expression of these genes is undetermined. A decrease in gene expression could be attributable to an increased expression of a repressor or to the sequestration

of a transcriptional activator, however, these possibilities were not further investigated. The striking feature of the *sigK* regulon is not its size, however, it is its specificity. All 13 genes that are up-regulated comprise only two regions, one containing *sigK* itself and the second containing *mpb70* and *mpb83*. This indicates the large degree of specificity of this system. No other *M. tuberculosis* ECF sigma factor for which a regulon has been determined, has demonstrated a similar degree of specificity.

MPB70 and MPB83 exhibit striking amino acid sequence homology (Hewinson *et al.*, 1996) and both are exported, but the two proteins localize differently. MPB70 has been confirmed to be a secreted protein, with a high localization index indicating a highly efficient secretion process, while MPB83 has been localized mainly to the cell surface (Fifis *et al.*, 1991;Hewinson *et al.*, 1996;Harboe *et al.*, 1998). The structure of MPB70 has recently been solved and superimposition of MPB83 on the MPB70 structure confirmed the overall homology of the antigens (Carr *et al.*, 2003). Based on the conserved residues, which are both solvent exposed and in the core regions, Carr *et al.* suggested that these proteins interact with host cell proteins either to modulate signaling pathways in host cells or to act as attachment proteins. MPB70 displays both sequence and structural homology to the FAS1 domains of fasciclin, periostin and βig-h3 (Ulstrup *et al.*, 1995;Matsumoto *et al.*, 1995;Carr *et al.*, 2003), all of which have been characterized as cell adhesion molecules. βig-h3 has been found to bind to several integrins via the FAS1 domains (Kim *et al.*, 2000;Kim *et al.*, 2002) and, although the binding domains

were not identified, periostin has also been found to bind various integrins (Gillan *et al.*, 2002). A possible function of MPB70 and MPB83 could be to interact with selected integrins to increase the uptake of *M. tuberculosis*, a step required for pathogenesis, or to alter signaling in the macrophage. A *sigK*-knockout strain in *M. tuberculosis* H37Rv has recently been created (Said-Salim *et al.*, 2006) and this should allow for further elucidation of the function of the *sigK*-regulon. Studies of growth of the BCG Pasteur::*sigK* strain in mice have, however, provided some initial insight into the possible importance of this system. As shown in Figure 3.14, C57Bl/6 mice infected with Pasteur::*sig*KRussia have higher CFU in the spleen and lungs at day 1 compared to mice infected with Pasteur::pMV306, despite equal or slightly higher Pasteur::pMV306 infection inoculums, suggesting that increased expression of MPB70 and MPB83 conferred some advantage to BCG Pasteur early in infection. Similar to the *in vitro* growth curves, growth of Pasteur::pMV306 was faster than that of Pasteur::*sigK*Russia over the first three weeks of infection, at which point both strains reached their maximum CFU detected and the infection was brought under control. Interestingly, over the later time points, Pasteur::pMV306 decreased more rapidly and to lower levels in both the lungs and the spleen than Pasteur::*sigK*Russia. Long term studies would be required to determine if and to what extent Pasteur::*sigK*Russia persists longer than Pasteur::pMV306, but this early data again suggests that expression of a functional *sigK*-regulon may be advantageous during infection. As persistence of BCG is crucial for an effective protective response, expression of the *sigK*-regulon could be a beneficial characteristic of a BCG-based vaccine.

From immunologic studies, it is known that both MPB70 and MPB83 induce cellular and humoral responses in experimental infections of model hosts and in natural infections of humans (Miura *et al.*, 1983;Haslov, Andersen, and Bentzon, 1987;Roche *et al.*, 1994;Fifis *et al.*, 1994;Harboe *et al.*, 1995;Wiker *et al.*, 1996;Vordermeier *et al.*, 2000;Lyashchenko *et al.*, 2001). Based on these observations, both MBP70 and MPB83 have been developed as candidates for novel TB vaccine development (Chambers *et al.*, 2000;Morris *et al.*, 2000;Al Attiyah *et al.*, 2003;Tollefsen *et al.*, 2003;Xue *et al.*, 2004). Vaccination of cattle with both *mpb70* and *mpb83* DNA vaccines successfully induced strong cellular and humoral antigen-specific responses, although vaccination with *mpb83* was noted to produce a superior, more reliable response (Vordermeier *et al.*, 2000). Subcutaneous vaccination of C57Bl/6 mice with both BCG Pasteur::pMV306 and BCG Pasteur::*sigK*Russia induced a cellular immune response towards BCG, but only BCG Pasteur::*sigK*Russia induced a strong, specific response to MPB70. These results were encouraging as they indicate that the proteins are indeed produced *in vivo* and importantly, that these proteins stimulate an immune response.

Results of protection from *mpb83*-based vaccines, which have been more commonly tested than *mpb70*-based vaccines, have not been consistent between models. Protection against an intravenous *M. bovis* challenge in Balb/c mice vaccinated with an *mpb83* DNA vaccine was equivalent to that induced by BCG, with significant levels of protection in lungs and spleen being observed (Chambers *et al.*, 2000). In another study using an *mpb83* DNA vaccine, protection against a *M.*

tuberculosis aerosol challenge in C57Bl/6 mice revealed only moderate protection, measured as a reduction of CFU (only significant in lungs), that was not comparable to BCG (Morris *et al.*, 2000). Protection in guinea pigs was more ambiguous with no reduction in CFU being observed in the *mpb83*-DNA vaccinated animals, but an improvement of lung histopathology intermediate to the non-vaccinated and the BCG vaccinated animals was observed. This improvement was reported as a decrease in the number of lesions in the lungs and a reduction in the extent of granulomatous inflammation and caseation (Chambers *et al.*, 2002). A time to death experiment was not performed, but would have been informative as to whether the apparent histopathological improvement correlated with a decrease in disease severity and adverse outcomes. Vaccination of cattle with any of an *mpb83* DNA vaccine, an *mpb70* DNA vaccine or an *mpb70* DNA prime, MPB70 protein boost strategy did not effectively protect against an intratracheal challenge with *M. bovis*. Only BCG vaccination in this model reduced the number of animals with lung and lymph node lesions, the mean score of these lesions and the bacterial load in the lymph nodes (Wedlock *et al.*, 2003). Although this may be indicative that MPB70 and MPB83 may not be capable of solely inducing a strong protective immune response equivalent to or better than BCG, it does not suggest that either or both of these proteins cannot be an effective and beneficial component of strongly protective vaccine. There is no single *M. tuberculosis* antigen yet identified that, when used alone as a DNA or subunit vaccine, has been shown to have the capability of equaling the protective capacity of BCG vaccination in animal models. The vaccines that have shown an effective level of protection have either been combinations of antigens or BCG-based

vaccines. Since a moderate level of protection was induced by MPB70 and MPB83 in these previous challenge models, the inclusion of these genes in the strains of BCG used for vaccination may improve the amplitude and the range of the immune response induced by BCG. As extracellular antigens have consistently been implicated in the induction of a protective immune response against *M. tuberculosis*, it is remarkable that all BCG strains are unable to produce the antigenic proteins ESAT-6 and CFP-10 (lost with the RD1 deletion of 1908-1921), while strains obtained after 1927-31 are also deficient in the proteins MPB64 (Harboe *et al.*, 1986;Li *et al.*, 1993) and CFP-21 (Mahairas *et al.*, 1996;Weldingh *et al.*, 1998;Weldingh and Andersen, 1999) and are functionally deficient in production of MPB70 and MPB83 via the *sigK* mutation described here. Two challenge models were used to evaluate the efficacy of BCG Pasteur::*sigK*Russia compared to BCG Pasteur::pMV306. In both infection models, in which vaccinated mice were challenged with BCG Russia or *M. tuberculosis* H37Rv, equal protection was induced by BCG Pasteur::pMV306 and BCG Pasteur::*sigK*Russia at four weeks following infection. At eight weeks, however, the protective efficacy of BCG Pasteur::*sigK*Russia was slightly greater than BCG Pasteur::pMV306 in two of the three challenge studies. Although the overall differences between the vaccination strains were not significant, the trend shows that BCG Pasteur::*sigK*Russia confers slightly enhanced protection over that induced by BCG Pasteur::pMV306 and suggests that *sigK*, MPB70 and MPB83 should be considered beneficial elements of a BCG vaccine and of the protective immune response induced by such a vaccine. The ability of BCG Pasteur::*sigK*Russia to protect against an aerosol *M. tuberculosis*

challenge in guinea pigs was examined by collaborating researchers at Colorado State University. Although BCG Pasteur::*sigK*Russia did induce a significant level of protection against a *M. tuberculosis* challenge in this model, the level of protection observed was equivalent to that induced by BCG Pasteur::pMV306, as measured by histopathological determination of tissue damage in the lungs and by CFU enumeration in the lungs and the spleen of challenged animals (A. Izzo, personal communication). To further evaluate the protective ability of BCG Pasteur::*sigK*Russia vaccination, a *M. bovis* challenge experiment in cattle is currently in progress. MPB70 and MPB83 are constitutively expressed at high levels in *M. bovis* and as such, are classically considered as *M. bovis* antigens. Enhancing the protection against *M. bovis* infection in cattle would have enormous economic benefits to the cattle industry worldwide.

