DISSERTATION

# EXPORT MEDIATED ASSEMBLY OF MYCOBACTERIAL GLYCOPROTEINS PARALLELS EUKARYOTIC PATHWAYS

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY BRIAN CHRIS VANDERVEN ENTITLED EXPORT MEDIATED ASSEMBLY OF MYCOBACTERIAL GLYCOPROTEINS PARALLELS EUKARYOTIC PATHWAYS BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

EXPORT MEDIATED ASSEMBLY OF MYCOBACTERIAL GLYCOPROTEINS PARALLELS EUKARYOTIC PATHWAYS

Protein *O*-mannosylation is an essential and evolutionarily conserved posttranslational modification among eukaryotes. This form of protein modification is also described in *Mycobacterium tuberculosis*; however, the mechanism of mannoprotein assembly is fundamentally undefined. One *M. tuberculosis* H37Rv open reading frame (*Rv1002c*) was identified as a putative protein mannosyltransferase based on sequence similarities to known eukaryotic protein mannosyltransferases. To understand the initial stages of mycobacterial *O*-mannoprotein biosynthesis, *Rv1002c* was cloned and overexpressed in *M. smegmatis.* Cytoplasmic membrane extracts containing recombinant Rv1002c enzymes were assayed for protein mannosyltransferase activity establishing that Rv1002c functions as a protein mannosyltransferase. Two conserved amino acid residues of Rv1002c that comprise a putative active site of eukaryotic protein mannosyltransferases are also required for Rv1002c activity. Additionally, to determine what types of secreted proteins can potentially be mannosylated by *M. tuberculosis* a strategy utilizing differentially translocated chimeric proteins and mass spectrometry to monitor glycosylation was employed. Specifically, recombinant mycobacterial proteins secreted by the Sec (FbpC), SNM (ESAT-6) and SecA2 (SodA) pathways were fused to a native mycobacterial glycosylation domain from MPT32. These fusion proteins were expressed in mycobacterial hosts, purified, and analyzed for the presence of

mannosylation. This approach established that *M. tuberculosis* protein mannosylation is dependent upon Sec-translocation and does not involve the SNM or SecA2 protein secretion pathways. Lastly, non-secreted forms of these fusion proteins and native cytosolic *M. tuberculosis* proteins were not mannosylated providing strong evidence that mycobacterial protein mannosylation occurs extracellularly.

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DEDICATION

To my wife:

Mindy VanderVen

for giving so much

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# ABBREVIATIONS

| | |
|---|---|
| Å | Angstrom |
| AG | Arabinogalactan |
| Ala | Alanine |
| Amp | Ampicillin |
| APC | Antigen Presenting Cell |
| Ara | Arabinose |
| Arg | Arginine |
| Asp | Aspartic acid |
| Asn | Asparagine |
| ATP | Adenosine triphosphate |
| Bac | Bacillosamine |
| BCA | Bicinchoninic acid |
| bp | base pair |
| BSA | Bovine serum albumin |
| cDNA | Complementary DNA |
| CFP | Culture filtrate proteins |
| CID | Collisionally induced dissociation |
| ConA | ConcanavalinA |
| CV | Column volume |
| CW | Cell wall |
| Cys | Cysteine |
| CYT | Cytosol |
| Da | Dalton |
| DAP | meso-diaminopimelic acid |
| Dol-P-Glc | Dolichol-P-Glucose |
| Dol-P-Man | Dolichol-P-Mannose |
| dTDP-Rha | deoxyribosylthymine 5'-diphosphate |
| DNA | Deoxyribonucleic acid |
| DTT | Dithiothreitol |
| EDL | Electron dense layer |
| ER | Endoplasmic Reticulum |
| ESI-MS | Electrospray Ionization Mass Spectrometry |
| ETOH | Ethanol |
| ETZ | Electron Transparent Layer |
| *f* | furanose |
| FucNAc | N-fucosamine |
| G | gram |
| Gal | Galactose |
| GalNAc | N-acetylgalactosamine |
| Glc | Glucose |
| GlcNAc | N-acetylglucosamine |

| | |
|---|---|
| Glu | Glutamic acid |
| HBHA | Heparin binding hemagglutinin |
| His | Histidine |
| HPLC | High Pressure Liquid Chromatography |
| Hr | hour |
| Hyg | Hygromycin |
| IFN-γ | Interferon-γ |
| IL-12 | Interlukin-12 |
| Kan | Kanamycin |
| KDa | kilodalton |
| KV | kilovolt |
| LAM | Lipoarabinomannan |
| LB | Luria Broth |
| LC | Liquid Chromatography |
| LM | Lipomannan |
| Lys | Lysine |
| M | Molar |
| MAGP | mycolic acid-arabinogalactan-peptidoglycan |
| Man | Mannose |
| ManNAc | N-acetylmannosamine |
| ManLAM | Mannosylated LAM |
| mg | milligram |
| MHC | Major Histocompatibility Complex |
| Min | minute |
| mL | milliliter |
| mm | millimeter |
| mM | millimolar |
| MMR | Macrophage Mannose Receptor |
| mRNA | messenger RNA |
| MS | Mass Spectrometry |
| OL | Outer layer |
| ORF | Open Reading Frame |
| OST | Oligosaccharyltransferase |
| *p* | pyranose |
| PBS | Phosphate buffered saline |
| PCR | Polymerase Chain Reaction |
| PDIM | Phthiocerol dimycocerosates |
| PGL | Phenolic glycolipids |
| PIM | Phosphatidylinositol mannosides |
| pmol | picomole |
| PMT | Protein O-mannosyltransferase |
| Pro | Proline |
| Rha | Rhamnose |
| RNA | Ribonucleic acid |
| RT-PCR | Reverse Transcription PCR |
| Ser | Serine |

| | |
|---|---|
| Sp-A | Surfactant protein A |
| SRP | Signal Recognition Particle |
| TAT | Twin Arginine Translocation |
| TDM | trehalose dimycolate |
| Thr | Threonine |
| TLR | Toll Like Receptor |
| TNF-α | Tumor Necrosis Factor-α |
| TMM | trehalose monomycolate |
| Tyr | Tyrosine |
| μg | microgram |
| WHO | World Health Organization |

# Chapter I

## Literature Review Part I: History, Tuberculosis Disease, and the Unique Characteristics of the *Mycobacterium tuberculosis* Surface.

### 1.1 Historical perspective of tuberculosis

Skeletal remains suggest that *Mycobacterium tuberculosis* has plagued human beings for thousands of years. Disfigured skeletons with malformed vertebrae consistent with skeletal tuberculosis pathology (Potts disease) were discovered and carbon dated to 5,400 years in age (36). These bone samples tested positive for the presence of mycobacterial DNA suggesting that the skeletal deformities were mycobacterial in orgin (36). Additionally, *M. tuberculosis* DNA was extracted from mummified tissue resembling pulmonary granulomas dating back to 4,000 years in age (211). Documentation of pulmonary tuberculosis symptoms first appeared around 600 B.C. in European medical references (73). These early medical descriptions accurately described tuberculosis symptoms and how the disease could be potentially spread without description of the causative agent. In 1868 the French surgeon, J. Villemin, demonstrated that tuberculosis was transmissible from humans to rabbits by inoculation, providing the first evidence that tuberculosis was in fact a contagious disease (73). In the years following J. Villemins' research Robert Koch discovered the tubercle bacilli, and in 1882 provided evidence that the acid-fast bacilli *M. tuberculosis* was indeed the etiologic agent of tuberculosis (73).

Despite plaguing mankind throughout history, tuberculosis remains one of the world's most destructive diseases. Presently the World Health Organization (WHO) estimates that nearly one-third of the world's population is infected with *M.*

*tuberculosis* and in the year 2003 approximately 1.75 million deaths resulted from tuberculosis worldwide (www.who.int/mediacentre/factsheets/fs104/en). Threatening our current control measures is the recent emergence of multi-drug resistant strains of *M. tuberculosis* (MDR-TB) and the HIV-associated tuberculosis epidemic. In the year 2000 alone 273,000 new cases of MDR-TB were observed, and tuberculosis accounted for 13% of the AIDS related deaths in 2003 (49). It is unlikely that TB will be eradicated by chemotherapy, which is continuously hampered by a large reservoir of latently infected individuals and delays in diagnosis. However, even a vaccine of modest efficacy when combined with chemotherapy could potentially reduce the global burden of TB. Accordingly, current tuberculosis research is focused on identifying new strategies to better control *M. tuberculosis*.

## 1.2 The bacillus

Mycobacteria are non-motile, rod-shaped bacteria of the order *Actinomycetales* belonging to the family *Mycobacteriaceae*, which encompasses the lone genus, *Mycobacterium*. Mycobacteria are distinguished by having a high DNA G+C content (62-70 mol%), and cell walls containing long chain mycolic acid that retain the carbol fushin dye following an acid alcohol wash thus are termed acid fast. The genus *Mycobacterium* is principally comprised of nonpathogenic environmental bacteria, however, *M. tuberculosis* and *M. leprae*, the causative agent of leprosy, are well-adapted human pathogens. *M. tuberculosis*, *M. africanum*, *M. canettii*, *M. bovis*, and *M. microti* comprise the *M. tuberculosis* complex sharing identical 16s RNA sequences and are often associated with human and animal disease. *Mycobacterium* spp. can also be divided into slow- and fast-growing bacteria. *M. tuberculosis*, *M.*

*bovis, M. canettii,* and *M. africanum* grow slowly (12-24 hour doubling time) and typically cause disease, while fast growers (1-4 hour doubling time) such as *M. smegmatis* and *M. phlei,* are widely considered to be nonpathogenic. Members of the *M. tuberculosis* complex are also traditionally classified based on specific host tropisms. *M. tuberculosis, M. africanum,* and *M. canettii* are common human isolates while *M. microti* was originally isolated from field voles, and *M. bovis* from cattle. *M. bovis* has an expanded host range, and is capable of infecting several mammalian species including man. Thus, *M. bovis* is believed to be the primary cause of tuberculosis in humans prior to widespread pasteurization of milk. Recent genome sequencing efforts and comprehensive cross genome comparisons have now confirmed that the *M. tuberculosis* complex evolutionarily stands alone within the genus and each member of the complex has co-evolved from a "*M. tuberculosis* like" common ancestor while *M. bovis* most likely evolved directly from an *M. tuberculosis* organism (21).

## 1.3 Host response

### 1.3.1 Pathogenesis

*M. tuberculosis* is primarily transmitted by the respiratory route, and although the bacterium can initiate disease in multiple organs, pulmonary tuberculosis remains the most common form of disease. Pulmonary tuberculosis initiates when airborne particles containing *M. tuberculosis* are deposited in terminal alveoli and the bacilli are phagocytosed by scavenging macrophages, dendritic cells, or invade epithelial pneumocytes (15, 54). Following infection host cells initiate the formation of granulomatous lesions in an attempt to prevent bacterial dissemination. The formation

of human granulomas is a complex process, requiring activated macrophages, giant cells, T cells, B cells, and fibroblasts (58). Characteristic of an *M. tuberculosis* granuloma is a central region of caseous necrosis rich in acellular debris (43). Within human granulomas, *M. tuberculosis* primarily localizes to peripheral macrophages although some bacilli are present within the central caseous region (43). Successful containment of mycobacteria occurs when the granuloma develops, becoming fibrotic and/or calcified and encloses the bacilli. Non-fibrotic, non-calcified mycobacterial granulomas are thought to be responsible for transmission once they rupture. Release of bacteria-loaded, liquefied caseum or infected host cells into adjacent airways or blood vessels allows for airborne transmission of the bacilli or dissemination to other organs, respectively (43). Furthermore, release of bacilli into neighboring lung tissues initiates new granuloma formation, leading to the subsequent pathology and the consequent morbidity associated with tuberculosis.

### 1.3.2 Host cell binding

As mentioned, *M. tuberculosis* has the unique ability to successfully thrive and multiply within macrophages, an environment typically lethal to microorganisms. Specifically, *M. tuberculosis* lives in macrophage phagosomes where it prevents normal phagosome maturation and blocks fusion events between bacterial phagosomes and lysosomes (159). Because *M. tuberculosis* primarily exists intracellularly, efficient phagocytosis of the bacilli is a crucial event, and may occur many times throughout the course of infection. Phagocytosis events are orchestrated by a variety of mycobacterial surface ligands and host cell receptor pairings. Various host cell surface receptors including the CR1, CR3, and CR4 complement receptors,

mannose receptor, surfactant receptor, dendritic cell-specific intercellular adhesion molecule-3 grabbing nonintegrin (DC-SIGN) and Fc receptors mediate *M. tuberculosis* phagocytosis (3, 48, 62, 170, 173, 210).

Following activation of the alternative complement pathway *M. tuberculosis* can be opsonized by C3b and iC3b leading to phagocytosis via the complement receptors CR1, CR3, and CR4 with 80% of complement-mediated phagocytosis events occurring via CR3 (171). *M. tuberculosis* binds to CR3 through two distinct sites on the receptor. Opsonized *M. tuberculosis* binds CR3 at its C3bi binding domain, while nonopsonized bacteria interact with the β-glucan binding site of the CR3 α-subunit (CD11b) (37, 38). The CR3 β-glucan binding domain binds mannose, *N*-acetyl-D-glucosamine, or glucose moieties with high affinity (38, 188). Interestingly, pathogenic mycobacterial species, such as *M. tuberculosis, M. avium*, and *M. kansasii*, can be phagocytosed in a non-opsonized state by CR3 while the non-pathogenic species *M. smegmatis* cannot (37, 38). The ligands recognized by the β-glucan binding domain of CR3 remain undefined, but there is some speculation that mycobacterial surface glucan or capsular mannan participate as ligand (38).

Additionally, phagocytosis of *M. tuberculosis* occurs by the macrophage mannose receptor (MMR) (170). MMR is a pattern recognition receptor belonging to the family of C-type (calcium-dependent) lectins which bind mannose- and fucose-containing glycoconjugates (186). Binding and subsequent phagocytosis by the MMR can discriminate between virulent and attenuated strains of *M. tuberculosis* (170, 172). Mannose caps present on the major surface lipoglycan, mannosylated lipoarabinomannan (ManLAM), of *M. tuberculosis* serve as a ligand of the MMR

accounting for 50% of MMR mediated phagocytosis (172). Several additional mycobacterial glycosylated MMR ligands have been identified including phosphatidylinositol mannosides (PIMs) (80, 88, 196, 210). Specifically, exogenously added PIM molecules (PIM2 and PIM6) block uptake of *M. tuberculosis* and, when coated on latex beads, PIM2 directs MMR dependent particle phagocytosis (195).

Human surfactant protein-A receptor and the human class-A scavenger receptor both may participate in mycobacterial phagocytosis. The macrophage protein-A receptor binds surfactant protein-A (Sp-A) complexed to a ligand, promoting macrophage phagocytosis. Sp-A enhances macrophage binding and uptake of *M. tuberculosis*, however, these mechanisms are not fully elucidated (48). Purified Sp-A binds directly to proteins of the *M. tuberculosis* cell envelope, and in membrane overlay experiments, Sp-A interacted with a 60-kDa protein (141). Macrophage phagocytosis of *M. bovis* BCG was blocked with an antibody specific to the Sp-A receptor (197). Human class-A scavenger receptors bind polyanionic macromolecules of bacteria (99). Blocking CR1, CR3, and CR4 with monoclonal antibodies and MMR with a competitive inhibitor, mannosylated bovine serum albumin, demonstrated that the class-A scavenger receptor mediates phagocytosis of *M. tuberculosis* (209). Purified class A scavenger receptors bind *M. tuberculosis* and sulfolipids, a molecule restricted to pathogenic mycobacteria (48, 210). It is unknown if scavenger receptors directly mediate phagocytosis or simply bind bacteria and phagocytosis is driven by other receptors.

Lastly, phagocytosis of mycobacteria by dendritic cells is an important event and the DC-SIGN receptor is implicated in this process (111, 184). DC-SIGN like the

MMR is a C-type lectin possessing a high affinity towards mannose-containing glycoconjugates. DC-SIGN mediated entry is specific to dendritic cells given that macrophages do not express these receptors (184). DC-SIGN mediated phagocytosis by dendritic cells can be driven by the mycobacterial ligand ManLAM (65, 111, 184). Blocking DC-SIGN by antibody interactions inhibits up to 90% of *M. tuberculosis* binding to human dendritic cells while anti-CR3 and -MMR antibodies had no effect on binding (184). In total, a variety of glycosylated mycobacterial surface molecules are involved as ligands in phagocytic events. Understanding of the phagocytic receptors utilized by *M. tuberculosis in vivo* and whether or not these receptors provide a beneficial route of entry into phagocytic cells are both matters of current research and speculation.

### 1.3.3 Acquired immunity

Host cell control of mycobacterial infections is focused at the granuloma, where T cells provide much of the protective immune response. Successful T cell control of *M. tuberculosis* infections requires several populations, including $CD4^+$ and $CD8^+$ T cells, γδ T cells, and CD1 restricted T cells (134, 157). During *M. tuberculosis* infection T cells are presented the proper antigen and secrete interferon-γ (IFN-γ), which synergizes with tumour necrosis factor-α (TNF-α) as central mediators of a Th1 immune response (9, 30, 56, 59, 102, 136). TNF-α is produced in high levels by T cells and infected macrophages or dendritic cells. Furthermore, Interleukin-12 (IL-12) produced by macrophages and dendritic cells in response to *M. tuberculosis* infection dramatically enhances IFN-γ secretion by T cells and appears to participate in granuloma formation (101). Activated macrophages in turn eliminate

intracellular mycobacteria through the generation of reactive nitrogen intermediates and/or reactive oxygen intermediates via the oxidative burst (58). Additionally, T cells secrete perforin and granulysin to directly kill *M. tuberculosis* and the infected macrophages (180). It is well established that proper Th1 type cytokine signaling events are required for the control of infection and formation of granulomas. Gene-targeted knockout mice lacking the cytokines IFNγ and IL-12 fail to control mycobacterial infections and form poor granulomas (30, 31). Likewise, TNF-α production is also required for control of acute *M. tuberculosis* infection given that mice deficient in TNF-α or the TNF-α receptor succumb rapidly when infected with *M. tuberculosis* (9, 59). Additionally, TNF-α deficient mice form granulomas that are disorganized and contain fewer activated macrophages (59).

$CD4^+$ is the major T cell phenotype involved in defense against tuberculosis (58). $CD4^+$ T cells secrete high levels of IFN-γ following recognition of antigen, which activates infected macrophages. Direct cytolytic activity of $CD4^+$ T cells directed to mycobacteria-pulsed monocytes *in vitro* has been described, but the contribution of cytolytic $CD4^+$ T cells in controlling mycobacterial infections remains unknown (23, 185). $CD4^+$ T cells recognize antigen presented in the context of Major Histocompatibility Complex (MHC) II. Mycobacterial protein and peptide antigens present in endocytic or phagocytic compartments of activated Antigen Presenting Cells (APC) encounter competent MHC class II molecules through direct fusion with endocytic vesicles (190). MHC class II molecules are made competent by compartment-specific glycosylation and proteolytic removal of the invariant chain-derived peptide, a process that is catalyzed by HLA-DM (H2-M in mice) (127). After

competent MHC class II molecules bind peptide antigen the MHC class II/peptide complex reaches the plasma membrane via a poorly defined exocytic process (128). It remains unknown how mycobacterial antigens are loaded into MHC class II molecules by resting APCs (190).

$CD8^+$ T cells also contribute in controlling mycobacterial infections since $\beta_2$-microglobulin deficient mice, lacking mature $CD8^+$ T cells, exhibit increased susceptibility to *M. tuberculosis* infections (57). $CD8^+$ T cell populations contribute to controlling mycobacteria infections by antigen-specific IFN-γ secretion and direct cytolytic activity (102, 180, 185). MHC class I dependent antigen presentation to $CD8^+$ T cells is observed primarily with cytoplasmic viral antigens, but recently MHC class I restricted $CD8^+$ T cell clones were isolated specific to the MPT32, 38 kDa, 19 kDa, 65-kDa, 85a, 85b, ESAT-6, and CFP10 *M. tuberculosis* antigens (87, 100, 102, 123, 176, 177, 200). MHC class I antigens require cytoplasmic processing by the host cell proteasome and the resulting peptides are transported into the lumen of the endoplasmic reticulum (ER) by TAP (transporter associated with antigen presentation) (129). In the ER lumen a mature MHC class I molecule associates with the luminal face of TAP where it is presumably loaded with peptide antigen (181). After the MHC class I molecule binds a peptide, it is exocytosed to the cell surface for presentation to T cells (203). How mycobacterial protein antigens gain access to MHC class I presentation is not understood and could involve a phagosomal pore or active trafficking of specific proteins into the host cell cytosol (11, 167).

γδ T cells can also secrete IFN-γ potentially leading to the activation of APCs and can be directly cytolytic (46, 189). These T cells specifically recognize non-

peptide, small-phosphorylated antigens in an antigen-specific manner, and how these antigens are processed and presented to γδ T cells is unknown. *M. tuberculosis* specific human γδ T cell clones that display cytolytic activity towards infected APCs *in vitro* have been identified (140). The role of γδ T cells in mycobacterial infections is not understood, but evidence suggests that γδ T cells participate in inflammation or cellular trafficking events of the lung (165).

CD1 restricted T cells belong to a newly discovered class of T cells capable of recognizing lipid and glycolipid antigens presented in the context of CD1 molecules. Like the $CD4^+$, $CD8^+$ T cells, and γδ T cells, CD1 restricted T cells activate macrophages through the secretion of IFN-γ and can display cytolytic phenotypes when presented the proper non-peptide antigen (10, 148, 175). CD1a-d are antigen-presenting molecules closely related to MHC class I proteins and possess a distinct hydrophobic antigen-binding groove allowing presentation of lipids to T cells (206). Exactly how and where in the host cell lipid antigens are loaded into CD1 molecules remains poorly understood.

### 1.3.4 Innate immunity

It is clear that T cell mediated immunity is essential for controlling mycobacterial infections, however, certain aspects of the innate immune response also direct outcomes of disease (93). Toll-like receptors (TLRs) are a family of membrane bound receptors that recognize conserved ligand patterns and initiate specific intracellular responses. Several microbial ligands interact with TLRs resulting in cytokine and chemokine production or alteration, which in turn regulates the host immune response (76). TLRs recognize several *M. tuberculosis* antigens

resulting in activation and/or modulation of the host immune response (19). Specifically, LAM, PIM, 19-kDa lipoprotein, and lipomannan (LM) are recognized by TLR2 or TLR4 resulting in the modulation of TNF-α secretion by macrophages (19, 66, 85, 86, 150, 194). Precisely how TLR mediated cytokine modulation impacts *M. tuberculosis* infections *in vivo* is poorly understood and is currently under evaluation by a number of laboratories.

Understanding how *M. tuberculosis* is recognized and controlled by the human immune system is a prerequisite for constructing and evaluating new vaccine candidates and strategies. Effective vaccination represents an important goal in tuberculosis control, and is perhaps the best hope for the eventual eradication of tuberculosis. The importance of cytokines such as IFN-γ is well established, and the role of key lymphocyte effector cells has begun to be elucidated. Despite advances in our understanding of tuberculosis immunology, how the bacillus gains access and lives in host cells or granulomas, and how each T cell population contributes to controlling the infection requires further elucidation. Comparable to all infectious diseases, the clinical manifestations of tuberculosis are caused by a balance between certain bacterial factors and the human host immune response.

For many years the lipid-rich cell envelope of *M. tuberculosis* was implicated in virulence and this idea is supported by numerous studies demonstrating immuno-modulatory activities of purified surface components. In addition to the activities elicited by mycobacterial lipids, several proteins have been identified that alter the immune response and direct the outcome of disease. Undoubtedly the complex and unique physiology of *M. tuberculosis* contributes directly to the success of this

organism in overcoming both the innate and acquired immune mechanisms of the host.

## 1.4 Pathogenic mycobacterial physiology

Comprehensive understanding of *M. tuberculosis* physiology in terms of pathogenesis and overcoming host immune systems is lacking, and how the bacillus persists indefinitely within the human host remains enigmatic. It is clear that the elaborate *M. tuberculosis* cell envelope provides a robust physical barrier while at the same time directly influences pathogenesis (17, 18, 98). Also, numerous extracellular *M. tuberculosis* molecules contribute to the survival of the bacillus while having extraordinary roles in pathogenesis (149, 192). Thus, considerable efforts have been devoted to understanding the structural characteristics of the mycobacterial cell envelope constituents and their biosynthesis.

### 1.4.1 Cell wall core

Classic and recent studies employing negative staining, freeze fracture, electron microscopy, X-ray diffraction, electron spin resonance, and differential scanning calorimetry revealed a highly ordered mycobacterial cell envelope structure (7, 83, 108, 109, 133). Traditionally, the asymmetric mycobacterial cell envelope is divided into four sections based on appearances by thin section electron microscopy: 1) the plasma membrane, 2) the electron-dense layer (EDL), 3) the electron-transparent zone (ETZ), and 4) the outer layer (OL) (Fig. 1.1).

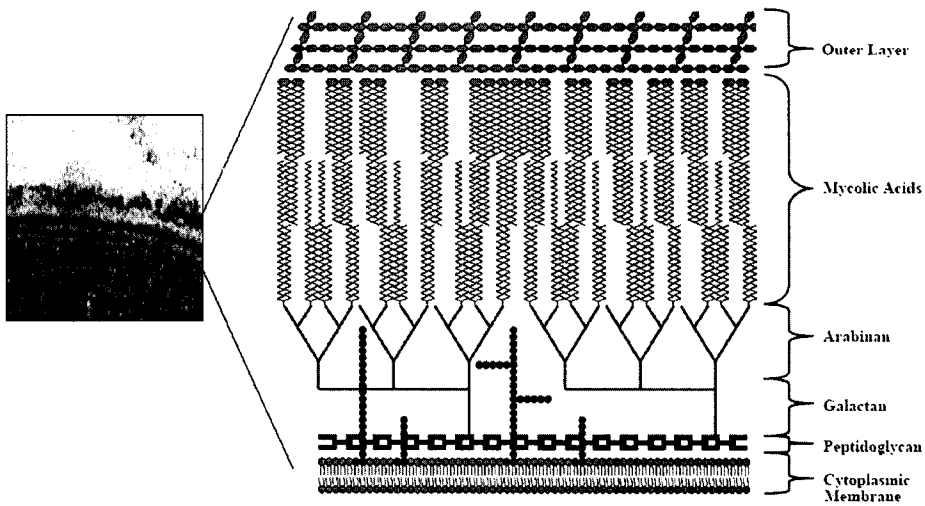


**Fig 1.1 Electron micrograph of *M. phlei* cell envelope adapted from Brennan and Nikaido (18).** Schematic representation of mycobacterial cell wall, depicting the major molecules believed to be responsible for the electron micrograph appearance.

Mycobacterial plasma membranes are typical phospholipid bilayers containing the integral and peripheral membrane proteins required for electron transport, ATP synthesis, solute transfer, protein translocation, and a variety of other biosynthetic processes (18). *M. tuberculosis* plasma membranes contain typical membrane phospholipids, tuberculostearic (10-methyloctadecanoic) acid, and phosphatidylinositol mannosides whose distribution is restricted to mycobacteria and related genera. External to the plasma membrane is the mycolic acid-arabinogalactan-peptidoglycan (mAGP) complex that spans the EDL and the ETZ layers. mAGP is comprised of peptidoglycan covalently linked to polymeric arabinogalactan (AG), which is esterified at the non-reducing termini with mycolic acids (17). Mycobacterial peptidoglycan is of the typical A1γ type, consisting of a repeated β-1,4-linked *N*-acetylmuramic acid (MurNAc) and *N*-acetylglucosamine (GlcNAc) disaccharide unit. Unlike other bacteria the muramic acid residues of the mycobacterial peptidoglycan can be oxidized forming *N*-glycolyl muramic acid (MurNGly) (103, 151). The

muramic acid of Mycobacterial peptidoglycan is joined to the tetrapeptide sidechain comprised of (L-alanyl-D-isoglutaminyl-meso-diaminopimelyl-D-alanine). Cross-links in mature peptidoglycan occur between either two meso-diaminopimelic acid (DAP) residues or between the D-alanine residues (199).

AG is a tripartite carbohydrate molecule covalently attached to peptidoglycan and consists of a proximal linker, medial galactofuranosyl, and distal arabinofuranosyl regions (Fig. 1.2). The AG proximal linker region is comprised of the α-L-Rha*p*-(1→3)-α-D-Glc*N*Ac-(1→P) disaccharide, attached to mycobacterial peptidoglycan by a phosphodiester linkage at the C6 position of MurNAc/MurNGly residues (34). Linear polymeric galactan comprises the medial portion of AG and consists of approximately 30 alternating (1→5) and (1→6)-linked β-D-Gal*f* residues (Fig. 1.2) (39). The arabinan component of AG is a linear α(1→5)-linked α-D-Ara*f* chain branched with a α(1→3)-linked α-D-Ara*f* unit and a α(1→5)-linked α-D-Ara*f* unit (39, 112). Ara6 domains or ([β-D-Ara*f*-(1→2)-α-D-Ara*f*]$_2$-3,5-α-D-Ara*f*-(1→5)-α-D-Ara*f*) terminate the nonreducing termini of the arabinan segment (Fig. 1.3) (39, 112). The non-reducing end of the Ara6 motif is terminated with the disaccharide β-D-Ara*f*-(1→2)-α-D-Ara*f*-(1→) which serves as the attachment point of mycolic acids.

