BIOCHEMICAL AND MOLECULAR CHARACTERISATION OF CELL WALL GLYCOSYLTRANSFERASES IN *MYCOBACTERIUM TUBERCULOSIS*

By

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Dedicated to my both MUMMI and PAPA

ABSTRACT

The human pathogen and aetiological agent of tuberculosis, *Mycobacterium tuberculosis* has a cell wall architecture similar to the non-pathogenic bacterium *Corynebacterium glutamicum*. The availability of their genome sequences has enabled the utilisation of *C. glutamicum* as a model for the identification and study of essential mycobacterial genes involved in the synthesis of cell wall components such as lipomannan (LM), lipoarabinomannan (LAM) and arabinogalactan (AG).

In this study, we have analysed several uncharacterised open reading frames, which encode for putative glycosyltransferases from *M. tuberculosis* and deleted their respective orthologues in *C. glutamicum*. Mutant phenotypes were characterised biochemically using two-dimensional-thin layer chromatography, SDS-polyacrylamide gel electrophoresis, gas-chromatography-mass spectrometry, nuclear magnetic resonance spectrometry and *in vitro* enzyme assays. Mutants with altered phenotypes were complemented with their respective mycobacterial orthologues to characterise their functions. In this thesis we have identified and characterised several putative glycosyltransferases and established their role in *M. tuberculosis* cell wall biogenesis.

One of these ORFs, *Rv2174*/*NCgl2093*, was identified to encode for an $\alpha(1\rightarrow6)$ mannosyltransferase [MptA] involved in the later stages of the biosynthesis of the $\alpha(1\rightarrow6)$ mannan core of LM/LAM, while Rv1459c/NCgl1505 [MptB] was shown to be involved in the early stages of the biosynthesis of the $\alpha(1\rightarrow6)$ mannan core of LM/LAM. The disruption of *NCgl2106* [*Rv2188c*] has shown its role in synthesis of phosphatidyl-*myo*-inositol dimannoside (Ac_1PIM_2) and also sheds further light on the synthesis of a Mannosyl-α-**D**-glucopyranosyluronic acid-$(1\rightarrow3)$-glycerol ($ManGlcAGroAc_2$) anchored LM (Cg-LM-B).

Furthermore, three different glycosyltransferases from *C. glutamicum* were characterised and on the basis of biochemical analysis of mutants, NCgl2100 and NCgl2097 were identified as α(1→2) mannopyranosyltransferases [MptC and MptD], and NCgl2096 as an α(1→2) arabinofuranosyltransferase [AftE], involved in LM/LAM biosynthesis. Altogether, these studies have shed further light on the complex cell wall biosynthesis in *Corynebacterineae* and identified several potential new drug targets for tuberculosis.

DECLARATION

The work presented in this thesis was carried out in the School of Biosciences at the University of Birmingham, U.K., B15 2TT during the period October 2006 to September 2009. The work in this thesis is original except where acknowledged by references.

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

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This thesis is truly dedicated to my parents Mr and Mrs A. K. Mishra and teachers Prof B. C. Goswami and his wife. It's my father's hard work, his dream and vision that finally came true. I am grateful to all my family members for providing me their all time support and guidance during ups and down during the period. The word 'thank you' is very small for the unconditional love from Babu, Laxmi, and Mantu. And of course in the last but not the least I would like to acknowledge the heartiest blessings of my DADI.

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LIST OF ABBREVIATIONS

A	Adenosine
AftA	Arabinofuranosyltransferase A
AftB	Arabinofuranosyltransferase B
AftC	Arabinofuranosyltransferase C
AftD	Arabinofuranosyltransferase D
AftE	Arabinofuranosyltransferase E
AG	Arabinogalactan
AIDS	Acquired immuno-deficiency syndrome
Ara*f*	Arabinofuranose
AraLAM	Uncapped LAM
BCA	Bicinchoninic protein assay
BCG	Bacille Calmette-Guérin
BHI	Brain heart infusion
BHIS	Brain heart infusion sorbitol
C	Cytosine
CDP-DAG	Cytidine diphosphate-diacylglycerol
Cg	*C. glutamicum*
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
Ci	Curie
CID	Collision induced dissociation
COSY	Correlation spectroscopy
DAG	Diacylglycerol

DAP	Diaminopimelic acid
DAT	Diacyl trehalose
DC	Dendritic cell
DC-SIGN	DC specific intercellular adhesion molecule-3 grabbing non-integrin
DEAE	Diethylaminoethyl
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
Dol-P	Dolichyl phosphate
DPA	Decaprenyl-phosphate-**D**-arabinose
DPG	Diphosphatidyl glycerol
DPM	Dolichyl-phospho-mannose
DPPR	Decaprenylphosphoryl-5-phosphoribose
DPR	Decaprenyl-phosphate-**D**-ribose
EDTA	Ethylenediaminetetraacetic acid
EMB	Ethambutol
ER	Endoplasmic reticulum
f	Furanose
FAB	Fast atom bombardment
g	Grams
G	Guanine
Gal*f*	Galactofuran
GC/MS	Gas chromatography-mass spectrometry
GDP-Man*p*	Guanosine diphospho-mannose pyranose
Gl-A	1,2-di-*O*-C_{16}/$C_{18:1}$-(α-**D**-glucopyranosyl uronic acid)-(1→3)-glycerol
GlcAGroAc2	1,2-di-*O*-C_{16}/$C_{18:1}$-(α-**D**-glucopyranosyl uronic acid)-(1→3)-glycerol

GlcNAc	N-acetylglucosamine
Gl-X	1,2-di-*O*-$C_{16}/C_{18:1}$-(α-**D**-mannopyranosyl)-(1→4)-(α-**D**-glucopyranosyl uronic acid)-(1→3)-glycerol
GMCM	Glucose monocorynomycolate
GPI	Glycosylphosphatidyl inositol
GPLs	Glycopeptidolipids
Gro	Glycerol
h	Hour
HCl	Hydrochloric acid
HIC	Hydrophobic interaction chromatography
HIV	Human immuno-deficiency virus
HMBC	Heteronuclear multiple bond connectivity spectroscopy
HMQC	Heteronuclear multiple quantum correlation spectroscopy
Hz	Hertz
IL	Interleukin
INF-γ	Interferon gamma
Ins	Inositol
IPP	Isopentenyl pyrophosphate
IPTG	Isopropylthio-β-**D**-galactoside
kDa	Kilo Dalton
kPa	Kilo Pascal
L	Litre
LAM	Lipoarabinomannan
LB	Luria-Bertani
LM	Lipomannan

LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
LU	Linkage unit
M	Molar
mAGP	Mycolyl-arabinogalactan-peptidoglycan complex
MALDI-TOF	Matrix assisted laser desorption ionisation-time of flight
$ManGlcAGroAc_2$	1,2-di-*O*-$C_{16}/C_{18:1}$-(α-**D**-mannopyranosyl)-(1→4)-(α-**D**-glucopyranosyl uronic acid)-(1→3)-glycerol
ManLAM	LAM with mannosyl caps
Man*p*	Mannopyranose
MDR	Multi-drug resistant
mg	Milligram
MgtA	α-Mannosyl-glucopyranosyluronic acid-transferase A
min	Minutes
ml	Millilitre
mM	Millimolar
MOPS	4-Morpholine propane suphonic acid
MPA	Molybdophosphoric acid
MPI	Mannosyl-phosphatidyl-*myo*-inositol
MptA	α(1→6) mannospyranosyltransferase A
MptB	α(1→6) mannospyranosyltransferase B
MptC	α(1→2) mannospyranosyltransferase C
MptD	α(1→2) mannospyranosyltransferase D
MS	Mass spectrometry
Ms	*M. smegmatis*

MSX	5-Deoxy-5-methylsulfoxy-xylofuranose
Mt	*M. tuberculosis*
MTX	5-Deoxy-5-methylthio-xylofuranose
Mur	Muramic acid
NAP	Naphthol
NMR	Nuclear magnetic resonance
NOESY	Nuclear Overhauser and exchange spectroscopy
OD	Optical density
P	Phosphate
PAT	Pentaacyl trehalose
PBP	Penicillin-binding proteins
PCR	Polymerase chain reaction
PDIM	Phthiocerol dimycoserosate
PG	Peptidoglycan
PGls	Phenolic glycolipids
PI	Phosphatidyl-*myo*-inositol
PILAM	LAM with phosphoinositide caps
PIM	Phosphatidyl-*myo*-inositol mannoside
Pol-P	Polyisoprenoid phosphate
PPM	Polyprenyl monophosphate
pRpp	5-Phosphoribofuranose pyrophosphate
psi	Pounds per square inch
Rha*p*	Rhamnose pyranose
SDS	Sodium dodecyl sulphate
sec	Second

t	Terminal
T	Thymine
TAE	Tris-acetate EDTA
TB	Tuberculosis
TDM	Trehalose dimycolate
TetR	Tetracycline repressor
TFA	Trifluoroacetic acid
TLC	Thin-layer chromatography
TLR	Toll-like receptor
TMCM	Trehalose monocorynomycolate
TMM	Trehalose monomycolate
TMS	Trimethyl-silyl
UDP-GlcA	UDP-D-glucuronic acid
v/v	Volume/volume
w/v	Weight per volume
µg	Microgram
µl	Microlitre
µM	Micromolar

PUBLISHED WORK ASSOCIATED WITH THIS THESIS

1. **Mishra, A. K., Batt, S., Krumbach, K., Eggeling, L. & Besra, G. S. (2009).** Characterization of the *Corynebacterium glutamiucm*Δ*pimB'*Δ*mgtA* double deletion mutant and the role of *Mycobacterium tuberculosis* orthologues Rv2188c and Rv0557 in glycolipid biosynthesis. *J Bacteriol* **191** (13), 4465-4472.

2. **Mishra, A. K., Alderwick, L. J., Rittmann, D., Wang, C., Bhatt, A., Jacobs, W. R. Jr, Takayama, K., Eggeling, L. & Besra, G. S. (2008).** Identification of a novel α(1→6) mannopyranosyltransferase MptB from *Corynebacterium glutamicum* by deletion of a conserved gene, *NCgl1505*, affords a lipomannan- and lipoarabinomannan-deficient mutant. *Mol Microbiol* **68** (6), 1595-613.

3. **Mishra, A. K., Klein, C., Gurcha, S. S., Alderwick, L. J., Babu, P., Hitchen, P. G., Morris, H. R., Dell, A., Besra, G. S. & Eggeling, L. (2008).** Structural characterization and functional properties of a novel lipomannan variant isolated from a *Corynebacterium glutamicum pimB'* mutant. *Antonie Van Leeuwenhoek*. **94** (2), 277-87.

4. **Mishra, A. K., Alderwick, L. J., Rittmann, D., Tatituri, R. V., Nigou, J., Gilleron, M., Eggeling, L. & Besra, G. S. (2007)**. Identification of an α(1→6) mannopyranosyltransferase (MptA), involved in *Corynebacterium glutamicum* lipomannan biosynthesis, and identification of its orthologue in *Mycobacterium tuberculosis*. *Mol Microbiol* 65 (6), 1503-17.

WORK TO BE COMMUNICATED

1. **Mishra, A. K., Driessen, N. N., Appelmelk, B. J. & Besra, G. S. (2009).** Lipoarabinomannan and related glyco-polymers: Structure, biogenesis and their role in *Mycobacterium tuberculosis* physiology and host interactions. *Manuscript in preparation*.

2. **Mishra, A. K., Krumbach, K., Eggeling, L. & Besra, G. S. (2009).** Lipomannan and lipoarbinomannan biosynthesis in *Corynebacterineae*: The role of two α(1→2) mannopyranosyltransferases (MptC and MptD) in *Corynebacterineae. Manuscript in preparation*.

3. **Mishra, A. K., Geurtsen. J., Krumbach, K., Appelmelk, B. J., Eggeling, L. & Besra, G. S. (2009).** A novel mechanism of mannan priming and immunological properties of hypermannosylated lipomannan from *C. glutamicum*Δ*aftE*. *Manuscript in preparation*.

1. INTRODUCTION

Tuberculosis (TB) is a major cause of death worldwide. Approximately, 9.27 million cases were registered in 2007, out of which 1.37 million were HIV-positive. Of these 9.27 million new cases, an estimated 4.1 million were new smear positive cases, and 2 million deaths were reported, of which 0.45 million were in HIV-positive individuals. To compound this situation 0.5 million cases were multi-drug resistant TB (MDR-TB) and it is estimated that 55 countries globally had reported at least one case of extensively-drug resistant TB (XDR-TB) (Fig. 1.1) (WHO, 2009).

Mycobacterium tuberculosis, which is a Gram-positive bacterium, is the causative agent of TB. *M. tuberculosis* enters the host in the form of tiny airborne particles expelled by an infectious person (droplet infection), whereby the bacterium has evolved a complex system allowing it to evade the immune system of the host. It has an unusual lipid rich cell wall which is unique to the order Actinomycetes including the genera *Mycobacterium*, *Rhodococcus*, *Corynebacterium* and *Nocardia* (Brennan & Nikaido, 1995). It is surprising that *M. tuberculosis* has been classified as a Gram-positive organism since it weakly takes up the Gram stain (Minnikin, 1982). The mycobacterial cell wall is composed of a mycolyl-arabinogalactan-peptidoglycan (mAGP) complex (Besra *et al.*, 1995; Brennan, 2003; Daffe *et al.*, 1990; Dover *et al.*, 2004; McNeil *et al.*, 1990; McNeil *et al.*, 1991), which forms the inner part of the cell wall. Other additional lipids such as phthiocerol dimycocerosates, glycopeptidolipids, menaquinones, and glycosylated phenolpthiocerols intercalate to the mycolic acid layer and form the outer region of the cell wall (Bhowruth *et al.*, 2008; Brennan & Nikaido, 1995; Brennan & Crick, 2007). Such an unique arrangement in the outer envelope results in a highly impermeable barrier for the penetration of chemical drugs, such as penicillin and sulphonamides (Amberson *et al.*, 1931; Minnikin *et al.*, 2002).

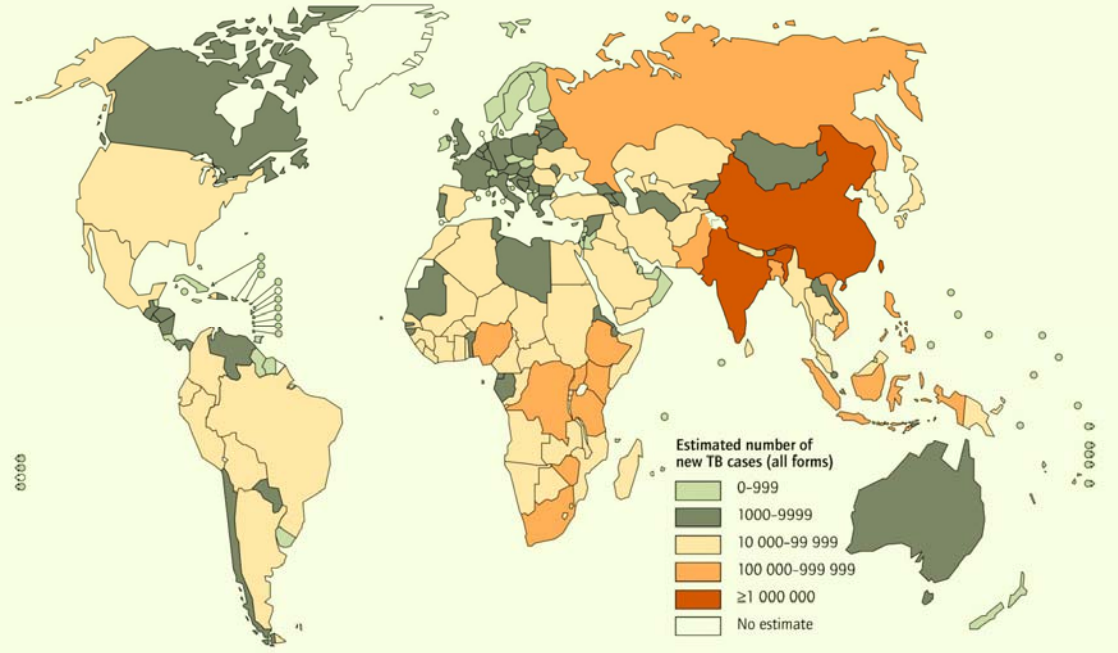

Fig. 1.1: Estimated number of TB cases in the year 2008 (WHO, 2009).

Other cell wall associated lipids, such as phosphatidyl-*myo*-inositol mannosides (PIMs) and lipoglycans, termed lipomannan (LM) and lipoarabinomannan (LAM) are also found in the outer leaflet of the cell wall (Besra *et al.*, 1997; Brennan & Ballou, 1967; Brennan & Ballou, 1968b; Brennan & Nikaido, 1995; Hill & Ballou, 1966; Morita *et al.*, 2004). In addition to their physiological function, these glycoconjugates play a key role in the modulation of the host response during infection (Chatterjee & Khoo, 1998; Maeda *et al.*, 2003; Nigou *et al.*, 2002; Schlesinger *et al.*, 1994). Most of the genetic elements involved in their biosynthesis are essential for the survival of *M. tuberculosis* (Kordulakova *et al.*, 2002), therefore they represent potential drug targets. In this thesis, we will review the history of tuberculosis, followed by the invention of BCG as a vaccine, the adoption of chemotherapy regimens, the emergence of drug-resistant TB; and recent studies centred on the mycobacterial cell wall as a drug target.

1.1 History of TB

TB has plagued human kind throughout its recorded history and has possibly resulted in more deaths than any other microbial pathogen. The disease is caused by the etiological agent *M. tuberculosis* (Koch, 1932; Koch, 1952; Koch, 1982), which evolved from East Africa around 3 million years ago and infected early hominids of that time (Gutierrez *et al.*, 2005). The, availability of genome sequences of various strains of the *M. tuberculosis* complex suggest that all modern strains, including *M. tuberculosis*, *Mycobacterium africanum*, *Mycobacterium canettii* as well as *Mycobacterium bovis*, had a common African ancestor, approximately 35, 000- 15, 000 years ago (Table 1.1) (Brosch *et al.*, 2002; Sreevatsan *et al.*, 1997a).

On the basis of molecular markers on the Y chromosome, the origin of Asian and Oceania populations has been traced to Africa 35,000 to 89,000 years ago (Ke *et al.*, 2001). It is quite possible that these migrants carried diseases with them including TB to other parts of the world. Physiological changes due to TB in humans have been reported from Egypt (3500-2650 BC) (Zink *et al.*, 2001), Sweden (3200-2300 BC), the Eastern Mediterranean (7000 BC) (Hershkovitz *et al.*, 2008) and from the first half of the fourth millennium BC in Italy (Formicola *et al.*, 1987), while written text describing TB are available in India and China from as early as 3300 and 2300 years ago, respectively (Daniel, 2006).

TB was well known in classical Greece, where it was called phthisis or consumption. Hippocrates (460-370 BC), the 'father of medicine' had clearly described the clinical signs of TB and recommended good food, milk and physical exercise for its treatment (Coar, 1982). However, it was Aristotle (384-322 BC), who described 'scrofula' and believed it to be infectious rather than

hereditary, which was a common belief (Garrison, 1913). In the 5th century AD, Caelius Aurelianus, a Roman physician, brilliantly described the diagnosis of a disease with latent fever, coughing with purulent sputum, breathing difficulty, and loss of appetite with few physiological changes in the body (Herzog, 1998). Aretaeus of Capadocia described pulmonary consumption as a disease with chronic sputum with poor prognosis, while Galen (131-201) re-emphasised the contagious nature of the disease (Guthrie, 1945).

For many centuries, there was no further addition to the knowledge of phthisis or consumption probably due to the down-surge of the disease due to unknown reasons. The work of Johannes Gutanberg (Germany, 1398-1468), Girolamo Fracastro (Italy, 1478-1553), and Andreas Vesalius (Holland, 1514-1564), contributed towards the understanding of the disease to some extent (Daniel, 2006). However, it was the era of pathological anatomy, which enhanced the knowledge of the disease considerably. Sylvius de la Boe (Amsterdam, 1617-1655) was the first to describe tubercles as specific characteristics of lungs and its progression into cavities and ulcers. In addition, he also established the correlation between scrofula and consumption, later supported by Richard Morton (London, 1637-1698) (Keers, 1978; Keers, 1982), while Thomas Willis (UK, 1621-1771) and John Jacobus Manget in France shed light on miliary-TB. However, it was the less well-known English physician Benjamin Marten (1690-1751) who suggested the involvement of small living creatures in phthisis. Furthermore, he also shed light on the air-borne infectious nature of that living organism (Doetsch, 1978). Ironically, his work got recognition only after 150 years after the discovery of *M. tuberculosis* by Robert Koch (Koch, 1932).

Invaluable contributions made by Giovanni Battista in Padua (Italy, 1682-1771) and French giants Marie-Francois-Xavier Bichat (1771-1802), Gaspard Laurent Bayle (1774-1816), and Jean

Time	Event/Discovery	References
3 million years ago	**Evolution of *M. tuberculosis***	(Gutierrez *et al.*, 2005)
35,000-15,000 years ago	All strains of *M. tuberculosis* complex evolved from a common African Ancestor	(Brosch *et al.*, 2002; Sreevatsan *et al.*, 1997a)
7000 BC	Physiological changes from tuberculosis like disease were reported from Eastern Mediterranean	(Hershkovitz *et al.*, 2008)
460-370 BC	Hippocrates described the clinical signs of TB	(Coar, 1982)
384-322 BC	**Aristotle described TB as infectious**	(Garrison, 1913)
5th Century	Caelius Aurelianus complete diagnosis of TB	(Herzog, 1998)
1600-1700	Tubercles were recognized as specific characteristics of lungs	(Keers, 1978)
1705	Benjamin Marten established the role of a small air-borne living infectious creature in TB	(Doetsch, 1978)
1781-1826	**Concept of pulmonary or extrapulmonary TB** (By Rene Theophile Hycinthe Laennec)	(Laennec, 1962)
1834	**Schonlein coined the term 'tuberculosis'**	(Ferlinz, 1995)
1840s	**Henle-Koch postulates**	(Evans, 1976)
1865	Role of a specific microorganism (Jean Antonie Villemin)	(Herzog 1998)
1880-1940	Treatment in sanatorium	
1881	**Discovery of *M. tuberculosis* and tuberculin** (Robert Kotch)	(Koch, 1932)
1907-1926	**Tuberculin skin test** (Clemens Freiherr von Pirquet, Charles Mantoux, Florence Seibert)	(von Pirquet, 1907, Lebedeva, 1977, Seibert, 1926)
1888-1960	Lungs collapse therapy by artificial pneumothorax, phrenicectomy or thoracoplasty	(Sharpe, 1931, Sakula, 1983)
1920s	**Vaccine 'BCG'** (Albert Calmette and Camille Guerin)	(Calmette and Guérin, 1924, Calmette, 1928)
1950	**Discovery of Streptomycin** and other antibiotics	(Schatz *et al.*, 1944, Jones *et al.*, 1944)
1950-60	Development of DOTS procedure	
1990	MDR-and XDR-TB pandemic	(Pablos-Mendez *et al.*, 1998)

Table 1.1: History of TB. Bold sentences marked for major milestones in field of TB research.

Nicolas Corvisart (1755-1821) were followed by Rene Theophile Hycinthe Laennec's work (1781-1826), which explained TB pathogenesis and gave the concept of pulmonary and extrapulmonary TB (Laennec, 1962). The field of TB research was revolutionised with the entry of German scientists. Johann Lukas Schonlein of Wurzberg (1793-1864) who had different

thoughts regarding scrofula, tubercles and phthisis coined the term 'tuberculosis' to describe the affliction with tubercles (Ferlinz, 1995). In 1840, Jakob Henle (1809-1885) in Gottingen suggested that phthisis could be contagious only under certain circumstances and gave three postulates for categorising a disease as infectious: (1) the causative agent must be found in every case of disease; (2) it must not occur in another disease; (3) its application must always result in the same disease; later re-stated by his pupil Robert Koch which is also known as Henle-Koch postulates in bacteriology (Evans, 1976).

Later in 1865, Jean Antonie Villemin (1827-1892) demonstrated the transmission of phthisis from blood or sputum from diseased human and cattle to rabbits and guinea pigs and showed that a specific microorganism causes the disease. Years later in 1877, Theodor Klebs (1834-1913), was able to maintain the causative agent in artificial medium, however he was unable to recognise the true nature of the agent (Herzog, 1998).

1.2 Discovery of *Mycobacterium tuberculosis*

On 24th March 1881, Robert Koch (1843-1910) delivered a famous lecture entitled '*Die Ätiologie der Tuberkulose*' to the Physiological Society at the Charité Hospital in Berlin. He used microscopic techniques and identified the causative agent from tuberculous tissue as a rod-shaped bacilli which he called '*Mycobacterium tuberculosis*' and also verified the Henle-Koch postulates regarding the disease causing microorganism (Koch, 1932). Later, he introduced the concept of primary and secondary infection in guinea pigs (Koch's phenomenon) and discovered 'tuberculin'-a glycerin extract of dead tubercle bacilli, which was later utilised for tuberculin skin test. For his contribution, Robert Koch was awarded with the Noble Prize in Medicine or

Physiology in 1905.

In 1907, Clemens Freiherr von Pirquet (Austria, 1874-1929) established that 'tuberculin' can be injected intra-cutaneously and resulted in an immune response (von Pirquet, 1907). Further, Charles Mantoux (France, 1877 - 1947), invented a more safe cannulated needle and syringe for tuberculin injection (Lebedeva, 1977) and Florence Seibert (USA, 1898-1991) isolated the active substance from tuberculin: Purified Protein Derivative (PPD) which further improved the accuracy of the tuberculin skin test (Seibert, 1926; Seibert & Dufour, 1948).

1.3 Initial attempts of TB treatment

The discovery of *M. tuberculosis* as the causative agent and the tuberculin skin test marked key milestones in TB research. However, thousands of individuals were dying due to the absence of a treatment. In the absence of antibiotics, the concept of public health came into existence. Hermann Brehmer (Germany, 1826-1889) emphasised the necessity of an immune balance and the concept of sanatoria: an environment with fresh air, good food, and rest and devoid of any known consumptives. In the following years, this regimen was practiced all over the world for the treatment of TB. Initially, recovery was surprising; however, the long-term results were not encouraging. However, it helped in the restriction of TB transmission, as infected patients were confined to certain locations, minimising contact transmission (Daniel, 2006).

In patients where bed-rest alone was not sufficient, the localised collapse therapy by artificial pneumothorax, phrenicectomy or thoracoplasty, which consists of introducing clean and filtered air into the pleural space of the lung was introduced (Sharpe, 1931).

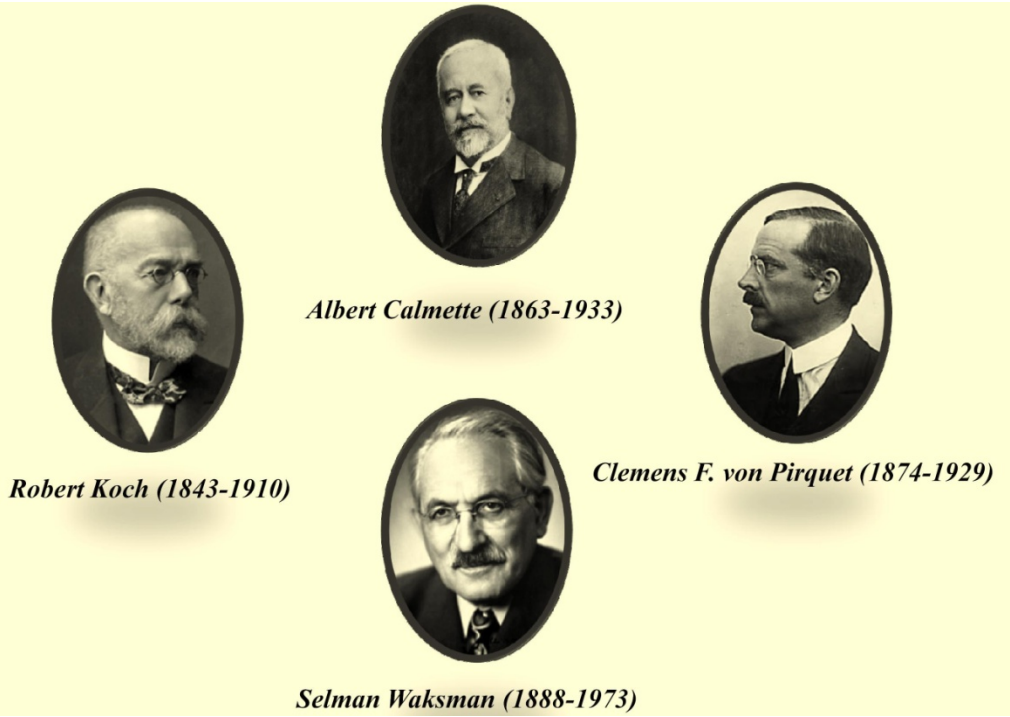

Fig. 1.2: Few of the main contributors in TB research.

In 1888, Carlo Forlanini (Italy, 1847-1918) performed the first artificial pneumothorax (Sakula, 1983). While collapse therapy yielded successful results; with closure of cavities, conversion to negative sputa, and low-operative mortality, it also produced numerous complications which included tissue infection, conversion into acute TB, fistula formation and empyma (Gaensler, 1982).

1.4 Invention of 'BCG'

The concept of a vaccine was introduced by Edward Jenner (England, 1749-1823) in 1796, when he found that the use of pus from a hand of a milkmaid with cow pox, when administered to healthy individuals provided protection against smallpox (Baxby, 1999). Based on similar experiments, Frenchmen Albert Calmette (1863-1933) and Camille Guerin (1872-1961), obtained

an attenuated strain of *M. bovis* BCG which could induce protective immunity in humans, especially at an early age (Calmette & Guérin, 1924; Calmette, 1928). Soon a worldwide campaign against TB was started with the involvement of WHO, UNICEF and the Red Cross, based on the use of the tuberculin skin test followed by BCG vaccination (Comstock, 1994).

1.5 Final combat: The discovery of streptomycin and other drugs against TB

Selman Waksman (USA, 1888-1973) and colleagues at the University of California demonstrated that streptomycin, an antibiotic, isolated from cultures of *Streptomyces griseus* had the ability to inhibit tubercle growth with low toxicity in laboratory animals (Jones *et al.*, 1944; Schatz *et al.*, 1944). Subsequently, Selman Waksman was awarded the Nobel Prize in Physiology or Medicine in 1952. This success was short-lived due to the appearance of resistant *M. tuberculosis* mutants. However, the problem of drug resistance could be overcome by using combinations of two or three drugs under a drug regimen (Table 1.2).

Following the discovery of streptomycin, *p*-aminosalicylic acid (Nagley, 1949; Nagley & Logg, 1949), isoniazid [INH] (Steenken & Wolinsky, 1952a; Steenken & Wolinsky, 1952b), pyrazinamide (Mc *et al.*, 1954; Muschenheim *et al.*, 1954; Tompsett *et al.*, 1954), cycloserine (Morton *et al.*, 1955), ethambutol [EMB] (Forbes *et al.*, 1962), and rifampin (Furesz & Timball, 1963) were developed as anti-TB drugs. There was an improvement in the disease condition with the recommended clinical regimen, involving these drugs (Table 1.2). However, due to patient compliance and non-adherence to the drug regimen the number of cases has increased and now the situation has led to the emergence of MDR- and XDR-TB (Pablos-Mendez *et al.*, 1998).

Drug	Prescribed Dose
	Initial phase (2 months)
Isoniazid	5 mg/kg/day (max. dose 300 mg/day)
Rifampin	10 mg/kg/day (max. dose 600 mg/day)
Pyrazinamide	30 mg/kg/day (max. dose 2 g/day)
Ethambutol	15–25 mg/kg/day (max. dose 2 g/day)
	Consolidation phase (4–6 months)
Isoniazid	as above
Rifampin	as above
	or
Isoniazid	twice a week 14 mg/kg (max dose 1 g/day)
Rifampin	twice a week 10 mg/kg (max. dose 600 mg/day)

Table 1.2: Chemotherapy protocol for the treatment of TB (WHO, 2009). First-line drugs are isoniazid, rifampin, ethambutol, pyrazinamide, and streptomycin.

1.6 Drug resistant TB

During mid-1950s, three-drug combination therapies, consisting of isoniazid, rifampin, pyrazinamide, and ethambutol initially for two months, followed by a two-drug phase of isoniazid and rifampin lasting for four months, were introduced (Table 1.2) (WHO, 2009). The advent of such a program, almost eradicated TB from many countries. However, the occurance of HIV and the emergence of MDR- and XDR-TB has led to an uncontrollable spurt in TB cases (Snider & La Montagne, 1994).

The complex nature and length of therapy, drug supply, and the tendency of patients to feel well before completion of the course of treatment has accelerated and promoted MDR- and XDR-TB (Munro *et al.*, 2007). MDR-TB is a form of drug-resistant TB in which *M. tuberculosis* can no longer be killed by at least two of the front line antibiotics, isoniazid and rifampin, and XDR-TB involves resistance against second-line drugs, including fluoroquinolone, and at least one of the other three injectable anti-TB drugs; amikacin, kanamycin, and capreomycin (WHO, 2009).

MDR-TB requires up to two years of multi-drug treatment and XDR-TB is untreatable so far. Therefore, there is a need for rapid and continued progress in the development of new antimicrobial compounds against *M. tuberculosis*.

1.7 The cell wall of *M. tuberculosis* as a drug target

In the case of MDR and XDR strains of *M. tuberculosis*, the antibacterial compounds which can act on actively growing *M. tuberculosis* may help towards the treatment of TB (Bhowruth *et al.*, 2007). In this regard, cell wall inhibitors have been one of the most active agents of chemotherapy. Agents such as, INH and EMB, inhibitors of mycolic acid and arbinogalactan (AG) biosynthesis, respectively, have proved highly successful against combating the disease (Bhatt *et al.*, 2007b; Bhowruth *et al.*, 2008; Brennan & Crick, 2007). However, due to the development of drug resistant strains (Heymann *et al.*, 1998; Sreevatsan *et al.*, 1997b; Telenti *et al.*, 1997), there is a need for the identification of novel drug targets and development of active compounds against them. In this respect the biosynthetic machinery of mycobacterial cell wall represents an attractive target (Bhatt *et al.*, 2007b; Bhowruth *et al.*, 2007; Brennan & Crick, 2007; Dover *et al.*, 2008).

1.8 Structural features of the mycobacterial cell wall

D. E. Minnikin first proposed a model of the mycobacterial cell wall in 1982, which was subsequently modified by McNeil and Brennan (Fig. 1.3) (McNeil & Brennan, 1991; Minnikin, 1982; Minnikin *et al.*, 1982). The mycobacterial cell wall core consists of a peptidoglycan (PG) layer, followed by AG, mycolic acids, and additional lipids and lipoglycans attached to the

plasma membrane (Dover *et al.*, 2004; McNeil & Brennan, 1991; Minnikin, 1982; Minnikin *et al.*, 1982). The PG layer is covalently attached to the AG through an unique linker unit (Amar & Vilkas, 1973; McNeil *et al.*, 1990; Vilkas *et al.*, 1973). The AG consists of a highly-ordered galactan attached to a branched arabinan which is esterified at the distal end to PG by a family of long-chain unusual lipids, termed mycolic acids. This entire structure is known as the mycolyl-arabinogalactan-peptidoglycan (mAGP) complex (Fig. 1.3) (Besra & Brennan, 1997).

1.8.1 Mycolyl-arabinogalactan-peptidoglycan (mAGP) complex

Mycobacterial PG is very similar to the PG of *Escherichia coli* (Janczura *et al.*, 1981; Petit *et al.*, 1969; Wietzerbin *et al.*, 1974). The glycan part in mycobacteria consists of alternating *N*-acetylglucosamine (GlcNAc) and a modified muramic acid (Mur) (Azuma *et al.*, 1970), which is oxidised to *N*-glycolyl units to form MurNGly (Mahapatra *et al.*, 2005a; Mahapatra *et al.*, 2005b). Further modification of the MurNGly unit with a tetrapeptide unit, -L-alanyl-**D**-*iso*-glutaminyl-*meso*-diaminopimelyl-**D**-alanine-, results in a stem-peptide which is further cross-linked to form the complete structure of PG (Petit *et al.*, 1969; Takayama *et al.*, 1970; Wietzerbin-Falszpan *et al.*, 1970; Wietzerbin *et al.*, 1974). The muramic acid residue provides the key attachment point to the galactan segment of AG *via* the disaccharide bridge, α-L-Rha*p*-(1→3)-**D**-GlcNAc-(1→P), at C-6 position of MurNGly (Azuma *et al.*, 1970; Takayama *et al.*, 1970).

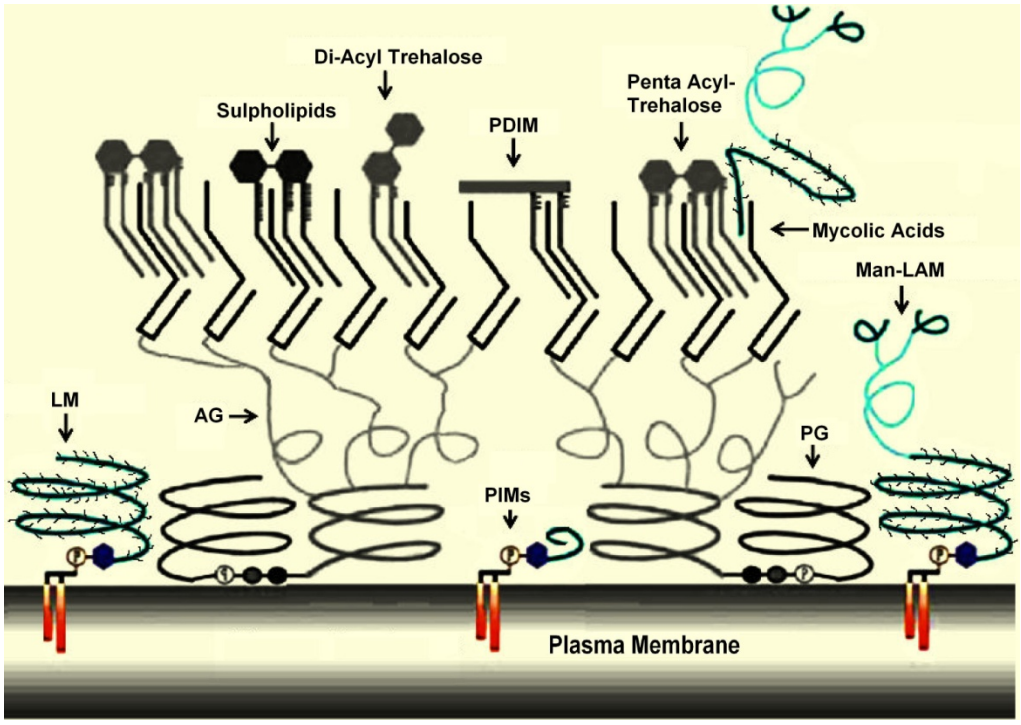

Fig. 1.3: Structural representation of cell wall core of *M. tuberculosis* (Adapted from Dover et al., 2004). The M. tuberculosis cell wall possesses covalently attached mycolic acid residues intercalated with TMM and TDM glycolipids and a diverse repertoire of complex lipids. The AG domain consists of arabinan and galactan that is further intercalated with the coiled glycan domains of PG via linker unit Rha-GlcNAc-phosphate linker. Glycolipids such as PIM6, anchored to plasma membrane through PI, followed by a string of mannose (LM), which is further branched by arabinan in LAM.

The galactan domain of AG is made up of a linear chain of approximately 30 residues of β-**D**-Gal*f* linked together by alternating β(1→5) and β(1→6) linkages resulting in a helical-shaped polysaccharide chain (Daffe *et al.*, 1990; Dmitriev *et al.*, 2000). Approximately three chains made up of α-**D**-Ara*f* units are linked to the C-5 position of β(1→6)-linked galactose residues towards the non-reducing end of the galactan backbone (Besra *et al.*, 1995; Daffe *et al.*, 1990). Interestingly, most of the arabinan of AG consists of a linear α(1→5)-**D**-Ara*f* residues which are branched with 3,5-linked α-**D**-Ara*f* units which are further extended with α(1→5)-**D**-Ara*f* residues towards the non-reducing end (Besra *et al.*, 1995). The non-reducing termini is further decorated with a branched hexa-arabinofuranosyl (Ara-6) motif, [t-β-**D**-Ara*f*-(1→2)-α-**D**-Ara*f*]$_2$-

3,5-α-**D**-Ara*f*- (1→5)-α-**D**-Ara*f*, which are covalently linked to mycolic acids (Besra *et al.*, 1995; McNeil *et al.*, 1991; McNeil *et al.*, 1994).

The mycolic acids are α-alkyl-β-hydroxy fatty acids (C_{70}-C_{90}) and are unique to the genus *Mycobacterium* which makes the outer layer of bacterium highly lipid rich and impermeable to hydrophilic antibiotics (Fig. 1.3) (Minnikin, 1982). Apart from esterified mycolates, mycobacteria also possesses free, solvent-extractable trehalose conjugates of mycolic acids; trehalose mono- and di-mycolates (TMM and TDM, respectively) (Asselineau & Lederer, 1950; Minnikin, 1982; Minnikin *et al.*, 1982). Based on their structure, mycolic acids are classified into three classes; α-mycolic acids, ketomycolates and methoxymycolates (Minnikin, 1982). Interspersed within the hydrophobic environment provided by mycolic acids of the mAGP complex are the lipids, which form the outer layer of the pseudomembrane (Brennan & Nikaido, 1995; Minnikin, 1982). These include phthiocerol dimycerosate, menaquinones, glycosylated phenolpthiocerols, TMM, TDM, sulpholipids, glycopeptidolipids, and phosphoinositolmannosides (Fig. 1.3) (Bozic *et al.*, 1988; Chatterjee *et al.*, 1988a; Chatterjee *et al.*, 1988b; Cosma *et al.*, 2003; Fujiwara *et al.*, 1984; Glickman *et al.*, 2001; Huang *et al.*, 2002; Minnikin, 1982; Minnikin *et al.*, 2002).

1.8.2 Structural features of PIMs/LM/LAM

PIMs, LM, and LAM are believed to be non-covalently attached to the cell membrane *via* the lipid portion of the PI anchor (Hunter & Brennan, 1990), while phosphatidyl-*myo*-inositol dimannoside (PIM_2) serves as the scaffold at which higher mannosylated PIMs and lipoglycans are built (Fig. 1.4). The mannosylated core in LM consists of a linear α-(1→6)-linked mannan

which extends from the mannose (Man*p*) residue linked to the *O*-6 position of inositol in PIM_2 (Besra *et al.*, 1997; Chatterjee *et al.*, 1991) and is further decorated by singular α-(1→2)-linked Man*p* residues. In LAM, this mannan backbone is further elaborated by the addition, through a currently unknown linkage, of an arabinan domain similar to that found in AG, and finally decorated by Man caps (Man-LAM) (Chatterjee *et al.*, 1993; McNeil *et al.*, 1994).

1.8.2.1 Phosphatidyl-*myo*-inositol mannosides (PIMs)

During a study to identify novel phospholipids in mycobacteria, PIMs were accidently discovered and after a detailed chemical analysis they were categorised as glycolipids with fatty acids attached to glycerol, linked by a phosphodiester to *myo*-inositol and α-**D**-Man*p* residues (Vilkas & Lederer, 1956). It was later shown that the glycerol phosphate moiety was attached to the **L**-l-position of *myo*-inositol (Ballou *et al.*, 1963). This phosphate-*myo*-inositol (PI) is based on a *sn*-glycero-3-phospho-(1-**D**-*myo*-inositol) unit, where hydroxyl moieties at *O*-2 and *O*-6 positions of *myo*-inositol are substituted with α-**D**-mannopyranose (α-**D**--Man*p*) units in case of PIM_2 (Lee & Ballou, 1964; Nigou *et al.*, 2003). The anchor is heterogeneous, with variations occurring with respect to the number, the location, and the nature of the fatty acids. There are four potential sites of acylation with different fatty acids (e.g. palmitic, stearic and tuberculostearic acid) at positions 1 and 2 of the glycerol unit in the anchor, position 3 of *myo*-inositol and position 6 of the Man*p* unit linked at *O*-2 of *myo*-inositol (Khoo *et al.*, 1995b; Nigou *et al.*, 2003). In mycobacteria, palmitic and tuberculostearic (10-methyl-octadecanoic) acids are predominant, while myristic and octadecenoic acids are also found in large amounts, with traces of stearic, hexadecenoic and heptadecanoic acids (Ballou & Lee, 1964; Lee & Ballou, 1964; Nigou *et al.*, 2003).