Comparison of BCG Pasteur::*sigK*Russia with BCG Pasteur in mice pre-sensitized to environmental mycobacteria may elucidate any potential benefit of a strain that persists longer and that expresses two immunodominant antigens that are not expressed in mycobacteria outside of the *M. tuberculosis* complex. From studies in Malawi, the species of environmental mycobacteria to which there is a higher prevalence of responsiveness than to *M. tuberculosis* include *M. avium*, *M. intracellulare* and *M. scrofulaceum*. Similarly, in England, the same species had the highest prevalence of responsiveness, albeit with a much lower prevalence than in Malawi. These strains, as with almost all environmental mycobacteria, do not express *mpb70* or *mpb83*. Animal studies looking at the effects of pre-sensitization by

environmental mycobacteria on a BCG-induced immune response and BCG proliferation have used two of these strains (*M. avium, M. scrofulaceum,)*, as well as *M. fortuitum, M. vaccae and M. kansasii.* Proliferation of BCG in mice pre-sensitized to environmental bacteria is hampered to varying degrees depending on the sensitizing strain, with the greatest inhibition being induced by *M. avium* (Demangel *et al.*, 2005). Inhibition of BCG growth was either reduced or not significant, depending on the pre-sensitizing strain, when the BCG strain used was BCG::RD1. The ability of the BCG::RD1 strain to grow in pre-sensitized mice was attributed to two factors: first, BCG::RD1 persists longer in immunocompetant mice and second, infection with BCG::RD1 generates a strong ESAT-6 response (Demangel *et al.*, 2005). These same two factors may be applicable to BCG Pasteur::*sigK*. Our *sigK*-complemented strain appears to have increased persistence (to be further examined in a long term infection model) and expresses two strongly immunogenic antigens that are unique to the *M. tuberculosis* complex. Vaccination with strains expressing *mpb70* and *mpb83*, like BCG::RD1, may be able to overcome the inhibition of BCG induced by environmental mycobacteria. In countries where there is a high prevalence of immune responses to environmental mycobacteria, vaccination with antigen expressing recombinant BCG, such as BCG::*sigK,* may prove to be highly advantageous.

The data presented here explain the difference in expression between high-producing and low-producing BCG strains. However, MPB70 and MPB83 are also differentially produced by *M. tuberculosis* and *M. bovis*. Although these organisms

have identical, wild type AUG *sigK* start codons, *in vitro* expression is low (albeit inducible) in *M. tuberculosis* and constitutively high in *M. bovis* (Wiker *et al.*, 1996). The constitutive *in vitro* production of MBP70 and MPB83 observed in *M. bovis* may therefore stem from unregulated activity of SigK. Activity of an ECF sigma factor can be mediated by a second protein, the anti-sigma factor, that functions post-translationally as a negative regulator to prevent constitutive expression of the target regulon (Helmann, 2002;Manganelli *et al.*, 2004b). The sigma/anti-sigma pair are usually adjacent and co-transcribed genes. For instance, in *M. tuberculosis*, RshA (*Rv3221A*) is the anti-sigma factor for SigH (*Rv3223c*), UsfX (*Rv3287c*) is the anti-sigma factor for SigF (*Rv3286c*) (Beaucher *et al.*, 2002;Song *et al.*, 2003) and RslA (*Rv0736*) has been demonstrated to act as an anti-sigma factor for sigL (*Rv0735*) (Hanh 2005, Dainese 2006). Consistent with this pattern, recent investigations, based on the presence of two non-synonymous SNPs in *Rv0444c* restricted to *M. tuberculosis* complex species presenting constitutively high MPB70 production, have demonstrated that *Rv0444c* is the anti-sigma factor of SigK (*Rv0445c*) (Said-Salim *et al.*, 2006).

As BCG vaccines are given to an estimated 2 million infants per week, there are important practical implications of these findings. The *sigK* mutation described here impairs the production of two immunodominant antigens, MPB70 and MPB83, as well as transcription of *mpb53*. The importance of this deficit for TB immunization is unknown because strains of BCG that produce these antigens have never been utilized in a randomized clinical trial, although results from some

observational studies have suggested a greater potency to some of the high-producing strains (Kroger *et al.*, 1994;Vitkova *et al.*, 1995). Based on the number of documented differences between BCG strains obtained before 1927 and those obtained after 1931, there is a compelling rationale to perform a human trial comparing BCG strains from these two groups. Furthermore, these results are also applicable towards efforts to develop improved vaccines against TB. Recent advances have demonstrated that recombinant strains of BCG expressing *M. tuberculosis* antigens provide an important avenue towards more effective vaccines (Horwitz *et al.*, 2000;Pym *et al.*, 2003). These constructs may benefit from the inherent MPB70 and MPB83 expression of the high-producing strains obtained before 1931, or alternatively, by correcting the *sigK* mutation in later strains to achieve the same result.

4.3. General Discussion

Although the use of BCG as a vaccine for tuberculosis is often criticized for the lack of reproducible protective efficacy, two facts remain true: first, BCG is given to millions of individuals every year and is beneficial in certain populations and second, perhaps more importantly, tuberculosis continues to kill millions of people every year, indicating that a vaccine that induces a greater immune response is not necessarily what is required, but a vaccine that provides a more reliable and ubiquitous protective response is what is necessary.

It has been clearly and extensively demonstrated that BCG is not one uniform vaccine. This has been further demonstrated here with the characterization of a single nucleotide polymorphism in the start codon of an ECF sigma factor that profoundly affects the expression of two immunodominant antigens. However, even though it may be conceptually simple to rationalize that the loss of both regulatory and antigenic proteins from various strains of BCG could impact upon how each strain interacts with its environment and its host during infection, demonstrating this potential impact *in vivo* and particularly in terms of vaccine efficacy has been challenging. Comparison of the protective efficacy of BCG strains in animal models has identified varying abilities of the strains to protect against an *M. tuberculosis* challenge, however, no clear trend has been established and variations between experiments makes direct comparisons difficult (Lagranderie *et al.*, 1996;Castillo-Rodal *et al.*, 2006). In addition, comparison of protection induced by vaccination with the same strains of BCG, but in different animal models, failed to produce a consensus regarding the ranking of the protective efficacy of the strains tested, even within the same testing laboratory (Smith, Wiegeshaus, and Balasubramanian, 2000). Consequently, this confusion has contributed to the impression that strain selection is insignificant and to date, there was perhaps not sufficient evidence to warrant the selection of one particular strain over another. As our knowledge of the differences between the strains increases, however, we should be able to tailor our choice of strain to maximize the protection induced by vaccination. For example, persistence of BCG is necessary for the induction of a protective immune response. Selecting a strain of BCG that persists longer or whose replication is not inhibited by

environmental mycobacteria, may prove to be more protective. As an example, the recombinant strain of BCG expressing the RD1 region was shown to persist longer, both in unsensitized animals and in animals previously sensitized with environmental mycobacteria, and was capable of surpassing the level of protection induced by BCG (Pym *et al.*, 2002;Pym *et al.*, 2003;Demangel *et al.*, 2005). As suggested by the *in vivo* growth data presented here, BCG Pasteur::*sigK* may persist longer in both the lungs and spleen of infected C57BL/6 mice than BCG Pasteur not expressing the *sigK* regulon, and although this needs further examination, it is a potentially beneficial characteristic that needs to be considered when selecting a BCG strain for use in vaccination programs.

Similarly to selecting a strain that is not growth inhibited by environmental mycobacteria, selection of a strain with limited antigenic crossreactivity to environmental mycobacteria may produce a broader response by inducing an immune response to antigens to which the vaccinated individual has not been previously exposed. This would in effect use the environmental mycobacteria to increase the breadth of the immune response, given that there will be less similarity between the response stimulated by this exposure and the immune response induced by the vaccine. As previously described, vaccination of animals pre-sensitized with environmental mycobacteria was most effective when there was the least crossreactivity between the sensitizing and the vaccinating strains (Orme and Collins, 1984). Given that exposure to environmental mycobacteria will likely remain a persistent factor, the strain of BCG used for vaccination should be selected such that

it induces the maximum immune response that would not be acquired by sensitization to environmental mycobacteria. This would not only enhance the level of protection attributable to BCG, but it would take advantage of the immunity stimulated by natural exposure to mycobacteria. BCG Pasteur::*sigK* is a promising candidate to fulfill such a role, as it expresses two immunodominant antigens that are not present in mycobacteria species outside of the *M. tuberculosis* complex. Alternatively, using a strain of BCG that naturally expresses the *sigK* regulon (ie. early strains of BCG) also has the advantage of having the expression of MPT64, another *M. tuberculosis* complex specific antigen.

