Studies into the Role of Two Mycobacterial Proteins in Stress Response and Survival Inside Macrophages

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ABSTRACT

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The success of *M. tuberculosis* lies in its ability to stay alive and persist in a potentially hostile environment represented by the macrophage phagosome. Hence there is a desperate need to identify the molecular mechanisms and associated proteins enabling mycobacterial survival and replication inside macrophages. Recent studies have shown that several mycobacterial proteins may play distinct roles during different stages of infection. This thesis was focused on investigation of the biological function of two mycobacterial proteins, Rv1219c and Rv3136 (PPE51 protein) during macrophage infection and stress response.

A recent study has established that RaaS (Rv1219c in *M. tuberculosis* and BCG_1279c in *M. bovis* BCG) mediates mycobacterial survival in stationary phase and during mouse infection. RaaS (for regulator of antimicrobial-assisted survival) controls expression of ATP-dependent efflux pumps Bcg_1278c/1277c and DrrC and mediates survival of *M. bovis* BCG in growth non-permissive conditions. One of the aims of this project was investigating the role of RaaS in mycobacterial replication and persistence in macrophages. The result showed that Δ*raaS* mutant of *M. bovis* BCG was significantly impaired in initial survival in macrophages. Moreover, the mutant was extremely sensitive to H_2O_2 and low pH, the stress factors, which probably influence mycobacterial viability upon their uptake into macrophages. Treatment with reserpine, an inhibitor of ATP-dependent efflux pumps, prior to stress exposure had managed to improve the survival of the mutant suggesting that the impaired stress response of the *raaS* mutant was due to dysregulation of efflux pumps. While BCG_1279c and its orthologous in *M. tuberculosis*, Rv1219c, are important for survival inside macrophages and in stationary phase, published data has shown that Rv3136 is up-regulated during mycobacterial replication in macrophages. To establish the role of Rv3136 in macrophage infection and virulence, an *rv3136* deletion mutant was generated in *M. tuberculosis* H37Rv. The mutant did not show any growth defect in laboratory medium. Although macrophage infection experiments did not link *rv3136* to survival in macrophages, preliminary data from mouse infection indicated that Rv3136 could potentially play a role in survival inside the host.

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ABC	ATP-binding cassette
ADC	Albumin dextrose catalase
AFB	Acid fast bacilli
AG	Arabinogalactan
ANOVA	Analysis of variance
ART	Antiretroviral therapy
ATP	Adenosine triphosphate
BCG	Bacille Calmette-Guerin
BCIP/NBT	5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt/nitro-blue tetrazolium chloride
Bp	Base pair
cAMP	Cyclic Adenosine Monophosphate
CFU	Colony forming unit
CFP10	10 kDa culture filtrate antigen
CRP	Cyclic adenosine monophosphate receptor proteins
C	Cytosine
DIG	Digoxigenin
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOTS	Directly observed treatment, short-course
EMSA	Electromobility shift assay
ESAT-6	Early secreted antigenic target 6
FCS	Foetal calf serum
G	Guanosine

HIV	Human immunodeficiency virus
H_2O_2	Hydrogen peroxide
hsps	Heat shock proteins
icl	Isocitratelyase
IGRAs	Interferon-gamma release assays
IFN γ	Interferon γ
INH	Isoniazid
iNOS	Inducible nitric oxide synthase
Kb	Kilobase
LAM	Lipoarabinomannan
LB	Lysogeny broth
LM	Lipomannan
MDR	Multi-drug resistant
MDG	Millennium Development Goal
M	*Mycobacterium*
ml	Millilitre
mM	Millimolar
MOI	Multiplicity of infection
MQ	Macrophage
MT	Mutant
MTBC	*Mycobacterium tuberculosis* complex
Mtb	*Mycobacterium tuberculosis*
mAGP	mycolyl Arabinogalactan–peptidoglycan
NK cells	Natural killer cells
NO	Nitric oxide

NRP	Non-replicating persistence
NTM	Non-tuberculosis mycobacterial
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDIMs	Phthiocerol dimycocerosates
PIMs	Phosphatidylinositol mannosides
PG	Peptidoglycan
PMA	Phorbol 12-myristate 13-acetate
pM	Picomolar
RaaS	Regulator of antimicrobial assisted survival
RIF	Rifampicin
RNI	Reactive nitrogen intermediates
Rpm	Rotation per minute
ROI	Reactive oxygen intermediates
rpfA	Resuscitation-promoting factor A
SEM	Standard error of the mean
SN	Culture supernatant
ssDNA	Single stranded DNA
TACO	Tryptophan aspartate containing coat protein
TAG	Triacylglycerol
TB	Tuberculosis
TCA	Tricarboxylic acid
T-cells	T-lymphocytes
TLR	Toll like receptor

TNFα	Tumour necrosis factor α
WHO	World health organisation
WT	Wild type
XDR	Extensively-drug resistant

Chapter 1 **:Introduction**

1.1 General Introduction

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* (*M. tuberculosis*). WHO reports have identified TB as the second leading cause of death from a single infectious agent, after the human immunodeficiency virus (HIV) (WHO, 2014).The TB burden has been complicated by the failure to develop an efficient TB vaccine, the prolonged period of treatment, TB/HIV co-Infection, the emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains of *M. tuberculosis* (Sakamoto, 2012). Many of the challenging physiological characteristics of *M. tuberculosis* such as resistance to antimicrobial agents and host defence mechanisms are attributed to its unique cell wall structure. The cell wall contains mycolic acids which are covalently attached to the underlying peptidoglycan-bound polysaccharide arabinogalactan (Lee et al., 2005).

TB is an ancient disease that has affected mankind for over thousands of years. Evidence for this assumption came from a study which has documented pulmonary and osseous TB in a mummy of an Egyptian child which dates back to more than 4000 years ago (Zimmerman, 1979). However, a subsequent study used morphological and molecular methods to provide evidence of TB in humans which dates back to over 8,000 years ago in a Neolithic settlement in the Eastern Mediterranean (Hershkovitz et al., 2008). *M. tuberculosis* was discovered in 1882 when the German physician and microbiologist Robert Koch announced the discovery of the bacterium which caused what was considered to be at that time as the most important disease of mankind (Murray, 1989). At that time, he referred to the causative agent of TB as Tuberkelbazillus. It wasn't until 1891, when Lehmann and Neumann used the term *Mycobacterium tuberculosis* (Grange, 2009).

One in every three people is thought to be infected with *M. tuberculosis* during their life time. However, the majority of those people will never show overt symptoms of the disease. The risk for developing active TB for latent carriers is estimated to be 2-23% unless the immune system is suppressed in which the risk increases to 10% (Zahrt, 2003).

TB is usually transmitted through inhalation of air borne droplets containing tubercle bacilli from an infected person. Among other infectious diseases, TB is thought to be the only communicable disease which exclusively transmits through the air (Roy and Milton, 2004). Once inside the lungs, the tubercle bacilli travel to the alveoli where they are phagocytosed by alveolar macrophages. Factors which influence the transmission of TB include number of bacilli expelled into the air, length of exposure, space of ventilation and the immune status of the exposed person (Knechel, 2009).

Although, pulmonary TB is the most common form of the disease, other organs such as gastrointestinal tract, bones/ joints, lymph nodes, genitourinary tract and central nervous system can also be affected with a condition known as extrapulmonary TB (Kruijshaar and Abubakar, 2009). Clinical symptoms, radiological assessment and isolation of *M. tuberculosis* from clinical samples are all used in the diagnosis of active TB (Parrish and Carroll, 2011).

Methods based on nucleic acid amplification are also used in diagnosis. Although being sensitive, these methods cannot differentiate between live and dead bacilli which imply that they can't be used to monitor the efficacy of treatment. Blood based tests which measure host biomarkers have also been employed for diagnosis of TB. An example of such tests is the IFN-γ release assays (IGRAs). These assays are designed to measure IFN-γ^+ production in response to stimulation with *M. tuberculosis* specific antigens ESAT6 (6 kDa early secretory antigenic target) and CFP10 (10 kDa culture filtrate antigen). They are preferred over the traditional tuberculin skin test that has the possibility of giving false positive results. However, IGRAs are inadequate in distinguishing between active and latent TB (Thillai et al., 2014). A recent study has identified a number of biomarkers in T cells of TB patients (CD38, HLA-DR and the intracellular proliferation marker Ki-67); these markers can be instrumental in monitoring treatment response and distinguishing between active and latent forms of TB (Moon et al., 2015).

1.2 The Global Burden of Tuberculosis

In the early nineties, the WHO declared TB as a global emergency and subsequently introduced the internationally recommended TB control strategy which was named as directly observed treatment, short-course (DOTS). DOTS implementation achieved remarkable progress in TB control. However, by 2005 the progress was insufficient to reach the target set by the WHO. Consequently, the WHO launched the second Global Plan to Stop TB (2006–2015) which has a medium-term goal to achieve reductions in incidence, prevalence and mortality rates of TB by 2015. The longer term goal is to eliminate TB by reducing the yearly incidence to less than 1 case per 1000 000 population by 2050 (Onozaki and Raviglione, 2010).

Although the last decade has seen a slow decline in TB due to early diagnosis and effective treatment but TB is still considered as a global emergency with an estimated 9.0 million new TB cases and 1.5 million deaths in 2013. Of those deaths, 360 000 were HIV-positive with a large majority being from sub-Saharan Africa. TB cases from the South-East Asia and Western Pacific regions accounts for 56% of the total cases estimated in 2013. The highest rates of cases and deaths relative to population were from the African region which accounted for 25% of the total cases worldwide. Incidence rates in India and China represents 24% and 11% of total global cases respectively (WHO, 2014). It is thought that TB can be one of the leading causes of death in women during their productive age. Furthermore, untreated TB is one of the most common non-obstetric causes of death in pregnant women with an estimated mortality rate of 40% in non-HIV patients and 49% in HIV-infected pregnant women (Mathad and Gupta, 2012). Figure 1 shows the estimated TB incidence in 2013.

Statistics published in the WHO Global TB report for 2014 showed that the global TB incidence fell at an average rate of about 1.5% per year between 2000 and 2013 achieving the 2015 Millennium Development Goal (MDG) of halting and reversing TB incidence worldwide. However, the same report indicated more progress is needed to reach the target of 50% reduction in prevalence and mortality rates. The reduction in Incidence, prevalence and mortality rates seen in the African, Eastern Mediterranean and European regions was not sufficient to reach the targets of 2015 (WHO, 2014). Data on TB burden among children less

than 15 years have recently become available as part of the annual WHO TB reports. The latest report published in 2014 estimated 550 000 cases and 80 000 deaths among HIV negative children. These figures are worrying given the fact that death from TB can be prevented if appropriate health care and treatment are provided (WHO, 2014).

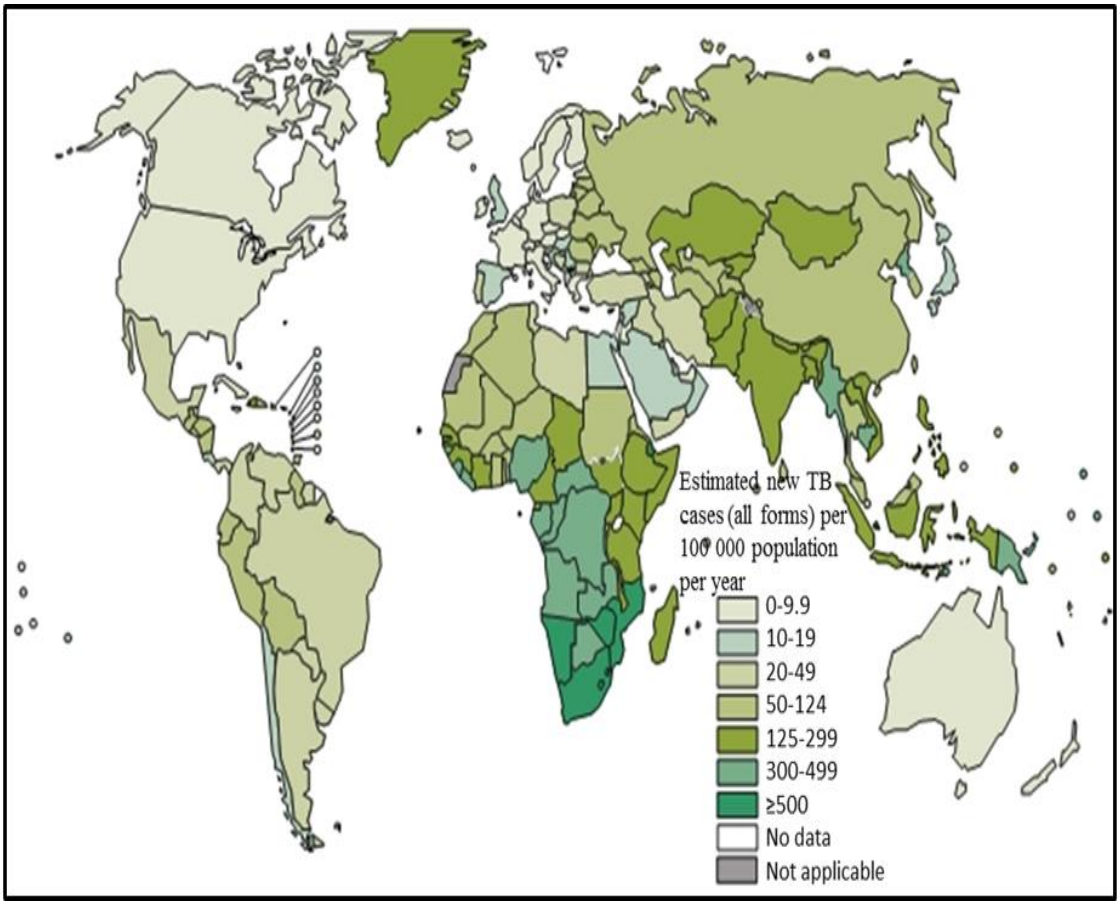


Figure 1: Estimated TB incidence rates, 2013.
Geographical distribution of the estimated TB incidence for the year 2013. Figure adapted from the Global tuberculosis report 2014 (WHO, 2014).

Until recently, TB control programs have not given enough priority to childhood TB probably due to difficulties in isolation of mycobacterium in children as most cases are sputum-smear negative on microscopy. In children, recent exposure to the bacilli is thought to be the cause behind active disease (Jurcev-Savicevic et al., 2011). Therefore childhood TB burden is thought to be a good indicator of the efficiency and progress achieved by TB control programs in a certain setting. TB control programs have recently recognised the importance of defining guidelines for treatment and management of childhood TB. In addition, developments in

treatment and diagnosis of childhood TB have been included as an important element in global TB control efforts (WHO, 2014).

Of the major challenges facing global TB control, is the emergence and spread of MDR TB and XDR TB. MDR strains are defined as those which are resistant to at least rifampicin (RIF) and isoniazid (INH), while XDR strains are defined as MDR-TB which has developed additional resistance to fluoroquinolones and at least one second-line injectable drug (Zhang and Yew, 2009). One of the factors leading to the emergence of TB drug resistance is poor adherence to therapy (Berti et al., 2014). In 2013, the WHO estimated that 20.5% of previously treated patients and 3.5% of new cases were MDR-TB. The proportion of XDR-TB among those patients with MDR-TB was 9%. Treatment outcome for patients with MDR-TB who started in 2011 showed a low global cure rate (48%). Insufficient resources, ineffective treatment regimens and lack of adequate funding are all thought to have contributed to this worryingly low cure rate (WHO, 2014). Improved diagnostics of anti TB drug resistance, rapid enrolment on second line treatment and development of new drugs are important measures needed to bridge the growing diagnosis- treatment gaps in some countries (Zhang and Yew, 2009).

Another major challenge which imposes huge impact on global TB control is the TB/HIV co-epidemic. Data from the 2010 Global TB control report showed that the African region has the highest TB/HIV burden. TB patients from the sub-Saharan Africa region accounted for 82% of the global burden of HIV associated TB cases and 71% of the related deaths (WHO, 2010). The high mortality rates amongst HIV-associated TB patients are thought to be either due to TB not being diagnosed in HIV patients, not offering HIV test to all TB patients resulting in missing the diagnosis of HIV in TB patients or late intervention for patients diagnosed with HIV-associated TB. Tackling the TB/HIV co-epidemic starts with HIV testing for all TB patients. It is thought that long term reduction in TB incidence and mortality can partly be achieved through expanded use of antiretroviral therapy (ART) among those with HIV (Chindelevitch et al., 2015). Another important intervention is the use of new sensitive and specific diagnostic tools. One of the important and revolutionary diagnostic developments is the commercially available Xpert MTB/RIF assay (Cepheid, Inc., Sunnyvale, CA, USA). This assay is a rapid and sensitive nucleic acid amplification test capable

of detecting *M. tuberculosis* and its rifampicin resistant strains in sputum and other body specimens (Boehme et al., 2010).

Other TB risk factors which are thought to partially influence Global TB control programs include malnutrition, smoking, alcoholism, diabetes and indoor air pollution (Lonnroth and Raviglione, 2008). Finally, providing quality health care to all patients regardless of age, gender and socio-economic status will greatly help in implementing the guidelines of TB control programs. In addition, elimination of TB can only be achieved when sufficient support is provided for development of new diagnostics, drugs, and vaccines.

1.3 General characteristics of *Mycobacterium tuberculosis*

Mycobacteria are non-spore forming, non-flagellate, weakly gram positive, straight or slightly curved rods (Grange, 2009). Depending on the growth rate, the genus *Mycobacterium* is generally divided into slow growers and fast growers (based on the ability of strains to develop visible colonies in less or more than 7 days, respectively). The slow growers are thought to be closely related to the genus *Nocardia* (Tortoli, 2003). The genus *Mycobacterium* includes either obligate pathogens (*M. tuberculosis* complex and *M. leprae*) or environmental mycobacteria (found in marshes, lakes, and rivers) which causes what is known as non-tuberculosis mycobacterial (NTM) infections (Grange, 2009).

The approximate generation time for *M*. *tuberculosis* in synthetic media is 24 hours. This lengthy generation time is one of the factors contributing to long duration of treatment regimens. Dormancy is one of the primary characteristic features of tubercle bacilli (Cole et al., 1998). Dormant bacilli are viable cells in a state of low metabolic activity. Certain dormant bacteria have no ability to form colonies on solid media but can be resuscitated when appropriate conditions become available. Reactivation of dormant bacilli may occur when the host immune system become compromised resulting in an active disease (Kell et al., 1998).

One of the most distinguishing characteristics of *M. tuberculosis* is acid fastness. Components of the mycobacterial cell wall are responsible for this staining property. It has been shown that the mycobacterial lipid-rich cell envelope could

be penetrated by phenol-based stains but resists decolonization by acid-alcohol. Hence the term acid fast bacilli (AFB) are often used to describe mycobacteria. However, the exact component responsible for this property remains to be elucidated (Yamada et al., 2012).

Many components of mycobacterial cell wall are involved in *M. tuberculosis* pathogenicity. The mycobacterial cell wall is composed of an inner part and an outer section. The inner part is composed of peptidoglycan (PG) covalently attached to arabinogalactan (AG) which in turn is attached to the mycolic acids forming the mycolyl arabinogalactan–peptidoglycan (mAGP) complex. This complex forms the cell wall core. The upper section is composed of free lipids, cell-wall proteins, the phosphatidylinositol mannosides (PIMs), lipomannan (LM), lipoarabinomannan (LAM) and the phthiocerol-containing lipids (Brennan, 2003). *M. tuberculosis* complex (MTBC) is a term used to describe the different species that cause TB in humans and mammals. Members of the MTBC that cause TB in humans include *M. tuberculosis*, *M. africanum* and *M. canetti.* Members of the MTBC are genetically very closely related but differ in their principal host range (Grange, 2009). Table 1 show the species name and host range of MTBC.

Table 1: Members of the MTBC and their hosts

Mycobacterial species	**Hosts**
M. tuberculosis	Human
M. canetti	Human
M. africanum	Human
M. caprae	Goat
M. microti	Vole
M. bovis	Cattle, deer, elk, bison, badger, opossum
M. pinnipedii	Seal

Complete genome sequencing of the *M. tuberculosis* H37Rv laboratory strain was done in 1998. The results showed that the genome has about 4000 genes and a high guanine + cytosine (G+C) content (65.6%). Another important feature that was revealed through this sequencing is the presence of two unique protein families, the PE and PPE families. Both PE and PPE proteins are glycine rich, while the PPE family also contain abundant amount of asparagine (Cole et al., 1998).

1.4 Phases of Tuberculosis

Human TB infection usually happens upon exposure to aerosol droplets from person with open pulmonary disease. It has been reported that the infectious dose can range from 1 to 200 bacilli (Balasubramanian et al., 1994). The bacilli are immediately killed by alveolar macrophages. However in some cases the bacilli can escape intracellular destruction and is able to replicate inside macrophages and may result in active disease (Koul et al., 2011).

The outcome of infection by *M. tuberculosis* is regulated by a number of factors. The most important of which is the cross talk of the host's immune system and the survival strategies employed by the bacilli (Zahrt, 2003). In General, an encounter with *M. tuberculosis* is thought to generate four possible outcomes: complete elimination, active primary TB, latent TB and post primary TB (Stewart et al., 2003). Figure 2 illustrates the potential outcome of *M. tuberculosis infection*.

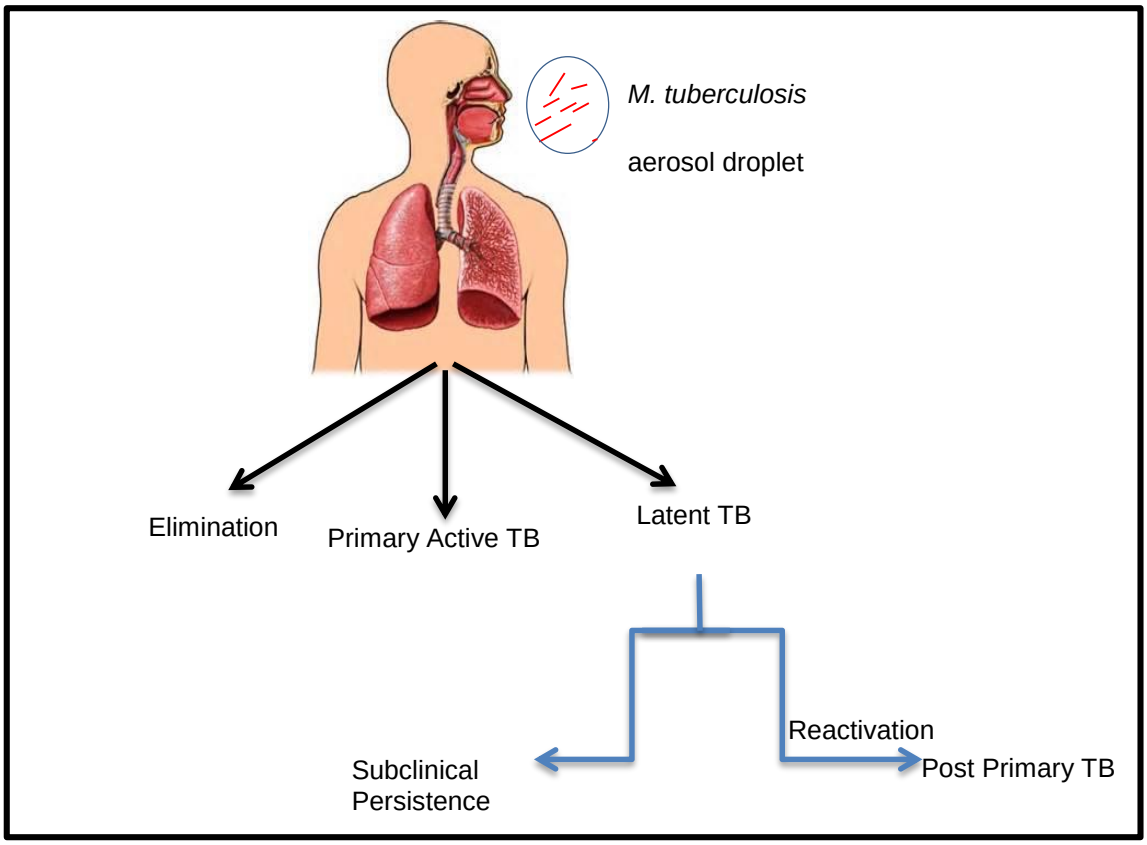


Figure 2: Potential outcomes after exposure to *M. tuberculosis*.
Exposure to *M. tuberculosis* has four potential outcomes depending on the fate of the microbe inside the macrophages. These include elimination, active disease, latent disease when the bacteria become dormant, and reactivation.

1.4.1 Active primary TB

A minority of people (5%) develop a rapid progressive disease after exposure to aerosol droplets harbouring tubercle bacilli expectorated from an infected person. High bacterial load, increased bacterial virulence, immunosuppression, or genetic susceptibility, are all elements which lead to this outcome. The risk of developing active primary TB is higher in infants and immune compromised individuals. Once inside the lungs, *M. tuberculosis* is engulfed by phagocytic cells such as alveolar macrophages and dendritic cells. *M. tuberculosis* employs a number of survival strategies which enables it to survive destruction and even manages to replicate inside phagocytic cells resulting in establishment of an acute infection. This stage is characterised by uncontrolled bacillary replication and dissemination. Patients at this stage suffer from low-grade fever, weight loss, persistent fatigue, anorexia, and chronic cough (Zahrt, 2003). Diagnosis of active

TB relies on clinical symptoms and sputum smear positivity. Confirmation of diagnosis can be done through isolation of *M. tuberculosis* in culture and more recently through the nucleic acid amplification testing (NAAT). However, both techniques require obtaining sputum which is not always easy. A new diagnostic approach which relies on studying the blood transcriptomic signature has recently been proposed. The aim of this approach is to develop immune biomarkers capable of determining people who suffer from active disease and aid in treatment monitoring (Cliff et al., 2015). Management of active tuberculosis is done through directly observed therapy (DOT). A 6 month long regimen consisting of rifampicin, isoniazid, pyrazinamide, and ethambutol is used to resolve infection (Choby and Hunter, 2015).

1.4.2 Latent TB

The immune system in the majority of healthy individuals who get exposed to aerosol droplets containing tubercle bacilli will resolve the infection or prevent further proliferation. Hence, an infected individual may remain asymptomatic for years with the persistent bacilli held in check by the immune system. In this phase alveolar macrophages are unable to eliminate the bacilli leading to a moderate increase in bacterial burden. However, the majority of infected individuals develop an effective immune response which results in the formation of granulomas. In addition to activated macrophages, both $CD4^+$ T cells and $CD8^+$ T cells are recruited to the site of infection (Zahrt, 2003). When the acquired host immune response emerges, the tubercle bacilli will stabilise in number leading to control of bacillary growth in a state known as latent TB in which there is no detectable growth of bacteria. Latent TB is characterised by the absence of any clinical signs of active disease in the presence of immunological sensitisation by mycobacterial proteins (Barry et al., 2009). Previously, sensitisation was defined by a positive reaction in the tuberculin skin test. The reagent used in this test (purified protein derivate) has shown cross reactivity with some non-pathogenic mycobacteria which lead to its replacement by the more specific antigen-specific interferon-gamma (IFN-γ) release assays. These assays have shown high specificity and unlike the tuberculin skin test are not affected by prior exposure to the Bacille Calmette Guérin (BCG) vaccine (Pai et al., 2008). However, both tests fail to distinguish between active and latent TB (Barry et al., 2009).

1.4.3 Post Primary TB

An estimated 10% of individuals with latent TB may relapse into active disease and develop what is known as post primary TB at some point during their lives (Tufariello et al., 2003). Weakening of the host immune response through HIV infection, the use of immunosuppressive drugs, malnutrition or old age are the most probable factors leading to reactivation (Flynn and Chan, 2001). It has been estimated that HIV patients face an annual reactivation risk of 10% and this has been documented by the increase in HIV/TB co-infection (Shen et al., 2004). Much of the success of *M. tuberculosis* as a pathogen is attributed to its ability to cause asymptomatic latent disease followed by reactivation and transmission. Reactivation of the tubercle bacilli after a period of latency in asymptomatic carriers is thought to be the primary mechanism by which the bacilli are transmitted in the population (Zahrt, 2003). It has been estimated that 80% of clinical TB cases and almost 100% of transmission results from reactivation in latent TB patients. It is not known whether reactivation is a result of reinfection or if the bacilli are responsible for reactivation (Sakamoto, 2012). It has been found that most cases of post primary TB are self-limited and resolve prior to the appearance of clinical symptoms. An estimated 5% of people who develop post primary TB are thought to develop active disease (North and Jung, 2004). Most cases of post primary TB are restricted to the upper lobe of the lungs. Patients who recover from post primary TB are at higher risk of developing recurrent disease which probably is a result of new infection. This suggests that post primary TB does not provide immunity for future infection (den Boon et al., 2007).

1.5 Model organisms of *M. tuberculosis*

Studying *M. tuberculosis* directly is important to understand its mechanisms of virulence, interaction with the host and to improve methods used for diagnosis and treatment. However, there are a number of reasons which makes the use of this pathogen in research not always feasible. The most important of which is the fact that *M. tuberculosis* is regarded as a biosafety level 3 pathogen which requires a biosafety level three laboratory and animal facilities, appropriate training in handling this pathogen and preventive measures which minimises any risk of accidental exposure among laboratory workers (Alderton, 2001). In

addition, the slow growing nature of this pathogen (>20 hours generation time) has made experimentation with this pathogen time consuming. To overcome the associated experimental limitations researchers have successfully used other mycobacterial species as model organisms to study *M. tuberculosis* (Shiloh and Champion, 2010). The most widely used mycobacterial species in the literature are *M. smegmatis*, *M. marinum* and *M. bovis* BCG. The use of these Mycobacterial species as model organisms for *M. tuberculosis* has helped researchers to further understand the physiology and genetic basis of TB. Nevertheless, any observations made in *any of these models* should be verified in *M. tuberculosis*.

1.5.1 *Mycobacterium smegmatis*

M. smegmatis is a fast-growing (doubling time is approximately 3 hours), non-pathogenic and soil dwelling saprophytic mycobacterium. Genomic comparisons have shown that approximately 30% of *M. tuberculosis* proteins lack conserved orthologues in *M. smegmatis* (Altaf et al., 2010). During the last few decades, *M. smegmatis* has been extensively used as a host for mycobacteriophage propagation and as a host for the introduction of recombinant DNA (Snapper et al., 1990). *M. smegmatis* has also been used to construct gene replacement mutants via a technique known as recombineering. This technique was developed to enhance recombination frequencies by employing mycobacteriophage that facilitate recombination from PCR products (van Kessel and Hatfull, 2008). Researchers have also used *M. smegmatis* in optimising molecular techniques and systems which were subsequently applied to *M. tuberculosis*. A good example is the development of inducible systems which enabled researchers to construct conditional mutants of essential genes (Guo et al., 2007). The literature is full of examples of how utilisation of *M. smegmatis* as a mycobacterial model has contributed in further understanding of TB pathogenesis. On the other hand, there have been some questions raised about the suitability of *M. smegmatis* as mycobacterial model (Reyrat and Kahn, 2001).This controversy has been raised mainly due to its non-pathogenic nature and its inability to survive in both mice and macrophages (Bange et al., 1999, Jordao et al., 2008) However, others have argued that the *M. smegmatis* model can still be used to study certain aspects of *M. tuberculosis* pathogenesis such

as the mechanism of the adaptive response to hypoxia (Tyagi and Sharma, 2002).

1.5.2 *Mycobacterium marinum*

Another widely used model organism is the animal and human pathogen *M. marinum*. The optimal growth temperature for *M. marinum* ranges from 25 to 35 °C (Pozos and Ramakrishnan, 2004). Genomic sequencing has shown that *M. marinum* holds an average identity of 85% with the *M. tuberculosis* genome (Stinear et al., 2008). Although considered as one of the slow growing members of the genus *Mycobacterium* but it has a shorter generation time when compared to that of *M. tuberculosis.* It also has the ability to survive and replicate inside macrophages (Barker et al., 1997). *M. marinum* is a fish pathogen capable of causing a chronic progressive TB-like disease characterised by nodules with central caseous necrosis which resemble granulomas caused by *M. tuberculosis* in the lungs of humans. The affected organs include liver, kidney and spleen. Little is known about the mode of transmission of *M. marinum* among fish. Possible routes of transmission are oral, through injuries in fish suffering from suppression in the immune system and uptake via the grills (Stamm and Brown, 2004). Studies on both mammalian macrophages and fish monocytes found that *M. marinum* was localised in non-acidified phagosomes which suggest that it can survive in the host by arresting phago-lysosome fusion (Barker et al., 1997). Studies on *M. marinum* have reported that this strain has the ability to escape from the phagosome to the cytosol (Stamm et al., 2003). However, the question whether this observation also holds true for *M. tuberculosis* has been a matter of dispute with some studies reporting results that confirm this observation and other studies presenting contradicting results (McDonough et al., 1993, Clemens et al., 2002). Recent advanced imaging approaches have confirmed that *M. tuberculosis* has the capability of escaping the phagosome (Simeone et al., 2015). The presence of both innate and adaptive branches of the immune system in zebrafish has made the zebrafish embryo infection model a suitable mean to study the early events following mycobacterial infection (Davis et al., 2002). Insights from the *M. marinum*/zebrafish model have been used to conclude the role of granulomas in restricting mycobacterial infection (Tobin and Ramakrishnan, 2008). Unlike humans, zebrafish does not seem to have the

ability to clear mycobacterial infection suggesting that it cannot be used to study latent TB (Swaim et al., 2006). The genetic and pathogenic similarities between *M. marinum* and *M. tuberculosis* have made this organism an appealing model to study TB. However, *M. marinum* has some unique characteristics such as photochromagenicity and actin polymerization which are absent in *M. tuberculosis.* It is thought that studying these unique characteristics in detail will help in revealing their role in adaptations to specific niches (Stamm and Brown, 2004).

1.5.3 *Mycobacterium bovis* BCG

Another widely used *in vitro* screening model for TB is Bacillus Calmette-Guerin (BCG) which is an attenuated strain of *Mycobacterium bovis* (*M. bovis*). *M. bovis BCG* is a member of the MTBC developed by serial passage of the virulent *M. bovis* 230 times in liquid culture (Frothingham et al., 1994). For over than 7 decades BCG has been used as a vaccine for TB. Although its potency to protect against TB is variable, previous data showed that the risk of developing TB was reduced by 50% in new born babies and infants (Colditz et al., 1994). *M. bovis* BCG is widely used as an *in vitro* screening model for TB due to the large sequence homology (99.9%) between *M. bovis* BCG and *M. tuberculosis* (Mattow et al., 2001). Another common feature between *M. tuberculosis* and *M. bovis* BCG is the ability to persist in the host and reactivation in immune compromised individuals (Rosenfeldt et al., 1997).

Although, *M. bovis* BCG is a biosafety level 2 organisms but its slow growth rate means that experimentation using this organism can be time consuming. Recent work has indicated that 3% of *M. tuberculosis* proteins lack conserved orthologues in *M. bovis BCG* which makes *M. bovis BCG* a good *in vitro* screening model for TB drug discovery (Altaf et al., 2010). However, Myrvik and colleagues proposed that *M. tuberculosis* can actually disrupt the phagosomal membrane and can be found in the cytosol whereas later work concluded that *M. bovis* BCG remained inside the membrane encoded vesicle (Myrvik et al., 1984, McDonough et al., 1993). A recent study has confirmed that *M. tuberculosis* can be found in the cytosol of macrophages after three days (Simeone et al., 2012). Studies of the transcriptional response of both *M. tuberculosis* and *M. bovis BCG*

to macrophage phagosome showed that 24 genes were similarly up-regulated in both species, however after 2 hours of macrophage entry there were up regulation of a subset of species specific genes (Rohde et al., 2007b).
Comparative and genomic studies have shown that the ESX-1 system is deleted in *M. bovis* BCG and it has been concluded that its loss has contributed to the attenuation of *M. bovis* BCG (Simeone et al., 2009).

1.6 Immune response against *M. tuberculosis* infection

Information gained from clinical experience and the increasing body of literature on TB research had indicated that host immunity is a major player in the host pathogen interaction. Hence, a comprehensive knowledge of the basic elements of host response will have a substantial impact on understanding the pathogenesis of the disease (Schluger and Rom, 1998). As mentioned previously, inhalation of droplets containing tubercle bacilli is the rout of entry of *M. tuberculosis* into the host. Only small droplets (≤ 2 μm) are able to pass through the lower respiratory tract as larger ones are cleared out of the respiratory tract via the physical action of the cilia (Riley et al., 1995). Once inside the lungs, the tubercle bacilli are usually engulfed by alveolar macrophages which represent the first line of defence against the infection. In addition to alveolar macrophages, dendritic cells are also thought to have a role in augmenting the innate immune response against *M. tuberculosis* as they were found to be highly present at sites of infection (Giacomini et al., 2001). As with any other phagocytosis process, the first step is engulfing the invading microbe. This occurs through the pseudopods which fuse to contain the microbe in a membrane-bound tight vacuole named the phagosome. *M. tuberculosis* binds to the macrophage via complement receptors CR1, CR3, CR4, the surfactant protein A receptor (SP-A), Scavenger receptors and mannose receptors (Schlesinger et al., 1994).There is also gathering evidence that CD14 may also have a role in mediating the binding of *M. tuberculosis* to the alveolar macrophages (Hoheisel et al., 1995). Many *in vitro* studies have focused on the role of CR3 in facilitating the entry of *M. tuberculosis* into the macrophage (Boom et al., 2003). The mycobacterial surface glycoprotein LAM seems to have a role in the interaction between mannose receptors and mycobacteria. The mannose receptor is of particular importance

as down regulation of this receptor seems to decrease the binding of virulent mycobacteria (Schlesinger et al., 1994). Inside the macrophage, *M. tuberculosis* is subjected to a number of bactericidal factors which may eventually lead to the killing of the microorganism as shown in figure 3. Other immune cells such as cytotoxic T lymphocytes help macrophages by ingestion of macrophages which have engulfed mycobacteria (Stenger et al., 1997).

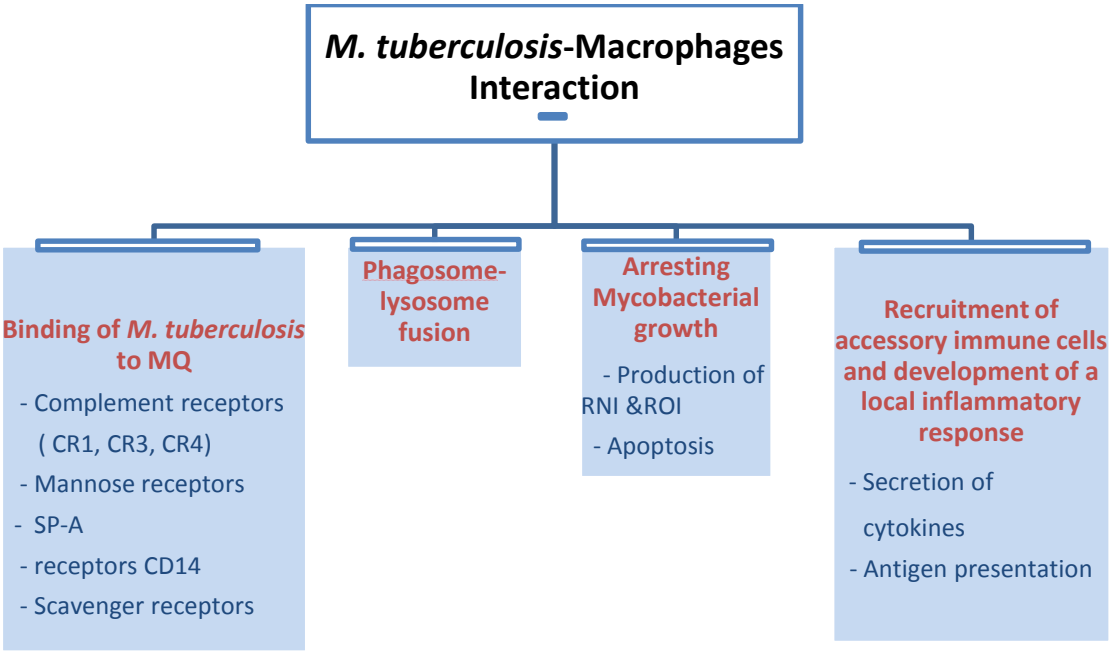


Figure 3: Schematic presentation showing a summary of the main events resulting from *M. tuberculosis* - Macrophages Interaction.
Resident Alveolar macrophages phagocytose inhaled tubercle bacilli via a number of different receptors. The interaction between Macrophages and *M. tuberculosis* via leads to a pro-inflammatory response and recruitment of cells of the innate and adaptive immune system. The above figure was adapted from a table published in Schluger and Rom, 1998 (Schluger and Rom, 1998).

Natural killer cells (NK) are important players in the host immune response against *M. tuberculosis.* They exert their role during both innate and adaptive immune response against TB. Natural killer cells have the ability to recognise and lyse *M. tuberculosis* infected monocytes and alveolar macrophages. Natural killer cells which are activated by *M. tuberculosis* are capable of producing interferon gamma (IFN-γ) which elicits the production of IL-12, IL-15 and IL-18 by monocytes, (Vankayalapati et al., 2005). The T cell cytokine, IFN-γ is thought to be one of the most important cytokines in the host response to mycobacteria

(Martinez et al., 2009a). Studies have shown that patient who have defects in the expression of IFN-γ receptor or in the production of IFN-γ are more susceptible to mycobacterial infection (Ottenhoff et al., 1998). Another important cytokine in maintaining the host immunity against *M. tuberculosis* is tumour necrosis factor α (TNF-α) which is produced by infected and activated macrophages and proinflammatory T cells. The importance of TNF-α is highlighted by the role it plays in enhancement of macrophage activation, recruitment of immune cells to the site of infection and the regulation of granuloma function (Harris et al., 2008, Zhou et al., 2007, Clay et al., 2008). Lin and colleagues have shown that neutralisation of TNF-α can lead to reactivation of latent TB (Lin et al., 2010). Although neutrophils are recruited to the sight of infection through the secretion of IL-8 from infected alveolar macrophages, but experiments done on murine models have suggested that they have a regulatory non phagocytic role in *M. tuberculosis* infection (Pedrosa et al., 2000). The percentage of active TB in HIV patients is inversely proportional to the number of CD4+ T lymphocytes, which means that these cells have an important role in the host response to *M. tuberculosis* (Shafer and Edlin, 1996). Specific CD8+ T lymphocytes response (include classical (MHC Class I restricted) and non-classical CD8 T cells) has also been documented in TB patients (Lin and Flynn, 2015). Pleural fluid and bronchoalveolar lavage from TB patients have been shown to contain large amounts of IL-6 which was found to prevent the binding of TNF-α to macrophages resulting in hindering its antimicrobial activity (Xirouchaki et al., 2002). The inflammatory process resulting from interaction of the tubercle bacilli with macrophages in the lung leads to the recruitment of other cells such as monocytes, neutrophils, T cells and B cells to the site of infection. The accumulation of these cells in the lung leads to the formation of the granuloma. Although a granuloma consists of many cell types but the macrophages are the major constituent. The granuloma represents a niche for the mycobacterium and also a site in which the immune cells can interact to prevent the spread of the bacilli (Flynn et al., 2011). To sum up, it is evident that the coordinated response of both the innate and acquired arms of the immune system plays an important role in controlling mycobacterial infection.

1.6.1 The Role of macrophages in the host immune response to *M. tuberculosis*

In order to survive in the host cells some pathogens such as *M. tuberculosis, Legionella pneumophila* and *Salmonella typhimurium*, enter macrophages through a number of different receptors and interfere with vacuolar maturation (Aderem and Underhill, 1999). Alveolar macrophages present within the lung are the main cellular host for mycobacteria (McDonough and Kress, 1995). Macrophages play a unique role in *M. tuberculosis* infection because they represent both the host cells and the primary effecter cells which the immune system employs for bacterial killing. The success of this pathogen is mainly due to its ability to overcome the host's immune response and survive in the hostile environment of the macrophage phagosome (Gandhi et al., 2006). Macrophages have a number of surface receptors that enables them to phagocytise and internalise bacteria into the phagosome (Schlesinger, 1996). Once inside the phagosome, the microbe is subjected to acids, lytic enzymes and reactive oxygen and nitrogen intermediates (ROI and RNI) which eventually lead to the killing of the microbe (Aderem and Underhill, 1999). There is a controversy regarding which one of these chemically reactive micromolecules is of greater importance in mycobacterial infection with some studies proposing that RNI are more important. One study has reported that replication of *M. tuberculosis* was faster in mice lacking inducible nitric oxide synthase (iNOS) when compared with their wild type littermates (MacMicking et al., 1997). In addition, high expression of iNOS was found in macrophages of active TB patients (Nicholson et al., 1996). Cooper and colleagues have hypothesized that the role of nitric oxide is not restricted to limiting bacterial growth but extends to minimising the immunopathological damage seen in chronic TB(Cooper et al., 2002). On the other hand, Kuo and coworkers were able to demonstrate that alveolar macrophages from TB patients showed higher capacity of releasing ROI (Kuo et al., 1996). Collectively these studies emphasize on the important role of these two reactive micromolecules in limiting mycobacterial growth regardless of which one is most potent during infection.

Mycobacterial antigens are processed and presented to T lymphocytes which lead to activation of the adaptive immune response and INFγ is released resulting in enhancement of the antimicrobial defense capacity of the host such as NO production and restriction of mycobacterial growth. Three different outcomes are expected when *M. tuberculosis* infects the macrophage, cell necrosis which is mostly seen in virulent strains, cell apoptosis which is mostly seen in avirulent strains or macrophage survival (Behar et al., 2011). Work carried out by Molloy and colleagues have proposed that apoptosis is another potential defense mechanism employed by macrophages to limit mycobacterial replication as their findings have shown that apoptosis resulted in reducing the viability of mycobacteria (Molloy *et al.,* 1994). This concept has been supported by the findings of Behar and coworkers who reported that apoptosis reduce the viability of *M. tuberculosis* and is an important link between the innate immune response and the adaptive T-cell immunity (Behar, 2011). Macrophages infected with mycobacteria produce IL-12 which is thought to be an important regulator of the antimycobacterial immune response. IL-12 is believed to enhance the production of IFN-γ, facilitate the development of Th1 cells and augment the cytotoxicity of antigen-specific T cells and NK cells (Zhang et al., 1994). Figure 4 shows a summary of the main defense mechanisms employed by macrophages to combat the growth of mycobacteria.

A recent study has reported that in the advanced stages of *M. tuberculosis* infection a certain type of macrophage population is generated. These populations of macrophages possess a strong immune suppressor activity (M2 phenotype) against the hosts T cell function. It is proposed that these immunosuppressive macrophages may have a role in the persistence of *M. tuberculosis* in the host (Tomioka et al., 2012). Lopes and colleagues have recently suggested that mycobacterial DnaK polarises macrophages to the M2-like phenotype and could constitute a virulence factor (Lopes et al., 2014).

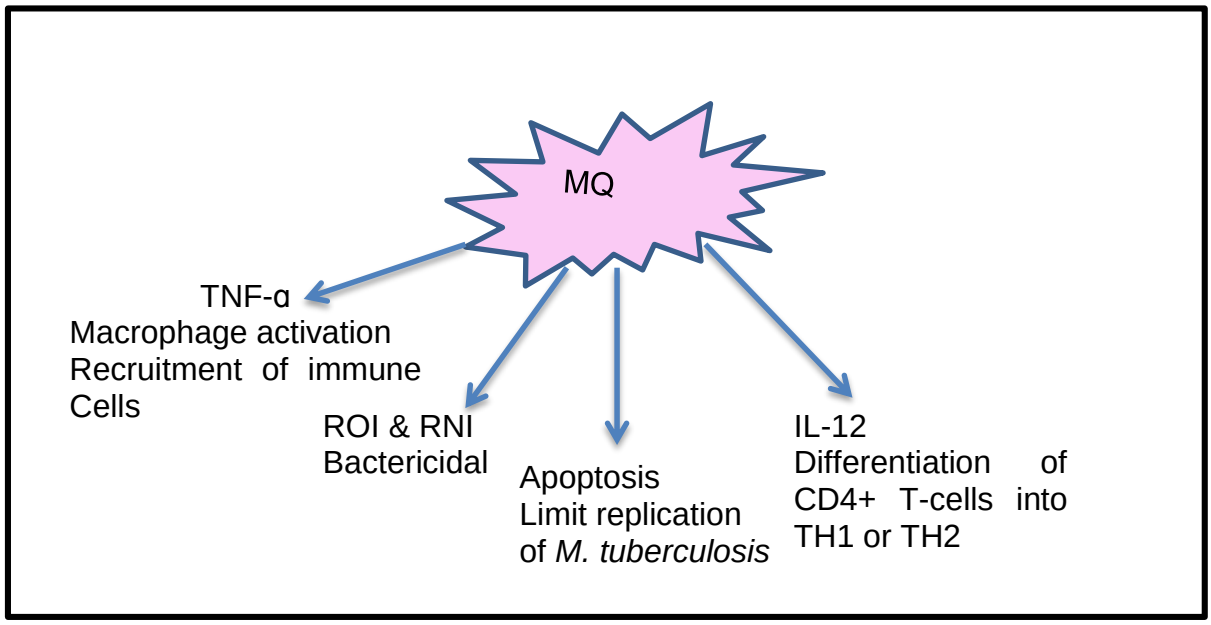


Figure 4: Summary of the main defence mechanisms utilised by macrophages to cease /limit replication of *M. tuberculosis*

1.6.2 The Granuloma

Granulomas are aggregates of immune cells that form as a result of persistent stimuli. TB granulomas are a hallmark of *M. tuberculosis* infection and are believed to be a host protective element which prevents *M. tuberculosis* from spreading (Ramakrishnan, 2012). On the other hand, TB granulomas represent a niche in which the bacilli are sequestered with possible dissemination in the presence of favourable conditions (Ehlers and Schaible, 2012). Inhaled tubercle bacilli are phagocytosed by alveolar macrophages in which they replicate by subverting the killing mechanisms of infected cells. Infected cells migrate into deeper tissue where they aggregate to form granulomas. In addition to macrophages, TB granulomas also contain T lymphocytes, B lymphocytes, dendritic cells, neutrophils and fibroblasts (Flynn and Chan, 2001). One characteristic of Mycobacterium granulomas is differentiation of macrophages into epithelioid cells and multinucleated giant cells. Within the granuloma, *M. tuberculosis* is in a state of little or no replication with full capability of energy-generation (Ehlers and Schaible, 2012). The transparent zebrafish embryo infection model has revealed that early granuloma which is formed during the innate immune response may aid in the local expansion and systemic dissemination of virulent mycobacteria. It has been proposed that the ESX-1/RD1

virulence locus induce the recruitment of new macrophages which in turn phagocytose apoptotic infected macrophages and lead to rapid expansion of infected macrophages and an increase in bacterial numbers.

Upon the initiation of the adaptive immune response, bacterial expansion is halted as a result of a complex interplay between the host and pathogen (Davis and Ramakrishnan, 2009). Upon the onset of the adaptive immune response and the recruitment of pathogen specific T-cells, the granuloma become organised with the infected macrophages in the core surrounded by a rim of lymphocytes. Granulomas can be found in active, reactivation and latent TB. In active TB, there are multiple granulomas which have limited ability to control bacillary proliferation leading to spread of infection. On the other hand, latent TB is usually characterised by the formation of few granulomas in lungs or lymph nodes, these granulomas have the ability to contain the infection (Flynn et al., 2011).

1.7 Survival strategies of Mycobacteria inside the host

In order to survive and replicate inside the host cells, *M. tuberculosis* must have a number of robust survival mechanisms which enables it to resist killing by macrophages. Some pathogens can survive inside the harsh environment of the phagosome by inhibition of the phagosome maturation and adapting itself to the environment inside the host cells (Finlay and Falkow, 1997). There have been a number of studies which addressed the survival mechanisms utilised by *M. tuberculosis* in order to survive inside the host and a number of these mechanisms has been unravelled (Scherr et al., 2009). Understanding the survival mechanisms whereby mycobacteria are able to circumvent the microbicidal activities imposed by the host will have a huge impact on finding successful novel approaches for treatment and prevention. Below is a summary of some of the survival mechanisms employed by *M. tuberculosis:*

1.7.1 Blocking of phagosome maturation

Several studies have reported that *M. tuberculosis* phagosome has two main characters which are incomplete luminal acidification and low content of mature lysosomal hydrolases (Oh and Straubinger, 1996, Malik et al., 2003). One of the most important survival mechanisms of *M. tuberculosis* is inhibition of

phagosome-lysosome fusion and eventually escaping lysosomal killing. *M. tuberculosis* can alter the maturation of the phagosomal compartment leading to the inhibition of phagosome-lysosome fusion (Russell, 2001). Inhibition of phagosome-lysosome fusion was first discovered in 1971 by Armstrong and Hart when they found that phagosomes containing live *M. tuberculosis* do not fuse with lysosomes (Armstrong and Hart, 1971). Since then, blocking of phagosome maturation has been considered as a hallmark of *M. tuberculosis* infection and one of the major survival strategies. Two studies have shown that the ESX-1 secretion system in *M. tuberculosis* has a role in blocking phagosome maturation (MacGurn and Cox, 2007, Brodin et al., 2010). Gatfield and Pieters have reported that prevention of phagosome-lysosome fusion requires the presence of cholesterol at the mycobacterial phagosome (Gatfield and Pieters, 2000). Retention of the host protein coronin 1, also known as TACO (tryptophan aspartate containing coat protein) on the wall of the mycobacterial phagosome has a proposed role in preventing the delivery of bacteria to the lysosome (Ferrari et al., 1999). Hasan and colleagues have suggested that mycobacteria retain TACO/Coronin at the phagosomal membrane to mimic the plasma membrane and subsequently prevent lysosomal delivery (Hasan et al., 1997). On the other hand, contradicting results were reported by Schüller and coworkers who showed that human coronin is associated with bacterial uptake rather than phagosome maintenance (Schuller et al., 2001). Another factor which contributes to the blocking of phagosome lysosome fusion is PknG which is a soluble serine/threonine protein kinase. Studies have shown that mycobacteria deficient in PknG cannot alter phagosome maturation (Cowley et al., 2004). The lipid phosphatase, SapM secreted by *M. tuberculosis* was reported to play a role in blocking phagosome maturation. SapM seems to exert its role by exclusion of a membrane trafficking regulatory lipid called Phosphatidylinositol 3-phosphate (PI3P) which plays a prominent role in phagosome maturation (Vergne et al., 2005). The surface exposed mycobacterial lipid Phthiocerol dimycocerosates (PDIM) was also found to contribute in interference with phagosome maturation by preventing acidification of the phagosome (Astarie-Dequeker et al., 2009).

Phagosome maturation can also be blocked by LAM which is one of the key virulence factors in *M. tuberculosis.* LAM has been shown to interfere with phagosome maturation by insertion into the rafts of the host cell membrane (Welin et al., 2008). Another mycobacterial product which interfere with phagosomal maturation and leads to persistence of virulent mycobacteria in macrophages is manLAM. It has been found that manLAM exerts its action by inhibiting the increase in cytosolic Ca^{2+} levels of macrophages thereby blocking the Ca^{2+}-binding protein calmodulin pathway, an essential mediator of phagosome maturation (Vergne et al., 2003a, Vergne et al., 2003b). A number of proteins which are required for blocking phagosome maturation have been identified and they include proteins which are involved in biosynthesis of surface lipids which suggests that blocking phagosome maturation is a multifactorial process (Pethe et al., 2004).

1.7.2 Factors involved in mycobacterial persistence in macrophages:

1.7.2.1 Alteration of metabolism and nutritional requirements

Several proteins have been found essential for *M. tuberculosis* replication and persistence in macrophages. Some of them are involved in the alteration of mycobacterial metabolism in macrophages such as isocitratelyase (icl) which is a key enzyme of the glyoxylate pathway (McKinney et al., 2000). In bacteria the glyoxylate pathway is one of two pathways used to utilise fatty acids (Clark, 1996). More recently, icl has also been identified as one of the metabolic mediators of intrinsic resistance/tolerance to currently used TB drugs in replicating *M. tuberculosis* populations (Nandakumar et al., 2014). It has been suggested that during chronic infection of the lungs, *M. tuberculosis* alters its metabolism by utilising fatty acids as the major source of carbon and energy (Segal, 1984). *M. tuberculosis* also has the ability to acquire iron from the host by the production of siderophores which are small high-affinity iron chelators (Braun et al., 1998). De voss and colleagues have proven that siderophores are essential for the optimal growth of *M. tuberculosis* in macrophages. Deletion of the *mbtB* gene which is involved in the biosynthesis of siderosphores resulted in a mutant suffering from a growth defect in iron depleted conditions and a low survival rate in macrophages (De Voss et al., 2000). Metabolic adaptation has long been

identified as one of the defining features of *M. tuberculosis* pathogenicity; the above are just examples of how this pathogen alters its metabolism in order to survive in the host.

1.7.2.2 Stress Defence and Detoxification

M. tuberculosis has a number of genes which encode for proteins associated with defence against the stressful conditions which the bacilli encounter inside the host such as *acg* which is one of the DosR regulated genes. The results of macrophage infection experiments showed that the *acg* mutant was unable to grow and survive inside macrophages implying that *acg* is associated with virulence (Hu and Coates, 2011). Heat shock proteins (hsps) are important for maintaining the function of the cells during normal and stressful conditions. In *M. tuberculosis,* the syntheses of hsp are increased after infection (Young et al., 1988). It has been found that over expression of some of *M. tuberculosis* hsps leads to improvement in survival of the organism at higher temperatures when compared to the wild type (Stewart et al., 2001). One member of the heat shock regulon which is Hsp 22.5 was shown to have an important role in survival inside macrophages (Abomoelak et al., 2011). Another example of an important Hsp in *M. tuberculosis* is ClpX which was proven to be essential for *in vivo* survival (Ribeiro-Guimaraes and Pessolani, 2007).

