Mannolipids and mannose metabolism in *Mycobacterium tuberculosis*

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By

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2011

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MY BELOVED

MOTHER AND LATE FATHER

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CERTIFICATE

This is to certify that the work embodied in the thesis entitled, ***"*Mannolipids and mannose metabolism in *Mycobacterium tuberculosis"*** has been carried out by Mr. Shyam Krishna under my supervision and the same has not been submitted elsewhere for a degree.

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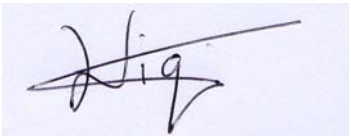
During his PhD, Mr Shyam Krishna has worked 8 months (April to December 2009) under my guidance at Institut de Pharmacologie et de Biologie Structurale, UMR 5089 CNRS / Université Paul Sabatier, Toulouse in the context of a "Sandwich PhD fellowship" from the French Embassy in India. His phD work was entitled "Mannolipids and mannose metabolism in M. tuberculosis". He is an excellent student and he has obtained very good results that should lead to several publications very soon.

During the time he has spent in our lab, he has learned several techniques, including gene knock-out in mycobacteria and biochemical analyses of mycobacterial cell wall compounds, most particularly lipoglycans.



Dr Jérôme Nigou,
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DECLARATION

I hereby declare that the Ph. D. thesis entitled, **"Mannolipids and mannose metabolism in *Mycobacterium tuberculosis*"** is an independent work carried out by me at the Biotechnology Division, National Institute for Interdisciplinary Science and Technology-CSIR, Trivandrum, under the supervision of Dr. K. Madhavan Nampoothiri and it has not been submitted elsewhere for any other degree, diploma or title.

In keeping with the general practice of reporting scientific observations, due acknowledgement has been made wherever the work described is based on the findings of other investigators.

Shyam Krishna

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PREFACE

Mycobacterium tuberculosis, the causative agent of tuberculosis, has plagued mankind since the beginning of medical history. It is currently the second leading cause of mortality due to a single infectious agent. All mycobacteria are surrounded by a compositionally unique cell wall that is essential for the survival of pathogenic species in the phagolysosomes of human macrophages. The mycobacterial cell wall comprises a core complex of covalently linked peptidoglycan, arabinogalactan polysaccharide and long chain mycolic acids. Additional glycol and phospho-lipids may be incorporated into this structure or form a distinct outer lipid layer that intercalates in to the layer of mycolic acids. Mannose-containing glycolipids, including the phosphatidylinositol mannosides (PIMs) and their hypermannosylated derivatives, lipomannan (LM) and lipoarabinomannan (LAM) are a major class of cell-wall glycolipids in all mycobacteria. The PIMs, LM and LAM are thought both to be incorporated into the plasma membrane and to be exposed on the cell surface. PIM, LAM and LM are of the mycobacterial cell wall recognized by cells involved in the innate immune response and have been found to modulate the cytokine response. Structural elucidation, functional relationship with innate immune response and identification of enzymes involved in the biosynthesis of LAM and LM may provide insight on the immunomodulatory activities of LM and LAM of mycobacteria during primary infection.

Present study focused to analyse the role of lipoarabinomannan (LAM) and lipomannan (LM) in the pathogenicity of Mycobacterium by moderately increasing its deposition in mycobacterial cell wall. To better decipher mannose metabolism in mycobacteria, the study aims to use genetic manipulation of mannose biosynthesis pathway to construct mycobacterial mutants with an altered production of lipoglycans and by this way to determine the contribution of the latter in detecting mycobacteria by the innate immune system.

The thesis has been framed into six chapters. Chapter 1 gives an introduction about the tuberculosis disease followed by a detailed review of literature on mycobacterial cell wall characteristics such as its structure, biosynthesis and interaction of mannolipids with human immune system. Chapter 2 describes the construction of *Mycobacterium smegmatis* mutants by cloning and overexpressing the *Mycobacterium tuberculosis* genes such as *manA* (Rv 3255c), *manB* (Rv 3264c), *pmmB* (Rv 3308) and *manC* (Rv 3264) involved in the mannose metabolism. This chapter also describes the creation of a *pmmB* deleted *M. smegmatis* mutant. Chapter 3 deals with the characterization of the wild and mutant strains which include (i) assay of various enzymes involved in the mannose biosynthesis coded by the above genes in wild type strain as well as in mutant strains (ii) isolation and characterization of various mannolpids from the wild type strain and also from the mutant strains (iii) quantification of the lipoglycans in various constructs (iv) Cell surface properties such as hydrophobicity, aggregation etc. in all constructs. Chapter 4 explains the immune response of mycobacterial mutants with Human Embryonic Kidney (HEK) cells and Human acute monocytic leukemia (THP-1) cells. Chapter 5 explains the attempts to over express the *M.tb* genes involved in mannose biosynthesis in *E. coli* and the purification of the recombinant proteins. The last chapter summarizes the total work and conclusion. This is followed by a bibliographic section and two annexures. Annexure I show the list of major equipments and the composition of various culture media used in the present study. List of publications and awards are attached as Annexure II.

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ABBREVIATIONS

%	Percentage
° C	Degree celsius
µ	Micro
µA	Micro Ampere
µF	Microfarad
µg	Microgram
µg l^{-1}	Microgram per litre
µg ml^{-1}	Microgram per microlitre
µl	Microlitre
µM	Micro molar
µm	Micrometre
ADP	adenosine diphophate
AP	activator protein
APTS	1-aminopyrene-3, 6, 8-trisulfonate
ATP	adenosine triphosphate
bp	base pair
BSA	Bovine serum albumin
CD	cluster of differentiation
cm	Centimetre
CO_2	carbon dioxide
CZE	Capillary Zone Electrophoresis
DMEM	Dulbecco's Modified Eagle Medium

DMSO	Dimethyl Sulphoxide
DNA	deoxyribo Nucleic acid
DNase	Deoxyribonuclease
dNTP	deoxynecleotide phosphate
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
F6P	fructose-6-phosphate
FBS	Fetal bovine serum
FSL	fibroblast-stimulating lipopeptide
g	Gram
g	Gravity
g l^{-1}	Gram per litre
G6P	glucose-6-phosphate
G6PDH	D-glucose-6-phosphate dehydrogenase
GDP	guanosine di phoshate
GDPMP	GDP-mannose pyrophosphorylase
GMP	Guanosine monophosphate
GPI	Glycophosphatidylinositol
h	Hour
H_2SO_4	Suphuric acid
HCl	Hydrochloric acid
HEK	Human Embryonic Kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IFN	interferon

IgA	immunoglobilun A
IgG	immunoglobulin G
IL	interleukin
KV	kilo volt
LAM	lipoarabinomannan
LB	Luria Bertani
LIF	laser-induced fluorescence
LM	lipomannan
LTA	lipoteichoic acid
M1P	D-mannose-1-phosphate
M6P	mannose-6-phosphate
MAMPs	microbe associated molecular patterns
ManTs	mannosyltransferases
mbar	millibar
mg	milligram
mg ml^{-1}	milligram per millilitre
$MgCl_2$	Magnesium chloride
min	Minute
ml	millilitre
mM	milli molar
MOI	multiplicity of infection
mU mg^{-1}	milliunits per milligram
mW	milli Watts
N	Normal
$NaBH_3CN$	cyanoborohydride

NADP	nicotinamide adenine dinucleotide phosphate
NaF	sodium fluoride
NaPPi	sodium pyrophosphate
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
ng	nano gram
nM	nano molar
NOD	nucleotide-binding oligomerization domain
OD	optical density
Pa	pascal
PBS	phosphate bufferd saline
PCR	Polymerase Chain Reaction
PGI	phosphoglucose isomerase
PGM	phosphoglucomutase
pH	Hydrogen ion concentration
PIM	phosphotidylinositol mannosides
PMI	phosphomannose isomerase
PMM	phosphomannomutase
PMSF	Phenyl methane sulphonyl fluoride
PPM	Polyprenyl- monophosphoryl-mannose
PRRs	pattern recognition receptors
RMT RSD	relative migration time reproducibility
RPMI	Roswell Park Memorial Institute medium
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEAP	secreted embryonic alkaline phosphatase
sec	Second

TEA	Tris EDTA Acetic acid
TFA	Tri Fluoro Acetic Acid
THF	tetrahydrofuran
THP	Human acute monocytic leukemia
TLR	Toll-Like Receptor
U g^{-1}	Units per gram
v/v	volume/volume
w/v	weight/volume

CHAPTER 1

Introduction and Review of Literature

CHAPTER 1

Introduction and Review of Literature

1.1 INTRODUCTION

Tuberculosis is an old disease but remains one of the main threats to mankind. It is caused by *Mycobacterium tuberculosis*, one of the most effective bacterial human pathogens. The disease affects 1.8 billion people per year which is equal to one-third of the entire world population (Caws et al., 2008). Mycobacteria are extraordinarily successful pathogens with the remarkable ability to persist intracellularly even in the presence of an intact immune system. One major challenge that the intracellular bacteria face is overcoming cell-mediated mechanisms of immunity that detect signals originating from infected cells. An important key to the success of pathogenic mycobacteria is likely to be their unusual cell wall structure and its interactions with the immune system (Chatterjee, 1997; Flynn and Chan, 2003). This cell envelope consists of a highly complex array of distinctive lipids, glycolipids and proteins (Brennan and Nikaido, 1995). These complex molecules are believed to play important roles in the physiology of the bacterium as well as in the modulation of the host response during infection (Karakousis et al., 2004).

Several mannose-containing molecules, actually mannolipids, of the cell-wall are thought to play a crucial role in the interplay between *M. tuberculosis* and host cells. Indeed, the lipoglycans: lipoarabinomannan (LAM) and its related precursors, lipomannan (LM) and phosphatidyl-*myo*-inositol mannosides (PIMs) as well as a mannolipoprotein (the so-called 19 kDa lipoprotein) are powerful immunomodulatory molecules (Briken et al., 2004; Chatterjee and Khoo, 1998; Nigou et al., 2002).

PIMs, LM and LAM are major glycolipids and lipoglycans that are non-covalently

attached to the cell wall. The main activities associated to LAM from *M. tuberculosis* (called ManLAM) are their ability to inhibit, (i) activation of the macrophages, (ii) the production of the Th1 pro-inflammatory cytokines IL-12 and TNF-α and (iii) *M. tuberculosis*-induced macrophage apoptosis (Chatterjee and Khoo, 1998; Nigou et al., 2002). Moreover, ManLAM is a key ligand in the interaction between *M. tuberculosis* and macrophages and dendritic cells (DCs) (Malaga et al., 2003; Schlesinger et al., 1994). Mannose Receptor and DC-SIGN are likely to favour the intra-macrophagic survival of the bacilli (Tailleux et al., 2003). So, ManLAM appear as a virulence factor that contributes, via an immunosuppressive effect, to the persistence of slow-growing mycobacteria in the human reservoir. In contrast, PIM, LM and the 19 kDa lipoprotein are able to induce the release a variety of pro-inflammatory cytokines through the activation of Toll-like 2 receptors (TLR-2) (Brightbill et al., 1999; Gilleron et al., 2003; Quesniaux et al., 2004; Vignal et al., 2003). LM and the 19 kDa lipoprotein, which are very powerful pro-inflammatory stimuli, are therefore likely to favour the killing of the mycobacteria by activating the macrophages.

PIM, LM and LAM share a common mannosyl-phosphatidyl-*myo*-inositol (MPI) anchor (Khoo et al., 1995). PIM are predominantly found as di-and hexa-mannoside forms (PIM2 and PIM6, respectively) (Gilleron et al., 2003). LM correspond to multimannosylated forms of PIM, with a mannan core that consists of an 1,6-α-linked Man*p* backbone, which is substituted at O-2 by single Man*p* units in all the mycobacterial species investigated so far, except *M. chelonae* (Guerardel et al., 2002) where it occurs at O-3. LAM corresponds to a LM with a branched arabinan polymer and, in some cases; cap motifs decorate the termini of the branched arabinan (Nigou et al., 2003). The arabinan of LAM consists of a linear (α1→5)-linked arabinofuranosyl backbone punctuated with branched hexa-arabino furanosi des and linear tetra-arabinofuranosides (Chatterjee et al., 1991; Chatterjee et al., 1993). LAM can be classified into three families according to the capping motifs present on the non-

reducing termini of the arabinosyl side-chains. Mannooligosaccharide units cap the LAM from the slow growing mycobacteria, *M. tuberculosis*, *M. leprae*, *M. bovis* BCG, *M. bovis*, *M. kansasii* and *M. avium* (Chatterjee et al., 1992; Nigou et al., 2003) ; these LAM have been called ManLAM. Phosphoinositides units cap the LAM from the fast growing mycobacteria, *M. smegmatis* and *M. fortuitum* (Gilleron et al., 1997), these LAM have been termed PILAM. In addition, a LAM devoid of caps has been identified in *M. chelonae* defining the AraLAM family (Guerardel et al., 2002).

Contrarily to ManLAM that show an anti-inflammatory effect, PILAM are efficient pro-inflammatory stimuli (Gilleron et al., 1997; Means et al., 1999). This paradigm provides an interesting correlation between LAM structure and their immunomodulatory effect on one hand, and the intramacrophagic fate of the corresponding mycobacteria on the other hand (Nigou et al., 2002). Indeed, the inability of *M. smegmatis* to survive inside activated macrophages correlates with the pro-inflammatory effect of PILAM. Likewise, the capacity of *M. tuberculosis* and *M. bovis* BCG to survive and multiply inside macrophages is in agreement with the anti-inflammatory effect of ManLAM.

The structure/function relationships underlying the activities of the different lipoglycans, LAM, LM and PIM have been roughly deciphered from the study of chemically or enzymatically modified lipoglycans from various mycobacterial species (Barnes et al., 1992; Gilleron et al., 1997; Nigou et al., 2001; Vignal et al., 2003) as well as the use of LAM structural variants originating from genera related to mycobacteria, ie *Rhodococcus*, *Amycolatopsis* or *Tsukamurella*. A linear ($\alpha 1 \rightarrow 6$)-Man*p* chain, linked to the MPI anchor, such as in PIM for example, is sufficient in providing pro-inflammatory activity to the lipoglycan (Gibson et al., 2004). However adding and increasing the size of side chains such as in LM dramatically increases this activity. Concerning LAM, it is now clear that the arabinan domain, by masking the mannan core of LAM, inhibits the pro-inflammatory

activity of its LM portion on macrophages (Gibson et al., 2004; Vignal et al., 2003). Moreover, the presence of mannose caps substituting the arabinan domain allows ManLAM binding to C-lectins and provides an inhibitory effect to the molecule whereas the presence of phosphoinosite caps provides an epitope directing the molecule towards a pro-inflammatory activity (Nigou et al., 2002; Nigou et al., 2001).

Therefore, in *M. tuberculosis*, LM and PIM to a lesser extent are proinflammatory factors whereas ManLAM are powerful anti-inflammatory molecules. So, it would seem important for virulent mycobacteria to minimize the amount of LM present in the cell wall in order to reduce the host's proinflammatory response. Consequently, mycobacterial virulence could be modulated by the LAM/LM ratio as well as by variations in LAM and LM fine structure that defines their biological activity.

To date, a thorough investigation of the roles of PIMs, LM and LAMs in mycobacterial virulence has been hampered by a lack of defined mutants that fail to synthesize these specific cell surface components. Consequently, analysis of the fine structure and immunomodulatory properties of LAM and LM as well as their relative ratio in the cell walls of different virulent mycobacteria would help to gain better understanding on the role of these cell-wall components.

Understanding the biosynthesis of these lipoglycans has been the focus of numerous genetic and biochemical studies that are extensively reviewed in Briken et al., (2004) and Nigou et al., (2003). The complexity of LAM structure suggests that several acyl-, mannosyl- and arabinosyl-transferases are involved in its biogenesis. So far, only the enzymes involved in the addition of the first 3 Man*p* units on the MPI anchor, an acyl-transferase and an arabinosyltransferase involved in (α1→3)-Ara*f* branching have been identified. However, the sugar donors for mannan and arabinan biosynthesis are well characterized. GDP-Man*p* and polyprenol-monophosphoryl-mannose (C_{35}/C_{50}-P-Man*p*) are the donors of Man*p* for

mannosyltransferases. GDP-Man is used during the early steps of PIM biosynthesis whereas C_{35}/C_{50}-P-Man*p* is used later in LM synthesis from PIM precursors (Besra and Brennan, 1997; Brennan and Ballou, 1967). It is noteworthy that C_{35}/C_{50}-P-Man*p* is synthesized from GDP-Man*p* and polyprenol by the polyprenol monophosphomannose synthase (*ppm1*) (Gurcha et al., 2002). Polyprenol-monophosphoryl-β-D-Ara*f* (C_{35}/C_{50}-P-Ara*f*) is the only Ara*f* sugar donor identified so far (Wolucka et al., 1994).

Mannose is a common constituent of these very important mannolipids, ie the lipoglycans as well as of the 19 kDa mannolipoprotein, and GDP-Man*p* has been shown to act as the archetypal mycobacterial mannose donor (Brennan and Ballou, 1967). Possible homologues of the genes encoding enzymes involved in the *de novo* synthesis of the activated form of mannose, GDP-Man*p*, occur in the genome of *M. tuberculosis* (Cole et al., 1998). This biosynthesis pathway (Fig. 1.1) involves a phosphomannose isomerase (PMI; *ManA, Rv3255c*) that catalyzes the interconversion of fructose-6-phosphate (F6P) to mannose-6-phosphate (M6P). Then phosphomannose mutase (PMM; *manB, Rv3257c*; *pmmB, Rv3308*) catalyzes the interconversion of mannose-6-phosphate and mannose-1-phosphate (M1P). Finally, GDP-Man*p* is synthesized from mannose-1-phosphate and GTP by GDP-mannose pyrophosphorylase (GMP; *manC, Rv3264c). manA, manB* and *manC* have been found to be essential for growth of *M. tuberculosis* as defined by high density mutagenesis whereas *pmmB* was found non-essential (Sassetti et al., 2003). Recently, a PMI deletion mutant has been generated in *M. smegmatis* (Patterson et al., 2003). When this mannose auxotroph mutant was suspended in media without mannose, ongoing synthesis of the mannolipids was halted and a pronounced hyperseptation phenotype appeared. In addition these changes preceded a dramatic loss of viability after 10 h in mannose-free media. Altogether, these results show that mannose metabolism is required for growth of mycobacteria and that one or more mannose-containing molecules may play a role in regulating septation and cell division

in these bacteria (Patterson et al., 2003). Mannose thus appears as a key metabolite of mycobacteria and its metabolism deserves to be investigated in details.

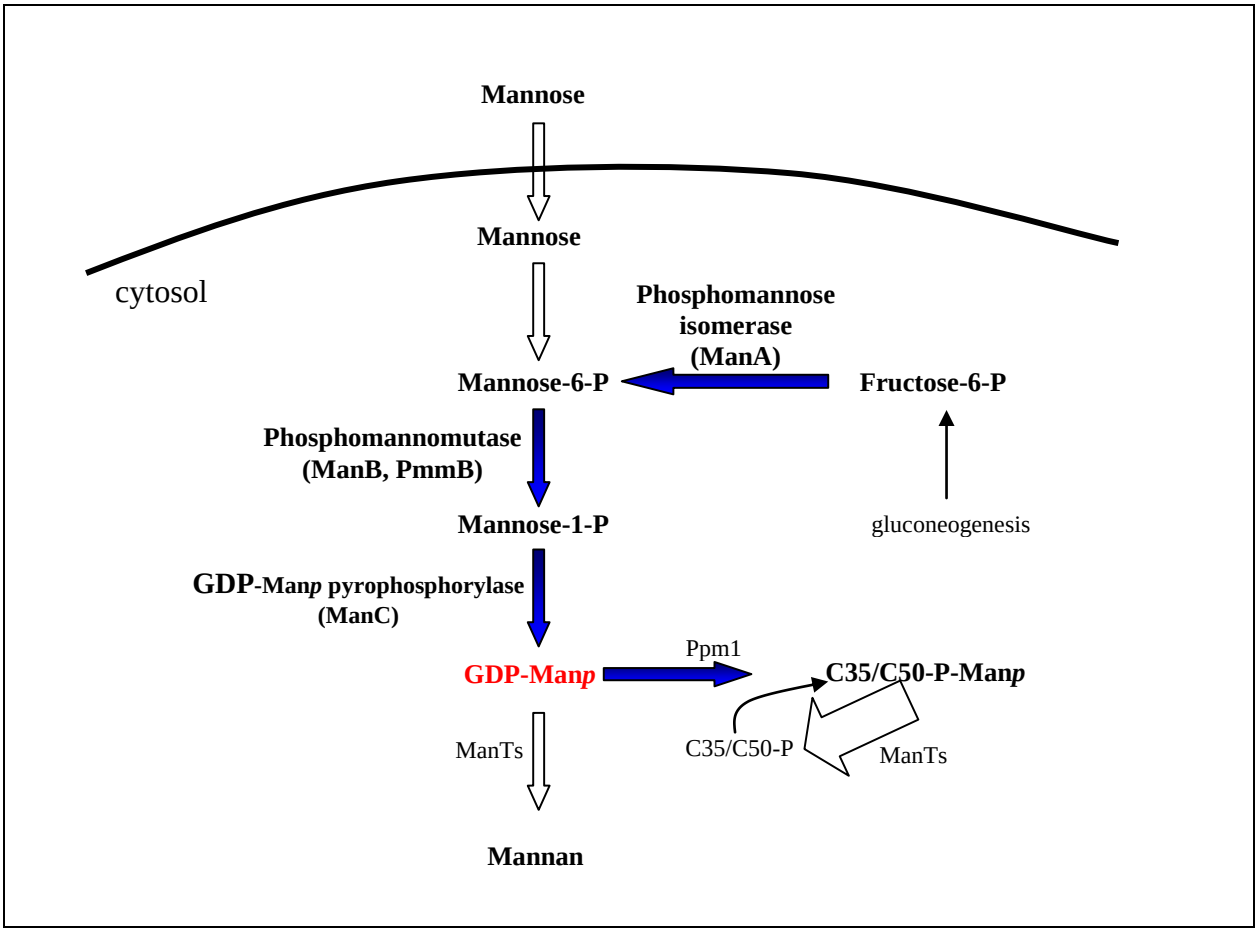


Fig. 1.1 Proposed pathway of mannose biosynthesis in mycobacteria

Enzymatic steps considered in the present project are indicated in blue and underlined.

ManTs, mannosyltranferases; P, phosphate.

1.2 OBJECTIVES OF THE STUDY

To study mannose metabolism in mycobacteria, and particularly in *M. tuberculosis* by,

a) Characterization of *M. smegmatis* mutants, either over expressing *M. tuberculosis* genes or knocked out for the genes involved in the biosynthetic pathway of GDP-manno pyranose (GDP-Man*p*).
b) Purification and quantification of mannolipids, the immuno modulators from wild and mutants strains of *M. smegmatis.*
c) Determining the pro-or anti-inflammatory effects in human monocyte/macrophages by the mannolpids.
d) Cloning and heterologous expression of the putative enzymes involved the biosynthesis pathway of GDP-Man*p* in *E.coli.*

1.3 REVIEW OF LITERATURE

1.3.1 Tuberculosis

Tuberculosis (TB) is the leading cause of death in the world from a bacterial infectious disease. The disease affects 1.8 billion people per year which is equal to one-third of the entire world population (Caws et al., 2008). *M. tuberculosis* is the etiologic agent of tuberculosis in humans. *M. bovis* is the etiologic agent of TB in cows and rarely in humans. Humans can also be infected by the consumption of unpasteurized milk. This route of transmission can lead to the development of extrapulmonary TB, exemplified in history by bone infections that led to hunched backs. Other human pathogens belonging to the Mycobacterium genus include *M. avium* which causes a TB-like disease especially prevalent in AIDS patients, and *M. leprae,* the causative agent of leprosy.

1.3.1.1 TB Global perspective

In 2008, there were an estimated 9.4 (range, 8.9–9.9 million) million incident cases (equivalent to 139 cases per 100000 population) of TB globally (Fig. 1.2). This is an increase

from the 9.3 million TB cases estimated to have occurred in 2007, as slow reductions in incidence rates per capita continue to be outweighed by increases in population. Estimates of the number of cases broken down by age and sex are being prepared by an expert group (http://www.who.int/tb/dots/r_and_r_forms/en/ index.html) as part of an update to the Global Burden of Disease study, (http://www.who.int/tb/dots/r_and_r_forms/en/ index.html). From the reports published by WHO, the five countries that rank first to fifth in terms of total numbers of incident cases in 2008 are India (1.6–2.4 million), China (1.0–1.6 million), South Africa (0.38–0.57 million), Nigeria (0.37–0.55 million) and Indonesia (0.34–0.52 million). India and China alone account for an estimated 35 % of TB cases worldwide. Of the 9.4 million incident cases in 2008, an estimated 1.2–1.6 million (13–16 %) were HIV-positive, with a best estimate of 1.4 million (15 %). In 2008, an estimated 1.3 million (range, 1.1–1.7 million) deaths, including 0.5 million (range, 0.45–0.62 million) deaths among women, occurred among HIV-negative incident cases of TB.

1.3.1.2 MDR-TB and XDR-TB

There were an estimated 0.5 million cases of MDR-TB in 2007. There are 27 countries (15 in the European Region) that account for 85 % of all such cases; these countries have been termed the 27 high MDR-TB burden countries. The countries that ranked first to fifth in terms of total numbers of MDR-TB cases in 2007 were India (131000), China (112000), the Russian Federation (43000), South Africa (16000) and Bangladesh (15000). By November 2009, 57 countries and territories had reported at least one case of XDR-TB (http://www.who.int/tb/dots/r_and_r_forms/en/ index.html).

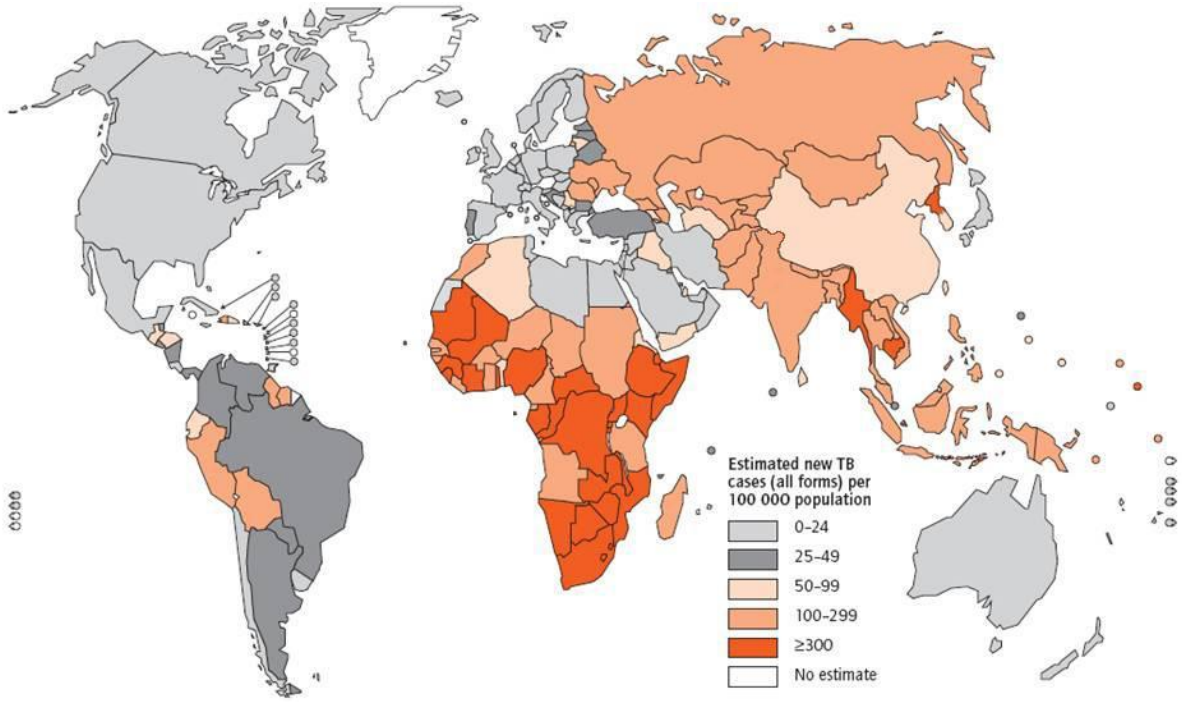


Fig. 1.2 Estimated TB incidence rates, 2008 (WHO 2009)

1.3.2 Mycobacteria-General characteristics and classification

Mycobacteria belong to the family *Mycobacteriaceae* and are members of the CMN group (*Corynebacteria, Mycobacteria and Nocardia*). The family *Mycobacteriaceae* is grampositi ve, nonmotile, catalase-positive, have a rodlike to filamentous morphology and can be pleomorphic. Mycobacteria are acid-fast rods of variable appearance, approximately 0.2-0.6 by 1-10 µm. The genus Mycobacterium consists of 127 species (excluding subspecies) according to the latest approved list of bacterial species (http://www.dsmz.de/microorganisms /bacterial_nomenclature.php). Mycobacteria other than *M. tuberculosis* are commonly referred to as atypical or non-tuberculous mycobacteria (NTM). Two of these cause disease in normal hosts and are thus primary pathogenic: *M. leprae, M. ulcerans*. They are often not regarded as NTM. The remaining species are considered nonpathogenic or opportunistic pathogens and cause disease when host-defences are compromised.

Mycobacteria can be arranged into four groups according to the Runyon classification (Sommers, 1985).

- Group 1 - Photochromogens: slow growers and form pigment when exposed to light (eg, *M. kansasii, M. marinum, M. simiae*)
- Group 2 - Scotochromogens: slow growers and form pigment in the dark (eg, *M. scrofulaceum, M. szulgai, M. gordonae*)
- Group 3 - Nonphotochromogens: slow growers and not pigmented (eg, *M. malmoense, M. xenopi, M. avium*-complex, *M. ulcerans, M. haemophilum*)
- Group 4 - Slow growers (eg, *M. tuberculosis, M. chelonae, M. abscessus*)
- Group 5 - Fast growers (eg, *M. smegmatis, M. bovis*)

Most slow-growing species have been associated with disease in humans while only few species of group 4 (the ones mentioned above) are disease associated.

M. tuberculosis is an obligate aerobe, which is a member of slow growing group. The bacterium is a facultative intracellular parasite, usually of macrophages, and has a slow generation time 15-20 h, a physiological characteristic that may contribute to its virulence. Two media are used to grow MTB Middle brook's medium which is an agar based medium and Lowenstein-Jensen medium which is an egg based medium. *M. tuberculosis* colonies are small and buff colored when grown on either medium. It takes 4-6 weeks to get visual colonies on either type of media. Mycobacterium species are classified as acid-fast bacteria due to their impermeability by certain dyes and stains (http://www.textbookofbacteriology.net/tuberculosis.html). One acid-fast staining method for *M. tuberculosis* is the Ziehl-Neelsen stain. When this method was used, the *M. tuberculosis* smear is fixed, stained with carbol-fuchsin (a pink dye), and decolorized with acid-alcohol. The smear is counterstained with methylene-blue or certain other dyes. Acid-fast bacilli appeared in pink clour in a

contrasting background. Fig. 1.3 a and b shows the colony morphology and acid-fast stained cells of *M. tuberculosis* respectively

Fig. 1.3 a Colonies of *Mycobacterium tuberculosis* on Lowenstein-Jensen medium.

(http://www.textbookofbacteriology.net/tuberculosis.html)

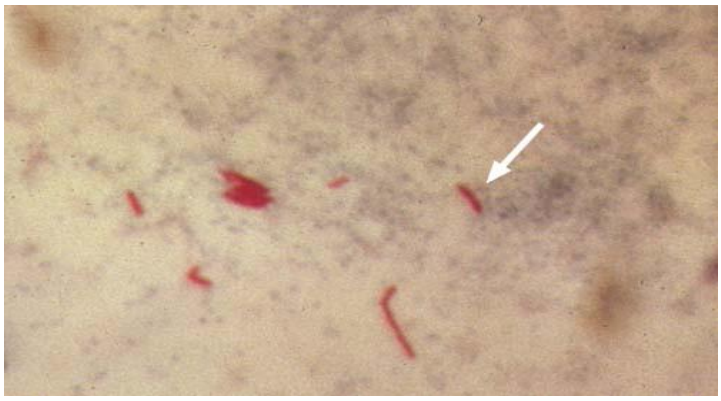


Fig. 1.3 b *Mycobacterium tuberculosis.* Acid-fast stain

(http://www.textbookofbacteriology.net/tuberculosis.html)

1.3.2.1 Evolution

Devulder et al., (2005) developed a multigene sequence database incorporating four genes (16S rRNA, hsp65, rpoB, sod) within the genus Mycobacterium. The final phylogenetic tree agrees with pre-existing, generally accepted phylogenetic relationships; in particular, the partition between slowly and rapidly growing mycobacteria is strongly supported (Fig.1.4).

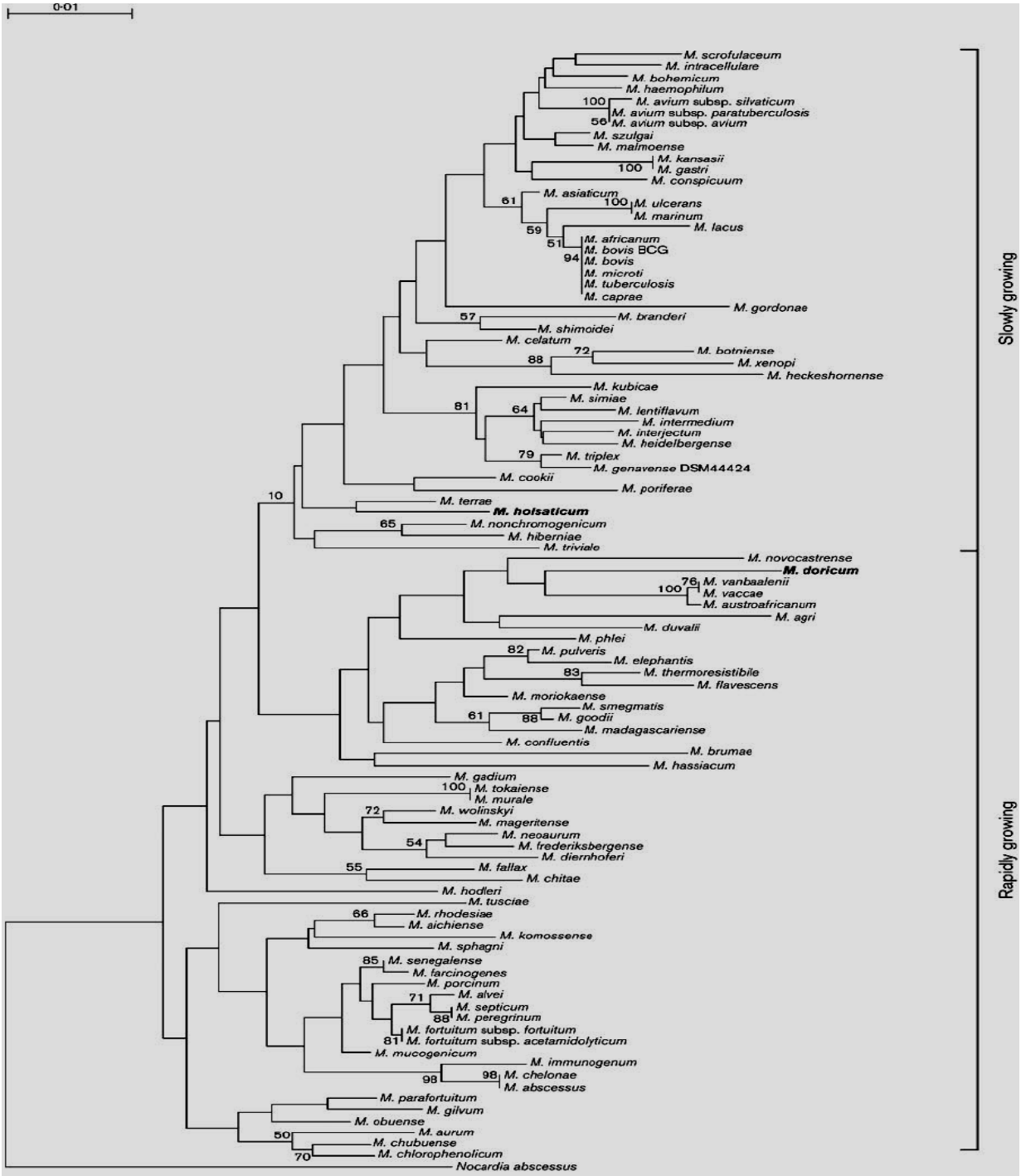


Fig. 1.4 Phylogenetic tree of the genus Mycobacterium computed from the concatenation of 16S rRNA gene, hsp65, sod and rpoB sequences by the neighbour-joining method and Kimura's two-parameter model as the substitution model (Devulder et al., 2005).

1.3.3 Structure of mycobacterial cell wall

The *M. tuberculosis* envelope is an essential feature in regulating pathogen interactions with the host (Brennan and Nikaido, 1995). The cell envelope is comprised of plasma membrane and a surrounding cell wall. The cell wall (Fig. 1.5) is composed of peptidoglycan (PG) in covalent attachment to arabinogalactan (AG), which in turn is attached to the mycolic acids with their long meromycolate and short α-chains. This is termed the cell wall core-the mycolyl arabinogalactan-peptidoglycan (mAGP) complex. Peptidoglycan, which forms the backbone of the mAGP, consists of alternating units of N-acetylglucosamine and N-glycolylmuramic acid (Besra and Brennan, 1997). The tetrapeptide side chains are attached to muramic acid which is crosslinked to AG via a phosphodiester link to position 6 of a proportion (about 10-12 %) of the muramic acid residues (Lederer et al., 1975). The tetrapeptide side chains consist of L-alaninyl-D-isoglutaminyl-meso-diaminopimelyl-D-alanine (Petit et al., 1969) with the diaminopimelic acids being further amidated (Wietzerb.J et al., 1970). Mycobacterial peptidoglycan is notoriously resistant to lysozyme, and, although the evidence is not conclusive, it appears that the N-glycolyl group on the muramic acid residue in the peptidoglycan may protect the organism from degradation. Arabinogalactan is formed of repeating units of 11–16, α-1-4- linked galactopyranose or 1-5-linked galactofuranose (Vilkas et al., 1973). The mycolic acids are present as tetramycolyl pentaarabinofuranosyl clusters on the arabinan portion of mAG (Besra GS, 1996). Mycolic acids are large (C_{70}-C_{80}), α-alkyl branched, and β-hydroxylated (Besra et al., 1997) fatty acids. Two families of mycolic acids are known: α mycolates without any oxygenated functional groups and the oxygenated mycolates. Slowgrowing pathogenic mycobacteria such as *M. tuberculosis* modify their acids by cyclopropanation, whereas rapid growing saprophyte species such as *M. smegmatis* do not. The proportion of mycolic acid containing trans-substituents at proximal positions of the meromycolate chain is an important determinant of

fluidity of the mycobacterial cell wall, and is also related to the sensitivity of mycobacterial species to hydrophobic antibiotics (Yuan et al., 1997). Interspersed somehow in the mAGP there are cell-wall proteins, phosphatidylinositol mannosides (PIMs), the phthiocerol containing lipids, lipomannan (LM), and lipoarabinomannan (LAM). This integrated region which is composed of lipoproteins, proteins, and lipoglycans are the signaling, effector molecules in the disease process, whereas the insoluble core is essential for the viability of the cell and should be addressed in the context of new drug development.

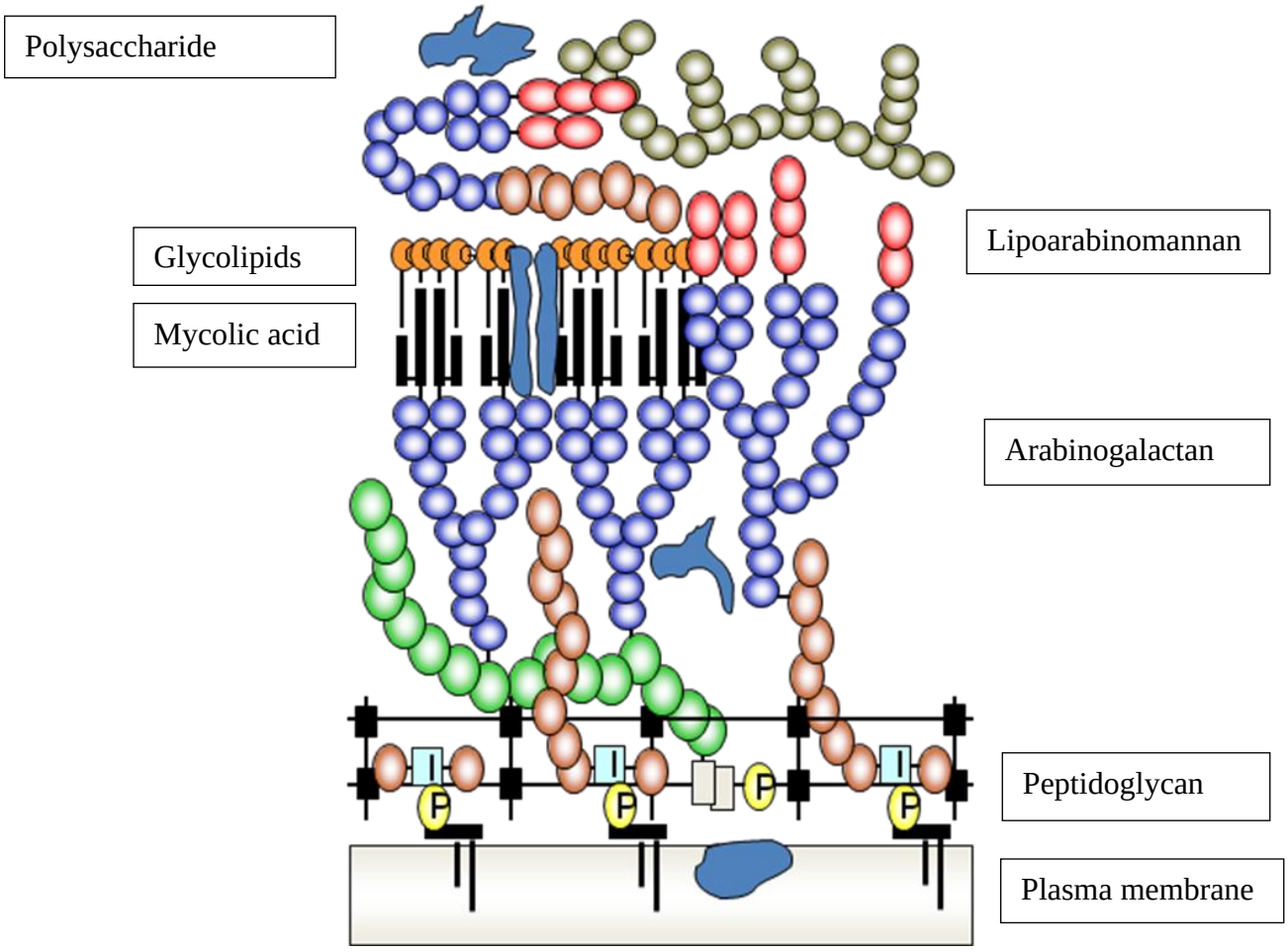


Fig. 1.5 Cell wall of Mycobacterium

1.3.4 Potential drug targets on Mycobacterial cell wall

The cell wall of *M. tuberculosis* is essential for its growth and survival in infected host, and thus, contributes to the resistance to most commonly-used antibiotics and chemotherapeutic agents. Cell wall is characterized as a preferred source of molecular targets because the

biosynthetic enzymes do not have homologues in mammalian system (Chatterjee, 1997; Mdluli and Spigelman, 2006). Most of the successful antibiotics against TB used were targeting the enzymes involved in the synthesis of cell wall components of Mycobacterium like ethonamide and isoniazide (Inh) targeting mycolic acids synthesis and enthambutol (Emb) inhibit arabinogalactan synthesis proved the significance of detailed analysis of cell wall (FG, 1962; Mikusova et al., 1995; Takayama and Kilburn, 1989).

Based on the crystal structure available (LeMagueres et al., 2005), alanine racemase from *M. tuberculosis*, plays an essential role in peptidoglycan synthesis is expected to be a potent target for drug design against TB; furthermore, the related studies provided valuable insights about the precise mechanism of D-cycloserine inhibition against drug-resistant TB (Feng and Barletta, 2003; LeMagueres et al., 2005).

AG and LAM biosynthesis pathways are crucial in finding drug targets against Mycobacterium as AG and LAM responsible for the cell wall integrity and latter have physiological role (Chatterjee, 1997). For example, ribosyltransferase is key to catalyze decaprenyl-phosphoryl-D-atabinose synthesis (Huang et al., 2005) ; UDP-galactopyranose mutase (Kremer et al., 2001), galactofuranosyl transferase (Brunger et al., 1998), dTDP-6-deoxy-L-lyxo-4-hexulose reductase (RmID) (Nakano et al., 2000), RmlB and RmlC are also essential for mycobacterial growth (Li et al., 2006; Ma et al., 2001). These enzymes can be the potential targets for anti- *M. tuberculosis* drug discovery.