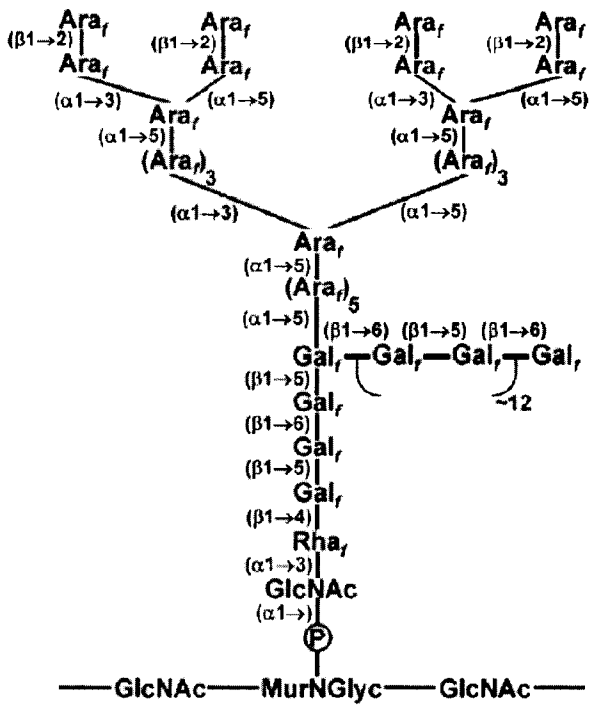


**Fig 1.2 Major subunit of mycobacterial cell envelope.** Linkages between the adjacent residues is denoted in parentheses. Note, two additional arabinan domains exist attached to the linear galactan molecule that are not shown.

Mycolic acids are located on approximately two-thirds of the nonreducing terminal arabinofuranosides and are anchored by ester bonds at the C5 positions of the arabinose residues (Fig. 1.3) (34). Mycolic acids are the (C60-C90) α-branched, β-hydroxy fatty acids extending out from the cell wall comprising the majority of the cell envelope structure. Mycolic acids contain two fatty acyl chains. The main acyl chain or mero chain is 50-60 carbon atoms in length and possesses unsaturations, keto, methoxy, and/or cyclopropane modifications (Fig. 1.3) (8). The second acyl chain or α-chain is a saturated fatty acyl function typically of 24 carbons in length (8). Recent analyses indicate that the mycolic acids may be arranged in parallel by hydrophobic interactions and this configuration likely accounts for the low permeability of the mycobacterial cellular envelope (108). Exterior to and/or

intercalated within the covalently bound mAGP structure lies the less well-defined OL capsular material.

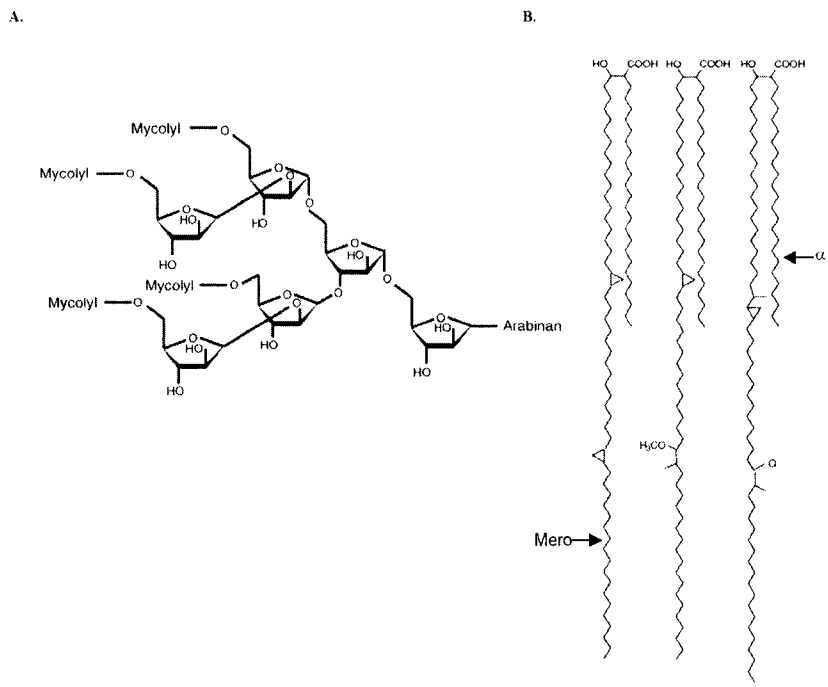


**Fig 1.3 Major mycobacterial cell envelope constituents. (A)** Non-reducing terminal Ara6 motif found on the arabinogalactan molecule. The points of mycolic acid attachment are denoted by mycolyl. **(B)** Three major forms of mycolic acids found in the mycobacterial cell envelope.

### 1.4.2 Peripheral cell wall components

The *M. tuberculosis* OL material consists primarily of polysaccharides, free lipids, lipoglycans, and proteins loosely bound to the cellular envelope. The principal polysaccharide component of the OL is a glycogen-like polysaccharide with an apparent molecular mass of 100 kDa termed glucan (106). Glucan is composed of linear (1→4)-α-D-glucosyl residues periodically branched at the C6 position with mono or di-(1→4)-α-D-glucosyl units (106, 137). The OL free lipids and glycolipids

are believed to be intercalated with the mycolic acids through hydrophobic interactions. Free lipids and glycolipids of the *M. tuberculosis* OL include phthiocerol dimycocerosates (PDIM), phosphatidylinositol mannosides, trehalose-6,6'-dimycolate (TDM), trehalose-6,6'-monomycolate (TMM), sulfolipid, other acylated trehalose molecules, and phenolic glycolipids (18, 107).

The hydrophobic PDIM molecules are commonly referred to as true mycobacterial waxes and can be found as phthiocerol dimycocerosate A and B and phithiodilone dimycocerosates based on their respective diol function (4). PDIM waxes consist of two multi-methyl branched mycocerosic acids esterified to phthiocerol. PDIM serves as the lipid core of the glycosylphenolphthiocerol dimycocerosates, known as phenolic glycolipids (PGL) (122). PGL molecules are present in *M. bovis*, *M. canettii* variants, and *M. tuberculosis* clinical isolates (HN878 and W-Beijing strain 210) (29, 153). It is hypothesized that PDIM molecules serve to stabilize the outer layer of the mycobacterial cell envelope (121). PDIM molecules are clearly associated with the pathogenicity of *M. tuberculosis* since mutants lacking these structures are attenuated in animal models of infection (22, 32). Additionally, *M. tuberculosis* PGL inhibits the release of TNF-α and IL-12 resulting in a hyper-lethal phenotype observed in mice (153).

### 1.4.3 Phosphatidylinositol mannosides

Phosphatidylinositol mannosides such as PIM, lipomannan (LM), lipoarabinomannan (LAM), and ManLAM are dominant glycolipids found in either the plasma membrane and/or the cell envelope and are based on an acylated phosphatidylinositol core (25, 90). Phosphatidylinositol mannosides are essential

molecules found in all *Mycobacterium* spp. Evidence is now accumulating suggesting that the phosphatidylinositol mannosides of *M. tuberculosis* are important immunomodulatory molecules (132). However, the presence of phosphatidylinositol mannosides in saprophytic mycobacterial species and their apparent essentiality for *in vitro* growth in *M. tuberculosis* suggests that these molecules have a more fundamental role in mycobacterial physiology. It is postulated that certain polar PIM species function to maintain membrane bilayer integrity but this remains a matter of speculation and current research. PIM based glycolipids are also believed to be the direct intermediates involved in LM/LAM/ManLAM biosynthesis (16).

The PIM phosphatidylinositol core is comprised of an inositol residue covalently attached to a diacylglycerol moiety through a phosphodiester bond (90). The acyl functions on PIMs interact within the plasma membrane and/or cell wall lipids through hydrophobic interactions. The inositol anchors a polysaccharide composed of one to six mannopyranose residues corresponding to six distinct molecules termed PIM1-6 (Fig. 1.4) (131). Additionally, the first mannose residue attached to the PI unit (PIM1) may be esterified at the C6 position with an acyl function and these triacylated molecules are referred to as AcPIM1-6 (89). Of the PIM family PIM5 and PIM6 serve as metabolic end-products containing linkages between the fourth and fifth mannose not found in LM or LAM, therefore, PIM4 likely functions as the precursor required for LM/LAM synthesis (89).

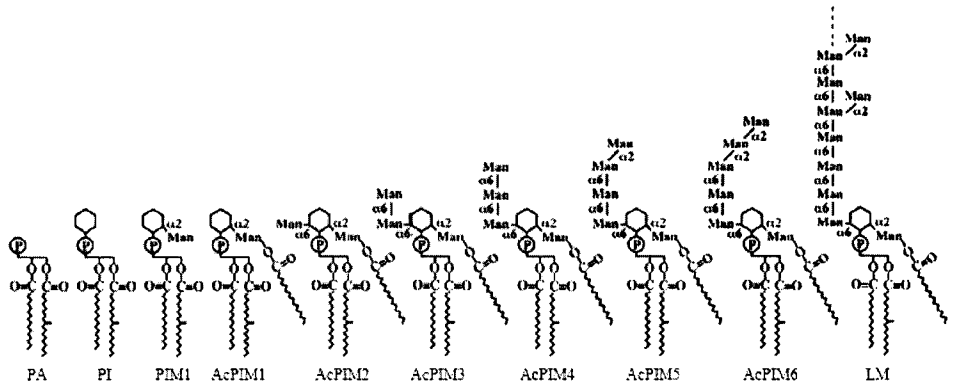


**Fig 1.4 Major phosphatidylinositol mannoside constituents of the mycobacterial cell envelope.** PA refers to phosphatidic acid and PI refers to phosphatidylinositol, which serves as the core of all PIM molecules. Mannosylated forms of PI are termed PIM1-6 based on the number of mannose residues present. Each PIM intermediate can be present in a diacylated form or a triacylated form indicated by the prefix Ac.

LM is simply PIM4 with a mannopyranose polymer estimated to be around 20 mannose units in length consisting of α-(1→6)-linked mannan with alternating α-(1→2)-linked mannose branches (25). LAM is LM that has undergone further elaboration by arabinosylation (26, 191). The arabinan segments of LAM, like the arabinans of mAGP, consist of linear α(1→5)-linked α-D-Ara*f* residues branched with a α(1→3)-linked α-D-Ara*f* unit and a α(1→5)-linked α-D-Ara*f* residue, both of which anchor nonreducing terminal arabinan domains (Fig. 1.5) (24, 39, 112). Non-reducing terminal regions of LAM arabinans contain an Ara6 motif, like mAGP, in addition to a linear Ara4 (β-D-Ara*f*-(1→2)-α-D-Ara*f*-(1→5)-α-D-Ara*f*-(1→5)-α-D-Ara*f*) motif (Fig. 1.5) (24). In *M. tuberculosis* LAM, mannose caps are attached to termini of the arabinan domain and is termed ManLAM. The mannose caps consist of either a single Man*p* residue, a dimannoside (α-D-Man*p*(1→2)-α-D-Man*p*→) or a trimannoside (α-D-Man*p*-(1→2)-α-D-Man*p*-(1→2)-α-D-Man*p*→), and these

mannose residues are attached through an α(1→5) linkage to the terminal Ara*f* residue (91).

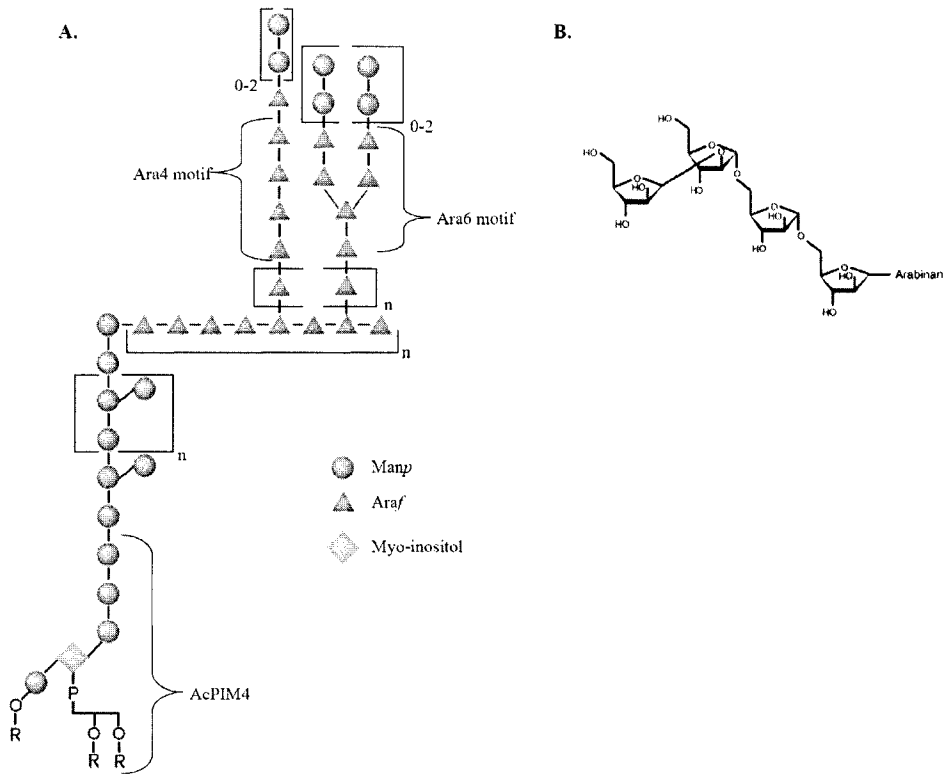


**Fig 1.5 Lipoarabinomannan structural features. (A)** Proposed structure of LAM; R, represents fatty acyl functions; n, indicates a repeated subunit of unknown length. The Ara6 motif is found in the cell wall bound mAGP and LAM structures. (B) Structure of the Ara4 motif found in LAM.

TDM or cord factor is the molecule responsible for the distinguishing serpentine cord forms *M. tuberculosis* takes when grown *in vitro* and observed by light microscopy (178). TDM is α,α' trehalose diesterified at both C6 positions with mycolic acids while TMM is α,α' trehalose monoesterified at a single C6 position with mycolic acids, and both of these glycolipids are highly abundant in the mycobacterial cell envelope. It is believed that the mycolic acids of TMM and TDM tether these glycolipids by hydrophobic interactions within the AG bound mycolic

acids of the cell envelope affording the bacterium some yet undefined benefit, perhaps to stabilize the outer mycolic acid layer.

Sulfolipids are also members of the acylated trehalose family of *M. tuberculosis* glycolipids isolated and chemically characterized in early studies (70, 71, 117). *M. tuberculosis* sulfolipids are 2,3,6,6'-tetraacyl trehalose-2'-sulphates where the acyl functions are typically a mixture of C14-C19 fatty acids frequently containing methyl branches. Sulfolipids are only present in *M. tuberculosis*, thus considerable effort was put forth toward understanding their role in pathogenesis. In early work sulfolipids were hypothesized to disrupt normal phagocytic functions and inhibit activation of macrophages (138, 207). Additionally, sufolipids are also highly immunogenic since they are recognized by sera from infected patients (35, 155). In contradiction to these results, recent work utilizing *M. tuberculosis* strains deficient in sulfolipids show no attenuation nor altered pathology in mouse or guinea pig models of infection (158). Exactly how sulfolipids contribute to mycobacterial physiology and pathogenesis is unknown.

## 1.5 Aspects of *M. tuberculosis* cell envelope biosynthesis

A current rationale for studying the *M. tuberculosis* cell wall is the need to identify targets for development of new antimycobacterial drugs. Therefore, aspects of contemporary mycobacterial research have focused on the biosynthesis of unique components required in mycobacterial pathogenesis and/or are essential molecules for bacterial viability. Carbohydrate structures of the mycobacterial cell envelope and peripheral components are quite well defined, including mAGP, which separates *M. tuberculosis* from most other prokaryotes. For this reason the biosynthetic origins of

mAGP have been scrutinized as potential drug targets revealing several unique enzymes all of which are essential for *in vitro* growth (164).

### 1.5.1 Peptidoglycan biosynthesis

Many specific details are unknown concerning *M. tuberculosis* peptidoglycan biosynthesis; however, it is generally assumed to proceed in a fashion similar to that found in *E. coli*. Peptidoglycan genesis initiates when enoylpyruvate derived from phosphoenoylpyruvate is linked to the 3 position of the GlcNAc residue of the UDP-GlcNAc and, subsequently, the enoylpyruvate function is reduced to form UDP-MurNAc. Unique to *Mycobacterium* spp., UDP-MurNAc can be further oxidized forming MurNGly (50, 151). L-alanine is linked to UDP-MurNAc/MurNGly followed by the sequential addition of D-glutamic acid, DAP and a D-alanyl-D-alanine dipeptide, forming the UDP-MurNAc/MurNGly pentapeptide precursor (50). The phosphoryl-MurNAc/MurNGly pentapeptide is transferred to the lipid carrier, polyprenyl phosphate and termed Lipid I (82). A single GlcNAc residue is subsequently added to Lipid I forming GlcNAc-MurNAc/MurNGly (pentapeptide)-diphosphoryl-polyprenol or Lipid II (114). Next, Lipid II is inverted through the cell membrane, exposing the disaccharide-glycopeptide portion of the lipid-linked molecule to the exterior face of the membrane where it can be utilized for the final stages of peptidoglycan assembly. The concluding stages of peptidoglycan assembly occur extra-cytoplasmically and involve transglycosylation and transpeptidation reactions catalyzed by a family of penicillin-binding proteins (67).

### 1.5.2 Arabinogalactan biosynthesis

Synthesis of the *M. tuberculosis* AG portion of the mAGP structure commences with a transfer of GlcNAc-1-phosphate from UDP-GlcNAc to the lipid carrier polyprenyl phosphate forming polyprenyl diphosphoryl-GlcNAc (118). Next, WbbL/Rv3265c, a α-3-L-rhamnosyltransferase, transfers a single rhamnose from deoxyribosylthymine 5'-diphosphate (dTDP-Rha) to polyprenyl diphosphoryl-GlcNAc forming α-L-Rha-(1→3)-α-D-GlcNAc-diphosphoryl polyprenol (120). Galactose residues are subsequently built on the α-L-Rha-(1→3)-α-D-GlcNAc-diphosphoryl polyprenol with UDP-galactofuranose (UDP-Gal*f*) serving as the donor for these reactions (Fig. 1.6) (198).

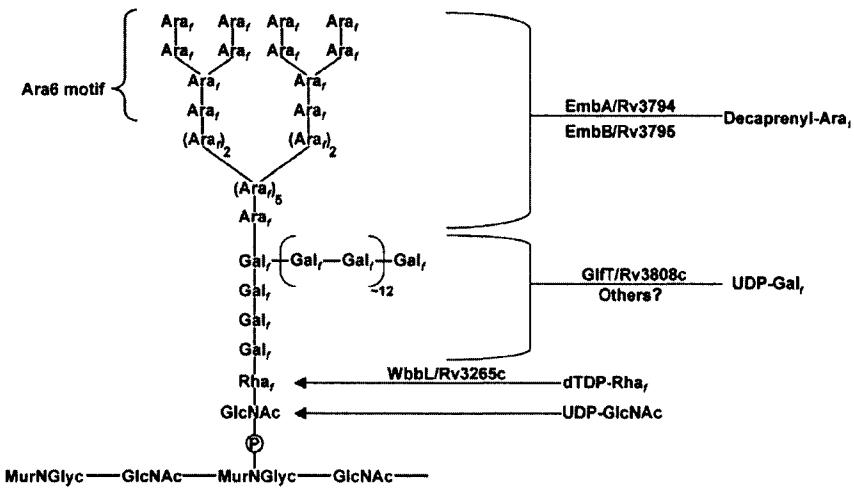


**Fig 1.6 Major polymeric subunit of the mycobacterial cell envelope indicating the precursors and enzymes involved in biosynthesis.** A single arabinan domain is shown; two additional chains exist attached near the reducing end of the galactan chain. UDP, uridine 5'-diphosphate; dTDP, deoxyribosylthymine 5'-diphosphate; Decaprenyl-Ara, decaprenylphosphoryl-D-arabinose. Reactions utilizing nucleotide sugar precursors are presumed to occur within the cytosol prior to transport of the whole lipid linked molecule. Arabinosylation reactions are hypothesized to occur extracellularly following transport.

Approximately 30 Gal*f* residues are built on a α-L-Rha-(1→3)-α-D-GlcNAc-diphosphoryl polyprenol molecule and one glycosyltransferase GlfT/Rv3808c is identified as being capable of adding Gal*f* to either the 5-position or the 6-position of another Gal*f* residue (96, 119). Distal to the galactan portion of the AG complex is the arabinan component, which is entirely built with polyprenyl phosphoryl-Ara*f* serving as the donor (168, 169). Substantial evidence has implicated EmbC,A,B/Rv3793,Rv3794,Rv3795 as the glycosyltransferases responsible for *M. tuberculosis* arabinosylation reactions, however, exactly how the Emb family of proteins function remains poorly understood. Most of our understanding regarding the Emb proteins is obtained from studies with *M. smegmatis* and it is widely accepted that similar processes occur in *M. tuberculosis*. Mutants lacking *embA* or *embB* are viable and arabinosylation of AG is diminished while arabinosylation of LAM remains unaffected in these mutants (51). It seems as if EmbA and EmbB operate as α-(1→3) arabinosyltransferases while EmbC is involved in some yet uncharacterized arabinosylation reaction of LAM (12, 14, 51, 208).

PIM/LM/LAM biosynthesis requires several distinct mannosyltransferases and can be distinguished by the specific mannose donor utilized; GDP-Man*p* for PI→PIM1→PIM2→PIM3 biosynthesis, or polyprenyl monophosphoryl-Man*p* in later →PIM4→PIM5→PIM6→LM→LAM→ManLAM synthesis. PIM biosynthesis is initiated by a distinct mannosyltransferase PimA/Rv2610c that uses GDP-Man*p* as the sugar donor, and transfers a mannose residue to the 2-position of the *myo*-inositol ring of phosphatidyl-*myo*-inositol (PI) forming PIM1 (94). The second mannosylation of PIM1 is catalyzed by PimB/Rv0557 which transfers another Man*p* residue from

GDP-Man*p* to the 6-position of the *myo*-inositol ring forming PIM2 (166). A final Man*p* unit is finally incorporated from GDP-Man*p* to the growing PIM molecule forming PIM3. In *M. bovis* the mannosyltransferase responsible for GDP-Man dependent PIM2→PIM3 extension is carried out by PimC/Mb1785c, an enzyme absent from the *M. tuberculosis* genome (97). Assembly of PIM1→PIM3 is believed to occur at the internal face of the cytoplasmic membrane due to the requirement for GDP-Man*p*, a strict cytosolic precursor. *M. tuberculosis*, like other bacteria, lack nucleotide sugar transporters in their cytoplasmic membranes. Thus extracellular assembly of carbohydrate containing molecules typically utilizes lipid-linked precursors.

Several mannosyltransferases likely extend PIM3 to PIM4-6/LM/LAM/ManLAM extracellularly utilizing the alkali-stable polyprenyl monophosphoryl-Man*p* molecule as the Man*p* donor (126). Polyprenyl monophosphoryl-Man*p* is synthesized from GDP-Man*p* and polyprenols by the polyprenol monophosphomannose (Ppm1) synthase, encoded by the gene Rv2051c (72). The polyprenol monophosphoryl-Man*p* dependent mannosyltransferases responsible for the extension of PIM3→6 remain largely undefined, however, PimF encoded by the gene Rv1500 appears to mannosylate PIM5 leading to the formation of early linear LM molecules (1). Mannosyltransferases involved in the formation of the α(1→6) mannose polymer comprises much of the linear LM molecule is currently unknown but these polymerization steps are understood to utilize a monophosphoryl-Man*p* donor (126). In addition to linear LM, a second LM variant is described in *M. tuberculosis* termed mature LM which is linear LM with single α(1→2)-linked Man*p*

residues branching from the α(1→6) mannose polymer main chain (20). Specific genes encoding the monophosphoryl-Man*p* dependent mannosyltransferases responsible for the α(1→2) branching of mature LM remain to be identified. Mature LM is subsequently arabinosylated with multiple D-Ara*f* residues to form LAM. Unlike the mAGP terminal Ara6 arabinan domains, the LAM arabinan domains terminate with either an Ara4 or Ara6 motif (24). As mentioned above, EmbA and EmbB are reported to participate in the arabinosylation of the AG Ara6 motif. Inactivation of the *M. smegmatis embC* gene abolishes arabinosylation of LAM while having no effect on AG arabinosylation (208).

## 1.6 Mycobacterial protein posttranslational modifications

Posttranslational modification of proteins is a continually expanding field of interest and it now is known that posttranslationally modified *M. tuberculosis* proteins govern several physiologic processes. Certain *M. tuberculosis* proteins possess modifications common amongst bacteria such as adenylylation, acylation, and phosphorylation. Additionally, it is apparent from our growing understanding of the *M. tuberculosis* proteome that protein modification systems typically found in eukaryotic organisms are conserved by *M. tuberculosis*. Specifically, *M. tuberculosis* proteins can possess rare modifications such as acetylation, methylation and glycosylation. Modification by adenylylation, acylation, and phosphorylation have distinct and well-known functions, however, it is largely unknown what functions acetylation, methylation and glycosylation serve.

### 1.6.1 Protein acylation

N-terminal acylation of bacterial lipoproteins is a universal mechanism enabling proteins to associate with membrane structures. Bacterial lipoproteins possess a highly conserved structure comprised of a tri-acylated N-terminal Cys residue (182). N-terminal lipoprotein Cys residues possess a one acyl function linked by an amide bond and a diacylglycerol unit coupled by a thioether bond at the thiol sidechain. Acyl functions found on bacterial lipoproteins are derived from the same fatty acid pool that supplies phospholipid biosynthesis (27). Therefore, the acylated amino termini of bacterial lipoproteins are believed to behave much the same as membrane phospholipids. Biosynthesis of bacterial lipoproteins depends on the type II signal peptides that conserve the consensus sequence ([LV][ASTVI][GAS]C) (163). Type II signal peptide sequences direct Sec-dependent translocation through the cytoplasmic membrane where the diacylglycerol unit is transferred by the prolipoprotein diacylglyceryl transferase Lgt (162). Following Lgt dependent lipidation the type II signal peptide is cleaved by the signal peptidase II Lsp, at a cleavage site preceding the acylated Cys, that consequently becomes the N-terminus of the mature lipoprotein (154, 202). In Gram-negative bacteria a third enzyme, lipoprotein N-acyl transferase (Lnt), adds the amide-linked acyl function to the amino terminus of the mature lipoprotein (60). Members of the above biosynthetic pathway Lgt/Rv1614 and Lsp/Rv1539 were identified in the *M. tuberculosis* genome and the activity of Lsp was partially characterized (28, 161). The identity of a *M. tuberculosis* Lnt needs to be confirmed since Rv2051c, originally annotated as a putative Lnt, was

recently shown to function as a polyprenol monophosphomannose synthase, Ppm1 (72).

Annotation and analyses of the *M. tuberculosis* genome has identified 89-99 genes encoding proteins containing type II signal peptides (28, 183). Putative lipoproteins represent an abundant and functionally diverse sub-set of the *M. tuberculosis* proteome. Immuno-localization has demonstrated surface localization of several lipoproteins in *M. tuberculosis* including SodC/Rv0432, PstS1/Rv0934 and LpqH/Rv3763 (52, 130, 201). Likewise, the lipoproteins MPB83/Mb2898, PstS1/Mb0959, and LppX/Mb2970c localize to the surface of *M. bovis* (74, 104, 105). Thus, acylation may serve to retain proteins within either the cytoplasmic membrane or the mycolic acid layer but following degradation lipoproteins may localize to other subcellular compartments of the bacterium.

