CHARACTERIZATION OF A *MYCOBACTERIUM TUBERCULOSIS RV3351C* MUTANT

by

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(Under the Direction of Frederick David Quinn)

ABSTRACT

The primary mode of transmission for *Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB) is through inhalation of aerosolized bacilli. It is well established that alveolar macrophages are important in the ultimate success or failure of an *M. tuberculosis* infection, however, for other lung pathogens, type II alveolar epithelial pneumocytes also pay a significant role in disease outcome. Our research demonstrated that within hours of an *M. tuberculosis* aerosol infection of mice and rabbits, intracellular mycobacteria were readily detected in type I and type II pneumocytes and alveolar macrophages. Concomitantly, two *M. tuberculosis* genes were examined whose products appear to interact exclusively with pneumocytes: *hbhA*, which encodes a heparin-binding hemagglutinin adhesin, and *Rv3351c*, which when deleted from an *M. tuberculosis* strain, produces significantly less pneumocyte killing compared with controls.

A recently identified hypervirulent clinical strain of *M. tuberculosis*, RD^{RIO}, was found to possess a 10-gene deletion including *Rv3351c*. However, deleting the entire region in *M. tuberculosis* strain Erdman did not produce a hypervirulent phenotype in type II pneumocytes, but instead showed reduced virulence similar to infections with ΔRv3351. In the lungs of aerosol-infected mice, ΔRv3351c replicated more slowly and disseminated less efficiently compared with the parent strain, the opposite of what has been reported with strain RD^{RIO} in humans. In examining transcription levels from in vitro-grown strain Erdman, genes comprising the RD^{RIO} deletion region did not demonstrate transcriptional overlap. These data indicate that *Rv3351c* may not be transcribed as part of an operon, at least *in vitro*. However, the four genes that constituted a putative *Rv3351c* operon were constitutively expressed. This suggests that the encoded gene products are available throughout the bacterial growth cycle and thus, may be available for the attachment process or early endocytosis by epithelial cells.

Mechanisms responsible for observed virulence differences among strain ΔRv3351c and the RD^{RIO} clinical strain have not yet been defined. An understanding of these processes *in vivo* could ultimately help define a potential new aspect of *M. tuberculosis* virulence and a more robust understanding of the role of epithelial cells in pulmonary tuberculosis.

INDEX WORDS: *Mycobacterium tuberculosis* strain Erdman, *M. tuberculosis* strain RD^{Rio}, alveolar type II pneumocytes, cellular necrosis, epithelial cell, early infection, A549 cell line, H441 cell line, J774A.1 cell line, mouse tuberculosis aerosol infection model, rabbit tuberculosis aerosol infection model, *Mycobacterium microti*, *M. tuberculosis* gene *Rv3351c, M. tuberculosis* gene *hbhA.*

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CHAPTER 1

INTRODUCTION

Mycobacterium tuberculosis, the causative agent of tuberculosis (TB), is estimated to reside primarily as a latent infection in approximately 2 billion people worldwide. Additionally, nearly 9 million cases of active TB occur each year with a majority of these cases located in impoverished nations. The World Health Organization estimates that 1.7 million deaths resulted from TB in 2007 [38, 39]. The highest number of deaths occurred in the sub-Saharan region of Africa where co-infection with HIV has fuelled the TB epidemic [20, 39].

The current understanding of the events that take place in the lungs after inhalation of *M. tuberculosis* bacilli are as follows: alveolar macrophages and dendritic cells phagocytose the inhaled bacilli and initiate an adaptive immune response either in the lung or draining lymph nodes [1, 5, 12, 21]. *In vitro* studies indicate that bacilli encountering activated macrophages are killed [2]. In contrast, bacilli encountering non-activated macrophages avoid destruction by blocking fusion of lysosomes with mycobacteria-containing phagosomes or possibly by escaping directly into the cytoplasm [5, 21, 28, 31, 40]. It is hypothesized that if the host innate immune response does not kill the inhaled bacilli, they will replicate within the non-activated macrophages and eventually escape to infect and replicate within subsequent waves of recruited macrophages. Typically, however, a signalling cascade is initiated by the first infected macrophages, and the recruited phagocytic cells will arrive already activated. T-cells may also be recruited, and the infection, ultimately, will be contained. However, if the initial infection is not controlled, a disseminated infection is the likely outcome [3].

Previous studies asserted that proper macrophage function and maturation are solely responsible for the control and elimination of *M. tuberculosis* bacilli after aerosol infection of the alveoli [21]. However, more recent studies have shown that *M. tuberculosis* infection and dissemination occur at a similar rate in mice deficient in macrophage function as compared to wild-type mice [8, 22]. Other bacterial pathogens, including *Legionella pneumophila* and *Yersinia pestis*, infect alveolar epithelial cells as part of the disease process. Taken together, these observations suggested that macrophages and dendritic cells are not the sole alveolar cell types of importance during early infection, and that *M. tuberculosis* bacilli can infect, survive and replicate within alveolar type II and potentially type I pneumocytes [1, 8, 22, 32, 36].

To date, most identified mycobacterial virulence genes have been associated with macrophage invasion and survival [3, 20, 21, 27, 28, 33, 34, 35, 37]. Only a handful of genes, virulence-associated or not, have been associated or presumed to be involved in the mycobacterial-epithelial cell interaction [4, 9, 22]. Since the publication of the complete genomic sequence of *M. tuberculosis* strain H37Rv [6], several potential virulence genes have been analysed for their contribution to the early infection process [20]. For example, in 1996, Menozzi and colleagues reported that the *M. tuberculosis* heparin-binding hemagglutinin (HbhA) could bind to epithelial cells but not to macrophages [29]. This was the first publication suggesting that *M. tuberculosis* may have evolved a mechanism to specifically target nonphagocytic cells. Upon deletion of the *hbhA* gene, the bacteria were found to be deficient in the ability to infect and survive within type II pneumocytes and disseminate from the lungs of aerosol-infected mice. This was an important finding since it is believed that the bacteria may use type II cells as a reservoir for immune evasion and dissemination to the blood. Other *M. tuberculosis* genes such as *esat6/cfp10* have also been identified that, upon deletion, lead to a decreased cytotoxicity phenotype in type II pneumocytes [5, 16, 22, 27, 31, 33]. The importance of *esat6/cfp10* as a virulence factor is well documented in the literature, and deletion of this gene is believed to be a major cause of the attenuated phenotype seen in the BCG vaccine strains [16, 31]. Adhesion and invasion of type II cells by this mutant is significantly reduced and necrosis is reduced by up to 50% during *in vitro* infections [22, 33].

In collaboration with C.H. King of Emory University, our laboratories published a series of papers describing the mechanism of killing of A549 human alveolar type II pneumocyte cells by *M. tuberculosis* as necrosis as opposed to macrophage apoptosis [8]. Anticipating the role of a specific *M. tuberculosis* gene(s) in the induction of type II cell necrosis, a transposon library was constructed for the purpose of detecting mutants impaired in this ability. In the assay, A549 human type II cell monolayers were infected and necrosis was measured spectrophotometrically by release of the lactose dehydrogenase (LDH) cytoplasmic enzyme. Several mutants producing varied reduced levels of LDH compared with the parent strain were identified. One transposon mutant in particular, targeting *Rv3351c,* reduced LDH release by up to 70% compared to controls. BLAST searches of *Rv3351c* against DNA and protein databases did not reveal significant homology to known genes or proteins. Other transposon mutants that produced a decreased LDH response on A549 cells included insertions in *hbhA* and *esat6*. Infections with these mutants resulted in decreased LDH releases of approximately 60% compared with controls. Although similar phenotypes were observed between *Rv3351c*::TN5370, *hbhA*::TN5370 and *esat6*::TN5370, it was hypothesized that the underlying mechanisms used by these mutants differed since preliminary data indicated that unlike *hbhA*::TN5370, *Rv3351c*::TN5370 and *esat6*::TN5370 still internalized in A549 cells [16, 22].

A recently identified hypervirulent clinical strain of *M. tuberculosis*, $\mathrm{RD^{RIO}}$, was found to possess a 26.3-kb deletion encompassing a putative four-gene *Rv3351c* operon and several flanking genes. This deletion was replicated in laboratory strain Erdman and assayed for virulence in epithelial cells. The four genes representing the putative *Rv3351c* operon are of particular interest. Determining if these genes are constitutively expressed or induced specifically in epithelial cells could ultimately help define a potential new aspect of *M. tuberculosis* virulence and a more robust understanding of the role of epithelial cells in pulmonary tuberculosis.

The work presented in this thesis describes survival of ΔRv3351c within various pneumocyte and macrophage cell lines and aerosol-infected animal models, and compares those results to similar experiments performed with ΔhbhA. Preliminary gene regulation experiments were also be performed on the putative *Rv3351c* operon as were initial assays on defining virulence associated with the large chromosomal deletion found in clinical strain $\mathrm{RD^{RIO}}$. Thus, detailed specific aims include:

Specific Aim1: Determine if *M. tuberculosis Rv3351c* is associated with survival specifically within human type II pneumocytes, similar to what has previously been determined for *hbhA*. Also, determine if *Rv3351c* is part of a four-gene operon. The *working hypothesis* is that the deletion of the *Rv3351c*, like that of *hbhA*, will negatively impact the ability of the mutant bacilli to survive within type II pneumocytes, but not within macrophages, and that *Rv3351c* is a critical gene within an operon.

Specific Aim 2: Determine if the *Rv3351c* deletion mutant is attenuated *in vivo*. The *working hypothesis* is that the *Rv3351c* deletion will significantly attenuate the bacterium in both the murine and rabbit infection models.

Specific Aim 3: Determine if deletion of the 26.3-kd region surrounding the *Rv3351c* gene is important for virulence in alveolar epithelial cells. The working hypothesis is that deletion of this entire region will lead to hypervirulence *in vitro* and *in vivo*. Studying this region of the *M. tuberculosis* genome will provide us a better understanding of the regulation of *Rv3351c* and the other important epithelial cell-specific virulence genes in the operon.

These specific aims were designed to evaluate the importance of the *M. tuberculosis Rv3351c* gene as a virulence factor and to examine its contribution to the infection process within alveolar type II pneumocytes during the initial stages of infection. This research will lead to a better general understanding of early *M. tuberculosis* infection and contribution of type II pneumocytes.

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CHAPTER 2

LITERATURE REVIEW

OVERVIEW OF *MYCOBACTERIUM TUBERCULOSIS*

Tuberculosis (TB), a multisystemic disease with a myriad of presentations and manifestations, is the most common cause of mortality worldwide due to a single infectious disease agent [107]. The World Health Organization estimates that two billion people are infected and that globally, the disease kills 1.7 million people annually. TB is most common in Africa, the West Pacific, and Eastern Europe. These regions are plagued by factors that contribute to the spread of TB, including the presence of limited resources, HIV infection, and multidrug-resistance.

Mycobacterium tuberculosis is the causative agent of TB. Infection is spread via bacilli-laden droplets and can cause an acute active disease or remain latent within the body for many years [4]. The cell wall of *M. tuberculosis* possesses a high lipid content composed primarily of mycolic acids. This cell wall structure is primarily responsible for observed resistance to disinfection and the acid-fast staining capability [96]. Routine detection of infection occurs via skin testing using purified protein derivative injected intradermally, or via blood testing using at least two bacterial antigens as immune stimulators. Both of these tests screen for immunological memory responses to *M. tuberculosis*-specific antigens [4, 73, 96]. If positive, chest X-rays, sputum staining, gamma-interferon release blood tests and culture are used to confirm the presence of an active infection [2, 73]. Multi-drug regimens for 6-9 months have proven very successful in the treatment of this infection [107].

HISTORY: PHARAOHS TO 2011

Significant archaeological evidence has shown that tuberculosis-like disease has been present within the human population for at least 6,000 years and may have been present in the animal population previous to its introduction to man [6, 18, 27, 28, 33, 79, 93]. References have been found in ancient Assyrian and Egyptian scrolls of patients being struck down by the gods with a curse causing them to cough up blood and have severe pains in the chest [33, 79]. Greek and Roman doctors such as Galen, Aristotle and Hippocrates wrote of patients with phthisis (Greek term for consumption) that caused them to waste away with severe chest pain and coughing. Various sources have lead researchers to believe that Indo-European cattle farmers may have been the initial carriers of the disease, spreading it to Europe and Asia during the migration of their herds [6, 42]. In the fifth century B.C., Hippocrates wrote about this disease and its devastating effects upon the population, although he believed it was an inherited disease and was not contagious [6]. By the time of Galen in the second century A.D., TB was well established in the population and the idea that it might be caused by an outside contagion was gaining popularity [27].

Over the centuries this disease has had a significant effect upon the development of art, literature and even architecture [18]. It has been linked to the defeat of the Roman Empire and may have been a contributing factor to the downfall of monarchies in many European countries. It has often been depicted as “the white death” due in part to the wasting and gaunt pallor that victims present during the end stages of active disease [41, 53]. It was not until 1882, when Robert Koch first isolated the tubercle bacilli in pure culture that progress could effectively begin to be made on defining this disease, and initial steps implemented towards prevention and control. With the overall improvement in public health beginning in the latter half of the 19th century, specific steps such as isolation in sanatoria and granuloma extraction surgeries aided significantly in the overall decline of the incidence of TB [41]. This trend continued and accelerated through the first half of the 20th century, primarily due to the discovery of antibiotics and to some extent the use of the BCG vaccine [2, 6, 34]. This positive trend, however, halted in the 1980s due to co-infection with the Human Immunodeficiency Virus (HIV) and funding lapses for public surveillance [2, 49]. With the implementation of directed drug therapy efforts for AIDS and TB, and the re-establishment of public surveillance programs in the developed world, rates of TB in these specific locations are again on the downward slope [107]. Unfortunately, due to the misuse of antibiotics, the lack of sufficient anti-AIDS medication programs and poor protection afforded by the BCG vaccine, the under-developed world has continued to experience an increase in the number of TB cases each year [49]. It is estimated that there is one TB related death every 15 seconds in the world, leading to approximately 2 million preventable deaths per year [78]. If nothing is done to reverse this trend, the number of deaths will likely continue to increase [2]. Clearly, *M. tuberculosis* infection continues to be a significant problem, and novel strategies must be developed to deal with this scourge [6, 27].

TAXONOMY OF *MYCOBACTERIUM TUBERCULOSIS*

M. tuberculosis is a member of the genus *Mycobacterium*, which like other actinomycetes has evolved from soil microbes to be able to live in mammals [42]. It has often been hypothesized that *Mycobacterium bovis* was the evolutionary precursor to *M. tuberculosis*, but genetic testing using single-nucleotide polymorphisms has shown that *M. tuberculosis* and the other members of the *M. tuberculosis* complex (*M. africanum, M. bovis* and *M. microti*) more than likely evolved from a common precursor [100]. All members of the *M. tuberculosis* complex show a greater than 99% DNA sequence similarity and more than likely evolved around the same time [42, 100].

PATHOPHYSIOLOGY AND EPIDEMIOLOGY OF *MYCOBACTERIUM TUBERCULOSIS*

Since the implementation of pasteurization, the most common method of infection with *M. tuberculosis* has been through inhalation of bacilli-laden aerosol droplets. The droplets must be of the correct diameter to successfully traverse to the lower recesses of the lungs. In animal models, an infectious dose is generally around 2-3 bacilli per droplet [4, 38, 40, 44, 69, 73, 85, 96]. Although infection rates vary to some extent with the *M. tuberculosis* strain, dosage, duration of contact, health of the exposed patient and perhaps host genetic susceptibility all contribute to the success of an infection. In some professions and populations within the United States, risk of exposure to infected individuals is elevated [2, 49, 73, 96]. In these instances, individuals are regularly screened for exposure to *M. tuberculosis* with the tuberculin skin test.

The standard recommended tuberculin test, known as the Mantoux test, is administered by injecting intradermally 0.1 mL containing 5 TU (tuberculin units) of purified protein derivative – an emulsion of *M. tuberculosis* cell wall material - into the top layers of skin on the forearm. Often within a few hours, but up to 72-hours post-injection, a positive result will present itself as an inflamed, sometimes painful, circular zone at the site of testing. The diameter of the inflamed raised zone and the presence of induration will assist in the determination of exposure to *M. tuberculosis*. Unfortunately, the test will not discriminate among active, latent or cleared infections, or if false-positive cross-reactions are occurring against the BCG vaccine or exposure to closely related *Mycobacterium* species [4, 73, 96]. Other considerations for interpretation include the immune status of children, the elderly, and patients who are severely immune compromised (such as those with HIV co-infection) [2, 6, 34, 100]. Positive results must be followed with either a second skin test using a different preparation of PPD, gamma-interferon release blood tests, or by other tests such as chest X-rays and sputum testing [73, 100]. Negative results can also be misinterpreted since six weeks is typically required after infection before a positive reaction for the PPD test or gamma-interferon release assay is detected [53, 100].

VACCINES, THERAPEUTICS AND DIAGNOSTICS

Prior to Robert Koch's discovery of the causative agent of TB, Herman Brehmer a Silesian botany student suffering from TB introduced the idea of the sanatorium [18, 100]. This provided the first modern step in the fight against the spread of TB. While working on his thesis in botany, he was instructed by his doctor to seek out a healthier climate. After traveling to the Himalayan Mountains and remaining for a number of years, he eventually returned home with only a latent case of TB. He subsequently gave up the study of botany and turned to medicine [27, 100]. In 1854, he presented his doctoral dissertation titled, "Tuberculosis is a Curable Disease". After receiving funds from various benefactors, he constructed an institution in the mountains of eastern Germany, now Poland, where patients were put under strict regiments of diet, rest and open air exposure [100]. This institute became the gold standard for subsequent sanatoria and helped change the way consumption was treated [27, 100]. By the early 1900s there were thousands of sanatoria, strategically located throughout the world. Since many of these institutions were located in mountainous or seaside regions, they provided a dual function: they kept the sick quarantined, while the enforced hospital regimen assisted the latency-development process [18, 100]. Additionally, while in sanatoria, many patients were used in researching treatments for this disease [18]. For example, the Italian physician Forlanini was able to show that collapsing the lung of patients tended to help the curative process. This treatment called an "artificial pneumothorax" was soon in wide use, as were other surgical methods, to reduce the lung volume and starve the organism of oxygen [18, 73, 100].

Ten years after the Brehmer Institute was opened, a French military doctor, Jean-Antoine Villemin performed experiments leading to the determination that TB could be passed from humans to cattle and from cattle to rabbits via lung tissues from diseased humans and animals [27, 41, 100]. On the basis of this experiment, he postulated that a specific microorganism must be the causative agent of TB and that it was not passed by hereditary or developed spontaneously [41]. Finally, in 1882, Robert Koch discovered a staining technique that enabled him to microscopically identify *M. tuberculosis* bacilli, and a culture technique that permitted him to grow the organism outside of a human or animal host [6, 41]. These two discoveries revolutionized the approach to diagnosing and studying *M. tuberculosis*, and would play a vital role in the development of the first generation of specific chemotherapeutics and vaccines.

In 1921, French bacteriologists Calmette and Guerin used a multiple-passage technique to attenuate a virulent strain of *Mycobacterium bovis*, the causative agent of TB in cattle and other ruminants. This attenuated strain was the basis for the BCG vaccine, and although it has been generally ineffective in the treatment of pulmonary tuberculosis in adults, BCG remains the most widely used vaccine in the world today, and is useful in the prevention of pediatric meningeal TB and the treatment of bladder cancer [49, 107].

The first true antibiotics were discovered in the 1920s. These first drugs, including penicillin, were used successfully but sporadically in the treatment of various bacterial diseases.

Unfortunately, none of the original drugs were useful against *M. tuberculosis*. In 1940, Selman A. Waksman along with his team at Rutgers University identified and purified actinomycin, the first effective anti-*M. tuberculosis* antibiotic. However, in clinical trials, this compound proved to be too toxic for use in humans or animals [100]. In 1943, streptomycin was isolated and shown to be effective against *M. tuberculosis* bacilli in animals with manageable toxicity levels [2, 6, 27, 53]. In a desperate attempt to save a critically ill TB patient, streptomycin was administered for the first time on November 20th, 1944 [27]. The patient recovered quickly and the bacteria disappeared from his sputum within days. However, in large doses, the new drug had some serious side effects such as hearing loss and organ malfunction. Also, the development of resistance to streptomycin occurred almost immediately after the drug's introduction. The hunt for newer and safer drugs quickly intensified [6, 27, 53].

In the 1950s, additional antibiotics were introduced for the treatment of TB including p-aminosalicylic acid, isoniazid, pyrazinamide and cycloserine, and in the 1960s, ethambutol and rifampicin [6, 53, 73, 96, 97]. Second-line drugs were also developed during this time. These antibiotics are typically somewhat less effective than the front-line choices but useful as alternatives against multi-drug or extremely-drug resistant strains of *M. tuberculosis*. These second-line drugs include capreomycin, viomycin, kanamycin, and amikacin, and the newer quinolones (e.g. moxifloxacin, levofloxin, ofloxacin, and ciprofloxacin) [2, 73]. When front-line and second-line drugs are used in combination, treatment programs such as D.O.T.S (Directly Observed Treatment Short-course) [107] are very effective.

