Identification of a Novel Mycobacterial Gene Involved in the Synthesis of a Phenolic Glycolipid and its Role in the Prevention of Phagosome Maturation

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Abstract

Pathogenic Mycobacteria persist in an early endosome-like compartment by interfering with late endosomal fusion mediating factors. Studies have unraveled some of the mechanisms employed by mycobacteria to create a niche for themselves in macrophages, but it is widely accepted that they possess an arsenal of weapons to impede phagosome genesis.

M. marinum has gained importance in recent years, as a model organism to study mycobacterial pathogenesis due to its phylogenetic closeness to *M. tuberculosis*. The infection it causes in its natural hosts display characteristic features of tuberculosis, exhibiting blocking of phagosome maturation and granuloma formation.

To gain insight into the genes required for the inhibition of phagosome maturation, *M. marinum* transposon mutant library representing knock outs covering the entire genome was sifted for mutants defective in inhibiting phagosome maturation by designing an elegant screen, which employs magnetic separation. In this process we identified a number of mutants unable to inhibit phagosome maturation and characterised in detail one of these mutants (mutant P1). The colony morphology and sequence analysis revealed that the interrupted gene of mutant P1 *(pmiA)* is likely to be involved in lipid metabolism. The mutant also had a reduced intracellular survival as inferred from the *in vitro* bacterial survival experiments in HMDM and using mice as an *in vivo* model. The mutant completely reverted to its wild-type phenotype when complemented with the respective gene from wild-type *M. marinum*. Thin layer chromatography on the lipids isolated from the mutant showed that the disruption of the gene *pmiA* in mutant P1 leads to the loss of a glycolipid of the outer envelope of *M. marinum* (Robinson N et al., Infect Immun. 2007 Feb;75(2):581-91).

The missing glycolipid was further characterised to be a phenolic glycolicpid (PGL) using mass spectrometry and nuclear magnetic resonance spectroscopy. In order to prove that the lipid is capable of inhibiting phagosome maturation, it was extracted from wild-type *M. marinum*, coated on to hydrophobic beads and chased into human monocyte derived macrophages (HMDM). Characterising the phagosomes containing the beads by western blot analysis and immunofluorescence microscopy proved the lipid to be a key molecule employed by virulent mycobacteria to inhibit phagosome maturation.

Phagosomes were characterised employing an efficient adenoviral transfection system harbouring Rab-GFP fusion proteins to transfect primary phagocytes. This transfection

system enables phagosome maturation to be studied efficiently by fluorescence microscopy in live cells, in contrast to immunostaining which can be performed only on fixed cells.

The gene *pmiA* involved in the biosynthesis of the phenolic glycolipid shows little homology with the gene sequences available through genome databases. It also does not display any signature sequences of proteins with known functions. Therefore, an attempt was made to study its interacting proteins by using Histidine-tag pull down assay. Proteins interacting with pmiA were analyzed by mass spectrometry. A methyl transferase and an isocitrate lyase, both enzymes critically involved in lipid biosynthesis were found to interact with pmiA. Our results prove that genes involved in the synthesis of this phenolic glycolipid are ideal pharmacological targets to design drug interventions against tuberculosis.

1 Introduction

1.1 Tuberculosis

Tuberculosis (TB) is a chronic infectious disease which has afflicted humanity for over 35,000 years. Its etiological agent *Mycobacterium tuberculosis* (*Mtb*) has accounted for more human deaths than any other pathogen to date. TB is also an old disease in terms of its documentation. Descriptions of the pulmonary lesions in patients who had died of consumption were clearly documented by the French physician Rene Theophile Hyacinthe Laennec in his landmark work "A treatise on disease of the chest" in 1821. Another French physician Jean-Antoine Villemin published in 1868 "Etudes sur la Tuberculosis" (studies on tuberculosis) identifying the infectious nature of tuberculosis. Villemin had also provided proof of transmissibility of the disease. The job of identifying the causative agent of TB was left to Robert Koch, who announced his discovery at a meeting of the Physiological Society of Berlin on 24 March 1882. Koch's contributions to the study of TB were enormous. The staining technique developed by Koch still remains an important tool in diagnosing TB. These documentations prove the antiquity of the TB (Daniel, 2006). TB continues to have a devastating impact globally, claiming the lives of approximately 2 million people yearly worldwide. Every year 8 million people become newly infected with the *Mtb* and has been noted that a person becomes infected with the bacilli every second (WHO, 2006). These staggering statistics remain true despite the fact that TB is one of the first infectious diseases for which a vaccine and drugs became available.

1.1.1 TB Pathogenesis

TB predominantly develops in the lung. *Mtb* is transmitted by the expulsion of nasal droplets from an infected individual to an uninfected one. These droplets containing the bacilli are able to reach the alveoli of the respiratory tract. Alveolar macrophages and probably dendritic cells (DC), which screen the mucosa for foreign organisms, ingest the tubercle bacilli. The alveolar macrophages are usually equipped to destroy any invading pathogen. Virulent mycobacteria have devised mechanisms to survive in the hostile environment of these macrophages. Therefore, these alveolar macrophages serve as mobile habitat. It is widely postulated that alveolar macrophages in the airways, are stimulated to invade the lung epithelium following internalization of inhaled bacteria.

The invading macrophages loaded with the bacilli produce tumour necrosis factor alpha (TNF-α) and other inflammatory chemokines, resulting in the recruitment of successive waves of neutrophils, natural killer (NK) T-cells, CD4+ T-cells and CD8+ T-cells. Each of these cells produces their own complement of cytokines and chemokines that amplify cellular recruitment, resulting in the remodelling of the infection site. This inflammatory cascade is regulated and superseded by a specific cellular immune response that is linked to the production of interferon gamma (IFN-γ). At this stage the bacteria multiply inside the macrophages, leading to the recruitment of more and more macrophages and inflammatory cells. This marks the formation of stable granuloma. More mature phase granulomas show marked neo-vascularization and develop an extensive fibrotic capsule that delineates the margin between the macrophages, granulocytes, foamy macrophages and giant cells. The granulomas thus formed can be either progressive or non-progressive. Progressive granulomas in the later stages lose their vascular appearance and become necrotic leading to the development of caseous necrosis. Caseous necrosis starts in the centre of the granuloma, as a result of killing of macrophages in which the bacilli multiply. The size of the caseous lesion is proportional to the bacillary load. One of the most intriguing observations is the relative distribution of bacteria and bacterial products. Several studies noted that although bacteria were found in the central necrotic region of the lesion, a significant proportion of bacteria were associated with macrophages in the peripheral leukocytic infiltrate. These macrophages have been found bordering the necrotic region and outside the fibrotic capsule. One possible explanation could be that the macrophages walling the caseous center ingest the bacteria escaping from the edge of the caseum.

The human tuberculosis granuloma is the product of a cellular immune response to various mycobacterial components. In AIDS patients diminished capacity to mount a CD4+ dependent T-cell response correlates with reduced granuloma forming capacity and consequently a reduced ability to prevent metastasis of infection. Although studies indicate that granuloma structures deal with the containment of infection and transmission as a breakdown of immunoregulation, mycobacteria have a vested interest in driving transmission (Russell, 2007). How does *Mtb* influence this process to maximize its survival and subsequent transmission under strong immune pressure? The propensity of virulent mycobacteria to invade host macrophages by inhibiting

phagosome maturation (PM) is considered central for the pathogen's effective pathogenicity, latency and transmission.

1.2 Virulent Mycobacteria Inhibit Phagosome Maturation

Mtb and other virulent mycobacteria lik *Mycobacterium bovis, Mycobacterium avium* and *Mycobacterium marinum* dwell in macrophages by modifying the phagosome according to their requirements and thus preventing PM. This is a signature mechanism of virulent mycobacteria. A deeper understanding of the different signalling events involved in PM is necessary for the evaluation of mycobacterial principles that participate in the inhibition of PM.

1.2.1 Phagosome Maturation

The process by which cells internalize large foreign particulate matter is termed phagocytosis, which was first popularized by Ilya Metchnikoff by the end of the 19th century. Immunity to many microbial infections depends on the phagocytosis of invading bacteria by specialised phagocytes (Macrophages, DCs and neutrophils) into a plasma membrane derived intracellular vacuole or phagosome. The resulting phagosomes then undergo a series of fission and fusion events that modify the composition of the limiting membrane and their contents by a sequence that resembles the progression of the endocytic pathway. This process is termed phagosome maturation.

Immediately after phagosome formation, the limiting membrane of the phagosome resembles the plasma membrane. Recently proteomic analysis of latex bead containing phagosomes has identified endoplasmic reticulum being another source of membrane, but this proposal is still under debate (Gagnon *et al.*, 2002); (Desjardins, 2003); (Touret *et al.*, 2005). As such the phagosome does not evince bactericidal activity. It undergoes a complete overhaul, resulting in massive changes in its composition due to a progressive maturation process. This process depends critically on the interaction of the phagosome with the endocytic pathway; which is a continuum of organelles ranging from early endosomes (EE) to lysosomes. Phagosomes undergo transient fusion/fission events with the organelles of the endocytic pathway, which are accompanied by selective transfer of tracers from the lumen of endosomes to phagosomes. These observations have led to the proposal of a "kiss and run" hypothesis (Desjardins, 1995).

The first endocytic organelle phagosomes engage with is the EE or the sorting endosomes (SE). Several studies indicate that the early phagosomes and EE coalesce. These vacuoles are often tubulovesicular and can be typically recognised by the presence of Rab5 GTPase, early endosomal antigen 1 (EEA1) and transferin receptors (Tfr). The lumen of EE is relatively poor in proteases and is mildly acidic with a pH of 6.0. From EE or SE cargo can be either sorted out to the recycling endosomes earmarked by the presence of Rab11 GTPase or to the late endosome (LE). Killing of pathogens and MHCII presentation of antigens to T-cells can be accomplished only if the cargo is sorted to the late phagosomes which resemble the LE. These LEs are more acidic with a pH of 5.5 and are comparatively enriched in hydrolytic enzymes. LEs can be identified by their multivesicular nature, the presence of Rab7 GTPase, Rab9 GTPase, lysobisphosphatidic acid and lysosome associated membrane protein (LAMP) (Vieira *et al.*, 2002). Rab7 is considered vital for the interaction of phagosomes with LE by associating itself with dynein-dynactin (microtubule associated motor complex), through Rab7 interacting lysosomal protein (RILP). The motors promote the extension of tubules towards the late endocytic compartment (Jordens *et al.*, 2001). The final stage in the phagosomal degradation pathway is the formation of phagolysosomes by fusing with lysosomes. The lysosomes contain the bulk of the proteases and lipases and are extremely acidic (pH < 5.5). Lysosomes contain LAMPs and hydrolytic enzymes such as cathepsin-D, β-galactosidase etc. These enzymes facilitate the degradation of the pathogen and in the loading of peptides on to MHCII. NADPH oxidase also assembles in the phagosomes catalysing the formation of reactive oxygen intermediates. It also contributes an optimal environment for the activation of proteases. Due to different stages PM appears as a complex biological process that is nevertheless performed elegantly (Vieira *et al.*, 2002).

1.2.2 Methods to Study Phagosome Maturation

Different stages of phagosome maturation have been analysed by investigating the proteins that are present on the phagosome/endosome. As described earlier (1.2.1), each stage is marked by the presence of different Rab GTPases. Therefore, Rab GTPases can be used as markers to study PM (Deretic *et al.*, 1997). PM has been studied on isolated phagosomes of infected phagocytes, using autoradiography and western blot analysis. However, fluorescence microscopy is at the present time, probably the most widely

used imaging technique to study association of stage-specific proteins with the phagosomes/endosome, as it enables the molecular composition of the structures being observed to be identified through the use of fluorescently-labelled probes of high chemical specificity such as antibodies. These bind stably and specifically to their corresponding antigen, they are invaluable as probes for identifying a particular molecule in cells, tissues or biological fluids. Antibody molecules can be used to locate their target molecules accurately in cells. When the primary antibody itself or the secondary anti-immunoglobulin antibody is labelled with a fluorescent dye the technique is known as immunoflurescence microscopy. However, use of antibodies is mainly confined to studies of fixed specimens because of the difficulties of introducing antibody complexes into living specimens. Non-specific binding of antibodies to different proteins of the cell is also possible, which can mislead the investigator. For proteins that can be extracted and purified in reasonable amounts, these difficulties can be circumvented by directly conjugating a fluorophore to a protein and introducing this back into a cell. It is assumed that the fluorescent analogue behaves like the native protein and can therefore serve to reveal the distribution and behaviour of this protein in the cell.

An exciting new development in the use of fluorescent probes for biological studies has been the development of the use of naturally fluorescent proteins as fluorescent probes. The jellyfish Aequorea victoria produces a naturally fluorescent protein known as green fluorescent protein (GFP). The gene for this protein has been cloned and can be transfected into other organisms. This can provide a very powerful tool for localizing regions in which a particular gene is expressed in an organism, or in identifying the location of a particular protein. Surprisingly, in many cases these chimeric proteins preserve their original function. It is therefore often possible to use this technique to visualize the intracellular distribution of a protein. An outstanding advantage of the GFP technique is that living, unstained samples can also be observed. There are presently several variants of GFP which provide spectrally separable emission colours (Heim and Tsien, 1996).

Gene delivery into primary macrophages is a major hurdle in the use of GFP-fusion proteins to study protein distribution in these cells. As they are terminally differentiated cells and do not divide, retroviral vectors fail to transfect efficiently (Haddada *et al.*, 1993). Hence PM has not been studied using protein markers fused to fluorescent

proteins in primary macrophages. A better transfection protocol would greatly enable researchers to study PM in primary phagocytes.

1.2.3 Intracellular Signals Targeted by Virulent Mycobacteria to Inhibit Phagosome Maturation

The fact that pathogenic mycobacteria dwell in a hostile environment of the macrophages, occupying a naive phagosomal compartment and avoiding the default pathway of phagosome maturation was first demonstrated by Armstrong and Hart using tracers that end up in the phagolysosomes (Armstrong and Hart, 1975). Phagocytosis of mycobacteria involves multiple receptors. The mode of entry has been considered to predetermine the subsequent fate of mycobacteria. Mycobacteria opsonised with specific antibodies and taken up via Fcγ receptors end up in phagolysosomes. However, experiments blocking individual receptors did not show differential trafficking. Early studies have defined the markers of phagosomes containing mycobacteria. Table: 1 gives a detailed compilation of proteins and lipids studied till date. The most salient features of mycobacterial phagosomes are that they fail to acidify below pH 6.2 and are characterised by the absence of lysosomal associated membrane proteins (LAMP), lysosomal hydrolases, reduced levels of ATPase and retention of early endosomal markers Rab5 (Russell, 2001); (Via *et al.*, 1997). Phagosomes containing mycobacteria were found to tether TACO or mouse coronin. This was thought to be a reason for the defective PM. Other independent groups either found coronin insufficient to halt mycobacterial phagosomes from maturing or could not confirm the role of coronin. Recent studies indicate that mycobacteria effectively arrest phagolysosomal biogenesis by suppressing sphingosine 1-phosphate regulated cytosolic Ca2+ rise ((Malik *et al.*, 2001); (Malik *et al.*, 2003)), which in turn hinders recruitment of Phosphoinositol-3-kinase (PI3K) or yeast hVPS34 to the phagosomal membrane. Aberrant mustering of hVPS34 inhibits tethering of EEA-1 or Hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) to the mycobacterial phagosome (MP), leading to an obstruction in the delivery of lysosomal ingredients from trans golgi network to the MP (Deretic *et al.*, 2004). Actin is an important factor that mediates the fusion of phagosomes with the organelles of the endocytic pathway. Pathogenic mycobacteria are capable of disrupting the actin filament network surrounding the mycobacterial phagosome (Guerin and de Chastellier, 2000). This disruption of actin nucleation surrounding the MP has been

observed to be complemented by the addition of selected lipids (arachidonic acid, phosphoinositol-4,5-bisphosphate (PtdIns(4,5)P_2), ceramide, sphingosine and sphingosine-1-phosphate) at low and high ATP concentrations. Another important molecule in PM is cAMP. Levels of cAMP in phagosomes are regulated by protein kinaseA (PknA). Lowering cAMP or inhibiting PknA leads to increased actin nucleation and phagosome-lysosome fusion and thereby increased killing of pathogenic mycobacteria (Kalamidas *et al.*, 2006). *M. avium* and *Mtb* containing phagosomes also become gradually depleted for selective cell surface derived glycoproteins and probably mycobacteria themselves revert back to reside in immature phagosomes from phagolysosome (Pietersen *et al.*, 2004). Mycobacteria thus target multiple signalling events in a disciplined fashion using their complex machinery. Understanding the armoury used by mycobacteria to prevent PM is imperative in designing new therapeutics or vaccines to tackle TB.

Table 1 Regulators of membrane trafficking and maturation markers on mycobacterial phagosomes

Protein or lipid	Latex Bead phagosome	Mycobacter Phagosome	Cellular function or location	References
Maturation markers				
Cathepsin D	+	+[a]	Lysosomal hydrolase	(Sturgill-Koszycki *et al.*, 1996)
CD63 (LIMP-1)	+	−	Lysosomal marker	(Clemens and Horwitz, 1995)
V_1 H^+ATPase (E subunit)	+	−	Head group of vacuolar H^+ATPase (ATP hydrolysis), acidification of phagosome	(Sturgill-Koszycki *et al.*, 1994)
V_o H^+ ATPase (16-kDa proteolipid; 110-kDa accessory protein)	+	−	Transmembrane portion of vacuolar H^+ATPase (H^+ translocation), acidification of phagosome	(Fratti *et al.*, 2003b); (Sturgill-Koszycki *et al.*, 1996)
LAMP-1, LAMP-2	+	+/−[b]	Lysosomal markers	(Clemens and Horwitz, 1995); (Xu *et al.*, 1994)

Table 1 Regulators of membrane trafficking and maturation markers on mycobacterial phagosomes

Protein or lipid	Latex Bead phagosome	Mycobacter Phagosome	Cellular function or location	References
Mannose 6 phosphate receptor	+	−	Lysosomal hydrolase trafficking	(Xu *et al.*, 1994); (Barker *et al.*, 1997)
Transferrin receptor (TfR) and transferrin	+[c]	+[c]	Recycling endosome markers and iron delivery	(Clemens and Horwitz, 1996); (Sturgill-Koszycki *et al.*, 1996); (Via *et al.*, 1997); (Kelley and Schorey, 2003)
Small GTP-binding proteins				
ARF6	+[c]	+[c]	Endosomal trafficking, actin remodeling, cell shape	(Niedergang *et al.*, 2003)
LRG-47	+[d]	+[d]	Specifically induced by IFNγ	(MacMicking *et al.*, 2003)
Rab2	+[e]	ND	ER to golgi traffic	(Garin *et al.*, 2001)
Rab4	–[e,f,g]	–[e,f]	Recycling endosome, fast recycling	(Garin *et al.*, 2001), (Via *et al.*, 1997)[f]
Rab3c	+[e]	ND	Unknown	(Garin *et al.*, 2001)
Rab5	+[h]	+[h]	Early endosome fusion	(Via *et al.*, 1997)
Rab7	+	−	Late endosome transport	(Via *et al.*, 1997)
Rab9	+[f]	–[f]	Late endosome to TGN retrograde transport	(Fratti *et al.*, 2003b); (J. Chua et al., unpublished data)[f]
Rab10	+[e,f]	+[f]	Unknown	(Garin *et al.*, 2001)[f]

Table 1 Regulators of membrane trafficking and maturation markers on mycobacterial phagosomes

Protein or lipid	Latex Bead phagosome	Mycobacter Phagosome	Cellular function or location	References
Rab11a	+	+	Recycling endosome	(Fratti *et al.*, 2003b)
Rab11b	+[e]	ND	Recycling endosome	(Garin *et al.*, 2001)
Rab14	+[e,f,i]	+[f,i]	Unknown	(Garin *et al.*, 2001); (J. Chua et al.,unpublished data)[f]
Rab22	+[f,i]	+[f,i]	Unknown	(J. Chua et al., unpublished data)[f]
Rab interacting proteins				
Guanine nucleotide dissociation inhibitor (GDI)	−	+	Maintains Rab5 in GDP-bound (inactive) form	(Fratti *et al.*, 2003a)
EEA1	+	−	Early endosome tethering molecule	(Fratti *et al.*, 2001)
Rabaptin-5	−	−	Rab5 effector	(Fratti *et al.*, 2001)
Rab7-interacting lysosomal protein (RILP)	+[j]	ND	Dynein motor endosomal movement	(Harrison *et al.*, 2003)
SNAREs and interacting proteins				
Cellubrevin	+[k]	+[k]	Recycling endosome v-SNARE	(Fratti *et al.*, 2002)
Endobrevin	+	+	Recycling endosome v-SNARE	(R.A. Fratti et al., unpublished data)

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Protein or lipid	Latex Bead phagosome	Mycobacter Phagosome	Cellular function or location	References
N-ethylmaleimide-sensitive fusion protein (NSF)	+	+	ATPase essential for membrane fusion	(Fratti *et al.*, 2002)
alpha-soluble NSF attachment protein (αSNAP)	+	+	NSF adaptor essential for membrane fusion	(Fratti *et al.*, 2002)
SNAP23	+	+	Plasma membrane, early endosome t-SNARE	(Fratti *et al.*, 2002)
Syntaxin 3	+	+	Plasma membrane t-SNARE	(Fratti *et al.*, 2003b)
Syntaxin 4	+[c]	+[c]	Plasma membrane and endosome t-SNARE	(Vergne *et al.*, 2004b); (R.A. Fratti et al., unpublished data)
Syntaxin 6	+	−	TGN t-SNARE	(Fratti *et al.*, 2003b)
Syntaxin 7	+	+	Late endosome t-SNARE	(Defacque *et al.*, 2000); (R.A. Frattiet al., unpublished data)
Syntaxin 8	+	+	Early endosome, late endosome t-SNARE	(Fratti *et al.*, 2003b)
Syntaxin 13	+	+	Early endosome t-SNARE	(Fratti *et al.*, 2001), (Defacque *et al.*, 2000)
Cytoskeleton proteins and motors				
Actin	+[e]	−	Microfilaments	(Anes *et al.*, 2003), (Garin *et al.*,

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Protein or lipid	Latex Bead phagosome	Mycobacter Phagosome	Cellular function or location	References
				2001)
Annexins	+[l]	+/–[l]	Unknown	(Diakonova *et al.*, 1997), (Pittis *et al.*, 2003)
Coronin/TACO	+[m]	+[m]	Actin-binding protein participating in phagocytosis	(Pieters, 2001)[n]; (Tailleux *et al.*, 2003)
Dynein and dynactin	+	ND	Microtubule motor	(Blocker *et al.*, 1997)
Ezrin/moesin	+	ND	Actin assembly	(Defacque *et al.*, 2000)
Kinesin	+	ND	Microtubule motor	(Blocker *et al.*, 1997)
Myosins	+	ND	Actin motor	(Al-Haddad *et al.*, 2001)
Tubulin	+[e]	ND	Microtubules	(Garin *et al.*, 2001)
Signaling proteins				
Calmodulin	+[n]	–	Ca^{2+}-binding sensor	(Malik *et al.*, 2001)
CaMKII/Phospho CaMKII	+[n]	–	Calmodulin effector protein kinase	(Malik *et al.*, 2001)
Lipids and lipid modifying enzymes				
Cholesterol	ND	+[o]	Lipid rafts	(Gatfield and Pieters, 2000)

Table 1 Regulators of membrane trafficking and maturation markers on mycobacterial phagosomes

Protein or lipid	Latex Bead phagosome	Mycobacter Phagosome	Cellular function or location	References
Diacylglycerol (DAG)	+[j]	ND	Signaling lipid on plasma membrane	(Botelho *et al.*, 2000)
Lysobisphosphatidic acid (LBPA)	+	−	Late endosome, multivesicular bodies	(Fratti *et al.*, 2001), (Fratti *et al.*, 2003a)
Phosphatidylinositol 3-phosphate (PI3P)	+[p]	+/−[p]	Signaling lipid on endosomes	(Vieira *et al.*, 2001); (J. Chua et al., unpublished data)[p]
Phosphatidylinositol 4-phosphate (PI4P)	−[f]	−[f]	Precursor of PI(4,5)P_2	(J. Chua et al., unpublished data)[f]
Phosphatidylinositol 4,5-bisphosphate (PI(4,5)P_2)Z	+[f,q]	+[f,q]	Signaling lipid on plasma membrane	(Botelho *et al.*, 2000); (J. Chua et al., unpublished data)[f]
Phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P_2)	+[f,q,r]	+[f,q,r]	Signaling lipid on plasma membrane	(Marshall *et al.*, 2001); (J. Chua et al., unpublished data)[f,r]
MTM1	−	−	PI3P phosphatase	(J. Chua et al., unpublished data)[f]
MTMR3	+	+	PI3P phosphatase	(J. Chua et al., unpublished data)[f]
Phosphatidylinositol 3-Kinase (p85 unit), Type I	+[j,q]	ND	Regulatory subunit, synthesis of PI(3,4,5)P_3	(Marshall *et al.*, 2001)
Phosphatidylinositol 3-Kinase (hVPS34), Type III	+[s]	+[s]	Catalytic subunit, synthesis of PI3P	(Fratti *et al.*, 2001),(Vieira *et al.*, 2001)
Phosphatidylinositol phosphate kinase	+[j,q]	ND	Synthesis of PI(4,5)P2	(Botelho *et al.*, 2000)

Table 1 Regulators of membrane trafficking and maturation markers on mycobacterial phagosomes

Protein or lipid	Latex Bead phagosome	Mycobacter Phagosome	Cellular function or location	References
(PIKIα), Type I				
Phospholipase C (PLCγ)	$+^{j,q}$	ND	Signaling enzyme recruited to the plasma membrane	(Botelho *et al.*, 2000)
PTEN	$-^{j}$	ND	Terminates PI(3,4,5)P_3 signal, produces PI(4,5)P_2	(Marshall *et al.*, 2001)
SHIP1	$+^{j,q}$	ND	Terminate PI(3,4,5)P_3 signal, produces PI(3,4)P_2	(Marshall *et al.*, 2001)
Biosynthetic pathway				
Calnexin	+	+	ER chaperone	(Gagnon *et al.*, 2002), (Tailleux *et al.*, 2003)
Calreticulin	$+^{t}$	ND	ER chaperone	(Gagnon *et al.*, 2002), (Henry *et al.*, 2004))^{t}
Sphingomyelin	+	−	Sphingolipid made in Golgi	(Fratti *et al.*, 2003b)
Antigen presentation and recognition				
CD1a, b, and c	$+^{u}$	$+^{u}$	Lipid antigen presentation	(Schaible *et al.*, 2000)
MHC class I	$+/-^{v}$	$+/-^{v}$	Cytosolic antigen presentation	(Clemens and Horwitz, 1995)
MHC class II	$+/-^{v}$	$+/-^{v}$	Endosomal antigen presentation	(Clemens and Horwitz, 1995), (Ullrich *et al.*, 2000)

Table 1 Regulators of membrane trafficking and maturation markers on mycobacterial phagosomes

Protein or lipid	Latex Bead phagosome	Mycobacter Phagosome	Cellular function or location	References
Toll-like receptors (TLR)	+	ND	Pathogen-associated molecular patterns recognition and initiation of innate immune responses	(Underhill *et al.*, 1999)

[a]Immature intermediate form.

[b]Variable results.

[c]Syntaxin 4, ARF6, and TfR accumulate on mycobacteria phagosomes, whereas they are transiently present on latex bead phagosomes.

[d]LRG-47 expression is induced in IFNγ-activated macrophages.

[e]Proteomic analysis of purified latex bead phagosomal preparations (Garin *et al.*, 2001).

[f]Time-lapse 4D confocal microscopy with GFP fusion protein in RAW-transfected cells.

[g]Rab4 was not detected on mycobacteria and latex bead phagosomes by Western blot on isolated phagosomes (Via *et al.*, 1997). However, it was detected on latex bead phagosomes using a proteomic approach (Garin *et al.*, 2001).

[h]Rab5 accumulates on mycobacterium phagosomes but not on latex bead phagosomes.

[i]Different dynamics between latex bead and mycobacterium phagosomes.

[j]IgG-opsonized 3-μm beads used for model phagosome (instead of 1-μm beads).

[k]Cellubrevin is present on both mycobacteria and latex bead phagosomes, but a putative degradation product is present on mycobacteria phagosomes.

[l]Less annexin I, VI, VII, and XI on mycobacteria phagosomes than on latex bead phagosomes.

[m]Coronin, termed TACO by Pieters and colleagues, appears to be selectively accumulated on mycobacterial phagosomes (Pieters, 2001).

[n]Dead mycobacteria used for model phagosome (instead of 1-μm latex beads).

[o]Cholesterol enrichment during mycobacteria entry into macrophages.

[p]Time-lapse confocal microscopy with GFP fusion protein probe for PI3P in RAW-transfected cells. Different dynamic and profiles between 1-μm latex bead and mycobacteria phagosomes (J. Chua et al., unpublished data).

[q]Present on phagocytic cups but absent upon completion of the phagosomal closure.

[r]PI(3,4,5) P_3 is present on phagocytic cups of mycobacteria and IgG-opsonized 3-μm beads but not on complement-opsonised 1-μm bead phagosomes.

[s]Western blot of isolated phagosomes shows that hVPS34 is present on latex bead and mycobacteria phagosomes but at different time points.

[t]YFP-tagged ER targeting and retrieval sequence of calreticulin failed to colocalize with erythrocyte phagosomes (Henry *et al.*, 2004).

[u]CD1a and c are found on mycobacterial phagosomes, whereas CD1b is found only on phagolysosomes.

[v]Different profiles depending on the macrophages (human versus murine and resting versus IFNγ-activated).

Adapted from (Vergne *et al.*, 2004a)

1.3 Genetic Tools to Study Mycobacterial Virulence Factors

The first condition of Koch's molecular postulates was to isolate a mutant bacterium with a phenotype that differs from wild-type phenotype. This postulate was fulfilled with the isolation of BCG (bacillus Calmette-Guèrin) by Calmette and Guèrin in 1908. To prove that a phenotype such as virulence is caused by the presence and expression of specific gene, it is necessary to (i) isolate a mutant bacterium with a phenotype that differs from the wild-type phenotype, (ii) to clone the wild-type gene and (iii) by introducing the wild-type gene back into the mutant bacterium, to reproduce the wild-type phenotype. These points have been considered by molecular biologists as Koch's molecular postulates. Methods to fulfil molecular postulates of Koch, namely to clone the wild-type gene and introducing the wild-type gene back into the mutant bacterium, to reproduce the wild-type phenotype were not possible until quite recently. Unique characteristics of mycobacteria like that of its slow growth rate and unusually rich lipid cell envelope were major obstacles in the development of genetic methodologies to study this organism (Jacobs and Bloom, 1994).

Approaches for studying mycobacterial pathogenesis include (a) analysis of mycobacterial gene expression under specific growth and environmental conditions, (b) the use of information derived from genome determinations to identify homologues of virulence genes of other organisms, (c) generation of mutations in specific genes hypothesised to be involved in pathogenesis and comparison of intracellular survival and growth between mutant and wild-type strains, and (d) screening of libraries of randomly generated mutants for altered ability to survive and grow intracellularly.

1.3.1 Gene Expression Analysis

In past years studies on mycobacterial gene expression during its growth in different environments have augmented our understanding of mycobacterial physiology. Many of

these studies have been performed on mycobacteria grown under *in vitro* conditions designed to simulate *in vivo* situations. However, advancements in molecular biological techniques have enabled investigators to use animals or human cells in culture to analyze *in vivo* gene expression.

Investigators have used complementary DNA (cDNA) subtractive hybridization to identify genes differentially expressed by bacteria grown in various environments or closely related bacterial species. This method subtracts out common cDNA molecules and leaves cDNA molecules corresponding to genes that are differentially expressed or uniquely expressed by mycobacteria in a specific environment. cDNA-RNA subtractive hybridization was used to identify differentially expressed genes by comparing messenger RNA (mRNA) levels in virulent *Mtb* strain (H37Rv) with that of an avirulent *Mtb* strain (H37Ra) (Kinger and Tyagi, 1993). In another study, cDNA-cDNA subtractive hybridization was used to compare differential gene expression patterns of *M. avium* grown in macrophages compared to that of *M. avium* grown in broth. This is the only published study in which c-DNA subtractive hybridization was performed on cultured primary macrophages (Plum and Clark-Curtiss, 1994). Selective capture of transcribed sequences, an improvement of c-DNA subtractive hybridization was developed for analysing bacterial gene expression during growth in HMDM (Graham and Clark-Curtiss, 1999). This technique was also used to study the genes expressed by *Mtb* and *M. avium* in common that are important for survival and growth in HMDM (Hou *et al.*, 2002).