The continued use of BCG has been debated both in countries with low prevalence of tuberculosis and in countries where there is a high burden of disease. In many countries with low prevalence, the widespread use of BCG has been discontinued, with selective vaccination of high risk individuals being favoured. In countries with high prevalence of tuberculosis, widespread use generally continues to be supported due to the effectiveness of the vaccine in the prevention of morbidity and mortality from disseminated disease in children and has recently been reported to be a highly cost effective intervention for control of tuberculosis in children (Trunz, Fine, and Dye, 2006). However, the protection observed in children has very little effect on the prevention of propagation of the disease, as tuberculosis in young children is rarely a significant source of transmission. It is for these two reasons that it is argued that BCG vaccination is both effective and ineffective; it protects children against the most serious forms of the disease, but it is minimally effective at

combating disease in adults and reducing the propagation of the disease. It is this latter aspect on which a large portion of future vaccine development should focus. The use of a prime-boost strategy has promising potential to be an effective mechanism to induce and maintain a protective response. Encouraging results were obtained in a Phase I clinical trial conducted by McShane *et al.* (McShane *et al.*, 2004) examining the safety and immunogenicity of a modified vaccinia virus expressing Ag85A (MVA85A). Testing MVA85A as a booster in individuals previously vaccinated with BCG (mean of 18y prior) produced an immune response with greater magnitude and of longer duration than that observed by vaccination with either BCG or MVA85A alone. Although these results are encouraging that a prime-boost strategy using BCG as the prime could be effective, it is difficult to assess the efficacy of any tuberculosis vaccine strategy without undertaking the long term study looking at disease incidence. Immune correlates of protection have not been well established and this poor understanding of what responses are predictive of a protective vaccine induced immune response has made the evaluation of new vaccines difficult.

The selection of which strain of BCG to use will be an important aspect of a prime-boost strategy that includes BCG. Testing of new vaccines as boosters to a BCG prime has the advantage of having millions of individuals who have already been vaccinated with BCG, however, to some extent, this is may also work as a disadvantage, as the choice of which strain of BCG to use is eliminated. Designing a combination of a BCG prime and a boosting vaccine will maximize the individual

benefits of both vaccines. Both DNA and subunit vaccines incorporating MPT70 and MPT83 have been shown to have the capacity to stimulate a certain degree of protection in animal models and should be tested as booster vaccines to a strain of BCG expressing these antigens.

4.4. Conclusions and Summary

The strains of BCG currently in use have previously been shown to be genotypically distinct from each other, however, how those genetic differences have impacted on the phenotypes of each of those strains has received little attention.

Deletion of the RD2 region in late strains of BCG, which resulted in the loss of antigenic and regulatory proteins, was hypothesized to have contributed to further attenuation of these strains. The deletion of this region in a virulent strain of *M. tuberculosis* did not lead to an obvious attenuation in the models in which it was examined, however, studying this deletion in combination with other deletions and mutations that contribute to the attenuation of BCG strains (such as RD1), may reveal a degree of attenuation not observed in this study.

Comparison of global gene expression between early and late strains of BCG, however, led to the identification of the cause of the decreased expression of the antigenic proteins MPB70 and MPB83 in late strains of BCG. Two regions were identified as consistently down-regulated in late strains of BCG compared to early strains, one of which contained the gene encoding the ECF sigma factor, *sigK*, and the second of which contained the genes encoding MPB70 and MPB83. Sequencing of the *sigK* gene revealed a single nucleotide polymorphism at the third base pair of the start codon, which was hypothesized to limit the translation of *sigK* mRNA and thus decrease the expression of SigK-controlled genes. Through complementation of *sigK* from strains of BCG that produce high amounts of MPB70 and MPB83 to a low-producing strain, we revealed that SigK controls a strikingly small regulon, consisting of the *sigK* region itself and the *mpb70/mpb83* region.

Characterization of BCG Pasteur::*sigK* revealed both *in vitro* and *in vivo* differences compared to control strains of BCG Pasteur. The control strains demonstrated an *in vitro* growth advantage over the complemented strains, suggesting that an *in vitro* advantage was gained by the loss of expression of the *sigK* and *mpb70/mpb83* regions. *In vivo* studies, however, suggested that expression of MPB70 and MPB83 is beneficial to *in vivo* conditions. Specific characteristics of BCG Pasteur::*sigK* that would be advantageous to BCG as a vaccine include stimulation of a strong antigen-specific immune response, increased deposition in organs following infection and prolonged persistence. In the challenge studies completed here, protection induced by vaccination with BCG Pasteur::*sigK* was equal or greater than that induced by vaccination with BCG Pasteur::pMV306. Taken together, these results suggest that expression of MPB70 and MPB83 is a beneficial characteristic of a BCG-based vaccine. Further use of BCG strains as vaccines for TB should take into consideration the characteristics of the BCG strain used, as it has been demonstrated here that the deletions and mutations that BCG strains have sustained through *in vitro* propagation have impacted upon the characteristics that are important for BCG as a vaccine.

5. *Appendices*

5.1. Approval Forms for Animal Use Protocols

5.2. Journal Article

Charlet,D., Mostowy,S., Alexander,D., Sit,L., Wiker,H.G., and Behr,M.A. (2005) Reduced expression of antigenic proteins MPB70 and MPB83 in *Mycobacterium bovis* BCG strains due to a start codon mutation in *sigK* *Molecular Microbiology* **56**: 1302-1313.

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Reduced expression of antigenic proteins MPB70 and MPB83 in *Mycobacterium bovis* BCG strains due to a start codon mutation in *sigK*

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Summary

***Mycobacterium bovis* Bacille Calmette–Guérin (BCG) strains are genetically and phenotypically heterogeneous. Expression of the antigenic proteins MPB70 and MPB83 is known to vary considerably across BCG strains; however, the reason for this phenotypic difference has remained unknown. By immunoblot, we separated BCG into high- and low-producing strains. By quantitative reverse transcription polymerase chain reaction (RT-PCR), we determined that transcription of the antigen-encoding genes, *mpb70* and *mpb83*, follows the same strain pattern with mRNA levels reduced over 50-fold in low-producing strains. Transcriptome comparison of the same BCG strains by DNA microarray revealed two gene regions consistently downregulated in low-producing strains compared with high-producing strains, one including *mpb70* (*Rv2875*) and *mpb83* (*Rv2873*) and a second that includes the predicted sigma factor, *sigK*. DNA sequence analysis revealed a point mutation in the start codon of *sigK* in all low-producing BCG strains. Complementation of a low-producing strain, BCG Pasteur, with wild-type *sigK* fully restored MPB70 and MPB83 production. Microarray-based analysis and confirmatory RT-PCR of the complemented strains revealed an upregulation in gene transcription limited to the *sigK* and the *mpb83/mpb70* gene regions. These data demonstrate that a mutation of *sigK* is responsible for decreased expression of MPB70 and MPB83 in low-producing BCG strains and provide clues into the role of *Mycobacterium tuberculosis* SigK.**

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Introduction

Mycobacterium bovis Bacille Calmette–Guérin (BCG) strains have been given to billions of people as vaccines against tuberculosis (TB) since their derivation at the Pasteur Institute between 1908 and 1921. While BCG immunization reliably provides protection in animal models, their protection in human clinical trials has been inconsistent, leading to a number of hypotheses to explain these variable findings (Fine, 1995; Agger and Andersen, 2002). One theory that has been the subject of recent investigation pertains to the heterogeneity of BCG preparations used in an era before standardized vaccines. With the advent of comparative genomic tools has come the recognition that BCG strains are distinct from each other and different from the vaccines first provided in the early 20th century (Mostowy *et al.*, 2003). Moreover, analysis of the elements implicated in BCG evolution indicates that genes encoding regulatory elements and antigenic proteins are over-represented in the genomic deletions incurred by BCG strains (Behr, 2002).

The importance of antigenic proteins in TB pathogenesis and vaccine development has been well established through their use in generating immunity to TB and the demonstration that disruption of the ESAT-6 region contributed to the derivation of BCG (Andersen, 1994; Harboe *et al.*, 1996; Mahairas *et al.*, 1996; Pym *et al.*, 2002; Lewis *et al.*, 2003; Brodin *et al.*, 2004; Doherty *et al.*, 2004). Therefore, the observation that BCG strains have suffered loss of antigenic proteins during *in vitro* passage is consistent with a potential impairment in their capacity to serve as immunizing agents. Of the described antigenic proteins of the *Mycobacterium tuberculosis* complex, the *M. bovis* antigens MPB70 and MPB83 (also known as MPT70 and MPT83 when studied in *M. tuberculosis*) figure prominently as candidates for vaccine development (Fifis *et al.*, 1994; Mustafa *et al.*, 1998; Chambers *et al.*, 2002; 2004). Although the genes encoding these proteins

have not been deleted in BCG evolution, production of these proteins by BCG vaccines during *in vitro* growth varies considerably. In certain BCG strains, such as BCG Tokyo, MPB70 represents the most abundant protein in the culture filtrate (Nagai *et al.*, 1981). In other BCG strains, such as BCG Pasteur, production of MPB70 is markedly reduced, leading to the division of BCG strains into high-producers or low-producers (Miura *et al.*, 1983; Harboe and Nagai, 1984). The same pattern of antigen production across BCG strains has also been observed for MPB83, although the differences have generally not been as dichotomous (Wiker *et al.*, 1996).