Essential enzymes in type II fatty acid biosynthetic (FAS-II) pathway (Mdluli and Spigelman, 2006) that are responsible for large fatty acid synthesis are crucial for mycolic acid synthesis (Chatterjee, 1997). Hence FAS-II can be the potential targets for drug discovery. Polyketide synthase Pks13, which is crucial for mycobacterial growth, catalyzes the final condensation step in mycolic acid synthesis in FAS-II pathway (Bhatt et al., 2007; Raman et al., 2005; Takayama et al., 2005). Acyl-AMP ligase (Portevin et al., 2005; Trivedi

et al., 2004), FadD32 (Leger et al., 2009; Portevin et al., 2005) and the AccD4-containing acyl-coenzyme A (CoA) carboxylase (Portevin et al., 2005), are also proved to be essential for mycobacterial growth. Moreover, Fab (He and Reynolds, 2002; Musayev et al., 2005; Scarsdale et al., 2001), MabA (Cohen-Gonsaud et al., 2002; Ducasse-Cabanot et al., 2004) and InhA (Dessen et al., 1995; Ducasse-Cabanot et al., 2004; Nunn et al., 2002; Oliveira et al., 2006; Sharma et al., 1998) have indispensible roles in mycolic acid synthesis.

1.3.5 Mannolipids on Mycobacterial cell wall

Exposed on the cell wall surface and interspersed somehow in the mAGP there are cell-wall proteins, phosphatidylinositol mannosides (PIMs), the phthiocerol containing lipids, lipomannan (LM), and lipoarabinomannan (LAM). In which, PIM, LM and LAM have potent immunomodulatory properties (Crellin et al., 2008) by acting as ligands for host cell receptors (Chatterjee and Khoo, 1998; Nigou et al., 2004). Following section will be detailing PIM, LM and LAM with their structure, biosynthesis and physiological role as modulator of innate immunity.

1.3.5.1 Structure of PIM, LM and LAM

Mycobacteria contain a family of lipoglycans whose archetypes are phosphatidyl-myo-inositol mannosides (PIM), lipomannan (LM), and lipoarabinomannan (LAM). The structures of these molecules were established over 30 years ago (Brennan and Nikaido, 1995). LAM are amphipathic molecules presenting a tripartite structure (Fig.1.6) including a mannosyl-phosphatidyl-*myo*-inositol (MPI) anchor (Fig.1.6), a polysaccharide backbone composed of D-mannan and D-arabinan, and finally caps (Chatterjee and Khoo, 1998; Gilleron et al., 2001; Nigou et al., 2003).

MPI anchor structure is heterogeneous, with variations in the number and location of fatty acid residues. MPI anchor of LAM contains four potential sites of acylation: positions 1 and 2 of the glycerol unit, position 6 of the Man*p* unit linked at O-2 of the myo-inositol, and

position 3 of the myo-inositol. O-6 of myo-inositol can be glycosylated by one or five Man*p* units, yielding PIM2 and PIM6. LMs correspond to polymannosylated PIMs and are built from a conserved (α-1→6)- Man*p* backbone in which some units are substituted, generally at O-2, by single α-Man*p* units. LAMs correspond to LMs with an attached D-arabinan domain (Fig.1.6). In some mycobacterial species, the non reducing termini of the arabinosyl side-chains can be modified by a cap motive consisting of either oligomannosyl or phospho-myo-inositol units. LM (Quesniaux et al., 2004; Vignal et al., 2003) and phospho-myoinositol-capped LAM (PILAM) (Means et al., 1999; Underhill et al., 1999) have been described as strong TLR2 agonists, whereas PIMs (Gilleron et al., 2006; Gilleron et al., 2003; Jones et al., 2001) were found to be weak agonists. Lipoglycans identified in other genera such as *Rhodococcus, Corynebacterium, Tsukamurella, Turicella,* and *Saccharothrix* also consisted of a MPI anchor glycosylated by a (α1→6)-Man*p* backbone. However, in most cases, they are simpler in structure. Most particularly, the arabinan moiety of the molecule can be reduced to single arabinosyl susbtituents (Garton et al., 2002; Gibson et al., 2004; Gibson et al., 2003; Gibson et al., 2005; Gilleron et al., 2005). These different lipoglycan variants, either LAM or LM, show variable TLR2-dependent proinflammatory activities (Gibson et al., 2004; Gibson et al., 2005). Thus far the molecular bases underlying the ability of lipoglycans to induce signaling via TLR2 are poorly understood. We have recently shown, using *M. bovis* bacillus Calmette-Gue´rin (BCG) LM as a model, that the MPI anchor must be at least triacylated (Gilleron et al., 2006).

The polysaccharide backbone is composed of two homopolysaccharides, D-mannan and D-arabinan. The structure of this backbone is highly conserved among different mycobacterial species. The mannan core is composed of approximately 30–35 Man*p* residues. These residues form a linear *α* (1→6)-Man*p* backbone with various single *α* (1→2)-Man*p* side chains. The arabinan domain comprises approximately 60 arabinofuranose

residues (Ara*f*) linked in a linear $\alpha(1\rightarrow5)$- Ara*f* fashion, with branching occurring via the 3-position of some residues. These lateral branches occur in two arrangements: a linear tetra-arabinofuranoside [Ara*f*-($\beta1\rightarrow2$)-Ara*f*-($\alpha1\rightarrow5$)-Ara*f*-($\alpha1\rightarrow5$)-Ara*f*-($\alpha1\rightarrow$)] and a bi-antennary hexa-arabinofuranoside [Ara*f*-($\beta1\rightarrow2$)-Ara*f*-($\alpha1\rightarrow$]$2\rightarrow3$and$\rightarrow5$)-Ara*f* -($\alpha1\rightarrow5$)-Ara*f* -($\alpha1\rightarrow$)].

The terminal residues of the arabinan domains are decorated with various 'caps'. Two capping motifs have been identified for LAM. Mannooligosaccharides cap the LAM of slow-growing mycobacteria, such as *M. tuberculosis* which have now been termed ManLAM (Chatterjee et al., 1993). Phosphoinositide units cap LAM from fast growing mycobacteria, such as *M. smegmatis* (Gilleron et al., 1997; Khoo et al., 1995). The LAM of *M. chelonae* has been shown to be devoid of any caps and has been termed AraLAM (Guerardel et al., 2002). ManLAM and PILAM (phospho-*myo*-inositol-capped LAM) exhibit a broad spectrum of immunomodulatory activities. The main immune response activities of ManLAM derive from its ability to inhibit the activation of macrophages and to inhibit the production of the Th1 pro-inflammatory cytokines IL-12 and TNFα. *M. tuberculosis* ManLAM is also able to induce apoptosis in macrophages (Dao et al., 2004; Nigou et al., 2001).

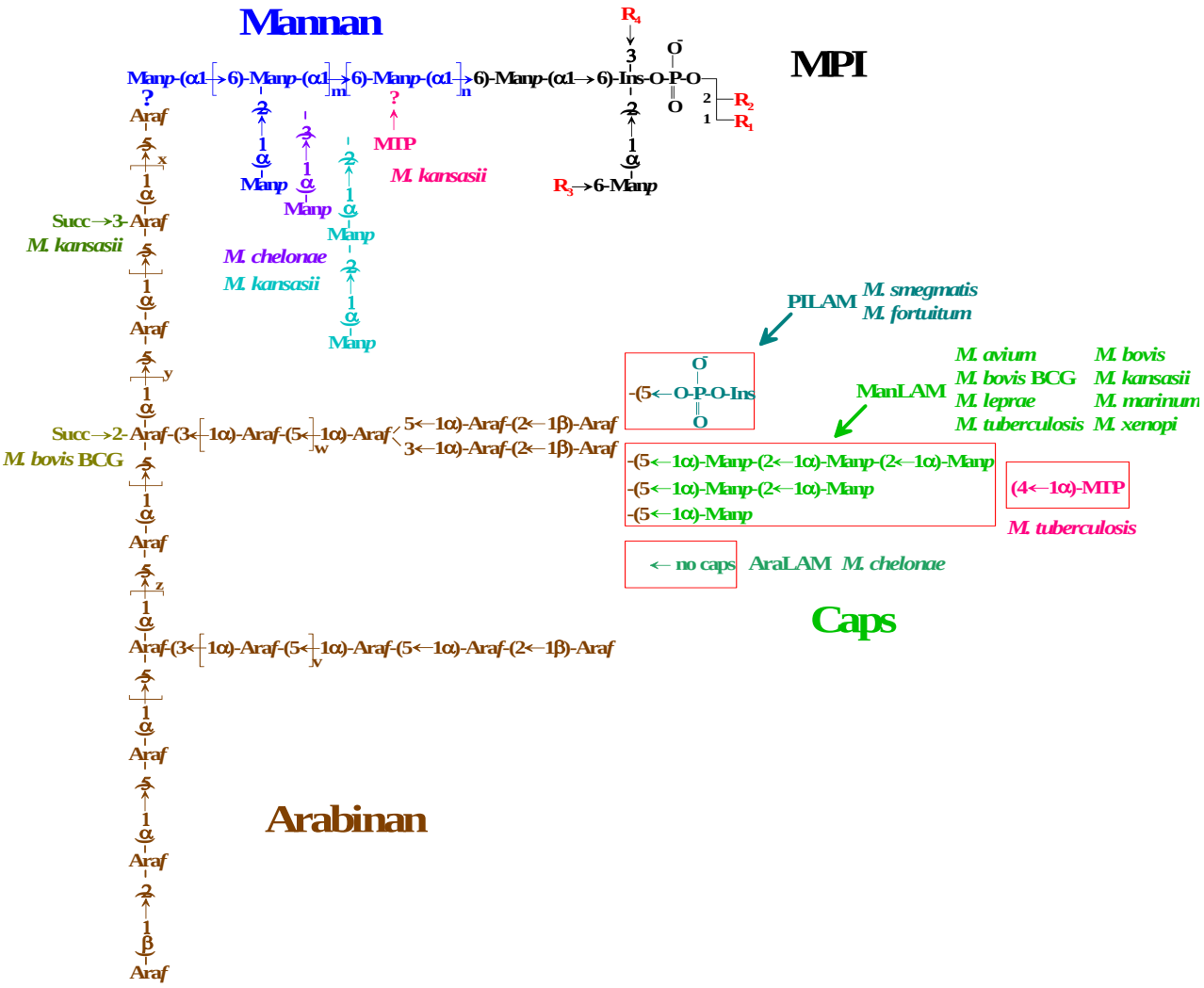


Figure 1.6 Structural model of LM and LAM

Ara*f*, arabinofuranose; Ins, *myo*-inositol; Man*p*, mannopyranose; MPI, mannosyl-phosphatidyl-*myo*-inositol; MTP, 5-deoxy-5-methylthio-xylofuranose ; R_n, acyl group; Succ, succinyl group.

1.3.5.2 Biosynthesis of PIM, LM and LAM

Recent studies have elucidated a number of the key steps in LAM biosynthesis, in which PIM and LM form the intermediate products (Fig.1.7); however, our knowledge of its complete synthesis is still somewhat fragmented. The biosynthetic scheme for the biosynthesis of this membrane molecule has been proposed as PI→PIM (phosphatidyl-*myo*inositol mannoside)→LM→LAM. Recent studies have led to the identification of several key *α*-

mannosyltransferease, PimA, PimB (and PimB') and PimC. Kordulakova et al., (2002) identified PimA which catalyses the transfer of a mannose residue from GDP-Man to the 2-position of PI to form PIM_1. PIM_1 is then further glycosylated by PimB (Rv0557), which may occur before, or after acylation of PIM_1 by Rv2611c (Kordulakova et al., 2003) and results in the formation of Ac1PIM2 (mono-acylated PIM_2) (Schaeffer et al., 1999). However, recently, this second mannosylation step in the biosynthesis of $Ac1PIM_2$ has been shown to be catalysed by PimB' (Rv2188c and NCgl2106) (Lea-Smith et al., 2008; Mishra et al., 2007), whereas PimB now termed MgtA is involved in the synthesis a novel mannosylated glycolipid, 1,2-di-*O*-$C_{16}/C_{18:1}$-(α-D-mannopyranosyl)-(1→4)-(α-D-glucopyranosyluronic aci d)-(1→3)-glycerol ($ManGlcAGroAc_2$) and a hypermannosylated variant (Tatituri et al., 2007). Previous studies have shown that RvD2-ORF1 from *M. tuberculosis* CDC1551, designated as PimC, catalysed further mannosylation of Ac_1PIM_2, resulting in Ac_1PIM_3 (Kremer et al., 2002). Further elaboration of this pathway has revealed the presence of a redundant pathway from mono-acylated PIM_4, by the addition of Man*p* residues from the alkali-stable sugar donor C50/C35- PPM (polyprenol monophosphomannose) (Gurcha et al., 2002) and PimE to form α(1→2)-branched 'higher' PIMs in the form of mono-acylated PIM_5 (Morita et al., 2004; Morita et al., 2006). Subsequently, mono-acylated PIM_5 is then mannosylated further to mono-acylated-PIM_6. An additional parallel biosynthetic pathway extends monoacylated PIM_4 to linear LM via a PPM-dependent α(1→6)-mannosyltransferase MptA and subsequently to a branched-LM via an α(1→2)-mannosyltransferase (Rv2181) (Kaur et al., 2006; Mishra et al., 2007). The transition of LM to LAM has recently been shown to occur exclusively by EmbC. An *M. smegmatis* embC mutant was found to be devoid of LAM (Zhang et al., 2003). Furthermore, AG biosynthesis was unaffected by the mutation, indicating the essentiality of EmbC towards LAM biosynthesis. Recently a novel α-mannosyltransferase, MT1671 (and Rv3265c), has been

shown to add terminal Man*p* residues to the mature LAM in *M. tuberculosis* to form ManLAM (Appelmelk et al., 2008; Dinadayala et al., 2006).

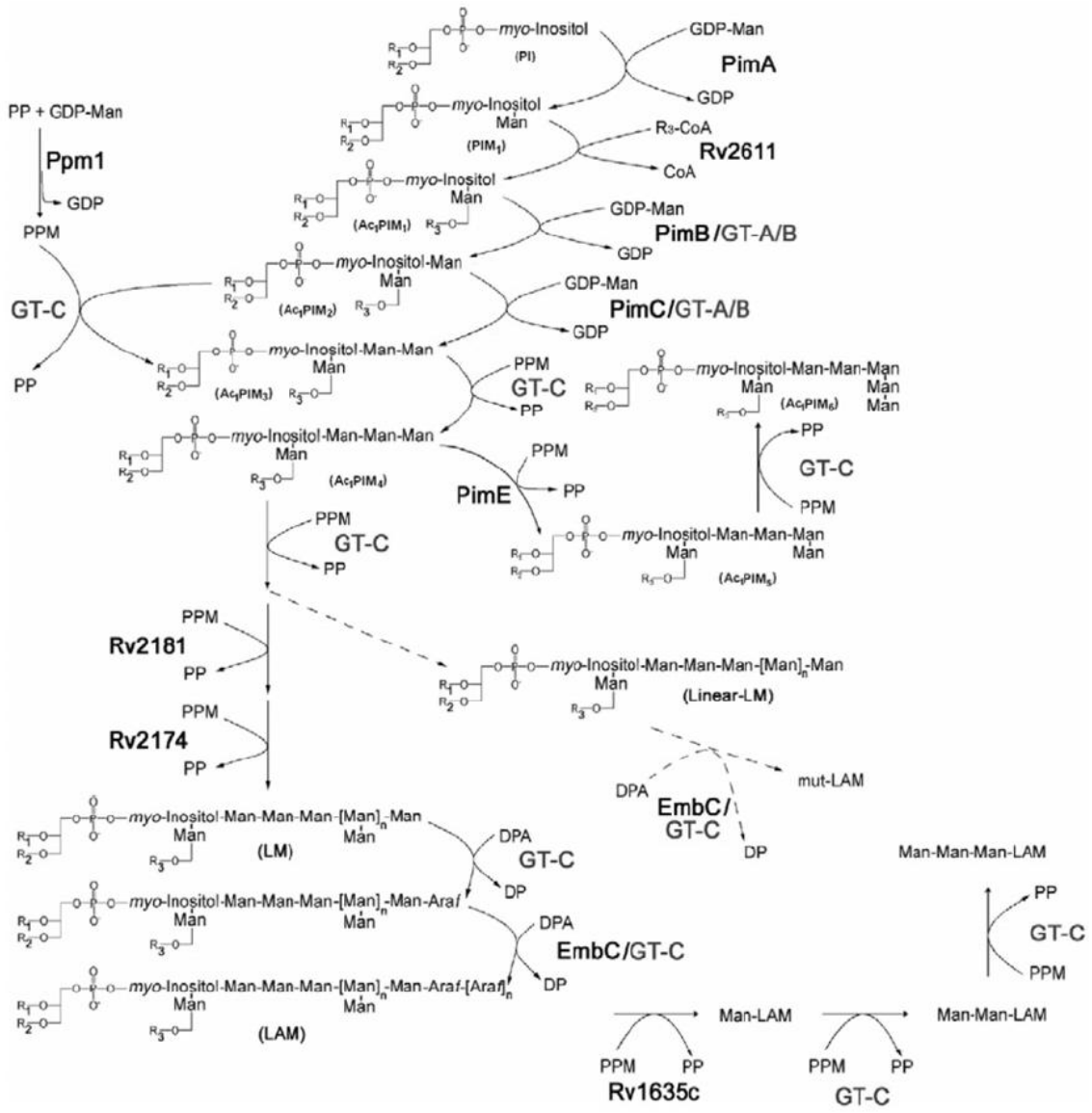


Fig. 1.7 Schematic representation of the lipoarabinomannan (LAM) biosynthesis pathway in mycobacteria GT-C, glycosyltransferase family C enzyme; PI, phosphatidylinositol; PP, polyprenyl phosphate (Bhowruth et al., 2008).

1.3.5.3 Modulation of the immune response by PIM/LM/LAM

PIM, LM and LAM activate innate immunity by inducing events leads to inflammation and inhibit various host cellular response enable the survival of pathogen intracellularly.

1.3.5.3.1 Inflammatory-inducing activity

Studies based on checking the inflammatory lipoglycans given initial evidence PILAM, but not ManLAM or AraLAM, significantly induces IL-12 expression and apoptosis (Dao et al., 2004). PILAM, but neither ManLAM nor AraLAM, consistently induces the secretion of the proinflammatory cytokines IL-8 and TNF-α (Guerardel et al., 2002; Vignal et al., 2003). These results support the hypothesis that mannose caps do not inhibit the proinflammatory activities of LAM, but rather that the phosphoinositol caps of PILAM are potent proinflammatory constituents.

Characterization of LAM from the facultative pathogenic mycobacteria *M. kansasii* and *M. chelonae* provided information that neither ManLAM from *M. kansasii* nor AraLAM from *M. chelonae* had any activity, the addition of LM from either species induced potent secretion of IL-8 and TNF-a (Vignal et al., 2003) and significant expression of IL-12 and apoptosis (Dao et al., 2004). LM purified from *M. smegmatis*, *M. tuberculosis* and *M. bovis* BCG also induced proinflammatory responses (Dao et al., 2004). Moreover, LM but not the corresponding LAM induced macrophage activation characterized by cell surface expression of CD40 and CD86, as well as NO secretion (Quesniaux et al., 2004). Gradual chemical reduction in the amount of arabinan domain of the *M. kansasii* ManLAM correlated with increased proinflammatory cytokine expression of the truncated LAM molecules, thus revealing the proinflammatory activity of the LM core (Vignal et al., 2003). In addition one LAM termed ‘simpler’ LAM molecule, which resembles LM, having a single terminal a-D-Ara*f* residues capping was found to induce an early macrophage proinflammatory response (Garton et al., 2002). LAM from *Tsukamurella paurometabola* was recently demonstrated to

induce the secretion of TNF-α in murine and human macrophages (Gibson et al., 2004). Interestingly, this activity was dramatically increased after removal of the arabinan chains by mild acidic treatment, which exposed the LM core. These results supporting the notion that an extended arabinan domain may hinder the LM-dependent inflammatory response.

Several reports demonstrated that highly purified PIMs like PIM2 and PIM6 isolated from *M. tuberculosis* are able to induce TNF-α and IL-8 secretion by human and murine macrophages (Barnes et al., 1992; Gilleron et al., 2003; Jones et al., 2001; Zhang et al., 1995).This is supported by two studies (Barnes et al., 1992; Zhang et al., 1995) reporting the strongest activity of PIMs on cytokine secretion used either primary human peripheral blood mononuclear cells or primary human alveolar macrophages respectively.

1.3.5.3.2 Inhibition of cellular responses

The first demonstration of the capacity of LAM to inhibit a host response involved in defense against bacterial infection was conducted by Sibley et al., (1988), who reported the inhibition of the interferon (IFN)-γ response of macrophages by ManLAM. Subsequently, live *M. tuberculosis* infection was shown to inhibit IFN-γ signaling, as demonstrated by the reduction in the IFN-γ-mediated cell surface expression of MHC class II and receptors for the Fc portion of IgG after infection of macrophages with *M. tuberculosis* (Hmama et al., 1998; Hussain et al., 1999; Pai et al., 2003; Ting et al., 1999). Furthermore, ManLAM from *M. tuberculosis* inhibited the *M. tuberculosis* infection induced apoptosis of macrophages (Rojas et al., 1997; Rojas et al., 2000) and the secretion of IL-12 induced by lipopolysaccharide (LPS) in DCs (Nigou et al., 2001) and macrophages (Knutson et al., 1998). The activity of ManLAM reflects the capacity of whole *M. tuberculosis* bacteria to inhibit infection-induced apoptosis (Keane et al., 2000) and Il-12 secretion of macrophages (Giacomini et al., 2001; Hickman et al., 2002; Li et al., 2002). Contradictory results show that, in DCs, *M.*

tuberculosis seems either to induce secretion of IL-12 (Giacomini et al., 2001) or to inhibit IL-12 production (Demangel et al., 2002; Johansson et al., 2001).

1.3.5.4 Isolation and purification of mannolipids from mycobacterial cell wall

The mannoconjugates can be classified in two groups, lipoglycans and polysaccharides. The lipoglycans are lipoarabinomannan (LAM), lipomannan (LM) and phosphatidyl inositol mannosides (PIMs). Polysaccharides are restricted to arabinomannan (AM) and mannan. D-arabinomannan exist in two forms, lipid-free, namely AM and acylated, namely LAM (Nigou et al., 1997; Sugden et al., 1987). Likewise, mannan also exists in an acylated form, the LM.

Initially, mannoconjugates were extracted after vigorous alkali-treatments of the cells prior solvent extraction (Azuma et al., 1968; Misaki et al., 1977) called delipidation. Consequently LAM and LM were deacylated leading to the formation of AM and mannan. In order to isolate the lipoglycans in their native form, the cells are extracted by solvents without any alkali treatment. The LAM purification from complex mixture of structurally related molecules such as PIMs, LM and AM is achieved by their intrinsic molecular heterogeneity. This molecular heterogeneity was first illustrated by their behavior in SDS-PAGE, showing a broad band around 30 kDa (Hunter et al., 1986) and more precisely defined by matrix-assisted laser desorption/ionization mass spectrometry analysis. The average molecular weight of LAM from *M. bovis* BCG was determined at 17 kDa and the heterogeneity estimated at 6 kDa (Venisse et al., 1993).

From delipidated mycobacteria LAM has been isolated following two kinds of extraction procedures (Fig. 1.8). The main difference between these two methods is that LAM arises from ethanol/water extracts of either disrupted (Khoo et al., 1995) or non-disrupted (Hunter et al., 1986; Venisse et al., 1993) delipidated mycobacteria. Recently, a new method, resulting in a combination of the two previously described procedures, was developed (Delmas et al., 1997). According to the extraction mode, two types of LAMs, the

parietal and the cellular LAMs, were identified (Delmas et al., 1997). The parietal LAM containing fraction results from ethanol/water extraction of delipidated mycobacteria. Afterwards, the resulting cells are disrupted, and then extracted again by ethanol/water, to give the fraction containing the cellular LAM. The routine separation steps for LAM purification from ethanol/water extracts employed in both procedures are similar, i.e., phenol/water biphasic wash to remove proteins, enzymatic degradation of the contaminants (glucan, nucleic acids, proteins), gel permeation to separate LAM and LM.

LAM, LM and AM have been tentatively separated by gel filtration and/or anion exchange chromatography. However, the aggregate formation between AM and, LAM and LM hinders their separation, even using detergents. In order to eliminate AM, prior gel filtration, the triton X-114 phase separation technique was successfully applied (Nigou et al., 1997). This non-ionic detergent favours the dissociation of amphipathic and hydrophilic molecules, and forms, at temperatures beyond the cloud point, a hydrophilic detergent-depleted phase and an amphipathic detergent-rich phase. AM is found in the former phase and LAM and LM in the latter one. A similar approach, allowing the extraction of lipoglycans by Triton X-114, was developed at the same time by another group in New-Zealand (Severn et al., 1997). Another alternative, to separate lipoglycans and glycans, is the use of hydrophobic interaction chromatography on octyl-Sepharose. This technique allows the retention of lipoglycans and the elution of hydrophilic compounds (Leopold and Fischer, 1993).

The purification protocol described in the figure 1.8, devoted to the obtention of the so-called parietal and cellular LAMs, was applied to *M. bovis* BCG cells (Nigou et al., 1997). Parietal LAM represents only 8 % of the total LAMs. The amount of parietal and cellular LMs counts as the half of the amount of the corresponding LAMs. So, parietal lipoglycans represent a minor quantity of the total lipoglycan fraction. Likewise, the parietal

polysaccharides AMs are found in lower abundance compared to the cellular one's (half), but polysaccharides relatively to lipoglycans, are more abundant in the parietal fraction than in the cellular one. The epithets, parietal and cellular, used to characterize the two fractions of LAMs do not necessarily reflect a difference in the LAM localization in the mycobacterial envelope. Indeed, the localization of LAMs in the mycobacterial envelope is not yet established. LAM is not covalently attached since it can be extracted by solvents. At present, two models are proposed. Rastogi et al., (1991), hypothesized that LAM could be inserted, through their phosphatidyl-myo-inositol unit, in an outer leaflet composed by different lipids. McNeil and Brennan, (1991) proposed that LAM could be anchored, still by their phosphatidyl-myo-inositol unit, in the plasma membrane. In a recent review (Chatterjee and Khoo, 1998) privileged the second hypothesis relying on the relatively strong conditions required for LAM release from mycobacteria. This supposition has to be moderated since parietal LAM, contrarily to the cellular one, is obtained by ethanol/water extract without cell disruption. It can therefore be speculated that the major amount of LAM, i.e. the cellular one, could be inserted into the plasma membrane, while a minor amount, i.e. the parietal one, could be localized near the surface. It is noteworthy that the two previously proposed models concerning LAM localization are speculations since they are based on any convincing experimental support. Indeed, antibodies against LAM cross-react with AM precluding LAM identification, and so AM and LAM respective localization by immunocytochemistry experiments.

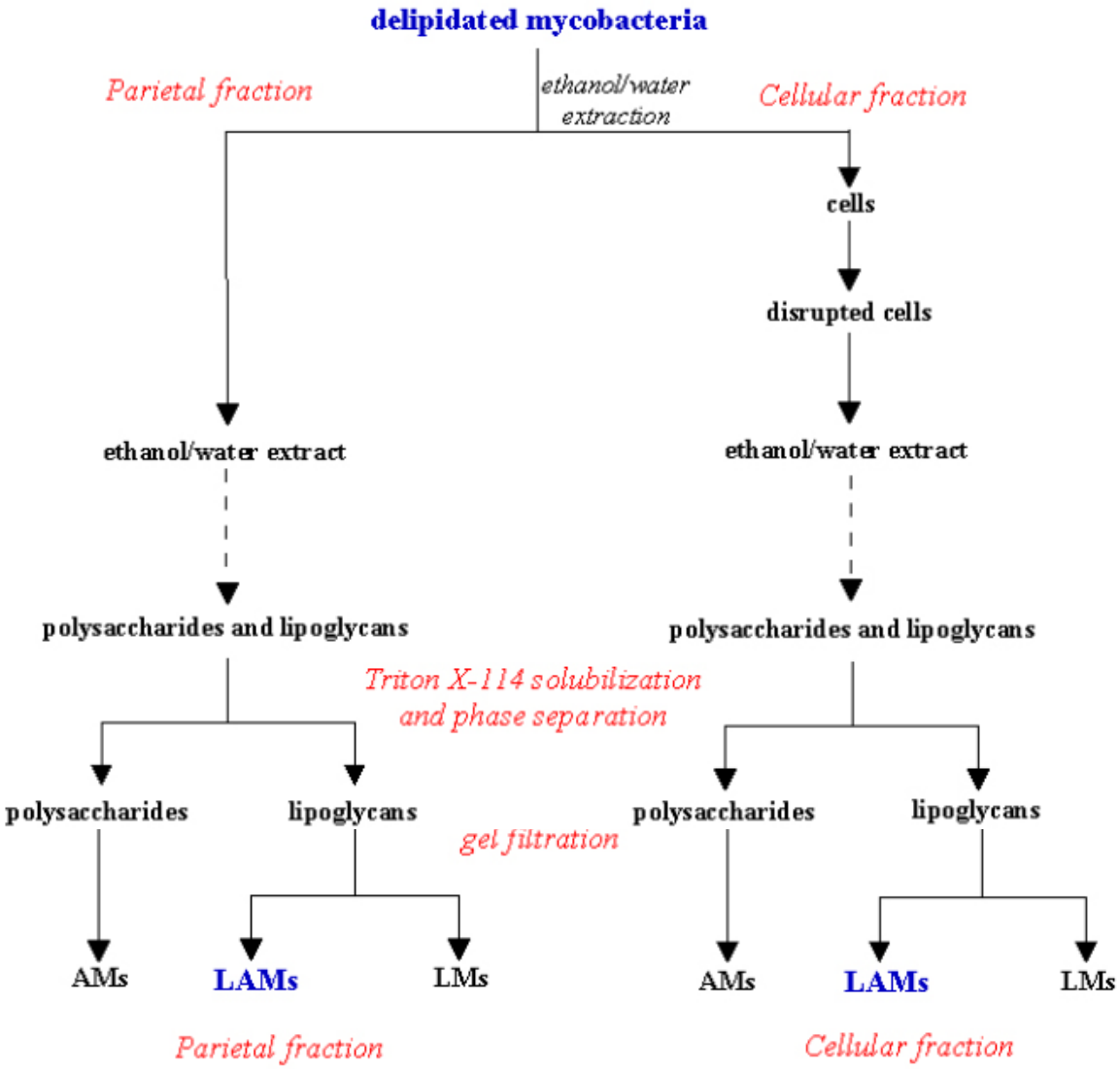


Fig. 1.8 Purification scheme for LAM (Vercellone et al., 1998)

1.3.6 Mannose metabolism in Mycobacterium

The complete genome sequence of *M. tuberculosis* H37Rv was determined by Sanger Centre and the Institute Pasteur in 1998 (Camus et al., 2002). The genome comprises 4411529 bp and has an average G + C content of 65.6 % although some areas with an exceptionally high G + C content (80 %) were detected and found to correspond to a novel gene family. A

significant portion of the genome is devoted to families, genes involved in lipid metabolism. An overview of the broad classification of genes of *M. tuberculosis* is shown in the table 1.1

Table 1.1 Broad classification of *M. tuberculosis* genes (Cole, 1999)

Class	Function	Gene Number	%Total	Total length (kb)%	Total coding
1	Lipid metabolism	225	5.7	372	9.3
2	Information pathways	207	5.2	243	6.1
3	Cell wall and cell process	517	13.0	620	15.5
4	Stable RNAs	50	1.3	10	15.5
5	Insertion sequences and phages	137	3.4	100	2.5
6	PE and PPE Protein	167	4.2	283	7.1
7	Intermediary metabolism and respiration	877	22.0	985	24.6
8	Proteins of unknown function	607	15.3	396	9.9
9	Regulatory proteins	188	4.7	162	4.0
10	Conserved hypothetical proteins	911	22.9	739	18.4
11	Virulence, detoxification, adaptation	91	2.3	95	2.4
12	Non-coding sequences			434	

Biosynthesis of lipoglycans starts from GDP-mannose has been shown to be the only donor species for PIM_1 - PIM_3 biosynthesis via the mannosyltransferases *pimA* (Kordulakova et al., 2002), *pimB* (Schaeffer et al., 1999) and *pimC* (Kremer et al., 2002). *pimF* has been identified as the mannosyltransferase responsible for the biosynthesis of a higher order PIM; however, the nature of the mannose donor species used in this reaction remains unknown (Alexander et al., 2004). Other PIMs, LM and the terminal mannose capping motifs of LAM

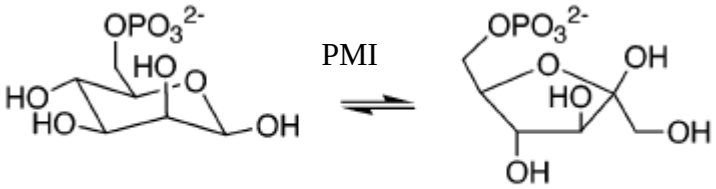
are synthesized through as yet unidentified mannosyltransferases, with either GDP-mannose or its derivative polyprenyl-phosphate mannose as the primary donor species.

The GDP-mannose used in these reactions can be produced in two distinct ways (Fig. 1.1), either through the conversion of exogenously acquired mannose to mannose-6-phosphate (M6P) by a hexokinase, or from the glycolytic pathway by converting fructose-6-phosphate (F-6-P) to M6P by a phosphomannose isomerase (PMI). M6P is then converted to mannose-1-phosphate (M1P) by a phosphomannomutase (PMM) and then to GDP-mannose by a GDP-mannose pyrophosphorylase (GDPMP). In the case of mycobacteria, PMI activity has been confirmed from *manA* (Rv3255c), an enzyme that has been shown to be essential for mycobacterial growth *in vitro* (Patterson et al., 2003), while *manC* (Rv3264c) has been defined as a GDPMP (Ma et al., 2001; Ning and Elbein, 1999). PMMs have been shown to be centrally involved in biosynthesis of the alginate capsule of *Pseudomonas aeruginosa* as well as the biosynthesis of lipopolysaccharide (LPS) from *P. Aeruginosa* and *E. coli*, among others (Goldberg et al., 1993; Marolda and Valvano, 1993; Ye et al., 1994; Zielinski et al., 1991). In addition, PMMs are presumed to play an important role in mannose-donor biosynthesis in mycobacteria. The PMMs from many Gram-negative bacteria also contain phosphoglucomutase (PGM) activity, accounting for their essential role in biosynthesis of the core polysaccharide of LPS (Coyne et al., 1994). PGM activity in mycobacteria may be required for nucleotide sugar synthesis for glycopeptidolipid (GPL) biosynthesis. Based on this information, we have hypothesized that *M. tuberculosis* enzymes PMI, PMM and GDPMP plays important role in the biosynthesis of mannosylated glycoconjugates that are involved in the host/pathogen interaction. Details of the enzymes involved in the synthesis of mannolipids are described below.

1.3.6.1 PHOSPHOMANNOSE ISOMERASE

1.3.6.1.1 Enzyme action

Phosphomannose isomerase (PMI) is an essential enzyme in the early steps of the protein glycosylation pathway in both prokaryotes and eukaryotes. It catalyzes the reversible interconversion (Fig. 1.9 a and b) of F-6-P and M-6-P (Cleasby et al., 1996). This reaction links M-6-P into the mannose metabolism pathway resulting in the generation of GDP-mannose (GDP-Man), an important precursor of many nucleotide sugars such as GDP-rhamnose and GDP-fucose, and for mannosylation of various bacterial structural components such as lipopolysaccharides and glycoproteins (Rocchetta et al., 1998; Wu et al., 2002). In mycobacteria PMI is essential as it is catalyzing the first step in the synthesis of GDP-mannose, which is an activated form of mannose, supplies mannose for the synthesis of mannolipids through various mannosyl transferases, which helps mycobacteria to survive and persists inside alveolar macrophages (Patterson et al., 2003). It was first identified that in yeast and shown to be a 50 kDa metalloprotein containing an essential zinc ion (Gracy and Noltmann, 1968).



M6P F6P

Fig. 1.9 a Reversible interconversion of F6P and M6P catalyzed by phosphomannose isomerases. Only the β-pyranose anomers are reported to be substrates of the enzymes

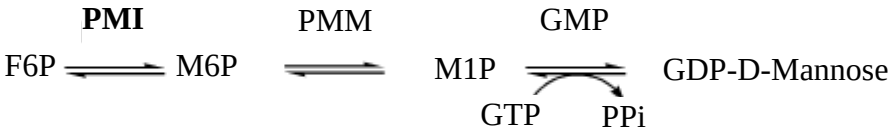


Fig. 1.9 b GDP-D-mannose biosynthesis pathway from F-6-P. PMI activity is indicated in bold letters.

1.3.6.1.2 Enzyme occurrence

PMIs are considered to be potential therapeutic targets because of their role in survival and pathogenesis in several microbes, and in many cases, there are significant differences in amino acid sequence between the PMIs from pathogens and humans. Indeed, unless the growth medium is supplemented with D-mannose, PMI has been found to be essential for the survival of cells from *Saccharomyces cerevisiae* (Payton et al., 1991), *Candida albicans* (Smith et al., 1995), and *M. smegmatis* (Patterson et al., 2003)and important for the virulence of the protozoan parasite *Leishmania mexicana* (Garami and Ilg, 2001). PMI is also needed for the development of mucoid strains of *P. aeruginosa* that cause recurrent and life-threatening lung infections in cystic fibrosis (CF) patients. The serious lung infections are a major cause of the decreased life expectancy of CF patients. PMI has been found to be essential in the production of the exopolysaccharide alginate (Shinabarger et al., 1991), which coats the bacteria and protects them from antibiotics and the host's immune system. In most human tissues, the bulk of the central metabolite M-6-P that is utilized for glycoprotein synthesis is likely not derived from G-6-P but originates from efficient uptake of D-mannose in serum through a specific mannose transporter, followed by phosphorylation by hexokinase (Panneerselvam et al., 1997). In humans, a deficiency of PMI activity leads to carbohydrate-deficient glycoprotein syndrome type 1b, a severe metabolic disorder with hepatic-intestinal

presentation (de Koning et al., 1998; Jaeken et al., 1998), that is however today successfully treated by oral D-mannose (Hendriksz et al., 2001).

1.3.6.1.3 Enzyme classification

From sequence alignments, physicochemical and kinetic characterization, PMIs has been classified into (Proudfoot et al., 1994) Type I PMIs, which include proteins from *Aspergillus nidulans, C. albicans, E. coli, Homo sapiens, Salmonella enterica, S. cerevisiae* (Proudfoot et al., 1994; Wells et al., 1994), *Caenorhabditis elegans*, *Streptococcus mutans* (Jensen and Reeves, 1998), and *Cryptococcus neoformans* (Wills et al., 2001), are homologous monofunctional enzymes catalyzing the single isomerization reaction. The type I PMI isolated from *S. cerevisiae* in 1968 (Gracy and Noltmann, 1968) has been shown to be a zinc dependent metalloenzyme, with one metal atom per molecule of the 45 kDa monomer (Gracy and Noltmann, 1968). Type II PMIs are bifunctional enzymes possessing both PMI and GMP activities in separate catalytic domains. In some species the PMI and GMP domains are found as separate proteins (Jensen and Reeves, 1998). Type II PMIs reported so far are found only in some bacteria, including *P. Aeruginosa* (Shinabarger et al., 1991), *Xanthomas campestris* (Papoutsopoulou and Kyriakidis, 1997), *Acinetobacter calcoaceticus* (Jensen and Reeves, 1998), *Rhodospirillum rubrum* (Ideguchi et al., 1993), *Acetobacter xylinum* (Griffin et al., 1997), *Salmonella typhimurium* (Collins and Hackett, 1991; Jiang et al., 1991), and *Helicobacter pylori* (Wu et al., 2002). PMI activity in type II enzymes uses Zn^{2+} as the metal cofactor but also can use other divalent cations, Co^{2+}, Mg^{2+}, Mn^{2+}, Ca^{2+}, or Ni^{2+}, depending on the enzyme source. So far, only one type III PMI has been reported, from *Rhizobium meliloti* (Schmidt et al., 1992).

1.3.6.1.4 Enzyme structure

From all the three classes of PMI, the structure of Type 1 PMI from *C. albicans* (Cleasby et al., 1996) and *S. typhimurium* (Gowda et al., 2008) are elucidated by X-ray crystallography to

date. The type I PMI isolated from *S. cerevisiae* in 1968 (Gracy and Noltmann, 1968) has been shown to be a zinc dependent metalloenzyme, with one metal atom per molecule of the 45 kDa monomer. A pocket on the surface that is very likely to be the active site and a zinc metal cofactor binding site have been identified; however, the roles of the individual active site amino acids and zinc ion in the catalytic mechanism are still not known. Type II PMIs are bifunctional enzymes possessing both PMI and GMP activities in separate catalytic domains. In some species the PMI and GMP domains are found as separate proteins (Jensen and Reeves, 1998). There is no sequence identity is found between type I and type II enzymes, except for a very small conserved amino acid sequence motif, which makes up part of the active site in the crystal structure (Jensen and Reeves, 1998)**.** Previous studies (Ritter and Schmitt, 1973; Vanheyningen et al., 1975) indicated that, in humans, phosphomannose isomerase is encoded by a single genetic locus with no evidence for different isoforms.

1.3.6.2 PHOSPHOMANNOMUTASE

1.3.6.2.1 Enzyme action

The enzyme phosphomannomutase is a member of the α-D-phosphohexomutase enzyme superfamily (Shackelford et al., 2004). It will catalyse the reversible conversion of M1P to M6P (Regni et al., 2006). It requires a phosphorylated serine on the enzyme 108 residue and the reaction proceed via the formation of biphosphorylated sugar intermediate i.e.,mglucose-1,6 biphosphate. The enzyme can utilize either glucose or mannose based phosphosugar as substrates and entails two phosphoryl transfer reactions. Figure 1.10; demonstrate the reaction catalyzed by phosphomannomutase.

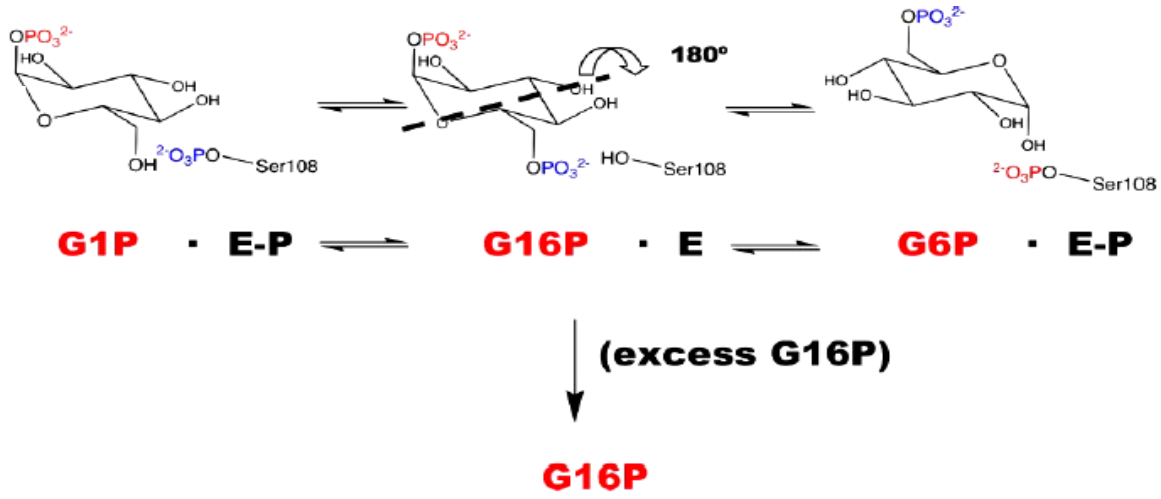


Fig. 1.10 Reaction catalyzed by Phosphomannomutase (Regni et al., 2006).

1.3.6.2.2 Enzyme occurance

Phosphomannomutase enzyme is found in all organisms from bacteria to human. PMM form *P. aeruginosa* is the first enzyme to be structurally characterized in the phosphohexomutase family. The enzyme phosphomannomutase is required for the biosynthesis of two bacterial exopolysaccharides: alginate and lipopolysaccharide (LPS). The key role of the enzyme in the biosynthesis of the virulence factors of *P. aeruginosa* makes it an attractive target for inhibitor design. In plants, PMM activity has been detected in *Amorphophallus konjac*, *Cassia corymbosa*, the red algae *Galdieria sulphuraria*, spinach, maize, *Arabidopsis* and *Cucurbita pepo* (Hancock et al., 2003). PMM from *G. sulphuraria* has both PMM and phosphoglucomutase activities. PMM catalyzed reaction that provides GDP-mannose, which is an essential sugar nucleotide in plants, being vital for the synthesis of D-mannose-containing cell wall polysaccharides (galactomannans and glucomannans), glycolipids and glycoproteins (Conklin et al., 1999). GDP-mannose is also an intermediate for the biosynthesis of the important antioxidant L-ascorbic acid through the Smirnoff-Wheeler pathway in plant cells (Smirnoff et al., 2001; Wheeler et al., 1998). Human α-PMM catalyses the conversion of D-Mannose-6-Phosphate to α-D-Mannose-1-Phosphate, which is required

for GDP-Mannose and dolichol-phosphate mannose biosynthesis. Two isoforms are found; PMM2 is expressed in all tissues, where as the expression of PMM1 is restricted to the brain and lungs (Pirard et al., 1999). α-PMM is distinguished from the bacterial β-phosphogluco mutase (β-PGM) in its C(1) anomer specificity as well as in the position and fold of the cap domain which serves as a lid over the conserved catalytic core domain. PMM defeiciency in humans leads to Carbohydrate-deficient glycoprotein (CDG) syndromes hence it is used in the treatment of CDG in humans (Matthijs et al., 1998).

