Molecular and biochemical characterisation of novel glycosyltransferases in *Mycobacterium tuberculosis*

By

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A thesis submitted to the

UNIVERSITY OF BIRMINGHAM

for the degree of

DOCTOR OF PHILOSOPHY

School of Biosciences
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The University of Birmingham

January 2011
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ABSTRACT

The cell wall mycolyl-arabinogalactan-peptidoglycan complex is essential in mycobacterial species, such as Mycobacterium tuberculosis and is the target of several antitubercular drugs. Arabinofuranosyltransferase enzymes, such as EmbA, EmbB, and AftA, play pivotal roles in the biosynthesis of arabinogalactan. The anti-tuberculosis agent ethambutol (EMB) targets arabinogalactan biosynthesis through inhibition of Mt-EmbA and Mt-EmbB and also targets the biosynthesis of the important immunomodulatory molecule lipoarabinomannan (LAM), through inhibition of Mt-EmbC.

A bioinformatics approach identified putative integral membrane proteins in Mycobacterium smegmatis, M. tuberculosis and the closely related species Corynebacterium glutamicum, with features common to the GT-C superfamily of glycosyltransferases. A novel arabinofuranosyltransferase, AftC, was deleted from both M. smegmatis and C. glutamicum and shown to be an internal branching α(1→3) arabinofuranosyltransferase involved in arabinogalactan biosynthesis. Further studies revealed a truncated LAM whereby the arabinan domain was severely reduced and consisted of a simple linear arabinan of approximately 12-15 α(1→5) linked Araf residues. This mutant LAM was also shown to be a potent stimulator of TNF-α production using a human macrophage cell line, thus illustrating that masking of the mannan core by arabinan in wild type LAM alters its ability in the production of this cytokine. We also describe a further arabinofuranosyltransferase, AftB. Deletion of its orthologue in C. glutamicum resulted in a viable mutant and biochemical analysis revealed the complete absence of terminal β(1→2)-linked arabinofuranosyl residues. Further analysis confirmed AftB as a terminal β(1→2) arabinofuranosyltransferase, which was also insensitive to EMB.

The bioinformatic search for cell wall glycosyltransferases led to the identification of a rhamnosyltransferase in C. glutamicum, RptA. Deletion resulted in a reduction of terminal-rhamnopyranosyl linked residues and as a result, a corresponding loss of branched 2,5-linked arabinofuranosyl residues. Furthermore, analysis of base-stable extractable lipids from C. glutamicum revealed the presence of decaprenyl-monophosphorylrhamnose, a putative substrate for the cognate cell wall transferase. Altogether, these studies have shed further light on the complexities of Corynebacterianae cell wall biosynthesis, and represent potential new drug targets.
DECLARATION

The work presented in this thesis was carried out in the School of Biosciences at the University of Birmingham, U.K., B15 2TT during the period October 2006 to October 2010. The work in this thesis is original except where acknowledged by references.

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

I would like to take this opportunity to thank all the people who have made this thesis possible. First and foremost, I would like to express my sincere gratitude to my supervisor, Professor Gurdyal S. Besra, for granting me the opportunity to study my MRC funded PhD and whose expertise, understanding, and patience, made this thesis possible.

A special thanks goes out to Dr. Luke Alderwick for his invaluable guidance, motivation and technical support and Dr. Lynn Dover, my undergraduate tutor, for encouraging me to pursue my post-graduate studies. Further thanks go to Dr. Apoorva Bhatt for sharing his expertise so readily and patiently.

I would like to thank Dr. Lothar Eggeling and his lab from the Institute for Biotechnology in Juelich, for performing some of the molecular biology that appears in this thesis and Dr. Ben Appelmelk from the University Medical Center in Amsterdam, for his valuable immunological data. Also, a massive thanks to Graham Burns and Peter Ashton for their technical superiority in performing GC and GC/MS.

I would also like to thank all of my colleagues and fellow PhD students for their support: Becci, Justyna, Mimi, Sarah, George, Sid, Albel, Arun, Oona, Hemza, Veemal and Natacha.

Last but not least I would also like to thank my parents, brother and sisters, Robert and Diane and my close friends for all the support they provided me with throughout. Most importantly, I want to acknowledge my partner and best friend, Andy, without his love and encouragement, I would not have finished this thesis.
This thesis is dedicated to Andy
# TABLES OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>i</td>
</tr>
<tr>
<td>Declaration</td>
<td>ii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>iii</td>
</tr>
<tr>
<td>Dedication</td>
<td>iv</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>v</td>
</tr>
<tr>
<td>List of Figures</td>
<td>ix</td>
</tr>
<tr>
<td>List of Tables</td>
<td>xi</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>xii</td>
</tr>
<tr>
<td>Published work associated with this thesis</td>
<td>xviii</td>
</tr>
<tr>
<td>1. Introduction</td>
<td>1</td>
</tr>
<tr>
<td>1.1. Mycobacterial Classification and Phylogenetic Analysis</td>
<td>2</td>
</tr>
<tr>
<td>1.1.1. The Genus <em>Mycobacterium</em></td>
<td>2</td>
</tr>
<tr>
<td>1.2. History of tuberculosis</td>
<td>4</td>
</tr>
<tr>
<td>1.2.1. Discovery of the tubercle bacillus and initial therapies</td>
<td>5</td>
</tr>
<tr>
<td>1.3. Pathogenesis of tuberculosis</td>
<td>6</td>
</tr>
<tr>
<td>1.4. Epidemiology of tuberculosis</td>
<td>9</td>
</tr>
<tr>
<td>1.5. TB drug treatments</td>
<td>11</td>
</tr>
<tr>
<td>1.5.1. TB Drug resistance</td>
<td>12</td>
</tr>
<tr>
<td>1.6. The mycobacterial cell wall</td>
<td>13</td>
</tr>
<tr>
<td>1.6.1. The cell wall of <em>M. tuberculosis</em> as a drug target</td>
<td>15</td>
</tr>
<tr>
<td>1.6.2. Plasma membrane</td>
<td>15</td>
</tr>
<tr>
<td>1.6.3. Structure and biosynthesis of peptidoglycan</td>
<td>16</td>
</tr>
<tr>
<td>1.6.4. Biosynthesis of the linker unit</td>
<td>18</td>
</tr>
<tr>
<td>1.6.5. Arabinogalactan</td>
<td>23</td>
</tr>
<tr>
<td>1.6.5.1. Structural features of arabinogalactan</td>
<td>23</td>
</tr>
<tr>
<td>1.6.5.2. Synthesis of arabinogalactan biosynthetic precursors</td>
<td>25</td>
</tr>
<tr>
<td>1.6.5.2.1. Galactan precursor synthesis</td>
<td>25</td>
</tr>
<tr>
<td>1.6.5.2.2. Arabinan precursor synthesis</td>
<td>26</td>
</tr>
<tr>
<td>1.6.5.3. Galactan biosynthesis</td>
<td>29</td>
</tr>
<tr>
<td>1.6.5.4. Arabinan biosynthesis</td>
<td>31</td>
</tr>
<tr>
<td>1.6.5.4.1. Ethambutol inhibition and its use in the identification of the Emb proteins</td>
<td>31</td>
</tr>
<tr>
<td>1.6.5.4.2. EMB resistance</td>
<td>34</td>
</tr>
<tr>
<td>1.6.5.4.3. Identification of novel arabinofuranosyltransferases and the use of <em>C. glutamicum</em> as a model organism</td>
<td>35</td>
</tr>
<tr>
<td>1.6.5.4.4. Identification and functional role of AftA</td>
<td>37</td>
</tr>
<tr>
<td>1.6.6. Mycolic acids</td>
<td>38</td>
</tr>
<tr>
<td>1.6.6.1. Biosynthesis of mycolic acids</td>
<td>39</td>
</tr>
<tr>
<td>1.6.7. Structural features of lipoarabinomannan (LAM) and related biosynthetic precursors (LM and PIMs)</td>
<td>42</td>
</tr>
<tr>
<td>1.6.7.1. Phosphatidyl-&lt;i&gt;myo&lt;/i&gt;-inositol mannosides (PIMs)</td>
<td>43</td>
</tr>
<tr>
<td>1.6.7.2. Characterisation of LM and LAM</td>
<td>43</td>
</tr>
<tr>
<td>1.6.7.3. Characterisation of LAM capping</td>
<td>45</td>
</tr>
<tr>
<td>1.6.7.4. Biosynthesis of PIMs, LM and LAM</td>
<td>47</td>
</tr>
<tr>
<td>1.6.7.4.1. Synthesis of precursors</td>
<td>47</td>
</tr>
</tbody>
</table>
1.6.7.4.2. GDP-Manp biosynthesis .......................................47
1.6.7.4.3. β-D-mannosyl-1-monophosphoryldecaprenol (PPM) biosynthesis ..................................................48
1.6.7.4.4. Synthesis of PI .......................................................49
1.6.7.5. Biosynthesis of PIIs ..................................................49
1.6.7.6. Biosynthesis of LM and LAM ......................................54
1.6.7.6.1. The role of MptA, MptB and Rv2181 .....................54
1.6.7.7. Arabinan biosynthesis in lipoarabinomannan .................56
1.6.7.7.1. The role of EmbC ..................................................56
1.6.7.8. Mannose - capping of LAM .......................................57
1.6.7.9. Immunomodulatory properties of LAM & LM ..............58
1.6.7.9.1. Phagosome maturation arrest ...............................58
1.6.7.9.2. Lipoglycan interaction with the host cell ..................61
1.6.7.9.3. LAM and the Mannose receptor .........................61
1.6.7.9.4. Modulation of DC-SIGN activity .........................62
1.6.7.9.5. Toll-like receptors ..............................................63

1.7. Project aims ...................................................................................................................65

2. Identification and characterisation of AftB and AftC arabinotransferases involved in arabinogalactan biosynthesis ....... 68

2.1. Introduction ...................................................................................................................68
2.2. Results ...........................................................................................................................72
  2.2.1. Genome comparison of the aftB locus .................................................................72
  2.2.2. Construction of \(C.\ glutamicum\Delta aftB\) .................................................................74
  2.2.3. In vitro growth analysis of \(C.\ glutamicum\Delta aftB\) ....................................................75
  2.2.4. Analysis of cell wall associated lipids and bound corynomycolic acid ...............76
  2.2.5. Cell wall glycosyl compositional and linkage analysis of cell walls ....................78
  2.2.6. Endogenous in vitro arabinofuranosyltransferase activity of \(C.\ glutamicum\), \(C.\ glutamicum\Delta aftB\) and \(C.\ glutamicum\Delta aftB\) complemented with Mt-aftB and product analysis. ....81
  2.2.7. ES-MS and GC/MS analysis of product A and B ..............................................83
  2.2.8. Genome comparison of the AftC locus ...............................................................85
  2.2.9. Construction and growth of mutants .................................................................86
  2.2.10. Analysis of cell wall bound mycolic acids ......................................................89
  2.2.11. Glycosyl compositional analysis of cell walls from \(M.\ smegmatis\), \(M.\ smegmatis\Delta aftC\) and complemented strains .................................................................91
  2.2.12. Glycosyl linkage analysis of cell walls .............................................................93
  2.2.13. In vitro arabinofuranosyltransferase activity with extracts of \(M.\ smegmatis\), \(M.\ smegmatis\Delta aftC\) and complemented strains .................................................................95
  2.2.14. Discussion ........................................................................................................101

3. Identification and characterisation of a crucial branching \(\alpha(1\rightarrow3)\) arabinofuranosyltransferase involved in LAM bisynthesis .......... 109

3.1. Introduction ..................................................................................................................109
3.2. Results ........................................................................................................................111
  3.2.1. Effects of aftC inactivation on LM/LAM biosynthesis ............................................111
  3.2.2. Structural characterisation of AftC-LAM .............................................................112
  3.2.3. Effect of ethambutol on AftC-LAM formation .....................................................117
3.2.4. AftC-LAM displays pro-inflammatory properties .....................................118
3.2.5. Discussion...................................................................................................122

4. Identification of a Rhamnopyransolyltransferase (RptA) which utilises a novel decaprenolphosphorhamnose substrate ...............................128

4.1. Introduction .................................................................................................................128
4.2. Results .........................................................................................................................129
  4.2.1. Genome comparison of the NCgl0543 (rptA) locus ..................................................129
  4.2.2. Construction of C. glutamicum ΔrptA ..................................................................131
  4.2.3. In vitro growth phenotype of C. glutamicum ΔrptA ..............................................131
  4.2.5. Glycosyl linkage analysis of cell walls from C. glutamicum, C. glutamicum ΔrptA, C. glutamicum ΔrptA pVWEx-Cg-rptA, and C. glutamicum ΔrptA pVWEx-Rv3779 .................................................................134
  4.2.6. Recognition of a rhamnose lipid-linked sugar donor, decaprenyl-P-rhamnose 135

4.3. Discussion....................................................................................................................140

5. Conclusion and future work ................................................................... 144

6. Materials and Methods ........................................................................... 151

6.1. Chemicals, reagents and growth conditions .................................................................151
6.2. Bacterial growth conditions ..........................................................................................151
  6.2.1. Construction of C. glutamicum mutants ..............................................................152
    6.2.1.1. Construction of C. glutamicum ΔaftB and complementing strains .........................152
    6.2.1.2. Construction of C. glutamicum ΔaftC and complementing strains .......................154
    6.2.1.3. Construction of C. glutamicum ΔrptA and complementing strains .......................154
  6.2.2. Construction of M. smegmatis mutants ..............................................................155
    6.2.2.1. Construction of M. smegmatis ΔaftC and complementing strains .......................155
  6.2.3. mAGP purification procedures .................................................................................157
    6.2.3.1. Acid hydrolysis and alditol acetate derivatisation .............................................157
    6.2.3.2. Glycosidic linkage analysis .............................................................................158
  6.2.4. GC and GC/MS ........................................................................................................158
  6.3. Extraction and visualisation of lipids ..........................................................159
    6.3.1. Cell wall associated lipid extraction ..................................................................159
    6.3.2. Exported (media filtrate) lipid extraction ..........................................................160
    6.3.3. Cell wall bound lipid extraction .......................................................................160
    6.3.4. Analysis of AftB muteins .................................................................................161
  6.4. Extraction and Purifications of lipoglycans ...............................................................162
    6.4.1. NMR spectroscopic analysis of WT-LAM and AftC-LAM ..................................163
    6.4.2. SDS PAGE ........................................................................................................163
6.5. Cell free [14C]-labeling assays ................................................................. 164
  6.5.1. Preparation of *C. glutamicum* and *M. smegmatis* membranes .......... 164
  6.5.2. Preparation of *C. glutamicum* and *M. smegmatis* cell wall material .... 164
  6.5.3. Estimation of protein concentration ................................................... 165
  6.5.4. Arabinofuranosyltransferase assays .................................................. 165
    6.5.4.1. Arabinofuranosyltransferase activity with membrane preparations of
              *C. glutamicum*, *C. glutamicum*Δ*aftB*, and *C. glutamicum*Δ*aftB* pMSX-Mt-aftB ...................................................... 165
    6.5.4.2. Analysis of arabinofuranosyltransferase reaction products prepared
              from *C. glutamicum* and *C. glutamicum*Δ*aftB* membranes .......................................................... 167
    6.5.4.3. Arabinofuranosyltransferase activity with membrane preparations of
              *M. smegmatis*, *M. smegmatis* pMV261-Mt-aftC, *M. smegmatis*Δ*aftC* and *M. smegmatis*Δ*aftC* pMV261-Mt-aftC .......................................................... 167
    6.5.4.4. Characterization of a(1→3)-arabinofuranosyltransferase activity with membranes prepared from *M. smegmatis*, *M. smegmatis*Δ*aftC* and *M. smegmatis*Δ*aftC* pMV261-Mt-aftC .......................................................... 169
  6.5.5. Treatment of *M. smegmatis* and *M. smegmatis*Δ*aftC* with sub-inhibitory
         concentrations of EMB and subsequent lipoglycan analysis. .................. 173
  6.5.6. Treatment of WT-LAM, AftC-LAM and Pam3CSK4 with H2O2 .............. 173
  6.5.7. Cell culture ....................................................................................... 174
  6.5.8. Cell stimulation assays ....................................................................... 174
  6.5.9. DC-SIGN-Fc ELISA ........................................................................... 174

7. References .................................................................................................. 177
| Figure 1.1 | The intracellular existence of *M. tuberculosis* | 7 |
| Figure 1.2 | Overview of TB infection and host defence | 8 |
| Figure 1.3 | Estimated TB incidence rates in the year 2009 (WHO, 2010) | 10 |
| Figure 1.4 | Estimated HIV prevalence in new TB cases in the year 2009 (WHO, 2010) | 10 |
| Figure 1.5 | Schematic model of the mycobacterial cellwall | 14 |
| Figure 1.6 | Biosynthesis of peptidoglycan in *M. tuberculosis* | 18 |
| Figure 1.7 | The Rha-GlcNAc linker unit. The molecular structure of the LU, the conduit between AG and PG | 19 |
| Figure 1.8 | Formation of linker unit glycolipid-1 (GL-1) and glycolipid-2 (GL2) | 20 |
| Figure 1.9 | Biosynthetic pathway of dTDP-rhamnose | 22 |
| Figure 1.10 | Structural features of arabinogalactan | 24 |
| Figure 1.11 | Formation of galactan precursors | 26 |
| Figure 1.12 | Formation of arabinan precursor DPA | 28 |
| Figure 1.13 | Polymerisation of galactan - biosynthesis of GL-4 | 29 |
| Figure 1.14 | Polymerisation of galactan | 30 |
| Figure 1.15 | Schematic presentation of arabinogalactan biosynthesis | 33 |
| Figure 1.16 | Early arabinogalactan formation | 37 |
| Figure 1.17 | Structures of representative mycolic acids from *M. tuberculosis* | 39 |
| Figure 1.18 | Mycolic acid biosynthesis in *M. tuberculosis* | 41 |
| Figure 1.19 | Structure of TDM | 42 |
| Figure 1.20 | Current structural model of mycobacterial LAM | 44 |
| Figure 1.21 | Structural model of mycobacterial ManLAM, PILAM and AraLAM highlighting the different capping motifs found in all mycobacterial LAM | 46 |
| Figure 2.1 | Comparison of the *aftB* locus within Corynebacterianeae | 72 |
| Figure 2.2 | Construction and characteristics of *C. glutamicum ΔaftB* | 74 |
| Figure 2.3 | Quantitative analysis of extractable [14C]lipids from *C. glutamicum, C. glutamicum ΔaftB* and *C. glutamicum ΔaftB* pMSX-Mt-ΔaftB | 76 |
| Figure 2.4 | Quantitative analysis [14C]CMAMEs from *C. glutamicum, C. glutamicum ΔaftB* and *C. glutamicum ΔaftB* pMSX-Mt-ΔaftB | 77 |
| Figure 2.5 | Glycosyl linkage analysis of cell walls of *C. glutamicum* (A), *C. glutamicum ΔaftB* (B), *C. glutamicum ΔaftB* pMSX-Mt-ΔaftB | 79 |
| Figure 2.6 | Formation of Mt-AftB in *C. glutamicum* | 80 |
| Figure 2.7 | Arabinofuranosyltransferase activity in membranes prepared from *C. glutamicum, C. glutamicum ΔaftB* and *C. glutamicum ΔaftB* pMSX-Mt-ΔaftB | 81 |
| Figure 2.8 | ES-MS and GC/MS characterisation of products A and B | 83 |
| Figure 2.9 | Comparison of the *aftC* locus within the Corynebacterianeae | 84 |
| Figure 2.10 | Generation of a MSMEG2785 null mutant | 86 |
| Figure 2.11 | Colony morphology of wild-type *M. smegmatis* and *M. smegmatis ΔaftC* | 87 |
| Figure 2.12 | Strategy to delete Cg-ΔaftC using the deletion vector pK19mobsacBΔaftC | 88 |
| Figure 2.13 | Analysis of cell wall bound MAMES from *M. smegmatis, M. smegmatis ΔaftC, M. smegmatis ΔaftC* pMV261-Ms-ΔaftC and *M. smegmatis ΔaftC* pMV261-Mt-ΔaftC | 89 |
| Figure 2.14 | Analysis of cell wall bound CMAMES from *C. glutamicum* and *C. glutamicum ΔaftC* | 90 |
Figure 2.15 GC analysis of cell walls of *M. smegmatis*, *M. smegmatis*Δ*aftC*, *M. smegmatis*Δ*aftC* pMV261-Ms-*aftC* and *M. smegmatis*Δ*aftC* pMV261-Mt-*aftC*…………………………………………………………………………….91

Figure. 2.16 GC/MS analysis of cell walls of *M. smegmatis*, *M. smegmatis*Δ*aftC*, *M. smegmatis*Δ*aftC* pMV261-Ms-*aftC* and *M. smegmatis*Δ*aftC* pMV261-Mt-*aftC*…………………………………………………………………………….92

Figure 2.17 GC and GC/MS analysis of cell walls of *C. glutamicum* and *C. glutamicum*Δ*aftC*…………………………………………………………………………….93

Figure 2.18 Arabinofuranosyltransferase activity utilising an Ara2 acceptor and membranes prepared from *M. smegmatis*, *M. smegmatis*Δ*aftC* and *M. smegmatis*Δ*aftB* pMV261-Mt-*aftC*……………………………………………………………………95

Figure 2.19 Arabinofuranosyltransferase activity utilising an Ara3 acceptor and membranes prepared from *M. smegmatis*, *M. smegmatis*Δ*aftC* and *M. smegmatis*Δ*aftC* pMV261-Mt-*aftC*……………………………………………………………………97

Figure 2.20 GC/MS characterisation of in vitro synthesised product X from the arabinofuranosyltransferase assays utilising the Ara5 acceptor…………………………………………………………………………………98

Figure 2.21 Proposed mycobacterial arabinan biosynthesis and the role of AftB and AftC………………………………………………………………………………………………………103

Figure 3.1 SDS-PAGE analysis of lipoglycans extracted from *M. smegmatis* and *M. smegmatis*Δ*aftC*………………………………………………………………………………110

Figure 3.2 MALDI-TOF-MS analysis of purified WT-LAM (A) and AftC-LAM (B) extracted from *M. smegmatis* and *M. smegmatis*Δ*aftC*………………………………………………………………………………111

Figure 3.3 GC and GC/MS analysis of purified lipoglycans extracted from *M. smegmatis* (A and B), *M. smegmatis*Δ*aftC* (C and D), *M. smegmatis*Δ*aftC* pMV261-Ms-*aftC* (E and F), and *M. smegmatis*Δ*aftC* pMV261-Mt-*aftC* (G and H).112

Figure 3.4 Two-dimensional NMR spectra of WT-LAM and AftC-LAM purified from *M. smegmatis* and *M. smegmatis*Δ*aftC*…………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………113

Figure 3.5 Structural representation of WT-LAM and AftC-LAM………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………………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### LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1.1</td>
<td><em>Mycobacterium</em> genus with representative slow-growing and fast-growing bacilli</td>
<td>4</td>
</tr>
<tr>
<td>Table 1.2</td>
<td>Commonly used TB drugs and their targets</td>
<td>11</td>
</tr>
<tr>
<td>Table 1.3</td>
<td>Genes involved in biosynthesis of LAM and related glycoconjugates</td>
<td>54</td>
</tr>
<tr>
<td>Table 1.4</td>
<td>Primers used for the generation of knock-out mutants and complemented strains</td>
<td>154</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
<td></td>
</tr>
<tr>
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<td>-----------</td>
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GL  glycolipid
GlcNAc  N-acetylglucosamine
GMCM  glucose monocorynomycolate
GPI  glycosylphosphatidyl inositol
GPLs  glycopeptidolipids
Gro  glycerol
h  hour
HCl  hydrochloric acid
HIC  hydrophobic interaction chromatography
His-tag  6 histidine residue tag
HIV  human immuno-deficiency virus
Hz  hertz
IL  interleukin
INF-γ  interferon gamma
Ins  inositol
IPTG  isopropylthio-β-D-galactoside
kDa  kilo Dalton
kPa  kilo Pascal
L  litre
LAM  lipoarabinomannan
LB  Luria-Bertani
LM  lipomannan
LOSs  lipooligosaccharides
LPS  lipopolysaccharide
LU  linkage unit
M  molar
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</tr>
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</tr>
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Chapter 1
1.1. **Mycobacterial Classification and Phylogenetic Analysis**

Tuberculosis (TB) has been a prominent human disease for several thousand years. Mycobacterium tuberculosis, the aetiological agent of TB, belongs to the genus Mycobacterium, which is presumed to have originated more than 150 million years ago (Daniel, 2006; Hayman, 1984). Phylogenetic analysis of bacilli isolated from patients living in remote East African villages, suggest that these isolates predate the modern M. tuberculosis complex (MTBC) members, representing the ancestral species from which these modern members may have evolved (Gutierrez et al., 2005). This has led to the assumption that an ancient predecessor co-existed and co-evolved nearly 3 million years ago with early East African Hominids. The organism which later underwent an “evolutionary bottleneck”, some 20,000 years ago, resulting in the present strains that account for the majority of TB worldwide (Brosch et al., 2002; Daniel, 2006; Kapur et al., 1994; Sreevatsan et al., 1997a).

1.1.1. **The Genus Mycobacterium**

Members of the MTBC include \( \text{M. tuberculosis, Mycobacterium bovis, Mycobacterium bovis BCG, Mycobacterium africanum, Mycobacterium canettii, Mycobacterium leprae and Mycobacterium pinnipedii} \) (Brosch et al., 2002), which belong to the genus Mycobacterium. At present over 100 species have been recognised in this genus, the majority of which are saprophytic soil species (Brown-Elliott et al., 2002); however, a minority are pathogenic to humans, such as \( \text{M. tuberculosis and M. leprae} \), causing TB and leprosy, respectively. In addition, Mycobacterium kansasii, Mycobacterium fortuitum and Mycobacterium avium complex (MAC) members cause illness and fatalities in immunocompromised individuals (Kiehn et al., 1985). The Mycobacterium genus belongs to the family Mycobacteriaceae, positioned in the suborder Corynebacterineae, which is included in the suprageneric Acinomycete taxon. Corynebacterineae also includes Corynebacterium, Nocardia and Rhodococcus, all of which are classified as Gram-positive, non-motile, aerobic, rod-shaped
bacteria, with characteristically high proportions of guanine and cytosine in their genomes. One of the foremost defining features of this suborder is the existence of mycolic acids (Minnikin & Goodfellow, 1980), unique $\beta$-hydroxy-$\alpha$-alkyl branched long chain fatty acids, which are specific constituents of the cell envelope (Daffé & Draper, 1998; Dover et al., 2004). This distinctive ‘waxy’ cell wall is highly impermeable and is the basis for M. tuberculosis’ intrinsic resistance to common antibiotics (Brennan & Nikaido, 1995; Nguyen et al., 2006) and its formidable strength against the bactericidal activities of the macrophage.

Species within the Mycobacterium genus exhibit variable growth rates and are classified as either slow or rapid growers (Table 1.1) (Lewin & Sharbati-Tehrani, 2005). A characteristic feature of highly pathogenic species such as M. tuberculosis and M. leprae is their slow growth rate and fastidious culturing processes. M. tuberculosis divides every 15 to 20 hours, taking between 4-6 weeks to obtain visual colonies, which is extremely slow compared to other bacteria, for instance Escherichia coli has a doubling time of 20 minutes (Lewin & Sharbati-Tehrani, 2005).

Mycobacterium smegmatis is classified as a fast-growing species, with a doubling time of approximately 3 hours, producing visible colonies in 3-5 days. It is generally considered non-pathogenic in immunocompetent individuals, and thus does not require the Category 3 safety laboratory. There are many similarities between M. smegmatis and the much more virulent pathogens, with more than 2000 homologous genes shared with M. tuberculosis. These properties make it a very useful model organism for M. tuberculosis and other mycobacterial pathogens.


Table 1.1: Mycobacterium genus with representative slow-growing and fast-growing bacilli.

<table>
<thead>
<tr>
<th>Slow Growers</th>
<th>Fast Growers</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. africanum</em></td>
<td><em>M. marinum</em></td>
</tr>
<tr>
<td><em>M. avium</em></td>
<td><em>M. microti</em></td>
</tr>
<tr>
<td><em>M. bovis</em></td>
<td><em>M. scrofulaceum</em></td>
</tr>
<tr>
<td><em>M. intracellulare</em></td>
<td><em>M. simiae</em></td>
</tr>
<tr>
<td><em>M. farginogenes</em></td>
<td><em>M. szulgai</em></td>
</tr>
<tr>
<td><em>M. gastri</em></td>
<td><em>M. tuberculosis</em></td>
</tr>
<tr>
<td><em>M. kansasii</em></td>
<td><em>M. ulcerans</em></td>
</tr>
<tr>
<td><em>M. leprae</em></td>
<td><em>M. xenopi</em></td>
</tr>
</tbody>
</table>

1.2. History of tuberculosis

Mycobacterial DNA has been detected in numerous archaeological specimens, proving that TB is an ancient disease, which plagued the prehistoric man. Molecular identification of human TB from 4500 year old Egyptian mummies (Zink et al., 2001) and further palaeomicrobiological evidence (Donoghue et al., 2004) from the United Kingdom (Taylor et al., 2005), South America (Salo et al., 1994), Borneo (Donoghue et al., 2004) and numerous other world-wide locations, illustrate the wide geographical distribution of the disease.

During 460 BC, the first documented account of TB emerged; the term phthisis (to waste away) was introduced into Ancient Greek literature by Hippocrates, who recognised how widespread and prevalent the disease was (Coar, 1982). It wasn’t until the writings of Clarissimus Galen (131-201 AD) that Phthisis or ‘consumption’ was described as ulceration of the lungs, along with wasting away of the body, fever and cough. TB persisted throughout the ages, surging into 17th century Europe, becoming the principal cause of death. Mortality rates reached their pinnacle in the 18th and 19th centuries and TB gained the title the “Great
White Plague”. The name ‘tuberculosis’ was introduced into the medical language after Gaspard Laurent Bayle (1774-1816) proved that tubercles present on the lungs of TB sufferers were the cause of illness.

1.2.1. Discovery of the tubercle bacillus and initial therapies

In 1720, Benjamin Marten, a prominent English Physician, published work proposing that TB was infectious, stating that these ‘minute, living creatures’ enter the body and induce formation of lesions, producing the symptoms of Phthisis (Doetsch, 1978). In 1882, Robert Koch provided unequivocal proof that TB was in fact caused by a bacterium, *M. tuberculosis*, which could be isolated and stained (Koch, 1931). This significant discovery awakened the scientific community to the prospect that this age-old disease could be treated, and research concentrated on producing effective chemotherapeutic agents.

The mid 19th century was accompanied by a decrease in TB mortality rates and a lesser incidence of disease, which may have coincided with the establishment of Hermann Brehmer’s sanatoriums in 1859. These establishments promoted good nutrition, exercise, rest and fresh air (Cox, 1923; Daniel, 2006), providing the first widely practiced anti-tuberculosis treatment. The introduction of active therapy soon followed in the form of pneumothorax and thoracoplasty procedures that collapsed part of the infected lungs with the intention of closing the cavities (Daniel, 2006). Nonetheless, these invasive early therapies seemed to do little to alleviate the symptoms, and are now deemed dangerous and controversial. Albert Calmette and Camille Guerin, inspired by Edward Jenner’s discovery, that humans could be immunised against smallpox following cowpox infection, began developing a vaccine against *M. tuberculosis* using *M. bovis*, the aetiological agent of bovine TB. In 1921, after successful attenuation of the strain, the Bacille Calmette-Guerin (BCG) vaccine was administered to humans (Calmette, 1928) and remains the only vaccine in use
today regardless of reports on its variable efficacy (Brewer & Colditz, 1995; Colditz et al., 1994).

1.3. Pathogenesis of tuberculosis

Transmission of M. tuberculosis begins with the inhalation of infective droplet nuclei expelled from the respiratory tract of an active TB sufferer. The nasopharynx and the upper respiratory tract trap larger particles; however, droplets with a diameter as large as 5 µm may enter the alveoli, whereby they are ingested by the alveolar macrophages (Lurie, 1950). Receptor-mediated phagocytosis of the invading bacteria involves several major host cell surface receptors, such as, complement receptors (CR1, CR2, and CR3), mannose receptors (MR), surfactant protein receptors, CD14, and scavenger receptors (Ernst 1998). Following phagocytosis, the bacteria continue to reside within the membrane-bound vacuole called the phagosome, which matures to form the phagolysosome (Figure 1.1). This process subjects the internalised bacilli to attack from hydrolytic enzymes delivered by the lysosome. These initial microbicidal defenses may destroy the invading bacilli, yet some are able to evade this outcome by inhibiting the maturation process of the phagosome, as well as modulating the internal environment allowing logarithmic growth, leading to lysis of the immune cell. The released bacteria, chemoattractants, and cellular debris draw in other macrophages and monocytes from the bloodstream. The logarithmic bacterial growth terminates when T-lymphocytes, attracted by interleukin-8, arrive at the lesions. The lesions then undergo caseous necrosis due to the amplified immune response, resulting in a build up of acidic necrotic material in the centre, predominantly made up of macrophage debris (Dannenberg & Rook, 1964; Dannenberg & Sugimoto, 1976). This acidic, anoxic environment does not support bacterial growth, although the bacteria can survive in the destroyed tissue. Eventually, the copious amounts of released antigen stimulate the cell-mediated immune response; cytokines activate macrophages, which are then capable of killing bacilli ingested
from the edges of the lesion. In most cases the disease enters a period latency with the formation of a granulomatous lesion, named a ‘tubercule’.

**Figure 1.1: The intracellular existence of M. tuberculosis.** This figure illustrates the influence of M. tuberculosis on the phagosome and endosome maturation processes. Specialised cell types — the so-called ‘professional’ phagocytes, engulf larger particles. Numerous cell-surface receptors participate in the interaction between M. tuberculosis and the macrophages, facilitated by Cholesterol. M. tuberculosis resides in a phagosome, the maturation of which is inhibited at an early stage. The early mycobacteria-containing phagosome retains tryptophan-aspartate containing coat protein TACO, which apparently prevents its further maturation. M. tuberculosis inhibits phagosomal acidification (which normally occurs via recruitment of V_o-H^+ ATPase) and prevents fusion with the endosomal pathway. Rab5 is involved in regulation of the fusion with vesicles of the endosomal-lysosomal pathway. Phagosomal maturation is not always successfully halted, allowing some phagosomes to mature into phagolysosomes. Phagosome maturation is promoted in activated macrophages, particularly after stimulation by IFN-γ. CR, complement receptor; FcR, receptor for the constant fragment of immunoglobulin; LAMP-1 lysosomal-associated membrane protein 1; LBPA, lysobiphosphatic acid; MR, mannose receptor; Rab5 and Rab7, members of the small GTPase family; SPR, surfactant protein receptor; TACO, tryptophane, aspartate-containing coat protein; TLR, Toll-like receptor; V-H^+ATPase, vacuolar ATP-dependent proton pump (Adapted from Kaufmann, 2001).
Figure 1.2 Overview of TB infection and host defence. There are three potential outcomes of infection of the human host in *M. tuberculosis*. 

a) The frequency of spontaneous healing is unknown, but is assumed to be rare.

b) Disease progression immediately after infection in the immunocompromised host.

c) In the majority of cases, mycobacteria are initially contained and disease progresses later due to reactivation. The granuloma is the major site of infection, which develops from infected macrophages that become surrounded by lymphocytes. Effector T cells (CD4+ and CD8+ T cells, and double-negative or CD4/CD8 single-positive T cells that recognise antigen presented by CD1) and macrophages participate in the control of TB. Crucial macrophage activators interferon-γ (IFN-γ) and tumour-necrosis factor-α (TNF-α) are produced by T cell and macrophages respectively. Macrophage activation permits phagosomal maturation and the production of antimicrobial molecules such as reactive nitrogen intermediates (RNI) and reactive oxygen intermediates (ROI). LT-3, lymphotoxin-3 (Adapted from Kaufmann, 2001).
It is estimated that only 1 in 10 infected individuals will go on to develop clinical symptoms due to reactivation of TB, and the lifetime risk of this is 5-10% (Figure 1.2). The strongest risk factor for reactivation of TB is an impaired immune system, for instance, as a result of HIV infection, malnutrition, advanced malignancy or old age (Kaufmann, 2001). HIV infected individuals have a 7-10% annual risk of latent disease progressing into active TB. Their incompetent immune cells ingest the bacilli from the edges of the caseous regions, yet they are unable to halt growth, thus the delayed-type hypersensitivity response continues to destroy tissue. This results in lymphohematogenous dissemination, whereby escaping bacteria are free to form multiple lesions throughout the body (Kaufmann, 2001; Raupach & Kaufmann, 2001).