When the macrophage becomes activated, *M. tuberculosis* is rapidly transferred to lysosomes where it is destroyed by ROI and RNI. Although these substances are toxic to the bacterium but *M. tuberculosis* also have a number of strategies which enables it to stay alive even in the presence of such intermediates. Li and colleagues have reported that *M. tuberculosis* produce KatG, a catalase-peroxidase which can inactivate reactive oxygen within phagosomes. There is also a proposed role of the mycobacterial proteasome in providing resistance against RNI by functioning in the elimination of proteins damaged by RNI (Li et al., 1998, Darwin et al., 2003). Over expression of a protein in the virulent Erdman strain enables it to be less susceptible to peroxidase (Yuan et al., 1995). *In vitro* studies have identified a number of genes whose products seems to have a role in the resistance against ROI and RNI (Piddington et al., 2001, Edwards et al., 2001).

M. tuberculosis is also predicted to have a number of genes which products may be involved in the adaptation to the toxic environment inside macrophages. The metal cation transporting P-type ATPase CtpC (Rv3270) in *M. tuberculosis* was shown to be important for survival upon exposure to zinc and inside macrophages (Botella et al., 2011). *M. tuberculosis* has at least two independent copper inducible pathways which are thought to play an important role during copper mediated stress during infection. These pathways are controlled by the RicR and CsoR regulons which are thought to play a protective role during copper toxicity (Liu et al., 2007, Festa et al., 2011).

1.7.2.3 Evasion of the host immune response

One of the most important survival mechanisms employed by *M. tuberculosis* is the ability to evade the host immune response (Gupta et al., 2012). Lipoprotein A (LprA) which is one of the 100 putative lipoproteins in *M. tuberculosis* has been found to have the ability to reduce the antigen presenting function of macrophages and thus lead to evasion of immune surveillance (Pecora et al., 2006). Other lipoproteins play important roles in the evasion from the immune response such as LpqH and LprG which have both stimulatory and inhibitory effect on the antigen presenting cells (Brightbill et al., 1999, Gehring et al., 2004). Another strategy adopted by *M. tuberculosis* to evade the host immune response is enhancement of the expression of the CC chemokine receptor 5 (CCR5) in infected macrophages which leads an increase in the production of the immunosuppressive cytokine IL-10 (Das et al., 2014). *M. tuberculosis* was found to express agonists of both Toll like receptor 9 (TLR9) and TLR2. Expression of both agonists is thought to be a mechanism by which *M. tuberculosis* limits T cell activation and antigen presentation. This mechanism may elucidate why IFN-α/β has limited contribution to host immunity in TB patients (Simmons et al., 2010).

1.8 Mycobacterial PPE and PE Protein Families

PPE and PE are a unique family of proteins found only in mycobacteria. These were designated the pe and ppe genes, after highly conserved Proline-Glutamate and Proline-Proline-Glutamate residues near the start of their encoded proteins. These proteins are so named due to the presence of Proline-Glutamate (PE) and Proline-Proline-Glutamate (PPE) signature motifs near the N-terminus of their gene products (Gordon et al., 2001).

These families of genes represent 7% of *M. tuberculosis* genome. There are approximately 107 PPE genes and 69 PE genes; a PE gene is usually followed by a PPE gene. The PE genes are classified into PE and PE_PGRS (polymorphic GC-rich-repetitive sequence); the latter has a conserved PE terminal domain and a variable C terminal domain (Tundup et al., 2006). The PE and PPE proteins are antigenic in nature and most of them are localized in the cell surface. Although the function of these families of proteins is unknown, they may have an important role in the pathogenesis and virulence of *M. tuberculosis* (Akhter et al., 2012). Studying the evolutionary history of the PE and PPE gene families has linked their evolution with the ESAT-6 (esx) gene cluster regions. The PE and PPE genes were predicted to be inserted into region 1 of the ESAT-6 (esx) gene cluster and duplications of the ESAT-6 gene clusters lead to subsequent expansion of these two gene families (Gey van Pittius et al., 2006). Published data have reported that 128 of the 169 PE/PPE genes were differentially expressed under 15 different conditions. Among those conditions were non-replicating persistence (NRP), stationary phase, hydrogen peroxide (H_2O_2), potassium cyanide (KCN), carbonyl-cyanide 3-chlorophenylhydrazone (CCCP), ethambutol, palmitic acid, starvation, and heat shock. Interestingly, data analysis revealed that seven PPE proteins were differentially expressed under at least 5 conditions as shown in table 2.

These findings may indicate that these proteins might have a role in pathogenesis suggesting that these members of the PPE family deserve further investigations. It has been suggested that the expression of PE/PPE proteins is likely to change when the bacilli encounter different microenvironmental conditions (Voskuil et al., 2004a, Voskuil et al., 2004b). Researchers have found evidence that members

of these families of proteins have variable expression in different strains of *M. tuberculosis* depending on the environmental conditions (Flores and Espitia, 2003, Voskuil et al., 2004b). Kohli and colleagues have found several differences between H37Rv and H37Ra which were only found in certain characteristics specific to the PE/PPE family and not in any of the other families which means that they may have an important role in the virulence and pathogenesis of *M. tuberculosis* (Kohli et al., 2012).

Table 2: PPE genes differentially expressed (at least 2 folds) in at least 5 different environmental conditions[1]

Gene	Rv.no	Conditions
PPE17	Rv1168c	- DETA/NO 0.5M 40mins (repressed) - 50µM Palmitic acid for 4H (induced) - Starvation for 24 hours (induced) - High iron (induced) - SDS (0.05%) for 1 hour (induced)
PPE18	Rv1196c	- INFɣ activated macrophages after 24 hours (repressed) - H_2O_2 (10mM) for 40mins (repressed) - After 14 days of stationary phase (repressed) - 20 days of NRP (repressed) - DETA/NO (0.5M) for 40mins (repressed) - SDS (0.05%) for 1 hour (induced) - KCN (0.5mM) for 1 hour (repressed).
PPE32	Rv1808	- After 14 days of stationary phase (induced) - H_2O_2 (10mM) for 40mins (induced) - Palmitic acid (50µM) for 4hours (induced) - 20 days of NRP (repressed) - Starvation for 24 hours (induced) - SDS (0.05%) for 1 hour (induced)
PPE33	Rv1809	- H_2O_2 (10mM) for 40mins (induced) - Palmitic acid (50µM) for 4hours (induced) - 20 days of NRP (repressed) - Starvation for 24 hours (induced) - SDS (0.05%) for 1 hour (induced)

[1] Table adapted from Voskuil et al., 2004b

Table 2 continued:

Gene	Rv.no	Conditions
PPE50	RV3135	- Hypoxia (0.20%) for 2 hours (induced) - Palmitic acid (50µM) for 4hours (induced) - H_2O_2 (10mM) for 40mins (repressed) - CCCP (0.5mM) for 1 hour (repressed) - Ethambutol (10µM) for 24 hours (induced) - KCN (0.5mM) for 1 hour (repressed)
PPE51	Rv3136	- Palmitic acid (50µM) for 4hours (induced) - H_2O_2 (10mM) for 40mins (repressed) - DETA/NO (0.5M) for 40mins (repressed) - 20 days of NRP (repressed) - Stationary phase for 14 days (repressed)
PPE60	Rv3478	- INFɣ activated macrophages after 24 hours (repressed) - DETA/NO (0.5M) for 40mins (repressed) - 20 days of NRP (repressed) - Starvation for 24 hours (repressed) - KCN (0.5mM) for 1 hour (repressed)

Some studies have reported findings which indicate that certain members of the PPE/PE families have a vital role in the virulence of *M. tuberculosis*. For example PPE18 (Rv1196) which is expressed on the surface binds to the toll like receptor 2 (TLR2) on the macrophage, this leads to the modulation of the host innate immune response and represses the production of IL-12 and promotes IL-10 production (Nair et al., 2009). Another example of an important member of the PPE family is PPE31 (Rv1807) which is required for the growth of the bacterium in mice (Sassetti et al., 2003). One member of the PPE family which is PPE51 (Rv3136) was found to be highly up regulated after 2 hours of macrophage infection suggesting that this protein may have a role in the virulence of *M. tuberculosis* (Rohde et al., 2007b). However the exact function of this protein is still unknown but a previous study has shown higher expression of *rv3136* in the wild type when compared to *rv3676* knock out mutant which means that Rv3136 is possibly regulated by Rv3676 (Rickman et al., 2005). The *M. tuberculosis* Rv3676 protein is a member of the cyclic AMP (adenosine monophosphate) receptor proteins (CRP family) which are transcriptional factors that respond to cAMP by binding at target promoters when cAMP concentrations increase

(Stapleton et al., 2010). Rv3676 was classified as a global transcriptional regulator because it regulates the expression of a large number of genes. Among the genes which are regulated by Rv3676 is *rpfA* which encodes one of five Resuscitation-promoting factor proteins. This refers that Rv3676 may have an important role in the persistence and/or emergence from the dormant state (Rickman et al., 2005).

1.9 Putative transcriptional regulators and efflux pumps in the genus *Mycobacterium*

1.9.1 Putative transcriptional regulators

When a bacteria senses a change in its environment it responds either by increasing the synthesis of some proteins or down regulating other proteins and this is mainly done by DNA binding proteins which are termed either transcriptional repressors or activators (Fontan et al., 2004). In order to survive in the host *M. tuberculosis* must adapt itself to the changing environment which the organism encounters at each stage of infection. The genomic sequence of *M. tuberculosis* revealed many putative transcriptional regulators which imply that these regulators are probably involved in the adaption to environmental changes (Cole et al., 1998).

1.9.1.1 Regulator of antimicrobial assisted survival RaaS (Rv1219c)

Studying the survival of both *M. tuberculosis* and *M. bovis* BCG during prolonged stationary phase has revealed that *M*. *bovis* BCG has a survival defect when compared to *M. tuberculosis* H37Rv (Turapov et al., 2014a). Interestingly, continuous exposure of stationary phase *M. bovis* BCG cultures to antimicrobial compounds targeting cell wall biosynthesis (ethambutol, isoniazid, and cerulenin) for 2 months resulted in a significant improvement in survival when compared to drug free cultures or cultures treated either with antimicrobials targeting replicating bacteria or antimicrobials inhibiting translation (Turapov et al., 2014a). This observation was only found in stationary phase cultures indicating that it is growth phase dependent and only happens when the bacteria are in non-growth

permissive conditions. Transcriptomic analysis of 1-month-old *M. bovis* BCG cultures revealed that bacteria treated with either ethambutol, isoniazid, or cerulenin had a common set of differentially expressed genes that was not found in drug free cultures or cultures treated with antimicrobial compounds inhibiting translation (streptomycin). Among those genes was the predicted transcriptional regulator of unknown function, *bcg_1279c* (*rv1219c* in *M. tuberculosis*). Turapov and co-workers predicted that this transcriptional regulator which was later designated as RaaS (for Regulator of antimicrobial assisted survival) was mediating the antimicrobial improvement in survival. This prediction was investigated by overexpression of *raaS* in *M. bovis* BCG and studying the effect it would have on survival during extended stationary phase. Mycobacteria overexpressing *raaS* was found to survive better during extended stationary phase when compared to the wild type confirming that *raaS* plays a role in persistence during extended stationary phase. The functional role of RaaS in antimicrobial-assisted survival was also explored by the construction of an *in-frame raaS* deletion mutant (Δ*raaS*) and the corresponding complemented strain (Δ*raaS* $_{com}$) in *M. bovis* BCG. The *raaS* deletion mutant showed a defect in survival during drug free extended stationary phase when compared to the wild type and the complemented strains. Furthermore, Δ*raaS*$_{com}$ strain which had a 2.6 fold increase in expression of *raaS* survived even better than the wild type during extended stationary phase further confirming the significant role of *raaS* in survival during extended stationary phase. Further evidence on the importance of RaaS in survival during non-permissive growth conditions came from the finding that the *raaS* deletion mutant of *M. bovis* BCG was attenuated in an *in vivo* model of mouse infection (Turapov et al., 2014a). As with overexpression studies, complementing Δ*raaS of M*. *bovis* BCG with either by *M. tuberculosis* or *M. bovis* BCG versions of *raaS* gave similar results indicating that RaaS from either mycobacteria play similar roles. Similar to *M. bovis* BCG, deletion of *raaS* from *M. tuberculosis* lead to a decrease in survival in extended stationary phase. Furthermore, deletion of *raaS* from *M. tuberculosis* resulted in decreased intracellular survival after 72 hours of macrophage infection. These findings imply that RaaS is important for persistence of *M. tuberculosis* during extended stationary phase and inside macrophages (Turapov et al., 2014a).

The regulator of antimicrobial assisted survival RaaS encoded by *rv1219c* is a predicted transcriptional regulator in *M*. *tuberculosis* with orthologues in *M. bovis* BCG (BCG_1279c), *M. avium* (MAV_1363), *M. smegmatis* (MSMEG_5074) and in *M. bovis* (MT1257). It is highly conserved in *M. tuberculosis* complex bacteria. The only difference between Rv1219c and BCG_1279c is one amino acid (W113C) due to a single G to C substitution which implies that these proteins have similar roles. Structure simulation (Figure 5) and analysis of the published structure of RaaS from *M. tuberculosis* suggests that the residue 113 (tryptophan or cysteine) is not in direct contact with DNA or ligand (Turapov et al., 2014; Cohen-Gonsaud, personal communication; Kumar et al., 2014).

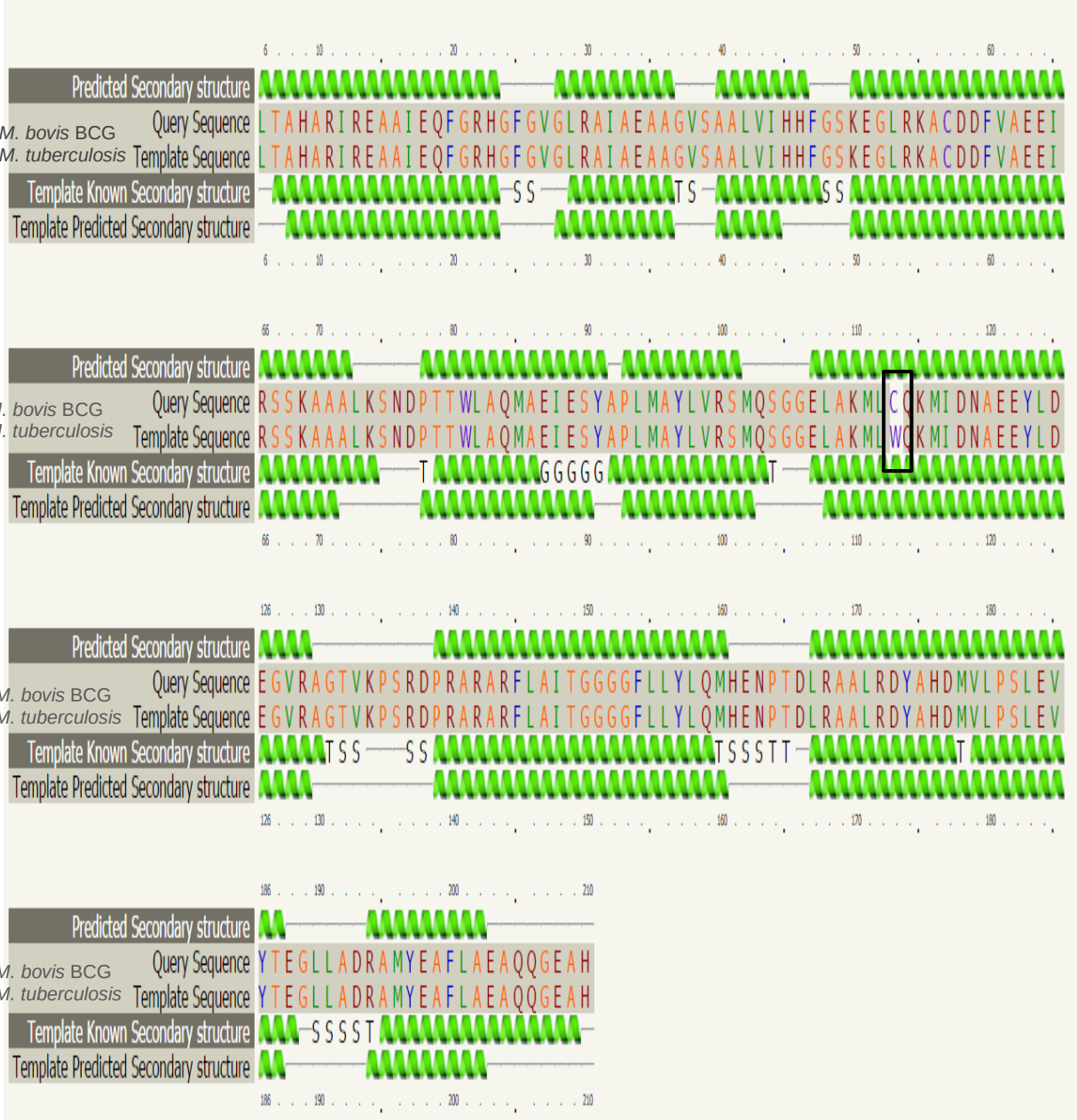


Figure 5: Sequence alignment of *M. bovis* BCG RaaS protein (BCG_1279) with *M. tuberculosis* RaaS protein (Rv1219c).

This alignment was generated using the Phyre2 web portal (Kelley et al., 2015). Residues are colored as following: yellow (for A,S,T,G,P: small/polar), green (for M,I,L,V: hydrophobic), red (for K,R,E,N,D,H,Q: charged and purple (for W,Y,F,C: aromatic + cysteine). The secondary structure prediction is as following: Green helices represent α-helices, faint lines indicate coils, G = 3-turn helix (310 helix), T = hydrogen bonded turn and S = bend. Identical residues in the alignment are highlighted with a gray background

Whole genome transcriptional analysis of the Δ*raaS* mutant strain relative to the complemented mutant strain and wild type *M. bovis* BCG identified seven genes significantly differentially expressed in the mutant compared either to the wild type or the complemented strain, thus defining the putative RaaS regulon. This analysis showed that one gene which is *bcg_3553* (homologous to *rv3489*) was repressed in the mutant. On the other hand, 5 genes which are *drrC* and b*cg_1278c/ bcg_1277c/ bcg_1276c/ bcg_1275c* (corresponding to *rv1218c/ rv1217c/ rv1216c/ rv1215c*) were induced in the mutant (Turapov et al., 2014a). This indicated that RaaS acts as a repressor of *drrC* (*bcg_2960*), which encodes an annotated efflux pump, as well as the cluster of genes located immediately downstream from itself. *Bcg_1279c* is the first gene in the *bcg_1279c-bcg_1275c* operon which also includes two genes (*bcg_1278c and bcg_1277c*) encoding subunits of predicted ATP dependent pump and two genes (*bcg_1276c* and *bcg_1275c*), encoding proteins of unknown function. It has been suggested that antimicrobial induction of this transcriptional regulator represses the expression of efflux pumps and subsequently leads to improvement in survival during extended stationary phase. This prediction has been confirmed by the finding that exposure of stationary phase cultures of *M. bovis* BCG to the efflux pump inhibitor reserpine or the proton ionophore CCCP resulted in increased survival (Turapov et al., 2014a).

Electrophoretic mobility shift assays (EMSA) using oligonucleotides that comprised the intergenic region upstream of *rv1219c* showed that oligonucleotides containing two 12-bp imperfect direct repeats separated by the predicted *raaS* -10 promoter element were bound by RaaS. The second direct repeat included the predicted start codon of *raaS* implying that RaaS represses the expression of its own gene in addition to other genes in the operon. Truncation of the sequence or substitution of three thymidines in one repeat with adenosines led to complete loss of binding indicating that both repeats were necessary for RaaS binding. Furthermore, RaaS was found to strongly bind the identified DNA sequence with an estimated binding constant of 31 nM ± 6 nM (Turapov et al., 2014a).

Rv1219c belongs to the TetR family of transcriptional repressors which are present in many species of Bacteria and Archaea. This family of transcriptional repressors is involved in regulating a variety of functions such as osmotic stress, catabolic pathways, homeostasis, biosynthesis of antibiotics, efflux pumps, multidrug resistance, and virulence of pathogenic bacteria (Ramos et al., 2005). However the most frequent function that they are involved in is regulation of efflux pumps and transporters involved in antibiotic resistance and tolerance to toxic chemical compounds (Ramos et al., 2005).

As many TetR repressors, RaaS contains a ligand binding domain. Bioinformatics analyses was used to predict the putative RaaS ligand. This approach did not detect direct binding of RaaS to any of the antimicrobial compounds that enhanced mycobacterial survival. However, sequence similarity with distal proteins (<20% sequence identity) revealed that these proteins share a strong hydrophobic ligand-binding pocket within the C-terminal domain. Among those proteins was the transcriptional regulator YsiA (FadR) in *Bacillus subtilis*, whose structure has been previously solved in complex with stearoyl-CoA. Further analyses led to the prediction that CoA derivatives of fatty acids could possibly regulate the binding of RaaS to DNA. The abundance of oleate in both growth media and eukaryotic cells, and the fact that Mycobacteria convert oleate to oleoyl-CoA by fatty acyl-CoA ligases suggested that oleoyl-CoA influence the RaaS-DNA interaction. This prediction has been confirmed by the finding that the long chain acyl-CoA derivative, oleoyl-CoA (and to a lesser extent, stearoyl-CoA) regulates the binding of RaaS to DNA. Furthermore, it was found that Arg-140, Arg-144, and Tyr-174 were important for acyl-CoA binding in RaaS as replacement of these residues lead to a decrease in the DNA binding affinity (Turapov et al., 2014b). Thus it has been demonstrated that oleoyl-CoA regulates the binding of RaaS to DNA and subsequent expression of the downstream genes which encode efflux pumps. An increase in the concentration of oleoyl-CoA blocks the binding of RaaS to DNA resulting in an increase in expression of RaaS efflux regulon in non-replicating mycobacteria. Furthermore, the addition of oleic acid to 1-month old stationary phase cultures of *M. bovis* BCG resulted in inhibition of the antimicrobial survival-enhanced effect (Turapov et al., 2014b). It is proposed that increased accumulation of free acyl-CoA abolish the binding of

RaaS to its DNA binding site and subsequently results in increased expression of the down-stream genes encoding efflux pumps. On the other hand, depletion of free acyl CoA results in binding of RaaS to its DNA binding site and subsequent repression of the downstream genes encoding efflux pumps as shown in Figure 6.

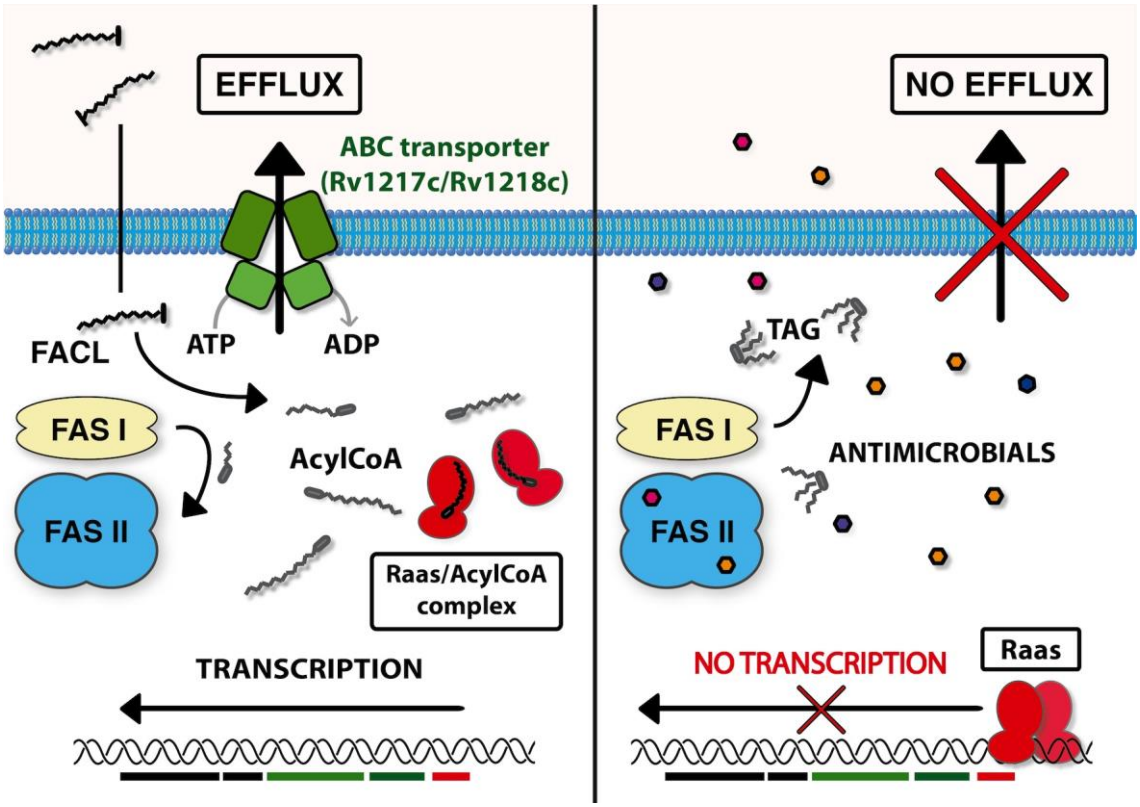


Figure 6: Overview of the proposed RaaS function

During active growth (left panel), RaaS is bound by acyl-CoA, and the Rv1218c/Rv1217c-encoded ATP-dependent pump is expressed, while antimicrobial treatment of stationary phase *M. bovis* BCG cells (right panel) leads to inhibition of residual cell wall biosynthesis and depletion of acyl-CoA. In the absence of circulating acyl-CoA, RaaS binds to its recognition sequence, preventing expression of the downstream genes encoding the ATP-binding cassette (*ABC*) transporter. *FAS*, fatty acid synthase; *FACL*, fatty acyl-CoA ligase; *TAG*, triacylglycerols. (Figure adapted from Turapov et al., 2014b).

The crystal structure of RaaS has revealed the existence of a very large hydrophobic pocket which points out that other complex lipids may fit and thus contribute in regulating the binding of RaaS to DNA (Kumar et al., 2014). Thus RaaS is thought to be an important regulator of metabolite-regulated shutdown of efflux and subsequent improvement of mycobacterial survival. The precise physiological role of the Rv1218c/Rv1217c (Bcg_1278c/Bcg_1277c) pump is unknown but it has been predicted that it could possibly transport a lipid molecule(s) (Turapov et al., 2014b).

According to the published CHIP-seq data available in TB database [http://genome.tbdb.org/], RaaS has 107 predicted regulatory interactions and a total of 44 binding sites. Of those predicted interactions, is an interaction to the coding region of the histidine kinase response regulator DosT (*rv2027c*). DosT has been implicated in induction of the DosR regulon. This prediction implies that RaaS could possibility be involved in the regulation of the family of genes involved in the survival during latency.

1.9.2 Efflux pumps in Mycobacteria

Efflux pumps are proteins localised in the cell membrane of cells capable of extrusion of metabolites and toxic compounds accumulated inside the cells to the extracellular environment via an energy dependent process. Depending on the energy source, efflux pumps can either be primary transporters or secondary transporters. Primary transporters which are known as ATP-binding cassette (ABC) transporters use Adenosine triphosphate (ATP) as an energy source. On the other hand, proton or sodium gradient are used as a source of energy in secondary transporters (Marquez, 2005). In general, efflux pumps can be divided into five super families which include four secondary transporters; major facilitator super-family (MFS), resistance nodulation division (RND), small multidrug resistance (SMR) and multidrug and toxic-compound extrusion (MATE). The fifth is the ABC binding cassette superfamily which is a primary transporter (da Silva et al., 2011). Genomic sequencing of the *M. tuberculosis* H37Rv strain has revealed that is contains a number of putative efflux pumps (Viveiros et al., 2003). It has been predicted that these efflux pumps play an important physiological role. These pumps protect the bacillus from low levels of toxic intracellular molecules

and have been shown to transport harmful metabolites and toxins to the extracellular environment (Pasca et al.,2005). In addition, a number of efflux pumps have been linked to drug resistance with some efflux pumps being specific to a certain antibiotic while others pump out structurally and functionally unrelated toxic compounds suggesting that drug efflux is a nonspecific secondary role (Pasca et al., 2005, Marquez, 2005). Some studies on *M. tuberculosis* have described and characterised a number of efflux pumps belonging to the ABC superfamily, MFS and SMR families. Identification of these efflux pumps was mainly done using laboratory generated mutants, with little research done on clinical isolates. Substrates and the specific induction conditions for efflux pumps are mainly unknown. However, it has been documented that these pumps need to be tightly regulated in order to perform their physiological roles (Nikaido, 2001).

Efflux pumps substrates include drugs, lipids, sugars, proteins, synthetic compounds, host defence molecules and toxic metabolites (Li and Nikaido, 2009). A number of antibiotic efflux transporters have been described in *M. tuberculosis* and other mycobacterial species. An example is *lfrA* which encodes an efflux pump that confers resistance to hydrophilic fluoroquinones. Another example is *EfpA* in *M. smegmatis* (*M. tuberculosis* homologue *rv2846c*) which confers resistance to fluoroquinolones, rifamycins, chloramphenicol and isoniazid (Takiff et al., 1996). The genomic sequence of *M. tuberculosis* revealed the presence of 13 transmembrane proteins belonging to the RND superfamily. One of those proteins which is mycobacterial membrane proteins, large 7 (MmpL7) was found to be involved in the export of phthiocerol dimycocerosate (PDIM) in *M. tuberculosis* (Camacho et al., 2001).

In addition to their proposed role in drug resistance, some efflux pumps of *M. tuberculosis* have been linked to virulence, growth and oxidative response (Ramon-Garcia et al., 2009).

It is thought that 2.5% of *M. tuberculosis* genomic capacity encodes ABC transporters which can either be exporters or importers. Sequence analysis has

identified 12 genes (*Rv0194, Rv1218c-Rv1217c, Rv1273c-Rv1272c, Rv1348-Rv1349, Rv1456c-Rv1457c-Rv1458c, Rv1473, Rv1667c-Rv1668c, Rv1686c-Rv1687c, Rv1819, Rv2477, Rv2688c-Rv2687c-Rv2686c* and *drrA-drrB-drrC*) which encode efflux pumps. One of the major efflux pumps in *M. tuberculosis* is the Rv1218c-Rv1217c pump which is composed of two copies of Rv1218c and one copy of Rv1217c (Braibant et al., 2000). Studies on this pump have shown that Rv1218c is involved in the efflux of diverse classes of compounds, none of which are frontline antimicrobials (Balganesh et al., 2010). In addition, a study carried out on clinical isolates has shown that over-expression of this pump correlated with multidrug resistance (Wang et al., 2013). A study designed to identify mutants defective in intracellular survival has shown that transposon inactivation of Rv1218c resulted in the highest fitness cost (Stewart et al., 2005). To date, the exact nature of the molecule(s) transported by the Rv1218c-Rv1217c pump and the precise physiological role is still unknown. However, it has been predicted that this efflux pump might possibly transport a lipid molecule(s) necessary for early stages of infection but may become toxic during non-replicating persistence (Turapov et al., 2014b).

1.10 Aims and Objectives of this Study

The general aim of this thesis was to study some proteins which might play a role in the survival and persistence of *M. tuberculosis* inside macrophages. Based on published data, two proteins were selected to be investigated; the first is the regulator of antimicrobial assisted survival, RaaS (Rv1219c) and the second is a member of the PPE family of proteins which is PPE51 (Rv3136).

Published data has already shown that RaaS (RV1219c/BCG_1279c) is important for prolonged persistence in stationary phase and inside macrophages. Hence, a part of this thesis was aimed to investigate the importance of RaaS during exposure to some of the stressful conditions which are likely to be experienced by mycobacteria during infection.

The up regulation of *rv3136* during macrophage infection and the unique expression levels during different conditions suggested that it deserved further investigation. Hence the following objectives were undertaken:

- Investigating the survival of the RaaS deletion mutant during exposure to different stresses.
- Complementing the RaaS deletion mutant with different constructs to investigate the importance of tight regulation of efflux pumps during exposure to stress conditions.
- Generation of a Δ*rv3136 M. tuberculosis* mutant to characterise its growth phenotype and investigate the role that it might play in persistence inside macrophages.

Chapter 2 : **Materials and Methods**

2.1 Bacterial strains and plasmids:

The strains and plasmids used in this study are listed in table 3:

Table 3: Strains and plasmid used

Strain or plasmid	Description	Reference or source
M. bovis BCG Glaxo strain	Wild type	Laboratory strain obtained from GSK, Stevenage
M. bovis BCG Δ*raaS*	An in-frame deletion mutant missing *bcg_1279c*	Turapov et al., 2014a
M. bovis BCG Δ*raaS*$_{com1}$	*M. bovis* BCG deletion mutant missing *bcg_1279c* *complemented* with full length *rv1219c*	Turapov et al., 2014a
M. bovis BCG Δ*raaS*$_{com2}$	*M. bovis* BCG deletion mutant missing *bcg_1279c* complemented with full length *rv1219c*, binding site and the predicted promoter region	This study
M. bovis BCG Δ*raaS*$_{com3}$	*M. bovis* BCG deletion mutant missing *bcg_1279c* complemented with full length *rv1219c* and binding site	This study
M. tuberculosis CDC1551	Wild type	BEI resources (Lamichhane et al., 2003)
M. tuberculosis CDC1551	MT3221 transposon mutant (*rv3136*::Tn)	BEI resources (Lamichhane et al., 2003)
M. tuberculosis H37Rv	Wild type	laboratory stocks obtained from W. Jacobs
M. tuberculosis H37Rv Δ*rv3136*	An in-frame deletion mutant missing *rv3136*	This project
pGem-T Easy	*E.coli* cloning vector carrying Ampicillin resistance	Promega
p2NIL	Suicide vector for manipulation of the gene of interest, kanamycin resistant.	Parish and Stoker (2000)
pGoal19	*Hyg* P_{Ag85}-*lacZ* P_{hsp60}-*sacB* PacI cassette vector, ampicillin resistant	Parish and Stoker (2000)
E.coli DH5α	*E.coli* chemically competent cells used for cloning	Invitrogen, Bioline
pRBexint	Integrating plasmid harbouring hygromycin resistance	Bottai et al., 2011

2.2 Cultivation of Bacteria

2.2.1 Cultivation of mycobacteria

2.2.1.1 Cultivation of *M. bovis BCG*

All handling of *M. bovis* BCG was done in a Class 2 safety cabinet. Starter cultures of *M. bovis* BCG were set up by inoculating 5ml of Middlebrook 7H9 broth (Becton, Dickinson and Company) supplemented with 10% (v/v) ADC (see appendix) and 0.05% (v/v) Tween 80 (designated as supplemented 7H9 medium) with 100µl of frozen stocks and incubating them at 37 °C (without shaking) until they reached the required optical density (OD_{600nm}). *M. bovis BCG* were also grown on Midddlebrook 7H10 agar (Becton, Dickinson and Company) supplemented with 10 % (v/v) ADC (designated as supplemented 7H10 agar). When necessary, appropriate antibiotics were added to the cultivation media for selection of resistance markers (Hygromycin 50µg/ml).

2.2.1.2 Cultivation of *M. tuberculosis*

All the experimental procedures which required manipulation of live *M. tuberculosis* were carried out in either class1 or 2 microbiology safety cabinet within the category 3 facility. The established SOPs and Code of Practise were followed during carrying out these procedures. *M. tuberculosis,* CDC1551 and H37Rv strains were grown in either Sauton's medium or in supplemented 7H9 medium or Middlebrook 7H10 agar plates. For the maintenance of *M. tuberculosis* CDC1551 MT2551 (Rv3136) transposon mutant 25µg/ml of kanamycin was added to the cultivation media. The cultures were incubated at 37 °C with shaking (100 rpm) until they reached the required OD_{600nm}. One litre roller bottles (Greiner, Stonehouse, UK) were used for growing large scale cultures (100-200ml). These cultures were incubated at 37 ºC on a rolling mechanism with constant mixing until they reached the required OD_{600nm}.

2.2.2 Cultivation of *E. coli*

Bacterial starter cultures were set up by inoculating 5ml of LB broth containing the appropriate antibiotic with either one colony picked from a LA agar plate or 100µl of growing culture. Then the cultures were incubated overnight at 37°C with

shaking (220 rpm). This starter culture was used to inoculate larger volumes when larger scale cultures were required.

2.3 Cell line and culture conditions

2.3.1 J774.1 cells

J774.1 is a semi adherent murine macrophage-like cell line. The cells were grown in RPMI 1640 (Sigma Aldrich) supplemented with 10% (v/v) foetal calf serum (FCS), 2 mM L-glutamine, Penicillin 100 U/ml and Streptomycin 100 µg/ml. The cells were sub cultured twice a week by gently dislodging them from the flask with a cell scraper. Then a sample was taken for counting and viability assessment. After that the cells were split at a density of approximately 2-3 x 10^5 cells /ml by adding fresh media. The cells were incubated in a humidified incubator (5% CO_2) at 37°C.

2.3.2 THP-1 cells

THP-1 cell line is a human acute monocytic leukemia cell line. The cells were grown in RPMI 1640 (Sigma Aldrich) supplemented with 10% FCS, 2 mM L-glutamine, 1% non-essential amino acids, Penicillin 100 U/ml and Streptomycin 100 µg /ml. The cells were maintained twice a week by counting them in a haemocytometer and splitting them at a density of 2 x 10^5 cells /ml through the addition of fresh supplemented RPMI media as mentioned above. The cells were incubated in a humidified incubator (5% CO2) at 37°C.

2.4 Viability determination

2.4.1 Trypan blue exclusion method

The aim of this method was to count the total number of cells and assess the viability of macrophages by using trypan blue exclusion. This was done by mixing 10µl of cell suspension with an equal volume of trypan blue in a 0.6ml eppendorf tube. Then 10µl of the mixed suspension was loaded in a haemocytometer. Using the 10X objective lenses, the total number of cells and the number of dead blue cells were counted. The total number of cells and the percentage of viable cells were calculated as following:

Number of cells/ml= number of cells counted X 2 X 10^4

Viability of cells (%) = number of live cells / total number of cells X 100

2.4.2 Colony forming unit counting

This method gives an estimate of the number of live bacteria based on their ability to form visible colonies on agar plates. The viability of both *M. tuberculosis* and *M. bovis* BCG was determined by serially diluting (10- fold) bacterial cultures in an appropriate diluent (either PBS or 7H9) followed by spotting three 20µl drops on duplicate segments of supplemented Middlebrook 7H10 agar plates. After drying, the plates were placed in either one bag (*M. bovis* BCG) or in double bags (*M. tuberculosis*) and incubated in a static incubator at 37°C until visible colonies appear (usually 2-3 weeks). The average number of colony forming units (CFU) was calculated as following:

CFU/ml = average number of CFU in 20µl X 50 X dilution factor

2.5 Preparation of frozen stocks

2.5.1 Preparation of bacterial frozen stocks

Bacterial frozen stocks were prepared for long term preservation by adding 850µl of growing culture to 150µl of 100%(v/v) of glycerol in a suitable cryogenic vial. Then they were carefully mixed and stored at -80 ºC.

2.5.2 Preparation of frozen stocks of *M. bovis BCG* for macrophage infection

Starter cultures were prepared as previously mentioned in 2.2.1.1 until they reached an OD_{600nm} of 0.5-0.8. Once the cultures reached the desired OD, 2ml of the starter culture was used to set up a large culture volume by inoculating 180 ml of supplemented 7H9 medium. This culture was incubated at 37°C without shaking until the culture reached an OD_{600nm} of ~0.5. After this the cells were harvested by centrifugation at 1000xg for 15 minutes, then washed once in 50ml RPMI and centrifuged again at 1000xg for 15 minutes to concentrate them. Then the mycobacterial pellet was resuspended in 18ml of RPMI with 10% (v/v) of autoclaved glycerol and 0.1% bovine serum albumin. Aliquots of the bacterial suspension in cryogenic vials (500 µl in each tube) were frozen and stored at -80ºC. After one week of freezing, a vial was thawed at room temperature and the number of CFUs was assessed by serial dilution and plating on supplemented 7H10 agar plates as described in 2.4.2

2.5.3 Preparation of frozen stocks of *M. tuberculosis* for infection

One hundred microliters of frozen stocks from each of the wild type and *rv3136* deletion mutant of *M. tuberculosis* H37Rv were inoculated in 5ml of supplemented 7H9 medium. The cultures were incubated at 37 °C with shaking (100rpm) until they reached an OD_{600nm} of 0.5. This starter culture was used to inoculate a 30 ml culture of supplemented 7H9 medium. These cultures were incubated at 37 °C with shaking (100rpm) until they reached an OD_{600nm} of approximately 0.5. After that the cultures were centrifuged at 1000xg for 15 minutes followed by removal of supernatant and the pellet was resuspended in 30 ml of 10% (v/v) glycerol. Then the cultures were centrifuged as previously mentioned to wash any remaining 7H9 medium. This washing step was repeated twice and after the last washing step the supernatant was removed and the pellet resuspended in 3 ml of 10% (v/v) glycerol. Then the cell suspension was aliquoted in cryo vials and stored at -80 °C. In the next day, one vial was thawed from each strain for CFU counts.

2.5.4 Preparation of frozen stocks of eukaryotic cells

Three flasks ($75cm^3$ flasks) of actively dividing macrophages were used to prepare frozen stocks for storage. Cells were centrifuged at 100xg for 5 minutes at room temperature, then the supernatant was discarded and the pellet resuspended in 10ml of the following freezing medium:

The normal growth medium for the cell line	8ml
FCS	1ml
Sterile DMSO	1ml

Then the cells were aliquoted in screw-top cryo vials (with rubber sealing O rings in the lid to prevent liquid Nitrogen entry). The tubes were placed into a small polystyrene box and the container stored in -80C freezer for at least 4 hours (or overnight). In the next day the vials were transferred to liquid nitrogen. After a few days, one vial was taken out and thawed quickly in warm tap water. Then the contents of the vial was emptied in a centrifuge tube containing 10ml of prewarmed RPMI and centrifuged briefly (100xg for 3 minutes) to pellet the cells. The supernatant was discarded and the pellet resuspended in 10ml of normal growth medium and transferred to a tissue culture flask to check the cells ability to recover.

2.6 Genetic Manipulation (for generation of deletion and complementation strains)

2.6.1 Polymerase Chain Reaction (PCR)

The desired gene coding sequences were amplified by usually using high fidelity platinum *tag* DNA polymerase (Invitrogen). In the cases of high GC regions, either Phusion high-fidelity or Q5 high-fidelity DNA polymerase (NEB, UK) were used for amplification of the desired region. GoTaq® polymerase (Promega) was used in the case of diagnostic PCR. Depending on the polymerase used, the components listed in table 4 were mixed in a 0.2ml PCR tube (Fisher, UK).

Table 4 : Components of PCR reaction

Components	**Polymerase**			
	Platinium tag	Phusion	Q5	Go tag
dNTP mix	40µM	40µM	40µM	40µM
Buffer	10X buffer	5X GC buffer	5X Q5 buffer	5X green buffer
50 mM MgSO4	2mM	-	-	-
Forward primer	0.25µM	0.5 µM	0.5 µM	0.25µM
Reverse primer	0.25µM	0.5 µM	0.5 µM	0.25µM
DMSO[2]	10%v/v	10%	-	10%
Q5® high GC Enhancer	-	-	1X	-
polymerase	0.2µl (1.0U)	0.5µl (1.0U)	0.5µl (0.02/ µl)	0.25µl (1.25U)
Template DNA	1ng	1ng	1ng	1ng
DNase free H_2O	To 50µl	To 50µl	To 50µl	To 50µl

[2] DMSO was used as required.

After mixing, the PCR tubes were placed in a thermocycler. The following cycling conditions were applied when either high fidelity platinum *tag* DNA polymerase or Go tag polymerase was used:

Initial denaturation	94 ºC	5 min	
Denaturation	94 ºC	30 sec	30 cycles
Annealing[3]	55 ºC	30 sec	
Extension[4, 5]	68 ºC	1 min	
Final extension	68 ºC	5 min	

The following cycling conditions were used when either Phusion or Q5 polymerase was used:

Initial denaturation	98 ºC	30 sec	
Denaturation	98 ºC	10 sec	30 cycles
Annealing[6]	65 ºC	30 sec	
Extension[7]	72 ºC	15 sec	
Final extension	68 ºC	2-5 min	

2.6.2 Restriction digestion of DNA

PCR products and cloning vectors were digested using suitable restriction enzymes. All buffers and enzymes were purchased from NEB (UK) and the digestion was performed according to manufacturer's instructions. The total reaction volume ranged from 10 to 50 µl, depending on the amount of DNA digested. The reaction was incubated at 37ºC and then the DNA fragments were checked by running the samples on an agarose gel (0.8-2%). The size of DNA fragments was checked by exposing gel to UV light. Then the DNA fragments were excised and gel purified using QIAquick Gel Extraction Kit (Qiagen).

[3] For each primer pair the annealing temperature was usually -5°C of the Tm of the primer pair.
[4] One minute was used for each 1 Kb being amplified.
[5] The extension temperature was 72 ºC, when Go Tag polymerase was used.
[6] For each primer pair the annealing temperature was usually -5°C of the Tm of the primer pair.
[7] 15 seconds were used for each Kb being amplified.

2.6.3 DNA ligation

The cloning vector and PCR product were ligated by setting up a ligation reaction in a total volume of 10µl. the reaction was performed by mixing the following components:

Plasmid DNA	100ng
PCR product	100ng
2x Rapid ligation buffer[8]	5µl
T4 DNA ligase[9]	1µl
Nuclease free H_2O	up to 10 µl

Then the reaction was incubated overnight at room temperature. The next day the reaction was either transformed into competent *E. coli* cells (DH5α) or stored at -20ºC until further use.

2.6.4 Transformation

The ligated plasmids were transformed into either DH5α competent cells (Invitrogen) or α-select DH5α gold efficiency competent cells (Bioline Reagents Ltd, London). Aliquots (25µl) of competent cells were mixed with 2µl of ligation mixture followed by incubation on ice for 30 minutes. Then the cells were heat shocked by heating them to 42 ºC for 45 seconds. Then the mixture was placed on ice for 2 minutes followed by adding 900 µl of LB medium containing 10mM MgSO4 and incubating the mixture at 37 ºC with shaking (220 rpm) for 1 hour. After that the mixture was plated on LA agar plates containing appropriate antibiotics and incubated overnight.

2.6.5 Plasmid DNA preparation

A number of single colonies were picked and each was used to inoculate 5ml of LB broth supplemented with the appropriate selective antibiotic. Then the cultures were incubated over night at 37 ºC with shaking (220 rpm). In the next day, the cells were harvested by centrifugation at 3000xg for 20 minutes. Plasmid DNA was extracted using GeneElute™ miniprep kit (Sigma-Aldrich) according to manufacturer's instructions. This kit employs a modified alkaline-SDS lysis

[8] 10X rapid ligation buffer was used when the volume of DNA was more than 2µl used.
[9] Promega

procedure in which DNA is adsorbed under appropriate high salt conditions. After the removal of contaminant by a simple washing step, the DNA was eluted in DNase and RNase free H2O. Then the purified plasmid DNA was checked for the presence of insert by performing restriction digestion followed by gel electrophoresis.

2.6.6 Agarose gel electrophoresis

Agarose gel electrophoresis was performed to separate and visualise DNA fragments. Depending on the size of the DNA fragment, the concentration of agarose used ranged from 0.8% to 2%. TAE buffer (see appendix) was used to dissolve agarose and ethidium bromide was added to a final concentration of 0.2µg/ml. DNA samples mixed with 6x gel loading buffer (Fermentas) were loaded into wells. Five microliters of GeneRuler™ 1 kb ladder (Fermentas) or 100bp (NEB) were used to estimate the size of the separated DNA fragments. Electrophoresis was done at 90 volts for 45 minutes and visualisation of DNA was carried out under UV light from a transilluminator. The gel was photographed using a gel doc system (ImageQuant 100, GE Healthcare).

2.6.7 Electroporation

For the transformation of *M. tuberculosis* H37Rv and *M. bovis* BCG cells by electroporation the method described by Parish and Stoker was followed (Parish and Stoker, 1998). The mycobacterial culture was prepared by inoculating 100µl of either *M. tuberculosis* H37Rv or *M. bovis* BCG into 5 ml of supplemented 7H9 medium. The culture was then incubated at 37°C with shaking (100rpm) or statically for two weeks. After that 100ml of supplemented 7H9 medium was inoculated with 5 ml of the previously prepared growing culture and incubated at 37°C with shaking (100 rpm) or statically. The cultures were left to grow until it reached an OD_{600nm} of 1 in the case of *M. tuberculosis* and an OD_{600nm} of 0.6-0.8 in the case of *M. bovis* BCG. This culture was used to prepare electrocompetent cells. Electrocompetent cells were prepared by transferring 100ml of the mycobacterial culture into four 50ml falcon tubes. The cells were harvested by centrifugation at 2500xg for 15 minutes at room temperature. After centrifugation, the supernatant was discarded and the cells were washed by resuspending the

pellet in 25ml of 10% (v/v) glycerol and centrifugation at 2500xg for 15 minutes. The washing steps were repeated two more times and the volume of bacterial culture was reduced at each washing step. After the last washing step the pellet was resuspended in 1ml of 10% (v/v) glycerol. The electroporation was performed in 2mm electrode-gap electroporation cuvettes (Eppendorf, UK) by mixing 400µl of the freshly prepared competent cells with 3µl of DNA[10]. The cuvette was then placed in the electroporation chamber of Bio-Rad Gene Pulser and subjected to one single pulse of 2.5 kV, 25µF with the pulse controlled resistance at 1000Ω. Then the cuvette was immediately taken out of the chamber and 1ml of supplemented 7H9 medium was added to the cuvette. Then the contents of the cuvette were transferred into a 30ml universal tube and incubated at 37°C overnight (with shaking for *M. tuberculosis* and statically for *M. bovis* BCG). In the next day, different dilution of the cells (neat, 10^{-1} and 10^{-2}) were plated on supplemented 7H10 agar plates. Competent cells with no DNA were also plated on supplemented 7H10 agar plates as a negative control. The plates were sealed with Nescofilm and incubated at 37°C for 4-5 weeks.

2.7 Infection of Macrophages with Mycobacteria

2.7.1 Infection of THP-1 cell

2.7.1.1 Preparation of THP-1 for infection

Actively dividing THP-1 cells maintained as previously mentioned in 2.3.2 were used for infection. The cells were prepared for infection by washing them twice to remove antibiotics. Washing was done by transferring the cell culture from tissue culture flasks to a 50ml falcon tube and the cells were pelleted by centrifugation at 100xg for 5 minutes. Then the supernatant was removed and the pellet resuspended in 10ml of fresh warm antibiotic free RPMI followed by centrifugation as before. Then the cells were counted in a haemocytometer and seeded into a 6 well tissue culture plates. THP-1 cells were differentiated in to macrophages by phorbol 12-myristate 13-acetate (PMA) at a concentration of 100ng/ml. The cells were left to differentiate into macrophages by incubating them at 37 ºC for 48 hours in a humidified incubator (5% CO_2). After this

[10] 5-10µg of DNA was used for suicide plasmids and 1µg for replicating plasmids.

incubation step, the cells had become adherent. Then the cells were washed twice with warm RPMI to remove the PMA. The cells were resuspended in fresh antibiotic free RPMI media and left to rest overnight in a 37 ºC in a humidified incubator (5% CO_2).

2.7.1.2 Infection of THP-1 cell with *M. bovis* BCG

Differentiated THP-1 cells were infected with *M. bovis* BCG (wild type and *ΔraaS*). The cells were then incubated for 2 hours at 37˚C. Amikacin (200 µg/ml) was then added to the wells with infected cells for 1 hour to kill extracellular bacteria. After that Amikacin was washed twice with warm antibiotic free RPMI. Then the cells in each well were resuspended in 2ml of fresh antibiotic free RPMI. The cells were then incubated at 37˚C for 48, 96, 144 and 192 hours. Control wells were included which contained RPMI with bacteria only and wells with PMA treated THP-1 cells only. At each incubation time 0.2% (v/v) of Triton X-100 was added to lyse the cells. Then the number of bacteria was assessed by serial dilution and plating on supplemented 7H10 agar plates.

2.7.2 Infection of J774.1

2.7.2.1 Preparation of J774.1 for infection

J774.1 cells were detached from the tissue culture flasks by gentle scraping with a cell scraper. Then the cells were transferred to a 50ml falcon tube and washed twice with warm antibiotic free RPMI as previously mentioned in 2.7.1.1. Then the cells were counted in a haemocytometer and seeded into tissue culture plates. The cells were incubated overnight in a 37˚C humidified incubator (5% CO_2) to allow the cells to adhere to the wells.

2.7.2.2 Infection of J774.1 cells

J774.1 cells were infected with either *M. tuberculosis* H37Rv (wild type and *rv3136* deletion mutant) or with *M. bovis* BCG (wild type and *ΔraaS*). Then the wells were incubated at 37˚C for 4 hours[11]. Control wells were included which contained RPMI with bacteria only and wells with J774.1 cells only. After that the wells which contained J774.1 cells were washed two times by removing the old

[11] The incubation time was 6 hours when the cells were infected with *M. bovis BCG*.

medium and adding 2ml of warm antibiotic free RPMI. After the last washing step the cells were maintained in 2ml of RPMI containing 10% (v/v) FCS. Then the cells were further incubated for 24, 48, 72 and 96 hours. After each time point, the cells were lysed with 0.2% (v/v) of Triton X-100 (Sigma-Aldrich). Then the number of bacteria was assessed by serial dilution and plating on supplemented 7H10 agar plates.

2.8 Preparation of culture supernatant

Culture supernatant (SN) was prepared from *M. bovis* BCG (wild type and *ΔraaS*) by growing them in supplemented 7H9 medium until they reached an OD_{600nm} of 0.5 - 0.7. Then the cells were harvested by centrifugation at 2000xg for 15 minutes. The supernatant was carefully removed and subsequently filtered by passing through a 0.2µm filter. After this step the resulting SN was either used for experiments immediately or freeze dried and stored at - 80ºC until needed.

2.9 Statistical analyses

Statistical analyses and significance tests were performed using Prism 6 (GraphPad Software, Inc.) statistical software, whereas calculations were carried out using Excel 2010 (Microsoft Corp.). Two tailed, unpaired students T- test was used to determine the P value unless otherwise stated. The statistical significance between results was denoted by either * ($P \leq 0.05$), ** ($P \leq 0.01$) or *** ($P \leq 0.001$).

Chapter 3 ：**The role of RaaS during stress conditions**

3.1 Introduction

The regulator of antimicrobial assisted survival RaaS (encoded by *rv1219c* in *M. tuberculosis* and *bcg_1279c* in *M. bovis BCG*) has been previously demonstrated to possess an important role in prolonged survival in stationary phase and during mouse infection perhaps by dysregulating function of the efflux pumps (Turapov et al., 2014a). This transcriptional regulator controls the expression of several genes. Among those are two genes that encode subunits of predicted ATP dependant efflux pumps (Rv1218c/1217c in *M. tuberculosis* and BCG_1278c/1277c in *M. bovis BCG* and DrrC). It appears that a broader investigation would provide further insight into the importance of this predicted transcriptional regulator in survival during exposure to stressful conditions which are likely to be experienced by mycobacteria during infection. Hence this chapter was focused on further investigating the survival of the RaaS deletion mutant during exposure to different stresses (macrophage infection, nutrient starvation, acidic stress, nitrosative stress and exposure to H_2O_2). *M. bovis* BCG Glaxo strain was chosen for these investigations as the difference between *bcg_1279c* and its *M. tuberculosis* and *M. bovis* homologues is one amino acid (W113C) due to a single guanosine to cytosine substitution. Therefore it was reasonable to assume that both proteins probably play similar roles in these closely related organisms.

3.1.1 Macrophage infection models

Human and murine derived monocyte cultures have been traditionally employed to study the intracellular growth rates of various strains of mycobacteria (usually comparing wild type and mutant strains). These models are used to evaluate various aspects such as initial mycobacterial uptake, intracellular growth, intracellular survival and induction of cytokine release or production. Both primary and *in vitro* differentiated cells have been employed in an attempt to resemble the *in vivo* situation and investigate host-pathogen interactions. Macrophage-like cell lines have been widely used mainly because of practical (easy to maintain) and standardisation aspects (Riendeau and Kornfeld, 2003). Of the available macrophage cell lines, THP1 cells have been an attractive model used by many researches for mycobacteria related infection studies (Theus et al., 2004). It has been shown that this particular cell line has the ability to develop macrophage

function after the addition of stimulators such as PMA. Furthermore, it has been suggested that differentiated THP-1 cells closely resemble the behaviour of both human alveolar macrophages (Riendeau and Kornfeld, 2003) and human monocyte derived macrophages (Stokes and Doxsee, 1999).

A variety of murine macrophage models have also been available to use in mycobacterial infection studies. Of those, the J774 macrophage cell line has been the most commonly utilised cell line (Lewin et al., 2008). Parallel studies in mouse infection models with the same genetic background can complement the results obtained in this cell line and assist in prediction of intercellular activity in humans.