1.3.6.2.3 Enzyme structure

Phosphomannomutase is a heart shaped protein with 463 amino acid residues comprising four domains three of which have a similar three dimensional fold. The residues that are present in all the four domains contribute to the large active site cleft in the centre of the molecule (Regni et al., 2002). The crystal structure of PMM/PGM from *P. aeruginosa* was determined by MAD phasing using crystals of the selenomethionine-substituted protein and has been refined to 2.2 A°. The crystal structure of PMM shows that the active site is present in the center of the protein, in a deep cleft formed by atoms in 67 residues distributed across all four domains. The structural studies of these proteins had shown that the binding of the substrate causes a rotation of the C-terminal domain, changing the active site from an open cleft in the apoenzyme into a deep, solvent inaccessible pocket where phosphoryl transfer takes place (Schramm et al., 2008).

1.3.6.3 GDP-MANNOSE PYROPHOSPHORYLASE

1.3.6.3.1 Enzyme action

GDP-mannose pyrophosphorylase is an important enzyme in eukaryotic and prokaryotic cells, doing the synthesis of GDP-mannose from M-1-P and GTP (Szumilo et al., 1993). Since GDP-Man, the activated form of mannose, is essential for synthesis of both N- and O-linked oligosaccharides as well as of GPI anchors, its formation should be vitally important.

Biosynthesis of GDP-Man, like that of other sugar nucleotides, occurs in the cytoplasm. The final reaction is the transfer of M-1-P to GTP, catalyzed by GDP-mannose pyropho sphorylase (Fig. 1.11). This activated form of mannose is the major mannosyl donor for the synthesis of glycoproteins (Kornfeld and Kornfeld, 1976), glycosylpho sphatidylinositol (GPI) membrane anchors (Ferguson and Williams, 1988), and various bacterial and lower eukaryotic cell wall polymers (Roberts, 1996; Tanner and Lehle, 1987). GDP-mannose plays a key role in the biosynthetic pathway that produces the N-linked oligosaccharides of many membrane and secretory glycoproteins of eukaryotic cells (Chen and Lennarz, 1977). The mannose component of D-P-Man is also derived from GDP-Man.

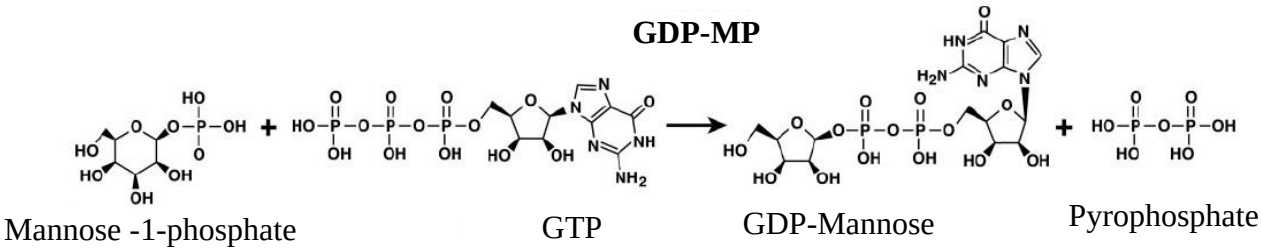


Fig. 1.11 GDP-mannose pyrophosphorylase catalyzed reaction.

1.3.6.3.2 Enzyme occurrence

Ning and Elbein, (1999) were purified GDPMP from *M. smegmatis* and the size was found to be 37 kDa. GMPP was partially purified and characterized from *Athrobacter sp*. (Preiss and Wood, 1964). Since then, the enzyme has been purified from a number of organisms and tissues such as *P. aeruginosa* (Shinabarger et al., 1991), *M. smegmatis* (Ning and Elbein, 1999), mammary gland (Verachte.H et al., 1966) and pig liver (Szumilo et al., 1993). The gene for this enzyme was cloned from *Saccaromyces cerevisiae* and expressed in *E. coli*

(Hashimoto et al., 1997). The GMPPs from *S. cerevisiae* and *M. smegmatis* are quite specific in that they only utilize GDP-Man as a substrate in the reverse direction and are not active with other mannose or glucose nucleotides. On the other hand, the pig liver enzyme is most active with GDP-glucose as the substrate (assayed in the reverse direction), followed by IDP-mannose and then GDP-mannose. On SDS-PAGE, the purified pig liver GMPP shows two protein bands: one corresponding to a molecular mass of 43 kDa and a second of 37 kDa. However, the enzyme from either *S. cerevisiae* or *M. smegmatis* is a single protein, having a molecular mass of 37 kDa (Hashimoto et al., 1997; Ning and Elbein, 1999). In plants, three nucleotide interconversion pathways have been described in which GDP-D-mannose gives rise to other guanosine-containing sugar nucleotides. GDP-L-galactose, GDP-L-fucose and GDP-D-rhamnose are believed to be synthesized using GDP-D-mannose as a precursor (Barber, 1968; Baydoun and Fry, 1988; Reiter, 1998). GDP-L galactose is a further intermediate in the pathway of ascorbate formation and cell wall synthesis, whereas the other GDP-containing sugar nucleotides are exclusively needed for protein glycosylation and cell wall formation.

1.3.6.3.3 Enzyme structure

Pelissier et al., (2010)elucidated the crystal structure of GDP-mannose pyrophosphorylase from (TmGMP) *Thermotoga maritime,* a thermophylic bacterium. TmGMP forms a homo dimer in solution and it is having an approximate molecular weight of 80 kDa. TmGMP monomer is made of two separate domains (N-terminal and C-terminal) and has overall dimensions of ~ 45 Å x ~ 40 Å x ~ 60Å, in which the active center lies in a deep pocket located in the N-terminal domain.

1.3.7 Innate immunity and TLR

The mammalian immune system is comprised of two branches: innate and acquired immunity. The innate immune system is the first line of host defense against pathogens and is

mediated by phagocytes including macrophages and dendritic cells (DCs). Acquired immunity is involved in elimination of pathogens in the late phase of infection as well as the generation of immunological memory. Innate immunity is the immediate response against the entry of pathogen by the interaction between certain broad specific germline-encoded Pattern Recognition Receptors (PRRs), present on the effecter cells and pathogen-associated molecular patterns (PAMPs) on the surface of the pathogen (Janeway, 1989). PAMPs represent a limited number of conserved molecular structures produced by micro-organisms in which they play a vital role for their survival and replication. Another prerequisite for bacterial PAMPs is that they be exclusively derived from the microbe and not present in the multicellular host (Janeway and Medzhitov, 1999). Although now well accepted in innate immunity research, the term PAMP has received criticism, since molecules of microbial origin and not patterns interact with their receptors in a specific way (Beutler, 2003). Furthermore, recognition processes are modulated by co-receptors, thus increasing the efficiency and reliability of PAMPs. During the past decade effort has emphasized defining the specific interactions as to how PAMPs are recognized by their PRRs. Extracellular so-called Toll-like receptors (TLRs) (Beutler, 2004) in addition to intracellularly binding nucleotide-binding and oligomerization domain (Nod) receptors (NLRs) have been identified (Girardin et al., 2002). Following chapters enables as to get some information about innate immunity, their components, innate activation of adaptive immunity, and their interaction with various MAMPs present on different organisms.

1.3.7.1 Innate immunity and their components

Innate immune system is equipped a collection of distinct subsystems, or modules that appeared at different stages of evolution and carry out different functions in host defense. Some of the main modules found in mammals and how these function in innate host defense are described in this section (Table 1.2).

Table 1.2 Modules of innate immunity

Innate host-defence module	Primary sensors (PRR)	Prototypical responses
Mucosal epithelia	TLRs and NOD proteins	Production of antimicrobial peptides Production of mucins
Phagocytes	TLRs, dectins and NOD proteins	Production of antimicrobial peptides Production of IL-1β, IL-6 and TNF
Acute-phase proteins and complement system	Collectins, pentraxins and ficolins	Lysis or opsonization of pathogens Chemotactic attraction of leucocytes
Inflammasomes	NALPs and NAIPs	Production of IL-1 family members Apoptosis of infected host cells
NK cells	ND	Apoptosis of infected host cells
Type-1-IFN induced antiviral proteins	RIG-1, MDA5, DAI and TLRs	Induction of antiviral state Apoptosis of infected host cells
Eosinophils and basophils	ND	Contraction of smooth muscle Production of mucins Peristalsis Production of biogenic amines Production of cytokines
Mast cells	ND	Contraction of smooth muscle Production of mucins Peristalsis Production of biogenic amines Production of cytokines

1.3.7.2 Innate control of adaptive immune responses

In addition to direct activation of innate host-defence mechanisms, some PRRs are coupled to the induction of adaptive immune responses. The basic principle of innate control of adaptive immunity is based on establishing an association between the antigens recognized by

lymphocytes and the microbial products (that is, PAMPs) recognized by PRRs (Janeway, 1989).

For T cells, this association is interpreted by dendritic cells *via* various PRRs. Dendritic cells processed the protein constituents of the pathogen phagocytosed and are presented at the cell surface by MHC class I and/or class II molecules as antigenic peptides. Thus the internalization of pathogen was in response to the triggering of TLRs or other PRRs (Blander and Medzhitov, 2006) for both class I and class II MHCs. PRRs also activate dendritic cells, to produce cytokines which results in T-cell activation and, in the case of T_H ($CD4^+$) cells, differentiation into one of several types of effector T_H cell (Banchereau and Steinman, 1998).

For B cells, the association between an antigen and a PAMP occurs directly through co-engagement of a B-cell receptor and a PRR. TLR ligands such as lipopolysaccharide or flagellin is itself recognized by the B-cell receptor and TLR expressed on B cell surface, activates both innate and adaptive immune recognition, are called T-independent antigens.

Finally, the adaptive immune response results in an antigen-specific activation of the effector mechanisms of the innate immune system. Thus, the effector T_H cells produce the appropriate effector cytokines that activate a specific module of the innate immune system (Fig. 1.12), including activation of macrophages by T_H1 cells, activation of neutrophils by T_H17 cells and activation of eosinophils, mast cells and basophils by T_H2 cells (Reinhardt et al., 2006; Shinkai et al., 2002). Similarly to NK cells, cytotoxic T cells induce apoptosis of infected cells, except that the T-cell response is antigen specific. Likewise, antibodies activate the modules of the innate immune system in a class-dependent (and antigen-dependent) manner. IgG activates complement and opsonizes pathogens to aid their phagocytosis by macrophages and neutrophils, whereas IgE activates mast cells and basophils. Each of the innate effector responses can therefore be activated either directly, by

the appropriate PRRs at the early stages of infection, or indirectly, by T cells and antibodies (in an antigen-specific manner) at the later, effector, stages of the immune response (Fig. 1.12). Furthermore, each effector mechanism of the adaptive immune system might have evolved to activate the appropriate host-defence module of the innate immune system.

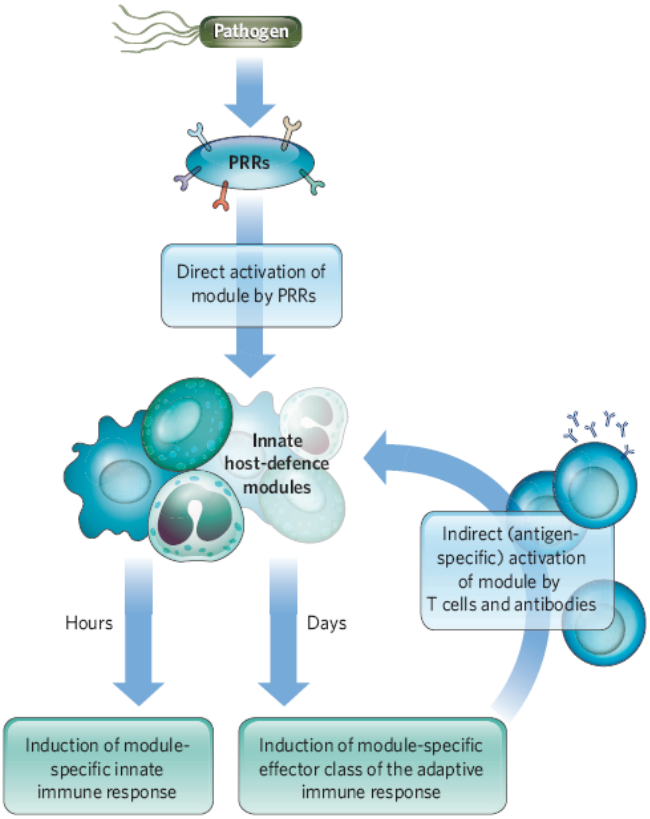


Fig. 1.12 Activation of host-defence mechanisms (Medzhitov, 2007)

1.3.7.3 PRR and MAMPs of Innate immunity

TLRs are the most prominent PRRs recognize various ligands (MAMPs) from a wide variety of microorganisms. MAMPs represent microbial components that are essential for the survival of the microorganism and are therefore difficult for the microorganism to alter. MAMPs interaction with a constitutively active form of TLR could activate the transcription factor nuclear factor-kappa B (NF-κB), leading to the expression of proinflammatory genes encoding interleukin (IL)-1, IL-6, and IL-8 and the upregulation of costimulatory molecules

(Medzhitov, 2007), immediately activates innate immunity and later activates the components of adaptive immunity.

1.3.7.3.1 TLRs as PRRs

TLRs are type I (N-terminal portion is outside, C-terminal portion projected into the cytoplasm) transmembrane proteins of the Interleukin-1 receptor (IL-1R) family that possess an N-terminal leucine-rich repeat (LRR) domain for ligand binding, a single transmembrane domain, and a C-terminal intracellular signaling domain. The TLR C-terminus is homologous to the intracellular domain of the IL-1R and is thus referred to as the Toll/IL-1 receptor (TIR) domain. TLRs are widely expressed in many cell types, including nonhematopoietic epithelial and endothelial cells; although most cell types express only a select subset of these receptors. Hematopoietically derived sentinel cells, such as macrophages, neutrophils, and dendritic cells (DCs), however, express most of the TLRs, with some variation in different subsets, e.g., between conventional DCs and plasmacytoid DCs. Thus far, 13 mammalian TLRs, 10 in humans and 13 in mice, have been identified (Beutler, 2004). TLRs 1-9 are conserved among humans and mice, yet TLR10 is present only in humans and TLR11 is functional only in mice. Following sections explain the structure, mechanism and signaling of TLRs as PRR.

1.3.7.3.1.1 Toll/IL-1R domain

TLRs and IL-1Rs have a conserved region of ~ 200 amino acids in their cytoplasmic tails, which is known as the Toll/IL-1R (TIR) domain (Slack et al., 2000). Within the TIR domain, the regions of homology comprise three conserved boxes, which are crucial for signalling (Fig. 1.13). The crystal structures of the TIR domains of human TLR1 and TLR2 have been obtained and analysed; they contain a central five stranded parallel β-sheet, which is surrounded by five α-helices on each side (Xu et al., 2000).

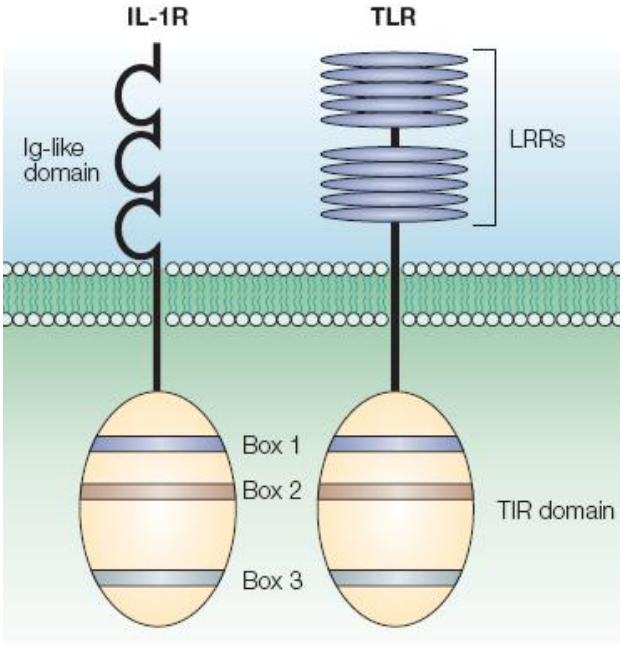


Fig. 1.13 Toll-like receptors domain organization (Akira et al., 2004**)**

1.3.7.3.1.2 Leucine-rich repeats

The extracellular domain of TLRs contains 19–25 tandem copies of the LRR motif. Each repeat consists of 24–29 amino acids and contains the leucine-rich sequence XLXXLXLXX, and another conserved sequence $X\text{Ø}XX\text{Ø}X_4FXXLX$ (Bell et al., 2003) where X denotes any amino acid and Ø a hydrophobic amino acid. The repeats comprise a β-strand and an α-helix connected by loops. The LRR domains of TLRs form a horseshoe structure, and it is thought that the concave surface of the LRR domains is involved directly in the recognition of various pathogens.

1.3.7.3.2 TLR2 -a major PRR recognize mycobacterial MAMPs

Among the TLR ligands given in the table 1.3 TLR2 together with TLR1 interacts with mycobacterial components and activates innate immunity. Purified mycobacterial cell-wall components have been shown to preferentially activate TLR2 and, to a lesser extent, TLR4.

LM and LAM has immunomodulatory functions. PILAM is a potent TLR2 stimulator that has been identified in nonpathogenic, fast-growing species such as *M. smegmatis* (Gilleron et al., 2003). In contrast, ManLAM is a powerful anti-inflammatory molecule that is found in slow-growing virulent mycobacteria, such as *M. tuberculosis*, *M. bovis* BCG, and *M. avium*. Since LMs from both pathogenic and nonpathogenic mycobacterial species, independent of their origin, induce inflammatory cytokines in a TLR2-dependent manner, the ultimate response against virulent mycobacteria may be determined by the ManLAM/LM ratio in the cell wall (Quesniaux et al., 2004).

In addition, TLR2 in association with TLR1 can recognize a 19 kDa cell-wall-associated lipoprotein, a secreted antigen of *M. tuberculosis* that is also a potent cytokine inducer of macrophages (Thoma-Uszynski et al., 2001), and TLR9 can be activated by mycobacterial DNA, which may be released during endolysosomal degradation.

The role of individual TLRs in mycobacterial infections has been examined using TLR-deficient mice. In the case of the nonpathogenic species *M. smegmatis*, TLR2 is indispensable for effective clearance of *M. smegmatis* from the pulmonary compartment. In contrast, in the case of infections with virulent mycobacteria, such as *M. avium* and *M. tuberculosis*, mice deficient in TLR2, TLR4, or TLR6 appear to show minor or no defects in the control of infection, although conflicting results have been reported to date. *M. leprae* is the causative bacterium of leprosy, whose clinical manifestations depend on the host cell-mediated immune response against the pathogen. Tuberculoid leprosy patients manifest a strong Th1 response, resulting in a few localized and often self-healing paucibacillary lesions. In contrast, lepromatous leprosy patients manifest a predominant Th2 response, leading to a disseminated disease involving extended multibacillary lesions of the skin and nerves. The TLR2/TLR1 heterodimers are responsible for the cellular activation mediated by *M. leprae* as well as its triacylated 19 kDa and 33 kDa lipoprotein (Krutzik et al., 2003). TLR2 and TLR1

are strongly expressed on monocytes and DCs in lesions from tuberculoid leprosy patients but not in those from lepromatous leprosy patients. It is reported that a mutation in the intracellular domain of hTLR2 (Arg677Trp) is associated with lepromatous leprosy in a Korean population (Malhotra et al., 2005). These findings demonstrate that TLR2 plays a critical role in the innate immune response to *M. leprae*.

Table. 1.3 TLR recognition of microbial compounds.

	Microbial compounds	Species	TLR usage
Bacteria	LPS	Gram-negative bacteria	TLR4
	Diacyl lipopeptides	Mycoplasma	TLR6/ TLR 2
	Triacyl lipopeptides	Bactria and mycobacteria	TLR1/ TLR2
	LTA	Group B *Streptococcus*	TLR6/ TLR2
	PG	Gram-positive bacteria	TLR2
	Porins	*Neisseria*	TLR2
	Lipoarabinomannan	Mycobacteria	TLR2
	Flagellin	Flagellated bacteria	TLR5
	CpG-DNA	Bactria and mycobacteria	TLR9
	ND	Unpathogenic bacteria	TLR11
Fungus	Zymosan	*Saccaromyces cerevisiae*	TLR6/ TLR 2
	Phospholipomannan	*Candida albicans*	TLR2
	Mannan	*Candida albicans*	TLR4
	Glucoronoxylomannan	*Cryptococcus neoformans*	TLR2 and TLR4
Parasites	tGPI-mutin	*Trypanosoma*	TLR2
	Glycoionositol phospholpids	*Trypanosoma*	TLR4
	Hemozoin	*Plasmodium*	TLR9
	Profilin-like molecule	*Toxoplasma gondii*	TLR11
Viruses	DNA	Viruses	TLR9
	dsDNA	Viruses	TLR3
	ssDNA	RNA viruses	TLR7 and TLR 8
	Envelope proteins	RSV, MMTV	TLR4
	Hemeagglutinin proteins	Measles virus	TLR2
	ND	HCMV, HSV 1	TLR2
Host	Heat shock protein 60, 70		TLR4
	Fibrinogen		TLR4

1.3.7.3.3 TLR2 function as TLR1-TLR2 functional complex

Jin et al., (2007) crystallized the human TLR1-TLR2-lipopeptide complex and of the mouse TLR2-lipopeptide complex. Binding of the tri-acylated lipopeptide, Pam_3CSK_4, induced the formation of an ‘‘m’’ shaped heterodimer of the TLR1 and TLR2 ectodomains whereas binding of the diacylated lipopeptide, Pam_2CSK_4, did not. Two of the three lipid chains of the ligand interact with a pocket in TLR2, and the remaining amide-bound lipid chain is inserted into a narrow channel in TLR1. The TLR1 channel and TLR2 pocket are connected at the dimer interface, forming a long and continuous lipid-binding site. The TLR1-fused and TLR2-fused VLR fragments in the complex do not interact with each other or with the TLR fragments since their closest atoms are more than 9 A° apart. An extensive hydrogen-bonding network, as well as hydrophobic interactions, between TLR1 and TLR2 further stabilize the heterodimer.

The lipopeptide-binding site of TLR2 is found in a highly unusual region. In TLR2, the convex region formed at the border of the central and C-terminal domains opens into a crevice that is connected to a large internal pocket (Fig. 1.14 and Fig. 1.15); the two ester-bound lipid chains are inserted into the TLR2 pocket in extended conformation, and the amide-bound lipid chain and peptide head group are exposed to the outside of the pocket, interacting with TLR1 and hydrophilic interfacial residues (Fig. 1.14). The surface of the internal pocket is completely lined with hydrophobic residues from LRR modules 9~12. The two ester-bound lipid chains occupy over 90 % of the total solvent-accessible volume of the pocket (Fig. 1.15), and the remaining 10 % of the pocket may permit minor structural variation of the lipopeptide ligands.

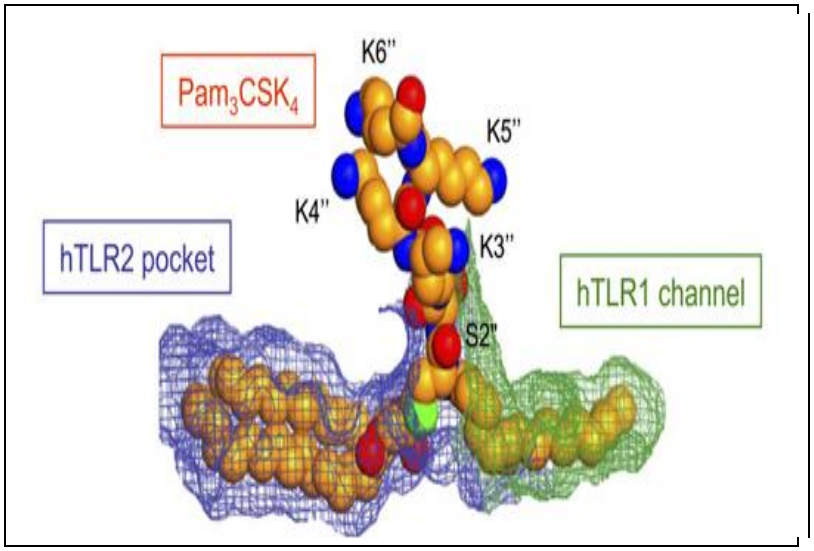


Fig. 1.14 The Lipopeptide-Binding Site of the Human TLR1- TLR2 Complex. The shape of the Pam3CSK4-binding pocket is shown in mesh. Molecular surfaces that belong to TLR1 and TLR2 are drawn in green and blue, respectively (Jin et al., 2007).

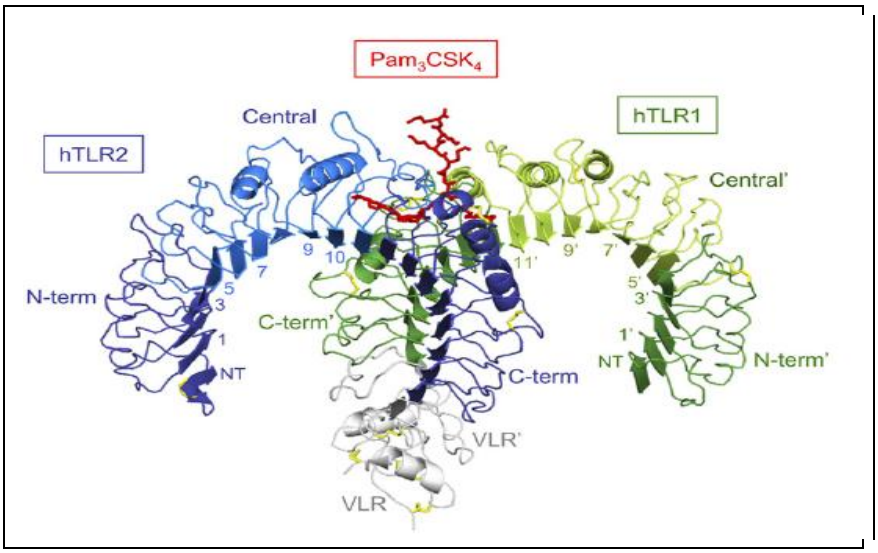


Fig. 1.15 Overall Structure of the Human TLR1-TLR2-Pam3CSK4 Complex. The central domains are colored in light green or light blue, and the Pam_3CSK_4 lipopeptide in red (Jin et al., 2007).

1.3.7.3.4 TLR mechanism of action

Jin et al., (2007) demonstrated the mechanism of TLR action by crystallizing TLR1 and TLR2 extracellular domains bound to a synthetic lipopeptide agonist Pam3CSK4. Their study

revealed that binding of triacylated lipopeptide to TLR 2 occurs in a lipid-binding pocket that is formed at the convex face of the junction between the central and C-terminal LRR domains. This pocket accommodates two of the acyl chains of the synthetic TLR agonist, Pam3CSK4, whereas a similar lipid-binding pocket in TLR1-also at the junction between the central and C-terminal LRR domains-accommodates the third acyl chain. This structure explains the requirement for TLR2-TLR1 heterodimerization for the response to triacylated lipopeptides (Fig. 1.16). Kajava and Vasselon, (2009), supported the above data by providing evidence for the existence of functional TLR2-TLR1-Pam3CSK4 through extensively mutating the ectodomain of TLR2, and complex and the mutants were assessed for their ability to bind and to mediate cellular responses to triacylated lipopeptide Pam3CSK4.

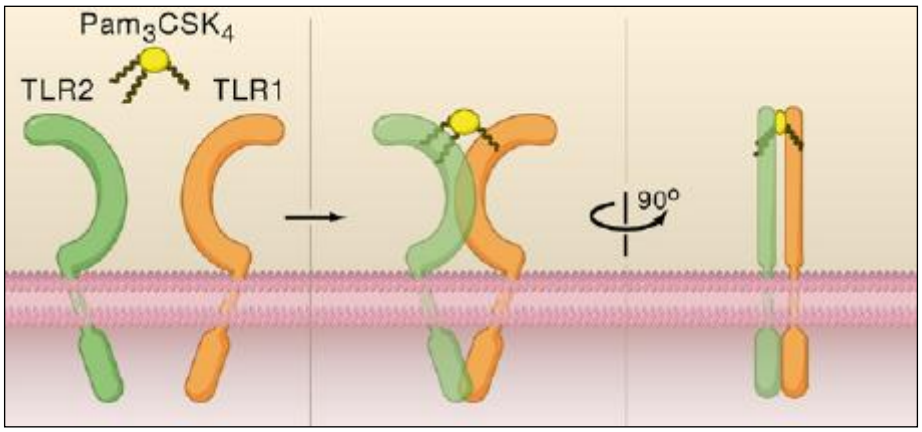


Fig. 1.16 TLR mechanism of action. Binding of the synthetic TLR2 agonist Pam3CSK4 to TLR2 and TLR1 occurs at the convex face of the extracellular LRR domain (Jin et al., 2007).

1.3.7.3.5 TLR signaling pathways

TLRs and IL-1R share common signaling pathways in general. All TLRs activate a signalling pathway that culminates in the activation of nuclear factor-κB (NF-κB) transcription factors, as well as the mitogen-activated protein kinases (MAPKs) extracellular signal-regulated kinase (ERK), p38, and C-Jun N-terminal kinase (JNK) (Fig. 1.17). Stimulation with their

ligands recruits TIR-domain-containing adaptors including MyD88 and TIRAP to the receptor, and the subsequent formation of a complex of IRAKs, TRAF6, and IRF-5 is induced. TRAF6 acts as an E3 ubiquitin ligase and catalyzes the K63-linked polyubiquitin chain on TRAF6 itself and NEMO with E2 ubiquitin ligase complex of UBC13 and UEV1A. This ubiquitination activates the TAK1 complex, resulting in the phosphorylation of NEMO and activation of the IKK complex. Phosphorylated IkB undergoes K48-linked ubiquitination and degradation by the proteasome. Freed NF-kB translocates into the nucleus and initiates the expression of proinflammatory cytokine genes. Simultaneously, TAK1 activates the MAP kinase cascades, leading to the activation of AP-1, which is also critical for the induction of cytokine genes. TLR4 triggers the MyD88-independent, TRIF-dependent signaling pathway via TRAM to induce type I IFNs. TRIF activates NF-kB and IRF-3, resulting in the induction of proinflammatory cytokine genes and type I IFNs. TRAF6 and RIP1 induce NF-kB activation and TBK1/IKK-i phosphorylate IRF-3, which induces the translocation of IRF-3. The entire above signaling pathways end up with the secretion of proinflammatory cytokines, which initially activates the innate immune system and finally activates the adaptive immunity for the successful removal or prevention from multiplication of pathogen (Akira and Takeda, 2004).

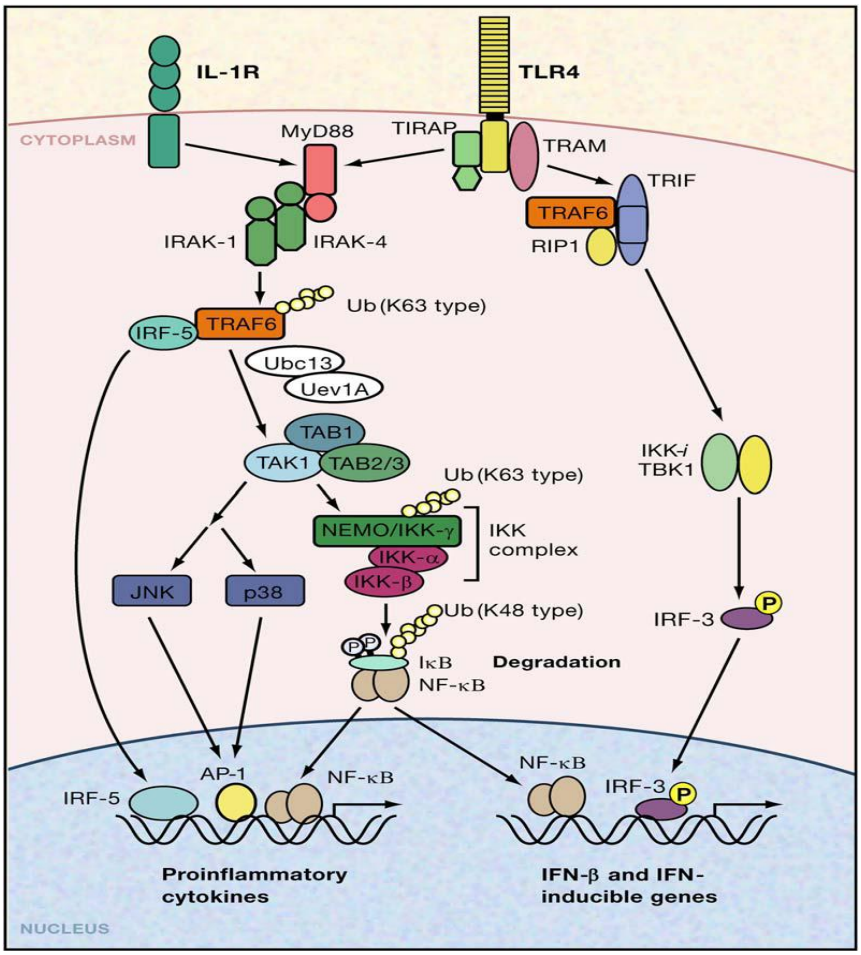


Fig. 1.17 TLR signaling pathways (Akira et al., 2006)

1.3.8 Heterologous expression of proteins

Heterologous expression of recombinant proteins in prokaryotic system is well established years before. It includes cloning of genes of interest in suitable host via certain carriers called vectors. Of which most commonly used vector systems are pET series as they are having a strong T7-promoter, which can be easily induced by IPTG. Among the host used for cloning the best one is *E.coli*, as its ability to grow rapidly and at high density, its well characterized genetics and the availability of an increasingly large number of vectors and host strains (Baneyx, 1999; Verma et al., 1998; Yang et al., 2005). One drawback among the most referred of using *E.coli* as host for expressing the recombinant proteins is the chance of forming inclusion bodies because of unfolding. Inclusion body formation can be minimized

by adopting various strategies like modifying the growth conditions, like expressing the proteins at different inducer concentration, growing the bacterium at different temperatures, and growing the cells in different medium. In addition to this co-expression of recombinant proteins with chaperonins are also successful methods. The following section detailed the literature about various vectors used for cloning, various expression systems and finally the various methods adopted for getting the protein in active form from inclusion bodies

1.3.8.1 Gene cloning: Vectors and hosts

The T7 RNA polymerase based plasmid vector, pET expression system is one of the most powerful and widely used prokaryotic expression systems available today (Pan and Malcolm, 2000). Unlike *E. coli* promoters, the pET system uses the bacteriophage T7 promoter to direct the expression of target gene. Since *E. coli* RNA polymerase does not recognize the T7 promoter, there is virtually no transcription of the target gene in absence of a source of RNA polymerase. The cloning step is thus effectively uncoupled from the expression step (NOVAGEN manual). Many genes that have been difficult to establish in *E. coli* have been stably cloned and expressed in the pET system.

A wide variety of pET vectors is available. All except the specialized pSCREENo-1b (+) vector are derived from the pBR322 and vary in leader sequences, expression signals, relevant restriction sites and other features. There are two major categories of pET plasmids known as transcription vectors and translation vectors.

1. The transcription vectors (including pET-21, pET-23 and pET-24) express target RNA but do not provide translation signals. They are useful for proteins from target genes that carry their own translation signals.
2. The translation vectors contain efficient translation initiation signals that are designed for protein expression. Most contain cloning sites in reading frames a, b or c that

correspond to the GGA, GAT, or ATC triplet of the BamH1 site respectively (NOVAGEN pET system).

Zawalik et al., (2004) used pET28a (+) vector to over-express the *M. tuberculosis* Dna A as histidine tagged protein. pET systems can be induced by the varying concentration of IPTG from 0.1-1mM (Fig. 1.18). The recombinant protein when over-expressed in *E. coli* BL21*(DE3)* cells was found to come in the soluble fraction when a low concentration of IPTG was used for induction (0.05m M) and by use of a reduced temperature (30º C). Hsieh et al., (1996) worked on the cloning expression and characterization of polyphosphate glucokinase from *M. tuberculosis*. BL21 pLysS cells and BL21 cells were used as hosts and pET23a vector which harbored the recombinant plasmid. It was observed that polyphosphate glucokinase was expressed as a soluble protein and no activity of the protein was found in the pellet fraction. Jamil and Drews, (1999) worked on the expression and characterization of *M. tuberculosis* serine/threonine protein kinase (PKn B). This gene was cloned and expressed with the control of T7 promoter in *E. coli* expression vector pET-22b. It was found that PKnB was present in the form of insoluble inclusion bodies. Mukherjee et al., (2005) did some work on the cloning of the gene encoding a protective *M. tuberculosis* secreted protein shed by the pathogen during early phase of infectious process. The PCR amplified gene for the protein was cloned into p ET-17b vector and the expression vector used for transformation was *E. coli* BL-21(DE3) pLysS. The recombinant protein was found entirely in inclusion bodies. Korepanova et al., (2005) worked on the cloning and expression of multiple integral membrane proteins from *M. tuberculosis* in *E. coli*. pET29b (+) vector was used for cloning and for protein expression *E. coli* BL21 (DE3) codon plus-RP (Strategene) or C43 (DE3) strains were used. It was found that more than 94 % of the expressed protein formed some degree of insoluble aggregates. Cloning into some of the pET vectors is reported to bring certain proteins to the soluble fraction.

Some fusion tags such as thioredoxin tag, gst tag etc are used to increase the soluble expression of recombinant proteins. Liew et al., (2005) adopted this fusion tag approach to achieve high level of soluble expression of C type natriuretic protein. When the peptide was linked at its N terminus to a thioredoxin carrier protein, it was found that more than 50% of total protein content was obtained in the soluble fraction. The influence of solubility enhancing fusion proteins-Glutathione S-transferase and Nus A on the heterologous expression of carotenoid cleavage dioxygenase of *Arabidopsis thaliana* was investigated by Schilling et al., (2007). It was observed that the specific activity in the cellular extract was increased by two fold by use of GST as a carrier protein whereas it was decreased by 70 % when fused with Nus A.

Several *E. coli* hosts are used for expression of recombinant proteins such as BL21*(DE3)* Rosetta, ER2556 *etc* are available. Bukhiyarova et al., (2004) reported that there is significant increase in the yield of p38 mitogen activated protein kinase in soluble fraction when genetically modified *E. coli* BL21*(DE3)* Rosetta was used. For stringent control of the degree of transcription of desired genes in uninduced state, *E. coli* hosts carrying the pLysS or pLysE plasmids are available. The pLysS plasmids encode the T7 lysozyme which is a natural inhibitor of T7 RNA polymerase and thus reduces its ability to transcribe genes in uninduced cells. pLysS hosts produce a low level of T7 RNA polymerase while pLysE hosts produce much more enzyme and thus help in a more stringent control over the transcription of target gene (NOVAGEN).

It is also reported that the T7 RNA polymerase based pET system has been widely used for the expression of mycobacterial proteins in *E. coli* (Poletto et al., 2004). The mutant forms of *M. tuberculosis* 2 trans enoyl ACP CoA reductase and 3 ketoacyl ACP CoA reductase enzyme in *E. coli* BL21*(DE3)* host strain. The absolute requirement for IPTG

induction to obtain protein expression in the host cell suggests that no inherent defect in the transcriptional activity of T7 promoter is present.

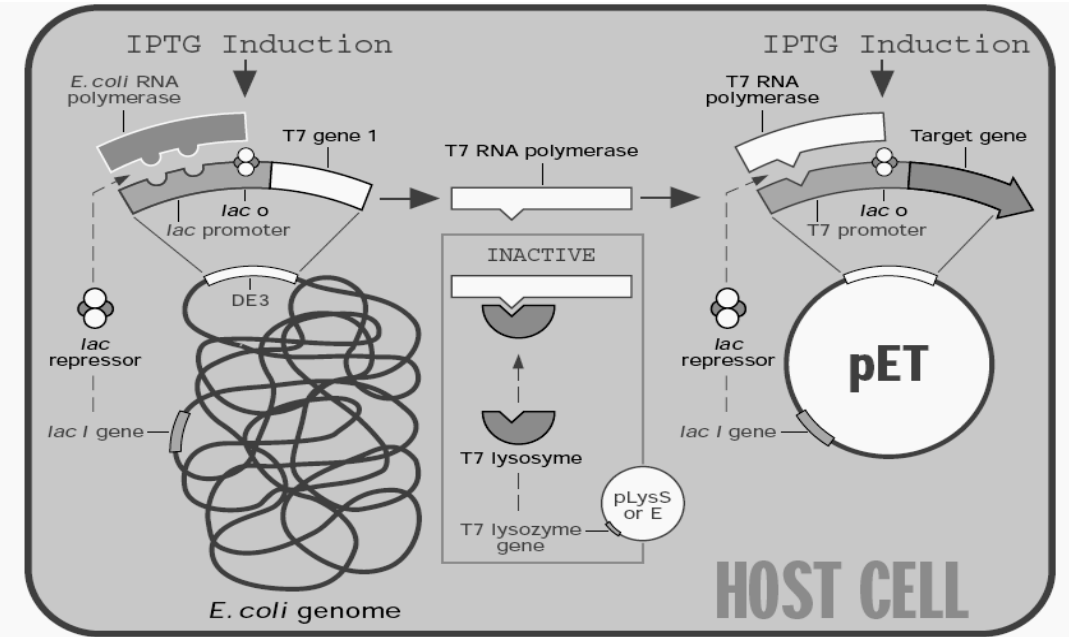


Fig. 1.18 Control elements in pET system (Zawilak et al., 2004).

1.3.8.2 Soluble expression of recombinant proteins

Although prokaryotic expression systems are widely used, proteins obtained in this way are typically found to be insoluble. And to refold these inactive proteins so that they can show biological activity is indeed a difficult task. No universal approach has been established for the efficient folding of aggregation prone recombinant proteins.

A well known technique to limit the *in vivo* aggregation of recombinant proteins consists of cultivation at reduced temperatures (Schein, 1989). This strategy has worked for a number of proteins such as subtilisin E, bacterial luciferase, beta lactamase etc. (Vasina and Baneyx, 1997). The aggregation reaction is in general favoured at higher temperatures due to strong temperature dependence of hydrophilic interactions that determine aggregation. A

direct consequence of temperature reduction is the partial elimination of heat shock proteases that are induced under overexpression conditions (Chesshyre and Hipkiss, 1989). Furthermore, the activity and expression of *E. coli* chaperons are increased at temperatures around 30 ^{o}C (Ferrer et al., 2004; Mogk et al., 2002).

E. coli mutant strains like C41*(DE3)* and C43*(DE3)* have contributed significantly to the soluble expression of recombinant proteins (Miroux and Walker, 1996) like globular and membrane proteins which is unable to be expressed at high levels in the parent strain BL21*(DE3).*

Folding of some proteins require existence of a specific cofactor. Addition of such cofactors or binding partners to the cultivation media may increase the yield of soluble protein dramatically. This was demonstrated for a recombinant mutant of hemoglobin for which accumulation of soluble product was improved when heme was in excess (Weickert et al., 1999).

1.3.8.3 Isolation and solubilization of inclusion bodies

Expression of genetically engineered proteins in bacteria often leads to the accumulation of protein in inactive, insoluble deposits inside the cell called as inclusion bodies (Baneyx, 1999). Researchers usually attempt to improve solubility by a variety of methods such as growing at lower temperature, co-expressing the protein of interest with chaperons or foldases (Baneyx, 1999). However, expressing the proteins as inclusion body can be advantageous-the proteins are highly enriched, protected from proteolytic degradation and the best available method if the protein is toxic or lethal to the host cell (Rudolph and Lilie, 1997). But it still remains a challenge to convert the inactive and misfolded proteins into soluble, bioactive proteins.

Structural characterization studied using Attenuated Total Reflectance Fourier Transformed Infrared Spectroscopy (ATR-FTIR) have shown that insoluble nature of

inclusion bodies might be due to increased levels of non native intermolecular beta sheet content compared with native and salt precipitated protein (Fink, 1998; Seshadri et al., 1999). The resulting suspension is treated by either low speed centrifugation or filtration to remove soluble proteins from the particulate containing inclusion bodies

After isolation, inclusion bodies are solubilized using high concentration of chaotropic agents such as urea and guanidium hydrochloride. They show concentration dependant binding to proteins (Muralidhara and Prakash, 1997). Although expensive, guanidium hydrochloride is preferred because of its superior chaotropic properties. Moreover, urea solutions may contain and spontaneously form cyanate (Hagel et al., 1971) which carbamylate the aminogroups of protein (Cejka et al., 1968). In addition, inclusion body solubilization is pH dependant and optimum pH conditions should be determined for each protein (Estape and Rinas, 1996). There are reports that inclusion bodies can be solubilized at extreme pH in the presence or absence of low concentration of denaturants (Iwakura et al., 1992). However, extreme pH treatment can result in irreversible protein modifications such as deamidation and alkaline desulphuration of cysteine residues (Thatcher D R, 1994). These low pH solubilization processes may not be applicable to many proteins, particularly those that undergo irreversible chemical modifications at these conditions or those susceptible to acid cleavage (Reddy et al., 1998).