1.4. Epidemiology of tuberculosis

By the mid 20th century, with the onset of improved public health services, the BCG vaccine and the use of specific drug therapies, TB prevalence had declined dramatically in industrialised countries, so much so, that the disease was considered to be close to elimination. The resurgence of TB occurred during the 1980s, exacerbated by several factors, such as the HIV/AIDS pandemic, the variable effectiveness of the BCG vaccine, lax drug adherence resulting in the emergence of multi-drug resistant strains (MDR-TB) and increased immigration from developing countries where TB has continued to be endemic (Figures 1.3 & 1.4). In fact, the concept of TB’s imminent eradication cannot be further from reality (Murray & Salomon, 1998). In 1993 the World Health Organisation declared TB a global emergency, estimating that a third of the world’s population are infected, equating to 2 billion people. With 8 million new cases reported each year and 2 million fatalities annually, M. tuberculosis is the biggest global killer amongst infectious diseases (Dye & Raviglione, 2005). These alarming statistics and the emergence of MDR-TB strains and extensively-
drug resistant (XDR-TB) strains have highlighted the urgency for the development of newer, more efficient antimicrobial agents (Blanchard, 1996; Pablos-Mendez et al., 1998).

Figure 1.3: Estimated TB incidence rates in the year 2009 (WHO, 2010).

Figure 1.4: Estimated HIV prevalence in new TB cases in the year 2009 (WHO, 2010).
1.5. TB drug treatments

The most commonly used TB drug regimens include the so-called front-line drugs, rifampicin (RIF), isoniazid (INH), pyrazinamide (PZA) and ethambutol (EMB) as shown in Table 1.2.

Table 1.2: Commonly used TB drugs and their targets. Adapted from Kremer & Besra, (2002); Nachega & Chaisson, (2003).

<table>
<thead>
<tr>
<th>Drug</th>
<th>Drug mechanism of action</th>
<th>Genes involved in resistance</th>
<th>Function of gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoniazid (INH) (1952)</td>
<td>Inhibition of mycolic acid biosynthesis and other multiple effects on DNA, lipids, carbohydrates and NAD metabolism</td>
<td>katG, inhA, ndh, oxyR-aphC</td>
<td>Catalase-peroxidase, Enoyl-Acp reductase, NADH dehydrogenase II, Alkyl hydroperoxidase</td>
</tr>
<tr>
<td>Ethionamide (ETH) (1956)</td>
<td>Inhibition of mycolic acid biosynthesis</td>
<td>ethA, inhA</td>
<td>Flavin mono-oxygenase, Enoyl-Acp reductase</td>
</tr>
<tr>
<td>Ethambutol (EMB) (1961)</td>
<td>Inhibition of cell wall arabinogalactan (AG) biosynthesis</td>
<td>embCAB</td>
<td>Arabinosyltransferases</td>
</tr>
<tr>
<td>Rifampicin (RIF) (1966)</td>
<td>Inhibition of transcription</td>
<td>rpoB</td>
<td>RNA polymerase</td>
</tr>
<tr>
<td>Pyrazinamide (PYR) (1952)</td>
<td>Acidification of cytoplasm and de-energising the membrane</td>
<td>pncA</td>
<td>Nicotinamidase/pyrazinamide</td>
</tr>
<tr>
<td>Streptomycin (STREP) (1944)</td>
<td>Inhibition of protein synthesis</td>
<td>rpsL, rrs</td>
<td>S12 ribosomal protein, 16S rRNA</td>
</tr>
</tbody>
</table>
In cases where INH resistance has occurred, streptomycin (STRP) is used as a replacement, although it is not preferred since there is a higher toxicity and a high incidence of STRP-resistant TB. The most effective drug regimen is endorsed by the WHO and is called Directly Observed Treatment, Short-course (DOTS), developed to fully supervise those patients deemed high-risk for non-drug adherence. This course consists of a 6-month treatment plan, where the initial phase is 2 months, involving the administration of INH, RIF, EMB and PZA. The remaining four months is known as the continuation phase, during which time different regimens may be followed depending on any resistance profiles, but generally involves taking RIF and INH (Farmer & Kim, 1998).

1.5.1. TB Drug resistance

The global problem of TB has been worsened by the development of drug resistant M. tuberculosis strains and is generally attributed to poor compliance with treatment. Lax drug adherence is associated with either lengthy duration of TB therapy, the tendency of patients to feel free of previous disease-like symptoms before course completion, therapy termination due to certain side effects, or unreliable drug supplies, all of which have accelerated MDR- and XDR-TB strains (Munro et al., 2007). MDR-TB is defined as TB that is resistant to at least INH and RIF, the two most powerful first-line anti-TB drugs. Treatment in the case of MDR-TB can exceed 2 years, in comparison to drug-sensitive TB, which can be effectively treated with a 6-month regimen, thus significantly increasing costs and side effects. The incidence rates are increasing with 424,000 cases in 2004, up from 273,000 in 2000 (Aziz et al., 2006; Zignol et al., 2006), and a mortality rate of 27% in those infected with MDR-TB compared to 18% for drug-sensitive TB infections (World Health Organisation (WHO) 2007). XDR-TB has now disturbingly emerged; defined as MDR-TB that has developed additional resistance to three injectable second-line drugs (aminoglycosides) and one of the fluoroquinolones (CDC, 2006; World Health Organisation (WHO) 2007). Worryingly,
XDR-TB has been detected in 45 countries as disclosed by the WHO/IUATLD report for 2008, and is untreatable so far. This shows that control of drug-resistant TB incidence is somewhat inadequate, especially in regions with a high prevalence of HIV cases, where TB-HIV co-infections increase the susceptibility to re-infection, relapse and treatment failure (Nations et al., 2006; Omerod, 2005). Therefore, there is a necessity to discover new specific antimicrobial compounds against M. tuberculosis.

1.6. The mycobacterial cell wall

The compositional complexity of the mycobacterial cell wall (Figure 1.5) distinguishes Mycobacterium species from the majority of other prokaryotes. Classified as Gram-positive organisms, their envelopes do in fact share notable features with Gram-negative cell walls, such as an outer permeability barrier acting as a pseudo-outer membrane (Brennan & Nikaido, 1995; Minnikin, 1982). Much of the early structural definition of the cell wall was conducted in the 1960s and 1970s (Lederer et al., 1975; Petit et al., 1969; Adam et al., 1969). Minnikin, in 1982, proposed the currently accepted structural model for the cell wall architecture that was later supported by McNeil and Brennan (McNeil & Brennan, 1991; Minnikin, 1982; Minnikin et al., 2002) and numerous further investigations (Besra & Brennan, 1995; Brennan & Nikaido, 1995; Minnikin, 1991; Nikaido et al., 1993).

The structural features of the cell envelope can be divided into three major components;

1. the plasma membrane

2. the mycolyl-arabinogalactan-peptidoglycan (mAGP) complex (Besra & Brennan, 1997), consisting of three covalently linked macromolecules, peptidoglycan (PG), arabinogalactan (AG) and hydrophobic mycolic acids which decorate the non-reducing terminus of the arabinan domains
3. lipoarabinomannan (LAM), lipomannan (LM), non-covalently bound lipids and glycolipids.
1.6.1. **The cell wall of *M. tuberculosis* as a drug target**

Mycobacterial diseases are especially problematic to treat owing to this unique cell wall architecture. This tightly packed, lipid rich envelope accounts for the inherent resistance of *M. tuberculosis* to various hydrophilic antimicrobials, as well as lipophilic molecules that have difficulty passing through this highly ordered mycolic acid layer. The cell wall is essential for growth and survival and cell wall inhibitors have been one of the most active agents of chemotherapy, and as such, the majority of front-line drugs target its biosynthesis (Zhang, 2005). Drug regimens employ at least one cell wall biosynthetic inhibitor, for instance INH and ETH, which target mycolic acid biosynthesis or EMB which inhibits arabinan biosynthesis, taken in combination with other chemotherapeutic agents that have intracellular targets (Nikaido & Jarlier, 1991). However, due to the emergence of various drug resistant strains as discussed previously, there is a need for the discovery of novel drug targets and the development of active compounds against them (Heymann et al., 1999; Sreevatsan et al., 1997b; Telenti et al., 1997). In this regard, the biosynthetic machinery of mycobacterial cell wall represents an attractive target and numerous research studies have been and are being conducted, delving into the intricacies of the mycobacterial cell wall assembly (Bhatt et al., 2007b; Bhowruth et al., 2007; Brennan & Crick, 2007; Dover et al., 2008).

1.6.2. **Plasma membrane**

The plasma membrane acts as a boundary between the cytosol and the periplasmic space, but it also harbours a number of substrates required by cell wall biosynthetic enzymes (Berg et al., 2007), thus playing a crucial role in the biogenesis of the cell wall. A group of glycosylphosphoprenols associated with the plasma membrane have been identified and operate as sugar donor substrates for many of the TB glycosyltransferases. For instance, activated forms of D-ribofuranose (Wolucka et al., 1994; Wolucka & de Hoffmann, 1995),
D-mannopyranose (Takayama & Goldman, 1970) and D-arabinofuranose (Wolucka & de Hoffmann, 1994; Wolucka et al., 1994) have been identified. Many of the involved glycosyltransferases are either embedded in the lipid bilayer as integral membrane proteins, or associated with the membrane surface by hydrophobic and/or electrostatic interactions. For instance, topology studies of the EmbCAB proteins, the putative arabinofuranosyltransferases revealed 13 transmembrane domains (Telenti et al., 1997). In addition, Alderwick et al. (2006b) recently discovered a further arabinofuranosyltransferase AftA, which also contains integral transmembrane spanning domains. It is believed that glycosyltransferases involved in the biosynthesis of the arabinans of both AG and LAM are all integral membrane proteins and are therefore classified as members of the glycosyltransferase family C (GT-C) (Berg et al., 2007). With the exception of phosphatidylinositol mannosides (PIMs), which are constrained to Actinomycetales, the lipid composition of this biomembrane is similar to that of other prokaryotes, suggesting that the general properties of lipid bilayers are applicable for mycobacteria.

1.6.3. Structure and biosynthesis of peptidoglycan

Peptidoglycan (PG) is a complex macromolecular structure situated on the outside of the plasma membrane of almost all eubacteria (Schleifer & Kandler, 1972; van Heijenoort, 2001a, 2001b). Its mesh-like arrangement confers rigidity to the cell, allowing it to withstand osmotic pressure maintaining cell integrity and cellular shape. Relatively little is known about M. tuberculosis PG synthesis, although it is generally assumed to be akin to that of E. coli (van Heijenoort, 2001a, 2001b), also being classified as A1γ according to the classification system of Schleifer & Kandler (1972). Mycobacterial PG forms the backbone of the mAAGP complex, composed of alternating N-acetylglucosamine (GlcNAc) and modified muramic acid (Mur) residues, linked in a β(1→4) configuration (Lederer et al., 1975). Unlike E. coli PG, the muramic acid residues in M. tuberculosis and M. smegmatis
for instance, contain a mixture of N-acetyl and N-glycolyl derivatives, whereby the N-acetyl function has been oxidised to a N-glycolyl function to form MurNGly (Mahapatra et al., 2005a; Mahapatra et al., 2005b; Raymond et al., 2005). These additional glycolyl containing residues result in extra hydrogen bonding, strengthening the mesh-like structure (Brennan & Nikaido, 1995), as well as possibly protecting the organism from lysozyme degradation. Tetrapeptide side chains consisting of L-alaninyl-D-isoglutaminyl-meso-diaminopimelyl-D-alanine (Petit et al., 1969) cross-link with identical short peptides of neighbouring glycan chains (van Heijenoort, 2007). These cross-links include the expected meso-diaminopimelic acid (DAP) and D-alanine bond, common to most prokaryotes, but also a high degree of bonds between two residues of DAP (Ghuysen, 1968; Wietzerkin et al., 1974). The proportion of cross-linking in Mycobacterium species is 70-80%, significantly more so than E. coli, with only 50% (Vollmer, 2004). An additional deviation from E. coli PG, is the use of the muramic acid residues as attachment sites for the galactan domain of the arabinogalactan, whereby carbon-6 of some of the muramic acid residues form a phosphodiester bond and are linked to the \( \alpha-L\)-rhamnopyranose-(1→3)-\( \alpha-D\)-GlcNAc(1→P) linker unit of AG (Hancock et al., 2002; McNeil et al., 1990).

One model proposed for the three-dimensional topology of the mAGP complex, consistent with the traditional models of PG architecture (Ghuysen, 1968; McNeil & Brennan, 1991), suggests that the PG and the galactan moiety run parallel to the plasma membrane. However, an opposing model put forward by other modeling studies predict that the PG and the AG polymers may in fact be coiled and are thus orientated perpendicular to the plane of the plasma membrane (Dmitriev et al., 2000; Dmitriev et al., 2003). Minnikin and colleagues (2002) proposed that AG as well as LAM form coiled strands and integrate with PG. Although, recent published nuclear magnetic resonance (NMR) data concluded that the PG glycan strand is orthogonal to the plane of the membrane, thus the overall three-dimensional
structure and topology remains open to debate (Meroueh et al., 2006). The biosynthesis of mycobacterial PG is comparable to that of E. coli and is shown in Figure 1.6.

![Figure 1.6: Biosynthesis of peptidoglycan in M. tuberculosis](image)

The synthesis of the disaccharide-peptide monomer unit is initiated in the cytoplasm, catalysed by six enzymes, MurA to MurF. MurA transfers enolpyruvate from phosphoenolpyruvate to the 3-OH of UDP linked GlcNAc, which is reduced to form UDP-MurNAc by MurB (De Smet et al., 1999; Goffin & Ghuysen, 2002). The UDP-MurNAc-pentapeptide is then formed via the sequential addition of L-alanine, D-glutamic acid, DAP and a D-alanyl-D-alanine dipeptide, to MurNAc, by MurC, MurD, MurE and MurF, respectively (Mahapatra et al., 2000). Lipid I synthesis involves MraY (MurX) transferring the modified MurNAc residue to a polyisoprenyl phosphate carrier lipid, followed by addition of GlcNAc by MurG (Ikeda et al., 1991). The product, GlcNAc-MurNAc(pentapeptide)-diphosphoryl-undecaprenol (Lipid II) is then translocated across the membrane and incorporated into the growing PG by transglycosylases and further modified by transpeptidases (Bhakta & Basu, 2002; Goffin & Ghuysen, 2002).

**1.6.4. Biosynthesis of the linker unit**

Mycobacterial viability rests heavily on the structural integrity of the cell wall, thus the attachment of which the AG proper is hinged to the PG layer is pivotal. Amar & Vilkas (1973), initially reported that AG is tethered to PG at intervals by a phosphodiester bond,
supported by the presence of muramyl-6-phosphate in the cell wall preparations from several mycobacterial species (Kanetsuna, 1968; Liu & Gotschlich, 1967). The fundamental question of the chemical nature of this link wasn’t answered until 20 years later when oligosaccharides containing galactofuranose (Gal\(\text{f}\)) from the galactan domain were isolated along with rhamnose (Rha) residues (McNeil et al., 1990). The further discovery of the disaccharide \(\text{L}-\text{Rha}\cdot(1\rightarrow3)\cdot\text{D}-\text{GlcNAc}\) (Figure 1.7) led to the conclusion that these constituents make up the linkage unit and the inference that the GlcNAc is directly attached to the 6-position of a proportion of the muramyl residues of PG (McNeil et al., 1990).

![Figure 1.7: The Rha-GlcNAc linker unit. The molecular structure of the LU, the conduit between AG and PG.](image)

The crucial structural role of the linker unit (LU) (Figure 1.7) in the attachment of AG to PG, as well as the presence of \(\text{L}-\text{Rhamnose}\), a sugar absent in humans, makes the biosynthetic machinery of the mycobacterial LU (Figure 1.8) an attractive drug target (Ma et al., 2002).

In \(M.\) tuberculosis, synthesis is initiated on the acceptor decaprenyl-phosphate (Dec-P or \(C_{50}\)-P), whereby GlcNAc-phosphate is transferred from UDP-GlcNAc, forming \(C_{50}\)-P-P-GlcNAc, referred to as glycolipid 1 (GL-1) (Mikusova et al., 1996). The enzyme formerly known as Rfe (Rv1302, WecA) has been implicated as a decaprenyl-phosphate \(\alpha\)-N-acetylglucosaminyltransferase by its significant homology to the E. coli WecA protein (Amer
& Valvano, 2002; Dal Nogare et al., 1998), although there are no biochemical data as yet to definitively prove this supposition. The linkage region is completed by the subsequent addition of the Rha residue by the recently defined rhamnosyltransferase, \textit{WbbL} (Rv3265c), to the 3-position of the GlcNAc of GL-1, forming glycolipid 2 (GL-2) (Figure 1.8) (Mills et al., 2004). The key to these studies was with the successful complementation of an \textit{E. coli} mutant with Rv3265c (\textit{wbbL}) and subsequent restoration of rhamnosyltransferase activity (McNeil, 1999). Moreover, investigations from \textit{M. smegmatis} have highlighted the essentiality of \textit{wbbL} for bacterial viability (Mills et al., 2004).

\textbf{Figure 1.8: Formation of the linker unit - glycolipid-1 (GL-1) and glycolipid-2 (GL-2).} \(\alpha\text{-GlcNAc-1-P}\) is transferred from UDP-GlcNAc to decaprenol (DP) by the GlcNAc-1-phosphate transferase Rfe (Rv1302, WecA), followed by the attachment of a rhamnosyl residue from dTDP-Rha catalysed by WbbL (Rv3265c) (Mills et al., 2004), resulting in Rha-GlcNAc-P-P-decaprenyl.

The \textit{wbbL} gene product utilises the nucleotide donor dTDP-rhamnose for the formation of GL-2, so it stands to reason that the rhamnosyl biosynthetic pathway has come under scrutiny and a number of inhibitors have been described (Babaoglu et al., 2003; Kantardjieff et al.,
2004; Ma et al., 2001). Synthesis of dTDP-Rha occurs via a linear 4-stage pathway utilising the gene products of \textit{rmlABCD} as depicted in Figure 1.9.

Recognition of the genes involved transpired by comparison to known polysaccharide biosynthetic enzymes found in other bacteria, namely \textit{E. coli} (Ma et al., 1997; Stevenson et al., 1994; Weston et al., 1997). \textit{RmlA} (Rv0334) sets in motion the sequence of reactions, converting dTTP + $\alpha$-D-glucose 1-phosphate to dTDP-glucose + PP$_i$ (Ma et al., 1997). The enzyme was cloned from \textit{M. tuberculosis} and transformed into an \textit{E. coli} strain devoid of four TDP-Rha biosynthetic genes. Cellular extract analysis revealed an abundance of $\alpha$-D-Glc-P thymidylyltransferase activity confirming its proposed function (Ma et al., 1997). The product of \textit{RmlA} activity is then shuttled through three sequential reactions catalysed by dTDP-D-glucose 4,6-dehydratase (Rv3464, RmlB), dTDP-4-keto-6-deoxy-D-glucose 3,5 epimerase (Rv3465, RmlC) and dTDP-Rha synthase (Rv3266, RmlD) (Hoang et al., 1999; Ma et al., 2001; Stern et al., 1999). Li et al (2006) demonstrated that \textit{rmlB} and \textit{rmlC} genes are also essential for mycobacterial growth.
A series of enzymes converts glucose-1-phosphate (α-D-Glc-1-P) to dTDP-Rha. RmlA (Rv0334) = α-D-glucose-1-phosphate thymidylyl transferase (Ma et al., 1997), RmlB (Rv3464) = dTDP-D-glucose 4,6-dehydratase (Ma et al., 2001), RmlC (Rv3465) = dTDP-4-keto-6-deoxy-D-glucose (Stern et al., 1999), and RmlD (Rv3266c) = dTDP-rhamnose synthetase (Hoang et al., 1999).

Figure 1.9: Biosynthetic pathway of dTDP-rhamnose. A series of enzymes converts glucose-1-phosphate (α-D-Glc-1-P) to dTDP-Rha. RmlA (Rv0334) = α-D-glucose-1-phosphate thymidylyl transferase (Ma et al., 1997), RmlB (Rv3464) = dTDP-D-glucose 4,6-dehydratase (Ma et al., 2001), RmlC (Rv3465) = dTDP-4-keto-6-deoxy-D-glucose (Stern et al., 1999), and RmlD (Rv3266c) = dTDP-rhamnose synthetase (Hoang et al., 1999).
1.6.5. Arabinogalactan

1.6.5.1. Structural features of arabinogalactan

Exclusive to the Actinomycetales, the mAG complex is a key structural component which makes up the bulk of the cell wall (Minnikin et al., 2002). Arabinogalactan (AG), the unique heteropolysaccharide, is covalently tethered to the PG via a phosphodiester bond to approximately 10-12% of the muramic acid residues of PG (Amar & Vilkas, 1973; Misaki et al., 1974). Collectively, PG and AG, form a covalently linked network positioned between the plasma membrane and the mycolic acid layer, resulting in an exceptionally robust cell wall. Early work demonstrated that the polymer was composed predominantly of arabinose and galactose (Azuma et al., 1968; Kanetsuna, 1968; Kanetsuna et al., 1969; McNeil et al., 1987; Misaki et al., 1966). Both sugars are in the furanoid ring form, D-galactofuranosyl (Gal\f) and D-arabinofuranosyl (Araf), which are extremely rare in nature (McNeil et al., 1987). Detailed characterisation of oligomers generated from partial depolymerisation of per-O-alkylated AG and the use of methylation analysis, gas-chromatography-mass spectrometry (GC-MS), fast atom bombardment-mass spectrometry (FAB-MS) and NMR spectroscopy established the detailed structure of this complex and the fact that unlike most bacterial polysaccharides, AG lacks repeating units being composed of a few distinct structural motifs (Besra et al., 1995; Daffé et al., 1990; McNeil et al., 1990; McNeil et al., 1991; Vilkas et al., 1973). The use of endo-arabinofuranosidases secreted from a Cellomonas species provided additional support for the obtained structural data as well as revealing further insights (McNeil et al., 1994). AG consists of a linear galactan composed of 30 linear alternating β(1→5) and β(1→6) Gal\f residues (Abou-Zeid et al., 1982; McNeil et al., 1987; Vilkas et al., 1973), attached to the rhamnosyl residue of the linker unit. Three apparently similar D-arabinan chains comprised of 22 or 23 Araf residues are affixed to the C-5 of the β(1→6) linked Gal\f (Besra et al., 1995). Recent studies by Alderwick et al (2005) ascertained that the arabinan chains are attached specifically to the 8\[^{th}\], 10\[^{th}\] and 12\[^{th}\] Gal\f
residue, data congruent with Besra et al. (1995) who recognised long stretches of unbranched galactan, leading to the prediction that the chains are attached near the reducing end. It seems that the arabinan domain is present as a highly branched network built on a backbone of $\alpha(1\rightarrow5)$ linked sugars with a number of $\alpha(1\rightarrow3)$ linked residues forming 3,5-Araf branch points (Daffé et al., 1990). Further $\alpha(1\rightarrow5)$ linked Araf sugars are attached subsequent to this branch point with the non reducing ends terminated by $\beta(1\rightarrow2)$ Araf residues. The final structural motif is the distinctive hexa-arabinoside (Ara$_6$) (McNeil et al., 1994) present as $(\text{Araf-}\beta(1\rightarrow2)\text{-Araf-}\alpha(1\rightarrow5)\text{-Araf-}\alpha(1\rightarrow5))$, of which two-thirds are mycolated (Figure 1.10).

Figure 1.10: Structural features of arabinogalactan. Pictorial representation of the arabinogalactan of M. tuberculosis as defined by McNeil et al. (1987), Daffé et al. (1990), McNeil et al. (1994) and Besra et al. (1995). Three chains of arabinan are attached to each galactan chain, of which two-thirds are substituted with mycolic acids.
Research emphasis has now shifted somewhat from structural analysis to biosynthesis. The complete sequencing of the mycobacterial genome has provided major impetus in the identification and the study of the enzymes involved in the biosynthesis of this exceptional structure, with the hope of uncovering new drug targets.

1.6.5.2. Synthesis of arabinogalactan biosynthetic precursors

1.6.5.2.1. Galactan precursor synthesis

D-gal residues, the main constituents of the galactan component of the mAEP complex, do not exist in mammalian metabolism, thus their biosynthesis constitutes an appealing target for chemotherapy without any deleterious side effects. Gal residues of the galactan domain are incorporated from the high-energy sugar nucleotide donor UDP-Galp, which is formed via two reactions. In E. coli, galactosyl residues in the pyranose ring form (UDP-Galp) are synthesised by the action of UDP-glucose 4-epimerase on UDP-glucopyranose (UDP-Glcp), the protein of which is encoded by the galE gene (Lemaire & Müller-Hill, 1986). Weston et al. (1997) assayed for the reverse reaction in M. smegmatis using radiolabeled UDP-Galp and UDP-glucose 4-epimerase activity was observed, of which the purified protein and its N-terminal sequence was shown to be similar to M. tuberculosis Rv3634. Conversion of UDP-Galp to the furanose form occurs via ring contraction catalysed by the enzyme UDP-galactopyranose mutase (Glf) that was first recognised in E. coli (Nassau et al., 1996) and subsequently in M. smegmatis and M. tuberculosis (Rv3809c) (Weston et al., 1997) and is summarised in Figure 1.11. Allelic exchange experiments of glf in M. smegmatis highlighted the essentiality of this gene (Pan et al., 2001).
1.6.5.2.2. Arabinan precursor synthesis

Endeavours to uncover sugar nucleotides of Araf have proven unsuccessful. Arabinan biosynthesis in the Actinomycetales involves β-D-arabinofuranosyl-1-monophosphodecaprenol (DPA), the only known donor of Araf residues (Alderwick et al., 2005; Wolucka et al., 1994; Xin et al., 1997). Synthetically derived DP[14C]A and an array of synthetic acceptors have determined that DPA provides Araf units in the in vitro formation of 2-linked and 5-linked arabinofuranosyl linkages present in the arabinans of AG and LAM (Belanger et al., 1996; Lee et al., 1997; Xin et al., 1997). The use of DPA and endogenous acceptors from membrane preparations produced a structure identical to the natural polymer, with equal distribution of radiolabel, highlighting that the lack of 3-linked linkages was not due to another unidentified activated donor and most likely the inability of the α(1→3) AraT
to recognise the acceptor. Subsequent assays discovered other DPA dependent AraTs, the so-called “priming” enzyme AftA (Rv3792), was responsible for the addition of the first AraT to the galactan domain, as well as demonstrating conclusively that DPA is the only donor in the related organism C. glutamicum (Alderwick et al., 2005).

Due to the important role of DPA, elucidation of its biosynthetic pathway has received much attention. The recognised biosynthesis of such polyprenylphosphate sugars chiefly involves the donation of a glycosyl residue from a sugar nucleotide to the polyprenylphosphate, interestingly the sugar nucleotides of arabinose (UDP-Ara or GDP-Ara) have not been identified. The carbon skeleton of the arabinosyl residues (Figure 1.12) are derived from the non-oxidative pentose shunt (Scherman et al., 1995), after which, ribose 5-phosphate and ATP react with the assistance of a pRpp synthetase to produce pRpp (Wolucka et al., 2008). The 5-phospho-\(\alpha\)-D-ribose 1-pyrophosphate:decaprenyl phosphate 5-phosphoribosyl transferase (Rv3806c), or UbiA transfers ribose-5-phosphate from pRpp to decaprenylphosphate to form DPPR (Huang et al., 2005). The final stages involve dephosphorylation of DPPR and epimerisation at the C-2 anomeric hydroxyl of the ribose moiety by Rv3790 (DprE1) forming the putative intermediate, decaprenylphosphoryl-2-keto-\(\beta\)-D-erythro-pentofuranose (DPK) (Mikusova et al., 2005). Subsequent reduction by Rv3791 (DprE2) results in the completed arabinose donor DPA. Studies on the enzymes involved in the complete pathway to D-arabinose await thorough biochemical characterisation.
Figure 1.12: Formation of arabinan precursor DPA. The carbon atoms of the Ara residues are derived from the pentose shunt pathway. α-D-ribose-5-phosphate (R5P) is then acted on by PrsA a pRpp synthetase, which catalyses the addition of a diphosphate moiety from ATP to the carbon-1-OH (Alderwick et al., 2010). The formation of decaprenylphosphoryl-D-arabinose (DPA) then proceeds with the transfer of ribose-5-phosphate from pRpp to decaprenylphosphate to form decaprenylphosphoryl-5-phosphoribose (DPPR). DPPR then undergoes dephosphorylation to decaprenol-1-monophosphoribose (DPR) and epimerisation of the ribosyl unit at carbon 2-OH position. DprE1 (Rv3790), a FAD-containing oxidoreductase is responsible for oxidising the ribosyl carbon-2-OH producing the keto sugar decaprenol-1-monophosphoryl-2-keto-β-erythro-pentofuranose (DPX). This is the reduced to DPA DprE2 (Rv3791, a decaprenylphosphoryl-2-keto-D-erythropentose reductase (Mikusova et al., 2005).
1.6.5.3. **Galactan biosynthesis**

The assortment of glycosyl linkages within the galactan moiety leads to the supposition that at least two to four GalT transferases (GalTs) are involved in its biosynthesis (Crick et al., 2001). It was predicted that one GalT could be specifically designed to recognise the LU (C$_{50}$-P-P-GlcNAc-Rha or GL2) catalysing the β(1→4) linkage allowing galactan formation to begin and then another to add the following GalT units. Indeed, Mikusova et al. (2000, 2006) recognised Rv3782 (GlfT1) as the putative GalT responsible for the initial transfer of possibly two GalT residues from UDP-GalT, forming C$_{50}$-P-P-GlcNAc-Rha-GalT (GL-3) and C$_{50}$-P-P-GlcNAc-Rha-GalT-GalT (GL-4) (Alderwick et al., 2008; Mikusova et al., 2006).

**Figure 1.13: Polymerisation of galactan - biosynthesis of GL-4.** The first two galactosyl sugars are added to the rhamosyl residue of the linker unit via GlfT1 (Rv3782) (Mikusova et al., 2006).
Rv3782 was implicated as a possible GalT due to the observations that there is significant sequence identity with portions of Rv3808c a known GalT (discussed below), which is classified as an inverting glycosyltransferase-2 (GT-2) of the GT-A superfamily. In addition, Rv3782 is also situated in the putative “AG biosynthetic gene cluster” (Mikusova et al., 2006). Kremer et al. (2001a) reported the first GalT with the identification of Rv3808c (GIFT1) through the use of a novel neoglycolipid acceptor assay, together with UDP-Galf and isolated E. coli membranes expressing the aforementioned gene (Kremer et al., 2001a). It was demonstrated that the enzyme has dual functionality, acting both as a UDP-Galf:β-D-(1→5) GalT and the UDP-Galf:β-D-(1→6) GalT, responsible for the polymerisation of approximately 30 Galf residues with alternating β(1→5) and β(1→6) linkages (Kremer et al., 2001; Rose et al., 2006).

**Figure 1.14: Polymerisation of galactan.** The bifunctional galactosylfuranosyl transferase GIFT2 (Rv3808c) adds the remaining alternating β(1→5) and β(1→6) galactosyl residues to GL-4.
1.6.5.4. Arabinan biosynthesis

1.6.5.4.1. Ethambutol inhibition and its use in the identification of the Emb proteins

The arabinan homopolymer is a pivotal structure in both AG and LAM, the two major cell wall polysaccharides. Accordingly, it is considered one of the most prominent mycobacterial structures being targeted by the frontline drug EMB ((S,S’)-2,2′(ethylenediimino)di-1-butanol). EMB is a synthetic compound that was first recognised as an anti-mycobacterial agent in 1961 (Thomas, 1961). Early work by Kilburn & Greenberg observed an unanticipated increase in viable cells during the initial four hours after addition of EMB to M. smegmatis cultures. It was postulated that large bacillary clusters disaggregated due to a possible reduction in lipid content, which would lead to the apparent increase in colony-forming units (CFU) (Kilburn & Greenberg, 1977). This theory was supported by Takayama and coworkers who conducted a series of early studies into the effects of the EMB on M. smegmatis, reporting that inhibition of mycolic acid transfer into the cell wall and the simultaneous accumulation of trehalose-monomycolate (TMM), trehalose-dimycolate (TDM), and free mycolic acids occurred within 15 minutes of drug administration (Kilburn & Takayama, 1981; Takayama et al., 1979). This suggested that the target might be a mycolytransferase responsible for the transfer of mycolic acids onto the arabinan polymer. However, it was later discovered that the earliest point of drug inhibition occurred during arabinan synthesis demonstrated by the immediate inhibition of incorporated label from [14C]-glucose into the cell wall D-arabinose monomers (Takayama & Kilburn, 1989), whilst synthesis of the galactan of AG remained unaffected (Mikusova et al., 1995). Furthermore, the arabinans of both AG and LAM were disturbed, although inhibition of label into the latter was less pronounced and at a later stage of its biosynthesis (Deng et al., 1995; Mikusova et al., 1995).
The generation of EMB-resistant M. smegmatis mutants greatly aided the discovery of the primary EMB target, implicating it as an arabinan specific inhibitor. This was illustrated by Mikusova et al., (1995), who subjected an EMB-resistant strain to sub-inhibitory levels of EMB (10µg/ml) showing that the resistant mutant produced “normal” cell wall AG but a truncated version of LAM due to arabinan inhibition. Extending this observation using higher concentrations of EMB, Khoo et al. (1996) reported that the degree of truncation in LAM was dose dependent and at higher concentrations, the arabinan of AG was also impaired. Collectively, these studies indicate that the effects of EMB on the synthesis of both arabinan moieties are uncoupled, and the time difference of inhibition implies that synthesis occurs via distinct pathways, involving multiple AraT targets with varying EMB sensitivities. A number of concurrent EMB studies provided evidence of an accumulation of DPA, the source of Ara residues in arabinan biosynthesis, confirming that EMB effects were not due to inhibition of Ara donor synthesis but rather its utilisation (Lee et al., 1995; Wolucka et al., 1994). Taken together, all the evidence points to arabinan polymerisation, specifically the arabinan of AG, as the primary target of EMB.

A major breakthrough in the discovery of the precise EMB cellular target arose through exploitation of a moderately resistant strain from the related M. avium species. The genomic library from the aforementioned strain was screened and over-expressed in an otherwise susceptible M. smegmatis host, leading to the identification of a resistance conferring region encompassing three complete open reading frames (ORFs), embR, embA and embB (Belanger et al., 1996). Moreover, use of an EMB-sensitive cell-free arabinan biosynthetic assay demonstrated that arabinosyltransferase activity was restored with embAB over-expression. Interestingly, neither embA or embB alone was sufficient to confer multi-copy resistance, thus supporting the supposition that they are translationally coupled forming a multienzyme complex (Belanger et al., 1996). EMB resistance was also used to identify the embCAB gene
cluster from *M. smegmatis*, which was subsequently characterised in *M. tuberculosis* and *M. leprae*, all of which possess the same syntenic organisation (Lety et al., 1997; Telenti et al., 1997) and encode homologues of the *embA* and *embB* genes from *M. avium*. Escuyer et al. (2001) created individual genetic knockouts in *M. smegmatis*, *embC*, *embA* and *embB*, all of which were viable, with the most profound affects observed in the *embB* mutant. Individual inactivation of *embA*, and *embB* resulted in the diminished incorporation of arabinose into AG, specifically, the terminal disaccharide $\beta$-D-raf - (1→2)-$\alpha$-D-raf, normally situated on the 3-OH of the 3,5-linked Araf residue.

Figure 1.15: Schematic presentation of arabinogalactan biosynthesis.
Chapter 1

Introduction

Thus, a substantial amount of the otherwise Ara₆ motifs were present as terminal linear Ara₄ structures akin to the terminal motif of LAM (Escuyer et al., 2001), also leading to a loss of cell wall bound mycolates. Based on the above observations, it appears that the Emb proteins could be involved in Ara₆ biosynthesis, with the authors suggesting that the EmbA/EmbB proteins could both act as α(1→3) AraTs. Indeed, Khasnobis et al. (2006), assayed for the putative activity of EmbA/EmbB, using cell-free preparations from wild-type M. smegmatis and demonstrated the formation of the non-reducing terminal disaccharide, which were absent in both embA and embB mutants. Moreover, the transferase activity was re-established upon mixing the membrane preparations from the disrupted strains with wild-type membranes.

1.6.5.4.2. EMB resistance

Identification of the emb gene cluster has provided the opportunity to analyse the molecular basis of resistance of mycobacteria to EMB. Telenti et al. (1997) demonstrated that high-level resistance to EMB in M. smegmatis could be related to either overproduction of the Emb protein(s), a structural mutation in a conserved region of EmbB, or both. A number of reports presented additional genetic evidence for a key role of the EmbB protein in cell wall biosynthesis highlighting the fact it is the most EMB-sensitive protein in the gene cluster (Alcaide et al., 1997). Mutations in EmbB have been recorded in up to 65% of EMB resistant clinical isolates of M. tuberculosis, with the majority of mutations present at codon 306 or in the immediate surrounding area (Lety et al., 1997; Ramaswamy et al., 2000). This region is highly conserved amongst mycobacteria and topological analysis of the Emb proteins (Telenti et al., 1997) positioned this EMB resistance-determining region (ERDR) in the second intracellular loop of EmbB (Sreevatsan et al., 1997b). Five distinct mutations have been recognised at codon 306, resulting in a substitution of the wild-type methionine with isoleucine, leucine or valine (Sreevatsan et al., 1997b). Other mutations have been identified
in the second intracellular loop region and the large-C terminal globular region of EmbB (Ramaswamy et al., 2000). It should be noted that there are a number of EMB resistant strains that do not possess ERDR mutations, thus other genes may be involved in EMB resistance.