Another approach for identifying genes that are differentially expressed in specific environments has been the generation of libraries of DNA fragments cloned into plasmids upstream of a promoterless reporter gene. The principle of this assay is that some of the clones in the library will possess promoter sequences which can be induced in response to specific environmental conditions resulting in the expression of the reporter gene. The reporter gene should encode a product which is stable and easy to assay in mycobacteria. Reporter genes namely, *cat*, *lacZ*, *phoA* and *xylE* have been used by different groups (Kinger and Tyagi, 1993). More recently gene encoding GFP has been extensively used by several groups to assess induction of the respective genes in response to various environmental conditions. In contrast to other reporter genes, GFP does not require the addition of substrates or cofactors, thus allowing this reporter to be used when studying live bacteria. In addition to facilitating studies on broth grown

bacteria, GFP reporter systems have been used to study gene expression in mycobacteria growing in cultured cells and in animal tissues. A GFP reporter system was also used in promoter trap libraries of *M. marinum* DNA fragments, leading to the identification of virulent genes expressed specifically in granulomas (Ramakrishnan *et al.*, 2000).

The most desirable way to assess gene expression in *Mtb* growing in a specific environment would be to analyse every possible ORF in the genome. This capability is the premise and the promise of DNA microarray analysis (Clark-Curtiss and Haydel, 2003). These microarray based studies typically use expression data to make inferences about the biological functions of regulated genes – a strategy proven to be fruitful in other organisms. Most microarray based mycobacterial studies have focused on *in vitro* conditions thought to mimic infections (i.e. starvation, iron limitation, exposure to nitric oxide or hypoxia (Betts *et al.*, 2002); (Ohno *et al.*, 2003); (Hampshire *et al.*, 2004); (Bacon *et al.*, 2004). DNA microarray analyisis for studying mycobacterial gene expression *in vivo* is technically challenging due to the requirement of large amounts of total RNA in order to generate cDNA probes. However, a few groups have succeeded in overcoming technical hurdles, enabling comprehensive analysis of genes expressed *in vivo*. *Mtb* expression patterns in the context of macrophage were first examined by Schnappinger and colleagues (Schnappinger *et al.*, 2003). More recently the transcriptome signature of *Mtb* in human pulmonary tuberculosis was elegantly studied on clinical lung samples using DNA microarray analysis (Rachman *et al.*, 2006).

Although gene expression studies are of value and interest, there are important limitations in the design and interpretation of expression experiments. (i) These studies represent snapshots in time. Altering the expression of a gene might have consequences at a much later point during the course of infection. (ii) Transcriptional changes in a gene could be responses to a variety of stimuli. (iii) Although the upregulation of a gene might suggest an important role in infection, this is certainly not always true. Therefore complementary approaches are needed to dissect the roles of individual genes (Murry and Rubin, 2005).

1.3.2 Comparative Genomics

Determination of the complete nucleotide sequence of *Mtb* H37Rv (Cole *et al.*, 1998), *Mtb* CDC1551, *M. avium* (http://www.tigr.org), *M. bovis*, *M. marinum*

(http://www.sanger.ac.uk) and *M. leprae* (Cole *et al.*, 2001) genomes has provided a means to compare these genomes and has revealed features of the genomes that were previously unknown. Comparison of genomes has also provided information that should be useful for further understanding of the basis for attenuation of strains such as *Mtb* H37Ra and *M. bovis* BCG. By identifying sequences that have been deleted or disrupted in the attenuated strains and the subsequent knock out of specific genes in these sequences in wild-type *Mtb* or *M. bovis* or introducing these genes into the attenuated strains, investigators were able to evaluate the contributions of specific genes to virulence (Clark-Curtiss and Haydel, 2003). Recent work has suggested that strain comparisons will continue to yield significant insights into pathogenesis. Genomic deletions and alterations are likely to be present in most clinical strains and there are evidences that these alterations might affect the severity of the disease outcome (Kato-Maeda *et al.*, 2001a; Kato-Maeda *et al.*, 2001b); (Tsolaki *et al.*, 2004). Studies have been carried out to show that some strains, such as the Texas HN878 strain, as well as several other Beijing strains, kill mice more rapidly than the sequenced reference strains (Manca *et al.*, 1999); (Lopez *et al.*, 2003). These results complement epidemiological evidence, supporting the increased prevalence of Beijing strains (Glynn *et al.*, 2002).

Although strain variability has important biological consequences, limited knowledge is available on the mechanisms of pathogenesis that are common to all strains. Analysis of variation has limited ability to illuminate shared characteristics (Murry and Rubin, 2005).

1.3.3 Generation of Mutations in Specific Genes

The tubercle bacillus has been extremely successful at holding its own virulence secrets hostage. However, development of several efficient mutagenesis strategies has greatly assisted in identifying *Mtb* virulence factors. In order to decipher the roles of genes identified through gene expression analysis or comparative genomics approach, candidate genes have to be mutated as a complementary approach.

Targeted knock out of specific genes in mycobacteria has been achieved by using linear substrates, counterselectable plasmid delivery systems and phage delivery systems. Earlier long linear substrate: ~ 20 kb of homologous flanking DNA was used to create a mutant. More recently, allelic exchange mutants have been created by electroporating

short, linear plasmids harbouring the respective genes interrupted by Kanamycin or Hygromycin resistant markers (Clark-Curtiss and Haydel, 2003). Counterselectable plasmid delivery systems have greatly improved the efficiency of isolating targeted mutations in mycobacteria. The most common allelic exchange mutagenesis procedure currently being used with mycobacteria involves a two-step selection method employing the counterselectable properties of the *sacB* gene, which is lethal to mycobacteria in the presence of sucrose (Pelicic *et al.*, 1996a, 1996b). After the first selection on an antibiotic containing solid medium, clones are propagated in liquid medium to facilitate second crossover needed for allelic exchange followed by selection on sucrose. This procedure has been used extensively to generate numerous mutants.

The development of conditionally replicating mycobacteriophages provides another genetic strategy to generate targeted deletion mutations via allelic exchange. The mycobacteriophage delivery system involves the use of temperature-sensitive shuttle phasmids that can replicate as plasmids in *E. coli* and as phages in mycobacteria. These phasmids are able to infect and replicate as phage particles in *M. smegmatis* at 30°C (permissive temperature), but fail to replicate in bacterial host cells (e.g. *Mtb*) at the nonpermissive temperatures, 37°C (TM4-based) or 38.5°C (D29-based), thereby allowing transposon delivery or generation of defined deletion mutations by allelic exchange (Bardarov *et al.*, 1997).

1.3.4 Analysis of Mutant Library

Transposon mutagenesis is a procedure that generates stable, single insertions which can be subsequently linked to the disrupted gene. Transposon mutagenesis is achieved by using a transposon, a DNA element that can move from its original site on a DNA molecule to a random site in the same or different DNA molecule. The transposition event, i.e., the transfer of the transposon from one site to another is mediated by a gene encoding for transposase. In addition to carrying the transposase, the transposon carries a selectable marker gene, such as an antibiotic resistance gene. Thus to generate a library of transposon-inserted mutants, the transposon is introduced into the host bacterium by means of a delivery vector that cannot replicate in the host organism. The transposon will hop from the delivery vector to the chromosome of the host bacterium. The bacterial cells in which the delivery vector is introduced are plated on medium containing selective agent. Only those cells in which the transposon has stably inserted

will give rise to colonies. By collecting large sets of colonies from such an experiment, a library of transposon mutants can be assembled. Because transposon-mediated mutagenesis can potentially allow for insertions in every gene of a bacterial chromosome, transposon mycobacterial mutant libraries have been constructed in different mycobacterial strains and species. Several advances in molecular mycobacteriology have enabled analysis of multiple mutants rather than performing individual experiments with each mutant.

Signature tagged mutagenesis (STM) marked a new era in the generation of transposon mutant libraries and in the subsequent screening of bacterial virulence gene mutants in appropriate animal models. This novel system uses a pool of uniquely oligonucleotide-tagged transposons for mutagenesis, thus allowing individual transposons to be identified by hybridization to the signature oligonucleotide tag after insertion into the genome. Therefore, pools of mutants can be used to infect animals in an effort to identify strains with attenuated virulence. STM has been successfully used to identify numerous virulence genes in numerous pathogens including *Mtb* (Clark-Curtiss and Haydel, 2003).

Additional approaches DeADMAn (Designer arrays for defined mutant analysis) and TraSH (transposon site hybridization) for screening transposon pools have been recently developed. These methods rely on microarray hybridization for the analysis of mutant abundance, which provides a more quantitative approach than radiolabelling used by STM. All these screens have been so far employed to study genes involved in the fitness of mycobacteria in the host. They use a very narrow definition of virulence. These screens identify genes that are required for bacterial survival and growth in the host. Survival and growth, though necessary to cause disease, are by no means the only functions associated with causing it (Murry and Rubin, 2005). Specific screens to identify virulent genes involved in arresting PM in macrophages, which is considered central for the TB pathogenesis, will greatly assist in understanding the virulence mechanisms of mycobacteria.

1.4 Mycobacterial Factors Disrupting Phagosome Maturation

Though several host cell mechanisms in the inhibition of phagosome maturation have been proposed, the explanation of how mycobacteria accomplish a safe haven for themselves in the macrophages remains elusive. Generating genetic tools to study

mycobacteria have lead to identification of virulence factors involved in the inhibition of PM. Early studies by Goren and colleagues have shown that *Mtb* uses its sulphur-containing-lipid to inhibit PM (Goren *et al.*, 1976), but this was later attributed to a technical problem. Cord factor or trehalose dimycolate (TDM) a characteristic lipid of *Mtb* caused a delay in phagosomal acidification (Indrigo *et al.*, 2003). Phosphoinositols are part of another mycobacterial lipid family which includes lipoarabinomanan (LAM) and phosphoinositol mannoside (PIM). Phagocytised LAM coated beads have similar properties as that of MP, including reduced acidification and reduced acquisition of late endosomal markers. LAM also inhibits PI3kinase dependent pathway between TGN and phagosomes. LAM also prevents the recruitment of EEA-1 another important factor in PM. Improper recruitment of EEA-1 leads to reduced Ca2+ flux (Vergne *et al.*, 2003). This explains in part the mycobacterial inhibition of Ca2+/calmodulin complex as shown by Kusner and colleagues (Malik *et al.*, 2001; Malik *et al.*, 2003). Phosphoinositol mannosides (PIM) are similar to the mammalian phosphoinositol lipids and hence preferentially enhance fusion of MPs with early endosomes by competing with phosphoinositol-3-phosphate (PI3P) (Vergne *et al.*, 2004b). Studies have also postulated possible roles for mycobacterial urease (Gordon *et al.*, 1980). In addition, a secreted mycobacterial lipid phosphatase (SapM) was identified by Vergne and colleagues which is held responsible for hydrolyzing PI3P, leading to inhibition of PM (Vergne *et al.*, 2005). Eukaryotic-like serine/threonine mycobacterial protein kinase G is said to prevent transfer of mycobacteria to lysosomes, suggesting that virulent mycobacteria have evolved eukaryotic-like signal transduction mechanisms (Walburger *et al.*, 2004). It is reasoned that close apposition of mycobacteria to the phagosomal membrane hampers MP maturation (de Chastellier and Thilo, 1998). This concept was expanded by the observation that cholesterol depletion with methyl-β-cyclodextrin loosened the close apposition of phagosomal membrane and bacterium and resulted in fusion with lysosomes (de Chastellier and Thilo, 2006). ESAT-6 and CFP-10 might also have additional roles in inhibiting PM as evinced from studies conducted using *M. marinum* (Tan *et al.*, 2006). Discrepancies arising from different groups and recent screens to identify genes involved in the inhibition of PM suggest involvement of more than one effector molecule in the retardation of MP maturation (Pethe *et al.*, 2004); (Stewart *et al.*, 2005). Most of the studies emphasise the participation of the mycobacterial cell envelope lipids in the inhibition of PM.

1.5 Unique Cell Envelope and Lipids of Mycobacteria

Mycobacteria are a family of eubacteria that belong to a group of Gram-positive bacteria containing GC-rich DNA (Cole *et al.*, 1998). These bacteria produce cell walls of a unique structure, containing meso-diaminopimelic acid as the diamino acid in the peptidoglycan. Interestingly the muramic acid residue is N-glycosylated in mycobacterium, in contrast to the N-acetlyation found in most other bacteria. An important feature of the cell wall is the presence of a unique polysaccharide, arabinogalactan (AG), which is substituted by characteristic long chain fatty acids, namely the mycolic acids containing 70-90 carbon atoms (Brennan and Nikaido, 1995).

Mycolic acids

Mycolic acids are high molecular weight α-alkyl, β-hydroxy fatty acids. They primarily appear as esters of AG, but are also in extractable lipids namely as trehalose 6-6'-dimycolate (TDM or cord factor). Mycolic acid structures can be separated into meromycolate moiety and the α-branch. They have the largest α-branch (C_{20} to C_{25}). In the main chain (the meromycolic acid moiety), they contain one or two groups which may be double bonds or cyclopropane rings that are capable of producing "kinks" in the molecule; they may contain oxygen functions additional to the β-hydroxy group; and they may have methyl branches in the main carbon backbone (Fig: 1). The cyclopropane rings in the meromycolate chain are mediated by specific methyl transferases (Barry *et al.*, 1998); (Glickman *et al.*, 2000). Knocking out the genes coding for these methyltransferases leads to loss of oxygenated mycolic acids and cyclopropanation on major mycolic acids, difference in colony morphology and reduced virulence (Dubnau *et al.*, 2000); (Glickman *et al.*, 2000). These observations also implicate the fine structure of mycolic acid subclasses in the interaction of mycobacteria with the host immune system.

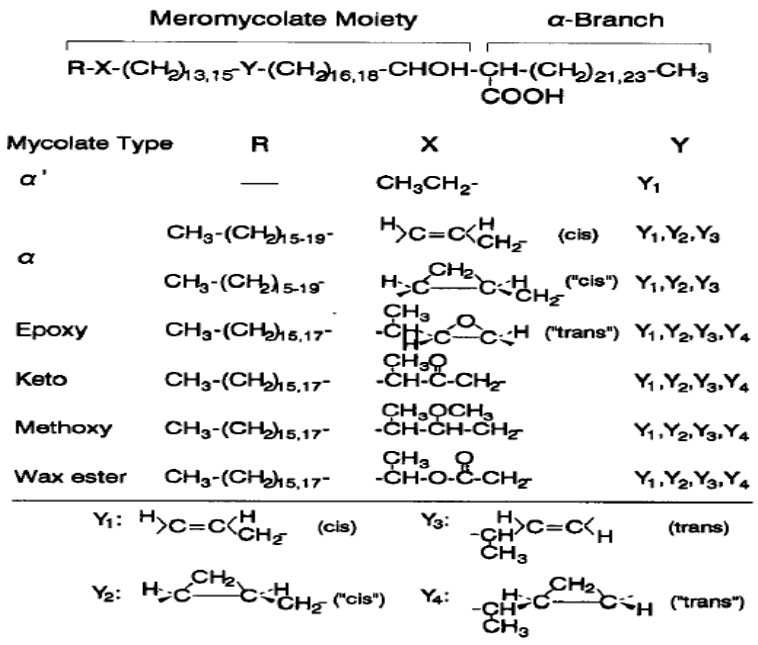

Fig1: Structure of mycolic acids from mycobacteria adapted from (Brennan and Nikaido, 1995)

Lipoarabinomanan (LAM)

Mycobacteria also contain soluble immunologically active arabinomanan (Azuma *et al.*, 1970). Studies showed that LAM contains glycerol, inositol and phosphate in addition to arabinose, mannose, lactate, succinate, palmitate and tuberculostearate, which were identified much earlier (Hunter and Brennan, 1990). LAM is a multiglycosylated extension of PIMs. The arabinose termini of LAM from *Mtb* is capped with mannose residues and termed as mannose lipoarabinomanan (ManLAM) (Chatterjee *et al.*, 1992). In contrast, the LAM of the rapidly growing species *M. smegmatis* is devoid of mannose caps and is termed arabinose lipoarabinomanan (AraLAM) (Prinzis *et al.*, 1993). LAM exhibits a wide spectrum of immunoregulatory functions. Earlier data using LAM of *M. leprae* and AraLAM from a rapidly growing mycobacterium species were interpreted as a suggestion that LAM suppresses immune responses, thus contributing to pathogenesis of tuberculosis and leprosy. These data include LAM-induced abrogation of T-cell activation (Kaplan *et al.*, 1987), inhibition of γ-interferon mediated activation of murine macrophages (Sibley *et al.*, 1988), scavenging of potentially cytotoxic oxygen free radicals, and inhibition of protein kinase C activity (Chan *et al.*, 1991). Although AraLAM evoked a large array of cytokines associated with macrophages, such as TNF-α (Chatterjee *et al.*, 1992), granulocyte macrophage colony stimulating factor, and interleukins-la, lb, 6, and 10 (Barnes et al., 1992), this was frequently interpreted as a

contributor to the disease processes: for example, the production of fever, weight loss, and tissue necrosis was emphasized in the case of TNF-α. More recently, however, ManLAM, which is present in strains of *Mtb*, was found to be much less potent in evoking TNF-α, in contrast to AraLAM found in non-virulent species (Chatterjee *et al.*, 1992). Similarly, AraLAM, but not ManLAM, was found to activate the early response genes (including c-fos and the genes for TNF-α) in macrophages (Roach *et al.*, 1993). Additionally, ManLAM could stimulate phagocytosis by interacting with the Man receptor (Schlesinger, 1993). These results now suggest that *Mtb* strains become phagocytised efficiently but survive within the host macrophages because their ManLAM does not activate these phagocytes (Brennan and Nikaido, 1995). Moreover, recently it was shown that *Mtb* LAM inhibited cytosolic calcium rise thereby blocking the acquisition of late endosomal and lysosomal markers.

Waxes, Acylated Trehaloses and Sulpholipids

Several slowly growing mycobacteria contain an array of waxes, generally long-chain diols [phthiocerols A and B, phthiodiolone, phthiotriol; phthiocerol A is a mixture of 3-methoxy-4-methyl-do-(and tetra)-triacontane-9,11-diols] in which long chain fatty acids namely mycocerosic acid or its isomer phthioceranic acid are esterified to both hydroxyl groups. Three other families of trehalose-based lipids have been implicated in the pathogenesis of tuberculosis (Brennan and Nikaido, 1995), (i) cord factor, (ii) the simpler acylated trehaloses [containing a combination of saturated straight-chain C_{16}-C_{19}, C_{21}-C_{25} mycocerosate, C24-C2s mycolipanolic, and C25-C27 mycolipenic fatty acids: for example, 2, 3-di-O acylated trehalose isolated from *Mtb*, (iii) and the sulfolipids (trehalose 2′-sulfate acylated with hydroxyphthioceranic, phthioceranic, and saturated straight-chain fatty acids). The cord factor can produce granulomatous inflammation and thymic atrophy when injected into mice (Ozeki *et al.*, 1997) in addition to affecting membrane fusion in model systems (Spargo *et al.*, 1991) and toxicity for mitochondria (Glickman and Jacobs, 2001). Mutants that failed to produce or secrete the phthiocerol containing PDIM (Phthiocerol di mycocerosate) failed to replicate in the lungs and were hypothesized to be necessary for organ specific replication (Camacho *et al.*, 1999); (Cox *et al.*, 1999). Sulfolipid of *Mtb* was the first lipid to be shown to inhibit PM. Thus, all these waxes are thought to be significant for the successful pathogenesis.

The Extractable Lipids of Cell Wall

The search for dominant antigens on the surfaces of various mycobacteria, especially "atypical" (or nontuberculous saprophytic) mycobacteria, was stimulated by the infections caused by these bacteria in immunocompromised patients (Horsburgh and Selik, 1989). This led to the definition of a remarkable army of cell wall glycolipids (Brennan, 1989). The major classes of such extractable glycolipids include lipooligosaccharides (LOS), phenolic glycolipids (PGLs), and glycopeptidolipids (GPLs)

Members of the LOS class of glycolipids were first found in *M. kansasii* and later in *M. malmoense, M. szulgai, M. gordonae, and M. butyricum*. They are composed of variable residues of xylose, 3-O-methyl rhamnose, fucose and a novel N-acylamino sugar (N-acylkansosamine) linked to a common tetra glucose core, which itself contains an α,α´-trehalose moiety at the end (Figure: 2). The terminal glucose residue of the α,α´-trehalose unit is usually acylated at positions 3, 4, and 6 by 2,4-dimethyl tetra decanoic acid residues ("R" in Figure2) (Brennan and Nikaido, 1995). Very recently LOS biosynthetic gene cluster was identified in *M. marinum* and it was also shown that LOSs play an important role in sliding motility, biofilm formation, and infection of host macrophages (Ren *et al.*, 2007).

Another class of mycobacterial glycolipids are glycopeptidolipids (GPLs) [described as "C-mycosides" in earlier literature]. As shown in Figure 5, the head group is a short peptide, D-Phe-D-*allo* Thr-D-Ala-L-alaninol and the alaninol is substituted by a 3, 4-di-O-methyl-L-rhamnose. The hydroxyl group of the D-*allo*threonine residue carries an oligosaccharide substituent; its most proximal portion is usually α-L-rhamnopyranosyl-(1-2)-6-deoxy-L-talopyranose. The amino group of D-phenylalanine residue is substituted by a fatty acid residue. GPLs are the major cell surface antigens of the *M. avium, M. intracellulare, M. scrofulaceum* group, and they can be subdivided into 31 distinct serotypes based on the serospecific GPLs. GPL has been suggested to protect mycobacterial cells within the phagolysososmes. It is also shown that GPL from *M. avium* serovar 4 inhibits PM whereas GPL from serovar 9 does not inhibit PM. Additionally it is also reported that the inhibition is mediated through mannose receptor (Shimada *et al.*, 2006).

Another class of glycolipids is more correctly termed glycosylphenolphthiocerol dimycocerosates, although the term PGL is generally used (Brennan, 1989); (Besra *et al.*, 1991); (Dobson *et al.*, 1990); (Gaylord and Brennan, 1987). This class includes "mycoside A" of *M. kansasii,* "mycoside G" of *M. marinum,* and "mycoside B" of *M. bovis* in the earlier literature. Their structure (Figure: 2) is characterized by a very large hydrophobic moiety, containing a C_{36} phenolic diol substituted by two molecules of typically C_{34} fatty acid, mycocerosate. The oligosaccharide part contains from one to four sugar residues, and the sugars are usually not very hydrophilic, often consisting of deoxy sugars that are multi-O-methylated. PGL is thought to contribute to the intracellular survival of *M. leprae* within macrophages of individuals with lepromatous leprosy through its ability to scavenge oxygen radicals. The variable oligosaccharide constituents of these glycolipid antigens are usually of sufficient antigenicity as to evoke corresponding specific antibodies and thereby allow serodiagnosis of individual mycobacterioses and leprosy (Gaylord and Brennan, 1987). PGL is apparently not found in the sequenced strains of *Mtb* thought to be due to a frame shift mutation between two genes encoding polyketide synthases *pks1-15* (Constant *et al.*, 2002). Although not produced by the laboratory strains of *Mtb*, epidemiologically more prevalent Beijing strains produce PGL and are shown to be more virulent. PGL was also shown to inhibit production of proinflammatory cytokines in bone marrow derived mouse macrophages, which were previously stimulated with apolar lipids of an *Mtb pks1-15* mutant (Reed *et al.*, 2004).

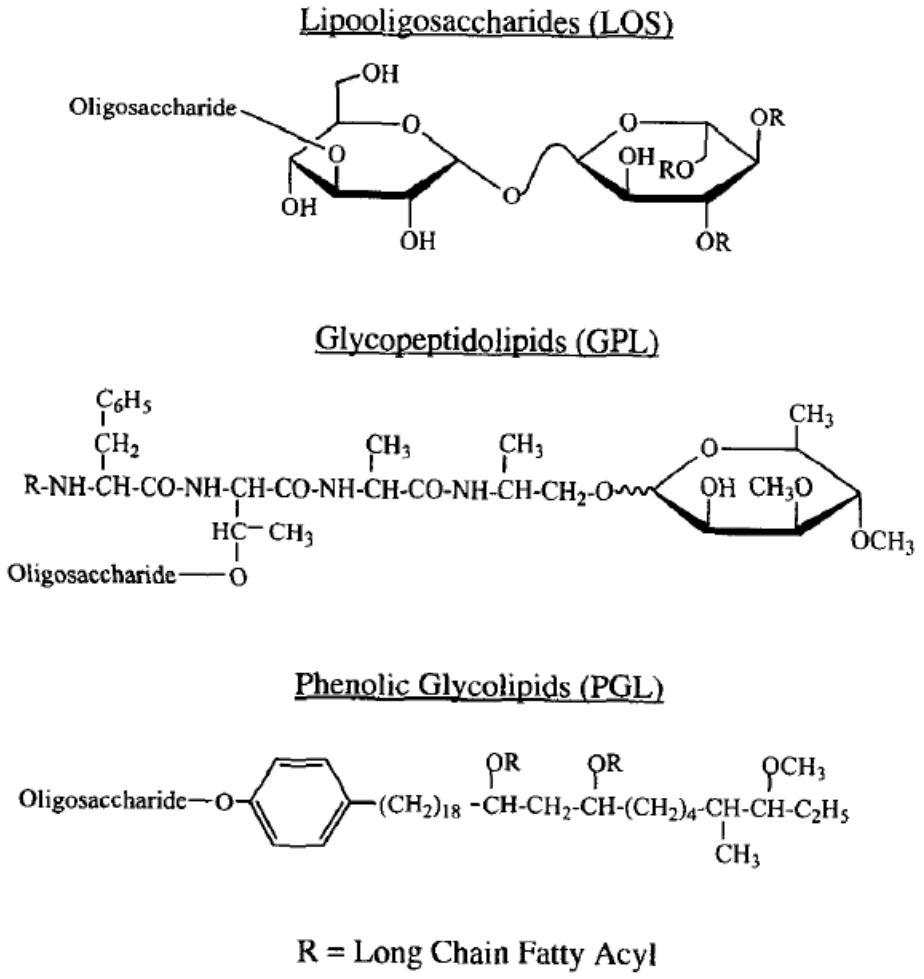

Fig 2: Structure of three major classes of extractable glycolipids of the mycobacterial cell wall adapted from (Brennan and Nikaido, 1995).

1.6 Analysis of Mycobacterial Lipids

The introduction of the new spectroscopic and chromatographic techniques since the early 1960s has revolutionised the approaches to the analysis of these relatively intractable natural compounds. Thin layer (TLC), gas and high performance liquid chromatographic techniques (HPLC) allow the preparation of reproducible lipid profiles and the rapid small-scale isolation of individual components. Radio labelling of lipids with specific radiolabelled substrates combined with chromatography aids in the biochemical characterisation of the lipid. Mycobacterial lipids are radiolabelled by growing mycobacteria in a medium containing specific substrates which are radioactive. Lipids are radiolabelled when bacteria uses the radioactive substrate to synthesise the

lipid. These lipids can be extracted using organic solvents and analysed by chromatography.

Thin Layer Chromatography

TLC is the most commonly used chromatographic technique to generate lipid profiles. It is a simple, quick, and inexpensive procedure that gives a quick answer as to how many lipid components are in a mixture. TLC is also used to support the identity of a compound in a mixture by comparing the retention factor (RF) value of the sample with that of a standard. A TLC plate is a sheet of glass, metal, or plastic which is coated with a thin layer of a solid adsorbent (usually silica or alumina). A small amount of the mixture to be analysed is spotted near the bottom of this plate. The TLC plate is then placed in a shallow pool of a solvent in a developing chamber so that only the very bottom of the plate is in the liquid. This liquid, or the eluent, is the mobile phase, and it slowly rises up the TLC plate by capillary action. As the solvent moves past the spot that was applied, equilibrium is established for each component of the mixture between the molecules of that component which are adsorbed on the solid and the molecules which are in solution. In principle, the components will differ in solubility and in the strength of their adsorption to the adsorbent and some components will be carried farther up the plate than others. When the solvent has reached the top of the plate, the plate is removed from the developing chamber, dried, and the separated components of the mixture are visualised. Visualisation is done using reagents that react with the lipid or the sugars of glycolipids etc to give colours to the spots. When the samples are coloured, spots can be seen with naked eye. Alternatively plates can be viewed under UV-light. In the case that lipids are radiolabelled X-ray films can be placed over the plate and later developed to reveal the spots.

Mass spectrometry and Nuclear magnetic resonance spectroscopy facilitate the precise determination of the masses and functional groups of the lipid to be analysed. Most of the mycobacterial lipids have been structurally characterized employing these two techniques.

Mass Spectrometry

Mass spectrometry is unlike most other forms of spectroscopy or spectrometry that are concerned with non-destructive interactions between molecules and electromagnetic

radiation. A mass spectrometer converts sample molecules into ions in the gas phase, separates them according to their mass to charge ratio (m/z) and sequentially records the individual ion current intensities at each mass - the mass spectrum. The principle of the mass spectrometer is simple though details vary enormously. Atoms from the material to be studied are ionized, accelerated to a known energy by passing through a potential difference, and then passed through a magnetic field which separates particles with different momentary. The ions can be produced by bombarding atoms in the residual gas with an electron beam in the ion source. The (positively charged) ions are then accelerated by the voltage which can be adjusted to choose ions of the desired mass. Some of the ions pass through a slit and enter a region with a uniform magnetic field. If voltage is chosen correctly, ions of the appropriate mass are deflected by the magnetic field, pass through a second slit, and are detected in an ion collector. The amplified signal from the ion collector is a measure of the ion current. These ion current intensities are drawn in histogram form taking the most intense ion current as 100%, the values of m/z versus percentage relative intensity. The mass peaks can be compared with standards to identify compounds or with the theoretical mass of a compound.

Nuclear Magnetic Resonance Spectroscopy

Nuclear magnetic resonance, or NMR, is a phenomenon which occurs when the nuclei of certain atoms are immersed in a static magnetic field and exposed to a second oscillating magnetic field. Some nuclei experience this phenomenon, and others do not, dependent upon whether they possess a property called spin. All nuclei that contain odd numbers of protons or neutrons have an intrinsic magnetic moment and angular momentum. The most commonly measured nuclei are hydrogen-1 (the most receptive isotope at natural abundance) and carbon-13, although nuclei from isotopes of many other elements can also be observed. NMR studies magnetic nuclei by aligning them with a very powerful external magnetic field and perturbing this alignment using an electromagnetic field. The resulting response to the external perturbing electromagnetic magnetic is the phenomenon that is exploited in nuclear magnetic resonance spectroscopy.

1.7 *Mycobacterium marinum* as a Model

Although some genetic tools for studying *Mtb* are now available, it remains a difficult organism to work with. This has turned many investigators towards related organisms.

M. marinum has become increasingly useful as a model organism. It causes fatal infection in fresh and salt water fish as well as amphibians. In humans it is the causative agent of a disease called swimming pool granuloma (Wolinsky, 1992); (Collins *et al.*, 1985). A recent case study expands the spectrum of infection caused by *M. marinum* to granulomatous pulmonary disease in humans (Lai *et al.*, 2005). Signature mechanisms of tuberculosis disease initiation, namely retardation of PM and granuloma formation are conserved between *M. marinum* and *M. tuberculosis* (Barker *et al.*, 1997). Faster growth of *M. marinum* compared to that of *M. tuberculosis* and its phylogenetic closeness to *M. tuberculosis* complex have been exploited to some extent. Utilising *M. marinum* as a model, granuloma-specific expression of virulence proteins from the glycine rich PE-PGRS family (Ramakrishnan *et al.*, 2000) and *MmW04* gene involved in intracellular survival and pigmentation have been identified (Gao *et al.*, 2003a). Studies using *M. marinum* mutants helped to identify *kasB* as a novel drug target in mycobacteria. This gene is required for full elongation of mycolates. The respective mutants have increased permeability of their cell walls with the consequence of impaired growth within macrophages (Gao *et al.*, 2003b) and loss of acid fastness in *Mtb* (Bhatt *et al.*, 2007). A *M. marinum* virulence factor as yet not found in other virulent mycobacteria is the escape from their phagosome to the cytosol and a capability for cell to cell spreading by polymerising actin of the host cell (Stamm *et al.*, 2003). The archetypical mycobacterial pathological feature of granuloma formation in tissues has been addressed in an elegant study using *M. marinum*. It was shown that existing granulomas fail to eliminate invading naïve mycobacteria that traffic to them (Cosma *et al.*, 2004). More recently additional roles for ESAT-6 (Early secreted antigen) and CFP-10 (Culture filtrate protein) in impeding PM and the essentiality of Erp (exported repetitive protein) in mounting an initial infection have been established using *M. marinum* (Cosma *et al.*, 2006); (Tan *et al.*, 2006). The diversity of possible approaches used in these studies validates the utilization of *M. marinum* as a model to study mycobacterial pathogenesis. It was one of the aims of this work to apply a genetic screen to *M. marinum* and ask whether the genes involved in the important mycobacterial virulence trait, namely inhibition of PM, can be identified and further studied.

