The role of CwlM in peptidoglycan synthesis and remodelling in Mycobacteria

Thesis submitted for the degree of
Doctor of Philosophy at the University of Leicester

By

Baleegh Abdulzahra Kadhim (MSc)

Department of Infection, Immunity and Inflammation

University of Leicester

September 2019
Declaration

I, Baleegh A. Kadhim declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy at the University of Leicester. It has not been submitted before for any degree or examination at this or any other University.

_________________________   _______________________
Baleegh Kadhim               Prof. Galina Mukamolova

____________________     Day of   __________September____  2019
9th                      2019
Papers and presentations from this thesis


**Conference** : Microbiology Society Annual Conference in Birmingham

**Presentation** : Poster

**Year** : 2018

**Conference** : World TB Day Conference in Leicester

**Presentation** : oral

**Year** : 2019
Abstract

The role of CwlM in peptidoglycan synthesis and remodelling in Mycobacteria

Baleegh Abdulzahra Kadhim

Tuberculosis remains a major global health problem, claiming around 1.5 million lives annually, and its causative agent *Mycobacterium tuberculosis* (*Mtb*) can persist in humans for decades. *Mtb* has a complex cell wall, biosynthesis and remodelling of which is controlled by serine/threonine protein kinase signalling. Protein kinase B (PknB) is indispensable for mycobacterial growth and it phosphorylates CwlM, a predicted N-acetylmuramyl-L-alanine amidase. However, recent findings suggest that CwlM has a non-enzymatic function and regulates biosynthesis of peptidoglycan precursors via activation of MurA.

This study was focused on investigation of the enzymatic and non-enzymatic roles of CwlM, its localization in mycobacterial cells and interactions with other proteins.

Recombinant *Mtb* CwlM and its forms (mutated and truncated) were purified and used for investigation of peptidoglycan-cleaving activity by application of zymogram and digestion of FITC-labeled PG. Full-length CwlM showed no detectable PG-cleaving activity, however a shorter CwlM form corresponding to the predicted amidase domain was active in both assays. The site directed mutagenesis of the catalytic residue D339A completely abolished the PG-activity. PknB-mediated phosphorylation of CwlM or phosphomimetic mutations had no effect on peptidoglycan cleaving activity, however improved CwlM stability.

CwlM was found in the cytoplasmic and membrane fractions isolated from growing mycobacteria. Moreover, the phosphorylated CwlM localised in the cytoplasm, and non-phosphorylated CwlM was associated with the plasma membrane. CwlM was not detectable in cell wall and culture filtrate preparations.

Immunoprecipitation assays using CwlM-IgG-sepharose confirmed that phosphorylated CwlM interacted with FhaA, a fork-head-associated domain protein, whereas non-phosphorylated CwlM bound to the intracellular domain of MurJ<sub>ICD</sub> (MviN), a proposed Lipid II flippase.

A model of CwlM-mediated regulation of peptidoglycan biosynthesis was proposed based on the findings of this study and previously published observations.
Acknowledgements

I would like to massively thank Prof Galina Mukamolova and Prof Russell Wallis for their supervisions, expertise, guidance, encouragement, positive criticism and also for giving me the freedom to try different experiments during the project journey allowing me to learn how critically to think ‘out of the box’.

I would like to say a great big thank to Dr Obolbek Turapov who was to me like a father, an older brother and a best friend throughout the project giving me a complete support of everything I do, and for training me, making my experimental work so easy. Words cannot express how much I am grateful to him for everything that he has done for me. I am grateful to Hui Wen Lim for initial demonstration of activity in AmiR1, Oliver Sampson for optimisation of MurJ_{cod} expression and Evan Mitchell for help with isolation of CwlM.

I would like to acknowledge our collaborators Francesca Forti and Daniela Ghisotti at the University of Milan, Martin Cohen-Gonsaud and Philippe Barthe at the University of Montpellier, Andrew Bottrill at University of Warwick, Paul Ajuh at the Gemini Biosciences Ltd, Waldemar Vollmer at Newcastle University.

I would like also to thank my colleagues in the laboratory: Kawther, Ohoud, Mariam and Joss for sharing the funny time especially when nothing was working; this allowed me to overcome many challenges that faced me throughout my studies.

I would like to gratefully thank the Higher Committee for Education Development in Iraq (HCED) for funding the project that allowed me to undertake this PhD.

Special thanks to my family Mother, Father and my five older Brothers for encouraging and reminding me what makes me happy not just what makes me money.

To my small beloved family Wife (Sura), my son (Mohammed) and daughter (Zahraa) thank you very much for sharing me the time and place throughout my PhD journey; this thesis would have never been possible without you.
### List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADC</td>
<td>Albumin Dextrose Catalase</td>
</tr>
<tr>
<td>AG</td>
<td>Arabinogalactan</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired Immunodeficiency Syndrome</td>
</tr>
<tr>
<td>Amp</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>AmiR1</td>
<td>Amidase domain only</td>
</tr>
<tr>
<td>AmiR2-DD</td>
<td>Amidase with tail - phosphomimetic</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>BCIP/NBT</td>
<td>5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt/nitro-blue tetrazolium chloride</td>
</tr>
<tr>
<td>B-cell</td>
<td>Bursa dependent lymphocyte</td>
</tr>
<tr>
<td>BCG</td>
<td>Bacille Calmette-Guerin</td>
</tr>
<tr>
<td>Bp</td>
<td>Base pair(s)</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin Fraction V</td>
</tr>
<tr>
<td>CD</td>
<td>Circular Dichroism</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>CV</td>
<td>Column Volumes</td>
</tr>
<tr>
<td>D</td>
<td>Aspartate</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DAT</td>
<td>diaclyltrehalose</td>
</tr>
<tr>
<td>D-Glu</td>
<td>D-iso-Glutamic acid</td>
</tr>
<tr>
<td>dH2O</td>
<td>Distilled water</td>
</tr>
<tr>
<td>DPG</td>
<td>diphasphatidylglycerol</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DOTS</td>
<td>Directly observed therapy short-course</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>E</td>
<td>Glutamate</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbant assay</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast protein liquid chromatography</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione-S-transferase</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescence protein</td>
</tr>
<tr>
<td>H</td>
<td>Histidine</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HIV</td>
<td>Human I immunodeficiency Virus</td>
</tr>
<tr>
<td>His-tag</td>
<td>Histidine Tag</td>
</tr>
<tr>
<td>HPLC</td>
<td>High pressure liquid chromatography</td>
</tr>
<tr>
<td>hrs</td>
<td>hours</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>INH</td>
<td>Isoniazid</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>Kan</td>
<td>Kanamycin</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>KH2PO4</td>
<td>Potassium dihydrogen phosphate</td>
</tr>
<tr>
<td>KOH</td>
<td>Potassium hydroxide</td>
</tr>
<tr>
<td>L</td>
<td>Litre</td>
</tr>
<tr>
<td>LA</td>
<td>Luria agar</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>LAM</td>
<td>Lipoarabinomannan</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny broth</td>
</tr>
<tr>
<td>LM</td>
<td>Lipomannan</td>
</tr>
<tr>
<td>log</td>
<td>Logarithmic</td>
</tr>
<tr>
<td>M</td>
<td>Marker</td>
</tr>
<tr>
<td>MA</td>
<td>Mycolic acid</td>
</tr>
<tr>
<td>mAGPc</td>
<td>Mycolyl-arabinogalactan-peptidoglycan complex</td>
</tr>
<tr>
<td>mDAP</td>
<td>meso-Diaminopimelic acid</td>
</tr>
<tr>
<td>MDP</td>
<td>Muramyl Dipeptide</td>
</tr>
<tr>
<td>MIM</td>
<td>mycobacterial inner membrane</td>
</tr>
<tr>
<td>Min</td>
<td>minutes</td>
</tr>
<tr>
<td>MOM</td>
<td>mycobacterial outer membrane</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger Ribonucleic acids</td>
</tr>
<tr>
<td>Mtb</td>
<td><em>Mycobacterium tuberculosis</em></td>
</tr>
<tr>
<td>MTBC</td>
<td>Mycobacterium tuberculosis complex</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NAG</td>
<td><em>N</em>-Acetylglucosamine acid</td>
</tr>
<tr>
<td>NAM</td>
<td><em>N</em>-Acetylmuramic acid</td>
</tr>
<tr>
<td>NamH</td>
<td>N-acetylmuramic acid hydroxylase</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>Ni-NTA</td>
<td>Nickel Nitrilotriacetic Acid</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometre</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>OD600</td>
<td>Optical density reading at 600nm</td>
</tr>
<tr>
<td>PAT</td>
<td>polyacyltrehalose</td>
</tr>
<tr>
<td>PBP</td>
<td>Penicillin Binding Proteins</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDIM</td>
<td>Phthiocerol dimycocerosates</td>
</tr>
<tr>
<td>PE</td>
<td>phosphatidylethanolamine</td>
</tr>
<tr>
<td>PHA</td>
<td>Polyhydroxyalkanotes</td>
</tr>
<tr>
<td>PHE</td>
<td>Public Health England</td>
</tr>
<tr>
<td>PG</td>
<td>Peptidoglycan</td>
</tr>
<tr>
<td>PGRS</td>
<td>Peptidoglycan recognition proteins</td>
</tr>
<tr>
<td>PI</td>
<td>Isoelectric point</td>
</tr>
<tr>
<td>PI</td>
<td>phosphatidyl-myoinositol</td>
</tr>
<tr>
<td>PIMS</td>
<td>Phosphatidylinositol mannosides</td>
</tr>
<tr>
<td>PknB</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>POA</td>
<td>pyrazinoic acid</td>
</tr>
<tr>
<td>PZA</td>
<td>Pyrazinamide</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>Rpf</td>
<td>Resuscitation promoting factor</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RIF</td>
<td>Rifampicin</td>
</tr>
<tr>
<td>RipA</td>
<td>Rpf interacting protein A</td>
</tr>
<tr>
<td>SEC</td>
<td>Size exclusion chromatography</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecylsulphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SL</td>
<td>Sulfolipids</td>
</tr>
<tr>
<td>TB</td>
<td>Tuberculosis</td>
</tr>
<tr>
<td>TEV</td>
<td>Tobacco Etch Virus</td>
</tr>
<tr>
<td>T-cell</td>
<td>Thymus dependent lymphocyte</td>
</tr>
<tr>
<td>TDM</td>
<td>Trehalose dimycoclates</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour Necrosis Factor</td>
</tr>
<tr>
<td>TMM</td>
<td>Trehalose monomycolate</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris (hydroxymethyl) aminomethane</td>
</tr>
<tr>
<td>Tween</td>
<td>Polyoxyethylene sorbitan monooleate</td>
</tr>
<tr>
<td>UDP</td>
<td>Uridine diphosphate</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume per volume</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight per volume</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
</tbody>
</table>
List of Figures

Figure 1. The world map showing global incidence rates of tuberculosis, 2018 ........................................... 19
Figure 2. Estimated incidence cases of Tuberculosis in England (2016) .................................................... 20
Figure 3. The mechanism of D-cycloserine in inhibition of PG synthesis .................................................. 26
Figure 4. Vancomycin structure and its mode of action .............................................................................. 27
Figure 5. The structure of mycobacterial cell wall ....................................................................................... 29
Figure 6. Peptidoglycan biosynthesis of the Mtb cell wall ....................................................................... 34
Figure 7. Peptidoglycan hydrolase enzymes and their cleavage sites ....................................................... 36
Figure 8. Mtb NAM-amidases ............................................................................................................... 38
Figure 9. The elongation and division proteins complex in mycobacteria ................................................ 41
Figure 10. The putative structure of protein kinase B (PknB) .................................................................. 42
Figure 11. Calibration of the SEC column (HiLoad 16/600 Superdex 200 pg) using Bio-Rad’s SEC Standard ................................................................. 53
Figure 12. The putative structure of an annotated NAM-amidase CwlM .................................................. 58
Figure 13. Alignment of PG amidase domains of mycobacteria and other bacterial species .................... 59
Figure 14. Hayashi test to estimate the SDS concentration in the PG solution ....................................... 61
Figure 15. Expression trials of the recombinant CwlM using 0.5 mM IPTG .............................................. 64
Figure 16. Expression trials of the recombinant CwlM using 0.1 mM IPTG ............................................. 65
Figure 17. Purification of recombinant Mtb CwlM from E. coli C41 ........................................................... 66
Figure 18. The predicted secondary structure of CwlM based on amino acid sequences using Jpred 4 server ................................................................................................................................. 67
Figure 19. CD spectra and thermal denaturation curve of purified recombinant CwlM ........................... 68
Figure 20. Purification of recombinant MtB PknB .................................................................................. 69
Figure 21. Phosphorylation of CwlM by PknB in vitro ............................................................................ 70
Figure 22. SDS-PAGE shows the fresh and stored CwlM purified by IMAC (Ni-NTA) and SEC ......... 71
Figure 23. Purification of the phosphomimetic CwlM–DDD protein expressed in E.coli C41 strains 72
Figure 24. Investigation CwlM stability In vitro using different concentration of imidazole .......... 73
Figure 25. The peptidoglycan-cleaving activity of non-phosphorylated and phosphomimetic CwlM 74
Figure 26. Peptidoglycan-cleaving activity of phosphorylated and non-phosphorylated CwlM ...... 75
Figure 27. PG-cleaving activity of the recombinant CwlM forms (wild type, DDD, phosphorylated) using FITC labelling peptidoglycan of E. coli LMG ................................................................. 76
Figure 28. Zymogram showed CwlM-E. coli C41 lysates ................................................................. 77
Figure 29. The mutated forms of CwlM that used in this study .............................................................. 78
Figure 30. E. coli C41 lysates of CwlM forms ......................................................................................... 79
Figure 31. The truncated and mutated forms of CwlM used in this study .............................................. 80
Figure 32. Protein expression trials of AmiR1 ......................................................................................... 81
Figure 33. PG-cleaving activity of AmiR1 (amidase domain only) ............................................................ 82
Figure 34. The PG-cleaving activity of the recombinant AmiR1 using FITC-labelled peptidoglycan of E. coli LMG ............................................................................................................... 83
Figure 35. PG-cleaving activity of AmiR2-DD (Phosphomimetic domain) ............................................. 84
Figure 36. PG-cleaving activity of the recombinant AmiR2-DD using FITC-labelled peptidoglycan of E. coli LMG ............................................................................................................... 85
Figure 37. Expression and purification the mutated active sites of AmiR1 ............................................. 86
Figure 38. PG-hydrolysis activity of mutated AmiR1 forms (H204A, E217A and D339A) using FITC-labelled peptidoglycan of E. coli LMG ................................................................. 87
Figure 39. Graphical abstract of PG-cleaving activity of recombinant CwlM forms. .............................. 92
Figure 40. Schematic of Anti-Phospho and Anti-Non-phospho CwlM antibodies production. ........... 96
Figure 41. Purification of Anti-CwlM antibodies from final bleeds serum. ........................................ 99
Figure 42. Dot Blot assays of anti-CwlM polyclonal antibodies. .......................................................... 100
Figure 43. Validation of anti-CwlM antibody using Western Blot analysis............................................. 101
Figure 44. Validation of purified anti-CwlM antibodies against phosphorylated-dephosphorylated recombinant CwlM using Western Blot.................................................................................. 102
Figure 45. Validation of anti-CwlM antibodies against CwlM in mycobacteria by Western Blot. .......... 103
Figure 46. Detection of CwlM in cytoplasm and membrane fractions of M. smegmatis...................... 104
Figure 47. Detection of CwlM in the mycobacterial cell wall and filtered culture supernatant preparations.................................................................................................................. 105
Figure 48. PknB-mediated phosphorylation of T382 determines the distribution of CwlM in cytoplasmic and membrane fractions of M. smegmatis.................................................................................................. 107
Figure 49. Detection of CwlM in cytoplasm and membrane fractions of M. smegmatis during different growth stages.................................................................................................................... 108
Figure 50. Expression and purification of recombinant Mtb Wag31 from E. coli C41 ............................. 123
Figure 51. Expression and purification of recombinant Mtb FhaA ........................................................... 125
Figure 52. Expression and purification of recombinant Mtb MurJicd..................................................... 127
Figure 53. Expression and purification of recombinant Mtb MurJicdE541-F680 .................................... 128
Figure 54. Validation of CwlM phosphorylation using specific Anti-CwlM antibody. ....................... 129
Figure 55. Binding assays for Wag31 and CwlM forms using a CwlM-IgG-Sepharose ......................... 130
Figure 56. Binding assays for FhaA and CwlM forms using a CwlM-IgG-Sepharose......................... 131
Figure 57. Binding assays of MurJicd and CwlM forms using a CwlM-IgG-Sepharose...................... 132
Figure 58. CwlM interacts with an E541-F680 linker of MurJicd................................................................. 133
Figure 59. Protein-protein interaction using size-exclusive chromatography (SEC)............................ 135
Figure 60. Crystallisation trials for solving the recombinant CwlM structure........................................ 136
Figure 61. Crystallisation trials for solving the recombinant CwlM-DDD structure............................... 137
Figure 62. Trials for solving the recombinant CwlM-DDD structure after removing the His-tag. ....... 138
Figure 63. Crystallisation trials for solving the structure of proteins complex DDD-FhaA.................. 139
Figure 64. Crystallisation trials for solving the structure of proteins complex CwlM-MurJicd............ 140
Figure 65. Schematic of immunoprecipitation assay using a CwlM-IgG-Sepharose ......................... 142
Figure 66. The proposed model of CwlM functions in the peptidoglycan biosynthesis in Mtb .......... 150
List of Tables

Table 1: Reagents used in this study ................................................................. 46
Table 2: Bacterial strains used in this study .................................................... 48
Table 3: Identification of potential CwIM partners by immuno-precipitation and protein fragment complementation assays. Y refers to Yes; N refers to No; N/A refers to not assessed .......... 121
# Table of Contents

Abstract ..................................................................................................................................... 4  
Acknowledgements .................................................................................................................. 5  
List of Abbreviations ............................................................................................................... 6  
List of Figures ........................................................................................................................... 9  
List of Tables .......................................................................................................................... 11  
Table of Contents ................................................................................................................... 12  
Chapter 1 ................................................................................................................................ 17  
  1. General Introduction ......................................................................................................... 17  
  1.1 The global burden of Tuberculosis ................................................................................. 18  
  1.2 Overview of Mtb biology and tuberculosis ..................................................................... 20  
  1.3 Treatment of tuberculosis ............................................................................................... 23  
    1.3.1 Inhibitors that target PG synthesis ............................................................................. 25  
  1.4 Cell wall of mycobacteria ............................................................................................... 28  
  1.5 PG function and structure ............................................................................................... 31  
  1.6 PG synthesis and remodelling ......................................................................................... 32  
    1.6.1 PG synthesis ................................................................................................................... 32  
    1.6.2 PG remodeling in mycobacterial cell growth and division ......................................... 35  
  1.7 N-acetylMuramyl-L-alanine amidases (NAM-amidases) ............................................. 36  
  1.8 Coordination of PG biosynthesis with bacterial growth and division ....................... 39  
  1.9 Regulation of mycobacterial growth and PG biosynthesis by serine/threonine kinase signaling .................................................................................................................................. 41  
  1.10 Hypothesis of this project ............................................................................................... 43  
  1.11 Aims of this project: ........................................................................................................ 43  
Chapter 2 ................................................................................................................................ 45  
  2. General Materials and Methods ....................................................................................... 45
2.1 Regents ............................................................................................................................. 46
Phospho-Threonine Antibody (P-Thr Polyclonal) ..................................................................... 46
Monoclonal Anti-polyHistidine antibody ................................................................................ 46
Anti-rabbit IgG, HRP-linked antibody .................................................................................... 46
BCIP®/NBT Liquid Substrate System ..................................................................................... 46
SignalFire™ Elite ECL Reagent .............................................................................................. 46
Cyanogen bromide-activated-Sepharose® 4B ........................................................................ 46
cOmplete Ultra Tablets Protease Inhibitor Cocktail ............................................................. 47
PhosSTOP™ phosphatase inhibitor tablets .......................................................................... 47
Ni-NTA agarose .................................................................................................................... 47
Glutathione Sepharose 4B .................................................................................................... 47
HiLoad 16/600 Superdex 200 pg prepacked column ............................................................ 47
SERVA GelTMTG Prime™ 4-20% 10 samples wells ............................................................. 47
SERVA GelTMTG Prime™ 12% 12 samples wells ............................................................... 47
2.2 Media .................................................................................................................................. 47
2.3 Bacterial strains ............................................................................................................... 47
2.4 Preparation bacterial stocks ........................................................................................... 50
2.5 Cultivation of bacteria ..................................................................................................... 50
  2.5.1 Escherichia coli C41 and BL21 (DE3) strains ............................................................... 50
  2.5.2 Mycobacterium smegmatis strains ............................................................................. 50
  2.5.3 Micrococcus luteus Fleming 2665 strain ..................................................................... 50
2.6 Buffers and solutions ....................................................................................................... 50
2.7 Preparation of proteins purification columns ............................................................... 52
2.8 Expression and purification of His-tagged proteins ..................................................... 53
2.9 Expression and purification of GST-tagged proteins ................................................... 54
2.10 Protein dialysis ............................................................................................................... 54
2.11 Protein concentration .................................................................................................... 54
2.12 Measurement of protein concentration ........................................................................ 54
Chapter 2 - Methods

2.13 SDS-PAGE ...................................................................................................................... 55
2.15 Identification of proteins by mass-spectrometry ........................................................ 55
2.16 ImageJ analysis .............................................................................................................. 56
2.17 Statistical analysis .......................................................................................................... 56
2.18 Preparation of Figures ................................................................................................... 56

Chapter 3 - Investigation of the Enzymatic Activity of CwlM

3.1 Introduction ...................................................................................................................... 57
3.2 Materials and methods .................................................................................................... 59
  3.2.1 Expression of CwlM proteins .................................................................................. 59
  3.2.2 Expression and Purification of Protein kinase B (PknB) ............................................. 60
  3.2.3 In vitro phosphorylation of the recombinant CwlM by PknB ...................................... 60
  3.2.4 Circular dichroism of the recombinant CwlM protein ............................................. 60
  3.2.5 Purification of E. coli peptidoglycan ....................................................................... 60
  3.2.6 Hayashi test and digestion cell wall proteins ......................................................... 61
  3.2.7 Labelling of peptidoglycan with Fluorescein isothiocyanate (FITC) ....................... 62
  3.2.8 Zymography ............................................................................................................. 62
  3.2.9 Digestion of PG-FITC by CwlM ............................................................................. 63
3.3 Results ............................................................................................................................... 63
  3.3.1 Expression of recombinant CwlM .......................................................................... 63
  3.3.2 Purification of the recombinant CwlM .................................................................. 65
  3.3.3 Secondary structure and protein folding of CwlM .................................................... 66
  3.3.4 PknB phosphorylates CwlM in vitro .................................................................... 68
  3.3.5 Phosphorylation or phosphomimetic mutations improve CwlM stability ............... 71
  3.3.6 Full-length CwlM has no PG-cleaving activity ......................................................... 73
  3.3.7 PG-cleaving activity of truncated CwlM proteins ..................................................... 80
3.4 Discussion.......................................................................................................................... 87

Chapter 4 - Investigation of CwlM localisation in mycobacteria during different stages of growth

4. Investigation of CwlM localisation in mycobacteria during different stages of growth
                                                                                           93
4.1 Introduction ................................................................................................................................. 94

4.2 Materials and Methods .................................................................................................................. 95
  4.2.1 Organisms and media .................................................................................................................. 95
  4.2.2 Generation of anti-CwlM Antibody .......................................................................................... 96
  4.2.3 Generation Anti-Phospho and Anti-Non-phospho CwlM antibodies .............................................. 96
  4.2.4 Purification of Anti-CwlM polyclonal Antibody from sera ........................................................... 97
  4.2.5 Dot Blot to determine sensitivity to recombinant Mtb CwlM .......................................................... 97
  4.2.6 Mycobacterial cell fractionation .................................................................................................. 97
  4.2.7 Culture supernatant preparation ................................................................................................ 98

4.3. Results ........................................................................................................................................... 99
  4.3.1 Production anti-CwlM antibody .................................................................................................. 99
    4.3.1.1 Purification of anti-CwlM antibody using FliQ Protein A column .............................................. 99
    4.3.1.2 Validation of AB against recombinant CwlM using a dot blot and Western blot ...................... 99
    4.3.1.3 Recognition of phosphorylated and non-phosphorylated recombinant CwlM ............................... 101
    4.3.1.4 AB recognize the native CwlM in mycobacterial lysates ......................................................... 102
  4.3.2 Localisation studies of CwlM in M. smegmatis ............................................................................ 103
    4.3.2.1 CwlM localised in cytoplasm and membrane fractions but missing in culture filtrate and cell wall preparations .......................................................................................................................... 103
    4.3.2.2 Phosphorylation of T382 determines CwlM localisation in the cytoplasm ................................. 105
    4.3.2.3 CwlM is expressed only during active growth ......................................................................... 107

4.4 Discussion ...................................................................................................................................... 108

4.5 Conclusions and future work ....................................................................................................... 113

Chapter 5 .............................................................................................................................................. 115

5. Identification of protein partners interacting with CwlM in Mycobacteria ..................................... 115
  5.1 Introduction ..................................................................................................................................... 116

5.2 Materials and Methods ................................................................................................................. 118
    5.2.1 Preparation of anti-CwlM antibody sepharose .......................................................................... 118
    5.2.2 Expression and purification of recombinant Mtb proteins ......................................................... 118
    5.2.3 Cleavage of Poly-His and GST tags ........................................................................................... 118
    5.2.4 Preparation of proteins and screens for crystallisation trials .................................................... 119
    5.2.5 Immunoprecipitation assays .................................................................................................... 119
    5.2.6 Densitometry analysis .............................................................................................................. 120
5.3 Results .............................................................................................................................................. 120

5.3.1 Phosphorylated and non-phosphorylated CwlM proteins have different protein partners .............................................................................................................................................. 120

5.3.2 Generation of recombinant Mtb proteins .................................................................................................................. 121

5.3.2.1 Expression and purification of Wag31 .................................................................................................................. 121

5.3.2.2 Expression and purification of recombinant FhaA .................................................................................................. 124

5.3.2.3 Expression and purification recombinant MurJ_{icd} .............................................................................................. 126

5.3.2.4 Expression and purification recombinant E541-F680 linker of MurJ_{ICD} ................................................................ 128

5.3.2.5 Generation of phosphorylated CwlM .................................................................................................................. 128

5.3.3 Co-purification of candidate proteins and CwlM forms using Anti-CwlM-IgG-sepharose .................................................................................................................................................. 129

5.3.3.1 Phosphorylated and non-phosphorylated CwlM do not bind Wag31 ........................................................................ 129

5.3.3.2 Phosphorylated CwlM mainly binds to FhaA ......................................................................................................... 130

5.3.3.3 Non-phosphorylated CwlM interacts with MurJ_{icd} .................................................................................................. 131

5.3.3.4 Non-phosphorylated CwlM interacts with an E541-F680 linker of MurJ_{icd} ................................................................ 132

5.3.4 Application of size exclusion chromatography (SEC) for confirmation of FhaA and CwlM interactions .............................................................................................................................................. 134

5.3.4 Crystallisation trials .............................................................................................................................................. 135

5.3.4.1 Recombinant CwlM full-length (wild type) .............................................................................................................. 135

5.3.4.2 Phosphomimetic CwlM (DDD) ......................................................................................................................... 136

5.3.5 Crystallisation trials of complexes (FhaA-DDD; MurJ-CwlM) .................................................................................... 138

5.4 Discussion .............................................................................................................................................. 140

5.5 Conclusion and Future Work .............................................................................................................................................. 146

Chapter 6 .............................................................................................................................................. 147

6. Final conclusion and Future work .............................................................................................................................................. 147

6.1 Final conclusion .............................................................................................................................................. 148

6.2 Future work .............................................................................................................................................. 151

Appendix ...................................................................................................................................................... 153

References ...................................................................................................................................................... 156
Chapter 1

1. General Introduction
1.1 The global burden of Tuberculosis

Tuberculosis (TB) is an ancient infectious disease that has plagued humankind for millennia as researchers provided molecular evidence of *Mycobacterium tuberculosis* (*Mtb*) infection in a 3000 years old Egyptian mummy (Nerlich *et al.* 1997). The global burden of TB rapidly increased during the industrial revolution, and by the 19th century, it was the main cause of death in Europe and North America (Daniel 2006; Barberis *et al.* 2017). With the discovery of anti-*Mtb* drugs in the 20th century, the incidence cases of tuberculosis significantly decreased in developed countries (Murray *et al.* 2015). However, the emergence of the Human Immunodeficiency Virus (HIV) resulted in a rapid increase of TB cases, making the World Health Organization (WHO) in 1993 announce TB a global emergency (Getahun *et al.* 2010; Nathavitharana and Friedland 2015).

Although TB incidence has been steadily decreasing since 2006, WHO still estimated 10 million new cases of active TB in 2017 (WHO 2018). The most recent WHO report revealed that around 1-2 million individuals die every year from TB and nearly 1.7 billion are latently infected with *Mtb* (Houben and Dodd 2016; WHO 2018). The highest TB burden was recorded in low-income countries such as India, China, Pakistan and South Africa (*Figure 1*) (WHO 2018). In Iraq, the TB incidence was 25-99 per 100,000 populations which accounted for 9 % of the estimated global number of incident cases in 2017 (*Figure 1*) (WHO 2018).

TB also affects developed countries in North America, Australia and Europe (*Figure 1*). The high number of immigrants from developing countries to developed countries contributed to the high TB burden (Glaziou *et al.* 2018). According to Public Health England (PHE) about 5,664 TB cases were estimated in England in 2016 and the majority of TB cases were notified in London, with an incidence rate of 25.1 per 100,000 population (*Figure 2*) (PHE 2017).
Figure 1. The world map showing global incidence rates of tuberculosis, 2018.

Dark green countries have the highest TB incidence rates ≥300 per 100,000 population whereas the lower distribution of TB 0-24 per 100,000 population was more seen in developed areas such as Europe, Australia and the United States of America. The Figure was taken from Global tuberculosis control (WHO 2018).

The rate and distribution of TB are significantly affected by the Human Immunodeficiency Virus (HIV) and acquired immunodeficiency syndrome (AIDS). It has been estimated that 5–15% of HIV-negative individuals who are latently infected with Mtb will develop active TB during their lives, but the TB reactivation rate in HIV-infected individuals could increase up to 50%. According to WHO, 1.2 million new TB cases were notified in areas with higher HIV prevalence and 71% of affected individuals were from Kenya, Namibia, Tanzania, Zambia, Zimbabwe and South Africa (WHO 2018; Gelaw et al. 2019).

The WHO report emphasised that there is an urgent need to reduce the current incidence (10 million) of TB worldwide to 50% by the year 2023 (WHO 2018). However, eradication of TB is complicated by several factors. These include the ability of Mtb to establish latent and active infections. Mtb resistance to the host immune system (innate and acquired), and Mtb resists to drugs, which is aided by the presence of multiple drug efflux pumps, an impermeable thick cell wall and the occurrence of chromosomal mutations (Antonova et al. 2018; Iketleng et al. 2018; Hamblion et al. 2019; Zhai et al. 2019).
The London region has the highest TB cases with an incidence rate of 25.1 per 100,000 populations. This figure adapted from the Public Health England report of Tuberculosis (PHE 2017).

1.2 Overview of Mtb biology and tuberculosis

In 1882, the causative agent of tuberculosis was discovered by Robert Koch. He observed a rod-shaped bacterium under a microscope isolated from human lesions, later named as Koch's bacillus or Mtb (Murray 2004; Cambau and Drancourt 2014).