### 1.6.2 Phosphorylation

Phosphorylation of proteins is a conserved mechanism by which environmental signals are translated into specific cellular responses. In bacteria two component regulatory systems composed of specific protein kinases coupled to autophosphorylation and dephosphorylation reactions drive these processes. *M. tuberculosis* conserve numerous proteins containing the highly predictable modules involved in the bacterial two-component regulatory systems. Typically this system involves a sensor kinase that senses a stimulus and changes conformation enabling autophosphorylation of a His residue within a cytosolic domain of the protein. In turn the phosphate group is donated to a Asp residue of a second protein termed a

response regulator (33). In a phosphorylated state the response regulator drives gene expression or specific protein function. Typically the acceptor His and Asp residues of the sensor kinase and the response regulator, respectively, occur in highly conserved domains and are easily discerned by genome annotation efforts. A total of 11 sensor kinase and response regulator pairs have been identified in the *M. tuberculosis* genome (summarized in Table 1.1). Additionally, several orphan sensor kinase and response regulators have been identified in the *M. tuberculosis* genome (28). In addition to the ubiquitous two-component systems *M. tuberculosis* conserves a family of regulatory proteins typically found in eukaryotic organisms, termed Ser/Thr kinases (5). In eukaryotes, phosphorylation of Ser, Thr or Tyr residues by eukaryotic protein kinases and dephosphorylation reactions by phosphatases function as the primary signal transduction pathways.

**Table 1.1 Summary of identified and putative mycobacterial phosphorylated proteins.**

| Protein/gene# | Function | Signal or response | Reference(s) |
|---|---|---|---|
| DevR/Rv3133c | Response regulator[1] | Hypoxia | (44, 160) |
| DevS/3132c | Sensor kinase[1] | Hypoxia | (44, 160) |
| KdpE/Rv1027c | Response regulator (putative)[1] | ? | (28) |
| KdpD/Rv1028c | Sensor kinase (putative)[1] | ? | (28) |
| MprB/Rv0982 | Response regulator[1] | ? | (204, 205) |
| MprA/Rv0981 | Sensor kinase[1] | ? | (204, 205) |
| MtrA/Rv3246c | Response regulator[1] | ? | (193) |
| MtrB/Rv3245c | Sensor kinase[1] | ? | (193) |
| PrrA/Rv0903c | Response regulator (putative)[1] | ? | (28) |
| PrrB/Rv0902c | Sensor kinase (putative)[1] | ? | (28) |
| RegX3/Rv0491 | Response regulator[1] | ? | (79) |
| SenX3/Rv0490 | Sensor kinase[1] | ? | (79) |
| NarL/Rv844c | Response regulator (putative)[1] | ? | (28) |
| NarS/Rv0845 | Sensor kinase (putative)[1] | ? | (28) |
| TcrX/Rv3765c | Response regulator (putative)[1] | ? | (28) |
| TcrY/Rv3764c | Sensor kinase (putative)[1] | ? | (28) |
| TrcR/Rv1033c | Response regulator (putative)[1] | ? | (28) |
| TrcS/Rv1032c | Sensor kinase (putative)[1] | ? | (28) |
| PhoP/Rv0757 | Response regulator (putative)[1] | ? | (28) |
| PhoR/Rv0758 | Sensor kinase (putative)[1] | ? | (28) |
| Rv0601c | Response regulator (putative)[1] | ? | (28) |
| Rv0600c | Sensor kinase (putative)[1] | ? | (28) |
| PknB/Rv0014c | Ser/Thr kinase[2] | ? | (6) |
| PknD/Rv0931c | Ser/Thr kinase[2] | ? | (142) |
| PknE/Rv1743 | Ser/Thr kinase[2] | ? | (124) |
| PknF/Rv1746 | Ser/Thr kinase[2] | Cell growth? | (45, 95) |
| PknH/Rv1266c | Ser/Thr kinase[2] | Stress response? | (125, 174) |
| PknI/Rv2914c | Ser/Thr kinase[2] | ? | (69) |

[1]Members of two-component regulatory systems. Note, phosphorylation is not demonstrated for each two-component member listed. [2]The Ser/Thr kinases listed have all been shown to autophosphorylate.

### 1.6.3 Methylation

*M. tuberculosis* produces a heparin-binding hemagglutinin adhesin (HBHA) that is required for extrapulmonary dissemination and cytoadherence via laminin (115, 144, 145). Initially it was believed that HBHA was glycosylated due an apparent molecular mass shift and an aberrant mobility by SDS-PAGE. Complete biochemical characterization of HBHA from *M. tuberculosis* and *M. bovis* revealed mono- and dimethylations of specific Lys residues (146). This modification was

confirmed to be mycobacteria specific by mass spectrometry analysis of the native and *E. coli* produced recombinant forms of HBHA, and *S*-adenosylmethionine-dependent HBHA methyltransferase activity was detected in mycobacterial cell-wall fractions (146). Methylated Lys residues are common on eukaryotic histones, cytochromes, and ribosomal proteins, and the methylation of histones appears to be involved in control of eukaryotic gene expression (143). Lys methylation of HBHA appears to enhance the stability of the protein by protecting it from trypsin-like proteases and those proteases found in human lungs (146). Proper methylation of HBHA is essential for effective T cell immunity to this antigen in infected healthy humans and in mice (187). *M. tuberculosis* HBHA is involved in dissemination from the lungs to the spleen by binding to non-phagocytic cells such as epithelial cells (144). Additionally, the *M. smegmatis* LBP protein possess Lys rich sequences similar to the methylated sequence of HBHA, and recombinant HBHA produced in *M. smegmatis* is methylated, suggesting that this modification may not be protein specific (144, 147).

### 1.6.4 Adenylylation and acetylation

*M. tuberculosis* and pathogenic mycobacteria, but not nonpathogenic mycobacteria, export large quantities of the glutamine synthetase enzyme GlnA/Rv2220 (152). Glutamine synthetases function as complex dodecameric oligomers and are involved in converting ammonia and glutamate to glutamine (75). Several bacterial glutamine synthetases are regulated by adenylylation of the

monomeric subunits within the complex (179). *M. tuberculosis* glutamine synthetase GlnA undergoes adenylylation and the catalytic activity of this complex is inversely proportional to the extent of adenylylation (2, 92). Adenylylation and deadenylylation of the *M. tuberculosis* glutamine synthetases are presumably catalyzed by the adenylyl-transferase GlnE/Rv2221c, an essential enzyme (139). GlnA/Rv2220 undergoes adenylylation at $Tyr_{406}$ and functions as a substrate for an *E. coli* adenylyl-transferase (113). Lastly, the extent of adenylylation present on GlnA/Rv2220 is regulated by the availability of glutamine in the culture medium (113). Acetylation is also reported to occur at the N-terminus of the secreted dominant T cell antigen ESAT-6 (135). ESAT-6 acetylation at $Thr_2$ prevents proper recognition by CFP10, a co-transcribed, secreted protein that interacts directly with ESAT-6 (135). Little is understood about the acetylation of bacterial proteins and it remains to be seen if acetylation is a common modification of mycobacterial proteins and how this modification may affect the proteome.

### 1.6.5 Glycosylation

In 1978, Daniel and Anderson first described purification of the *M. tuberculosis* secreted 38 kDa antigen, antigen 5 (PstS1 homolog) (41). Further characterization suggested that the *M. tuberculosis* antigen 5 was intimately associated with carbohydrate and potentially was a glycoprotein sharing common characteristics with the mycobacterial cell wall arabinomannan (40, 42). The same year, several other *M. tuberculosis* putative glycoprotein antigens corresponding to the molecular weights 55 kDa (MPT32), 50 kDa (MPT32), and 38 kDa (antigen 5/PstS1) were identified (53). In 1991 Fifis *et al.* reported that the *M. bovis* secreted

proteins MPB70, 39 kDa (antigen 5/ PstS1) and the *M. tuberculosis* 19 kDa antigen exist as glycoproteins (55). In 1993 Garbe *et al.* provided more evidence that the 19 kDa antigen is posttranslationally modified by glycosylation when expressed in *M. smegmatis* (61). Each of the above-mentioned studies depended heavily on Western blotting with the lectin Concanavalin A, a technique susceptible to cross reactivity with non-protein contaminating glycosylated molecules and thus potentially misleading. For this reason the early reports of *M. tuberculosis* glycoproteins went without complete acceptance due to the heavy abundance of glycosylated molecules found in the cell envelope. Acknowledging the limitations of lectin based conclusions, Dobos *et al.* (47) in 1996 provided the first definitive chemical proof of mycobacterial glycoproteins by describing the full structure of the *M. tuberculosis* MPT32 glycoprotein antigen. Employing Fast-Atom-Bombardment Mass Spectrometry, N-terminal sequencing, Gas-Chromatography Mass Spectrometry, and Electrospray-Ionization Mass Spectrometry (ESI-MS), Dobos *et al.* defined four sites of glycosylation on MPT32, the carbohydrate constituents, and the glycan linkage configuration (47). MPT32 was found to contain a mannose, α-D-(1→2) mannobiose, and α(1→2),α(1→2) mannotriose chains *O*-linked to four separate threonine residues of the protein (Fig. 1.7). ESI-MS evaluation of MPT32 by Romain *et al.* revealed heterogeneity of glycosylation varying between 0-10 mannose residues with 7 attached mannose residues being the most dominant form of MPT32 (156). Utilizing a molecular approach in 1996, Herrmann *et al.* defined two threonine clusters occurring near the amino terminus of the *M. tuberculosis* 19 kDa antigen as being glycosylated when expressed in *M. smegmatis* (78). In 2000, Herrmann *et al.* further

employed a *M. smegmatis* cassette expression system to confirm that mycobacterial protein glycosylation sequences identified by *in silico* prediction could be glycosylated (77).

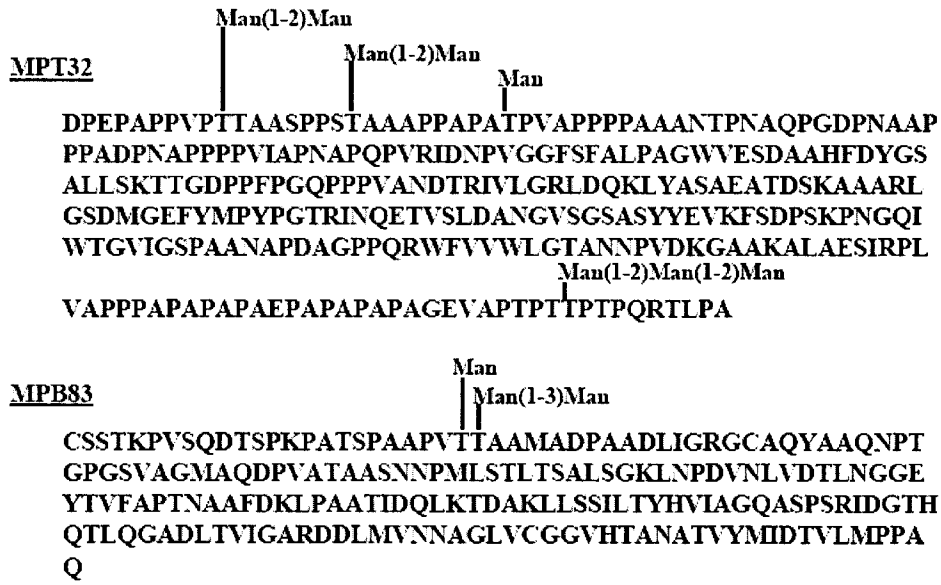


**Fig 1.7 Complete chemical structures of MPT32 and MPB83.** All carbohydrate linkages are in the α-configuration and the amino acid sequences depicted correspond to the mature form of each protein following Sec-translocation.

This study employed the neural network NetOglyc, developed for eukaryotic glycoprotein prediction, to identify potential mycobacterial glycosylated polypeptide sequences. Of the 35 proteins predicted to contain putative glycosylation sequences, 11 sequences were fused to the C-terminus of the secreted protein PhoA and expressed in *M. smegmatis*. These PhoA fusion proteins were then purified and evaluated by ConA western blotting to determine if the proteins were glycosylated. Select peptide sequences derived from MPT83, 38 kDa/PstS1, 19 kDa/LpqH, LppN, LppQ, SodC, GlnH, and MPT32 (8 out of 11) were found to be glycosylated based on ConA reactivity (summarized in Table 1.2). Thus, the neural network NetOglyc

proved to be a dependable predictor of mycobacterial *O*-glycosylation sites based on the presence of a proline residue ±1 or ±2 relative to a Thr residue, similar eukaryotic to mucin-like proteins (68).

**Table 1.2 Summary of identified mycobacterial glycoproteins.**

| Protein/gene# | Evidence | Species | Sites[3] | Signal sequence | Reference(s) |
|---|---|---|---|---|---|
| MPT32/Rv1860 | ConA[1], MS[2] | *Msm*, Mtb, Mbv* | 4 | Yes | (47) |
| MPB83/Mb2898 | ConA[1], MS[2] | *Msm*, Mbv* | 2/? | Yes | (77, 116) |
| PstS1/Rv0934 | ConA[1] | *Msm*, Mtb, Mbv* | ? | Yes | (53, 77) |
| LpqH/Rv3763 | ConA[1] | *Msm*, Mtb, Mbv* | ? | Yes | (61, 77) |
| LppN/Rv2270 | ConA[1] | *Msm** | ? | Yes | (77) |
| LppQ/Rv2341 | ConA[1] | *Msm** | ? | Yes | (77) |
| SodC/Rv0432 | ConA[1] | *Msm** | ? | Yes | (77) |
| GlnH/Rv0411c | ConA[1] | *Msm** | ? | Yes | (77) |
| LppZ/Rv3006 | ConA[1], MS[2] | *Mtb* | ? | Yes | (13) |
| PstS2/Rv0928 | ConA[1], MS[2] | *Mtb* | ? | Yes | (13) |
| LppC/Rv1911c | ConA[1], MS[2] | *Mtb* | ? | Yes | (13) |
| Rv0315 | ConA[1], MS[2] | *Mtb* | ? | Yes | (13) |
| LprG/Rv1411c | MS[2] | *Mtb* | ? | Yes | (64) |

[1]ConA denotes Concanavalin A reactivity by Western blotting. [2]MS refers to mass spectrometry confirmed glycosylation. *Msm*, *M. smegmatis*; *Mtb*, *M. tuberculosis*; *Mbv*, *M. bovis*. [3]Sites, refers to the number of amino acids in the whole protein that are modified by glycosylation. Asterisk indicates recombinant glycoproteins produced in *Msm*.

Recently, Michell *et al.* reported the chemical structure of the secreted *M. bovis* glycoprotein antigen MPB83 (116). MPB83 was found modified with three mannose units *O*-linked to two Thr residues, and the mannose residues of the mannobiose chain are joined by an α(1→3) linkage, in contrast to the α(1→2) linkages observed between the mannose units of MPT32 (Fig. 1.7).

Whole proteome analyses have recently expanded the list of known native *M. tuberculosis* glycoproteins. Specifically, Keen and Belisle utilized two-dimensional gel electrophoresis, Con-A affinity purification, N-terminal sequencing, ConA western blotting, and ESI-MS analyses to identify several secreted *M. tuberculosis* glycoproteins including four novel [Rv3006, Rv0928 (PstS2), Rv0315, Rv1911] and

two known [MPT32, Rv0923 (PstS1)] glycoproteins (summarized in Table 1.2) (13). More recently, an approach using Electrospray Ionization Fourier Transform Mass Spectrometry to analyze secreted proteins enriched by ConA affinity chromatography was used to detect the presence of a 9 kDa product with seven hexose residues and two 20 kDa products with 20 hexose residues each (63). However, it should be noted that no protein sequence was identified in this study.

In eukaryotes protein glycosylation serves to enhance antigen recognition, facilitate receptor/chaperone binding, act as sorting determinants, regulate enzymatic activity, and protect proteins from proteolysis (84, 110). In general the function of protein glycosylation in mycobacteria and prokaryotes is less well understood. Glycosylation of the *M. smegmatis* recombinant LpqH/19 kDa protected this protein from proteolysis while mutated, non-glycosylated forms of this protein were rapidly degraded (78). For at least one mycobacterial protein (LpqH/19 kDa), protein glycosylation seems to enhance the protein's stability. Further glycosylation of MPT32 influences host interactions; Marchal and colleagues demonstrated the importance of mannosylation for antigen specific T cell recognition of MPT32 (81, 156).

## 1.7 Concluding remarks

Traditional mycobacterial physiology research has focused on the characterization of the *M. tuberculosis* cell envelope molecules. These efforts have identified numerous glycoconjugates, many of which are implicated in pathogenesis. For this reason, the biosynthesis of key mycobacterial cell surface molecules has received suitable attention. Likewise, this research seeks to characterize the initial

stages of mycobacterial protein glycosylation by identifying protein glycosyltransferase(s) and defining substrate translocation requirements.

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# Chapter II
# Literature Review Part II: Structure and Biosynthesis of Prokaryotic Glycoproteins.

## 2.1 Introduction and perspectives

Covalent modification of proteins with carbohydrates remains the most complex and important posttranslational modification described in biological systems. Protein glycosylation directly influences the physical and chemical properties of proteins by providing increased resistance to denaturation, protection from proteolytic degradation, and enhanced solubility (52, 65, 134). Protein glycosylation also regulates important biological functions, such as immunogenicity, enzymatic activity and receptor-ligand interactions (102, 129, 130). At one time, protein glycosylation was considered exclusive to eukaryotic organisms; however, it is now accepted that glycoproteins are ubiquitous throughout biology (103). Since the discovery of genuine glycoproteins in Archaea surface or S-layers in 1976, glycoproteins have been identified in numerous prokaryotes, thus confirming the presence of *bona fide* glycoprotein structures in all domains of life (74, 76). It is also understood that prokaryotic glycoproteins are involved in numerous key physiological processes including pathogenesis and virulence (103, 124). With this in mind, it is necessary to characterize relevant prokaryotic glycoprotein structures and to identify the species-specific systems responsible for their genesis.

Until recently our basic biochemical understanding regarding glycoprotein structures and biosynthesis was derived from studies with eukaryotic cells (49). From the limited prokaryotic glycoprotein biochemical and structural data it is evident that there are some parallels between eukaryotic and prokaryotic glycoproteins. Both

eukaryotic and prokaryotic organisms modify proteins with carbohydrates *N*-linked to Asn residues and/or *O*-linked to Ser/Thr residues. Certain elements of the glycans attached to prokaryotic proteins bear resemblance to those found on eukaryotic proteins. However, prokaryotic glycoproteins typically contain uncommon carbohydrates and are often more complex than their eukaryotic counterparts. Eukaryotic cells utilize distinct enzyme systems to form *N*- and *O*-linked glycoproteins, and homologous components of these systems are present in select prokaryotic organisms (126). Additionally, eukaryotic glycoprotein biosynthesis is predominantly a membrane-associated process occurring exterior to the cytosol. Similarly, prokaryotic glycoproteins are principally extracellular and include flagellar subunits, surface proteins, pili subunits, lipoproteins and soluble secreted proteins. The spatial aspects of prokaryotic glycoprotein biosynthesis is beginning to be addressed and like eukaryotic systems evidence is now accumulating linking glycosylation to specific bacterial physiological processes (10, 55, 126). The purpose of this chapter is to review the known structures of prokaryotic glycoproteins, and to highlight our understanding regarding prokaryotic glycoprotein biosynthesis.

## 2.2 Prokaryotic glycoprotein structures

Numerous prokaryotic glycoproteins are now formally characterized demonstrating a diverse assortment of protein-glycosidic linkages and carbohydrate content (Table 2.1). *Campylobacter* spp. modifies assorted proteins with both *N*- or *O*-glycosidically linked glycans (120). *Campylobacter* spp. *O*-glycosylate the flagellar subunit FlaA on Ser/Thr residues with pseudaminic acid derivatives (123). *Campylobacter* spp. also *N*-glycosylate Asn residues of select secreted proteins with a

Glc-GalNAc unit linked to proteins through a 2,4-diacetamido-2,4,6-trideoxyglucopyranose or bacillosamine (Bac) (140). *Helicobacter pylori* also *O*-glycosylates FlaA subunits on Ser/Thr residues with single a pseudaminic acid residue (110), while *Listeria monocytogenes O*-glycosylates several Ser/Thr residues of the flagellar subunit FlaA with a single GlcNAc residue (109). *Pseudomonas aeruginosa* strains PAK and JJ692 *O*-glycosylate Ser/Thr residues of their A-type flagellar subunits with Rha containing glycans (108). Pili of *P. aeruginosa* strain 1244 are *O*-glycosylated on Ser residues with a trisaccharide of FucNAc, Xyl, and 5-N-β-hydroxybutyryl-7-N-formylpseudamic acid (15, 20). Type IV pili of *Neisseria meningitidis* strain C311 are glycosylated with Gal-Gal-Bac *O*-linked to a single Ser residue (114). *Mycobacterium* spp. *O*-glycosylate Thr residues of secreted antigens with Man residues of variable length (24, 79). *Flavobacterium meningosepticum O*-glycosylate Ser/Thr residues with methylated heptasaccharides of Glc, Man, Rha, uronyl Glc and GlcNAc (94, 100). *Clostridium thermocellum* modifies proteins with a methylated tetrasaccharide 3-O-Me-GlcNAc-[Gal]-Gal-Gal (36). *Bacteroides cellulosolvens* glycosylates proteins with a pentasaccharide composed of Gal and GlcNAc (36). *Clostridium thermosaccharolyticum* strain E207-71 *O*-glycosylates Tyr residues of S-layer proteins with Glc, Rha, Gal, Man, and 3-amino-3,6-dideoxy glucose residues (4). *Geobacillus stearothermophilus* NRS 2004/3a (formerly *Bacillus stearothermophilus* NRS 2004/3a) glycosylates S-layer proteins with a Rha and Gal rich glycan (19, 78, 105). The S-layer glycoprotein from *Aneurinibacillus thermoaerophilus* (formerly *Bacillus thermoaerophilus*) strain DSM 10155/$G^+$ is *O*-glycosylated with a Rha and glycero-D-manno-heptose rich glycan (64, 135). *A.*

*thermoaerophilus* strain L420-91$^T$ modifies a S-layer protein with a methylated Rha, FucNAc containing glycan (63, 104). S-layer proteins of *Bacillus alvei* CCM 2051 (formerly *Paenibacillus alvei* CCM 2051) are glycosylated with a Gal, Glc, Rha, ManNAc, and glycerol containing glycan, attached through Gal *O*-linked to Tyr residues (3, 77). *Methanococcus voltae* glycosylates proteins with a (ManNAc-GlcNAc3NAcA-GlcNAc) trisaccharide, and the non-reducing ManNAc is derivatized with a Thr residue at the C6 position (125). S-layer proteins of *Thermoanaerobacter thermohydrosulfuricus* strains L111-69 and L110-69 are glycosylated with a Rha, Man, Gal unit *O*-linked to Tyr residues (13). *Thermoanaerobacter thermohydrosulfuricus* S102-70 glycosylates a S-layer protein with Gal, Rha, Man glycans *O*-linked to Tyr residues (18). *Lactobacillus buchneri* 41021/251 glycosylates S-layer proteins with a linear Glc unit *O*-linked to Ser (81). *Haloferax volcanii* (formerly named *Halobacterium volcanii*) *N*-glycosylates a S-layer proteins on Asn residues with a linear Glc unit (73). The dominant S-layer protein of *Halobacterium halobium* is *N*-glycosylated at a single Asn residue with a sulfated and methylated Gal, GalA, GlcNAc, glycan (91, 92).

In a few cases, structural elements are conserved between the glycans of certain prokaryotic and eukaryotic glycoproteins. *N*-glycosylation of Asn in the amino acid sequence Asn-X-Ser/Thr is common to eukaryotic and prokaryotic glycoproteins (62, 126). Likewise, GlcNAc and Man *O*-glycosidically linked to Ser or Thr residues is common to both prokaryotic and eukaryotic glycoproteins (24, 41, 115, 123). Beyond shared protein glycosidic linkages, there is little in common between prokaryotic and eukaryotic glycoproteins. Predominantly the glycan

**Table 2.1. Glycosylated prokaryotic proteins with known structures and linkage units.**

| Location/ Function | Secretion | Organism | Protein Linkage | Ref |
|---|---|---|---|---|
| Flagella | Type III | *Campylobacter* spp. | Pse*-*O*-Ser/Thr | (70, 123) |
| Flagella | Type III | *H. pylori* | Pse-*O*-Ser/Thr | (110) |
| Flagella | Type III | *L. monocytogenes* | GlcNAc-*O*-Ser/Thr | (109) |
| Flagella | Type III | *P. aeruginosa* | Rha-*O*-Ser/Thr | (108) |
| Flagella/ S-layer | Sec | *M. voltae* | GlcNAc-*N*-Asn | (125) |
| Pili | Sec | *P. aeruginosa* | FucNAc-*O*-Ser | (15, 20) |
| Pili | Sec | *N. meningitidis* | Bac-*O*-Ser | (114) |
| Secreted | Sec | *Campylobacter* spp. | Bac-*N*-Asn | (86, 126, 140) |
| Secreted | Sec | *Mycobacterium* spp. | Man-*O*-Thr | (24, 79) |
| Secreted | Sec | *F. meningosepticum* | Man-*O*-Ser/Thr | (94, 100) |
| Secreted | Sec | *C. thermocellum* | Gal-*O*-Thr | (36) |
| Secreted | Sec | *B. cellulosolvens* | Gal-*O*-Thr | (36) |
| S-layer | Sec | *C. thermosaccharolyticum* | Glc-*O*-Tyr | (4) |
| S-layer | Sec | *G.stearothermophilus* | Gal-*O*-Ser/Thr | (78, 105) |
| S-layer | Sec | *A. thermoaerophilus* DSM 10155/G+ | GlcNAc-*O*-Ser/Thr | (135) |
| S-layer | Sec | *A. thermoaerophilus* L420-91$^T$ | GalNAc-*O*-Thr | (63) |
| S-layer | Sec | *B. alvei* CCM 2051 | Gal-*O*-Tyr | (3, 77) |
| S-layer | Sec | *T. thermohydrosulfuricus* L111-69 & L110-69 | Gal-*O*-Tyr | (13) |
| S-layer | Sec | *T. thermohydrosulfuricus* S102-7 | Glc-*O*-Tyr | (18) |
| S-layer | Sec | *L. buchneri* 41021/251 | Glc-*O*-Ser | (81) |
| S-layer | Sec | *H. volcanii* | Glc-*N*-Asn | (73, 118) |
| S-layer | Sec | *H. halobium* | GalNAc-*N*-Asn | (91, 92) |

Pse* indicates pseudaminic acid with acetamidino and hydroxyproprionyl substitutions and C5 and C7 positions. Pse, represents pseudaminic acid, Bac represents bacillosamine, and Sec denotes Sec translocated proteins

structures found on prokaryotic glycoproteins far exceed the diversity of their eukaryotic counterparts. For example, eukaryotic glycoproteins do not contain the exotic sugars pseudaminic acid or bacillosamine and are rarely methylated or sulfated (70, 103, 114, 140). In addition, carbohydrates *O*-linked to Try residues are restricted to prokaryotic organisms (4). In general, eukaryotic glycoproteins seldom are modified with single sugar residues as seen with the bacterial flagella subunits (70, 108, 114, 123).

In two cases prokaryotic glycoproteins contain structural elements common to other extracellular glycoconjugates from the same bacterium. As discussed above *P. aeruginosa* strain 1244 modifies its pili with a trisaccharide containing 5-N-β-hydroxybutyryl-7-N-formylpseudamic acid (15). 5-N-β-hydroxybutyryl-7-N-formylpseudamic acid is a rare sugar and also occurs in the O-antigens of certain *P. aeruginosa* serotypes (61). Additionally, the linear mannans attached to the secreted antigens of *Mycobacteria* spp. resemble the linear Man capping motifs found on the major surface lipoglycan, mannosylated lipoarabinomannan (ManLAM) (82). These observations strongly suggest bacteria predominantly utilize dedicated anabolic pathways for the assembly of protein glycosylation precursors and glycoproteins.

It is clear from the reported prokaryotic glycoprotein structures that glycoprotein assembly varies widely between prokaryotic organisms. Only in the case of *H. pylori* and *Campylobacter* spp. flagella glycosylation does the process of protein glycosylation seem to be conserved between bacterial organisms (120). To date, only the *N*-linked eukaryotic glycoprotein biosynthetic model has been extended to the *N*-linked protein glycosylation system of *C. jejuni* (126). Before discussing what is understood regarding bacterial glycoprotein biosynthesis, the central mechanisms of eukaryotic protein glycosylation will first be addressed.