2 Objectives

The objective of this study was to identify and characterise mycobacterial virulence factors that are involved in the inhibition of PM.

The availability of mycobacterial genomes have facilitated several genetic screens, designed to identify genes implicated in the infection process. Although it is likely that the resulting list of genes from such screens contain mediating factors arresting PM, the lists are too complex to identify such components directly. Moreover, the lysosomal milieu is clearly bacteriostatic and only weakly bacteicidal for *Mtb*. Therefore, using bacterial death to identify mutants defective in arresting PM will be misleading and will be masked by other deleterious mutations. Hence, the primary objective was to design a screening technique to sift through a pool of mycobacterial mutants for mutants particularly defective in inhibiting PM. This objective was to be addressed using *M. marinum* as a model for other virulent mycobacteria, namely *Mtb*.

As with any other screening techniques validity of the screen can be assessed only by demonstrating atleast some of the mutants have the predicted phenotype. In order to study the phenotype of the mutants the maturation status of the phagosomes containing mutants has to be analysed. Therefore one of the aims of this thesis was also to develop necessary tools to study phagosome maturation.

Finally, the resulting lists of genes from genetic screens are less informative unless the effector molecules the genes directly or indirectly codes for is known. Therefore, investigations were carried out to pin down the effector molecules encoded by the affected genes of the mutants that directly inhibit PM.

3 Materials and Methods

3.1 Materials

3.1.1 Instruments Used

Name of the Instrument	Brand Name	Manufacturer
β-Scintillation counter	1217 Rackbeta	LKB Wallac
Agarose gel electrophoresis apparatus	Mini-sub Cell GT	Bio-Rad
Capillary electrophoresis sequencer	ABI Prism™ 310 Genetic Analyzer	Applied Biosystems
Cell mill	MM2000	Retsch
Centrifuge	Table top: Sepatech 15Biofuge	Heraeus
CO_2 Incubator	Hera cell 240	Heraeus
Electroporator	*E. coli* pulser	Biorad
Flowcytometer	FACScan™	Becton Dickinson
Fluorescence microscope	IX81	Olympus
Gel documentation apparatus	Geldoc 2000	Bio-Rad
Heating Plate (TLC)	Thermoplate S	Desaga/Sarstadt
Hybridization oven	OV1	Biometra
Laminar air flow bench	Herasafe KSP15	Heraeus
Mass spectrometer	Q-TOF 2	Micromass

NMR spectrometer	AMX 500	Bruker
pH meter	Multical	WTW
Shaker incubator	Innova™ 4200	New Brunswick Scientific
Sodium dodecyl sulphate - Polyacrylamide agarose gel electrophoresis (SDS-PAGE) apparatus	Mini Protean II	Bio-Rad
Sonicator bath	Sonorex TK30	Bandelin
Spectrophotometer	SmartSpec™ Plus	Bio-Rad
Thermal block	Thermomixer Comfort	Eppendorf
Thermal Cycler	PTC200	MJ Research
Tissue culture microscope	CKX41	Olympus
UV-Cross Linker	UVC500 UV cross linker	Hoefer
Vacuum blot apparatus (Southern Blot)	Vacuum Blotter	Apligene
Vortexer	Reax2000	Heidolph
Water Bath	Type: 1002	Geselschaft für Labortechnik
Weighing Balance	Genius	Sartorius
Western Blot apparatus	Mini Trans Blot	Bio-Rad
X-ray film developer	Agfa Curix 60	Agfa

3.1.2 Chemicals, Media and other Accessories Used

Chemicals and accessories	Manufacturer
β-galactosidase assay kit	Boehringer Mannheim, Germany
^{14}C palmitate supplemented Middlebrook 7H12 medium	BACTEC, Becton Dickinson, MD, USA
Acetic acid	Merck KGaA, Darmstadt, Germany
Agarose	Seakem ME, Lonza GmbH, Wupertal, Germany
Alexa568 dextran	Molecular Probes, Invitrogen GmbH, Karlsruhe, Germany
Alexa568 transferrin	Molecular Probes, Invitrogen GmbH, Karlslruhe, Germany
Alexa594 anti mouse IgG	Molecular Probes, Invitrogen GmbH, Karlsruhe, Germany
Ampicillin	Sigma-Aldrich Chemie, Steinheim, Germany
Carboxyl- ^{14}C *p*-hydroxy benzoic acid	American Radiochemicals, Cologne, Germany
Cationic lipid transfection reagent (Lipofectamine)	Invitrogen GmbH, Karlsruhe, Germany
CDP star detection kit	GE Healthcare Europe GmbH, Munich, Germany
Cell scrapper	Greiner bio-one, Frickenhausen, Germany
Chloroform	Merck, Darmstadt, Germany
Copper sulphate	Roth, Karlsruhe, Germany
Cover slip	Engelbrecht, Edermünde, Germany

Cytochalasin B	Sigma-Aldrich Chemie, Steinheim, Germany
Di chloromethane	Merck KGaA, Darmstadt, Germany
DIG DNA labelling kit	Roche Applied Science, Mannheim, Germany
DMEM (Dulbecos minimum essential medium)	Gibco, Invitrogen GmbH, Karlsruhe, Germany
DTT (dithiothreitol)	Sigma-Aldrich Chemie, Steinheim, Germany
EDTA (ethylene diamine tetraacetic acid)	Roth, Karlsruhe, Germany
EGTA (ethylene glycol tetraacetic acid)	Roth, Karlsruhe, Germany
Enhanced chemiluminescence (ECL) Kit	GE Healthcare Europe GmbH, Munich, Germany
Entellan	Merck KGaA, Darmstadt, Germany
Eppendorf tubes	Sarstedt, Nümbrecht, Germany
Ethanol	Roth, Karlsruhe, Germany
FITC (fluorescein isothiocyanate)	Sigma-Aldrich Chemie, Steinheim, Germany
Foetal bovine serum (FBS)	Sigma-Aldrich Chemie, Steinheim, Germany
Gateway cloning system	Invitrogen GmbH, Karlsruhe, Germany
Gelatine	Merck KGaA, Darmstadt, Germany
Glass slide	Engelbrecht, Edermünde, Germany
High fidelity DNA polymerase from *Thermococcus kodakaraensis* (KOD DNA polymerase)	Novagen, Merck KGaA, Darmstadt, Germany
High performance thin layer chromatography	Merck KGaA, Darmstadt, Germany

(HPTLC) Plate, Silica gel 60 20x10cm	
Histopaque (ficoll for isolating peripheral blood monocytes)	Sigma-Aldrich Chemie, Steinheim, Germany
Hydrochloric acid	Merck KGaA, Darmstadt, Germany
Hygromycin	Boehringer Mannheim
Hyper film	GE Healthcare Europe GmbH, Munich, Germany
Imidazole	Merck KGaA, Darmstadt, Germany
Infusion cloning Kit	Clontech, CA, USA
Iodomethane	Merck KGaA, Darmstadt, Germany
Kanamycin	Sigma-Aldrich Chemie, Steinheim, Germany
Klenow polymerase	New England Biolabs GmbH, Frankfurt, Germany
Lysozyme	Sigma-Aldrich Chemie, Steinheim, Germany
MACS (Magnetic cell sorting) columns	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
Magnesium chloride	Sigma-Aldrich Chemie, Steinheim, Germany
Magnet	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany Dynal, Biotech ASA, Oslo, Norway
Magnetic agarose beads	Qiagen, Hilden, Germany
Maleic acid	Merck, Darmstadt, Germany
Mannitol	Sigma-Aldrich Chemie, Steinheim, Germany

Methanol	Roth, Karlsruhe, Germany
Middlebrook 7H10 agar	Becton Dickinson, MD, USA
Middlebrook 7H9 broth	Becton Dickinson, MD, USA
Miltenyi basic beads (iron dextran micro beads)	Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
Molybdate phosphoric acid	Roth, Karlsruhe, Germany
Mouse anti-EEA-1	BD Biosciences, Heidelberg, Germany
Mouse anti-LAMP1	BD Biosciences, Heidelberg, Germany
Mouse anti-LAMP2	Southern Biotech, Alabama, USA
Mouse anti-Rab5	BD Biosciences, Heidelberg, Germany
Mouse-anti-β galactosidase	Abnova, Taipei City, Taiwan
Multi-well tissue culture plates	Nunc, IL, USA
My one beads (hydrophillic beads)	Dynal Biotech ASA, Oslo, Norway
Ni-NTA (Nickel-nitrilotriacetic acid)	Qiagen, Hilden, Germany
N-lauryl sarcosine	Serva, Heidelberg, Germany
Oleic acid albumin dextrose complex (OADC)	Becton Dickinson, MD, USA
Opti-MEM 1 medium	Invitrogen GmbH, Karlsruhe, Germany
Parafilm	Pechiney Plastic Packaging, IL, USA
Paraformaldehyde	Sigma-Aldrich Chemie, Steinheim, Germany

PBS (phosphate buffer saline)	Biochrom, Berlin, Germany
PCR purification kit	Qiagen, Hilden, Germany
Perchloric acid	Merck, Darmstadt, Germany
Petriperm	Greiner Bio-one, Frickenhausen, Germany
Petroleum ether	Merck KGaA, Darmstadt, Germany
PIPES (piperazine-N,N'- bis ethansulfonic acid)	Sigma-Aldrich Chemie, Steinheim, Germany
Plasmid mini-prep kit	Qiagen, Hilden, Germany
PMSF (phenylmethylsulphonyl fluoride)	Sigma-Aldrich Chemie, Steinheim, Germany
Polystyrene fluorescent beads	Bangs Laboratories inc, IN, USA
Potassium chloride (KCl)	Merck KGaA, Darmstadt, Germany
Potassium hydroxide (KOH)	Merck KGaA, Darmstadt, Germany
Primers	MWG Biotech, Martinsried, Germany
Prolong anti-fade gold	Molecular Probes, Invitrogen GmbH, Karlsruhe, Germany
Protease inhibitor	Roche Applied Science, Mannheim, Germany
Protein estimation kit	Perbio Science GmbH, Bonn, Germany
PVDF membrane	GE Healthcare Europe GmbH, Munich, Germany
Rabbit anti-EGFP	Molecular Probes, Invitrogen GmbH, Karlslruhe, Germany
Restriction endonucleases	New England Biolabs, Frankfurt, Germany

RPC-18 beads (hydrophobic beads)	Dynal Biotech ASA, Oslo, Norway
RPMI-1640 medium (Roswell Park Memorial Institute medium)	Biochrom, Berlin, Germany
Saponin	Sigma-Aldrich Chemie, Steinheim, Germany
Scintillation fluid	Zinsser Analytic, Berkshire, UK
Serum Free medium for macrophages	Gibco, Invitrogen, USA
Sodium bi carbonate	Merck KGaA, Darmstadt, Germany
Sodium chloride (NaCl)	Merck KGaA, Darmstadt, Germany
Sodium dodecyl sulphate (SDS)	Serva, Heidelberg, Germany
Sodium hydroxide (NaOH)	Merck KGaA, Darmstadt, Germany
Sucrose	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Sulphuric acid (H_2SO_4)	Merck KGaA, Darmstadt, Germany
Syringe	Omnifix, B Braun, Melsungen, Germany
Syringe needle	BD Microlance, Becton Dickinson
Taq polymerase	Qiagen, Hilden, Germany
Tetra-n-butyl ammonium hydrogen sulphate	Merck KGaA, Darmstadt, Germany
Thinlayer chromatography (TLC) plate silica gel 60 20x20cm	Merck KGaA Darmstadt, Germany
Tissue culture flasks	Nunc, IL, USA

Toluene	Roth, Karlsruhe, Germany
Triethanolamine	Sigma-Aldrich Chemie, Steinheim, Germany
Tris	Roth, Karlsruhe, Germany
TRITC (tetramethylrhodamine-5-isothiocyanate)	Sigma-Aldrich Chemie, Steinheim, Germany
Tween 20	Sigma-Aldrich Chemie, Steinheim, Germany
Whatmann3 Paper	Schleicher and Schüll, Germany
X-ray film	Fujifilm, Düsseldorf, Germany
X-ray film cassette	Siemens, Germany
Zwittergent 3-12	Calbiochem, Merck KGaA Darmstadt, Germany

3.1.3 Oligonucleotides

Name	Sequence	Use
ARB1	5′-GGCCACGCGTCGACTAGTAC NNNNNNNNNN-‘3	Sequencing of Mutants
ARB2	5′-GGCCACGCGTCGACTAGTAC -3′	Sequencing of Mutants
P1_1064F1	5′-TGCGGCCGCTCTAGATGCGG TCAGGTATGTCAGCA–3′	Trans complementation of mutant P1
P1_8007R2	5′-GGGGGATCCACTAGTCTATC GACGCTGGCGCAT–3′	Trans complementation of mutant P1
P1N_del_F1	5′-TGCGGCCGCTCTAGACGATG AGTTGTGGGCGAA-3′	Construction of Nested deletion plasmids

P1N_del_F2	5′-TGCGGCCGCTCTAGAATGCG ACATCAGTTCGGG-3′	Construction of Nested deletion plasmids
P1N_del_F3	5′-TGCGGCCGCTCTAGAATGAT AGAATCTCACTAC-3′	Construction of Nested deletion plasmids
P1N_del_R1	5′-GGGGGATCCACTAGTGTGAG CGGCGGCGTCCCG-3′	Construction of Nested deletion plasmids
P1N_del_R2	5′-GGGGGATCCACTAGTGTGGA GTCGCATCGGCGC-3′	Construction of Nested deletion plasmids
P1N_pQE10_BD_F2	5′-TCACCATACGGATCCGATAG AATCTCACTAC - 3′	Cloning pmiA into an expression vector
P1N_pQE10_BD_R1	5′-CAGCTAATTAAGCTTTTAAT TACGTCGGGCGCC-3′	Cloning pmiA into an expression vector
P1N_pQE18_BD_F1	5′-ACTATGAGAGGATCCATGAT AGAATCTCACTAC-3′	Cloning pmiA into an expression vector
P1N_pQE18_BD_R2	5′-GAGATCGGAAGATCTCAGGG GTATAGGTGTAAT-3′	Cloning pmiA into an expression vector
Rab_BD-infusion_fwd1	5′-TCTTGTCGACCTCGAGCAGG TCGTTACATAACT-3′	Cloning of Rab5- and Rab7-EGFP fusion proteins into pENTRY vector
Rab_BD-infusion_rev1	5′-TAGCGAGCTCTCTAGCCAGA CATGATAAGATAC-3′	Cloning of Rab5- and Rab7-EGFP fusion proteins into pENTRY vector
RPCRa1	5′-CTTGCTCTTCCGCTTCTTCT C-3′	Sequencing of Mutants
RPCRa2	5′-CTCTACACCGTCAAGTGCGAAGAG-3′	Sequencing of Mutants
RPCRb1	5′-CAGGCACGTCGAGGTCTTTC -3′	Sequencing of Mutants
RPCRb2	5′-CTTTCAGATGGATGGCGTAG -3′	Sequencing of Mutants

3.1.4 Plasmids

Name	Marker	Description	Source
pGPC330 (pEGFP-Rab11b)	Kana^r	Plasmid containing Rab-11b GFP fusion protein construct	(Rzomp *et al.*, 2003)
pGPC15 (pOLYG)	Hyg^r	*E.coli* – Mycobacteria shuttle vector with a Hygromycin resistance cassette	(Ó Gaora *et al.*, 1997)
pGPC331 (pENTRY)	Kana^r		Invitrogen
pGPC335 (pGreen Lantern-Rab5a)	Amp^r	Plasmid containing Rab-5a GFP fusion protein construct	Generously provided by Craig Roy, Yale University, New Haven, Connecticut
pGPC336 (pGreen Lantern-Rab7)	Amp^r	Plasmid containing Rab-7 GFP fusion protein construct	Generously provided by Craig Roy.
pGPC352	Hyg^r	*pmiA* spanning region cloned into pOLYG used for trans-complementation	From this work
pGPC358	Amp^r	Rab11b-GFP containing adenoviral vector	From this work
pGPC359	Amp^r	Rab5a-GFP containing adenoviral vector	From this work
pGPC360	Amp^r	Rab7 containing adenoviral vector	From this work
pGPC363	Amp^r	*pmiA* cloned into pQE10 (n-terminal his-tag)	From this work
pGPC364	Amp^r	*pmiA* cloned into pQE18 (c-terminal his-tag)	From this work
pGPC369	Hyg^r	PCR Nested deletion fragments of *pmiA* spanning region cloned into pOLYG	From this work

pGPC370	Hyg^r	PCR nested deletion fragments of *pmiA* spanning region cloned into pOLYG	From this work
pGPC371	Hyg^r	PCR Nested deletion fragments of *pmiA* spanning region cloned into pOLYG	From this work
pGPC372	Hyg^r	PCR Nested deletion fragments of *pmiA* spanning region cloned into pOLYG	From this work
pGPC373	Hyg^r	PCR Nested deletion fragments of *pmiA* spanning region cloned into pOLYG	From this work
pGPC374	Hyg^r	PCR Nested deletion fragments of *pmiA* spanning region cloned into pOLYG	From this work
pGPC81	Amp^r	Adenoviral vector	Kindly provided by Andreas Untergasser
pQE10	Amp^r	His tag expression vector	Qiagen
pQE16	Amp^r	DHFRS (dihydrofolate reductase)-His-tag ; expression vector	Qiagen
pQE18	Amp^r	His-tag expression vector	Qiagen
pUC4K	kana^r	Vector with kanamycin resistance cassette	Pharmacia

3.1.5 Bacterial Strains

Strain	Characteristics	Source
E. coli DH5α	F$^-$(ϕ80dΔlacZM15) Δ (lacZYA-argF)U169 deoR	Invitrogen GmbH, Karlsruhe, Germany
E. coli NM554	MC1061 *recA13*	NEB, Frankfurt, Germany

M. marinum (ATCC 927)	Mycobacterium marinum strain isolated from fish	Dept Microbiologie, Instituut voor Tropische Genes Kunde, Antwerpen, Belgium
M. marinum mutants	Transposon mutant library generated in *M. marinum* (ATCC927)	(Rybniker *et al.*, 2003)

3.1.6 Cell Lines

Cell Line	Characteristics	Source
HEK 293	Human embryonic kidney cell line	(Graham *et al.*, 1977)

3.2 Methods

3.2.1 Culturing Human Monocyte Derived Macrophages (HMDM)

Buffy coats were obtained from the University clinic blood bank, University of Cologne. The buffy coat obtained from a 500ml blood donation of the same day was split into two 50ml tubes. It was further diluted 1:1 with RPMI medium. The diluted buffy coat was layered on a 15ml histopaque gradient. The discontinuous gradient was centrifuged at 1600rpm for 40 minutes and the centrifuge was brought to halt without applying brakes. Monocytes layered in the interface were carefully transferred out into a fresh 50ml tube. The isolated monocytes were then washed with RPMI twice by centrifuging at 900rpm for 10 minutes. The monocytes were counted using a Neubauer chamber and adjusted to a density of $3x10^6$cells/ml with tissue culture (TC) medium [RPMI containing 5% foetal bovine serum (FBS)]. These cells were plated on serum opsonised TC flasks, dishes or petriperms as required. The monocytes were allowed to adhere on to the base overnight at 37^oC in a 5% CO_2 atmosphere. After an overnight incubation the cells were washed with pre-warmed RPMI medium to remove the non-adherent cells. The adherent cells were allowed to differentiate into macrophages for another 6 days. Cells were renewed with TC

medium (RPMI containing 5% FBS) every second day. The differentiated macrophages were used for further experiments.

3.2.2 Screening for Mutants Incompetent in Retarding Phagosome Maturation

3.2.2.1 Preparation of Single Cell Suspension of Mycobacteria for Infection

More than 4000 colonies of transposon mutants grown on a 7H10 agar plate were scraped off using a cell scraper and suspended in 7H9 broth in a 15ml tube. Bacteria were washed with phosphate buffer saline (PBS) three times by centrifuging at 5000rpm. The bacterial pellet was then resuspended in 1ml of RPMI. A small amount of glass beads of 0.2mm diameter were added and shaken on a mill for 5min to disrupt the clumps. Glass beads and the bacterial clumps were removed by centrifuging at a low speed of 600rpm for 5min. After centrifugation the supernatant was collected without disturbing the pellet. The collected supernatant was then passed through a 27 gauge needle three times. The bacterial suspension was then centrifuged at 600rpm for 5min to make sure that there were no bacterial clumps left. Optical density (OD) was measured on a spectrophotometer at 600nm. The OD values were used as a measure for the number of bacterial cells. This bacterial suspension was used to infect HMDM.

3.2.2.2 Endocytosis of Iron Dextran Beads

$3x10^6$ HMDM were seeded on 10 petriperms (Tissue culture dish with a hydrophilic membranous base) and pulsed with 0.5ml of 1:10 diluted colloidal iron dextran particles (Miltenyi basic beads) suspended in TC medium (RPMI). Micro beads (Miltenyi basic beads) were pulsed for one hour at 37^oC in a CO_2 incubator. After one hour HMDM were washed with RPMI repeatedly (three times) to remove the non-endocytosed beads. The endocytosed beads were further chased for another 2hrs. After the chase HMDM monolayers were rinsed with RPMI once and used for infection.

3.2.2.3 Infection of Iron Dextran Pre-fed HMDM with *M. marinum* Transposon Mutants

HMDM that were pre-fed (3.2.2.2) with microbeads infected with $3x10^7$ transposon mutants prepared as described in section 3.2.2.1. HMDM were pulsed with bacterial suspension in TC medium (RPMI) for 2hrs at 37^oC. After 2hrs the macrophage culture was washed with RPMI to remove the non-phagocytosed bacteria. Three repeated

washings were done. HMDM were replenished with RPMI containing 5% FBS. The phagocytosed bacteria were then chased overnight at 37^{o}C in a 5% CO_2 atmosphere.

3.2.2.4 Isolation of Phagolysosomes Containing *M. marinum* Mutants and Phagolysosomal Marker

Buffers Used

Equilibration Buffer

50mM Pipes buffer pH7.0; 50mM KCl; 2mM $MgCl_2$; 5mM EGTA; 1mM DTT and 10µM Cytochalasin B.

Lysis Buffer

50mM Pipes buffer pH7.0; 50mM KCl; 2mM $MgCl_2$; 5mM EGTA; 220mM Mannitol; 68mM Sucrose.

Wash Buffer

50mM Pipes buffer pH7.0; 50mM KCl; 2mM $MgCl_2$; 5mM EGTA; 1mM DTT.

After an overnight chase HMDM were rinsed twice with PBS. 750µl of equilibration buffer was added to the cells followed by incubation on ice for 20min. After the incubation 250µl lysis buffer was added and HMDM were scrapped off using a rubber policeman and collected in a 2ml tube (eppendorf). The collected cell suspension was passed through a 23 gauge needle at least 15 times. 100µl of the homogenized cell suspension was kept aside for β-galactosidase assay. The remaining fraction was applied on a mini-MACS column mounted on a magnet. The MACS column was pre-equilibrated with wash buffer before applying the sample. A fraction of the flow-through was saved for β-galactosidase assay. The column was washed three times with wash buffer. The washings were collected and saved. The column was next removed from the magnet to release the bound organelles and 1ml of 7H9 medium was flushed through using a piston. The flow-through containing the mutant phagosomes was collected and a sample was kept aside for β-galactosidase assay and the remaining centrifuged at 15,000rpm for 2min. The pellet was resuspended in 200µl of 7H9 broth and 100µl of the suspension was plated on to two 7H10 agar plates. The inoculated agar plates were then incubated at 30^{o}C for 5 days until the colonies became just visible. Colonies were scraped off and treated as described to obtain a single cell suspension (3.2.2.1) and used for HMDM infection. The screen was repeated a total of three times. From the third screen 100 individual clones

were picked and inoculated individually on 7H10 agar plates. Subcultures of these clones were again inoculated on 7H10 agar plates and used to prepare frozen stocks at -80^{o}C.

3.2.3 Southern Blot Analysis

3.2.3.1 Extraction of Mycobacterial Chromosomal DNA

Buffers

Lysis Buffer

25% Sucrose; 50mM Tris pH 8.0; 1mM EDTA.

TE Buffer

10 mM Tris-HCl pH 7.5; 1 mM EDTA.

A loop of mycobacteria grown on 7H10 agar plate was taken and suspended in 400µl lysis buffer in an eppendorf tube and vortexed well. A small amount of glass beads was added to the bacterial suspension which was then agitated in a cell mill (Retsch MM2000) for 2min at maximum amplitude. The tubes were removed from the mill 10µl lysozyme (40mg/ml) was added to each followed by incubation on ice for 5min. After the incubation 4µl ProteinaseK (10mg/ml), 40µl 0.5M EDTA and 16µl sarcosyl (35%) were added and incubated on ice for further 1hr, followed by an overnight incubation at 50°C. Next morning the cell debris was removed by spinning at 15,000rpm for 5min discarding the pellet. The supernatant was collected in a separate tube and 0.5 volume of 7.5M ammonium acetate was added before centrifugation at 15,000rpm for 30min to precipitate DNA. The DNA pellet was washed with 500µl 70% ethanol by centrifuging at 15,000rpm for another 30min. The pellet was dried and resuspended in 50µl TE buffer. OD was measured to quantify the DNA obtained.

3.2.3.2 Restriction Enzyme Digestion and Blotting

Buffers

Denaturation Buffer

1.5M NaCl and 0.5M NaOH

Neutralization Buffer

0.5M Tris/HCl pH 7.0 and 3M NaCl

20X SSC Buffer

3M NaCl; 0.3M sodium citrate and pH adjusted to 7

Hybridization Buffer

Formamide 50%; 5X SSC; 2% Blocking reagent; 0.1% N-Lauryl sarcosine; 0.02% SDS.

4µg of extracted chromosomal DNA of wild-type (WT) *M. marinum* or mutants isolated and was digested with *BamHI* endonuclease. The restricted DNA was separated by electrophoresis on an agarose gel. The DNA in the gel was fragmented by submerging the gel in 0.25M HCl for 10min followed by a brief washing with distilled water. After fragmentation DNA was denatured by incubating the gel in denaturation buffer for 15min at RT. After 15min the solution was changed and a fresh solution of denaturation buffer was added and incubation continued for another 30min at RT followed by a washing with distilled water. After denaturation the gel was neutralised by placing it in neutralisation buffer at RT for 15min. The incubation was repeated with a fresh change of neutralisation buffer.

Whatman3 paper and nylon membrane (NytranN) were cut to the size of the gel and equilibrated by dipping in 2X SSC buffer and placed on a vacuum blot apparatus. The gel was placed over the membrane and 55-60mbar vacuum was applied to initiate transfer for 60min. The gel was not allowed to dry by adding 20X SSC buffer on top of the gel. After the transfer the membrane was removed, washed once with 2X SSC buffer and the DNA was crosslinked in a UV cross linker apparatus (UVC500 UV cross linker) before pre-hybridization by incubating the membrane in hybridization buffer for 4hrs at 42°C.

3.2.3.3 Digoxigenin (DIG) Labelling and Hybridization

A probe specific for the *aph* gene was prepared by restricting the aph gene from PUC4K plasmid with *pstI*. Restriction fragments were separated by electrophoresis on a 0.7% agarose gel. A band of approximately 1.5kb size corresponding to the *aph* gene was sliced out of the gel. DNA was extracted from the gel slice and purified using Qiagen gel extraction kit following the manufacturer's protocol. The extracted DNA was denatured by boiling for 10min and immediately cooled down to -20°C. Hexanucleotide mix, dNTP labelling mix and klenow polymerase were added to the DNA and incubated at 37°C overnight. The reaction was stopped by adding 2µl of 0.2M EDTA. The DIG labelled DNA was further purified using Qiagen PCR purification kit. Following the addition of the probe, hybridization to the filter cross-linked DNA was done in hybridization buffer for a brief period at 80°C and overnight at 42°C. Thereafter the hybridization membrane

was washed with 2X SSC buffer containing 0.1%SDS twice at RT (5min each wash), followed by washing with 0.2X SSC buffer containing 0.1%SDS twice at 68°C (15min each wash). Finally the membrane was developed using CDP star luminescence detection reagent.

3.2.3.4 Enhanced Chemiluminescence (ECL) Detection

Buffers

Buffer 1

Maleic acid 0.1 M, NaCl 0.15 M in water and pH adjusted to 7.5 with 1N NaOH.

Wash Buffer

Buffer 1 + 0.3% Tween 20

Buffer 2

Blocking buffer diluted 1:10 in buffer1

Buffer 3

Tris/HCl 0.1M; NaCl 0.1M, $MgCl_2$ 50 mM in water and pH adjusted to 9.5

The hybridized membrane was washed with wash buffer and then incubated in buffer2 for 30min. Anti-DIG-alkaline phosphatase conjugated antibody was diluted 1:10000 in buffer2 and the membrane was incubated in this solution for 30min. After the incubation the membrane was washed with wash buffer twice (15minutes each). Washing was followed by equilibration in buffer3 for 5min. The blot was transferred on to a polythene sheet and the ready to use CDP star substrate solution was added and incubated at 37°C briefly. The excess substrate solution was removed and washed gently with buffer3. The polythene sheet was sealed and exposed on to X-ray film in the dark for different time periods.

3.2.4 Cloning by Homologous Recombination using "Infusion Kit"

Buffers

TE Buffer

10 mM Tris-HCl pH 7.5; 1 mM EDTA.

3.2.4.1 Cloning

Plasmid was restricted with suitable restriction enzymes and mixed with fragments of PCR amplified using a high fidelity polymerase from *Thermococcus kodakaraensis* (KOD

DNA Polymerase) in a 2:1 vector/insert molar ratio according to the recommendations of the manufacturer (Clontech). The final volume was made up to 10µl. The 10µl mixture was added to an "Infusion" reaction tube. The reaction mix was incubated at 42^{o}C for 30min and then immediately stopped on ice. The reaction mix was diluted by adding 40µl of TE buffer.

3.2.4.2 Transformation

3µl of the diluted reaction mix was transformed into "Infusion blue" (Clontech) competent cells. The transformation was done by heat shocking the cells at 42^{o}C in a water bath for 45sec followed by incubation on ice for one minute. Then 450µl of SOC medium was added to the tube and it was incubated at 37^{o}C for 1hr. After 1hr 100µl of the cells were plated on a LB agar plate containing 50µg/ml ampicillin. The plates were incubated at 37^{o}C overnight. Next day the colonies grown on the plate were picked and inoculated into LB broth containing 50µg/ml ampicillin for plasmid DNA preparation.

3.2.4.3 Plasmid Preparation

Bacterial culture was centrifuged to obtain the bacterial pellet and the plasmid was isolated using Qiagen mini prep kit as per the manufacturer's protocol. The plasmids were checked for the inserted gene fragment by restricting them with appropriate restriction enzymes and electrophoresing on 0.7% agarose gel with appropriate size standards.

3.2.5 Western Blot Analysis

Buffer

Transfer Buffer

Methanol 400ml; Glycine 28.8g; Tris 6.6g

Blocking Buffer

PBS containing 0.5% Gelatin and 0.1% Tween20

Protein samples were separated on a SDS-PAGE. Hybond-PVDF membrane was pre-wetted in 100% methanol for 5sec followed by water for 5min and then equilibrated in transfer buffer for 10min. Membrane was not allowed to dry. The SDS gel was also equilibrated in transfer buffer for 10min. A fibre pad was wetted in transfer buffer and placed on the transfer chamber. Over the pad two Whatman3 paper sheets of the size of the gel dipped in transfer buffer was placed. The gel was placed upon the stack and the

membrane over the gel. The membrane and gel were sandwiched with another 2 sheets of whatman3 paper wetted with transfer buffer. The transfer chamber was closed and placed on ice. Transfer was done at 250mA for 90min. The membrane was then washed with PBS, blocked by incubating in blocking buffer overnight on an orbital shaker and then was washed three times (5min each wash) with PBS containing 0.1% Tween20 at RT. Primary antibody diluted appropriately in PBS containing 0.1% Tween20 was added to the membrane and incubated for 60min at RT. Membrane was washed three times (5min each wash) with PBS containing 0.1% Tween20 at RT. Following the washing the secondary antibody was diluted appropriately in PBS containing 0.1% Tween 20 and added to the membrane and incubated for 60min at RT with constant agitation. Membrane was washed three times (5min each wash) with PBS containing 0.1% Tween20 at RT. Membrane was placed on a polythene sheet and ECL reagents mixed in a 1:1 ratio were added on to it and the polythene sheet was sealed at all sides. Membrane was developed by placing polythene bag containing the membrane on a cassette and exposed to an X-ray film (Hyperfilm) for 5-60min in dark.