In general, mycobacteria are rod-shaped, obligate aerobic, non-motile bacteria. They are commonly categorized as Gram-positive bacteria according to their phylogenetic position and cell wall structure (Fu and Fu-Liu 2002; Humphreys et al. 2002). However, they are called "acid fast bacteria" because of the high lipid contents of their cell wall that resist de-colorisation with acid-alcohol once they are stained with “Ziehl-Neelsen” (Bishop and Neumann 1970; Bhatt et al. 2007).
Mycobacteria belong to the phylum of *Actinobacteria*. This phylum is characterized by a high guanosine–cytosine content in the genomic DNA and an abundance of lipid components in the cell wall (Shaila *et al.* 1973; Wayne and Kubica 1986). Mycobacteria appear under a microscope as filaments, curved-rods or cocci depending on growth conditions and the culture age (Farnia *et al.* 2018). The colonies have a ‘wrinkled’ appearance with white or yellowish colour due to the high lipid component (Fregnan and Smith 1962; Gonzalez *et al.* 1998; Shleeva *et al.* 2004). Based on their growth properties, mycobacteria can be generally classified into fast and slow growers (Fregnan and Smith 1962). *Mtb* is categorized as a slow-growing bacterium with a generation time of 24 hours, while the generation time of *Mycobacterium smegmatis* is 3 hours (Wheeler and Ratledge 1994; Klann *et al.* 1998; Delogu *et al.* 2013). Therefore, *M. smegmatis* is often used in research as a model organism due to its fast-growing and non-pathogenic characteristics (Smith 2003; Shiloh and Champion 2010).

*The Mycobacterium tuberculosis* complex (tubercle bacilli) includes *Mtb*, *M. africanum*, *M. canettii*, *M. caprae*, *M. bovis*, *M. pinnipedii*, and *M. microti* (Sinha *et al.* 2016). These pathogenic mycobacteria species are genetically-related with (85 to 100% homology), however, they differ in their virulence and epidemiology (Frothingham *et al.* 1994; Malone and Gordon 2017). *Mtb*, *M. africanum* and *M. canettii* cause tuberculosis in humans (Supply and Brosch 2017; Yeboah-Manu *et al.* 2017). *M. caprae* infects goats; *M. bovis* causes TB in a wide spectrum of hosts including cattle and human (Pesciaroli *et al.* 2014); *M. pinnipedii* is a TB causative agent in seals (Cousins *et al.* 2003) and *Mycobacterium microti* is a rodent pathogen (Brosch *et al.* 2002; Homolka *et al.* 2010).

*Mtb* spreads through the air as droplet particles (1-5 µm) by coughing or sneezing of infected persons (Fennelly and Jones-López 2015). Inhalation of these particles by healthy individuals could start a new cycle of infection (Philips and Ernst 2012). *Mtb* can pass through the physical barriers of innate immunity such as mucus and cilia to enter the alveoli (Sia *et al.* 2015). There, the immune system initiates a strong inflammatory response by recruiting phagocytic alveolar macrophages, monocytes, dendritic cells, natural killers, and T, B lymphocytes to form a granuloma (Peddireddy *et al.* 2017). In this cellular structure, phagocytosis leads to a cascade of events where proteolytic enzymes and cytokines control the bacterial infection, and the majority of patients are cured or develop latent TB infection (Philips and Ernst 2012). Indeed, around one-fourth of the human population is latently infected with *Mtb* (Getahun *et al.* 2015; WHO 2018). However, *Mtb* could adapt and survive in the environment with low oxygen, low pH and low nutrition availability within a granuloma (Russell *et al.* 2010; Gengenbacher and
Additionally, the cell wall component lipoarabinomannan (LAM) is a key virulence factor for \textit{Mtb} by interacting with the macrophage mannose receptor and inhibiting the maturation of phagosome (Welin \textit{et al.} 2008). Blocking phagocytosis activity enables \textit{Mtb} to survive and replicate in macrophages which in turn develop active TB infection (Jamwal \textit{et al.} 2016). Around 10\% of patients infected with \textit{Mtb} could develop active TB in their lives (Narasimhan \textit{et al.} 2013; Bloom \textit{et al.} 2017).

Symptoms of active TB include persistent coughing, fatigue, loss of appetite and weight, respiratory distress, mild fever and night sweats (Sherman \textit{et al.} 1999; Breen \textit{et al.} 2008) which are often accompanied by necrosis and severe damage of lungs and other organs (Miller \textit{et al.} 2000).

Rapid TB diagnosis is a core factor for an effective treatment (Pai \textit{et al.} 2008). Current TB diagnostic methods include microscopic tests, culture method, chest X-ray, skin test (tuberculin test), PCR-based methods (e.g. Gene-X-pert) and Interferon gamma release assays (IGRAs) (Andersen \textit{et al.} 2000; Birhanu and Ejeta 2015; Chin \textit{et al.} 2018). However, the accuracy, sensitivity, specificity and cost of these methods can vary. For example, microscopic test is low cost and it is commonly used to detect \textit{Mtb} in Ziehl-Neelsen stained sputum smears but it may have poor sensitivity and it is not used for detection of an extra pulmonary TB (Laifangbam \textit{et al.} 2009; Abdelaziz \textit{et al.} 2016). Some methods like a chest x-ray and the serological test could be used for rapid diagnosis of active TB, however poor sensitivity and specificity were also reported with the use of these methods (Steingart \textit{et al.} 2007). Polymerase chain reaction (PCR) test based on insertion sequence IS1081 could be used for rapid and sensitive detection of active \textit{Mtb} in clinical samples (Ahmed \textit{et al.} 1998). Other methods that can be used for confirmation of latent TB infection and active TB are tuberculin skin test and interferon-\(\gamma\) assays (Al-Orainey 2009; Moon and Hur 2013). However, most of these methods are expensive and therefore current efforts are focused on developing accurate and cheap tests that could be effectively used for diagnosis TB especially in poor and high TB burden countries.

Bacillus Calmette–Guérin (BCG) is an attenuated strain of \textit{Mycobacterium bovis} that is commonly used as vaccine against TB (Hart and Sutherland 1977). This vaccine shows efficiency in reducing morbidity rates of TB in children (Mendez-Samperio 2018), and therefore WHO recommends vaccination of children with the BCG vaccine at birth (B. Zhu \textit{et al.} 2018). However, BCG does not provide sufficient protection against active pulmonary TB in adults (Khoshnood \textit{et al.} 2018; Nieuwenhuizen and Kaufmann 2018). Additionally, the BCG
vaccine cannot be given to the immunocompromised group such as HIV-infected individuals (Hashimoto 1997; Vasconcelos-Junior et al. 2009). Therefore, there is also a need to develop more effective vaccine against *Mtb* (Mendez-Samperio 2018).

### 1.3 Treatment of tuberculosis

Since the discovery of the causative agent of tuberculosis, attempts to find an effective treatment against *Mtb* have not stopped yet. In 1940, the first regimen effective against *Mtb* was developed by Albert Schatz, Selman Waksman and others. They produced streptomycin, an agent that inhibits protein synthesis (Waksman et al. 1945). Unfortunately, this treatment has not been used for a long time because of the emergence of drug-resistant strains (Shaila et al. 1973; Murray et al. 2015).

The next challenge after the first application of streptomycin was to find an alternative anti-TB agent. A further four drugs were discovered and introduced for tuberculosis treatment; isoniazid in 1952, pyrazinamide in 1954, rifampicin in 1957 and ethambutol in 1961. These anti-*Mtb* agents are known as first-line anti-tuberculosis drugs and they are currently used for the treatment of active TB (Mitchison and Davies 2012; Zumla et al. 2013; Murray et al. 2015).

**Rifampicin (RIF)** or rifampin, is a bactericidal antibiotic that targets both replicating and non-replicating bacilli (Maggi et al. 1966; Campbell et al. 2001). RIF binds to the β-subunit of RNA polymerase encoded by *rpoB* and inhibits transcription of mycobacterial DNA to RNA (Levin and Hatfull 1993; Miller et al. 1994).

However, *Mtb* frequently develops resistance to RIF either by mutation in the promoter region of *rpoB* gene or by altering residues of the RIF binding site on RNA polymerase which reduce affinity for rifampicin (Telenti et al. 1993; Feklistov et al. 2008; Pierre-Audigier and Gicquel 2012).

**Pyrazinamide (PZA)** is a pro-drug that is commonly used in combination with other agents to shorten the treatment time for active TB therapy (Mitchison 1985; Njire et al. 2016). The active form of the drug is pyrazinoic acid (POA), and it has been shown that PZA is more active against slower or non-replicating bacilli (Zhang et al. 2003; Zhang et al. 2013). However, the precise mechanism of action for PZA is obscure (Anthony et al. 2018). PZA targets the component of 30S ribosomal subunit S1 protein (RpsA) to inhibit the trans-translation process in slow-growing mycobacteria under stressful conditions (Butler and Kilburn 1983; W. Shi et
In vitro, the activity of PZA can be enhanced by acid pH 5.6 (Heifets and Lindholm-Levy 1990) or under stressful conditions like hypoxia (Iacobino et al. 2017). This may therefore explain the efficiency of PZA in killing slow replicating or dormant *Mtb* bacterium that other TB agents fail to kill.

Resistance to PZA is usually associated with mutations in the *pncA* gene encoding pyrazinamidase that plays a key role in the conversion of the prodrug PZA to the active form POA (Hirano et al. 1998).

**Isoniazid (INH)** is a prodrug that targets InhA, 2-trans-enoic-acyl carrier protein reductase (ENR) (Metcalfe et al. 2008; Chollet et al. 2018). This enzyme belongs to the Fatty Acid Synthase II (FAS-II system), and therefore it is involved in mycolic acid biosynthesis machinery in *Mtb* (Musser 1995; Marrakchi et al. 2000). INH could be activated by catalase-peroxidase KatG and its mode of action includes generating NAD+/NADP+ adducts that are potent inhibitors of InhA (Mahapatra et al. 2012; Unissa et al. 2016). InhA inactivation leads to bacterial lysis by the destruction of the membrane and cell fragmentation (Marrakchi et al. 2000). Thus, INH is believed to kill actively growing bacteria (Takayama et al. 1972), however, it is also used to prevent reactivation of latent tuberculosis (Stagg et al. 2017).

Resistance to INH is caused by a mutation in KatG or reduced affinity for NADH by inhibiting the activity of NADH dehydrogenase (Zhao et al. 2006; Stagg et al. 2017).

**Ethambutol (EMB)** is an anti-mycobacterial drug that is usually used in combination with other drugs against actively growing TB bacilli and to minimize the emergence of drug resistance (D. Shi et al. 2011; Favrot and Ronning 2012). However, the exact mechanisms for the potential synergistic action of EMB with other anti-TB drugs remain unclear (C. Zhu et al. 2018). EMB disrupts arabinogalactan biosynthesis by inhibiting the enzyme arabinosyl transferase encoded by *embAB* which inhibits formation of arabinogalactan and leads to cell lysis (Belanger et al. 1996; Ghiraldi-Lopes et al. 2018). A recently published article found a new molecular mechanism for the synergistic activity of EMB and INH against replicating *Mtb*. The authors identified a putative transcription factor (EtbR) encoded by the *Rv0273c* as a target of EMB. EMB alleviates EtbR-mediated repression of *inhA* which in turn increases the bactericidal effect of INH (C. Zhu et al. 2018).
*Mtb* usually has mutations in an *embB* gene and it therefore develops EMB-resistant (Zhang and Yew 2015). Furthermore, mutations in the promoter region of either *embC* or *embA* also contribute to EMB resistance in *Mtb* (Cui *et al.* 2014).

Globally, the first line anti-*Mtb* drugs (INH, PZA, EMB and RIF) are currently a standard anti-tuberculosis drug regimen. These drugs are not given separately to patients as the use only single anti-*Mtb* drugs results in the rapid development of mycobacterial resistance and treatment failure (Laurenzi *et al.* 2007). Therefore, the WHO has introduced Directly Observed Therapy, Short–Course (DOTs) programme to reduce the risk of emergence of drug resistance, and to prevent TB relapse (WHO 2009). DOTs is usually 6 months long with a variation of the drugs prescribed for TB treatment. In the early stage of treatment INH, RIF, EMB and PZA given for two months, while during the final four months only INH and RIF are prescribed to eliminate active and latent tuberculosis infections (WHO 2009; Hoppe *et al.* 2016). From 1995 to 2008, the DOTS programs were successful in curing 8 million TB patients throughout the world (WHO 2009; Glaziou *et al.* 2011). This regimen is also can be used for both HIV-infected and HIV-negative TB patients (De Cock and Chaisson 1999). However, DOTs is difficult to implement and expensive, particularly in developing countries. Thus, there is a pressing global need to develop new drugs, reduce treatment duration and prevent TB relapse (Out 2013; Karumbi and Garner 2015).

Other TB drugs are classified as second-line antimicrobials. These include several types such as Ethionamide, Fluoroquinolones, P-aminosalicylic acid, Kanamycin, Amikacin, Capreomycin, etc. The majority of these drugs are given to the patients by injection whereas front line drugs administered orally (Zumla *et al.* 2013). Additionally, inhibitors of peptidoglycan biosynthesis can be used as alternative drugs

### 1.3.1 Inhibitors that target PG synthesis

The peptidoglycan (PG) component of the mycobacterial cell wall is distinctive and its synthesis involves many essential enzymes that could be significant targets for TB drugs.

*D-cycloserine* has a similar structure of D-alanine and it is a second-line drug used to treat TB (Zhang 2005). It inhibits PG biosynthesis by preventing the conversion of L-alanine to D-alanine and through inhibition of D-alanine racemase (Alr) activity (see Figure 3) (Prosser and de Carvalho 2013). However, it has recently been shown that *Mtb* arises resistance to the drug
by mutation in alr (rv3423c) promoter region encoding alanine racemase (Chen et al. 2017). Overexpression of alr, or mutations in the promoter region, lead to D-cycloserine resistance (Cáceres et al. 1997). D-cycloserine is toxic to the central nervous system and its use for TB treatment is therefore limited (W E Sanders and Sanders 1979).

\[
\begin{align*}
\text{L-alanine} & \xrightarrow{\text{alr}} \text{D-alanine} \\
\text{H}_3\text{C} & \text{NH}_2 \hspace{2cm} \text{HO} \hspace{2cm} \text{HO} \hspace{2cm} \text{CH}_3 \\
& \text{O} \hspace{2cm} \text{O} \\
& \text{NH}_2
\end{align*}
\]

**Figure 3.** The mechanism of D-cycloserine in inhibition of PG synthesis.

D-cycloserine binds to D-alanine racemase (Alr) and deactivates the conversion of L-alanine to D-alanine during PG biosynthesis. This Figure was drawn using ChemDraw Pro software 15.1.

**Vancomycin** is a glycopeptide antibiotic that binds with the two terminal D-alanine residues during peptidoglycan biosynthesis, thereby it inhibits crosslinking between the 4\textsuperscript{th} D-alanine residue and adjacent meso-DAP residue (see **Figure 4**) (Barna and Williams 1984). Although vancomycin is generally used against Gram-positive bacteria, the combination of vancomycin with cerulenin (lipid synthesis inhibitor) was synergistically effective in killing *Mtb* (Soetaert et al. 2015).
Figure 4. Vancomycin structure and its mode of action.

(A) The chemical structure of vancomycin was taken from (Schäfer et al. 1996). (B) Vancomycin inhibits peptidoglycan polymerisation by binding with the two terminal D-alanine residues, thus it prevents crosslinking between the 4th D-alanine residue and adjacent meso-DAP residue.

The combination of Carbapenems, a class that belongs to β-lactam antibiotics with clavulanate (β-lactamase inhibitor) target non-classical and classical transpeptidases (L, D- and D, D- transpeptidases) (Bianchet et al. 2017; Mattoo et al. 2017). The action of these antibiotics displays high tolerance to mycobacterial β-lactamase induced degradation of their β-lactam ring unit and inhibits the crosslinking of stem peptides (Veziris et al. 2011; Bianchet et al.
However, β-lactams are generally not efficient in killing *Mtb* due to the presence of 3-3 cross-links (Erdemli *et al.* 2012) and the production of β-lactamases (Chambers *et al.* 1995; Flores *et al.* 2005).

TB treatment takes a long-time resulting in low compliancy and side effects of drugs (Wade and Zhang 2004; Yang *et al.* 2017). Thus, this motivates drug discovery programmes to develop nontoxic, more effective and low-cost anti-tuberculosis agents.

### 1.4 Cell wall of mycobacteria

Mycobacterial cell wall structure differs from other bacterial types in several molecular features. For example, in contrast to the Gram-positive bacteria such as *Bacillus subtilis*, mycobacteria possess high lipid contents with a very low permeability (Alderwick *et al.* 2015). Unlike Gram-negative bacteria such as *E. coli*, mycobacteria lack a typical outer membrane (Mahapatra *et al.* 2008). However, the periplasmic space of the mycobacterial cell wall is similar to that in Gram-negative bacteria that locates where the arabinogalactan (AG) and the peptidoglycan (PG) layers are situated (Alderwick *et al.* 2015; Touchette and Seeliger 2017).

The complex cell envelop of mycobacteria consists of distinct multiple layers: mycobacterial outer membrane (MOM), mycobacterial inner membrane (MIM) and mycolic-arabinogalactan-peptidoglycan (mAGP) cell wall core (see Figure 5).

**Mycobacterial outer membrane (MOM)** consists of acyl lipids and glycolipids including phthiocerol dimycocerosate (PDIM), trehalose monomycolate (TMM), trehalose dimycolates (TDM), sulfoglycolipid (SGL), diacyltrehalose (DAT) and polyacyltrehalose (PAT) (Jankute *et al.* 2015; Abrahams and Besra 2018). These components play a significant role in mycobacterial pathogenesis (Briken *et al.* 2004; Mishra *et al.* 2011; Lee *et al.* 2012; Singh *et al.* 2018).

The outer membrane is a highly hydrophobic layer due to the lipid structure and therefore hydrophilic nutrients or drugs can only cross this layer by porins or transporters. Pore proteins (porins) are involved in transition of nutrients from the surroundings (Kartmann *et al.* 1999; Niederweis 2003; Niederweis 2008). *Mtb* encodes around 140 outer membrane proteins (Niederweis *et al.* 2010). These proteins have a variety of functions such as transport virulence factors and extracellular materials across the outer membrane for capsule and biofilm formation, which are required for intracellular survival and persistence within hosts (Daffe and Etienne 1999; Niederweis *et al.* 2010).
**Mycobacterial inner membrane (MIM)** also known as cytoplasmic membrane of *Mtb* that consists of phospholipids including phosphatidyl-myo-inositol mannosides (PIMs), phosphatidylethanolamine (PE), phosphatidyl-myo-inositol (PI) and diphosphatidylglycerol (DPG) as well as glycolipids like lipomannan (LM) and lipoarabinomannan (LAM) (Jankute *et al.* 2015; Abrahams and Besra 2018). Lipomannan (LM) and lipoarabinomannan (LAM) are non-covalently attached into mycobacterial membranes (MIM and MOM) of the cell wall via the PIMs anchor (Ortalo-Magné *et al.* 1996; Abrahams and Besra 2018). In *Mtb* lipoarabinomannan also called Mannose-capped LAM (ManLAM) acts as a virulence factor by interacting with the macrophage mannose receptors, C-lectins and Toll-like receptors which result in inhibition of the maturation of phagosome during host-pathogen interactions (Mishra *et al.* 2011; Fukuda *et al.* 2013).

**Figure 5.** The structure of mycobacterial cell wall based on Minnikin model of mycobacterial cell envelope (Minnikin *et al.* 2002).

Mycobacterial outer membrane (MOM) composed of acyl lipids and glycolipids including phthiocerol dimyccocerosate (PDIM), trehalose monomycolate (TMM), trehalose dimycolates (TDM), sulfoglycolipid (SGL), diacyltrehalose (DAT) and polyacltyrehalose (PAT). The core of the mycobacterial cell wall mainly consists of mycolic acids-arabinogalactan-peptidoglycan complex (mAGPc). Mycobacterial inner membrane (MIM) consists of phospholipids including phosphatidylethanolamine (PE), phosphatidyl-myo-inositol (PI) and diphosphatidylglycerol (DPG). PG displayed several modifications such as a 3-3 cross-link, Amidation and Glycolylation. The capsular material is not illustrated.
The mycolic-arabinogalactan-peptidoglycan (mAGP) complex includes mycolic acids, arabinogalactan and peptidoglycan (Brennan 2003), and represents a cell wall core that is essential for Mtb viability (Alderwick et al. 2015).

Mycolic acids (MA) mainly consist of two branched fatty acid chains. A short α-alkyl chain of around 30 carbon atoms and a long β-hydroxyl chain (also known as meromycolate) composed of 60 carbon atoms long (Sridharan et al. 2007; Schiebel et al. 2013). The MA chains form tetramycolyl-penta-arabinofuranosyl clusters, which are hydrophobic and play an important role in reducing the permeability of small molecules to mycobacteria (Hett and Rubin 2008). The MA layer is covalently linked via ester bonds to the arabinogalactan (AG) layer (Crick et al. 2001).

Though mycolic acids can be found in other species of actinobacteria such as Rhodococcus and Corynebacteria (Ochi 1995), in mycobacteria, they contain double bonds (C=C) and cyclopropane rings (Marrakchi et al. 2014). These distinct structures of mycolic acid may also play a role in the cell wall integrity, antimicrobial resistance and suppression of immune response within macrophages (George et al. 1995; Korf et al. 2005; Marrakchi et al. 2014).

Another important layer, arabinogalactan (AG), is a polysaccharide of two unusual furanose forms (D-galactofuranose and D-arabinofuranose), which are the major saccharides of the mycobacterial cell wall (Daffe et al. 1990; McNeil et al. 1990). The galactan residues are a linear chain of around 30 β-D-galactofuranose units that are alternately linked in a 1-5 and 1-6 (Hett and Rubin 2008, Abrahams and Besra 2018). The arabinan component has three branched chains composed of 23 alternative α-D-arabinofuranose units that are linked to galactan at the 5th position in 1-5 linkages (Besra et al. 1995; Alderwick et al. 2015). Arabinan has also important modification such as succinyl or galactosamine (GalN) residues, which are mostly present in pathogenic mycobacteria (Kaur et al. 2009; Kieser and Rubin 2014). AG enables the peptidoglycan layer to covalently connect the mycolic acids layer and maintains the integrity of mycobacterial cell envelop (Birch et al. 2008). Furthermore, AG can be an anchor for the impermeable mycolic acid layer and therefore it plays a key role in regulating the cell permeability of mycobacteria (Hett and Rubin 2008).
1.5 PG function and structure

Peptidoglycan (PG) also known as murein is a “mesh” or scaffold of the bacterial cell wall that is critical for cell integrity (Brennan and Nikaido 1995). It is a rigid structure that forms the basal layer of the mAGP complex in mycobacteria (Alderwick et al. 2015). PG is also involved in cell signaling, antibacterial resistance, cell growth, division, virulence, resistance to osmotic stress and maintenance of cell shape and size (Hett and Rubin 2008, Raghavendra et al. 2018, Squeglia et al. 2018).

Mycobacterial PG belongs to the A1γ type, which is very common and present in Escherichia coli (Schleifer and Kandler 1972; Raghavendra et al. 2018). It is composed of two long glycan strands and a short peptide stem. Each glycan strand is a polymer of two disaccharides N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) that are alternately linked by β-1, 4-glycosidic bonds (Botella et al. 2017). The peptide stem consists of L-alanyl–D-iso-glutaminyl-meso-diaminopimelic acid-D-alanyl-D-alanine, which plays a role in crosslinking of peptidoglycan strands (Heijenoort 2001; Alderwick et al. 2015). This pentapeptide is attached to the NAM residue by an amide bond of the L-alanyl moiety (Brennan 2003; Vollmer et al. 2008).

*Mtb* PG contains diverse modifications (see Figure 5). For example, muramic acid residues can be N-glycolylated by N-Ac-Muramic hydroxylase (NamH) to produce an N-glycolated form (Raymond et al. 2005). This modification has been detected in all mycobacteria excluding Mycobacterium leprae in which *namH* is a pseudogene (Mahapatra et al. 2008). This modification is thought to increase antimicrobial resistance to lysozyme and β-lactam antibiotics (Raymond et al. 2005; Davis and Weiser 2011). Previous studies have revealed that D-cycloserine increased the formation of N-glycolated PG (Hett and Rubin 2008). Though, NamH is not essential for *Mtb* growth *in vitro* (Raymond et al. 2005), and its depletion also did not affect the pathogenicity of *M. tuberculosis* in infected mice, N-glycolylated PG formation could provoke NOD2-mediated host responses and therefore plays a role in immunogenicity of *Mtb* (Hansen et al. 2014).

Another important modification is the occurrence up to 80% of peptide crosslinking between meso-Diaminopimelic acid in a 3-3 position, rather than the typical 4-3 cross-linkage, produced by L-D transpeptidases (Consaul et al. 2005; Gupta et al. 2010; Raghavendra et al. 2018). This makes PG more rigid and assists to survive in stressful conditions and during the stationary phase of growth (Lavollay et al. 2008, Vollmer 2008, Gupta et al. 2010). A recent study has
been shown that a mutant of *M. smegmatis* lacking all L-D transpeptidases did not form 3-3 crosslinks and highly increased the formation of 4-3 crosslinks, thus, it was susceptible to β-lactam antibiotics and resulted in rapid cell death (Baranowski et al. 2018). 3-3 cross-links are believed to be critical for PG maturation (Baranowski et al. 2018).

Mycobacterial PG can also be amidated at the carboxylic acid group of *meso*-Diaminopimelic acid or D-iso-Glutamic acid in the peptide stem (Kumar et al. 2012; Kieser and Rubin 2014). This modification might play an important role in the regulation of the peptides cross-linking and increase resistance to endopeptidases (Hett and 2008, Abrahams and Besra 2018).

1.6 PG synthesis and remodelling

Regulation of cell wall biosynthesis is essential for mycobacterial viability, antibiotic resistance and pathogenesis (Kieser and Rubin 2014). However, compared with other bacteria, current knowledge about the PG synthesis in mycobacteria is limited. There are several enzymes involved in biosynthesis, remodelling and modification of the peptidoglycan. They include PG synthesising and PG cleaving enzymes, which are located either in the cytoplasmic or periplasmic compartments of the cell (Lee and Huang 2013). The well-balanced activity of PG synthesis versus PG degradation ensures that *Mtb* continues to synthesise a new PG during cell growth but at the same time, it cleaves and remodels the existing PG in a regulated mode to allow for cell elongation and cell division (Egan et al. 2017; Squeglia et al. 2018).

1.6.1 PG synthesis

PG biosynthesis is a multistep process, occurring in three overall stages (see Figure 6). The first stage starts in the cytoplasm with the synthesis of PG monomeric precursors (Typas et al. 2011). It begins with the generation of uridine-diphospho-N-acetylglucosamine (UDP-NAG) unit which is synthesised from D-fructose-6-phosphate through the action of three different enzymes (Alderwick et al. 2015). GlmS is an aminotransferase that catalyses and converts D-fructose-6-P to D-glucosamine-1-P, which in turn converts to glucosamine-1-P by a mutase enzyme, GlmM (Durand et al. 2008; Li et al. 2011). The final building step of UDP-NAG is achieved by GlmU, an essential enzyme with bifunctional activity (acetylation and uridylation) (Zhang et al. 2009). The UDP-NAG residue is then transformed to the UDP-N-acetylmuramic acid (UDP-NAM) by linking enoyl pyruvate at the C5 position of NAG (Bouhss et al. 2007; Kumar et al. 2011). This is catalysed by MurA, a UDP-NAG enolpyruvyl

The stem peptide is then attached to the UDP-NAM residue to form what has known as the Park’s nucleotide (Barreteau et al. 2008). This is performed by enzymes of the Mur family (C-F), which are ATP-dependent ligases that sequentially incorporate the amino acids beginning with the L-alanine (L-Ala), then D-glutamine (D-iso-Glu), meso-diaminopimelic acid (m-DAP) and finally adding the two terminal D-alanine (D-Ala) residues (Kurosu et al. 2007; Abrahams and Besra 2018) (Figure 6). MurA-F enzymes are essential for mycobacterial viability and could be targeted for developing new anti-Mtb drugs (Kouidmi et al. 2014).

The second stage is the translocation of PG monomeric precursors across the membrane. The UDP of the Park’s nucleotide is transferred to generate the first membrane-anchored PG precursor (Lipid I) by MurX (Kurosu et al. 2007; Chen et al. 2013). The NAG is then added to the NAM residue via β (1→4) linkage by the glycosyltransferase MurG to form Lipid II (Mengin-Lecreulx et al. 1991; Jha et al. 2012). Lipid II precursors are transported through the cytoplasmic membrane and incorporated into the existing PG. Two different proteins FtsW and MurJ (MviN) have been proposed to possess Lipid II Flippase activity (Butler et al. 2013; Ruiz 2015). The study of Mohammadi et al. (2011) has provided an in vitro biochemical evidence and suggested that FtsW promotes the translocation of fluorescence of nitrobenzoxadiazole (NBD)-labeled lipids (a Lipid II homolog) across the membrane. Additionally, the depletion of FtsW is affected and reduced the transportation of Lipid II, therefore, the authors assumed that FtsW has a flippase activity (Mohammadi et al. 2011). MurJ is another essential enzyme has flippase activity and its depletion resulted in reduced PG synthesis and increased the accumulation of PG precursors in the cytoplasm (Inoue et al. 2008; Ruiz 2015). Further, a recent study in E.coli showed that MurJ binds to lipid II using a native mass spectrometry (Bolla et al. 2018). In the Gram-positive bacterium B. subtilis it has been shown that MurJ with Amj, an alternate lipid II flippase is important for regulating the biosynthesis of the cell wall (Meeske et al. 2015) (Figure 6).

The last stage is the PG polymerisation. At the final stage, PG synthases play a key role in PG polymerisation and modification (Squeglia et al. 2018). Class A PBPS like PonA1 and PonA2 (PBP1 in E. coli) are the most prominent polymerising enzyme group with bifunctional activities: glycosyltransferase (GT) and transpeptidase (TP) (Alderwick et al. 2015; Egan et al. 2015; Egan and Vollmer 2015; Kieser et al. 2015). The GT domain with glycosyltransferase
activity polymerises PG units and cross-links the NAM-peptide stem to the adjacent NAG residue at multi-layered PG strands (Abrahams and Besra 2018). The TP domain with DD-transpeptidase activity forms classical (3-4) cross-linkages between m-DAP and D-Ala of the adjacent stem peptide (Kumar et al. 2012). However, the significant proportion of the peptide cross-links in the *Mtb* PG is of a 3-3 type (as described above) (Lavollay et al. 2008). This is catalysed by LD-transpeptidases, which are required for cell wall integrity and maintaining mycobacteria rod-shaped particularly during asymmetric polar growth (Baranowski et al. 2018). This type of cross-link is attributed to the resistance of *Mtb* to β-lactam antibiotics (Fisher and Mobashery 2016). The deletion of PG synthetic enzymes PonA1, PonA2 and LdtB increased the susceptibility of *Mtb* to β-lactam antibiotics (Kieser et al. 2015).

The members of monofunctional class B PBPs such as PbpA and PbpB (PBP2 in *E. coli*) have a transpeptidase domain (Arora et al. 2018) and carry out DD-transpeptidation activity by releasing a D-Ala precursor (Goffin and Ghysen 2002; Egan et al. 2015). The DD-transpeptidase domain reacts with β-lactam antibiotics (penicillin) structurally similar to D-Ala–D-Ala (Wivagg et al. 2014).

**Stage 3 PG polymerisation**

Figure 6. Peptidoglycan biosynthesis of the *Mtb* cell wall.

**Stage 1** – the cytoplasmic stage where PG monomeric units (lipid II) produced. **Stage 2** - transport (flipping) of lipid II precursors across the cytoplasmic membrane to the periplasm. **Stage 3** - PG polymerisation by transglycosylation and transpeptidation processes. The functions of the important enzymes that participated in synthesising of PG are illustrated.
1.6.2 PG remodeling in mycobacterial cell growth and division

PG is a polymer that forms a mesh-like sacculus of glycan strands and cross-linking peptides that surrounds the cytoplasmic membrane. This sacculus must be rebuilt and simultaneously cleaved during the separation of daughter cells (Raghavendra et al. 2018). A finely tuned balance between synthases and hydrolases ensures PG integrity and prevents defects within the cell wall that might increase susceptibility to antibiotics and cell lysis (Typas et al. 2011; Egan et al. 2015). Therefore, PG turnover is an essential process in which mycobacteria regulate their own growth, division, and resuscitation from dormancy (Squeglia et al. 2018).

PG hydrolases have many biological functions. They play a key role in cell wall biosynthesis, remodeling of peptidoglycan throughout bacterial growth, septation of daughter cells during cell division, bacterial signaling and autolysis (Shockman and Höltinge 1994; Lee and Huang 2013). Nevertheless, the actions of these enzymes in mycobacteria are not fully understood.