To explore the reasons underlying these differences in production, sequence-based analysis has been performed, but no mutations in the encoding genes or their upstream promoter regions have been detected (Hewinson *et al.*, 1996; Vosloo *et al.*, 1997). Complementation of BCG Pasteur with *mpb70* from BCG Tokyo did not restore levels of MPB70 to those observed with BCG Tokyo, suggesting differences in expression inherent to the parent BCG strain (Matsumoto *et al.*, 1995). By targeted expression analysis, using reverse transcription polymerase chain reaction (RT-PCR) and Northern blots, an obvious difference in *mpb70* transcription was observed between BCG Tokyo (high-producer) and BCG Pasteur (low-producer) (Matsuo *et al.*, 1995). However, the reason for this difference has remained unknown.

Because the history of BCG strain dissemination has been recorded, it has been possible to precisely determine the chronology of specific genetic changes in BCG strains (Behr and Small, 1999). A number of these mutations affect putative regulatory genes (Behr *et al.*, 1999; Brosch *et al.*, 2000; Spreadbury *et al.*, 2005), so we hypothesized that a mutation in a regulatory gene was likely responsible for the variable production of MPB70 and MPB83. We have therefore determined the production of MPB70 and MPB83 across a panel of BCG strains, in order to assign the chronology of this phenotypic change and thereby guide studies towards identifying the responsible mutation. Our data implicate a start codon mutation in the *M. tuberculosis* sigma factor K (*Rv0445c* or *sigK*) and point to a highly specific link between *sigK* and expression of MPB70 and MPB83.

Results

Immunoblotting M. bovis *BCG culture filtrate proteins and whole-cell extracts*

To determine the production of MPB70 and MPB83 across BCG strains, we analysed culture filtrate proteins and whole-cell extracts blinded to strain identity. Upon decoding the samples, MPB70 was detected in substantial amounts in the culture filtrates of BCG Russia, Birkhaug, Sweden, Japan and Moreau and not in the remaining strains (Fig. 1A). In the whole-cell extracts, MPB70 was not detectable in any strain (Fig. 1B). MPB83 was detected in both the culture filtrate proteins and the whole-cell extracts of BCG Russia, Birkhaug, Sweden, Japan and Moreau (Fig. 1C). In the remaining strains of BCG, MPB83 could be detected in low amounts in the whole-cell extracts (Fig. 1D). These results are in agreement with previous results from a subset of these strains (Miura *et al.*, 1983; Harboe and Nagai, 1984; Wiker *et al.*, 1996) and indicate a clear delineation between strains obtained from the Pasteur Institute until 1927 (high-producers) versus strains obtained, either directly or indirectly, in 1931 or later (low-producers).

Transcription of mpb70 *and* mpb83 *in BCG strains*

To determine whether transcriptional differences might correlate with variations in protein production, we

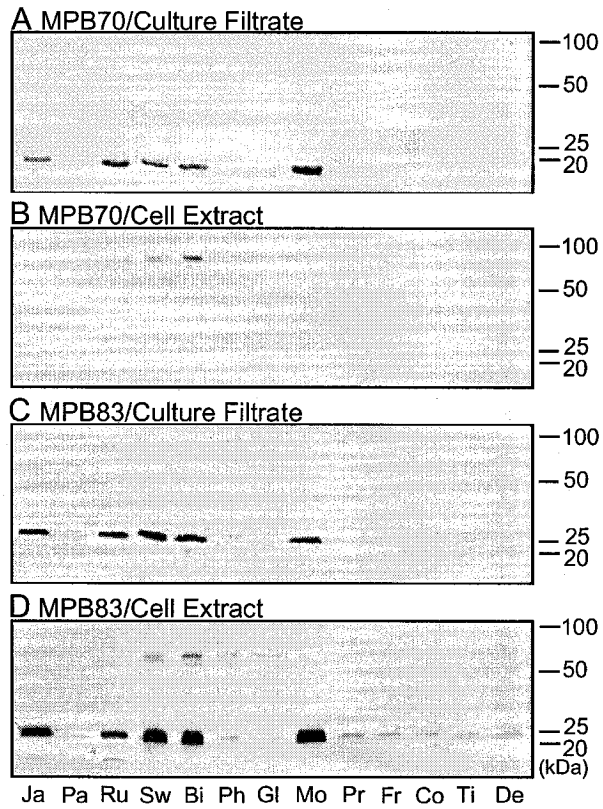

Fig. 1. SDS-PAGE and immunoblotting of culture filtrate proteins (A and C) and cell extracts (B and D) from BCG strains, using monoclonal antibodies 1-5c for MPB70 (A and B) and MBS43 for MPB83 (C and D). The strains used were as follows: Ja, BCG Japan; Pa, BCG Pasteur; Ru, BCG Russia; Sw, BCG Sweden; Bi, BCG Birkhaug; Ph, BCG Phipps; Gl, BCG Glaxo; Mo, BCG Moreau; Pr, BCG Prague; Fr, BCG Frappier; Co, BCG Connaught; Ti, BCG Tice; De, BCG Denmark.

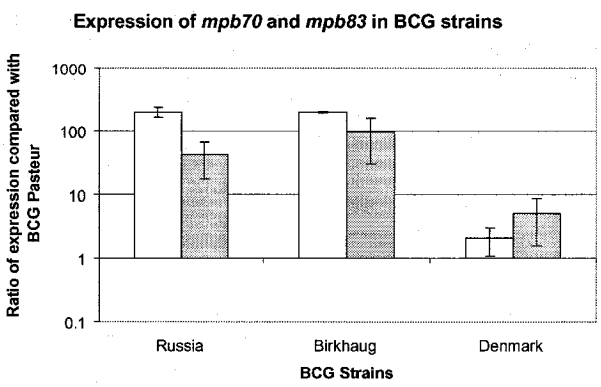

Fig. 2. Expression of *mpb70* (white) and *mpb83* (grey) in *M. bovis* BCG strains. Ratio of expression is to that of BCG Pasteur. All values were normalized to the levels of *sigA* mRNA.

employed quantitative RT-PCR with molecular beacons to estimate relative mRNA levels for *mpb70* and *mpb83*. As immunoblotting results pointed to distinctions between strains obtained before or after the interval 1927–1931, we selected the first and last strain obtained from each group (BCG Russia and BCG Birkhaug for the earlier/high-producing group and BCG Danish and BCG Pasteur for the later/low-producing group). Consistent with previous reports indicating different mRNA expression (Matsuo *et al.*, 1995), measured levels of *mpb70* and *mpb83* mRNA were profoundly lower in BCG Danish and BCG Pasteur as compared with BCG Birkhaug and BCG Russia with a calculated difference greater than 50-fold (Fig. 2).

Microarray analysis of BCG strains

To look for other differences in gene expression that might coincide with transcription of *mpb70* and *mpb83*, we studied the same four BCG strains by whole genome microarray, directly comparing BCG Russia with BCG Pasteur and BCG Birkhaug with BCG Danish (Table 1). Consistent with the RT-PCR data, levels of *mpb70* and *mpb83* were significantly lower in BCG Pasteur and BCG Danish, as compared with BCG Russia and BCG Birkhaug. Also in this region, *Rv2876* and *Rv2878c* showed decreased expression in late/low-producing strains compared with early/high-producing strains. A second region that showed consistent downregulation in the low-producing strains was *Rv0445c–Rv0449c*. Of these genes, the only putative transcriptional regulator is *Rv0445c* (*sigK*), which is predicted to encode an alternate sigma factor, prompting further analysis.

Sequence analysis of sigK

Comparison of *sigK* across sequenced genomes indicated two polymorphisms in BCG Pasteur compared with *M. tuberculosis* H37Rv. First, located at nucleotide –31 upstream from the *sigK* start codon, the adenine residue is replaced by a thymidine residue in BCG Pasteur. Sequencing this region across members of the *M. tuberculosis* complex and 13 BCG strains revealed that this mutation represents an *M. tuberculosis* polymorphism; *Mycobacterium canettii*, *Mycobacterium microti*, *Mycobacterium caprae*, *M. bovis* and all BCG strains have the thymidine residue at the –31 upstream position while

Table 1. Microarray analysis of *Mycobacterium bovis* BCG strains.