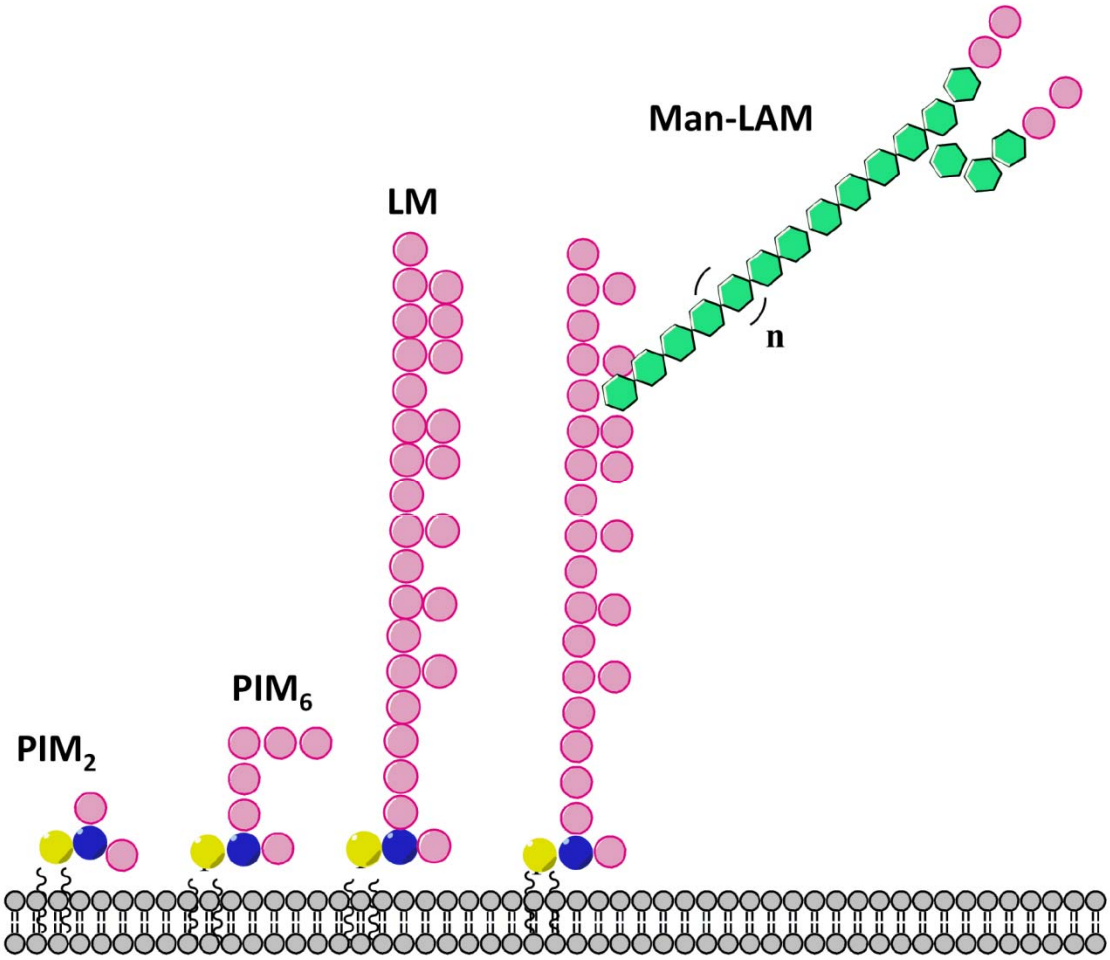

Fig. 1.4: Structure of LAM and related glyco-conjugates found in the cell wall of *M. tuberculosis*. Structural analysis of the mycobacterial cell wall suggests that only di- and hexa-mannosylated versions of PIMs, and the higher glycosylated polymers LM and LAM accumulate in the cell wall. In these glyco-conjugates, PI (yellow and blue circles) acts as anchor to the plasma membrane and further glycosylated at 2-OH and 6-OH positions of inositol by Man*p* residues (pink) which results in the synthesis of PIM_2. In case of PIM_6, Man*p* at 6-OH position of inositol is linked to three and two residues of α(1→6)-Man*p* and α(1→2)-Man*p*, respectively, while in LM and the mannan core of LAM, PIM_2 is linked to another 17-19 residues of Man*p* in α(1→6) direction and 7-9 singular branched α(1→2)-Man*p* units. Mature LM is further linked *via* an unknown linkage to an arabinan domain made up of 70 arabinan residues (green). The majority of the arabinan domain consists of a linear α(1→5)-Ara*f* polymer branched at certain positions with α(3→5)-Ara*f* residues towards its non-reducing end resulting in a linear (Ara-4) or/and branched (Ara-6) arabinan domain which in turn is terminated by β(1→2)-Ara*f* and capped by α(1→2)-Man*p* units. n= to 55 units of Ara*f*.

Further, Lee and Ballou (1965) discovered a pentamannoside, Man*p*-α(1→2)-Man*p*-α(1→2)-Man*p*-α(1→6)-Man*p*-α(1→6)-Man*p*-α(1→.) attached to position *O*-6 of the *myo*-inositol of PI and identified it as phospho-*myo*-inositol-hexamannoside (PIM_6) (Lee & Ballou, 1965). They also reported a biosynthetic relationship between PIM_1 and PIM_2, and suggested the involvement of a stepwise glycosylation of phosphatidyl-*myo*-inositol (PI), first at the *O*-2 position and then at the *O*-6 position of the inositol ring (Ballou & Lee, 1964). With the use of fast atom bombardment-mass spectrometry (FAB-MS) and gas chromatography-mass spectrometry (GC-MS) analysis of acylated versions of PIMs, LM and LAM, Khoo and colleagues (1995) suggested that a C_{16} fatty acyl substituent is attached to the 6-OH position of the *O*-2 mannose attached to the inositol of PIM_2 and also present in LM and LAM from *M. tuberculosis* and *Mycobacterium leprae* (Khoo *et al.*, 1995b). Furthermore, it was suggested that the acylated version of PIM_2 i.e. Ac_1PIM_2 is both a metabolic end-product and an intermediate in Ac_1PIM_6 and LM/LAM synthesis (Besra *et al.*, 1997; Khoo *et al.*, 1995b).

Recently, the presence of 'glucuronic acid diacyl-glycerol ($GlcAGroAc_2$)' based glycolipids, ($GlcAGroAc_2$ and $ManGlcAGroAc_2$) and a novel lipomannan (Cg-LM-B) were reported in *Corynebacterium glutamicum*, and to date this lipoglycan has not been identified in mycobacteria (Lea-Smith *et al.*, 2008; Mishra *et al.*, 2008b; Mishra *et al.*, 2009; Tatituri *et al.*, 2007b). However, Rv0557 [MgtA] of *M. tuberculosis* has shown the ability to synthesise these novel lipids and lipoglycan in *in vitro* and *in vivo* (Mishra *et al.*, 2009; Tatituri *et al.*, 2007b), and all members of the genus *Mycobacterium* possess the orthologue of MgtA. Therefore, the possibility remains for the identification of a glucoronic acid based-LM in mycobacteria.

1.8.2.2 Discovery of polysaccharides containing arabinan and mannan

In 1930, Masucci and colleagues isolated a polysaccharide with high serological activity from a mycobacterial culture medium and showed that it contained **D**-arabinose and **D**-mannose (Masucci *et al.*, 1930). At the same time, Chargaff and Schaefer (1935) reported the identification of two polysaccharides prepared from defatted *M. bovis* BCG. One of the polysaccharides contained **D**-mannose and **D**-arabinose together with a small amount of inositol (Chargaff & Schaefer, 1935). Later, Seibert and Watson (1941) separated these polysaccharides using electrophoresis (Seibert & Watson, 1941). However, it was the work of Nobel laureate Sir Norman Haworth at the University of Birmingham, UK, which described the structural features of these polysaccharides from heat-killed cells of *M. tuberculosis* (Haworth *et al.*, 1948). Polysaccharides were methylated using methyl sulphate in the presence of sodium hydroxide and identified as highly branched structures composed of Man*p*, Ara*f*, amino-sugar, and rhamnopyranose (Rha*p*) units forming terminal residues (Haworth *et al.*, 1948).

Later, Misaki and Yukawa at the University of Osaka, Japan, identified one of the polysaccharides as arabinogalactan (Misaki & Yukawa, 1966), in which the majority of the arabinans were covalently linked by α(1→5)-glycosidic linkages with minor α(1→3) and α(1→2) linkages (later re-annotated as β(1→2)), whilst the other was the immunologically active arabinomannan (Misaki *et al.*, 1977). They described the arabinomannan as a polysaccharide consisting of α(1→6) mannan backbone with short chains of α(1→2)-Man*p* and α(1→5)-linked **D**-arabinose residues. In addition to arabinomannan, an immunologically inactive α-**D**-mannan was also identified whose structure resembled that of the core mannan of the arabinomannan (later identified as LM) (Misaki *et al.*, 1977).

1.8.2.3 Identification of LM and LAM

After these initial studies, Hunter *et al.* (1986) purified a polysaccharide from both *M. leprae* and *M. tuberculosis* using anion exchange and gel filtration chromatography. Apart from Man*p* and Ara*f* the polysaccharide contained glycerol, *myo*-inositol phosphate and was acylated by lactate, succinate, palmitate, and 10-methyloctadecanoate (Hunter *et al.*, 1986). Further biochemical analyses established that "arabinomannan" of the genus *Mycobacterium* in its native state is acylated, contains the substituents of PI, which was later shown to be membrane bound (Hunter *et al.*, 1986; Hunter & Brennan, 1990). They also isolated the mannan of *M. tuberculosis* as the native LM and proposed these polysaccharides as a multiglycosylated version of the mycobacterial PIMs (Hunter and Brennan 1990), which was verified later (Chatterjee *et al.*, 1992a).

Hunter and Brennan (1990) proposed that LM and LAM are covalently and/or non-covalently attached to the cell membrane *via* the lipid portion of PI, while PIM_2 serves as the scaffold at which LM and LAM are built (Hunter & Brennan, 1990). The *O*-6 position of inositol in PIM_2 serves as the attachment point for the synthesis of the α(1→6)-mannan backbone which is composed of around 25-30 residues of Man*p* and decorated by singular α(1→2)-Man*p* units, resulting in the formation of LM (Chatterjee *et al.*, 1992a). The mannan core is further elaborated by the addition of an arabinan domain consisting of approximately 60-70 Ara*f* residues in a linear α(1→5)-**D**-Ara*f* fashion with 3,5-α-**D**-Ara*f* branches. The linear chain is highly branched and conserved (Chatterjee *et al.*, 1991; Chatterjee *et al.*, 1992a; Chatterjee *et al.*, 1992b; Chatterjee *et al.*, 1992c) with two types of chain arrangements. Firstly, linear tetraarabinofuranosides (Ara-4) of the structure β-**D**-Ara*f*(1→2)-α-**D**-Ara*f*(1→5)-α-**D**-Ara*f*(1→5)-α-**D**-Ara*f*, and secondly,

branched (Ara-6) motifs with the structure [β-**D**-Ara*f*(1→2)-α-**D**-Ara*f*]$_2$-3,5-α-**D**-Ara*f*(1→5)-α-**D**-Ara*f* (Chatterjee *et al.*, 1991). In both the cases, the non-reducing end is characterised by the disaccharide unit, Ara*f*-β(1→2)-Ara*f*-α(1→.) (Chatterjee *et al.*, 1991; McNeil *et al.*, 1994).

1.8.2.4 Characterisation of non-reducing termini of LAM

The arabinan termini of LAM from the virulent, Erdman strain of *M. tuberculosis* was shown to be capped with α(1→2)-**D**-Man*p* residues following analysis after arabinase treatment (Chatterjee *et al.*, 1992b). It was established that the tetra/hexaarbinofuranoside unit was further extended by mono-, di- and tri-α(1→2)-**D**-Man*p* units (Chatterjee *et al.*, 1992b; Chatterjee *et al.*, 1993). At the same time, Puzo and colleagues (1993) reported the structure of Man-LAM from *M. bovis* BCG Pasteur, using 2-D Nuclear Magnetic Resonance (NMR) spectroscopy and per-*O*-methylation studies revealing that the Man-LAM from *M. bovis* BCG contained two types of terminal Man*p* and 2-*O*-linked Man*p* residues (Venisse *et al.*, 1993). The number of mannose caps is species specific with *M. tuberculosis* H37Rv and *M. bovis* BCG Man-LAM equally capped with around seven caps *per* molecule (Khoo *et al.*, 1995b; Nigou *et al.*, 2003). Surprisingly, LAM from a fast growing *Mycobacterium sp.*, was devoid of any Man*p* caps (termed as Ara-LAM) (Chatterjee *et al.*, 1992b) and in turn, a novel inositol phosphate capping motif was identified on the arabinan termini of LAMs from *Mycobacterium smegmatis* ATCC 14468 and mc^{2}155 (PI-LAM) (Khoo *et al.*, 1995a).

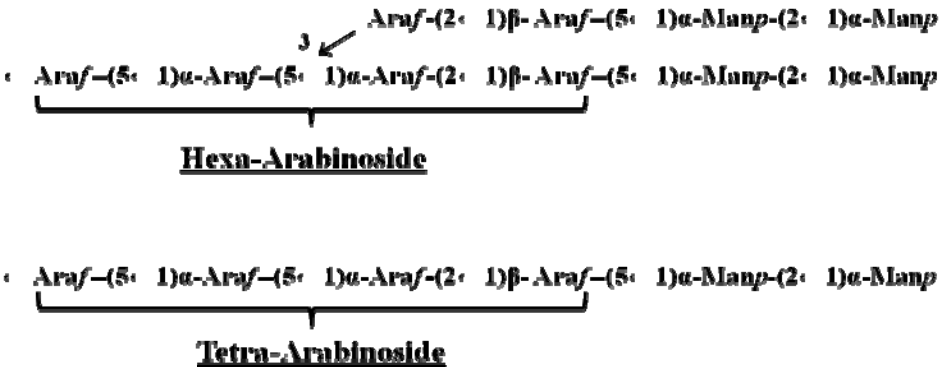

Fig. 1.5: The non-reducing termini of the arabinan domain of Man-LAM from *M. tuberculosis*. The arabinan polymer is a linear α(1→5)-linked Ara*f* backbone punctuated with branched hexa-Ara*f* or/and tetra-Ara*f* motifs. The mannose caps, which terminate the arabinan domain, consist of a single Man*p* residue, a dimannoside [α-**D**-Man*p*-(1→2)-α-**D**-Man*p*-(1→.)] or a trimannoside [α-**D**-Man*p*-(1→2)α-**D**-Man*p*-(1→2)α-**D**-Man*p*-(1→.)].

1.8.2.5 Further modifications of LAM

Nigou and colleagues (1997) established that *M. bovis* BCG contains two types of Man-LAM, namely parietal and cellular, which are different in terms of the percentage of Man*p* caps and the lipid anchor moiety (Nigou *et al.*, 1997). Parietal Man-LAM had a novel fatty acid assigned as 12-*O*-(methoxypropanoyl)-12-hydroxystearic acid, esterified at *C*-1 of the glycerol residue of PI, while cellular Man-LAMs were largely heterogeneous with palmitic and tuberculostearic acid (Nigou *et al.*, 1997). Later, they also reported that these *iso*-forms of Man-LAM occur in *M. tuberculosis* (Gilleron *et al.*, 2000). In different *M. bovis* BCG strains (Pasteur, Glaxo, Copenhagen, and Japanese strains), the presence of succinyl groups on *O*-2 of the 3,5-di-α-**D**-Ara*f* of arabinan domain of Man-LAM were also reported (Delmas *et al.*, 1997). Recently, Treumann and colleagues (2002) identified a 5-methylthiopentose substituent on the terminal Man*p* in the cap structure of Man-LAM in several strains of *M. tuberculosis* (Treumann *et al.*, 2002) which was later characterised as 5-deoxy-5-methylthio-xylofuranose (Joe *et al.*, 2006).

1.9 Biogenesis of cell wall skeleton

The importance of studying the biosynthesis of mycobacterial cell wall is necessary with regards to the development of new drugs against TB, which can target the novel enzymes involved in its synthesis (Bhatt *et al.*, 2007b; Bhowruth *et al.*, 2007; Brennan & Crick, 2007; Dover *et al.*, 2008). The following section highlights key steps of mycobacterial cell wall biogenesis.

1.9.1 Biogenesis of mAGP complex

1.9.1.1 Mycobacterial PG

Biosynthesis of mycobacterial PG is very similar to *E. coli*. N-acetylmuramic acid (MurNAc) bound to an undecaprenyl carrier lipid *via* a phosphodiester is the main precursor of PG. In *M. tuberculosis,* UDP-MurNAc is synthesised from UDP-GlcNAc catalysed by MurA [Rv1315] and MurB [Rv0482] similar to *E. coli* (De Smet *et al.*, 1999; Goffin & Ghuysen, 2002). Initially, UDP-MurNAc is converted into UDP-MurNGly and UDP-MurNGly-Ala, which is later modified by series of enzymes: MurC [Rv2152c] (Mahapatra *et al.*, 2000), MurD [Rv2155c] (Crick *et al.*, 2001), MurE [Rv2158c], and MurF [Rv2157c] for the sequential addition of **D**-glutamate, diaminopimelate and **D**-alanyl-**D**-alanine dipeptide, respectively (Goffin & Ghuysen, 2002; van Heijenoort, 2001a; van Heijenoort, 2001b). However, MurX (Rv2156c) catalyses the transfer of phosphoryl-MurNGly-(pentapeptide) to its DPP acceptor which affords decaprenyl-diphosphoryl-MurNGly-(pentapeptide) (Dover *et al.*, 2004). The final step in PG biosynthesis involves transpeptidation, i.e. the cross linking of glycan chains (Bhakta & Basu, 2002; Goffin & Ghuysen, 2002).

1.9.1.2 Biosynthesis of AG

Mycobacterial AG is attached to PG by a linker unit which is synthesised by transfer of GlcNAc from UDP-GlcNAc onto a C_{50}-polyprenol carrier by a decapaprenyl-phosphate α-N-acetylglucosaminyltransferase, Rfe (UDP-GlcNAc transferase, Rv1302) (Mikusova *et al.*, 1996). The synthesised lipid, C_{50}-P-P-GlcNAc, is further glycosylated to form C_{50}-P-P-GlcNAc-Rha*p* by a rhamnosyltransferase, WbbL [Rv3265c] which utilises dTDP-Rha*p* as a donor (Fig. 1.6) (Mikusova *et al.*, 2000; Mills *et al.*, 2004). C_{50}-P-P-GlcNAc-Rha*p* then further serves as an acceptor for the sequential polymerisation of the galactan segment of AG with Gal*f* residues from UDP-Gal*f* (Lee *et al.*, 1996) initially mediated by an unique galactosyltransferase, GlfT1 [Rv3782] (Mikusova *et al.*, 1996), which transfers the first two Gal*f* residues, followed by further polymerisation by another bifunctional galactosyltransferase, GlfT2 [Rv3808c] which adds approximately a further 30 Gal*f* residues in alternating β(1→5) and β(1→6) fashion (Kremer *et al.*, 2001a).

The C_{50}-P-P-GlcNAc-Rha*p*-Galf_{30} is further primed by AftA [Rv3792] using decaprenol-P-arabinofuranose (DPA) as an Ara*f* donor at precisely three positions along the galactan at the 8th, 10th, and 12th Gal*f* residue (Alderwick *et al.*, 2006b). C_{50}-P-P-GlcNAc-Rha*p*-Galf_{30}-Araf_3 is further extended by concerted action of EmbA and EmbB, encoded by the *emb* operon (Telenti *et al.*, 1997). Recently an α(1→3) arbinofuranosyltransferase, AftC [Rv2673], has been shown to add a single Ara*f* unit at the 3-OH position of the α(1→5)-linked arabinan segment of AG (Birch *et al.*, 2008) which in turn is further extended by an unknown α(1→5) arabinofuranosyltransferase. The non-reducing end of the arabinan chain of AG is terminated by

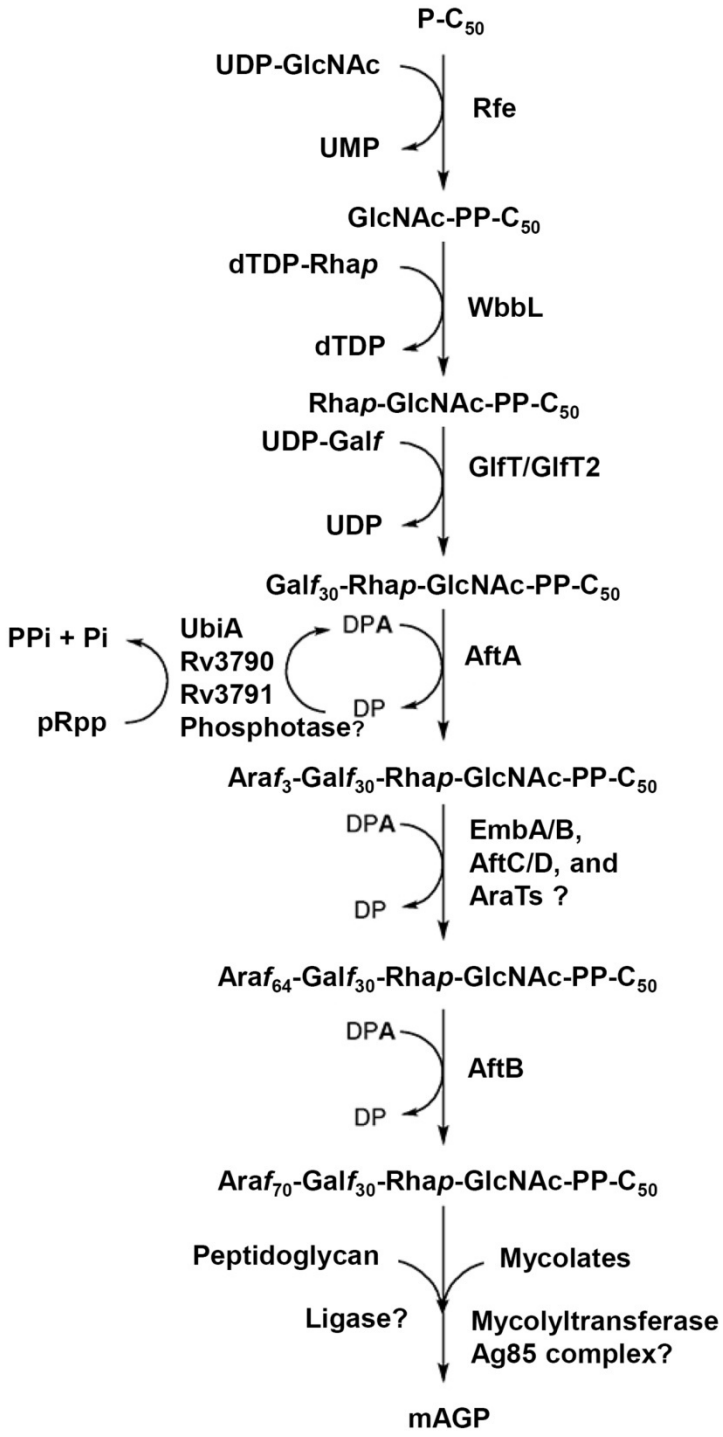

Fig. 1.6: Proposed biosynthetic pathway of AG synthesis. Biosynthesis of AG begins with synthesis of the linker unit by Rfe which transfers GlcNAc from UDP-GlcNAc to afford C_{50}-P-P-GlcNAc followed by transfer of Rha*p* by WbbL. Gal*f* is then transferred to C_{50}-P-P-GlcNAc-Rha from UDP-Gal*f* by GlfT/RfbE followed by galactan polymerisation by GlfT2. Matured C_{50}-P-P-GlcNAc-Rha-Gal_{30} is further primed by AftA and arabinan is synthesised by the action of EmbA, EmbB, AftC, AftD and AftB.

a novel β(1→2) arabinofuranosyltransferase, AftB [Rv3805c] (Seidel *et al.*, 2007a). The mature C_{50}-P-P-GlcNAc-Rha*p*-Gal*f*$_{30}$-Ara*f*$_{70}$ is then transglycosylated to the PG and mycolated (Fig. 1.6) (Yagi *et al.*, 2003).

1.9.1.3 Mycolic acid synthesis

Mycolates are synthesised by two discrete elongation systems, the Type I and Type II fatty acid synthases (FAS-I and FAS-II, respectively) in *Mycobacterium sp.* (Fig. 1.7) (Bloch, 1977; Bloch & Vance, 1977). FAS-I [Rv2524c] is a multi-domain enzyme with multiple catalytic activities (including acyltransferase, enoyl reductase, dehydratase, malonyl/palmitoyl transferase, acyl carrier protein, β-ketoacyl reductase and β-ketoacyl synthase), responsible for the synthesis of fatty acid acyl-CoA derivatives of C_{14} to C_{26} (Smith *et al.*, 2003). The C_{14} acyl-CoA derivative is further utilised by FAS-II for the synthesis of meromycolates, which are later condensed with a C_{26} acyl-CoA. Initially, a β-ketoacyl-ACP synthase III, MtFabH [Rv0533c] (Choi *et al.*, 2000) channels the C_{14}-CoA, FAS-I, product and extends this substrate using malonyl-AcpM, synthesised by a malonyl-CoA:AcpM transacylase, (MtFabD, Rv2243) (Kremer *et al.*, 2001b), affording a C_{16} acyl-AcpM, which is then elongated and processed *via* FAS-II (Fig. 1.7) (Bhowruth *et al.*, 2008; Takayama *et al.*, 2005).

Mycobacterial FAS-II consists of four enzymatic activities, which ensure the C_{16} acyl-AcpM is extended to C_{56}-AcpM meromycolates. In the first cycle, C_{16} acyl-AcpM is directly reduced by a β-ketoacyl-AcpM reductase, FabG1 [MabA, Rv1483] reduction *via* an enoyl-AcpM reductase, InhA [Rv1484] (Gurvitz *et al.*, 2008; Kremer *et al.*, 2003; Quemard *et al.*, 1995a; Quemard *et al.*, 1995b). After the first cycle, MtFabH is replaced by KasA/KasB [Rv2245/Rv2246]

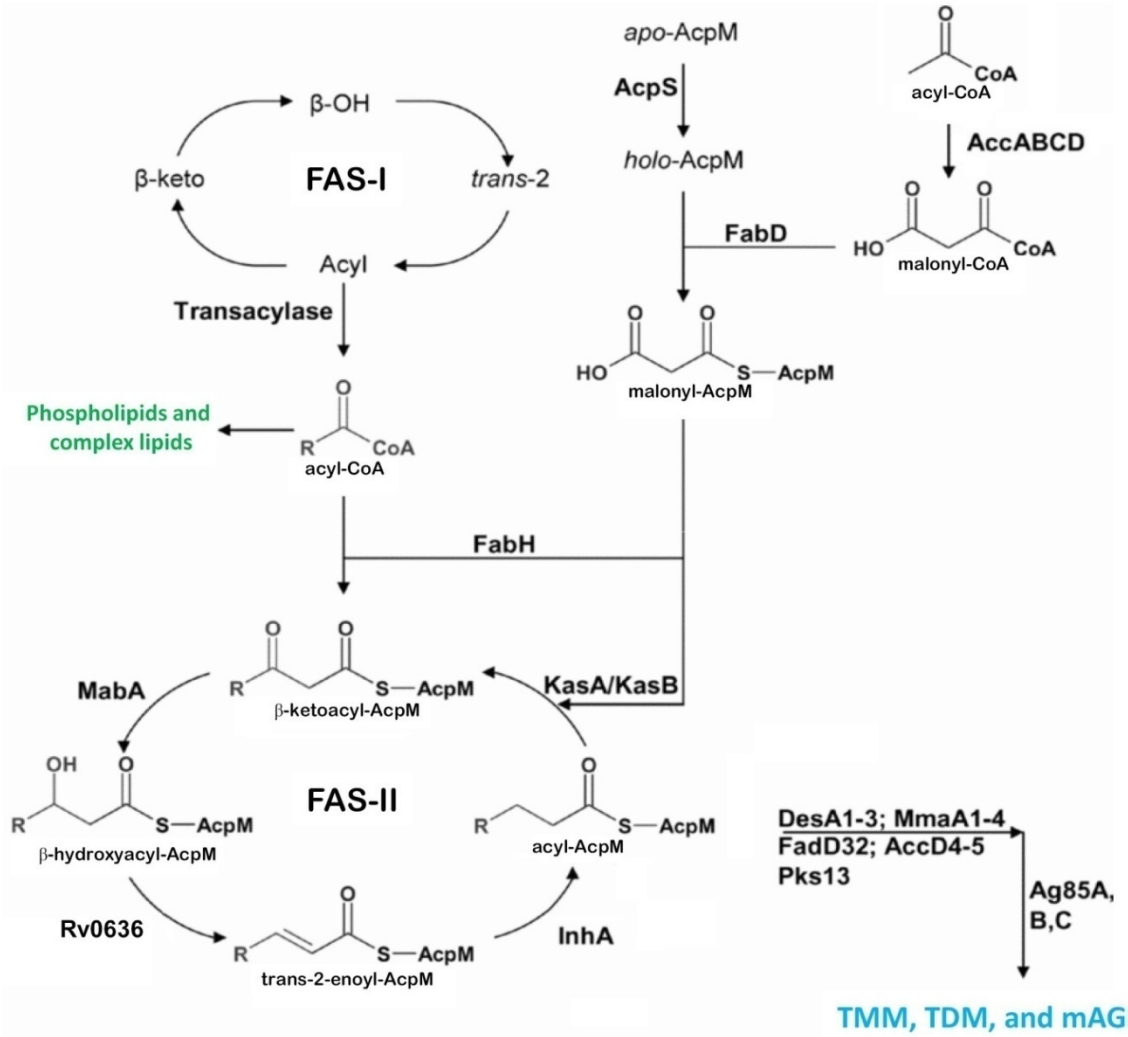

Fig.1. 7: Mycolic acid biosynthesis in *M. tuberculosis* (Adapted from Bhowruth *et al.*, 2008). Malonyl-CoA is converted to malonyl-AcpM by mtFabD, which is then ligated by mtFabH to C_{14} -CoA synthesised by FAS-I. The C_{16} acyl-AcpM product is further processed by FAS-II (KasB/A, MabA, InhA, and Rv0636) and converted to mercomycolates (C_{56}). The meromycolic acids precursors are ligated to a C_{26} fatty acid synthesised by FAS-I that constitutes the α-branch of the final mycolic acid. Finally, the polyketide synthase Pks13 catalyses the condensation of the α-branch and the meromycolate to produce mycolic acids.

(Banerjee *et al.*, 1998; Gurvitz, 2009; Marrakchi *et al.*, 2002) which is followed by dehydration catalysed by a β-hydroxyacyl-AcpM dehydratase [Rv0636] (Brown *et al.*, 2007a; Brown *et al.*, 2007b). KasA and KasB catalyse the condensation of the acyl-AcpM and malonyl-AcpM, which results in a further increment of two carbon units (Fig. 1.7) (Bhatt *et al.*, 2005; Bhatt *et al.*,

2007a; Kremer *et al.*, 2002a; Schaeffer *et al.*, 2001; Slayden & Barry, 2002). After about 20 cycles, FAS-II produces a meromycolic acid (C_{56}) which then undergoes a Claisen-type condensation reaction to form a pre-mycolic acid (Bhatt *et al.*, 2007b; Bhowruth *et al.*, 2008; Qureshi *et al.*, 1978; Takayama *et al.*, 1978; Takayama *et al.*, 2005).

The acyl-CoA carboxylases, AccD4 [Rv3799c] and AccD5 [Rv3280], carboxylate C_{26}-S-CoA after its synthesis by FAS-I and affords 2-carboxyl-C_{26}-CoA. Simultaneously, a fatty acyl-AMP ligase, FadD32 [Rv3801c] converts meromycolic acids (C_{56}) derived from the FAS-II to meromycolyl-AMP (Trivedi *et al.*, 2004). The 2-carboxyl-C_{26}-CoA and mature meromycolyl-AMP undergo the final Claisen-type condensation (Qureshi *et al.*, 1978; Takayama *et al.*, 1978) catalysed by polyketide synthase-13, Pks13 [Rv3800] (Gande *et al.*, 2004; Gokhale *et al.*, 2007; Takayama *et al.*, 2005). Similar to FAS-I, Pks-13 also has a multi-domain structure with two phosphopantotheine-binding (PPB) domains, a ketoacyl synthase (KS), acyl transferase (AT) and thioesterase (TE) domain. The condensation reaction results in the formation of 3-oxo-C_{78}-mycolate which is then reduced by a reductase [Rv2509] and generates the mature C_{78}-mycolic acid (Fig. 1.7) (Bhatt *et al.*, 2008; Lea-Smith *et al.*, 2007).

The mature mycolic acid then reacts with trehalose-6-phosphate to yield phosphorylated trehalose monomycolate (TMM-P) (Shimakata & Minatogawa, 2000) which is transported outside of the cell by an ABC transporter [Rv1458c-Rv1456c] after its dephosphorylation by a phosphatase through a mycolic acid carrier (Besra *et al.*, 1994; Takayama *et al.*, 2005). The mycolic acids are then transferred to the arabinogalactan-peptidolycan complex, as well as TDM and TMM by the antigen 85 complex to produce arabinogalactan-mycolate and trehalose dimycolate, respectively (Fig. 1.7) (Belisle *et al.*, 1997; Sathyamoorthy & Takayama, 1987; Takayama *et al.*, 2005).

1.9.2 Biosynthesis of PIMs, LM, and LAM

1.9.2.1 Biosynthesis of substrates

1.9.2.1.1 GDP-Man*p* biosynthesis

Apart from being the part of glycolipids and lipoglycans, mannose is also involved in the synthesis of a number of glycosylated proteins and a key component of several intracellular molecules in mycobacteria. These molecules are synthesised by both pathogenic and non-pathogenic species, raising the possibility of yet undefined, 'housekeeping' functions in these organisms. Patterson *et al.* (2003) demonstrated that mannose metabolism is essential for growth in *M. smegmatis* and suggested that apart from glycolipid and lipoglycan biosynthesis mannose-containing molecules may also have a role in regulating septation and cell division (Patterson *et al.*, 2003).

In mycobacteria, mannose can be produced by two distinct pathways, first by transport of extracellular mannose from the medium or extracellular environment, where free mannose is phosphorylated by a hexokinase [Rv2702] (Hsieh *et al.*, 1996), and transported inside the cell in the form of mannose-1-phosphate, which is further converted into GDP-Man*p* by ManC [Rv3264c] (Fig. 1.8) (Ma *et al.*, 2001; Ning & Elbein, 1999). Secondly, in absence of extracellular mannose, it can be derived from glucose and other sugars *via* the glycolytic pathway, where fructose-6-phosphate is converted to mannose-6-phosphate by an essential enzyme, phosphomannose isomerase (PMI), encoded by *manA* [Rv3255c] (Patterson *et al.*, 2003). A PMI deletion mutant in *M. smegmatis* was unable to synthesise mannose-containing molecules in the absence of an exogenous source of mannose which suggested the essentiality of this enzyme in mycobacteria (Patterson *et al.*, 2003).

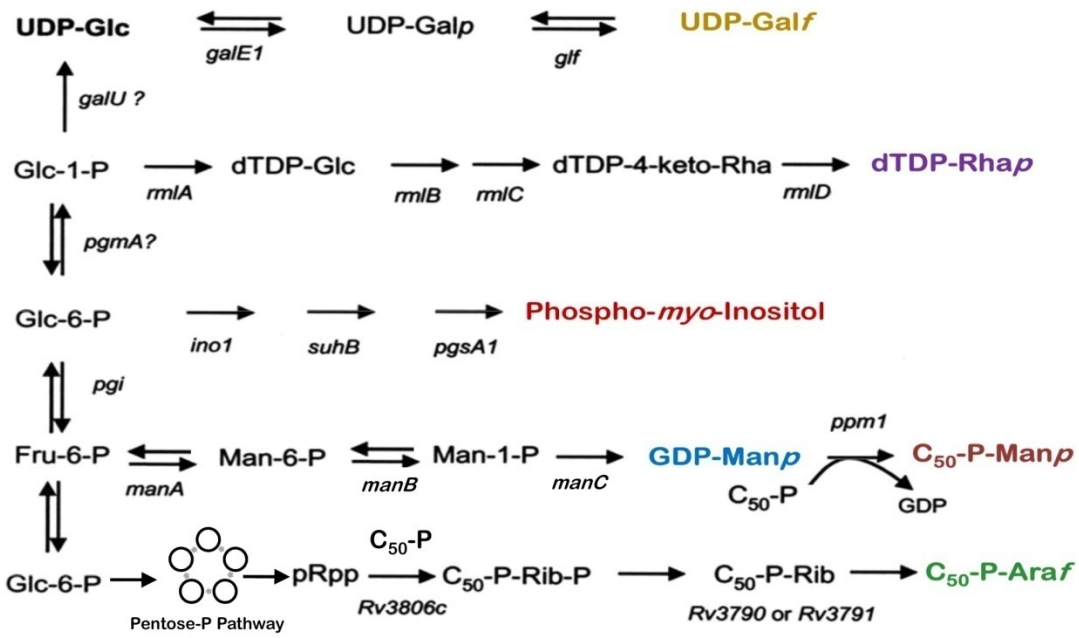

Fig. 1.8: Biosynthetic pathways of important nucleotide and lipid-linked sugar donors in *M. tuberculosis*. Most of the sugars utilised by mycobacteria are derived from glycolytic intermediates or glucose as major carbon source. Apart from glycolytic pathway, GDP-Man*p*, PPM, DPA and PI are also derived from exogenous sources.

The mannose-6-phosphate is then converted to mannose-1-phosphate by a phosphomannomutase (PMM). In search of a putative PMM, McCarthy *et al.* (2005) identified four *M. tuberculosis* open reading frames (ORF) which were similar to known PMMs (McCarthy *et al.*, 2005). They utilised two PMM and phosphoglucomutase (PGM) deficient strains of *Pseudomonas aeruginosa* and expressed putative PMMs from *M. tuberculosis*. Based on complementation studies and *in vitro* enzyme assays, it was established that Rv3257c [ManB] from *M. tuberculosis* possesses both PMM and PGM activity. Overexpression of mycobacterial ManB in *M. smegmatis* led to the accumulation of PIMs, LM, and LAM, which suggested its role in the biosynthesis of these mannosylated molecules (McCarthy *et al.*, 2005). Finally, mannose-1-phosphate is converted to the nucleotide sugar donor GDP-Man*p* by GDP-mannose pyrophosphorylase, ManC [Rv3264c] (Ma *et al.*, 2001; Ning & Elbein, 1999).

1.9.2.1.2 Synthesis of β-D-mannosyl-1-monophosphoryldecaprenol (PPM)

Takayama and Goldman (1970) were the first one to show the presence of C_{50}-polyprenol based mannolipid (C_{50}-P-Man) biosynthesis in *M. tuberculosis* (Takayama & Goldman, 1970). Later on, they identified a second *M. smegmatis* specific alkali stable, C_{35}-octahydroheptaprenyl-phospho-mannose (C_{35}-P-Man). Based on similarities to the known eukaryotic dolichol monophosphomannose (DPM) synthases, Gurcha *et al.* (2002) identified a polyprenol monophosphomannose synthase, Rv2051 [Ppm1] from *M. tuberculosis* (Fig. 1.8). Surprisingly Ppm1 possesses an unusual two-domain architecture in *M. tuberculosis* of which the second domain, *viz.* Mt-Ppm1/D2, is sufficient for PPM synthesis (Gibson *et al.*, 2003; Gurcha *et al.*, 2002). Interestingly, *M. smegmatis*, *M. avium* and *M. leprae* produce two distinct proteins, which are similar to the two domains found in Mt-Ppm1, with Ms-Ppm2 and Ma-Ppm2 having similar catalytic activity to Mt-Ppm1/D2. Due to the essentiality of PIMs, LM, and Man-LAM the PPM synthase represents an attractive target for drug development (Gibson *et al.*, 2003; Gurcha *et al.*, 2002).

1.9.2.1.3 Origin and synthesis of decaprenyl-phospho-arabinose (DPA)

The arabinofuranose sugar donor was identified by Wolucka *et al.* (1994) as lipid linked decaprenyl-phospho-arabinose (C_{50}-P-Ara*f*, DPA) (Wolucka *et al.*, 1994). In mycobacteria, DPA can be synthesised *via* two routes. First, by exogenous **D**-arabinose which can be reduced by an NADPH-dependent **D**-arabinose dehydrogenase to **D**-arabinotol followed by oxidation to **D**-xylulose by an NAD-dependent **D**-arbinotol dehydrogenase [Rv1928], which is further phosphorylated by a **D**-xylulose kinase [Rv0729], and enters into the non-oxidative pentose

phosphate pathway *via* **D**-xylulose 5-phosphate 3-epimerase [Rv1408] (Fig. 1.8) (Wolucka, 2008). Second, through the glycolytic pathway, where **D**-glucose-6-phosphate is reduced by glucose 6-phosphate-1-deydrogenase [Rv0407] (Bashiri *et al.*, 2007) into 6-phosphoglucono-1,5-lactone, which is further converted to 6-phosphogluconate by a lactonase [Rv1445] and entering the pentose phosphate pathway with the activity of an unidentified decarboxylase and 6-phosphogluconate dehydrogenase [Rv1122, Rv1844] (Fig. 1.8) (Wolucka, 2008).

The majority of DPA synthesised in mycobacteria comes from the pentose shunt pathway. A vitamin B_1 dependent transketolase [Rv1449] links the glycolytic and pentose shunt pathway and transfers a keto-group from the pentose pathway intermediate, sedoheptulose 7-phosphate to **D**-glyceraldehyde 3-phosphate and produces ribose 5-phosphate. Alternatively, **D**-ribulose 5-phosphate can be isomerised by ribose 5-phosphate isomerase [Rv2465] (Roos *et al.*, 2004; Roos *et al.*, 2005) into ribose 5-phosphate. However, Rv2465 is non essential in *M. tuberculosis*, while the putative transketolase, Rv1449 is essential (Sassetti *et al.*, 2003) which indicates that isomerisation of ribulose 5-phosphate plays a very minor role towards DPA biosynthesis. In Gram-negative bacteria, ribose 5-phosphate diphosphokinase (pRpp synthase, PrsA) adds a pyrophosphate group to ribose 5-phosphate and converts it into 5-phosphoribosyl-α-1-pyrophosphate (pRpp) with the expense of an ATP molecule (Eriksen *et al.*, 2000). In *M. tuberculosis*, an essential putative ribose-5-phosphate diphosphokinase, Rv1017c, exists which possesses a conserved pRpp binding motif (Eriksen *et al.*, 2000; Wolucka, 2008).

On the basis of time course, feedback, and chemical reduction experiments, Mikusová *et al.* (2005) proposed the dephosphorylation of pRpp by a phosphatase as the first committed step in decapolyprenol ribose (DPR) and DPA biosynthesis (Fig. 1.8) (Mikusova *et al.*, 2005). In the

genome of *M. tuberculosis* an unknown PAP2-family phospholipid phosphatase [*Rv3807c*] exists which is present in the AG biosynthetic cluster (*Rv3779-Rv3809c*) and next to the *Rv3806c*.

Scherman *et al.* (2006) demonstrated the synthesis of DPA and decaprenylphosphate-[^{14}C]-Ribose (DPR) from pRpp (Scherman *et al.*, 1996). Furthermore, it was shown that DPA is formed from pRpp *via* a two-step pathway, with an additional epimerisation step that converts DPR to DPA (Mikusova *et al.*, 2005; Scherman *et al.*, 1996). Recently, Huang *et al.* (2005) identified an ORF encoding for a 5-phospho-α-**D**-ribose-1-diphosphate:decaprenyl-phosphate 5-phospho-ribosyltransferase, UbiA [Rv3806c] in *M. tuberculosis* (Fig. 1.8) (Huang *et al.*, 2005). Deletion of *ubiA* in *C. glutamicum* produced a mutant that possessed a galactan core consisting of alternating β(1→5)-Gal*f* and β(1→6)-Gal*f* residues and was completely devoid of arabinan and cell-wall-bound corynomycolic acids, confirming its role in the synthesis of DPR and DPA biosynthesis in *Corynebacterineae* (Alderwick *et al.*, 2006a).

More recently, Mikusová *et al.* (2005) identified an epimerase, which is involved in the epimerisation of DPR to DPA (Fig. 1.8). It was established that the 2-OH of ribose is oxidised to decaprenylphosphoryl-2-keto-β-**D**-*erythro*-pentofuranose, which is reduced to form DPA. These activities are encoded by *Rv3790* and *Rv3791* and simultaneous expression of both is required for complete activity of the epimerase reaction (Fig. 1.8) (Mikusova *et al.*, 2005). Interestingly, Rv3790 has been shown to be a target of benzothiazinones, thus a potential TB drug (Makarov *et al.*, 2009).

1.9.2.1.4 Synthesis of PI

PIMs, LM and LAM are attached to the cell membrane with the linker unit composed of phosphatidyl-*myo*-inositol. Inositol is an essential metabolite in *Mycobacterium*, *Corynebacterium*, *Nocardia*, *Micromonospora*, *Streptomyces*, and *Propionibacterium* (Brennan & Ballou, 1968a; Brennan & Lehane, 1971; Kataoka & Nojima, 1967; Tabaud *et al.*, 1971; Yano *et al.*, 1969). In mycobacteria, inositol is essential for growth and derived directly *via* glycolysis (Jackson *et al.*, 2000). Glucose-6-phosphate is cyclised by an inositol-1-phosphate synthase, Ino1 [Rv0046c] (Bachhawat & Mande, 1999; Movahedzadeh *et al.*, 2004), and converted to *myo*-inositol-1-phosphate followed by dephosphorylation of *myo*-inositol-1-phosphate by an inositol monophosphatase (IMP) (Fig. 1.8). On the basis of homology, the *M. tuberculosis* genome shows four ORFs exhibiting an IMP signature. Rv2701c [SuhB] possesses the highest homology with human IMP and has been shown to possess inositol monophosphatase activity (Fig. 1.8) (Nigou & Besra, 2002a; Parish *et al.*, 1997).

The first step in the production of many phospholipids, including PI, is the phosphorylation of diacylglycerol (DAG) by a DAG Kinase [Rv2252] to form phosphatidic acid (Owens *et al.*, 2006). Phosphatidic acid is then activated by CTP to form cytidine diphosphate-diacylglycerol (CDP-DAG) by a CDP-DAG synthase [Rv2881c], a homologue of which has been characterised in *M. smegmatis* (Nigou & Besra, 2002b). Furthermore, Salman *et al.* (1999) have shown that cell wall fraction (P_{60}) from *M. smegmatis* is able to synthesise P-[^{3}H]-I in presence of exogenous substrate, CDP-dipalmitoyl-DAG (Salman *et al.*, 1999). *Myo*-inositol reacts with CDP-DAG and forms PI (Fig. 1.8). Recently, the gene encoding PI synthase [*Rv2612c*] has been identified and shown to be essential in *M. tuberculosis* (Jackson *et al.*, 2000).

1.9.2.2 Overview of PIM biosynthesis

The current model of mycobacterial PIM biosynthesis supported by biochemical and genetic studies, follows a linear pathway from PI → PIM_2 → PIM_4 → PIM_6 (Fig. 1.9) (Besra & Brennan, 1997). PI is glycosylated by an α-Man*p* residue catalysed by PimA [Rv2610c], which transfers Man*p* from GDP-Man*p* to the 2-position of PI to form PIM_1 (Kordulakova *et al.*, 2002). PIM_1 is acylated by Rv2611c (Kordulakova *et al.*, 2003) and the resulting Ac_1PIM_1 is further glycosylated by PimB [Rv2188c] at the 6-OH position of inositol (Lea-Smith *et al.*, 2008; Mishra *et al.*, 2008b; Mishra *et al.*, 2009). RvD2-ORF1 from *M. tuberculosis* CDC1551, designated as PimC, catalyses the further α-mannosylation of Ac_1PIM_2 resulting in Ac_1PIM_3 (Kremer *et al.*, 2002b). Recently, a novel mannopyranosyltransferase PimE [Rv1159], utilising PPM has been identified and shown to be involved in the biosynthesis of Ac_1PIM_5 (Morita *et al.*, 2006). However, the enzyme responsible for the synthesis of Ac_1PIM_4 from Ac_1PIM_3 still remains to be identified (Fig. 1.9).

1.9.2.2.1 Conversion of PI into Ac_1PIM_1

The enzymes involved in the synthesis of early PIMs are part of a conserved cluster of six ORFs in an operon, which is found in all members of *Corynebacterineae* (Fig. 1.10) (Cole & Barrell, 1998; Cole *et al.*, 1998). The first ORF of this cluster, *Rv2614c*, encodes for an aminoacyl-transfer RNA synthetases class-II signature and similar to *E. coli* threonyl-*t*-RNA synthetases. The second ORF, *Rv2613c*, encodes for an unknown protein with similarity to the proteins involved in nucleotide biosynthesis. The third ORF, *Rv2612c*, has been identified to encode for a phosphatidylinositol synthase, PgsA and is essential in *M. tuberculosis* (Jackson *et al.*, 2000).