PG hydrolases can be classified into four basic groups according to their cleaving sites. Muramidases (lysozymes), N-acetylglucosaminidases (glucosaminidases), peptidases (endopeptidase and carboxypeptidase) and N-acetylmuramoyl- L-alanine amidases (amidases) as shown in (Figure 7) (Volmer et al. 2008).

Muramidases cleave the glycosidic bond (1-4) between NAG and NAM residues. They play a role in the cell expansion by hydrolysing the septal of PG and insertion of new PG precursors during bacterial growth (Holtje 1995; Vollmer 2012). Glucosaminidases also target the glycosidic bond between the N-acetyl-beta-D-glucosamine residue and the adjacent monosaccharide substrates in the peptidoglycan (Frirdich and Gaynor 2013). They are involved in the PG turnover and production of new PG precursors (Karamanos 1997). Another important PG hydrolases group is peptidases. These enzymes cleave amide bonds between amino acids in the stem peptide (Wyckoff et al. 2012). Depending on their cleavage sites, they are categorized into carboxypeptidases (CPases) and endopeptidases (EPases) (Sauvage et al. 2008). CPases cleave the carboxy group of DD-alanine. Thus, these enzymes play a crucial role in PG biosynthesis (Vollmer et al. 2008; Domínguez-Gil et al. 2016). EPases target (4-3) crosslinks and non-terminal amide bonds in the stem peptide (Holtje 1995; Vollmer et al. 2008).

N-acetylmuramoyl- L-alanine amidases (amidases) will be discussed in the next (section 1.7).
Peptidoglycan hydrolases include muramidases, N-acetylglucosaminidases (glucosaminidases), peptidases (endopeptidase and carboxypeptidase) and N-acetylmuramoyl-L-alanine amidases (amidase).

1.7 N-acetylmuramyl-L-alanine amidases (NAM-amidases)

N-Acetylmuramyl-L-alanine amidases (NAM-amidases) belong to the zinc metalloenzyme family. They are responsible for eliminating the entire stem peptide from glycan strands by cleaving the amide bond between lactyl group of the N-acetylmuramic acid moiety and the L-Alanine, the first amino acid in the stem peptide (Figure 7) (Heidrich et al. 2001; Vollmer et al. 2008).

Depending on the type of amidase domain present in the protein, NAM-amidases can be divided into two main families, amidase_2 and amidase_3 domains (Bateman and Rawlings 2003; Vollmer et al. 2008). These enzymes can be found in all organisms, and their physiological functions have been implicated in the cleavage of cell wall materials (Firczuk and Bochtler 2007).

Bacteriophages often produce cell wall amidases to hydrolyse the bacterial cell wall and release the host DNA during phage infection (Cheng et al. 1994).
In eukaryotes, NAM-amidases are known as peptidoglycan recognition proteins (PGRPs) (Steiner 2004). They contain the bacterial amidases 2 domain and have an amidase activity by cleaving the stem peptide of the bacterial peptidoglycan (Dziarski and Gupta 2006). It was shown that PGRPs could activate or inhibit Toll-I receptors through binding to degraded muropeptides (Dziarski and Gupta 2006), and therefore these NAM-LA-amidases play an important role in the regulation of immune responses, which in turn reduce the risk of bacterial infection (Girardin et al. 2003; Guan and Mariuzza 2007; Dziarski and Gupta 2010).

In bacteria, NAM-amidases participate in peptidoglycan remodeling, antimicrobial resistance, virulence, cell signaling and septation of daughter cells during cell division (Machowski et al. 2014). For example, *Streptococcus pneumonia* has a peptidoglycan NAM-amidase LytA. The precise role of this enzyme is unknown. However, it is thought to be important for the virulence of *S. aureus* releasing the pneumolysin toxin (Martner et al. 2008; Eldholm et al. 2009). Previous studies showed that knocking out this enzyme in *Streptococcus pneumoniae* resulted in increased susceptibility of the bacterium to antibiotics (Berry and Paton 2000; Dalia and Weiser 2011).

Most bacteria encode a large number of NAM-amidases with the same substrate specificity, however, their functions may differ. For example, *Bacillus subtilis* contains three amidases CwlB, CwlC and CwlD (Firczuk and Bochtler 2007). These enzymes are believed to play distinct roles in sporulation, germination, cell communication and peptidoglycan turnover (Smith et al. 2000; Shida et al. 2001). *Escherichia coli* has five amidases (AmiA, AmiB, AmiC, AmiD and AmpD) (Priyadarshini et al. 2007). AmpD is localized in the cytoplasm; while all others are translocated into the periplasm, and their amidase activity depends on the conformation of PG (Machowski et al. 2014). AmiA, AmiB, and AmiC can only hydrolyse a mesh-like PG conformation of alternating NAG and NAM residues bound to the stem peptide when they are found in the periplasm (Heidrich et al. 2001). AmpD can hydrolyse cytoplasmic anhydromuropeptides and it is important for PG recycling (Heidrich et al. 2001; Uehara and Park 2007). AmiD was found to degrade PG regardless of substrate conformation (Uehara and Park 2007; Pennartz et al. 2009). An *E. coli* mutant lacking AmiA, AmiB and AmiC formed long cell chains as bacteria could not be divided into daughter cells; it was also more sensitive to antibiotics (Heidrich et al. 2001; Priyadarshini et al. 2007). NAM-amidases (AmiA, AmiB and AmiC) require activation by special partner proteins (Uehara et al. 2010). According to the proposed model these amidases contain a special loop which closes the active site and prevents the protein binding to the bacterial peptidoglycan (Yang et al. 2012). Interaction of EnvC and
NlpD with AmiA, AmiB and AmiC amidases alters the native conformation of the enzymes by opening the active sites and facilitates the attachment to and hydrolysis of PG (Uehara et al. 2010; Yang et al. 2012). The same mode of activation for NAM-amidases was also found in other bacteria such as Neisseria gonorrhoeae (Lenz et al. 2016) and Xanthomonas campestris (Yang et al. 2018).

*Mtb* contains at least four NAM-amidases, two with an amidase_2 type domain, Ami3 (Rv3811) and Ami4 (Rv3594), whereas Ami1 (Rv3717) and Ami2 (Rv3915) contain an amidase_3 type domain (Figure 8) (Machowski et al. 2014).

**Figure 8.** *Mtbc* NAM-amidases.

Ami1 (Rv3717) and Ami2 (Rv3915) contain an amidase_3 type domain while Ami3 (Rv3811) and Ami4 (Rv3594) possess an amidase_2 type domain. The NAM–amidase Rv3915 (CwlM) has two putative PG binding domains and one amidase domain with no signal peptide or a transmembrane domain. This Figure was adapted from (Machowski et al. 2014).

Rv3717 and Rv3915 are highly conserved in mycobacteria (Machowski et al. 2014). However, only Rv3915 (CwlM) is essential for *Mtbc* growth and viability (Boutte et al. 2016, Turapov et al. 2018).

Our knowledge on the enzymatic activity of *Mtbc* NAM amidase is limited. The crystal structure of Rv3717 has been recently solved and its enzymatic activity has been characterised (Kumar et al. 2013; Prigozhin et al. 2013). Rv3717 lacks the PG-binding domain, and therefore the interaction of Rv3717 with cell wall PG is questionable (Kumar et al. 2013). Rv3717 has a typical signal peptide which facilitates its translocation across the membrane to the periplasm (Machowski et al. 2014). Rv3717 activity depends on Zn$^{2+}$ which is coordinated by the active sites of His101 and Glu46 (Kumar et al. 2013; Prigozhin et al. 2013).
The function of another predicted NAM amidase, CwlM remains a controversial issue. Deng et al., (2005) purified recombinant CwlM from E. coli strain HMS174 (DE-3) and showed that it was able to cleave Micrococcus luteus and M. smegmatis cell wall preparations in zymogram assays (Deng et al. 2005). Additionally, the authors showed the purified recombinant CwlM could digest the artificial substrate N-acetylmuramoyl-l-alanyl-disoglutamine (MAG) and its enzymatic products were analysed using HPLC and LC/MS analysis, confirming that CwlM has NAM-amidase activity (Deng et al. 2005). However, Boutte et al questioned the enzymatic function of CwlM and demonstrated a regulatory role for PG biosynthesis in mycobacteria (Boutte et al. 2016). Further studies of this enzyme are needed such as the investigation of its biochemical function, molecular signaling mechanisms involved in regulation of CwlM activity, protein-protein interaction and the crystal structure of CwlM.

1.8. Coordination of PG biosynthesis with bacterial growth and division

In Mtb, cell division is synchronized with the genome replication and chromosome segregation into two daughter nucleoids (Szwedziak and Löwe 2013; Squeglia et al. 2018). Unlike other bacteria, elongation and division of Mtb generates asymmetric daughter cells (Hett and Rubin 2008, Kieser and Rubin 2014). The reason for asymmetric septation in mycobacteria is obscure but it is believed to increase the population heterogeneity and resistance to antibiotics (Aakre and Laub 2012; Kieser and Rubin 2014).

The division process of Mtb begins with possible interaction and cross-talk between the elongasome and divisome, multi-protein complexes at the mid-cell (Logsdon and Aldridge 2018). FtsZ, a tubulin-like protein, is localised in the mid cell to form Z-ring at the septum (Hong et al. 2013) and FtsZ depletion leads to the long filamentous cells (Li et al. 2013). After the formation of Z-ring, other proteins are assembled to form the divisome complex. There are many regulatory proteins like ClpX, an integral protease that controls the Z-ring polymerization by direct interaction with FtsZ (Dziedzic et al. 2010). It was shown that the overexpression of ClpX results in reducing the production of Z rings and hence cells seem to be elongated (Camberg et al. 2009). Another important regulatory protein is ChiZ that contains a lysin motif (LysM) domain (Chauhan et al. 2006; Kieser and Rubin 2014). This protein showed a similar phenotype of ClpX as a result of ChiZ overexpression, and therefore it might also coordinate Z-ring formation (Chauhan et al. 2006; Vadrevu et al. 2011). The elongation complex represented by interaction of CrgA with CwsA is also recruited at the septum to coordinate cell elongation and division (Plocinski et al. 2011; Plocinski et al. 2012). These proteins are guided
to the cell poles by \textit{Mtb} structural protein Wag31, acting as a scaffold which is a homolog of DivIVA in \textit{B. subtilis} (Plocinski \textit{et al.} 2012; Szwedziak and Löwe 2013). The complex CrgA–CwsA known as a (cell wall synthesis protein A) binds to Wag31, and they are therefore key players in PG synthesis and cell shape maintaining (Plocinski \textit{et al.} 2012). As previously mentioned, the PG layer of \textit{Mtb} is highly cross-linked by a 3-3 cross-links and, therefore, Ldts might be also recruited to the elongation complex during mycobacteria growth stages (Kieser and Rubin 2014). These structural components are collectively involved in stabilising the divisome and promoting the synthesis of a septum (Figure 9).

After septum formation is finished and shared between daughter cells, PG needs to be degraded to complete the bacterial division. As mentioned above, Mycobacteria have multiple PG hydrolases. One of them is RipA has been shown to localise at the septum of dividing cells and its depletion results in a growth defect and an abnormal phenotype (Ruggiero \textit{et al.} 2010). RipA interacts with PonA1 (Hett \textit{et al.} 2010) and Resuscitation-promoting factor B (RpfB) (Hett \textit{et al.} 2008). PonA1 synthesises PG and its activity might be regulated by interaction with RipA, while RipA-RpfB complex is critical for cleavage of PG in the septum (Hett \textit{et al.} 2007; Hett \textit{et al.} 2010).
This Figure was taken from (Kieser and Rubin 2014). A group of enzymes and proteins are involved in cell elongation and division including PG synthases (purple), PG hydrolases (green), structural proteins (yellow) and regulatory proteins (blue). A. The elongasome complex of multi-proteins controls the polar growth of mycobacteria. Wag31 acts as a scaffold for elongation proteins. A cell wall synthesis protein A (CwsA) plays a key role in stabilising Wag31 whereas PG synthases (e.g. PonA1) incorporates new peptidoglycan precursors into existing PG of the bacterial cell wall. PG hydrolases contribute to the turnover of PG units by creating spaces for insertion new synthesised PG subunits. PonA2 and other L, D-transpeptidases are important for cross-linking stem peptides of PG strands (4–3 and 3–3 types). B. The cell division process depends on the divisome complex of multi-proteins. The septation of elongated cells begins with the formation of the Z ring formed by FtsZ. Regulatory proteins including ClpX, ChiZ, FhaB and Rv3660c play a key role in stabilising Z ring. Phosphorylation by protein kinase A (PknA) regulates the activity of FtsZ. Structural proteins FtsW/Q, CrgA and CwsA are recruiting to assemble the divisome. The divisome complex with PBPA/B, PonA1 and Ldts generate the septal ring. The hydrolases including RipA, RpfB and possibly other unknown hydrolases degrade the septum to separate the daughter cells.

1.9 Regulation of mycobacterial growth and PG biosynthesis by serine/threonine kinase signaling

Two of them PknA and PknB are essential for growth and maintenance of cell shape (Fernandez et al. 2006; Nagarajan et al. 2015; Squeglia et al. 2017; Khan et al. 2018).

PknB consists of one intracellular kinase domain at the N-terminus, one transmembrane domain (juxtamembrane) and four extracellular domains called PASTA (penicillin-binding protein and serine/threonine kinase associated) at the C-terminus (Figure 10) (Barthe et al. 2010; Turapov et al. 2015). All these domains have been shown to be essential for PknB functions (Chawla et al., 2014; Prigozhin et al., 2016).

It has been shown that the extracellular PASTA domains are able to recognise peptidoglycan units and are involved in PknB localization (Mir et al. 2011). A recent study has shown that the PASTA domain of PknB interacts with lipid II particularly at a meso-diaminopimelic acid residue and this interaction activates and controls the localisation of PknB in the cell (Kaur et al. 2019).

The juxtamembrane domain has been shown to recruit FhaA and perhaps other proteins that regulate peptidoglycan biosynthesis (Roumestand et al. 2011). PknB phosphorylates many substrates, mainly proteins participating in the regulation of PG biosynthesis and remodelling (Prigozhin et al. 2016; Squeglia et al. 2017). For example, PknB phosphorylates and activates the peptidoglycan synthase PonA1, a penicillin binding protein (PBP) that plays a vital role in the regulation of mycobacterial polar growth and cell wall biosynthesis (Kieser et al. 2015).

PknB also phosphorylates N-acetylglucosamine-1-phosphate uridylytransferase (GlmU) and control its acetyltransferase activity, which is required for the formation of UDP-NAG subunit.

---

**Figure 10.** The putative structure of protein kinase B (PknB).
It consists of the intracellular kinase domain (279 amino acids), transmembrane domain, TM (331 amino acids) and four extracellular PASTA domains (354-627 amino acids). This Figure is adapted from (Turapov et al. 2015).
during the PG biosynthesis process (Parikh et al. 2009). Additionally, PknB controls the activity of ATP-dependent Mur ligases (MurE, MurC, MurD and MurF) of Mtb which play essential roles in the cytoplasmic steps of the PG synthesis (Figure 6) (Munshi et al. 2013).

PknB also plays an important role in regulating protein localisation and protein-protein interaction. FhaA is a PknB-phosphorylated protein that has a C-terminal Fork Head Associated (FHA) domain and could interact with other phosphorylated proteins (Roumestand et al. 2011). Gee et al., (2012) show that PknB phosphorylates MurJ (MviN) that interacts with a regulatory FHA protein, and this interaction has been shown to be important for regulating cell wall biosynthesis (Gee et al. 2012). Recently published work has revealed that CwlM is phosphorylated by PknB in the cytoplasm (Boutte et al. 2016). The authors suggested that the phosphorylated CwlM was essential for regulating the activity of MurA, the primary enzyme in PG precursor’s biosynthesis (Boutte et al. 2016). PknB also interacts and phosphorylates proteins involved in lipid metabolism such as EchA7 and WbbL2 (Wu et al. 2017).

1.10 Hypothesis of this project

CwlM is essential for mycobacterial growth and its depletion resulted in cell lysis and loss of viability. This led to a hypothesis that CwlM might play a key role in peptidoglycan biosynthesis either by direct enzymatic cleavage or by regulating activity of enzymes involved in peptidoglycan biosynthesis. Furthermore, PknB mediated phosphorylation could control CwlM localisation and interaction with partner proteins.

1.11 Aims of this project:

The overall aim of this project was to investigate the function of CwlM in peptidoglycan biosynthesis and remodeling in mycobacteria. Project objectives included.


2. Investigate CwlM enzymatic activity by zymography and assessing cleavage of FITC-labelled PG.


4. Investigate localisation of CwlM in mycobacteria by western blot using anti-CwlM antibody.
5. Identify protein partners interacting with phosphorylated and non-phosphorylated CwlM using immunoprecipitation and protein fragment complementation assays.

6. Optimise conditions for crystallisation of CwlM using commercially available screens and attempt to solve the CwlM structure by using X-ray crystallography method.
Chapter 2

2. General Materials and Methods
2.1 Regents

All reagents used in this project are listed in the Table 1.

<table>
<thead>
<tr>
<th><strong>Table 1: Reagents used in this study</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Reagent</strong></td>
</tr>
<tr>
<td><strong>Antibodies</strong></td>
</tr>
<tr>
<td>Phospho-Threonine Antibody (P-Thr Polyclonal)</td>
</tr>
<tr>
<td>Monoclonal Anti-polyHistidine antibody</td>
</tr>
<tr>
<td>Monoclonal anti-GlnA antibody</td>
</tr>
<tr>
<td>Monoclonal anti-GroEL antibody</td>
</tr>
<tr>
<td>Polyclonal anti-PknB antibody</td>
</tr>
<tr>
<td>Polyclonal anti-GarA antibody</td>
</tr>
<tr>
<td>Custom polyclonal anti-CwlN antibody raised in rabbit</td>
</tr>
<tr>
<td>Custom polyclonal antibodies raised against GKNDRPT-phosphoGT in rabbit (anti-T382-P)</td>
</tr>
<tr>
<td>Custom polyclonal antibodies raised against GKNDRPTGT in rabbit (anti-T382)</td>
</tr>
<tr>
<td>Alkaline-phosphatase conjugated anti-rabbit IgG</td>
</tr>
<tr>
<td>Anti-rabbit IgG, HRP-linked antibody</td>
</tr>
<tr>
<td><strong>Chemicals</strong></td>
</tr>
<tr>
<td>BCIP®/NBT Liquid Substrate System</td>
</tr>
<tr>
<td>SignalFire™ Elite ECL Reagent</td>
</tr>
<tr>
<td>Cyanogen bromide-activated-Sepharose® 4B</td>
</tr>
<tr>
<td>cOmplete Ultra Tablets</td>
</tr>
<tr>
<td>-------------------------------</td>
</tr>
<tr>
<td>Protease Inhibitor Cocktail</td>
</tr>
<tr>
<td>PhosSTOP™ phosphatase inhibitor tablets</td>
</tr>
<tr>
<td>Ni-NTA agarose</td>
</tr>
<tr>
<td>Glutathione Sepharose 4B</td>
</tr>
<tr>
<td>HiLoad 16/600 Superdex 200 pg prepacked column</td>
</tr>
<tr>
<td>SERVA Gel™TG Prime™ 4-20% 10 samples wells</td>
</tr>
<tr>
<td>SERVA Gel™TG Prime™ 12% 12 samples wells</td>
</tr>
</tbody>
</table>

2.2 Media

**Lysogeny Broth (LB)** was prepared by dissolving 10 g tryptone, 5 g yeast extract and 10 g NaCl in 1 L dH₂O; pH was adjusted to 7.5 before sterilisation by autoclaving at 121°C.

**Power Prime Broth™** (Molecular Dimensions) was prepared according to the manufacturer’s instruction and sterilised by autoclaving at 121°C for 15 min.

**Middlebrook 7H9 Broth** (Beckton Dickenson Difco) was prepared according to manufacturer’s instruction and sterilised by autoclaving at 121°C for 15 min. This medium was supplemented with 10% (v/v) Albumin-Dextrose complex (ADC) and 0.05% (w/v) Tween 80. ADC was prepared by dissolving 50 g bovine serum albumin, 20 g D-glucose and 8.5 g NaCl in 1 L of water and filter sterilized.

**Sauton’s medium** was prepared by dissolving 0.5 g KH₂PO₄, 0.5 g MgSO₄, 4 g L-asparagine, 0.05 g ferric ammonium citrate, 2 g citric acid, 10 ml glycerol and 0.1 ml of 1% (w/v) ZnSO₄ in 1 L of water. The pH was adjusted to 7.2-7.4. Medium was supplemented by 0.05% (w/v) Tween 80.

2.3 Bacterial strains

A list of the bacterial strains used in this project are given in Table 2.
Table 2: Bacterial strains used in this study.

Phosphorylation sites of CwlM T43, T382 and T386 were replaced with Aspartate (D) (phosphomimetic) or Alanine (A) (phosphoablative). AmiR1 represents the amidase domain only; AmiR2-DD represents the amidase domain with phosphomimetic tail. Sites-directed mutagenesis of CwlM catalytic residues included H204, E217 and D339 were replaced with Alanine (A) to abolish the activity of CwlM.

<table>
<thead>
<tr>
<th>Strain name</th>
<th>Strain description</th>
<th>Plasmid</th>
<th>Comments</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Escherichia coli strains of CwlM forms (full length)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> C41 (DE3) pET</td>
<td>Empty plasmid strain</td>
<td>pET15bTEV</td>
<td>Control</td>
<td>Lab stock (Bioline)</td>
</tr>
<tr>
<td><em>E. coli</em> C41 (DE3) pET CwlM</td>
<td>6xHis-CwlM expression strain</td>
<td>pET15bTEV::cwlM</td>
<td>Protein expression &amp; Purification</td>
<td>This study</td>
</tr>
<tr>
<td><em>E. coli</em> C41 (DE3) pET DDD</td>
<td>6xHis-DDD expression strain</td>
<td>pET15bTEV:: cwlM DDD</td>
<td>Protein expression &amp; Purification</td>
<td>This study</td>
</tr>
<tr>
<td><em>E. coli</em> C41 (DE3) pET H204A</td>
<td>6xHis-H204A expression strain</td>
<td>pET15bTEV:: cwlM H204A</td>
<td>Protein expression &amp; Purification</td>
<td>This study</td>
</tr>
<tr>
<td><em>E. coli</em> C41 (DE3) pET D339A</td>
<td>6xHis-D339A expression strain</td>
<td>pET15bTEV:: cwlM D339A</td>
<td>Protein expression &amp; Purification</td>
<td>This study</td>
</tr>
<tr>
<td><em>E. coli</em> C41 (DE3) pET E217A</td>
<td>6xHis-E217A expression strain</td>
<td>pET15bTEV:: cwlM E217A</td>
<td>Protein expression &amp; Purification</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Escherichia coli strains of CwlM forms (truncated)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> C41 (DE3) pET AmiR1</td>
<td>6xHis- AmiR1$_{S183-1375}$ expression strain</td>
<td>pET15bTEV:: amiR1</td>
<td>Protein expression &amp; Purification</td>
<td>This study</td>
</tr>
<tr>
<td><em>E. coli</em> C41 (DE3) pET AmiR2-DD</td>
<td>6xHis- AmiR2$_{D2183-S407}$ expression strain</td>
<td>pET15bTEV:: amiR2-DD</td>
<td>Protein expression &amp; Purification</td>
<td>This study</td>
</tr>
<tr>
<td><em>E. coli</em> C41 (DE3) pET AmiR1H204A</td>
<td>6xHis- H204A expression strain</td>
<td>pET15bTEV:: amiR1H204A</td>
<td>Protein expression &amp; Purification</td>
<td>This study</td>
</tr>
<tr>
<td><em>E. coli</em> C41 (DE3) pET AmiR1 E217A</td>
<td>6xHis- E217A expression strain</td>
<td>pET15bTEV:: amiR1 E217A</td>
<td>Protein expression &amp; Purification</td>
<td>This study</td>
</tr>
<tr>
<td><em>E. coli</em> C41 (DE3) pET AmiR1 D339A</td>
<td>6xHis- D339A expression strain</td>
<td>pET15bTEV:: D339A</td>
<td>Protein expression &amp; Purification</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Escherichia coli strains of CwlM partners proteins</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> C41 (DE3) pET MurJicd</td>
<td>6xHis- MurJicd expression strain</td>
<td>pET15bTEV::murJicd</td>
<td>Protein expression &amp; Purification</td>
<td>This study</td>
</tr>
<tr>
<td>E. coli C41 (DE3) pET MurJ E541-F680</td>
<td>6xHis-MurJ E541-F680 expression strain</td>
<td>pET15bTEV::murJ E541-F680</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>---------------------------------------</td>
<td>----------------------------------------</td>
<td>--------------------------</td>
<td>------------</td>
<td></td>
</tr>
<tr>
<td>E. coli C41 (DE3) pGEX Wag31</td>
<td>GST-Wag31 expression strain</td>
<td>pGEX TEV::wag31</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>E. coli C41 (DE3) pGEX FhaA</td>
<td>GST-FhaA expression strain</td>
<td>pGEX TEV::fhaA</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>E. coli BL21 (DE3) pGEX PknB</td>
<td>GST-PknBkinase domain expression strain</td>
<td>pGEX::pknBkinase</td>
<td>This study</td>
<td></td>
</tr>
</tbody>
</table>

**Mycobacterium smegmatis strains**

**Mycobacterium smegmatis mc^2155 strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Phenotype</th>
<th>Genetic Modification</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>cwlM-CM</td>
<td>CwlM conditional mutant</td>
<td>pAZI9479::cwlM</td>
<td>Lab stock</td>
</tr>
<tr>
<td>cwlM-CM_pMV</td>
<td>Empty plasmid control</td>
<td>pMV306</td>
<td>Lab stock</td>
</tr>
<tr>
<td>CwlM-pMIND</td>
<td>Empty plasmid control</td>
<td>pMIND::cwlM</td>
<td>Lab stock</td>
</tr>
<tr>
<td>DDD-pMIND</td>
<td>DDD-overexpression</td>
<td>pMIND::cwlM DDD</td>
<td>Lab stock</td>
</tr>
<tr>
<td>AAA-pMIND</td>
<td>AAA-overexpression</td>
<td>pMIND::cwlM AAA</td>
<td>Lab stock</td>
</tr>
</tbody>
</table>

**Mycobacterium tuberculosis strains**

**Mycobacterium tuberculosis H37Rv strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Phenotype</th>
<th>Genetic Modification</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>cwlM-CM_pMV</td>
<td>Empty plasmid control</td>
<td>pMV306</td>
<td>Lab stock</td>
</tr>
<tr>
<td>cwlM-CM T42A</td>
<td>T42A CwlM variant</td>
<td>pMV306::cwlMT4 2A</td>
<td>Lab stock</td>
</tr>
<tr>
<td>cwlM-CM T382D</td>
<td>T382D CwlM variant</td>
<td>pMV306::cwlMT3 82D</td>
<td>Lab stock</td>
</tr>
</tbody>
</table>

**Bacterial strains used for PG digestion activity of CwlM forms**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Phenotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli LMG 194</td>
<td>Empty plasmid</td>
<td>Lab stock</td>
</tr>
<tr>
<td><em>M. luteus</em> Fleming strain 2665</td>
<td>WT strain</td>
<td>Lab stock</td>
</tr>
</tbody>
</table>

This study
2.4 Preparation bacterial stocks

A sterile 75% v/w glycerol (250 μl) was added to 750 μl of overnight bacterial culture in 1.5 ml cryogenic tubes (Nalgene, Fisher). After careful mixing, stocks were stored at -80°C.

2.5 Cultivation of bacteria

2.5.1 *Escherichia coli* C41 and BL21 (DE3) strains

_E. coli_ starter cultures were prepared by inoculating a frozen culture into 5 ml LB media containing ampicillin (100 µg/ml). Tubes were then incubated overnight at 37°C, with shaking (200 rpm) in a shaking incubator (New Brunswick™ Innova® 44/44R).

2.5.2 *Mycobacterium smegmatis* strains

_M. smegmatis_ stock culture was inoculated into 7H9 media supplemented with ADC and Tween 80. When necessary, antibiotics were added as follows: tetracycline (20 ng/ml), kanamycin or hygromycin (50 µg/ml). Tubes were then incubated at 37°C, with shaking (200 rpm) for 3 days or until they reached a desirable OD$_{600nm}$.

2.5.3 *Micrococcus luteus* Fleming 2665 strain

_M. luteus_ starter cultures were inoculated from frozen stocks into 5 ml of LB (without antibiotics). Tubes were then incubated at 32°C, shaking at 200 rpm for 2 days.

2.6 Buffers and solutions

Electrophoresis Buffers

Typical Tris-HCl resolving gels were prepared at basic pH using a stock solution of Acrylamide/Bis-acrylamide (30%/0.8% w/v). The final mixture contained 12% polyacrylamide, 0.39 M Tris HCl, pH 8.8 and 0.1% SDS. Stacking gel contained 4% polyacrylamide, 0.125 M Tris- HCl, pH 6.8 and 0.1% SDS.

SDS Running buffer 10x was prepared by dissolving 144g glycine, 36.3g Tris base and 10g of SDS in 1 L of dH$_2$O; pH 8.3.

SDS Running buffer 1x was prepared by adding 100 ml of 10x SDS running buffer to 900 ml of dH2O.
SDS sample buffer 4x contained 10% (w/v) SDS, 10 mM dithiothreitol, 20 % (v/v) glycerol, 0.2 M Tris-HCl, pH 6.8 and 0.05% (w/v) bromophenol blue dissolved in dH2O.

Colloidal Coomassie staining solution was prepared using Coomassie Brilliant Blue (CBB) G250. Staining stock solution contained 0.1 % (w/v) CBB G250, 2 % (w/v) ortho-phosphoric acid and 10 % (w/v) ammonium sulphate. Working solution was prepared using 80 % (v/v) staining stock solution and 20 % (v/v) methanol.

**Western blots buffers**

Transfer buffer 10x was prepared by dissolving 144g of glycine and 30.3g Tris base in 1L of distilled water.

TBS buffer 1x was prepared by dissolving 20mM Tris pH8.3, 20mM KCl and 5mM NaCl in 1L of Milli-Q water.

Washing buffer (TBST) was prepared using TBS buffer 1x and 1% Tween-20 in 1L Milli-Q water.

PBS (Phosphate Buffered Saline) was prepared by dissolving one PBS tablet (Sigma-Aldrich) in 200 ml Milli-Q water. The resultant buffer consists of 10 mM KH$_2$PO$_4$ (pH 7.4), 2.7 mM KCl and 137 mM NaCl. The PBS buffer was then autoclaved.

Blocking buffer contained 2% skimmed milk PBS buffer.

**De-phosphorylation NEB Buffer 3 (1X)** was used for dephosphorylation of CwlM. This buffer contained 100 mM NaCl, 50 mM Tris-HCl pH 8.2, 10 mM MgCl$_2$ and 1 mM DDT

**Chromatography buffers for proteins purification**

**Buffers for purification of His-tagged proteins on Ni-NTA Sepharose (Qiagen).**

Loading buffer contained TrisHCl 20 mM, pH 8.5, 500 mM NaCl.

Washing buffer contained 45 mM imidazole in loading buffer.

Elution buffer contained 250 mM imidazole in loading buffer.

**Buffers for purification of GST-tagged proteins on Glutathione Sepharose 4B (Generon).**

Loading or washing buffer consisted of 140 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, 1.8 mM KH$_2$PO$_4$, pH 7.5.
Elution buffer composed of 33 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0.

Buffers for purification of antibodies

Phosphate buffered saline (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.3) was used as a loading and washing buffers.

Elution buffer contained 100 mM glycine, pH 2.8.

Tris base buffer (1 M, pH 8.5) was used for neutralization of the elution buffer.

Size-exclusion chromatography (SEC) buffer contained 150 mM NaCl, 20 mM KCl and 20 mM Tris-HCl pH 8.5.

2.7 Preparation of proteins purification columns

The type of chromatography column is chosen depending on the tag, size and isoelectric point (pI) of the protein. For purification of 6xHis-tagged proteins 1 ml of Ni-NTA-agarose (Qiagen) was packed into the column, washed with loading buffer and charged with 20 column volumes of 40 mM NiSO₄. The column was washed with 10 volumes of loading buffer before adding the filtered E. coli lysate. For purification of GST-tagged proteins, 1ml of Glutathione Sepharose™ 4B (GE Health Care) was loaded into a column, washed with PBS before loading the filtered cells lysate onto the column. Columns were not allowed to run dry during any step of the preparation. The protein purification procedure was done in the cold room.