		Russia versus Pasteur		Birkhaug versus Denmark		
ORF	Gene name	Fold change	*z*-score	Fold change	*z*-score	Gene product
Rv0445c	*sigK*	–5.29	3.56	–7.14	3.04	Sigma factor
Rv0446c		–5.79	3.82	–8.34	3.36	Conserved transmembrane protein
Rv0447c	*ufaA1*	–6.91	3.79	–8.14	3.17	Cycolpropane-fatty-acyl-phospholipid synthase
Rv0448c		–15.82	4.22	–11.09	4.68	Conserved hypothetical protein
Rv0449c		–14.46	5.35	–19.16	5.07	Conserved hypothetical protein
Rv2627c		3.10	2.93	5.02	2.18	Conserved hypothetical protein
Rv2707		–3.53	2.42	–3.93	2.28	Conserved transmembrane protein
Rv2873	*mpt83*	–13.71	6.47	–36.87	5.00	Cell surface lipoprotein
Rv2875	*mpt70*	–17.51	6.31	–33.99	5.45	Major secreted immunogenic protein
Rv2876		–10.04	4.64	–12.81	4.21	Conserved transmembrane protein
Rv2878c	*mpt53*	–3.55	3.92	–26.41	2.25	Soluble secreted antigen
Rv3681c	*whiB4*	2.73	2.08	3.06	2.22	Transcriptional regulatory protein

Predicted genes whose *in vitro* expression was significantly dysregulated between early versus late BCG. The *z*-score, indicative of how many standard deviations a data point lies from the population mean, is the average for that gene across replicate ($n = 2$) microarrays of Russia (early BCG) versus Pasteur (late BCG) and Birkhaug (early BCG) versus Denmark (late BCG). Genes with average *z*-scores of 2 or greater for both early versus late BCG comparisons are presented and the fold change is calculated from a normalized log ratio of that gene. The fold change in late BCG expression is expressed as a ratio, with early BCG expression for that gene providing the reference value of 1. Genes for which expression was decreased in late BCG are indicated by a negative sign; genes with increased expression in late BCG are reported by a positive sign. Open reading frame (ORF) and gene name are listed by Rv number as annotated for *M. tuberculosis* H37Rv (http://genolist.pasteur.fr/TubercuList/). Genes from the same region of the genome are grouped and presented in a darker shade.

sequenced *M. tuberculosis* strains (H37Rv, 210 and CDC1551) have the adenine residue. Second, in *M. tuberculosis* H37Rv, the start codon of *sigK* is the predominant start codon sequence AUG, while in BCG Pasteur there is a G → A mutation at the third nucleotide, resulting in an altered AUA start codon. The AUG was observed in all members of the *M. tuberculosis* complex except for the eight low-producing BCG strains obtained after 1927 in which the altered AUA start codon was observed (Table 2). While the codon AUA has been identified as a functional start codon in *Escherichia coli*, *Bacillus subtilis* and *Salmonella* spp., levels of translation are substantially reduced with this codon compared with the conventional start codon AUG (Romero and Garcia, 1991; Sussman *et al.*, 1996). Because the start codon mutation correlated precisely with the BCG strains having decreased *sigK* and *mpb83/mpb70* expression, we proceeded to examine the functional consequence of the *sigK* mutation.

Effect of sigK *complementation on transcription in BCG Pasteur*

We first determined the effect of *sigK* complementation on its own expression by quantitative RT-PCR. Complementation with the empty vector or with the mutant *sigK* resulted in no change of *sigK* expression, as seen with BCG Pasteur::pMV306 (empty vector) and BCG Pasteur::pPAST. This latter result indicated that a second copy of the mutant gene did not alter levels of transcription. In contrast, complementation with wild-type *sigK*, demonstrated with BCG Pasteur::pH37Rv, BCG Pasteur::pRUSS and BCG Pasteur::pBIRK, showed a marked increase in transcription of *sigK* (Fig. 3A). Similar results were obtained with a second clone of each of the same strains (data not shown). As the mutation in *sigK* is predicted to impair translation, not transcription, and complementation of wild-type, but not mutant-type, *sigK* served to markedly increase *sigK* expression, these results signify that expression of this gene appears to be autoregulated, as has been described for other *M. tuberculosis* sigma factors (Helmann, 2002; Manganelli *et al.*, 2004a).

Table 2. Sequence Analysis of *sigK*.

Strains	31 bp before start codon	3 bp of start codon
M. canettii	T	G
M. tuberculosis H37Rv	A	G
M. tuberculosis H37Ra	A	G
M. africanum	T	G
M. microti	T	G
M. caprae	T	G
M. bovis	T	G
BCG Russia	T	G
BCG Moreau	T	G
BCG Japan	T	G
BCG Sweden	T	G
BCG Birkhaug	T	G
BCG Prague	T	A
BCG Glaxo	T	A
BCG Denmark	T	A
BCG Tice	T	A
BCG Connaught	T	A
BCG Frappier	T	A
BCG Phipps	T	A
BCG Pasteur	T	A

Sequence analysis of *sigK* for two representative isolates of different *M. tuberculosis* complex members, with the exception of *M. canettii* where only one isolate was sequenced. The only polymorphisms detected are presented.

Next, we determined the effect of *sigK* complementation on *mpb70* and *mpb83* levels, using the same clones and mRNA preparations. In BCG Pasteur::pPAST and BCG Pasteur::pMV306, levels of mRNA were comparable to those previously demonstrated in low-producing strains of BCG. The same strains in which we observed increased *sigK* expression, BCG Pasteur::pH37Rv, BCG Pasteur::pRUSS and BCG Pasteur::pBIRK, manifested highly increased transcription levels for *mpb70* and *mpb83*, comparable to the levels observed with high-producing strains of BCG (Fig. 3B). The same results were obtained with a second clone of the same strains (data not shown).

Effect of sigK *complementation on MPB70 and MPB83 production*

To determine the effect of expression of wild-type *sigK* in BCG Pasteur on protein synthesis, culture filtrate proteins and whole-cell lysates from the *sigK*-complemented strains of BCG Pasteur were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. MPB70 was detected in the culture filtrate proteins in BCG Pasteur::pH37Rv, BCG Pasteur::pRUSS and BCG Pasteur::pBIRK, but could not be detected in Pasteur::pPAST or BCG Pasteur::pMV306 (Fig. 3C). Similarly, MPB83 was detected in the whole-cell lysates of the same clones where MPB70 was abundantly detected in the culture filtrates (Fig. 3D). Upon complementation of wild-type *sigK*, BCG Pasteur was able to produce MPB70 and MPB83 in a pattern consistent with high-producing strains.

Further expression analysis of genes neighbouring mpb70/mpb83 *and* sigK

Based on observed differences in expression of the genes neighbouring *sigK* and *mpb70/mpb83* by microarray-based analysis of four BCG strains, we used quantitative RT-PCR to determine the effect of wild-type *sigK* complementation on transcription in these regions. As shown in Fig. 4A, expression of *Rv0443c-Rv0449c* was increased

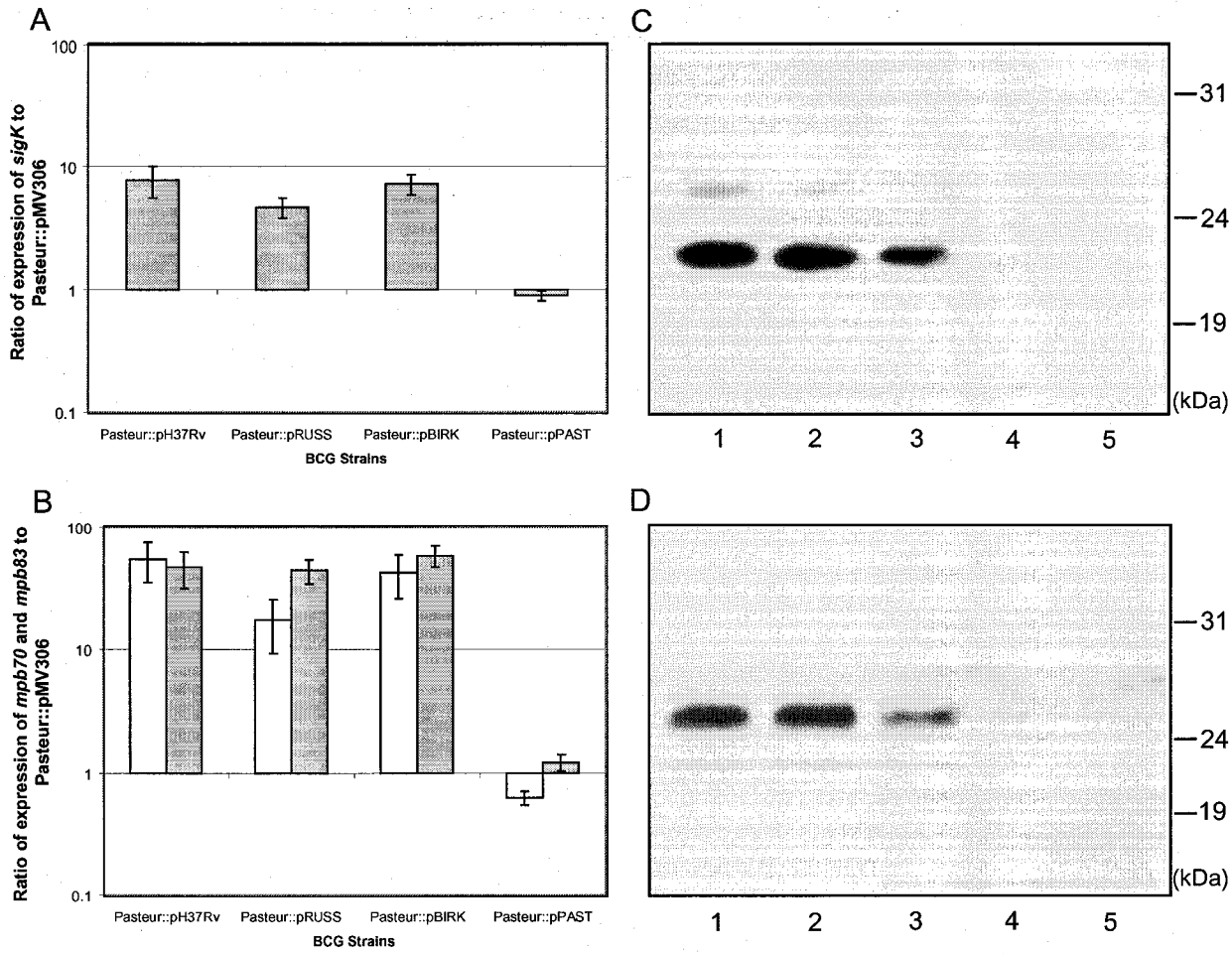

Fig. 3. A and B. Expression of *sigK* (A) and *mpb70* (white) and *mpb83* (grey) (B) upon complementation of BCG Pasteur with *sigK* from *M. tuberculosis* H37Rv, BCG Russia, BCG Birkhaug and BCG Pasteur. Values are expressed as a ratio of mRNA copies in complemented strains compared with BCG Pasteur::pMV306. All values were normalized to the levels of *sigA* mRNA and error bars represent the standard error of the mean.