3.1.2 Oxidative and Nitrosative stress in mycobacteria

In order to survive within the host, *M. tuberculosis* and other species of mycobacteria must cope with a variety of host mediated stresses. Of those stresses, are the ones resulting from reactive oxygen and nitrogen species (ROS and RNS) produced by macrophages via NADPH oxidase and inducible nitric oxide synthase (Ehrt and Schnappinger, 2009). ROS are defined as oxygen derived molecules which consist of both oxygen radicals; superoxide ($O_2^{\bullet-}$), hydroxyl ($^{\bullet}OH$), peroxyl ($RO_2^{\bullet}$), and alkoxyl) and non-radicals; hypochlorous acid (HOCl), ozone (O_3), singlet oxygen (O_2), and hydrogen peroxide (Thannickal and Fanburg, 2000). In general, ROS is produced as a result of a number of subsequent reactions which starts with the production of superoxide that eventually converts to hydrogen peroxide (H_2O_2) either spontaneously or as a result of catalysis by superoxide dismutase. It has been shown that ROS have the ability to interact with a number of molecules such as proteins, carbohydrates, nucleic acids and lipids resulting in the destruction of such molecules. These interactions may lead to alteration of the function of target molecules and are thought to facilitate the damage to invading organisms (Bedard and Krause, 2007). As mentioned previously, RNS has been extensively reported as one of the major antimicrobial molecules which are produced within the macrophage. Nitric oxide (NO) is generated by nitric oxide synthase isoforms in a reaction which involves NADPH-dependent oxidation of L-arginine to citrulline (Nathan and Shiloh, 2000). Their production is part of the host complex response which target to limit the survival and proliferation of invading microorganisms. High

levels of markers associated with NO have been reported in lesions from lungs of TB patients (Choi et al., 2002). Furthermore, it has been found that low, non-toxic concentrations of NO have the ability to inhibit respiration, slow growth, and induce the dormancy regulon (Voskuil et al., 2003).

3.1.3 Acidic stress in mycobacteria

During bacterial infection, macrophages are activated by cytokines (such as IFN-γ and TNF-α) resulting in a number of events specifically aimed to induce killing of the invading microorganisms. Among those events is gradual acidification of phagosomes and phagolysosome fusion. In order to survive and thrive inside host macrophages, the TB bacilli manipulate the intraphagosomal environment resulting in phagosomes which fuse poorly with host cell lysosomes and barely acidify (pH~6.5). However, once macrophages have been activated by immune cell-derived cytokines, the pH of the mycobacterial phagosome drops below 5.5, and mycobacterial growth is restricted to some extent (Vandal et al., 2009). It is thought that the initial acid stress response enables mycobacteria to survive in the harsh intracellular environment at subsequent stages of infection. One study has found that blocking acidification of *M. tuberculosis* containing phagosomes abolished the up-regulation of 80% of the genes normally up-regulated upon macrophage entry which proves that pH is a vital signal which the bacterium responds to (Rohde et al., 2007a). In addition, transcriptional profiling has led to the identification of 23 *M. tuberculosis* genes which are induced by acidic pH in the macrophage phagosome (Rohde et al., 2007b). This suggests that acid adaptation plays a vital role in the survival of mycobacteria within macrophages.

3.1.4 Models of nutrient starvation in mycobacteria

Several *in vitro* models have been developed to mimic the persistent state of *M. tuberculosis.* These models were required for testing and identification of potential novel drugs to target non-replicating and stressed mycobacteria. It has been well established that *M. tuberculosis* may be exposed to low-nutrient concentrations within host granulomatous lesions (Timm et al., 2003). Models using nutrient deprivation have demonstrated that *M. tuberculosis* has the ability to survive for extended periods in a non-growing state. In 1933, Loebel and colleagues used a nutrient starvation model where cultures were transferred from

nutrient rich medium into PBS in an attempt to investigate the effect of nutrients predicted to be available in a granuloma on the metabolism of *M. tuberculosis*. Nutrient starvation resulted in a gradual shutdown of respiration to minimal levels, however bacilli remained viable and were able to recover and grow when inoculated into rich medium (Loebel et al., 1933). In 1974, Nyka found that *M. tuberculosis* cultures starved in distilled water displayed the same properties of altered morphology and staining as those bacilli isolated from lung lesions (Nyka, 1974). Based on Loebels nutrient starvation model, Betts and coworkers have developed an *in vitro* nutrient starvation model which causes the microbe to arrest growth, limit aerobic metabolism and become resistant to existing drugs but maintain viability. It is thought that this model mimics certain features of persistent *M. tuberculosis* and may provide a useful tool for testing potential anti- TB drugs (Betts et al., 2002).

3.1.5 The importance of studying stress response in mycobacteria

Improvement of the efficiency of anti-TB drug requires a more comprehensive understanding of the mechanisms of action and kinetics of how drugs kill *M. tuberculosis in vitro.* In order to accomplish this goal, it is vital to provide a definition on how drugs penetrate and work effectively within TB lesions. This must be preceded by a detailed knowledge of the factors dictating the tissue microenvironment which are influenced by both microbe and host responses (Ehlers and Schaible, 2012). During growth in the host, *M. tuberculosis* encounters a number of environmental and/or physiological stresses. In persistent stages, *M. tuberculosis* faces stresses such as nutrient limitation, oxygen and nitrogen intermediates, hypoxia as well as low pH (Manganelli et al., 2004). Thus gaining more information on how *M. tuberculosis* responds to the environment of the lung will aid in developing more effective strategies for treating acute manifestations of the disease, as well latent and chronic infections.

3.2 Methods

3.2.1 Organism and Media

The organisms used in this chapter were wild type *M. bovis* BCG and *raaS* deletion mutant. Both were cultured in either supplemented 7H9 medium or Sauton's medium at 37° C without shaking. The *raaS* mutant is an in-frame deletion mutant in which 573-bp was deleted from *M. bovis* BCG as previously described (Turapov et al., 2014a).

3.2.2 *In vitro* growth characteristics of wild type *M. bovis* BCG and *raaS* deletion mutant.

A starter culture was set as previously mentioned in 2.2.1.1 and once this starter culture reached an OD_{600nm} of 0.6 -0.8, it was used to inoculate 5ml of supplemented 7H9 medium. This culture was incubated at 37°C without shaking until it reached an OD_{600nm} of 0.5. At this point, 50ml of supplemented 7H9 medium was inoculated with the previously prepared secondary culture and incubated at 37°C without shaking in a 250ml flask at an initial OD_{600nm} of 0.01. The OD_{600nm} was measured every 2-4 days, and 10-fold serial dilutions were plated on supplemented 7H10 agar plates for CFU counts.

3.2.3 Infection of macrophages with *M. bovis* BCG and *raaS* deletion mutant

Cells were prepared and infected as previously mentioned in 2.7.1 and 2.7.2. The infection was carried out using $4x10^5$ of macrophages at a multiplicity of infection (MOI) of 30 for THP-1 cells and 1 for J774.1 cells. The experiment was done in 6 well tissue culture plates. Controls wells containing macrophages only were included. At every time point, macrophages were inspected by microscopy to ensure continued confluence and adherence to the wells.

3.2.4 Exposure of wild type *M. bovis* BCG and *raaS* deletion mutant to stressful conditions (acidic, nitrosative and H_2O_2 treatment)

These experiments were carried out to test the effect of the above stress conditions on the survival of *M. bovis BCG* Glaxo strain (wild type and *raaS* deletion mutant). Starter cultures of wild type *M. bovis* BCG and *raaS* deletion

mutant were prepared as previously mentioned in 2.2.1.1. The starter culture was incubated at 37°C without shaking until it reached an OD_{600nm} of 1. For nitrosative and H_2O_2 treatment, this starter culture was used to inoculate 50ml of supplemented 7H9 medium in a 250ml flask at a starting OD_{600nm} of approximately 0.01. The flasks were incubated at 37°C without shaking until the cultures reached an OD_{600nm} of 0.4-0.6. At this stage, the cultures were transferred into 30ml universal tubes (5 ml of culture in each tube) and they were subjected to either nitrosative stress or treated with H_2O_2 as an oxidative stress mediator. The effect of nitrosative stress was tested by subjecting the bacilli to two nitric oxide donors (compound number 10126069 at a concentration of 15 µM and compound number 10626173 at a concentration of 45 µM). Both compounds were kindly provided by Dr Vadim Makarov (Bach Institute of Biochemistry, Moscow). These two compounds have the property of spontaneous and enzymatic release of nitric oxide. Triplicate tubes of each strain were treated with two nitric oxide donors. Negative controls were included which had no nitric oxide donors. Then the samples were incubated at 37°C without shaking for 72 hours. At 0, 24 and 48 hours of incubation samples were taken from each tube for CFU counting.

For H_2O_2 treatment, triplicate tubes (each containing 5ml of culture) of each strain were treated with 0, 1 and 10mM of H_2O_2 (Fisher scientific). At 0, 1, 6 and 24 hours of incubation, samples were taken from each tube for CFU counting. The pH of the cultures was checked by a pH indicator paper during each time point. In some experiments, reserpine was added at a final concentration of 20µg/ml immediately after H_2O_2 treatment in order to test the effect of ABC efflux pump inhibitors on the survival of both strains during H_2O_2 treatment.

The effect of acidic stress was tested by subjecting the bacteria to a range of pH (pH=4.5, pH=5.5 and pH=7). Starter cultures were prepared and incubated as mentioned above, then used to inoculate 100 ml of Sauton's medium (pH 7) supplemented with 10% (v/v) ADC and 0.05% (v/v) Tween 80 in a 500 ml flask. This culture was incubated at 37°C without shaking until it reached an OD_{600nm} of approximately 0.5. When the culture reached the desired OD, it was transferred into 50ml falcon tubes (10 ml of culture in each tube). Then the tubes were centrifuged at 2500xg for 15 minutes to pellet the cells. Following centrifugation

the supernatant was discarded and the cells were resuspended in 10ml of freshly prepared Sauton's medium at a range of pH (pH=4.5, pH=5.5 and pH=7). Then the cultures were incubated at 37 °C without shaking for 72 hours. At 0, 6, 24 and 72 hours of incubation, a sample was takes from each tube for CFU counting. The percentage of survival after each of the above stresses was calculated relative to live bacilli prior to treatment.

3.2.5 Exposure of wild type *M. bovis* BCG and *raaS* deletion mutant to nutrient starvation

The nutrient starvation model used was that previously described by Betts and colleagues which is starvation in PBS (Betts et al., 2002). Briefly, the starting cultures were prepared as previously described in 2.2.1.1[12] and the cultures were incubated at 37 °C without shaking and left to grow until they reached late log phase. After that the cultures were diluted 1:100 into a 250 ml flask which contained 50 ml of supplemented 7H9 broth[12]. The flasks were incubated at 37 °C without shaking and left to grow for 7 days. After 7 days of growth to log phase, the cultures were transferred to a 50ml falcon tube and centrifuged at 2500xg for 15 minutes to pellet the cells. Then the supernatant was removed and the cells were washed twice by resuspending the pellet in 50ml of PBS and centrifugation as mentioned previously. After the last washing step, the cultures were resuspended in PBS and each 5ml of culture was transferred to a 30ml universal tube. Sterile methylene blue solution at a final concentration of 1.5mg/ml was added to 5ml cultures maintained under starvation conditions as a visual indication of oxygen depletion. In addition, control tubes which contained either PBS or supplemented 7H9 medium and methylene blue but lacking bacteria were also included. The viability of bacteria during the nutrient starvation period was determined by taking samples and doing serial dilutions which were then plated on supplemented 7H10 agar plates at several time points (day 0, weeks 1, 2, 3 and 6).

[12] The final concentration of Tween 80 was 0.025%.

3.2.6 The Effect of culture supernatant from *raaS* deletion mutant on eukaryotic cells and wild type *M. bovis* BCG

The possible toxic effect exerted by culture supernatant (SN) obtained from *raaS* deletion mutant was investigated via two strategies. First, the effect of SN on the viability of eukaryotic cells (J774.1 in this case) was investigated. This was done by preparing SN from both wild type and *raaS* deletion mutant as previously mentioned in 2.8 which was subsequently freeze dried and stored in -80 ºC. Supplemented 7H9 medium (without Tween 80) was also freeze dried in the same manner to serve as a control. In the next day, one tube of SN from each of the wild type and mutant was thawed and subsequently resuspended in sterile H_2O. The volume of H_2O used for resuspension was 10 times lower than the original one before freeze drying. This SN was incubated on ice for 30 minutes. J774.1 macrophages ($4x10^5$ cells) were seeded in each well of a 6 well tissue culture plate and different volumes (100, 50, 25 and 5 µl) of SN were added to the cells. Then the cells were incubated overnight at 37 ºC in a humidified incubator (5% CO_2). In the next day, the plates were taken out of the incubator and the supernatant was carefully mixed with a pipette without disturbing the monolayers. Next samples were taken to estimate the number of floating dead cells by trypan blue exclusion method as mentioned in 2.4.1. This was done by counting the number of dead blue cells in a 4.5mm field of view using a 40x objective lens. Viability was measured after 24 and 48 hours of incubation. Second, the effect of SN obtained from the *raaS* deletion mutant on the survival of wild type *M. bovis* BCG during oxidative stress was investigated using the following protocol. Mid log phase cultures (triplicate tubes with 5ml of culture in each) of wild type *M. bovis* BCG were centrifuged to pellet the cells and the supernatant was discarded. The cells were washed once by resuspending in 5ml of 7H9 and centrifugation at 2000xg for 15 minutes. Then the supernatant was discarded and the resulting pellet was resuspended in 5ml of freshly prepared *raaS* deletion mutant SN. Afterwards this culture was exposed to 10mM H_2O_2 and incubated at 37º C without shaking. At 1, 6 and 24 hours of incubation, a sample was taken from each tube, serially diluted and plated on supplemented 7H10 agar plates for viability determination as previously mentioned in 2.4.2.

3.2.7 Level of intracellular ATP in growing cultures of *M. bovis* BCG (wild type and *raaS* deletion mutant)

The BacTiter-Glo Microbial Cell Viability Assay Kit (Promega) was used to quantify the level of intracellular ATP in wild type *M. bovis* BCG and *raaS* deletion mutant according to manufacturer's instructions. This was done by growing both strains in supplemented 7H9 medium and collecting 250µl samples at various optical densities (0.3, 0.5 and 1). These samples were stored at -80 ºC until use. The assay was performed by mixing 25 µl of each sample with an equal volume of freshly prepared BacTiter-Glo reagent in a 96 well white flat bottom plate (Corning). Then the plate was incubated in the dark for 5 minutes with constant shaking. The emitted luminescence was recorded by a plate reader and expressed as relative light units (RLU). ATP standards ranging from 1µM to 10pM were included to generate a standard curve. The ATP content for each sample was extrapolated from the standard curve.

3.3 Results

3.3.1 Growth of wild type *M. bovis* BCG and *raaS* deletion mutant in 7H9 medium

As mentioned previously, one of the first steps was to confirm the *in vitro* growth characteristics of the strains. This was done by growing both wild type *M. bovis* and *raaS* deletion mutant in supplemented 7H9 medium and measuring both OD_{600nm} and CFU counts over a period of 22 days. The results represented in Figure 7A showed that the OD_{600nm} values of the deletion mutant were consistent with that of the wild type in the majority of the time points. In addition, there was no big difference in CFU values (Figure 7B). These results are consistent with the previous published data which had shown that the *raaS* deletion mutant did not display any significant growth defect during logarithmic growth phase in supplemented Sauton's medium (Turapov et al., 2014a).

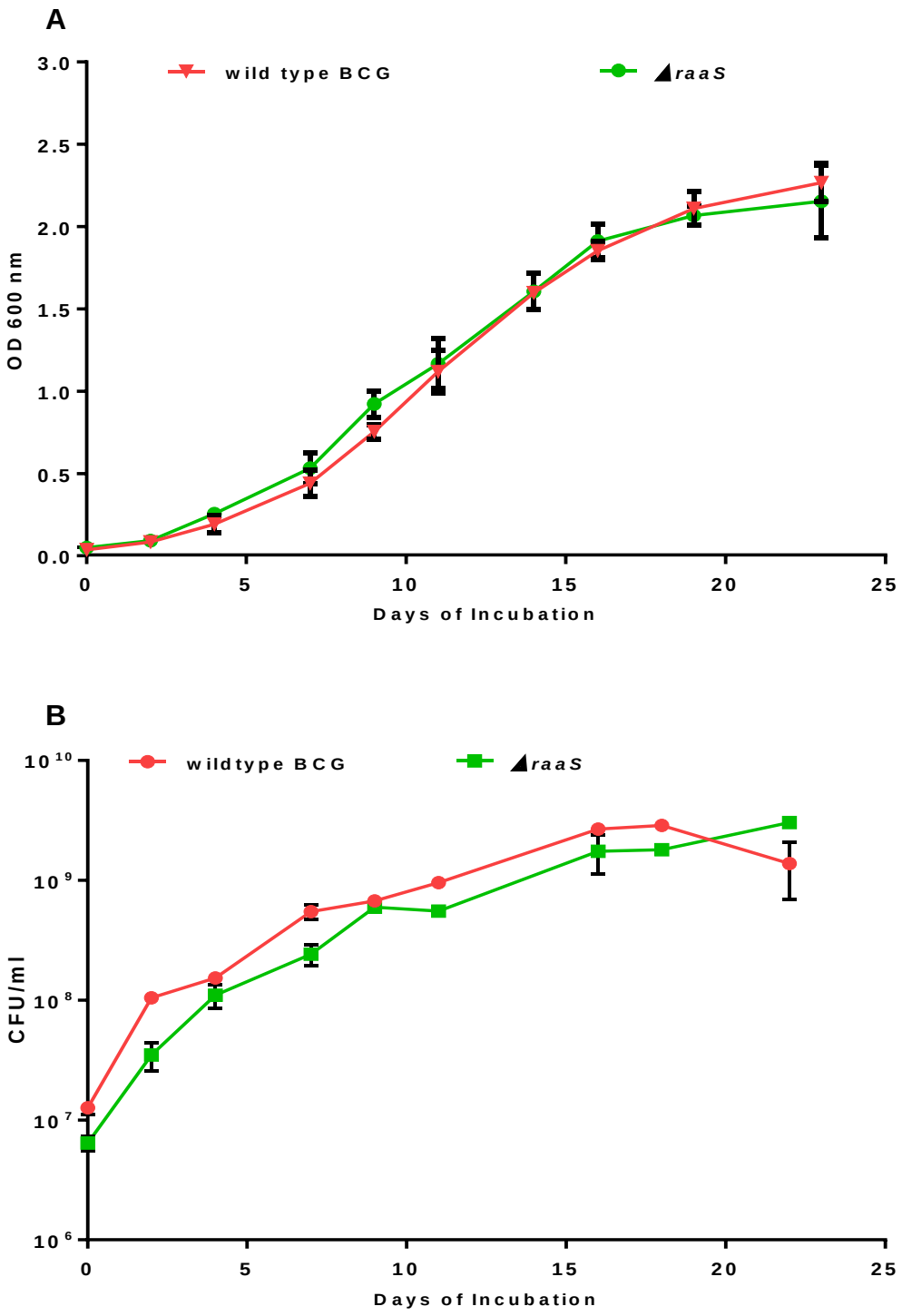


Figure 7: Growth of wild type *M. bovis* BCG and *raaS* deletion mutants *in vitro.*
M. bovis BCG strains (*M. bovis* BCG and *raaS* deletion mutant) were inoculated in supplemented 7H9 medium and incubated at 37°C without shaking. The growth rate was followed over a period of 22 days by taking OD_{600nm} measurements and doing CFU counts. A: OD measurements B: CFU counts. Data points are average of two biological replicates (two flasks). Data are expressed as mean ± SEM.

3.3.2 The deletion of *raaS* causes a defect in intracellular survival

The role of *raaS* during intracellular survival was assessed in a macrophage infection model. For that purpose two cell lines have been used, one of human origin (THP-1 cells) and the other of murine origin (J774.1 cells). Both of these cell lines have been shown to be efficiently infected with *M. tuberculosis* (Mehta et al., 1996, Theus et al., 2004). The first cell line used was THP-1 cell and the infection was carried out using PMA differentiated cells at an MOI of 30. It has been previously reported that treatment of human monocytic THP-1 cells with PMA results in differentiation of these cells into mature macrophage like cells. It is thought that PMA treatment mimics the effect exerted by diacylglycerol which mediates natural macrophage differentiation (Riendeau and Kornfeld, 2003). The infection was carried out for two hours and this was followed by an attempt to remove extracellular bacteria by treatment with amikacin which is an aminoglycoside antimicrobial agent. Published data has shown that PMA differentiated and adherent THP-1 cells apparently do not replicate (Stokes and Doxsee, 1999). Taking these data into account, the intracellular survival of mycobacteria was assessed every 48 hours up to 192 hours (8 days). The monolayers were washed twice at every time point and the number of intracellular bacteria was assessed by lysing the macrophage monolayers and taking samples for CFU determination. The number of intracellular bacteria recovered after 2 hours of infection was similar for both wild type and the deletion mutant, indicating that there was no difference in uptake between both strains (Figure 8). However, after 48 hours of infection, the intracellular survival of the mutant seems to be significantly decreased when compared with that of the wild type. This significant difference in intracellular survival was also observed at 144 and 192 hours of infection ($p=0.03$ and $p=0.02$ respectively). Thus it can be concluded that the *raaS* deletion mutant suffers from a significant survival defect inside THP-1 cells.

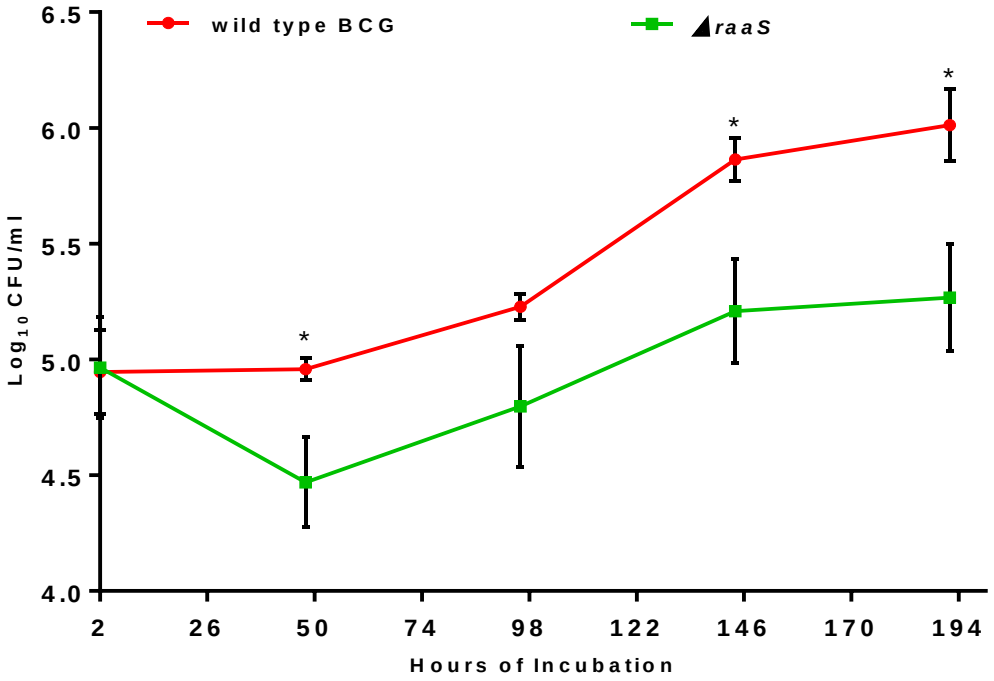


Figure 8: Intracellular survival of wild type *M. bovis* BCG and *raaS* deletion mutant in THP-1 cell.

PMA treated THP-1 cells were infected with both wild type *M. bovis* BCG and *raaS* deletion mutant for 2 hours. Then Amikacin was added (200µg/ml) for 1 hour followed by two washing steps. The wells were further incubated for 48, 96, 144 and 192 hours. At each time point, the cell monolayers were washed twice, lysed with 0.2% (v/v) of Triton X-100 and samples were taken for CFU determination. Result is an average of two independent experiments, each including three technical replicates (three individual wells). Data are expressed as mean ± SEM.

The intracellular survival of wild type *M. bovis* BCG and *raaS* deletion mutant was further investigated in the murine macrophage cell line J774.1 which is a well-established model for studies of mycobacterial pathogenesis (Lewin et al., 2008). J774.1 cells were infected with both strains at an MOI of 1 for 6 hours as it has been previously reported that the percentage of *M. tuberculosis* inoculum associated with J774.1 monolayers after 6 hours of addition of infection is about 13% which is considered a good rate (Mehta et al., 1996). However it is likely that *M. bovis* BCG would have a lower association rate. The number of intracellular bacteria was assessed at 6, 24, 48, 72 and 96 hours by determining the number of CFU/ml in the macrophage lysate.

After the initial 6 hour infection period, the number of intracellular bacteria was comparable for both the wild type and the mutant indicating that the extent of phagocytosis was similar and that uptake was independent of strain type as shown in Figure 9. After 24 hours of infection, there was a sharp decline in the intracellular survival rate of both wild type and *raaS* deletion mutant. However, when comparing survival rate between both strains, it was noted that the wild type survived significantly better than the *raaS* deletion mutant (P=0.0008).

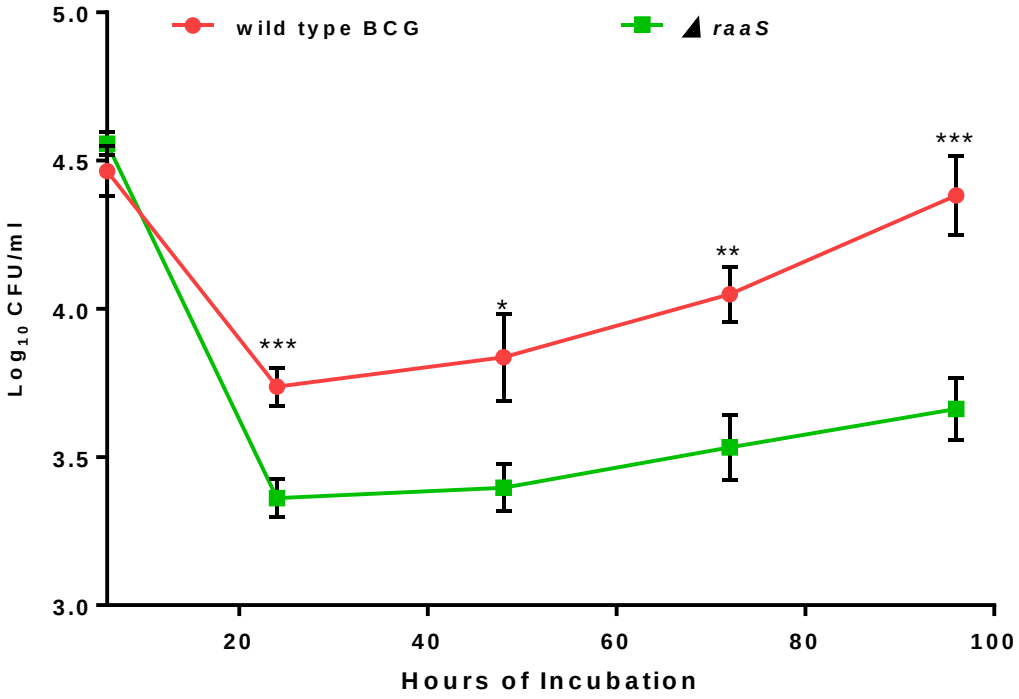


Figure 9: Intracellular survival of wild type *M. bovis* BCG and *raaS* deletion mutant in J774.1 cell.

The murine macrophage cell line J774.1 was infected with both wild type *M. bovis* BCG and *raaS* deletion mutant for 6 hours. Then infected monolayers were washed three times to remove extracellular bacteria. Wells were further incubated for 24, 48, 72 and 96 hours. At each time point, the cell monolayers were washed twice, lysed with 0.2% (v/v) of Triton X-100 and samples were taken for CFU determination. Result is an average of three independent experiments, each including three technical replicates (three individual wells). Data are expressed as mean ± SEM.

Furthermore, a significant difference in survival was also seen after 48, 72 and 96 hours of infection (P=0.018, 0.002 and 0.0006 respectively). Taken together, the data obtained from both cell lines clearly indicate that the *raaS* deletion mutant suffers from an intracellular survival defect which is seen from early stages of infection.

3.3.3 The *raaS* deletion mutant showed a survival defect in a nutrient starvation model

It was interesting to investigate whether the deletion of *raaS* had an influence on survival during nutrient starvation as it has been previously shown that during infection there are limited nutrients available to the bacteria (Sassetti and Rubin, 2003, Munoz-Elias and McKinney, 2005, Russell et al., 2009). Several lines of evidence have shown that during infection *M. tuberculosis* shifts to lipids and host derived cholesterol as nutrient sources (Garay et al., 2015). It has been established that *M. tuberculosis* adapts to nutrient starvation by arresting growth and minimising aerobic metabolism (Schnappinger et al., 2003).

The survival of both wild type and *raaS* deletion mutant during starvation in PBS was followed over a period of 6 weeks after transferring the strains from nutrient rich media to PBS. After 1 week of starvation, both wild type and mutant managed to maintain their viability as shown in Figure 10. However, comparison of the number viable bacteria at the beginning of starvation with that of 6 week starved cultures showed that the *raaS* deletion mutant suffered from a sharp decline in CFU values. On the other hand, data for the wild type showed that there was a slight drop in viability after 6 weeks of starvation. These data clearly indicate that this nutrient starvation model highlighted the survival defect of the mutant during nutrient deprivation.

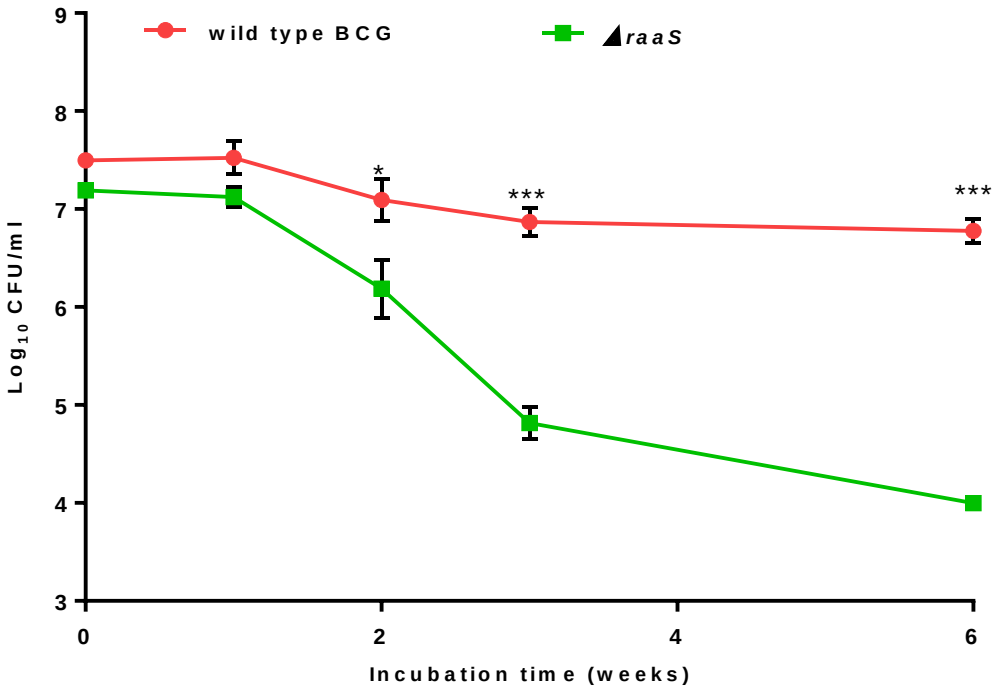


Figure 10: Survival of *M. bovis* BCG (wild type and *raaS* deletion mutant) during starvation in PBS.
Cultures of wild type *M. bovis BCG* and *raaS* deletion mutant were transferred from nutrient-rich medium into PBS. The number of colony forming unit were followed over a period of 6 weeks. Data shown are average of $\log_{10}$ CFU/ml from two independent experiments, each including three biological replicates. Data are expressed as mean ± SEM.

3.3.4 The role of RaaS during acidic stress

It is well established that *M. tuberculosis* encounters acidity once inside the host. *M. tuberculosis* has the ability to block phagosome acidification but this block maybe incomplete and the phagosome can still undergo a drop in pH (Deretic and Fratti, 1999). The acidic pH can either be toxic killing the microbe or inhibiting by restricting the ability to replicate. The survival of both wild type and *raaS* deletion mutant during acidic stress was investigated by growing them in supplemented Sauton's medium which has been buffered to a range of pH (7, 5.5 and 4.5). In the beginning, the cultures were first grown in supplemented 7H9 medium until they reached an OD_{600nm} of 0.4-0.5. At this stage, the cultures were pelleted, supernatant removed and resuspended in an equal volume of supplemented Sauton's medium at a range of pH (7, 5.5 and 4.5) and the viability was measured after 6, 24 and 72 hours. The percent of survival was calculated

based on the number of CFU/ml at 0 hours in pH 7. The results represented by Figure 11 shows that after 6 hours of incubation there was no difference in survival between the two strains in any of the tested pHs. However the results obtained after 24 hours of incubation showed that both of the wild type and the *raaS* deletion mutant did show a decrease in survival at pH=4.5. After 72 hours of incubation, there was a decrease in the survival of the *raaS* deletion mutant at pH=4.5.

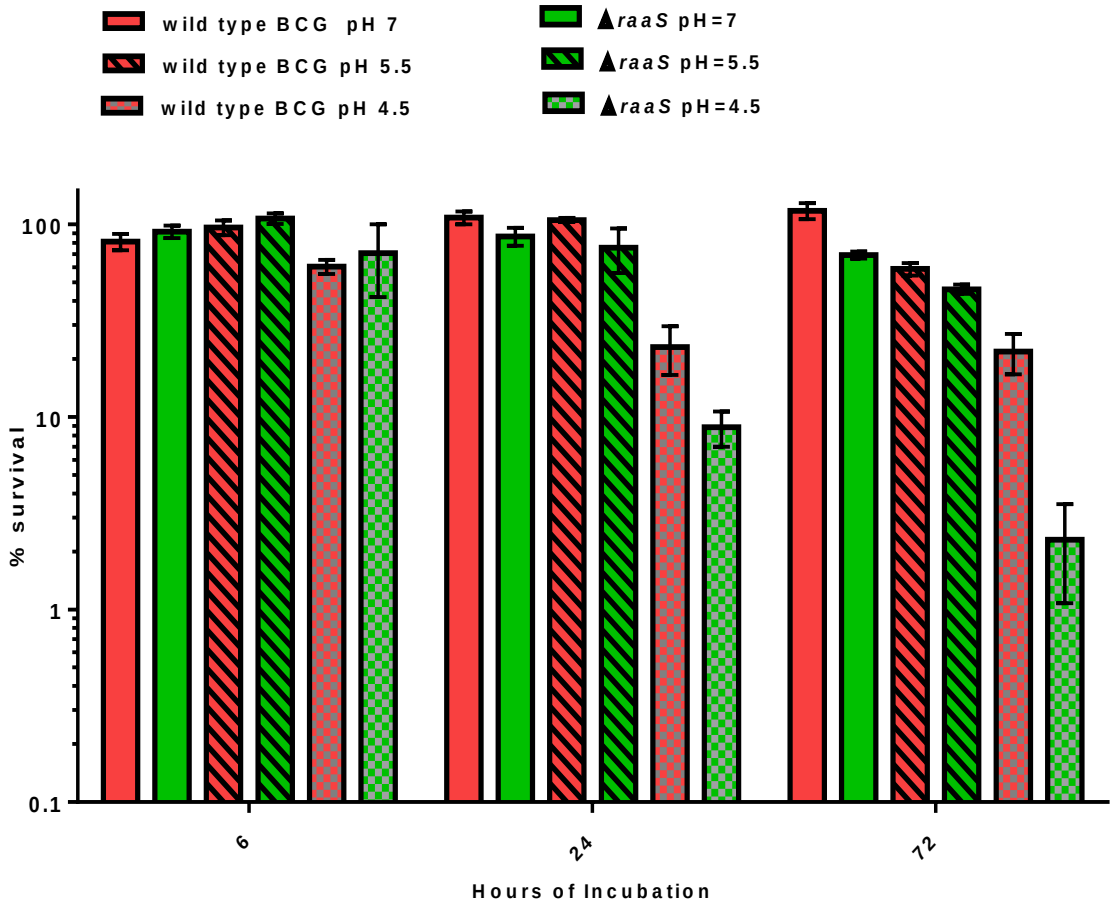


Figure 11: Survival of *M. bovis BCG* (wild type and *raaS* deletion mutant) after applying acidic stress.
Cultures of wild type *M. bovis BCG* and *raaS* deletion mutant were grown in supplemented 7H9 medium until they reached an OD_{600nm} of 0.4-0.5. Then the cultures were centrifuged at 1000xg for 15 minutes. After that the supernatant was discarded and the pellet was resuspended in supplemented Sauton's medium at a range of pH (pH=4.5, pH=5.5 and pH=7.0). Then the cultures were incubated at 37 °C for 72 hours. At 6, 24 and 72 hours of incubation, a sample was taken for CFU counting. Survival was calculated depending on the CFU/ml at pH 7.0 at 0 hours. Data represent mean ± SEM of three technical replicates (three individual tubes).

During the initial 72 hours of incubation in supplemented Sauton's medium (pH 7.0) both the wild type and the mutant did not show much replication, suggesting that *M. bovis* BCG needs time to adapt when transferred from 7H9 medium (which is an enrichment media) to Sauton's medium. Therefore the protocol was modified by growing the strains first in supplemented Sauton's medium at pH=7 until they reached an OD_{600nm} of 0.4-0.5 and then pelleting the cells and resuspending in an equal volume of supplemented Sauton's medium at a range of pH (7.0, 5.5 and 4.5). The results of this protocol represented by Figure 12 revealed that after 24 hours of incubation the survival did not change for both strains in any of the tested pHs.

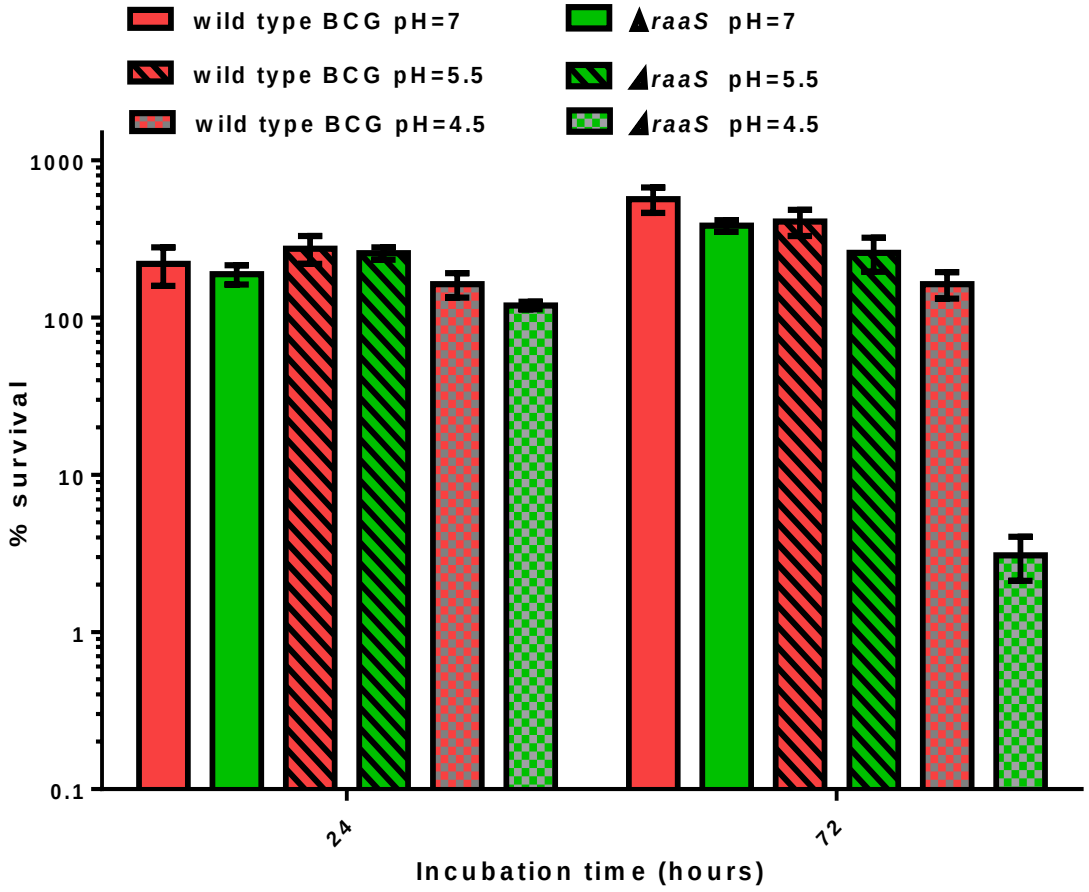


Figure 12: Survival of *M. bovis* BCG (wild type and *raaS* deletion mutant) after applying acidic stress with modified protocol.
Cultures of wild type *M. bovis BCG* and *raaS* deletion mutant were grown in supplemented Sauton's broth until they reach an OD_{600nm} of 0.4-0.5. Then the cultures were centrifuged at 1000xg for 15 minutes. After that the supernatant was discarded and the pellet was resuspended in supplemented Sauton's medium at a range of pH (pH=4.5, pH=5.5 and pH=7). Then the cultures were incubated at 37 °C for 72 hours. At 24 and 72 hours of incubation, a sample was taken for CFU counting. Survival was calculated depending on the CFU/ml at pH 7 at 0 hours. Data represent mean ± SEM of three technical replicates (three individual tubes).

On the other hand, after 72 hours of incubation the *raaS* deletion mutant showed a defect in survival at pH 4.5. This result was consistent with the results of the previous experiment (Figure 11) which also showed a decrease in mutant survival after 72 hours of exposure to a pH of 4.5. Together, these results indicate that the *raaS* deletion mutant may also have a survival defect when subjected to acidic *in vitro* growth conditions.

3.3.5 The role of RaaS in survival during nitrosative stress

Within the host, *M. tuberculosis* is subjected to RNS in an attempt to eliminate or limit the replication of the microbe. Thus it was important to investigate the survival of the *raaS* deletion mutant during nitrosative stress and whether it could be linked to the defect in intracellular survival. The NO donors (compounds number 10126069 and 10626173) used were compounds that have the ability to enzymatically and spontaneously release NO. The minimal inhibitory concentration (MIC) in *M. smegmatis* for donor number 10126069 and number 10626173 were 16µg/ml (15µM) and 4µg/ml (45µM) respectively (Sarah Glenn, personal communication). Thus, these compounds were used in concentrations equivalent to the MIC for each one. Both wild type *M. bovis* BCG and *raaS* deletion mutant were treated with compound number 10126069 for 24, 48 and 72 hours as shown in Figure 13. Statistical comparison between survival of wild type only and wild type treated with compound 10126069 showed a significant difference after 24 and 72 hours of incubation ($P<0.01$ and $P<0.0001$ respectively), but not after 48 hours of incubation. On the other hand, statistical comparison between survival of treated and untreated *raaS* deletion mutant showed a significant decrease in survival after 72 hours of treatment ($P<0.05$) but not after 24 or 48 hours.

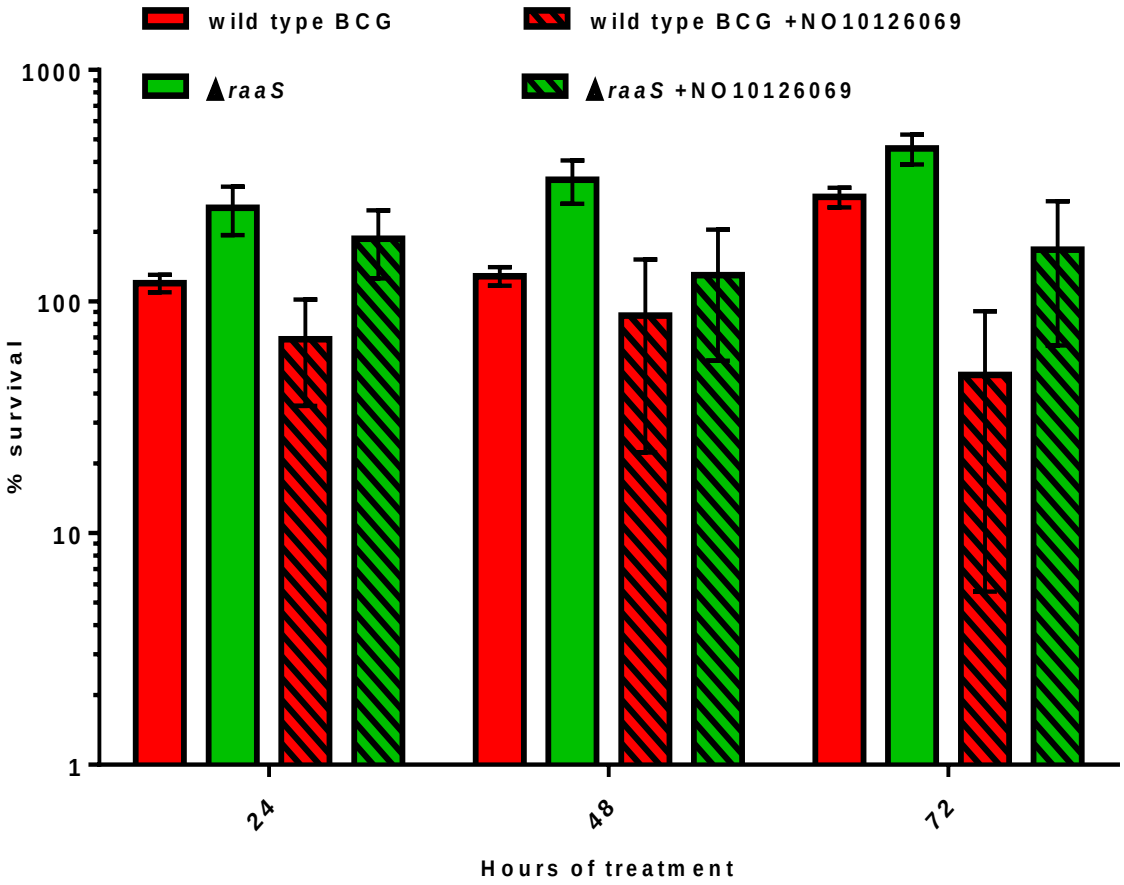


Figure 13: Survival of *M. bovis* BCG (wild type and *raaS* deletion mutant) after treatment with nitric oxide donor compound 10126069 (15µM).
Cultures of wild type *M. bovis BCG* and *raaS* deletion mutant were grown in supplemented 7H9 medium until they reach an OD_{600nm} of 0.4-0.5. Then the cultures were treated with NO donor number 10126069 (15µM). Cultures were incubated at 37 °C for 72 hours and at 0, 24, 48 and 72 hours of incubation, a sample was taken for CFU counting. Survival was calculated depending on the CFU/ml before treatment. Result is an average of three independent experiments, each including three technical replicates (three individual tubes). Data are expressed as mean ± SEM.

Treatment of wild type *M. bovis* BCG and *raaS* deletion mutant with compound number 10626173 at a final concentration of 45µM resulted in decrease in survival of both strains as seen in Figure 14. Interestingly, statistical comparison between treated and untreated strains of wild type *M. bovis* BCG showed a highly significant decrease in survival of treated strain after 24, 48 and 72 hours of incubation ($P<0.001$). Statistical comparison between mutant only and treated mutant showed a significant defect in survival after 24, 48 and 72 hours of

treatment (P<0.01). This apparent discrepancy in the results obtained with the two NO donors means that it is not possible to reach a conclusion regarding the effect of nitrosative stress on the mutant. Thus further experiments using one of the commercially available NO donors might help in assessing if deletion of *raaS* has any effect on survival during nitrosative stress.

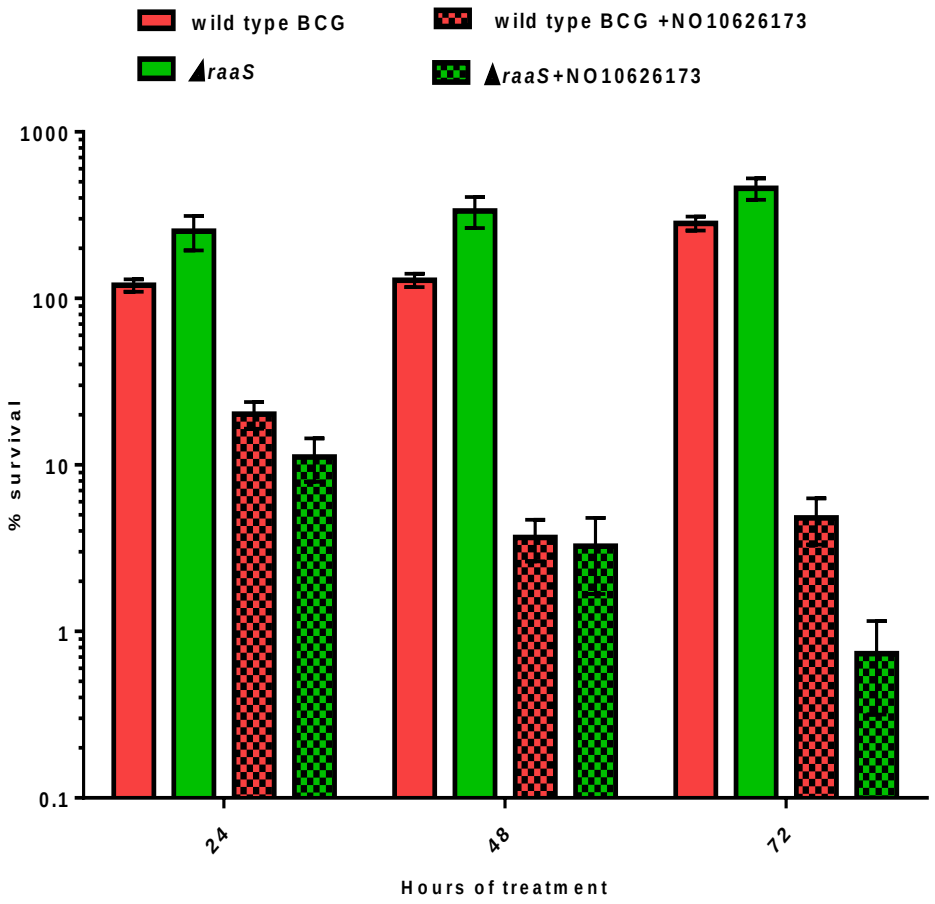


Figure 14: Survival of *M. bovis* BCG (wild type and *raaS* deletion mutant) after treatment with nitric oxide donor compound 10626173 (45µM).

Cultures of wild type *M. bovis BCG* and *raaS* deletion mutant were grown in supplemented 7H9 until they reached an OD_{600nm} of 0.4-=0.5. Then the cultures were treated with NO donor number 10626173 (45µM). After that the samples were incubated at 37 °C for 72 hours. At 0, 24, 48 and 72 hours of incubation, a sample was taken for CFU counting. Survival was calculated depending on the CFU/ml before treatment. Results are average of three independent experiments, each including three technical replicates (three individual tubes). Data are expressed as mean ± SEM.

3.3.6 The role of RaaS during H_2O_2 exposure

Exposure to ROS is one of the stresses that mycobacteria encounter once inside the macrophage, thus I next investigated if RaaS (BCG1279c) has a role in survival during oxidative stress. This was done by using H_2O_2 as an oxidative stress mediator. Of the *M. bovis* BCG strains, Glaxo strain has been previously reported to be the most sensitive to H_2O_2 treatment (Hayashi et al., 2010). Therefore a preliminary experiment was carried out to investigate the concentrations of H_2O_2 which enables studying the survival of bacteria without complete elimination. In the beginning two different concentrations of H_2O_2 were tested, the first was 1mM and the second was 10mM. Exposure of both wild type and mutant to these concentrations of H_2O_2 showed that 1mM concentration had no effect on the survival. On the other hand, exposure to 10mM of H_2O_2 resulted in decrease in viability of both wild type and *raaS* deletion mutant without complete elimination of the bacteria as shown in Figure 15.

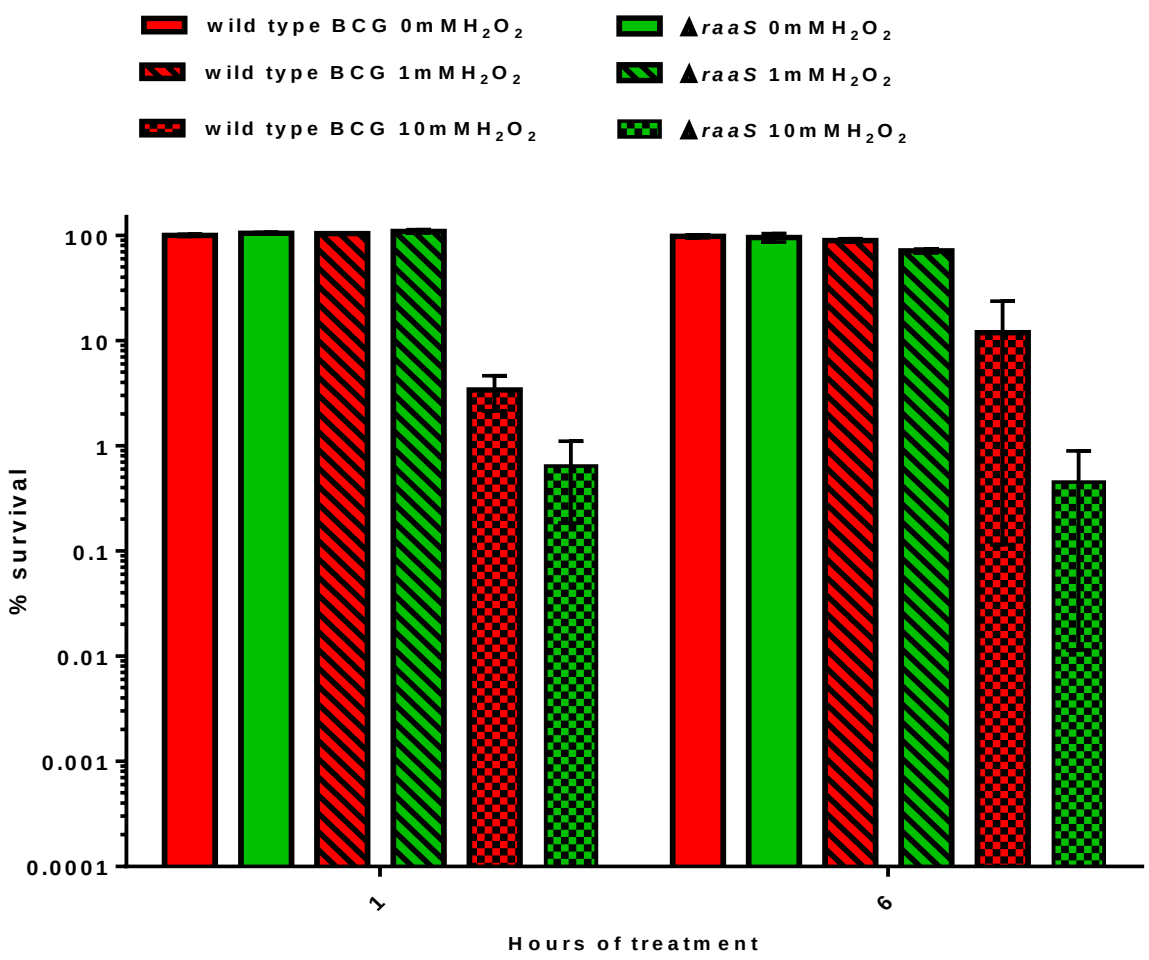


Figure 15: Survival of wild type *M. bovis* BCG and *raaS* deletion mutant after treatment with H_2O_2.
Cultures of wild type *M. bovis BCG* and *raaS* deletion mutant were grown in supplemented 7H9 medium until they reach an OD_{600nm} of 0.4-0.6. Then the cultures were treated with 1 and 10 mM of H_2O_2. After that the samples were incubated at 37 °C without shaking. At 0, 1 and 6 hours of incubation, a sample was taken for CFU counting. Survival was calculated depending on the CFU/ml before treatment. Result is from one experiment with three technical replicates (three individual tubes). Data are expressed as mean ± SEM.

Therefore subsequent experiments were done using 10mM of H_2O_2 as a mediator of oxidative stress. Treatment with 10mM of H_2O_2 resulted in a decreased survival of both wild type *M. bovis* BCG and the *raaS* deletion mutant. However, statistical analysis showed that treatment for 6 hours resulted in a significant decrease ($p=0.02$) in the deletion mutant when compared to the wild type as shown in Figure 16. Thus it is clear that RaaS plays a role in survival during oxidative stress. It is worth noting that the colonies of *raaS* deletion mutant treated with

10mM H_2O_2 took longer to recover on supplemented 7H10 plates when compared with treated wild type (21-26 days for mutant versus 18-21 days for wild type).