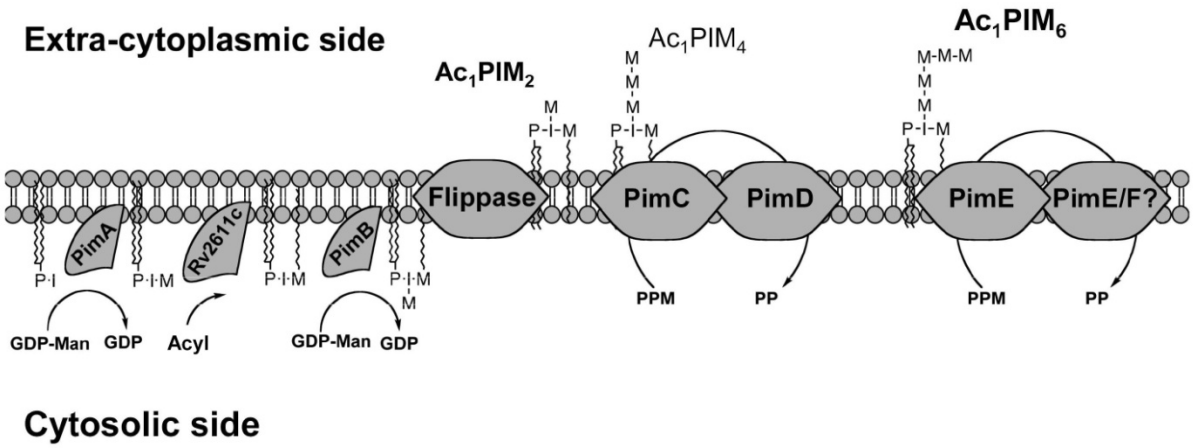

Fig. 1.9: Overview of PIM biosynthesis in *M. tuberculosis*. On the cytosolic side PI is glycosylated by PimA, PimB and an acyltransferase to form Ac_1PIM_2, which is then transported across the plasma membrane by an unidentified flippase where it is further mannosylated by PimC and PimD to form Ac_1PIM_4, an intermediate for Ac_1PIM_6 and LM. Ac_1PIM_4 is further mannosylated by α(1→2) mannosyltransferases, PimE and/or another unidentified enzyme to form Ac_1PIM_6 . The species shown in bold accumulate on the mycobacterial cell wall.

The fourth ORF, *Rv2611c*, encodes an acyltransferase which acylates the 6-position of Man*p* residue linked to 2-OH position of *myo*-inositol (Kordulakova *et al.*, 2003). A *Rv2611c* mutant of *M. smegmatis* exhibited severe growth defects and accumulation of non-acylated PIM_1 and PIM_2. Furthermore, in a cell-free assay utilising membrane preparations from *M. smegmatis*, overexpression of Rv2611c increased the incorporation of [^{14}C]-palmitate into PIMs (Kordulakova *et al.*, 2003). *PimA* [*Rv2610c*] is the fifth ORF of the operon and is essential in *M. smegmatis* (Kordulakova *et al.*, 2002). In cell-free assays with partially purified Rv2610c and/or membranes from *M. smegmatis* overexpressing *pimA* and GDP-[^{14}C]-Man*p*, Kordulakova *et al.* (2002) identified the incorporation of radioactivity into Ac_1PIM_1 and Ac_2PIM_1 (Kordulakova *et al.*, 2002). They deduced that *Rv2610c* encodes for an α-mannopyranosyltransferase, and PimA was responsible for the formation of PIM_1 from PI and GDP-Man*p* (Kordulakova *et al.*, 2002). The structure of PimA in complex with GDP-Man*p* from *M. smegmatis* has a two-domain

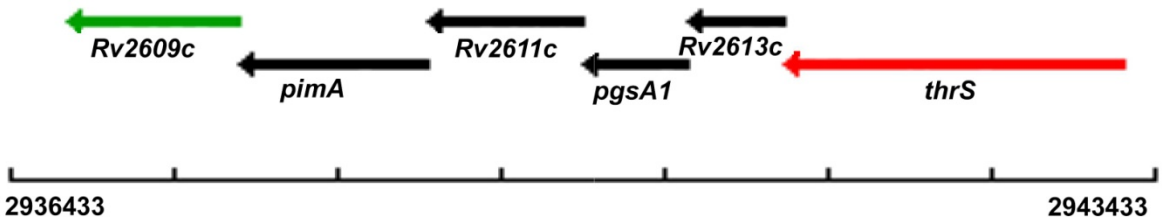

Fig. 1.10: PIM biosynthetic cluster in *M. tuberculosis*. Enzymes involved in the early steps of biosynthesis of PIMs are part of a conserved operon consists of six ORFs. *Rv2614c* encodes for an aminoacyl-transfer RNA, while *Rv2613c* is probably involved in nucleotide biosynthesis. *Rv2612c* and *Rv2611c* encode for a phosphatidylinositol synthase [PgsA] and an acyltransferase, respectively. Last two ORFs, *Rv2610c* and *Rv2609c*, encode for PimA and an uncharacterised GDP-Man*p* hydrolase. Out of these six ORFs, three have been identified in the synthesis of PIMs.

organisation and the catalytic machinery typical of GT-B glycosyltransferases (Guerin *et al.*, 2007). The sixth ORF, *Rv2609c* encodes for a putative GDP-Man*p* hydrolase carrying a mutT domain signature. The remarkable conservation of this cluster of genes among genus *Mycobacterium* may be related to an essential feature dedicated for mycobacterial viability and growth which is attributed to this class of glycolipids (Fig. 1.10) (Kordulakova *et al.*, 2003).

1.9.2.2.2 Ac_1PIM_2, a key regulatory molecule in the biosynthesis of PIMs

Schaeffer *et al.* (1999) proposed Rv0557 as an α-**D**-mannose-α(1→6)-phosphatidyl-*myo*-inositol-mannopyranosyltransferase [PimB] that transfers mannose from GDP-Man*p* to Ac_1PIM_1 to form Ac_1PIM_2. The study was based on the utilisation of cell-free assays using GDP-[^{14}C]-Man*p*, Ac_1PIM_1, *M. smegmatis* membranes and/or partially purified recombinant Rv0557 (Schaeffer *et al.*, 1999). However, on disruption of *Rv0557* in *M. tuberculosis*, the biosynthesis of PIMs remain unaffected (Torrelles *et al.*, 2009) suggesting either gene duplication or that Rv0557 performed another function in *M. tuberculosis*. Interestingly, Rv0557 has also shown to be involved in the

biosynthesis of a novel mannolipid, ManGlcAGroAc_2 and a LM-like molecule in *C. glutamicum* (Tatituri *et al.*, 2007b).

Furthermore, recently, Rv2188c was also proposed as an α-**D**-mannose-α(1→6)-phosphatidyl-*myo*-inositol-mannopyranosyltransferase [PimB'] (Lea-Smith *et al.*, 2008; Mishra *et al.*, 2008b) which has augmented ongoing confusion in the field. The synthesis of PIM_2, is followed by synthesis of PIM_4, PIM_6, LM and LAM (Besra & Brennan, 1997; Besra *et al.*, 1997). However, biochemical characterisation of the cell wall shows the accumulation of different acylated versions of PIM_2, PIM_6, LM and LAM. Herein, PIM_6 and LAM have been assigned as the metabolic end-products of the pathway, while PIM_2 has been characterised as a precursor of Ac_1/Ac_2- PIM_6 and LM, and LM as a precursor of LAM (Besra *et al.*, 1997; Mishra *et al.*, 2008a). In spite of the commitment of Ac_1/Ac_2PIM_2 towards PIM_6 and LM biosynthesis, there is substantial accumulation of Ac_1/Ac_2PIM_2 in the mycobacterial cell wall, which suggests that upstream and downstream steps are very tightly regulated.

1.9.2.2.3 Synthesis of higher PIMs

RvD2-ORF1 from *M. tuberculosis* CDC1551 was identified as an Ac_1PIM_2:α-**D**-mannose-α(1→6)-phosphatidyl-*myo*-inositol-mannopyranosyltransferase [PimC], involved in the addition of Man*p* from GDP-Man*p* to the 6-OH of mannose at the non-reducing end of Ac_1/Ac_2PIM_2 (Kremer *et al.*, 2002b). PimC belongs to Family 4 of the CAZy classification of glycosyltransferases (Cantarel *et al.*, 2009). RvD2-ORF1 and PimA, as well as six other putative α-mannopyranosyltransferases found in the *M. tuberculosis* H37Rv contain a conserved EXE

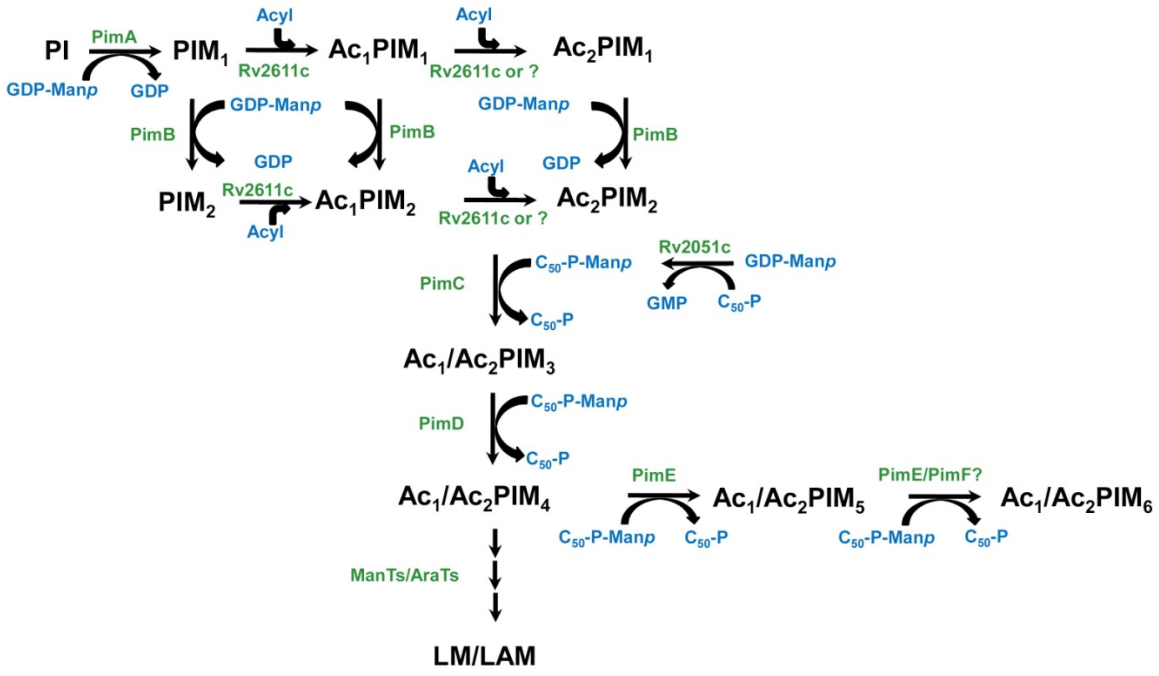

Fig. 1.10: Early and late steps of PIM biosynthesis in *M. tuberculosis*. PI is converted to Ac_1/Ac_2PIM_2 by the action of cytosolic glycosyltransferases and acyltransferases. Furthermore, Ac_1/Ac_2PIM_2 are transported across extracellular face by a flippase where it is converted to Ac_1/Ac_2PIM_6 by trans-membrane glycosyltransferases. Towards the cytosolic side glycosyltransferases use nucleotide-linked mannose donor, whilst on the extracellular side lipid-linked mannose is used as a sugar donor.

motif (Glu- X-Glu) found in most known mannopyranosyltransferases (Berg *et al.*, 2007). The use of a cell-free assay containing GDP-Man*p*, amphomycin and membranes from *M. smegmatis* over-expressing PimC led to the synthesis of Ac_1/Ac_2PIM_3. However, inactivation of *pimC* in *M. bovis* BCG did not affect the production of higher PIMs, LM, and LAM suggesting the existence of redundant gene(s) or an alternate pathway that may compensate for PimC deficiency (Kremer *et al.*, 2002b).

Ac_1/Ac_2PIM_3 are further α(1→6) mannosylated at the non-reducing termini by an unidentified α(1→6) mannopyranosyltransferase [PimD] which results in the formation of Ac_1/Ac_2PIM_4. This

step in the biosynthesis of higher PIMs, LM and LAM, as Ac_1/Ac_2PIM_4 have been predicted as a key branching point towards the synthesis of Ac_1/Ac_2PIM_6, LM and LAM (Mishra *et al.*, 2008a; Morita *et al.*, 2004; Morita *et al.*, 2006). It has been proposed that at this point, a transition occurs from glycosyltransferases, utilising nucleotide-derived sugar substrates characterised by the GT-A/B superfamily to glycosyltransferases utilising polyprenylphosphate sugars, the GT-C superfamily (Liu & Mushegian, 2003), for the elongation and branching of LM and LAM (Morita *et al.*, 2006). Rv1159 [PimE] has been identified as an α(1→2)-mannopyranosyltransferase which utilises PPM as a substrate and adds an α(1→2)-Man*p* to the Ac_1/Ac_2PIM_4 resulting in the synthesis of Ac_1/Ac_2PIM_5 (Morita *et al.*, 2006). However, it is not clear whether PimE is also responsible for the synthesis of both Ac_1/Ac_2PIM_5 and Ac_1/Ac_2PIM_6.

Morita *et al.* (2005) suggested that enzymes involved in the biosynthesis of early PIM intermediates (PIM_1 and Ac_1PIM_1) are localised to a membrane sub-domain termed PM_f in the plasma membrane, while the majority of Ac_1/Ac_2PIM_2 and enzymes involved in higher PIMs (Ac_1/Ac_2PIM_4 and Ac_1/Ac_2PIM_6) are localised to a denser fraction that contained both plasma membrane and cell wall markers (PM-CW) (Morita *et al.*, 2005). On the basis of various cell free assays, they concluded that higher PIM biosynthesis occurs in the plasma membrane rather than the PM-CW fraction followed by their transport to the cell wall (Morita *et al.*, 2005). The relative amount of higher PIMs and lipoglycans across the membrane was suggested to be regulated by a recently identified lipoprotein [LpqW] of *M. smegmatis* (Kovacevic *et al.*, 2006; Marland *et al.*, 2006). However, the exact mechanism of the regulation of PIM flux and its segregation for Ac_1/Ac_2PIM_6 or LM biosynthesis is unknown. Furthermore, Ac_1/Ac_2PIM_4 was suggested as key regulatory products involved in the biosynthesis of Ac_1/Ac_2PIM_6 and/or LM biosynthesis (Morita *et al.*, 2004; Morita *et al.*, 2006). While PimE directs Ac_1/Ac_2PIM_4 towards Ac_1/Ac_2PIM_6

synthesis, LpqW helps in the commitment of Ac_1/Ac_2PIM_4 for LM synthesis (Crellin *et al.*, 2008). The mature Ac_1/Ac_2PIM_4 is transported by a flippase or sugar transporter across the plasma membrane where mannosylation occurs by distinct mannopyranosyltransferases belonging to GT-C family.

1.9.2.3 Synthesis of LM, LAM and Man-LAM

1.9.2.3.1 Synthesis of mannan core of LM/LAM

The α(1→6) mannan backbone in LM/LAM is synthesised by the cumulative action of two α(1→6) mannopyaranosyltransferases, MptA and MptB, which have been identified and characterised in this thesis and therefore will be discussed in more detail later (Chapter 2). The α(1→6) mannan core is further decorated by single α(1→2)-Man*p* branches (Chatterjee *et al.*, 1992a; Hunter & Brennan, 1990) from the action of an α(1→2) mannopyranosyltransferase (Fig. 1.11). On the basis of known polyprenol-dependent glycosyltransferases, Kaur *et al.* (2006) identified Rv2181 as an α(1→2) mannopyranosyltransferase involved in the synthesis of α(1→2)-Man*p* side chains. Rv2181 has a DID motif, characteristic of most known glycosyltransferases, and contain 10 predicted transmembrane-spanning domains. A *M. smegmatis* mutant [*Rv2181*] showed absence of LM/LAM and synthesised a truncated version of LAM with less α(1→2)-Man*p* residues (Kaur *et al.*, 2006). However, *M. tuberculosis*Δ*Rv2181* possessed truncated versions of LM and Man-LAM (Kaur *et al.*, 2008), illustrating that regulation of LM/LAM biosynthesis in *M. smegmatis* is slightly different with *M. tuberculosis*.

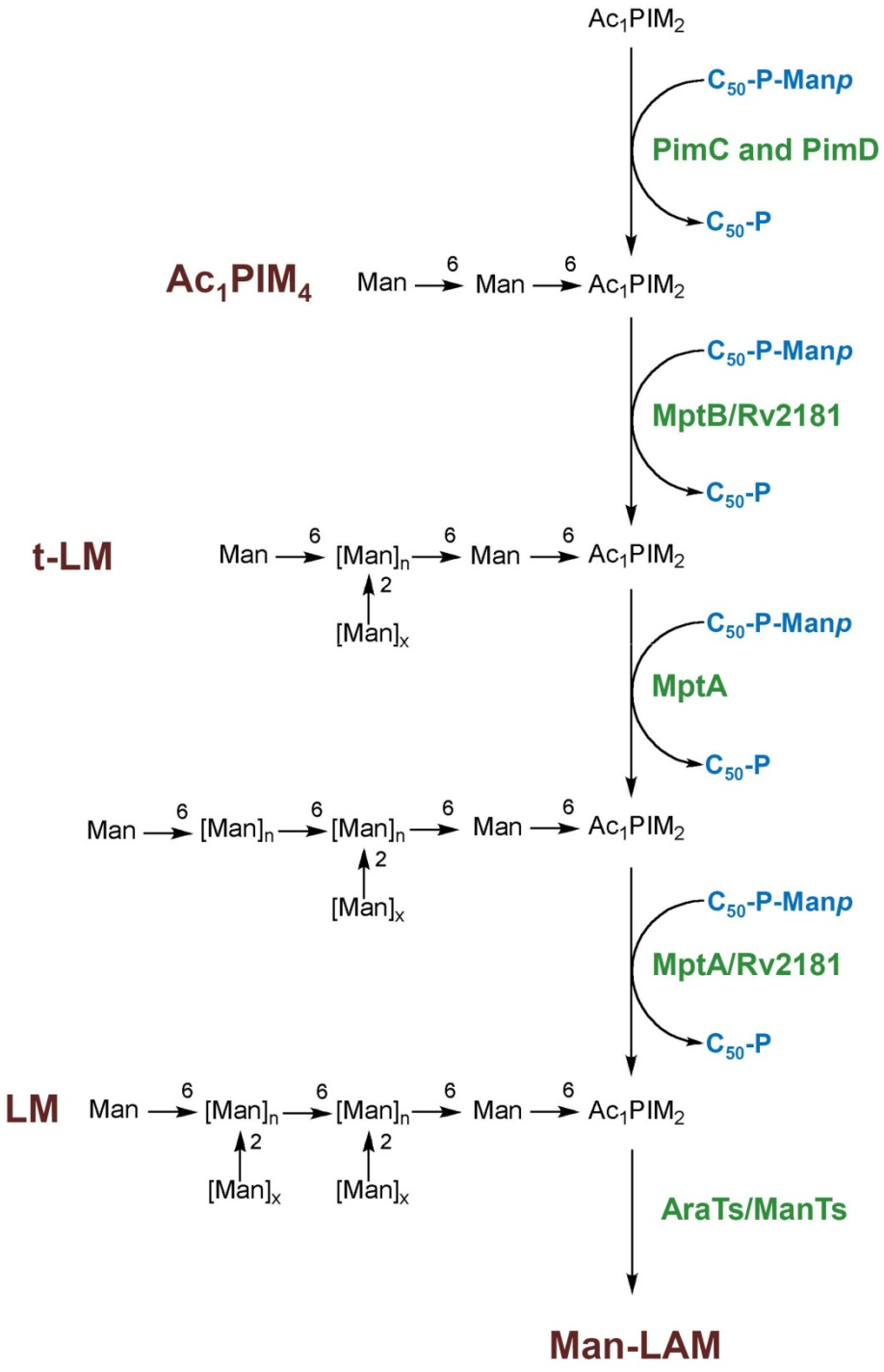

Fig.1.11: Synthesis of LM and the mannan core of LAM in *M. tuberculosis*. Ac_1/Ac_2PIM_4 is polymerised by MptB and MptA and decorated by singular α(1→2)-Man*p* by Rv2181. The mature LM is further utilised by arabinofuranosyltransferases to form LAM which is later capped by mannopyranosyltransferases forming Man-LAM. n = 20-25; and x=0-2.

1.9.2.3.2 The assembly of the arabinan domain

Mature LM is further glycosylated with α-**D**-Ara*f* units by arabinofuranosyltransferases to produce LAM, which is further capped by mannopyranosyltransferases (Besra *et al.*, 1997). The number of arabinofuranosyltransferases required for mycobacterial arabinan biosynthesis depends on the arabinan linkages present in mycobacterial LAM and one would suggest the activity of at least five distinct arabinofuranosyltransferases for mature LAM (Fig. 1.12). The EmbC protein is involved in arabinan biosynthesis of LAM, although the exact activity is unknown (Zhang *et al.*, 2003). It contains 11-13 transmembrane helices (TMH) and a large carboxyl-terminal globular region which controls chain length extension of the arabinan domain of LAM (Shi *et al.*, 2006). It is also speculated that the GT-C motif of EmbC maybe involved in the non-branched chain elongation of α(1→5)-Ara*f* residues, either through an initial polymerisation, where the arabinan units are linked to each other creating 3,5-α-Ara*f* branches on 5-linked chains or by transferring the units directly on to the mannan backbone (Fig. 1.12) (Berg *et al.* 2005).

Recently, utilising bioinformatics Birch *et al.* (2008) identified an α-arabinofuranosyltransferase, AftC in *M. smegmatis* (MSMEG_2785) and *M. tuberculosis* (Rv2673) with a glycosyltransferase motif (DDX) that is common to the GT-C family of glycosyltransferases (Birch *et al.*, 2008). Deletion of *aftC* from *M. smegmatis* resulted in a truncated AG and LAM. Cell free assays using the sugar donor DPA and a synthetic α(1→5)-linked-[Ara*f*]$_5$ acceptor, identified AftC as a branching α(1→3) arabinofuranosyltransferase. On that basis, it was concluded that AftC catalyses the addition of α(1→3)-linked Ara*f* residues on a linear α(1→5) arabinan domain resulting in 3,5-Ara*f* residues (Birch *et al.*, 2008).

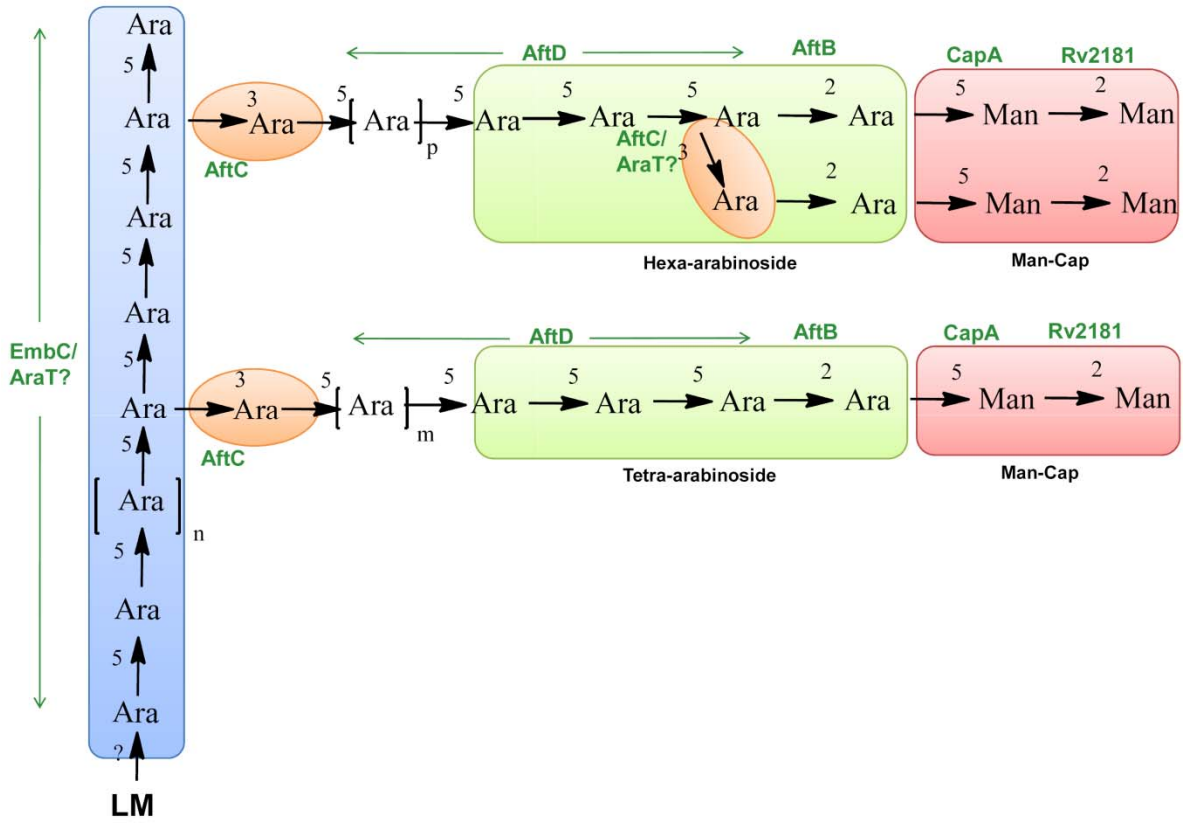

Fig.1.12: Synthesis of the arabinan domain and capping motif of Man-LAM in *M. tuberculosis*. Mature LM is primed by a singular **D**-Ara*f* through an unknown linkage which is extended by EmbC or unidentified α(1→5) arabinofuranosyltransferases. The linear α(1→5)-**D**-Ara*f* chain is further primed by AftC which is subsequently extended by AftD and terminated by the action of AftC/AftB or unidentified AraTs to form linear Ara-4 or branched Ara-6. The penultimate Ara*f* of the arabinan domain is further capped by Man*p* residues by CapA and Rv2181 to form Man-LAM. n, m, and p represent different numbers of Ara*f* residues.

Interestingly, Skovierova *et al.* (2009) have recently identified Rv0236c as another α(1→3) arabinofuranosyltransferase involved in the addition of α(1→3) arabinan residues towards the non-reducing end of LAM and AG. Although, they also suggested that AftD may have another unknown function e.g. regulation of arabinan assembly in AG/LAM or in fact even an α(1→5) arabinan transfer activity (Fig. 1.12) (Skovierova *et al.*, 2009).

1.9.2.3.3 Mannan priming and Man-LAM synthesis

All pathogenic strains of the genus *Mycobacterium* are known to possess mannose-capped LAM (Man-LAM), which is responsible for some of the immuno-modulatory properties of these strains (Briken *et al.*, 2004). Close inspection of the *M. tuberculosis* genome in comparison with *M. smegmatis* which possesses LAM without mannose caps, provided the first indication of the role of Rv1635c in Man-LAM synthesis. On that basis, the homologue of Rv1635c in *M. tuberculosis* CDC1551 was identified as a glycosyltransferase that could be involved in Man-LAM capping (Dinadayala *et al.*, 2006). Biochemical analysis of the cell wall of a transposon mutant strain of *M. tuberculosis* CDC1551 deficient in MT1671 (Rv1635c) yielded a smaller version of Man-LAM. Further biochemical analyses established that the Man-LAM of the mutant strain was in fact devoid of Man*p* capping (Dinadayala *et al.*, 2006). Furthermore, heterologues expression of MT1671 in a *M. smegmatis* strain resulted in a hybrid LAM, capped with a single mannose residues. Simultaneously, *Rv1635c* mutants in *M. marinum* and *M. bovis* BCG showed that *Rv1635c* encoded for an α(1→2) mannopyranosyltransferase involved in the addition of the first Man*p* residue on the non-reducing arabinan termini of LAM (Appelmelk *et al.*, 2008). More recently, Kaur *et al.* (2008) have shown that Rv2181c also has an ability to add α(1→2)-Man*p* residues not only onto α(1→6) mannan backbone but also at the non-reducing end of LAM in combination with Rv1635c (Kaur *et al.*, 2008). This was the first report confirming a mycobacterial glycosyltransferase having varied substrate specificity (Fig. 1.12).

1.10 Aims and objectives

With the advent of MDR- and XDR-TB there is a need to identify novel drug targets in *M. tuberculosis*. The unusual lipid rich cell wall of *M. tuberculosis* inhibits the entry of hydrophilic drugs into the cell. Interestingly, many of the available front-line drugs (EMB, INH, and ethionamide) target cell wall biosynthetic machinery. In this respect, the unexplored biosynthetic machinery of the mycobacterial cell wall represents an attractive target for the development of new drugs.

According to the CAZy (Carbohydrate-Active Enzymes) database (Cantarel *et al.*, 2009), forty one loci of *M. tuberculosis* H37Rv have been shown to encode for putative glycosyltransferases on the basis of sequence similarity and alignment. Liu and Mushegian (2003) categorised these loci into three large superfamilies; GT-A and GT-B (soluble and peripheral membrane proteins which use NDP-sugars as donor substrates) and GT-C (integral membrane proteins) (Liu and Mushegian, 2003). At the start of this thesis only a handful have been characterised experimentally: EmbA, B and C (Telenti *et al.*, 1999), AftA (Alderwick *et al.*, 2006), AftB (Seidel *et al.*, 2007a), MgtA (Tatituri *et al.*, 2007b), Rv2181 (Kaur *et al.*, 2006) and CapA (Appelmelk *et al.*, 2008, Dinadayala *et al.* 2006). The major aim of this thesis was to determine the role of other uncharacterised putative glycosyltransferases in cell wall biosynthesis of *M. tuberculosis* with the view of identifying new drug targets for MDR- and XDR-TB.

2. BIOSYNTHESIS OF THE MANNAN BACKBONE OF *C. GLUTAMICUM* AND *M. TUBERCULOSIS*

2.1 Introduction

The current paradigm of mycobacterial lipoglycan biosynthesis follows a linear pathway, PI → PIM_2 → LM → LAM (Besra & Brennan, 1997), with each individual step synthesising an increasingly glycosylated molecule catalysed by specific glycosyltransferases. In this pathway, PI acts as a substrate for the α-mannopyranosyltransferase PimA [Rv2610c], which transfers a Man*p* to PI to form PIM_1 (Kordulakova *et al.*, 2002), followed by acylation of PIM_1 by Rv2611c (Kordulakova *et al.*, 2003) and the second mannosylation step by PimB [Rv2188c] (Lea-Smith *et al.*, 2008, Mishra *et al.* 2008b, Mishra *et al.*, 2009). PimE (Rv1159) has been implicated in the synthesis of Ac_1/Ac_2PIM_5 (Morita *et al.*, 2006), however, the enzyme responsible for the synthesis of the intermediate Ac_1/Ac_2PIM_4 from Ac_1/Ac_2PIM_3, remains elusive.

Ac_1/Ac_2PIM_4 has been suggested as a branching point and a likely precursor to LM/LAM (Morita *et al.*, 2006), with a transition in type of glycosyltransferases involved in LM/LAM synthesis (Liu & Mushegian, 2003). The mature Ac_1/Ac_2PIM_4 are flipped from the extracytoplasmic side of the membrane by an unidentified flippase, followed by polymerisation *via* distinct mannopyranosyltransferases and arabinofuranosyltransferases (Morita *et al.*, 2004, Morita *et al.*, 2006). Recently, Kaur *et al.* (2006, 2008) reported the involvement of Rv2181 in the synthesis of the α(1→2)-Man*p* linked branches on the mannan core of LM/LAM (Kaur *et al.*, 2006; Kaur *et al.*, 2008). However, the enzyme activity required for the synthesis of the core linear LM/LAM mannan domain through an α(1→6) mannopyranosyltransferase remains to be identified.

Herein, we have used *C. glutamicum* as a surrogate system to study the putative α(1→6)

mannopyranosyltransferases of *M. tuberculosis*. Apart from *C. glutamicum* and *M. tuberculosis* sharing a similar cell wall architecture, the availability of completed genome sequences for both organisms has enabled the use of *C. glutamicum* as a suitable model to study the essential mycobacterial genes involved cell wall biosynthesis (Alderwick *et al.*, 2005; Alderwick *et al.*, 2006b; Gande *et al.*, 2004; Gibson *et al.*, 2003; Seidel *et al.*, 2007a). In this Chapter, we have examined two ORFs from *C. glutamicum*, *NCgl2093* (homologue of *Rv2174*) and *NCgl1505* (homologue of *Rv1459c*), which encode putative GT-C glycosyltransferases. Based on molecular and biochemical studies, NCgl2093/Rv2174 were assigned as $\alpha(1\rightarrow6)$ mannopyranosyltransferases involved in the distal mannan backbone, while NCgl1505/Rv1459c represents $\alpha(1\rightarrow6)$ mannopyranosyltransferases involved in the proximal mannan backbone synthesis of LM in *C. glutamicum* and *M. tuberculosis*.

2.2 Materials and methods

2.2.1 Bacterial strains and growth conditions

C. glutamicum ATCC 13032 and *E. coli* DH5α*mcr* were grown in Luria-Bertani broth (LB, Difco) at 30°C and 37°C, respectively. The recombinant strains were grown on rich BHI medium (Difco), and the salt medium CGXII used for *C. glutamicum* as described (Eggeling & Bott, 2005). Kanamycin and ampicillin were used at a concentration of 50 µg/ml. Samples for lipid analysis were prepared by harvesting cells at an OD of 10-15, followed by a saline wash and freeze drying. *M. smegmatis* strains were grown in Tryptic Soy broth (TSB, Difco) containing 0.05 % Tween80. The concentrations of antibiotics used for *M. smegmatis* were 100 µg/ml for hygromycin and 20 µg/ml for kanamycin. All other chemicals were of reagent grade and obtained from Sigma-Aldrich.

2.2.2 Construction of plasmids and strains

2.2.2.1 Construction of *C. glutamicum*Δ*mptA* and complemented strains

All mutant strains in *C. glutamicum* and their complimented strains were constructed at Institute for Biotechnology Research Centre, Juelich, Germany. In order to enable deletion of the gene with the locus tag *C. glutamicum NCgl2093* (Cg-*mptA)* the primer pair P1, CGCTTCTAGACAACGCGCTGATAAGCAATCTCC, (all primers given in 5´to 3´direction) and P2rev, CCCATCCACTAAACTTAAACACGTTGAAAAAGTGT**CAT**AC GCG, were used with start codon in bold and restriction endonuclease sites underlined to generate a 288 bp fragment upstream of *NCgl2093*. Similarly, the pair P3, TGTTTAAGTTTAGTGGATGGGACTGACCCTGCAACAAC, and P4rev, GCGGGAATTCG AAGGAAAACACCAACCGTTTCATC was used to generate a 340 bp downstream fragment. Using both isolated fragments cross over PCR was applied with primers P1 and P4rev to generate a 628 bp fragment which was cloned into EcoRI-XbaI-cleaved pK19mobsacB (Schafer *et al.*, 1994) resulting in pK19mobsacBΔ*mptA*. For the chromosomal deletion of Cg-*mptA*, plasmid pK19mobsacBΔ*mptA* was used, taking advantage of the kanamycin resistance gene *aph,* to select for plasmid integration in the first round of homologues recombination and the sucrose gene *sacB*, to select for loss of vector in the second round of homologues recombination (Jager *et al.*, 1992). The successful deletion in the resulting strain *C. glutamicum*Δ*mptA* was verified by use of two different primer pairs (P1 and P4).

To enable plasmid encoded expression of *C. glutamicum NCgl2093* (Cg-*mptA*), the gene was amplified using the primer pairs 2093rev, GTAATGGA**TCC**TAGGAAACGGTATGCGGG GAG and 2093RBSfor, GCGCGGTTAACAGGGAGATATAG**ATG**ACACTTTTTCAACGTT

TAAC were as used with start and stop codons in bold and restriction endonuclease sites underlined. The resulting fragment was cloned into pGEM-T, excised as an HpaI-SpeI fragment and cloned into the *E. coli*-*C. glutamicum* shuttle vector pVWEx resulting in pVWEx-Cg-*mptA*. To clone *Rv2174* of *M. tuberculosis* (Mt-*mptA*) the primer pairs Rv2174rev, CAGTGAGAT **CTC**TATGGCGTATTGACCACCG and 2174RBSfor, CACTAGTTAACAGGGAGATATAG **ATG**ACTACTCCGAGCCATG were used. The resulting fragment was cloned as above into pGEM-T and sub-cloned resulting in pVWEx-Mt-*mptA*. All plasmids used were confirmed by sequencing for integrity. Plasmid pVWEx-Cg-*mptA* and pVWEx-Mt-*mptA* were introduced into *C. glutamicum*Δ*mptA* by electroporation with selection to kanamycin resistance (25 µg/ml).

2.2.2.2 Construction of *C. glutamicum*Δ*mptB*, *C. glutamicum*Δ*mptB*Δ*mptA*, and complemented strains

The genes analysed were *Rv1459c* and *NCgl1505* from *M. tuberculosis* and *C. glutamicum*, respectively, termed *mptB*. The vectors made were pVWEx-Mt-*mptB*, pVWEx-Cg-*mptB*, pET-Mt-*mptB*, pET-Cg-*mptB*, and pK19mobsacBΔ*mptB*. To construct the deletion vector pK19mobsacBΔ*mptB*, crossover PCR was applied with primer pairs AB (A, CGTTAAGCTTCC AAAGGTAACCTTATTTATGCTGGCCACAGG; B, CCCATCCACTAAACTTAAACAC GATGCGCGGCAAAGT) and CD (C, TGTTTAAGTTTAGTGGATGGGGAGTTTGAGGCGG AATCC; D, GCATGGATCCGCGGTAAAACCTTCGCACATTTCAATG) and *C. glutamicum* genomic DNA as template. Both amplified products were used in a second PCR with primer pairs AD to generate a 597 bp-fragment consisting of sequences adjacent to Cg-*mptB*, which was ligated with HindIII-BamHI-cleaved pK19mobsacB.

To enable expression of Cg-*mptB* in *C. glutamicum* the primer pair EF was used (E, CGAATTGGATCCTCAGTGTAAACCAAAGGTTGGATTCC; F, GATATGTTAACAGGG AGATATAGTTGCCGCGCATCGG) to amplify *C. glutamicum mptB*, which was ligated with SalI-, BamHI-cleaved pVWEx to generate pVWEx-Cg-*mptB*. To enable expression of Mt-*mptB* in *C. glutamicum* the primer pair HJ was used (H, GATATGTTAACAGGGAGATATAGATG GCAGCCCGCCAC; J, GGAATTGGATCCTCACGTGGAATCAGCGTAGGCG) to amplify *M. tuberculosis mptB*, which was ligated with SalI-, BamHI-cleaved pVWEx to generate pVWEx-Mt-*mptB*.

All plasmids were confirmed by sequencing. The chromosomal deletion of Cg-*mptB* was performed as described previously using two rounds of positive selection (Schafer *et al.*, 1994), and its successful deletion was verified by use of primer pairs A,B and the additional primer pair LM (L, GCGCGTATCACCGTCTCCGGTGTG; M, GCTGTTGGCCACCTGACAGACGTCG). Due to the similarity of MptB with MptA *C. glutamicum*Δ*mptB* was transformed with and used together with pK19mobsacBΔ*mptA* (Mishra *et al.,* 2007) to give the double mutant *C. glutamicum*Δ*mptB*Δ*mptA*. Plasmids pVWEx-Cg-*mptB*, pVWEx-Cg-*mptA*, pVWEx-Mt-*mpt*, and pVWEx-Ms-*mptB* were introduced into *C. glutamicum*Δ*mptB* and *C. glutamicum*Δ*mptB*Δ*mptA* by electroporation with selection to kanamycin resistance (25 μg/ml).

2.2.2.3 Construction of *M. smegmatis*Δ*mptB*

*M. smegmatis*Δ*MSMG3120* was constructed at Howard Hughes Medical Institute, Albert Einstein College of Medicine, New York, USA and was gift from Prof. K. Takayama. For details, please refer to Mishra et al. (2008a).

2.2.3 Extraction and biochemical analysis of lipids and lipoglycans

Methodology covering extraction and biochemical analysis of lipids and lipoglycans with two dimensional-thin layer chromatography (2D-TLC), SDS-PAGE, GC-MS, MALDI-TOF-MS and NMR are covered separately as part of General Materials and Methods in Chapter 5.

2.2.4 Preparation of enzymatically active membranes and cell envelope fraction

M. smegmatis and C. glutamicum strains were cultured to mid-logarithmic growth phase in 1 L BHIS medium supplemented with kanamycin (25 μg/ml) and IPTG (0.2 mM) where appropriate. Cells were harvested by centrifugation, resuspended in 20 ml of buffer A (50 mM MOPS pH 7.9, 5 mM β-mercaptoethanol and 5 mM $MgCl_2$) and lysed immediately by sonication (60 sec ON, 90 sec OFF for a total of 10 cycles). The lysate was clarified by centrifugation at 27,000 x *g* (4°C, 30 min) and membranes were deposited by centrifugation of the supernatant at 100,000 x *g* (4°C, 90 min). The membranes were resuspended in buffer A to a final protein concentration of 20 mg/ml. The 27,000 x *g* pellet was resuspended in 10 ml of buffer A and 15 ml of Percoll (Pharmacia, Sweden) and centrifuged at 27,000 x *g* for 60 min at 4°C. The particulate, upper diffuse band, containing both cell walls and membranes, was removed, collected by centrifugation, washed three times in buffer A, and finally resuspended in 1 ml of buffer A. The final concentration of this Percoll-60 cell envelope fraction (P_{60}) was 20 mg/ml.

2.2.5 *In vitro* incorporation of radiolabeled mannan from GDP-[^{14}C]-Man*p* into membrane lipids

Initial assays involved incubation of membranes (0.5 mg of protein), P-60 fraction (0.5 mg of protein) in buffer A, containing 1 mM ATP and 0.25 μCi of GDP-[^{14}C]-Man*p* (Amersham Pharmica Biotech, Uppsala, Sweden, 303 mCi/mmol) in a final volume of 100 μl incubated at 37°C for 60 min as described (Besra *et al.*, 1997). The reactions were terminated by the addition of $CHCl_3/CH_3OH$ (6 ml, 2:1, v/v), centrifuged and the pellet re-extracted thrice using $CHCl_3/CH_3OH$ (6 ml, 2:1, v/v). The resulting insoluble pellet was sequentially washed three times with 0.9 % NaCl in CH_3OH (2 ml), CH_3OH/H_2O (2 ml, 1:1, v/v) and CH_3OH (2 ml) to remove residual GDP-[^{14}C]Man*p* before extracting three times with $CHCl_3/CH_3OH/H_2O$ (2 ml, 10:10:3, v/v/v). The $CHCl_3/CH_3OH/H_2O$ (10:10:3)-soluble lipids were dried and resuspended in 200 μl of $CHCl_3/CH_3OH/H_2O$ (10:10:3, v/v/v) and an aliquot (10 %) of the resulting [^{14}C]-labeled mannoligosaccharide polymer [α(1→6)linear-LM] quantified by liquid scintillation counting using 5 ml of EcoScintA (National Diagnostics, Atlanta, GA). The remaining aliquot analysed by SDS-PAGE/autoradiography.

The original combined $CHCl_3/CH_3OH$ (2:1) organic extracts were dried and resuspended in $CHCl_3/CH_3OH/H_2O$ (4 ml, 10:10:3, v/v/v) followed by the addition of 1.75 ml of $CHCl_3$ (1.75 ml) and H_2O (0.75 ml). The reaction mixture was vortexed, centrifuged and the upper aqueous phase removed. The organic phase was washed three times with $CHCl_3/CH_3OH/H_2O$ (2 ml, 3:47:48, v/v/v), and the final organic extract dried under a stream of nitrogen to afford C_{50}-PP-[^{14}C]-M, Ac_1PI-[^{14}C]-M_2 and [^{14}C]-$Man_1GlcAGroAc_2$. Alternatively, the combined $CHCl_3/CH_3OH$ (2:1) organic extracts were dried and resuspended in $CHCl_3/CH_3OH$/0.8 M

NaOH (4 ml, 10:10:3, v/v/v) and heated at 50^{o}C for 30 min, followed by the addition of 1.75 ml of $CHCl_3$ (1.75 ml) and H_2O (0.75 ml) and processed as described above to afford C_{50}-PP-[^{14}C]-M. The resulting C_{50}-PP-[^{14}C]-M, Ac_1PI-[^{14}C]-M_2 and [^{14}C]-$Man_1GlcAGroAc_2$ products were subjected to TLC/autoradiography using silica gel plates (5735 silca gel $60F_{254}$, Merck) developed in $CHCl_3$:CH_3OH:H_2O:NH_4OH (65:25:3.6:0.5, v/v/v/v) and the products visualised and quantified by phosphorimaging (Kodak K Screen).

2.2.6 *In situ in vitro* synthesis of [^{14}C]-labeled mannoligosaccharide polymers

The lipopetide amphomycin (2 mg) was dissolved in 500 µl of 0.1 M acetic acid and the solution adjusted to 0.05 M sodium acetate (pH 7.0) with 0.1 M NaOH to a final concentration of 2 mg/ml (Gurcha *et al.*, 2002). Membranes/cell envelope (5 mg) in 500 µl of buffer A were pre-incubated with amphomycin (1µg/10µL reaction mixture) at 37^{o}C for 15 min resulting in inhibition of PPM synthesis, prior to a further short 15 min pulse incubation with 1.25 µCi of GDP-[^{14}C]-Man. A 20 % aliquot of the reaction mixture was processed as described above to afford Ac_1PI-[^{14}C]-M_2, [^{14}C]-$Man_1GlcAGroAc_2$ and $CHCl_3$/CH_3OH/H_2O (10:10:3)-soluble lipids. The remaining amphomycin-treated membranes/cell envelope containing *in situ* [^{14}C]-Man-labeled lipids [Ac_1PIM_2 and $Man_1GlcAGroAc_2$] were diluted with buffer A and recovered by re-centrifugation at 100,000 x *g* for 60 min, carefully washed and re-centrifuged with cold buffer A twice, thereby ensuring complete removing of unused GDP-[^{14}C]-Man*p* (Besra *et al.*, 1997).

The [^{14}C]-Man-labeled membranes were then carefully resuspended in 400 µl buffer A prior to the addition of 0.5 mg C_{50}-PPM in 1% IgePal CA-630 (40 µl, Sigma Aldrich) (Gurcha *et al.*, 2002), incubated further at 37^{o}C for 60 min and a 100 µl aliquot processed/analysed as described

above to provide the $CHCl_3/CH_3OH$ (2:1) and $CHCl_3/CH_3OH/H_2O$ (10:10:3)-soluble [^{14}C]-labeled mannose containing products.

