

Identifying Biosynthetic Pathways for Mycobacterial Cell Wall Components Using Transposon Mutagenesis

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degree of Doctor of Philosophy

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Abstract

Mycobacterium tuberculosis, the causative agent of the infectious disease tuberculosis, has a distinct lipid-rich cell wall which not only provides physical protection to the bacilli, but also plays an important role in virulence. Several currently used anti-TB drugs target cell wall biosynthetic pathways. Therefore, a good understanding of the structure and biosynthesis of the cell wall will provide helpful clues for the development of novel anti-*Mycobacterium* drug targets, as well as a better understanding of *Mycobacterium*-host cell interactions.

To study cell wall biosynthesis in mycobacteria, a strategy based on random transposon (*Tn*) mutagenesis with two different screening criteria, altered colony morphology and mycobacteriophage resistance, was developed. Two model systems, non-pathogenic *Mycobacterium smegmatis* and the fish pathogen *Mycobacterium marinum* were used for generating *Tn*-mutant libraries. From the colony morphology screen, two out of eight genes identified from *M. smegmatis* *Tn*-mutants and eleven out of twenty from *M. marinum* *Tn*-mutants with altered colony morphology were directly involved in cell wall synthesis. Genes with an indirect impact on colony morphology were also identified. One mutant from each species was chosen for further study. First was the *M. smegmatis* 3D9 *Tn*-mutant with the only isocitrate dehydrogenase (*icd*) gene disrupted. Although there were no differences in lipid profiles, fatty acids and mycolic acids, it was an ideal surrogate to study different orthologues of *M. tuberculosis* *icds*. The second mutant described in these studies was the *M. marinum* 8G10 mutant with a *Tn* inserted into the promoter region of *MMAR0978* with a deficiency in methoxymycolates production. The virulence of the 8G10 mutant was tested using murine bone marrow derived macrophages and the zebrafish infection model, with the mutant found to be attenuated in the latter.

The generalised transducing phage I3 was chosen for isolating mycobacteriophage resistant *M. smegmatis* *Tn*-mutants. Four I3-resistant mutants

were identified with a *Tn* insertion in a gene cluster involved in the biosynthesis of the cell wall associated glycopeptidolipids (GPLs) and loss of GPLs in the mutants was confirmed, demonstrating the potential of using phage-resistant mutants for identifying cell wall biosynthetic genes. The minimal phage I3 receptor in *M. smegmatis* was further identified by using a set of previously described *M. smegmatis* mutants that produced truncated GPL intermediates.

Declaration

The work carried out in this thesis was carried out in the School of Biosciences at the University of Birmingham, Birmingham, UK, B15 2TT during the period October 2007 to September 2010. The work in this thesis is original except where acknowledged by reference.

No portion of the work is being, or has been submitted for a degree, diploma or any other qualification at any other university.

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When I first thought about studying abroad, I never had UK in my mind. When I decided to stop my first PhD six years ago, I never thought that one day I would throw myself into it again. So, here I am, standing at the end of my 3-year PhD and looking back, nothing I thought before has come true. Life is full of surprises and changes always run faster than plans. I guess this is the very first lesson I have learned from my PhD. However, no matter what the plan was or what happened, my father was always there and is still there, with supports and encouragements. Without him, I would never go this far.

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List of Abbreviations

°C	degree centigrade
ACP	acyl carrier protein
AG	arabinogalactan
AprR	apramicin resistant
Araf	arabinofuranosyl
ATP	adenosine triphosphate
BCG	Bacillus Calmette-Guérin
Bp	base pair
BSL	biosafety level
Cfu	colony forming unit
Cg	<i>Corynebacterium glutamicum</i>
CoA	coenzyme A
Cpm	counts per minute
DAT	diacyl trehaloses
DCs	dendritic cells
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
DOTs	directly observed therapy, short-course
EMB	ethambutol
FAME	fatty acid methyl ester
FATP	fatty acyl-tetrapeptide
FBS	fetal bovine serum
G	gram
Galf	galactofuranose
GlcNAc	N-acetylglucosamine
GMM	glucose monomycolate
GPLs	glycopeptidolipids
HDH	homocitrate dehydrogenase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	human immunodeficiency virus
<i>Hyg</i>	hygromycin resistance cassette
HygR	hygromycin resistant
IDH/ICD	isocitrate dehydrogenase
IFN- γ	interferon-gamma
IL	interleukin
IMDH	isopropylmalate dehydrogenase
INH	isoniazid
IS	insertion sequence
KanR	kanamycin resistant
L	Litre

LAM	Lipoarabinomannan
LM	Lipomannan
LOS	Lipooligosaccharide
MAC	<i>Mycobacterium avium</i> complex
mAGP	mycolyl-arabinogalactan-peptidoglycan
MAME	mycolic acid methyl ester
mDAP	D-isoglutamine and meso-diaminopimelic
MDR	multidrug resistant
Me	Methyl
Min	Minute
ml	Millilitre
mM	Millimolar
MOI	Multiplicity of infection
NAD ⁺	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide, reduced
NADP ⁺	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate, reduced
NAG	N-acetylglucosamine
NAM	N-acetylmuramic acid
OD	optical density
<i>Ori</i>	replication origin
PBS	phosphate buffer solution
PCR	polymerase chain reaction
PDIM	phthiocerol dimycocerosate
Pfu	plaque forming units
PG	Peptidoglycan
PGL	phenolic glycolipid
PI	phosphatidylinositol
PIMs	phosphatidylinositol mannoside
PL	Phospholipid
PMA	phorbol myristate acetate
PZA	Pyrazinamide
RD1	region of difference-1
Rha	Rhamnose
RMP	Rifampicin
RNA	ribonucleic acid
Rpm	revolutions per minute
SL	Sulfolipids
SSC	saline-sodium citrate buffer
STM	Streptomycin
TAG	Triacylglycerol
Tal	Talose
TB	Tuberculosis
TCA cycle	tricarboxylic acid cycle

TDH	tartrate dehydrogenase
TDM	trehalose dimycolates
TLR	toll-like receptors
TMM	trehalose monomycolates
Tn	Transposon
TNF	tumor necrosis factor
Ty	Tyloxapol
v/v	volume per volume
w/v	weight per volume
WHO	World Health Organization
XDR	extensively drug resistant
μg	Microgram
μl	Microlitre
μm	Micrometre

Chapter 1

General Introduction

1.1 History

Tuberculosis (TB) is an infectious disease caused by the bacterium *Mycobacterium tuberculosis* that primarily affects the lungs, but may also spread to other parts of the body. It is an ancient disease that has plagued mankind for thousands of years. The oldest case of human TB, from Atlit-Yam in the Eastern Mediterranean (9250-8160 years old), was confirmed by PCR analysis for *M. tuberculosis* genetic loci and high performance liquid chromatography for *M. tuberculosis* specific mycolic acid lipid biomarkers (HersHKovitz *et al.*, 2008). Through history, TB has been referred to by different names. Phthisis was used in Greek literature around 460 BC for its characteristics of fever, cough and loss of appetite. It was also referred to as the white plague in Europe for its association with anaemia and high mortality in the 17th century epidemic which lasted for about 200 years and caused more than 1 billion deaths (Dormandy, 1999).

Records of theories for the cause of TB can be also found throughout history. While ancient Greeks believed that TB was hereditary, Aristotle suggested that it was contagious (Madkour, 2004). Girolamo Fracastoro, a Venetian physician in the 16th century, first proposed that it was caused by transferable tiny particles *via* direct or indirect contact (Brock, 1998). In 1819, the French physician Rene Laennec published his book explaining the pathogenesis of TB, the correspondence between pulmonary lesions and respiratory symptoms in TB patients (Daniel, 2000). In 1869, Jean Antoine Villemin proved that TB was contagious using animal experimental models

(Barnes, 1995). However, the defining moment in the history of the disease came in 1882, when Robert Koch presented his discovery that the bacterium, *M. tuberculosis*, was the causative agent of TB at the Physiological Society of Berlin (Koch, 1982). Later in 1890, he also developed tuberculin, a protein preparation from *M. tuberculosis*, which was ineffective for immunisation, but was subsequently found useful in the diagnosis of TB by Charles Mantoux in 1908. The Mantoux Test, as it is called now, is still widely used as a TB diagnostic method (Madkour, 2004).

1.2 TB chemotherapy and vaccination

TB is treated with a combination of drugs because of the high risk of developing drug resistance when using single drugs (Wang *et al.*, 2006). A standard treatment for TB includes: 2 months of isoniazid (INH), rifampicin (RMP), pyrazinamide (PZA) and ethambutol (EMB), which is then followed by 4 months of INH and RMP. As for latent TB, 6-9 months treatment of INH and RMP after 2-month standard treatment is recommended by WHO (World Health Organization). Because of the long course of therapy, patients show poor adherence/compliance which is also one of the causes of the appearance of drug resistance. WHO launched DOTs (directly observed therapy, short-course) to help ensure patient's compliance, as a part of a global TB eradication program, which includes government commitment to control TB, diagnosis based on sputum-smear microscopy tests done on patients who actively report TB symptoms, a supply of drugs and standardised reporting and recording of cases and treatment outcomes (Elzinga *et al.*, 2004). Statistical studies revealed that DOTs can

successfully prevent the emergence of multi-drug resistance (MDR-TB) strains and their recurrence.

Anti-TB drugs belong to three categories according to WHO guidelines (WHO, 2003) based on their mode of action and side-effects. The first-line (essential) drugs include INH, RMP, PZA, EMB and streptomycin (STM). The second-line (reserved) drugs, which are less effective or with more side-effects compared to first-line drugs, include six classes: aminoglycosides (*e.g.* kanamycin), polypeptides (*e.g.* capreomycin), fluoroquinolones (*e.g.* ciprofloxacin), thioamides (*e.g.* ethionamide), cycloserine, *p*-aminosalicylic acid. Third-line drugs which are less effective (or the efficacy has not been proven yet) include rifabutin and macrolides. Structures of some of the anti-TB drugs in use are shown in Fig. 1.1.

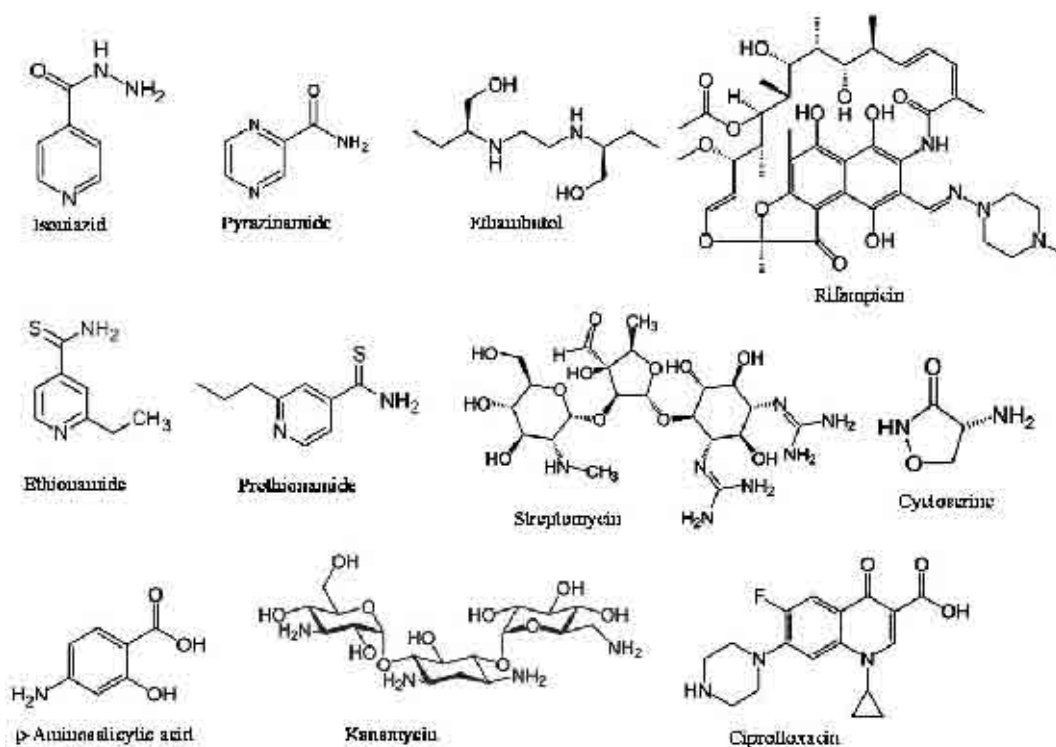


Fig. 1.1 Structures of some commonly used TB drugs.

A majority of them target mycobacterial cell wall biosynthetic pathways as shown in Table 1.1 (Zhang, 2005).

Table 1.1 Commonly used TB drugs and their mechanisms (Zhang, 2005)

Drug	Mechanisms of Action
Isoniazid	Inhibition of cell wall mycolic acid synthesis; effects on DNA, lipids, carbohydrates and NAD metabolism
Rifampin	Inhibition of RNA synthesis
Pyrazinamide	Disruption of membrane transport and energy depletion
Ethambutol	Inhibition of cell wall arabinogalactan synthesis
Streptomycin	Inhibition of protein synthesis
Kanamycin	Inhibition of protein synthesis
Quinolones	Inhibition of DNA synthesis
Ethionamide	Inhibition of mycolic acid synthesis
PAS	Inhibition of folic acid and iron metabolism
Cycloserine	Inhibition of peptidoglycan synthesis

While the first anti-TB drug streptomycin was discovered in 1944, and it was followed with a golden era in the 1950s and 1960s of antibiotic discovery, no more new anti-TB drugs have been launched during the last 50 years. Utilising genomic information, several potential new drug targets have been identified and have been used for lead compound screening. For example, R207910, a diarylquinoline, is an inhibitor of the membrane bound ATP synthase and shows activity against both drug-sensitive and MDR- *M. tuberculosis* (Andries *et al.*, 2005; Koul *et al.*, 2007);

inhibitors of the two component system *DosR/S*, which is responsible for the response to the host immune defence mechanisms may have an effect against W-Beijing hypervirulent strains of *M. tuberculosis* (Reed *et al.*, 2007). Furthermore, several new drug candidates are currently in clinical trials (Barkan *et al.*, 2009; Guy & Mallampalli, 2008), such as TMC-207 (former R207910); nitroimidazopyran PA-824 which blocks cell wall protein and lipid synthesis and is active against both replicating and non-replicating *M. tuberculosis* (Tyagi *et al.*, 2005); SQ 109, a derivative of ethambutol, with potent activity against drug-sensitive and MDR- *M. tuberculosis* (Chen *et al.*, 2006).

To fight against infectious disease, apart from medication after its establishment, vaccination is another important method to protect humans from being infected. Facing human immunodeficiency virus (HIV) co-infection and the emergency of untreatable MDR and extensively drug resistant (XDR) - *M. tuberculosis*, an effective TB vaccine is essential. BCG, an attenuated live vaccine, is currently the only available vaccine against TB. In the early 1900s, Albert Calmette and Camille Guerin noticed the reduction of virulence of *M. bovis*, a strain that caused bovine TB, during passage under laboratory conditions. After a 13-year continual *in vitro* passaging of this strain and trials on different animal models, they did the first human trial in 1921, which showed protection against TB (Behr, 2002; Oettinger *et al.*, 1999). Later this BCG strain was distributed to laboratories around the world. A subsequent sub-culture across the globe resulted in a continued evolution of BCG, and worldwide, BCG now represents not a single strain but a group of sub-strains with varying degrees of

deletion (Behr & Small, 1999; Behr *et al.*, 1999).

Despite that, BCG has already been used worldwide for decades; its protective efficacy remains contentious, especially in adults. BCG can provide more than 80% protection against severe forms of TB, like miliary TB in children (Trunz *et al.*, 2006), but its protection against pulmonary TB in adults ranges from 0-80% (Brewer, 2000).

A better understanding of the mechanism in BCG attenuation can be helpful to address its variation in protective efficacy. With the advance of genomic techniques and knowledge of pathogenicity of *M. tuberculosis*, the mechanisms underlying BCG attenuation begin to be unveiled. Compared with *M. tuberculosis* and *M. bovis* virulent strains, BCG contains a deletion, termed Region of Defference-1 (RD1). Results based on studies in *M. tuberculosis* show that RD-1 includes genes encoding proteins essential for growth in macrophages or important for virulence (Guinn *et al.*, 2004; Stanley *et al.*, 2003). In addition to the loss of RD-1, further studies have shown that other changes are also involved, for instance the loss of specific cell-wall lipids (PDIM, phthiocerol dimycocerosate and PGL, phenolic glycolipid) (Rao *et al.*, 2006) and mutations in virulence related two-component systems, *phoP-phoR* (Leung *et al.*, 2008).

In addition to the numerous genome differences between BCG and *M. tuberculosis* revealed by comparative genomic analysis, studies have also provided evidence which shows differences in the immune response between BCG vaccination and *M. tuberculosis* infection, especially *M. tuberculosis* induced CD8⁺ T cell

responses, which has been considered as a major contribution against the disease (Caccamo *et al.*, 2009; Woodworth & Behar, 2006). Therefore, there are two main strategies used for developing new vaccines based on our current understanding of *M. tuberculosis* infection, and the use of specific genetic tools. One approach is to improve the efficacy of BCG by expressing recombinant *M. tuberculosis* antigenic proteins (known as recombinant BCG, rBCG) (Bastos *et al.*, 2009; Grode *et al.*, 2005; Horwitz, 2005) or boosting BCG vaccination using *M. tuberculosis* antigen protein/protein units or antigen encoding viral vectors (Reed *et al.*, 2009; Tchilian *et al.*, 2009). The second strategy is to generate *M. tuberculosis* attenuated strains by knocking out genes involved in replication or virulence (Aguilar *et al.*, 2007; Waters *et al.*, 2007). Two rBCG candidates are in phase 1 clinical trials (Walker *et al.*, 2010), whilst another rBCG expressing *M. tuberculosis* Antigen 85B has entered phase I clinical trials in 2004 (Horwitz, 2005).

1.3 Resurgence of TB

After improvement in public health and sanitation, and introduction of the BCG vaccine and antibiotics in clinical treatment, the incidence and mortality of TB steadily declined in developed countries until the 1980s. However, in the mid-1980s, the incidence of TB increased in North America and Western Europe (Raviglione *et al.*, 1993; Rieder *et al.*, 1989), as well as in developing areas, such as Africa and Southern Asia (Corbett *et al.*, 2003; Raviglione *et al.*, 1995).

The WHO announced TB as a global epidemic in 1993 and estimated that more

than 2 billion people, approximately a third of the global population, were infected with the TB bacillus. In 2008, there were about 9.4 million new cases of TB, of which 55% occurred in Asia and 30% in Africa (Fig. 1.2). India, China and South Africa ranked among the top three countries for incidence of TB. Of the new cases, 13-16% were HIV-positive. Meanwhile, there were 11.1 million cases worldwide in 2008, a decrease from 13.7 million in 2007 and 13.9 million in 2006. In 2008, about 1.8 million people died of TB, of which 0.5 million were HIV-positive (WHO, 2009a; WHO, 2009b).

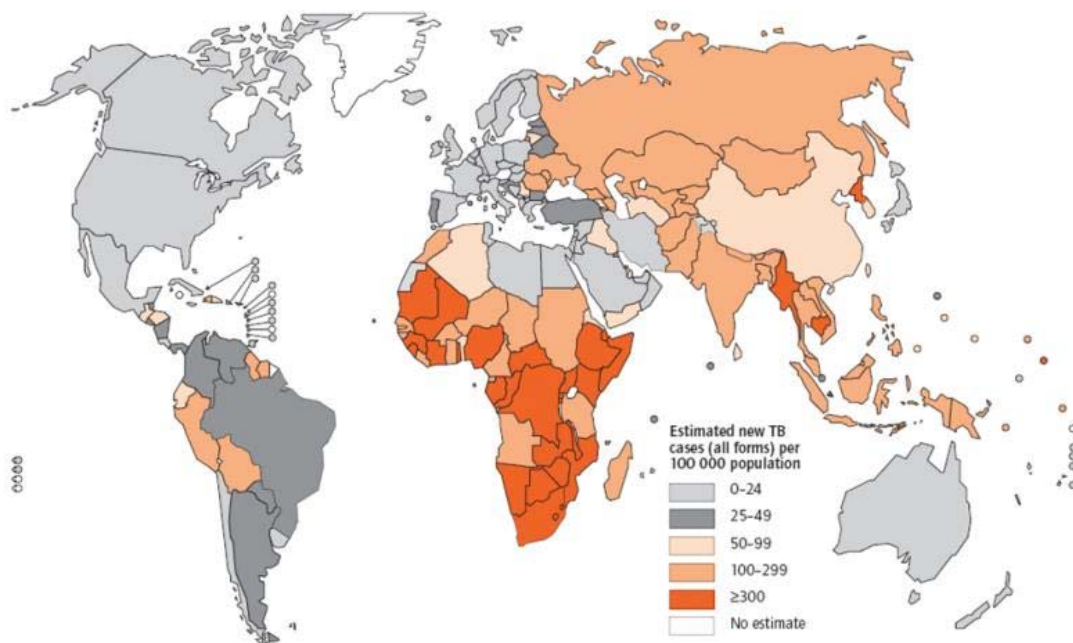


Fig. 1.2 Estimated TB incidence rates, 2008 (WHO, 2009a).

A major factor, for the resurgence of TB, is the spread of HIV. HIV co-infection raised the risk of developing active TB in a population with latent TB, from 5-10% during their lifetime, to the same risk in one year (CDC, 1998; Cole *et al.*, 1998; Corbett *et al.*, 2003).

An additional factor is the emergence of MDR - *M. tuberculosis* strains in the past several decades. Incomplete/inadequate treatment and poor adherence/compliance to therapy are two main reasons for the appearance of MDR - *M. tuberculosis* strains. In WHO 2008 report, it was estimated that about 490,000 cases of MDR-TB emerged every year and caused more than 110,000 deaths; nearly half of the burden occurred in China and India (WHO, 2008). Amongst new TB cases, 2.9% were MDR-TB, compared with 15.3% amongst previous treated cases (WHO, 2008) (Fig. 1.3).

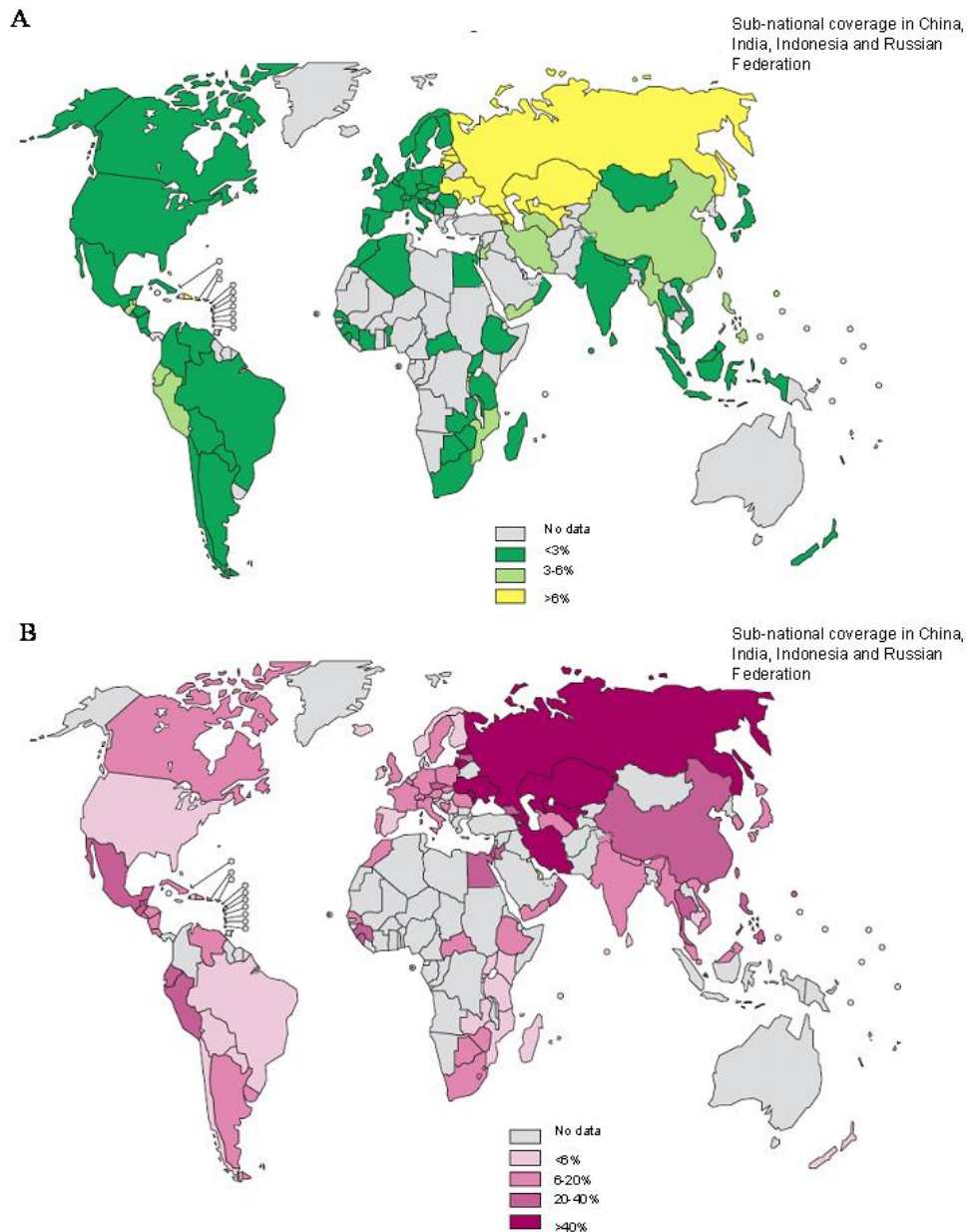


Fig. 1.3 Global incidence of MDR-TB. A. MDR-TB among new cases 1994-2007; B. MDR-TB among previously treated cases 1994-2007 (WHO, 2008)

Furthermore, this problem has been compounded by the recent emergence of XDR-TB, which are resistant to RIF, INH, fluoroquinolones and at least one of the three injectable second-line drugs (capreomycin, kanamycin, and amikacin). This form of TB, which is almost incurable, was first reported in 2005, when 52 out of 53

patients died within a median period of 16 days (from TB test to death) (Gandhi *et al.*, 2006). Now, 58 countries have at least one XDR-TB case confirmed (Fig. 1.4) (WHO, 2010).

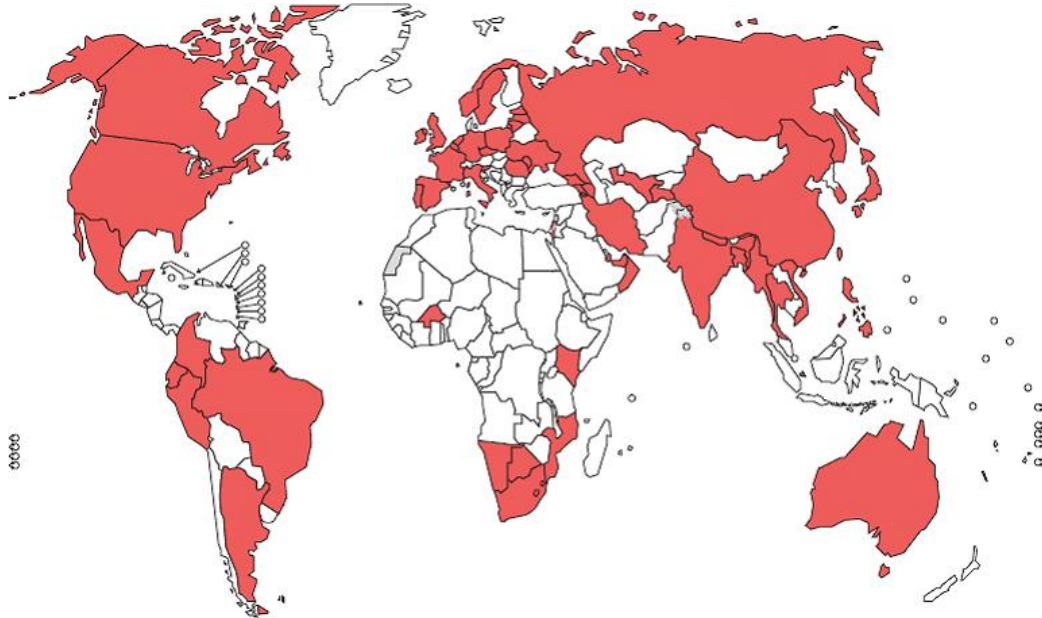


Fig. 1.4 Distribution of countries and territories (in red) reporting at least one case of XDR-TB as of January 2010 (WHO, 2010).

1.4 The biology of pulmonary *M tuberculosis* infection

TB is an airborne disease and thus the primary site of TB infection is the lung (pulmonary TB). Symptoms of pulmonary TB are chest pain, prolonged cough, fever, and appetite and weight loss. Extrapulmonary TB can occur in the central nervous system, lymphatic system, bones and joints (Be *et al.*, 2009; Kaufmann, 2001). An extreme form of extrapulmonary TB with a close to 100% mortality rate is disseminated TB, also known as miliary TB, in which the bacteria enter the blood stream and seed infection in several organs *via* the circulatory system (Bologna *et al.*,

2008; Lessnau & Luise, 2009).

Not all *M. tuberculosis* infections can lead to active TB. Following inhalation, a number of fates are possible for the inhaled bacteria. In most cases, the host immune response is strong enough to ‘clean’ the bacilli, resulting in no infection. The bacteria could also proceed to the alveoli where they are engulfed by macrophages, which triggers the innate immune response followed by an adaptive immune response. Then activation of the host immune system initiates the development of a granuloma. A granuloma represents a dynamic interaction between different immune cells, such as T cells, macrophages and dendritic cells (DCs). If the host is weak, the bacilli can not be eliminated or restrained, leading to active TB. If the host immune system is competent, the bacteria can be withheld in granulomas, causing latent infection with no symptoms. Once the host immune condition changes that breaks the balance in the granuloma, thus the alive bacteria will be released and cause TB reactivation.

In TB infection, after the entry into the lung by airborne transmission of tiny droplets produced by an active TB patient (Cole, 2005; Zahrt, 2003), *M. tuberculosis* is engulfed by alveolar macrophages from the innate immune response using mannose receptors (MR) and Toll-like receptors (TLR) that are considered as the first line of defence against infection. To survive and replicate in macrophages, *M. tuberculosis* adopts several strategies to escape the antimicrobial defence mechanisms of macrophages. First, it can arrest phagosome maturation (Fratti *et al.*, 2003; Vergne *et al.*, 2003; Vergne *et al.*, 2005) and block phagosome-lysosome fusion (Walburger *et*

al., 2004) to avoid being degraded. Second, genes in the bacilli, such as *katG* and *sodC*, as well as cell wall components, such as lipoarabinomannan (LAM), can help *M. tuberculosis* resist intracellular oxidative stress (Chan *et al.*, 1991; Piddington *et al.*, 2001; Stover *et al.*, 1991; Wu *et al.*, 1998). Also, *M. tuberculosis* can maintain access to essential nutrients for intracellular replication by intercepting with the endocytic system (Sturgill-Koszycki *et al.*, 1996).

Besides the innate immune response, the adaptive immune response is also important. *M. tuberculosis* is also exposed to dendritic cells (DCs), antigen presenting cells which are important for activating T cell-related adaptive immune responses at the site of infection. Although, DCs can not eliminate engulfed *M. tuberculosis*, unlike macrophages, DCs do not allow it to grow intracellularly (Tailleux *et al.*, 2003). To activate naïve T cells, DCs have to migrate to drainage lymph nodes which help to further disseminate the bacilli (Banchereau *et al.*, 2000; Meya & McAdam, 2007; Wolf *et al.*, 2008). During TB infection, CD1b molecules responsible for presenting lipid antigens play an important role. Virulence related cell wall components, such as LAM and phosphatidylinositol mannosides (PIMs), have been reported to be presented by CD1 (Sieling *et al.*, 1995). However, the exact function of CD1b-restricted T cells in anti-TB immune response is not clear yet. As for B cell induced humoral response, there is still no consistent results for its contribution against TB, although it has been demonstrated that B cells can contribute to the fight against some intracellular bacteria and parasites (Maglione & Chan, 2009) and modulate T cell responses *via* antigen-presenting, priming memory T cells and

cytokines (Andersen *et al.*, 2007; Harris *et al.*, 2000; Igietseme *et al.*, 2004; Lund *et al.*, 2006). Data from human and animal models suggests that anti-TB immunity is based mainly on CD4⁺ Th1-mediated activation of phagocytes to destroy intracellular bacilli through the IFN- γ and IL-12 pathways (Boom, 1996; Cooper *et al.*, 1993; Cooper *et al.*, 1997; Flynn *et al.*, 1993; Flynn & Chan, 2001). However, the Th1 immune response is important as it stops the expansion of infection by taking part in the formation of granulomas (Russell, 2007), although it is not enough for eliminating the infection. In contrast to the CD4⁺ T cell response, the CD8⁺-mediated cytotoxic response to TB is still not fully understood.