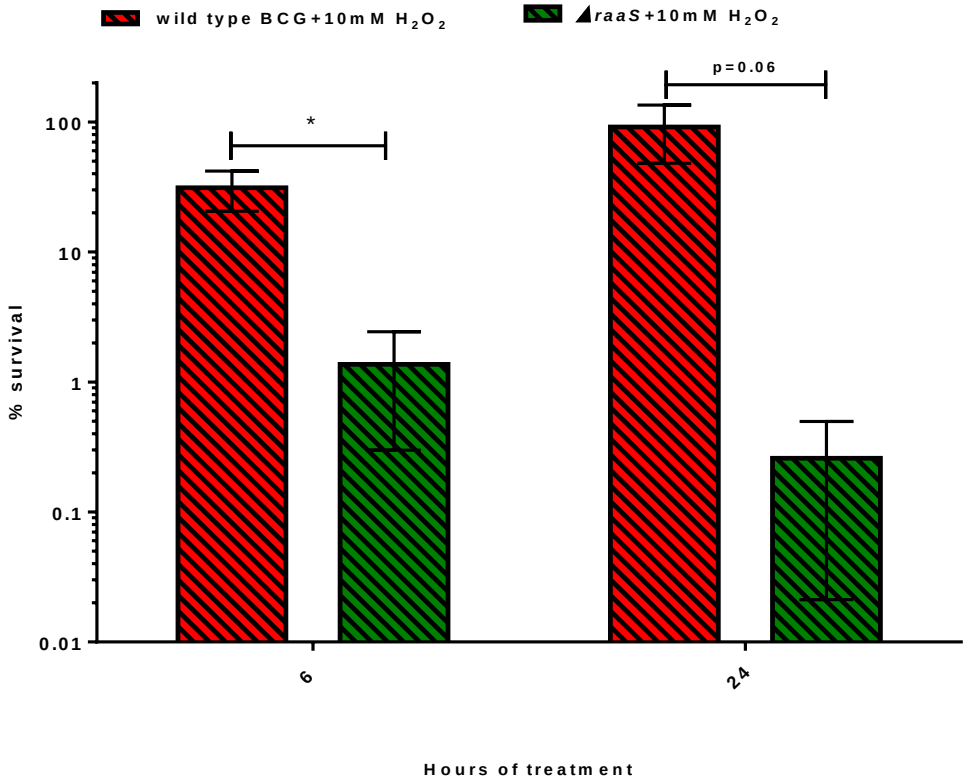


Figure 16: Survival of wild type *M. bovis* BCG and *raaS* deletion mutant after treatment with 10mM H_2O_2.
Cultures of wild type *M. bovis BCG* and *raaS* deletion mutant were grown in supplemented 7H9 medium until they reach an OD_{600nm} of 0.4-0.6. Then the cultures were treated with 10mM of H_2O_2. After that cultures were incubated at 37 °C without shaking. At 0, 6 and 24 hours of incubation, a sample was taken for CFU counting. Survival was calculated depending on the CFU/ml before treatment. Result is an average of three independent experiments, each including three technical replicates (three individual tubes). Data are expressed as mean ± SEM.

A previous study has established that RaaS acts as a repressor of *drrC/bcg_2960* (which encodes an annotated efflux pump) and the cluster of genes located immediately downstream from itself (Turapov et al., 2014a). It is predicted that these genes encode ABC transporters (Bcg_1278c/ Bcg_1277c) and are associated with integral membrane proteins. However the exact role of the Rv1218c/Rv1217c (Bcg_1278c/Bcg_1277c) pump has not been discovered yet. Therefore it was reasonable to predict that dysregulation of the pump as a result of deletion of its repressor could account for the survival defect of the *raaS*

deletion mutant during H_2O_2 treatment. One way to investigate this hypothesis is to test if blocking of the pumps via a pump inhibitor can improve the survival of the *raaS* deletion mutant during exposure to H_2O_2. The plant alkaloid reserpine is an ATP-dependent efflux pump inhibitor and has previously been used in bacterial efflux inhibition studies (Stavri et al., 2007, Huang et al., 2013, Turapov et al., 2014a). The concentration of reserpine used was taken from previous studies which showed that it did not exert any inhibitory effect on growth (Brown and Parish, 2008, Turapov et al., 2014a). The results represented by Figure 17 confirmed the findings of these previous studies.

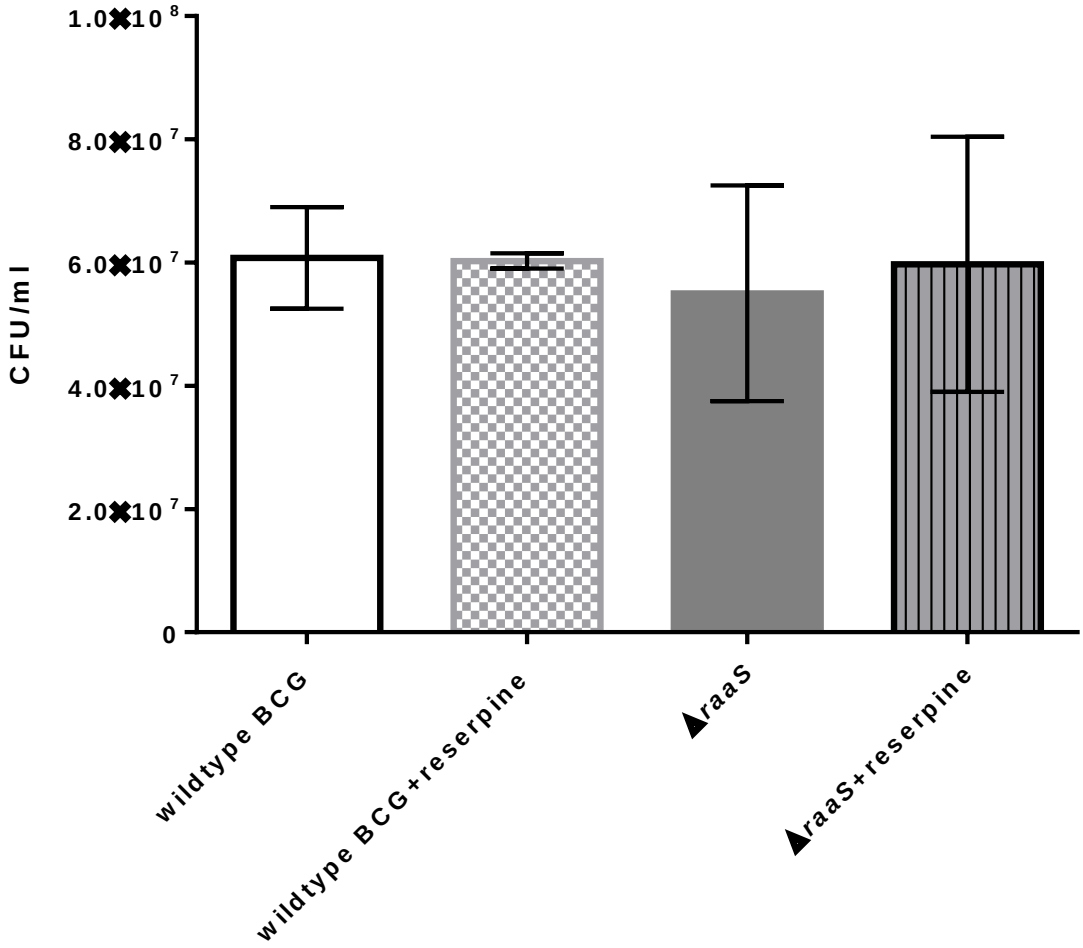


Figure 17: Effect of reserpine on the growth of wild type *M. bovis* BCG and *raaS* deletion mutant

Cultures of wild type *M. bovis BCG* and *raaS* deletion mutant were grown in 250ml flasks containing 50ml of supplemented 7H9 medium until they reached an OD_{600nm} of 0.4-0.5. Then the culture in each flask was divided into 4 falcon tubes, and reserpine (20µg/ml) was added to two of these tubes. The tubes were incubated at 37 °C without shaking for 24 hours and samples were taken for CFU determination. Error bars represent mean ± SEM of duplicate tubes.

The effect of reserpine at a final concentration of 20µg/ml on the survival of both strains during H_2O_2 treatment was tested and the results are shown in Figure 18 and Figure 19. These results clearly show that reserpine had an effect on increasing the survival of the mutant during H_2O_2 (10mM) treatment when

compared with H_2O_2 treated cells only with a significant difference seen after 1 hour of reserpine addition (p=0.007).

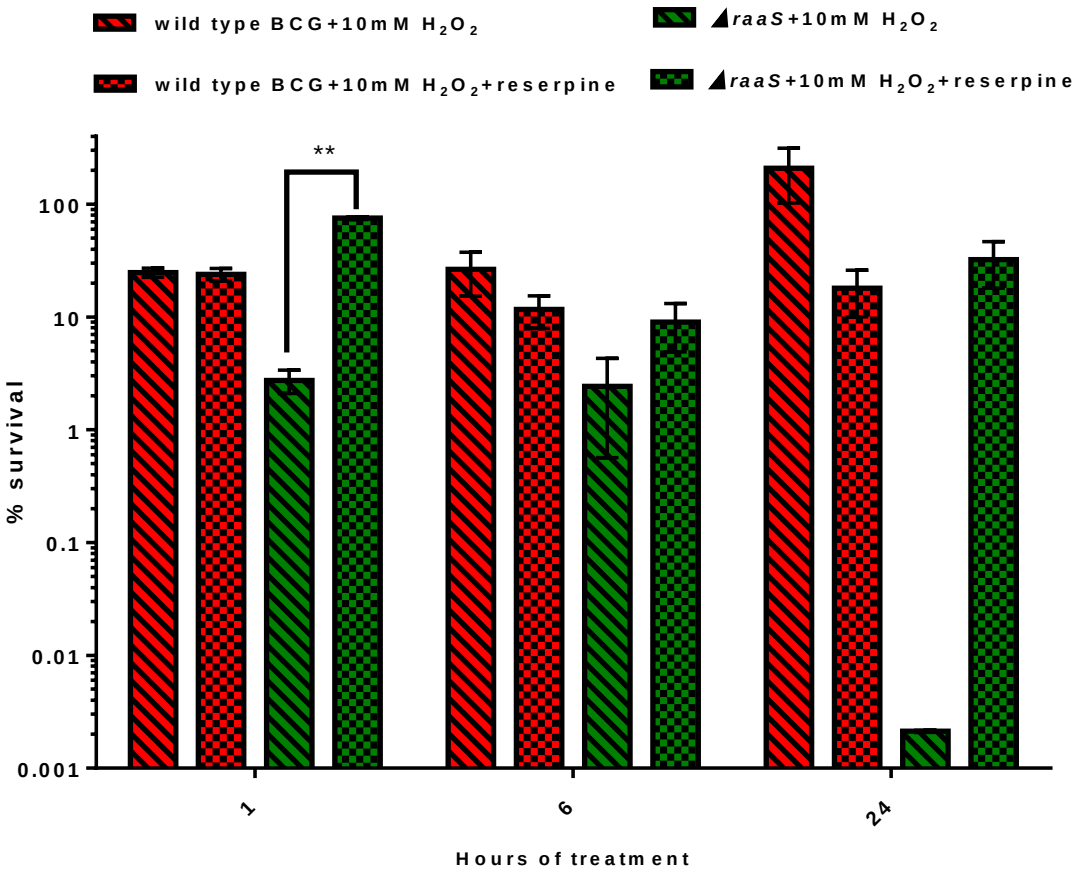


Figure 18: The effect of reserpine on the survival of wild type *M. bovis* BCG and *raaS* deletion mutant during treatment with 10mM H_2O_2.
Cultures of wild type *M. bovis BCG* and *raaS* deletion mutant were grown in supplemented 7H9 medium until they reach an OD_{600nm} of 0.4-0.6. Then the cultures were treated with 10 mM of H_2O_2 and reserpine (20µg/ml). After that the samples were incubated at 37 °C without shaking. At 1, 6 and 24 hours of incubation, a sample was taken for CFU counting. Survival was calculated depending on the CFU/ml before treatment. Result is an average of two independent experiments, each including three technical replicates (three individual tubes). Data are expressed as mean ± SEM.

On the other hand, reserpine treatment did not increase the survival of the wild type during H_2O_2 treatment. These results indicate that inhibition of efflux pumps might be the cause behind improved survival of the mutant during H_2O_2 treatment. It is worth noting that there was no drop in pH during the course of treatment indicating that acidity was not the cause behind any decrease in survival.

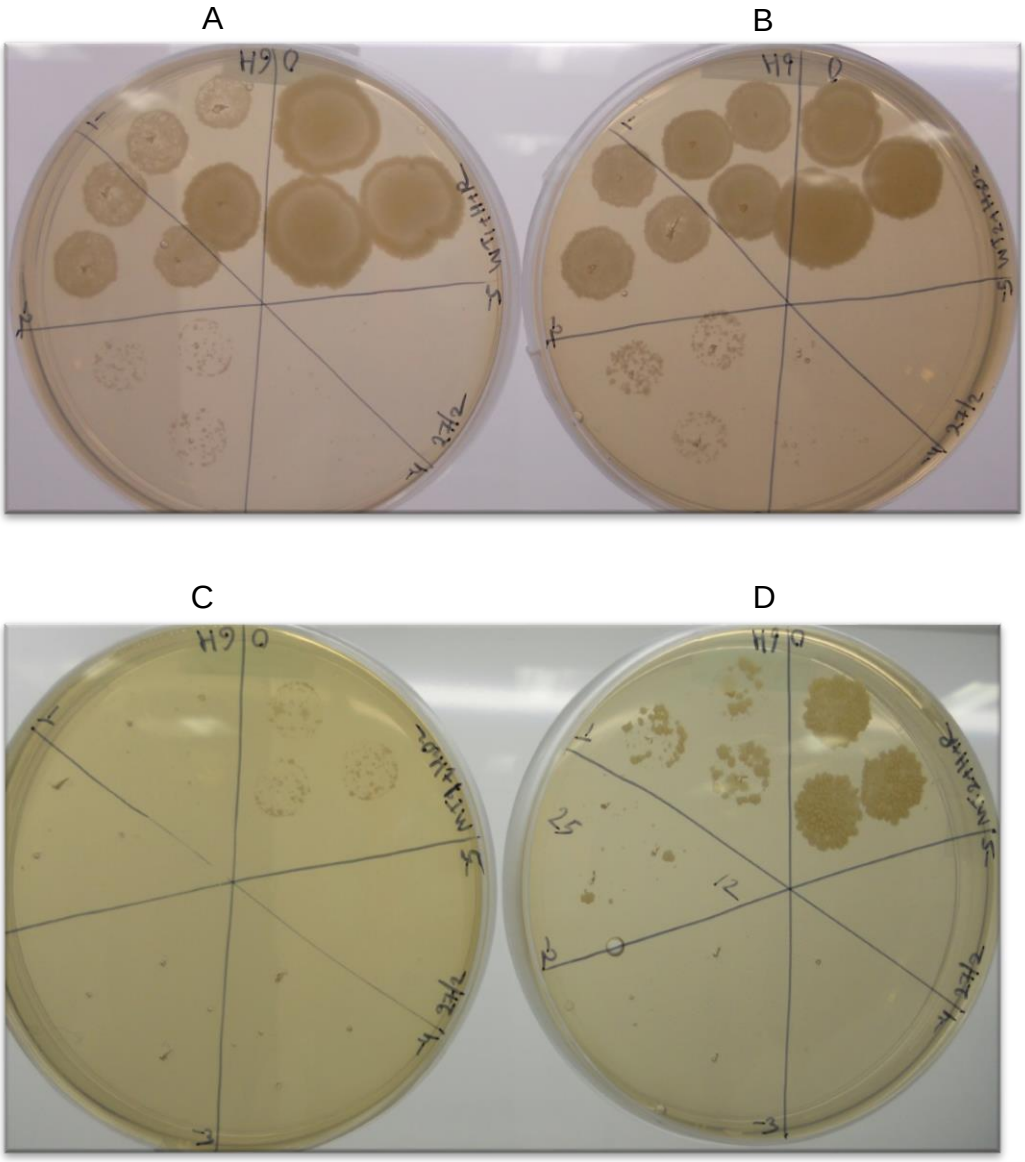


Figure 19: The effect of reserpine on the growth of wild type *M. bovis* BCG and *raaS* deletion mutant after 6 hours of 10mM H_2O_2 treatment.

Representative images of supplemented 7H10 agar plates showing growth of wild type *M. bovis* BCG and *raaS* deletion mutant (with and without reserpine) after 6 hours of H_2O_2 treatment. A: wild type treated with 10 mM H_2O_2 and reserpine; B: wild type treated with 10 mM H_2O_2 C: *raaS* deletion mutant treated with 10 mM H_2O_2; D: *raaS* deletion mutant treated with 10mM H_2O_2 and reserpine.

3.3.7 The effect of culture supernatant from the *raaS* deletion mutant on the survival of wild type *M. bovis BCG* during H_2O_2 treatment

Culture supernatant (SN) was prepared from the *raaS* deletion mutant to further investigate the role of the dysregulated efflux pumps and the possible effect of the unknown molecules transported by Rv1218c/Rv1217c (Bcg_1278c/Bcg_1277c) on the survival of the mutant during H_2O_2 treatment. Culture supernatant was harvested from growing cultures of *raaS* deletion mutant at two different optical densities (0.5 and 0.7). The effect of *raaS* deletion mutant SN on the survival of wild type *M. bovis* BCG during H_2O_2 treatment was assessed by pelleting mid-log phase cultures of wild type *M bovis* BCG, discarding the supernatant and resuspending the pellet in an equal volume of SN. This was followed by exposure to 10mM H_2O_2 and assessing the viability. The first SN tested was harvested from *raaS* deletion mutant cultures growing at an OD_{600nm} of 0.7. The results represented by Figure 20 showed that wild type *M. bovis* BCG growing in the presence of SN did not show any significant reduction in survival when compared with the control cultures containing untreated wild type *M. bovis* BCG. In addition, H_2O_2 treatment of the wild type in the presence of mutant SN for 6 hours did not result in a significant change in the survival when compared with cultures treated with H_2O_2 only. However treatment of the wild type with 10mM H_2O_2 in the presence of mutant SN for 24 hours resulted in a marginal difference in survival when compared with H_2O_2 only but this did not reach statistical significance.

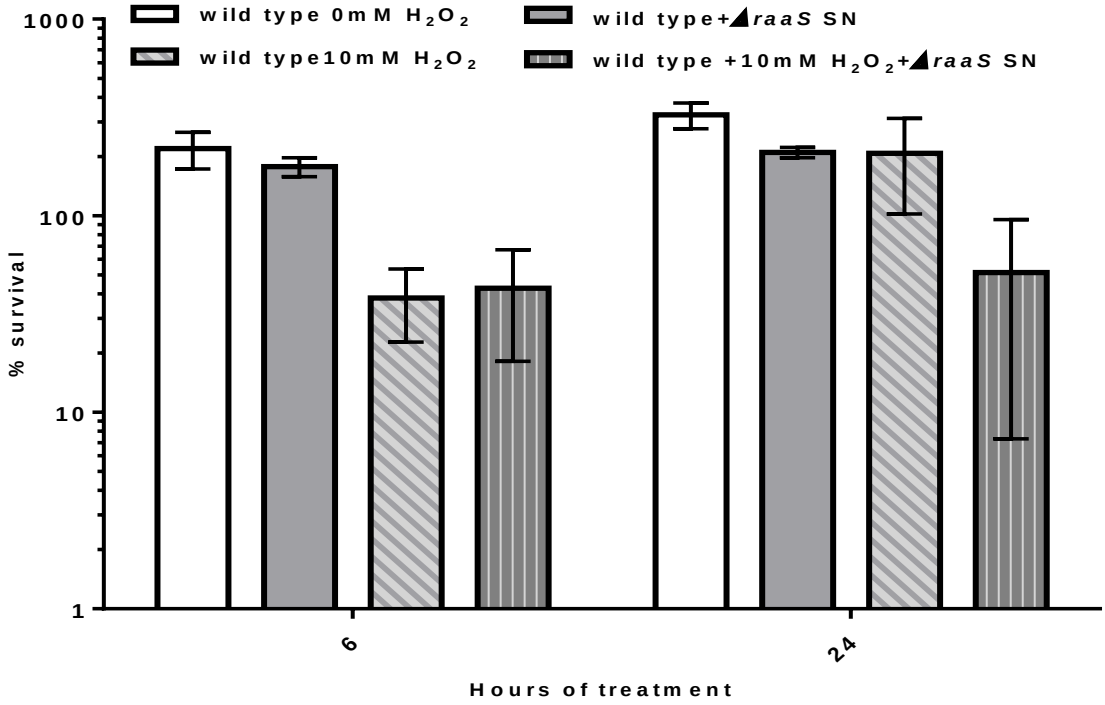


Figure 20: Survival of wild type *M. bovis BCG* during H_2O_2 treatment in the presence of Δ*raaS* culture supernatant harvested at an OD_{600nm} of 0.7.
Wild type *M. bovis* BCG cultures were exposed to 10mM H_2O_2 for 6 and 24 hours, both in the presence and absence of mutant SN. Survival was calculated depending on the CFU/ml before treatment. Result is average of three technical replicates. Data are expressed as mean ± SEM.

Next, the survival of wild type *M. bovis* BCG during H_2O_2 treatment in the presence of *ΔraaS* SN harvested at an OD_{600nm} of 0.5 was investigated. This time the survival was evaluated after 1, 6 and 24 hours of incubation and the results showed that the survival of wild type *M. bovis* BCG during H_2O_2 treatment in the presence of mutant SN was impaired compared with the survival of the wild type as shown in Figure 21. The same figure also shows that there is a trend towards decreased survival rate of the wild type during H_2O_2 treatment in the presence of mutant SN when compared with survival during H_2O_2 treatment only after 6 and 24 hours of incubation. These data clearly show that the combined effect of the mutant SN harvested at an OD_{600nm} of 0.5 and H_2O_2 treatment was more toxic on the wild type than H_2O_2 treatment only. In this experiment wild type cultures only were used as a control. Additional controls should include wild type cells

resuspended in wild type SN which has been prepared in the same manner as the mutant SN.

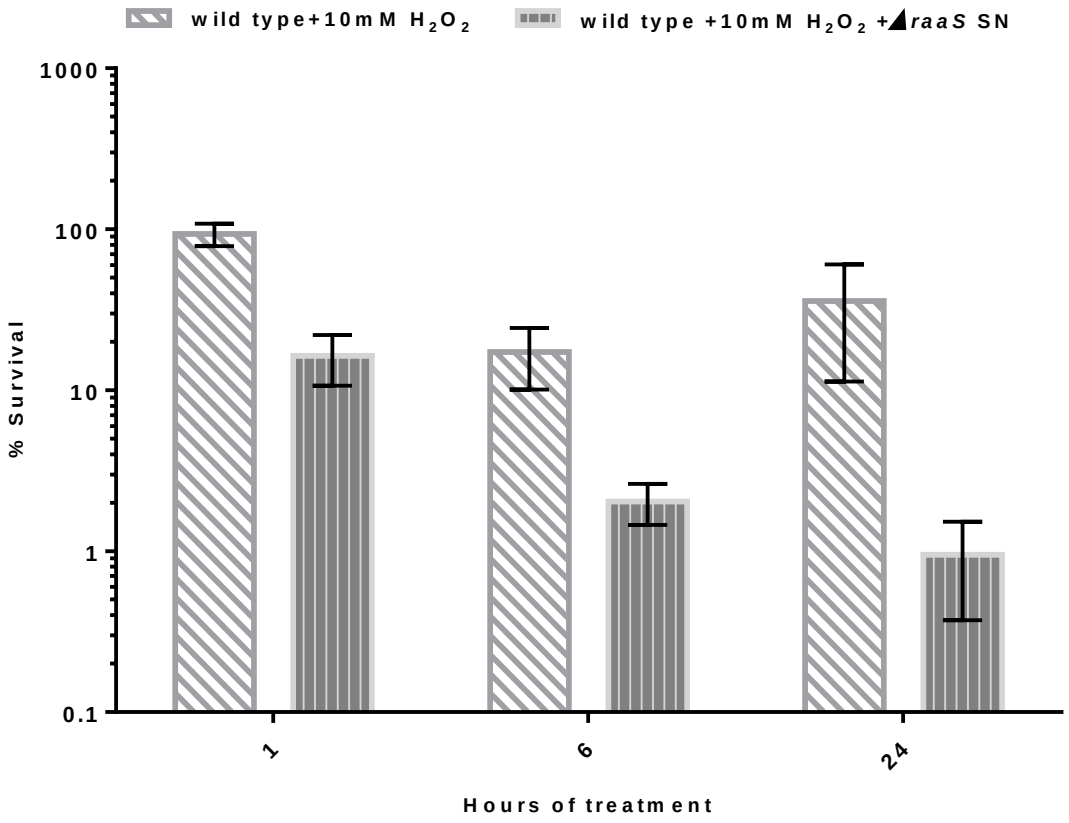


Figure 21 : Survival of wild type *M. bovis* BCG during H_2O_2 treatment in the presence of Δ*raaS* culture supernatant harvested at an OD_{600nm} of 0.5.
Wild type *M. bovis* BCG cultures were exposed to 10mM H_2O_2 for 1, 6 and 24 hours, both in the presence and absence of mutant SN. Survival was calculated depending on the CFU/ml before treatment. Result is a representative of three technical replicates. Data are expressed as mean ± SEM.

3.3.8 The effect of *raaS* deletion mutant culture supernatant on the viability of eukaryotic cells

The results described previously indicate that chemicals accumulated in the mutant supernatant during logarithmic phase may possibly be toxic for mycobacteria exposed to H_2O_2. The defect in survival of the *raaS* deletion mutant during both macrophage infection and H_2O_2 treatment prompted further investigations into the possible toxic effect of the unknown molecules transported by Rv1218c/Rv1217c (Bcg_1278c/Bcg_1277c) on eukaryotic cells. One possible

explanation for the decreased survival of the *raaS* deletion mutant during macrophage infection is that macrophages were killed by the unknown molecule secreted by the mutant. Thus the effect of SN harvested from both wild type *M. bovis* BCG and the *raaS* deletion mutant on the viability of J774.1 cells was estimated. The results showed that after 24 hours of incubating the macrophages in the presence of 100 µl of SN, there were comparable numbers of dead cells in wells treated with either mutant SN or 7H9 media only (control) as shown in Figure 22. Although the number of dead cells in wells treated with mutant SN was higher than wells treated with wild type SN, but this difference did not reach statistical significance.

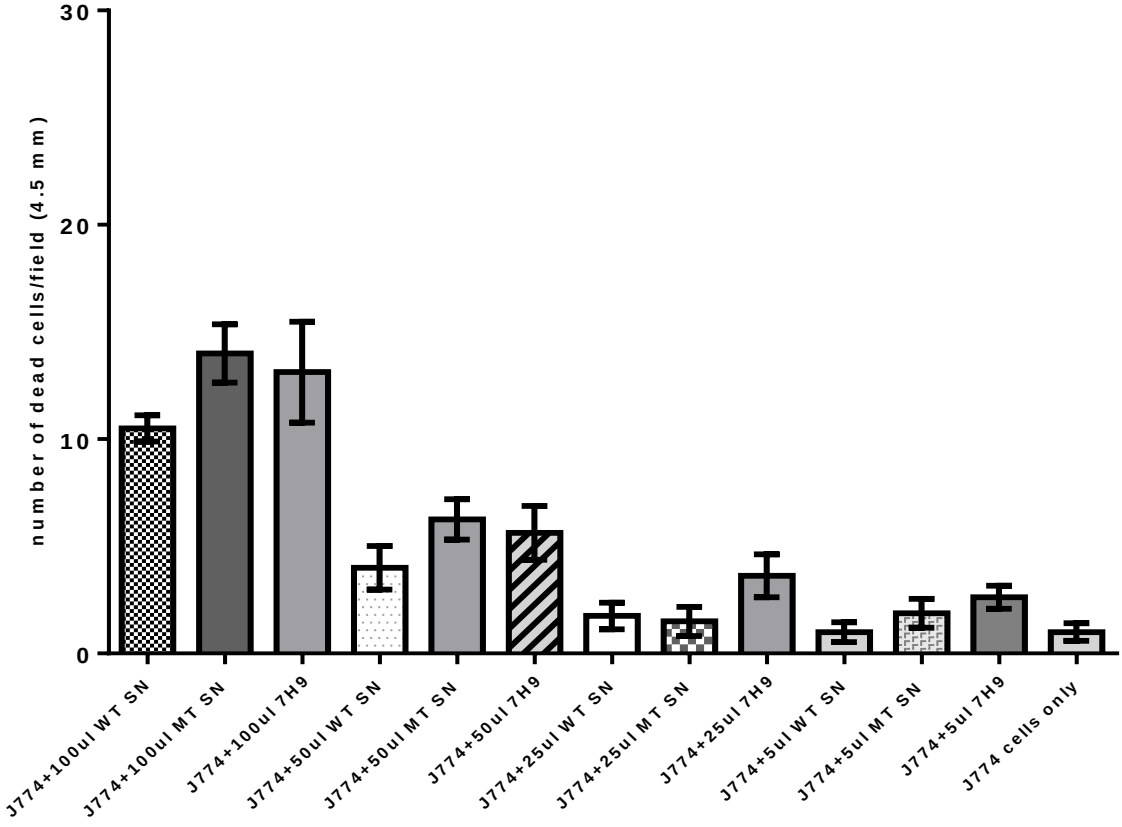


Figure 22 : The effect of culture supernatant on the viability of eukaryotic cells after 24 hours of incubation.

J774.1 macrophages were incubated at 37 ºC in the presence of different concentrations of SN harvested from both wild type *M. bovis* BCG and *raaS* deletion mutant, in addition to 7H9 medium. After 24 hours of incubation, the dead cells were counted by taking samples from the wells and performing the trypan blue exclusion method. WT= wild type, MT= ∆ *raaS* mutant. Result is an average of ≥6 technical replicates. Data are expressed as mean ± SEM.

Incubation of J774.1 cells in the presence of SN (from wild type, mutant or 7H9 only) for another 24 hours resulted in an increased number of dead cells when 100µl of SN was used as shown in Figure 23. However, incubation of J774.1 cells in the presence of 7H9 medium only caused the highest number of dead cells. The data at both 24 and 48 hours of incubation gives an indication that 7H9 medium only seems to cause death of eukaryotic cells. This means that this experiment suffers from the limitation caused by death of cells with 7H9 medium only.

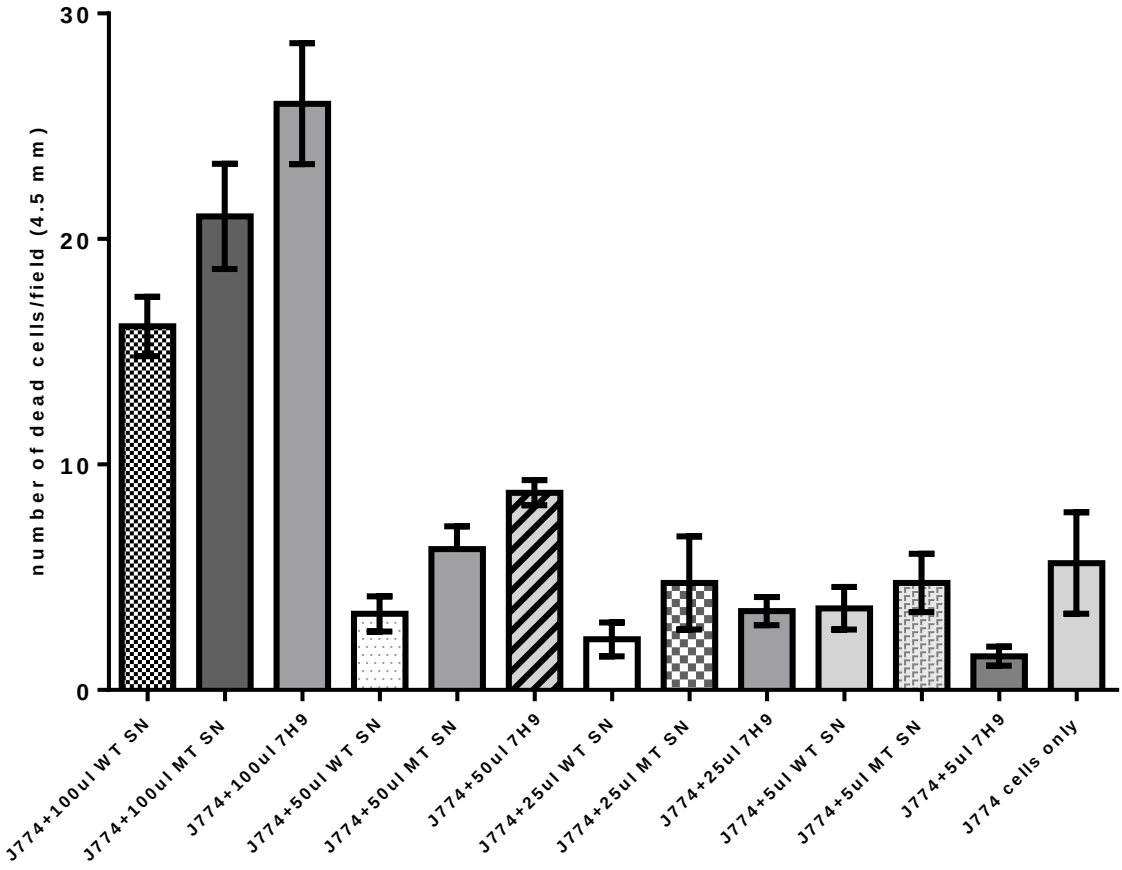


Figure 23 : The effect of culture supernatant on the viability of eukaryotic cells after 48 hours of incubation.

J774.1 macrophages were incubated at 37 ºC in the presence of different concentrations of SN harvested from both wild type *M. bovis* BCG and *raaS* deletion mutant, in addition to 7H9 medium as a control. After 48 hours of incubation, the dead cells were counted by taking samples from the wells and performing the trypan blue exclusion method. WT= wild type, MT= ∆ *raaS* mutant. Result is an average of at least 6 technical replicates. Data are expressed as mean ± SEM.

3.3.9 Level of intracellular ATP in wild type *M. bovis* BCG and *raaS* deletion mutant

The level of intracellular ATP was measured in both wild type and mutant to assess if over expression of the efflux pump (encoded by bcg_1277c/1278c) leads to depletion of intracellular ATP. Aliquots of growing cultures from both strains were collected at three different OD_{600nm} (0.3, 0.5 and 1). ATP levels were measured in these samples and the results represented by Figure 24 show that both strains had similar levels of ATP at OD_{600nm} of 0.3 and 0.5. On the other hand, the mutant growing at OD_{600nm} of 1 seems to show lower levels of ATP when compared with the wild type.

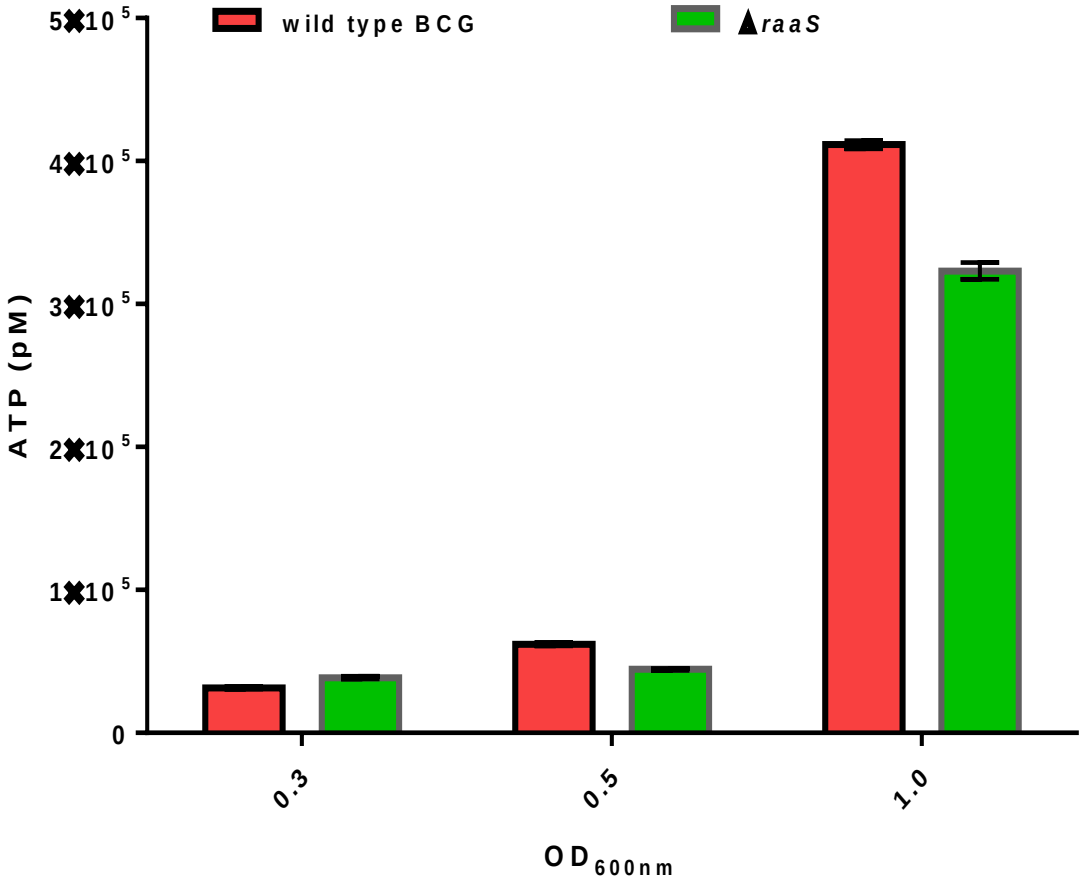


Figure 24: Intracellular levels of ATP in wild type *M. bovis* BCG and *raaS* deletion mutant. ATP levels were measured in both wild type *M. bovis* BCG and *raaS* deletion mutant growing at three different optical densities (0.3, 0.5 and 1). Samples were serially diluted in supplemented 7H9 broth and ATP levels were evaluated using the BacTiter-Glo Microbial Cell Viability assay. Result is an average of three technical replicates. Data are expressed as mean ± SEM.

3.4 Discussion and conclusions

3.4.1 Discussion

M. tuberculosis remarkable ability to survive and replicate inside host cells is mainly attributed to its capacity to respond to different stressful conditions such as nutrient deprivation, hypoxia, ROI, RNI, toxic lipid moieties and acidic pH (Warner and Mizrahi, 2007). It is thought that this is mainly achieved via transcriptional reprogramming. The genomic sequence of *M. tuberculosis* revealed many putative transcriptional regulators which are probably involved in the adaption to environmental changes (Cole et al., 1998).

Of those transcriptional regulators, is the regulator of antimicrobial assisted survival RaaS (encoded by *rv1219c* in *M. tuberculosis* and *bcg_1279c* in *M. bovis* BCG). Published data have shown that this transcriptional regulator plays an important role in prolonged survival in stationary phase and during mouse infection. The previously published *in vitro* growth characteristics (Turapov et al., 2014a) and the *in vitro* growth curve presented in this chapter clearly indicate that the *raaS* deletion mutant had no growth abnormalities during logarithmic growth phase. These data confirm the results of a study aimed to identify genes required for mycobacterial growth by high density mutagenesis which defined *rv1219c* (*bcg_1279c*) as non-essential for mycobacterial growth *in vitro* (Sassetti et al., 2003). A number of *in vitro* stress conditions were utilised in order to gain a more comprehensive understanding of the role played by this predicted transcriptional regulator during non-growth permissive conditions. The results reported in this chapter have shown that the *raaS* deletion mutant is more sensitive to stress.

Data on *M. tuberculosis* entrance and trafficking in macrophages have been obtained due to the availability of both primary and *in vitro* differentiated cells. Therefor macrophage infection models were used to study the importance of RaaS during infection. Macrophage infection studies, both in J774.1 and THP-1 cells revealed that both wild type and the *raaS* deletion mutant had similar uptake rates. However it is obvious that the mutant suffers from a significant defect in intracellular survival at early stages of infection when compared with the wild type. A screening of a transposon library in *M. bovis* BCG designed to identify mutants with decreased intracellular fitness found that Rv1218c was important for initial stages of infection (Stewart et al., 2005). Previously published data on

macrophage infection with the *rv1219c* deletion mutant have shown that both wild type and the mutant replicate in the early stages of infection with no distinguishable differences during this stage. However, the mutant shows a defect in persistence during later stages of macrophage infection when compared with the wild type. The difference in persistence between wild type *M. tuberculosis* and the *raaS* deletion mutant only started to appear after 72 hours of infection (Turapov et al., 2014a). It is obvious that *M. tuberculosis* and *M. bovis* BCG (Glaxo strain) behave differently during macrophage infection; *M. tuberculosis* apparently replicates very well, while BCG seems not to replicate well. Hayashi and co-workers have also published data which revealed that *M. bovis* BCG Glaxo strain has reduced intracellular survival activity (Hayashi et al., 2010). It can be concluded from macrophage infection results that deletion of *raaS* (*rv1219c/bcg_1279c*) resulted in a defect in intracellular survival and persistence. The cause for this defect may be multifactorial involving more than one element. Overexpression of the Rv1218c/Rv1217c (Bcg_1278c/Bcg_1277c) pump as a result of deletion of their repressor may lead to consumption of more ATP leading eventually to exhaustion of the bacterium. The level of intracellular ATP is considered as an indicator of cell energy and thus can determine the rate of the various intracellular reactions. It has been observed that high intracellular ATP levels during antibiotic exposure was correlated with survival and post antibiotic recovery of mycobacterial cells (Maglica et al., 2015). Thus the level of intracellular ATP was measured in *M. bovis* BCG wild type and *raaS* deletion mutant to investigate whether low ATP level was associated with reduced intracellular survival of the mutant. The results showed that the mutant had lower levels of ATP at an OD_{600nm} of 1, when compared with the wild type. This result is consistent with the predication that overexpression of the pumps as a result of deletion of its repressor would lead to more consumption of ATP. This experiment was done in the last week of this project, thus time limitations prevented repeating this experiment. Additional experiments are needed to confirm this result. It would have been useful if the level of ATP was also measured in stationary phase cultures as the mutant has previously shown a defect in survival during prolonged stationary phase (Turapov et al., 2014a) and it is possible that the decreased availability of intracellular ATP levels is one factor contributing to this defect.

Another possible element which might contribute to the intracellular defect of the mutant is that the unknown molecule(s) transported by Rv1218c/Rv1217c (Bcg_1278c/Bcg_1277c) pump might be toxic to eukaryotic cells. Mycobacteria have been previously shown to possess lipids which can be toxic to eukaryotic cells. An example is the cell wall associated mycobacterial glycolipid LAM which was found to be cytotoxic to human mononuclear cells. This cytotoxic potential is attributed to LAMs ability to inhibit protein kinase C and subsequently interfere with the phosphorylation of a number of proteins (Ghosh et al., 1998).

An attempt has been made to investigate if SN harvested from the mutant can be toxic to eukaryotic cells. Culture supernatant was harvested from cultures grown in the absence of tween 80 to exclude its toxic effect on eukaryotic cells. The results obtained from this attempt suggested a trend for increased cell killing with mutant SN when compared to wild type SN. However this attempt suffered from a drawback due to the limitations caused by killing of eukaryotic cells by 7H9 media supplemented with 10% ADC which indicate that there is a substance in the composition of this media which is toxic for eukaryotic cells. Therefore a more refined approach which excludes any toxic effect other than that of the SN is needed to confirm any toxicity caused by mutant SN.

Studying the behaviour of *M. tuberculosis* following exposure to various *in vitro* simulated phagosomal conditions, may help in understanding how the bacterium behaves during infection. Although exposure to one stress condition does not effectively reflect what happens *in vivo* where the bacteria may simultaneously encounter multiple stresses, but it still may aid in understanding the behaviour of the bacterium during intracellular infection. Of the defence mechanisms employed by the host 'macrophage' upon infection with mycobacteria, are acidic stress and the production of RNS. Therefore the intracellular survival defect of the *raaS* deletion mutant was further investigated by studying the survival of the mutant during these two stress conditions. Depending on the activation state of the macrophage in which *M. tuberculosis* resides in, the pH of the macrophage compartment usually ranges from 6.2 to 4.5 (Via et al., 1998). The mutant showed a significant survival defect after 72 hours of exposure to an acidic pH of 4.5. Previous experimental data have reported that the high sensitivity of the bacterium to low pH in growth medium is attributed to the release of oleic acid or

other fatty acids from tween 80 and albumin which are toxic to mycobacteria (Kondo and Kanai, 1976, Lynn et al., 1979). Moreover, it has been suggested that *M. tuberculosis* resists phagolysosomal concentrations of acid when grown in simple buffer (Vandal et al., 2008). Therefore one possible explanation for the survival defect of the *raaS* deletion mutant is that the unknown molecules transported by Rv1218c/Rv1217c (Bcg_1278c/Bcg_1277c) might become mycobactericidal when exposed to acidic conditions. However it is not known if acidity by itself was directly toxic or whether decreased survival was due to a combination of sensitivity to low pH and other antimicrobial factors.

Previous work on *M. bovis* BCG Glaxo strain has shown that it is moderately susceptible to nitrosative stress (Hayashi et al., 2010). Exposure to nitrosative stress by treatment with two different NO donors showed that compound number 10626173 had the highest killing potential. Both of the NO donors used were compounds that are designed to spontaneously and enzymatically release NO. However, it was noticed that the *raaS* deletion mutant suffered from a survival defect after 72 hours of treatment with compound number 10626173. The survival of the mutant was higher than the wild type upon treatment with compound number 10126069 but this did not reach statistical significance as there was large variation between the results of the three independent experiments. Thus it was not possible to make any conclusion on susceptibility of the mutant to NO due to high variability of data. Experimental variables such as the type of RNI mediator, concentration, duration of exposure and the growth phase of the culture when the stress is applied are thought to influence the response of mycobacteria to nitrosative stress (Florio et al., 2006). Further experiments using one of the commercially available NO donors as mediators of nitrosative stress may be more useful in assessing if RaaS played any role in survival during this stress.

Published data have suggested that *M. tuberculosis* can limit the host's antimicrobial defense via resistance mechanisms which enable the bacterium to counter the phagocyte respiratory burst (Ng et al., 2004). In a study which was conducted to investigate the biochemical characters among *M. bovis* BCG strains, Glaxo strain showed the highest reduction in CFU (>3 fold reduction in log CFU) after 6 hours of treatment with 10mM H_2O_2 when compared with other strains of *M. bovis* BCG (Russia, Japan, Sweden, Danish and Pasteur) (Hayasi et al., 2010). Results presented in this chapter have shown that the *raaS* deletion

mutant had a significant survival defect during exposure to 10mM H_2O_2 treatment. The survival of *raaS* deletion mutant after 24 hours of H_2O_2 treatment ranged between 0.002 and 0.3%. The variability in survival rate after H_2O_2 treatment observed in this study is probably attributed to different stages of growth. Published data have demonstrated that cells at different stages of the growth cycle display dramatic differences in their sensitivity to H_2O_2 (Dowds et al., 1987). Furthermore, it has been shown that exponential-phase cells are generally more sensitive to stress than stationary-phase cells (Yoshpe-Purer and Henis, 1976). Previous work has proposed that resistance to H_2O_2 was related to the survival in host cells (Hayashi et al., 2010). This is consistent with what was obtained in this study, as the *raaS* deletion mutant has shown a defect during intracellular survival and H_2O_2 treatment. Dysregulation of the pump Bcg_1278c/Bcg_1277c (Rv1218c/Rv1217c) as a result of deletion of its repressor could account for the survival defect of the *raaS* deletion mutant during H_2O_2 treatment. One way to investigate this assumption is to inhibit the pump and explore the effect that it would exert on the survival of the mutant during H_2O_2 treatment. As with previous studies, the results presented in this chapter showed that the concentration of reserpine used was not inhibitory for growth (Brown and Parish, 2008, Turapov et al., 2014a). Inhibition of the pumps via reserpine did significantly improve the survival of the mutant during H_2O_2 treatment, although it did not manage to fully restore the phenotype of the wild type which means that other factors may be contributing to the survival defect. Overexpression of the MDR pump SmeDEF in *Stenotrophomonas maltophilia* was associated with decreased fitness and metabolic impairment (Alonso et al., 2004).

The role of the dysregulated efflux pumps and the possible effect of the unknown molecule(s) transported by Rv1218c/Rv1217c (Bcg_1278c/Bcg_1277c) pump on the survival of the mutant during H_2O_2 treatment were further investigated by testing the effect of SN harvested from the *raaS* deletion mutant on the survival of the wild type during H_2O_2 treatment. Results have indicated that mutant SN harvested at an OD of 0.7 had no toxic effect either on the wild type growing in normal conditions or during H_2O_2 treatment after 6 hours of incubation. A 24 hour exposure period of the wild type to mutant SN during H_2O_2 treatment did result in lower survival percentage when compared to exposure to H_2O_2 only but this did

not reach statistical significance. The findings obtained with mutant SN harvested at an OD_{600nm} of 0.5 showed that survival of the wild type during treatment with this SN and 10mM H_2O_2 for 1, 6 and 24 hours resulted in a decrease in survival rate when compared to exposure to 10mM H_2O_2 only. The obtained results (Figure 21) indicate that a combination of H_2O_2 treatment and mutant SN was more toxic to the wild type. This may possibly suggest that this pump is secreting a substance which is toxic upon reaction with H_2O_2. One approach to test this assumption would be to harvest SN from cultures of *raaS* deletion mutant growing in the presence of a pump inhibitor and subsequently testing its effect on the survival of the wild type during H_2O_2 treatment. Unfortunately this approach could not be implemented due to time limitations. The high resistance of *M. tuberculosis* to oxidative and nitrosative stress has been previously attributed to expression of genes that encode ROS and RNS scavenging functions, properties of the cell wall, induction of DNA repair genes (Voskuil et al., 2011). Furthermore, coordination between oxygen radical detoxification system and thiol homeostasis contributes to survival during the host's oxidative stress (Nambi et al., 2015). Published data have also demonstrated that Lsr2 in mycobacteria plays a protective role against ROI. It is thought that Lsr2 forms a physical barrier by binding bacterial DNA and thus protects DNA from the damaging effect of hydroxyle radicals (Colangeli et al., 2009). Further investigation into the nature of the molecule transported by Rv1218c/Rv1217c (Bcg_1278c/Bcg_1277c) pump would aid in elucidation of the mechanism by which Rv1219c/ raaS contributes to survival of the bacterium during non-permissive growth conditions.

It was interesting to explore how the *raaS* deletion mutant behaves in a model of nutrient deprivation. The nutrient starvation model employed was that based on Loebels work. *M. tuberculosis* in this model is in a state of low respiration with little or no replication but it still maintains long term viability (Betts et al., 2002). The growth rate of wild type *M. bovis* BCG and *raaS* deletion mutant was indistinguishable during the first week of starvation. However, the mutant seemed to suffer from a decrease in growth rate from 2 weeks onwards suggesting that BCG_1279c/RaaS does play a role in survival during nutrient starvation. This result supports the assumption that this transcriptional regulator plays an important role in survival during non-growth permissive conditions. The results

presented in this chapter indicate that deletion of *raaS* rendered *M. bovis* BCG more susceptible to stressful conditions. The improvement in survival of the *raaS* mutant after reserpine treatment indicate that dysregulation of the pump is a possible factor influencing the survival of the mutant. It has been speculated that overexpression of some efflux pumps may cause undesirable efflux of metabolites or other signalling molecules resulting in a damaging effect on cell physiology (Sun et al., 2014).

As mentioned in chapter one of this thesis, CHIP-seq data available in TB database [http://genome.tbdb.org/] has revealed that RaaS has 107 predicted regulatory interactions highlighting the possibility that deletion of RaaS could dysregulate the expression of genes other than those encoding the Rv1218c/Rv1217c (Bcg_1278c/Bcg_1277c) pump. Many genes from the RaaS regulon encode transport proteins and pumps. Apart of Rv1218c-Rv1217c discussed above these include MmpL7, Rv1250, CysA1, CysT, SugA-C. MmpL7 belongs to the MmpL (mycobacterial membrane protein large) family of proteins in *M. tuberculosis.* MmpL7 and DrrC (which is also regulated by RaaS) were found to be required for transport of PDIM to the cell surface (Cox et al., 1999). PDIM is one of *M. tuberculosis* key virulence factors, therefore it is attractive to speculate that deletion of RaaS may potentially disrupt transport of PDIM and thus result in attenuation of *M. tuberculosis.* Another set of genes regulated by RaaS is an *mce1* operon *rv0171* (*mce1C*), *rv0172* (*mce1D*) and *rv0174* (*mce1F*). Mce1 is one of 4 operons (mce1-4) which are thought to encode lipid transporters (Cole et al., 1998). It has been suggested that Mce1 functions as a mycolic acid re-importer in non-permissive growth conditions (Forrellad et al., 2014). Based on this prediction, it would be reasonable to assume that any dysregulation of the Mce1 operon would result in fitness cost and thus would explain the attenuation of the *raaS* mutant. RaaS also regulates the histidine kinase response regulator DosT which contributes to the control of the dormancy response mediated by DosR (Sivaramakrishnan and de Montellano, 2013). This implies that RaaS could possibility be involved in the regulation of the family of genes involved in the survival during latency. The transmembrane serine/threonine-protein kinase D (pknD) is also regulated by RaaS. PknD was found to mediate bacillary invasion of the blood-brain barrier and thus any disruption of this protein would probably result in attenuation (Be et al., 2012). Furthermore, RaaS is also a regulator of

RpfC, RpfD, FbpB and DacB2. The first two are resuscitation-promoting factors whom have been linked to cell growth and cell survival under different physiological stresses, while the third is responsible for covalent attachment of mycolates to arabinogalactan and the last is a low molecular mass penicillin binding protein with a potential role in regulation of peptidoglycan biosynthesis (Mukamolova et al., 2006, Backus et al., 2014, Bourai et al., 2012). It would be attractive to speculate that any changes in cell wall composition resulting from the disruption of the aforementioned genes would eventually lead to a survival defect.

3.4.2 Conclusions

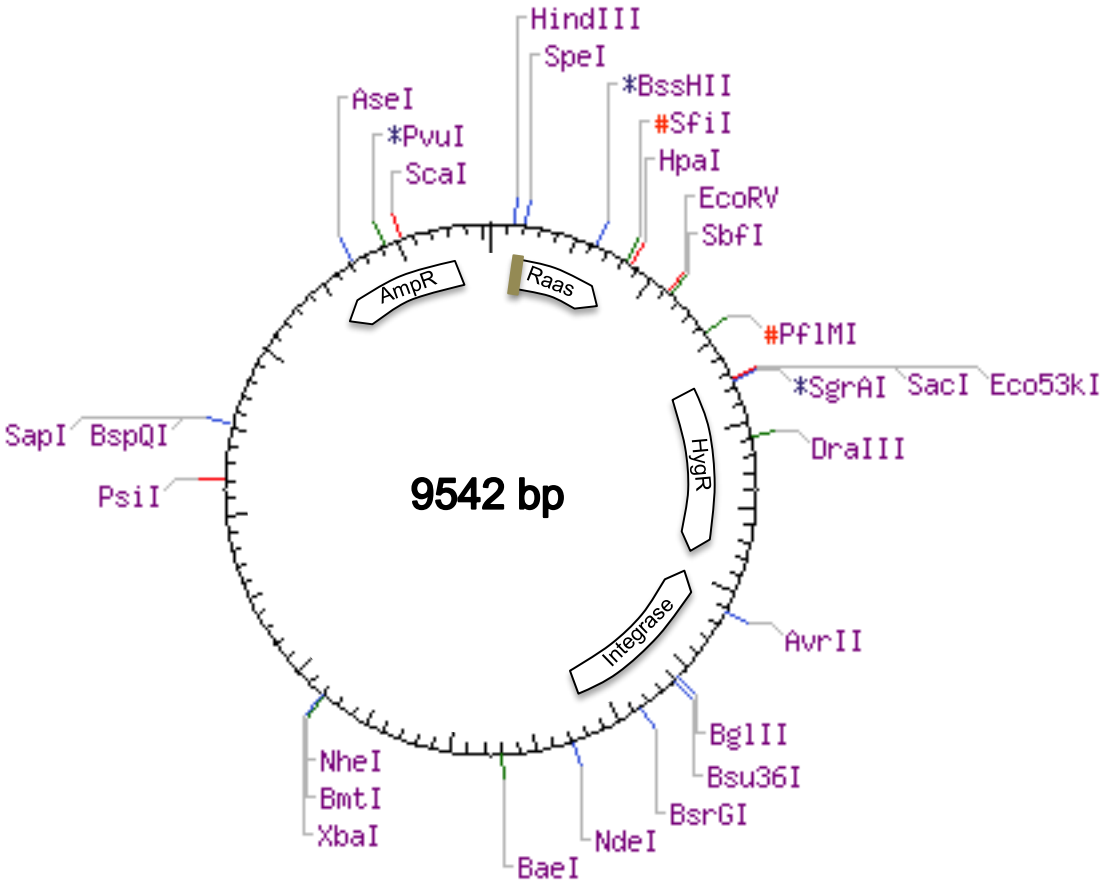
One of the aims of this chapter was to investigate the role of Rv1219c/ RaaS in mycobacterial replication and persistence in macrophages. The results presented have established that the *raaS* deletion mutant of *M. bovis* BCG was significantly impaired in initial survival in macrophages confirming published data which proposed that Bcg_1278c/Bcg_1277c (Rv1218c/Rv1217c) likely transports molecule(s) that are important for the initial stages of infection. In addition, the role of BCG_1279c/ RaaS during various stress condition has been explored and the results have shown that deletion of this transcriptional regulator resulted in a defect in survival during nutrient starvation, H_2O_2 treatment and acidic stress, the stress factors, which probably influence mycobacterial viability upon their uptake into macrophages. Treatment with reserpine, an inhibitor of ATP-dependent efflux pumps, during stress exposure had managed to improve the survival of the mutant suggesting that the impaired stress response of the *raaS* deletion mutant was due to overexpression of efflux pumps that resulted from deleting their repressor (BCG_1279c). Preliminary results have also shown that the decrease in the level of intracellular ATP might also contribute to the survival defect seen in the deletion mutant. Overall, the results presented in this chapter indicate that tight regulation of efflux pumps is critical for survival under stressful conditions.

Chapter 4 : Complementation of *M. bovis* BCG Δ*raaS* with $raas_{Mtb}$

4.1 Introduction

The results presented in the previous chapter have clearly shown that deletion of *raaS* resulted in a significant survival defect during various stress conditions (macrophage infection, acidic stress and H_2O_2 treatment). The next obvious step would be to complement the deletion mutant and subsequently test if complementation was able to restore the phenotype of the wild type strain. In previously published studies the *raaS* deletion mutant was complemented by integration of a single *raaS* copy into the mutant genome (Turapov et al., 2014a). A full-length $raaS_{Mtb}$ was cloned into *SpeI* and *HpaI* sites of the integrating vector pRBexint. This plasmid enables constitutive expression of a target gene from a *dnaK* promoter (Bottai et al., 2011).