2.2.7 *In vitro* analysis of α(1→6) mannopyranosyltransferase activity

The neoglycolipid acceptor α-**D**-Man*p*-(1→6)-α-**D**-Man*p*-O-C_8 (stored in C_2H_5OH) and C_{50}-P-[^{14}C]-M (stored in $CHCl_3/CH_3OH$, 2:1, v/v) were prepared as described elsewhere (Gurcha *et al.*, 2002), and separated into aliquots into 1.5 ml eppendorf tubes to a final concentration of 2 mM and 0.25 μCi (0.305 Ci/mmol), respectively, and dried under nitrogen. IgePal CA-630 (8 μl) was added and the tubes sonicated to resuspend the lipid-linked components, and the remaining assay components in a final volume of 80 μl were added, which included: 1 mM ATP, 1 mM NADP, and membrane protein (1 mg) from either *M. smegmatis*, *C. glutamicum*, *C. glutamicum*Δ*mptA*, *C. glutamicum*Δ*mptA* pVWEx-Cg-*mptA* and *C. glutamicum*Δ*mptA* pVWEx-Mt-*mptA*, *C. glutamicum*Δ*mptB*, *C. glutamicum*Δ*mptB* pVWEx-Cg-*mptB*, *C. glutamicum*Δ*mptB*Δ*mptA*, *C. glutamicum*Δ*mptB*Δ*mptA* pVWEx-Cg-*mptB, C. glutamicum*Δ*mptB*Δ*mptA* pVWEx-Cg-*mptA, C. glutamicum*Δ*mptB*Δ*mptA* pVWEx-Mt-*mptB*, and *C. glutamicum*Δ*mptB*Δ*mptA* pVWEx-Ms-*mptB*. Assays were incubated at 37°C for 1 h and then quenched by the addition of $CHCl_3/CH_3OH$ (533 μl, 1:1, v/v).

The reaction mixtures were then centrifuged at 27, 000 x *g* for 15 min at 4°C, the supernatant removed and dried under nitrogen. The residue was resuspended in C_2H_5OH/H_2O (700 μl, 1:1, v/v) and loaded onto a 1-ml SepPak strong anion exchange cartridge (Supleco) pre-equilibrated with C_2H_5OH/H_2O (1:1, v/v). The column was washed with 2 ml of C_2H_5OH, and the eluate

collected, dried, and partitioned between the two phases arising from a mixture of *n*-butanol (3 ml) and water (3 ml). The resulting organic phase was recovered after centrifugation at 3,500 x *g*, and the aqueous phase again extracted twice with 3 ml of water saturated-butanol. The pooled extracts were back-washed twice with *n*-butanol saturated water (3 ml). The *n*-butanol fraction was dried and resuspended in 200 μl of *n*-butanol.

The extracted radiolabeled material was quantified by liquid scintillation counting using 10% of the labeled material and 5 ml of EcoScintA. The incorporation of [^{14}C]-Man*p* was determined by subtracting counts present in control assays (incubations in the absence of acceptor), which were typically less than 100 cpm per assay. The remaining labeled material was subjected to TLC using silica gel plates developed in $CHCl_3$:CH_3OH:H_2O:NH_4OH (65:25:3.6:0.5, v/v/v/v) and the products visualised by phosphorimaging.

2.3 Results

2.3.1 Genome comparison of the *NCgl2093*/*Rv2174* locus

In order to advance further our understanding of glycosyltransferases in *Corynebacterineae*, we focused on the genes annotated by *NCgl2093* and *Rv2174* from *C. glutamicum* and *M. tuberculosis*, respectively, which are recognised as glycosyltransferases of unknown function (Liu & Mushegian, 2003). The genomic organisation of these genes in all *Corynebacterineae* analysed is syntenic, and even in *M. leprae* the locus organisation is retained, indicating an apparent fundamental function of its product (Fig. 2.1).

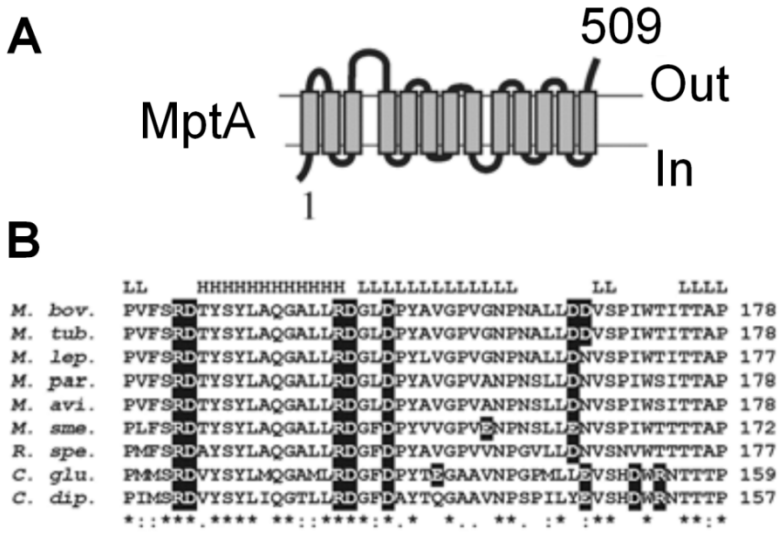

Fig. 2.1 Comparison of the *mptA* locus within the *Corynebacterineae* and structural analysis of putative protein. (**A**) MptA spans the membrane 13 times and a large loop connects TMH 3 and 4. Part of the loop sequence is given, where acid and basic residues are highlighted. On top of the sequence comparison the predicted secondary structure is given, with *H* indicating a helical structure, and *L* a loop region. The entire region has a high solvent accessibility, which indicates together with the conserved aspartyl residues their functional significance. (**B**) The locus in the bacteria analysed consists of *mptA* which in *C. glutamicum* has the locus tag *NCgl2093* and in *M. tuberculosis R*v2174. A conserved ORF is present upstream of *mptA* and probably forms a transcriptional unit with *mptA*, and predicted to encode a polyprenyl synthetase. The genomic region displayed encompasses 6 kb, and orthologous genes are highlighted accordingly. *M. bov.*, *Mycobacterium bovis*; *M. tub*. *Mycobacterium tuberculosis*; *M. lep.*, *Mycobacterium leprae*; *M. par.*, *Mycobacterium paratuberculosis*; *M. avi*., *Mycobacterium avium; M. sme., Mycobacterium smegmatis; R. spe., Rhodococcus sp. (strain RHA1); C. glu., Corynebacterium glutamicum; C. dip., Corynebacterium diphtheriae.*

A pfam analysis (Bateman *et al.*, 2004) of the ORFs upstream of *Rv2174*, revealed that the gene product derived bears structural similarities to polyprenyl synthetases, which could be functionally related to the glycosyltransferase, and both genes might form a transcriptional unit.

In *C. glutamicum* and *M. tuberculosis,* a number of orthologous GT-C family glycosyltransferases have been identified by us and others, which transverse the membrane (Alderwick *et al.*, 2006b; Kaur *et al.*, 2006; Morita *et al.*, 2006; Seidel *et al.*, 2007a; Seidel *et al.*,

2007b). Indeed, NCgl2093 and its *M. tuberculosis* orthologue (Rv2174) are putative membrane bound GT-C glycosyltransferases. Although, both orthologues have 13 TMHs, they differ from the α-mannosyltransferase (Rv1635c) involved in the mannose capping of LAM (Appelmelk *et al.*, 2008; Dinadayala *et al.*, 2006) and the arabinofuranosyltransferases AftA, AftB and the Emb proteins (Alderwick *et al.*, 2005; Alderwick *et al.*, 2006a; Alderwick *et al.*, 2006b; Alderwick *et al.*, 2007; Seidel *et al.*, 2007a; Seidel *et al.*, 2007b) by the absence of a periplasmatic extension at the carboxy terminus. The degree of conservation, with respect to topology and sequence among the orthologues of NCgl2093, is high within the *Corynebacterineae*. For instance, the similarity of the *C. glutamicum* and *M. tuberculosis* protein is 58 %, and with the most distant pairs among the genus *Corynebacterium*, *C. glutamicum* and *C. jeikeium*, the similarity is approximately 64%.

One of the most conserved regions is the loop between TMH 3 and 4 (Fig. 2.1 A). This sequence is reminiscent to the glycosyltransferase family GT-C modified DXD motif, since it contains a number of basic and acidic residues, the latter shown in mutational studies to be essential for glycosyl transfer from polyprenylated phospho-sugar donors (Berg *et al.*, 2005; Seidel *et al.*, 2007b). Based on the results described below, the *Rv2174* gene and its orthologues were designated as *mptA* (acronym for mannopyranosyltransferase A).

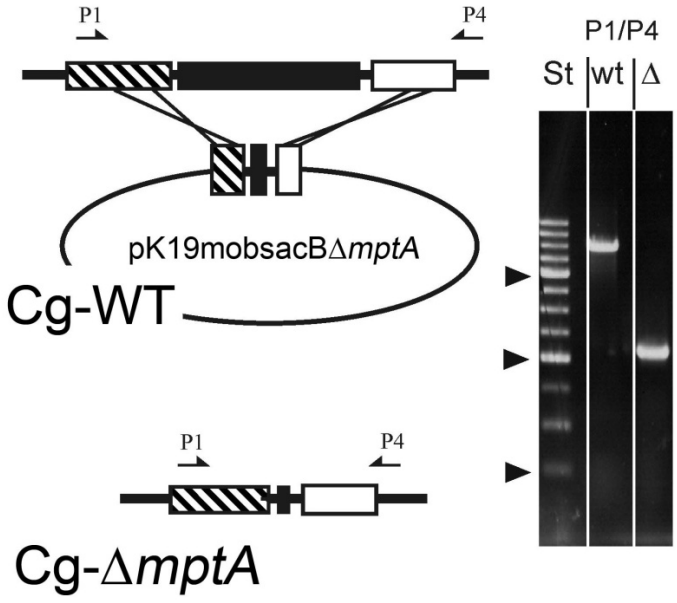

Fig. 2.2: In-frame deletion of Cg-*mptA* using the deletion vector pK19mobsacBΔ*mptA*. pK19mobsacBΔ*mptA* carries 18 nucleotides of the 5'-end of Cg-*mptA* and 36 nucleotides of its 3'-end thereby enabling the in-frame deletion of almost the entire Cg-*mptA* gene. The arrows marked P1 and P4 locate the primers used for the PCR analysis to confirm the absence of Cg-*mptA*. Distances are not drawn to scale. The results of the PCR analysis with the primer pair P1/P4 are shown on the right. Amplification products obtained from the wild type (wt) were applied in the left Lane and that of the deletion mutant (Δ) in the right Lane. *St* marks the standard, where the arrowheads located at 1.5, 1, and 0.5 kilobases.

2.3.2 Construction and growth of *C. glutamicum*Δ*mptA*

In an attempt to delete *mptA* in *C. glutamicum*, the non-replicative plasmid pK19mobsacBΔ*mptA* was constructed carrying sequences adjacent to Cg-*mptA* (Fig. 2.2). The vector was introduced into *C. glutamicum* and in several electroporation assays kanamycin resistant clones were obtained, indicating integration of the vector into the genome by homologous recombination. The *sacB* gene enables for positive selection of a second homologous recombination event, which can result either in the original wild-type genomic organisation or in clones deleted of *mptA*. Twenty-four clones exhibiting the desired phenotype of vector-loss (Kan^S, Suc^R) were analysed by PCR

and eighteen of them were found to have Cg-*mptA* excised. These numbers indicate that the loss of Cg-*mptA* is apparently not a disadvantage for viability. As a result, one clone was subsequently termed *C. glutamicum*Δ*mptA* and confirmed by PCR to have Cg-*mptA* deleted, whereas controls with *C. glutamicum* wild type resulted in the expected larger amplification product (Fig. 2.2). Growth of wild type *C. glutamicum* and *C. glutamicum*Δ*mptA* were compared in BHI medium as well as salt medium CGXII. Both strains exhibited comparable growth rates and final cell densities grown on CGXII of 0.31 ± 0.2 h^{-1} and 29.4 ± 2.3 (OD_{600}) for the two strains, respectively. Thus, *C. glutamicum*Δ*mptA* does not exhibit an apparent growth defect under the conditions assayed indicating a degree of tolerance to the deletion of Cg-*mptA*. *C. glutamicum*Δ*mptA* was transformed with pVWEx-Mt-*mptA* and pVWEx-Cg-*mptA*. As expected with these complemented strains, no alteration in growth phenotype was apparent.

2.3.3 Chemical analysis of extracted lipoglycans from *C. glutamicum*Δ*mptA* and complemented strains

Extracted lipoglycans from *C. glutamicum, C. glutamicum*Δ*mptA*, *C. glutamicum*Δ*mptA* pVWEx-Cg-*mptA* and *C. glutamicum*Δ*mptA* pVWEx-Mt-*mptA* were examined on 15 % SDS-PAGE (Fig. 2.3). Extracts from wild type *C. glutamicum* showed the presence of Cg-LAM and Cg-LM, while both of these lipoglycans were absent from *C. glutamicum*Δ*mptA*. Interestingly, a lower molecular weight lipoglycan, now termed truncated (t)-LM, could be observed in *C. glutamicum*Δ*mptA*. Complementation of *C. glutamicum*Δ*mptA* by either pVWEx-Cg-*mptA* or pVWEx-Mt-*mptA* restored the wild type phenotype (Fig. 2.3). Cg-t-LM was purified by hydrophobic interaction chromatography (HIC) and compared with wild type LM. Total sugar

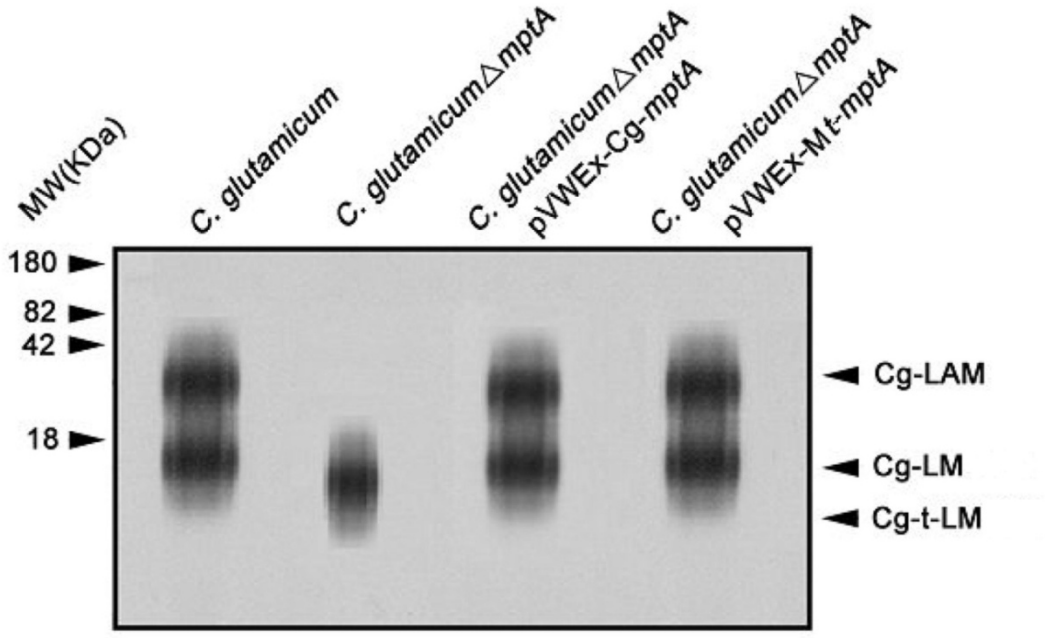

Fig. 2.3: Lipoglycan profiles of *C. glutamicum*, *C. glutamicum*Δ*mptA*, *C. glutamicum*Δ*mptA* pVWEx-Cg-*mptA*, and *C. glutamicum*Δ*mptA* pVWEx-Mt-*mptA*. Lipoglycans were analysed using SDS-PAGE and visualised using a Pro-Q emerald glycoprotein stain (Invitrogen) specific for carbohydrates. The three major bands represented by Cg-LAM, Cg-LM and Cg-t-LM are indicated. The STD Lane contains CandyCane glycoprotein molecular weight standards (Invitrogen). The four major bands represent glycoproteins of 180, 82, 42, and 18 kDa, respectively.

analysis of alditol aceteate derived sugars from Cg-t-LM by GC, identified the presence of only mannose and traces of inositol. Glycosyl linkage analysis of the per-*O*-methylated alditol acetate derivatives from Cg-t-LM indicated the presence of t-Man*p*, 2-Man*p,* 6 Man*p*, and 2,6-Man*p* similar to wild type LM (Fig. 2.4 A, B), but with an overall decrease in 6-Man*p* and 2,6-Man*p* linkages, when compared to 2-Man*p* in *C. glutamicum*Δ*mptA* (Fig. 2.4 A,B). This tentatively suggested that NCgl2093 is probably involved in the synthesis of the α(1→6) mannan core *via* an α(1→6) mannopyranosyltransferase, whereby deletion results in a shorter backbone and in turn branching sites. Furthermore, the analysis of Cg-t-LM in comparison to Cg-LM also suggests that the distal end to the PI of LM is probably more heavily branched. This phenotype is in contrast to studies of other mannosyltransferase (Kaur *et al.*, 2006), whereby inactivation resulted in the

complete loss of

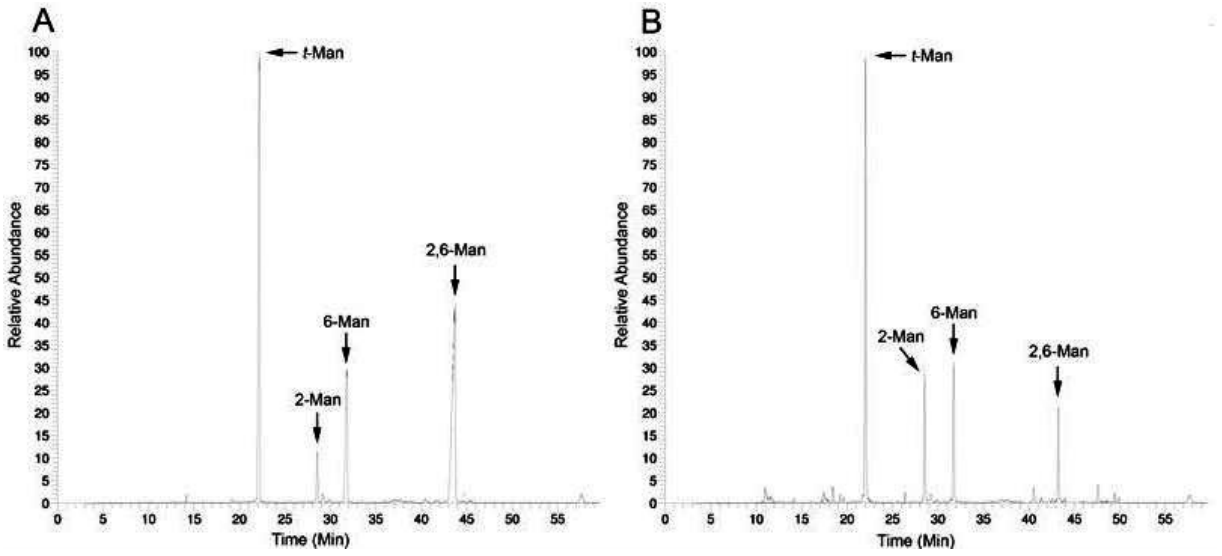

Fig. 2.4: GC-MS analysis of LM from *C. glutamicum* and *C. glutamicum*Δ*mptA*. For GC-MS analysis, per-*O*-methylated samples were hydrolysed using 2M trifluoroacetic acid, reduced and per-*O*-acetylated. The resulting partially per-*O*-methylated, per-*O*-acetylated alditol acetates from *C. glutamicum* LM (**A**) and *C. glutamicum*Δ*mptA* Cg-t-LM (**B**) were analysed by GC/MS.

2-Man*p* residues and a mut-LAM structure possessing a linear α(1→6) mannan core devoid of α(1→2)-Man*p* branches and as a result the characterisation of Rv2181 as an α(1→2) mannopyranosyltransferase. The extracted LM from *C. glutamicum* and *C. glutamicum*Δ*mptA* were analysed by MALDI-TOF-MS (Fig. 2.5). The negative MALDI-TOF-MS spectrum of Cg-LM showed a broad unresolved peak centered at *m/z* 5700 (Fig. 2.5 A), indicating a molecular mass of approximately 5.7 kDa for the major molecular species of this lipoglycan. Analysis of Cg-t-LM from *C. glutamicum*Δ*mptA* (Fig. 2.5 B) produced a lower average molecular mass of approximately around 3.3 kDa, proposing a composition based on extension of Ac_1PIM_2 (*m/z* 1398) (Tatituri *et al.*, 2007b) to afford Cg-t-LM as an average molecule centred on Ac_1PIM_{14}.

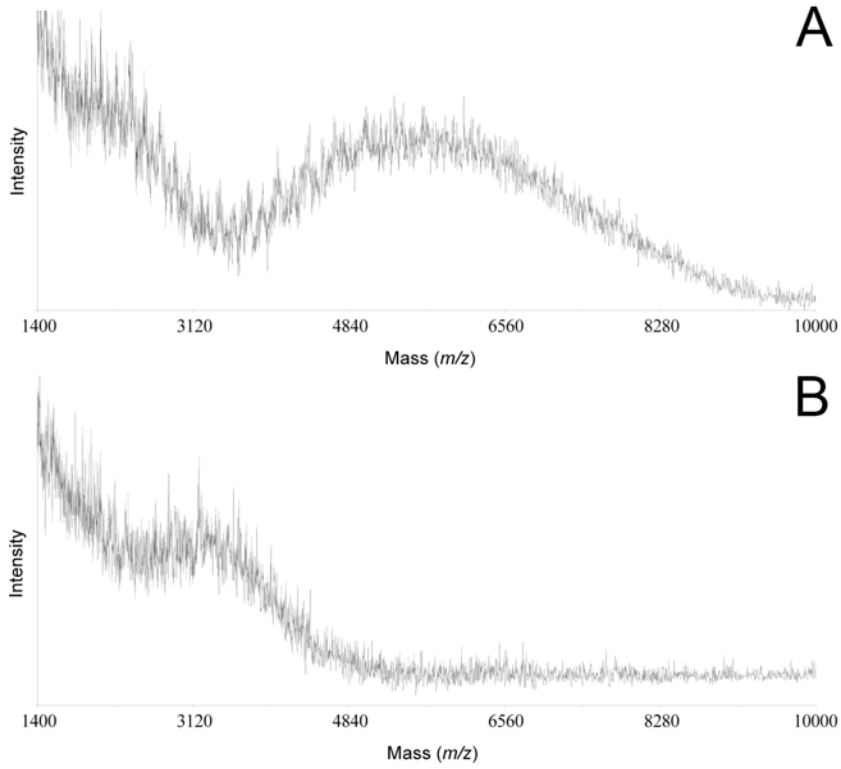

Fig. 2.5: MALDI-TOF-MS spectra of LM from *C. glutamicum* (A) and *C. glutamicum*Δ*mptA* (B). The spectra were acquired in the linear negative mode with delayed extraction using 2,5-dihydrobenzoic acid as a matrix.

As highlighted in our previous studies, the carbohydrate backbone of Cg-LM has been shown to be composed of an α(1→6)-Man*p* backbone substituted at most of the *O*-2 positions by t-Man*p* and t-Man*p*-α-**D**-(1→2)-Man*p* units (Tatituri *et al.*, 2007b). The different NMR spin systems of Cg-LM and Cg-t-LM were further characterised by one dimensional ^{1}H and two dimensional ^{1}H-^{13}C HMQC NMR (Fig. 2.6). The Cg-t-LM from *C. glutamicum*Δ*mptA* possessed the same spin systems (Fig. 2.6C, D) as Cg-LM (Fig. 2.6 A, B) and their anomeric resonances were attributed as follows: δH_1C_1 5.12/101.2 (I_1) to 2,6-Man*p*, 5.05/105.2 (II_1) to t-Man*p*, 5.00/104.9 (III_1) to 2-Man*p* and 4.92/102.6 (VII_1) to 6-Man*p* units, respectively. However, integration of the 1D ^{1}H

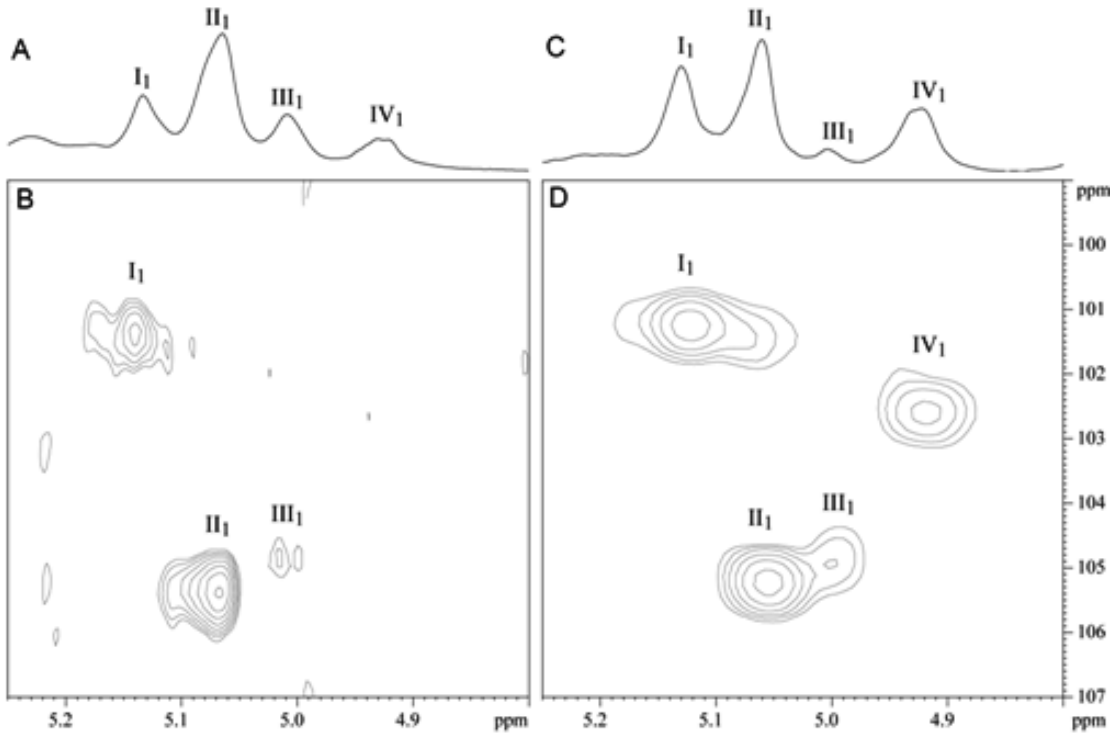

Fig. 2.6: Structural characterisation of LM from *C. glutamicum* (A, B) and Cg-t-LM from *C. glutamicumΔmptA* (C, D) using NMR. 1D ^{1}H (**A,C**) and 2D ^{1}H-^{13}C HMQC (B,D) NMR spectra of Cg-LMs in D_2O at 313K. Expanded regions (δ ^{1}H: 4.80-5.25) (**A,C**) and (δ ^{1}H: 4.80-5.25, δ ^{13}C: 99-107) (**B,D**) are shown. Glycosyl residues are labelled in roman numerals and their carbons and protons in arabic numerals. I, 2,6-α-Man*p*; II, t-α-Man*p*; III, 2-α-Man*p*; IV, 6-α-Man*p*.

resonances, supporting our earlier glycosyl linkage analysis indicated a reduced branching degree, approximately 50 % for Cg-t-LM, as compared to 78 % for Cg-LM. Altogether, the data indicate that Cg-t-LM in *C. glutamicumΔmptA* occurs possibly as a result of inactivation of a core α(1→6) mannopyranosyltransferase, presumably involved in assembly of the distal portion of Cg-LM, thereby rendering a substrate possessing reduced sites for branching.

2.3.3 *In vitro* analysis of α(1→6) mannopyranosyltransferase activity with different strains

Initial attempts to develop an *in vitro* assay using either purified recombinant expressed Mt-MptA, Cg-MptA, or *E. coli* membranes expressing the proteins, have proved unsuccessful. In an alternative approach, we assessed the capacity of membrane preparations from *C. glutamicum*, *C. glutamicum*Δ*mptA*, *C. glutamicum*Δ*mptA* pVWEx-Cg-*mptA* and *C. glutamicum*Δ*mptA* pVWEx-Mt-*mptA* to catalyse α(1→6) mannopyranosyltransferase activity in a previously defined neoglycolipid acceptor assay utilising an exogenous α-**D**-Man*p*-(1→6)-α-**D**-Man*p*-*O*-C_8 acceptor and PP-[^{14}C]-M as a sugar donor (Brown *et al.*, 2001) (Fig. 2.7 A). TLC analysis of radio-labeled products, when assayed with *C. glutamicum* membranes, resulted in the formation of two products, X and Y (Fig. 2.7 A, B). Control assays when performed in the absence of acceptor afforded background counts, typically <100 cpm per assay (Brown *et al.*, 2001).

The enzymatic synthesis of product X and Y using membranes from *C. glutamicum* relates to the biosynthesis of the radiolabeled trisaccharide α-**D**-[^{14}C]-Man*p*-(1→6)-α-**D**-Man*p*-(1→6)-α-**D**-Manp-*O*-C_8 (97864 cpm) and the tetrasaccharide α-**D**-[^{14}C]-Man*p*-(1→6)-α-**D**-[^{14}C]Man*p*-(1→6)-α-**D**-Man*p*-(1→6)-α-**D**-Manp-*O*-C_8 (5915 cpm), respectively, and is consistent with previous studies (Brown *et al.*, 2001). However, when assays were performed using membranes prepared from *C. glutamicum*Δ*mptA*, a drastically reduced amount of product X (1385 cpm) could be observed and a complete absence of product Y, indicating that Cg-MptA provides the majority of α(1→6) mannopyranosyltransferase activity utilising the α-**D**-Man*p*-(1→6)-α-**D**-Man*p*-*O*-C_8 neoglycolipid acceptor (Fig. 2.7).

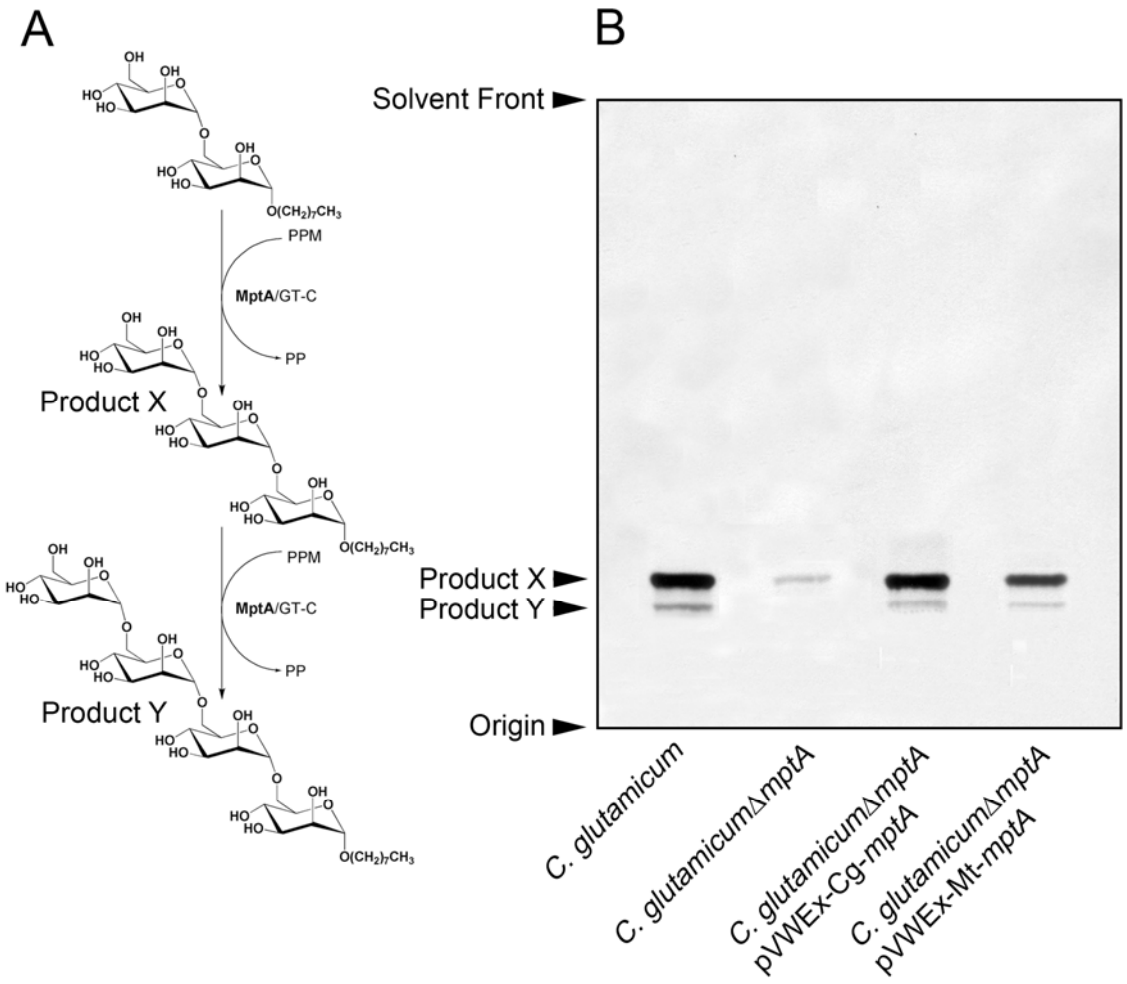

Fig. 2.7: α(1→6)-Mannopyranosyltransferase activity in membranes prepared from *C. glutamicum*, *C. glutamicum*Δ*mptA*, *C. glutamicum*Δ*mptA* pVWEx-Cg-*mptA*, and *C. glutamicum*Δ*mptA* pVWEx-Mt-*mptA*. (**A**) Biosynthetic reaction scheme of products formed in the α(1→6)-mannopyranosyltransferase assay utilising α-D-Man*p*-(1→6)-α-D-Man*p*-*O*-C_8 and C_{50}-PP-[^{14}C]-M. (**B**) α(1→6)-Mannopyranosyltransferase activity determined using the synthetic α-D-Man*p*-(1→6)-α-D-Man*p*-*O*-C_8 neoglycolipid acceptor in a cell free assay. The products of the assay were resuspended in *n*-butanol before scintillation counting. The incorporation of [^{14}C]-Man*p* was determined by subtracting counts present in control assays (incubations in the absence of acceptor). The labeled material were also subjected to TLC using silica gel plates developed in $CHCl_3$:CH_3OH:H_2O;NH_4OH (65:25:3.6:0.5, v/v/v/v) and the products visualised by phosphorimaging. The results represent triplicate assays in two independent experiments.

In addition, these results also suggest the existence of a second α(1→6) mannopyranosyltransferase presumably affording the weak activity seen within the membrane preparations of *C. glutamicum*Δ*mptA* and involved in the synthesis of the α(1→6) mannan core proximal to the PI of LM. Membranes assayed with *C. glutamicum*Δ*mptA* complemented with either pVWEx-Cg-*mptA* (X, 62953 cpm; Y, 1947 cpm) or pVWEx-Mt-*mptA* (X, 26145 cpm; Y, 1174 cpm) restored product formation to that of wild type *C. glutamicum*, albeit at a lower rate of transfer (Fig. 2.7).

2.3.4 Genome locus and structural features of Rv1459c/NCgl1505

Residual α(1→6) mannopyranosyltransferase activity with *C. glutamicum*Δ*mptA* membrane suggested the presence of another α(1→6) mannopyranosyltransferase in *C. glutamicum* which may be involved in the synthesis of the α(1→6) mannan core proximal to the PIMs of LM. *NCgl1505* in *C. glutamicum* shows the highest homology with *NCgl2093* and *Rv2174*, and may encode for a potential α(1→6) mannopyranosyltransferase and for the remainder of the text will be referred to as MptB. Therefore, *mptB* from *C. glutamicum* was deleted in order to study its role in LM/LAM synthesis. Rv1459c and NCgl1505 both are members of the GT-C family of glycosyltransferases and present in all *Mycobacterium* and *Corynebacterium* species as well as the sequenced *Nocardia farcinica* IFM 10152 and *Rhodococcus sp.* RHA1 strains (Fig. 2.8A). In addition, this gene is retained in *M. leprae*, supporting the hypothesis that *Rv1459c* encodes for a protein possessing a vital function inherent to this group of bacteria.

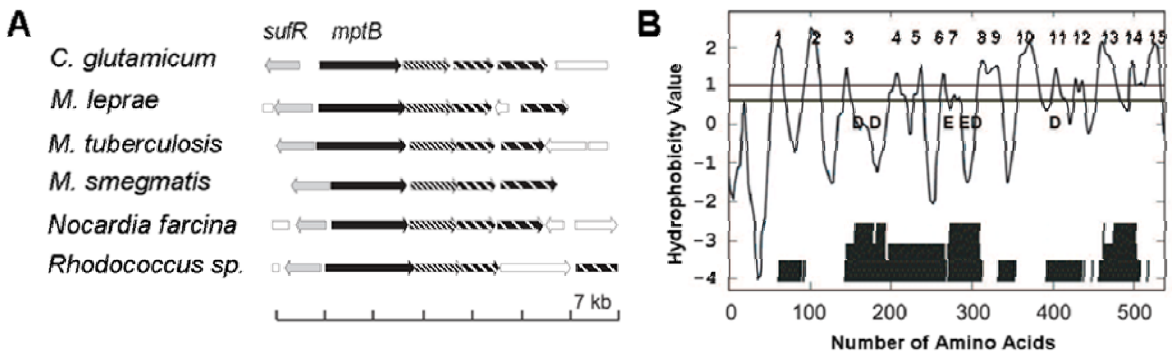

Fig. 2.8: The analysis of *mptB* locus and its homologoues and structural features of putative protein. (**A**) The locus in the bacteria analysed consists of *mptB* which has in *C. glutamicum* the locus tag *NCgl1505* and in *M. tuberculosis Rv1459c*. *sufR* encodes a transcriptional regulator in front of an operon of the SUF machinery of [Fe-S] cluster synthesis. The genomic region displayed encompasses 7 kb, and orthologous genes are highlighted accordingly. *Nocardia farcina*, *Nocardia farcina* IFM 10152; *Rhodococcus*, *Rhodococcus* sp. strain RHA1. (**B**) MptB is a hydrophobic protein predicted to span the membrane 15 times and the TMH are numbered accordingly. The lower part of the figure shows the degree of conservation of the orthologues given in A as analysed by the DIALIGN method. Also shown is the approximate position of the fully conserved aspartyl (D) and glutamyl (E) residues.

The glycosyltransferase encoded by *NCgl1505* is a polytopic membrane protein, which is comprised of 558 amino acid (aa) residues, and is predicted to encode 15 hydrophobic segments, HS (Fig. 2.8 B). Rv1459c constitutes 591 aa, with the additional length mostly due to an extended loop between HS 7 and 8. This loop extension is not present in *Mycobacterium paratuberculosis* or *M. smegmatis*. It contains a number of repeated Pro and Arg residues, and similarly highly charged repeat sequences are found in loop regions of other transporters, without having a specific function (Eng *et al.*, 1998; Vrljic *et al.*, 1999). The sequence identity of the orthologues NCgl1505 and Rv1459c is 37% (52% similarity) and can therefore be considered very high. The strongest conserved regions are found in loops connecting HSs and adjacent regions with intermediate hydrophobicity, like those between HS 3-4, HS 7-8 and HS 13-14. Within the highest conserved regions; 5 of the 6 fully conserved acidic Asp and Glu residues are

located (Fig. 2.8 B), which are known to play important roles as general bases and nucleophiles in enzyme catalysis. They are also retained in the MptB orthologue in *N. farcinica* IFM 10152 and *Rhodococcus sp.* RHA1 and are therefore likely to be involved in catalysis, or in interactions with the sugar donor or acceptor (Liu & Mushegian, 2003). Interestingly, among the glycosyltransferases of *M. tuberculosis* and *C. glutamicum* previously identified (Alderwick *et al.*, 2006b; Dinadayala *et al.*, 2006; Kaur *et al.*, 2006; Morita *et al.*, 2006; Seidel *et al.*, 2007a), NCgl1505 and Rv1459c possess the highest identities to the mannosyltransferase MptA (Mishra *et al.*, 2007), and based on the results described below, the *Rv1459c* gene and its orthologues have been designated as *mptB*, as an acronym for mannopyranosyltransferase B.

2.3.5 Construction and growth of *C. glutamicum*Δ*mptB*, *C. glutamicum*Δ*mptB*Δ*mptA*, and complemented strains

In order to delete *mptB* in *C. glutamicum*, the non-replicative plasmid pK19mobsacBΔ*mptB* was constructed carrying sequences adjacent to Cg-*mptB* (Fig. 2.9). Using this vector, *C. glutamicum* was transformed to kanamycin resistance, indicating integration of the vector into the genome by homologous recombination. The *sacB* gene enables for selection of loss of vector in a second homologous recombination event, which can result either in the original wild-type genomic organisation or in clones deleted of *mptB*. Ninety clones exhibiting the desired phenotype of vector-loss (Kan^s, Suc^r) were analysed by PCR but only one single colony was found to have Cg-*mptB* excised, whereas the others resulted in a wild type genotype. The low number of recombinant knock outs indicates that the loss of Cg-*mptB* is apparently a disadvantage for cell viability, similar to that of previously observed mutants with altered mycolate

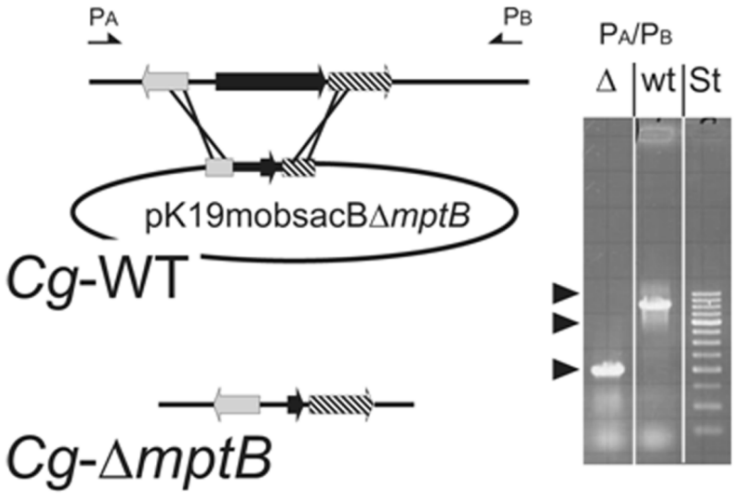

Fig. 2.9: Strategy to delete Cg-*mptB* using the deletion vector pK19mobsacBΔ*mptB*. This vector carries 18 nucleotides of the 5' end of Cg-*mptB* and 36 nucleotides of its 3' end thereby enabling the in-frame deletion of almost the entire Cg-*mptB* gene. The arrows marked PA and PB locate the primers used for the PCR analysis to confirm the absence of Cg-*mptB*. Distances are not drawn to scale. The results of the PCR analysis with the primer pair PA/PB are shown on the right. Amplification products obtained from the wild type (wt) were applied in the middle Lane and that of the deletion mutant (Δ) in the left Lane. 'St' marks the standard, where the arrowheads are located at 1.5, 1 and 0.5 kb.

(Gande *et al.*, 2004) or arabinogalactan biosynthesis (Alderwick *et al.*, 2006a). The resulting clone was subsequently termed *C. glutamicum*Δ*mptB* and confirmed by PCR with different primer pairs to have Cg-*mptB* deleted, whereas controls with *C. glutamicum* wild type resulted in the expected larger amplification product (Fig. 2.9).

In liquid culture, growth of *C. glutamicum*Δ*mptB* was very poor. Only when rich medium BHI was used a growth rate of 0.13 h^{-1} was obtained in comparison to wild type *C. glutamicum* growth rate of 0.31 h^{-1} (Mishra *et al.*, 2007), and on the same medium supplemented with 500 mM sorbitol (BHIS), the growth rate was 0.51 h^{-1}, which is still lower than that of the wild type on this medium (0.70 h^{-1}). *C. glutamicum*Δ*mptB* was transformed with pVWEx-Cg-*mptB* and the

resultant complemented strain exhibited a growth rate of 0.66 h^{-1}, almost superimposable to that of the wild type in BHIS medium.

Due to the similarity of *mptB* with *mptA*, we wanted to exclude any possible interference and constructed a strain of *C. glutamicum* deficient in both *mptB* and *mptA*. For this purpose *C. glutamicum*Δ*mptB* was transformed with plasmid pK19mobsacBΔ*mptA* (Mishra *et al.*, 2007) and processed to afford the double mutant, *C. glutamicum*Δ*mptB*Δ*mptA*. Analysis of *C. glutamicum*Δ*mptB* and *C. glutamicum*Δ*mptB*Δ*mptA* showed a similar growth profile with a delayed lag-phase and slow growth-rate in comparison to wild type *C. glutamicum*. For further analysis of *C. glutamicum*Δ*mptB* and *C. glutamicum*Δ*mptA*Δ*mptB* the strains were transformed with plasmid-encoded Cg-*mptB*, Cg-*mptA*, Mt-*mptB* and Ms-*mptB*.

2.3.6 Polar lipid analysis of *C. glutamicum* and *C. glutamicum*Δ*mptB*

Lyophilised cells were extracted using petroleum-ether and methanolic saline to initially recover apolar lipids. Further processing of the methanolic extract afforded the polar lipid fraction which was examined by 2D-TLC (Fig. 2.10). In both, the wild type *C. glutamicum* and *C. glutamicum*Δ*mptB*, Ac_1PIM_2 and $Man_1GlcAGroAc_2$ (Tatituri *et al.*, 2007b) were visualised either by α-naphthol/sulfuric acid (Fig. 2.10), 5% ethanolic molybdophosphoric acid or Dittmer and Lester reagent. In both *C. glutamicum* and *C. glutamicum*Δ*mptB*, no products could be observed which correspond to higher PIMs (i.e. Ac_1PIM_3 through to Ac_1PIM_6) or higher mannose variants of $Man_1GlcAGroAc_2$ (Tatituri *et al.*, 2007b). The presence of only Ac_1PIM_2 and $Man_1GlcAGroAc_2$, and the inability to synthesise Cg-LAM, Cg-LM by *C. glutamicum*Δ*mptB*

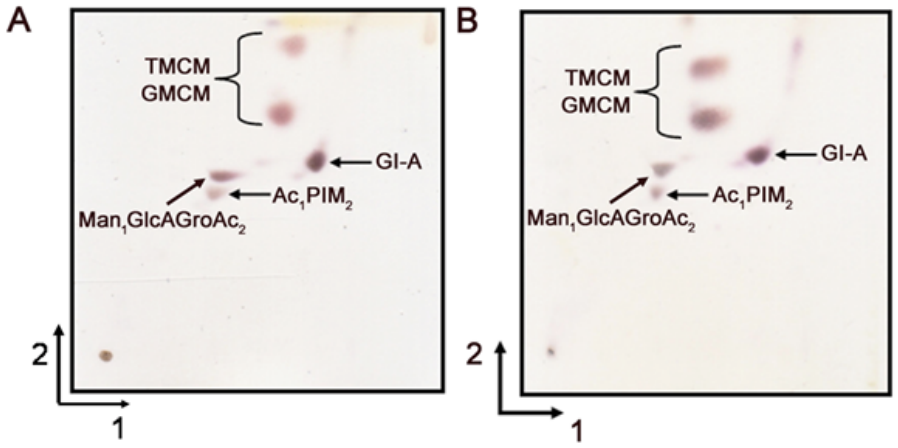

Fig. 2.10: Analysis of polar lipids in *C. glutamicum* (A) and *C. glutamicum*Δ*mptB* (B). The polar lipid extract was examined by 2D-TLC, using $CHCl_3/CH_3OH/H_2O$ (60:30:6, v/v/v) in the first direction and $CHCl_3/CH_3COOH/CH_3OH/H_2O$ (40:25:3:6, v/v/v/v) in the second direction. Glycolipids were visualised by spraying plates with α-naphthol/sulfuric acid, followed by gentle charring of the plates. Abbreviations: Gl-A, α-**D**-glucopyranosyluronic acid-(1→3)-glycerol; GMCM, glucose monocorynomycolate; TMCM, trehalose monocorynomycolate.

demonstrated that MptB is involved in the early steps of α(1→6) mannan core biosynthesis by extending the substrates Ac_1PIM_2 and $Man_1GlcAGroAc_2$.