1.6.5.4.3. Identification of novel arabinofuranosyltransferases and the use of C. glutamicum as a model organism

Efforts to generate viable embA/embB mutants in M. tuberculosis and an embAB double mutant in M. smegmatis have so far proven unfruitful, highlighting their essentiality (Mills et al., 2004; Pan et al., 2001; Vilcheze et al., 2000). The Corynebacterianeae taxon, as previously discussed, encompasses Mycobacterium species as well as Corynebacterium species, such as C. diphtheriae and C. glutamicum. The corynebacteria possess a comparable cell wall core and have previously been shown to serve as a useful model organism in the study of essential orthologous M. tuberculosis genes (Gande et al., 2004; Gibson et al., 2003; Radmacher et al., 2005). Centred on this observation, Alderwick et al. (2005) successfully constructed a C. glutamicum mutant with its singular emb gene disrupted (Cg-emb). Corynebacterium are deemed the archetype of Corynebacterianeae since they maintain a low frequency of gene duplications and modifications, thus it is reasonable that C. glutamicum possesses only one emb gene. Surprisingly, Cg-emb exhibited higher identity to embC, even though C. glutamicum lacks an elaborately arabinosylated LM product (Dover et al., 2004; Tatituri et al., 2007a). Chemical analyses of the tolerable Cg-emb deletion mutant revealed an almost total loss of cell wall arabinan, except terminal t-Araf residues decorating the galactan backbone (Alderwick et al., 2005). Moreover, EMB treatment of wild-type C. glutamicum produced an identical profile to that of the mutant, illustrating that Cg-emb is indeed the target of EMB and furthermore, there is another AraT responsible for “priming” the galactan backbone. Disruption of the decaprenyl transferase orthologue Cg-ubiA, involved in formation of the lipid-linked sugar donor DPA, resulted in total ablation of Araf
By virtue of DPA’s role as the only arabinose donor, it follows that all of the AraTs will be dependent on this polyprenyl-linked sugar. The Emb proteins, although novel, possess membrane topologies consistent with other glycosyltransferases (GTs) that use lipid-linked precursors, and do not resemble the more typical nucleotide-diphosphate (NDP) sugar donor requiring GTs. The Carbohydrate-Active enZymes (CAZy) have classified GTs into approximately 87 families with 3 large structural superfamilies, GT-A, GT-B and GT-C (Berg et al., 2007; Unligil and Rini, 2000; Liu and Mushegian, 2003). GT-A and GT-B are NDP-sugar utilising, predominantly soluble and peripheral membrane proteins. The GT-C superfamily embody polyprenyl-linked sugar donor dependent, integral membrane proteins, all of which contain 8-13 predicted transmembrane (TM) domains, with typically low sequence similarity, but a conserved amino acid motif, called the DxD motif, generally positioned in the first or second predicted extracytoplasmic loop. The modified DxD motif (e.g., DxE, ExD, DDx, DEx, or EEx) lies upstream of a conserved proline motif, both of which are located in the same predicted loop and have been so called the “GT-C motif” (Berg et al., 2007). The actual mechanism is unknown but this motif may be responsible for the binding of a lipid-linked sugar donor and/or catalytic activity. The Emb proteins have been classified as GT-Cs, comprising approximately 1100 amino acids and 12-13 TM spanning regions (Berg et al., 2005). Considering the other putative AraTs will utilise the same substrate, it follows that they will share these structural similarities.
1.6.5.4.4. Identification and functional role of AftA

The identity of the novel “priming” AraT, arabinofuranosyltransferase AftA (Rv3792), which is responsible for priming the 8th, 10th and 12th Gal\(\alpha(1\rightarrow5)\)-linked Ara\(\alpha\) units, was addressed by Alderwick et al. (2006b).

![Diagram](image)

**Figure 1.16: Early arabinogalactan formation.** The biosynthesis of the arabinan of AG is initiated by AftA (Rv3792), an integral membrane protein responsible for priming the decaprenyl-PP-galactan for further elaboration by arabinosyltransferases (Alderwick et al., 2005). The Ara\(\alpha\) residues derived from DPA are affixed to the carbon-5 hydroxyl of the 8th, 10th and 12th \(\beta\)-(1\→\6) linked Gal\(\beta\) residues of the galactan chain.

Genome comparisons of the Corynebacterianeae emb locus revealed a highly conserved gene situated after the DPPR epimerising enzymes and adjacent to embC. Although AftA proteins show no significant sequence identity to the Emb proteins, they do possess a notably similar domain organisation and localisation to that of EmbC, with an array of conserved amino acids comparable to the GT-C motif. Contrary to the Emb proteins, AftA only encodes for 643 amino acids with 11 predicted TM domains; however, it is proposed to contain an Emb analogous C-terminal region directed towards the periplasm. AftA is essential for M. tuberculosis (Sassetti et al., 2003); hence its orthologue was successfully disrupted in C.
glutamicum producing a cell wall lacking Ara units (Alderwick et al., 2005). Furthermore, E. coli membranes expressing M. tuberculosis AftA exhibited AraT activity, specifically transferring arabinose from DPA to a galactan acceptor, which was shown to be EMB resistant (Alderwick et al., 2006b).

1.6.6. Mycolic acids

Mycolic acids and their homologs have been defined as high molecular weight α-alkyl, β-hydroxy fatty acids (Asselineau & Lederer, 1950). These fatty acids differ in length across species, consisting of 70-90 carbons in mycobacteria (Goodfellow & Minnikin, 1981; Lechevalier et al., 1986) and 22-38 carbons in their corynebacterial counterparts, the corynomycolic acids. In M. tuberculosis, these long chain fatty acids are extremely hydrophobic, containing a meromycolate chain (up to C₅₆) with a saturated α-side chain (C₂₄-C₂₆). Studies have shown that there are three distinct structural categories of mycolic acids, depending on the chemical modifications present in the meromycolate chain. These include the α-mycolates, which are the most abundant form, containing no oxygenated functional groups in the meromycolate branch and two cyclopropane rings that are in the cis configuration (Minnikin et al., 2002; Qureshi et al., 1978; Yuan et al., 1995). Ketomycolates and methoxymycolates on the other hand, are oxygenated species with only one cyclopropane ring in the cis and trans configuration, respectively (George et al., 1995). The cyclopropane rings contribute to the structural integrity of the cell wall complex (George et al., 1995), and protect the bacillus from oxidative stress by hydrogen peroxide (Yuan et al., 1995). Deletion of the methoxy- and ketomycolates (Dubnau et al., 2000) and removal of the proximal cyclopropane ring of the α-mycolic acids (Glickman et al., 2000) leads to considerable attenuation of virulence in mouse models. Thus, the fine structure of mycolic acids is coupled to the virulence of M. tuberculosis.
1.6.6.1. Biosynthesis of mycolic acids

The fatty acid synthase-I (FAS-I) and fatty acid synthase-II (FAS-II) pathways are the two systems responsible for the biosynthesis of the mycolic acids (Bloch, 1977; Bloch & Vance, 1977). FAS-I is a multifunctional polypeptide encoded by the gene $fas$ ($Rv2524c$) (Smith et al., 2003). This single polypeptide contains all the functional domains required for the generation of short chain acyl-Coenzyme A (acyl-CoA) primers, which are either fed into the FAS-II pathway or become the C$_{26}$ α-alkyl branch of the mycolic acids. FAS-I begins de novo fatty acid synthesis by utilising acetyl-CoA and malonyl-CoA, elongating the acetyl group by two carbons in each cycle (Heath & Rock, 2002). These substrates are only available for the FAS-I pathway upon activation via a thioester linkage to the prosthetic group of Coenzyme A. The intermediates remain associated to the polypeptide during the complete process; transacylation passes them from one active site of one domain to the active site of the next in the following sequence: acyltransferase, enoyl reductase, dehydratase, malonyl/palmitoyl transferase, acyl carrier protein (ACP), β-ketoacylreductase and β-keto synthase (Smith et al., 2003).
The FAS-II system is not capable of de novo fatty acid synthesis, thus relying on FAS-I for its acyl-CoA substrate. An additional FAS-II substrate is malonyl-AcpM, an activated primer (Kremer et al., 2001; Schaeffer et al., 2001). Malonyl-AcpM is synthesised by the malonyl-CoA:AcpM transacylase mtFabD from malonyl-CoA and phosphopantothenylated holo-AcpM. AcpM has the vital role of steering acyl intermediates between enzymes, initially shuttling malonyl-AcpM to an acyl-CoA primer derived from the FAS-I pathway, allowing the two substrates to undergo a condensation reaction catalysed by mtFabH (Choi et al., 2000). The AcpM-acyl precursor, β-ketoacyl-ACP is then reduced by MabA/FabG1 to β-hydroxyacyl-AcpM (Banerjee et al., 1998; Marrakchi et al., 2002), which is subsequently dehydrated by a β-hydroxyacyl-AcpM dehydratase (Rv0636) (Sacco et al., 2007; Brown et al., 2007). InhA, an enoyl-AcpM reductase; the target of the INH, performs the enoyl-reduction, resulting in an AcpM-bound acyl chain, which is now two carbon units longer through the complete enzyme cycle. The cycle is then repeated several times, except for the substitution of mtFabH with the β-ketoacyl-AcpM synthases, KasA (Rv2245) or KasB (Rv2246), which catalyse the condensation of the acyl-AcpM and the new malonyl-AcpM, further elongating the chain by two carbon units (Figure 1.15) (Bhatt et al., 2005; Bhatt et al., 2007; Gao et al., 2003; Kremer et al., 2002a; Schaeffer et al., 2001b; Slayden & Barry, 2002).

The final step in the synthesis of the mycolic acids was proposed by Walker et al. (1973) using a C. diptheriae model, and later in M. tuberculosis by Takayama & Qureshi (1975). The proposed Claisen-type condensation of C_{26}-S-CoA and meromycolyl-AMP has been described as the final step carried out by Pks13, the type I polyketide synthase family protein. Prior to the participation of Pks13, each meromycolyl-S-AcpM from the FAS-II pathway are converted into meromycolyl-AMP by FadD32 (Triverdi et al., 2004), a specific fatty acyl-AMP ligase.
Chapter 1

Introduction

Figure 1.18: Mycolic acid biosynthesis in M. tuberculosis (Adapted from Bhowruth et al., 2008). mtFabD converts malonyl-CoA to malonyl-AcpM, which is then ligated by mtFabH to FAS-I derived C14-CoA. The FAS-II system (KasB/A, MabA, InhA, and Rv0636) continues to process the C16 acyl-AcpM product producing mercomycolates (C56). The meromycolic acid precursors are ligated to a FAS-I synthesized C26 fatty acid that constitutes the α-branch of the final mycolic acid. Mycolic acids are then formed via the condensation of the α-branch and the meromycolate by polyketide synthase Pks13.

The C26-S-CoA released from FAS-I is carboxylated by AccD4 (Rv3799c) and AccD5 (Rv3280), the acyl-CoA carboxylases, yielding 2-carboxyl-C26-CoA. This is followed by Pks13 thioester binding of the two substrates, C52-meromyceryl and 2-carboxyl-C26-acyl group to the N-terminus and C-terminus PPB domains, respectively (Takayama et al., 2005). Subsequent reactions occur generating the mature C78-mycolate (Lea-Smith et al., 2007) (as
shown in Figure 1.18). A series of mycolyltransferases are believed to be involved in the transfer of mycolic acids to their outer cell wall position. Mycolates are also present in free, solvent-extractable trehalose conjugates TMM and TDM (Asselineau & Lederer, 1950; Minnikin, 1982; Minnikin et al., 2002), of which TMM is present both intra- and extracellularly, leading to the possible implication that it is responsible for the transport of extracellular mycolates and mycolyl components of the mAGP (Takayama et al., 2005). TMM, a key precursor for the biosynthesis of TDM is dephosphorylated and translocated across the membrane, whereby three mycolyltransferases Ag85A, Ag85b and Ag85c synthesise TDM and transfer mycolic acids to the arabinan termini (Belisle et al., 1997; Takayama et al., 2005).

![Figure 1.19: Structure of TDM](image)

1.6.7. Structural features of lipoarabinomannan (LAM) and related biosynthetic precursors (LM and PIMs)

The mycobacterial cell wall's core framework, the mAGP complex, provides a template for the insertion of an abundance of mannosylated molecules, such as lipoarabinomannan (LAM) and its structurally related glycolipids, lipomannan (LM) and phosphatidylinositol mannosides (PIMs) (Besra et al., 1997; Brennan & Ballou, 1967; Brennan & Ballou, 1968b; Brennan & Nikaido, 1995; Hill & Ballou, 1966; Morita et al., 2004). These highly complex immunomodulatory lipoglycans are found ubiquitously in the envelopes of all mycobacterial
species, non-covalently associated to the plasma membrane and/or the mycolic acid layer via a conserved phosphatidyl-myoinositol (PI) anchor (Hunter & Brennan, 1990), which extends to the exterior of the cell wall (Besra & Brennan, 1997; Belanger & Iamine, 2000; Nigou et al., 2003). LAM is composed of numerous structural domains, the aforementioned PI anchor, as well as a polysaccharidic backbone consisting of D-mannan and D-arabinan and several distinct capping motifs.

1.6.7.1. Phosphatidyl-myoinositol mannosides (PIMs)

The PI linker is based on a sn-glycero-3-phospho-(1-D-myoinositol) unit, whereby, the glycerol phosphate component is attached to the L-1-position of myo-inositol (Ballou et al., 1963; Ballou & Lee, 1964) and mannopyranosyl (Manp) residues decorate positions C-2 and C-6 of the inositol ring, constituting phosphatidylinositol dimannoside (PIM\(_2\)). This anchor shows a high degree of heterogeneity with regard to its multiple acylation states, with four potential sites, two of which are present on the glycerol unit, one on the Manp unit linked to C-2 of myo-inositol and the fourth at the C-3 position of myo-inositol (Brennan & Ballou, 1968; Khoo et al., 1995). A\(_c\)\(_1\)PIM\(_2\) and A\(_c\)\(_1\)PIM\(_6\) are the major PIM species, existing as a diacylglycerol with a further fatty acid attached to the Manp residue at C-2 of myo-inositol. A\(_c\)\(_2\)PIM\(_2\) and A\(_c\)\(_2\)PIM\(_6\) also exist, but A\(_c\)\(_1\)PIM\(_2\) is believed to be the preferred precursor upon which higher PIM\(_{3-6}\)s are built, as well as LAM, and its arabinose-free counterpart LM (Chatterjee et al., 1992a).

1.6.7.2. Characterisation of LM and LAM

The mannan of LM and LAM is an extension of the PIMs, containing on average 20-30 residues, emanating from the C-6 position of the inositol ring (Chatterjee et al., 1991; Khoo et al., 1996). This \(\alpha(1\rightarrow6)\)-linked mannan backbone is adorned with single Manp sugars at
C-2 of the occasional α(1→6)-linked mannose in all mycobacterial species examined, with the exception of M. chelonae, which contains C-3-linked branching (Guerardel et al., 2002).

**Figure 1.20: Current structural model of mycobacterial LAM.** The MPI anchor is linked to a mannann backbone with an α(1→6)Man core branched frequently at the 2-position with a single α-Man. Attached to the mannann core is an arabinan domain (approximately 50–70 residues in total). The point of attachment of arabinan to the mannann has not been elucidated. The non-reducing termini of the arabinan are capped with species specific sugars (Lee et al., 2005).
L M is further glycosylated with an arabinan domain similar to, but more variable than, that in A G. Shi et al. (2006) identified the occurrence of an Ara_{18-22} motif in M. smegmatis that resembled the internal structure of the arabinan of A G, however, the terminal extensions at the non-reducing end varied. The basic structure of the polymer consists of a linear α(1→5)-linked arabinosylfuranosyl backbone with branched hexa-Araf (Ara_6) and linear tetra-Araf (Ara_4) terminal moieties (Chatterjee et al., 1991; 1993), equating to between 50-80 Araf residues (Khoo et al., 1996).

1.6.7.3. Characterisation of LAM capping

Mycobacterial species differ in the nature and extent of the capping motifs modifying the nonreducing termini of the arabinan chains, specifically, the β(1→2)-linked terminal Araf units. To date, three structural families have been recognised, mannose capped LAM (Man-LAM), PI capped LAM (PI-LAM) and non capped LAM (Ara-LAM), of which “Man-caps” are an important feature of the LAM of pathogenic species or slow growing mycobacteria, such as M. tuberculosis, M. bovis, M. bovis BCG, M. leprae, M. avium, M. xenopi, M. marinum, and M. kansasii. The caps may be present as single Manp capping residues, di or tri-mannosides, of which di-mannosides predominate. (Delmas et al., 1997; Guerardel et al., 2003). Fast growing mycobacteria such as M. smegmatis and M. fortuitum possess PI caps, and M. chelonae is the only known example of Ara-LAM.
Figure 1.21: Structural model of mycobacterial ManLAM, PILAM and AraLAM highlighting the different capping motifs found in all mycobacterial LAM. The non-reducing termini of arabinan are decorated with species specific sugars; 1–3 mannose units in M. tuberculosis and M. bovis, phosphatidylinositol in M. smegmatis (Nigou et al., 2003).
1.6.7.4. **Biosynthesis of PIMs, LM and LAM**

1.6.7.4.1. **Synthesis of precursors**

1.6.7.4.2. **GDP-Man biosynthesis**

Mannose is a key component in numerous cell wall and intracellular molecules present in mycobacteria, including the manno lipids PIMs, LM and LAM and has been shown to be essential for the growth and viability of *M. smegmatis* (Patterson et al., 2003). Mycobacteria can obtain mannose via two distinct pathways (Figure 1.21). The first relies on phosphorylation by a hexokinase (Rv2702) to produce mannose-6-phosphate from exogenously obtained mannose (Hsieh et al., 1996). The second is carried out by ManA (Rv3255c), a phosphomannose isomerase (PMI), which synthesises mannose-6-phosphate by converting fructose-6-phosphate obtained from the glycolytic pathway. Patterson et al. (2003), demonstrated the essentiality of PMI in vitro in an *M. smegmatis* mutant, which was unable to synthesise mannose-containing molecules in the absence of an extracellular source of mannose (Patterson et al., 2003). Mannose-6-phosphate is subsequently converted into mannose-1-phosphate by a phosphomannomutase (PMM) encoded by manB (Rv3257c) (McCarthy et al., 2005). Indeed, accumulation of PIMs, LM, and LAM was observed in a *M. smegmatis* strain overexpressing *M. tuberculosis* ManB, which intimates its role in the biosynthesis of these manno lipids (McCarthy et al., 2005). Finally, mannose-1-phosphate is modified by GDP-mannose pyrophosphorylase, ManC (Rv3264c) to GDP-Man, the nucleotide sugar donor of mannose (Ma et al., 2001; Ning & Elbein, 1999).
Takayama & Goldman (1970) presented initial reports characterising mannolipids potentially involved in mannan biosynthesis. They identified a C<sub>50</sub>-polyisoprenyl based mannolipid (C<sub>50</sub>-P-Man) in M. tuberculosis, followed by the characterisation of a M. smegmatis specific alkali stable, C<sub>35</sub>-octahydrooctaprenyl-phospho-mannose (C<sub>35</sub>-P-Man) (Takayama & Goldman, 1970). It was later demonstrated by Besra et al. (1997) that Ac<sub>1</sub>PIM<sub>2</sub> is specifically extended by the addition of Manp residues from the alkali-stable sugar donor, polyisoprenol-monophosphomannose (PPM), to form higher PIM<sub>2</sub>s and linear LM. Gurcha et al. (2002) identified a polyisoprenol monophosphomannose synthase, Rv2051 (Ppm1) from M. tuberculosis by means of a combined genomics and biochemical approach, derived from similarities to the known eukaryotic dolichol monophosphomannose (DPM) synthases.
(Gibson et al., 2003; Gurcha et al., 2002). It was discovered that PPM is generated from GDP-Man and the corresponding polyprenol phosphate via Ppm1. Therefore the PPM synthase is a key enzyme in the generation of PPM, which is subsequently utilised in the biosynthesis of LM and LAM, thus making it a potential drug target.

1.6.7.4.4. **Synthesis of PI**

Phosphatidyl-myoinositol (PI) is the component upon which PIMs, LM and LAM are built and is the linkage to the plasma membrane. The initial step in the synthesis of PI is the phosphorylation of diacylglycerol (DAG) by a DAG Kinase (Rv2252) to form phosphatidic acid (Owens et al., 2006; Salman et al., 1999). A CDP-DAG synthase (Rv2881c), activates phosphatidic acid with CTP forming cytidine diphosphate-diacylglycerol (CDP-DAG) (Nigou & Besra, 2002b), which later reacts with myo-inositol forming PI. Recently, the gene encoding the PI synthase (Rv2612c) was identified and shown to be essential in M. tuberculosis (Jackson et al., 2000).

1.6.7.5. **Biosynthesis of PIMs**

The currently accepted model for PIM biogenesis follows a linear pathway PI → PIM₂ → PIM₄ → PIM₆ (Figure 1.23). In M. tuberculosis PimA (Rv2601) catalyses the glycosylation of PI by the transference of Manp from GDP-Manp to the 2-position of PI forming PIM₁ (Kordulakova et al., 2002), which is subsequently acylated by Rv2611c at the 6-position (Kordulakova et al., 2003). A Rv2611c mutant of M. smegmatis exhibited severe growth defects and accumulation of non-acylated PIM₁ and PIM₂. Furthermore, in a cell-free assay utilising membrane preparations from M. smegmatis, overexpression of Rv2611c increased the incorporation of [¹⁴C]-palmitate into PIMs (Kordulakova et al., 2003). Both of these enzymes are part of a conserved gene cluster of six ORFs in an operon, which is present in all members of Corynebacterineae (Cole & Barrell, 1998; Cole et al., 1998).
Figure 1.23: Pictorial depiction of PIM, LM and LAM biosynthesis. The three first mannosylations of PI in biosynthesis of PIMs involve the GDP-Man dependent PimA, PimB, and PimC, which are believed to occur on the cytoplasmic face of the plasma membrane. Rv2611c is responsible for the acylation of PIM1. PIM3 or PIM4 is translocated across the bilayer by an unidentified flippase, where it then acts as a substrate for PimE towards biosynthesis of the polar PIM6 and as a precursor in the formation of LM. The ManT(s) involved in the synthesis of the mannan backbone of LM (MptA and MptB) are believed to be C50-P-M an dependent, as was suggested for Rv2181, the α1,2-ManT responsible for the LM branching. LAM is generated via the further elaboration of LM with Ara f units forming an arabinan domain. As yet, only the EmbC protein has been recognised, but the exact activity remains to be ascertained.

PimB (Rv0557) was originally proposed as an α-D-mannose-α(1→6)-phosphatidyl-myo-
inositol-mannopyranosyltransferase (PimB) responsible for the formation of Ac$_1$PIM$_2$ through the transference of mannose from GDP-Man to Ac$_1$PIM$_1$ (Schaeffer et al., 1999). However, it was later found that disruption of pimB in M. tuberculosis did not affect the biogenesis of PIMs (Torrelles et al., 2009) suggesting that either complementary activities existed in the form of gene duplications or PimB is responsible for another function (Kremer et al., 2002a). Recent investigations have identified Rv2188c, now termed PimB’, as the protein involved in the second mannosylation step responsible for Ac$_1$PIM$_2$ formation (Lea-Smith et al., 2008; Mishra et al., 2008b; Mishra et al., 2009). Indeed, work carried out by Tatituri et al. (2007) demonstrated PimB’s role in the biosynthesis of a novel mannolipid, ManGlcA GroAc$_2$ and a LM-like molecule in C. glutamicum, and has since been renamed MgtA (Tatituri et al., 2007b). Ac$_1$PIM$_2$ can remain unmodified, accumulating as an end product or be acylated to Ac$_2$PIM$_2$ and/or mannosylated forming the higher PIMs (Ac$_1$PIM$_3$-Ac$_1$PIM$_6$/Ac$_2$PIM$_3$-Ac$_2$PIM$_6$), or LM and LAM. Research using cell free extracts has identified the mannosyltransferase PimC from M. tuberculosis strain CDC1551 implicating it as the ManT responsible for Ac$_1$PIM$_3$/Ac$_2$PIM$_3$ formation from Ac$_1$PIM$_2$/Ac$_2$PIM$_2$ (Kremer et al., 2002a). However, a M. bovis BCG pimC deletion mutant continued to produce normal levels of PIMs/LM/LAM implying redundancy of genes or compensatory pathways (Kremer et al., 2002b). In fact, there is no strong homologue of pimC in M. tuberculosis H$_{37}$Rv or M. smegmatis, thus it remains to be identified along with the ManT accountable for α(1→6) mannosylation of Ac$_1$/Ac$_2$PIM$_3$ yielding Ac$_1$/Ac$_2$PIM$_4$. This product is believed to be an intermediate at the branch point at which polar PIMs and LM/LAM biosynthesis diverges (Mishra et al., 2008a; Morita et al., 2004; Morita et al., 2006). It is proposed that up until this branch point, mannose residues have been provided by the nucleotide-derived sugar substrate GDP-Man, characterising the earlier ManTs as members of the GT-A/B superfamily of glycosyltransferases. It has been hypothesised that Ac$_1$/Ac$_2$PIM$_4$ is translocated across the plasma membrane, where it becomes available for Rv1159 (PimE), the recently identified
\(\alpha(1\rightarrow2)\)-mannopyranosyltransferase which utilises the polyprenylphosphate sugars \(\text{PPM} / \text{C}_{50}\)-P-Man as a substrate, adding an \(\alpha(1\rightarrow2)\)-Manp residue resulting in the synthesis of Ac\(_1/Ac_2\)PIM\(_5\) (Morita et al., 2006). This transition to the GT-C superfamily of glycosyltransferases, which utilise polyprenylphosphate sugars (Liu & Mushegian, 2003), has been supported by a number of inhibition studies in which amphomycin, an inhibitor of the mannose donor \(\text{PPM} / \text{C}_{50}\)-P-Man, was shown to halt the synthesis of PIM\(_4\)-PIM\(_6\) (Besra et al., 1997; Morita et al., 2006) and effect elongation and branching of LM and LAM (Morita et al., 2006). The biosynthesis of the final product Ac\(_2/Ac_2\)PIM\(_6\) has not been deduced, although the involvement of PimE cannot be ruled out.
Figure 1.24 Biogenesis of PIM, LM and LAM. A schematic representation of the current understanding of the PIM, LM and LAM biosynthetic pathways in M. tuberculosis.
1.6.7.6. Biosynthesis of LM and LAM

1.6.7.6.1. The role of MptA, MptB and Rv2181

The mannan of LM/LAM is believed to be an extension of the PIMs, specifically Ac1Ac2PIM4, and is composed of a linear $\alpha(1\rightarrow6)$ mannan backbone, punctuated occasionally at C-2 with single $\alpha(1\rightarrow2)$-linked mannoses, resulting in a mannan of approximately 25-30 residues (Figure 1.24) (Chatterjee et al., 1991; Khoo et al., 1996). Mishra et al. (2007, 2008a) recently identified two $\alpha(1\rightarrow6)$ mannopyranosyltransferases, mannopyranosyltransferase A and B, named MptA and MptB, using C. glutamicum as a model organism. It was elucidated that MptA (NCgl2093 and M. tuberculosis homolog, Rv2174) are concerned with the biosynthesis of the distal end of the $\alpha(1\rightarrow6)$ mannan backbone of LM (Mishra et al., 2007; Kaur et al., 2007) and MptB (Rv1459c) is involved in the synthesis of the proximal end of the mannan backbone. A reduction in $\alpha(1\rightarrow6)$-mannopyranosyltransferase activity, with complete loss of LM and LAM, was observed in a MptB-deletion mutant in C. glutamicum. Further evidence from cell free C. glutamicum assays utilising C$_{50}$-P-Man and expressing Rv1459c and/or its M. smegmatis homologue MSMEG_3120 showed that these enzymes possess $\alpha(1\rightarrow6)$-mannopyranosyltransferase activity. However, M. tuberculosis and M. smegmatis MptB proteins are unable to complement the C. glutamicumΔmptB mutant, suggesting substrate specificity differences. It is proposed that MptB catalyses the addition of further Manp residues (12-15 sugars) to Ac1PIM4, which has been transported across the plasma membrane by an as yet unidentified flippase (Mishra et al., 2008a).

Kaur et al. (2006) recently identified the putative integral membrane proteins, MSMEG4250 in M. smegmatis and Rv2181 in M. tuberculosis as potential polyprenol-dependent glycosyltransferases based on shared characteristics with previously identified enzymes. A knock-out of MSMEG4250 in M. smegmatis possessed a truncated version of LAM with a
decrease in the number of α(1→2)-Manp branching residues and altered growth with an inability to synthesise LM. Complementation of the mutant with the corresponding ortholog of M. tuberculosis (Rv2181) restored normal LM/LAM synthesis. However, regulation of LM and LAM biosynthesis in M. smegmatis appears to differ somewhat with M. tuberculosis, as M. tuberculosisΔRv2181 produced truncated versions of LM and Man-LAM (Kaur et al., 2008).

Table 1.3: Genes involved in biosynthesis of LAM and related glycoconjugates (Mishra et al., 2009)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
<th>Role</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>PgsA (Rv2612c)</td>
<td>PL synthase</td>
<td>PL synthase</td>
<td>Jackson et al. 2000</td>
</tr>
<tr>
<td>PimA (Rv2610c)</td>
<td>Synthesis of PIM1</td>
<td>α(1→2)-Mannopyranosyltransferase</td>
<td>Kordulakova et al. 2003</td>
</tr>
<tr>
<td>Rv2611c</td>
<td>Synthesis of Ac1/Ac2PIM1</td>
<td>Acyl transferase</td>
<td>Kordulakova et al. 2003</td>
</tr>
<tr>
<td>PimB (Rv2188c)</td>
<td>Synthesis of Ac1/Ac2PIM2</td>
<td>α(1→6)-Mannopyranosyltransferase</td>
<td>Lea-Smith et al. 2008; Mishra et al. 2008; 2009</td>
</tr>
<tr>
<td>MgtA (Rv0557)</td>
<td>Synthesis of GI-X and Ac1/ Ac2PIM2</td>
<td>α(1→6)-Mannopyranosyltransferase</td>
<td>Tatituri et al. 2007a; Mishra et al. 2008</td>
</tr>
<tr>
<td>PimC (RvD2-ORF1)</td>
<td>Synthesis of Ac1/ Ac2 PIM5</td>
<td>α(1→2)-Mannopyranosyltransferase</td>
<td>Kremer et al., 2002</td>
</tr>
<tr>
<td>PimE (Rv1159)</td>
<td>Synthesis of Ac1/ Ac2 PIM5</td>
<td>α(1→2)-Mannopyranosyltransferase</td>
<td>Morita et al., 2006</td>
</tr>
<tr>
<td>MptB (Rv1459c)</td>
<td>Synthesis of proximal mannan backbone i.e. Ac1/ Ac2PIM 12-17</td>
<td>α(1→6)-Mannopyranosyltransferase</td>
<td>Mishra et al., 2008</td>
</tr>
<tr>
<td>MptA (Rv2174)</td>
<td>Synthesis of distal mannan backbone i.e. Ac1/ Ac2PIM 22-25</td>
<td>α(1→6)-Mannopyranosyltransferase</td>
<td>Mishra et al., 2007.</td>
</tr>
<tr>
<td>MptC (Rv2181)</td>
<td>Adds α(1→2)-Manp units on mannan backbone, and also adds second mannose cap on ManLAM</td>
<td>α(1→2)-Mannopyranosyltransferase</td>
<td>Kaur et al., 2008; 2010</td>
</tr>
<tr>
<td>EmbC (Rv3793)</td>
<td>Involved in the synthesis of α(1→5)-arabinan backbone</td>
<td>α(1→5)-Arabinofuranosyltransferase</td>
<td>Zhang et al., 2003</td>
</tr>
<tr>
<td>AfcC (Rv2673)</td>
<td>Adds Araf on α(1→5)-arabinan backbone in α(3→5)-direction</td>
<td>α(1→3)-Arabinofuranosyltransferase</td>
<td>Birch et al., 2010</td>
</tr>
<tr>
<td>AfdD (Rv0236c)</td>
<td>Either adds α(1→3)-Araf units to the non-reducing end of α(1→5)- arabinan branch or synthesises α(1→3) or α(1→5)-Arabinofuranosyltransferase</td>
<td>α(1→3)- or α(1→5)-Arabinofuranosyltransferase</td>
<td>Skovierova et al., 2009</td>
</tr>
<tr>
<td>CapA (Rv1635c)</td>
<td>Adds first mannose cap on ManLAM</td>
<td>α(1→5)-Mannopyranosyltransferase</td>
<td>Appelmelk et al., 2008</td>
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1.6.7.7. **Arabinan biosynthesis in lipoarabinomannan**

1.6.7.7.1. **The role of EmbC**

LAM is generated via the further elaboration of LM with Araf units forming an arabinan domain akin to that found in AG (Besra et al., 1997). The linkage pattern of Araf residues and the Ara6 non-reducing end are also present in AG (as discussed in section 1.6.5.1); however, the arabinans also differ in a number of ways. Firstly, LAM also contains a linear tetra-arabinoside (Ara4) as well as the Ara6 motif, with a major difference being that the non-reducing end of the LAM arabinan is substituted with α-mannosyl residues rather than with mycolic acids (Chatterjee et al., 1992b). The arabinan structure is also more variable in LAM, with a recent study suggesting that the precise Ara$_{38}$ arrangement is not always present, but rather chains of differing lengths extend from the interior 3,5-Araf residues (Shi et al., 2006). It is theorised that at least five distinct arabinofuranosyltransferases are required for the generation of mature LAM, based on the linkages present; as yet only the EmbC protein has been recognised, but the exact activity remains to be ascertained (Zhang et al., 2003). Although EmbC is an essential enzyme in M. tuberculosis (Goude et al., 2008), inactivation of the gene in M. smegmatis produced a viable mutant, with lack of arabinosylation in LAM but AG remained largely unaffected (Escuyer et al., 2001; Zhang et al., 2003). This knockout strain did possess two to three single Araf residues secured to the mannan backbone, suggesting the existence of an additional AraT, analogous to the priming enzyme AftA (Zhang et al., 2003). However, despite extensive analyses, the arabinan chain attachment sites have not been defined (Chatterjee & Khoo, 1998).

The EmbC protein is predicted to contain 13 transmembrane helices and a modified DxD motif, which is expected to play a role in carbohydrate binding and is thus classified as a member of the GT-C super-family of integral membrane glycosyltransferases (Berg et al., 2005). The characteristic GT-C motif of EmbC may be responsible for the chain elongation
of $\alpha(1\rightarrow5)$-Araf residues, where the arabinan units are linked to each other creating 3,5-$\alpha$-Araf branches on 5-linked chains or by transferring the residues directly on to the mannan backbone (Berg et al. 2005). Thus, embC inhibition appears to be parallel to the emb mediated inhibition of arabinosylation of galactan in corynebacterial AG, conferring the $\alpha(1\rightarrow5)$ transferase activity. Point mutations introduced into the conserved proline motif proximal to the large carboxyl-terminal globular region, resulted in the biosynthesis of shorter arabinan domains thus this region seems to control chain length extension of the arabinan domain of LAM (Shi et al., 2006).

1.6.7.8. Mannose - capping of LAM

The arabinan domain of LAM is further modified by the addition of $\alpha$-mannosyl residues, resulting in capping with either one, two or three Manp residues. The total extent of capping is about 70%, with a di-mannoside cap being the most abundant (Chatterjee et al., 1992; Chatterjee et al., 1993). It is predicted that synthesis would require at least two ManTs, one that should recognise the arabinan domain and thus add the primary $\alpha(1\rightarrow5)$-mannose and a further ManT for the elongation of this mannose with a second $\alpha(1\rightarrow2)$-mannose. Mannose-capped LAM (Man-LAM) is a feature of all pathogenic strains of the Mycobacterium genus and is, hence, responsible for some of the immuno-modulatory properties of these strains (Briken et al., 2004). Subtractive genomics of the M. tuberculosis genome, with those species of the genus that do not contain mannose-capped LAM, such as M. smegmatis, highlighted the GT-C enzyme Rv1635c (Dinadayala et al., 2006). A transposon mutant strain of M. tuberculosis CDC1551 with an ineffective copy of MT1671 (Rv1635c), did indeed produce LAM devoid of Manp capping (Dinadayala et al., 2006). Moreover, an M. smegmatis strain expressing MT1671 resulted in a hybrid LAM, possessing single mannose caps. Further studies supported this finding, with Rv1635c mutants in M. marinum and M. bovis BCG showing that Rv1635c encoded for a mannoypyranyosyltransferase involved in the addition of
the first Manp residue on the non-reducing arabinan termini of LAM (Appelmelk et al., 2008). More recently, Kaur et al. (2008) have shown that Rv2181c possesses varied substrate specificity, capable of adding $\alpha(1\rightarrow2)$-Manp residues onto the mannan backbone, as well as the non-reducing end of LAM in combination with Rv1635c (Kaur et al., 2008).