Granuloma formation represents a critical manifestation in TB infection not only as a pathological symptom but as determination of disease progression. It starts when the bacteria are phagocytosed by alveoli macrophages. At this early stage, the bacilli replicate at a rapid rate within macrophages to trigger a cellular immune response, while the infected macrophages initiate a proinflammatory response at the site of infection and start to differentiate into different types, such as giant cells and foamy macrophages. Tubercles are formed, composed of infected macrophages surrounded by foamy macrophages and other phagocytes in the middle and further surrounded by lymphocytes. Then, the bacilli stop the rapid replication followed by the development of fibrous capsule to separate the tubercle and lymphocytes which represents the maturation of granulomas (Fig. 1.5) (Russell, 2007; Russell *et al.*, 2009). Up till this stage, the granuloma functions as a container of the bacteria and prevents its dissemination which leads to latent infection. When the immune status of the host

changes, for instance old age or poor immunity, such as HIV infection, the granuloma breaks down and the live bacilli are released into the airway and transmitted, as is termed ‘reactivation of TB’.

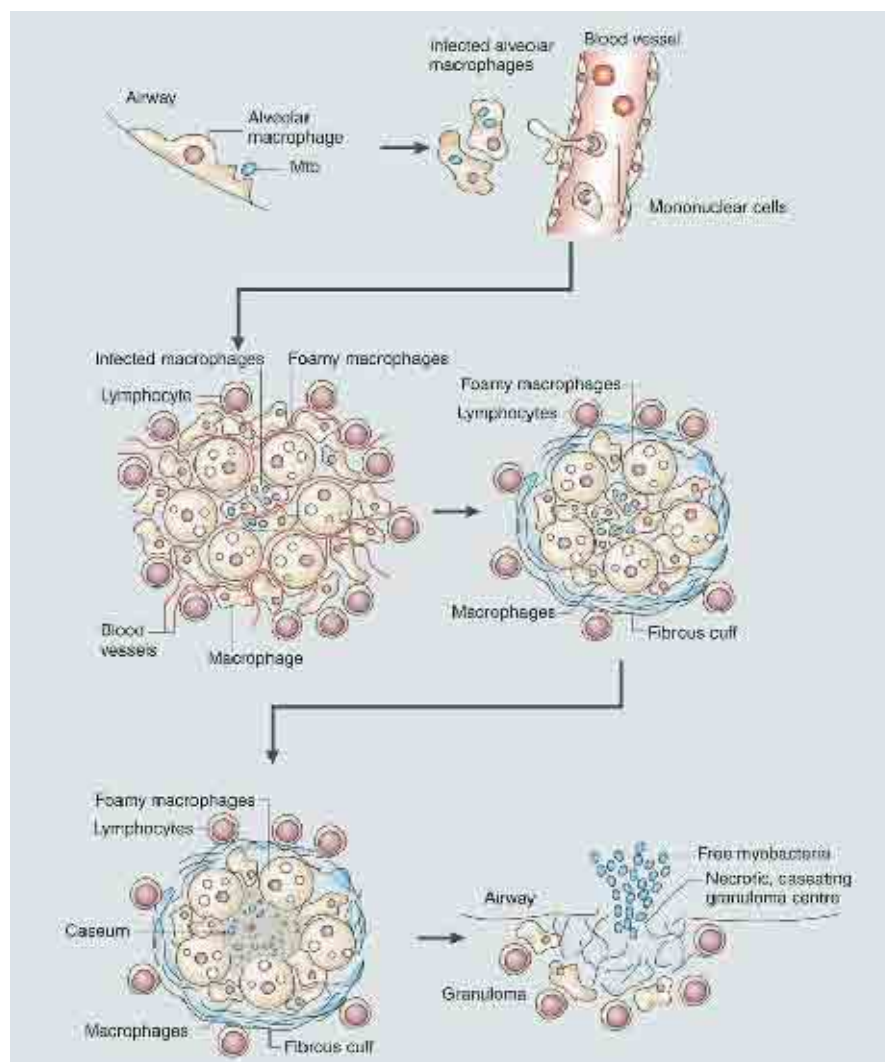


Fig. 1.5 Progression of the human tuberculosis granuloma (Russell, 2007; Russell *et al.*, 2009).

Research to understand the formation of the granuloma and its impact has been carried out for decades using different *in vivo* and *in vitro* models. Although the whole picture is still vague, some parts have been well established. As a

proinflammatory response, many cytokines and chemokines are involved, such as TNF (tumor necrosis factor) (Algood *et al.*, 2005; Saunders & Britton, 2007) and IFN- γ (Cooper *et al.*, 1993; Pearl *et al.*, 2001).

1.5 Mycobacterial cell wall

Mycobacteria are classified as Gram-positive, but have an unusual and distinct lipid-rich cell wall. The mycobacterial cell wall functions as a permeability barrier for many molecules, including many antibiotics (Brennan & Nikaido, 1995). It also provides a physical protection that helps the bacteria survive from the harsh environment inside infected macrophages (Wang *et al.*, 2000). This atypical cell wall confers a characteristic acid-fast staining property to the bacteria; cells stained with a primary dye (basic fuchsin) are resistant to acid-alcohol decolourisation (Brennan & Nikaido, 1995; Danilchanka *et al.*, 2008). Additionally, in pathogenic mycobacteria, due to its location as the outermost component, the cell wall also interacts with the host cell and is involved in immune modulation and virulence (Cox *et al.*, 1999; Kan-Sutton *et al.*, 2009; Strohmeier & Fenton, 1999). The cell wall biosynthetic pathways have been targets for several anti-mycobacterial drugs too. INH targets enoyl-ACP reductase (InhA) in mycolic acid biosynthesis (Rozwarski *et al.*, 1998; White *et al.*, 2005) and EMB targets an arabinosyltransferase involved in cell wall arabinan biosynthesis (Belanger *et al.*, 1996; Telenti *et al.*, 1997). Therefore, a good understanding of the structure and biosynthesis of the cell wall will help give clues not only to the development of novel anti-mycobacterial drug targets, but also further

our understanding of *Mycobacterium*-host cell interactions. The various components of the mycobacterial cell wall are shown in Fig. 1.6.

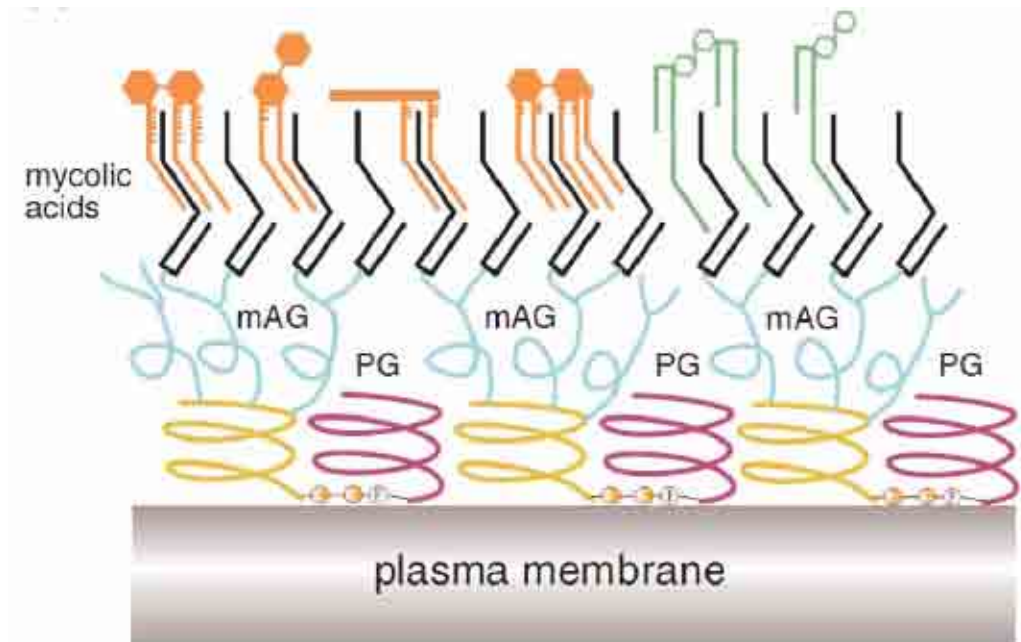


Fig. 1.6 Schematic representation of the mycobacterial cell wall (Dover *et al.*, 2004). Burgundy, peptidoglycan (PG); yellow, galactan domains of mycolylarabinogalactan (mAGP) ; turquoise, arabinan of mAGP; black, mycolic acid residues of mAGP; green, trehalose mono- and dimycolates (TMM and TDM); orange, a diverse repertoire of complex lipids.

1.5.1 Peptidoglycan (PG):

The peptidoglycan (PG) of mycobacteria is similar to that of other bacteria, consisting of β -(1,4) linked N-acetylglucosamine (NAG), N-acetylmuramic acid (NAM) residues, and a tetra-peptide chain. The tetra-peptide chain usually includes *L/D*-alanine, *D*-isoglutamine and *meso*-diaminopimelic (mDAP), and can be cross-linked to form a three dimensional mesh-like layer (Madigan & Martinko, 2005). In *Mycobacterium*, the cross-link occurs between two mDAP residues or between

mDAP and *D*-alanine (Brennan, 2003; Lavollay *et al.*, 2008). The biosynthesis of PG is well defined in *Escherichia coli* and the biosynthetic process is considered to be similar between the two species (Goffin & Ghuysen, 2002; van Heijenoort, 2001).

1.5.2 Arabinogalactan (AG):

Situated immediately outside the PG, the arabinogalactan (AG) layer is a branched polymer of galactose and arabinose. The galactose containing non-reducing end of AG is covalently bound to the NAG residue of PG by a unique linker, a disaccharide bridge α -L-Rhap-(1→3)-D-GlcNAc-(1→P) (McNeil *et al.*, 1990). AG biosynthesis is essential for the growth and survival of mycobacteria. AG has a linear galactan chain of about 30 β -D-galactofuranose (β -D-Galf) residues which is further elaborated by arabinan chains. The arabinan chain is composed of linear (1→5)- α -D-arabinofuranosyl (α -D-Araf) residues and branches to form a 3,5- α -D-Araf linked fork (Alderwick *et al.*, 2007; Besra *et al.*, 1995; Daffe *et al.*, 1990). In addition, almost two-thirds of the arabinan chains are esterified with mycolic acids at the non-reducing termini of the arabinan chain (Daffe *et al.*, 1990). The entire complex, encompassing PG and AG are esterified with mycolic acids, is also known as the mycolyl-arabinogalactan-peptidoglycan (mAGP) complex.

1.5.3 Membrane bound lipoglycans and glycophospholipids:

LAM, together with its precursors, lipomannan (LM) and PIMs are major lipoglycans found in the mycobacterial cell wall. They are important not only for the structure of the cell wall but also for modulating the host response during infection. Core structures are shown in Fig. 1.7 (Guerardel *et al.*, 2003).

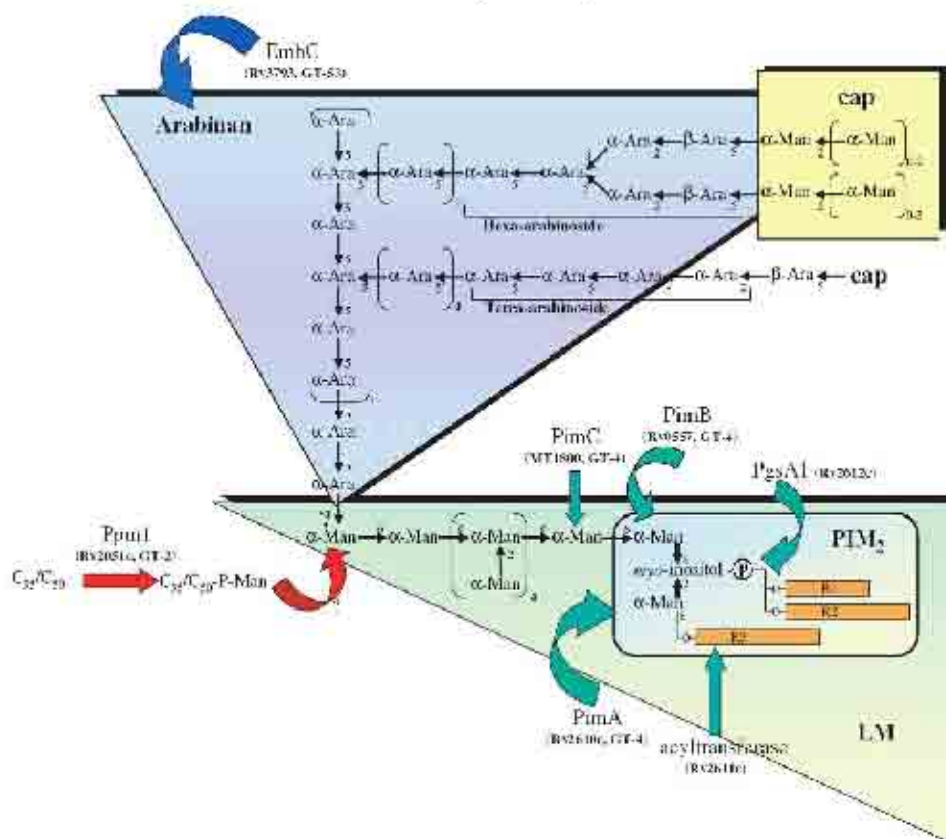


Fig. 1.7 General structure of ManLAM from *M. tuberculosis* and structural relationship between PIMs, LM and LAM (Guerardel *et al.*, 2003).

All lipoglycans contain a phosphatidyl-*myo*-inositol moiety (PI anchor), which is acylated. The acyl chains insert into the cell membrane, attaching the molecule directly to the membrane (Brennan & Besra, 1997; Hunter & Brennan, 1990). A previous study revealed that the PI anchor in LAM derived from *M. bovis* BCG plays

an important role in stimulating cytokine secretion of human DCs (Nigou *et al.*, 1997). Structural analysis shows that the *O*-3 position of the *myo*-inositol unit can also be acylated with fatty acids (Hsu *et al.*, 2007). The smallest lipoglycan precursor is PIM₁ which has a mannose residue attached to the 2-position of the *myo*-inositol ring of the PI anchor which can be further acylated at the 6-position of the mannose residue (Hsu *et al.*, 2007; Kordulakova *et al.*, 2003). Addition of another mannose residue to the 6-position of the *myo*-inositol ring of the PIM₁ results in the formation of PIM₂. Primed from the second mannose, the molecule can be elongated up to five mannose residues at position-6 to form PIM₆ (Briken *et al.*, 2004). LM from most mycobacteria including *M. tuberculosis* possesses an $\alpha(1\rightarrow6)$ -linked mannan backbone extended from PIMs with $\alpha(1\rightarrow2)$ substitution of single mannose, while *Mycobacterium chelonae* possesses $\alpha(1\rightarrow3)$ substitution of single mannose residues (Guerardel *et al.*, 2002). LAM, based on the structure of LM, has a branched arabinan polymer attached to the mannan, which is further decorated by terminal mannose caps (Nigou *et al.*, 2003). It can be classified into three groups based on the capping motif: mannose-capped LAM (ManLAM), in pathogenic strains such as *M. tuberculosis*, has caps for single, di- or tri-mannosides (Chatterjee & Khoo, 2001; Guerardel *et al.*, 2003); inositol phosphate-capped LAM (PILAM), found in the fast-growing non-pathogenic strains such as *M. smegmatis*, has inositol phosphate caps (Khoo *et al.*, 1995); AraLAM, found in *M. chelonae*, has neither mannose caps or inositol phosphate caps (Guerardel *et al.*, 2002; Guerardel *et al.*, 2003).

1.5.4 Mycolic acids:

Mycolic acids are one of the major components of the cell wall and can be found attached either to AG or as components of other free lipids, such as trehalose monomycolates (TMM) and dimycolates (TDM), and glucose monomycolate (GMM). Mycolic acids are long chain β -hydroxy- α -alkyl fatty acids. They consist of a long mero-chain ($> C_{50}$, various in different species) and a saturated α -chain (C_{24} - C_{26}) (Brennan & Nikaido, 1995). The mero-chain can be modified with different functional units, such as double bonds, methoxy or keto groups, *cis/trans*-cyclopropanations and epoxidations (Barry *et al.*, 1998), which vary in different species (Fig. 1.8 and Table 1.2).

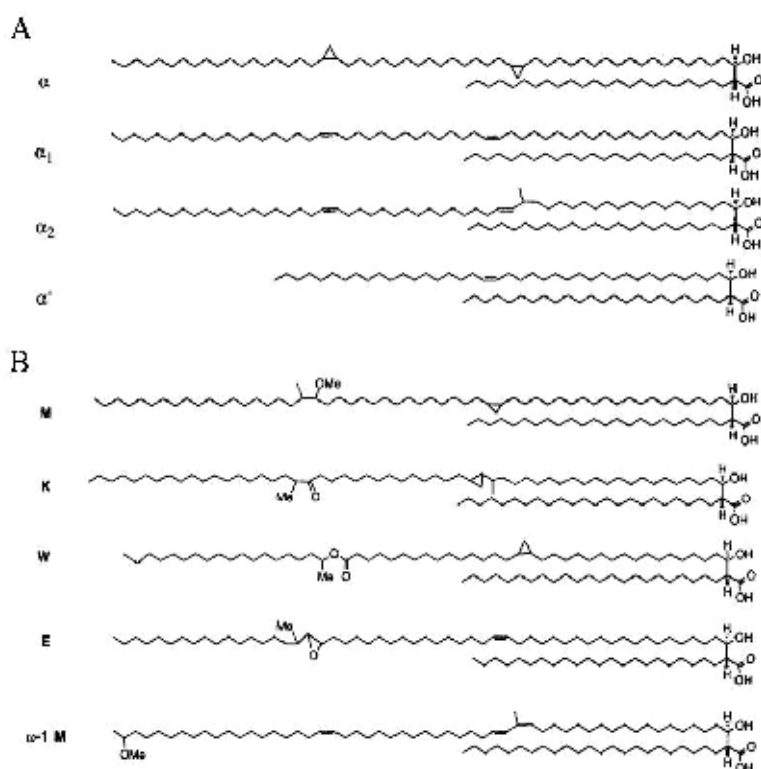


Fig. 1.8 Representative structures of mycolic acids (Barry *et al.*, 1998). A. Some representative examples of α - and α' -mycolic acids, lacking oxygen functions in addition to the 3-hydroxy acid unit. B. Oxygenated mycolic acids, M-methoxy, K-keto, W-wax ester, E-epoxy, ω -1 M- ω -1 methoxy.

Table 1.2 Variation of mycolic acid classes in *Mycobacterium* species

Species	α^*	α'	Mycolic Acid Classes			
			M	K	E	W
<i>M. tuberculosis</i>	+		+	+		
<i>M. bovis</i>	+		+	+		
<i>BCG</i>	+		+	+		
<i>M. avium</i>	+			+		+
<i>M. smegmatis</i>	+	+			+	
<i>M. kansasii</i>	+		+	+		
<i>M. marinum</i>	+		+	+		

* α and α' lacking oxygen functions in addition to the 3-hydroxy acid unit, M-methoxy, K-keto, E-epoxy, W-wax ester (Barry *et al.*, 1998)

The structure of mycolic acids also relate to the function of these lipids. Small changes in the structure can have profound effects on the biology of the bacilli (Dubnau *et al.*, 2000; Ueda *et al.*, 2001). For example, cyclopropanation of mycolic acids is essential for cord formation and virulence (Glickman *et al.*, 2000). *Cis*-cyclopropane modification can help bacteria with early growth in macrophages (Rao *et al.*, 2005); and *trans*-cyclopropane modification shows suppressive effects on *M. tuberculosis*-induced inflammation and virulence (Rao *et al.*, 2006) .

1.5.5. Intercalation of free-outer lipids:

As mentioned above, the mycobacterial cell envelope is intercalated with various 'free' glycolipids that are attached to the mAGP complex through hydrophobic interactions. This glycolipid-rich outer layer is composed of PDIMs, PGLs, mycolic acid containing lipids (TMM, TDM and GMM) and other kinds of complex lipids, such as glycopeptidolipids (GPLs) and lipooligosaccharides (LOSs).

i. Phthiocerol dimycocerosate (PDIM):

PDIM is composed of a long-chain phthiocerol or phthiodiolone di-esterified with multimethyl-branched long-chain mycocerosic acids (Minnikin *et al.*, 2002). PDIM deficiency causes attenuation of virulent strains in mice (Camacho *et al.*, 2001; Cox *et al.*, 1999; Simeone *et al.*, 2007). It participates in the early stage of *M. tuberculosis* infection with macrophages, including receptor-dependent phagocytosis, phagosome acidification arrest, protection of bacilli from reactive nitrogen intermediates and also modification of host cell membranes (Astarie-Dequeker *et al.*, 2009; Rousseau *et al.*, 2004). In addition, PDIM biosynthesis can be negatively selected under laboratory conditions (Domenech & Reed, 2009).

ii. Phenolic glycolipid (PGL):

PGL was originally termed as a type of “mycoside” in mycobacteria (Gastambide-Odier *et al.*, 1967) and was only observed in slow growing pathogenic strains, such as *M. kansasii*, *M. gastri*, *M. ulcerans*, *M. leprae*, *M. marinum* and *M. tuberculosis* (Daffe & Laneelle, 1988). PGL and PDIM share a similar lipid structure, whilst PGL has a glycosylated phenolic moiety at the end of the phthiocerol chain. PGL has been reported with various immunomodulatory properties (Fournie *et al.*, 1989; Prasad *et al.*, 1987; Vachula *et al.*, 1989), for example inhibiting the release of proinflammatory chemokines and cytokines (Reed *et al.*, 2004). The deficiency of PGL production can lead to attenuation in virulence of *M. bovis* in a Guinea pig infection model (Collins *et al.*, 2005).

iii. Mycolic acid containing glycolipids:

TDM, also known as cord factor, contributes not only to the cord-like serpentine growth characteristics of *M. tuberculosis* but also to virulence (Ryll *et al.*, 2001). Purified TDM induces a wide range of chemokines and cytokines which contributes to its immunomodifying function (Lima *et al.*, 2001). TDM can help the bacilli to enter macrophages (Yasuda, 1999) and induce the formation of granulomas (Behling *et al.*, 1993; Borders *et al.*, 2005; Guidry *et al.*, 2007). As a structural component of TDM, mycolic acids with different modifications and chain lengths can impact on the toxicity and other biological functions of TDM (Dubnau *et al.*, 2000; Fujita *et al.*, 2007). Besides, TDM shows diverse immunologic activities like adjuvant and antitumor properties (Silva *et al.*, 1985). TMM is a precursor in TDM biosynthesis and plays a role in the transfer of mycolic acids onto the cell wall AG (Belisle *et al.*, 1997; Katsube *et al.*, 2007). GMM is synthesised by using host-derived glucose and can be an indicator of infection. GMM can be recognised by CD1-T cells and mediate a T cell response (Enomoto *et al.*, 2005; Moody *et al.*, 1997).

iv. Sulfolipid (SL):

SL is a glycolipid composed of a trehalose 2-sulfate core modified with four fatty acyl substituents (Goren *et al.*, 1976). It is found in some virulent strains of *M. tuberculosis*, although its function remains unclear. For example, it is reported that SL can block the release of reactive oxygen and enhance the secretion of IL-8 and TNF- α in phorbol myristate acetate (PMA)-stimulated macrophages (Brozna *et al.*, 1991).

More recent studies with SL have shown its inhibitory effect on TDM induced TNF- α secretion in macrophages and granuloma formation in mice (Okamoto *et al.*, 2006). In addition, the loss of SL shows no impact on bacilli replication in a mouse model of infection (Kumar *et al.*, 2007).

v. Glycopeptidolipids (GPLs):

GPLs are a major constituent of the outer layer cell wall of several species of non-tuberculosis *Mycobacterium* including the saprophytic *M. smegmatis* and the opportunistic pathogen *Mycobacterium avium*. A common structure of fatty acyl-tetrapeptide (FATP) are shared between GPLs, while the glycosylations are different between non-pathogenic and pathogenic mycobacteria (Chatterjee & Khoo, 2001; Patterson *et al.*, 2000). In non-serovar-specific GPLs (nsGPLs) from non-pathogenic *Mycobacterium*, such as *M. smegmatis*, the FATP is modified with 6-deoxytalose (6-*d*-Tal) at the threonine residue and *O*-Me-Rha (*O*-methyl-rhamnose) at the *L*-alaninol residue. In serovar-specific GPLs (ssGPLs) from pathogenic mycobacteria, such as *M. avium*, more complicated glycosylations are found based on the structure of nsGPLs (Aspinall *et al.*, 1995; Belisle *et al.*, 1993). Although, ssGPLs are from non-tuberculosis *Mycobacterium*, their virulence-related and immunomodulating properties have been identified (Barrow *et al.*, 1995; Krzywinska *et al.*, 2005; Sweet & Schorey, 2006; Torrelles *et al.*, 2002). Recently, it was reported that GPLs from *M. smegmatis* participate in receptor-dependent phagocytosis of *Mycobacterium* by macrophages (Villeneuve *et al.*, 2005), and *M. avium*-GPLs core

antigen can be used for the clinical serodiagnosis of pulmonary *M. avium* disease (Kitada *et al.*, 2005; Kitada *et al.*, 2008).

vi. Lipooligosaccharides (LOSs):

LOSs can be found in *M. kansasii*, *M. gastri*, *M. marinum* and the Canetti variant of *M. tuberculosis* (Burguiere *et al.*, 2005b; Daffe *et al.*, 1991b; Gilleron & Puzo, 1995). LOSs are highly antigenic glycoconjugates on the cell surface and are used as targets for serotyping. It has been reported that LOSs are involved in biofilm formation and infection of macrophages in *M. marinum* (Ren *et al.*, 2007). Recently several genes have been identified involved in LOS biosynthesis (Burguiere *et al.*, 2005b; Etienne *et al.*, 2009; Ren *et al.*, 2007). However, little is known about the biosynthesis and function of LOSs.

1.6 Surrogate systems for TB research

Although much effort has been made to understand the mechanism of pathogenesis for the discovery of novel drugs and development of better vaccines, there are still several problems associated with *M. tuberculosis* research. Firstly, *M. tuberculosis* grows very slowly and the average generation time is about 20 hours. Secondly, since *M. tuberculosis* is an airborne human pathogen it has to be handled in a Biosafety Level 3 (BSL-3) laboratory. Thirdly, genetic manipulation, till recently, has been lagging behind due to lack of available genetic tools. To overcome these problems, a number of other *Mycobacterium* and related species have been used as

surrogate models to study TB. Furthermore, the whole genome information of *M. tuberculosis* was released in 1998, with the size of 4.4Mb and about 4000 open reading frames (Camus *et al.*, 2002; Cole *et al.*, 1998). Later, when more mycobacterial species have also been sequenced, it will be possible to compare genomes between *M. tuberculosis* and surrogate systems to help better understand the mechanism of *M. tuberculosis* virulence and choose the right surrogate system for certain studies.

1.6.1 *Corynebacterium glutamicum*

C. glutamicum is non-pathogenic and is classified into *Corynebacterineae* of the *Actinobacteria* phylum, and is thus closely related to *M. tuberculosis*. *C. glutamicum* has been used industrially for amino acid production (Kinoshita, 1985). It has a distinct lipid rich cell wall, similar to *M. tuberculosis* with mAGP (Dover *et al.*, 2004) but simpler than *M. tuberculosis*. The genome sequencing of *C. glutamicum* was completed in 2003, showing a genome half the size of *M. tuberculosis* and less paralogous genes in the *C. glutamicum* genome (Kalinowski *et al.*, 2003). Although *C. glutamicum* shows a broad metabolic diversity, compared to *M. tuberculosis*, it is used as a model system for mycobacterial cell wall biosynthesis studies (De Sousa-D'Auria *et al.*, 2003; Gande *et al.*, 2004; Gibson *et al.*, 2003). This is because of its better tolerance to the deletion of genes whose homologues are essential in *M. tuberculosis*, such as *Cg-emb* (Seidel *et al.*, 2007).

1.6.2 *M. smegmatis*

M. smegmatis is non-pathogenic and fast-growing, with an average generation time of about 4-5 hours, and thus overcomes the two major hurdles of working with *M. tuberculosis*. While *M. smegmatis* makes some lipids that are different, most cell wall components like PG, AG and mycolic acids are common to both species. *M. smegmatis* can tolerate mutations altering cell wall biosynthesis better than *M. tuberculosis*, for example, *embC* and *afiC* can be deleted in *M. smegmatis* but not in *M. tuberculosis* (Birch *et al.*, 2010). The size of *M. smegmatis* genome is 7.0 Mb, larger than that of *M. tuberculosis*, and it shows high genomic divergence in that only 69.8% of the protein-coding genes of *M. tuberculosis* have been found with orthologues in *M. smegmatis* (Altaf *et al.*, 2010). Given its non-pathogenicity, *M. smegmatis* has limited use as surrogate model for virulence studies and is usually restricted to the study of metabolic processes. However, it is still a useful system for cell wall biosynthesis, because it can easily be genetically manipulated compared to *M. tuberculosis*. The most popular *M. smegmatis* strain used in TB research is *M. smegmatis* mc²155, which originated from *M. smegmatis* ATCC 607 (Snapper *et al.*, 1990). It shows a high efficiency in plasmid transformation (electroporation), which is invaluable in the analysis of mycobacterial gene function, expression and replication.

1.6.3 *Mycobacterium marinum*

M. marinum is a fish pathogen and can also cause lesions on human skin (Wolinsky, 1992). It grows faster than *M. tuberculosis* with an average generation

time of 4 hours under an optimal growth temperature of 25°C to 35°C (Clark & Shepard, 1963). Additionally, *M. marinum* can be handled in a BSL-2 laboratory. The size of the *M. marinum* genome is 6.6Mb, about 1.5 times the size of *M. tuberculosis*. *M. marinum* shares 85% identity to the orthologous regions of *M. tuberculosis* (Stinear *et al.*, 2008). The cell wall lipids of *M. marinum* are related to those of *M. tuberculosis*, except for the altered stereochemistry of PDIM components (Onwueme *et al.*, 2005). Thus, while *M. marinum* has most of the advantages of *M. smegmatis*, it can also be evaluated for virulence, as it is a pathogenic *Mycobacterium*, without the need for BSL-3 containment. Another advantage of *M. marinum* is that it can be used to study the pathogen-host interaction using zebrafish model as the host (Pozos & Ramakrishnan, 2004; Stamm & Brown, 2004). Zebrafish are one of the natural hosts of *M. marinum* and infected zebrafish can develop granulomas, a key feature of TB infection in humans (Prouty *et al.*, 2003; Tobin & Ramakrishnan, 2008). Although the fish immune system is still not fully understood, zebrafish have both innate and adaptive immune systems (Traver *et al.*, 2003) and the outcome of infection, acute or chronic, depends on the infection dose (Prouty *et al.*, 2003). There are several other advantages of the zebrafish model, such as transparent fish embryos allow real-time analysis by using fluorescent labeled *M. marinum* (Davis *et al.*, 2002).

1.7 Molecular genetic tools in TB research

To control TB, a better understanding of mycobacteria, the bacilli itself and its interaction with host as well, is very important. Genetic manipulations of the genome,

such as gene knock-outs, introduction of other genes, or the generation of random *Tn*-mutant libraries, are very powerful methods. Although genetic tools used in mycobacteria are less well developed compared with those in other species, such as *E. coli*, much progress has been made in past 30 years, especially after the release of the complete genome sequence of *M. tuberculosis*.

1.7.1 Plasmid

A plasmid is a DNA molecule outside the bacterial chromosome and can replicate independently. It plays an important role in bacterial biology. Several *Mycobacterium* species have been found containing naturally occurring plasmids, for instance the MAIS complex (*M. avium*, *Mycobacterium intracellulare*, *Mycobacterium scrofulaceum*) and *Mycobacterium fortuitum* (Crawford & Bates, 1979; Crawford & Bates, 1986). A small plasmid has been found in the widely used *M. smegmatis* mc²155 strain (Beggs *et al.*, 1995), although its original strain, *M. smegmatis* ATCC607, shows no sign of a plasmid (Crawford *et al.*, 1981). Conversely, *M. tuberculosis* do not possess a plasmid (Zainuddin & Dale, 1990). Naturally occurring plasmids in mycobacteria are considered to correlate with virulence (Gangadharam *et al.*, 1988; Mizuguchi *et al.*, 1981). For example, *M. ulcerans*, the causative agent of the devastating skin disease, Buruli ulcer, contains a 174-kb plasmid (pMUM001) bearing a cluster of genes responsible for the biosynthesis of a macrolide toxin, which is cytotoxic and immunosuppressive and can cause massive tissue damage (Stinear *et al.*, 2004).

As a genetic tool, plasmids are used to multiply gene copies or to express proteins for functional studies. Plasmids isolated from mycobacteria are very helpful for developing vectors for genetic manipulation in mycobacteria. Plasmid pAL5000, recovered from *M. fortuitum* (Labidi *et al.*, 1985), is the most widely used replicon in many *Mycobacterium* species. Thus, many currently used mycobacterial vectors are developed on pAL5000. Shuttle plasmids made by cloning pAL5000 into *E. coli* plasmids can replicate in not only fast-growing mycobacteria but also slow-growing mycobacteria, including *M. bovis* BCG and *M. tuberculosis* (Ainsa *et al.*, 1996; Garbe *et al.*, 1994; Snapper *et al.*, 1988). Later, with the understanding of their replication mechanisms, small fragments with only genes essential for replication in *Mycobacterium* are used as vectors. An example is pMV206 carrying only *orf1*, *orf2* and part of *orf5*, from which derived pMV261 with the *M. bovis* BCG *hsp60* promoter, to help the expression of foreign proteins in mycobacteria (Stover *et al.*, 1991). Derivatives of pAL5000 are applied to general cloning, reporter genes, delivery systems and protein expression. Shuttle vectors derived from pAL5000 maintain low copy in *Mycobacterium* (Stolt & Stoker, 1996; Stover *et al.*, 1991) with 2-5 copies *per* cell and *E. coli* is used as an intermediate host for DNA manipulation. Besides pAL5000, other naturally occurring plasmids are also used for vector construction, such as pMSC262 from *M. scrofulaceum* (Qin *et al.*, 1994). To avoid possible effects in phenotype caused by gene over expression, sometimes an integration system is utilised. Vectors carrying L5 phage integrase were later developed as pMV361 which is a derivative of pMV261 (Stover *et al.*, 1991).