With the available arsenal of drugs and DOTS therapy, TB could be effectively eliminated from the world today [107]. However, not considering financial, political and logistical issues, one of the most difficult problems associated with the treatment of TB is the requirement for 6-9 months of continuous daily multi-drug antibiotic therapy. Compliance problems due to unpleasant side effects, drug fatigue, and a general lack of understanding of antibiotic function by the patient have plagued TB eradication programs since their inception in the 1950s [2, 6, 73, 100, 107]. The current recommended course of treatment for active disease involves an initial daily combination of isoniazid, rifampin, pyrazinamide, and/or ethambutal for 2 months, followed by a daily treatment of isoniazid and rifampin for 5-10 months. If the drugs are taken as recommended, rapid clinical improvement and a significant decrease in disease transmission are typically observed within two weeks [107]. After one month, fever diminishes, and the patient generally feels well and stops losing weight. Coughing and chest congestion also decrease, as do the number and severity of lung lesions visible on chest X-rays [73]. Although bacteria may still be present in sputum smears, they will become more difficult to culture [96]. If noticeable improvements are not observed within the first two months, drug resistance may have developed or been present from the beginning. Re-evaluation of drug susceptibility patterns should be performed routinely. Patient compliance is the most complicated variable in the treatment process and must be monitored throughout the 6-9 month time-frame. To this end, many countries have had great success using programs such as DOTS, which reward patients for compliance [107].

If a patient with latent TB developed a reactivation case, there is a high probability that the infecting strain of *M. tuberculosis* will be drug resistant [73, 107]. In cases of reactivation TB, the rapid initiation of drug susceptibility assays is vital to the successful treatment of this disease [73].

STRUCTURE AND FUNCTION OF THE HUMAN LUNG

The function of the lung is to provide efficient gas exchange with the external environment while also providing barrier protection from chemical and infectious contaminants [12, 26, 32, 58, 61, 63, 65, 68, 70, 82, 83, 91]. The lung structure is divided into upper and lower airways. The upper airway consists of ciliated cells, some of which produce mucus, all of which are involved in capturing particulate matter, including infectious agents, before reaching the lower airway [82]. The lower airway consists of hundreds of small sacks referred to as pulmonary alveoli, spherical outcroppings of the respiratory bronchioles [10, 12]. With this structure, significantly more surface area is made available for gas exchange with the blood. This construction also dramatically improves lung capacity, but also provides significantly more area for infectious agents to target.

The lung alveolus consists of five major and several minor cell populations. The major populations include epithelial pneumocytes which constitute >99% of the alveolar cell surface, endothelial cells which line the capillary walls, and fibroblasts which are found within the fibrous basement membrane separating the epithelial and endothelial layers [68]. Minor populations include macrophages and dendritic cells associated with the alveolar surfaces and in the adjacent capillary beds, and migrating monocytes and lymphocytes often observed moving freely between the blood and epithelial surface [8, 12, 53, 68, 85, 100].

The cells that constitute the alveolar epithelium consist of two types: type I, and type II pneumocytes [12, 82, 112]. Both of these cell types possess distinctly different morphological and functional characteristics [12]. Type I cells represent a small percentage of overall alveolar cell number, however, due to their large flat and squamous morphology they constitute a majority of the surface area. Functionally, these cells are involved in gas exchange [12, 98].

Type II cells are more numerous but columnar in shape and thus cover less surface area. These cells are involved in surfactant production, a fluid that provides a barrier against the entry of infectious agents and other particulates into the blood, and provides lubrication for the expansion and contraction of lung tissues during respiration [12, 94]. Few pathogens appear to directly interact with type I cells, however, several important lung bacterial pathogens including *Streptococcus pneumoniae, Legionella pneumophila, Pseudomonas aeruginosa* and *Yersinia pestis* (pneumonic form) have been shown to target type II cells. It is hypothesized that these cells are a site for intracellular replication and are used by these pathogens as a portal for gaining access to the bloodstream of the host [13, 19, 25, 45, 56, 60, 64, 80, 95]. During an infection, type II cells can terminally differentiate into type I cells to maintain an intact epithelial barrier, and secrete cytokines and chemokines to recruit macrophages and T-cells [8, 10, 12, 26, 32, 85, 94].

HOST IMMUNE RESPONSE TO MYCOBACTERIAL INFECTION

The mammalian host response to *M. tuberculosis* aerosol challenge can be divided into three stages [38, 97, 100, 106]. The first stage begins within hours or a few days after inhalation of the bacilli [38]. In animal models, this stage lasts approximately three weeks and will be discussed in detail in the subsequent section. The second stage lasts 3-12 weeks after inhalation, with recruitment of macrophages and T-cells to the foci of infection and measurable dissemination of bacilli from the lungs through the lymphatic circulation to regional lymph nodes [3, 8, 12, 14, 20, 55]. These first two stages are often asymptomatic and the only indicating factor is patient conversion to skin test positive. The third stage is often the most obvious and can result in death if left untreated. This stage lasts 3-7 months and is marked by dissemination of the bacilli from the lungs to many organs, including the liver, kidneys, spleen, and back to the upper lungs [4, 12, 109]. Occasionally, the bacilli migrate to the brain, causing acute and often fatal meningitis [96]. During this stage, massive amounts of inflammation occur on the pleural surfaces of the lungs causing severe chest pain as well as coughing and wheezing. This inflammation is thought to occur because secreted bacterial components interact with CD4 T lymphocytes that proliferate and release inflammatory cytokines [29].

Alternatively, the infection can be contained and the bacilli remain dormant (or latent) for many years [102]. This is the most common form of the infection worldwide [107]. Individuals with latent infections have a 3-5% chance of reactivating and presenting an acute infection. The most common reason for reactivation is immune system suppression, typically caused by co-infection with HIV or steroid therapy [2]. During this third “inflammatory stage” of disease, significant amounts of host cell death occur in the lungs allowing for cavitation and a general tissue necrosis of the protective barriers [4, 40, 69]. There are two primary pathways for host cell death that can result upon *M. tuberculosis* infection. Macrophages are preferentially killed by apoptosis [7]. Epithelial and endothelial cells are killed primarily by necrosis [26, 32]. Other cell types have not been extensively examined for their activity during the infection process.

Apoptosis, often referred to as programmed cell death, occurs naturally in mammalian cells but can be induced prematurely by some microbes or can be prevented from occurring by others [39, 56]. It is characterized by condensation of the chromatin, DNA cleavage and fragmentation, and plasma membrane blebbing, all leading to phagocytosis of the cell by circulating activated macrophages without inducing an inflammatory response [83]. When apoptosis is initiated in activated macrophages through mycobacterial infection, mitochondrial permeability is increased and cytochrome c is released. This release subsequently induces Apaf-1 (apoptosis activating factor-1), which activates the caspase cascade via caspase-9, and ultimately leads to cell death [89, 94]. Apoptosis of infected resting macrophages is likely part of the immune defence mechanism to prevent dissemination of a localized bacterial infection [8, 112]. However, *M. tuberculosis* has been shown to have the ability to control apoptotic induction within resting macrophages, thereby promoting intracellular survival, immune evasion and potentially systemic dissemination [10, 11, 39].

Necrosis is defined as cell death due to injury, intoxication or severe membrane perturbation [26, 32]. During acute *M. tuberculosis* infection, tissue necrosis results in cell membrane permeation and release of cytoplasmic contents ultimately inducing inflammation and further lung tissue destruction [26, 32, 63, 69]. This process is less orderly than apoptosis but as our laboratory previously demonstrated, it is the standard method of epithelial cell killing by *M. tuberculosis* bacilli *in vitro* [32]. Our laboratory also demonstrated that *M. tuberculosis* bacilli can spread cell-to-cell in epithelial cell monolayers with concomitant necrosis of the infected cells leaving a plaque-like area of clearance [88, 89]. The role for mycobacterial-induced necrosis of primary epithelial cells and “plaque formation” during human or animal infection has yet to be determined.

HOST-PATHOGEN INTERACTION DURING EARLY INFECTION

The first alveolar cell type to interact with inhaled *M. tuberculosis* bacilli has been hypothesized to be the alveolar macrophage. Dendritic cells may also play an important role upon initial infection due to their antigen presenting capabilities and their ability to migrate from the site of infection [100]. More recently, it has been demonstrated that the bacilli also may interact with alveolar pneumocytes. As with other respiratory pathogens, it is hypothesized that this interaction can be used by *M. tuberculosis* as a method of immune evasion or location for enhanced bacterial replication and access to the bloodstream through a damaged epithelial cell lining [3, 8, 10, 11, 12, 14, 26, 29, 32, 38, 55, 63, 69, 85, 94, 100, 112]. Although investigators, including our group, have demonstrated *M. tuberculosis*-pneumocyte interactions *in vitro*, little is known about these interactions *in vivo*.

A significant portion of published host cell-*M. tuberculosis* literature is dedicated to the interaction with macrophages [8, 10, 32]. Upon contact between the bacterium and macrophage mannose and complement receptors, phagocytosis is initiated [12, 44, 59, 99]. Binding and uptake can be upregulated by the presence of the glycoprotein, surfactant protein A. This protein is generally found on alveolar surfaces and unlike surfactant protein D has a positive effect on phagocytosis [100]. The human toll-like receptors 2 (TLR2) and 4 (TLR4) also play an important role in this initial binding and phagocytic process [7, 104]. Once phagocytosis occurs, the bacillus usually resides within and traffics through the phagosome. *M. tuberculosis* possesses the ability to arrest phagosome-lysosome fusion and prevent exposure to acidic pH, reactive oxygen intermediates, lysosomal enzymes and toxic peptides, all detrimental to the survival of *M. tuberculosis* bacilli [17, 44, 99]. Like other pathogens such as *Shigella flexneri, Yersinia pestis, Listeria monocytogenes* and *Legionella pneumophila* that can prevent phago-lysosome fusion, *M. tuberculosis* bacilli use this phagosomal vesicle to mature, multiply and avoid immune surveillance [19, 24, 36, 37, 46, 48, 56, 60, 64, 71, 74, 75, 76, 81, 100]. Although the exact mechanism by which *M. tuberculosis* bacilli block this maturation process has not yet been determined, it is suspected that recruitment of Rab proteins such as Rab 5 and Rab 7 as well as a mechanism for decreasing Ca^{2+} levels may be important for that process [39, 55, 100, 103].

VIRULENCE FACTORS AFFECTING THE COURSE OF DISEASE

Recent technical advancements in genetics, immunology, microscopy, animal models and many other disciplines have provided an unprecedented opportunity to identify and define *M. tuberculosis* virulence mechanisms that are used constitutively or intermittently throughout the course of the disease. Many of the virulence genes that have been identified thus far focus on the interaction between the bacilli and macrophages [11, 20, 26, 38, 50, 55, 100]. The gene products are generally divided into four groups involved in bacterial secretion, envelope formation and function, cellular metabolism and transcriptional regulation. Many of these factors, such as Esat6/CFP10 and FbpA, have been examined as possible enhancements to the current BCG vaccine, or deletions as part of an attenuated *M. tuberculosis* vaccine strain [1, 15, 17, 39, 51, 66, 100, 109, 110]. However, few genes have been identified that specifically target interactions with epithelial cells. *M. tuberculosis hbhA* transcribes a protein that affects the ability of *M. tuberculosis* bacilli to enter and survive within type II pneumocytes *in vitro* and *in vivo* [1, 16, 31, 35, 72, 86, 100]. Other pneumocyte-specific virulence genes are currently being studied. One in particular, *Rv3351c*, was identified as a possible virulence factor during a wide transposon-based screen in A549 cells [58]. This gene encodes a product of unknown function. Although disruption of this gene seems to have a detrimental effect upon the ability of bacteria to induce necrosis in epithelial cell lines, various clinical isolates, such as the RD^{RIO} strain, lacking genes in this region have shown an increased virulence in human populations [105]. This gene is the primary focus of the studies set forth in this thesis.

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CHAPTER 3

THE *MYCOBACTERIUM TUBERCULOSIS RV3351C* GENE IS ASSOCIATED WITH ALVEOLAR EPITHELIAL CELL PATHOGENESIS

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ABSTRACT

Upon inhalation, it is hypothesized that most *Mycobacterium tuberculosis* bacilli are engulfed by patrolling alveolar phagocytes. However, Mycobacterial factors with roles in accessing alveolar epithelial cells (AECs) are being discovered. The *hbhA* gene product, the heparin-binding hemagglutinin adhesin, is known to bind sulphated glycoconjugates abundant on the surface of AECs but not on phagocytes. In this report, we identify another gene involved in mycobacterial utilization of AECs, *Rv3351c*. A *M. tuberculosis Rv3351c* mutant (ΔRv3351c) exhibited attenuation following infection of AECs compared to either the parental strain *M. tuberculosis* Erdman or the complemented mutant (CMΔRv3351c). Infection of A549 AECs with ΔRv3351c bacilli resulted in significantly reduced leakage of host cytoplasmic lactate dehydrogenase (LDH) into culture medium over time compared to infections with CMΔRv3351c or Erdman. The LDH released following ΔRv3351c infection in A549 host cells was comparable to that observed for infections with an Erdman *hbhA* mutant (ΔhbhA). Neither mutant was attenuated in macrophages. Reduced pathogenesis in AECs by either mutant was confirmed by observations of increased host cell resistance to trypan blue penetration and by reduced intracellular bacterial loads when compared to infections with parent strain Erdman or complemented mutants. Electron microscopy of AECs infected with ΔRv3351c, ΔhbhA, or strain Erdman after 96 hours revealed host cell necrosis without hallmarks of apoptosis. This indicates that loss of *Rv3351c* or *hbhA* delays mycobacterial killing of AECs but not the fundamental mechanism by which death occurs. The phenotypic similarities between the *Rv3351c* and *hbhA* mutants suggest that *Rv3351c* is also an important factor in alveolar epithelial cell infection.

INTRODUCTION

Mycobacterium tuberculosis is an intracellular pathogen that typically establishes infection after inhalation of the bacilli into the lung alveoli [5, 20]. This bacterium remains a leading cause of morbidity and mortality; 9.4 million new cases of tuberculosis (TB) and 1.8 million deaths were estimated for 2008 [44]. Although much is known of the human immune response during later stages of disease, little is known of the interactions between the bacterium and host cells during the first hours or days after infection, where it is thought that less than 100 organisms are sufficient to establish infection [16, 20, 37]. Early interactions of *M. tuberculosis* bacilli with alveolar epithelial cells (AECs) and the contribution of these cells to the initial infection and ultimate dissemination of *M. tuberculosis* bacilli from the lungs are poorly defined [38].

The alveolar epithelium consists primarily of large flat type I pneumocytes which cover a majority of the alveolar surface area, and smaller cuboidal type II pneumocytes which account for 80% of the alveolar cells [5]. Type I cells are terminally-differentiated and function in gas exchange, while the primary functions of type II cells are to secrete surfactants to prevent alveolar collapse during exhalation and to differentiate into type I cells to replace damaged cells [5, 7, 38, 57]. The lung epithelium has been shown to play a significant role in the outcome of various respiratory diseases due to the nature of the interactions and immunological responses generated. Several bacterial pathogens including *Streptococcus pneumoniae*, *Legionella pneumophila*, *Yersinia pestis*, *Chlamydia pneumoniae*, *Staphylococcus aureus*, *Bacillus anthracis* and *Haemophilus influenzae* have been reported to invade and replicate within a variety of lung-derived non-professional phagocytes including fibroblasts, endothelial, and epithelial cells *in vitro* and *in vivo* [8, 14, 15, 22, 27, 31, 38, 41, 45, 54, 56]. It has also been hypothesized that anti-bacterial chemotherapy may fail to eradicate bacteria within epithelial cells causing such cells to be reservoirs for chronic or persistent infections [2, 13].

Regardless of the outcome of an epithelial cell infection, physiological mediators including cytokines are known to be secreted and released. For example, infection of the lung epithelium by *C. pneumoniae* bacilli triggers secretion of IL-6, IL-8 and plasminogen activator inhibitor-1, resulting in recruitment of inflammatory cells and increased procoagulant activity [18, 28, 42]. It has also been demonstrated that epithelial cells infected with *P. aeruginosa* bacilli can augment the expression of molecules that promote adherence and migration of lymphocytes and transendothelial migration of phagocytic cells [30].

Previous work demonstrates that *M. tuberculosis* bacilli replicate more rapidly in cultured type II human AECs than in human macrophages, and that AECs exhibit necrotic death rather than apoptosis as observed in macrophages [1, 4, 17, 20, 37, 55]. However, internalization of *M. tuberculosis* bacilli into human AECs was reported to be slower than that observed in murine or human macrophages derived from peripheral blood [20, 38]; this observation is consistent with the barrier function of epithelial cells and the phagocytic nature of macrophages. The heparin-binding hemagglutinin adhesion (HbhA) from *M. tuberculosis* was shown to bind epithelial cells but not macrophages; furthermore, epithelial-cell binding could be blocked by the addition of sulfated carbohydrates or antibodies to HbhA [40]. Deletion of *hbhA* reduced bacterial dissemination from the lungs to other organs [51].

In the present study, we identify a second *M. tuberculosis* gene involved in targeting AECs. We observed that a *M. tuberculosis* mutant lacking the *Rv3351c* gene exhibited attenuated phenotypes similar to those with an *hbhA* mutant in type II pneumocytes but not macrophages.

These findings suggest that *Rv3351c* either functions in conjunction with HbhA or represents a novel system in *M. tuberculosis* to utilize AECs as hosts.

MATERIALS AND METHODS

CELL CULTURE

A549 human type II alveolar epithelial cells were obtained from ATCC (CCL-185) and maintained in EMEM (Eagle's Minimum Essential Medium; Gibco) with 5% FBS [38]. H441 human type II alveolar epithelial cells were obtained from ATCC (HTB-174) and maintained in RPMI 1640 (Gibco) with 15% FBS [11]. J774A.1 murine macrophages were obtained from ATCC (TIB-67) and maintained in DMEM (Dulbecco's Modified Eagle Medium; Gibco) with 10% FBS [53]. During viable count, lactate dehydrogenase (LDH) and trypan blue assays, cells were seeded at 1 x 10^6 cells/ml in either 6- or 24-well Costar® dishes and allowed to adhere overnight. Media were changed prior to experimentation. For transmission electron microscopy (TEM), cells were harvested and seeded into T25 flasks at a density of 5 x 10^6 cells/ml. For all microscopy experiments, the cell cultures were synchronized by incubation at 4°C: 1 hr after seeding and 1 hr post infection (pi).

BACTERIAL STRAINS

Mycobacterium tuberculosis strains Erdman and derivatives were cultured in Middlebrook 7H9 broth supplemented with 0.05% Tween 80, 0.5% glycerol and 10% ADS or plated on 7H10 agar supplemented with 10% ADS, 0.5% glycerol and 0.05% Tween 80 (TH9tgADS)[9]. *M. tuberculosis* Erdman mutants were generated by deleting either *Rv3351c* or *hbhA* and replacing with a hygromycin resistance cassette using published methods [3, 9, 33]. The strain ΔRv3351c was constructed as follows: chromosomal regions flanking *Rv3351c* were amplified from genomic DNA using primers KpnI-683upRv3351c-F and XbaI-1upRv3351c-R for the upstream region and primers HindIII-Rv3351c877-F and SpeI-624downRv3351c-R for the downstream region. The sequences of these primers and all primers used in this study can be found in Table 1. The upstream and downstream regions were digested with *Kpn*I/*Xba*I and *Hin*dIII/*Spe*I, respectively, and cloned sequentially into plasmid pYUB854. The resulting plasmid was digested with *Pac*I and concatenated with *Pac*I-digested phasmid phAE87, inserted into bacteriophage lambda heads using Gigapack III Gold Packaging Extract (Stratagene, Inc.), and transfected using the manufacturer's protocol into *E. coli* strain HB101. The resulting cosmid DNA was isolated and electroporated into *Mycobacterium smegmatis* strain mc^{2}155 which was then cultured at 30°C on a soft agar overlay, allowing amplification of the phage and plaque formation. Phages were amplified in *M. smegmatis* before transfection of *M. tuberculosis* strain Erdman and plating on 7H10 agar supplemented with 10% ADS and hygromycin (50 µg/ml). Primers HindIII410upRv3353c (which anneals to the chromosome upstream of the *Rv3351c* upstream-flanking region) and hyg30-R (which anneals within the hygromycin cassette) were used to screen candidates for replacement of *Rv3351c* with *hyg*. Confirmation of the *Rv3351c* mutant was obtained by Southern blotting using a probe that hybridizes upstream of *Rv3351c*. The probe template was generated with primers 450upRv3351cF and 24upRv3353cR.

The ΔRv3351c-complementing strain (CMΔRv3351c) was constructed by amplifying the region from 338 bp upstream to 14 bp downstream of *Rv3351c* from *M. tuberculosis* strain H37Rv with primers HindIII338upRv3351cF and EcoRI-14downRv3351c-R and insertion into the chromosome-integrating plasmid pMV306 digested with *Hin*dIII and *Eco*RI. The resulting plasmid was electroporated into the ΔRv3351c strain and maintained by selecting for resistance to kanamycin (Braunstein *et al*., 2002). Complementation was confirmed via PCR.

For construction of the ΔhbhA deletion strain, *hbhA*-flanking regions were amplified from genomic DNA using upstream primers 875uphbhA-F and 27uphbhA-R and downstream primers hbhA572-F and 834downhbhA-R. Each product was TA cloned into plasmid pCR2.1topo. The upstream and downstream regions were then excised using enzyme pairs *Kpn*I/*Xba*I and *Nco*I/*Avr*II, respectively, and sequentially cloned into pYUB854 digested with the same enzymes. The resulting plasmid was concatenated with phAE87 and used to construct ΔhbhA using similar methodology as described for strain ΔRv3351c. Homologous replacement was determined by colony PCR using primer 902uphbhA (which anneals to the chromosome upstream of the *hbhA* upstream-flanking region) and hyg30-R to detect replacement of *hbhA* by the hygromycin cassette, and with internal *hbhA* primers hbhA78F and hbhA517R to confirm loss of the gene in the ΔhbhA mutant. Results were confirmed by Southern blotting with a probe to a 549 bp region upstream of *hbhA* amplified with primers 1625upHbhA-F and 1076upHbhA-R.