### 1.6.7.9. Immunomodulatory properties of LAM & LM

LAM and related lipoglycans are not only essential for mycobacterial growth and survival (Haites et al., 2005; Kovacevic et al., 2006), but have also been implicated in a broad spectrum of immunomodulatory activities. During the last decade, investigators have studied LAM and LM isolated from a multitude of mycobacterial organisms with various capping motifs, hoping to gain insight into the nature of these host-pathogen interactions and the importance of these complex heteropolysaccharides in pathogenesis (Dao et al., 2004; Garton et al., 2002; Gibson et al., 2003; Gibson et al., 2004; Gibson et al., 2005; Maeda et al., 2003; Quesniaux et al., 2004a, 2004b).

The ability of mycobacteria to persist in host tissues is central to the disease. One strategy for survival is phagosomal maturation inhibition (Armstrong & Hart, 1971; Nguyen & Pieters, 2005; Russell, 2001); another is the suppression of the cell-mediated host immune response after infection. Several studies using purified LAM have highlighted a major role for this lipoglycan in both of these phenomena.

#### 1.6.7.9.1. Phagosome maturation arrest

ManLAM has been implicated in inhibition of phagosomal maturation (Jo, 2008; Quesniaux et al., 2004a) and infection induced apoptosis (Rojas et al., 2000). Man-LAM blocks the increase of macrophage cytosolic $\text{Ca}^{2+}$ that normally occurs upon infection, thereby inhibiting $\text{Ca}^{2+}$/calmodulin-dependent PI3-kinase hVPS34 (Chua and Deretic, 2004; Malik et
PI3-Kinase is necessary for the production of PI3-phosphate on the phagosomal membrane, which is subsequently involved in the recruitment of the Rab5 effector EEA1 to the early endosome. This is required for the delivery of lysosomal components from the trans-Golgi network (TGN) to the phagosome and regulation of fusion with vesicles of the endosomal-lysosomal pathway, as discussed in section 1.3 (Simonsen et al., 1999; Vergne et al., 2003). ManLAM is able to suppress cytosolic Ca\textsuperscript{2+}-increase and therefore restrict generation of PI3-phosphate (Fratti et al., 2003; Vergne et al., 2003b). Indeed, Fratti et al. (2001) reported that ManLAM-coated beads behaved similarly to M. bovis BCG LAM, in that they resided in early endosomes that failed to recruit EEA1. Thus, M. tuberculosis ManLAM may contribute to the maturation arrest of bacillus-containing phagosomes by virtue of its ability to attenuate hVPS34 and to inhibit EEA1 recruitment. The inhibition of Ca\textsuperscript{2+} increase appears to be specific for ManLAM, as LAM, lacking terminal mannose capping, from non-pathogenic Mycobacterium does not affect Ca\textsuperscript{2+} levels (Vergne et al., 2003). At present, the mechanism by which ManLAM alters Ca\textsuperscript{2+} fluxes remains to be established (figure 1.25).

It has also been reported that p38 mitogen-activated protein kinase (p38 MAPK) contributes to phagosome maturation arrest in a way distinct from the inhibition of cytosolic Ca\textsuperscript{2+} increase. ManLAM has been implicated as a trigger for an increase in p38 MAPK activation, which contributes to phagosome maturation arrest through modulation of EEA1 recruitment (Fratti et al., 2003) and downstream trafficking events. However, Welin et al. (2008) recently published contrasting results demonstrating that ManLAM did not effect p38 MAPK activation.
Chapter 1

Introduction

Figure 1.25: The role of ManLAM and PIMs in phagosome maturation arrest. Fusion with early endosomes required for nutrient acquisition is promoted by PIMs. ManLAM inhibits lysosomal fusion and acidification by inhibiting cytosolic-Ca$^{2+}$ increase and thereby blocks the successive steps of hVPS34 kinase activity at the phagosomal membrane, the recruitment of Rab5, EEA1 and Syn6 to the phagosome, and the delivery of cathepsins and VoH$^+$ ATPase. TGN = trans-Golgi network, CaM = calmodulin, PI(3P) = phosphatidylinositol (3-phosphate), Syn = syntaxin, EEA1 = early endosome autoantigen.

Another process of maturation arrest involves the incorporation of ManLAM, via its GPI anchor, into so-called lipid rafts or membrane rafts of the macrophage cell membrane. These are cholesterol and glycosphingolipid-rich domains that act as docking sites for cell signalling processes (Welin et al., 2008). The insertion of ManLAM is reported to reorganise the phagosomal membrane in such a way as to disrupt access to the molecules essential for maturation (Hayakawa et al., 2007). The overall effect is that the mycobacteria can survive in their self-created phagosomal niche.

In contrast to ManLAM, its biosynthetic precursor LM does not affect phagolysosomal development (Kang et al., 2005; Vergne et al., 2004b). PIMs, however, have been found to specifically stimulate early endosomal fusion through incorporation into the membrane rafts.
and thus competitively inhibiting insertion of LAM (Welin et al., 2008). Therefore PIM generates a bypass mechanism allowing endosome fusion necessary for nutrient delivery to the bacilli residing in the phagosome (Kelley and Schorey, 2003; Vergne et al., 2004b). Torrelles et al. (2006), reported that the MR has a high affinity for higher-order PIMs (PIM_5 and PIM_6) while lower-order PIMs (PIM_2) are not recognised. Thus, although ManLAM and PIMs influence the phagosome maturation by distinct mechanisms, both may involve ligation to the mannose receptor (MR) (Vergne et al., 2004b).

1.6.7.9.2. Lipoglycan interaction with the host cell

While LM is mainly associated with toll-like receptor (TLR)-signalling, the higher-order PIMs and ManLAM are recognised by the C-type lectins DC-SIGN and the macrophage MR (Tailleux et al., 2005). Both C-type lectins show comparable recognition specificities for the mannosylated glycolipids, and, as receptors CD14 and pulmonary surfactant protein (SP)-A and SP-D also bind ManLAM (Ernst, 1998; Pugin et al., 1994; Sidobre et al., 2000; Torrelles et al., 2008), interactions of mycobacteria with all these pattern recognition receptors may enhance or dampen inflammatory signals and thereby determine the nature of the immune response. Anti-inflammatory signaling, via the interaction of the glycolipids with the host immune system, can be regarded as strategies of mycobacteria to escape immune surveillance (Torrelles & Schlesinger, 2010), but may be also vital in prevention of an exaggerated inflammatory response (Jo, 2008).

1.6.7.9.3. LAM and the Mannose receptor

The ManLAM-mediated phagosome maturation delay is dependent on the MR present on the macrophage membrane. Indeed, Kang et al. (2005) demonstrated the reversal of delayed phagolysosome fusion by use of a MR-blocking antibody alongside LAM-coated
microspheres. Further supporting evidence was presented by Ferguson et al. (2006), demonstrating that coating M. tuberculosis with the mannose cap binding C-type lectin surfactant protein D (SP-D), increases phagolysosome fusion in infected macrophages. Given that the macrophage MR only recognises the mannose-capped ManLAM and not AraLAM or PILAM (Schlesinger et al., 1994), this is further evidence that ligation to the macrophage MR is required for the phagosome maturation block, which appears to be restricted to the more virulent Mycobacterium species. This receptor is distinguished by the fact that it mediates the engulfment of microbes without necessarily inciting a proinflammatory response and thereby has long been postulated to enhance early intracellular survival of the microbe. This receptor has also been implicated in intracellular trafficking of LAM to the late endosomal compartments for loading onto CD1b molecules for LAM presentation to T cells (Prigozy et al., 1997). Some species of PIMs expressed by M. tuberculosis have been shown to engage the MR and illicit an MR-dependent delay in phagolysosome fusion (Torelles et al., 2006), whereas PILAM and AraLAM did not effect maturation (Kang et al., 2005).

1.6.7.9.4. Modulation of DC-SIGN activity

Immature DCs are seeded throughout peripheral tissues to act as sentinels against invading pathogens. Indeed, immature DCs internalise M. tuberculosis derived LAMs and present these structures via the CD1b-presentation pathway to LAM specific T cells. DC-SIGN has a high affinity for mannose-containing carbohydrates. It was demonstrated by Geijtenbeek et al. (2003), that mycobacteria specifically target DC-SIGN through ManLAM to impair DC maturation and to induce production of the anti-inflammatory cytokine IL-10. These conditions promote immunosuppression.

DC-SIGN only recognises LAM if it is mannose-capped (Geijtenbeek et al., 2003; Maeda et al., 2003) and it has been shown to have a strong affinity for increasing chain lengths of
α(1→2)-linked mannosyl residues (Koppel et al., 2004). Unsurprisingly, PIM5 and PIM6 are recognized by DC-SIGN with a higher affinity than lower order PIMs, most likely due to terminal α(1→2)-linked mannosyl residues similar to the mannose cap of ManLAM (Boonyarattanakalin et al., 2008; Driessen et al., 2009). On the other hand, a BCG mutant producing a mannose-cap devoid LAM still bound DC-SIGN to the same extent as wild-type BCG (Appelmelk et al., 2008); thus, other DC-SIGN ligands must be present in the mycobacterial cell envelope.

ManLAM binding to LPS-activated DCs increases the secretion of pro-inflammatory IL-12 and IL-6 as well as IL-10 (Gringhuis et al., 2009); therefore, this suggests that DC-SIGN binding may function primarily in the protection of the host (Ehlers, 2009).

1.6.7.9.5. Toll-like receptors

PILAM and LM stimulate innate immunity via signaling through TLR2, which differs from that of ManLAM. TLR2 signalling has been demonstrated for PI-anchored mannosylated lipoglycans; however, it is dependent on their degree of acylation and mannosylation (Doz et al., 2007; Gilleron et al., 2006; Nigou et al., 2008). For instance, lipoglycan-induced signaling occurs via the TLR1/TLR2-heterodimer complex, which recognizes tri-acylated lipoglycans (Elass et al., 2005; Gilleron et al., 2006; Nigou et al., 2008). The degree of mannosylation also affects the ability of the lipoglycan to activate TLR2, with a higher level of activation accompanying an increasing length of mannan chain (Nigou et al., 2008). LM presents the largest accessible mannan chain, since it does not possess a masking arabinan domain; it did indeed show increased TLR2-signalling (Quesniaux et al., 2004b), although this activity is restricted to the tri- and tetra-acylated forms (Ac1/Ac2-LM) (Doz et al., 2007; Gilleron et al., 2006). Ac1/Ac2PIM2, PILAM and AraLAM have been shown to be poor inducers of TLR2-signalling in comparison to LM and Ac1/Ac2PIM6 (Nigou et al., 2008), highlighting the need for a large unmasked mannan chain. Vignal et al. (2003) also presented
supporting evidence, showing that in contrast to LM, neither AraLAM from M. chelonae nor ManLAM and Ac1/Ac2PIM2 from M. kansasii mediate a TLR2-dependent activation (Vignal et al., 2003). Moreover, chemical degradation of the arabinan domain of ManLAM from M. kansasii restored its ability to induce cytokine secretion via TLR2, which suggests that the arabinan domain prevents proper interaction of ManLAM with TLR2 (Vignal et al., 2003). This was confirmed in this thesis (Chapter 3) in which LAM containing a truncated arabinan domain from a M. smegmatis AftC knock-out mutant, showed enhanced TLR2-signalling as compared to wild-type LAM (Birch et al., 2010). Overall, the data indicate that LM, and in a minor respect PIM6, are the only significant TLR2-ligands from this group of mycobacterial lipoglycans.
1.7. Project aims

Mycobacterial diseases are especially problematic to treat owing to the unique lipid-rich cell wall architecture. The cell wall is essential for growth and survival and the majority of front-line drugs target its biosynthesis (Zhang, 2005). In particular, EMB and INH inhibit biosynthesis of arabinan and mycolic acids, respectively. Nonetheless, the global problem of TB has been worsened in recent years by the emergence of MDR- and XDR-TB cases, leading to a necessity to discover novel drug targets and specific antimicrobial compounds against them (Sreevatsan et al., 1997b; Telenti et al., 1997). In this regard, the biosynthetic machinery of the mycobacterial cell wall and associated lipoglycans represent attractive targets (Bhatt et al., 2007b; Bhowruth et al., 2007; Brennan & Crick, 2007; Dover et al., 2008).

The structural basis of the arabinan of both AG and LAM is now well defined (Besra et al., 1995; Daffé et al., 1990; McNeil et al., 1990), conversely, aspects of its biosynthesis remained poorly resolved. Upon commencing this study, only EmbA, EmbB and the newly identified AftA were implicated in AG arabinan biosynthesis and only EmbC as the AraT involved in LAM biosynthesis (Alderwick et al., 2006; Mikusova et al., 1995; Zhang et al., 2003). Given that the arabinan domains utilise several different Araf linkages clearly suggests that additional AraTs must be required to form a fully matured arabinan. All of these recognised AraTs are classified as members of the GT-C superfamily of integral membrane proteins. Liu and Mushegian (2003) identified members of this superfamily, representing potential candidates involved in the biosynthesis of cell wall related components. The major hindrance for the study of these genes, adopting a “reverse-genetics” approach, is the essentiality of arabinan in M. tuberculosis. For instance, a direct knock out embA, embB or embC resulted in non-viable mutants of M. tuberculosis. Furthermore, the study of M. tuberculosis has posed a formidable challenge as a result of its long generation time,
fastidious growth requirements, and high risk of contagion. M. smegmatis and C. glutamicum share similar genomic organization and cell wall biosynthetic machinery, and are nonpathogenic, fast-growing organisms that can tolerate deletion of some cell wall biosynthetic genes that are essential in M. tuberculosis (Alderwick et al., 2005b; Alderwick et al., 2006c; Gande et al., 2004). The aim of this thesis was to study the direct effects of Corynebacteriaceae glycosyltransferases which represent the orthologous genes and enzymes of M. tuberculosis, in the model organisms C. glutamicum and M. smegmatis. More specifically:

- Bioinformatic investigation of the published Corynebacterineae genomes using comparative genomics to identify putative glycosyltransferases involved in cell wall AG and LAM biosynthesis.

- Deletion of selected genes from M. smegmatis and C. glutamicum that encode putative GT-C glycosyltransferases involved in AG and LAM biosynthesis.

- Subsequent phenotypic characterization of strains deleted of putative GT-C glycosyltransferase encoding genes which includes
  
  - Carbohydrate and lipid chemical compositional analysis of purified cell wall material.
  
  - Biochemical investigation into consequential effects of AraT activity in isolated membranes using a range of neoglycolipid acceptor analogues.
Chapter 2
2. Identification and characterisation of \textit{AftB} and \textit{AftC} arabinotransferases involved in arabinogalactan biosynthesis

2.1. Introduction

TB is currently the leading cause of mortality from a single infectious agent, responsible for 1.8 million fatalities annually, as well as latently infecting a third of the world’s population. Mycobacterial diseases, such as TB and leprosy, consequently still represent a global health problem (Gupta et al., 2001). For instance, the recent emergence of MDR-TB strains and, more recently, XDR-TB clinical isolates (Singh et al., 2007; Zignol et al., 2006), has prompted the need for new drugs and drug targets. The causative agent of these diseases, \textit{M. tuberculosis} and \textit{M. leprae}, respectively, are members of the \textit{Corynebacterianeae}, a group of atypical Gram-positive bacteria characterised by an intricate cell envelope (Besra et al., 1995; McNeil et al., 1990; McNeil et al., 1991).

This distinctive mycobacterial cell envelope is composed of three macromolecules, LAM, mycolyl-AG and PG (Besra et al., 1995; Chatterjee et al., 1991; McNeil et al., 1990; McNeil et al., 1991). The galactan domain of AG is linked to PG via a specialised “linker unit”, L-Rhap-(1→4)-\(\alpha\)-D-GlcNAc, and its distal arabinan domain to mycolic acids, forming mAGP complex (Besra et al., 1995; McNeil et al., 1990; McNeil et al., 1991). The arabinan domain contains \(\alpha(1\rightarrow5)\), \(\alpha(1\rightarrow3)\) and \(\beta(1\rightarrow2)\) Araf linkages, arranged in several distinct structural motifs (Alderwick et al., 2005b; Besra et al., 1995; Daffé et al., 1990). The non-reducing arabinan termini of AG consists of t-Araf, 2-Araf, 5-Araf, and 3,5-Araf residues arranged into a characteristic terminal Ara\(_6\) motif, with the 5-OH of the t-Araf and 2-Araf residues representing sites of mycolylation (McNeil et al., 1991). The packing and ordering of mycolic acids within the mAGP and additional lipids within the outer envelope results in a highly impermeable barrier (Minnikin et al., 2002), which is essential for bacterial survival.
It is interesting to note that several front-line anti-tubercular drugs, such as ethambutol (EMB) (Belanger et al., 1996; Takayama & Kilburn, 1989; Telenti et al., 1997) and isoniazid (INH) (Banerjee et al., 1994; Winder & Collins, 1970), target aspects of the biosynthesis of the mA GP complex.

The structural basis of AG is now well defined (Besra et al., 1995; Daffé et al., 1990; McNeil et al., 1990), conversely, aspects of its biosynthesis remain poorly resolved. The biosynthesis of AG involves the formation of a linear galactan chain, with alternating $\beta(1\rightarrow5)$ and $\beta(1\rightarrow6)$-$D$-galactofuranosyl (Gal$\f$) residues of approximately 30 residues in length, from the specialised ‘linker unit’, $L$-Rhap-(1$\rightarrow4$)-$\alpha$-$D$-GlcNAc (Kremer et al., 2001; Mikusova et al., 2000), attaching the AG to the PG via the C-6 of some of the MurNGly residues. MALDI-TOF MS analyses of per-O-methylated AG of C. glutamicum, deleted of its single arabinofuranosyltransferase Cg-emb, revealed that the 8th, 10th and 12th Galf residue possessed singular Araf residues (Alderwick et al., 2005b). These specific Araf residues were recently shown to be transferred by a specialised arabinofuranosyltransferase AftA, whose gene in all Corynebacterianeae analysed to date is adjacent to the emb cluster (Alderwick et al., 2006c). These initial Araf residues “prime” the galactan backbone for further attachment of $\alpha(1\rightarrow5)$ linked Araf residues. These reactions require the arabinofuranosyltransferase activities of Mt-EmbA and Mt-EmbB, or Cg-Emb, respectively; these are also targets of EMB (Alderwick et al., 2005b; Radmacher et al., 2005; Telenti et al., 1997), which eventually result in mature AG.

Disruption of either embA or embB, in M. smegmatis, produced an impairment of the terminal Ara6 motif, resulting in a linear terminal motif (Escuyer et al., 2001). The Emb and AftA proteins utilise the specialised sugar donor, $\beta$-$D$-arabinofuranosyl-1-monophosphoryl-

69
decaprenol (DPA) (Lee et al., 1995b; Lee et al., 1997; Wolucka et al., 1994b), and is a characteristic feature found only in Corynebacterianeae (Alderwick et al., 2006a; Huang et al., 2005; Mikusova et al., 2005). In addition, these proteins also belong to the GT-C superfamily of integral membrane glycosyltransferases (Liu & Mushegian, 2003). A recent topological analysis of Cg-Emb (Seidel et al., 2007c), together with a mutational study of Mt-EmbC (Berg et al., 2005), revealed for the first time a clear domain organisation of these proteins. The glycosyltransferase DDX signature is evident in the extracellular loop which connects helixes III-IV and the chain elongation “Pro-motif”, is in the extracellular loop connecting helixes XIII-XIV (Berg et al., 2005).

It is interesting to note that the arabinan domain of AG utilises several different Ara linkages, which suggests that additional arabinofuranosyltransferases must be required to form a fully matured AG. Moreover, initial Ara residues at branching sites could require specialised arabinofuranosyltransferases, as already observed for AftA (Alderwick et al., 2006c). Clearly additional arabinofuranosyltransferases still remain to be identified in Corynebacterianeae. Liu and Mushegian (2003) identified fifteen members of the GT-C superfamily, representing candidates involved in the biosynthesis of cell wall related glycans and lipoglycans in M. tuberculosis. In order to study the direct effects of Corynebacteriaceae arabinofuranosyltransferases, we have attempted to identify and delete genes, which represent the orthologous genes and enzymes of M. tuberculosis, in the model organisms C. glutamicum and M. smegmatis. The study of M. tuberculosis has posed a formidable challenge as a result of its long generation time, fastidious growth requirements and high risk of contagion. M. smegmatis and C. glutamicum, on the other hand, are non-pathogenic, fast-growing organisms that can tolerate deletion of some cell wall biosynthetic genes that are essential in M. tuberculosis (Alderwick et al., 2005b; Alderwick et al., 2006c; Gande et al., 2004). Herein, we present the identification of two novel arabinofuranosyltransferases of the
GT-C superfamily, arabinofuranosyltransferase B (AftB) and arabinofuranosyltransferase C (AftC). The latter is responsible for the transfer of Ara residues from DPA to the arabinan domain to form $\alpha(1\rightarrow3)$-linked Ara residues, which result in the branched arabinan domain distal to the non-reducing terminal Ara motif characteristic of mycobacterial AG. AftB is responsible for the transfer of Ara residues from DPA to the arabinan domain to form terminal $\beta(1\rightarrow2)$ linked Ara residues, which marks the “end-point” for AG arabinan biosynthesis before decoration with mycolic acids.
2.2. Results

2.2.1. Genome comparison of the aftB locus

With the recent identification of AftA as a novel arabinofuranosyltransferase present in Corynebacterianeae (Alderwick et al., 2006c), and based on the fact that it is present in a highly conserved cell wall locus (Alderwick et al., 2006c), we concentrated our studies to identify other cell wall related genes. Subsequently, we identified Rv3805c (Figure 2.1A), which is located in close proximity to the antigen 85 complex-encoding genes fbpA and fbpD (Belisle et al., 1997). Furthermore, Rv3805c is likely to form an operon together with ubiA, which is required for prenyl transfer to 5-phosphoribose pyrophosphate (PRPP) to form decaprenylphosphoryl-5-phosphoribose (DPPR), before conversion to DPA (Huang et al., 2005; Mikusova et al., 2005), and glfT, which is responsible for establishing the galactan backbone of AG (Kremer et al., 2001; Mikusova et al., 2000). The apparent fundamental function of aftB is indicated by the fact that the genome organisation of this particular region is syntenic in Corynebacterianeae, including all Mycobacterium and Corynebacterium species analysed to date (Figure 2.1A, B) and also in Norcardia farcinica IFM 10152 and Rhodococcus sp. RHA1.

The gene product of Rv3805c, termed AftB, is predicted to form nine transmembrane (TM) spanning helixes, in its amino-terminal part, whereas a 237 amino acid carboxy-terminal part is directed towards the periplasm (Figure 2.1C). Interestingly, AftB shows no obvious sequence similarity to the previously identified arabinofuranosyltransferases, such as Emb (Alderwick et al., 2005b) and AftA (Alderwick et al., 2006c), although the topology, with the C-terminus directed towards the periplasmic side, is to some degree comparable. However, the intrageneric similarity of the AftB proteins is very high, even for the most distant pairs, M. tuberculosis and C. diphtheriae, exhibiting 33% identity over the entire length of
Figure 2.1: Comparison of the aftB locus within Corynebacteriaceae. A) The locus consists in M. tuberculosis (M. tub.) of aftB with the upstream located ubiA gene product catalysing prenylation of 5-phosphoribose pyrophosphate (PRPP) (Huang et al., 2005), and glfT (Kremer et al., 2001) and UDP-Galp mutase enzyme glf (Pan et al., 2001). Downstream of aftB the genes fbpA, and fbpD are located which encode mycolyltransferases (Belisle et al., 1997). The organisation of these genes is largely retained in a number of Corynebacteriaceae indicative for a basic functional unit. In N. farcinica (N. far.), a third paralogous mycolyltransferase is present, and in C. glutamicum (C. glu.) a transposon is inserted between the two mycolyltransferases. Orthologous genes are shaded accordingly. M. bovis (M. bov.), M. avium paratuberculosis (M. av. p.), M. leprae (M. lep.), Rhodococcus sp. RHA1 (R. spe.), C. efficiens (C. eff.), and C. diphtheriae (C. dip.). B) Partial sequence comparison of the first loop region of AftB. The conserved charged residues possibly involved in glycosyltransferase activity are shaded in grey, and the adjacent aspartate residues possibly directly involved in glycosyl transfer are in white on black background (Liu & Mushegian, 2003). On top are the predicted structural properties of the peptide with E indicating β-sheet, and H α-helix structure. C) Topology of Mt-AftB based on dense alignment surface (DAS) analysis (Cserzo et al., 1997). The membrane spanning helixes are given in Roman numbers, and their amino acyl residues in Arabic.
the proteins. Even stronger conservation is found in the first periplasmic loop region (Figure 2.1B), exhibiting a modified motif of the GT-C superfamily of glycosyltransferases consisting of two adjacent aspartic acid residues (Liu & Mushegian, 2003). Also, the periplasmic loop regions following helix V and VII are strongly conserved, which may play a role in presenting the nascent arabinose domain to the catalytic glycosyltransferase site. Taken together, the features of AftB and the locus where the gene is localised suggests that it represents a glycosyltransferase involved in AG biosynthesis.

2.2.2. Construction of C. glutamicum ΔaftB

In an attempt to delete aftB in C. glutamicum the non-replicative plasmid pK19mobsacB ΔaftB was constructed carrying sequences adjacent to Cg-aftB. The vector was introduced into C. glutamicum and in several electroporation assays kanamycin resistant clones were obtained, indicating integration of pK19mobsacB ΔaftB into the genome by homologous recombination (Figure 2.2A). The sacB gene enables for positive selection of a second homologous recombination event, which can result either in the original wild-type genomic organisation or in clones deleted of aftB (Schafer et al., 1994). Forty-eight clones exhibiting the desired phenotype of vector-loss (Kan^S, Suc^R) were analysed by PCR and twenty-one of them were found to have Cg-aftB excised. These numbers indicate that the loss of Cg-aftB is apparently not a serious disadvantage for viability, in contrast with Cg-aftA, where deletion was rather difficult to obtain (Alderwick et al., 2006c). As a result, one clone was subsequently termed C. glutamicum ΔaftB and confirmed by PCR to have Cg-aftB deleted, whereas controls with C. glutamicum wild type and genes adjacent to Cg-aftB resulted in the expected amplification products (Figure 2.2A).
Figure 2.2: Construction and characteristics of \textit{C. glutamicum}\textsuperscript{ΔaftB}. A) Genomic illustration of Cg-aftB with its adjacent genes \textit{ubiA} and \textit{cmt2}, which is the orthologue of mycobacterial \textit{fbpA}, and the strategy to delete Cg-aftB using the deletion vector pK19mobsacB\textsuperscript{ΔaftB}. This vector carries 18 nucleotides of the 5´-end of Cg-aftB and 36 nucleotides of its 3´-end thereby enabling the in-frame deletion of almost the entire Cg-aftB gene. The arrows marked P2 locate the primers used for the PCR analysis to confirm the absence of Cg-aftB. Primers P1 were used to detect \textit{ubiA}, and P3 to detect \textit{cmt2}. Distances are not drawn to scale. The results of the PCR analysis are shown on the right, where the results obtained with the corresponding primer pairs are marked accordingly. Samples were applied pair wise with the amplification products obtained from the wild type applied in the left lane, and that of the deletion mutant in the right lane. St marks the standard, where the arrowheads are located at 10, 3, 2, 1, and 0.5 kb, respectively. B) Phenotype of \textit{C. glutamicum}\textsuperscript{ΔaftB} cells spread on BHI medium and incubated for 3 days. On the left is shown wild type \textit{C. glutamicum} (Cg-WT) and on the right the deletion mutant \textit{C. glutamicum}\textsuperscript{ΔaftB} (Cg-ΔaftB). The picture shows an area of about 1.5 cm square.

2.2.3. \textit{In vitro} growth analysis of \textit{C. glutamicum}\textsuperscript{ΔaftB}

Growth of wild type \textit{C. glutamicum} and \textit{C. glutamicum}\textsuperscript{ΔaftB} were compared in BHI medium as well as salt medium CGXII (Eggeling & Bott, 2005). Both strains exhibited comparable growth rates and the final cell densities reached were comparable. Single colonies of the deletion mutant appeared less glossy. In streak-outs on BHI plates, the surface of the deletion mutant appeared rough with a coarsely granular surface, as compared to wild type \textit{C. glutamicum} (Figure 2.2B). Taken together \textit{C. glutamicum}\textsuperscript{ΔaftB} possesses only a slight growth defect, under the conditions assayed, indicating a degree of tolerance to the deletion of Cg-aftB. Complementation of \textit{C. glutamicum}\textsuperscript{ΔaftB} with either pMSX-Cg-aftB or pMSX-M\textit{t-aftB} restored the mutant to a wild type phenotype. For the purpose of significance, C.
Chapter 2

glutamicumΔaftB complemented with M t-aftB was used throughout this investigation to study the corresponding mutant phenotype; however, similar results were also obtained with C. glutamicumΔaftB complemented with Cg-aftB.

2.2.4. Analysis of cell wall associated lipids and bound corynomycolic acid

The initial qualitative investigations involved the analysis of cell wall associated lipids and release of bound corynomycolic acids as corynomycolic acid methyl esters (CMAMEs) following by TLC analysis. Analysis of free lipids from other previously identified cell wall mutants, such as C. glutamicumΔemb (Alderwick et al., 2005b) and C. glutamicumΔaftA (Alderwick et al., 2006c), highlighted an apparent increase in trehalose monocorynomycolate (TMCM) indicating a defect in cell wall biosynthesis. This phenotype was also consistently observed for the aftB deletion mutant (Figure 2.3). Quantitative free lipid analysis used [14C]-acetate labeled cultures and equal loading of radioactivity based on the extractable free lipids for C. glutamicum, C. glutamicumΔaftB and the complemented C. glutamicumΔaftB pM SX-M t-aftB strains. Typically, C. glutamicum exhibited the known free lipid profile for wild type C. glutamicum, including phospholipids (3945 cpm), TMCM (3217 cpm), trehalose dicorynomycolate (TDCM) (8619 cpm) and non-polar lipids migrating at the solvent front (8753 cpm) (Figure 2.3, lane 1). In contrast, following equivalent loading of radioactivity and quantitative analysis by phospoimager analyses, C. glutamicumΔaftB possessed an approximate significant three-fold increase in TMCM (10185 cpm) and a decrease in TDCM (6539 cpm), phospholipids (1275 cpm) and non-polar lipids (5439 cpm) (Figure 2.3, lane 2). Complementation of C. glutamicumΔaftB with pM SX-M t-aftB, reverted the deletion mutant back to a phenotype similar to the wild type, TMCM (3331 cpm), TDCM (9123 cpm), phospholipids (4011 cpm) and non-polar lipids (8901 cpm) (Figure 2.3, lane 3).
Chapter 2

Figure 2.3: Quantitative analysis of extractable $^{14}$C lipids from C. glutamicum, C. glutamicumΔaftB and C. glutamicumΔaftB pM SX-M t-aftB. Lipids were extracted from cells by a series of organic washes as described in "Materials and methods". An aliquot (25,000 cpm) from each strain was subjected to TLC using silica gel plates (5735 silica gel 60F254, Merck) developed in CHCl$_3$/CH$_3$OH/H$_2$O (60:16:2, v/v/v) and either charred using 5% molybdophosphoric acid in ethanol at 100°C to reveal the extracted lipids and compared to known standards (Alderwick et al., 2005; Gande et al., 2004) or quantified using a phosphorimager following exposure to Kodak X-Omat film for 24 h. The TLC-autoradiogram is representative of 3 independent experiments. Lane 1, C. glutamicum; lane 2, C. glutamicumΔaftB; and lane 3, C. glutamicumΔaftB pM SX-M t-aftB.

To relate the above growth phenotypic changes of C. glutamicumΔaftB to its cellular composition, C. glutamicumΔaftB and C. glutamicumΔaftB pM SX-M t-aftB, along with wild type C. glutamicum, were analysed for arabinogalactan esterified corynomycolic acids released from the above $^{14}$C-delipidated cells. As expected, the wild type exhibited a typical profile of CMAMEs (Figure 2.4, lane 1, 28562 cpm), whereas these products were significantly reduced in C. glutamicumΔaftB (Figure 2.4, lane 2, 8947 cpm). In addition, complementation of C. glutamicumΔaftB with pM SX-M t-aftB (Fig. 2.4, lane 3, 27523 cpm) led to the restoration of normal ‘levels’ of cell wall bound corynomycolic acids. These results suggested that Mt-t-aftB was involved in a key aspect of arabinan biosynthesis, whereby
deletion perturbs tethering of corynomycolic acids to AG, but not as severely as in C. glutamicumΔemb and C. glutamicumΔaftA mutants (Alderwick et al., 2005b; Alderwick et al., 2006c).

2.2.5. Cell wall glycosyl compositional and linkage analysis of cell walls

Alditol acetate derivatives of highly purified mAGP from C. glutamicum, C. glutamicumΔaftB and C. glutamicumΔaftB pM SX-M t-aftB were prepared for glycosyl
compositional analysis. All strains exhibited a similar Ara:Gal ratio of 3.7:1. However, glycosyl linkage analysis of per-O-methylated alditol acetate derivatives of mA GP extracted from these strains highlighted an obvious difference in linkage profiles (Figure 2.5).

All glycosyl linkages could be accounted for in wild type \textit{C. glutamicum} (Figure 2.5A) as described previously (Alderwick et al., 2005b; Alderwick et al., 2006c), however, mA GP from \textit{C. glutamicum}\text{Δ}aftB was devoid of $\beta(1\rightarrow2)$ Ara linkages (Figure 2.5B). Complementation of \textit{C. glutamicum}\text{Δ}aftB with pMSX-Mt-aftB restored the $\beta(1\rightarrow2)$ Ara linkage thus reverting the deletion mutant to a wild type phenotype (Figure 2.5C). Further to this, we analysed the cell wall glycosyl composition of \textit{C. glutamicum}\text{Δ}aftB complemented with either pMSX-Mt-aftB-D29A or pMSX-Mt-aftB-D30A. Each of these complemented stains exhibited a phenotype identical to that of \textit{C. glutamicum}\text{Δ}aftB, with a complete loss of $2$-Ara linkages.

As confirmed by SDS-PAGE (Figure 2.6) the Mt-AftB muteins are synthesised in vivo and the failure to establish the $\beta(1\rightarrow2)$ Ara linkage is therefore most likely due to a catalytically inactive AftB, thus highlighting the importance of these particular aspartic acid residues in enzyme function.
Figure 2.5: Glycosyl linkage analysis of cell walls of \textit{C. glutamicum} (A), \textit{C. glutamicum}\textsuperscript{\Delta aftB} (B), \textit{C. glutamicum}\textsuperscript{\Delta aftB} pM SX-M\textsuperscript{t-aftB} (C). Cell walls were per-O-methylated, hydrolysed using 2M TFA, reduced and per-O-acetylated. The resulting partially per-O-methylated, per-O-acetylated glycosyl derivatives were analysed by GC/MS as described previously (Alderwick et al. 2005; Besra et al., 1995; Daffé et al., 1990).
Figure 2.6. Formation of Mt-AftB in C. glutamicum. Extracts of C. glutamicum ΔaftB expressing His-tagged M. tuberculosis AftB and AftB muteins were subjected to Ni²⁺-NTA chromatography and analysed by SDS-PAGE. Lane 1, 20 µg of clarified extract of C. glutamicum ΔaftB pM SX-Mt-aftB prior to chromatography. Lane 2-5 received the entire protein isolated from a 2 L culture of the respective recombinant strain via Ni²⁺-NTA chromatography, which was approximately 20 µg in each case. Lane 2 C. glutamicum ΔaftB pM SX-Mt-aftB; lane 3, C. glutamicum ΔaftB pM SX-Mt-aftB-D29A; lane 4, C. glutamicum ΔaftB pM SX-Mt-aftB-D30A; and lane 5, C. glutamicum ΔaftB pM SX (control). Standards (Std) along with their molecular weights in kDa are shown. The expected molecular weight for Mt-AftB is 72 kDa and the faint band at this location in lanes 2-4 is shown by an arrow and was verified by peptide mass fingerprinting as M. tuberculosis AftB.