Finally, inclusion bodies can be solubilized with different types of detergents (Kurucz et al., 1995; Puri et al., 1992). For example, detergents such as SDS and CTAB help in the solubilization of recombinant proteins (Singh and Panda, 2005). Inclusion body proteins solubilized under these conditions can possess a native secondary structure (Umetsu et al., 2004) and may even show some biological activity (Tokatlidis et al., 1991). It has also been demonstrated that utilization of a milder solubilization condition can give a higher refolding

yield compared to solubilization by high concentration of guanidium hydrochloride or urea (Puri et al., 1992).

In addition to solubilizing agent, the presence of low molecular weight thiols such as dithiothreitol (DTT) or 2-mercaptoethanol is generally required. These thiols will reduce non native inter and intramolecular disulphide bonds possibly formed during air oxidation during cell disruption and keep the cysteines in the reduced state (Clark, 1998). Chelating agents can be added to the solubilization solution to prevent metal catalyzed oxidation of cysteines. Alternatively, reduced cysteines may be protected from oxidation by formation of S-sulphonate derivatives or mixed disulphides (Mukhopadhyay, 2000).

When expression levels are high, a competitive alternative method is to add solubilization agents to the broth at the end of the fermentation process. This *in situ* solubilization method has been used to recover insulin like growth factor using urea under alkaline conditions and antifungal recombinant peptides using a combination of low pH (<2.6) and high temperature (85º C) (Gavit and Better, 2000). The main disadvantage of this method is that it causes release of both proteinaceous and non-proteinaceous contaminants before renaturation. It has been shown that protein refolding in the presence of contaminants will result in decreased yield (Futami et al., 2000). The main advantage of this method is the elimination of time consuming and energy consuming mechanical disruption and centrifugation steps.

Solubilization of inclusion bodies may be accompolished using high hydrostatic pressures (1-2 kbar) in the presence of reducing agents and low concentration of solubilization agents (St John et al., 1999).

1.3.8.4 Chaperone assisted protein refolding

The *in vivo* competition between folding and aggregation is modulated by chaperons and foldases. So, these proteins can also affect the *in vitro* competition between folding and

aggregation (Mayer et al., 2000). The strategy of chaperone assisted refolding aims to mimic the function of the natural GroEL-GroES chaperonin (Fig. 1.19). In vivo, GroEL forms part of a tunnel through which the newly synthesized proteins pass to help them form a native structure (Ellis, 2003). GroEL first captures the non native protein substrate by binding to the exposed hydrophobic surfaces. In a second step, GroEL interacts with ATP and the protein substrate is released in the folded form. GroES also plays a role in the release of the protein substrate.

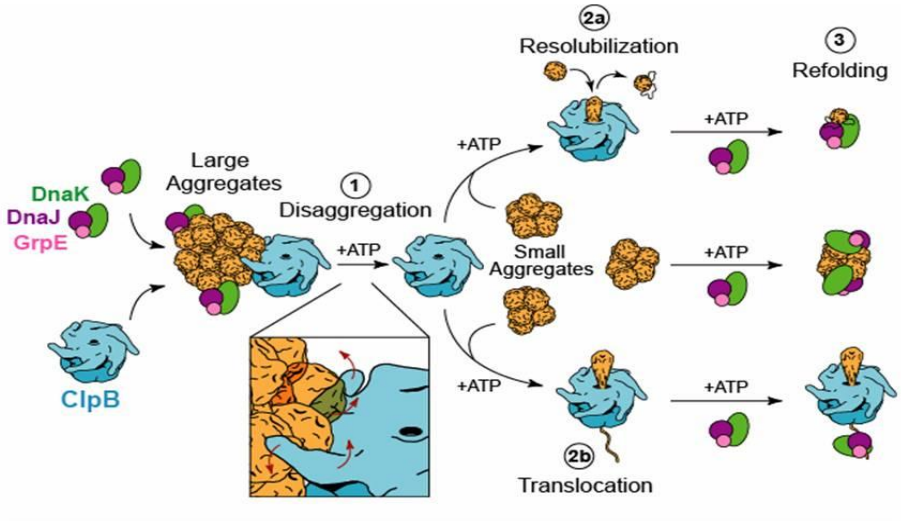


Fig. 1.19 Chaperone assisted protein folding (Ellis, 2003).

Based on this scheme there are two methods of chaperone assisted refolding; one involving the passage of partially denatured substrate through a column that contains immobilized protein chaperone and the other involving passage of removable chemical 'artificial chaperons', to prevent the aggregation of immobilized protein substrate after elution. Altamirano et al., (1999) developed a reusable molecular chaperone system for oxidative refolding chromatography that utilizes a GroEL mini chaperone that can prevent

aggregation. Recently, a chaperone assisted refolding bioreactor that uses a stirred cell membrane system to immobilize the cells has been developed (Kohler et al., 2000).

The main disadvantages in chaperone assisted refolding are that chaperons and foldases need to be removed from the renaturation solution at the end of the refolding process and that they are difficult to produce. So, their commercial use will largely depend on their recovery-reuse scheme.

CHAPTER 2

Construction of *M. smegmatis* mutants expressing *M. tuberculosis* genes involved in mannose biosynthesis

CHAPTER 2

Construction of *M. smegmatis* mutants expressing *M. tuberculosis* genes involved in mannose biosynthesis

2.1 INTRODUCTION

In Mycobacterium mannolipids like PIMs and LAM can act as ligands for host-cell receptors and contribute to the pathogenesis of *M. tuberculosis* (Chatterjee and Khoo, 1998; Ernst et al., 1998; Russell et al., 2002). However, the presence of these glycolipids in saprophytic species of mycobacteria suggest that they may have more general, but as yet undefined, roles in cell-wall biogenesis and growth. Mannose metabolism is essential for the growth and viability of Mycobacterium, by supplying mannose moieties for the synthesis of various mannolipids like PIM, LM and LAM from GDP-mannose, the activated form of mannose, or from its derivative polyprenyl-phosphate mannose and the deposition is carried out through a group of mannosyl transferases those are not yet identified completely. This notion is supported by the finding that enzymes involved in inositol metabolism, phosphatidylinositol synthesis and PIM synthesis appear to be essential for the viability of the rapidly growing species *M. smegmatis* (Jackson et al., 2000; Kordulakova et al., 2002; Parish et al., 1997). GDP-mannose used in these reactions can be produced in two distinct ways (Fig. 1.1), either through the conversion of exogenously acquired mannose to mannose-6-phosphate (M6P) by a hexokinase, or from the glycolytic pathway by converting fructose-6-phosphate (F6P) to M6P by a phosphomannose isomerase (PMI). M6P is then converted to mannose-1-phosphate (M1P) by a phosphomannomutase (PMM) and then to GDP-mannose by a GDP-mannose pyrophosphorylase (GDPMP) (Patterson et al., 2003).

As shown in the figure 1.1, PMI is an essential gene coded by *manA* (Rv3255c), PMM is coded by an essential gene *manB* (Rv3257c), *pmmB* (Rv3308) codes a hypothetical PMM and GDPMP is coded by *manC* (Rv3264c) (http://genolist.pasteur.fr/TubercuList/). The absence of GDP mannose biosynthesis would be expected to lead to decreased production of cell wall-associated mannose containing lipids which in turn could destabilize the cell wall. Further, there is considerable evidence (Nigou et al., 2003; Schlesinger et al., 1994) for the critical role of mycobacterial mannosylated glycoconjugates in the pathogenesis of mycobacterial species. McCarthy et al., (2005) have identified a *M. tuberculosis* gene, *manB* encoding both PMM and PGM activity. The enzymatic activity of *manB* from *M. tuberculosis* strain Erdman (*M. tb manB*) was demonstrated through expression in two PMM deficient strains of *P. aeruginosa* and the use of an *in vitro* enzyme assay (McCarthy et al., 2005). In addition McCarthy et al., (2005) showed that the over expresssion of *manB* in *M. smegmatis* resulted in an increased amounts of the major cell wall mannosylated glycoconjugates, LAM, LM and higher order PIMs. But these studies are incomplete for providing information about the role and essentiality of the rest of the genes, which are supposed to be essential according to Sassetti et al., (2003). In addition, GDP-mannose pathway (Fig. 1.1) provides the evidence for the existence of a hypothetical PMM coded by *pmmB* (*Rv3308*). *pmmB*, which is having an ortholog, *MSMEG1695* found in *M. smegmtis* added further interest for checking the *pmmB* essentiality in Mycobacterium as *M. smegmatis* is a member of non pathogenic group of mycobacteria (http://www.dsmz.de/microorganisms /bacterial nomenclature.php). *M. smegmatis* can be a potent model host for the overexpression of these genes and the altered cell wall composition can be monitored by analysing the cell wall components. Patterson et al., (2003)proved that the overexpression of *manB* in *M. smegmatis* can modify the cell wall by depositing lipoglycans.

In the present study, the mutants of *M. smegmatis,* over expressing *M. tuberculosis* genes involved in mannose metabolism were constructed by the use of pMV261 which is an *E. coli*- mycobacterial shuttle vector (Quandt and Hynes, 1993). Making knock out mutants is a powerful way to dissect the function of each gene and the tools for making *M. smegmatis* knock outs were well standardized (Portevin et al., 2005). This chapter also explain the construction of *pmmB* knock out mutant and its complement of *M. tuberculosis pmmB* ortholog in *M. smegmatis* to prove the essentiality and involvement of *pmmB,* which is a hypothetical PMM in mycobacteria in lipoglycan synthesis.

2.2 MATERIALS AND METHODS

2.2.1 Bacterial strains and culture conditions

E. coli -DH5α and HB101 used for cloning were from Invitrogen, France. *E. coli* strains were grown on Luria-Bertani broth or agar. *M. smegmatis* MC^2 155 obtained from University of Montpellier, France. According to the requirement, *M. smegmatis* was cultured either in LB medium or in Middle brook 7H9 medium supplemented with 0.05 % tween 80, to prevent cellular aggregation. The strains were grown on LB agar slants for 18 h (*E. coli*) and 72 h (*M. smegmatis*) at 37º C and sub-cultured every two weeks. To grow the plasmid bearing strains, kanamycin was added when required to the final concentration of 30 µg ml^{-1}.

2.2.2 Plasmids and Vectors

Shuttle vector, pJQ200 used for making deletion mutant was purchased from ATCC. pBSK (+) was from Stratagene, La Jolla, CA, USA. pMV 361 and pMV 261 are the vectors used for overexpression and gene integration respectively in Mycobacterium. pCG 121 used as the source of kanamycin cassette is a derivative of pBSK (+).

2.2.3 Chemicals and reagents

Primers for Polymerase Chain Reaction (PCR) and sequencing were synthesized from Integrated DNA technologies, USA. Primers for *pmmB* knock out generation were

synthesized from Milligen, France. The PCR cloning kit, plasmid isolation kit and PCR clean up kits were obtained from Qiagen, Germany. H37Rv genomic DNA is obtained through TB Vaccine Testing and Research Materials Contract, NIH, NIAID NO1-AI-40091. *Taq* DNA polymerase, restriction enzymes, T4 DNA Ligase, Phosphatase, Polynucleotide kinase and ATP were purchased from New England Biolabs, France. *Pfu* DNA polymerase was procured from Promega, France. Middlebrook 7H9 medium was procured from Difco, France. Kanamycin is purchased from Sigma-aldrich, India. All other molecular biology grade chemicals used in the present study were procured from either Sigma, Merck, USA or Hi-media, India. Other chemicals were of the analytical or molecular biology grade obtained from locally available commercial sources.

2.2.4 PCR amplification of genes

All DNA manipulations were performed using standard protocols as described by Sambrook et al., (1989). The web site used for the genome search of *M. tuberculosis* H37 Rv was http://genolost.pasteur.fr/TubercuList.

From the reports available (Patterson et al., 2003), four genes namely *manA, manB, manC* and *pmmB* are involved in mannose metabolism of *M. tuberculosis* H37Rv and are given below,

1. *M. tuberculosis* H37Rv|Rv3255c|manA: 1227 bp - Probable Mannose-6-Phosphate Isomarase manA (PhosphomannoseIsomerase) (PMI) (Phosphohexoisomerase) (Phosphohexomutase)
2. *M. tuberculosis* H37Rv|Rv3257c|manB: 1398bp – Probable Phosphomannomutase PMMA(PMM) (Phosphomannosemutase)
3. *M. tuberculosis* H37Rv|Rv3264c|manC: 1080 bp –D-Alpha-D-Mannose-1-Phosphate Guanyl transferase manB (D-Alpha-D-Heptose-1-Phoaphate Guanyl transferase)

4. *M. tuberculosis* H37Rv|Rv3308|pmmB: 1605 bp – Probable Phosphomannomutase pmmB (Phophomannose mutase)

Standard PCR strategies using the primers listed in table 2.1 and the reaction mixture specified in table 2 .2 with *Pfu* DNA polymerase were used to amplify the above genes from *M. tuberculosis* H37Rv genomic DNA.

Table 2.1 Genes and primers used to amplify *M. tuberculosis* genes to construct the over-expressing *M. smegmatis* mutants

No	Name of the gene	Primer used	Restriction site
1	*manA*	MAFB: 5'-AGCTAG**GATATC**CGGTGGAACTGCTACGTGGCGCGT-3' MARB: 5'-AGCTAG**AAGCTT**CAA CCCGACGGTCGCCCT-3,	EcoR V Hind III
2	*manB*	PAFB: 5'-AGCTAG**TGGCCAG C**ATGTCTTGGCCCGCCGC-3' PAR: 5'-AGCTAG**AAGCTT**CGGTCCGGCCTT CGCAT-3'	Bal I Hind III
3	*manC*	MBFB: 5'-AGCTAG**GATATCAG**TTGGCAACTCACCAAGT-3' MBR: 5'-AGCTAG**AAGCTT**AACGTCGGACGAGTAAC-3'	EcoR V Hind III
4	*pmmB*	PBFB: 5'-AGCTAG**CCCGGG**CCGTGACGCCAGAGAATTGGA-3' PBR2: 5'-AGCTAG**AAGCTT**CCACCACCGCCGCA-3'	Sma I Hind III

Table 2.2 PCR reaction components

Reaction component	Volume (µl)	Final concentration
10X PCR buffer	2.5	1X
dNTP mix (25mM each nucleotide)	0.2	200 µM (each nucleotide)
Forward primer (25 pmoles μl^{-1})	0.4	0.4 µM
Reverse primer (25 pmoles μl^{-1})	0.4	0.4 µM
DMSO	0.5	2 %
Pfu DNA polymerase	0.2 Units	1Unit/25 µl
Genomic DNA (100 ng μl^{-1})	1	100 ng 25 μl^{-1}
MilliQ water	19.8	-
TOTAL	**25µl**	

2.2.4.1 Amplification of *manA*

manA (Rv3255c) was amplified by PCR from *M. tuberculosis* H37Rv genomic DNA using the primers specified in the table 2.1, containing EcoR V and Hind III restriction sites (underlined), respectively. PCR amplification consisted of one cycle of denaturation (95° C, 5 min), followed by 35 cycles of amplification that included denaturation (95° C, 40 sec), annealing (63° C, 30 sec), primer extension (72° C, 1 min 30 sec) and final extension (72° C, 10 min). The 1227 bp PCR product was purified (Fig. 2.7 a, Lane 3) and kept at -20 for further cloning studies.

2.2.4.2 Amplification of *manB*

manB (Rv3257c) was amplified by PCR from *M. tuberculosis* H37Rv genomic DNA using the primers specified in the table 2.1 containing Bal I and Hind III restriction sites (underlined), respectively. PCR amplification consisted of one cycle of denaturation (95° C, 5

min), followed by 35 cycles of amplification that included denaturation (95° C, 40 sec), annealing (59° C, 30 sec), primer extension (72° C, 1 min 30 sec) and final primer extension (72° C, 10 min). The 1398 bp PCR product was purified (Fig. 2.7 a, Lane 6) and kept at -20 for further cloning studies.

2.2.4.3 Amplification of *manC*

manC (Rv3264c) was amplified by PCR from *M. tuberculosis* H37Rv genomic DNA using the following primers specified in the table 2.1, containing EcoR V and Hind III restriction sites (underlined), respectively. PCR amplification consisted of one cycle of denaturation (95° C, 5 min), followed by 35 cycles of amplification that included denaturation (95° C, 40 sec), annealing (56.9° C, 30 sec), primer extension (72° C, 1 min) and final extension (72° C, 10 min). The 1080 base pair PCR product was purified (Fig. 2.7 a, Lane 4) and kept at -20 for further cloning studies.

2.2.4.4 Amplification of *pmmB*

pmmB (Rv3308) was amplified by PCR from *M. tuberculosis* H37Rv genomic DNA using the primers specified in the table 2.1, containing Sma I and Hind III restriction sites (underlined), respectively. PCR amplification consisted of one cycle of denaturation (95° C, 5 min), followed by 35 cycles of amplification that included denaturation (95° C, 40 sec), annealing (58° C, 30 sec), primer extension (72° C, 2 min) and final extension (72° C, 10 min). The 1605 bp PCR product was purified (Fig. 2.7 a, Lane 7) and kept at -20 for further cloning studies.

2.2.5 Cloning and expression of genes

2.2.5.1 Ligation of genes into pMV261

For cloning the genes initially in *E. coli* and latter in *M. smegmatis*, pMV261 (Stover et al., 1991), an *E. coli* - mycobacterial shuttle vector (Fig. 2.1) was used. This vector permits the

stable and moderate expression of the genes cloned under to BCG '*hsp*' promoter when the *M. smegmatis* mutants containing the constructs were grown at 37º C for 3-5 days.

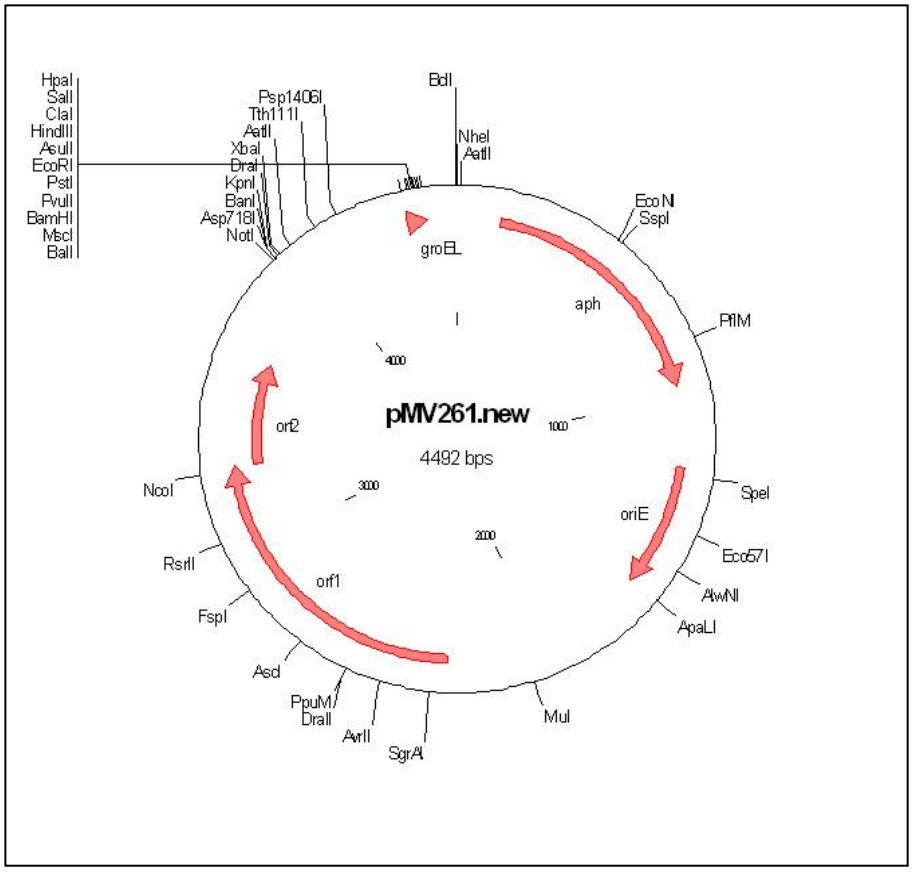


Fig. 2.1 pMV261 shuttle vector

Amplified PCR products and the vector were purified, eluted and digested with the respective restriction enzymes specified in the table 2.1. Digested PCR products were ligated into pMV261 vector which is already digested with Bal I and Hind III using the cloning kit (Qiagen) following manufactures procedure to make *manA::*pMV, *manB::*pMV, *manC::*pMV and *pmmB::*pMV respectively.

2.2.5.2 Transformation of the cloned vector plasmids into *M. smegmatis*

*manA::*pMV, *manB::*pMV, *manC::*pMV and *pmmB::*pMV were transformed individually into *M. smegmatis* using an electroporator (Eppendorf - 2510). Electro competent cells of *M. smegmatis* was prepared by washing the cells with 10 % ice cold glycerol after harvesting the cells at A_{600} of 0.8-1.0 (Parish T, 1998). Electroporation was carried out at 2.5KV and 25µF with 10ng of pMV 261 which is mixed with 100 µl of *M. smegmatis* competent cells which is placed in a 0.2 cm electroporation cuvette. pMV 261 carries an aminoglycoside phosphotransferase gene '*aph'*, which allows the selection of transformants on LB kanamycin plates after 3-5 days at 37º C. Mutants were confirmed by comparing the mobility of constructs run through 0.7 % agarose gel electrophoresis, double restriction with different set of restriction enzymes and sequencing using the primers provided in the table 2.1, which is specific for '*hsp'* of the pMV 261 shuttle vector.

2.2.6 Construction of *M. smegmatis* knockout mutant and its complement strain

Two DNA fragments, each ≈ 1 kb in length, flanking *pmmB* ortholog, *MSMEG1695* were amplified by PCR from *M. smegmatis* genomic DNA by using primers PBFP and PBRP (Table 2.3) and cloned (Fig. 2.3) in pBSK (+), which is a plasmid vector routinely used for cloning (Fig. 2.2). These fragments excised from pBSK (+) and were inserted (Fig. 2.5) into pJQ200 (Fig. 2.4), which is a suicidal vector permits gene replacement by homologous recombination (Quandt and Hynes, 1993). A kanamycin resistance cassette derived from pCG122 (derivative of pBSK(+), Malaga et al., (2003), was inserted between the two PCR fragments to give pPBA2 (Fig. 2.5). This plasmid was transferred into *M. smegmatis* by electroporation and the transformants were selected on plates (Fig. 2.5) containing kanamycin (25 µg ml^{-1}) and 5 % sucrose. Transformants in which pPBA2 had been integrated by double crossover between the WT (Wild Type) gene and *pmmB* gene flanking region in pPBA2 were identified by PCR using the primers RES1+PBR and RES 2+PBF (Table 2.3) against the

genomic DNA isolated from mutants (Belisle, 1998). Out of 4 colonies, one strain named as PMBK4 was shown positive signal towards PCR and it was selected for further studies. In order to produce the complementation plasmid pPBC2, the full length *pmmB gene* (*MSMEG1695*) was amplified by PCR from *M. smegmatis* genomic DNA by using primers PBFP and PBRP (Table 2.3) and was inserted into pMV361(Fig. 2.6) integration plasmid (Stover et al., 1991). Complement strain PMBC4 was made by transforming pPBC2 into PMBK4 (Knockout strain) and was selected on Kanamycin and Hygromycin plates. The three strains WT (Wild type)**,** PMB4 (knockout) and PMBC4 (complement) were used for further characterization studies.

Table 2.3 Primers used to make *M. smegmatis* knockout

Primers	Restriction site
PBFP: 5'-ATGCCCATGGTGACGCCAGAGAATTGGATCGCCC-3'	NcoI
PBRP: 5'-ATGCGAATTCTCACCACCACCGCCGCACGCTAG-3'	EcoRI
FPB : 5'-CTCTGCAGTTATCCCGACCTCGCCA-3'	-
RPB : 5'-CACCGT TCCAGATCGGCGATCGAC-3'	-
PMFP:5'- ATGCATATGACGTCGACCGCGACAACAGC-3'	NdeI
PMRP:5'- ATGCAAGCTTTCACCAGCC TTTCGCGGCGC -3'	HindIII
RES1:5'-GCTCTAGAGCAACCGTCCGAAATATTATAAA-3'	-
RES2:5'-GCTCTAGATCTCATAAAAATGTATCCTAAATCAAATATC-3'	-
RPI :- 5-GGT TTC GTC GCC CGA GAG CAT G -3	-

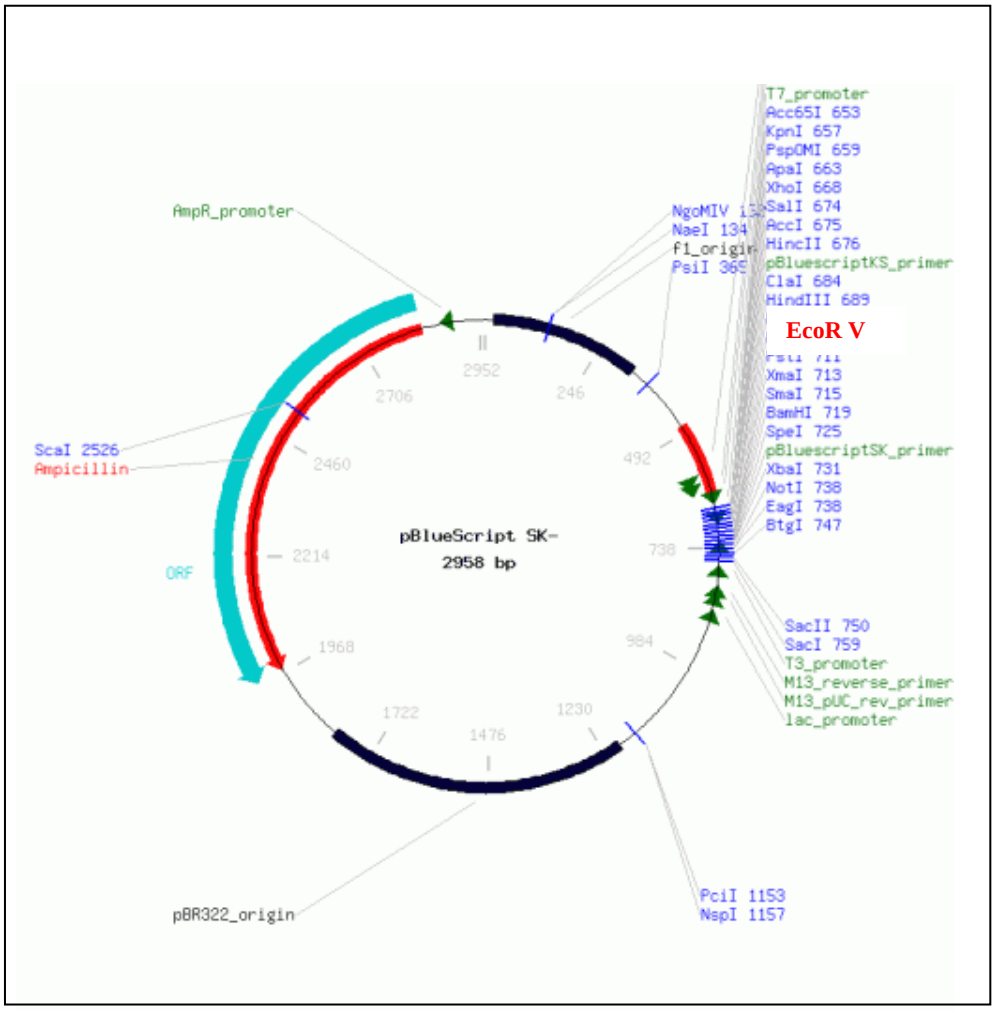


Fig. 2.2 Map of pBSK(+)

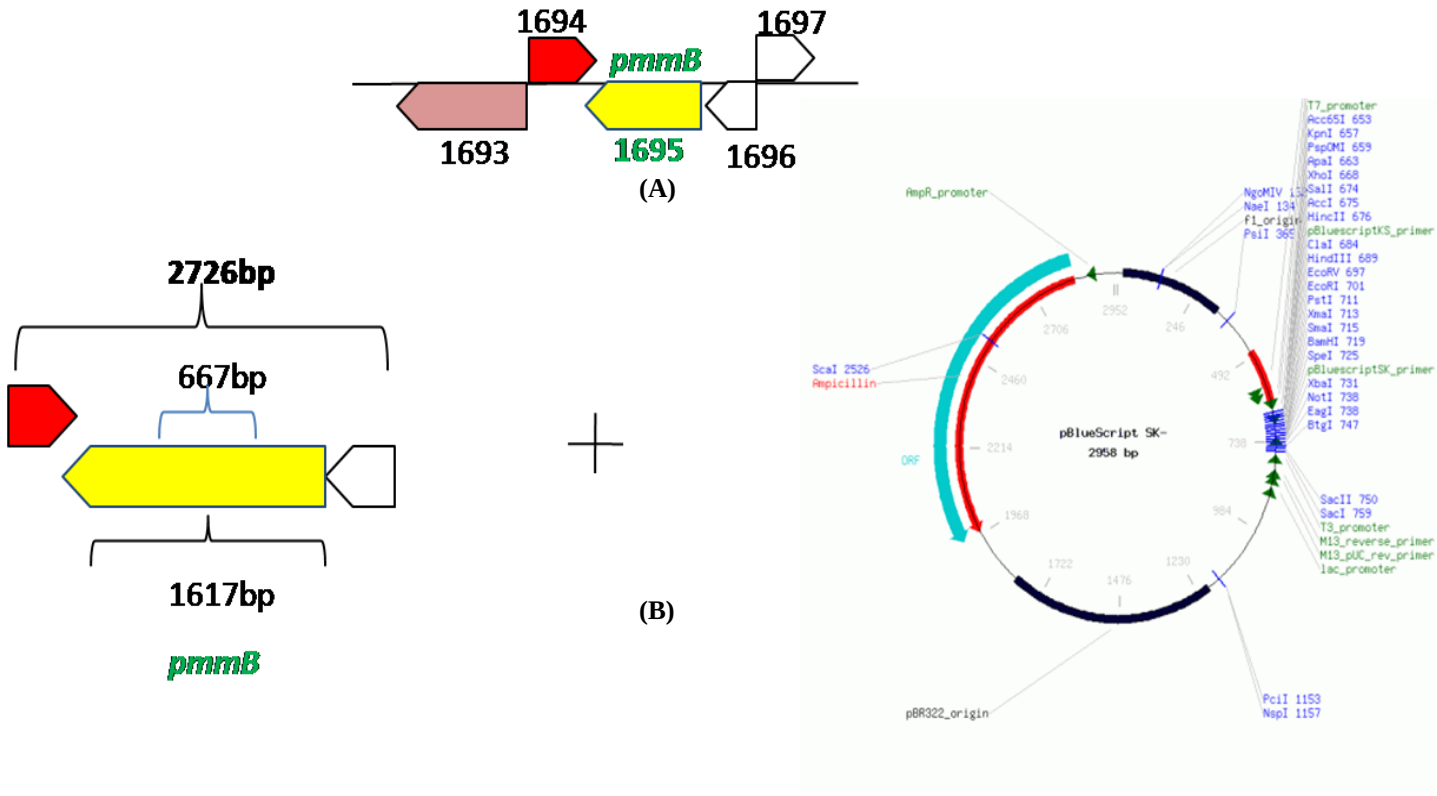
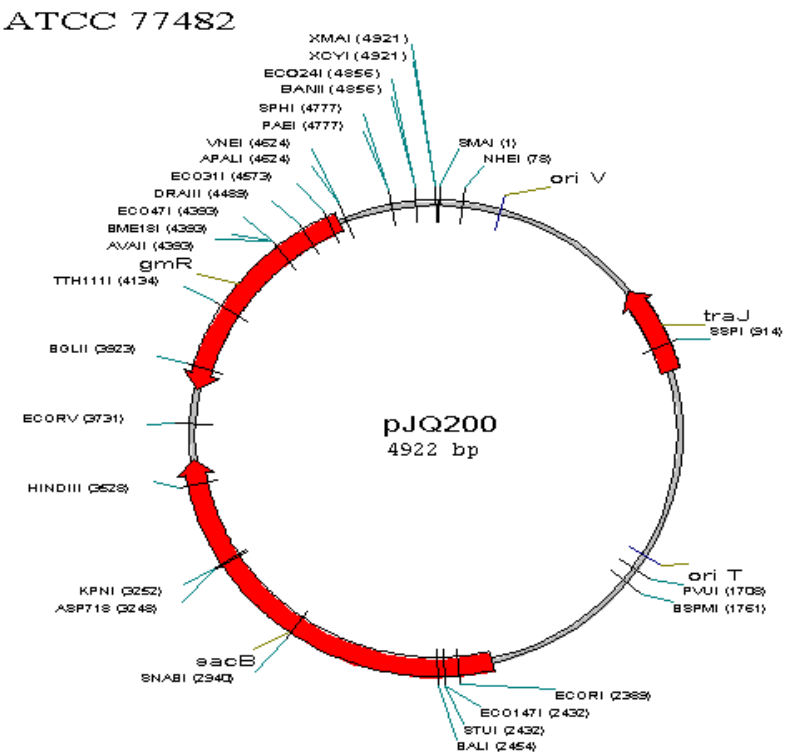


Fig. 2.3 Cloning of *MSMEG1695* region into pBSK (+)

(A) *MSMEG1695* region on *M.smegmatis* genome (B) PCR amplification and ligation of *MSMEG1695* region from *M. smegmatis* genome into pBSK (+)



- Suicidal vector permiting gene replacement
- Induced by 5% sucrose, sacB is lethal to gram negative bacteria, and thus permits selection for loss of the vector
- Order of features
  gtmR-SmaI-P15A-traJ-oriT-sacB

Fig. 2.4 Map of pJQ 200

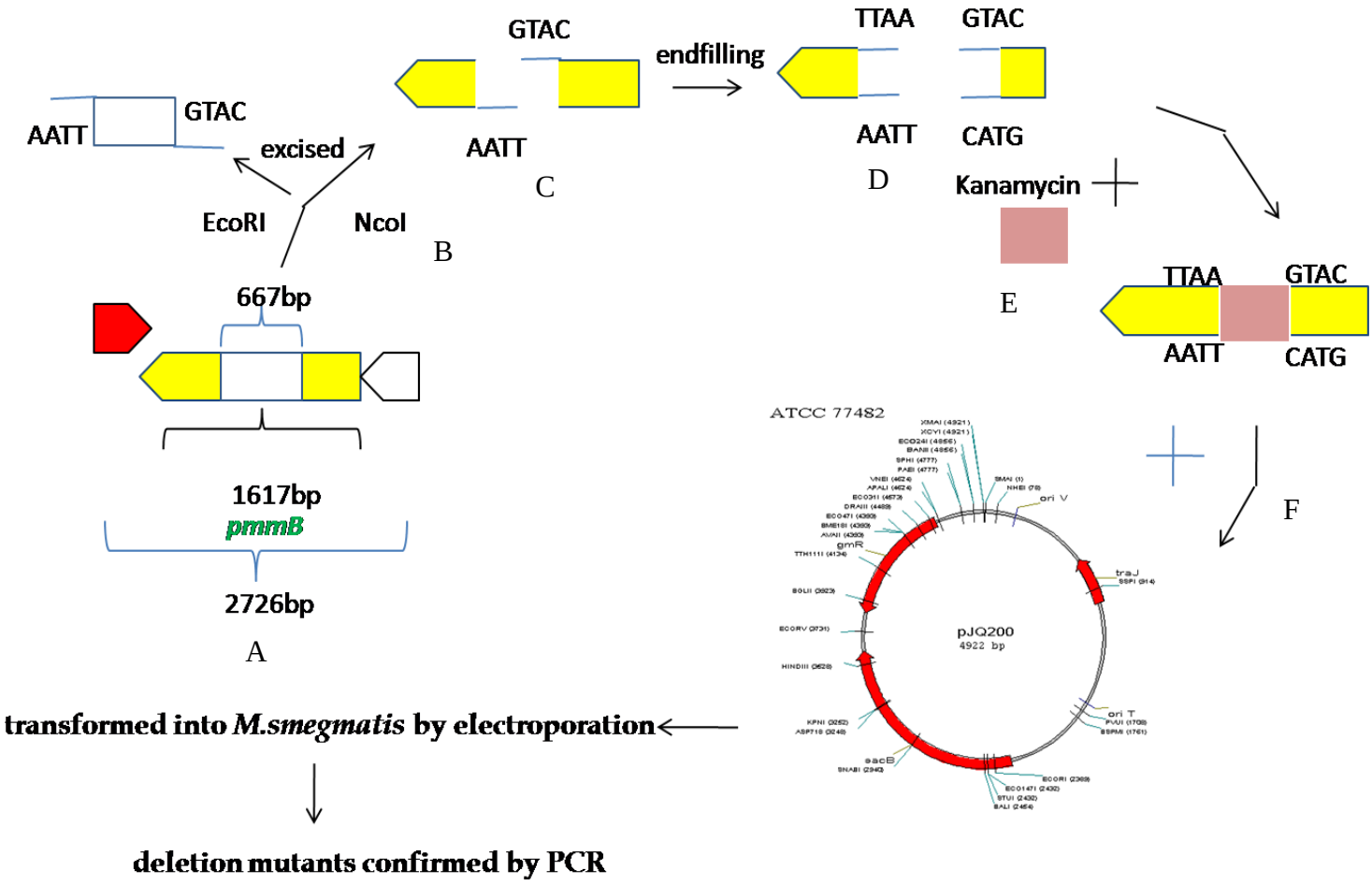
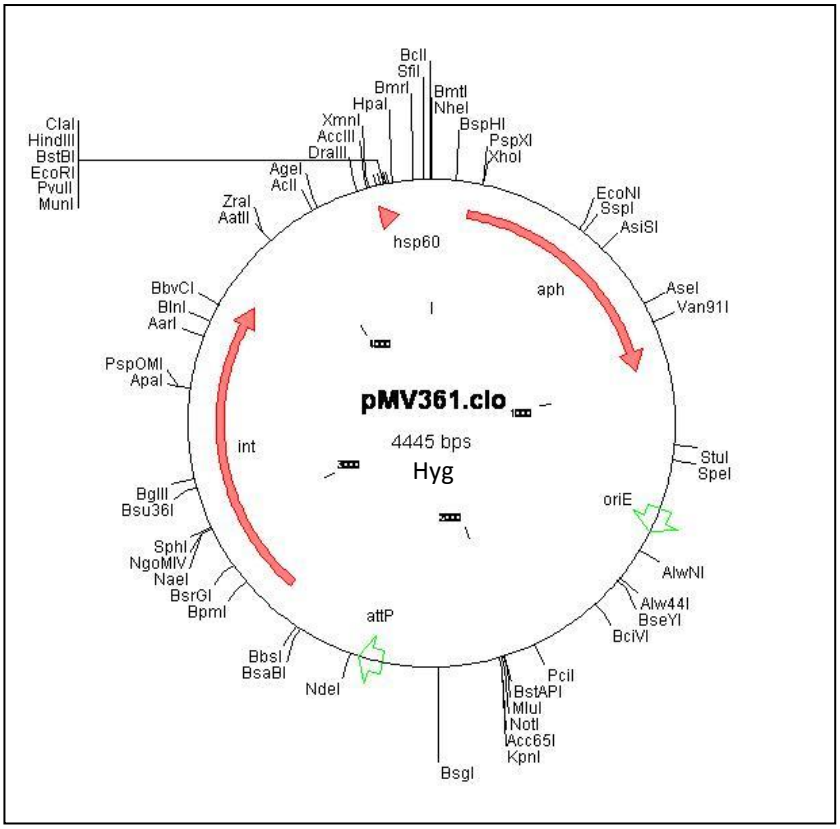


Fig. 2.5 *pmmB* deletion strategy

(A) *pmmB* region on pBSK (+), (B) *pmmB* excision from the region by EcoR I and Nco I (C) *pmmB* excised region of pBSK (+), (D) end filled *pmmB* region for blunt end ligation, (E) insertion of kanamycin cassette into the end filled *pmmB* region, (F) ligation of kanamycin cassette flanked with *pmmB* border region into pJQ 200 vector.



- Nonreplicatiove integrative shuttle vector
- *groELp int attP*, helps to integrate into the *Mycobacterium* genome

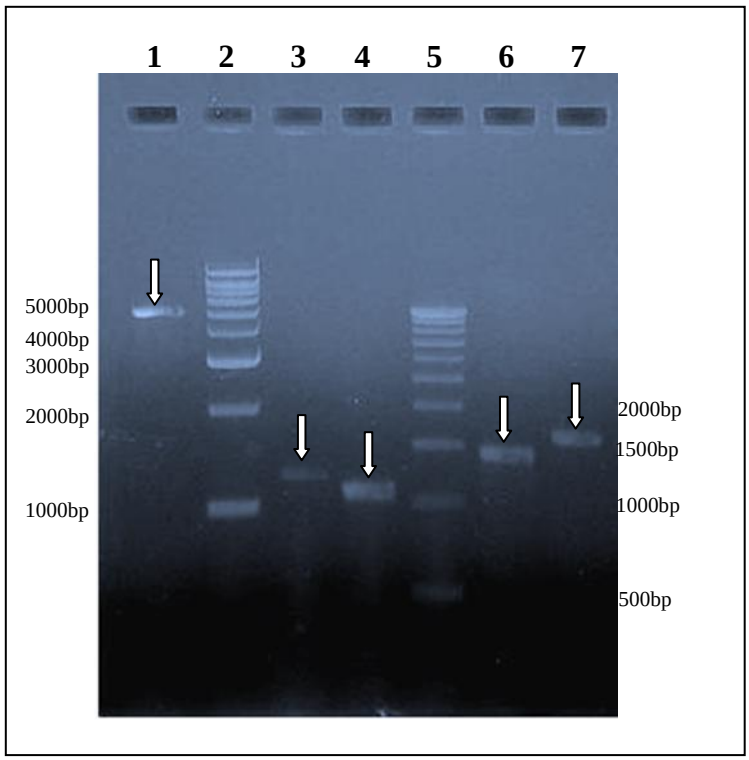
Fig 2.6 Map of pMV361

2.3 RESULTS AND DISCUSSION

2.3.1 Cloning of genes in *M. smegmatis* via pMV 261

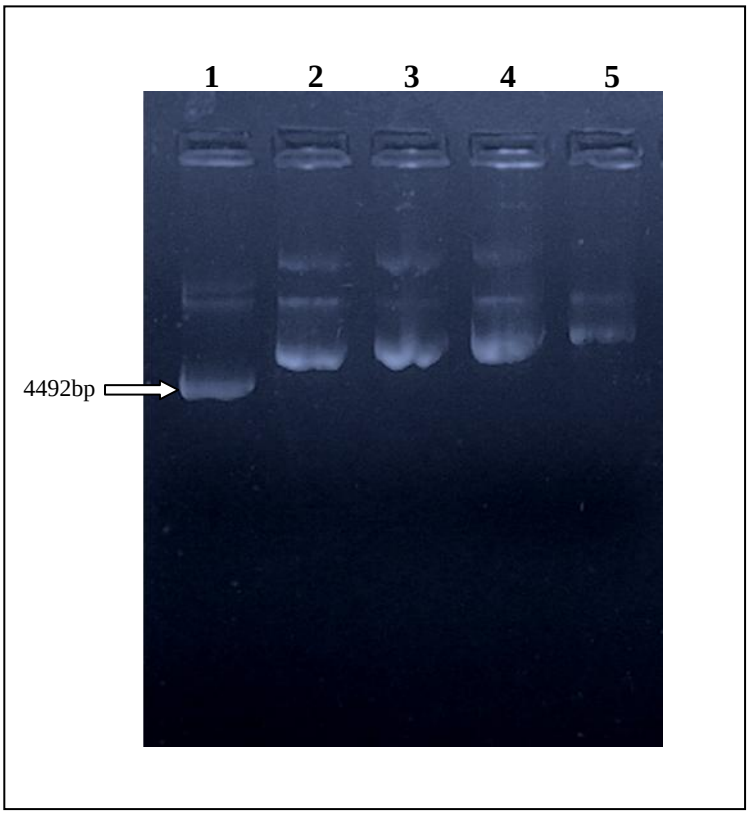
In the present study, to determine whether lipoglycans are *bona fide* TLR2 ligands sensed in the context of a bacterium infection, *M. smegmatis* mutants were generated which were altered for the production of lipoglycans. Since the biosynthesis of the latter cannot be abrogated (Kordulakova et al., 2002), some strains were constructed with either an augmented or a reduced production of lipoglycans, by manipulating the mycobacterial GDP-mannose biosynthesis pathway. *M. smegmatis* mutants of Rv3255c, Rv3257c and Rv3264c were generated as these gene have been shown to encode PMI (Patterson et al., 2003), PMM (McCarthy et al., 2005) and GDPMP (Ma et al., 2001) enzymes respectively. Mutants were made by amplifying *manA*, *manB*, *manC* and *pmmB* gene copies from *M. tuberculosis* H37Rv (Cole et al., 1998) and inserted them separately in the *E. coli*-mycobacterial shuttle vector pMV261. *manA* of 1227 bp, *manB* of 1398 bp, *manC* of 1080 bp and *pmmB* of 1605 bp were amplified (Fig. 2.7 a) from H37Rv genomic DNA and it was analysed by agarose gel electrophoresis and found have the correct size by comparing the size of each construct with the DNA marker run along with the samples. Fig. 2.7 b confirms the clones by comparing the size of native pMV 261 with the different constructs those are loaded in the native form without digestion with restriction enzymes. In which, Lane 1 occupied by pMV261 having a size of 4492 bp in its super coiled form, Lane 3, 4, 6 and 7 are pMV::*manA,* pmV::*manC,* pmV::*manB and* pmV::*pmmB* clones of different constructs respectively. Fig. 2.7 c represents the clone confirmation by restriction digestion with a set of enzymes (HindIII and XbaI) and tried to release the insert from the construct. Fig. 2.7 c, Lane 1 occupied by pMV::*manC,* released a fragment of 1080 bp, Lane 2 of pMV::*manA* relased a fragment of 1227 bp , Lane 3 released a fragment of 1398 bp and Lane 4 released a fragment of 1605 bp during digestion with restriction enzymes. Constructs were further analysed for any kind of mutations by

sequencing with a set of specific primers (Table 2.3) against *'hsp'* of pMV 261 that is upstream and downstream of the gene in the construct. Table 2.4 a, b, c and d showed each gene sequence and the genes were properly inserted in the vector and were of expected size.