The complemented ΔhbhA strain, CMΔhbhA, was constructed by amplifying the *hbhA* region from *M. tuberculosis* strain H37Rv using primers Comp.hbhA-forward-SalI and Comp.hbhA-reverse-SalI and inserting the fragment into plasmid pMV306 digested with *Sal*I. The resulting plasmid was electroporated into the ΔhbhA strain selecting for resistance to kanamycin. Complementation was confirmed via PCR.

Bacterial strains were grown in 7H9gtADS at 37°C / 5% CO_2 with occasional shaking to an OD_{600} of 1.0. Cultures were mixed by vortexing and dispensed as frozen aliquots. For infection of host cells, thawed bacterial stocks were transferred to fresh tubes, vortexed vigorously for several minutes, and a microscopic bacterial count was performed using a Petroff-Hausser counting chamber. The bacteria were then diluted to equivalent densities in PBS and passed once through a tuberculin syringe directly into culture wells. Viable counts were determined by serial dilution in 0.05% Tween 80, plating on 7H10tgADS, and incubation at 37°C for 3 weeks.

Growth curves were performed with all mycobacterial strains used in this study. Bacteria were cultured in 7H9gtADS broth at 37^{o} C in 5% CO_2 with occasional shaking. Optical density (OD_{600nm}) was measured in triplicate at each time point and colony forming units (CFUs) determined by serial dilution plating.

SOUTHERN ANALYSES

For *Rv3351c* analysis, genomic DNA was digested with *Avr*II and *Ear*I enzymes. For *hbhA* analysis, genomic DNA was digested with *Pml*I. The resulting DNA fragments were resolved on a 1.0% agarose gel. The DNA fragments were transferred to a positively-charged Nylon membrane (GE Healthcare Systems) as described in the manufacturer's instructions. Following transfer, the DNA was UV-crosslinked to the membrane using a Stratalinker. Probes were prepared by PCR amplification of sequences upstream of the target gene region or internally within the hygromycin resistance gene. The Chemiluminescent Nucleic Acid Detection kit was used for probe labelling following manufacturer’s instructions (Thermo-Fisher). Visualization was performed using autoradiography.

BACTERIAL CELL VIABILITY

A549, H441 and J774A.1 cells were infected at an MOI of 100 for 6 hr as described above. Monolayers were washed 3 times prior to the addition of fresh medium containing 200 µg/ml amikacin and incubated at 37^{o}C for 2 hr. Culture medium was then replaced with medium without antibiotic. This was considered the zero time point. To count intracellular bacteria, at the indicated time point monolayers were washed 3 times with PBS, lysed in 0.1% Triton X-100, and the mixture serially-diluted in 0.05% Tween 80 and plated on 7H11 agar supplemented with 0.5% glycerol, 0.05% Tween 80, and 10% ADS. Bacterial colonies were counted after 3-week incubation at 37°C. Assays for most strains were performed in triplicate and repeated twice. CMΔhbhA was assayed once in triplicate.

LACTATE DEHYDROGENASE (LDH) ASSAYS

A549, H441 and J774A.1 cells were seeded at 1.0 x 10^{6} cells/ml in 6-well Costar® dishes and allowed to adhere overnight. Media were changed prior to experimentation. Cells were infected with the indicated *M. tuberculosis* strains at an MOI = 100. Culture supernatants were removed at the indicated time points and passed through 0.22 µm PVDF filters prior to storage at 4°C and assay using the Cytotoxicity Detection Kit (Roche, Inc.)[20]. Percent LDH release was calculated by the following formula: [(Release from Strain - Background)/(Max release – Background)] x 100. Max release refers to lysis obtained by incubation with 0.1% Triton X-100 for 15 minutes. Due to the intrinsic resistance of pneumocytes to the detergent, 100% lysis of the monolayer did not always occur during that time frame. All infections were performed in triplicate and repeated twice.

TRYPAN BLUE HOST CELL VIABILITY ASSAY

A549 and H441 cell monolayers were infected at an MOI of 100 as described earlier. At the specified time points, culture media were removed and stored on ice. Monolayers were then treated with 0.25% trypsin (Gibco) for 15 min at 37°C. An aliquot of the suspended cells was mixed with an equal volume of 0.4% trypan blue (Invitrogen) prior to microscopic enumeration with a hemocytometer. Numbers of viable and dead host cells in the spent culture medium were also counted. Cell viability in each sample was calculated as the percentage of unstained cells from the culture medium and from the monolayers relative to all stained and unstained cells. Infection studies for most strains were performed in triplicate and the experiments were repeated at least once. Assays with CMΔhbhA were performed once in duplicate.

TRANSMISSION ELECTRON MICROSCOPY

Glutaraldehyde-fixed infected and control cell culture specimens were treated with 1% osmium tetroxide for 45 min after being scraped and pelleted. An ethanol series was used to dehydrate the specimens prior to infiltration using a solution of propylene oxide: resin (Epon-araldite) at three different ratios (2:1, 1:1, and 1:2) as described [43]. Specimens were incubated 1 hr in each resin mixture followed by an exchange and overnight rotation at room temperature. After an additional resin exchange, samples were embedded and polymerized overnight at 60°C. Staining was performed using 4% uranyl acetate and lead citrate. A Tecnai BioTwin (FEI Company, Hillsboro, OR) electron microscope operating at 80 or 120 kV was used to image the specimens. Digital images were captured using a 2K x 2K camera (AMT, Danvers, MA) and edited using Microsoft® Picture Manager and Adobe® Photoshop 7.0.

ZYMOGRAPHY

Matrix metalloproteinase activities in culture supernatants of uninfected and infected A549 and H441 cells were analyzed by polyacrylamide gel zymography. Each cell culture supernatant was mixed with an equal volume of 3x sample buffer (12% SDS, 15% glycerol, 30 mM Tris-HCl pH 6.8, 0.03% bromo-phenol blue) and 20 µl samples separated on 10% polyacrylamide gels containing gelatin ([1 mg/ml], porcine skin, 300g Bloom, Sigma). The gels were then immersed in Tris-buffered saline (TBS) containing 2.5% Triton X-100 for 1 hr at 25^{o}C prior to immersion in TBS containing 10 mM $CaCl_2$ for 16 hr at 37^{o}C. The gels were stained with 0.5% Coomassie blue R-250 in water/methanol/acetic acid (45:45:10) and destained with 50% methanol/1% acetic acid. The gels were dried and bands measured using a densitometer.

TRANSCRIPTION STUDIES

M. tuberculosis strain Erdman was cultured to an OD_{600} = 1.0 in 7H9tgADS. Cells were harvested and RNA isolated using the method of Voskuil [59]. For qRT-PCR studies, cDNA was synthesized from 400 ng total RNA using ImProm-II reverse-transcriptase (Promega Corp.). Titrated amounts of cDNA were used in Fast SYBR Green real-time PCR assays (Applied Biosystems) with region-specific primer pairs. Primers were selected using PrimeTime qPCR program (Integrated DNA Technologies, Inc.). PCR parameters were as follows: 95°C 2 min, followed by 40 cycles of 95 °C 15s, 65°C 30s, 72°C 25s. Fluorescence was measured during the annealing step using an iCycler (BioRad). Transcript copy numbers were determined by comparison of the average cycle (C_t) threshold to a standard curve of C_t values generated from assays with titrated amounts of genomic DNA using the same region-specific PCR primer pairs. The *sigA* gene encoding the primary sigma factor was examined in parallel. Assays were performed in triplicate.

STATISTICAL ANALYSIS

The unpaired t-test and one-way ANOVA were used for analysis of statistical significance with GraphPad Prism version 5 software (GraphPad Software, San Diego, CA, USA). A P-value of $\leq$0.05 was considered statistically significant.

RESULTS

EXAMINATION OF A GENETIC LOCUS OF *M. TUBERCULOSIS* GENES EVOLUTIONARILY LINKED TO *RV0063*

To help discern the function of the PPE family of very large proline/glutamate rich proteins, an unstudied region of the *M. tuberculosis* genome containing a potential operon consisting of the *Rv3353c*, *Rv3352c*, *Rv3351c*, and *PPE56* genes was examined (Figure 3.1A). The *Rv3353c*, *Rv3352c*, and *Rv3351c* genes appear to have evolved from the *Rv0063* gene; the entirety of the first two encoded gene products and the amino terminal 190 amino acids of Rv3351c have high sequence identity (53%, 72%, 51%, respectively) to regions of Rv0063 (amino acids 2-91, 141-263, and 292-479, respectively, Figure 1A). No specific functions have been ascribed to Rv0063, Rv3353c, Rv3352c, or Rv3351c, however, BLAST analysis indicated limited similarity of portions of Rv0063 to oxidoreductases and FAD-binding proteins. DNA sequence analysis suggested the possibility of an 80 aa polypeptide encoded between *Rv3351c* and the downstream *PPE56* gene if a +1 frame-shift mutation occurred at the initiation codon of the PPE56 gene (Figure 3.1B).

To test for possible transcription linkage of the *Rv3353* to *PPE56* region, quantitative RT-PCR analysis was performed with cDNA generated from RNA isolated from *M. tuberculosis* strain Erdman cultured to A_{600}=1.0. Primer sets that amplify within each gene and across the

Rv3353c-Rv3352c, *Rv3352c-Rv3351c*, and *Rv3351c-PPE56* junctions were utilized. Total transcript copy numbers per 10 ng starting RNA were determined to be 2700 for *Rv3353c*, 758 for *Rv3352c*, 6170 for *Rv3351c*, and 357 for *PPE56*. For the intergenic regions, 233 transcript copies were obtained for *Rv3353c-Rv3352c*, 140 for *Rv3351c-PPE56*, and 0 for *Rv3352c-Rv3351c*. As copy numbers vary several-fold between adjacent genes, the data suggest that none of these genes are transcriptionally linked under the growth condition tested. For comparison, primers specific to the housekeeping sigma factor gene, *sigA*, resulted in 11,000 copies per 10 ng starting RNA. This value is low compared with published values (4 x 10^5/10 ng RNA) measured using molecular beacons [35]. Additional studies will be performed with increased denaturation time and temperature to optimize assay conditions. The lack of transcriptional linkage between *Rv3351c* and PPE56 in vitro does not preclude the possibility that transcription of these genes may be coupled *in vivo*.

AN *RV3351C* MUTANT GROWS NORMALLY *IN VITRO* AND IN MACROPHAGES, BUT EXHIBITS REDUCED REPLICATION IN A549 HUMAN ALVEOLAR EPITHELIAL CELLS

An internal deletion mutant (ΔRv3351c) of the *Rv3351c* gene was generated in *M. tuberculosis* strain Erdman as described in Methods and confirmed by RT-PCR (Figure 3.2) and Southern analysis (Supplemental Figure 3.1). *In vitro* growth rates of ΔRv3351c in Middlebrook 7H9 medium were not impaired relative to the complemented mutant (CMΔRv3351c) or strain Erdman (Figure 3.3). To determine if Rv3351c was required for bacterial survival in host cells, infections of macrophages (J774.A1) and alveolar epithelial cell lines (A549 and H441) were examined. Following macrophage infection and treatment with amikacin to kill extracellular bacteria, no significant differences in numbers of intracellular viable bacilli were observed among strains ΔRv3351c, CMΔRv3351c, or Erdman (Figure 3.4C). In contrast, infections of A549 cells revealed reduced survival of ΔRv3351c compared to the complemented mutant or strain Erdman (Figure 3.4). At 72 hr pi, the number of viable mutant bacilli (1.5 x 10^7 ΔRv3351c) was approximately half of that observed for parent strain Erdman (3.3 x 10^7) or the complemented *Rv3351c,* Figure 3.4A). Reduced survival of the mutant was also observed following infection of H441 cells, although differences were not statistically significant until 120 hr pi, when 1.7 x 10^7 ΔRv3351c and 2.9 x 10^7 viable Erdman bacilli were counted (Figure 3.4B).

An *hbhA* deletion mutation (ΔhbhA) was developed in strain Erdman because the encoded protein was previously characterized as an adhesin for glyco-conjugates present on the surface of alveolar epithelial cells [51]. ΔhbhA grows somewhat slower than the complemented mutant (CMΔhbhA) *in vitro* at higher cell densities, but this may be the result of enhanced bacterial clumping (Figure 3.3). Bacterial survival of ΔhbhA following infections of macrophages or type II pneumocytes are similar to those observed for ΔRv3351c (Figure 4). It is unclear if the delayed killing of either ΔhbhA or ΔRv3351c bacilli in H441 relative to A549 cells is due in part to enhanced surfactant production which may slow the rate of attachment and internalization of H441 cells.

To compare the efficiency of host cell lysis following infection with the different *M. tuberculosis* strains, release of host cytoplasmic enzyme lactate dehydrogenase (LDH) into the culture medium was monitored. Release of LDH is expected in cells in late stage apoptosis or following cellular necrosis. A549 and H441 cells infected with strain ΔRv3351c or ΔhbhA released less LDH over time than cells infected with Erdman, CMΔRv3351c or CMΔhbhA (Figure 3.5A and B). Infection of A549 cells demonstrated significant differences between mutants and parent as early as 48 hr pi (Figure 5A). At 72 hr following infection of A549 host cells, cells infected with deletion mutants ΔRv3351c or ΔhbhA resulted in significantly lower LDH release (42% and 29%, respectively) compared to LDH released from cells infected with strain Erdman (approaching 100%, Figure 3.5A). In H441 host cells, significant reductions in LDH release by ΔRv3351c or ΔhbhA infection relative to strain Erdman were not observed until 120 hr pi (Figure 3.5B). In J774.A1 macrophages, no significant differences in LDH release were observed following infections with the same *M. tuberculosis* strains (Figure 3.5C).

Trypan blue exclusion was used to confirm attenuation of ΔRv3351c and ΔhbhA in type II pneumocytes. A549 cells infected with ΔRv3351c or ΔhbhA bacilli for 120 hours resulted in staining of 13% and 10% of the cells, respectively, versus 51% staining with strain Erdman, 41% with the *Rv3351c* complement, and 46% with the *hbhA* complement (Figure 3.6A). Trypan blue staining of infected H441 cells also demonstrated attenuation of ΔRv3351c or ΔhbhA at 120 hr pi; only 14% and 13% staining was observed by cells infected with ΔRv3351c and ΔhbhA, respectively, versus 42% and 25% in cells infected with strain Erdman and the *Rv3351c* complement, respectively, with significant differences observed at 168 hr pi (Figure 3.6B). No significant differences were detected in J774.A1 macrophage viability following infection with the same strains over time (Figure 3.6C).

Transmission electron microscopy demonstrated death of A549 cells infected with strains Erdman, ΔRv3351c, or ΔhbhA at 96 hours pi (Figure 3.7). Although the number of dying A549 cells was significantly lower in cells infected with the mutant strains versus strain Erdman, characteristics associated with apoptosis (e.g. membrane bleebing, loss of membrane asymmetry, cell shrinkage, nuclear fragmentation, chromatin condensation, or chromosomal DNA fragmentation) were not observed. Cell membrane rupture with cytoplasmic streaming and organelle degeneration was visible, signs more often associated with cellular necrosis.

Matrix metalloproteinases (MMPs), particularly MMP-2 and MMP-9, are known to be involved in alveolar remodelling during *M. tuberculosis* infection [10, 21, 36, 47, 48, 52, 58, 60]. Type II pneumocytes infected with other lung pathogens were shown to be contributors to this process through the secretion of various MMPs [34]. Potential differences in MMP-2 and -9 secretion profiles were examined following infection of A549 cells with the mutant and parent *M. tuberculosis* strains; however, no significant differences in secreted levels of MMP-2 and MMP-9 were observed from cells infected with any of the *M. tuberculosis* strains examined (data not shown).

DISCUSSION

Alveolar macrophages are primarily responsible for the control or dissemination of *M. tuberculosis* bacilli from the initial site of pulmonary infection [23, 25, 29, 50, 63]. However, during the early phase of the disease process, other cells in the alveolar microenvironment including various types of epithelial cells, endothelial cells and fibroblasts may directly or indirectly interact with the invading bacilli and ultimately influence the local innate immune response [24]. These non-professional phagocytes could also be involved in the process of mycobacterial dissemination from the lung or serve as additional reservoirs for persistent bacilli. For example, it is known that types I and II pneumocytes infected with *Streptococcus pneumoniae*, *Legionella pneumophila*, and *Yersinia pestis* can recruit leukocytes and potentially serve as an intracellular refuge from alveolar macrophages [8, 14, 15, 22, 27, 31, 41, 45, 56].

Mycobacteria-induced apoptosis of alveolar macrophages has been well documented as a possible host strategy to avoid dissemination of *M. tuberculosis* bacilli from the lung [6, 16, 23, 29, 63]. This process may also be important for recruiting other immune cells to the site of infection with minimal tissue destruction and inflammation [16]. Previous *in vitro* work has shown that *M. tuberculosis* bacilli can readily infect type II pneumocytes (A549 cells) and induce cytokine and chemokine release with eventual necrosis of the cell [17, 33, 37, 55, 64]. Cellular necrosis is known to produce a stronger localized inflammatory immune response with more tissue destruction than that resulting from apoptosis. Necrotic disruption of the alveolar lining may provide a greater opportunity for bacterial dissemination from the lungs. In addition, studies have shown that virulent *M. tuberculosis* bacilli that survive within epithelial cells may be better able to subsequently survive within macrophages as well as other epithelial and endothelial cells [6].

Although many *M. tuberculosis* genes have been identified as having roles in replication or survival of the pathogen within macrophages [12, 61, 62], few mutants that impair bacterial internalization and/or intracellular replication in alveolar epithelial cells have been described. An *hbhA* mutant, deficient in the production of the heparin-binding adhesion molecule filamentous hemagglutinin was reported to be defective in interactions with alveolar epithelial cells [51]. This mutant was found to disseminate less effectively than the parent strain from the lungs to other organs following mouse aerosol lung infection suggesting that *M. tuberculosis* may utilize the alveolar epithelium in the dissemination process. In this study, we determined that mutations in a second gene, *Rv3351c*, provided *M. tuberculosis* bacilli with traits similar to those described for ΔhbhA including reduced killing of infected epithelial cells. However, *Rv3351c* encodes a product of unknown function possessing no obvious adhesion or secretory signal sequences.

Both *Rv3351c* and *hbhA* [40] are expressed *in vitro*, but their functions appear to be specifically required in alveolar pneumocytes; strains with mutations in these genes possess no decreased survival advantage or deleterious phenotypes in macrophages [51, current study].

During the course of A549 and H441 cell infections performed in this study, intracellular ΔRv3351c and ΔhbhA viable counts significantly decreased compared to parent and complemented strains. The decrease in ΔhbhA bacterial numbers in both cell lines may be due to an attachment defect or attachment via an alternate receptor resulting in internalization of the bacilli via a less-favorable pathway; previous research by Pethe and others [19, 40, 49, 51] provided *in vitro* and *in vivo* evidence to support this hypothesis. Enhanced surfactant production by H441 cells may exacerbate the ΔhbhA attachment defect and contribute to the slower rate of internalization compared to infections in A549 cells. Intracellular trafficking patterns of ΔRv3351c bacilli differ from those of the parent strain suggesting that the *Rv3351c* gene product is involved in an as yet undefined bacterially-induced epithelial cell-specific endosomal trafficking process leading to intracellular bacterial replication and host cell necrosis [Fine et al., submitted]. Thus, these two genes may be virulence factors involved in a novel epithelial cell-specific component of *M. tuberculosis* pathogenesis potentially leading to bacterial dissemination from the lungs.

Although *M. tuberculosis* strain Erdman possessing mutations in the *Rv3351c* or *hbhA* genes produced less virulent phenotypes, independently, these mutations were not sufficient to completely eliminate epithelial cell death. It is likely that *Rv3351c* and *hbhA* are only two components of a large complex of genes required for the bacterium to interact successfully with epithelial cells. *Shigella flexneri* is an example of an intracellular bacterial pathogen that possesses numerous genes that interact specifically with the organ- or tissue-appropriate epithelium [26, 39, 46]. Our future work will include analyzing other genes in the *Rv3351c* genomic region, defining the function of the *Rv3351c* gene product, identifying other interacting factors in the endosomal trafficking process, and more thoroughly defining the role of the alveolar epithelium in pulmonary tuberculosis using primary cells and animal models.