We assessed the capacity of membrane preparations from C. glutamicum, C. glutamicum ΔaftB and C. glutamicum ΔaftB, complemented with pM SX-Mt-aftB, to catalyse arabinofuranosyltransferase activity in the presence of an exogenous synthetic α-D-Araf-(1→5)-α-D-Araf-0-C8, neoglycolipid acceptor (Lee et al., 1997) and DP[¹⁴C]A (Lee et al., 1998). TLC analysis of the products, when assayed with wild type C. glutamicum membranes, resulted in the formation of two products (A and B) (Figure 2.7A) when analysed by TLC (Figure 2.7B). The enzymatic synthesis of products A and B are consistent with previous studies (Lee et al., 1997) using mycobacterial membrane preparations resulting in trisaccharide products as a result of the addition of α(1→5) and β(1→2) linked Araf residues to the disaccharide acceptor (Figure 2.7A) (Lee et al., 1997). Addition of EMB in several experiments, even at high concentrations of up to 1 mg/ml to the reaction mixture, resulted in the complete loss of only product A. However, when assays were performed using...
Figure 2.7: Arabinofuranosyltransferase activity in membranes prepared from C. glutamicum, C. glutamicumΔaftB and C. glutamicumΔaftB pMSX-Mt-aftB. A, Biosynthetic reaction scheme of products formed in arabinofuranosyltransferase assays using α-D-Araf-(1→5)-α-D-Araf-O-C₈. B, Arabinofuranosyltransferase activity was determined using the synthetic α-D-Araf-(1→5)-α-D-Araf-O-C₈ acceptor in a cell-free assay as described previously (Lee et al., 1997). The products of the assay were resuspended prior to scintillation counting and subjected to TLC using silica gel plates (5735 silica gel 60F₂₅₄, Merck) in CHCl₃:CH₃OH:H₂O:NH₄OH (65/25/3.6/0.5, v/v/v/v) with the reaction products visualised by autoradiography.
Membranes, prepared from C. glutamicum\(\Delta\)aftB, only a single band migrating to a position akin to that of product A could be observed and no product formation could be identified upon the addition of 100 \(\mu\)g/ml of EMB (Figure 2.7B). Membranes prepared from C. glutamicum\(\Delta\)aftB, complemented with pMSX-Mt-aftB, restored product A and B formation back to that of the wild type (Figure 2.7B) and only product B was synthesised when EMB (up to 1 mg/ml) was added to the reaction mixture.

2.2.7. ES-MS and GC/MS analysis of product A and B

Newly synthesised products A and B prepared using C. glutamicum treated with EMB and C. glutamicum\(\Delta\)aftB membranes, as described above, were further characterised. ES-MS analysis of the reaction products A and B extracted through preparative TLC (Figure 2.8A), revealed a strong molecular ion \(m/z\) 549.3 (M+Na\(^{+}\)), which corresponds to a trisaccharide product Ara\(\f\)-(1\(\rightarrow\)?)-Ara\(\f\)-(1\(\rightarrow\)5)-\(\alpha\)-D-Ara\(\f\)-O-C\(\beta\). GC/MS analysis of the partially per-O-methylated, per-O-acetylated alditol acetate derivative of product A, synthesised in assays with C. glutamicum\(\Delta\)aftB membranes revealed the addition of only an \(\alpha\)(1\(\rightarrow\)5) linked Ara\(\f\) residue (Figure 2.8B and Figure 2.7A) (Lee et al., 1997). However, GC/MS analysis of the partially per-O-methylated, per-O-acetylated alditol acetate derivative of product B, synthesised in enzyme assays utilising membranes from C. glutamicum and EMB, identified the new glycosyl linkage as a \(\beta\)(1\(\rightarrow\)2)-linked Ara\(\f\) residue (Figure 2.8C and Figure 2.7A). By analogy, this new glycosidic linkage corresponds to a terminal \(\beta\)(1\(\rightarrow\)2) linked Ara\(\f\) residue (Lee et al., 1997). Finally, the results clearly establish, both from in vivo and in vitro experiments, that Mt-AftB catalyses the addition of a \(\beta\)(1\(\rightarrow\)2) Ara\(\f\) unit, and that this enzyme is resistant to EMB (Figure 2.7B).
Figure 2.8: ES-MS and GC/MS characterisation of products A and B. 

A, ES-MS analysis of products from assays containing membranes prepared from C. glutamicum treated with EMB and C. glutamicumΔaftB. 

B, GC/MS analysis of the partially per-O-methylated, per-O-acetylated alditol acetate derivative of product A obtained from assays containing membranes prepared from C. glutamicumΔaftB. 

C, GC/MS analysis of the partially per-O-methylated, per-O-acetylated alditol acetate derivative of product B obtained from assays containing membranes and EMB prepared from C. glutamicum.
2.2.8. Genome comparison of the AftC locus

In silico analysis of one of the putative glycosyltransferases of M. tuberculosis, Rv2673, highlighted that orthologues are present in a range of species belonging to the sub-order Corynebacterianeae, including the families Mycobacteriaceae, Corynebacteriaceae and Nocardiaceae (Figure 2.9A).

Figure 2.9: Comparison of the aftC locus within the Corynebacterianeae. A) The locus in the bacteria analysed consists of aftC which in M. tuberculosis has the locus tag Rv2673 and in C. glutamicum NCgl1822. The genomic region displayed encompasses 7 kb, and orthologous genes are highlighted accordingly. Abbreviations: M. marium, Mycobacterium marium; M. av subsp. par., Mycobacterium avium subsp. paratuberculosis; C. efficiens, Corynebacterium efficiens; C. jeikeium, Corynebacterium jeikeium; Nocardia farcina, Nocardia farcina IFM 10152; Rhodococcus, Rhodococcus sp. strain RHA1. B) AftC is a hydrophobic protein predicted to span the membrane 10 times and the transmembrane helices are numbered accordingly. The lower part of the figure shows the degree of conservation of the orthologues given in A as analysed by the DIALIGN method (Brudno et al., 2003). Also shown is the approximate position of the fully conserved aspartyl and glutamyl residues.

Furthermore, the organisation of the gene locus is largely retained. The adjacent genes are largely of unknown function. RibD encodes a bifunctional deaminase-reductase domain, followed by a gene product containing a hydrolase domain, which is however absent in Corynebacterium, and downstream of Rv2673 a domain of unknown function is present. The wide distribution of Rv2673, its syntenic organisation, and the fact that it is retained even in...
M. leprae, strongly indicates a fundamental function of its product. According to our experimental analysis we annotated this gene arabinofuranosyltransferase C (aftC).

AftC of M. tuberculosis is 433 amino acid residues long. It is a hydrophobic protein and is predicted to possess 10 transmembrane-spanning segments (Figure 2.9B). However, in contrast to AftA, AftB or EmbC, it is characterised by the absence of a periplasmic carboxyterminal extension. The amino acid sequence among the Corynebacterianeae is very well conserved, and there are 43% identical residues shared by the M. tuberculosis and C. glutamicum proteins. The degree of conservation is particularly high in the loop regions, for instance between helix 1 and 2, 3 and 4, or 6 and 7 (Figure 2.9B). The fully conserved aspartyl and glutamyl residues, which we propose to be involved in catalysis or substrate binding, are located in the first extended loop region (Liu & Mushegian, 2003) as demonstrated earlier with AftB. Interestingly, the long transmembrane helix 8 is well conserved and it is within this region that there is a strong identity to a membrane protein of Vibrio parahaemolyticus (CpsG). Furthermore, this gene is located in a gene cluster involved in the biosynthesis of a capsular polysaccharide within this pathogen (Guvener & McCarter, 2003).

2.2.9. Construction and growth of mutants

In order to delete aftC and study possible consequences, we generated a null mutant of M. smegmatis mc2155 MSMEG2785 (homologue of Rv2673) using specialised transduction (Figure 2.10A). In contrast to our C. glutamicum studies (see below), growth of M. smegmatisΔaftc, in comparison to M. smegmatis, was poor in liquid media (Figure 2.10B) and sensitive to the addition of Tween-20 on agar plates (>0.005%). In addition, on solid media, M. smegmatisΔaftc had a smooth appearance in comparison to the typical crenulated colony morphology found for wild type M. smegmatis (Figure 2.11). Complementation of M.
Chapter 2

smegmatisΔaftC with either pMV261-M s-aftC or pMV261-M t-aftC restored the mutant to a wild type phenotype (Figure 2.10B).

**Figure 2.10: Generation of a MSMEG2785 null mutant.** A) A map of the MSMEG2785 region in the parental M. smegmatis strain and its corresponding region in the ΔMSMEG2785 mutant. res, γδ resolvase site; hyg, hygromycin resistance gene from Streptomyces hygroscopicus; sacB, sucrose counter-selectable gene from Bacillus subtilis. Digoxigenin-labelled probes were derived from ~1kb upstream and downstream flanking sequences that were used to construct the knockout plasmid, and are indicated by thick lines with square ends. SacI digested bands expected in a Southern blot are indicated in roman numerals with sizes in brackets. The inset shows the Southern blot of SacI digested genomic DNA from the two strains with expected bands indicated by arrows. B) Growth of wild type of M. smegmatis (◇), M. smegmatisΔaftC (□), M. smegmatisΔaftC pMV261-M s-aftC (△), and M. smegmatisΔaftC pMV261-M t-aftC (○) on TSB medium.
To study the function of the corynebacterial AftC the non-replicative plasmid pK19mobsacBΔaftC was constructed. This was used to transform C. glutamicum to kanamycin resistance, indicating integration in its chromosome (Figure 2.12). Loss of vector was obtained by selection for sucrose-resistance yielding clones with aftC deleted. A PCR analysis with primer pairs P5 and P6 resulted in the expected fragment of 2160 bp for the wild type and of 1065 bp for the deletion mutant, which was termed C. glutamicumΔaftC (Figure 2.12A). In contrast to M. smegmatisΔaftC, the growth of the C. glutamicumΔaftC mutant on the salt medium CGXII possessed only a slightly reduced growth rate of 0.32 h⁻¹, whereas, that of the wild type C. glutamicum was 0.39⁻¹h (Figure 2.12B).
Chapter 2

Figure 2.12: Strategy to delete Cg-aftC using the deletion vector pK19mobsacB ΔaftC. A) This vector carries 18 nucleotides of the 5' end of Cg-aftC and 36 nucleotides of its 3' end thereby enabling the in-frame deletion of almost the entire Cg-aftC gene. The arrows marked P5 and P6 locate the primers used for the PCR analysis to confirm the absence of Cg-aftC. Distances are not drawn to scale. The results of the PCR analysis with the primer pair P5/P6 are shown on the right. Amplification products obtained from the wild type (wt) were applied in the middle lane and that of the deletion mutant in the right lane. ‘St’ marks the standard, where the arrowheads are located at 1 and 0.5 kb. B) Growth of the wild type of C. glutamicum (▲) and C. glutamicum ΔaftC (Δ) on salt medium CGXII with glucose as carbon source.

2.2.10. Analysis of cell wall bound mycolic acids

To study the function of mycobacterial AftC deletion, defatted cells were analysed qualitatively for AG esterified mycolic acids from an equivalent starting amount of biomass for each strain, due to differences in growth rate (Figure 2.12B). As expected, M. smegmatis exhibited a typical profile of α, α’ and epoxy-mycolic acid methyl esters (MAMEs), whereas, these products were drastically reduced in M. smegmatisΔaftC (Figure 2.13). In addition, complementation of M. smegmatisΔaftC with either pMV261-Ms-aftC or pMV261-Mt-aftC (Figure 2.13), led to the restoration of normal ‘levels’ of cell wall bound mycolic acids. These results demonstrated that Ms-aftC and Mt-aftC are involved in a key aspect of arabinan biosynthesis, whereby deletion substantially perturbs tethering of mycolic acids to AG.
Figure 2.13: Analysis of cell wall bound MAMES from M. smegmatis, M. smegmatisΔaftC, M. smegmatisΔaftC pMV261-Ms-aftC and M. smegmatisΔaftC pMV261-Mt-aftC. The bound mycolic acids from an equivalent amount of freeze-dried cells (100 mg), which were initially de-lipidated using two consecutive extractions of CHCl₃:CH₃OH:H₂O (10/10/3; v/v/v/v) at 50°C for 4 h, were released by the addition of tetra-butylammonium hydroxide at 100°C overnight, and methylated as described in the “Materials and methods” Chapter 6. An equivalent aliquot from each strain was subjected to TLC using silica gel plates (5735 silica gel 60F₂₅₄, Merck), and developed in petroleum ether/acetone (95:5, v/v) and charred to reveal MAMES and compared to known standards (Gande et al., 2004).

In contrast to the mycolic acid studies performed with the mycobacterial AftC deletion mutant, C. glutamicumΔaftC cells were analysed quantitatively for AG esterified corynmycolic acids due to similar growth rates between strains (Figure 2.11B). Wild type C. glutamicum exhibited the known profile of corynmycolic acid methyl esters (CMAMEs, 35,345 cpm) (Figure 2.14), whereas, cell wall bound CMAMEs were significantly reduced in C. glutamicumΔaftC (8023 cpm). The above data were reassuring, as the qualitative (M. smegmatisΔaftC) and quantitative (C. glutamicumΔaftC) analyses were comparable in terms of a reduction in cell wall bound mycolic acids (Figure 2.13 and Figure 2.14). Importantly,
these results have also shown that Cg-aftC is involved in a key aspect of arabinan biosynthesis, whereby deletion perturbs tethering of corynomycolic acids to AG.

![Figure 2.14: Analysis of cell wall bound CMAMES from C. glutamicum and C. glutamicum ΔaftC.](image)

**Figure 2.14: Analysis of cell wall bound CMAMES from C. glutamicum and C. glutamicum ΔaftC.** The bound [14C]-labeled corynemycolic acids from de-lipidated [14C]-labeled cell of pulse-labeled 5 ml cultures were released by the addition of tetra-butyl ammonium hydroxide at 100°C overnight and methylated as described in the “Materials and methods”. An equivalent aliquot from each strain was subjected to TLC/autoradiography using silica gel plates (5735 silica gel 60F254, Merck), and developed in petroleum ether/acetone (95:5, v/v) reveal CMAMES and compared to known standards.

2.2.11. Glycosyl compositional analysis of cell walls from M. smegmatis, M. smegmatis ΔaftC and complemented strains

The cell wall core (mAOG) was prepared from M. smegmatis and M. smegmatis ΔaftC as described (Alderwick et al., 2005b; Besra et al., 1995; Daffé et al., 1990) and the ratio of Ara to Gal in mAOG determined by gas chromatography (GC) analysis of alditol acetates (Alderwick et al., 2005b; Besra et al., 1995; Daffé et al., 1990) (Figure 2.15). The glycosyl compositional analysis revealed an Ara:Gal ratio of 2.45:1 for M. smegmatis. The M.
smegmatisΔaftC yielded an AG with a significant reduction in cell wall Ara with an Ara:Gal ratio of 0.45:1. Complementation of M. smegmatisΔaftC with either pMV261-Ms-aftC or pMV261-Mt-aftC, restored the Ara:Gal ratio to that of the wild type M. smegmatis (Figure 2.15, Ara:Gal, 2.40:1).

Figure 2.15: GC analysis of cell walls of M. smegmatis, M. smegmatisΔaftC, M. smegmatisΔaftC pMV261-Ms-aftC and M. smegmatisΔaftC pMV261-Mt-aftC. Samples of purified cell walls were hydrolysed with 2M TFA, reduced, per-O-acetylated and analysed as described under “Materials and Methods” (Alderwick et al., 2005b; Besra et al., 1995).
2.2.12. Glycosyl linkage analysis of cell walls

Gas chromatography mass spectrometry (GC/MS) analysis of per-O-methylated alditol acetate derivatives prepared from *M. smegmatis* and *M. smegmatis*ΔaftC indicated the complete absence of 3,5-Araf branching residues, with a significant reduction in t-Araf, 2-Araf and 5-Araf-linkages (Figure 2.16). Complementation of *M. smegmatis*ΔaftC with either plasmid encoding Ms-aftC or Mt-aftC restored the glycosyl linkage profile to that of wild type *M. smegmatis* (Figure 2.16). These results demonstrate that MSMEG2875 and Rv2673, are functionally equivalent and are involved in the synthesis of 3,5-Araf branching residues.

The GC/MS profiles of per-O-methylated alditol acetate derivatives of *C. glutamicum* and *C. glutamicum*ΔaftC are shown in Figure 2.17 with *C. glutamicum*ΔaftC also clearly devoid of 3,5-Araf branching residues.

![Figure 2.16: GC/MS analysis of cell walls of M. smegmatis, M. smegmatisΔaftC, M. smegmatisΔaftC pMV261-Ms-aftC and M. smegmatisΔaftC pMV261-Mt-aftC. Per-O-methylated cell walls were hydrolysed with 2M TFA, reduced, per-O-acetylated and analysed as described under "Materials and methods" (Alderwick et al., 2005b; Besra et al., 1995).](image-url)
Figure 2.17: GC and GC/MS analysis of cell walls of *C. glutamicum* and *C. glutamicum* ΔaftC. Samples of either purified cell walls or per-O-methylated, hydrolysed using 2M TFA, reduced, per-O-acetylated and analysed as described under "Materials and methods".
2.2.13. *In vitro* arabinofuranosyltransferase activity with extracts of *M. smegmatis*, *M. smegmatis*Δ*aftC* and complemented strains

Initial attempts to develop an *in vitro* assay using either purified recombinant expressed AftC or *E. coli* membranes expressing aftC, have thus far proved unsuccessful, probably due to the hydrophobic nature of the protein. In an alternative approach, we assessed the capacity of membrane preparations from *M. smegmatis*, *M. smegmatis*Δ*aftC* and *M. smegmatis*Δ*aftC* complemented with pMV261-M t-aftC to catalyse arabinofuranosyltransferase activity in the presence of exogenous synthetic acceptors (Lee et al., 1997; Seidel et al., 2007b).

We first assessed whether *M. smegmatis*Δ*aftC* was deficient in $\alpha(1\rightarrow5)$ and $\beta(1\rightarrow2)$ arabinofuranosyltransferase activity, using an $\alpha$-D-Araf-(1→5)-$\alpha$-D-Araf-O-(CH$_2$)$_3$CH$_3$ (Araf$_2$) synthetic acceptor (Lee et al., 1997) and DP[$^{14}$C]A as a sugar donor based on an established assay format for determining $\alpha(1\rightarrow5)$ and $\beta(1\rightarrow2)$ arabinofuranosyltransferase activities (Lee et al., 1998). TLC/autoradiographic analysis of the products, which were only synthesised in the presence of Araf$_2$, when assayed with *M. smegmatis* membranes resulted in the formation of two products (A and B) (Figure 2.18A and B). The enzymatic synthesis of products A and B are consistent with previous studies using mycobacterial (Lee et al., 1997) membrane preparations resulting in trisaccharide products as a result of $\alpha(1\rightarrow5)$ and $\beta(1\rightarrow2)$ Araf linkages to the Araf$_2$ acceptor (Figure 2.18A) (Lee et al., 1997). Addition of EMB in several experiments, even at high concentrations of up to 1 mg/ml, to the reaction mixture, resulted in a decrease in only the *in vitro* synthesised $\alpha$-D-$[^{14}$C]Araf-(1→5)-$\alpha$-D-Araf-(1→5)-$\alpha$-D-Araf-O-(CH$_2$)$_3$CH$_3$ product A (Figure 2.18A and B). Assays performed with membranes from *M. smegmatis*Δ*aftC* and the pMV261-M t-aftC complemented strain using the Araf$_2$ synthetic acceptor gave a similar profile to that of wild type *M. smegmatis* (Figure 2.18B). The data clearly show that the *M. smegmatis*Δ*aftC* strain possesses comparable levels of EMB-sensitive $\alpha(1\rightarrow5)$ and EMB-resistant $\beta(1\rightarrow2)$ arabinofuranosyltransferase activity.
Figure 2.18: Arabinofuranosyltransferase activity utilising an Ara₂ acceptor and membranes prepared from M. smegmatis, M. smegmatis Δ aftC and M. smegmatis Δ aftB pMV261-Mt-aftC. A) Biosynthetic reaction scheme of products A and B formed in arabinofuranosyltransferase assays using the neoglycolipid Ara₂ acceptor. B) Arabinofuranosyltransferase activity was determined using the synthetic Ara₂ acceptor in a cell-free assay with and without EMB (1 mg/ml) as previously described (Lee et al., 1997). The products of the assay were resuspended prior to scintillation counting (10 %) and the remaining subjected to TLC using silica gel plates (5735 silica gel 60F₂₅₄, Merck) in CHCl₃:CH₃OH:H₂O:NH₄OH (65/25/3.6/0.5, v/v/v/v) with the reaction products visualised by autoradiography. The TLC autoradiogram is representative of several independent experiments.
We then developed an arabinofuranosyltransferase assay using the synthetic acceptor $\alpha$-D-Araf-(1→5)-$\alpha$-D-Araf-(1→5)-$\alpha$-D-Araf-(1→5)-$\alpha$-D-Araf-O-(CH$_2$)$_8$NH$_2$ (Ara$_5$) (See figure 2.20B) and DP[$^{14}$C]A as a sugar donor (Lee et al., 1998). TLC/autoradiographic analysis of the products, which are only synthesised in the presence of Ara$_5$, when assayed with M. smegmatis membranes resulted in the formation of a single product X (Figure 2.19A) through the transfer of a single [$^{14}$C]Araf residue, with a retardation factor ($R_f$) consistent with a synthetic Ara$_6$ acceptor (Appelmelk et al., 2008) standard (Figure 2.19B). Interestingly, addition of EMB in several experiments, even at high concentrations of up to 1 mg/ml to the reaction mixture did not inhibit the synthesis of this in vitro synthesised [$^{14}$C]Araf-Ara$_5$ (Figure 2.19A, Product X), illustrating that the Ara$_5$ acceptor was not extended via an EMB-sensitive $\alpha$(1→5) arabinofuranosyltransferase. Interestingly, membranes prepared form the M. smegmatisΔaftC strain were unable to synthesise the in vitro product to the same level of activity that was observed with wild type membranes prepared from M. smegmatis (Figure 2.19A). This was not surprising, since our earlier in vivo and in vitro studies would have expected residual Ara$_6$ product formation, considering that M. smegmatisΔaftC possesses β(1→2) arabinofuranosyltransferase activity. Assays performed with membranes from the M. smegmatisΔaftC pMV261-Mt-aftC complemented strain, gave a similar profile to that of wild type M. smegmatis (Figure 2.19).
Figure 2.19: Arabinofuranosyltransferase activity utilising an Ara$_5$ acceptor and membranes prepared from M. smegmatis, M. smegmatis$\Delta$aftC and M. smegmatis$\Delta$aftC pMV261-Mt-aftC. A) AraT activity was determined using the synthetic Ara$_5$ acceptor in a cell-free assay with and without EMB (1mg ml$^{-1}$). The products reflective of three independent enzymes and assayed were resuspended prior to scintillation counting (10%) and the remaining subjected to TLC using silica gel plates (5735 silica gel 60F254 Merck) in isopropanol/acetic acid/water 8/1/1 v/v/v), with the reaction product X visualised with autoradiography. The TLC autoradiogram is representative of three different independent experiments. B) Ara$_5$ and Ara$_6$ (Appelmelk et al., 2008) acceptor standards were subjected to TLC as above, and the reaction products were visualised by staining with $\alpha$-naphthol followed by charring.

To establish that the Ara$_5$ acceptor is being utilised by two different arabinofuranosyltransferases, presumably establishing $\beta$(1→2) and $\alpha$(1→3) linkages, assays similar to that used before were scaled up and product X extracted and purified through preparative TLC for each membrane preparation. GC/MS analysis of the partially per-O-methylated, per-O-acetylated alditol acetate derivatives of product X in assays preformed with M. smegmatis membranes revealed the addition of $\beta$(1→2) (R$_t$ 15.52 min; m/z 129, 130, 161, 190) and $\alpha$(1→3) (R$_t$ 15.83 min; m/z 118, 129, 130, 190, 202, 233) linked Araf residues (Figure 2.20A and B). Therefore, the product migrating below Ara$_5$ and co-incident
Figure 2.20: GC/MS characterisation of in vitro synthesised product X from the arabinofuranosyltransferase assays utilising the Ara5 acceptor. A) GC/MS analysis of the partially per-O-methylated, per-O-acetylated alditol acetate derivative of product X obtained from assays containing membranes prepared from either *M. smegmatis*, *M. smegmatis* Δ*aftC* or *M. smegmatis* Δ*aftB* pMV261-Mt-aftC. B) Panel illustrates the structure(s) of product X.

with the Ara6 acceptor standard on TLC (Figure 2.19A and B) is in fact a mixture of two products. The addition of β(1→2)-linked Ara residues can be attributed to the function of AftB. The presence of α(1→3)-linked Ara residues in this assay using an Ara5 acceptor clearly highlights the role of a novel arabinofuranosyltransferase(s) capable of functioning in an α(1→3) capacity. Importantly, there is a complete removal of α(1→3) activity when the Ara5 acceptor is incubated with membranes prepared from *M. smegmatis* Δ*aftC* (Figure
2.20A). However, β(1→2) activity is clearly present in M. smegmatisΔaftC (Figure 2.20A).
In addition, M. smegmatisΔaftC complemented with pMV261-Mt-aftC restores α(1→3) arabinofuranosyltransferase activity to wild type M. smegmatis (Fig 2.20A). The results clearly establish both from in vivo and in vitro experiments that AftC catalyses the addition of an α(1→3)-Araf unit via an α(1→3) arabinofuranosyltransferase and that this enzyme is also resistant to EMB (Figure 2.19A).
2.2.14. Discussion

The mAAGP represents one of the most important cell wall components of the Corynebacterianeae and is essential for the viability of M. tuberculosis (Gande et al., 2004; Mills et al., 2004; Pan et al., 2001; Vilcheze et al., 2000). It is therefore not surprising that one of the most effective anti-mycobacterial drugs, EMB, targets its synthesis through inhibition of AG biosynthesis. However, the emergence of MDR-TB and XDR-TB has accelerated the need to discover new drug targets (Brennan & Nikaido, 1995). One of the strategies is to identify genes involved in AG biosynthesis. This strategy was previously used to identify the presence of a new "priming" enzyme, now termed AftA, which would link the initial Araf unit with the C-5 OH of a β(1→6) linked Gal of a pre-synthesised galactan core (Alderwick et al., 2005b).

Although, the previously described Emb (Alderwick et al., 2005b) and AftA (Alderwick et al., 2006c) are arabinofuranosyltransferases, the proteins cannot functionally replace each other. Thus, despite some functional relationship, these glycosyltransferases have inherent specific features, as evident from the insensitivity of AftA towards EMB. The single Cg-Emb (Alderwick et al., 2005b; Radmacher et al., 2005) and Mt-Emb proteins are sensitive towards EMB (Belanger et al., 1996; Telenti et al., 1997). The number of arabinofuranosyltransferases that are required for mycobacterial arabinan biosynthesis has been a matter of speculation to date, depending on how the arabinan chains are assembled. The primary structure of AG (Besra et al., 1995; Daffé et al., 1990) would suggest at least five distinct arabinofuranosyltransferases are required for the complete formation of AG. Interestingly, M. smegmatis embA and embB mutants were found to possess reduced amounts of the non-reducing terminal disaccharide β-D-Araf-(1→2)-a-D-Araf and result in the removal of the dominant terminal non-reducing Ara6 branched motif in the mutant being
replaced by a linear Ara₄ motif. The authors of this study concluded that the \( M. \) smegmatis \( \text{embA} \) and \( \text{embB} \) mutants result in a lack of 3-arm branching off the main \( \alpha(1\rightarrow5) \)-arabinan chain proximal to the non-reducing and attachment site of mycolic acids in AG (Escuyer et al., 2001). Initially, it was proposed that the \( \beta-D-A\ra-(1\rightarrow2)-\alpha-D-A\ra \) disaccharide was assembled using \( \text{EmbA} \) and \( \text{EmbB} \). However, the identification of AftB in this study and the development of an in vitro assay (Lee et al., 1997) suggests that \( \text{EmbA/B} \) act as \( \alpha(1\rightarrow5) \) arabinofuranosyltransferases.

This study has identified Rv3805c, which has now been termed AftB, as a novel retaining arabinofuranosyltransferase, and is likely to form a new family which is distinct from the inverting arabinofuranosyltransferase enzymes (\( \text{EmbA, B, C, and AftA} \)) in GT-83/85 families (Coutinho & Henrissat, 1999). More precisely, AftB adds to the non-reducing end of the arabinan domain of AG \( \beta(1\rightarrow2) \) Araf residues, as shown through both in vivo and in vitro experiments. For instance, incubation of membranes prepared from \( C. \) glutamicum with DP\[^{14}\text{C}\]A and the disaccharide neoglycolipid acceptor resulted in the appearance of two trisaccharide products (A and B), which equate to the transfer of both \( \alpha(1\rightarrow5) \) and \( \beta(1\rightarrow2) \) Araf residues, respectively. Through further chemical characterisation of the products by TLC, ES-MS, and glycosyl linkage analyses, an \( \alpha(1\rightarrow5) \) linked trisaccharide product could only be identified in assays conducted with membranes prepared from \( C. \) glutamicum\( \Delta \)aftB. This clear loss of \( \beta(1\rightarrow2) \) Araf activity corroborates the cell wall analysis of the \( C. \) glutamicum\( \Delta \)aftB mutant, where the loss of \( \beta(1\rightarrow2) \) linked Araf residues could also be observed. We also attempted to inhibit AftB activity by incubation of the assay components in the presence of high concentrations of EMB (up to 1 mg/ml), a known inhibitor of the Emb proteins in \( M. \) tuberculosis and \( C. \) glutamicum. In doing so, analysis of the corresponding products synthesised from \( C. \) glutamicum membranes following EMB
Chapter 2

treatment clearly show evidence of an EMB resistant \( \beta(1 \rightarrow 2) \) arabinofuranosyltransferase activity and an EMB sensitive \( \alpha(1 \rightarrow 5) \) arabinofuranosyltransferase activity. In addition, since it has previously been established that the EMB-resistant AftA introduces the priming Araf residue at the 8\(^{th}\), 10\(^{th}\) and 12\(^{th}\) Galf residue of the galactan backbone, it can be concluded that the bulk \( \alpha(1 \rightarrow 5) \) Araf stems of AG represent the primary target of EMB. It is interesting to note that EMB resistance is simply not due to AftB being a retaining arabinofuranosyltransferase, in contrast to the inverting arabinofuranosyltransferase Mt-EmbA and Mt-EmbB, since AftA, which is also an inverting arabinofuranosyltransferase, is also EMB resistant (Alderwick et al., 2006c). It is noteworthy that deletion of aftB in C. glutamicum results in only a weak phenotype (Figure 2.2B). In M. tuberculosis, mycolic acids are attached to the terminal \( \beta(1 \rightarrow 2) \) Araf and penultimate \( \alpha(1 \rightarrow 5) \) Araf residue of the Araf\(_6\) motif of AG (McNeil et al., 1991). This appears to be similar in C. glutamicum since, in the absence of terminal \( \beta(1 \rightarrow 2) \) Araf residues, mycolic acids are still bound to AG, thus emphasizing, in this respect, the cell wall similarity of these bacteria. However, in C. glutamicum a maximal 5% of the mycolic acids are covalently attached to AG (Puech et al., 2001), whereas this value is about 10% in M. tuberculosis (McNeil et al., 1991). The fact that the aftB deletion mutant of C. glutamicum possesses less AG bound mycolic acids, also results in an increased abundance of TMCM. This situation can be entirely different in M. tuberculosis due to the essentiality of aftB in M. tuberculosis (Sassetti et al., 2003) and requires further investigation.

A modified scheme for terminal cell wall arabinan biosynthesis in Corynebacterianeae is presented in Figure 2.21. It is possible that the AftB protein is responsible for the successive addition of two \( \beta(1 \rightarrow 2) \) Araf residues at a 3,5-Araf branched residue. Although, this may be a reasonable inference from the in vivo structural work with the aftB deletion strain, it has not
been completely verified by our in vitro assay. Therefore, it is formally possible that the AftB-dependent addition of one β(1→2) Ara residue is required, before a second GT-C related arabinofuranosyltransferase adds the second terminal β(1→2) Ara residue as shown in Figure 2.21.

Figure 2.21: Proposed mycobacterial arabinan biosynthesis and the role of AftB and AftC. For reasons of simplicity it is shown that one of the β(1→2) linked Ara residues is added by AftB, whilst the second β(1→2) linked Ara residue may be catalysed by AftB or via an unknown GT-C arabinofuranosyltransferase (AftD), presumably closely related to AftB.

This study has also identified MSMEG2785 (also Rv2673 and NCgl1822), which we have termed AftC, as a novel branching arabinofuranosyltransferase. More precisely, AftC
catalyses the addition of $\alpha(1\rightarrow3)$ Ara residues as shown through both in vivo and in vitro experiments, ultimately resulting in 3,5-Araf residues after further $\alpha(1\rightarrow5)$ extension, characteristic of AG. For instance, incubation of membranes prepared from M. smegmatis with DP$[^{14}C]$A and a linear $\alpha(1\rightarrow5)$-Ara$_5$ neoglycolipid acceptor resulted in the synthesis of an Ara$_6$ product. Further chemical characterisation of the product, by glycosyl linkage analysis, established that the $\alpha(1\rightarrow5)$-Ara$_5$ acceptor was extended via an EMB resistant $\alpha(1\rightarrow3)$ arabinofuranosyltransferase, giving rise to 3-linked Ara residues and corroborating our earlier cell wall analysis of the M. smegmatis$\Delta$aftC mutant. Since, it is now established that only $\alpha(1\rightarrow5)$ arabinofuranosyltransferase(s) are EMB-sensitive it can be further speculated that EmbA and EmbB function in the assembly of the linear $\alpha(1\rightarrow5)$ arabinan segments as presented in Figure 2.21. This is in accordance with previous data (Besra et al., 1995; Daffé et al., 1990) and phenotypes of EmbA and EmbB (Escuyer et al., 2001; Zhang et al., 2003) and Cg-Emb (Alderwick et al., 2005b) mutants. It is clear that further studies are required to establish the precise role of EmbA and EmbB in mycobacteria.

The analysis of the M. smegmatis$\Delta$aftC mutant to date and based on the Ara:Gal ratio would suggest that the residual arabinan segment in the mutant consists of approximately five Ara residues: $\beta$-D-Araf-(1$\rightarrow$2)$-\alpha$-D-Araf-(1$\rightarrow$5)$-\alpha$-D-Araf-(1$\rightarrow$5)$-\alpha$-D-Araf-(1$\rightarrow$5)$-\alpha$-D-Araf- located at three branches on the galactan chain (Alderwick et al., 2005b; Besra et al., 1995). This is consistent with the recent primary structure of AG (Bhamidi et al., 2008), with a ‘non-variable’ terminal non-reducing Ara$_{17}$ motif, introduction of a 3,5-Araf residue distal to this non-reducing end by AftC and further extension by a linear $\alpha(1\rightarrow5)$Araf domain (Figure 2.21). The latter appears to be variable (up to 12/13 residues). However, based on M. smegmatis$\Delta$aftC and the subsequent Ara:Gal compositional analysis, a dominant Ara$_{22}$/Ara$_{23}$ motif would be consistent with recent (Bhamidi et al., 2008) and previous (Besra et al.,
1995) structural data on AG and this is represented in terms of biosynthetic considerations in Figure 2.21.

The arabinofuranosyltransferases of the Emb family (EmbC, EmbA and EmbB) (Belanger et al., 1996; Berg et al., 2005; Escuyer et al., 2001; Telenti et al., 1997), AftA (Alderwick et al., 2006c), AftB and AftC, possess some sequence similarity. This relates to a modified glycosyltransferase motif, which is defined in the GT-C glycosyltransferase superfamily as either DXD, EXD, DDX, or DEX (Liu & Mushegian, 2003). The most distant is probably AftA with only one negatively charged D residue, however possessing an adjacent polar Q residue (Alderwick et al., 2006c). In AftB there are two adjacent D residues (Figure 2.1B), which due to our mutational study are likely to be directly involved in glycosyl hydrolysis and transfer. Also, the high number of charged amino acyl residues of the strongly conserved loop region following the first TM helix might contribute to the proper orientation of substrates at the catalytic centre. The glycosyltransferase motif of arabinofuranosyltransferases so far identified is always located in a periplasmic loop region, which connects TM III-IV in EmbC, TM III-IV in AftA, and TM I-II in AftB (Figure 2.1B & C). A further feature common of the Emb, AftA and AftB proteins is that they consist of an N-terminal region, which has a number of hydrophobic segments spanning the TM, and a large C-terminal domain, which in Emb has been demonstrated to be located towards the periplasmic side (Seidel et al., 2007c). The number of TMs is different amongst these proteins, but the involvement of these TMs could be considered as being important for the translocation of DPA, the lipid linked substrate of these glycosyltransferases. The weak structural identities of the membrane embedded part of the arabinofuranosyltransferases, indicates that transport and presentation of DPA to the catalytic site might be different for these enzymes. A “Pro-motif” as identified in the Emb proteins (Berg et al., 2005) is not present in AftB and AftA. This motif is typical for polysaccharide co-polymerases and is
assumed to control the chain length in polysaccharide biosynthesis. Its absence in AftA and AftB seems plausible, since these enzymes add only singular Ara residues, but the Emb proteins presumably add a number of $\alpha(1\rightarrow5)$ linked Ara residues to form the inner chain of the AG domain.

