Genetic basis of survival of *Mycobacterium bovis* inside *Acanthamoeba castellanii*

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Dedication

This thesis is dedicated to my affectionate parents who motivated me for the PhD and to my loving husband who encouraged me to accomplish it.
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Summary

Tuberculosis remains a major threat to human health accounting for 2 million annual deaths worldwide. *M. bovis* causes TB in cattle which is a serious issue in the UK. Mycobacteria are widely distributed in the environments that are also colonized by free living amoebae. In the present study, free-living amoeba (*A. castellanii*) has been used to study the genetic factors required for the intracellular survival of *M. bovis*. Role of region of difference 1 (RD1), isocitrate lyase (Rv0467), ClgR (Rv2745) and the VapC (Rv2548) toxin-antitoxin system was examined for survival in amoebae. While the role of RD1 in mycobacterial survival in amoebae could not be observed, isocitrate lyase and a transcriptional regulator (*ClgR*) might play some part in survival of *M. bovis* in *A. castellanii*. It was found that although the mycobacteria were able to remain inside the amoebae vacuoles, they were unable to control the pH as the vacuoles remained acidic. This is very interesting as it is in contrast to macrophages where the mycobacteria are controlling and keeping the phagosomal pH only weakly acidic. A library of ~2500 *M. bovis* mutants was also created and TraSH mutagenesis was performed to provide a systematic assessment of the importance of mycobacterial genes for intra-amoebic survival. The results indicate that perhaps Rv3087 plays some role in the ability of *M. bovis* to grow inside amoebae. Rv3087 is considered to be essential for growth in murine infection model and induced when tubercle bacilli are exposed to acidic conditions *in vitro* and in macrophages. However, these are preliminary findings that need to be confirmed by further research.
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<th>Description</th>
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<tbody>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>BCG</td>
<td>Bacillus Calmette Guerin</td>
</tr>
<tr>
<td>bTB</td>
<td>bovine tuberculosis</td>
</tr>
<tr>
<td>CCAP</td>
<td>Culture Collection For Algae And Protozoa</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster Of Differentiation</td>
</tr>
<tr>
<td>CFP</td>
<td>Culture Filtrate Proteins</td>
</tr>
<tr>
<td>CR</td>
<td>Complement Receptor</td>
</tr>
<tr>
<td>CTAB</td>
<td>Cetyltrimethylammonium bromide</td>
</tr>
<tr>
<td>Cy</td>
<td>Cytidine</td>
</tr>
<tr>
<td>DEFRA</td>
<td>Department of the Environment, Food, and Rural Affairs</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
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<tr>
<td>ESAT-6</td>
<td>Early Secreted Antigenic Target-6</td>
</tr>
<tr>
<td>ESX-1</td>
<td>Early Secretory Antigenic Target 6 System 1</td>
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<td>G+C</td>
<td>Guanine+Cytosine</td>
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<td>Hygromycin</td>
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<td>Interferon Gamma</td>
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<td>Interleukin 12</td>
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<tr>
<td>iNOS</td>
<td>inducible Nitric Oxide Synthase</td>
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<tr>
<td>Kan</td>
<td>Kanamycin</td>
</tr>
<tr>
<td>LAM</td>
<td>lipoaribomannan</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertoni</td>
</tr>
<tr>
<td>MR</td>
<td>Mannose Receptor</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NCTC</td>
<td>National Collection of Type Cultures</td>
</tr>
<tr>
<td>OADC</td>
<td>Oleic Albumin Dextrose Catalase</td>
</tr>
<tr>
<td>ORF</td>
<td>Open Reading Frame</td>
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<tr>
<td>PAS</td>
<td>Pages Amoeba Saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
</tbody>
</table>
PPG  Proteose Peptone Glucose
RD1  Region of Difference 1
RNI  reactive nitrogen intermediates
SDS  Sodium Dodecyl Sulphate
Spp. Species'
SSC  Saline sodium citrate
STM  Signature Tagged Mutagenesis
TNF α  Tumor Necrosis Factor Alpha
TraSH  Transposon Site Hybridization
TSM  Transposon Screen by Microarray
WT  Wild-Type

units

°C  degree centigade
μF  microfarad, a unit of capacitance
μl  micro liter
bp  Base Pairs
cfu  colony forming units
da  daltons
kb  kilo bases
kV  kilo voits
Mb  mega base
mg  milligrams
Mins  minutes
ml  milliliters
mM  milli molar
rpm  revolutions per minute
Ω  ohm, SI unit of electrical resistance
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Thank you all!
Chapter 1

Introduction

1.1. The genus *Mycobacterium*

The genus *Mycobacterium* comprises Gram-positive aerobic bacteria and is the only member of the family *Mycobacteriaceae* within the order *Actinomycetales*. It shares an unusually high genomic DNA G+C content (62-70%) and production of mycolic acids with closely related genera, *Nocardia* and *Corynebacterium* within the *Actinomycetales*. In addition, within the genus *Mycobacterium*, various species are grouped into complexes (e.g., *M. avium* and *M. tuberculosis* complexes) based on a high degree of genetic similarity as well as the ability to cause similar diseases. The *M. tuberculosis* complex comprises *M. tuberculosis*, *M. bovis*, *M. canetti*, *M. microti* and *M. africanum*, of which only *M. tuberculosis* and *M. bovis* are significant sources of human infection (Saviola and Bishai, 2006).

Mycobacteria are straight or slightly curved non-motile rods (approximately 0.2-0.6 μm wide by 1.0-10 μm long) and are aerobic, non-spore formers which are defined by two remarkable features. Firstly, a cell wall rich in mycolic acids which are long chain fatty acid esters attached to the cell wall through arabinogalactan. Mycolic acids give mycobacterial colonies their characteristic waxy appearance and the cells a tendency to clump and resist dispersion. The mycolic and fatty acids are essential for the resistance of *M. tuberculosis* to drug therapies (Barkan *et al*, 2009). The second characteristic feature is the slow growth rate of all mycobacteria ranging from generation times of 2hrs for *M. smegmatis* to 12 days for *M. leprae* (Saviola and Bishai, 2006).
1.2. **Tuberculosis – the disease**

In 2010, there were 8.8 million (range, 8.5–9.2 million) incident cases of TB, 1.1 million (range, 0.9–1.2 million) deaths from TB among HIV-negative people and an additional 0.35 million (range, 0.32–0.39 million) deaths from HIV-associated TB (World Health Organization Report, 2011). It is a contagious disease, affecting both animals and humans, caused by the bacteria of the *M. tuberculosis* complex. The disease was known as ‘*phthisis*’, derived from the Greek word for ‘wasting away’ from the time of Hippocrates. Vertebral fusion and deformity of the spine, the characteristic features of Potts disease or TB of the spine led historians to date back the disease to 2000–4000 B.C., following the evidence of TB in mummified remains (Bloom and Murray, 1992). However, despite the development of a vaccine, Bacillus Calmette-Guerin (BCG), by French bacteriologists Albert Leon Calmette and Alphense F.M. Guerin in 1924 (Calmette and Guerin, 1924), TB remains a major infectious disease of global proportion.

Mostly the disease is pulmonary (affecting the lungs only). However, in some cases, TB can spread from the lungs to other parts of the body (extra-pulmonary TB) such as lymph nodes, genitourinary tract, gastrointestinal tract, the skeletal and the central nervous systems. Active disease patients with sputum smear-positive pulmonary TB are the main source of infection.

The symptoms for pulmonary TB, that can take months or even years to surface, commonly include a persistent cough usually lasting longer than three weeks, night sweats for weeks or months, weight loss, fatigue, high temperature and breathlessness.

Treatment of active pulmonary TB always involves a combination of many drugs (usually Isoniazid, Rifampin, Pyrazinamide and Ethambutol). However, the drugs may cause certain side effects, including liver problems, changes in vision, orange- or brown-coloured tears and urine, and rash. Furthermore, the development of multidrug-resistant tuberculosis and extensively drug-resistant tuberculosis, which are infections that cannot be treated with first-line and second-line drugs, respectively, continue to keep *M. tuberculosis* a concern in the developed countries.
1.3. Bovine tuberculosis

Bovine tuberculosis (bTB) is an infectious disease of cattle. It is caused by the bacterium *Mycobacterium bovis*. It is a zoonotic disease, which means it can be transmitted from affected animals to people, causing a condition very similar to that caused by *M. tuberculosis*. *M. bovis* has the largest host range of any member of the *M. tuberculosis* complex, including wildlife, captive wildlife, domestic livestock, non-human primates and humans (Biet et al, 2005). Humans may get infected by inhaling air contaminated by an infected animal, eating undercooked infected meat or by drinking *M. bovis* contaminated milk but owing to pasteurization of milk, this incidence has decreased significantly. Among cattle, *M. bovis* may spread by an aerosol route (Saviola and Bishai, 2006). In humans *M. bovis* may cause intra-abdominal TB, cervical lymphadenitis or pulmonary TB (Adler and Rose, 1996). Although *M. bovis* is considered to be an obligate intracellular pathogen, there have been reports of its survival for long periods in the environment (Taylor et al, 2003; Young et al, 2005).

Bovine TB is a serious issue in the UK as the incidence of the disease has been rising. According to the latest bovine TB statistics from the Department for Environment, Food and Rural Affairs (DEFRA), Bovine TB is GB’s biggest endemic animal health issue, costing the taxpayer around £90 million in 2010/11 in England with 3,622 new TB incidents in 2010 (a 7.5% increase on 2009). Around 25,000 cattle were slaughtered for TB control in England in 2010 (www.defra.gov.uk).

Control of bovine tuberculosis in cattle has proven particularly challenging where reservoirs of infection exist in wildlife populations. In Britain and Ireland, control is hampered by a reservoir of infection in *Eurasian badgers (Meles meles)*. Badger culling has positive and negative effects on bovine TB in cattle and is difficult, costly and controversial (Chambers et al, 2011).
Since both *M. bovis* and *M. tuberculosis* are part of the MTB complex sharing many similarities and since *M. tuberculosis* is more extensively studied as compared to *M. bovis*, the survival mechanisms of *M. tuberculosis* are discussed here.

### 1.4. Events in the TB infection process

In pulmonary TB, *M. tuberculosis* spreads via droplets (1-5μm in diameter) from an actively infected person. Droplets of a larger size are efficiently excluded from the lower respiratory tract by the physical barriers of the nasopharynx and upper respiratory tract. Once inhaled, the alveolar macrophages readily phagocytose the bacteria by interaction with mannose receptors (MR) and/or complement receptors (CR1, CR3 and CR4) (Schlesinger, 1993). The interaction between mannose receptors on phagocytic cells and mycobacteria seems to be mediated through the mycobacterial surface glycoprotein lipoarabinomannan (LAM), which is present on the cell wall of the mycobacteria, including virulent strains of *M. tuberculosis*, and is capped by a mannose residue (Schlesinger et al., 1994). As a general phenomenon, phagocytosis usually begins with the phagocytic cell engulfing the invading microbe by engulfing it in a membrane-bound tight vacuole, which is created when pseudopods surround the bacterium and fuse distally.

Upon entry into a host macrophage, *M. tuberculosis* initially resides in an endocytic vacuole called the phagosome. If these macrophages are activated (capable of controlling mycobacterial replication), the mycobacteria-containing phagosome fuses with lysosomes and the bacteria are killed by the hostile environment that includes low pH, reactive nitrogen and oxygen intermediates, lysosomal enzymes (protease, lipase, amylase, nuclease) and toxic peptides. If, on the other hand, the alveolar macrophages are not activated, the bacilli survive within these phagosomes by resisting the host defenses. The bacilli multiply within these phagosomes until the macrophages lyse, probably because of the bacterial burden, and the mycobacteria are released into the surrounding lung tissue where they are taken up by tissue macrophages. The fate of the newly phagocytosed bacteria again depends on the activation state of the macrophage. At some
point during this cycle, an immunocompetent infected human will detect the presence of the mycobacteria and begin to launch an immune response (Russell, 2011).

1.5. Immune response to *M. tuberculosis*

The encounter with tuberculosis causing bacteria can have three possible outcomes.

1. The immune system kills the bacteria with complete eradication of the pathogen.
2. The immune system cannot kill the bacteria, but manages to build a defensive barrier around the infection (the granuloma). This means bacteria will remain in the body but without disease symptoms. This is known as latent TB but could develop into active TB at a later stage if the immune system is compromised.
3. The immune system fails to kill or contain the infection and it slowly spreads to the lungs causing actual disease with symptoms. This is known as active TB.

Initially, the immune response against *M. tuberculosis* is a non-specific inflammatory response (i.e., innate immunity) that destroys both *M. tuberculosis* and the surrounding cells. Lysis of mycobacteria-containing macrophages results in the presentation of *M. tuberculosis* specific antigens to the host immune system, leading to the generation of specific humoral and cell-mediated immune responses.

The initial interaction between mycobacteria and macrophage stimulates specific T-cells; including CD4+ and CD8+ cells. The CD4+ cells are T-helper cells that express the protein CD4 on their surface and whose function is to assist other immune cells in immunologic processes. CD8+ are cytotoxic T-cells that express the CD8 glycoprotein at their surface. They are responsible for the destruction of pathogen infected, tumorous or otherwise damaged and dysfunctional cells. Both CD4+ and CD8+ cells are important in protection against tuberculosis (Serbina and Flynn, 2001).
Although T-cells are the major mediators of protection, effector functions are mostly provided by macrophages. The T-cells and macrophages communicate via various cytokines, notably interleukin 12 (IL-12), interferon gamma (IFNy) and tumor necrosis factor alpha (TNFα). This cross talk first results in macrophage activation. Secondly, this cross talk causes the formation of well organised granulomas, where macrophages, dendritic cells, and different T-cell populations exist in near vicinity. As long as this cross talk is well balanced, productive granulomas develop which contain the bacterium. Once any element of this complex interplay is impaired, the balance is tipped and the productive granuloma can no longer be sustained. Caseous (cheese-like) lesions develop which fail to contain the bacteria. Consequently, bacteria can be disseminated to other tissue sites, to other organs, and even to the environment: active disease develops and the patient becomes contagious (Russell, 2001).

1.6. Survival of *M. tuberculosis* in macrophages

*M. tuberculosis* is a resilient pathogen whose success lies in its ability to evade the host defenses. Following is a brief description of some of the mechanisms that make *M. tuberculosis* a successful pathogen.

1.6.1 Inhibiting phago-lysosomal maturation

A vital component of *M. tuberculosis* pathogenesis is its ability to parasitize macrophages. The macrophage is also the first line of defense against microbial pathogens. For the vast majority of microbes, internalization and exposure to the acidic, hydrolytically active environment of the phagosome is sufficient to bring about their demise (Russell, 2005). However, *M. tuberculosis* is able to resist the destruction by the phagosome. After infecting an alveolar macrophage, *M. tuberculosis* shifts into an infectious state. *M. tuberculosis* also accumulates mycolic and fatty acids on its cell wall in order to survive a very hostile phagosome environment that is nutrient poor, hypoxic, nitrosative, and oxidative (Schnappinger et al, 2003).
The major functions of the macrophage are all dependent on the biology of the phagocytic compartment. Biochemical analysis of the phagocytic compartment shows an increasing abundance of lysosomal constituents as the phagosome matures following internalization of bacteria (Stuart et al., 2007; Garin et al., 2001). Pathogens capable of surviving within macrophages use a range of differing strategies to avoid delivery to the lysosome and subsequent death. Some pathogens such as *Listeria* and *Shigella* escape into the cytosol, others like *Leishmania* and *Coxiella* actually survive and replicate within the lysosomal milieu. Many, like *Legionella, Brucella, Erlichia*, and *M. tuberculosis* and *M. bovis*, subvert the normal progression of their phagosomal compartment and prevent it from fusing with or maturing into an active lysosomal compartment (Russell, 2005).

On phagocytosis, nascent phagosomes acquire the GTPase Rab5 either from the plasma membrane or by fusion with early endosomes. Rab5 recruits phosphatidylinositol-3-kinase (PI3K), which generates phosphatidylinositol-3-phosphate (PI3P); PI3P mediates the recruitment of early endosomal antigen (EEA1) from endosomes. EEA1 is a Rab5 effector that triggers fusion of phagosomes with late endosomes. During the course of phagosomal maturation, early endosomal markers, such as Rab5 and EEA1, are lost from the intermediate phagosome, which then fuses with late endosomes and thereby acquires a second GTPase, Rab7. Late phagosomes fuse with lysosomes to form phagolysosomes, which are characterized by the presence of hydrolytic proteases, such as cathepsin D, and an acidic pH. Phagosomal maturation takes less than one hour. Ca2+ is a key regulator of phagosome maturation because it activates calmodulin and the calmodulin-dependent protein kinase CaMKII — which are necessary for recruitment of PI3K. To prevent phagosomal maturation, pathogenic mycobacteria block the rise in cellular Ca2+ concentration, and thereby affect the association of phosphorylated CaMKII with the phagosomal membrane (Koul, 2004).

The *M. tuberculosis*-containing phagosome is a dynamic compartment and behaves as though it has been arrested at an early stage of its maturation (Russell et al., 2005). As such, it appears to retain all the characteristics of a normal phagosomal
compartment shortly after internalization. The vacuole maintains a lumenal pH of 6.4 (Sturgill-Koszycki *et al.*, 1996), it retains Rab5 (Clemens *et al.*, 2000), the small GTPase associated with membrane fusion events in the early endosome (Sturgill-Koszycki *et al.*, 1996). Several *M. tuberculosis* molecules have been proposed to modulate phagosome maturation. The cell wall lipids lipoarabinomannan (Hmama *et al.*, 2004), trehalose dimycolate (Indrigo *et al.*, 2003), and the sulfolipids (Goren *et al.*, 1976) have all been implicated in blocking phagosome/lysosome fusion. In addition, the bacterial phosphatase SapM (Vergne *et al.*, 2005) and the serine/threonine kinase PknG (Cowley *et al.*, 2004) are thought capable of regulating phagosome maturation. Secreted acid phosphatase M (SapM) is proposed to function through dephosphorylation of phosphatidylinositol 3-phosphate (PI3P) and protein kinase G (PknG) through the phosphorylation of an unknown host protein (Rhode *et al.*, 2007).

### 1.6.2 Escape from phagosome to the cytosol

It was believed for a long time that *M. tuberculosis* resides exclusively in membrane-enclosed vacuoles within the macrophage, gaining access to nutrients through endosomal trafficking. However, some studies reported *M. tuberculosis* that appeared free of a phagosomal membrane after a few days of infection (Leake *et al.*, 1984; McDonough *et al.*, 1993; Myrvik *et al.*, 1984). On the contrary, many studies have reported no evidence of extra-phagosomal *M. tuberculosis* (Clemens *et al.*, 2002; Jordao *et al.*, 2008; Beatty *et al.*, 2000; Pietersen and de Chastellier; 2004; Xu *et al.*, 1994; Mwandumba *et al.*, 2004). It was discovered that *M. marinum* was able to escape from the phagosome into the cytosol, moving around by the propulsion of actin through Arp2/3 complex-mediated actin reorganization (Stamm *et al.*, 2003). This feature was later found to be dependent on a functional RD1 region. It was suggested that ESAT-6 secreted by *M. marinum* ESX-1 could play a direct role in producing pores in *Mycobacterium*-containing vacuole membranes, facilitating *M. marinum* escape from the vacuole and cell-to-cell spread (Smith *et al.*, 2008).

The publications about *M. marinum* initiated a new debate about whether *M. tuberculosis* is also able to also escape from the phagosome. Van der Wel *et al* (2007)
reported that \emph{M. tuberculosis} could be found in increasing numbers in the cytosol of dendritic cells and macrophages after at least two days of infection, and that escape from the phagosome was dependent on ESAT-6 and led to increased replication rates in the cell cytoplasm. The phenomenon was distinct from that observed in \emph{M. marinum} as \emph{M. tuberculosis} did not form actin comet tails. The authors argued that this phenomenon was not merely due to the bacteria outgrowing their phagosome and that it was not a result of membrane disruption during apoptotic cell death.

The contradicting results concerning escape from the phagosome could be explained due to different types of cells and techniques used and the different experimental set ups in these studies. Furthermore, the mycobacterial cell wall can be mistaken for a phagosomal membrane due to its lipid-rich composition, giving false-negative results (Zuber \textit{et al.}, 2008). Another explanation for the presence of cytosolic \emph{M. tuberculosis} in some preparations is that the phagosomal membrane is lysed in the process of lysing the cell (de Chastellier, 2009).

\subsection*{1.6.3 Resisting phagosomal acidic pH.}

The pH of the macrophage compartment, in which \emph{M. tuberculosis} resides, ranges from pH 6.2 to 4.5, depending on the activation state of the macrophage (MacMiking \textit{et al.}, 2003; Schaible \textit{et al.}, 1998; Via \textit{et al.}, 1998). In macrophages that have not yet been immunologically activated, many species of mycobacteria inhibit the fusion of phagosomes with lysosomes and thereby reside in an environment that is only very mildly acidic with a pH of about 6.2 (MacMiking \textit{et al.}, 2003). Lack of acidification of the mycobacterial phagosome is likely to be due to the absence of the vacuolar proton ATPase (Huynh \textit{et al.}, 2007). However, after immunologic activation of the macrophage, such as by exposure to gamma interferon (IFN-\(\gamma\)), the fusion block is relieved and the phagosomal compartment acidifies to pH 4.5 to 5.0 (MacMiking \textit{et al.}, 2003; Schaible \textit{et al.}, 1998; Via \textit{et al.}, 1998; Sibley \textit{et al.}, 1985).

The mycobacterial cell wall also plays a critical role in resistance to acid. Recent work has demonstrated the existence of an additional outer lipid bilayer surrounding mycobacteria (Zuber \textit{et al.}, 2008). This complex cell envelope acts as a formidable permeability barrier for antibacterial effectors, including protons.
The outer membrane protein A (OmpATb) of M. tuberculosis is a pore-forming protein or porin and is important for acid resistance and virulence. The ompATb mutant showed delayed growth at pH 5.5 in vitro, and reduced growth in macrophages and in mice. However, the exact mechanism of resistance remains unknown (Raynaud et al., 2002). It is suggested that the channel is pH-sensitive and has a propensity to close at low pH (Molle et al., 2006). Decreasing cell envelope permeability at low pH may represent an adaptive mechanism used by Mtb to survive in the low pH of the phagosome. Vandal et al., (2008) used transposon mutagenesis to identify genes responsible for M. tuberculosis's acid resistance. A strain disrupted in Rv3671c, a previously uncharacterized gene encoding a membrane-associated protein, was sensitive to acid and failed to maintain intrabacterial pH in acid in vitro and in activated macrophages. Growth of the mutant was also severely attenuated in mice suggesting some role of Rv3671c in acid resistance as well as virulence.

1.6.4 Evading toxicity by reactive oxygen and nitrogen intermediates (RNIs)

Two key antimycobacterial mechanisms of macrophages include the generation of reactive oxygen species or ROS (particularly hydrogen peroxide and superoxide anion) and reactive nitrogen intermediates (RNI) (such as nitric oxide) which exert toxic effects on the bacilli. T cell-derived cytokines, mainly IFN-γ and TNF-α, activate macrophages, which then generate nitric oxide and other RNIs which are mycobactericidal (Scanga et al., 2001). Studies have shown that M. tuberculosis has evolved several strategies to evade this toxicity. It has been shown that inducible nitric oxide synthase (iNOS), a cytoplasmic protein, may be recruited to the phagosomes and this recruitment may be inhibited by M. tuberculosis (Miller et al., 2004). M. tuberculosis is also known to produce catalase to inactivate reactive oxygen species.

M. tuberculosis utilizes a range of mechanisms to defend against ROS and RNS including direct scavenging of the reactive species and the repair and protection
of proteins and DNA (Ehrt and Schnappinger, 2009). The resistance of \textit{M. tuberculosis} to ROS is partly due to the thick \textit{M. tuberculosis} cell wall containing lipoarabinomannan (LAM) and cyclopropanated mycolic acids, as well as phenolic glycolipid I (PGL-I), which act as potent scavengers of oxygen radicals (Flynn and Chan, 2001). In addition, \textit{M. tuberculosis} produces the ROS scavenging enzymes catalase (KatG; Manca \textit{et al}, 1999; Ng \textit{et al}, 2004), superoxide dismutases (SodA and C; Piddington \textit{et al}, 2001; Tullius \textit{et al}, 2001), and the peroxidase and peroxynitrite reductase complex of alkyl hydroperoxide reductase (AhpC), dihydrolipoamide succinyltransferase (SucB), and dihydrolipoamide dehydrogenase (Lpd) (Bryk \textit{et al}, 2002). It is also reported that ingestion of \textit{M. tuberculosis} via complement receptor three (CR3) is advantageous to the bacillus as it prevents macrophage activation (Caron and Hall, 1998). However, contradicting studies have shown no effect of CR3 deletion on replication and survival of \textit{M. tuberculosis} in macrophages (Hu \textit{et al}, 2000).

Another potential mechanism for blunting the toxic effects of RNI is the presence of two haemoglobin-like proteins encoded by \textit{glbN} and \textit{glbO} in \textit{M. tuberculosis} (Quellet \textit{et al}, 2002). Furthermore, inhibition of respiration by nitric oxide induces a dormancy program in \textit{M. tuberculosis} that leads to increased survival and persistence of the pathogen in immune cells (Voskuil \textit{et al}, 2003).