3.2.6 Rab5-, Rab7- and Rab11-GFP Adenoviral Vector Construction

3.2.6.1 Cloning of Rab-GFP into Adenoviral Vectors

Mammalian expression plasmids containing N-terminal fusions of human Rab-GTPases tagged to green fluorescent protein (p-Greenlantern-Rab5 and –Rab7) constructs were a kind gift from Craig Roy (Boyer centre for Molecular Microbiology and Immunology, Yale University School of Medicine, New Haven, Connecticut). Fusion constructs pEGFP-Rab11b/Rab11a (Rzomp *et al.*, 2003) was generously provided by Marci A. Scidmore (Dept of Microbiology and Immunology, Cornell University, Ithaca, New York). Rab5- and Rab7-GFP adenoviral constructs were made by homologous recombination (In-fusion kit) (3.2.4.1). Rab5- and Rab7-GFP gene constructs were amplified from plasmids pGPC335 and pGPC336 using primers Rab_BD-infusion_fwd1 and Rab_BD-infusion_rev1. High fidelity polymerase with a proof reading activity (KOD DNA Polymerase) was used to amplify the fragments by PCR. The amplified Rab5- and Rab7-GFP fusion genes were purified using Qiagen PCR purification kit and cloned into pENTRY vector (pGPC331) by homologous recombination (In-fusion cloning kit) (2.2.4.l).

A Rab11-GFP gene fragment was excised from the plasmid received from M. Scidmore using restriction enzymes *AseI* and *MluI*. The purified fragment and the *XbaI* and *XhoI* digested pENTRY vector were blunt ended with klenow polymerase before ligation using T4 DNA ligase at RT and transformation into *E.coli* NM554. The cloned Rab-GFP fusions in pENTRY vectors were recombined into an adenoviral vector pGPC81 using Gateway system and transformed into *E.coli* DH5α.

Following were the adenoviral vectors obtained after recombining the individual pENRY vectors containing Rab-GFP constructs into pGPC81.

pGPC358; Rab11b

pGPC359; Rab5a

pGPC360; Rab7

3.2.6.2 Transfection of HEK 293 Cells to Produce Adenoviral Rab-GFP Constructs

Human embryonic kidney cell line (HEK 293 cell line) generated by transformation human embryonic kidney cell cultures with sheared adenovirus 5 DNA was used to generate Rab-GFP fusion adenoviral constructs (Graham *et al.*, 1977). 3µg of plasmids pGPC358, pGPC359 and pGPC360 were digested with *PacI* restriction enzyme. Restricted plasmids were purified using Qiagen PCR purification kit. Purified linearised plasmids were mixed with 500µl of TC medium (Opti-MEM l) and incubated at RT for 5 min. At the same time 45µl of cationic lipid transfection reagent (Lipofectamine^{TM}) was suspended in 2.5ml of Opti-MEM l and incubated at RT for 5min. Opti-MEM l containing the restricted plasmid and lipofectamine were mixed and incubated at RT for 20min. The mixture was carefully added on to a monolayer of 293 cells in a 25cm^2 TC flask and the cells were incubated overnight at 37^{o}C in a 5% CO_2 atmosphere. Following overnight incubation the medium was removed and renewed with fresh medium and incubation continued for another day at 37^{o}C in a 5% CO_2 incubator. The following day the monolayer was dispersed and transferred to a 75cm^2 TC flask. The cells were incubated until the monolayer started disrupting and cells were found floating in the medium. Cells were scrapped off using a cell scrapper and the cell suspension was transferred to a 50ml centrifuge tube. Cells were frozen and thawed repeatedly for three times to release the virus particles. After freezing and thawing, cell debris was removed by centrifugation at

4000 rpm for 10min. Virus titre in the supernatant was determined and virus stocks were frozen in aliquots at -80^{o}C.

3.2.7 Transfection of HMDM and Mouse Dendritic Cells with Rab-GFP Adenoviral Vectors

1 x 10^6 monocytes isolated from buffy coat, dendritic cells isolated from mouse or mouse peritoneal macrophages in TC medium (RPMI containing 5% FBS) were seeded on glass coverslips placed in 6 well plates. HMDM were used for transfection on the 6th day of culture, dendritic cells were transfected on the 4th day and activated mouse peritoneal macrophages on the same day of isolation. Cells cultured on glass coverslips were transfected with respective Rab-adenovirus particles suspended in RPMI without FBS at an MOI of 100:1. Followed by the addition of virus particles, plates containing the cell seeded glass coverslips were centrifuged on a plate centrifuge at 2500rpm for 120min. After centrifugation the medium was removed and the cells were replenished with RPMI containing 5% FBS. Cells along with virus particles were incubated overnight at 37^{o}C in a 5% CO_2 atmosphere. Cells were viewed under a fluorescence microscope (IX81 Olympus) for detection of GFP-fluorescence. Cells were also analysed by flowcytometry (FACS Calibur) to determine the transfection efficiency. The transfected cells were further used to study mycobacterial PM.

3.2.8 Staining using Antibodies and Immunofluorescence Microscopy

Buffers

Permeabilisation/Wash Buffer

5% BSA and 2% saponin or 0.5% poly ethylene glycol in PBS

Fixation Buffer

4% Paraformaldehyde in PBS

HMDM differentiated on glass cover slips were infected with FITC or TRITC labelled mycobacteria at an MOI of 1:1 or pulsed with fluorescent beads (approx 10beads/cell) and then fixed using fixation buffer for 20min at RT. Fixation buffer was removed and the cells were washed with PBS three times. Cells were permeabilised by incubating in permeabilisation buffer at RT for 40min. A 10µl drop of antibody diluted in wash buffer containing PBS (antibody dilutions were chosen as per manufacturer's instruction) was placed on a strip of parafilm. Glass coverslips containing permeabilised cells were

inverted on the drop of antibody placed on parafilm. Cells were incubated with antibody at RT for 1hr. Cells were then washed with wash buffer three times. After washing secondary antibody conjugated to a fluorophore (Alexa594, Alexa488) was diluted in wash buffer and a 10µl drop of the dilution was placed on a strip of parafilm. Again glass coverslips containing the cells were placed on the drop and incubated at RT for 1hr followed by washing with wash buffer thrice. The cover slips were then mounted on a glass slide over a drop of anti-bleach reagent (Entellan or Prolong anti-gold) and left overnight at RT. The following day cells were viewed under an Olympus IX80 immunofluorescence microscope using a 60X oil immersion lens.

Rab-GFP adenovirus transfected cells were infected with mycobacteria or pulsed with beads, fixed with fixation buffer, washed and mounted on glass slides as described above before being viewed under the microscope.

3.2.9 Staining of Mycobacterium with FITC or TRITC

Buffers

Sodium bicarbonate Buffer pH 9.0

8.4g sodium bicarbonate in 1 litre distilled water pH adjusted with 1N NaOH

$5x10^7$ WT or mutant *M. marinum* cells were suspended in 200µl of 0.1M sodium bicarbonate buffer pH 9.0, containing 1mg/ml FITC or 0.1mg/ml TRITC. Bacteria were incubated with the fluorescent dye at 30°C for 30 minutes. After incubation the excess dye was removed by three repeated washings with PBS. Bacteria were then resuspended in TC medium (RPMI) treated as described earlier to make single cell suspension (3.2.2.1) and then used for macrophage infections.

3.2.10 Growth Rate of *M. marinum*

6 well plates were seeded with $2x10^6$ monocytes isolated from buffy coat and allowed to differentiate into HMDM for 7 days. HMDM cultures were then infected with WT, P1 and P1 (pGPC352) at a MOI of 10:1. The number of bacteria was calculated from the OD values obtained and a predetermined standard curve. HMDM infected with bacteria were incubated at 37°C for 2hrs. Cells were then washed thoroughly with TC medium (RPMI) to remove extracellular bacteria and further incubated at 37°C. At different time points (2hrs, 24hrs, 48hrs, 72 hrs and 96hrs) cells infected with all three bacterial strains were lysed using 0.1% SDS for 10min, neutralized using 20%BSA and plated on 7H10 agar

plates. 7H10 agar plates were incubated at 30°C for 5 days for enumerating colony forming units (CFU). The CFU count obtained after 2hrs was considered as time zero post infection.

3.2.11 Flowcytometric Analysis of Cells Expressing EGFP-Rab

Rab-GFP adenoviral vector infected HMDM, mouse macrophages or mouse dendritic cells were washed and collected in tubes. The cells were incubated with antibodies specific for markers of macrophages and dendritic cells at concentrations recommended by the manufacturer at 4°C for 30min in PBS with 5% FBS. The cells were then washed with PBS. Cells were then analysed on a fluorescence activated cell sorter (FACScan^{TM}). The results were recorded using software (CELLQuest^{TM}) and expressed as percentage of fluorescent cells.

3.2.12 Trans-Complementation of the Mutant P1

A 3.8 kb region from *M. marinum* harbouring the putative *pmiA* gene was PCR amplified using primers P1_1064F1 and P1_8007R2. The PCR product was purified using Qiagen PCR purification kit and eluted with 30µl of water. 5µl of the product was electrophoresed on a 0.7% agarose gel to verify the product size. This PCR fragment was cloned into *XbaI* and *SpeI* digested pOLYG plasmid by homologous recombination technique (In-fusion cloning kit; See section 2.2.4.1) to yield pGPC352 conferring resistance to hygromycin. Plasmid pGPC352 was transformed into electroporation competent P1 mutant cells by applying an electric pulse of 2.5Kv. Following electroporation the cells were suspended in 7H9 medium and incubated at 30°C for 4hrs on a shaker incubator. Then the cells were selected on a 7H10 agar plate containing 30µg/ml kanamycin and 50µg/ml hygromycin. Colonies were picked from the plates and analysed for the presence of the plasmid pGPC352 by a PCR reaction using primers P1_1064F1 and P1_8007R2.

Nested-deletion DNA fragments were generated from the 3821bp *M. marinum* fragment in pGPC352 using a combination of internal PCR primers. The amplified fragments were cloned into pOLYG in the same way as described earlier to yield pGPC369, 370, 371, 372, 373 and pGPC374 (Fig-3). These plasmids were transformed into the mutant P1 as described before for analysis of functional complementation.

3.2.13 Radiolabelling of Mycobacterium

M. marinum WT and mutants were metabolically labelled with [1-^{14}C] palmitic acid by growing bacteria in 7H12 medium (BACTEC) containing 1μCi [1-^{14}C] palmitic acid. Bacteria were cultured for five days at 30^{o}C before being used for experiments. Phenolic glycolipids of mycobacteria were labelled by pulsing a three day old mycobacterial culture with [carboxyl – ^{14}C] *p*-hydroxy benzoic acid (0.7μCi/ml). After pulsing with [carboxyl – ^{14}C] *p*-hydroxy benzoic acid the culture was further grown for another two days before it was harvested.

3.2.14 Quantitative Analysis of Mutants Reaching the Phagolysosmes

Bacterial cultures were transferred to 15ml centrifuge tubes and centrifuged at 5000rpm for 10min. The bacterial pellets obtained were suspended in 5ml TC medium (RPMI) and washed by centrifugation at 5000rpm for 10 min. The washing was repeated once again. Supernatants were discarded and the pellets resuspended in 1ml of 7H9 broth. To the bacterial suspensions a little amount of sterile glass beads was added and the mycobacterial clumps were dispersed on a cell mill (Retsch MM2000) for 5min followed by centrifugation at 600rpm for 5min to remove the remaining bacterial clumps. The supernatants were aspirated and passed through a 27G needle 3 times. The CFU was of bacteria was calculated as described previously from the OD measurements and a standard curve. The density of the bacterial suspensions was adjusted as required using TC medium (RPMI).

HMDM grown as described previously (see section 3.2.1) were infected with radiolabelled bacteria at an MOI of 1:1 and chased overnight into PL along with iron dextran beads as PL marker. Following the chase, bacteria present in PL along with the iron dextran beads were selected on a MACS column (for a detailed protocol see section 3.2.2). 100μl of the isolated PL fraction was added to 2ml of scintillation fluid in scintillation vials. The scintillation vials were then placed in a β-scintillation counter (1217 Rackbeta) to determine the counts per minute (CPM).

3.2.15 Mice Infection

Specific pathogen-free C57BL/6 mice were infected intravenously with 4x10^{5} viable *M. marinum* WT, mutants P1 and complemented P1 (pGPC352). At 1 and 2 weeks after

infection five mice from each group were sacrificed by cervical dislocation and their liver and spleen excised aseptically. Liver and spleen were weighed and small equal samples of each liver and spleen were fixed in 4% formaldehyde for histopathology. Remaining liver and spleen samples were taken in a mortar and a little amount of white sand was added and the organs homogenized with a pestle. 5ml of sterile water was added to the homogenized suspension and transferred to a 15ml tube. The homogenized organ suspension was centrifuged at 400rpm to remove the tissue debris. The supernatant was transferred to another 15ml tube and centrifuged at 5000rpm to pellet the bacteria. The bacterial pellet was resuspended in 1ml 7H9 broth. Serial dilutions were made in 7H9 medium and 20µl of the diluted samples dropped on 7H10 agar plates. The inoculated 7H10 agar Plates were incubated for 5 days and CFU were counted.

3.2.16 Mycolic Acid Extraction and Analysis

3.2.16.1 Solvent Extraction

Methanolic esters of mycolic acids were prepared from [1-^{14}C] palmitate labelled bacteria. Bacteria (WT or mutant) grown in [1-^{14}C] palmitate containing medium were collected in 15ml tube. To the bacterial pellet 1ml methanol, 1ml 30% aqueous KOH and 0.1ml toluene were added. The mixture was incubated at 75^{o}C overnight, was cooled and acidified to pH1 by adding 3.6% HCl. Three extractions were performed with 1ml petroleum ether each time. The petroleum ether layers were pooled and evaporated to dryness by passing over a mild stream of nitrogen. Once dried the mycolic acids were methanolated by adding 1ml of Dichloromethane, 1ml catalyst solution (0.8g NaOH and 3.39g Tetra-n-butyl ammonium hydrogen sulphate in 100ml water) and 25µl Iodomethane. The mixture was mixed for 30min and the upper layer was removed and the bottom layer dried under nitrogen. The dried fatty acids were dissolved in 3:1 chloroform and methanol (Valero-Guillen *et al.*, 1986).

3.2.16.2 TLC Analysis

Mycolic acid samples were spotted on a silica high performance thin layer chromatography (HPTLC) plates (20x10cm silica gel 60 HPTLC plates). Petroleum ether (bp 60-80°C) and Acetone in a 95:5 v/v ratio were used as mobile phase to chromatograph the samples. After the mobile phase reached 3/4th of the TLC plate, the plate was removed from the chamber, dried and placed in a cassette and an X-ray film was placed over the

plate. The cassette containing the plate was left undisturbed for 4 days and then the film was removed in the dark and developed using an X-ray film developing machine (Agfa Curix60).

3.2.17 Non-Polar Lipid Analysis

3.2.17.1 Extraction

Bacteria were grown in 10ml 7H9 middle brook liquid broth for 5 days. The culture was centrifuged at 5000rpm for 10min and the medium was removed. 1ml of methanol – 0.3% NaCl (100: 10) was added to the cell pellet. Non polar lipids were extracted from the bacterial suspension using petroleum ether (3x 1ml). The petroleum ether extracts were pooled and dried under nitrogen. The dried lipid was dissolved in $CHCl_3$ – CH_3OH (3:1) (Dobson *et al.*, 1985).

3.2.17.2 Analysis by TLC

The lipid samples were spotted on a silica HPTLC plate (20x10cm silica gel 60 HPTLC plates) and chromatographed using $CHCl_3$ – CH_3OH – water (90:10:1) as mobile phase. Lipid spots were visualized by dipping the chromatographed TLC plate in a solution containing CuSO4 (10%) and 8% perchloric acid or in a solution containing 0.2% anthrone in concentrated sulphuric acid (to detect glycolipids) followed by charring at 180°C over a hot plate (Thermoplate S).

3.2.18 Extraction of a Lipid Species from WT *M. marinum* Missing in Mutant P1

WT *M. marinum* was grown in 100ml of 7H9 Middle Brook broth for 5 days. The cells were harvested by centrifuging at 5000rpm for 10min. The bacterial cells were left in $CHCl_3$–CH_3OH (2:1 v/v) for 2 days. Then cells were pelletted down by centrifugation and the chloroform – methanol supernatant was stored separately from the cells. Cells were extracted twice with $CHCl_3$–CH_3OH (1:1); each extraction was carried out for 24hrs. The extractions were pooled and washed twice with normal saline (0.9% NaCl in water). The aqueous layer was removed and the organic layer was dried under nitrogen.

The extracted lipids were re-dissolved in $CHCl_3$–CH_3OH (3:1 v/v) and chromatographed on a preparative silica gel TLC plate (20x20cm silica gel 60 TLC plate). $CHCl_3$ – CH_3OH – water (90:10:1 v/v) was used as mobile phase (Constant *et al.*, 2002). After

chromatography staining for the lipids was performed by dipping the sides of the TLC plate in a solution containing $CuSO_4$ (10%) and perchloric acid (8%) followed by charring at 80°C over a hot plate (Thermoplate S) to visualize the lipid spots. The lipid spot corresponding to the lipid missing in the mutant was marked and the corresponding silica gel band was scraped off from the unstained region of the TLC plate using a scalpel and taken in a 50ml tube.

The lipid was further extracted from the silica matrix using $CHCl_3$ – CH_3OH (1:1 v/v). The extraction was performed three times. The pooled extracts were centrifuged at 5000rpm to remove any silica gel. The extracts were dried under nitrogen and dissolved 1ml of $CHCl_3$ – CH_3OH (3:1 v/v). This solution was transferred to a 1.5ml eppendorf tube and centrifuged at 15,000rpm for 2 min to remove any silica particles. The remaining solution was transferred to another pre-weighed 2ml eppendorf and dried under nitrogen. Once dried the lipid present was weighed and stored at -20°C until use.

3.2.19 Structural Elucidation of the Lipid Missing in Mutant P1 by Mass and NMR Spectrometry

ESI-TOF (Electro spray ionisation- time of flight) -mass spectra were recorded in positive ion mode on a Q-TOF 2 mass spectrometer (Micromass, Manchester, UK) equipped with a nanospray source. Analytes were dissolved in chloroform : methanol and were injected into the mass spectrometer by glass capillaries using a capillary voltage of 1000 V and a cone voltage of 50 V. Instrument calibration was done with a mixture of sodium iodide and caesium iodide dissolved in 50% aqueous 1-propanol.

1H-NMR Spectra were recorded in CDCl3 on a Bruker AMX 500 Instrument at 500 MHz

3.2.20 Coating of Beads with Mycobacterial Lipids.

10µl (0.5mg) of RPC-18 beads (Dynal) or fluorescent polystyrene beads were placed in a 1.5ml eppendorf tube and 500µl 0.1M $NaHCO_3$ buffer pH 9 was added. Beads were washed by pipetting up and down carefully and the tube was placed on magnet (Dynal) for 2min. With beads captured to the wall next to the magnet, the buffer was removed. This procedure was repeated twice. The bead suspension was added to tubes in which PGL-1 or PGL-2 lipids were dried (1mg of lipid), followed by sonication in a sonicator bath for 10min. The suspension was further incubated at 37^{o}C for 2hrs with intermittent

sonication for 5min. The tube containing beads and lipid was placed again on magnet to remove buffer. The lipid coated beads were suspended in 1ml of PBS containing 5% BSA and incubated at 37^{o}C for 1hr to block the unbound sites of the beads. The lipid coated beads were then washed with 1ml of serum free medium (SFM) twice as described above. The lipid coated beads were then suspended in 1ml of SFM.

Fluorescent polystyrene beads were coated with lipid using the same procedure described above but the washing of the beads was done by centrifugation at 1200g for 15min instead of the magnetic separation.

3.2.21 Characterisation of Phagosomes Containing Lipid Coated Beads.

Buffers

Equilibration Buffer

50mM Pipes buffer pH7.0; 50mM KCl; 2mM $MgCl_2$; 5mM EGTA; 1mM DTT and 10μM Cytochalasin B.

Lysis Buffer

50mM Pipes buffer pH7.0; 50mM KCl; 2mM $MgCl_2$; 5mM EGTA; 220mM Mannitol; 68mM Sucrose.

Wash Buffer

50mM Pipes buffer pH7.0; 50mM KCl; 2mM $MgCl_2$; 5mM EGTA; 1mM DTT.

Elution Buffer

10mM triethanolamine; 10mM acetic acid; 1mM EDTA; 0.25M sucrose

HMDM were cultured in TC flasks as described earlier (see section 2.2.1). Cells were washed with SFM twice and placed in 2ml of SFM medium. A suspension of 100μl PGL-1 coated beads, PGL-2 coated beads (prepared as described above), uncoated RPC-18 beads (hydrophobic) or Myone beads (hydrophilic) was added to the cells in each TC flask. Uncoated beads were treated in the same way as coated beads except that no lipid was added. Cells with beads were incubated at 37^{o}C in a 5% CO_2 atmosphere for 1hr. After 1hr cells were washed with SFM three times to remove non-phagocytosed beads. HMDM along with beads were further chased by incubating overnight at 37^{o}C in a 5% CO_2 atmosphere. Macrophages were then washed with PBS once followed by the addition of 1.5ml equilibration buffer. The cells were then incubated on ice for 20min before 500μl lysis buffer was added. HMDM were then scraped off using a rubber policeman and collected in a 2ml eppendorf tube. The collected cell suspension was passed through a 23

gauge needle at least 15 times. 100µl of the homogenized cell suspension was kept aside for analysis. The eppendorf tubes containing the cell lysate were then placed on a magnet stand (Dynal) for 2min. Phagosomes containing the beads were captured on the wall of the tube, while the remaining fraction was removed and stored for analysis. The captured bead fraction was washed twice with 500µl of elution buffer and was finally suspended in 200µl of phagosome elution buffer.

The fractions stored were subjected to western blot analysis (section 3.2.5). After transferring the proteins on to PVDF membrane the blots were probed for Rab5 and β-galactosidase using the respective antibodies. Fluorescent polystyrene beads coated with PGL-1 and PGL-2 were chased (as described earlier in this section 3.2.21) into HMDM grown on glass cover slips and the cells were marked for different phagosomal markers (see section 3.2.8 for protocol) and investigated under a fluorescence microscope.

3.2.22 Cloning, Expression and Purification of PmiA-Protein

3.2.22.1 Cloning

Gene *pmiA* was amplified from WT *M. marinum* using primers P1N_pQE18_BD_F1 and P1N_pQE18_BD_R2 for cloning into vector pQE10 and primers P1N_pQE10_BD_F2 and P1N_pQE10_BD_R1 for vector pQE18. The amplified fragments were purified using Qiagen PCR purification kit and cloned in frame with the 6X Histidine (His) tag into plasmids pQE10 (N-terminal His-tag) and pQE18 (C-terminal His-tag) by homologous recombination (In-fusion kit) to yield plasmid pGPC363 and pGPC364 respectively. The recombination reactions were transformed into BD-fusion blue cells supplied along with the kit (see section 3.2.4). The clones were selected on LB plates containing 50µg/ml ampicillin. Clones were picked and analysed for inserts. Clones containing inserts were sequenced to confirm the correct fusion of the *pmiA* ORF to the His tag. Clones C777 (*pmiA* in pQE10) and C778 (*pmiA* in pQE18) were chosen for further use.

3.2.22.2 Expression

To express the gene *pmiA* in *E. coli*, a 10ml LB broth with 50µg/ml ampicillin was inoculated with a colony of C777 and C778 containing the *pmiA* plasmid and grown at 37°C overnight. The overnight culture was transferred to a 100ml LB broth containing 50µg/ml ampicillin and incubated at 37°C until the culture reached an OD of 0.8. *E. coli*

cells were induced by adding 100µl of IPTG (Isopropyl β-D-1-thiogalactopyranoside) and further incubated for 3hrs. The cells were harvested by centrifuging for 15min at 5000rpm. The cell pellets were stored at -80°C until use.

3.2.22.3 Purification

Buffers

Buffer A

1M NaCl, 10mM Imidazole and 50mM Tris pH 8.0

To purify the protein the cell pellet was thawed, suspended in 5ml of Buffer A, 300µl of lysozyme (40mg/ml stock solution) was added and incubated on ice for 30min. A small amount of glass beads of 0.2mm diameter were added and the suspension was agitated on a mill for 5min to disrupt the cells. Glass beads were allowed to sediment and the supernatant was transferred to another tube and centrifuged for 20min at 10000rpm/min. The pellet was suspended in 5ml buffer A containing 1% Zwittergent 3-12, 1mM PMSF and incubated for 1hr at 20°C and centrifuged again at 10000rpm for 10min. Pellets were stored at -20°C. The supernatant was incubated with 1ml 50% Ni-NTA for 1hr at RT. Ni-NTA was washed twice with 4ml buffer A containing 1% Zwittergent 3-12 and twice with buffer B (same as buffer A but the pH is 6.0) with 1% Zwittergent 3-12. Elution was done in a cold room (+4°C). The protein was eluted from Ni-NTA resin using 0.5ml buffer B containing 1% zwittergent and 250mM imidazole. Elution was repeated 5 times. The amount of protein was estimated using Pierce assay following the protocol of the manufacturer. The protein was further analysed on a 10% SDS-PAGE.

3.2.23 Capturing Proteins Interacting with PmiA.

3.2.23.1 Preparation of Proteins from Mycobacterial Lysate

Buffers

Interaction Buffer

50mM NaH_2PO_4; 300mM NaCl; 20mM Imidazole; 0.005% Tween20 and pH adjusted to 8.0.

Wash Buffer

50mM NaH_2PO_4; 300mM NaCl; 20mM Imidazole; 0.1% Tween20 and pH adjusted to 8.0.

M. marinum protein whole cell soluble protein solution was prepared from a cell pellet obtained from a 5 day old 500ml culture. The liquid culture was centrifuged at 5000rpm for 15min to obtain the bacterial pellet. The pellet was suspended in 9ml interaction buffer with 1ml EDTA free protease inhibitor cocktail. The cell suspension was distributed into eppendorf tubes and a small amount of glass beads was added and agitated on a mill for 30min to completely disrupt the cells. Unbroken cells and glass beads were removed by centrifuging at 600rpm for 5min. The supernatant was collected and stored at -80°C until use.

3.2.23.2 His-Tag Pull Down Assay

Native proteins expressed and purified from C777 and C778 were immobilized on to magnetic agarose beads by incubating 30µg of protein with 100µl magnetic agarose beads in protein binding buffer at 22°C in a hybridization oven. The tubes were then placed on a magnet stand (Dynal) to separate the magnetic beads immobilized with protein. The supernatant was removed and the beads were washed once with 500µl interaction buffer. 500µl of *M. marinum* protein solution was mixed with pmiA protein bound to the magnetic agarose beads and incubated for 1hr at 22°C in a hybridization oven. The tubes were again placed on a magnet and the supernatant was removed. The beads were washed twice with 500µl wash buffer. The agarose beads left in the tube were stored at -20°C until use. The proteins interacting with pmiA were analyzed by separating the proteins on a SDS-PAGE. His-tag-DHFRS (dihydrofolate reductase) protein (expressed and purified from pQE16 plasmid) was used as a negative control in these experiments.

4 Results

4.1 Magnetic Separation of Phagolysosomes from Subcellular Components

In order to isolate phagolysosomal fractions iron dextran micro beads which are proved to be delivered to the phagolysosomes were chased into HMDM. HMDM containing iron dextran beads were lysed carefully and the cell components were passed through a MACS column under strong magnetic field. The cellular fraction that adhered to the magnetic column was eluted and further analysed. Experiments were performed to prove that the micromagnetic beads reach the lysosomal vacuoles and the isolated fractions were phagolysosomes. The isolated PL fraction was subjected to western blot analysis by probing with LAMP- 1 and EEA-1 antibody. As expected the PL fraction isolated did not contain EEA-1 but LAMP-1 effectively marked the isolated PL fraction as depicted in (Fig-3).

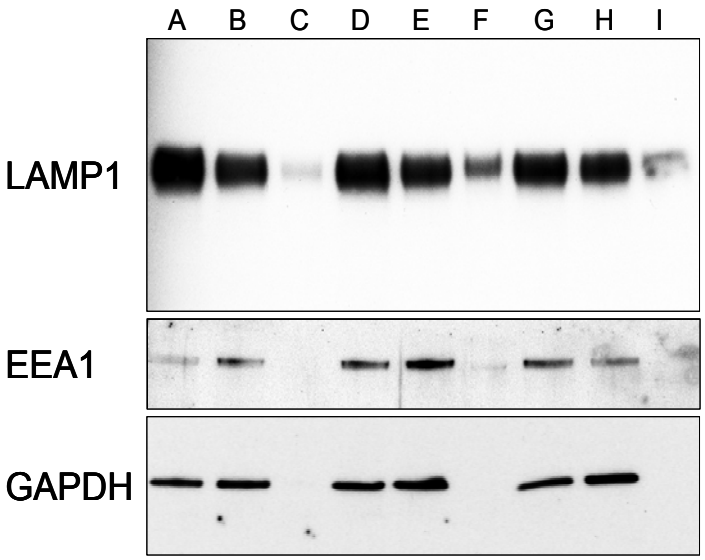

Figure 3: Western Blot Analysis of lysosomal fraction recovered by magnetic separation.

HMDM were pulsed with 'basic microbeads' (Miltenyi Biotech) for one hour followed by a chase for two hours (lanes D-F) or overnight (lanes G-I); lanes A-C: control macrophages without beads. After the chase, macrophages were lysed (lanes A, D, G) and magnetic separation of subcellular compartments was done following the same procedure as for the selection of *M. marinum* mutants incompetent in retarding phagosome maturation. Lanes B, E, H: macrophage lysate fraction not binding to magnetic column (flow-through). Lanes C, F, I: organelle fraction binding to magnetic column in the absence (C) or presence (F, I) of 'basic microbeads'. Following SDS-PAGE and blotting, membranes were probed with LAMP-1, EEA-1 or GAPDH antibody and developed by enhanced-chemiluminescence analysis.

4.2 Screening for *M. marinum* Mutants Permitting Phagosome Maturation

To decipher the mycobacterial genes ascribed to inhibit PM, a screen to isolate Tn*5367* transposon inserted mutants defective in inhibiting PM was established (Fig-4). We designed the screen using our own experience that *M. marinum* can endure the hostile phagolysosomal milieu for a long duration (24 hours, data not shown). The screen was based on the presumption that mutants disabled to inhibit PM would be enriched in the phagolysosome (PL) along with the PL markers. Transposon insertion mutants were chased together with magnetic microbeads into the phagocytic pathway of human monocyte derived macrophages (HMDM). After an overnight chase the plasma membrane of HMDM was lysed under carefully controlled conditions, preserving the intracellular organelles. The homogenate containing the subcellular components was then passed through MACS columns under a magnetic field which retained the magnetic beads in the PL inside the column. The column was next removed from the magnetic field and the mutants were eluted along with the PL marker. A β-galactosidase assay of the eluted and the flow-through fractions revealed that the eluted fraction was strongly enriched in phagolysosomes (Fig-5). The lysosomal fraction was plated on 7H10 agar and incubated for five days. Bacterial colonies grown on the plate were scraped off, pooled, carefully dispersed to yield a single cell suspension and used to infect a fresh culture of HMDM. The selection was repeated three times for further enrichment of mutants allowing PM. After the final selection 100 individual colonies were picked for further investigation.

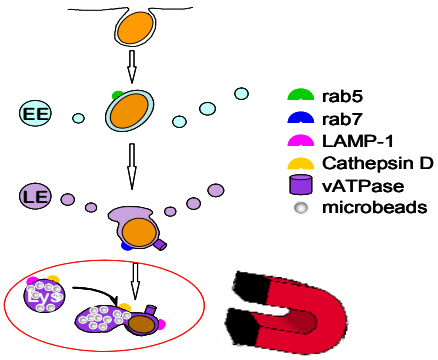

Figure 4: Screening for mycobacterial mutants permitting phagosome maturation.

Phagosomal processing of mycobacterial mutants with defects in PM inhibition. EE, early endosome; LE, late endosome; WT bacteria will remain in a Rab5-positive vacuole, whereas heat killed bacteria and mutants with defects in PM will be processed into a LAMP1-positive, vATPase-positive and cathepsin D-positive phagolysosome (PL). *M. marinum* transposon mutant library was chased together with magnetic microbeads to the PL and those mutants proceeding to the PL were selected on a MACS column under a strong magnetic field.