1.7.2 Transposon

A transposable element is a DNA fragment that can move from one position in the genome to another independently. They are found widely in prokaryotes and eukaryotes. About 50 transposable elements have already been discovered in several *Mycobacterium* species based on sequence similarity, and most of them are host restricted and inactivated (Hatfull & Jacobs, 2000).

Transposition can cause different genetic rearrangements among which interruption of the gene in the transposition site is the most common. According to this, transposable element- based genetic tools were developed. As a class II transposable element, transposon (*Tn*) is composed of a transposition essential element (invert repeat sequence, transposase and resolvase) and genes not required for transposition, such as an antibiotic resistant gene. Thus *Tn* can be used for functional studies by either insertion mutagenesis or by activation through induction of promoter and protein expression under certain conditions by adding promoterless reporter genes. There are two kinds of *Tn* used in TB research, *Tn* derived from naturally occurring mycobacterial transposon elements and from *mariner* transposon elements. Since there are six activate insertion sequences (*ISs*) in *Mycobacterium*, *Tn* were developed based on them, such as *Tn61i* from *IS6100* (Martin *et al.*, 1990) and *Tn5370* from *IS1096* (Cox *et al.*, 1999). The horn fly *Haematobia irritans* original *mariner* transposable element belongs to a superfamily whose members can be found in a wide range of eukaryotic hosts. It can be used in both *E. coli* and *M. smegmatis* (Rubin *et*

al., 1999).

1.7.3 Delivery systems for mutagenesis

Random *Tn* mutagenesis and specialised mutagenesis are two useful methods. In case of mycobacteria, especially for slow growing mycobacteria, because of the frequency of transposition is very low, to get enough random mutants, a large amount cells with DNA transferred are necessary. Similarly, to obtain a specific knock-out mutant, a large amount cells with DNA transferred can increase the odds of successful homologous recombination. Therefore, an efficient delivery system is critical for successful mutagenesis (Pelicic *et al.*, 1997). In mycobacteria mutagenesis, two delivery systems are most often used, temperature-sensitive plasmid and phage (Hatfull & Jacobs, 2000).

Temperature-sensitive plasmids are plasmids that can replicate at 30°C to 32°C but not at 39°C to 40°C. The first thermosensitive plasmid used in mycobacteria was derived from pAL5000 and tested in *M. smegmatis* (Guilhot *et al.*, 1992). Further, by carrying a *Tn*, it has succeeded in *Tn*-mutagenesis (Perez *et al.*, 1998). Later two modified plasmids were constructed based on this, pPR27 and pPR23, with a *SacB* gene, for use in slow growing mycobacteria. *SacB* encodes a levan sucrose, which is lethal to mycobacteria if sucrose is added to the media. It provides a counter-selection for mutagenesis in addition to temperature pressure (Pelicic *et al.*, 1997).

Phage is a bacterial virus and it infects host cells *via* a receptor-dependent process.

Phage can also specifically infect mycobacteria. Due to high infection efficiency, it has been used as a delivery system. A random library of phage genome was constructed into an *E. coli* as plasmid and selected for the chimeric molecule that could replicate in *E. coli* as a plasmid and infect mycobacterial cells as a phage (Jacobs *et al.*, 1989a; Jacobs *et al.*, 1989b). The final molecule was termed phasmid. Later, a temperature-sensitive shuttle phasmid system was developed, phAE70 derived from D29 mycobacteriophage and phAE87 from TM4 (Bardarov *et al.*, 1997). It can be manipulated as plasmid or cosmid in *E. coli* and replicate as a lytic phage in mycobacteria at the permissive temperature, while it can only infect mycobacteria but not form plaques at the non-permissive temperature.

1.9 Aims and objectives

The main objective of my studies is to study cell wall biosynthesis in mycobacteria. Considering the advantages and disadvantages of different models in TB research, I plan to use *M. smegmatis* and *M. marinum* as models to isolate mutants of each species that are defective in the biosynthesis of the mycobacterial cell wall with the aim of identifying genes involved in its synthesis. There are three genetic strategies that have been used to study cell wall biosynthetic pathways.

The first is specialised knock-out, generating mutants of specific genes in surrogate systems and complementing the mutants with the gene and also its *M. tuberculosis* homologue, for functional studies. As mentioned above, due to the difficulties of genetic manipulation in *M. tuberculosis*, surrogate systems are widely

used to study cell wall biosynthesis in *Mycobacterium*. For instance, *aftA* which is involved in AG biosynthesis in *M. tuberculosis* was identified using bioinformatics analysis and later confirmed by complementing the mutant in *C. glutamicum* with the *M. tuberculosis* orthologue (Alderwick *et al.*, 2006). Similarly, the function of *aftC* of *M. tuberculosis* in LAM biosynthesis was also confirmed in *M. smegmatis* (Birch *et al.*, 2010).

Another strategy is to screen for mutants with morphology changes from random *Tn*-mutant libraries. From previous studies, it is known that the disruption of genes involved in the biosynthesis or the transportation of cell wall components, usually results in an altered physical morphology. For example, deletion of *kasB*, responsible for elongation of mycolic acids, results in a small colony morphology in *M. tuberculosis* (Bhatt *et al.*, 2007). Dysfunction of *pcaA*, a gene responsible for cyclopropane ring synthesis of mycolic acids, causes no cording forming and fails to persist in infected mice (Glickman *et al.*, 2000). Inactivation of *pstA* in *M. avium* leads to the loss of a specific lipopeptide and an inability to form biofilms (Wu *et al.*, 2009). Thus, it is assumed that a morphology change can *vice versa* indicate changes in cell wall components.

Finally, based on the generation of a *Tn*-mutant library, screening for mycobacteriophage-resistant mutants was also developed as a tool. Mycobacteriophages can infect host cells by attaching to specific cell surface receptor molecules, *e.g.* proteins, glycans or lipids. These receptors would be expected to be

the outermost components of the mycobacterial cell wall. Thus, any changes in those surface molecules may cause resistance to phages. For example, two phage resistant mutants of *M. smegmatis* result in structural changes of acyltrehalose-containing lipooligosaccharides (Dao *et al.*, 2008). The difference in phage sensitivity can be caused by the disruption of host genes involved in phage infection processes, one of which associates with the receptor on cell wall. Therefore phage sensitivity can also be used to study cell wall biosynthesis.

In this study, the last two strategies will be utilised and the aims are as follows:

1. To generate a *Tn*-mutant library of *M. smegmatis* mc²155 and *M. marinum* 1218R (ATCC 927).
2. To screen for mutants based on colony morphology changes on different agar media and phage-sensitivity.
3. To biochemical characterise selected mutants by lipid analysis and complementation experiments.
4. To examine the virulence of *M. marinum* mutants *in vitro* in murine bone marrow derived macrophages and the zebrafish model of infection.

Chapter 2

Screening Transposon Libraries of *M. smegmatis* and *M. marinum* for Mutants with Defective Cell Walls

2.1 Introduction

As outlined in Chapter 1, the mycobacterial cell wall is made up of complex lipids and carbohydrates that play important roles in determining permeability and virulence (Aspinall *et al.*, 1995; Cox *et al.*, 1999). Our understanding of the mycobacterial cell wall structure is evolving along with advances in the analysis techniques for observing the physical structure of the cell wall. Minnikin first proposed in 1982 that the mycobacterial cell envelope was an asymmetric structure, with the inner layer formed by the cell membrane, PG and AG-attached mycolic acids, with the outer layer consisting of ‘free’ lipids intercalated within the AG-attached mycolate layer (Minnikin, 1982). Further, X-ray diffraction studies by Nikaido revealed that mycolic acids were stacked parallel to each other and perpendicular to the cell membrane (Nikaido *et al.*, 1993). Much finer details were subsequently revealed by cryo-electron tomography that confirmed the multilayer structure of the mycobacterial cell wall, but proposed a modified model with an complete coverage of mycolic acids and free lipids in the inner layer binding to the cytoplasm membrane directly (Hoffmann *et al.*, 2008; Zuber *et al.*, 2008).

Thus a complex mix of covalent bonds and hydrophobic interactions results in a uniquely ‘packed’ cell wall. Any changes in the classical composition of the cell wall constituents could impact upon interactions. These alterations often result in changes of cell wall structure and as a result in colony morphology. Changes include either a complete loss of a cell wall component (Kan-Sutton *et al.*, 2009) or the accumulation

of an intermediate with altered structure (Ren *et al.*, 2007). For example, (i) a deficiency in elongation of mycolic acids caused by the deletion of *kasB* resulted in small colonies of *M. tuberculosis* (Bhatt *et al.*, 2007), (ii) the absence of cyclopropanation of mycolic acids in *M. bovis* BCG and *M. tuberculosis* resulted in loss of cord formation (Glickman *et al.*, 2000), (iii) the deletion of a reductase responsible for reducing β -oxo acyl intermediates to form mature mycolic acids in *M. smegmatis*, changes the colony morphology from a smooth surface to a dry texture (Bhatt *et al.*, 2008), and (iv) the loss of GPLs in either *M. smegmatis* or *M. avium* leads to a rough colony morphology (Billman-Jacobe *et al.*, 1999; Riviere *et al.*, 1996).

The aim of the studies described in this chapter was to generate random *Tn*-mutant libraries of *M. smegmatis* and *M. marinum* (two mycobacterial species that are used as surrogates to study *M. tuberculosis*), and to use these as a resource to screen for mutants with altered cell walls. Two different, but complementary approaches were used to isolate mutants from these two libraries. In one approach, *Tn*-mutant libraries of *M. smegmatis* and *M. marinum* were plated onto various agar media to isolate *Tn*-mutants that displayed altered colony morphology. In a second parallel approach, the *M. smegmatis* library was screened for resistance to mycobacteriophages. This second screen was based on the hypothesis that the outer cell wall components would also be receptors for mycobacteriophages and that loss of a specific receptor (due to a *Tn* insertion in one of its biosynthetic genes) would result

in resistance to the mycobacteriophage. The results obtained from this second screen are discussed separately in Chapter 3.

Tn-mutagenesis has been a powerful tool for studying different aspects of *M. tuberculosis* biology. It has been used to identify metabolic pathways and virulence factors (Guilhot *et al.*, 1994; Kleckner *et al.*, 1991; McAdam *et al.*, 2002) as well as to provide information for global screens to predict essential genes in genomes of mycobacteria (Lamichhane *et al.*, 2003; Sassetti *et al.*, 2001). Although *Tns* derived from naturally occurring insertion sequences (*ISs*) in mycobacterial genomes have often been used for mutagenesis (Cirillo *et al.*, 1991; Martin *et al.*, 1990; McAdam *et al.*, 1995), more recently a mariner-based *Tn*, which is derived from a *Tn* in the horn fly *Haematobia irritans* has been used for *Tn* mutagenesis in both fast-growing and slow-growing mycobacteria (Rubin *et al.*, 1999; Rybniker *et al.*, 2003; Sassetti *et al.*, 2001). Mariner-based *Tns* offer the advantage of being non-indigenous (no homologous recombination with *IS* insertion) and having a high transposition frequency. In addition, the efficiency of *Tn*-mutagenesis has been improved by the use of a temperature sensitive phage delivery systems (Bardarov *et al.*, 1997; Rybniker *et al.*, 2003).

In this study, phAE181, a recombinant phage that contains a derivative of the mariner-based *Tn5371* was used. The recombinant phage phAE181 is derived from a temperature sensitive mutant of the lytic mycobacteriophage TM4, which can infect many slow and fast growing *Mycobacterium* species, including *M. tuberculosis*, *M.*

smegmatis and *M. marinum*. The phage can replicate inside a mycobacterial host (with a productive lytic cycle) at 30°C, but not at the non-permissive temperature of 37°C. *Tn5371*, which contains a hygromycin resistance gene (*hyg*), can thus be delivered by infecting the target *Mycobacterium* host at 37°C, followed by selection of *Tn*-mutants on hygromycin-containing plates at 37°C. *Tn5371* mutagenesis libraries were generated for *M. smegmatis* and for *M. marinum* using phAE181 and the data included in this chapter describe the isolation and identification of *M. smegmatis* and *M. marinum* *Tn*-mutants with altered colony morphology.

2.2 Materials and Methods

2.2.1 Bacterial strains, phages and growth conditions

Bacterial strains, plasmids, and phages used in this study are listed in Table 2.1. *M. smegmatis* was grown in TSB at 37°C and *M. marinum* was grown in 7H9+OADC at 30°C, both with 0.05% Tween 80. *E. coli* was grown in LB broth at 37°C. Hygromycin B was added at concentrations of 100µg/ml, 75µg/ml and 150µg/ml, respectively, for *M. smegmatis*, *M. marinum* and *E. coli* where required.

Table 2.1 Phage and bacterial strains used in this study

Strains and Phage	Description	Source or Reference
Phage		
phAE181	Conditionally replicating phage TM4 derivative carrying <i>Tn5371</i>	(Kriakov <i>et al.</i> , 2003)
Bacteria		
<i>E. coli</i> cc118λpir		
<i>M. smegmatis</i> mc ² 155	Parental (wild type strain) strain, Ept mutant	(Snapper <i>et al.</i> , 1990)
<i>M. marinum</i> 1218R	Parental (wild type strain) strain	ATCC927

2.2.2 Generation of high titre phage lysate

For generating a high titre phage lysate, the titre of an existing phAE181 lysate was first checked as follows. First, 200µl of a late log-phase culture of *M. smegmatis* mc²155 was mixed with molten 7H9 soft agar (5ml, 50°C) and overlaid on a basal layer of 7H9 agar in a petri-dish and the agar was allowed to solidify. In parallel, the existing stock of phAE181 was ten-fold serially diluted (10^{-1} - 10^{-10}) in MP buffer (50mM Tris·HCl pH7.8, 150mM NaCl, 10mM MgSO₄, 2mM CaCl₂). Then 10µl of the dilutions were spotted on the overlay of *M. smegmatis* mc²155. Plates were incubated at 30°C for 2-3 days till plaques were observed on a lawn of *M. smegmatis* mc²155. The plaque forming units (pfu) were counted and phage titre was calculated according to the dilution (pfu/ml=number of pfu in spot×dilution factor×100). To prepare the high titre lysate, the phage suspension was diluted to obtain an estimated 500-1000 pfu *per* plate and mixed with 200µl of late log-phase *M. smegmatis* mc²155 culture and molten 7H9 soft agar overlaid on 7H9 agar. Plates were incubated at 30°C for 2-3 days to obtain plaques in a lacy pattern. MP buffer (4-5 ml) was added to each plate and plates were left at 4°C overnight. The phage soaked buffer was then collected and filtered through a 0.45µm filter to obtain the phage lysate. Phage lysate was kept at 4°C and the titre was checked as described above. The optimal titre required for transduction was 10^{10} pfu/ml (Stover *et al.*, 1991).

2.2.3 Transposon mutagenesis

M. smegmatis or *M. marinum* cultures were grown in 50ml of TSB (0.05% Tween

80) or 7H9+OADC (0.05% Tween 80) to an optical density (OD_{600nm}) of 0.8. Cells were harvested by centrifugation at room temperature and washed with 50 ml MP buffer. Cells were resuspended in 5 ml MP buffer and mixed with high titre phage lysate (10^{10} to 10^{11} pfu/ml) at a multiplicity of infection (MOI) of 10, while 0.5 ml cells were mixed with 0.5 ml MP buffer as control. After incubation at 37°C for 1 hour static culture, cells were harvested and resuspended in 25 ml TSB with 0.05% Tween 80 (for *M. marinum*, 7H9 broth with 0.05% Tween 80). Cells were recovered at 37°C overnight before being plated on TSB agar plates with 100µg/ml hygromycin B (for *M. marinum*, 7H10 agar plates with 75µg/ml hygromycin B were used). Plates were incubated at 37°C for either 3 to 4 days for *M. smegmatis* or 2 weeks for *M. marinum*.

2.2.4 Isolation and sequencing of *Tn* insertion sites

Genomic DNA was digested with *Bss*HIII (NEB) and ligated with T4 ligase (NEB). To select for self-ligated fragments of genomic DNA containing *Tn5371* (containing *hyg* and the R6K *ori*) the ligation mix was transformed into *E. coli* cc118λpir. *Hyg*^R colonies were grown up in LB with hygromycin to obtain plasmid DNA. Plasmid DNA isolated from *Hyg*^R transformants was then sequenced using the primers *mariner*KM (5'-GCTTACAATTTAGGTGGCACT-3') and *mariner*Hyg (5'-TAGACAGATCGCTGAGATAGG-3') to obtain sequences of the left or right regions flanking the *Tn* insertion.

2.3 Results

2.3.1 Isolation of *M. smegmatis* *Tn*-mutants with altered colony morphology and phage-resistance

A total of 715 *M. smegmatis* strains with *Tn* insertions were initially inoculated into 96-well plates. The cultures were then inoculated using a replicator onto rectangular agar media plates. A number of media combinations were used, these included TSB, TSB-T (0.05% Tween 80 plus), 7H10, 7H11-T (0.05% Tween 80 plus), LB and LB-T (0.05% Tween 80 plus). Eight mutants displayed colony morphology changes on at least one of the six agar media compared with wild type strain (Fig. 2.1). Under the same growth conditions and similar inoculum volumes, alterations were of different kinds, for example, some had smaller zones of growth (1G2 on LB agar), while others spread as bigger colonies compared to the wild type strain (2H11 on LB agar). Some had smooth surfaces (8D5 on 7H10-T agar), while the wild type strain had rough surface (8B7 on TSB-T agar).

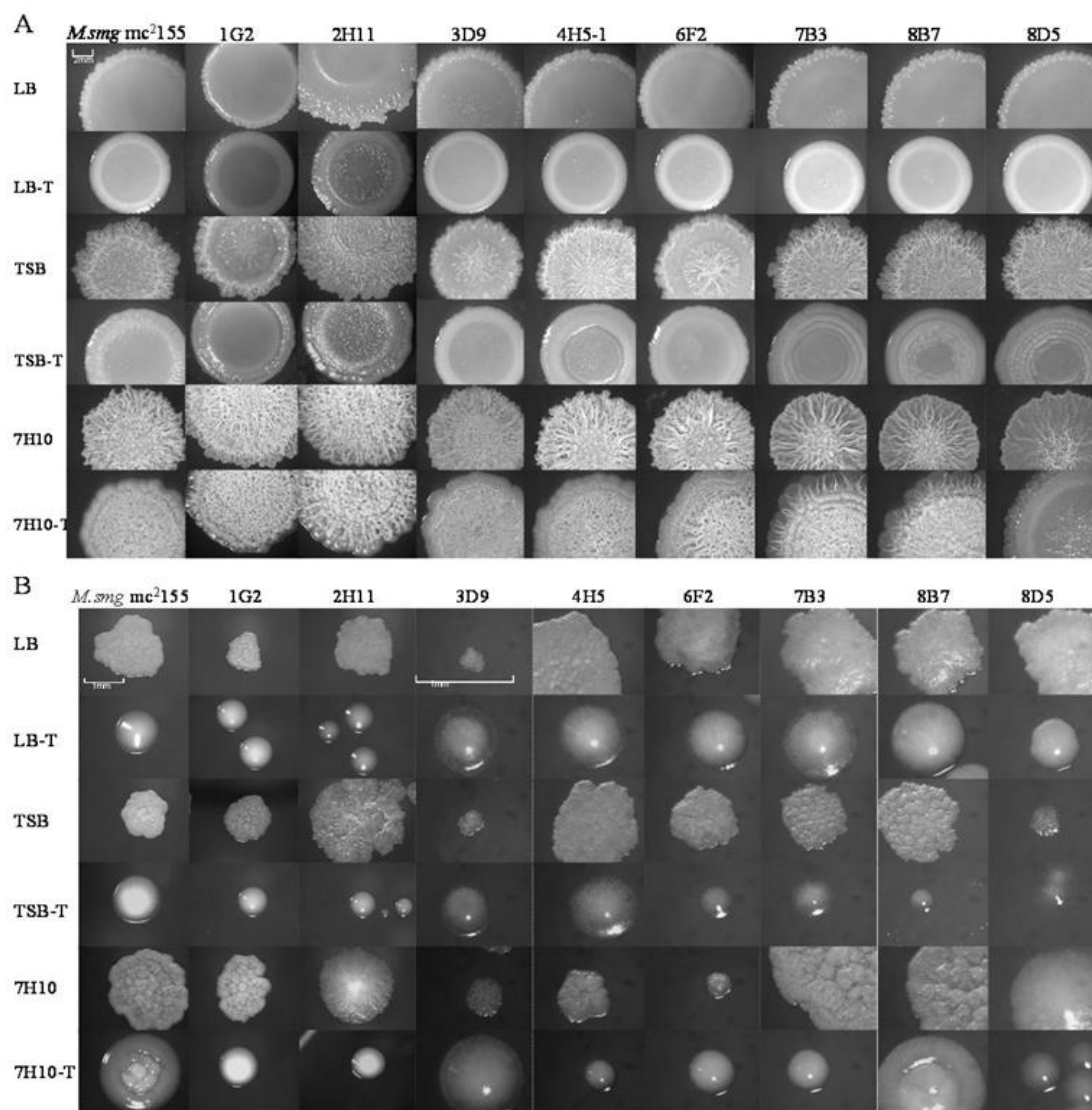


Fig. 2.1 Colony morphology of *M. smegmatis* *Tn*-mutants on different media (denoted on the left). A. 10µl spot of a stationary phase culture; B. Single colony of each *Tn*-mutant

2.3.2 Isolation of *Tn* insertion sites and identification of disrupted genes in *M. smegmatis* mutants with altered colony morphology.

When genomic DNA from a *Tn*-mutant is digested with *Bss*HII, an enzyme that has no sites in *Tn3571*, one of the genomic *Bss*HII fragments contains the intact *Tn5371* insertion. When this fragment is circularised by ligation, it replicates as a

plasmid (due to the presence of R6K *ori*) in *E. coli* cc118 λ pir. Thus, *Bss*HIII digested genomic DNA can be added to a ligation reaction and subsequently transformed into *E. coli* cc118 λ pir to select for *Tn*-containing recircularised fragments by plating the transformants on hygromycin plates. This strategy was used to isolate the *Tn*-insertion as part of a plasmid in *E. coli*. The isolated plasmids were sequenced using either marinerKM (5'-GCTTACAATTTAGGTGGCACT-3') or marinerHyg (5'-TAGACAGATCGCTGAGATAGG-3'). The sequence obtained was then used as a query sequence for BLAST (Altschul *et al.*, 1990) to search the Xbase genome database (<http://xbase.bham.ac.uk/mycodb/>).

As shown in the Table 2.2, amongst the genes that were disrupted by *Tn* insertions in the eight *M. smegmatis* *Tn*-mutants with colony morphology changes, three were genes in the cell wall and cell processes category, two were genes with a role in intermediary metabolism and respiration, two were regulatory genes and one was a conserved hypothetical protein with unknown function.

Table 2.2 Genes disrupted by *Tn* insertion in *M. smegmatis* colony morphology mutants

Mutant No.	Gene No.	Gene Name	Homologue in <i>M. tuberculosis</i> H37Rv	Function
1G2	<i>MSMEG0031</i>	Penicillin binding protein, transpeptidase domain protein	<i>Rv0016c(pbpA)</i>	Involved in final stages of peptidoglycan synthesis
2H11	<i>MSMEG0035</i>	FHA domain protein	<i>Rv0020c(fhaA)</i> : conserved hypothetical protein with FHA domain	Signal transduction
3D9	<i>MSMEG1654</i>	Isocitrate dehydrogenase, NADP-dependent	<i>Rv0066c(icd2)</i> : Probable isocitrate dehydrogenase [NADP] Icd2	Involved in the TCA cycle
4H5-1	<i>MSMEG3327</i>	Hypothetical protein		Unknown
6F2	<i>MSMEG5102</i>	ABC transporter ATP-binding protein	<i>Rv1473</i> : Probable macrolide-transport, ATP-binding protein ABC transporter	Involved in active transport of macrolide across the membrane (export).
7B3	<i>MSMEG0268</i>	Transcriptional regulator, GntR family protein	<i>Rv0792c</i> : Probable transcriptional regulatory protein (probably GntR-family)	Involved in transcriptional mechanism
8B7	<i>MSMEG4265</i>	MmpS3 protein	<i>Rv2198c(mmpS3)</i> : probable conserved membrane protein MMPS3	Unknown
8D5	<i>MSMEG6756</i>	Glycerol kinase, glpK	<i>Rv3696c(glpK)</i> : Probable glycerol kinase GlpK	Key enzyme in the regulation of glycerol uptake and metabolism

2.3.3 Isolation of *M. marinum* *Tn*-mutants with altered colony morphology.

Using a similar approach as above, an *M. marinum* *Tn*-mutant library was screened for mutants with altered colony morphology. In this case, studies were limited to 7H10 agar with or without Tween 80 (7H10/7H10 T) due to the inability of *M. marinum* to grow on LB/TSB agar.

A total of 960 *M. marinum* *Tn*-mutants were screened, 23 had colony morphology changes either on 7H10 or 7H10 T agar plates compared to the wild type strain (Fig. 2.2). Under the same growth conditions and similar inoculum volumes, one mutant lacked an orange pigment (3E5), some were smaller (8D9-1) or bigger in colony size (16F6) and others had different types of colony border/fringes (5G3 and 9D9).

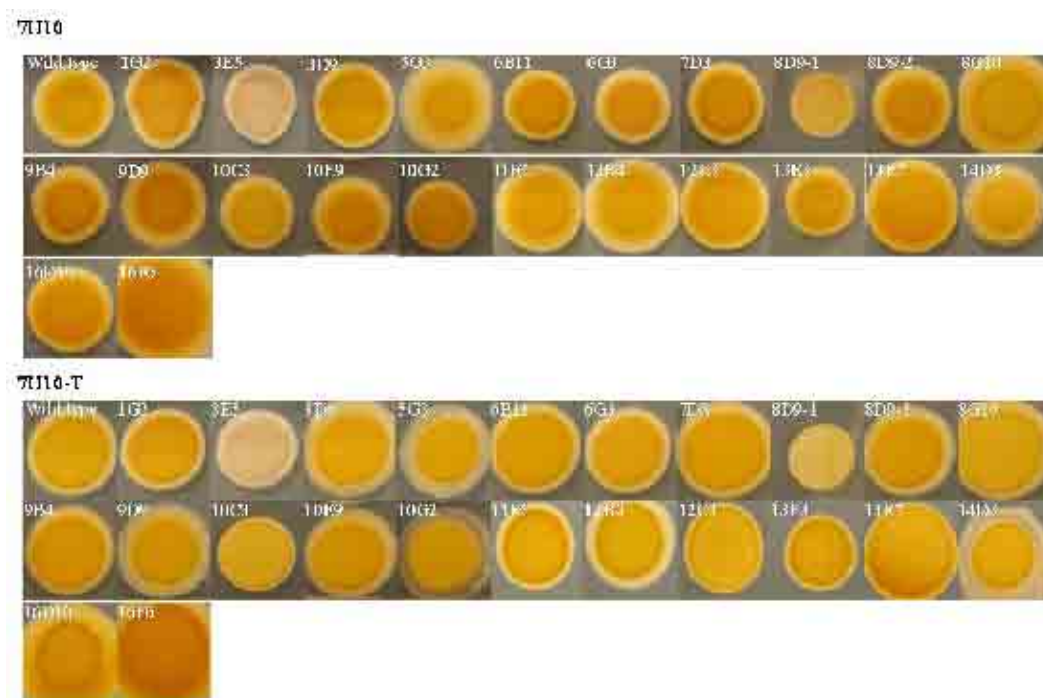


Fig. 2.2 Colony morphology of *M. marinum* *Tn*-mutants on 7H10 and 7H10 T agar. 7H10 T: 7H10 with 0.05% Tween 80

2.3.4 Identification of the *Tn* disrupted genes in *M. marinum* mutants with altered colony morphology

Using the same strategy that was used for *M. smegmatis*, the *Tn* insertions in the *M. marinum* mutants were isolated in *E. coli* as plasmids and sequenced. The genes disrupted in the twenty *M. marinum* *Tn*-mutants with colony morphology changes are listed in Table 2.3. Amongst the 20 genes, five belonged to the cell wall and cell processes category, with only one with known function (*MMAR2913*, *betP*, in mutant 10E9, is a glycine betaine transporter). Amongst the others, one was involved in intermediary metabolism and respiration category and seven in lipid metabolism. For example *mmaA3* in mutant 8G10 is involved in mycolic acid synthesis, whilst *ppsB* and *ppsC* in mutants 6G3 and 16F6 are involved in PGL and PDIM biosynthesis. The remaining disruptions were a putative regulator, a virulence/detoxification/adaptation

related gene and five conserved hypotheticals.

Table 2.3 Genes disrupted by *Tn* insertion in *M. marinum* colony morphology mutants

Mutant No.	Gene No.	Gene Name	Homologue in <i>M. tuberculosis</i> H37Rv	Function
1G2	<i>MMAR1767</i>	Multifunctional mycocerosic acid synthase membrane-associated Mas	<i>Rv2940c(mas)</i> : probable multifunctional mycocerosic acid synthase membrane-associated Mas	Catalyzes the elongation of N-fatty acyl-CoA with methylmalonyl-CoA to form mycocerosyl lipids
3E5	<i>MMAR4806</i>	Phytoene dehydrogenase CrtI (lipid metabolism)	<i>Rv3829c</i> : probable dehydrogenase	Function unknown: Probably involved in cellular metabolism
6B11	<i>MMAR5449</i>	EsaT-6 like protein, EsxB	<i>Rv3874(esxB)</i> : 10 kDa culture filtrate antigen EsxB (LHP) (CFP10)	Function unknown
6G3	<i>MMAR1775</i>	Phenolphthiocerol synthesis type-I polyketide synthase PpsB	<i>Rv2932(ppsB)</i> : phenolphthiocerol synthesis type-I polyketide synthase PpsB	Involved in phenolphthiocerol and phthiocerol dimycocerosate (DIM) biosynthesis
7D3	<i>MMAR5440</i>	Hypothetical protein	<i>Rv3865</i> : conserved hypothetical protein	Function unknown.
8D9-1	<i>MMAR4214</i>	Serine protease HtrA (DegP protein)	<i>Rv1223(htrA)</i> : probable serine protease HtrA (DegP protein)	Possibly hydrolyzes peptides and/or proteins
8D9-2	<i>MMAR1180</i>	Fatty acyl-AMP ligase FadD28_1 and polyketide synthase	<i>Rv1662(pks8)</i> : probable polyketide synthase Pks8	Potentially involved in some intermediate steps for the synthesis of a polyketide molecule
8G10	Promoter of <i>MMAR0978</i>	Methoxymycolic acid synthase 3	<i>Rv0643c(mmaA3)</i> : methoxymycolic acid synthase 3 MMAA3	Involved in mycolic acid modification
9B4	<i>MMAR5457</i>	ESX-1 secreted protein, EspB	<i>Rv3881c</i> : conserved hypothetical alanine and glycine rich protein	Function unknown.
9D9	<i>MMAR2327</i>	Hypothetical protein	<i>Rv1508c</i> : probable membrane protein	Function unknown.
10C3	<i>MMAR5457</i>	ESX-1 secreted protein, EspB	<i>Rv3881c</i> : conserved hypothetical alanine and glycine rich protein	Function unknown.
10E9	<i>MMAR2913</i>	Glycine betaine transport integral membrane protein BetP	<i>Rv0917(betP)</i> : possible glycine betaine transport integral membrane protein BetP	High-affinity uptake of glycine betaine
10G2	<i>MMAR2341</i>	Fatty acyl-AMP ligase FadD25	<i>Rv1521(fadD25)</i> : probable fatty-acid-CoA ligase FadD25	Involved in lipid degradation.
11F5	promotor region of <i>MMAR3235</i>	Hypothetical protein	<i>Rv2191</i> : conserved hypothetical protein	Function unknown.