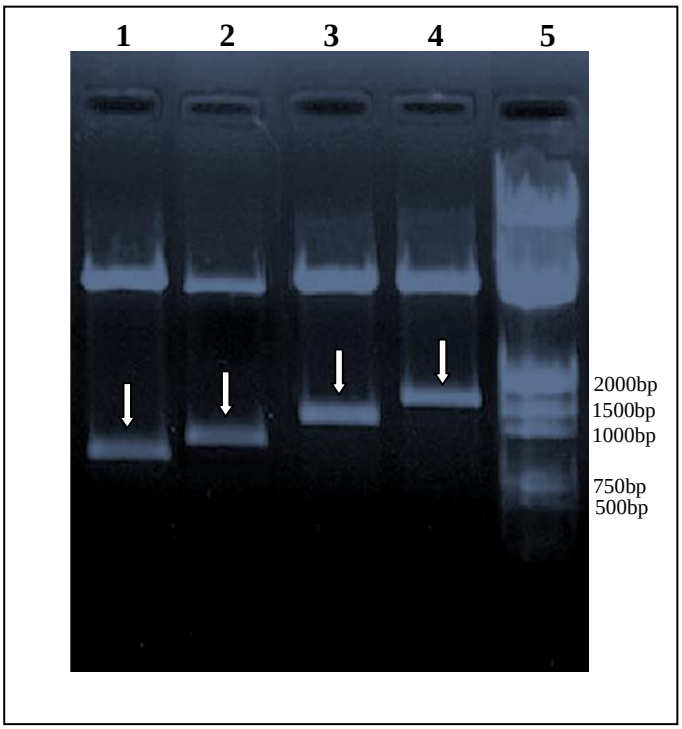
Lane 1-Lineasrised pMV 261, Lane 2&5-DNA marker, Lane 3-manA, Lane 4-manC, Lane 6-manB, 7-pmmB

Fig. 2.7 a Vector and PCR amplified genes



Lane1-pMV, Lane2-manC, Lane3-manA, Lane4-manB, Lane5-pmmB

Fig. 2.7 b Cloning of genes into pMV261



Lane1-manC+pMV, Lane 2-manA+pMV, Lane 3- manB+pMV , Lane4-pmmB+pMV, Lane5 -1 Kb marker

Fig. 2.7 c pMV clone confirmation by double digestion

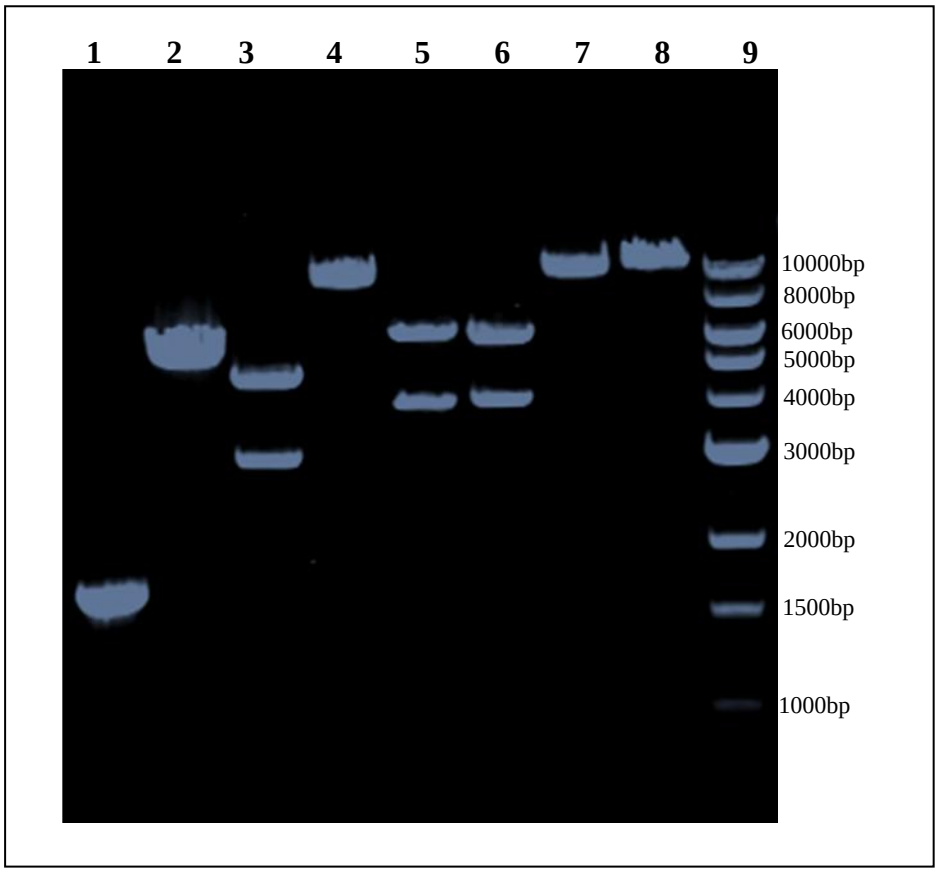
2.3.2 Construction of pmmB deletion and its complement mutant in *M. smegmatis*

pmmB codes for a PMM that acts on Mannose-6-Phosphate and convert it into Mannose-1-phosphate, which will be further utilized for the synthesis of GDP- Mannose (Patterson et al., 2003). *pmmB* putative gene product showed some homology with known PMMs (Cole et al., 1998; McCarthy et al., 2005). High density mutagenesis previously proved that this gene was non-essential in *M. tuberculosis* (Sassetti et al., 2003). Present study thus investigated whether the deletion of its ortholog in *M. smegmatis* (*MSMEG1695*) could result in a strain producing reduced amount of lipoglycans. For that a *M. smegmatis* mutant was constructed

by exchanging the wild-type allele of *MSMEG1695* with a kanamycin resistance cassette-disrupted allele.

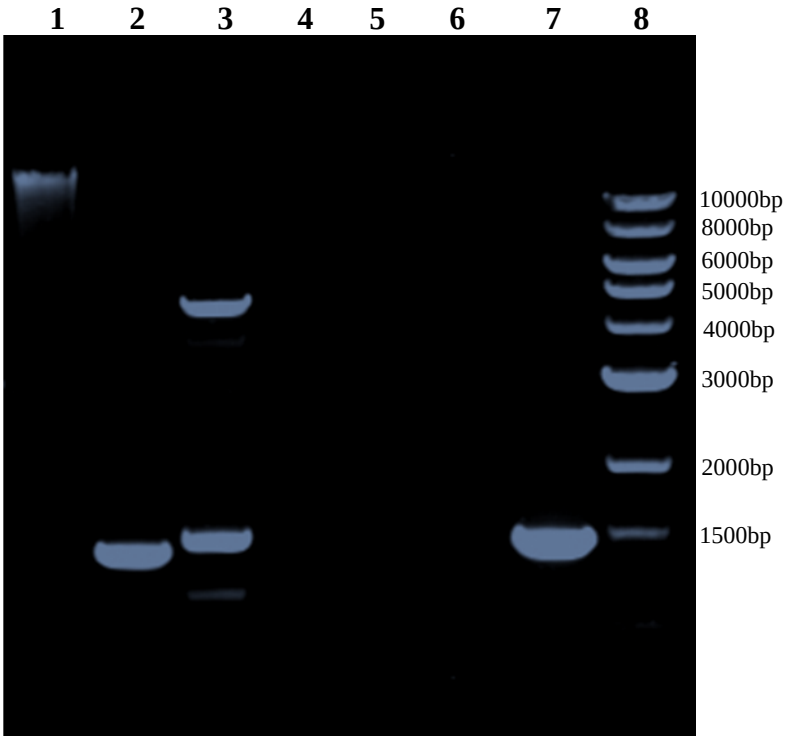
Fig. 2.8 a demonstrate the pPBA2 clone confirmation by (i) by digesting *MSMEG1695* including region of 2760 bp with a double cutter EcoRV released a fragment of size 2760 bp represents the insert (*MSMEG1695*, flanked by a size of 1000 bp on both sides) and a bigger fragment of 4998 bp indicate the size of pBSK(+) (Lane 2) (ii) verifying the size of kanamycin resistance cassette derived from pCG121 in pBSK (+) and size was near to 11000 bp which represents pBsk(+)+pmmB+Kan (Lane 3) (iii) by double digestion with a set of XhoI::BamHI and NcoI::Sac II, released a fragment of ~ 4000 bp and ~ 6000 bp (Lane 4 and 5) and by digesting with single cutters (Sma I and Xba I) produced a fragment of ~ 10000 conclude that Lane 4 and Lane 5 represents pBA2 (*pmmB*::Kan::*pmmB* construct of pJQ200).

Fig. 2.8 b demonstrate the confirmation of PMBK4 (knockout) by (i) PCR amplification with RES1 and RES 2 primers against *pmmB*::Kan::*pmmB* cassette, which is supposed to be absent in wild type, and the amplification was found only in PMBK (Lane 2 and 3) where as it is absent in the PCR against wild type DNA (Lane 4 and 5) (ii) PCR amplification against *MSMEG1695* with the primers RPI and FPB (Table 2.3), and it was observed that the amplification was found only in wild type (Lane 7) and absent in PMBK4 (Lane 4) . PCR amplification with the primers (Table 2.3) against *MSMEG1695* using the genomic DNA isolated from PMBC4 (complement) had given a band size of 1617 bp (Fig. 2.8 a, Lane 1) proved that PMBC4 was complemented with *MSMEG1695*.



Lane 1- PCR of PMBC4 , Lane 2-*pmmB*+pBSK(+), Lane 3 -*pmmB*+ pBSK +Kan, Lane 4, 5, 6, 7 and 8- *pmmB*+pJQ200 double digests, Lane 9- 1Kb marker

Fig. 2.8 a Generation of *pmmB* deletion mutant



Lane 1-Genomic DNA of PMBK4, Lane 2, 3 and 4- PCR PMBK4, Lane 5, 6 and 7- PCR of WT, Lane 8-1Kb DNA marker

Fig 2.8 b Confirmation of *pmmB* deletion mutant by PCR

2.4 CONCLUSION

M. smegmatis mutants having *manA, manB, manC* and *pmmB* genes of *M. tuberculosis* were constructed via pMV 261 shuttle vector. M. smegmatis mutant strain with *pmmB* and its complement with *pmmB* regained activity were constructed. Vector pJQ200 was used for making knockout strain and pMV 361 was used for complementation.

Table 2.4 a Sequence of *manA*

M. tuberculosis H37Rv|Rv3255c|manA: 1227 bp

gtggaactgctacgtggcgcgttacgcacctacgcttggggatcgcgcaccgctatcgccgaattcaccgggcgtccggtgccggc
cgctcaccccgaggccgaactatggttcggtgcacacccgggtgatccggcttggctgcagacgccgcatggccaaacctcgttgct
cgaagcgttggtcgcggatccggaggggcagctcggctccgcgtcgcgcgcgcgattcggcgatgtgttgccgttcttggtcaaggt
gttggcggccgacgagccactatcgttgcaggcccatccgagcgccgagcaggcggttgagggctacctgcgggaagagcgaatg
ggcattccggtgtcctcacccgtccgcaactaccgcgacaccagtcacaagccagagttattggtggcgctgcagccgttcgaggcg
ctggccggattccgggaggcggctcgcaccaccgagctgctgcgggcgctggccgtatccgacctcgacccgttcatcgacttgctg
agcgaggggtccgatgccgatggtttgcgtgcgctgttcaccacctggattaccgcaccccagcccgacatcgacgtgctggtgcct
gccgtgctggacggcgctatccagtacgtcagctccggcgcaacggaatttggcgccgaagccaagacagtgctggaactcggcg
aacgttatcccggcgacgccggtgtgctggcggcgttgttgctcaaccgcatcagcttggctcctggggaggcgatcttcctgccggc
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cacgtcgatgtgcccgagttgttgcgggtgctggacttcgcccccacgccgaaggctcggctgcggcccccgatccggcgcgagg
ggctggggctggtctttgagacgcccaccgatgagttcgcggccacgctactggtgctcgacggcgatcacctcggccacgaggtc
gacgcgtcgtccggccatgacggtccacagatcttgttatgcaccgagggttcggcgacggtgcacgggaagtgcgggtcgctcac
gctacagcgcggcacggccgcctgggtggcggccgacgacggcccgatccggctgaccgccggccaacccgccaagctgttca
gggcgaccgtcgggttg**tga**

Table 2.4 b Sequence of *manC*

M. tuberculosis H37Rv|Rv3264c|manC: 1080 bp

ttggcaactcaccaagtcgatgcggtggtcctggtcggtggcaagggtacccgactgcggccgttgacgctgtcggcgcccaagcc
aatgctgcctaccgccggactgccgttcctcacccatctgctgtcgcggatcgccgcagcgggcatcgagcacgtgatcctgggtac
gtcctacaaacccgcagtcttcgaagcggagttcggcgacgggtccgcactgggcctacagatcgaatacgtgaccgaggagcatc
ccttggggactggcggcggcatcgccaacgttgccggcaagctgcgcaacgacaccgcgatggtgtttaacggcgatgtgctctcg
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gggcattcggctgcgtacccaccgacgaggaggaccgcgtagtcgcctttctggagaagacggaggatccgccgaccgaccagat
caatgccggctgctatgtcttcgaacgcaacgtcatcgaccggattccgcagggccgggaggtttcggtggaacgcgaggtgttccc
ggccttgctcgccgacggcgactgcaagatctacggctatgtcgatgccagctattggcgggacatgggcacaccggaagacttcgt
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tgtctttcttcccgacggcgggatccgttactcgtccgacgtt**tga**

Table 2.4 c Sequence of *manB*

M. tuberculosis H37Rv|Rv3257c|manB: 1398 bp

atgtcttggcccgccgcggctgtggaccgcgttatcaaggcttacgacgtacgcgggctggtcggcgaagagatcgacgagtcgct
ggttaccgatctcggcgccgcattcgcgcggttgatgcggaccgaggatgcgcgaccagtggtgatcggtcacgacatgcgggaca
gttcgccgtcgctggccgacgcgttcgcggccggggtgaccgggcagggcctcgacgtggtgcgagttggtttggcgtccaccgat
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tgctggccgcactgcatgtgctggccgccctcggtgagcagagcagaccgctgtcggagttgaccgcggactaccaacgctatgaat
cctccggcgagatcaacttcaccgtggtcgactcttcggcctgtgtggaggccgtgttgaaatcgttcggcaaccggattgtcagtattg
atcacctcgatggcgtgaccgttgacttaggcgacgacagctggttcaacctgcgcagctcaaacaccgagccgttgctgcggctcaa
tgtggagggccgcagcgtcggggacgttgacgcggtggtacgtcaggtcagcgctgaaatcgctgcccagagcgcacatgcgaag
gccggaccg**tga**

Table 2.4 d Sequence of *pmmB*

M. tuberculosis H37Rv|Rv3308|pmmB: 1605 bp

gtgacgccagagaattggatcgcccacgacccggacccgcagacggccgccgagctcgccgcctgcggccccgacgagctgaa
agcgcggttcagccgcccactggcgttcggcaccgcggggttgcgcgggcacctgcggggcgggccggacgcgatgaacctgg
cggtggtgttgcgcgccacctgggcggtggcacgggtgctcacggatcgaggtctggctggttcgccggtgatcgtggggcgcgac
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cacccacccccggtggtggcgttcgcggtgcggcacaccggcgccgccgctgggatacagatcacggcgtcacacaacccggcga
ccgacaacggctacaaggtctatgtcgacggcggccttcagctcctcgcccctaccgaccggcagatcgaagccgcgatggccacc
gcgcccccggccgatcagatcgccaggaagaccgtcaaccccagtgaaaaccgcgcctccgatctgatcgaccgttatatccagcg
tgcggccggggtccgaaggtgcgccggttcggtccgggtggccctgacgccgctgcacggggttggcggggcgatggccgtcga
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ccagctgcccgacagctggtgcgggcgaggatcgatgagctgtcggctagcgtgcggcggtggtgg**tga**

CHAPTER 3

Biochemical characterization of the *M.smegmatis* wild and mutant strains

CHAPTER 3

Biochemical characterization of the *M. smegmatis* wild and mutant strains

3.1. INTRODUCTION

Lipoglycans are synthesized via the sequential addition by mannosyl transferase (ManTs) of mannosyl units on phosphatidyl-*myo*-inositol, yielding phosphatidyl-*myo*-inositol mannosides (PIM) and LM (lipomannan) (Gilleron, 2008). LM can be further arabinosylated to give lipoarabinomannan (LAM). ManTs involved in the first mannosylation step use the soluble sugar nucleotide GDP-mannose as mannose donor whereas those catalyzing later steps require the polyprenyl-phosphate based sugar donor, polyprenyl-monophosphoryl-mannose. However, the latter derives from GDP-mannose, which is thus the primary mannose donor in mycobacteria (Patterson et al., 2003). GDP-mannose can be produced in three steps from either exogenously acquired mannose or gluconeogenesis-derived fructose-6-phosphate (Fig. 1.1). Mannose and fructose-6-phosphate are converted to mannose-6-phosphate by the action of a hexokinase and a phosphomannose isomerase (PMI), respectively. Further, mannose-6-phosphate is transformed to mannose-1-phosphate by a phosphomannomutase (PMM) and finally to GDP-mannose by a GDP-mannose pyrophosphorylase (GDPMP). In the *M. tuberculosis* genes, *Rv3255c*, *Rv3257c* and *Rv3264c* have been shown to encode PMI (Patterson et al., 2003), PMM (McCarthy et al., 2005) and GDPMP (Ma et al., 2001) enzymes respectively and accordingly have been named *manA*, *manB* and *manC* (McCarthy et al., 2005). *manA* was proved to be essential for mycobacterial growth *in vitro* in the absence of an exogenous source of mannose (Patterson et al., 2003). In this context, it was

assumed that boosting the GDP-mannose pathway in mycobacteria could result in a subsequent increased production of lipoglycans. The proof of that concept was demonstrated by McCarthy et al., (2005) who showed that *M. smegmatis* overexpressing *M. tuberculosis manB* gene produced increased levels of PIM, LM and LAM. *pmmB* putative gene product shows some homology with known PMMs (Cole et al., 1998; McCarthy et al., 2005).

Mycobacteria are gram-positive, acid-fast bacteria possessing a cell envelope composed of highly characteristic constituents (Goren MB, 1979). The Tubercle bacillus and most other species of the genus Mycobacterium are known to form large clumps, especially in stationary liquid culture. Although the chemical basis of mycobacterial cell aggregation is not known, it is assumed that the postulated large amounts of surface-exposed lipids render the mycobacterial surface hydrophobic, which given the property of acid fastness to the cell wall. Such properties of mycobacteria are considered to be due to the existence of miscellaneous lipophilic molecules, such as trehalose dimycolate, lipomannan, lipoarabinomannan and cell wall skeleton (Goren MB, 1979). Studies by Ortalo et al., (1995) and Lemassu et al., (1996), provided information on the implication of the major surface-exposed lipoglycans of *M. tuberculosis* and most other non-tuberculosis mycobacterial species, i.e., the glycogen-like glucan, arabinomannan, and mannan.

Cell surface properties like hydrophobicity, sliding motility or clumping have been previously demonstrated to reflect profound alterations of the cell envelope composition (Etienne et al., 2002). Hence we reasoned to analyse the cell surface properties of *M. smegmatis* mutants, which is supposed to be having a modified cell wall with increased lipoglycan content by the altered expression levels of genes involved in the mannose biosynthetic pathway. In this chapter, the detailed biochemical characterization of the mutants of *M. smegmatis* strains having *manA, manB, manC* and *pmmB* gene copies from *M. tuberculosis* H37Rv and a *pmmB* deletion mutant and its complement were described. Apart

from that, attempts were also made to analyse the cell surface properties of mutants which is expected to have an altered amount of lipoglycan mainly LM and LAM on the cell wall surface.

3.2. MATERIALS AND METHODS

3.2.1 Characterization of enzymes involved in the mannose biosynthetic pathway

3.2.1.1 Chemicals and reagents

GDP-D-mannose, adenosine diphosphate (ADP), D-mannose-1-phosphate (M1P), D-mannose-6-phosphate (M6P), D-glucose-1,6-bisphosphate, nicotinamide adenine dinucleotide phosphate (NADP), D(+) glucose, phosphomannose isomerase (PMI), phosphoglucose isomerase (PGI), hexokinase, sodium fluoride (NaF), sodium pyrophosphate (NaPPi), Tris buffer and magnesium chloride were purchased from Sigma, USA. D-glucose-6-phosphate dehydrogenase (G6PDH) was procured from Roche Diagnostics, Germany. Bradford's reagent used for total soluble protein quantification was procured from Bio-Rad, France. Other chemicals were of the analytical or molecular biology grade obtained from locally available sources.

3.2.1.2 Preparation of cell free extract

To prepare cell free intracellular extracts, all the *M. smegmatis* mutants, were grown in LB medium containing 25 µg ml^{-1} kanamycin and 0.05 % tween 80 at 37° C until OD 600 reached 1.0. Cultures were harvested by centrifugation at 6361*g* for 10 min at 4° C and was followed by the washing of the pellets with phosphate buffered saline (PBS). The washed bacterial pellets (≈ 2 g wet weight of cells) were resuspended in the specific extraction buffers at a concentration of ≈ 1 g of cell paste/5 ml. The buffer used for PMI extraction was 25 mM Tris-HCL (pH-7.6) containing 1mM PMSF and 5mM DTT (Gracy and Noltmann, 1968), for GMP, the buffer was 25 mM Tris-HCL (pH-7.6) containing 10 % glycerol, 1 mM EDTA, 1 mM DTT and 1 mM PMSF (Munch-Petersen, 1962) and for the PMM the buffer

was 50 mM HEPES (pH-7.1) containing 1 mM DTT, 0.5 mg ml^{-1} BSA, 0.1mM EDTA and 1mM PMSF (Pindar and Bucke, 1975). The cells were disintegrated by sonic oscillation with a probe tip sonicator at 80 % of maximum output for a total of 30 min (Sonics, USA). The sample was sonicated for 5 min in an ice bath and then cooled for 5 min. Sonication and cooling was repeated six times. At the completion of this procedure, the suspension was cleared by centrifugation at 4° C for 30 min at 27000 *g*. Protein concentrations of the cell free extracts were measured by Bradford's method using the BIO-RAD protein assay kit. The cleared cell lysates (4 mg of protein) was used as the source of enzyme.

3.2.1.3 Enzyme assays

The activity of all the enzymes were assayed using the respective cell lysate having 4 mg of total protein at 37° C in 1 ml^{-1} reaction volume for 1 h coupled with other auxiliary enzymes such as phosphoglucose isomerase (PGI), glucose-6-phosphate dehydrogenase (G6PDH), hexokinase and phosphomannose isomerase (PMI) . 1U is defined as the amount of enzyme producing one µ mol NADPH min^{-1} at 37° C.

Phosphomannose isomerase activity was measured by the coupled assay, in which the product fructose-6-phosphate is formed from M6P by the action of PMI is converted into glucose-6-phosphate (G6P) by the action of coupled enzyme PGI. G6P is then oxidized to 6-phosphogluconate by the action of glucose-6-phosphate dehydrogenase, the second coupled enzyme , and the reduction of $NADP^+$ to NADPH is measured by the change in absorption at 340 nm. Enzyme activity was assayed in 25 mM Tris-HCL (pH 7.6) containing 5 mM $MgCl_2$, 1 mM NADP, 3 mM M6P, 0.5 U of PGI, 1.0 U of glucose-6-phosphate dehydrogenase. The reaction was started by the addition of 3 mM M6P. Background levels were subtracted from enzyme assays in which M6P was omitted (Gracy and Noltmann, 1968).

GMP assay was coupled with hexokinase and in which the substrate GDP-mannose acted upon by the enzymes leads to the synthesis of NADPH, which is measured at 340 nm (Munch-Petersen, 1962). Assay of GMP reverse reaction was performed in 25 mM Tris-HCL (pH 7.6) containing 10 mM sodium flouride, 1 mM $MgCl_2$, 0.4 mM glucose, 0.1 mM ADP, 0.1 mM GDP- mannose, 1 mM NADP, 12 U of hexokinase and 1mM sodium pyrophosphate. The reaction was started by the addition of NaPPi (sodium pyrophosphate, freshly prepared) to give a final concentration of 1 mM. Background levels were subtracted from enzyme assays in which PPi (pyrophosphate) was omitted.

In the PMM assay, M1P was converted into M6P, and then the reaction was coupled with three enzymes: glucose-6-phosphate dehydrogenase, phosphoglucose isomerase and phospho mannose isomerise which results in the formation of NADPH and was measured at 340 nm. Enzyme activity was analyzed in 50 mM HEPES (pH 7.1) containing 5 mM $MgCl_2$ 0.025 mM NADP, 10 µg ml^{-1} glucose-6-phosphate dehydrogenase, 0.1 mM mannose-1-phosphate, 1µM glucose 1,6- bisphosphate , 10 µg ml^{-1} phosphoglucose isomerase and 3.5 µg ml^{-1} phosphomannose isomerase (Pindar and Bucke, 1975). Enzyme units were calculated after subtracting background levels from parallel assays in which mannose-1-phosphate was omitted.

3.2.2. Carbohydrate quantification and lipoglycan analysis

3.2.2.1. Chemicals and reagents

Mannoheptose, 1-aminopyrene-3,6,8-trisulfonate (APTS) and sodium cyanoborohydride ($NaBH_3$ CN) used for capillary electrophoresis were procured from Sigma-aldrich, France. All other chemicals and reagents used for lipoglycan extraction were purchased from locally available sources. TEA running buffer used was filtered through a 0.45 µ pore size filter and carefully degassed at 100 mbar.

3.2.2.2 Isolation of lipoglycan

Enriched lipoglycan fractions containing glycans and proteins were prepared as previously described (Fig. 3.1) (Nigou et al., 2000; Patterson et al., 2003). In this protocol, *M. smegmatis* cells of both mc^2155 and mutants were grown at 37° C in Middlebrook 7H9 medium supplemented with 25 μg ml^{-1} kanamycin and 0.05 % tween 80 for 3 days or until the OD 600 nm is 1.0. Properly grown cells were harvested by centrifugation at 6361 *g* for 10 min. The cells were delipidated with methanol and chloroform in the ratio of 2 : 1 for three times at 70º C with constant stirring (usually for 2 g of wet cells from 500 ml of culture was de-lipidated with 40 ml of methanol and 20 ml of chloroform). Chloroform: methanol fractions were collected after centrifugation at 400 *g* for 10 min at 21º C and used for the isolation of PIMs. The remaining pellets were dried, weighed and stirred with ethanol : water in the ratio of 1 : 1 for three times at 70º C for extracting the LM and LAM from the supernatant after centrifugation at 400 *g* for 10 min at 21º C. The remaining pellets were subjected to sonication with a probe tip sonicator at 80 % of maximum output for a total time of 30 min 'on ' cycle and 30 min 'off 'cycle and the lysate was treated with ethanol : water for three times to get cytosolic LM and LAM. The disrupted and non disrupted ethanol: water extracts were pooled, evaporated, weighed and used as the source of LM and LAM. Dried fractions were suspended in 20 mM Tris-HCL pH 7.4 containing 1mM $MgCl_2$, 0.02 % sodium azide for enzymatic digestion. For removing the nuclear content, the fractions were incubated with DNase and RNase in the concentration of 1 U g^{-1} of dry weight of ethanol : water extract for 18 h at 37º C. Freeze dried the samples after dialysis against water for 24 h at 4º C. Starch and protein contamination in the samples were removed by incubating it first with α-amylase for 18 h at 37º C and then with different proteases including trypsin and chymotrypsin for 18 h at 25º C sequentially but were not removed from the fractions to keep them as an internal reference between the different strains. Arabinose and mannose were quantified from an

equivalent amount of 1 µg of proteins by capillary electrophoresis after total acid hydrolysis (Nigou et al., 2000).

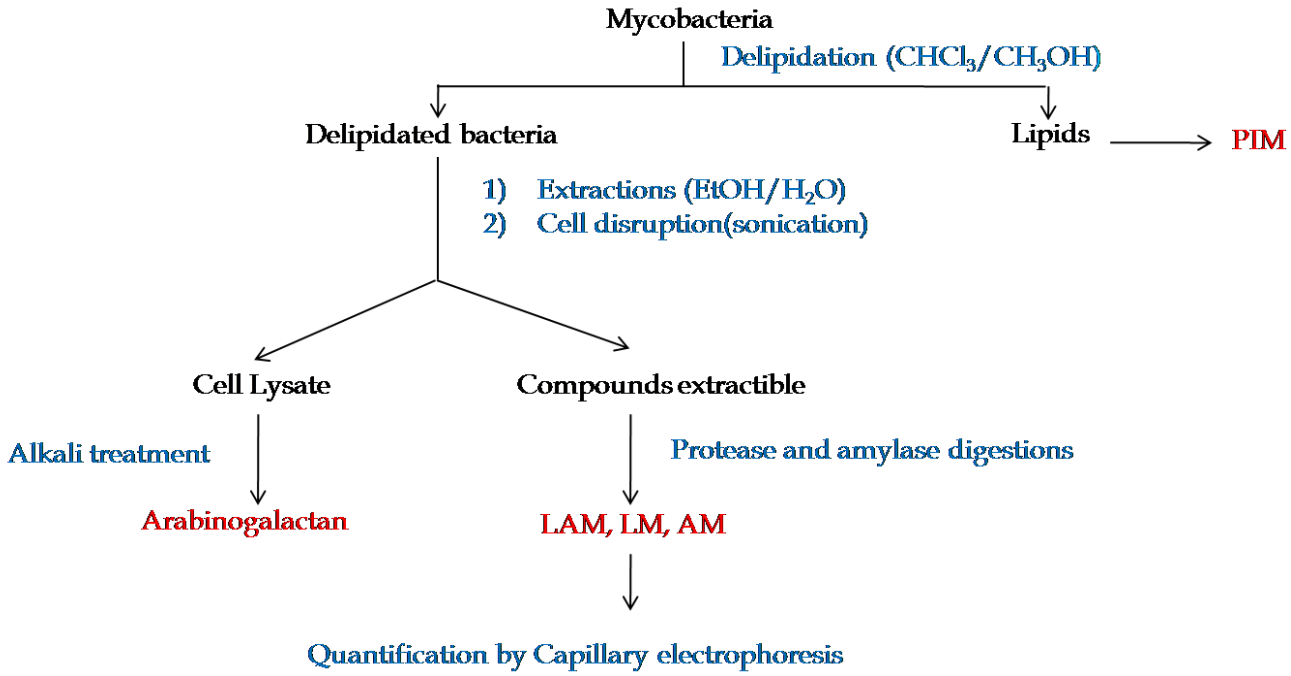


Fig. 3.1 Isolation of lipoglycan fractions from mycobacterial cells

3.2.2.3 Carbohydrate quantification by Capillary Zone Electrophoresis (CZE)

3.2.2.3.1 Principle

Capillary electrophoresis separations were performed on a P/ACE 5000 capillary electrophoresis system (Beckman Instruments, Inc.) with the cathode on the injection side and the anode on the detection side (i.e., reversed polarity), since the negatively charged, APTS-labelled carbohydrate molecules migrate toward the anode under the influence of the electric field and an injection pressure of 0.5 psi for 5 sec. The separations were monitored on column with a Beckman laser-induced fluorescence (LIF) detection system using a 4-mW argon-ion laser with the excitation wavelength of 488 nm and emission wavelength filter of 520 nm. The temperature of the capillary in the P/ACE instrument was controlled at 25º C

(Fig.3.2). The electropherograms were acquired and stored on an IBM 486/66 computer using the System Gold software package (Beckman Instruments, Inc.).

3.2.2.3.2 Arabinose and mannose quantification by capillary electrophoresis

Arabinose and mannose were quantified from an equivalent amount of 1 µg of proteins by capillary electrophoresis after total acid hydrolysis (Nigou et al., 2000). Dried and cleared cell lysate containing 25 µg of protein (for direct quantification of LM and LAM in the cell lysate) or purified LM and LAM containing 1µg protein in a 0.5 ml microfuge tube in a centrifuge vacuum concentrator at 25º C for acid hydrolysis. The carbohydrates were hydrolysed by adding 2 N of Tri Fluoro Acetic Acid (TFA) and 1 nm of mannoheptose for 2 h at 110º C in a sand bed. Hydrolysed samples were dried and were labelled through reductive amination by the addition of APTS in 15 % acetic acid and $NaBH_3CN$ in tetra hydrofuran (THF) for 1 h 30 min in a water bath set at 55º C. The samples were dried and mixed with 20 µl of double distilled water and was diluted 20 times with water and was injected into Capillary Zone Electrophoresis (CZE) for analysis.

Capillary Zone Electrophoresis separations were carried out on a 47 cm × 50 µm (I.D) uncoated fused-silica capillary column of 40 and 50 cm effective length (47 and 57 cm total length) (Sigma, Division Supelco, St.Quentin, France) with Beckman laser indused fluorescence (LIF) detection. The capillary was flushed with 0.1 N HCL before each injection. In all the separations, 25 mM TEA (pH 4.75) containing a polymeric additive was used as running buffer. The use of this buffer resulted in very high relative migration time reproducibility (RMT RSD. 0.1 %) and low and stable current (<20 µA). The labelled samples were injected by the pressure injection mode of the system, typically for 5–10 sec at 0.5 psi (3447.38 Pa). The electropherograms were acquired, stored and compared with the peaks obtained from 1 nm mannoheptose standard as showed in Fig. 3.2 and calculated the amount of arabinose and mannose in the total cell lysate and purified LM and LAM.

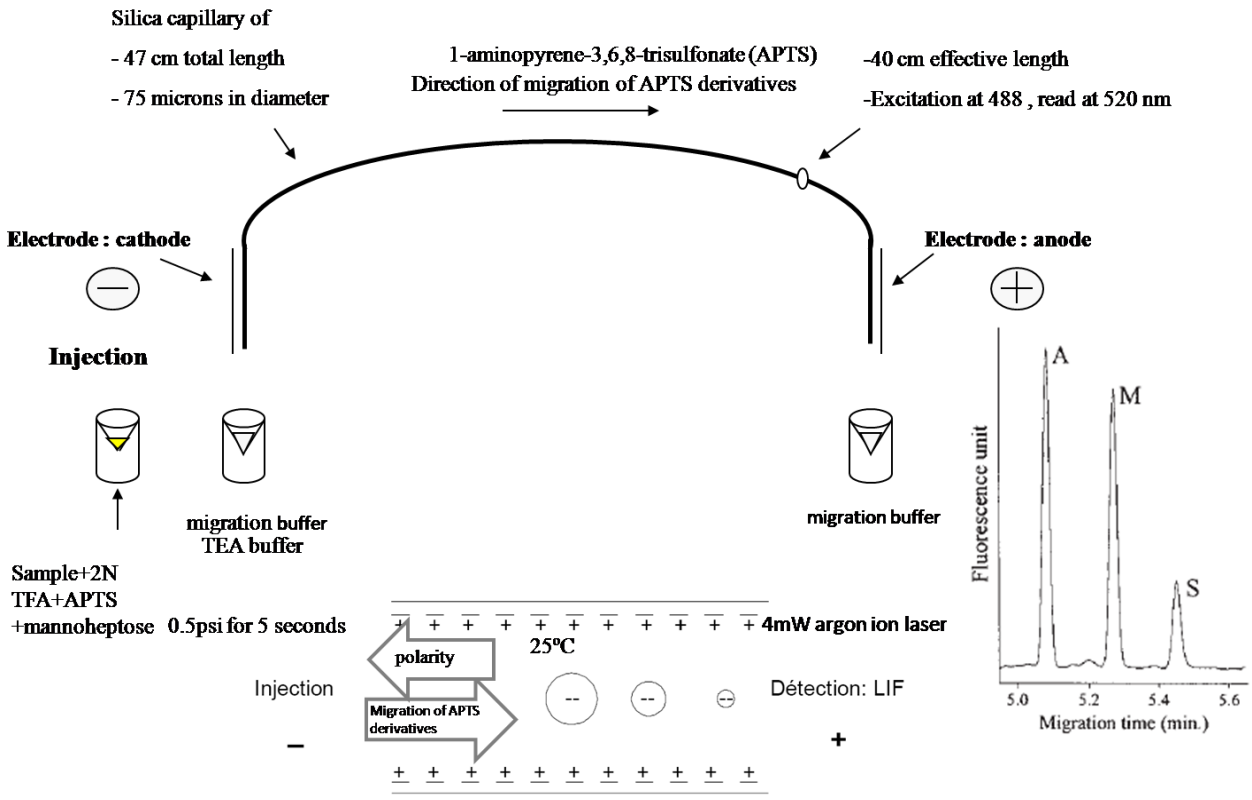


Fig. 3.2 Capillary electrophoresis - principle

3.2.2.4 Lipoglycan analysis

Lipoglycan fractions isolated containing glycans and proteins were prepared by the protocol specified in the chapter section 3.2.2.2. Contaminating proteins which were not removed after protease digestion was considered as an internal reference between the different strains. Lipoglycans were analyzed by SDS-PAGE (10 µg of proteins were loaded on the gel for each strain) followed by periodic acid-silver nitrate staining explained in the paper by Azuma et al., (1968).

3.2.3 Lipoprotein quantification

Mycobacterial cells (1.5 g) were disrupted by sonication and unbroken cells were removed by gentle centrifugation. Lipoproteins were extracted from the cleared lysate by a phenol/water

partition (Ozinsky et al., 2000; Zahringer et al., 2008). The phenol phase containing lipoproteins was dialyzed against water, dried and weighed.

3.2.4 Cell-surface properties of *M. smegmatis* mutants

M. smegmatis mutants were grown in LB broth with agitation for preparing the inoculums in bottles and as surface pellicles on plates of Sauton's medium (Sigma-aldrich, France), at 37º C without agitation, when information was needed on extracellular materials.

Mycobacterial cell surface properties were determined as described by Gilles et al., (2002). A single-cell suspensions were prepared with late exponential phase cultures incubated in 100 ml Sauton's medium. The pellicles were harvested by pouring off the medium and were gently shaken for 30 sec with 5 g glass beads (4 mm diameter). The declumped cells resulting from this treatment were suspended in 10 ml LB plus 0.05 % tween 80 and centrifuged for 10 min 100 g. The OD_{650} of the supernatant, which contains mainly single cells, was adjusted to 1 and directly used to inoculate the media (100 µl suspensions for 50 ml broth) for the determination of some of the cell surface properties. Alternatively, cells were washed three times with PBS to give a PBS-washed single cell suspension, or stored at -80° C in the presence of 20 % (w/v) glycerol for further inoculation. 50 ml of cells grown with or without tween 80 after 3 days was used for the determination of the following cell surface studies,

(i) Cellular aggregation was quantified by cultivating mycobacteria in LB broth without tween 80. The unicellular mycobacteria were separated from the aggregates by differential centrifugation (Cougoule et al., 2002) and the cellular aggregation was calculated as the percentage of aggregate-containing pellets versus total cell weight.

(ii) For congored accumulation assay (Cougoule et al., 2002), mycobacteria were cultivated in LB broth plus 100 mg congo red ml^{-1} and 0.05 % tween 80. The cells were then washed extensively with distilled water and the congored that remained associated with the cells was

extracted with acetone. The congo red binding index was defined as the A_{488} of the acetone extracts divided by the dry weight of the cell pellet.

(iii) The relative hydrophobicity was assessed by the hexadecane partition procedure (Rosenberg, 1980). A single cell suspension of each strain (OD_{650} 1) was mixed with 0.3 ml hexadecane and the hydrophobicity index was defined as the percentage reduction in the OD_{650} of the aqueous phase after complete separation of the two phases.

3.3 RESULTS AND DISCUSSION

3.3.1 Characterization of the *M. smegmatis* wild and mutant strains

There are three enzymes directly engaged in the pathway leads to GDP-mannose, PMI, PMM and GDPMP (Fig.1.1). In the present study PMI, PMM and GDPMP enzymatic activities in the different recombinant strains using *in vitro* assays on bacterial lysates coupled with auxiliary enzymes were done. Use of coupled assays for determining the activities of PMI, PMM and GDPMP were well established (Gracy and Noltmann, 1968; Munch-Petersen, 1962; Pindar and Bucke, 1975) and it is sensitive at microgram levels of substrate concentration. In coupled assays the specific activities of all the enzymes were calculated at microgram levels of the NADPH product that is measured at 340 nm.

Overexpression of *manA* resulted in more than 6-fold increase in PMI activity as compared to the control strain whereas PMM and GDPMP activities were not affected (Fig. 3.3 or table 3.1). Similarly, GDPMP activity was increased by around 2.5-fold in the *manC* over expressing strain (*Msmeg*/pMVmanC) but not in the other ones (Fig. 3.3 or table 3.1). In the present conditions, no PMM activity could be detected in the control or non-relevant strains. However, an activity of around 0.6 mU mg^{-1} of total proteins was measured in both strains overexpressing *manB* (*Msmeg*/pMVmanB) or *pmmB* (*Msmeg*/pMVpmmB) (Fig. 3.3 or table 3.1). The study revealed that, the recombinant strains such as *Msmeg*/pMVmanA,

Msmeg/pMVmanB, *Msmeg*/pMVmanC and *Msmeg*/pMVpmmB showed an individual increase of each enzymatic activity involved in GDP-mannose biosynthesis as expected.

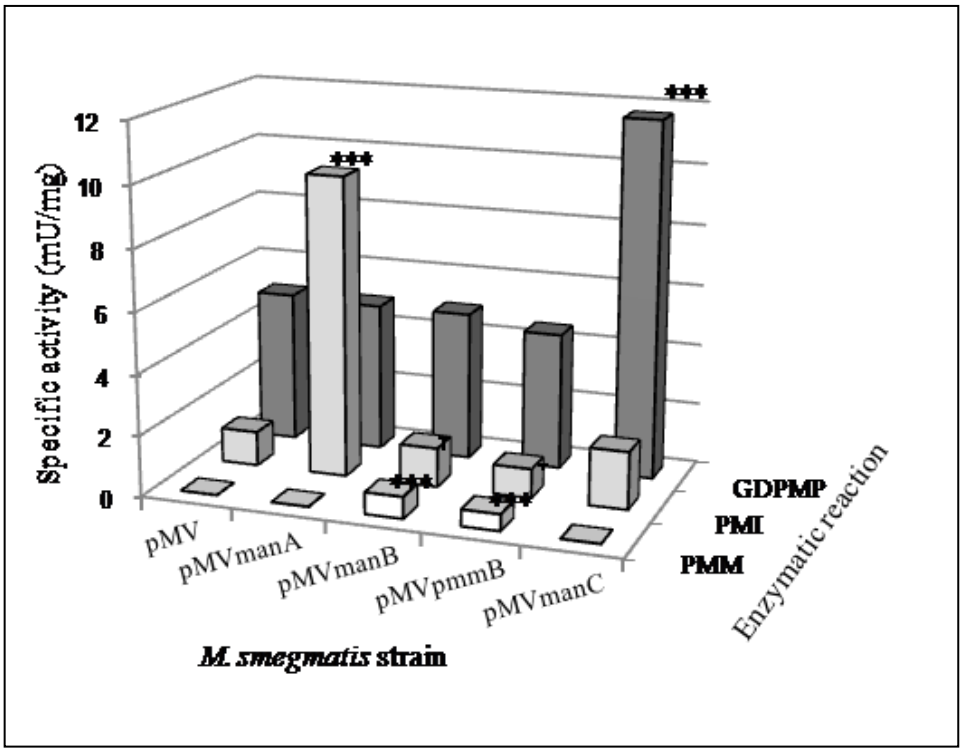


Fig. 3.3 Enzyme activity of *M. smegmatis* mutants

Table 3.1 Enzyme activity of *M. smegmatis* mutants

	pMV	pMVmanA	pMVmanB	pMVmanC	pMVpmmB
PMI	1.127	9.867***	1.280	1.887	0.956
PMM	0	0	0.707***	0	0.540***
GDPMP	5.067	4.933	4.867	11.7***	4.5

Several genes involved in mannoglycoconjugate biosynthesis have been shown to be involved in virulence due to their central role in the biosynthesis of major surface-associated glycoconjugates. In the case of *Leishmania mexicana*, an intracellular pathogen of macrophages (Handman and Bullen, 2002), disruption of genes encoding PMI (Garami and Ilg, 2001), PMM (Garami et al., 2001), GDPMP (Garami and Ilg, 2001) and dolichol phosphate mannose synthetase (Garami et al., 2001) activity led to decreases in the amount of surface-associated mannosylated lipoglycans. These results indicate the importance of manno glycoconjugate biosynthesis, the reaction catalysed by the enzymes involved in GDP-mannose synthesis, later supplies mannose for the synthesis of mannoglycoconjugates, specifically lipoglycans.

McCarthy et al., (2005) demonstrated that the overexpression of *M. tuberculosis manB* in *M. smegmatis* have produced mycobacterial strain with increased amounts of the major cell wall mannosylated glycoconjugates, LAM, LM and higher order PIMs, the latter of which leads to increased association of this strain with human macrophages.