Figure 25 illustrates the construct used to generate this complementation strain which was designated as $\Delta raaS_{com1.}$ Data obtained by Quantitative RT-PCR have shown that *raas* (*rv1219c/bcg_1279c*) was 2.6-fold overexpressed in the complemented strain compared to the wild type (Turapov et al., 2014a). Interestingly, $\Delta raaS_{com1}$ showed better survival in extended stationary phase when compared to the wild type which is likely to be attributed to overexpression of *raas* in the complemented strain. *In vivo* experiments revealed that $\Delta raaS_{com1}$ managed to restore the wild type phenotype in murine lungs. In murine spleen, $\Delta raaS_{com1}$ managed to partially restore the wild type phenotype at 17 days with full complementation seen at 21 days of infection (Turapov et al., 2014a). The same construct complemented $raaS_{Mtb}$ mutant phenotype in prolonged stationary phase and macrophage infection.



$\Delta raaS_{com1}$: **ATG**CGTTCAGCCGATCTGAC**...**

Figure 25: Schematic representation illustrating the construct used for generation of Δ*raaS*com$_1$ in *M. bovis* BCG.

Full-length *raaS*$_{Mtb}$ was cloned into *SpeI* and *HpaI* sites of the integrating vector pRBexint. Constitutive expression of a *raaS* was driven from a *dnaK* promoter. Start codon of *raaS* (red).

RaaS belongs to the autorepressor proteins and control expression of its own gene (Rubinstein et al., 2011). The *raaS* upstream region contains a RaaS binding site which consists of the two 12-bp imperfect direct repeats separated by the predicted *raaS* -10 promoter element as the DNA binding sites of RaaS (Figure 26).

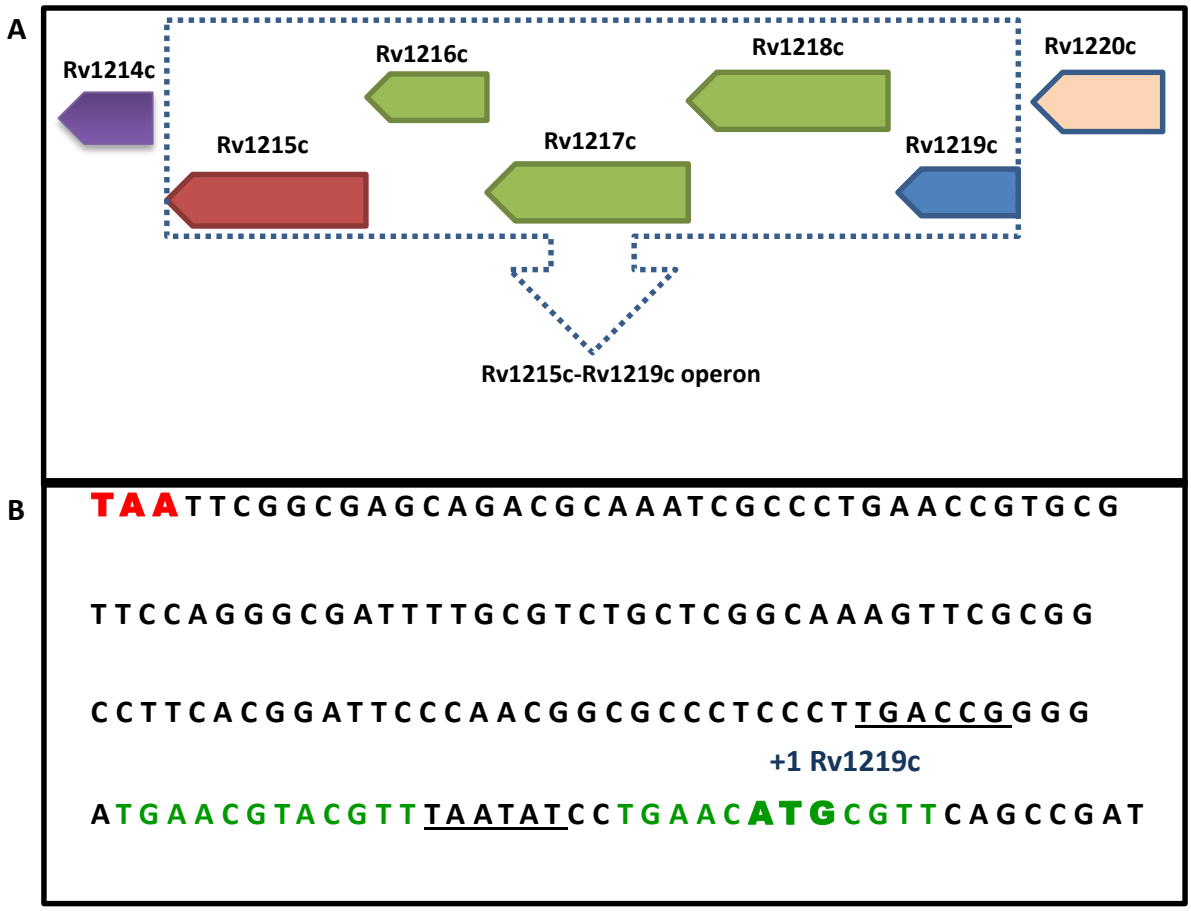


Figure 26: Schematic of the *raaS* (*rv1219c*) operon and the sequence of the RaaS (Rv1219c) binding site.

A: Representation of the *raaS* (*rv1215c-rv1219c*) operon B: sequence of the Intergenic region between *raaS* and *rv1220c* showing the RaaS-binding site (Green), the predicted -10 and -35 promoter elements (underlined), the *raaS* start codon (Bold Green) and the *rv1220c* stop codon (Bold Red). Figure adapted from Turapov et al., 2014a.

Replacement of three thymidines in one repeat with adenosines led to complete loss of binding indicating that both were necessary for RaaS binding (Turapov et al., 2014a). The localisation of the binding site within the promoter and coding sequence of the gene suggest the tight regulation of *raaS* expression. However its biological significance remains unknown. To investigate the importance of tight regulation of *raaS* expression in stress response, additional complemented strains were constructed and their survival was investigated in two different stressful conditions (nutrient starvation and H_2O_2 treatment). Thus this chapter

will describe the construction of two new complementing strains and assess their survival during stress.

As mentioned in the previous chapter, the only difference between *raaS*$_{Mtb}$ (*rv1219c*) and *raaS*$_{BCG}$ (*bcg_1279c*) is one amino acid (W113C) indicating that both proteins most probably play similar roles. Furthermore, the *M. bovis* BCG *raaS* deletion mutant complemented with either *M. tuberculosis* or *M. bovis BCG* versions of *raaS* has previously shown the same phenotype during extended stationary phase confirming that RaaS plays the same role in these closely related organisms (Turapov et al., 2014a).

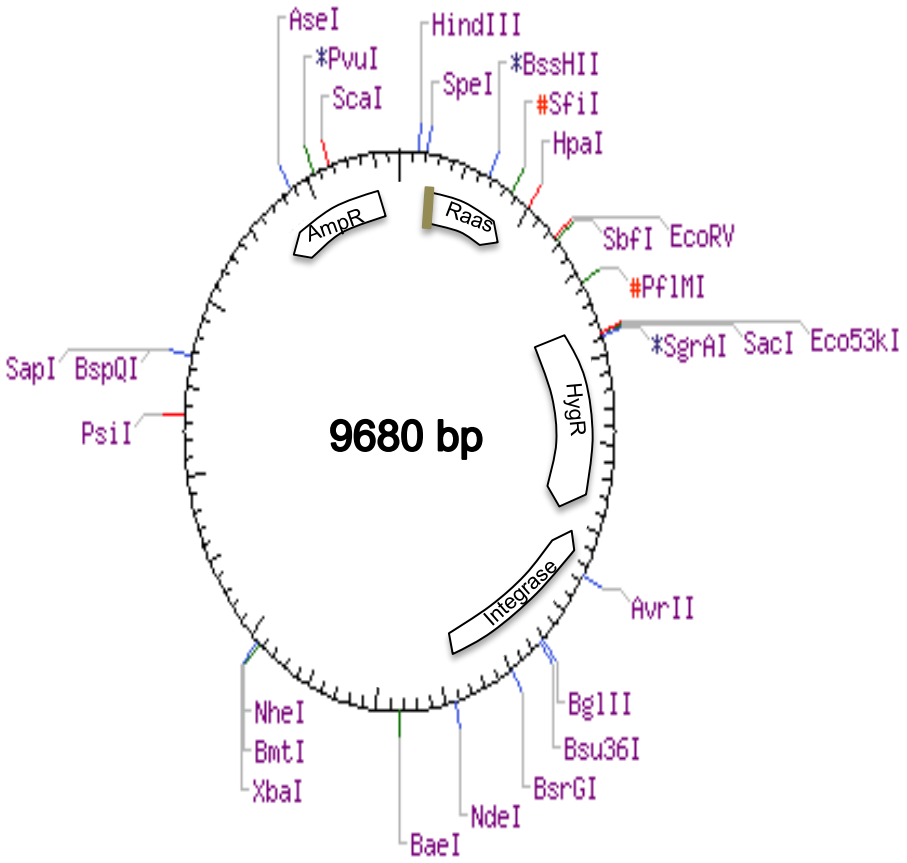
4.2 Methods

4.2.1 Organisms and media

The strains used in this chapter were *M. bovis* BCG (wild type, Δ*raaS*, Δ*raaS*$_{com1}$, Δ*raaS*$_{com2}$ and Δ*raaS*$_{com3}$). These strains were grown in supplemented 7H9 medium. Hygromycin (50µg/ml) was added in the case of complemented strains.

4.2.2 Generation of complemented *raaS* deletion mutants

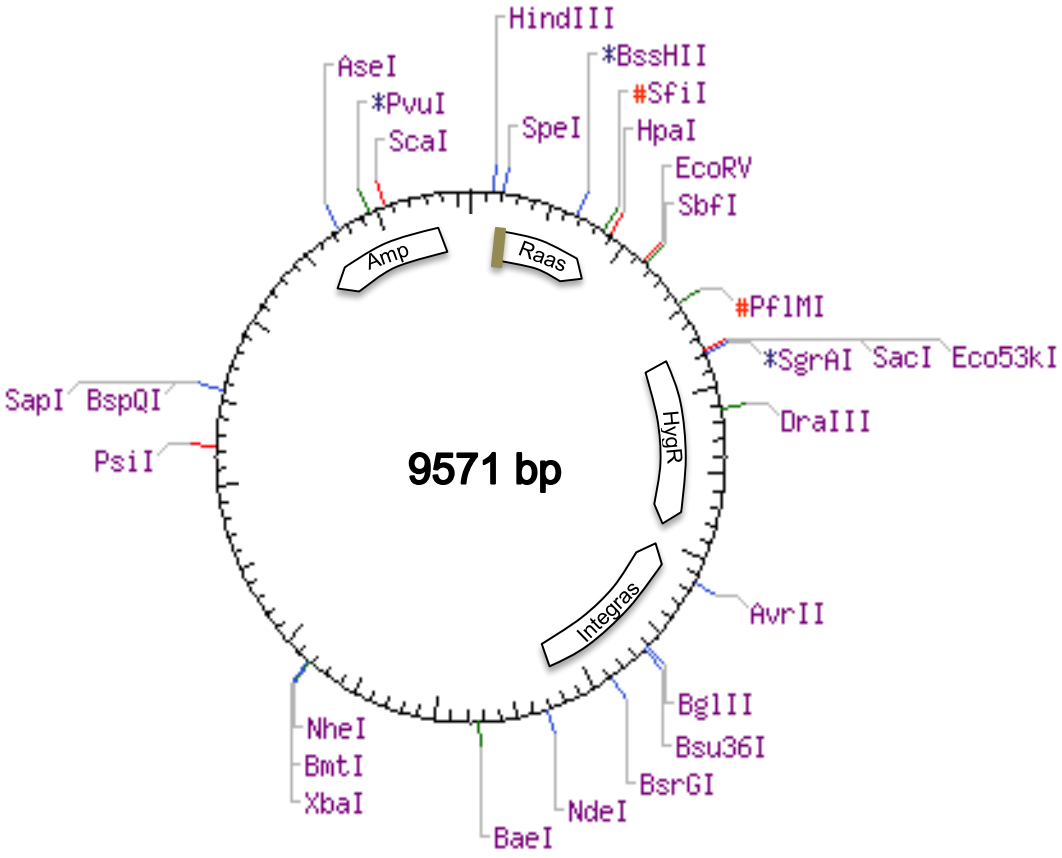
To complement *M. bovis* BCG Δ*raaS*, *raaS*$_{Mtb}$ with its upstream region was PCR amplified from *M. tuberculosis* genomic DNA using two sets of primer pairs harbouring *SpeI* and *HpaI* restriction sites. The first primer pair was designed to amplify full-length *raaS*$_{Mtb}$, binding sites and the predicted -10 and -35 promoter elements (for long version which will be designated as Δ*raaS*$_{com2}$). The second primer pair was designed to amplify full-length *raaS*$_{Mtb}$ and the binding site only (for short version which will be designated as Δ*raaS*$_{com3}$). The resulting PCR products were purified using PCR purification kit and subsequently digested using both *SpeI* and *HpaI* restriction enzymes (NEB, UK). Each one of the resulting PCR products were cloned into SpeI and HpaI sites of pRBexint (kindly provided by R. Brosch), which is an integrative cosmid vector derived from pYUB412 (Ottai et al., 2011). The resulting construct pRBexint-raaS$_{com2}$ (Figure 27) was used to generate *ΔraaS*$_{com2}$ (long version), while pRBexint-raaS$_{com3}$ (Figure 28) was used for the generation of (*ΔraaS*$_{com3)}$ (for short version).



Δ*raaS*$_{com2}$: **TGACCG**GGGATGAACGTACGTT**TAATAT**CCTGAAC **ATG**CGTTCAGCCGATCTGAC

Figure 27: Schematic representation illustrating the construct used for generation of *Δraa*S$_{com2}$ (Long version) in *M. bovis* BCG.

Full-length *raaS*$_{Mtb}$, binding sites and the predicted -10 and -35 promoter elements were cloned into *SpeI* and *HpaI* sites of the integrating vector pRBexint.. *raaS* start codon (red), Binding site (underlined), -10 promotor (green), -35 promotor (blue).



Δ*raaS*$_{com3}$: GGGATGAACGTACGTTTAATATCCTGAAC
ATGCGTTCAGCCGATCTGAC

Figure 28: Schematic representation illustrating the construct used for generation of Δ*raaS*com3 (Short version) in *M. bovis* BCG.

Full-length *raaS*$_{Mtb}$, binding sites and the predicted -10 were cloned into *SpeI* and *HpaI* sites of the integrating vector pRBexint.. *raaS* start codon (red), Binding site (underlined), -10 promotor (green).

The resulting constructs were sent for sequencing to GATC. After verification of the constructs, 3µg of each of pRBexint-raaS$_{com2}$ and pRBexint-raaS$_{com3}$ DNA were electroporated into *M. bovis* BCG *⊿raaS* mutant and the bacteria were plated on 7H10 agar plates supplemented with 50 µg/ml of hygromycin. After 25-30 days, hygromycin resistant colonies were picked for further screening. The primers used in this chapter are listed in Table 5.

Table 5: Primers used for complementation studies

Primer	Sequence (5'-3')	Application
Rv1219Fexint2	Ggt*ACTAGT*ccttgaccggggatgaacgta	Amplification of *raaS$_{Mtb}$* with the binding site and the containing the predicted -10 and -35 promoter elements for cloning in pRBexint; *SpeI* site introduced
Rv1219Fexint3	Ggt*ACT AGT*gggatgaacgtacgtttaata	Amplification of *raaS$_{mtb}$* with the binding site in pRBexint; *SpeI* site introduced
Rv1219Rexint	Aca*GTTAAC*tcagccgacatgtgcttctcc	Amplification of *raaS$_{mtb}$* for cloning in pRBexint; *Hpa*II site introduced
1219F4	atcctgaacatgcgttcagccgatctgacc	Test primers for *raaS$_{BCG}$* deletion mutants
1219R4	caaccgcac gcttccgccgtcggccttcac	Test primers for *raaS$_{BCG}$* deletion mutants
1219F5	aaggaaggtctccgcaaggcgtgcgac	Gene specific primers for *raaS$_{BCG}$* deletion mutants
1219R5	caggccttcggtgtagacctccaggga	Gene specific primers for *raaS$_{BCG}$* deletion mutants

4.2.3 Diagnostic PCR

After transformation, three hygromycin resistant colonies were picked and each one was used to inoculate 5ml of supplemented 7H9 medium (with ADC, tween 80 and hygromycin). The cultures were incubated at 37 ºC without shaking. After 3 weeks of incubation, 100µl of each culture was centrifuged at 10000xg for 10

minutes to pellet the cells. Afterwards, the supernatant was discarded and the pellet resuspended in 50µl of H_2O. This suspension was subsequently heated at 95 ºC for 30 minutes and after cooling centrifuged for 10000xg. Pellets were discarded and supernatants were applied for diagnostic PCR using two sets of diagnostic primers (test and gene specific primes).

4.2.4 Complementation studies

Two stresses were chosen for complementation studies, the first was survival during nutrient starvation in PBS and the second was survival during exposure to 10mM of H_2O_2. In both conditions the deletion mutant showed a significant survival defect. Exposure of *M. bovis* BCG (wild type, *raaS* deletion mutant and the three complemented strains) to treatment with 10mM H_2O_2 and nutrient starvation in PBS was carried out as mentioned in 3.2.4 and 3.2.5 respectively.

4.3 Results

4.3.1 Generation and Confirmation of Complementation Strains

Figure 29 demonstrated the PCR products which were generated as a result of amplification of full length $raaS_{Mtb}$, binding sites and the predicted -10 and -35 promoter elements for the long complementation construct and full length $raaS_{Mtb}$ and the binding site for the short complementation construct.

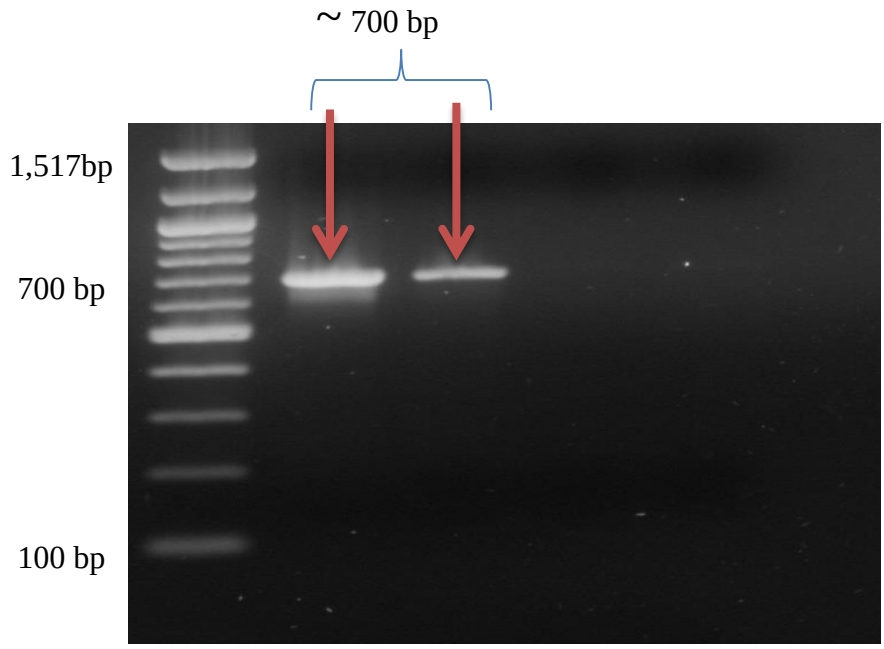


Figure 29: 1% agarose gel electrophoresis of the PCR products for *M. bovis* BCG Δ*raaS* long and short complementation constructs.

Lanes1-5 (left to right) , 1; 100 bp DNA ladder, 2; PCR product for long construct, 3; PCR product for short construct, 4&5 negative control (H_2O).

The PCR products were subsequently cloned into *SpeI* and *HpaI* sites of pRBexint. Successful transformants were subjected to restriction digestion with *SpeI* and *HpaI* and subsequently analysed using 1% (w/v) agarose gel to confirm that they contain the insert as shown in Figure 30.

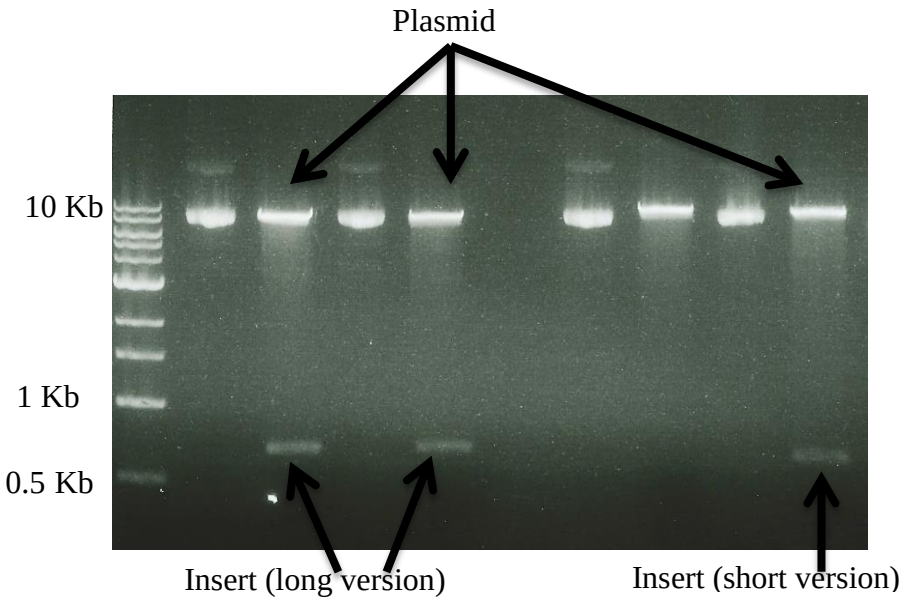


Figure 30: 1% agarose gel showing restriction digestion for candidate *M. bovis* BCG Δ*raaS* long and short complementation constructs.
Lanes1-10 (left to right), 1; 10 Kb DNA ladder, 2, 4, 7 & 9; undigested candidate plasmids, 3, 5 &10: plasmids with the right insert.

Constructs with no PCR errors were subsequently electroporated into *M. bovis* BCG Δ*raaS* and candidate long and short complementation constructs were tested using test and gene specific primers. A band around 274 bp is expected for complementation strains using test primers. On the other hand, a band around 420 bp is expected with gene specific primers.

Figure 31 shows the candidate transformants tested with test and gene specific primers.

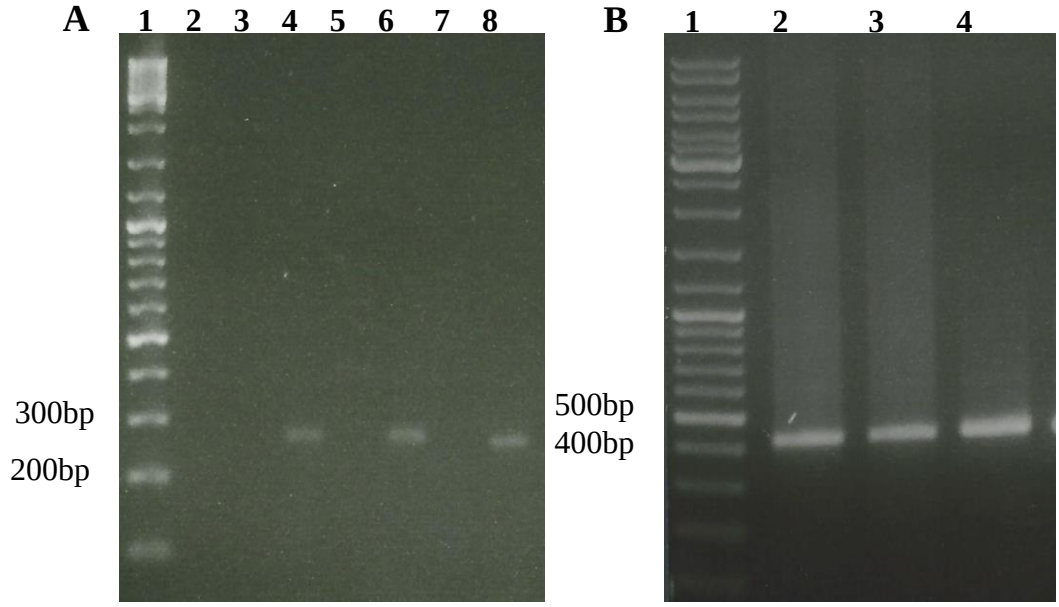


Figure 31: 1.5% agarose gel showing PCR products with Δ*raaS* test and gene specific primers.

A) Lanes1-8 (left to right), 1; 10 Kb DNA ladder, 4, 6 & 8; candidate transformants tested with test primers, B) Lanes1-4 (left to right), 1; 10 Kb DNA ladder, 2,3 & 4; candidate transformants tested with gene specific primers.

4.3.2 Survival of *M. bovis* BCG (wild type, *raaS* deletion mutant and complemented strains) during nutrient starvation

Data presented in chapter three showed that the *M. bovis* BCG *raaS* deletion mutant showed a drastic growth defect during nutrient starvation. Thus the three complemented strains were tested in this condition to assess which one of the three complemented strains is able to restore the phenotype of the wild type. Data represented by Figure 32 shows that both the wild type and Δ*raaS*$_{com2}$ had similar growth rates during most of the time points (except at 6 weeks of starvation where Δ*raaS* $_{comp2}$ shows a slight decrease). On the other hand, there is a significant decrease in growth rates of each of Δ*raaS*, Δ*raaS*$_{com1}$, and Δ*raaS*$_{com3}$ when compared to that of both wild type and Δ*raaS* $_{com2}$ after 3 weeks of starvation ($p < 0.01$). After 6 weeks of starvation, both Δ*raaS* and Δ*raaS*$_{com1}$ show a further decrease in growth rate with a significant difference with that of wild type and Δ*raaS*$_{com2}$ ($p < 0.01$, one way ANOVA). Thus it is clear from the aforementioned data that Δ*raaS* $_{com2}$ was the strain which was able to restore the phenotype of the wild type.

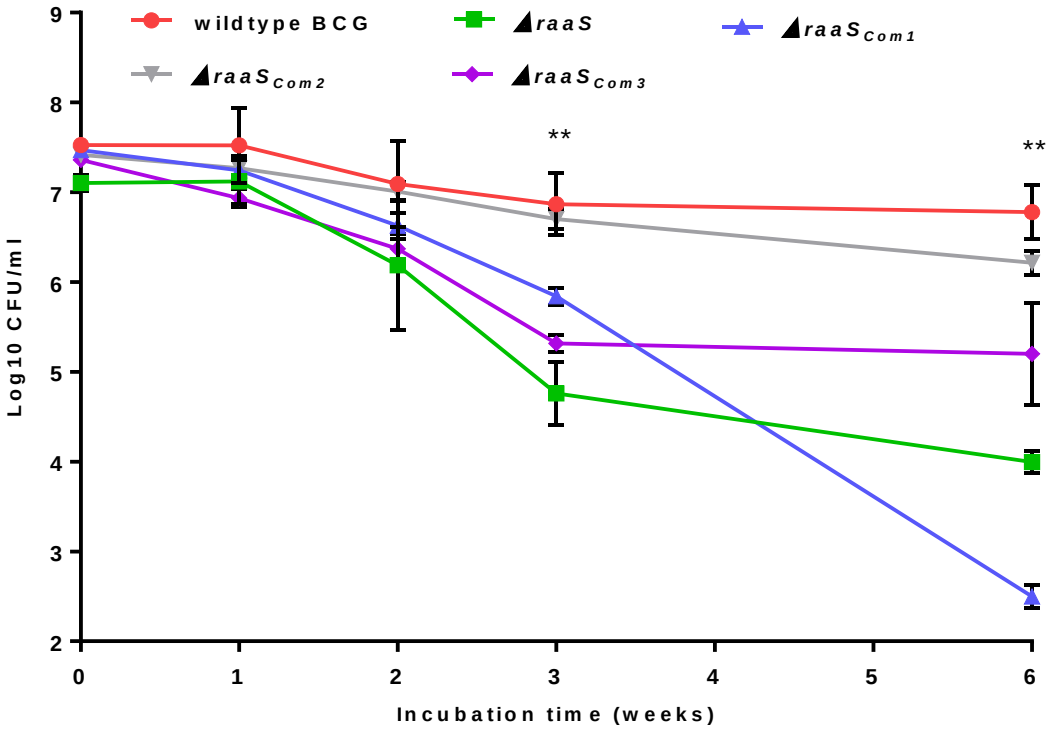


Figure 32: Survival of *M. bovis* BCG (wild type, Δ*raaS* and the three complemented strains) during starvation in PBS.
Cultures of wild type *M. bovis BCG*, Δ*raaS*, Δ*raaS* com1, Δ*raaS* com2 and Δ*raaS* com3 were transferred from nutrient-rich medium into PBS. The number of colony forming unit were followed over a period of 6 weeks. Data shown are average of two independent experiments, each including three biological replicates. Data are expressed as mean ± SEM.

Statistical analysis using one way ANOVA showed that $\log_{10}$ CFU/ml for each of Δ*raaS*, Δ*raaS* com1, and Δ*raaS* com3 were statistically lower than that of the wild type and Δ*raaS* com2 after 3 weeks of starvation ($P < 0.01$). After 6 weeks of starvation, $\log_{10}$ CFU/ml for Δ*raaS,* Δ *raaS* com1 and Δ *raaS* com3 were statistically lower than that of the of the wild type ($P < 0.01$), while $\log_{10}$ CFU/ml for Δ *raaS* and Δ *raaS* com1 were statistically lower than that of the of Δ *raaS* com2 ($P < 0.01$).

4.3.3 Survival of *M. bovis* BCG (wild type, *raaS* deletion mutant and complemented strains) during H_2O_2 treatment

Exposure to 10mM of H_2O_2 was the second stressful condition used to assess the survival of the complemented strains as mentioned earlier in this chapter. Prior to the construction of the new complemented strains *(*Δ*raaS* com2 and Δ *raaS* com3), a preliminary experiment was carried out to test if Δ*raaS* com1 was able to complement the phenotype of the wild type during treatment with 10mM of H_2O_2. The result of this experiment represented by Figure 33 show that this complemented strain showed a defect in survival rate after both 1 and 4 hours of treatment and failed to show a survival rate similar to that of the wild type.

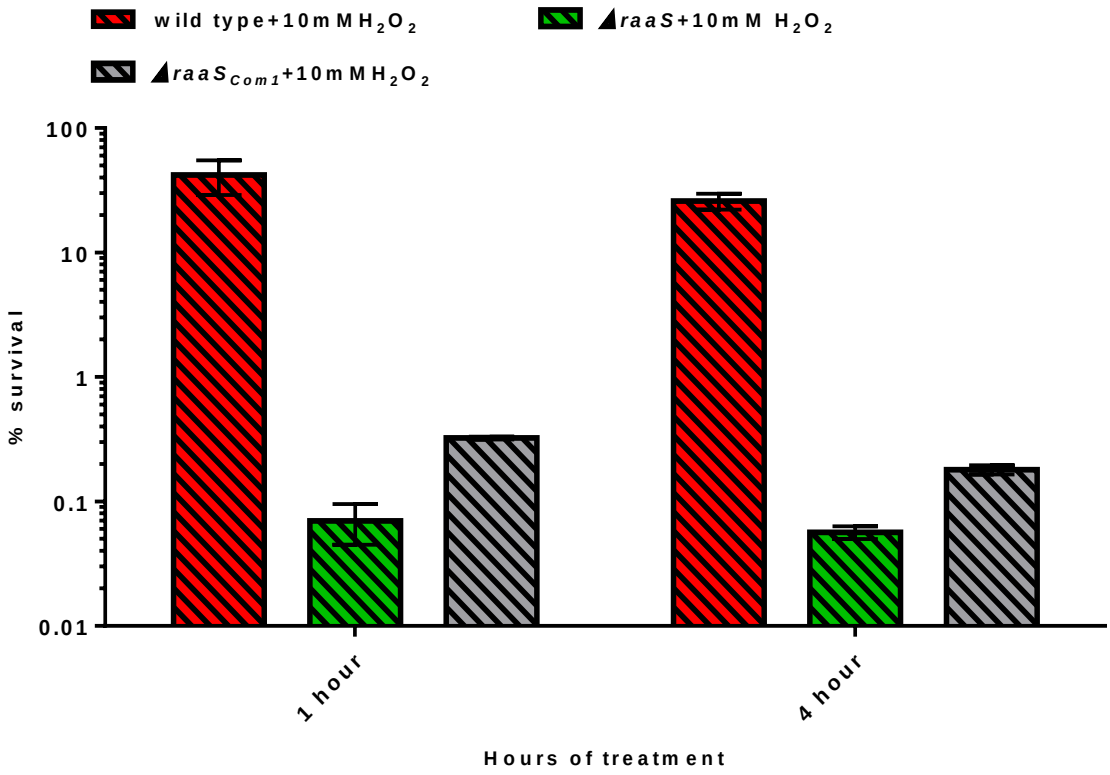


Figure 33: Survival of wild type *M. bovis* BCG, Δ*raaS* and Δ*raaS*$_{Com1}$ after treatment with 10mM H_2O_2.

Cultures of wild type *M. bovis BCG*, Δ*raaS* and Δ*raaS*$_{Com1}$ were grown in supplemented 7H9 medium until they reached an OD of 0.5. Then the cultures were treated with 10mM of H_2O_2. After that the samples were incubated at 37 °C without shaking. At 1 and 4 hours of incubation, a sample was taken for CFU counting. Survival was calculated depending on the CFU/ml before treatment. Result is an average of three technical replicates (triplicate tubes). Data are expressed as mean ± SEM.

It is worth noting that this experiment was carried out when the OD_{600nm} of the cultures was 0.5. After the construction of the new complemented strains (Δ*raaS*$_{com2}$ and Δ*raaS* $_{com3}$), an experiment was carried out to assess the survival of all three complemented strains during treatment with 10 mM H_2O_2. This experiment was done when the cultures were at an OD_{600nm} of 0.4 and the results showed that both Δ*raaS*$_{com1}$ and Δ*raaS*$_{com3}$ failed to survive after 6 hours of treatment. On the other hand, Δ*raaS*$_{com2}$ showed a very low survival rate as shown in Figure 34.

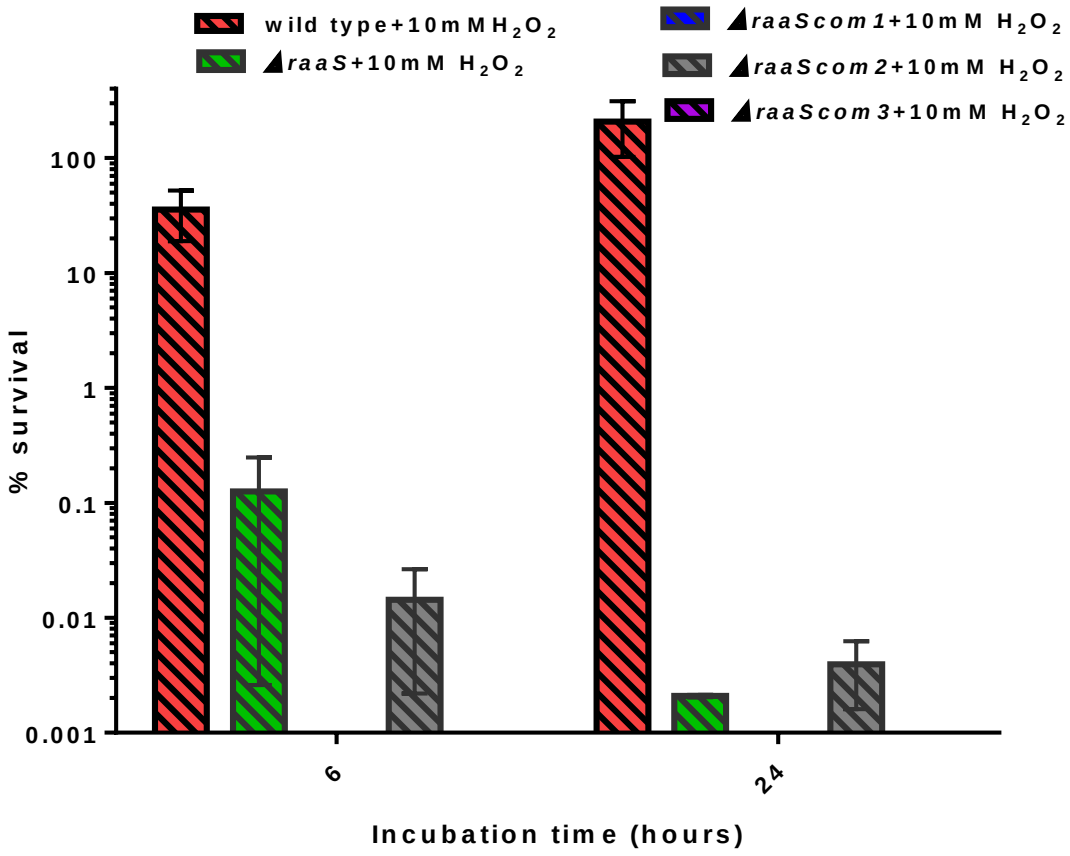


Figure 34: Survival rates of *M. bovis* BCG cultures (wild type, Δ*raaS*, ΔraaS_{Com1}, ΔraaS_{Com2} and ΔraaS_{Com3}) at an OD of 0.4 after treatment with 10mM H_2O_2.

Cultures were grown in supplemented 7H9 broth until they reach an OD_{600nm} of 0.4. Then the cultures were treated with 10mM of H_2O_2. After that the samples were incubated at 37 °C. At 6 and 24 hours of incubation, a sample was taken for CFU counting. Result is an average of three technical replicates (triplicate tubes). Data are expressed as mean± SEM.

Then a second experiment of 10mM H_2O_2 treatment was carried out using cultures at an OD_{600nm} of approximately 0.7. The results of this experiment represented by Figure 35 shows that Δ*raaS*$_{com2}$ was able to show a survival rate similar to that of the wild type after both 6 and 24 hours of treatment. Attempts were made to repeat the aforementioned experiment but complementation experiments could only be carried out when all strains were in the same growth phase. This standardisation criteria and time limitation prevented the H_2O_2 stress from being repeated to confirm the results obtained with all three complemented strains.

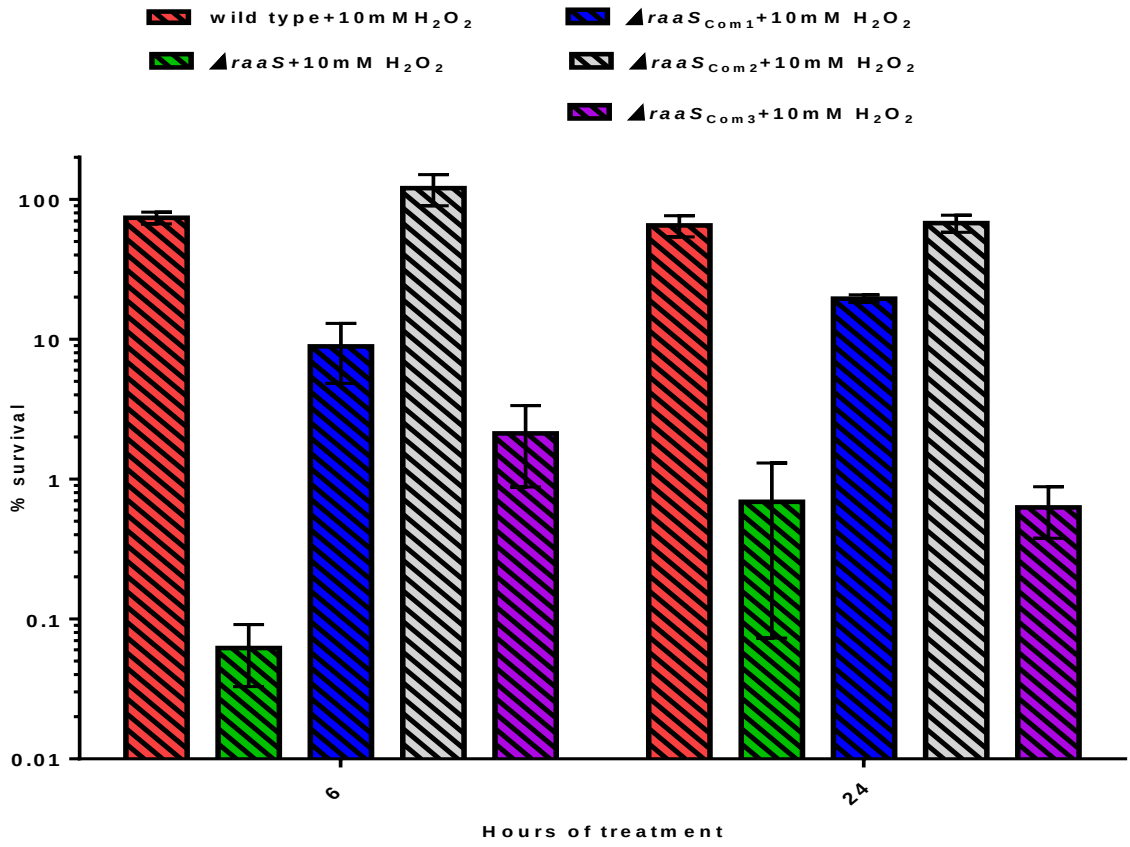


Figure 35: Survival rates of cultures (wild type *M. bovis* BCG, Δ*raaS*, Δ$raaS_{Com1}$, Δ$raaS_{com2}$ and Δ$raaS_{Com3}$) at an OD of 0.7 after treatment with 10mM H_2O_2.

Cultures were grown in supplemented 7H9 broth until they reach an OD of 0.7. Then the cultures were treated with 10mM of H_2O_2. After that the samples were incubated at 37 °C. At 6 and 24 hours of incubation, a sample was taken for CFU counting. Result is an average of three technical replicates (triplicate tubes). Data are expressed as mean ± SEM.

4.4 Discussion and Conclusions

4.4.1 Discussion

Mounting evidence has suggested that efflux pumps also have physiological functions in bacteria and their expression is subject to tight regulation in response to various environmental and physiological signals (Nikaido, 2001). It has been well known that efflux pumps can mediate the resistance to some antibiotics. Furthermore, resistance to certain antibiotics may result from a constitutive increase in expression of the efflux-pump protein. Recent data have proposed that efflux pumps may play an important role in INH acquired resistance in MDR *M*. *tuberculosis* (Li et al., 2015). Nevertheless, emerging data have shown that in some cases, antibiotic resistance is not the primary function of pumps. It must be noted that overexpression of pumps in some cases might be disadvantageous. An example is that overexpression of MexABOprM and MexCDOprJ pump in *Pseudomonas aeruginosa* resulted in a fitness cost (Sanchez et al., 2002).
RaaS is a member of the TetR family of transcriptional regulators. Members of this family function via binding of an inducer molecule to the non-conserved domain of a TetR family member leading to conformational changes which results in release of the repressor and subsequently leads to transcription of target genes. Furthermore, it has been found that TetR family members usually regulate their own synthesis (Ramos et al., 2005). In stressful conditions free RaaS binds to its DNA binding site and results in repression of the cluster of efflux pump genes and itself (Turapov et al., 2014a).

The results presented in the previous chapter have revealed that the deletion of *raaS* and subsequent overexpression of efflux pumps resulted in a mutant with a defect in survival during exposure to the stressful conditions which are likely to be experienced by mycobacteria during infection. Thus, it was necessary to undertake complementation studies to confirm the important role of *raaS* (*bcg_1279c/rv1219c*) during exposure to these stressful conditions. The *raaS* deletion mutant has shown a significant survival defect during both nutrient starvation and exposure to 10mM of H_2O_2, therefore these two conditions were

chosen for complementation studies. It is important to note that *M. bovis* BCG *raaS* deletion mutant was generated by in-frame deletion of 573 bp with the retention of the region which forms part of the predicted RaaS binding site. Among the three complemented strains tested, Δ*raaS*$_{com2}$ was the strain which showed a phenotype most similar to that of the wild type. Treatment with 10mM of H_2O_2, revealed that Δ*raaS*$_{com2}$ was able to restore the wild type phenotype when cultures were at an OD_{600nm} of 0.7 but not 0.4. This apparent discrepancy in the results between the two experiments is probably attributed to different growth phases. Published data revealed that cultures from early to mid-log phase were found to be more sensitive to H_2O_2 treatment than cultures grown to mid- to late log phase, whilst stationary phase cultures were more resistant to H_2O_2 (Dowds et al., 1987). Nutrient starvation results showed that Δ*raaS*$_{com2}$ was able to restore the wild type phenotype indicating that the presence of both the genes own promoter and the binding site were necessary for restoring the wild type phenotype. The complemented strain Δ*raaS*$_{com1}$ failed to restore the phenotype of the wild type in the two stress conditions tested. The expression of complemented strain Δ*raaS*$_{com1}$ was constitutively expressed from a *dnaK* promoter which probably was the reason behind overexpression of *raaS* (*rv1219c)* in the complemented strain (2.6 fold). Overexpression of the gene is the probable reason behind failure of Δ*raaS*$_{com1}$ in restoring the wild type phenotype. It is expected that overexpression of *raaS* will lead to an increase in repression of the operon and subsequently result in increased intracellular accumulation of the molecule(s) transported by the BCG_1278c/ 1277c (Rv1218c-Rv1217c) pump which might be toxic above certain level. The results presented in this chapter also demonstrated that Δ*raaS*$_{com3}$ failed to restore the phenotype of the wild type which might indicate that the presence of the binding site without the genes own promoter did not result in the right level of expression of the transcriptional regulator. Although Δ*raaS*$_{com1}$ was previously reported to complement the wild type phenotype during certain conditions such as extended stationary phase, antibiotic treatment and macrophage infection, but it seems that this strain fails to complement when the bacteria experience sudden transition to non-permissive growth conditions. For example in the nutrient starvation model, the bacteria are suddenly transmitted from nutrient rich media to nutrient deprived media (PBS). In this condition the bacteria did not have enough time to adapt and

hence it seems that tight regulation of efflux pumps is necessary for the bacilli to be able to tolerate this stress as RaaS mediates mycobacterial survival in non-permissive growth conditions by controlling expression of ATP-dependent efflux pumps. The same may also apply when the bacteria is suddenly exposed to the damaging effect of H_2O_2. Due to time restrictions, it was not possible to measure the level of expression of *raaS* in the two newly constructed complemented strains ($\Delta raaS_{com2}$ and $\Delta raaS_{com3}$). These data would have been important in confirming that both the RaaS binding site and predicted promoter are critical in conferring the right level of expression. RaaS is the first gene in the represses the operon in which it is encoded and thus was classified as an autorepressor (Turapov et al., 2014a). It has been hypothesized that the localisation of autorepressors in the first operon position as applicable here to RaaS minimises energy waste and leads to tight regulation of operon expression (Rubinstein et al., 2011).

Interestingly, published data has shown that oleoyl-CoA regulates the binding of RaaS to DNA and thus the RaaS mediated mycobacterial survival in non-permissive growth conditions (Turapov et al., 2014b). However, Oleoyl-CoA did not exert any significant effect on mycobacterial growth in logarithmic phase. It has been predicted that during stress conditions, free RaaS binds to its DNA recognition sequence resulting in repressing of transcription of itself and the cluster of efflux pump genes. Moreover, the addition of oleic acid which is a precursor of oleoyl-CoA, results in suppression of the RaaS mediated antimicrobial assisted survival in mycobacteria (Turapov et al., 2014b). This suggests that the regulation is more important when bacteria suddenly experience growth limiting stresses. In mycobacterial cells, oleic acid is converted to oleoyl-CoA resulting in an increase in intracellular acyl-CoAs concentration (Daniel et al., 2011). It has been suggested that *M. tuberculosis* responds to stress by accumulation of TAG (triacylglycerol) which results in lowering of growth and metabolic activity. Under stressful conditions, *M. tuberculosis* incorporates oleoyl-CoA into TAG leading to growth restriction via redirecting carbon away from growth-promoting pathways such as the TCA (tricarboxylic acid) cycle (Baek et al., 2011). Depletion of acyl-coA derivatives would allow binding of RaaS to DNA resulting in repression of downstream genes.

Over all, the results presented here highlight the importance of tightly regulated expression of efflux pumps in response to nutrient starvation and exposure to H_2O_2. Constitutive expression of otherwise tightly regulated pump genes above normal levels leads to impaired survival during the aforementioned stress conditions. In both, of these conditions, stress is applied suddenly and thus the bacilli will not have enough time to adapt and here comes the importance of tight regulation of efflux pumps. The results presented in this chapter are in accordance with previous studies which have stressed the importance of tightly regulated expression of efflux pumps (Martinez et al., 2009b). Published data has demonstrated that P55 which is a mycobacterial efflux pump belonging to the MFS of membrane transporters, plays a vital role in drug resistance, maintenance of cellular redox balance and late stages of infection. Furthermore, deletion of *p55* resulted in attenuation of *M. tuberculosis in vivo* (Bianco et al., 2011, Ramon-Garcia et al., 2015). In *M. smegmatis*, inactivation of the MFS transporter LfrA, revealed the involvement of this pump in the intrinsic resistance to cationic dyes, fluoroquinolones, and tetracycline. LfrA expression is regulated by the putative TetR family transcriptional repressor LfrR (Li et al., 2004). Deletion of this repressor resulted in high resistance to the aforementioned drugs. The above examples highlights the importance of pump regulation in mycobacteria.

4.4.2 Conclusions

The data reported in this chapter clearly indicate that both binding sites and promoter region were important in restoring the wild type phenotype with regards to survival during both nutrient starvation and treatment with 10mM H_2O_2. Moreover, the failure of complementation constructs (Δ*raaS*$_{com1}$ and Δ*raaS*$_{com3}$) in which constitute expression was driven from a *dnaK* promoter in restoring the wild type phenotype suggest that overexpression of the transcriptional regulator might be the cause behind this failure. Indeed, this is the probable cause behind the inability of Δ*raaS*$_{com1}$ to complement the wild type phenotype as published data has shown that this strain showed a 2.6 fold overexpression of *raaS* (*rv1219c)* when compared to the wild type. It seems that the right level of expression of efflux pumps is necessary for survival during stress conditions as over expression of the pumps as a result of deletion of their repressor (*bcg_1279c*/*rv1219c*) or overexpression of their repressor resulted in defective survival during both nutrient starvation and treatment with 10mM H_2O_2. Thus, it is important that any future work should include measuring the level of expression of *raaS* in the complementing strains to confirm this assumption.

The importance of efflux pumps has been highlighted by the observation that expression of efflux transporters are modulated in phagocytosed, non-replicating persistent and multi-drug resistant bacilli (Sarathy et al., 2012). Although the physiological role of the Bcg_1278c/Bcg_1277c (Rv1218c/Rv1217c) pump is not known but it has been proposed that this pump might be involved in transporting lipid molecule(s) important for the initial stages of infection and may become toxic or are dispensable during non-replicating persistence (Turapov et al., 2014b).

The finding that over expression of their repressor in the case of Δ*raaS*$_{com1}$ resulted in impaired survival during the tested stressful conditions seems to support this proposal. This might imply that dysregulation of the pumps by either overexpression or repression might be used to kill mycobacteria under non-permissive growth conditions. Another important point to highlight is that tight regulation of efflux pumps is more important when the bacilli is exposed to sudden stress conditions such as nutrient starvation and H_2O_2 treatment.

Chapter 5 : RV3136 (PPE51) a member of the PPE family with a possible role in intracellular replication

5.1 Introduction

Despite being discovered almost two decades ago, the precise biological function of the PE/PPE protein family is still unknown. Published data have shown that about 7% of *M. tuberculosis* and 9.1% of *M. marinum* genomes encode proteins belonging to these two families (Lew et al., 2011, Stinear et al., 2008). Their unique presence in pathogenic mycobacteria strongly suggests a possible role in virulence. Recent data indicate that *pe/ppe* genes are differently expressed during infection, suggesting that the proteins of these families may act as virulence factors and facilitate the establishment of successful infection (Betts et al., 2002, Mukhopadhyay and Balaji, 2011). Multiple transcriptional regulators have been implicated in the regulation of *pe/ppe* genes (Voskuil et al., 2004a). Among those is the cAMP (Cyclic adenosine monophosphate) dependent regulator Rv3676 (CRP), which is an important transcription factor involved in regulation of the persistence and reactivation of *M. tuberculosis.* Rv1675c is putative cAMP responsive transcription factor of a member of the PE family and is important for intramacrophage gene regulation (Gazdik et al., 2009). It has been suggested that studying selected PE/PPE family members as cAMP mediated virulence factors might aid in gaining further insights into the possible role of some PE/PPE family members in virulence (Fishbein et al., 2015). Thus this chapter was aimed to investigate whether one of the PPE family members (PPE51) predicted to be regulated by a cAMP dependent regulator could be important for intracellular survival and virulence.

5.1.1 RV3136 (PPE51); a member of the PPE family regulated by a cAMP dependent regulator with a possible role in virulence

As mentioned in chapter one, genes encoding some members of the PE/PPE family of proteins have shown variable expression as the bacilli experience different environmental changes. Of those, is *rv3136* which encodes PPE51 protein. *Rv3136* (*ppe51*) is among the *ppe* genes predicted to be regulated by the cAMP dependent regulator Rv3676. The *ppe51* was among eight genes whose transcripts were >2.4-fold more abundant in wild type *M. tuberculosis*

H37Rv than the Rv3676 deletion mutant. The intergenic DNA sequence of *rv3136* was analysed to identify the likely binding site for Rv3676 in *M. tuberculosis* (Rickman et al., 2005). This analysis identified a possible binding site for Rv3676 situated ~100 bp upstream of *rv3136* as shown in Figure 36.

Figure 36: Schematic representation showing the upstream genomic region of *rv3136* in *M. tuberculosis* showing the predicted Rv3676 binding site.

Representation of *rv3136* and upstream region; *rv3136* start codon (Red); the sequence of the putative Rv3676 binding site (Green)

Another potential regulator of *rv3136* is the regulatory hub Rv0081. A putative binding site for Rv0081 is predicted to be within the coding sequence of *rv3136*. Together with DosR, Rv0081 is thought to mediate the initial response to hypoxia (Galagan et al., 2013).

Examining the transcriptional response of both *M. tuberculosis* and *M. bovis* BCG to the macrophage phagosome environment revealed a set of species specific pattern at both 2 and 24 hours post infection. Interestingly, *rv3136* was among the set of genes being up regulated (1.74 fold) in *M. tuberculosis* but not in *M. bovis* BCG at 2 hours post infection (Rohde et al., 2007b). Another study which

was conducted to explore the *M. tuberculosis* proteome *in vivo* using a guinea pig model of TB identified PPE51 in the lungs of infected animals after 30 days of infection (Kruh et al., 2010). Furthermore, a comparison of transcriptomics signatures of *M. bovis* and *M. tuberculosis* during exponential phase (OD_{540nm} ~0.25) of growth identified 18 genes of the pe/ppe families differentially expressed in *M. tuberculosis* compared to 3 genes in *M. bovis*. In this study bacteria were grown in 7H9 media supplemented with 10% ADC and 0.05% tween 80. For *M. tuberculosis*, 0.2% glycerol was also added to the media, while for *M. bovis* the media was supplemented with 0.4% sodium pyruvate. *Rv3136* was found to be significantly up regulated (47.2 fold) in *M. tuberculosis* compared with *M. bovis* (Rehren et al., 2007). In addition, *rv3136* showed higher expression (9.6 fold) in *M. tuberculosis* H37Rv than *M. bovis* under steady-state growth (Golby et al., 2007). Interestingly this gene is among 22 genes which are consistently up regulated in *M. tuberculosis* H37Rv when compared with its avirulent counterpart H37Ra (Mostowy et al., 2004). However, the biological significance of the variation in the expression of *rv3136* remains unknown. Studying the adaptation of *M*. *tuberculosis* to anaerobic conditions and the non-replicating persistence (NRP) state revealed that *rv3136* was up regulated by 2.6 times during NRP1 in which the oxygen concentration reaches 1% of normal saturation. This value was found to be significant (p=0.005) when compared to aerobically grown cultures (Muttucumaru et al., 2004). On the other hand, Voskuil and co-workers reported that *rv3136* is repressed at day 20 of NRP which was regarded to represent middle or NRP2 (Voskuil et al., 2004b). It has been postulated that variation in gene expression is likely to be the cause behind the distinctive adaptive responses to changes in environmental conditions (Rehren et al., 2007). In their nutrient starvation model, Betts and co-workers identified *rv3136* as being down regulated after 4 and 96 hours of starvation (Betts et al., 2002). It is expected that the ability of *M. tuberculosis* to regulate virulence factors, acquire nutrient and modulate the host cell would correlate with its virulence. Although there is no definitive link, all these published data suggest that *rv3136* might be playing a role in intracellular survival and virulence. An in frame deletion mutant was constructed in *M. tuberculosis* H37Rv background to investigate the possible role of *rv3136* in intracellular survival and to study any other phenotypes conferred by this gene.