12B4	<i>MMAR0992</i>	Transcriptional regulatory protein	<i>Rv0653c</i> : possible transcriptional regulatory protein (probably TetR-family)	Involved in transcription mechanism.
12C3	<i>MMAR5092</i>	Lipoprotein LpqF	<i>Rv3593(lpqF)</i> : probable conserved lipoprotein, LpqF	Function unknown.
13E3	<i>MMAR3223</i>	Hypothetical protein	<i>Rv2179c</i> : conserved hypothetical protein	Function unknown.
14D3	<i>MMAR2352</i>	Hypothetical protein	<i>Rv1517</i> : conserved hypothetical transmembrane protein	Function unknown.
16D10	<i>MMAR3250</i>	Transmembrane protein	<i>Rv2206</i> : probable conserved transmembrane protein	Function unknown.
16F2	<i>MMAR1774</i>	Phenolphthiocerol synthesis type-I polyketide synthase PpsC	<i>Rv2933(ppsC)</i> : phenolphthiocerol synthesis type-I polyketide synthase PpsC	Involved in phenolphthiocerol and phthiocerol dimycocerosate biosynthesis

2.4 Discussion

According to our hypothesis, inactivation of genes related directly to cell wall components, for instance, membrane proteins or lipids, would lead to colony morphology changes. In the twenty-eight genes identified from both *M. smegmatis* and *M. marinum* morphology mutants this was indeed the case; fifteen were annotated genes involved in cell wall or cell wall-lipid metabolism. However, six were in regulator genes or genes involved in intermediary metabolism and it was likely that alterations in the colony morphology were indirect effects.

In *M. smegmatis*, eight mutants with colony morphology changes were isolated from the *Tn*-mutant library by using different agar media, either with or without Tween 80. Amongst these mutants, two had insertions in a gene cluster associated with cell division genes. The mutants 1G2 and 2H11 had insertions in *pbpA* (*MSMEG0031*) and *fhaA* (*MSMEG0035*). The former is a penicillin binding protein and a previous report of a *M. smegmatis* *pbpA* mutant showed that cells of the

knockout strain are longer in shape (Dasgupta *et al.*, 2006). The *fhaA* gene on the other hand encodes a forkhead-associated (FHA) domain protein. The FHA domain can recognise phosphothreonine epitopes on proteins which have been found in prokaryotic and eukaryotic organisms (Durocher & Jackson, 2002); the phosphorylation of proteins is critical for signal transduction. Interestingly, the protein kinase *pknB* is located in the same operon (Fernandez *et al.*, 2006). Thus, the *fhaA* domain protein could be associated with a signalling process in cell division, perhaps forming a scaffold for *pbpA* and other proteins. Although both *pbpA* and *fhaA* mutants had morphology changes, there were differences between them which indicated different functions had been inactivated. Thus, the observed morphology changes were likely due to altered cell division patterns. Another mutant with altered colony morphology was GntR (*MSMEG0268*), a regulatory protein. This gene was named after the *Bacillus subtilis* transcription regulator, a repressor of the gluconate operon (Haydon & Guest, 1991). According to sequence analysis, GntR (*MSMEG0268*) belongs to the HutC-like regulator sub-family and this sub-family had been reported to respond to a number of molecules including long chain fatty acids and trehalose 6-phosphate (Allison & Phillips, 1990; Matthijs *et al.*, 2000; Quail *et al.*, 1994; Rao *et al.*, 2006). Interestingly, the latter is a putative intermediate in TDM biosynthesis.

Two disruptions isolated were in genes involved in metabolism, *MSMEG1654* (isocitrate dehydrogenase, *icd*) and *MSMEG6756* (glycerol kinase, *glpK*). Isocitrate dehydrogenase is an enzyme of the tricarboxylic acid cycle (TCA cycle). It is highly likely that these metabolic pathways indirectly affect the biosynthetic pathways that

contribute to cell wall components. In addition to the above categories of genes, mutants having *Tn* insertions in an ABC transporter (*MSMEG5102*) and two genes with unknown function (*MSMEG4256* and *MSMEG3327*) were identified.

As for the *M. marinum* colony morphology mutants, we identified twenty genes with *Tn* insertions (Table 2.3). Amongst the five genes identified involved in cell wall and cell process, *MMAR2327* (9D9) is a hypothetical protein with a homologue of a probable membrane glycosyltransferase, which is non-essential in *M. tuberculosis* (Lamichhane *et al.*, 2003). *MMAR2913* (10E9) is a glycine betaine transport integral membrane protein, in *M. tuberculosis* starvation model, the transcription of its homologue has been found to be up-regulated (Betts *et al.*, 2002). *MMAR5092* (12C3) is annotated as a lipoprotein, LpqF, its homologue in *M. tuberculosis* is identified as essential for growth but with an unknown function (Sasseti *et al.*, 2003). *MMAR2352* (14D3) is a hypothetical protein with unknown function. *MMAR325* (16D10) is a transmembrane protein, its homologue in *M. tuberculosis* has been identified to be required for survival in murine macrophages using *Tn* mutagenesis (Rengarajan *et al.*, 2005).

Amongst the six genes identified involved in lipid metabolism, *MMAR1767* (1G2), membrane associated mycocerosic acid synthase (*mas*), *MMAR1775* (6G3), phenolphthiocerol synthesis type-I polyketide synthase (*ppsB*) and *MMAR1774* (16F2), phenolphthiocerol synthesis type-I polyketide synthase (*ppsC*), are all involved in biosynthesis of PDIMs, a cell wall lipid (Azad *et al.*, 1997; Kruh *et al.*, 2008; Mathur

& Kolattukudy, 1992). *MMAR1180* (8D9-2) is annotated as a fatty acyl-AMP ligase *FadD28_1*, while its homologue in *M. tuberculosis* is polyketide synthase *pks8* (*Rv1662*), which has been reported together with *pks17* (*Rv1663*) to be responsible for the production of monomethyl branched unsaturated fatty acids (Dubey *et al.*, 2003); *MMAR0978* (8G10, in promoter region) is methoxymycolic acid synthase 3, responsible for transferring a methyl group from *S*-adenosyl methionine (SAM) to form methoxymycolic acids, one of the three sub-groups of mycolic acids (Yuan & Barry, 1996); *MMAR2341* (10G2) is annotated as fatty acyl-AMP ligase (*fadD25*), although the exact function is unknown, its homologue in *M. tuberculosis* has been suggested to be involved in lipid degradation (Betts *et al.*, 2002).

Amongst the two genes identified involved in intermediary metabolism and respiration, *MMAR4806* (3E5) is a phytoene dehydrogenase (*crtI*) involved in carotene biosynthesis, which is a group of coloured terpenoids providing protective functions against photo-oxidative damage (Runyon, 1959). As shown in Fig. 2.2, mutant 3E5 lost the orange colour compared with the wild type strain, which is consistent with these observations. *MMAR4214* (8D9-1) is serine protease (*htrA* or *degP*), whose homologue in *M. tuberculosis* has been predicted to be essential for pathogenicity (Ribeiro-Guimaraes & Pessolani, 2007).

Among the remaining seven genes, one (*MMAR5449*, 6B11) is an Esat-6-like protein involved in virulence (Gao *et al.*, 2004) and *MMAR0992* (12B4) is a regulator gene with the remaining five conserved hypothetical proteins with unknown functions.

One mutant from each species was then selected for further studies as part of this thesis work.

While the *pbp* cluster related mutants in *M. smegmatis* looked the most promising, these were not pursued any further due to the existence of already published literature on this topic (Dasgupta *et al.*, 2006; Fernandez *et al.*, 2006). Instead, the 3D9 (*icd*, *MSMEG1654*) mutant was chosen for further analysis for two reasons. Firstly, isocitrate dehydrogenase is one of the key enzymes in the central metabolism of the citric acid cycle (TCA cycle) which controls the isocitrate flux between the TCA cycle and glyoxylate bypass (LaPorte, 1993; Zheng & Jia, 2010). The first enzyme in glyoxylate shunt pathway in *M. tuberculosis*, isocitrate lyase (*icl*), has been reported to be involved in virulence (Munoz-Elias & McKinney, 2005). Secondly, in non-pathogenic mycobacteria, such as *M. smegmatis*, only one *icd* presents in their genomes, whilst *M. tuberculosis* and some other pathogenic mycobacteria have two copies. Phylogenetic analysis shows that in *M. tuberculosis* one is similar to prokaryotic *icds*, and the other to eukaryotic versions, indicating different function roles possibly related to virulence or intracellular survival.

From the set of *M. marinum* mutants, the 8G10 (*mmaA3*, *MMAR0978*) mutant was also chosen for further study as it is the homologue of *mmaA3* for *M. tuberculosis* and potentially involved in the biosynthesis of methoxymycolic acids. This provided an opportunity to study the role of methoxymycolic acids in virulence.

Chapter 3

Use of Mycobacteriophage Resistant Strains to Identify Cell Wall Biosynthesis Pathways in Mycobacteria

3.1 Introduction

Bacteriophages have played an important role in the development of bacterial genetics and their utility as genetic tools for cloning and manipulation of genes is one of the most significant (Fitzgerald & Gasson, 1988; Kurzepa *et al.*, 2009; Maloy & Gardner, 2007). Indeed in mycobacteria, phages have made an enormous contribution towards developing genetic tools for the manipulation of mycobacteria (Bardarov *et al.*, 1997; Jacobs *et al.*, 1989b; van Kessel *et al.*, 2008). Attachment of a phage by binding to its receptor on the mycobacterial cell surface is important for initiating infection, and the study of phage-resistant mutants defective in phage adsorption is a useful approach to identifying these receptors. Phage receptors would be expected to be the outer components of the bacterial cell wall, and loss of this component would lead to phage resistance. Thus phage-resistant mutants would also be an extremely useful resource for the identification of biosynthetic pathways of mycobacterial cell wall components. Indeed, studies of interactions of bacteriophages with host cells have often led to the discovery of bacterial cell surface receptors and transporters that play important roles in microbial physiology. In *E. coli*, many membrane proteins and transporters were initially identified in phage resistant mutants (Heller, 1992), such as the maltose receptor (LamB) as the receptor for phage λ (Szmelcman & Hofnung, 1975), the F-pilus for phage f1, the outer membrane ferrichrome (FhuA, formerly TonA) for phages T5, T1, and Φ 80 (Braun, 1995) and vitamin B12 transporter (BtuB) for phage BF23 (Bradbeer *et al.*, 1976). In other bacteria species, phage resistant

mutants have been used to identify cell wall components like polysaccharides and glycolipids, for instance the modified O-antigen lipopolysaccharides in *Shigella* (Verma *et al.*, 1991), galactosamine-containing teichoic acids in *Bacillus subtilis* (Estrela *et al.*, 1991) and *N*-acetylglucosamines of *Listeria monocytogenes* (Tran *et al.*, 1999).

More than 250 mycobacteriophages have been identified since the 1950s (Hatfull, 1994), some of them can infect a broad range of mycobacteria, as D29 which can infect fast-growing as well as slow-growing mycobacteria, and some of them have a limited range of hosts, as DS6A which can only infect the *M. tuberculosis* complex. Several mycobacteriophages have been well characterised and genetically modified for application in clinical diagnosis and research. For example, phage D29 was first isolated in 1954 (Froman *et al.*, 1954) and its host includes *M. smegmatis* and *M. tuberculosis*. D29 is a lytic phage which kills the host cells when releasing progeny particles and forms clear plaques on the lawn of its host strain. It has been used as a typical model for understanding of mycobacteriophage infection process (David *et al.*, 1984a; David *et al.*, 1984b; Jones & David, 1971). A D29 luciferase phage has been constructed for detecting mycobacteria (Ford *et al.*, 1998). The genome of D29 is the second mycobacteriophage genome that has been sequenced after the temperate mycobacteriophage L5 (Ford *et al.*, 1998). Comparative studies of both genomes revealed that D29 is a close relative to L5 but with a large deletion. These results can give us information of the biology, as well as the evolution of this group of mycobacteriophages. Phage TM4 is different from L5/D29. It was initially isolated

from a *M. avium* culture following mitomycin C treatment (Timme & Brennan, 1984). Although, TM4 can lyse *M. smegmatis* cells, the plaques it forms are different from those of lytic phages or temperate phages (Hatfull, 1994). TM4 has been used to construct the first mycobacterial phasmid which can grow as a plasmid in *E. coli* and as phage in mycobacteria (Jacobs *et al.*, 1987), which has been widely used as a foreign DNA delivery system. Phage I3 is a temperate phage belonging to the *Myoviridae* family. It is also one of only two mycobacteriophages exhibiting generalised transduction and has been used as a genetic tool for transducing auxotrophic markers, as well as drug resistance markers (Lee *et al.*, 2004; Raj & Ramakrishnan, 1970; Saroja & Gopinathan, 1973). However, I3 can only infect fast-growing mycobacteria but not slow-growing mycobacteria, such as *M. bovis* BCG and *M. tuberculosis* which restricts its uses as a genetic tool. A better understanding of the mechanism of its generalised transduction will be helpful for general phage biology, as well as developing new genetic tools. Recently, several new mycobacteriophages have been isolated, such as another generalised transducing phage BxZ1 (Lee *et al.*, 2004) which forms clear plaques on *M. smegmatis* compared to the turbid plaques of phage I3.

Despite a vast amount of work on mycobacteriophages, relatively little is known about mycobacteriophage receptors. Whilst, biochemical characterisation of cell extracts and phage adsorption experiments have shown that surface glycolipids of *M. phlei* are involved in the adsorption of mycobacteriophages D4 and D29 (Bisso *et al.*, 1976), resistance of *M. smegmatis* to phage D29 results in the emergence of new

pyruvylated, glycosylated acyltrehaloses (Besra *et al.*, 1994), C-mycosides (PGL) and glycopeptidolipids (GPLs) are important for phage D4 adsorption (Dhariwal *et al.*, 1986), there have been no previous reports of a phage resistant mutant that is missing a cell wall component. There is only one report pertaining to a genetic basis for phage resistance which demonstrated that the overexpression of a multicopy phage resistant (*mpr*) gene in *M. smegmatis* led to resistance to phage D29 and L5 (Barsom & Hatfull, 1996). Another study described the appearance of a new cell wall lipid species in a *M. smegmatis* strain spontaneously resistant to phage D29, rather than the disappearance of an existing cell wall component (Besra *et al.*, 1994).

As a case in study for demonstrating the utility of mycobacteriophage resistant mutants, the generalised transducing phage I3 was selected for screening an *M. smegmatis* transposon library for phage resistant mutants. This chapter describes the isolation and characterisation of phage I3 resistant *M. smegmatis* *Tn*-mutants, studies which were further extended to identify the minimal receptor for phage I3. Therefore, phage resistance was used as one of the screening strategies to study mycobacterial cell wall biosynthetic pathways.

Additionally, a parallel line of studying examining *mpr* (multicopy phage resistant, *MSMEG1236*) was explored. *Mpr* was previously shown to confer an increased resistance to mycobacteriophage D29 and L5 when overexpressed in wild type *M. smegmatis* (Barsom & Hatfull, 1996). Phage D29 and L5 are two closely related mycobacteriophages that share similar mechanisms in infecting *M. smegmatis*

(Ford *et al.*, 1998). Colony morphology changes were also observed in the recombinant strain and studies showed that the D29/L5 resistance may be caused by the inhibition of phage DNA injection after *mpr* overexpression. These combined results suggest that the function of the unknown *mpr* gene was possibly involved in modifying the cell wall. These early studies were followed by an analysis of the cell wall of a Δmpr mutant and strains overexpressing *mpr* using an inducible vector.

3.2 Materials and Methods

3.2.1 Bacterial strains, plasmids, phages and growth conditions

Bacterial strains, mycobacteriophages and plasmids used in this study are listed in Table 3.1. *M. smegmatis* strains were grown at 37°C on Middlebrook 7H10 agar (Difco) and in Middlebrook 7H9 broth (Difco), supplemented with 0.2% glycerol and 0.05% Tween 80. *M. smegmatis* was also grown on TSB (Difco) agar, in TSB broth supplemented with 0.05% Tween 80 and Sauton medium (for 1L, K₂HPO₄ 0.5g, Mg₂SO₄·7H₂O 0.5g, citric acid 2.0g, ferric ammonium citrate, 0.05g, glycerol 60ml, L-asparagine 4.0g, water to 1L) supplemented with 0.05% Tween 80. For phage infection experiments Tween 80 was omitted in all media. The antibiotics kanamycin and hygromycin B were added at concentrations of 25µg/ml and 100µg/ml for selection of *M. smegmatis* mc²155. Acetamide was added at a concentration of 0.2% for *M. smegmatis* transformants using the plasmid pSD26. *E. coli* was routinely grown in LB medium at 37°C. Mycobacteriophages were propagated on Middlebrook

7H9 with 0.2% glycerol and plaque forming units (pfu) of each phage and its multiplicity of infection (MOI) were estimated as described (Sarkis & Hatfull, 1998).

Table 3.1 Plasmids, phages and bacterial strains used in this study

Plasmids, phages and strains	Description	Source or Reference
Plasmids		
pMV261	<i>E.coli</i> -mycobacterial shuttle plasmid, Kan ^R	(Stover <i>et al.</i> , 1991)
pMV <i>gtf2</i>	<i>gtf2</i> cloned in pMV261, Kan ^R	(Miyamoto <i>et al.</i> , 2006)
pSD26	<i>E.coli</i> -mycobacterial shuttle plasmid with acetamidase promoter, Kan ^R	(Parish & Brown, 2008)
pSD26MSMEG1236	<i>mpr</i> (MSMEG1236) cloned in pSD26, Kan ^R	This study
Phages		
phAE185	Temperature sensitive derivative of mycobacteriophage TM4 containing the <i>IS1096</i> -derived transposon <i>Tn5370</i>	(McAdam <i>et al.</i> , 2002)
phAE181	Temperature sensitive derivative of mycobacteriophage TM4 containing the Himar1-derived transposon <i>Tn5371</i>	(Kriakov <i>et al.</i> , 2003)
I3	Generalized transducing mycobacteriophage	(Raj & Ramakrishnan, 1970)
D29	Lytic mycobacteriophage	(Froman <i>et al.</i> , 1954)
BXZ1	Generalized transducing mycobacteriophage	(Lee <i>et al.</i> , 2004)
Bacteria		
<i>E. coli</i> cc118λpir		
<i>M. smegmatis</i> mc ² 155	Parental (wile type strain) strain, Ept mutant	(Snapper <i>et al.</i> , 1990)
<i>M. smegmatis</i> Δ <i>gtf1</i>	<i>gtf1</i> disruptant of mc ² 155	(Miyamoto <i>et al.</i> , 2006)
<i>M. smegmatis</i> Δ <i>gtf2</i>	<i>gtf2</i> disruptant of mc ² 155	(Miyamoto <i>et al.</i> , 2006)
<i>M. smegmatis</i> Δ <i>gtf3</i>	<i>gtf3</i> disruptant of mc ² 155	(Miyamoto <i>et al.</i> , 2006)
<i>M. smegmatis</i> Δ <i>gtf2</i> /pMV <i>gtf2</i>	Δ <i>gtf2</i> complemented with a <i>gtf2</i> -containing plasmid	(Miyamoto <i>et al.</i> , 2006)
<i>M. smegmatis</i> Δ <i>mpr</i>	<i>mpr</i> disreptant of mc ² 155, Hyg ^R	This study
<i>M. smegmatis</i> mc ² 155/pSD26	Wild type strain with pSD26, Kan ^R	This study
<i>M. smegmatis</i> mc ² 155/pSD26MSMEG1236	Wile type strain with <i>mpr</i> overexpressed, Kan ^R	This study

3.2.2 Isolation of mycobacteriophage resistant mutants

Individual *M. smegmatis* clones from the *Tn*-mutant library were spotted with 96-spike replicator onto plates with TSB agar, containing 10^{10} pfu *per* plate of mycobacteriophage I3. All phage resistant candidates were purified for single colonies and retested for resistance to phage I3 by a phage sensitivity test.

3.2.3 Phage sensitivity test

M. smegmatis mc²155 and I3 phage mutants were grown to an OD₆₀₀ of 1 and 100µl of fresh culture of each strain was mixed with molten 7H9 soft agar (0.6%) and overlaid onto a basal 7H9 agar plate. A high titre phage lysate was ten-fold serially diluted to about 100pfu/ml. On each plate, 10µl of each phage lysate dilution was spotted on the plates overlaid with *M. smegmatis* cells. Plates were incubated at 37°C for 1-2 days to visualise plaques. D29 and BxZ1 phages were used as controls in this study.

3.2.4 Biochemical characterisation of GPLs

Polar lipids were extracted as described in Chapter 7 and then subjected to alkali treatment to remove acetyl/acyl substituents as described previously (Burguiere *et al.*, 2005a). The dried polar lipids were resuspended in 4 ml of CHCl₃/CH₃OH/0.8M NaCl (10:10:3, v/v/v) and incubated at 55°C for 15 min. The mixture was allowed to cool at room temperature. After CHCl₃ (1.75ml) and H₂O (0.25ml) were added, the mixture was mixed on rotary for 10 min and centrifuged. The lower organic layer was washed

twice with 2ml of CHCl₃/CH₃OH/H₂O (3:47:48, v/v/v). The lower organic layer, containing base stable polar lipid, was dried and resuspended in 200µl of CHCl₃/CH₃OH (2:1, v/v); 5µl was dried in a scintillation vial and then mixed with 5ml of scintillation fluid and had its radioactivity measured. Equivalent amounts (10,000 cpm) of each sample were spotted on TLC plates (5735 silica gel 60F524; Merck) for further one dimensional (1D-) TLC in CHCl₃/CH₃OH/H₂O (90:10:1, v/v/v) as described (Burguiere *et al.*, 2005a). TLC results were visualised by exposing to Kodak X-Omat film overnight.

3.2.5 Phage adsorption assay

Wild type and mutant strains of *M. smegmatis* were grown to OD₆₀₀ of 1. Cells were washed with MP buffer and were infected with I3 phage with MOI of 1 for 1 hour. After infection, cells were treated with DNaseI to digest extracellular phage DNA and washed four times with PBS with 0.1% Tween 80. Subsequently, total DNA was extracted from cells. The presence of injected I3 phage DNA in *M. smegmatis* cells was detected by PCR using primer specific for I3 phage structural protein (gi|15097|emb|X68296.1, primer forward 5'GTACAACCCGCCAACCCAC 3' and primer reverse 5'CAGGCGGACGAGATAGGTG 3') performed with 10µg of total DNA extracted from cells as template.

3.2.6 Construction of phasmid for knocking out *mpr* in *M. smegmatis*

Left and right flank sequences of *mpr* (*MSMEG1236*) were amplified by PCR using primers listed in Table 3.2. PCR products were purified (QIAquick PCR Purification Kit, QIAGEN) and digested with *Van9II* (NEB). Digested left and right flank fragments were ligated with P0004s *Van9II*-digested fragments. The ligation mixture was then transformed into *E. coli* Top10 and selected onto LB agar with hygromycin at 37°C overnight. Plasmids recovered from *E. coli* Top10 were digested by *PacI* and ligated with *PacI*-digested phAE159 DNA after confirmation by sequencing. Ligated knock-out plasmid and phAE159 DNA were packaged into λ phage heads and transduced into *E. coli* HB101 and selected on LB agar with hygromycin at 37°C overnight.

Table 3.2 Primers used for knocking out *mpr* in *M. smegmatis*

Name	Sequences (5'->3')	Product	Size
MSMEG1236_LL	<u>TTTTTTTTCCATAAAATTGG</u> CATCCC GCCCTCCCGTCG *	left side flank	961
MSMEG1236_LR	<u>TTTTTTTTCCATTICTTGG</u> CCGGGGTGTGATTGTTGC	sequence of <i>mpr</i>	bp
MSMEG1236_RL	<u>TTTTTTTTCCATAGATTGG</u> CAGCCC GTCGAATCAGAAG	right side flank	1051
MSMEG1236_RR	<u>TTTTTTTTCCATCTTTTGG</u> CGGGCTGCTCGAGTCGC	sequence of <i>mpr</i>	bp

*Sequence underlined represents *Van9II* enzyme site.

3.2.7 Preparation of recombinant deletion of *mpr* in *M. smegmatis*

Cosmids recovered from *E. coli* HB101 were then transformed into *M. smegmatis* by electroporation after being confirmed and *M. smegmatis* cells were recovered in TSB at 30°C for 3-4 hours. Cells were then harvested and mixed with 200 μ l of fresh

late-log phase *M. smegmatis* culture and 4 ml of molten 7H9 soft agar. Cells were overlaid onto basal 7H9 agar plates and incubated at 30°C for 2-3 days to form plaques. Phage plaques were patched for making high titre phage lysate for future transductions.

3.2.8 Phage transduction

M. smegmatis cultures were grown in 50ml of TSB (0.05% Tween 80) to an OD_{600nm} of 0.8. Cells were harvested by centrifugation at room temperature and washed with 50 ml MP buffer. Cells were resuspended in 5 ml MP buffer and mixed with high titre phage lysate (10^{10} to 10^{11} pfu/ml) at an MOI of 10, while 0.5ml cells were mixed with 0.5ml MP buffer as control. After overnight incubation at 37°C, cells were harvested and resuspended in 25ml TSB with 0.05% Tween 80. Cells were recovered at 37°C for 24 hours before being plated onto TSB agar plates with hygromycin B. Plates were incubated at 37°C for 1-2 weeks. Colonies were inoculated at 10ml of TSB (0.05% Tween 80) with hygromycin B for further study.

3.2.9 Southern Blot

SacI digested genomic DNA from *M. smegmatis* wild type and Δmpr mutant strains were applied to gel electrophoresis to separate DNA fragments by size. In the mutant, the *mpr* gene was replaced with a *hyg^R* gene. There were four *SacI* cutting sites in *hyg^R* while none in *mpr*. PCR products of left and right flank sequences of *mpr* (*MSMEG1236*) were used as probes. Thus, after *SacI* digestion, there would be only

one fragment (~4.5kb) from wild type genomic DNA that can be detected, while two (2.2kb and 1.8kb) from genuine Δmpr mutants. The agarose gel was then applied to depurination (0.2M HCl, 15 min), denaturation (1.5M NaCl, 0.5M NaOH, 15 min) and neutralisation (0.5M Tris-HCl pH7.2, 1M NaCl, 15 min). Then DNA was transferred onto nylon membrane (positively charged) by capillary transfer with 20×SSC (3M NaCl, 0.3M sodium citrate, pH7.0). DNA on membrane was fixed by UV-crosslinking. The membrane was rinsed with distilled water and air-dried.

DIG High Prime DNA Labeling and Detection Start Kit II (Roche) was used for probe labeling, hybridisation and detection. The procedure was performed as described in the instruction manual. Briefly, digoxigenin (DIG), a steroid hapten, was used to label DNA probes according to the random primed labeling technique. The hybridized probes were immunodetected with anti-digoxigenin-AP, Fab fragments and were then visualized with the chemiluminescence.

3.3 Results

3.3.1 Isolation of phageI3-resistant *M. smegmatis* *Tn* mutants

Based on the hypothesis that changes in cell wall components can lead to changes in susceptibility of mycobacteriophage infection, *M. smegmatis* *Tn*-mutants were spotted onto phage I3 seeded agar plates to select phage-resistant mutants. Two *Tn*-mutant libraries were used for screening, one generated with *HimarI*-derived *Tn5371* and other with *IS1096*-derived *Tn5370* (Cirillo *et al.*, 1991; McAdam *et al.*,

2002). Altogether four I3-resistant mutants were identified, three from the *Tn5371* library and one from the *Tn5370* library. The I3-resistance of the mutants was confirmed twice after single colony purification. As shown in Fig. 3.1, compared with the wild type strain the mutants were resistant to I3 phage.

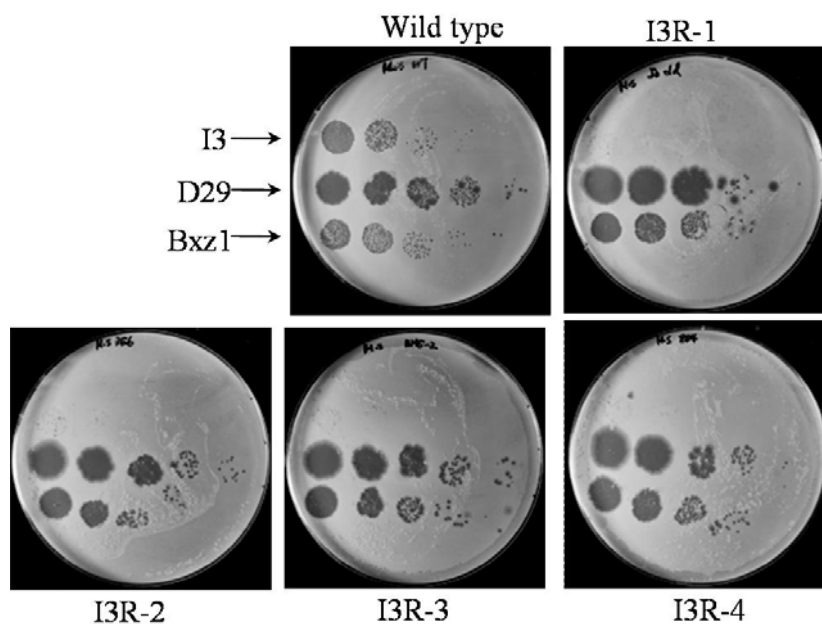


Fig. 3.1 Sensitivity of *M. smegmatis* GPL-associated glycosyl transferase mutants to phages I3, D29 or BxZ1. Ten fold dilutions of phages ($10\mu\text{l}$, 10^{-2} to 10^{-6}) spotted from left to right on soft agar overlays on 7H9-agar plates containing either wild type strain or I3-resistant mutants

3.3.2 Phage I3 fails to inject DNA into I3-resistant *M. smegmatis* *Tn*-mutants

In order to differentiate whether the resistance was caused by failure to initiate infection or due to a later replication stage specific defect, an assay was performed to test if phage DNA could be detected in the cells following phage adsorption. The results showed that intracellular phage I3 genomic DNA could be detected by PCR from *M. smegmatis* mc²155 cells pre-adsorbed with phage I3, but not from phage resistant mutants indicating that the phage failed to inject DNA into the phage

resistant mutants (Fig. 3.2, lane 4).

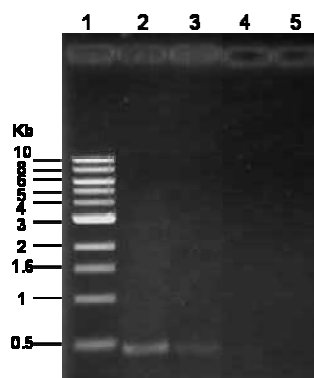


Fig. 3.2 PCR amplification of part (461 bp) of a phage I3 gene encoding a 17kD structural protein. Lane 1, DNA size markers; lane 2, PCR positive control using phage I3 DNA as template; lanes 3, 4 and 5, PCR reaction using total DNA extracted from *M. smegmatis* wild type strain, I3R-1 and *gtf2* respectively, each of which was pre-adsorbed with phage I3.

3.3.3 Identification of disrupted genes in phage I3-resistant *M. smegmatis*

Tn-mutant

Using the *Tn*-insertion rescue method described in chapter 2, genes disrupted in I3-resistant *M. smegmatis* *Tn*-mutants were identified. As shown in Table 3.3, all four phage I3-resistant mutants had a *Tn* insertion in genes associated with a cluster involved in the biosynthesis of GPLs, the outermost ‘free’ lipids found in *M. smegmatis*. Glycosyl transferase (*gtf2*, MSMEG0392) is responsible of transferring *O*-methyl-rhamnose onto the fatty acyl-tetrapeptide core (FATP) core, the inactivation of GTF2 leads to the accumulation of fatty acyl-tetrapeptide core (FATP) core with

6-deoxy-talose-FATP (Miyamoto *et al.*, 2006). *MSMEG0400* is a hypothetical protein with a conserved MbtH-like domain, although its function is not clear, it has been reported that this MbtH-like protein is found in almost all non-ribosomal peptide synthetase (NRPS) biosynthetic gene clusters (Stegmann *et al.*, 2006). *MSMEG0400* (*mps1*) and *MSMEG0401* (*mps2*) are responsible of synthesis of the dipeptide and the amino acid alcohol in FATP core, respectively (Ripoll *et al.*, 2007).

Table 3.3 Genes disrupted in phage I3-resistant *M. smegmatis* *Tn*-mutants

Mutant	Disrupted gene	Function
I3R-1	<i>MSMEG0392</i>	Glycosyl transferase (<i>gtf2</i>)
I3R-2	<i>MSMEG0399</i>	MbtH-like protein
I3R-3	<i>MSMEG0400</i>	Non-ribosomal peptide synthase (<i>mps1</i>)
I3R-4	<i>MSMEG0401</i>	Non-ribosomal peptide synthase (<i>mps2</i>)

3.3.4 Phage I3-resistant *M. smegmatis* mutant strains are defective in GPL biosynthesis

As mentioned above, all I3-resistant *Tn*-mutants had insertions in genes previously identified to play a role in GPL biosynthesis. GPLs are a group of complex lipids abundant in the outer envelope of *M. smegmatis* and other mycobacteria (Billman-Jacobe, 2004; Schorey & Sweet, 2008). *M. smegmatis* produces six sub-classes of GPLs each of which has a common fatty acyl-tetrapeptide (FATP) core consisting of a tetrapeptide amino alcohol (*D*-Phe-*D*-allo-Thr-*D*-Ala-*L*-alaninol) linked to a C26–C34 fatty acyl chain *via* an amide bond (Miyamoto *et al.*, 2006) (Fig. 3.3).