To examine the consequences in terms of lipoglycan production, the amount of pentose sugars such as mannose and arabinose which are known to compose the various lipoglycans were quantified in each strain. An increase of around 70% of these sugars was observed for *Msmeg*/pMVmanB, *Msmeg*/pMVpmmB and *Msmeg*/pMVmanC as compared to the control strain (*Msmeg*/pMV). On the other hand, no change was detected in *Msmeg*/pMVmanA (Fig. 3.4). These results indicated an increase of lipoglycan production in *Msmeg*/pMVmanB, *Msmeg*/pMVpmmB and *Msmeg*/pMVmanC mutants.

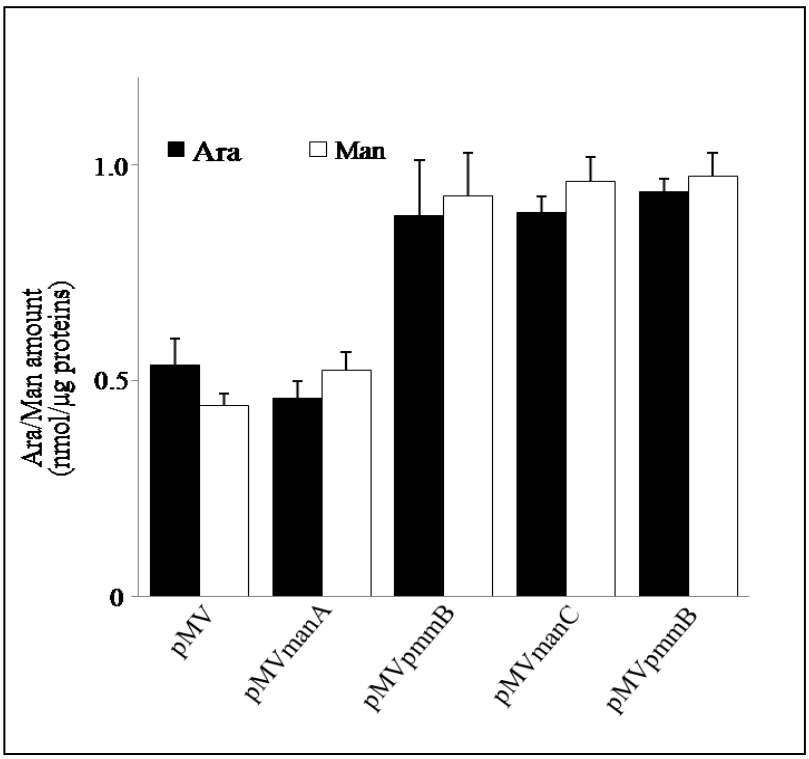


Fig. 3.4 Sugar quantification of *M. smegmatis* mutants by capillary electrophoresis

Alternatively, the lipoglycans were analysed by SDS-PAGE followed by silver nitrate staining. The samples were loaded on the gel according to a fixed amount of proteins (10µg of protein /well). The gels showed the presence of a higher amount of lipoglycans, with a slight increase in the apparent molecular weight in the three strains over expressing ManB, PmmB and ManC which showed an increased enzyme activity of the respective enzymes (Fig. 3.5 a). Enhancing GDP-mannose synthesis supplies activated form of mannose to the pathway which leads to the synthesis of PIM, LM and LAM (Patterson et al., 2003). However the chance of mannosylation of the proteins is not neglected. Hence, in order to support the hypothesis that the activated mannose will be utilized by the various mannosyl transferases and form PIM, LM and LAM, studies conducted to isolate and analyze the lipoproteins from

the cell free lysate of the mutants. Fig. 3.5 b showed the lipoprotein distribution of the mutants and all the strains showed more or less same amount (55±3 mg per 1.5 g cells) as showed by the wild type strain. This observation provided additional information that the increased production of recombinant enzymes doesn't influence the synthesis of lipoprotein synthesis, on the other hand it supply activated mannose was mostly used towards the synthesis of lipoglycans.

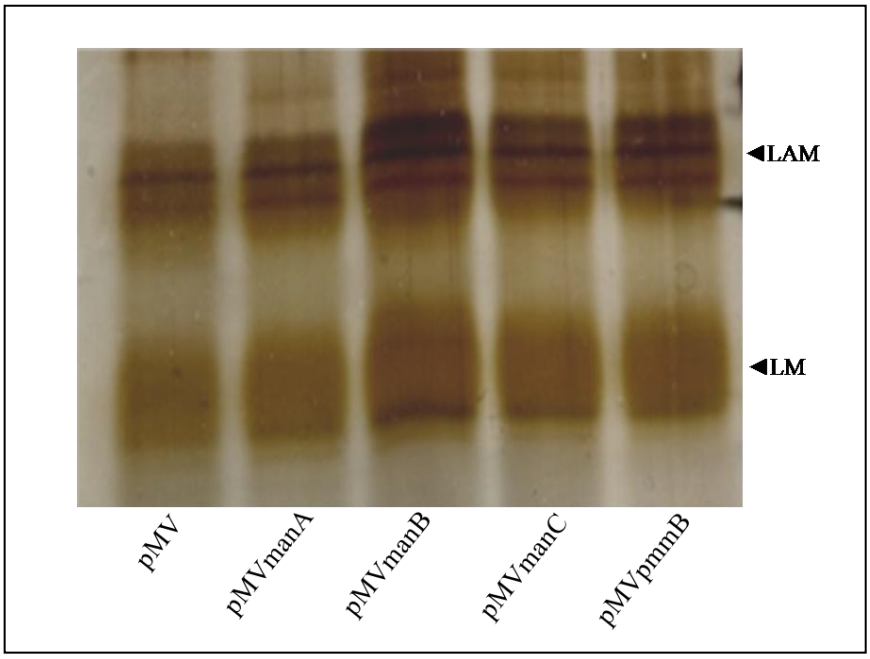


Fig. 3.5 a Lipoglycan quantification in *M. smegmatis* mutants by 12 % SDS-PAGE

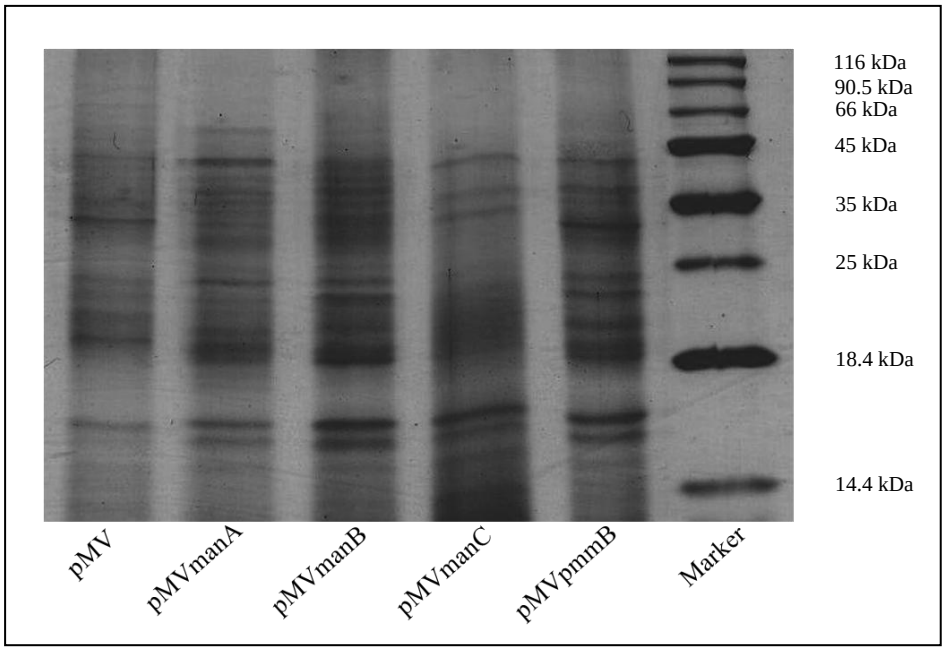


Fig. 3.5 b Lipoprotein distribution on 12 % SDS-PAGE

20µg of protein was loaded on each well

It was demonstrated above that *pmmB* gene product encodes an enzyme with PMM activity and that the latter affects lipoglycan biosynthesis (Fig. 1.1). High density mutagenesis previously proved that this gene was non-essential in *M. tuberculosis* (Sassetti et al., 2003). Hence effects were made to investigate whether the deletion of its ortholog *MSMEG1695* in *M. smegmatis* could result in a strain producing reduced amount of lipoglycans. One *M. smegmatis* mutant was constructed by exchanging the wild-type allele of *MSMEG1695* with a kanamycin resistance cassette-disrupted allele. One clone, named *Msmeg*/ΔpmmB, exhibiting an amplification pattern consistent with an allelic exchange at the *MSMEG1695* locus, was retained for further analysis. Carbohydrate quantification, as shown in the Fig. 3.6 a showed that the lipoglycan-enriched fraction of *Msmeg*/ΔpmmB contained only half amount of arabinose and mannose as compared to the wild-type strain. SDS-PAGE

analysis confirmed that *Msmeg*/ΔpmmB produced a reduced amount of LAM and LM as compared to the wild-type strain (Fig. 3.6 b). It was interesting to note that the lipoglycan production was restored (Fig. 3.6 b) in the *M. smegmatis* mutant upon complementation with pMV/*MSMEG1695* (*Msmeg*/ΔpmmB/CL).

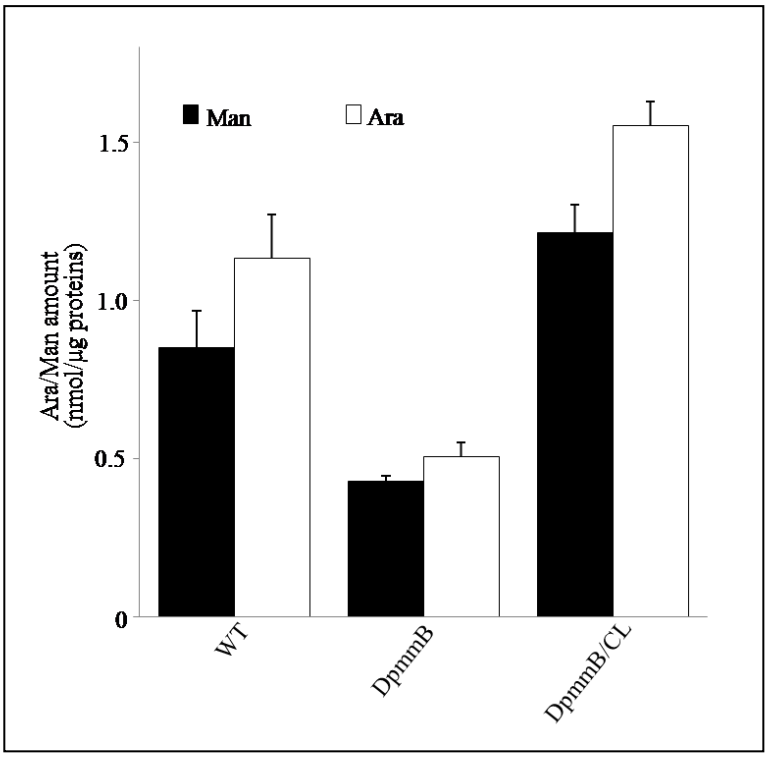


Fig. 3.6 a Sugar quantification of wild and *pmmB* mutants by capillary electrophoresis

WT-wild type, DpmmB-pmmB deletion mutant and DpmmB/CL-pmmB complement

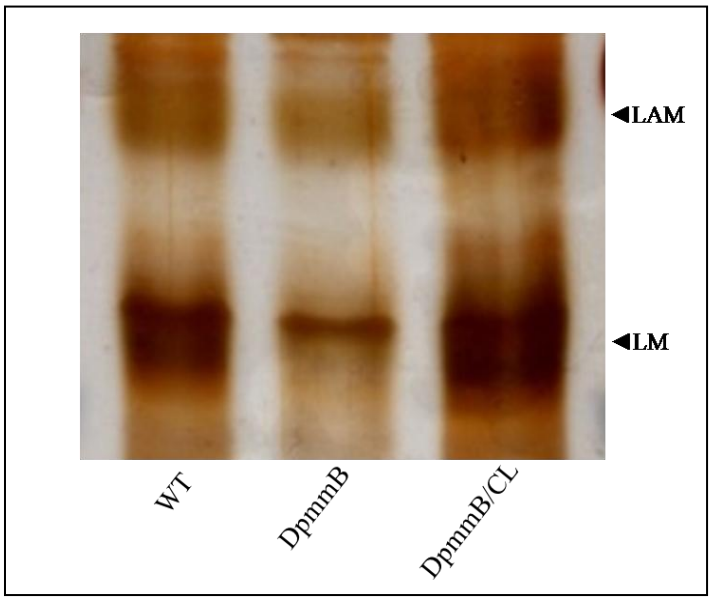


Fig. 3.6 b Lipoglycan quantification in PmmB mutants by SDS-PAGE

WT-wild type, DpmmB-pmmB deletion mutant and DpmmB/CL-pmmB complement

McCarthy et al., (2005) reported that *manB* overexpression deposited LM and LAM on the mycobacterial cell wall. Lipoglycan quantification and analysis on *M. smegmatis* mutants of manB, manC and pmmB were proved that there is an increased deposition of LM and LAM on the surface of cell wall. Even though, McCarthy et al., (2005) analysed and proved that there is an increased deposition of lipoglycans on the cell wall, the study never tried to analyse the nature of the cell wall, which is supposed to be modified with the high lipid content. Since mycobacterial cell surface properties like hydrophobicity, cellular aggregation are having relation to pathogenecity, studies focussing on the cell wall properties can provide information on the role of these molecules on mycobacterial invasion and pathogenesis. In the present study cell surface properties of all the mutants were analysed and compared and the following observations were made,

(i) In mutants, *Msmeg*/pMVmanB, *Msmeg*/pMVpmmB and *Msmeg*/pMVmanC the cellular aggregation was more compared to control (Fig. 3.7 a)

(ii) Mutants, *Msmeg*/pMVmanB, *Msmeg*/pMVpmmB and *Msmeg*/pMVmanC showed more affinity towards Congo red which is a hydrophobic dye (Fig. 3.7 b)

(iii) Mutants, *Msmeg*/pMVmanB, *Msmeg*/pMVpmmB and *Msmeg*/pMVmanC were more hydrophobic towards hexadecane (Fig. 3.7 c).

These results suggested that the lipoglycan deposition on the cell wall of Mycobacterium is able to modify cell wall properties, which in turn plays some role in making the cell more pathogenic, as the properties like cellular aggregation, hydrophobicity are helpful in adhere to the cell surface which is an important first step in microbial colonization in new environments.

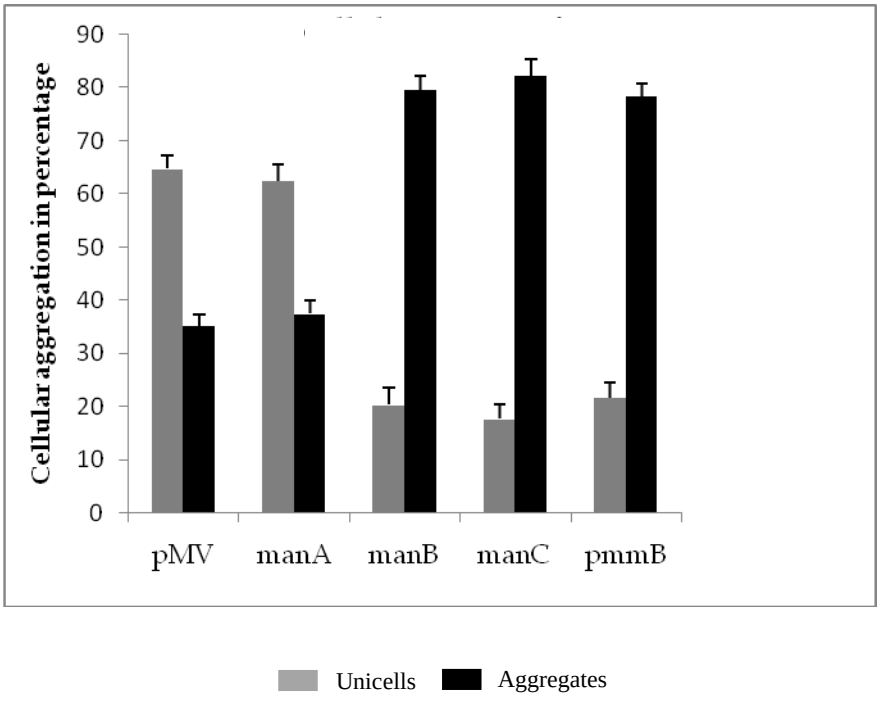


Fig. 3.7 a Cell surface properties of *M. smegmatis* mutants - Aggregation

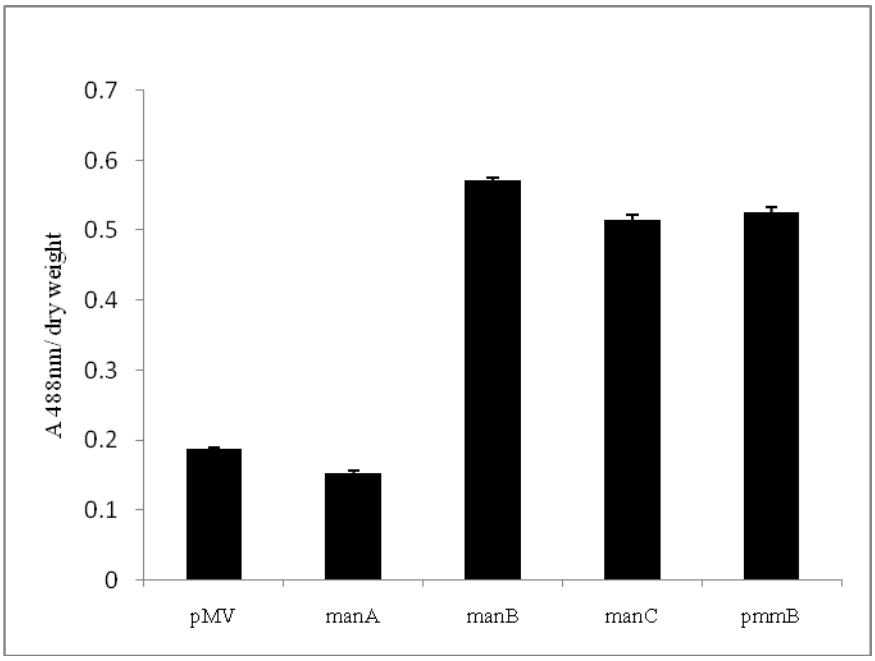


Fig. 3.7 b Cell surface properties of *M. smegmatis* mutants - Congored accumulation

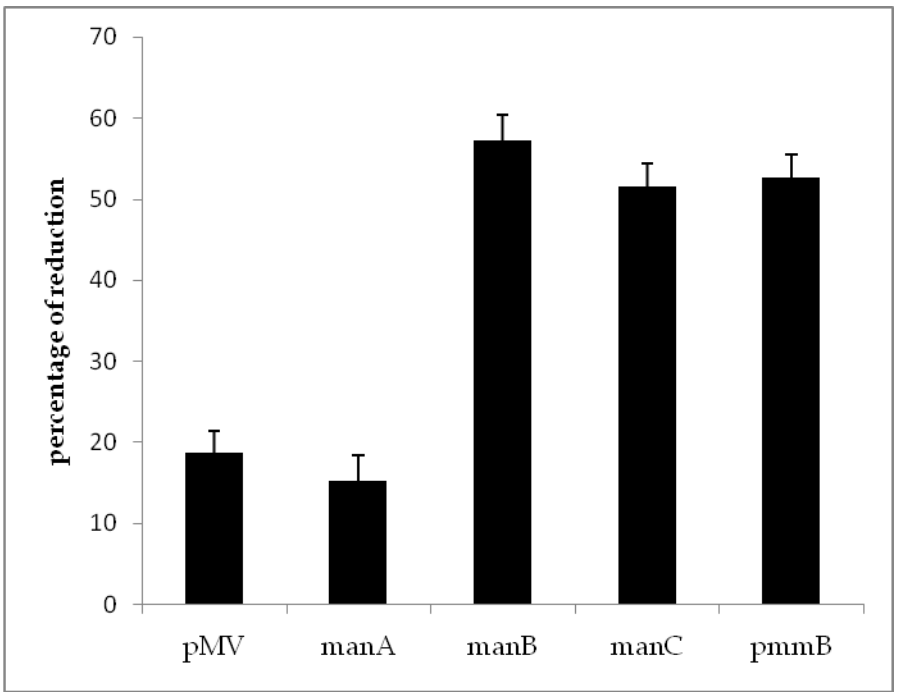


Fig. 3.7 c Cell surface properties of *M. smegmatis* mutants - Hydrophobicity

3.4 CONCLUSION

Overexpression of PMM and GDPMP resulted in an increased deposition of lipoglycans mainly LM and LAM on the surface of Mycobacterium, whereas PMI overexpression has no effect on the lipoglycan deposition. These results suggested that the genes coding PMM (*manB*-Rv3257c and *pmmB*-Rv3308) and gene coding GDPMP (*manC*-Rv3264c) are the rate limiting genes in mannose metabolism in *M. tuberculosis.* Since *pmmB* deletion reduces the lipoglycan deposition, it gives the additional information that PmmB could be an additional phoshomanno mutase coded by *pmmB* in Mycobacterium and it is simultaneously expressed along with *manB.* Cell surface studies like affinity towards congo red and hydrophobicity provided information on the fact that enhanced lipoglycan deposition makes the cell surface more hydrophobic. Mutants of *manB*, *manC* and *pmmB* were more aggregative compared to the wild *M. smegmatis* mc^2 155.

CHAPTER 4

Immune response of *M. smegmatis* mutants towards TLR 2 mediated innate immunity

CHAPTER 4

Immune response of *M. smegmatis* mutants towards TLR 2 mediated innate immunity

4.1 INTRODUCTION

Innate immune recognition is based on the detection of molecular structures that are unique to microorganisms (Medzhitov, 2007). It involves a limited number of germline-encoded pattern recognition receptors (PRRs) that recognize conserved molecules of microbes, referred to as microbe associated molecular patterns (MAMPs). MAMPs follow three criteria: i) they have an invariant core structure among a given class of microorganisms, ii) they are products of pathways that are unique to microorganisms and iii) they are essential for the survival of the microorganism and are therefore difficult for it to alter (Medzhitov, 2007). The best characterized class of PRR is Toll-Like Receptors (TLRs). TLRs trigger innate immune responses through NF-κB-dependent and IFN regulatory factor-dependent signalling pathways (Akira et al., 2006). Among all TLRs, TLR 2 is most probably the receptor that recognizes the structurally broadest range of MAMPs (Zahringer et al., 2008). Indeed, its ligands are as diverse as lipoproteins, lipopeptides, lipoteichoic acid (LTA), peptidoglycan, zymosan, GPI anchors or lipoglycans (Zahringer et al., 2008). This high diversity in ligand recognition has been proposed to possibly arise, at least in part, from its capacity to function as a heterodimer with either TLR 1 or TLR 6 (Ozinsky et al., 2000). However, because some of these molecules are structurally unrelated, their real nature as TLR 2 ligands is a matter of controversy (Zahringer et al., 2008). Moreover, no obvious structure-function relationship can be drawn as one could expect from an ordinary receptor-ligand interaction (Beutler,

2004). This chaotic situation results from both the use of incompletely defined agonist preparations and the lack, until very recently, of high resolution structural data defining these interactions at the atomic level (Zahringer et al., 2008). For example, the TLR 2 activity originally found in some commercially available LPS preparations was subsequently demonstrated to arise from endogenous contaminating lipoproteins (Hirschfeld et al., 2000; Tapping et al., 2000). This assumption is reinforced by the recently published crystal structure of a TLR 1 - TLR 2 heterodimer in complex with the model lipopeptide Pam_3CSK_4 (Jin et al., 2007). It clearly showed the importance of ligand acyl chains to bind and induce heterodimerization of the receptors and to provide a rationale to tentatively understand the ligand structure-function relationships, although the presence of binding sites other than that of lipopeptides cannot be excluded (Pathak et al., 2007). LTA, that bears two acyl chains, has been unambiguously proved, using chemically synthesized analogs, to stimulate TLR 2 (Morath et al., 2002). However, its role as a physiological TLR 2 ligand is not clearly established (Hashimoto et al., 1997; Zahringer et al., 2008). A set of studies focusing on *Staphylococcus aureus* and using cell wall-derived compounds as well as a mutant lacking acylated lipoproteins, demonstrated that LTA is much less active than lipoproteins and suggested that not LTA but lipoproteins are the dominant immunobiologically active compounds in this Gram-positive bacterium (Hashimoto et al., 2006; Zahringer et al., 2008). As a consequence, in a recent review, Zahringer et al., (2008) proposed that lipoproteins/ lipopeptides are the only compounds of microorganisms sensed at physiological concentrations by TLR 2.

Mycobacteria and related genera of the order *Actinomycetales* are Gram-positive bacteria that do not express LTA but rather lipoglycans (Sutcliffe, 2005), which are surface-exposed (Pitarque et al., 2008) and have been described by others (Gilleron, 2008) and also by us to act as TLR 2-TLR 1 agonists. Their structure is based on a mannosyl-phosphatidyl-

myo-inositol anchor (Fig. 1.6), which, although very similar to the GPI anchors found in eukaryotic cells, is specific of genera *Actinomycetales* which include mycobacteria (Gilleron, 2008). The biosynthesis of the mannosyl-phosphatidyl-*myo*-inositol anchor is essential in mycobacteria (Gilleron, 2008; Kordulakova et al., 2002). The most active lipoglycan, lipomannan (LM), is sensed at concentrations similar to that of mycobacterial lipoproteins and showed recently that it can compete for lipopeptide binding to TLR 2, suggesting that it shares at least in part the same binding site (Nigou et al., 2008). Assuming that it is the case, straightforward structure-function relationships can account for the observed TLR 2-stimulatory capacity of the various purified LM acyl-forms (Gilleron et al., 2006; Nigou et al., 2008). Nevertheless, a contamination of lipoglycan fractions by highly active lipopeptides is formally difficult to rule out. Moreover, a *M. tuberculosis* mutant deficient for lipoprotein processing is dramatically altered in its capacity to stimulate TLR 2 (Banaiee et al., 2006), suggesting, as for *S. aureus*, a predominant role of lipoproteins in mycobacteria sensing by TLR 2.

In order to determine whether lipoglycans are indeed: i) *bona fide* TLR 2 ligands and ii) sensed at physiological concentrations in the context of the whole bacterium, we used the model organism, *M. smegmatis* to generate mutants altered for the production of lipoglycans. Since the biosynthesis of the latter is difficult to abrogate (Kordulakova et al., 2002), attempts were made to construct some strains with an augmented production of lipoglycans and a deletion mutant of *pmmB* and were analysed biochemically in detail in the previous chapters. In the present chapter, the focus was to look into the interaction between lipoglycans deposited on the cell surface of *M. smegmatis* mutants, and thus to evaluate whether lipoglycans are MAMPs involved in the detection of mycobacteria through TLR 2.

4.2 MATERIALS AND METHODS

4.2.1 HEK-TLR 2 experiments

4.2.1.1 Cell lines and media

HEK-Blue™-hTLR 2 cells along with HEK-Blue™, Normocin™ (50 mg ml^{-1}) QUANTI-Blue™, alkaline phosphatase detection medium were procured from Invivogen, France. Dulbecco's Modified Eagle Medium (DMEM), glucose, fetal bovine serum, penicillin, streptomycin, L-glutamine and DMSO were purchased from sigma Aldrich, France.

4.2.1.2 Principle

HEK-Blue™-hTLR 2 cells are designed for studying the stimulation of human TLR 2 (hTLR 2) by monitoring the activation of NF-κB. HEKBlue ™-hTLR 2 cells were obtained by co-transfection of the hTLR 2 and CD 14 co-receptor genes and an optimized secreted embryonic alkaline phosphatase (SEAP) reporter gene was placed under the IFN-b minimal promoter fused to five NF-κB and AP-1 binding sites into HEK 293 cells. Stimulation with a TLR 2 ligand activates NF-κB and AP-1 which induces the production of SEAP (Fig. 4.1). Levels of SEAP can be easily determined with QUANTI-Blue™ a detection medium that turns purple/blue in the presence of alkaline phosphatase (Fig. 4.2). Alternatively, HEK Blue ™ Detection, a cell culture medium that allows for real-time detection of SEAP can be used (Sandor et al., 2003). HEK 293 cells express endogenous levels of TLR 1, TLR 3, TLR 5, TLR 6 and NOD 1. TLR 2 is involved in the recognition of a wide array of microbial molecules. Simultaneous expression of the extracellular and intracellular domains of both TLR 1 and TLR 2 is essential for ligand recognition and subsequent ligand induced signal activation (Sandor et al., 2003).

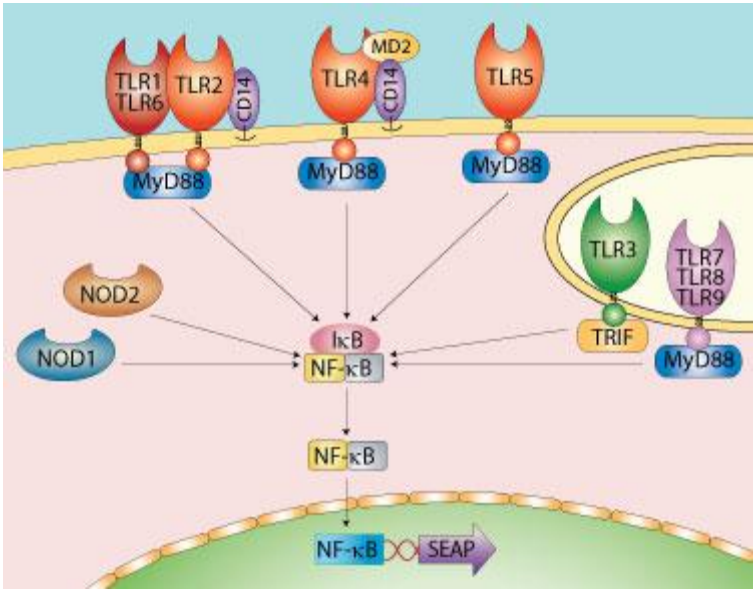


Fig.4.1 Monitoring of TLR 2 activation via NF-κB activated SEAP (Invivogen)

TLR2 activates the synthesis of NF-κB, which in turn induce the transcription of SEAP

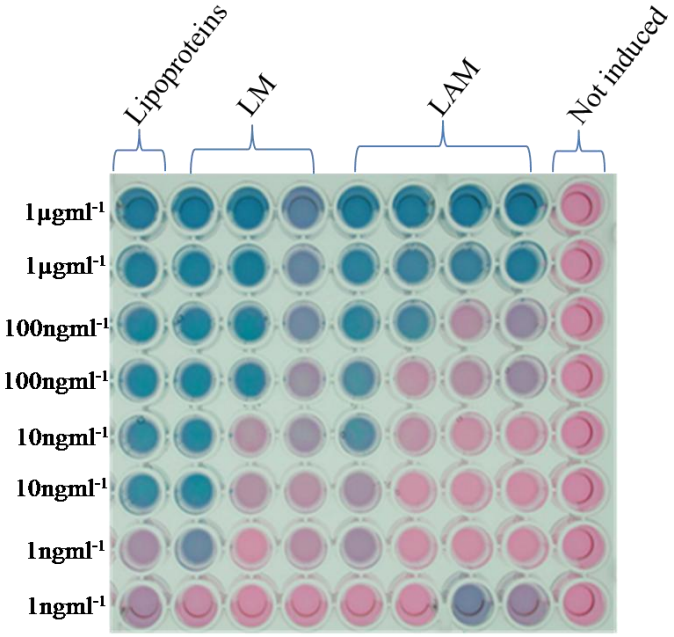


Fig. 4.2 Quantification of SEAP with QUANTI-Blue™ a detection medium

4.2.1.3 Procedure

4.2.1.3.1 Initial culture preparation

For preparing the initial culture for propagation, the cells thawed slowly by gentle agitation in a water bath 37° C. The cells were decontaminated by washing with 70 % (v/v) ethanol. After decontamination, cells were transferred in a larger vial containing 15 ml of pre-warmed growth medium (DMEM, 4.5 g l^{-1} glucose, 10 % (v/v) fetal bovine serum, 50 U ml^{-1} penicillin, 50 mg ml^{-1} streptomycin, 100 mg ml^{-1} Normocin™, 2 mM L-glutamine). After removing the supernatant containing the cryoprotective agent by centrifugation at 300 g for 5 min the cells were resuspended in growth medium without selective antibiotics. Cells were then diluted by transferring the vial contents to a 25 cm^2 tissue culture flask containing 5 ml of growth medium without selective antibiotics and incubated the cells at 37˚ C in 5 % CO_2.

4.2.1.3.2 Cell maintenance

The cells were maintained and subcultured in growth medium supplemented with 1X HEK-Blue™ selection was done for every 2 weeks. When 70-80 % confluency is reached, the cells were passaged. For use, the cells were detached in the presence of PBS by tapping the flask or by using a sterile cell scraper.

4.2.1.3.3 HEK-TLR 2 Stimulation

A cell suspension of HEK-Blue™-hTLR 2 cells at ~ 280,000 cells/ml was prepared in test medium which contains 10 % (v/v) heat inactivated FBS. For TLR 2 stimulation, HEK-Blue™-2 cells were added in the HEK-Blue™ detection medium, which contains a substrate for alkaline phosphatase, at 5×10^4 cells per well in 96-wells plates and the samples were incubated with a positive control such as FSL-1, 1 mg ml^{-1}, a negative control such as sterile, endotoxin-free water and different *M. smegmatis* recombinant strains harbouring the plasmids such as *pMV* (control), *pMV manA, pMV manB, pMV manC, pMV DpmmB* and *DpmmB/CL* at multiplicity of infection (MOI) ranging from 1 to 0.1. Alkaline phosphatase activity was

measured after 18 h of incubation at 37° C in a CO_2 incubator for 20-24 h by reading OD at 630 nm.

4.2.2 THP-1 experiments

4.2.2.1 Cell lines and media

THP1-XBlue™ cells (5-7×10^6 cells) in freezing medium along with Normocin™ (50 mg ml^{-1}), Zeocin™ (100 mg ml^{-1}), QUANTI-Blue™ and alkaline phosphatase detection medium were procured from Invivogen, France. L-glutamine, sodium bicarbonate, glucose, HEPES, sodium pyruvate, fetal bovine serum and DMSO were procured from Sigma Aldrich, France.

4.2.2.2 Principle

THP1-X Blue™ cells were derived from the human monocytic THP-1(Human acute monocytic leukemia) cell line. They were obtained by stable transfection of THP-1 cells with a reporter plasmid expressing a secreted embryonic alkaline phosphatase (SEAP) gene under the control of a promoter inducible by the transcription factors NF-κB and AP-1. Upon TLR stimulation, THP1-XBlue™ cells will activate the transcription factors and subsequently the secretion of SEAP THP1-XBlue™ cells will express all the TLRs, as determined by RT-PCR, but respond only to ligands for TLR 2, TLR 1/2, TLR 2/6, TLR 4, TLR 5 and TLR 8. THP1-XBlue™ cells are resistant to the selectable marker Zeocin™.

4.2.2.3 Procedure

4.2.2.3.1 Initial Culture preparation

Cells were propagated initially by thawing immediately the cell containing vials by gentle agitation in a water bath at 37° C. The vials were removed from the water bath as soon as the contents are thawed, and decontaminated by washing with or by spraying with 70 % ethanol. The cells were diluted in 15 ml of pre-warmed growth medium (RPMI 1640 (2 mM L-glutamine, 1.5 g l^{-1} sodium bicarbonate, 4.5 g l^{-1} glucose, 10 mM HEPES and 1.0 mM sodium pyruvate) with 10% fetal bovine serum (FBI), 100 mg ml^{-1} Normocin™, Pen-Strep (50 U ml^{-}

^{1}-50 μg ml^{-1}). The supernatant containing cryoprotective agent was recovered by centrifugation of the vial at 200 - 300 g for 5 min and suspended the vial contents to a 25 cm^{2} tissue culture flask containing 5 ml of growth medium. The cell suspension was incubated at 37˚ C in 5 % CO_2.

4.2.2.3.2 Cell maintenance

Once the cells were recovered and grown well, they were maintained and subcultured in growth medium supplemented with 200 μg ml^{-1} of Zeocin™. The cells were passed every 3 days by inoculating 5×10^{5} cells/ml.

4.2.2.3.3 Detection of TLR stimulation

The THP-1 monocyte/macrophage human cells were pelleted by centrifugation at 200 - 300 *g* for 5 min and resuspended at 2-4× 10^{6} cells/ml in fresh assay medium (RPMI 1640 with 10% heat-inactivated fetal bovine serum). Cell suspensions were added at 10^{5} cells per well in 96-wells plates in HEK-Blue™ detection medium or differentiated with 20 ng ml^{-1} of PMA for 24 h in RPMI 1640 culture medium (Lonza, Verviers, Belgium). The different recombinant *M. smegmatis* strains, *pMV* (control), *pMV manA, pMV manB, pMV manC, pMV DpmmB* and *DpmmB/CL* were added at MOI ranging from 10 to 1 and, after 18 h, NF-κB activation was measured either by determine the SEAP levels using a spectrophotometer at 630 nm or by cytokines were assayed in the supernatant by sandwich ELISA using commercially available kits (Diaclone, Besançon, France). In the kit, mouse anti-human IL-8 act as the capture antibody, and the biotinylated goat anti-human IL-8 act as detection antibody and the activity were detected with Streptavidin-HRP **(**horseradish peroxidise labelled), incubated with its substrate H_2O_2 along with Tetramethyl benzidine followed by reading the absorbance at 450 nm after inactivating the reaction with 2 N H_2SO_4. To investigate TLR 2 dependence, cells were pre-incubated for 30 min at 37° C, before adding the bacteria along with 5 μg ml^{-1} of

anti-TLR 2 antibodies (IgG2a clone TL2.1 or IgA1, Invivogen) or isotype controls (IgG2a, eBioscience or IgA1, Invivogen).

4.3 RESULTS AND DISCUSSION

In the present study, three *M. smegmatis* recombinant strains moderately overproducing lipoglycans, *Msmeg*/pMVmanB, *Msmeg*/pMVpmmB and *Msmeg*/pMVmanC, and a knock-out mutant with a reduced production of lipoglycans, *Msmeg*/ΔpmmB (Fig. 3.5 a and Fig. 3.6 b), were available to evaluate the possible implication of these compounds in mycobacteria sensing by TLR 2. It is noteworthy that these strains contained wild-type amount of lipoproteins (55 ± 3 mg/1.5 g cells) as determined by analysis and quantification of the phenol extracts (Fig. 3.5 b). First of all, tested the relative ability of the different strains to stimulate HEK 293 cells stably transfected with human TLR 2 and 14 genes (HEK-TLR 2 cells) and a NF-κB-inducible reporter system. All the strains CD induced NF-κB activation in HEK-TLR 2 cells (Fig. 4.3 a and 4.3 b) but not in the parent HEK cells, and it indicated that the activation was specific for TLR 2. It was noted that, *Msmeg*/pMVmanB, *Msmeg*/pMVpmmB and *Msmeg*/pMVmanC were more stimulatory with reproducibility than the control strain (*Msmeg*/pMV), and *Msmeg*/pMVmanA mutant (Fig. 4.3 a). On the other hand, *Msmeg*/ΔpmmB was less stimulatory than the wild-type strain (Fig. 4.3 b). This experiment demonstrated that there is an association between the level of lipoglycan production on one hand and the magnitude of TLR 2 signaling on the other hand.

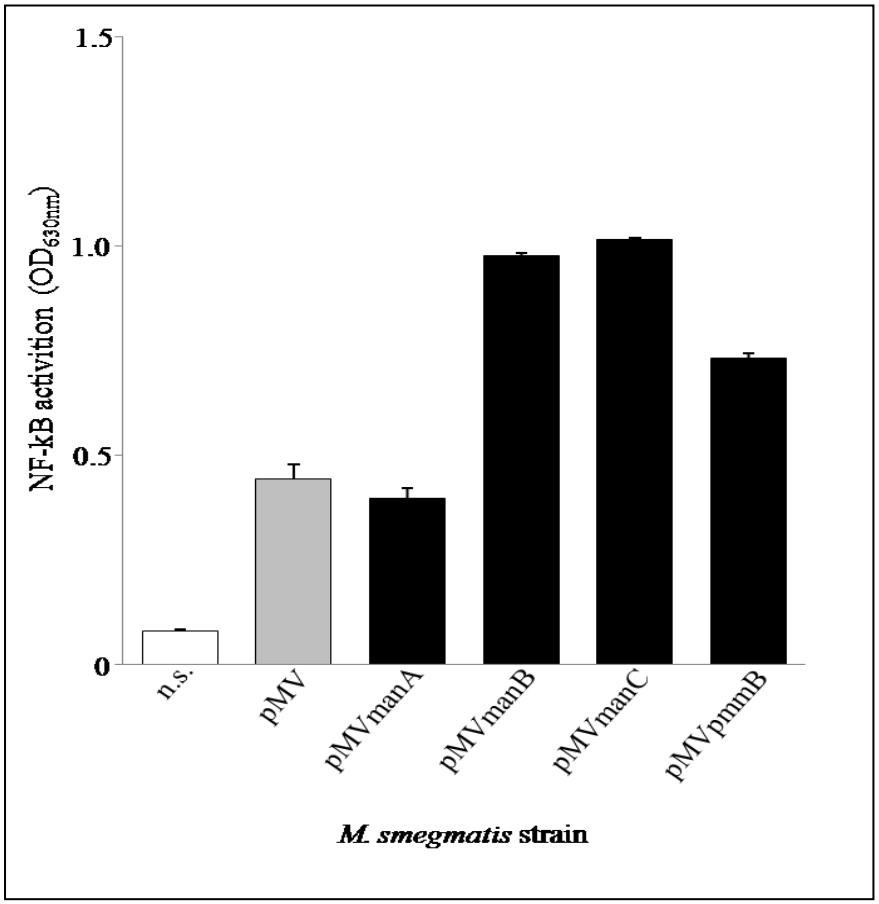


Fig. 4.3 a Increased lipoglycan production is associated with an enhancement in TLR 2 signalling and cytokine production-NF-κB activation in HEK-TLR 2, (n.s.- not stimulated)

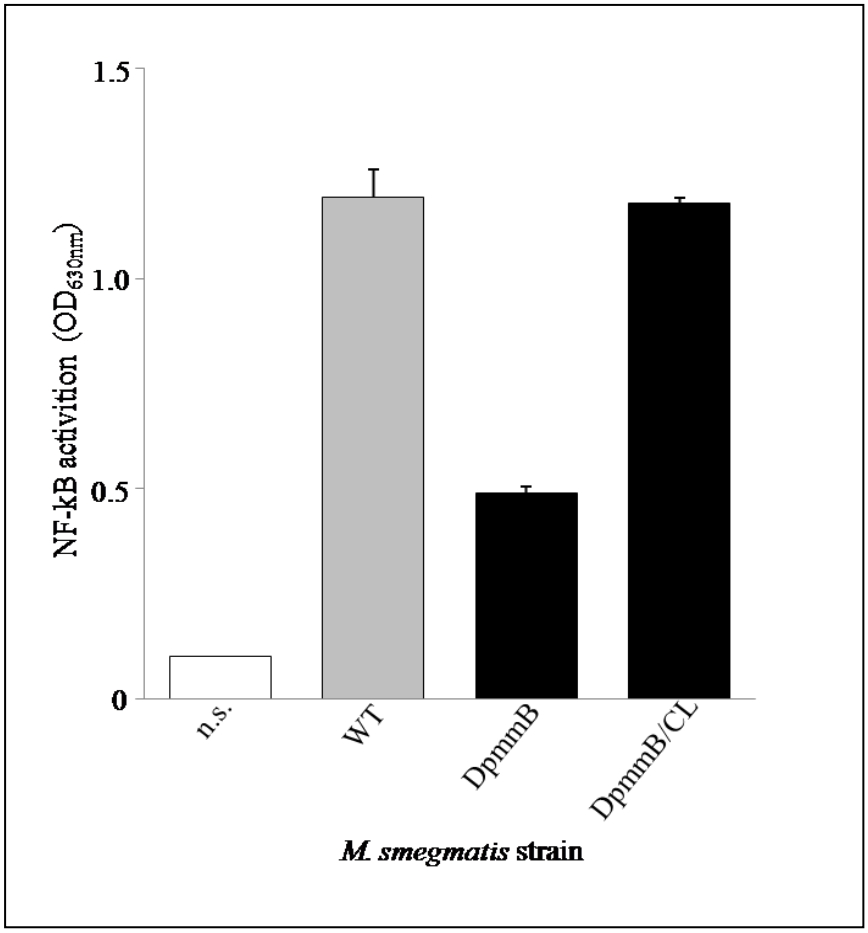


Fig. 4.3 b Reduced lipoglycan production is associated with a decrease in TLR 2 signalling and cytokine production- NF-κB activation in HEK-TLR 2

To confirm it in more physiological cells, it was decided to investigate the relative capacity of the different strains to activate the human THP-1 monocyte/macrophage cell line, using a cell line derivative that stably expresses a NF-κB-inducible reporter system. Again, it was found that *Msmeg*/pMVmanB, *Msmeg*/pMVpmmB and *Msmeg*/pMVmanC were more potent than the control strains in their ability to induce NF-κB activation (Fig. 4.4 a) and IL-8

release (Fig. 4.4 b). The IL-8 release was found to be mainly depended on TLR 2 signaling as determined by antibody blocking experiments (Fig. 4.4 c).