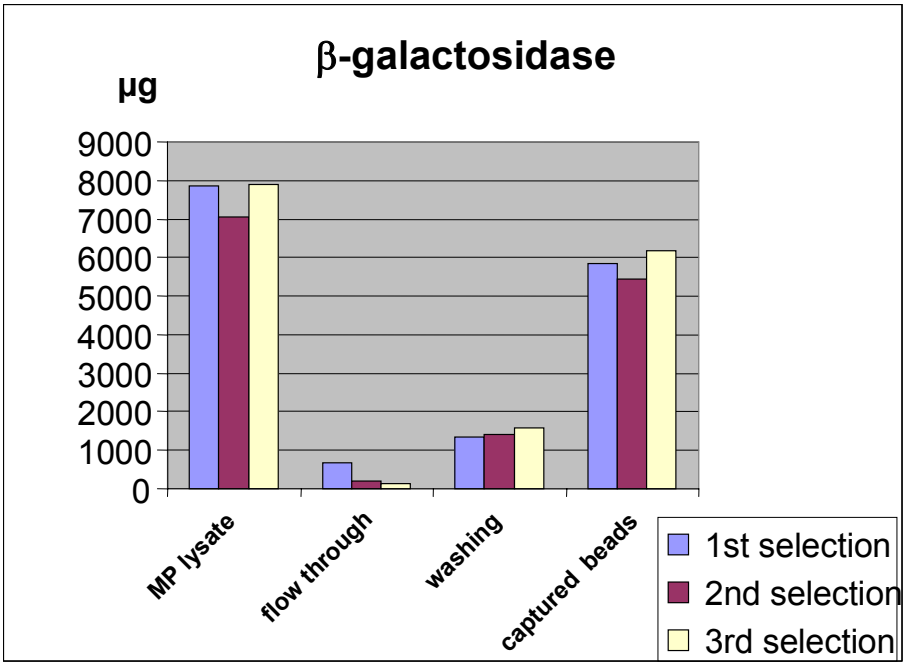

Figure 5: β-galactosidase assay performed with macrophage lysate (MP lysate), flow through after passing the lysate through MACS column, the washings of the column and the eluted phagosomal fraction for all three selections.

4.3 Defining Transposon Insertion Sites by Sequence and Southern Blot Analysis

In order to define transposon disrupted regions and to identify independent insertions in identical genes or loci, 79 of the 100 mutants selected for their inability to prevent PM were analysed by sequencing the transposon inserted locus. Southern blot analysis was also performed to confirm that each mutant had only a single transposon insertion and to identify mutants with transposon insertions in the same gene (Fig-6). The sequences obtained were BLASTed against the available *M. marinum* sequence database of Sanger (www.sanger.ac.uk). Since *M. marinum* genes are not annotated, a homology search was done against the *Mycobacterium tuberculosis* database available through TIGR CMR using the BLASTX function (www.tigr.org). 'Rv' numbers are used to represent the genes identified. Analysis revealed that the insertions were not spread across the genome (Fig-5). The most striking feature was that transposon inserted regions in 15 of the mutants could not be mapped to the available *M. marinum* genome database of Sanger, although similar genes, albeit with low probability scores were identified in other mycobacterial genomes. Interestingly, 50% of the insertions were mapped to genes influencing the

constitution of the cell envelope of mycobacteria (Listed in Table-2). The significance of the mutations is highlighted by the fact that insertions in the same gene or related genes in independent mutants were frequently found. Our results in general describing the diversity of mycobacterial genes involved in retarding PM are in accordance with previous reports. Genes coding for fadD proteins, transporters and genes of the PE/PPE gene family also appeared in our screen as was the case in previous screens (Pethe *et al.*, 2004); (Stewart *et al.*, 2005). Interestingly a mutant having a transoposon insertion in a gene involved in isoprenol biosynthesis as identified by Pethe et al was also identified through our screen. Moreover, we had taken utmost care to disperse bacterial clumps into single bacilli. This enabled us to identify genes coding for membrane proteins and secretory proteins. Under representation of such proteins in genetic screens has been speculated to be due to cross presentation by Stewart et al. Our screen also identified genes contributing to the resistance of mycobacteria to the killing by macrophages as reported by others independently (Sassetti and Rubin, 2003); (Miller and Shinnick, 2001); (Gao *et al.*, 2004); (Raynaud *et al.*, 2002); (Haydel and Clark-Curtiss, 2006); (He *et al.*, 2003) (see Table-2 for details).

Table – 2: List of Mutants obtained through the screen, their putative function and their *M. tuberculosis* homologue.

mutant	putative function	*Mtb* H37Rv hom.
N68	mmpL4	Rv0450c
N4R	mmpS4	Rv0451c
N43	chorismate pyruvate-lyase	Rv2949c
N33	fadD22, Probable Acyl-CoA Synthetase	Rv2948c
N8	hypothetical protein Rv1259	Rv1259
N93	PPE family	Rv0355c
N29	PPE family	Rv1918c
N67	galT gal-1-P uridylyltransferase	Rv0618
N34	POSSIBLE TRANSMEM-BRANE PROTEIN	Rv0514
N69	hypothetical protein Rv1134	Rv1134
N95	hypothetical protein Rv0007	Rv0007
N21	PPE family	Rv1918c
N10	PPE family	Rv1918c
N61	PPE family	Rv2356c
N32	hypothetical protein Rv0326	Rv0326
N47	hypothetical protein Rv3829c	Rv3829c
P3	PPE family	Rv1548c
N5	PPE family	Rv1135c
N75	PPE family	Rv1135c

N76	Unknown function	Rv0213c
N77	Unknown function	Rv0213c
N27	unknown function	Rv3897c
N40	cell division protein ftsh	Rv3610c
N66	unknown function	Rv0365c
N6	unknown function	Rv0365c
N9	unknown function	Rv0365c
N15	unknown function	Rv1735c
N13L	PE_PGRS family	Rv3388
N70	hyp. prot. toxin prod & resist.	Rv2959c
N28	unknown function	Rv1776c
N48	hyp. protein, electron transport	Rv1882c
N45	possible transmem. prot.	Rv1888c
N30	unknown function	Rv0037c
N16	PPE family	Rv1918c
N37	rnhB, ribonuclease HII	Rv2902c
N71	unknown function	Rv1111c
N78	hyp. prot. metalloendopeptidase	Rv1977
N18	possible membrane protein	Rv0677c
N81	PE_PGRS family	Rv3652
P1	unknown function	Rv2229c
N100	hyp. prot. stress response	Rv2624c
N39	unknown function	Rv0004
N13R	PE-PGRS family protein	Rv3812
N24	fadD22 Acyl-CoA Synthetase	Rv2948c
N63	polyketide synthase	Rv0405
N20	hyp. prot. helicase activity	Rv1179c
N64	unknown function	Rv0538
N84	fadE25 acyl-coA dehydrogenase	Rv3274c
N83	fadD35, Acyl-CoA Synthetase	Rv2505c
N14	unknown function	Rv1291c
N44	PPE protein family	Rv3533c
N82	unknown function	Rv0841c
N65	hyp. prot Cell envelope	Rv1145
N22	cell envelope, toxin production	Rv1115
N73	PGRS family	Rv0335c
N97	hyp. prot. cell envelope	Rv1057
N23	unknown function	Rv2347c
N99	Transport and binding proteins	Rv3239c
N12	Cysteine synthase/cystathio-ninebeta-synthase	Rv1336
N74	ompATb	Rv0899
N38	plcB: phospolipases C	Rv2350c
N92	bifunctional short chain isoprenyl diphosphate synthase	Rv3398c
N4L	unknown function	Rv3860
N36	hyp. prot. cell envelope	Rv3881c
N1	hypothetical phage protein	spyM18_0725
N2	unknown function	no homology
N19	prob. cyclase histidine biosynthesis	Rv1605

N3	hyp. prot. transport	Rv0849
N31	unknown function	no homology
N35	hyp. prot. transport	Rv0849
N41	putative transporter Nocardia	pnf1810
N42	peptide synthetase	Rv0101
N46	unknown function	Rv2100
N62	hyp. prot. fatty acid and phospholipid metabolism	Rv2800
N7	unknown function	no homology
N72	unknown function	Rv0523c
N91	unknown function	Rv1944c
N94	unknown function	Rv3668c

Hit within 500bp distance

Hit within 5000bp distance

Hit within 10000bp distance

No strict homology found in *M. marinum* M strain genome

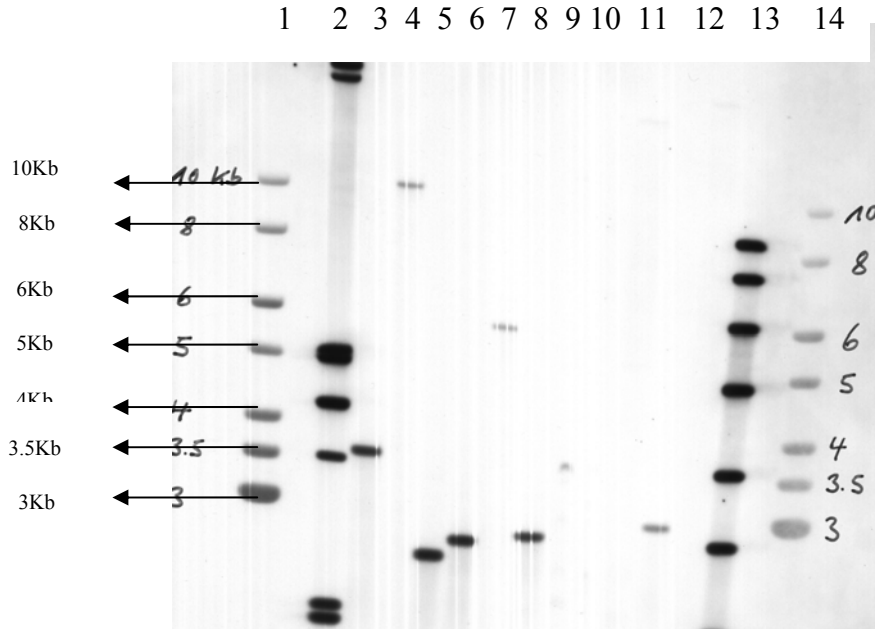

Figure 6: Southern blot analysis of *M. marinum* transposon mutants. DNA was isolated from mutants separated on agarose gel, transferred onto nitrocellulose membrane and hybridized to an *aph* gene. Lanes 6, 8 and 11 show mutants having transposon insertion in the same gene.

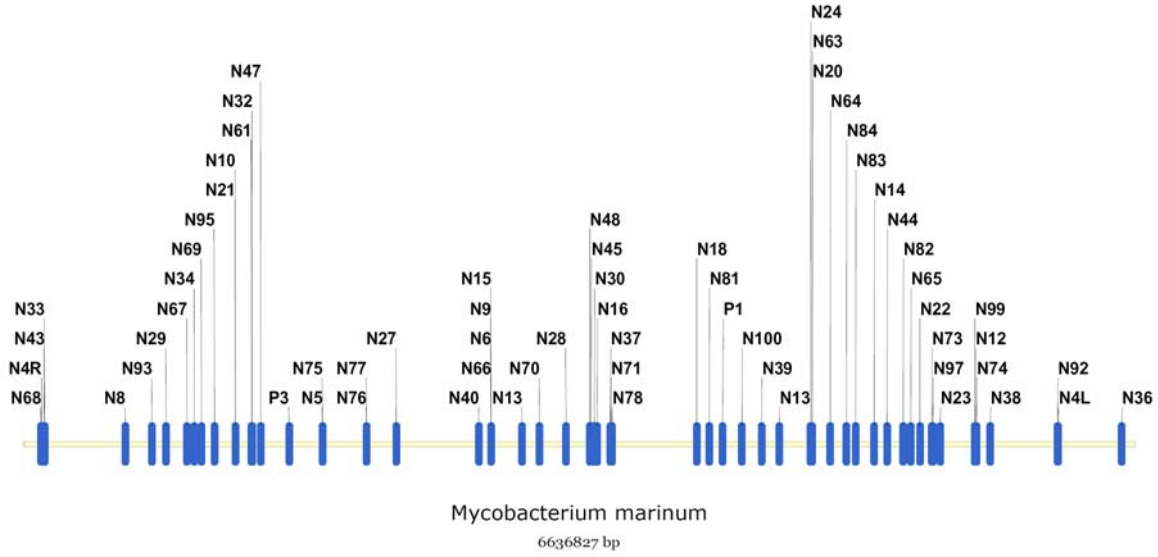

Figure 7: Distribution of selected mutants on the *M. marinum* M strain genome map. Transposon insertion sites and genomic locations were determined by sequencing and BLAST analysis (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/m_marinum).

4.4 Adenoviral Vectors to Track the Endocytic / Phagocytic Pathway

Rab5 and Rab7 Green fluorescent protein constructs were received from Dr. Craig Roy and Rab11 GFP construct was received from Dr. Marci Scidmore. These genes were re-cloned and packed into an adenoviral vector. Adenoviral vectors harbouring the Rab-GFP fusion proteins were used to transfect HMDM. An efficient transfection in HMDM was achieved with an MOI of 100:1 when the cells were spin infected. The cells were scanned under a fluorescence microscope. Cells expressing GFP were also quantified by FACS (Fig-9). In order to confirm that the Rabs were expressed as GFP tagged proteins, western blot analysis was performed. The blots were probed with an anti-GFP antibody followed by ECL detection. Analysis proved that the Rab-GTPases were expressed as GFP-fusion proteins as shown in figure (Fig-8)

The functionality of the Rab GTPases was assessed by chasing fluorescent dextran and transferin (Tf) in HMDM transfected with respective Rab-GFP adenoviral vectors, followed by evaluation under a fluorescent microscope. When fluorescent dextran was chased for 2 minutes followed by fixation, vacuoles containing dextran were marked by the presence of Rab5-GFP indicating early endosomes (Fig-9), whereas in Rab7-GFP transfected HMDM, dextran chased for 30min was present in compartments marked with Rab7-GFP signifying late endosomes (Fig-9). Similarly Rab11-GFP marker for recycling

endosomes was present in organelles containing Tf, when Tf was chased in HMDM for 5 minutes (Fig-9). These observations ascertain that the Rab-GFP fusion proteins expressed in HMDM using adenoviral transfections were functional or at least co-localised with the functional wild-type proteins. The same was also confirmed in mouse DCs and macrophages (Fig-9).

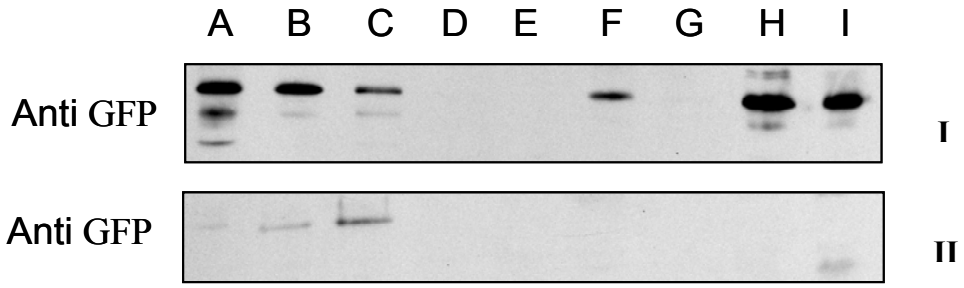

Figure 8: HMDM were transfected with Rab5 (I: A-C), Rab7 (I: D-F), Rab11a (I: G-IRab11b (II: A-C) adenoviral constructs for different time points (overnight, 48hrs and 72 hrs). Following transfection, cells were lysed and equal amounts of protein was loaded on SDS-PAGE. Gel was blotted onto PVDF membrane and probed with antibody against GFP. Cell lysate from cells transfected with an empty adenoviral vector (II: D-F) and macrophage lysate (II: G-I) without adenoviral transfection were used as negative controls.

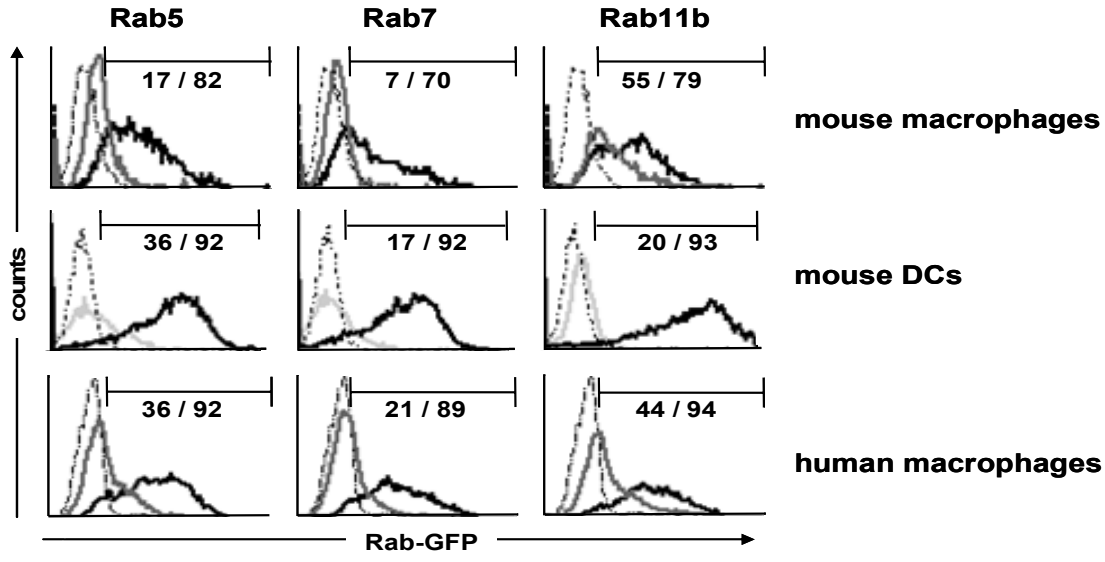

Fig 9: FACS analysis of Rab-GFP fusion transfected cells

Mouse macrophages, Mouse dendritic cells and HMDM were transfected with Rab-GFP adenoviral constructs by spin and without spin infection and analysed by FACS. Black curve indicates transfection by spin, grey curve indicates transfection without spin and dotted lines indicate non-transfected cells. Numbers over the histogram indicate percentage of cells transfected by spin/ percentage of cells transfected without spin.

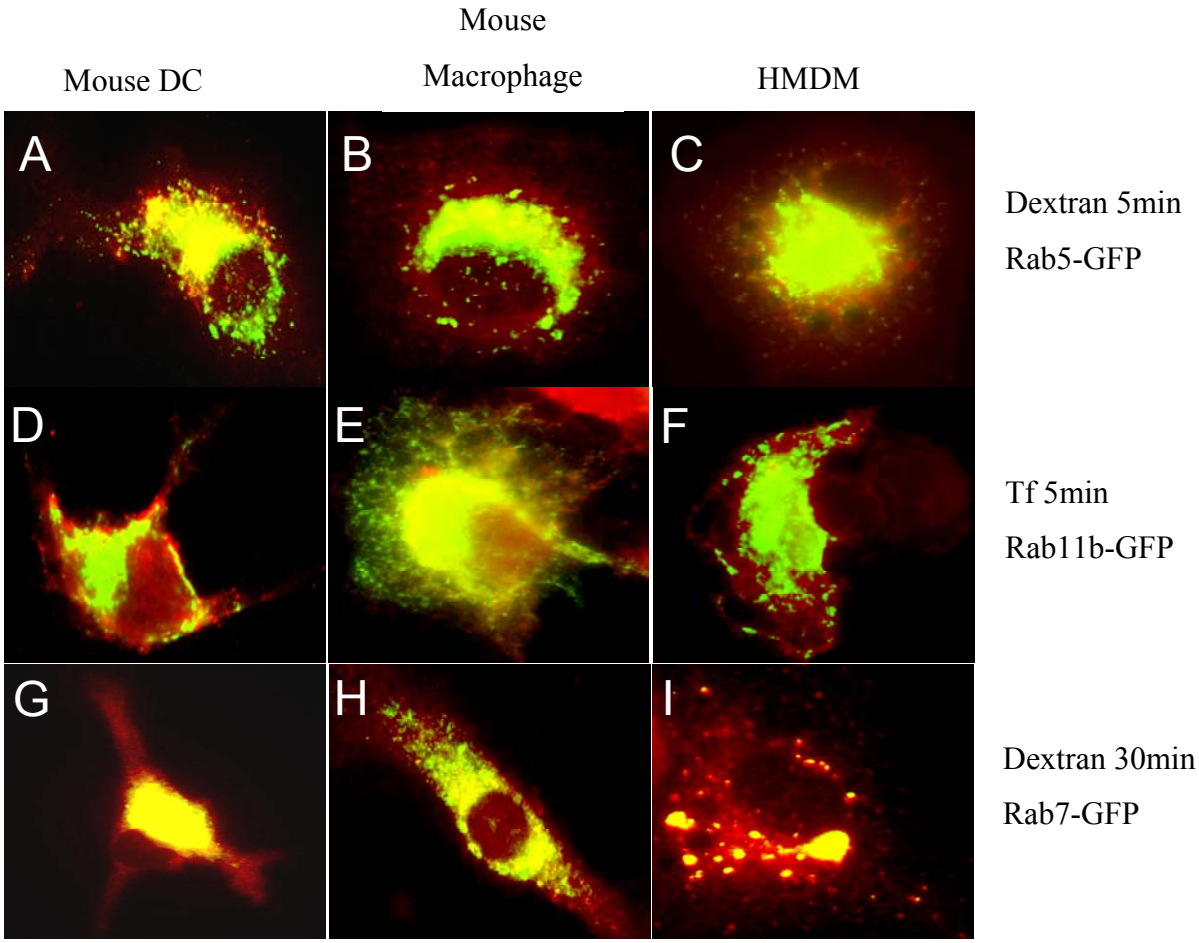

Figure 10: Functional characterisation of adenoviral Rab constructs by fluorescent microscopy

Mouse dendritic cells, macrophages and HMDM were transfected with Rab-GFP adenoviral constructs and chased with dextran and transferin. Fig depicts co-localisation of dextran with Rab5-GFP after a 5min chase (A-C), Transferin (Tf) with Rab11b-GFP after a 5min chase (D-F) and Dextran with Rab7-GFP after a 30min chase (G-I)

4.5 Immunofluorescence Microscopy of HMDM Infected with Mutants

We employed the above described Rab5- and Rab7-GFP fusion proteins and LAMP-1 antibody staining to characterize the wild-type *M. marinum* (WT) and mutant containing phagosomes. Immunofluorescence micrographs revealed that WT containing phagosomes were indeed found to retain Rab5 (Fig-11A) even after an overnight chase, whereas LAMP-1 (Fig-11C) and Rab7 (Fig-11E) were excluded. In contrast, phagosomes containing mycobacteria were observed to co-localise with Rab7 and LAMP-1 (Fig-11F and 11D). One of the six randomly selected mutants screened (P1) had severely reduced capacity to resist PM. 69 $\pm$ 4% of the P1 phagosomes acquired Rab7 and 73 $\pm$ 3% LAMP-1 compared to 13 $\pm$ 2%, 22 $\pm$ 2% respectively of WT bacteria, when 100 phagosomes of WT and P1 mutant were enumerated each (see Fig–12 and table-3 for details). This indicated that the interrupted gene in this mutant has a vital function in preventing PM. The gene has been given the provisional name *phagosome maturation inhibition A* (*pmiA*).

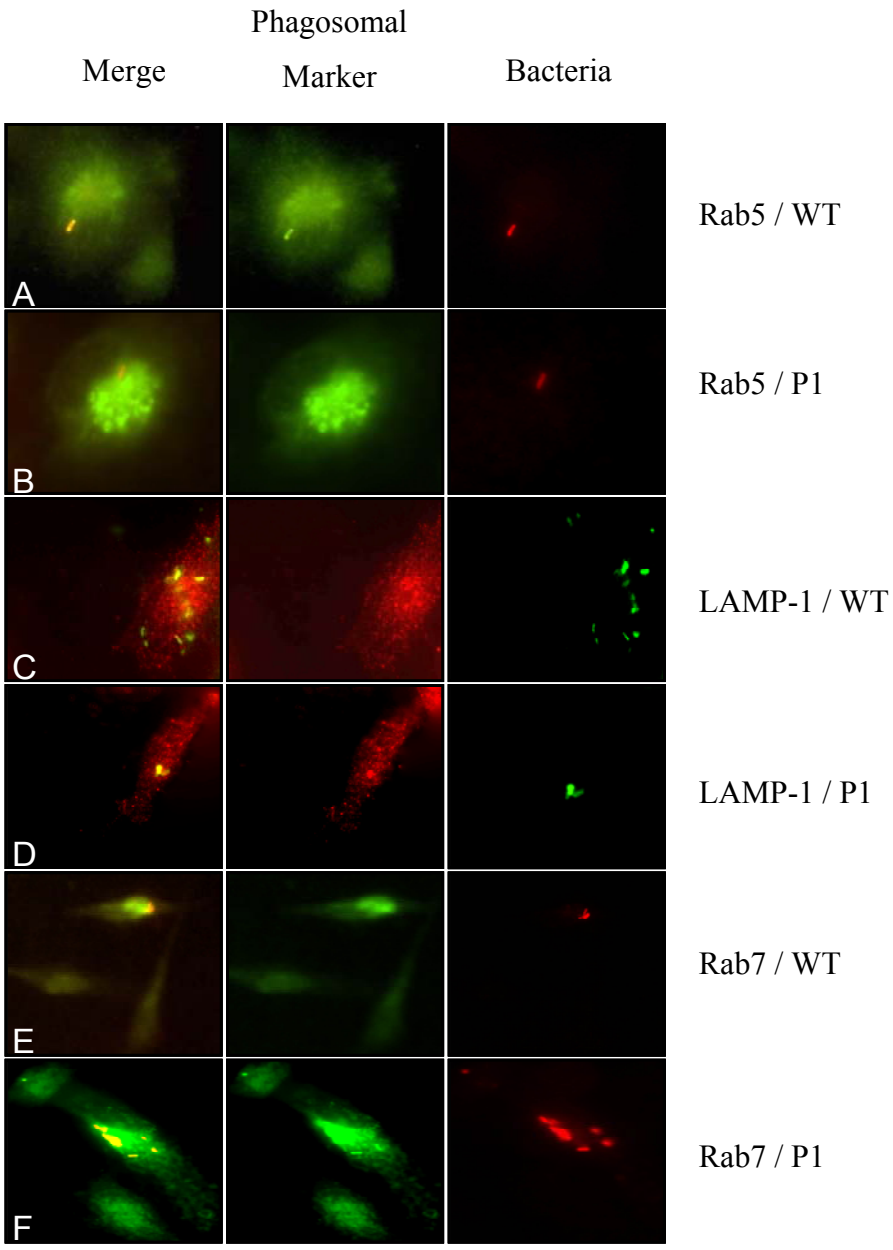

Figure 11: Phagosome-phenotypic characterization by fluorescence microscopy.

HMDM expressing Rab5-GFP (A and B) and Rab7-GFP (E and F) were infected with WT and mutant P1labelled with TRITC and observed for co-localisation of the mycobacterial phagosome with the phagosomal markers. LAMP-1 staining was done on HMDM infected with WT and mutant P1 labelled with FITC (C and D). Phagosomes containing mutant P1 co-localise with late endosomal markers Rab7 and LAMP-1 (D and F) but do not co-localise with the early endosomal marker Rab5 (B) whereas phagosomes containing WT do not co-localise with the late endosomal markers(C and E) but co-localise with Rab5 (A).

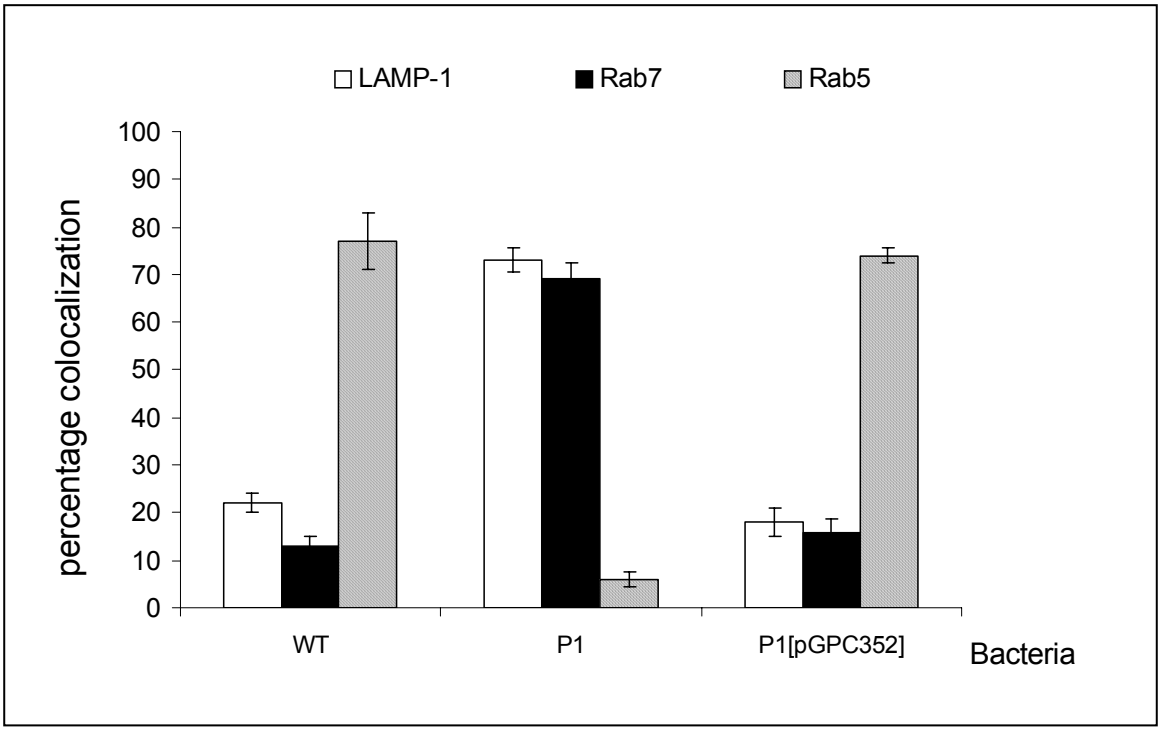

Figure 12: Analysis of co-localisation data of WT and mutant *M. marinum* with endocytic markers. Values indicate the means and standard deviations of percentages of phagosomes containing WT- , P1- and complemented P1[pGPC352] *M. marinum* co-localised with LAMP-1 (white boxes), Rab7 (black boxes) and Rab5 (grey boxes). Data are the mean of three independent experiments, with a minimum of 100 phagosomes counted per experiment for each sample.

Table-3: p values of percentage bacteria co-localising with phagosomal markers

	WT			**P1**			**P1[pGPC352]**		
	Lamp-1	Rab7	Rab5	Lamp-1	Rab7	Rab5	Lamp-1	Rab7	Rab5
P1	<0.001	<0.001	<0.001	-	-	-	<0.001	<0.001	<0.001
P1[pGPC352]	0.138	0.165	0.576	redundant			-	-	-

Statistical analyses of data from each time point were performed by one-way ANOVA post hoc range test and pairwise multiple comparisons with Tamhane's T2 corrections assuming nonequal variances. P-values are indicated.

4.6 Mutant P1 is Attenuated in HMDM

Our results prove that the WT was able to multiply in HMDM. WT *M. marinum* did not grow well nor was there a drastic cell death until day three. This observation is in correlation with the recent study by Kent et al (Kent *et al.*, 2006). After 72hrs a rapid increase in multiplication of WT was observed, whereas the virulence of the mutant P1 was severely attenuated in HMDM. After a slight increase during the first 24hours a rapid decrease in viability of the mutant P1 was observed. By contrast the complemented mutant P1(pGPC352) had a similar growth pattern to that of the WT (Fig-13). Decline in the survival of the mutant was as expected and could be attributed to the defect in the inhibition of PM. Although a significant reduction in growth of P1 was observed in HMDM, P1 growth in 7H9 broth was not inhibited, as shown in Fig-14 neither was a growth difference between P1 and WT observed on 7H10 agar medium.