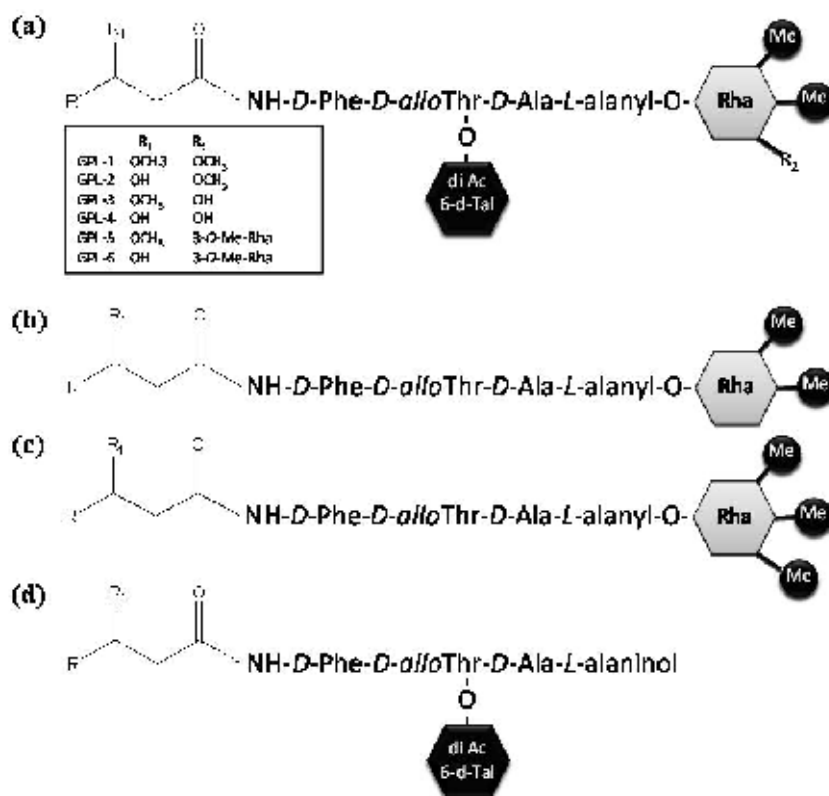


Fig. 3.3 Simplified representations of the structures of GPLs found in *M. smegmatis*. (a) GPLs in wild type strain, (b, c) biosynthetic intermediates that accumulate in Δ *gtf1*, (d) biosynthetic intermediates that accumulate in Δ *gtf2*. Rha, rhamnose; di-Ac 6-d-Tal, di-acetylated 6-deoxy talose; Me; methyl; Variations in the fatty acyl chain (R) are not shown.

GPL-1, 2, 3 and 4 all contain a rhamnose (with varying levels of *O*-methylation) and 6-deoxy-talose as sugar residues (Miyamoto *et al.*, 2006). GPL-5 and GPL-6 contain an additional rhamnose or *O*-methyl rhamnose attached to the original rhamnose residue. Given the association of the *Tn* insertion in the phage I3-resistant mutants with genes involved in GPL biosynthesis, it was likely that the mutants were affected in GPL biosynthesis. However, to have a comprehensive picture of lipid biosynthesis in the mutants, a complete analysis of polar and non-polar lipids from the I3-resistant mutants was performed using 2D-TLC (Dobson *et al.*, 1985). The analysis

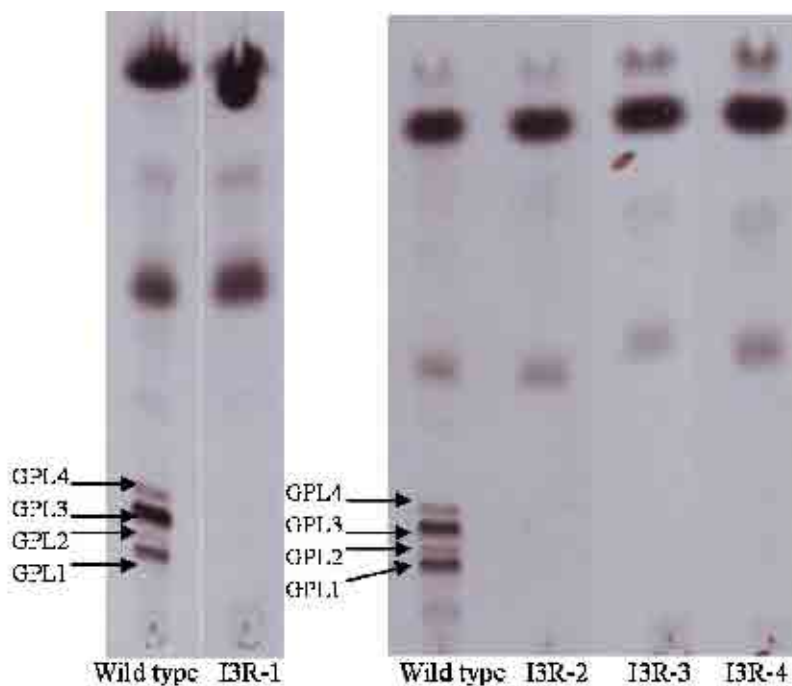


Fig. 3.5 TLC analysis of deacetylated GPLs of I3-resistant *M. smegmatis* *Tn*-mutants. Deacetylated polar lipids were separated using the solvent system $\text{CHCl}_3:\text{CH}_3\text{OH}:\text{H}_2\text{O}$ (90:10:1, v/v) to visualise GPLs 1, 2, 3 and 4

3.3.5 Loss of GPLs correlates specifically to phage I3 resistance

GPLs are one of the outermost lipids of the mycobacterial envelope. Given this location, it is likely that phage resistance was not due to the loss of a phage I3 receptor, but due to a non-specific effect affecting a pre-adsorption stage of mycobacteriophages. If this was true, the isolated mutants would be resistant to a wider range of mycobacteriophages. To test if this was the case, sensitivity of the I3-resistant mutants to two other lytic mycobacteriophages, D29 and BxZ1, was examined. Both phages were able to form plaques on lawns of wild type and phage I3-resistant mutants indicating that GPL-deficiency did not affect the normal course of infection for these phages (Fig. 3.1). Therefore, GPL deficiency specifically caused

resistance to phage I3 and, thus, this class of lipids were likely the receptors for phage I3.

Given that one of the *Tn* insertions was in a glycosyl transferase, *gtf2*, we also tested an independently generated targeted knockout mutant of *gtf2* (Miyamoto *et al.*, 2006) for sensitivity to phage I3. The strain $\Delta gtf2$ was also resistant to phage I3, and the I3 sensitivity was restored in the complemented strain, $\Delta gtf2/pMV261gtf2$ (Fig. 3.6). Also similar to the *Tn*-mutant, $\Delta gtf2$ was sensitive to BxZ1 and D29.

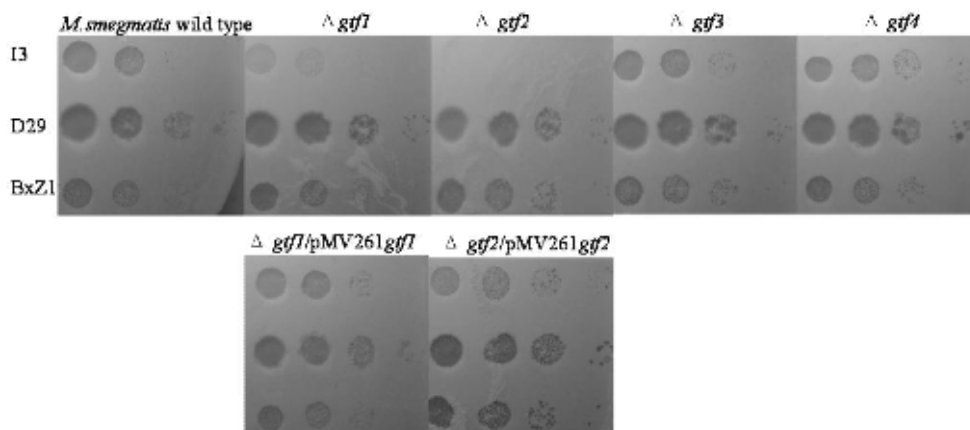


Fig. 3.6 Sensitivity of *M. smegmatis* GPL-associated glycosyl transferase mutants to phages I3, D29 or BxZ1. Ten fold dilutions of phages ($10\mu\text{l}$, 10^{-2} to 10^{-5}) spotted from left to right on soft agar overlays on 7H9-agar plates containing either wild type or glycosyl transferase mutants

3.3.6 Minimal structural requirements for the phage I3 receptor

As mentioned above, there are sub-classes of GPLs (Fig. 3.3), with deficiencies in levels of glycosylation. In order to further elaborate on the minimal structure required for phage adsorption, a set of previously described *M. smegmatis* mutants that produced truncated GPL intermediates was examined. The sequence of assembly

of sugar residues in GPLs was identified by Miyamoto *et al.* by specifically deleting *gtf1*, *gtf2*, *gtf3* and *gtf4*, the four genes encoding glycosyltransferases in *M. smegmatis* mc²155 (Miyamoto *et al.*, 2006). Gtf1 was involved in the transfer of deoxytalose to the FATP core and consequently the $\Delta gtf1$ mutant lacked all classes of GPLs and instead accumulated the intermediates FATP-3,4-di-*O*-methyl rhamnose and FATP-2,3,4-tri-*O*-methyl rhamnose (FATP-di-*O*-Me-Rha and FATP-tri-*O*-Me-Rha; Fig. 3.3 b, c). Similarly, Gtf2 was involved, independently, in the transfer of rhamnose to the FATP core and the $\Delta gtf2$ mutant, which also lacked all classes of GPLs, accumulated FATP-6-deoxytalose (Fig. 3.3 d). On the other hand, Gtf3 was involved in the transfer of the second rhamnose residue and subsequently only GPL-5 and GPL-6 were missing in the $\Delta gtf3$ mutant. There were no differences in lipid profiles of the $\Delta gtf4$ mutant compared with wild type strain. The accumulation of different intermediates of GPL biosynthesis in these mutants allowed these strains to identify the specific structural components of GPL that defined the receptor of phage I3. The $\Delta gtf1$, $\Delta gtf3$ and $\Delta gtf4$ mutants were all sensitive to infection by phage I3, although the plaquing efficiency of phage I3 on a lawn of the $\Delta gtf1$ mutant was diminished (Fig. 3.6). On the other hand, the $\Delta gtf2$ mutant was found to be phage-resistant, and complementation of the $\Delta gtf2$ mutant with plasmid encoded *gtf2* restored phage sensitivity (Fig. 3.6). This was consistent with the phage resistance phenotype of the *Tn*-mutant I3R-1 which had a *Tn* insertion in *gtf2*. Also, resistance of the $\Delta gtf2$ mutant to phage I3 correlated with the inability to detect intracellular phage DNA by PCR following adsorption with phage (Fig. 3.2). Also, like the I3-resistant

Tn-mutants, the $\Delta gtf2$ mutant retained sensitivity to phages D29 and BxZ1 (Fig. 3.6). These results indicated that addition of the first rhamnose residue to the FATP was critical for phage I3 infection and thus the FATP containing a single di or tri-*O*-methylated rhamnose was sufficient to allow binding of phage I3. However, the reduced plaquing of phage I3 on lawns of the $\Delta gtf1$ mutant (which accumulates FATP-di-*O*-Me-Rha and FATP-tri-*O*-Me-Rha) indicated that while talose was not required for phage binding, it likely affects binding efficiency.

3.3.7 Deletion of *mpr* in *M. smegmatis* and phage sensitivity of Δmpr

To identify the function of *mpr* and its role in phage D29 and L5 binding and consequently to understand the resulting changes in the cell wall, two approaches were undertaken. Firstly, a *M. smegmatis* Δmpr mutant, and secondly, overexpression of *mpr* in *M. smegmatis* using an inducible acetamide promoter. In both approaches, cell wall lipids would be analysed and compared to a control strain (the wild type strain and the wild type strain with an empty vector control).

According to the results from the previous study (Barsom & Hatfull, 1996) using overexpression of *mpr* in *M. smegmatis*, which suggested that mycobacteriophage resistance could be involved in a cell wall component biosynthetic process or other related function. The Δmpr mutant was confirmed by Southern blot using DIG-labeled (digoxigenin-dUTP, alkali-labile, Roche) PCR fragment of left and right side flanks. As shown in Fig. 3.7, one band (~4.5kb) was detected in *SacI* digested genomic DNA from the wild type strain and two bands (2.2kb and 1.8kb) from both Δmpr mutants

indicating that both mutants were genuine. The *M. smegmatis* $\Delta mpr1$ was used for further studies. Phage sensitivity was compared between the wild type and *M. smegmatis* Δmpr mutant strain. As shown in Fig 3.8, there was no distinctive difference in sensitivity to phage D29, I3 and BxZ1, between the wild type and the mutant strain.

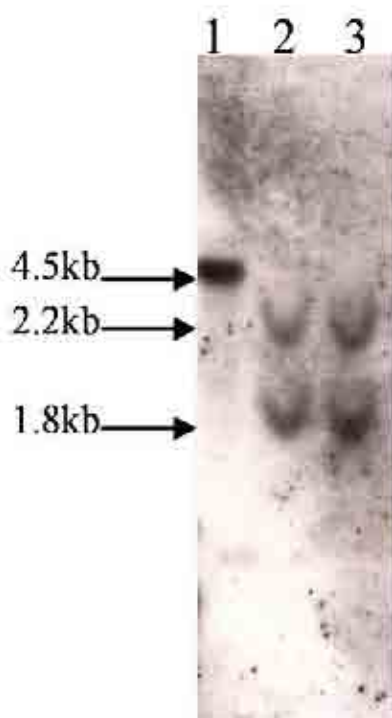


Fig. 3.7 Confirmation of Δmpr mutants by Southern blot. *SacI* digested genomic DNA from wild type strain (lane 1), $\Delta mpr(1)$ (lane 2) and $\Delta mpr(2)$ (lane 3) digested with *SacI* and probed with the same flank sequences used for constructing knock-out phasmid.

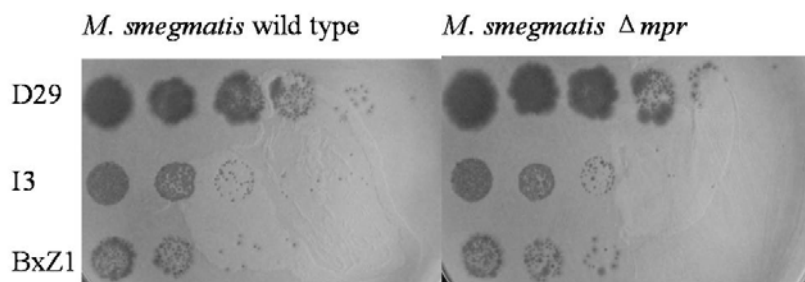


Fig. 3.8 Sensitivity of *M. smegmatis* Δmpr mutant to phages I3, D29 or BxZ1. Ten-fold dilutions of phages ($10\mu\text{l}$, 10^{-2} to 10^{-7}) spotted from left to right on soft agar overlays on 7H9-agar plates containing either wild type or Δmpr mutant.

Since the phage resistant phenotype was observed under the condition of overexpression of *mpr*, deletion of *mpr* may not have the same effect. Although, it was still possible that the cell wall structure could be changed if *mpr* was involved in any function related to cell wall composition, lipid profiles, as well as fatty acids and mycolic acids of wild type strain and mutant were analysed by [^{14}C]-acetate labeling. The results of TLC analysis showed no distinct difference between the wild type and the *M. smegmatis* Δmpr mutant strain (Fig. 3.9), when cultured in either TSB with Tween 80 or Sauton medium with Tween 80 respectively. However, there was a difference in System C of non polar lipid, when comparing the same strain in different media (Fig. 3.9 labelled with circle). When cultured in Sauton medium with Tween 80, both *M. smegmatis* wild type and Δmpr mutant gave one spot in System C, of non polar lipid, running behind the normal fatty acid spot. Because this pattern was consistent between wild type and mutant strain, it might be caused by the different medium used for culturing. However, these spots need to be further identified.

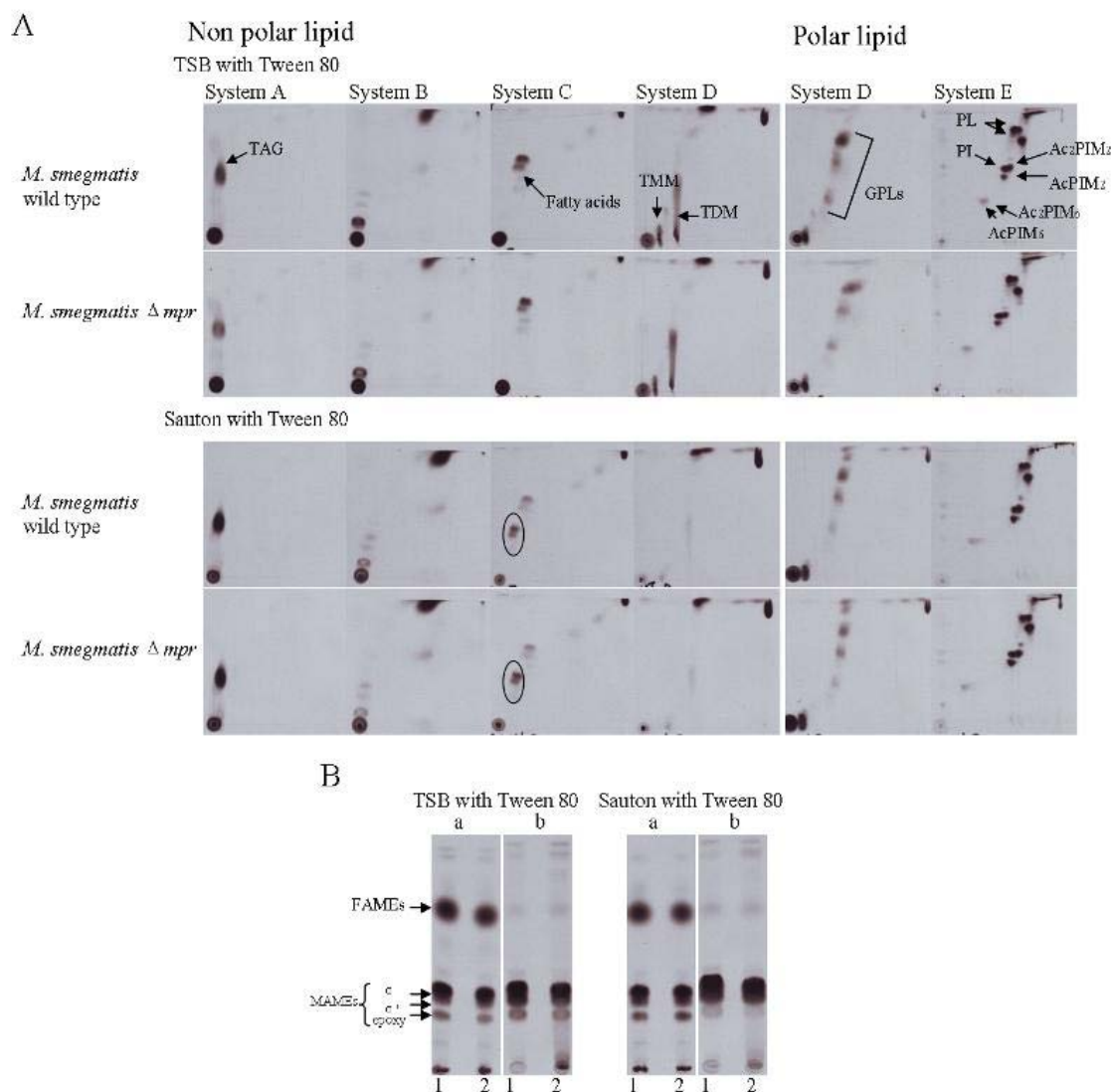


Fig. 3.9 Lipids profiles and FAMES/MAMES analysis of *M. smegmatis* Δmpr mutant. A. 2D-TLC analysis of nonpolar and polar lipids of *M. smegmatis* cultured in TSB with Tween 80 or Sauton with Tween 80. B. 1D-TLC analysis of FAMES/MAMES of *M. smegmatis* cultured in TSB with Tween 80 or Sauton with Tween 80. 1-*M. smegmatis* wild type, 2- *M. smegmatis* Δmpr ; a-whole FAMES/MAMES, b-cell wall bound FAMES/MAMES. Ac₂PIM₂ and AcPIM₂ di- and monoacyl phosphatidylinositol dimannosides, Ac₂PIM₆ and AcPIM₆ di- and monoacyl phosphatidylinositol hexamannosides, GPLs glycopeptidolipids, PI phosphatidyl inositol, PL phospholipid, TDM trehalose dimycolates, TMM trehalose monomycolates, TAG-triacylglycerol, FAME fatty acid methyl ester, MAME mycolic acid methyl ester.

3.3.8 The effect on phage sensitivity and cell wall components of *mpr* overexpressed in *M. smegmatis*

The phage resistance phenotype was observed in *M. smegmatis* when *mpr* was overexpressed in a previous study (Barsom & Hatfull, 1996). Thus, the acetamide inducible pSD26 vector was used to construct pSD26MSMEG1236 to increase the expression of Mpr in the wild type strain and then test for phage sensitivity. As shown in Fig. 3.10, the phage sensitivity to D29/I3/BxZ1 of the Mpr-overexpressed strain was similar compared to the wild type strain.

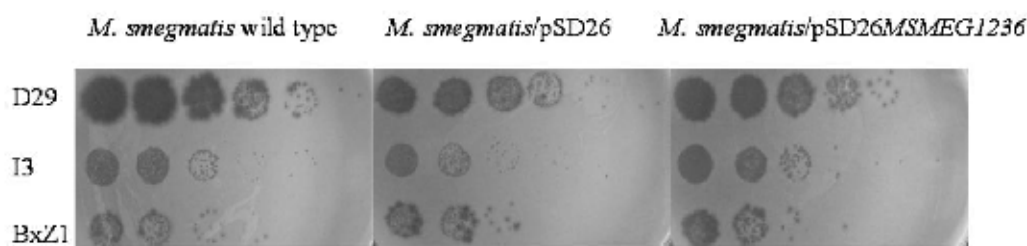


Fig. 3.10 Sensitivity of *M. smegmatis mpr* overexpressed strain to phages I3, D29 or BxZ1 Ten fold dilutions of phages (10µl, 10^{-2} to 10^{-7}) spotted from left to right on soft agar overlays on 7H9-agar plates containing either wild type or Δmpr mutant

The cell wall components were further analysed from cultures grown in 7H9 broth without Tween 80. There were bands in System C of non polar lipid of both pSD26 and *mpr* overexpressed strain, compared to wild type strain (Fig. 3.11 labelled in circle). These bands were at the similar position to fatty acids and need to be further identified. However, because the pattern was consistent between wild type/pSD26 and *mpr* overexpressed strain, this change may be caused by the pSD26 vector or the acetamide used for protein overexpression rather than the overexpression

of *mpr*. No other differences were found either in the lipid profiles, fatty acids or mycolic acids (Fig. 3.11).

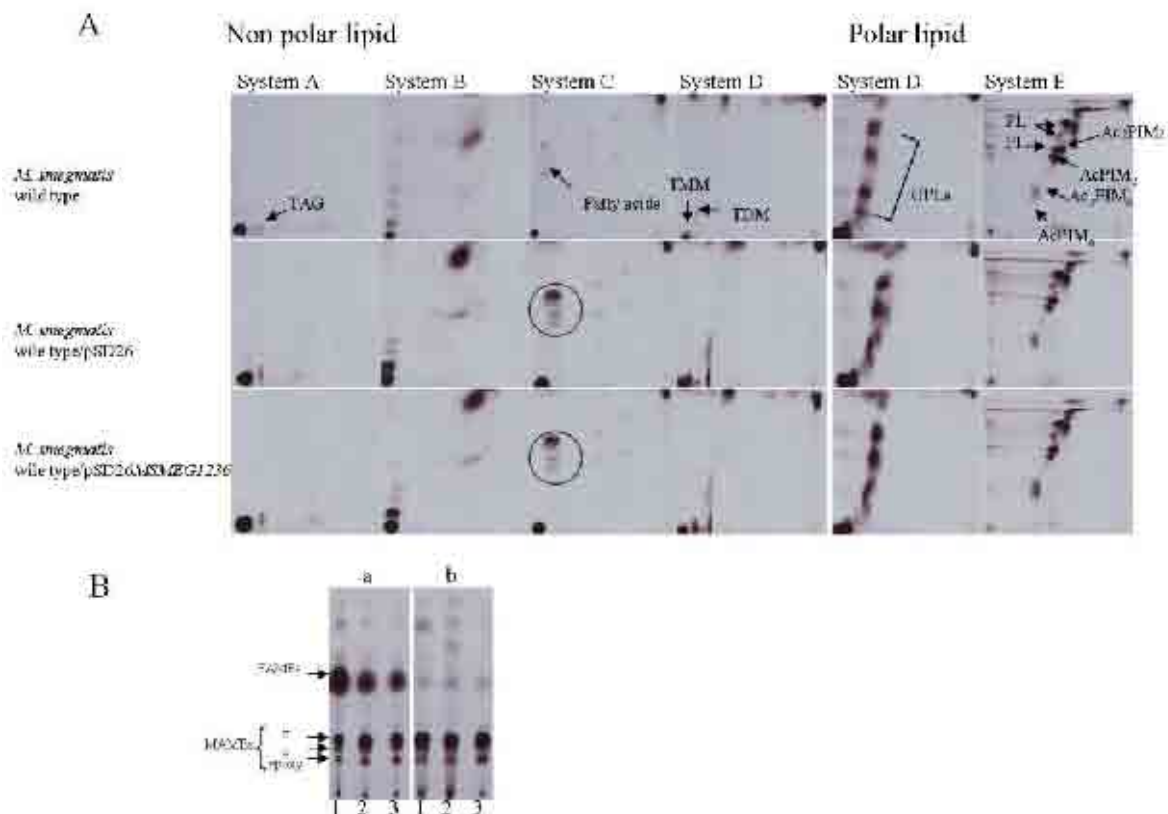


Fig. 3.11 Lipid profiles and FAMES/MAMES analysis of *M. smegmatis mpr* overexpressed strain.

A. 2D-TLC analysis of non-polar and polar lipids of *M. smegmatis* cultured in 7H9 broth without Tween 80. B. 1D-TLC analysis of FAMES/MAMES *M. smegmatis* cultured in 7H9 broth without Tween 80. 1-*M. smegmatis* wild type, 2- *M. smegmatis*/pSD26, 3- *M. smegmatis*/pSD26MSMEG1236; a-whole FAMES/MAMES; b-cell wall bound FAMES/MAMES Ac₂PIM₂ and AcPIM₂ di- and monoacyl phosphatidylinositol dimannosides, Ac₂PIM₆ and AcPIM₆ di- and monoacyl phosphatidylinositol hexamannosides, GPLs glycopeptidolipids, PI phosphatidyl inositol, PL phospholipid, TDM trehalose dimycolates, TMM trehalose monomycolates, TAG triacylglycerol, FAME fatty acid methyl ester, MAME mycolic acid methyl ester.

3.4 Discussion

Most mycobacteriophages belong to the family *Myoviridae* or *Sophoviridae*, encompassing phages with either contractile or long tails (Ackermann, 2001). It is

speculated that this tail morphology may be particularly relevant to mycobacteriophages, helping the injection of phage DNA through an exceptionally thick, carbohydrate and lipid-rich cell wall (Aspinall *et al.*, 1995). Interestingly, the sequencing of a number of mycobacteriophage genomes has revealed that the phage-tail associated tape measure proteins from mycobacteriophages contain a number of domains, some of which are similar to those involved in host-encoded PG degrading enzymes (Piuri & Hatfull, 2006), suggesting that these additional domains possibly aid viral DNA injection *via* localised cell wall degradation and thus play an important role in initialising infection. However, these early events in phage infection are dependent on, and pre-ceded by, the specific binding of phage to its cell surface receptor on the bacterial envelope. In this study we have used *Tn*-mutants to identify GPLs as the *M. smegmatis* cell surface receptor for phage I3, which belongs to the family *Myoviridae*. Phage resistance was not due to a non-specific effect caused by alterations in the cell wall, since the phage I3 resistant mutants retained sensitivity to other mycobacteriophages, such as D29 and BxZ1. This work highlights the utility of phage resistance screens to identify genes involved in the biosynthesis/transport of cell envelope components. Whilst in this case, sequencing of *Tn* insertion sites in the phage I3-resistant mutants revealed insertions in a well studied gene cluster involved in GPLs biosynthesis, the availability of numerous mycobacteriophages would allow screening for mutants defective in their corresponding phage receptors *viz* a mycobacterial cell wall components. Indeed, this work showed that the phages D29 and BxZ1 have receptor(s) are distinct from for phage I3, as the I3-resistant mutants

retained sensitivity to these phages. More than 250 mycobacteriophages have been isolated to date (Barksdale & Kim, 1977; Hatfull, 1994) and only a few have been studied in detail (Hatfull, 2005). Mycobacteriophages show diversity in genome sequences, morphology and in host range (Pedulla *et al.*, 2003) and it is quite likely that this diversity is also reflected in the nature of their corresponding receptors in the cell wall of mycobacteria. Besides, studies on phage receptors may also aid the further development of phage typing as a diagnostic tool for the identification of strains of pathogenic mycobacteria that produce variants of cell surface components.

Further analysis using defined *M. smegmatis* mutants that accumulated GPL intermediates showed that a FATP with 3,4-di or 2,3,4-trimethylated rhamnose (FATP-di-*O*-Me-Rha and FATP-tri-*O*-Me-Rha) was the minimal structural requirement for phage I3 binding. However, the lower phage adsorption efficiencies observed with the $\Delta gtf1$ mutant (which accumulates FATP-di-*O*-Me-Rha and FATP-tri-*O*-Me-Rha) indicated that while the 6-d-Tal residue was not essential for phage infection to occur, the presence of this sugar had an effect on phage binding efficiency. On the other hand, the second rhamnose residue seemed non-essential for phage binding as the $\Delta gtf3$ mutant (which lacks the second rhamnose-containing GPL-5 and GPL-6) did not show any differences in sensitivity to phage I3 as compared to the wild type strain.

Interestingly, *M. avium*, which also produces GPLs seems naturally resistant to phage I3 (Lee *et al.*, 2004). This may be either because a rough variant of *M. avium*

(that does not produce GPLs) was used to assay infectivity, or because the glycosylation of the deoxytalose residue in *M. avium* GPLs may have a 'masking' effect that prevents the recognition of methylated rhamnose by phage I3.

Previous studies have shown that the elevated expression of *mpr* (MSMEG1236) gene in *M. smegmatis* leads to the resistance of the recombinant strain to both mycobacteriophage D29 and L5 (Barsom & Hatfull, 1996). Although the exact function of *mpr* is unknown, the change of colony morphology, as well as the inhibition of phage DNA infection indicates that this gene is involved in cell wall component biosynthesis or modifying a structure but not as the receptor for phage infection considering the resistance appears when it is overexpressed. Initially, a *M. smegmatis* Δmpr mutant was used to study whether there would be any change in phage sensitivity or cell wall composition, lipid profile, fatty acids and mycolic acids. No difference was observed in both scenarios. Given the condition of elevated expression of *mpr* when the phage resistant phenotype is observed in previous study, an inducible expression vector pSD26 was used to elevate expression levels of *mpr* in *M. smegmatis* for further phage sensitivity and cell wall composition studies. Unlike the previous study, phage resistance in the overexpression strain to phage D29 was not observed. In addition, no distinct difference was observed in cell wall composition. According to the previous study, one extra copy of *mpr* is not sufficient to confer a phage-resistant phenotype (Barsom & Hatfull, 1996). Thus, one possible reason for failing to repeat the phage resistant phenomenon, in this study, might be the different

expression systems used in both studies which could affect the level of expression and further affect the phenotype.

Chapter 4

Impact of Mycolic Acid Modification on the Virulence of *Mycobacterium marinum*

4.1 Introduction

This chapter describes the characterisation of a *M. marinum* *Tn*-mutant 8G10 isolated from the colony morphology screen described in Chapter 2. The mutant contains a *Tn* insertion in the promoter region of *MMAR0978*, a gene encoding a *O*-methyl transferase. The region surrounding *MMAR0978* showed synteny with the *M. tuberculosis* cluster encoding genes involved in the biosynthesis of oxygenated mycolic acids. Mycolic acids are major components of the mycobacterial cell envelope. These unique long chain α -alkyl, β -hydroxy fatty acids are found either covalently linked to the cell wall mAGP complex or in the outer layers of the cell envelope as free glycolipids. Structural diversity in sub-classes of mycolic acids include variations in mero-chain length and mero-chain modifications (*e.g.* unsaturations, cyclopropanations, methylation) (Yuan & Barry, 1996). *M. tuberculosis* and *M. bovis* synthesise three sub-classes of mycolates: α -, keto and methoxy. The α -mycolic acids contain two *cis*-cyclopropane rings in the mero-chain, whilst the oxygenated mycolates contain either a proximal *cis* or *trans* cyclopropane ring (Yuan & Barry, 1996). Additionally, keto and methoxymycolates contain a distal keto and methoxy group, respectively (Yuan & Barry, 1996). Modifications of the mero-chain play an important role in virulence (Dao *et al.*, 2008; Dubnau *et al.*, 2000; Glickman *et al.*, 2000; Rao *et al.*, 2006; Ueda *et al.*, 2001) and also affect susceptibility to drugs (Barkan *et al.*, 2009; Glickman *et al.*, 2000).