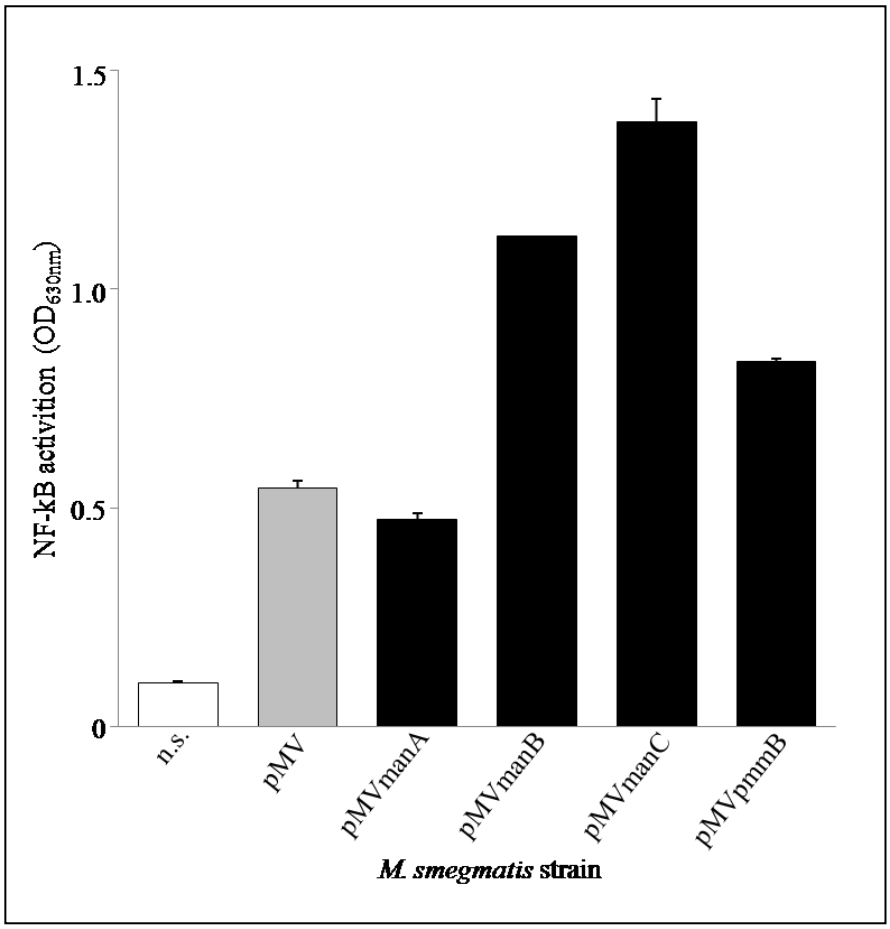


Fig. 4.4 a Increased lipoglycan production is associated with an enhancement in TLR 2 signalling and cytokine production-monocyte/macrophage THP-1 cells -NFκB production

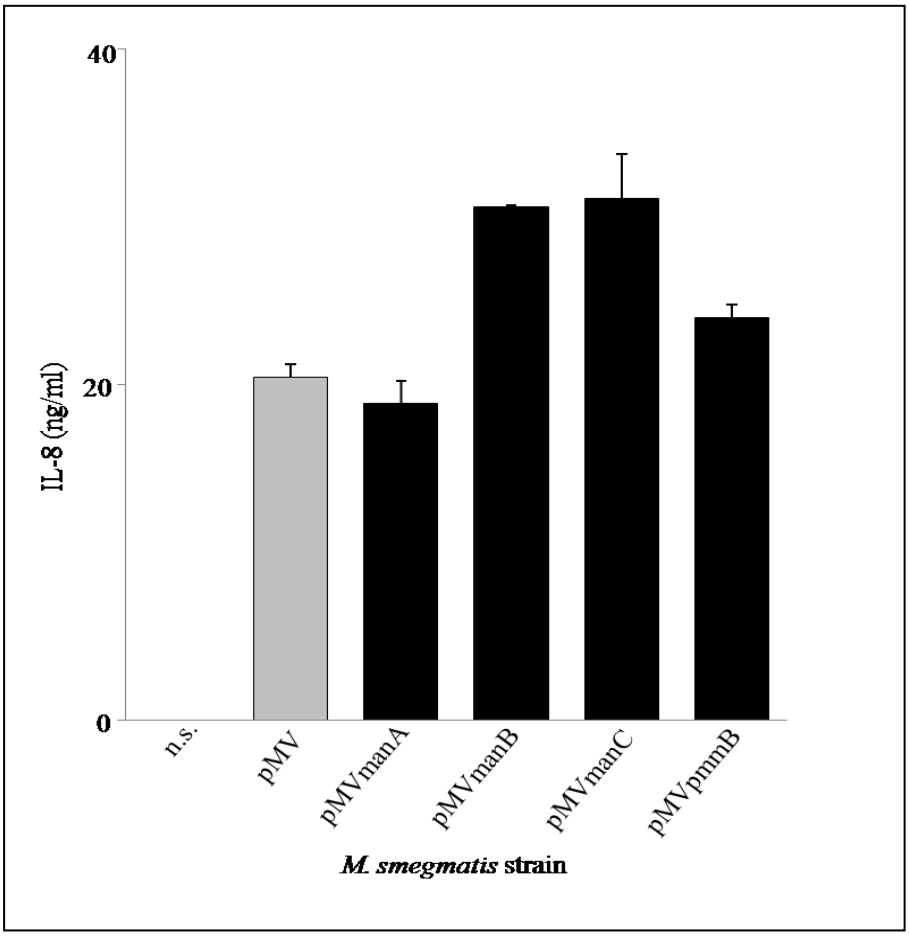


Fig. 4.4 b Increased lipoglycan production is associated with an enhancement in TLR 2 signalling and cytokine production-IL-8 production by THP-1 cells

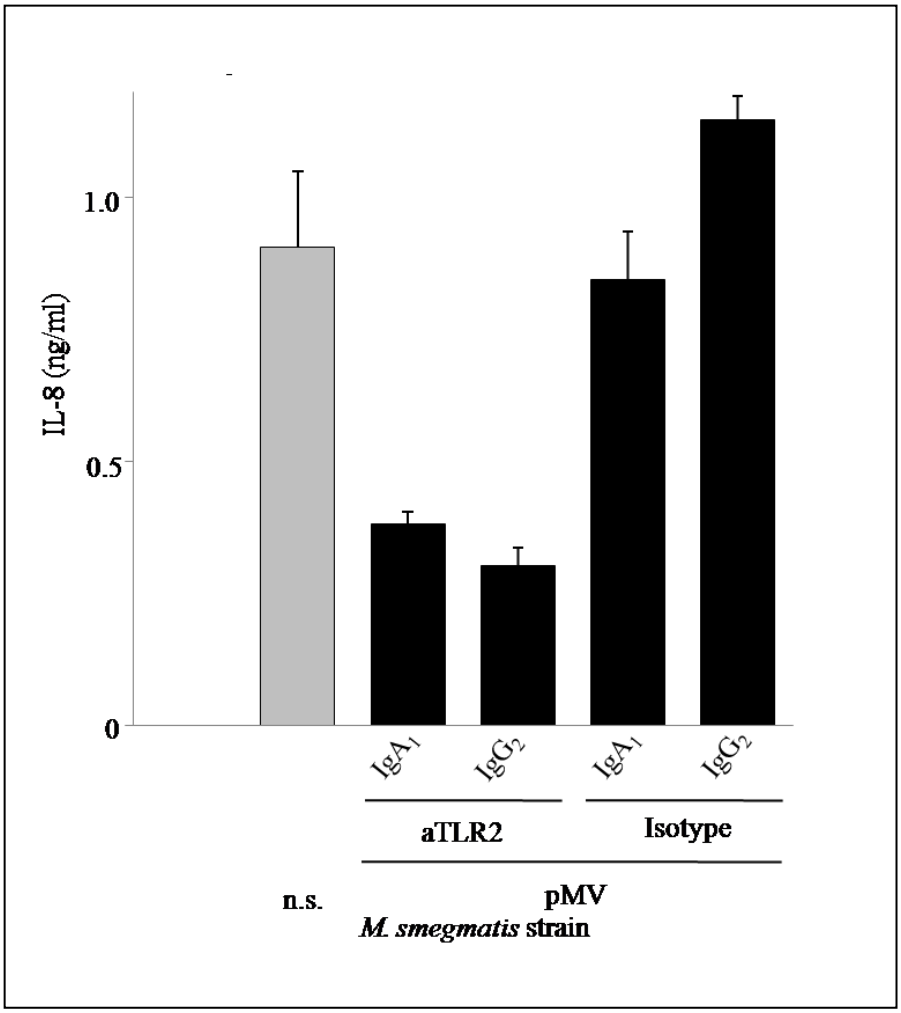


Fig. 4.4 c Increased lipoglycan production is associated with an enhancement in TLR 2 signalling and cytokine production-IL-8 production by THP-1 cells.

Cells were pre-incubated for 30 min at 37º C before the addition bacteria along with various antibodies, anti TLR isotype controls, at a concentration of 5 µg ml^{-1}

Similar results were obtained for IL-6 or TNF-α production (Fig. 4.5 a and Fig. 4.5 b). Interestingly, *Msmeg*/ΔpmmB was less potent than the wild-type strain in its ability to induce NF-κB activation (Fig. 4.5 c) and IL-8 release (Fig. 4.5 d) in THP-1 cells. However, wild-type activation was restored in the complemented strain. These results were confirmed in human monocyte-derived dendritic cells with *Msmeg*/ΔpmmB mutant inducing a reduced amount of IL-8 (Fig. 4.5 e).

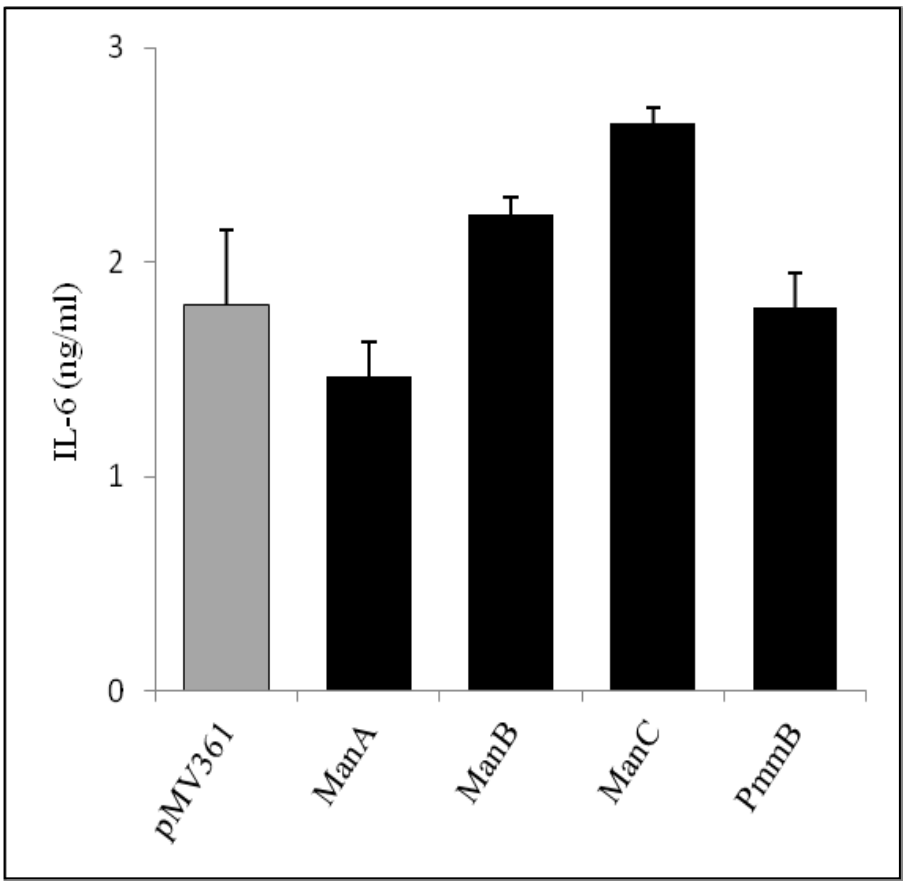


Fig. 4.5 a Increased lipoglycan production is associated with an enhancement in TLR 2 signalling and cytokine production-IL-6 production by THP-1 cells

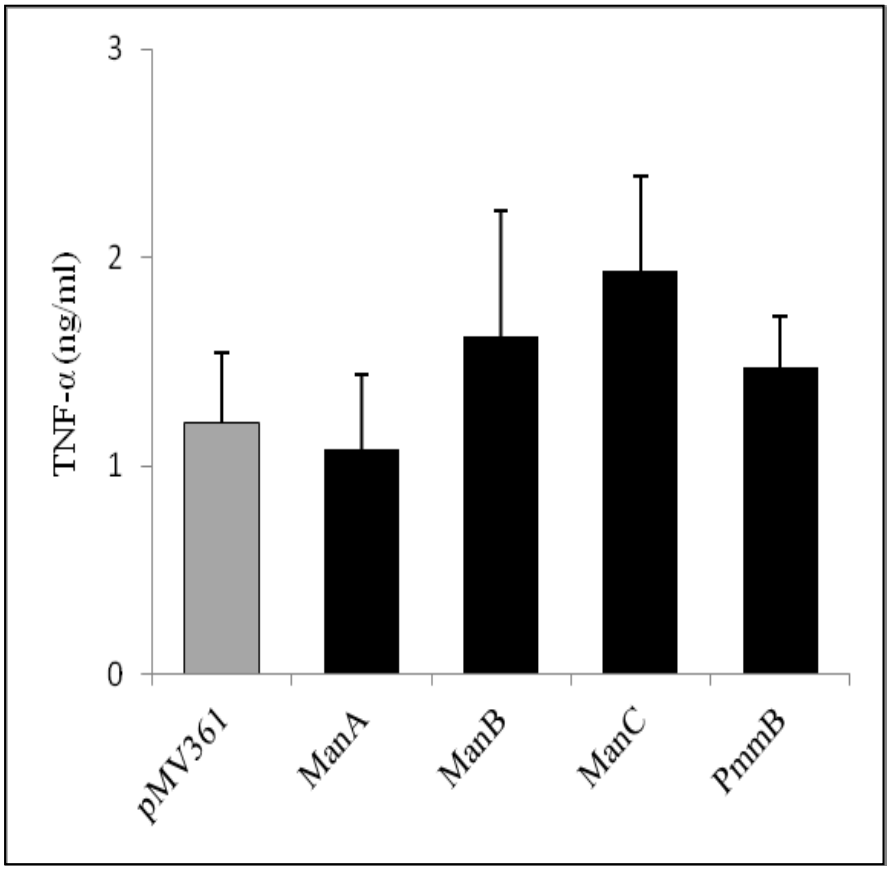


Fig. 4.5 b Increased lipoglycan production is associated with an enhancement in TLR 2 signalling and cytokine production-TNF-α production by THP-1 cells

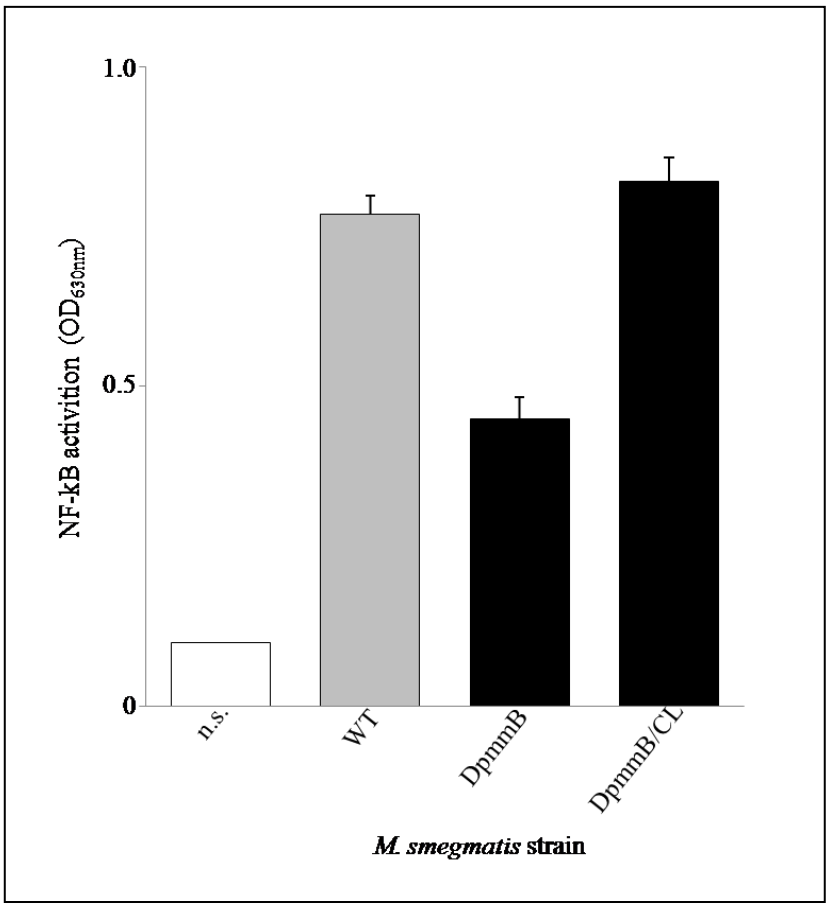


Fig. 4.5 c Reduced lipoglycan production is associated with a decrease in TLR 2 signalling and cytokine production- monocyte/macrophage THP-1 cells-NFκB production

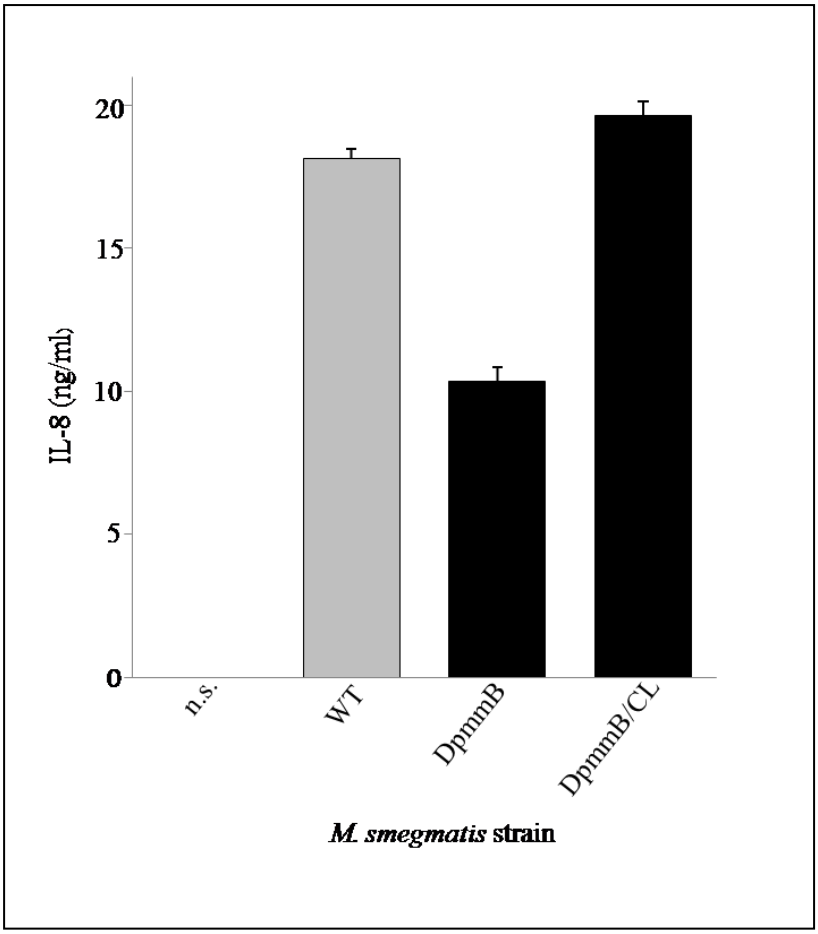


Fig. 4.5 d Reduced lipoglycan production is associated with a decrease in TLR 2 signalling and cytokine production-IL-8 production by THP-1 cells

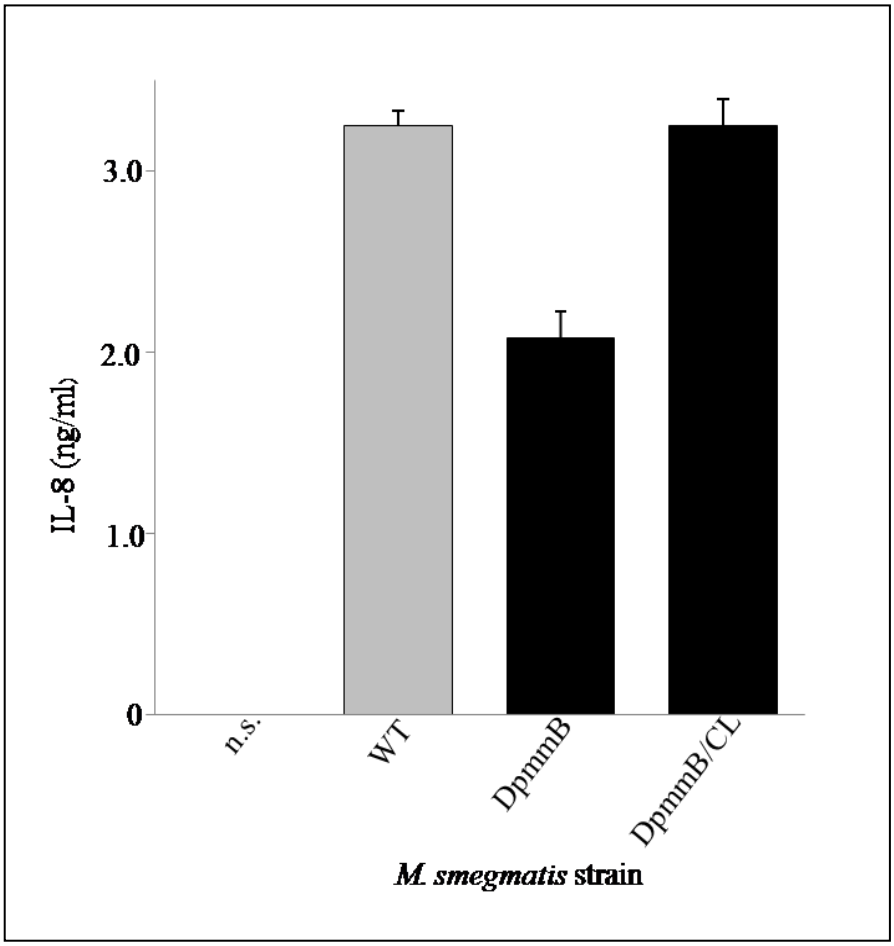


Fig. 4.5 e Reduced lipoglycan production is associated with a decrease in TLR 2 signalling and cytokine production-IL-8 production by monocyte derived dendritis cells

Innate immune detection of mycobacteria involves several PRRs that cooperate to mediate both uptake of the bacilli into host cells and activation of intracellular signaling cascades that signal the presence of the bacterial invader (Korbel et al., 2008). Amongst these receptors, TLR 2 plays a key role in initiating the production of pro-inflammatory cytokines and chemokines that are crucial in eliciting the protective adaptative immune response (Lyakh et al., 2008). TLR 2 recognizes lipoproteins that are ubiquitously found in bacteria,

including mycobacteria. Accordingly, several purified mycobacterial lipoproteins have been found to activate TLR 2 (Brightbill et al., 1999) and a *M. tuberculosis* mutant deficient for lipoprotein processing is dramatically altered in its capacity to stimulate TLR 2 (Banaiee et al., 2006). Lipoglycans are another family of TLR 2 agonists found in the cell envelope of mycobacteria (Gilleron, 2008) and the most active ones, LM, are sensed by the receptor at concentrations similar to that of lipoproteins (Nigou et al., 2008). However, so far, their activity has been demonstrated using purified molecules only and a contamination by highly active lipopeptides is formally difficult to rule out. Moreover, their role as physiological ligands in the context of a whole bacterium infection is not clearly established (Zahringer et al., 2008). All the results in correlation with literature suggested that, lipoglycans are bonafied ligands in activating the innate immunity through TLRs specifically TLR 2 and activation of TLR 2 is directly proportional to the lipoglycan deposition on the cell wall of Mycobacterium.

4.3 CONCLUSION

Studies on cell lines such as HEK and THP-1 enlighten the fact that a slight increase of lipoglycan amount (~1.7 fold) resulted in an enhancement of mycobacteria-induced TLR 2 signaling, demonstrated that lipoglycans could be the *bona fide* TLR 2 ligands that can be sensed at physiological concentrations in the context of the whole bacterium. *pmmB* gene deletion experiments supported the above result and in addition it gives information about the *pmmB* essentiality in lipoglycan biosynthesis. Hence, besides the immunodominant lipoproteins/ lipopeptides, lipoglycans were also considered as MAMPs contributing to the innate immune detection of mycobacteria by TLR 2.

CHAPTER 5

Heterologous expression of the mannose biosynthetic genes of *M. tuberculosis* in *E.coli*

CHAPTER 5

Heterologous expression of the mannose biosynthetic genes of *M. tuberculosis* in *E.coli*

5.1 INTRODUCTION

Recombinant expression systems have been developed for the expression of proteins coded by the gene of interest with considerable ease and high yield. These expression systems can be divided into bacterial, yeast, insect cell lines and mammalian expression systems with each having specific purposes. Bacterial expression systems are commonly used because these are considered to be the cheapest and fastest (Swietnicki, 2006). Among the various bacterial expression systems, *E. coli* remain to be the most suitable host for the expression of foreign proteins because of its relative simplicity, inexpensive cultivation and well known genetics (Sorensen et al., 2003).

The *E. coli* system has some drawbacks, like the proteins which are produced as inclusion bodies are often inactive, insoluble and require refolding. In addition, there is a problem producing proteins with many disulfide bonds and refolding these proteins is extremely difficult. The *E. coli* system produces unmodified proteins without glycosylation which is the reason why some of the antibodies produced fail to recognize mammalian proteins (Jenkins and Curling, 1994). However to improve the *E. coli* expression, the following measures can be taken: (i) use of different promoters to regulate expression (ii) use of different host strains (iii) co-expression of chaperones and/ or foldases (iv) lowering of temperature (v) secretion of proteins into the periplasmic space or into the medium (vi) reducing the rate of protein synthesis (vii) changing the growth medium (viii) addition of a

fusion partner (ix) expression of a fragment of the protein and (x) in vitro denaturation and refolding of the protein (Choi and Lee, 2004; Chou, 2007; Maldonado et al., 2007; Mergulhao et al., 2005; Shiloach and Fass, 2005; Swartz, 1996; Wong et al., 2008).

The recombinant protein in general represents the major fraction of inclusion body proteins (Singh and Panda, 2005). Purification of recombinant proteins in the form of inclusion bodies can be achieved by 'N' and/or 'C' terminal histidine tagging of proteins by nickel affinity chromatography (Hengen, 1995).

Mycobacteria contain a variety of mannose-containing compounds including lipoarabinomannan, lipomannan (Hunter et al., 1986), mannophosphoinositides (Subrahma.D, 1965) and other glycolipids and polysaccharides (Brennan, 1989). LM and LAM were proved to have physiological activities and these are considered as MAMPs which can interact with human TLRs in the initial process of pathogenesis. LAM and LM synthesis starts from GDP-mannose, and these can be produced in two distinct ways, either through the conversion of exogenously acquired M6P by a hexokinase or from the glycolytic pathway by converting F6P to M6P by a phosphomannose isomerase. M6P is then converted to M1P by a phosphomannomutase and then to GDP-mannose by a GDP-mannose pyrophosphorylase (Fig. 1.1). Studies focussing on the pathways lead to the synthesis of these molecules are critical to development of new and novel chemotherapeutic approaches.

In mycobacteria, PMI activity has been confirmed from ManA (Rv3255c), an enzyme that has been shown to be essential for mycobacterial growth *in vitro* (Patterson et al., 2003), while ManC1 (Rv3264c) has been defined as a GDPMP (Ma et al., 2001; Ning and Elbein, 1999). Indeed, unless the growth medium is supplemented with D-mannose, PMI has been found to be essential for the survival of cells from *Saccharomyces cerevisiae* (Payton et al., 1991), *Candida albicans* (Smith et al., 1995), and *M. smegmatis* (Patterson et al., 2003) and important for the virulence of the protozoan parasite *Leishmania* (Garami and Ilg, 2001).

Proudfoot et al., (1994) successfully solubilised PMI from *C. albicans* in *E. coli* by the assistance of chaperones. McCarthy et al., (2005), overexpressed ManB in *M. smegmatis* and it resulted in an increased lipoglycan deposition on cell wall gives basic information that PMM is also actively involved in mannose metabolism. The genomes of *M. tuberculosis* and other mycobacteria contain four ORFs that are annotated as putative PMM or PGM encoding genes. The true function of each of the three remaining ORFs [*pmmB* (*Rv3308*), *mrsA* (*Rv3441c*), and *pgmA* (*Rv3068c*)] remains to be characterized. In which *pmmB* (*Rv3308*) is a hypothetical PMM, supposed to play a significant role in GDP-mannose synthesis. GDP-mannose pyrophosphorylase has also been purified to homogeneity from various bacteria, and in some of these organisms the enzyme has multiple activities. Thus, in *Pseudomonas aeruginosa* this is a multifunctional enzyme that has both GDP-mannose pyrophosphorylase and phosphomannose isomerase (PMI 2) activities (Shinabarger et al., 1991). The purpose of having these two activities together in a bifunctional enzyme is not clear, but some studies have shown that overexpression of this enzyme leads to an increase in phosphomannomutase (PMM) activity (Shankar et al., 1995). GDP-mannose pyrophosphorylase gene (ManC) of *E. coli* O157 was cloned and expressed as a highly soluble protein in *E. coli* BL21 *(DE3)* (Yang et al., 2005). Ning and Elbein, (1999), purified GDPMP from *M. smegmatis* about 2300-fold to near homogeneity from the soluble fraction.

LM and LAM deposition is peculiar to Mycobacterium (Brennan and Nikaido, 1995) and absent in humans. The enzymes involved in mannose synthesis (Fig. 1.1) are expected to be the target of antitubercular drugs. Current studies are limited in explaining the structure and role of enzymes in lipoglycan biosynthesis. Hence biochemical characterization of enzymes has scope towards to find suitable drug targets.

In the present study attempts were made to express the mannose biosynthetic genes of *M. tuberculosis* as recombinant proteins in *E. coli* that can help to study the biochemical

properties in future. Efforts were also made to activate the over expressed proteins by different approaches like modifying the conditions of expression and adopting various strategies to solubilise the expressed proteins after purifying the proteins.

5.2 MATERIALS AND METHODS

5.2.1 Bacterial strains, vectors, chemicals and reagents

E. coli-DH5α was procured from Invitrogen, CA, USA for initial cloning studies. BL21 *(DE3)* used for expression studies was purchased from Novagen, USA. Chaperonins, pGro7 and pTf16 used for solubilising the over expressed proteins were procured from Takara, Japan. pTZ57R/T used for cloning the genes used for sequencing was purchased from MBI Fermentas, USA. The expression vector, pET28a was purchased from Invitrogen, for cloning the genes. The PCR cloning kit, QIAprep Miniprep kit, QIAquick gel extraction kits were obtained from Qiagen, Germany. Restriction enzymes, T4 DNA ligase and *Taq* DNA polymerase were procured from New England Biolabs and MBI Fermentas, USA. The primers were procured from Integrated DNA technologies, USA. The strains were grown on LB broth or LB agar, (Sigma-aldrich, India) slants at 37º C for 24 h and subcultured every two weeks. Antibiotics, kanamycin of 30 µg ml^{-1} and/or ampicillin of 50 µg ml^{-1} for pTZ57R/T were added when required to the final concentration. All other molecular biology grade chemicals used in the present study were procured from Sigma, USA or from Hi-media, India.

5.2.2 PCR amplification of genes

Standard PCR strategies with *Taq* DNA polymerase were used to amplify the *M. tuberculosis* H37Rv *manA, manB, manC* and *pmmB* genes. All the protocols of molecular biology were adopted from Sambrook, (1989).

5.2.2.1 Amplification of *manA* gene

manA (Rv3255c) was amplified by PCR from *M. tuberculosis* H37Rv genomic DNA using the primers (Table 5.1) having NdeI and XhoI restriction sites. PCR amplifications consisted of one cycle of denaturation (95° C, 5 min), followed by 35 cycles of amplification that include denaturation (95° C, 40 sec), annealing (57° C, 30 sec), primer extension (72° C, 1 min) and final extension (72° C, 10 min). The 1227 bp PCR product was purified from 0.8 % agarose gel and kept at -20 for further cloning studies.

5.2.2.2 Amplification of *manB* gene

manB (Rv3257c) was amplified by PCR from *M. tuberculosis* H37Rv genomic DNA using the primers (Table 5.1) having NdeI and XhoI restriction sites. PCR amplifications consisted of one cycle of denaturation (95° C, 5 min), followed by 35 cycles of amplification that include denaturation (95° C, 40 sec), annealing (75° C, 2 min), and final primer extension (75° C, 10 min). The 1398 bp PCR product was purified from 0.8 % agarose gel and kept at -20 for further cloning studies.

5.2.2.3 Amplification of *manC* gene

manC (Rv3264c) was amplified by PCR from *M. tuberculosis* H37Rv genomic DNA using primers (Table 5.1) having NdeI and XhoI restriction sites. PCR amplifications consisted of one cycle of denaturation (95° C, 5 min), followed by 35 cycles of amplification that include denaturation (95° C, 40 sec), annealing (55° C, 1 min), primer extension (72° C, 2 min) and final extension (72°C, 10 min). The 1080 bp PCR product was purified from 0.8% agarose gel and kept at -20 for further cloning studies.

5.2.2.4 Amplification of *pmmB* gene

pmmB (Rv3308) was amplified by PCR from *M. tuberculosis* H37Rv genomic DNA using the primers (Table 5.1) having NdeI and XhoI restriction sites. PCR amplifications consisted of one cycle of denaturation (95° C, 5 min), followed by 35 cycles of amplification that include

denaturation (95° C, 40 sec), annealing (58° C, 1.5 min), primer extension (72° C, 1.3 min) and final extension (72° C, 10 min). The 1605 bp PCR product was purified and kept at -20 for further cloning studies.

Table 5.1 Primers designed for amplifying the genes used for cloning in *E.coli*

Name of the gene	Primer used	Restriction site
manA (Rv3255c)	FP1:5'-GGG**CATATG**GTGGAACTGCTACGTGGCG-3' RP1:5'-GGG**CTCGAG**TCACAACCCGACGGTCG-3'	Nde I Xho I
manB (Rv3257c)	FP3:5-'GGGG**CATATG**TCTTGGCCCGCCGCGG-3' RP3:5'-GGG**CTCGAG**TCA CGGTCCGGCCTTCG-3'	Nde I Xho I
manC (Rv3264c)	FP2:5'-GGG**CATATG**TTGGCAACTCACCAAGTCGATG-3' RP2:5'-GGG**CTCG AG**TCAAACGTCGGACGAGTAACGG-3'	Nde I Xho I
pmmB (Rv3308)	FP4: 5'-GGG**CATATG**GTGACGCCAGAGAATTGGATCG-3' RP4:5'-GGGGG**CTCGAG**TCACCACCACCGCCG-3'	Nde I Xho I

5.2.3 Cloning of genes

5.2.3.1 Cloning of genes into pTZ57R/T

Genes were cloned in pTZ57R/T having a M13/pUC promoter which permits the sequencing of the genes with M13 primer (Fig. 5.1). Amplified PCR products and vector were purified, eluted and were ligated into pTZ57R/T to make *manA::*pTZ57R/T, *manB::*pTZ57R/T, *manC::*pTZ57R/T and *pmmB::*pTZ57R/T respectively. Ligated products were transformed into *E. coli* -DH5α. Clones were confirmed by restriction digestions and finally sequences were verified by double strand sequencing services from Axygen, India.

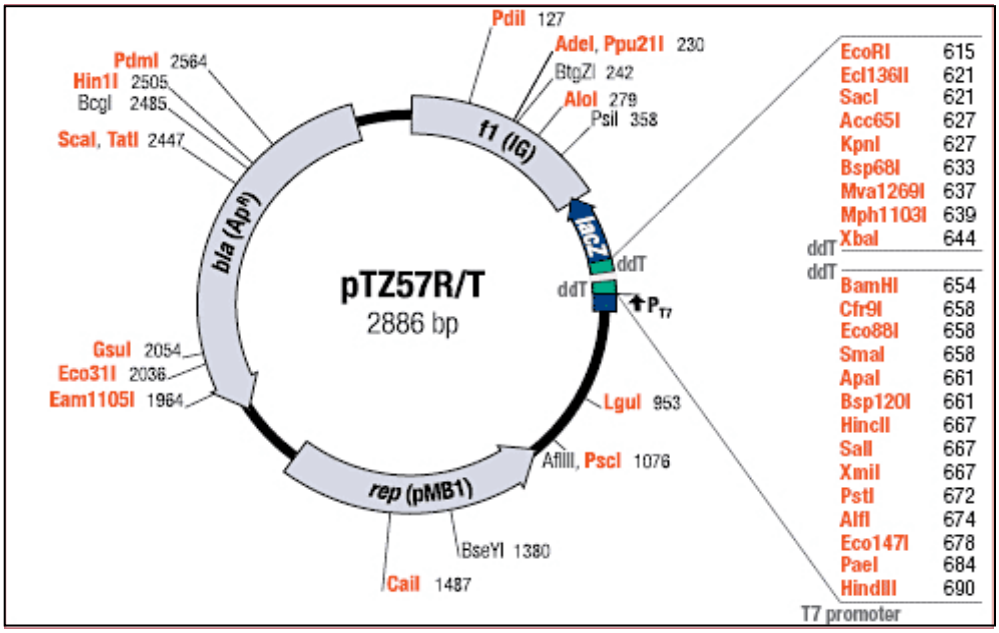


Fig 5.1 Map of pTZ57R/T vector

5.2.3.2 Cloning of genes into pET28a

For expression, the genes from the confirmed clones in pTZ57R/T were released by double digestion with a set of specific enzymes, and the released gene fragment was ligated into pET28a (Fig. 5.2) having an IPTG inducible '*lacI*' operator. The clone confirmation of *manA::*pET28a, *manB::*pET28a, *manC::*pET28a and *pmmB::*pET28a were done by digestion with different set of restriction enzyme (Nde I and Xho I) and the confirmed clones were transformed into BL21 *(DE3)* for overexpression.

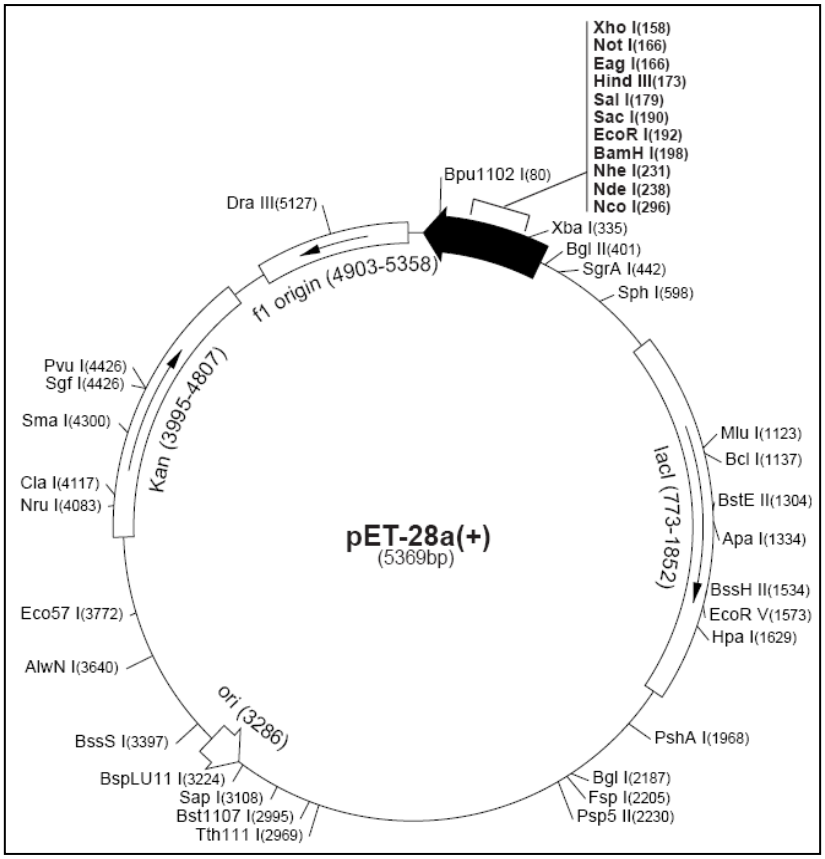


Fig. 5.2 Restriction map of pET28a

5.2.4 Overexpression of recombinant proteins

1 ml overnight culture of *E. coli*-BL21 *(DE3)* cells harbouring recombinant plasmids was inoculated into 100 ml of LB-kanamycin broth. The cells were incubated at 37º C for 3 h or until the OD_{600} was about 0.6. The expression of recombinant proteins was induced by the addition of 1 mM IPTG to the culture medium. The cells were further incubated at 37º C for 3 h. The cells were then pelleted by centrifugation at 19,320 g for 15 min at 4º C. The supernatant was discarded and to the pellet 5 ml of cold 20 mM Tris-HCl buffer (pH 7.2) was added. Protease inhibitor cocktail (1mM) was added and vortexed well. Lysozyme was added at concentration of 0.5 mg ml^{-1}, vortexed well and kept in ice for about 30 min. The cells were then subjected to sonication on ice at 45 % amplitude for 3 min with 10 sec 'on' and 10 sec 'off' cycle. Cell free lysate was recovered from the total cell lysate by centrifugation at

19,320 *g* for 15 min at 4º C, and the fractions were checked for phosphomannose isomerase, phosphomannomutase and GDP-mannose pyrophosphorylase activities with their specific coupled assays mentioned in the chapter 3-section 3.2.1.3. The remaining pellet was extracted with 1ml of ice cold Tris-HCl (pH-7.2) contains 8 M urea to get all the recombinant proteins if they formed inclusion bodies in the total cell lysate (Fischer et al., 1993; Rudolph and Lilie, 1997). Highly hygroscopic nature of the urea helps to solubilze the inclusion bodies of over expressed recombinant proteins (Baneyx, 1999). Both soluble and pellet fractions were analysed in a 12 % SDS- PAGE as described by (Laemmli et al., 1970). Molecular weight of the recombinant proteins loaded in equivalent amount was verified by comparing the bands with a protein molecular weight marker which was loaded along with sample each time. The concentration of protein in various samples was determined according to the method of Bradford et al., (1976), using Bradford's reagent with bovine serum albumin as the standard (595 nm) and also by using NANODROP-Spectrophotometer (ND-1000), by reading absorbance at 280 nm.

5.2.5 Solubilisation studies on recombinant proteins

Based on the above expression studies, it was observed that most of the over-expressed proteins were in the insoluble fraction. Hence, attempts were made to bring over expressed proteins to the soluble fraction by using different IPTG concentrations, use of different growth media and use of different temperatures for incubation.

5.2.5.1 Effect of different IPTG concentrations

Different IPTG concentrations were screened for induction as per the methods described in various literatures (Chalmers et al., 1990; Harcum and Bentley, 1993; Katayama et al., 1998; Winograd et al., 1993; Yildir et al., 1998). One ml overnight culture of recombinant BL21 *(DE3)* cells was inoculated into 100 ml of sterile LB-kanamycin broth. The cells were incubated at 37^{o} C for 3 h till an OD_{600} value of 0.6 was reached. The different IPTG

concentrations used for induction were 0.1, 0.25, 0.5, 0.75 and 1 mM. Similarly, each culture was inoculated in to 100 ml LB broth without adding IPTG and used as uninduced control for each gene. After induction, the cells were incubated at 37^{o}C for 3 h and then pelleted by centrifugation at 19, 320 g for 15 min. Soluble as well as urea fractions were obtained after sonication and subsequent centrifugation. Each fraction was checked separately for the presence of over expressed protein and the enzyme activity.

5.2.5.2 Effect of different growth media

The method of using different growth media in solubilising recombinant proteins was described by Riesenberg et al., (1991) and Stanier, (1960). One ml of the overnight culture of recombinant BL21 *(DE3)* cells was inoculated into three different growth media such as LB broth, 2xYT and Terrific broth. The cells were incubated at 37 ^{o}C till the OD_{600} value reached 0.6 and then induced with 0.5 mM IPTG. The cells were further incubated at 37 ^{o}C for 3 h and then pelleted by centrifugation. The soluble fractions and urea fractions obtained after sonication and subsequent centrifugation were checked for enzyme activity.

5.2.5.3 Effect of different induction temperature

The idea of using a lower temperature for solubilizing recombinant proteins was reported by many researchers (Maldonado et al., 2007; Schein, 1989). 1 ml of overnight culture of recombinant BL21 *(DE3)* cells was diluted into 100 ml of LB-kanamycin in three separate flasks. The cells were incubated at 37º C till the OD_{600} reached 0.6 and then induced with 0.5 mM IPTG at different temperatures (one was induced at 37º C referred as control, one was at 30º C and the third one was at 16º C) for 18 h. Induced cells were pelleted, suspended, sonicated and extracted the soluble and urea fractions for checking the enzyme activities with equal amount of total soluble protein (10 mg).