### 1.6.5 Avoidance of killing by ubiquitin-derived peptides

Another component of the antimicrobial repertoire of macrophages is lysosomal killing of \textit{M. tuberculosis} mediated by ubiquitin-derived peptides (Alonso \textit{et al}, 2007). The ubiquitination destroys tubercle bacilli by autophagy as a ubiquitin-derived peptide impairs the membrane integrity of \textit{M. tuberculosis} that allows nitric oxide to kill more efficiently. On the contrary, decreased outer membrane permeability protects \textit{M. tuberculosis} from killing by ubiquitin-derived peptides (Purdy \textit{et al}, 2009).
1.6.6 Inhibiting the apoptosis of MTB-containing macrophages

Apoptosis (programmed cell death or cell suicide) of infected macrophages is one of the several host defenses against M. tuberculosis infection. M. tuberculosis modulates apoptosis by targeting caspases; one of the main executors of the apoptotic process. The M. tuberculosis components that inhibit apoptosis include cell wall components (Lopez et al., 2003), virulence-related secretion system encoded by secA2 that transports superoxide dismutase (encoded by sodA) to control reactive oxygen intermediates, and nicotinamide adenine dinucleotide dehydrogenase (encoded by nuoG) (Hinchey et al., 2007; Velmurugan et al., 2007). By inhibiting apoptosis of macrophages, M. tuberculosis avoids host defenses and escapes from infected cells by causing necrotic cell death (Gan et al., 2008). Derrick and Morris (2007) demonstrated that ESAT6 (early secreted antigenic target-6) capable of disrupting artificial lipid bilayers (Hsu et al., 2003), induces apoptosis via the receptor-mediated extrinsic pathway in human macrophages depending on the dose of the protein and the expression of caspase genes. Persson et al., (2009) found that p38 mitogen-activated protein kinase (p38-MAPK) was a key mediator in M. tuberculosis-induced apoptosis in human neutrophils.

1.6.7 Non-replicating intracellular M. tuberculosis

Another feature of M. tuberculosis is its ability to go into the phenotypically drug-resistant non-replicating dormant state in latent infection. It is a major impediment to curing the disease since currently available drugs cannot kill latent M. tuberculosis. Granulomas are thought to limit M. tuberculosis growth by restricting bacterial access to oxygen and nutrients and exposing the bacilli to acidic pH and immune effectors, such as nitric oxide. However, the bacilli are not necessarily eradicated and can persist for many years.

DosR is widely considered essential for TB latency. Boon and Dick (2002) showed that the disruption of the dosR (for dormancy survival regulator) gene resulted in a more than 1,000-fold loss of viability in oxygen-starved non-growing culture. This shows that dosR function is essential for adaptation to hypoxic survival.
as the genetic loss of dosR resulted in loss of induction of the other dormancy-induced proteins, indicating that expression of these proteins is under the control of DosR. Park et al (2003) also showed that nearly all the genes regulated by hypoxia require DosR transcriptional factor for their induction. On the other hand, it was shown that loss of dosR produced no discernible phenotype in mice, even though expression of DosR regulon genes was perturbed, suggesting that additional genes may be essential for entry into and maintenance of bacteriostasis. (Rustad et al., 2008). Others have found very modest phenotypes of the ΔdosR mutant in guinea pigs, mice and rabbits (Malhotra et al., 2004; Converse et al., 2009). Hypervirulence of the ΔdosR mutant in severe combined immunodeficiency (SCID) and immunocompetent mice is also documented (Parish et al., 2003).

1.7. Role of Region of Difference 1 (RD1) in mycobacterial virulence

Gene deletion was an important parameter of the attenuation that led to the generation of the avirulent vaccine strain BCG. Comparative studies have identified genes that are present in the pathogenic strains of mycobacteria but absent in the vaccine strain BCG (Brodin et al., 2004; Mahairas et al., 1996). Initial studies identified three regions of difference (also known as region of deletion (RD1, RD2, RD3) that were present in M. tuberculosis but absent in M. bovis BCG (Mahairas et al., 1996). Subsequent studies identified 16 RD regions within the genomes of M. tuberculosis complex (Behr et al., 1999).

One of the most active areas of mycobacterial research is the regulation, selection and transport of protein virulence factors that are likely to interact with the host cell during infection. It was observed that in contrast to vaccination by live BCG, immunization with heat-killed BCG confers little or no protection against TB, indicating that active secretion of antigenic proteins induces appropriate protective T-cell responses (Horwitz et al, 1995). Therefore short-term culture filtrate proteins (CFP) were purified from early growth phase of M. tuberculosis and were assessed for antigenicity. It was found that some of the low molecular weight proteins were able to induce immune response with 6kDa early secreted antigenic target-6 (ESAT-6) being the most potent
among such proteins (Sorenson et al, 1995). Upon molecular characterization of various culture filtrate proteins, it was reported that some proteins did not display any secretion signals to be recognized by the general secretory pathway (GSP). Among these were ESAT-6 and a 10kDa culture filtrate protein (CFP-10) (Berthet et al, 1998). They are thought to be secreted via the Esx-1 secretion system [early secreted antigen 6 kilodaltons (Esat-6) secretion system 1], or more recently called type VII secretion systems (T7S system) (Abdallah et al, 2007). This secretion system is primarily encoded by genes within and adjacent to, the region of difference 1 (RD1). Fascinatingly, upon surveying other members of the M. tuberculosis complex for RD1, it was found that this region was missing from all BCG vaccine strains, yet was present and expressed in all fully virulent members (Gordon et al, 1999; Behr et al, 1999). Studies have shown RD1 to be at least partly responsible for the attenuated phenotype and reduced pathogenicity (Lewis et al., 2003; Pym et al., 2002).

1.8. Acanthamoeba castellani

Protozoa (from Greek words ‘proto’ meaning first and ‘zoa’ meaning animals) are unicellular heterotrophs that belong to the Kingdom Protista according to the latest system of classification (Cavalier-Smith, 2004). The term ‘amoebae’ represent the largest group of organisms in protists that have been studied. In 1930, Castellani discovered an amoeba as a contaminant in a fungal culture (Castellani, 1930). This amoeba exhibited pseudopodia in the trophozoite stage while the encysted form exhibited a double wall. So the term acanth (Greek for ‘spikes’) was added to the term ‘amoeba’ to indicate the presence of spine-like structures (known now as ‘acanthopodia’) and hence it is called ‘Acanthamoeba’.

Acanthamoeba are ubiquitous and have been isolated from as diverse environments as sea water, ocean sediments, beaches, pond water, stagnant water, soil, mud, shoreline soils, fresh water lakes, river water, contact lenses, surgical instruments, air-water interface, air, factory discharges, cooling towers of electric and nuclear power plants, humidifiers, air conditioning units, sewage, compost, fruits and vegetables and body surfaces and tissues (Khan, 2006).
There are two stages in the *Acanthamoeba* life cycle: a vegetative (trophozoite) stage that feeds mainly on bacteria and detritus present in the environment and reproduces by binary fission, and a dormant but resistant cyst stage. The trophozoites range in size from 15 to 50 μm depending upon the species (Khan, 2006). The trophozoites exhibit acanthopodia which play an important role in adhesion to surfaces, movements and capturing prey. When food becomes scarce or when it is facing desiccation or other environmental stresses like temperature, pH and osmotic pressure changes, the amoebae round up and encyst. The cysts are double walled and have an average diameter of 9 to 12 μm. The ectocyst or outer cyst wall is wrinkled with folds and ripples and contains protein and lipid. The inner cyst wall is the endocyst and contains cellulose. The endocyst varies in shape: it may be stellate, polygonal, oval, or spherical. Pores or ostioles that are covered by convex–concave plugs or opercula are present at the junction of the ectocyst and the endocyst. Upon return to favorable growth conditions, the dormant amoeba is activated to leave the cyst by dislodging the operculum and reverting to a trophic form (Page, 1967).

*Acanthamoeba* have the basic features of eukaryotic cells including membrane bound organelles like rough endoplasmic reticulum, a small amount of smooth endoplasmic reticulum, Golgi complex, free ribosomes, mitochondria, food vacuoles, contractile vacuole, microtubules and a nucleus. It has been observed that amoebae and human phagocytic cells share several unique functions, such as the ability to crawl on a substrate or to ingest microorganisms by phagocytosis. Just like macrophages they can ingest particles such as bacteria that are trafficked into lysosomes for digestion/killing. there are many experimental advantages in working with amoebae. For example, one of the most studied amoebae *Dictyostelium* (Cosson and Soldati, 2008) grow at room temperature in a simple and cheap medium or in the presence of bacteria, with no need for a CO₂-enriched atmosphere. They can be grown in a shaken suspension, allowing large-scale cultures and biochemical analysis. Their small, haploid, sequenced, and annotated genome (Eichinger *et al.*, 2005) makes them so easily amenable to genetic analysis.
1.9. *Acanthamoeba and bacteria interactions*

*Acanthamoeba* are known to interact with various Gram-positive and Gram-negative bacteria resulting in a range of outcomes. *Acanthamoeba* may act as bacterial predators, bacterial reservoirs or bacterial Trojan horses.

1.9.1 *Acanthamoeba as bacterial predators*

*Acanthamoeba* utilize bacteria as a food source, ingesting them by phagocytosis and subsequently lysing them in the phagolysosome. Although *Acanthamoeba* consume both Gram-positive and Gram-negative bacteria, they preferentially graze upon Gram-negative bacteria (Bottone *et al.*, 1994). However, the ability of *Acanthamoeba* to feed on bacteria depends on the virulence properties of bacteria and also the environmental conditions. Alsam *et al.*, (2005) showed that in the absence of nutrients, virulent strains of *E. coli* K1 invade *Acanthamoeba* and remain viable intracellularly. And upon availability of nutrients, K1 escapes *Acanthamoeba*, grows exponentially and lyses the amoebae. On the contrary, in the absence of nutrients, the avirulent strains of *E. coli* K12 are phagocytosed by *Acanthamoeba* and are used as a food source.

1.9.2 *Acanthamoeba as bacterial reservoirs*

The importance of amoeba as bacterial reservoirs is highlighted even more since most of these interactions involve human pathogens such as *Legionella pneumophila* (causes Legionnaires’ disease) (Rowbotham, 1980); *Escherichia coli* 0157 (causes diarrhea) (Barker *et al.*, 1999); *Coxiella burnetii* (causes Q fever) (La Scola and Raoult, 2001); *Pseudomonas aeruginosa* (causes keratitis) (Michel *et al.*, 1995); *Vibrio cholera* (causes cholera) (Thorn *et al.*, 1992); *Helicobacter pylori* (causes gastric ulcers) (Winiecka-Krusnell *et al.*, 2002); *Listeria monocytogenes* (causes listeriosis) (Ly and Muller, 1990); and *Mycobacterium avium* (an opportunistic pathogen of severely immunocompromised human immunodeficiency virus (HIV)-infected individuals) (Krishna-Prasad and Gupta, 1978; Steinert *et al.*, 1998). These pathogens not only survive intracellularly but multiply within the amoebae allowing the bacteria to transmit throughout the environment, evade host defenses and/or chemotherapeutic drugs and reproduce in sufficient numbers to produce disease.
1.9.3 *Acanthamoeba* as bacterial Trojan horse

The success of many bacterial pathogens lies in their ability to survive harsh environmental conditions during transmission from one host to another. In this scenario, *Acanthamoeba* may act as a ‘Trojan horse’ for bacteria. The term ‘Trojan horse’ is used to describe bacterial presence inside *Acanthamoeba* as opposed to a ‘carrier’ function, which may be mere attachment/adsorption to the surface (Khan, 2006). Studies have shown that *Burkholderia cepacia* (a causative agent of lung infection) (Landers *et al.*, 2000) and *Chlamydia pneumonia* (causative agent of respiratory disease) (Essig *et al.*, 1997) remain viable within *Acanthamoeba* but do not multiply. These findings suggest that *Acanthamoeba* facilitate bacterial transmission and/or provide protection against the human immune system. Moreover, *Acanthamoeba* is able to resist harsh environmental conditions such as extreme temperatures, pH and osmolarity by forming a cyst which makes them useful as bacterial vectors.

1.10. The effect of bacteria on amoeba

Little is known about the effect of bacterial infection on amoeba compared to the effect of amoeba on bacteria. However, there are a few reports where symbiotic bacteria bring about gene switching (Jeon and Jeon, 2004), changes in protein expression (AbuKwaik *et al.*, 1994) and the inhibition of cyst formation (Michel *et al.*, 2000) in the host amoeba. Co-incubation with *P. aeruginosa* altered amoeba survival, and maximum survival occurred in the presence of the invasive strain of *P. aeruginosa* (Cengiz *et al.*, 2000). It was also found that the contamination of contact lens care systems with amoeba and certain bacterial species favoured the rapid growth of the amoeba which led to the induction of keratitis (Botzone *et al.*, 1994). Similar results were shown where the attachment of *Acanthamoeba* to hydrogel lenses was enhanced by the presence of *P. aeruginosa* (Gorlin *et al.*, 1996). On the contrary, the growth and survival of amoeba was suppressed by *P. aeruginosa* at higher densities (Wang and Ahearn, 1997). The extensive changes in the pH due to a large number of bacteria, the presence of toxic bacterial pigments and/or production of inhibitory by-products like phospholipases (Qureshi *et al.*, 1993) may be responsible for the decline of amoeba.
1.11. The importance of Protozoa as a model system for investigation

The first clearly identified reports of *Acanthamoeba* pathogenesis came out in the early 1970s when it was associated with granulomatous encephalitis (Jager and Stamm, 1972) and keratitis. *Acanthamoeba* was first shown to be infected with bacteria in 1954 (Drozanski, 1956) and later was shown to harbour bacteria as endosymbionts (Proca-Ciobanu et al, 1975) and also acting as a reservoir for pathogenic facultative mycobacteria (Krishna-Prasad and Gupta, 1978). Since the association of *Acanthamoeba* with Legionnaires' disease (Rowbotham, 1980), there has been an exponential increase in the interest in *Acanthamoeba* research worldwide.

*Acanthamoeba* are gaining much attention in research worldwide due to their diverse roles in the nutrient and energy turnover in the ecosystem, their ability to phagocytose like macrophages, their roles as vectors and Trojan horses of microbial pathogens and their action to cause serious human disease. The present understanding of survival strategies in amoeba come from the extensive studies of *Legionella* associations with *Acanthamoeba* and *Hertmannella*. *Legionella* has complex growth requirements but it is able to survive in nutritionally deficient conditions by associating with a protist. Rowbotham (1980, 1983, 1986, 1993) not only demonstrated the intracellular multiplication of *L. pneumophila* in free-living amoebae but he also suggested that humans become infected with *Legionella* by inhaling vesicles or amoebae filled with bacteria. Dispersion of *Legionella* from lysed amoebae may occur in the environment or within a human host. Therefore, amoebae play an important role in disease spreading.

Approximately 20-24% of *Acanthamoeba* isolated from clinical settings or the environment harbor intracellular bacteria (Fritsche et al, 2000). Nonetheless, the relationship between amoeba and bacteria may differ depending on the environmental conditions such as temperature. For example, *Candidatus Odyssella thessalonicensis* incubated with *Acanthamoeba* at 30°C to 37°C lyse the amoebae while coincubation at 22°C appeared to result in a symbiotic relationship and lysis did not occur (Birtles et al, 2000).
Perhaps the most important aspect of amoebae-bacteria interactions is the adaptation to invasion of mammalian cells. Studies on *Legionella* associations with protozoans strongly suggest that the mechanisms for recognition, entrance and intracellular proliferation of bacteria in the amoebic and mammalian host cells are similar (Table 1.1). Same genes are shown to be utilized by *Legionella* to grow within protozoan and mammalian cells. However, the identification of novel genes which are required for the infection of macrophages but not the protozoan hosts suggests that adaptation to intracellular habitats in free-living amoebae has preceded acquisition of genes specific for survival and replication in macrophages (Gao *et al.*, 1998).

**Table 1.1. Similarity of *Legionella*’s mechanisms in both amoebae and macrophages (Adapted from Greub and Raoult, 2004).**

<table>
<thead>
<tr>
<th>Stage</th>
<th>Amoeba</th>
<th>Macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Entry</td>
<td>Coiling phagocytosis</td>
<td>Coiling phagocytosis</td>
</tr>
<tr>
<td>Traffic</td>
<td>No phagosome-lysosome fusion</td>
<td>No phagosome-lysosome fusion</td>
</tr>
<tr>
<td>Phagosome</td>
<td>Associated with rough endoplasmic reticulum</td>
<td>Associated with rough endoplasmic reticulum</td>
</tr>
<tr>
<td>Replication</td>
<td>Intraphagosomal</td>
<td>Intraphagosomal</td>
</tr>
<tr>
<td>Exit</td>
<td>Host cell lysis</td>
<td>Host cell lysis</td>
</tr>
</tbody>
</table>

### 1.12. Mycobacterial survival inside amoebae

One of the first reports of mycobacterial survival in amoebae came from Jadin (1975) and by Krishna Prasad and Gupta (1978). Since then there have been many reports of mycobacterial survival and growth within amoebae (Cirillo *et al.*, 1997; Steinert *et al.*, 1998; Skriwan *et al.* 2002). *A. castellanii* was reported to accumulate several mycobacterial species in their cytoplasm although, whether the authors could differentiate phagosomal or cytoplasmic localization is debatable (Krishna Prasad and Gupta, 1978). There has also been evidence of mycobacterial survival inside amoebae for several years without any detrimental effects on the host (Yu *et al.*, 2007). Interestingly, pathogenic species of mycobacteria, like *M. avium* and *M. ulcerans* survived intracellularly without any apparent injury to the host cells. However, amoebae incubated
with non-pathogenic species like *M. smegmatis*, *M. fortuitum* and *M. phlei*, were lysed within 5 days. Whether it was due to accumulation, intracellular multiplication or toxic affects remains unclear (Thomas and McDonnell, 2007).

In the late 1990s there was increasing interest in amoebae-mycobacteria associations as numerous mycobacterial species were demonstrated to survive ingestion by free living amoebae (Table 1.2). *M. avium* was shown to interact with *A. castellanii*, *A. polyphaga* (Cirillo *et al*, 1997; Steinert *et al* 1998; Whan *et al*, 2006) *Tetrahymena pyriformis* and *Dictyostelium discoideum* (Strahl *et al* 2001; Skriwan *et al*, 2002). Mycobacteria can survive inside trophozoites as well as in cysts (Adekambi *et al*, 2006).
Table 1.2. Some *Mycobacterium* species reported to survive within *Acanthamoeba*.

<table>
<thead>
<tr>
<th><em>Mycobacterium</em> Species</th>
<th>Reference (survival in <em>Acanthamoeba</em>)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. leprae</em></td>
<td>Jadin, 1975</td>
</tr>
<tr>
<td><em>M. smegmatis</em></td>
<td>Krishna Prasad and Gupta, 1978)</td>
</tr>
<tr>
<td><em>M. phlei</em></td>
<td>Krishna Prasad and Gupta, 1978)</td>
</tr>
<tr>
<td><em>M. abscessus</em></td>
<td>Krishna Prasad and Gupta, 1978)</td>
</tr>
<tr>
<td><em>M. avium subsp. avium</em></td>
<td>Krishna Prasad and Gupta, 1978)</td>
</tr>
<tr>
<td><em>M. marinum</em></td>
<td>Cirillo <em>et al.</em>, 1997</td>
</tr>
<tr>
<td><em>M. fortuitum</em></td>
<td>Cirillo <em>et al.</em>, 1997</td>
</tr>
<tr>
<td><em>M. avium</em></td>
<td>Cirillo <em>et al.</em>, 1997</td>
</tr>
<tr>
<td><em>M. bovis</em></td>
<td>Taylor <em>et al.</em>, 2003</td>
</tr>
<tr>
<td><em>M. bovis BCG</em></td>
<td>Taylor <em>et al.</em>, 2003</td>
</tr>
<tr>
<td><em>M. massiliense</em></td>
<td>Adekambi <em>et al.</em>, 2006</td>
</tr>
<tr>
<td><em>M. lentiflavum</em></td>
<td>Adekambi <em>et al.</em>, 2006</td>
</tr>
<tr>
<td><em>M. intracellulare</em></td>
<td>Adekambi <em>et al.</em>, 2006</td>
</tr>
<tr>
<td><em>M. gastri</em></td>
<td>Adekambi <em>et al.</em>, 2006</td>
</tr>
<tr>
<td><em>M. porcinum</em></td>
<td>Adekambi <em>et al.</em>, 2006</td>
</tr>
<tr>
<td><em>M. avium subsp. paratuberculosis</em></td>
<td>Whan <em>et al.</em>, 2006</td>
</tr>
<tr>
<td><em>M. xenopi</em></td>
<td>Drancourt <em>et al.</em>, 2007</td>
</tr>
<tr>
<td><em>M. kansasii</em></td>
<td>Goy <em>et al.</em>, 2007</td>
</tr>
</tbody>
</table>

Adekambi *et al* (2006) investigated the ability of 26 *Mycobacterium* spp. to survive in the trophozoites and cysts of *Acanthamoeba polyphaga*. All the species of *Mycobacterium* tested were able to enter *A. polyphaga* and were able to survive in the intracellular milieu of the trophozoites for the duration of the experiment (5 days). All the *Mycobacterium* spp. tested also survived inside *A. polyphaga* cysts for 15 days at room temperature. They also examined the fate of mycobacteria inside *A. polyphaga* cysts by transmission electron microscopy and found *M. chelonae* and *M. abscessus* visible in the spaces between the two walls (i.e., inner and outer walls) of the cyst. Some species (e.g.,
M. septicum) were observed to have been present on the inner side of the outer wall and in the cytoplasm of the cyst.

Ben Salah and Drancourt (2010) studied the intra-ameobal location of all members of the Mycobacterium avium complex (MAC) and demonstrated the engulfment and replication of mycobacteria into vacuoles of A. polyphaga trophozoites. Mycobacteria were further entrapped within amoebal cysts and all MAC members typically resided within the exocyst.

*M. avium, M. tuberculosis* and *M. bovis* survived in *A. polyphaga* cysts for up to 18 days. *M. bovis* and a few *M. avium* organisms were observed on the inner side of the outer wall and in the cytoplasm of the *A. polyphaga* cysts (Mba Medie et al., 2011). Taylor et al (2003) demonstrated the survival of *M. bovis* in *A. castellanii* for 14 days. In contrast, *M. bovis* BCG numbers were below the detection limit at the end of the experiment. This study highlighted the apparent disparity in the abilities of *M. bovis* and the BCG vaccine strains to survive within *A. castellanii*. Therefore, further studies are needed to understand the reasons for this effect, which would be invaluable information for attempts to develop an improved vaccine against tuberculosis. The present study aims to investigate the genetic factors that contribute to the mycobacterial survival in amoebae.

Mycobacteria are intrinsically resistant to disinfection in comparison to other vegetative bacteria (McDonnell and Russell 1999; McDonnell, 2007) especially chlorine- (Le Dantec et al 2002), heat- (Blackwood et al, 2005) and gluteraldehyde- (Griffiths et al. 1999) tolerant strains of mycobacteria have been reported. While the exact mechanism remains to be validated, the resistance can be attributed to the complex cell wall structure of mycobacteria (Thomas and McDonnell, 2007). Once the mycobacteria are internalized inside amoebae, resistance profiles are likely to be promoted further as Whan et al (2006) demonstrated that *A. polyphaga* trophozoites protected *M. avium* against chlorine at 2mg/l for 30-60 mins. Also 26 mycobacterial species in encysted amoebae were protected against chlorine exposure at 1.5mg/l for 24 hrs (Adekambi et al, 2006).
Similarly, intracellular mycobacteria were protected from antibiotics as \( M. \ avium \) strains inside \( A. \ castellanii \) had increased resistance to antibiotics as compared to macrophages (Miltner and Bermudez, 2000).

### 1.13. Genetic requirements for intracellular survival of mycobacteria in amoebae

Amoebae ingest bacteria including pathogens and there are two ways by which the protozoa eliminate pathogens from their cells: expulsion as vesicles and killing. The formation of vesicles has been observed with \( L. \ pneumophila \) (Berk et al., 1998), \( Salmonella \ enterica \) (Brandl et al., 2005), \( Francisella \ tularensis \) (Abd et al., 2003) and \( M. \ septicum \) (Adekambi et al., 2006).

However, the pathogens have evolved ways to survive within the protozoa. For example, the \( pmi \) (protozoan and macrophage infection) gene (Miyake et al., 2005) and \( mip \) (macrophage infectivity potentiator) gene (Cianciotto and Fields, 1992) of \( L. \ pneumophila \) are required for survival both inside amoeba and macrophages. In \( L. \ pneumophila \), the Dot/icm system (a secretion system essential for intracellular growth) was also found to be a shared determinant for survival in protozoa and macrophages (Segal and Shuman, 1999). \( L. \ pneumophila \) utilizes the Dot/icm system to inject bacterial effector molecules into the host cell cytosol to survive and replicate in the intracellular compartment through modulation of phagosome biogenesis (Nagai et al., 2002). A candidate similar to the Dot/icm for the survival of mycobacteria in amoebae could be the ESX-1 secretion system encoded by the region of difference 1 (RD1). ESAT-6 and CFP-10 play an important role in the translocation of \( M. \ marinum \) and \( M. \ tuberculosis \) from the phagosome to the cytosol (Smith, 2003; Hogedorn et al., 2009).

Various \( M. \ avium \) genes are shown to be required for survival in macrophages. These include genes encoding proteins for transport/binding, synthesis, modification and degradation of macromolecules (Danelishvili et al., 2004). Tenant and Bermudez (2006) showed that many \( M. \ avium \) genes are commonly upregulated following macrophage and
amoeba infection. Danelishvili et al (2007) showed that \textit{M. avium} mutants that were defective for macrophage invasion were also defective for the invasion of amoeba, suggesting that \textit{M. avium} might have evolved mechanisms that are used to enter amoebae and human macrophages.

The availability of the complete sequence of the 4,345,492 bp genome of \textit{M. bovis} (Gamier et al, 2003) allowed detailed insight into the genetic basis of key phenotypic traits of the bovine tubercle bacillus. The single, circular chromosome with an average G+C content of 65.63% and containing 3,952 genes encoding proteins bears striking similarity (>99.95% identical at the nucleotide level) to \textit{M. tuberculosis}. (Gamier et al, 2003). Table 1.3 shows a broad classification of \textit{M. tuberculosis} genes according to their function.