In *M. tuberculosis*, *mmaA3* encodes an *O*-methyltransferase responsible for the biosynthesis of methoxymycolic acids. Functional evidence of this came from *mmaA3* overexpression studies in *M. tuberculosis* and *M. bovis* BCG, where overproduction of *M. tuberculosis* MmaA3 resulted in a complete replacement of ketomycolates by methoxymycolates, and from *in vitro* assays using purified MmaA3 (Yuan *et al.*, 1998). Additionally, the failure of several strains of *M. bovis* BCG to produce methoxymycolic acids correlated with a mutation in *mmaA3*. Furthermore, methoxymycolate production could be restored in these *M. bovis* BCG strains following introduction of a wild type copy of *M. bovis* BCG *mmaA3* (Belley *et al.*, 2004). While the role of oxygenated mycolic acids (as a group) in *M. tuberculosis* virulence has been studied using a mutant strain that is unable to synthesise both keto and methoxymycolates (Dubnau *et al.*, 2000), the specific effects of loss of only methoxymycolates in pathogenic strains remains to be studied. Whilst, *M. marinum* produces the same sub-classes of mycolic acids as *M. tuberculosis*, unlike in the latter, oxygenated mycolates from *M. marinum* are not cyclopropanated and contain mero-chain double bonds (Daffe *et al.*, 1991a).

M. marinum is a pathogen of fish and amphibians and shares many virulence traits of *M. tuberculosis* infection, such as the ability to survive and replicate within macrophages, formation of granulomatous lesions and reactivation (Pozos & Ramakrishnan, 2004). *M. marinum* has thus been used extensively as a surrogate for studying mycobacterial pathogenesis using the zebrafish model of infection (Stamm & Brown, 2004). The virulence of *M. marinum* can be assessed in a zebrafish model,

that infected embryos have been used to observe macrophage aggregation and granuloma formation, as well as infected adult fish have been used to study both innate and adaptive immune responses and granuloma formation (Pozos & Ramakrishnan, 2004). The studies described in this chapter include the biochemical characterisation of the 8G10 *Tn*-mutant and also an assessment of the effects of the mutant on virulence using a zebrafish infection model.

4.2 Materials and Methods

4.2.1 Bacterial strains, plasmids and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 4.1. Mycobacterial strains were grown in 7H9 broth with 0.05% Tween 80 and on 7H10 agar at 30°C. *E. coli* was grown in LB broth at 37°C. The antibiotics hygromycin, kannamycin and apramycin were added either at concentrations of 75µg/ml, 25µg/ml and 30µg/ml, respectively for *M. marinum*, or 150µg/ml, 50µg/ml and 30µg/ml for *E. coli* where required.

Table 4.1 Plasmids and bacterial strains used in this study

Plasmids, phages and strains	Description	Source or Reference
Plasmid		
pMV261	<i>E. coli</i> -mycobacterial shuttle plasmid, Kan ^R	(Stover <i>et al.</i> , 1991)
pMV261MMAR0978	<i>M. marinum</i> MMAR0978 was cloned in to pMV261, Kan ^R	This study
pMV261Rv0643c	<i>M. tuberculosis</i> Rv0643c was cloned in to pMV261, Kan ^R	This study
pMSP12-dsRed-Apr	<i>E. coli</i> -mycobacterial shuttle plasmid, containing dsRed and Apr ^R	This study
Bacteria		
<i>E. coli</i> TOP10		
<i>M. marinum</i> 1218R	Wild type strain	ATCC927
<i>M. marinum</i> 8G10	Transposon mutant, MMAR0978 promoter:: <i>Tn5371</i> Hyg ^R	This study
<i>M. marinum</i> 8G10/pMV261	Transposon mutant, MMAR0978 promoter:: <i>Tn5371</i> , transformed with vector pMV261, Hyg ^R and Kan ^R	This study
<i>M. marinum</i> 8G10/pMV261MMAR0978	Transposon mutant, MMAR0978 promoter:: <i>Tn5371</i> , complemented with pMV261MMAR0978, Hyg ^R and Kan ^R	This study
<i>M. marinum</i> 8G10/pMV261Rv0643c	Transposon mutant, MMAR0978 promoter:: <i>Tn5371</i> , complemented with pMV261Rv0643c (methoxy mycolic acid synthase 3 in <i>M. tuberculosis</i> H37Rv), Hyg ^R and Kan ^R	This study

4.2.2 Complementation of *M. marinum* 8G10

MMAR0978 was PCR-amplified from *M. marinum* 1218R genomic DNA using the primers MMAR0978-F (5'-CGCGGATCCAATGTCCGGTAAAGCAGTAGC-3') and MMAR0978-R (5'-CCCAAGCTTCTACTTGGCCAGAGTGAAGT-3'). The complementation plasmid pMV261MMAR0978 was obtained by cloning *Bam*HI-*Hind*III digested PCR product (sites incorporated in the primers are underlined) into pMV261. A similar approach was used to construct pMV261Rv0643c,

the complementation plasmid containing the *M. tuberculosis* homologue: *Rv0643c* was PCR amplified from *M. tuberculosis* H37Rv genomic DNA using the primers Rv0643-F (5'-CCGGAATTCATGTCTGATAACTCAACGGGC-3') and Rv0643-R (5'-CCCAAGCTTTCTACTTGGCCAGCGTGAAC-3') and cloned into the *EcoRI-HindIII* digested pMV261. Electroporation of *M. marinum* 8G10 was performed as described in the general protocols for mycobacteria by Larsen *et al* (Larsen *et al.*, 2007) and the complemented strains were obtained following electroporation of *M. marinum* 8G10 with pMV261MMAR0978 and pMV261Rv0643, respectively.

4.2.3 Extraction and culturing of murine bone marrow derived macrophages

Mouse (Balb/c) were sacrificed and the skin removed from the lower part of the body. Tissue was removed and legs were dissected. The pelvic and femoral bones were cleaned and separated at the knee joint. The bone was cut off at each end and bone marrow was expelled with 5ml of bone marrow medium (Dulbecco's modified Eagle's medium [DMEM], supplemented with 4.5g/L glucose, L-glutamine, pyruvate and 10% heat-inactivated fetal bovine serum [FBS]), from both ends by using a syringe. Cells were collected and harvested by centrifuging at 1,500rpm for 5 minutes. Cells were resuspended gently in 10ml of fresh DMEM and gently aspirated to break down cell aggregates. The cell suspension was adjusted to 10^6 cells/ml and seeded into 12 well plates with 10^6 cells/well. Cells were incubated for 5-7 days at 37°C under 10% (v/v) CO₂ and were fed every 2-3 days. The medium was changed on day 6.

4.2.4 Macrophage infections

Late log phase bacteria were washed with PBS and resuspended in infection medium (DMEM with 10% FBS) to 10^7 cfu/ml. Murine bone marrow macrophages were infected by *M. marinum* with MOI of 10 and incubated at 37°C under 10% (v/v) CO₂ for 2-4 hours. The infection medium was removed and the cells were washed twice with PBS and once with complete medium (DMEM with 10% FBS and 50µg/ml gentamicin). Cells were incubated in 1ml complete medium at 37°C under 10% (v/v) CO₂ overnight. On Day 1, the supernatant was removed and cells were washed once with PBS. Fresh complete medium without gentamicin was added. Cells were incubated at 37°C under 10% (v/v) CO₂ for 1 hour. The supernatant was collected as Day 1 for future cytokine studies and cells were lysed with 1ml of PBS with 0.1% TritonX-100. Samples were collected on Day 3 and Day 5. The infection medium and cell lysates were 10 fold serial diluted and plated out on 7H10 agar plates. The plates were incubated at 30°C for 1 week before counting colonies (cfu). Survival percentage was calculated using $100 \times (\text{cfu}_{\text{DayX}} / \text{cfu}_{\text{Infection}})$. Each experiment was repeated in triplicate.

4.2.5 Construction of a *M. marinum* strain encoding dsRed for visualisation in zebrafish embryo

pMSP12-dsRed-Apr: *aacC41* gene was amplified from plasmid pMV261-Apr (with primers: 5'CCAATGCATATGGGCACTCGGCATAGGCGAGTG3' and 5'CCAATGCATATGGTGGATCCCCCGTGTTGCCCC3',) and cloned into NsiI

digested vector pMSP12-dsRed. After transformation into *E. coli* TOP10, cells were selected on LB agar with 30µg/ml apramycin at 37°C, overnight.

4.3 Results

4.3.1 Colony morphology of *M. marinum* 8G10 *Tn*-mutant

As described in Chapter 2, the strain 8G10 was isolated from a *Tn*-mutant library of *M. marinum*, as a mutant with altered colony morphology. The most apparent changes for 8G10 were observed on 7H10 agar plates that contained Tween 80 as shown in Fig. 4.1, with a smoother and glossier surface compared to the wild type strain.

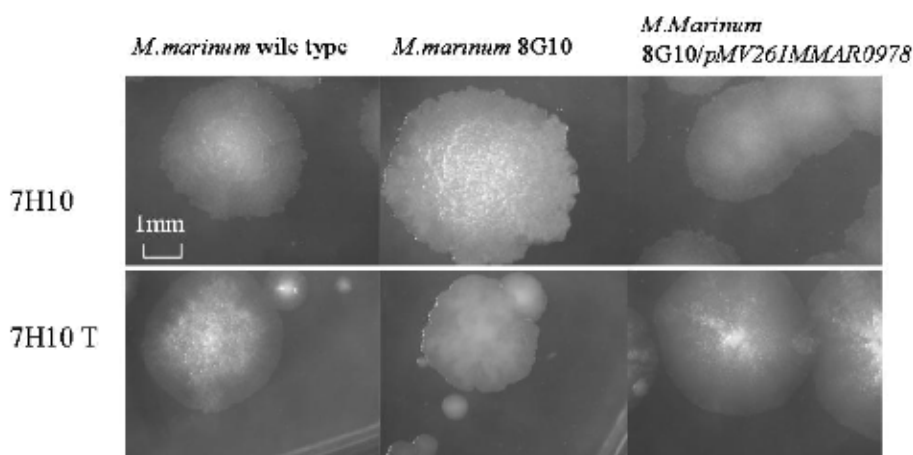


Fig. 4.1 Single colony morphology of *M. marinum* 8G10 mutant on 7H10 agar (with or without 0.05% Tween 80). Late-log phase culture was streaked out on agar plate and then incubated at 30°C to form single colony.

4.3.2 Effect of *mmaA3* on *M. marinum* mycolic acid profile

Sequencing of the *Tn5371* insertion site in 8G10 mutant revealed that the *Tn* inserted into the promoter region, upstream of *MMAR0978* encoding a mycolic acid

O-methyl transferase similar to *Rv0643c*, a homologue found in *M. tuberculosis* responsible for the biosynthesis of methoxymycolic acids. This suggested that the observed alteration in colony morphology was likely due to disruption of transcription of *MMAR0978*, thus affecting *MMAR0978* function. It could cause the loss of methoxymycolic acid production that might change the lipid and mycolic acid profile and alter the colony morphology. Initially, total lipid analysis by 2D-TLC revealed no substantial change in the mutant's non-polar and polar lipid profiles compared with wild type *M. marinum* (Fig. 4.2A). However, analysis of the sub-classes of mycolates from the mutant revealed a dramatic decrease in methoxymycolate production (Fig. 4.2B). This defect could be complemented by introduction of a pMV261-cloned copy of *M. marinum* *mmaA3* (*MMAR0978*) and the *M. tuberculosis* homologue (*Rv0643c*), respectively. Both complementation experiments restored methoxymycolate synthesis more than wild type levels with less ketomycolate than the wild type (Fig. 4.2B).

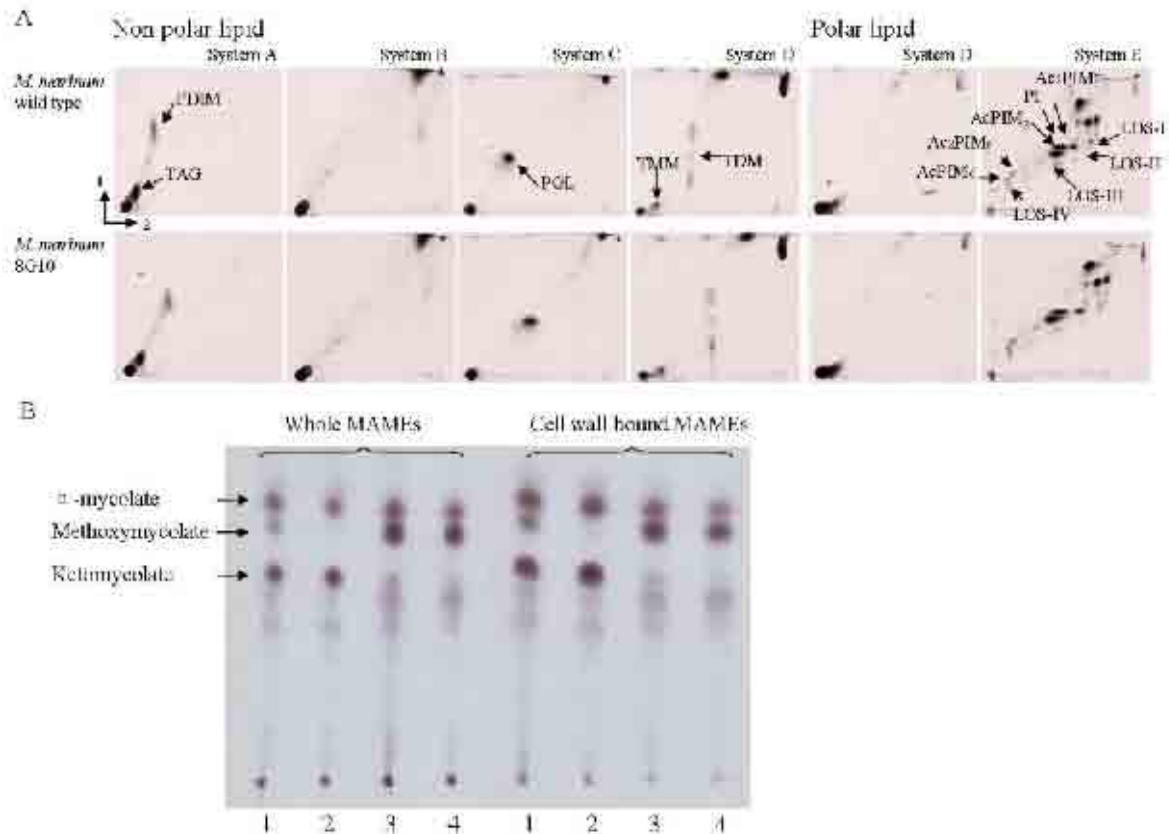


Fig. 4.2 Lipid and mycolic acid analysis of *M. marinum* 8G10 mutant and the complementation strains. A. 2D-TLC of non-polar and polar lipids of *M. marinum*. B. 1-D TLC analysis of MAMEs of *M. marinum*. 1-*M. marinum* wild type; 2-*M. marinum* 8G10; 3-*M. marinum* 8G10/pMV261MMAR0978; 4-*M. marinum* 8G10/pMV261Rv0643c. Ac₂PIM₂ and AcPIM₂ di- and monoacyl phosphatidylinositol dimammosides, Ac₂PIM₆ and AcPIM₆ di- and monoacyl phosphatidylinositol hexammosides, LOS-I LOS-II LOS-III and LOS-IV lipooligosaccharide I II III and IV, PI phosphatidyl inositol, PDIM phthiocerol dimycocerosate PGL phenolic glycolipid, PL phospholipid, TDM trehalose dimycolates, TMM trehalose monomycolates, TAG triacylglycerol, MAME mycolic acid methyl ester.

4.3.3 Effect of loss of methoxymycolic acids on *M. marinum* virulence

To assess the virulence of the methoxymycolic acid deficient mutant, an intracellular survival assay using murine bone marrow macrophages was performed. The results showed no distinct differences between wild type *M. marinum* and the mutant suggesting no changes in the ability to replicate in macrophages (Fig. 4.3).

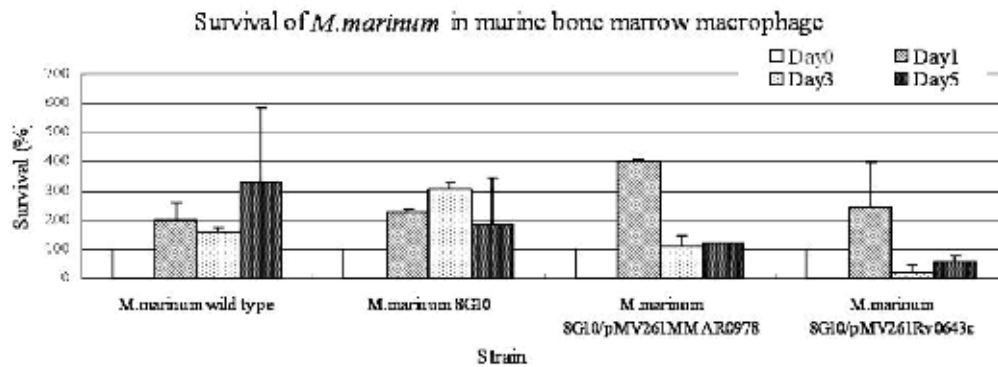


Fig. 4.3 Survival of *M. marinum* in murine bone marrow macrophages

Given that this cultured macrophage can only represent an intracellular environment during disease progression, but not the complete host immune system, the zebrafish infection model was used to examine if there would be any difference in terms of virulence. *E. coli*-mycobacterial shuttle plasmid containing dsRed, pMSP12-dsRed-Apr, was electroporated into *M. marinum* wild type, 8G10 *Tn*-mutant and two complemented strains to generate Red fluorescence-labeled bacteria. These red fluorescence-labeled bacteria were then used for infection in order to observe the spread of the bacteria in real-time and the amount of living bacteria could be quantified by the increase of red fluorescence. A representative experiment is shown in Fig. 4.4, five days after infection, strong red fluorescence was detected in zebrafish embryos infected with wild type *M. marinum* from head to tail, suggesting that the bacteria survived and disseminated all over the embryo, while only weak fluorescence could be seen in embryos infected with the 8G10 mutant, representing less live bacteria in the zebrafish embryos. This experiment is currently being repeated to confirm the initial data and adult zebrafish will also be used for infection studies to

examine the formation of granulomas.



Fig. 4.4 Real-time analysis of *M. marinum* infection in zebrafish embryos

4.4 Discussion

M. tuberculosis has a special lipid-rich cell wall providing protection from harsh environments which is crucial for the successful infection process. As the outmost part of the bacilli, the cell wall components are the first things encountered by host cells during infection; therefore, they play important roles in disease progression. Several cell wall components, such as TDM and LAM, have been identified as immunomodulators (Guerardel *et al.*, 2003; Kan-Sutton *et al.*, 2009). Information of how mycobacterial cell wall components interact with the host immune system can help understand the disease and provide clues for the development of novel drugs as well as vaccines.

Based on the hypothesis that a change of cell wall composition will lead to a change in colony morphology, the *M. marinum* *Tn*-mutant library was screened for

colony morphology altered mutants. From the 20 *Tn*-mutants with altered colony morphology, the *MMAR0978* promoter disrupted mutant was chosen for further study. From the annotation data, *MMAR0978* was in the region of genes involved in mycolic acid biosynthesis. Its homologue in *M. tuberculosis* was *Rv0643c* (*mmaA3*), an *O*-methyltransferase responsible for transferring a methyl group from SAM to form methoxymycolic acids. Although, the *Tn* insertion of the mutant was in the promoter but not the gene itself, it blocked the function of *MMAA3* confirmed by FAMEs and MAMEs analysis that the production of methoxymycolate decreased dramatically in the mutant and was restored by complementing the mutant with *MMAR0978* and its *M. tuberculosis* homologue *Rv0643c*, respectively, indicating that these two genes shared the same function. As shown in Fig. 4.2B, there was altered mycolate composition, increased methoxymycolates and decreased ketomycolates, as described previously (Yuan & Barry, 1996). This may be due to the fact that pMV261 is a multi-copy plasmid, which causes overexpression of *mmaA3* and leads to the overproduction of methoxymycolic acids. As keto and methoxymycolic acids share the same precursor (Dubnau *et al.*, 2000), this causes a decrease of ketomycolic acid production.

In both *M. tuberculosis* and *M. marinum*, there are three sub-classes of mycolic acids based on the modification on the mero-chain, α -, methoxy and keto (Barry *et al.*, 1998). The last two are also known as oxygenated mycolic acids and share the same precursor in biosynthesis (Dubnau *et al.*, 2000). It has been reported that the loss of both keto and methoxymycolic acids in *M. tuberculosis* leads to a decrease in cell wall permeability to small molecules and growth in a mouse model. Recent research

suggests that these oxygenated mycolic acids play an important role in macrophages differentiating into lipid-accumulated foamy macrophages, which are found in granulomas and may be the nutrient source for the bacilli in the persistent phase of growth (Peyron *et al.*, 2008). However, the loss of methoxymycolic acids was reported and related to the attenuation of *M. bovis* BCG and the restoration of methoxymycolic acids made no difference in *M. bovis* BCG sensitivity to antibiotics and survival in macrophages, as well as in mice (Belley *et al.*, 2004). Given the situation that *M. bovis* BCG has lost the virulence related RD1 during the first phase of attenuation; it is difficult to draw any conclusions that methoxymycolic acids are unrelated to virulence. Besides, up till now, there is no research evaluating the virulence using keto or methoxymycolic acids, respectively, in pathogenic mycobacteria. Thus, this methoxymycolic acid deficient *M. marinum* 8G10 mutant isolated in this study provided an opportunity to study the effects of loss of methoxymycolates on virulence in a strain that still produced comparable levels of α - and ketomycolates.

Intracellular growth in macrophages and survival from acid stress, as well as oxidative stress are crucial for virulence. No difference was found between the wild type *M. marinum* and the methoxymycolic acid deficient 8G10 *Tn*-mutant in the murine bone marrow macrophage infection assay. Considering that cultured macrophages can only partially mimic the intracellular environment but not infection in the context of the complete host immune system and that intracellular survival is just the start of successful infection of the disease, a different animal model of

infection and granuloma formation in an intact host was examined.

M. marinum is a fish pathogen and has similar mycolic acid components as *M. tuberculosis*. Recently, the *M. marinum*-zebrafish infection model was used for pathogen-host studies (Pozos & Ramakrishnan, 2004; Stamm & Brown, 2004). As one of the natural hosts of *M. marinum*, the zebrafish infection model has one specific advantage, the formation of granulomatous lesions which are similar to TB infection in humans. Therefore, the zebrafish was used for virulence studies using the methoxymycolic acid deficient 8G10 *Tn*-mutant. The initial data showed that the wild type *M. marinum* could successfully survive, replicate and further migrate within the zebrafish embryos five days after infection, whilst the majority of the methoxymycolic acid deficient 8G10 mutant was eliminated. These preliminary results indicated that the loss of methoxymycolic acids may cause the attenuation of *M. marinum* in terms of infecting zebrafish embryos.

Recent studies demonstrate that oxygenated mycolic acids, which include methoxymycolic acids, can induce the differentiation of macrophages to foamy macrophages, which are found in granulomas. These foamy macrophages are not only involved in the cavitation of granulomas which leads to the release of living bacilli but also as a reservoir for bacilli persistence in TB patients (Peyron *et al.*, 2008; Russell *et al.*, 2009). Considering that the studies refer to oxygenated mycolic acids as a whole group, including keto, methoxy and hydroxyl-mycolic acids, methoxymycolic acids may be involved in the later stage of TB progression, such as persistence or

granuloma formation/destruction. Given that zebrafish embryos have not developed an adaptive immune system, only the innate immune response is involved in this infection model. However, adult zebrafish, which have both innate and adaptive immune systems, would be used for the aims mentioned above in future studies.

Chapter 5

Functional Studies on Mycobacterial Isocitrate Dehydrogenase

5.1 Introduction

Three major carbon metabolism pathways have been reported in prokaryotic organisms, which are the citric acid cycle (also known as tricarboxylic acid or TCA cycle), and two anaplerotic pathways, the glyoxylate cycle and the methylcitrate cycle. The TCA cycle is essential in cell metabolism and in eukaryotes. It uses acetyl-CoA derived from carbon sources, such as sugars and fatty acids, to provide reducing equivalents for energy and also intermediates for the biosynthesis of some amino acids. As for prokaryotes, under anaerobic conditions or limited carbon sources, the glyoxylate cycle and methylcitrate cycle are involved in replenishing intermediates for TCA cycle (Fig. 5.1). Isocitrate dehydrogenase (ICD) is one of the key regulatory enzymes in the TCA cycle and is located at the junction between the TCA cycle and the glyoxylate cycle. It is responsible for catalysing oxidative decarboxylation of *D*-isocitrate to α -ketoglutarate (α -KG) and CO₂, using NAD⁺ or NADP⁺ as a cofactor. In *E. coli*, the activity of ICD is regulated by a bi-functional isocitrate dehydrogenase kinase/phosphatase, AceK, from the glyoxylate operon. ICD is inactivated by phosphorylation resulting in isocitrate flux through the glyoxylate bypass (LaPorte, 1993; Zheng & Jia, 2010), which is important for bacteria when growing under sole carbon sources, such as acetate (El-Mansi *et al.*, 2006).

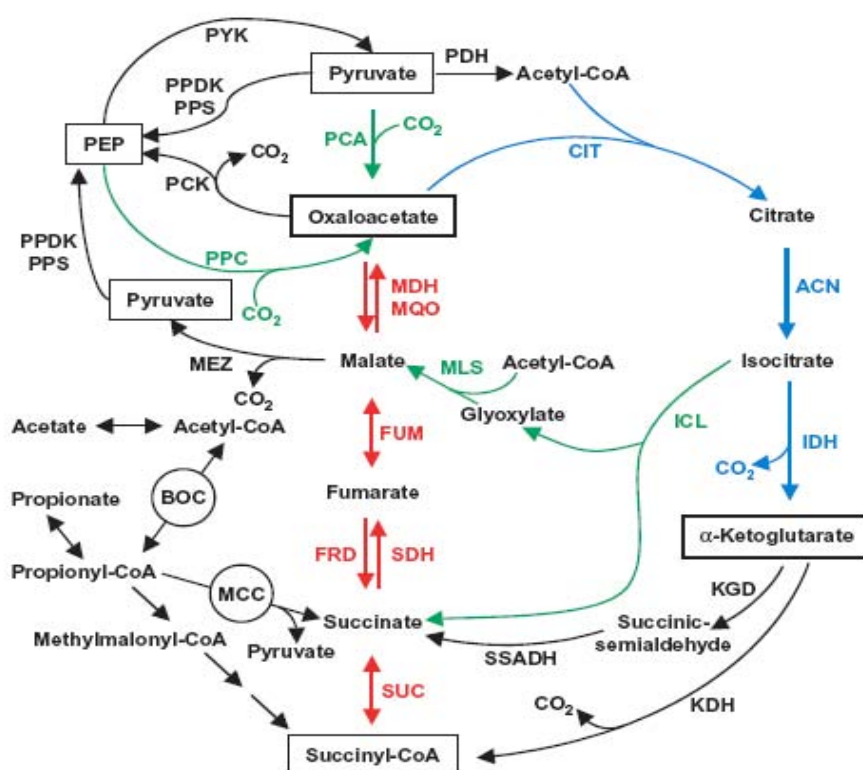


Fig. 5.1 Citric acid cycle and related anaplerotic pathway. The bifurcated biosynthetic mode of operation of the citric acid cycle, comprising oxidative (blue) and reductive (red) branches, is employed by bacteria growing under aerobic conditions in excess glucose or anaerobically on any carbon substrate, and by bacteria that lack KDH/KGD. Some intermediates of glycolysis and the citric acid cycle (boxed) are also diverted into biosynthetic pathways. Depleted intermediates are replenished via anaplerotic reactions (green), catalysed by PPC or PCA during growth on excess carbohydrate, or by ICL and MLS during growth on fatty acids or limiting carbohydrate. MEZ, malic enzyme; PPC, PEP carboxylase; PCK, PEP carboxykinase; PPS, PEP synthase; PPK, pyruvate phosphate dikinase; PYK, pyruvate kinase; PCA, pyruvate carboxylase; PDH, pyruvate dehydrogenase; CIT, citrate synthase; ACN, aconitase; IDH, isocitrate dehydrogenase; KGD, α -ketoglutarate decarboxylase; SSADH, succinic semialdehyde dehydrogenase; KDH, α -ketoglutarate dehydrogenase; SUC, succinyl-CoA synthetase; SDH, succinate dehydrogenase; FRD, fumarate reductase; FUM, fumarase; MDH, malate dehydrogenase; MQO, malate:quinone oxidoreductase; ICL, isocitrate lyase; MLS, malate synthase; BOC, β -oxidation cycle; MCC, methylcitrate cycle. (Munoz-Elias & McKinney, 2005)

While playing an important role in central metabolism, the TCA cycle and associated bypass pathways also play an important role in the ability of pathogenic bacteria to survive inside the infected host. The product of ICD in the TCA cycle,

α -KG, has been recently reported to have a role in the detoxification of reactive oxygen species (ROS), such as hydrogen peroxide and superoxide (Mailloux *et al.*, 2007). In addition, NADP⁺-dependent ICD is also one of the resources of the critical molecule NADPH. It is the common co-factor as electron donor in various mechanisms of detoxification, for example in the reduction of NO⁻ to NO₃ by Fhb1 (Gardner *et al.*, 2000) and demutation of H₂O₂ to O₂ and water by catalase (Kirkman & Gaetani, 1984; Sadecky *et al.*, 1975). Meanwhile, NADH, the cofactor of NAD⁺-dependent ICD, acts as a pro-oxidant. The ratio of NAD⁺ and NADH determines the rate of superoxide (one species of ROS) formation. NADH:ubiquinone oxidoreductase (complex I) plays a significant contribution in cellular oxidative stress as a major source of superoxide in mitochondria. Research, using complex I isolated from bovine heart mitochondria, shows superoxide is produced by transferring electrons from reduced flavin to O₂. The rate is determined by the portion of competent flavin which is set by a pre-equilibrium and controlled by the dissociation constants of NADH and NAD⁺, as well as, the reduction potentials of flavin and NAD⁺ (Kussmaul & Hirst, 2006). Studies in *E. coli* demonstrate that the ICD knock-out mutant is more susceptible to oxidative stress (Kriakov *et al.*, 2003). However, studies in *Cryptococcus neoformans*, an opportunistic fungal pathogen, suggests that ICD is important for resistance to nitrosative stress but not oxidative stress (Brown *et al.*, 2010).

M. tuberculosis is an intracellular bacterium that can replicate and survive in macrophages (Vergne *et al.*, 2004). The mechanisms of its survival in macrophages

and its ability to circumvent the immune defence have been studied for some time. Modulation of the immune response by mycobacterial cell wall components (Pieters, 2008) includes arresting phagosome maturation (Fratti *et al.*, 2003; Vergne *et al.*, 2005), evading intracellular oxidative stress (Bhatt *et al.*, 2007; Gao *et al.*, 2004; Piddington *et al.*, 2001) and metabolic adaptation to the harsh intracellular environment (Boshoff & Barry, 2005; McAdam *et al.*, 2002). It has been reported that *M. tuberculosis* metabolises fatty acids rather than carbohydrates (Boshoff & Barry, 2005). The expression of genes involved in fatty acid metabolism increase *in vivo* (Dubnau & Smith, 2003), indicating anaplerotic pathways are important for *in vivo* growth. Isocitrate lyase (ICL) is the first enzyme in the glyoxylate shunt, an anaplerotic pathway for the TCA cycle to help replenish intermediates in certain environments, such as using acetate as the only carbon source. In *M. tuberculosis* two *icls* (*icl1* Rv0647, and *icl2a/b* Rv1915-Rv1916) have been reported and both are required for *in vivo* growth and virulence (Munoz-Elias & McKinney, 2005). However, *icl1* shows more virulence related functions compared to *icl2*. Firstly, only the loss of *icl1* causes the defect in persistence of the bacilli in a chronic infection mouse model (Munoz-Elias & McKinney, 2005). Secondly, *icl1* can also function as 2-methylisocitrate lyase (*mcl*), an enzyme in the methylcitrate shunt, which is important for utilising propionyl-CoA, a product of the β -oxidation of odd-chain fatty acids when using fatty acids as a carbon source (Gould *et al.*, 2006). Besides, *M. tuberculosis* also shows several unique features in terms of the composition of its central carbon metabolism pathways. Biochemical experiments on the TCA cycle

enzyme activities have shown that *M. tuberculosis* lacks α -ketoglutarate dehydrogenase (KDH) which is responsible for decarboxylation of α -ketoglutarate, producing succinyl-CoA. Instead, it uses α -ketoglutarate decarboxylase (KGD), and succinic semialdehyde dehydrogenase (SSADH) to produce succinate directly (Fig. 5.1) (Munoz-Elias & McKinney, 2005; Tian *et al.*, 2005a; Tian *et al.*, 2005b). In addition, two ORFs (*Rv3339c-icd1*, *Rv0066c-icd2*) in the *M. tuberculosis* genome are annotated as genes encoding isocitrate dehydrogenases. Protein sequence alignment shows that *M. tuberculosis* ICD1 is closer to eukaryotic NADP⁺-dependent ICDs, while ICD2 clusters with other prokaryotic NADP⁺-dependent ICDs (Banerjee *et al.*, 2005; Steen *et al.*, 2001). Both of them have been confirmed to be functional in *in vitro* enzymatic assays (Banerjee *et al.*, 2005) and reported to be able to elicit a B cell response in TB patients, respectively (Banerjee *et al.*, 2005). Interestingly, most pathogenic mycobacteria, like *M. bovis*, *M. tuberculosis* and *M. marinum*, have two *icds* annotated, while non-pathogenic mycobacteria, like *M. smegmatis*, have only one.