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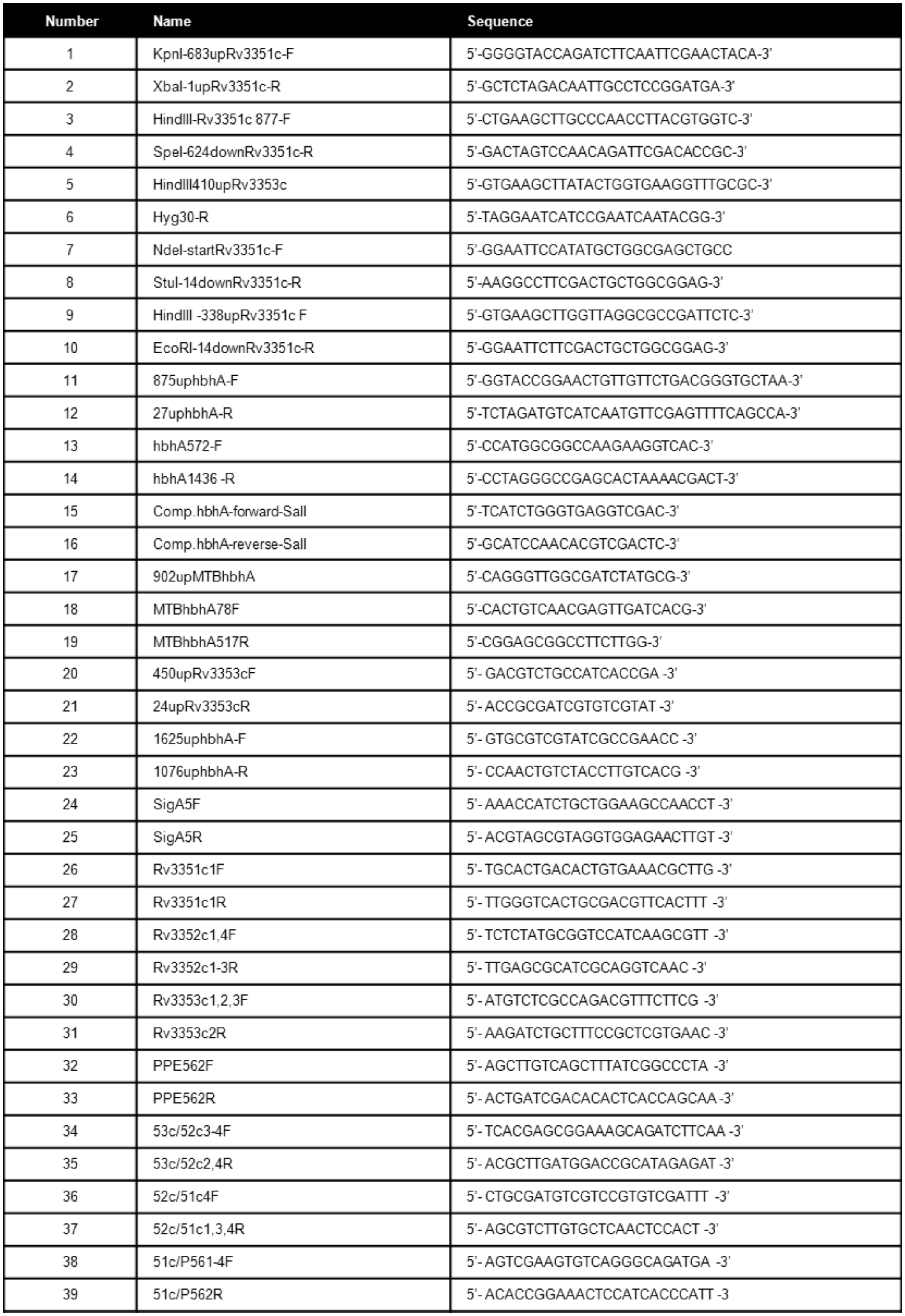
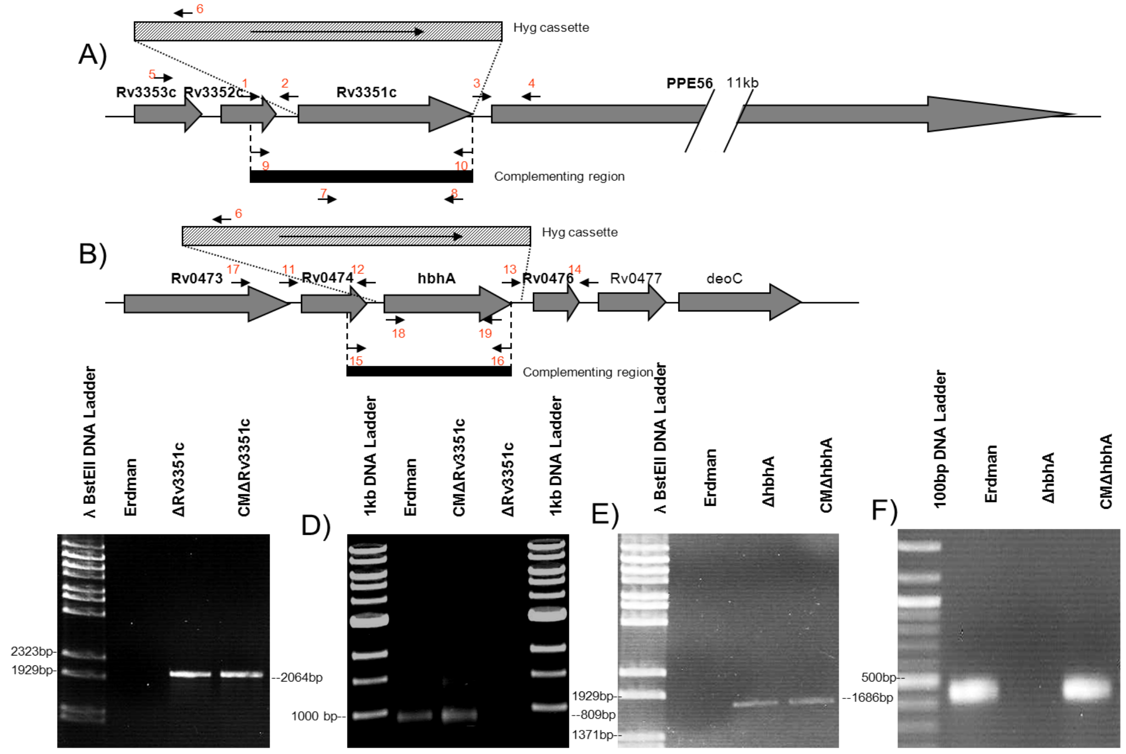
Table 3.1: Primers used in this study



Number	Name	Sequence
1	KpnI-683upRv3351c-F	5'-GGGGTACCAGATCTTCAATTCGAACTACA-3'
2	XbaI-1upRv3351c-R	5'-GCTCTAGACAATTGCCTCCGGATGA-3'
3	HindIII-Rv3351c 877-F	5'-CTGAAGCTTGCCCAACCTTACGTGGTC-3'
4	SpeI-624downRv3351c-R	5'-GACTAGTCCAACAGATTCGACACCGC-3'
5	HindIII410upRv3353c	5'-GTGAAGCTTATACTGGTGAAGGTTTGCGC-3'
6	Hyg30-R	5'-TAGGAATCATCCGAATCAATACGG-3'
7	NdeI-startRv3351c-F	5'-GGAATTCCATATGCTGGCGAGCTGCC
8	StuI-14downRv3351c-R	5'-AAGGCCTTCGACTGCTGGCGGAG-3'
9	HindIII -338upRv3351c F	5'-GTGAAGCTTGGTTAGGCGCCGATTCTC-3'
10	EcoRI-14downRv3351c-R	5'-GGAATTCTTCGACTGCTGGCGGAG-3'
11	875uphbhA-F	5'-GGTACCGGAACTGTTGTTCTGACGGGTGCTAA-3'
12	27uphbhA-R	5'-TCTAGATGTCATCAATGTTCGAGTTTTCAGCCA-3'
13	hbhA572-F	5'-CCATGGCGGCCAAGAAGGTCAC-3'
14	hbhA1436 -R	5'-CCTAGGGCCGAGCACTAAAACGACT-3'
15	Comp.hbhA-forward-SalI	5'-TCATCTGGGTGAGGTCGAC-3'
16	Comp.hbhA-reverse-SalI	5'-GCATCCAACACGTCGACTC-3'
17	902upMTBhbhA	5'-CAGGGTTGGCGATCTATGCG-3'
18	MTBhbhA78F	5'-CACTGTCAACGAGTTGATCACG-3'
19	MTBhbhA517R	5'-CGGAGCGGCCTTCTTGG-3'
20	450upRv3353cF	5'- GACGTCTGCCATCACCGA -3'
21	24upRv3353cR	5'- ACCGCGATCGTGTCGTAT -3'
22	1625uphbhA-F	5'- GTGCGTCGTATCGCCGAACC -3'
23	1076uphbhA-R	5'- CCAACTGTCTACCTTGTCACG -3'
24	SigA5F	5'- AAACCATCTGCTGGAAGCCAACCT -3'
25	SigA5R	5'- ACGTAGCGTAGGTGGAGAACTTGT -3'
26	Rv3351c1F	5'- TGCACTGACACTGTGAAACGCTTG -3'
27	Rv3351c1R	5'- TTGGGTCACTGCGACGTTCACTTT -3'
28	Rv3352c1,4F	5'- TCTCTATGCGGTCCATCAAGCGTT -3'
29	Rv3352c1-3R	5'- TTGAGCGCATCGCAGGTCAAC -3'
30	Rv3353c1,2,3F	5'- ATGTCTCGCCAGACGTTTCTTCG -3'
31	Rv3353c2R	5'- AAGATCTGCTTTCCGCTCGTGAAC -3'
32	PPE562F	5'- AGCTTGTCAGCTTTATCGGCCCTA -3'
33	PPE562R	5'- ACTGATCGACACACTCACCAGCAA -3'
34	53c/52c3-4F	5'- TCACGAGCGGAAAGCAGATCTTCAA -3'
35	53c/52c2,4R	5'- ACGCTTGATGGACCGCATAGAGAT -3'
36	52c/51c4F	5'- CTGCGATGTCGTCCGTGTCGATTT -3'
37	52c/51c1,3,4R	5'- AGCGTCTTGTGCTCAACTCCACT -3'
38	51c/P561-4F	5'- AGTCGAAGTGTCAGGGCAGATGA -3'
39	51c/P562R	5'- ACACCGGAAACTCCATCACCCATT -3



Primers 5 and 6

Primers 7 and 8

Primers 17 and 6

Primers 18 and 19

Figure 3.1:Drawing and PCR of mutants. Schematic of *M. tuberculosis* Rv3351c (panel A) and hbhA (panel B) genomic regions based on information obtained from the Tuberculist Web Server (http://genolist.pasteur.fr/TubercuList/). Open reading frames and orientations are shown. The regions deleted and replaced with the hygromycin-resistance cassette and the regions used for complementation are marked. Annealing sites for primers (small, numbered arrows) used in this study are shown. Primer sequences can be found in Table 3.1. Results of agarose gel electrophoresis of PCR products used to screen for mutants are shown (panels C-F). DNA templates are indicated atop the lanes. Primer pairs used for PCR are indicated below each gel. DNA ladders are indicated with selected band sizes shown on the left of each gel. The expected PCR products are indicated on the right side of each gel.

A. Alignment of Rv0063 (479 aa) with Rv3353c (86 aa), Rv3352c (123 aa), and Rv3351c (264 aa)

```
       10        20        30        40        50        60        70        80
LAREISRQTFLRGAAGALAAGAVFGSVRATADPAASGWEALSSALGGKVLQPDDGPQFATAKQVFNTNYNGYTPAVIVTP
.    ::::::::.:: :: :.::: .. : : :. .:: :.:.:.::.:: : .: :.. .::.::.::.: .::..:.::
M----SRQTFLRGAVGAPATSAVFPTILARATPG-DGWASLASSIGGQVLLPANGRAFTSGKQIFNSNYSGLNPAAVVTV
         10        20        30         40        50        60        70

       90       100       110       120       130       140       150       160
TSQLDVQKAMAFAAANNLKVAPRGGGHSYVGASTANGAMVLDLRQLPGDINYDATTGRVTVTPATGLYAMHQVLAAAGRG
.:: ::.::..                                   :. :: :::.::.:: .:.
ASQADVRKAVS-------------------------------------------------VSAATDLYAVHQALAGESRA
  80                                                          10        20

      170       180       190       200       210       220       230       240
IPTGTCPTVGVAGHALGGGLGANSRHAGLLCDQLTSASVVLPSGQAVTASATDHPDLFWALRGGGGGNFGVTTSLTFATF
::::.:::::::: .::::::.:::::: :: : ::.::::.:.::.:::.:: .:::::::::::::::::.::: :
IPTGSCPTVGVAGLTLGGGLGADSRHAGLTCDALKSATVVLPGGDAVSASADDHAELFWALRGGGGGNFGVTTSMTFARF
      30        40        50        60        70        80        90       100

      250       260       270       280       290       300       310       320
PSGDLDVVNLNFPPQSFAQVLVGWQNWLRTADRGSWALADATVDPLGTHCRILATCPAGSGGSVAAAIVSAVGTQPTGTE
:..: ::: ..: :.. :::::                  .::.::: ::..:: :: :::.:.::.:.:
PTADCDVVRVDFAPSAAAQVLVG----------------------------VLASCPARSGAAVADAIKSAVGVQPSGVE
      110       120                                  10        20

      330       340       350       360       370       380       390       400
NHTFNYLDLVRYLAVGNLNPSPLGYVGGSDVFTTITPATAQGIASAVDAFPRGAGRMLAIMHALDGALATVSPGATAFPW
..:.  .:::::: :. .  : :.:.::::. : .::.::.:.:.: ..: .:::  :.. .: ::.. ..: ..::::
HKTLRRMDLVRYLAGGHTTYPPEGFVAGSDVIGTTNPAAAQAIVAAIGTWPPAAGRASALIDSLGGAVGDMDPEGSAFPW
30       40        50        60        70        80        90       100       110

      410        420       430       440       450       460       470
RRQSALVQWYVET--SGSPSEATSWLNTAHQAVRAYSVGGYVNYLEVNQPPARYFGPNLSRLSAVRQKYDPSRVMFSGLN
::::.::::.:  .:. .  :.::: ::.:: ::::::::::.:   ..::: :::::  .::::::::.:.:.:.:::
CRQSAVVQWYVNTPSDGQVATANKWLSDAHHAVQHFSVGGYVNYLEANAAASQYFGANLSRLTTVRRKYDPDRIMYSGLD
      120       130     140       150       160       170       180      190

F-----------------------------------------------------------------------
:
FSTRQVAERLLPALGFRVRFGVLVIRCALCTDTVKRLGTLPNLTWSRLKVNVAVTQEQAGVMDLPALPVRRTPRR

       200       210       220       230       240       250       260
```

B. A potential 80 aa peptide encoded between *Rv3351c* and *PPE56*

Stop Rv3351c

TGAgcatgctcgcggactccgccagcagtcgaagtgtcagggcagatgacgccctgccgctcgccggtctggacagggcatatgtg

catttttgctgcggcgtctgtgcaaagtgcacccagtacccgcgattatcggggaatcgttcttgggccgttggcaatgcgagcaaaa

ttatcggggcgtcgacacgtcatgtgggggtgtcgggtgggggattggtgtcgggctgggaagggggaatgggtgATGgag

Start *PPE56*

Figure 3.2:Genomic alignment. Sequence analysis of Rv3351c genomic region. A) BLAST comparison of the translated Rv0063 gene to those of Rv3353c (blue font), Rv3352c (orange font), and Rv3351c (green font). Amino acid identity (:) and similarity (.) are indicated. B) DNA sequence between the stop codon of Rv3351c (TGA, red font) and the start codon of PPE56 (ATG, green font). Alternating codons (starting from the atg located 2 nucleotides downstream of the Rv3351c stop codon are shown (alternating blue and black) may represent an 80 aa peptide that could couple transcription of Rv3351c to PPE56 under conditions in which a +1 frame-shift is induced.

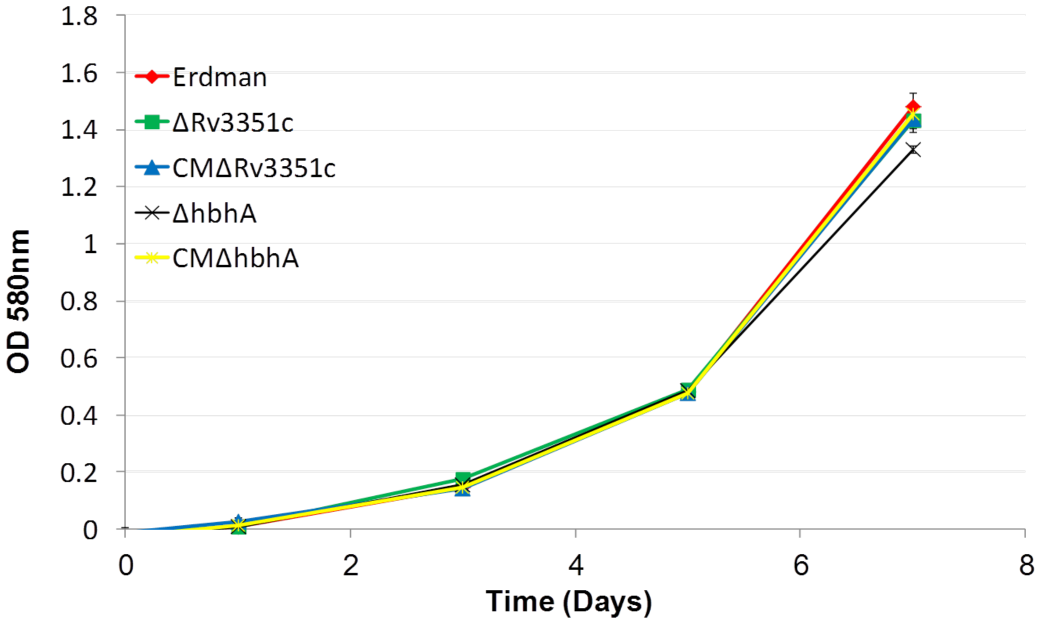


Figure 3.3:Growth Curve under broth conditions. Growth of *M. tuberculosis* strains in Middlebrook 7H9tgADS broth. Optical density (580 nm) was monitored in triplicate at each time point. Experiments were repeated with similar results.

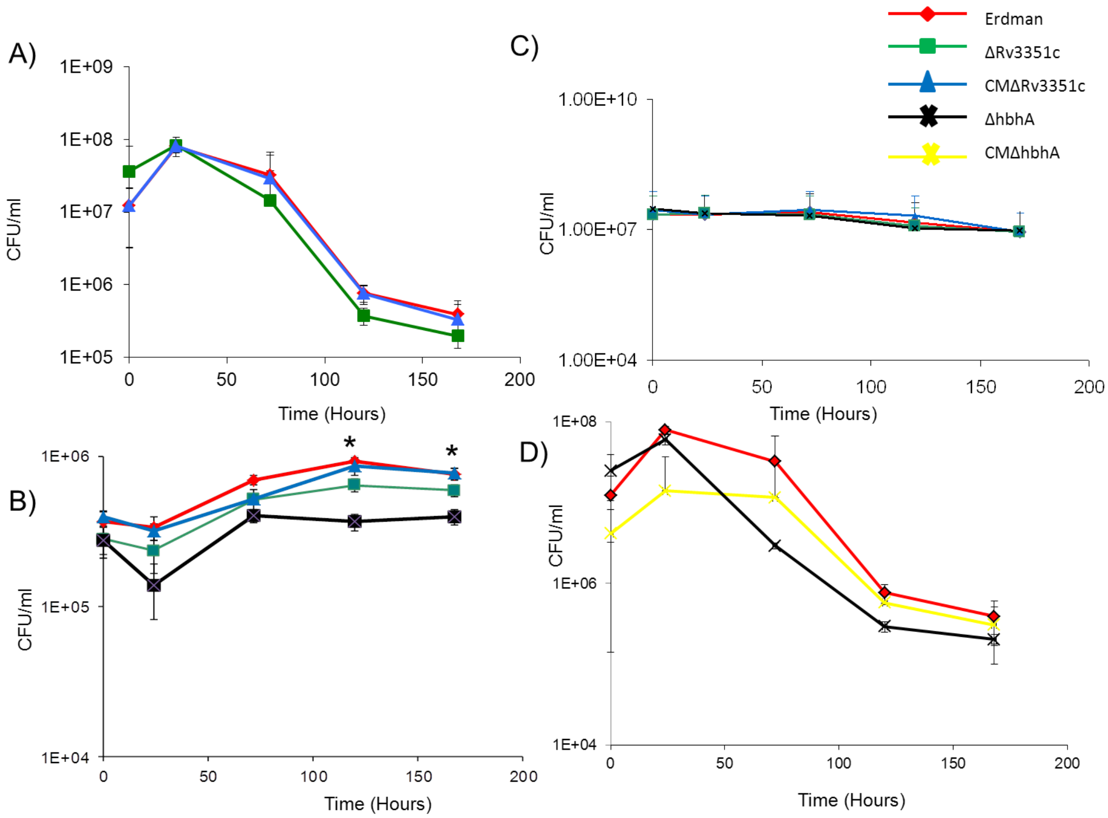


Figure 3.4:Intracellular viability of *Mtb* bacilli. Intracellular viability (colony forming units per ml [CFU/ml]) in: A) A549, B) H441, C) J774A.1 and D) A549 cells. Data shown are the average of three experiments performed in triplicate. Significant differences (P values <0.05) between mutant strains and strain Erdman and CMΔRv3351c are indicated (*).