C and D. SDS-PAGE and immunoblotting of culture filtrate proteins (C) and cell extracts (D) from complemented BCG Pasteur, using monoclonal antibodies 1-5c for MPB70 (C) and MBS43 for MPB83 (D). The strains used were as follows: 1, BCG Pasteur::pH37Rv; 2, BCG Pasteur::pRUSS; 3, BCG Pasteur::pBIRK; 4, BCG Pasteur::pPAST; 5, BCG Pasteur::pMV306.

over twofold with *sigK* complementation. Transcription of *Rv2874*, *Rv2876* and *Rv2877c* was increased over 10-fold in the *sigK*-complemented strains compared with the control strain. *Rv2878c* (*mpt53*) was also increased, although to a lesser extent, with fourfold increase seen in the *sigK*-complemented strains. Contrary to expectations from previous study indicating that *Rv2871–Rv2874* are cotranscribed (Juarez *et al.*, 2001), we did not detect any increased expression of either *Rv2871* or *Rv2872*, suggesting that these two genes are under separate transcriptional control. Based on the differences in transcription, this *sigK*-regulated gene region includes *Rv2873* through *Rv2878*, but not *Rv2872* or *Rv2879* (Fig. 4B).

Microarray-based analysis of sigK *complementation*

To further examine the role that *sigK* plays in global gene expression, we analysed global transcription in BCG Pasteur::pH37Rv, BCG Pasteur::pRUSS and BCG Pasteur::pBIRK compared with BCG Pasteur::pMV306 by DNA microarray. Results revealed increased expression of the majority of genes presented in Fig. 4, with significant changes in expression as measured by both induction ratios and *z*-scores. Because of the stringency of the analysis performed, genes whose expression was induced in three of four arrays, but whose expression could not be optimally quantified on the fourth, were not included in the table (e.g. *mpb83*). Only four genes were

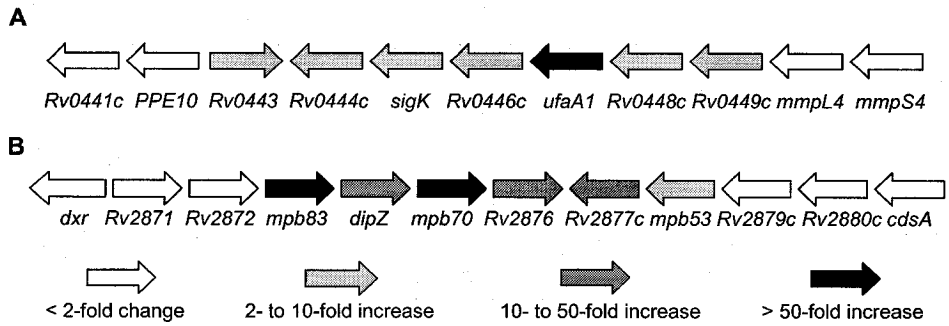

Fig. 4. A. Expression analysis of the genes *Rv0441c* to *Rv0450c*.
B. Expression analysis for the genes *Rv2870c* to *Rv2881c*. Levels presented represent the ratio of mRNA copies in *sigK*-complemented strain to the control strain BCG Pasteur::pMV306 (empty vector). All values were normalized to the levels of *sigA* mRNA and the ratio presented represents the mean of results from different clones, specifically BCG Pasteur::pH37Rv, BCG Pasteur::pRUSS and BCG Pasteur::pBIRK.

repressed with introduction of wild-type *sigK*; genes whose expression was increased were restricted to the *sigK* and the *mpb70/83* regions (Table 3).

Discussion

Comparative genomic studies have demonstrated the substantial genome decay experienced by BCG strains during a half-century of *in vitro* passage (Mostowy *et al.*, 2003). While DNA microarray-based comparisons have efficiently uncovered genomic deletions as the most evident form of evolution, these tools overlook other forms of genomic variability, such as duplications and single nucleotide polymorphisms (SNPs). With targeted study of specific genes has come the recognition of numerous loss-of-function SNPs during BCG evolution, including a SNP that impairs synthesis of methoxymycolic acids (Behr *et al.*, 2000), a SNP predicted to decrease the DNA binding ability of a cAMP receptor protein (CRP) homologue (Spreadbury *et al.*, 2005) and the start codon mutation described in this report. Remarkably, along with the loss of the RD2 region (Mahairas *et al.*, 1996), these three SNPs all coincide with the interval 1927–1931, suggesting either a period of considerable *in vitro* evolution or the replacement of one BCG stock with another at the Pasteur Institute in the late 1920s.

Our data indicate that the *sigK* SNP occurred between 1927 and 1931 at the Pasteur Institute and resulted, either directly or indirectly, in a major drop in production of the antigenic proteins MPB70 and MPB83. These two pro-

Table 3. Genes whose expression was changed upon complementation of BCG Pasteur with wild-type *sigK*.

		BCG Pasteur::pBIRK versus BCG Pasteur::pMV306		BCG Pasteur:: pH37Rv versus BCG Pasteur::pMV306		
ORF	Gene name	Fold change	*z*-score	Fold change	*z*-score	Gene product
Rv0445c	*sigK*	2.7	2.6	2.6	2.4	Sigma factor
Rv0446c		6.1	5.3	7.9	5.2	Conserved transmembrane protein
Rv0447c	*ufaA1*	5.0	4.5	5.0	4.0	Cycolpropane-fatty-acyl-phospholipid synthase
Rv0448c		15.1	7.8	9.4	5.6	Conserved hypothetical protein
Rv0449c		11.8	6.5	8.3	5.2	Conserved hypothetical protein
Rv1884c	*rpfC*	–3.0	3.2	–3.6	3.3	Probable resuscitation-promoting factor C
Rv1886c	*fbpB*	–2.1	2.2	–3.2	2.9	Secreted antigen 85-B
Rv2031c	*hspX*	–2.1	2.2	–2.6	2.4	Heat shock protein X
Rv2874	*dipZ*	836.6	15.0	88.8	9.5	Possible integral membrane protein
Rv2875	*mpt70*	62.2	11.7	17.7	7.2	Major secreted immunogenic protein
Rv2876		12.5	7.4	13.1	6.2	Conserved transmembrane protein
Rv2878c	*mpt53*	37.6	8.1	9.7	3.3	Soluble secreted protein
Rv3681c	*whiB4*	–2.2	2.1	–2.2	2.0	Transcriptional regulator protein

Predicted genes whose *in vitro* expression was significantly dysregulated between BCG Pasteur::pMV306 (empty vector) and BCG Pasteur::*sigK* (wild type). The *z*-score, indicative of how many standard deviations a data point lies from the population mean, is the average for that gene across replicate ($n = 2$) microarrays of BCG Pasteur::pBIRK versus BCG Pasteur::pMV306 and BCG Pasteur::pH37Rv versus BCG Pasteur::pMV306. Genes with average *z*-scores of 2 or greater for both comparisons are presented and the fold change is calculated from a normalized log ratio of that gene, with BCG Pasteur expression for that gene providing the reference value of 1. Open reading frame (ORF) and gene name are listed by Rv number as annotated for *M. tuberculosis* H37Rv (http://genolist.pasteur.fr/TubercuList/). Genes from the same region of the genome are grouped and presented in a darker shade.

teins exhibit striking amino acid sequence homology (Hewinson *et al.*, 1996) and both are exported, but localize differently. The single form of MPB70 is secreted into culture media while MPB83 is present in two forms, a 26 kDa lipoprotein which remains associated with the mycobacterial cell wall and a 23 kDa form which is found in the culture media (Harboe *et al.*, 1998). The structure of MPB70 has recently been solved and superimposition of MPB83 on the MPB70 structure confirmed the overall homology of the antigens (Carr *et al.*, 2003). From immunologic studies, it is known that both proteins induce cellular and humoral responses in experimental infection of model hosts and natural infection of humans (Miura *et al.*, 1983; Haslov *et al.*, 1987; Fifis *et al.*, 1994; Roche *et al.*, 1994; Harboe *et al.*, 1995; Wiker *et al.*, 1996; Vordermeier *et al.*, 2000; Lyashchenko *et al.*, 2001). Based on these observations, both MBP70 and MPB83 have been developed as candidates for novel TB vaccine development (Chambers *et al.*, 2000; Morris *et al.*, 2000; Al Attiyah *et al.*, 2003; Tollefsen *et al.*, 2003; Xue *et al.*, 2004). As extracellular antigens have consistently been implicated in the induction of a protective immune response against *M. tuberculosis*, it is remarkable that all BCG strains are unable to produce the antigenic proteins ESAT-6 and CFP-10 (lost with the RD1 deletion of 1908–1921), while strains obtained after 1927–1931 are also deficient (through the deletion of RD2) in the proteins MPB64 (Harboe *et al.*, 1986; Li *et al.*, 1993) and CFP-21 (Mahairas *et al.*, 1996; Weldingh *et al.*, 1998; Weldingh and Andersen, 1999) and functionally deficient in production of MPB70 and MPB83 via the *sigK* mutation described here.