2.3.7 Chemical analysis of lipoglycans from *C. glutamicum*, *C. glutamicum*Δ*mptB*, *C. glutamicum*Δ*mptB*Δ*mptA* and complemented strains

Lipoglycans were extracted by refluxing delipidated cells in ethanol, followed by hot-phenol extraction, protease digestion and dialysis to remove impurities. The extracted lipoglycans were examined initially on 15 % SDS-PAGE (Fig. 2.11). Extracts from wild type *C. glutamicum* showed the presence of Cg-LAM and Cg-LM, while they were absent from *C. glutamicum*Δ*mptB*. Complementation of *C. glutamicum*Δ*mptB* by transformation with plasmid

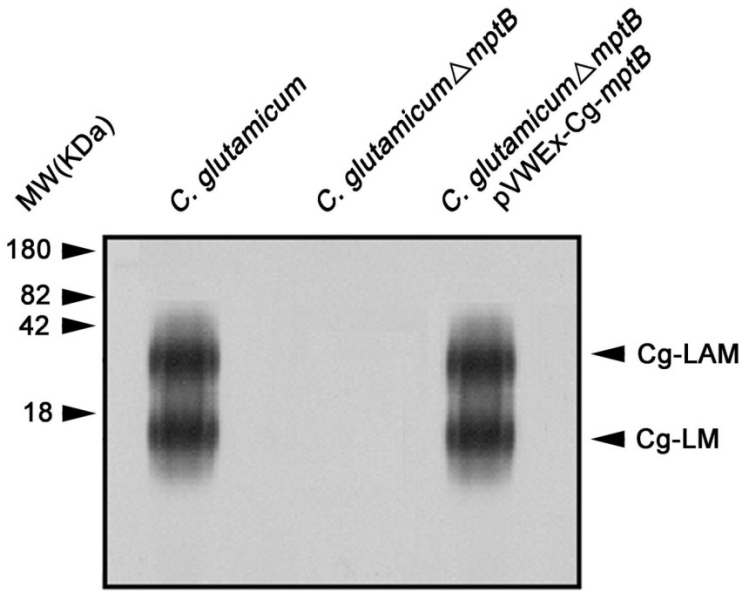

Fig. 2.11: SDS-PAGE analysis of lipoglycans of *C. glutamicum* strains. Lipoglycans extracted from *C. glutamicum*, *C. glutamicum*Δ*mptB* and *C. glutamicum*Δ*mptB* pVWEx-Cg-*mptB*. The major bands represented by Cg-LAM and Cg-LM are indicated. The four major bands represent glycoproteins of 180, 82, 42 and 18 kDa respectively.

pVWEx-Cg-*mptB* restored the wild type phenotype (Fig. 2.11). In addition, transformation of *C. glutamicum*Δ*mptB* with plasmid pVWEx-Cg-*mptA* failed to restore the wild type phenotype.

To study the *in situ* specificity of MptA and MptB, lipoglycans were extracted from *C. glutamicum*Δ*mptB*Δ*mptA*, and from the same strain carrying either pVWEx-Cg-*mptB* or pVWEx-Cg-*mptA* and analysed by 15 % SDS-PAGE (Fig. 2.12). Extracts from *C. glutamicum*Δ*mptB*Δ*mptA* indicated that, as expected, no lipoglycans were present, whereas the presence of pVWEx-Cg-*mptB* resulted in formation of a truncated (t) version of Cg-LM, as shown previously. However, lipoglycan extracts from *C. glutamicum*Δ*mptB*Δ*mptA* carrying pVWEx-Cg-*mptA* were identical to that of *C. glutamicum*Δ*mptB*Δ*mptA*, indicating that MptA fails to substitute for MptB in the double mutant (Fig. 2.12). Since, pVWEx-Cg-*mptA* results in

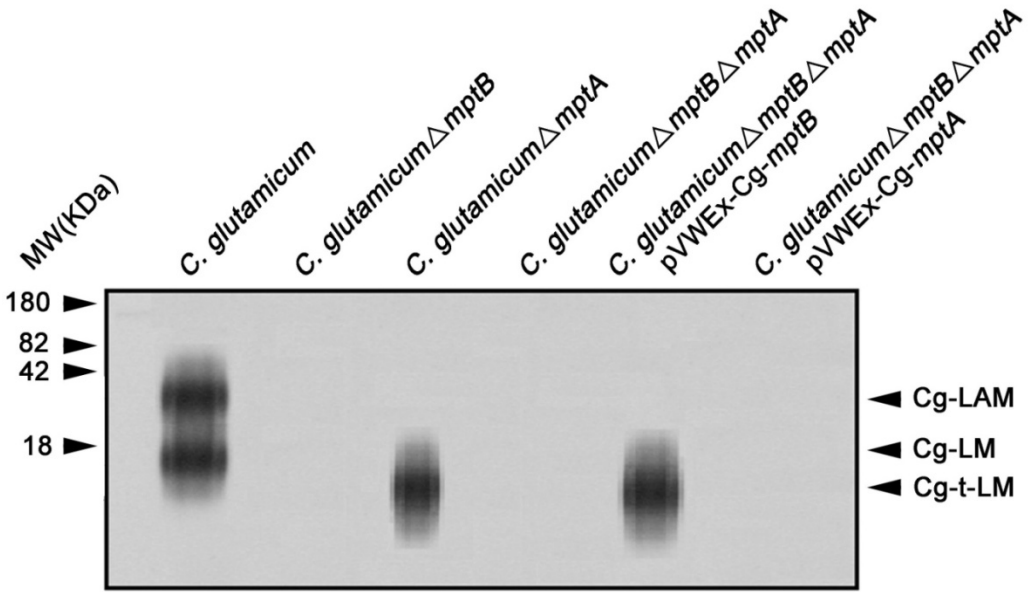

Fig. 2.12: SDS-PAGE analysis of lipoglycans from *C. glutamicum* strains. Lipolglycans extracted from *C. glutamicum*, *C. glutamicumΔmptB*, *C. glutamicumΔmptA*, *C. glutamicumΔmptBΔmptA*, *C. glutamicumΔmptBΔmptA* pVWEx-Cg-*mptB*, and *C. glutamicumΔmptBΔmptA* pVWEx-Cg-*mptA*. The major bands represented by Cg-LAM, Cg-LM, and Cg-t-LM are indicated. The four major bands represent glycoproteins of 180, 82, 42 and 18 kDa respectively.

functional MptA this result shows that MptA is unable to substitute *in vivo* for MptB.

Therefore, both MptA and MptB are distinct and MptB is involved in the initial steps of Cg-LAM and Cg-LM biosynthesis, prior to MptA. Furthermore, analysis of *C. glutamicumΔmptBΔmptA* carrying either pVWEx-Mt-*mptB* or pVWEx-Ms-*mptB* resulted in a complete lack of lipoglycan biosynthesis indicating that Mt-MptB and Ms-MptB does not function *in vivo* as the initial $\alpha(1\rightarrow6)$ mannopyranosyltransferase probably due to an inability to extend Ac_1PIM_2 and $Man_1GlcAGroAc_2$ by mannose residues as shown below through *in vitro* chase experiments.

2.3.8 *In vitro* incorporation of radiolabeled mannose from GDP-[^{14}C]-Man*p* into membrane lipids utilising *C. glutamicum*, *C. glutamicum*Δ*mptB* and complemented strains

Incorporation of [^{14}C]-Man*p* from GDP-[^{14}C]-Man*p* into $CHCl_3/CH_3OH$ (2:1) and $CHCl_3/CH_3OH/H_2O$ (10:10:3)-soluble lipids was examined using membrane/cell envelope extracts prepared from *C. glutamicum* as described previously utilising mycobacterial membrane/cell envelope fractions (Besra *et al.*, 1997). TLC-autoradiography (Fig. 2.13 A, 1) of the $CHCl_3/CH_3OH$ (2:1)-soluble lipids synthesised by wild type *C. glutamicum* membrane/cell envelope extracts contained as expected C_{50}-PP-[^{14}C]-M, [^{14}C]-$Man_1GlcAGroAc_2$ and Ac_1PI-[^{14}C]-M_2. The identity of the three labeled lipids was established by (i) base treatment i.e. degradation of Ac_1PI-[^{14}C]-M_2 and [^{14}C]-$Man_1GlcAGroAc_2$ (Fig. 2.13 A, 2); (ii) addition of amphomycin, which specifically chelates polyprenyl phosphates in the presence of Ca^{2+} and thus inhibiting the transfer of Man*p* from GDP-Man*p* to polyprenyl carriers (Fig. 2.13 A, 3); and (iii) in comparison with known standards (Tatituri *et al.*, 2007b). As expected from the analysis of whole cells *C. glutamicum*Δ*mptB* synthesised comparable levels of all three radiolabeled lipids using membrane/cell envelope extracts prepared from *C. glutamicum*Δ*mptB* (Fig. 2.13 A, 4).

The above reaction mixtures were then further processed as described in the experimental procedures section to provide the $CHCl_3/CH_3OH/H_2O$ (10:10:3)-soluble lipids initially using membrane/cell envelope extracts prepared from *C. glutamicum* to provide [^{14}C]-mannooligosaccharides (Fig. 2.13 B, 1), which were further characterised by a series of degradation experiments.

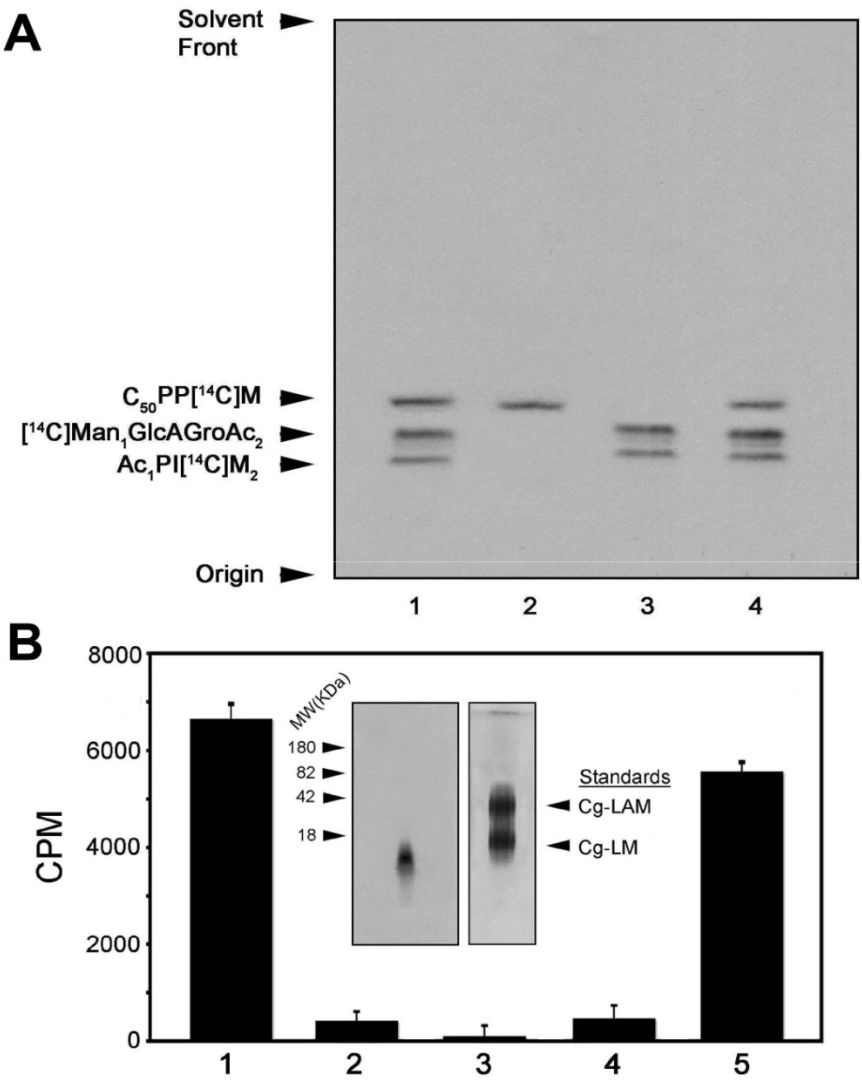

Fig. 2.13: Incorporation of [^{14}C]-Man*p* from GDP-[^{14}C]-Man*p* into corynebacterial membrane/cell envelope lipids. (**A**) TLC-autoradiography of labeled $CHCl_3/CH_3OH$ (2:1)-soluble lipids, C_{50}-PP-[^{14}C]-M, [^{14}C]-$Man_1GlcAGroAc_2$ and Ac_1PI-[^{14}C]-M_2 using GDP-[^{14}C]-Man*p* and membrane/cell envelope extracts from *C. glutamicum* and *C. glutamicum*Δ*mptB*. *C. glutamicum* $CHCl_3/CH_3OH$ (2:1)-soluble lipids (Lane 1), base-treatment of $CHCl_3/CH_3OH$ (2:1)-soluble lipids (Lane 2), amphomycin treatment (Lane 3) and *C. glutamicum*Δ*mptB* $CHCl_3/CH_3OH$ (2:1)-soluble lipids (Lane 4). (**B**) Characterisation of $CHCl_3/CH_3OH/H_2O$ (10:10:3)-soluble lipids as α(1→6)-linear mannooligosaccharides. The insoluble pellet from the above reaction mixtures following extraction with $CHCl_3/CH_3OH$ (2:1) were sequentially washed with 0.9 % NaCl in 50 % CH_3OH, 50 % CH_3OH and CH_3OH, prior to extraction with $CHCl_3/CH_3OH/H_2O$ (10:10:3) and an aliquot (10%) taken for scintillation counting and the remaining product analysed by SDS-PAGE/autoradiography (left-panel inset). *C. glutamicum* (No. 1), amphomycin treatment (No. 2) and acetolysis treatment of $CHCl_3/CH_3OH/H_2O$ (10:10:3)-soluble lipids (No. 3), *C. glutamicum*Δ*mptB* (No. 4) and *C. glutamicum*Δ*mptB* pVWEx-Cg-*mptB* (No. 5) as described in the experimental procedures.

The [^{14}C]-mannooligosaccharides were sensitive to acetolysis (Fig. 2.13 B, 3), thus establishing a core α(1→6)-linear mannan backbone within the $CHCl_3/CH_3OH/H_2O$ (10:10:3)-soluble lipids. In separate experiments the addition of amphomycin to block C_{50}-PP-[^{14}C]-M synthesis also inhibited the synthesis of the α(1→6)-linear mannan lipids demonstrating that the synthesis of these $CHCl_3/CH_3OH/H_2O$ (10:10:3)-soluble lipids is PPM dependent (Fig. 2.13 B, 2) and similar to the previously characterised *in vitro* synthesised mycobacterial products (Besra *et al.*, 1997). SDS-PAGE and subsequent autoradiography of the dried gels demonstrated that the $CHCl_3/CH_3OH/H_2O$ (10:10:3)-soluble lipids (Fig. 2.13 B, 1) had slightly reduced mobility indicating that they were smaller in size (Fig. 2.13 B, left-panel inset), presumably due to their lack of α(1→2)-branching characteristic of Cg-LM (Tatituri *et al.*, 2007b). As expected, synthesis of $CHCl_3/CH_3OH/H_2O$ (10:10:3)-soluble lipids using membranes from *C. glutamicum*Δ*mptB* was completely abolished (Fig. 2.13 B, 4). Furthermore, complementation with pVWEx-Cg-*mptB* restored synthesis of $CHCl_3/CH_3OH/H_2O$ (10:10:3)-soluble lipids (Fig. 2.13 B, 5).

2.3.7 Chase of *in situ* labelled glycolipids into α(1→6)-linear Cg-LM utilising membranes

Amphomycin treated wild type *C. glutamicum* membrane/cell envelope extracts were initially pulsed with GDP-[^{14}C]-Man*p* during a short incubation period (15 min) which was shown earlier to inhibit the synthesis of the $CHCl_3/CH_3OH/H_2O$ (10:10:3)-soluble α(1→6)-linear [^{14}C]-mannan lipids, but instead of extracting with $CHCl_3/CH_3OH$ (2:1), the [^{14}C]-Man-labeled membranes were re-harvested by ultracentrifugation at 100,000 x *g*, carefully washed and re-centrifuged

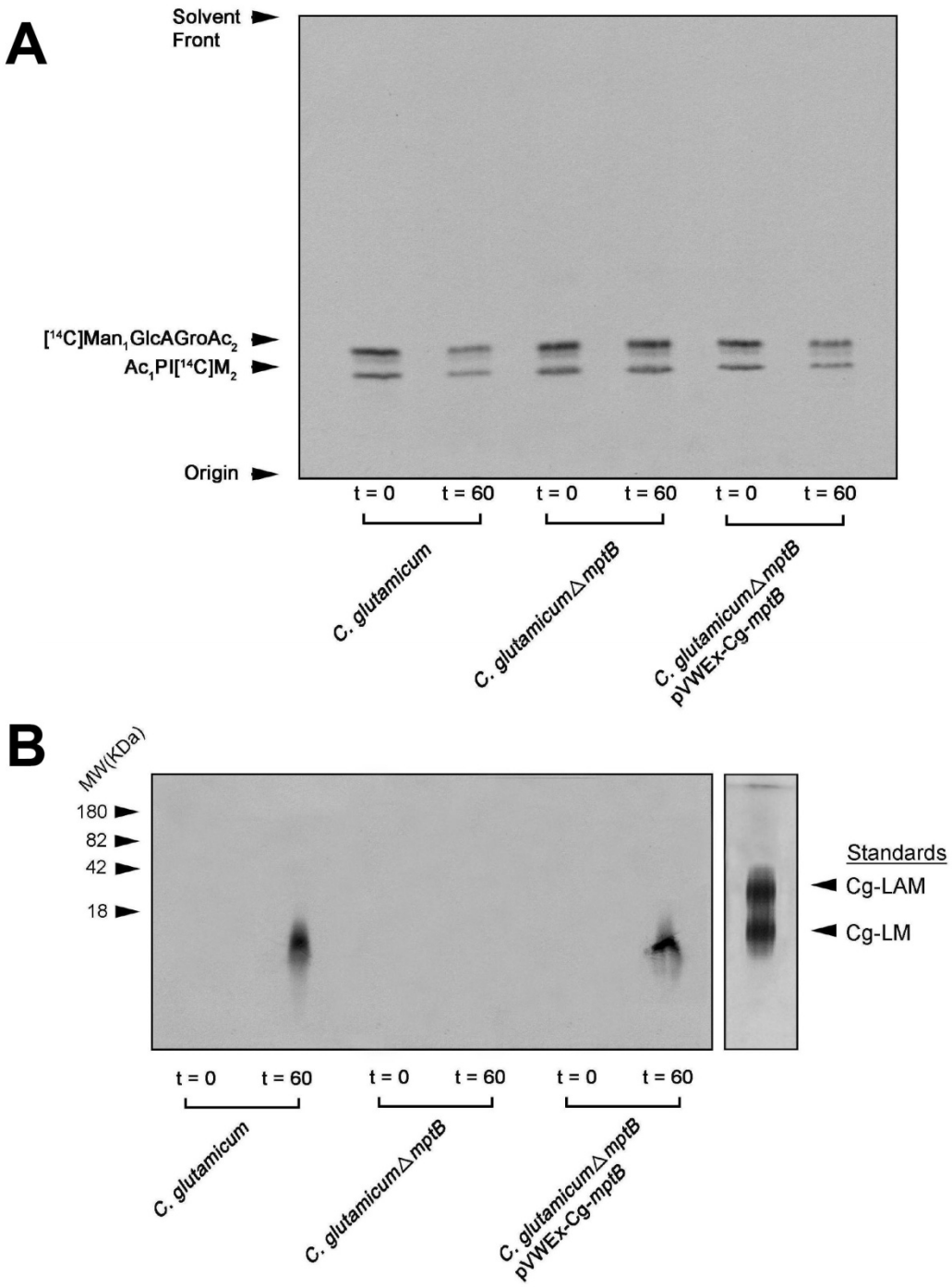

Fig. 2.14: Incorporation of *in vitro in situ* Ac_1PI-[^{14}C]-M_2 and [^{14}C]-$Man_1GlcAGroAc_2$ into α(1→6)-linear mannooligosaccharides with either *C. glutamicum*, *C. glutamicumΔmptB* or *C. glutamicumΔmptB* pVWEx-Cg-*mptB* membrane preparations. Membranes were initially pre-treated with amphomycin, labelled using GDP-[^{14}C]-Man*p*, and was processed for different time intervals as described in the experimental procedures for $CHCl_3/CH_3OH$ (2:1)-soluble lipids and analysed by TLC/autoradiography using $CHCl_3/CH_3OH/NH_4OH/H_2O$ (65:25:0.4:3.6, v/v/v/v) (**A**) and $CHCl_3/CH_3OH/H_2O$ (10:10:3)-soluble lipids by SDS-PAGE/autoradiography (**B**).

twice using cold buffer, to remove unused GDP-[^{14}C]-Man*p*. An aliquot of the [^{14}C]-Man-labelled membranes were extracted with $CHCl_3/CH_3OH$ (2:1) and contained as expected solely Ac_1PI-[^{14}C]-M_2 (3329 cpm) and [^{14}C]-$Man_1GlcAGroAc_2$ (5474 cpm) at t = 0 chase time as determined by TLC-autoradiography and phosphorimaging (Fig. 2.14 A). The $CHCl_3/CH_3OH/H_2O$ (10:10:3)-soluble lipids at t=0 gave 226 cpm.

The [^{14}C]-Man-labeled membranes were then further incubated for 60 min following the addition of excess exogenous cold C_{50}-PPM (Gurcha *et al.*, 2002) prior to the standard extraction method to provide $CHCl_3/CH_3OH$ (2:1) and $CHCl_3/CH_3OH/H_2O$ (10:10:3)-soluble lipids. The t = 60 chase time revealed a loss of radioactivity from both Ac_1PI-[^{14}C]-M_2 (1709 cpm) and [^{14}C]-$Man_1GlcAGroAc_2$ (2530 cpm) as determined by TLC-autoradiography and phosphorimaging, and incorporation into $CHCl_3/CH_3OH/H_2O$ (10:10:3)-soluble α(1→6)-linear [^{14}C]-mannoligosaccharide lipids (2895 cpm) (Fig. 2.14 B). The *in vitro in situ* chase experiment demonstrated that the α(1→6)-linear [^{14}C]-mannoligosaccharide lipids synthesised were elongation products of both Ac_1PI-[^{14}C]-M_2 and [^{14}C]-$Man_1GlcAGroAc_2$.

Similar experiments repeated with *C. glutamicum*Δ*mptB in situ* prepared [^{14}C]-labeled membranes as above resulted in comparable products at t = 0 and t = 60 for $CHCl_3/CH_3OH$ (2:1)-soluble lipids (Ac_1PI-[^{14}C]-M_2 [t = 0, 3345 cpm; t = 60, 2968 cpm] and [^{14}C]-$Man_1GlcAGroAc_2$ [t = 0, 5840 cpm; t = 60, 5025 cpm]) and a lack of the synthesis of α(1→6)-linear [^{14}C]-mannoligosaccharide lipids (240 cpm) from the elongation primers Ac_1PI-[^{14}C]-M_2 and [^{14}C]-$Man_1GlcAGroAc_2$ following the 'chase period' (Fig. 2.14 A and B). Complementation of *C. glutamicum*Δ*mptB* by transformation with plasmid pVWEx-Cg-*mptB* resulted in Ac_1PI-[^{14}C]-M_2

[t = 0, 3229 cpm; t = 60, 1725 cpm] and [^{14}C]-$Man_1GlcAGroAc_2$ [t = 0, 5367 cpm; t = 60, 2550 cpm]) and *in vitro in situ* synthesis of α(1→6)-linear [^{14}C]-mannoligosaccharide lipids (2471 cpm) to levels comparable to wild type *C. glutamicum* (Fig. 2.14 A and B). The data clearly demonstrates that Cg-MptB functions *in vivo* and *in vitro* as the initial α-mannopyranosyltransferase, which extends Ac_1PIM_2 and $Man_1GlcAGroAc_2$. However, under the same *in vitro in situ* chase conditions, *C. glutamicum*Δ*mptB* pVWEx-Mt-*mptB* (or pVWEx-Ms-*mptB*) failed to elongate the primers Ac_1PI-[^{14}C]-M_2 and [^{14}C]-$Man_1GlcAGroAc_2$ and restore synthesis of the α(1→6)-linear [^{14}C]-mannoligosaccharides.

In addition, experiments conducted with *C. glutamicum*Δ*mptB* pVWEx-Mt-*mptB* and *C. glutamicum*Δ*mptB* pVWEx-Ms-*mptB* and the addition of the exogenous primer Ac_1PI-[^{14}C]-M_4 isolated from a *M. bovis* BCG PimE mutant, also failed to restore the synthesis of the α(1→6)-linear [^{14}C]-mannoligosaccharides.

2.3.8 In vitro analysis of α(1→6) mannopyranosyltransferase activity

Initial attempts to develop an *in vitro* assay using either purified recombinant expressed MptB, or *E. coli* membranes harbouring the protein, have thus far proved unsuccessful. Alternatively, we assessed the capacity of membrane preparations from *M. smegmatis*, *C. glutamicum* and its recombinant strains to catalyse α(1→6) mannopyranosyltransferase activity in a previously defined acceptor assay utilising the neoglycolipid acceptor α-**D**-Man*p*-(1→6)-α-**D**-Man*p*-*O*-C_8 and C_{50}-PP-[^{14}C]-M as a sugar donor (Brown *et al.*, 2001). The TLC-autoradiography of products from *in vitro* assays when assayed with wild type *C. glutamicum* resulted in the formation of

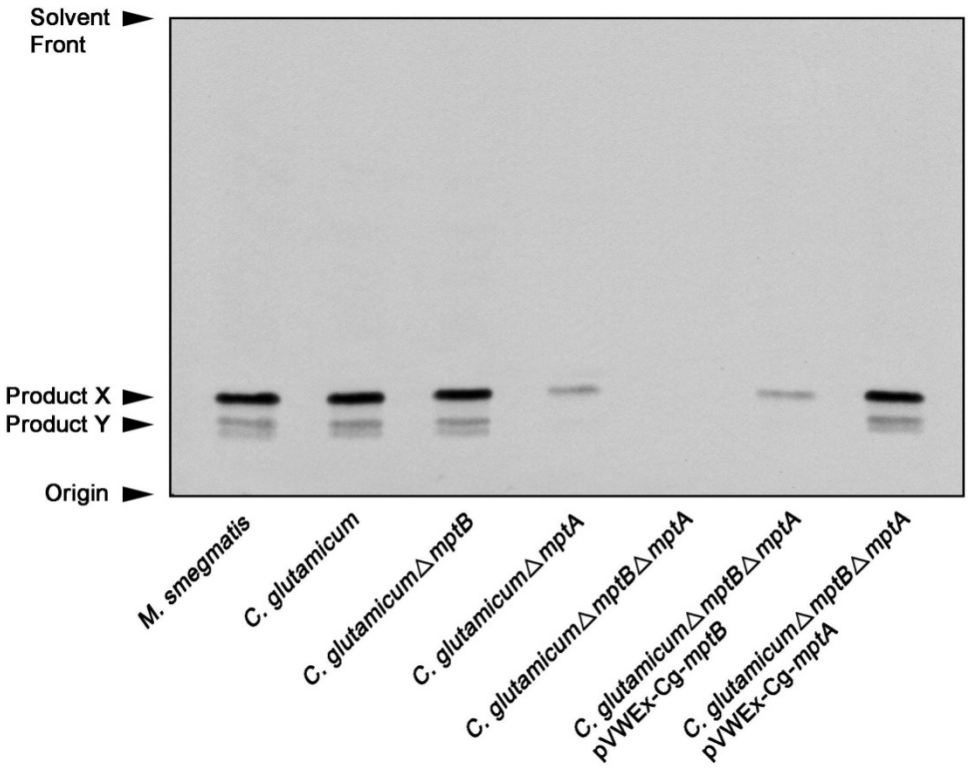

Fig. 2.15: Analysis of products obtained in a cell free assay for detecting α(1→6)-mannopyranosyltransferase activity. TLC analysis of products obtained in a cell free assay for detecting α(1→6)-mannopyranosyltransferase activity with membranes prepared from *M. smegmatis*, *C. glutamicum, C. glutamicum*Δ*mptB, C. glutamicum*Δ*mptA, C. glutamicum*Δ*mptB*Δ*mptA, C. glutamicum*Δ*mptB*Δ*mptA* pVWEx-Cg-*mptB,* and *C. glutamicum*Δ*mptB*Δ*mptA* pVWEx-Cg-*mptA*.

product X a trisaccharide, α-**D**-[^{14}C]-Man*p*-(1→6)-α-**D**-Man*p*-(1→6)-α-**D**-Manp-*O*-C_8, and product Y a tetrasaccharide, α-**D**-[^{14}C]-Man*p*-(1→6)-α-**D**-Man*p*-(1→6)-α-**D**-Man*p*-(1→6)-α-**D**-Manp-*O*-C_8 (Fig. 2.15). These products co-migrated on TLC-autoradiography with the corresponding products previously chemically characterised and prepared using mycobacterial membranes, and were cleaved by acetolysis demonstrating that they were α(1→6)-linked [^{14}C]-Man*p* products (Fig. 2.15) (Brown *et al.*, 1997; Brown *et al.*, 2001). The intensity of the major product X, a trisaccharide α-**D**-[^{14}C]-Man*p*-(1→6)-α-**D**-Man*p*-(1→6)-α-**D**-Manp-*O*-C_8, was consistently slightly reduced in the case of *C. glutamicum*Δ*mptB* (89217±4269 cpm) in

comparison to wild type *C. glutamicum* (92325±5017 cpm) (Fig. 2.15). This reduction in activity corresponded to the residual α(1→6) mannopyranosyltransferase activity observed in *C. glutamicum*Δ*mptA* (2053±604 cpm).

These results suggested the presence of two α(1→6) mannopyranosyltransferase activities utilising this neoglycolipid acceptor, catalysed by MptA and MptB, with the former more efficiently utilising the neoglycolipid acceptor as a substrate. Assays containing membrane preparations from *C. glutamicum*Δ*mptB*Δ*mptA* showed no product formation on TLC, indicating a complete abrogation of both α(1→6) mannopyranosyltransferase activities from *C. glutamicum* (Fig. 2.15). Analysis of the double mutant with pVWEx-Cg-*mptB* revealed a significant but weak band (2682±940 cpm) corresponding to product X on TLC analysis, however, when complemented with pVWEx-Cg-*mptA*, a similar phenotype to that of *C. glutamicum*Δ*mptB* could be observed (80614±4135 cpm for X) albeit at a slower transfer rate. The data confirmed that NCgl1505 is an α(1→6) mannopyranosyltransferase, however, the specific α(1→6) mannopyranosyltransferase, activity is much lower in comparison to MptA, under the assay conditions utilising the neoglycolipid acceptor.

To study the function of the mycobacterial MptB, we transformed the *C. glutamicum*Δ*mptB*Δ*mptA* double mutant with a plasmid containing either *M. tuberculosis Rv1459c* (pVWEx-Mt-*mptB*) or *M. smegmatis MSMEG_3120* (pVWEx-Ms-*mptB*). Membrane preparations of these strains restored *in vitro* α(1→6) mannopyranosyltransferase activity (Fig. 2.16) by formation of the trisaccharide product X (Mt-MptB, 3159±456 cpm; and Ms-MptB 2949±378 cpm) to a similar level to that of the isogenic strain with pVWEx-Cg-*mptB* showing

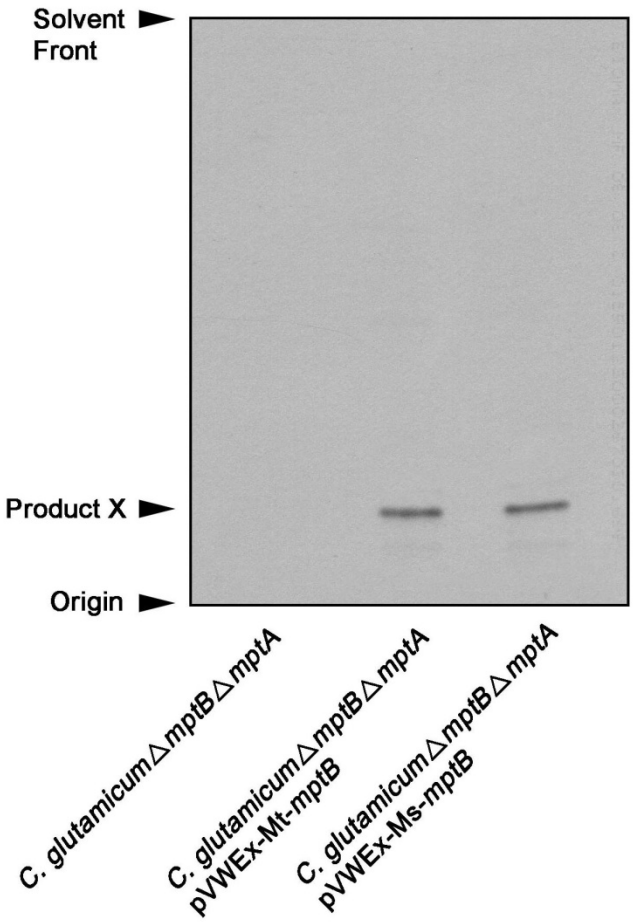

Fig. 2.16: Analysis of products obtained in a cell free assay for detecting α(1→6)-mannopyranosyltransferase from mycobacterial homologues. TLC analysis of products obtained in a cell free assay for detecting α(1→6)-mannopyranosyltransferase activity with membranes prepared from *C. glutamicum*Δ*mptB*Δ*mptA, C. glutamicum*Δ*mptB*Δ*mptA* pVWEx-Mt-*mptB*, and *C. glutamicum*Δ*mptB*Δ*mptA* pVWEx-Ms-*mptB*. Assays were performed using the synthetic α-D-Man*p*-(1→6)-α-D-Man*p*-*O*-C_8 neoglycolipid acceptor in a cell free assay as described previously.

that the *M. tuberculosis* and *M. smegmatis* gene(s) could restore activity in an *in vitro* cell free assay with the *C. glutamicum* double mutant.

2.3.9 Mutational analysis of the *M. smegmatis* MptB

Homologue of *Rv1459c* was deleted in *M. smegmatis* (*MSMEG_3120*) using specialised transduction, and polar lipid and lipoglycan content were analysed in the mutant strain (Mishra *et al.*, 2008a). The mutant strain *M. smegmatis*Δ*mptB* had a lipid content identical to the parental wild type strain *M. smegmatis* mc^2155 (Fig. 2.17 A) and no change was observed in the polar lipid profile similar to *C.glutamicum*Δ*mptB.* Surprisingly, lipoglycan profile of *M. smegmatis*Δ*mptB* was also identical to wild type strain *M. smegmatis* mc^2155 (Fig. 2.17 B), unlike *C.glutamicum*Δ*mptB* where the synthesis of Cg-LM and Cg-LAM was abolished. These results suggested that Ms-MptB, unlike its corynebacterial counterpart, is redundant and it is likely that MptA or another unidentified α(1→6) mannopyranosyltransferase compensated for the loss of its function in the *M. smegmatis*Δ*mptB*.

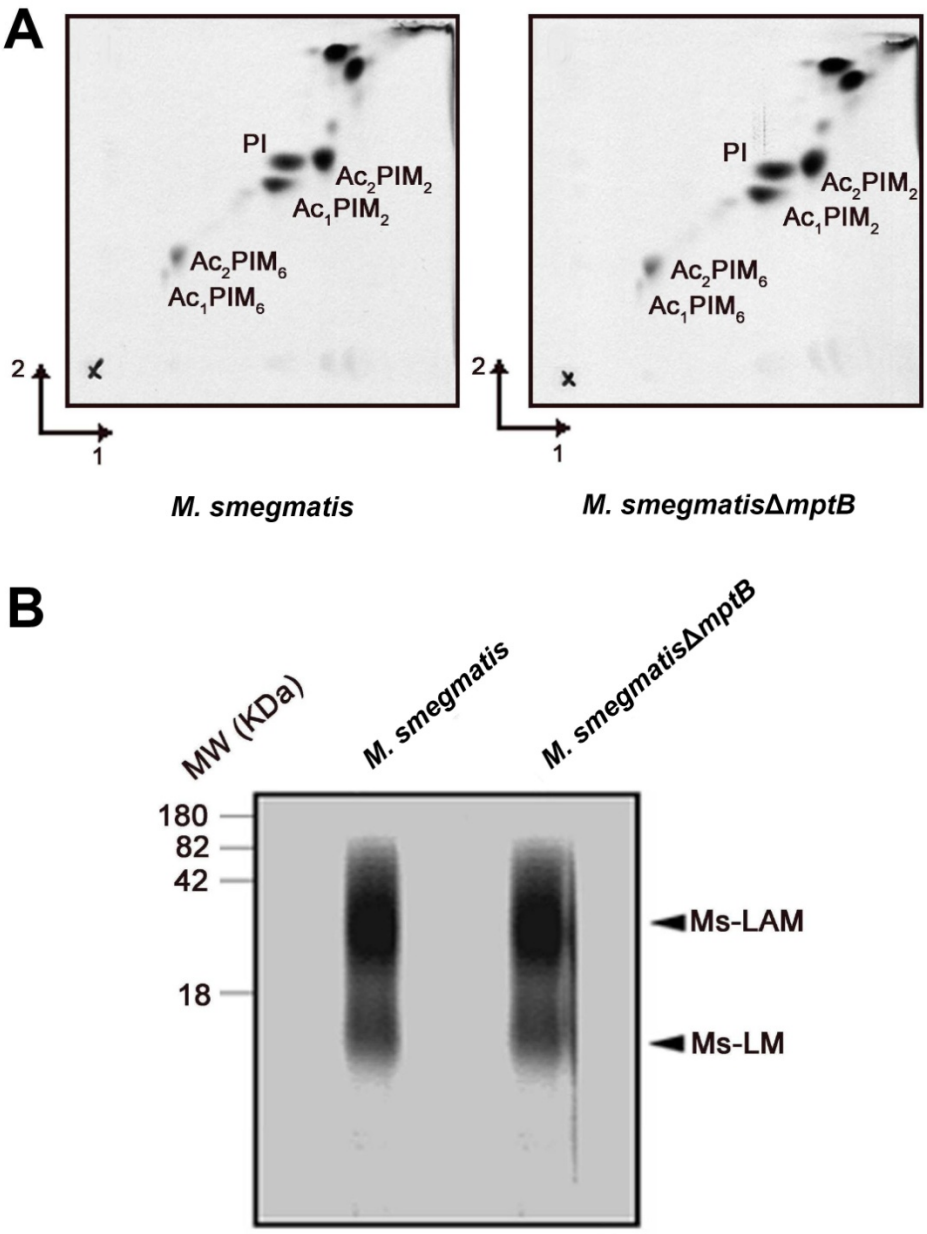

Fig. 2.17: Characterisation of a *M. smegmatis*Δ*mptB* mutant. (**A**) 2D-TLC analysis of the [^{14}C]-labelled (50,000 cpm) polar lipids fraction from *M. smegmatis* (WT) and *M. smegmatis*Δ*mptB*. (**B**) Lipoglycan analysis of wild type *M. smegmatis* and *M. smegmatis*Δ*mptB* using SDS-PAGE and visualised using a Pro-Q emerald glycoprotein stain. The four major bands represent glycoproteins of 180, 82, 42 and18 kDa respectively.

2.4 Discussion

Apart form belonging to the supragenic taxon *Corynebacterineae*, *M. tuberculosis* and *C. glutamicum* share common cell wall features and biosynthetic machinery. Many of the genes involved in *M. tuberculosis* cell wall biosynthesis have been shown essential for the growth, survival and pathogenicity of the bacillus (Belisle *et al.*, 1997; Bhatt *et al.*, 2005; Mills *et al.*, 2004; Movahedzadeh *et al.*, 2004). Due to the essentiality of such genes in mycobacteria (Sassetti *et al.*, 2003), we have previously demonstrated the inherent usefulness of *C. glutamicum* in the identification of genes involved in indispensable biochemical pathways (Alderwick *et al.*, 2005; Alderwick *et al.*, 2006b; Gande *et al.*, 2004; Seidel *et al.*, 2007a).

In this study, we sought to characterise the role of few putative glycosyltransferases (Rv2174 and Rv1459c) belonging to the GT-C superfamily of glycosyltransferases (Liu & Mushegian, 2003) by virtue of genomic deletion of their orthologues (NCgl2093 and NCgl1505, respectively) in *C. glutamicum*. Herein, we present MptA (Rv2174) and MptB (Rv1459c) as PPM dependent $\alpha(1\rightarrow6)$ mannopyranosyltransferases, involved in mannan backbone synthesis of LM biosynthesis, which then serves as a template for further $\alpha(1\rightarrow2)$ branching by other α-mannopyranosyltransferases, presumably Rv2181 (Kaur *et al.*, 2006).

Our initial investigation of the extractable glycolipids from *C. glutamicum*Δ*mptA* highlighted no apparent change in the profiles compared to those from *C. glutamicum*, which indicated that MptA was not involved in PIMs or $ManGlcAGroAc_2$ biosynthesis. This was not unsurprising, since PIMs and $ManGlcAGroAc_2$ biosynthesis is completely unique to enzymes belonging to the GT-A/B glycosyltransferase family, which utilise GDP-Man*p* as a substrate (Liu & Mushegian,

2003). However, examination of lipoglycans from *C. glutamicumΔmptA* afforded a complete loss of Cg-LM and Cg-LAM, and the appearance of a new smaller product (Cg-t-LM) as observed on a SDS-PAGE.

Interestingly, complementation of *C. glutamicumΔmptA* with a plasmid encoding Cg-*mptA* and Mt-*mptA*, restored the lipoglycan profiles to that of wild type *C. glutamicum*. Taken together with chemical analysis of Cg-t-LM, indicated that Cg-t-LM in *C. glutamicumΔmptA* occurs as a result of inactivation of a core α(1→6) mannopyranosyltransferase involved in assembly of the distal portion of LM, thereby rendering a substrate possessing reduced sites for branching sites. The enzymatic activity of NCgl2093 and Rv2174 were confirmed as bona fide α(1→6) mannopyranosyltransferase in a specific neoglycolipid acceptor assay (Brown *et al.*, 2001).

The apparent residual glycosyltransferase activity in membranes extracted from *C. glutamicumΔmptA* in the neoglycolipid assay could be attributed to another C_{50}-PPM dependent GT-C mannopyranosyltransferase. This situation is entirely plausible since our evidence suggests that there are at least two α(1→6) mannopyranosyltransferases, which utilise PPM as a substrate for glycosyl-transfer, inferring that both belong to the GT-C family of glycosyltransferases (Liu & Mushegian, 2003) and are therefore involved in LM-backbone synthesis. NCgl1505 in *C. glutamicum* showed the highest homology with NCgl2093 and Rv2174, and may be responsible for the residual activity in the neoglycolipid assay, thus potential α(1→6) mannopyranosyltransferase, MptB. On that basis *mptB* from *C. glutamicum* was deleted in order to study its role in LM/LAM synthesis.

Our initial *in vivo* and *in vitro* studies of PIMs and $Man_1GlcAGroAc_2$ biosynthesis in *C. glutamicum*Δ*mptB* highlighted no apparent change in lipid profiles, compared to those from wild type *C. glutamicum*. It is reasonable to conclude from the data that MptB is not involved in early PIM biosynthesis. However, due to absence of MptB, *C. glutamicum*Δ*mptB* is unable to synthesise Cg-LAM and Cg-LM *in vivo*, which is in contrast to our earlier results on MptA, where a truncated Cg-LM species was synthesised (Mishra *et al.*, 2007). Based on *in vitro in situ* chase experiments we found that Ac_1PIM_2 and $Man_1GlcAGroAc_2$ are acceptors for Cg-MptB and it is the first GT-C α-mannopyranosyltransferase committed to Cg-LM biosynthesis.

These crucial observations, together with the presence of Ac_1PIM_2 and $Man_1GlcAGroAc_2$, completely supports the hypothesis that Cg-MptB mannosylates Ac_1PIM_2 and $Man_1GlcAGroAc_2$. Our previous residual α(1→6) mannopyranosyltransferase activity in *C. glutamicum*Δ*mptA* membrane assay can now be attributed to the presence of MptB, since upon its deletion in *C. glutamicum*, a partial depletion in α(1→6) mannopyranosyltransferase activity is observed and a complete loss of activity is found upon deletion of both Cg-*mptA* and Cg-*mptB*. These data together with the *in vivo* analyses identifies MptB as a bona fide α(1→6) mannopyranosyltransferase. Interestingly, α(1→6) mannan extension is more complex in mycobacteria based on the evidence that Mt-MptB and Ms-MptB fail to complement the *C. glutamicum*Δ*mptB* mutant and suggests a slightly different substrate specificity of the MptB orthologues of *M. tuberculosis* and *M. smegmatis*.

Given the high degree of homology between the *C. glutamicum* and mycobacterial orthologues of MptB and the similar organisation of neighbouring genes in the two genera we expected deletion

of *M. smegmatis mptB* (*MSMEG_3120*) to have the same effect as that in *C. glutamicum*. However, surprisingly, the *M. smegmatis*Δ*mptB* still synthesised LM and LAM indicating that another, yet unidentified α-mannopyranosyltransferase could substitute for MptB in the *M. smegmatis*Δ*mptB*. It has been shown that a high degree of functional redundancy exists in key enzymes involved in mycobacterial cell wall assembly, for instance PimB and MgtA (Schaeffer *et al.*, 1999, Tatituri *et al.*, 2007, Lea-Smith *et al.,* 2008, Mishra *et al.,* 2008), PimC (Kremer *et al.*, 2002), and EmbA and EmbB (Berg *et al.*, 2007) in PIM/LM/LAM and AG biosynthesis, and the antigen 85 complex in mycolic acid biosynthesis (Puech *et al.*, 2002). In this particular case the *C. glutamicum* mutant study enabled the assignment of function to the GT-C glycosyltransferase Rv1459c and NCgl1505, which would have otherwise not been possible if similar studies would have concentrated solely on mycobacterial species.