<table>
<thead>
<tr>
<th>Gene function</th>
<th>% of total \textit{M. tuberculosis} genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid metabolism</td>
<td>5.7</td>
</tr>
<tr>
<td>Information pathways</td>
<td>5.2</td>
</tr>
<tr>
<td>Cell wall and cell processes</td>
<td>13.0</td>
</tr>
<tr>
<td>Stable RNA’s</td>
<td>1.3</td>
</tr>
<tr>
<td>ISs and phages</td>
<td>3.4</td>
</tr>
<tr>
<td>PE and PPE proteins</td>
<td>4.2</td>
</tr>
<tr>
<td>Intermediary metabolism and respiration</td>
<td>22.0</td>
</tr>
<tr>
<td>Proteins of unknown function</td>
<td>15.3</td>
</tr>
<tr>
<td>Regulatory proteins</td>
<td>4.7</td>
</tr>
<tr>
<td>Conserved hypothetical proteins</td>
<td>22.9</td>
</tr>
<tr>
<td>Virulence, detoxification, adaptation</td>
<td>2.3</td>
</tr>
</tbody>
</table>

Although deciphering the exact roles of these genes in mycobacterial virulence is a hot topic of research all over the world, these studies mostly involve the use of macrophages and other immune cells. The relationship between \textit{Legionella pneumophila} and \textit{A. castellanii}, in which the protozoa was used as a virulence model suggests that the virulence mechanisms of bacterial pathogens evolved through interaction with protozoa in the environment and the protozoa provide an environmental host for the bacterial pathogens that currently cause disease in humans. Therefore, amoebae hold great potential as a model system for investigating the secrets of some of the successful pathogens like \textit{M. tuberculosis}/\textit{M. bovis}.

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1.14 Transposon Site Hybridization (TraSH)

One of the most effective ways of determining gene function is to study the mutants that either lack that gene or express an altered version of it. Determining which cellular processes have been disrupted or compromised in such mutants will then frequently provide a window to a gene's biological role. However, studying single genes at a time can be labour-intensive. DNA microarrays prove to be an effective alternative that allows systematic analysis of thousands of genes at one time.

Transposon site hybridization (TraSH) is a technique that was developed by two independent research groups; Sassetti and coworkers and Badarinarayana and his team, almost simultaneously in 2001 (Sassetti et al., 2001; Badarinarayana et al., 2001). It combines transposon mutagenesis and microarray hybridization that enables the comprehensive identification of genetic elements required for bacterial survival under various selective conditions. In this technique, the mycobacterial genome is randomly mutagenized by transposon insertions, genomic DNA is extracted and the DNA regions flanking the transposon are selectively amplified, fluorescently labeled and quantified and identified by microarray hybridization.

Before TraSH, two general approaches were used to monitor the survival of individual mutants in a pool; signature tagged mutagenesis (STM) and genetic footprinting. STM, originally developed by David Holden and his colleagues in 1995, utilizes a number of transposons which are each tagged with a unique nucleotide sequence, allowing simultaneous detection of each mutant in a pool. However, it is limited due to the relatively small size of each pool and the labour required to perform a comprehensive screen. Also, for STM to work, the tags need to be pre-selected on the basis of their efficient amplification, labeling and lack of cross-hybridization to other tags.

The other strategy genetic footprinting is based on PCR amplification of the transposon insertions relative to a fixed site on a chromosome, followed by gel electrophoresis. Loci that are unable to sustain the insertion leave a 'footprint' on the gel and hence are regarded as essential for survival under that particular condition (Smith et
al., 1995). Although this technique allows the analysis of much larger pools than STM, only a small number of genes can be screened in each PCR reaction.

In this project, TraSH is also used as a high throughput screening tool to study the survival genes of *M. bovis* in protozoa.
1.15. Aims and objectives of the project

Although the survival of *M. bovis* inside amoebae has been reported (Taylor *et al.*, 2003; Young *et al.*, 2005), the genes responsible for the persistence of mycobacteria inside amoeba have not been identified yet. Based on *Legionella*-amoeba interactions and its usefulness in identifying the *Legionella* virulence genes, this study is designed to screen for genes that might play a role in mycobacterial survival inside the protozoa, which in turn can be applied to a better understanding of the mycobacterial-mammalian cell interactions.

The aim of the project is to highlight the genetic factors that contribute to the survival of *Mycobacterium bovis* inside *Acanthamoeba castellanii*.

Firstly, the role of Region of Difference 1 (RD1) in bacterial survival in amoebae was investigated using an RD1 knock-in strain of BCG.

Secondly, strains carrying deletions/interruptions in individual genetic regions/genes of *M. tuberculosis* were examined to have any role in survival inside the amoebae. The genes investigated encode isocitrate lyase (Rv0467), CtgR (Rv2745) and the VapC (Rv2548) toxin-antitoxin system.

Lastly, whole genomes of the avirulent *M. bovis* BCG and virulent *M. bovis* were screened by a TraSH mutagenesis system to provide a more systematic assessment of the importance of mycobacterial genes for intra-amoebic survival.
Chapter 2

General Materials and Methods

2.1 Strains and growth conditions

*M. tuberculosis* H37Rv and *M. bovis* NCTC 10772 were grown in MiddleBrook 7H9 broth with 0.05% sodium pyruvate, 25% OADC enrichment and 0.05% Tween80.

*Acanthamoeba castellanii* (Neff CCAP 1501/1A) was grown in proteose peptone glucose (PPG) medium in Nunc tissue culture flasks at 25°C for 5 days to get 90% confluency.

2.2 Counting amoebae

Total number of amoebae was determined by counting in a hemacytometer. The hemocytometer (Invitrogen) chamber was filled with 10μl of amoebae culture. Amoebae in 4 big squares (1mm² area each) in each of the two chambers were counted and the average was taken. The number of amoebae/ml was calculated by multiplying the average number of amoebae counted by dilution factor and volume of the big square.

2.3 Genomic DNA extraction

10ml of freshly grown log phase bacterial culture was centrifuged at 3000 xg for 10 mins. Supernatant was discarded and the cell pellet was resuspended in 1ml of GTE (Glucose, Tris, EDTA) solution. Contents were transferred into a 2-ml centrifuge tube and centrifuged for 10 minutes (18000 xg). Supernatant was discarded and the cell pellet was resuspended in 450μl of GTE solution. 50μl of a 10mg/ml lysozyme solution was added, mixed gently and incubated at 37°C overnight. As *M. bovis* and *M. tuberculosis* are class 3 pathogens, growth and manipulation of virulent mycobacteria was carried out in containment level 3 laboratory. Further steps were completed in containment level 2 laboratory however, it was essential that the strains are heat killed before they can be
taken out of the containment level 3 laboratory. Therefore, DNA of *M. bovis* was heat inactivated at 80°C for 2 hours before proceeding further. 100 μl of 10% sodium dodecyl sulphate (SDS) was added to the overnight suspension and mixed gently. 50 μl of 10mg/ml Proteinase K solution was added to above, mixed gently and incubated at 55°C for about an hour. 200 μl of 5M NaCl was added and mixed gently. Cetyltrimethylammonium bromide (CTAB) solution was preheated to 65°C and 160 μl was added into the above solution. Solution was mixed gently and incubated at 65°C for 20 mins.

An equal volume (1ml) of chloroform-isoamyl alcohol (24:1, v/v) was added, shaken gently to mix and was centrifuged for 5 mins at 18000 xg. Nine hundred μl of the upper aqueous phase was transferred into a fresh microcentrifuge tube. The extraction with chloroform-isoamyl alcohol (above two steps) was repeated. Eight hundred μl of supernatant was transferred into a fresh 1.5 ml microcentrifuge tube. To 800 μl of the aqueous layer, 560 μl (0.7x volume) of isopropanol was added and mixed gently by inversion until the DNA precipitated out of solution. After the DNA was precipitated, it was incubated at room temperature for 5 mins and then was centrifuged for 10 mins at 2300 xg. The supernatant was aspirated and 1ml 70% ethanol was added to wash the DNA pellet, mixed gently by inversion and centrifuged for 10mins at 2300 xg. Supernatant was aspirated and the DNA pellet was air-dried for 15 mins, taking care not to over-dry. Dried DNA was covered with 50 μl of elution buffer and stored at 4°C to allow pellet to dissolve overnight. DNA was stored at -20°C.

### 2.4 Generation of competent mycobacteria

50ml of bacterial culture was grown to mid-log phase and was centrifuged at 2100 xg for 10 mins. The pellet was resuspended in 40ml 10% sterile glycerol solution and centrifuged at 2100 xg for 10 mins. The supernatant was discarded. The pellet was resuspended in 40ml 10% sterile glycerol solution and centrifuged at 2100 xg for 10 mins. The supernatant was discarded. The final pellet was resuspended in 1ml 10% sterile glycerol solution.
2.5 Transformation of mycobacteria by electroporation

1μl each of sample and control DNA and sterile H₂O was added into 1.5ml eppendorf tubes. 200μl of freshly prepared competent cells were added into each microcentrifuge tube and mixed gently by pipetting and finally transferred to warm 0.2ml, 2mm gap electroporation cuvettes. Electroporation was done at 25μF, 1000Ω and 25kV using Biorad electroporator. One ml of warm MiddleBrook 7H9 was added to each cuvette and mixed. The contents of the cuvettes were transferred into bijou tubes and incubated overnight in a shaking incubator at 37°C. The overnight culture of each electroporation was plated onto MiddleBrook 7H11 plates with and without hygromycin as appropriate. The plates were incubated at 37°C for three to four weeks.

2.6 QIAquick PCR purification kit protocol (using a microcentrifuge)

This protocol is designed to purify single or double stranded DNA fragments from PCR and other enzymatic reactions. Fragments ranging from 100bp to 10kb are purified from primers, nucleotides, polymerases and salts using QIAquick spin columns in a microcentrifuge.

Five volumes of Buffer PBI were added to one volume of the PCR sample and mixed. The color of the mixture was yellow. The QIAquick spin column was placed in a provided 2ml collection tube. The sample was applied to the QIAquick column and centrifuged* for 30 to 60s to bind DNA. The flow-through was discarded and the QIAquick column was placed into the same tube. To wash, 0.75ml Buffer PE was added to the QIAquick column and centrifuged for 30-60s. The flow-through was discarded and the QIAquick column was placed into the same tube and the column was centrifuged for an additional 1 min. The QIAquick column was placed in a clean 1.5ml microcentrifuge tube.

To elute DNA, 50μl of Buffer EB (10mM Tris-Cl, pH 8.5) or water (pH 7.0-8.5) was added to the centre of the QIAquick membrane and the column was centrifuged for about 1min.

* All centrifugations were performed at 15700xg in a conventional table top microcentrifuge at room temperature.
2.7 MinElute PCR purification kit protocol (using a microcentrifuge)

This protocol is designed to purify double stranded DNA fragments from PCR reactions resulting in high end-concentrations of DNA. Fragments ranging for 70bp to 4kb are purified from primers, nucleotides, polymerases and salts using MinElute spin columns in a microcentrifuge.

Ninety-six percent ethanol was added to Buffer PE before use. Five volumes of Buffer PB were added to one volume of the PCR sample and mixed. The color of the mixture was yellow. The MinElute column was placed in a provided 2ml collection tube in a suitable rack. The sample was applied to the MinElute column and centrifuged* for 1min to bind DNA. The flow-through was discarded and the column was placed into the same tube. To wash, 0.75ml Buffer PE was added to the MinElute column and centrifuged for 1min. The flow-through was discarded and the MinElute column was placed into the same tube and the column was centrifuged for an additional 1 min at maximum speed. MinElute column was placed in a clean 1.5ml microcentrifuge tube.

To elute DNA, 10µl of Buffer EB (10mM Tris-Cl, pH 8.5) or water was added to the centre of the membrane. The column was allowed to stand for 1min and then centrifuged for about 1min.

* All centrifugations were performed at 15700xg in a conventional table top microcentrifuge at room temperature.

2.8 QIAquick Gel extraction kit protocol (using a microcentrifuge)

This protocol is designed to extract and purify DNA of 70bp to 10kbp from standard agarose gels in TAE or TBE buffer. Up to 400mg agarose can be processed per spin column. This kit can also be used for DNA clean up from enzymatic reactions.

DNA fragments were excised from the agarose gel with a clean, sharp scalpel. The gel slice was weighed in a colorless tube and three volumes of Buffer QG was added to 1 volume of gel. The tube was incubated at 50°C for 10mins to dissolve the gel completely. After the gel had completely dissolved, 1 gel volume of isopropanol was added to the sample and mixed. The QIAquick spin column was placed in a provided 2ml
collection tube. The sample was applied to the column and centrifuged* for 1min to bind DNA. The flow-through was discarded and the QIAquick column was placed into the same tube. To wash, 0.75ml Buffer PE was added to the QIAquick column and centrifuged for 1min. The flow-through was discarded and the QIAquick column was placed into the same tube and the column was centrifuged for an additional 1 min at maximum speed. QIAquick column was placed in a clean 1.5ml microcentrifuge tube.

To elute DNA, 50µl of Buffer EB (10mM Tris-Cl, pH 8.5) or water was added to the centre of the membrane. The column was allowed to stand for 1min and then centrifuged for about 1min.

* All centrifugations were performed at 15700×g in a conventional table top microcentrifuge at room temperature.

2.9 Media and solutions recipes

**Proteose Peptone Glucose (PPG) medium**
15.0 g proteose peptone, 18.0 D-glucose, 1.0 litre Page's Amoeba Saline Solution (PAS)

**MiddleBrook 7H9 broth**
4.7g Middlebrook 7H9, 50ml OADC, 8.0 ml sodium pyruvate stock solution, 500µl Tween80

**MiddleBrook 7H11 agar**
21.0 g Middlebrook 7H11, 50.0 ml OADC, 8.0ml sodium pyruvate stock solution

**Luria Bertani (LB) broth**
10g tryptone, 5g yeast extract, 10g NaCl, 1L dH2O pH adjusted to 7.0, autoclaved

**Page's Amoeba Saline Solution (PAS)**
Stocks per 500 ml
(1) 12.0 g NaCl, 0.40 g MgSO4.7H2O, 0.60 g, CaCl2.6H2O
(2) 14.20 g Na2HPO4, 13.60 g KH2PO4
Medium per litre: Stock solutions (1) and (2) 5.0 ml each in up to 1 litre water.
Glucose, Tris, EDTA (GTE) solution
25mM Tris-HCl, 10mM EDTA, 50mM glucose

Cetyltrimethylammonium bromide (CTAB) solution
4.1g sodium chloride in 90ml distilled water and adding 10g cetrimide at 65°C.

Sodium pyruvate stock solution
4.1g sodium pyruvate in 20ml distilled water; sterilized through a 0.2μm filter unit

Sodium dodecyl sulphate (SDS) solution
1.0g SDS in 100ml distilled water

Amikacin solution
0.01g in 100ml PAS (100μg/ml); sterilized through a 0.2μm filter unit

4% paraformaldehyde (PFA)
4.0g paraformaldehyde in 100ml water

50X TAE Buffer
121g Tris base, 28.6ml acetic acid, 50ml 0.5M EDTA pH 8.0, dH2O to 500ml

1X TAE Buffer
10mL 50X TAE buffer, 490mL ddH2O

1% agarose gel in TAE
1g agarose, 100mL 1X TAE
Chapter 3

The importance of Region of Difference 1 (RD1) and other genes in the survival of *Mycobacterium bovis* inside *Acanthamoeba castellanii*

3.1. Introduction

3.1.1 *M. bovis* BCG

The only successful vaccine against TB is BCG which was first generated by Calmette and Guerin who passaged a strain of *M. bovis* 230 times in ox bile medium between 1908 and 1921. The resulting vaccine was believed to have struck a balance between preserved immunogenicity and reduced virulence (Calmette and Guerin, 1924). BCG is currently given to most infants worldwide but the clinical efficacy in adults varies from 0-80% (Fine, 1995).

How BCG originally lost its virulence remained a mystery until Mahairas and colleagues showed that BCG lacks several regions of MTB chromosome (Mahairas *et al*, 1996; Behr *et al*, 1999; Gordon *et al*, 1999) but the mutation responsible for its attenuation has never been identified. Although it seems likely that many genetic alterations contribute to the avirulent phenotype. It has been estimated that *M. bovis* BCG is missing at least 61 genes with respect to *M. tuberculosis* (Table 3.1).
Table 3.1: Deleted or truncated genes in the RD regions of BCG.

<table>
<thead>
<tr>
<th>Region of deletion</th>
<th>ORF gene</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>RD1</td>
<td>Rv3871-Rv3879c</td>
<td>PE, PPE, ESAT-6, other hypothetical proteins</td>
</tr>
<tr>
<td>RD2</td>
<td>Rv1978-Rv1988</td>
<td>Methyltransferases, permease, MPT64, ribonucleotide reductase, conserved hypothetical, membrane and secreted proteins, LysR transcriptional regulator</td>
</tr>
<tr>
<td>RD3</td>
<td>Rv1573-Rv1586c</td>
<td>phiRv1 prophage</td>
</tr>
<tr>
<td>RD4</td>
<td>Rv1505c-Rv1516cc</td>
<td>Membrane proteins, enzymes for exopolysaccharide synthesis</td>
</tr>
<tr>
<td>RD5</td>
<td>Rv2346c-Rv2353c</td>
<td>ESAT-6, PE, PPE family members, phospholipases C</td>
</tr>
<tr>
<td>RD6</td>
<td>Rv3425-Rv3428c</td>
<td>PPE proteins, IS1532</td>
</tr>
<tr>
<td>RD7</td>
<td>Rv1964-Rv1977</td>
<td>Many integral membrane proteins, MceP invasins</td>
</tr>
<tr>
<td>RD8</td>
<td><em>ephA-lpqG</em></td>
<td>Epoxide hydrolase, monooxygenase, lipoprotein, ESAT-6, PE, PPE protein family</td>
</tr>
<tr>
<td>RD9</td>
<td><em>cobL-Rv2075</em></td>
<td>Precorrin methylase, oxidoreductase, exported protein</td>
</tr>
<tr>
<td>RD10</td>
<td>Rv0221-Rv0223c</td>
<td>Enoyl CoA hydratase, aldehyde dehydrogenase</td>
</tr>
<tr>
<td>RD11</td>
<td>Rv2645-Rv2695c</td>
<td>phiRv prophage</td>
</tr>
<tr>
<td>RD12</td>
<td>sseC-Rv3121</td>
<td>Thiosulfate sulfurtransferase, molybdopterin converting factor, methyltransferase cytochrome P450</td>
</tr>
<tr>
<td>RD13</td>
<td>Rv1255c-Rv1257c</td>
<td>Transcriptional regulator, cytochrome P450, dehydrogenase</td>
</tr>
<tr>
<td>RD14</td>
<td>Rv1765c-Rv1773c</td>
<td>PE-PGRS, conserved hypothetical, IclR transcriptional regulator</td>
</tr>
<tr>
<td>RD15</td>
<td>Rv0309-Rv0312c</td>
<td>Conserved hypotheticals</td>
</tr>
<tr>
<td>RD16</td>
<td>Rv3400-3405c</td>
<td>Transcriptional regulator, conserved hypotheticals, phosphoglucomutase</td>
</tr>
</tbody>
</table>

3.1.2. Region of difference 1 (RD1)

Of all the deletions, RD1 is the only region that is missing from all BCG strains but is present in every virulent MTB (Mostowry et al., 2004; Brosch et al., 2002). Also two naturally attenuated members of the MTB, the Vole bacillus and Dassie bacillus were found to have deletions in the RD1 region (Brodin et al., 2002; Mostowry et al., 2004).
This deleted region is about 9.5 kb and removes 7 genes completely (Rv3872-Rv3878) and truncates 2 others (Rv3871 and Rv3879c) (Cole et al., 1998). To explore the underlying mutation responsible for the attenuation of BCG, many associations have been made regarding the origin of BCG, mycobacterial virulence and the RD1 region (Mahairas et al., 1996; Behr et al., 1999; Wards et al., 2000).

RD1 is part of a 15-gene locus known as ESX-1 that encodes a specialized secretion system dedicated to the secretion of two important genes encoding a 6kDa early secreted antigenic target, (ESAT-6) (Cole et al., 1998) and a 10kDa culture filtrate protein (CFP-10) (Berthet et al., 1998; Sorenson et al., 1995). These are two of the most potent immunogens in humans and rodents. ESAT-6 is thought to have a possible direct role in cytotoxicity by enhancing membrane permeability as has been shown with artificial membranes (Hsu et al., 2003). ESAT-6 and CFP-10 are capable of forming a 1:1 complex (Renshaw et al., 2002) which binds to the cell surface receptors, leading to the modulation of the host cell behaviour to the advantage of the pathogen (Renshaw et al., 2005).

In a study by Pym et al. (2002), restoration of RD1 in BCG by gene knock-in resulted in a marked change in colonial morphology towards that of virulent tubercle bacilli. The BCG::RD1 and M. microti::RD1 knock-ins grew more vigorously than controls in immunodeficient mice, inducing extensive splenomegaly and granuloma formation. Increased persistence was observed when immunocompetent mice were infected with the BCG::RD1 knock-in, whereas BCG controls were cleared. Knocking-in five other RD loci did not affect the virulence of BCG. It was also reported that mice and guinea pigs vaccinated with the recombinant strain BCG::RD1-2F9 were better protected against challenge with M. tuberculosis, showing less severe pathology and reduced dissemination of the pathogen, as compared with control animals immunized with BCG alone (Pym et al., 2002). This enhanced protection may be due to an increase in the number of antigens presented by BCG that lead to an increase in the number of CD4+ T cells primed by vaccination.
3.1.3 Studying the role of selected genes in the survival of mycobacteria

The regulation of cellular physiology and metabolic pathways is essential for survival and enables the pathogens to establish pathogenicity. A key aspect of the pathogenicity of *M. tuberculosis* is its ability to dramatically change its physiology and metabolism in different environments.

With the advent of molecular techniques, thousands of new gene sequences from different organisms are added to the databases everyday. However, this is raw information and needs to be refined in terms of gene function. One way of studying gene function is to compare the DNA sequence to the database for homology to existing sequences which in turn can provide information about the possible gene function. But sometimes this approach is not effective in deciphering the role of the gene in a cell or an organism. For this purpose, more sophisticated techniques are required. Often the real challenge is to explore what exactly a gene does inside the cell? The answer could be found using the gene knockout approach. By mutating a specific gene *in vitro* and then replacing the normal copy in the genome with a mutant form, scientists can assess its *in vivo* function. This technique is referred to as *gene-targeted knockout*, or simply “knockout” (Lodish *et al*, 2000). Three *M. tuberculosis* gene knock out strains Rv2548, Rv2547 and Rv0467 (Table 3.2) were selected from the laboratory strain collection and their survival was studied in *A. castellanii*. 
Table 3.2: Selected MTB gene knock outs for survival in *A. castellanii*.

<table>
<thead>
<tr>
<th>Gene knock outs</th>
<th>H37Rv:ΔRv0467</th>
<th>H37Rv:ΔRv2548</th>
<th>H37Rv:ΔRv2745c</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gene name</strong></td>
<td><em>icl1</em></td>
<td><em>vapC19</em></td>
<td><em>Rv2745c</em></td>
</tr>
<tr>
<td><strong>Functional category</strong></td>
<td>Intermediary metabolism and respiration</td>
<td>Virulence, detoxification, adaptation</td>
<td>Regulatory proteins</td>
</tr>
<tr>
<td><strong>Function</strong></td>
<td>Involved in glyoxylate bypass (at the first step), an alternative to the tricarboxylic acid cycle (in bacteria, plants, and fungi) [catalytic activity: isocitrate = succinate + glyoxylate]. Involved in persistence in the host.</td>
<td>Unknown</td>
<td>Possibly involved in transcriptional mechanism.</td>
</tr>
<tr>
<td><strong>Product</strong></td>
<td>Isocitrate lyase ICL (isocitrase) (isocitratase)</td>
<td>Possible VAPC19</td>
<td>Possible transcriptional regulatory protein</td>
</tr>
</tbody>
</table>

3.1.4. Rv0467

Rv0467 (gene name *icl*) codes for an enzyme isocitrate lyase which catalyzes the formation of succinate and glyoxylate from isocitrate. This is an important step in the glyoxylate shunt.

The phagosome is a difficult organelle to study because profound biochemical shifts accompany the host cell's effort to kill and degrade microbial pathogens. *M. tuberculosis* is among the microorganisms most successful at adapting to long-term residence in macrophage phagosomes. Metabolic processes that are critical for the pathogen to go into dormancy, survive under the non-replicating drug-resistant state, and get reactivated when the immune system of the host is weakened remain poorly understood. It has been recognized for more than half a century that the pathogen inside the host utilizes fatty acids as the major energy source and that glyoxylate cycle plays a critical role in the use of fatty acids as the main carbon source.
Since *M. tuberculosis* is able to persist inside macrophages (McKinney *et al.*, 2000) it must be able to adapt itself to the changing host cell environment. This essentially involves changes in the mycobacterial metabolism. It has been shown that fatty acids are the main source of carbon and energy during the persistent phase of mycobacterial infection (Boshoff and Barry, 2005; Munoz-Elias and McKinney, 2006). *M. tuberculosis* might obtain these fatty acids from lung surfactant (Munoz-Elias and McKinney, 2005), by hydrolysis of phagosome membrane lipids (Kondo *et al.*, 1985), or from the macrophage triacylglycerol stores (Mason *et al.*, 1982). Bacteria are able to use the fatty acids by beta oxidation and the glyoxylate cycle.

When fatty acids are the sole source of carbon, a functional glyoxylate cycle with its key enzymes isocitrate lyase (ICL) and malate synthase (MS) is necessary for bacterial survival. The glyoxylate cycle is an anaplerotic pathway metabolizing acetyl coenzyme A (acetyl-CoA), the end product of beta-oxidation. Icl cleaves isocitrate into succinate and glyoxylate, whereas malate synthase subsequently condenses glyoxylate with another molecule, acetyl-CoA, to malate. Overall, the glyoxylate cycle ensures the bypass of two oxidative steps of the tricarboxylic acid cycle, thereby allowing the synthesis of carbohydrates from fatty acids and anaplerosis of tricarboxylic acid cycle intermediates. *M. tuberculosis* contains two *icl* enzymes (*icl1*, a prokaryote-like isoform (Rv0467), and *icl2*, a eukaryote-like isoform) and one malate synthase (Micklinghoff *et al.*, 2009).