SigK is one of 10 extracytoplasmic function (ECF) sigma factors annotated in the *M. tuberculosis* H37Rv genome. As the name suggests, these regulatory elements mediate responses to changing external conditions (Manganelli *et al.*, 2001; 2004b; Ando *et al.*, 2003; Hu *et al.*, 2004), with a common feature being their control over relatively small regulons (Bashyam and Hasnain, 2004). For instance, by microarray analysis, the regulon of *sigC* has been estimated to contain 13, 14 and 18 genes, in exponential, early and late stationary phase growth, respectively (Sun *et al.*, 2004), consistent with our observations of two regions, comprising 13 genes, being consistently upregulated in wild-type *sigK*-complemented strains. Mutants of *sigC*, *sigD*, *sigE* and *sigH* all exhibit reduced virulence in animal models (Kaushal *et al.*, 2002; Calamita *et al.*, 2005; Raman *et al.*, 2004; Sun *et al.*, 2004), but to date, there have been no published papers looking specifically at *M. tuberculosis sigK*. In transposon site hybridization studies, neither *sigK* nor any of the other genes in the regulon we have identified was observed to be essential for *in vivo* growth in a murine model (Sassetti and Rubin, 2003). However, these experiments averaged the results for *M. tuberculosis* H37Rv and BCG Pasteur, and BCG Pasteur is now shown to be functionally deficient in this regulon, therefore the impact of mutations in these genes may have been minimized. An epidemiologic study of *M. tuberculosis* isolates in San Francisco used genomic hybridization studies to determine deletions in strains that had successfully caused TB, thereby generating a list of genes that are apparently nonessential for disease (Tsolaki *et al.*, 2004). Of 224 genes disrupted in at least one clinical isolate, none of the genes implicated in the *sigK* nor the *mpb83/70* regions is featured, suggesting that loss of these genes may be detrimental to disease causation. The relevance of *sigK*-regulated genes is supported by transcriptome analysis of *M. tuberculosis* during intracellular conditions, where *mpb70* and *mpb83* figure among the most highly induced genes, across time points and in both activated and non-activated macrophages (Schnappinger *et al.*, 2003). Additionally, in a microarray-based study of *M. tuberculosis* expression during murine infection, *sigK* and *mpt53* were among those genes noted as significantly dysregulated *in vivo* (Talaat *et al.*, 2004). Together, these results point to a potential role of the *sigK* regulon in the pathogenesis of TB that merits further attention.

The data presented here explain the difference in expression between high-producing and low-producing BCG strains. However, MPB70 and MPB83 are also differentially produced by *M. tuberculosis* and *M. bovis*. Although these organisms have identical, wild-type AUG *sigK* start codons, *in vitro* expression is low (although inducible) in *M. tuberculosis* and constitutively high in *M. bovis* (Wiker *et al.*, 1996). The constitutive *in vitro* production of MBP70 and MPB83 observed in *M. bovis* may therefore stem from unregulated activity of SigK. Activity of an ECF sigma can be mediated by a second protein, the anti-sigma factor, that functions post-translationally as a negative regulator to prevent constitutive expression of the target regulon (Helmann, 2002; Manganelli *et al.*, 2004a). The sigma/anti-sigma pair are usually adjacent and co-transcribed genes; for instance, in *M. tuberculosis*, RshA (*Rv3221A*) is the anti-sigma factor for SigH (*Rv3223c*) while UsfX (*Rv3287c*) is the anti-sigma factor for SigF (*Rv3286c*) (Beaucher *et al.*, 2002; Song *et al.*, 2003). Consistent with this pattern, *Rv0444c* may encode the anti-sigma factor for *sigK* (*Rv0445c*), and by extension, mutations in *Rv0444c* might result in unregulated expression of *sigK*. Ongoing investigations are pursuing this possibility, based on the presence of two non-synomyous SNPs in *Rv0444c* restricted to *M. tuberculosis* complex species presenting constitutively high MPB70 production (data not shown).

As BCG vaccines are given to an estimated 2 million infants per week, there are important practical implications of these findings. The *sigK* mutation described here impairs the production of two immunodominant antigens,

MPB70 and MPB83, as well as transcription of *mpb53*. The importance of this deficit for TB immunization is unknown because strains of BCG that produce these antigens have never been utilized in a randomized clinical trial, although results from some observational studies have suggested a greater potency to some of the high-producer strains (Kroger *et al.*, 1994; Vitkova *et al.*, 1995). Based on the number of documented differences between BCG strains obtained before 1927 and those obtained after 1931, these is compelling rationale to perform a human trial comparing BCG strains from these two groups. Furthermore, these results are also applicable towards efforts to develop improved vaccines against TB. Recent advances have demonstrated that recombinant strains of BCG expressing *M. tuberculosis* antigens provide an important avenue towards more effective vaccines (Horwitz *et al.*, 2000; Pym *et al.*, 2003). These constructs may benefit from the inherent MPB70 and MPB83 expression of the high-producing strains obtained before 1931, or alternatively, by correcting the *sigK* mutation in later strains to achieve the same result.

Experimental procedures

Bacterial cultures

Unless otherwise stated, BCG strains were grown at 37°C in Middlebrook 7H9 medium (Difco Laboratories, Detroit, MI) containing 0.05% Tween 80 (Sigma-Aldrich, St. Louis, MO) and 10% albumin-dextrose-catalase (Becton Dickinson and Co., Sparks, MD) supplement on a rotating platform (Wheaton). Transformed BCG strains were resuspended in 7H9 containing 15% glycerol and frozen in 1 ml aliquots at −80°C until needed. Frozen bacteria were thawed and diluted in fresh 7H9 medium containing 10% albumin-dextrose-catalase and grown with rotation at 37°C.

PCR amplification and sequencing across Rv0445c (sigK)

The sequence of *Rv0445c* (*sigK*) was determined by amplifying the gene and flanking regions from isolates of *M. canettii*, *M. tuberculosis* H37Rv, *M. tuberculosis* H37Ra, *Mycobacterium africanum* ($n = 2$), *M. microti* ($n = 2$), *M. caprae* ($n = 2$), *M. bovis* ($n = 2$) and 13 members of the BCG family – BCG Russia (ATCC 35740), BCG Moreau, BCG Japan, BCG Sweden, BCG Birkhaug (ATCC 35731), BCG Prague, BCG Glaxo (ATCC 35741), BCG Denmark 1331 (ATCC 35733), BCG Tice (ATCC 35743), BCG Frappier (ATCC 35735), BCG Connaught, BCG Phipps (ATCC 35744) and BCG Pasteur 1173. The sequence was amplified using left primer: 5′-agctcgagcagctcaaaatc-3′; and right primer: 5′-acgcgtcaccccaactact-3′ and amplicons were sequenced by di-deoxy terminal sequencing at the McGill University and Genome Quebec Innovation Center. To look for differences between the amplified sequence and the prototype genome sequences, results were compared by BLAST analysis to *M. tuberculosis* H37Rv using Tuberculist (http://genolist.pasteur.fr/TubercuList/), *M. tuberculosis* 210 and CDC1551 using the sequences provided at NCBI (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi), *M. bovis* AF2122/97 using Bovilist (http://genolist.pasteur.fr/BoviList/), and the assembly sequence of BCG Pasteur (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/m_bovis).

RNA extraction

BCG strains grown to an OD_{600} of 0.3–0.5 were pelleted by centrifugation, resuspended in 1 ml of wash buffer (0.5% Tween 80, 0.8% sodium chloride) and transferred to 1.5 ml screw-cap cryovials. RNA was extracted by a modified phenol-chloroform extraction protocol as previously described (Belley *et al.*, 2004). Genomic DNA contamination was removed by RNAeasy on-column digestion, following the manufacturer's protocol (Qiagen, Mississauga, Canada). The quality of RNA was confirmed by denaturing gel electrophoresis (formaldehyde).