5.1.2 Manipulation of *M. tuberculosis* genome

The global health impact of *M. tuberculosis* has motivated many researchers to put considerable efforts into developing tools which allows transfer, mutation, and expression of specific genes in a number of mycobacterial species. Such tools would aid in characterising genes that could be related to virulence and subsequently used as potential drug targets. Allelic exchange mutagenesis in a specific gene enabled legitimate site specific mutation of *M. tuberculosis* (Balasubramanian et al., 1996). Specific genes were interrupted using transposon mutagenesis which generated transposon mutant libraries in *M. tuberculosis.* However, this technique resulted in generation of thousands of mutations which would require large scale screening (Lamichhane et al., 2003). Another technique used is signature-tagged mutagenesis which involves modification of a specific gene using transposon systems with different tags (Hisert et al., 2004). Site-directed mutagenesis was used to generate mutation at a specific site within the *M. tuberculosis* chromosome. It involves the synthesis of a short DNA primer which is subsequently hybridised with a single-stranded DNA containing the gene of interest. DNA polymerase is used to extend the reaction yielding a double stranded molecule which is introduced into an expression vector. The resultant vector is subsequently transformed into *E. coli* (Huang et al., 2006). Based on homologous recombination, Parish and Stoker described a new efficient method (the flexible cassette method) which can be used to generate both marked and unmarked deletion mutants in *M. tuberculosis* (Parish and Stoker, 2000). It is worth noting that the process of generating unmarked deletion mutants has a number of advantages such as minimising any possible polar effect and the possibility of making a second mutation without the need to exclude any resistance marker (Pavelka and Jacobs, 1999). The flexible cassette method employs a suicide plasmid which lacks a mycobacterial origin of replication (*oriM*) to deliver the recombination substrate to the cell. The need of several cloning steps to include the relevant markers and finding appropriate restriction sites for insertion of genes was overcome by separating the process of construction of the disturbed version of the gene of interest from that of inclusion of selective markers. Two series of vectors were developed; the first allows manipulation of the gene of interest while the second contains the marker cassette (Parish and Stocker, 2000). This method involves the amplification of

each of the upstream and the downstream flanking regions and subsequently cloning them into a separate vector (pGemTeasy). The suicide delivery plasmid was constructed by cloning both fragments into p2NIL, followed by cloning the *PacI* cassette from pGoal19 as illustrated in Figure 37.

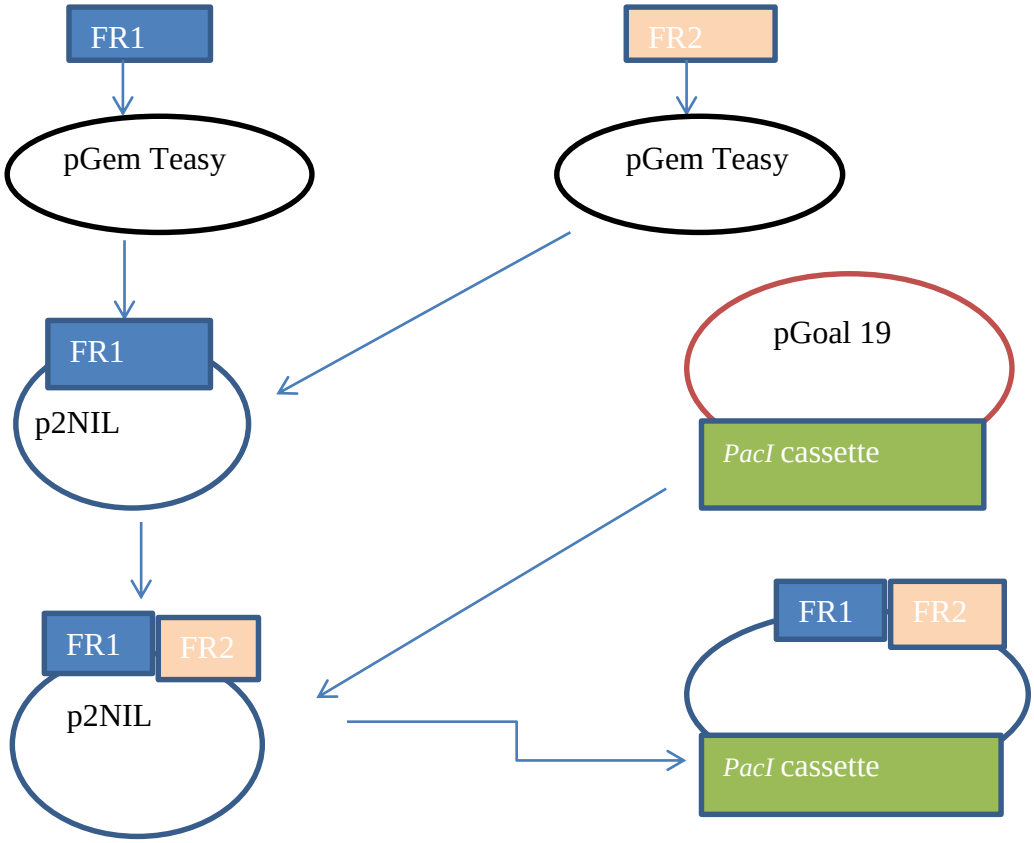


Figure 37: Schematic presentation of generating the suicide vector harbouring the upstream flanking region (FR1), the downstream flanking region (FR2) of the gene of interest and the PacI cassette from pGoal19.

5.2 Methods

5.2.1 Organisms and media

The strains used in this chapter were *M. tuberculosis* H37Rv (wild type and Δ*rv3136*) and wild type *M. tuberculosis* CDC1551 and *MT2551* (*rv3136*) transposon mutant (*rv3136*::Tn) kindly provided by BEI resources (BEI Resources, NIAID, NIH: *Mycobacterium tuberculosis*, Strain CDC1551, Transposon Mutant 3195 (MT3221, Rv3136), NR-18866). This transposon mutant was constructed by Lamichhane and co-workers who used random *Himar1* transposon insertion mutagenesis of *M. tuberculosis* CDC1551 to

generate a transposon Insertion mutant library. Mutants were characterised by PCR and DNA sequencing and data obtained showed that the transposon has integrated at nucleotide 132 of MT3221/Rv3136 coding region (Lamichhane et al., 2003). Strains were grown in either supplemented 7H9 medium or Sauton's medium; kanamycin (25µg/ml) was added to growth media to maintain the transposon mutant.

5.2.2 DNA Manipulation for Generation of an *rv3136* deletion mutant in *M. tuberculosis* H37Rv

The flexible cassette method was used to generate an *rv3136* deletion mutant in *M. tuberculosis* H37Rv in an attempt to elucidate the function of *rv3136.* Two fragments were amplified by PCR from *M. tuberculosis* genomic DNA (provided by Colorado State University, Contract No.HHSN266200400091C; NIH, NIAID N01-AI-40091) using the primers listed in table 6.

Table 6: Primers used to generate the *rv3136* deletion construct

Primer	Sequence (5`-3`)
3136FR1F	CAC ***AAG CTT***CGGATACATCCCGTCGGTTCA
3136FR1R	ATA ***GGA TCC*** TGCGAAATCCATTGTGTCTCA
3136 FR2F	TGA ***GGA TCC***TTCGGCTCACGGTGATGGCC
3136FR2R	CAT ***GGT ACC*** ATGGCGCTCTCGAGCGCCAGG

The first PCR product represents the upstream flanking region (FR1) of *rv3136* with an engineered *HindIII* and a *BamHI* restriction sites. The second PCR product represents the downstream flanking region (FR2) of *rv3136* with an engineered *BamHI and KpnI* restriction sites as shown in Figure 38.

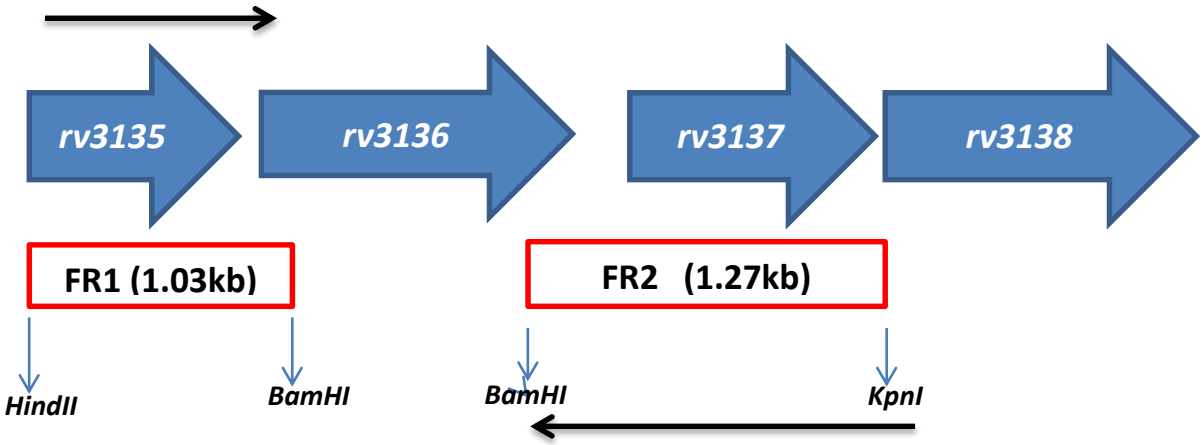


Figure 38: Schematic representation of *rv3136* genetic context showing the region of amplification of the upstream flanking region (FR1) and the downstream flanking region (FR2).

Each one of the PCR products was cloned into a separate vector (pGEM T Easy). This was followed by transforming the vectors harbouring the flanking regions into DH5α cells (Invitrogen). The transformation mixture was plated on LA agar plates containing ampicillin (50µg/ml) and X-gal (80µg/ml). Blue colonies were selected and three plasmid preps were prepared for each construct. These plasmids were sent for DNA sequencing to confirm the absence of any PCR errors. The next step was constructing the suicide delivery vector which initially involved assembling both flanking regions into the vector p2NIL. This was done by digesting both pGEMasy-FR1 and p2NIL with *HindIII* and *BamHI* and subsequent gel extraction. This was followed by ligating FR1 into p2NIL and transforming into DH5α cells producing the vector p2Nil-FR1. A number of digestions were carried out to confirm the presence of insert. Flanking region two was digested with *BamHI and KpnI* to release it from pGem T Easy followed by ligating it to the vector p2Nil-FR1 resulting in a vector which contains both flanking regions (p2NIL-FR1-FR2). Then a number of digestions were carried out to confirm the presence of both inserts into p2NIL. The next step was to digest pGoal19 with *PacI* to release the marker cassette which carries the hygromycin resistance, the *lac Z* and the *sac B* genes. To generate the suicide delivery vector, the released *PacI* cassette was then cloned into the *PacI* digested p2NIL-FR1-FR2. Successful transformants were selected and a number of digestions were carried out to confirm the Δ*rv3136* construct.

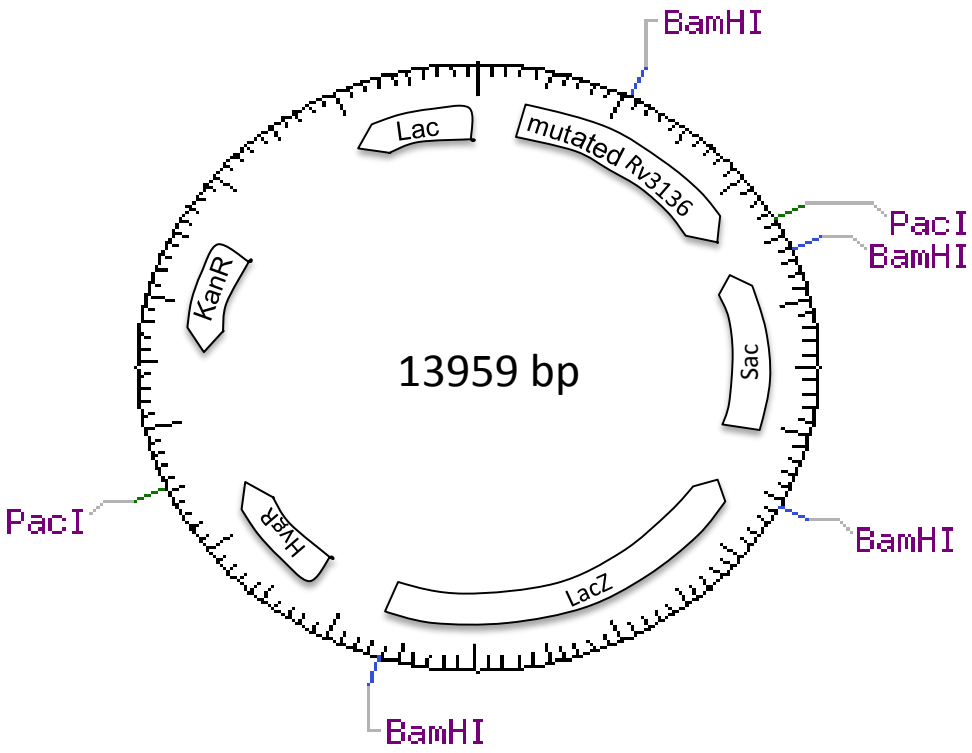


Figure 39: The construct used to generate the *rv3136* deletion mutant.

The mutated *rv316* was cloned into p2NIL vector and the required mutation generated. Then the *Pac*I cassette was excised from pGOAL19 and cloned into the unique *Pac*I site of the p2NIL/mutated *rv3136* vector, resulting in the final suicide delivery vector.

All subsequent steps were carried out in a Category 3 suite facility within class I or II microbiological safety cabinets according to the relevant Codes of Practice. Vector DNAs were used to electroporate *M. tuberculosis* H37Rv cells as previously mentioned in 2.6.7.

Following electroporation, samples were incubated overnight at 37 ºC with shaking. In the next day, different dilutions were plated on 7H10 agar plates supplemented with 10% (v/v) ADC, kanamycin (50µg/ml) and X-gal (80µg/ml). The plates were sealed with nescofilm, double bagged and incubated at 37 ºC for 4-5 weeks. Once colonies started to appear, blue colonies (single cross overs)

were selected and left until they became large enough to pick. Then each one of the blue colonies was inoculated on both 7H10 plates (supplemented with 10% (v/v) ADC and 50µg/ml of kanamycin) and supplemented 7H9 medium (with or without 50µg/ml of kanamycin). Liquid cultures growing in the presence of kanamycin were incubated 37 ºC until they reached an OD_{600nm} of approximately 0.5 and frozen stocks were made. On the other hand, cultures growing in the absence of kanamycin were incubated at 37 ºC until they reached stationary phase. These cultures were serially diluted (10^{-1}, 10^{-2} and 10^{-3}) and 100µl of suspension from neat, 10^{-1}, 10^{-2} and 10^{-3} dilutions were plated on 7H10 agar plates supplemented with 10% (v/v) ADC and 2% sucrose (w/v). These plates were incubated at 37 ºC until the appearance of colonies. After that 46 sucrose resistance colonies were picked and numbered from 1 to 46. Each one of these colonies were streaked on 7H10 agar plates supplemented with either 10% ADC (v/v) , X-gal (80µg/ml) and 2% (v/v) sucrose or 10% ADC (v/v) and kanamycin (50µg/ml). These plates were left to incubate at 37 ºC and after two weeks sucrose resistance and kanamycin sensitive colonies were selected. Out of theses, 10 colonies were chosen to be subsequently analysed by PCR using diagnostic primers.

5.2.3 Isolation of genomic DNA from *M. tuberculosis*

Genomic DNA was isolated from *M. tuberculosis* H37Rv (wild type and *rv3136* deletion mutant) according to a previously described standard protocol (Belisle and Sonnenberg, 1998). In Brief, stationary phase cultures were centrifuged at 2500xg and the pellet was resuspended of in 20ml of breaking buffer (see appendix). Then the cell suspension was centrifuged and the supernatant was carefully discarded and the pellet was resuspended in 0.5ml of breaking buffer followed by a 5 minute incubation step on ice. The mycobacterial suspension was transferred into a screw top tube containing ~300 µl of glass beads (Sigma – Aldrich) prewashed in breaking buffer. Then the tubes were placed in a mini bead-beater (Ribolyser, FastPrep FP120, ThermoSavant) followed by homogenising for 15 seconds at a speed of 6.5. The tubes were incubated on ice for 15 minutes. Then the tubes were briefly centrifuged and the lysates transferred into a sterile screw-cap microfuge tube. Proteins and other cellular contaminants were removed by adding 0.1 volume of 10% (w/v) SDS and 0.01 volume of proteinase

K solution (10mg/ml). The suspension was mixed carefully and incubated at 55 °C for 3 hours. The tubes were cooled on ice and the suspension was subjected to phenol: chloroform: isoamyl alcohol 25:24:1 extraction and chloroform: isoamyl alcohol 24:1 purification. Then precipitation of DNA was done by adding 1 volume of 3M sodium acetate and 1 volume of isopropanol to the aqueous layer. DNA was recovered by washing the pellet first with ice-cold 70% (v/v) ethanol and then with 96-100% ethanol. The pellet was left to air-dry subsequently dissolved in 200 µl of TE buffer.

5.2.4 Genotypic confirmation of candidate ∆ *rv3136* mutants

5.2.4.1 Diagnostic PCR

Sucrose resistant and kanamycin sensitive colonies were potential double cross overs, therefore 10 colonies were selected to be tested with diagnostic primers. Prior to that, heat inactivation of *M. tuberculosis* was carried out in the Category 3 suite facility. This was done by picking each colony and resuspending in 100µl of sterile H_2O. Then the samples were heat inactivated in a dry block at 95°C for 30 minutes. After this step, it was safe to remove the samples from the Cat3 facility and their subsequent testing by PCR using the diagnostic primers listed in Table 7.

Table 7 : Diagnostic primers used to confirm the deletion of *rv3136*

Primer	**Sequence (5`-3`)**
3136 test F	CATCGTCGTCGAAACGGCCAGTGC
3136 test R	ACTTCCGGTGGTGTACTCGCCGAA
3136 deletion F	ACTGATCGCGACGAACTTCT
3136 deletion R	GTCTCAATCAGCAGCGACAG

5.2.4.2 Southern hybridisation

Southern hybridisation was performed to confirm the deletion of *rv3136*. DIG High Prime DNA Labelling and Detection Starter Kit I (Roche) was used for this purpose. In addition to PCR, Southern hybridisation is commonly used to confirm gene deletion. A DNA sequence previously separated by gel electrophoresis is

detected via either a radioactive or chemically labelled probe which is similar to target DNA (Southern, 1975).

Non-radioactive detection systems include both biotin-streptavidin and the digoxigenin –antidigoxigenin system. In these systems, detection of the probe is usually carried out by chromogenic substrates, fluorescence or chemiluminescence (Holtke et al., 1995). The digoxigenin (DIG) labelling system is considered a highly sensitive system capable of detecting 0.01-0.03 pg of DNA. Labelling of probes is done by DIG molecules and detection is carried out through the addition of anti-DIG antibodies coupled to alkaline phosphatase (Holtke et al., 1990). This reaction is usually detected through the reaction of alkaline phosphatase with the substrates NBT (nitro-blue tetrazolium) and BCIP (5-bromo-4-chloro-3'-indolyphosphate) generating a purple/brown precipitate directly on the membrane (Holtke et al., 1992). Figure 40 illustrates the different stages of Southern hybridisation which involved the following steps:

5.2.4.2.1 Digestion of genomic DNA

1.5 µg of genomic DNA from *M. tuberculosis* H37Rv (wild type and *rv3136* deletion mutant) were digested with appropriate restriction enzymes. The digested samples were incubated at 37 ºC for 8 hours. After that, samples were separated by gel electrophoresis using 0.8% (w/v) agarose gel.

5.2.4.2.2 Transfer of DNA to a positively charged nylon membrane

Following separation, the gel was depurinated by soaking in HCL (0.25N). This was followed by denaturation in 0.5M NaOH, 1.5M NaCl and neutralisation by soaking in 0.5M Tris, 3M NaCl. DNA was transferred onto a positively charged nylon membrane via capillary action which takes several hours drawing the buffer up through the gel and into the membrane binding ssDNA. Transfer was done overnight in 20X SSC buffer (see appendix) after correct assembly of the capillary transfer as shown in Figure 40.

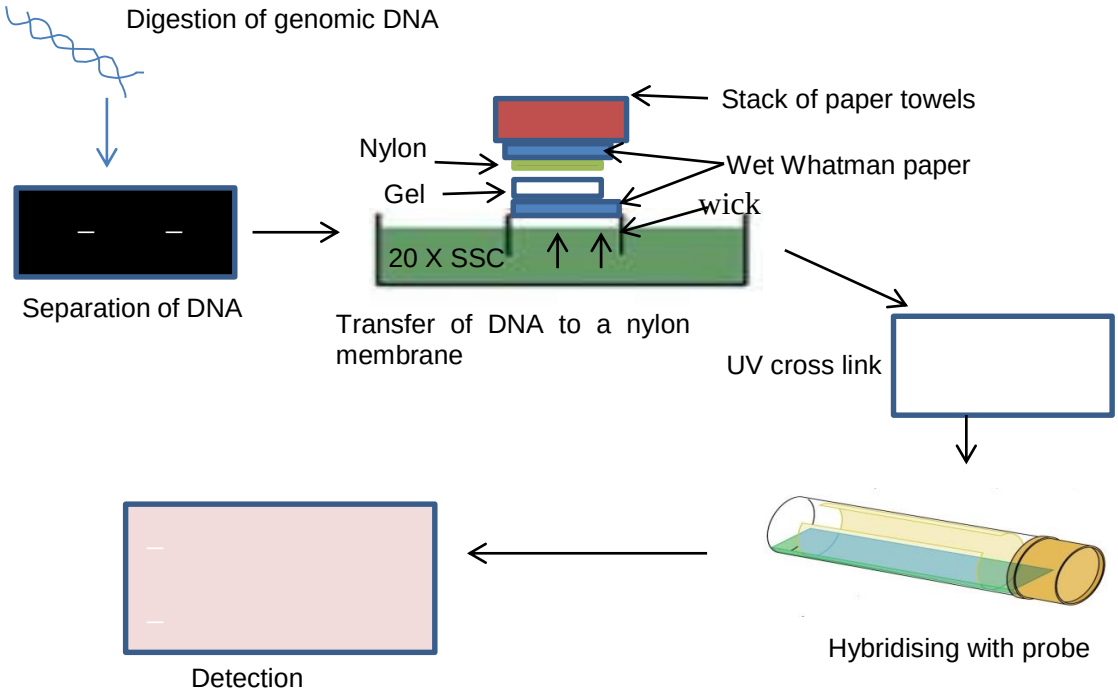


Figure 40 : An overview of Southern hybridisation steps.
Briefly, 1 µg of genomic DNA was digested separately with *XhoI* and *NheI*, incubated at 37°C for 8 hours. Then DNA fragments were separated by gel electrophoresis and transferred onto a positively charged nylon membrane. A DIG labelled probe was used for hybridization which was carried out at 65°C overnight using a roller bottle. Then hybridized membrane was exposed to alkaline phosphatase-conjugated anti-DIG antibody solution and detection was done using the substrate BCIP/NBT.

5.2.4.2.3 DIG labelling of the probe

The probe used was the upstream flanking region (FR1) of *rv3136* used to construct the Δ*rv3136* mutant. This was done by digestion of pGEM T Easy plasmid harbouring FR1 with *HindIII* and *BamHI* and subsequent gel extraction of FR1. 1µg of FR1 DNA was subsequently labelled according to manufacturer's instructions.

5.2.4.2.4 Fixation and hybridisation

After overnight transfer, the DNA was fixed to the positively charged nylon membrane by UV cross linking (Straterlinker™ 2400 -Invitrogen). After cross linking, the membrane was rinsed briefly in ddH_2O. Next was the pre-hybridisation step which was done be preheating the DIG Easy Hyb buffer (10ml/100cm2

membrane) to 65 ºC (hybridisation temperature) and then the nylon membrane was pre-hybridised by placing it in a glass roller bottle containing the preheated buffer with constant agitation for 30 minutes. The DIG labelled probe was denatured by heating to 95 ºC for 5 minutes followed by rapid cooling on ice. Then the prehybridisation solution was poured and replaced by the probe containing hybridisation solution. The hybridisation solution was prepared according to manufacturer's instructions. Hybridisation was done overnight at 65 ºC with constant gentle agitation. In the next day, the hybridisation solution was poured off and the membrane was washed twice as recommended by the manufacturer.

5.2.4.2.5 Immunological detection

The membrane was washed briefly and immunological detection was done using antibody solution (containing anti–DIG-Alkaline Phosphatase conjugated) according to manufacturer's instructions. Finally, the reaction was detected by incubating the membrane in 10ml of freshly prepared substrate solution for 16 hours. The reaction was stopped by washing in 50 ml of H_2O for 5 minutes.

5.2.5 Growth Studies in Laboratory Media

The growth characteristics of both *M. tuberculosis* H37Rv (wild type and *⊿rv3136*) and *M. tuberculosis* CDC1551 (wild type and *rv3136*::Tn transposon mutant) was studied by growing in supplemented 7H9 medium for H37Rv and in both kanamycin containing (25µg/ml) supplemented 7H9 and Sauton's medium for CDC1551 strain. Cultivation of *M. tuberculosis* was done in a Category 3 suite within microbiological safety cabinets (Class I and II) and following the relevant Codes of Practice and Standard Operating Procedures. Starter cultures were set up as previously mentioned in 2.2.1.2. Then these cultures were left to grow until they reached an OD_{600nm} of 0.6-0.8. These cultures were used to inoculate secondary cultures as before. Once these second-passage cultures had reached an OD_{600nm} of 0.5, they were used to inoculate either 25ml cultures in 125 ml flasks or 10ml cultures in 50ml falcon tubes. The cultures were left to grow for 2-3 weeks with optical density measurements taken every 1-2 days.

5.2.6 Infection of Macrophages with *M. tuberculosis* H37Rv (wild type *and rv3136* deletion mutant)

The intracellular survival and replication rate of *M. tuberculosis* H37Rv (wild type and *rv3136* deletion mutant) was assessed in the murine macrophage cell lines J774.1. Cells were prepared and infected as previously mentioned in 2.7.2. In addition to frozen stocks, growing cultures of *M. tuberculosis* from logarithmic phase (OD_{600nm} of 0.5) were also used for infection. Prior to infection cultures were washed twice with RPMI by resuspension and centrifugation at 10000xg for 10 minutes. J774.1 cells were infected with the appropriate number of bacilli. Infections were carried out at an MOI of 0.5, 1 and 5.

5.2.7 *In vivo* growth characteristics of *rv3136* deletion mutant

The *in vivo* growth characteristics of both wild type *M. tuberculosis* H37Rv and *rv3136* deletion mutant were investigated in a mouse model of TB infection. An *in vivo* experiment was carried out using female BALB/c mice (Charles River, UK). Prior to infection, mice were housed for a week in the Division of Biomedical Services, University of Leicester to acclimatise. The experiment was approved by the University of Leicester ethical committee and carried out in strict accordance with the Home Office project licence. Frozen titrated bacterial stocks prepared as previously mentioned in 2.5.3 were used for infection. On the day of infection, one vial from each strain was thawed followed by centrifugation at 12000xg for 10 minutes, then the supernatant was removed and the pellet was resuspended in PBS to give approximately $2x10^6$ CFU/ml. The mice were first anesthetised by isofluorane inhalation (2.5% over oxygen at 1.6-1.8 L/min). Then 50ul of bacterial suspension was gradually given into the nostrils of 8 to 10 week old female BALB/c mice. To monitor infection, mice were sacrificed to determine the initial pulmonary burden (as colony-forming units) 1 day after infection and at 1, 2, 4 and 6 week post infection to determine bacterial load in lungs, spleen and liver. Five mice were used for each group per time point. After removal, the lungs, spleen and liver were placed in individual pre-aliquoted 15ml falcon tube containing 5ml of PBS and 10 stainless steel beads (diameter ¼ inches; MP Bio). Homogenisation of organs was carried out at a speed of 6.0 for 30 sec; three

times in Fastprep-24 (MP Bio). The number of growing mycobacteria was quantified by serially diluting the resulting tissue homogenate in 7H9 medium and plating on supplemented 7H10 agar plates (with and without PANTA). During the duration of the experiment, infected mice were monitored daily for any signs of illness and disease scores were recorded two times a day (am and pm). The criteria used to score signs of illness in infected mice were hunched, starey (staring coat, bristling of hairs) or lethargic. Table 8 shows the scores used to assess disease severity.

Table 8: Scores used to assess disease severity

Condition	Severity[13],[14]	Given Score
Normal	N/A	0
Hunched	+	1
	++	2
Starey	+	3
	++	4
Lethargic	+	5
	++	6
Culled	N/A	7

5.3 Results

5.3.1 Generation of an unmarked *M. tuberculosis rv3136* deletion mutant

Both upstream (1036 bp) and downstream (1279 bp) flanking regions of *rv3136* were amplified and cloned into p2NIL generating the plasmid p2NIL-FR1-FR2. This plasmid was assessed by undertaking a number of diagnostic restriction digests to confirm the presence of both flanking regions. Table 9 shows the restriction enzymes used and the predicted fragment(s) size.

[13] + = Adverse effect visible
[14] ++= Adverse effect at critical stage

Table 9: Diagnostic restriction digests p2NIL-FR1-FR2

Restriction enzyme(s)	Predicted size (kb)	Confirmed
HindIII, BamHI and KpnI	1.03kb, 1.2kb and 4.43kb	yes
PacI	6.74kb	yes
EcoRI	5.04kb+ 1.7kb	yes
SalI	5.913kb+ 828bp	yes

1% (w/v) agarose gel electrophoresis of the digested DNA confirmed that both flanking regions of *rv3136* were cloned into p2NIL as shown in figure 34. This figure shows that digestion with *HindIII, BamHI and KpnI* (lane 3) yielded three bands which correspond to p2NIL and the flanking regions of *rv3136*. By comparing these bands with the relevant bands of the DNA marker it is obvious that the larger band is approximately 4.5 kb, while the other two smaller bands are approximately 1.2 and 1 kb respectively. Digestion with *PacI* (lane 4) resulted in one large band which is approximately 6.7 kb in size. The result of DNA digestion with *EcoRI* (lane 5) were one large band and one small band with the largest being approximately 5kb and the smallest approximately 1.7 kb. Lastly digestion with *SalI,* yielded one large band (approximately 6 kb in size) and one small band (approximately 800 bp). By comparing the expected band sizes in table 9 with the band sizes seen after restriction digests (Figure 41), it is obvious that all enzymes produced DNA bands of the expected sizes. Thus it can be concluded that the p2NIL-FR1-FR2 clone analysed had the correct insert.

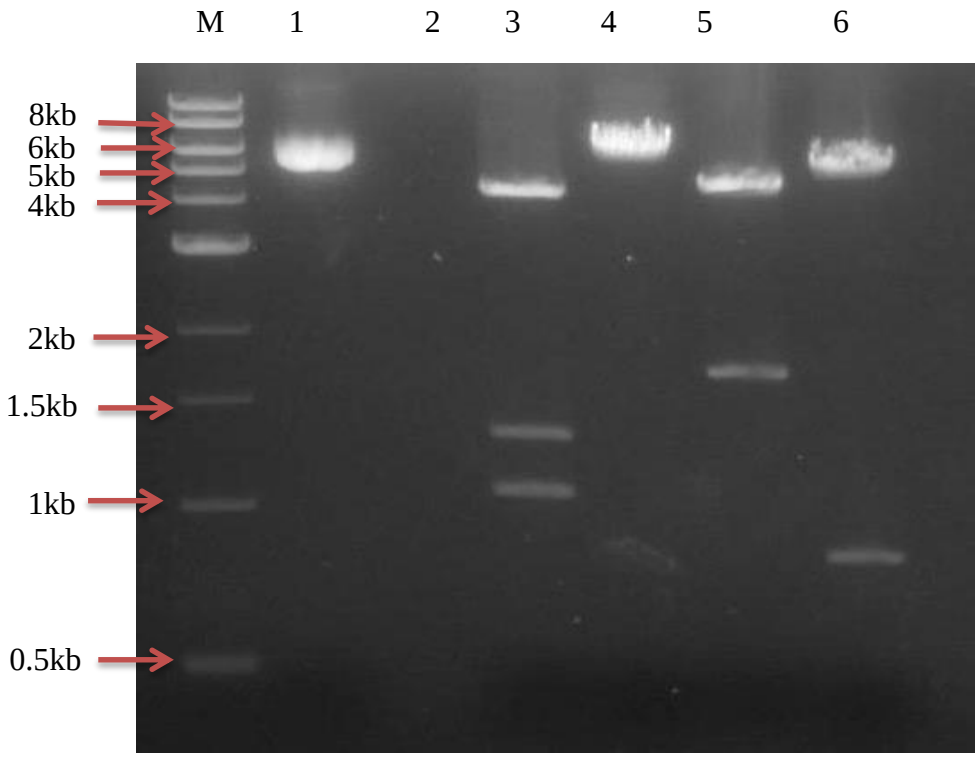


Figure 41: Confirmation of p2NIL-FR1-FR2 plasmid by diagnostic restriction digest
p2NIL-FR1-FR2 was digested using the restriction enzymes shown in table 9; the resulting fragments were analysed using 1% agarose gel to confirm the predicted fragment sizes. Lanes M-6; M: 1kb DNA ladder (NEB, UK), 1: undigested p2NIL-FR1-FR2, 3: digests with *HindIII, BamHI and KpnI,* 4: *PacI* digest, 5: *EcoRI* digest, 6: *SalI* digest.

After confirming that the *rv3136* deletion construct was correct, the *PacI* marker cassette (which carries the hygromycin resistance, *lac Z* and *sac B* genes) was inserted. The insertion was confirmed by digestion of the construct with *PacI* which resulted into the formation of two bands (7.9 and 6.7 Kb), corresponding to the *PacI* cassette and the deletion construct (data not shown). The resulting suicide delivery vector was electroporated into *M. tuberculosis* H37Rv and successful transformants represented by blue colonies were subsequently analysed for confirmation of deletion by PCR.

5.3.2 Confirmation of *M. tuberculosis rv3136* deletion mutant

In order to confirm the deletion of *rv3136*, candidate double crossover colonies were subjected to genotypic analysis. This was first done via diagnostic PCR and a successful clone was then subjected to Southern blot analysis.

Two primer pairs were used to confirm the deletion of *rv3136* from *M. tuberculosis* H37Rv. The first pair was test primers which were designed to amplify the sequences upstream and downstream of *rv3136*, therefore it is expected that wild type *M. tuberculosis* H37Rv would generate a larger PCR product than the *rv3136* deletion. The second primer pair was *rv3136* specific primers which were designed to amplify a sequence within *rv3136* and thus it is expected that the deletion mutant genome would not generate any PCR product. Table 10 illustrates the expected amplicon size in both wild type and *rv3136* deletion mutant using either test or gene specific primers.

Table 10: Expected Amplicon Size in Wild type and Δ*rv3136* Using Diagnostic Primers

Primer Pair	**Size in Wild type**	**Size in ∆*rv3136***
Test primers	1337 bp	227 bp
rv3136 specific primers	240 bp	N/A

With test primers it is expected that the wild type genome would generate a PCR product of 1337 bp in size. The same primers would generate a PCR product of 227 bp in size using *rv3136* deletion mutant genome. It is expected that gene (*rv3136*) specific primers would generate no PCR product when *rv3136* is deleted from the genome and a PCR product of 240 bp when using wild type genome.

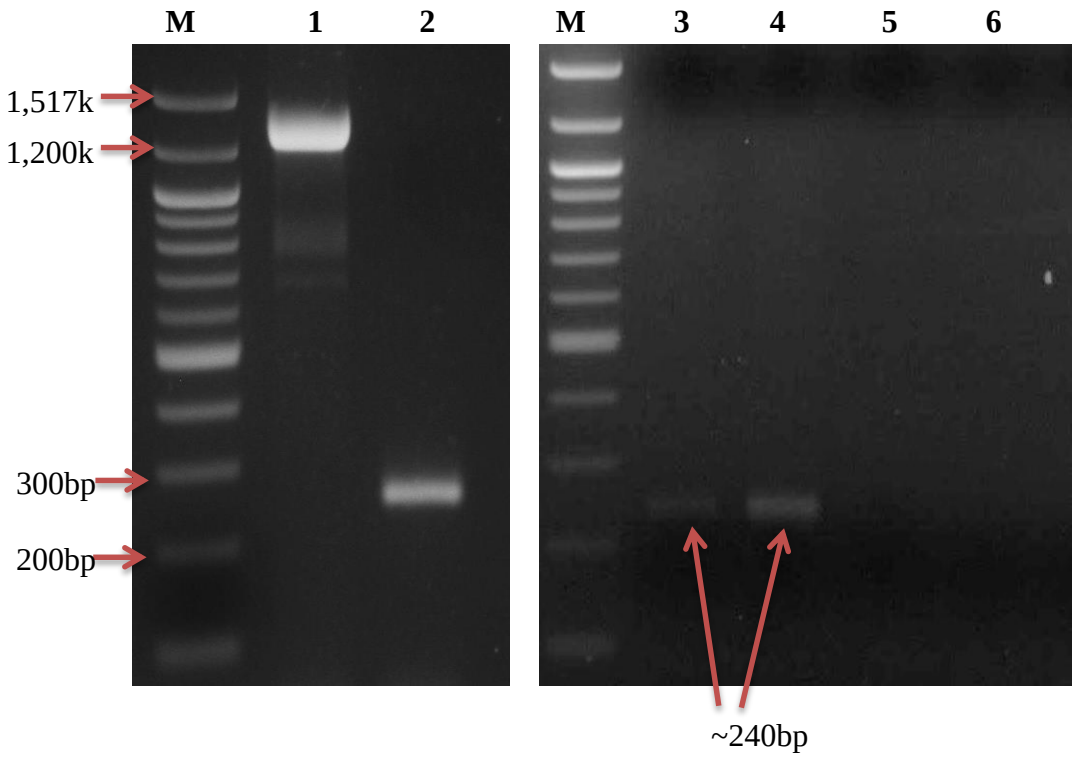


Figure 42: Confirmation of *rv3136* deletion in *M. tuberculosis* by PCR using *rv3136* test and gene specific primers.
Colony PCR was done for both wild type *M. tuberculosis* and Δ*rv3136* candidate 1 (double crossover) using test and gene specific primers. The PCR products were run on 0.8% agarose gel. Lanes M-6 (left to right) M; 100 bp NEB DNA ladder, 1; wild type with test primers, 2; Δ*rv3136* candidate 1 with test primers, 3&4; wild type with gene specific primers, 5; Δ*rv3136* candidate 1 with gene specific primers, 6; negative control (H_2O).

Figure 42 demonstrates that DNA from Δ*rv3136* candidate 1 generated a PCR product (lane 2) of approximately 250bp in size, which is the expected size using test primer. On the other hand, the same figure shows the absence of any PCR product (lane 5) when using *rv3136* gene specific primers indicating the deletion of *rv3136* from the genome of Δ*rv3136* candidate 1. The above results clearly indicate that Δ*rv3136* candidate 1 was the right clone. This clone was subsequently used in Southern hybridisation for further confirmation of the *rv3136* deletion.

Genomic DNA was extracted from wild type *M. tuberculosis* H37Rv and Δ*rv3136* candidate 1. These DNA samples were subsequently digested with either *XhoI* or *NheI*. The resulting fragments were analysed by Southern hybridisation using

the DIG High Prime DNA Labelling and Detection Starter Kit I (Roche). The sequence of both wild type *M. tuberculosis* H37Rv and the ∆*rv3136* were analysed and restriction enzymes were chosen based on the suitability of the fragment they produced. *XhoI* and *NheI* were chosen for their suitability and availability. Table 11 shows the enzymes used and the corresponding expected fragment size for each of the wild type and the mutant.

Table 11: Expected fragment size for each of wild type and ∆*rv3136* after digestion with either *XhoI* or *NheI.*

Enzyme	Fragment size	
	Wild type	∆*rv3136*
XhoI	~ 6 kb	~ 4.8 kb
NheI	~ 6.4 kb	~ > 8 kb

Sequence analysis showed that *XhoI* does not cut within *rv3136* and thus a smaller fragment is expected for the mutant. On the other hand, *NheI* cuts within ∆*rv3136* which means that the mutant should produce a fragment larger than that of the wild type when cutting with this restriction enzyme. Figure 43 shows the result of southern blot for both wild type and the candidate ∆*rv3136* clone. *XhoI* was predicted to produce a band of approximately 6 kb in the wild type and 4.8 kb in the mutant, and that is clearly seen in the blot (lane 1 and 3 respectively). Whereas, *NheI* is expected to produce a 6.4kb band in the wild type and a band which is larger than 8kb (roughly 9.5 kb) in the mutant. This is clearly observed in the blot which shows that restriction of the wild type genomic DNA with *NheI* resulted in a band (lane 4) situated between 6.1 and the 7.2 kb bands of the marker. On the other hand, a band which is larger than 8.5kb (lane 6) was the result of digestion of candidate ∆*rv3136* with *NheI.* The results of Southern hybridisation confirmed the previous results of diagnostic PCR which means that an *M. tuberculosis* H37Rv ∆*rv3136* has been successfully generated and thus can be used in future experiments aimed at characterising the role of *rv3136* in survival inside macrophages and virulence.

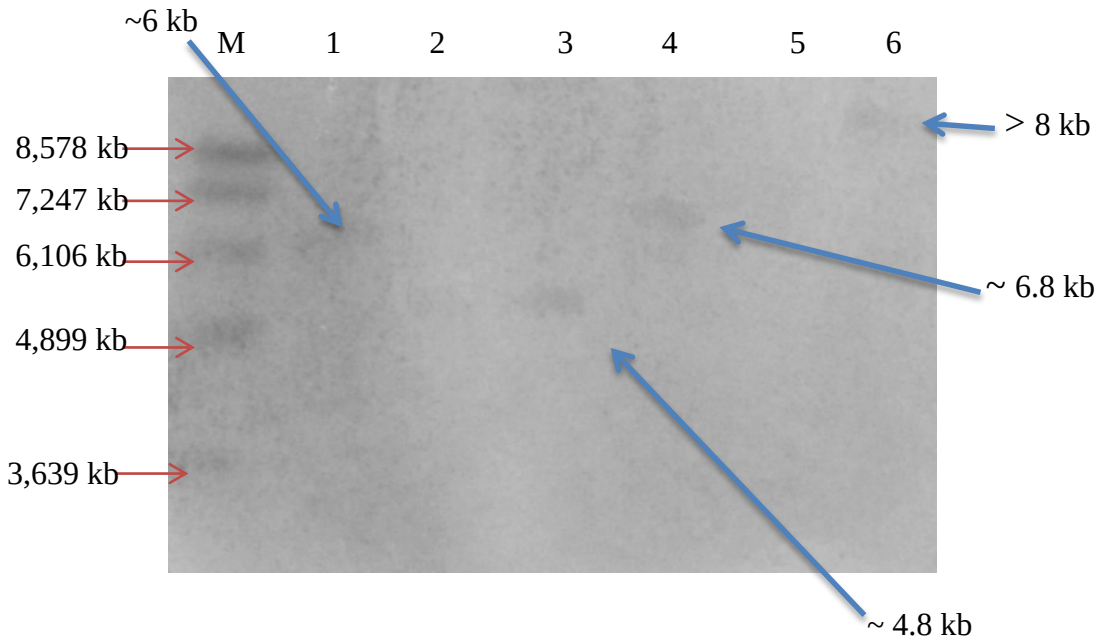


Figure 43: Southern blot of *M. tuberculosis* H37Rv (wild type and Δ*rv3136*) after digestion of genomic DNA with either *XhoI* or *NheI*.
1µg of genomic DNA from both wild type *M. tuberculosis* H37Rv and Δ*rv3136 were* digested for 8 hours with either *XhoI* or *NheI.* The resulting fragments were separated using 0.8% agarose, which was then denatured and neutralised. The DNA fragments were transferred to a positively charged nylon membrane (Hybond, GE healthcare) and subsequently probed with a DIG labelled DNA fragment. Detection was done using anti-DIG antibodies and enzyme-linked substrate. Lanes M-6 (left to right), M; DIG labelled DNA Molecular Weight Marker VII (Roche), 1; wild type digested with *XhoI,* 3; Δ*rv3136* digested with *XhoI,* 4; wild type digested with *NheI*, 6; Δ*rv3136* digested with *NheI,* arrows indicate bands of interest and predicted size.

5.3.3 Deletion of *rv3136* does not influence the growth of *M. tuberculosis* H37Rv in laboratory media

The first step in characterising the role of *rv3136* in virulence was to investigate if its deletion had any effect on growth of *M. tuberculosis* in laboratory media. As mentioned previously, BEI Resources kindly provided us with an *MT2551* (*rv3136*) transposon mutant (Lamichhane et al., 2003). The *M. tuberculosis* clinical strain CDC1551 has a homolog of Rv3136 which is MT2551 with 100% identity (TBDB). Therefore, plans were made to study the growth characteristics of the transposon mutant (*rv3136*::Tn) in laboratory media as these results might give a preliminary indication of the expected growth phenotype when *rv3136* is deleted. *In vitro* growth studies were done in both supplemented 7H9 and Sauton's medium using both 125ml flasks and 50ml falcon tubes as described in

5.2.5. Growth of *M. tuberculosis* CDC1551 in supplemented 7H9 medium represented by Figure 44 clearly indicate that *rv3136*::Tn grows slower than the wild type with a significant difference seen after 7,8 and 9 days of incubation. Similar results were obtained when growth was investigated in either 125ml flasks or 50 ml falcon tubes.

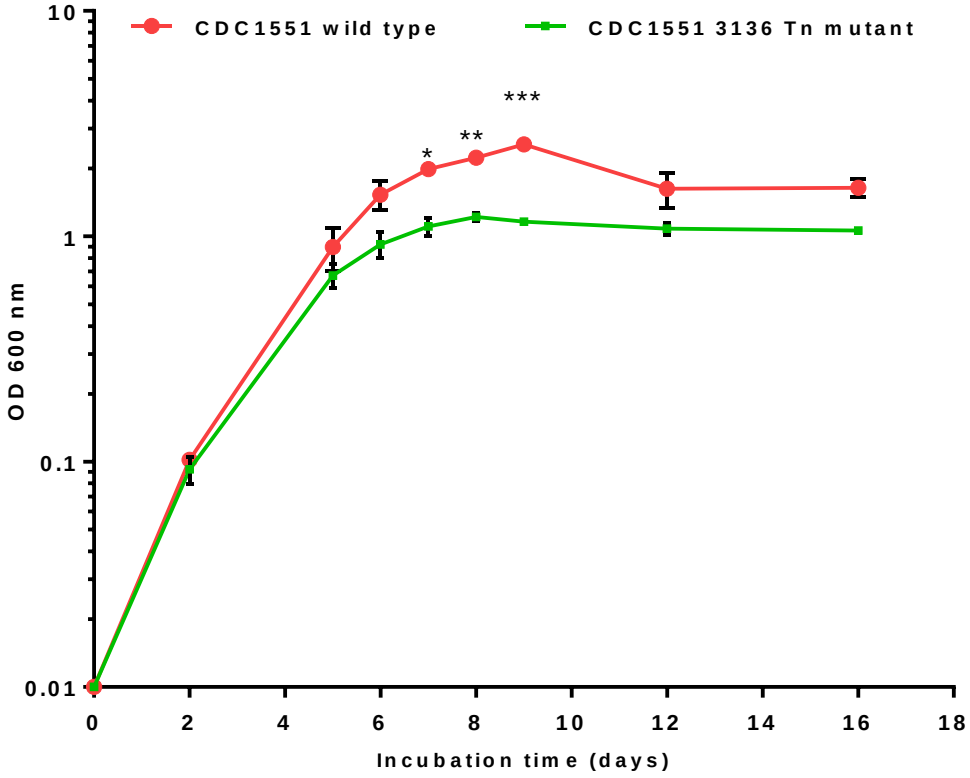


Figure 44: Growth of *M. tuberculosis* CDC1551 (wild type and *rv3136*::Tn) in supplemented 7H9 medium.
M. tuberculosis CDC1551 (wild type and *rv3136*::Tn) strains were inoculated in supplemented 7H9 medium and incubated at 37°C with shaking (100 rpm). The growth rate was followed over a period of 16 days by taking OD_{600} measurements. Data represent mean ±SEM of one out of four experiments (in duplicates) with similar results.

The growth of *M. tuberculosis* CDC1551 (wild type and *rv3136*::Tn mutant) was investigated in Sauton's medium and the results also show that the mutant grows significantly slower than the wild type as shown in Figure 45. This figure demonstrates that the transposon mutant did not grow above an OD_{600nm} of 0.5. It is worth noting that growth experiments were repeated both in the presence and absence of kanamycin to exclude any effect of antibiotic on bacterial growth. Growth studies in both supplemented 7H9 and Sauton's medium clearly indicate

that the *rv3136*::Tn suffer from a significant growth defect during exponential phase.

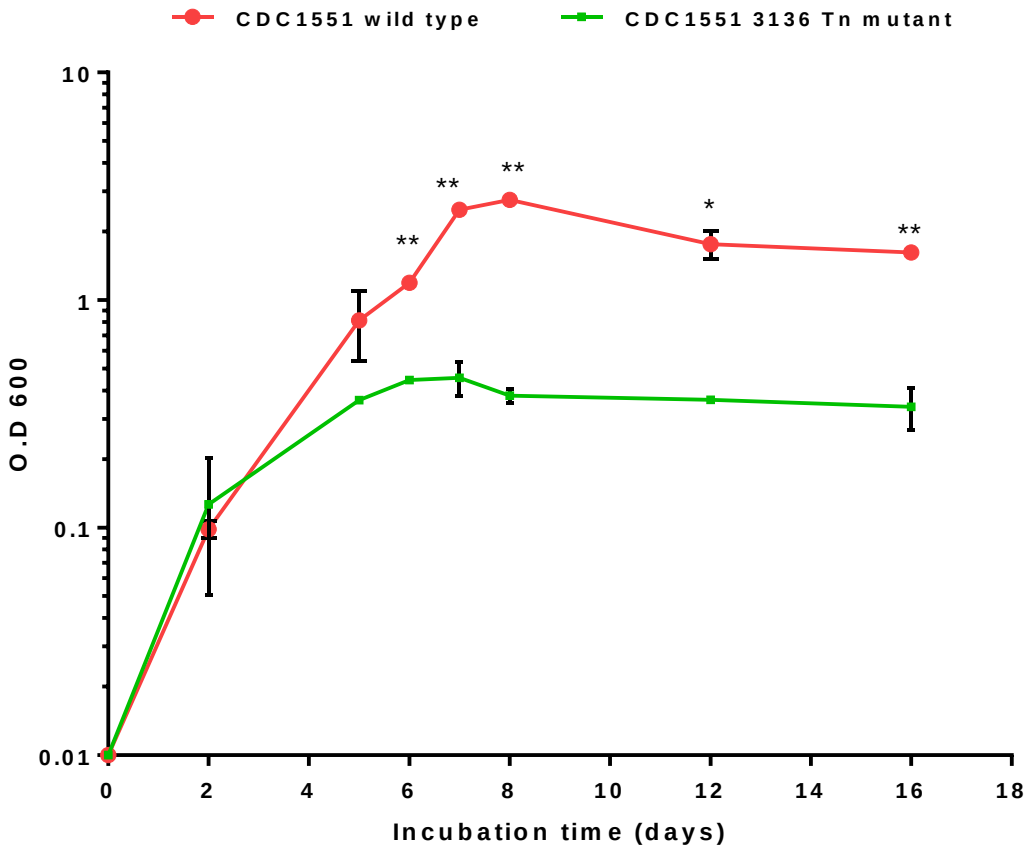


Figure 45: Growth of *M. tuberculosis* CDC1551 (wild type and 3136 *rv3136*::Tn) in Sauton's medium.
M. tuberculosis CDC1551 (wild type and *rv3136*::Tn) strains were inoculated in Sauton's medium and incubated at 37°C with shaking (100 rpm). The growth rate was followed over a period of 16 days by taking OD_{600nm} measurements. Data represent mean ±SEM of two biological replicates.

It has been reported that the insertion of a transposon within a gene does not necessarily result in gene inactivation (de Berardinis et al., 2008). To avoid any polar effect resulting from this type of mutagenesis, plans were made to generate an in frame deletion mutant in *M. tuberculosis* and to investigate if deletion of *rv3136* would result in an *in vitro* growth pattern similar to that seen with the transposon mutant (*rv3136*::Tn). Once the construction of an *M. tuberculosis* H37Rv *rv3136* deletion mutant has been completed and confirmed, the growth curves were repeated using the deletion mutant. Figure 46 shows the growth of both wild type *M. tuberculosis* H37Rv and *rv3136* deletion mutant in supplemented 7H9 medium. In contrast to the *rv3136*::Tn mutant, it is clear that

the *rv3136* deletion mutant did not suffer from any growth defect. Similar results were obtained using either 125ml flasks or 50ml falcon tubes.

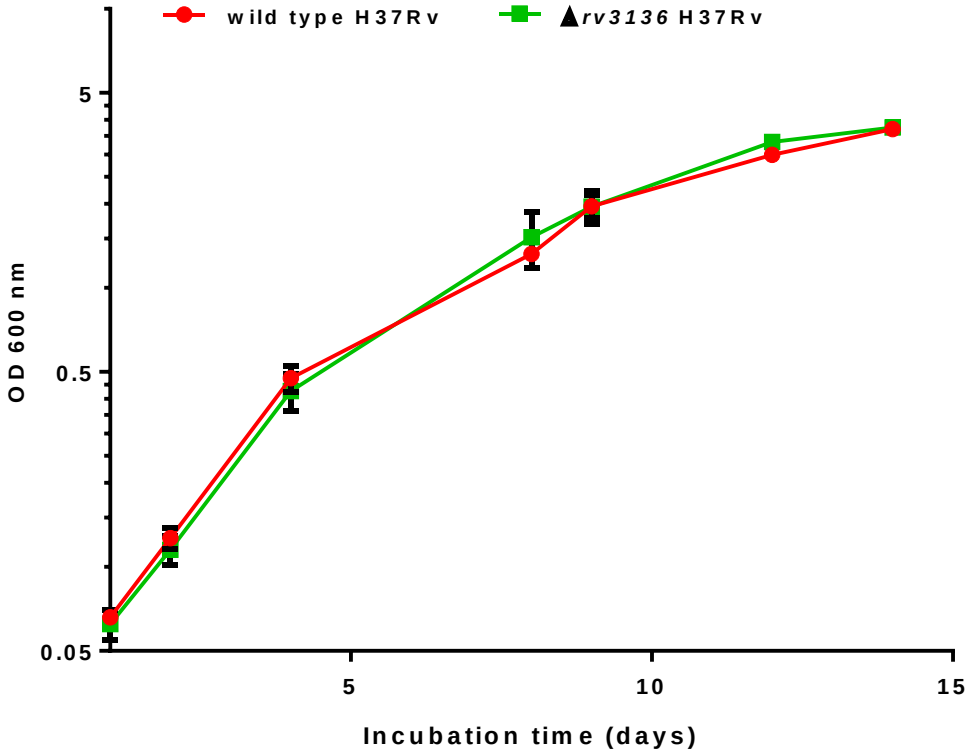


Figure 46: Growth of *M. tuberculosis* H37Rv (wild type and Δ*rv3136*) in supplemented 7H9 medium.

M. tuberculosis H37Rv (wild type and Δ*rv3136*) strains were inoculated in supplemented 7H9 medium and incubated at 37°C with shaking (100 rpm). The growth rate was followed over a period of 14 days by taking OD_{600} measurements. Data represent mean ±SEM of one out of four experiments (in triplicates) with similar results.

5.3.4 Does deletion of *rv3136* affect the intracellular survival and replication of *M. tuberculosis?*

A key feature of *M. tuberculosis* is its ability to survive and replicate within host macrophages. It is thought that evasion of the host immune response enables the survival of *M. tuberculosis* within host macrophages. Some members of both PE and PPE families are believed to play vital roles in antigenic variation, immune evasion and induction of B cell response (Brennan and Delogu, 2002, Li et al., 2005, Choudhary et al., 2003). *Rv3136* (*ppe51)* was among the genes highly up-regulated 2 hours post macrophage infection and hence it may play a role in

survival and/replication inside macrophages. The role of PPE51 (Rv3136) during macrophage infection was investigated using J774.1 murine macrophages. Four macrophage infection experiments were carried out at different MOIs. In three of these experiments, frozen bacterial stocks were used for infection, while growing bacteria was used in the fourth. All four experiments showed that there was no difference between wild type *M. tuberculosis* H37Rv and the *rv3136* deletion mutant. Figure 47 shows one of these experiments in which macrophages were infected at an MOI of 1 and the course of infection was followed over a period of 96 hours. Control wells which included macrophages only were included. The results presented in this figure demonstrate that there was no difference between wild type and mutant.