Much attention has been paid to this gene over the past decade. Numerous studies have shown that *icl* activity is necessary for virulence of *M. tuberculosis* in mice and bacterial growth in macrophages (Munoz-Elias and McKinney, 2005), and *icl1*-deficient *M. tuberculosis* are unable to sustain a chronic, persistent infection in mice (McKinney *et al.*, 2000). However, the role of *icl* in survival of *M. bovis* inside amoebae has not been investigated before.
Rv2548 (gene name vapC19) in *M. tuberculosis* encodes a possible toxin VapC19 as a part of toxin-antitoxin (TA) operon with Rv2547 (or vapB19 which encodes the antitoxin). Toxin-antitoxin (TA) systems are ubiquitous in prokaryotic genomes and have been proposed to play a role in several important cellular functions. These systems typically consist of a two-gene operon encoding a toxic protein that targets an essential cellular function and an antitoxin that binds to and inhibits the toxin.

Toxin-antitoxin gene cassettes appear to be involved in bacterial survival as well as in the formation of persistent pathogenic infections in hostile host-cell environments (Zhu et al., 2006). Toxin-antitoxin (TA) loci were first characterized as plasmid-borne genes involved in bacterial plasmid maintenance by eliminating the plasmid-free progeny cells but they are chromosomally encoded as well (Gerdes, 2000). Chromosomal TA modules have also been proposed to function as regulators of cell growth in response to environmental stress (Gerdes, 2000). Ramage et al. (2009) showed that some *M. tuberculosis* TA system genes are upregulated during hypoxia or macrophage infection, providing evidence that these systems are activated during specific stresses likely to be encountered in the host.

In the majority of individuals infected with *M. tuberculosis*, the bacteria initially grow and then establish a latent, asymptomatic infection that can persist for decades with the potential to reactivate later in life (Stewart et al., 2003). These persistent bacteria are thought to adopt a slowly or non-replicating state in response to environmental stresses encountered in the host (Gill et al., 2009), yet the mechanisms by which this non-replicating state is achieved are unknown. Because the majority of current antimicrobials require bacterial growth to exert their killing action, these non-replicating persistent bacteria are thought to comprise an important subpopulation of bacteria that is refractory to antibiotic therapy (Gomez and McKinney, 2004). A similar antibiotic-tolerant state is elicited by TA system activation in other bacteria (Keren et al., 2004), suggesting that TA systems may contribute to the long duration of antibiotic therapy required to cure
tuberculosis. Pandey and Gerdes (2005) found that *M. tuberculosis* H37Rv and *M. tuberculosis* CDC1551 have 38 (Table 3.3) and 36 TA loci, respectively. *M. bovis* contains more than 50 putative TA systems (Van Melder and Saavedra De Bast, 2009).
Table 3.3. MTB H37Rv chromosomal TA loci.

<table>
<thead>
<tr>
<th>Sl. no.</th>
<th>TA locus</th>
<th>Toxin</th>
<th>Protein</th>
<th>Antitoxin</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>relBE1</td>
<td>RelE1</td>
<td>Rv 1246c</td>
<td>RelB1</td>
<td>Rv 1247c</td>
</tr>
<tr>
<td>2</td>
<td>relBE2</td>
<td>RelE2</td>
<td>Rv 2866</td>
<td>RelB2</td>
<td>Rv 2865</td>
</tr>
<tr>
<td>3</td>
<td>relBE3</td>
<td>RelE3</td>
<td>Rv 3358</td>
<td>RelB3</td>
<td>Rv 3357</td>
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<td>4</td>
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<td>Rv 1956</td>
<td>HigA1</td>
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<tr>
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<td>MazF1</td>
<td>Unannotated</td>
<td>MazE1</td>
<td>Unannotated</td>
</tr>
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<td>MazF2</td>
<td>Rv 0659c</td>
<td>MazE2</td>
<td>Rv 0660c</td>
</tr>
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<td>Rv 1102c</td>
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<td>Protein</td>
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3.1.6 Rv2745

The Rv2547c is a putative transcriptional regulator which has high similarity (54–60% identity) to the Clp protease gene regulator (ClgR), a protein that activates transcription of Clp proteases in the *M. tuberculosis* related actinomycetes *Streptomyces coelicolor* and *Corynebacterium glutamicum* (Engels *et al.*, 2005; Bellier and Mazodier, 2004). Several independent studies have reported modulation of *clpP* gene transcription upon alteration of Rv2745c mRNA levels (Sherrid *et al.*, 2010; Barik *et al.*, 2010; Mehra and Kaushal, 2009). Transcription of ClgR, and members of its regulon, is induced following phagocytosis of *M. tuberculosis* by macrophages (Schnappinger *et al.*, 2003). Estorninho *et al.* (2010) showed that Rv2745c also activates expression of other proteases and chaperone systems and provided evidence for the ClgR dependent regulation of at least 10 genes, of which the majority was involved in protein stabilization, disassembly or degradation. They demonstrated that the induction of these genes is an essential element of the successful parasitism of the macrophage host cell.

In a recent study, Sherrid *et al.*, (2010) utilized reaeration from a defined hypoxia model to characterize the adaptive response of MTB following a return to favorable growth conditions. They identified several genes involved in reordering bacterial metabolism, including a ribonucleotide reductase gene essential for aerobic growth along with genes involved in fatty acid metabolism, sugar transport, and a triacylglycerol lipase (*lipY*), which may facilitate utilization of stored triacylglycerols
during reaeration (Dawes et al, 2003; Low et al, 2009), chaperones and proteases, as well as the transcription factor Rv2745c. It is up-regulated in late hypoxia, and is further induced upon reaeration (Sherrid et al, 2010). Expression of Rv2745c is also upregulated upon phagocytosis by macrophages and is essential for optimal growth in the macrophages (Estominho et al., 2010). However, the role of Rv2745 has not been studied in protozoan infection.

3.2. Aims and objectives

The aims of this chapter were to identify M. tuberculosis complex genes that are important for survival in amoebae.

Specific objectives:

BCG has been demonstrated to have reduced survival in amoebae compared to M. bovis. RD1 represents one of the major genetic differences between BCG and M. bovis. Thus, objective is to investigate the role of RD1 by comparing the survival of BCG carrying a wild type RD1 region on an integrating cosmid (BCG::RD1) with BCG::pYUB412 (empty vector), M. bovis BCG and M. bovis in amoebae and examining their localization inside amoebae after infection.

This objective aims to identify if M. tuberculosis genes known to be important for virulence in macrophages are also important for M. bovis survival in A. castellaniii. Investigate the roles of icl (Rv0467), a transcriptional regulator (Rv2745) and VapC toxin (Rv2548) in the survival of MTB in amoebae.
3.3. Materials and Methods

3.3.1. Strains and growth conditions:

All of the Mycobacterium tuberculosis knock-out strains H37Rv: Δ0467, H37Rv:Δ2548 and H37Rv:Δ2745 and the control strains M. tuberculosis H37Rv and M. bovis NCTC 10772 were grown in MiddleBrook 7H9 broth with 0.05% sodium pyruvate, 10% oleic acid-albumin-dextrose-catalase (OADC) enrichment and 0.05% Tween80.

M. bovis BCG::RD1 and BCG::pYUB412 were grown on 7H11 with 10% OADC and 50μg/ml Hygromycin.

A. castellanii (Neff CCAP 1501/1A) was grown as described in Chapter 2, section 2.1.

3.3.2. Electroporation of ElectroMax DH10B E.coli Cells

1μl of plasmid 2F9 and 1 μl of empty plasmid pYUB412 (both constructs from Institute of Pasteur, Paris) were added into microfuge tubes. Also 1 μl of water and 1 μl of pSMT1 (Snewin et al., 1999) was added to separate tubes to serve as negative and positive controls respectively. The construct 2F9 had an inserted RD1 region (henceforth called as pRD1) with an ampicillin and hygromycin selectable marker-genes. Construct pYUB412 did not have the gene inserted and was an empty construct (Appendex 1). Plasmid pSMT1 is a shuttle vector in E. coli and mycobacteria and employs hygromycin as a selectable marker (Snewin et al., 1999).

ElectroMax DH10B (Invitrogen) are commercially prepared E.coli cells which can be transformed by electroporation for high efficiency transformation. These cells were thawed on wet ice and were mixed gently by tapping. 200 μl of cells were added to each microfuge tube and gently pipetted. The cell/DNA mixtures were carefully transferred to chilled cuvettes. The cuvettes were gently tapped to ensure that the cell/DNA mixture makes contact to the electrodes all the way across the bottom of the cuvette chamber. Care was also taken to avoid any bubble formation.

The samples and control were electroporated at 20 μF, 1000Ω and 2.5kV using the Biond electroporator. 1ml of S.O.C medium (Sigma-Aldrich) was added to each cuvette and the contents were transferred into 50ml Falcon centrifuge tubes and
incubated at 37°C for 1 hour in a shaking incubator (225rpm). Aliquots (100 µl) of each sample and control were plated on nutrient agar with and without Ampicillin and incubated overnight at 37°C.

3.3.3. Plasmid purification

Plasmid was purified from *E. coli* cultures using the Qiagen Maxiprep Kit.

Transformation of *E. coli* with 2F9 produced an *E. coli* strain carrying RD1 and is now known as *E.coli::RD1*. The transformed *E.coli::RD1* and *E.coli::pYUB412* were streaked onto LB agar-Ampicillin (100µg/ml) selective plates and incubated at 37°C for 24 hrs. A single colony of each of *E.coli::RD1* and *E.coli::pYUB412* was picked from the plates and was used to inoculate a 5ml starter culture (LB containing Ampicillin). The culture was incubated for approximately 8 hrs at 37°C with vigorous shaking (300rpm).

The starter culture was diluted 1/1000 into selective LB medium and grown at 37°C for 12-16 hrs with vigorous shaking (300rpm). The bacterial cells were harvested by centrifugation at 6000xg for 15 minutes at 4°C. Bacterial pellets were resuspended in 10ml Buffer P1 (resuspension buffer) by pipetting for efficient cell lysis. 10ml Buffer P2 (lysis buffer) was added and mixed thoroughly by vigorously inverting the sealed tube 4-6 times and incubated at room temperature (15-20°C) for 5 mins.

During the incubation period, QIAfilter cartridges (QIAGEN) were prepared. The caps of the QIAfilter Maxi cartridges (QIAGEN) were screwed onto the outlet nozzle and were placed onto a convenient rack. 10 ml chilled Buffer P3 (neutralization buffer) was added to the lysate and mixed immediately and thoroughly by vigorously inverting 4-6 times. A fluffy white precipitate containing genomic DNA, proteins and cell debris became visible. The lysate was poured into the barrel of the QIAfilter cartridge and incubated at room temperature for 10 mins. A precipitate containing proteins, genomic DNA and detergent floated and formed a layer on top of the solution.

HiSpeed Maxi tips (QIAGEN) were equilibrated by applying 10ml Buffer QBT (equilibration buffer) and columns were allowed to empty by gravity flow. Flow of
buffer began automatically by reduction in surface tension due to the presence of detergent in the equilibrium buffer. The HiSpeed tip was allowed to drain completely.

The caps were removed from the QIAfilter outlet nozzle. Plunger was gently inserted into QIAfilter Maxi cartridge and the lysate was filtered into the previously equilibrated HiSpeed Tip. The cleared lysate was allowed to enter the resin by gravity flow. The HiSpeed Maxi tip was washed with 60ml Buffer QC (wash buffer). DNA was eluted in 15ml Buffer QF (elution buffer). DNA was precipitated by adding 10.5ml room temperature isopropanol to the eluted DNA, mixed and incubated at room temperature for 5 mins.

During the incubation, plungers were removed from 30ml syringe and the QIAprecipitator Maxi Module was attached onto the outlet nozzle. The QIAprecipitator was placed over a waste bottle and the eluate/isopropanol mixture was transferred into the 30ml syringe and plunger was inserted. The eluate/isopropanol mixture was filtered through the QIAprecipitator using constant pressure.

The QIAprecipitator was removed from the syringe and the plunger was pulled out. The QIAprecipitator was reattached and 2ml 70% ethanol was added to the syringe. DNA was washed by inserting the plunger and pressing the ethanol through the QIAprecipitator using constant pressure.

The QIAprecipitator was removed from the syringe and the plunger was pulled out. The QIAprecipitator was attached to the syringe again and the plunger was inserted. The membrane was dried by pressing the air through the QIAprecipitator quickly and forcefully. This step was repeated.

The outlet nozzle of the QIAprecipitator was dried with absorbent paper to prevent ethanol carryover. The plunger was removed from a 5ml syringe and the QIAprecipitator was attached onto the outlet nozzle. The outlet of the QIAprecipitator was held over a 1.5ml collection tube and 1ml of Buffer TE was added to the syringe. Plunger was inserted and the DNA was eluted into the collection tube using constant pressure.
The QIAprecipitator was removed from the 5ml syringe and the plunger was pulled out and the QIAprecipitator was reattached to the 5ml syringe. The eluate from step 24 was transferred to the 5ml syringe and eluted for a second time into the same 1.5 ml tube. Plasmid yield was determined by agarose gel electrophoresis and spectrophotometry. Both plasmids were stored at -20°C.

3.3.4. BCG electroporation with pRD1 and pYUB412

BCG competent cells were made with 50ml of BCG culture as described in Chapter 2, section 2.4).

Purified plasmids pRD1 and pYUB412 were thawed on ice. 1µl each of pRD1, pYUB412 and sterile H₂O was electroporated into 200µl of freshly prepared BCG competent cells as described in Chapter 2, section 2.5). About 100µl of each electroporation reaction was plated onto 7H11 plates with and without hygromycin (50µg/ml) and incubated at 37°C for three to four weeks.

3.3.5. Infecting A. castellanii with BCG::pRD1 and BCG::pYUB412

3.3.5.1 Growing amoebae

1 ml of fresh A. castellanii culture was added into each well of the 24-well tissue culture plates. Four such plates were prepared and were incubated at 25°C overnight to help amoebae attach onto the surface of the wells.

All plates were examined under the inverted microscope to observe the confluent layers of the amoebae.

3.3.5.2 Growing mycobacterial strains

BCG::pRD1 and BCG::pYUB412 were grown in 7H9 + OADC with hygromycin (50µg/ml) while the control strains M. tuberculosis H37Rv and M. bovis were grown in the same medium but without the antibiotic to reach an O.D of 0.7.

3.3.5.3 Infecting A. castellanii with BCG::pRD1 and BCG::pYUB412

Supernatant was removed from all the tissue culture plate wells to get rid of the non-adherent amoebae. 1ml of each strain was added into the respective wells of the plates and incubated at 20°C for 2 hours. Supernatant was removed and 1ml of PAS-amikacin (100µg/ml) was added in each well to get rid of the non-adherent bacteria.
Plates were incubated at 20°C for 2 hours. Supernatant was removed and the wells were washed with sterile PAS twice. 1 ml of sterile PPG was added into each well of three of the four plates. The plates were carefully sealed and incubated at 20°C for four, seven and fourteen days. To the remaining plate, 1 ml of sterile 0.5% SDS was added. The contents of each well were serially diluted and plated. Five replicates of each strain were processed at each time point. Bacterial counts were determined by Miles and Misra (Drop Count) technique. The previous two steps were repeated for the remaining three plates after four, seven and fourteen days.

3.3.6 Visualizing the BCG::pRD1, BCG::pYUB412, BCG and M. bovis interactions with A. castellanii using Confocal scanning laser microscopy

3.3.6.1 Growing amoebae
Round glass coverslips (20 mm) were sterilized by dipping in 70% ethanol. A coverslip was placed in the centre of each of the wells of the 6 well tissue culture plate (Nunc). 1 ml of autoclaved water was added into each well to wash away the ethanol from the coverslips. 2 ml fresh amoebae culture was added into each well and incubated overnight at 20°C. A. castellanii culture was examined under the microscope next day to see whether the amoebae had attached to the coverslips. Also the number of amoebae per millilitre was determined by counting in a hemocytometer (Chapter 2, section 2.2).

3.3.6.2 LysoTracker Red (LR) staining of amoebae

LysoTracker Red DND99 (Molecular probes, Invitrogen) was used to fluorescently stain acidic compartments within amoebae. A 50 nM solution of LysoTracker Red (LR) was made in DMSO. 45 μl of the LR solution was mixed with 40 ml pre-warmed PPG and 2 ml was added into each well of the tissue culture plates and incubated at 20°C for 2 hrs.

The PPG media containing LysoTracker Red was aspirated from the tissue culture plate wells. The A. castellanii culture in the wells was washed with sterile PAS twice. 1 ml of each of the FITC-stained mycobacteria were added into the respective wells. The tissue culture plates were wrapped up in an aluminium foil to protect from light and incubated at 20°C for 4 hrs, 24 hrs and 48 hrs.
3.3.6.3 Fluorescein Isothiocyanate (FITC) staining of mycobacteria

All of the process was performed in containment level 3 laboratory. Optical density at 600nm was measured for BCG::pRD1, BCG::pYUB412, BCG and M. bovis cultures. 10ml of each was centrifuged at 3700xg for 10 mins. The supernatant was discarded and the pellet was resuspended in 1ml carbonate buffer with Tween80.

100mg/ml solution of FITC was prepared in DMSO. 10µl of the freshly prepared FITC solution was added to each suspension and was incubated at 37°C for 15-20 mins without shaking. The stained suspensions were centrifuged at 3700xg for 5 mins. The supernatant was discarded and pellet was resuspended in 1ml PBS-Tween80 and was centrifuged at 3700xg for 5 mins. This step was repeated twice to wash off excess dye from the cells. The final pellet was resuspended in 7ml of PAS. Optical density at 600nm was taken for each suspension.

3.3.6.4 Infecting A. castellanii with BCG::pRD1, BCG::pYUB412, BCG and M. bovis

The PPG media containing LysoTracker Red was aspirated from the tissue culture plate wells. The A. castellanii culture in the wells was washed with sterile PAS twice. 1ml of each of the FITC-stained mycobacteria were added into the respective wells. The tissue culture plates were wrapped up in an aluminium foil to protect from light and incubated at 20°C for 4hrs, 24hrs and 48hrs.

3.3.6.5 Preparing the slides for Confocal microscopy

After 4 hours, one of the three tissue culture plates was taken inside the safety cabinet and the mycobacterial culture inside the wells was aspirated and the wells were washed with PAS. 1ml freshly prepared, cold 4% paraformaldehyde (PFA) was added into each well to fix the cells and incubated at 4°C for 1hr. After 1 hour, PFA was aspirated and the wells washed with PBS twice. 1-2 drops of mounting medium (Dako) was added onto a microscope slide. The glass coverslip with the fixed cells was inverted onto microscope slide with the help of sterile forceps. When the slide was dried, the coverslips were sealed with clear nail varnish and observed using a Zeiss Axiovert LSM510 confocal microscope.
3.3.7. Infecting *A. castellanii* with *M. tuberculosis* knock out strains

3.3.7.1 Growing amoebae

1 ml of fresh *A. castellanii* culture was added into each well of the 24-well tissue culture plates. Five such plates were prepared and were incubated at 25°C overnight to help amoebae attach onto the surface of the wells.

All plates were examined under the inverted microscope to observe the confluent layers of the amoebae.

3.3.7.2 Growing mycobacterial strains

All mutant and control strains (*M. tuberculosis* H37Rv::Δ2548, *M. tuberculosis* H37Rv::Δ2745, *M. tuberculosis* H37Rv::Δ0467, *M. tuberculosis* H37Rv and *M. bovis*) were grown in 7H9 with OADC to reach an O.D of 0.7.

3.3.7.3 Infecting *A. castellanii* with the mycobacterial strains

Supernatant was removed from all the tissue culture plate wells to get rid of the non-adherent amoebae. 1ml of each strain was added into the respective wells of the plates and incubated at 20°C for 2 hours. Supernatant was removed and 1ml of PAS-amikacin (100μg/ml) was added in each well to get rid of the non-adherent bacteria. Plates were incubated at 20°C for 2 hours. Supernatant was removed and the wells were washed with sterile PAS twice. 1ml of sterile PPG was added into each well of three of the four plates. The plates were carefully sealed and incubated at 20°C for four, seven and fourteen days. To the remaining plate, 1ml of sterile 0.5% SDS was added. The contents of each well were serially diluted and plated. Bacterial counts were determined by Miles and Misra (Drop Count) technique. The previous two steps were repeated for the remaining three plates after four, seven and fourteen days.
3.4. RESULTS

3.4.1. RD1 is not the sole virulence factor

The RD1 locus has been considered crucial for the pathogenesis of virulent mycobacteria since this region is not found in the naturally attenuated strains of vole bacillus and Dassie bacillus and was deleted from all BCG strains during its propagation. In this study, I have compared the survival of *M. bovis* BCG, *M. bovis*, BCG::RD1 and BCG::pYUB412 in amoebae.

The attenuated strain BCG shows decreased survival as compared to the pathogenic *M. bovis* and *M. tuberculosis* H37Rv (Figure 3.1). Previous studies have reported similar findings in which BCG struggles to survive in amoebae and shows a 5 log reduction as compared to *M. bovis* (3 log reduction) (Taylor, 2003; Ahmed, 2007).

In addition to this, a control strain of BCG carrying the empty construct pYUB412 was also used to infect *A. castellanii* in the same experiment. This construct was used to ensure that the effects observed of survival in the BCG transformed with RD1 strain was due to the 10kb fragment and not the effects of the construct itself. Figure 3.1 shows that the BCG::pYUB412 survives in a similar manner to BCG which means that the empty construct pYUB412 has no effect on the survival of BCG in amoebae.

Interestingly, the BCG::RD1 also shows a reduced survival like BCG and BCG::pYUB412 (figure 3.1). The RD1 fragment was re-introduced into the attenuated strain BCG to observe if the RD1 provides any survival advantage to the BCG::RD1. On the contrary, Ahmed (2007) showed that the re-introduction of RD1 region into BCG enabled the BCG::RD1 to survive better than BCG in *A. castellanii*. However, the re-introduction of RD1 into BCG did not make it as virulent as *M. bovis* and so the survival of BCG::RD1 was observed to be somewhere in between the survival of BCG (reduced survival) and *M. bovis* (increased survival) (Ahmed, 2007). However, in this study, there is no conclusive evidence that BCG::RD1 survived better than BCG as both were undetectable at 3 days (figure 3.1).
Figure 3.1. A comparison of the survival of BCG::RD1 and BCG::pYUB412 strains along with control strains *M. tuberculosis* H37Rv and *M. bovis* and *M. bovis* BCG. The results shown are representative of two independent experiments with five replicates of each strain at each time point. Error bars represent standard error (SE).

Note: BCG, BCG::RD1 and BCG::pYUB412 were below the detection limit at days 3, 7 and 14.

Figure 3.1 also shows that *M. tuberculosis* H37Rv likewise survives in amoebae as *M. bovis*. It suggests that perhaps any of the genetic differences between *M. tuberculosis* H37Rv and *M. bovis* are not important for survival in amoebae.

### 3.4.2 Mycobacteria are retained by the amoebae vacuoles

In order to see the fate of the mycobacteria inside amoebae, both were stained with fluorescent dyes and infections were performed. Bacteria were stained with FITC and acidic compartments which would include ‘normal’ phagosomes were stained with LysoTracker Red. In preliminary studies it was found that the majority of *M. tuberculosis* phagosomes in the *A. castellanii* cells strongly associated with the acidotrophic dye Lysotracker Red immediately after infection. This is in contrast to macrophages where the majority of *M. tuberculosis* phagosomes exclude lysotracker red. Slides were prepared at 4hrs, 24hrs and 48hrs post infection followed by observing under the confocal microscope (Figure 3.2 and 3.3). The number of mycobacteria that colocalized with the lysosomes and those that did not was counted in hundred amoebae at each time point.
Figure 3.2. Confocal microscopy images of *M. bovis* and acidic compartments. The images (4 hrs post infection) of *M. bovis* (green), acidic vacuoles of amoebae (red), and the colocalized bacteria inside the vacuoles (yellow) using Confocal microscopy with different lasers a) green laser b) red laser c) dark field d) combination of green laser, red laser and dark field.
Figure 3.3. Confocal microscopy images of *M. bovis* BCG::RD1 and acidic compartments. Images showing *M. bovis* BCG::RD1 (green), acidic vacuoles of amoebae (red) and the colocalized bacteria in vacuoles (yellow) after 24 hrs of infection using the confocal microscope with different lasers a) green laser b) red laser c) dark field d) combination of green laser, red laser and dark field.
Figure 3.4. Comparison of *M. bovis*, *M. bovis* BCG, *M. bovis* BCG::pRD1 and *M. bovis* BCG::pYUB412 for colocalization with amoeba vacuoles at 4hrs, 24hrs and 48hrs. The number of mycobacteria that colocalized with the acidic vacuoles (inside vacuole) and those that were not inside acidic vacuoles (outside vacuole) was counted in hundred amoebae at each time point.

The results (Figure 3.4) show that after 4 hours, all four strains of mycobacteria were mostly inside the acidic vacuoles. Figure 3.4 also shows that majority of all the four strains remain inside the acidic vacuoles at 24 and 48hrs.

This is a very interesting finding. It is widely believed that controlling the phagosomal pH is one of the important mechanisms by which virulent mycobacteria are able to survive in macrophages (MacMiking *et al.*, 2003; Huynh *et al.*, 2007). Although mycobacteria in this study remain inside the vacuoles, they are not able to change the pH near neutral as the mycobacteria-containing vacuoles stain strongly with the acidotropic dye, lysotracker red. This indicates that mycobacteria are able to maintain themselves inside the acidic vacuoles of the amoebae and they do not escape to the cytosol.

3.4.3. Survival of different *M. tuberculosis* knock-out strains in amoebae

In order to reveal the survival mechanisms of mycobacteria in amoebae, three different *M. tuberculosis* knock out strains were used to infect amoebae. These genes
encode isocitrate lysase (Rv0467), an important enzyme in the glyoxylate shunt, a possible VapC19 (Rv2548) and a putative transcriptional regulator (Rv2745).