To conclude, AftB and AftC represent novel arabinofuranosyltransferases in Corynebacterianeae, such as M. tuberculosis, which are responsible for the addition of the terminal $\beta(1\rightarrow2)$ linked Ara residues and branching $\alpha(1\rightarrow3)$ Ara residues, respectively. The genomic organisation in the genomes of the Corynebacterianeae sequenced is intriguing, revealing high synteny of the M. tuberculosis aftB and aftC locus to the maps of all other Mycobacterium and Corynebacterium species. The identification of new cell wall biosynthetic drug targets is of great importance, especially with the emergence of MDR-TB and XDR-TB. These newly discovered DPA dependent arabinofuranosyltransferases represent, along with straightforward in vitro enzyme assays, promising candidates for further exploitation as potential drug targets.
Chapter 3
3. Identification and characterisation of a crucial branching $\alpha(1\rightarrow3)$ arabinofuranosyltransferase involved in LAM bisynthesis

3.1. Introduction

TB affects a third of mankind and causes 1.7 million fatalities annually (Dye et al., 2006). The spread of TB has been facilitated in recent decades, due to the susceptibility of HIV infected individuals to Mycobacterium tuberculosis (Kaye & Frieden, 1996). The problem has been compounded by the emergence of MDR-TB and XDR-TB strains (Shah et al., 2007). M. tuberculosis resides within the family of Corynebacteriaceae. A common feature of this family is that they possess an unusual cell wall architecture dominated by an essential heteropolysaccharide termed AG, which is linked to both mycolic acids and peptidoglycan, forming the mA GP complex (Besra et al. 1995; Daffé et al., 1993; McNeil et al., 1991). The formation of the arabinan domain of AG results from the subsequent addition of Araf residues by a set of unique AraTs. The front line drug EMB has been shown to target at least three AraTs (EmbA, EmbB and EmbC) (Belanger et al., 1996; Telenti et al., 1997), but shows no inhibitory effects against the other recently identified cell wall AraTs, such as AftA (Alderwick et al., 2006), AftB (Chapter 2) and AftC (Chapter 2). In the previous chapter, we successfully deleted MSMEG2785 (Ms-aftC) (Birch et al., 2008), and showed that this leads to expression of a severely truncated AG structure with branching defects in its arabinan domain.

Apart from AG, mycobacteria contain several other glycoconjugates. LAM, which contains an arabinan domain that is structurally similar to that of AG, is a major component of the cell wall. It consists of a core mannan domain covalently linked to a mannosyl-phosphatidylmyo-inositol anchor, which makes it structurally similar to its biochemical precursor LM (Berg et al., 2007; Besra et al., 2007; Morita et al., 2004). Both LAM and LM exhibit
immunomodulatory functions that may influence the host immune response (Jozefowski et al., 2007). Species-specific differences in the “capping-motifs” of the non-reducing termini of the arabinan domain, for which three variants exist, i.e. AraLAM, PI-LAM and ManLAM (Gilleron et al., 1997; Kho et al., 1995; Kho et al., 2001; Nigou et al., 1997; Nigou et al., 1999), have been shown to be important for this function.

The arabinan domain of LAM is attached to an, as yet unidentified, region of the mannan backbone and is thought to be synthesised in a similar manner to that of arabinan found in AG (Berg et al., 2007; Besra et al., 2007; Morita et al., 2004). To date, only one AraT has been implicated in the biosynthesis of LAM. This enzyme, EmbC, has also been shown to be targeted by EMB, but to a lesser extent than the cell wall core AraTs, EmbA and EmbB (Mikusova et al., 1995; Zhang et al., 2003). The formation of the arabinan domain of LAM requires an α(1→3) AraT in a similar manner to AG, thus resulting in the branched motif of LAM. Here we investigated the potential role of AftC in LAM biosynthesis. By structural analysis of LAM from a M. smegmatisΔaftC mutant we demonstrate that AftC carries dual functionality and is responsible for introducing 3,5-Araf branches into LAM, in addition to AG. Furthermore, we show, by treating an M. smegmatisΔaftC mutant with EMB, that EmbC is involved in the very early steps of the LAM arabinan core synthesis and that truncation of this domain modulates the immunological properties of the molecule.
3.2. Results

3.2.1. Effects of aftC inactivation on LAM/LAM biosynthesis

M. smegmatis wild type (WT) (Figure 3.1, Lane 1) and M. smegmatis ΔaftC lipoglycans (Figure 3.1, Lanes 2-4) were purified using conventional methods (Nigou et al., 1997), resulting in the recovery of a highly purified lipoglycan with an intermediate size between M. smegmatis LAM and LM, now termed AftC-LAM (Figure 3.1, Lane 3). Complementation of M. smegmatis ΔaftC with Ms-aftC restored the lipoglycan profile to WT M. smegmatis (Figure 3.1, Lane 5). Plasmid borne Mt-aftC also resulted in complementation of the mutant (Figure 3.1, Lane 6). The molecular weight of AftC-LAM was investigated by negative-ion matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS). The mass of WT-LAM and AftC-LAM exhibited broad unresolved peaks centered at m/z 15000 and 8000, respectively, indicating a weight decrease of ~7 kDa for the mutant LAM (Figure 3.2, A and B).

Figure 3.1: SDS-PAGE analysis of lipoglycans extracted from M. smegmatis and M. smegmatis ΔaftC. Lane 1, lipoglycans extracted from M. smegmatis; Lane 2, lipoglycans extracted from M. smegmatis ΔaftC; Lane 3, purified AftC-LAM from M. smegmatis ΔaftC and Lane 4, purified LM from M. smegmatis ΔaftC; Lane 5, lipoglycans extracted from M. smegmatis ΔaftC pM V261-Ms-aftC and Lane 6, lipoglycans extracted from M. smegmatis ΔaftC pM V261-Mt-aftC.
Figure 3.2: MALDI-TOF-MS analysis of purified WT-LAM (A) and AftC-LAM (B) extracted from *M. smegmatis* and *M. smegmatis ΔaftC* respectively. MALDI-TOF-MS spectra were acquired in the linear negative mode with a delayed extraction using 2,5-dihydrobenzoic acid as a matrix.

3.2.2. Structural characterisation of AftC-LAM

The ratio of Ara to Man in WT-LAM and AftC-LAM was determined using gas chromatography (GC) of alditol acetate derivatives (Birch et al., 2008) (Figure 3.3, A and C). WT-LAM, had a molar ratio of Ara:Man of 2.7:1, which is consistent with previously reported data (Zhang et al., 2003), whereas for AftC-LAM the Ara:Man ratio was 0.59:1 (Figure 3.3, A and C). Complementation of *M. smegmatis ΔaftC* with either Ms-aftC or Mt-aftC, restored the Ara:Man ratio to that of the wild type LAM (Fig. 3.3, E and G). In *M. smegmatis*, LAM consists approximately of 71 Ara, 27 Man, and 1 Ins units (Petzold et al., 2005; Zhang et al. 2003). The loss of 7kDa for AftC-LAM equates to 45 Ara residues, suggesting that AftC-LAM contains one or more short arabinan domains of up to ~16 Ara residues.
Figure 3.3: GC and GC/MS analysis of purified lipoglycans extracted from M. smegmatis (A and B), M. smegmatis ΔaftC (C and D), M. smegmatis ΔaftC pMV261- Ms-aftC (E and F), and M. smegmatis ΔaftC pMV261-Mt-aftC (G and H). The glycosyl composition of lipoglycans extracted were analyzed by GC after derivatization to alditol acetates. Glycosyl linkage analysis was performed after per-O-methylation and derivatization to partially per-O-methylated alditol acetates. Briefly, lipoglycan samples were per-O-methylated using dimethyl sulfinyl carbamion, hydrolyzed using 2 M TFA, reduced using NaBD₄, and per-O-acetylated. The resulting per-O-methylated alditol acetates were solubilized in CHCl₃ before analysis by GC/MS.
The $^1$H-NMR spectrum of WT-LAM (Figure 3.4A) was much more complex than the anomeric region of AftC-LAM (Figure 3.4B). Indeed, the AftC-LAM 1D $^1$H spectrum exhibits three major well-defined resonances characterised by several overlapping resonances arising from six different classes of glycosidic residues.

Figure 3.4: Two-dimensional NMR spectra of WT-LAM and AftC-LAM purified from M. smegmatis and M. smegmatisΔaftC. Structural characterisation of WT-LAM A) and AftC-LAM B). $^1$H, $^{13}$C HSQC NMR spectra were acquired in D$_2$O at 313K. Expanded regions ($\delta$ $^1$H: 5.0-5.30, $\delta$ $^{13}$C: 101-111) are shown.
Based on our data for WT-LAM and previously published work (Nigou et al., 1997), the $^{13}$C resonance at $\delta$101 ppm that correlated to an anomic proton at $\delta$5.15 ppm with a $^{1}J_{H1,C1}$ coupling constant of approximately 170 Hz was assigned as 2,6-Manp. The resonances at $\delta$105 and $\delta$102.3, correlating to protons at $\delta$5.07 and $\delta$4.90 were assigned as t-Manp and 6-Manp, respectively. The t-β-Araf residues corresponded to $\delta$103.4 with $^{1}H$ at $\delta$5.16. The well-separated spin systems for 2-α-Araf attached to the 3-position (2-α-Araf→3, $\delta$108.2, $\delta$5.27 ppm) and 5-position (2-α-Araf→5, $\delta$108.5, $\delta$5.20) of the 3,5-Araf were also visible in the spectra of WT-LAM. Several spin systems were observed ($\delta$110.3 and $\delta$5.19 ppm, $\delta$110.3 and $\delta$5.14 ppm and $\delta$110.3 and $\delta$5.11 ppm) assigned to 5-α-Araf in different chemical environments, with one overlapping set of 3,5-α-Araf ($\delta$110.2-$\delta$5.12) for WT-LAM. As reported two distinct chemical shifts of t-α-Araf could occur for the respective arms of a branched 3,5-α-Araf, as observed for 2-α-Araf→3 and 2-α-Araf→5 (Nigou et al., 1997). In AftC-LAM (Figure 3.4B) the resonances associated with the mannan core (t-Manp, 6-Manp and 2,6-Manp) are conserved but resonances associated with Araf residues are notably less abundant. In particular, 2-α-Araf→3 is absent and the complex set of signals for the different 5-α-Araf are now much more simplified due to the loss of 3,5-α-Araf (Figure 3.4B). GC/MS of per-O-methylated alditol acetate derivatives confirmed this result in that it showed an unaltered glycosidic linkage profile for the mannan backbone of both WT- and AftC-LAM and a complete loss 3,5-Araf and significant reduction in t-Araf, 2-Araf and 5-Araf-linkages for the latter LAM (Figure 3.3B and D). Complementation of M. smegmatisΔaftC with either Ms-aftC or Mt-aftC restored the glycosyl linkage profile to that of WT (Figure 3.3, F and H), demonstrating that MSMEG2785 (Ms-aftC) and Rv2673 (Mt-aftC) are functional orthologues. Overall, the compositional analysis suggests that, as compared to WT-LAM, AftC-LAM has an unaltered mannan domain composed of an α(1→6)-Manp backbone substituted by t-Manp units at O-2 positions, and to which is attached one or more short
\( \alpha(1\rightarrow5) \)-linked linear arabinan chain (in total representing approximately 12-15 Ara units) terminating in a single \( \beta(1\rightarrow2) \) Ara residue (Figure 3.5).

Figure 3.5: Structural representation of WT-LAM and AftC-LAM. Mannose and arabinose residues are displayed in green and blue, respectively. Lipoglycans are represented in their triacylated form and capping motifs have been removed for clarity.
3.2.3. Effect of ethambutol on AftC-LAM formation

EMB blocks cell wall arabinan biosynthesis by targeting the Emb proteins (Belanger et al., 1996; Telenti et al., 1997). Although EmbC is known to be involved in LAM arabinan biosynthesis (Zhang et al., 2003), its precise role is as yet inconclusive. We exploited the phenotype of M. smegmatisΔaftC and the structural properties of AftC-LAM to investigate this role. For this, we treated [14C]-glucose labeled cultures of M. smegmatis with sub-inhibitory concentrations of EMB, extracted the lipoglycans and performed a radiochemical quantification of [14C]-incorporated sugars. SDS-PAGE analysis of the [14C]-LM/LAM pool of WT M. smegmatis revealed 2 broad bands corresponding to LAM and LM migrating to their expected sizes (Figure 3.6A, Lane 1) and an Ara:Man ratio of 1.2:1 (Figure 3.6B, Lane 1). Subsequent analysis of the [14C]-LM/LAM lipoglycan pool from M. smegmatisΔaftC, resulted in labeling of AftC-LAM and LM (Figure 3.6A, Lane 2) and an Ara:Man ratio of 0.5:1 (Figure 3.6B, Lane 2). Addition of EMB at 0.5 µg ml⁻¹ resulted in a decrease in the size of the AftC-[14C]-LAM, an accumulation of [14C]-LM (Figure 3.6A, Lane 3) and a corresponding change in the Ara:Man ratio of 0.3:1 (Figure 3.6B, Lane 3). Since AftC-LAM contains an intact mannan core, the only possible effect of EMB, is by directly inhibiting EmbC activity (addition of the terminal β(1→2) Ara residues by AftB is insensitive to EMB (Alderwick et al., 2006)), thus showing its involvement in the very early stages of LAM arabinan biosynthesis and inhibition of an α(1→5) AraT. Notably, we observed that residual [14C]-Ara labeling always remained upon EMB treatment (Figure 3.6B, Lane 3), suggesting that another AraT possibly adds the first Ara residue to LM, akin to AftA in AG biosynthesis (Alderwick et al., 2006).
Figure 3.6: SDS-PAGE A) and total sugar analysis B) of [14C]-labeled lipoglycans extracted from M. smegmatis and M. smegmatisΔaftC treated with EMB. Growing cultures of M. smegmatis and M. smegmatisΔaftC were labeled with [14C]-glucose and their lipoglycans extracted and analysed by SDS-PAGE (A). Lane 1, M. smegmatis, Lane 2, M. smegmatisΔaftC and Lane 3, M. smegmatisΔaftC + 0.5 µg ml⁻¹ EMB. The above lipoglycans were hydrolysed in 2 M TFA and analysed by TLC to determine the total sugar composition (B). Lane 1, M. smegmatis, Lane 2, M. smegmatisΔaftC and Lane 3, M. smegmatisΔaftC + 0.5 µg ml⁻¹ EMB.

3.2.4. AftC-LAM displays pro-inflammatory properties

LMs, in contrast to LAMs, are potent pro-inflammatory lipoglycans (Doz et al., 2007; Nigou et al., 2008; Quesniaux et al., 2004b; Vignal et al., 2003). This difference has been attributed
to the presence of the arabinan domain in LAM which masks the “bioactive” mannan core (Guerardel et al., 2002; Vignal et al., 2003). To investigate the consequence of the aftC mutation on the pro-inflammatory activity of LAM, we compared WT- and AftC-LAM for release of TNF-α by human THP1 cells. Consistent with earlier studies (Vignal et al., 2003), truncated AftC-LAM, as compared to WT-LAM, exhibited an increased pro-inflammatory activity (Figure 3.7A). LM is a known agonist for TLR2 (Briken et al., 2004). Therefore, we investigated the ability of WT- and AftC-LAM to activate this receptor. HEK293 cells expressing TLR2 were stimulated with increasing amounts of LAM after which TLR2-dependent IL-8 production was determined. As shown in Figure 3.7B, AftC-LAM induced a much stronger TLR2 activation than did WT-LAM (~10-fold). This result demonstrates that the presence of the full-length arabinan domain somehow hampered TLR2 activation. Previously, it has been reported that immune stimulatory activity of some M. smegmatis LAM preparations may have been caused by lipopeptide contamination (Nigou et al., 2008). To investigate this issue, both WT and AftC-LAM were pre-treated with H₂O₂ (a procedure that inactivates lipopeptides (Morr et al., 2002; Zahringer et al., 2008)), after which they were re-tested for their activity on HEK293 TLR2 cells. As shown in Figure 3.7, pre-treatment with H₂O₂ substantially reduced the activity of both WT- and AftC-LAM. However, in contrast to WT-LAM, which lost all of its activity, a substantial part of the activity arising from AftC-LAM was sustained for up to 168 h of treatment (Figure 3.7C). This was not due to the inactivation of the H₂O₂ itself, since addition of fresh H₂O₂ after 96 h did not further reduce the activity (Figure 3.7C). Therefore, we conclude that both LAM preparations were probably contaminated with lipopeptides to a certain extent. Nevertheless, inactivation of these molecules with H₂O₂, clearly demonstrated that AftC-LAM was able to activate TLR2, whereas WT-LAM (PILAM) became completely inactive for concentrations up to 100 µg/ml (Figure 3.7D).
Figure 3.7: TNF-α production by human THP-1 cells and IL-8 production by HEK 293 TLR-2 cells in response to WT-LAM and AftC-LAM. A) Human THP-1 cells were incubated with 1 μg ml⁻¹ (black bars) or 10 μg ml⁻¹ (white bars) WT- or AftC-LAM. TNF-α was quantified after 24 h by ELISA. (B-D) HEK 293 cells transfected with TLR2 were stimulated with: B) increasing amounts of WT- or AftC-LAM, with LPS (50 ng ml⁻¹), with Pam₃C₅K₄ (50 ng ml⁻¹) or with H₂O as a negative control; C) 50 μg ml⁻¹ of WT- or AftC-LAM (with and without 1% hydrogen peroxide (±H₂O₂) for the indicated time period), with LPS (50 ng ml⁻¹), Pam₃C₅K₄ (50 ng ml⁻¹) (±H₂O₂ for 48 h) or with H₂O as a negative control; D) with increasing amounts of WT- or AftC-LAM (treated with H₂O₂ for 168 h). In all cases, cells were stimulated for 24 h at 37°C, after which the supernatants were harvested and analysed for IL-8 by ELISA. E) Binding of DC-SIGN-Fc to WT- (▲) or AftC-LAM (■) as determined by ELISA. Absorption was measured at 490 nm. In all panels, data are expressed as the mean ± s.d. from 1 representative of 3 independent experiments.
To determine whether the increased pro-inflammatory activity of AftC-LAM indeed coincided with a more exposed mannan domain, the ability of WT- and AftC-LAM to interact with an Fc construct harboring the extracellular domain of the C-type lectin DC-SIGN was investigated. DC-SIGN is highly expressed on dendritic cells and recognizes high-mannose structures (Appelmelk et al., 2003; Feinberg et al., 2001) including LM (Pitarque et al., 2005) but not PILAM (Maeda et al., 2003). As shown in Figure 3.7E, the reactivity of DC-SIGN-Fc towards AftC-LAM was stronger as compared to the reactivity against WT-LAM, demonstrating that the mannan core of AftC-LAM was more accessible.
Chapter 3

3.2.5. Discussion

The data described unequivocally demonstrate that AftC is responsible for introducing 3,5-Araf branching in the LAM arabinan domain of M. smegmatis and proposes a new model for LAM biosynthesis (Figure 3.8). Furthermore, we show, for the first time, that EmbC is involved in the very early stages of LAM arabinan biosynthesis and that with the truncation of the arabinan domain LAM gains the ability to activate TLR2. Although the structure of the arabinan domain of LAM is well understood, less is known of the enzymes involved in its biosynthesis. One important reason is that C. glutamicum, a preferred model organism to study mycobacterial AG and LM biosynthesis (Alderwick et al., 2006; Seidel et al., 2007; Birch et al., 2008) does not produce a convoluted LAM, as present in mycobacteria. During our investigation of various putative GT-C glycosyltransferases, we deleted msmeg2785 (AftC) from M. smegmatis, which resulted in a phenotype that displayed a severely truncated AG (Chapter 2) (Birch et al., 2008). In this chapter, we re-visited this mutant in an attempt to investigate the potential role of AftC in LAM biosynthesis. Chemical analysis revealed that LAM isolated from the aftC mutant contained an unaltered mannan core, but with one or more simple arabinan moieties of approximately 12-16 Araf units composed of \( \alpha(1\rightarrow5) \)-linkages terminating in a single \( \beta(1\rightarrow2) \) fashion. It is reasonable to conclude from our data that AftC is not involved in early arabinan LAM biosynthesis, since a more pronounced truncation of LAM would then be expected. AftC has dual functionality, in terms of its involvement in the biosynthesis of both AG and LAM. Attempts to generate an aftC deficient strain of M. tuberculosis has this far proved unsuccessful, highlighting the essentiality of aftC in M. tuberculosis (Sassetti et al., 2003) and the species’ intolerance to cell-wall changes. Hence, AftC represents an excellent drug target.
EmbC has long been implicated in the biosynthesis of mycobacterial LAM and has been shown to be a target of the front line drug EMB (Mikusova et al., 1995; Zhang et al., 2003). However, speculation around its precise enzymatic function has remained controversial. By utilising the unique phenotype of M. smegmatisΔaftC, we now provide unequivocal evidence that EmbC is an α(1→5) AraT, which is inhibited by EMB (Figure 3.8).

![Figure 3.8: Mycobacterial LAM biosynthesis and the role of AftC.](image)

The GT-A/B family of glycosyltransferases perform sequential glycosidic transfer of mannose residues utilising the high energy nucleotide GDP-Man, to a PI based anchor in the cytoplasm. The GT-C family of glycosyltransferases then continue LM and LAM biosynthesis by elaborating AcPIM₄ with mannose and arabinose residues. MptA, MptB and Rv2181 are the only known ManTs involved in core mannan biosynthesis, for which they utilise the lipid linked substrate polyprenylphosphomannose (PPM) as the sugar donor. Decaprenyl-1-monophosphoarabinose (DPA) is utilised by an unknown AraT to prime LM at an, as yet, unidentified position. EmbC and AftC are the other two identified AraTs which mature the final LAM molecule before species specific capping occurs.

123
We now propose a working model, whereby an as yet unidentified GT-C AraT, primes the mannann backbone with singular Ara f residues in much the same way was as AftA primes the galactan backbone of AG (Alderwick et al., 2006). Subsequently, EmbC extends the arabinan chain in an α(1→5) fashion before AftC branches the polysaccharide with an α(1→3) Ara f residue and the domain is further matured by a combination of AraTs including AftC, AftB and perhaps other as yet unidentified enzymes (Figure 3.8).

Several mycobacterial cell-wall lipoglycans are pro-inflammatory stimuli of the host immune response. Both LM and LAM are members of a family of mycobacterial lipoglycans that all contain a conserved MPI anchor (Briken et al., 2004). Interestingly, LM, besides being a potent inducer of pro-inflammatory cytokines, also displays strong anti-inflammatory properties and represses lipopolysaccharide (LPS)-induced cytokine responses in macrophages (Quesniaux et al., 2004b). Recently, by making use of LM preparations, separated by their degree of acylation, it was demonstrated that these pro- and anti-inflammatory properties are, at least in part, dependent on the degree of acylation (Doz et al., 2007; Gilleron et al., 2006). Whereas, tri- and tetra-acylated LM forms displayed strong pro-inflammatory properties, mono- and di-acylated molecules did not (Gilleron et al., 2006).

Besides the degree of acylation, additional structural features of MPI-anchored lipoglycans determine their biological activity. Recently, it was shown that the chain length of the mannann core of MPI-anchored lipoglycans directly correlated with their ability to activate TLR2 (Nigou et al., 2008). Whereas lipoglycans with short oligomannopyranosyl backbones, such as the PIMs, were marginally active, the TLR2-activating potency of lipoglycans with longer backbones, i.e. LM, was strongly enhanced (Nigou et al., 2008). In addition to mannann chain length, the type of substitution of the mannann core was found to be critical, with activity being retained in the case of Man p substitutions but absent when the core was substituted with Araf residues (Nigou et al., 2008). This was evidently clear for
mycobacterial LAMs, which carry a bulky arabinan domain. Although their LM core would in principal allow for TLR2 activation, the LAMs were found to be inactive (Nigou et al., 2008). The assumption that the arabinan domain is directly involved in “silencing activity” is further sustained by the observation that chemical degradation of the arabinan domain restores the pro-inflammatory properties of LAM (Vignal et al., 2003). Our results are in agreement with these findings as AftC-LAM, which expresses a severely truncated arabinan domain, exhibited an increased pro-inflammatory activity (Figure 3.7). Since WT and AftC-LAM did not differ in their pattern of acylation or core mannan composition, the increased pro-inflammatory activity must be directly related to the shortening of the arabinan domain. Exactly how the arabinan domain prevents TLR activation is currently not fully understood. One thought is that the arabinan domain exerts its inhibitory effect by steric hindrance (Vignal et al., 2003). Although several lines of evidence point to this direction, the exact mechanism and the level at which this occurs remains unclear. In several mycobacterial species, the non-reducing termini of the branched arabinan domain are modified by capping motifs consisting of either oligomannosyl, i.e. ManLAMs, or PILAM units such as in M. smegmatis (Khoo et al., 1995). Whereas reports on the immune stimulatory properties of ManLAMs are consistent and unambiguously demonstrate that these types of LAMs are inactive (Nigou et al., 2008; Vignal et al., 2003), some controversy exists on the activity of PILAMs. Early reports demonstrate that PILAM is pro-inflammatory and signals via TLR2 (Chatterjee et al., 1992; Gilleron et al., 1997; Tapping et al., 2003; Underhill et al., 1999; Vignal et al., 2003). However, the mechanism by which a low degree of inositol-phosphate capping would confer this pro-inflammatory activity has never been explained. One hypothesis is that the myo-inositol-phosphate motif in the MPI-anchored lipoglycans may mimic the inositol-phosphate caps on PILAMs thereby conferring activity. However, the notion that the unsubstituted PI caps are unlikely to mimic the acylated MPI anchor plus the
observation that PIMs are only marginally active, clearly demonstrate that the PI motif alone does not confer activity (Vignal et al., 2003). Interestingly, in a more recent study, Nigou et al. (2008) demonstrated that highly purified PILAMs from M. smegmatis and M. fortuitum were equivalent in activity to ManLAMs. Therefore, in contrast to what was previously suggested, the authors concluded that the presence of PI caps did not make LAM pro-inflammatory and they suggested that the activity in earlier experiments was probably due to lipopeptide contamination. Even though contaminant lipopeptides may not be detected by the current analytical methods, trace amounts of these molecules (<0.1%) are enough to influence the outcome of much more sensitive biological assays. We therefore determined the effect of H$_2$O$_2$ treatment on the TLR2-stimulating activity of both WT and AftC-LAM. As shown in Figure 3.7C, treatment with H$_2$O$_2$ severely reduced the activity of WT-LAM (PILAM) suggesting that the earlier observed activity was indeed due to lipopeptide contamination. In contrast, although the activity of AftC-LAM was also substantially reduced, a stable part of the activity was insensitive to H$_2$O$_2$ treatment (Figure 3.7C). Although, we cannot exclude that H$_2$O$_2$ also acts on LAM and may thereby abrogate its activity, the observation that the residual activity of AftC-LAM stabilised after 4 days of treatment, instead of gradually declining further, argues against this notion. Furthermore, H$_2$O$_2$ treatment fully inactivated the lipopeptide Pam$_3$CSK$_4$, demonstrating that inactivated lipopeptides do not display any residual activity (Figure 3.7C). Overall, these data strongly suggest that the pro-inflammatory activity observed in some PILAM preparations was indeed the result of contaminating lipopeptides. Nevertheless, based on these experiments, we conclude that truncation of the arabinan domain by the aftC mutation resulted in an increased pro-inflammatory activity of AftC-LAM.
Chapter 4
4. Identification of a rhamnopyranosyltransferase (RptA) which utilises a novel decaprenophosphorhamnose substrate

4.1. Introduction

A particularly interesting feature of *C. glutamicum* is the presence of terminal rhamnopyranose (t-Rhap) residues attached to the C2 position of α(1→5) linked Araf residues in the arabinan domain of AG (Alderwick et al., 2005). The biological function of these residues remains to be clarified, nevertheless, they are a feature of the corynebacterial cell wall and the biosynthesis of which, needs to be addressed. The current paradigm of AG biosynthesis follows a linear pathway, which is built upon a decaprenyl pyrophosphate lipid carrier. The unique disaccharide linker and galactan domain is synthesised by a variety of GT-A and GT-B family of glycosyltransferases, all of which utilise a nucleotide diphosphate activated sugar substrate for transferase activity. It has been hypothesised (Alderwick et al., 2007; Alderwick et al., 2006; Berg et al., 2007), that a major shift in the biosynthetic machinery takes place upon the initiation of arabinan polymerisation. AftA, Emb, AftC and AftB all belong to the GT-C family of glycosyltransferases and all of which utilise DPA as the sole lipid activated phospho-sugar donor for arabinose transfer into the cell wall. Since, t-Rhap residues are present in the arabinan component of the cell wall, the enzyme(s) responsible for its addition are likely to belong to the GT-C family of glycosyltransferase, and through deduction, one which utilises a lipid-phosphate derived rhamnose substrate, similar to DPA. Herein, we present the putative protein NCgl0543 as a distinct t-rhamnopyranosyltransferase of the GT-C superfamily, which is responsible for the transfer of t-Rhap residues to the arabinan domain to form the branched 2,5-linked Araf motifs of *C. glutamicum*. In addition, we have identified a novel decaprenol-mono-phosphorylhamnose and discuss its role in substrate presentation for AG biosynthesis in *C. glutamicum*. 
4.2. Results

4.2.1. Genome comparison of the NCgl0543 (rptA) locus

A detailed examination of the genome sequence of *C. glutamicum* revealed that there are fourteen glycosyltransferases of the GT-C family. The function of AftA has been identified (Alderwick et al., 2006), AftB, AftC, MptA (Mishra et al., 2007), and that of MptB (Mishra et al., 2008) which act as membrane-bound glycosyltransferases using polyrenylated-phospho-arabinosyl- and mannosyl-sugar donors, respectively, in the synthesis of AG and LAM. The above mentioned genes are also present in *M. tuberculosis* illustrating that they are required to build the elementary cell wall structure of the Corynebacterianeae (Dover et al., 2004).

As yet, an uncharacterised GT-C glycosyltransferase of *C. glutamicum* is encoded by the putative protein NCgl0543. Orthologues of this gene, with identities exceeding 34%, are present in *C. glutamicum* R, *Corynebacterium efficiens*, *Corynebacterium glucuronalyticum* (ATCC 51867), and *Corynebacterium amycolatum* SK46, the latter two saprophytic organisms being human skin pathogens (Letek et al., 2006; Tauch et al., 2008). The genomic organisation surrounding NCgl0543 is largely syntenic and is preceded by a putative tRNA pseudouridine synthase and downstream of NCgl0543 is a membrane protein of unknown function (pfam class DUF690). There are no orthologues of NCgl0543 present in *Corynebacterium diphtheriae*, *Corynebacterium jeikeium* nor *Corynebacterium urealyticum* DSM 7109 (Cerdeno-Tarraga et al., 2003; Tauch et al., 2005; Tauch et al., 2008).

NCgl0543 of *C. glutamicum* is a large polytopic membrane protein of 799 amino acid residues and is predicted to possess 13 transmembrane-spanning helices (TMH) (Figure 4.1A). It is further characterised by a periplasmic carboxyterminal extension of 237 aa, similar to the C-terminal features of AftA (Alderwick et al., 2006), AftB, and the Emb
proteins (Alderwick et al., 2006; Berg et al., 2005; Seidel et al., 2007; Telenti et al., 2007). A particularly highly conserved region is present at the end of the long loop connecting TMH 3 and TMH 4. This region is schematically shown in Figure 4.1A, as is part of its sequence (Figure 4.1B). This sequence resembles the glycosyltransferase GT-C family DXD motif (Liu & Mushegian, 2003), as it contains a number of basic and acidic residues, with the latter being shown in mutational studies to be essential for glycosyltransferase activity using polyrenylated phospho-sugar donors (Berg et al., 2007; Seidel et al., 2007b).

**Figure 4.1: Hypothetical spatial organisation and partial sequence of the putative protein NCgl0543.**

The hypothetical schematic organisation of the putative protein NCgl0543 is shown spanning the membrane 13 times with the carboxy terminal end consisting of 237 amino acids and located in the periplasm. Also shown is the long loop of 168 amino acids connecting TMH 3 and TMH 4. The black star locates the putative glycosyltransferase region given in B and the white star a conserved region, and also present in the putative protein Rv3779, in the periplasmic part of the protein. B) Illustrates the part of the loop region, where acidic and basic residues are highlighted. Although, the overall topology of the M. tuberculosis putative protein Rv3779 and its orthologs are somewhat different to that of the corynebacterial proteins, the loop region is strongly conserved in the Corynebacterianeae and this is indicated for selected mycobacterial species underneath the sequence alignment. Shown are aa 127 - 188 of the M. tuberculosis sequence, and aa 184 - 235 of the C. glutamicum sequence. On top of the sequence comparison the predicted secondary structure is given, with L indicating a loop region, H a helical structure, and E an extended sheet structure. The abbreviations are as follows: C. glu., Corynebacterium glutamicum ATCC13032; C. glR., Corynebacterium glutamicum strainR; C. eff., Corynebacterium efficiens; C. gly., Corynebacterium glucuronalyticum; C. amy., Corynebacterium amycolatum; M. mar., Mycobacterium marinum (ATCC BAA -535); M. tub., Mycobacterium tuberculosis; M. par., Mycobacterium paratuberculosis; M. lep., Mycobacterium leprae.
4.2.2. Construction of \(\text{C. glutamicum}\Delta \text{rptA}\)

In an attempt to delete NCgl0543 in \(\text{C. glutamicum}\), the non-replicative vector pK19mobsacB\(\Delta\)NCgl0543 was constructed. This was introduced into \(\text{C. glutamicum}\) via electroporation and kanamycin-resistant clones obtained indicating integration in the chromosome by homologous recombination (Schafer et al., 1994). Using the sucrose resistance provoked by the sacB gene a second homologous recombination event was selected. A total of 9 clones were analysed by PCR and in 2 of them a wild type reversion at the NCgl0543 locus was restored, whereas a deletion of NCgl0543 was obtained in the 7 remaining clones. These numbers indicate that loss of NCgl0543 is not detrimental to cell growth or viability. As a result, and based on the results described below, one clone was subsequently termed \(\text{C. glutamicum}\Delta \text{rptA}\) and confirmed by PCR to have Cg-rptA deleted, whereas controls with \(\text{C. glutamicum}\) wild type resulted in the expected larger amplification product.

4.2.3. In vitro growth phenotype of \(\text{C. glutamicum}\Delta \text{rptA}\)

Growth of wild-type \(\text{C. glutamicum}\) and \(\text{C. glutamicum}\Delta \text{rptA}\) was compared in liquid mineral salt medium CGXII and rich medium BHIS (Eggeling & Bott, 2005). Both strains exhibited comparable growth rates of \(0.36 \pm 0.03 \text{ h}^{-1}\) on mineral salt medium CGXII and \(0.60 \pm 0.05 \text{ h}^{-1}\) on rich medium BHIS. Thus, \(\text{C. glutamicum}\Delta \text{rptA}\) does not exhibit an apparent growth defect indicating some degree of tolerance to the deletion of Cg-rptA. For further analyses, \(\text{C. glutamicum}\Delta \text{rptA}\) was transformed with a plasmid encoding Cg-rptA, as well as with a plasmid encoding Rv3779 of \(\text{M. tuberculosis}\) to result in \(\text{C. glutamicum}\Delta \text{rptA pVWEx-Cg-rptA}\) and \(\text{C. glutamicum}\Delta \text{rptA pVWEx-Rv3779}\).
Figure 4.2: Strategy to delete Cg-aftC using the deletion vector pK19mobsacBΔrptA. This vector carries 18 nucleotides of the 5' end of Cg-rptA and 36 nucleotides of its 3' end thereby enabling the in-frame deletion of the entire Cg-rptA gene. The arrows marked P1 and P2 locate the primers used for the PCR analysis to confirm the absence of Cg-rptA. Distances are not drawn to scale. The results of the PCR analysis with the primer pair P1/P2 are shown on the right. Amplification products obtained from the wild type (wt) were applied in the middle lane and that of the deletion mutant in the right lane. ‘St’ marks the standard.


To study the function of a corynebacterial rptA deletion mutant, defatted cells were analysed quantitatively for AG esterified corynemycolic acids. Wild type C. glutamicum exhibited the known profile of CMAMES as previously described (Alderwick et al., 2005). Furthermore, cell wall bound CMAMES were not significantly altered in C. glutamicumΔrptA, which is contrary to the deletion of other GT-C glycosyltransferases in C. glutamicum (Alderwick et al., 2005; Alderwick et al., 2006). Analysis of cell wall associated lipids in several independent experiments highlighted no change in the lipid profiles of the Cg-rptA deletion mutant compared to that of C. glutamicum. This was confirmed quantitatively through [14C]acetate labeling of cultures and equal loading of radioactivity of extractable free lipids.
from C. glutamicum, C. glutamicum∆rptA and the complemented C. glutamicum∆rptA strain using plasmid pVWEx-Cg-rptA. These results demonstrate that, unlike Cg-emb or Cg-aftA (Alderwick et al., 2005; Alderwick et al., 2006), Cg-rptA has little or no involvement in the structure or biosynthesis of cell wall bound/extractable lipids in C. glutamicum.