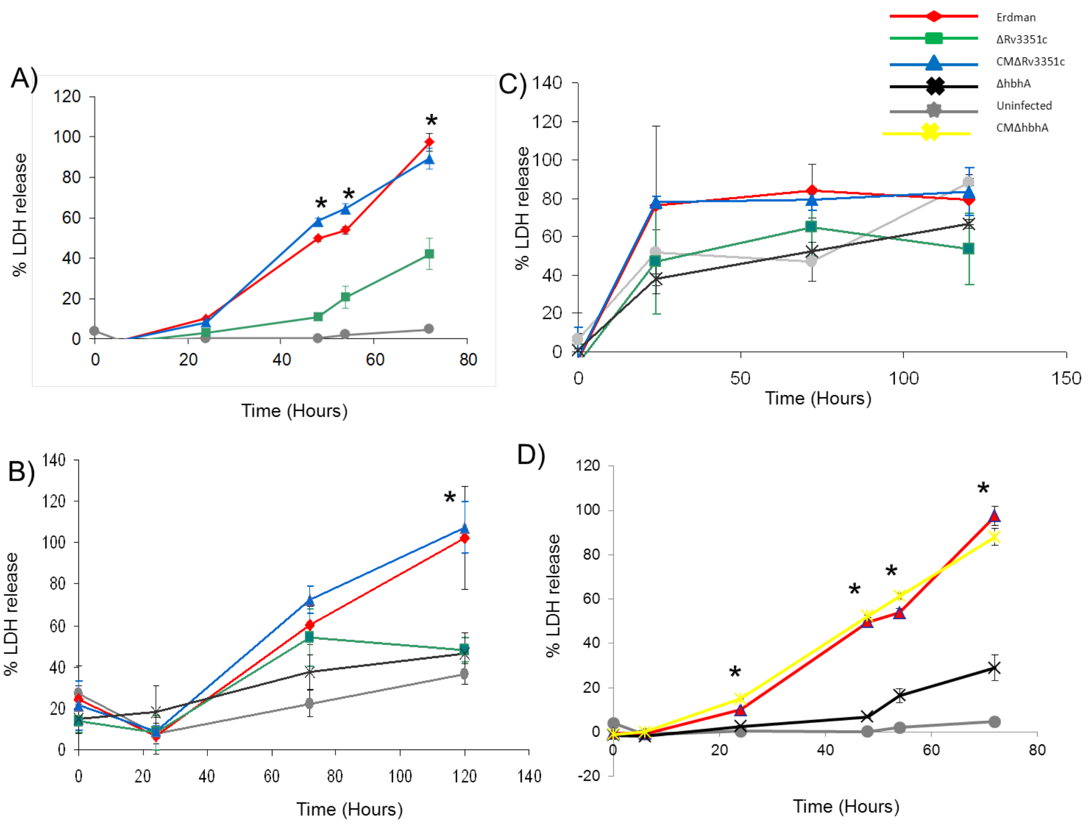


Figure 3.5:Lactate Dehydrogenase assay in various cell lines. Examination of lactate dehydrogenase (LDH) release from host cells following *M. tuberculosis* infection. A549 AECs (panel A), H441 AECs (panel B), J774.A1 macrophages (panel C) and A549 AECs (panel D) were infected as described in methods with the indicated *M. tuberculosis* strains at an MOI=100. Host cell toxicity was monitored by assaying for release of cytosolic enzyme LDH into culture supernatant over time. Data shown are the average of three experiments performed in triplicate. Significant differences (P values <0.05) between mutants and strain Erdman are indicated (*).

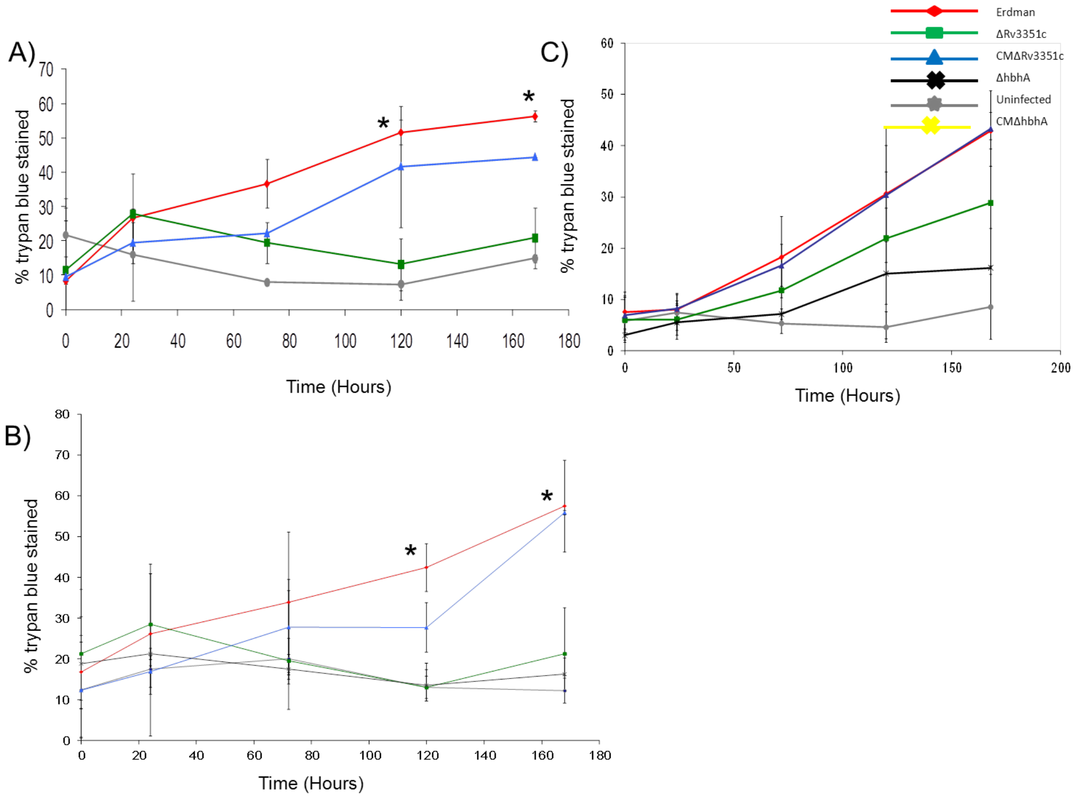


Figure 3.6:Trypan blue assay in various cell lines. Trypan blue viability assays for: A) A549, B) H441, and C) J774A.1 cells infected with mutant and control strains of *M. tuberculosis*. Assays were performed in duplicate and the experiment repeated twice. Significant differences (P values <0.05) between mutant strains and strain Erdman are indicated (*).

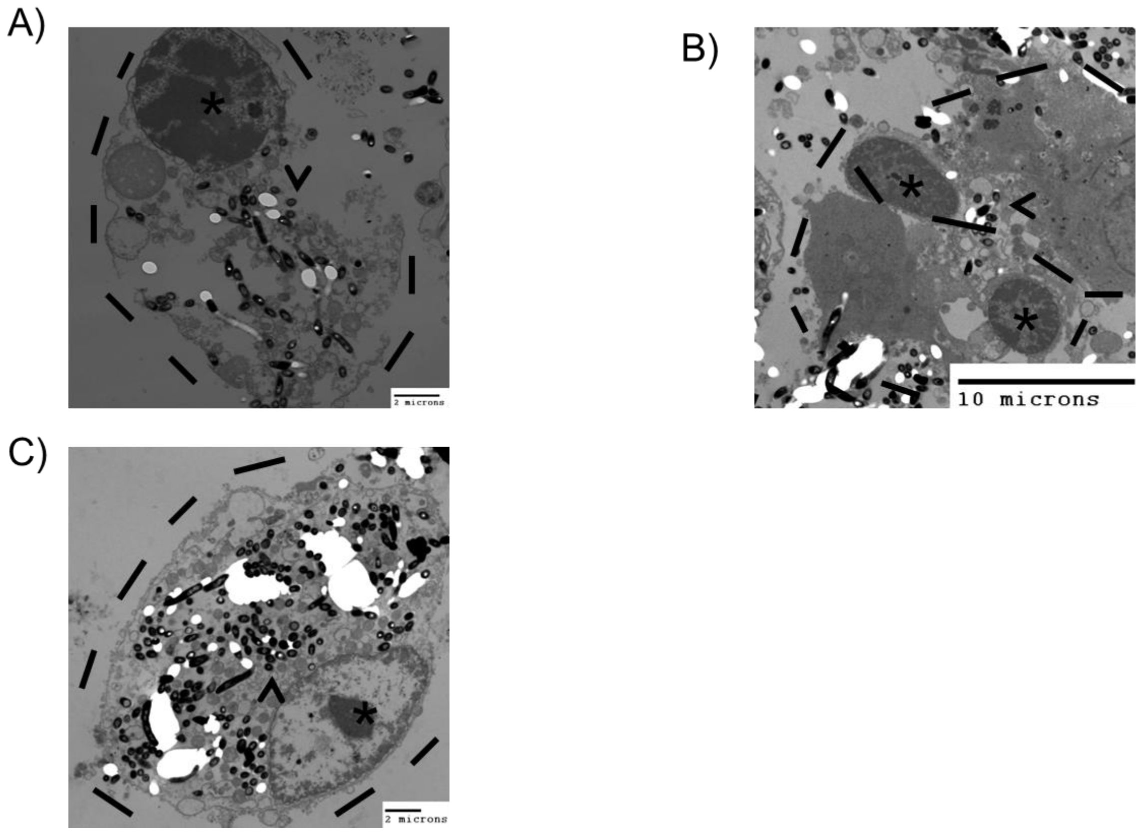
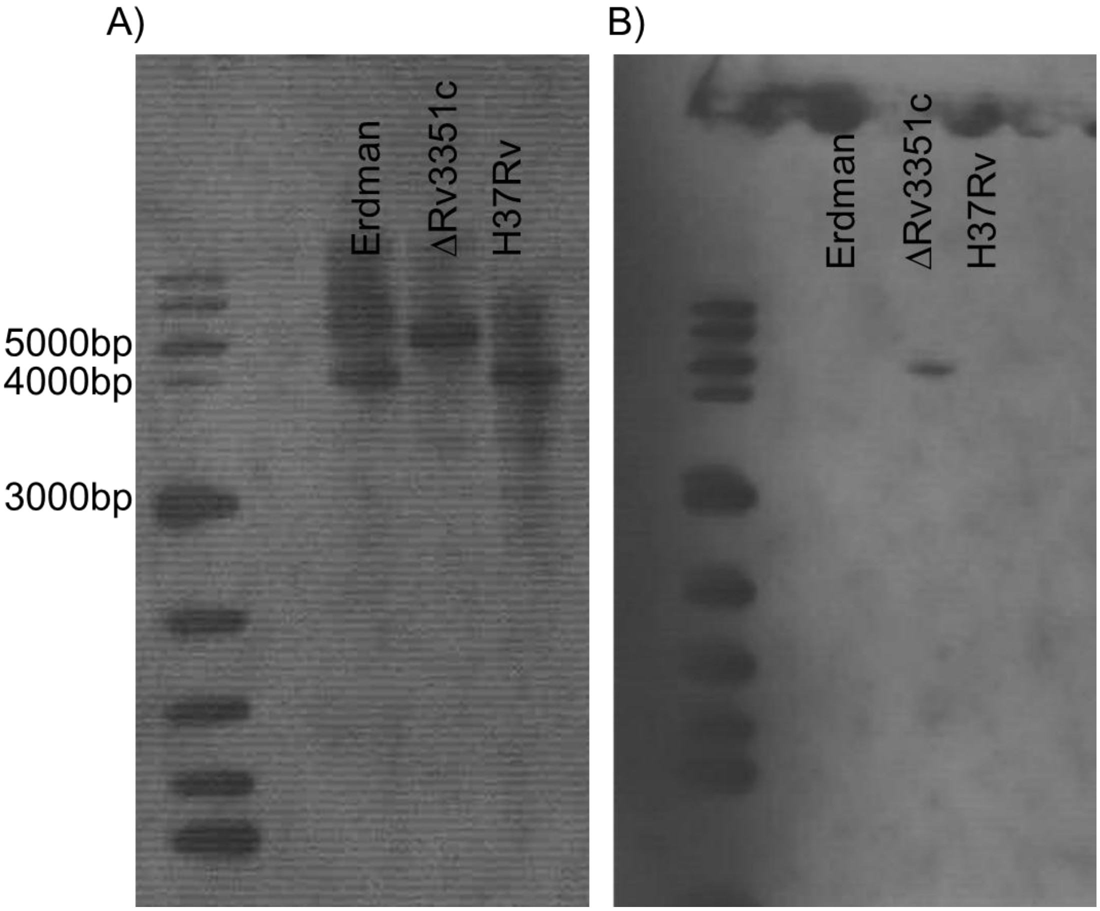


Figure 3.7:Electron micrographs of necrosing A549 cells. A549 cells 96 hr pi with: A) *M. tuberculosis* Erdman, B) ΔRv3351c, and C) ΔhbhA (41,250X). The host cells are outlined (dash-lines) and intact nucleus indicated (asterisks). Bacteria (electron-dense rods, the arrowheads indicate sample bacteria) are found throughout the dying host cells. Images are representative of three experiments; each strain repeated in triplicate. An average of 3 grids was examined for each embedded specimen.



Supplemental Figure 3.1:Southern blot of mutant. A) Southern blot results with a probe to genomic region upstream of Rv3351c: as expected for replacement of Rv3351c with the larger hygromycin-resistance gene, a band up-shift is observed with chromosomal DNA from ΔRv3351c relative to that observed for parental strain (Erdman) or a control M. tuberculosis strain (H37Rv). B) Southern blot of the membrane used in panel A after stripping and hybridizing to a hyg probe. As expected, only the shifted band in ΔRv3351c hybridizes to the hyg probe.

CHAPTER 4

ASSESSING VIRULENCE AND DISSEMINATION OF A *MYCOBACTERIUM TUBERCULOSIS RV3351C* DELETION MUTANT IN VARIOUS *IN VITRO* AND *IN VIVO* INFECTION MODELS

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(To be submitted to Microbiology.)

ABSTRACT

Many lung pathogens interact with epithelial cells lining the alveoli. However, this association has not been conclusively demonstrated for *Mycobacterium tuberculosis*. Our previous *in vitro* studies using type II pneumocytes infected with *M. tuberculosis* bacilli demonstrated that the mycobacteria are readily internalized within 30 minutes post infection and replicate to high numbers resulting in death of the host cell via necrosis. Results presented here demonstrated that *M. tuberculosis* bacilli were also able to attach to and internalize within type II and type I pneumocytes 24 to 48 hours after aerosol infections in mice and rabbits. A *M. tuberculosis Rv3351c* deletion mutant, compared with the complemented strain, replicated 10-fold less effectively in the lungs of mice and was significantly less efficient in disseminating to the spleen and liver by three weeks post-infection. In addition, histopathologic analysis of rabbit lung sections showed fewer mutant than parent bacilli associated with the pneumocytes by 96 hours post-infection. Interestingly, however, overall lung pathology was similar in mice and rabbits infected with the mutant, complemented, or parent strains between 24 hours and 3 weeks post-infection. These *in vivo* and previously reported *in vitro* data indicate that *M. tuberculosis* bacilli successfully infect and replicate within alveolar epithelial cells in the hours and days immediately following inhalation of the bacilli. Efficient internalization, intracellular replication and killing of alveolar pneumocytes by *M. tuberculoisis* bacilli are associated with successful dissemination of the mycobacteria from the lungs.

INTRODUCTION

Mycobacterium tuberculosis is a leading cause of morbidity and mortality worldwide. Approximately 9.4 million new cases of tuberculosis (TB) and 1.8 million deaths were estimated for 2008 [23]. Although research has been ongoing for over 100 years, efficacious vaccines, highly specific, sensitive, and rapid diagnostic tests, and less toxic and simple therapeutic regimens remain unavailable [4, 15, 23].

Much is known of the human immune response during later stages of disease and, in particular, the involvement of macrophages [7, 25]. However, little is known of the interactions between the bacterium and other cells of the innate immune system, particularly the lung epithelial cells, during the first hours or days after infection [18, 20, 21]. Researchers, including our group, have identified several *M. tuberculosis* genes that function specifically to enhance survival and replication of mycobacteria within macrophages [20, 21]. Interestingly, *M. tuberculosis* genes *hbhA* and *Rv3351c*, when deleted, were found to decrease the survival and replication rate of the mutant bacteria and enhance the survival of infected alveolar type II epithelial cells when compared to the parent or complementing strains [20, 21]. While both mutant strains show a similar decrease in bacterial survival within pneumocytes, the *hbhA* mutant (ΔhbhA) possesses an attachment defect that, in part, may account for their decreased ability to disseminate upon murine infection [20]. The *Rv3351c* mutant (ΔRv3351c) has not shown a similar defect on type II pneumocyte infections, and both mutant strains and the parent strain demonstrated no phenotypic differences in infected macrophages [21]. In addition, because of what has been observed with other bacterial pathogens, this interaction with epithelial cells likely occurs early in the infection, shortly after inhalation of the bacilli into the lungs [21].

A major difficulty in tuberculosis research has been the lack of a convenient and inexpensive animal model that accurately emulates the human infection process [6, 9, 10, 11, 12, 18, 19, 22, 24]. Because of its relative simplicity, availability of inbred strains, reagent bounty and low cost, the mouse has been the most widely used of the TB model systems. Unfortunately, there are a number of physiological and immunological problems that will always make the mouse a generally poor model for emulating human disease [8,9, 10, 11, 24]. The guinea pig and rabbit models follow the mouse model in relative convenience and low cost, and although reagent availability has improved and these models are more physiologically and immunologically relevant, the non-human primate remains the gold standard [2, 6, 9, 13, 14, 18, 19, 22, 24]. Nonetheless, the mouse and rabbit models were found to be adequate for efforts presented here to examine cell-to-cell interactions and pathophysiological responses to aerosol challenge with *M. tuberculosis* bacilli [1, 6, 8, 13, 17]. From the results of this study and associated projects, it is our belief that novel vaccines, diagnostics, and therapies against TB could be developed that target host and bacterial gene products and processes that are active, critical, and specific to the first hours and days after initial aerosol infection.

MATERIALS AND METHODS

EPITHELIAL CELL CULTURE

A549 human type II alveolar epithelial cells were obtained from ATCC (CCL-185) and maintained in EMEM (Eagle's Minimum Essential Medium; Gibco) with 5% Fetal Bovine Serum (FBS).

BACTERIAL STRAINS

Mycobacterium tuberculosis strain Erdman, and mutant strains ΔRv3351c and ΔhbhA, the ΔRv3351c-complementing strain, CMΔRv3351c, and the ΔhbhA-complementing strain, CMΔhbhA, were generated as previously described [21]. Bacterial strains were cultured in Middlebrook 7H9 broth supplemented with 0.05% Tween 80, 0.5% glycerol, and 10% ADS, or plated on 7H10 agar supplemented with 10% ADS, 0.5% glycerol, and 0.05% Tween 80 (7H9gtADS)[3, 21]. Bacterial stocks were grown in 7H9gtADS at 37°C and 5% CO_2 with occasional shaking to an OD_{600} of 1.0. Cultures were mixed by vortexing and dispensed as frozen aliquots. For infection of host cells, thawed bacterial stocks were transferred to fresh tubes, vortexed vigorously for several minutes, and a microscopic bacterial count was performed using a Petroff-Hausser counting chamber. The bacteria were then diluted to equivalent densities in PBS and passed once through a tuberculin syringe directly into culture wells. Viable counts were determined by serial dilution in 0.05% Tween 80, plating on 7H10gtADS, and incubation at 37°C for 3 weeks.

Growth curves were performed with all mycobacterial strains used in this study. Bacteria were cultured in 7H9gtADS broth at 37^{o} C and 5% CO_2 with occasional shaking. Optical density (OD_{600nm}) was measured in triplicate at each time point and colony forming units (CFUs) determined by serial dilution plating.

PLAQUE ASSAY

A549 cells were grown to a 70% confluent monolayer and then infected with strains Erdman, ΔRv3351c**,** ΔHBHA, or the complemented strains at an MOI of 0.1. Cells were incubated for 24 hr and then washed twice with Hank's balanced salts solution (HBSS). Trypan blue was added with the fresh media. Cells were then overlayed with 2 ml MEM containing 1% FBS and 1% agarose cooled to 42˚C before application, as previously described [5]. Zones of clearing within the monolayers were visualized and diameters measured at various time points over a 9-day period.

ANIMAL PROCUREMENT AND HOUSING

Specific pathogen-free outbred female rabbits (Myrtle's Rabbitry, Thompson Station, TN), SCID and BALB/c mice (Charles River Laboratories, Wilmington, MA) were procured for this study. Mice were 6 to 8 weeks old and were housed five per cage in HEPA-filtered containment units. The rabbits were approximately 3.0 - 3.5 kg and were housed individually in stainless steel cages strapped to Rubbermaid carts. All the cages were kept inside a negatively-pressured BioBubble (Colorado Clean Room Company, Fort Collins, CO) located within an approved ABSL-3 suite. All rabbit procedures were performed within the BioBubble. All the animal procedures were approved by the Institutional Biosafety Committee (IBC) and the Institutional Animal Care and Use Committee (IACUC) at the University of Georgia. The University of Georgia is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International (AAALAC).

SCID MOUSE INFECTION

SCID mice were anesthetized and intratracheal infections with *M. tuberculosis* strains Erdman, ΔRv3351c or CM ΔRv3351c were performed. Doses of 1 x 10^4 CFU/ml were given in 0.025 ml PBS + 0.01% Tween 80. Mice were sacrificed at the first sign of declining health up to 140 days. After being euthanized, tissues were removed for viable bacterial counts, and hematoxylin and eosin (H&E) and acid-fast staining.

BALB/C MOUSE HIGH DOSE INFECTION

BALB/c mice were anesthetized and intratracheal infections with *M. tuberculosis* strains Erdman, ΔRv3351c or CM ΔRv3351c were performed. Doses of 1 x 10^7 CFU/ml were given in

0.025 ml PBS + 0.01% Tween 80. Mice were sacrificed on days 1 and 21 and tissues were removed for viable bacterial counts, and H&E and acid-fast staining. Lesion scoring was performed blind as described below.