The studies described in this chapter were initiated by the isolation of a *M. smegmatis* *Tn*-mutant 3D9 with an insertion in *icd* (*MSMEG1654*) which displayed an apparent altered colony morphology on 7H10 agar plates (either with or without Tween 80). Given the role of *icd* in central carbon metabolism and the utilisation of fatty acids as a carbon source in *Mycobacterium*, it was presumed that the 3D9 mutant appeared to have altered colony morphology due to defects in cell wall lipid content, resulting in a perturbed carbon flux in cells. The first aim of this study was to identify

alterations in the cell wall lipid components. Additionally, the availability of a *M. smegmatis icd* mutant provided an opportunity to study the role of this enzyme in the above mentioned processes that are essential for virulence. Furthermore, given that *M. smegmatis* has only one *icd* gene, this mutant was an ideal surrogate to transform with different orthologues of *M. tuberculosis icds* to test for functional complementation. The main aims of this study were to (1) assess the effects of *icd* inactivation on mycobacterial lipid biosynthesis; (2) demonstrate functionality for both *icd* orthologues from *M. tuberculosis* by the use of enzymes assays with extracts from complemented strains and (3) study the effects of *icd* in terms of anti oxidative stress, nitrosative stress and acid stress.

5.2 Materials and Methods

5.2.1 Bacterial strains and plasmid growth conditions

Bacterial strains, plasmids, and phages used in this study are listed in Table 5.1. *M. smegmatis* was grown in TSB with 0.05% Tween 80 at 37°C and *E. coli* in LB broth at 37°C. The antibiotics hygromycin and kannamycin were added at concentrations of 100µg/ml and 25µg/ml, respectively, for *M. smegmatis* and 150µg/ml and 50µg/ml for *E. coli*, where required. Enriched mineral salt agar (Na₂HPO₄ 3.57g/L, KH₂PO₄ 1.5g/L, NH₄Cl 1.0g/L, MgSO₄·7H₂O 0.2g/L, CaCl₂·2H₂O 20.0mg/L, Fe(III)NH₄-Citrate 1.2mg/L, NaCl 0.85g/L pH 7.0, 1.5% agar w/v) supplemented with 0.1% glycerol, 0.1% glucose, 0.1% potassium acetate, 0.002% oleic acid or 0.002% palmitic acid was used for single carbon source test for *M.*

smegmatis.

Table 5.1 Plasmids and Bacterial strains used in this study

Strains or Plasmids	Discription	Source or Reference
Plasmid		
pMV261	<i>E.coli</i> -mycobacterial shuttle plasmid, Kan ^R	(Stover <i>et al.</i> , 1991)
pMV261MSMEG1654	MSMEG1654 cloned in pMV261, Kan ^R	This study
pMV261Rv3339c	Rv3339c cloned in pMV261, Kan ^R	This study
pMV261Rv0066c	Rv0066c cloned in pMV261, Kan ^R	This study
Bacteria		
<i>E.coli</i> Top10		
<i>M. smegmatis</i> mc ² 155	Parental (wild type strain) strain, Ept mutant	(Snapper <i>et al.</i> , 1990)
<i>M. smegmatis</i> 3D9::Tn5371	Transposon mutant, MSMEG1654::Tn5371 Hyg ^R	This study
<i>M. smegmatis</i> 3D9/pMV261	Transposon mutant, MSMEG1654::Tn5371, transformed with vector Pmv261, Hyg ^R and Kan ^R	This study
<i>M. smegmatis</i> 3D9/pMV261MSMEG1654	Transposon mutant, MSMEG1654::Tn5371, complemented with pMV261MSMEG1654, Hyg ^R and Kan ^R	This study
<i>M. smegmatis</i> 3D9/pMV261Rv3339c	Transposon mutant, MSMEG1654::Tn5371, complemented with pMV261Rv3339c(<i>icd1</i> in <i>M.tuberculosis</i> H37Rv), Hyg ^R and Kan ^R	This study
<i>M. smegmatis</i> 3D9/pMV261Rv0066c	Transposon mutant, MSMEG1654::Tn5371, complemented with pMV261Rv0066c(<i>icd2</i> , homologue in <i>M.tuberculosis</i> H37Rv), Hyg ^R and Kan ^R	This study

5.2.2 Growth curve and viability test

Fresh cultures of *M. smegmatis* were diluted in TSB with 0.05% Tween 80 (antibiotics were added where required) to OD₆₀₀=0.1 and incubated at 37°C shaking at 180 rpm. The growth of cultures was monitored by OD₆₀₀ every 6 hours. At every 6 hour monitoring point, 0.5ml culture was collected and a viability test conducted. Each sample was ten-fold serially diluted in TSB upto 10⁻⁶ and 10µl of each dilution was spotted on a TSB agar plate. Samples were performed in triplicate. TSB agar plates were incubated at 37°C for 2-3 days, and single colonies counted for calculating cfu/ml (colony forming units per ml) at each time point.

5.2.3 Preparation of bacterial lysate for assaying ICD activity

A mycobacterial cell pellet from a 5L culture was resuspended in 5ml of Buffer A (MOPS 50mM pH 7.9 with KOH, MgCl₂ 10mM, β-mercaptomethanol 5mM) and sonicated with 60 sec on and 90 sec off, 10 cycles. After centrifugation (27,000g, 30 min, 4°C), the supernatant was collected and centrifuged (100,000g, 3 hours, 4°C). The supernatant was collected and the protein concentration was determined using the BCA protein assay (Thermo scientific, Pierce).

5.2.4 Enzyme assay for isocitrate dehydrogenase

An isocitrate dehydrogenase enzyme assay was performed as described (Tian *et al.*, 2005a). The reaction mixture (1ml) contained 50mM HEPES (pH 8.0), 0.25mM NADP⁺, 10mM MgCl₂, 1 mM isocitrate and *M. smegmatis* whole cell lysate (50µg

protein). The reaction was monitored by production of NADPH at 340nm. The extinction coefficient factor of $6223\text{M}^{-1}\cdot\text{cm}^{-1}$ was used to calculate rate.

5.2.5 Assay of acid sensitivity

Mid-log-phase bacterial cultures were washed with pic-Ty-4.5 (phosphate-citrate buffer pH 4.5, tyloxapol 0.02% v/v). Cells were suspended in pic-Ty-4.5 at $\sim 5 \times 10^6$ cfu/ml and incubated at 37°C for 1, 2 and 3 doubling times. Viable cell numbers were determined by plating 10-fold serial dilutions of the cell suspension on TSB (for *M. smegmatis*).

5.2.6 Assay for oxidative stress

Late-log-phase bacterial cultures were mixed with molten 7H9 soft agar (50°C) and overlaid onto 7H9 agar plate. After the softer agar was solidified, a paper disc (diameter: 6mm) soaked with H₂O₂ (60mM and 100mM) was attached to the surface. Plates were incubated at 37°C to form inhibition zones.

5.2.7 Assay for nitrosative stress

Late-log-phase bacterial cultures were 10-fold diluted in PBS and spotted onto 7H9 agar plates containing different concentrations of sodium nitrite (0mM, 2.5mM, 5mM, 10mM). Plates were incubated at 37°C for 3 days.

5.3 Results

5.3.1 Colony morphology changes of the *M. smegmatis* 3D9 mutant

As described in Chapter 2, the strain 3D9 was isolated from a *Tn*-mutant library of *M. smegmatis*, as a mutant with altered colony morphology. The most apparent changes of 3D9 were observed on 7H10 agar plates (either with or without Tween 80) in single colony morphology as shown in Fig. 5.2. Compared to the wild type strain, on 7H10-T agar, 3D9 mutant had a smoother surface on 7H10-T agar, but a smaller colony with a smoother surface on 7H10 agar plates.

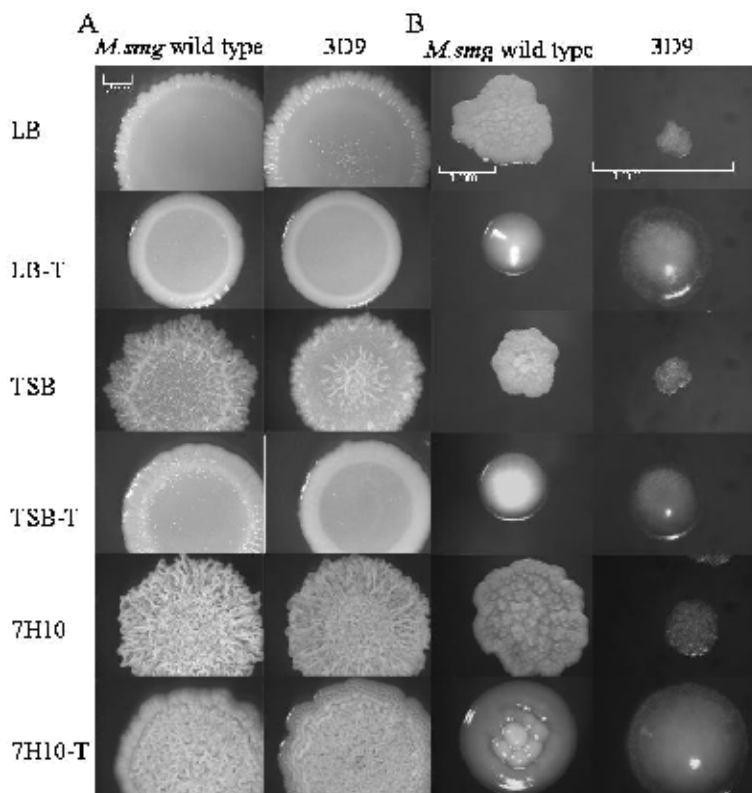


Fig. 5.2 Colony morphology of *M. smegmatis* 3D9 mutant on different agar media (denoted on the left). A. 10µl spot of a stationary phase culture; B. Single colony of each *Tn*-mutant

5.3.2 Lipid analysis of *M. smegmatis* 3D9 mutant

To identify changes (if any) in the lipid profiles of the *M. smegmatis* 3D9 mutant, wild type and mutant strains were labeled with [14 C] acetate and analysed by TLC for polar lipids, non-polar lipids, fatty acids and mycolic acids. Both TSB (plus 0.05% Tween 80) and 7H10 (plus 0.05% Tween 80) broth were used for culturing. As shown in Fig. 5.3, no apparent differences were observed in any of these species.

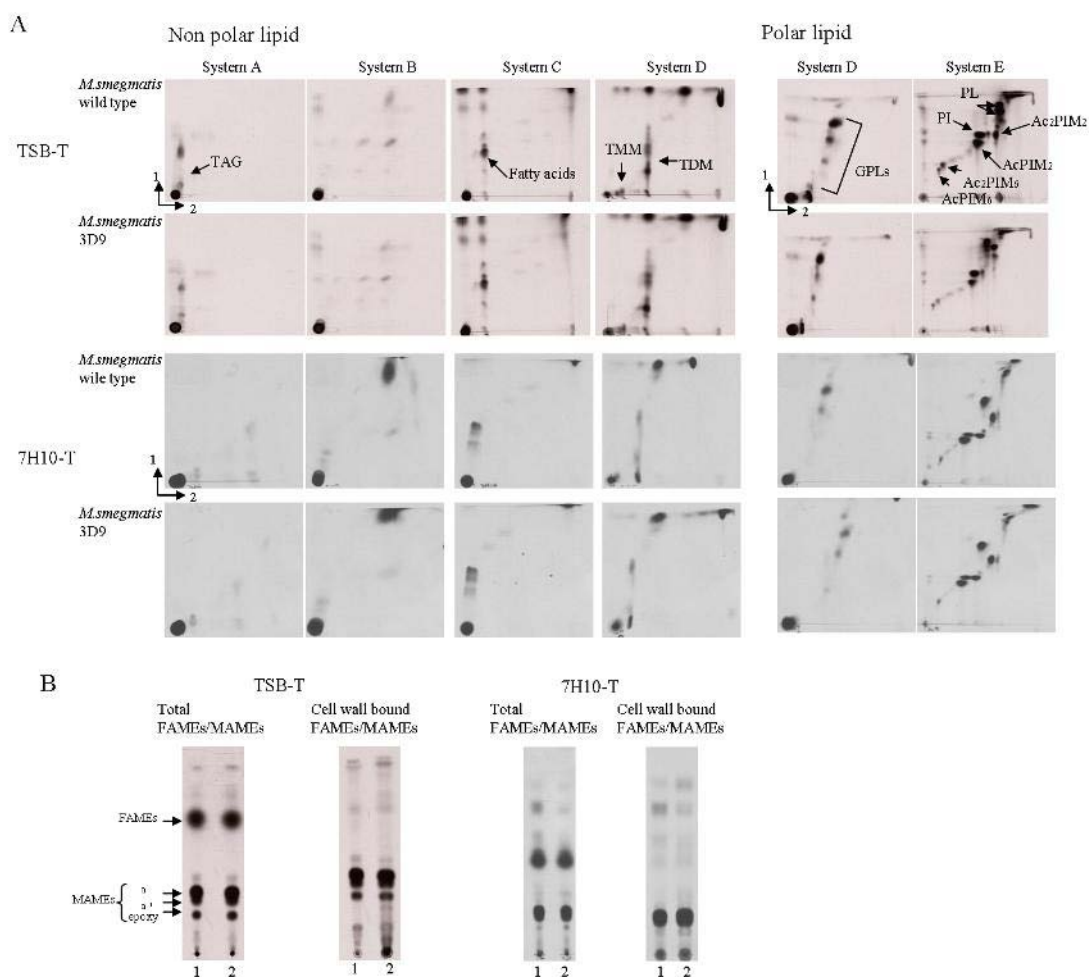


Fig. 5.3 Lipid and FAMES/MAMES analysis of *M. smegmatis icd* (3D9) mutant (TSB-T and 7H10-T). A. 2D-TLC analysis of non-polar and polar lipids. B. 1-D TLC analysis of FAMES/MAMES. 1-*M. smegmatis* wild type, 2-*M. smegmatis* 3D9. Ac₂PIM₂ and AcPIM₂ di- and monoacyl phosphatidylinositol dimannosides, Ac₂PIM₆ and AcPIM₆ di- and monoacyl phosphatidylinositol hexamannosides, GPLs glycopeptidolipids, PI phosphatidyl inositol, PL phospholipid, TDM trehalose dimycolates, TMM trehalose monomycolates, TAG triacylglycerol; FAME fatty acid methyl ester, MAME mycolic acid methyl ester, 1- *M. smegmatis* wild type, 2- *M. smegmatis* 3D9 mutant

5.3.3 Growth characteristics of *M. smegmatis* 3D9 mutant

Apparent colony morphology changes may often be a result of altered growth rates, rather than cell wall alterations. As mentioned above, isocitrate dehydrogenase participates in the TCA cycle which generates usable energy in form of ATP. Thus, it was likely that loss of ICD function in *M. smegmatis* may have affected growth. To test whether this was the case with the *M. smegmatis* 3D9 mutant, the growth rate of the mutant with that of wild type strain was compared. On examination of the growth curve it can be seen that compared with wild type strain, 3D9 mutant achieved a slower rate of growth (Fig. 5.4). When the mutant was complemented with the *M. smegmatis icd* gene, it showed the same growth pattern comparable to that of the wild type strain.

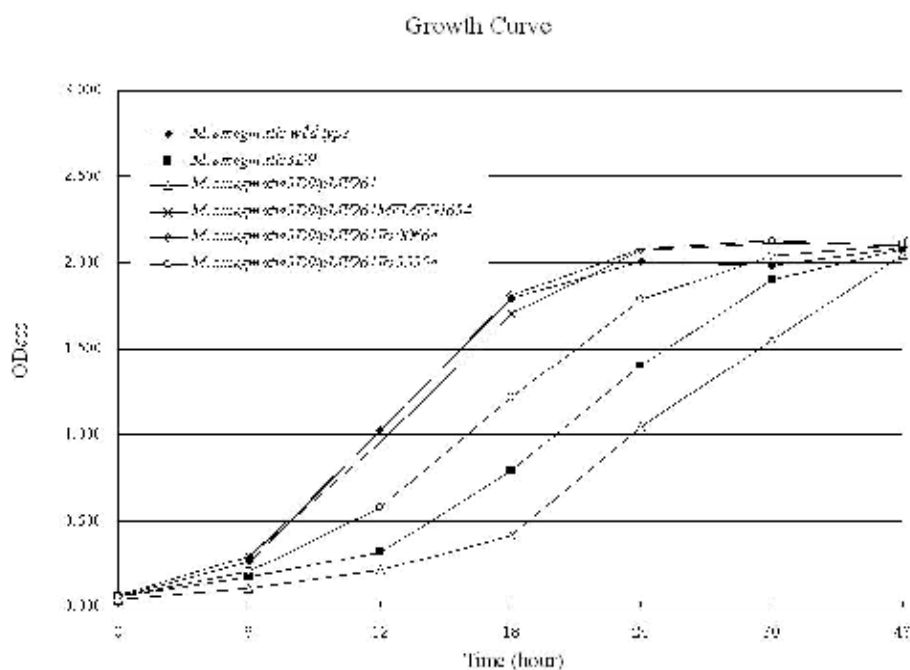


Fig. 5.4 Growth curve of *M. smegmatis* wild type strain, 3D9 mutant and complemented strains. Fresh culture of *M. smegmatis* were diluted in TSB with 0.05% Tween 80 (antibiotics were added when required) to OD₆₀₀=0.1 and incubated at 37°C shaking at 180rpm. The growth of the cultures was monitored by OD₆₀₀ every 6 hours.

Thus, it was likely that the observed altered colony morphology was due to a slower growth rate, rather than an altered cell wall. The possibility remains that AG, LAM or capsular components of the mutant were altered and these remained to be analysed. However, this remains highly unlikely as alterations in these cell wall components are often fatal, or result in extremely drastic changes in colony morphology, none of which were observed in this mutant.

5.3.4 The *M. smegmatis* 3D9 mutant as surrogate for studying mycobacterial *icds*

While the 3D9 mutant did not show any apparent cell wall alterations, it still presented an opportunity to study ICD in a mutant background, and as a surrogate for the *M. tuberculosis* orthologues. Unlike non-pathogenic mycobacteria which have only one *icd* in their genome, *M. tuberculosis* and some other pathogenic mycobacteria have two copies. Phylogenetic analysis based on protein sequence reveals that *M. tuberculosis* ICD1 is much closer to eukaryotic ICDs (Fig. 5.5 A) (Banerjee *et al.*, 2005) while *M. tuberculosis* ICD2 is closer to prokaryotic ones (Fig. 5.5 B) (Steen *et al.*, 2001). However, both of them are NADP⁺-dependent with different physio-chemical properties, such as ionic cofactor, optimal pH and temperature for activity *in vitro* (Banerjee *et al.*, 2005). Current bioinformatics and enzymology data indicates that it is highly possible these two *icd* genes in *M. tuberculosis* may overlap in function in the TCA cycle while play different roles under different conditions. The two *M. tuberculosis icd* orthologues were amplified by PCR and cloned into the vector pMV261. Constructs were confirmed by sequencing before

used in further studies with the *M. smegmatis* 3D9 mutant for functional complementation.

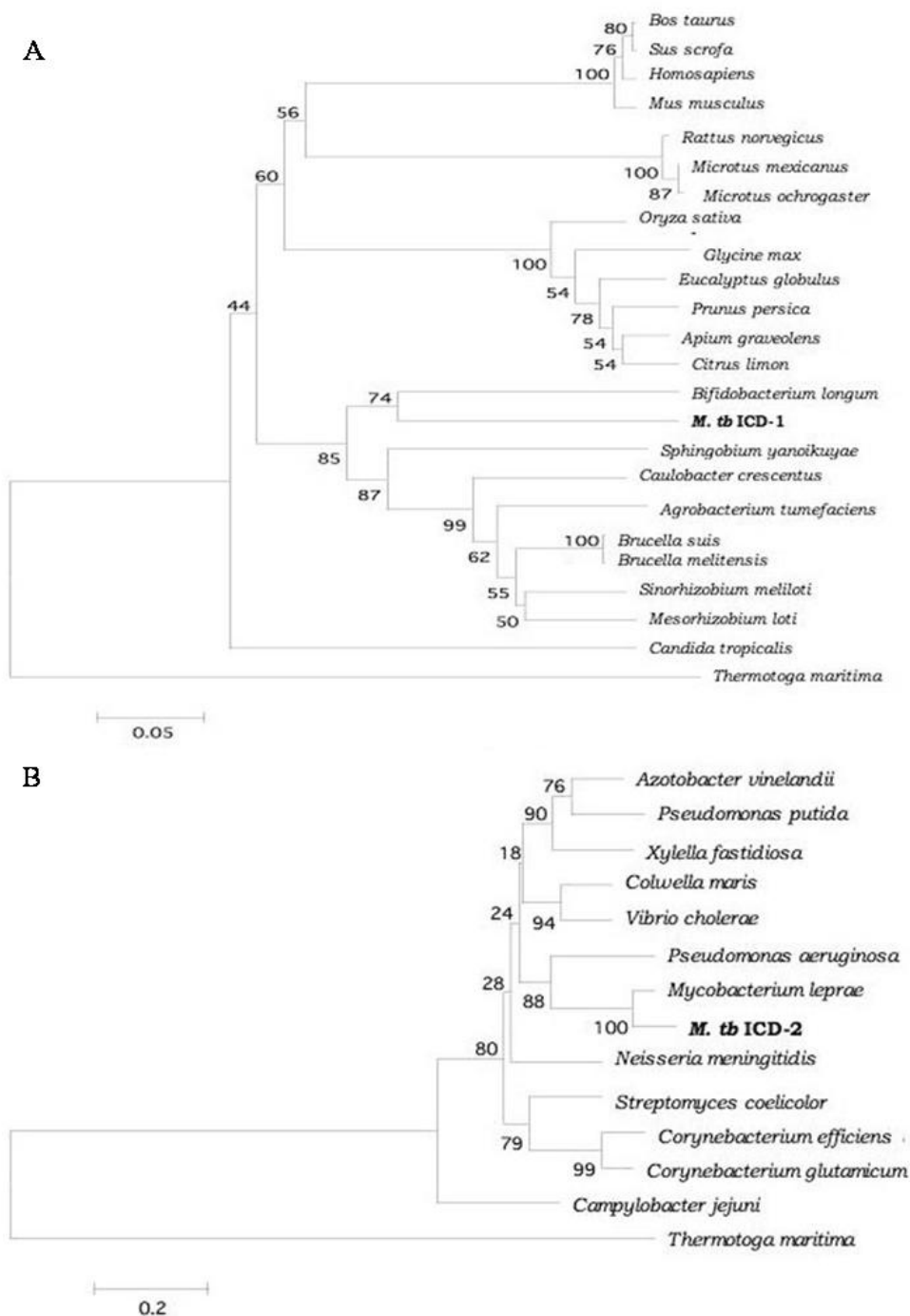


Fig. 5.5 Phylogenetic analysis of *M. tuberculosis* ICD1 and ICD2 (Banerjee *et al.*, 2005). A. ICD1 B. ICD2

5.3.5 The *M. smegmatis* 3D9 mutant as surrogate for studying mycobacterial *icds*: restoration of growth pattern in different carbon sources

To test for functional complementation with the *M. tuberculosis icd* orthologues, the growth curve of the wild type, 3D9 mutant and complemented strains were examined. Results showed that *M. smegmatis* 3D9 mutant complemented with *M. tuberculosis icd2* had a similar growth pattern as the wild type strain, while the mutant complemented with *M. tuberculosis icd1* still grew more slowly than the wild type strain but faster than the mutant (Fig. 5.4). The ability of the 3D9 mutant to grow on single carbon sources, such as sugars and fatty acids, and to observe whether the loss of ICD in *M. smegmatis* would affect the use of different carbon sources was examined. Results showed that when using either glycerol, glucose, acetate, oleic acid or palmitic acid as only carbon source, respectively, *M. smegmatis* wild type strain could grow as normal while the 3D9 mutant and the mutant transformed with pMV261 vector could not. When the mutant was complemented with *M. smegmatis icd* or *M. tuberculosis icd1/icd2*, its ability to grow on each carbon source was restored comparable to the wild type strain (Fig. 5.6).

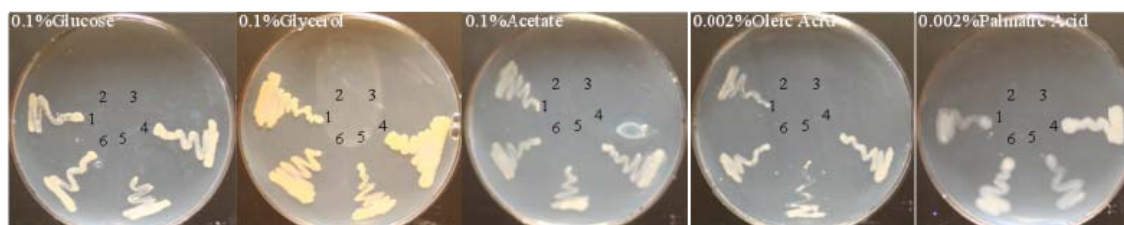


Fig. 5.6 Growth of *M. smegmatis icd* mutant and complementation strains on minimum medium with only carbon source 1-*M. smegmatis* wild type; 2-*M. smegmatis* 3D9, 3-*M. smegmatis* 3D9/pMV261, 4- *M. smegmatis* 3D9/pMV261MSMEG1654, 5- *M. smegmatis* 3D9/pMV261Rv0066c, 6- *M. smegmatis* 3D9/pMV261Rv3339c.

5.3.6 The *M. smegmatis* 3D9 mutant as surrogate for studying mycobacterial *icds*: restoration of ICD activities in *M. smegmatis* lysates

To detect the isocitrate dehydrogenase activity directly in *M. smegmatis*, whole cell lysates of wild type, 3D9 mutant and complemented strains were examined in an *in vitro* enzyme assay. From the results, no isocitrate dehydrogenase activity was detected in the *M. smegmatis* 3D9 mutant and the mutant with only pMV261 vector, whilst the mutant complemented with *M. smegmatis icd* showed high activity. Isocitrate dehydrogenase activity was also detected by using whole cell lysates of the *M. smegmatis* 3D9 mutant complemented with *M. tuberculosis* orthologues. Contrary to expectations, low activity was detected in the mutant complemented with *M. tuberculosis icd2* which was the homologue of *M. smegmatis icd*. In addition, high background (without substrate isocitrate) was also detected compared with the wild type strain and other complemented strains, while the mutant with *M. tuberculosis icd1* showed the highest activity, higher than that of the mutant with *M. smegmatis icd*. The enzyme activity dropped about 90% when MgCl₂ was removed (Table 5.2).

Table 5.2 ICD enzymatic activities in *M. smegmatis* wild type, 3D9 mutant and complemented strains

Strain	Rate($\mu\text{M}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$) 37°C		
	10mM MgCl ₂	No MgCl ₂	No Isocitrate
<i>M. smegmatis</i> wild type	727.37	28.73	N/D
<i>M. smegmatis</i> 3D9	N/D	N/D	N/D
<i>M. smegmatis</i> 3D9/pMV261MSMEG1654	2162.62	328.20	N/D
<i>M. smegmatis</i> 3D9/pMV261Rv0066c*	12.57	2.03	9.08
<i>M. smegmatis</i> 3D9/pMV261Rv3339c	2574.13	658.14	N/D

Reaction condition (1ml): 50mM HEPES (pH 8.0), 0.25mM NADP⁺, 10mM MgCl₂, 1mM isocitrate and *M. smegmatis* whole cell lysate with 50 μg protein, 37°C. *Whole cell lysate with 500 μg protein was used for *M. smegmatis* 3D9/pMV261Rv0066c

5.3.7 Optimal conditions for ICD activity

As reported by Banerjee *et al* (Banerjee *et al.*, 2005), *M. tuberculosis* ICD1 (*Rv3339c*) and ICD2 (*Rv0066c*) both are functional enzymes but with different physio-chemical and kinetic properties. Both of them are NADP⁺-dependent. The eukaryotic clustered ICD1 can use both Zn²⁺ and Mg²⁺ as ionic cofactors, whilst the prokaryotic clustered ICD2 can only use Mg²⁺ with lower affinity as compared to ICD1. ICD1 has a wider range of pH for activity than ICD2. The ICD activity of cell lysates from different *M. smegmatis* strains used in this study were performed under different pH conditions as well as different concentrations of MgCl₂. As shown in Fig. 5.7, except *M. smegmatis* 3D9/pMVRv3339c (Fig. 5.7 D), the cell lysates from other three strains showed higher enzymatic activity with high concentrations of MgCl₂ (Fig. 5.7 A B C), which is consistent with the previous report that ICD1 has higher affinity with this cofactor. When different pH conditions were used for activity assay, lysates from *M. smegmatis* wild type (Fig. 5.7 A) and 3D9 mutant complemented strain with its own ICD (Fig. 5.7 B), demonstrated an increasing ICD activity along with an increase in pH. However, lysate of *M. smegmatis* 3D9/pMVRv0066c (the prokaryotic ICD2 in *M. tuberculosis*) showed higher ICD activity at low pH and decreased along with an increase of pH. Meanwhile, the ICD activity from lysate of *M. smegmatis* 3D9/pMVRv3339c (the eukaryotic ICD1 in *M. tuberculosis*) increased along with an increase of pH from 6.5 to 7.5 and started to decrease at pH 8.0.

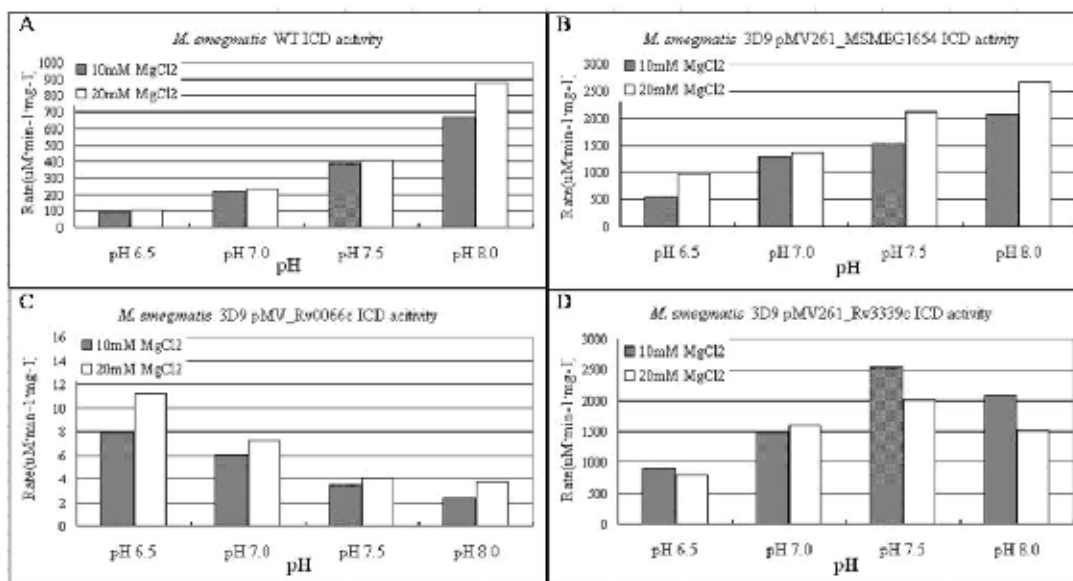


Fig. 5.7 ICD enzymatic activities in *M. smegmatis* wild type strain and complemented *icd* mutant strains under different conditions. *M. smegmatis* whole cell lysate with 50 μ g protein was used except *M. smegmatis* 3D9/pMV261Rv0066c (500 μ g). Data for *M. smegmatis* 3D9 are not included because no ICD activity detected.

5.3.8 Sensitivity of *M. smegmatis* mutant and complemented strains to oxidative and nitrosative stress

It has been reported that ICD contributes to either anti-oxidative stress or nitrosative stress depending on species (Brown *et al.*, 2010; Kriakov *et al.*, 2003). Therefore, the sensitivity to oxidative stress using H₂O₂ was tested by comparing the diameter of the inhibition zone formed by a filter paper disc soaked with different concentrations of H₂O₂ on a lawn of each strain. Inhibition zones could be seen at 40mM H₂O₂, but no distinctive difference between *M. smegmatis* wild type and 3D9 mutant, or complemented strains (Table 5.3).

Table 5.3 Sensitivity to H₂O₂ of *M. smegmatis* mutant and complemented strains

	Diameter of clear zone (cm)	
	60mM H ₂ O ₂	100mM H ₂ O ₂
<i>M. smegmatis</i> wild type	1.0	1.2
<i>M. smegmatis</i> 3D9	1.2	1.4
<i>M. smegmatis</i> 3D9/pMV261MEMEG1654	1.0	1.4
<i>M. smegmatis</i> 3D9/pMV261Rv3339c	1.0	1.2
<i>M. smegmatis</i> 3D9/pMV261Rv0066c	1.2	1.4

Discs soaked with different concentration of H₂O₂ solution were placed on bacterial lawn. Sensitivity was compared by the diameter of clear zone.