## 2.3 Endoplasmic Reticulum protein glycosylation processes

### 2.3.1 *N*-linked glycosylation

Asn or *N*-linked glycosylation is the most common type of eukaryotic protein glycosylation. *N*-linked glycans mediate numerous biological functions of secreted and integral membrane proteins and are required for eukaryotic cell viability. *N*-

linked glycosylation initiates with an *en bloc* transfer of a complex oligosaccharide from the lipid carrier dolichyl phosphate to proteins during translocation across the Endoplasmic Reticulum (ER) membrane. Eukaryotic cells utilize a conserved *N*-linked oligosaccharide biosynthetic pathway to first form the lipid-linked precursor ($Glc_3Man_9GlcNAc_2$-PP-dolichol) and then to transfer the oligosaccharide to proteins within the ER. The $Glc_3Man_9GlcNAc_2$-PP-dolichol lipid-linked precursor is synthesized while anchored in the ER membrane. One GlcNAc-P, one GlcNAc residues and five mannose residues are sequentially linked to a P-dolichol at the cytosolic face of the ER membrane using the substrates UDP-GlcNAc, UDP-GlcNAc, and GDP-Man, respectively (Fig 2.1). After the initial seven sugars are added to the lipid-linked precursor in the cytosol the oligosaccharide moiety is translocated into the lumen of the ER by the integral membrane protein RFT1, an ATP-independent, bi-directional flippase (45). Four additional mannose residues and three glucose residues are added on the luminal side of the ER with Man-P-dolichol (Dol-P-Man) and Glc-P-dolichol (Glc-P-Man) serving as the monosaccharide donors, respectively. The $Glc_3Man_9GlcNAc_2$ oligosaccharide is then transferred *en bloc* to nascent secretory proteins by the oligosaccharyltransferase complex (OST). Once the oligosaccharide is transferred, three glucoses and a specific mannose residue are trimmed by Golgi located, species-specific processing glucosidases (101). The OST activity in the ER membrane of eukaryotic cells is highly conserved. However eukaryotic cells utilize a variety of Golgi processing enzymes such that the final glycans vary considerably from species to species (101).

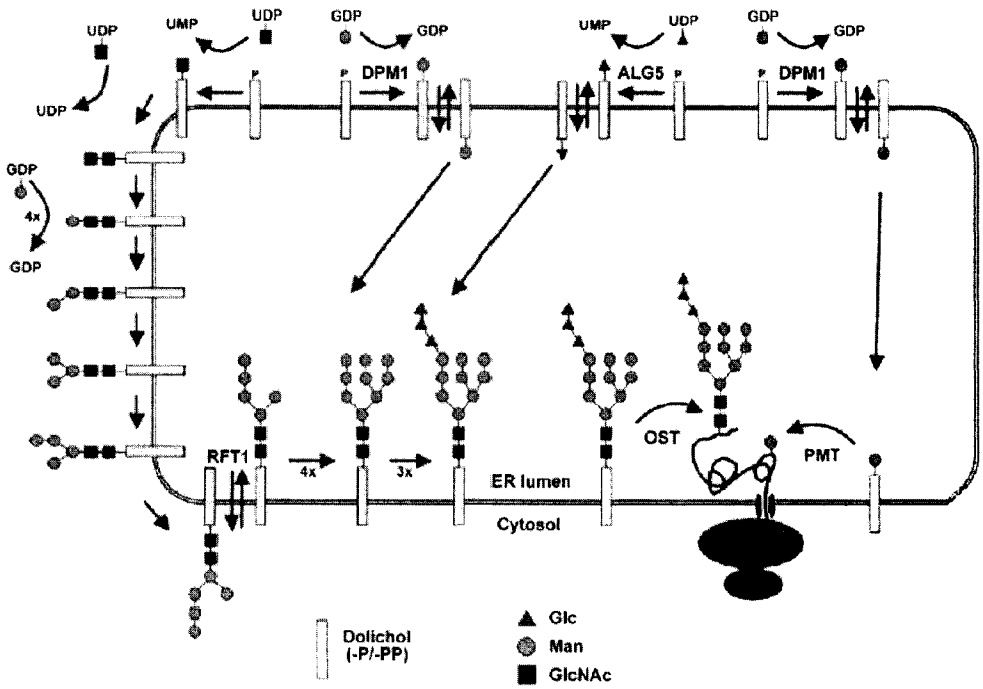



**Fig 2.1 Yeast ER protein glycosylation events.** Illustration of the compartmentalized yeast protein glycosylation processes, adapted from (44).

The OST transfers the oligosaccharide from a lipid-linked precursor to Asn acceptor sites of secretory proteins within the Asn-X-Ser/Thr consensus sequence where X is any amino acid but Pro. The OST catalyzes the formation of an N-C bond between the nitrogen of Asn and the C1 position of GlcNAc of the oligosaccharide. It is proposed that the oligosaccharide transfer to the inert side chain of the Asn requires formation of a slight bend in the acceptor protein allowing contact between the adjacent hydroxyl sidechains of Ser or Thr with the Asn amide nitrogen rendering it nucleophilic (51). The bending requirement of the protein substrate explains why the residue X in the consensus sequence cannot be a Pro. Pro residues would prevent local protein bending and this may also explain why folded polypeptides are poor *N*-linked glycosylation substrates *in vitro*.

The OST complex consists of at least nine major integral membrane subunits Ost1-6p, Wbp1p, Stt3p, and Swp1p (58). Ost1-2p, Wbp1p, Stt3p, Swp1p subunits are essential for eukaryotic cell viability (57). The nine member OST complex has a total of 31 transmembrane domains and is assembled from several subcomplexes (56, 122, 136). It is difficult to assign functions to individual OST subunits due to the essentiality and multiple interactions within the OST complex. Cross-linking experiments suggest that Stt3p, Ost1, and Wbp1p subunits make direct contact with the nascent protein substrate (90, 137, 138). The largest and most conserved OST subunit, Stt3p, contains 10-12 transmembrane domains and is probably involved in substrate recognition and/or glycosyl transfer (137, 141). Recent discovery that a bacterial Stt3p homolog from *C. jejuni* is sufficient for OST activity imply that the Stt3p subunit is the OST catalytic subunit (126). Although genetic and biochemical studies have yielded considerable information about the OST complex, the enzymatic mechanism of *N*-glycosylation remains unknown. Specifically, the substrate recognition domains and/or catalytic sites of each subunit, and how the subunits interact structurally, remain to be fully defined.

### 2.3.2 *O*-linked glycosylation

Protein O-mannosylation is another common process that occurs within the ER lumen. Like *N*-linked glycosylation, the attachment of *O*-linked mannosyl residues to secretory proteins is required for yeast cell viability (34). Protein *O*-mannosylation is also important in mammals. For example, defects in protein *O*-mannosylation is linked to several forms of congenital muscular dystrophies and improper neuronal development (9, 71, 132). This form of glycosylation is

extensively studied in *Saccharomyces cerevisiae* and the findings have been extended to mammalian cell processes (71). Biosynthesis of *S. cerevisiae* *O*-mannosylated proteins initiates in the ER lumen by an evolutionarily conserved family of dolichyl phosphate-β-D-mannose:protein *O*-α-D-mannosyltransferases (PMT). PMT enzymes catalyze the transfer of a single mannosyl residue from Dol-P-Man to Ser and Thr residues of secretory proteins (Fig 2.1) (115). Once the single mannose is transferred, the secretory protein is transported to the Golgi apparatus where the lone mannose is elongated to an oligosaccharide by a series of species-specific Golgi mannosyltransferases.

*S. cerevisiae* PMTs are a redundant family of enzymes consisting of seven highly homologous members; *Sc*Pmt1-7 proteins share an overall amino acid sequence identity of 50-55% (116). PMTs are complex integral ER membrane glycoproteins containing multiple transmembrane domains, and of the seven *S. cerevisiae* PMTs, *Sc*Pmt1 is the best characterized (116). Protein *O*-mannosyltransferase activity is confirmed for Pmt1-4p and Pmt6p (35, 50). Unique to *S. cerevisiae* PMT enzymes (Pmt1-7p) are the near identical hydropathy profiles suggesting that PMT enzymes possess similar transmembrane topologies (115). The transmembrane structure of *Sc*Pmt1 was experimentally established using localization dependent protein fusions and engineered *N*-linked glycosylation sites (116). Additionally, several amino acids of *Sc*Pmt1 that are invariantly conserved in all *S. cerevisiae* PMTs were confirmed to be involved in recognition and/or binding of protein substrates, and/or catalysis (38). *Sc*Pmt1p and *Sc*Pmt2p form an active heterodimeric enzyme complex that was isolated with an anti-Pmt1p antibody using

immunoprecipitation (33). *S. cerevisiae* PMT enzymes are divided into three subfamilies (PMT1, PMT2 and PMT4) based on dimerization capacity. The *S. cerevisiae* PMT1 subfamily is comprised of *Sc*Pmt1p and *Sc*Pmt5p, the PMT2 subfamily is made of *Sc*Pmt2p and *Sc*Pmt3p, lastly *Sc*Pmt4p comprises the PMT4 subfamily. Active *S. cerevisiae* enzyme complexes form when PMT1 subfamily members heterodimerize with PMT2 subfamily members or when *Sc*Pmt4p forms homodimers (37).

Amino acid sequences that act as signals for directing the *O*-mannosylation of specific Ser and Thr residues are unknown. However, most *O*-mannosylated proteins are modified in regions rich in Ser, Thr, and Pro clusters. *In vitro* yeast studies using synthetic peptides as acceptors has revealed that adjacent Pro residues at positions −1 enhance glycosylation and Thr generally is a better acceptor than Ser for *O*-mannosylation (7, 117). It remains to be seen if the seemingly redundant yeast PMTs with potentially different substrate specificities account for the lack of a clear *O*-mannosylation consensus sequence.

### 2.3.3 Protein translocation

To appreciate the complexity of the ER localized protein glycosylation process, one must understand the context in which protein glycosyltransferases operate. Eukaryotic proteins destined for secretion are synthesized as a signal peptide and are translocated into the ER by the Sec protein-conducting channel. The Sec protein-conducting channel is ubiquitous in biology and operates to circumvent the universal difficulty of translocating proteins across a membrane bilayer. The Sec system operates as a pore comprised of SecY, SecE, and SecG in *E. coli*; Sec61p,

Sbh1p, and Sss1p in yeast; and Sec61α, Sec61β, Sec61γ, in conjunction with the translocation-associated membrane protein (TRAM), in mammalian cells (99). SecY and SecE dimerize in *E. coli* cytoplasmic membranes forming a stable 1:1 complex and co-purify with SecG forming the minimal protein-conducting channel (54, 85). Crystallography studies of the Sec complex from *Methanococcus jannaschii* have confirmed that the SecYEG subunits interact directly, and SecY comprises the actual protein-conducting channel. The Sec protein-conducting channel is simply a passive pore and associates with other protein components to provide the energy and substrates for translocation.

Targeting nascent protein substrates to the Sec protein-conducting channel occurs by two mechanisms. The first is co-translational, whereby nascent proteins are delivered from the ribosome to the Sec pore before translation is complete, and the second is post-translational where the nascent protein is fully translated and released from the ribosome before engaging the Sec protein-conducting channel. Co-translational protein translocation occurs when the secretory protein moves directly from the ribosome into the membrane located Sec translocase. Co-translational protein translocation is mediated by the signal recognition particle (SRP) which aligns the ribosomal exit tunnel to the protein-conducting channel allowing the unfolded protein substrates entrance into the Sec pore. Alternatively, post-translational protein translocation initiates when pre-proteins bind to cytosolic chaperones independent of the SRP pathway (25). Chaperones bind nascent proteins as they exit the ribosome preventing folding and aggregation before delivering the substrate to the Sec translocase. Various accessory proteins are involved in delivering

the chaperone/preprotein complex to the Sec translocase such as Sec62p, Kar2p/BiP, Sec63p, Sec71p, and Sec72p in yeast, or SecA and SecB in *E. coli* (22, 26, 88). SecA is specific to prokaryotes and mediates protein translocation through the SecYEG translocase (42). It is believed that SecA co-inserts with the signal peptide into the SecYEG complex then withdraws and binds a distal region of the preprotein, and through multiple rounds of ATP hydrolysis, pushes the preprotein though the SecYEG pore. In yeast the Sec complex plus Kar2p/BiP is sufficient for the posttranslational protein translocation into reconstituted proteoliposomes *in vitro* and is required for SRP dependent cotranslational protein translocation (88, 139).

Cleavable amino terminal signal peptides direct proteins to the prokaryotic Sec translocase. The amino terminus of the signal peptide (N-domain) contains at least one positively charged Arg or Lys residue. It is proposed that the positively charged N-domain interacts with the Sec translocase and/or membrane phospholipids during translocation (1). Flanking the N-domain is a region of hydrophobic amino acids commonly surrounding a single Gly or Pro residue termed the H-domain. Gly or Pro residues found within the H-domain are believed to promote signal peptidase cleavage while the hydrophobicity of this region conceivably participates with insertion of the signal peptide into membrane bilayers. Adjacent to the H-domain is the C-domain, that functions as the cleavage site for signal peptidases, which remove signal peptides from secretory proteins during or shortly after translocation. Signal peptides undergo cleavage by one of two signal peptidases on the external side of the cytoplasmic membrane. Signal peptidase I cleaves signal sequences of soluble

proteins while Signal peptidase II cleaves membrane bound lipoprotein signal peptides.

Protein translocation and glycosylation are understood to occur simultaneously in eukaryotes (83). Coordination of these processes is required to ensure all potential glycosylation sites are modified before they are made inaccessible due to protein folding. For this reason, OST and PMT enzymes are believed to be peripheral components of the Sec translocase, but do not contribute to the formation of the protein-conducting channel. The Sec translocase and the OST copurify (107, 127), and secretory proteins are believed to be glycosylated within 30–40 Å from the lumen face of the ER membrane (27, 84). Other processes of glycosylation and translocation are also interdependent, for example, the glycosylation of sites near signal peptides requires signal peptidase activity (17). Hydrophobic signal peptides probably stay embedded in the membrane and sequester substrate away from protein glycosyltransferases preventing glycosylation. Direct protein interactions between OST subunits and the Sec translocase has been reported and the molecular organization of these interactions is now under current investigation (16, 107). In yeast, *O*-mannosylation of Ccw5 by Pmt4 prevents *N*-glycosylation by blocking a *N*-glycosylation site present in Ccw5, suggesting that the OST and PMT enzymes compete for Ccw5 and *O*-mannosylation may precede *N*-glycosylation (27).

## 2.4 Prokaryotic glycoprotein biosynthesis

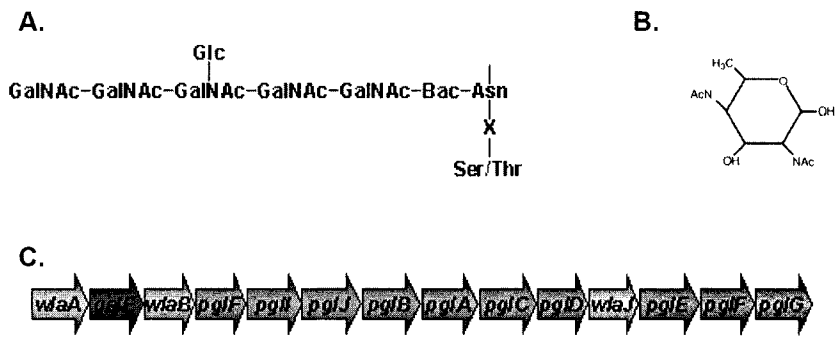
The limited genetic and biochemical studies concerning prokaryotic glycoprotein formation indicate that prokaryotes have evolved unusual strategies to glycosylate proteins and in some cases have conserved systems related to those found

in eukaryotic organisms (126). An interesting finding unique to prokaryotic organisms is that substrates and the systems responsible for glycosylation are typically encoded within the same genetic locus (5, 96, 126). In general, prokaryotic protein glycosyltransferases remain largely undefined, however, genetic evidence for their presence exists in some cases (14, 23, 80, 108, 121). Prokaryotic protein glycosylation systems use both lipid linked precursors and sugar nucleotides as donors for protein glycosylation (21, 29, 30, 43, 74, 113). Additionally, bacteria lack the organelle compartments required for eukaryotic glycoprotein formation signifying some differences at the biosynthetic level. Surprisingly, prokaryotic glycoproteins are predominantly extracytoplasmic but, unlike eukaryotic cells, there appears to be no specific protein secretory route associated with protein glycosylation in prokaryotic organisms.

### 2.4.1 Campylobacter *N*-linked protein glycosylation

To date, the *N*-linked protein glycosylation process of *C. jejuni* is the best-studied prokaryotic glycoprotein biosynthetic system. The *N*-linked protein glycosylation system enzymes are encoded in a 12-gene protein glycosylation (PGL) locus that is highly conserved among *Campylobacter* spp. (133). Systematic characterization of glycoproteins produced by mutants deficient in genes within this locus has identified the glycosyltransferases involved in the synthesis of a lipid linked oligosaccharide precursor and the protein glycosyltransferase (69). Uniquely, the glycan found on *Campylobacter* spp. glycoproteins contains bacillosamine (Bac) which is also found on the *N. meningitidis O*-glycosylated Type IV pili (Fig 2.2) (114). It is proposed that *Campylobacter* spp. convert UDP-HexNAc to UDP-Bac via

sequential modification by PglF (a dehydratase), PglE (an aminotransferase), and PglD (an acetyltransferase) in the cytoplasm (120). PglC then transfers the Bac residue to a lipid carrier from UDP-Bac (Fig 2.2) (69). PlgA, PlgH, PlgI and PlgJ function as specific glycosyltransferases responsible for sequential addition of monosaccharides, forming the lipid linked oligosaccharide (Fig 2.2). In *C. jejuni* it is proposed that PglA adds the GalNAc to lipid linked Bac, then PlgH and PlgI are responsible of the next four GalNAc additions. The final glycosyltransferase in the pathway, PglI, adds the branched glucose from UDP-Glc (Fig 2.2) (69). Orthologous proteins involved in synthesis of the Bac precursor in *Neisseria* spp. (PglBCD) were identified in *Campylobacter* spp. (PglDEF) (120). It is further proposed that analogous to *N*-linked protein glycosylation in *S. cerevisiae*, the *C. jejuni* lipid linked precursor is produced in the cytosol prior to translocation from the cytoplasm into the periplasm.



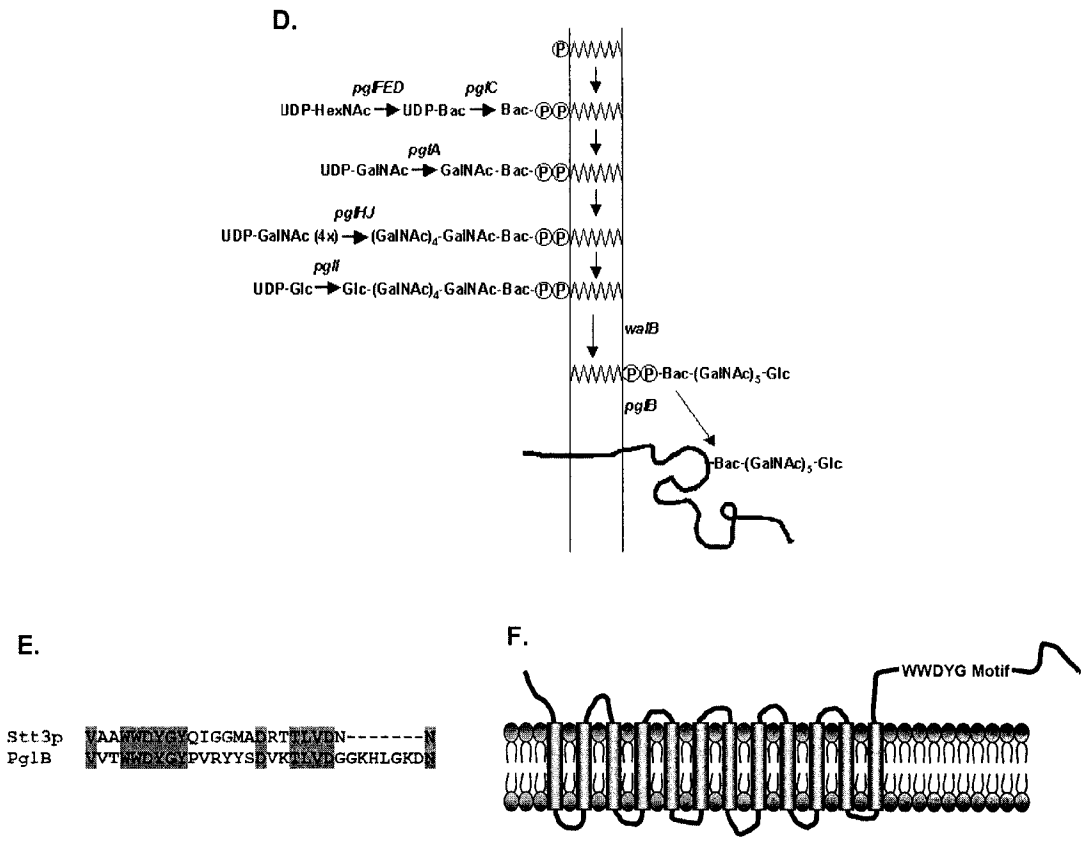



**Fig 2.2 The Campylobacter *N*-linked protein glycosylation system.** *N*-linked glycan found on *Campylobacter* spp. glycoproteins **(A)**. Chemical structure of bacillosamine (Bac) or DADTH (2,4-diacetamido-2,4,6-trideoxyglucose) **(B)**. Genetic arrangement of the protein glycosylation genetic locus **(C)**. Proposed pathway of *N*-linked glycoprotein formation **(D)**. Partial alignment of the putative active sites of the OST component Stt3p and PglB **(E)**. Predicted transmembrane topology of PglB indicating the position of the putative active site **(F)**.

It is also proposed that the entire lipid linked oligosaccharide is flipped into the periplasm by the putative ABC transporter, WlaB (120). The key enzyme of the *Campylobacter* spp. PGL locus is PglB which transfers the oligosaccharide to the Asn residues from the lipid linked precursor (30, 69, 126). PglB contains 12 putative transmembrane domains and shares 17 % amino acid identity with the yeast OST component Stt3 (Fig 2.2) (31). PglB contains a conserved, putative active site domain

(WWDYG) within the C-terminus which is required for both PglB and Stt3 activity (126).

### 2.4.2 Flagellin *O*-linked protein glycosylation

Flagellin proteins from *Campylobacter* spp., *H. pylori*, *L. monocytogenes*, and *P. aeruginosa* are all modified with *O*-linked carbohydrate (70, 108-110, 123). *C. jejuni* *O*-glycosylates multiple Ser and Thr residues within the central surface-exposed domain of the flagellar filament subunit, FlaA (123). A flagellar glycosylation locus identified in *C. jejuni* contains roughly 50 genes, yet, how FlaA is glycosylated remains poorly understood. (120). Sequence annotation of the flagellar glycosylation locus indicates that numerous genes are involved in polysaccharide biosynthesis, linked to motility, or encode flagella structural proteins (120). Nearly all of the putative proteins encoded within the flagellar glycosylation locus have unknown functions and no protein glycosyltransferase has been characterized. Mutations in the specific genes within this locus (*Cj1293*, *Cj1311*, and *Cj1317*) result in nonmotile phenotypes and non-glycosylated flagella (39, 123). It is presumed that *Cj1293*, *Cj1311*, and *Cj1317* encode proteins involved in the formation of precursors required for protein glycosylation and are not protein glycosyltransferases. This idea is supported by the finding that *Cj1311* and *Cj1317* are orthologous to the *H. pylori* genes involved in the formation of precursors for flagellin glycosylation (see below).

*Helicobacter* spp. glycosylate their unipolar sheathed flagella at several sites within the central core region of FlaA in a manner similar to *Campylobacter* spp. (110). Likewise, *H. pylori* possesses a flagellar glycosylation locus and encoded within this locus are the flagellin glycosylation related genes (*Hp114*, *Hp178*,

*Hp326a*, and *Hp326b*) which are orthologous to the *C. jejuni* genes (*Cj1318*, *Cj1317*, *Cj1311*, *and Cj1312*), respectively. *H. pylori* with mutations in the genes *Hp114*, *Hp178*, *Hp326a*, and *Hp326b* display non-motile phenotypes and produce non-glycosylated flagella (55, 110). A novel approach using liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) to identify sugar nucleotide precursors from intracellular metabolic pools of parent and mutant strains revealed that Hp178, Hp326a, and Hp326b are involved in the biosynthesis of CMP-Pse, a presumed precursor of *Helicobacter* spp. and *Campylobacter* spp. protein glycosylation (113). Like *Campylobacter* spp. the identity of the protein glycosyltransferase involved in *Helicobacter* spp. flagellin glycosylation remains elusive.

The type-A flagella of *P. aeruginosa* primarily consists of a FliC subunit. FliC is *O*-glycosylated with a variable glycan attached to the protein by a Rha residue (108). *P. aeruginosa* strain PAK possesses a cluster of 14 genes that encode the determinants of the FliC glycosylation (5). Studies confirmed that inactivation of the *orfA* or *orfN* genes within this locus altered flagellin glycosylation and heterologous expression of the entire locus demonstrated it contains all of the genes required for glycosylation of FliC (5). More recent examination of the glycans present on FliC produced by isogenic mutant strains lacking *orfA* or *orfN* has confirmed that *orfA* is involved in the variable glycan assembly while the protein encoded by *orfN* is potentially the protein rhamnosyltransferase responsible for addition of the Rha to FliC (108). The OrfN protein product has no putative transmembrane domains and shares 25% amino acid identity to human UDP-GalNAc:polypeptide *N*-

acetylgalactosaminyltransferase (pp-GalNAc-T) (40). The Golgi lumen pp-GalNAc-T enzyme transfers GalNAc from UDP-GalNAc to either Ser or Thr residues forming mucin-type *O*-linked glycoproteins (40). It remains to be established if the product encoded for by *orfN* is indeed a protein glycosyltransferase or is simply involved in the formation of a precursor required for protein glycosylation.

### 2.4.3 Pilin *O*-linked protein glycosylation

Pili of *P. aeruginosa* are crucial for motility, and the pilin of *P. aeruginosa* strain 1244 (O7 serotype) is *O*-glycosylated with a trisaccharide on specific Ser residues. The pilin trisaccharide is identical to the O-antigen repeat unit found in the O7 serotype lipopolysaccharide (LPS) (15). The *P. aeruginosa* strain 1244 pilin glycan and the O7 repeating unit are formed by the same biosynthetic pathway and potentially diverge at the lipid linked precursor (23). Certain strains of *P. aeruginosa* posses a *pilO* gene in a operon with the pilin structural gene (*pilA*) (14, 23). PilO is required for glycosylation of PilA and *P. aeruginosa* strains that express non-glycosylated pilin and lack the *pilO* gene (15). PilO shows poor homology with characterized proteins and contains 13 putative transmembrane domains. The predicted transmembrane topology of PilO and the strong possibility that lipid linked precursors are the protein glycosylation donors suggest that PilO is a protein glycosyltransferase.

*Neisseria* spp. *O*-glycosylate type IV pili, which is involved in the adhesion of capsulated *N. meningitidis* to eukaryotic cells (128). Six genes are identified as participating in the formation of the type IV pili trisaccharide glycan. PglB-D and PlgI are involved in the biosynthesis of the precursor form of bacillosamine that is

used for pili glycosylation. PglB-D share considerable homology with the products involved in the formation of Bac in *C. jejuni* and likely function similarly by being an acetyltransferase, aminotransferase, dehydratase, and an acyltransferase, respectively. *Neisseria* spp. mutants deficient in *pglB-D* and *pglI* fail to glycosylate pili but are otherwise normal, and so the exact function of these gene products remains unclear. PglA is responsible for transferring the first Gal residue to Bac, and PglE transfers the third and final Gal residue forming the mature Gal-Gal-Bac glycan (53, 98). The protein glycosyltransferase that transfers the glycan to the pili has yet to be identified in *Neisseria* spp.

### 2.4.4 *Streptococcus gordonii* and *Escherichia coli* glycoprotein biosynthesis

It is important to mention that *Streptococcus gordonii* strain M99 appears to glycosylate the cell surface glycoprotein GspB, yet the attached glycans are unknown (10). Lectin binding and carbohydrate analysis suggest the predominate carbohydrates associated with GspB are GlcNAc and Glc, and the linkage to the protein is uncharacterized (10). The glycosylation of GspB appears to be dependent on the proteins Gly, Nss, Gtf, and Orf4 which are encoded within the GspB locus (121). Secretion of GspB occurs in a SecA2 dependent manner and the SecA2 proteins required for this system to function are also encoded in the same locus (11). Cytosolic pools of GspB appear to be glycosylated in SecA2 mutants, suggesting the protein glycosyltransferase is cytosolic (10).

Two separate autotransporter adhesion proteins produced by *Escherichia coli* strains appear to be glycosylated but the nature of glycosylation remains partially characterized. In the case of the plasmid-borne autotransporter adhesin, AIDA-I, of *E.*

*coli* strain 2787 (O126:H27), glycosylation depends on the Aah protein encoded immediately upstream on the same plasmid (12). Partial characterization of AIDA-I suggested that the sole carbohydrate associated with the protein was a heptose; the linkage to the protein remains unknown (12). Presently the function of Aah remains unknown, but it is conceivable that this protein functions as a protein glycosyltransferase or is involved in the production of a precursor. Similarly, *E. coli* strain H10407 appears to glycosylate the chromosomally encoded autotransporter TibA (68), and the nature of the glycan and the protein-glycosidic linkage on TibA are uncharacterized. Both the protein bound glycan and the processes of TibA glycosylation are very similar to AIDA-1, since TibA is encoded directly adjacent to the putative glycosyltransferase (TibC) and isogenic TibC mutants are complemented by heterologous expression of Aah (80).