A HiLoad 16/600 Superdex 200 pg prepacked column (GE Health Care) and an ÄKTA FPLC™ system (GE Health care) were used for size exclusion chromatography (SEC). The column was pre-equilibrated with SEC loading buffer containing 150 mM NaCl, 20 mM KCl and 20 mM Tris-HCl pH 8.5 before use. The SEC standards (Bio-Rad) with different molecular weight markers (1,350 to 670,000 Da) were used for column calibration according to manufacturer instructions (Figure 11).
Figure 11. Calibration of the SEC column (HiLoad 16/600 Superdex 200 pg) using Bio-Rad’s SEC Standard. A mixture of molecular weight markers ranging from 1,350 to 670,000 Da was loading onto SEC column using an ÄKTA FPLC™ system. The protein markers were separated according to their molecular weight (from high to low M.W) Thyroglobulin (bovine) 670,000, gamma-globulin (bovine) 158,000, Ovalbumin (chicken) 44,000, Myoglobin (horse) 17,000 and Vitamin B12 1,350 Da.

2.8 Expression and purification of His-tagged proteins

For large scale protein production 2 ml of overnight E. coli (C41) starter culture was inoculated into 600 ml LB or Power Broth containing 100 µg/ml ampicillin. The culture was incubated at 37°C with shaking (200rpm) until OD\textsubscript{600nm}= 0.6, cooled on ice for 30 min. Protein expression was then induced by addition of 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). This culture was incubated overnight at 16°C with shaking (200 rpm). The cell culture was then harvested by centrifugation at 8000 x g for 25 minutes at 4°C using Beckman F10 rotor in Beckman Coulter Avanti J-30I centrifuge. The pellet was re-suspended in loading buffer. For bacterial lysis the pellet was sonicated using a number of short pulses (10-12 sec) with pausing for 5-10 min, to control the temperature (soniprep 150 Plus, MSE). The cell lysate was
centrifuged at 20,000 x g for 45 minutes using Beckman JA 25.5 rotor in Beckman Coulter Avanti J-30I centrifuge. The bacterial lysate was loaded on the Ni-NTA resin (Qiagen) column which was prepared as described above. The column was washed with 20 column volumes of loading buffer, 20 volumes washing buffer containing 45mM imidazole. The protein was eluted with elution buffer containing 250 mM imidazole. Further purification was done in SEC column using ÄKTA™ Purifier system (GE Healthcare).

2.9 Expression and purification of GST-tagged proteins

Growth conditions, induction of expression and preparation of lysate were similar to those described in the section 2.8. After loading cleared lysate, Glutathione Sepharose™ 4B (GE Healthcare) column was washed extensively with 20 column volumes of PBS. GST-tagged proteins were eluted with 33 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0. The elution fraction was loaded onto the SEC column as described above.

2.10 Protein dialysis

For some applications, purified protein was dialysed against the desired buffer (300 mM NaF, 20 mM NaH₂PO₄, pH 7.5). A semi-permeable membrane with 3,000 Da cut off (Thermo Fisher Scientific) was used for buffer exchange and removal of imidazole or glutathione. The first step of dialysis was performed by cutting the desired length of the membrane and hydrate it in ddH₂O for a few minutes. One end of the bag was then sealed using dialysis membrane clamps (Fisher scientific). A protein solution was gently transferred into the dialysis membrane and the other end was also sealed using membrane clamps. The dialysis bag containing protein solution was placed in a large volume of the freshly prepared buffer of the desired composition and left on stirrer with gentle mixing overnight in cold room. To increase dialysis efficiency, dialysis buffer was changed three times a day.

2.11 Protein concentration

The protein was concentrated using 3,000 Da cut off filters (EMD Millipore) by centrifugation at 4000 x g, and concentrated protein solutions were used for experiments or stored at -80°C.

2.12 Measurement of protein concentration

Concentration of purified protein was determined using a Nanodrop spectrophotometer (Thermo Scientific) according to manufacturer’s instructions. The extinction coefficient of protein was determined using Expasy-Protparam tool (https://web.expasy.org/protparam/). The
Beer-Lambert formula: \( c = \frac{A}{e \cdot b} \) was used to estimate the protein concentration; \( c \) - concentration (mg/ml); \( A \) - Absorbance at 280 nm; \( e \) - extinction coefficient; \( b \) - path length in centimeters.

2.13 SDS-PAGE

Ready-made polyacrylamide gels (12% or 4-20%, SERVA) were used for separating proteins according to their molecular weight. The samples of protein were prepared by mixing the desired amounts of proteins with sample buffer and heated to 90°C for 5 minutes. Samples and protein ladder (Geneflow) with desirable volumes were then loaded onto a gel. SDS_PAGE gels were run at 200 V for 1 hour using SDS running buffer. Gels were then either used immediately for Western Blot analysis or stained with Colloidal Coomassie blue stain (BIO-RAD) overnight to visualise the protein bands. Gels were washed with water and scanned using GS 710 BIO-RAD scanner for further analysis.

2.14 Western Blot

The Western Blot was applied for detection of proteins using specific antibodies. Proteins were separated by SDS-PAGE as mentioned above. Gels were placed on a nitrocellulose membrane (Amersham) and covered with three sheets of Whatman filter papers on each side. The protein transferring onto the membranes was performed at 1 mA per 1cm² for 2 hours using the Trans-Blot® Turbo Transfer System (BIO-RAD). Upon transfer, completion membranes were blocked by incubation in blocking buffer for 1 hour. After washing with TBST buffer, membranes were incubated with blocking buffer containing a primary antibody overnight at 4°C. Next day membranes were washed with TBST for 15 minutes 3 times; then incubated in blocking buffer containing a secondary antibody for 2 hours at room temperature. After washing with TBST 3 times, membranes were incubated with BCIP®/NBT Liquid Substrate for band visualisation. The reaction was then stopped by washing membranes in water and scanned using GS 710 BIO-RAD scanner for further analysis.

2.15 Identification of proteins by mass-spectrometry

Mass spectrometry analysis was done by the Protein Nucleic Acid Chemistry Laboratory at the University of Leicester (PNACL). Attained SDS-PAGE gels were washed in 10% methanol and in Nano pure water. Protein bands of interest were excised from gels and digested by trypsin and analysed using a LTQ-Orbitrap-Velos mass-spectrometer. The database search was performed with MASCOT (version 2.3.2, Matrix Science, London, UK).
2.16 ImageJ analysis

Densitometry analysis of protein bands was performed using ImageJ version 1.51 software. Data from three independent experiments were analysed by using Graphpad Prism 7 and Microsoft Excel 2013 software.

2.17 Statistical analysis

The data of current study were statistically analysed using Graphpad Prism 7 and Microsoft Excel 2013 software unless otherwise stated.

2.18 Preparation of Figures

The illustrated Figures that used in this study was drawn using PowerPoint 2013 unless otherwise stated.
Chapter 3

3. Investigation of the enzymatic activity of CwIM
3.1 Introduction

Expression and purification of soluble recombinant proteins is a key aspect for investigating the functions of these proteins in in vivo. It is also important for studying protein-protein interaction and solving the protein structure. However, the generation and purification of heterologous proteins can be challenging for a number of reasons. A relevant host should be selected to produce soluble recombinant proteins which remain functionally active (Young et al. 2012; Ahmad et al. 2018). Cell wall enzymes can be particularly challenging for expression in heterologous hosts (Young et al. 2012). Some of them are toxic for the host such as resuscitation promoting factor (Rpf) and other muralytic enzymes overexpressed in E. coli strains and led to bacterial PG lysis (Fischer et al. 1993; Ehlert et al. 1995; Mukamolova et al. 2006). Most PG hydrolysing enzymes possess a typical signal peptide and are secreted via a SecA or TAT-secretion systems (Bernhardt and de Boer 2003). A secretion process often contributes to proper protein folding and formation of S-S bonds (Green and Mecsas 2016).

Another challenge is the generation of active enzymes. A major focus of this chapter is to investigate the enzymatic activity of CwlM, an annotated NAM-amidase that has several unique features (Figure 12). CwlM consists of two putative peptidoglycan binding domains and one catalytic domain (Deng et al. 2005, Machowski et al. 2014), however, it lacks a typical signal sequence or a transmembrane domain, and therefore, its translocation across the cytoplasmic membrane to the periplasm is questionable (Deng et al. 2005, Machowski et al. 2014).

Moreover, the two catalytic histidine residues which are critical for PG hydrolysing activity in other NAM-amidases are replaced with arginines in CwlM Figure 13 (Boutte et al. 2016). CwlM is phosphorylated by PknB and four distinct phosphorylation sites (T42, T43, T382 and T386) were detected in vivo Figure 12 (Turapov et al. 2018).
Figure 13. Alignment of PG amidase domains of mycobacteria and other bacterial species.

This figure was taken from (Boutte et al. 2016). The Zn$^{2+}$-coordinating residues are boxed as following: The two catalytic histidine residues which are critical for PG hydrolysing activity in other NAM-amidases are replaced with arginines in CwlM (red box). The conserved catalytic residues aspartate (D) and glutamates (E) were shown in a green box.

Deng et al showed the PG-hydrolysing activity of CwlM using zymography and the cleavage of muramyl-dipeptide while Boutte and colleagues could not reproduce these results and concluded that the protein has no PG hydrolysing activity but has a regulatory activity by stimulating MurA, a primary enzyme in the PG synthesis (Deng et al. 2005; Boutte et al. 2016).

I investigated CwlM activity independently and this chapter will describe the results of my experiments. The PG-cleaving activity of CwlM and its forms was studied using two common methods, zymography and fluorescein isothiocyanate labelled peptidoglycan digestion (PG-FITC) assays.

The chapter will also cover the optimisations for overcoming challenges of the production of recombinant CwlM and its truncated forms as well as confirmation of protein folding by using circular dichroism analyses. The effect of CwlM phosphorylation by PknB and phosphomimetic mutations on the CwlM stability and activity will be also reported.

3.2 Materials and methods

3.2.1 Expression of CwlM proteins

Bacteria were grown as described in (section 2.5). Recombinant CwlM forms (wild type, mutated and truncated) were cloned in the pET15b-TEV and transformed into E. coli C41 (DE3) strains (see Table 2). Figure 29 and Figure 31 show schematic presentation of CwlM forms investigated in this project. Protocols for expression and purification of His-tagged proteins are described in (section 2.8). The large-scale protein production was performed using a Ni-NTA resin (Qiagen), followed by size-exclusion chromatography (SEC) on a 16/600 Superdex 200 pg column using ÄKTA™ Purifier system (GE Healthcare Life Sciences).
3.2.2 Expression and Purification of Protein kinase B (PknB)

The construct for the generation of GST-tagged PknB was kindly provided by Dr Virginie Molle and transformed into *E. coli* BL21 (DE3) (Table 2). The protein was expressed and purified on Glutathione Sepharose 4B column (GE Healthcare) and SEC as described in (section 2.9).

3.2.3 *In vitro* phosphorylation of the recombinant CwlM by PknB

Phosphorylation of recombinant CwlM (10 µg) was performed in kinase buffer containing 20 mM Tris–HCl, pH 8.0, 0.5 mM DTT, 10 mM MgCl₂, 0.1 mM ATP (final volume 100 µl). PknB (5 µg) was added and the mixture was incubated at room temperature for 2 hours. CwlM phosphorylation was confirmed by Western Blot using anti-phosphothreonine antibody (Cell Signalling Technology) and mass spectrometry. In control samples ATP and PknB were absent.

3.2.4 Circular dichroism of the recombinant CwlM protein

The purified recombinant CwlM (3 mg/ml) was dialysed overnight against 2 L of sodium fluoride buffer (300 mM NaF, 20 mM NaH₂PO₄, pH 7.5) as described in section (2.10). The buffer was changed three times. The protein was then centrifuged at 20,000 x g, for 15 min at 4°C using Eppendorf 5417R Refrigerated Centrifuge to remove insoluble protein. CwlM (300 µg/ml) in the buffer of 100 mM NaF and 25 mM NaH₂PO₄, pH 7.5 was loaded on the cell holder (0.1 cm length) of a Chirascan plus spectrometer system (Applied Photophysics). The CD spectra of CwlM were recorded between 195 and 255 nm at room temperature. The thermal denaturation curve of CwlM was achieved by increasing the temperature over a range from 5 to 95°C. The obtained spectra were analysed using Pro-Data Viewer (Applied Photophysics) and then normalized using Prism 7 (GraphPad).

3.2.5 Purification of *E. coli* peptidoglycan

Peptidoglycan purification was performed according to the protocol developed by Glauner’s group (Glauner 1988; Vollmer and Bertsche 2008). *E. coli* LMG 194 starter culture was inoculated in 2 litres of LB and grown to OD₆₀₀nm 0.8 at 37°C. Flasks with bacterial culture were rapidly cooled on ice for 10 minutes. The culture was harvested at 6000 x g for 20 min using Beckman Coulter Avanti J-30I centrifuge. The pellet was resuspended in 6-10 ml of pre-chilled Nano pure water. SDS solution (10 %) was prepared and heated in Duran® Erlenmeyer
flask using hotplate magnetic stirrers. The cell culture was added to the boiled SDS dropwise and rigorously stirred for half an hour.

The boiled bacterial suspension was left at room temperature for cooling, then harvested at 20000 x g for 1 hr using Beckman JA 25.5 rotor of Beckman Coulter Avanti J-30I centrifuge. The supernatant was removed, and the pellet was resuspended in hot Nano pure water (60°C). The SDS solution was removed using several cycles of ultracentrifugation at 100,000 x g for 1 hr.

3.2.6 Hayashi test and digestion cell wall proteins
The concentration of SDS in solution was checked using Hayashi test (Hayashi 1975). The following reagents were added in a 1.5 ml Eppendorf tubes: 335 μl of the sample, 7 μl of 0.5% methylene blue, 170 μl of 700 mM NaPO₄ pH 7.2 and 1 ml of chloroform. The tubes with SDS solutions were vortexed for 20 sec and centrifuged at 8000 x g. The blue colour in the chloroform layer shows the presence of SDS, whereas clear or slightly pinkish layer indicates that the solution is free of SDS (Figure 14).

![Figure 14. Hayashi test to estimate the SDS concentration in the PG solution. The blue colour (blue arrow) in the chloroform layer refers to the presence of SDS whereas clear or slightly pinkish layer (pink arrow) indicates that the solution is free of SDS.](image)

Once the concentration of SDS reduced below 0.01%, α-amylase (0.75 mg/ml) was added to the sample and incubated at 37°C for 2 hours to degrade the glycogen in the saccule. Pronase E (10 mg/ml) was pre-incubated at 60°C for 2 hours. Pre-incubated Pronase E (100μl) was added to the sample and incubated at 60°C for 1 hour to digest cell wall-bound proteins. Further boiling in 4% SDS was performed for 15 minutes to extract digested proteins. The sample was
washed (6-10 times) using ultracentrifugation to remove remaining proteins and SDS. After this procedure, the solution of peptidoglycan should be free of SDS.

3.2.7 Labelling of peptidoglycan with Fluorescein isothiocyanate (FITC)

Purified PG was labelled with FITC according to previously published protocol (Glauner 1988, Vollmer and Bertsche 2008). PG was re-suspended in 100 mM sodium carbonate buffer, pH 8.5. FITC was dissolved in DMSO at 1 mg/ml. For 1 ml of PG solution, 50 μl of FITC solution was added and gently mixed with PG. The cross-linking reaction of PG with FITC was conducted overnight in the dark cold room. The solution was ultra-centrifuged at 100,000 x g for 1 hr and the supernatant with unbound FITC was removed. The labelled PG was washed several times (6-10 washes) with Nano pure water by ultracentrifugation. The FITC labelled PG was stored in the dark at 4°C with 0.2% sodium azide.

3.2.8 Zymography

Zymography was done as previously described (Mukamolova et al. 2006). M. luteus starter cultures were inoculated in 1 litre of LB and incubated at 37°C, with shaking 200 rpm to grow to OD_{600nm} 2. The culture was then harvested at 7000 x g for 25 min. using Beckman Coulter Avanti J-30I centrifuge. The pellet was re-suspended in 10 ml of PBS and transferred into a sterile tube (TubeSpin® Bioreactor, 50 ml). Further centrifugation was done at 4500 x g for 15 min using Eppendorf 5804R refrigerated centrifuge to remove PBS buffer and the tube with pellet was kept at -20 °C. The frozen pellet then lyophilized by using Virtis Benchmark Freeze Dryer (SP Scientific).

For zymography 0.2 % w/v of lyophilised M. luteus was incorporated in the solution for SDS-PAGE gel and polymerised. The samples of protein were prepared by mixing sample buffer with a protein but without boiling the protein and loaded onto a gel. After running electrophoresis, gels were washed for 2 hours using renaturing buffers containing 20 mM KH₂PO₄ with a range of pH. Triton X-100 (2 % v/v) was used to remove the SDS from the gels. Gels were incubated overnight either at 37°C or room temperature. Gels were stained with 0.1% (w/v) methylene blue in 0.05% KOH. Gels were washed with water and placed on a shaker for 2 hours. The PG-cleaving activity appeared as clearance bands on a blue background.
3.2.9 Digestion of PG-FITC by CwlM

Recombinant CwlM full-length or truncated forms (5 μg) in the buffer of 20 mM KH₂PO₄, pH 8.0 were mixed with 4μl of FITC-PG substrate to make a total volume of 100μl and incubated at 30°C with shaking at 100 rpm for 24-48 hours. PG (50μl) was labelled with FITC-Cyanogen bromide using a kit Fluorescein isothiocyanate isomer I (Sigma-Aldrich) to produce 54 μl of PG-FITC. The tubes with samples were centrifuged at 20,000xg for 30 min. Supernatants were carefully collected and passed through 0.22μm syringe filters (Merck Millipore). The filtered samples were transferred to 96 well microplates (Greiner Bio One). The amount of FITC released was measured at 540 nm in a VarioSkan plate reader (Thermo Fisher Scientific). Lysozyme (5 μg) was used as a positive control, negative controls (samples without proteins) were also done. Protein samples were denaturated by heating in a thermocycler at 95°C for 10 minutes and used for measurement of background.

3.3 Results

3.3.1 Expression of recombinant CwlM

Optimal conditions for CwlM expression were carried out by trialling different IPTG concentrations, incubation times and temperatures. Figure 15 shows an SDS–PAGE gel and Western Blot membrane, confirming expression of full-length CwlM by detecting protein bands of predicted CwlM size (~47 kDa) in soluble fractions. However, most of protein was found in insoluble fractions as protein aggregates (~75 kDa) or protein degradations (~25 kDa) when bacteria were induced with IPTG (0.5 mM) regardless incubation temperature or time (Figure 15A and B), while single bands of CwlM were detected at molecular weight of ~47 kDa in all fractions when bacteria were induced with IPTG (0.1 mM) (Figure 16).
Figure 15. Expression trials of the recombinant CwlM using 0.5 mM IPTG.

A. 12% SDS-PAGE  B. Western Blot using anti poly-His antibodies shows that CwlM was produced as a soluble protein, but the protein was also found as insoluble as (aggregation and degradation bands) when bacteria were grown for 1 hr and 3 hrs at 37°C or overnight at 16°C with 0.5 mM of IPTG.

Thus, optimal conditions for CwlM expression prior to large-scale protein purification are to use a lower concentration of IPTG (0.1 mM) for protein induction and grow bacteria overnight at 16°C with shaking 200 rpm.
Figure 16. Expression trails of the recombinant CwlM using 0.1 mM IPTG. A. SDS-PAGE B. Western Blot using anti poly-His antibodies shows that CwlM was produced as a soluble protein with single bands, but the protein was also found as insoluble when bacteria were grown for 1 hr and 3 hrs at 37°C or overnight at 16°C with 0.1 mM of IPTG.

3.3.2 Purification of the recombinant CwlM

CwlM was successfully purified as a soluble protein using IMAC and SEC. Figure 17A prepacked column as a single peak at 84 min corresponding to the monomeric form of the protein. A small peak at 50 minutes probably contained proteins aggregates, while smaller peaks at 120 min had degraded protein and imidazole. In Figure 17B, SDS-PAGE gel shows collected fractions of CwlM obtained from SEC and confirms the purity of protein as single bands at the right size 47 kDa, however, smaller bands were found in fractions 83, 84 and 85.

Figure 17C Western Blot using anti poly-His antibodies confirmed that fractions 79 and 84 had purified CwlM which appeared as a single band of expected molecular weight. The identity of recombinant CwlM was further confirmed by mass spectrometry analysis (Figure 17D).
**Figure 17.** Purification of recombinant *Mtb* CwlM from *E. coli* C41

A. SEC shows the monomeric peak of CwlM eluted at 84 minutes  
B. SDS-PAGE shows fractions of CwlM obtained from SEC.  
C. Western Blot using anti poly-His antibodies  
D. Mass spectrometry analysis confirms the identity of recombinant CwlM. The yellow-highlighted peptides identified by mass spectrometry. NC: negative control (lysate of *E. coli* containing empty plasmid pET).

### 3.3.3 Secondary structure and protein folding of CwlM

The secondary structure of CwlM was predicted using Jpred 4 server, showing that CwlM is mainly α-helical, with some β-sheet (Figure 18) and it was in agreement with CD data (Figure 19A).
Figure 18. The predicted secondary structure of CwlM based on amino acid sequences using Jpred 4 server. The data shows mixed alpha helices and beta sheet structure of Mtb CwlM. ‘H’ represents predicted helix; ‘E’ indicates predicted beta sheet. The bioinformatics software Jpred 4 server used online http://www.compbio.dundee.ac.uk/jpred/.

The spectra of CwlM were recorded in the UV range of 195 and 240 nm at room temperature (Section 3.2.5). The spectra indicate that the estimated secondary structure of the recombinant CwlM was predominantly alpha-helices with some beta-sheet motifs (Figure 19A).

The thermal stability of CwlM was assessed by monitoring the protein denaturation as the temperature was increased up to 95 °C (Section 3.2.5). The spectra showed a rapid unfolding of CwlM when the temperature reached 37°C (Figure 19B). These results suggest that CwlM was found as folded protein prior thermal denaturation. However, rapid denaturation of the protein at 37°C indicated that CwlM is unstable protein.
3.3.4 PknB phosphorylates CwlM in vitro

Recombinant PknB was expressed and purified from *E. coli* BL21 (DE3) using Glutathione Sepharose 4B and SEC (section 3.2.3). As shown in **Figure 20A**, PknB was eluted from the SEC column as a soluble monomeric protein at 56 min with little aggregation and degradation. **Figure 20B** shows ~63 kDa bands of PknB in fractions 55 and 56 but some aggregation bands and potential impurities were also observed. **Figure 20C** Western Blot using anti-GST antibody detected a single band of PknB with MW of ~63 kDa, as expected.
PknB was used for in vitro phosphorylation of recombinant CwlM (section 3.2.4). Figure 21 confirms that PknB was able to phosphorylate Mtb CwlM in vitro. The phosphorylated CwlM was assessed by Western Blot using anti-phosphothreonine antibody. The Blot shows that in the absence of ATP, there is no band of CwlM was detected, while in the presence of ATP the protein band of phosphorylated CwlM was detected at the expected molecular weight of 47 kDa (Figure 21B). The protein band of PknB was also detected in the presence of ATP at ~63 kDa (Figure 21B). SDS-PAGE shows loaded proteins with or without ATP (Figure 21B). The phosphorylation sites of CwlM were identified using mass spectrometry (Figure 21C). In addition to the four sites (T42, T43, T382 and T386) that had been identified previously by phosphoproteomics studies (Turapov et al., 2018), two additional sites were detected in vitro, Thr94 and Thr384 (Figure 21A). Thus, PknB can phosphorylate six threonine residues in CwlM in vitro. Complementation studies revealed that phosphoablative variant T382A was not able to complement Mtb cwlM-CM, while a double phosphoablative mutant T382A and T386A was very toxic for mycobacteria (Turapov et al. 2018).
**Figure 21.** Phosphorylation of CwlM by PknB *in vitro.*

Recombinant CwlM was phosphorylated by the enzymatic domain of PknB. **A.** Schematic representation of phospho-sites detected *in vitro* (top) and in phosphoproteomics study (bottom) (Turapov *et al.*, 2018). **B.** Detection of phosphorylated proteins by Western Blot analysis using anti-phospho-threonine antibody in the presence of ATP (+) and in the absence of ATP (-), lane 1 CwlM only, lane 2 CwlM with PknB, lane 3 CwlM with PknB. **C.** Phosphopeptides detected by mass spectrometry.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Phospho residue</th>
<th>Observed mass (charge)</th>
<th>Actual mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>AALTALGMLDHQEDLTGGR</td>
<td>T43</td>
<td>1111.52 (2)</td>
<td>2221.03</td>
</tr>
<tr>
<td>RLGARTLYHQFAPLYGDDVATLQAR</td>
<td>T94</td>
<td>938.47 (3)</td>
<td>2814.4</td>
</tr>
<tr>
<td>RLGARTLYHQFAPLYGDDVATLQAR</td>
<td>Y96</td>
<td>704.11 (4)</td>
<td>2812.4</td>
</tr>
<tr>
<td>EYGLAADGCICPETLR</td>
<td>Y144</td>
<td>595.93 (3)</td>
<td>1784.78</td>
</tr>
<tr>
<td>NDRPTGTFTFAELLAHESVER</td>
<td>T382</td>
<td>882.08 (3)</td>
<td>2583.22</td>
</tr>
<tr>
<td></td>
<td>T384</td>
<td>646.82 (4)</td>
<td>2583.22</td>
</tr>
<tr>
<td>NDRPTGTFTFAELLAHESVER</td>
<td>T384</td>
<td>646.81 (4)</td>
<td>2583.22</td>
</tr>
<tr>
<td>NDRPTGTFTFAELLAHESVER</td>
<td>T382</td>
<td>888.74 (3)</td>
<td>2663.2</td>
</tr>
<tr>
<td>NDRPTGTFTFAELLAHESVER</td>
<td>T384</td>
<td>888.41 (3)</td>
<td>2662.2</td>
</tr>
<tr>
<td>NDRPTGTFTFAELLAHESVER</td>
<td>T386</td>
<td>861.76 (3)</td>
<td>2582.26</td>
</tr>
<tr>
<td>NDRPTGTFTFAELLAHESVER</td>
<td>T384</td>
<td>888.41 (3)</td>
<td>2662.20</td>
</tr>
</tbody>
</table>
3.3.5 Phosphorylation or phosphomimetic mutations improve CwlM stability

Recombinant *Mtb* CwlM was purified as a soluble protein and its folding confirmed by circular dichroism (CD). However, CwlM is an unstable protein. The protein was easily degraded in the presence of high concentration of imidazole (>250 mM). Likewise, imidazole free CwlM was degraded when it was stored at 4°C or at -20°C (Figure 22).

![Figure 22](image)

**Figure 22.** SDS-PAGE shows the fresh and stored CwlM purified by IMAC (Ni-NTA) and SEC. The freshly purified CwlM was more intact than the stored one, as the latter was degraded to lower bands (shorter forms).

Therefore, I investigated the effect of phosphorylation by PknB on stability of CwlM. To address this, the phosphorylated sites of CwlM (T43, T382 and T386) were substituted with the acidic amino acid (aspartate) to mimic a phosphorylated protein. Triple phosphomimetic form, designated as CwlM-DDD was generated and used for investigation of protein stability. CwlM-DDD was expressed and purified from *E. coli* C41 (DE3) as described for the CwlM wild type in (section 3.2.1).

SEC analysis showed that CwlM-DDD was a soluble monomeric protein eluted at 84 min (Figure 23A). SDS-PAGE and Western Blot using anti poly-His antibodies confirmed the expression and purification of CwlM-DDD at the calculated molecular mass of 47 kDa as
single bands without aggregation and degradation (Figure 23B, C). These results suggested that CwlM-DDD was more stable than the wild type version.

![Figure 23](image)

**Figure 23.** Purification of the phosphomimetic CwlM–DDD protein expressed in E.coli C41 strains

A. Size-exclusion chromatography (SEC) analysis shows the monomeric peak of CwlM-DDD eluted at 84 minutes  B. SDS-PAGE and C. Western Blot using anti poly-His antibodies show CwlM fractions obtained from SEC at the expected molecular weight of ~47 kDa as a single band with no aggregation and degradation.

Recombinant CwlM forms (Wildtype, DDD, and phosphorylated) were mixed with imidazole (250 and 500 mM). The protein samples were incubated at 37°C for 1, 2, 3, 4 and 24 hrs. After incubation, the samples were analysed using SDS-PAGE. CwlM-WT was rapidly degraded in two hours in the presence of 250 mM imidazole and in half an hour with 500 mM of imidazole (Figure 24A, B). However, PknB-phosphorylated CwlM and the triple phosphomimetic version were more stable compared with WT version (Figure 24A, B).

Densitometry analysis of 3 independent gels indicates that 50% of phosphorylated and phosphomimetic proteins were degraded during 2 hours incubation at 37°C, whilst 99% of WT CwlM was degraded within first two hours (Figure 24A, B). These results suggest that PknB-mediated phosphorylation stabilises CwlM.
Figure 24. Investigation CwlM stability *In vitro* using different concentration of imidazole.

A and B. 12% SDS-PAGE shows phosphorylated CwlM is more stable than non-phosphorylated CwlM. Imidazole (250-500mM) was mixed with CwlM forms and incubated at 37°C. Densitometry analysis of 3 independent gels was performed using ImageJ software (1.51).

### 3.3.6 Full-length CwlM has no PG-cleaving activity

The PG-cleaving activity of recombinant *Mtb* CwlM forms (full length) was investigated using zymogram method (Mukamolova *et al.* 2006). The purified CwlM was run on an SDS-PAGE gel containing lyophilised *M. luteus* (*section 3.2.9*). The following renaturation buffers were used to remove SDS and refold CwlM: 25 mM (citrate phosphate, sodium phosphate, potassium phosphate and Tris-HCl) with different pH range (4.5-9). Figure 25 shows that wild type CwlM was inactive in zymogram, as no clearance band of the protein was observed in the gel.

Since CwlM belongs to a zinc metalloenzymes group, ZnSO₄ was added to the renaturation buffers. The results show that using different concentrations of ZnSO₄ (0.5-5 mM) in renaturation buffers had no effect on the activity of CwlM. Furthermore, removal of the poly-His tag also did not improve the activity of the recombinant protein.
Further, we aimed to investigate whether phosphorylation might affect the activity of CwlM. The PG-hydrolysing activity of phosphomimetic (CwlM-DDD) and phosphorylated CwlM forms was investigated by zymogram. The results indicate that CwlM-DDD (Figure 25) and the phosphorylated CwlM (Figure 26) were also inactive in zymogram. Addition of metal ion co-factor (0.5-5 mM ZnSO₄) also did not influence the activity of the phosphorylated CwlM.

**Figure 25.** The peptidoglycan-cleaving activity of non-phosphorylated and phosphomimetic CwlM  
A. SDS-PAGE shows the recombinant CwlM forms (non-phosphorylated and phosphomimetic)  
B. Western Blot shows the purified CwlM (non-phosphorylated and phosphomimetic) using anti-His antibody  
C. Zymogram at pH 7 shows no clearance bands for CwlM forms. Identical results were found using a range of renaturation buffers pH 4.4, 5.0 (citrate phosphate); pH 6.0, 7.0 and 8.5 of 25 mM (KH₂PO₄ & Na₂PO₄). The PBS buffer was used as a negative control while lysozyme was used as a positive control in zymogram. The zymogram gels contained 0.2% w/v *M. luteus* cell walls.
Figure 26. Peptidoglycan-cleaving activity of phosphorylated and non-phosphorylated CwlM

A. SDS-PAGE analysis of the recombinant CwlM forms (phosphorylated and non-phosphorylated) B. Western Blot shows PknB-phosphorylated CwlM using anti-phosphothreonines antibody (Cell Signalling) C. Zymogram at pH 7 shows no clearance bands for CwlM forms. Identical results were found using a range of renaturation buffers pH 4.4, 5.0 (citrate phosphate); pH 6.0, 7.0 and 8.5 of 25 mM (KH2PO4 & Na2PO4). The PBS buffer was used as a negative control while lysozyme was used as a positive control in zymogram. The zymogram gels contained 0.2% w/v M. luteus cell walls. Recombinant PknB was removed using a GTH affinity column.