Besides oxidative stress, the other mechanism macrophages adopt to eliminate bacterial infection is nitrosative stress. Studies in the fungal pathogen *Cryptococcus neoformans* showed the importance of ICD in resistance to nitrosative stress rather than oxidative stress (Brown *et al.*, 2010). Therefore, a sensitivity test to see whether ICD has a similar function in *Mycobacterium* was performed. As shown in Fig. 5.8, after 2 days, the growth of the 3D9 mutant was inhibited at 2.5mM NaNO₂ by one log less compared to that of wild type strain and the three complemented strains, whilst, at higher concentration, the wild type strain was also inhibited. After 3 days, the growth defect of 3D9 mutant on plates containing NaNO₂ was more distinct, suggesting that the loss of ICD activity may reduce the tolerance to nitrosative stress in *M. smegmatis*. Although the wild type strain can grow on plates containing 5mM or 7.5mM, it still showed one log less compared to complemented strains. Furthermore, on both day 2 and day 3, the mutant complemented with *M. tuberculosis icd2* grew better than the other two complemented strains.

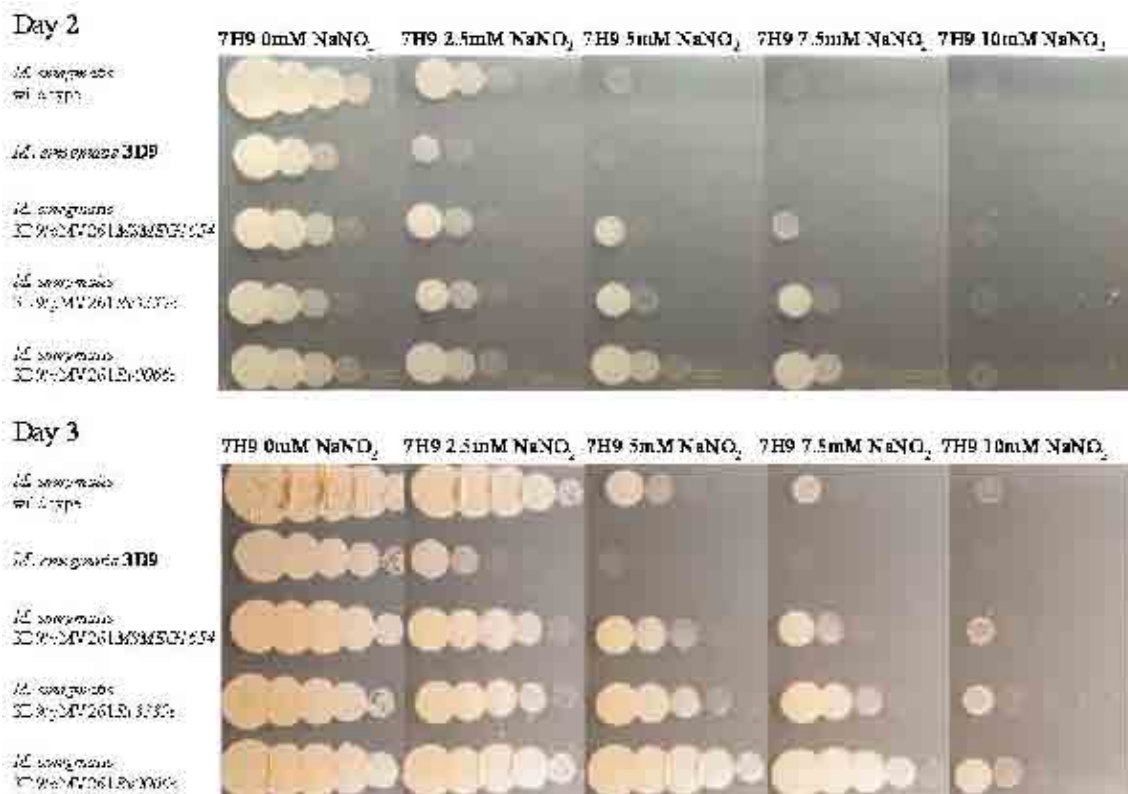


Fig. 5.8 Sensitivity of *M. smegmatis* 3D9 mutant and complemented strains to nitrosative stress (NaNO₂)

5.3.9 Sensitivity of *M. smegmatis* mutant and complemented strains to acid stress

Apart from oxidative/nitrosative stress, acid stress is another important strategy that a macrophage uses to eliminate engulfed pathogens. An *in vitro* assay was done to evaluate the sensitivity to acid stress of the *M. smegmatis* 3D9 mutant and complemented strains with different *icd* genes comparing to the wild type strain. Bacterial cells were incubated in phosphate-citrate buffer (pH4.5) at 37°C and samples were collected after one doubling time and twice the doubling time. Cells were counted by plating out serial dilutions on TSB agar plates. Results showed that more than 70% of the bacteria were killed at pH 4.5 after 8 hours for all strains;

however, no distinct differences between the wild type strain and the mutant, as well as mutant complemented strains with different *icd* gene were observed (Fig. 5.9).

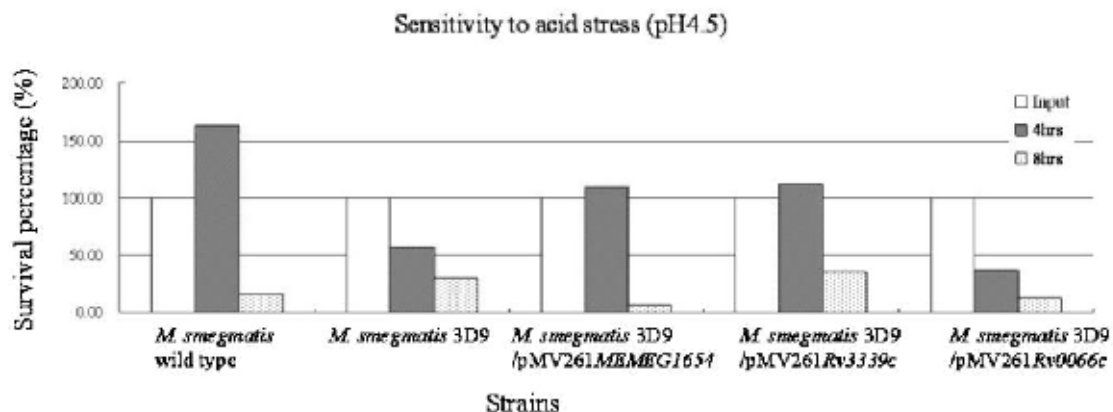


Fig. 5.9 Sensitivity of *M. smegmatis* 3D9 mutant and complemented strains to acid stress (pH 4.5)

5.4 Discussion

Although bacterial metabolic pathways have been studied for some time in the artificial environment of the laboratory, little was known about the metabolism of intracellular bacteria during infection (Munoz-Elias & McKinney, 2005). It was assumed that there should not be any differences between the metabolism of bacteria *in vivo* and *in vitro*. More recent studies have suggested that this may not be the case, since pathogenic bacteria use different nutrients and metabolic pathways during infection (Munoz-Elias & McKinney, 2005; Nathan, 2004). The TCA cycle is a central metabolic pathway in cells. The inactivation of *icd* in *M. smegmatis* resulted in a slow-growing phenotype in a rich medium (TSB) and disabled the growth in minimal media using glucose, glycerol or fatty acid as the sole carbon source. At the

same time, there was no obvious change found in lipids, FAMES and MAMES. Thus, the effect of inactivation of ICD function on growth may be the reason for colony morphology change, but not on changes in cell wall components.

ICL, the first enzyme of the glyoxylate cycle, has been found with two copies in *M. tuberculosis* and reported to be essential for *in vivo* growth and virulence (Gould *et al.*, 2006; Munoz-Elias & McKinney, 2005). Similarly, two ICDs were annotated in the *M. tuberculosis* genome, as well as other pathogenic mycobacteria, but only one in non-pathogenic mycobacteria (Banerjee *et al.*, 2005). It has been shown by *in vitro* enzymatic assays that both have identical function but different biochemical properties and phylogenic affiliations (Banerjee *et al.*, 2005), which suggests that they may play different roles during growth stages and environmental adaptation. The complementation experiments revealed that under the same growth conditions, the function of *M. smegmatis* ICD could be replaced by *M. tuberculosis* ICD2, while only partially by *M. tuberculosis* ICD1. At the same time, results of enzymatic assays with whole cell lysates from the wild type, mutant and different complemented strains showed that: (1) the activity of *M. tuberculosis* ICD2 was much lower than that of *M. tuberculosis* ICD1 and that of *M. smegmatis* ICD in certain *in vitro* assay conditions, with high background when without isocitrate as a substrate; (2) *M. tuberculosis* ICD2 had lower affinity to Mg^{2+} compared to *M. tuberculosis* ICD1 and *M. smegmatis* ICD, which was consistent with previous reports (Banerjee *et al.*, 2005).

It has been reported that ICD may be involved in anti-oxidative stress or other

harsh conditions, although the results in different species are not yet conclusive (Brown *et al.*, 2010; Kriakov *et al.*, 2003). Thus we studied the ability of the *M. smegmatis* wild type, 3D9 mutant and complemented strains with different *icd* genes to survive under, oxidative stress, nitrosative stress, and acid stress (pH 4.5), which are major mechanisms used by macrophages to kill bacteria. The results showed no differences between the wild type and the mutant, or complemented strains with different *icd* genes in terms of resistance to oxidative stress and acid stress, whilst the mutant was more susceptible to nitrosative stress compared to the wild type strain. Besides, a better tolerance of the complemented strains than that of wild type at higher concentrations of NaNO₂ was also observed, which could be the result of over expression of different *icds* by the pMV261 vector, respectively, in complemented strains, similar to the situation found in the enzymatic assays. Interestingly, when comparing the three complemented strains with each other, the mutant complemented with *M. tuberculosis icd2*, the prokaryotic *icd*, showed an even better tolerance.

In previous studies in the fungi pathogen, *C. neoformans*, several enzymes essential for survival from oxidative or nitrosative stress in macrophages have been identified (Chow *et al.*, 2007; de Jesus-Berrios *et al.*, 2003; Giles *et al.*, 2006) and regardless of the mechanism, a common requirement for these enzymes to function is the cofactor NADPH as an electron donor. Further studies revealed that as one source of NADPH production, the ICD, which can be found in cytosol, peroxisomes or mitochondria, is important for resistance to nitrosative stress and suggests that it is the compartmentalisation of NADPH but not the recycling of oxidised antioxidants that is

critical for resistance (Brown *et al.*, 2010). Although, *M. tuberculosis* is a prokaryote, it is still highly possible that the ICD-dependent NADPH production is the mechanism of resistance to nitrosative stress observed in this study.

The *M. smegmatis* 3D9 mutant with a transposon insertion in *icd* provided a useful surrogate for studying the function of *M. tuberculosis* ICD orthologues *in vivo*. *M. tuberculosis* eukaryotic like ICD1 only partially complemented the mutant in growth rate but at the same level as the wild type strain in a nitrosative test, although it had a high enzymatic activity in an *in vitro* assay. On the other hand, the prokaryotic like ICD2 fully complemented the mutant in growth rate and gave better tolerance in the nitrosative test, whilst showed low activity in the *in vitro* assay. Although, no conclusion of the exact function of these two ICDs in *M. tuberculosis* infection can be drawn, these results indicate that these enzymes are involved in mycobacterial survival to nitrosative stress and that the function of these two ICDs, although different, often overlap. To further understand their roles in virulence and intracellular survival, specific knock-out strains of the two *icds* in *M. bovis* BCG will be made, respectively, and complemented with *M. tuberculosis icds*. These strains should prove valuable resources for further probing the individual roles of the two *icd* orthologues in slow growing mycobacteria.

Chapter 6

General Discussion

The distinct lipid-rich cell wall of mycobacteria can not only provide physical protection to the bacilli but can also play an important role in virulence. Several currently used anti-TB drugs target cell wall biosynthetic pathways. Thus a good understanding of the structure and biosynthesis of the cell wall will give helpful clues for the development of novel anti-mycobacterial drug targets, as well as a better understanding of mycobacterium-host cell interactions.

The main objective of the studies reported in this thesis was to study cell wall biosynthesis in mycobacteria. To achieve this, a strategy based on random *Tn* mutagenesis with two different screening criteria, altered colony morphology and mycobacteriophage resistance, were adopted. Two model systems, non-pathogenic *M. smegmatis* and the fish pathogen *M. marinum*, were used for generating *Tn*-mutant libraries. *M. smegmatis* was used for both the colony morphology screen and mycobacteriophage resistance screen, whilst *M. marinum* was used only for morphology screen. A temperature sensitive phage with a mariner-based *Tn5371* was used for *Tn* mutagenesis. The *Tn* insertion site was identified by sequencing and blast searching.

By using different agar media (TSB, LB and 7H10, with or without Tween 80), eight mutants with colony morphology changes from the *M. smegmatis* *Tn*-mutant library were isolated. Among these mutants, two had *Tn* insertions in a gene cluster associated with cell division genes, *pbpA* (penicillin binding protein, *MSMEG0031*) and *fhaA* (*MSMEG0035*, forkhead-associated domain protein). PBPA has been

reported to be involved in PG biosynthesis and regulated by phosphorylation (Dasgupta *et al.*, 2006), whilst the FHA domain can recognise phosphothreonine epitopes on proteins (Durocher & Jackson, 2002). Both of them are in the same operon together with a kinase PknB and a phosphatase PstP. The difference of morphology changes between these two mutants indicates different roles in cell division. Another mutant had a *Tn* insertion in GntR (*MSMEG0268*), a HutC-like sub-family regulator protein, which has been reported to respond to a number of molecules including long chain fatty acids and trehalose 6-phosphate (Allison & Phillips, 1990; Chen *et al.*, 1990; Matthijs *et al.*, 2000; Quail *et al.*, 1994). Two disruptions were found in genes involved in metabolism, *MSMEG1654* (isocitrate dehydrogenase, *icd*) and *MSMEG6756* (glycerol kinase, *glpK*). It is highly likely that these metabolic pathways indirectly affect the biosynthetic pathways that contribute to cell wall components and it is also possible that central metabolic pathways can affect growth which further affects colony morphology. In addition, one mutant with a *Tn* insertion in an ABC transporter (*MSMEG5102*) and another two in unknown membrane proteins (*MSMEG4256*) and a hypothetical protein (*MSMEG3327*) were also isolated.

By using 7H10 agar (with or without Tween 80), twenty *M. marinum* colony morphology mutants were isolated. Amongst the genes identified with a *Tn* insertion, five were involved in cell wall and cell processes, including (i) a hypothetical protein with a homologue of a probable membrane glycosyltransferase in *M. tuberculosis* (*MMAR2327*), (ii) a glycine betaine transport integral membrane protein

(*MMAR2913*), (iii) a lipoprotein LpqF (*MMAR5092*) whose homologue is essential for growth in *M. tuberculosis* but with a unknown function (Sasseti *et al.*, 2003), (iv) a hypothetical protein with a unknown function (*MMAR2352*), and (v) a transmembrane protein (*MMAR325*) whose homologue in *M. tuberculosis* is required in survival in murine macrophages (Rengarajan *et al.*, 2005). Six genes are involved in lipid metabolism. Three of them (*MMAR1767*, *mas*; *MMAR1775*, *ppsB*; *MMAR1774*, *ppsC*) are in the biosynthesis pathway of cell wall lipid/virulence factor, PDIM (Azad *et al.*, 1997; Kruh *et al.*, 2008; Mathur & Kolattukudy, 1992). Two genes are involved in the biosynthesis of fatty acid, a fatty acyl-AMP ligase (*FadD28_1*, *MMAR1180*) whose homologue in *M. tuberculosis* is responsible for production of monomethyl branched unsaturated fatty acids (Dubey *et al.*, 2003) and a methoxymycolic acid synthase 3 (*MMAR0978*) involved in the modification of mycolic acids (Yuan & Barry, 1996). The last one is a fatty acyl-AMP ligase (*MMAR2341*, *fadD25*) that has been suggested involved in lipid degradation in *M. tuberculosis* (Betts *et al.*, 2002). Besides, another two genes involved in intermediary metabolism and respiration, including a phytoene dehydrogenase (*MMAR4806*, *crtI*) involved in the biosynthesis of carotene that has protective functions against photo-oxidative damage (Runyon, 1959) and a serine protease (*MMAR4214*, *htrA* or *degP*), which might be essential for virulence in *M. tuberculosis*, were isolated. One virulence-related Esat-6-like protein (*MMAR5449*), one regulator (*MMAR0992*) and five hypothetical proteins with unknown function were also identified.

Morphology screening used here for mycobacterial cell wall biosynthetic

pathways based on the hypothesis that changes in the composition of the cell wall components would alter the structure and then result in a different colony morphology. According to the results, two out of eight genes identified from the *M. smegmatis* *Tn*-mutants with altered colony morphology, were directly involved in cell wall biosynthesis, whilst eleven out of twenty in the *M. marinum* *Tn*-mutants. Although, the rest of them were not involved directly, they could affect cell wall components indirectly, such as intermediates for cell wall biosynthesis and transporters. Using a random approach based on morphology to screen for cell wall biosynthetic pathways can help to identify not only genes responsible for cell wall components but also genes which have an indirect impact, therefore providing more information for understanding cell wall biosynthesis as a whole picture.

One mutant from each species was then selected for further studies as part of this study. As for *M. smegmatis*, the 3D9 (*icd*, *MSMEG1654*) mutant was selected, since, firstly, this enzyme involved in central metabolism pathways could also be involved in pathogenesis, such as *icl* (Munoz-Elias & McKinney, 2005). Secondly, compared to only one copy in non-pathogenic mycobacteria, pathogenic mycobacteria have two copies of *icd* indicating different functional roles, possibly related to virulence or intracellular survival. From the set of *M. marinum* mutants, the 8G10 (*mmaA3*, *MMAR0978*) mutant was chosen, as it was the homologue of *mmaA3* for *M. tuberculosis* and was potentially involved in the biosynthesis of methoxymycolic acids. This provided an opportunity to study the role of methoxymycolic acids in virulence.

M. smegmatis 3D9 *Tn*-mutant had only *icd* gene disrupted and showed distinct colony morphology changes on 7H10 agar, however no difference was observed in its lipid profile when cultured in TSB, as well as 7H10 broth. Considering that *icd* is a key enzyme in central metabolism cycle, a growth test and a sole carbon source assay were performed and the results showed a slow-growth pattern compared to the wild type strain and a deficiency of growth on a single carbon source medium. Thus, the colony morphology change could be caused by the slow-growth pattern, rather than changes in cell wall components. Since there are two genes annotated as *icd* in *M. tuberculosis*, which have been confirmed with an enzymatic assay *in vitro* (Banerjee *et al.*, 2005), the *M. smegmatis* 3D9 mutant was complemented with *M. smegmatis icd*, as well as both *M. tuberculosis icds* for functional studies *in vivo*. The results showed that both *M. tuberculosis icds* can complement the activity of *M. smegmatis icd* in both growth curve tests and sole carbon source assays, whilst the *M. tuberculosis icd1* (*Rv3339c*) could only partially complement the mutant in the growth curve test. To study further the enzymatic activity of ICD in complemented strains, an enzymatic assay using whole cell lysates was performed. The lysate of the complemented strains all had NADP⁺-dependent isocitrate dehydrogenase activity compared to the mutant, whilst lysates of both *M. smegmatis icd* and *M. tuberculosis icd1* complemented strains showed higher activities than that of the wild type strain, which could be because of the overexpression of ICD by the multi-copy vector pMV261 used for complementation. Although, the lysate of *M. tuberculosis icd2* (*Rv0066c*) showed enzymatic activity, it was much lower than the wild type and the other two

complemented strains and also required more protein for the assays. Interestingly, according to phylogenetic analysis of these two *M. tuberculosis icds*, *icd1* is closer to eukaryotic and *icd2* closer to prokaryotic versions. The above results indicate that the two *icds* in *M. tuberculosis* might have different functions in terms of growth. ICD locates at the junction between the TCA cycle and the glyoxylate shunt. It controls the isocitrate flux between the TCA cycle and the glyoxylate shunt by phosphorylation/inactivation (LaPorte, 1993; Zheng & Jia, 2010). Studies in other species demonstrates that ICD can also be involved in the response to oxidative or nitrosative stress, which is important for intracellular pathogens to survive after phagocytosis by alveolar macrophages (Brown *et al.*, 2010; Kriakov *et al.*, 2003). In this study, the sensitivity to oxidative stress, nitrosative stress, as well as acid stress (pH 4.5) of the *M. smegmatis* wild type, mutant and different complemented strains was examined. There was no differences between the wild type and the mutant or complemented strains with different *icd* genes, in terms of resistance to oxidative stress and acid stress, whilst the mutant was more susceptible to nitrosative stress (NaNO₂) compared to the wild type strain. Furthermore, better tolerance to higher concentrations of NaNO₂ was observed in all the complemented strains and the strain with the *M. tuberculosis icd2* (prokaryotic version of *icd*), showed an even better tolerance.

M. marinum Tn-mutant 8G10 contains a Tn insertion in the promoter region of *MMAR0978*, a gene encoding an O-methyl transferase. Its homologue in *M. tuberculosis*, *mmaA3* (*Rv0643c*), is responsible for the biosynthesis of

methoxymycolic acids (Yuan & Barry, 1996). Lipid analysis of this *M. marinum* 8G10 mutant showed no difference in lipid profiles, whilst the analysis of the sub-classes of mycolates demonstrated a dramatic decrease in methoxymycolate production. This decrease was restored by complementing the mutant with *M. marinum* MMAR0978 and *M. tuberculosis mmaA3 (Rv0643c)*, respectively, indicating MMAR0978 has the same function as *mmaA3* for methoxymycolic acid production. Methoxymycolates together with ketomycolates have been reported to be necessary for virulence in *M. tuberculosis* (Dubnau *et al.*, 2000), although these two groups have never been studied separately. As a pathogenic *Mycobacterium* and fish pathogen, a *M. marinum* methoxymycolic acid deficient mutant will allow us to study the function of methoxymycolic acid in virulence. A murine bone marrow derived macrophage was used to see if there was any difference in intracellular survival between the wild type strain and the mutant and no differences were observed. However, by using the zebrafish infection model, the 8G10 mutant was deficient in infecting zebrafish embryos. This experiment is still ongoing and the mutant will be further analysed in adult fish for virulence and granuloma formation.

In mycobacteria, phages have made an enormous contribution towards developing genetic tools for manipulation of mycobacteria (Bardarov *et al.*, 1997; Jacobs *et al.*, 1989b; van Kessel *et al.*, 2008). Mycobacteriophage infection starts with its binding to a receptor on the bacterial cell surface, given that phage receptors are expected to be cell wall components, the study of phage-resistant mutants defective in phage adsorption is a promising strategy to identify phage receptors, as well as genes

in their biosynthetic pathways. The generalised transducing phage I3 was chosen for screening two *M. smegmatis* *Tn*-mutant libraries for phage resistant mutants, and altogether four I3-resistant mutants were identified. Compared to the wild type strain, phage I3-resistant mutants failed to inject DNA after pre-adsorption with I3 phage, suggesting that the resistance was caused by the failure of initiating infection. Sequencing results showed that all four mutants had *Tn* insertion in the same cluster that were involved in the biosynthesis of GPLs. Lipid analysis further confirmed the loss of GPLs in all four mutants. Meanwhile, by using another two mycobacteriophages, D29 and BxZ1, the resistance to phage I3 was specifically caused by the loss of GPLs, indicating GPLs may be the receptor for phage I3 infection in *M. smegmatis*. GPLs can be sub-classified according to the levels of glycosylation. To further identify the minimal phage I3 receptor in *M. smegmatis*, a set of previously described *M. smegmatis* mutants that produced truncated GPL intermediates (Miyamoto *et al.*, 2006), by specifically deleting three glycosyltransferases *gtf1*, *gtf2*, or *gtf3* in *M. smegmatis*, were analysed. The results showed that the product of *gtf1*, a FATP core containing a single di or tri-*O*-methylated rhamnose, was sufficient for phage I3 binding and initiate the infection as the minimal receptor. This study demonstrated the potential use phage-resistance mutants for cell wall biosynthetic studies. Currently, more than 250 mycobacteriophages have been isolated (Barksdale & Kim, 1977; Stover *et al.*, 1991). Their diversity in genome sequences, morphology and host range (Pedulla *et al.*, 2003) indicate different cell wall components are used as receptors. In addition, a better

understanding of phages receptors may also aid the further development of phage as diagnostic tools in clinical and genetic tools for basic research. A previously reported phage resistance related gene, *mpr* (*MSMEG1236*), was chosen for this study. Although, this gene has been reported to confer phage resistance to phage D29 and L5 when overexpressed in *M. smegmatis* (Barsom & Hatfull, 1996), results indicate that it must be involved in cell wall composition related processes. Unfortunately, there were no differences in the phage resistance phenotype, as well as cell wall components, in both *mpr* knock-out mutant and the *mpr* overexpressed strain.

By using *Tn* mutagenesis, two different strategies, colony morphology and phage resistance have been used to screen for cell wall deficient mutants, using both *M. smegmatis* and *M. marinum* (only for morphology screen). The hypothesis is that altered colony morphology indicates changes in cell wall components and the failure of phage infection is possibly related to the loss of specific cell wall components as receptors. Amongst the genes identified in both screens, the majority were related to cell wall biosynthesis or lipid metabolism, as expected. However, some of them appeared not to be related to the cell wall directly, which offered opportunities to follow up the study and understand colony morphology and cell wall biosynthesis from a different perspective. Therefore, these studies have demonstrated that both strategies showed prospects for mycobacterial cell wall biosynthesis analyses.

Chapter 7

General Material and Methods

7.1 Extraction of genomic DNA - cetyltrimethyl ammonium bromide (CTAB)-lysozyme method

Cells were harvested from a 10ml overnight bacterial culture by centrifugation and washed with a glucose Tris-EDTA (GTE) solution (25mM Tris-HCl pH 8.0, 10mM EDTA, 50mM glucose). Cells were resuspended in 500µl GTE solution with 10 mg/ml lysozyme and incubated at 37°C overnight. After 10% sodium dodecyl sulfate (100µl) and 50µl of 10mg/ml Proteinase K (Sigma, cat. P4914) were added, the mixture was incubated at 55°C for 20 - 40 min. 5M NaCl (200µl) and preheated CTAB solution (160µl) were added and the mixture incubated at 65°C for 10 min. Chloroform extraction was performed twice to exclude protein before DNA was precipitated with 560µl of isopropanol (0.7 volume of total mixture). The DNA pellet was washed with 70% ethanol and air-dried before dissolved in Tris buffer (10 mM Tris-Cl, pH 8.5) or water.

7.2 Preparation of chemical competent *E. coli* cells

A single colony of *E. coli* was inoculated into 2.5ml LB medium and incubated at 37°C overnight. The overnight culture was then used to inoculate 250ml LB medium with 20mM MgSO₄. Cells were grown to OD₆₀₀ of 0.4-0.6 and then harvested by centrifugation at 4500g for 5 min at 4°C. Cell pellets were gently resuspended in 0.4 volume of ice-cold TFB1 (30mM potassium acetate, 10mM CaCl₂, 50mM MnCl₂, 100mM RbCl, 15% glycerol, filter-sterilised and stored at 4°C) and resuspended cells were incubated on ice for 5 min. Cells were harvested by centrifugation as described

previously and were then gently resuspended in 1/25 of the volume of ice-cold TFB2 (10mM MOPS or PIPES, pH 6.5, 75mM CaCl₂, 10mM RbCl, 15% glycerol, filter-sterilised and stored at 4°C). Cells were incubated on ice for 15-60 min and then stored as 100µl aliquots at -70°C.

7.3 Transformation of *E. coli* competent cells

E. coli competent cells were thawed on ice and then the ligation reaction (5µl) was added to the cells. Cells and the ligation reaction were mixed gently and then incubated on ice for 30 min. After heat shock at 42°C for 90 sec, the cells were placed on ice to cool. LB medium (1ml) was added into the tube and incubated at 37°C for about 45 min with shaking. The cells were then plated onto selection plates.

7.4 Preparation of mycobacterial electrocompetent cells

Mid-log phase mycobacterial cells were washed with 1 volume (original volume of culture) of the 10% glycerol (filter-sterilized, pre-cooled) twice and harvested by centrifugation at 4°C. Cells were resuspended in 1/10 volume of 10% glycerol and aliquoted (200µl) for storage at -70°C.

7.5 Electroporation of mycobacteria

Electrocompetent cells were thawed on ice and no more than 5µl of plasmid dissolved in water was added. Cells were placed into a 1mm cuvette and electroporated at 1800kv. TSB (1ml) (for *M. marinum*, 7H9 was used) was added

and cells were recovered at 37°C (for *M. marinum* at 30°C) for at least one generation time before plated on TSB (*M. smegmatis*) or 7H10 (*M. marinum*) agar with antibiotics.

7.6 Radioactive labeling of lipids

Mid-log phase mycobacterial cultures (5ml) were labeled with 50 μ Ci of [¹⁴C] acetate acid (50mCi/ml, Amersham Pharmacia Biotech) and incubated for 4-6 hours. The [¹⁴C]-labeled cells were harvested by centrifugation and washed with PBS before freeze-drying.

7.7 Lipid extraction

[¹⁴C]-labeled polar and non-polar lipids were extracted using the methods described by Dobson *et al* (Dobson *et al.*, 1985). The dried [¹⁴C]-labeled cells were mixed with 2ml of CH₃OH/0.3% NaCl (100:10, v/v) and 1ml of petroleum ether (b.p. 60-80°C) for 15 min. The mixture was centrifuged and the upper layer removed and stored. Petroleum ether (1ml) was added to the lower fraction and the mixture was mixed and centrifuged as previously described. The combined upper layers containing the non-polar lipids were dried and dissolved in 200 μ l of CHCl₃/CH₃OH (2:1, v/v); 5 μ l was dried in a scintillation vial and then mixed with 5 ml scintillation fluid and radioactivity measured. The polar lipids were further extracted by adding 2.3ml of CHCl₃/CH₃OH/0.3 % NaCl (90:100:30, v/v/v) to the lower aqueous layer. The solution was mixed for 1 hour and centrifuged and the supernatant removed and stored.

The above step was repeated twice by the addition of 0.75ml of $\text{CHCl}_3/\text{CH}_3\text{OH}/0.3\%$ NaCl (50: 100: 40, v/v/v) and mixing for 30 min. Defatted cells were kept for further analysis. A solution of $\text{CHCl}_3/0.3\%$ NaCl (1:1, v/v) (2.6ml) was added to the pooled supernatants, mixed for 5 min, centrifuged and the lower layer recovered and dried. The polar lipids were dissolved in 200 μl of $\text{CHCl}_3/\text{CH}_3\text{OH}$ (2:1, v/v); and 5 μl was dried in a scintillation vial and then mixed with 10 ml scintillation fluid and its radioactivity measured.

7.8 Thin layer chromatography (TLC) analysis for lipids

Equivalent amounts (10,000 to 20,000 cpm) of each sample were spotted on TLC plates (5554 silica gel 60F524; Merck) for further 1D- or 2D-TLC analysis using following solution systems. [^{14}C]-labeled lipids were revealed by overnight exposure to Kodak X-Omat AR film.

Table 7.1 Developing system of 2D- TLC analysis for lipid

System ¹	Direction 1	Direction 2
A ²	Petroleum Ether (b.p. 60-80°C)/ Ethyl Acetate	Petroleum Ether (b.p. 60-80°C)/Acetone
	98 : 2 (v/v)	92 : 8 (v/v)
B ²	Petroleum Ether (b.p. 60-80°C)/Acetone	Toluene/Acetone
	92 : 8 (v/v)	95 : 5 (v/v)
C	Chloroform/Methanol	Toluene/Acetone
	96 : 4 (v/v)	80 : 20(v/v)
D	Chloroform/ Methanol /Water	Chloroform/Acetone/ Methanol /Water
	100 : 14 : 0.8 (v/v/v)	50 : 60 : 2.5 : 3 (v/vv/v)
E ³	Chloroform/ Methanol /Water	Chloroform/Acetic Acid/ Methanol /Water
	60 : 30 : 6 (v/v/v)	40 : 25 : 3 : 6 (v/vv/v)

1. System A, B, C and D are for non-polar lipid analysis; System D and E are for polar lipid analysis.
2. Direction 1 of both system A and B was run for 3 times and allow plates to dry between each run.
3. At least 1 hour drying period for direction 1 of system E.

7.9 FAMES and MAMES extraction from defatted cells and whole cells

The [^{14}C]-labeled cells or defatted cells were subjected to alkaline hydrolysis using 15% aqueous tetrabutylammonium hydroxide at 100°C overnight. CH_2Cl_2 (4ml), CH_3I (300 μl) and water (2ml) was added to the reaction mixture followed by mixing for 30 min. The upper aqueous phase was discarded, and the lower organic phase was washed twice with water and evaporated to dryness. MAMES were redissolved in diethyl-ether, and the solution was evaporated to dryness. The final residue was dissolved in CH_2Cl_2 (200 μl) and 5 μl of the resulting solution was used for liquid scintillation counting.

7.10 TLC analysis for FAMES and MAMES

The FAMES/MAMES mixture (10,000 cpm) was subjected to TLC (5554 silica gel 60F524; Merck), developed in petroleum ether (b.p. 60°C-80°C):acetone (95:5, v/v). [^{14}C]-labeled FAMES/MAMES were revealed by overnight exposure to Kodak X-Omat AR film.

Chapter 8

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