BALB/C MOUSE LOW DOSE INFECTION

BALB/c mice were infected by aerosol in a Madison Chamber using a nebulized dose of 3 x 10^6 CFU/ml *M. tuberculosis* strains Erdman, ΔRv3351c, or CM ΔRv3351c diluted in PBS + 0.01% Tween 80. One additional group of mice was mock-infected with PBS + 0.01% Tween 80. Each mouse received an average dose of 7 x 10^5 CFU. Groups were nebulized for 15 minutes and allowed to rest in the chamber for 30 minutes before being returned to their cages. Mice were sacrificed at 24 hr, 1 week, 2 weeks, and 5 weeks. The remainder of the mice were humanely euthanized at 18 weeks.

RABBIT INFECTION

Prior to infection, rabbits were pre-medicated with glycopyrrolate (0.01 mg/kg) (Baxter Healthcare Corp.) and anesthetized with a combination of ketamine (35 mg/kg) (Lloyd Laboratory), xylazine (5.0 mg/kg) (Lloyd), and acepromazine (0.25 mg/kg) (Fort Dodge Animal Health). Once anesthetized, the end of a sterile, 2.5mm diameter endotracheal (ET) tube was coated with lidocaine jelly (Akorn Pharmaceutical Products, Inc.) and inserted orally using a "blind technique" described by Morgan and Glowaski [16]. Once successfully intubated, the tube was tied with gauze around the rabbit's head. The rabbit was then transported to the BioBubble inside the BSL-3 lab and placed in a biosafety cabinet. With the rabbit laterally recumbent, a PE-100 tube (Becton Dickinson) was passed through the entire length of the ET tube and exited just proximal to the tracheal bifurcation. A 20G (Intramedic) Luer Stub adapter was attached to the distal end of a PE-100 tube. *M. tuberculosis* strains Erdman or ΔRv3351c (delivered at 2-3 x 10^6 CFU/rabbit), or PBS, all suspended in 1 ml of 0.05% Tween 80 (PBS-T) was slowly instilled into the tube. Once infected, the rabbits were given an IV injection of yohimbine (0.2 mg/kg) (Lloyd). Animals were closely monitored post-infection by a trained veterinary technician until recovered. Rabbits were sacrificed at 48 hr and 96 hr post-infection, and lung tissue was removed and fixed for H&E and acid-fast staining. As described below, lesion scoring was performed blind.

RABBIT PRIMARY TYPE II PNEUMOCYTE ISOLATION, CULTURE, AND INFECTION

Uninfected rabbits were humanely euthanized, and the lungs were aseptically removed and perfused with 20 ml of sterile PBS (Cellgro). A 15 gauge adapter was inserted into the trachea, and the lungs were lavaged three times with 10 ml PBS to remove alveolar macrophages. Twenty ml of dispase was then slowly injected followed by 20 ml of 1% low-melting agarose. Lung tissue was removed and cut into small pieces. These sections were then put in a 50 ml falcon tube and shaken at 150 rpm for 45 min. Tissues were pulled apart using sterile forceps in EMEM with 10% FBS and 0.1% DNase. After shaking again at 150 rpm for 10 min, the liquid-containing tissue was filtered through a 1 ply sterile gauze and then through a 100 µm filter. The filtrate was then centrifuged at 1000 x g for 10 minutes. The pellet was resuspended in 30 ml EMEM with 10% FBS, L-glutamine and 1x pen-strep (complete EMEM) and cells were counted using a hemocytometer. Cells at 1 x 10^6 per ml in complete EMEM were added to 24-well cell culture plates. Empty wells were filled with sterile HBSS. Cells were incubated overnight at 37°C with 5% CO_2 before being washed three times with sterile HBSS. Complete EMEM was added to each well and cells were then incubated for 3 days, washed three times with HBSS, and fresh EMEM without pen-strep was added prior to infection.

Infections of transformed cell lines (e.g. A549) were performed routinely at 70% or 100% confluence. Due to technical limitations, infections of primary cells were performed at 30% confluency, but with an MOI of 100 for 6 hr as described previously [21]. Culture medium was then replaced with medium without antibiotic. This was considered the zero time point. To count intracellular bacteria, monolayers were washed 3 times with PBS at the indicated time point, lysed in 0.1% Triton X-100, and the mixture serially-diluted in 0.05% Tween 80/PBS and plated on 7H11 agar supplemented with 0.5% glycerol, 0.05% Tween 80, and 10% ADS. Bacterial colonies were counted after 3-week incubation at 37°C. Assays were performed in triplicate and repeated twice.

LACTATE DEHYDROGENASE (LDH) ASSAY

After the infection described above, culture supernatants were removed at the indicated time points and passed through 0.22 μm PVDF filters prior to storage at 4°C and assayed using the Cytotoxicity Detection Kit (Roche, Indianapolis, Ind.) [20]. Percent LDH release was calculated by the following formula: [(Release from Strain - Background)/(Max release – Background)] x 100. Max release refers to lysis obtained by incubation with 0.1% Triton X-100 for 15 minutes. Due to the intrinsic resistance of pneumocytes to the detergent, 100% lysis of the monolayer did not always occur during that time frame. All infections were performed in triplicate and repeated at least two times.

HISTOLOGY

Rabbit and mouse lung tissues for histology were fixed in 10% phosphate-buffered formalin for 2 days and then embedded in paraffin wax. Sections of 5 μm width were cut and stained with H&E by the Histology Laboratory at the University of Georgia, College of Veterinary Medicine. Ziehl-Neelsen staining (for acid-fast bacilli) was performed using standard protocols. Photomicrographs were taken using an Olympus BX41 Light Microscope and Olympus DP70 Camera, with DPController Imaging Software. The sections were evaluated in a blinded fashion by a board-certified veterinary pathologist. The number of granulomas and percentage of the lung section affected were noted. Severity of bronchiolitis, alveolitis, bronchiolar-associated lymphoid tissue (BALT), edema, eosinophilic infiltration, and type II pneumocyte hyperplasia were scored. Scoring was performed as indicated in Table 4.2.

RESULTS

IN VITRO PLAQUE ASSAY MAY MIMIC *IN VIVO* INFECTION.

Previous research published by our laboratory demonstrated that *M. tuberculosis* strain ΔRv3351c produces a decreased cytotoxic response but a similar replication rate in human type II pneumocytes compared to its parent strain [21]. A similar phenotype was not observed in murine type II pneumocytes upon *in vitro* infection (Figure 4.1). Other work by our group demonstrated that *M. tuberculosis* Erdman was capable of infecting confluent pneumocyte monolayers and successfully disseminating cell to cell, ultimately creating a zone of clearing (plaque) [5]. Confluent monolayer infections with ΔRv3351c produced significantly smaller plaques than observed from infections with Erdman (data not shown). This and other preliminary *in vitro* data led us to believe that ΔRv3351c attenuation may be receptor-related as the bacilli were not as efficient as the parent strain in causing cytotoxicity of the monolayer.

M. TUBERCULOSIS BACILLI DETECTED INTRACELLULARLY AND ASSOCIATED WITH *IN VIVO* MOUSE AND RABBIT TYPE I AND II PNEUMOCYTES

Within 24 – 48 hours after intratracheal infection of mice and rabbits, acid fast- stained lung sections (Figures 4.3 and 4.5) demonstrated the presence of associated and potentially intracellular mycobacteria in type I and type II pneumocytes and alveolar macrophages. Electron microscopic images confirmed the intracellular location of *M. tuberculosis* Erdman bacilli in rabbit type II pneumocytes (Figure 4.6). These results demonstrate early association of the bacilli with both type II pneumocytes and macrophages in the mouse and rabbit infection models.

DIFFERENT RESPONSES BY ΔRV3351C IN SCID AND BALB/C MOUSE MODELS

SCID mice are defective in the adaptive immune response. We hypothesized that the innate immune system, particularly resting macrophages and epithelial cells, could potentially provide protection against aerosol challenge with the ΔRv3351c mutant strain. This conclusion was based on our previous *in vitro* data that showed type II pneumocytes infected with the ΔRv3351c strain versus the parent strain were more resistant to cellular necrosis and slowed ΔRv3351c intracellular replication over time [21]. However, in the current study, both *M. tuberculosis* strains were equally virulent in the SCID model (Figure 4.2), demonstrating a vital role for activated macrophages and the adaptive immune response in controlling even this mutant strain.

Levels of lung tissue destruction were examined in BALB/c mice infected with either the parent, mutant, or complementing strains at various time points up to 21 days post- infection. No significant differences in gross lung lesions were observed in any of the infected animals (Figure 4.3B). Bacterial replication rates were also examined over the same time-frame and CFU from the lungs of mice infected with the mutant strain were 10-fold lower than from animals infected with the complemented strain. CFU from the spleen were more than two-fold less, and viable bacilli disseminating to the liver were below detectable levels, compared to infections with the complemented strain (Table 4.1). This demonstrated that although the mutant bacilli were able to kill the SCID mice in a similar fashion to the wild-type, mutant replication and dissemination rates were significantly decreased in the BALB/c model. This supports the hypothesis that the bacilli may use epithelial cells as an immunological refuge early in the infection process.

DECREASED ASSOCIATION OF ΔRV3351C COMPARED TO ERDMAN BACILLI IN RABBIT MODEL

The rabbit model was next employed to determine if pathogenic differences between mutant and parent strains observed in the BALB/c model were observed in the physiologically more appropriate rabbit model. New Zealand white rabbits were infected via the intratracheal route with *M. tuberculosis* Erdman or ΔRv3351c strains and sacrificed at 48 and 96 hours post-infection. As with the BALB/c model, no significant differences in lung lesions at either time point were observed from rabbits infected with the two strains (Figure 4.5A-B and Table 4.2). However, through a detailed multi-point lesion scoring system, fewer intracellular and associated mutant bacilli were observed associated with type I and type II pneumocytes by 96 hours, compared with the parent strain (Table 4.2). We were able to identify numerous type II pneumocytes that contained internalized bacilli demonstrating that a percentage of the bacteria do interact with type II cells during the initial infection process, at least in the rabbit and mouse models.

INCREASED VIRULENCE IN RABBIT PRIMARY TYPE II PNEUMOCYTES INFECTED WITH STRAIN ΔRV3351C.

In vitro pneumocyte infection studies have proven to be extremely helpful in defining aspects of this study. However, all cells examined have been transformed, and prone to biological artifacts associated with studies of immortalized cells. The isolation, short-term culture, and study of primary type II pneumocytes from mice and rabbits are routinely performed in many laboratories. However, due to lung volume issues, routine use of mouse tissues is impractical. The isolation, culture, and infection of rabbit tissues were thus utilized in this study.

Rabbit type II pneumocytes were isolated and cultured to 30% confluency in EMEM with 1x pen-strep. Cells were washed three times with PBS and then infected with *M. tuberculoisis* strains Erdman or ΔRv3351c at an MOI of 100 for 6 hr. Subsequently, the cells were thoroughly washed and EMEM without antibiotics was added for up to 96 hr. Supernatants were then collected and tested for LDH release. Interestingly, unlike the previously reported A549 assays, the level of necrosis produced by the mutant strain was actually higher than the Erdman strain after the 96 hr infection (Figure 4.4). When A549 cell monolayer confluency was decreased to 30%, the LDH release was similar to that observed here with the rabbit primary cells (data not shown).

DISCUSSION

Our goal was to infect mice and rabbits via the intratracheal route with a sufficient dose of *M. tuberculosis* Erdman and *Rv3351c* deletion mutant bacilli to confirm direct interaction between the bacilli and alveolar pneumocytes *in vivo* during the initial hours and days following infection. Using a high dose infection, we demonstrated that *M. tuberculosis* bacilli in fact interact with lung pneumocytes and may use them to replicate, escape from the innate immune system, and/or gain access to the blood stream.

An additional goal here was to examine biological activity of ΔRv3351c compared to a strain of *M. tuberculosis* with this gene intact and transcriptionally active. We demonstrated that the ΔRv3351c mutant is not attenuated with regards to host survival and tissue lesions in the models tested, however, the mutant did show a significant attenuation in its ability to persist and disseminate from the lungs to other organs in the murine model. This may be an important finding for future vaccine research since the inability to disseminate from the lungs may be an important attenuation that could be incorporated into vaccine strain candidates.

Overall these studies should raise a note of caution, since phenotypes observed during human type II pneumocyte infections may be missed in animal model infections due to differences in human and animal bacterial association. This could be an important consideration for human vaccine studies, and further demonstrates that there is no perfect animal model that meets the needs of current *M. tuberculosis* vaccine research.

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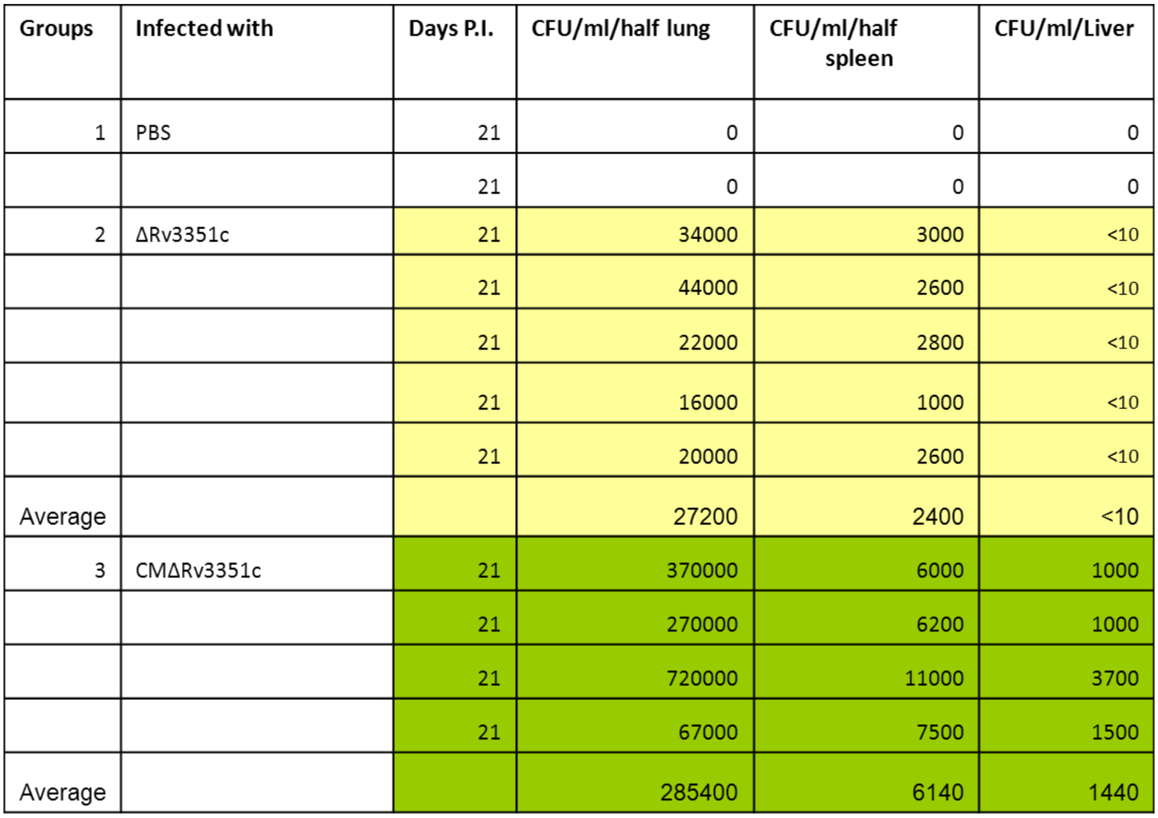
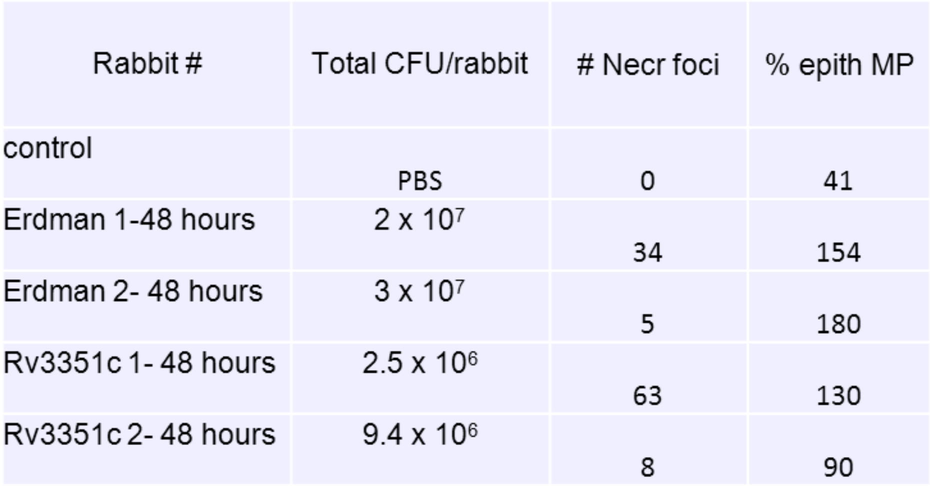
Table 4.1:BALB/c bacterial burden. Bacterial burden was significantly reduced in the lungs, liver, and spleen of *M. tube*rculosis ΔRv3351c-infected BALB/C mice when compared to the complemented strain, 21 days post-infection.



Groups	Infected with	Days P.I.	CFU/ml/half lung	CFU/ml/half spleen	CFU/ml/Liver
1	PBS	21	0	0	0
		21	0	0	0
2	ΔRv3351c	21	34000	3000	<10
		21	44000	2600	<10
		21	22000	2800	<10
		21	16000	1000	<10
		21	20000	2600	<10
Average			27200	2400	<10
3	CMΔRv3351c	21	370000	6000	1000
		21	270000	6200	1000
		21	720000	11000	3700
		21	67000	7500	1500
Average			285400	6140	1440

Table 4.2:Rabbit bacterial burden. Histopathologic scoring of rabbit sections indicated fewer ΔRv3351c bacilli within and associated with type I and type II pneumocytes compared with the parent strain by 96 hours post-infection. Scoring was performed on a graduated scale.



Rabbit #	Total CFU/rabbit	# Necr foci	% epith MP
control	PBS	0	41
Erdman 1-48 hours	2×10^{7}	34	154
Erdman 2- 48 hours	3×10^{7}	5	180
Rv3351c 1- 48 hours	2.5×10^{6}	63	130
Rv3351c 2- 48 hours	9.4×10^{6}	8	90

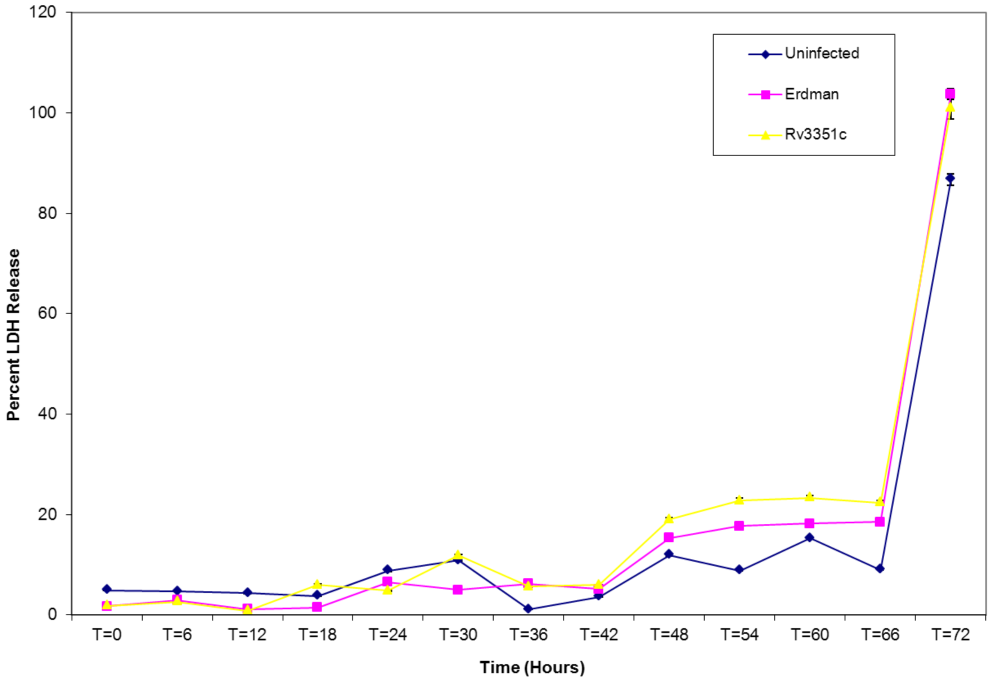


Figure 4.1:Lactate Dehydrogenase assay of murine type II cells. Examination of lactate dehydrogenase (LDH) release from MLE-15 murine alveolar type II pneumocytes following *M. tuberculosis* infection demonstrates a variant phenotype from human pneumocyte cell infections previously shown [21]. MLE-15 monolayers were infected as described in Materials and Methods, with the indicated *M. tuberculosis* strains at an MOI=100. Host cell toxicity was monitored by assaying for release of the cytosolic enzyme LDH into the culture supernatant over time. Data shown are the average of three experiments performed in triplicate. Significant differences (*P* values < 0.05) between mutants and strain Erdman are indicated (*).