PG-cleaving activity of recombinant CwlM proteins was also investigated using the PG-FITC assay (Glauner 1988; Vollmer and Bertsche 2008) which is based on measurement fluorescence intensity of soluble labelled muropeptides released from the digested PG.

Freshly purified recombinant CwlM proteins (CwlM\text{WT}, CwlM\text{DDD} and phospho-CwlM) were mixed with PG-FITC substrate and incubated at 30°C for 24-48 hours (section 3.2.10). Lysozyme was used as a positive control whereas the negative control included the boiled CwlM proteins. However, the CwlM proteins tested were inactive in PG-FITC assay (Figure 27).

The results of zymogram and PG-FITC assays were inconsistent with a published work of Deng et al., where authors identified the PG-hydrolysing activity of recombinant CwlM (Deng et al. 2005). However, the findings of the current study are consistent with a recently published work of Boutte et al., (2016). The authors proposed that the protein lost its enzymatic function.
because of mutation of two catalytic histidines which are important for hydrolytic activity in other NAM-amidases (Boutte et al., 2016).

**Figure 27.** PG-cleaving activity of the recombinant CwlM forms (wild type, DDD, phosphorylated) using FITC labelling peptidoglycan of *E. coli* LMG.

Five micrograms of purified proteins were used per assay. All samples were assayed from three independent experiments. Boiled proteins were used as negative controls. Data shown as means + SEM, (N=3). ns- P>0.05 as judged by unpaired T-test where boiled and non-boiled samples were compared.

We observed that *E. coli* lysates prepared from cultures over-expressing CwlM often had a prominent clearance band of lower molecular weight (~15 kDa), which was not seen in the lysate of empty plasmid strain (**Figure 28**). This observation suggests that a shorter form of CwlM may possess peptidoglycan cleaving activity.
Figure 28. Zymogram showed CwlM-E. coli C41 lysates.

The CwlM (wild type) presented a clearance band with estimated MW of ~15 kDa using a renaturation buffer of 25 mM KH$_2$PO$_4$ pH 7 (indicated with an arrow). The mutated amidase (CwlM-H204A) and pET (empty plasmid) did not have this clearance band. The clearance band at ~25 kDa was seen in all strains suggesting that it is unspecific activity of E. coli protein. The zymogram gels contained 0.2% w/v M. luteus cell walls.

Therefore, we aimed to investigate the PG-hydrolysing activity of CwlM forms in bacterial lysates as a protein will probably be susceptible to cleavage either by itself or by unknown proteinases. The presumptive catalytic residues (H204, E217 and D339A) of CwlM were predicted using the bioinformatics software “COACH” (http://zhanglab.ccmb.med.umich.edu/COACH/) Figure 29. These residues were replaced with alanine using site-directed mutagenesis (SDM) and the constructs of the full-length mutated CwlM were generated by Protein Expression Laboratory (Protex) University of Leicester (Table 2).
The full-length of CwlM with site directed mutagenesis

**Figure 29.** The mutated forms of CwlM that used in this study. The “COACH” software was used to detect the predicted catalytic sites (H204, E217 and D339) [https://zhanglab.ccmbr.med.umich.edu/COACH/](https://zhanglab.ccmbr.med.umich.edu/COACH/). The predicted structure of CwlM (full-length with predicted catalytic residues) were determined using PHYRE2 Protein Fold Recognition Server.

The mutated forms (H204A, E217A and D339A) were expressed using the protocol described in the **section 3.2.1**. The expression of the proteins was confirmed by Western Blot using anti poly-His antibody (**Figure 30B**). The Western Blot confirmed that all proteins were expressed. Moreover, CwlM-DDD and CwlM-E217A were less degraded compared with CwlM-WT, CwlM-H204A and CwlM-D339A (**Figure 30B**).

Lysates prepared from these strains were used for zymography. **Figure 30C** shows clearance bands (MW~18 kDa) were detected in CwlM-WT and CwlM-E217A lysates but not in CwlM-DDD, CwlM-H204A, CwlM-D339A and control empty plasmid strains. All samples had a clearance band at ~25kDa which was probably caused by an *E. coli* protein as a pET control was also shown this clearance band.

The replacing of glutamate residue (E217) by alanine did not completely abolish the PG-hydrolysing activity of CwlM. Proteins with mutations of other residues (H204A and D339A) had no clearance bands. Those residues may play an important role in the hydrolytic activity of the protein.
Figure 30. *E. coli* C41 lysates of CwlM forms.

A. SDS-PAGE and B. Western Blot using anti poly-His antibody indicate CwlM wild-type, CwlM-DDD, CwlM- H204A, CwlM- E217A and CwlM- D339A were expressed at 47 kDa. C. Zymogram shows clearance bands as shorter forms for CwlM-wild type and CwlM- E217A. The control pET (empty plasmid), CwlM- DDD, CwlM- H204A, and CwlM- D339A had no shorter bands of clearance. The clearance bands at ~25kDa in control and in all CwlM forms were determined as an unspecific hydrolysing activity.

*E. coli* lysates with degraded CwlM showed multiple clearance bands, suggesting that shorter CwlM form might cleave PG. The lack of activity in D339A mutant indicated that this activity might be associated with the predicted amidase domain. Therefore truncated forms of this protein CwlM-AmiR1 (amidase domain only), AmiR2 (amidase domain with the tail) and AmiR2-DD (amidase domain+tail with phosphomimetic mutations) were generated for further investigation of the PG-cleaving activity (Figure 31: Table 2).
Figure 31. The truncated and mutated forms of CwlM used in this study.

The “COACH” software was used to predict catalytic sites (H204, E217 and D339) [https://zhanglab.ccmb.med.umich.edu/COACH/]. The predicted structure of CwlM (amidase domain only with predicted catalytic residues) were determined using Phyre2 server [http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index].

3.3.7 PG-cleaving activity of truncated CwlM proteins

Western Blot analysis was used to confirm the expression of proteins using anti poly-His antibody. The results show that AmiR1 was expressed at the expected size of ~23 kDa under all conditions (Figure 32B). We can see the higher bands of protein on Western Blot, it indicates that protein is aggregated.
Figure 32. Protein expression trials of AmiR1.

E. coli C41 (DE3) containing pET15b-amiR1 vector were grown to the optical density of 0.6. IPTG with concentration 0.1 mM was used to induce the protein expression. Bacterial culture was incubated either at 37°C for 1 hr and 3 hrs or overnight at 16°C, only soluble fractions were used for the analysis. A: SDS-PAGE and B. Western Blot was done using anti–His antibody.

The truncated AmiR1 was easily precipitated when it is purified at a pH range of around 8.0 that is close to its isoelectric point. To overcome this issue, the protein was purified in a buffer with pH 6.5. Further purification was done by using gel filtration to remove high and low molecular weight.

The successful purification of AmiR1 was confirmed by SDS-PAGE and Western Blot using anti-His antibody. Figure 33 A, B shows that the recombinant AmiR1 was a pure protein and had single bands at the molecular mass of 23 kDa.

The PG-cleaving activity of AmiR1 was confirmed using zymogram. Figure 33C shows clearance bands of AmiR1 were at the molecular weight of 23 kDa, suggesting that the protein was active in zymogram using the renaturation buffer (20 mM KH2PO4, pH 7.0). However, it was difficult to have a reproducible activity of AmiR1 on zymogram. The stored enzyme never had activity despite appearing intact by SDS-PAGE. Therefore, the protein was always freshly purified and used immediately to investigate the activity of AmiR1 on PG-FITC assay.
Figure 33. PG-cleaving activity of AmiR1 (amidase domain only).
A. SDS-PAGE gel of purified AmiR1. B. Western Blot demonstrated the purified proteins at 24 kDa using anti-His antibody. C. Zymogram showed that recombinant AmiR1 (5 µl and 10 µl) was active at pH 7.0. The PBS buffer was used as a negative control in all methods. The zymogram gels contained 0.2% w/v *M. luteus* cell walls.

The activity of AmiR1 was confirmed by PG-FITC assay, and this was reproducible. A freshly purified AmiR1 was mixed with PG-FITC substrate and incubated at 30°C for 24 hours (section 3.2.10). Lysozyme was used as a positive control whereas PBS buffer and boiled AmiR1 were used as negative control. Figure 34 shows that AmiR1 cleaved soluble muropeptides from the FITC-labelled PG.
Figure 34. The PG-cleaving activity of the recombinant AmiR1 using FITC-labelled peptidoglycan of *E. coli* LMG.

The purified AmiR1 (5 µg) was added to the PG-FITC (5 µl) with 25 mM KH2PO4 pH 7 buffer (100 µl final volume) and incubated at 30°C for 24 hrs. AmiR1 was significantly active compared to the negative control (PBS buffer). The data from three independent experiments was statistically analysed by prism7. Data are presented as mean ± SEM, (N=3). ***P < 0.001 vs control (PBS buffer) by T-test.

Attempts to investigate activity of AmiR2 form (the amidase domain with the tail) were also made, however the protein was very unstable and could not be purified. Therefore, a phosphomimetic form of this protein AmiR2-DD was generated (Table 2). Expression and purification of recombinant AmiR2-DD were performed as described for AmiR1.

AmiR2-DD was successfully purified as shown in Figure 35A, B. A single protein band of expected size (MW~ 26 kDa) was observed in SDS-PAGE gel and it was recognised by anti-His antibody.

PG-cleaving activity of AmiR2-DD was assessed in zymogram (Figure 35C). The protein was unable to produce a clearance band, suggesting that it did not digest *M. luteus* cell wall.
Figure 35. PG-cleaving activity of AmiR2-DD (Phosphomimetic domain).

A. SDS-PAGE displays purified AmiR2-DD as a single band. B. Western Blot confirms that the band was recognised by anti-His antibody. C. Zymogram shows recombinant AmiR2-DD was inactive in renaturation buffer (KH$_2$PO$_4$, pH 7.0). The PBS buffer was used as a negative control in all methods. Lysozyme was used as a positive control. The zymogram gels contained 0.2% w/v *M. luteus* cell walls.
The PG-cleaving activity of AmiR2-DD was also investigated by PG-FITC assay (Figure 36). The protein was unable to digest PG-FITC of *E. coli* LMG strain, suggesting that it is not active protein.

**Figure 36.** PG-cleaving activity of the recombinant AmiR2-DD using FITC-labelled peptidoglycan of *E. coli* LMG.

Purified AmiR2-DD (5 µg) was added to PG-FITC (5 µl) with 25 mM KH₂PO₄ pH 7 buffer (100 µl final volume) and incubated at 30°C for 24 hrs. Activity of AmiR2-DD was similar to the negative control (pET). Data from three independent experiments were analysed using Prism7. ***P < 0.001 vs control pET (empty plasmid) by unpaired t-test. Data presented as mean ± SEM, N=3.

Since recombinant AmiR1 was consistently active and stable compared with full-length CwlM, mutated versions of AmiR1 were generated to confirm catalytic residues. H204A, E217A and D339A variants were cloned in the pET15b-TEV vector with a 6xHis-tag at the N-terminus and transformed to *E. coli* C41 (DE3) strains. Protein expression trials of mutated AmiR1 forms were done as previously described for AmiR1 (wildtype). The mutated proteins were successfully purified and showed no sign of degradation after purification (Figure 37).
Figure 37. Expression and purification the mutated active sites of AmiR1
A. SDS-PAGE and B. Western Blot confirm the identity of the purified proteins (AmiR1-WT, AmiR1-H204A, AmiR1-E217A and AmiR1-D339A) at calculated molecular weight ~24 kDa using anti-His antibody. The empty plasmid (pET) was used as a negative control.

PG-cleaving activity of AmiR1 mutants was investigated using assay of FITC-labelled peptidoglycan. As in previous experiments AmiR1 sample showed higher fluorescence compared with the buffer sample and this activity was completely abolished by boiling AmiR1 (Figure 34). H204A and E217A samples had lower activity compared with AmiR1, while D339A mutant had no activity (Figure 38). These results confirm that AmiR1 was able to cleave FITC-labelled PG and D339 was critical for this activity.
Figure 38. PG-hydrolysing activity of mutated AmiR1 forms (H204A, E217A and D339A) using FITC-labelled peptidoglycan of *E. coli* LMG.

Purified AmiR1 or mutants (5 µg) were mixed with PG-FITC (5 µl) and 25 mM KH2PO4 pH 7 buffer (100 µl final volume) and incubated at 30°C for 24 hrs. Data from three independent experiments were analysed using Prism7. Data are mean ± SEM, N=3. *P, **P, ***P, ns < 0.001 vs control (boiled protein) by unpaired t-test.

### 3.4 Discussion

The aim of this section was to assess enzymatic activity of CwlM. To achieve this aim, sufficient amount of recombinant CwlM were required. Recombinant CwlM proteins were also used for generation of antibody and structural studies described in chapters 4. Expression and purification of recombinant CwlM was challenging due to its high instability and propensity to aggregate. Thus, many different conditions were tested for optimisation of expression and purification. Firstly, different expression host were tried and *E. coli* C41 (DE3) supported synthesis of soluble protein. This host has been previously used for expression of toxic and membrane proteins (Dumon-Seignovert et al. 2004). *E. coli* grows fast in inexpensive media and can be genetically manipulated using various expression vectors (Terpe 2006; Mühlmann et al. 2017). However, many aspects should be taken into consideration when large amounts of recombinant proteins produced in *E. coli*. These include protein's toxicity, stability of mRNA (Mahalik et al. 2014), post translational modifications (Kaur et al. 2018), protein degradation (Ryan and Henehan 2013), protein aggregation and formation of inclusion bodies (Ahmad et al. 2018).
Protein expression trials of full length-CwIM (wild type and mutated forms) showed that only a small proportion of protein was soluble during expression at 37°C. Most of the protein was found in pellet fractions in the form of inclusion bodies. Difficulties with production of sufficient amount of CwIM have been also reported by other authors (Deng et al. 2005). Therefore, I used lower temperature for expression of CwIM. Moreover, Bacterial culture at the mid log phase (OD 0.6) was kept on ice for 30 minutes before the induction of protein expression by IPTG in order to delay the rapid production of CwIM. Another important factor was lowering the concentration of IPTG to (0.05-0.1 mM) which helped to reduce protein aggregation and prevent the formation of inclusion bodies (Lebendiker and Danieli 2014; Rizkia et al. 2015). Trial experiments established that induction of CwIM expression by low concentration of IPTG (0.05-0.1 mM) and incubation overnight at 16°C produced soluble proteins and prevented the formation of inclusion bodies.

A large amount of CwIM was expressed and purified using Ni-NTA affinity chromatography and size-exclusion chromatography. However, purified CwIM was very unstable, especially in the presence of imidazole. While the precise mechanism of this phenomenon is poorly understood, imidazole is known to affect protein stability (Hamilton et al. 2003; Lee et al. 2007). In our experiments, protein eluted from a Ni-NTA column was immediately diluted in binding buffer to reduce the concentration of imidazole and minimise degradation of the protein.

Remarkably, truncated forms AmiR1 (amidase domain only) and AmiR2-DD (amidase domain with phosphomimetic C-terminus) were more stable than the full-length of recombinant CwIM. However, these proteins were prone to aggregation and easily precipitated. I used buffer with pH 8.0, as recommended by the resin manufacturer because of the highest capacity of Ni-NTA resin for binding of tagged protein (Bornhorst and Falke 2000). However isoelectric point of AmiR1 was 8 and I used elution buffer with pH 6.5 to prevent protein precipitation (Kirkwood et al. 2015).

It is generally agreed that a well-folded recombinant protein that purified in its native form is critical for studying protein functions such as enzymatic activity and protein-protein interaction (Yao et al. 2018). Furthermore, it is also important to ensure that the polyclonal antibody raised against a correct conformational structure and can recognise specific epitopes of the native protein (Kelly and Price 2000; Martin and Bayley 2002). CD spectroscopy is a powerful tool for evaluating the secondary structure, binding properties and folding of proteins (Greenfield
2006; Clarke 2011). In this study, the CD spectroscopy results demonstrated that recombinant CwlM was well-folded. However, the rapid denaturation of protein at 37°C indicated that CwlM is a highly unstable protein.

Although CD spectroscopy offers a low-resolution structural information compared to X-ray crystallography and NMR, it is widely used to study the secondary structure of protein (Micsónai et al. 2015; Miles and Wallace 2016). The protein structure of CwlM has not been solved. According to the CD results the estimated secondary structure of the CwlM predominantly represented by alpha-helices with some beta-sheet motifs. That is in agreement with the predicted secondary structure of CwlM shown on Jpred 4 server (http://www.compbio.dundee.ac.uk/jpred/).

As mentioned above recombinant CwlM was very unstable and it could not be stored, complicating production of CwlM for crystallisation studies. It is currently unknown whether CwlM possesses self-proteolytic activity or it is very sensitive to action of proteases found in E. coli. In eukaryotes, limited proteolysis is used as an important regulatory mechanism in growth and development of organisms. For example, in plants degradation of Ore9 protein was important for control of leaf senescence (Woo et al. 2001). In bacteria proteolysis play regulatory roles in stress responses, pathogenesis and growth (Gottesman 2003; Konovalova et al. 2014). Proteolysis may also lead to the complete or partial degradation of specific sites of the protein. This mechanism occurs in order to modify or alter the protein activity (Jenal and Hengge-Aronis 2003). Additionally, proteolysis activities may control the intracellular and extracellular localisation of proteins (Konovalova et al. 2014).

Protein stability is essential for functional studies where protein could be active for a long period of time in a wide range of pH, temperatures and salts (Bommarius and Paye 2013). Factors such as unfolding, degradation and proteolytic cleavage may affect the stability of protein (Deller et al. 2016). Posttranslational modifications such as phosphorylation play crucial roles in proteins functions. Importantly, phosphorylation may increase protein stability by inhibiting proteolytic cleavage (Miranda et al. 2004). I therefore investigated the effect of CwlM phosphorylation by PknB on its stability. I also tested phosphomimetic forms of CwlM. I have established that phosphorylated CwlM and phosphomimetic CwlM were more stable than non-phosphorylated CwlM. Thus, bacteria can use phosphorylation to prevent CwlM degradation during growth, while the protein would be quickly degraded in non-replicating conditions when PknB is not produced.
To access PG-cleaving activity of CwlM and its variants I used zymography and PG-FITC assay. Zymogram is a qualitative method that used for initial assessment of the PG-cleaving activity of a protein. *M. luteus* is commonly used as a substrate impregnated within an SDS-PAGE gel because of its high sensitivity to muralytic enzymes. It produces an opaque background in gels, and it is very easy to prepare from relatively small number of bacteria. The main drawback of zymogram is denaturation of the protein for SDS-PAGE. Therefore, the method predominantly depends on the successful replacement of SDS by Triton and refolding of the protein in the polyacrylamide gel.

Another assay used in this study, digestion of PG-FITC measures fluorescence intensity of soluble muropeptides that released from the digested FIC-labelled PG. The essay is very sensitive and does not require protein denaturation/refolding.

Using zymography and PG-FITC assays, I established that full-length CwlM protein had no muralytic activity. I then investigated whether phosphorylation could activate CwlM. For this, recombinant CwlM was phosphorylated by PknB* in vitro*. I also used a phosphomimetic form of CwlM where phosphosites T43, T382 and T386 were substituted with Aspartate (D). However phosphorylated CwlM and phosphomimetic CwlM were also inactive in zymogram and PG-FITC assays. As CwlM belongs to the zinc metalloenzymes group, I tested the effect of Zn$^{2+}$ on CwlM activity by adding ZnSO$_4$ in renaturation buffers. However, CwlM still was inactive. Furthermore, removal of the poly-His tag did not result in improving activity of CwlM. Thus, I could not confirm the previously published study of Deng* et al.* (2005) where authors demonstrated that the full-length CwlM had NAM-amidase activity (Deng* et al.* 2005). However, my results are consistent with the prediction of Boutte* et al.* (2016) that CwlM had no enzymatic activity. The authors proposed that the protein lost its enzymatic function because of mutation of two catalytic histidines which are important for hydrolytic activity in NAM-amidases. Furthermore, the authors indicated that overexpression of CwlM had no effect on the viability of *M. smegmatis*, while overexpression of active PG hydrolases usually results in bacterial lysis (Shida* et al.* 2001, Uehara and Bernhardt 2011, Kumar* et al.* 2013, Prigozhin* et al.* 2013, Boutte* et al.* 2016).

Our results suggested that the lysates from *E. coli* cultures overexpressing CwlM produced an additional clearance band of lower molecular weight, which was not seen in the lysate from empty plasmid cultures. To prove that this activity was associated with CwlM I tested lysates from CwlM mutants where predicted catalytic residues (H204, E217 and D339) were
substituted with alanine using site-directed mutagenesis (SDM). Alanine is a small non polar, non-hydrophobic and inert amino acid that would not affect protein function by conformational changes or unwanted alternative chemical reactions (Betts and Russell 2003; Yampolsky and Stoltzfus 2005; Creixell et al. 2012). The results of zymography showed that E217A mutation did not abolish the clearance band, suggesting that the mutant was still active. However, lysates from H204A and D339A E. coli expressing cultures had no clearance bands. In control E.coli lysates, recombinant CwlM (wild type) consistently produced an active bands with MW ~18 kDa.

Since the predicted catalytic residues are in the amidase domain of CwlM truncated forms of CwlM-AmiR1 (corresponding to the amidase domain) and AmiR2-DD (phosphomimetic form of amidase domain with tail) were generated and assessed for muralytic activity. The result showed that AmiR1 was active in both zymogram and PG-FITC assay, while AmiR2-DD had no activity. Notably, recombinant AmiR1 was rapidly losing its activity upon storage. Therefore, fresh protein preparation was always used to investigate AmiR1 activity. My results suggest that CwlM may require proteolytic cleavage for activation.

There are some known examples of hydrolase activation by proteolytic cleavage. RipA, a putative endopeptidase, is produced as a zymogen that has a unique loop which close the active site (Ruggiero et al. 2010). RipA was only active and able to cleave the bacterial peptidoglycan when this loop was degraded via proteolytic cleavage (Ruggiero et al. 2010).

To confirm that the observed activity of AmiR1 was mediated by the predicted catalytic residues I also tested activity of H204A, E217A and D339A variants of AmiR1 using PG-FITC assays. AmiR1 D339A mutant had no detectable activity, confirming zymogram results obtained with E. coli lysates. Thus, findings of this study demonstrate that the amidase domain of CwlM has peptidoglycan cleaving activity and it can be completely abolished by D339A mutation. However additional experiments require to characterise this activity.

3.5 Conclusion and Future work

The successful expression and purification of recombinant CwlM and its truncated forms were described in this Chapter. According to CD spectroscopy, CwlM was a well-folded protein as its native form and mainly consists of alpha helices and some beta sheets. Protein stability experiments indicated that CwlM was extremely unstable protein and easily degraded particularly in the presence of imidazole. The data showed that phosphorylation by PknB improved CwlM stability and prevented its degradation.
In this chapter, the PG-cleaving activity of CwlM was investigated using zymogram and FITC labelled PG assays. Both non-phosphorylated and phosphorylated CwlM (full length) were inactive proteins. However, recombinant AmiR1 (the amidase domain) of CwlM was active and this activity was abolished by D339A mutation. However, the precise mechanism that regulates this activity is still unknown. Characterization of the precise nature of AmiR1 enzymatic activity, the products of its action on bacterial peptidoglycan and their possible effect on mycobacterial growth are challenges for future studies.

**Figure 39.** Graphical abstract of PG-cleaving activity of recombinant CwlM forms.

Full-length CwlM proteins (Wild type, phosphomimic DDD, Phosphorylated and Site-directed mutagenesis SDM have no PG-cleaving activity using zymogram and PG-FITC assays. Truncated CwlM form (AmiR1 amidase domain only) has PG-cleaving activity using zymogram and PG-FITC assays.
Chapter 4

4. Investigation of CwlM localisation in mycobacteria during different stages of growth
4.1 Introduction

NAM-amidases are widely distributed in Gram-negative and Gram-positive bacteria. In *Escherichia coli*, there are six cell wall amidases, AmiA, AmiB, AmiC, AmpD, AmiD and AmpD which are genetically and biochemically well-characterised (van Heijenoort 2011). Based on bioinformatics analyses from the Pfam database, AmiA, AmiB, and AmiC belong to the Amidase_3 domain family, while AmiD and AmpD are members of the Amidase_2 domain family (Pennartz *et al.* 2009).

The Amidase_2 and Amidase_3 enzymes possess a typical signal peptide which is required for secretion and subsequent periplasmic localisation (Heidrich *et al.* 2001; Bernhardt and de Boer 2003). It has been shown that AmiA and AmiC are transported to the periplasmic septal ring by the Tat secretion system (Bernhardt and de Boer 2003; Ize *et al.* 2003), while AmiB is potentially secreted to the periplasm by the SecA dependent pathway (Bernhardt and de Boer 2003).

Certain NAM-amidases localise on the outer membrane. For example, AmiD (YbjR), a 1, 6-anhydro-N-acetylmuramic acid-l-alanine amidase contains a short signal peptide and a lipid anchor motif, suggesting an outer membrane localisation of this lipoprotein (Park and Uehara 2008; Pennartz *et al.* 2009; Kerff *et al.* 2010). In *Bacillus subtilis*, LytC (CwlB), a vegetative cell wall amidase was found to be localised on the entire cell surface after the middle of the exponential growth phase, suggested that LytC is not involved in the cell separation (Yamamoto *et al.* 2003).

Non-secreted cytoplasmic NAM-amidases are also produced by Gram-positive and Gram-negative bacteria (Priyadarshini *et al.* 2007). In *E. coli*, AmpD is localised in the cytoplasm, and it is able to hydrolyse 1, 6-anhydro-MurNAc-peptides as a key component of muropeptide recycling system (Lindquist *et al.* 1989; Park and Uehara 2008). In *Streptococcus pneumoniae*, LytA is N-acetylmuramoyl-l-alanine amidases involved in bacterial autolysis and fratricidal. This enzyme does not contain a signal sequence for the protein secretion, thus, LytA was mainly localized in the cytoplasm (Mellroth *et al.* 2012).

Other NAM-amidases could be localised in specific sites like septum or divisome. For example, AmiA and AmiC are transported to the septal ring during cell division and deletion these amidases in *E. coli* leads to abnormal growth in long chains of connected cells (Heidrich *et al.* 2001; Heidrich *et al.* 2002).
Mtb contains four predicted NAM-amidases (Machowski et al. 2014). Rv3717 belongs to the Amidase_3 family and possesses a signal sequence that allows the translocation of the protein to the periplasm by tat or SecA systems (Kumar et al. 2013; Prigozhin et al. 2013; Houben et al. 2014). Rv3717 has been recently shown to localise at the septum and lateral axis of dividing cells (Senzani et al. 2017).

CwlM is also annotated as amidase_3 domain-containing protein, however as discussed in chapter 3, it does not have a typical signal sequence or a lipobox motif (Deng et al. 2005, Machowski et al. 2014). It has recently been revealed that in Mycobacterium smegmatis CwlM is localised in the cytoplasm and can be phosphorylated by PknB (Boutte et al. 2016). The authors proposed that the phosphorylated CwlM was essential for regulating the activity of MurA, the primary enzyme in PG precursor’s biosynthesis (Boutte et al. 2016). However, possible functions and localisation of the non-phosphorylated CwlM have not been addressed by Boutte and colleagues.

This chapter will be focused on the investigation of CwlM cellular localisation during various growth phases. The effect of phosphorylation on CwlM localisation will be also studied. In this study, Western Blotting assays using anti-CwlM specific antibodies will be employed to investigate CwlM in mycobacterial fractions.

4.2 Materials and Methods

4.2.1 Organisms and media

M. smegmatis mc²155 (WT) and conditional mutant M. smegmatis (cwlM-CM) strains were grown in supplemented Middlebrook 7H9 (Becton, Dickinson and Company) and Sauton’s media with shaking (200 rpm) at 37°C. When required antimicrobials were added to media at the following concentrations: hygromycin 50; kanamycin at 50 μg/ml; pristinamycin 2 and tetracycline at 20 ng/ml. Bacterial growth was assessed by measurement of absorbance at 580 nm, using a spectrophotometer (Biochrom WPA CO8000). Overexpressing M. smegmatis strains were grown in 7H9 medium to the optical density 0.6, and for the protein expression tetracycline at 20 ng/ml was used to induce the expression of CwlM.
4.2.2 Generation of anti-CwlM Antibody

Recombinant *M. tuberculosis* CwlM was expressed and purified from *E. coli* C41 (DE3). 3 mg of protein was sent to Thermo Fisher Scientific, USA for the production of polyclonal Anti-CwlM antibody in rabbits. Rabbits were immunized with CwlM four times (0.5 mg per inoculum). Incomplete Freund’s adjuvant was used as a booster. Pre-immune and post-immunisation sera were provided and tested by Dot Blot and Western Blot. The antibody was purified from the final immunization serum and used for experiments.

4.2.3 Generation Anti-Phospho and Anti-Non-phospho CwlM antibodies

Further antibodies were generated to detect the phosphorylation state of CwlM in *M. smegmatis* fractions (Figure 40). These included custom polyclonal antibodies raised against CwlM phosphopeptide KNDRP T(p) GTFT designed as anti-T382-P and custom polyclonal antibody produced against CwlM non-phosphopeptide KNDRPTGT named as anti-T382. Gemini Biosciences Ltd, UK, generated both antibodies in rabbits. Polyclonal anti-T382 and anti-T382-P antibodies were purified on a cyanogen bromide-activated-Sepharose® 4B column crosslinked with either CwlM-P (Phosphopeptide) or CwlM (Non-phospho peptide). Fractions with the specific antibody were collected and aliquoted to be kept at -80 °C.

**Figure 40.** Schematic of Anti-Phospho and Anti-Non-phospho CwlM antibodies production.

Serum with polyclonal anti-T382 and anti-T382-P antibodies obtained from rabbits were purified on a cyanogen bromide-activated-Sepharose® 4B column crosslinked with either CwlM-P (Phosphopeptide) or CwlM (Non-phospho peptide). The specific antibody was collected from flow through and aliquoted at -80 °C.
4.2.4 Purification of Anti-CwlM polyclonal Antibody from sera

Anti-CwlM antibody was purified using FliQ Protein A column (Generon). This column was equilibrated with 10 column volumes (CV) of PBS, pH 7.4. The serum was diluted by mixing with PBS and loaded onto FliQ Protein A column. The flow-through was collected in a clean beaker before washing the column with 10 CV of PBS. The retained antibody was eluted using 5 column volume of 100 mM Glycine, pH 2.4, and fractions were collected in Eppendorf tubes containing 100 μl of buffer (1 M Tris-HCl, pH 8.5). Elution fractions were combined and concentrated using Amicon Ultra Centrifugal filters (100,000kDa). The Antibody was analysed by SDS-PAGE, Dot Blot and Western Blot. The purified antibody aliquoted in tubes and kept at -80°C.

4.2.5 Dot Blot to determine sensitivity to recombinant *Mtb* CwlM

Purified recombinant CwlM (1μg) was spotted onto the nitrocellulose membrane and left to air dry at room temperature. Nitrocellulose membranes were blocked with blocking buffer (5% (w/v) milk dissolved in TBS) for 1 hour and washed three times with TBS. Membranes were incubated with pre-immune serum and polyclonal anti-CwlM antibody overnight at 4°C. Different dilutions (1:10,000, 1:20,000; 1:40,000; 1:80,000; 1; 160,000) were used to determine the sensitivity of anti-CwlM antibody to recombinant *Mtb* CwlM. Membranes were further washed three times using washing buffer TBST (TBS + 0.01% Tween 20). A 1:10,000 dilution of the secondary anti-rabbit alkaline phosphatase conjugated antibody was added to membranes and incubated for two hours at room temperature. Membranes were washed again three times with TBST as above. BCIP/NBT reagent (Sigma-Aldrich) was used to visualize the dots. The reaction was stopped by washing the membrane with water.