### 2.4.5 S-layer glycoprotein biosynthesis

Despite advances in our knowledge of the glycan structures and protein glycosidic linkages of S-layer glycoproteins, our understanding regarding their biosynthesis is poorly understood. To date, no S-layer protein glycosyltransferase has been described and for the most part precursor molecules involved in protein glycosylation reactions remain unknown. Biochemical examination of *Halobacteria salinarum* and *Haloferax volcanii* systems has identified lipid linked donors probably involved in protein glycosylation reactions (67). Bacitracin interferes with glycosylation of the *H. salinarum* S-layer protein and has no effect on *H. volcanii* glycoprotein biosynthesis (29, 75). In *H. salinarum*, bacitracin is thought to interfere with phosphate cycling preventing the formation of di-phosphate-linked lipid

precursors, the presumed donor lipids for protein glycosylation (131). The inability of bacitracin to halt *H. volcanii* glycoprotein synthesis is supported by the finding that only mono-phosphate-linked lipid carriers are detected in this organism (66, 67). A tetrasaccharide-lipid linked donor probably utilized by *Bacillus alvei* CCM 2051 for S-layer protein glycosylation was characterized (43). The genes involved in the formation of GDP-D-Rha, a putative precursor required for the formation of *Aneurinibacillus thermoaerophilus* 10155 S-layer glycoproteins, were identified and partially characterized (59). Also, the genes involved in the formation of GDP-glyero-mannoheptose, a putative precursor for the formation of *Aneurinibacillus thermoaerophilus* L420-91$^T$ S-layer glycoproteins, were characterized (60). A 13 gene cluster was identified in *Geobacillus stearothermophilus* NRS 2004/3a that encodes the glycosylated S-layer protein SgsE (87). Within this locus are several genes directly involved in dTDP-L-Rha biosynthesis that are annotated as being glycosyltransferases, transporters, and methyltransferases of unknown function (87).

### 2.4.6 *Mycobacterium* spp. glycoprotein biosynthesis

Relatively little is understood concerning the glycosylation of mycobacterial proteins. Two secreted mycobacterial proteins (MPT32 and MPB83) have been formally characterized revealing short, linear, mannan chains *O*-linked to Thr residues (24, 79). Protein mannosyltransferase activity was detected in mycobacterial extracts and was detected by incorporating radiolabeled mannose from GDP-[$^{14}$C]mannose into peptide acceptors (21). Peptide acceptors in this assay were derived from known glycosylation sequences of the *M. tuberculosis* protein, MPT32/Rv1860 (24). This relatively simple assay was inhibited by amphomycin

indicating that mycobacterial protein mannosylation proceeds via lipid-linked C35/C50-polyprenol-Man intermediates (21). Native mycobacterial glycoproteins are modified in regions rich with Thr, Ser, and Pro sequences related to those found in eukaryotic *O*-linked glycoproteins, both of which are accurately predicted using the neural network Net-O-Glyc (47). To date no mycobacterial protein glycosyltransferase has been identified and it is unknown if a lipid-linked oligosaccharide or monosaccharide serves as a donor for mycobacterial protein mannosyltransferases.

## 2.5 Spatial aspects of prokaryotic glycoprotein biosynthesis

Structural, biochemical, and genetic evidence has confirmed the existence of protein glycosylation systems in bacteria, however, uncertainty remains as to the subcellular location of these glycosylation events. Prokaryotic glycoproteins are predominantly extracellular, being membrane, cell surface, or soluble proteins secreted into the culture medium (124). Many prokaryotic glycoproteins contain an identifiable Sec signal peptide and, although details of archaeal protein translocation are poorly defined, the majority of archaeal glycoproteins also possess what appears to be a signal peptide (28). Additionally, numerous other extracytoplasmic prokaryotic glycoproteins are secreted by mechanisms other that the Sec system.

### 2.5.1 Sec translocated prokaryotic glycoproteins

The bulk of characterized prokaryotic glycoproteins are translocated by the Sec translocase. Recently 23 glycoproteins predominantly annotated as Sec translocated periplasmic proteins were identified as glycoproteins in *C. jejuni* (126, 140). One of these glycoproteins is the Sec translocated lipoprotein AcrA (126).

Substituting the AcrA Type II signal peptide for a Type I signal peptide from the periplasmic protein (PelB) had no effect on glycosylation while removal of the signal peptide prevented glycosylation (86). All mycobacterial mannoproteins identified to date are Sec translocated (8, 24, 32, 47, 48, 79, 112). The glycosylated Type IV pilin subunits of gram negative organisms and archaeal flagellar subunits are secreted via the Sec translocase (28, 93). The glycosylated autotransporters of *E. coli* also pass the inner membrane via the Sec translocase and insert into the outer membrane by a distinct mechanism (46). It remains to be established if the Sec translocation of prokaryotic proteins is an equally fundamental component of protein glycosylation as it is with eukaryotic cells.

### 2.5.2 Sec independently translocated prokaryotic glycoproteins

There are several examples of prokaryotic glycoproteins that are secreted in a Sec independent manner. The glycosylated flagellins of *H. pylori*, *Campylobacter* spp., *L. monocytogenes*, and *P. aeruginosa* are all unique in that these proteins never pass through a membrane bilayer. Flagella proteins are transferred from the cytoplasm through the hollow flagella structure prior to incorporation at the distal tip of the filament (2). Thus, bacterial flagellins are never exposed to the periplasmic side of the inner membrane. It is presumed that flagella proteins are glycosylated during or immediately prior to translocation by a mechanism quite distinct from eukaryotic processes. The secreted glycoprotein GspB of *S. gordonii* is one of the few identified substrates translocated by the newly identified SecA2 protein translocation pathway (11). The mechanism by which GspB is translocated is unknown but mutants deficient in the SecA2 system appear to accumulate glycosylated forms of GspB

within the cytosol (10). This observation is unique since it is presumed that GspB passes through a modified Sec translocase (11). It now remains to be determined if the SecA2 translocase accommodates a modified protein. Previously it was demonstrated in a eukaryotic system that bulky *N*-linked glycans attached to proteins prevents passage though the Sec translocase *in vitro* (72). Since the bulk of prokaryotic glycoproteins are extracellular, how protein secretion contributes to protein glycosylation processes is an important but unexplored aspect of prokaryotic protein glycosylation. It is well established that the various prokaryotic protein translocation mechanisms transport proteins in a range of states, e.g., folded or linear. At present it is unknown if these mechanisms of secretion directs the ability of a protein to be glycosylated.

## 2.6 Functions of prokaryotic protein glycosylation

Not only are prokaryotic glycoproteins typically found in extracytoplasmic compartments, the glycans are commonly exposed to the environment. Archaeal and bacterial S-layer proteins are surface arrays of a single protein or peptide species (111). S-layers directly interact with the environment and may act to maintain cell shape or integrity, or as a molecular sieve (6). It is believed that the glycans on S-layer proteins extend from the cell surface analogous to the O-antigens of Gram negative organisms (106). The *Campylobacter* spp. flagella are glycosylated in a surface-exposed domain of the flagella filament (95). Glycosylated flagella monomers of *P. aeruginosa*, *L. monocytogenes*, and *H. pylori* are modified at sites localized to the central, surface-exposed domain of the filament (108-110). The pili subunits of *N. gonorrhoeae* and *P. aeruginosa* are glycosylated at surface-exposed sites (20, 89).

Glycosylation of the subunits of complex structures such as S-layers, pili, and flagella may enhance protein stability or assembly. The elaborate glycans found on S-layers, pili, or flagella may also contribute to the overall hydrophilic nature of these surface structures. Protein glycosylation is an essential process in eukaryotic cells and to date no prokaryotic glycosylation system has been found to be indispensable (14, 23, 97, 119, 140). This strongly suggests that prokaryotic glycoproteins play accessory roles *in vivo*. Under the proper environmental conditions prokaryotic glycoproteins could be required for cell viability, perhaps by maintaining cell wall integrity, driving cellular morphogenesis, protein stability, protein localization, or transport of proteins.

## 2.7 Rationale and objectives

The primary objective of this dissertation is to provide a critical examination of the biochemistry and genetics involved during early stages of mycobacterial *O*-mannoprotein biosynthesis. Specifically, this dissertation evaluates the role of protein secretion for mannoprotein biosynthesis, and provides the first identification of a mycobacterial protein mannosyltransferase. Understanding the genetics and biochemical processes involved in mycobacterial *O*-mannoprotein biosynthesis will permit future comprehension of how mannoproteins are implicated in mycobacterial physiology or pathogenesis. Additionally, mycobacterial *O*-mannoprotein biosynthesis may prove to be a viable drug target, and an understanding of the *M. tuberculosis* glycoprotein biosynthetic pathway(s) must first be established before novel drugs are rationally developed to inhibit their synthesis.

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# Chapter III

## Identification of a Mycobacterial Protein *O*-mannosyltransferase

Partially presented in Brian C. VanderVen, Jeffery D. Harder, Dean C. Crick, and John T. Belisle. 2005. Export Mediated Assembly of Mycobacterial Glycoproteins Parallels Eukaryotic Pathways. Science. Aug 5; Vol 309: 941-943.

### 3.1 Introduction

Structurally the glycoproteins of mycobacteria most closely resemble the *O*-mannosylated yeast secretory glycoproteins first identified in 1969 by Sentandreu and Northcote (34). The glycan core of yeast *O*-mannoproteins is comprised of a linear oligomannose chain with the reducing end α-linked to the hydroxy amino acids Ser or Thr (12). For many years it was believed that glycoproteins modified with an α-linked mannose to Ser or Thr residues were restricted to yeast. However, in the last 10 years it has been made clear that higher eukaryotic cells also modify Ser or Thr residues in a similar manner (5, 9, 50). Biosynthesis of *O*-mannosyl glycans has been studied most extensively in *Saccharomyces cerevisiae* and initiates in the Endoplasmic Reticulum (ER) lumen by an evolutionarily conserved family of dolichyl phosphate-β-D-mannose:protein *O*-α-D-mannosyltransferases (PMT) (37). PMT enzymes catalyze the transfer of a single mannosyl residue from dolichyl-phosphate-mannose (Dol-P-Man) to Ser and Thr residues of proteins entering the secretory pathway (37).

The genome of *Saccharomyces cerevisiae* encodes seven distinct PMTs that share little or no similarity to other known glycosyltransferases. Activity is confirmed for *Sc*Pmt1-4,6 and the *Sc*Pmt1-6 proteins share an overall amino acid sequence identity of 57% (14, 15). PMT homologues are also known in many multicellular

eukaryotes such as *Drosophila melanogaster*, mouse and, humans (21, 26, 47). In yeast the *O*-mannosylation of secretory proteins is essential for cell viability being required for cell wall integrity, cellular morphogenesis, protein stability, protein localization, and transport of misfolded proteins (13, 17, 32, 43). Protein *O*-mannosylation is also important in higher eukaryotes. In mammals, defects with protein *O*-mannosylation are linked to several forms of congenital muscular dystrophies and improper neuronal development (4, 48).

Although the biosynthetic pathway of *M. tuberculosis* protein glycosylation remains largely unknown, it is interesting to note the similarities between the *O*-mannosylation of *M. tuberculosis* proteins and the *O*-mannosylation of *S. cerevisiae* proteins. First, all mannoproteins of *M. tuberculosis* identified to date are exported (7, 10, 11, 27), and yeast protein *O*-mannosylation initiates in the lumen of the ER where nascent secretory proteins are glycosylated (37). Secondly, like *S. cerevisiae,* protein *O*-mannosyltransferase activity of *M. tuberculosis* is membrane associated and requires a lipid carrier, presumably mannosyl-phosphoryl-decaprenol for donation of mannose (6). These similarities between otherwise disparate organisms suggest that the enzymatic machinery for protein *O*-mannosylation might also be conserved. We hypothesized that the protein *O*-mannosylation events in *M. tuberculosis* occur in a similar manner to *S. cerevisiae.* This hypothesis was tested by identifying and evaluating a eukaryotic like PMT homologue of *M. tuberculosis* that shares several biochemical characteristics to the PMTs of *S. cerevisiae.*

## 3.2 Materials and methods

### 3.2.1 Bacterial strains and other materials

Recombinant clones of *Escherichia coli* DH5α and *M. smegmatis* $mc^2155$ were routinely selected on Luria-Bertani (LB) agar. Growth of *E. coli* strains for the propagation of recombinant plasmids was performed in LB broth containing 25 μg/ml kanamycin. Growth of *M. smegmatis* for isolation of subcellular fractions was achieved by propagation in 2 L of glycerol-alanine-salts medium (41) at 37°C with gentle shaking. Kanamycin at the concentration of 25 μg/ml was added for the selection of mycobacterial strains harboring recombinant plasmids. Cells were harvested after approximately two days at an optical density of 0.4-0.6 at 600nm. The cytoplasmic membrane subcellular fraction was isolated by ultra-centrifugation as previously described (20, 36). Briefly, the whole bacilli were resuspended (2 g/ml) and washed once in assay buffer [50 mM MOPS (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT] containing EDTA-Free Complete protease inhibitor cocktail according to the manufacturer's protocol (Roche, Basel, Switzerland). Following washing, the whole bacilli were suspended (2 g/ml) and washed once in assay buffer plus protease inhibitors and broken by probe sonication at 4°C. Sonication proceeded for eight 1 min cycles with 30 sec rests at 4°C. Unbroken cells and cell walls were removed by centrifugation at 27,000 x g for one hour. The 27,000 x g supernatant was subjected to a 100,000 x g centrifugation for two hours resulting in a cytoplasmic membrane pellet which was resuspended and termed the cytoplasmic membrane extract. Final protein concentrations were determined using the bicinchoninic acid (BCA) protein assay (35).

### 3.2.2 Construction of Rv1002c point mutations

To produce a recombinant Rv1002c enzyme for *in vitro* protein mannosylation assays a 2,206 bp gene fragment encoding Rv1002c was PCR amplified from *M. tuberculosis* H37Rv genomic DNA using the primer pair 1002cF and 1002cR (Table 3.1) and ligated into *Bam*HI and *Xba*I digested pMH29 creating pBV90 (18). Derivatives of pBV90 with point mutations in *Rv1002c* were generated by PCR-based, site directed mutagenesis (46). Briefly, site directed mutagenesis of plasmid DNA was achieved by PCR amplifying pBV90 with mutagenesis oligonucleotide primers listed in Table 3.1. PCR amplifications were carried out with an automated thermal cycler using the PFU polymerase (Stratagene, La Jolla, CA) for 10 cycles of denaturation at 94°C for 1 min, annealing at the specified temperature (Table 3.1) for 1 min, and extension at 72°C for 7 min. Following PCR amplification, *Dpn*I restriction endonuclease treatment was used to select against parental DNA molecules. The mutation-containing vector DNA was transformed into *E. coli* DHα.

Specifically, the plasmid that expresses Rv1002c with a $D_{55}E_{56}\rightarrow A_{55}A_{56}$ substitution (pBV90.1) was generated by PCR-based mutagenesis of pBV90 using the primer pair 1002cDE/AAF and 1002cDE/AAR. Likewise, the plasmid that expresses Rv1002c possessing a $E_{56}\rightarrow A_{56}$ substitution (pBV90.2) was generated by PCR-based mutagenesis of pBV90 with the primer pair 1002cDE/DAF and 1002cDE/DAR. Similarly, the plasmid that expresses Rv1002c possessing a $D_{55}\rightarrow A_{55}$ substitution (pBV90.3) was created by PCR-based mutagenesis of pBV90 with the primer pair 1002cDE/AEF and 1002cDE/AER. All plasmid constructs were confirmed by nucleotide sequencing through Macromolecular Resources (Colorado State

University, Fort Collins, CO). Overproduction of Rv1002c and its derivatives was achieved following electroporation of *M. smegmatis* $mc^2155$ with the plasmids pBV90, pBV90.1, pBV90.2, and pBV90.3.

**Table 3.1 Oligonucleotides used in the construction of the recombinant Rv1002c proteins and RT-PCR analysis.**

| Oligonucleotide | Annealing temp(°C) | Sequence |
|---|---|---|
| 1002cF | 60 | 5'GGATCCGGGCGAAACCCTCGAAGCAGAACT |
| 1002cR | 60 | 5'TCTAGACGGTGTCGTTGTCGCCTACG |
| 1002cDE/AAF | 75 | 5'ACCCCCATCTTCGCCGCGAAGCATTACGCA |
| 1002cDE/AAR | 75 | 5'TGCGTAATGCTTCGCGGCGAAGATGGGGGT |
| 1002cDE/DAF | 75 | 5'ACCCCCATCTTCGACGCGAAGCATTACGCA |
| 1002cDE/DAR | 75 | 5'TGCGTAATGCTTCGCGTCGAAGATGGGGGT |
| 1002cDE/AEF | 75 | 5'ACCCCCATCTTCGCCGAGAAGCATTACGCA |
| 1002cDE/AER | 75 | 5'TGCGTAATGCTTCTCGGCGAAGATGGGGGT |
| 1002c 450F | 60 | 5'CATATGGTGGTACCCGTCGTCAGCC |
| 1002c 450R | 60 | 5'GGATCCATCGGTGACGAAGCTGAC |
| RplJF | 65 | 5'GCAGCGACAGAGCAGGCG |
| RplJR | 65 | 5'CCGCCAAGGCGATCAAGA |

Engineered restriction sites are denoted by the underline

### 3.2.3 Gene expression analysis

Total bacterial RNA from *M. smegmatis* strains was isolated by a Trizol (Invitrogen, Carlsbad, CA) based method (24). Briefly, whole *M. smegmatis* $mc^2155$ cells were resuspended in (2 g/ml) PBS (pH 7.4) and washed once. Following washing, the whole bacilli were resuspended in Trizol (2 g/ml) and broken by probe sonication at 4°C. Sonication proceeded for eight 1 min cycles with 30 sec rests at 4°C. Unbroken cells and cell walls were removed by 27,000 x g centrifugation at 4°C and for one hour. The Trizol layer was removed and 0.2 volumes of chloroform was added and centrifuged at 27,000 x g for 30 min. The chloroform layer was removed to a new tube and 2.5 vol of isopropanol was added, vortexed and precipitated overnight at -70°C. Isopropanol precipitated material was centrifuged 27,000 x g, at 4°C for one

hour. Following centrifugation the isopropanol pellet was suspended in 2 mL of 80% ETOH and RNA was purified with an RNeasy purification kit according to the manufacturer's instructions (Qiagen, Valencia, CA). This purified material was then treated with RNAse free DNAse (MBI Fermentas, Hanover, MD) at 1 unit/μg total RNA for 30 min at 37°C. DNA free total RNA was repurified using an RNeasy purification kit according to the manufacturer's instructions (Qiagen, Valencia, CA). Synthesis of cDNA was performed with 800 ng RNA serving as the template using gene specific primers and the SuperScript first-strand synthesis system (Invitrogen, Carlsbad, CA). An aliquot (5-μl) of the cDNA reaction mixture then served as the template for PCR amplification using gene specific primers. For Rv1002c cDNA synthesis and PCR amplification the primer pair 1002c 450F and 1002c 450R were used. As a positive control the primers RplJF and RplJR were used to generate cDNA and amplify a segment of the *M. smegmatis* ribosomal subunit *rplJ* mRNA. All PCR products were visualized on a 1% agarose gel.

### 3.2.4 Protein *O*-mannosylation *in vitro* assays

To evaluate PMT activity an *in vitro* glycosylation assay was employed using cytoplasmic membrane extracts of recombinant *M. smegmatis* $mc^2155$ strains as the enzyme source (6). Specifically, membrane preparations (2 mg protein) were suspended in assay buffer (50 mM MOPS (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT) to which 1.9 nmol [$^{14}C$] GDP-mannose with a specific activity of 260 mCi/mmol (Perkin Elmer, Boston, MA) and 1 mmol of the synthetic peptide (AAAPPAPATPVAPPPPHHHHHH) were added. The final reaction volume was brought to 500 μL with assay buffer. The assay mixture was incubated at 37°C for 30

min and the enzymatic reaction was stopped by heating at 85°C for 2 min. The suspension was centrifuged at 13,000 x g for 30 min to remove particulate material, and the supernatant was recovered. Radiolabeled peptides were purified from the supernatant of the enzymatic reaction by nickel affinity chromatography. Briefly, the supernatant was applied to a 0.5 ml (bed vol) His-Bind resin (Novagen, Madison, WI). Ten column vol of 20 mM Tris-HCl (pH 7.9), 500 mM NaCl was added to the column and allowed to flow by gravity. The resin was then washed with six column vol of 20 mM Tris-HCl (pH 7.9), 500 mM NaCl, 60 mM imidazole to remove non-specifically bound material and the bound glycosylated his-tagged peptides were eluted with 6 column vol of 20 mM Tris-HCl (pH 7.9), 500 mM NaCl, 1M imidazole. Each fraction was added to 10 ml of EcoLume scintillation fluid (ICN, Costa Mesa CA) and counted for 1 min on a Beckman LS6500 Scintillation counter (Beckman, Fullerton CA). Specific activity was calculated as pmol of mannose incorporated per mg protein per hr.

### 3.2.5 Bioinformatic methods

BLAST based searches employed for PMT discovery were applied to the GenBank *M. tuberculosis* H37Rv complete genome sequence (Genbank accession number AL123456) using the BLASTP and TBLASTN programs, National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov/blast) (1). Initial queries for PMT searches used the amino acid sequences for *S. cerevisiae* ScPmt1-6, accession numbers NP_010188, NP_009379, NP_014966, NP_012677 NP_010190 NP_011715, respectively. Transmembrane domains were predicted by analyzing the Rv1002c amino acid sequence with TMHMM Version 2.0

(www.cbs.dtu.dk/services/TMHMM), and HMMTOP Version 2.0 (www.enzim.hu/hmmtop) (28, 44). Hydropathy prediction was also performed with the Rv1002c amino acid sequence according to Kyte-Doolittle (23). Amino acids predicted by hydropathy analysis displaying scores larger than zero were considered as being part of a transmembrane helix. Protein sequences were aligned using ClustalW Version 1.82 (www.ch.embnet.org/software/ClustalW.html) (19). Pairwise identity was established using the NCBI BLAST 2.0 algorithm (www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi) (42). BLAST 2.0 was run using the default BLOSUM62 matrix allowing 5 open gap penalties and 2 extension gap penalties with a word size setting of 3. For phylogenetic analysis, PMT amino acid sequences were aligned with AlignX, a component of the Vector NTI Advance 9.0 (Informax) sequence analysis program, and the phylogenetic tree was constructed using the neighbor-joining method of the multiple sequence alignment (31).

## 3.3 Results

### 3.3.1 Identification of a mycobacterial protein *O*-mannosyltransferase candidate

Recognizing the structural similarities between the *O*-mannoproteins of yeast and *M. tuberculosis*, the PMTs of *S. cerevisiae* served as model proteins for comparative bioinformatic analyses to identify a putative *M. tuberculosis* PMT. Comparing six *S. cerevisiae* PMTs to the *M. tuberculosis* H37Rv genome by BLAST analyses identified a single mycobacterial PMT candidate designated Rv1002c. The gene *Rv1002c* encodes a putative 55.5 kDa protein that shares 20 to 24% amino acid identity with the PMTs of *S. cerevisiae* and shares 24 to 27% amino acid identity with

known PMTs from higher eukaryotic organisms (Fig 3.1). Rv1002c also belongs to a small family of glycosyltransferases, designated as Family 39 by the CAZy classification (afmb.cnrs-mrs.fr/CAZY/). Lastly, Rv1002c conserves a distinct pfam02366.11 domain found in all PMT enzymes which is believed to be responsible for catalytic activity (16). The protein Rv1002c is highly conserved in *M. tuberculosis* CDC1551, *M. bovis* AF2122/97, *M. leprae* TN, *M. avium* str 104, *M. avium* subsp. *paratuberculosis* str K10, and *M. smegmatis* $mc^2155$ (Fig 3.1). Of the 23 completely sequenced and annotated *Actinomycete* genomes, Rv1002c homologs were detected in 16 organisms, 11 of which are not *Mycobacterium* spp (Fig 3.1).

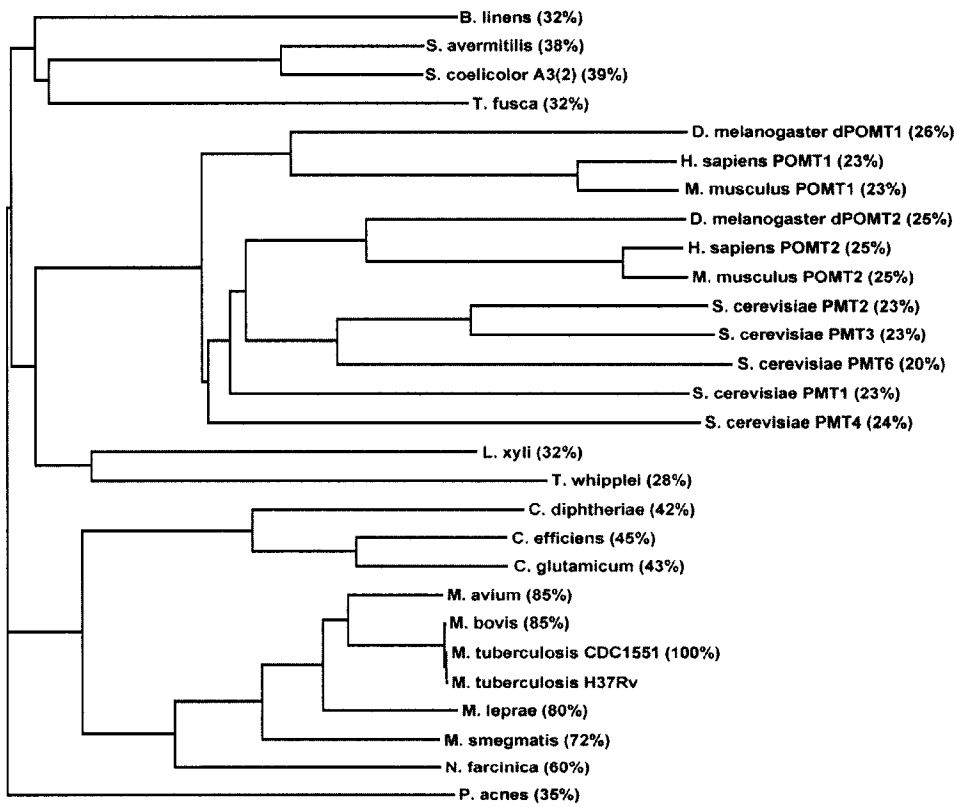



**Fig 3.1 PMT phylogenetic analysis.** Following alignment with AlignX, the phylogenetic tree was constructed using the neighbor-joining method of the multiple sequence alignment. Amino acid identities shared with Rv1002c are indicated in the parentheses.

*S. cerevisiae* PMTs are complex transmembrane proteins and display nearly identical hydropathy profiles. Of the seven *S. cerevisiae* PMTs only *Sc*Pmt1p has received in-depth characterization. *Sc*Pmt1p is an integral ER membrane glycoprotein with seven transmembrane domains (39). The N terminus of *Sc*Pmt1p resides in the cytoplasm, and the C terminus resides in the lumen of the ER (16). Two major hydrophilic domains are located between transmembrane domains one and two (Loop 1), and five and six (Loop 2) (16). These ER lumen located domains of *Sc*Pmt1p are believed to be responsible for enzymatic activity and substrate recognition, respectively. Rv1002c is predicted to be a putative integral membrane protein and presents a hydropathy profile similar to *S. cerevisiae* PMTs (Fig 3.2). Like the PMTs of *S. cerevisiae*, Rv1002c is also predicted to possess two major hydrophilic domains (Fig 3.3). Both the Loop 1 of *Sc*Pmt1p and the predicted Loop 1 of Rv1002c are found within the pfam02366.11 domain suggesting that this region is responsible for PMT activity. Additionally, the pfam02366.11 domain of both *Sc*Pmt1p and Rv1002c share similar putative transmembrane regions at comparable relative positions (Fig 3.3).