The cell wall core (mAGP) was prepared from C. glutamicum and C. glutamicum∆rptA as described (Alderwick et al., 2005; Besra et al., 1995; Daffé et al. 1990) and the ratio of Rha, Ara to Gal in mAGP determined by gas chromatography (GC) analysis of alditol acetates (Alderwick et al., 2005; Besra et al., 1995; Daffé et al. 1990 (Figure 4.2). The glycosyl compositional analysis of wild type C. glutamicum revealed a relative molar ratio of Rha:Ara:Gal of 21:71:31 which is in accordance with previous data (Alderwick et al., 2005) (Figure 4.2). The C. glutamicum∆rptA mutant yielded a molar ratio of Rha:Ara:Gal (1:71:31), with a significant reduction in Rha content and no relative change in the Ara:Gal ratio (Figure 4.2). Complementation of C. glutamicum∆rptA with pVWEx-Cg-rptA restored the Rha:Ara:Gal ratio to that of wild type C. glutamicum (Figure 4.2). Interestingly, complementation with pVWEx-Rv3779 did not complement and yielded a phenotype identical to that of C. glutamicum∆rptA.
Figure 4.3: GC analysis of cell walls of C. glutamicum, C. glutamicumΔrptA and C. glutamicumΔrptA pVWE x-Cg-rptA. Samples of purified cell walls were hydrolysed with 2M TFA, reduced, per-O-acetylated and analysed as described under “Materials and Methods” (Alderwick et al., 2005; Besra et al., 1995). Abbreviations: Ara, arabinose; Gal, galactose; Rha, rhamnose.

4.2.5 Glycosyl linkage analysis of cell walls from C. glutamicum, C. glutamicumΔrptA, C. glutamicumΔrptA pVWE x-Cg-rptA, and C. glutamicumΔrptA pVWE x-Rv3779

Gas chromatography mass spectrometry (GC/MS) analysis of per-O-methylated alditol acetate derivatives prepared from C. glutamicum and C. glutamicumΔrptA indicated a loss of 1-linked Rhap residues with a corresponding loss of 2,5-linked Araf residues (Figure 4.4).
Chapter 4

Complementation of C. glutamicumΔrptA with plasmid-encoded pVWEx-Cg-rptA restored the glycosyl linkage profile to that of wild type C. glutamicum (Figure 4.4). Furthermore, as demonstrated by our total sugar analysis and cell wall sugar linkage analysis, Rv3779 was unable to complement C. glutamicumΔrptA by pVWEx-Rv3779. These results demonstrate that the putative protein NCgl0543 is involved in the biosynthesis of C. glutamicum AG through the addition of t-Rhap residues to the C2 position of the 5-linked backbone domain of specific Ara residues.

4.2.6. Recognition of a rhamnose lipid-linked sugar donor, decaprenyl-P-rhamnose

Initial assays involved wild type membranes from C. glutamicum and either UDP[^14C]GlcNAc, dTDP-[^14C]Rha, UDP-[^14C]Gal or p[^14C]Rpp as sugar donors for AG biosynthesis. Samples of the radioactive lipids from each assay were applied to TLC plates which were then developed in CHCl₃/CH₃OH/NH₄OH/H₂O (65:25:0.5:3.6) and autoradiograms obtained (Figure 4.5).

As expected a series of polar glycolipids (GL-1 to -4) were observed corresponding to GlcNAc-P-polyprenyl (GL-1), Rha-GlcNAc-P-polyprenyl (GL-2), Gal-Rha-GlcNAc-P-polyprenyl (GL-3), Gal-Gal-Rha-GlcNAc-P-polyprenyl (GL-4) and DPA/DPR consistent with previous studies utilising M. smegmatis membranes (Mikusova et al., 1996). Interestingly, the inclusion of dTDP-[^14C]-Rha also resulted in the synthesis of a more apolar rhamnose-labeled lipid X product, in comparison to GL-2-4 (Figure 4.5).
Figure 4.4: GC/MS analysis of cell walls of C. glutamicum, C. glutamicumΔrptA and C. glutamicumΔrptA pVWEx-Cg-rptA. Samples of per-O-methylated cell walls were hydrolysed with 2M TFA, reduced, per-O-acetylated and analysed as described under "Materials and Methods" (Alderwick et al., 2005; Besra et al., 1995)
Figure 4.5: Analysis of $[^{14}\text{C}]\text{GlcNAc}$, $[^{14}\text{C}]\text{Rha}$, $[^{14}\text{C}]\text{Gal}$ and $[^{14}\text{C}]\text{Ara}$ labeled glycolipids in C. glutamicum. Membranes from C. glutamicum were prepared, mixed with decaprenyl-1-monophosphate and labeled with either UDP-$[^{14}\text{C}]\text{GlcNAc}$, dTDP-$[^{14}\text{C}]\text{Rha}$, UDP-$[^{14}\text{C}]\text{Gal}$ or p-$[^{14}\text{C}]\text{Rpp}$. Radiolabeled glycolipids were extracted, analysed by TLC and visualised by autoradiography as described in "Materials and Methods". Abbreviations: GL-1, GlcNAc-P-P-polyprenyl; GL-2, Rha-GlcNAc-P-P-polyprenyl; GL-3, Gal-Rha-GlcNAc-P-P-polyprenyl; GL-4, Gal-Gal-Rha-GlcNAc-P-P-polyprenyl; Lipid-X, putative decaprenol-1-monophosphate-rhamnopyranose; DPA, $\beta$-D-arabinofuranosyl-1-monophosphoryldecaprenol; DPR, $\beta$-D-ribofuranosyl-1-monophosphoryldecaprenol.

This lipid X was sensitive to acid and resistant to mild-base treatment indicating that this product was also a polyprenyl-P based lipid. Importantly, an increase in the synthesis of lipid X, based on TLC and densitometry, was found when assays were repeated with membranes prepared from C. glutamicum$\Delta$rptA (lipid X, 4300 cpm) compared to C. glutamicum (lipid X, 3057 cpm), indicating that lipid X was probably the lipid-linked sugar donor for the GT-C glycosyltransferase RptA in this study (Figure 4.6). Furthermore, the synthesis of lipid X was unaffected by the addition of tunicamycin (C. glutamicum lipid X, 3222 cpm; C. glutamicum$\Delta$rptA lipid X, 4300 cpm).
glutamicum∆rptA lipid X, 4203 cpm) which inhibits GlcNAc phosphotransferase activity of Rv1302, thus leading to a marked decrease in GlcNAc-P-polyprenyl (GL-1) and higher GLs (e.g. >GL-2) (Figure 4.6).

Figure 4.6: Analysis of [$^{14}$C]Rha base stable lipids in C. glutamicum and C. glutamicum∆rptA and the effect of tunicamycin. Membranes from C. glutamicum and C. glutamicum∆rptA were prepared, mixed with decaprenol-1-monophosphate and labeled with dTDP-[$^{14}$C]Rha. [$^{14}$C]Rha-labeled glycolipids were extracted, analysed by TLC and visualised by autoradiography as described in "Materials and Methods". Abbreviations: GL-1, GlcNAc-P-P-polyprenyl; GL-2, Rha-GlcNAc-P-P-polyprenyl; GL-3, Gal-Rha-GlcNAc-P-P-polyprenyl; GL-4, Gal-Gal-Rha-GlcNAc-P-P-polyprenyl; Lipid-X, putative decaprenol-1-monophosphate-rhamnopyranose.

The basic assay mixture was scaled-up containing cold dTDP-Rha and the lipid X extracted for subsequent characterisation by treatment with mild base. Negative-ion electro-spray mass spectrometry (ES-MS) revealed a major signal (M-H)$^-$ at m/z 777 for decaprenyl phosphate (Figure 4.7A inset), and a product ion at m/z 924 corresponding to decaprenyl-P-rhamnose (Figure 4.7A). We also observe mass ions at m/z 934 and m/z 936, which could indicate 5 and 6 fully saturated isoprene units out of a possible 10. Similar observations have previously been reported in other Corynebacteriaceae glycolipids, such as the apparent mycolate transporter Myc-PL (Besra et al., 1994). Altogether, the above dTDP-Rha labeling, sensitivity to acid, resistance to base and to tunicamycin, and analysis by ES-MS, confirmed
that lipid X was prenylated-P-rhamnose (Figure 4.7B), and is therefore the likely substrate of the membrane bound rhamnosyltransferase RptA.

**Figure 4.7: Mass spectrometry analysis and identification of decaprenyl-1-monophosphorylrhamnose. A) Electrospray mass spectrometry (in the negative mode) of decaprenol-1-phosphorylrhamnose. B) Structure of decaprenol-1-phosphorylrhamnose.**
4.3. Discussion

It is clear, from this present study, that Cg-rptA is non-essential and is involved in the addition of t-Rhap residues to a 5-linked Arf backbone giving rise to branched 2,5-linked Arf residues. We also report a Rha:Ara molar ratio of 21:71 in C. glutamicum AG (Figure 4.3), which equates to approximately 7 t-Rhap residues per arabinan tricosamer. This significant amount of rhamnose in the cell wall must not be dismissed, and more so, the functional significance must be elucidated. Rhamnose is present in the cell walls of many Gram-positive bacteria such as Staphylococcus, Streptococcus, Bacillus and Pseudomonas (Weidnemaier & Peschel, 2008). In Pseudomonas aeruginosa, L-rhamnose is present in the form of mono or di substituted rhamnolipids, and these virulence factors are thought to act by disrupting lipid membranes by acting as a surfactant (Van Delden & Iglewski, 1998). In other Gram-positive bacteria, rhamnose is present in the cell wall as part of the carbohydrate moieties of teichoic acids and other cell-wall glycopolymers (Baddiley, 1972). However, since C. glutamicum is non-pathogenic, the introduction of t-Rhap residues is probably related to either (i) structural integrity or (ii) a mechanism to cap and end arabinan synthesis and provide a control point for the extent of mycolation of the "terminal" arabinan units.

Cg-rptA shares approximately 40% sequence similarity with the putative M. tuberculosis protein Rv3779. Although, the total length of the proteins differ by 133 aa, there are remarkable structural similarities, such as the high identity at the sequence level in the GT-C loop, which is located between TMH 3 and 4 in C. glutamicum (Figure 4.1) and TMH 4 and 5 in M. tuberculosis Rv3779. This loop is followed by 10 TMHs in C. glutamicum, which are present and similarly arranged in the M. tuberculosis orthologue. Interestingly, within the final periplasmic region, there is also a similar stretch of aa residues indicated as an open star in Figure 4.1A. Since the carboxy terminal periplasmic domain is suggested to play a role in
substrate recognition, which in the case of the GT-C glycosyltransferases is a growing polysaccharide, we speculate that both Cg-RptA and the putative Rv3779 protein recognise a related substrate, such as an arabinan oligosaccharide and distinct sugar donors, since the putative protein, Rv3779, failed to complement \textit{C. glutamicum}\text_d\text{rptA}.

NCgl0543 is part of the \textit{rfb} locus and its genomic organisation is well retained in \textit{Corynebacterianeae}. Orthologues of this locus, which include \textit{rfbE}, NCgl0197, NCgl0195, \textit{rfbD} (NCgl0198), are present in \textit{C. glutamicum}. \textit{RfbE} has similarity to an ATP-dependent export carrier and is tentatively annotated as being a polysaccharide export ATP-binding protein, and \textit{rfbD} as a polysaccharide export ABC transporter permease (Cole et al., 1998). We have found that the galactosyltransferase NCgl0195 (Alderwick et al., 2008; Mikusova et al., 2006) is essential in \textit{C. glutamicum} and is involved in GL-3 and GL-4 biosynthesis. Due to the function of Cg-RptA, and its similarity to the putative protein Rv3779, we speculate that the \textit{rfb} locus is essential for polysaccharide biosynthesis and resultant export to the periplasm.

The biosynthesis of high energy nucleotide derived sugar substrates of \textit{Corynebacteriaceae} glycosyltransferases, such as UDP-\textalpha-D-Galf (Weston et al., 1997), dTDP-Rhap (Ma et al., 1997) has been well characterised. These substrates are utilised by GT-A/B glycosyltransferases in the cytoplasm of the cell to form a preliminary linear galactan polysaccharide before being exported to the periplasm, by an as yet unidentified flippase. At this point, further polysaccharide biosynthesis employs glycosyltransferases belonging to the GT-C family which make use of prenylated phosphosugar substrates (Liu & Mushegian, 2003; Berg et al., 2007). In \textit{Corynebacterianeae}, decaprenol (C\textsubscript{50}) and, to a lesser extent octahydroheptaprenol (C\textsubscript{35}), are the predominant lipid carriers for peptidoglycan, arabinogalactan and mannan biosynthesis. Decaprenyl phosphate is glycosylated by
arabinose and ribose to form $\beta$-D-arabinofuranosyl-1-monophosphoryldecaprenol (DPA) and $\beta$-D-ribofuranosyl-1-monophosphoryldecaprenol (DPR), respectively (Wolucka et al., 1994). It is also glycosylated by mannose to form $\beta$-D-mannosyl-1-monophosphoryldecapreol (DPM or PPM) (Gurcha et al., 2002; Yokoyama & Ballou, 1989) and glucose to form $\beta$-D-glucosyl-1-monophosphoryldecaprenol (GPM) (Schultz & Elbein, 1974). To date, this is the first report of a GT-C rhamnosyltransferase (RptA). Furthermore, this is also the first report of a rhamnosylated monophosphodecaprenol, which is the substrate for RptA and novel with respect to DPA, DPR and DPM. Finally, the discovery of Cg-rptA has shed new light on the glycosyltransferases which are key to building the cell wall AG of Corynebacterianeae.
Chapter 5
5. Conclusion and future work

*M. tuberculosis* is a major cause of illness and death worldwide and with the advent of MDR- and XDR-TB strains discovery of new chemotherapeutic agents is paramount (Brennan & Nikaido, 1995). Mycobacteria have an intricate cell wall and maintenance of its integrity is essential for the survival of the bacilli, and as such, numerous anti-tubercular drugs target its biosynthetic machinery. One such drug, EMB, inhibits synthesis of the arabinan, which forms an integral component of AG and LAM, the two dominating heteropolysaccharides located in the mycobacterial cell wall. In this regard, the last two decades have seen considerable effort concentrated towards the elucidation of the enzymes involved with in their biosynthetic pathways.

The number of arabinofuranosyltransferases that are required for mycobacterial arabinan biosynthesis has been a matter of speculation to date depending on how and where the arabinan chains are assembled. In terms of AG, the primary structure (Besra et al., 1995; Daffé et al., 1990) would suggest at least five distinct arabinofuranosyltransferases are required for the complete formation of the three AG arabinan domains. The availability of the complete genome sequence of *M. tuberculosis* H37Rv, as well as the decoding of other Actinomycetales genomes such as *C. glutamicum* proved crucial in forming our experimental hypothesis. Carrying out comparative genomic analysis of Corynebacterineae genome sequences, and employing surrogate experimental model systems such as *C. glutamicum* and *M. smegmatis*, has enabled us to study the molecular genetics of mycobacterial cell wall biosynthetic processes, which are otherwise essential in *M. tuberculosis*. This strategy was previously used to identify the presence of a new “priming” enzyme, now termed AftA, which would link the initial Ara unit with the C-5 OH of a β(1→6) linked Gal of a presynthesised galactan core (Alderwick et al., 2005b).
The studies reported in this thesis have identified Rv3805c, which has now been termed AftB, as a novel retaining arabinofuranosyltransferase, which adds to the non-reducing end of the arabinan domain of AG $\beta(1\rightarrow2)$ Araf residues. Deletion of its orthologue NCgl2780 in the closely related species C. glutamicum produced a viable mutant with a decreased abundance of cell wall bound mycolic acids, consistent with a partial loss of mycolylation sites. Glycosyl linkage analysis of arabinogalactan revealed the complete loss of terminal $\beta(1\rightarrow2)$ linked Araf residues which was fully restored upon complementation with a plasmid expressing Cg-AftB or Mt-AftB but not with muteins of Mt-AftB, where the two adjacent aspartic acid residues, which have been suggested to be involved in glycosyltransferase activity, were replaced by alanine. In addition, the use of C. glutamicum and C. glutamicumΔaftB in an in vitro assay utilizing the sugar donor DPA together with a neoglycolipid acceptor as substrate, confirmed AftB as a terminal $\beta(1\rightarrow2)$ arabinofuranosyltransferase, which was also insensitive to EMB.

Interestingly, Escuyer et al. (2001) reported, M. smegmatis embA and embB mutants possessing reduced amounts of the non-reducing terminal disaccharide $\beta$-D-Araf-(1→2)-$\alpha$-D-Araf, resulting in the absence of the dominant terminal non-reducing Araf branched motif in the mutant being replaced by a linear Araf motif. The authors of this study concluded that the M. smegmatis embA and embB mutants result in a lack of 3-arm branching off the main $\alpha(1\rightarrow5)$-arabinan chain proximal to the non-reducing and attachment site of mycolic acids in AG (Escuyer et al., 2001), leading to the supposition that the $\beta$-D-Araf-(1→2)-$\alpha$-D-Araf disaccharide was assembled using EmbA and EmbB.

The results presented in this thesis do not reflect the proposed roles of the known ArafTs propounded by Escuyer et al. (2001), in fact the complete biochemical and molecular characterisation of AftB in this study and the development of an in vitro assay (Lee et al.,
Chapter 5

1997) suggests that EmbA/B may act as $\alpha(1\rightarrow5)$ arabinofuranosyltransferases, although further definitive characterisation is required. Due to the inherent absence of a LAM-like molecule in C. glutamicum, we were unable to determine the involvement of Mt-AftB in both AG and LAM arabinan biosynthetic pathways. In terms of future studies, it would be interesting to generate an aftB mutant in M. smegmatis to assess whether or not AftB has dual functionality, with regard to LAM biosynthesis, and is responsible for $\beta(1\rightarrow2)$ capping in LAM, akin to our studies of M. smegmatis AftC (see below).

In addition, our current studies have also identified MSMEG2785 (Rv2673 and NCgl1822), which we have termed AftC, as a novel branching arabinofuranosyltransferase. In vivo and in vitro experiments demonstrated that AftC catalyses the addition of $\alpha(1\rightarrow3)$ Araf residues, ultimately resulting in 3,5-Araf residues after further $\alpha(1\rightarrow5)$ extension, characteristic of AG. It was shown that incubation of membranes prepared from M. smegmatis with DP$[^{14}$C]$A$ and a linear $\alpha(1\rightarrow5)$-Ara5 neoglycolipid acceptor resulted in the synthesis of an Ara6 product. Further chemical characterisation of the product by glycosyl linkage analysis established that the $\alpha(1\rightarrow5)$-Ara5 acceptor was extended via an EMB resistant $\alpha(1\rightarrow3)$ arabinosyltransferase, giving rise to 3-linked Araf residues. Since, it is now established that only $\alpha(1\rightarrow5)$ arabinofuranosyltransferase(s) are EMB-sensitive it can be further speculated that EmbA and EmbB function in the assembly of the linear $\alpha(1\rightarrow5)$ arabinan segments. This is in accordance with previous data (Besra et al., 1995; Daffé et al., 1990) and phenotypes of EmbA and EmbB (Escuyer et al., 2001; Zhang et al., 2003) and Cg-Emb (Alderwick et al., 2005b) mutants. It is clear that further studies are required to establish the precise role of EmbA and EmbB in mycobacteria.

Given that M. smegmatis possesses a LAM structure similar to M. tuberculosis, and the arabinan domain is structurally similar to that of AG, we investigated the potential dual
functionality of AftC. The formation of the arabinan domain of LAM requires an α(1→3) AraT resulting in the branched motif of LAM. By structural analysis of LAM from a M. smegmatis ΔaftC mutant, we demonstrated that AftC carries dual functionality and is responsible for introducing 3,5-Araf branches into LAM, in addition to AG. Furthermore, it was shown by treating an M. smegmatis ΔaftC mutant with EMB, that EmbC is involved in the very early steps of the LAM arabinan core synthesis and that truncation of this domain modulated the immunological properties of the molecule. AftC-LAM exhibited enhanced pro-inflammatory activity due to its ability to activate TLR2, thus further work using this truncated LAM could shed light on the structure-function relationship of TLR2 activation by mycobacterial lipoglycans. It would be interesting to examine whether the generation of aftC mutants in M. bovis BCG and M. marinum is in fact feasible and if these could be used in infection studies involving mice and zebrafish, respectively.

This study also characterised a further ORF from C. glutamicum encoding a distinct t-rhamnopyranosyltransferase from the GT-C superfamily of glycosyltransferases, now termed RptA. Disruption of NCgl0543 in C. glutamicum and subsequent biochemical and molecular analyses demonstrated that the enzyme is responsible for the transfer of t-Rhap residues to the arabinan domain to form the branched 2,5-linked Araf motifs of C. glutamicum. The introduction of t-Rhap residues is probably related to either structural integrity or a mechanism to cap and end arabinan synthesis, providing a control point for the extent of mycolation of the "terminal" arabinan units. Cg-rptA shares approximately 40% sequence similarity with the putative M. tuberculosis protein Rv3779 and there are remarkable structural similarities, such as the high identity at the sequence level in the GT-C loop. Interestingly, within the final periplasmic region, there is also a similar stretch of amino acid residues and given that the carboxy terminal periplasmic domain is suggested to play a role in substrate recognition, we speculated that both Cg-RptA and the putative Rv3779 protein
recognise a related substrate, such as an arabinan oligosaccharide but utilising distinct sugar donors, since the putative protein, Rv3779, failed to complement C. glutamicumΔrptA. A recent investigation by Scherman et al. (2009) presented evidence that Rv3779 exhibited polyprenyl-phosphomannopyranose synthase activity. However, a very recent investigation carried out in the same laboratory presented conflicting data, which clearly establishes Rv3779 as a polyprenyl-phospho-N-acetylgalactosamine transferase, the enzyme responsible for modifying the interior 3,5-Ara branch motifs by the addition of terminal GalN units (Skovierova et al., 2010). Based on the findings reported in this thesis for RptA (Chapter 4), the latter is the more likely role of Rv3779 since its natural substrate is polyprenyl-phospho-N-acetylgalactosamine (polyprenyl-P-GalNAc) for which the equivalent is polyprenyl-P-Rhap in C. glutamicum (Chapter 4). Furthermore, the absence of GalN units in the AG of M. smegmatis correlates with a clear lack of a Rv3779 homolog in the M. smegmatis genome. Our apparent negative observation of a clear phenotypic change upon over expression of Rv3779 in M. smegmatis and the inability of Rv3779 to complement the C. glutamicumΔrptA mutant, presents conclusive evidence that C. glutamicum and M. tuberculosis share similar AG modifying activities. The role of GalN modifications in the AG of M. tuberculosis remains unknown.

As noted previously, glycosyltransferases involved in galactan and early PIM biosynthesis utilise high-energy sugar donor substrates and belong to the GT-A/B family present in the cytoplasm of the cell. Upon transition from galactan to arabinan biosynthesis, and early PIM to higher PIM biosynthesis, there is a change to GT-C family of glycosyltransferases, which make use of polyprenol phosphate substrates (Liu & Mushegian, 2003; Berg et al., 2007). With this in mind and the fact that rhamnosyl residues are most likely added in the periplasm, we set about looking for lipid linked sugar donor akin to DPA (Wolucka et al., 1994) and
DPM (Gurcha et al., 2002; Yokoyoma & Ballou, 1989). This is the first report of a rhamnosylated monophosphodecaprenol, which is the substrate for RptA.

To conclude, AftB and AftC represent novel AraTs in Corynebacterianeae, which are responsible for the addition of the terminal $\beta(1\rightarrow2)$ linked Araf residues and branching $\alpha(1\rightarrow3)$ Araf residues into arabinan, respectively. The identification of new cell wall biosynthetic drug targets is of great importance, especially with the emergence of MDR-TB and XDR-TB. Since AftB and AftC are EMB insensitive, these newly discovered DPA dependent AraTs represent promising candidates for further exploitation as potential drug targets. The disruption of the biosynthesis of mycobacterial AG and LAM formation by small molecule inhibition would potentially have a catastrophic effect on the structural integrity, composition and permeability of the TB cell wall. The molecular mechanism and biochemistry of the GT-C superfamily of glycosyltransferases are poorly understood. Considering that 80% of all pharmacological drug targets are located in the cytoplasmic membrane of cells, further investigation into these polytopic membrane bound enzymes is warranted. It is envisaged that the studies reported in this thesis could be expanded in terms of future experiments exploring: (i) over expression of recombinant arabinofuranosyltransferases, (ii) the development of a sensitive and selective AraT assay suitable for high-throughput screening of a chemical library, (iii) X-ray crystallographic structural studies, and (iv) in silico drug design.
Chapter 6
6. Materials and Methods

6.1. Chemicals, reagents and growth conditions

All chemicals and solvents were purchased from Sigma-Aldrich (Dorset, UK), Biorad (CA, USA) and Fischer Chemicals (UK) unless otherwise stated, and were of AnalR grade or equivalent. Enzymes were obtained from Sigma-Aldrich (Dorest, UK), Roche (Lewes, UK) or New England Biolabs (Boston, USA) and were of the highest grade available.

6.2. Bacterial growth conditions

C. glutamicum ATCC 13032 and E. coli DH5αmcr were grown in Luria-Bertani broth (LB, Difco) at 30°C and 37°C, respectively. The recombinant strains generated in this thesis were grown on complex brain–heart infusion medium LBHIS (5 g tryptone, 5 g NaCl, 2.5 g yeast extract, 18.5 g brain heart infusion (Difco), and 90.1 g sorbitol per litre). Kanamycin and ampicillin were used at a concentration of 50 µg/ml. The minimal medium CGXII was used for C. glutamicum (Eggeling & Bott, 2005). Samples for lipid and cell wall analysis were harvested after reaching an OD 3, followed by a saline wash and freeze drying.

M. smegmatis strains were grown in tryptic soy broth (TSB; Difco), containing 0.05% Tween 80 (TSBT). M. smegmatis and mycobacteriophages were also propagated on Middlebrook 7H9 broth (Difco), supplemented with 0.2% glycerol and 0.05% Tween 80. For phage infection experiments Tween 80 was omitted in all media. Solid media were made by adding 1.5% agar to the above-mentioned broths. The concentrations of antibiotics used for M. smegmatis were 100µg/ml for hygromycin and 20µg/ml for kanamycin.
6.2.1. Construction of C. glutamicum mutants

6.2.1.1. Construction of C. glutamicum ΔaftB and complementing strains

M. tuberculosis H37Rv DNA was obtained from the NIH Tuberculosis Research Materials and Vaccine Testing Contract at Colorado State University. All mutant strains in C. glutamicum were constructed at Institute for Biotechnology Research Centre, Juelich, Germany.

The vectors made were pMSX-Cg-aftB (NCgl2780), pMSX-Mt-aftB (Rv3805c), and pK19mobsacΔaftB (NCgl2780). To enable deletion of Cg-aftB, cross-over PCR was applied to generate the fragments carrying fused sequences adjacent to the gene in question. Cross-over PCR was applied with primer pairs AB and CD (Table 1.4) and C. glutamicum genomic DNA as template. Both amplified products were used in a second PCR with primer pairs AD to generate a fragment consisting of sequences adjacent to Cg-aftB, which was blunt end-ligated with SmaI-cleaved pK19mobsacB. All plasmids were confirmed by sequencing. The chromosomal deletion of Cg-aftB was performed, using two rounds of positive selection (Schafer et al., 1994), and its successful deletion was verified by use of different primer pairs. Plasmid pMSX-Mt-aftB and pMSX-Cg-aftB were introduced into C. glutamicum ΔaftB by electroporation with selection to kanamycin resistance (25 µg/ml).

To express M. tuberculosis aftB in C. glutamicum, the primer pair EF was used, with the restriction sites NdeI and XhoI underlined, using M. tuberculosis H37Rv chromosomal DNA as a template. The purified PCR fragment was ligated with accordingly digested pMSX to give pMSX-Mt-aftB. pMSX was prepared from pEKEx2 (Eikmanns et al., 1991) to generate a derivative providing an appropriate ribosome binding site together with a C-terminal His tag. It was created by the individual cleavage of pEKEx2 with Ndel and Xhol, each followed by Klenow treatment and religation. The intermediate construct was SalI/Dral-cleaved,
treated with mung bean nuclease, and ligated with the XbaI/MroI fragment from pET22b, which before use was treated with the Klenow fragment to eventually yield pMSX. To overexpress Cg-aftB, the primer pair GH was used to amplify C. glutamicum aftB, which was ligated with NdeI- and XhoI-cleaved pMSX to generate pM SX-Cg-aftB.

<table>
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<th>Primer name</th>
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<tr>
<td>B</td>
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<td>E</td>
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Site-specific mutations were introduced in Mt-aftB using appropriate mutagenic primers and pMSX-Mt-aftB as the double-stranded template (QuikChange kit, Stratagene). After linear amplification of the newly synthesized strands and DpnI digestion of parental strands, plasmids pMSX-Mt-aftB-D29A and pMSX-Mt-aftB-D30A were generated carrying the mutations as indicated. All plasmids were verified by sequencing.
6.2.1.2. Construction of C. glutamicumΔaftC and complementing strains

The genes analysed were Rv2673 and NCgl1505 from M. tuberculosis and C. glutamicum, respectively, termed aftC. To construct the deletion vector pK19mobsacB ΔaftC (NCgl1822), cross-over PCR was applied with primer pairs IJ and KL and C. glutamicum genomic DNA as template. Both amplified products were used in a second PCR with primer pairs IL to generate a 656 bp fragment consisting of sequences adjacent to Cg-aftC, which was ligated with BamHI–HindIII-cleaved pK19mobsacB. All plasmids were confirmed by sequencing.

The chromosomal deletion of Cg-aftC was performed using two rounds of positive selection (Schafer et al., 1994) and its successful deletion was verified by use of two different primer pairs.

6.2.1.3. Construction of C. glutamicumΔrptA and complementing strains

The vectors made were pVWEx-Cg-rptA, pVWEx-Rv3779, and pK19mobsacBrptA. To construct the latter deletion vector, crossover PCR was applied with primer pairs MN and OP and C. glutamicum genomic DNA as a template. Both amplified products were used in a second PCR with primer pair AD to generate a 587-bp fragment consisting of sequences adjacent to NCgl0543, which was treated with EcoRI and XbaI and ligated with pK19mobsacB to yield pK19mobsacBrptA. To enable the expression of NCgl0543, the primer pair QR was used to amplify rptA of C. glutamicum, which was treated with XbaI and ligated to yield pVWEx-Cg-rptA. The primer pair ST was used to amplify Rv3779 from M. tuberculosis. The resulting fragment was treated with XbaI to yield pVWEx-Rv3779. The correct orientation and the sequence identities were confirmed for all plasmids by sequencing.

The chromosomal deletion of the NCgl0543 gene was performed, as described previously, using two rounds of positive selection (Schafer et al., 1994) and its successful deletion was verified by use of primer pair UV. This yielded the expected fragment of 837 bp in the
deletion mutant and was termed *C. glutamicum* rptA and a fragment of 3,179 bp in wild-type *C. glutamicum*.

### 6.2.2. Construction of *M. smegmatis* mutants

#### 6.2.2.1. Construction of *M. smegmatis*Δ*aftC* and complementing strains

Approximately 1 kb of upstream and downstream flanking sequences of *MSMEG2785* were PCR amplified from *M. smegmatis* mc.155 genomic DNA using the primer pairs MS2785LL/MS2785LR and MS2785RL/MS2785RR, respectively. Following restriction digestion of the primer incorporated Van91I sites, the PCR fragments were cloned into Van91I-digested p0004S. The ligation mixture was transformed into *E. coli* Top10 cells and selected on LB agar with hygromycin at 37° overnight. Recovered plasmids were digested with PacI and ligated with PacI-digested phAE159 to yield the knockout plasmid pDM SMEMEG2785. The ligated knock-out plasmid and the temperature-sensitive mycobacteriophage phAE159 were then packaged into λ phage heads and transduced into *E. coli* HB101, as described previously (Bardarov et al., 2002), to yield phasmid DNA of the knockout phage phDM SMEMEG2785. The recovered phasmid was then transformed into *M. smegmatis* via electroporation and cells grown at 30°C for 3 days to yield phage particles. Generation of high titre phage particles involved 200 µl of 0.8 OD₆₀₀nm culture of *M. smegmatis* mixed into 5 ml molten 7H9 soft agar and overlaid on a basal layer of 7H9 agar. The phage stock was serially diluted (10⁻¹ - 10⁻¹⁰) with MP buffer (50mM Tris.HCl pH7.8, 150mM MgSO₄, 2mM CaCl₂) and 10 µl of each dilution spotted onto the solidified overlay of *M. smegmatis*. Plates were incubated at the permissive temperature of 30°C for 3 days, after which, the plaque forming units were counted and phage titre calculated (pfu = number of pfu in spot x dilution factor x 100). Original phage stock was then diluted to yield approximately 500 plaques per plate and mixed with 200 µl *M. smegmatis* in molten 7H9 agar, which was overlaid on solid 7H9 agar.
Chapter 6  Materials and Methods

Plates were incubated at 30°C for 3 days, resulting in a “lacy pattern”. The high titre phage was then collected and filtered through a 0.45 µm filter (Stover et al., 1991).

To perform specialized transduction, M. smegmatis cultures were grown to an OD_{600nm} 0.8 in 50 ml of TSB (0.05% Tween 80). Harvested cells were then washed three times with 50ml MP buffer and finally resuspended in 5 ml MP buffer. 0.5 ml of cells was then mixed with 0.5 ml of the optimum high titre lysate (10^{10} pfu/ml) and incubated overnight at 37°C (Stover et al., 1991; Bardarov et al., 2002). Cells were harvested and resuspended in 25 ml TSB and recovered overnight at 37°C, followed by plating onto TSB agar plates with hygromycin B. Plates were incubated at 37°C for 1-2 weeks. Deletion of MSMEG2785 in one hygromycin-resistant transductant was confirmed by Southern blot. To enable expression of MSMEG2785 and Rv2673, in the deletion mutant, these were amplified using primer pairs designed for subsequent cloning into the mycobacterial-shuttle vector pHV261 (Stover et al., 1991). All cloned fragments were verified by sequencing.

6.2.2.2. Southern blot analysis

Southern blot analysis was used to confirm gene knock-out mutants and was performed as specified in the Roche Applied Science kit manual. Sacl digested genomic DNA from M. smegmatis wild-type and M. smegmatisΔaftC strains were subjected to gel electrophoresis to separate the fragments, producing a laddering effect. In the mutant strain the aftC gene had been replaced with a hyg^R gene. There were two Sacl restriction sites in the wild-type and three in the mutant due to the presence of the hyg^R gene marker. PCR products of 1 kb of aftC left and right flanking regions were used as probes. Following Sacl digestion, one fragment was observed for wild-type and two fragments for M. smegmatisΔaftC. Following gel electrophoresis, the gel was depurinated (0.2M HCL, 15 min) and denatured (1.5M NaCl, 0.5M NaOH, 15 min). The gel was then washed to return to pH 7.4 and the DNA
subsequently transferred to positively charged nitrocellulose membrane via capillary action with 20 x SSC (3M sodium citrate, pH 7.0). DNA on membrane was then covalently bound by UV-crosslinking. The membrane was finally rinsed with distilled water and air-dried. Probe preparation involved boiling, snap freezing and labeling with digoxigenin-dUTP, using the DIG High Prime DNA labeling and Detection Start Kit (Roche), as described in the kit manual. The membrane was exposed to the probe at 65°C overnight, followed by washing and finally immunodetected with the antibody for 30 min at 25°C. The hybridized probes were visualized with the chemiluminescence.

6.2.3. mAGP purification procedures

After cultivation the thawed C. glutamicum/M. smegmatis cells were resuspended in phosphate buffered saline containing 2% Triton X-100 (pH 7.2) and disrupted by sonicaton (MSE Soniprep 150, 12 micron amplitude, 60s ON, 90s OFF for 10 cycles, on ice). The cell debris was centrifuged at 27,000 xg (Besra et al., 1995; Daffé et al., 1990). The pelleted material was extracted three times with 2% SDS in phosphate buffered saline at 95°C for 1 hr to remove associated proteins, successively washed with water, 80% (v/v) acetone in water, and acetone, and finally dried under compressed N₂ to yield a highly purified cell wall preparation (Besra et al., 1995; Daffé et al., 1990).

6.2.3.1. Acid hydrolysis and alditol acetate derivatisation

Cell wall preparations (1 mg) were hydrolysed in 250 µl of 2 M trifluoroacetic acid (TFA) at 120°C for 2 h (Besra et al., 1995; Daffé et al., 1990). After drying under compressed N₂, sugar residues were reduced with 50 µl NaB₂H₄ (10 mg/ml in ethanol:1M NH₃ OH (1:1)) and the resultant alditols per-O-acetylated using 100µl acetic anhydride with heating at 100 °C for 1 hr. After cooling, the reaction mix was combined with 100µl toluene and dried under compressed N₂. The resulting alditol acetates were partitioned between 2 ml H₂O and 2 ml
CHCl₃, mixed, centrifuged and the organic phase dried under compressed N₂. Alditol acetates were examined by GC as described in section 6.2.4.