4.2.6 Mycobacterial cell fractionation

Cell fractionation was performed as previously described (Turapov *et al.* 2015). Briefly, mycobacterial cultures were grown to a different optical density (0.2-7.4) and cells were harvested by centrifugation at 4500xg for 15 min at 4°C. Bacterial pellets were washed two times with 10% (v/v) glycerol to remove any remaining media. Bacteria were lysed in Minilys personal homogenizer (Bertin instruments) using glass beads (Sigma-Aldrich) in lysis buffer (TrisCl 20 mM, pH 8.0, NaCl 150 mM, KCl 20 mM, MgCl₂, 10 mM) containing proteinase (cOmplete Ultra Tablets, Roche) and phosphatase inhibitors (PhosStop, Roche). Three cycles of homogenisation were done for 45 seconds. After each cycle, lysates were cooled on ice for
2 minutes. Lysates were centrifuged at 5000xg for 5 minutes and pellets were discarded. The supernatants were centrifuged using an ultracentrifuge (Beckman Coulter Centrifuge, rotor TLA-100) at 27,000 x g for 1 hour at 4°C. The pellets were washed in carbonate buffer (15 mM Na₂CO₃, 35 Mm NaHCO₃, 3.1 mM NaN₃, pH 10), and then collected as cell wall fractions. The supernatants were subjected to further 4-hours centrifugation at 100,000 x g. The supernatants contained cytoplasmic proteins (cytoplasmic fraction); the pellets (membrane fractions) were washed twice in lysis buffer, once in carbonate buffer, pH 10 and once in nano-pure water. Proteins from cellular fractions were separated on SDS PAGE. The purity of fractions was confirmed by detection of diagnostic proteins GarA (cytoplasmic protein) and GlnA (membrane protein). Tubes were kept on ice during the entire procedure. Mycobacterial cell fractions were aliquoted and stored at -80°C.

4.2.7 Culture supernatant preparation

Bacterial starter cultures were grown to exponential phase (OD= 0.8) in 7H9 supplemented with 10% ADC and 0.05% Tween80. When required antimicrobials were added to media as described in (section 4.2.1). Culture cells were centrifuged at 2000 x g for 15 min. Bacteria cells were washed three times in 10% glycerol to remove BSA that is in the ADC. Further centrifugation as above was done, and the supernatant was discarded. Bacterial pellets were inoculated in Sauton’s media supplemented with 0.2% glucose and 0.05% Tween80 but no ADC. Bacteria were grown at 37°C with shaking 200 rpm to exponential phase (OD=0.8). Bacteria were centrifuged at 2000 x g for 15 min at 4°C and supernatant was collected. The supernatant was filtered by using 0.22µm syringe filters (Merck Millipore). trNA (20 µg/ml) was added to the filtered supernatant as a carrier. The supernatant proteins were precipitated by adding 10% TCA. Further centrifugation was done at 2000 x g for 30 min at 4°C and the supernatant was discarded. Pellet samples were washed three times with 10% acetone by centrifuging 2000 xg for 15 mins. The samples were placed inside the fume hood cabinet to remove acetone from the pellet. The SDS sample buffer was added to the pellet and boiled at 90°C for 10 min to be used in SDS-PAGE and Western blot assays.
4.3. Results

4.3.1 Production anti-CwlM antibody

4.3.1.1 Purification of anti-CwlM antibody using FliQ Protein A column

As Figure 41A shows IgG fraction was eluted at 10 ml using protein A sepharose column. The collected fractions were confirmed by SDS-PAGE. The heavy (~50 kDa) and light chains (~25 kDa) of the purified immunoglobulin G (IgG) were visible on the gel at two distinct bands of predicted molecular weight (Figure 41B). All relevant fractions containing anti-CwlM antibodies were concentrated and aliquoted at -80°C to avoid numerous freeze-thaw cycles. Polyclonal anti-CwlM antibody purified from the final bleed could be used for CwlM localisation studies and immunoprecipitation assays in Chapter 5.

![Figure 41. Purification of Anti-CwlM antibodies from final bleeds serum.](image)

A: Anti CwlM serum was loaded onto a protein A sepharose on an AKTA FPLC system. A red arrow refers to IgG antibodies eluted from sepharose in low pH buffer (100 mM glycine pH 2.4). The eluted antibody was analysed by SDS-PAGE. B: SDS-PAGE indicates the heavy and light chains of denaturated IgG at ~50 kDa and ~25 kDa respectively.

4.3.1.2 Validation of AB against recombinant CwlM using a dot blot and Western blot

For validation of purified antibodies, initially a Dot Blot method was used; pre-immune and anti-CwlM sera were used as a control. Figure 42A shows that the pre-immune serum did not recognise recombinant CwlM at dilutions of 1:10 000 and 1:20 000, while anti-CwlM serum
could detect CwlM at 1: 20, 000 dilution (Figure 42B). Purification enriched anti-CwlM antibodies as evident from Figure 42C shows that the purified antibody recognised CwlM at the highest dilutions used (1:160, 000).

**Figure 42.** Dot Blot assays of anti-CwlM polyclonal antibodies.

Recombinant CwlM (1µg) and the alkaline phosphatase conjugated anti-rabbit antibody were used for all experiments. (A) A pre-immune serum was tested at a 1:10,000 and 1: 20,000 dilutions. (B) Anti-CwlM serum and (C) Anti-CwlM-IgG after purification was tested at dilutions of 1:20,000; 1: 40,000; 1:80,000 and 1:160,000.

The purified antibody was further validated using Western blot against recombinant CwlM (wild type). Western blot results (Figure 43) confirmed the high sensitivity of the purified antibody to recombinant CwlM at 1:160,000 dilution. Moreover, the purified antibody was able to recognize shorter forms of CwlM (probable products of degradation). The sensitivity of anti-CwlM at 1:200,000 dilutions were also determined by Thermo Fisher Scientific custom service using ELISA.
4.3.1.3 Recognition of phosphorylated and non-phosphorylated recombinant CwlM

The antibody was also tested against phosphorylated and de-phosphorylated recombinant CwlM. The recombinant CwlM was phosphorylated \textit{in vitro} by PknB using kinase buffer with 100 µM of ATP (see general Materials and Methods). As shown in Figure 44B1, the affinity of the anti-CwlM antibody to recombinant CwlM was not affected by phosphorylation. To dephosphorylate protein this was then incubated for 2 hours at 37°C in NEB3 buffer containing phosphatase. The phosphorylation state of CwlM was confirmed by Western blot using anti-phosphothreonine antibody (described in general materials and methods). Figure 44B2 shows that the anti-CwlM antibody was also able to recognize dephosphorylated CwlM. SDS-PAGE electrophoresis shows PknB at 63 kDa and CwlM at 47 kDa (Figure 44A).
Figure 44. Validation of purified anti-CwlM antibodies against phosphorylated-dephosphorylated recombinant CwlM using Western Blot.

Recombinant CwlM (10 µg) was phosphorylated by 1.5 µg of PknB in kinase buffer with 100 µM of ATP. Dephosphorylation was done by incubating for 2 hours at 37°C in NEB3 buffer containing phosphatase. (A) SDS-PAGE indicates loaded proteins, PknB at 63 kDa and CwlM at 47 kDa. (B) Western Blot shows in lane 1 phosphorylated CwlM, lane 2 dephosphorylated CwlM and lane 3 non-phosphorylated CwlM using anti-CwlM antibodies. M - Protein markers.

4.3.1.4 AB recognize the native CwlM in mycobacterial lysates

*Mtb* lysates (pMV, CwlM-Wild type, T382A and T382D) were prepared and kindly provided by Dr Obolbek Turapov. CwlM overexpressing *M. smegmatis* lysates pMind (empty plasmid), CwlM-Wild type, phosphoablative AAA (T43A, T382A, T386A) and phosphomimetic DDD (T43D, T382D, T386D) were prepared as described in (section 4.2.6). Figure 45A shows that anti-CwlM antibody detected bands of CwlM of predicted size ~47 kDa in *M. smegmatis* lysates overexpressing CwlM, while no band of the right size was detected in the empty plasmid pMind. Figure 45B shows that the AB also recognise a band of predicted size in wild type *Mtb* or *cwlM-CM* grown with pristinamycin. However, no bands of the right size were detected in CwlM-depleted mycobacteria (*cwlM-CM* grown without pristinamycin), confirming the purified antibody detects CwlM and therefore could be used for localization and immunoprecipitation studies.
Figure 45. Validation of anti-CwlM antibodies against CwlM in mycobacteria by Western Blot.

(A) CwlM overexpressing *M. smegmatis* lysates pMind (empty plasmid), CwlM-Wild type, phosphoablative AAA (T43A, T382A, T386A) and phosphomimetic DDD (T43D, T382D, T386D) (B) immunoblot of CwlM disrupted *Mtb* lysates transformed with pMV and derivatives that detected with anti-CwlM antibody (1:20,000 dilutions). Negative controls represented by pMIND and pMV. The anti-rabbit alkaline phosphatase conjugated antibody was used as a secondary antibody 1:10,000 dilution. M- Protein markers.

4.3.2 Localisation studies of CwlM in *M. smegmatis*

4.3.2.1 CwlM localised in cytoplasm and membrane fractions but missing in culture filtrate and cell wall preparations

To investigate the localisation of CwlM during exponential growth, bacteria were grown to OD 0.6, 0.8 and 1.0 and used for the preparation of cellular fractions (Section 4.2.6). The purity of mycobacterial fractions was confirmed using anti-GarA and anti-GlnA antibody (Figure 46C, D).

As shown in Figure 46B CwlM could be detected in cytoplasmic and membrane fractions of *M. smegmatis* in all samples. Analysis of membrane fractions shows a slight reduction in the amount of CwlM compared to cytoplasmic fractions. As shown on a (Figure 46A) it was used a higher amount of protein samples in order to see CwlM on Western Blot. The results also show that the optimal optical density for native CwlM expression in *M. smegmatis* is between 0.6 and 1.0.
Figure 46. Detection of CwlM in cytoplasm and membrane fractions of *M. smegmatis*.

Lysates obtained from *M. smegmatis* mc^{2155} grown in Sauton’s media were fractionated using the procedure of (Turapov et al. 2015). The optimal optical density for native CwlM expression in *M. smegmatis* is between 0.6 and 1.0. A. SDS-PAGE indicates loaded proteins of bacterial fractions from cytoplasm and membrane B. Western Blot analysis of CwlM in *M. smegmatis* fractions (cytoplasm and membrane) using anti-CwlM antibody. C and D Western Blot analysis of cellular compartments where GarA and GlnA were used as markers of cytoplasm and membrane respectively. 3-replicate experiments (N=3)

Separately I investigated the presence of CwlM in cell wall fractions and filtered culture supernatant. Mycobacterial fractions of *M. smegmatis* mc^{2155} wild type and CwlM conditional mutant (CwlM-CM) were prepared using cell fractionation procedure. The filtrated culture was prepared as described in (section 4.2.7) and examined by Western Blot using anti-CwlM antibody.
However, no bands with predicted CwlM size were detected. Smaller protein bands with a molecular weight of ~ 25 kDa were identified in cell wall fractions isolated from both wild-type \textit{M. smegmatis} and CwlM-depleted mutant (\textbf{Figure 47A}), suggesting that these were not derived from CwlM. Similar patterns were observed in mycobacterial culture filtrates (\textbf{Figure 47B}). Thus, our results suggest that CwlM is produced in mycobacteria in two distinct forms, cytoplasmic CwlM and membrane-associated CwlM.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure47.png}
\caption{Detection of CwlM in the mycobacterial cell wall and filtered culture supernatant preparations. \textbf{A} and \textbf{B} Western Blot using anti-CwlM antibody confirmed the existence of CwlM in cytoplasm and membrane fractions of both wild type and pristinamycin-induced CwlM-CM at the right size 47 kDa, however, non-specific recognition of smaller protein bands with molecular weight of ~ 25 kDa were identified in cell wall and culture filtrates preparations of all \textit{M. smegmatis} strains. 3-replicate experiments (N=3) }
\end{figure}

\subsection{4.3.2.2 Phosphorylation of T382 determines CwlM localisation in the cytoplasm}

Previous research in our lab with phosphoablative\textsuperscript{1} and phosphomimetic\textsuperscript{2} CwlM mutants as well as with PknB-depleted and CwlM-depleted mycobacteria strains shows that phosphorylation plays an important and distinct role in mycobacterial growth (Turapov \textit{et al.}, 2018). Our complementation studies showed that phosphorylation of threonine 382 by PknB is critical for mycobacterial viability, and the T382A phosphoablative mutant results in \textit{Mtb} lysis in standard media, while the T382D phosphomimetic mutant could grow in standard media (Turapov \textit{et al.}, 2018).

Therefore, we hypothesised that both phosphorylated and non-phosphorylated forms of CwlM are produced and present during growth. To investigate this, two separate antibodies generated
to detect the phosphorylation state of CwlM in *M. smegmatis* fractions. The first antibody generated against a peptide containing the phosphorylated threonine 382, designated as an anti-T382-P antibody. The second antibody generated against the same peptide but with non-phosphorylated threonine 382, designated as anti-T382 antibody (see section 4.2.3).

Figure 48B shows that anti-T382-P antibody could only recognise the cytoplasmic form of CwlM, confirming the previous finding that in the cytoplasm CwlM was phosphorylated (Boutte *et al.*, 2016). The non-phosphorylated CwlM form was identified only in membrane fraction using anti-T382 antibody (Figure 48C). Importantly, both forms were missing in the control CwlM-CMpmv306 grown without pristinamycin in all immunoblots (Figure 48).

As discussed previously there were multiple bands in the range of 20-50 kDa in all experiments using different anti-CwlM antibodies in cell-wall preparations. The purity of mycobacterial fractions was confirmed using anti-GarA and anti-GlnA antibodies (Figure 48D).

These results prove that the phosphorylation of CwlM determines its distribution between cytoplasm and membrane fractions. Moreover, recently published data from our laboratory strongly suggest that PknB-mediated phosphorylation on T382 is critical for this distribution (Turapov *et al.*, 2018).

---

1 **Phosphoablative CwlM**: The phosphorylation sites of CwlM (T43, T382 and T386) were blocked with amino acid Alanine (A) substitution.

2 **Phosphomimetic CwlM**: The phosphorylation sites of CwlM (T43, T382 and T386) were substituted with a negatively charged amino acid Aspartate (D) to mimic phosphorylated CwlM.
Figure 48. PknB-mediated phosphorylation of T382 determines the distribution of CwlM in cytoplasmic and membrane fractions of *M. smegmatis*.

Lysates obtained from wild type *M. smegmatis* and CwlM-CM were grown in Sauton’s media with or without pristinamycin were fractionated for immunoblots assay. A. SDS-PAGE shows loaded proteins of *M. smegmatis* fractions (cytoplasm, membrane and cell wall). The membrane fractions were loaded 5 times more than that in other fractions, as CwlM is less abundant in the membrane. B. Western Blot using anti-CwlM-382-P antibody detected phosphorylated CwlM-T382 form only in the cytoplasm fraction of both wild type and pristinamycin-induced CwlM-CM. C. Anti-CwlM-382 antibody identified non-phosphorylated CwlM form only in the membrane fraction of both wild type and pristinamycin-induced CwlM-CM. The control CwlM-CMpmv306 grown without pristinamycin had no CwlM forms in all experiments. Cell wall fractions indicate non-specific recognitions in all experiments using different anti-CwlM antibodies. D. The Western Blot of GarA and GlnA were used as markers to confirm the purity of bacterial fractions. M- Protein markers; “+” referrers to CwlM detection at 47kDa. 3-replicate experiments (N=3)

4.3.2.3 CwlM is expressed only during active growth

Next, I investigated the expression of CwlM during various growth phases in the wild type *Mycobacterium smegmatis* mc²155 strain. Mycobacterial cultures from four separate phases were used for these experiments, and their cytoplasmic and membrane fractions were prepared using fractionation procedure (see 4.2.6). In the logarithmic phase, CwlM was not detectable in cytoplasmic and membrane fractions (Figure 49B). In the exponential phase, CwlM is expressed and could be detected in both cytoplasmic and membrane compartments (Figure 49B). In the early stationary phase, degraded forms of CwlM could be found in the cytoplasm.
but not in the membrane fraction (Figure 49B). In the late stationary phase, CwlM was also not detectable in cytoplasmic and membrane fractions (Figure 49B).

These results suggested that CwlM was only detected in growing mycobacteria. CwlM was expressed as cytoplasmic phosphorylated CwlM and non-phosphorylated CwlM associated with membrane. As CwlM is a main substrate of PknB, phosphorylation by this kinase controls the distribution of CwlM.

**Figure 49.** Detection of CwlM in cytoplasm and membrane fractions of *M. smegmatis* during different growth stages.

WT *M. smegmatis* were grown in 7H9 medium at 37°C without antibiotics. The growth stages absorbance was measured at 600 nm, using a spectrophotometer as following: grown to O. D=0.2 (lag-phase, Lag.), OD= 0.8 (exponential phase, Exp.), O. D= 3.6 (early stationary phase, Early stat.) and O. D=7.4 (late stationary phase, Late stat.). A: 4-20% SDS-PAGE shows loaded proteins in bacterial fractions cytoplasm and membrane. B: Western Blot using anti CwlM antibodies detects CwlM in growing mycobacteria (exponential phase) in cytoplasm and membrane fractions. In the early stationary phase, degraded forms of CwlM found in the cytoplasm but not in the membrane fraction. In Lag and late stationary phases, CwlM was not detectable in cytoplasm or membrane fractions. M- Protein markers. 3-replicate experiments (N=3)

### 4.4 Discussion

Generation specific antibodies against CwlM is very important to study protein localisation. Also, it will be a powerful tool for the investigation of protein-protein interaction. It was opted to produce polyclonal antibodies instead of monoclonal antibodies despite the later may offer
higher specificity to CwlM. Monoclonal antibodies can recognize only one specific epitope on a protein and they may fail to interact with the protein processed during post-translational modifications, also protein-protein interaction that may lead to the epitope occlusion (Leder et al. 1994; Read et al. 2009). The polyclonal anti-CwlM antibody can recognise multiple epitopes on a protein which is useful for further experiments.

Polyclonal antibodies are commonly produced in rabbits for several reasons. The most important reason is the excellent immune response of the rabbit to a variety of antigens and production of a single primary immunoglobulin G isotype. Secondly, highly efficient purification of rabbit immunoglobulins G on protein A-sepharose (Drenckhahn et al. 1993; Page and Thorpe 2002; Stills 2012). It was essential to test the pre-immune serum of rabbits for reactivity prior to immunization. In this study, pre-immune sera from 4 different rabbits were tested against recombinant Mtb CwlM using Dot Blot assay. The results indicate that the pre-immune serum did not contain any specific antibody to CwlM. Therefore, it was possible to immunize rabbits with presented antigen and produce specific polyclonal anti-CwlM antibody.

Alongside anti-CwlM specific antibodies, there are some impurities in final bleed serum. It was also necessary to purify anti-CwlM from sera. Affinity chromatography is a widespread technique used in the purification of antibodies (Ayyar et al. 2012; Arora et al. 2017). It is well known that protein A from Staphylococcus aureus (SpA) has a good affinity to bind immunoglobulins IgG produced in rabbits (Page and Thorpe 2002; Hober et al. 2007). Subsequent to this, an anti-CwlM antibody from serum was purified using protein A-sepharose. In this study, the purity and specificity of immunoglobulin G produced against CwlM were confirmed. SDS-PAGE analysis showed that the heavy and light chains of denaturated IgG were present at the expected mass range. Dot Blot analysis showed that the reactivity of purified antibody to CwlM was higher than that of crude serum. This means anti-CwlM specific antibodies were more abundant after purification.

The purified antibodies showed a significant sensitivity to recombinant CwlM, recognising it at dilution of 1:160 000. Such a high titer is valuable for localisation experiments. Importantly, the antibody was also able to recognise the degradation bands of recombinant CwlM in Western Blot assays. Another important finding is that anti-CwlM antibody could detect the phosphorylated CwlM. It was crucial to understanding whether posttranslational modifications may alter the antigenic determinants of protein which in turn antibodies may fail to bind CwlM.
The phosphorylation state of protein did not abolish the affinity of the anti-CwlM antibody. This result can support the use of an anti-CwlM antibody to detect the potential interaction of phosphorylated CwlM with its partners in immunoprecipitation experiments.

The tagged proteins techniques such as GFP are commonly used to study the cellular localization of proteins (Beatty and Russell 2000; Cimino et al. 2006; Marx 2013; Vettiger 2014). For example, the periplasmic localization of AmiA and AmiC in E. coli was identified by the use of GFP (Bernhardt and de Boer 2003; Diestra et al. 2009). In mycobacteria, the subcellular localisation of Rv3717 was studied using fluorescent tags (Senzani et al. 2017). However, it was reported that tags could produce suspicious localisation artifacts (Sampson et al. 2001; Dziadek et al. 2002; Cimino et al. 2006; Margolin 2012). The main limitation of the use GFP is an aggregate formation or a cleavage tag of proteins (Lindow et al. 2002; Margolin 2012; Vettiger 2014). Additionally, tags could negatively impact on the study of protein interaction with its partners (Carrero et al. 2003; Xing et al. 2016). We attempted to use of GFP-fusions, but the fusion protein produced inclusion bodies and thus it cannot be used for localisation studies.

An alternative method, therefore, was needed to investigate the localisation of CwlM in mycobacterial cells. The specific antibody generated against target proteins is very important for localisation studies. One effective method described in the literature is mycobacterial cell fractionation procedure followed by detection of proteins using the specific antibodies (Koul et al. 2001; Turapov et al. 2015). Significantly, the polyclonal antibody raised in this study was able to detect CwlM in both M. smegmatis and Mtb, and thus could be used in localisation and protein-protein interaction studies.

The main aim of research in this chapter was to investigate a localisation of CwlM in M. smegmatis. Understanding where proteins are in the bacterium is often crucial to know their functions. Some proteins were found to have two different locations in the cell. For example, DivIVA of Bacillus subtilis localises both to the cell poles and to synthesised division septa (Edwards and Errington 1997; Rudner and Losick 2010), and therefore this protein has multiple functions importantly plays a key role in bacterial division and sporulation (Thomaides et al. 2001; Perry and Edwards 2004). Another protein that has two distinct subcellular sites is IcsA, a virulence factor of Shigella flexneri. This protein localises to the outer membrane of the bacterium and mediates the assembly of an actin tail that controls the Shigella flexneri movement in the cytoplasm of its mammalian host cell (Goldberg et al. 1993). IcsA also
localises to the division sites, suggesting that it plays an important role in the bacterial septation (Charles et al. 2001; Janakiraman and Goldberg 2004).

A little is known about where CwlM resides in mycobacteria. It has been shown that CwlM is localised in the cytoplasm (Boutte et al. 2016). However, the current study shows that CwlM was localised in cytoplasm and membrane fractions, but it was missing in culture filtrate and cell wall preparations of WT M. smegmatis mc²155 and CwlM-CM M. smegmatis mutant. A slight reduction of CwlM was observed in the membrane fraction because the concentration of proteins used for SDS-PAGE electrophoresis was high.

This study has revealed that CwlM forms were missing in the conditional mutant of M. smegmatis grown without pristinamycin. This strain contains the pmv306 plasmid, which allowed pristinamycin to regulate gene expression of CwlM (Forti et al. 2009). The lower bands in cell wall fractions and culture filtrate preparations can be observed in this strain using Western Blot analysis, demonstrating that those bands are unspecific recognised by different anti-CwlM antibodies. This can be the result of the usage of Freund’s adjuvant to boost the immune response of animals during the process of antibodies production. It is well known that Freund’s adjuvant composed of inactivated mycobacteria (Stills 2012).

CwlM is a putative amidase_3 domain, which does not contain a typical signal sequence and a lipid anchor motif (Deng et al. 2005; Machowski et al. 2014; Boutte et al. 2016), therefore it is not surprising that CwlM was not detected as a secreted protein in culture filtrate preparations. Thus, the current results suggest that CwlM is produced in mycobacteria in two distinct forms, cytoplasmic CwlM and membrane-associated CwlM.

Experiments using Mtb mutant strains have shown that CwlM is essential for mycobacterial growth (Turapov et al., 2018), and the T382A phosphoablative mutant results in Mtb lysis in standard media, while the T382D phosphomimetic mutant could grow in standard media (Turapov et al., 2018). Additionally, the phenotypes of Mtb mutant strains including PknB-depleted and CwlM-depleted have suggested that both phosphorylated and non-phosphorylated CwlM play distinct roles in mycobacterial growth (Turapov et al. 2018). Therefore, it was plausible to suppose that both phosphorylated and non-phosphorylated forms of CwlM are produced and present during growth. Here, two important questions were investigated during this study. The first question was whether phosphorylation by PknB could regulate the distribution of CwlM. The second one was about the roles of the two CwlM forms.
The first question was answered using anti-CwlM antibodies against either the full length or synthesised peptide containing the phosphorylated and non-phosphorylated T382 of CwlM. The present study demonstrates that the phosphorylated form of CwlM was mainly found in the cytoplasm, while the non-phosphorylated CwlM form associated with the membrane of wild type *M. smegmatis* and a CwlM-CM *M. smegmatis* mutant.

This is the first study to demonstrate that PknB mediated phosphorylation regulates the distribution of CwlM in *M. smegmatis*. There are many proteins could be found as phosphorylated and non-phosphorylated forms with a distinct localisation to perform a specific function in other organisms. As an example, for this behaviour, in human, the function of signal transducer and activator of transcription 1 (STAT1) is mainly controlled by its phosphorylation state that required for activating the interferons (IFNs) signalling in hepatocellular carcinoma. The authors showed that non-phosphorylated STAT1 is required for cell cycle arrest and apoptosis that was highly increased in hepatocellular carcinoma cells of patients and mainly expressed in the cytoplasm, whereas phosphorylated STAT1-p was absent. However, inhibiting the activity of non-phosphorylated STAT1 but not phosphorylated STAT1-p increased the susceptibility of hepatocellular carcinoma cells to interferons (IFNs) treatment, suggesting that STAT1 has dichotomous functions that could be coordinated via its phosphorylation state (Ma et al. 2019).

Localisations of CwlM forms (phosphorylated and non-phosphorylated) in different compartments of mycobacterial cells could suggest the different roles of CwlM depending on its location. CwlM could be recruited to specific subcellular sites, in order to regulate specific targets. Further experimental work will be done to investigate the biological functions of CwlM forms in mycobacteria. The raised antibodies in this study will also be used in immunoprecipitation experiments to investigate the potential partners of CwlM in cytoplasm and membrane fractions of mycobacteria. This will provide important information about how phosphorylation by PknB could regulate the CwlM interactions with its partners. Understanding the biological functions of CwlM is important for drug discovery.

The final aim of this Chapter was to examine CwlM expression levels during various growth phases of WT *M. smegmatis*. Here, I demonstrate that the CwlM could be detected only during exponential growth. In this phase, the expressed CwlM is stable in the cytoplasm, while in membrane fractions of mycobacteria it is slightly degraded. In contrast, degraded forms of CwlM were found in the cytoplasm and was not detectable in the membrane fraction during
early stationary phase. It has been shown that PknB is produced during exponential growth (Forti et al. 2009). It is possible that PknB plays an essential role in the stability and activity of CwlM forms during mycobacterial growth. That in agreement with the results of CwlM stability studies in vitro (section 3.3.5). PknB is not expressed during stationary phase and we can see that CwlM is not phosphorylated and degraded in the cytoplasm. Overall finely tuned balance between CwlM phosphorylated and non-phosphorylated forms are crucial for mycobacterial viability.

The current study also shows that CwlM forms were not detectable in cytoplasm or membrane fractions during lag and late stationary phases. This is another evidence that CwlM forms are essential in growing mycobacteria. They could be produced as a non-phosphorylated membrane-associated and a PknB-phosphorylated cytoplasmic CwlM. Importantly, PknB expression and its activity regulate the locations of CwlM via phosphorylation of T382. As a cytoplasmic CwlM is phosphorylated and potentially can be stable, CwlM overexpression in M. smegmatis can be used to purify a large scale of native protein for crystallization of CwlM in future studies.

### 4.5 Conclusions and future work

Mycobacterial fractionation method followed by the use of anti-CwlM specific antibodies in Western Blotting analysis facilitated the study of CwlM localisation in mycobacteria.

The successful generation of polyclonal anti-CwlM antibodies was described in this project. Recombinant Mtb CwlM was used for immunizations of rabbits. The sensitivity of anti-sera was characterised as pre-immune, non-purified and purified anti-CwlM antibody using Dot Blot and Western Blot assays. The purified anti-CwlM antibody showed very high sensitivity to CwlM. Also, this antibody recognized CwlM variants in M. smegmatis and Mtb.

This study indicates that two forms of CwlM exist in mycobacteria compartments. The phosphorylated CwlM by PknB is mainly found in the cytoplasm, while the non-phosphorylated CwlM is localised in the membrane. These two forms could only be detected in growing mycobacteria. Thus, they could play an essential role in mycobacterial growth. PknB could regulate the distribution of CwlM via phosphorylation of T382.

The raised antibodies in this study will also be used in immunoprecipitation assays to determine the probable partners of CwlM forms in mycobacteria. This will provide important information about how phosphorylation by PknB could regulate the CwlM interactions with its partners in
peptidoglycan-synthesising machinery. Localisations of CwlM in different compartments of mycobacterial cells could suggest the different roles of CwlM forms depending on their locations. CwlM could be recruited to specific subcellular sites, in order to regulate specific targets. Further experimental work will be done to investigate the biological functions of CwlM forms in mycobacteria. Understanding the biological functions of CwlM will be a valuable target for drug discovery.

In this study, Western Blotting assay using anti-CwlM specific antibody was employed to investigate CwlM in bacterial fractions. This chapter firstly describes the production and characterisation of polyclonal anti-CwlM antibodies and then mainly demonstrates the distinct localisations of CwlM forms in \textit{M. smegmatis}.

Custom polyclonal CwlM specific antibody was produced by Thermo Fisher Scientific, USA against recombinant \textit{Mtb} CwlM protein. The sensitivity and specificity of the anti-CwlM antibody to CwlM proteins were validated using Dot Blot and Western Blot assays. The purified antibody showed very high sensitivity to recombinant CwlM protein. Also, the antibody was found to recognise CwlM variants in \textit{M. smegmatis} and \textit{Mtb}.

The work described in this chapter provides a novel insight to the localisation of CwlM and its possible action in mycobacterial growth. The current study demonstrates that in growing mycobacteria two essential CwlM forms are produced: non-phosphorylated membrane-associated CwlM and PknB-phosphorylated cytoplasmic CwlM. Importantly, PknB regulates the localisations of CwlM via phosphorylation of T382. The putative roles of these two forms in mycobacterial growth will be extensively described in chapter 5.
Chapter 5

5. Identification of protein partners interacting with CwlM in Mycobacteria
5.1 Introduction

Peptidoglycan (PG) is a unique layer of the bacterial cell wall which plays an essential role in bacterial growth, division and survival within host. Thus, most enzymes involved in the PG biosynthesis are targets of currently used antibiotics or considered as candidates for development of novel drugs. PG biosynthesis has been studied for decades, mainly in two fast growing bacteria *E. coli* and *B. subtilis*. However, the precise regulatory mechanisms that govern PG synthesis in mycobacteria are not fully addressed.

As previously described in (section 1.6.1), PG biosynthesis has many steps including the production of PG precursors in the cytoplasm, their transporting across the membrane and inserting newly synthesised peptidoglycan in the existing cell wall (Hett and Rubin 2008; Typas *et al.* 2011; Kieser and Rubin 2014). This pathway depends on coordinated activities of PG synthesising and PG cleaving enzymes which are localised in different cellular compartments. Therefore, these enzymes engage in multiple protein-protein interactions either to form multi-enzyme complexes at the sites of PG synthesis or to control enzymatic activities (Egan *et al.* 2017).

The role of N-acetylmuramoyl-L-alanine amidases in the functioning of PG biosynthesis machinery was initially studied in *E. coli* by phenotypic characterisation of amidase mutants, characterisation of enzymatic activities and investigation of protein-protein interactions involved in activation of amidases. Periplasmic AmiA, AmiB and AmiC have been reported to be important for *E. coli* division and PG remodelling (Heidrich *et al.* 2001; Bernhardt and de Boer 2003).