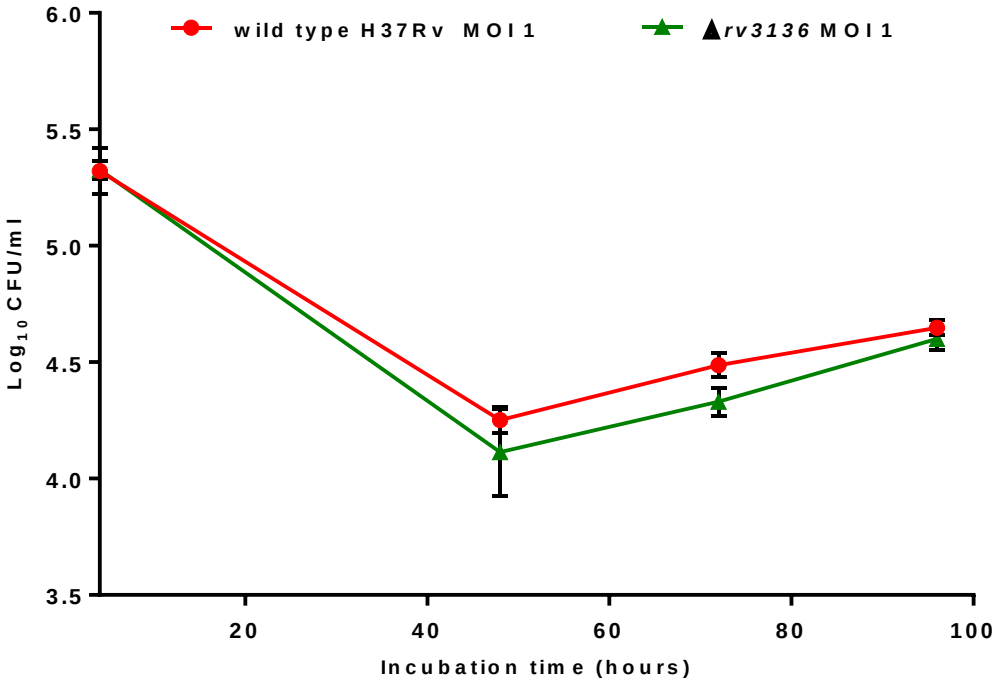


Figure 47: Infection of J774.1 macrophages with *M. tuberculosis* H37Rv (wild type and Δ*rv3136*) from frozen stocks.
J774.1 macrophages (1x10^6 cells) were infected with *M. tuberculosis* H37Rv (wild type and Δ*rv3136*) at an MOI of 1. Infection was carried out in a 24 well tissue culture plate and samples were taken after 4, 48, 72 and 96 hours of infection for CFU determination. At each time point, the cell monolayers in 3 wells were washed twice and lysed with 0.2% (v/v) of Triton X-100. The result from one independent experiment including 3 technical replicates. Data are expressed as mean ± SEM.

Another macrophage infection experiment was performed using log phase (OD_{600nm} 0.5) growing bacteria. In this experiment J774.1 macrophages were infected with both wild type *M. tuberculosis* and Δ*rv3136*. Infection was at an MOI of 0.5 and 5 and the course of infection was also followed over a period 96 hours. At each time point, the infected monolayers were inspected visually and compared with control wells for any signs of detachment. During the course of infection, no signs of detachment were observed in wells infected with the mutant at either MOI 0.5 or 5. However, it was clear that infection of macrophages at MOI of 5 with wild type *M. tuberculosis* for 96 hours resulted in detachment of monolayers. This indicates that wild type *M. tuberculosis* started to kill macrophages at this MOI. Figure 48 shows the survival of both wild type *M. tuberculosis* and Δ*rv3136* over a period of 96 hours.

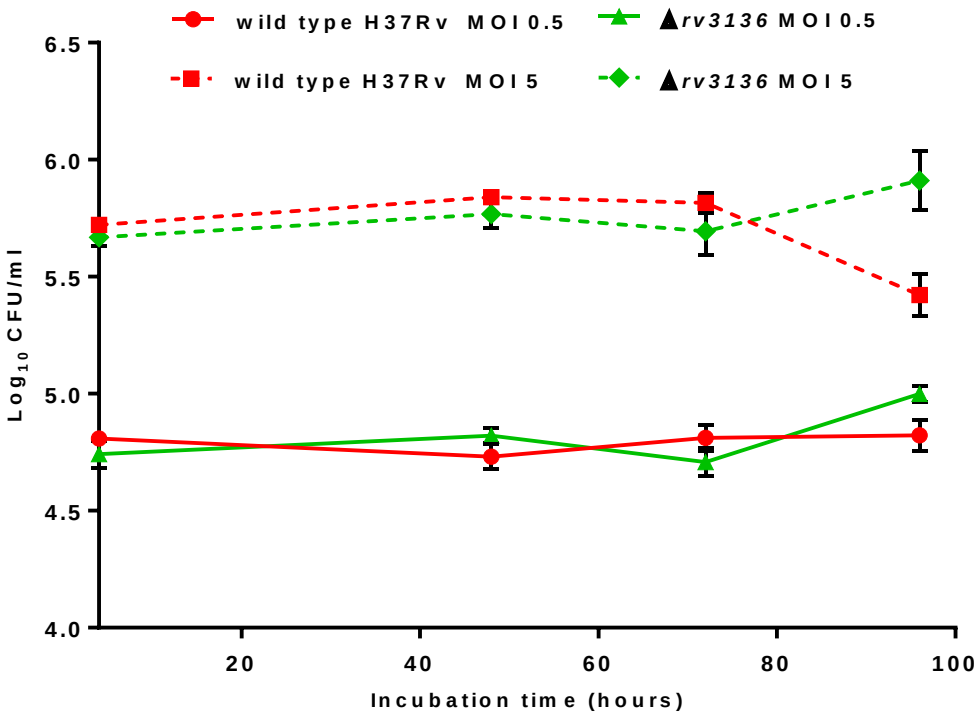


Figure 48: Infection of J774.1 macrophages with growing cultures of *M. tuberculosis* H37Rv (wild type and Δ*rv3136*)

J774.1 macrophages ($2x10^4$ cells) were infected with growing cultures of *M. tuberculosis* H37Rv (wild type and Δ*rv3136*) at MOI of 0.5 and 5. Infection was carried out in 24 well tissue culture plate and samples were taken after 4, 48, 72 and 96 hours of infection for CFU determination. At each time point, the cell monolayers in 3 wells were washed twice and lysed with 0.2% (v/v) of Triton X-100. The result is from one independent experiment. Data are expressed as mean ± SEM.

It is clear from this figure that there is a similar trend in survival at MOI of 0.5 in both wild type and mutant. However, it was noted that at MOI of 5, the wild type started to drop after 96 hours of infection. As mentioned earlier, the monolayers infected with the wild type had started to detach at this time point and hence this explains the drop in CFU counts seen at this time point as there is not enough macrophages to support the growth of the bacterium.

5.3.5 Survival of wild type *M. tuberculosis* and ∆*rv3136* in a mouse model of TB infection

Mouse models of TB are one of the most widely used animal models in mycobacterial research. It has been found that mouse models of TB are able to replicate some aspects of the human immune response and even detect very low numbers of the bacilli (Orme, 2003). However, this model is limited by the lack of granuloma formation and caseous necrotic centres similar to human infections. The relative resistance of mice to develop classic TB is another limitation to this model. Nevertheless, the data generated from mouse models has helped to broaden our knowledge of many aspects of the host immune response. As mentioned in 5.2.7, the *in vivo* growth characteristics of wild type *M. tuberculosis* H37Rv and *rv3136* deletion mutant were investigated in a mouse model of TB infection. The inoculum used for infection was plated on supplemented 7H10 agar plates and the results showed that 5.3 x10^4 CFU/mice was used for the wild type and 6.4 x10^4 CFU/mice was used for ∆*rv3136* (data not shown). After 24 hours of infection, 5 mice infected with each strain were culled, dissected and lung homogenate was plated for determining the initial pulmonary load. Lungs, spleen and liver homogenate were plated at 1, 2, 4 and 6 weeks of infection. Due to technical complications associated with the temperature (39 ºC) and humidity of the incubators, CFU counts were only recovered from lungs at 4 and 6 weeks post infection. Unfortunately, the temperature issue was only discovered after 4 weeks of infection and agar plates were moved to an incubator which showed the appropriate temperature. Figure 49 shows the average CFU/lungs after 4 and 6 weeks of infection. It is clear from this figure that the pulmonary load after 4 weeks

of infection was significantly higher in mice infected with the wild type as compared with those infected with the mutant (p=0.02). It is worth noting that at this time point colony forming units for the mutant took longer to appear on agar plates (approximately 4 weeks for the wild type and 6 weeks for the mutant). No significant difference in pulmonary burden was seen between strains after 6 weeks of infection.

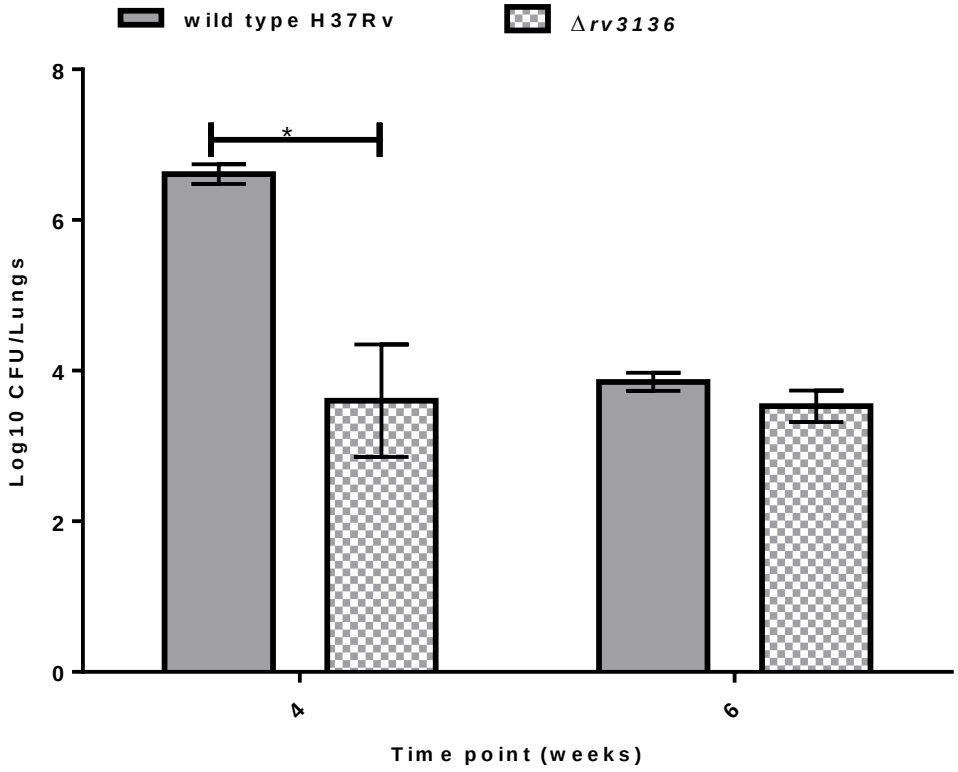


Figure 49: Pulmonary load in mice infected *M. tuberculosis* H37Rv (wild type and Δ*rv3136*) after 4 and 6 weeks.

Female BALB/c mice were inoculated intranasally with approximately $1x10^5$ CFU of *M. tuberculosis* H37Rv wild type and Δ*rv3136*. The survival and multiplication of each strain in the lungs was determined by CFU counts. Data represents the mean ± SEM of five mice per time point.

As for liver and spleen from infected mice, no colonies were recovered on plates for either strain except at 4 weeks post infection where one or two colonies from spleen homogenate were found on random agar plates for the wild type and at 6

weeks post infection where spleen homogenates from both strains showed one or two colonies.

As mentioned previously, infected mice were monitored over the time course of the infection and disease signs were recorded. Analysis of the disease signs was performed to give an alternative representation of the course of infection. Mice were assessed daily to track whether the mice showed overt signs of infection such as hunched posture, ruffled fur and lethargy, and the mice were scored according to the method described in 5.2.7. Scores were given for the most sever sign observed and average scores were calculated against time as shown in Figure 50. From this figure it is clear that both groups of mice did not show much disease signs during the first 3 weeks of infection. However, mice infected with the wild type started to show adverse disease signs from 4 weeks after infection. The appearance of disease symptoms in mice infected with the wild type at this time point coincided with the significant difference seen in pulmonary load. The disease scores for this group of mice continued to spike reaching up to 3 during the last few days of infection. On the other hand, mice infected with the mutant did not start to show adverse disease symptoms until the last week of infection with the average score reaching 1 at its highest.

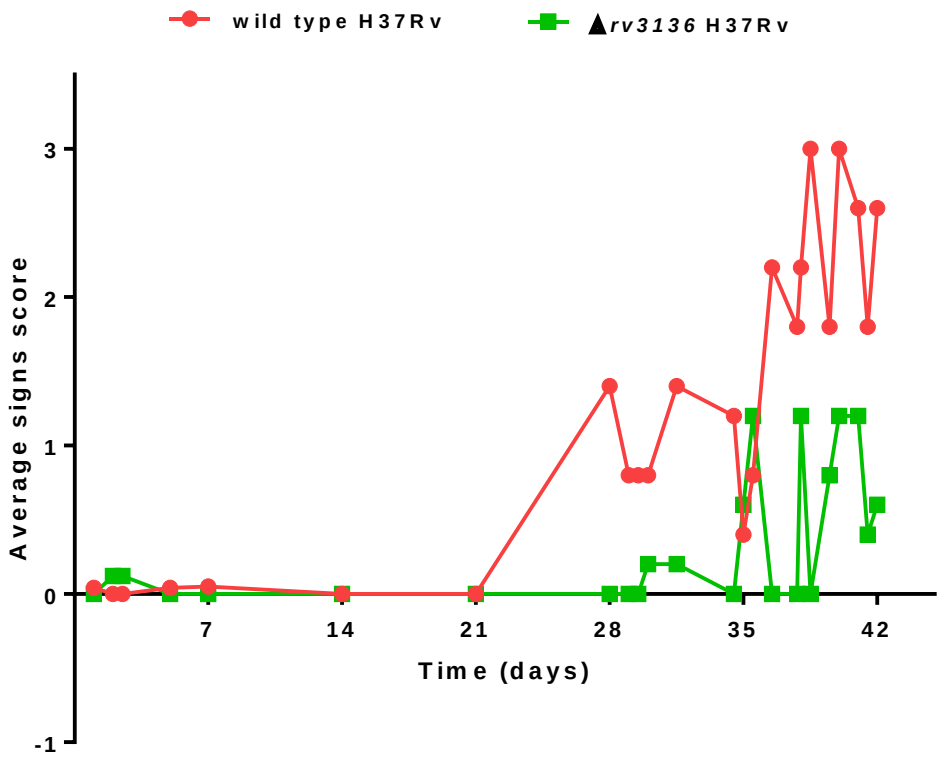


Figure 50: Average disease scores of mice infected either with wild type *M. tuberculosis* or Δ*rv3136.*
BALB/c mice infected with either wild type *M. tuberculosis* or Δ*rv3136* were monitored over a period of 6 weeks for any disease signs. Mice were scored according to the severity of disease signs. Data points represent the average symptoms score for mice at the indicated time points.

Phthiocerol dimycocerosates (PDIMs) are key virulence factors in *M. tuberculosis* and their loss has been previously reported to cause attenuation of mutants *in vivo* (Cox et al., 1999, Domenech and Reed, 2009, Hotter and Collins, 2011). Hence, it has become increasingly necessary to screen for the presence of PDIM to rule out that any *in vivo* virulence attenuation of mutants are related to loss of PDIM rather than to the gene understudy. Published data have demonstrated that *M. tuberculosis* H37Rv has the ability to spontaneously lose the ability for PDIM synthesis during *in vitro* culture (Domenech and Reed, 2009). The results of mouse infection experiment presented in this study showed that Δ*rv3136* could potentially be attenuated. However, Interpretation of any *in vivo* results showing potential virulence factors would require PDIM screening for both wild type and

mutant is to verify whether any attenuation in the mutant is due to loss of PDIM during *in vitro* culturing.

To address this issue, qualitative PDIM analysis of *M. tuberculosis* H37Rv Jacobs strain (wild type and ∆*rv3136*) was attempted by preparing apolar lipids from growing cultures of *M. tuberculosis* H37Rv (wild type and ∆*rv3136*) according to a previously described method (Besra, 1998). A control containing PDIM standards was also included as a positive control. Unfortunately, the results of PDIM analysis were inconclusive as the TLC (Thin Layer Chromatography) plate of the mutant showed a faint spot which could refer to PDIMs. On the other hand, this spot is absent in the TLC plate of the wild type (image not shown). One possible explanation is that the biomass of wild type cells was not enough to show a PDIM spot.

5.4 Discussion and Conclusions

5.4.1 Discussion

The unique presence of the PE/PPE family of proteins in mycobacteria and their predominance in pathogenic mycobacteria have provoked many researchers to study these proteins in detail. This extensive research has generated data which strongly support a role for the PE/PPE proteins in mycobacterial virulence. As mentioned earlier in this chapter published data demonstrated that PPE51 (Rv3136) has shown a distinctive expression pattern during different conditions (macrophage infection, nutrient starvation, guinea pig model of TB and NRP). These findings suggest that PPE51 deserves further study and made it tempting to explore this protein in detail due to its potential role in replication inside macrophage and adapting to the changes in environmental conditions. Hence, an attempt was made to generate an in frame deletion mutant in *M. tuberculosis* H37Rv background using the flexible cassette method (Parish and Stocker, 2000). This mutant was successfully generated and confirmed by application of two different methods. The first was colony PCR using diagnostic primers in which candidate double crossovers were analysed. Firstly with test primers designed to amplify the upstream and downstream flanking regions; and

secondly with gene specific primers. The results of these PCRs showed that the tested candidate produced the right bands with the test primers and as expected there was no band with the gene specific primers. This Δ*rv3136* candidate was subsequently confirmed by Southern hybridisation in which two enzymes were used. The sizes of the resulting bands were in accordance with calculated sizes confirming the deletion of *rv3136*. The next step was analysis of the mutant phenotype which provides a powerful tool in elucidating the function of a gene. Prior to the generation of the deletion mutant, BIE resources kindly provided this study with a *M. tuberculosis* transposon mutant created by disruption of a PPE family protein (MT3221, Rv3136) of the wild-type strain CDC1551. The transposon integrated at the beginning of the gene (POI 132) and probably resulted in inactivation of the gene. The *in vitro* growth characteristics of this transposon mutant was studied and the results showed a growth defect in laboratory media (7H9 and Sauton's media). The growth defect observed in Sauton's media was more pronounced as the *rv3136*::Tn only reached an OD_{600nm} of 0.5 in this medium. This obvious difference in the growth profile of the transposon mutant on Sauton's medium could probably be attributed to the difference in carbon and nitrogen sources available in these two media. The Middlebrook 7H9 broth is an optimal medium for growing mycobacteria which provides all the essential substances. In comparison, Sauton medium is a less nutrient rich synthetic medium.

The in frame deletion mutant of *rv3136* made in the H37Rv background showed a growth pattern similar to the wild type in supplemented 7H9 medium indicating that the mutant growth was not impaired. Construction of in frame deletion mutants minimises any possible polar effect and thus results obtained with this type of mutants are more reliable as it is well known that the insertion of a transposon within a gene does not necessarily lead to gene inactivation. Furthermore, this method of mutagenesis can result in a polar effect when the insertion is in essential genes distal to the site of disruption (McAdam et al., 2002). Therefore one possible reason for the growth defect seen in the transposon mutant is that one of these genes could have been affected. Another possible reason for the difference in the *in vitro* growth phenotype may be related to the different backgrounds in which these two mutants were generated in. This prediction could have been investigated by generating a deletion mutant in the

clinical strain *M. tuberculosis* CDC1551 using the same construct that was used to generate the deletion mutant in the H37Rv background. However due to the slow growing nature of *M. tuberculosis* and the limited time available this assumption could not be further explored. A recent study conducted a comparison of transposon and deletion mutants in *M. tuberculosis.* The results revealed that the *rv1248c* transposon mutant's growth profile differed from that of a deletion mutant in the same strain background. No obvious reason for this phenotypic discrepancies was found but it was predicted that epigenetic changes may have led to different growth phenotypes (Maksymiuk et al., 2015).

Selective expression of specific genes in host macrophages is thought to facilitate mycobacterial replication and persistence *in vivo*. Ramakrishnan and co-workers published data showing that *M. marinum* selectively expressed a number of genes once inside the macrophages. Among those genes were two homologs of *M. tuberculosis* PE/PE-PGRS genes (*Rv1651c* and *Rv3812*). Inactivation of these genes resulted in decreased mycobacterial replication in macrophages (Ramakrishnan et al., 2000). The gene encoding PPE51 (*Rv3136*) has been previously reported to be up regulated 2 hours post macrophage infection and therefore it was hypothesised that the deletion mutant of *rv3136* might show some degree of impairment within macrophages. To determine whether *rv3136* played a role in intracellular survival/replication, the multiplication capacity of both wild type and Δ*rv3136* were compared in the murine macrophage cell line J774.1. Analysis of the CFU counts obtained from those experiments showed no difference in survival. It was observed that infecting macrophages with wild type *M. tuberculosis* H37Rv at MOI of 5 for 96 hours resulted in cell detachment and hence this explains the drop in CFU seen at this time point. The transcriptional response of *M. tuberculosis* to the macrophage phagosome was investigated in the clinical strain CDC1551 (Rohde et al., 2007b). Published data have revealed that different *M. tuberculosis* strains use diverse strategies to survive in the hostile intracellular environment (Cappelli et al., 2001). Therefore it is not known whether the up-regulation of *rv3136* inside macrophages could be related to the background strain. This could have been investigated by conducting macrophage infection experiments using an *rv3136* deletion mutant created in the CDC1551

background. The strains and experimental conditions used in the current study did not reveal any evidence that the deletion of *rv3136* (*ppe51*) influenced mycobacterial survival/ replication inside macrophages. However, the literature is full of examples of certain PE/PPE family members whom have been implicated in establishing a successful macrophage infection. Of those examples are PPE24 which promotes survival inside macrophages and PPE53 which was required for association and survival inside macrophages (Mehta et al., 2006). *PE15* which is six-fold upregulated in *M. tuberculosis* when compared with *M. bovis,* was found to enhance survival inside infected macrophages (Tiwari et al., 2012). It has been suggested that some members of the PE/PPE family are involved in the adaptation to the host microenvironment and immune evasion, and thus may contribute in the process by which the bacilli persist in the host tissue (Sampson, 2011). Therefore it might be possible that PPE51 (Rv3136) is involved in the persistence of the bacilli inside the host.

To determine whether the deletion of *rv3136* affected multiplication within the host, the replication and persistence of Δ*rv3136* and wild type H37Rv were assessed in mice. Analysis of the disease signs in mice over the time course of infection showed that mice infected with the wild type had more adverse disease signs. Furthermore, the highest scores for disease severity was recorded mice infected with wild type at 6 weeks post infection. The technical complications experienced during this experiment resulted in CFU counts being only recovered from lungs of infected mice at 4 and 6 weeks post infection. The pulmonary load obtained after 4 weeks of infection was higher in mice infected with the wild type. This result indicate that the mutant might be attenuated in multiplication within the host especially that mice infected with the wild type started to show more sever disease symptoms at this time point. However, data obtained 6 weeks post infection shows that there was a decrease in the pulmonary load of mice infected with wild type *M. tuberculosis* H37Rv. This was unexpected as previous reports on the *in vivo* survival profile of *M. tuberculosis* H37Rv in BALB/c mice revealed that it multiplies in the lungs during the first 60 days of infection and stabilise thereafter (Rickman et al., 2005, Gutka et al., 2015). The technical complications related to the temperature and humidity of the incubators experienced during this experiment is probably the reason behind the decrease in bacterial load at 6

weeks post infection. Several studies using mouse models of TB infection have used histopathological techniques to assess the pathologic consequences of infection (Cooper, 2015). Inclusion of this parameter in the current study would have provided valuable information regarding the immunopathology and the level of tissue changes in each group of infected mice.

Previously published data found that deletion of *rv3676* which is predicted to regulate *rv3136* resulted in a growth defect in a mouse model of TB. In addition it was noted that the Δ*rv3676* strain grew very slowly on solid 7H11 agar taking approximately 5 weeks for Δ*rv3676* compared to 2–3 weeks for the wild type strain (Rickman et al., 2005). Interestingly, the results of the animal experiment showed that Δ*rv3136* took longer to grow on solid 7H10 agar plates. All these observations indicate that Δ*rv3136* might be attenuated *in vivo*. This can only be confirmed by constructing a complementing strain and repeating the *in vivo* infection experiment. Prior to constructing the complementation strain is it important to confirm the DNA binding site of the regulator as this site might possibly be of great importance to complement the gene. Published data have suggested that Rv3676 and Rv0081 are putative regulators of *rv3136* (Rickman et al., 2005, Galagan et al., 2013). The first protein is thought to play a key role in regulating the persistence and reactivation of *M. tuberculosis*, while the second is predicted to contribute in mediating the initial response to hypoxia. This suggests that *rv3136* could possibly play an important role during latent infection. Day and co-workers have shown that *M. tuberculosis* cells which lack PDIM have reduced total bacterial numbers in lungs of infected mice when compared to their wild type counterparts (Day et al., 2014). Therefore it was necessary that both wild type and Δ*rv3136* would be tested for the presence of this lipid as the loss of the virulence-associated surface lipid PDIM has been reported to occur spontaneously during in vitro manipulations of H37Rv strains (Domenech and Reed, 2009). Although the results of PDIM analysis were inconclusive but it did not show the presence of PDIM in the wild type and absence in the deletion mutant in which the attenuation of the mutant would have been attributed to the loss of PDIM.

5.4.2 Conclusions

An in frame deletion mutant of *rv3136* was generated in *M. tuberculosis* H37Rv to investigate whether this gene which encodes a member of the PPE family had any implications in virulence. Deletion of the gene was confirmed by using both PCR and Southern hybridisation. Unlike, the transposon mutant in the CDC1551 strain, the *rv3136* deletion mutant in H37Rv showed a growth pattern similar to that of the wild type indicating that it does not suffer from any growth defect in laboratory media. The role of *rv3136* in intracellular survival and multiplication was studied in J774.1 macrophages to investigate whether up-regulation of this gene during the first two hours of infection played any role in survival inside macrophages. Macrophage infection data showed that *rv3136* did not play a role in intracellular replication or survival. A mouse model of TB was used to investigate if *rv3136* played a role in survival inside the host. The results obtained after 4 weeks of infection showed that wild type mice had significantly higher pulmonary burden when compared to the mutant. In addition, mice infected with the wild type showed more severe adverse disease signs during the last two weeks of infection when compared to mice infected with the mutant.

Chapter 6 : **Final Conclusions and Future work**

6.1 Final Conclusions and Future work

Despite many years of global efforts to combat the disease, TB remains a worldwide health problem affecting millions of people. The ability to survive within host macrophages is thought to be the cornerstone facilitating the persistence of *M. tuberculosis* in humans. Macrophages play a central role in protecting the host from pathogens by clearing the invading pathogens and recruiting other immune cells to the site of infection. However, in the case of *M. tuberculosis*, macrophages are also the primary host cells for mycobacteria. To control *M. tuberculosis* infection, it is necessary to investigate possible mycobacterial virulence factors which may facilitate survival inside macrophages. These virulence factors could potentially be targeted in an attempt to develop more effective anti-TB drugs. This thesis investigated the role of two mycobacterial proteins (encoded by *rv1219c* and *rv3136*) in mycobacterial stress response, virulence and survival within macrophages. The results presented in this thesis have indeed demonstrated the importance of these two proteins in survival inside the host.

The first protein RaaS (Rv1219c/BCG_1279c) is a transcriptional regulator which controls the expression of two genes that encode subunits of predicted ATP dependant efflux pumps (Rv1218c/1217c in *M. tuberculosis*). The results presented here showed that the *bcg_1279c* (*raaS*) deletion mutant was attenuated in both murine and human macrophages; and survived less during the first 24 hours of infection. Previous published data have revealed that RaaS has an important role in improvement of mycobacterial survival in non-permissive growth conditions. RaaS has been proposed to mediate shutdown of efflux in conditions when ATP pool becomes depleted. It was further hypothesised that key metabolites, generated during growth, may regulate binding of RaaS and other transcription regulators to DNA and therefore serve as sensors of metabolic activity and physiological state of mycobacteria (Turapov et al., 2014a). This study was focused on the importance of RaaS-mediated efflux shutdown in stressful conditions that mimic the hostile *in vivo* environment. The findings described in this thesis have revealed that the *raaS* deletion mutant is impaired in survival during nutrient depletion, H_2O_2 treatment and acidic stress. Published data have shown that *raaS* acts as a repressor of the cluster of genes located immediately downstream from itself which includes genes encoding the

Rv1218c/Rv1217c (Bcg_1278c/Bcg_1277c) pump (Turapov et al., 2014a). Although the exact role of the Rv1218c/Rv1217c (Bcg_1278c/Bcg_1277c) pump and the precise nature of the molecule(s) transported by this pump have not been discovered yet, but dysregulation of the pump accompanied by excessive release of potentially toxic lipids is the most probable reason behind the defect seen in the mutant. Over expression of the pump may also lead to increased use of ATP and ultimately to exhaustion of the mutant which could be one of the causes lying behind the defect of the mutant under stressful conditions. The results described here indicate that presumably both factors contributed to the survival defect of the mutant. Preliminary results indicated that the mutant may have less ATP at late stages of growth. Moreover, the survival defect observed in the mutant under oxidative stress was significantly ameliorated by the addition of reserpine, a known inhibitor of ATP-dependent efflux pumps. Furthermore, the results presented in this thesis also gave an indication that SN prepared from the mutant could potentially be toxic to both mycobacteria and eukaryotic cells (represented by wild type *M. bovis* BCG and J774.1 cell respectively). These data collectively suggest that fine regulation of efflux pumps is an essential component of mycobacterial adaptation to changing environment. This was further supported by the observation that only the construct containing a full-length *raaS* and it regulatory elements (the promoter and binding site) was able to complement the mutant phenotype.

This means that dysregulated pump may become a lethal weapon against non-growing and stressed bacteria. Efflux pump inhibitors have recently become candidate tools for treatment of infectious diseases (Lomovskaya and Bostian, 2006), since they potentially may reduce acquired resistance to drugs (da Silva et al., 2011). However, the findings presented in this thesis clearly show that efflux pumps inhibitors may actually improve the survival of mycobacteria exposed to stressful conditions. However, further study of the precise role of this efflux pump and elucidating the nature of the substrate of this pump are needed to establish a complete understanding of the feasibility and significant potential for developing such approach.

RaaS has been shown to be an important element of the complex regulatory mechanisms which coordinated down regulation of energy-consuming processes

during growth non permissive conditions. This suggests that targeting this protein could potentially be used to kill mycobacteria.

As previously discussed, future work on RaaS will include identifying the nature of the substrate of the RaaS regulated efflux pump (Rv1218c/1217c in *M. tuberculosis*) which is an important element that could provide further guidance on the precise role of this efflux pump and how *M. tuberculosis* employs the RaaS-mediated mechanism for survival during various stressful conditions. Studying the complete lipid profile of SN extracted from both wild type and Δ*raaS* mutant might help in identifying the substrate of the Rv1218c/1217c pump as it has been previously predicted that this pump might transport lipid molecule(s). This would involve studying the structures, functions, interactions, and dynamics of cellular lipids. However, this is not a straightforward process as the conditions during which SN are collected should be carefully selected. In addition, it would be useful to try and narrow the search to include a certain group of lipids rather than conducting a general lipidomics study which probably might not provide us with a conclusive result. Another important point to consider is whether lipidomics investigation should be expanded to include cell lysates from both wild type and Δ*raaS* mutant. Identifying the nature of the substrate for the Rv1218c/1217c pump would be highly informative in expanding our understanding of the mechanism by which this efflux pump influence survival of mycobacteria during infection, persistence and non-growth permissive conditions. The results presented in this thesis highlight the importance of tightly regulated expression of efflux pumps in response to stressful conditions. This can be confirmed by measuring the level of expression of *raaS* in the three complemented strains. Interestingly, systems analysis of the *M. tuberculosis* regulatory and metabolic networks, predicted that RaaS could possibly bind to the coding sequence of DosT (Galagan et al., 2013). This prediction suggests that RaaS regulates DosT. One way of investigating this assumption is studying the protein-DNA interaction by Electromobility shift assay (EMSA). If the interaction is confirmed, this suggests a much wider role of RaaS in *M*. *tuberculosis* virulence than has been previously been established.

The second protein under study was a member of the PE and PPE family of proteins which is PPE51 (encoded by *rv3136*). Owing to their unique presence

and different expression pattern in various micro-environmental conditions, as well as their association with the ESAT-6 gene cluster, the role of the PE and PPE protein family members in virulence has been of major interest in the last couple of years. The gene encoding PPE51 has shown a unique expression pattern during different environmental conditions suggesting that it may be linked to virulence. Investigating this assumption requires studying the survival of *M. tuberculosis* in the absence of *rv3136* (during *in vitro* growth, macrophage and mouse infection). TB mouse infection model showed that it played a role in initial replication of *M. tuberculosis* inside lungs providing an indication that *rv3136* may play a role in virulence. Wild type mice showed higher pulmonary load and more adverse disease symptoms after 4 weeks of infection.

The *in vitro* growth characteristics were first investigated in *M. tuberculosis* CDC1551 background using a *MT2551* mutant (*rv3136*::Tn), an *rv3136* orthologue in *M. tuberculosis* H37Rv. This transposon mutant was constructed using random *Himar1* transposon insertion mutagenesis (Lamichhane et al., 2003). The results obtained from these investigations showed that the transposon mutant is attenuated during growth in both 7H9 and Sauton's medium.

Given that insertion of a transposon within a gene does not necessarily result in gene inactivation (de Berardinis et al., 2008) and to avoid any polar effect resulting from this type of mutagenesis, it was important to investigate the *in vitro* growth characteristics of *M. tuberculosis* when *rv3136* has been confirmed to be deleted from the genome. Hence, an in frame deletion mutant of *rv3136* was created in *M. tuberculosis* H37Rv. This deletion mutant showed a similar growth pattern to that of the wild type when grown in supplemented 7H9 medium. The up- regulation of *rv3136* during the first 2 hours of macrophage infection gave an indication that *rv3136* could be a potential player in virulence and survival of *M. tuberculosis* inside macrophages. However, macrophage infection studies showed no difference in survival between the wild type and the mutant indicating that *rv3136* does not seem to play a precise role in survival inside macrophages. Understanding how *M. tuberculosis* responds to its microenvironment will provide important information about what is required for survival and establishing a successful infection. However, the results obtained from mouse infection indicated that *rv3136* influenced survival inside the host. To confirm this result it would be necessary to construct a complemented strain and assess if

reintroducing *rv3136* into the deletion mutant would complement the phenotype of the wild type. Any attempt to construct a complementation strain should be preceded by confirming the binding site whereby Rv3676 is predicted to bind to the upstream region of *rv3136*. This can be done by performing EMSA using annealed forward and reverse primers containing the predicted Rv3676 binding site. Confirmation of the binding site might be of great importance as complementation may not happen unless this binding site is included in the construct used to complement Δ*rv3136.*

The unique expression pattern exhibited by *rv3136* during different environmental conditions suggests that it could be one of the virulence factors mediating the establishment of a successful infection inside the host. Thus testing the survival of Δ*rv3136* during different conditions such as H_2O_2 exposure and nutrient starvation might help in enhancing our understanding of the possible role of Rv3136 in mechanisms of virulence, pathogenesis and latency of TB.

The alarming rise in TB cases and in particular drug resistant mycobacteria has stressed the urgent need to identify new drug targets. Hence, characterisation of mycobacterial components important in the establishment of infection is of vital importance. In this perspective, the finding that *rv3136* is important for initial replication in mice lungs suggest that this protein could potentially be considered as a drug target.

Appendix

7H9 Middlebrook medium:

7H9 powder	4.7g
Glycerol	2.5g

Made up to 900 ml in ddH2O, sterilised by autoclaving and 0.05% (w/v) Tween 80 was added to 7H9 media prior to use.

7H10 Middlebrook agar:

7H10 powder	19g
Glycerol	6.25g

Dissolved in 900ml of ddH_2O, this was then boiled in a microwave prior to sterilisation by autoclave.

Sauton's medium:

KH_2PO_4	0.5g
$MgSO_4.7H_2O$	0.5g
L-asparagine	4g
Ferric ammonium citrate	0.05g
Citric acid	2g
Glycerol	10ml
1% (w/v) $ZnSO_4$ solution	0.1ml

Made up to 800ml ddH_2O and pH was adjusted to 7.2-7.4 using NaOH and the volume made up to 1L prior to sterilisation by autoclaving. Medium was supplemented by 0.05% (w/v) Tween 80 and in some cases by 10% (v/v) ADC.

LB Broth

Tryptone	10 g
Yeast extract	6 g
NaCl	10 g

Made up to 1 litre with dH2O and then sterilised by autoclaving.

LA agar

Tryptone	10 g
Yeast extract	6 g
NaCl	10 g
Agar	15 g

Dissolved in 1 litre of dH_2O, then boiled in a microwave and sterilised by autoclaving.

ADC

Bovine serum albumin	50 g/L
D-glucose	20 g/L
NaCl	8.5 g/L

Dissolved in 1 litre of dH_2O and sterilised by autoclaving.

TAE buffer 50X

Tris-HCl	2 M
Acetic Acid	2 M
EDTA	50 mM

Breaking buffer

Tris-HCl pH= 8.0	50mM
EDTA	10mM
NaCl	100mM

RNAse (DNAse- free) is added to a final concentration of 200µg/ml immediately before use.

TE buffer

Tris-HCl pH= 8.0	50mM
EDTA	1mM

Sterilization was done by autoclaving

20x SSC

NaCl	175.3g
Sodium citrate	88.2g

Dissolved in 800ml of water, then the pH was adjusted to 7.0 with HCl and the volume was adjusted to 1L.

REFERENCES

MCDONOUGH ABOMOELAK, B., MARCUS, S. A., WARD, S. K., KARAKOUSIS, P. C., STEINBERG, H. & TALAAT, A. M. 2011. Characterization of a novel heat shock protein (Hsp22.5) involved in the pathogenesis of *Mycobacterium tuberculosis*. *J Bacteriol,* 193**,** 3497-505.

ADEREM, A. & UNDERHILL, D. M. 1999. Mechanisms of phagocytosis in macrophages. *Annu Rev Immunol,* 17**,** 593-623.

AKHTER, Y., EHEBAUER, M. T., MUKHOPADHYAY, S. & HASNAIN, S. E. 2012. The PE/PPE multigene family codes for virulence factors and is a possible source of mycobacterial antigenic variation: perhaps more? *Biochimie,* 94**,** 110-6.

ALDERTON, H. S., D. 2001. Safety in the laboratory. *In:* PARISH, T. S., NG., (ed.) *Methods in molecular medicine: Mycobacterium tuberculosis protocols.* Totowa, NJ, USA: Humana press.

ALONSO, A., MORALES, G., ESCALANTE, R., CAMPANARIO, E., SASTRE, L. & MARTINEZ, J. L. 2004. Overexpression of the multidrug efflux pump SmeDEF impairs Stenotrophomonas maltophilia physiology. *J Antimicrob Chemother,* 53**,** 432-4.

ALTAF, M., MILLER, C. H., BELLOWS, D. S. & O'TOOLE, R. 2010. Evaluation of the *Mycobacterium smegmatis* and BCG models for the discovery of *Mycobacterium tuberculosis* inhibitors. *Tuberculosis (Edinb),* 90**,** 333-7.

ARMSTRONG, J. A. & HART, P. D. 1971. Response of cultured macrophages to *Mycobacterium tuberculosis*, with observations on fusion of lysosomes with phagosomes. *J Exp Med,* 134**,** 713-40.

ASTARIE-DEQUEKER, C., LE GUYADER, L., MALAGA, W., SEAPHANH, F. K., CHALUT, C., LOPEZ, A. & GUILHOT, C. 2009. Phthiocerol dimycocerosates of *M. tuberculosis* participate in macrophage invasion by inducing changes in the organization of plasma membrane lipids. *PLoS Pathog,* 5**,** e1000289.

BACKUS, K. M., DOLAN, M. A., BARRY, C. S., JOE, M., MCPHIE, P., BOSHOFF, H. I., LOWARY, T. L., DAVIS, B. G. & BARRY, C. E., 3RD 2014. The three Mycobacterium tuberculosis antigen 85 isoforms have unique substrates and activities determined by non-active site regions. *J Biol Chem,* 289**,** 25041-53.

BAEK, S. H., LI, A. H. & SASSETTI, C. M. 2011. Metabolic regulation of mycobacterial growth and antibiotic sensitivity. *PLoS Biol,* 9**,** e1001065.

BALASUBRAMANIAN, V., PAVELKA, M. S., JR., BARDAROV, S. S., MARTIN, J., WEISBROD, T. R., MCADAM, R. A., BLOOM, B. R. & JACOBS, W. R., JR. 1996. Allelic exchange in *Mycobacterium tuberculosis* with long linear recombination substrates. *J Bacteriol,* 178**,** 273-9.

BALASUBRAMANIAN, V., WIEGESHAUS, E. H., TAYLOR, B. T. & SMITH, D. W. 1994. Pathogenesis of tuberculosis: pathway to apical localization. *Tuber Lung Dis,* 75**,** 168-78.

BALGANESH, M., KURUPPATH, S., MARCEL, N., SHARMA, S., NAIR, A. & SHARMA, U. 2010. Rv1218c, an ABC transporter of *Mycobacterium tuberculosis* with implications in drug discovery. *Antimicrob Agents Chemother,* 54**,** 5167-72.

BANGE, F. C., COLLINS, F. M. & JACOBS, W. R., JR. 1999. Survival of mice infected with *Mycobacterium smegmatis* containing large DNA fragments from *Mycobacterium tuberculosis*. *Tuber Lung Dis,* 79**,** 171-80.

BARKER, L. P., GEORGE, K. M., FALKOW, S. & SMALL, P. L. 1997. Differential trafficking of live and dead *Mycobacterium marinum* organisms in macrophages. *Infect Immun,* 65**,** 1497-504.

BARRY, C. E., 3RD, BOSHOFF, H. I., DARTOIS, V., DICK, T., EHRT, S., FLYNN, J., SCHNAPPINGER, D., WILKINSON, R. J. & YOUNG, D. 2009. The spectrum of latent tuberculosis: rethinking the biology and intervention strategies. *Nat Rev Microbiol,* 7**,** 845-55.

BE, N. A., BISHAI, W. R. & JAIN, S. K. 2012. Role of Mycobacterium tuberculosis pknD in the pathogenesis of central nervous system tuberculosis. *BMC Microbiol,* 12**,** 7.

BEDARD, K. & KRAUSE, K. H. 2007. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiol Rev,* 87**,** 245-313.

BEHAR, S. M., MARTIN, C. J., BOOTY, M. G., NISHIMURA, T., ZHAO, X., GAN, H. X., DIVANGAHI, M. & REMOLD, H. G. 2011. Apoptosis is an innate defense function of macrophages against *Mycobacterium tuberculosis*. *Mucosal Immunol,* 4**,** 279-87.

BELISLE, J. T. & SONNENBERG, M. G. 1998. Isolation of genomic DNA from mycobacteria. *Methods Mol Biol,* 101**,** 31-44.

BERTI, E., GALLI, L., VENTURINI, E., DE MARTINI, M. & CHIAPPINI, E. 2014. Tuberculosis in childhood: a systematic review of national and international guidelines. *BMC Infect Dis,* 14 Suppl 1**,** S3.

BESRA, G. S. 1998. Preparation of cell-wall fractions from mycobacteria. *Methods Mol Biol,* 101**,** 91-107.

BETTS, J. C., LUKEY, P. T., ROBB, L. C., MCADAM, R. A. & DUNCAN, K. 2002. Evaluation of a nutrient starvation model of *Mycobacterium tuberculosis* persistence by gene and protein expression profiling. *Mol Microbiol,* 43**,** 717-31.

BIANCO, M. V., BLANCO, F. C., FORRELLAD, M. A., AGUILAR, D., CAMPOS, E., KLEPP, L. I., HERNANDEZ-PANDO, R., CATALDI, A. A. & BIGI, F. 2011. Knockout mutation of p27-p55 operon severely reduces replication of Mycobacterium bovis in a macrophagic cell line and survival in a mouse model of infection. *Virulence,* 2**,** 233-7.

BOEHME, C. C., NABETA, P., HILLEMANN, D., NICOL, M. P., SHENAI, S., KRAPP, F., ALLEN, J., TAHIRLI, R., BLAKEMORE, R., RUSTOMJEE, R., MILOVIC, A., JONES, M., O'BRIEN, S. M., PERSING, D. H., RUESCH-GERDES, S., GOTUZZO, E., RODRIGUES, C., ALLAND, D. & PERKINS, M. D. 2010. Rapid molecular detection of tuberculosis and rifampin resistance. *N Engl J Med,* 363**,** 1005-15.

BOOM, W. H., CANADAY, D. H., FULTON, S. A., GEHRING, A. J., ROJAS, R. E. & TORRES, M. 2003. Human immunity to *M. tuberculosis*: T cell subsets and antigen processing. *Tuberculosis (Edinb),* 83**,** 98-106.

BOTELLA, H., PEYRON, P., LEVILLAIN, F., POINCLOUX, R., POQUET, Y., BRANDLI, I., WANG, C., TAILLEUX, L., TILLEUL, S., CHARRIERE, G. M., WADDELL, S. J., FOTI, M., LUGO-VILLARINO, G., GAO, Q., MARIDONNEAU-PARINI, I., BUTCHER, P. D., CASTAGNOLI, P. R., GICQUEL, B., DE CHASTELLIER, C. & NEYROLLES, O. 2011. Mycobacterial p(1)-type ATPases mediate resistance to zinc poisoning in human macrophages. *Cell Host Microbe,* 10**,** 248-59.

BOTTAI, D., MAJLESSI, L., SIMEONE, R., FRIGUI, W., LAURENT, C., LENORMAND, P., CHEN, J., ROSENKRANDS, I., HUERRE, M., LECLERC, C., COLE, S. T. & BROSCH, R. 2011. ESAT-6 secretion-independent impact of ESX-1 genes espF and espG1 on virulence of *Mycobacterium tuberculosis*. *J Infect Dis,* 203**,** 1155-64.

BOURAI, N., JACOBS, W. R., JR. & NARAYANAN, S. 2012. Deletion and overexpression studies on DacB2, a putative low molecular mass penicillin binding protein from Mycobacterium tuberculosis H(37)Rv. *Microb Pathog,* 52**,** 109-16.

BRAIBANT, M., GILOT, P. & CONTENT, J. 2000. The ATP binding cassette (ABC) transport systems of *Mycobacterium tuberculosis*. *FEMS Microbiol Rev,* 24**,** 449-67.

BRAUN, V., HANTKE, K. & KOSTER, W. 1998. Bacterial iron transport: mechanisms, genetics, and regulation. *Met Ions Biol Syst,* 35**,** 67-145.

BRENNAN, M. J. & DELOGU, G. 2002. The PE multigene family: a 'molecular mantra' for mycobacteria. *Trends Microbiol,* 10**,** 246-9.

BRENNAN, P. J. 2003. Structure, function, and biogenesis of the cell wall of *Mycobacterium tuberculosis*. *Tuberculosis (Edinb),* 83**,** 91-7.

BRIGHTBILL, H. D., LIBRATY, D. H., KRUTZIK, S. R., YANG, R. B., BELISLE, J. T., BLEHARSKI, J. R., MAITLAND, M., NORGARD, M. V., PLEVY, S. E., SMALE, S. T., BRENNAN, P. J., BLOOM, B. R., GODOWSKI, P. J. & MODLIN, R. L. 1999. Host defense mechanisms triggered by microbial lipoproteins through toll-like receptors. *Science,* 285**,** 732-6.

BRODIN, P., POQUET, Y., LEVILLAIN, F., PEGUILLET, I., LARROUY-MAUMUS, G., GILLERON, M., EWANN, F., CHRISTOPHE, T., FENISTEIN, D., JANG, J., JANG, M. S., PARK, S. J., RAUZIER, J., CARRALOT, J. P., SHRIMPTON, R., GENOVESIO, A., GONZALO-ASENSIO, J. A., PUZO, G., MARTIN, C., BROSCH, R., STEWART, G. R., GICQUEL, B. & NEYROLLES, O. 2010. High content phenotypic cell-based visual screen identifies *Mycobacterium tuberculosis* acyltrehalose-containing glycolipids involved in phagosome remodeling. *PLoS Pathog,* 6**,** e1001100.

BROWN, A. C. & PARISH, T. 2008. Dxr is essential in Mycobacterium tuberculosis and fosmidomycin resistance is due to a lack of uptake. *BMC Microbiol,* 8**,** 78.

CAMACHO, L. R., CONSTANT, P., RAYNAUD, C., LANEELLE, M. A., TRICCAS, J. A., GICQUEL, B., DAFFE, M. & GUILHOT, C. 2001. Analysis of the phthiocerol dimycocerosate locus of Mycobacterium tuberculosis. Evidence that this lipid is involved in the cell wall permeability barrier. *J Biol Chem,* 276**,** 19845-54.

CAPPELLI, G., VOLPE, P., SANDUZZI, A., SACCHI, A., COLIZZI, V. & MARIANI, F. 2001. Human macrophage gamma interferon decreases gene expression but not replication of Mycobacterium tuberculosis: analysis of the host-pathogen reciprocal influence on transcription in a comparison of strains H37Rv and CMT97. *Infect Immun,* 69**,** 7262-70.

CHINDELEVITCH, L., MENZIES, N. A., PRETORIUS, C., STOVER, J., SALOMON, J. A. & COHEN, T. 2015. Evaluating the potential impact of enhancing HIV treatment and tuberculosis control programmes on the burden of tuberculosis. *J R Soc Interface,* 12.

CHOBY, B. A. & HUNTER, P. 2015. Respiratory infections: pulmonary tuberculosis. *FP Essent,* 429**,** 22-9.

CHOI, H. S., RAI, P. R., CHU, H. W., COOL, C. & CHAN, E. D. 2002. Analysis of nitric oxide synthase and nitrotyrosine expression in human pulmonary tuberculosis. *Am J Respir Crit Care Med,* 166**,** 178-86.

CHOUDHARY, R. K., MUKHOPADHYAY, S., CHAKHAIYAR, P., SHARMA, N., MURTHY, K. J., KATOCH, V. M. & HASNAIN, S. E. 2003. PPE antigen Rv2430c of *Mycobacterium tuberculosis* induces a strong B-cell response. *Infect Immun,* 71**,** 6338-43.

CLARK, D. P., AND J. E. CRONAN, JR 1996. Two-carbon compounds and fatty acids as carbon sources. *In:* F. C. NEIDHARDT, R. C. I., J. L. INGRAHAM, E. C. C. LIN, K. B. LOW, B. MAGASANIK, W. S. REZNIKOFF, M. RILEY, M. SCHAECHTER, AND H. E. UMBARGER (ed.) *Escherichia coli and Salmonella: cellular and molecular biology.* 2nd ed. Washington, D.C. : ASM Press,.

CLAY, H., VOLKMAN, H. E. & RAMAKRISHNAN, L. 2008. Tumor necrosis factor signaling mediates resistance to mycobacteria by inhibiting bacterial growth and macrophage death. *Immunity,* 29**,** 283-94.

CLEMENS, D. L., LEE, B. Y. & HORWITZ, M. A. 2002. The *Mycobacterium tuberculosis* phagosome in human macrophages is isolated from the host cell cytoplasm. *Infect Immun,* 70**,** 5800-7.

CLIFF, J. M., KAUFMANN, S. H., MCSHANE, H., VAN HELDEN, P. & O'GARRA, A. 2015. The human immune response to tuberculosis and its treatment: a view from the blood. *Immunol Rev,* 264**,** 88-102.

COLANGELI, R., HAQ, A., ARCUS, V. L., SUMMERS, E., MAGLIOZZO, R. S., MCBRIDE, A., MITRA, A. K., RADJAINIA, M., KHAJO, A., JACOBS, W. R., JR., SALGAME, P. & ALLAND, D. 2009. The multifunctional histone-like protein Lsr2 protects mycobacteria against reactive oxygen intermediates. *Proc Natl Acad Sci U S A,* 106**,** 4414-8.

COLDITZ, G. A., BREWER, T. F., BERKEY, C. S., WILSON, M. E., BURDICK, E., FINEBERG, H. V. & MOSTELLER, F. 1994. Efficacy of BCG vaccine in the prevention of tuberculosis. Meta-analysis of the published literature. *JAMA,* 271**,** 698-702.

COLE, S. T., BROSCH, R., PARKHILL, J., GARNIER, T., CHURCHER, C., HARRIS, D., GORDON, S. V., EIGLMEIER, K., GAS, S., BARRY, C. E., 3RD, TEKAIA, F., BADCOCK, K., BASHAM, D., BROWN, D., CHILLINGWORTH, T., CONNOR, R., DAVIES, R., DEVLIN, K., FELTWELL, T.,

GENTLES, S., HAMLIN, N., HOLROYD, S., HORNSBY, T., JAGELS, K., KROGH, A., MCLEAN, J., MOULE, S., MURPHY, L., OLIVER, K., OSBORNE, J., QUAIL, M. A., RAJANDREAM, M. A., ROGERS, J., RUTTER, S., SEEGER, K., SKELTON, J., SQUARES, R., SQUARES, S., SULSTON, J. E., TAYLOR, K., WHITEHEAD, S. & BARRELL, B. G. 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature,* 393**,** 537-44.

COOPER, A. M. 2015. Mouse model of tuberculosis. *Cold Spring Harb Perspect Med,* 5**,** a018556.

COOPER, A. M., ADAMS, L. B., DALTON, D. K., APPELBERG, R. & EHLERS, S. 2002. IFN-gamma and NO in mycobacterial disease: new jobs for old hands. *Trends Microbiol,* 10**,** 221-6.

COWLEY, S., KO, M., PICK, N., CHOW, R., DOWNING, K. J., GORDHAN, B. G., BETTS, J. C., MIZRAHI, V., SMITH, D. A., STOKES, R. W. & AV-GAY, Y. 2004. The *Mycobacterium tuberculosis* protein serine/threonine kinase PknG is linked to cellular glutamate/glutamine levels and is important for growth in vivo. *Mol Microbiol,* 52**,** 1691-702.

COX, J. S., CHEN, B., MCNEIL, M. & JACOBS, W. R., JR. 1999. Complex lipid determines tissue-specific replication of *Mycobacterium tuberculosis* in mice. *Nature,* 402**,** 79-83.

DA SILVA, P. E., VON GROLL, A., MARTIN, A. & PALOMINO, J. C. 2011. Efflux as a mechanism for drug resistance in *Mycobacterium tuberculosis*. *FEMS Immunol Med Microbiol,* 63**,** 1-9.

DANIEL, J., MAAMAR, H., DEB, C., SIRAKOVA, T. D. & KOLATTUKUDY, P. E. 2011. *Mycobacterium tuberculosis* uses host triacylglycerol to accumulate lipid droplets and acquires a dormancy-like phenotype in lipid-loaded macrophages. *PLoS Pathog,* 7**,** e1002093.

DARWIN, K. H., EHRT, S., GUTIERREZ-RAMOS, J. C., WEICH, N. & NATHAN, C. F. 2003. The proteasome of *Mycobacterium tuberculosis* is required for resistance to nitric oxide. *Science,* 302**,** 1963-6.

DAS, S., BANERJEE, S., MAJUMDER, S., CHOWDHURY, B. P., GOSWAMI, A., HALDER, K., CHAKRABORTY, U., PAL, N. K. & MAJUMDAR, S. 2014. Immune subversion by *Mycobacterium tuberculosis* through CCR5 mediated signaling: involvement of IL-10. *PLoS One,* 9**,** e92477.

DAVIS, J. M., CLAY, H., LEWIS, J. L., GHORI, N., HERBOMEL, P. & RAMAKRISHNAN, L. 2002. Real-time visualization of mycobacterium-macrophage interactions leading to initiation of granuloma formation in zebrafish embryos. *Immunity,* 17**,** 693-702.

DAVIS, J. M. & RAMAKRISHNAN, L. 2009. The role of the granuloma in expansion and dissemination of early tuberculous infection. *Cell,* 136**,** 37-49.

DAY, T. A., MITTLER, J. E., NIXON, M. R., THOMPSON, C., MINER, M. D., HICKEY, M. J., LIAO, R. P., PANG, J. M., SHAYAKHMETOV, D. M. & SHERMAN, D. R. 2014. Mycobacterium tuberculosis strains lacking surface lipid phthiocerol dimycocerosate are susceptible to killing by an early innate host response. *Infect Immun,* 82**,** 5214-22.

DE BERARDINIS, V., VALLENET, D., CASTELLI, V., BESNARD, M., PINET, A., CRUAUD, C., SAMAIR, S., LECHAPLAIS, C., GYAPAY, G., RICHEZ, C., DUROT, M., KREIMEYER, A., LE FEVRE, F., SCHACHTER, V., PEZO, V., DORING, V., SCARPELLI, C., MEDIGUE, C., COHEN, G. N., MARLIERE, P., SALANOUBAT, M. & WEISSENBACH, J. 2008. A complete collection of single-gene deletion mutants of *Acinetobacter baylyi* ADP1. *Mol Syst Biol,* 4**,** 174.

DE VOSS, J. J., RUTTER, K., SCHROEDER, B. G., SU, H., ZHU, Y. & BARRY, C. E., 3RD 2000. The salicylate-derived mycobactin siderophores of *Mycobacterium tuberculosis* are essential for growth in macrophages. *Proc Natl Acad Sci U S A,* 97**,** 1252-7.

DEN BOON, S., VAN LILL, S. W., BORGDORFF, M. W., ENARSON, D. A., VERVER, S., BATEMAN, E. D., IRUSEN, E., LOMBARD, C. J., WHITE, N. W., DE VILLIERS, C. & BEYERS, N. 2007. High prevalence of tuberculosis in previously treated patients, Cape Town, South Africa. *Emerg Infect Dis,* 13**,** 1189-94.

DERETIC, V. & FRATTI, R. A. 1999. *Mycobacterium tuberculosis* phagosome. *Mol Microbiol,* 31**,** 1603-9.

DOMENECH, P. & REED, M. B. 2009. Rapid and spontaneous loss of phthiocerol dimycocerosate (PDIM) from *Mycobacterium tuberculosis* grown in vitro: implications for virulence studies. *Microbiology,* 155**,** 3532-43.

DOWDS, B. C., MURPHY, P., MCCONNELL, D. J. & DEVINE, K. M. 1987. Relationship among oxidative stress, growth cycle, and sporulation in Bacillus subtilis. *J Bacteriol,* 169**,** 5771-5.