3. BIOGENESIS OF TRIACYLATED PHOSPHATIDYL-*MYO*-INOSITOL DIMANNOSIDE (AC_1PIM_2) IN *M. TUBERCULOSIS*

3.1 Introduction

The current model of mycobacterial PIM biosynthesis is supported by biochemical and genetic studies, follows a linear pathway from PI → PIM_2 → PIM_4 → PIM_6 (Besra & Brennan, 1997). In view of identification of genes involved in PIMs and LM/LAM biosynthesis, Schaeffer and colleagues (1999) proposed Rv0557 as an α-**D**-mannose-α-(1→6)-phosphatidyl-*myo*-inositol-mannopyranosyltransferase that transfers mannose from GDP-Man*p* to Ac_1PIM_1 to form Ac_1PIM_2 (Schaeffer *et al.*, 1999). The study was based on a cell-free assay using GDP-[^{14}C]-Man*p*, Ac_1PIM_1, *M. smegmatis* membranes and/or partially purified recombinant Rv0557. On the basis of these *in vitro* studies, Rv0557 was assigned as Mt-PimB and in the synthesis of Ac_1PIM_2.

However, on disruption of *Rv0557* in *M. tuberculosis*, PIM biosynthesis remain unaffected (Torrells *et al.*, 2008) suggesting either gene duplication or that Rv0557 performed another function in *M. tuberculosis*. Recently, it was demonstrated NCgl0452 of *C. glutamicum* (homologue of Rv0557), originally termed PimB and now termed MgtA (Tatituri *et al.*, 2007b), primarily acts as an α-mannopyranosyltransferase to add mannose to a novel glycolipid, $GlcAGroAc_2$ and is involved in the synthesis of 1,2-di-*O*-C_{16}/$C_{18:1}$-(α-**D**-mannopyranosyl)-(1→4)-(α-**D**-glucopyranosyluronicacid)-(1→3)-glycerol($ManGlcAGroAc_2$) (Tatituri *et al.*, 2007b). This study also highlighted the presence of a lipomannan, now termed Cg-LM-B, based on a $GlcAGroAc_2$ anchor rather than a PI-anchor. These studies suggested the involvement of another α-mannopyranosyltransferase in the synthesis of Ac_1PIM_2.

In view of the identification for another enzyme, which might be responsible for the synthesis of Ac_1PIM_2 and PI-based LM and LAM in *C. glutamicum* and *M. tuberculosis*, we searched for

unknown glycosyltransferases using nucleotide-activated sugars. Amongst others we identified *NCgl2106* in the genome of *C. glutamicum* with orthologues present in all *Corynebacterineae* including the genus *Mycobacterium*. In this study, we have examined an *NCgl2106* null mutant of *C. glutamicum* and established that NCgl2106 is a phosphatidyl-*myo*-inositol monomannoside mannopyranosyltransferase exclusively involved in the synthesis of Ac_1PIM_2 from Ac_1PIM_1. Furthermore, with the use of a *C. glutamicumΔpimB'ΔmgtA* double deletion mutant and expression of Rv0557 and Rv2188c coupled with *in vitro* enzyme assays we have clearly assigned the enzyme functions of Rv0557 and Rv2188c in terms of $Man_1GlcAGroAc_2$ and Ac_1PIM_2 synthesis, respectively.

3.2 Materials and methods

3.2.1 Strains and culture conditions

The wild type *C. glutamicum* was grown on either the complex medium BHI or CGXII at 30 °C. The *E. coli* strain DH5*αmcr* was grown on LB at 37 °C. Kanamycin and ampicillin were used at a concentration of 25 or 50 µg/ml, wherever appropriate. Samples for lipid analysis were prepared by harvesting the cells at an optical density 600 nm of 10-15 followed by a saline wash and freeze-drying.

3.2.2 Construction of plasmids and strains

All mutant strains in *C. glutamicum* and their complimented strains were constructed at Institute for Biotechnology Research Centre, Juelich, Germany. To construct the deletion vector pK19mobsacBΔ2106 crossover PCR was applied with primer pairs Nout2106/Nin2106

(Nout2106, AATCGGAGATCCGAGACCGGG; Nin2106, CCCATCCACTAAACTTAAACATTTTCGGGATGCAGACACAAAGA) (all primers in 5'-3'direction) and Cout2106/Cin2106 (Cout2106, ACCCAGTTGTCAGCGCCTTGAG; Cin2106, TGTTTAAGTTTAGTGGATGGGCGGTTGACCAATATTTTGCAGAG) with *C. glutamicum* genomic DNA as template. Both amplified products were used in a second PCR with primer pairs Nout2106/Cout2106 to generate a 1025bp fragment consisting of sequences adjacent to NCgl2106, which was made blunt, phosphorylated and ligated with SmaI-cleaved pK19mobsacB. The chromosomal deletion of *NCgl2106* was performed as described previously using two rounds of positive selection (Schafer *et al.*, 1994), and its successful deletion verified by use of two different primer pairs. Plasmids were introduced into *C. glutamicum* by electroporation with selection to kanamycin resistance (25 µg/ml) on BHI. To enable the expression of NCgl2106 in the deletion mutant, *NCgl2106* was amplified using the primer pair CGCGGATCCAAGGAG ATATAGATATGTCTGCATCCCGAAAAACTCTC and CGCGAATTCTCATCGTGGTTCA CTCTGC. The purified PCR fragment was digested with BamHI-EcoRI and ligated with pEKEx2 (Eikmanns *et al.*, 1991). All cloned fragments were verified by sequencing.

To enable expression of Rv2188c for complementation studies, primers Mt-*Rv2188c*-fw and Mt-*Rv2188c*-rev were used together with *M. tuberculosis* H37Rv DNA as a template. The resulting fragment was purified and digested with BamHI and EcoRI and ligated with similarly treated pEKEx2 (Eikmanns *et al.*, 1991) to result in pEKEx2-Mt-*Rv2188c*. This vector, as well as pEKEx2-Cg-*pimB´*, pEKEx3-Mt-*mgtA* and pEKEx3-Cg-*mgtA* were introduced into *C. glutamicum* via electroporation using 25 µg/ml kanamycin for primary selection of pEKE2-based vectors and 100 µg/ml spectinomycin for primary selection of pEKEx3 vectors.

To delete *NCgl2106* in *C. glutamicum*Δ*mgtA*, plasmid pK19mobsacBΔ*pimB´* was introduced by electroporation. One kanamycin resistant clone with vector integrated was selected for sucrose resistance as described (Schafer *et al.*, 1994), and clones with loss of vector were assayed *via* PCR to assess whether they had lost *mgtA* during the second recombination event or whether the wild type situation was restored. Deletion clones were finally verified *via* PCR analysis with a different primer set than that used for construction, and all plasmids constructed were finally verified by sequencing.

3.2.3 Extraction and biochemical analysis of glycolipids and lipoglycans

Methodology covering extraction and biochemical analysis of lipids and lipoglycans with 2D-TLC, SDS-PAGE, GC-MS, MALDI-TOF-MS and NMR are covered separately as part of General Materials and Methods in Chapter 5.

3.2.4 Permethylation of Cg-LM-B prior to MALDI-TOF analysis

Permethylation was performed using the sodium hydroxide procedure as described previously (Dell *et al.*, 1993). MALDI-TOF and TOF/TOF MS data on permethylated samples were acquired in the positive ion mode $(M+Na)^+$ using a 4800 (Applied Biosystems) mass spectrometer in the reflector mode with delayed extraction. The collision energy was set to 1 kV, and argon was used as collision gas for MS/MS data collection. Samples were dissolved in methanol, and 1 μl was mixed at a 1:1 ratio (v/v) with 2,5-dihydrobenzoic acid (20 mg/ml in 70 % methanol in water) as matrix.

3.2.5 GC-MS linkage analysis of Cg-LM-B

Partially methylated alditol acetates were prepared from permethylated samples for gas chromatography–mass spectrometry (GC–MS) linkage analysis as described in Chapter 5 (Dell *et al.*, 1993) and were analysed using a PerkinElmer Clarus 500 instrument fitted with a RTX-5 column (30 m x 0.25-mm internal diameter, Restek Corp.) The sample was dissolved in hexane and injected onto the column at 65 °C. The column was maintained at this temperature for 1 min and then heated to 290 °C at a rate of 8 °C per min.

3.2.6 *In vitro* analysis of glycolipid biosynthesis

Reaction mixtures containing 0.25 µCi of GDP-[^{14}C]Man*p*, 1 mM ATP, and membrane protein (1 mg) from wild type *C. glutamicum*, mutants and/or complemented strains in a final volume of 50 µl were incubated at 37°C for 30 min as described previously (Gibson *et al.*, 2003). The reactions were terminated by the addition of $CHCl_3/CH_3OH/H_2O$ (4 ml, 10:10:3, v/v/v) followed by the addition of 1.75 ml of $CHCl_3$ (1.75 ml) and H_2O (0.75 ml). The reaction mixture was vortexed, centrifuged and the upper aqueous phase removed. The organic phase was washed three times with $CHCl_3/CH_3OH/H_2O$ (2 ml, 3:47:48, v/v/v), and the final organic extract dried under a stream of nitrogen. The resulting products were resuspended in 200 µl of $CHCl_3/CH_3OH$ (2:1, v/v), and aliquots (20 µl) quantified by liquid scintillation counting using 5 ml of EcoScintA. Equivalent aliquots (20 µl) of labeled material were subjected to TLC using silica gel plates and developed in $CHCl_3/CH_3OH/H_2O/NH_4OH$ (65:25:3.6:0.5, v/v/v/v). The products were visualised by phosphorimaging.

3.2.7 Expression, purification and *in vitro* characterisation of Cg-PimB'

Cg-PimB' was over-expressed in *E. coli* BL21 (DE3). *E. coli*-pET16b-Cg-*pimB'* was grown at 30ºC in LB supplemented with ampicillin (100 µg/mL). The expression of Cg-*pimB'* was induced by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranose (IPTG) at an OD of 0.4-0.6 for 4 hrs. Cells were harvested by centrifugation, washed with saline and stored at -20ºC until use. Cells were thawed on ice and resuspended in 30 mL of buffer B (50 mM, NaH_2PO_4 (pH 8.0), 300 mM NaCl and 10 mM imidazole), supplemented with an EDTA-free protease cocktail tablet (Roche). The cell suspension was sonicated (12 µm amplitude, 20 s on, 40 s off for 10 cycles, at 4 ºC) and the lysate centrifuged at 27000 x g for 30 minutes at 4°C. The supernatant was passed through a pre-equilibrated (buffer B) Ni^{2+}-charged His-Trap column (1 mL) (GE Healthcare). The column was subsequently washed with 50 mL of buffer B and the protein eluted with a 50-300 mM gradient of imidazole with continuous monitoring using a UV spectrophotometer and further assessed using 12 % SDS-PAGE with coomassie blue staining. Fractions containing protein were dialysed against 20 mM Tris-HCl (pH 7.5), 10 mM NaCl, 10% glycerol and 1 mM DTT. After dialysis, proteins were concentrated to 3 mg/mL using a 10 kDa cut-off centriprep device (Millipore).

Purified Cg-PimB' was incubated with 0.25 µCi of GDP-[^{14}C]-Man*p*, 1 mM ATP, and either extracted or purified polar lipids or Ac_1PIM_1 from *C. glutamicum*Δ*pimB'*Δ*mgtA* in a final volume of 50 µl at 37°C for 30 min. The reactions were terminated and products extracted as described above The resulting Ac_1PI-[^{14}C]-M_2 and [^{14}C]-$Man_1GlcAGroAc_2$ products were subjected to TLC/autoradiography using silica gel plates developed in $CHCl_3$:CH_3OH:H_2O:NH_4OH (65:25:3.6:0.5, v/v/v/v) and the products visualised and quantified by phosphorimaging.

3.3 Results

3.3.1 Construction and growth of *C. glutamicumΔpimB′*

We found that the gene product of NCgl0452 of *C. glutamicum*, respectively its orthologue Rv0557 in *M. tuberculosis*, originally termed PimB and now termed MgtA (Tatituri *et al.*, 2007b), primarily acts as an α-mannopyranosyltransferase to add Man*p* to $GlcAGroAc_2$ (Tatituri *et al.*, 2007b) and is involved in the synthesis of a novel lipomannan, now termed Cg-LM-B, based on a $GlcAGroAc_2$ anchor rather than a PI-anchor. Further Characterisation of Cg-LM-B was hampered due to co-migration and co-elution following size exclusion chromatography with the PI-based Cg-LM-A (Tatituri *et al.*, 2007b). To investigate the structure and function of Cg-LM-B further we adopted a strategy based on the existence of two pathways to lipoglycan synthesis and to identify Cg-*pimB′* involved in Ac_1PIM_2 synthesis whereby disruption would block Cg-LM-A and Cg-LAM synthesis while Cg-LM-B would be unaffected. We searched for unknown glycosyltransferases using nucleotide-activated sugars. Amongst others we identified NCgl2106 in the genome of *C. glutamicum* with orthologues present in all *Corynebacterineae* and deleted it from *C. glutamicum* (Fig. 3.1).

The colony morphology of *C. glutamicumΔpimB′* was somewhat different in comparison to wild type. The colonies were sticky and formed threads upon grasping with an inoculating loop similar to a lysed colony, indicating a cell wall related function of the gene product. *C. glutamicumΔpimB′* was transformed with pEKEx2-Cg-*pimB′* and pEKEx2-Mt-*Rv2188c* and the resultant complemented strain exhibited a growth rate similar to that of the wild type in CGXII medium.

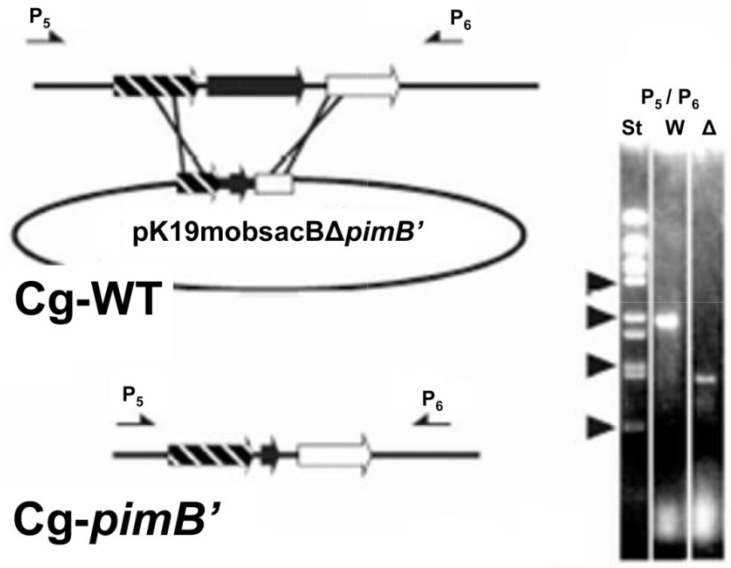

Fig. 3.1: Inframe deletion mutant of *pimB'* in *C. glutamicum*. Strategy to delete *pimB´* by use of vector pK19mobsacBΔ*pimB´* by two homologous recombination events with the wild type chromosome (Cg-WT). The deletion is demonstrated on the right *via* PCR using primer pairs P5/P6 showing the expected 1088 bp fragment for the deletion mutant in the Lane marked "Δ", and that of 2159 bp for the wild type marked "W". The Lane marked "St" is the standard consisting of BstEII-fragments of λ-DNA, with arrowheads positioned at 0.70, 1.37, 2.32, and 3.68 kb.

3.3.2 Chemical analysis of polar lipids and lipoglycans from *C. glutamicumΔpimB´*

Lyophilised cells were extracted using petroleum-ether and methanolic saline to recover apolar lipids. Further processing of the methanolic extract afforded the polar lipid fraction, which was examined by 2D-TLC. The extract from wild type *C. glutamicum* showed the presence of $ManGlcAGroAc_2$, $GlcAGroAc_2$ and Ac_1PIM_2, by α-naphthol/sulfuric acid staining (Fig. 3.2 A). Surprisingly, while the synthesis of $ManGlcAGroAc_2$ and $GlcAGroAc_2$ remained unaffected the component corresponding to Ac_1PIM_2 identified by negative ion mode MALDI-TOF-MS at *m/z* 1398 $[M-H]^-$ with the fatty acyl groups C_{16} and $C_{18:1}$ (Tatituri *et al.*, 2007b) was absent in *C. glutamicumΔpimB´* with the appearance of a new product, which was sugar and phosphate

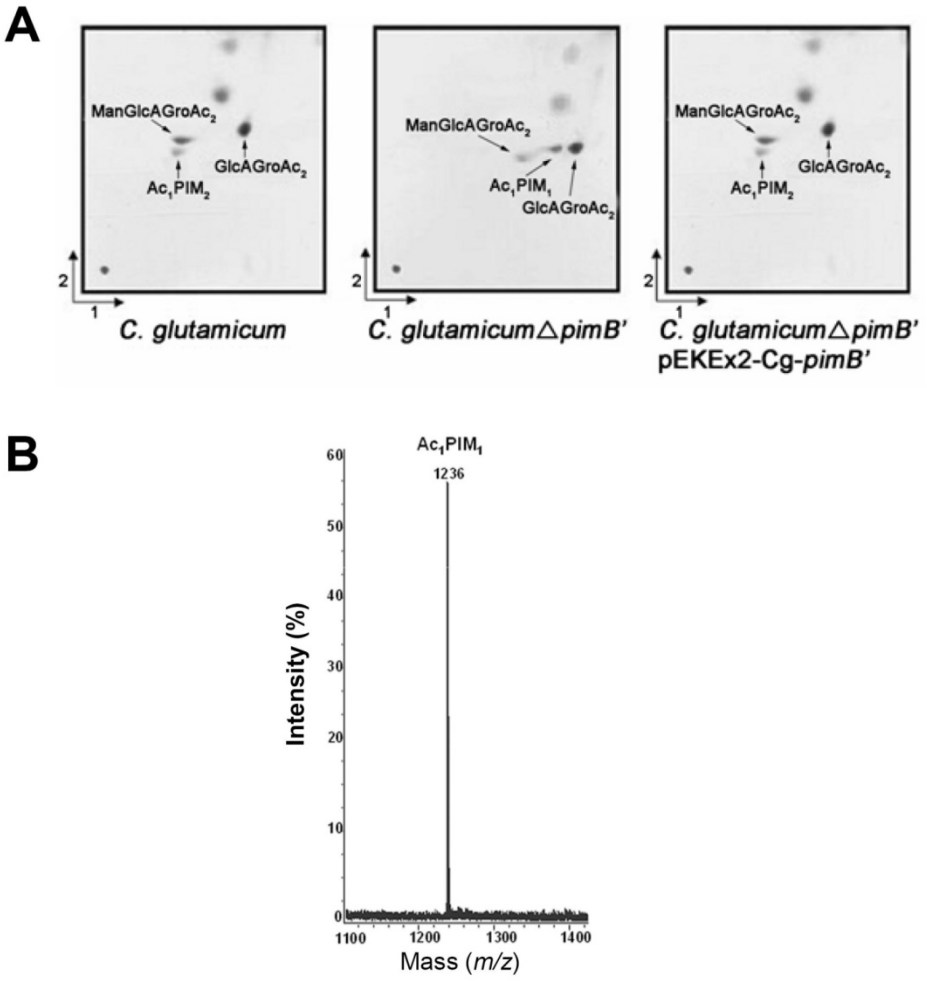

Fig. 3.2: Polar lipid profile of *C. glutamicum*, *C. glutamicumΔpimB'* and *C. glutamicumΔpimB'* pEKEx2-Cg-*pimB'*. (**A**) TLC-analysis of PIM biosynthesis in strains; glycolipids were visualised by spraying plates with α-naphthol/ sulfuric acid, followed by gentle charring of TLC plates. (**B**) Negative ion mode MALDI-TOF-MS of purified novel lipid product from *C. glutamicumΔpimB'*. Purified novel lipid was analysed on negative ion mode MALDI-TOF-MS as described by Tatituri *et al.* (2007b).

positive by specific staining (Fig. 3.2 A). This predominant lipid spot was purified and corresponded to Ac_1PIM_1, which was confirmed by negative ion mode MALDI-TOF-MS analyses due to the characteristic ion at *m*/*z* 1236 $(M-H)^-$ (Fig. 3.2 B).

Complementation of *C. glutamicumΔpimB′* by pEKEx2-Cg-*pimB′* restored the wild type phenotype (Fig. 3.2 A). Altogether, the data indicated that Ac_1PIM_1 in *C. glutamicumΔpimB′*

occurs possibly as a result of inactivation of a phosphatidyl-*myo*-inositol mannopyranosyltransferase, presumably which transfers a Man*p* residue from GDP-Man*p* to the 6-position of Ac_1PIM_1. In addition, this data also shed further light on the acylation step in PIM biosynthesis in *Corynebacterineae*. The accumulation of Ac_1PIM_1 in *C. glutamicumΔpimB′* showed that the acylation step (Kordulakova *et al.*, 2003) precedes the second mannosylation step in PIM biosynthesis, and results in the formation of Ac_1PIM_2 (Schaeffer *et al.*, 1999).

Lipoglycans were extracted by refluxing delipidated cells in ethanol, followed by hot-phenol extraction, protease digestion and dialysis to remove impurities. The extracted lipoglycans were examined initially on 15 % SDS-PAGE (Fig. 3.3). Extracts from wild type *C. glutamicum* showed the presence of Cg-LAM, Cg-LM-A and Cg-LM-B (Tatituri *et al.*, 2007b). As expected the lipoglycan extract from *C. glutamicumΔpimB′* showed the absence of Cg-LAM and Cg-LM-A, and the presence of a single species (Cg-LM-B) by chemical characterisation as described below. Complementation of *C. glutamicumΔpimB′* by transformation with plasmid pEKEx2-Cg-*pimB′* restored the wild type phenotype of Cg-LAM, Cg-LM-A and Cg-LM-B (Fig. 3.3). As predicated, the inactivation of Cg-*pimB′* abolished the synthesis of Cg-LAM and Cg-LM-A, and provided a strain that allowed the purification of Cg-LM-B for further chemical and functional characterisation. Cg-LM-B from *C. glutamicumΔpimB′* and Cg-LM-A from *C. glutamicumΔmgtA* (Tatituri *et al.*, 2007b) were purified by HIC and size exclusion chromatography followed by their chemical analysis using mass spectrometry.

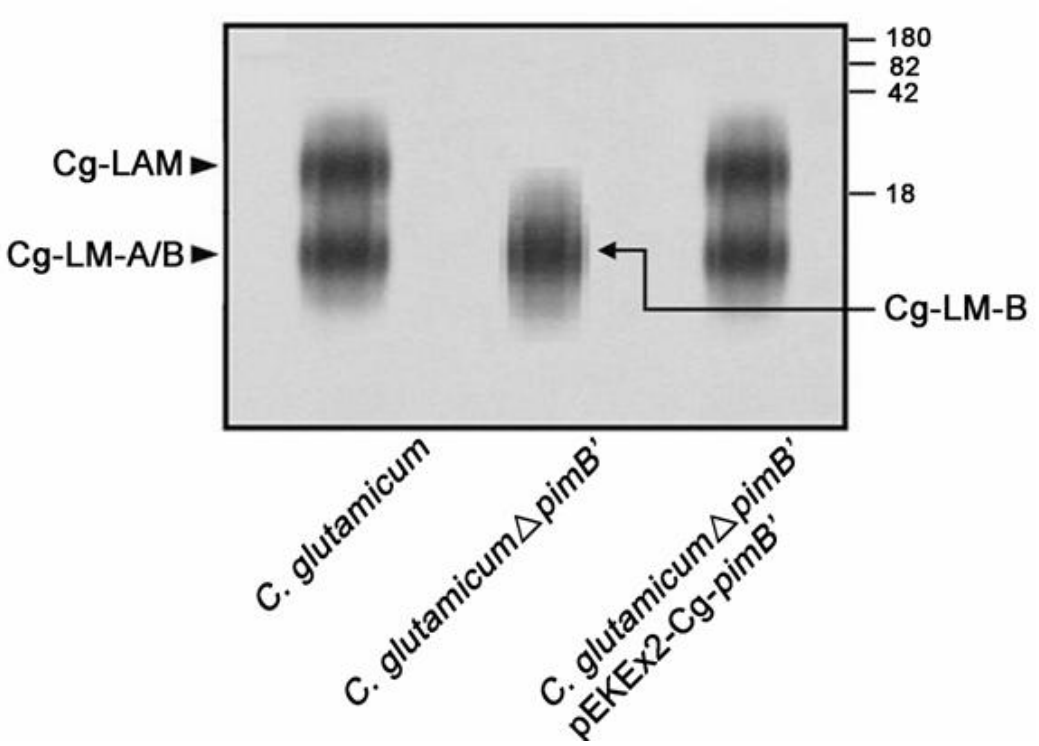

Fig. 3.3: Lipoglycan profile of *C. glutamicum, C. glutamicumΔpimB'* and *C. glutamicumΔpimB'* pEKEx2-Cg-*pimB'*. Lipoglycan profiles of *C. glutamicum* strains were analysed using SDS-PAGE and visualised using a Pro-Q emerald glycoprotein stain. The major bands represented by Cg-LAM, Cg-LM-A, and Cg-LM-B are indicated. The four major standard bands indicated on the side of the gel represent glycoproteins of 180, 82, 42 and18 kDa, respectively.

3.3.3 Analysis of Cg-LM-B by mass spectrometry

The Cg-LM-B was permethylated prior to detailed mass spectrometric analysis. During the permethylation step the acyl groups from the lipoglycan are liberated and the glycerol hydroxyl groups are methylated. The MALDI-TOF MS profile of the permethylated sample in the positive mode showed no signals for $ManGlcAGroAc_2$ (*m/z* 579) but, interestingly, a series of peaks $(M+Na)^+$ corresponding to the addition of 4-22 hexose residues to the $GlcAGroAc_2$ core structure (Fig. 3.4 A) were observed. In the lower mass region of the spectrum, signals consistent with the

4457.2 and 4865.6 with $Hex_{12}GlcAGroAc_2$ (*m/z* 2824.6) being most abundant (Fig. 3.4 A). The low abundance of signals attributable to components carrying an odd number (Hex11, 13, 15, 17, 19 and 21) of hexoses (*m/z* 2620.5, 3028.7, 3436.9, 3849.0, 4252.2 and 4660.4), as compared to even numbered, suggests that the hexose polymer is branched rather than linear. In addition, there are also signals present in the MS spectrum attributable to hexose oligomers. This may result from partial degradation of the sample during preparation but could also be due to contamination.

Each of the major signals in the spectrum was subjected to collision induced decomposition MS/MS (CID-MS/MS) analysis to establish their structures. For example, MS/MS spectra of the signal at *m/z* 2416, which has the composition $Hex_{10}GlcAGroAc_2$, showed peaks at *m/z* 2198, 1994, 1585.6, 1381.5, 1177.5, 769.3 and 361 (Fig. 3.4 B) which are due to the loss of 1, 2, 4, 5, 6, 8 and 10 hexose units from the molecular ion. The inset to Fig. 3.4 B shows the likely sequence that is consistent with this set of fragment ions. Furthermore, in order to confirm the nature of the hexose units and linkages, GC/MS analysis of partially methylated alditol acetates was carried out. This revealed the presence of t-Man*p*, 2-Man*p*, and 2,6-Man*p* in Cg-LM-B. The presence of t-Man*p* and 2,6-Man*p* convincingly establishes that the backbone of the oligomannans is heavily branched. The relative abundances of t-Man*p* (1.0), 6-Man*p* (0.5) and 2,6-Man*p* (0.98) are consistent with single mannose residues being appended at *O*-2 to the 6-linked mannosyl backbone. The glycosyl composition and nature of chemical linkages in Cg-LM-B are exactly similar to Cg-LM-A except the presence of a PI unit towards the reducing end of the Cg-LM-A instead of $GlcAGroAc_2$ of Cg-LM-B.

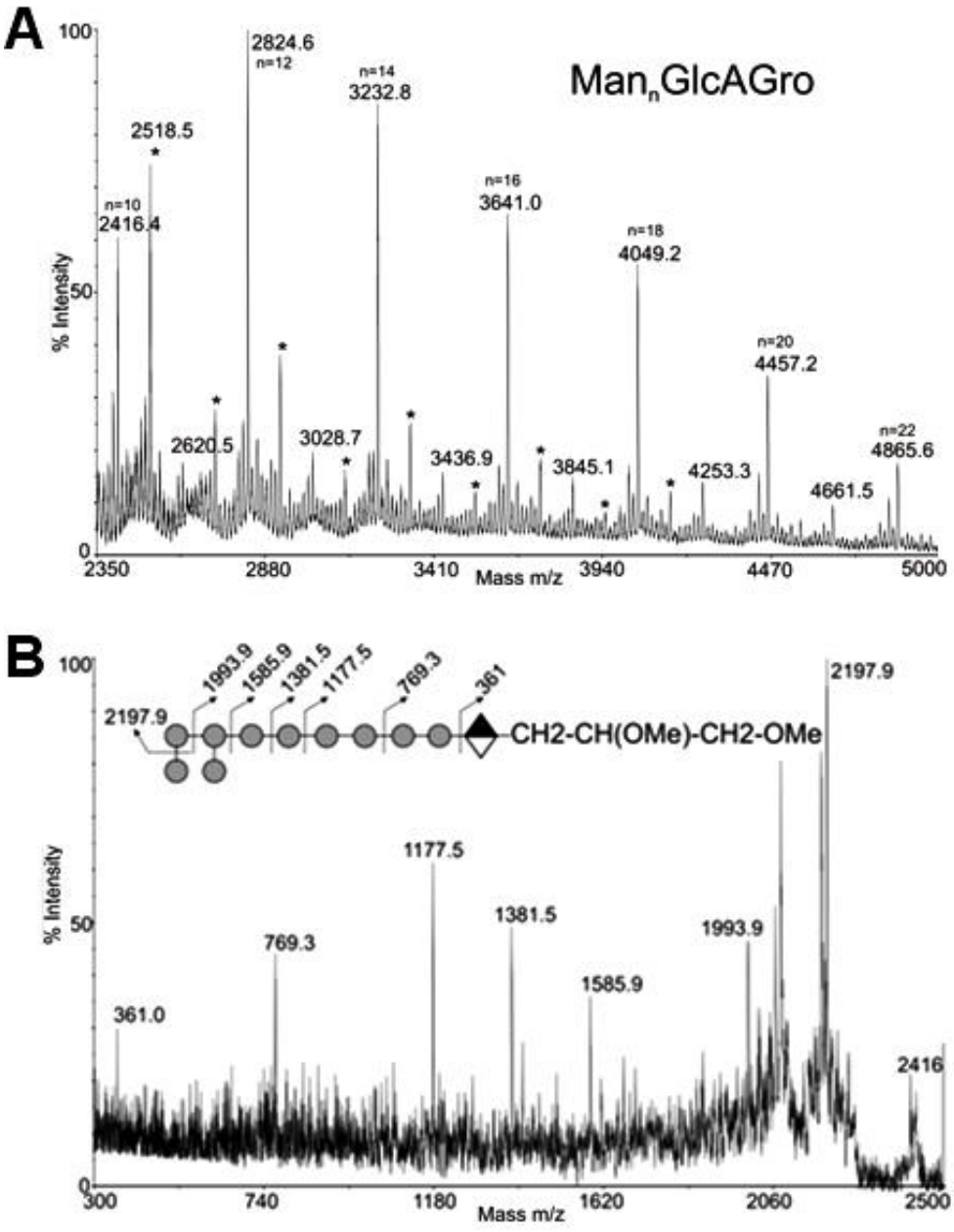

Fig. 3.4: MALDI-TOF/TOF analysis of permethylated Cg-LM-B [M + Na+] $ManGlcAGroAc_2$. (**A**) MS spectrum of derivatised Cg-LM-B. Unassigned peaks (*) are due to permethylation artifacts. (**B**) CID-MS/MS analysis of *m/z* 2416 $(M+Na)^+$ $Man_{10}GlcAGroAc_2$. A possible structure and CID fragmentation pattern of peak *m/z* 2416.4 is depicted in the cartoon representation, inset. Circle-mannose; diamond-glucuronic acid.

3.3.4 Construction and growth of *C. glutamicumΔpimB´ΔmgtA, C. glutamicumΔpimB´ΔmgtA* -pEKEx2-*Rv2188c* and *C. glutamicumΔpimB´ΔmgtA*-pEKEx3-*Rv0557*

Rv2188c and Rv0557 both belong to the GT-B family of glycosyltransferases (Liu and Mushegian), and in the CAZy classification system they are part of the subgroup GT4. Using MgtA as a query sequence in a blast comparison the next paralog among the 6 members of *M. tuberculosis* within the GT4 family is Rv2188c revealing the structural similarity of both proteins. Both proteins possess orthologs in *C. glutamicum* and the above genetic and biochemical studies confirmed that the orthologous proteins have identical functions (Tatituri *et al.*, 2007b). When either *pimB´*or *mgtA* in *C. glutamicum* ATCC13032 was deleted, no reliable growth rate defect was observed. We therefore transformed *C. glutamicumΔpimB´* with the deletion vector pK19mobsacBΔ*mgtA* (Tatituri *et al.*, 2007b) which resulted in Kan^r, and after two rounds of positive selection small colonies on BHI plates were obtained.

The *mgtA* locus was analysed *via* PCR and one of the 18 positive clones was identified as *C. glutamicumΔpimB´ΔmgtA*.The mutant has a reduced growth rate of 0.32 h^{-1} as compared to that of the wild type with a growth rate of 0.43 h^{-1}. As expected, plasmid encoded Cg-*mgtA* restored to some extent growth, as did Cg-*pimB´*, with the latter resulting in better growth restoration, and this might indicate that Cg-PimB´ is the more relevant to sustain the growth characteristics of the wild type. This is also in agreement with the fact that Cg-PimB´-derived LM-A and LAM is more abundant in *C. glutamicum* than the Cg-MgtA-derived LM-B (Tatituri *et al.*, 2007b). Also Mt-Rv0557 restored growth, whereas Mt-Rv2188c for unknown reasons was unable to reverse the growth effect, but is apparently expressed using 0.01 mM IPTG during growth.

3.3.5 *In vivo* glycolipid analysis of *C. glutamicumΔpimB´ΔmgtA*, *C.glutamicumΔpimB´ΔmgtA* pEKEx2-*Rv2188c* and *C. glutamicumΔpimB´ΔmgtA*-pEKEx3-*Rv0557*

Polar lipids containing PIMs and other glycolipids were extracted from *C. glutamicum*, *C. glutamicumΔpimB´*, *C. glutamicumΔmgtA, C. glutamicumΔpimB´ΔmgtA*, *C. glutamicumΔpimB´ΔmgtA*-pEKEx2-*Rv2188c* and *C. glutamicumΔpimB´ΔmgtA*-pEKEx3-*Rv0557* strains using an established chloroform-methanolic saline procedure (Dobson *et al.*, 1985). Extracted lipids were examined by 2D-TLC and MALDI-TOF-MS. The lipid extracts from *C. glutamicum* possessed a typical profile of $ManGlcAGroAc_2$, $GlcAGroAc_2$, Ac_1PIM_2, trehalose monocorynomycolate (TMCM) and glucose monocorynomycolate (GMCM) by α-naphthol/sulfuric acid staining (Fig. 3.5). As shown previously the corresponding Ac_1PIM_2 (negative ion mode MALDI-TOF-MS *m/z* 1398 $[M-H]^-$, fatty acyl groups C_{16} and $C_{18:1}$) and $ManGlcAGroAc_2$ (Gl-X) (positive ion mode MALDI-TOF-MS *m/z* 977 $[M-H+2Na]^+$, fatty acyl groups C_{16} and $C_{18:1}$) (Tatituri *et al.*, 2007b) were confirmed by mass spectrometry (Fig. 3.6).

In addition, as reported above Ac_1PIM_2 and $ManGlcAGroAc_2$ were completely absent in *C. glutamicumΔpimB'* and *C. glutamicumΔmgtA*, respectively. Lipid extracts from the *C. glutamicumΔpimB'ΔmgtA* double knock out were found to be devoid of both Ac_1PIM_2 and $ManGlcAGroAc_2$ (Fig. 3.5 and 3.6) with accumulation of Ac_1PIM_1 (negative ion mode MALDI-TOF-MS *m/z* 1236 $[M-H]^-$. Therefore, *C. glutamicumΔpimB'ΔmgtA* was utilised to study the role of the orthologous Rv2188c and Rv0557 proteins. Plasmid-borne over-expression of Rv2188c in *C. glutamicumΔpimB'ΔmgtA* restored the synthesis of Ac_1PIM_2 (Fig. 3.5, Fig. 3.6A), whilst $ManGlcAGroAc_2$ was still absent, which suggests that Rv2188c is solely involved in the

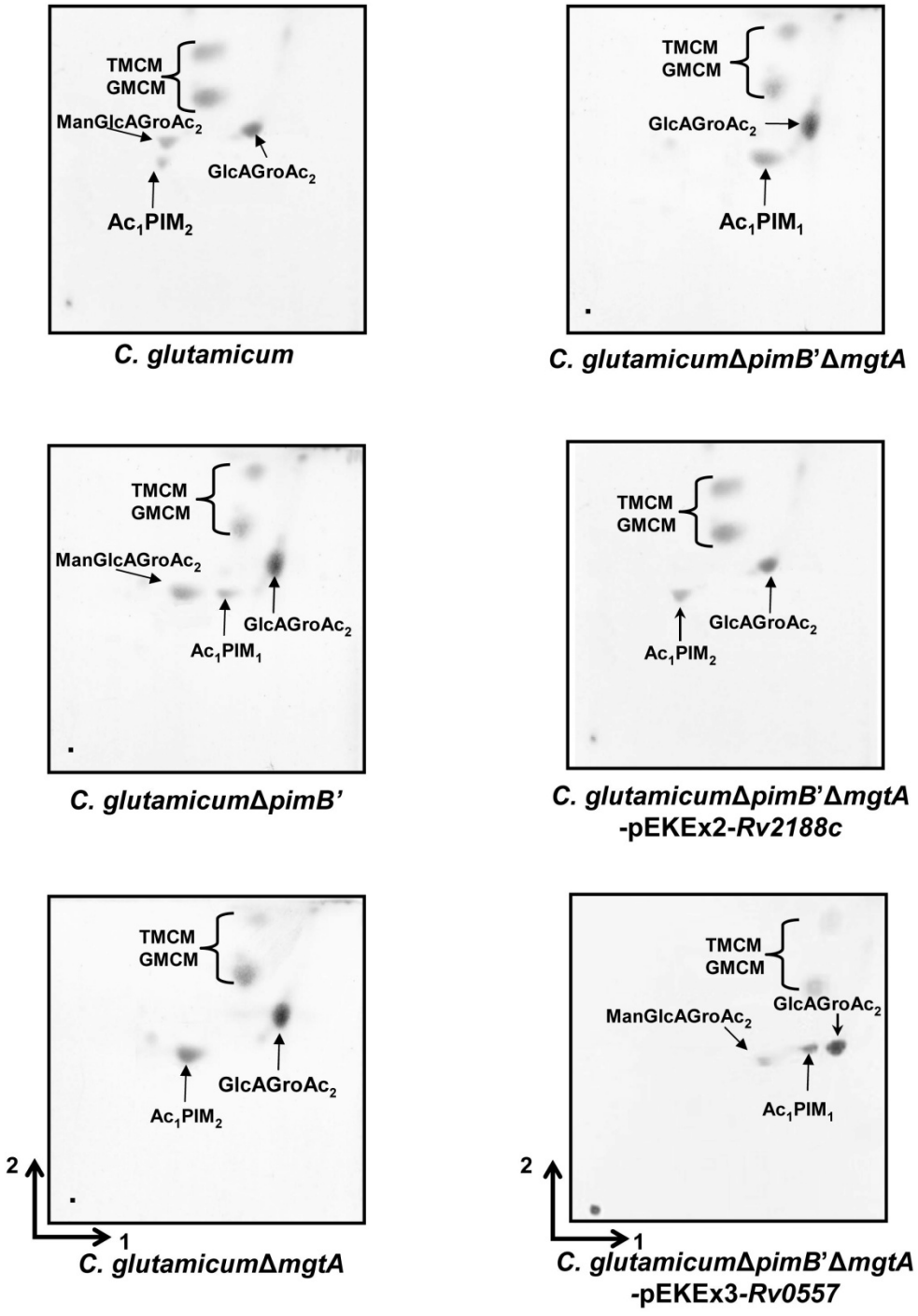

Fig. 3.5: Glycolipid profiles of different strains of *C. glutamicum*. The polar lipid extracts were examined by 2D-TLC on aluminum-backed plates of silica gel 60 F_{254}, using $CHCl_3/CH_3OH/H_2O$ (60:30:6, v/v/v) in the first direction and $CHCl_3/CH_3COOH/CH_3OH/H_2O$ (40:25:3:6, v/v/v/v) in the second direction. Glycolipids were visualised by spraying plates with α-naphthol/sulfuric acid, followed by gentle charring of the plates.

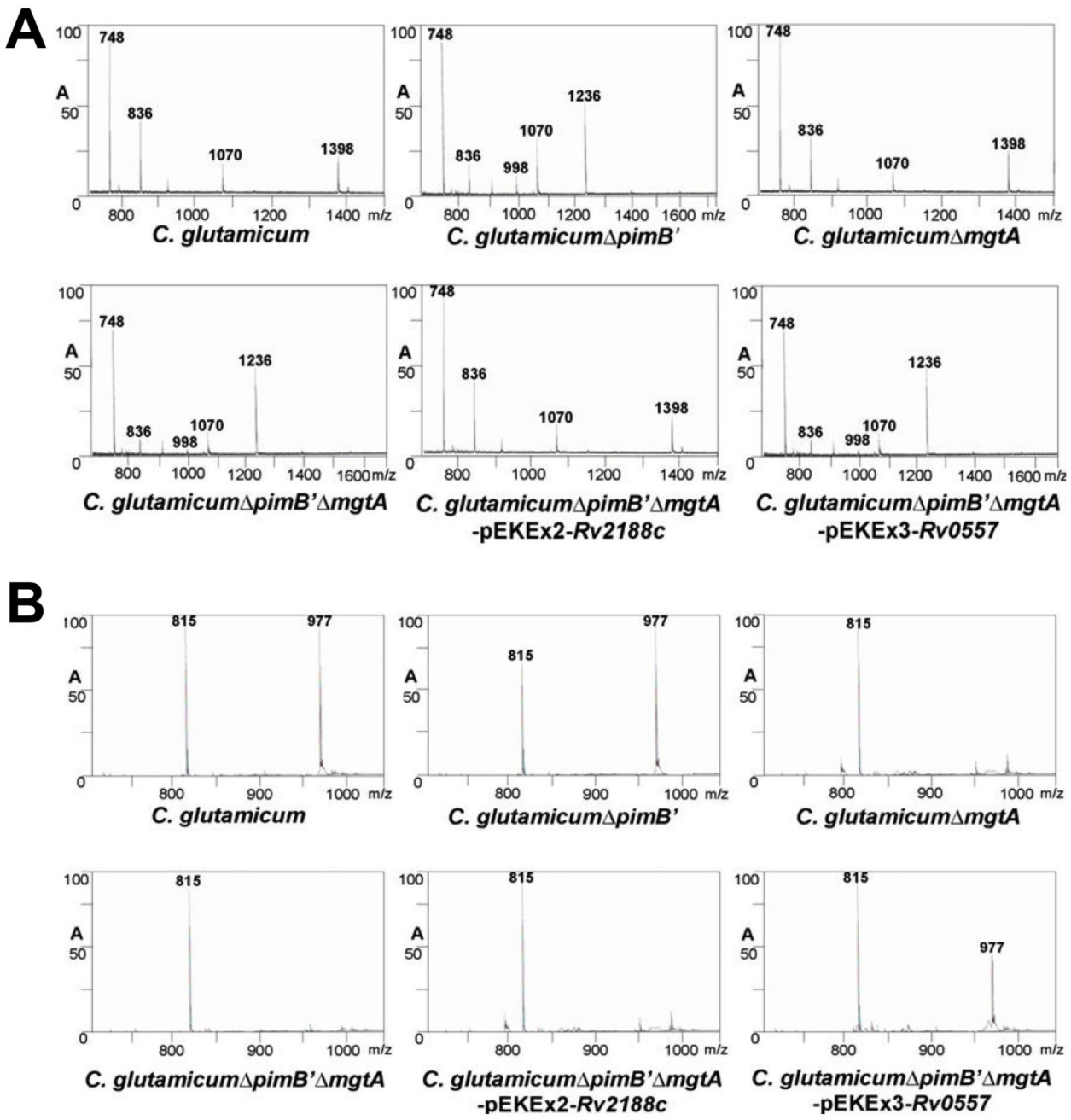

Fig. 3.6: MALDI-TOF MS analyses of glycolipids from different strains of *C. glutamicum* in negative- (A) and positive- (B) ion-mode. (**A**) The peaks observed are *m/z* 836 $(M-H)^-$, [PI with $C_{16}/C_{18:1}$ fatty acyl groups]; *m/z* 998 $(M-H)^-$, [PIM_1 with $C_{16}/C_{18:1}$ fatty acyl groups]; *m/z* 1236 $(M-H)^-$, [Ac_1PIM_1 with $2C_{16}/C_{18:1}$ fatty acyl groups]; and *m/z* 1,398 $(M-H)^-$, [Ac_1PIM_2 with $2C_{16}/C_{18:1}$ fatty acyl groups]. The peak *m/z* 748 was not attributable to any PIM species and, as such, may represent unidentified lipid species and/or plasticizer. (**B**) Positive-ion MALDI-TOF-MS spectrum of the cationized, sodiated precursor ion $(M-H+2Na)^+$ of $GlcAGroAc_2$ and $ManGlcAGroAc_2$ at *m/z* 815 and *m/z* 977, respectively.

synthesis of Ac_1PIM_2. In contrast, plasmid-borne over-expression of Rv0557 in *C. glutamicumΔpimB'ΔmgtA* restored the synthesis of only $ManGlcAGroAc_2$ (Fig. 3.5, Fig.3.6B), which also suggests a specific role in $ManGlcAGroAc_2$ synthesis (Tatituri *et al.*, 2007b). Surprisingly, it did not complement the synthesis of Ac_1PIM_2 a function previously assigned using *in vitro* studies (Schaeffer *et al.*, 1999).