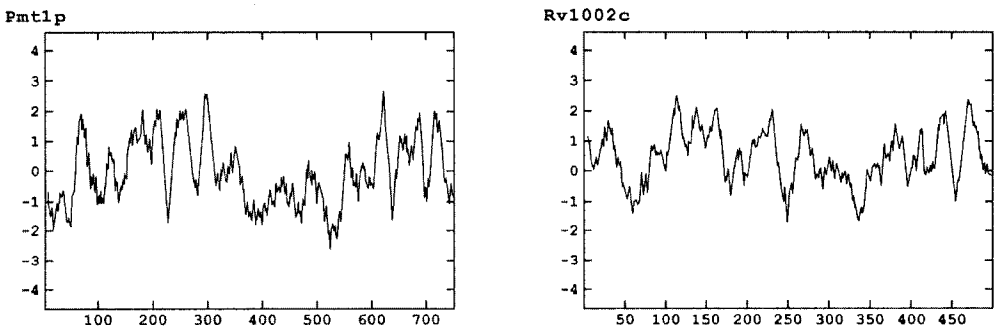
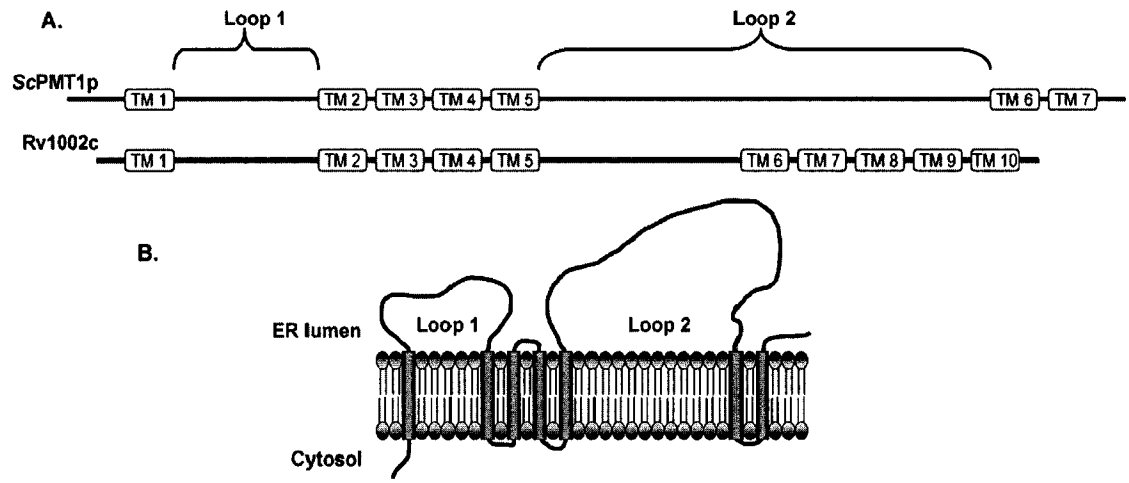



**Fig 3.2 Hydropathy profiles of ScPmt1 and Rv1002c.** Hydropathy profiles were established using the standard Kyte-Doolittle hydropathy scale (23).

More prognostic of potential PMT activity by Rv1002c is the presence of several key conserved residues within the pfam02366.11 domain. Rv1002c possesses a conserved $Arg_{39}$ in the first putative transmembrane domain and a distinct $Asp_{55}$-$Glu_{56}$ motif within Loop 1 (Fig. 3.3). Rv1002c also possesses a conserved $Arg_{106}$ within the second putative transmembrane domain (Fig. 3.3). It should be noted that these residues are found invariantly conserved in all PMTs listed in Fig 3.1. $Arg_{64}$ and $Arg_{138}$ of *Sc*Pmt1p lie near the membrane-water interface also within the transmembrane domains bracketing Loop 1 and are essential for transferase activity (16). $Arg_{64}$ of *Sc*Pmt1p appears to be important for PMT catalytic activity while $Arg_{138}$ contributes to PMT dimerization, probably by maintaining a local arrangement of the protein (16).




C.