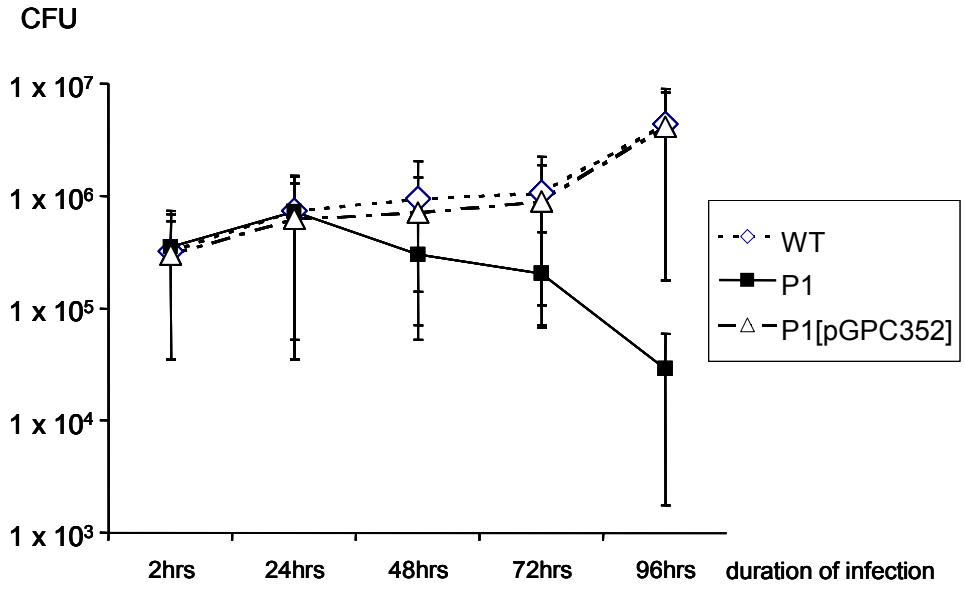

Figure 13: Survival of *M. marinum* WT, P1 and P1(pGPC352) in human monocyte derived macrophages. Macrophages were infected with a MOI of 10:1, incubated at 37°C and lysed for CFU counting at indicated time points. The graph shows the mean values of two independent experiments with duplicate determinations of each time point in each experiment. Error bars indicate the standard deviation.

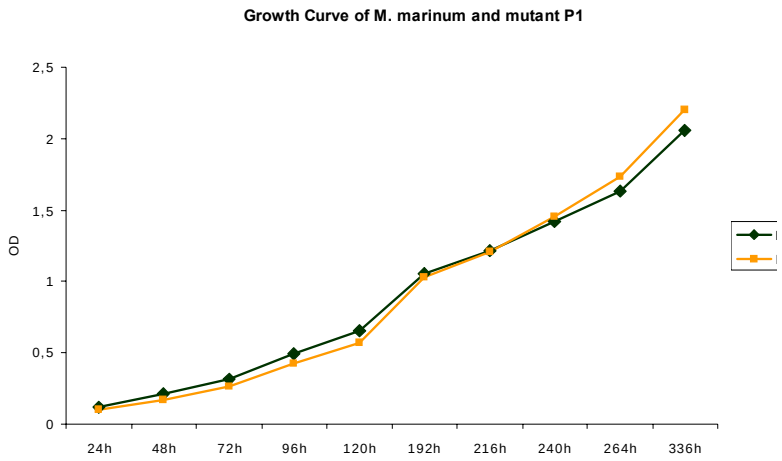

Figure 14: Growth of mutant P1 and WT M. marinum in 7H9 broth.

7H9 broth was inoculated with WT or mutant P1 and at different time interval OD was measured and the values plotted.

4.7 Bioinformatics Analysis of Transposon Inserted Gene of Mutant P1

Sequencing of the transposon insertion site in the P1 mutant showed that the transposon had inserted in a gene for which the function has not been described. The insertion site was mapped to a sequence highly similar (97% sequence identity) to ORF MM3386 in the *M. marinum* ATCC BAA-535 genome. Neither the putative peptide sequence of 203 amino acids of *M. marinum* ATCC 729 nor the putative 197 amino acids of MM3386 were found to have significant similarities to genes in any other genome in the current databases (NCBI, EBI and TIGR-CMR). No signature patterns, domains, repeats, motifs or other features could be predicted with confidence except for a RGD motif in the putative peptide sequence using the latest SMART (Simple Modular Architecture Research Tool) at EMBL, the latest BLAST engines at NCBI or the latest releases of the PROSITE search engine at http://www.expasy.ch/tools/scanprosite. RGD is the single letter code for arginine-glycine-aspartate. This tripeptide motif is normally found in proteins of the extracellular matrix. Since the mutant disrupted in this gene is unable to inhibit phagosome maturation the gene was named *pmiA* (phagosome maturation inhibition). Downstream to the gene *pmiA* are putative hydroxylase and carboxylase genes with opposite polarity, which could possibly be involved in fatty acid metabolism (Cole *et al.*, 1998). A sketch of the organisation of the genes is shown in figure (Fig-15).

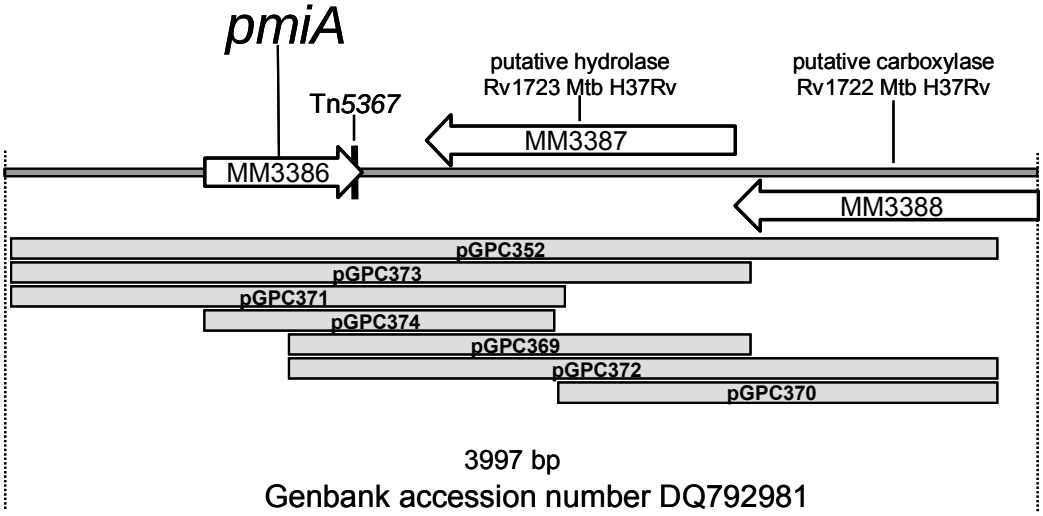

Figure 15: Physical map of *pmiA* (MM3386) in *M. marinum* ATCC 729 and the adjacent putative hydrolase (MM3387) and carboxylase (MM3388) genes. The numbering of the ORFs relates to the *M. marinum* ATCC BAA-535 strain sequenced at the Sanger Institute. The *M. marinum* ATCC 729 chromosomal fragments cloned in this study for complementation analysis of the mutation in mutant P1 are indicated in shaded bars below the gene graphs. The Genbank accession number is indicated.

4.8 Trans-complementation of P1 and Co-elution of ^{14}C Labelled Bacterial Cells with Lysosomal Marker

The region spanning the transposon insertion site was cloned into a pOLYG shuttle vector, electroporated into P1 and selected on plates containing hygromycin and kanamycin. To examine whether the reconstitution of the disrupted gene restored WT phenotype, HMDM were infected with ^{14}C palmitate labelled bacteria (WT, WT heat killed, P1 and P1[pGPC352]) then pulsed and chased with marker for phagolysosome (PL) and selected on MACS column under magnetic field as performed for transposon mutant screen. Bacteria that co-elute with the PL marker were quantified on a β-scintillation counter. As shown in figure (Fig-16) the majority of the heat killed *M. marinum* co-elute with the PL marker, whereas only a minor fraction of the WT co-elute with PL. In control a significant fraction of P1 was observed to co-elute with the PL marker whereas the trans-complemented P1[pGPC352] and P1[pGPC374] were restored to WT levels. We could infer from these observations that the mutant P1 was not competent enough to resist PM and when reconstituted with the respective gene was able to regain its lost phenotype. To narrow the range of genes affected by the transposon insertion and responsible for the phenotypic change in P1, a set of nested deletions in the

complementation plasmid was generated. The range of WT chromosomal sequences covered by the nested plasmids is also shown in figure (Fig-15). Plasmid pGPC374 harbouring just 1332 bp of WT sequence spanning *pmiA* and no other putative mycobacterial ORF is sufficient to restore the WT phenotype as less than 5% of P1 bacterial cells harbouring this plasmid co-eluted with the PL marker. These results confirm that the transposon interrupted gene *pmiA* itself is responsible for the inhibition of PM and eliminates the involvement of any polar effects on the neighbouring genes.

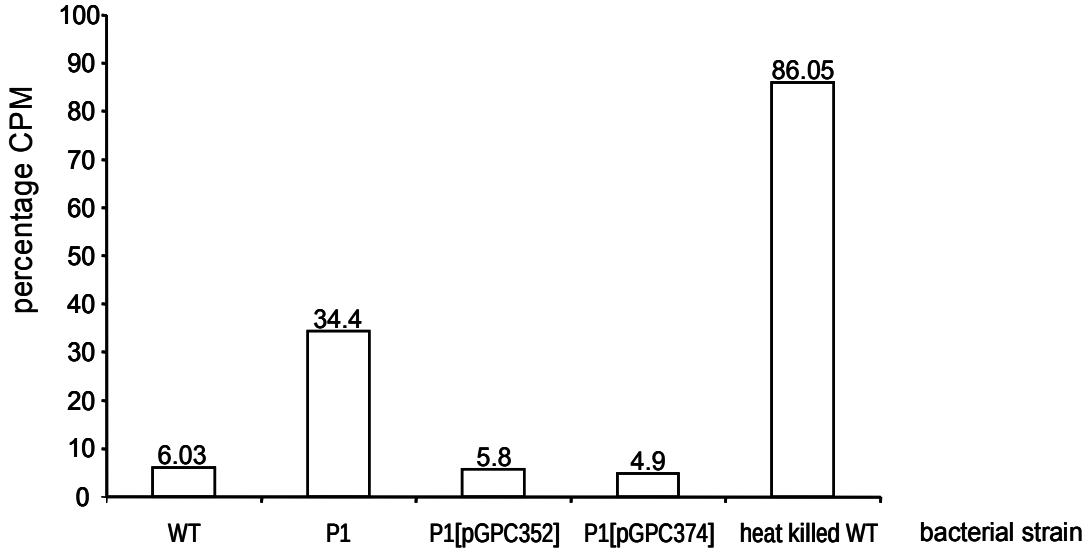

Figure 16: Co-elution of bacterial cells with lysosomal marker.

M. marinum WT, mutant P1 and complemented mutant P1[pGPC352] were metabolically labelled with ^{14}C palmitate. HMDM were infected with live strains and with heat killed wild-type (WTHK) as indicated. Macrophages were pulsed and chased with lysosomal marker (micro beads). Subcellular homogenate was applied to a MACS column under a strong magnetic field. After removing non-specifically retained organelles by washing the phagosomes containing lysosomal marker were eluted. The fraction of bacteria that co-eluted along with the PL marker were quantified on a β-scintillation counter. The values above the columns denote the percentage of cells co-eluting with lysosomal marker.

4.9 *In vivo* Survival of P1 in Mice

The ATCC 927 *M. marinum* strain used in this study has been shown to be able to cause systemic infection in mice (Kent *et al.*, 2006). Therefore a mouse model was used to evaluate the efficiency of the mutant P1 to survive *in vivo*. C57BL/6 mice were intravenously challenged with WT, P1 and P1[pGPC352]. Mice were sacrificed one and two weeks post infection and the number of bacteria in spleen and liver were determined (Fig-17A and 17B). Analysis of colony forming units recovered from infected mice by one way ANOVA for liver and spleen and each week separately showed that the capacity to maintain viable bacterial cells in the infected organs was significantly diminished in the mutant P1 compared to both WT and P1[pGPC352]; p-values, $p < 0.004$ for livers first and second week, $p < 0.001$ for spleens first week and $p < 0.011$ for spleens second week. In accordance with previous findings *M. marinum* WT undergoes a steady decline in viability, which was significantly more rapid in P1 infected animals. This is attributed to the loss of virulence in P1.

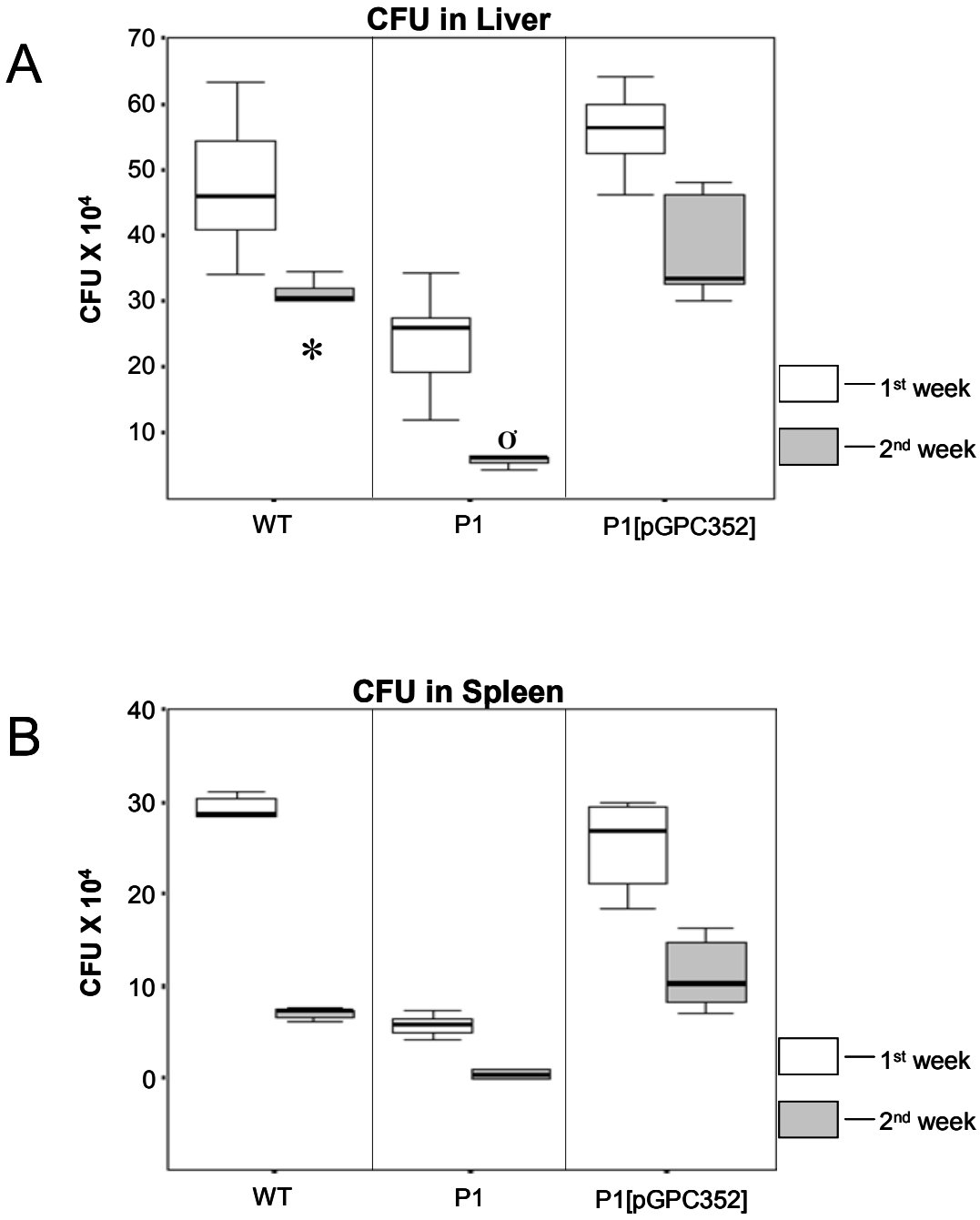

Figure 17: *In vivo* survival of *M. marinum* mutant P1 in mice.

C57BL/6 mice were challenged intravenously with an inoculum of 4 x 10^5 CFU per mouse of *M. marinum* WT, P1 and P1[pGPC352]. After infection, liver and spleen were excised, homogenized and the homogenate diluted and plated on 7H10 agar. Box plots of liver and spleen CFUs recovered from livers and spleen of 5 mice for each time point infected with WT, P1 and P1[pGPC352] are shown. The symbol (*) indicates an 'extreme' value and the symbol (*o*) an 'outlier'.

4.10 Histopathology of Infected Organs

Mycobacterium marinum mutant P1 showed significantly reduced pathological changes in C57BL/6 mouse livers. The capacity of P1 to induce granuloma formation was clearly attenuated, as the number and extent of the epitheloid granulomas was reduced in the mutant in comparison both to the WT and P1[pGPC352], especially in the second week. However, P1[pGPC352] infected animals demonstrated a more variable number of granulomas than WT. The majority of the granulomas were developed in the lobules and fewer in the portal tracts. Granulomas in P1 infected animals also showed more and stronger signs of inflammation around the granulomas; i.e. mainly lymphocytes and few granulocytes. A striking feature of the liver sections of WT and P1[pGPC352] infected animals was the severe endothelialitis which was only marginal in P1 infected animals (Fig.-18). One of the five WT infected livers was entirely necrotic with widespread hepatocyte damage. Necrosis in the granulomas was observed in none of the animals in the other groups. The spleens demonstrated pathological changes to a lesser extent. In the second week the red and white pulp of the spleen of WT infected showed in two out of five animals very few granulomas, in two out of five none and in one animal the entire spleen was necrotic. Among the P1[pGPC352] infected animals five out of five spleens demonstrated few small granulomas in the white pulp. In contrast, the P1 infected animals showed normal histology and no granulomas except in one animal.

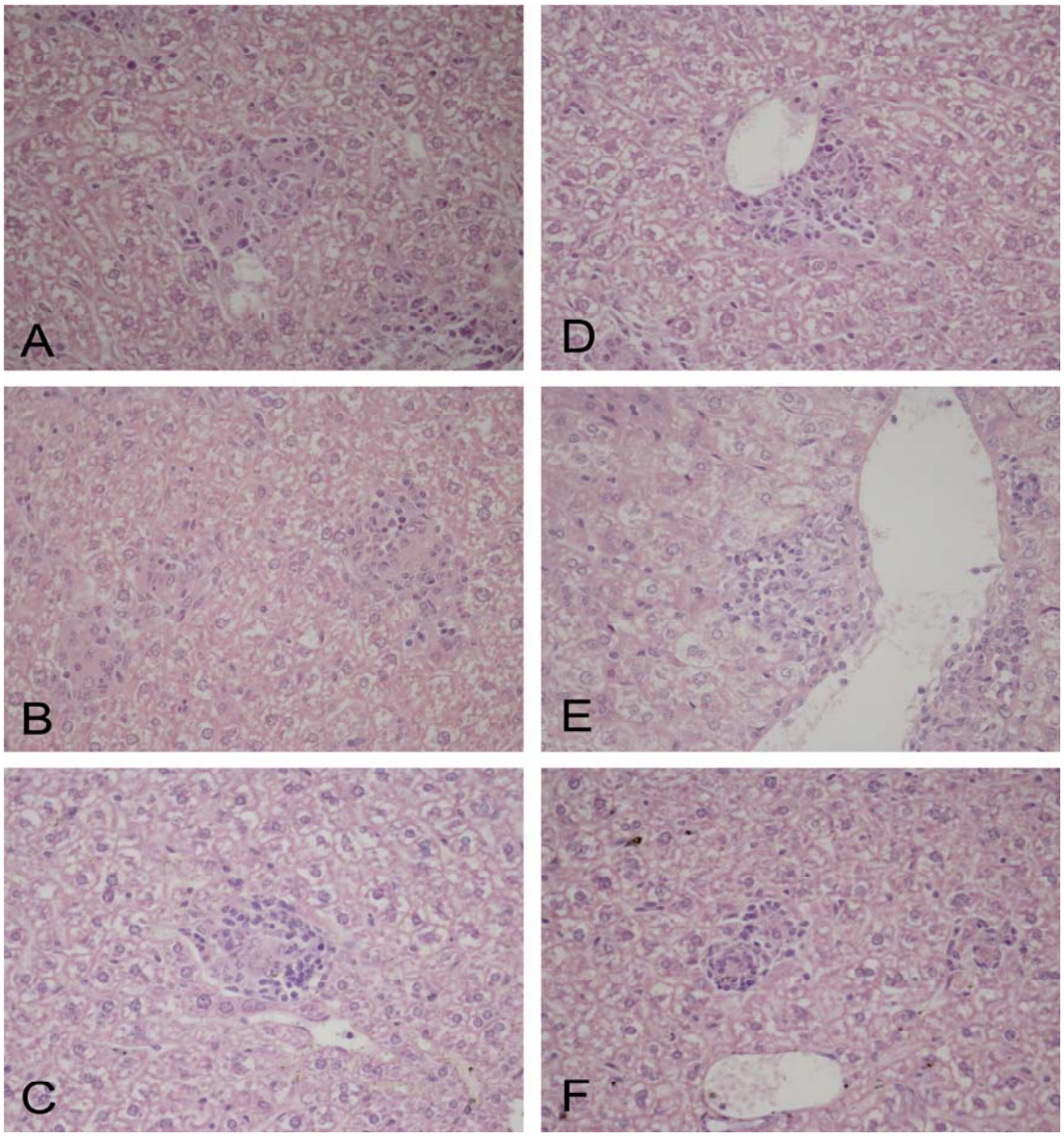

Figure 18: Representative micrographs of the histopathology of infected organs. Magnification of each panel is 400X. Granuloma morphology in *M. marinum*-infected C57BL/6 mice. Mice were intravenously infected with 4 x 10^5 CFU of *M. marinum* WT, P1, P1[pGPC352] and sacrificed 2 weeks post infection. Liver sections were stained with HE. (A) and (B) large epithelioid granulomas in a WT-infected and in a P1[pGPC352]- -infected mouse. (C) Smaller, less well organized granulomas in P1-infected mouse. (D) and (E) endothelialitis in a WT and P1[pGPC352] -infected mouse. (F) Signs of endothelialitis absent in a P1-infected mouse

4.11 Non-Cording Phenotype and Lipid Profile

Unexpectedly, P1 when recovered from the organs of mice produced flat, smooth and transparent colonies. WT bacteria retained their rough colony morphology and the complemented P1[pGPC352] reverted back to WT morphology to a greater extent (Fig-19), confirming that the altered colony morphology of P1 was due to the disruption of the gene *pmiA*. We investigated whether the mycolic acid profile of P1 was altered. The mycolic acid patterns were as described before and no significant differences were observed in P1 when compared to that of the wild-type (Fig-20). Hence we undertook a more systematic approach to study lipid profiles as explained by Dobson et al (Dobson *et al.*, 1985). When highly hydrophobic outer layers of lipids were extracted using petroleum ether and analysed on TLC a lipid moiety in WT could be resolved, which was missing in the mutant P1 and was restored in the complemented P1[pGPC352] (Fig-21). These results indicate a function for the gene *pmiA* in lipid metabolism or transport.

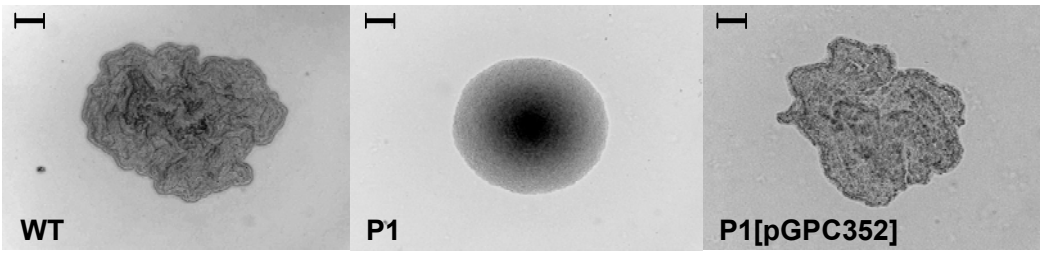

Figure 19: Colony morphology of mutant bacterial cells.

Bright field microscopy of *M. marinum* WT-, P1- and P1[pGPC352]-colonies; 7 days after recovery from mouse organs and seeding onto 7H10 agar. After passage through mouse organs P1 produced flat, smooth and translucent colonies. P1[pGPC352] regained WT characteristics to a greater extent. The scale bar in all images is equal to 0.1 mm.

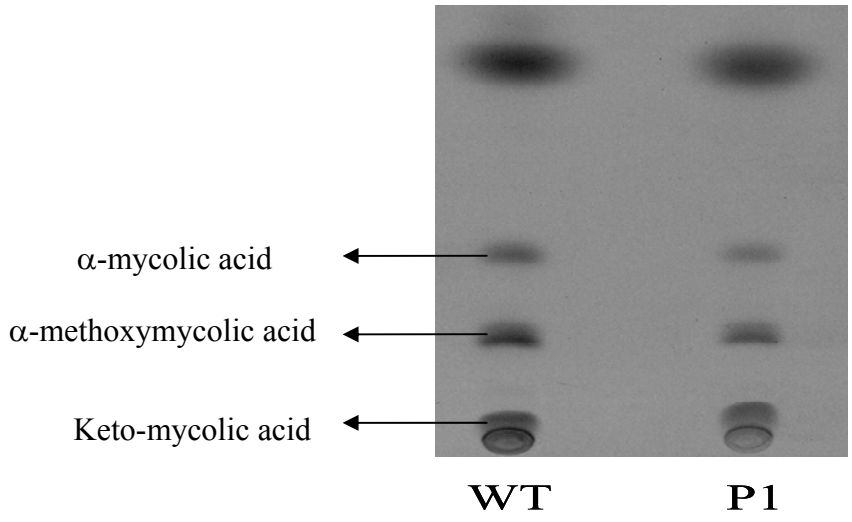

Figure 20: Mycolic acid profile of WT and mutant P1.

Mycolic acids were extracted from WT and mutant P1 labelled with ^{14}C palmitate and chromatographed on a TLC plate. Fig shows the different mycolic acids present in WT and mutant P1

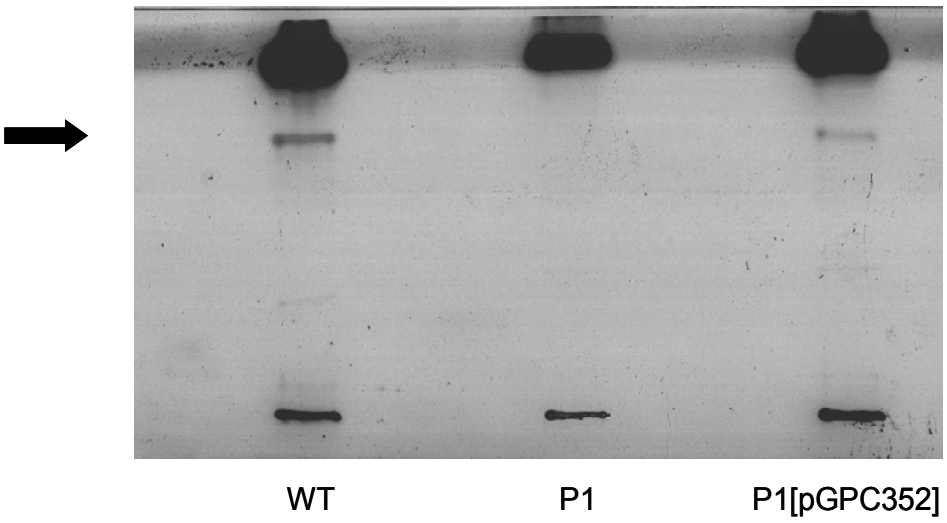

Figure 21: Lipid profile of mycobacterial cell wall.

Non-polar lipids non-covalently bound to the cell wall of *M. marinum* WT, P1 and P1[pGPC352] were isolated using petroleum ether and separated by thin layer chromatography (TLC). The chromatogram was developed by treating with phosphoric acid followed by charring. The black arrow indicates a lipid fraction present in WT and P1[pGPC352] but missing in the mutant P1.

4.12 Characterization of the Lipid Missing in Mutant P1

The lipid absent in mutant P1 was purified from a lipid extract of WT using preparative TLC. The purified lipid was subjected to electrospray mass spectrometry (ES/MS). This analysis revealed a series of sodiated molecular ion peaks at 1516, 1530, 1544, 1558, 1572, 1586, 1600, 1614 m/z (Fig-22). The same peaks were also observed in a study where two of the three terminal sugars were truncated by knocking out a gene involved in glycosylation of phenolic glycolipid (PGL) (Perez *et al.*, 2004). It is also know from earlier studies that PGL of *M. marinum* is 3-O-methyl rhamnose glycosylated phenolphthiocerol. In order to further confirm the nature of the sugar, PGL was subjected to mild acid hydrolysis and the sugar and the lipid moieties were separated on a preparative TLC plate. The sugar moiety was extracted, purified and analysed by MS. A peak was observed at 191m/z (Fig-24) which corresponds to the mass of the predicted sugar moiety. Using the structure derived from ES/MS analysis, a NMR spectrum was predicted for PGL using ChemDraw® 8.0 (Cambridgesoft Corp.). The NMR spectrum obtained (Fig 25B) from the purified PGL matched with that of the predicted spectrum (Fig-25A). Two unshielded doublets were observed at 6.97 and 7.10ppm corresponding to the proton resonance of the phenolic group of PGL-1. At least two singlets were observed in the region of the resonances of OCH3 protons linked to the sugar moiety between 3.5 and 3.6ppm. A signal at 1.25 ppm corresponding to the polymethylenic CH_2 units was also noted. The resonances of the terminal CH_3 protons were observed at 0.8-1.00 ppm. Also the resonance of the methine (CH) proton of the esterified β-glycol and the methoxy group of phenolphthiocerol were observed at 4.83 and 3.32 ppm respectively (Perez *et al.*, 2004). From these observations the chemical structure of PGL-1 was derived (Fig-23).

The lipid missing in the mutant P1 was also characterized biochemically. WT and P1 mutant were grown in liquid broth pulsed with [^{14}C] palmitate or [^{14}C] *p*-hydroxy benzoic acid, a known precursor of PGL. The lipids of the membrane and the cytoplasm were extracted separately from the bacterial pellet following a previously described protocol (Sulzenbacher et al., 2006). [^{14}C] palmitate labelled cells showed up many lipids of the outer envelope and the inner cytoplasm except for one lipid spot missing in the mutant P1 (Fig-26). When labelled with [^{14}C] *p*-hydroxy benzoic acid, very clearly only one lipid spot corresponding to the missing lipid spot of the mutant P1 was found to co-migrate with the *M. marinum* specific lipids labelled with [^{14}C] palmitate. This radioactive species

was again absent in the mutant P1 (Fig-27), confirming that the lipid contains a *p*-hydroxy benzyl moiety. These data collectively prove that the missing lipid spot in mutant P1 is a PGL.

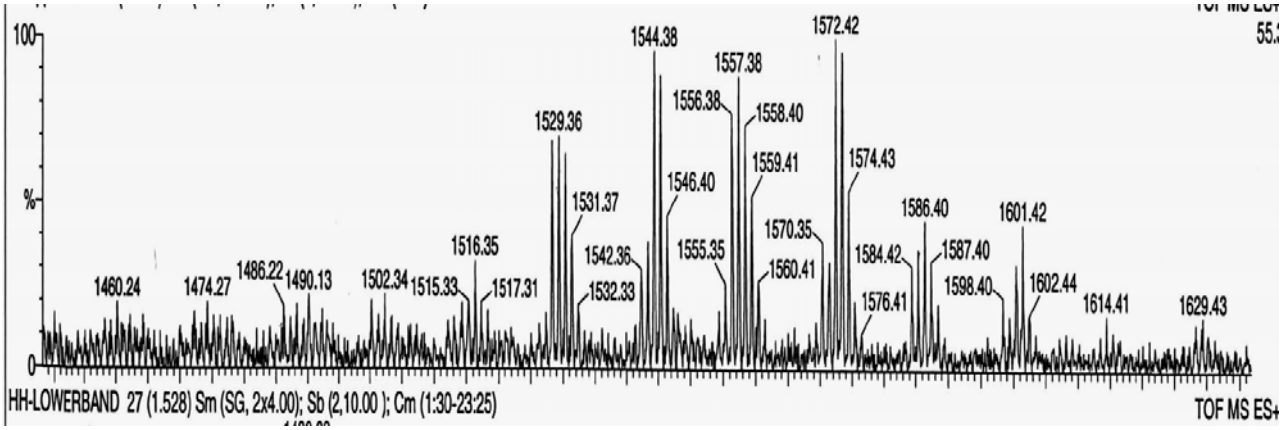

Figure 22: Mass spectrum of the lipid missing in mutant P1.

The lipid spot missing in mutant P1 was purified and subjected to ES/MS analysis. The figure represents the mass speaks corresponding to that of phenolic glycolipid.