3.3.4 Chemical analysis of lipoglycans in *C. glutamicumΔpimB'ΔmgtA*, *C. glutamicumΔpimB'ΔmgtA*-pEKEx2-*Rv2188c* and *C. glutamicumΔpimB'ΔmgtA*-pEKEx3-*Rv0557*

Lipoglycans were extracted by refluxing delipidated cells in 50% ethanol, followed by hot-phenol treatment, protease digestion and dialysis. The extracted lipoglycans were examined on 15% SDS-PAGE (Fig. 3.7) using a Pro-Q emerald glycoprotein stain. Extracts from *C. glutamicum* showed the presence of Cg-LAM and Cg-LM-A and Cg-LM-B (which co-migrate with Cg-LM-A as previously shown (Tatituri *et al.* 2007b). The lipoglycan extract from *C. glutamicumΔpimB'* showed the presence of a single species $ManGlcAGroAc_2$ based Cg-LM-B, whilst *C. glutamicumΔmgtA* showed the presence of PI based lipoglycans, Cg-LAM and Cg-LM-A. Interestingly, *C. glutamicumΔpimB'ΔmgtA* was shown to be devoid of all three species of lipoglycans (Fig. 3.7). The lipoglycans from *C. glutamicumΔpimB'ΔmgtA*-pEKEx2-*Rv2188c* were analysed and as expected the synthesis of PI-based Cg-LAM and Cg-LM-A was restored by Rv2188c supporting the *in vivo* lipid studies and the specific role of Rv2188c (Fig. 3.7).

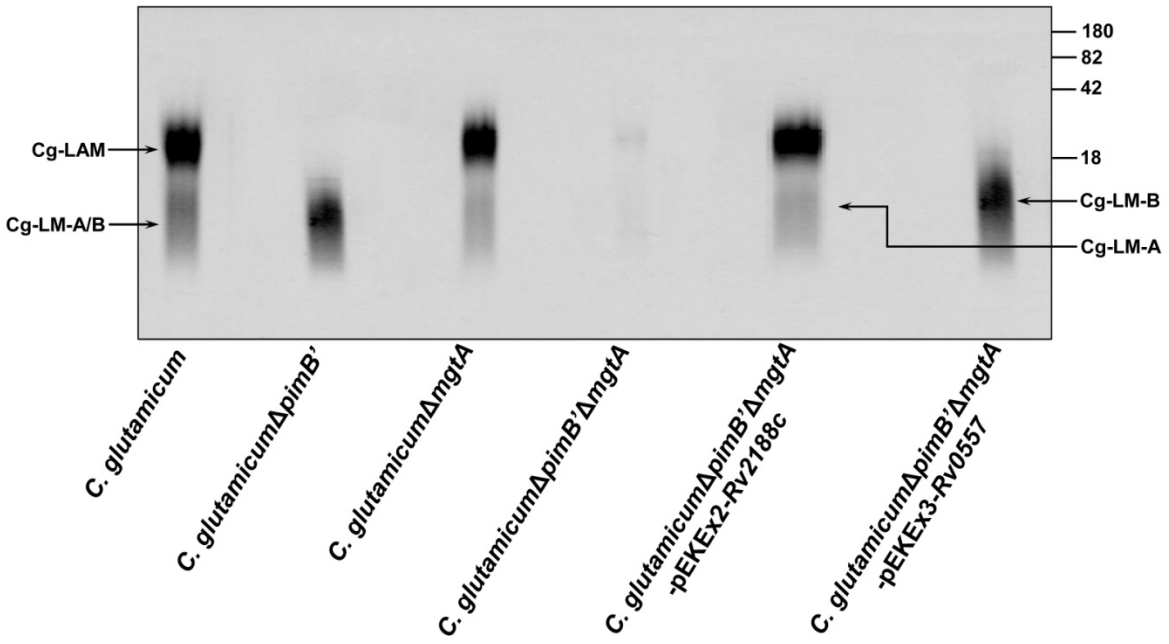

Fig. 3.7: Lipoglycan profiles of different strains of *C. glutamicum*. Lipoglycans were analysed using SDS-PAGE and visualised using a Pro-Q emerald glycoprotein stain specific for carbohydrates. The three major bands represented by Cg-LAM, Cg-LM-A, and Cg-LM-B (which co-migrates with Cg-LM-A) are indicated. The glycoprotein molecular weight standards are provided on the right for comparison. The four major bands represent glycoproteins of 180, 82, 42, and 18 kDa, respectively.

Similarly, *C. glutamicumΔpimB'ΔmgtA*-pEKEx3-*Rv0557* restored the synthesis of $ManGlcAGroAc_2$ based Cg-LM-B, again illustrating the specific role of Rv0557 with respect to $ManGlcAGroAc_2$ and Cg-LM-B synthesis.

3.3.5 Cell-free mannolipid biosynthesis

Membrane preparations from wild type *C. glutamicum* synthesise Ac_1PIM_2 and $ManGlcAGroAc_2$ utilising endogenous acceptors and GDP-[^{14}C]-Man*p* as a sugar donor as reported previously (Fig. 3.8 A) (Brown *et al.*, 2001; Mishra *et al.*, 2008a; Tatituri *et al.*, 2007a; Tatituri *et al.*, 2007b). The TLC analysis of radio-labeled products from *in vitro* assays revealed the synthesis of PP-[^{14}C]-M, Ac_1PI-[^{14}C]-M_2 and [^{14}C]-$ManGlcAGroAc_2$ in accordance with previous studies using wild type *C. glutamicum* membranes (Fig. 3.8 B) (Mishra *et al.*, 2008a). In assays performed with *C. glutamicumΔpimB'* membranes, an additional minor species migrating between Ac_1PI-[^{14}C]-M_2 and ^{14}[C]-$ManGlcAGroAc_2$ was observed and confirmed as PI-[^{14}C]-M_1 as shown previously (Kordulakova *et al.*, 2003). Surprisingly, a faint band corresponding to Ac_1PIM_2 was also detected, which may be due to a relaxed acceptor specificity of Cg-MgtA present in *C. glutamicumΔpimB'*. Assays utilising membrane preparations from *C. glutamicumΔmgtA* synthesised Ac_1PI-[^{14}C]-M_2, but surprisingly possessed a faint band corresponding to [^{14}C]-$ManGlcAGroAc_2$, possibly due to relaxed substrate specificity of Cg-PimB' present in membrane preparation of *C. glutamicumΔmgtA* (Fig. 3.8 B). Interestingly, the synthesis of Ac_1PI-[^{14}C]-M_2 and [^{14}C]-$ManGlcAGroAc_2$ was totally abrogated in assays with membranes prepared from *C. glutamicumΔpimB'ΔmgtA,* (Fig. 3.8 B), whilst accumulation of Ac_1PI-[^{14}C]-M_1 and PP-[^{14}C]-M was observed.

Similar results were also obtained using assays and membrane preparations from *C. glutamicumΔpimB'ΔmgtA*-pEKEx2-*Rv2188c* (Fig. 3.8 B). Herein, Rv2188c showed substrate specificity towards Ac_1PIM_1 and also a weak recognition for the substrate $GlcAGroAc_2$, resulting

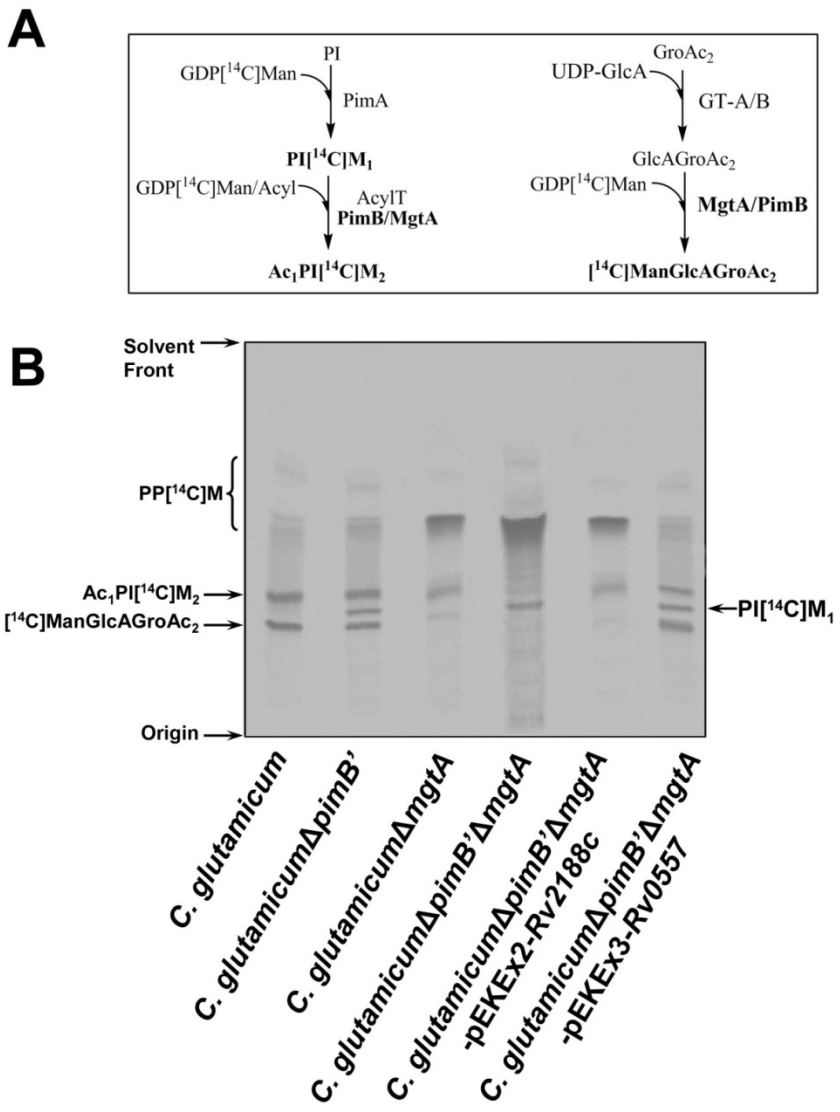

Fig. 3.8: ***In vitro*** **mannolipid biosynthesis**. (**A**) Biosynthetic reaction scheme of products formed in *in vitro* assays utilising GDP-[^{14}C]-Man*p* and corynebacterial membranes. Species represented in bold accumulate as reaction product in the assay. (**B**) TLC-autoradiography of synthesised mannolipids, using GDP-[^{14}C]-Man*p* and membrane extracts from different strains of *C. glutamicum*. Enzymatically synthesised products PP-[^{14}C]-M, [^{14}C]-Man-$GlcAGroAc_2$, Ac_1PI-[^{14}C]-M_2, and PI-[^{14}C]-M_1 were isolated and subjected to TLC/autoradiography using $CHCl_3/CH_3OH/NH_4OH/H_2O$ (65:25:0.4:3.6, v/v/v/v).

in the synthesis of Ac_1PI-[^{14}C]-M_2 and [^{14}C]-$ManGlcAGroAc_2$.Interestingly, and in contrast with the above studies of Rv2188c assays, membrane assays prepared from *C.*

glutamicumΔpimB'ΔmgtA-pEKEx3-*Rv0557* illustrated that Rv0557 possesses a broader relaxed substrate specificity as both Ac_1PIM_1 and $GlcAGroAc_2$ were equally efficient substrates for the enzyme affording Ac_1-[^{14}C]-PIM_2 and [^{14}C]-$ManGlcAGroAc_2$ synthesis (Fig. 3.8 B). This would explain the previous assignment based on *in vitro* data that Rv0557 was involved in the synthesis of Ac_1PIM_2 and annotated as PimB (Schaeffer *et al.*, 1999).

3.3.6 Mannolipid synthesis using recombinant Cg-PimB'

Initial attempts to develop an *in vitro* assay using either purified recombinant expressed Rv2188c or Rv0557 have thus far proved unsuccessful. Therefore, their *C. glutamicum* orthologues were cloned, expressed and purified. Whilst, Cg-PimB' (Rv2188c orthologue) was expressed as a soluble protein (Fig. 3.9 A) and shown to be active in an *in vitro* assay, Cg-MgtA resulted in an inactive protein. The activity of purified Cg-PimB' was initially determined in a pre-defined *in vitro* assay utilising GDP-[^{14}C]-Man*p* and purified polar lipid extracts from *C. glutamicumΔpimB'ΔmgtA* which possess Ac_1PIM_1 and $GlcAGroAc_2$.

The resulting products from the assay involving Cg-PimB' showed a high substrate specificity of the enzyme towards Ac_1PIM_1, and a relaxed specificity towards $GlcAGroAc_2$ (Fig. 3.9 B). In addition, assay performed with highly purified Ac_1PIM_1 (Fig. 3.9 B, 2) resulted in the formation of Ac_1PI-[^{14}C]-M_2, only. Altogether, the data supports the findings from the previous section and suggested the redundant features of these enzymes *in vitro* (Schaeffer *et al.*, 1999).

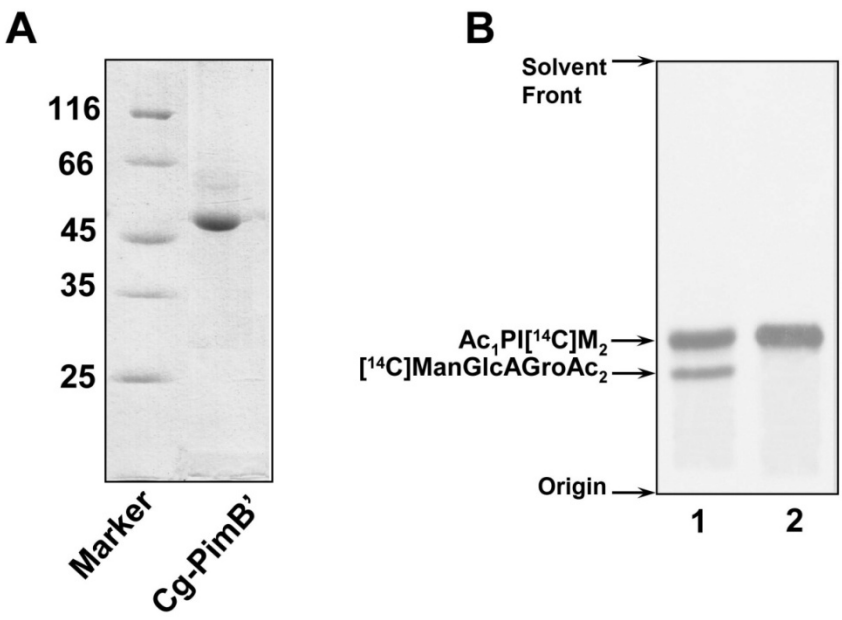

Fig. 3.9: Mannolipid synthesis using recombinant Cg-PimB'. (**A**) Recombinant Cg-PimB' was purified using Ni^{2+} affinity chromatography and purity determined on a 12% SDS-PAGE gel. (**B**) TLC-autoradiography of synthesised mannolipids, using GDP-[^{14}C]-Man*p* and lipid extracts from *C. glutamicum*Δ*pimB*Δ*mgtA* (Lane 1) and purified Ac_1PIM_1 (Lane 2) with purified Cg-PimB'. Enzymatically synthesised products were isolated and subjected to TLC/autoradiography using $CHCl_3/CH_3OH/NH_4OH/H_2O$ (65:25:0.4:3.6, v/v/v/v).

3.4 Discussion

The gene product of *NCgl0452* (initially termed *pimB* and now *mgtA*) of *C. glutamicum*, and orthologue of *Rv0557* in *M. tuberculosis*, is responsible for the synthesis of glycolipid $ManGlcAGroAc_2$ by the addition of a Man*p* unit to $GlcAGroAc_2$ (Tatituri *et al.*, 2007b). For the identification of the enzyme, which adds Man*p* to the 6-OH position of Ac_1PIM_1, to synthesise Ac_1PIM_2 (Schaeffer *et al.*, 1999), we identified *NCgl2106* in the genome of *C. glutamicum* with orthologues present in all *Corynebacterineae*. Our *in vivo* glycolipid and lipoglycan synthesis data suggested the correct *in vivo* functions of Rv2188c as an Ac_1PIM_1:mannopyranosyltransferase (originally termed Mt-PimB') and Rv0557 as a $GlcAGroAc_2$:mannopyranosyltransferase (originally termed Mt-PimB), which we have reassigned as Mt-PimB and Mt-MgtA, respectively, in *M. tuberculosis*.

Furthermore, SDS-PAGE analysis of purified lipoglycans from *C. glutamicumΔpimB'* showed the presence of a single species, which migrated akin to Cg-LM. Chemical characterisation of this novel species using MS-MS established it as an oligomer of hypermannosylated oligosaccharides linked to $GlcAGroAc_2$. It was previously established that these glycosylated diacylglycerols function as precursors/anchors for hyperglycosylated variants, such as the lipomannans, as found in the case of dimannosyl diacylglycerols in *Micrococcus* and lipoteichoic acids (Pieringer (Pakkiri *et al.*, 2004; Pakkiri & Waechter, 2005; Pieringer, 1989). These results also support the initial hypothesis of Tatituri *et al.* (2007b) that $ManGlcAGroAc_2$ participates in the biosynthesis of a novel Cg-LM-like molecule and that the Cg-LM most likely consists of two components, a Cg-LM based on $ManGlcAGroAc_2$ (Cg-LM-B) and a component akin to the characteristic mycobacterial PI-based LM (Cg-LM-A) (Tatituri *et al.*, 2007b).

The α-**D**-mannose-α(1→6)-phosphatidyl-*myo*-inositol-mannosyltransferase activity of Rv0557 reported by Schaeffer *et al.* (1999), and its participation in $ManGlcAGroAc_2$ and Cg-LM-B biosynthesis, and the identification of NCgl2106 and its mycobacterial homologue Rv2188c as another α-**D**-mannose-α(1→6)-phosphatidyl-*myo*-inositol-mannosyltransferase has augmented ongoing confusion in the field (Schaeffer *et al.*, 1999, Tatituri *et al.*, 2007b). To solve this puzzle, we have generated a double knock out of *C. glutamicum*, deficient in Cg-*pimB'* and Cg-*mgtA,* and subsequently over-expressed Rv2188c and Rv0557, individually to identify their true *in vivo* and *in vitro* activities.

As expected *C. glutamicum*Δ*pimB'*Δ*mgtA* mutant was devoid of Ac_1PIM_2 and $ManGlcAGroAc_2$. The *in vivo* complementation of PimB activity was restored using plasmid borne copies of Rv2188c resulting in the synthesis of Ac_1PIM_2 and Cg-LM-A in *C. glutamicum*Δ*pimB'*Δ*mgtA*. In addition, *in vitro* assays utilising membrane preparation from *C. glutamicum*Δ*pimB'*Δ*mgtA*-pVWEx2-*Rv2188c* and GDP-[^{14}C]-Man*p* revealed the synthesis of Ac_1PI-[^{14}C]-M_2. However, it also synthesises $ManGlcAGroAc_2$ albeit with a lower efficiency, demonstrating that Rv2188c has a leaky substrate specificity towards $GlcAGroAc_2$.

Mannolipid synthesis utilising purified recombinant Cg-PimB' has also shown similar activity like its homologue Rv2188c. The extractable lipid profile from *C.glutamicum*Δ*pimB'*Δ*mgtA*-pEKEx3-*Rv0557* contained $ManGlcAGroAc_2$, while no Ac_1PIM_2 was observed, suggesting that Rv0557 is a true α-mannosyl-glucopyranosyluronic acid transferase. However, *in vitro* assays utilising membrane preparations from *C.glutamicum*Δ*pimB'*Δ*mgtA*-pEKEx3-*Rv0557* possessed traces of Ac_1PIM_2 apart from $ManGlcAGroAc_2$, which explains the previous interpretation of the Rv0557 activity as phosphatidyl-*myo*-inositol mannosidase, PimB (Schaeffer *et al.*, 1999).

The presence of a similar branching pattern in LM-A (PI anchored) and LM-B ($GlcAGroAc_2$ anchored) suggests the involvement of similar enzyme machinery in the later stages of both of these pathways. However the presence of only PI anchored LAM and not $GlcAGroAc_2$ anchored, increases the amount of complexity in lipoglycan biosynthesis in *Corynebacterineae*. It is quite interesting to observe the specificity of arabinofuranosyltransferases involved in LAM synthesis towards the reducing end of lipomannan as Cg-LM-A and Cg-LM-B differ only in the structure at their reducing termini. It also opens the possibility of strict regulation of arabinosylation engaged in the synthesis of Cg-LAM from Cg-LM-A and not from Cg-LM-B.

4. SYNTHESIS OF BRANCHING MANNAN AND ARABINAN RESIDUES OF LAM IN *CORYNEBACTERINEAE*

4.1 Introduction

In the previous chapters, we have identified enzymes involved in the early and late stages of LM/LAM biosynthesis. We established that NCgl2106/Rv2188c is involved in the synthesis of Ac_1PIM_2, and NCgl1505/Rv1459c and NCgl2093/Rv2174 synthesise the proximal and distal α(1→6) mannan backbone in LM/LAM. However, enzymatic steps involved in the biosynthesis of mannan branching and arabinan domain of LAM are yet to be fully elucidated. Recently, Kaur *et al.* (2006) reported the involvement of Rv2181 in the synthesis of some if not all α(1→2)-Man*p* units on the α(1→6) mannan backbone of LM/LAM (Kaur *et al.*, 2006, Kaur *et al.*, 2008). They also established that this enzyme had dual functionality; apart from α(1→2)-Man*p* branches, a characteristics of LM and the mannan backbone of LAM, it also adds the second or possibly third Man*p* unit onto mature LAM after Rv1635c adds the first Man*p* unit to give rise to Man_2/Man_3-LAM (Appelmelk *et al.*, 2008, Kaur *et al.*, 2008). However, the possibility of involvement of one more enzyme towards synthesis of α(1→2)-Man*p* units on the α(1→6) mannan backbone has remained an open question.

In *C. glutamicum*, LM is further decorated by single α(1→2)-Ara*f* units on the mannan backbone which results in mature Cg-LAM (Tatituri *et al.*, 2007a). Whilst mycobacterial LM is branched by a long arabinan domain mainly composed of α(1→5)-Ara*f* with linear, Ara-4 or branched Ara-6 motifs (Chatterjee *et al.*, 1993; McNeil *et al.*, 1994). Therefore, in *C. glutamicum* a single arabinofuranosyltransferase activity is responsible for the entire arabinan, while in mycobacteria 5-7 different arabinofuranosyltransferases are required for arabinan synthesis.

In the current study we have used *C. glutamicum* to study the enzymes involved in the mannan

and arabinan branching of lipoglycans in *Corynebacterineae*. We have examined three different ORFs from *C. glutamicum*, which encode for putative GT-C glycosyltransferases based on homology alignment. The genes encoding for putative glycosyltransferases were disrupted and the cell wall phenotype of the mutant strains analysed. On the basis of the biochemical data we report that NCgl2100 and NCgl2097 act as α(1→2) mannopyranosyltransferases (MptC and MptD) and NCgl2096 as an α(1→2) arabinofuranosyltransferase (AftE) involved in LM/LAM biosynthesis in *Corynebacterineae.*

4.2 Materials and methods

4.2.1 Bacterial strains and growth conditions

C. glutamicum and *E. coli* DH5α*mcr* were grown in LB broth at 30°C and 37°C, respectively. The recombinant strains generated were grown on BHI and CGXII medium used for *C. glutamicum*. Kanamycin and ampicillin were used at a concentration of 50 μg/ml for selection of recombinants. Samples for lipid analyzes were prepared by harvesting cells at an OD of 10-15, followed by a saline wash and freeze drying.

4.2.2 Construction of plasmids and strains

All mutant strains in *C. glutamicum* and their complimented strains were constructed at Institute for Biotechnology Research Centre, Juelich, Germany. To delete *mptC* (NCgl2100), *mptD* (NCgl2097), and *aftE* (NCgl2096), respectively, the deletion vectors pK19mobsacBΔ-*mptC*, pK19mobsacBΔ-*mptD* and pK19mobsacBΔ-*aftE* were made. In each case cross-over PCR was

applied using genomic DNA as template and two different PCR´s with primer pairs AB and CD (Table 4.1). The resulting PCR product served as template for primer pairs AD. The PCR product contained 18 nucleotides (nt) of the 3´-end of the respective gene together with genomic upstream sequences, and 36 nt of the 5´-end together with genomic downstream sequences, thus theoretically resulting in a peptide with 6 amino acids of the amino-terminal end fused to 12 amino acids of the carboxy-terminal end but leaving the entire region otherwise intact. All plasmids used in this work were confirmed by sequencing. Genes were deleted in the wild type of *C. glutamicum* by first introducing plasmids prepared from *E. coli* via electroporation into *C. glutamicum* and then selection for sucrose resistance in a procedure as described (Schäfer *et al.*, 1994). Chromosomal deletions were confirmed using primer pairs AD, as well as the additional new primer pairs EF hybridizing outside of the regions used for plasmid constructions.

To construct pEKEx2-Mt-*mptC*, chromosomal DNA of *M. tuberculosis* served as template, and Rv2181 was amplified using primer pairs v2181-for and v2181-rev, the former providing the sequence CTGCAG as a ribosome binding site. The amplified product was treated with PstI and EcoRI and ligated with pEKEx2. Plasmid pEKEx2-Mt-*mptC* was transformed into electrocompetent cells of *C. glutamicum*Δ*mptD* to result in *C. glutamicum*Δ*mptD* pEKEx2-Mt-mptD.

4.2.3 Extraction and biochemical analysis of lipids and lipoglycans

Methods covering extraction and biochemical analysis of lipids and lipoglycans with 2D-TLC, SDS-PAGE, GC-MS, MALDI-TOF-MS and NMR are covered separately as part of General materials and methods in Chapter 5.

Primer	Sequences
A_{2100}	CGTTAAGCTTTGTGTGCTAACTGCGTAATACTCGCGAC
B_{2100}	*CCCATCCACTAAACTTAAACA*TCACCGTGCATGAAAAATAGTGTATCCG
C_{2100}	*TGT TTA AGT TTA GTG GAT GGG* TGG TTA CTGCCGTATTTAGTTGTG
D_{2100}	GTTTGGATCCATACCGTAGTCAAATGCAGAGTCTTGAGC
E_{2100}	CATTTGTGCCAACAACACGTGGTCATATGCG
F_{2100}	GCGGATGCGATCACCGTCAACGC
A_{2096}	CGTT AAGCTT TCG TGA CCG TCG AGC CTG AAT CC
B_{2096}	*CCCATCCACTAAACTTAAACA*CTGCAACGAGGGAACCAC
C_{2096}	*TGTTTAAGTTTAGTGGATGGG*TGGTTACTGCCGTATTTAGTTGTGG
D_{2096}	GCATGGATCCCCGCTGCAATGCCAAGAACTG
E_{2096}	CCAAGGTATCTACTGAGGTCAAGCGCG
F_{2096}	GTAGTGAAATATCACCCATATCAAACGGCTGCG
A_{2097}	CGCTTCTAGAGGATCATTCCACAATTTCCTACCCTCAG
B_{2097}	*CCCATCCACTAAACTTAAACA*ATGTGCTGTCTGGGGGTTC
C_{2097}	*TGTTTAAGTTTAGTGGATGGG*TTGGGTCTTGGGCTGAAAG
D_{2097}	GCGGGAATTCCAGGCGGAATCCACATTGAGTTC
E_{2097}	GTGATCATCGCAGTCGCAGTTGCTGC
F_{2097}	GCATGGGCCACGACAAGGTTCGC
v2181-for	GTGCTGCAGAAGGAGATATAGATATGAATTCGCCCTTGGTGGTCGGG
v2181-rev	CACGGATCCTTAGACGGTCACGGTCAGGCTG

Table 4.1: Primers used for plasmid and strain construction, respectively. Restriction sites are in bold, and overhangs required for hybridisation in the second PCR in italics. Primers are given in their 5´to 3´direction.

4.3 Results

4.3.1 Genome locus and structural features of MptC, MptD and AftE

We previously identified the α(1→6) mannosyltransferases MptA and MptB of *C. glutamicum* and *M. tuberculosis* (Mishra *et al.*, 2007; Mishra *et al.*, 2008a). These catalyse the α(1→6)-Man*p* transfer to distal and proximal parts of the Cg-LM-A/B backbone, respectively. On search of further glycosyltransferases building the elaborated structures of Cg-LM-A/B and Cg-LAM, we inspected the genome of *C. glutamicum* for additional glycosyltransferases of the GT-C family (Liu and Mushegian, 2003). Within a 16 kb genomic region containing MptA, three further GT-C

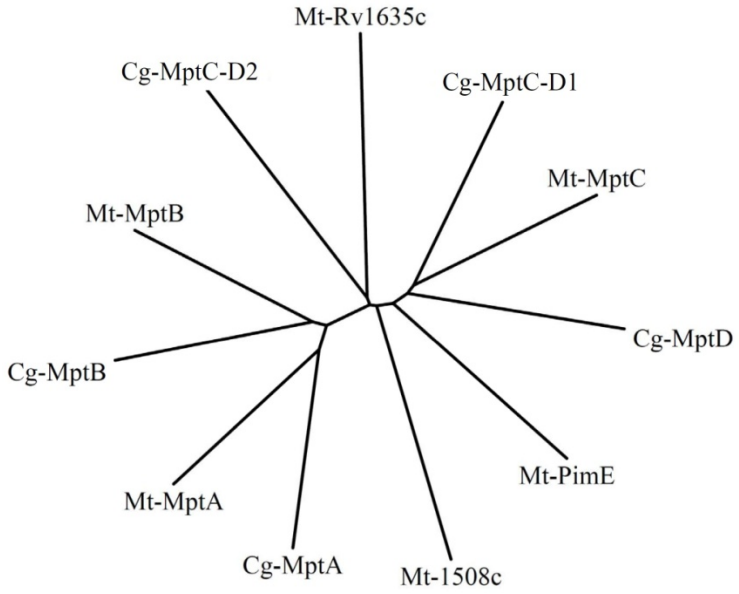

Fig. 4.1: Relatedness α(1→2) and α(1→6) mannopyranosyltransferase. The sequences of proteins identified in this work were used, as well as the prior identified Mt-MtpC (Kaur *et al.*, 2006; Kaur *et al.*, 2008), MptA and MptB (Mishra *et al.*, 2007, Mishra *et al.*, 2008a), PimE (Morita *et al.*, 2006), and Rv1635c (Appelmelk *et al.*, 2007). The functions are not identified for Cg-MptC-D2, which represents the second half of the large fusion protein encoded by *NCgl2100*, and for Rv1508c, both sharing features of GT-C transferases. Sequences were aligned using CLUSTAL and the tree drawn using the Phylip program.

transferases are located. These are NCgl2096 (Cg-AftE) and NCgl2097 (Cg-MptD) which are organised in tandem, as well as NCgl2100 (Cg-MptC). Furthermore, in several *Mycobacterium* species, a MptC orthologue is present close to MptA, which has been identified in *M. tuberculosis* as mannosyltransferase, Rv2181, of multiple function (Kaur *et al.*, 2006; Kaur *et al.*, 2008). Although the detailed organisation of genes within the *Corynebacterineae* is different, the syntenic organisation of additional genes like a serine/threonine protein kinase (*pknL*), hypothetical proteins (NCgl2099, Rv2179c; NCgl2094 , Rv2175c), and 3-deoxy-7-phosphoheptulonate synthase (*aroG*) is largely retained in the organisms inspected.

The NCgl2100 encoded protein is a hydrophobic polytopic membrane protein of 812 amino acid

residues. The first half from 1-417 shares as much as 37% identity (55% similarity) with the mycobacterial Rv2181, and 38% identities (57% similarity) to Cg-MptD, whereas the second half has no counterpart in *Mycobacterium* species. The protein is apparently a fusion of two membrane proteins, or a result of a duplication event. Both Cg-MptD, Cg-MptC, and their orthologue Rv2181 with Mt-PimE have similar size, high degree of hydrophobic similarity, and share amino acid regions of high identity, thus suggesting a closely related function (Fig. 4.1). The NCgl2097 encoded Cg-MptD is a hydrophobic polytopic membrane protein of 436 amino acid residues with 11 TMH. After TMH #1 and #7 larger loop regions are present probably localized in the periplasm. Very similar structural characteristics are shared by the GT-C transferase, Mt-PimE, an α(1→2) mannosyltransferase known to add the 5th Man*p* residue to Ac_1/Ac_2PIM_4 (Morita *et al.*, 2006).The NCgl2096 encoded *C. glutamicum*-AftE consists of 412 amino acid with 9 TMH. It has 2 large loops rich in negatively charged amino acids (6 Asp, 2 Glu) with the first following TMH #1 as required for glycosyl linkage activity for known GT-C members (Seidel *et al.*, 2007b).

4.3.2 Construction of deletion mutants and growth

Construct pK19mobsacBΔ-*mptC* was made containing 13 nt of the 3′-end of *mptC* together with genomic upstream sequences, and 36 nt of the 5′-end together with genomic downstream sequences. This non-replicative vector was used to transform *C. glutamicum* to Kan^r indicating chromosomal integration. Suc^r clones were selected in a second round of positive selection, indicating loss of the vector-bound *sacB* function (Schäfer *et al.*, 1994). From 12 Kan^s and Suc^r clones analysed *via* PCR, 7 had lost *mptC*, whereas in the remaining 5 the wild type phenotype was restored. One clone with deleted *mptC* was selected and referred as *C. glutamicum*Δ*mptC*. In

an analogous manner, pK19mobsacBΔ-*mptD* and pK19mobsacBΔ-*aftE* were made. They were used to select from the wild type by double cross-over events as described above for absence of the respective gene, eventually yielding *C. glutamicum*Δ*mptD* and *C. glutamicum*Δ*aftE*.

The mutants were inoculated in liquid BHI and CGXII and their growth pattern was studied. *C. glutamicum*Δ*aftE* had a growth rate of 0.32±0.01 h^{-1} in comparison to 0.39±0.02 h^{-1} of wild type on CGXII. A slightly retarded growth was also observed for *C. glutamicum*Δ*mptD* (0.37±0.02 h^{-1}), whereas growth of *C. glutamicum*Δ*mptC* was indistinguishable from that of the wild type. A similar trend was seen on BHI medium, but the differences were even smaller. This illustrates that the genes are not vital for growth as we have seen for other glycosyltransferases involved in cell wall synthesis (Emb and AftA) (Alderwick *et al.*, 2005; Alderwick *et al.*, 2006b). Nevertheless, the presence of *aftE* is necessary to ensure optimal growth of *C. glutamicum*.

4.3.2 Purification and general characteristics of lipoglycans

Lipoglycans from wild-type *C. glutamicum*, *C. glutamicum*Δ*mptC*, *C. glutamicum*Δ*mptD*, and *C. glutamicum*Δ*aftE* with complemented strains *C. glutamicum*Δ*mptC* pVWEx-Cg-*mptC*, *C. glutamicum*Δ*mptD* pVWEx-Cg-*mptD* and *C. glutamicum*Δ*aftE* pVWEx-Cg-*aftE* were extracted from delipidated cells by ethanol/water extraction followed by hot-phenol water treatment and enzymatic degradation of contaminants. The crude lipoglycan extract was subjected to HIC for removal of glycans and further applied on gel permeation chromatography for separation of Cg-LM-A/B and Cg-LAM. The extracted lipoglycans were examined for their size and mobility on

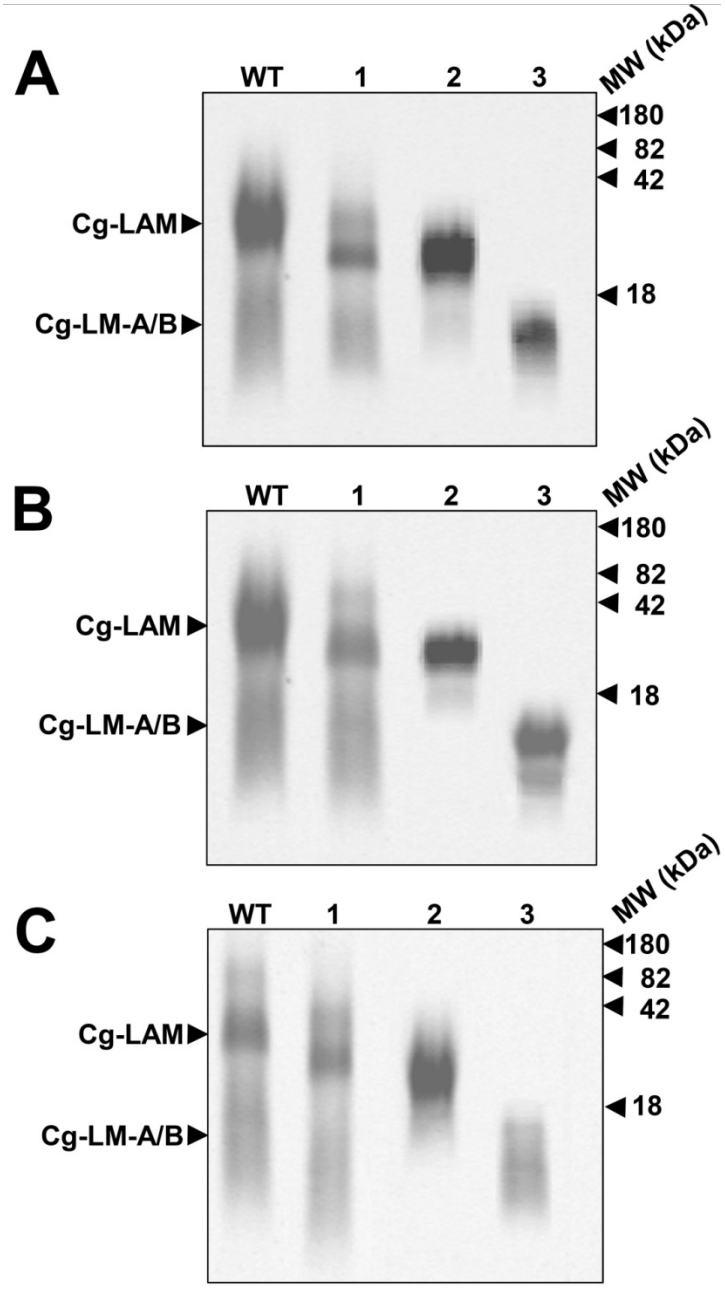

Fig. 4.2: Lipoglycan profiles of wild-type and mutant strains of *C. glutamicum*. Lipoglycans were extracted from wild-type and mutants and analysed using SDS-PAGE and visualised using a Pro-Q emerald glycoprotein stain. Lipoglycan profiles from *C. glutamicum*Δ*mptC*, (**A**); *C. glutamicum*Δ*mptD*, (**B**); and *C. glutamicum*Δ*aftE*; (**C**), are represented with standard molecular weight markers of glycoproteins of 180, 82, 42, and 18 kDa, respectively. In (**A**), (**B**) and (**C**), Lane WT, contains lipoglycans extracted from *C. glutamicum*; Lane 1, crude lipoglycans from mutant strains and Lane 2 and 3 have purified Cg-LAM and Cg-LM-A/B, respectively. The major bands represented by Cg-LAM and Cg-LM-A/B are indicated.

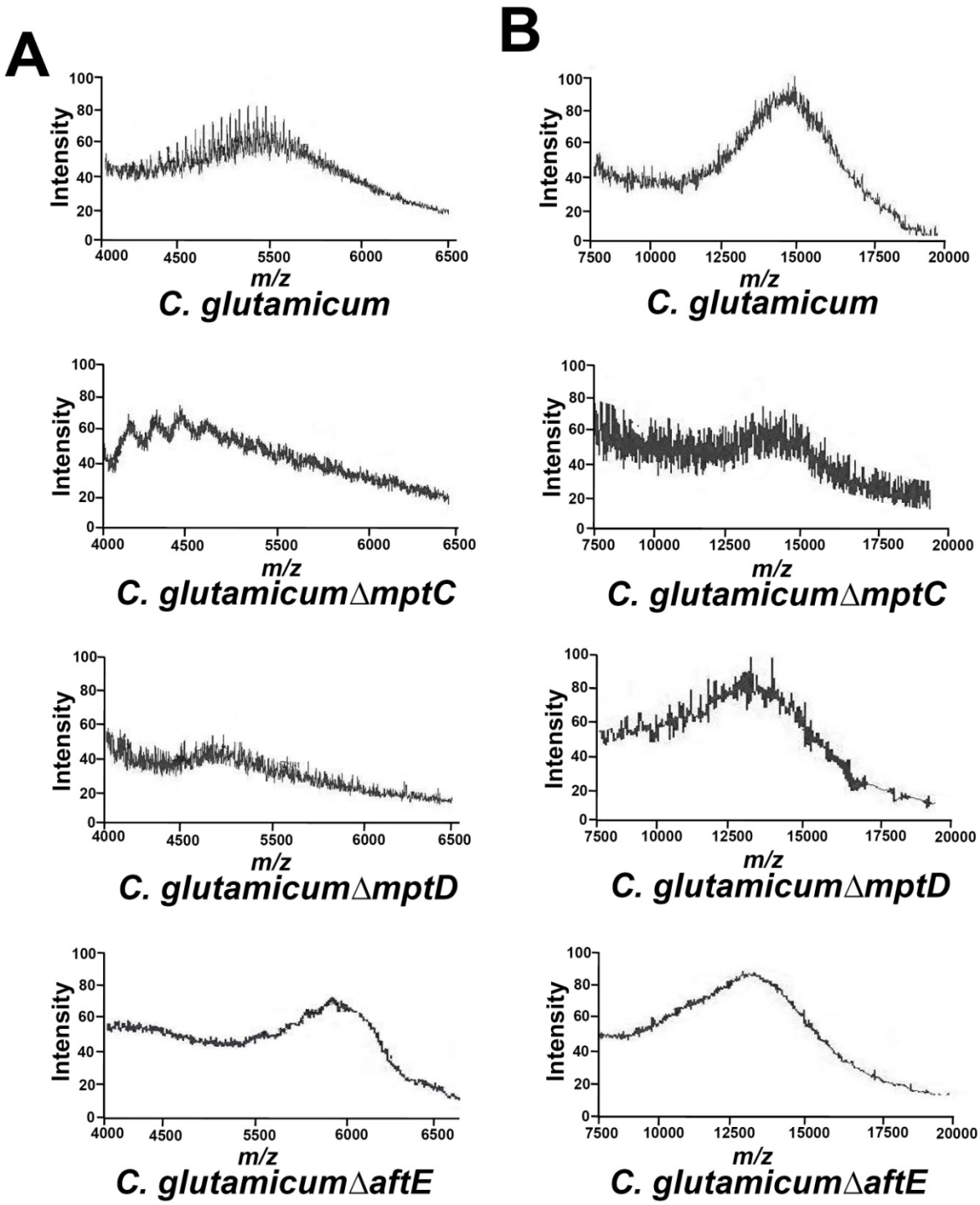

Fig. 4.3: MALDI-TOF-MS spectra of Cg-LM-A/B and Cg-LAM from different strains of *C. glutamicum*. Cg-LM-A/B (**A**) and Cg-LAM (**B**) were purified from different strains and their MALDI-TOF-MS was recorded. MALDI-TOF-MS spectra were acquired in the linear negative mode with delayed extraction using 2,5-dihydrobenzoic acid as a matrix.

15 % SDS-PAGE (Fig. 4.2). Extracts from wild type *C. glutamicum* showed the presence of Cg-LM-A and Cg-LM-B (both co-migrate) and Cg-LAM, while lipoglycans from mutant strains, *C. glutamicum*Δ*mptC*, *C. glutamicum*Δ*mptD* and *C. glutamicum*Δ*aftE* showed subtle differences in their migration on SDS-PAGE (Fig. 4.2).

All three species of lipoglycans from *C. glutamicum*Δ*mptC*, *C. glutamicum*Δ*mptD*, and *C. glutamicum*Δ*aftE* were different in their mobility on SDS-PAGE which directly suggested changes in their size and molecular weight in these mutants. Cg-LM-A, Cg-LM-B and Cg-LAM from all mutant strains migrated faster in comparison to wild-type *C. glutamicum* which implies that these lipoglycans were smaller in size and have low molecular weights. Furthermore, the molecular weight of the lipoglycans was investigated on MALDI-TOF-MS. The mass spectra of wild-type and mutant strains' lipoglycans were summarised in Table 4.2.

The Cg-LAM from wild-type *C. glutamicum* exhibited a broad unresolved peak centred at *m/z* 15,000, indicating a molecular weight of approximately 15 kDa for the major molecular species of this lipoglycan (Fig. 4.3). Interestingly, Cg-LAM species from *C. glutamicum*Δ*mptC* peaked at *m/z* 13,800, indicating a decrease of around 1.2 kDa for the mutant Cg-LAM isolated from *C. glutamicum*Δ*mptC* as compared to the wild-type Cg-LAM (Fig. 4.3 B). In addition, Cg-LM-A/B from *C. glutamicum*Δ*mptC* exhibited a broad unresolved peak centred at *m/z* 4,400, indicating a molecular weight of approximately 4.4 kDa, a decrease of 1.1 kDa in comparison to 5.5 kDa of Cg-LM-A/B from wild-type *C. glutamicum*. Similarly, Cg-LM-A/B and Cg-LAM from *C. glutamicum*Δ*mptD* are centred around *m/z* 4,700 and 13,000, respectively (Fig. 4.3 A). The difference in size of all three lipoglycan species, including Cg-LM-A, Cg-LM-B and Cg-LAM suggested that there is a difference in a common component of all three strains.

The MALDI-TOF mass spectra of *C. glutamicum*Δ*aftE* gave some surprising results in comparison to *C. glutamicum*Δ*mptC* and *C. glutamicum*Δ*mptD* mutants. The Cg-LAM from *C. glutamicum*Δ*aftE* showed an unresolved peak which centred around *m/z* 13,000, similar to *C. glutamicum*Δ*mptC*. However, Cg-LM-A/B from *C. glutamicum*Δ*aftE* peaked around *m/z* 6,000, in comparison to 5.5 kDa of Cg-LM-A/B of wild-type *C. glutamicum* (Fig. 4.3 A, B). The presence of a similar Cg-LM-A/B and shorter Cg-LAM in *C. glutamicum*Δ*aftE* in comparison to wild-type Cg-LM-A/B and Cg-LAM, suggested that AftE is involved in the synthesis of Cg-LAM only.

4.3.3 Glycosyl composition of purified lipoglycans

Lipoglycans from *C. glutamicum*, *C. glutamicum*Δ*mptC*, *C. glutamicum*Δ*mptD* and *C. glutamicum*Δ*aftE* were purified and separated as Cg-LM-A/B and Cg-LAM as described above. Purified lipoglycans were processed and converted into alditol acetates and their glycosyl composition was determined by GC (Fig. 4.4). The glycosyl composition of each lipoglycan species in all mutant strains is summarised in Table 4.2. GC analysis of mutant Cg-LM-A/B from *C. glutamicum*Δ*mptC* and *C. glutamicum*Δ*mptD* showed a relative decrease in amount of Man*p* in comparison to Man*p* content of Cg-LM-A/B from wild type *C. glutamicum* (Fig. 4.4 A).