*M. bovis* NCTC 10772 and *M. tuberculosis* H37Rv were used as control strains as *M. bovis* is reported to survive well inside *A. castellanii* (Taylor et al, 2003) while the knock out strains used in this study were of *M. tuberculosis* and hence for comparison with the wild type, H37Rv was also used. As shown in figure 3.5, *M. bovis* survived the ingestion by amoeba for the duration of the experiment with about 80% bacteria recovered at Day 14 compared to at Day0 (p-value =0.02, α<0.05). The figure also shows an interesting finding that *M. tuberculosis* H37Rv behaves like *M. bovis* in amoebae (p-value =0.05, α<0.05).

**Figure 3.5.** A comparison of the survival of three knock-out strains *M. tuberculosis* H37Rv:Δ2548, *M. tuberculosis* H37Rv:Δ2745, *M. tuberculosis* H37Rv:Δ0467 along with control strains *M. tuberculosis* H37Rv and *M. bovis*. The results shown are representative of two independent experiments with five replicates of each strain at each time point. Error bars represent standard error (SE).
3.4.3.1 Rv0467 contributes to mycobacterium survival in protozoa

Many studies have focussed on mycobacterial metabolism to unveil the virulence mechanisms of this persistent pathogen. Genes involved in fatty acid metabolism have undergone extensive duplication in the genus Mycobacterium. It is evidenced that the pathogenic mycobacteria primarily utilize fatty acids rather than carbohydrates as a source of carbon during infection. M. tuberculosis has two genes for isocitrate lyase; the most important enzyme of the glyoxylate cycle for utilization of fatty acids (Cole et al, 1998). The smaller isocitrate lyase gene (icl1) encodes an enzyme closely related to isocitrate lyase in other eubacteria while the product of the larger gene (icl2) is more homologous to the isocitrate lyases of eukaryotes (Munoz-Elias and McKinney, 2005). It has been shown that both isoforms of the enzyme isocitrate lyase are required for survival of M. tuberculosis in macrophages and mice implying a fundamental role of fatty acid catabolism during infection (Munoz-Elias and McKinney, 2005).

In the present study, the survival of M. tuberculosis H37Rv: Δicl1 mutant was studied in amoebae. Results (Figure 3.5) show that this mutant dies off fairly quickly (day 3, p-value =0.022, α≤0.05) indicating that mycobacteria needs icl to utilize lipids during survival in amoebae. Alternatively, Beste et al., 2009 measured the activity of isocitrate lyase in BCG cells grown at fast (doubling time of 23 hours) and slow (doubling time of 69 hours) growth rates in a chemostat. Specific isocitrate lyase activity was significantly higher (twofold change) in the slow growing cells suggesting that the enzyme was involved in maintaining growth at slow growth rates. Deb et al (2009) demonstrated a link between storage-lipid accumulation and development of drug-tolerance in the M. tuberculosis cells during the development of dormancy. M. tuberculosis accumulates storage lipids under multiple-stress like hypoxia (slow oxygen depletion), nutrient deprivation, NO treatment and growth in acidic media. It was shown that the application of these four stress factors in combination led to the accumulation of triacylglycerols and wax esters in M. tuberculosis cells (Deb et al., 2009).
3.4.3.2. Mycobacterium Rv2548 mutant is hypervirulent for survival in protozoa

Rv2548 in *M. tuberculosis* encodes a possible toxin VapC19 whose exact function is unknown. In this study the mutant with this region deleted was able to survive well inside amoebae (Figure 3.5). Interestingly, the deletion of this gene renders the mutant to survive better than the control strains.

It suggests that the toxin encoded by Rv2548 is involved in persistence of *M. tuberculosis*. Persistence is characterized by slow growth and the results presented in this study show that deletion of Rv2548 resulted in better survival of *M. tuberculosis*. Several putative toxin genes and have been proposed to play a role in promoting slow growth during stressful environments (Pandey and Gerdes, 2005). Also, mutants in two anti-toxin genes (Rv0596c and Rv1962) were identified as having reduced fitness at the fast growth rate suggesting some role of toxin-antitoxin systems in the persistence of *M. tuberculosis* (Beste et al., 2009).

3.4.3.3. Rv2745c might contribute to mycobacterium survival in protozoa

Rv2745c mutant shows (Figure 3.5) some evidence of a decreased survival within the amoeba with a reduced count (p-value = 0.003, α<0.05) at Day14. It is widely recognized that the intracellular oxygen tension plays an important role in cellular function and metabolism. *M. tuberculosis* enters a non-replicating state when exposed to low oxygen tension, a condition the bacillus encounters in granulomas during infection (Sherrid et al., 2010). Determining how mycobacteria enter and maintain this state is a major focus of research. Rv2745c is induced by many stresses including general stress such as heat shock. The attenuation of this mutant strain could be due to reduced resistance to a variety of stresses.
3.5. Conclusions

1. Reintroduction of RD1 in *M. bovis* BCG does not confer survival advantage to BCG::RD1 in amoebae to the level seen with *M. bovis*.

2. *M. bovis, M. bovis* BCG, *M. bovis* BCG::pRD1 and *M. bovis* BCG::pYUB412 are retained by the amoeba vacuoles.

3. *M. tuberculosis* H37Rv::Δicl (Rv0467) is attenuated for survival in amoebae.

4. *M. tuberculosis*::ΔVapC19 (Rv2548) is hypervirulent in amoebae.

5. There is some evidence that *M. tuberculosis* H37Rv::ΔClgR (Rv2745) is attenuated for survival in amoebae.
3.6 Discussion

3.6.1 Impact of RD1 on mycobacterial survival

Members of the *M. tuberculosis* complex are highly related mycobacteria exhibiting remarkable nucleotide sequence level homogeneity. With advances in genomics, the genetic basis of these differences can be interpreted. BCG, the vaccine strain has several genetic regions missing from its genome as compared to *M. bovis*. Although the precise genetic events that lead to the attenuation of BCG are not known, region of difference 1 (RD1) was a prime candidate due to the absence of this region from all the avirulent mycobacterial strains. RD1 encodes the two most potent mycobacterial antigens (ESAT-6 and CFP-10) as well as a novel secretion system for their export. More importantly, the association of mycobacterial virulence and the RD1 region has been made in several studies. Lewis *et al.* (2003) showed that deletion of RD1 from *M. tuberculosis* renders it less virulent in humans and mice. Conversely, Pym *et al.* (2002) demonstrated that *M. microti* and BCG became virulent upon incorporation of the genes from the MTB chromosome. The BCG::RD1 and *M. microti::RD1* knock-ins grew more vigorously than controls in immunodeficient mice, inducing extensive splenomegaly and granuloma formation. However, expression of the RD1 locus in both vaccine strains does not lead to full-blown pathogenesis, and this argues in favour of complete attenuation arising as the synergistic effect of multiple mutations. Gao *et al.* (2004) showed that there are multiple genes on the mycobacterial chromosome including RD1 and surrounding genes referred to as the ‘extended RD1’ (Rv3866-Rv3881c) that play an important role in virulence.

One of the reasons why RD1 does not restore virulence in this study could be that the BCG::RD1 was possibly not functional. The functional integrity of the RD1 locus has been previously documented (Pym *et al.*, 2002) and since the same plasmid has been used in both studies, only the presence of RD1 fragment in BCG::RD1 was confirmed here by PCR (data not shown). However, confirming the functionality of RD1 would have provided conclusive evidence whether the inability of RD1 to confer survival advantage to BCG in amoebae is simply due to a non-functional RD1.

Various studies have tried to investigate the role of major virulence factors of other pathogens in providing survival benefit within amoebae. Zhao *et al.* (2007) tested
the specific hypothesis that *L. monocytogenes* can survive predation by *A. castellanii* and that listeriolysin O (*hly* gene) plays a crucial role in this process. They found that listeriolysin O is not required to survive in amoebae because while the *hly* mutant was compromised in its ability to survive predation, the complemented strain did not fully recover its ability to survive. A similar study with *Salmonella enterica* also revealed that virulence factors are not required for invasion and replication within amoebae (Tezcan-Merdol *et al.*, 2004). Consistent with these findings, in the present study no definitive association could be observed between RD1 and enhanced survival in *A. castellanii*.

### 3.6.2 Mycobacteria are retained in the amoebae vacuoles

Upon bacterial invasion, the phagocytic cells are recruited at the site of infection by the immune system. This is followed by the engulfment and subsequent lysis of the bacteria by phagocytosis. Pathogens capable of surviving within macrophages use a range of differing strategies to avoid delivery to the lysosome and subsequent death. Some pathogens such as *Listeria* and *Shigella* escape into the cytosol, others like *Leishmania* and *Coxiella* actually survive and replicate within the lysosomal milieu. Many, like *Legionella, Brucella, Erlichia,* and *Mycobacterium* spp., subvert the normal progression of their phagosomal compartment and prevent it from fusing with or maturing into an active lysosomal compartment (Russell, 2005).

In this study, amoebae were infected with *M. bovis* BCG::RD1, *M. bovis* BCG::pYUB412 (empty plasmid), *M. bovis* BCG and *M. bovis* to determine if they colocalize with the vacuoles. It was observed that all four strains were taken up by amoebae vacuoles. About 70% of all strains were inside amoebae vacuoles as soon as 4hrs post infection and remained so through the duration of the experiment (figure 3.4). Gao *et al* (1998) showed the ability of *L. pneumophila* to form a phagosome within *A. polyphaga* at 4 hours post infection and the subsequent intracellular replication within the phagosome. However, there are contrasting observations in regards to whether mycobacterium species remain in the phagosomal or escape into the cytosol. Cirillo *et al* (1997) observed that *M. avium* was taken up by the amoeba into individual vacuoles during the first 15 minutes after infection. Many studies have reported similar findings in which *M. tuberculosis* was able to persist and replicate within the phagosomes of the infected macrophages (Rumsey *et al* 2006; MacMicking *et al*., 2003). *M. avium* was also
able to survive inside amoebae by inhibiting phagosome-lysosomal fusion (Cirillo et al., 1997). *M. tuberculosis* was able to persist and replicate within the phagosomes of the infected macrophages (Rumsey et al. 2006; MacMicking et al., 2003).

On the contrary, some mycobacterial species have been reported to escape into the host cytosol. Using the social amoeba *Dictyostelium* as a genetically tractable host for pathogenic mycobacteria, it was discovered that *M. tuberculosis* and *M. marinum* but not *M. avium* are ejected from the cell through an actin-based structure, the ejectosome. This conserved nonlytic spreading mechanism was dependent on an intact mycobacterial ESX-1 secretion system (Hagedorn et al., 2009). *Listeria monocytogenes* and *Shigella flexneri* however, lyse the phagosomal membrane and escape from the endocytic system into the host cytosol, where they replicate and are able to spread to neighboring cells via actin-based motility (Stevens et al., 2006).

Van Der Wel et al. (2007) showed that lysosomes rapidly fuse with the virulent *M. tuberculosis* - and *M. leprae*-containing phagosomes of human monocyte-derived dendritic cells and macrophages. After 2 days, *M. tuberculosis* progressively translocated from phagolysosomes into the cytosol in non-apoptotic cells. Cytosolic entry was also observed for *M. leprae* but not for vaccine strains *M. bovis* BCG or in heat-killed mycobacteria and is dependent upon secretion of the mycobacterial gene products CFP-10 and ESAT-6. The different observations could be due to different host cells and experimental conditions.

### 3.6.3 Role of Rv0467, Rv2548 and Rv2745c in mycobacterial survival

In this project, three different *M. tuberculosis* genes were selected for study in amoebae. These genes were involved in fatty acid metabolism (Rv0467), a transcriptional regulator that is upregulated during resumption of replication following hypoxia (Rv2745) and a toxin of unknown function (Rv2548).

Acquisition of essential nutrients by intracellular pathogens is an important research area. Fatty acids might be a major source of carbon and energy in *M. tuberculosis* metabolism in chronically infected lung tissues. In *E. coli* and *Salmonella typhimurium*, induction of the fatty acid catabolic regulon is essential for survival in stationary phase (Farewell et al., 1996; Spector et al., 1999).
In this study, the \textit{id} mutant was attenuated for growth in amoebae. Muñoz-Elías and John McKinney (2005) showed that isocitrate lyase (ICL) is required for fatty acid catabolism and virulence in \textit{M. tuberculosis}. When mycobacteria infect amoebae, the environmental changes could affect the bacterial metabolism and it might switch to a slower growth on fatty acids. This indicates the importance of glyoxylate pathway for \textit{M. tuberculosis} and may explain why the \textit{id} mutant in this study failed to survive in amoebae.

The Rv2548 \textit{M. tuberculosis} mutant was reported as hypervirulent in the present study. Hypervirulence has been reported previously for \textit{M. tuberculosis} where a disruption of the \textit{mcel} operon allowed the mutant to replicate and kill the mice more rapidly than the wild type strain. The \textit{mcel} operon may be involved in modulating the host inflammatory response in such a way that the bacterium is able to persist (Shimono \textit{et al}, 2003). Similarly, Catherinot \textit{et al} (2007) identified a \textit{M. abscessus} mutant that was hyperlethal to mice.

The presence of TA systems in \textit{M. tuberculosis} may contribute to its ability to undergo long term dormancy and consequently might prove to be essential for the persistence of the pathogen (Zhu \textit{et al}., 2006). This potential importance of TA systems in the life cycle of \textit{M. tuberculosis} is suggested by the presence of a remarkably large number of these TA genes in its genome (Cole \textit{et al}., 1998). It suggests that the toxin encoded by Rv2548 is involved in persistence of \textit{M. tuberculosis}. Persistence is characterized by slow growth and the results presented in this study show that deletion of Rv2548 resulted in fast growth of \textit{M. tuberculosis}. Several putative toxin genes and have been proposed to play a role in promoting slow growth during stressful environments (Pandey and Gerdes, 2005). Also, mutants in two anti-toxin genes (Rv0596c and Rv1962) were identified as having reduced fitness at the fast growth rate suggesting some role of toxin-antitoxin systems in the persistence of \textit{M. tuberculosis} (Beste \textit{et al}., 2009).

Chromosomal TA modules have also been proposed to function as regulators of cell growth in response to environmental stress (Gerdes, 2000). Ramage \textit{et al}, (2009) showed that some TA system genes are upregulated during hypoxia or macrophage infection, providing evidence that these systems are activated during specific stresses.
likely encountered in the host. Bacilli grown under hypoxia exhibit a tolerance to antimicrobial therapy (Rustad et al., 2008). Given that hypoxia results in a state of cell stasis and the formation of antibiotic-tolerant persisters, TA systems are prime candidates for mediating this transition both in vitro and in vivo.

There is some indication in this study that the Rv2745 mutant is attenuated for survival in amoebae. Recently, Estorninho et al., (2010) revealed that the clp gene regulator (CglR) of M. tuberculosis activates the transcription of at least ten genes involved in stabilization, disassembly or degradation of proteins. They also compared the survival of a CglR deleted M. tuberculosis mutant to that of a wild type strain in murine macrophages. It was found that the CglR mutant was at a disadvantage for intracellular survival and failed to inhibit phagosome acidification (Estorninho et al., 2010).

Moreover, in the present study, the experiment was performed at 20°C which is also a stress factor for the bacteria (M. bovis grows at 37°C). CglR also controls the expression of acr2 gene, a dominant antigen (Wilkinson et al., 2005) and a virulence factor (Stewart et al., 2005), whose function is to stabilize proteins during stress conditions (Van Montfort et al., 2001). Acr2 gene is also reported to be required for pathogenesis in a murine model of tuberculosis (Stewart et al., 2005). In another study, Sherrid et al (2010) observed that the Rv2745 was upregulated during late hypoxia and was further induced during reaeration. Since a putative transcriptional regulator is lacking in H37Rv: ΔRv2745, it becomes attenuated in amoebae. However, the evidence for the decreased survival is not strong and would need to be verified by further experiments.

This chapter investigated the importance of selected genes on survival of M. tuberculosis / M. bovis inside amoebae. Although the chosen genes were important candidates for survival studies, it is desirable to study every possible gene that might confer a survival advantage to mycobacteria. But the process of screening mutants individually for survival could be laborious and time consuming. However, new screening methods enabling the simultaneous study of pools of mutants have proved successful. Therefore, these global screening methods were incorporated in the present study to investigate the survival genes of M. bovis in amoebae.
Chapter 4

Screening the *M. bovis* BCG and *M. bovis* genomes for factors required during survival in *Acanthamoeba castellanii*.

4.1. Introduction

Understanding the pathogenesis of *Mycobacterium tuberculosis* complex bacteria requires the knowledge of specific virulence determinants. Studying the mutant strains provides important information about the virulence of a pathogen. While one approach is to generate and study mutations in a specific gene, advances in genetic tools have enabled the simultaneous analysis of multiple rather than the individual mutants. Transposons have proven to be one of the most versatile and useful genetic tools for molecular genetics research. They can be used to generate marked gene knockouts, create gene fusions, and act as mobile priming sites for DNA sequence analyses. More recently, the repertoire of transposon applications has expanded dramatically with the development of *in vitro* transposition systems.

4.1.1. Transposable elements

An important mechanism in generating genetic diversity is transposition which is the movement of mobile genetic elements from an original site on a DNA molecule to a new site on the same or on a different DNA molecule. These elements are present in almost all genomes and can bring about chromosomal rearrangements through deletion, insertion, inversion and chromosomal fusion mutations. Transposable elements are grouped on the basis of their mechanism of transposition; either replicative
or conservative. However, both mechanisms share some basic characteristics. Transposition requires a transposase protein and specific end sequences which define the transposable element. The success of the process is largely dependent on these end sequences (Reznikoff, 1993).

Transposition is a highly regulated process involving many host proteins and sometimes the transposable element itself is involved in regulation at a post-transcriptional level. Host proteins are involved in many processes like facilitating the end-sequence binding of the transposase, nucleating the high order structure in which the ends are brought together and various repair functions (Reznikoff, 1993). This tight regulation enables the host to allow proliferation of the transposable element without compromising its genetic integrity.

4.1.2. Tn5 transposition

Tn5 is a composite transposon in which two nearly identical insertion sequences IS50R and IS50L flank three antibiotic resistance genes (figure 4.1). While IS50L encodes an inactive protein, IS50R encodes the fully functional transposase (Tnp) (Isberg et al, 1982; Johnson and Reznikoff, 1984). Tn5 transposition is also down regulated by the inhibitor protein (Inh) which is encoded by IS50R as well. Both proteins are translated in the same reading frame but the inhibitor protein (Inh) lacks the N-terminal 55 amino acid whereas this region plays an important role in the activity of the transposase (Tnp). There is also a 19-bp end sequence at both ends of each IS50 which is recognized by the host functions and hence also plays a critical role in transposition (Reznikoff, 1993).
Figure 4.1: Structure of the Tn5 transposon. Two almost identical insertion sequences IS50L and IS50R flank the three antibiotic genes (kan=kanamycin, ble=bleomycin, str=streptomycin). IE = inside end, OE = outside end, Tnp = transposase, IS50L = insertion sequence 50 left, IS50R = insertion sequence 50 right). The IE sequence is 5'CTGTCTCTTGATCAGATCT3' and the OEs sequence is 5'CTGACTCTTATACACAAGT3' (Bhasin et al., 1999).

The transposase creates a staggered cut in the mycobacterial DNA and cleaves at the ends of the transposon inverted-repeat sequences (figure 4.2). The transposase catalyses the movement of Tn5 throughout the genome and joins the overhanging ends of the target DNA to the transposable element. The resulting gap in the target-site DNA is repaired by DNA synthesis, followed by ligation to the other strand of the transposon and finally creating a signature 9bp direct repeat of the target-site DNA flanking the integrated transposon.
TnS Integrated at donor site

Transposon creates a staggered cut in the target DNA and cleaves at the mosaic ends (ME) of the transposon (Tn)

Overhanging ends of target DNA are joined to the transposon

Gaps are filled in to create a signature 9bp repeat at both ends of the transposon

**Figure 4.2: Tn5 Transposition process**
4.1.3. Advantages of Ez::Tn transposon based mutagenesis

The Ez::Tn system was used in this study to create a transposon mutant library.

1. No vector delivery system is required as DNA can be introduced into the host by simple electroporation.
2. It is a simple process which relies on the ability to generate a stable EZ::TN Transposome.
3. The transposome can be generated in vitro using purified transposase protein and a DNA fragment flanked by the inverted repeats.
4. The system is highly flexible as any gene flanked by the inverted repeats can be used to form a transposome, as long as it is of a size that will permit synaptic complex formation.
5. Transposomes are unusually stable DNA complexes and, in particular, the EZ::TN (Tn5) complex has been shown to maintain its integrity throughout the electroporation process (Haniford et al. 1991; Chaconas et al. 1996).
6. It had already been used successfully to generate the BCG mutant library (Stewart et al., 2005).
7. It is the least biased transposon system available.

4.1.4. Transposon Site Hybridization (TraSH)

The generation of a mutant pool containing mutations in almost every non-essential gene becomes more useful when combined with a tool that provides simultaneous analysis of all the mutants in that pool. One such powerful tool is the DNA microarray.

Identifying the bacterial genes required for intracellular survival can be achieved in a systematic fashion by Transposon site hybridization (TraSH). This analysis is a high throughput method of screening for the presence of specific transposon mutants within bacterial pools subjected to different experimental conditions (Badarinarayana, et al. 2001; Sassetti et al, 2001), similar conceptually to the previously developed Signature Tag Mutagenesis (STM) (Hensel et al, 1995). In STM, each strain contains a unique molecular tag such that the presence of each mutant can be determined within a
complex pool. A group of strains can then be subjected to the experimental condition of interest, and all bacteria that are capable of surviving the treatment are isolated at the end of the experiment. Mutations in genes required for surviving under the experimental condition are those whose corresponding tags are underrepresented in the post-treatment population. However, in the original STM technique developed by Hensel et al. (1995), miniTn5 transposons that contained signature tags composed of random sequences of 40bp were randomly inserted into the bacterial genome, yielding a tagged mutant library. The individual mutants were pooled together. Then the tags of the pool were amplified with universal tag primers, radioactively labelled and hybridized on membranes spotted with DNA from the corresponding mutants. The input and output pools were compared where a weak or absent hybridization signal from the output pool compared to the input pool identified attenuated mutants.

In TraSH, the molecular tags used are the genomic DNA sequences directly adjacent to each transposon insertion. A PCR reaction from genomic DNA prepared from mutant pools will specifically amplify all of the transposon flanking sequences present. These are then fluorescently labelled so they can be hybridized to a microarray containing oligonucleotides complementary to these probes. In this manner, the relative fluorescence intensity for a particular oligonucleotide spot on the microarray corresponds to the relative number of bacteria present containing that tag. TraSH has been used successfully by the laboratories of George Church and Eric Rubin at Harvard Medical School to characterize growth preferences of E. coli and M. tuberculosis respectively (Badarinarayana, et al. 2001; Sassetti, et al., 2003).

To date, all of the experiments using pooled M. tuberculosis mutants have relied on the mouse model of infection. This is the first study using a library of M. bovis transposon mutants to identify the survival factors of mycobacteria in protozoa.
4.2. Aims and objectives

The aim of this chapter was to perform TraSH mutagenesis on BCG and *M. bovis* to provide a systematic assessment of the importance of mycobacterial genes for intra-amoebic survival.

Specific objectives were:

1. To perform TraSH screening of *M. bovis* BCG for further attenuation in *A. castellanii* using an existing BCG Tn5 transposom mutant library.

2. To construct a library of *M. bovis* Tn5 transposon mutants

3. To evaluate coverage of the *M. bovis* transposon mutant library

4. To screen the *M. bovis* genome for genes required for survival inside amoebae using TraSH.
4.3. MATERIALS AND METHODS

4.3.1 TraSH screening of BCG mutant library

4.3.1.1 Strains and growth conditions

The *M. bovis* BCG Ez:Tn-Hyg transposon mutant library (consisting of ~2500 mutants), obtained from Dr Graham Stewart (Stewart *et al.*, 2005) was maintained in MiddleBrook 7H9 broth, supplemented with 5% OADC, 0.05% Tween80 and Hygromycin (50μg/ml) for a week at 37°C.

Figure 4.3 shows the strategy by which only the DNA regions flanking the transposon insertion site were selectively amplified.
Genomic DNA extraction from BCG transposon mutant library

Digestion with restriction enzymes with 4 bp recognition site (BssHII/MluI)

Ligation to Y-linker (TA1/TA2)

Transposon specific sequence

1st round of PCR (TSM I) with transposon-specific (TA3) and Y-linker specific (TA4) primers

No transposon insert so no sequence available for Primer binding

Amplification of DNA sequences flanking the transposon.

2nd round of PCR (TSM II) using products from the first round as templates and Y-linker-specific primer (TA4) and nested transposon-specific primer (TA6)

Figure 4.3. Strategy for selective amplification of transposon flanking DNA.
In order to optimize selective amplification process, 3 different types of TA4 primers were used for the amplification of the BCG mutant library as well as the control BCG Pasteur DNA. These primers have different sequences and lengths as shown in table 4.1.

### 4.3.1.2 DNA Restriction digestion

The mutant library was screened by microarray to reveal insertion sites and relative abundance of mutants. For this purpose, genomic DNA was extracted by the phenol-chloroform method (Chapter 2, section 2.3) and digested by BssHII/MluI to generate an average fragment size of approximately 900bp. Digested DNA was purified using QIAquick PCR purification kit (QIAGEN) and run on a 1% agarose gel to analyze the digests.

### 4.3.1.3 Ligation with linkers

The TA1 and TA2 oligonucleotides (Table 4.1) were annealed to make a Y-shaped linker. TA1 and TA2 oligonucleotides were resuspended in TE buffer and 50Mm NaCl, mixed together and heated at 95°C for 2 mins on a heating block. After 5 mins, the power supply was disconnected and the linker was allowed to cool down at room temperature over 2-3 hrs. The Y-linker has a BssHII compatible 5' overhang and digested genomic DNA was ligated to it using T4 ligase and buffer. Reaction tubes were left at 8°C overnight and then purified into 10μl water using QIAquick PCR MinElute kit.