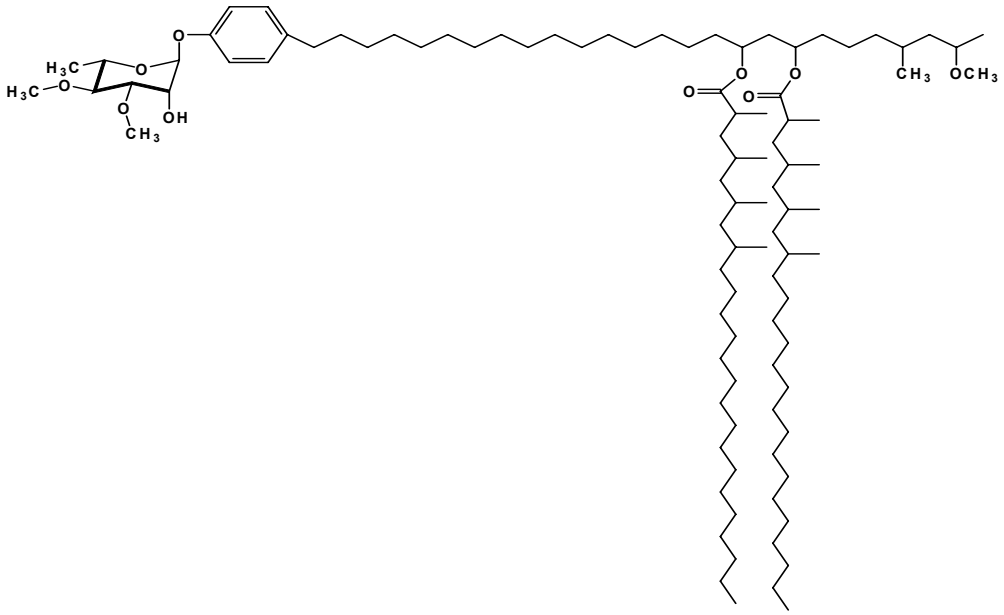

Figure 23: Chemical structure of the characterized Phenolic Glycolipid of *M. marinum*.

The chemical structure was derived from the mass values obtained from ES/MS analysis.

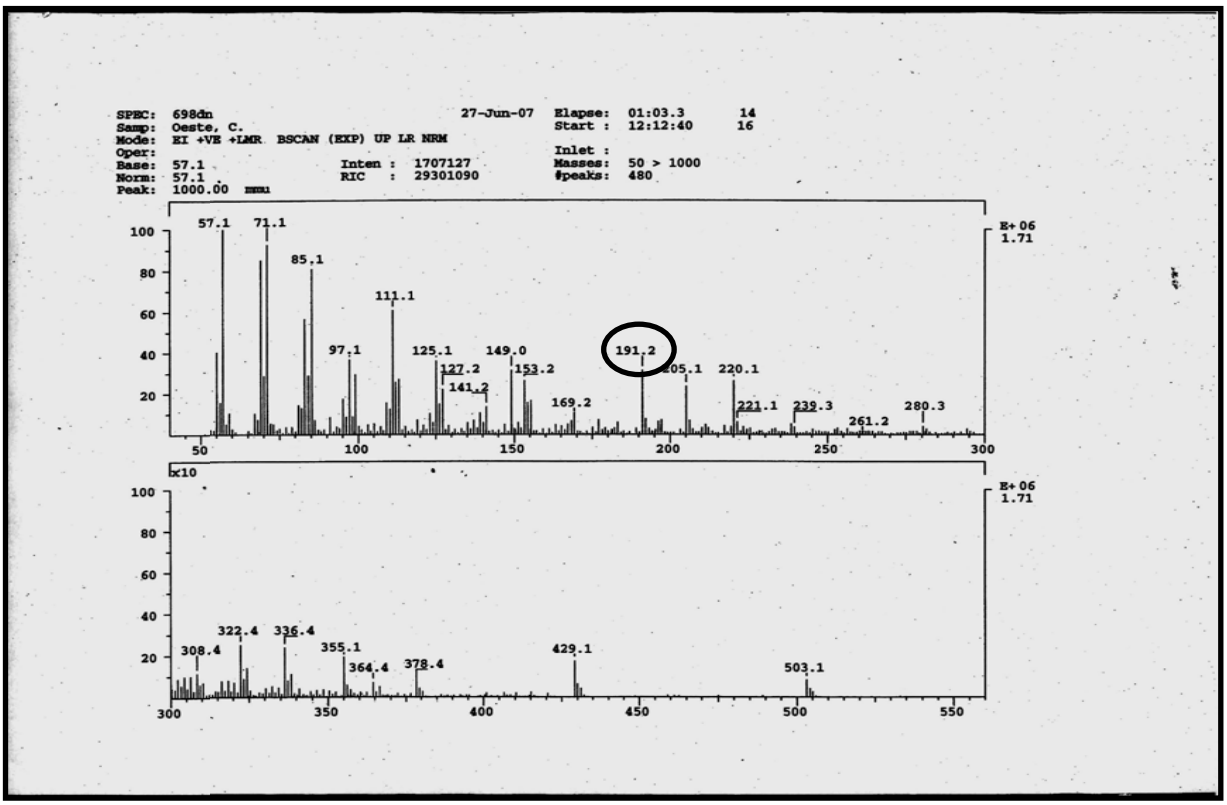

Figure 24: Mass spectrum of the sugar component of PGL-1

PGL-1 was subjected to acid hydrolysis to yield the sugar. The sugar moiety was purified from a preparative TLC plate and subjected to MS analysis. The figure shows a mass peak at 191.2 m/z corresponding to that of the sugar moiety present in PGL-1.

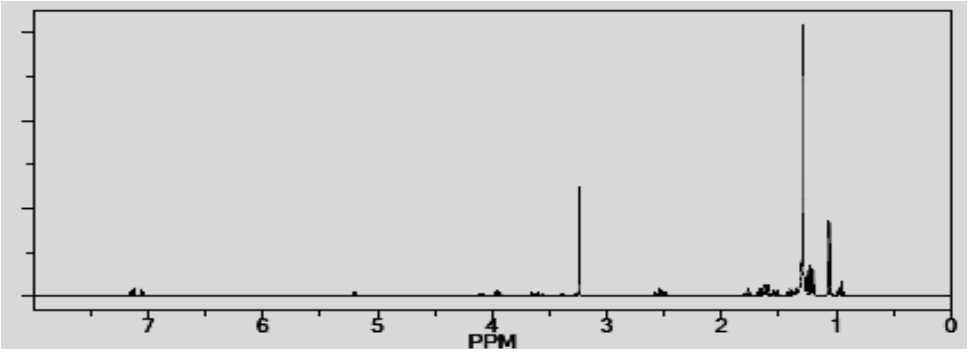

Fig – 25 A: Predicted NMR spectrum of PGL-1

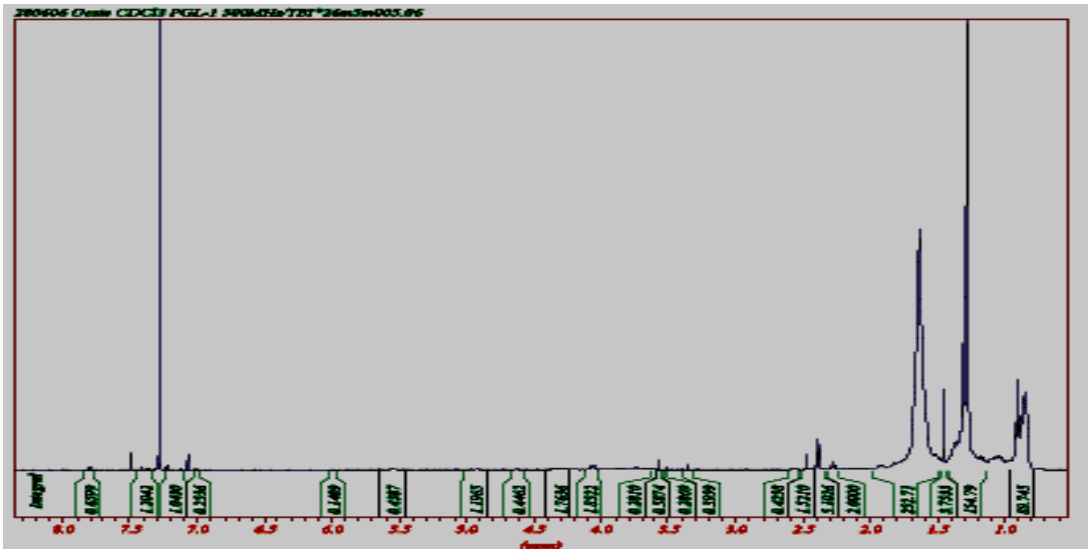

Fig – 25 B: Obtained NMR spectra of PGL-1

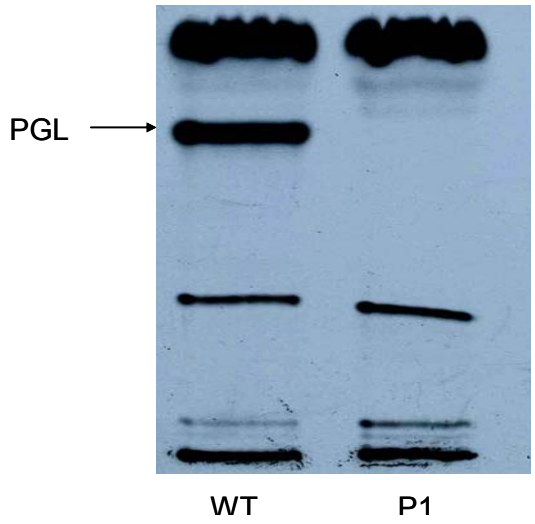

Figure 26: TLC profile of lipids of WT and mutant P1

Lipids were extracted from WT and P1 grown in 7H12 medium containing ^{14}C palmitic acid and chromatographed on TLC. Arrow indicates PGL-1 found missing in mutant P1

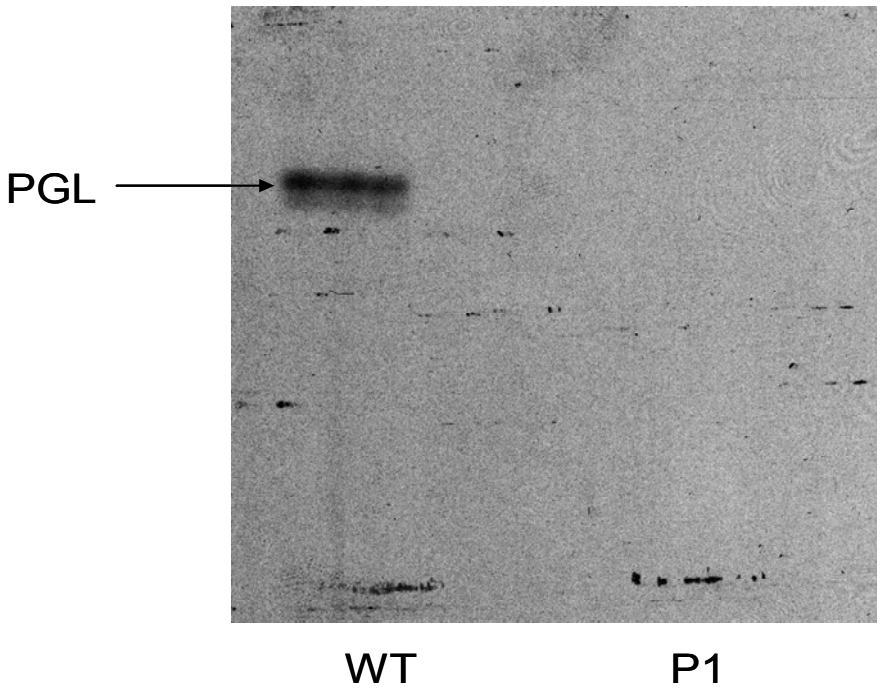

Figure 27: Lipids of WT and mutant P1 labelled with ^{14}C *p*-hydroxybenzoic acid

WT and mutant P1 were grown in 7H9 medium pulsed with ^{14}C *p*-hydroxybenzoic acid and lipids extracted and chromatographed on TLC plate. Arrow indicates PGL-1 of WT labelled with ^{14}C *p*-hydroxybenzoic acid whereas it is absent in mutant P1.

4.13 *M. marinum* PGL Inhibits Phagosome Maturation

The mutant P1 was unable to inhibit PM and failed to synthesise PGL. PGL analysis by radioactive labelling and TLC were also performed with other mutants (N33, N43, N68 and N70) obtained through the screen. These mutants had transposon insertions in genes involved or possibly involved in PGL biosynthesis. Interestingly these mutants also failed to produce PGL. Therefore, we asked if PGL on its own could inhibit PM. To prove this hypothesis, PGL purified from *M. marinum* was coated on to fluorescent polystyrene beads which were then chased in HMDM overnight. As controls uncoated beads and beads coated with a lipid migrating below the PGL on TLC which is also present in the mutant were chased in HMDM separately. Following the chase the cells were stained for LAMP-2 and Rab5 and viewed under a fluorescence microscope. As expected higher percentages of phagosomes containing plain beads and control lipid coated beads were LAMP-2 positive compared to that of PGL coated beads. In contrast, phagosomes containing PGL-1 coated beads contained Rab5 as demonstrated by immunofluorescence microscopy (Fig-28).

To further prove that *M. marinum* PGL inhibits PM, hydrophobic magnetic beads coated with PGL or control lipid, uncoated beads and hydrophilic magnetic beads were chased into HMDM and, using the same protocol used to isolate mutants ending up in the phagolysosomes, the cells were lysed and the phagosomes containing magnetic beads were isolated under a magnetic field. The isolated fractions were subjected to western blot analysis. Following electrophoretic transfer to polyvinylidene difluoride (PVDF) membrane, this was probed with antibody against Rab5 and β-galactosidase and further detected by electro-chemiluminescence (ECL) analysis. This revealed that the phagosomal fraction containing PGL coated beads were highly reactive with Rab5 antibody compared to that of the controls (Fig-29), denoting that the PGL coated beads are held up in an early endosome like phagosome. By contrast PGL phagosomal fraction did not react with anti-β-galactosidase antibody. These results indicate that *M. marinum* PGL is capable of inhibiting PM on its own. Moreover, a defect in the biosynthesis of PGL by the mutant P1 could be contributing for its inefficiency in restricting PM.

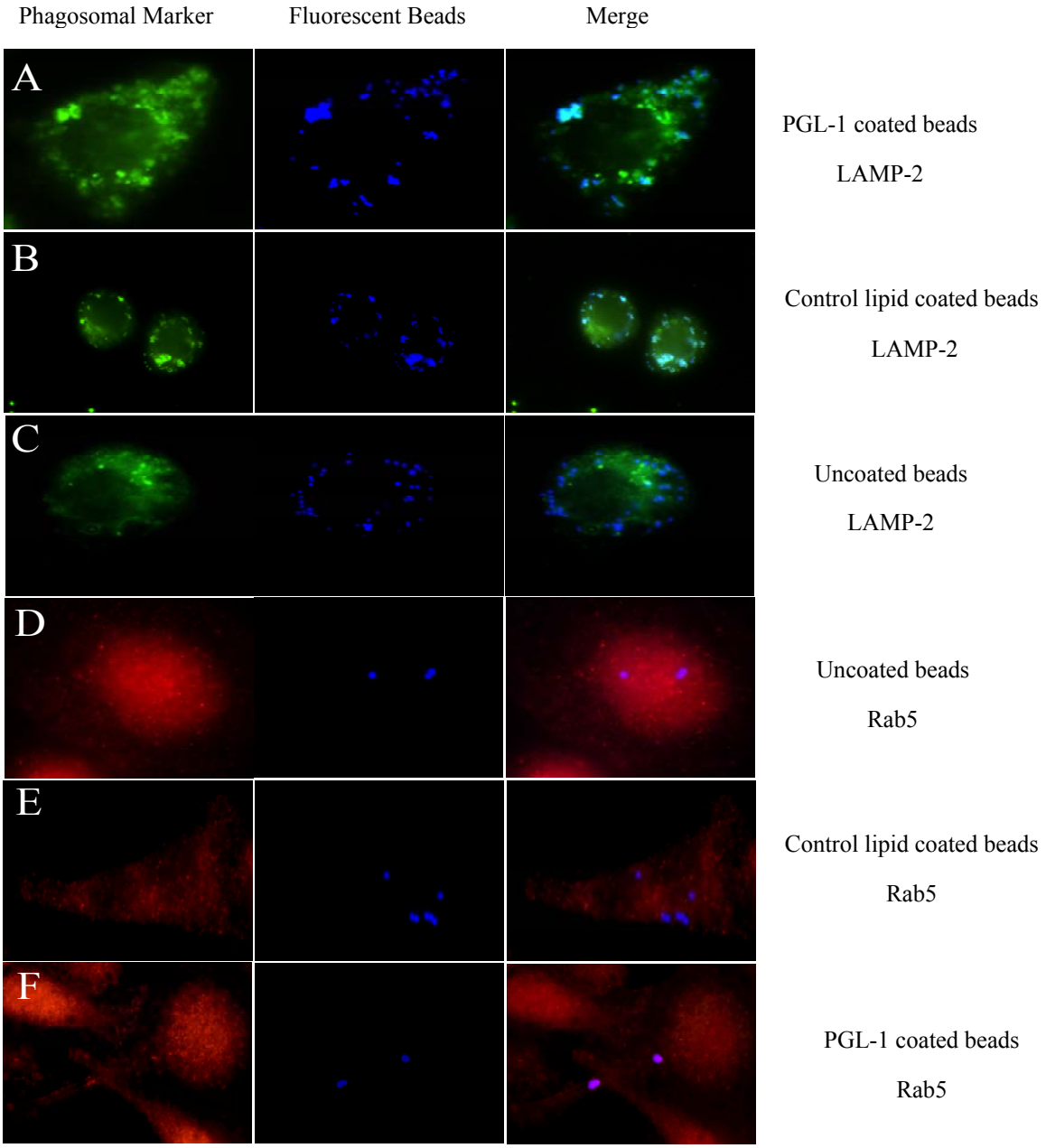

Figure 28: Characterization of phagosomes containing PGL-1 coated beads by fluorescence microscopy.

PGL-1-, control lipid-coated and uncoated fluorescent beads were chased into HMDM overnight and stained for PL marker LAMP-2 (A-C) and early phagosomal marker Rab5 (D-F). phagolysosomes containing PGL-1 coated bead stained for LAMP-2 but did not stain for early phagosomal marker Rab5 (F), whereas phagosomes containing uncoated beads and control lipid coated bead stained for LAMP-2 (A, B) but did not stain for Rab5 (D, E).

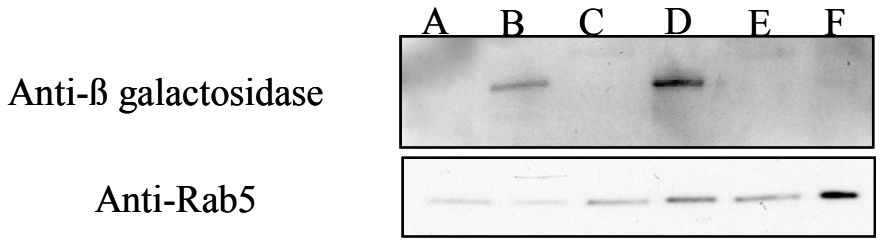

Fig-27

Figure 29: Western blot analysis of phagosomes containing PGL-1

Hydrophobic magnetic beads were coated with PGL-1 and chased into macrophages followed by isolation of phagosomes containing beads, which were lysed and separated on a SDS-PAGE, transferred on to PVDF membrane and probed with anti-β galactosidase and anti-Rab5 antibodies.

A- Macrophage lysate

B- Phagosomes containing plain beads

C- Lysate of macrophages chased with beads coated with a lipid of mycobacteria also present in mutant P1.

D- Phagosomes containing beads coated with a lipid of mycobacteria also present in mutant P1

E- Lysate of macrophages chased with beads coated with PGL-1

D- Phagosomes containing beads coated with a PGL-1

4.14 Functional Characterization of the Gene *pmiA*

Database search using the available search engines and analysis of the gene sequence and the predicted protein sequence using bioinformatics tools as described above did not return significant hits. Therefore, to study the involvement of gene *pmiA* in the biosynthesis of PGL an attempt was made to identify its binding partner proteins if any. The protein PmiA was expressed as a histidine fusion protein immobilized on magnetic agarose beads. These beads were used to pull out the interacting partner(s) of PmiA from a lysate of mycobacterial proteins. As a control DHFRS-His tag protein was expressed from a plasmid immobilized on to magnetic agarose beads and allowed to bind with mycobacterial proteins. Proteins bound to the beads were resolved on SDS PAGE. Interacting proteins specific for pmiA were excised from SDS PAGE gel and were given for mass finger printing (Fig-30). Interestingly, MALDI-MS results unveil the pmiA interacting proteins to be a methyl transferase and isocitrate lyase. A similar methyl

transferase has been earlier shown to be responsible for the cyclopropanation of mycolic acids, major fatty acids of mycobacterium species. Isocitrate lyase is considered important for the glyoxylate shunt pathway used by *M. tuberculosis* during its period of latency, in order to derive its energy through fatty acid metabolism. These results, though preliminary, substantiate the role of PmiA in lipid biosynthesis.

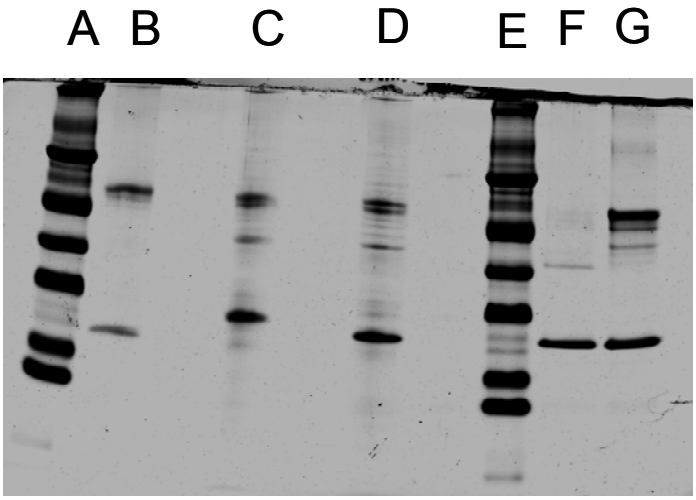

Figure 30: SDS-PAGE showing proteins possibly interacting with PmiA

PmiA was expressed as his-tag fusion protein, bound to magnetic agarose beads and allowed to interact with slurry of mycobacterial proteins. The proteins bound to PmiA were separated on SDS-PAGE.

5 Discussion

5.1 Mycobacterial Genes Possibly Involved in the Inhibition of Phagosome Maturation

Virulent mycobacteria hijack macrophages and interfere with the intracellular signalling to reside within a specialised phagocytic compartment. In order to understand the genes contributing to the inhibition of PM, we performed a screen to select for the transposon inserted *M. marinum* mutants localising in the PL. Although many genetic approaches have been used to elucidate the mechanisms of mycobacterial pathogenesis, studies using mutant strains are considered to be more effective than other strategies (Murry and Rubin, 2005). Investigations using transposon mutant pools have been successfully used by different groups to examine genetic needs for mycobacterial virulence *in vivo* in mice, survival in macrophages, to identify genes involved in arresting PM and acidification of phagosomes. Are there multiple genes involved in the inhibition of PM? Using our screen we identified genes related to lipid and protein metabolism, transport, cell wall associated proteins, genes belonging to PE/PPE (Proline-Glutamine / Proline-Proline-Glutamine domains) gene family and many unknown or hypothetical genes, implying diverse and multiple genetic requirement. Identification of mutants with insertions in genes involved in isoprenoid biosynthesis pathway by us and Pethe et al. indicates the extent of biochemical machinery employed by mycobacteria to facilitate the inhibition of PM (Pethe *et al.*, 2004).

5.1.1 Genes Belonging to PE/PPE Gene Family

Identification of genes belonging to the PE/PPE gene families has been a common feature in different genetic screens done to study different aspects of mycobacterial pathogenesis and our screen is not an exception. This family of genes is remarkable in that it is composed of 100 homologous genes only in mycobacteria (Brennan and Delogu, 2002). The PE and PPE gene families of *M. tuberculosis* encode large multi-protein families (99 and 69 members respectively) of unknown function. These protein families comprise about 10% of the coding potential of the genome of *M. tuberculosis*. The PE family is characterized by the presence of a proline-glutamic acid (PE) motif at positions 8 and 9 in a much conserved N-terminal domain of approximately 110 amino acids. Similarly, the PPE family also contains a highly conserved, but unique, N-terminal domain of

approximately 180 amino acids, with a proline-proline-glutamic acid (PPE) motif at positions 7–9. Although the N-terminal domains are conserved within each family, there is very little N-terminal homology between the two different families. The C-terminal domains of both of these protein families are of variable size and sequence. They also frequently contain repeat sequences of different copy numbers. Both the PE and PPE protein families can be divided into subfamilies according to the homology and presence of characteristic motifs in their C-terminal domains. The largest subfamily (65 members) of the PE family is the polymorphic GC-rich-repetitive sequence (PGRS) subfamily. It contains proteins with multiple tandem repeats of a glycine-glycine-alanine (Gly-Gly-Ala) or a glycine-glycine-asparagine (Gly-Gly-Asn) motif in the C-terminal domain (Cole *et al.*, 1998); Campus et al., 2002).

Although the function of the 168 members of the PE and PPE protein families has not been established, various hypotheses have been advanced. The fact that these genes encode about 4% of the total protein species in the organism (if all genes are expressed) suggests that they most probably fulfil important functions in the organism. Genes of PE/PPE gene family are speculated to have varied functions from antigenic variation to resistance to being killed in macrophages and granulomas (Li *et al.*, 2005); (Cole *et al.*, 1998); (Brennan and Delogu, 2002); (Ramakrishnan *et al.*, 2000). The most widely supported theory suggests the involvement of these proteins in antigenic variation due to the highly polymorphic nature of their C-terminal domains (Gey van Pittius *et al.*, 2006). In earlier studies using transposon mutant libraries and differential fluorescence induction (DFI), to screen for virulent genes associated with inhibiting PM, mycobacterial survival during infection and granuloma formation, a number of hits has been mapped to genes belonging to GC rich PE and PPE gene families (Pethe *et al.*, 2004); (Sassetti and Rubin, 2003); (Ramakrishnan *et al.*, 2000). A PPE gene of *M. avium* (Rv1787) was also shown to provide resistance to killing by macrophages and the mutant was reported to be delivered to PL. Using our screening technique we were able to identify four distinct interruptions in genes homologous to Rv1918c (PPE 35). Mutants N10 and N21 had independent insertions in the same locus and in N16 and N29 the transposon had inserted in a distant region but all of their sequences shared homology with Rv1918c and within their own group. Two separate insertions in N5 and N75 were mapped to another PPE gene (Rv1135c). In mutants N13 and N81 genes homologous to PE-PGRS genes were inactivated. PE-PGRS (proline-glutamine – polymorphic GC rich repetitive sequence)

gene interrupted in N13 shared homology with MAG24-1 (Rv2328), MAG24-2 and MAG24-3 which are postulated to contribute to the microbe's persistence in granulomas [Ramakrishnan, 2000]. Identification of PE/PPE genes in every aspect of mycobacterial pathogenesis demands extensive research to define the functional role of the genes belonging to this gene family which are abundantly distributed throughout the mycobacterial genomes.

5.1.2 Membrane Transporters

RND proteins (resistance, nodulation, and cell division proteins) are a family of multi-drug resistance pumps that recognize and mediate the transport of a great diversity of cationic, anionic, or neutral compounds, including various drugs, heavy metals, aliphatic and aromatic solvents, bile salts, fatty acids, detergents, and dyes. RND pumps are large proteins ranging from 800 to 1,100 amino acids that are characterized by the presence of 12 transmembrane domains (TMD) and two extracytoplasmic loops of approximately 300 amino acids located between the first and second TMD and the seventh and eighth TMD (Paulsen et al., 1996). RND proteins have been reported in the genomes of organisms from all major kingdoms of life. Mycobacterial membrane protein large (MmpL) family of proteins belongs to the RND (resistance, nodulation and cell division) permease superfamily transporters (Tseng *et al.*, 1999). The *M. tuberculosis* genome contains 13 genes coding for MmpLs (Cole *et al.*, 1998). A mutant with a transposon insertion in *mmpL7* has been shown to be defective in transporting PDIM (Cox *et al.*, 1999), (Camacho *et al.*, 2001) and MmpL8 transports a precursor molecule to outer cell envelope for the synthesis of sulfolipid (Domenech *et al.*, 2005). Of all the *mmpL* mutants previously evaluated in *M. tuberculosis* mutants, in *mmpL4* and *mmpL7* were the only mutants showing impaired growth during the initial phase of infection in mice. ORF Rv0450c codes for MmpL4 while the adjacent Rv0451c is a small gene termed *mmpS*. Both are associated with four of the 13 *mmpL* genes (*mmpL1, mmpL2, mmpL4* and *mmpL5*) identified so far (Domenech *et al.*, 2005). It is noteworthy that our screen isolated two mutants N68 and N4R in ORFs identical to Rv0450c and Rv0451c respectively. Isolation of mutants having insertions in *mmpL4* and its neighbour *mmpS* could imply that these two genes are together involved in transport of a molecule which could be involved in interfering with the early innate immune responses of the host i.e. arresting PM. This hypothesis is in agreement with the notion that the protein MmpS may be a functional

equivalent of the periplasmic membrane fusion protein (MFP), which are associated with RND efflux pumps of gram negative bacteria (Domenech *et al.*, 2005).

5.1.3 Porins

The porins of *M. tuberculosis* are medically the most relevant porins. Mycobacterial cell envelope is highly hydrophobic and porins are thought to be the key proteins for the uptake of hydrophilic drugs. This view is based on the fact that three out of the four current first line TB drugs, namely isoniazid, ethambutol and pyrazinamide, are small and hydrophilic molecules (Niederweis, 2003). An open reading frame (Rv0899), corresponding to the OmpA family of outer membrane proteins has been located and named as OmpATB (Senaratne *et al.*, 1998). OmpATB is postulated to be the major active porin at growth conditions with low pH. *ompATB* mutant had a reduced virulence in macrophages and in mice. The reduced virulence of *ompATB* mutant has been attributed to the acidic milieu of the phagocytic vacuoles and is reasoned that higher expression of OmpATB at lower pH could help the bacteria survive in such acidic conditions (Raynaud *et al.*, 2002). Selection of a mutant N74 having an insertion in a gene homologous to Rv0899 from our screen corroborates the above finding.

5.1.4 β-Propeller Protein

Our screen also identified mutant with a transposon insertion in a gene homologous to Rv1057. Rv1057 shares similarities with surface layer proteins of *Mehanosarcina*. Bioinformatic evaluation has found Rv1057 encoding for the only seven-bladed β-propeller in the *M. tuberculosis* genome. β-propeller proteins perform extremely diversified functions including enzyme catalysis, signal transduction, ligand binding, transport, mediation of protein-protein interactions, control of cell division, and modulation of gene expression. Using selective capture of transcribed sequence (SCOTS) Rv1057 was found to be expressed during early phase of *M. tuberculosis* growth in macrophages (Haydel and Clark-Curtiss, 2006).

5.1.5 FtsH

We also obtained mutants with transposon insertions in genes encoding for FtsH protease (N27 – Rv3610c). FtsH is an ATP dependent endopeptidase believed to have a role in the quality control of membrane proteins. *E.coli* FtsH protease is shown to maintain a balance

between SecY and SecE production in order to form a stable translocon (Ito and Akiyama, 2005). FtsH is also studied to be an important regulator for the production of membrane lipid components in *E.coli* (Ogura *et al.*, 1999). In *M. tuberculosis* FtsH is characterised as an immunodominant antigen (Amara *et al.*, 1998) and expression of the *M. smegmatis ftsH* gene in *E.coli*, led to a proteolytic active product that was toxic to *E. coli* and resulted in growth arrest and filamentation of the bacteria (Anilkumar *et al.*, 2004). In mycobacteria FtsH is thought to have diverse functions helpful for adaptation to life inside the host. Investigating the membrane proteins regulated by FtsH in mycobacteria and their possible role in regulating the membrane lipids of mycobacteria might result in the usage of FtsH as a potential antibiotic target.