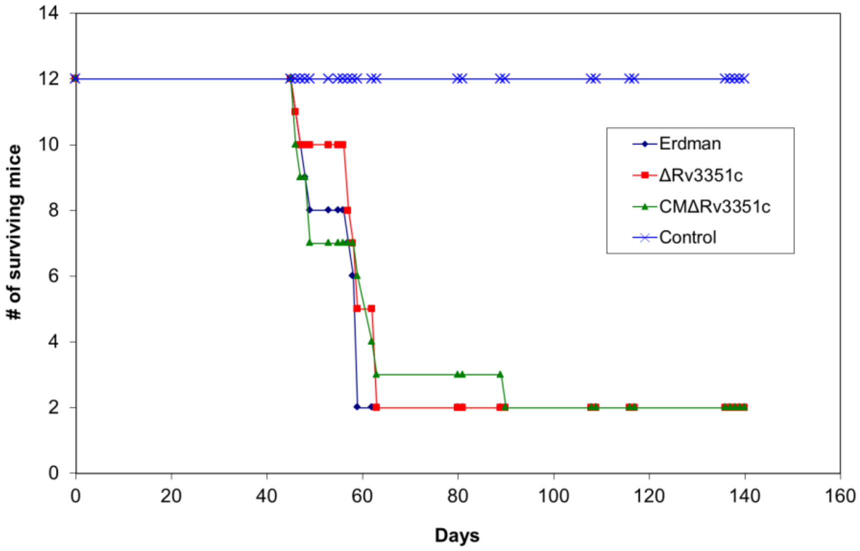


Figure 4.2:SCID mouse infection survival over time. The loss of *Rv3351c* does not confer attenuation of virulence in the SCID mouse model. Survival time of SCID mice intratracheally infected with 1 x 10^4 CFU of *M. tuberculosis* Erdman, ΔRv3351c or CMΔRv3351c up to 140 days post-infection.

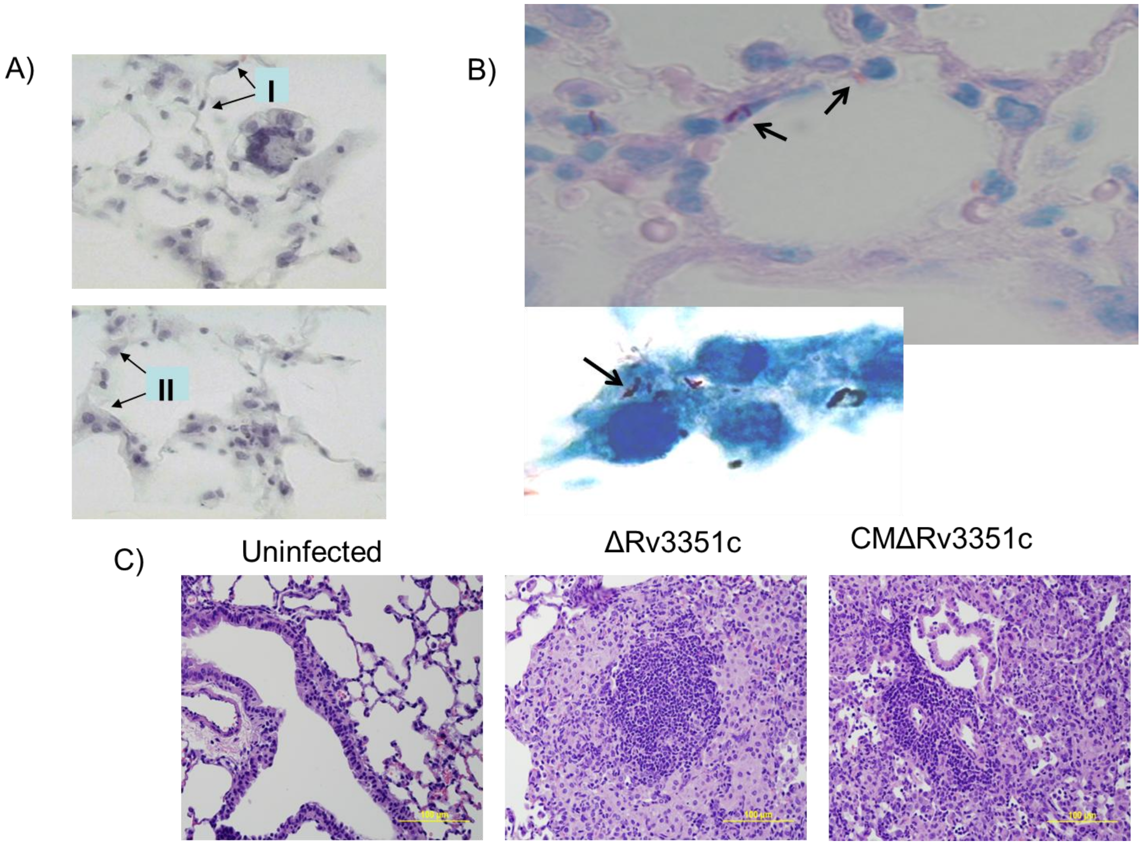


Figure 4.3:BALB/c mouse acid-fast and H&E stained sections. A) Representative photomicrographs of acid-fast stained infected and uninfected BALB/c lung sections, with arrows indicating type I and type II pneumocytes. Type I pneumocytes appear flat with small and elongated nuclei while type II pneumocytes have rounded nuclei. B) Acid-fast stained *M. tuberculosis* Erdman infected lung sections 24 hours post-infection. The bacilli are stained pink while type I and type II pneumocytes can be identified by their location and morphology. Arrows indicate bacteria associated with pneumocytes. C) Lymphocyte infiltration of BALB/c lung sections 21 days post-infection with ΔRv3351c or CMΔRv3351c.

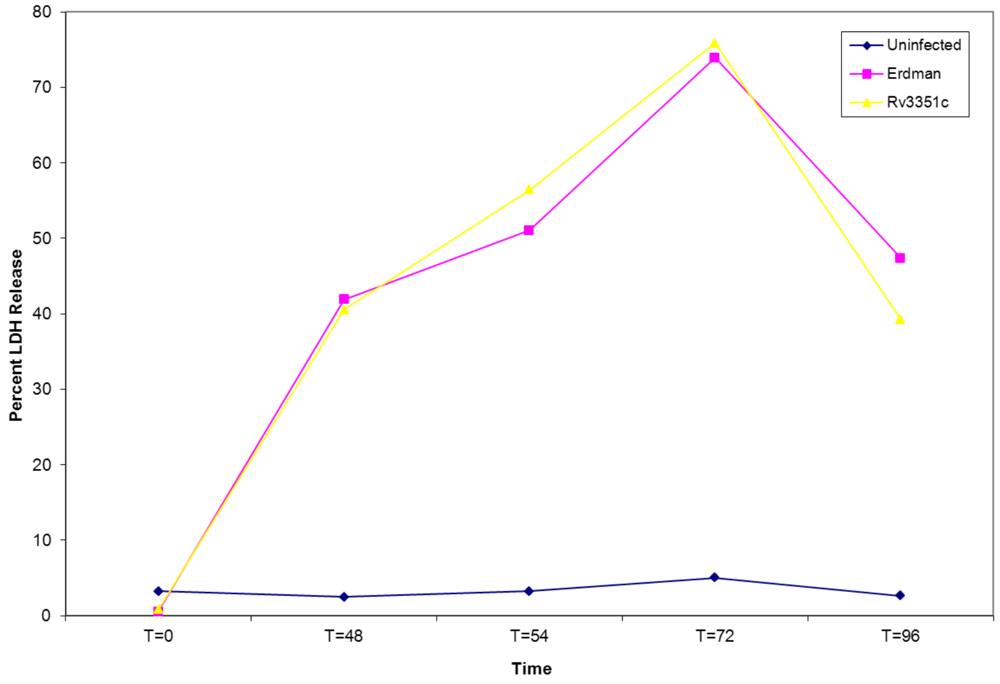


Figure 4.4:Lactate Dehydrogenase assay of primary rabbit type II cells. LDH release of infected primary type II pneumocytes up to 72 hours post- infection. Monolayers were infected with an MOI=100 with either *M. tuberculosis* Erdman or ΔRv3351c, and supernatants were analyzed for LDH release. Data shown are the average of two experiments performed in triplicate.

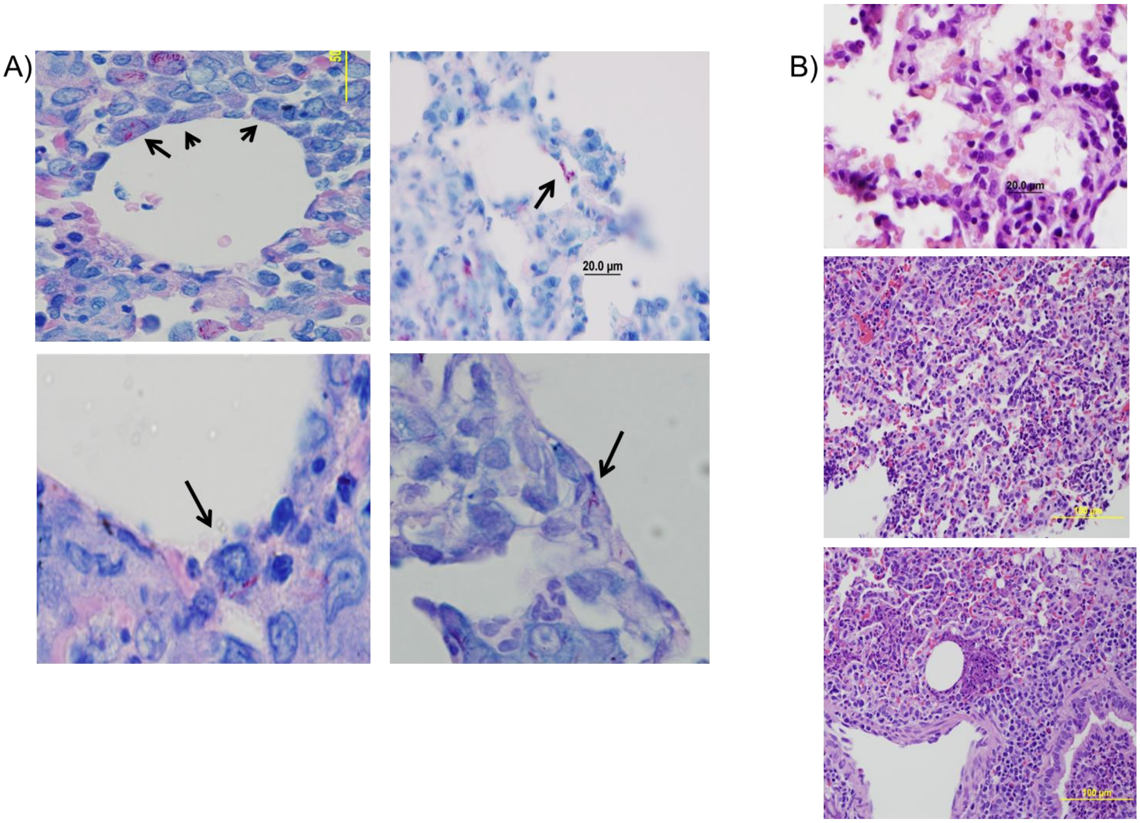


Figure 4.5:Rabbit infection acid-fast and H&E stained sections. A) Representative photomicrographs of acid-fast stained *M. tuberculosis* Erdman- infected rabbit lung sections 48 hours post-infection. The rabbits were intratracheally infected with 2 x 10^6 CFU as described in Methods. The bacilli are stained pink while type I, and type II pneumocytes can be identified by their location and morphology. Arrows indicate areas of bacterial association with pneumocytes. B) Photomicrographs of representative 5 µm sections stained with H&E showing lymphocyte infiltration 48 hours post-infection with *M. tuberculosis* Erdman.

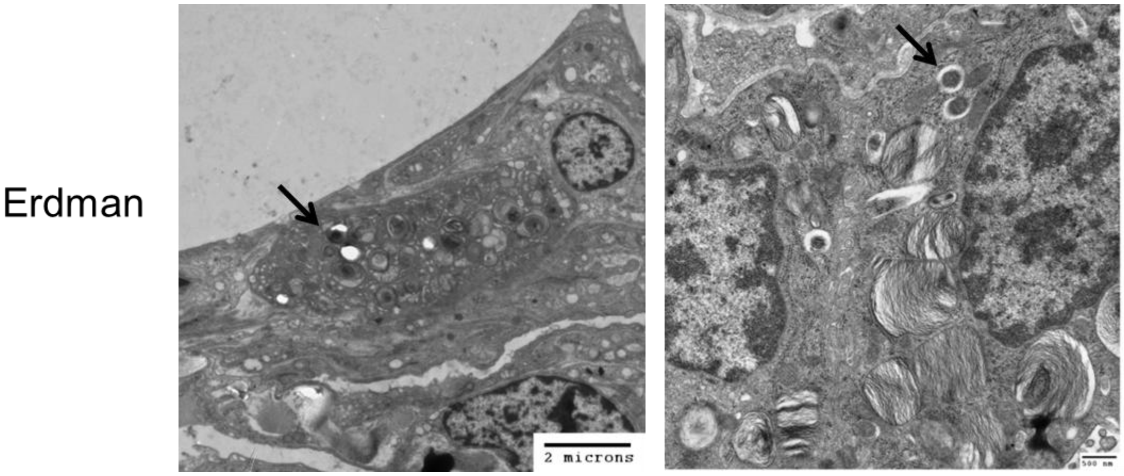


Figure 4.6:Electron micrographs of primary rabbit type II cells. Electron micrographs of intracellular bacilli in primary rabbit type II pneumocytes 72 hours post-infection with *M. tuberculosis* Erdman. Bacteria (electron-dense rods, indicated by arrows) are within membrane-limited vacuoles in the cytoplasm of host cells. Images are representative of one experiment performed in duplicate. An average of 3 grids were examined for each embedded specimen.

CHAPTER 5

EFFECT OF *MYCOBACTERIUM TUBERCULOSIS* RD^{RIO} DELETION MUTANTS ON ALVEOLAR EPITHELIAL CELL TOXICITY

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(To be submitted to Microbiology)

ABSTRACT

The RD^{RIO} strain of *Mycobacterium tuberculosis* has been identified as a member of the Latin American-Mediterranean (LAM) family. Infections with this strain produce an unusually hyper-virulent phenotype including high rates of severe cavitary disease, more rapid onset of acute disease symptoms, high sputum bacillary loads and higher than typical rates of multidrug-resistance compared to patients infected with typical LAM-family strains. The ability to induce hyper-virulence may result from a 26.3 kb, 10-gene deletion found in this strain. Four of the genes within the RD^{RIO} deletion region comprise the putative *Rv3351c* operon; *Rv3351c* has been shown to contribute to *M. tuberculosis* virulence specifically in alveolar pneumocytes. To determine if the 26.3 kb deletion or portions thereof are responsible for the enhanced virulence phenotype, we constructed three mutants in *M. tuberculosis* strain Erdman comprising deletions of the first four genes, the last six genes and the entire 10-genes of the RD^{RIO} region. Infection of A549 human pneumocytes with all three mutant strains showed attenuated virulence similar to that observed for ΔRv3351c when compared to strain Erdman. *Mycobacterium microti*, one of four species comprising the *M. tuberculosis* complex, also possesses a virtually identical RD^{RIO}-like deletion but shows an attenuated phenotype in humans. Like all of the *M. tuberculosis* mutants examined in this study, *M. microti* virulence was attenuated in infected A549 pneumocytes. Thus, the 26.3 kb deletion and the association with alveolar pneumocytes are not responsible for the hyper-virulence phenotype associated with the RD^{RIO} strain, at least under the examined conditions.

INTRODUCTION

Mycobacterium tuberculosis has infected nearly 2 billion people worldwide resulting in 1.7 million annual deaths [17, 27]. The live, attenuated *M. bovis* vaccine, Bacille Calmette-Guerin (BCG), offers at best variable protection against pulmonary tuberculosis (TB) in adults [15, 21, 25]. Co-infection with HIV and the increasing incidence of multidrug resistance are severely complicating control efforts.

Since the implementation of tools for rapid genomic analysis in the 1990s, epidemiological studies have demonstrated the long-term stability of the *M. tuberculosis* genome. Nonetheless, particular families of strains with minute genetic differences have come to predominate in specific geographic locations around the world [6, 16, 17, 29]. In most cases, these strains do not appear to possess enhanced virulence traits, and the genetic and environmental reasons for geographic predominance are not understood. The recent appearance of one particular strain, RD^{RIO}, is of particular concern to the public health community. The RD^{RIO} strain is derived from the Latin American-Mediterranean (LAM) family; infections with LAM strains account for 15% of the global TB burden [5, 6]. The RD^{RIO} strain was first identified in Brazil in the late 1990s, but now has been found on four continents and was estimated to be responsible for 8% of the TB cases in New York between 2001 and 2005 [17]. Human patients infected with this strain present with high rates of severe cavitary disease (80%), multidrug-resistance (12%), faster onset of acute disease, and higher than normal sputum bacillary loads compared with infections with the standard LAM strain [16, 17, 29].

The only known polymorphism between RD^{RIO} and other members of the LAM family is a 26.3-kb deletion (RD^{RIO} deletion) that extends from *Rv3346c* to *Rv3355c*. Two of these genes encode proteins belonging to the PPE family, surface-expressed antigens of immunologic importance [16, 17]. *Rv3351c* is currently under investigation for its association with virulence in alveolar epithelial cells [24]. This gene is potentially part of a four-gene operon including, *Rv3350c*, a gene encoding one of the PPE proteins. When *Rv3351c* is deleted, the bacilli produce a reduced level of necrosis in human epithelial cell monolayers but not in macrophages, when compared to infections with the parent strain [24]. All other genes in the region encode proteins of unknown function.

Mycobacterium microti, a member species in the *M. tuberculosis* complex, possesses >99% sequence homology with *M. tuberculosis*. It also possesses a virtually identical eight-gene, 23 kb RD^{RIO}-like deletion that includes the *Rv3351c* gene and putative operon. Voles infected with *M. microti* develop pulmonary TB, but the organism is avirulent and poorly immunogenic in humans [3, 4, 5, 7, 10, 14, 20, 22, 23, 25, 26]. This deceased human virulence may be due in part to an independent deletion in the *M. tuberculosis* RD1 chromosomal virulence region [1, 15, 18]. However, Pym et al. [2002] showed that re-introduction of this region only restored partial virulence in macrophage and mouse virulence models, indicating that the loss of RD1 is not solely responsible for *M. microti* attenuated virulence [25, 26]. Potentially, the re-introduction of *Rv3351c* and the remainder of the RD^{RIO} region could completely restore *M. tuberculosis*-like virulence.

The development of hypervirulence by *M. tuberculosis* deletion mutants is not a novel observation [6, 16]. Therefore, our hypothesis is that genes within the 26.3-kb RD^{RIO} region are responsible for regulating interactions between *M. tuberculosis* bacilli and alveolar pneumocytes, and that deletion of this region permits enhanced replication within lung pneumocytes, ultimately leading to a more rapid onset and robust clinical presentation. In this study, pneumocyte infections were compared among *M. tuberculosis* Erdman, three Erdman-RD^{RIO} deletion mutants, a *Rv3351c* deletion mutant, and *M. microti*. By comparing the abilities of these various strains to produce necrotic phenotypes in the human type II pneumocyte cell line A549 we may gain a better understanding of how pneumocyte infections are globally regulated, the precise role of pneumocytes in early infection, and potential new target genes for therapeutics and vaccines.

MATERIALS AND METHODS

EPITHELIAL CELL CULTURE

A549 human type II alveolar epithelial cells were obtained from ATCC (CCL-185) and maintained in EMEM (Eagle's Minimum Essential Medium; Gibco) with 5% FBS.

BACTERIAL SPECIES AND STRAINS

Mycobacterium microti and *M. tuberculosis* Erdman strains were obtained from the Mycobacteriology Laboratory Section, CDC. Mutants of *M. tuberculosis* Erdman were generated by deletion of selected genes (Figure 1A) and replacement with a hygromycin resistance cassette using the method of Braunstein et al., 2002 [2]. The generation of the three RD^{RIO} deletion mutants, the *Rv3351c* deletion mutant and the complementing strains was accomplished as detailed in Pavlicek, et al., 2011 [24]. The construction of the *Rv3346c/Rv3355c* chimeric gene identified by Lazzarini et al., 2007 [16] was constructed by insertion of a 1196 bp PCR product containing *folD* plus the identified portion of *Rv3355c* and a 237bp PCR product containing the C-terminal portion of *Rv3346c* into plasmid pMV261. The plasmid was then electroporated into *M. tuberculosis* Erdman and plated on 7H11gtADS containing 50µg/ul kanamycin.

BACTERIAL INFECTIONS

M. tuberculosis strains were grown in Middlebrook 7H9 broth containing 0.5% glycerol, 0.05% Tween 80 and 10% albumin-dextrose-sodium chloride (7H9gtADS) at 37°C in 5% CO_2 with occasional shaking to an OD_{600} of 1.0. Sub-culturing occurred 24 hr prior to infection, and optical density was calculated to be OD_{600} of 1.0 immediately prior to infection. The cultures were centrifuged at 1,000Xg for 5 minutes. Pellets were vortexed and suspended in complete EMEM, then passed through a tuberculin syringe and counted on a Petroff Hauser chamber. Dilutions were performed to infect at MOI of 100 when adding 500µl of bacterial suspension into tissue culture wells. Bacterial inocula were vortexed between each infection and added in a drop-wise fashion. *M. microti* was grown in a similar fashion in media lacking glycerol. During the Lactate Dehydrogenase Assay (LDH assay), all strains were grown in media containing glycerol with the addition of 2 mg per ml sodium pyruvate.

LACTATE DEHYDROGENASE (LDH) ASSAY

A549 cells were seeded at 1.0 x 10^6 cells/ml in 6-well Costar® dishes and allowed to adhere overnight. Medium was changed prior to experimentation. Cells were infected with the indicated *M. tuberculosis* strains or with *M. microti* at an MOI = 100. Culture supernatants were removed at the indicated time points and passed through 0.22 µm PVDF filters prior to storage at 4°C and eventual assay using the Cytotoxicity Detection Kit (Roche, Indianapolis, Ind.)[9]. Percent LDH release was calculated by the following formula: [(Release from Strain - Background)/(Max release – Background)] x 100. Max release refers to lysis obtained by incubation with 0.1% Triton X-100 for 15 minutes. Due to the intrinsic resistance of pneumocytes to the detergent, 100% lysis of the monolayer did not always occur during that time frame. All infections were performed in triplicate and repeated two times.