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    PMT1   (62) VIRLHGLA---WPDSVVFDEVHFGGFASQYIRGTYFMD-------VHPPLAKMLYAGVASLGGFQGDFDFENIGDSFPSTTP-YVLMRFFSAS
    PMT2   (77) FTRMYKIG---INNHVVWDEAHFGKFGSYYLRHEFYHD-------VHPPLGKMLVGLSGYLAGYNGSWDFP-SGEIYPDYLD-YVKMRLFNAS
    PMT3   (70) FTRMYKIG---RNNHVVWDEAHFGKFGSYYLRHEFYHD-------VHPPLGKMLVGLSGYLAGYNGSWDFP-SGEVYPDYID-YVKMRLFQAM
    PMT6   (78) YLRFQHID---QNNYVVWDEAHFGKFGSYYIKHEYYHD-------VHPPLGKMLIALSEWMAGFDGQFDFS-SNNAYPENVN-FKLMRQFNAT
    PMT4   (65) AARFYKIW---YPKEVVFDEVHFGKFASYYLERSYFFD-------VHPPFAKMMIAFIGWLCGYDGSFKFDEIGYSYETHPAPYIAYRSFNAI
  Rv1002c  (37) VTRFLNLGSLTDAGTPIFDEKHYAPQAWQVLNNHGVEDNPGYGLVVHPPVGKQLIAIGEAIFGYNG----------------FGWRFTGAL
  Consensus        R              DE H              D      VHPP  K         G  G                    R   A
```

**Fig 3.3 Predicted transmembrane topology of Rv1002c and partial alignment of putative PMT active site domains.** Approximate positions of the known

transmembrane domains (TM) of *Sc*PMT1 in relation to the predicted TM domains of Rv1002c **(A)**. Graphic representation of ER membrane topology of *Sc*PMT1 **(B)**. Partial alignment of the Loop 1 and flanking transmembrane domains of known yeast PMT enzymes and Rv1002c **(C)**. Dark shading represents invariant residues. The conserved $R_{64}$, $E_{78}$, and $R_{138}$ of *Sc*PMT1 required for PMT activity are indicated by triangles (16).

The conserved $Glu_{56}$ of Rv1002c also found in Loop 1 of *Sc*Pmt1p ($Glu_{78}$) is 100% conserved in all characterized PMT enzymes (Fig 3.3) and is absolutely required for enzymatic activity (16). It is proposed that the conserved Glu residue functions as a nucleophile involved in catalysis. A single $S_{N2}$ nucleophilic substitution could account for an inversion of the anomeric configuration of a β-linked mannose from a lipid carrier to an acceptor Ser or Thr residue (22). A mechanism of this type would also require donation of a proton likely from a His, Arg, or Lys residue and there are several potential residues conserved by both yeast PMTs and Rv1002c within Loop 1 that fulfill this requirement (Fig 3.3). The Rv1002c $Asp_{55}$ is also conserved in all characterized PMT enzymes and in terms of the acid-base chemistry proposed for these enzymes it could also serve as an active site nucleophile.

Comparison of yeast PMTs has identified the conserved Loop 2 motifs: LHSH-$X_3$-YP-$X_{2-9}$-S-$X_2$-QQ(V/I)T-X-Y-$X_3$-D-X-NN-X-W, L-X-H-$X_2$-T-$X_3$-L-$X_2$-H-$X_{11-14}$E, and P-X-W-X-F-X-Q-X-E-$X_{13-15}$-W-$X_2$-E corresponding to amino acid positions 345-373, 399-426, and 487-512 of *Sc*Pmt1, respectively (16). $Leu_{408}$ is essential for *Sc*Pmt1 activity and point mutations in other perfectly conserved residues within these regions are tolerated suggesting that the structure of Loop 2 is essential for *Sc*Pmt1p activity (16). Moreover, secondary structure predictions of *Sc*Pmt1 Loop 2 indicate this domain is composed of alternating α-helices and β-

strands which potentially bind protein substrates, probably in co-operation with Pmt2p (16). Rv1002c does not conserve the above-mentioned Loop 2 domains found in yeast PMTs and in general little overall homology exists between the Loop 2 regions of yeast PMTs and Rv1002c. Thus the sequence variability in Loop 2 of yeast PMTs and Rv1002c probably reflects the different substrate specificities of the PMT enzymes.

### 3.3.2 *In vitro* analysis of Rv1002c

Demonstration of PMT activity by Rv1002c was complicated by our inability to produce an *E. coli* recombinant form of this integral membrane protein and the previously observed essentiality of Rv1002c (33). Several strategies for the production of an epitope tagged recombinant Rv1002c were attempted in *E. coli*. Specifically, Rv1002c was cloned into pET23b and pET15 under control of a T7 promoter, creating an in-frame C- and N-terminal fusion to a hexa-his tag, respectively. The recombinant Rv1002c expression constructs were transformed into numerous *E. coli* BL21 variants and induced under conditions in which isopropyl-β-D-thiogalactopyranoside concentrations, duration of induction, and temperature varied. Western blot analysis was preformed on crude lysates using a monoclonal anti hexa-his tag antibody to confirm recombinant expression. Despite conditions or construct tested no recombinant Rv1002c was produced as determined by Western blot analysis. Rv1002c was also cloned into the mycobacterial expression vectors pVV16 and pVV2 under control of a *hsp60* promoter, creating in-frame C- and N-terminal fusions to a hexa-his tag, respectively. Recombinant expression of Rv1002c

was attempted in *M. smegmatis* and again no recombinant Rv1002c production was observed as determined by Western blot analysis.

Therefore, a strategy of overexpressing Rv1002c in *M. smegmatis* and assaying for increased enzymatic activity was employed. This approach was recently used to define the function of gene products (DesA3) involved in mycobacterial cell wall biosynthesis (30). In addition, a similar approach that overexpressed human PMTs was used to detect PMT activity in mammalian cells (25). To confirm that Rv1002c functions as a mycobacterial PMT, the wild type *Rv1002c* gene and three mutated forms conferring $D_{55}E_{56}\rightarrow A_{55}A_{56}$, $D_{55}E_{56}\rightarrow A_{55}E_{56}$, or $D_{55}E_{56}\rightarrow D_{55}A_{56}$ substitutions in the conserved $D_{55}E_{56}$ motif were cloned into the high copy mycobacterial expression vector pMH29 (18) and overexpressed in *M. smegmatis*. RT-PCR analyses confirmed expression of the *Rv1002c* recombinant genes in *M. smegmatis* (Fig 3.4). For RT-PCR total RNA purified from *M. smegmatis* strains containing the various expression plasmids served as a template for a single step cDNA synthesis reaction using *Rv1002c* and *RplJ* gene specific primers. An aliquot of the cDNA served as the template for conventional PCR using the same primers. For the PCR positive control *M. tuberculosis* H37Rv and *M. smegmatis* $mc^2155$ genomic DNA served as the template and were amplified using the gene specific primers for *Rv1002c* and *RplJ*, respectively. Contaminating DNA present in the total RNA preparation was removed by RNAse free DNAse pretreatment. To verify that no DNA contaminated the total RNA preparation, cDNA synthesis reactions lacking reverse transcriptase (No RT) were run and aliquots of these reactions served as a template for conventional PCR using the same gene specific primers.

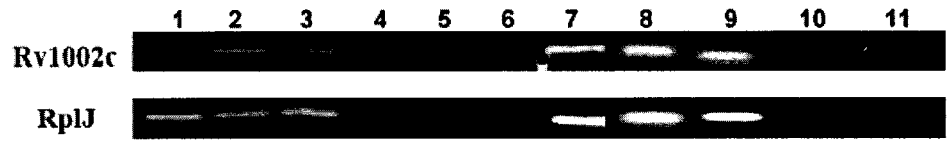


**Fig 3.4 RT-PCR analysis of *M. smegmatis* expressing recombinant forms of Rv1002c.** Ethidium bromide stained amplicons separated on a 1.0 % agarose gel. Lane 1, *M. smegmatis*/pMH29; Lane 2, *M. smegmatis*/pBV90; Lane 3, *M. smegmatis*/pBV90.1; Lane 4, No RT *M. smegmatis*/pMH29; Lane 5, No RT *M. smegmatis*/pBV90; Lane 6, No RT *M. smegmatis*/pBV90.1; Lane 7, Genomic DNA positive control; Lane 8, *M. smegmatis*/pBV90.2; Lane 9, *M. smegmatis*/pBV90.3; Lane 10, No RT *M. smegmatis*/pBV90.2; Lane 11, No RT *M. smegmatis*/pBV90.3.

Cytoplasmic membrane preparations derived from log-phase grown *M. smegmatis* expressing the various forms of recombinant *Rv1002c* were assayed for their ability to transfer radiolabeled mannose to a hexa-his tagged synthetic peptide (AAAPPAPATPVAPPPPHHHHHH). This synthetic peptide contained a 16 amino acid glycosylation sequence derived directly from the *M. tuberculosis* MPT32 antigen and spanned a single glycosylation site (7). Previously it was demonstrated that mycobacterial cytoplasmic membrane extracts catalyze the incorporation of radiolabeled mannose from GDP-[$^{14}$C]mannose onto peptide acceptors containing glycosylation sequences derived from MPT32 (6). This mycobacterial PMT assay is a composite assay requiring two distinct enzymatic activities. First GDP-[$^{14}$C]mannose is utilized by an endogenous polyprenol-monophosphomannose synthase forming polyprenyl monophosphoryl-Man*p*. Polyprenyl monophosphoryl-Man*p* is then utilized by a PMT for protein mannosylation, indicated by the sensitivity of this assay to amphomycin (6). Amphomycin specifically inhibits the transfer of monomeric carbohydrate units to polyprenol phosphate carriers (3).

Cytoplasmic membranes prepared from *M. smegmatis* containing the vector control first established a background level of PMT activity due to the endogenous *M. smegmatis* Rv1002c homologue (Fig 3.5). Membrane extracts prepared from *M. smegmatis* expressing the wild type *M. tuberculosis Rv1002c* gene had significantly increased (66.8%) PMT activity over background levels (Fig. 3.5). Recombinant Rv1002c proteins with $D_{55}E_{56}$ to $A_{55}A_{56}$ or $D_{55}A_{56}$ substitutions failed to significantly augment protein mannosylation activity of *M. smegmatis* membrane preparations over vector control levels. Thus, only wild type recombinant Rv1002c conferred increased PMT activity and the $D_{55}$ and $E_{56}$ residues conserved between Rv1002c and *Sc*Pmt1p are critical to Rv1002c PMT activity. Interestingly, membranes containing recombinant Rv1002c with a DE to AE substitution resulted in decreased (56.0%) mannosyltransferase activity compared to the *M. smegmatis* vector control. This suggests that altered forms of Rv1002c could compete for substrate binding without catalysis of glycosylation.

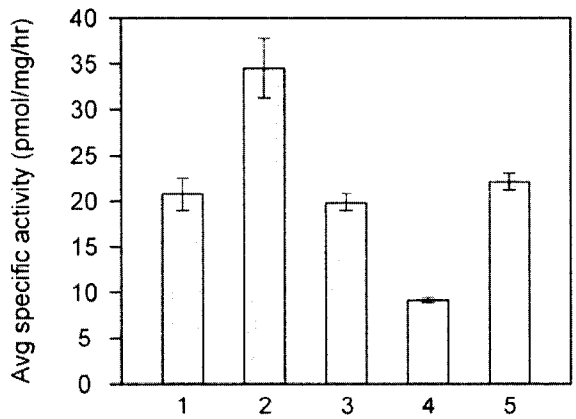


**Fig 3.5 *In vitro* protein mannosylation activity of cytoplasmic membranes containing recombinant *Mtb* Rv1002c derivatives.** Column 1, vector control; column 2, W.T. Rv1002c; column 3, Rv1002c $D_{55}E_{56}\rightarrow A_{55}A_{56}$ mutant; column 4, Rv1002c $D_{55}E_{56}\rightarrow A_{55}E_{56}$ mutant; column 5, Rv1002c $D_{55}E_{56}\rightarrow D_{55}A_{56}$ mutant.

## 3.4 Discussion.

In this study, we utilized an existing method to detect the PMT activity in *M. smegmatis* cytoplasmic membrane extracts (6). Using this method, we examined whether endogenous *M. smegmatis* PMT activity was enhanced by overexpressing *Rv1002c* in *M. smegmatis.* We found that PMT activity was significantly increased in extracts from cells expressing wild type *Rv1002c* but not in extracts from cells expressing point mutated forms of *Rv1002c.* Specifically, point mutated forms of Rv1002c in which the putative PMT active site $Glu_{56}$ and $Asp_{55}$ residues were substituted simultaneously and individually with Ala were inactive *in vitro.* Based on the acid-base catalytic mechanism proposed for PMTs either $Asp_{55}$ or $Glu_{56}$ could function as nucleophiles in the PMT glycosyltransfer reaction and this idea is supported by the finding that both residues are important for *M. tuberculosis* PMT activity.

The primary purpose of this study was to identify a *M. tuberculosis* PMT. Further biochemical characterization of Rv1002c will involve kinetic studies, requiring pure native or recombinant Rv1002c enzyme. Several techniques are commonly employed to purify active native yeast PMT enzymes, which can be directly applied to obtaining pure Rv1002c. Ion exchange chromatography and/or hydroxyapatite fractionation of detergent solubilized membrane extracts is a classic yeast PMT purification procedure (40). Immunoprecipitation of sodium deoxycholate solubilized membrane extracts is routinely employed to purify yeast PMTs (15, 16, 38). Recombinant expression and purification of yeast PMTs from *E. coli* is uncommon. One report exists in the literature, in which *Sc*Pmt1 was expressed in *E.*

*coli* resulting in PMT activity (38). It should be noted that this study simply assayed *E. coli* lysates for PMT activity and the recombinant *Sc*Pmt1 was not purified nor epitope tagged. Expression of recombinant integral membrane proteins in *E. coli* remains a difficult task due to the toxic effects exerted by hydrophobic domains of the recombinant protein on the host cell. To overcome this difficulty, strategies involving various epitope tagged fusion partners or designer strains of *E. coli* which produce more membrane material are often employed and could be applied to Rv1002c (2). Lastly, further studies of Rv1002c will also require purified mannosyl-phosphoryl-decaprenol and there are established procedures for obtaining this molecule (29).

The assay employed to detect PMT activity in *M. smegmatis* cytoplasmic membranes utilized a synthetic peptide acceptor derived from a *M. tuberculosis* mannoprotein. It is now becoming clear that PMT enzymes are substrate specific, able only to glycosylate particular target protein sequences (8, 14, 25, 32). The specificities of PMTs towards discrete protein substrates is probably mediated by structural elements contained within the variable Loop 2, present in all PMTs. This idea is supported by the finding that yeast glycosylation substrate peptides are not glycosylated by human PMTs and only human specific peptides are glycosylated by human PMTs (25). Accordingly, the poor homology of Loop 2 observed with Rv1002c and other PMTs is probably indicative of specific substrate requirements.

The *S. cerevisiae* PMT enzymes are divided into three subfamilies (PMT1, PMT2 and PMT4) based on sequence homology and dimerization capacity. The *S. cerevisiae* PMT1 subfamily is comprised of *Sc*Pmt1p and *Sc*Pmt5p, the PMT2 subfamily is made up of *Sc*Pmt2p and *Sc*Pmt3p, lastly *Sc*Pmt4p comprises the PMT4

subfamily. Active *S. cerevisiae* enzyme complexes form when PMT1 subfamily members heterodimerize with PMT2 subfamily members or when *Sc*Pmt4p forms homodimers (15). Recently, PMT activity was described from mammalian cells and this activity is dependent upon coexpression of both human PMT homologs (25). Consequently, with the absence of any other PMT homologs in the *M. tuberculosis* genome, Rv1002c may function as a homodimer.

For two decades, synthesis of *O*-mannosylated proteins has been studied exclusively in yeast. The discovery of an active PMT in *M. tuberculosis* confirms that protein *O*-mannosylation is conserved from bacteria to man. Our current data along with the recent discovery of an eukaryotic like OST *N*-glycosylation system in *C. jejuni* demonstrate a previously unrealized evolutionary conservation between protein glycosylation pathways of eukaryotes and those of specific bacterial species (45). Interestingly, recent saturating transposon mutagenesis experiments in *M. tuberculosis* has identified *Rv1002c* as an essential gene (33). Unlike the dispensable *N*-glycosylation system of *C. jejuni* (49), the apparent essentiality of protein *O*-mannosylation suggests a fundamental role of mannoproteins in *M. tuberculosis* physiology.

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# Chapter IV

## Localization of Mycobacterial Protein *O*-mannosylation and its Dependence on Sec-translocation.

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## 4.1 Introduction

Extracellular bacterial proteins are ideally positioned to participate in host–pathogen interactions. Key molecules of bacterial virulence are secreted proteins, their posttranslational modifications, and the systems responsible for protein export (7, 23, 28, 29, 32, 42). Strikingly, most glycoproteins produced by bacteria are extracytoplasmic including S-layer subunits, flagella, lipoproteins and fully secreted proteins (33). Only recently has the spatial and/or secretion requirements of bacterial glycoprotein biosynthesis been addressed. *Streptococcus gordonii* glycosylates the platelet binding cell surface protein GspB by an uncharacterized cytosolic glycosyltransferase(s) prior to translocation. (4). *Helicobacter pylori* and *Campylobacter jejuni* are hypothesized to *O*-glycosylate flagellin subunits by a membrane bound glycosyltransferase given that cytosolic protein preparations contain non-glycosylated flagellar subunits and the apparent donor for these reactions is a nucleotide-activated sugar (24, 37). PglB is the integral membrane protein responsible for the *N*-linked glycosylation of numerous *C. jejuni* secreted proteins and appears to catalyze the formation of glycoproteins within the periplasm of this organism (27, 43). Similarly, all mycobacterial *O*-mannoproteins identified to date are exported in a Sec-dependent manner, and *in vitro* protein *O*-mannosylation assays have

demonstrated the presence of an active protein *O*-mannosyltransferase enzyme(s) in mycobacterial cytoplasmic membrane extracts (8).

Protein glycosylation in eukaryotic organisms occurs during the Sec-translocation of nascent secretory proteins into the ER (19). Biosynthesis of yeast *O*-mannosylated glycoproteins initiates when a single mannose is linked to a Sec-translocated protein in the ER by an evolutionarily conserved family of dolichyl phosphate-β-D-mannose:protein *O*-α-D-mannosyltransferases (PMT) (39). Structure function analyses of the yeast integral membrane PMTs has revealed an orientation within the ER membrane that places the active site domain and the majority of the PMT enzyme in the ER lumen (14, 40). In this way, protein *O*-mannosylation in yeast directly depends on Sec-translocation into the ER lumen. Additionally, prokaryotic and eukaryotic organisms conserve the Sec-dependent system to translocate secretory proteins. Abundant evidence exists for the *O*-mannosylation of Sec-translocated proteins of *M. tuberculosis* and our identification of a *M. tuberculosis* PMT homolog (Rv1002c) strongly suggests that *O*-mannosylation of mycobacterial proteins may also depend on Sec-translocation (10, 12, 13, 17, 20, 25). Unlike eukaryotic organisms, prokaryotes have a variety of protein translocation pathways to transfer proteins across lipid bilayers. We hypothesized that protein mannosylation and Sec-translocation are linked in *M. tuberculosis*. The studies presented in this chapter address this hypothesis and whether other translocation mechanisms of *M. tuberculosis* support protein mannosylation

## 4.2 Materials and Methods

### 4.2.1 Bacterial strains, growth and subcellular fractionation

Recombinant clones of *E. coli* DH5α and *M. smegmatis* $mc^2155$ were selected on Luria-Bertani (LB) agar containing 100 μg/ml hygromycin. Recombinant clones of *M. tuberculosis* H37Rv were selected on Middlebrook 7H11 medium supplemented with oleic acid-dextrose catalase, and 100 μg/ml hygromycin. Growth of *E. coli* strains for the propagation of recombinant plasmids was performed in LB broth containing 100 μg/ml hygromycin. Growth of *M. tuberculosis* and *M. smegmatis* for isolation of subcellular fractions and protein purification was achieved by propagation in 1 L of glycerol-alanine-salts medium (41) at 37°C with gentle shaking. Hygromycin at a concentration of 100 μg/ml was added for selection of mycobacterial strains harboring recombinant plasmids. Cells and culture media were harvested after fourteen or three days of growth for *M. tuberculosis* or *M. smegmatis* strains, respectively. Individual subcellular fractions of culture filtrate, cytosol, membrane, and cell wall were isolated by centrifugation and ultra filtration as previously described (21, 36). Briefly, the culture filtrate was harvested from the actively growing cells by sterile filtration and concentration by Amicon ultrafiltration (Millipore, Billerica, MA) with a 10,000 Da molecular weight cutoff membrane. Following inactivation with 24,000 Gy of gamma-irradiation the whole bacilli were suspended (2 g/ml) in breaking buffer [50 mM Tris, 150 mM NaCl, 100 mM EDTA pH 7.4] containing 0.06% DNase, 0.06% RNase, 0.07% pepstatin, 0.05% leupeptin, and 20 mM phenylmethylsulfonyl fluoride (PMSF) and broken by probe sonication at $4^{o}$C. Sonication proceeded for ten 1 min cycles with 30 sec rests at 4°C between each rest. Unbroken cells were removed by low speed (3,000 x g) centrifugation and the cell wall was isolated by centrifugation at 27,000 x g for one hour. The 27,000 x g

supernatant was subjected to a 100,000 x g centrifugation for two hours resulting in a cytoplasmic membrane pellet and cytosolic supernatant. Final protein concentrations were determined using the bicinchoninic acid (BCA) protein assay (35).

Subcellular fractions were adjusted to a final concentration of 1.0 mg of protein per ml and an equal volume of PBS saturated phenol was added. After mixing, the phenol and aqueous phases were separated by centrifugation at 27,000 x g, 22°C for 60 min. The phenolic phase and interface were collected and extensively dialyzed against deionized water followed by dialysis against 10 mM ammonium bicarbonate. Protein concentration following dialysis was determined by BCA.(10, 12, 13, 17, 20, 25)

### 4.2.2 Plasmid construction for chimeric protein production.

Recombinant plasmid constructs were created according to standard molecular biology protocols and are summarized in Table 4.1 (31). All PCR amplifications of genes or gene fragments were performed with *M. tuberculosis* H37Rv genomic DNA serving as template except where noted. PCR amplifications were carried out with an automated thermal cycler using the PFU polymerase (Stratagene, La Jolla, CA) for 30 cycles of denaturation at 94°C for 1 min, annealing for 1 min, and extension at 72°C for 3 min. Specific oligonucleotides and annealing temperatures are listed in Table 4.2. PCR amplicons were analyzed by 1% agarose gel electrophoresis. To generate a mycobacterial expression plasmid encoding an exported form of FbpC fused at the C-terminus to a mannosylation domain of MPT32/Rv1860, a 411 bp gene fragment encoding 137 amino acids corresponding to the mature N-terminus of MPT32 was PCR amplified using the primer pair ApaF and ApaR. This 411 bp fragment with

*Bam*H1 and *Hin*dIII linker sequences was ligated into the 2,656 bp *Bam*HI and *Hin*dIII digested pUC19, creating pBV66. The 1,035 bp gene encoding FbpC/Rv0129c, including the signal peptide but lacking a stop codon, was amplified by PCR using the primer pair SP(+)F and FbpCR, then ligated into the 3,067 bp *Nde*I and *Bam*HI digested pBV66 creating the intermediate plasmid pBV67. To generate the final fused gene construct in a mycobacterial hexa-his tagged expression vector, the full length *fbpC* 1,035 bp gene and the fused 45 bp of the MPT32 fusion were amplified by PCR employing pBV67 as the template and the primer pair SP(+)F and TApaR. This 1,080 bp PCR product was then ligated into the 5,781 bp *Nde*I and *Hin*dIII digested mycobacterial expression vector pVV16, and named pBV68 (34). The plasmid pBV68, encodes a full length FbpC (including the signal peptide) fused at the C-terminus to both a mannosylation domain of MPT32 and a hexa-his tag; this recombinant fusion protein was termed SP(+)FbpC-MC. To produce a non-exported form of FbpC fused at the C-terminus to the mannosylation domain of MPT32, the gene fragment encoding FbpC possessing a $Ser_{125}$to $Ala_{125}$ active site mutation was PCR amplified employing pCSB9sa as the template with the primer pair SP(-)F and FbpCR (3). Additionally, to create the non-exported FbpC fusion, the 894 bp *fbpC* gene sequence was PCR amplified to truncate the DNA that coded for the signal sequence. The truncated *fbpC* gene sequence was then ligated into the 5,834 bp *Nde*I and *Bam*HI digested pBV68 creating pBV69. The recombinant plasmid pBV69 encoded the fusion SP(-)FbpC-MC which is defined as an inactive FbpC devoid of its signal sequence fused at the C-terminus to both a mannosylation domain of MPT32 and a hexa-his tag.

**Table 4.1 Plasmids used in this study and their useful features.**

| Plasmid | Size (bp) | Origin | Marker | Features | Reference |
|---|---|---|---|---|---|
| pUC19 | 2686 | OriE | $Amp^r$ | Cloning vector | (31) |
| pVV16 | 5822 | OriE/OriM | $Kan^r/Hyg^r$ | Expression vector, hsp60 promoter, and hexa-his tag | (34) |
| pCSB9sa | 4566 | OriE | $Amp^r$ | pET23b + the full length *fbpC* gene containing active site mutation | (3) |
| pBV66 | 3067 | OriE | $Amp^r$ | pUC19 + the *mpt32* gene containing the mannosylation domain | This Work |
| pBV67 | 4102 | OriE | $Amp^r$ | pBV66 + the full length *fbpC* gene fused to the *mpt32* gene | This Work |
| pBV68 | 6861 | OriE/OriM | $Kan^r/Hyg^r$ | pVV16 + the full length *fbpC* gene fused to 15 *mpt32* codons | This Work |
| pBV69 | 6728 | OriE/OriM | $Kan^r/Hyg^r$ | pVV16 + the truncated *fbpC* gene fused to 15 *mpt32* codons | This Work |
| pBV70 | 6458 | OriE/OriM | $Kan^r/Hyg^r$ | pVV16 + the full length *sodA* gene fused to 15 *mpt32* codons | This Work |
| pBV71 | 6122 | OriE/OriM | $Kan^r/Hyg^r$ | pVV16 + the full length *esat6* gene fused to 15 *mpt32* codons | This Work |
| pBV71.1 | 6371 | OriE/OriM | $Kan^r/Hyg^r$ | pVV16 + *fbpC* gene sequence encoding the FbpC amino terminus fused to the full length *esat6* gene fused to 15 *mpt32* codons | This Work |

OriE denotes an *E. coli* orgin of replication and OriM denotes a mycobacterial orgin of replication.

To generate a recombinant SodA/Rv3847 fused to the mannosylation domain of MPT32, the 624 bp *sodA* gene lacking a stop codon was amplified by PCR using the primer pair SodAF and SodAR and ligated into the 5,834 bp *Nde*I and *Bam*HI digested pBV68 resulting in pBV70. The plasmid pBV70 encodes a full-length SodA protein fused at the C-terminus to both a mannosylation domain and hexa-his tag, termed SodA-MC.

To produce an ESAT-6 protein fused to the mannosylation domain of MPT32, the 288 bp *esat-6*/Rv3875 gene lacking a stop codon was amplified using the primer pair Esat-6F and Esat-6R and ligated into the 5,834 bp *Nde*I and *Bam*HI digested pBV68 plasmid resulting in pBV71. The recombinant plasmid pBV71 encodes the fusion ESAT-6-MC which is defined as ESAT-6 fused at the C-terminus with both a mannosylation domain and a hexa-his tag. To create SP(+)-ESAT-6-MC the 249 bp

gene fragment encoding the native signal peptide and 34 amino acids of the mature N-terminus of FbpC was amplified using the primer pair FbpC(SP)F and FbpC(SP)R and ligated to the 6122 bp *Nde*1 digested pBV71 resulting in the plasmid pBV71.1. SP(+)-ESAT-6-MC is defined as having the 46 amino acid native FbpC signal peptide and 34 amino acid residues of the mature amino terminus of FbpC fused to ESAT-6-MC which contains a mannosylation domain and hexa-his tag. All plasmid constructs were confirmed by nucleotide sequencing through Macromolecular Resources (Colorado State University, Fort Collins, CO).

**Table 4.2 Oligonucleotides used in the construction of the chimeric fusion proteins.**

| Oligonucleotide | Annealing temp (°C) | Sequence |
|---|---|---|
| 32F | 58 | 5'GGATCCGATCCGGAGCCAGCGCCCCC |
| 32R | 58 | 5'AAGCTTTTGGTCTAGCCGGCCGAGCA |
| SP(+)F | 55 | 5'CATATGACGTTCTTCGAACAGGTGCG |
| SP(-)F | 55 | 5'CATATGTTCTCTAGGCCCGGTCTTCC |
| FbpCR | 55 | 5'GGATCCGGCGGCCGGCGCAGCAGGGG |
| MPT32R | 55 | 5'AAGCTTCGGCGAGGCGGCCGTTGTGG |
| SodAF | 55 | 5'CATATGGCCGAATACACCTTGCCAGA |
| SodAR | 55 | 5'GGATCCGAATATCAACCCCTTGGTCT |
| Esat-6F | 50 | 5'CATATGACAGAGCAGCAGTGGAAT |
| Esat-6R | 50 | 5'GGATCCTGCGAACATCCCAGTGACGTTG |
| FbpC(SP)F | 50 | 5'CATATGACGTTCT TCGAACAGG |
| FbpC(SP)R | 50 | 5'CATATGTACCGCGTGCGGTCCG |

The engineered restriction site is denoted by the underline

### 4.2.3 Affinity purification of hexa-histidine tagged fusion proteins

Hexa-his tagged fusion proteins were purified from subcellular fractions following dialysis against 10 mM ammonium bicarbonate to remove EDTA. Dialyzed fractions were dried completely by lyophilization and resuspended in urea binding buffer (5.0 mM imidazole, 0.5 M $NaCl_2$, 20 mM Tris HCl, 6.0 M urea, pH 7.9). Protein samples in binding buffer were loaded onto 2 mL column bed of 6 M urea

equilibrated His-bind resin (Novagen, San Diego, CA) and purified according to the manufacturers protocol. Briefly, ten column vol (CV) of urea binding buffer was added to the column and allowed to flow by gravity. Six CV of wash buffer (60 mM imidazole, 0.5 M $NaCl_2$, 20 mM Tris HCl, 6.0 M urea, pH 7.9) were added to the column and allowed to flow by gravity. Proteins were eluted from the column by the addition of six CV of elute buffer (1.0 M imidazole, 0.5 M $NaCl_2$, 20 mM Tris HCl, 6.0 M urea, pH 7.9). Each fractions were concentrated 10 fold by Centricon centrifugal filtration devices (Millipore, Billerica, MA).

### 4.2.4 Mass spectrometry and other analytical methods

Purified proteins were separated by SDS-PAGE 10-20% Tricine gels (Invitrogen, Carlsbad, CA) and stained with Coomassie Brilliant Blue R250. SDS-PAGE-separated protein bands were excised from the gel and subjected to in-gel digestion with trypsin (Roche, Basel, Switzerland), and the resulting peptides were extracted as described (16). Briefly, the minced gel slices were destained for 30 min in 0.2 M ammonium bicarbonate, 50% acetonitrile at 37°C. The destained gel slices were dried to completion in a speed-vac. Dried gel slices was suspended with 0.5 μg trypsin (Roche, Basel, Switzerland) in 0.2 M ammonium bicarbonate and allowed to swell for 20 min. Additional 0.2 M ammonium bicarbonate was added to cover the swollen gel slices and the samples were incubated overnight at 37°C. The digestion reaction was stopped by adding 0.1 vol the digest volume of 10% trifluoroacetic acid (TFA). Peptides were extracted with two 40 min treatments of 100 μl 0.1% TFA, 60% acetonitrile with mixing at 37°C. Extracted peptides were dried to completion and resuspended in 20 μl 5% acetonitrile, 0.1% acetic acid prior to mass

spectrometry. Extracted peptides were applied to a microcapillary 0.2 x 50 mm C18 reversed phase column (Michrom BioResources, Auburn, CA) and eluted with a linear increase of acetonitrile in 0.1% acetic acid using an Eldex MicroPro capillary HPLC system operated at 5 μl per min (Eldex, Napa, CA). The RP eluant was routed directly into a ThermoFinnigan LCQ electrospray mass spectrometer (ThermoFinnigan, San Jose, CA). The electrospray needle was operated at 4 kV with a sheath gas $N_2$ flow at 40 psi and a capillary temperature of 200°C. Tandem-MS was automatically preformed on the most dominant molecular ion from the previous scan with the normalized collision energy set at 25% or 40% to dissociate glycosidic or amide bonds, respectively. Glycosylated peptides were identified by neutral loss analysis of the tandem-MS data with the Bioworks software package (ThermoFinnigan, San Jose, CA) (44).

Purified proteins and subcellular fractions were subjected to SDS-PAGE and electrophoretically transferred to Trans-Blot nitrocellulose membranes (Bio-Rad, Hercules, CA) as previously described (36). Nitrocellulose membranes were blocked with 1.0 % BSA (Sigma, St. Louis, MO) in PBS pH 7.2 for one hour. Following three washes with PBS the membranes were incubated with either one unit peroxidase-conjugated ConA (Sigma, St. Louis, MO) in PBS or a PBS solution containing 1.0 μg of monoclonal mouse anti-His antibody (Qiagen, Valencia, CA) for one hour followed by three washes with PBS. Detection of ConA reactive proteins was achieved using a 5-bromo-chloronaphthol solution according to the manufacturer's recommendations (Sigma, St. Louis, MO). For the anti-His Western blots the secondary antibody, alkaline phosphatase conjugated goat anti-mouse (Sigma, St.

Louis, MO), was applied to the membrane and incubated for one hour followed by three washes in PBS and development with 5-bromo-4-chloro-3-indolyl SigmaFast tablets (Sigma, St. Louis, MO).

Protein standards employed were prestained See Blue markers (Invitrogen, Carlsbad, CA). N-terminal sequencing of the purified fusion proteins was performed by Edman degradation with a pulsed liquid-phase sequencer from Applied Biosystems by Macromolecular Resources, Fort Collins, CO.

## 4.3 Results

### 4.3.1 Glycoproteins are absent from *M. tuberculosis* cytosol

Previous identification of mycobacterial glycoproteins has exclusively described culture filtrate, cell wall and cytoplasmic membrane proteins. In most cases the identities of mycobacterial glycoproteins were determined by antibody reactivity, Edman degradation sequencing, or mass spectrometry (10, 12, 13, 17, 18, 25). Contained within the amino acid sequence of each identified mycobacterial glycoprotein is a typical Sec-dependent signal peptide or a canonical bacterial Sec-dependent lipoprotein-sorting motif L-(A/S/V)-(G/A)-C (15). Collectively, this data strongly suggest that only Sec-translocated mycobacterial proteins can be *O*-mannosylated. First, to assess whether protein mannosylation is restricted to the exported/secreted proteins of *M. tuberculosis*, subcellular fractions (culture filtrate, cytoplasmic membrane, and cytosol) were isolated (21) and subjected to a phenol-aqueous phase partitioning to remove contaminating lipoglycans. Detection of the *M. tuberculosis* subcellular fractions containing glycoproteins was established by Concanavalin A (ConA) Western blotting. The lectin ConA is employed for the

identification of mycobacterial mannoproteins based on its selective binding to terminal α-mannopyranosyl and α-glucopyranosyl residues. Native *M. tuberculosis* subcellular fractions resolved by SDS-PAGE were subjected to Western blot analysis using peroxidase conjugated ConA as the probe (Fig. 4.1). A subset of the membrane and culture filtrate proteins reacted with ConA, but no ConA binding was observed with the proteins from the cytosol, suggesting that only exported or secreted proteins of *M. tuberculosis* are mannosylated.

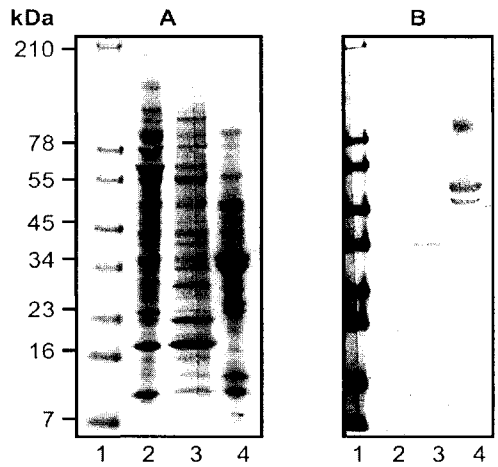


**Fig 4.1 Analysis of *M. tuberculosis* subcellular fractions for the presence of glycoproteins.** Subcellular fractions were separated by SDS-PAGE and **(A)** stained with Coomassie Brilliant Blue, or **(B)** electroblotted and probed with horseradish peroxidase conjugated ConA. Lane 1, molecular weight markers; lane 2, cytosol; lane 3, cell membrane; and lane 4, culture filtrate.

### 4.3.2 Sec-translocated proteins are mannosylation substrates

Evaluation of a link between protein *O*-mannosylation and Sec-dependent protein export in *M. tuberculosis* is complicated by the essentiality of the Sec translocase for viability (5). To overcome this obstacle, FbpC, the well-characterized Sec-translocated and non-glycosylated protein found in high abundance within the *M. tuberculosis* cell wall and culture filtrate, was used to generate fusion constructs (3).

Specifically, two recombinant fusion constructs containing the gene sequence encoding FbpC were fused in-frame at the C-terminus to a small gene fragment encoding a mannosylation cassette $_1$DPEPAPPVPTTAASP$_{15}$ derived from MPT32 and a hexa-his tag (Fig. 4.2A) (10). MPT32 is a well-characterized *O*-mannnosylated secretory protein of *M. tuberculosis* that has four distinct sites of glycosylation. The MPT32 sequence $_1$DPEPAPPVPTTAASP$_{15}$ spans a single glycosylation site (Thr$_{10}$) and contains the minimal structural motif required to accept *O*-mannosylation *in vitro* and *in vivo* (10, 17). The two FbpC fusion constructs differed in that the fusion construct SP(+)FbpC-MC possessed the native N-terminal Sec-dependent signal sequence of FbpC, and SP(-)FbpC-MC was truncated to eliminate this signal sequence (Fig. 4.2A). SP(-)FbpC-MC also possessed a single point mutation resulting in a S$_{125}$ to A$_{125}$ mutation, inactivating the mycolyltransferase activity of FbpC (3). This point mutation was introduced because initial expression studies demonstrated that production of a non-secreted form of active FbpC was toxic to *M. tuberculosis*. Following recombinant expression of these two chimeric proteins in *M. tuberculosis* and *M. smegmatis*, localization was evaluated by Western blot analysis of subcellular fractions (cytosol, membrane, cell wall and culture filtrate) obtained from late-log phase cultures.

As expected these two chimeric recombinant proteins differentially localized based on the presence or absence of a signal peptide. Specifically, the signal sequence containing fusion protein SP(+)FbpC-MC produced by *M. tuberculosis* and *M. smegmatis* localized to the membrane, cell wall, and culture filtrate, but not the cytosol (Fig. 4.2B). N-terminal sequencing of the cell wall and culture filtrate forms

of SP(+)FbpC-MC revealed the sequence $_{1}$FSRPGLPVEY$_{10}$ which corresponded to the mature amino terminus of FbpC (3), confirming that SP(+)FbpC-MC was indeed translocated and processed via the Sec translocase. In comparison, the recombinant fusion SP(-)FbpC-MC was present in the cytosol of *M. smegmatis* as expected, but was also found in the membrane and cell wall of this bacterium, and was predominantly in the cell wall of *M. tuberculosis* and as a truncated form in the membrane (Fig. 4.2B). The occurrence of SP(-)FbpC-MC in the membrane and cell wall fractions was likely due to mixing of protein pools during subcellular fractionation, and may be a direct result of the fatty acid binding domain in FbpC (30). This idea is further supported by the complete absence of SP(-)FbpC-MC in the culture filtrate which was harvested prior to breakage of the cells. Instability of SP(-)FbpC-MC in *M. tuberculosis* likely accounts for its absence in the cytosol fraction and the apparent molecular weight shift in the membrane fraction. In total these data clearly demonstrate the expression of SP(+)FbpC-MC and SP(-)FbpC-MC in mycobacterial hosts, and that these two recombinant chimeric proteins localized differentially, based on the presence or absence of a Sec-dependent translocation signal peptide.