### 4.3.1.4 First round of PCR

The Y-linker ligated DNA was used as a template for amplification of transposon-flanking regions using the transposon specific primer TA3 and 3 different Y-linker specific TA4 primers (Table 4.1) by running the following PCR cycle (figure 4.4).
4.3.1.5 Nested second round of amplification of Tn insertion sequences

Amplification products less than 500bp were gel purified using QIAGEN gel extraction kit and used in a second round of PCR between the Y-linker specific primer TA4 and a nested transposon-specific primer TA6 (Table 4.1) to further enrich for transposon flanking products and to incorporate Cy3 or Cy5-dCTP. Fourteen PCR cycles were run as shown below (figure 4.5).

Appropriate control (the BCG wild type DNA, labeled with Cy3) and library (BCG mutant library DNA labeled with Cy5) samples, each from the same set of PCR cycle were mixed together and purified using the MinElute PCR purification kit using a microcentrifuge.
Table 4.1. Sequences of oligonucleotides

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA1</td>
<td>5’-ACTAGCAGCGACGAGACGTAGC-3’</td>
</tr>
<tr>
<td>TA2</td>
<td>5’-CGCGAGCGCTACGCTGAGTTGTCGGTCCTG-3’</td>
</tr>
<tr>
<td>TA3</td>
<td>5’-GCCTTCACCTTCTGACACGACTTCGAGGT-3’</td>
</tr>
<tr>
<td>TA4</td>
<td>5’-ACGCACGCGACGAGACGTAGC-3’</td>
</tr>
<tr>
<td>TA4 medium</td>
<td>5’-ACTACGAGCAGCGACGAGACGTAGC-3’</td>
</tr>
<tr>
<td>TA4 long</td>
<td>5’-ACTACGACGAGCGACGAGACGTAGC-3’</td>
</tr>
<tr>
<td>TA6</td>
<td>3’-GTGTCGAGGAGCCCGCTGGATCTCTC-5’</td>
</tr>
</tbody>
</table>

4.3.1.6 Microarray

The commercially available microarrays are constructed by robotic spotting of short oligonucleotide sequences from all of the open reading frames (ORFs) of the *M. tuberculosis* genome on a glass slide. The fluorescently labeled transposon insertion sites were hybridized to whole-genome microarray slides (Operon) containing 4320 spots printed by Bacterial Microarray Group at St. Georges Hospital Medical School, made up of 70-mer oligonucleotide from open reading frames (ORFs) of *M. tuberculosis* H37Rv and CDC1551, along with some negative control spots. The microarray protocol was performed as follows:

4.3.1.7 Slide prehybridization

The pre-hybridization solution was prepared by mixing the reagents (Table 4.2) in a coplin jar and incubated at 65°C for 30-60 mins.

Table 4.2. Reagents of pre-hybridization solution

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>36 ml</td>
</tr>
<tr>
<td>20X SSC</td>
<td>8.75 ml (3.5X SSC)</td>
</tr>
<tr>
<td>20% SDS</td>
<td>250 µl (0.1% SDS)</td>
</tr>
<tr>
<td>BSA(100mg/ml)</td>
<td>5 ml (10mg/ml)</td>
</tr>
</tbody>
</table>
The microarray slide was put into the coplin jar containing the pre-heated pre-
hybridization solution at 65°C for 20 mins. The slide was rinsed thoroughly in 400ml 
H₂O for 1 min followed by rinsing in 400ml propan-2-ol for 1 min and then it was 
centrifuged at 550xg for 5 mins to dry. The slide was stored in a dark, dust-free box 
until hybridization (<1hr).

4.3.1.8 Wash A preparation

Wash A components (Table 4.3) were mixed in a sealed bottle and incubated at 
65°C along with an empty staining trough.

Table 4.3. Reagents of wash-A

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>20X SSC</td>
<td>20ml (1X SSC)</td>
</tr>
<tr>
<td>20% SDS</td>
<td>1ml (0.05% SDS)</td>
</tr>
<tr>
<td>H₂O</td>
<td>to 400ml</td>
</tr>
</tbody>
</table>

4.3.1.9 Hybridization of the microarray slide with Cy3/Cy5 labeled amplified Tn 
flanking DNA

The freshly pre-hybridized microarray slide was placed in the hybridization 
cassette and two 15μl aliquots of H₂O were added to the wells in the cassette. The 
Cy3/Cy5 labeled amplified transposon flanking DNA sample was mixed with the 
following hybridization solution (Table 4.4).

Table 4.4. Hybridization solution

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cy3/Cy5 labeled amplified Tn flanking DNA sample</td>
<td>10.5μl</td>
</tr>
<tr>
<td>filtered 20X SSC</td>
<td>3.2μl (4X SSC)</td>
</tr>
<tr>
<td>filtered 2% SDS</td>
<td>2.3μl (0.3% SDS)</td>
</tr>
</tbody>
</table>

The hybridization solution was heated at 95°C for 2 mins, allowed to cool 
slowly and briefly centrifuged. The hybridization solution was pipetted onto the slide at

78
the edge of the arrayed area avoiding bubble formation. Using fine forceps, the edge of the cover slip was dragged along the surface of the slide towards the arrayed area and into the hybridization solution at the edge of the array. The cover slip was carefully lowered down over the array avoiding any additional movement once in place. The hybridization cassette was sealed and submerged in the water bath at 65°C in the dark for 16-20hrs.

4.3.1.10 Washing the slide

Wash B (x2) was prepared by mixing the components together (Table 4.5).

**Table 4.5. Reagents of wash-B**

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>20X SSC</td>
<td>1.2 ml (0.06X SSC)</td>
</tr>
<tr>
<td>H₂O</td>
<td>to 400ml</td>
</tr>
</tbody>
</table>

Pre-heated wash A at 65°C was added to a pre-heated staining trough at 65°C. The microarray slide was removed from the hybridization cassette and initially washed carefully in staining trough of wash A at 65°C to remove the cover slip. Once the cover slip was displaced, the slide was placed in a slide rack and agitation was continued in wash A for a further 2 mins.

Slides were transferred to a clean slide rack and agitated in the first trough of wash B for 2 mins, followed by washing for 2mins in a second trough of wash B. Slides were placed into 50ml centrifuge tubes and centrifuged at 550xg for 5 mins to dry.

4.3.1.11 Scanning the slide

Slides were scanned using a dual-laser scanner Axon 4000B, signal intensities and the corresponding gene IDs were determined, followed by analyzing the transposon insertions with reference to the corresponding mycobacterium ORFs.
4.3.2 Infecting *Acanthamoeba castellanii* with the *M. bovis* BCG mutant library

### 4.3.2.1 Strains and growth conditions

*A. castellanii* was grown and counted as described in chapter 2, sections 2.1 and 2.2 respectively. The *M. bovis* BCG mutant library was grown in 7H9+OADC+Hyg50 at 37°C to reach an O.D of ~1.0.

### 4.3.2.2 Infection process

*A. castellanii* culture was infected with the BCG mutant library at an M.O.I of 10:1. Dilutions (10^-1 to 10^-7) of the BCG library were plated onto 7H11-Hyg50 plates to more accurately determine the number of bacteria used for infection. The PPG was removed from the flasks, which were then inoculated with the library and incubated at 20°C for 2 hrs. The BCG culture was removed, amoebae washed with sterile PAS and incubated with PAS-amikacin (100µg/ml) for 2 hrs at 20°C. The PAS-amikacin was removed and the culture washed with sterile PAS. PPG was added to all the flasks.

Two of the four flasks were processed further for bacterial uptake at time zero (T0) while the rest were incubated at 20°C. 0.5% SDS (w/v) was added to the flasks, the amoebae were scraped off and the whole culture was transferred to a universal tube and centrifuged at 3000rpm for 10 mins. The supernatant was discarded and the pellet resuspended in 7H9+OADC+Hyg50. Dilutions (10^-1 to 10^-5) were plated onto 7H11+OADC+Hyg50 to determine the total number of bacteria recovered after infection. The plates were incubated at 37°C for two weeks. The remaining recovered culture was grown in 7H9+OADC+Hyg50 for genomic DNA isolation. The above steps were repeated for taking readings on day 1 to day 7.

### 4.3.3 Making a transposon library of *M. bovis*

Transposon mediated DNA exchange in which a foreign DNA i.e. a transposable element can be introduced randomly into multiple sites in the bacterial genome provides the means for a 'global gene inactivation' method. Based on a similar principle, an attempt was made to create a mutant library of *M. bovis* using a Tn5 based transposon (Ez::Tn transposon). The plasmid pMOD-Hyg was digested with *Pvu*II to release the hygromycin fragment with mosaic ends which was mixed with transposase and finally electroporated into *M. bovis* competent cells to create mutants (figure 4.6).
Figure 4.6. Strategy for creating a transposon mutant library of *M. bovis*. The transposable element (hygromycin) is flanked by 19-bp mosaic ends (ME) that are specifically and uniquely recognized by EZ-Tn5 Transposase.
4.3.3.1 Strains and growth conditions

_**E. coli**_ DH5α was maintained in LB broth or LB agar with Hyg150 at 37°C. _M. bovis_ competent cells were made as described in chapter 2, section 2.4.

4.3.3.2 Transposon construction

_**E. coli**_ DH5α cells were transformed with plasmid pMOD-Hyg (Stewart et al, 2005) containing the transposon. Plasmid DNA was purified using a QIAprep Spin Miniprep Kit (QIAGEN) and a microcentrifuge. Restriction digested with _PvuII_ to release the transposon. The digested sample was run on 1% agarose gel and the Hyg fragment with mosaic ends (the transposon ends recognized by the transposase) extracted using the QIAquick Gel Extraction Kit Protocol (chapter 2, section 2.8) using a microcentrifuge.

4.3.3.3 Production of Ez-Tn Transposome

The transposome reaction mixture was prepared by adding in the following order:

- 2µl Ez-Tn transposon DNA (100 µg/ml in TE buffer)
- 4 µl Ez-Tn5 transposase (Epicentre)
- 2 µl 100% glycerol

The reaction mixture was mixed by vortexing and incubated for 30 mins at room temperature.

4.3.3.4 Electroporation of _M. bovis_ competent cells with the Ez-Tn transposome

Two microliters transposome was electroporated into 200 µl _M. bovis_ competent cells (x4) as described before (Chapter 2, section 2.5) and finally plated onto 7H11+OADC+Hyg50 plates. Plates were incubated at 37°C for 3-4 weeks.

4.3.4 Sequencing of individual _M. bovis_ library mutants

Eleven single colonies were randomly selected from the _M. bovis_ library and each was grown in 7H9-Hyg. DNA of all individual mutants was extracted as described before (Chapter 2, section 2.3).

Ten µl of each DNA was digested with _BssHII_ and _MluI_ (table 4.6)
Table 4.6. DNA digestion reaction

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Quantity (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>10.8</td>
</tr>
<tr>
<td>gDNA</td>
<td>10.0</td>
</tr>
<tr>
<td>RNase (10mg/ml)</td>
<td>0.2</td>
</tr>
<tr>
<td>NEB 3 Buffer</td>
<td>2.5</td>
</tr>
<tr>
<td>BssHII</td>
<td>1.5</td>
</tr>
</tbody>
</table>

The tubes containing the DNA and digestion mix were put in a 50°C water bath for 3 hrs. After that, 1µl MluI was added to each tube and incubated at 37°C.

DNA was cleaned using QIAQuick PCR Purification kit (QIAGEN) (chapter 2, section 2.7). 1µl of each mutant DNA was run on a 1% agarose gel to confirm the digestion reaction.

The purified DNA was then sent off to Eurofins MWG Operon for sequencing using Tseq1 primers.

4.3.5 Statistical Analysis of Microarray Data

Microarray data was normalised to the medium of each dye/channel in each array, and then mean T7/T0 ratios were calculated. P-values were calculated by paired t-test followed by multiple testing corrections.
4.4. RESULTS

4.4.1. TruSH screening of the Ez::Tn BCG mutant library

For initial analysis, an Ez::Tn (EpiCenter) BCG mutant library was obtained from the laboratory culture collection (Stewart et al., 2005). Microarray hybridization was used to estimate the position and relative abundance of mutants in the *M. bovis* BCG transposon library. It was important to selectively amplify the DNA sequences flanking the inserted transposon. The PCR based method (figure 4.3) used here requires the information of only transposon-specific sequences, consisting of two simple steps of ligation and amplification and does not exhibit non-specific background amplification (Stewart et al., 2005).

For selective amplification of transposon flanking DNA, the genomic DNA of BCG mutant library was *BssHl/MluI* digested, ligated to Y-shaped linkers TA1/TA2 and amplified using transposon specific primer TA3 and 3 different types of the Y-linker specific primer TA4. These TA4 primers differ in the number of bases as shown in table 4.1 and were compared in this study to improve transposon amplification which was not optimal using the published primer pair (Stewart et al., 2005). Digest sizes were analysed by running on 1% agarose gel (figure 4.7).
These results clearly demonstrate that only the transposon specific DNA regions were amplified (figure 4.7; lanes 3, 4, 5, 11, 12, 13) whereas no amplified product was obtained for the control or WT-BCG (without any transposon insert; figure 4.7; lanes 6, 7, 8, 14, 15, 16) signifying the efficiency of amplification strategy.

These PCR products were used as a template for a second round of PCR amplification between the Y-linker-specific primer TA4 and a nested transposon-specific primer TA6. The resulting labelled mutant BCG library products were mixed
with the appropriate labelled control (WT-BCG) products and eluted together using the Qiagen MinElute kit and finally hybridized to the whole-genome microarray slides (figure 4.8).

**Figure 4.8.** Microarray of *M. bovis* BCG transposon library. The library DNA was labelled with Cy5 (red) and control WT- BCG DNA with Cy3 (green). The presence of red spots indicate the genes flanking the transposon insert.

As the control was labelled with green fluorescent dye (Cy3) and mutants with red (Cy5), each red spot represents a gene that has been disrupted by the insertion of the transposon, and hence represents the mutated version of the specific gene as represented by the array. Hybridization from the wild type BCG control indicates non-transposon specific amplification and labelling.
Another way of showing the transposon incorporation into the mycobacterial genome is by measuring the signal intensities of the labelling dyes. The signal intensity of each spot/gene was quantified by subtracting the background dye from the foreground values of the dyes. Figure 4.9 shows the graphical representations of transposon insertions. It should be noted that the amplification of the control DNA (BCG-WT) by the TraSH procedure gave low hybridization signal intensities. Thus, these graphs represent the sites of Tn insertion across the mycobacterial genome. The x-axis represents the mycobacterial open reading frames against the signal intensities of the Cy3 or Cy5 dyes (y-axis). Three different types of TA4 primers and different number of amplification cycles (18 and 20) were used in order to optimize the methodology. These primers differ from each other in sequence and length (Table 4.1); with primer TA4 being the shortest (21 bases) as compared to TA4-long which is the longest (27 bases).
Figure 4.9: Graphs showing the transposon insertions at different ORFs, as indicated by the signal intensities. Signal intensities have been plotted against the ORFs of mycobacterium as represented on the array; each of the bars represents the transposon insertion at the corresponding ORF. These 6 profiles vary on the basis of different
number of PCR cycles and the type of TA4 primer. (18 PCR cycles: OP3 95-97; 22 PCR cycles: OP3 98- 100; TA4 primer: OP3 95, 98; TA4 medium: OP3 96, 99; TA4 long: OP3 97, 100). 

TA4-long Y-linker-specific primer shows more efficient amplification of the transposon flanking DNA as compared to the TA4 or TA4-medium primers. It might be due to primer length because at higher temperatures, smaller oligonucleotides (in this case TA4 and TA4-medium) dissociate more rapidly as compared to the longer ones (TA4-long).

Also, the melting temperature (Tm) of the primers plays an important part in the amplification process. The melting temperature of a nucleic acid duplex increases both with its length, and with increasing GC content. The melting temperatures of TA4, TA4-medium and TA4-long are predicted to be 73.8, 73.9 and 78.1 respectively. The annealing temperatures of these primers is critical for the efficiency of the amplification as the high annealing temperature (72°C) in the PCR cycles is necessary to maintain specificity. It appears that the shorter primers are not annealing well at 72°C despite their predicted melting temperatures. The predicted melting temperatures are approximate and will be affected by salt concentration and other additives in the buffer.

4.4.2. A. castellanii infection with BCG mutant library

In order to identify particular mutants defective for intra-amoebal survival, the pool of 2500 mutants in the BCG library was used to infect Acanthamoeba castellanii. Initially the infections were carried out in tissue culture plate wells but the recovered bacterial count dropped by day seven (results not shown) probably because not enough bacteria were taken up. Therefore, infections were done in big tissue culture flasks allowing larger volumes but keeping the same multiplicity of infection to see if increasing the scale of the infection had any effect on the outcome. The purpose was to infect amoebae with as many mutants as possible so that each individual mutant gets a chance to be internalized.
To rule out the possibility of variable conditions during these two different experiments, infections in both wells and flasks were carried out in a single experiment with uniform conditions.

Figure 4.10. BCG mutants (log no. of bacteria /ml) recovered after infection period. Infections were done in wells (W) and flasks (F) in a single experiment, at same M.O.I and under uniform conditions. Bacteria were recovered immediately after infection (T0) and at day 7 (T7).

Figure 4.10 shows duplicate results of the log number of bacteria recovered per millilitre after day zero (T0) and day seven (T7) of infection in flasks (F1, F2) and wells (W1, W2). There is a three-log drop in bacterial counts over the period of infection suggesting incapability of BCG mutants to survive well inside the protozoan for long.

It is also clear that the number of bacteria/ml recovered after infection remains almost same in both scales. However, if we calculate the total number of bacteria recovered, then the number recovered from flasks is about a log higher than from wells (figure 4.11).
Figure 4.11. The total number of BCG mutants recovered after infection at times T0 and T7 in flasks (F) and plate wells (W). F1, F2, W1, W2 represent duplicate flasks and wells respectively.

It is observed that increasing the scale of the experiment provides a little advantage but it is not significant enough to give higher counts of recovered bacteria probably because multiplicity of infection was kept the same.

Another important factor to consider here is that bacteria were recovered at day zero and then at day seven. In order to get the exact point where the numbers started to decrease, bacteria were recovered each day. It was observed (figure 4.12) that the bacterial counts starts to drop by day 1, dropping further on day 2 and 3 until very few or none could be recovered later.
Figure 4.12. Total bacteria recovered after 3 days of infection. Bacteria were recovered from amoebae flasks every day to analyse the pattern of log drop. It is shown that the number had already fallen by day 2 as there is a 0.7 log decrease in the number of total recovered bacteria.

The aim was to recover at least $10^5$ mutants at the end of infection period (day 7) in order to do TraSH. Initial experiments were performed in tissue culture plate wells which did not produce enough mutants at day 7. Therefore, the experiment was scaled up to tissue culture flasks but that also did not improve the number of recovered bacteria. Recovering bacteria at different time points (day 3, day 5) was also tried but unfortunately, the recovery was very low. Therefore, it was concluded that BCG was too attenuated to do further TraSH without doing impractically large infections.

4.4.3. Constructing a library of \textit{M. bovis} transposon mutants

As BCG infections did not work out, it was decided to apply the TraSH assay to study the survival of \textit{Tn} mutants of virulent \textit{M. bovis} in amoebae. Therefore, the Ez::Tn transposon based mutagenesis system (EpiCentre) was used to generate a library of \textit{M. bovis} random mutants (figure 4.6).

\textit{Tn5} is a bacterial genetic element that transposes via a cut-and-paste mechanism. Only three macromolecular components are required for this process; the transposase, the transposon and the target DNA into which the insertions are made (Goryshin and Reznikoff, 1998). The \textit{Tn5} transposition process begins with the
formation of a transposome. The transposase monomers bind to the 19bp end sequences of the transposon. These transposon end-bound transposase monomers then oligomerize to form a transposition synaptic complex. This is followed by the blunt end cleavage of the transposition synaptic complex from adjoining DNA, resulting in the formation of a released transposition complex or Transposome. The resulting transposome was electroporated into *M. bovis* electrocompetent cells where it binds randomly to the target mycobacterial DNA. The 3'end of the transposon is transferred into a staggered 9bp *M. bovis* target sequence where the transposase is removed by the host and the gaps are filled. Mutant clones with transposon insertions were finally selected by plating with hygromycin.

The total number of mutants generated in the library was estimated by counting individual colonies after selection on appropriate medium. A total of about 2500 individual mutants were generated.

**4.4.4. Evaluation of the *M. bovis* transposon mutant library**

**4.4.4.1. Confirming genuine transposon insertions by DNA sequencing**

In order to confirm that the mutants were genuine transposon insertions and to examine any insertion bias, the insertion sites of some individual transposon mutants were sequenced. For this purpose, DNA of 11 randomly selected individual transposon mutants was isolated and digested with *BssHII* and *MluI* which leaves a 4 bp overhang. The Y-shaped linker TA1/TA2 is complementary to this overhang and is easily ligated to the digested DNA. The transposon specific primer anneals to the transposon sequences during the first round of PCR and extends the DNA synthesis into the Y region of the ligated Y linker so that the Y linker specific primer can now anneal. Hence the two primers selectively amplify the fragments containing the transposon. Figure 4.13 shows the gel picture of PCR products of some individual mutants. All the 11 PCR products show a single DNA band of different size, indicating single transposon insertion in each clone and that the insertions were in different genomic positions.
Figure 4.13: Electrophoresis gel picture showing PCR products of individual transposon mutants (lane 2-12). Each sample shows a discrete DNA band of different size implying a unique individual mutant of the *M. bovis* transposon library.

The amplified products were sequenced by sending the purified PCR products to MWG sequencing company. The resulting sequences (9 out of 11) were used to run BLAST against the *M. bovis* genome to identify the genes disrupted by the transposon insertion (Table 4.7).
Table 4.7: List of *M. bovis* genes/regions disrupted due to transposon insertion as shown by DNA sequencing.

<table>
<thead>
<tr>
<th>Random mutant (RM) No.</th>
<th><em>M. bovis</em> gene disrupted</th>
<th>Function of the gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>RM 1</td>
<td>Between greC1 and htpX</td>
<td>Intergenic DNA (no known function)</td>
</tr>
<tr>
<td>RM2</td>
<td>Mb2700</td>
<td>Conserved hypothetical alanine rich protein</td>
</tr>
<tr>
<td>RM3</td>
<td>cstA</td>
<td>Probable carbon starvation protein a homolog cstA</td>
</tr>
<tr>
<td>RM4</td>
<td>Mb3232c</td>
<td>Conserved hypothetical protein</td>
</tr>
<tr>
<td>RM5</td>
<td>Between Mb 2713c and Mb 2714</td>
<td>Intergenic DNA (no known function)</td>
</tr>
<tr>
<td>RM6</td>
<td>Mb2804c</td>
<td>Possible alanine rich oxidoreductase</td>
</tr>
<tr>
<td>RM7</td>
<td>mmsA</td>
<td>Valine and pyrimidine metabolism</td>
</tr>
<tr>
<td>RM8</td>
<td>lpqZ</td>
<td>Putative lipoprotein lpqz</td>
</tr>
<tr>
<td>RM9</td>
<td>Mb1449</td>
<td>Conserved hypothetical protein</td>
</tr>
</tbody>
</table>

Sequencing of insertion sites within specific genes revealed 9-bp direct repeats flanking the transposon, thus indicating genuine Tn5 transposition (Berg *et al.*, 1983). Further analysis of the insertions revealed some site preference, with position 1 of the direct repeat biased to G in 80% cases and position 9 to C in 50% of events (figure 4.14 a).
Figure 4.14. Sequence logo showing the relative frequency of each base upstream and downstream of the transposon insertion. (a) Tn5 insertion bias observed in *M. bovis* mutant library (present study) and (b) in *M. bovis* BCG mutant library (Stewart *et al.*, 2003) created using the same EZ::TN system. The transposon inserts and creates a 9bp direct repeat (position 11 to 19, shown as a red box).

The preference of the Tn5 transposon to insert at the nucleotide G has also been observed previously. Stewart *et al.* (2005) used the Epicentre EZ::TN system to create a BCG mutant library and the sequence analysis of 30 mutants revealed a site preference with a G at position 1 and a C at position 9 of the direct repeat in more than 60% of the events (figure 4.14 b). This preference observed in both studies suggests that Epicentre EZ::TN is a very effective transposition system in GC-rich genomes such *M. bovis*. It contrasts with the mariner based transposon systems which are commonly used in mycobacteria (Sassetti *et al.*, 2003) and insert at TA sites only.
4.4.4.2. TraSH analysis of the *M. bovis* library

For TraSH, DNA was digested by *BssHII* and *MluI* which cut, on average, every 800 and 600 bp respectively. These digested DNA fragments are then ligated to TA1/TA2 linkers, followed by first round of PCR (also known as transposon screen by microarray or TSM 1) using transposon specific (TA3) and Y-linker specific (TA4) primers. Different numbers of amplification cycles are run so that appropriate sized product of first round of PCR is used as a template for the second PCR (or TSM 2). This stage is important as too much or too little of the template can lead to false positive or false negative labelling on the microarray.

In order to make sure that each labelled spot on the microarray represents a single gene disruption, and not due to amplification of adjacent genes, the TSM 1 products less than 1000 bp were cut out from the gel and purified for use in TSM 2. Microarray protocol was performed as described before (chapter 4, section 4.3.1.6).
Figure 4.15: Microarray scan showing different spots after the TSM 1 products less than 1000 bp were cut from the gel and then purified for use in TSM 2.
Figure 4.15 shows the results of the technique of cutting the gel smears of the TSM1 cycle. T0 DNA was labelled with Cy3 and T7 with Cy5. A mix of yellow, green and red coloured spots can be seen. Yellow spots imply the fact that the mutant population remains largely the same at T0 and T7 with a small fraction of mutants differing at the two time points.

Green spots demonstrate mutants that were present at T0 but were lost during the course of infection (T7), representing a mutation in an essential *M. bovis* gene required for survival in amoebae.

The red spots on this array (figure 4.15) are strange as they represent mutants that were not originally present at T0 and hence should not be present at T7. This peculiarity can be explained by the way in which DNA was processed after TSM-1. The DNA smears from TSM-1 were cut out from the gel, purified and then used in TSM-2. As the cutting was done manually, it might have resulted in unequal cutting out of the two DNAs. Since the two DNA smears cannot be cut with 100% accuracy every time, it can lead to unreliable results. Therefore, this cutting step was suspended.