5.1.6 Genes Encoding Unknown/Hypothetical Proteins

Another common appearance in genetic screens has been the category of genes of hypothetical/unknown functions. In a recent study of genes upregulated in pulmonary tissue samples from TB patients, the majority of upregulated genes in *Mtb* were those of unknown functions (Rachman *et al.*, 2006). This is paralleled by our study finding that more than 30% of the genes identified are also of unknown function. A closer look at these genes revealed that a significant number (10) of these genes have orthologues in the *M. tuberculosis* genome and are annotated there as putative membrane proteins. It is of particular interest that N66, N6, N9 and N15 had independent insertions in the same ORF of an unknown gene. The H37Rv homologue of this gene is designated as Rv0365c. The only study addressing this gene showed an increased resistance to killing by human macrophages when expressed in the avirulent *M. smegmatis* (Miller and Shinnick, 2001). Our study also yielded mutants of note, bearing transposon insertions in genes sharing homology with Rv3881c (N36) and Rv1038c (N23). Gao and colleagues had already pinned on a region between Rv3866 and Rv3881c in a screen to isolate *M. marinum* transposon mutants failing to cause haemolysis, Rv3881c is a conserved hypothetical alanine and glycine rich protein and the authors reported that disruption of the *M. marinum* homologue of Rv3881c led to a severely attenuated phenotype in macrophages and *in vivo* (Gao *et al.*, 2004). Rv1038c (Esxj) belongs to the ESAT-6 protein family mentioned above. The significance of the Rv1038c has been shown before in a study using 2D-Gel electrophoresis and mass spectrophotometry of secreted proteins of the virulent *Mtb* H37Rv and the less virulent H37Ra strain. One of the three spots identified

in this study shared homology with Rv1038c, Rv2347c, Rv2346c and Rv3620c. These genes carry multiple inactivating mutations in the less virulent H37Ra strain (He *et al.*, 2003).

5.1.7 Genes Involved in Biosynthesis of Cell Envelope Associated Lipids

The mycobacterial cell wall has high and complex lipid content. Consistently, a high proportion of mycobacterial genes are believed to be involved in lipid metabolism and transport (Cole *et al.*, 1998). A number of mycobacterial lipids has been implicated in mycobacterial pathogenesis (Asselineau and Laneelle, 1998). Numerous roles have been attributed to PGLs, including the resistance of *M. leprae* to killing by macrophages (Neill and Klebanoff, 1988) and the predilection of *M. leprae* to peripheral nerves (Ng *et al.*, 2000). Importantly hypervirulent *M. tuberculosis* W-Beijing strain produces PGL and inhibits innate immune responses and shows hyperlethality in mice [Reed, 2004]. In particular the genes *fadD26* and *fadD28* have been implicated in the biosynthesis of phthiocerol dimycocerosate (PDIM) (Camacho *et al.*, 2001) and have been isolated in a previous similar screen by Pethe et al (Pethe *et al.*, 2004). *fadD28* mutant was also found by Rengarajan et al., by screening *Mtb* for mutants unable to survive in macrophages (Rengarajan *et al.*, 2005). In an alternative screen using *M. bovis* BCG *fadD28* mutant was again found to have a reduced fitness in macrophages (Stewart *et al.*, 2005). In contrast, our screen did not yield mutants in *fadD28* itself, but in genes that in the *M. tuberculosis* genome are located in a proximal region, i.e. two of the mutants (N24, N33) in genes corresponding to *fadD22* (Rv2948c) were interrupted and a third mutant had transposon insertion in the gene immediately upstream of *fadD22* (Rv2949c). The gene *fadD22* is present upstream of a polyketide synthase (*pks15/1,* Rv2947c) which has been found to contribute to the elongation of *p*-hydroxybenzoate to *p*-hydroxyphenylalkonic acid in mycobacterial strains producing phenolic glycolipid. H37Rv and related clinical strains of *M. tuberculosis* do not produce phenolic glycolipid due to a frame shift mutation between *pks15* and *pks1*. The immediate upstream gene (Rv2949c) to *fadD22* was recently characterised as chorismate pyruvate-lyase responsible for the conversion of chorismate to *p*-hydroxybenzoate which is likely to be the sole source of *p*-hydroxybenzoic acid in mycobacteria (Stadthagen *et al.*, 2005). A role for *p*-hydroxybenzoic acid derivatives (*p*-HBAD) secreted by all virulent strains of mycobacteria including H37Rv in mediating virulence has been suggested (Constant *et al.*, 2002). Isolation of mutants with insertions

in *fadD22* and *Rv2949* (chorismate pyruvate-lyase) suggests that, *fadD22* and *Rv2949* along with *pks15/1* could be together involved in the production of phenolic glycolipids. It is also possible that *p*-HBAD synthesised by Rv2949 from chorismate could arbitrate the arrest of PM and thereby mediate virulence as proposed by Constant et al. (Constant *et al.*, 2002). Selection of multiple mutants with disruption in genes involved in PGL synthesis and its secreted precursor, *p*-HBAD, may indicate that PGL, *p*-HBAD or both could facilitate mycobacteria to reside in immature phagosomes. The screen also provided a mutant with a transposon insertion in a gene homologous to Rv2959c. This gene has been discovered to catalyze the *o*-methylation of the hydroxyl group located on carbon 2 of the rhamnosyl residue linked to the phenolic group of PGL and *p*-HBAD produced by *M. tuberculosis* (Perez *et al.*, 2004). Isolation of this particular mutant having an insertion in a gene identical to Rv2959c from the PL fraction prompts us to hypothesise that an unaltered structure of PGL or *p*-HBAD is required by mycobacteria to accomplish a niche in the phagosome. Though further understanding of these mutants and elucidation of the mechanism by which PGL, *p*-HBAD or both mediate the arrest of PM is needed, from these observations it seems highly likely that these glycolipids play a cooperative role in preventing PM.

5.2 Other Genes of Importance

We also obtained two mutants (N76 and N77) harbouring separate insertions in a gene encoding a probable methyltransferase which could be involved in fatty acid biosynthesis. In another mutant (N92) a gene homologous to Rv3398 (*idsA*) was interrupted. The encoded gene product of Rv3398 belongs to a novel unique class of, ω,E,E-farnesyl diphosphate synthases involved in isoprenoid biosynthesis pathways. It is claimed to be unique as it is the first eubacterial ω,E,E-farnesyl diphosphate synthase that does not have four amino acids between the aspartate residues of the FARM and that has features of the archaeal CLD region (Dhiman *et al.*, 2004). Sassetti et al., observed attenuated *in vitro* growth in a mutant having a disruption in Rv3398 (Sassetti and Rubin, 2003), but in our study a difference in *in vitro* growth could not be observed. Earlier Pethe et al., in their attempt to identify mutants unable to prevent PM identified three independent insertions in an operon of five genes having roles in isoprenol biosynthesis or modifications. One of the insertions was reported to be in an intergenic region upstream of *idsB* (Pethe *et al.*, 2004).

Another mutant N47 having an insertion in a gene homologous to an *Mtb* gene coding for a probable phytoene dehydrogenase was also identified. These results indicate that the isoprenol metabolic pathway is important for virulent mycobacteria in a phagolysosomal environment.

Mutants with transposon insertions in genes involved in amino acid metabolism and nucleotide metabolism were also observed. Upregulation of genes of the amino acid metabolism pathway has been attributed to a nutrient deprived environment and upregulation of nucleotide metabolism and nucleotide repair genes are considered to be the consequence of an atmosphere enriched in agents that damage nucleic acids (Rachman *et al.*, 2006).

Our results describing the diversity of mycobacterial genes involved in retarding of PM are in general in accordance with previous reports (Pethe *et al.*, 2004); (Stewart *et al.*, 2005). Any discrepancies in genes identified could be due to the difference in mycobacterial strains, cell culture system and infection or other experimental conditions used in the studies.

(1) Mycobacterial strains – for instance our screen yielded genes related to PGL synthesis in contrast to identification of genes related to PDIM synthesis using strains of *M. tuberculosis* [Pethe, 2004] and *M. bovis* BCG Pasteur (Stewart *et al.*, 2005). (2) Cell culture system used – Different intensities of staining for lysosomal membrane glycoproteins and cathepsin-D staining in different cell systems have been observed (Clemens, 1996); (Xu *et al.*, 1994). The discrepancies have been attributed to the different cell culture systems used (HMDM and bone marrow derived mouse macrophages). It is also reasoned that nitric oxide is readily induced in mouse macrophages but not in HMDM on stimulus with mycobacteria (Clemens, 1996). (3) Earlier studies have performed infections for shorter time periods; instead, our experiments were aimed at later stages of PM. This could lead to the identification of different subsets. Further *M. tuberculosis* phagosomes are said to exhibit heterogeneity in macrophages, even within the same macrophage, as observed from staining for different phagosomal markers (Clemens, 1996). Hence mutants were chased for longer duration in order to maintain maximum possible homogeneity. Moreover clumps of mycobacteria have been observed by us and others to proceed to the PL (Clemens and Horwitz, 1995). It has also been studied that phagosomes containing multiple hydrophobic beads mature in contrast to a

single hydrophobic bead, which possesses a close and tight apposition to the phagosomal membrane (de Chastellier and Thilo, 1997). We took utmost care to disperse the clumps into single bacilli to perform the screen. Therefore we were able to isolate PL mutants in which gene coding for secreted and many membrane proteins were disrupted. In the screens done by other groups, underrepresentation of such genes has been speculated to be due to cross complementation (Stewart *et al.*, 2005).

In spite of the differences observed many of the genes identified through our screen have been identified by others individually as virulent genes in other mycobacterial species including *Mtb*. Identified genes were also found to have an effect on the survival fitness of mycobacteria in macrophages. This proves the validity of our screen and the usage of *M. marinum* as a model organism to study mycobacterial virulence principles. Moreover, our genetic screen corroborates the hypothesis that multiple effectors cooperate in establishing a niche in the phagosomes of the host's phagocytes (Russell, 2001).

5.3 Efficient Transfection System to Study Phagosome Maturation

Different Rab-GTPases mark different stages of PM. Therefore Rab-GTPases are useful markers to study PM. Retroviral Rab-GFP fusion constructs have been used in J774 macrophage cell line and bone marrow derived mouse macrophages to study MP (Kelley and Schorey, 2003, 2004). Primary HMDM are terminally differentiated cells and they are unable to divide, thus it is impossible to deliver a gene using retroviral vectors. Instead, adenoviral viral vectors are attractive vehicles among other viral vectors for gene delivery, as they are able to transfect a large variety of cells including those in quiescent state. They have also been used in gene therapy experiments to transfect macrophages (Haddada *et al.*, 1993); (Schneider *et al.*, 1997). Therefore, Rab-GFP fusion adenoviral constructs were designed to transfect macrophages. Flowcytometric analysis on the transfected cells revealed excellent transfection potency of the Rab-GFP adenoviral constructs. Moreover, these constructs were also capable of transfecting primary mouse dendritic cells and activated peritoneal macrophages effectively.

5.4 Mutant P1 is Defective in Inhibiting Phagosome Maturation

The validity of a screen can be assessed only by demonstrating that at least some of the mutants have the predicted phenotype (Pethe *et al.*, 2004). We looked for the co-localisation of phagosomal markers Rab5 and Rab7 and LAMP1 with phagosomes

containing mutant as the arrest of PM has been shown to occur between stages controlled by Rab5 and Rab7 (Via *et al.*, 1997). Immunofluorescence microscopy identified a mutant P1 of which approximately 70% of the phagosomes acquired markers of late endosomes and were not found to retain the early endosomal marker Rab5. Co-elution of radiolabelled bacteria with PL marker showed enrichment of the P1 in PL compared to that of the WT and the P1[pGPC352], signifying PM. As expected for mutants defective in preventing PM, P1 has a diminished survival in macrophages and is severely attenuated *in vivo* in mice. Histopathological analysis of the P1 infected organs showed reduced pathological changes with fewer granulomas in liver and spleen, a macrophage mediated reaction. Together these results strongly suggest that the gene *pmiA* that is inactivated in P1 takes part in modulating PM.

5.5 Gene *pmiA* is Involved in Phenolic Glycolipid Biosynthesis

The most striking observation was that P1 when recovered from the organs of mice produced flat, smooth and transparent colonies. Lipid biosynthesis and fatty acid modifying genes have been found to be upregulated in an intracellular milieu up to twice the activity of the normal level. From these observations it has been inferred that *M. tuberculosis* undergoes immense changes in cell envelope composition upon infection and that these microbes are capable of mobilising mechanisms to evade host immune responses by modifying lipid and cell wall components (Rachman *et al.*, 2006). Our observations substantiate this hypothesis. Altered colony morphology of the mutant P1 was due to a defect in cording as observed microscopically. Correlation between virulence and cording has long been appreciated. Virulent mycobacteria form braided serpentine cords as noted by Koch. The cord forming capacity is attributed to multiple cell envelope lipids. In *M. tuberculosis* cording requires cyclopropanation of mycolic acids while in *M. marinum* disruption of *kasB* leads to non-cording colonies (Glickman *et al.*, 2000); (Gao *et al.*, 2003b). Although mycolic acid synthesis is unaffected in our mutant P1, a systematic lipid profiling identifies a lipid of the outer cellular envelope in the WT that is missing in the mutant P1. When complemented the mutant regains the lipid moiety. These results suggest a function for the gene *pmiA* in lipid metabolism or transport which would also correspond closely to the analogy based assumption that adjacent putative hydrolase (MM3387) and carboxylase (MM3388) genes that are presumably involved in fatty acid metabolism (Cole *et al.*, 1998). The mycobacterial cell envelope is composed of an

unparalleled variety of complex lipids. Very little knowledge is available on the genetics of these lipids and their physiological role. Our results reveal the involvement of a so far uncharacterised gene adjacent to a putative hydrolase and carboxylase genes in lipid metabolism. Moreover, biochemical characterization of lipids of bacteria grown in 7H9 broth containing [^{14}C] *p*-hydroxy benzoic acid and structural characterization of the lipid missing in mutant P1 by ES/MS and NMR reveal that the lipid is a phenolic glycolipid. *M. marinum* along with other virulent strains are known to produce phenolic glycolipids. Although not much is known about the physiological role of PGL produced by *M. marinum* in particular, numerous roles have been attributed to PGLs in general, including the resistance of *M. leprae* to killing by macrophages (Neill and Klebanoff, 1988) and the predilection of *M. leprae* to peripheral nerves (Ng *et al.*, 2000). Importantly hypervirulent *M. tuberculosis* W-Beijing strain produces PGL and inhibits innate immune responses and shows hyperlethality in mice (Reed *et al.*, 2004). Recently it was also shown that many of the clinical isolates possess an intact *pks15/1* region, which has been clearly shown to be important for the biosynthesis of PGL. Considering the fact that PGL is not secreted by all the strains of *M. tuberculosis* it is likely that an alternative molecule could be involved in hampering PM. In this regard, our screen identified genes related to PGL, whereas other screens prompted genes related to PDIM synthesis. These at first sight conflicting results can be brought together if it is considered that *M. marinum* utilizes PGL instead of PDIM for inhibition of PM. In a general sense, it has been suggested that lack of one virulence factor might be compensated by the over production of other factors (Constant *et al.*, 2002). Following that line of argument PGL and PDIM could be used interchangeably by virulent mycobacteria. The mutants defective in PGL biosynthesis were also found to be enriched in the PL. It is compelling to speculate from these results along with the identification of many more mutants defective in PGL synthesis through our screen that PGL could be an inhibitor of phagosome maturation.

5.6 PGL Inhibits Phagosome Maturation

Previous reports have shown that mycobacterial lipids including PGL can modify membranes (Sut *et al.*, 1990) and also prevent actin nucleation, a prerequisite for PM (Anes *et al.*, 2003). In addition, the glycolipids PIMs are shown to mediate selective fusion with early endosomes and yet another glycolipid LAM prevents mycobacterial phagosomes from fusing with late endosomes. All these reports implicate mycobacterial

lipids as key factors in the prevention of PM. As discussed earlier our screen identified mutants having transposon insertions in genes presumably involved in the synthesis of PGL or its precursors. Our work identifies a novel gene *pmiA* that has a role to play in the synthesis of PGL. These evidences prompted us to track the phagosomes containing PGL-coated beads. As expected, PGL-coated beads were restricted to early endosome like phagosomes. Phagosomes containing PGL-coated beads were marked by the presence of Rab5-GTPase, a marker for early endosomes and devoid of mature β-galactosidase a hydrolytic enzyme characteristic for lysosomes. Immunofluorescence microscopy for LAMP-2 confirmed that the PGL-coated beads retarded phagosome maturation. These data lead highly convincingly to the conclusion that *M. marinum* PGL is an important molecule employed by the pathogen to create a niche for itself in macrophages, an unknown function till date.

The cellular events in the host cell permitting this niche remain ill-defined. PGL of *M. leprae* has been found to scavenge oxygen radicals generated upon IFN-γ stimulation. This is considered to be important for the intracellular persistence of *M. leprae*. Infection of bone marrow derived macrophages with a *Mtb* Beijing strain that produces PGL and a mutant defective in the synthesis of PGL revealed an inverse correlation between PGL production and the level of secretion of the pro-inflammatory mediators TNF-α, IL-6, IL-12 and MCP-1 (monocyte chemotactic protein-1). Also, PGL from Beijing strain was able to inhibit innate immune responses by down regulating pro-inflammatory cytokines induced by apolar lipids of H37Rv (Reed *et al.*, 2004). Furthermore, it is known that activation of macrophages with IFN-γ leads to maturation of MP (Via *et al.*, 1998). Moreover, *M. avium* containing phagosomes acidify upon treatment with IFN-γ, resulting in PM (Schaible *et al.*, 1998). Taking these observations together it can be speculated that by down regulating pro-inflammatory cytokines PGL probably inhibits macrophage activation, thereby arresting PM.

Recently cytokines have been implicated in the modulation of phagosome maturation by modulating Rab-GTPase expression. IL-12 increases expression of Rab7 and IL-6 induces Rab-5 (Bhattacharya *et al.*, 2006). In antigen presenting cells cytokines are mainly regulated via Toll like receptor (TLR) signalling. It is also known that TLRs are recruited to phagosomes upon phagocytosis and they have a critical role in phagosomal processing through their downstream signalling (Underhill *et al.*, 1999). It has also been documented

that knock down of TLRs and its adaptor molecule Myd88 result in impaired PM (Blander and Medzhitov, 2004). Moreover, p38 MAP kinases stimulated upon TLR activation dissociate Rab5 from the phagosome through the activation of guanosine dissociation inhibitor (GDI) (Blander, 2007). Taking these findings and our observation that Rab5 does not dissociate from phagosomes containing PGL coated beads into consideration, it is perceivable that PGL interferes with TLR signalling cascade thereby arresting PM (Fig-31). *M. marinum* is one of the mycobacterial species producing PGL and was structurally characterised by Dobson and colleagues (Dobson *et al.*, 1990) but the functional characteristics of *M. marinum* PGL remained undefined to date. This work for the first time demonstrates inhibition of PM by PGL, an important trait of mycobacterial pathogenesis.

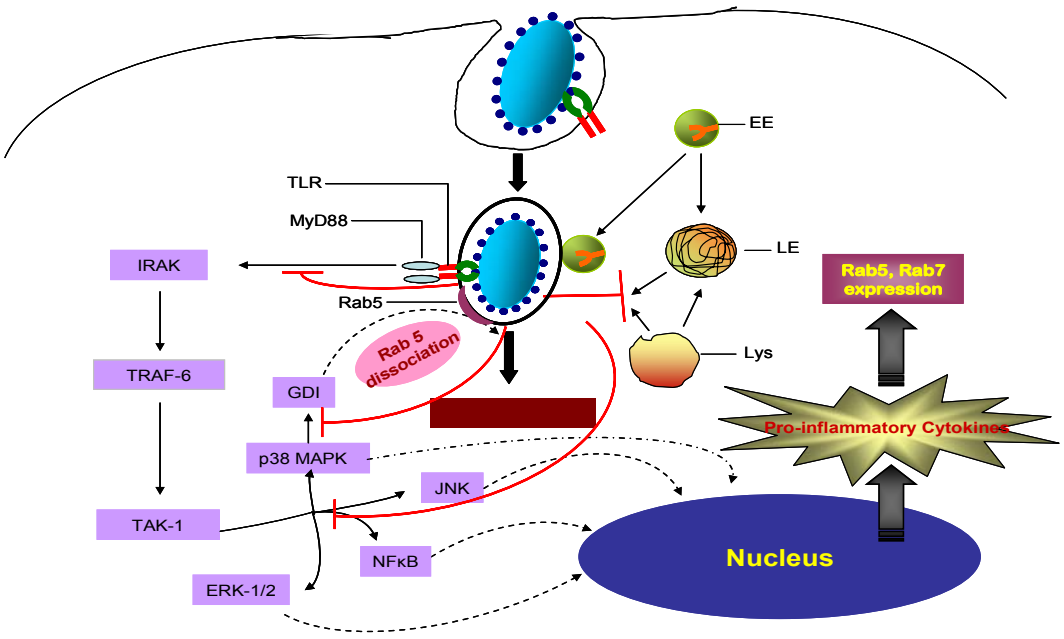

Fig 31: Possible intracellular targets of PGL.

Mycobacteria undergo receptor mediated phagocytosis, but the phagosomes containing mycobacteria or phenolic glycolipid (PGL) coated beads do not fuse with late endosomes (LE) and lysosomes (Lys). However, they interact with early endosomes (EE) and acquire markers of EE, namely Rab5. Toll like receptors (TLR) are the likely candidate receptors to recognise PGL. TLRs are recruited to the phagosomes and are activated on recognition of its ligand. Upon activation of the TLR adaptor protein namely myeloid differentiation factor 88 (MyD88) transduce a cascade of signalling events leading to the activation of p38 mitogen activated kinase (p38 MAPK), extracellular signal-regulated kinase 1 and 2 (ERK 1 / 2), c-Jun NH2-terminal kinase (JNK) and translocation of NF-κB (Nuclear factor-κB). The signalling cascade leads to the induction of pro-inflammatory cytokines. Pro-inflammatory cytokines modulate the expression of fusion mediating Rab-GTPases (Rab5 and Rab7). Moreover, p38 MAPK phosphorylates guanine dissociation factor which in turn dissociates Rab5 from the phagosome permitting maturation. It is possible that PGL interferes with TLR signalling at different levels in the TLR signalling cascade (shown using red lines), thus impairing phagosome maturation.

5.7 PmiA, its Interacting Partners and their Function in Lipid Metabolism

A novel gene has been identified which participates in the biosynthesis of PGL. The gene and its protein product do not share any significant homology with the known genes or proteins identified so far. Therefore, an attempt was made to identify its interacting partners using a His-tag pull down assay. His-tagged PmiA pulled down two proteins namely a methyl transferase and isocitrate lyase (ICL). A similar *Mtb* methyl transferase has been earlier proved to be responsible for the terminal cyclopropanation of mycolic acids. It is plausible that these methyl transferases are involved in the methylation of PGL.

There is accumulating evidence suggesting that mycobacteria subsist primarily on fatty acids rather than on carbohydrates. When bacteria are grown on fatty acids as the principal carbon source, replenishment of citric acid cycle intermediates (anaplerosis) occurs via the glyoxylate cycle, which converts acetyl-coA derived from β-fatty acid oxidation to oxaloacetate. ICL, the first enzyme in the glyoxylate cycle, is upregulated in dormant cultures of *Mtb* under low oxygen tension. In addition, *icl* expression is upregulated during infection of macrophages [Graham and Clark-Curtiss, 1999; Sturgill-Koszycki, 1997]. *Mtb* genome contains two genes coding for ICL *icl1* and *icl2*. An *icl1* knock out mutant was still able to cause acute infection but failed to establish a chronic infection. Mycobacteria lacking both *icl1* and *icl2* were unable to grow on fatty acids or in macrophages (Munoz-Elias and McKinney, 2005). Moreover, ICL1 is proposed to have dual function in glyoxylate cycle and in methyl citrate cycle through which propionyl-coA derived from β-fatty oxidation of odd chain carbon atoms is detoxified. A link between ICL and pmiA remains to be established. It has been reported that anaerobic bacteria such as *Pseudomonas sp* degrade phenol to *p*-hydroxybenzoate via carboxylation (Tschech and Fuchs, 1987). *p*-hydroxybenzoate is also a precursor for PGL synthesis (Stadthagen *et al.*, 2005). It is possible that ICL along with pmiA and other enzymes is also involved in the degradation of hydroxybenzoyl-coA derived upon phenol catabolism. These results demonstrate that PmiA along with other proteins could function as a complex in the biosynthesis of PGL.

6 Conclusion

The persistence of *Mycobacterium tuberculosis* in humans depends predominantly on its ability to survive within the host macrophages. This ability also assists the bacilli to survive in the host for extended time periods in a state of clinical latency. Virulent mycobacteria using their currently ill-defined pathogenic principles achieve this phenomenon by inhibiting phagosome maturation in macrophages.

This thesis demonstrates an elegant screen using *Mycobacterium marinum* to identify mycobacterial genes orchestrating the inhibition of phagosome maturation. A number of genes having possible roles in arresting fusion of phagosomes with lysosomes have been identified using this screening technique. Further, a novel gene *pmiA* involved in the biosynthesis of an outer envelope glycolipid has been discovered to play a major role in stalling phagosome maturation and this glycolipid is characterised to be a phenolic glycolipid (PGL). Moreover, for the first time PGL is shown to be a key factor in the modulation of phagosome maturation.

Mycobacterial lipids have long been thought to play key roles in the pathogenesis of tuberculosis. This study reemphasises mycobacterial lipids as dominant pathogenic principles in mycobacterial pathogenesis. Mycobacterial strains producing PGL downregulate pro-inflammatory cytokines therefore are considered to be more virulent than the strains that do not produce PGL. It is conceivable that PGL arrests phagosome maturation either by diverting intracellular signalling or by interfering with intracellular signalling leading to cytokine induction.

This finding that PGL inhibits phagosome maturation stimulates follow-up studies to identify receptors and signal transduction pathways targeted by PGL for immunomodulation and inhibition of phagosomal processing. This study also portrays genes participating in the synthesis of PGL as ideal targets for developing anti-tuberculosis drugs and also highlights PGL as a possible antigen for vaccine development.

7 Bibliography

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8 Abreviations

αSNAP	Alpha-soluble NSF attachment protein
AG	Arabinogalactan
AIDS	Acquired immune deficiency syndrome
Amp	Ampicillin
AraLAM	Arabinose Lipoarabinomanan
ATCC	American type culture collection
ATP	Adenosine tri phosphate
ATPase	Adenosine tri phosphatase
BCG	Bacillus Calmette-Guèrin
BSA	Bovine serum albumin
Ca2+	Calcium
CaMKII	Calmodulin complex II
cAMP	Cyclic adenosine mono phosphate
CFP-10	10 kilo dalton Culture filtrate protein
CFU	Colony forming units
CPM	Counts per minute
CuSO4	Copper Sulphate
DAG	Diacylglycerol
DC	Dendritic cells
DeADMAn	Designer arrays for defined mutant analysis
DFI	Differential fluorescence induction
DHFRS	Dihydrofolate reductase
DIG	Digoxigenin
DMEM	Dulbecos minimum essential medium
DNA	Deoxy ribo nucleic acid
DTT	Dithiothreitol
E.*coli*	*Escherichia coli*
ECL	Enhanced chemiluminescence (?)
EDTA	Ethylene diamine tetraacetic acid

EE	Early endosomes
EEA1	Early endosomal antigen 1
EGTA	Ethylene glycol tetraacetic acid
EMBL	European Molecular Biology Laboratory
ER	Endoplasmic Reticulum
ES/MS	Electrospray mass spectrometry
ESAT-6	Early secreted antigen
ESI-TOF	Electro spray inonisation – time of flight
FACS	Fluorescence activated cell sorter
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
GAPDH	Glycerinaldehyde-3-phosphate- dehydrogenase
GC	Guanosine Cytosine
GDI	Guanine nucleotide dissociation inhibitor
GFP	Green Fluorescent protein
Gly-Gly-Ala	Glycine-Glycine-alanine
Gly-Gly-Asn	Glycine-glycine-asparagine
GPLs	Glycopeptidolipidis
GTP	Guanosine tri phosphate
GTPase	Guanosine tri phosphatase
HCl	Hydrochloric acid
HEK	Human embryonic kidney
His	Histidine
HMDM	Human Monocyte Derived Macrophages
HPLC	High performance liquid chromatographic
HPTLC	High performance thin layer chromatography
Hrs	Hepatocyte growth factor-regulated tyrosine kinase substrate
H_2SO_4	Slulphuric acid
Hyg	Hygromycin
ICL	Isocitrate lyase

IFN-γ	Interferon gamma
IgG	Immunoglobulin G
IL	Interleukin
IPTG	Isopropyl β-D-1-thiogalactopyranoside
Kana	Kanamycin
Kb	Kilo bases
KCl	Potassium chloride
kDa	Kilo dalton
KOD	*Thermococcus kodakaraensis* polymerase
KOH	Potassium hydroxide
Kv	Kilo volt
LAM	Lipoarbinomanan
LAMP	Lysosome asscociated membrane protein
LB	Luria-Bertani
LBPA	Lysobisphosphatidi c acid
LE	Late endosome
LOS	Lipooligosaccharides
M. avium	*Mycobacterium avium*
MACS	Magnetic cell sorting
MALDI	Matrix assisted laser desorption/ionization
ManLAM	Mannose Lipoarabinomannan
MCP-1	Macrophage chemotactic protein-1
MFP	Membrane fusion protein
Mg	Magnesium
MgCl	Magnesium Chloride
MHC	Major Histocompatibility complex
MmpLs	Mycobaterial membrane protein large
mmpS	Mycobacterial membrane protein small
MOI	Multiplicity of Infection
MP	Mycobacterial phagosome

mRNA	Messenger RNA
MS	Mass spectrometry
Mtb	*Mycobacterium tuberculosis*
NaCl	Sodium Chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
$NaHCO_3$	Sodium bi carbonate
$NaHPO_4$	Sodium hydrogen phosphate
NaOH	Sodium hydroxide
Ni-NTA	Nickel-nitrilotriacetic acid
NK	Natural killer
NMR	Nuclear Magnetic Resonance
NSF	N-ethylmaleimide-sensitive fusion protein
OADC	Oleic acid albumin dextrose complex
OD	Optical density
OmpA	Outer membrane protein A
ORF	Open reading frame
PI3K	Phosphoinositol-3-kinase
PI3P	Phosphoinositol-3-phosphate
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PDIM	Phthiocerol di mycocerosate
PE	Proline-Glutamine
PE-PGRS	Proline-glutamine – polymorphic GC-rich repetitive sequence
PGL	Phenolic Glycolipid
PGRS	Polymorphic GC-rich-repetitive sequence (?)
p-HBAD	P-hydroxybenzoic acid derivatives
PI3P	Phosphatidylinositol 3-phosphate
PIM	Phosphoinositol mannoside
PIPES	Piperazine-N,N´-bis ethansulfonic acid (?)
PknA	Protein kinaseA

pks	Polyketide synthase
PL	Phagolsosome
PM	Phagosome maturation
pmiA	Phagosome maturation inhibition A
PMSF	Phenylmethylsulphonyl fluoride
PPE	Proline-Proline-Glutamine
ppm	Parts per million
PtdIns(4,5)P	Phosphoinositol-4,5-bisphosphate
PVDF	Polyvinylidene difluoride
RF	Retention factor
RGD	Arginine-glycine-aspargine
RILP	Rab7-interacting lysosomal protein
RNA	Ribo nucleic acid
RND	Resistance, nodulation, and cell division proteins
rpm	Rotations per minute
RPMI	Roswell Park Memorial Institute medium
RT	Room temperature
SapM	Secreted mycobacterial lipid phosphatase
SCOTS	Selective capture of trancribed sequence
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate Poly acrylamide gel electrophoresis
SE	Sorting endosomes
SFM	Serum free medium
SMART	Simple Modular Architecture Research Tool
STM	Signature tagged mutagenesis
TB	Tuberculosis
TC	Tissue culture
TDM	Trehalose dimycolate
TE	Tris EDTA
Tf	Transferrin

Tfr	Transferrin receptor
TGN	Trans Golgi network
TLC	Thinlayer chromatography
TLR	Toll like receptor
TMD	Transmembrane domains
Tn	Transposon
TNF-α	Tumor necrosis factor alpha
TraSH	Transposon site hybridization
TRITC	Tetramethylrhodamine-5-isothiocyanate
UV	Ultra violet
WHO	World health organisation
WT	Wild-type
WTHK	Wild-type heat killed
YFP	Yellow fluorescent protein

Declaration

I hereby declare that the work in this thesis is original and has been carried out by me at the Institut für Medizinische Mikrobiologie, Immunologie und Hygiene, Universität Köln, under the supervision of Priv. Doz. Dr. med. Georg Plum and in partial fulfilment of the requirements of the Doctor of Philosophy degree of the Universität Bonn. I further declare that this work has not been the basis for the awarding of any degree, diploma, fellowship, associateship or similar title of any university or institution.

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May 2007

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"No one who achieves success does so withot acknowledging the help of others. The wise and confident acknowledge this help with gratitude"

- *Alfred North Whitehead*

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Publications

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