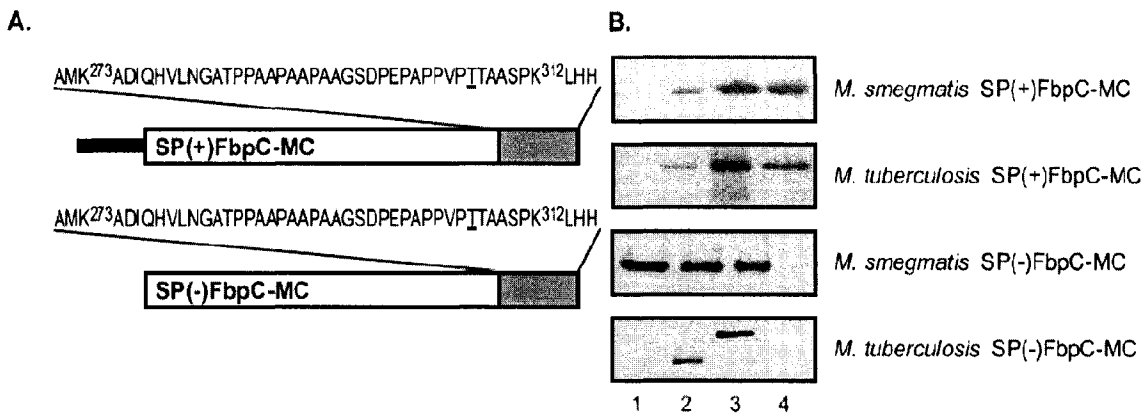


**Fig 4.2 Protein chimeras generated and their localization in *Mycobacterium* spp.** **(A)** Schematic representation of protein chimeras generated to assess the relationship between *O*-mannosylation and Sec-translocation. The numbered amino acid sequence shown for each chimer indicates the tryptic peptide containing the MPT32 mannosylation cassette with the known glycosylation site underlined. The heavy dark line depicts the signal sequence of FbpC, the open box depicts the mature FbpC, the gray box depicts the MPT32 mannosylation cassette and hexa-his tag. **(B)** Subcellular localization of each chimera produced in *M. tuberculosis* and *M. smegmatis* was determined by Western blot analysis using an anti-His antibody as the probe. Lane 1, cytosol; lane 2, cell membrane; lane 3, cell wall; and lane 4, culture filtrate.

The glycosylation status of SP(+)FbpC-MC and SP(-)FbpC-MC produced in *M. tuberculosis* and *M. smegmatis* was determined following affinity chromatography purification. Western blot analysis revealed that SP(+)FbpC-MC purified from the culture filtrate and cell wall *of M. tuberculosis* and *M. smegmatis* was ConA reactive. However, SP(-)FbpC-MC purified from *M. tuberculosis* cell wall was ConA non-reactive. To establish definitive proof of glycosylation, the purified SP(+)FbpC-MC and SP(-)FbpC-MC fusion proteins were digested with trypsin and the resulting peptides were analyzed by liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS). The *M. tuberculosis* culture filtrate form of SP(+)FbpC-MC yielded three major $[M+H]^{3+}$ molecular ions at 1219.0, 1273.1, and 1327.0 *m/z* corresponding to the mannosylation domain containing peptide

(ADIQHVLNGATPPAAPAAPAAGSDPEPA PPVPTTAASPK) with zero, one and two hexose residues, respectively (Fig. 4.3A). Tandem-MS fragmentation of the 1327.0 *m/z* molecular ion product generated the dominant $[M+H]^{3+}$ daughter ions of 1273.7 and 1219.6 *m/z* arising from neutral losses of 54 *m/z* (Fig. 4.3B), a diagnostic property for the dissociation of hexose residues from a triply charged precursor peptide (10). Further, tandem-MS analysis with an elevated collision energy sufficiently high enough for amide bond fragmentation confirmed that the parent ions 1219.0, 1273.1, and 1327.0 *m/z* corresponded to the peptide containing the mannosylation domain of SP(+)FbpC-MC. The relative abundance of parent ions corresponding to the glycosylated SP(+)FbpC-MC mannosylation domain containing peptide demonstrated semi-quantitatively that most of the SP(+)FbpC-MC protein population was glycosylated. The most abundant form of the glycosylated peptide of SP(+)FbpC-MC possessed a single hexose while the molecular ions corresponding to the non-glycosylated peptide accounted for a minor portion of the SP(+)FbpC-MC population (Fig. 4.3A). Identical experiments with the *M. tuberculosis* cell wall and *M. smegmatis* cell wall and culture filtrate forms of this protein demonstrated similar levels and patterns of glycosylation.

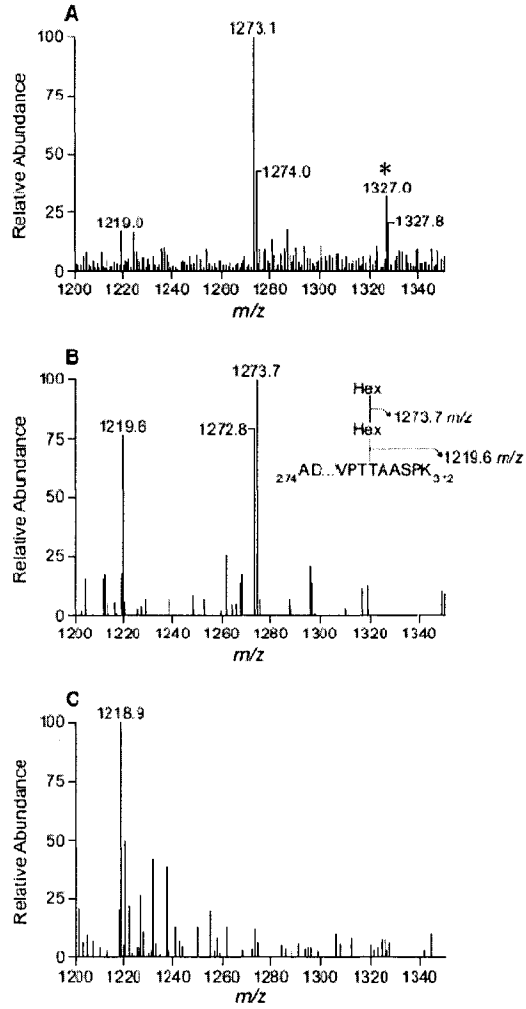


**Fig. 4.3 Mass spectrometry analysis of the peptide containing the mannosylation domain derived from trypsin digestion of SP(+)FbpC-MC and SP(-)FbpC-MC purified from the culture filtrate and cytoplasmic membranes of *M. tuberculosis.*** **(A)** ESI-MS spectrum averaged over five scans corresponding to the LC elution containing the mannosylation domain containing peptide of SP(+)FbpC-MC. The $[M+H]^{3+}$ 1327.0 *m/z* molecular ion marked by the asterisk was selected for CID tandem-MS fragmentation **(B)** to demonstrate the presence of two hexose residues. The predicted fragmentation pattern of the disaccharide linked to the peptide is shown in the insert. **(C)** ESI-MS spectrum averaged over five scans corresponding to the LC elution containing the mannosylation domain containing peptide of SP(-)FbpC-MC.

Likewise, to establish definitive proof for the absence of glycosylation, the SP(-)FbpC-MC fusion proteins from *M. tuberculosis* and *M. smegmatis* was digested with trypsin and the resulting peptides were analyzed by LC-ESI-MS/MS. Specifically, LC-ESI-MS/MS analysis of the trypsin digested SP(-)FbpC-MC derived

from the *M. tuberculosis* cell wall, and *M. smegmatis* cytosol, cell wall or culture filtrate revealed a single $[M+H]^{3+}$ molecular ion of 1218.9 *m/z*. However, in contrast to SP(+)FbpC-MC no significant molecular ions of 1273 or 1327 *m/z* were observed (Fig. 4.3C). Tandem-MS analysis demonstrated that the 1218.9 *m/z* ion corresponded to the tryptic peptide containing the non-glycosylated mannosylation cassette of SP(-)FbpC-MC. Further, a scan of the entire tandem-MS data set for a neutral loss of 54 *m/z* did not provide any evidence of glycosylation. It should be noted that SP(-)FbpC-MC harvested from the culture filtrate of *M. smegmatis* grown to late stationary phase remained non-glycosylated as determined by multiple MS analyses. The presence of SP(-)FbpC-MC in late stationary phase culture filtrate presumably accumulated due to bacterial lysis. These results demonstrated that only the fusion construct possessing the Sec-dependent signal sequence enabled efficient glycosylation. Further, data demonstrating the absence of glycosylation from cell wall and culture filtrate forms of SP(-)FbpC-MC obtained during late stationary phase provides additional evidence that release of this protein from the bacillus by a mechanism other than Sec-translocation does not allow glycosylation.

### 4.3.3 Sec-independently translocated proteins are not glycosylation substrates

*M. tuberculosis* produces several proteins that are secreted in a Sec-independent manner. Two such proteins, ESAT-6/Rv3875 and SodA/Rv3846, appear in the culture filtrate during early log-phase growth and lack signal peptides (1). Therefore, to evaluate if Sec-independently exported proteins could become glycosylated the recombinant fusion proteins containing full-length SodA and ESAT-6 proteins were fused at the C-terminus to the MPT32 mannosylation domain and a

hexa-his tag and expressed in *M. tuberculosis* (Fig. 4.4A). Additionally, to demonstrate that ESAT-6-MC can function as an authentic glycosylation substrate when presented in the proper context to protein mycobacterial mannosyltransferase(s) via the Sec-translocase, SP(+)-ESAT-6-MC was constructed. Others have demonstrated that altering ESAT-6 by adding a signal peptide can direct the protein to the Sec-translocon (2). SP(+)-ESAT-6-MC possesses a native FbpC Sec-dependent signal peptide fused in frame to the N-terminus of ESAT-6-MC (Fig 4.4A). To assure Sec-dependent translocation of SP(+)-ESAT-6-MC the *M. tuberculosis* ΔRD1 mutant was employed. The *M. tuberculosis* ΔRD1 mutant lacks the entire eight-gene locus responsible for the Sec-independent translocation of native ESAT-6 (22, 38).

Analysis of the subcellular localization of the SodA-MC and ESAT-6-MC recombinant gene products demonstrated that these proteins were abundant in all subcellular fractions including the culture filtrate and cell wall (Fig. 4.4B). Of note, native SodA and ESAT-6 proteins behave much like the mannosylation domain containing fusion proteins and localize to all subcellular fractions in *M. tuberculosis* (6, 38). SP(+)-ESAT-6-MC localized primarily to the cytoplasmic membrane and cell wall fractions of *M. tuberculosis* and *M. tuberculosis* ΔRD1 (Fig 4.4B). Additionally, truncated forms of SP(+)-ESAT-6-MC were observed in the culture filtrate fractions of both *M. tuberculosis* and *M. tuberculosis* ΔRD1. Altogether the SodA-MC and ESAT-6-MC fusion proteins were secreted much the same as native SodA and ESAT-6 and the fused signal peptide efficiently targeted the protein SP(+)-ESAT-6-MC for Sec-dependent translocation from both *M. tuberculosis* and *M. tuberculosis* ΔRD1.

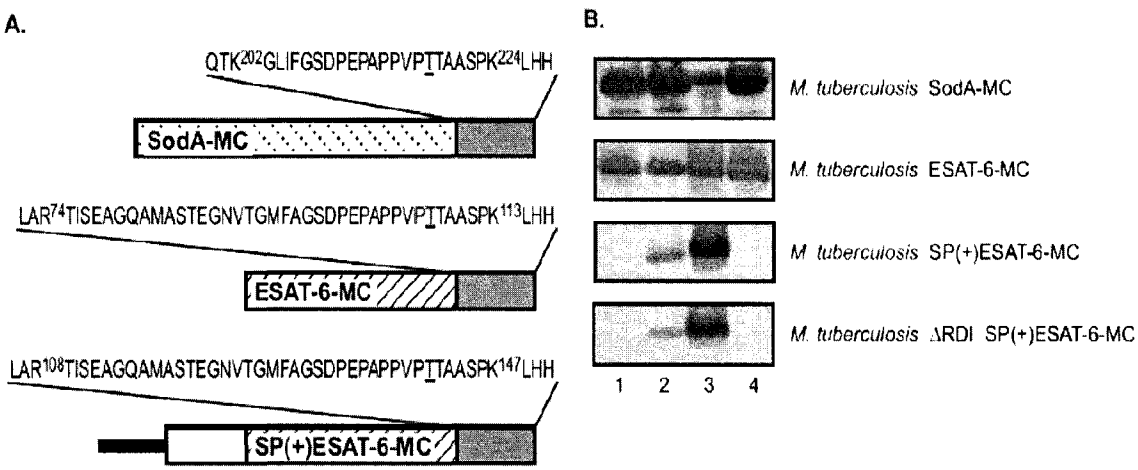


**Fig 4.4 Sec-independent and Sec-dependent protein chimeras generated and their localization in *M. tuberculosis*.** **(A)** Schematic representation of protein chimeras generated to assess the relationship between *O*-mannosylation and Sec-independent translocation. The amino acid sequence shown for each chimer indicates the trypsin cut sites and the fragment containing the MPT32 mannosylation domain with the known glycosylation site underlined. The heavy dark line depicts the signal sequence of FbpC, the open box depicts 34 amino acids of the mature FbpC, the gray box represents the MPT32 mannosylation domain and hexa-his tag, the dotted box depicts the full length SodA, and the slashed box depicts the full length ESAT-6. **(B)** The subcellular localization of each chimera produced in *M. tuberculosis* and *M. tuberculosis* ΔRD1 was determined by Western blot analysis using an anti-His monoclonal antibody as the probe. Lane 1, cytosol; lane 2, cell membrane; lane 3, cell wall; and lane 4, culture filtrate.

The glycosylation status of *M. tuberculosis*-produced SodA-MC and ESAT-6-MC was determined following affinity chromatography purification. Initially, Western blot analysis revealed that neither SodA-MC and ESAT-6-MC purified from the culture filtrate nor cytosol of *M. tuberculosis* were ConA reactive. As with the FbpC constructs, LC-ESI-MS/MS analysis was preformed on the mannosylation domain containing peptides of SodA-MC and ESAT-6-MC as a sensitive measure for the absence or presence of protein glycosylation. SodA-MC and ESAT-6-MC purified from the culture filtrate and cytosol of *M. tuberculosis* were digested with trypsin, and the resulting peptides were analyzed by mass spectrometry.

LC-ESI-MS/MS analysis of the culture filtrate and cytosolic form of SodA-MC yielded a single dominant $[M+H]^{2+}$ molecular ion at 1075.5 *m/z* corresponding to the non-glycosylated, mannosylation domain containing peptide (GLIFGSDPEPAPPVPTT AASPK) (Fig 4.5A). A minor molecular ion (1156.4 *m/z*) approximately 81 *m/z* greater than the 1075.5 *m/z* ion, that corresponds to the predicted $[M+H]^{2+}$ mass of a glycosylated peptide containing the engineered mannosylation domain, was detected. Tandem-MS analysis confirmed the 1156.4 *m/z* molecular ion as a true mannosylation domain containing peptide containing a single hexose, however, the relative abundance of the molecular ion corresponding to the glycosylated SodA-MC peptide was minor compared to the non-glycosylated form of the peptide. Likewise, LC-ESI-MS/MS analyses of the *M. tuberculosis* culture filtrate and cytosolic forms of ESAT-6-MC failed to identify a dominant glycopeptide. Specifically, ESAT-6-MC purified from the culture filtrate and cytosol were digested with trypsin and the resulting peptides analyzed by mass spectrometry yielded a dominant $[M+H]^{3+}$ molecular ion (1258.4 *m/z*) corresponding to the peptide containing the non-glycosylated mannosylation cassette (TISEAGQAMSTG NTGFAGSDPEPAPPVPTTAASPK) (Fig 4.5B). A minor molecular ion (1312.5 *m/z*) corresponding to the mannosylation cassette-containing tryptic peptide of ESAT-6-MC with a single hexose residue was detected. However, the relative abundance of the 1312.5 *m/z* ion was minor in comparison to the non-glycosylated peptide. SodA-MC and ESAT-6-MC were both ConA non-reactive by Western blotting, and this confirmed that the low relative abundance of ions corresponding to the glycosylated peptides detected by LC-ESI-MS/MS truly reflected the glycosylation status of the

whole protein. The scarcity of glycosyl modifications on secreted recombinant SodA-MC and ESAT-6-MC proteins demonstrated that Sec-independent export does not support robust protein glycosylation.

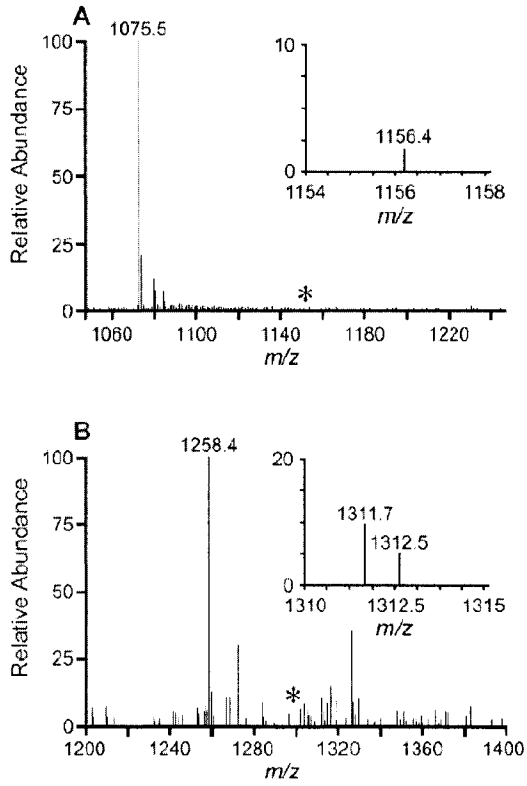


**Fig. 4.5 Mass spectrometry of the tryptic peptides containing the mannosylation cassette derived by digestion of SodA-MC and ESAT-6-MC purified from the culture filtrate of *M. tuberculosis*.** **(A)** ESI-MS spectrum averaged over six scans corresponding to the LC elution of the mannosylation cassette-containing peptide of SodA-MC The dominant $[M+H]^{2+}$ 1075.5 *m/z* molecular ion demonstrates the non-glycosylated peptide. The region of the spectrum denoted by the asterisk is provided as an enlarged image in the insert, and reveals a minor $[M+H]^{2+}$ molecular ion (1156.4 *m/z*) corresponding to the mannosylation cassette-containing tryptic peptide with a single hexose residue. **(B)** ESI-MS spectrum averaged over six scans corresponding to the LC elution containing the mannosylation cassette-containing tryptic peptide of ESAT-6-MC. The dominant $[M+H]^{3+}$ 1258.4 *m/z* molecular ion corresponds to the non-glycosylated tryptic peptide containing the mannosylation cassette. The region of the spectrum denoted by the asterisk is provided as an enlarged image in the insert, and reveals a minor $[M+H]^{3+}$ molecular ion (1312.5 *m/z*) corresponding to the mannosylation cassette-containing tryptic peptide with a single hexose residue.

To truly test the association between Sec-translocation and protein mannosylation SP(+)-ESAT-6-MC was purified by affinity chromatography, from *M. tuberculosis* and *M. tuberculosis* ΔRD1 cell wall fractions, digested with trypsin, and the resulting peptides were analyzed by LC-ESI-MS/MS. LC-ESI-MS of SP(+)-ESAT-6-MC produced by *M. tuberculosis* and *M. tuberculosis* ΔRD1 yielded two major $[M+H]^{3+}$ molecular ions of 1258.4 and 1312.2 *m/z* corresponding to the mannosylation domain-containing peptide (TISEAGQAMASTEGNVTGMFA GSDPEPA PPVPTTAASPK) with zero and one hexose residues, respectively (Fig. 4.6A).

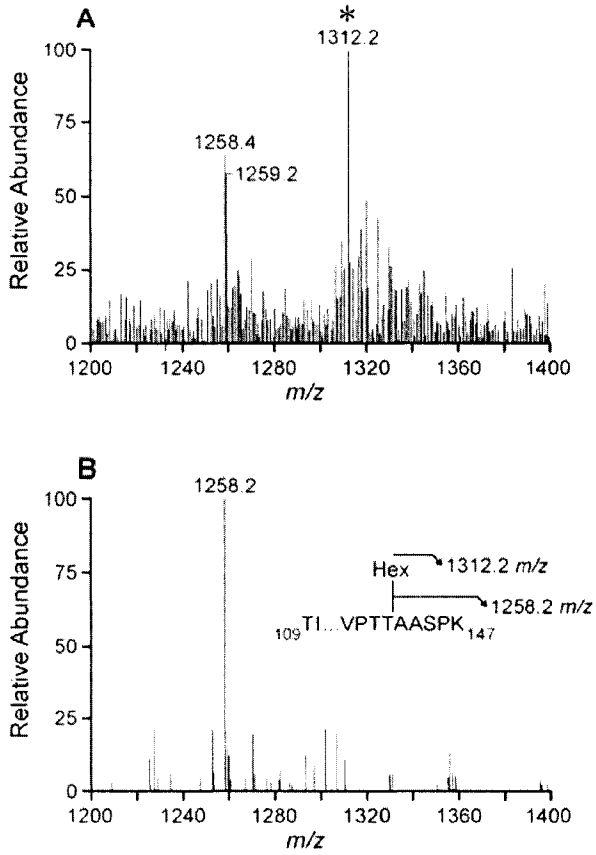


**Fig 4.6 Mass spectra of the peptide containing the mannosylation cassette derived by trypsin digestion of SP(+)ESAT-6-MC purified from the cell wall of *M. tuberculosis*.** **(A)** ESI-MS spectrum averaged over 5 scans corresponding to the LC elution containing the mannosylation cassette peptide of SP(+)-ESAT-6-MC. The 1312.2 *m/z* and 1258.4 m/z $[M+H]^{3+}$ molecular ions correspond to the glycosylated peptide and the non-glycosylated peptide, respectively. **(B)** The 1312.2 *m/z* molecular ion marked by an asterisk was selected for ESI-MS/MS to demonstrate the presence of one hexose residue. The fragmentation pattern of the monosaccharide linked to the peptide is shown in the insert.

Tandem-MS fragmentation of the 1312.2 *m/z* molecular ion producing the dominant $[M+H]^{3+}$ daughter ion of 1258.2 *m/z* that arose from neutral loss of 54 *m/z* (Fig. 4.6B) from a triply charged precursor peptide confirmed the presence of glycosylation. These results confirm that the SP(+)-ESAT-6-MC fusion can function as a glycosylation substrate when forced through the Sec-dependent translocation

system, thereby presenting the acceptor protein in the proper context to protein mannosyltransferase(s). In summary, regardless of the subcellular fraction or the species tested, the only fusions that could be efficiently glycosylated in this system were those containing Sec-dependent signal peptides (Table 4.3).

**Table 4.3 Summary of glycosylation of chimeric fusion proteins.**

| Fusion Protein | Species | Subcellular location | Glycosylation[1] |
|---|---|---|---|
| SP(+)FbpC-MC | *M. smegmatis* | CW, CFP | Yes |
| | *M. tuberculosis* | CW, CFP | Yes |
| SP(-)FbpC-MC | *M. smegmatis* | CYT, CFP | No |
| | *M. tuberculosis* | CW | No |
| SodA-MC | *M. tuberculosis* | CYT, CFP | No |
| ESAT-6-MC | *M. tuberculosis* | CYT, CFP | No |
| SP(+)ESAT-6-MC | *M. tuberculosis* | CW, CFP | Yes |

CW, cell wall; CFP, culture filtrate proteins; CYT, cytosol. Glycosylation[1] was determined by ConA binding and mass spectrometry.

## 4.4 Discussion

Data presented from this study provides the first direct evidence in a bacterial system for a protein glycosylation event being tied to a specific translocation system. The requirement of Sec-translocation for efficient protein *O*-mannosylation in *M. tuberculosis* may possibly be due to the export of unfolded proteins by the Sec translocase and the protein substrate structural requirements for *O*-glycosylation. It is well established that secondary structures surrounding sites of *O*-glycosylation are important for glycosyltransferase recognition (39). Prior to and during translocation, Sec translocated proteins are maintained in an unfolded state by chaperones or are directly co-translationally secreted by the Sec translocase (9). Therefore, linking protein mannosylation events to Sec translocation would allow for efficient transferase recognition of the *O*-glycosylation sites of unfolded proteins during or

immediately following export. This idea is further supported by observations that *O*-mannosylation sites of mycobacterial proteins are related to those found in *S. cerevisiae*, occurring within alanine, proline and serine/threonine rich regions that may adopt stiff extended structures (10). Additionally, the defined and predicted sites of *O*-mannosylation of mycobacterial proteins are clustered near the protein termini, regions unlikely to adopt higher order structures. The involvement of protein folding may also explain the lack of glycosylation on the SodA and ESAT-6 fusion proteins. These proteins lack signal sequences, and are exported by Sec-independent pathways (6, 38). Thus, poor glycosylation of the mannosylation cassettes fused to SodA and ESAT-6 likely resulted from protein folding prior to Sec-independent export. Alternatively, the efficient *O*-glycosylation of only Sec exported proteins could result from a physical interaction between the Sec complex and a protein mannosyltransferase of *M. tuberculosis*. Although there is no direct evidence to support this possibility in *M. tuberculosis*, the oligosaccharyltransferase (OST) complex and PMT's responsible for *N*- and *O*-glycosylation of *S. cerevisiae* are thought to localize within close proximity (30-40Å) to the Sec61 translocon pore (11, 26). In this regard, mycobacterial protein *O*-mannosylation may to some extent represent a functional equivalent to eukaryotic protein *O*-mannosylation events.

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## Chapter V

## Final Discussion

Pathogenic bacteria modify proteins with a variety of glycosyl residues, and these entities play significant roles in mediating a variety of host-pathogen interactions. Such activities include infectivity, adherence to host cells, and interference with complement mediated bacterial lysis (3, 30). Glycosylation of bacterial proteins also affects physiological functions such as enzymatic activity, transport, and motility (28, 34). In the *M. tuberculosis* complex two secreted glycoproteins (MPT32/Rv1860 and MPB83/Mb2898 ) are structurally characterized and are *O*-mannosylated on Thr residues with short linear oligomannosides (11, 21). As with other bacterial pathogens the glycosylation of *M. tuberculosis* proteins influences host interactions, as demonstrated by the requirement of proper *O*-mannosylation for antigen specific T cell recognition of MPT32 (19, 25). Additionally, the *O*-mannosylated lipoprotein LpqH is a dominant TLR-2 agonist (6).

Several decades of research have resulted in a firm understanding of eukaryotic glycoprotein biosynthesis and this information is now being applied to define prokaryotic glycoprotein biosynthetic pathways (13, 35). It is well recognized that protein *O*-mannosylation is an essential and highly conserved biological process (36). Relatively little is known regarding *M. tuberculosis* protein *O*-mannosylation processes; however, several similarities exist between the *O*-mannosylation of *M. tuberculosis* proteins and protein *O*-mannosylation in higher eukaryotic cells. This discussion will reiterate those aspects of mycobacterial *O*-mannoprotein biosynthesis

conserved throughout biology and will emphasize the observations made over the course of this work which may serve to guide future research directions.

## 5.1 Mycobacterial protein *O*-mannosylation

As discussed in chapter 3, direct sequence comparisons between known eukaryotic PMT enzymes and the *M. tuberculosis* H37Rv genome sequence identified the product encoded by *Rv1002c* as a mycobacterial protein PMT candidate. This identification was largely based upon the presence of a pfam02366.11 domain common to all characterized PMT enzymes. Additionally, a distinct putative transmembrane topology resembling eukaryotic PMTs is also conserved by Rv1002c (33, 36). Gene sequences homologous to *Rv1002c* are conserved in all of the sequenced *Mycobacterium* spp. genomes and *Rv1002c* appears to be essential for *M. tuberculosis* growth *in vitro* (27). Conserved within the pfam02366.11 or PMT domain of Rv1002c are multiple invariant residues corresponding to $Arg_{39}$, $Glu_{56}$, and $Arg_{106}$ which are required for eukaryotic PMT activity, in addition to $Asp_{55}$ of unknown function (15). To verify that Rv1002c functions as a *M. tuberculosis* PMT, wild type *Rv1002c* and three mutated forms of this gene were overexpressed in *M. smegmatis* and the resulting cytoplasmic membrane preparations were assayed for *in vitro* PMT activity. Wild type *M. tuberculosis Rv1002c* significantly increased (66.8%) PMT activity of the *M. smegmatis* membranes above the vector control levels and, similar to eukaryotic PMTs, $D_{56}$ and $E_{57}$ of Rv1002c were critical to proper function (15).

Select secreted *Mycobacterium* spp. proteins and lipoproteins are modified with *O*-linked mannosylation motifs suggesting a common glycosylation process for

these two groups of proteins. Both the soluble protein MPT32/Rv1860 and the lipoprotein MPB83/Mb2898 are Sec translocated and are modified on Thr residues with *O*-linked mannose (11, 21). Additionally, a recombinant *M. smegmatis* expression system is capable of glycosylating peptide sequences that are fused to a carrier protein possessing a *M. tuberculosis* Sec signal peptide (17). *M. tuberculosis*, like all bacteria, secrete proteins by Sec independent mechanisms and two Sec independent protein translocation systems (SNM, and SecA2) are known to function in this bacterium (5, 32). Additionally, the *M. tuberculosis* genome encodes a Twin Arginine Translocation (TAT) system, however, the TAT substrates are unconfirmed (9). The cytoplasmic membrane location of the *M. tuberculosis* PMT (Rv1002c) and the observation that certain *M. tuberculosis* Sec translocated proteins can be mannosylated suggest a link between protein export and glycosylation. As discussed in chapter 4, native *M. tuberculosis* subcellular fractions were isolated and analyzed by Concanavalin A (ConA) Western blotting. A subset of cytoplasmic membrane and culture filtrate proteins reacted with ConA, but no ConA reactive proteins were observed with cytosolic preparations. Based on this result it was concluded that *M. tuberculosis* protein mannosylation is restricted to exported/secreted proteins. To evaluate the association between protein *O*-mannosylation and Sec translocation a recombinant form of FbpC/Rv0129c was fused with and without a signal peptide to a mannosylation domain of MPT32 (2, 11). Secreted forms of the FbpC fusion produced in *M. tuberculosis* or *M. smegmatis* were glycosylated while non-secreted fusions were non-glycosylated. Analogous fusions were constructed utilizing the proteins ESAT-6/Rv3875 and SodA/Rv3846, both of which are secreted from *M.*

*tuberculosis* in a Sec independent manner via the SNM and SecA2 systems, respectively. The Sec independently secreted fusions produced in *M. tuberculosis* were not efficiently glycosylated. Lastly, a Sec dependent signal peptide was fused to the ESAT-6/Rv3875 fusion directing this construct through the Sec translocase, which allowed for efficient glycosylation. In total, these studies demonstrate a clear link between the mycobacterial PMT and the Sec translocase and it is probable that only proteins secreted by the Sec translocase can be mannosylated (Fig 5.1).

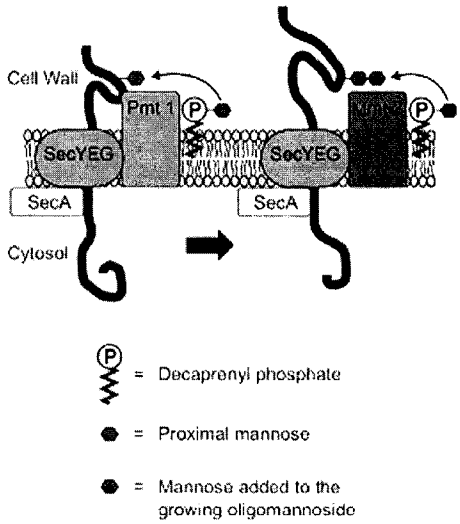


**Fig 5.1 Proposed pathway of protein *O*-mannosylation and the involvement of Sec translocation in *M. tuberculosis*.** The SecA-SecYEG complex is required for the translocation of unfolded proteins targeted for mannosylation. As the target protein emerges from the Sec translocon in an unfolded state a mannosylation domain interacts with the first protein mannosyltransferase (Rv1002c or Pmt1) and a mannose residue is transferred from mannosyl-phosphoryl-decaprenol to a Thr residue of the target protein. This proximal mannose unit serves as an acceptor for a second mannose donated from mannosyl-phosphoryl-decaprenol, and this transfer is catalyzed by a second mannoprotein mannosyltransferase, Mmt2.

Our conclusion that Rv1002c functions as a mycobacterial PMT was based on several lines of evidence. First, overexpression of wild type forms of the Rv1002c gene in *M. smegmatis* conferred increased *in vitro* PMT activity. Secondly, point mutations known to abolish eukaryotic PMT activity had similar affects when

introduced into Rv1002c and assayed *in vitro*. Expression of recombinant forms of *Rv1002c* was verified by RT-PCR analysis but was not confirmed at the protein level. Protein instability is an unlikely explanation for the reduction in enzymatic activity of the mutant forms of Rv1002c given the significant decrease in activity below vector control levels for the $D_{55}$ single point mutant.

An alternate interpretation of these results is that Rv1002c could be involved in the generation of a precursor required for protein glycosylation. Recent experimentation has established a related *in vitro* PMT assay utilizing mycobacterial cytoplasmic membrane enzyme preparations to form *O*-mannosylated synthetic peptides (10). This assay measures the active incorporation of radiolabeled [$^{14}$C]Man into glycopeptides when supplied as GDP-[$^{14}$C]Man. However, the donor molecule appears to be polyprenol-Man given that the assay is sensitive to amphomycin, an inhibitor of lipid linked donor formation (10). Thus, the mycobacterial PMT assay relies on two distinct enzymatic activities to first convert GDP-[$^{14}$C]Man to polyprenol-Man which in turn acts as the donor molecule for peptide glycosylation. Conversion of GDP-Man to polyprenol-Man is catalyzed by the *M. tuberculosis* integral membrane protein (Ppm1/Rv2051c), which possesses polyprenol-Man synthase activity (16). Rv1002c displays no homology to Rv2051c or other known polyprenol-Man synthase enzymes suggesting a role distinct from polyprenol-Man formation. Lastly, nucleotide sugars such as GDP-Man are not typically available outside of the bacterial cytoplasmic membrane where they diffuse rapidly into the environment. The putative active site of Rv1002c is predicted to be extracytoplasmic,

thus it would be sequestered away from GDP-Man, suggesting a role in protein glycosylation and not the cytosolic formation of polyprenol-Man.

## 5.2 Future directions

Our findings bring up some important points that will need to be addressed in future work. Two additional ORFs (Rv0539 and Rv3631) are present in the *M. tuberculosis* genome that encode putative polyprenol-Man synthases and have homology to Rv2051c (9). Saturating transposon mutagenesis experiments identified insertions in Rv0539, Rv3631, and Rv2051c, suggesting that individually these products are dispensable (27). Rv0539 and Rv3631 are predicted to be soluble cytosolic proteins while Rv2051c possesses multiple transmembrane domains (16). The apparent redundancy of these enzymes may reflect a heavy demand for the precursor polyprenol-Man in the genesis of mannoproteins, LAM, PIMs and ManLAM (4, 10). Alternatively, a particular biosynthetic pathway could favor a specific polyprenol-Man synthase. Supporting this idea is the finding that the *M. tuberculosis* ORF encoding Rv2051c is a natural genetic fusion between two adjacent genes (16). Specifically, Rv2051 contains a *M. tuberculosis* polyprenol-Man synthase domain fused in-frame to an apolipoprotein n-acyltransferase domain, and in other *Mycobacterium* spp. these two genes remain separate (16). The apolipoprotein n-acyltransferase (Lnt) domain of Rv2051c is likely responsible for the final acylation step in secreted lipoprotein biosynthesis (Chapter 1, Section 1.4.1). Immediately following Sec translocation diacyl-lipoproteins are cleaved by signal peptidase II (Rv1539) and are acylated at the amino terminal Cys residues by Lnt (14). This process is highly coordinated to ensure efficient acylation of lipoproteins and is likely

mediated by protein-protein interactions. In the unique case of *M. tuberculosis* the Lnt domain of Rv2051c may interact with the Sec translocase to govern protein acylation, directly linking the polyprenol-Man synthase domain of Rv2051c to the Sec translocase. Additionally, it has been proposed that PMT dimer complexes may possibly form a pore necessary for flipping Dol-P-Man across the ER membrane (15). This scenario would be analogous to the proposed model by which Alg8p and Alg10p flip the sugar donor Dol-P-Glc across the yeast ER membrane (7). Therefore it is reasonable to propose that Rv2051c synthesizes polyprenol-Man in close proximity to the Sec translocase and Rv1002c flips the polyprenol-Man across the membrane to glycosylate proteins.

To completely characterize the process of mycobacterial protein mannosylation future studies must examine interactions between components of the Sec translocase and Rv1002c. Due to the strong conservation between the mycobacterial *O*-mannosylation system and eukaryotic systems it is conceivable that the interactions are conserved as well. To direct efficient mannosylation of secreted proteins the mycobacterial PMTs may directly interact with components of the Sec translocase. In yeast, it was recently demonstrated that *O*-mannosylation potentially precedes *N*-linked glycosylation (12). Previously several subunits of the yeast OST complex were chemically cross-linked to the Sec translocase and two-hybrid screens have verified these interactions *in vivo* (8, 29). Likewise a combination of chemical cross-linking and two-hybrid analysis should shed light on Rv1002c and Sec translocase interactions. Cross-linking and two-hybrid approaches also have the

potential to identify additional mannosyltransferases and all the components of the Sec translocase and the *M. tuberculosis* protein glycosylation system.

The results presented in this dissertation contribute to our understanding regarding the initial steps of mycobacterial mannoprotein biosynthesis. One step not addressed by this work is the elongation of the protein bound mannose to a full-length oligomannoside, typically found on mycobacterial mannoproteins. Preliminary genetic comparisons of the *M. tuberculosis* genome identified Rv0696 as a potential mannosyltransferase responsible for this mannose chain extension. Identification of Rv0696 as a putative mannosyltransferase was based on the following criteria: strong conservation in *Mycobacterium* spp., annotation as being an inverting mannosyltransferase, and the candidate must be secreted or contain one or more putative transmembrane domains. The rationale for utilizing these criteria was based on the fact that all *Mycobacterium* spp. examined produce mannoproteins with elongated mannan chains (11, 21). The enzyme probably utilizes β-linked polyprenyl-Man as a donor to form the α-linked mannose chain, and the mannose transfer likely occurs extracellularly requiring the second enzyme to be secreted or membrane bound. Alternatively, Rv1002c could transfer a full-length oligomannose chain to the secretory protein and would not require a second extracytoplasmic mannosyltransferase. This scenario is unlikely given the homology of Rv1002c to eukaryotic PMTs, which transfer single mannose residues, and the fact that lipid linked oligomannose molecules have not been reported in mycobacteria.

Not addressed by this dissertation is the difference between the reported mannoproteins of *M. bovis* and *M. tuberculosis*. It appears as if the initial protein

mannosylation event catalyzed by Rv1002c is conserved in all mycobacteria; however, the extension of the mannan chain may differ between species or protein type. Dobos *et al.* reported a linear Man chain linked in the α(1→2) configuration bound to the *M. tuberculosis* secreted antigen MPT32 (11). Michell *et al.* reported a linear Man chain linked in the α(1→3) configuration bound to the *M. bovis* strain AN5 lipoprotein MPB83 (21). To date no lipo-mannoprotein of *M. tuberculosis* has been characterized, likewise no soluble secreted mannoprotein of *M. bovis* has been fully characterized. Based on this observation it is tempting to speculate that mannoproteins of *M. tuberculosis* differ from mannoproteins produced by other members within the genus by linkages in the protein-bound mannan chain. Alternatively, α(1→3) linked mannose residues may be restricted to lipo-mannoproteins and α(1→2) linked mannose residues may be unique to soluble secreted mannoproteins of *Mycobacterium* spp. From a biosynthetic point of view, both cases are equally intriguing and deserve further investigation.

The function of mycobacterial protein mannosylation remains an enigma. However, mycobacterial protein *O*-mannosylation protects the amino terminus of the lipoprotein LpqH (19 kDa antigen) from proteolysis (18). Also supporting this idea is the observation that the mycobacterial *O*-mannosylated lipoproteins MPB83 and PstS1 (38 kDa antigen) purified from the culture medium display proteolytic truncations at their amino termini (1, 21, 31). In addition, multiple forms of MPT32 with disparate molecular weights are commonly observed in mycobacterial culture filtrates (31). Collectively, these data suggest that mycobacterial protein *O*-mannosylation may function to stabilize select proteins that provide as yet unknown

functions to the bacillus. Interestingly, recent saturating transposon mutagenesis experiments in *M. tuberculosis* have identified *Rv1002c* as an essential gene (27). Null mutants deficient in known mannoproteins are viable *in vitro* suggesting that *O*-mannosylation may have a global effect by stabilizing a large group of proteins. Should *O*-mannosylation be essential for *in vitro M. tuberculosis* growth this would be a first for a prokaryotic organism.

Protein *O*-mannosylation is an essential process in *S. cerevisiae* with seemingly multiple functions. The *S. cerevisiae* plasma membrane proteins Wsc1p and Mid2p are sensors of the PKC-MPK1 cell wall compensatory system. The PKC-MPK1 system counteracts cell wall weakening by up-regulating the production of cell wall stabilizing products such as chitin (22, 23). Recently it was demonstrated that *O*-mannosylation protects Wsc1p and Mid2p from proteolysis, thereby facilitating their sensory functions (20). Additionally, the membrane protein Ax12p of *S. cerevisiae* is required for axial budding and *O*-mannosylation protects Ax12p from proteolysis, enhancing budding activity and localization of the protein (26). Lastly, proper localization of Fus1p to the plasma membrane of *S. cerevisiae* is dependent on *O*-mannosylation (24). It is tempting to speculate that mycobacterial protein *O*-mannosylation may play similar roles in regulating cell wall integrity by protecting select proteins from proteolysis or have protein-targeting functions.

In closing, the work presented in this dissertation has established and clarified some key issues regarding mycobacterial mannoprotein biosynthesis. Identification of the PMT (Rv1002c) has established how *M. tuberculosis* protein mannosylation initiates. Additionally, the present work defines the subcellular location of *M.*

*tuberculosis* protein *O*-mannosylation and the specificity of Rv1002c for Sec translocated proteins. Future biochemical characterization of the relatively simple *M. tuberculosis* protein mannosylation system may perhaps aid our understanding of eukaryotic PMT enzymes. Lastly, subsequent studies based on these results may allow future researchers to further define the molecular significance of *O*-mannoprotein structures in the physiology/pathogenesis of *M. tuberculosis.*

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