The microarray was repeated 8 times with independent amplification cycles and dye swapping. Samples with dye concentration of about 1.0 pmol/ul were selected for Microarray. Figure 4.15 shows the microarray image of the *M. bovis* library. The library was labelled with Cy5 (red) and the control DNA (WT-*M. bovis*) with Cy3 (green). The fact that most of the array is labelled with Cy5 shows that the transposon inserted well throughout the mycobacterial genome and also that the amplification strategy was successful.
Figure 4.16: Microarray scan of the *M. bovis* library showing the distribution of transposon insertion throughout the *M. bovis* genome (*M. bovis* library labelled with Cy5 and control *M. bovis* with Cy3).
As shown in figures 4.16 and 4.17, the transposon has inserted fairly randomly and evenly throughout the mycobacterial genome which clearly demonstrates the success of the transposon mutagenesis as a means of global gene inactivation and therefore an invaluable tool for various genetic studies. The genome-wide distribution of transposon insertion sites in the *M. bovis* library was estimated by TraSH in exactly the same manner as done with *M. bovis* BCG mutant library (described earlier).

![M.bovis library](image)

**Figure 4.17:** Transposon insertions at different ORFs, as indicated by the signal intensities. Signal intensities have been plotted against the ORFs of mycobacterium as represented on the array; each of the bars represents the transposon insertion at the corresponding ORF.

Although the genome sequence of *M. tuberculosis* and *M. bovis* is >99.95% identical (Garnier *et al.*, 2003), there are some genes which are missing from *M. bovis* as compared to *M. tuberculosis* H37Rv (Table 4.8). Since the microarray slide contains the oligonucleotides from *M. tuberculosis* ORFs and the library used in this study is *M. bovis* mutants, there is no hybridization from the missing *M. bovis* genes and hence very little signal (figure 4.18) could be obtained from those spots on the microarray. This also proves that the *M. bovis* library contains genuine transposon mutants.
Table 4.8. Genes missing from *M. bovis* as compared to *M. tuberculosis* H37Rv along with the corresponding signal intensities. Almost all of these values are lower than the cut-off value of 500.

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Signal intensities</th>
</tr>
</thead>
<tbody>
<tr>
<td>MtH37Rv-0221</td>
<td>119.5</td>
</tr>
<tr>
<td>MtH37Rv-1255c</td>
<td>422</td>
</tr>
<tr>
<td>MtH37Rv-1256c</td>
<td>405.5</td>
</tr>
<tr>
<td>MtH37Rv-1257c</td>
<td>39.5</td>
</tr>
<tr>
<td>MtH37Rv-1505c</td>
<td>909.2</td>
</tr>
<tr>
<td>MtH37Rv-1506c</td>
<td>-29</td>
</tr>
<tr>
<td>MtH37Rv-1507A</td>
<td>30</td>
</tr>
<tr>
<td>MtH37Rv-1507c</td>
<td>-25</td>
</tr>
<tr>
<td>MtH37Rv-1508A</td>
<td>175</td>
</tr>
<tr>
<td>MtH37Rv-1508c</td>
<td>-19</td>
</tr>
<tr>
<td>MtH37Rv-1509</td>
<td>74</td>
</tr>
<tr>
<td>MtH37Rv-1510</td>
<td>134</td>
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<tr>
<td>MtH37Rv-1511</td>
<td>23.5</td>
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<tr>
<td>MtH37Rv-1512</td>
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<tr>
<td>MtH37Rv-1513</td>
<td>-3</td>
</tr>
<tr>
<td>MtH37Rv-1514c</td>
<td>24</td>
</tr>
<tr>
<td>MtH37Rv-1515c</td>
<td>327</td>
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<tr>
<td>MtH37Rv-1516c</td>
<td>108.5</td>
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<td>132.5</td>
</tr>
<tr>
<td>MtH37Rv-1965</td>
<td>25.5</td>
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<tr>
<td>MtH37Rv-1966</td>
<td>12.5</td>
</tr>
<tr>
<td>MtH37Rv-1967</td>
<td>511</td>
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<tr>
<td>MtH37Rv-1968</td>
<td>74.5</td>
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<td>MtH37Rv-1970</td>
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<tr>
<td>MtH37Rv-1971</td>
<td>46</td>
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<td>MtH37Rv-1972</td>
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</tr>
<tr>
<td>MtH37Rv-1975</td>
<td>-18</td>
</tr>
<tr>
<td>MtH37Rv-1976c</td>
<td>36.5</td>
</tr>
<tr>
<td>MtH37Rv-1977</td>
<td>95.5</td>
</tr>
<tr>
<td>MtH37Rv-2346c</td>
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<tr>
<td>MtH37Rv-2347c</td>
<td>244</td>
</tr>
<tr>
<td>MtH37Rv-2348c</td>
<td>49</td>
</tr>
<tr>
<td>MtH37Rv-2349c</td>
<td>170</td>
</tr>
<tr>
<td>MtH37Rv-2350c</td>
<td>-8</td>
</tr>
<tr>
<td>MtH37Rv-2351c</td>
<td>64.5</td>
</tr>
<tr>
<td>MtH37Rv-2352c</td>
<td>477.5</td>
</tr>
<tr>
<td>MtH37Rv-2353c</td>
<td>26.5</td>
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<tr>
<td>MtH37Rv-3121</td>
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<td>MtH37Rv-3425</td>
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</tr>
<tr>
<td>MtH37Rv-3426</td>
<td>68.5</td>
</tr>
<tr>
<td>MtH37Rv-3427c</td>
<td>2</td>
</tr>
<tr>
<td>MtH37Rv-3428c</td>
<td>34</td>
</tr>
<tr>
<td>MtH37Rv-3619c</td>
<td>292.5</td>
</tr>
<tr>
<td>MtH37Rv-3620c</td>
<td>47</td>
</tr>
<tr>
<td>MtH37Rv-3905c</td>
<td>1364</td>
</tr>
</tbody>
</table>
Figure 4.18. Signal intensities of *M. tuberculosis* genes that are missing from and *M. bovis* as shown by microarray analysis. Since *M. bovis* library is hybridized to the *M. tuberculosis* microarray, there is very low/no signal on the corresponding gene spots, further verifying the genuine transposon library. Note that the y-axis scale is kept same as in figure 4.17.

4.4.5. Infecting *A. castellanii* with *M. bovis* library

*Acanthamoeba castellanii* was infected with *M. bovis* mutant library in the same manner as described for *M. bovis* BCG library (section 4.3.2) except that the multiplicity of infection was 100:1. 10^7 cfu/ml were recovered at T0 and 10^5 cfu/ml at T7 (figure 4.19). Therefore, DNA of both the recovered pools was extracted for mutant identification and quantitation by TraSH.
Figure 4.19. *M. bovis* mutants (log no. of bacteria /ml) recovered during the infection period (T0 and T7).

As shown in figure 4.20, the recovered bacteria at T0 and T7 were amplified and labelled with Cy3 and Cy5 respectively. If we observe visually, we cannot see any distinct red or green spots for the independent dyes. Rather, there are overall yellow toned spots representing the presence of both T0 and T7 pools. This indicates that perhaps most of the *M. bovis* mutants were of similar abundance in the input and output pools. However, collection of signal intensities from five independent replicate infections allowed for statistical analysis and the identification of any mutants with reduced survival in the amoebae as compared to the average survival of mutants in the pool.
Figure 4.20. Example of a microarray scan of the *M. bovis* mutants recovered at T0 (Cy3) and T7 (Cy5) post infection.
4.4.6 Statistical analysis of TraSH data

Data was normalised to the medium of each dye/channel in each array, and then mean T7/T0 ratios were calculated. Only these genes having the cut off value of less than 0.5 mean T7/T0 ratios were considered for further analysis as shown in Table 4.9. This cut off allows only those genes whose abundance has been reduced to 50% or lower at T7 days as compared to the beginning of the experiment i.e. T0 days.

Table 4.9: List of genes whose abundance is reduced by at least 50% or lower at T7 as compared to T0.

<table>
<thead>
<tr>
<th>ID</th>
<th>MEAN T7/T0 RATIO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rv-2187</td>
<td>0.312</td>
</tr>
<tr>
<td>Rv-0443</td>
<td>0.316</td>
</tr>
<tr>
<td>Rv-0193c</td>
<td>0.321</td>
</tr>
<tr>
<td>Rv-1728c</td>
<td>0.389</td>
</tr>
<tr>
<td>Rv-3087</td>
<td>0.400</td>
</tr>
<tr>
<td>Rv-2693c</td>
<td>0.421</td>
</tr>
<tr>
<td>Rv-0598c</td>
<td>0.428</td>
</tr>
<tr>
<td>Rv-0853c</td>
<td>0.430</td>
</tr>
<tr>
<td>Rv-0205</td>
<td>0.431</td>
</tr>
<tr>
<td>Rv-1414</td>
<td>0.433</td>
</tr>
<tr>
<td>Rv-1192</td>
<td>0.442</td>
</tr>
<tr>
<td>Rv-2462c</td>
<td>0.443</td>
</tr>
<tr>
<td>Rv-3420c</td>
<td>0.443</td>
</tr>
<tr>
<td>Rv-1223</td>
<td>0.447</td>
</tr>
<tr>
<td>Rv-0724</td>
<td>0.452</td>
</tr>
<tr>
<td>Rv-0901</td>
<td>0.463</td>
</tr>
<tr>
<td>Rv-0476</td>
<td>0.464</td>
</tr>
<tr>
<td>Rv-3104c</td>
<td>0.464</td>
</tr>
<tr>
<td>Rv-3133c</td>
<td>0.468</td>
</tr>
<tr>
<td>Rv-3230c</td>
<td>0.476</td>
</tr>
<tr>
<td>Rv-2047c</td>
<td>0.480</td>
</tr>
<tr>
<td>Rv-3243c</td>
<td>0.480</td>
</tr>
<tr>
<td>Rv-1399c</td>
<td>0.485</td>
</tr>
<tr>
<td>Rv-1181</td>
<td>0.498</td>
</tr>
<tr>
<td>Rv-1986</td>
<td>0.499</td>
</tr>
</tbody>
</table>
Although there has been a reduction in the number of clones with mutations in these genes (table 4.9) during the experiment, it is important to know if this decrease was statistically significant. For this purpose, p-values were generated by a paired t-test. At this point, there appears to be some significantly different mutants with a p-value less than or equal to the significance level ($\alpha$) of 0.05 ($\alpha \leq 0.05$) as highlighted in table 4.10.

**Table 4.10:** List of statistically significant genes by a paired t-test.

<table>
<thead>
<tr>
<th>ID</th>
<th>Mean T7/T0 Ratio</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rv-2187</td>
<td>0.312</td>
<td>0.151</td>
</tr>
<tr>
<td>Rv-0443</td>
<td>0.316</td>
<td>0.136</td>
</tr>
<tr>
<td>Rv-0193c</td>
<td>0.321</td>
<td>0.121</td>
</tr>
<tr>
<td>Rv-1728c</td>
<td>0.389</td>
<td>0.043</td>
</tr>
<tr>
<td>Rv-3087</td>
<td>0.400</td>
<td>0.017</td>
</tr>
<tr>
<td>Rv-2693c</td>
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</tr>
<tr>
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<tr>
<td>Rv-0853c</td>
<td>0.430</td>
<td>0.127</td>
</tr>
<tr>
<td>Rv-0205</td>
<td>0.431</td>
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<tr>
<td>Rv-1414</td>
<td>0.433</td>
<td>0.090</td>
</tr>
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</tr>
<tr>
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<td>Rv-0724</td>
<td>0.452</td>
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<td>Rv-0901</td>
<td>0.463</td>
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</tr>
<tr>
<td>Rv-0476</td>
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</tr>
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<tr>
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</tr>
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<td>0.480</td>
<td>0.184</td>
</tr>
<tr>
<td>Rv-3243c</td>
<td>0.480</td>
<td>0.107</td>
</tr>
<tr>
<td>Rv-1399c</td>
<td>0.485</td>
<td>0.154</td>
</tr>
<tr>
<td>Rv-1181</td>
<td>0.498</td>
<td>0.171</td>
</tr>
<tr>
<td>Rv-1986</td>
<td>0.499</td>
<td>0.143</td>
</tr>
</tbody>
</table>

Based on the results of the t-test alone, one can imply form table 4.8 that *M. bovis* clones with a mutation in seven genes (Rv1728c, Rv3087, Rv2693c, Rv0598c, Rv1192,
Rv2462c and Rv3104c) were less fit for intracellular survival, indicating that these genes might have a role in intracellular survival in amoebae.

**Rv1728c** is a non-essential gene (Sasseti et al., 2003), coding a conserved hypothetical protein of 256 amino acids.

**Rv3087** codes a conserved hypothetical protein of 472 amino acids. Rv3087 is part of the Rv3083-Rv3089 operon of *M. tuberculosis* that has been shown to be induced when tubercle bacilli were exposed to acidic conditions *in vitro* and also when inside macrophages. This indicates that the operon plays an important role in the ability of *M. tuberculosis* to grow inside host cells (Cheruvu et al., 2007). This gene is also considered to be essential in mouse model of infection (Sassetti et al., 2003). This is a very significant finding that this gene appears to be required during survival in amoebae as well as macrophages. Therefore, this gene is an attractive target for further research for confirming the role of this operon for *M. bovis* survival in amoebae.

**Rv2693c** is part of the Rv2693c-Rv2694c operon. Rv2693 codes a conserved alanine and leucine rich membrane protein while Rv2694c for a conserved hypothetical protein. It is a non-essential gene (Sassetti et al., 2003). In another study, results from the signature-tagged mutagenesis screen indicated that Rv2693c::Tn mutant showed some growth attenuation *in vivo* (MacGurn and Cox, 2007).

**Rv0598c** is also a non-essential gene of the Rv0598c-Rv0599c operon (Sassetti et al., 2003) for a conserved hypothetical protein of *M. tuberculosis*.

The **Rv1192** gene is for a conserved hypothetical protein, and is a part of the Rv1192-*fadD36* operon. FadD36 is fatty-acid-CoA ligase enzyme, encoded by the gene Rv1193.

**Rv2462c** is a non-essential gene (Sassetti et al., 2003) that codes for trigger factor protein (tig) in *M. tuberculosis*.

**Rv3104c** produces a conserved membrane protein. This non-essential gene is part of a Rv3103c-prfB operon where Rv3104c encodes a conserved membrane protein; Rv3103c, a hypothetical proline rich protein while Rv3105c codes for a peptide chain
release factor 2 (prfB) which directs the termination of translation in response to the peptide chain termination codons UGA and UAA (Cole et al., 1998).

4.4.7 RD1, Rv0467, Rv2548 and Rv2745 in TraSH data

As discussed in chapter 3, some evidence is presented here that RD1 might not enhance the survival of BCG in amoebae whereas isocitrate lyase (Rv0467) and CIGR transcriptional regulator (Rv2745) might be important for survival in amoebae. While these results were from infection of amoebae with single mutants, TraSH analysis of the same genes (when in a pool of mutants) produced different results. As mentioned in chapter 4, a cut off value of 50% decrease in abundance was used as a pre-selection criterion to look for genes important for survival. Three genes of the RD1 region Rv3871, Rv3873 and Rv3879c had a mean T7/T0 ratio of 1.5, 0.7 and 1.8 respectively. Guinn et al. (2004) examined mutants disrupted in five individual RD1 genes and observed that both in vitro and in vivo, each mutant displayed an attenuated phenotype very similar to a mutant missing the entire RD1 region. Genetic complementation of individual genes restored virulence. These data argue that RD1-region genes work in concert to form a single virulence entity, since individual gene mutants are phenotypically identical to deletion of the entire RD1 region.

This suggests that a mutation in any gene of the RD1 region would make the whole RD1 locus inefficient, resulting in a decrease in virulence. The fact that the abundance of the three RD1 mutants was not reduced at the end of the experiment might suggest the involvement of other genes that help M. bovis survive within amoebae.

The icl mutant in TraSH had a mean T7/T0 ratio of 0.8 and since only those genes were preselected whose abundance at T7 reduced by at least 50%, the icl gene was not analyzed further. However, this point out that when together in a pool of mutants, the function of icl might be trans-complemented by other mutants.
Also, it is not clear whether the transposon inserted within the open reading frame of \textit{id} gene or the neighboring genes. If so, the \textit{id} mutant might be functionally active to a certain degree which explains its sustained abundance at the two time points (T0 and T7).

Unfortunately, the other gene knock outs could not be analyzed by TraSH data due to poor hybridization signal on the microarray. Rv2548 knock out mutant was shown to be hypervirulent in amoebae. Unfortunately, it was not represented in TraSH data due to poor hybridization signal at that spot. However, TraSH identified other genes whose abundance increased over 100\% and hence were over represented at T7 (Appendix 3). These could also be called as hypervirulent.

\subsection*{4.4.8 Multiple testing corrections}

Multiple testing corrections refer to re-calculating probabilities obtained from a statistical test which was repeated multiple times. It is applied to correct for occurrence of false positives. A typical microarray experiment measures several thousand genes simultaneously across different conditions. When testing for potential gene differences across those conditions, each gene is considered independently from one another. In other words, a \textit{t}-test is performed on each gene separately. The incidence of false positives (genes that appear to be different at the two conditions) is proportional to the number of tests performed (about 4000 in the present study) and the critical significance level (0.05 in this project). This means that the chance that false positives are going to be sampled is higher the more genes we apply a statistical test on.

Here, a paired \textit{t}-test is applied on each gene. The probability by which the absence of a gene is considered to be significantly different at T7 and T0 is expressed by the \textit{p}-value. The \textit{p}-value is the probability that a gene is absent from the T7 population by chance alone. A \textit{p}-value of 0.05 signifies a 5\% probability that the gene is absent at T7 by chance. If 4000 genes are tested, 5\% genes (200 genes) might be called significant by chance alone. Hence, it is important to correct the \textit{p}-value of each gene when performing a statistical test on a large group of genes.
The Benjamini-Hochberg test is the least stringent of all corrections and provides a good balance between discovery of statistically significant genes and limitation of false positive occurrences (Benjamini and Hochberg, 1995). However, after applying the Benjamini and Hochberg to our data, none of the genes remained significant which means any mutants that are significantly less fit than average in the amoebae could not be recognized in this study.

4.5 DISCUSSION

With advances in technology and the data generated, it is vital that appropriate tools are developed to appreciate the scientific knowledge generated. The wealth of genomic data that is available for many disease causing organisms can only be appreciated if the necessary modern techniques are available to decipher the biology and hence the control of the pathogens. The time-consuming approach of studying single genes one at a time is simply not enough. TraSH is one such alternative to classical techniques. The aim of this project is to find out the genes that help *M. bovis* to survive in the amoebae. To highlight potential gene candidates out of a pool of about 4000, it was only logical to use rapid, high-through-put approaches like TraSH.

4.5.1 Optimization of TraSH

TraSH is an expensive and complicated process that requires expertise but once it is standardized, it can prove very useful. In this project, several variable factors were optimized to make use of this powerful tool.

4.5.1.1 Different types of Y-linker specific primer (TA4)

In this study, 3 different types of the Y-linker specific primer (TA4) were used (as stated in Chapter 4, section 4.3.1.1) to optimize the primer binding and DNA amplification.

Figure 4.8 shows that the TA4-long Y-linker-specific primer shows more efficient amplification of the transposon flanking DNA as compared to the TA4 or TA4-medium primers. It might be due to primer length because at higher temperatures, smaller
oligonucleotides (in this case TA4 and TA4-medium) dissociate more rapidly as compared to the longer ones (TA4-long).

Also, the melting temperature (Tm) of the primers plays an important part in the amplification process. The melting temperature of a nucleic acid duplex increases both with its length, and with increasing GC content. The melting temperatures of TA4, TA4-medium and TA4-long are 73.8, 73.9 and 78.1 respectively. The annealing temperature (Ta) should generally be about 5 degrees below the lowest Tm of the primers used. Here I used annealing temperature of 72°C, which is optimum for TA4-long primer so the amplification works better with TA4-long primer.

4.5.1.2 Quantification of template DNA

In order to make sure that each labelled spot on the microarray represents a single gene disruption, and not due to amplification of adjacent genes, during the first set of PCR (TSM-I), different numbers of amplification cycles was used (data not shown). In the light of these results, it was concluded that repeating the amplification cycle 18 and 20 times gave the optimum concentration (20ng/μl) of the target DNA.

4.5.1.3 Quantification of Cy dye incorporation.

The effectiveness of the microarray process is directly related to the presence of the fluorescent dyes present in the sample DNA. Preselecting the viable fluorescent-labelled DNA can eliminate the potentially flawed samples and improve research effectiveness. Therefore, in order to establish that the DNA is well labelled, both incorporated Cy3 and Cy5 dyes were quantified after TSM-II, using the NanoDrop 1000 Spectrophotometer (Thermo Scientific). Samples with dye concentration of about 1.0 pmol/μl were selected for Microarray.

4.5.2 M. bovis mutant library construction

The purpose of this study was to investigate the survival genes of M. bovis inside amoebae with the strategy that every non-essential gene be mutated so that the subsequent loss of intracellular survival marks that candidate gene as essential for survival. Therefore, the Ez::Tn transposon based mutagenesis system (EpiCentre) was
used to generate a pool of *M. bovis* random mutants. It is a simple and highly flexible system that is based on the *in vitro* generation of stable transposome molecules containing *Hygromycin* resistance gene flanked by inverted repeats and subsequent electroporation of these complexes into the target *M. bovis* DNA.

The total number of mutants generated in the library as determined by counting individual colonies was about 2500. The distribution of these individual mutants was found by microarray (figures 4.16 and 4.17). It was found that the transposon had successfully inserted randomly throughout the mycobacterial genome reflecting the success of Ez::Tn transposon mutagenesis system for constructing random mutant libraries. Further confirmation was obtained by sequencing of randomly selected individual mutants from the library. The transposon was able to disrupt, for example genes responsible for alanine rich oxidoreductase, putative lipoprotein and a probable carbon starvation protein A homolog.

4.5.3 *A. castellanii* infection with *M. bovis* mutants

After the creation of a mutant library, the next phase was to infect the amoebae and compare the input and output pools in order to identify survival genes. The infection period was seven days, so ideally we were looking for any gene (or genes) that was present at day one (T0) but that could not be recovered by the end of the infection period (T7). As a pre-selection criteria, only genes that had been reduced by at least 50% in abundance at the end of the infection (T7) as compared to the beginning (T0) were selected. Initially there was some success and seven potential survival genes which were statistically significant by a paired t-test were identified. Five out of seven genes were part of mycobacterial operons. This is quite interesting as it might mean inactivation of more genes because, if these genes are near the promoter of the operon, it can lead to inactivation of the downstream genes as well. For example, in case of Rv3087 operon, Singh *et al.*, (2005) found that the seven open reading frames Rv3083–Rv3089 are arranged in the same orientation in the *M. tuberculosis* genome, with a maximum distance of 38 bp between any two genes suggesting that these genes constitute a transcriptional unit. They also established that the genes Rv3083–Rv3089 are transcribed from a single promoter and constitute an operon referred to as “mymA operon” (named
after the first gene Rv3083, which is a homologue of monooxygenases and thus designated as mymA). The proteins encoded by mymA operon were involved in the modification of fatty acids required for the cell wall of *M. tuberculosis*.

Rv3087 is considered to be essential for growth in murine infection model (Sassetti *et al.*, 2003) and induced when tubercle bacilli are exposed to acidic conditions *in vitro* and in macrophages (Cheruvu *et al.*, 2007). In this study too, the abundance of Rv3087 mutant was reduced to more than half at the end of the experiment which might indicate that the operon plays some role in the ability of *M. bovis* to grow inside amoebae but further research is required to confirm these results.

Although the above outcomes were quite stimulating, unfortunately when the probabilities were re-calculated by applying multiple testing corrections none of the genes remained significant. This implies that any individual mutant was no less fit than the average mutants in the library. However true, this does point to some interesting findings. It is worth taking the genes in table 4.10 for further analysis individually. It will be quite interesting to see how these individual mutants behave when tested alone for survival in amoebae because when in a large pool, mutants may behave differently. The effect of single mutations may be masked or compensated by other mutants in the pool. Also, repeat experiments with more replicates can help produce a more consistent statistical data.
Chapter 5

General discussion

TB has haunted mankind for hundreds of years and it has been a long battle of survival between man and the TB pathogen. The most important weapon in the itinerary of this pathogen is the ability to persist in the host immune cells. Although many studies have investigated the interaction of mycobacteria with immune cells (both human and animal origin), there are still several unknown aspects of tuberculosis pathogenesis that make it difficult to eradicate completely. This calls for the development of new methods and techniques to aid in a better understanding of the pathogen. One of the model organisms offering new insights is the amoeba. In the present study, free-living amoeba (A. castellanii) has been used to study the genetic factors required for the intracellular survival of M. bovis.

5.1. Importance of amoebae.

Free-living amoebae, found in soil and water habitats, have a profound effect on their environment by interacting with other organisms such as bacteria. The interaction of amoeba with bacteria can result in destruction of the bacteria, destruction of the amoeba or alternatively there may develop a symbiotic relationship. Another important feature is the use of amoeba as a diagnostic culture system for non-culturable bacteria. Many of the endosymbiotic bacteria are non-culturable in standard media or on a variety of nutrient agars and can only be cultured in amoebae. Hall and Voelz (1985) isolated an Acanthamoeba sp. strain HN3 containing an endosymbiont that was non-culturable in liquid medium or on nutrient agar. This endosymbiont was later identified as Caedibacter acanthamoebae (Horn et al., 1999). Rowbotham (1993) isolated a
Legionella-like amoebal pathogen (LLAP) from the sputum of a patient with persistent pneumonia by culturing the sputum with Acanthamoeba.