Real-time quantitative RT-PCR

Targeted gene expression levels were determined using RT-PCR with molecular beacons or SYBR green, according to established protocols (Manganelli *et al.*, 1999; Mostowy *et al.*, 2004). To provide a normalization standard for mRNA expression, expression of *sigA* was also determined, and the level of expression of a gene of interest divided by that of *sigA* to normalize for differences in total mRNA extracted (Manganelli *et al.*, 1999). Sequences of the primers used for molecular beacon and sybr green analysis and the sequences of the molecular beacons used are as listed in Table 4.

Microarray analysis

Microarray hybridization and analysis were performed as previously described (Mostowy *et al.*, 2004). In brief, mRNA from BCG strains and complemented strains was extracted during log-phase *in vitro* growth and labelled with Cy3 or Cy5 dUTP by reverse-transcriptase (Amersham Biosciences). Labelled cDNA was hybridized to microarrays composed of oligonucleotide probes from the TB Array-Ready Oligo Set™ (Operon) that had been printed onto Sigmascreen™ microarray slides (Sigma). Initial comparisons were BCG Russia versus BCG Pasteur, and BCG Birkhaug versus Denmark. After complenting BCG Pasteur with wild-type *sigK*, comparisons of Pasteur::*sigK* versus Pasteur::pMV306 (empty vector) were also performed. In each case, duplicate hybridizations were performed for each dye combination (Cy3/Cy5 and Cy5/Cy3), resulting in four hybridizations per comparison. Hybridized arrays were scanned with Scanarray 5000XL (PerkinElmer, Freemont, CA) and hybridization results were quantified with Scanalyze software (http://rana.lbl.gov/EisenSoftware.htm/).

Array analysis was performed as previously described (Mostowy *et al.*, 2004) in order to determine a *z*-score, indicative of how many standard deviations a data point lies from the population mean, for each gene. *z*-scores for each gene were averaged across replicates within each experiment to minimize the probability of observing such variation by

Table 4. Sequences of primers and molecular beacons for quantitative RT-PCR.

Gene	Left primer (5′ to 3′)	Right primer (5′ to 3′)	Beacon
sigA	tgcagtcggtgctggaca	cgcgcaggacctgtgagc	cctcgcgtcgaagttgcgccatccgagcgagg
sigK	agtttgactccgccaaaggt	gcaccatagcgcacttcc	gcagcctgtcgaccgagtccgttggctgc
mpb70	ctcgaacaatccggagttgaca	acaccgtgtactgaccgctgtt	gcagccagctcaatccgcaagtaaacctgggctgc
mpb83	atcaactcaagactgacgccaaac	caccttgcagggtctgatgg	gcagccagcatcctgacctaccacgtgaggctgc
dxr	gggacttgaggtcatcgaaa	tatggaaatcacaggcagca	
Rv2871	gtatcgatgacgagctgtacc	ttggacgatagatcgacacc	
Rv2872	gactatcggggtttgcttga	tcaacatcggacgctaactg	
dipZ	tcggttggtatcaggcctac	ggtccaagtggcgtagttgt	
Rv2876	agtgggagttcgacgtcagt	acgtgatcaggaaccagtcc	
Rv2877c	ggttccatgtatggctacgg	agccagatagatcgctacgc	
mpb53	gttcggtctggccaatacac	cgtccagaaccacaacacc	
Rv2879c	gcgacgggtgtctattgagt	taccgtgcaggaaactcctt	
Rv2880c	aacacggtctgcatttcctc	cccatacccatgaacaccac	
cdsA	tggtcgttgtctgcatgatt	gagccattttccgggtagac	
Rv0441c	tgggtgccaaaaaggtagac	ccacaacagggtgacttcg	
PPE10	caattcggcactgatgtttg	caggctaggtactgggttgc	
Rv0443	cgggtgcaggatatacaggt	tagtaccccgacagcaggtc	
Rv0444c	ggccgagcaagttctgac	gcagccacatctgatacacg	
Rv0446c	tggcaactgtgggtattcaa	cagcaggtaggtcatcagca	
ufaA1	ccgacctttcgacctagttg	tgaacattgcgcacgaatac	
Rv0448c	cttctacgtttcgccgtttc	tcatcgcgatctgtcttgtc	
Rv0449c	cccacacccactatctggac	acatcgacatttccgactcc	
mmpL4	ctaaattcgcgaacgactcc	cttccagtgacgggacaaat	
mmpS4	cctggaaaacagcaaaccat	ccacgatatttcccatcacc	

chance alone and genes with average *z*-scores of 2 or greater are presented.

Complementation of sigK

To complement BCG strain Pasteur, the *sigK* region (including the complete gene and 288 bp upstream) was amplified via PCR from *M. tuberculosis* H37Rv, and BCG strains Russia and Birkhaug. As a control, the *sigK* from BCG Pasteur was also complemented to determine the effect of having a second copy of the mutated gene. To complement, PCR was performed using the following primers Rv0445cL and sigKR (left primer: 5′-agctcgagcagctcaaaatc-3′; right primer: 5′-acgcgtcaccccaactact-3′) and amplified products were cloned into the T-vector, pDRIVE (Qiagen). The *sigK* region was then removed by digestion with *Hin*dIII and *Kpn*I and ligated to the integrative mycobacterial vector pMV306 (Stover *et al.*, 1991) cut with the same restriction endonucleases. Integrity of the cloned genes was confirmed by DNA sequencing, then the resulting plasmids (pH37Rv, pRUSS, pBIRK and pPAST) were electroporated into *M. bovis* BCG Pasteur cells, using previously described methods (Belley *et al.*, 2004). The empty pMV306 vector was also included as a control. Transformants were grown at 37°C on Middlebrook 7H10 agar supplemented with 10% ADC [albumin (bovine fraction V), dextrose and catalase; BD/BBL media] and kanamycin (25 μg ml^{-1}). Complementation was PCR-confirmed by amplifying the *sigK* gene with primers specific for the regions of pMV306 flanking the *sigK* insert and these amplicons were sequence-confirmed for all transformants.

Protein preparation and immunoblot analysis

BCG strains. BCG strains were cultivated as surface pellicles on liquid synthetic Sauton medium for 3 weeks at 37°C. The bacteria were washed and disrupted by a bead beater to yield a cellular extract and the culture medium was filtered to remove residual bacteria and concentrated by ammonium sulphate precipitation at 80% saturation. The antigens were separated under reducing conditions by horizontal SDS-PAGE in precast 8–18% gradient Excel gel using a Multiphor II unit 2117 (Amersham Pharmacia). After separation, the proteins were transferred to a nitrocellulose membrane (pore size, 0.2 mm) by diffusion blotting (Olsen and Wiker, 1998) and the gel was stained with CBB. The membranes were blocked with PBS containing 2% bovine serum albumin (BSA) and 1% gelatin and incubated with antibodies overnight. Bound antibodies were recognized by horseradish peroxidase (HRP)-labelled anti-rabbit or anti-mouse Ig. As substrate, 3,3-diaminobenzidine was added to visualize the bound antibodies.

BCG Pasteur complemented strains. Cultures were grown at 37°C in 7H9 with 10% ADC, supplemented with kanamycin (25 μg ml^{-1}) for 7 days. The cultures were the centrifuged and the supernatant was filtered with a 0.22 μm membrane filter and concentrated with an Amicon Ultra-15 Centrifugal Filter Unit, 10 000 MWCO. Cell pellets were frozen and whole-cell lysates were prepared by resuspending the cell pellet in 100 μl of PBS and boiling for 20 min. The culture filtrate proteins (CFP) were precipitated by the following protocol: 1 volume of sample was mixed with 3 volumes of methanol, 1 volume of chloroform, 4 volumes of water. Samples were centrifuged at max speed (~13 200 rpm) for 1 min. The upper phase was removed and replaced with 4 volumes of methanol and mixed briefly. Protein was pelleted at max speed for 15 min (Wessel and Flugge, 1984). Protein was resuspended in 100 μl of PBS and the final concentration was determined using Coomassie Plus Protein Determination Kit (Pierce) following standard protocol.

A total of 10 μg of CFP for each sample or 2 μl of cell lysate was loaded in each lane. Samples were added to SDS-loading buffer and heated to 80°C for 5 min. SDS-PAGE was performed under reducing conditions using the Mini-PROTEAN 3 electrophoresis system (Bio-Rad) with 12% polyacrylamide gels. Proteins were transferred to a polyvinylidene difluoride membrane. Membranes were blocked in PBS containing 2% BSA and 0.05% Tween 20, then probed with primary antibodies for 1 h at room temperature. Bound antibodies were recognized by HRP-labelled anti-mouse Ig. All antibodies were diluted in PBST with 1% BSA. Mouse monoclonal antibodies 1-5C (anti-MPB70) and MBS43 (anti-MPB83) (Wiker *et al.*, 1998) were used at a dilution of 1/500 and the HRP-conjugated anti-mouse antibody was used at a dilution of 1/10 000. Protein bands were detected using ECL Plus™ Western Blotting Detection Reagents (Amersham).

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