5.2.5.4 Chaperone assisted refolding of recombinant proteins

Several groups have shown that recombinant proteins can fold properly in the presence of over expressed chaperones (Azem et al., 1995; Cole, 1996; Goloubinoff et al., 1989). All the experiments were performed with chaperones (pGro& and pTf16) according to the manufactures protocol (Takara, Japan). BL21 *(DE3)* (pGro7, pTf16) carrying recombinant plasmid was inoculated into 50 ml of LB containing kanamycin (25 µg ml^{-1}), chloramphenicol (34 µg ml^{-1}) and arabinose (0.5 mg ml^{-1}) for induction of chaperone and it was incubated at 37º C. When the OD of the cells at 600 nm reached 0.6, the cells were induced with 1mM IPTG and incubated at 30º C for 3 h. The cell pellet was obtained by centrifuging at 6361 g for 15 min. Both the soluble and urea fraction were collected. The protein concentration of both the soluble and urea fraction was measured. Soluble fractions were assayed for the enzyme activity of PMI, PMM and GDPMP by the respective enzyme assays (Fig. 1.1). Urea fractions were analysed on 12 % SDS-PAGE.

5.2.6 Dot blot immunodetection of soluble recombinant proteins

The presence or absence of the His tagged recombinant proteins in the soluble fractions were confirmed by dot blot immunodetection test based on the manufactures protocol (Qiagen). The soluble fractions obtained from different solubilization experiments (different IPTG concentration, growth media, temperature and chaperone assisted cells-pGro7 and pTf16) were loaded on to the nitrocellulose membrane at a normalized concentration of 10 µg ml^{-1} protein. 3M urea extract sample containing the over expressed proteins in the same concentration was kept as control. Briefly the protocol was as follows. The membrane was washed with TBS buffer at room temperature and was incubated for 1h in blocking buffer. The membrane was washed twice for 10 min each time in TBS-tween-triton buffer followed by a wash with TBS buffer for 10 min at room temperature. The membrane was incubated in anti-histidine IgG antibody solution (Qiagen) (1/1000-1/2000 dilution) in blocking buffer at

room temperature for 1 h. The membrane was washed for 10 min each time in TBS-tween-triton buffer at room temperature followed by a wash with TBS buffer at room temperature for 10 min. The membrane was then incubated with secondary anti-rabbit IgG antibody conjugated with alkaline phosphatase solution (Sigma) (1/5000 dilution in blocking buffer) for 1 h at room temperature followed by four washes (10 min each time) with TBS-tween-triton buffer at room temperature. Staining was done with BCIP-NBT (Sigma) until signal was cleared (5-15 min). The reaction was stopped by rinsing with distilled water. The membrane was dried and photographed.

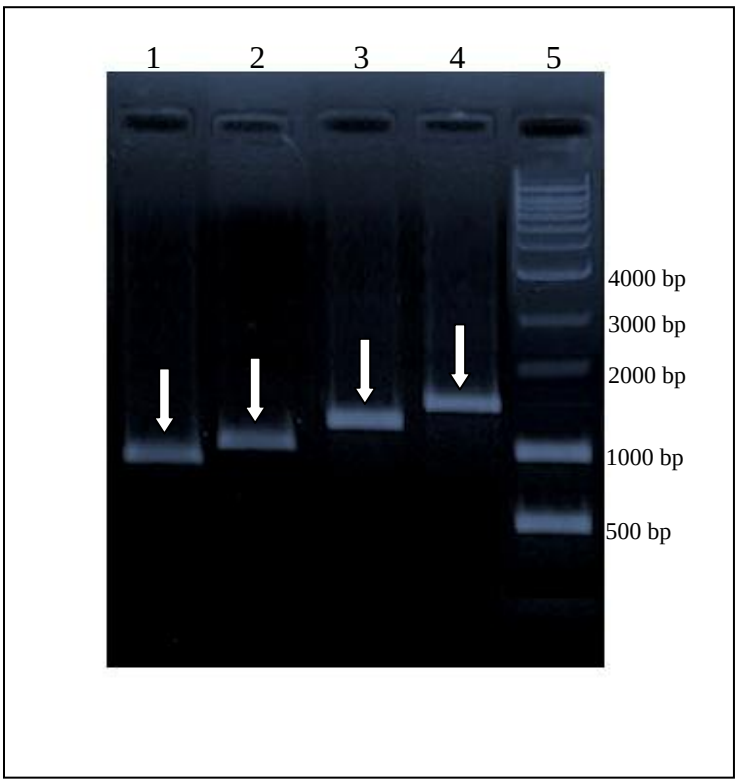
5.2.7 Purification of recombinant proteins

The purification using His Trap column was done based on the manufacturer's protocol (Amersham Biosciences). The column was first washed with 10 ml distilled water followed by a wash with 10 ml stripping buffer. The column was further washed with 2 ml of 1M NaOH for 2 h. 5-10 ml of stripping buffer was again used to wash the column followed by a wash with 1ml nickel sulphate. As a final step of pre treatment, the column was washed with 5 ml distilled water. The column was washed with binding buffer (50 mM Tris (pH 8.0), 0.5 M KCl, 3 M urea, 5 mM imidazole). 1ml of 3 M urea extract (≈3 mg protein) was loaded onto the column and the flow through was collected. Elution of the recombinant protein from the column was done using buffers with increased imidazole concentration of 150 mM in 50 mM tris, 0.5 M KCl, 3M urea, pH 8.0. The protein content in all the eluted fractions was determined by NANODROP and then by Barford's method and the proteins of equivalent quantity was loaded on SDS-PAGE for verifying their respective sizes.

5.3 RESULTS AND DISCUSSION

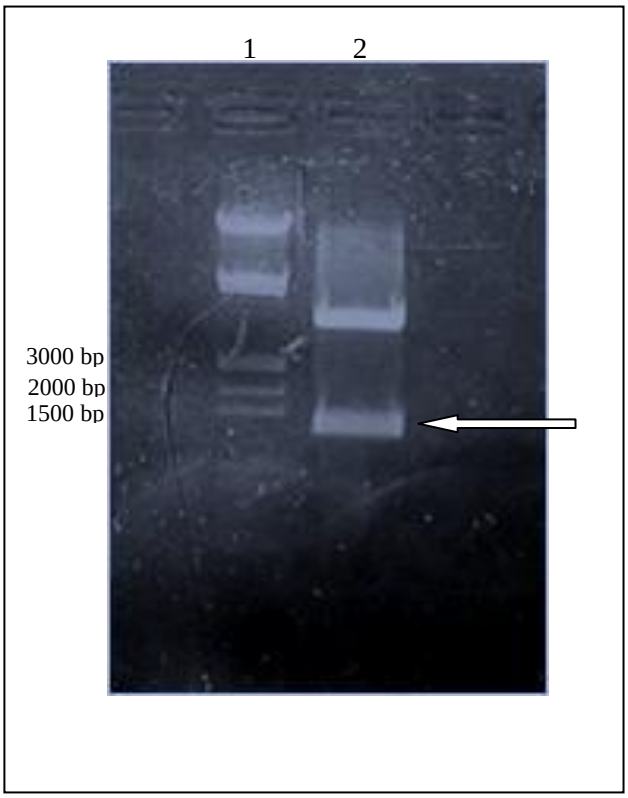
manA (Rv3255c) of 1227 bp , *manB* (Rv3257c) of 1398 bp, *manC* (Rv3264c) of 1080 bp and *pmmB* (Rv3308) of 1605 bp were amplified from *M. tuberculosis* genomic DNA (Fig. Fig 5.3). PCR amplica were cloned into pTZ57R/T for sequencing. Fig. 5.4 a, 5.4 b, 5.4 c and

5.4 d showed the clone confirmation of *manA*, *manB*, *manC* and *pmmB* respectively by verifying the insert size after double digesting with NdeI and XhoI, and the size of all the genes were found to be correct as expected. Clones were sequenced by M13 primers, sequences were verified and the ORF analysis proved that the amplica were complete without any kind of mutation. Sequence verified genes were cloned into pET28a and the ligation was confirmed by double digestion with NdeI and Xho I and the size of the insert size of the respective constructs were found to be correct as expected. Fig. 5.5 a showed the clone confirmation of *manA*::pET28a and the insert size was 1227 bp, *manC*::pET28a released a fragment of 1080 bp (Fig. 5.5 b) *manB*::pET28a insert size was 1398 bp (Fig. 5.5 c), and *pmmB*::pET28 a released a fragment of 1605 bp upon digestion with Nde I and Xho I (Fig 5.5 c).



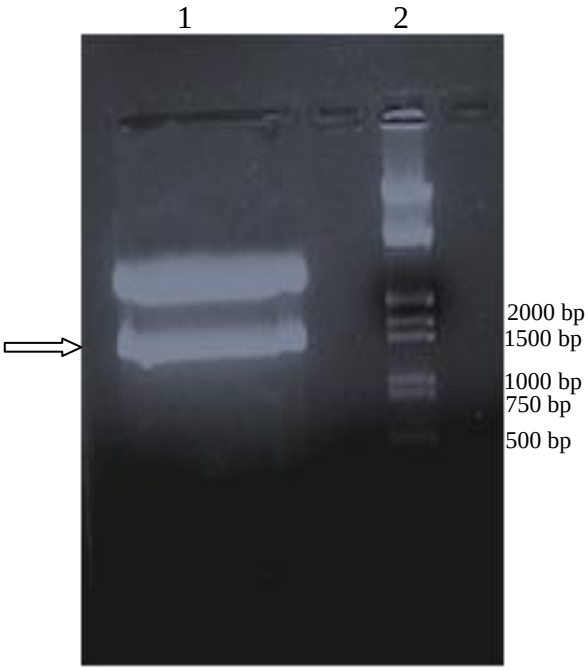
Lane 1-manC, Lane 2- manA, Lane 3- manB, lane 4- pmmB, Lane 5- DNA marker

Fig. 5.3 PCR amplification of genes



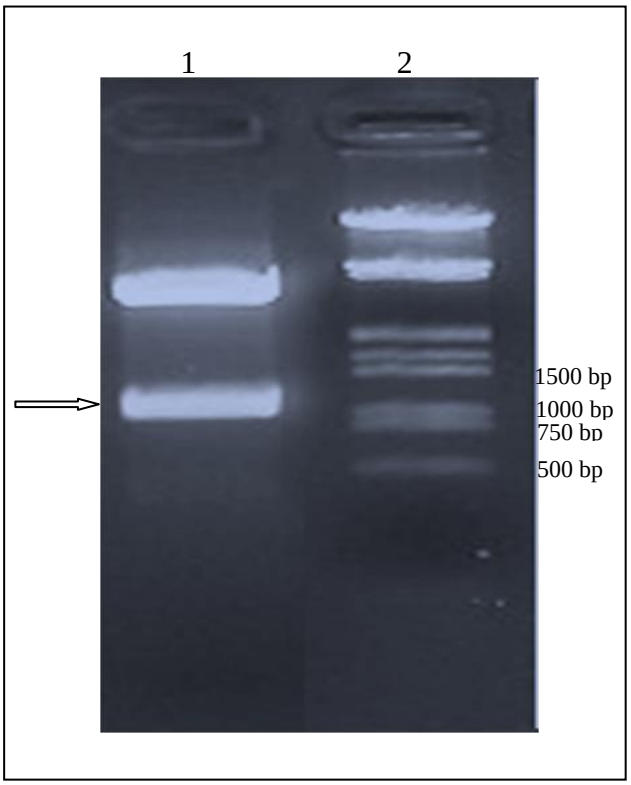
Lane 1-DNA marker, Lane 2-*manA* insert release marked (1227 bp)

Fig. 5.4 a Cloning of *manA* into pTZ57R/T



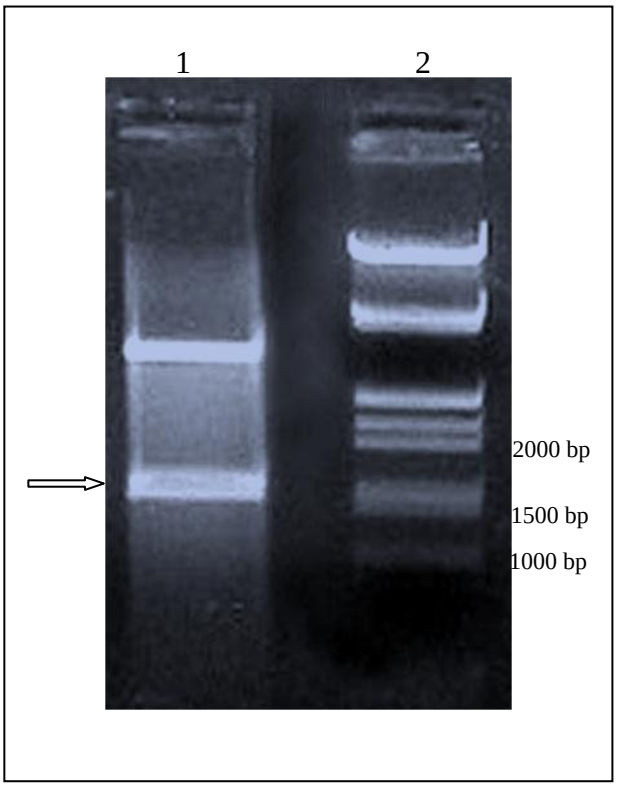
Lane 1-*manB* release (1398 bp) marked, Lane 2-DNA marker

Fig. 5.4 b Cloning of *manB* into pTZ57R/T



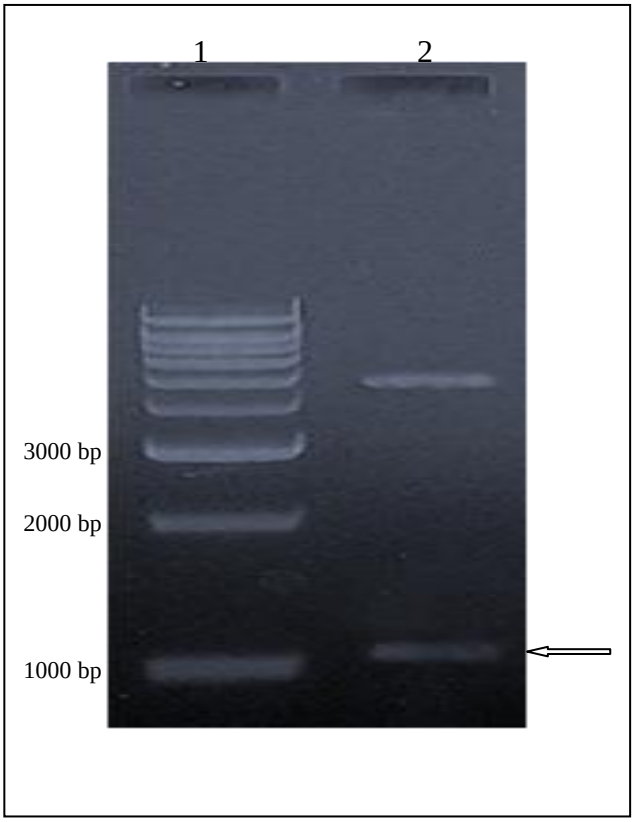
Lane 1-*manC* release (1080 bp) marked,
Lane 2-DNA marker

Fig. 5.4 c Cloning of *manC* into pTZ57R/T



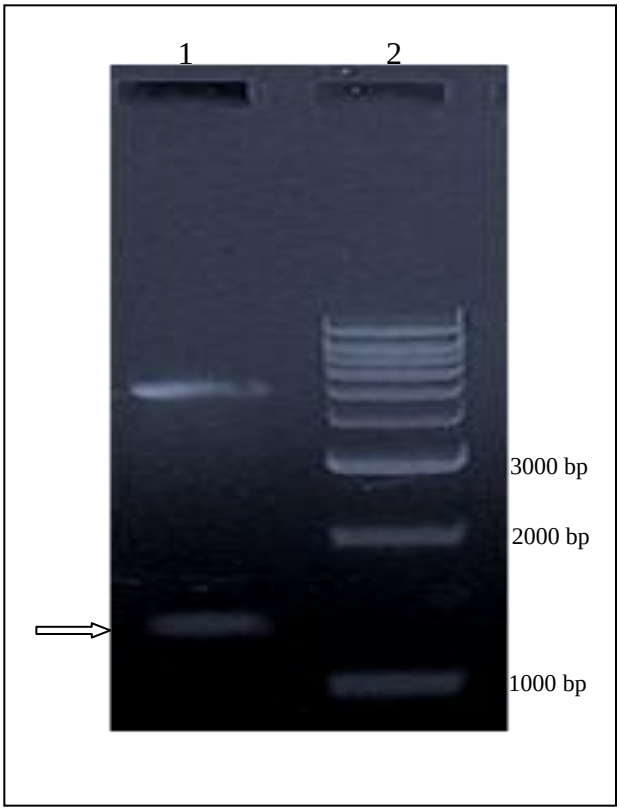
Lane 1-*pmmB* release (1605 bp) marked,
Lane 2-DNA marker

Fig. 5.4 d Cloning of *pmmB* into pTZ57R/T



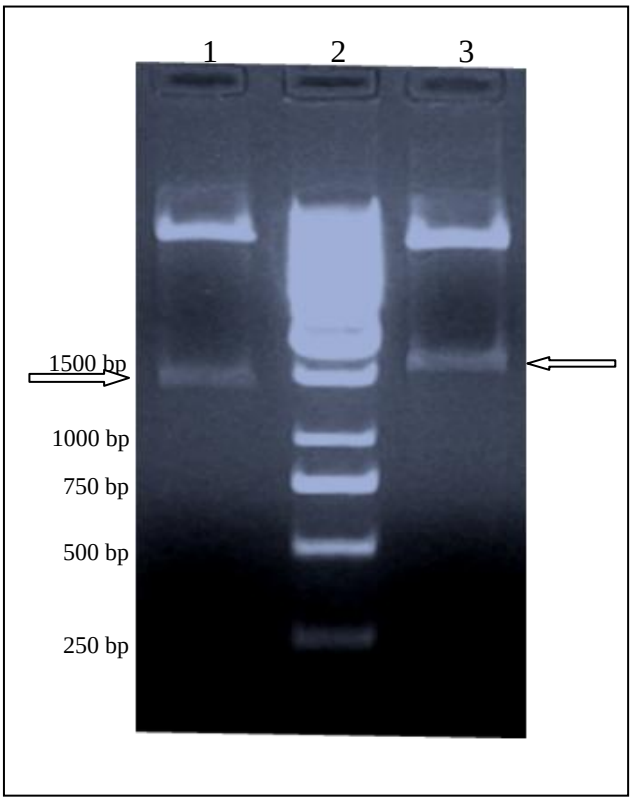
Lane 1-DNA marker, Lane 2-*manA* release (1227 bp) marked

Fig. 5.5 a Cloning of *manA* into pET28a



Lane 1-*manC* release (1398 bp) marked,
Lane 2-DNA marker

Fig. 5.5 b Cloning of *manC* into pET28a

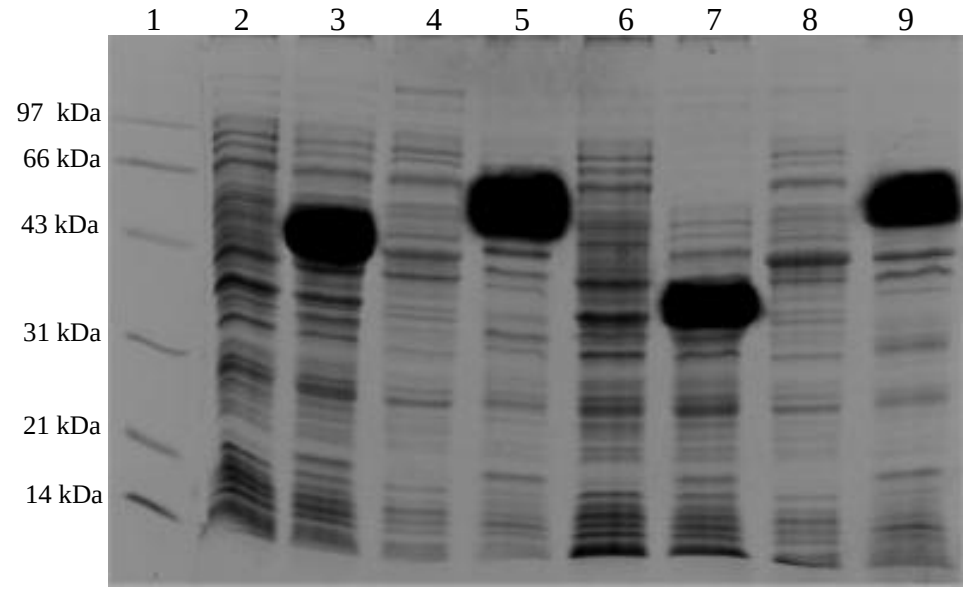


Lane 1-*manB* release (1398 bp) marked, Lane 2-DNA marker, Lane 3- *pmmB* release (1605 bp) maked

Fig. 5.5 c Cloning of *manB and pmmB* genes into pET28a

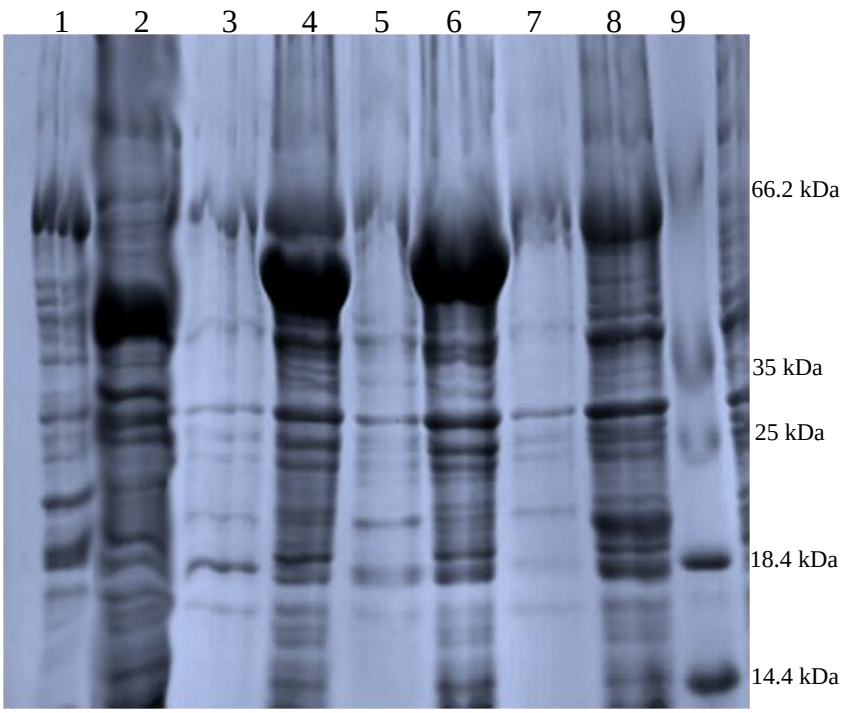
Fig 5.6 showed the SDS-PAGE separation of ManA, ManB, ManC and PmmB recombinant proteins with and without IPTG induction. The figure showed that all the respective recombinant proteins were expressed in their correct sizes only in IPTG induced fractions. Fig. 5.7 showed the SDS-PAGE analysis of the chaperon assisted cells, of different clones in C41*(DE3)*, in which all the recombinant proteins were found to be expressed in 3 M

urea fraction and the soluble fractions were have no such respective bands and it showed almost similar to the soluble fraction of negative controls ie., pGro7 and pTf16. Lane 1, 3, 5 and 7 of Fig. 5.7 were loaded with the soluble fractions of ManC, ManA, ManB and PmmB respectively and it is showed a similar band distribution and it indicated that the proteins were not properly folded to found in soluble fractions of the same. In Fig. 5.7, Lane 2, 4 6 and 8 were loaded with the 3 M urea fractions of ManC, ManA, ManB and PmmB respectively, in which Lane 2 (manC) of size 37.8 kDa, Lane 4 (manA) of size 43.3 kDa, Lane 6 (manB) of size 49 kDa and Lane 8 (PmmB) of size 56 kDa proves that the recombinant proteins are expressed in their respective sizes as expected. The results indicated that the all the recombinant proteins were expressed as inclusion bodies in their correct sizes. Fig 5.8 was loaded with the recombinant proteins purified from the 3 M urea fractions, in which Lane 1 is ManC (37.8 kDa), Lane 2 is ManA (43.3 kDa), Lane 3 is ManB (49 kDa) and Lane 4 is PmmB (56 kDa) and it was observed that all the recombinant proteins were purified of homogeneity from their respective 3 M urea fractions.



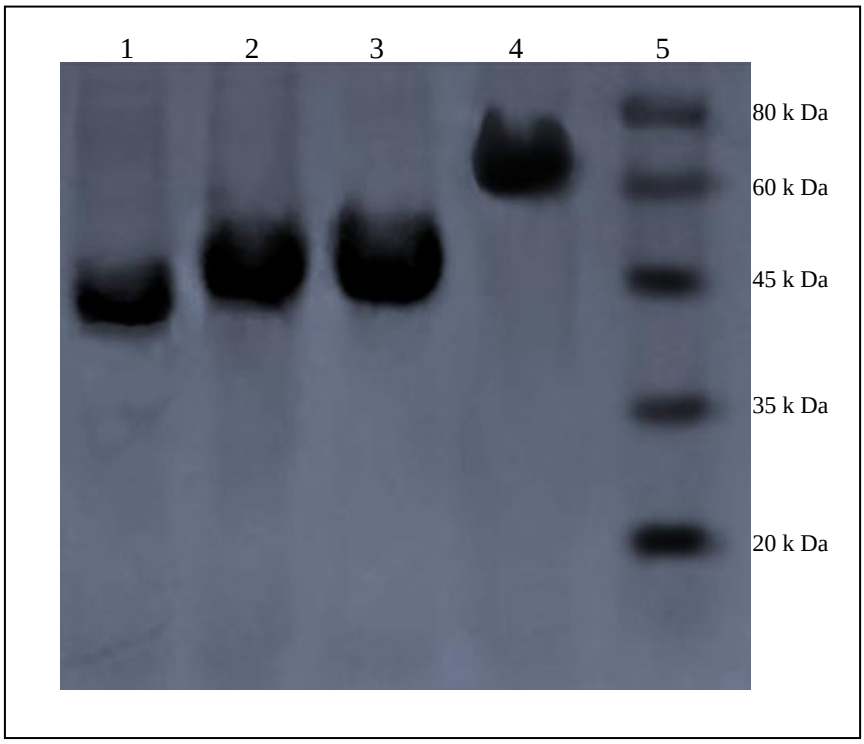
Lane 1-marker, Lane 2- ManA (wo IPTG), Lane 3-ManA (w IPTG), Lane 4-ManC (wo IPTG), Lane 5-ManC (w IPTG), Lane 6-ManB (wo IPTG), Lane 7-ManB (w IPTG), Lane 8-PmmB (wo IPTG), Lane 9-PmmB (w IPTG)
wo-with out, w- with

Fig. 5.6 Overexpression of recombinant proteins with or without IPTG



Lane 1-ManC sol, Lane 2-ManC urea, Lane 3-ManA sol, Lane 4-ManA urea, Lane 5-ManB sol, Lane 6-ManB urea, Lane 7-PmmB sol, Lane 8-PmmB urea, Lane 9- Marker

Fig. 5.7 Overexpression of recombinant proteins with chaperones



Lane 1-ManC, Lane 2-ManA, Lane 3-ManB, Lane 4-PmmB, Lane 5- Marker

Fig. 5.8 Purification of recombinant proteins

Dot blot immune detection for identifying the soluble recombinant enzymes obtained from different solubilisation experiments (different IPTG concentration, growth medium, low temperature induction and chaperone based co-expression) found in the total cell free lysate was negative while soluble fractions were produced no positive signal towards anti-histidine antibodies whereas the respective control reactions performed with 3 M urea was produced signal towards antibodies (Fig. 5.9), which supported the SDS-PAGE analysis providing

additional information about the inclusion body formation of the recombinant proteins. Enzyme assays using the fractions with 10 - 50 mg of total proteins obtained after different solubilisation experiments turned to be negative which confirmed that most of the proteins form inclusion bodies when over expressed in *E.coli* via pET28a and were in inactive form.

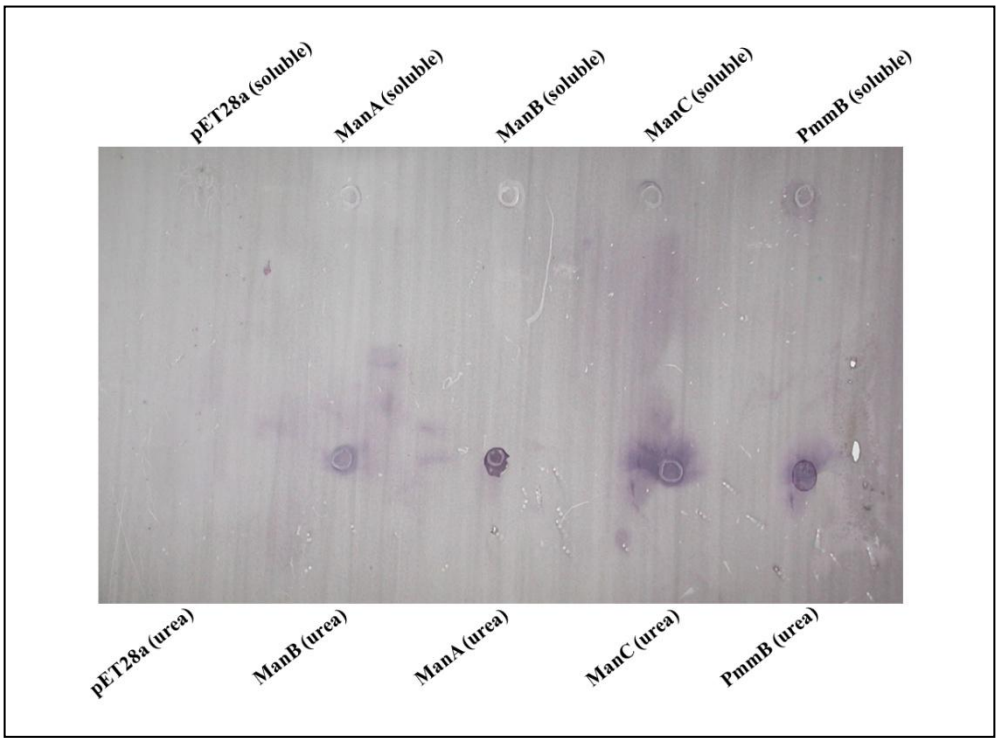


Fig. 5.9 Dot blot immune detection

Protein production for biochemical characterization presents one of the most difficult and challenging tasks for heterologous expression in *E. coli*. Generally, the protein must be native, active, soluble, pure and concentrated. A large majority of genes cloned from a variety of organisms and expressed well in *E. coli*. Among them 78 % express to a level greater than or equal to 10 mg l^{-1}, 17 % express less than 10 mg l^{-1} and 5 % express at a level that was undetectable, in which only 44 % form soluble proteins and 37 % were partially soluble, and for 19 % of the expressed proteins, no soluble product was detected (Goulding and Perry,

2003). It has been also estimated that one-third to one-half of prokaryotic proteins cannot be expressed in soluble form in *E. coli*, and likely a higher percentage for eukaryotic proteins (Edwards et al., 2000). Certainly, it has been our experience, as well as that of others, that protein solubility represents a major bottleneck for recombinant protein production which can be used for biochemical characterization (Goulding and Perry, 2003; Yee et al., 2002). At this time there is no reliable way of predicting the solubility of a recombinant protein. Several studies then opted with *M. smegmatis* as the host organism for *M. tuberculosis* proteins that were insolubly expressed in *E. coli* have been solubly expressed in *M. smegmatis* (Garbe et al., 1993; Harth and Horwitz, 1997; Triccas et al., 1998). It is attractive to speculate that a mycobacterial host may provide the optimal system for expression of such genes which need post translational modifications (Espitia and Mancilla, 1989; Fifis et al., 1991; Matsuo et al., 1990; Young, 1990; Young and Garbe, 1991). In addition, it was found that the mycobacterial system allowed expression of functionally active superoxide dismutase, in contrast to the enzymatically inactive recombinant product in E. coli (Zhang et al., 1991). Results from our studies (Section 3.3) also reminded us to use *M. smegmatis* as the host organism for expressing *M. tuberculosis* genes, in which all the genes found to be expressed in soluble form under '*hsp*' promoter in the moderate expression vector pMV261. Results from the present chapter also suggest either to renature the inclusion bodies by the effective use of renaturation kits or the use an alternate host.

5.4 CONCLUSION

PMI, PMM, and GDPMP were over expressed in *E. coli* via pET28a expression vector. Strategies like induction under different IPTG concentration, growing the culture under different temperature and media were adopted to make the recombinant proteins active were not successful. Co-expression of proteins with chaperones for getting soluble protein was also failed to produce active proteins. All the recombinant proteins were purified and analysed on

SDS-PAGE and the size was found to be correct. The use of inclusion body solubilisation kit for high through put screening of renatured forms using different buffer system could be an option to work it out further.

CHAPTER 6

Summary and Conclusion

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Analysis of *Mycobacterium tuberculosis* genome revealed 4 possible genes encoding enzymes involved in mannose metabolism: *manA* (Rv3255c), *manB* (Rv3257c), *manC* (Rv3264c) *and pmmB* (Rv3308), all being essential for the viability of the bacilli, with the exception of *pmmB*. We used the model organism, *Mycobacterium smegmatis*, to construct recombinant strains overexpressing the above genes via pMV 261 shuttle vector and a pmmB deletion mutant via pJQ 200 suicidal vector. As anticipated, overexpression of PMM and GDPMP resulted in an increased deposition of lipoglycans, mainly LM and LAM, on the surface of Mycobacterium, whereas PMI overexpression has no effect on the lipoglycan deposition. Deletion of *pmmB* orthologue in *M. smegmatis* resulted in a reduced lipoglycan deposition on cell envelope. Transfection studies on cell lines such as HEK and THP-1 with *M. smegmatis* mutants enlighten the fact that a slight increase of lipoglycan amount (~1.7 fold) resulted in an enhancement of mycobacteria-induced TLR 2 signalling, demonstrating that lipoglycans could be the *bona fide* TLR 2 ligands that can be sensed at physiological concentrations in the context of the whole bacterium. *pmmB* gene deletion experiments supported the above result by giving a reduced TLR 2 mediated signalling and in addition it gives information about the *pmmB* essentiality in lipoglycan biosynthesis. Hence, besides the immunodominant lipoproteins/lipopeptides, lipoglycans can also considered as MAMPs contributing to the innate immune detection of mycobacteria by TLR 2. Cell surface properties like hydrophobicity and affinity towards congored were found to be more in *M. smegmatis* mutants (*manB, manC* and *pmmB*). Similarly the mutants (*manB, manC* and *pmmB*) also showed tendency to form cellular aggregate compared to *M. smegmatis* mc^2 155.

ManA, ManB, ManC and PmmB were expressed in *E. coli* as recombinant proteins with the goal to better characterize their enzymatic activities. However most of the recombinant proteins were expressed as inclusion bodies. Studies were conducted to improve the solubility of the proteins produced by varying different conditions, expression temperature, IPTG concentration, *E. coli* strain, co-expression with chaperones. However, none of them were successful.

Mannose metabolism in Mycobacterium starts with fructose-6-phosphate, proceed via mannose-6-phosphate and mannose-1-phosphate, ended up with a mannose donor GDP-mannose, which further supplies mannose for the synthesis of lipoglycan. In the pathway proposed, phosphomannomutase coded by *manB* and *pmmB* and GDP-mannose pyrophosphorylase coded by *manC* are the rate limiting enzymes in mannose biosynthesis. Increased LM and LAM deposition on mycobacterial cell surface can activate the innate immune response via TLR2. Hence LM and LAM are proved to be considered as potent Pathogen Associated Molecular Patterns (PAMPs) on mycobacterium and it specifically interacts with TLR2. *pmmB* deletion reduced the amount of LM and LAM deposition on mycobacterial cell wall, shed some light on the constitutive nature of *pmmB* in mannose biosynthesis. It also aided to modify the mannose biosynthesis by adding *pmmB* gene in the pathway as a constitutive gene that simultaneously go along with *manB* (Fig.6.1). All these studies formally demonstrated for the first time that lipoglycans are *bona fide* TLR2 ligands involved in innate immune detection of mycobacteria.

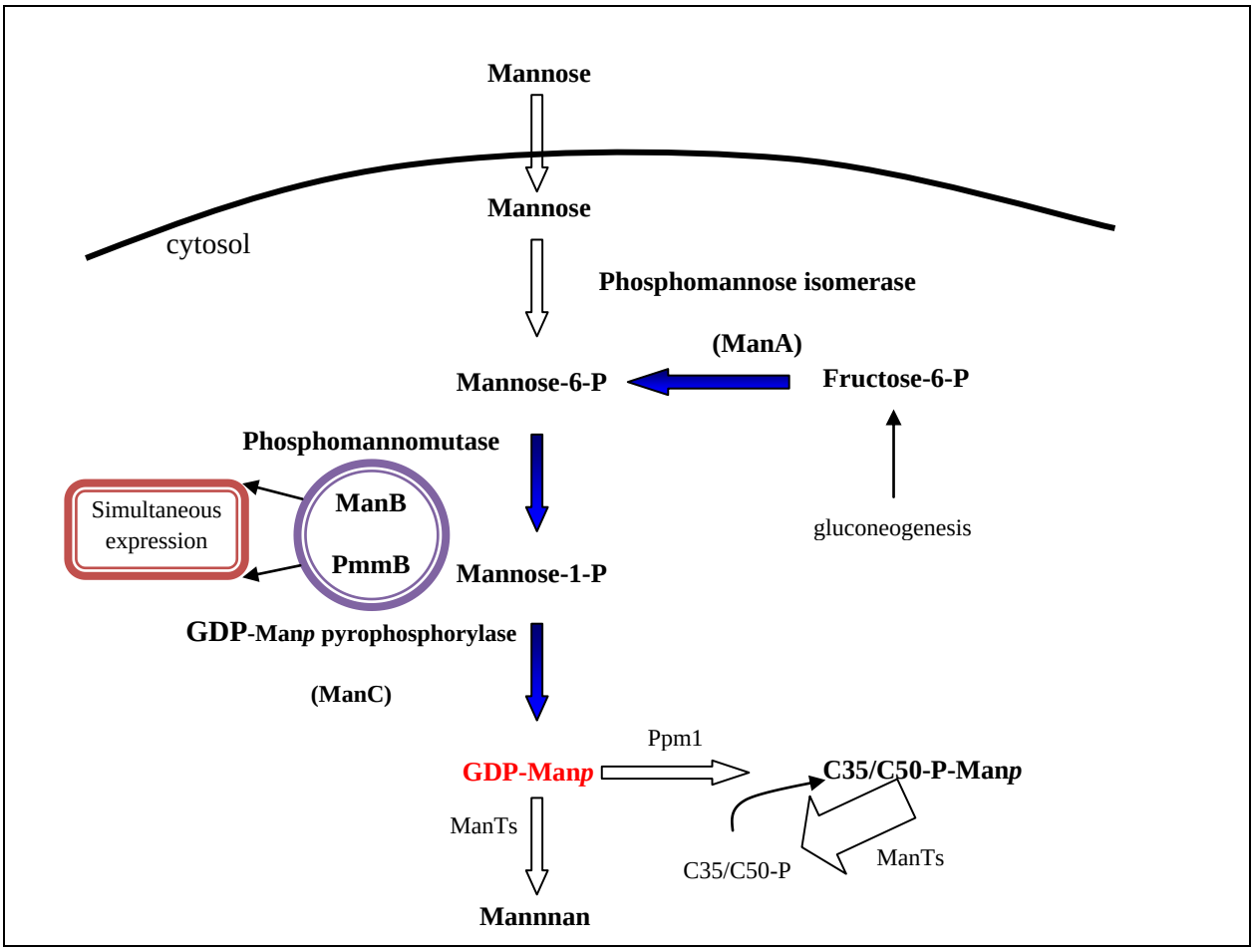

Fig. 6.1 Modified lipoglycan biosynthesis pathway

Cell surface studies proved that LM and LAM can act as molecules that can modify the cell surface properties of Mycobacterium. Tendency to form aggregates give indirect evidence for the role of LM and LAM in cell adhesion which is an essential property used for pathogenesis by most of the microbes like mycobacteria.

Most of the experiments performed to solublize the inclusion bodies were not successful, hence in future the appropriate use of inclusion body solubilization kits to get the enzymes active is advisable.

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Zielinski N.A., Chakrabarty A.M., Berry A., Characterization and regulation of the pseudo onas-aeruginosa algc gene encoding phosphomannomutase. Journal of Biological Chemistry, 266, 9754-9763, [1991].

ANNEXURE I

A1 - List of major instruments used

Equipment	Model
Cappillary Elctrophosresis	P/ACE 5000-Beckman Coulter, France
PCR machine	ep*gradient*- Eppendorf, India
Gel documentation system	G-box-Syngene, India
Electrophoresis unit	Biorad, India
Electroporator	2510- Eppendorf, India
Sonicator	VCX-750, Sonics, USA
Nanodrop	Thermo Scientific, USA
Mini bead beater	Qiagen, France
Vacuum concentrator	5301-Eppendorf, India
Rotavapour	Buchi, Switzerland
Lyophiliser	hDB-5503, Operon, Korea
Laminar Air Flow Chamber	Clean Air System, India
UV-VIS Spectrophotometer	UV-160A, Shimadzu, Japan
Centrifuge	Eppendorf, Germany; Hitachi, Japan
Autoclave	Tomy, Japan
Incubator	Sanyo, Japan; Innova 4230, New Brunswick Scientific, USA
Balance	Mettler Toledo, Mumbai, India
pH meter	Systronics, India
Deep freezer	Operon, Korea
Hot air Oven	Sanyo, Japan Kemi Instruments, India
Incubating water bath	Julabo, Germany

Table A2 Composition of Nutrient broth

Constituents	Concentration (g l^{-1})
Beef extract	10
Peptone	10
NaCl	5

Table A3 Composition of Luria Bertani medium

Constituents	Concentration (g l^{-1})
Yeast extract	5
Tryptone	10
NaCl	10
Agar	15

Table A4 Composition of Sauton's medium

Constituents	Concentration (g l^{-1})
KH_2PO_4	0.5 g
$MgSO_4.7H_2O$	0.5 g
Citric acid	2.0 g
Ferric ammonium citrate	0.05 g
Glycerol	60 ml
Asparagine	4.0 g

Table A5 Composition of Middlebrook 7H9 Broth Base

Constituents	Concentration (g l^{-1})
Ammonium Sulfate	0.5
Disodium Phosphate	2.5
Monopotassium Phosphate	1.0
Sodium Citrate	0.1
Magnesium Sulfate	0.05
Calcium Chloride	0.0005
Zinc Sulfate	0.001
Copper Sulfate	0.001
Ferric Ammonium Citrate	0.04
L-Glutamic Acid	0.5
Pyridoxine	0.001
Biotin	0.0005

ANNEXURE II

LIST OF PUBLICATIONS

1. Scientific Papers

1. **Shyam Krishna.**, Aurélie Ray., Shiv K. Dubey., Gérald Larrouy-Maumus., Christian Chalut, Audrey Noguera., Alain Vercellone., Martine Gilleron., Germain Puzo., K. Madhavan Nampoothiri and Jérôme Nigou., Lipoglycans contribute to innate immune detection of mycobacteria. PloS ONE, [2010]. ***In revision***.
2. Nampoothiri K.M., Rubex R., Patel A.K., Narayanan S.S., **Krishna S**., Das S.M., Pandey A., Molecular cloning, overexpression and biochemical characterization of hypothetical beta-lactamases of Mycobacterium tuberculosis H37Rv. *Journal of Applied Microbiology*, 105, 59-67, [2008].
3. Narayanan S.S., Ramanujan A., **Krishna S**., Nampoothiri K.M., Purification and Biochemical Characterization of Methionine Aminopeptidase (MetAP) from Mycobacterium smegmatis mc(2)155. *Applied Biochemistry and Biotechnology*, 151, 512-521, [2008].

1.1. Oral / Poster presentations in National / International Conference

1. **Shyam Krishna.** , Sai shyam N., K.Madhavan Nampoothiri., Cloning and overexpression of genes involved in the mannose metabolism of *Mycobacterium tuberculosis* H37 Rv. International Symposium on Emerging Trends in tuberculosis

research:biomarkers,drugs and vaccines (ITBS 2008) , December 1-3, 2008 , ICGEB, New Delhi P 134, [2008].

2. **Shyam Krishna.,** K.Madhavan Nampoothiri, Molecular cloning , overexpression and purification of *PMMA* (Rv3257c) from *Mycobacterium tuberculosis* , H37 Rv, Proceedings of 3 rd International congress on Bioprocesses in Food Industries & 5 th Convention of the Biotech Research Society of India, November 6-8, 2008, Osmania University, Hyderabad. P 165, [2008].
3. Sai shyam N , **Shyam Krishana**., K.Madhavan Nampoothiri, Peptide processing enzymes of Mycobacterium tuberculosis H37 Rv as potential drug targets , International Symposium on Emerging Trends in tuberculosis research:biomarkers,drugs and vaccines (ITBS 2008) , December 1-3, 2008 , ICGEB, New Delhi P 68, [2008].

AWARDS

1. CSIR-UGC Lectureship in Life Sciences, Exam held on December, 2005
2. Sandwich Ph.D. Fellowship 2009, offered by French Embassy, New Delhi, India.