Further studies have revealed that these NAM-amidases are inactive enzymes, and they, therefore, require an activator to promote their hydrolases activities (Priyadarshini *et al.* 2007). EnvC and NlpD are LytM-domain proteins that play a key role in activating NAM-amidases of *E. coli* (Uehara *et al.* 2010; Peters *et al.* 2011). EnvC was found to interact and activate AmiA and AmiB whereas NlpD binds and activates AmiC (Priyadarshini *et al.* 2007, Uehara *et al.* 2010, Yang *et al.* 2012). The authors proposed a model suggesting that binding of EnvC or NlpD with periplasmic NAM-amidases is essential for activating and recruiting theses amidases to the septum which in turn interact with divisome complex proteins to hydrolyse the septal PG during peptidoglycan remodelling and cell division (Yang *et al.* 2012; Peters *et al.* 2013).
This activation mechanism is not unique for *E. coli*. In *Neisseria gonorrhoeae*, NlpD also interacts with AmiC and stimulates the amidase activity to aid cell separation and PG remodelling (Lenz *et al.* 2016). PG amidases also interact with other PG enzymes. For examples, in *Bacillus subtilis*, the proposed SpoIIP amidase interacts with a lytic transglycosylase SpoIID to form IIP-IID complex at the hydrolysis sites of the cell wall, and this interaction was shown to be important for stimulating the PG amidase activity of SpoIIP during pores morphogenesis (Morlot *et al.* 2010).

Very little is known about mycobacterial amidases and their putative partners. It has recently shown that PknB-phosphorylated CwlM binds and activates MurA, the primary enzyme in the biogenesis of peptidoglycan precursors in the cytoplasm of mycobacteria. Therefore, CwlM might contribute to the regulation of peptidoglycan precursor production (Boutte *et al.* 2016). However, there is no available information on the possible role of non-phosphorylated CwlM.

Another study suggested that CwlM may interact with Wag31, a scaffold protein involved in the regulation of PG biosynthesis (Kang *et al.* 2008, Senzani *et al.* 2017). Wag31 is localised on cell poles and it is critical for the shape maintenance and recruitment of peptidoglycan synthesis materials during mycobacterial polar growth (Kang *et al.* 2008; Jani *et al.* 2010; Plocinski *et al.* 2012; Meniche *et al.* 2014; Senzani *et al.* 2017). Both cwlM depleted and wag31 depleted *M. smegmatis* strains produce prominent cell bulges potentially indicating that the two proteins are involved in the same pathway and may interact. However, actual interaction between Wag31 and CwlM has not been demonstrated (Kang *et al.* 2008; Senzani *et al.* 2017).

In Chapter 4, I described two distinct forms of CwlM, the phosphorylated cytoplasmic CwlM and membrane-associated non-phosphorylated CwlM. It is plausible to suggest that both forms interact with other proteins. This chapter will be focused on the identification of putative partners of phosphorylated and non-phosphorylated CwlM. Immunoprecipitation assays will be used for initial identification of CwlM partners. A cyanogen bromide-activated-sepharose will be crosslinked with the anti-CwlM antibody to allow for enrichment of CwlM from mycobacterial fractions and co-purification of putative CwlM partners. Co-purified proteins will be identified by mass spectrometry and selected interactions will be confirmed by protein fragment complementation assays. Recombinant CwlM partners will be generated for confirmation of direct interaction between candidate proteins and CwlM forms *in vitro*. Finally, crystallisation trials of CwlM forms with or without their partners will be discussed.
5.2 Materials and Methods

5.2.1 Preparation of anti-CwlM antibody sepharose

Anti-CwlM-IgG sepharose was prepared by crosslinking anti-CwlM antibody to Cyanogen bromide-activated-Sepharose® 4B (Sigma). The cyanogen-bromide activated resin was washed in cold 1 mM HCl for 30 minutes. The supernatant with lactose, a stabiliser agent of beads was removed by gentle filtration. The resin was washed further with distilled water and then with a coupling buffer (0.1 M NaHCO₃, 0.5 M NaCl, pH 8.3). The sepharose was transferred quickly to a Falcon tube and mixed with purified anti-CwlM antibody overnight at 4°C. The unbound anti-CwlM antibody was washed away using coupling buffer. The unreacted active groups were blocked with 0.2 M glycine, pH 8.0, for 1 hour at room temperature. Further steps of washing were done to remove the blocking buffer using a coupling buffer and 0.1 M acetate buffer, with 0.5 M NaCl pH 4. The anti-CwlM-IgG-sepharose was stored at 4°C with 0.2% sodium azide.

5.2.2 Expression and purification of recombinant Mtb proteins

Recombinant CwlM, MurJ icd and MurJ E541-F680 (linker) were cloned in pET15b-TEV whereas recombinant FhaA and Wag31 were cloned in pGEX2T-TEV. All constructs were confirmed by sequencing prior to transformation into E. coli OverExpress™ C41 (DE3) Competent Cells (Lucigen). E. coli strains were grown to OD-0.6 in LB as previously described in general methods. Proteins expression was induced with 0.1-0.5 mM IPTG followed by overnight incubation at 16 °C. The purification of recombinant CwlM, MurJ icd and MurJ linker with affinity poly-His tags were achieved using Ni-NTA resin (Qiagen). Recombinant FhaA and Wag31 with GST-tags were purified using a Glutathione Sepharose 4B column (GE Healthcare). All recombinant proteins were further purified using SEC on a 16/600 Superdex 200 pg column using ÄKTA™ Purifier system (GE Healthcare Life Sciences).

5.2.3 Cleavage of Poly-His and GST tags

Poly-His and GST tags were removed by cleavage with a TEV-protease (PROTEX). Digestion of tags was conducted at room temperature for 2 hours or overnight at 4°C. The cleaved tag of poly-His was removed using Ni-NTA resin (Qiagen) whereas GST-tag was removed using a Glutathione Sepharose 4B column (GE Healthcare). Flow through with isolated proteins were further purified on a 16/600 Superdex 200 pg column using ÄKTA™ Purifier system. Purity
and identity of proteins were analysed by SDS PAGE and Western Blot methods as described in Chapter 2.

5.2.4 Preparation of proteins and screens for crystallisation trials

Recombinant *Mtb* proteins, CwlM forms, FhaA and MurJ were purified as described above. Fractions with pure proteins were collected and analysed by SDS-PAGE. Proteins with the highest concentration and minimal degradation were concentrated to ~5mg/ml using a 10,000Da cut off filter (Merck Millipore). Multiple stock screens included: PACT premier™ HT-96 (Molecular Dimension), JCSG-plus™ HT-96 (Molecular Dimension), MIDAS™ (Molecular Dimension), ProPlex HT-96™ (Molecular Dimension) and MORPHEUS™ (Molecular Dimension) were prepared by adding 1 ml of the reservoir solutions to 96-well deep round plates (Porvair sciences) which then sealed and stored at 4°C. From the stocks, 80μl of screens (mentioned above) were added to 96-well plates (Hampton research). For the initial crystallisation trials, 0.1μl of the concentrated protein (6 mg/ml) mixed with 0.1μl of reservoir solution (commercial screens above) using Mosquito® Crystal machine. The plates with drops (1:1 ratio of protein-buffer) were sealed and incubated either at room temperature or at 4°C.

5.2.5 Immunoprecipitation assays

**Immunoprecipitation assays using *Mtb* fractions**

*Mtb* H37Rv lysates were prepared and fractionated as described in (section 4.2.6). The CwlM-IgG-Sepharose was equilibrated with binding buffer (20 mM Tris pH 8.5, 50 mM NaCl). The membrane fraction was dissolved in this binding buffer containing 0.5% (w/v) Triton X-100. Proteinase and phosphatase inhibitors were added to binding buffer (20 mM Tris pH 8.5, 50 mM NaCl) with 0.5% (w/v) Triton X-100. The protein samples of mycobacterial fractions (100 μg/ mL) were mixed with 10μl of a CwlM-IgG-Sep and gently mixed on a laboratory rotator for 60 minutes at room temperature. Tubes with proteins mixture were centrifuged for 10 min at 500 g and supernatants were discarded. The pellets with the resin were washed 3 times with binding buffer. Proteins were eluted from a CwlM-IgG-Seph with 40 μl of phosphoric acid (50 mM, pH 2.0) and then neutralised by adding sodium phosphate buffer (40 mM NaH2PO4, pH 8). Eluted proteins were analysed using SDS PAGE, Western blot and mass spectrometry. Lysates of CwlM-depleted mutant were used as a control for non-specifically binding proteins. The mass-spectrometry analysis was done at PNACL, University of Leicester as previously described in (section 2.15).
Immunoprecipitation assays using recombinant *Mtb* proteins

For immunoprecipitation assays poly-His-tag of MurJ<sub>icd</sub> and GST-tag of FhaA, Wag31 were removed by cleavage with a TEV-protease as described above. The CwlM-IgG-Seph was equilibrated with binding buffer (20 mM Tris pH 8.5, 100 mM NaCl, 10 mM KCl). A small amount (10 µg) of CwlM and one of the partner proteins were mixed equally in binding buffer with a total volume of 200 µl. The proteins were incubated at room temperature for 30 minutes, then 10 µl of Anti-CwlM-IgG-sepharose was added. This mixture was gently rotated at room temperature for a further 30 minutes and centrifuged at 500 xg for 10 minutes. Supernatant with unbound proteins was collected in a separate tube for a further analysis. The resin with bound proteins was washed twice in binding buffer. Proteins of both fractions were analysed by SDS-PAGE and Western blot. The control sample contained the candidate protein with a CwlM-IgG-Sephe only.

5.2.6 Densitometry analysis

Densitometry analysis of protein bands was performed using ImageJ version 1.51 software. Gels from three independent experiments were used. Protein abundance bound to anti-CwlM IgG sepharose was expressed as a percentage of the total amount used for immunoprecipitation assays which correspond to line 5 on each gel. Data were analysed using Microsoft Excel 2016 for windows 10.

5.3 Results

5.3.1 Phosphorylated and non-phosphorylated CwlM proteins have different protein partners

In initial experiments, we used cytoplasmic and membrane fractions isolated from growing *Mtb* cultures to enrich native CwlM proteins bound to potential partners. CwlM-depleted lysates were used as a control and allowed to exclude non-specifically bound proteins.

Table 3 shows proteins co-purified resin with CwlM on Anti-CwlM-IgG resin from cytoplasm and membrane fractions of CwlM-expression of *Mtb*. Cytoplasmic proteins included FhaA, FtsZ, DnaA, Wag31 and previously described MurA, whereas membrane proteins were MurJ (MviN), FtsE and CwsA. Application of mycobacterial protein fragment complementation (M-PFC) narrowed down the list of CwlM partners and confirmed FhaA, MurJ, and CwsA as proteins interacting with CwlM (Turapov *et al.* 2018) (see Table 3). For a demonstration of
direct interaction with CwlM recombinant Wag31, FhaA and MurJ (highlighted) were generated. MurA and CwsA were not investigated in this study.

**Table 3:** Identification of potential CwlM partners by immuno-precipitation and protein fragment complementation assays. Y refers to Yes; N refers to No; N/A refers to not assessed.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
<th>Fraction detected</th>
<th>Lysate Immuno-precipitation</th>
<th>M-PFC (Turapov et al. 2018)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MurA</td>
<td>UDP-N-acetylglucosamine 1-carboxyvinyltransferase</td>
<td>Cytoplasm</td>
<td>Y</td>
<td>N/A</td>
</tr>
<tr>
<td>FhaA</td>
<td>Conserved protein with FhaA</td>
<td>Cytoplasm</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>Wag31</td>
<td>DivIVa family protein</td>
<td>Cytoplasm</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>FtsZ</td>
<td>Cell division protein</td>
<td>Cytoplasm</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>DnaA</td>
<td>Chromosomal replication initiator protein</td>
<td>Cytoplasm</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>FtsE</td>
<td>Putative cell division ATP-binding protein</td>
<td>Membrane</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>CwsA</td>
<td>Membrane protein involved in division</td>
<td>Membrane</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>MurJ</td>
<td>Lipid II flippase</td>
<td>Membrane</td>
<td>Y</td>
<td>Y</td>
</tr>
</tbody>
</table>

5.3.2 Generation of recombinant *Mtb* proteins

5.3.2.1 Expression and purification of Wag31

Optimal conditions for Wag31 expression were established by trialling different incubation times and temperatures. **Figure 50A** indicates that there was a little aggregation of Wag31 in the insoluble fraction when bacteria were grown overnight at 16°C with 0.5 mM IPTG. Therefore, these conditions were used for Wag31-GST expression and purification using affinity chromatography and gel filtration (as described in Methods, section 5.2.2). **Figure 50B** shows SDS-PAGE gel with purified Wag31-GST. While fractions 41 contained multiple bands and potential impurities fractions 47 contained a single protein band which matched the calculated molecular weight 55 kDa. The Wag31-GST yielded as a single peak at 45 min corresponding to the monomeric form of the protein (**Figure 50D**), and pure fractions with single bands of the protein were collected.
The pure Wag31-GST fractions obtained from gel filtration were concentrated and analysed by SDS-PAGE (Figure 50C, lane1). GST-tag was removed by cleavage with a TEV-protease as described in Methods (sections 5.2.3). Figure 50C, lane 2 displays an SDS-PAGE gel with digested Wag31-GST. Two bands of ~27 and 28 kDa corresponds to GST-tag and Wag31. To remove the tag, a Glutathione Sepharose 4B column was used for separation tagged and untagged Wag31: GST-Wag 31 was retained on the column, while Wag31 went to flow-through fraction. Fractions Wag31 were collected and used for immunoprecipitation assays (Figure 50C, lane 3, 4 and 5). The identity of purified Wag31 was identified by mass-spectrometry as described in Materials and Methods (Figure 50E).
Figure 50. Expression and purification of recombinant *Mtb* Wag31 from *E. coli* C41

A. SDS-PAGE analysis shows that optimal conditions for Wag31 are an overnight expression at 16°C in the presence of 0.5 mM IPTG. B. SDS-PAGE displays fractions of Wag31-GST obtained from SEC at expected molecular mass 55 kDa. C. SDS-PAGE shows the concentrated Wag31-GST in a lane 1, the digestion of Wag31 using a TEV-protease in a lane 2 and the isolated Wag31 only in lanes 3, 4 and 5. D. Gel filtration analysis shows a monomeric peak of Wag31-GST at 45 minutes. E. Mass spectrometry analysis confirmed the identity of purified Wag31; blue font indicated peptides detected by mass-spectrometry.
5.3.2.2 Expression and purification of recombinant FhaA

FhaA expression trials were carried out to optimise protein yield. SDS-PAGE gel reveals that FhaA was expressed as a soluble protein when *E. coli* C41 strain was grown overnight at 16°C; FhaA appeared in soluble fraction only as a band of MW~100 kDa which corresponds to the predicted size (Figure 51A). In contrast, the majority of FhaA was expressed as aggregated protein when bacteria were grown at 37°C for 3 and 5 hours (Figure 51A). Therefore, overnight incubation of bacteria at 16°C with 0.5 mM IPTG yielded the highest amount of soluble recombinant FhaA.

Soluble FhaA protein was purified using Glutathione Sepharose 4B and SEC. SDS-PAGE gel shows the collected fractions of FhaA-GST from affinity chromatography with little degraded bands of a GST-tag at ~27 kDa (Figure 51B). The predicted size of FhaA-GST is 83 kDa but it migrated as ~100 kDa protein (Figure 51B). The GST-tag was cleaved by a TEV-protease at room temperature for 2 hours. The digested FhaA was separated from GST-tag by using SEC on an ÄKTA™ Purifier system. The FhaA was eluted at 80 min as a single peak whereas GST-tag was eluted at 88 min (Figure 51D); Figure 51C demonstrates the presence of pure FhaA with MW ~67 kDa obtained from SEC. Mass spectrometry analysis confirmed the identity of the purified FhaA (Figure 51E).
Figure 51. Expression and purification of recombinant *Mtb* FhaA

A. SDS-PAGE shows optimal expression condition for FhaA is 16°C with 0.5 mM IPTG. The FhaA bands were found in the soluble fractions only at 16°C. B. Purified FhaA-GST migrates as 100 kDa protein on SDS-PAGE. C. SDS-PAGE shows the digested FhaA fractions by TEV-protease and purified on SEC. D. SEC analysis displays the single peak of FhaA (80 min) and GST (88 min). E. Mass spectrometry analysis shown the identified peptides of trypsin-digested FhaA (a blue font).
5.3.2.3 Expression and purification recombinant MurJ_{icd}

To evaluate the optimal expression conditions for MurJ_{icd}, *E. coli* C41 (DE3) containing pET15bTEV-*murJ_{icd}* with an N-terminal poly-His tag was grown either at 37°C for 1hr and 3hr or overnight at 16°C (described in methods, section 5.2.2).

**Figure 52A and B** SDS-PAGE and Western Blot show that at 37°C the majority of MurJ_{icd} was expressed as insoluble protein. Thus, the optimal expression conditions for MurJ_{icd} was to grow bacteria overnight at 16°C after induction with 0.1 mM IPTG. MurJ_{icd} was successfully purified from soluble fraction on IMAC and SEC. The protein was purified using affinity chromatography and SEC. **Figure 52D** shows that MurJ_{icd} was eluted at 83 min as a single peak on an SEC column. The collected fractions of MurJ_{icd} were analysed on an SDS-PAGE gel. The expected size of the MurJ_{icd} is 48.7 kDa, it appears that it migrates as 60 kDa protein in SDS-PAGE (**Figure 52B, C**). Poly-His tag was removed from purified MurJ_{icd} by the TEV-protease. The identity of recombinant MurJ_{icd} was further confirmed by mass spectrometry analysis with highlighted blue peptides (**Figure 52E**).
Figure 52. Expression and purification of recombinant *Mtb* MurJ$_{ICD}$

A. The SDS-PAGE analysis of MurJ$_{ICD}$ expression under different conditions. B. Western Blot analysis using anti poly-His antibody shows that MurJ$_{ICD}$ produced as a soluble protein when bacteria were grown overnight at 16°C with 0.1 mM IPTG. C. SDS-PAGE analysis shows MurJ$_{ICD}$ fractions obtained from SEC at 60 kDa. D. SEC shows a monomeric peak of MurJ$_{ICD}$ at 83 minutes. E. Mass spectrometry analysis further confirms the identity of purified MurJ$_{ICD}$ with blue font peptides.
5.3.2.4 Expression and purification recombinant E541-F680 linker of MurJ icd

A poly His-tagged recombinant MurJ\textsubscript{E541-F680} was expressed using the same conditions as described for MurJ\textsubscript{icd}. The protein was purified using affinity chromatography and SEC (described in section 5.2.2). The SDS-PAGE gel shows the purified MurJ\textsubscript{E541-F680} at molecular weight \( \sim 23 \) kDa (Figure 53A). The identity of the purified MurJ\textsubscript{E541-F680} was confirmed by Western Blot using anti-His antibodies (Figure 53B). The empty plasmid (pET) was used as a control.

![Figure 53. Expression and purification of recombinant Mtb MurJ\textsubscript{E541-F680}](image)

\textit{E. coli} C41 (DE3) was induced with 0.1 mM IPTG and grown overnight at 16\(^\circ\)C. SDS-PAGE and Western Blot analysis using anti-poly-His antibody show that MurJ\textsubscript{icd} produced as a soluble protein at \(\sim 23\) kDa. M indicates marker. The negative control includes a PBS buffer.

5.3.2.5 Generation of phosphorylated CwlM

CwlM was purified and phosphorylated by PknB as described in section 3.3.4. Confirmation of phosphorylation was done using anti-CwlM antibodies previously described in chapter 4. Figure 54 shows none of these antibodies could recognise recombinant FhaA, MurJ\textsubscript{icd} and Wag31, confirming their specificity to CwlM. As predicted anti-CwlM antibody recognised both phosphorylated and non-phosphorylated CwlM (Figure 54D), while Anti-T382-P antibody could only detect phosphorylated CwlM (Figure 54B), and anti-T382 antibody could
recognise non-phosphorylated CwlM (Figure 54C). Phosphorylated and non-phosphorylated CwlM forms were used in Co-immunoprecipitation experiments.

Figure 54. Validation of CwlM phosphorylation using specific Anti-CwlM antibody.

A. SDS-PAGE analysis shows recombinant M. tuberculosis proteins, CwlM, CwlMphos, FhaA, MurJCD and Wag31. B. Western Blots analysis using anti-T382-P antibody detected the phosphorylated CwlM (C) anti-T382 antibody recognised non-phosphorylated CwlM only (D) Phosphorylated and non-phosphorylated CwlM were detected by the anti-CwlM antibody. Protein partners of CwlM were not detected in all experiments by any of three antibodies (anti-T382-P, anti-T382 and anti-CwlM).

5.3.3 Co-purification of candidate proteins and CwlM forms using Anti-CwlM-IgG-sepharose

After verifying the identity of recombinant proteins, CwlM forms, Wag31, FhaA, and MurJ, co-immunoprecipitation assays were used for confirmatory interacting of CwlM forms with their partners. To investigate protein-protein interaction equal amount of each recombinant proteins was mixed with either phosphorylated or non-phosphorylated CwlM and incubated 30 minutes at room temperature. The proteins mixture was loaded onto Anti-CwlM-IgG sepharose (section 5.2.5). The control represented a candidate protein incubated with Anti-CwlM-IgG sepharose only. Co-purification of CwlM and its partners was analysed by SDS-PAGE.

5.3.3.1 Phosphorylated and non-phosphorylated CwlM do not bind Wag31

As shown in Figure 55, Wag31 did not bind to either phosphorylated or non-phosphorylated CwlM. This result confirmed our results in Table 3 using protein fragment complementation (M-PFC) assays. Densitometry analysis from three independent gels was done by ImageJ.
software. The results show that 96±6% of Wag31 did not bind to non-phosphorylated CwlM as well as 95±4% of Wag31 did not interact with phosphorylated CwlM (Figure 55).

**Figure 55.** Binding assays for Wag31 and CwlM forms using a CwlM-IgG-Sepharose. The purified Wag31 was equally mixed with either (A) Phosphorylated CwlM or with (B) non-phosphorylated CwlM and incubated with gentle rotation for 30 min. The proteins mixture was loaded onto a CwlM-IgG-Seph and further incubated for 30 min. SDS-PAGE analyses indicate that Wag31 do not bind to CwlM forms. Controls represent Wag31 with or without a CwlM-IgG-Seph. M-protein markers; “+” - protein added; “−” - unbound fractions. Densitometry analysis of protein bands was performed using ImageJ version 1.51 software. Data from three independent experiments were analysed by using Microsoft Excel 2013 software. Protein abundance bound to anti-CwlM IgG Sepharose was expressed as a percentage of total amount used for immunoprecipitation assays, which corresponds to lane 5 on each gel.

5.3.3.2 Phosphorylated CwlM mainly binds to FhaA

Co-immunoprecipitation assays show that FhaA mainly binds to phosphorylated CwlM and shows a weaker binding to the non-phosphorylated form (Figure 56). ImageJ software (1.51) was used for densitometry analysis of gels from three independent experiments. The data confirmed that 84±4% of FhaA binds to phosphorylated CwlM while only 19.7±9% bound to non-phosphorylated CwlM (Figure 56).
Figure 56. Binding assays for FhaA and CwlM forms using a CwlM-IgG-Sepharose
The purified FhaA was mixed with either (A) Phosphorylated CwlM or with (B) non-phosphorylated CwlM and incubated for 30 min. The proteins mixture was loaded onto aCwlM-IgG-Seph and further incubated for 30 min. SDS-PAGE analyses indicate that FhaA interacts with phosphorylated CwlM. In control experiments, FhaA was incubated without CwlM. M-protein markers; “+” – reagent added; “-” unbound fractions. Densitometry analysis of protein bands was performed using ImageJ version 1.51 software. Data from three independent experiments were analysed by using Microsoft Excel 2013 software. Protein abundance bound to anti-CwlM IgG Sepharose was expressed as a percentage of total amount used for immunoprecipitation assays, which corresponds to lane 5 on each gel.

5.3.3.3 Non-phosphorylated CwlM interacts with MurJ_{ecd}

As can be observed in Figure 57B, SDS-PAGE shows that the recombinant MurJ_{ecd} mainly co-precipitated with non-phosphorylated CwlM. Densitometric analysis from three independent gels was done by ImageJ software. The results show that 85±6% of MurJ_{ecd} was bound to non-phosphorylated CwlM, and only 12±6% of phosphorylated version interacted with CwlM (Figure 57).
Figure 57. Binding assays of MurJ<sub>icd</sub> and CwlM forms using aCwlM-IgG-Seph

The purified MurJ<sub>icd</sub> was equally mixed with either (A) phosphorylated or with (B) non-phosphorylated CwlM and incubated for 30 min. The protein mixture was loaded onto aCwlM-IgG-Seph and further incubated for 30 min. SDS-PAGE analyses indicate that MurJ<sub>ICD</sub> binds to non-phosphorylated CwlM. the control represents MurJ<sub>icd</sub> with CwlM-IgG sepharose only. M-protein markers; “+” - proteins added; “-” – unbound fractions. Densitometry analysis of protein bands was performed using ImageJ version 1.51 software. Data from three independent experiments were analysed by using Microsoft Excel 2013 software. Protein abundance bound to anti-CwlM IgG Sepharose was expressed as a percentage of total amount used for immunoprecipitation assays, which corresponds to lane 5 on each gel.

5.3.3.4 Non-phosphorylated CwlM interacts with an E541-F680 linker of MurJ<sub>icd</sub>

Further, we assumed that the binding of CwlM with MurJ linker is crucial for mycobacterial growth. The study of Gee <em>et al.</em>, (2012) demonstrated that not all MurJ domains are essential for <em>Mtb</em> growth. Although they generated viable deletion mutants by truncating MurJ at phenylalanine 715, shorter truncated MurJ forms did not sustain mycobacterial viability. The authors show the domain E541-F680 of MurJ<sub>ICD</sub> that links the 14th transmembrane helix with the pseudokinase domain could be essential for mycobacterial growth. Also, they revealed that the D681-R963 region of non-essential pseudokinase domain might have a regulatory function by recruiting FHA domain of the FhaA protein (Gee <em>et al.</em> 2012). The role of the E541-F680 region was not clear, and thus we investigated whether this region able to interact with CwlM.
Recombinant MurJ_{E541-F680} was expressed and purified and used for the binding assays as described for MurJ_{ICD}. **Figure 58** shows that MurJ linker binds CwlM. Densitometry analysis from three independent gels was performed by ImageJ software. The results show that 87±3\% of MurJ_{ICD} was bound to non-phosphorylated CwlM (**Figure 58**).

**Figure 58.** CwlM interacts with an E541-F680 linker of MurJ_{ICD}.

Phosphorylated and non-phosphorylated recombinant CwlM were mixed with MurJ_{E541-F680} and incubated for 30 min. The protein mixture was loaded onto a CwlM-IgG-Seph and further incubated for 30 min. Sepharose bound and unbound samples were analysed on SDS-PAGE. M-protein markers; “+” – protein added; “-” - unbound fractions. Densitometry analysis of protein bands was performed using ImageJ version 1.51 software. Data from three independent experiments were analysed by using Microsoft Excel 2013 software. Protein abundance bound to anti-CwlM IgG Sepharose was expressed as a percentage of total amount used for immunoprecipitation assays, which corresponds to lane 5 on each gel.
5.3.4 Application of size exclusion chromatography (SEC) for confirmation of FhaA and CwlM interactions

The direct interaction between DDD-CwlM (phosphomimetic) and FhaA was also investigated using SEC to provide a successful crystallisation. It was difficult to phosphorylate a high concentration of CwlM (3-5 mg/ml) by PknB as the protein was precipitated at this level. Therefore, a phosphomimetic form of CwlM was used to mimic the phosphorylation state of a protein. The recombinant FhaA without GST-tag was purified and loaded onto a 16/600 Superdex 200 pg column using ÄKTA™ Purifier system (Figure 59A). The collected fractions of recombinant FhaA were concentrated to 5 mg/ml at 4000xg using a cut off filter (10,000Da). The phosphomimetic form of CwlM-DDD was used for direct interaction with FhaA due to its stability and reliability during protein purification. The recombinant CwlM-DDD was purified and loaded onto a 16/600 Superdex 200 pg column using ÄKTA™ Purifier system (Figure 59B). Fractions with the highest concentration of CwlM-DDD were collected and also concentrated to 5 mg/ml at 4000xg using a cut off filter (10,000Da). To achieve protein-protein interaction, Equal amounts of CwlM-DDD and FhaA were mixed and incubated at room temperature for two hours. The proteins mixture was loaded onto a 16/600 Superdex 200 pg column using ÄKTA™ Purifier system to indicate a proteins interaction (Figure 59C). The peak of eluted proteins at 76 min suggests that CwlM-DDD interacted with FhaA. Fractions with CwlM-DDD were collected and analysed by SDS-PAGE (Figure 59C). However, the peak of 80 min indicated that proteins FhaA-DDD are not entirely interacted.
5.3.4 Crystallisation trials

5.3.4.1 Recombinant CwlM full-length (wild type)

To this date, the structure of CwlM has not been solved. To achieve this aim, 0.1µl of concentrated CwlM (6 mg/ml) was mixed with 0.1µl reservoir solution (commercial screens) using mosquito® Crystal machine (Section 5.2.4). The sealed plates were stored at room temperature and 4°C. Despite four attempts were made to crystallise CwlM using different screens, we were unable to obtain crystals of the protein. The microscopic analysis of drops (CwlM: buffer) within sealed plates shows rapid precipitation and degradation of recombinant CwlM (Figure 60).

Although CwlM was purified with a high concentration and purity that required for successful crystallization, CwlM is an unstable protein (section 3.3.5). To overcome this issue, Muramyl dipeptide (MDP) is a synthetic peptide was used as a substrate and mixed with concentrated CwlM to stabilise and facilitate the protein crystallisation (Prigozhin et al. 2013). Unfortunately, MDP also failed to stabilise CwlM and therefore no protein crystals were obtained.
Figure 60. Crystallisation trials for solving the recombinant CwlM structure.

A. SEC analysis shows a monomeric peak of the eluted CwlM at 84 minutes. B. SDS-PAGE indicates the collected fractions of purified CwlM at molecular weight 47 kDa. The protein was concentrated to 6 mg/ml at 4000xg using a cut off filter (10,000Da). C. Different screens were used for initial crystallisation trials (1:1 ratio of protein: buffer). Microscopic analysis shows the recombinant CwlM was precipitated or degraded and failed to grow as protein crystals.

5.3.4.2 Phosphomimetic CwlM (DDD)

Other efforts were made to solve the CwlM structure using phosphomimetic (CwlM-DDD). Protein stability studies of CwlM showed that phosphorylation by PknB plays a key role in CwlM stability and preventing its degradation (section 3.3.5). Therefore, the phosphorylated sites of CwlM (T43, T382 and T386) were substituted with the amino acid Aspartate (D) to mimic a phosphorylated protein. This version (DDD-CwlM) showed to be more stable and reliable than CwlM (wild type) (section 3.3.5). Thus, the recombinant CwlM-DDD was purified using affinity chromatography and SEC (section 5.2.2). The purity of CwlM-DDD was demonstrated by 12% SDS-PAGE, which showed single bands (without aggregation or
degradation) after staining with Coomassie blue (Figure 61A, B). The purified CwlM-DDD was concentrated to 8 mg/ml and mixed with the reservoir buffer of different screens (protein: buffer) as described in (section 5.2.4). The attempts of five independent experiments were also unsuccessful, and no crystals of CwlM-DDD were obtained (Figure 61C).

![Figure 61. Crystallisation trials for solving the recombinant CwlM-DDD structure.](image)

A. SEC analysis shows a monomeric peak of the eluted CwlM-DDD at 84 minutes. B. SDS-PAGE indicates the collected fractions of purified CwlM-DDD at molecular weight 47 kDa. The protein was concentrated to 8 mg/ml at 4000xg using a cut off filter (10,000Da). C. Different screens were used for initial crystallisation trials (1:1 ratio of protein: buffer). Microscopic analysis shows the recombinant CwlM-DDD was precipitated or degraded and failed to grow as protein crystals.

Although the recombinant CwlM was purified as a soluble protein and its well-folded structure was indicated by Circular Dichroism (section 3.3.3), it was important to remove the His-tag from purified CwlM-DDD as an attempt to crystallise the protein in its native form. The concentrated CwlM-DDD was incubated with TEV-protease at room temperature for two hours to cleave the His-tag. The digested protein was loaded onto a Ni-NTA affinity chromatography column and SEC to remove the His-tag and TEV-proteinase (Figure 62). SDS-PAGE analysis shows CwlM-DDD with some degraded bands suggesting that TEV-protease affected the stability of CwlM-DDD (Figure 62B).
Fractions with the highest concentration of CwlM-DDD were collected from SEC and concentrated to 5mg/ml using a 10,000Da cut off filter (Merck Millipore). Crystallisation trials for CwlM-DDD without His-tag was achieved by mixing 0.1µl of the concentrated protein with 0.1µl of reservoir solution (commercial screens) using mosquito® Crystal machine (section 5.2.4). After incubation at room temperature or at 4°C for two weeks, plates with drops (1:1 ratio of protein-buffer) were examined under a microscope for growing protein crystals. Unfortunately, removing the His-tag from the recombinant CwlM-DDD also did not help to gain crystals of the recombinant protein (Figure 62C).