RESULTS

DELETION OF VARIOUS PORTIONS OF THE 26.3-KB GENE REGION RESULT IN LOWER CYTOTOXICITY LEVELS DURING TYPE II PNEUMOCYTE INFECTION

Infection of A549 pneumocytes with all three *M. tuberculosis* RD^{RIO} mutant strains resulted in reduced LDH release at 72 hours post infection when compared to the wild-type Erdman strain (Figure 5.1). *M. tuberculosis* mutant strains, Rio2 and Rio3, showed a significantly greater reduction in LDH release than was observed with strain Rio1; this mutant strain does not contain a deletion of the *Rv3351c* gene. LDH release levels from infections with Rio2 and Rio3 were similar to results obtained previously using the *Rv3351c* deletion mutant. An alternative hypothesis for the lack of observed hypervirulence is that introduction of the chimeric fusion that has been created in the RD^{RIO} clinical isolate upregulates as yet unidentified virulence factors. Preliminary results introducing the chimeric fusion into wild-type Erdman demonstrated an increase in cytotoxicity compared to the parent strain via LDH release during *in vitro* infections (data not shown). The chimera has yet to be transformed into the Erdman-Rio deletion strains.

MYCOBACTERIUM MICROTI DEMONSTRATES SIMILAR HOST CELL CYTOTOXICTY WHEN COMPARED TO THE *RV3351C* DELETION MUTANT

Mycobacterium microti is well documented as having low virulence and poor immunogenicity in humans, likely due to large genomic deletions, including RD1 and a significant portion of the 26.3-kb RD^{RIO} region. Initial studies indicated that *M. microti* grows as rapidly as *M. tuberculosis* Erdman in 7H9 broth medium containing 0.5% glycerol, 0.05% Tween 80, 10% albumin-dextrose-sodium chloride and 2mg per ml sodium pyruvate (Figure 5.2). *In vitro* infection of A549 pneumocytes with either *M. microti* or *M. tuberculosis* ΔRv3351c showed a similar reduced LDH release at 72 hours post-infection when compared to a wild-type Erdman strain (Figure 5.3). This decreased necrosis phenotype in infected type II pneumocytes may be a result of the natural deletion of *Rv3351c* that is present in *M. microti* but not in other members of the *M. tuberculoisis* complex. Re-introduction of RD1 and *Rv3351c* genes may restore virulence and is currently under investigation in our laboratory.

DISCUSSION

Mycobacterium tuberculosis and *Mycobacterium microti* are both members of the *M. tuberculosis* complex but have significantly different host specificity and virulence phenotypes in humans [3, 4, 5]. Attenuation of *M. microti* may be due in part to a naturally occurring deletion of the RD1 region, which is known to be important for the attenuation seen in *M. bovis* BCG vaccine strains [20]. However, Pym et al. [2002] demonstrated using a knock-in model of the RD1 gene region that virulence was increased but not fully restored in macrophages and mice using their complemented *M. microti* strain [25, 26]. This has led to the search for other deleted or modified genes that might be responsible for the decreased *in vitro* and *in vivo* virulence and immunogenicity observed with *M. microti* [21, 25] and resulted in discovery of a second large eight-gene deletion that includes *Rv3351c* and its putative operon [24].

It is hypothesized that the development of new, emerging and re-emerging bacterial pathogens stem from gross and subtle genomic modifications of existing strains. Examining *M. tuberculosis* genomic data, a clinical isolate designated RD^{RIO} was identified that possesses a multi-gene deletion similar to that identified in *M. microti*, including *Rv3351c*, but produces a drastically different virulence phenotype in infected humans [1, 4, 16]. *M. tuberculosis* RD^{RIO} is a highly-virulent strain which presents with severe pulmonary disease, greater lung bacterial burdens, enhanced capability to garner antibiotic resistance mutations and is highly-transmissible [13, 16, 17, 29]. It is a member of the Latin-American-Mediterranean family (LAM) and contains a large sequence polymorphism (RD^{RIO} deletion) that extends from *Rv3346c* to *Rv3355c* [29]. This 10-gene region encodes several hypothetical genes, and the deletion of this region produced a chimeric fusion of *Rv3346c* and *Rv3355c*.

Lactate Dehydrogenase (LDH) release is used as an indicator of cellular stress and membrane permeation and can be used as an indicator of cell death and monolayer destruction [9]. It has been well documented that during *M. tuberculosis in vitro* infections, type II pneumocytes are killed via necrosis as opposed to apoptosis [9]. In this study, it was observed that infections with *M. microti* and strains possessing deletions of the RD^{RIO} region resulted in significantly reduced type II cellular necrosis as measured by LDH release [24]. This is likely directly related to the deletion of the *Rv3351c* gene [24]. Interestingly, decreases in cytotoxicity were no greater between infections with the 10-gene or single gene deletion mutants. We hypothesize that re-introduction of the *Rv3351c* gene may restore the virulent phenotype in the three *M tuberculosis* deletion mutants (Rio1, Rio2 or Rio3) and the *M. microti*-Pym-RD1 complemented strain.

Although our initial hypothesis that deletion of this 26.3-kb region would lead to hypervirulence in pneumocytes was not proven correct under the conditions examined, more experimentation is required in order to identify the responsible component and definitively demonstrate the role of the RD^{RIO} deletion. *In vitro* tissue culture analysis may not be sufficient, and rabbit aerosol studies with the RD^{RIO} clinical strain and Erdman mutants Rio 1-3 will be needed to truly mimic and define the hypervirulent phenotype. Alternatively, introduction of the *Rv3346c/Rv3355c* chimeric fusion into the Erdman Rio 1, 2 or 3 mutants may result in the production of a hypervirulent phenotype *in vitro* and *in vivo*. Complementing the 26.3-kb deletion in the RD^{RIO} strain and *M. microti*-Pym-RD1 complemented strain are also planned.

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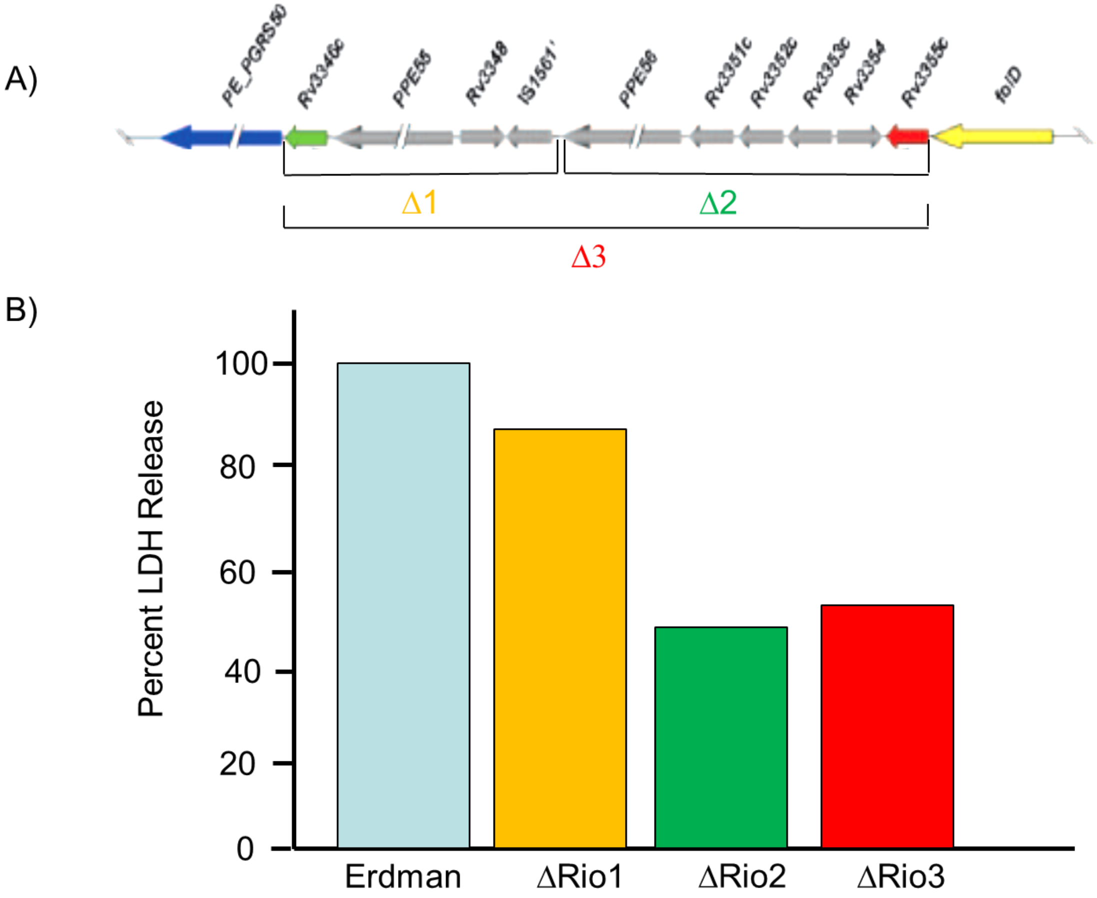

Figure 5.1:Drawing of deletion region and Lactate Dehydrogenase assay. A) Schematic of *M. tuberculosis* RD^{RIO} deletion region. Open reading frames and orientations are shown. Genes deleted are indicated for the three deletion mutants. B) Examination of lactate dehydrogenase (LDH) release from host cells 72 hours following *M. tuberculosis* RD^{RIO} deletion mutant infection. A549 cells were infected as described in Methods with the indicated *M. tuberculosis* strains at an MOI=100. Host cell toxicity was monitored by assaying for release of cytosolic enzyme LDH into culture supernatant. Data shown are the average of two experiments performed in triplicate.

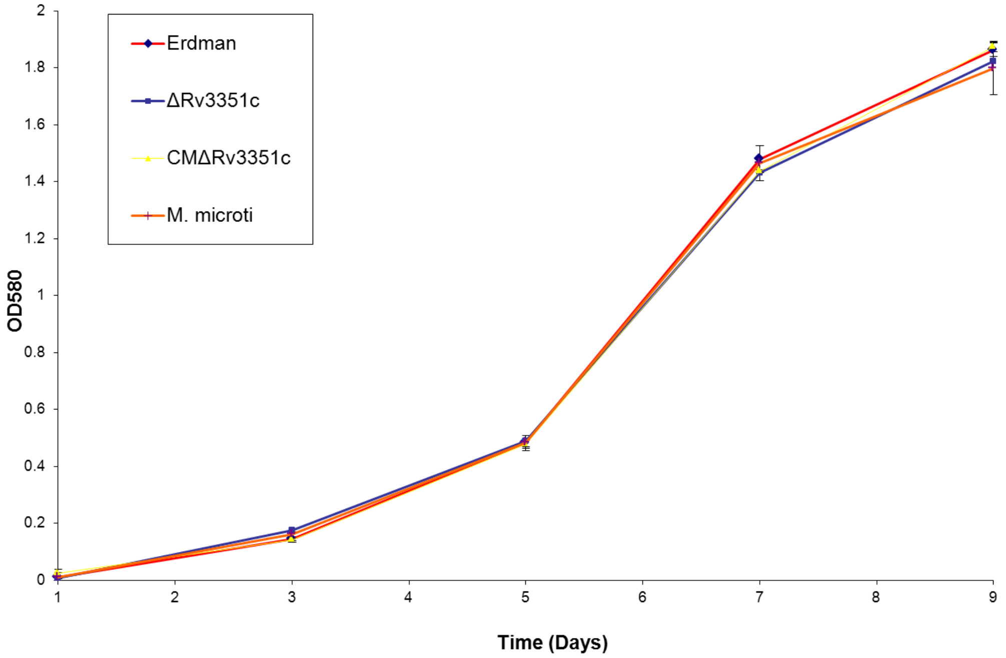

Figure 5.2:Growth curve of various mycobacterial species. Growth of *M. tuberculosis* strains or *M. microti* in Middlebrook 7H9 broth. Optical density (580 nm) was monitored in triplicate at each time point. Experiment was performed in duplicate and repeated with similar results.

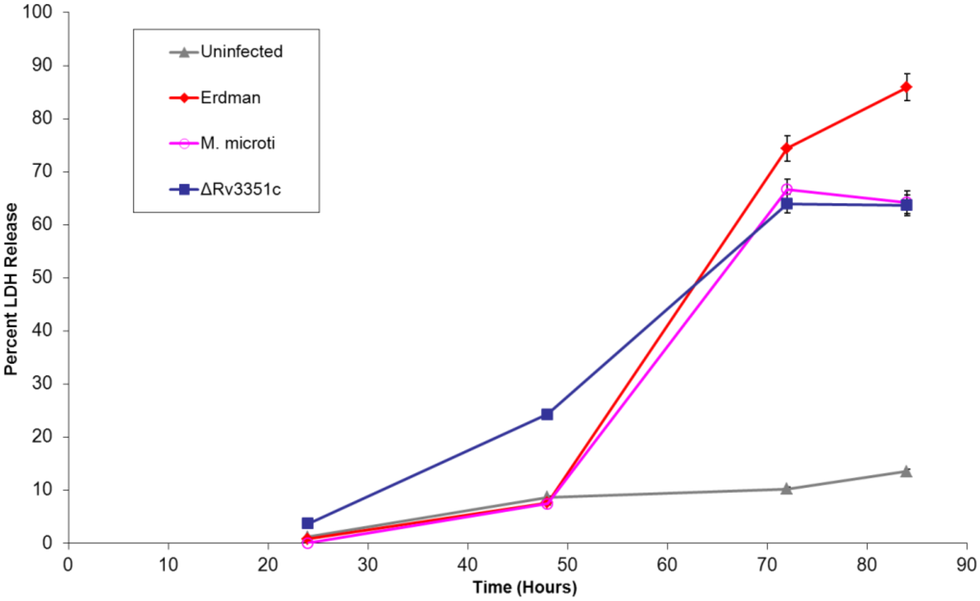

Figure 5.3:Lactate Dehydrogenase assay of various mycobacterial species. Examination of lactate dehydrogenase (LDH) release from host cells following *M. tuberculosis* or *M. microti* infection. A549 AECs were infected as described in Methods with the indicated *M. tuberculosis* or *M. microti* strains at an MOI=100. Host cell toxicity was monitored by assaying for release of cytosolic enzyme LDH into culture supernatant over time. Data shown are the average of three experiments performed in triplicate.

CHAPTER 6

CONCLUSIONS

Tuberculosis (TB) has evolved over the millennia as the single most important human respiratory disease. Events in our history likely have been altered due to the debilitating and ultimately fatal attributes of *Mycobacterium tuberculosis* infections. Unfortunately, this disease continues unabated as one of the world's top public health and economic nemeses.

Despite over 130 years of research, the existing vaccine, diagnostic test and treatment regimens for TB are sub-optimal. The prevention and control tools currently in use were developed through trial and error between 1880 and 1970 and have tended to target the acute phase of the disease. This is the most well-studied stage and occurs after the bacteria replicate to high numbers in the lungs, manipulate host defences, disseminate to the blood and other organs, and induce the "classical" TB clinical symptoms. A new generation of vaccine, drug and diagnostic candidates that were decades in the making are now being slowly deployed in the US and worldwide. These novel tools are designed around new understandings of the interactions between the host and pathogen. However, these novel reagents again target acute disease.

The course of a *M. tuberculosis* infection can roughly be divided into three stages: colonization, acute, and persistence/transmission. Most successful vaccines and therapies currently available for other bacterial diseases target the colonization stage; when pathogen numbers are low and most easily controlled. Due primarily to technical limitations, lung colonization by *M. tuberculosis* bacilli traditionally has been the most understudied stage of the disease. Within seconds or minutes after the bacilli are inhaled, appropriately sized particles transit through the upper airways and are deposited in the lung alveoli. Dogma states that resident non-activated alveolar macrophages and likely dendritic cells phagocytose and kill most of the invading bacilli. Occasionally, some intracellular mycobacteria survive, replicate, disseminate and ultimately proceed into the subsequent stages of the infection. If dogma were correct, this hypothetical course of disease would make *M. tuberculosis* unique among all other studied bacterial, viral and fungal lung pathogens. In known instances, alveolar pneumocytes and phagocytic cells both internalize the infecting organisms. Pneumocytes are considered part of the innate immune system, and thus also play an important role in the ultimate success or failure of these infections.

The experiments presented within this study were designed to identify the interactive mechanisms that are used specifically between the *M. tuberculosis* bacilli and alveolar epithelial cells within the first hours and days after aerosol infection, and determine their ultimate importance in dissemination of the bacilli from the lungs. The research hypothesis addressed was that *M. tuberculosis* bacilli can infect, survive and replicate within alveolar type II and potentially type I pneumocytes, and that these events provide an opportunity for the bacteria to avoid phagocytic cells capable of activating the host adaptive immune response. The specific aims that address the hypothesis were as follows:

Specific Aim1: Determine if the *M. tuberculosis Rv3351c* gene is associated with survival specifically within human type II pneumocytes. The *working hypothesis* was that the deletion of the *Rv3351c* gene, in a similar fashion to what has been demonstrated previously with the *hbhA* gene, will negatively impact the ability of the mutant bacilli to survive within type II pneumocytes, but not within macrophages.

The data shown in chapter 3 demonstrate that the *Rv3351c* gene is associated with the ability of the bacilli to induce a necrotic phenotype after infection of type II pneumocytes but that persistence/survival of the bacilli in these cells was not negatively affected by the deletion of this gene. It was confirmed that the *hbhA* deletion mutant also produces a significantly decreased cytotoxic effect on type II pneumocytes, but it could not be determined from the experiments performed here if the virulence mechanism employed by the *hbhA* gene product is similar to that of Rv3351c. We were able to demonstrate that decreased necrosis after infection with either mutant strains was specific to type II pneumocytes; macrophage virulence was identical after infections with the mutant or control strains. Lastly, it was hypothesized that *Rv3351c* was part of a four-gene operon, however, i*n vitro* experiments suggest that the genes constituting this putative operon are independently regulated.

Specific Aim 2: Identify *M. tuberculosis* bacilli associated with type I and type II pneumocytes within hours or a few days after aerosol infection *in vivo*. Determine if the *Rv3351c* deletion mutant is attenuated *in vivo*. The *working hypothesis* is that *M. tuberculosis* bacilli associate with type I and type II cells soon after inhalation of the bacilli into the alveoli, and that the *Rv3351c* deletion will significantly attenuate the bacterium in both the murine and rabbit infection models.

The results shown in chapter 4 demonstrate that the ΔRv3351c mutant was not attenuated in the SCID mouse model. However, in BALB/C mice, ΔRv3351c bacilli replicated 10-fold less and disseminated to the spleen and liver several fold less than the corresponding control strain bacteria. Interestingly, there were no significant pathological differences observed in lung tissues from mice infected with the mutant or control strains at any of the time points post infection. These findings are consistent with *in vivo* data using the *hbhA* mutant strain. In addition, rabbits, like the BALB/C mice, infected with the ΔRv3351c and control strains demonstrated very similar lung pathology over the course of the infection, however, visual scoring clearly demonstrated fewer ΔRv3351c than parent bacilli associated with the rabbit type I and type II cells by 96 hours post infection.

Specific Aim 3: Determine if a 26.3-kb deletion detected in the RD^{RIO} clinical strain is responsible for its observed hyper-virulent phenotype in human lungs. This deletion is of particular interest since *Rv3351c* and the genes comprising its putative operon are located within this region. The working hypothesis is that this region of the chromosome is affiliated with pneumocyte interactions, and that deletion of this entire region in laboratory strain Erdman will lead to induction of hypervirulence in the pneumocyte tissue culture infection model. Examining this region of the *M. tuberculosis* genome will provide a greater understanding of the regulation of *Rv3351c* and other important pneumocyte-specific virulence genes.

Results from chapter 5 demonstrate that through genetic recombination, the 26.3-kb region surrounding the *Rv3351c* gene was successfully deleted in strain Erdman. Pneumocyte infections with this mutant strain demonstrated that deletion of this region alone is not sufficient to produce a hypervirulent phenotype in this cell type. In fact, deletion of this region caused a similar decrease in cytotoxicity levels during pneumocyte infection consistent with what was previously measured with ΔRv3351c infections. Additionally, *M. micoti*, a species in the *M. tuberculosis* complex, causes TB in voles but is avirulent in humans. This species also is missing much of the RD^{RIO} region. Infection of type II pneumocytes with *M. microti* also produces a less necrotic phenotpye in pneumocyte cultures, similar to the *Rv3351c* mutant, but different than *M. tuberculosis* Erdman and $\mathrm{RD}^{\mathrm{RIO}}$ strains. More work needs to be done, but strains possessing this deletion alone do not induce a hypervirulent phenotype in infected pneumocytes. A more complex *in vivo* analysis may be required in order to truly mimic the phenotype. Alternatively, other subtle differences in the genome of the $\mathrm{RD}^{\mathrm{RIO}}$ clinical isolate await identification and study.

Together, these *in vitro* and *in vivo* findings provide compelling evidence for the existence of a previously unidentified *M. tuberculosis* virulence mechanism that specifically targets type II and potentially type I pneumocytes. By further studying the *Rv3351c* and *hbhA* mutants, we may gain a better understanding of how the bacteria are able to successfully colonize the lung alveoli and potentially devise vaccine or therapeutic candidates that target this disease stage.