Furthermore, it revealed a molar ratio of Ara*f*:Man*p* of 0.47:1.0 in Cg-LAM from wild type *C. glutamicum*. The mutant Cg-LAM from *C. glutamicum*Δ*mptC*, *C. glutamicum*Δ*mptD* yielded a significant reduction in Man*p* content concomitant with a relative increase in the amount of Ara*f* (Fig. 4.4 B). The *C. glutamicum*Δ*mptC* yielded a mutant Cg-LAM with an Ara*f*:Man*p* ratio of 0.7:1.0, while *C. glutamicum*Δ*mptD* Cg-LAM has an Ara*f*:Man*p* ratio of 0.54:1.0. This data

S. No.	Strain	Cg-LAM (MALDI-MS) kDa	Cg-LM-A/B (MALDI-MS) kDa	Sugar Analysis (Cg-LAM)	Sugar Analysis (Cg-LM-A/B)	Linkage Analysis (Cg-LAM)	Linkage Analysis (Cg-LM-A/B)
1	*C. glutamicum*	15	5.5	Ara*f* and Man*p*	Man*p* and Inositol	-	-
2	*C. glutamicum* Δ*aftE*	13	6.0	Ara*f* missing	Like WT	t-Ara Missing and increased ratio of α(1→2)-Man*p*	Increased α(1→2)-Man*p*
3	*C. glutamicum* Δ*mptC*	13.8	4.4	Reduced Man*p*	-	α(1→2)-Man*p* reduced	α(1→2)-Man*p* reduced
4	*C. glutamicum* Δ*mptD*	13	4.7	Reduced Man*p*	-	α(1→2)-Man*p* reduced	α(1→2)-Man*p* reduced

Table 4.2: Summary of biochemical analysis of lipoglycans from different strains of *C. glutamicum*. The numbers given are in approximate values.

suggested that both MptC and MptD are involved in the synthesis of mannan portion of Cg-LAM with MptA and MptB. Additionally, the higher Ara*f*:Man*p* ratio in *C. glutamicum*Δ*mptC* in comparison to *C. glutamicum*Δ*mptD* suggested that MptC synthesises the majority of mannan in comparison to MptD.

Surprisingly, the Man*p* content in Cg-LM-A/B from *C. glutamicum*Δ*aftE* showed no apparent change, however, mutant Cg-LAM showed complete absence of an arabinan peak in GC analysis of alditol acetates (Fig. 4.4 A) suggesting the involvement of AftE in the synthesis of the arabinan branches in Cg-LAM of *C. glutamicum*. It also suggested that the arabinan branches in *C. glutamicum* Cg-LAM are entirely contributed by a single enzyme unlike mannan branches where more than one enzyme is required.

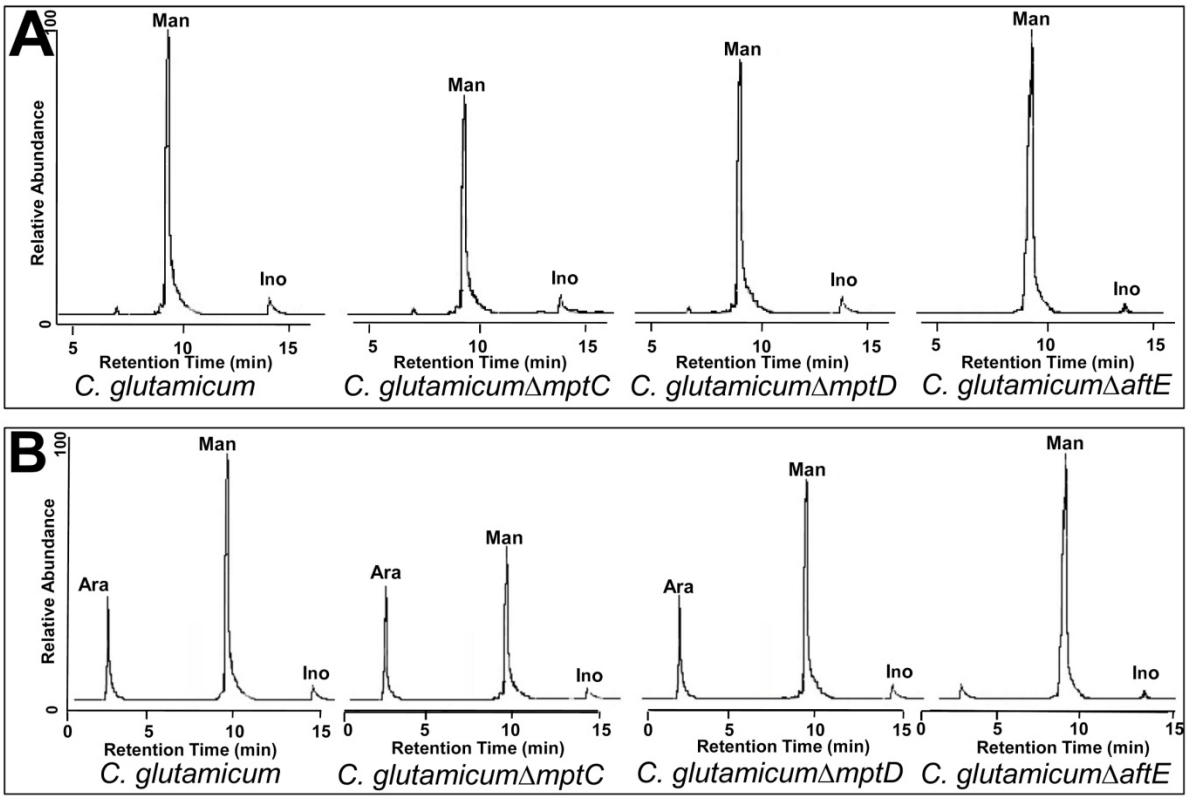

Fig. 4.4: Glycosyl compositional analysis of purified Cg-LM-A/B (A) and Cg-LAM (B) from different strains of *C. glutamicum*. Samples of individually purified lipoglycans were hydrolysed with 2M TFA, reduced and per-*O*-acetylated and subjected to GC analysis.

4.3.4 Glycosyl linkage of purified lipoglycans

The glycosyl linkages present in the lipoglycans from wild-type and mutant strains of *C. glutamicum* were analysed on GC-MS by per-*O*-methylated alditol acetate derivatives prepared from purified lipoglycans. Cg-LM-A/B from *C. glutamicum* possessed a normal profile of glycosidic linkages corresponding to t-Man*p*, 6-Man*p*, 2-Man*p*, and 2,6-Man*p* (Fig. 4.5 A). However, relative ratios of different linkages in Cg-LM-A/B from *C. glutamicum*Δ*mptC* and *C. glutamicum*Δ*mptD* indicated changes in mannan domain in Cg-LM-A/B from these strains. The

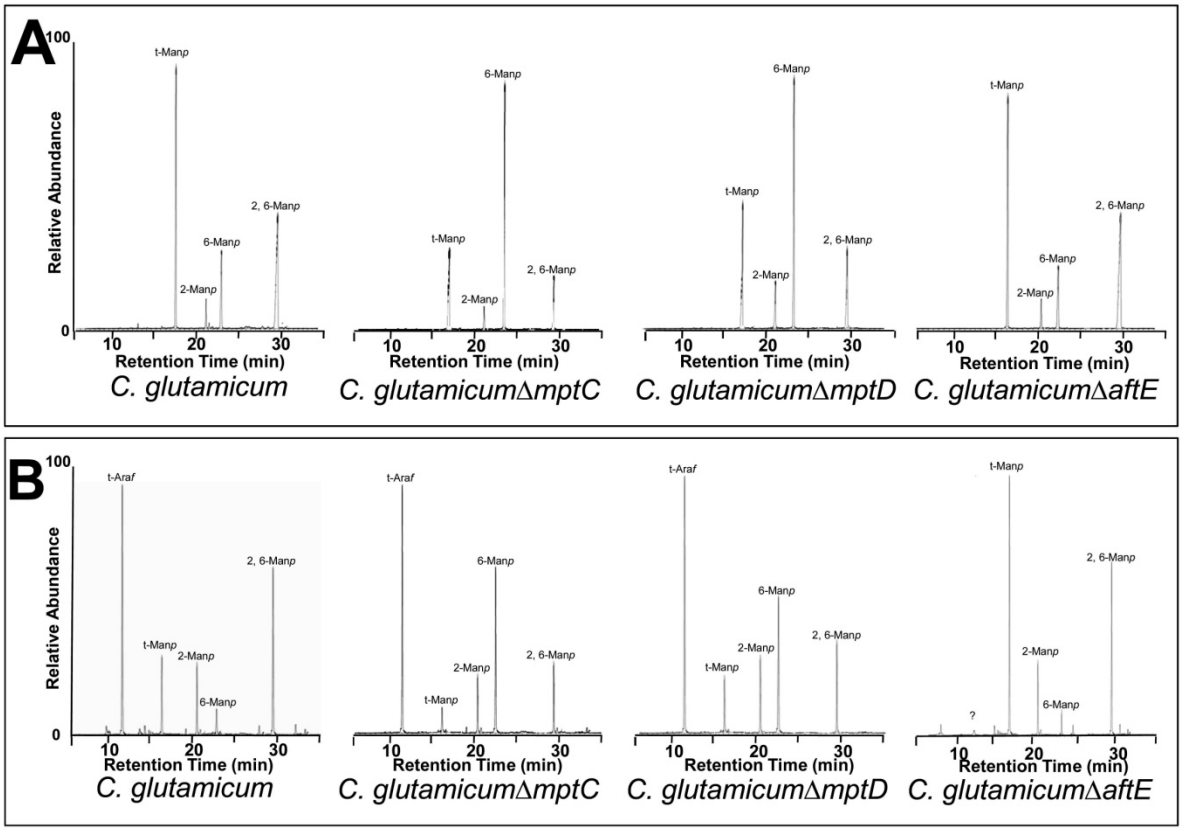

Fig. 4.5: Glycosyl linkage analysis of Cg-LM-A/B (A) and Cg-LAM (B) from different strains of *C. glutamicum*. Per-*O*-methylated samples were hydrolysed using 2M trifluoroacetic acid, reduced and per-*O*-acetylated. The resulting partially per-*O*-methylated, per-*O*-acetylated alditol acetates were analysed on GC-MS.

relative abundance of t-Man*p,* 2-Man*p*, and 2,6-Man*p* were reduced with a concomitant increase in the abundance of 6-Man*p* in Cg-LM-A/B from these strains (Fig. 4.5 A). Similarly, per-*O*-methylated alditol acetate derivatives of Cg-LAM from these strains showed a reduction in relative abundance of t-Man*p,* 2-Man*p*, and 2,6-Man*p* (Fig. 4.5 B).

The complementation of *C. glutamicum*Δ*mptC and C. glutamicum*Δ*mptD* with pVWEx-Cg-*mptC* and pVWEx-Cg-*mptD,* respectively restored the wild-type phenotype in these strains. These data suggested that Cg-LM-A/B and Cg-LAM from *C. glutamicum*Δ*mptC* and *C. glutamicum*Δ*mptD* have reduced α(1→2)-Man*p* units, and MptC and MptD are involved in the synthesis of these

residues in Cg-LM-A/B and Cg-LAM in *C. glutamicum*. In contrast, GC-MS analysis of per-*O*-methylated alditol acetate derivatives prepared from Cg- LM-A/B of *C. glutamicum*Δ*aftE* showed no major differences in the relative abundance of t-Man*p,* 2-Man*p*, and 2,6-Man*p*. However, mutant Cg-LAM from *C. glutamicum*Δ*aftE* was found to be completely devoid of t-Ara*f* branching residues and possess a significant increase in amount of t-Man*p*, 2-Man*p*, 2,6-Man*p*, and 6-Man*p.*

Complementation of *C. glutamicum*Δ*aftE* with plasmid pVWEx-Cg-*aftE* containing Cg-*aftE* restored the glycosyl linkage profile to that of wild type *C. glutamicum*. These results demonstrated that NCgl2100 and NCgl2097 are involved in synthesis α(1→2)-Man*p* residues in Cg-LM-A/B and Cg-LAM, and NCgl2096 is involved in the synthesis of t-Ara*f* residues in Cg-LAM in *C. glutamicum*.

4.3.5 Complementation of *C. glutamicum*Δ*mptC*, *C. glutamicum*Δ*mptD*, and *C. glutamicum*Δ*aftE* with their mycobacterial homologues

All three ORFs from *C. glutamicum* (*NCgl2100*, *NCgl2097* and *NCgl2096*) were used as query sequences and aligned against *M. tuberculosis* genome in search of their homologues. *NCgl2100* or *mptC* showed 55% similarity to *Rv2181* of *M. tuberculosis*, while *NCgl2097* and *NCgl2096* showed 47.9% and 40.8%, respectively, similarity to *Rv2181*. A plasmid encoding *Rv2181* was able to restore the wild-type phenotype in *C. glutamicum*Δ*mptC* (Fig. 4.6). However, *Rv2181* was quite specific for *C. glutamicum*Δ*mptC* and unable to complement mutant phenotypes in *C. glutamicum*Δ*mptD* and *C. glutamicum*Δ*aftE* (Fig. 4.6). The data suggested that *Rv2181* is a true

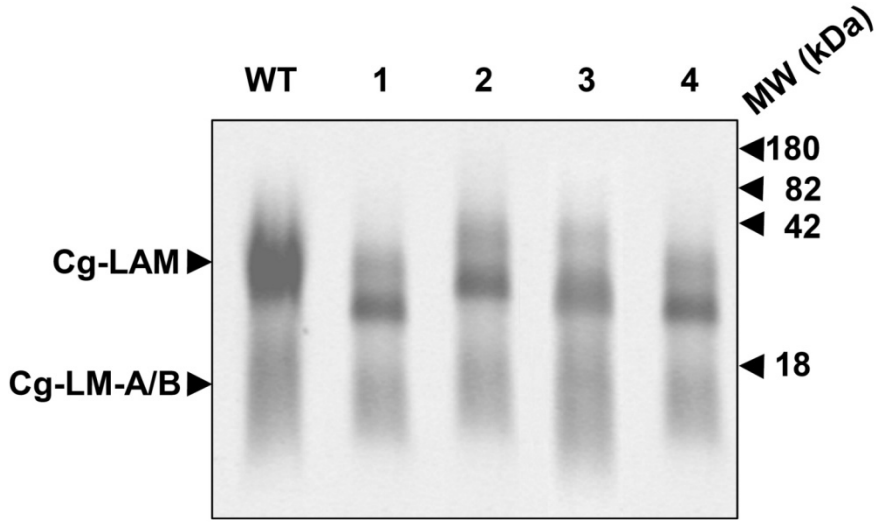

Fig. 4.6: Complementation of *C. glutamicum*Δ*mptC*, *C. glutamicum*Δ*mptD*, and *C. glutamicum*Δ*aftE* with *Rv2181*. Lipoglycans were extracted and analysed using SDS-PAGE and visualised using a Pro-Q emerald glycoprotein stain. Lane WT, contains lipoglycans extracted from *C. glutamicum*; Lane 1, crude lipoglycans from *C. glutamicum*Δ*mptC*; Lane 2, *C. glutamicum*Δ*mptC* pVWEx-Mt-*Rv2181*; Lane 3, *C. glutamicum*Δ*mptD* pVWEx-Mt-*Rv2181*; and Lane 4, *C. glutamicum*Δ*aftEC* pVWEx-Mt-*Rv2181*. The major bands represented by Cg-LAM and Cg-LM-A/B are indicated.

homologue of *NCgl2100* and both encode for an α(1→2) mannopyranosyltransferase and it was concluded that MptC is involved in the synthesis of on α(1→2)-Man*p* branches of Cg-LM-A/B and Cg-LAM in *Corynebacterineae*.

4.4 Discussion

Apart from sharing a similar cell wall architecture with *M. tuberculosis*, *C. glutamicum* also has similar genetic loci encoding for cell wall biogenesis. Furthermore, its usage in the study of essential genetic elements of *M. tuberculosis*, makes it an attractive model system for the functional genomics of *M. tuberculosis* (Alderwick *et al.*, 2006a; Alderwick *et al.*, 2006b). In addition, the non-pathogenic and fast growing nature of *C. glutamicum* has enabled its use as a suitable model for the identification and functional study of mycobacterial genes involved in mycolic acid, arabinogalactan, and LAM biosynthesis (Alderwick *et al.*, 2005; Alderwick *et al.*, 2006a; Alderwick *et al.*, 2007; Birch *et al.*, 2008; Birch *et al.*, 2009; Gande *et al.*, 2004; Gande *et al.*, 2007; Gibson *et al.*, 2003; Mishra *et al.*, 2007; Mishra *et al.*, 2008a; Mishra *et al.*, 2008b; Mishra *et al.*, 2009; Seidel *et al.*, 2007a; Seidel *et al.*, 2007b; Tatituri *et al.*, 2007a; Tatituri *et al.*, 2007b)

Previously, we reported the identification of enzymes involved in the early and late stages of LM/LAM biosynthesis using *C. glutamicum* as a model system (Mishra *et al.*, 2007; Mishra *et al.*, 2008a; Mishra *et al.*, 2008b; Mishra *et al.*, 2009). It was established that MptA and MptB synthesised the distal and proximal α(1→6) mannan backbone of LM/LAM, respectively (Mishra *et al.*, 2007; Mishra *et al.*, 2008a). Herein, we have continued our earlier studies to identify genes required for the biosynthesis of the core structural elements of the mycobacterial lipoglycans by studying mutants of *C. glutamicum* and the orthologous genes and enzymes of *M. tuberculosis.* Herein, we have identified the genetic loci responsible for the synthesis of the mannan and arabinan branches of LM and LAM in *Corynebacterineae*. On the basis of our genetic and biochemical analysis of mutants in *C. glutamicum* deficient in putative glycosyltransferases, we

report NCgl2096 as a novel arabinofuranosyltransferase, AftE, which is involved in priming of mannan backbone of Cg-LM-A/B with singular t-Ara*f* which results in the synthesis of the complete structure of Cg-LAM in *C. glutamicum*. Furthermore, NCgl2097 and NCgl2100 have been identified as two novel α(1→2)mannopyranosyltransferases, MptD and MptC, respectively. One of them adds α(1→2)-Man*p* residues to the proximal backbone of Cg-LM-A/B, whilst the other adds α(1→2)-Man*p* residues to the distal backbone of Cg-LM-A/B.

SDS-PAGE and MALDI-TOF-MS analysis of all three lipoglycan species, including Cg-LM-A, Cg-LM-B and Cg-LAM from *C. glutamicum*Δ*mptC* and *C. glutamicum*Δ*mptD* suggested that there is a difference in a common component of all three species. These lipoglycans share a similar anchor motif with an α(1→6) mannan backbone primed with α(1→2)-Man*p* units. 2D-TLC analysis supplemented with negative MALDI-TOF-MS of extracted glycolipids from the mutants have shown no difference in the precursors, and presence of MptA and MptB in these mutants negates the possibility of a change in the α(1→6) mannan backbone of these lipoglycans which left the possibility of a modification in the α(1→2)-Man*p* branches and suggested that MptC and MptD are involved in the synthesis of these α(1→2)-Man*p* units in these lipoglycans. In addition, glycosyl composition and linkage analysis suggested a reduction in the relative abundance of t-Man*p,* 2-Man*p* and 2,6-Man*p* with a concomitant increase in the abundance of 6-Man*p* in Cg-LM-A/B and 6-Man*p* and t-Ara*f* in Cg-LAM from these strains. The cumulative biochemical analysis of *C. glutamicum*Δ*mptC* and *C. glutamicum*Δ*mptD* suggested that MptC and MptD are involved in synthesis of α(1→2)-Man*p* units in Cg-LM-A/B and Cg-LAM in *C. glutamicum*.

Recently, Kaur *et al.* (2006; 2008) reported the involvement of Rv2181 in the synthesis of some if not all α(1→2)-Man*p* units on the α(1→6) mannan backbone of LM and LAM in mycobacteria (Kaur *et al.*, 2006, Kaur *et al.*, 2008). In addition, all three ORFs from *C. glutamicum* (*NCgl2100*, *NCgl2097* and *NCgl2096*) showed maximum similarity with *Rv2181* of *M. tuberculosis*. Therefore, a plasmid encoding *Rv2181* was transformed in these mutants, which was able to restore the wild-type phenotype in *C. glutamicum*Δ*mptC*, however, was unable to complement the mutant phenotypes in *C. glutamicum*Δ*mptD* and *C. glutamicum*Δ*aftE*. The data suggested that *Rv2181* is a true homologue of *NCgl2100* and both encode for α(1→2)mannopyranosyltransferase(s), and MptC is involved in the synthesis of the α(1→2)-Man*p* branches of LM/LAM in *Corynebacterineae*. Herein, we propose MptD as a homologue of another unidentified α(1→2)mannopyranosyltransferase responsible for synthesis of the residual α(1→2)-Man*p* units in *M. tuberculosis*Δ*Rv2181* (Kaur *et al.* 2008).

The number of α(1→2)-Man*p* residues in lipoglycans of *C. glutamicum*Δ*mptD* is more in comparison to *C. glutamicum*Δ*mptC*, which is due to presence of MptC in *C. glutamicum*Δ*mptD* which suggests that MptC is enzymatically more active than MptD. One of the important questions in lipoglycan biosynthesis is the division of LM and LAM abundance in the cell wall. The separation of LM and LAM in *C. glutamicum*, biosynthetically occurs after half of the mannan backbone is primed with α(1→2)-Man*p* residues by one of the α(1→2)mannopyranosyltransferases, probably MptC. Thereafter, in case of LM synthesis, the second α(1→2)mannopyranosyltransferase MptD primes the rest of the backbone, while in case of LAM; MptD and AftE both decorate the mannan backbone with α(1→2)-Man*p* and t-Ara*f* residues, respectively. The presence of two α(1→2)mannopyranosyltransferases, MptC and MptD, with different activities with two other α(1→6)mannopyranosyltransferases, MptA and

MptB, suggests the possibility of enzymatic complexes in lipoglycan biosynthesis similar to FAS-II in mycolic acid biosynthesis (Takayama *et al.*, 2005; Bhatt *et al.*, 2008). One of the α(1→2)mannopyranosyltransferases might be acting in complex with MptB, while other one with MptA. MptC might be the first priming enzyme which works in complex with MptB till PIM_{15-18} or Cg-t-LM (Mishra *et al.*, 2007). Thereafter, MptD takes over the priming in complex with MptA and gives a full length LM. Sequence and topology of MptC and MptD are quite similar to each other which supports the argument that both act on similar substrates (partial branched Cg-t-LM) (Mishra *et al.*, 2007), which indicates that they are involved in the synthesis of distal part of LM/LAM with MptA.

The biochemical analysis of *C. glutamicum*Δ*aftE* showed a Cg-LM-A/B similar to wild-type with a shorter mutant Cg-LAM. The glycosyl composition and linkage analysis of mutant Cg-LAM suggested the complete loss of arabinan or t-Ara*f* from Cg-LAM. Absence of arabinan residues in this lipoglycan suggested it as a novel LM with higher α(1→2)-Man*p* units probably due to the action of MptC and MptD on branching sites created due to the absence of branched t-Ara*f* residues. A comparison of the glycosyl linkage profile of the novel mutant lipoglycan from *C. glutamicum*Δ*aftE* with Cg-LM-A/B from wild-type *C. glutamicum*, suggested it as a higher Cg-LM-A/B with α(1→6) mannan backbone and highly decorated with α(1→2)-Man*p* unit.

5. GENERAL MATERIALS AND METHODS

5.1 Chemicals, reagents and enzymes

All chemicals and solvents were purchased from Sigma-Aldrich (Dorset, UK), Bio- Rad (Ca, USA) and Fisher Chemicals (UK) unless otherwise stated, and were of AnalR grade or equivalent. Enzymes were obtained from Sigma-Aldrich (Dorset, UK) or Roche (Lewes, UK) and were of the highest grade available.

5.2 Lipid extraction and analysis

Polar and apolar lipids were extracted as described by Dobson *et al.* (1985). Briefly, 6 g of dry *C. glutamicum* cells were treated in 220 ml of methanolic saline (20 ml 0.3% NaCl and 200 ml CH_3OH) and 220 ml of petroleum ether for 2 h (Dobson *et al.*, 1985). The suspension was centrifuged and the upper layer containing apolar lipids was separated. The step was repeated twice. The two upper petroleum ether fractions were combined and dried. For polar lipids, 260 ml $CHCl_3$/CH_3OH/0.3% NaCl (9:10:3, v/v/v) was added to the lower aqueous phase and stirred for 4 h. The mixture was filtered and the filter cake re-extracted twice with 85 ml of $CHCl_3$/CH_3OH/0.3% NaCl (5:10:4, v/v/v). Equal amounts of $CHCl_3$ and 0.3% NaCl (145 ml each) were added to the combined filtrates and stirred for 1 h. The mixture was allowed to settle, and the lower layer containing the polar lipids recovered and dried. The polar lipid extract was examined by two dimensional thin-layer chromatography (2D-TLC) on aluminum backed plates of silica gel 60 F_{254} (Merck 5554), using $CHCl_3$/CH_3OH/H_2O (65:25:4, v/v/v) in the first direction and $CHCl_3$/CH_3COOH/CH_3OH/H_2O (40:25:3:6, v/v/v/v) in the second direction. The thin-layer chromatographic plates sprayed with the appropriate staining solution to detect the presence of lipids, glycolipids or phospholipids as outlined below.

5.2.1 α-Naphthol-sulfuric acid (α-NAP)

α-NAP was used for the detection of carbohydrate containing lipids. The spray solution was prepared from 6 g of α-napthol dissolved in 25 ml of sulfuric acid and 450 ml of ethanol (Dobson *et al.*, 1985). Carbohydrate positive lipids were revealed by gentle charring of the plates with a heat gun until purple spots appeared which indicates the presence of carbohydrate moiety in the lipid.

5.2.2 Phosphate stain

The Dittmer and Lester reagent (Dittmer & Lester, 1964) was used for the detection of phosphate containing lipids with a modified procedure (Muthing & Radloff, 1998). Briefly, solution A was prepared from 40 g of molybdenum VI oxide dissolved in a 1 L of boiling sulfuric acid. Solution B contained 1.7 g of solid molybdenum dissolved into 0.5 L of solution A. Solutions A and B were then mixed with H_2O to give a final mixture in the ratio of solution A:solution B:H_2O, 1:1:4. Phosphate positive lipids were revealed by spraying the plates which were then left to develop for ten min without charring revealing light blue spots on a white background.

5.2.3 Molybdophosphoric acid (MPA)

MPA was used for the detection of all lipids. Spray solution was prepared from 5% (w/v) ethanolic molbdophosphoric acid (Dobson *et al.*, 1985). Lipid species were revealed by charring the plates with a heat gun.

5.3 Extraction and purification of lipoglycans

Lipoglycans were extracted from delipidated cells as previously described (Ludwiczak *et al.*, 2001; Ludwiczak *et al.*, 2002; Nigou *et al.*, 1997). Briefly, cells were broken by sonication (MSE Soniprep 150, 12 micron amplitude, 60s ON, 90s OFF for 10 cycles, on ice) and the cell debris refluxed 5 times with 50% C_2H_5OH at 68°C, for 12 h intervals. The cell debris was removed by centrifugation and the supernatant containing lipoglycans, neutral glycans and proteins dried. This dried extract was then treated with hot phenol–H_2O. The aqueous phase was dialyzed and dried, followed by extensive treatments with α-amylase, DNase, RNase chymotrypsin and trypsin. The fraction was dialyzed once again to remove residual impurities and enzymes.

The crude lipoglycan extract was dried and resuspended in buffer A (50 mM ammonium acetate and 15% propan-1-ol) and subjected to Octyl Sepharose CL- 4B HIC (2.5 cm x 50 cm) (Leopold & Fischer, 1993). The column was washed initially with 4 column volumes of buffer A to ensure removal of neutral glycans followed by buffer B (50 mM ammonium acetate and 50% propan-1-ol). The eluent was collected and concentrated to approximately 1 ml and precipitated using 5 ml of C_2H_5OH. The sample was dried using a Savant Speedvac and then resuspended in buffer C (0.2 M NaCl, 0.25% sodium deoxycholate (w/v), 1 mM EDTA and 10 mM Tris-HCl, pH 8) to a final concentration of 200 mg/ml.

The sample was gently mixed and left to incubate for 48 h at room temperature. The sample was then loaded onto a 200 ml Sephacryl S-200 column previously equilibrated with buffer C. The sample was eluted with 400 ml of buffer C at a flow rate of 3 ml/h, collecting 1.5 ml fractions. The fractions were monitored by SDS-PAGE using either a silver stain utilizing periodic acid and

silver nitrate (Hunter *et al.*, 1986) or a Pro-Q emerald glycoprotein stain and individual fractions pooled and dialyzed extensively against buffer D (10 mM Tris-HCl, pH 8, 0.2 M NaCl, 1 mM EDTA) for 72 h with frequent changes of buffer. The samples were further dialyzed against deionized water for 48 h with frequent changes of water, lyophilized and stored at - 20ºC.

5.3.1 SDS-PAGE

For the separation of lipoglycans and proteins SDS-polyacrylamide gel electrophoresis technique was used (Laemmli, 1970). Resolving gels of either 12% or 15% (w/v) acrylamide and a stacking gel of 6% were prepared and casted in the electrophoresis apparatus according to the manufacturer's instructions. Lipoglycan samples (usually 5 -20 μg) were analysed on 15% polyacrylamide gels, while protein fractions (25 μg) were run on 12% polyacrylamide gels. The ingredients used to cast the resolving and stacking gels are given in Table 5.1 and 5.2.

The running buffer was made up with 25mM Tris, 190mM glycine, and 4mM SDS. Lipoglycan samples were mixed with an appropriate amount of loading buffer (360mM Tris-HCl, pH 8.8, 9% (w/v) SDS, 0.9% (w/v) bromophenol blue, 15% (w/v) β-mercaptoethanol, and 30% glycerol) and boiled for 5 min before loading onto the gel. Pro-Q emerald glycoprotein stain (Invitrogen) was used for visualising the lipoglycan content in the samples as explained in the manufacturer's handbook.

Ingredients	Amount for 2 mini gels
4 % Resolving gel buffer	3.75 ml
Acrylamide/bis-Acrylamide/Water	11.25 ml
TEMED (Sigma)	30 µl
10 % Ammonium per sulphate	75 µl

Table 5.1: Ingredients used to make resolving gel of 12 % SDS-PAGE. Resolving gel buffer: 1.5M Tris-HCl (Roche), 0.4% SDS (BDH) pH 8.8; The acrylamide/bis/water mix:. Acrylamide mix (Protogel):water (6ml/5.25ml).

Ingredients	Amount for 2 mini gels
4 % Resolving gel buffer	1.25 ml
Acrylamide/bis-Acrylamide	0.65 ml
Water	3.05 ml
TEMED (Sigma)	15 µl
10 % Ammonium per sulphate	75 µl

Table 5.2: Ingredients used to make stacking gel of 12 % SDS-PAGE. Stacking gel buffer: 0.5M Tris-HCl (Roche), 0.4% SDS (BDH) pH 6.8.

5.3.2 Chromatography of lipoglycans

All glass columns were purchased from Sigma (Dorset, UK) with a bed size of either 2.5 x 50 cm or 1.5 x 30 cm. The matrices (Octyl sepharose CL-4B and Sephacryl S-200) used were obtained from Sigma and used processed according to the manufacturers' instructions. The chromatographic buffers used were filtered with Corning 0.22 µm CA (cellulose acetate) filter system and degassed with helium.

5.3.3 Dialysis of lipoglycans

Dialysis of lipoglycans was performed using a low molecular weight cut off dialysis tubing, Spectra/Por membrane 6, MWCO 3,500 kDA, and PIMs on Spectra/Por membrane 6, MWCO 1,000 kDA, purchased from Spectrum Labs, according to manufacturers instructions.

5.4 Glycosyl compositional and linkage analysis

Lipoglycans were hydrolyzed using 2M trifluoroacetic acid, reduced with NaB_2H_4, and the resultant alditols per-*O*-acetylated before examination by GC (Tatituri *et al.*, 2007b). Glycosyl linkage analyses were performed as described previously (Tatituri *et al.*, 2007b). Briefly, lipoglycan samples were per–*O*-methylated using dimethyl sulfinyl carbanion, hydrolysed using 2M trifluoroacetic acid, reduced using NaB_2H_4 and per-*O*-acetylated. The resulting per-*O*-methylated alditol acetates were solubilised in $CHCl_3$ before analysis by gas chromatography/mass spectrometry (GC/MS) (Tatituri *et al.*, 2007b). GC analysis was performed using a Thermoquest Trace GC 2000. Samples were injected in the split-less mode. The column used was a DB225 (Supelco). The oven was programmed to hold at an isothermal temperature of 275 °C for a run time of 15 min. GC/MS was carried out on a Finnigan Polaris/GCQ PlusTM. The column used was a BPX5 (Supleco). Injector temperature was set at 50ºC, held for 1 min and then increased to 110ºC at 20ºC/min. The oven was held at 110ºC then ramped to 290ºC at 8ºC/min and held for 5 min to ensure all the products had eluted from the column. All the data were collected and analysed using Xcaliber (v.1.2) software.

5.5 MALDI-TOF-MS analysis

The matrix used was 2,5-dihydroxybenzoic acid at a concentration of 10 µg/µl, in a mixture of water/ethanol (1:1, v/v), 0.1% trifluoroacetic acid. LM samples (0.5 µl) at a concentration of 10 µg/µl, were mixed with 0.5 µl of the matrix solution. Analyses were performed on a Voyager DE-STR MALDI-TOF instrument (PerSeptive Biosystems, Framingham, MA) using linear mode detection. Mass spectra were recorded in the negative mode using a 300 ns time delay with a grid voltage of 80% of full accelerating voltage (25 kV) and a guide wire voltage of 0.15%. The mass spectra were mass assigned using external calibration.

5.6 NMR spectroscopy

NMR spectra of LM samples were recorded on a Bruker DMX-500 equipped, with a double resonance (1H/X)-BBi z-gradient probe head. All samples were exchanged in D_2O (D, 99.97% from Euriso-top, Saint-Aubin, France), with intermediate lyophilization, and then dissolved in 0.5 ml D_2O and analysed at 313K. The ^{1}H and ^{13}C NMR chemical shifts were referenced relative to internal acetone at 2.225 and 34.00 ppm, respectively. All the details concerning NMR sequences and experimental procedures were described previously (Gilleron *et al.*, 1999; Gilleron *et al.*, 2000).

6. CONCLUSION

Sudden upsurge in the number of cases of MDR- and XDR-TB, imposes a need for the identification of novel drug targets and development of active compounds against them. In this respect, the biosynthetic machinery of the mycobacterial cell wall (target for first line drugs) represents an attractive target. In this regard, we have experimentally characterised several glycosyltransferases involved in the biosynthesis of the mycobacterial cell wall of *M. tuberculosis*. Herein, we used *C. glutamicum* as a 'a proof of principle' to study the role of glycosyltransferases in the biosynthesis of LAM and related glycoconjugates. We have analysed several ORFs, which encode for putative glycosyltransferases from *M. tuberculosis* and deleted their respective orthologs in *C. glutamicum*. Complete biochemical characterisation of these mutant phenotypes have shown their role in the synthesis of PIMs and LM/LAM. Furthermore, mutants were complemented with their respective mycobacterial homologous to identify and characterise their functions.

One of these ORFs, *NCgl2093/Rv2174*, categorised as a GT-C which utilises the polyprenyl based sugar donor, C_{50}-PP-Man*p*, was identified to encode for an $\alpha(1\rightarrow6)$ mannosyltransferase (MptA) which is involved in the later stages of the biosynthesis of the $\alpha(1\rightarrow6)$ mannan core of LM and LAM (Fig. 6.1). The homologue of Rv1459c [MptB] in *C. glutamicum* was shown to be involved in the early stages of the biosynthesis of the $\alpha(1\rightarrow6)$ mannan core of LM and LAM. MptB accepts Ac_1PIM_2 as substrate and adds 10-13 Man*p* residues in a $\alpha(1\rightarrow6)$ fashion which is further extended by MptA (Fig. 6.1). The MptB in *M. tuberculosis* and *M. smegmatis* possess similar $\alpha(1\rightarrow6)$ mannosyltransferase activity, however, failed to complement the mutant phenotypes either *in vivo* or *in vitro*, which suggests a subtle substrate specificity for MptB in *M. tuberculosis* and *M. smegmatis*. Furthermore, Ac_1PIM_2 and/or Ac_1PIM_4 have been suggested as

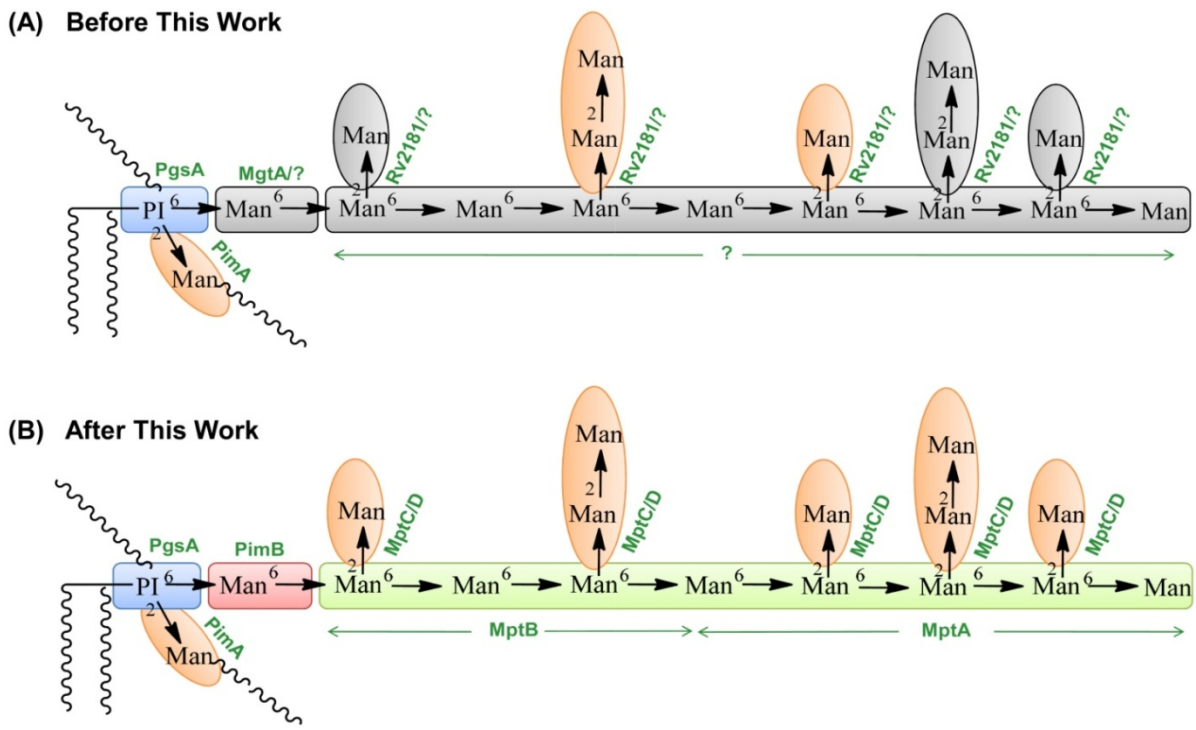

Fig.6.1: Biosynthetic pathway of LM biogenesis in *C. glutamicum* and *M. tuberculosis* before (A) and after (B) this work. (A) Before this work PgsA, PimA, acyltransferase (Rv2611c) and MgtA have been shown to be involved in the synthesis of PIMs, while Rv2181 in the synthesis of α(1→2)-Man*p* units in LM/LAM. However, enzymes involved in the synthesis of α(1→6)-Man*p* backbone were not known and have been identified in this study (**B**).

end products of the biosynthetic pathway on cytoplamic face and is later on flipped on the periplasmic side by an unidentified flippase.

Interestingly, α(1→6) mannan extension is more complex in *Mycobacterium* based on the evidence that Mt-MptB and Ms-MptB fail to complement the *C. glutamicum*Δ*mptB* mutant and suggests a slightly different substrate specificity of the MptB orthologues of *M. tuberculosis* and *M. smegmatis*. Redundency of Ms-MptB in *M. smegmatis*Δ*mptB*, indicate another, yet unidentified, mannosyltransferase that may substitute for MptB in the mutant or MptA itself or substituting for the deficiency of MptB. Creation of an M. *smegmatis* strain devoid of MptA and

MptB may shed further light on this aspect. However, usage of the *C. glutamicum* mutants have enabled the assignment of function and activity to the GT-C glycosyltransferases NCgl1505 and Rv1459c, which would have otherwise not been possible if similar studies would have concentrated solely on mycobacterial species (Fig. 6.1).

Disruption of *NCgl2106* has shown that this gene is involved in the synthesis of Ac_1PIM_2 and subsequently, PI-based LM and LAM in *C. glutamicum*. The study also highlighted the synthesis of a glycolipid, Man-α-**D**-glucopyranosyluronic acid-(1→3)-glycerol ($ManGlcAGroAc_2$) and $ManGlcAGroAc_2$ based LM (Cg-LM-B) in *C. glutamicum* by MgtA (Rv0557). The *M. tuberculosis* homologue of NCgl2106 (Rv2188c) has been identified as a novel phosphatidyl-*myo*-inositol mannosyltransferase, PimB and Rv0557 has been ruled out as a PimB candidate (Fig. 6.1 and Fig. 6.2). Herein, the use of *C.glutamicumΔpimB'ΔmgtA* mutant study enabled the assignment of true functions to mycobacterial glycosyltransferases Rv0557 and Rv2188c. Surprisingly, a lipoglycan similar to Cg-LM-B has not been identified in mycobacteria, and the precise role of Rv0557 in *M. tuberculosis*. However, it is possible that Rv0557 might supplement for 'loss of function' of Rv2188c, as suggested by our *in vitro* mannolipid synthesis study and recent studies (Torrelles *et al.*, 2009). To identify the true role of Rv0557 in *M. tuberculosis*, we can either create conditional mutants in *M. tuberculosis* devoid of Rv0557 and Rv2188c or try to develop methods to fractionate polar lipids and lipoglycans from *M. tuberculosis* in search for such novel glycolipids.

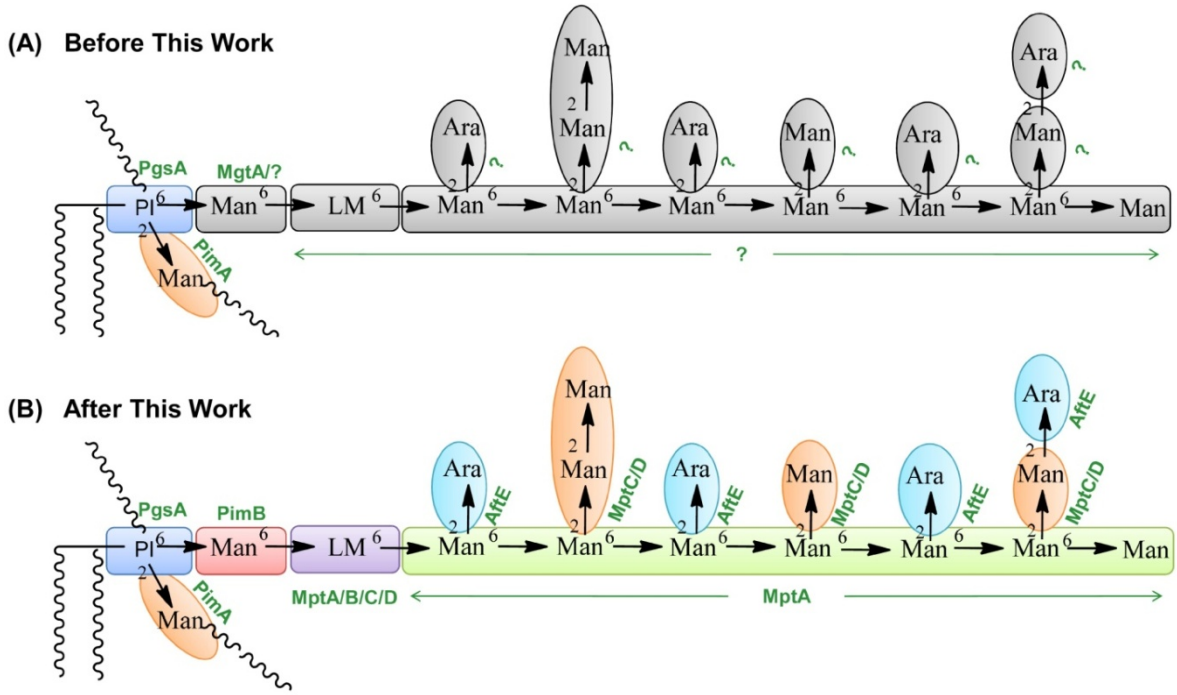

Fig.6.2: Biosynthetic pathway of Cg-LAM biogenesis in *C. glutamicum* before (A) and after (B) this work. (A) Before this work, only MgtA has been characetrised, which was shown to be involved in the synthesis of a mannolipid that is not involved in the synthesis of Cg-LAM. (**B**) The entire pathway of Cg-LAM biogenesis has been characterised in this work by assigning functions to the five different ORFs (*NCgl1505*, *NCgl2093*, *NCgl2096*, *NCgl2097* and *NCgl2100*) (**B**).

Characterisation of three different ORFs from *C. glutamicum* encoding putative glycosyltransferases from GT-C family, has suggested their role in cell wall biogenesis. On the basis of biochemical analysis of mutants, NCgl2100 and NCgl2097 are identified as α(1→2) mannopyranosyltransferases (MptC and MptD), and NCgl2096 as an α(1→2) arabinofuranosyltransferase (AftE), involved in LM/LAM biosynthesis. Furthermore, complementation of mutant phenotypes with their mycobacterial homologues suggested that Rv2181 is a true functional homologue of NCgl2100 (Fig. 6.1 and Fig. 6.2) and involved in the synthesis of singular α(1→2)-Man*p* units of LM and LAM.

The function of these glycolipids and lipoglycans is still a controversial topic in mycobacterial research. In that regard, lipoglycans from these *C. glutamicum* mutant strains provide elegant substrates for immunological studies, which can shed further light on the role of these lipoglycans and host pathogen interactions. In addition, the generation of *M. tuberculosis* mutants deficient in the enzymes identified in this study may be utilised to study their functional role in mycobacteria.

The entire reportire of enzymes involved in the biogenesis of PIMs and LM/LAM have almost been identified and some of them reperesent execellent drug targets. However, the participation of cytoplasmic and periplasmic biosynthetic machinary suggests the involvement of active transport of cytoplamic content to the extracellular part. This requires an in-depth inspection of the biochemistry behind the transport of these glycolipids and identification of molecular components involved which can be utilised as novel drug targets. Similarly, the concept of host-pathogen interaction involving PIMs and LM/LAM is still unexplored and therefore, further study is required utilsing these molecules, their receptors and interaction between them. PIMs and LM interact with Toll like receptor-2 and LAM interacts with Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin, DC-SIGN and Mannose receptor (Briken *et al.*, 2005). Study involving these molecular level interactions in which PIMs and LM/LAM could be co-crystalised with their respective receptors may shed further light on the molecular mechanism beneath host pathogen interactions.

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