Although most amoebae are free-living, some species have been associated with human disease. *Naegleria fowleri*, is the causative agent of primary amoebic meningoencephalitis, a rapidly fatal disease of the central nervous system. Several species of *Acanthamoeba* cause amoebic keratitis, granulomatous amoebic encephalitis or cutaneous acanthamoebiasis (Marciano-Cabral and Cabral, 2003). *Balamuthia mandrillaris*, also, has been associated with fatal amoebic encephalitis (Martinez *et al.*, 2001). However, the focus of research has shifted from the direct pathogenic effects of free-living amoebae towards their role as carriers of pathogenic bacteria. It has been shown that amoebae host several intracellular pathogens including *Legionella* spp., *Chlamydia* spp., *Parachlamydia* spp., *Listeria* spp., *Burkholderia* spp., *Campylobacter jejuni*, *Helicobacter pylori*, *Pasteurella multocida*, *Salmonella enterica*, *Francisella tularensis*, and *Simkania negevensis* (Greub and Raoult, 2004; Axelsson-Olsson *et al.*, 2005; Hundt and Ruffolo, 2005).

Thus, protozoa appear to play a central role in the transition of bacteria from the environment to mammals. In essence, protozoa may be viewed as a 'biological fitness center', within which intracellular bacterial pathogens train for their encounters with the more evolved mammalian cells (Harb *et al.*, 2000). Nonetheless, it is important to recognize that the virulence determinants responsible for bacterial invasion of *Acanthamoeba*, their intracellular survival via inhibition of formation of phagolysosomes or growth at acidic pH, and their escape from *Acanthamoeba* vary between different bacteria.

### 5.2. Survival tools of Mycobacteria

Mycobacteria are one of the most resilient pathogens. They possess some of the most unique features. Therefore, it is vital to develop techniques which help in understanding the continued existence of this rapidly evolving pathogen.
M. tuberculosis is an unusual microbe with respect to its cell-wall composition. In contrast to many other bacteria, M. tuberculosis harbours a thick cell wall made up of unique lipid and glycolipid moieties (Brennan and Nikaido, 1995) which constitutes an efficient permeability barrier and plays a crucial role in the intrinsic drug resistance and survival under harsh conditions. Loss of mycobacterial cell wall components is typically correlated with reduced virulence, suggesting that the pathogen's cell wall is important for mycobacterial survival inside the host cell (Makinoshima and Glickman, 2005). Also, the recent discovery of an outer membrane in mycobacteria, harboring proteins of unknown structures and functions, adds to the many potential virulence determinants (Hoffman et al., 2008).

Apart from intrinsic physical defenses, sometimes mycobacteria exploit other organisms for their survival. This is particularly important for environmental mycobacteria. They are known to exist and survive in environments which are also colonized by other organisms like protozoa (Nwachuku and Gerba 2004). A remarkable feature of amoebae is the ability to encyst under unfavourable conditions. The encystment process involves the reduction and exocytosis of the cellular content. Ben Salah and Drancourt (2010) reported that all Mycobacterium avium complex (MAC) members typically resided within the exocyst of A. polyphaga. Recently it has been observed that members of the MTC can survive in A. polyphaga cysts. (Mba Medie et al., 2011). This is another way by which mycobacteria are able to survive, even in severe conditions.

Such exploitation is also true in case of human immune cells where macrophages are taken hostage by M. tuberculosis/ M. bovis and therefore, the pathogen remains protected from host defences. Infection with M. tuberculosis occurs in the lung through phagocytosis of the pathogens by pulmonary macrophages. After infection, virulent M. tuberculosis blocks phagosome maturation by interrupting acidification and lysosome fusion, which creates a protected niche in the cell for bacterial replication. Ultimately, intracellular infection leads to macrophage death by necrosis, a process characterized by plasma membrane lysis and escape of the pathogens into the surrounding tissue for a new cycle of infection. M. leprae (Sibley et al, 1987), M. avium (Sturgill-Koszycki, 1996) and M. tuberculosis (MacMiking et al, 2003) all have the ability to prevent maturation of the
macrophage phagosome. Therefore, development of new techniques is indispensable for overcoming the survival strategies of TB pathogen.

5.3. Tools for studying the survival of mycobacteria

In this study, basically three experimental strategies have been employed to identify *M. bovis* genes which promote persistence of the pathogen in protozoa. One approach involves cloning of *M. tuberculosis/M. bovis* genes (RDI) in non-pathogenic mycobacteria and studying their survival in amoebae while the other approaches uses knockout mutants of *M. tuberculosis* (for selected genes) as well as random transposon mutagenesis of the *M. bovis* genome for studying the genetic basis of the survival of the pathogen in *A. castellanii*.

5.3.1 Studying the role of Region of difference 1 (RD1)

Many *Mycobacterium* species have been shown to be phagocytosed including *M. tuberculosis* (Hagedorn *et al.*, 2009), *M. bovis* (Taylor *et al.*, 2003), *M. leprae* (Lahiri and Krahenbuhl, 2008), *M. marinum* (Cirillo *et al.*, 1997), *M. avium* (Steinert *et al.*, 1998; Cirillo *et al.*, 1997), *M. avium* subsp. paratuberculosis (Whan *et al.*, 2006), *M. xenopi* (Drancourt *et al.*, 2007) *M. fortuitum* (Cirillo *et al.*, 1997), *M. smegmatis* (Sharbati-Tehrani *et al.*, 2005). All these *Mycobacterium* species have been shown to persist in trophozoites, with the exceptions of *M. smegmatis* (Cirillo *et al.*, 1997; Sharbati-Tehrani *et al.*, 2005) and the two BCG strains (Pasteur and Japan) of *M. bovis*, which were killed within *A. castellanii* trophozoites, contrary to the *M. bovis* parent strain (Taylor *et al.*, 2003).

These findings raise an interesting point that the non-pathogenic mycobacterium strains did not survive in amoebae. Although there could be other genetic factors, this study focused on RD1, the region of difference 1 which is present in all virulent mycobacteria but absent in avirulent ones. RD1 was introduced into BCG and its survival was investigated in *A. castellanii*. It was observed that the presence of RD1 did not confer any survival advantage to BCG and it died fairly quickly as compared to the
control strains. However, one limitation of the present study was that the functionality of the RD1 was not confirmed. The plasmid construct used in this study has been used in other projects where it was shown that the construct is fully functional (Pym et al., 2002). Although we confirmed the presence of RD1 in the plasmid pYUB412 by PCR, confirmation of RD1 activity would have been useful in concluding if RD1 contributes to the enhanced survival of BCG.

RD1 encodes ESX-1 (type VII secretion system) which is involved in the export of several M. tuberculosis proteins including ESAT-6 (encoded by exxA) and (CFP-10) (encoded by exxB). Region of difference 1 (RD1), encodes proteins that form this novel protein secretion system.

In this study, three mutants from M. bovis library that had transposon insertions in the RD1 genes were also obtained. The mutants were Rv3871, Rv3873 and Rv3879. TraSH results show that none of the three mutants showed decrease survival in amoebae. (Rv3871, Rv3873 and Rv3879 mutants had a p-value of 0.11, 0.52 and 0.44 respectively by a t-test). This means that the mutants with deletion in the RD1 region were no less fit than average mutants in the pool. This is in line with the other findings in this study where it was shown that the reintroduction of RD1 region into BCG did not enhance its survival in amoebae. Hence, it suggests that genetic regions other than RD1 also contribute to the attenuation of BCG or conversely, to the virulence of M. bovis and other virulent mycobacteria.

In a comprehensive study in order to identify mycobacterial genes essential for in vivo survival, Sassetti et al (2003) defined 194 growth-attenuating mutations. Prominent genes required in vivo were those involved in lipid metabolism and those involved in transport or metabolism of inorganic ions and carbohydrates. However, they observed that nearly all genes of the RD1 region are required for infection of mice.

ESX-1 secretion system is quite similar to the L. pneumophila’s Icm/Dot system. L. pneumophila is a facultative intracellular pathogen which replicates within macrophages and monocytes and finally causes a severe pneumonia known as
Legionnaires’ disease. The interaction of *Legionella pneumophila* with *Acanthamoeba* spp is extensively studied. An important hallmark of the pathogenesis of this bacterium is their ability to manipulate host cell processes, creating a specified replicative niche within host cells. An *L. pneumophila*-containing phagosome (LCP) is allowed to associate sequentially with smooth vesicles, mitochondria, and the rough endoplasmic reticulum (RER) to form a compartment called a replicative phagosome. LCPs are biologically characterized by delayed acidification and a low tendency to fuse with lysosomes. The establishment of these specialized phagosomes is mediated by the Icm/Dot Type IV secretion system, which is essential for the intracellular growth of *L. pneumophila*. *L. pneumophila* utilizes the Icm/Dot system to inject bacterial effector molecules into the host cell cytosol to survive and replicate in the intracellular compartment through modulation of phagosome biogenesis.

The importance of ESX-1 secreted proteins in virulence of *M. tuberculosis* has been shown by deletion of RD1 from *M. tuberculosis* genome that resulted in reduced virulence (spreading) both, in cultured macrophages and in mice (Lewis et al., 2003). Furthermore, the introduction of RD1 genes in *M. bovis* BCG resulted in altered colonial morphology, increased virulence in severely combined immune deficient mice including the formation of granuloma, and longer persistence in immunocompetent mice (Pym et al., 2002). However, the reintroduction of RD1 in vaccine strain does not make it as virulent as *M. tuberculosis* which indicates that other genes may be involved in conferring virulence along with RD1 (Gao et al, 2004). Also, studies with other pathogens indicate that the virulence factors do not necessarily provide the survival advantage to the bacteria (Zhao et al, 2007; Tezcan-Merdol et al., 2004). The results in the present study also indicate that the mere re-introduction of RD1 region into BCG is probably not enough to make it virulent or enable it to survive better. However, the result could also be due to a non-functional RD1 and further research is required to confirm it.

On the contrary, some mycobacterial species have been reported to escape into the host cytosol. Using the social amoeba *Dictyostelium* as a genetically tractable host for pathogenic mycobacteria, it was discovered that *M. tuberculosis* and *M. marinum* but
not *M. avium* are ejected from the cell through an actin-based structure, the ejectosome. This conserved nonlytic spreading mechanism was dependent on an intact mycobacterial ESX-1 secretion system (Hagedorn *et al.*, 2009). *Listeria monocytogenes* and *Shigella flexneri* however, lyse the phagosomal membrane and escape from the endocytic system into the host cytosol, where they replicate and are able to spread to neighboring cells via actin-based motility (Stevens *et al.*, 2006).

Van Der Wel *et al* (2007) showed that lysosomes rapidly fuse with the virulent *M. tuberculosis*- and *M. leprae*-containing phagosomes of human monocyte-derived dendritic cells and macrophages. After 2 days, *M. tuberculosis* progressively translocated from phagolysosomes into the cytosol in non-apoptotic cells. Cytosolic entry was also observed for *M. leprae* but not for vaccine strains *M. bovis* BCG or in heat-killed mycobacteria and is dependent upon secretion of the mycobacterial gene products CFP-10 and ESAT-6.

### 5.3.2. Mycobacteria are unable to control the pH of the acidic vacuoles in amoebae

The intracellular fate of the RD1 knock-in strain of BCG was investigated with the control strains. It was observed that all strains including BCG::RD1, BCG::pYUB412, BCG and *M. bovis* were ingested by amoebae and remained in the amoebae vacuoles. These findings are in agreement with the previous observations that *M. tuberculosis* was able to persist and replicate within the phagosomes of the infected macrophages (Rumsey *et al* 2006; MacMicking *et al*, 2003). However, mycobacteria are able to control the phagosomal pH and keep it only weakly acidic. On the contrary, in this study it was observed that mycobacteria were unable to control the pH of the acidic vacuoles in amoebae. It is a very interesting finding of the present study.

Lysotracker red is an acidotrophic fluorescent dye which is used to stain the intracellular acidic compartments. In macrophages that have not yet been immunologically activated, many species of mycobacteria inhibit the fusion of phagosomes with lysosomes and thereby reside in an environment that is only very
mildly acidic with a pH of about 6.2 (MacMiking et al., 2003). The fact that mycobacteria colocalized with the well-stained acidic vacuoles in amoebae, indicates that perhaps mycobacteria are not able to alter the pH of these compartments.

5.3.3 Studying selected *M. tuberculosis* gene knock-out mutants

In this project, three different *M. tuberculosis* genes were selected for study in amoebae. These genes were involved in fatty acid metabolism (Rv0467), a transcriptional regulator that is upregulated during resumption of replication following hypoxia (Rv2745) and a toxin of unknown function (Rv2548).

The survival of *M. tuberculosis* inside macrophages is a stressful event where the pathogen is under continuous attack by the immune system. In response, mycobacterium has to make use of all possible defence strategies that will help it survive. The primary purpose of TB research is to identify the genes that are involved in this reaction. Here, the survival of three *M. tuberculosis* gene knock-outs were studied in amoebae. It was found that isocitrate lyase enzyme might be important for survival of *M. bovis* inside *A. castellanii*. It has been observed that the *icl* mutant was attenuated in macrophages as well (Munoz-Elias and McKinney, 2005). It can be hypothesized that mycobacterium utilizes lipids for its survival as *icl* is mutually required for survival in macrophages and amoebae.

*M. tuberculosis* H37Rv and *M. tuberculosis* CDC1551 have 38 and 36 TA loci, respectively (Pandey and Gerdes, 2005). *M. bovis* contain more than 50 putative TA systems (Van Melderen and Saavedra De Bast, 2009). Here, VapC19 toxin gene (Rv2548) was used to infect amoebae and its survival was studied. It was found that the Rv2548 mutant was hypervirulent in amoebae.

*M. tuberculosis* TA systems are activated during specific stresses likely encountered in the host (Ramage *et al.*, 2009). In response to environmental stresses encountered in the host, these persistent bacteria are thought to adapt to a slowly or non-replicating state (Gill *et al.*, 2009). Although mechanism of this transition is unknown, it
can be assumed that TA systems might play some part as many toxins of TA systems function as RNases and result in translation inhibition when activated (Zhang et al., 2003).

The third gene knock-out studied is Rv2745 which is the putative transcriptional regulator with a high similarity to the Clp protease gene regulator (ClgR). Proteases play an important role by regulating various proteins in the cell. In a recent study it was revealed that ClgR of *M. tuberculosis* activates the transcription of at least ten genes involved in stabilization, disassembly or degradation of proteins (Estorninho et al., 2010). Some evidence about attenuation of Rv2745 mutant in amoebae is reported in the present project which highlights the fact that stable protein content of the cell depends on ClgR. Expression of Rv2745c is also up-regulated in late hypoxia, and is further induced upon reaeration (Sherrid et al., 2010) when the cell needs to remodel according to favorable conditions.

**5.3.4 Screening *M. bovis* whole genome using TraSH**

Use of mutant libraries to study bacterial genes provides a global overview of all the genes involved under a particular environmental condition. In this study, a similar approach to that of Badarinarayanan et al (2001) was used with some modifications. Firstly, a mutant library of *M. bovis* was created by using a Tn5 transposon carrying a hygromycin resistance determinant. The distribution of the transposon was determined using microarray. Further confirmation of transposon insertion was obtained by sequencing of random individual mutants from the *M. bovis* library. Finally, *A. castellanii* was infected with the library to screen for mutants at a survival disadvantage in amoebae.

TraSH data analysis was able to shortlist a few genes that could have a possible role in survival of *M. bovis*. While most of them code for hypothetical proteins, one gene in particular seems very interesting (Rv3087). Rv3087 is part of the Rv3083-Rv3089 operon of *M. tuberculosis* that has been shown to be induced when tubercle bacilli were exposed to acidic conditions *in vitro* and also when inside macrophages. This indicates that the operon plays an important role in the ability of *M. tuberculosis* to grow inside host cells (Cheruvu et al., 2007).
Besides *M. tuberculosis* H37Rv, this operon is also present in *M. tuberculosis* CDC1551 and *M. bovis*. Homologs of the genes of the operon are present individually in *M. leprae*, *M. avium* and *M. smegmatis* (Cheruvu et al., 2007).

The Rv3083–Rv3089 operon is a non-essential gene for growth in laboratory media because no phenotypic differences were found between a mutant with a knockout of the entire operon and its otherwise-isogenic parental strain, when grown on various media and various growth conditions (Wayne and Hayes, 1996). Conversely, this gene is considered to be essential in mouse model of infection (Sassetti et al., 2003). Singh et al. (2002) reported that deletion of this operon leads to altered composition of mycolic acids, increased sensitivity to detergent and acidic stress, decreased intracellular survival in activated macrophages. Fisher et al. (2002) suggested that the operon might form a type II non-ribosomal peptide synthetase and that a bioactive product made by this hypothetical multicomponent enzyme could play a role in altering phagosomal maturation or in another facet of intracellular survival.

Various research projects have successfully used mutant libraries to identify genes required in a particular set of conditions. *M. avium* survival genes have been studied in protozoa. Tenant and Bermudez (2006) studied the impact of amoeba infection on the regulation of *M. avium* genes using an *M. avium* GFP promoter library. They found many genes that were up-regulated upon *Acanthamoeba* infection, including those belonging to metabolic pathways, transcriptional regulators, DNA replication, energy metabolism, protein translation and modification, and degradation of macromolecules. A number of genes also had unknown functions. Several genes were found to be common between *M. avium* infection of macrophages and amoebae. These genes were involved in protein translation, energy metabolism, as well as some of unknown function, suggesting that *M. avium* uses some mechanisms for survival mutually in amoeba and macrophage.

Danelishvili et al., (2007) screened a *M. avium* transposon mutant library for the inability to enter macrophages. They identified a pathogenicity island (PI) unique for *M.
avium which is not present in \textit{M. tuberculosis} or \textit{M. paratuberculosis}. The PI region contains possible transport proteins or chaperones. The mutants were also evaluated for \textit{A. castellanii} invasion. The defect in the ability of the mutants to invade both cells was highly similar, suggesting that \textit{M. avium} might have evolved mechanisms that are used to enter amoebas and human macrophages.

\textit{Legionella} spp. also uses many of the same genes to establish themselves in \textit{Acanthamoeba} spp. and in mammalian cells (Gao \textit{et al.}, 1997; Segal and Shuman, 1999). Several genes required for the intracellular survival of \textit{L. pneumophila} in macrophages, such as the \textit{dot/icm} (\textit{dot} for defect in organelle trafficking; \textit{icm} for intracellular multiplication) genes (Segal and Shuman, 1999), the \textit{pmi} (protozoan and macrophage infectivity) genes (Gao \textit{et al.}, 1997), the \textit{pilD} (prepilin peptidase) gene (Liles \textit{et al.}, 1999) and the \textit{mip} (macrophage infectivity potentiator) gene (Cianciotto and Fields, 1992), are also required for survival within amoebae.

Similarly, the findings presented in this study would help in a better understanding of the survival and pathogenesis of \textit{M. bovis} in humans as macrophages and amoebae uses same mechanisms to deal with pathogens and using amoebae as a model for investigation may discover important genes which have not been discovered using macrophages.

\subsection*{5.3.4 Effect of temperature}

Temperature is one of the most important factors affecting bacterial growth. Pathogens in particular have a restricted growth temperature. \textit{M. bovis} has an optimum growth temperature of 37°C. In this study, the \textit{M. bovis}-amoebae infections were performed at 20°C which is the temperature of the environments in which amoebae naturally occur but this low temperature is also a stress condition for mycobacteria. An important aspect of \textit{M. tuberculosis} pathogenesis is its response to stress conditions whether those encountered in granuloma or during transmission of the pathogen. The transmission of the pathogen occurs via droplets released from an infected person. Even though these bacteria are exposed to severe stress conditions, such as a sudden drop in
temperature, a change in oxygen pressure and a limitation of nutrients, they remain viable within these droplets (Loudon et al., 1969) suggesting the presence of a temperature stress response.

5.3.6 Interaction between two versatile organisms

This project involves two versatile systems; mycobacteria and amoebae. Both are quite sensitive in terms of growth, responding to minor changes in the medium as well as the environment. Therefore, a system studying the interaction of *M. bovis* and *A. castellanii* could be very interesting but difficult at the same time as it is hard to replicate the experimental conditions exactly in the same manner.

5.3.7 Effect of multiplicity of infection (M.O.I)

Multiplicity of infection (M.O.I) also plays an important role in infections. Generally enhanced intracellular multiplication is reflected by an increase in parasitism of the organism. It is a ratio of the infectious agent (bacteria or virus) to the infection target (e.g. human cells). Generally with an increase in M.O.I, the number of target cells infected with at least one bacterium also increases.

M.O.I is difficult to control and depends on the number of infectious agent and the target cells. Optical density (OD) the amount of light scattered by a suspension of cells, is used generally as a measure of number of bacteria. However, it is a mere estimate and also it does not differentiate between viable and non-viable cells. A more reliable method is to determine colony forming units (cfu/ml) by plating dilutions of the inoculum on appropriate media. This technique assumes that each colony is derived from an individual cell and that the incubation conditions allowed the recovery of all cells present. While this method works very well with many bacteria, mycobacterial colonies are hard to count with certainty because of the tendency of the cells to clump. This could also affect the reproducibility of the experiments.
6. Conclusions

RDI region is probably not important for survival of *M. bovis* in *A. castellanii*. However, the evidence provided in this study is not conclusive and needs to be confirmed by further research.

In this study, we have been able to highlight some of the genes that play a role in survival of *M. bovis* in amoebae like isocitrate lyase and a transcriptional regulator (ClgR) which are documented as important for survival in macrophages as well (Munoz-Elias and McKinney, 2005; Estorninho et al., 2010) which underlines the role of amoebae in understanding the virulence determinants of pathogens.

Although TraSH did point out some candidate survival genes (Rv1728c, Rv3087, Rv2693c, Rv0598c, Rv1192, Rv2462c and Rv3104c) unfortunately, these genes were significant by a t-test alone. In a microarray data analysis, a t-test is applied individually to each gene. This could mean repeating the t-test thousands of times. To decrease the incidence of false positives and correct the probabilities, a multiple testing correction is applied on the data. Unfortunately, these potentially significant genes were dismissed by the Benjamini and Hochberg correction. Nonetheless, these are preliminary findings which are quite interesting and would require further confirmation.

In general, it can be concluded that at least some virulence mechanisms Genes important for growth in animals are also important for growth in amoebae. This begs the questions; were they learned in amoebae and does *M. bovis* use these for environmental survival in amoebae.
7. Future work

The work presented in the present study could be extended as follows:

The findings obtained in this study for survival of individual mutants could be verified by complementation experiments. This would enable a better understanding of the role of the genes in mycobacterial survival in amoebae.

The attenuation of H37Rv:Δ0467 could be followed up by investigating other genes involved in lipid metabolism. This would be interesting as utilization of fatty acid plays an important part in mycobacterial adaptation to intracellular survival and slow growth.

Slow growth is one of the most characteristic features of M. tuberculosis. In this study, H37Rv:Δ2548 survives better than controls in amoebae. It might indicate that this gene is required for slow growth. Therefore, further research is required to confirm this finding.

Preliminary evidence of contribution of Rv2745c in mycobacterial survival in amoebae in this study could be followed up as ClgR is involved in the regulation of at least 10 genes, of which the majority was involved in protein stabilization, disassembly or degradation.

TraSH can be improved by including more replicates for a better statistical analysis and also by using high density microarrays.

TraSH does highlight some interesting genes which could be studied in detail to determine their association with mycobacterial survival in amoebae. One such interesting candidate is Rv3087 as it is shown to be required for survival in protozoan and mammalian cells. Further research on this gene would be able to confirm its role in M. bovis survival in amoebae.
A. castellanii (and other protozoans) serves as a model investigation system in understanding the virulence genes of pathogens. Further research using protozoa can provide valuable information about *M. tuberculosis* pathogenesis in mammalian cells.
8. References


de Chastellier C (2009). The many niches and strategies used by pathogenic mycobacteria for survival within host macrophages. *Immunobiol* 14(7):526-42


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9. Appendices

APPENDIX 1
Restriction map of the construct PYUB412. It is a shuttle vector containing ampicillin and Hygromycin selectable marker genes. The construct is derived from *E.coli* containing a Xbal site flanked by two cos sites on either side, attB, Ori and int sites. RD1 construct was created by inserting the RD1 locus into this construct at the *BclI* site.
APPENDIX 2

pMOD-Hyg romycin sequence. The transposon sequence is highlighted in grey box.

```
TGGGGGGGGG
```
2041 CTGTGTGCAC GAACGCCGCG TTGAGGGGGA GGGGTGGGGG TTATGGGGTA AGTATGGTGT
2101 TGAGTGGAAG GGGGTAAGAG AGGAGTTATG GGGAGTGGGA GGAGGGAGTG GTAAGAGGAT
2161 TAGGAGAGGG AGGTATGTAG GGGGTGGTAG AGAGTTGTTG AAGTGGTGGG GTAAGTAGGG
2221 G TAG GAGTA GA AGGAGAGTAT TTGGTATGTG GGGTGTGGTG AAGGGAGTTA GGTTGGAAA
2281 AAGAGTTGGT AGGTGTTGAT GGGGGAAAGA AAGGAGGGGT GGTAGGGGTG G I  I  ITTTTGT
2341 TTGGAAGGAG GAGATTAGGG GGAG A A A A A A  AGGATGTGAA GAAGATGGTT TGATGTTTTG
2401 TAGGGGGTGT GAGGGTGAGT GGAAGGAAAA GTGAGGTTAA GGGATTTTGG TGATGAGATT
2461 ATG AAAA AGG ATGTTGAGGT AGATGGTTTT A A A TTA A A A A  TGAAGTTTTA AATG A A TG TA
2521 AAG TATATAT GAGTAAAGTT GGTGTGAGAG TTAGGAATGG TTAATGAGTG AGGGAGGTAT
2581 GTGAGGGATG TGTGTATTTG GTTGATGGAT AGTTGGGTGA GTGGGGGTGG TGTAGATAAG
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3481 ATGATTTTAG AAAATAAAC AAATAGGGGTT CCGGCGCGA TTTCCCGGAA AAGTGCGAAC
3541 TGACGTCTAA GAACAGTTA TTATCGTAC ATTAACCTAT AAAATAAGGC GTATCAGGAG
APPENDIX 3

Genes identified by Trash whose abundance increased over 100% and were over represented at T7

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