Figure 62. Trials for solving the recombinant CwlM-DDD structure after removing the His-tag. A. SEC analysis shows a monomeric peak of the eluted CwlM-DDD without His-tag at 80 minutes. B. SDS-PAGE indicates the collected fractions of digested CwlM-DDD at molecular weight ~45 kDa. The protein was concentrated to 5 mg/ml at 4000xg using a cut off filter (10,000Da). C. Different screens were used for initial crystallisation trials (1:1 ratio of protein: buffer). Microscopic analysis shows the recombinant CwlM-DDD was precipitated or degraded and failed to grow as protein crystals.

5.3.5 Crystallisation trials of complexes (FhaA-DDD; MurJ-CwlM)

It is well known that protein-protein interaction enhances the stability of proteins (Macdonald et al. 2015; Deller et al. 2016). Therefore, it would be important to exploit CwlM interaction with its partners for successful crystallisation. The CwlM protein partners (FhaA and MurJ) were purified using affinity chromatography and SEC as described in (section 5.2.2). The GST-tag of recombinant FhaA was cleaved by TEV-protease and removed using a Glutathione Sepharose 4B column (section 5.3.3.1).
For crystallisation experiments, different screens were prepared and used to crystallise a protein-complex of DDD-FhaA. This was achieved by mixing 0.1µl of reservoir solutions with 0.1µl of proteins-complex using mosquito® Crystal machine. The plates were sealed and incubated at room temperature or at 4°C for two weeks (Section 5.2.5).

Again, the four crystallisation attempts of DDD-FhaA were unsuccessful. However, the binding of phosphomimetic form with FhaA enhanced the solubility and stability of CwlM. The microscopic analysis of drops (proteins complex: buffer) within sealed plates shows a mixture of precipitated and soluble proteins due to a weaker binding between CwlM-DDD and FhaA (Figure 63). Our colleagues also showed the weaker binding between the phosphomimetic tail of CwlM and FhaA (synthetic peptides) by using two-dimension NMR spectroscopy (Turapov et al. 2018).

Co-crystallisation of CwlM and MurJ_{ICD} complex was also attempted. Recombinant proteins CwlM and MurJ_{icd} were purified using affinity chromatography and SEC as described in section 5.2.2. Co-crystallisation experiments of CwlM-MurJ_{icd} were performed by mixing 0.1µl of reservoir solutions with 0.1µl of proteins-complex using mosquito® Crystal machine. The plates were sealed and incubated at room temperature or at 4°C for two weeks (Section 5.2.4).

Unfortunately, co-crystallisation attempts of CwlM-MurJ_{icd} were unsuccessful as a higher concentration of proteins complex (CwlM-MurJ_{icd}) will be needed (up to 10 mg/ml). However, the binding of non-phosphorylated CwlM form with MurJ_{icd} enhanced the solubility and...
stability of CwlM. The microscopic analysis of drops (proteins complex: buffer) within sealed plates shows a mixture of solubilised proteins (Figure 64).

![Image of microscopic analysis](image)

**Figure 64.** Crystallisation trails for solving the structure of proteins complex CwlM-MurJ\textsubscript{icd}.

Different screens were used for initial crystallisation trials (1:1 ratio of proteins complex: buffer). Microscopic analysis shows the interaction of CwlM-MurJ\textsubscript{icd} increased the solubility and stability of proteins but failed to grow into protein crystals as a higher concentration of proteins complex will be needed (up to 10 mg/ml).

### 5.4 Discussion

The main aim of this Chapter is to identify putative partners of phosphorylated and non-phosphorylated CwlM by immunoprecipitation approach. Multiple methods can be used to investigate protein-protein interactions that important for studying the cell-signalling and biological functions of proteins (Rao et al. 2014). They include *in vivo* methods such as protein fragment complementation assay (M-PFC). In this application, the interaction of interests (A and B) is performed by fusing each protein independently to complementary fragments and then transformed into *M. smegmatis*. Bacteria could grow on trimethoprim (TRIM) plates when proteins are interacted, whereas proteins that do not interact will not allow transformants to survive on TRIM plates for not activating the mDHFR enzyme and conferring resistance to TRIM (Singh et al. 2006). For confirmation of protein-protein interactions *in vitro*, pull-down assays, Co-immunoprecipitation assays and SEC can be used (Berggard et al. 2007).

Immunoprecipitation (IP) is a popular method that depends on the use of a specific antibody (monoclonal or polyclonal) to bind and isolate a particular protein from cell lysates together with its interacting partner(s) (Phizicky and Fields 1995). To facilitate isolation of protein partners, the antibody can be crosslink to sepharose. In this study, a polyclonal anti-CwlM antibody was crosslinked to cyanogen bromide-activated-sepharose for the use in immunoprecipitation assays. This resin was used for purification of native CwlM and its
interacting partners from *Mtb* lysates and for the confirmation of interactions between recombinant proteins.

In initial experiments, cytoplasmic and membrane fractions of *Mtb* H37Rv were used for immunoprecipitation assays. Fractions from CwlM-depleted mutant were used as a control for non-specific binding. Proteins eluted from CwlM-antibody resin were analysed by mass spectrometry. Proteins retained by CwlM-antibody sepharose from cytoplasm were MurA, FhaA, FtsZ, DnaA, and Wag31, while MurJ, FtsE and CwsA were enriched on the sepharose from the membrane fraction.

Application of mycobacterial protein fragment complementation (M-PFC) assays (Turapov *et al.* 2018) confirmed interactions between CwlM and FhaA, MurJ, and CwsA. The binding between CwlM and MurA was not investigated as the latter had been previously described (Boutte *et al.* 2016). The authors have suggested that phosphorylated CwlM regulates the production of PG precursors in the cytoplasm by activating MurA. However, a possible role of non-phosphorylated CwlM has not been investigated.

In mycobacteria, MurJ has a unique intracellular domain (MurJ_{icd}) which contains a pseudokinase domain (KHD) (Gee *et al.* 2012). MurJ_{icd} can be phosphorylated by PknB on threonine (T947) and this phosphorylated form binds to the Fork Head Associated domain of FhaA. The precise function of this interaction is currently unknown, however, indirect evidence suggest that it may inhibit MurJ flippase activity, which is important for transporting PG subunits across the cytoplasmic membrane to the periplasm (Gee *et al.* 2012). I showed that phosphorylated CwlM also interacts with FhaA, while non-phosphorylated CwlM binds to the essential part of MurJ_{ICD}. Previously proposed CwlM partner Wag31, however did not interact with phosphorylated or non-phosphorylated CwlM (*Figure 65*).
Figure 65. Schematic of immunoprecipitation assay using a CwlM-IgG-Sepharose.
Recombinant Mtb proteins, FhaA, Wag31 and MurJ were mixed either with phosphorylated CwlM or non-phosphorylated CwlM. CwlM forms bound to a CwlM-IgG-Sepharose. Proteins interacting with CwlM forms retained in the column whereas unbound proteins were washed away. Phosphorylated CwlM interacts with FhaA whereas non-phosphorylated CwlM binds with MurJ. Wag31 did not bind with both CwlM forms.

Interaction of FhaA and CwlM was further confirmed by application of NMR spectroscopy to assess binding between FHA domain and synthetic peptides that represented the C-terminal tail of CwlM T382 and T386 (Turapov et al. 2018). The strongest interaction was detected between FHA and a peptide containing phospho-T382 and phospho-T386. A weaker binding was detected between FhaA and phosphomimetic peptide, whereas non-phosphorylated and phosphoablative peptides did not bind FhaA (Turapov et al. 2018).

A previous study (Gee et al. 2012) has shown that a small linker (E541-F680) between the transmembrane domain and KHD was essential for Mtb viability; attempts to produce a MurJ linker-lacking mutant in our laboratory were also unsuccessful (Turapov et al., 2018). My results confirm that non-phosphorylated CwlM interacts with this essential linker and this interaction may potentially regulated (or activate) MurJ, since CwlM-depleted Mtb did not incorporate BODIPY™ FL vancomycin and had impaired PG biosynthesis (Turapov et al., 2018).
Understanding protein-protein interaction is essential for studying cell-signalling, cellular machinery pathways and biological functions of proteins (De Las Rivas and Fontanillo 2010; Rao et al. 2014). Therefore, we could predict and understand the function of CwlM by interacting with its partners. Here, some important examples of the complex proteins.

Wag31 (a homologue of DivIVA family in Gram-positive bacteria) is a structural protein with localisation at the pole that responsible for synthesising peptidoglycan, cell wall integrity and maintaining cell shape of mycobacteria (Kang et al. 2008). Wag31 plays an essential role in mycobacterial growth by interacting with other proteins during the machinery pathways of cell wall biosynthesis. It has been reported that Wag31 interacts with CwsA, a synthase enzyme which is important for PG biosynthesis. This binding could recruit and stabilise Wag31 at the pole (Plocinski et al. 2012). Likewise, the interaction of CwsA with CrgA, a cell division protein plays a key role in mycobacterial peptidoglycan synthesis (Plocinski et al. 2012). Wag31 can be phosphorylated by PknB and PknA (Kang et al. 2008). Wag31 phosphorylation was apparently essential for polar localisation of Wag31 and PG biosynthesis, however, the precise mechanism of Wag31-mediated control of PG biosynthesis is unknown (Jani et al. 2010). Wag31 can also interact with PbpB (PBP3, FtsI) and protect PbpB from oxidative stress-induced cleavage (Mukherjee et al. 2009).

FhaA is a PknB-phosphorylated protein that has a C-terminal Fork Head Associated (FHA) domain (Roumestand et al. 2011). Phosphorylation by PknB recruits FhaA to the cell pole and septal division sites suggesting that FhaA plays an important role in regulating PG biosynthesis and maintaining mycobacterial cell wall integrity (Gee et al. 2012). The C-terminal FHA domain found in prokaryotic and eukaryotic organisms that was shown to interact with phosphorylated proteins by recognising phosphothreonine (Mahajan et al. 2008). In human, FHA domain of different proteins regulates cellular signalling pathways by monitoring the phosphothreonine of target proteins involved in the cell DNA repair (Liang and Van Doren 2008). In plants, proteins contained FHA domain recruits to the plasma membrane and regulate the signalling pathways of receptor-like protein kinase (Li et al. 2000). In bacteria, FHA domain proteins play different roles in bacterial cell wall regulation, sporulation, maintain bacterial shape, secretion, and signal transduction pathways (Pallen et al. 2002). In mycobacteria, FhaA interacts with penicillin binding protein A (PbpA), a PG-synthase, and improves its stability (Viswanathan et al. 2017).
Bacterial growth requires cell elongation and division. For both processes PG precursors also known as lipid II subunits are needed for synthesis of new cell wall (Kieser and Rubin 2014). MurJ (MviN) is a transmembrane protein with proposed flippase activity that translocates PG-precursor (Lipid II) across the membrane for incorporation into expanding the peptidoglycan layer in *E. coli* (Ruiz, 2015). A recent study has been shown that MurJ interacts with DivIB–DivIC–FtsL complex in *Staphylococcus aureus* and this interaction is important for the localization of these PG biosynthesis proteins in the septum (Monteiro *et al.* 2018).

The current study revealed novel interaction between CwlM and MurJ, however further studies required to elucidate its biological importance and function. The activation of MurJ by CwlM poses a significant technical challenge to demonstrate directly, because the possible flippase activity of MurJ has not been detected *in vitro*. CwlM forms might have other important functions that contribute to control different intracellular signalling pathways either by binding to CwsA, or they could have other enzymatic activity. Consequently, future studies will be important to highlight the precise role of CwlM, which is essential for cellular mechanisms during mycobacterial growth.

Solving the protein structure of CwlM or its complex with MurJ would make important breakthrough in understanding of CwlM function in mycobacteria. There are many techniques could be used to solve the structure of proteins such as X-ray crystallography, NMR spectroscopy and cryo-Electron Microscopy (Breda *et al.* 2007; Monroe *et al.* 2017). X-ray crystallography is a popular and powerful method as can provide a high resolution of the protein structure (McPherson and Gavira 2013).

Crystallisation trails of the recombinant CwlM were unsuccessful even with the use of large amounts of protein and different buffers. CwlM is extremely unstable protein which cannot withstand the long-period incubation either at room temperature or 4°C during the process of protein crystallisation.

The phosphorylation by PknB was shown positively impact on the stability of CwlM as described in Chapter 3. Therefore, the phosphomimetic form (DDD) was used for crystallisation trials by substituting the three phosphorylation threonines of CwlM with the amino acid aspartate to mimic a phosphorylation protein. Although the recombinant CwlM-DDD was purified as soluble and pure protein, the four attempts with using high concentration of protein and different screens were failed to obtain CwlM-DDD crystals. It is important to note that producing pure protein as a single band with the highest concentration is sometimes
not enough to gain crystals due to the compositional stability of the protein (Deller et al. 2016). Removing the N-terminal poly-His tag from CwlM-DDD as an attempt to crystallise the protein in its native form was also unsuccessful.

Understanding protein stability is an essential step for successful expression, purification, and structural studies of proteins (Deller et al. 2016; Ishchenko et al. 2017). Since protein crystallization is a long time process (takes 3-14 days), the compositional stability of protein should be maintained within screen conditions (Smialowski and Wong 2016). It is generally agreed that protein-protein interaction could increase the stability of proteins (Lu et al. 2013; Macdonald et al. 2015; Deller et al. 2016). Therefore, the co-crystallisation trials of CwlM with its partners were exploited to solve the protein structure. Despite the four crystallisation attempts of DDD-FhaA were unsuccessful, the binding of phosphomimetic form with FhaA enhanced the stability of CwlM and prevented the rapid degradation of the protein. The microscopic analysis of drops (proteins complex: buffer) within sealed plates shows a mixture of precipitated and soluble proteins due to a weaker binding between CwlM-DDD and FhaA which was shown previously by (Turapov et al. 2018) using NMR spectroscopy.

Co-crystallisation of CwlM and MurJ was promising because addition of MurJicd apparently increased stability and solubility of CwlM. Unfortunately, co-crystallisation attempts of CwlM-MurJicd were unsuccessful as a higher concentration of proteins complex (above 10 mg/ml) will be necessary for generation of CwlM-MurJ crystals. This concentration was required for crystallisation MurJicd-FhaA complex (Gee et al. 2012).

Successful crystallization requires highly concentrated and pure protein. Physical, chemical and biochemical conditions such as temperature, pH, precipitant type, precipitant concentration, ionic strength, the degree of supersaturation, aggregation state of the macromolecule, isoelectric point, the stability of the macromolecule also affect crystallisation (McPherson 2004). Therefore, finding conditions for protein crystallisation remains a challenging experiential task that requires multiple trials and modifications (Acharya and Lloyd 2005).

To this date, the structure of the recombinant Mtb CwlM protein has not been successfully solved by X-ray crystallography. Other efforts will be needed to optimise and solve the CwlM structure using a high concentration of proteins complex (CwlM-MurJ). Furthermore, the truncated forms of AmiR1 (amidase domain only) and CwlM AmiR2-DD (amidase domain
with phosphomimetic tail) were stable proteins and therefore they could be used for protein crystallography studies.

5.5 Conclusion and Future Work

In this chapter, the protein partners of CwlM forms in cytoplasm and membrane fractions of *Mtb* were identified by immunoprecipitation assays using CwlM-IgG-sepharose and mass spectrometry analyses. Application of mycobacterial protein fragment complementation (M-PFC) confirmed FhaA, MurJ, and CwsA as proteins interacting with CwlM.

*Mtb* recombinant proteins, CwlM, PknB, MurJ, Wag31 and FhaA were generated and successfully expressed and purified from *E. coli* strains using affinity chromatography and SEC applications.

This research has addressed the hypothesis that CwlM might interact with FhaA and an essential domain of MurJICD, which are playing a key role in peptidoglycan biosynthesis of mycobacteria.

PknB-phosphorylated CwlM was demonstrated to bind with FhaA, a fork-head-associated domain protein whereas non-phosphorylated CwlM was shown to interact with an essential domain of MurJ, a proposed Lipid II flippase. However, Wag31, an integral membrane protein was not co-precipitated with either phosphorylated or non-phosphorylated CwlM proteins.

Consequently, our results suggested a novel model on the proposed CwlM activity that may regulate the biosynthesis of peptidoglycan precursors in the cytoplasm and their transport across the cytoplasmic membrane for incorporation into the growing peptidoglycan layer.

To this date, the structure of CwlM has not been solved. It is well known that protein-protein interaction enhances the stability of proteins (Macdonald *et al.* 2015). Therefore, it would be important to exploit CwlM interaction with its partners for successful crystallisation and solving the structure of CwlM in future studies by providing a higher concentration.
Chapter 6

6. Final conclusion and Future work
6.1 Final conclusion

Tuberculosis remains a serious disease in worldwide for the success of its etiological agent, *M. tuberculosis* in human. *M. tuberculosis* has a distinct cell wall enables to resist antibiotics. It is composed of rigid and impermeable multilayers including myco-membranes and the mycolyl-arabinogalactan-peptidoglycan (mAGP) complex. Cell wall biosynthesis and remodelling is essential for *Mtb* viability within the host. Many antimicrobial agents that are currently used for TB treatment target essential enzymes involved in cell wall biosynthesis. However, the excessive use of antibiotics and the long treatment period for TB have led to an increase in the number of drug-resistant bacteria. Therefore, there is a need to discover or develop new drugs that should be more effective and low cost. Enzymes involved in PG biosynthesis remain excellent targets for design of non-toxic antibiotics, and there are many essential enzymes inhibition of which by novel antimicrobial would kill mycobacteria. For example, CwlM is essential for *Mtb* growth (Boutte *et al.* 2016; Turapov *et al.* 2018). It is annotated as N-acetylmuramyl-L-alanine amidase which is responsible for eliminating the entire stem peptide from glycan strands by cleaving the amide bond between lactyl group of the N-acetylmuramic acid moiety and the L-Alanine, the first amino acid in the stem peptide. CwlM is a major PknB substrate and phosphorylation of T382 is critical for *Mtb* viability (Turapov *et al.* 2018). It has been recently shown that phosphorylated CwlM activates MurA, a primary enzyme of PG precursor’s generation in the cytoplasm (Boutte *et al.* 2016). However, this interaction requires further biochemical and functional characterisation.

In this study the following objectives were addressed by application of multidisciplinary approaches: **Firstly**, the effect of PknB–mediated phosphorylation on CwlM stability and activity. This was analysed by expressing and purifying sufficient amounts of recombinant *Mtb* proteins from *E. coli* using affinity chromatography and SEC. Folding of recombinant CwlM was proved using CD; purified recombinant CwlM was used for investigation of enzymatic activity and generation of polyclonal anti-CwlM antibodies. This study demonstrated that CwlM is unstable protein and its degradation is stimulated by imidazole. However, phosphorylation by PknB or phosphomimetic mutations improved CwlM stability and prevented its degradation.

For investigation of PG- cleaving activity of CwlM, zymogram and FITC labelled PG assays were used. However, full-length CwlM showed no activity regardless its phosphorylation state. A shorter form of CwlM was detected in the *E. coli* lysate from CwlM-overexpressing strains
which was active in zymogram. Subsequently, AmiR1 corresponding to the amidase domain was produced and showed PG-cleaving activity in zymogram and PG-FITC assays. Importantly, D339A mutation completely abolished this activity. Further studies are required to understand the mechanism and specificity of this activity.

**Secondly**, this thesis described the generation of anti-CwlM antibodies for investigation CwlM localisation in mycobacteria fractions (cytoplasm, membrane, cell wall). Purified anti-CwlM antibody showed a very high sensitivity to CwlM. Also, this antibody recognized CwlM variants in *M. smegmatis* and *Mtb*.

The work described in this study provides a novel insight to the localisation of CwlM in mycobacterial compartments. The phosphorylated CwlM is mainly found in the cytoplasm, while the non-phosphorylated CwlM is associated with the membrane. These two forms could only be detected in growing mycobacteria. Thus, they could play an essential role in mycobacterial growth. PknB could regulate the distribution of CwlM via phosphorylation of T382. However, CwlM was not detected in both cell wall and culture filtrate preparations.

**Thirdly**, this study identified the protein partners of phosphorylated and non-phosphorylated CwlM by immunoprecipitation assays using CwlM-IgG-sepharose. These interactions were investigated by application of mycobacterial protein fragment complementation (M-PFC); FhaA, MurJ, and CwsA were confirmed as proteins interacting with CwlM.

Recombinant CwlM, PknB, MurJ, Wag31 and FhaA were generated and successfully expressed and purified from *E. coli* strains using affinity chromatography and SEC applications. The recombinant CwlM was phosphorylated by PknB *in vitro*. The direct interaction between recombinant CwlM and its partners was analysed by immunoprecipitation assay. This research has addressed the hypothesis that CwlM might interact with FhaA and an essential domain of MurJICD, which play key roles in peptidoglycan biosynthesis.

PknB-phosphorylated CwlM was demonstrated to bind with FhaA, a fork-head-associated domain protein whereas non-phosphorylated CwlM was shown to interact with an essential domain of MurJ, a proposed Lipid II flippase. However, Wag31, a membrane protein was not co-precipitated with either phosphorylated or non-phosphorylated CwlM proteins.

This study proposed a novel model for CwlM-mediated regulation of PG biosynthesis in mycobacteria (**Figure 66**). The interaction between non-phosphorylated CwlM and MurJ could be important for regulating MurJ flippase activity that is required for transporting PG
precursors across the cytoplasmic membrane to the periplasm and stimulating the PG polymerisation. On the other hand, phosphorylated CwlM binds with FhaA and MurA previously described by (Boutte et al. 2016), and this interaction is important for controlling PG precursor’s production in the cytoplasm. The balance work between phosphorylated and non-phosphorylated CwlM proteins is maintained by FhaA, which acts as a regulatory hub to control the interactions of CwlM forms with its partners, MurJ and MurA.

Figure 66. The proposed model of CwlM functions in the peptidoglycan biosynthesis in Mtb
This Figure adapted from (Turapov et al., 2018). A. Non-phosphorylated CwlM binds to MurJ at the essential linker domain, which in turn facilitates or stimulates MurJ flippase activity to transport peptidoglycan precursors across membrane to existing PG. This activity therefore might also stimulate PG polymerisation. B. Here, the PASTA domain of PknB plays role in senescing un-crosslinked peptidoglycan and triggering the auto-phosphorylation of PknB to stimulate its activity. Then, CwlM and MurJ could be phosphorylated by PknB to interact collectively with FhaA. PknB-Phosphorylated CwlM also binds MurA but not included in this model for clarity. The fine- balance between phosphorylated and non-phosphorylated CwlM proteins is maintained by FhaA, which acts as a regulatory hub to regulate CwlM potential interactions with its partners, MurJ and MurA during mycobacterial growth. “P” – refers to the state of PknB phosphorylation, CwlM and MurJ.

Finally, crystallisation trials for solving CwlM structure were described in this thesis. Sufficient amounts of recombinant CwlM were purified and used for crystallisation experiments. Different commercial buffers were tested for crystallisation of CwlM. However, all attempts were unsuccessful and failed to obtain a protein crystal. Addition of Muramyl
dipeptide (MDP) or tri-NAG did not facilitate crystallisation. Other efforts were made to solve the CwlM structure using phosphomimetic (CwlM-DDD). The attempts of five independent experiments were also unsuccessful, and no crystals of CwlM-DDD were obtained. Removing poly His-tag from recombinant CwlM-DDD by TEV-protease did not enhance the protein crystallisation of CwlM. Crystallisation trials of CwlM depending on protein-protein interaction approaches were also unsuccessful. However, the binding of non-phosphorylated CwlM with MurJ enhanced the solubility and stability of CwlM but failed to grow into protein crystals as a higher concentration of proteins complex will be needed (above 10 mg/ml).

To this date the structure of CwlM has not been solved. However, this study indicated the secondary structure of CwlM using Circular dichroism (CD) and Jpred 4 server, which are shown that CwlM is mainly α-helical, with some β-sheet.

### 6.2 Future work

As previously discussed, phosphorylated and non-phosphorylated CwlM had no muralytic activity except the short form (Amidase domain only). However, the precise mechanism that regulates this activity is still unknown. Determining the reason behind the PG-hydrolysing activity of CwlM would elucidate the multi-roles of this protein during growth and pathogenesis of *Mtb*. Characterization the products of CwlM hydrolysing activity by digestion bacterial peptidoglycan and determine the cleaved muropeptides fragments by using HPLC analysis. Furthermore, investigation of the hydrolysing activity of the mutated catalytic residues of CwlM and their possible effect on mycobacterial growth are challenges for the future studies.

Future work could investigate other protein partners of CwlM like CwsA, a membrane protein involved in cell division and confirm its interaction with CwlM using immunoprecipitation assay. This will provide a new insight into different functions of CwlM during PG synthesis and remodelling in mycobacteria and it would be seen whether CwsA is regulated in a similar way to FhaA, MurJ and MurA.

In order to achieve a successful protein crystallisation for CwlM, large amounts of soluble protein would be required. One possible way to increases the concentration and solubility of CwlM is to try an alternative tag, such as glutathione-S-transferase (GST), or Maltose-binding tag (MBP), or to use an alternative expression host like Rosetta™ or Origami™ B that could provide a more level of protein expression (Long *et al.* 2015; Kosobokova *et al.* 2016).
Additionally, future work to generate a construct co-overexpressing CwlM and MurJ or FhaA to improve the solubility of the recombinant protein could be investigated (Ikura et al. 2002). As a cytoplasmic CwlM is phosphorylated and potentially can be stable, CwlM overexpression in *M. smegmatis* can be also used to purify a large scale of native protein for crystallization of CwlM in future studies. Solving the structure of CwlM will allow to determine the catalytic and binding sites which in turn could find an active inhibitor able to inactivate the essential CwlM and kill *Mtb*. 
Appendix
Protein sequences

CwlM wild type with phosphorylation sites

MPSPRPREDGDALRCGDRSAAVTEIRAALTLGMLDHQEDDTTGRNVALELFDAQL DQAVRAFQQHRGLLVDGIVGEATRYRALKEASYRLGARTLYHQFGAPLYGDDVATL QARLQQLGFYTLVGDHGFQTHNALMSYQREYGLAADGICGEPETRLSFLSSRV SGGSHPAIREEELVRSSPGKLSGKRIIDPGGRGGVDHGGLIAQGPAGPISEADLLWDLAS RLEGRMIAIGMETHLRSRPNRPSDAERAAATANAVGADLMLSRCEQTSLAANGV ASFHFGNSHSVSTIGRNLADFIQREVARTGLRDCRVHGRTWDLRLTRMPTVQV DIGYITNPHDRGLVSTQTRDAIAEGILAAVKRLYLLGKNDRPTGTFTFAELLAHELS VERAGRLGGS

CwlM-DDD (Phosphomimetic)

MPSPRPREDGDALRCGDRSAAVTEIRAALTLGMLDHQEDDLTDGRNVALELFDAQL DQAVRAFQQHRGLLVDGIVGEATRYRALKEASYRLGARTLYHQFGAPLYGDDVATL QARLQQLGFYTLVGDHGFQTHNALMSYQREYGLAADGICGEPETRLSFLSSRV SGGSHPAIREEELVRSSPGKLSGKRIIDPGGRGGVDHGGLIAQGPAGPISEADLLWDLAS RLEGRMIAIGMETHLRSRPNRPSDAERAAATANAVGADLMLSRCEQTSLAANGV ASFHFGNSHSVSTIGRNLADFIQREVARTGLRDCRVHGRTWDLRLTRMPTVQV DIGYITNPHDRGLVSTQTRDAIAEGILAAVKRLYLLGKNDRPTGTFTFAELLAHELS VERAGRLGGS

Predicted catalytic residues: H204A, E217A, D339A (Full-length)

MPSPRPREDGDALRCGDRSAAVTEIRAALTLGMLDHQEDDTTGRNVALELFDAQL DQAVRAFQQHRGLLVDGIVGEATRYRALKEASYRLGARTLYHQFGAPLYGDDVATL QARLQQLGFYTLVGDHGFQTHNALMSYQREYGLAADGICGEPETRLSFLSSRV SGGSHPAIREEELVRSSPGKLSGKRIIDPGGRGGVDHGGLIAQGPAGPISEADLLWDLAS RLEGRMIAIGMETHLRSRPNRPSDAERAAATANAVGADLMLSRCEQTSLAANGV ASFHFGNSHSVSTIGRNLADFIQREVARTGLRDCRVHGRTWDLRLTRMPTVQV DIGYITNPHDRGLVSTQTRDAIAEGILAAVKRLYLLGKNDRPTGTFTFAELLAHELS VERAGRLGGS

Truncated forms of CwlM

AmiR1 (amidase domain only)

SSGPKLSGKRIIDPGGRGGVDHGGLIAQGPAGPISEADLLWDLASRLEGRMAAIGMETHLRSRPNRPSDAERAAATANAVGADLMLSRCEQTSLAANGVASFHFGNSHSVSTI
AmiR2-DD (amidase with tail - phosphomimetic)

Predicted catalytic residues: H204A, E217A, D339A (truncated forms)
References


Anthony, R.M., den Hertog, A.L. and van Soolingen, D. (2018) 'Happy the man, who, studying nature's laws, Thro' known effects can trace the secret cause.' Do we have enough pieces to solve the pyrazinamide puzzle?', J Antimicrob Chemother, 2018/03/13, available: http://dx.doi.org/10.1093/jac/dky060.


Brosch, R., Gordon, S.V., Marmiesse, M., Brodin, P., Buchrieser, C., Eiglmeier, K., Garnier, T., Gutierrez, C., Hewinson, G., Kremer, K., Parsons, L.M., Pym, A.S., Samper, S., van Soolingen, D. and Cole,


Girardin, S.E., Philpott, D.J. and Lemaître, B. (2003) 'Sensing microbes by diverse hosts. Workshop on pattern recognition proteins and receptors', *EMBO reports*, 4(10), 932-936, available: [http://dx.doi.org/10.1038/sj.embor.embor940](http://dx.doi.org/10.1038/sj.embor.embor940).


Lee, T.K. and Huang, K.C. (2013) 'The role of hydrolases in bacterial cell-wall growth', *Curr Opin Microbiol*, 16(6), 760-6, available: [http://dx.doi.org/10.1016/j.mib.2013.08.005](http://dx.doi.org/10.1016/j.mib.2013.08.005).


Raymond, J.B., Mahapatra, S., Crick, D.C. and Pavelka, M.S. (2005) 'Identification of the namH Gene, Encoding the Hydroxylase Responsible for the N-Glycoylation of the Mycobacterial...
Peptidoglycan', *Journal of Biological Chemistry*, 280(1), 326-333, available: [http://dx.doi.org/10.1074/jbc.M411006200](http://dx.doi.org/10.1074/jbc.M411006200).


Senzani, S., Li, D., Bhaskar, A., Ealand, C., Chang, J., Rimal, B., Liu, C., Joon Kim, S., Dhar, N. and Kana, B. (2017) 'An Amidase_3 domain-containing N-acetylmuramyl-L-alanine amidase is required for mycobacterial cell division', *Scientific Reports*, 7(1), 1140, available: [http://dx.doi.org/10.1038/s41598-017-01184-7](http://dx.doi.org/10.1038/s41598-017-01184-7).


Supply, P. and Brosch, R. (2017) 'The Biology and Epidemiology of Mycobacterium canettii', *Adv Exp Med Biol*, 1019, 27-41, available: [http://dx.doi.org/10.1007/978-3-319-64371-7_2](http://dx.doi.org/10.1007/978-3-319-64371-7_2).


Uehara, T., Parzych, K.R., Dinh, T. and Bernhardt, T.G. (2010) 'Daughter cell separation is controlled by cytokinetic ring-activated cell wall hydrolysis', *The EMBO Journal*, 29(8), 1412-1422, available: [http://dx.doi.org/10.1038/emboj.2010.36](http://dx.doi.org/10.1038/emboj.2010.36).