EDWARDS, K. M., CYNAMON, M. H., VOLADRI, R. K., HAGER, C. C., DESTEFANO, M. S., THAM, K. T., LAKEY, D. L., BOCHAN, M. R. & KERNODLE, D. S. 2001. Iron-cofactored superoxide dismutase inhibits host responses to *Mycobacterium tuberculosis*. *Am J Respir Crit Care Med,* 164**,** 2213-9.

EHLERS, S. & SCHAIBLE, U. E. 2012. The granuloma in tuberculosis: dynamics of a host-pathogen collusion. *Front Immunol,* 3**,** 411.

EHRT, S. & SCHNAPPINGER, D. 2009. Mycobacterial survival strategies in the phagosome: defence against host stresses. *Cell Microbiol,* 11**,** 1170-8.

FERRARI, G., LANGEN, H., NAITO, M. & PIETERS, J. 1999. A coat protein on phagosomes involved in the intracellular survival of mycobacteria. *Cell,* 97**,** 435-47.

FESTA, R. A., JONES, M. B., BUTLER-WU, S., SINSIMER, D., GERADS, R., BISHAI, W. R., PETERSON, S. N. & DARWIN, K. H. 2011. A novel copper-responsive regulon in *Mycobacterium tuberculosis*. *Mol Microbiol,* 79**,** 133-48.

FINLAY, B. B. & FALKOW, S. 1997. Common themes in microbial pathogenicity revisited. *Microbiol Mol Biol Rev,* 61**,** 136-69.

FISHBEIN, S., VAN WYK, N., WARREN, R. M. & SAMPSON, S. L. 2015. Phylogeny to function: PE/PPE protein evolution and impact on *Mycobacterium tuberculosis* pathogenicity. *Mol Microbiol*.

FLORES, J. & ESPITIA, C. 2003. Differential expression of PE and PE_PGRS genes in *Mycobacterium tuberculosis* strains. *Gene,* 318**,** 75-81.

FLORIO, W., BATONI, G., ESIN, S., BOTTAI, D., MAISETTA, G., FAVILLI, F., BRANCATISANO, F. L. & CAMPA, M. 2006. Influence of culture medium on the resistance and response of Mycobacterium bovis BCG to reactive nitrogen intermediates. *Microbes Infect,* 8**,** 434-41.

FLYNN, J. L. & CHAN, J. 2001. Immunology of tuberculosis. *Annu Rev Immunol,* 19**,** 93-129.

FLYNN, J. L., CHAN, J. & LIN, P. L. 2011. Macrophages and control of granulomatous inflammation in tuberculosis. *Mucosal Immunol,* 4**,** 271-8.

FONTAN, P.A., WALTERS, S. & SMITH, I. 2004. Cellular signalling pathways and transcriptional regulation in *Mycobacterium,tuberculosis*: Stress control and virulence. *Current Science,* Vol. 86(1):**,** 122-134.

FORRELLAD, M. A., MCNEIL, M., SANTANGELO MDE, L., BLANCO, F. C., GARCIA, E., KLEPP, L. I., HUFF, J., NIEDERWEIS, M., JACKSON, M. & BIGI, F. 2014. Role of the Mce1 transporter in the lipid homeostasis of Mycobacterium tuberculosis. *Tuberculosis (Edinb),* 94**,** 170-7.

FROTHINGHAM, R., HILLS, H. G. & WILSON, K. H. 1994. Extensive DNA sequence conservation throughout the *Mycobacterium tuberculosis* complex. *J Clin Microbiol,* 32**,** 1639-43.

GALAGAN, J. E., MINCH, K., PETERSON, M., LYUBETSKAYA, A., AZIZI, E., SWEET, L., GOMES, A., RUSTAD, T., DOLGANOV, G., GLOTOVA, I., ABEEL, T., MAHWINNEY, C., KENNEDY, A. D., ALLARD, R., BRABANT, W., KRUEGER, A., JAINI, S., HONDA, B., YU, W. H., HICKEY, M. J., ZUCKER, J., GARAY, C., WEINER, B., SISK, P., STOLTE, C., WINKLER, J. K., VAN DE PEER, Y., IAZZETTI, P., CAMACHO, D., DREYFUSS, J., LIU, Y., DORHOI, A., MOLLENKOPF, H. J., DROGARIS, P., LAMONTAGNE, J., ZHOU, Y., PIQUENOT, J., PARK, S. T., RAMAN, S., KAUFMANN, S. H., MOHNEY, R. P., CHELSKY, D., MOODY, D. B., SHERMAN, D. R. & SCHOOLNIK, G. K. 2013. The *Mycobacterium tuberculosis* regulatory network and hypoxia. *Nature,* 499**,** 178-83.

GANDHI, N. R., MOLL, A., STURM, A. W., PAWINSKI, R., GOVENDER, T., LALLOO, U., ZELLER, K., ANDREWS, J. & FRIEDLAND, G. 2006. Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. *Lancet,* 368**,** 1575-80.

GARAY, C. D., DREYFUSS, J. M. & GALAGAN, J. E. 2015. Metabolic modeling predicts metabolite changes in Mycobacterium tuberculosis. *BMC Syst Biol,* 9**,** 57.

GATFIELD, J. & PIETERS, J. 2000. Essential role for cholesterol in entry of mycobacteria into macrophages. *Science,* 288**,** 1647-50.

GAZDIK, M. A., BAI, G., WU, Y. & MCDONOUGH, K. A. 2009. Rv1675c (cmr) regulates intramacrophage and cyclic AMP-induced gene expression in *Mycobacterium tuberculosis*-complex mycobacteria. *Mol Microbiol,* 71**,** 434-48.

GEHRING, A. J., DOBOS, K. M., BELISLE, J. T., HARDING, C. V. & BOOM, W. H. 2004. *Mycobacterium tuberculosis* LprG (Rv1411c): a novel TLR-2 ligand that inhibits human macrophage class II MHC antigen processing. *J Immunol,* 173**,** 2660-8.

GEY VAN PITTIUS, N. C., SAMPSON, S. L., LEE, H., KIM, Y., VAN HELDEN, P. D. & WARREN, R. M. 2006. Evolution and expansion of the *Mycobacterium tuberculosis* PE and PPE multigene families and their association with the duplication of the ESAT-6 (esx) gene cluster regions. *BMC Evol Biol,* 6**,** 95.

GHOSH, S., PAL, S., DAS, S., DASGUPTA, S. K. & MAJUMDAR, S. 1998. Lipoarabinomannan induced cytotoxic effects in human mononuclear cells. *FEMS Immunol Med Microbiol,* 21**,** 181-8.

GIACOMINI, E., IONA, E., FERRONI, L., MIETTINEN, M., FATTORINI, L., OREFICI, G., JULKUNEN, I. & COCCIA, E. M. 2001. Infection of human macrophages and dendritic cells with *Mycobacterium tuberculosis* induces a differential cytokine gene expression that modulates T cell response. *J Immunol,* 166**,** 7033-41.

GOLBY, P., HATCH, K. A., BACON, J., COONEY, R., RILEY, P., ALLNUTT, J., HINDS, J., NUNEZ, J., MARSH, P. D., HEWINSON, R. G. & GORDON, S. V. 2007. Comparative transcriptomics reveals key gene expression differences between the human and bovine pathogens of the *Mycobacterium tuberculosis* complex. *Microbiology,* 153**,** 3323-36.

GORDON, S. V., EIGLMEIER, K., GARNIER, T., BROSCH, R., PARKHILL, J., BARRELL, B., COLE, S. T. & HEWINSON, R. G. 2001. Genomics of *Mycobacterium bovis*. *Tuberculosis (Edinb),* 81**,** 157-63.

GRANGE, J. M. 2009. The genus Mycobacterium and the *Mycobacterium tuberculosis* complex. *In:* S SCHAAF, A. Z. (ed.) *Tuberculosis: A Comprehensive Clinical Reference.* Philadelphia, PA: Saunders,.

GUO, X. V., MONTELEONE, M., KLOTZSCHE, M., KAMIONKA, A., HILLEN, W., BRAUNSTEIN, M., EHRT, S. & SCHNAPPINGER, D. 2007. Silencing *Mycobacterium smegmatis* by using tetracycline repressors. *J Bacteriol,* 189**,** 4614-23.

GUPTA, A., KAUL, A., TSOLAKI, A. G., KISHORE, U. & BHAKTA, S. 2012. *Mycobacterium tuberculosis*: immune evasion, latency and reactivation. *Immunobiology,* 217**,** 363-74.

GUTKA, H. J., WANG, Y., FRANZBLAU, S. G. & MOVAHEDZADEH, F. 2015. glpx Gene in Mycobacterium tuberculosis Is Required for In Vitro Gluconeogenic Growth and In Vivo Survival. *PLoS One,* 10**,** e0138436.

HARRIS, J., HOPE, J. C. & KEANE, J. 2008. Tumor necrosis factor blockers influence macrophage responses to *Mycobacterium tuberculosis*. *J Infect Dis,* 198**,** 1842-50.

HASAN, Z., SCHLAX, C., KUHN, L., LEFKOVITS, I., YOUNG, D., THOLE, J. & PIETERS, J. 1997. Isolation and characterization of the mycobacterial phagosome: segregation from the endosomal/lysosomal pathway. *Mol Microbiol,* 24**,** 545-53.

HAYASHI, D., TAKII, T., MUKAI, T., MAKINO, M., YASUDA, E., HORITA, Y., YAMAMOTO, R., FUJIWARA, A., KANAI, K., KONDO, M., KAWARAZAKI, A., YANO, I., YAMAMOTO, S. & ONOZAKI, K. 2010. Biochemical characteristics among *Mycobacterium bovis* BCG substrains. *FEMS Microbiol Lett,* 306**,** 103-9.

HERSHKOVITZ, I., DONOGHUE, H. D., MINNIKIN, D. E., BESRA, G. S., LEE, O. Y., GERNAEY, A. M., GALILI, E., ESHED, V., GREENBLATT, C. L., LEMMA, E., BAR-GAL, G. K. & SPIGELMAN, M. 2008. Detection and molecular characterization of 9,000-year-old *Mycobacterium tuberculosis* from a Neolithic settlement in the Eastern Mediterranean. *PLoS One,* 3**,** e3426.

HISERT, K. B., KIRKSEY, M. A., GOMEZ, J. E., SOUSA, A. O., COX, J. S., JACOBS, W. R., JR., NATHAN, C. F. & MCKINNEY, J. D. 2004. Identification of *Mycobacterium tuberculosis* counterimmune (cim) mutants in immunodeficient mice by differential screening. *Infect Immun,* 72**,** 5315-21.

HOHEISEL, G., ZHENG, L., TESCHLER, H., STRIZ, I. & COSTABEL, U. 1995. Increased soluble CD14 levels in BAL fluid in pulmonary tuberculosis. *Chest,* 108**,** 1614-6.

HOLTKE, H. J., ANKENBAUER, W., MUHLEGGER, K., REIN, R., SAGNER, G., SEIBL, R. & WALTER, T. 1995. The digoxigenin (DIG) system for non-radioactive labelling and detection of nucleic acids--an overview. *Cell Mol Biol (Noisy-le-grand),* 41**,** 883-905.

HOLTKE, H. J., SAGNER, G., KESSLER, C. & SCHMITZ, G. 1992. Sensitive chemiluminescent detection of digoxigenin-labeled nucleic acids: a fast and simple protocol and its applications. *Biotechniques,* 12**,** 104-13.

HOLTKE, H. J., SEIBL, R., BURG, J., MUHLEGGER, K. & KESSLER, C. 1990. Non-radioactive labeling and detection of nucleic acids. II. Optimization of the digoxigenin system. *Biol Chem Hoppe Seyler,* 371**,** 929-38.

HOTTER, G. S. & COLLINS, D. M. 2011. *Mycobacterium bovis* lipids: virulence and vaccines. *Vet Microbiol,* 151**,** 91-8.

HU, Y. & COATES, A. R. 2011. *Mycobacterium tuberculosis* acg gene is required for growth and virulence in vivo. *PLoS One,* 6**,** e20958.

HUANG, T. S., KUNIN, C. M., WANG, H. M., YAN, B. S., HUANG, S. P., CHEN, Y. S., LEE, S. S. & SYU, W. J. 2013. Inhibition of the *Mycobacterium tuberculosis* reserpine-sensitive efflux pump augments intracellular concentrations of ciprofloxacin and enhances susceptibility of some clinical isolates. *J Formos Med Assoc,* 112**,** 789-94.

HUANG, Y., GE, J., YAO, Y., WANG, Q., SHEN, H. & WANG, H. 2006. Characterization and site-directed mutagenesis of the putative novel acyl carrier protein Rv0033 and Rv1344 from *Mycobacterium tuberculosis*. *Biochem Biophys Res Commun,* 342**,** 618-24.

JORDAO, L., BLECK, C. K., MAYORGA, L., GRIFFITHS, G. & ANES, E. 2008. On the killing of mycobacteria by macrophages. *Cell Microbiol,* 10**,** 529-48.

JURCEV-SAVICEVIC, A., MULIC, R., KLISMANIC, Z. & KATALINIC-JANKOVIC, V. 2011. [Childhood tuberculosis: an ancient disease in the youngest generation in the 21st century from epidemiological point of view]. *Acta Med Croatica,* 65**,** 3-10.

KELL, D. B., KAPRELYANTS, A. S., WEICHART, D. H., HARWOOD, C. R. & BARER, M. R. 1998. Viability and activity in readily culturable bacteria: a review and discussion of the practical issues. *Antonie Van Leeuwenhoek,* 73**,** 169-87.

KNECHEL, N. A. 2009. Tuberculosis: pathophysiology, clinical features, and diagnosis. *Crit Care Nurse,* 29**,** 34-43; quiz 44.

KOHLI, S., SINGH, Y., SHARMA, K., MITTAL, A., EHTESHAM, N. Z. & HASNAIN, S. E. 2012. Comparative genomic and proteomic analyses of PE/PPE multigene family of *Mycobacterium tuberculosis* H(3)(7)Rv and H(3)(7)Ra reveal novel and interesting differences with implications in virulence. *Nucleic Acids Res,* 40**,** 7113-22.

KONDO, E. & KANAI, K. 1976. Further studies on the lethal effect of long-chain fatty acids on mycobacteria. *Jpn J Med Sci Biol,* 29**,** 25-37.

KOUL, A., ARNOULT, E., LOUNIS, N., GUILLEMONT, J. & ANDRIES, K. 2011. The challenge of new drug discovery for tuberculosis. *Nature,* 469**,** 483-90.

KRUH, N. A., TROUDT, J., IZZO, A., PRENNI, J. & DOBOS, K. M. 2010. Portrait of a pathogen: the *Mycobacterium tuberculosis* proteome in vivo. *PLoS One,* 5**,** e13938.

KRUIJSHAAR, M. E. & ABUBAKAR, I. 2009. Increase in extrapulmonary tuberculosis in England and Wales 1999-2006. *Thorax,* 64**,** 1090-5.

KUMAR, N., RADHAKRISHNAN, A., WRIGHT, C. C., CHOU, T. H., LEI, H. T., BOLLA, J. R., TRINGIDES, M. L., RAJASHANKAR, K. R., SU, C. C., PURDY, G. E. & YU, E. W. 2014. Crystal structure of the transcriptional regulator Rv1219c of *Mycobacterium tuberculosis*. *Protein Sci,* 23**,** 423-32.

KUO, H. P., HO, T. C., WANG, C. H., YU, C. T. & LIN, H. C. 1996. Increased production of hydrogen peroxide and expression of CD11b/CD18 on alveolar macrophages in patients with active pulmonary tuberculosis. *Tuber Lung Dis,* 77**,** 468-75.

LAMICHHANE, G., ZIGNOL, M., BLADES, N. J., GEIMAN, D. E., DOUGHERTY, A., GROSSET, J., BROMAN, K. W. & BISHAI, W. R. 2003. A postgenomic method for predicting essential genes at subsaturation levels of mutagenesis: application to *Mycobacterium tuberculosis*. *Proc Natl Acad Sci U S A,* 100**,** 7213-8.

LEE, R. E., LI, W., CHATTERJEE, D. & LEE, R. E. 2005. Rapid structural characterization of the arabinogalactan and lipoarabinomannan in live mycobacterial cells using 2D and 3D HR-MAS NMR: structural changes in the arabinan due to ethambutol treatment and gene mutation are observed. *Glycobiology,* 15**,** 139-51.

LEW, J. M., KAPOPOULOU, A., JONES, L. M. & COLE, S. T. 2011. TubercuList--10 years after. *Tuberculosis (Edinb),* 91**,** 1-7.

LEWIN, A., BAUS, D., KAMAL, E., BON, F., KUNISCH, R., MAURISCHAT, S., ADONOPOULOU, M. & EICH, K. 2008. The mycobacterial DNA-binding protein 1 (MDP1) from *Mycobacterium bovis* BCG influences various growth characteristics. *BMC Microbiol,* 8**,** 91.

LI, G., ZHANG, J., GUO, Q., JIANG, Y., WEI, J., ZHAO, L. L., ZHAO, X., LU, J. & WAN, K. 2015. Efflux pump gene expression in multidrug-resistant Mycobacterium tuberculosis clinical isolates. *PLoS One,* 10**,** e0119013.

LI, X. Z. & NIKAIDO, H. 2009. Efflux-mediated drug resistance in bacteria: an update. *Drugs,* 69**,** 1555-623.

LI, X. Z., ZHANG, L. & NIKAIDO, H. 2004. Efflux pump-mediated intrinsic drug resistance in Mycobacterium smegmatis. *Antimicrob Agents Chemother,* 48**,** 2415-23.

LI, Y., MILTNER, E., WU, M., PETROFSKY, M. & BERMUDEZ, L. E. 2005. A *Mycobacterium avium* PPE gene is associated with the ability of the bacterium to grow in macrophages and virulence in mice. *Cell Microbiol,* 7**,** 539-48.

LI, Z., KELLEY, C., COLLINS, F., ROUSE, D. & MORRIS, S. 1998. Expression of katG in *Mycobacterium tuberculosis i*s associated with its growth and persistence in mice and guinea pigs. *J Infect Dis,* 177**,** 1030-5.

LIN, P. L. & FLYNN, J. L. 2015. CD8 T cells and *Mycobacterium tuberculosis* infection. *Semin Immunopathol*.

LIN, P. L., MYERS, A., SMITH, L., BIGBEE, C., BIGBEE, M., FUHRMAN, C., GRIESER, H., CHIOSEA, I., VOITENEK, N. N., CAPUANO, S. V., KLEIN, E. & FLYNN, J. L. 2010. Tumor necrosis factor neutralization results in disseminated disease in acute and latent *Mycobacterium tuberculosis* infection with normal granuloma structure in a cynomolgus macaque model. *Arthritis Rheum,* 62**,** 340-50.

LIU, T., RAMESH, A., MA, Z., WARD, S. K., ZHANG, L., GEORGE, G. N., TALAAT, A. M., SACCHETTINI, J. C. & GIEDROC, D. P. 2007. CsoR is a novel *Mycobacterium tuberculosis* copper-sensing transcriptional regulator. *Nat Chem Biol,* 3**,** 60-8.

LOEBEL, R. O., SHORR, E. & RICHARDSON, H. B. 1933. The Influence of Adverse Conditions upon the Respiratory Metabolism and Growth of Human Tubercle Bacilli. *J Bacteriol,* 26**,** 167-200.

LOMOVSKAYA, O. & BOSTIAN, K. A. 2006. Practical applications and feasibility of efflux pump inhibitors in the clinic--a vision for applied use. *Biochem Pharmacol,* 71**,** 910-8.

LONNROTH, K. & RAVIGLIONE, M. 2008. Global epidemiology of tuberculosis: prospects for control. *Semin Respir Crit Care Med,* 29**,** 481-91.

LOPES, R. L., BORGES, T. J., ARAUJO, J. F., PINHO, N. G., BERGAMIN, L. S., BATTASTINI, A. M., MURARO, S. P., SOUZA, A. P., ZANIN, R. F. & BONORINO, C. 2014. Extracellular mycobacterial DnaK polarizes macrophages to the M2-like phenotype. *PLoS One,* 9**,** e113441.

LYNN, M., WILSON, A. R. & SOLOTOROVSKY, M. 1979. Role of bovine serum albumin in the nutrition of *Mycobacterium tuberculosis*. *Appl Environ Microbiol,* 38**,** 806-10.

MACGURN, J. A. & COX, J. S. 2007. A genetic screen for *Mycobacterium tuberculosis* mutants defective for phagosome maturation arrest identifies components of the ESX-1 secretion system. *Infect Immun,* 75**,** 2668-78.

MACMICKING, J. D., NORTH, R. J., LACOURSE, R., MUDGETT, J. S., SHAH, S. K. & NATHAN, C. F. 1997. Identification of nitric oxide synthase as a protective locus against tuberculosis. *Proc Natl Acad Sci U S A,* 94**,** 5243-8.

MAGLICA, Z., OZDEMIR, E. & MCKINNEY, J. D. 2015. Single-cell tracking reveals antibiotic-induced changes in mycobacterial energy metabolism. *MBio,* 6**,** e02236-14.

MAKSYMIUK, C., IOERGER, T., BALAKRISHNAN, A., BRYK, R., RHEE, K., SACCHETTINI, J. & NATHAN, C. 2015. Comparison of transposon and deletion mutants in Mycobacterium tuberculosis: The case of rv1248c, encoding 2-hydroxy-3-oxoadipate synthase. *Tuberculosis (Edinb),* 95**,** 689-94.

MALIK, Z. A., THOMPSON, C. R., HASHIMI, S., PORTER, B., IYER, S. S. & KUSNER, D. J. 2003. Cutting edge: *Mycobacterium tuberculosis* blocks Ca2+ signaling and phagosome maturation in human macrophages via specific inhibition of sphingosine kinase. *J Immunol,* 170**,** 2811-5.

MANGANELLI, R., PROVVEDI, R., RODRIGUE, S., BEAUCHER, J., GAUDREAU, L. & SMITH, I. 2004. Sigma factors and global gene regulation in *Mycobacterium tuberculosis*. *J Bacteriol,* 186**,** 895-902.

MARQUEZ, B. 2005. Bacterial efflux systems and efflux pumps inhibitors. *Biochimie,* 87**,** 1137-47.

MARTINEZ, F. O., HELMING, L. & GORDON, S. 2009a. Alternative activation of macrophages: an immunologic functional perspective. *Annu Rev Immunol,* 27**,** 451-83.

MARTINEZ, J. L., SANCHEZ, M. B., MARTINEZ-SOLANO, L., HERNANDEZ, A., GARMENDIA, L., FAJARDO, A. & ALVAREZ-ORTEGA, C. 2009b. Functional role of bacterial multidrug efflux pumps in microbial natural ecosystems. *FEMS Microbiol Rev,* 33**,** 430-49.

MATHAD, J. S. & GUPTA, A. 2012. Tuberculosis in pregnant and postpartum women: epidemiology, management, and research gaps. *Clin Infect Dis,* 55**,** 1532-49.

MATTOW, J., JUNGBLUT, P. R., SCHAIBLE, U. E., MOLLENKOPF, H. J., LAMER, S., ZIMNY-ARNDT, U., HAGENS, K., MULLER, E. C. & KAUFMANN, S. H. 2001. Identification of proteins from *Mycobacterium tuberculosis* missing in attenuated *Mycobacterium bovis* BCG strains. *Electrophoresis,* 22**,** 2936-46.

MCDONOUGH, K. A. & KRESS, Y. 1995. Cytotoxicity for lung epithelial cells is a virulence-associated phenotype of *Mycobacterium tuberculosis*. *Infect Immun,* 63**,** 4802-11.

MCDONOUGH, K. A., KRESS, Y. & BLOOM, B. R. 1993. Pathogenesis of tuberculosis: interaction of *Mycobacterium tuberculosis* with macrophages. *Infect Immun,* 61**,** 2763-73.

MCKINNEY, J. D., HONER ZU BENTRUP, K., MUNOZ-ELIAS, E. J., MICZAK, A., CHEN, B., CHAN, W. T., SWENSON, D., SACCHETTINI, J. C., JACOBS, W. R., JR. & RUSSELL, D. G. 2000. Persistence of *Mycobacterium tuberculosis* in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. *Nature,* 406**,** 735-8.

MEHTA, P. K., KING, C. H., WHITE, E. H., MURTAGH, J. J., JR. & QUINN, F. D. 1996. Comparison of in vitro models for the study of *Mycobacterium tuberculosis* invasion and intracellular replication. *Infect Immun,* 64**,** 2673-9.

MEHTA, P. K., PANDEY, A. K., SUBBIAN, S., EL-ETR, S. H., CIRILLO, S. L., SAMRAKANDI, M. M. & CIRILLO, J. D. 2006. Identification of Mycobacterium marinum macrophage infection mutants. *Microb Pathog,* 40**,** 139-51.

MOON, H. W., HUR, M., KIM, J. Y. & YUN, Y. M. 2015. Comparison of three molecular assays for the detection of rifampin resistance in *Mycobacterium tuberculosis*. *J Clin Lab Anal,* 29**,** 142-5.

MOSTOWY, S., CLETO, C., SHERMAN, D. R. & BEHR, M. A. 2004. The *Mycobacterium tuberculosis* complex transcriptome of attenuation. *Tuberculosis (Edinb),* 84**,** 197-204.

MUKAMOLOVA, G. V., MURZIN, A. G., SALINA, E. G., DEMINA, G. R., KELL, D. B., KAPRELYANTS, A. S. & YOUNG, M. 2006. Muralytic activity of Micrococcus luteus Rpf and its relationship to physiological activity in promoting bacterial growth and resuscitation. *Mol Microbiol,* 59**,** 84-98.

MUKHOPADHYAY, S. & BALAJI, K. N. 2011. The PE and PPE proteins of *Mycobacterium tuberculosis*. *Tuberculosis,* 91**,** 441-447.

MUNOZ-ELIAS, E. J. & MCKINNEY, J. D. 2005. Mycobacterium tuberculosis isocitrate lyases 1 and 2 are jointly required for in vivo growth and virulence. *Nat Med,* 11**,** 638-44.

MURRAY, J. F. 1989. The white plague: down and out, or up and coming? J. Burns Amberson lecture. *Am Rev Respir Dis,* 140**,** 1788-95.

MUTTUCUMARU, D. G., ROBERTS, G., HINDS, J., STABLER, R. A. & PARISH, T. 2004. Gene expression profile of *Mycobacterium tuberculosis* in a non-replicating state. *Tuberculosis (Edinb),* 84**,** 239-46.

MYRVIK, Q. N., LEAKE, E. S. & WRIGHT, M. J. 1984. Disruption of phagosomal membranes of normal alveolar macrophages by the H37Rv strain of *Mycobacterium tuberculosis*. A correlate of virulence. *Am Rev Respir Dis,* 129**,** 322-8.

NAIR, S., RAMASWAMY, P. A., GHOSH, S., JOSHI, D. C., PATHAK, N., SIDDIQUI, I., SHARMA, P., HASNAIN, S. E., MANDE, S. C. & MUKHOPADHYAY, S. 2009. The PPE18 of *Mycobacterium tuberculosis* interacts with TLR2 and activates IL-10 induction in macrophage. *J Immunol,* 183**,** 6269-81.

NAMBI, S., LONG, J. E., MISHRA, B. B., BAKER, R., MURPHY, K. C., OLIVE, A. J., NGUYEN, H. P., SHAFFER, S. A. & SASSETTI, C. M. 2015. The Oxidative Stress Network of Mycobacterium tuberculosis Reveals Coordination between Radical Detoxification Systems. *Cell Host Microbe,* 17**,** 829-37.

NANDAKUMAR, M., NATHAN, C. & RHEE, K. Y. 2014. Isocitrate lyase mediates broad antibiotic tolerance in *Mycobacterium tuberculosis*. *Nat Commun,* 5**,** 4306.

NATHAN, C. & SHILOH, M. U. 2000. Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. *Proc Natl Acad Sci U S A,* 97**,** 8841-8.

NG, V. H., COX, J. S., SOUSA, A. O., MACMICKING, J. D. & MCKINNEY, J. D. 2004. Role of KatG catalase-peroxidase in mycobacterial pathogenesis: countering the phagocyte oxidative burst. *Mol Microbiol,* 52**,** 1291-302.

NICHOLSON, S., BONECINI-ALMEIDA MDA, G., LAPA E SILVA, J. R., NATHAN, C., XIE, Q. W., MUMFORD, R., WEIDNER, J. R., CALAYCAY, J., GENG, J., BOECHAT, N., LINHARES, C., ROM, W. & HO, J. L. 1996. Inducible nitric oxide synthase in pulmonary alveolar macrophages from patients with tuberculosis. *J Exp Med,* 183**,** 2293-302.

NIKAIDO, H. 2001. Preventing drug access to targets: cell surface permeability barriers and active efflux in bacteria. *Semin Cell Dev Biol,* 12**,** 215-23.

NORTH, R. J. & JUNG, Y. J. 2004. Immunity to tuberculosis. *Annu Rev Immunol,* 22**,** 599-623.

NYKA, W. 1974. Studies on the effect of starvation on mycobacteria. *Infect Immun,* 9**,** 843-50.

OH, Y. K. & STRAUBINGER, R. M. 1996. Intracellular fate of *Mycobacterium avium*: use of dual-label spectrofluorometry to investigate the influence of bacterial viability and opsonization on phagosomal pH and phagosome-lysosome interaction. *Infect Immun,* 64**,** 319-25.

ONOZAKI, I. & RAVIGLIONE, M. 2010. Stopping tuberculosis in the 21st century: goals and strategies. *Respirology,* 15**,** 32-43.

ORME, I. M. 2003. The mouse as a useful model of tuberculosis. *Tuberculosis (Edinb),* 83**,** 112-5.

OTTENHOFF, T. H., KUMARARATNE, D. & CASANOVA, J. L. 1998. Novel human immunodeficiencies reveal the essential role of type-I cytokines in immunity to intracellular bacteria. *Immunol Today,* 19**,** 491-4.

PAI, M., ZWERLING, A. & MENZIES, D. 2008. Systematic review: T-cell-based assays for the diagnosis of latent tuberculosis infection: an update. *Ann Intern Med,* 149**,** 177-84.

PARISH, T. & STOKER, N. G. 1998. Electroporation of mycobacteria. *Methods Mol Biol,* 101**,** 129-44.

PARISH, T. & STOKER, N. G. 2000. Use of a flexible cassette method to generate a double unmarked *Mycobacterium tuberculosis* tlyA plcABC mutant by gene replacement. *Microbiology,* 146 (Pt 8)**,** 1969-75.

PARRISH, N. M. & CARROLL, K. C. 2011. Role of the clinical mycobacteriology laboratory in diagnosis and management of tuberculosis in low-prevalence settings. *J Clin Microbiol,* 49**,** 772-6.

PASCA, M. R., GUGLIERAME, P., DE ROSSI, E., ZARA, F. & RICCARDI, G. 2005. mmpL7 gene of *Mycobacterium tuberculosis* is responsible for isoniazid efflux in *Mycobacterium smegmatis*. *Antimicrob Agents Chemother,* 49**,** 4775-7.

PAVELKA, M. S., JR. & JACOBS, W. R., JR. 1999. Comparison of the construction of unmarked deletion mutations in Mycobacterium smegmatis, Mycobacterium bovis bacillus Calmette-Guerin, and Mycobacterium tuberculosis H37Rv by allelic exchange. *J Bacteriol,* 181**,** 4780-9.

PECORA, N. D., GEHRING, A. J., CANADAY, D. H., BOOM, W. H. & HARDING, C. V. 2006. *Mycobacterium tuberculosis* LprA is a lipoprotein agonist of TLR2 that regulates innate immunity and APC function. *J Immunol,* 177**,** 422-9.

PEDROSA, J., SAUNDERS, B. M., APPELBERG, R., ORME, I. M., SILVA, M. T. & COOPER, A. M. 2000. Neutrophils play a protective nonphagocytic role in systemic *Mycobacterium tuberculosis* infection of mice. *Infect Immun,* 68**,** 577-83.

PETHE, K., SWENSON, D. L., ALONSO, S., ANDERSON, J., WANG, C. & RUSSELL, D. G. 2004. Isolation of *Mycobacterium tuberculosis* mutants defective in the arrest of phagosome maturation. *Proc Natl Acad Sci U S A,* 101**,** 13642-7.

PIDDINGTON, D. L., FANG, F. C., LAESSIG, T., COOPER, A. M., ORME, I. M. & BUCHMEIER, N. A. 2001. Cu,Zn superoxide dismutase of Mycobacterium tuberculosis contributes to survival in activated macrophages that are generating an oxidative burst. *Infect Immun,* 69**,** 4980-7.

POZOS, T. C. & RAMAKRISHNAN, L. 2004. New models for the study of Mycobacterium-host interactions. *Curr Opin Immunol,* 16**,** 499-505.

RAMAKRISHNAN, L. 2012. Revisiting the role of the granuloma in tuberculosis. *Nat Rev Immunol,* 12**,** 352-66.

RAMAKRISHNAN, L., FEDERSPIEL, N. A. & FALKOW, S. 2000. Granuloma-specific expression of Mycobacterium virulence proteins from the glycine-rich PE-PGRS family. *Science,* 288**,** 1436-9.

RAMON-GARCIA, S., MARTIN, C., THOMPSON, C. J. & AINSA, J. A. 2009. Role of the *Mycobacterium tuberculosis* P55 efflux pump in intrinsic drug resistance, oxidative stress responses, and growth. *Antimicrob Agents Chemother,* 53**,** 3675-82.

RAMON-GARCIA, S., STEWART, G. R., HUI, Z. K., MOHN, W. W. & THOMPSON, C. J. 2015. The mycobacterial P55 efflux pump is required for optimal growth on cholesterol. *Virulence,* 6**,** 444-8.

RAMOS, J. L., MARTINEZ-BUENO, M., MOLINA-HENARES, A. J., TERAN, W., WATANABE, K., ZHANG, X., GALLEGOS, M. T., BRENNAN, R. & TOBES, R. 2005. The TetR family of transcriptional repressors. *Microbiol Mol Biol Rev,* 69**,** 326-56.

REHREN, G., WALTERS, S., FONTAN, P., SMITH, I. & ZARRAGA, A. M. 2007. Differential gene expression between *Mycobacterium bovis* and *Mycobacterium tuberculosis*. *Tuberculosis (Edinb),* 87**,** 347-59.

REYRAT, J. M. & KAHN, D. 2001. Mycobacterium smegmatis: an absurd model for tuberculosis? *Trends Microbiol,* 9**,** 472-4.

RIBEIRO-GUIMARAES, M. L. & PESSOLANI, M. C. 2007. Comparative genomics of mycobacterial proteases. *Microb Pathog,* 43**,** 173-8.

RICKMAN, L., SCOTT, C., HUNT, D. M., HUTCHINSON, T., MENENDEZ, M. C., WHALAN, R., HINDS, J., COLSTON, M. J., GREEN, J. & BUXTON, R. S. 2005. A member of the cAMP receptor protein family of transcription regulators in *Mycobacterium tuberculosis* is required for virulence in mice and controls transcription of the rpfA gene coding for a resuscitation promoting factor. *Mol Microbiol,* 56**,** 1274-86.

RIENDEAU, C. J. & KORNFELD, H. 2003. THP-1 cell apoptosis in response to Mycobacterial infection. *Infect Immun,* 71**,** 254-9.

RILEY, R. L., MILLS, C. C., NYKA, W., WEINSTOCK, N., STOREY, P. B., SULTAN, L. U., RILEY, M. C. & WELLS, W. F. 1995. Aerial dissemination of pulmonary tuberculosis. A two-year study of contagion in a tuberculosis ward. 1959. *Am J Epidemiol,* 142**,** 3-14.

ROHDE, K., YATES, R. M., PURDY, G. E. & RUSSELL, D. G. 2007a. *Mycobacterium tuberculosis* and the environment within the phagosome. *Immunol Rev,* 219**,** 37-54.

ROHDE, K. H., ABRAMOVITCH, R. B. & RUSSELL, D. G. 2007b. *Mycobacterium tuberculosis* invasion of macrophages: linking bacterial gene expression to environmental cues. *Cell Host Microbe,* 2**,** 352-64.

ROSENFELDT, V., PAERREGAARD, A. & VALERIUS, N. H. 1997. Disseminated infection with *Bacillus Calmette-Guerin* in a child with advanced HIV disease. *Scand J Infect Dis,* 29**,** 526-7.

ROY, C. J. & MILTON, D. K. 2004. Airborne transmission of communicable infection--the elusive pathway. *N Engl J Med,* 350**,** 1710-2.

RUBINSTEIN, N. D., ZEEVI, D., OREN, Y., SEGAL, G. & PUPKO, T. 2011. The operonic location of auto-transcriptional repressors is highly conserved in bacteria. *Mol Biol Evol,* 28**,** 3309-18.

RUSSELL, D. G. 2001. *Mycobacterium tuberculosis*: here today, and here tomorrow. *Nat Rev Mol Cell Biol,* 2**,** 569-77.

RUSSELL, D. G., CARDONA, P. J., KIM, M. J., ALLAIN, S. & ALTARE, F. 2009. Foamy macrophages and the progression of the human tuberculosis granuloma. *Nat Immunol,* 10**,** 943-8.

SAKAMOTO, K. 2012. The Pathology of *Mycobacterium tuberculosis* Infection. *Veterinary Pathology Online,* 49**,** 423-439.

SAMPSON, S. L. 2011. Mycobacterial PE/PPE proteins at the host-pathogen interface. *Clin Dev Immunol,* 2011**,** 497203.

SANCHEZ, P., LINARES, J. F., RUIZ-DIEZ, B., CAMPANARIO, E., NAVAS, A., BAQUERO, F. & MARTINEZ, J. L. 2002. Fitness of in vitro selected Pseudomonas aeruginosa nalB and nfxB multidrug resistant mutants. *J Antimicrob Chemother,* 50**,** 657-64.

SARATHY, J. P., DARTOIS, V. & LEE, E. J. 2012. The role of transport mechanisms in *mycobacterium tuberculosis* drug resistance and tolerance. *Pharmaceuticals (Basel),* 5**,** 1210-35.

SASSETTI, C. M., BOYD, D. H. & RUBIN, E. J. 2003. Genes required for mycobacterial growth defined by high density mutagenesis. *Mol Microbiol,* 48**,** 77-84.

SASSETTI, C. M. & RUBIN, E. J. 2003. Genetic requirements for mycobacterial survival during infection. *Proc Natl Acad Sci U S A,* 100**,** 12989-94.

SCHERR, N., JAYACHANDRAN, R., MUELLER, P. & PIETERS, J. 2009. Interference of *Mycobacterium tuberculosis* with macrophage responses. *Indian J Exp Biol,* 47**,** 401-6.

SCHLESINGER, L. S. 1996. Role of mononuclear phagocytes in *M tuberculosis* pathogenesis. *J Investig Med,* 44**,** 312-23.

SCHLESINGER, L. S., HULL, S. R. & KAUFMAN, T. M. 1994. Binding of the terminal mannosyl units of lipoarabinomannan from a virulent strain of *Mycobacterium tuberculosis* to human macrophages. *J Immunol,* 152**,** 4070-9.

SCHLUGER, N. W. & ROM, W. N. 1998. The host immune response to tuberculosis. *Am J Respir Crit Care Med,* 157**,** 679-91.

SCHNAPPINGER, D., EHRT, S., VOSKUIL, M. I., LIU, Y., MANGAN, J. A., MONAHAN, I. M., DOLGANOV, G., EFRON, B., BUTCHER, P. D., NATHAN, C. & SCHOOLNIK, G. K. 2003. Transcriptional Adaptation of Mycobacterium tuberculosis within Macrophages: Insights into the Phagosomal Environment. *J Exp Med,* 198**,** 693-704.

SCHULLER, S., NEEFJES, J., OTTENHOFF, T., THOLE, J. & YOUNG, D. 2001. Coronin is involved in uptake of *Mycobacterium bovis* BCG in human macrophages but not in phagosome maintenance. *Cell Microbiol,* 3**,** 785-93.

SEGAL, W. 1984. *In:* KUBICA, G. P. W., L. G. (ed.) *The Mycobacteria, A Sourcebook,.* Dekker, New York.

SHAFER, R. W. & EDLIN, B. R. 1996. Tuberculosis in patients infected with human immunodeficiency virus: perspective on the past decade. *Clin Infect Dis,* 22**,** 683-704.

SHEN, Y., SHEN, L., SEHGAL, P., HUANG, D., QIU, L., DU, G., LETVIN, N. L. & CHEN, Z. W. 2004. Clinical latency and reactivation of AIDS-related mycobacterial infections. *J Virol,* 78**,** 14023-32.

SHILOH, M. U. & CHAMPION, P. A. 2010. To catch a killer. What can mycobacterial models teach us about *Mycobacterium tuberculosis* pathogenesis? *Curr Opin Microbiol,* 13**,** 86-92.

SIMEONE, R., BOBARD, A., LIPPMANN, J., BITTER, W., MAJLESSI, L., BROSCH, R. & ENNINGA, J. 2012. Phagosomal rupture by *Mycobacterium tuberculosis* results in toxicity and host cell death. *PLoS Pathog,* 8**,** e1002507.

SIMEONE, R., BOTTAI, D. & BROSCH, R. 2009. ESX/type VII secretion systems and their role in host-pathogen interaction. *Curr Opin Microbiol,* 12**,** 4-10.

SIMEONE, R., SAYES, F., SONG, O., GROSCHEL, M. I., BRODIN, P., BROSCH, R. & MAJLESSI, L. 2015. Cytosolic access of *Mycobacterium tuberculosis*: critical impact of phagosomal acidification control and demonstration of occurrence in vivo. *PLoS Pathog,* 11**,** e1004650.

SIMMONS, D. P., CANADAY, D. H., LIU, Y., LI, Q., HUANG, A., BOOM, W. H. & HARDING, C. V. 2010. *Mycobacterium tuberculosis* and TLR2 agonists inhibit induction of type I IFN and class I MHC antigen cross processing by TLR9. *J Immunol,* 185**,** 2405-15.

SIVARAMAKRISHNAN, S. & DE MONTELLANO, P. R. 2013. The DosS-DosT/DosR Mycobacterial Sensor System. *Biosensors (Basel),* 3**,** 259-282.

SNAPPER, S. B., MELTON, R. E., MUSTAFA, S., KIESER, T. & JACOBS, W. R., JR. 1990. Isolation and characterization of efficient plasmid transformation mutants of *Mycobacterium smegmatis*. *Mol Microbiol,* 4**,** 1911-9.

SOUTHERN, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol,* 98**,** 503-17.

STAMM, L. M. & BROWN, E. J. 2004. *Mycobacterium marinum*: the generalization and specialization of a pathogenic mycobacterium. *Microbes Infect,* 6**,** 1418-28.

STAMM, L. M., MORISAKI, J. H., GAO, L. Y., JENG, R. L., MCDONALD, K. L., ROTH, R., TAKESHITA, S., HEUSER, J., WELCH, M. D. & BROWN, E. J. 2003. *Mycobacterium marinum* escapes from phagosomes and is propelled by actin-based motility. *J Exp Med,* 198**,** 1361-8.

STAPLETON, M., HAQ, I., HUNT, D. M., ARNVIG, K. B., ARTYMIUK, P. J., BUXTON, R. S. & GREEN, J. 2010. *Mycobacterium tuberculosis* cAMP receptor protein (Rv3676) differs from the *Escherichia coli* paradigm in its cAMP binding and DNA binding properties and transcription activation properties. *J Biol Chem,* 285**,** 7016-27.

STAVRI, M., PIDDOCK, L. J. & GIBBONS, S. 2007. Bacterial efflux pump inhibitors from natural sources. *J Antimicrob Chemother,* 59**,** 1247-60.

STENGER, S., MAZZACCARO, R. J., UYEMURA, K., CHO, S., BARNES, P. F., ROSAT, J. P., SETTE, A., BRENNER, M. B., PORCELLI, S. A., BLOOM, B. R. & MODLIN, R. L. 1997. Differential effects of cytolytic T cell subsets on intracellular infection. *Science,* 276**,** 1684-7.

STEWART, G. R., PATEL, J., ROBERTSON, B. D., RAE, A. & YOUNG, D. B. 2005. Mycobacterial mutants with defective control of phagosomal acidification. *PLoS Pathog,* 1**,** 269-78.

STEWART, G. R., ROBERTSON, B. D. & YOUNG, D. B. 2003. Tuberculosis: a problem with persistence. *Nat Rev Microbiol,* 1**,** 97-105.

STEWART, G. R., SNEWIN, V. A., WALZL, G., HUSSELL, T., TORMAY, P., O'GAORA, P., GOYAL, M., BETTS, J., BROWN, I. N. & YOUNG, D. B. 2001. Overexpression of heat-shock proteins reduces survival of *Mycobacterium tuberculosis* in the chronic phase of infection. *Nat Med,* 7**,** 732-7.

STINEAR, T. P., SEEMANN, T., HARRISON, P. F., JENKIN, G. A., DAVIES, J. K., JOHNSON, P. D., ABDELLAH, Z., ARROWSMITH, C., CHILLINGWORTH, T., CHURCHER, C., CLARKE, K., CRONIN, A., DAVIS, P., GOODHEAD, I., HOLROYD, N., JAGELS, K., LORD, A., MOULE, S., MUNGALL, K., NORBERTCZAK, H., QUAIL, M. A., RABBINOWITSCH, E., WALKER, D., WHITE, B., WHITEHEAD, S., SMALL, P. L., BROSCH, R., RAMAKRISHNAN, L., FISCHBACH, M. A., PARKHILL, J. & COLE, S. T. 2008. Insights from the complete genome sequence of Mycobacterium marinum on the evolution of Mycobacterium tuberculosis. *Genome Res,* 18**,** 729-41.

STOKES, R. W. & DOXSEE, D. 1999. The receptor-mediated uptake, survival, replication, and drug sensitivity of *Mycobacterium tuberculosis* within the macrophage-like cell line THP-1: a comparison with human monocyte-derived macrophages. *Cell Immunol,* 197**,** 1-9.

SUN, J., DENG, Z. & YAN, A. 2014. Bacterial multidrug efflux pumps: mechanisms, physiology and pharmacological exploitations. *Biochem Biophys Res Commun,* 453**,** 254-67.

SWAIM, L. E., CONNOLLY, L. E., VOLKMAN, H. E., HUMBERT, O., BORN, D. E. & RAMAKRISHNAN, L. 2006. *Mycobacterium marinum* infection of adult zebrafish causes caseating granulomatous tuberculosis and is moderated by adaptive immunity. *Infect Immun,* 74**,** 6108-17.

TAKIFF, H. E., CIMINO, M., MUSSO, M. C., WEISBROD, T., MARTINEZ, R., DELGADO, M. B., SALAZAR, L., BLOOM, B. R. & JACOBS, W. R., JR. 1996. Efflux pump of the proton antiporter family confers low-level fluoroquinolone resistance in Mycobacterium smegmatis. *Proc Natl Acad Sci U S A,* 93**,** 362-6.

THANNICKAL, V. J. & FANBURG, B. L. 2000. Reactive oxygen species in cell signaling. *Am J Physiol Lung Cell Mol Physiol,* 279**,** L1005-28.

THEUS, S. A., CAVE, M. D. & EISENACH, K. D. 2004. Activated THP-1 cells: an attractive model for the assessment of intracellular growth rates of *Mycobacterium tuberculosis* isolates. *Infect Immun,* 72**,** 1169-73.

THILLAI, M., POLLOCK, K., PAREEK, M. & LALVANI, A. 2014. Interferon-gamma release assays for tuberculosis: current and future applications. *Expert Rev Respir Med,* 8**,** 67-78.

TIMM, J., POST, F. A., BEKKER, L. G., WALTHER, G. B., WAINWRIGHT, H. C., MANGANELLI, R., CHAN, W. T., TSENOVA, L., GOLD, B., SMITH, I., KAPLAN, G. & MCKINNEY, J. D. 2003. Differential expression of iron-, carbon-, and oxygen-responsive mycobacterial genes in the lungs of chronically infected mice and tuberculosis patients. *Proc Natl Acad Sci U S A,* 100**,** 14321-6.

TIWARI, B. M., KANNAN, N., VEMU, L. & RAGHUNAND, T. R. 2012. The Mycobacterium tuberculosis PE proteins Rv0285 and Rv1386 modulate innate immunity and mediate bacillary survival in macrophages. *PLoS One,* 7**,** e51686.

TOBIN, D. M. & RAMAKRISHNAN, L. 2008. Comparative pathogenesis of *Mycobacterium marinum* and *Mycobacterium tuberculosis*. *Cell Microbiol,* 10**,** 1027-39.

TOMIOKA, H., TATANO, Y., MAW, W. W., SANO, C., KANEHIRO, Y. & SHIMIZU, T. 2012. Characteristics of suppressor macrophages induced by mycobacterial and protozoal infections in relation to alternatively activated M2 macrophages. *Clin Dev Immunol,* 2012**,** 635451.

TORTOLI, E. 2003. Impact of genotypic studies on mycobacterial taxonomy: the new mycobacteria of the 1990s. *Clin Microbiol Rev,* 16**,** 319-54.

TUFARIELLO, J. M., CHAN, J. & FLYNN, J. L. 2003. Latent tuberculosis: mechanisms of host and bacillus that contribute to persistent infection. *Lancet Infect Dis,* 3**,** 578-90.

TUNDUP, S., AKHTER, Y., THIAGARAJAN, D. & HASNAIN, S. E. 2006. Clusters of PE and PPE genes of *Mycobacterium tuberculosis* are organized in operons: evidence that PE Rv2431c is co-transcribed with PPE Rv2430c and their gene products interact with each other. *FEBS Lett,* 580**,** 1285-93.

TURAPOV, O., WADDELL, S. J., BURKE, B., GLENN, S., SARYBAEVA, A. A., TUDO, G., LABESSE, G., YOUNG, D. I., YOUNG, M., ANDREW, P. W., BUTCHER, P. D., COHEN-GONSAUD, M. & MUKAMOLOVA, G. V. 2014a. Antimicrobial treatment improves mycobacterial survival in nonpermissive growth conditions. *Antimicrob Agents Chemother,* 58**,** 2798-806.

TURAPOV, O., WADDELL, S. J., BURKE, B., GLENN, S., SARYBAEVA, A. A., TUDO, G., LABESSE, G., YOUNG, D. I., YOUNG, M., ANDREW, P. W., BUTCHER, P. D., COHEN-GONSAUD, M. & MUKAMOLOVA, G. V. 2014b. Oleoyl coenzyme A regulates interaction of transcriptional regulator RaaS (Rv1219c) with DNA in mycobacteria. *J Biol Chem,* 289**,** 25241-9.

TYAGI, J. S. & SHARMA, D. 2002. *Mycobacterium smegmatis* and *tuberculosis*. *Trends Microbiol,* 10**,** 68-9.

VAN KESSEL, J. C. & HATFULL, G. F. 2008. Mycobacterial recombineering. *Methods Mol Biol,* 435**,** 203-15.

VANDAL, O. H., NATHAN, C. F. & EHRT, S. 2009. Acid resistance in *Mycobacterium tuberculosis*. *J Bacteriol,* 191**,** 4714-21.

VANDAL, O. H., PIERINI, L. M., SCHNAPPINGER, D., NATHAN, C. F. & EHRT, S. 2008. A membrane protein preserves intrabacterial pH in intraphagosomal *Mycobacterium tuberculosis*. *Nat Med,* 14**,** 849-54.

VANKAYALAPATI, R., GARG, A., PORGADOR, A., GRIFFITH, D. E., KLUCAR, P., SAFI, H., GIRARD, W. M., COSMAN, D., SPIES, T. & BARNES, P. F. 2005. Role of NK cell-activating receptors and their ligands in the lysis of mononuclear phagocytes infected with an intracellular bacterium. *J Immunol,* 175**,** 4611-7.

VERGNE, I., CHUA, J. & DERETIC, V. 2003a. *Mycobacterium tuberculosis* phagosome maturation arrest: selective targeting of PI3P-dependent membrane trafficking. *Traffic,* 4**,** 600-6.

VERGNE, I., CHUA, J. & DERETIC, V. 2003b. Tuberculosis toxin blocking phagosome maturation inhibits a novel Ca2+/calmodulin-PI3K hVPS34 cascade. *J Exp Med,* 198**,** 653-9.

VERGNE, I., CHUA, J., LEE, H. H., LUCAS, M., BELISLE, J. & DERETIC, V. 2005. Mechanism of phagolysosome biogenesis block by viable *Mycobacterium tuberculosis*. *Proc Natl Acad Sci U S A,* 102**,** 4033-8.

VIA, L. E., FRATTI, R. A., MCFALONE, M., PAGAN-RAMOS, E., DERETIC, D. & DERETIC, V. 1998. Effects of cytokines on mycobacterial phagosome maturation. *J Cell Sci,* 111 (Pt 7)**,** 897-905.

VIVEIROS, M., LEANDRO, C. & AMARAL, L. 2003. Mycobacterial efflux pumps and chemotherapeutic implications. *Int J Antimicrob Agents,* 22**,** 274-8.

VOSKUIL, M. I., BARTEK, I. L., VISCONTI, K. & SCHOOLNIK, G. K. 2011. The response of *mycobacterium tuberculosis* to reactive oxygen and nitrogen species. *Front Microbiol,* 2**,** 105.

VOSKUIL, M. I., SCHNAPPINGER, D., RUTHERFORD, R., LIU, Y. & SCHOOLNIK, G. K. 2004a. Regulation of the *Mycobacterium tuberculosis* PE/PPE genes. *Tuberculosis (Edinb),* 84**,** 256-62.

VOSKUIL, M. I., SCHNAPPINGER, D., VISCONTI, K. C., HARRELL, M. I., DOLGANOV, G. M., SHERMAN, D. R. & SCHOOLNIK, G. K. 2003. Inhibition of respiration by nitric oxide induces a *Mycobacterium tuberculosis* dormancy program. *J Exp Med,* 198**,** 705-13.

VOSKUIL, M. I., VISCONTI, K. C. & SCHOOLNIK, G. K. 2004b. *Mycobacterium tuberculosis* gene expression during adaptation to stationary phase and low-oxygen dormancy. *Tuberculosis (Edinb),* 84**,** 218-27.

WANG, K., PEI, H., HUANG, B., ZHU, X., ZHANG, J., ZHOU, B., ZHU, L., ZHANG, Y. & ZHOU, F. F. 2013. The expression of ABC efflux pump, Rv1217c-Rv1218c, and its association with multidrug resistance of *Mycobacterium tuberculosis* in China. *Curr Microbiol,* 66**,** 222-6.

WARNER, D. F. & MIZRAHI, V. 2007. The survival kit of Mycobacterium tuberculosis. *Nat Med,* 13**,** 282-4.

WELIN, A., WINBERG, M. E., ABDALLA, H., SARNDAHL, E., RASMUSSON, B., STENDAHL, O. & LERM, M. 2008. Incorporation of *Mycobacterium tuberculosis l*ipoarabinomannan into macrophage membrane rafts is a prerequisite for the phagosomal maturation block. *Infect Immun,* 76**,** 2882-7.

WHO 2010. WHO global tuberculosis control report 2010. Summary. *Cent Eur J Public Health,* 18**,** 237.

WHO 2014. Global tuberculosis Report 2014.

XIROUCHAKI, N., TZANAKIS, N., BOUROS, D., KYRIAKOU, D., KARKAVITSAS, N., ALEXANDRAKIS, M. & SIAFAKAS, N. M. 2002. Diagnostic value of interleukin-1alpha, interleukin-6, and tumor necrosis factor in pleural effusions. *Chest,* 121**,** 815-20.

YAMADA, H., BHATT, A., DANEV, R., FUJIWARA, N., MAEDA, S., MITARAI, S., CHIKAMATSU, K., AONO, A., NITTA, K., JACOBS, W. R., JR. & NAGAYAMA, K. 2012. Non-acid-fastness in *Mycobacterium tuberculosis* DeltakasB mutant correlates with the cell envelope electron density. *Tuberculosis (Edinb),* 92**,** 351-7.

YOSHPE-PURER, Y. & HENIS, Y. 1976. Factors affecting catalase level and sensitivity to hydrogen peroxide in Escherichia coli. *Appl Environ Microbiol,* 32**,** 465-9.

YOUNG, D., LATHIGRA, R., HENDRIX, R., SWEETSER, D. & YOUNG, R. A. 1988. Stress proteins are immune targets in leprosy and tuberculosis. *Proc Natl Acad Sci U S A,* 85**,** 4267-70.

YUAN, Y., LEE, R. E., BESRA, G. S., BELISLE, J. T. & BARRY, C. E., 3RD 1995. Identification of a gene involved in the biosynthesis of cyclopropanated mycolic acids in *Mycobacterium tuberculosis*. *Proc Natl Acad Sci U S A,* 92**,** 6630-4.

ZAHRT, T. C. 2003. Molecular mechanisms regulating persistent *Mycobacterium tuberculosis* infection. *Microbes Infect,* 5**,** 159-67.

ZHANG, M., GATELY, M. K., WANG, E., GONG, J., WOLF, S. F., LU, S., MODLIN, R. L. & BARNES, P. F. 1994. Interleukin 12 at the site of disease in tuberculosis. *J Clin Invest,* 93**,** 1733-9.

ZHANG, Y. & YEW, W. W. 2009. Mechanisms of drug resistance in *Mycobacterium tuberculosis*. *Int J Tuberc Lung Dis,* 13**,** 1320-30.

ZHOU, Z., CONNELL, M. C. & MACEWAN, D. J. 2007. TNFR1-induced NF-kappaB, but not ERK, p38MAPK or JNK activation, mediates TNF-induced ICAM-1 and VCAM-1 expression on endothelial cells. *Cell Signal,* 19**,** 1238-48.

ZIMMERMAN, M. R. 1979. Pulmonary and osseous tuberculosis in an Egyptian mummy. *Bull N Y Acad Med,* 55**,** 604-8.