6.2.3.2. Glycosidic linkage analysis

Cell wall preparations (10 mg) were suspended in 0.5 ml dimethyl sulfoxide (anhydrous) and 100 µl of 4.8 M dimethyl sulfinyl carbanion (generated by mixing NaH (2.4 g) with anhydrous DM SO (20 ml) and heated at 50°C with stirring for 3 h) (Besra et al., 1995; Daffé et al., 1990). The reaction mixture was stirred at room temperature for 1 hr and then 50 µl of CH₃I was slowly added and the suspension stirred for a further 1 hr. A further 100 µl of 4.8 M dimethyl sulfinyl carbanion and 50 µl CH₃I was added and the process repeated for a total of three times. The reaction mixture was then diluted with an equal volume of water and the entire contents dialysed against 10 L of water, overnight (1000 kDa MWCO Spectra/Por membrane 6, Spectrum labs, UK). The dialysed retentate was dried in a Savant SpeedVac SC110. The resulting per-O-methylated cell wall samples were resuspended in 2 ml of H₂O/DM SO (1:1, v/v) and applied to a C₁₈ Sep-Pak cartridge (Alltech, Deerfield, USA), which was pre-equilibrated sequentially with 20 ml ethanol, 5 ml H₂O, 5 ml acetonitrile, 5 ml ethanol and 10 ml H₂O. The material bound to the Sep-Pak was washed with 5 ml H₂O, 8 ml H₂O/acetonitrile (4:1, v/v) and then eluted with 2 ml acetonitrile and 2 ml ethanol, the latter eluants were pooled and dried in a Savant SpeedVac SC110. The per-O-methylated cell walls were hydrolysed, reduced and per-O-acetylated as described in chapter 6.2.3.1. Per-O-methylated and per-O-acetylated alditol acetates were examined by GC/MS as described in chapter 6.2.4

6.2.4. GC and GC/MS

GC analysis was performed using a Thermoquest Trace GC 2000. Samples were injected in the splitless mode. The column used was a DB225 (Supelco) ID 0.10 mm, length 10 m and df
0.05 µm. The oven was programmed to hold at an isothermal temperature of 275 °C for a run time of 15 min.

GC/MS was carried out on a Finnigan Polaris/GCQ PlusTM. The column used was a BPX-5 (Supelco). Injector temperature was set at 50°C, held for 1 min and then increased to 110°C at 20°C/min. The oven was held at 110°C then ramped to 290°C at 8°C/min and held for 5 min to ensure all the products had eluted from the column. All the data were collected and analyzed using X calibor (v.1.2) software.

6.3. Extraction and visualisation of lipids

6.3.1. Cell wall associated lipid extraction

Cells were grown as described in section 6.2, harvested, washed and freeze-dried. Cell wall associated free lipids were extracted twice from 100 mg of dried cells using 2 ml of CHCl₃/CH₃OH/H₂O (10:10:3, v/v/v) for 3 hr at 50 °C. Organic extracts were combined with 1.75 ml CHCl₃ and 0.75 ml H₂O, mixed and centrifuged at 1500 x g for 5 mins. The lower organic phase was recovered, back washed twice with 2 ml of CHCl₃/CH₃OH/H₂O (3:47:48, v/v/v), dried and resuspended with 200 µl of CHCl₃/CH₃OH/H₂O (10:10:3, v/v/v). A 10 µl aliquot was subject to TLC analysis, using silica gel plates (5554 silica gel 60F₂₅₄, Merck) developed in CHCl₃/CH₃OH/H₂O (60:16:2, v/v/v). TLC plates were charred, using 5% molybdophosphoric acid (MPA) in ethanol at 100°C to reveal free lipids. MPA was used for the detection of all lipids. Lipid species were revealed by charring the plates with a heat gun. α-Naphthol (α-NAP) was used for the detection of all glycolipids. α-NAP solution was prepared from 6 g of α-naphthol dissolved in 25 ml of sulfuric acid and 450 ml ethanol. Glycolipid species were revealed by gently charring the plates with a heat gun. The Dittmer and Lester reagent was prepared according to the modified procedure of Muthing & Radloff (1998) for the detection of phospholipids and glycopospholipids. Solution A consisted of 40
g molybdenum VI oxide dissolved in 1 L of boiling sulfuric acid, solution B contained 1.7 g of solid molybdenum dissolved into 0.5 L of solution A. Solutions A and B were then mixed with H₂O to give a final solution in the ratio of solution A:solution B:H₂O, 1:1:4. Plates were sprayed with the stain and left to develop for 5 min at room temperature.

### 6.3.2. Exported (media filtrate) lipid extraction

Exported lipids were analysed in a similar manner to that in C. glutamicum cells were cultured as described above. Once the A₆₀₀ reached ~0.5, cultures were labelled with 1 μCi [1,2-¹⁴C]acetic acid (specific activity 50-62 mCi/mmol, GE Healthcare, UK) and further incubated for 8 hr. Cells were harvested by centrifugation at 27,000 x g for 30 min and the supernatant carefully removed and dried using a Savant SpeedVac SC110. The residue was resuspended into 2 ml of CHCl₃/CH₃OH/H₂O (10:10:3, v/v/v) for 3 hr at 50 °C. Organic extracts were combined with 1.75 ml CHCl₃ and 0.75 ml H₂O, mixed and centrifuged at 1,500 x g for 5 mins. The lower organic phase was recovered, back washed twice with 2 ml of CHCl₃/CH₃OH/H₂O (3:47:48, v/v/v), dried and resuspended with 200 μl of CHCl₃/CH₃OH/H₂O (10:10:3, v/v/v). An aliquot of each extraction was subjected to scintillation counting using 10 ml EcoScint and analysed by TLC using silica gel plates (5554 silica gel 60F₂₅₄, Merck) developed in CHCl₃/CH₃OH/H₂O (60:16:2, v/v/v). TLCs were exposed to X-ray film (Kodak X-Omat) for 2 days to visualise radiolabelled lipids by autoradiography.

### 6.3.3. Cell wall bound lipid extraction

Cells were grown as described, harvested, washed and freeze-dried. Cells (100 mg) were extracted by two consecutive extractions with 2 ml of CHCl₃/CH₃OH/H₂O (10:10:3, v/v/v) for 3 hr at 50°C. The bound lipids from the de-lipidated extracts were released by the addition of 2 ml of 5% aqueous solution of tetra-butyl ammonium hydroxide (TBAH),
followed by overnight incubation at 100 °C. After cooling, water (2 ml), CH$_2$Cl$_2$ (4 ml) and CH$_3$I (500 µl) were added and mixed thoroughly for 30 min. The lower organic phase was recovered following centrifugation and washed three times with water (4 ml), dried and resuspended in diethyl ether (4 ml). After centrifugation, the clear supernatant was again dried and resuspended in CH$_2$Cl$_2$ (100 µl). An aliquot (10 µl) from each strain was subjected to TLC, using silica gel plates (5554 silica gel 60F$_{254}$, Merck), developed in petroleum ether/acetone (95:5, v/v) and charred using 5% MPA in ethanol at 100 °C to reveal CMAMES/MAMES and compared to known standards (Gande et al., 2004).

### 6.3.4. Analysis of AftB muteins

Recombinant C. glutamicum strains deleted of the chromosomal Cg-aftB copy, but carrying either pM SX, pM SX-M t-aftB, pM SX-M t-aftB-D29A or pM SX-M t-aftB-D30A were each grown in LB up to an OD of 4. Cells were harvested by centrifugation, washed and resuspended in 30 ml of 50 mM TrisHCl pH 7.4 buffer, containing 200 mM NaCl and 50 mM imidazole, and disrupted by probe sonication. Centrifugation at 27,000 x g resulted in a clear supernatant, which was applied to a 1 ml HiTrap™ Chelating HP column (GE Healthcare) using an ÅTKA chromatography system. The column was initially washed with 10 ml of the aforementioned buffer and bound proteins subsequently eluted with 2 ml of the same buffer, but containing 500 mM imidazole. Eluted proteins were precipitated, dried, and resuspended in 10 µl loading buffer and SDS-PAGE carried out on a 10% polyacrylamide gel, which was subsequently stained using 0.05% Coomassie-G250 in 10% acetic acid and 25% isopropanol. Bands of interest were excised and subjected to in-gel digestion with trypsin before peptide mass fingerprinting. Peptides were extracted by sequential addition of water (12 µl) and 0.1% (v/v) trifluoroacetic acid (TFA) in 30% (v/v) acetonitrile (10 µl), and
analyzed manually, using an Applied Biosystems Voyager STR MALDI-TOF mass spectrometer.

6.4. Extraction and Purifications of lipoglycans

Lipoglycans were extracted from delipidated cells as described previously (Nigou et al., 1997; Mishra et al., 2008). Dried cells were resuspended in water, disrupted by sonication (MSE Soniprep 150, 12 micron amplitude, 60s ON, 90s OFF for 10 cycles, on ice) and an equal volume of ethanol was added and the mixture heated under reflux, followed by centrifugation and recovery of the supernatant. This extraction process was repeated five times and the combined supernatants, containing lipoglycans, neutral glycans and proteins were dried, followed by hot-phenol-H$_2$O treatment. The aqueous phase was dialysed against water, performed using a low molecular weight cut off dialysis tubing, (Spectra/Por membrane 6, MWCO 3,500 kDA from Spectrum Labs). The retentate was dried, resuspended in water and digested with $\alpha$-amylase, DNase, RNase, chymotrypsin and trypsin, and dialysed once again to remove residual impurities and enzymes.

The crude lipoglycan extract was dried and resuspended in buffer A (50 mM ammonium acetate and 15% propan-1-ol) and subjected to Octyl Sepharose CL- 4B HIC (2.5 cm x 50 cm) (Leopold & Fischer, 1993). The column was washed initially with 4 column volumes of buffer A to ensure removal of neutral glycans followed by buffer B (50 mM ammonium acetate and 50% propan-1-ol). The eluent was collected and concentrated to approximately 1 ml and precipitated using 5 ml of ethanol. The sample was dried using a Savant Speedvac and then resuspended in buffer C (0.2 M NaCl, 0.25% sodium deoxycholate (w/v), 1 mM EDTA and 10 mM Tris-HCl, pH 8) to a final concentration of 200 mg/ml.
The sample was gently mixed and left to incubate for 48 h at room temperature. The sample was then loaded onto a 200 ml Sephacryl S-200 column previously equilibrated with buffer C. The sample was eluted with 400 ml of buffer C at a flow rate of 3 ml/h, collecting 1.5 ml fractions.

The fractions were monitored by SDS-PAGE using Pro-Q emerald glycoprotein stain and individual fractions pooled and dialyzed extensively against buffer D (10 mM Tris-HCl, pH 8, 0.2 M NaCl, 1 mM EDTA) for 72 h with frequent changes of buffer. The samples were further dialyzed against deionized water for 48 h with frequent changes of water, lyophilized and stored at -20°C.

6.4.1. NMR spectroscopic analysis of WT-LAM and AftC-LAM.

NMR spectra of LAM samples were recorded on a Bruker DMX-500 equipped, with a double resonance (1H/X)-BBi z-gradient probe head. All samples were exchanged in D$_2$O (D, 99.97% from Euriso-top, Saint-Aubin, France), with intermediate lyophilization, and then dissolved in 0.5 ml D$_2$O and analyzed at 313K. The $^1$H and $^{13}$C NMR chemical shifts were referenced relative to internal acetone at 2.225 and 34.00 ppm, respectively. All the details concerning NMR sequences used and experimental procedures were described previously (Gilleron et al., 1999; Gilleron et al., 2000).

6.4.2. SDS PAGE

The method used in this study was the discontinuous gel method using a Hoefer “mighty small” SE200 vertical slab gel system. Resolving gels of either 12 % or 15% acrylamide (w/v) and a stacking gel of 6 % (w/v) acrylamide were prepared and cast in the apparatus according to the manufacturer’s specifications and and run at 300 mV and 20 mA/gel until completion. Lipoglycan samples (10 µg) were analysed on 15% polyacrylamide gels and
visualised using Pro-Q Emerald Glycoprotein stain (Invitrogen) according to the manufacturer's protocol. Protein samples (usually 10–20 µg) were run on 12% polyacrylamide gels and visualised by immersing gels in 20 ml Coomassie brilliant blue R-250 (CBB) stain (5% glacial acetic acid, 0.0025% CBB (w/v), 50% methanol) and left for 3-4 hr on a rotating platform. Excess stain was removed by rinsing in destaining solution (10% glacial acetic acid in 40% methanol).

6.5. Cell free [14C]-labeling assays

6.5.1. Preparation of C. glutamicum and M. smegmatis membranes

Pelleted cells, wet weight 5 g were resuspended in 50 mM MOPS (pH 7.9), 5 mM β-mercaptoethanol and 10 mM MgCl₂. Resuspended cells were sonicated (MSE Soniprep 150, 12 micron amplitude, 60s ON, 90s OFF for 10 cycles, on ice), centrifuged at 23000 x g for 20 mins at 4°C, and the resulting supernatant re-centrifuged at 100,000 x g for 90 mins at 4°C to isolate cell membranes which were collected and concentrated to a protein concentration of 15-20 mg/ml in 50 mM MOPS (pH 7.9), 10 mM MgSO₄, 5 mM β-mercaptoethanol (Chapter 6.5.3).

6.5.2. Preparation of C. glutamicum and M. smegmatis cell wall material

Cells (10 g) were resuspended in 35 ml of 50 mM MOPS (pH 7.9), 10 mM MgSO₄, 5 mM β-mercaptoethanol and subject to probe sonication (MSE Soniprep 150, 12 micron amplitude, 60s ON, 90s OFF for 10 cycles, on ice). The cell slurry was centrifuged at 27,000 x g for 20 min at 4 °C, the pellet was recovered and the resulting supernatant further centrifuged at 100,000 x g for 90 min at 4 °C. The pellet from the 27,000 x g spin was resuspended in 24 ml 50 mM MOPS (pH 7.9), 10 mM MgSO₄, 5 mM β-mercaptoethanol and 32 ml of Percoll (Sigma-Aldrich), mixed thoroughly and centrifuged at 27,000 x g for 60 min at 4 °C. The
upper band (corresponding to *C. glutamicum* cell wall “P60” material) was removed, washed with 50 mM MOPS (pH 7.9), 10 mM MgSO₄, 5 mM β-mercaptoethanol with further centrifugation to remove Percoll and the final cell wall fraction resuspended in 50 mM MOPS (pH 7.9), 10 mM MgSO₄, 5 mM β-mercaptoethanol to a final protein concentration of 8 – 10 mg/ml (Chapter 6.5.3).

### 6.5.3. Estimation of protein concentration

The concentration of total protein present in cell wall material and membranes were measured using the Bicinchoninic protein assay (BCA) kit (Pierce). The assay was performed according to the manufacturer’s protocol. The assay was calibrated over the range 2.5 – 5.0 µg/ml by using a range of standards derived from a 2 mg/ml stock solution of bovine serum albumin (BSA) (Pierce). Samples, standards and blanks were assayed in triplicate. Samples were also diluted 1 in 5 to ensure a reading was obtained within the limits of the standard curve. The assay was incubated at 37 °C for 30 min and the colorimetric change was measured at 562 nm.

### 6.5.4. Arabinofuranosyltransferase assays

#### 6.5.4.1. Arabinofuranosyltransferase activity with membrane preparations of *C. glutamicum*, *C. glutamicum*ΔaftB, and *C. glutamicum*ΔaftB pM SX-M t-aftB

Membranes were prepared as described previously (Alderwick et al., 2006; Lee et al., 1997) and resuspended in 50 mM MOPS (pH 7.9), containing 5 mM β-mercaptoethanol and 10 mM MgCl₂ (buffer A) to a final concentration of 15-10 mg/ml. The neoglycolipid acceptor α-D-Araf-(1→5)-α-D-Araf-O-C₈ (stored in C₂H₅OH) and DP[^14C]A (Lee et al., 1995; Lee et al., 1998) (stored in CHCl₃/CH₃OH, 2:1, v/v) were aliquoted into a 1.5 ml Eppendorf tube to a final concentration of 2 mM and 200,000 cpm (90 µM), respectively, and dried under nitrogen. The basic arabinofuranosyltransferase assay was carried out as described previously.
(Lee et al., 1997) with modifications. IgePal™ (Sigma-Aldrich) was added (0.1%, v/v) with the appropriate amount of buffer A (final volume 80 µl). Tubes were sonicated for 15 min to resuspend lipid linked substrates and then mixed with the remaining assay components, which included membrane protein (1 mg) from either C. glutamicum, C. glutamicum ΔaftB or C. glutamicum ΔaftB pMSX-M t-aftB, 1 mM ATP, 1 mM NADP and in some cases EMB (0-1 mg/ml). Assays were incubated for 1 h at 37 °C and quenched by the addition of 533 µl CHCl₃/CH₃OH (1:1, v/v). After mixing and centrifugation at 27,000 x g for 15 min at 4 °C, the supernatant was removed and dried under nitrogen. The residue was then resuspended in 700 µl of CH₃CH₂OH/H₂O (1:1, v/v) and loaded onto a 1 ml SepPak strong anion exchange cartridge (Supelco), pre-equilibrated with CH₃CH₂OH/H₂O (1:1, v/v). The column was washed with 2 ml CH₃CH₂OH and the eluate collected, dried and partitioned between the two phases, arising from a mixture of n-butanol (3 ml) and water (3 ml). The resulting organic phase was recovered following centrifugation at 3,500 x g and the aqueous phase again extracted twice with 3 ml of water-saturated n-butanol. The pooled extracts were back-washed twice with n-butanol-saturated water (3 ml). The n-butanol fraction was dried and resuspended in 200 µl n-butanol. The extracted radiolabeled material was quantified by liquid scintillation counting using 10% of the labelled material and 5 ml of EcoScintA (National Diagnostics, Atlanta). The incorporation of [14C]Araf was determined by subtracting counts present in control assays (incubations in the absence of acceptor). The remaining labeled material was subjected to TLC using silica gel plates (5735 silica gel 60F₂₅₄, Merck) developed in CHCl₃:CH₂OH:H₂O:NH₄OH (65:25:3.6:0.5, v/v/v/v). TLC-autoradiograms were obtained by exposing TLCs to Kodak X-Omat film for 3 days.
6.5.4.2. Analysis of arabinofuranosyltransferase reaction products prepared from C. glutamicum and C. glutamicumΔaftB membranes

Large-scale reaction mixtures containing cold DPA (200 µg, 0.75 mM) (Lee et al., 1997) and 50 mM of the acceptor α-D-Araf-(1→5)-α-D-Araf-O-C₈ were mixed and given an initial incubation at 37 °C with membranes prepared from either C. glutamicum (EMB also added to reaction mixtures at a concentration of 100 µg/ml) or C. glutamicumΔaftB for 1 h. The assays were replenished with fresh membranes (1 mg) and re-incubated for 1 h at 37 °C with the entire process repeated thrice. Products were extracted from reaction mixtures by n-butanol/water phase separation as described earlier to extract products. Products were applied to preparative TLC plates, developed in CHCl₃:CH₃OH:H₂O:NH₄OH (65:25:3.6:0.5, v/v/v/v) and sprayed with 0.01% 1,6-diphenylhexatriene in petroleum-ether:acetone (9:1, v/v), and the products localized under long-wave (366 nm) UV light (Lee et al., 1997). The plate was then re-developed in toluene to remove the reagent and the bands recovered from the plates by extraction with n-butanol. The butanol phases were washed with water saturated with n-butanol and the dried products subjected to ¹H-NMR, ES-MS and GC/MS as previously described (Lee et al., 1997).

6.5.4.3. Arabinofuranosyltransferase activity with membrane preparations of M. smegmatis, M. smegmatis pM V261-M t-aftC, M. smegmatisΔaftC and M. smegmatisΔaftC pM V261-M t-aftC

Membranes were prepared as described previously (Lee et al., 1997; Alderwick et al., 2006a) and re-suspended in 50 mM MOPS (pH 7.9), containing 5 mM β-mercaptoethanol and 10mM MgCl₂ (buffer A) to a final concentration of 15-10 mg/ml. The neoglycolipid acceptors used in this study were α-D-Araf-(1→5)-α-D-Araf-(1→5)-α-D-Araf-(1→5)-α-D-Araf-(1→5)-α-D-Araf-(1→5)-α-D-Araf-(1→5)-α-D-Araf-O-(CH₂)₈NH₂ (Ara5,) and α-D-Araf-(1→5)-α-D-Araf-O-(CH₂)₉NH₂ (Ara₅₉) and α-D-Araf-(1→5)-α-D-Araf-O-(CH₂)₉CH₃ (Ara₆) (Lee et al., 1995; 1998). The acceptors (either Ara₂ or Ara₅₉) and DP[¹⁴C]A (Lee et al., 1995; 1998) (stored in CHCl₃/CH₃OH, 2:1, v/v) were aliquoted into 1.5 ml Eppendorf tubes
to a final concentration of 2 mM and 200 000 cpm (90 mM), respectively, and dried under nitrogen. The arabinofuranosyltransferase assay was carried out as described previously (Lee et al., 1997) with modifications. IgePal™ (Sigma-Aldrich) was added (0.1%, v/v) with the appropriate amount of buffer A (final volume 80 ml). Tubes were sonicated for 15 min to re-suspend lipid linked substrates and then mixed with the remaining assay components, which included membrane protein from either M. smegmatis, M. smegmatisΔaftC, M. smegmatis pMV261-Mt-aftC and M. smegmatisΔaftC pMV261-Mt-aftC.

M. smegmatis pMV261-Mt-aftC M. smegmatisΔaftC or M. smegmatisΔaftC pMV261-Mt-aftC (1 mg), 1 mM ATP, 1 mM NADP and in some cases EMB (0–1 mg ml⁻¹). Assays were incubated for 1 h at 37°C and quenched by the addition of 533 ml CHCl₃/CH₃OH (1:1, v/v). After mixing and centrifugation at 27 000 g for 15 min at 4°C, the supernatant was removed and dried under nitrogen. The residue was then re-suspended in 700 ml of CH₂OH/H₂O (1:1, v/v) and loaded onto a 1 ml SepPak strong anion exchange cartridge (Supelco), pre-equilibrated with CH₂OH/H₂O (1:1, v/v). The column was washed with 2 ml CH₂OH and the eluate collected, dried and partitioned between the two phases arising from a mixture of n-butanol (3 ml) and water (3 ml). The resulting organic phase was recovered, following centrifugation at 3500 g, and the aqueous phase again extracted twice with 3 ml of water-saturated n-butanol. The pooled extracts were back-washed twice with n-butanol-saturated water (3 ml). The n-butanol fraction was dried and re-suspended in 200 ml butanol. The extracted radiolabelled material was quantified by liquid scintillation counting, using 10% of the labelled material and 5 ml of EcoScintA (National Diagnostics, Atlanta). The incorporation of [¹⁴C]AraF was determined by subtracting counts present in control assays (incubations in the absence of acceptor). The remaining labelled material was subjected to thin-layer chromatography (TLC) using either isopropanol/ acetic acid/water (8:1:1, v/v/v) for the assays utilizing the Ara₅ acceptor or CHCl₃/CH₂OH/H₂O/NH₄OH.
(65:25:3.6:0.5, v/v/v/v) in the case of the Ara acceptor on aluminum-backed Silica Gel 60 F254 plates (Merck, Darmstadt, Germany). Autoradiograms were obtained by exposing TLCs to X-ray film (Kodak X-Omat) for 3 days.

6.5.4.4. Characterization of a\((1\rightarrow3)\)-arabinofuranosyltransferase activity with membranes prepared from \(M.\ \text{smegmatis}\), \(M.\ \text{smegmatis}\Delta\text{aftC}\) and \(M.\ \text{smegmatis}\Delta\text{aftC}\ pM\ V261-\text{Mt-aftC}\)

Large-scale reaction mixtures containing cold DPA (200 mg, 0.75 mM) (Lee et al., 1997) and 50 mM of the acceptor Ara\(_5\) were mixed and given an initial incubation at 37°C with membranes prepared from either \(M.\ \text{smegmatis}\), \(M.\ \text{smegmatis}\Delta\text{aftC}\) or \(M.\ \text{smegmatis}\Delta\text{aftC}\ pM\ V261-\text{Mt-aftC}\) for 1 h. The assays were replenished with fresh membranes (1 mg) and re-incubated for 1 h at 37°C with the entire process repeated thrice. Products were extracted from reaction mixtures by n-butanol/water phase separation as described earlier to extract products. Products were applied to preparative TLC plates, developed in isopropanol/acetic acid/water (8:1:1, v/v/v) and sprayed with 0.01% 1,6-diphenylhexatriene in petroleum-ether/acetone (9:1, v/v), and the products localised under long-wave (366 nm) UV light (Lee et al., 1997). The plate was then re-developed in toluene to remove the reagent and the bands recovered from the plates by extraction with n-butanol. The n-butanol phases were washed with water saturated with n-butanol and the dried products subjected to GC (Sassaki et al., 2005) and GC/M S as described (Lee et al., 1997; Alderwick et al., 2006a).

6.5.4.5. \([^{14}\text{C}]\)-carbohydrate labeling of \(C.\ \text{glutamicum}\) and \(C.\ \text{glutamicum}\Delta\text{rptA}\) glycolipids.

Cells (10 g) from \(C.\ \text{glutamicum}\) and \(C.\ \text{glutamicum}\Delta\text{rptA}\) were resuspended in 35 mL of 50 mM MOPS (pH 7.9), 10 mM MgSO\(_4\), 5 mM β-mercaptoethanol (buffer A) and subjected to probe sonication for 60 s on and 90 s off (repeated for a total of 10 cycles). The cell slurry was centrifuged at 27,000 \(\times\) g for 20 min at 4°C; the pellet was recovered and the resulting
supernatant further centrifuged at 100,000 \times g for 90 min at 4°C. Purified C. glutamicum membranes were recovered and resuspended in buffer A to a final concentration of 15-20 mg/mL. The pellet from the 27,000 \times g spin was resuspended in 24 mL of buffer A and 32 mL of Percoll, mixed thoroughly, and centrifuged at 27,000 g for 60 min at 4°C. The upper band (corresponding to C. glutamicum cell wall “P60” material) was removed, washed with buffer A with further centrifugation to remove Percoll, and the final cell wall fraction resuspended in buffer A to a final concentration of 8-10 mg/mL.

The initial, standard reaction to measure incorporation of [14C]GlcNAc from UDP-[14C]GlcNAc, [14C]Rha from dTDP-[14C]Rha, [14C]Gal from UDP-[14C]Gal, and [14C]Ara from p[14C]Rpp were as follows. Decaprenol phosphate (50 µg, 5 mg/mL stored in ethanol, 10 µL) was dried under nitrogen and was resuspended by the addition of 50 µL of a 1% IgePal CA-630 (Sigma Aldrich) solution in buffer A and sonicated. The basic assay mix consisted of 2 mg of membranes and 0.5 mg cell wall “P60” from either C. glutamicum or C. glutamicumΔrptA, 1 mM ATP, and 1 mM NADP in a final volume of 160 µL of buffer A and initiated by the addition of either 100,000 cpm UDP-[14C]GlcNAc (ammonium salt; Amersham, Bucks, United Kingdom, specific activity 214 mCi/mmol), 200,000 cpm dTDP-[14C]Rha (enzymatically synthesized as described in Mikusova et al. (1996) specific activity 300 µCi/µmol kindly donated by Prof Michael McNeil, Colorado State University), UDP-[14C]Gal (ammonium salt; Amersham, Bucks, United Kingdom, specific activity 257 mCi/mmole) and 50,000cpm p[14C]Rpp (enzymatically synthesized as described in section 6.6.2, specific activity 250 µCi/µmol). Reactions were incubated at 37°C for 2 hr and quenched by the addition of CHCl3/CH3OH (1066 µL, 1:1, v/v) and mixed at room temperature for 30 min. The samples were then centrifuged at 27,000 \times g at room temperature for 20 min and the supernatant was removed from the pelleted material and H2O
(230 µl) and CHCl₃ (537 µl) added to the previously extracted supernatant, mixed at room temperature for 30 min and then centrifuged at 5,000 × g. The lower organic fraction was recovered and washed twice with CHCl₃/CH₃OH/H₂O (613 µl, 3:47:48, v/v/v). After centrifugation at 5,000 × g for 10 min, the lower organic fraction was recovered and dried under compressed nitrogen. The resulting extract was resuspended in CHCl₃/CH₃OH (100 µl, 2:1, v/v). An aliquot (10 µl) from each assay was subjected to scintillation counting and the remainder of the sample analyzed by TLC using silica gel plates (5735 silica gel 60F₂₅₄, Merck), developed in CHCl₃/CH₃OH/NH₄OH/H₂O (65:25:0.5:3.6, v/v/v/v) and visualized by autoradiography by exposure of Kodak X-Omat AR film to the TLC plates to reveal incorporation of either [¹⁴C]GlcNAc, [¹⁴C]Rha, [¹⁴C]Gal or [¹⁴C]Ara into solvent extractable lipids.

6.5.4.5.1. Preparation of p[¹⁴C]Rpp

Radiolabelled p[¹⁴C]Rpp was prepared as described (Hove-Jensen, 1985), but with modifications. 200 µCi [¹⁴C]-D-glucose (0.7 µmoles, Amersham) was dried under compressed N₂ and resuspended in 400 µl of 50 mM HEPES (pH 7.6), 5 mM MgCl₂ and 0.5 mM MnCl₂. ATP (1 mM) and β-NADP (4 mM) was added to the reaction mixture before the addition of 5 units of hexokinase powder (Sigma Aldrich, UK) and the reaction mixture was incubated at room temperature for 5 min. Glucose-6-phosphate dehydrogenase (5 units, Sigma Aldrich, UK) was added and incubated for 5 min at room temperature before the addition of 6-phosphogluconate dehydrogenase (5 units, Sigma Aldrich, UK) and further incubated at 37 °C for 5 min. Phosphoriboseisomerase (5 units, Sigma Aldrich, UK) was then added and the reaction mixture incubated at 30 °C for 5 min. PRPP synthetase was prepared as described in Huang et al. (2005).
6.5.4.6. Chemical identification of decaprenol-1-monophosphate-rhamnopyranose.

Membranes from *C. glutamicum* or *C. glutamicum*ΔrptA were assayed as described above, but with or without the addition of 50 µg/mL tunicamycin. Decaprenylmonophosphate (50 µg, 5 mg/ml stored in CHCl₃/CH₃OH (2:1, v/v)), was dried under nitrogen and resuspended by the addition of 107 µl of a 1% IgePal CA-630 (Sigma Aldrich) solution in buffer A and sonicated. The basic assay mix consisted of 2 mg of membranes, 0.5 mg cell wall “P60”, 1 mM ATP, 1 mM NADP in a final volume of 160 µl and initiated by the addition of 5 mM of dTDP-Rha or 200,000 cpm of dTDP-[¹⁴C]Rha chemically synthesized as previously described (enzymatically synthesized as described in Mikusova et al., (1996) specific activity 300 µCi/µmol kindly donated by Prof Michael McNeil, Colorado State University) and incubated at 37 °C for 1 h. Assays performed using dTDP-[¹⁴C]Rha were processed as described above, whereas assays performed using cold dTDP-Rha were replenished by the addition of a further 2 mg of membranes and 0.5 mg of cell wall “P60” with further incubation at 37°C for 1 h. This process was repeated for a total of three additions with a final assay volume of 250 µL. Assays were quenched by the addition of CHCl₃/CH₃OH (3330 µl, 1:1, v/v), 1.6 M NaOH (250 µl) and mixed at room temperature for 30 min. The samples were then centrifuged at 27,000 × g at room temperature for 30 min and the supernatant was removed from the pelleted material. H₂O (720 µl) and CHCl₃ (1678 µl) was added to the previously extracted supernatant, mixed at room temperature for 30 min and then centrifuged at 5,000 × g. The lower organic fraction was recovered and washed twice with 1.92 ml of CHCl₃/CH₃OH/H₂O (1.92 ml, 3:47:48, v/v/v). After centrifugation at 5,000 × g for 10 min, the lower organic fraction was recovered and dried under nitrogen. The resulting extract was resuspended in CHCl₃/CH₃OH (100 µl, 2:1, v/v) and analyzed by electrospray mass spectrometry (ES-MS) in the negative mode on a Micromass LCT mass spectrometer.
6.5.5. Treatment of *M. smegmatis* and *M. smegmatisΔaftC* with sub-inhibitory concentrations of EMB and subsequent lipoglycan analysis.

*M. smegmatis* and *M. smegmatisΔaftC* were grown in 100 mL TSB as described above. Once an O.D$_{600}$ of 0.4 was reached, EMB was added to cultures of *M. smegmatisΔaftC* at a concentration of 0 and 0.5 and 1.0 µg ml$^{-1}$. After a further 2 h incubation, 66 µCi of [U-$^{14}$C]-D-glucose (specific activity 300 mCi/mmol, ARC radiochemicals) was added to all cultures and incubated for a further 48 h. Cells were harvested by centrifugation and the lipoglycans were extracted and analysed by SDS-PAGE, as described above. Further analysis on the [$^{14}$C]-incorporated sugars was performed as described. Equal counts of extracted [$^{14}$C]-labelled lipoglycans (50,000 cpm) from each growing culture were then hydrolyzed by using 100 µL of 2 M TFA for 1 h at 120°C, dried, resuspended in 2 mL of CHCl$_3$/H$_2$O (1:1, v/v), and the upper aqueous phase recovered and dried. The residual radiolabeled sugars were resuspended in 20 µL of H$_2$O and subjected to TLC using cellulose-coated aluminum plates (HPTLC-Aluminum Cellulose, Merck) and developed three times in formic acid/H$_2$O/tert-butanol/methyl ethyl ketone (3:3:8:6, v/v/v/v). Sugars were visualized by TLC exposure to X-ray film (Kodak X-Omat) and quantified by densitometry.

6.5.6. Treatment of WT-LAM, AftC-LAM and Pam$_3$CSK$_4$ with H$_2$O$_2$

All immunomodulatory analyses were conducted by Jerome Nigou at the Department of Medical Microbiology and Infection Control, Amsterdam, Netherlands. WT-LAM, AftC-LAM and Pam$_3$CSK$_4$ (Invivogen) were treated with H$_2$O$_2$ as described (Morr et al., 2002; Zahringer et al., 2008). WT-LAM and AftC-LAM (0.2 mg ml$^{-1}$) and Pam$_3$CysK$_4$ (1 µg ml$^{-1}$) were incubated in the dark at 4°C in the absence or presence of 1% H$_2$O$_2$. After incubation for various periods of time, the samples were snap-frozen, using liquid nitrogen, and lyophilized.
6.5.7. Cell culture

The human myeloid THP-1 monocyte/macrophage human cell line was maintained in continuous culture with RPMI 1640 medium (Invitrogen), 10% fetal calf serum (Lonza) in an atmosphere of 5% CO$_2$ at 37 °C, as non-adherent cells. Purified native or modified lipoglycans as well as the other stimuli were added in duplicate or triplicate to monocyte/macrophage cells and then incubated for 20 h at 37 °C. HEK 293 cells transfected with TLR2 (Mambula et al., 2002) were kept in DMEM (Invitrogen) containing 10% FCS, 100 units ml$^{-1}$ penicillin, 100 µg ml$^{-1}$ streptomycin, 0.5 mg ml$^{-1}$ GW418, 2 mM L-glutamine, and 110 mg L$^{-1}$ pyruvate.

6.5.8. Cell stimulation assays

Cells (HEK 293 cells were first released by trypsinisation) were washed with and resuspended in culture medium at 1.25x10$^6$ cells ml$^{-1}$. 80 µl (1x10$^5$ cells) was transferred to a 96-well U-bottom plate (Greiner) and left for 2 h, followed by incubations in triplicate with H$_2$O$_2$-treated WT-LAM, AftC-LAM, Pam$_3$CSK$_4$ or with LPS (from Salmonella enterica serovar Abortus equi (Sigma-Aldrich L 5886)) in a final stimulation volume of 100 µl. Unstimulated cells served as controls. Culture supernatants were harvested after 24 h by centrifugation and stored at -80°C for cytokine measurements using enzyme-linked immunosorbent assay (ELISA). Human IL-8 and TNF-α concentrations were determined in ELISA according to the manufacturer’s instructions (Invitrogen and R&D systems, respectively).

6.5.9. DC-SIGN-Fc ELISA

WT-LAM and AftC-LAM (1 µg ml$^{-1}$ in saline 100 µL) were coated on NUNC Maxisorp plates (Roskilde) overnight at 4°C. Plates were blocked with 1% bovine serum albumine (BSA) and serially diluted DC-SIGN-Fc (van Die I et al., 2003) was added for 2 h at room
temperature. Then the plates were washed four times with Tris-buffered saline with 1 mM MgCl₂, 2 mM CaCl₂, 0.05% Tween-80 (TSMT) and incubated with goat-anti-human IgG antibody conjugated with peroxidase (Jackson Immunoresearch). Plates were washed eight times with TSMT and developed using o-phenylenediamine dihydrochloride and absorption measured at 490 nm.
Chapter 7
7. References


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196


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