Dissection of the molecular mechanisms underpinning the threat posed by antibiotic resistance of MRSA

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## Table of contents

1. Introduction .................................................................................................................. 1
   1.1. Antimicrobial resistance ......................................................................................... 1
       1.1.1. The discovery of the first antibiotics ................................................................. 1
       1.1.2. Antibiotics mode of action ................................................................................ 2
           1.1.2.1. DNA replication and repair ........................................................................ 2
           1.1.2.2. Protein translation ....................................................................................... 5
           1.1.2.3. Peptidoglycan biosynthesis ....................................................................... 5
       1.1.3. Mechanisms of antibiotic resistance ................................................................. 6
           1.1.3.1. Antibiotic modification and degradation ....................................................... 7
           1.1.3.2. Increased efflux of antibiotics ..................................................................... 8
           1.1.3.3. Sequestering antibiotics .............................................................................. 8
           1.1.3.4. Modification of antibiotic targets ............................................................... 9
           1.1.3.5. What can be done about antibiotic resistance? .......................................... 10
   1.2. The bacterial cell wall ............................................................................................. 11
       1.2.1. The synthesis of peptidoglycan ....................................................................... 14
           1.2.1.1. The cytoplasmic stage ................................................................................. 14
           1.2.1.2. The lipid-linked stage ................................................................................. 16
           1.2.1.2.1. Variation in the pentapeptide stem ......................................................... 16
           1.2.1.2.2. FtsW or MurJ as the lipid-II flippase? .................................................... 19
           1.2.1.3. The extracellular stage ................................................................................. 21
   1.3. Penicillin-binding proteins ...................................................................................... 23
       1.3.1. Inhibitors of PBPs ............................................................................................. 24


2. Materials and methods

1.4. S. aureus ................................................................. 27
1.4.1. Methicillin-resistant S. aureus ................................... 27
1.4.1.1. The emergence of livestock-associated MRSA ........... 28
1.4.2. Peptidoglycan synthesis in MRSA ............................. 29
1.4.2.1. MRSA and the Fem ligases .................................. 29
1.4.2.2. MRSA and the penicillin binding proteins ................. 31
1.4.3. Is peptidoglycan synthesis a realistic target for antibiotic therapy in MRSA? .... 34

1.5. Aims of thesis ............................................................ 34

2. Materials and methods .................................................. 35

2.1 Buffers and solutions ................................................... 35
2.2 Bacterial strains .......................................................... 35
2.2.1. Escherichia coli strains .............................................. 35
2.2.2. Staphylococcal strains .............................................. 35
2.2.3. Bacterial growth media ............................................ 35
2.2.3.1. Luria-Bertani (LB)-agar plates ................................ 35
2.2.3.2. Growth media .................................................... 36
2.2.4. Preparation of E. coli competent cells ......................... 37
2.2.5. Transformation of E. coli competent cells .................... 38
2.2.6. Preparation of glycerol stocks ................................... 38
2.2.7. Determining bacterial cell count ................................ 38
2.2.8. Bacterial turbidity assays ......................................... 38

2.3. Preparation of Staphylococcal tRNA ................................ 39
2.3.1. Growth of Staphylococcus aureus ATCC 25923 .............. 39
2.3.2. Extraction of tRNA .................................................. 39
2.3.2.1. Initial tRNA extraction ......................................... 39
2.3.2.2. tRNA deacetylation ............................................. 40
2.3.2.3. tRNA purification ............................................... 40
2.3.2.4. Quantification of tRNA ......................................... 41
2.3.2.5. Visualisation of tRNA ........................................... 41
2.3.2.5.1. Urea-Polyacrylamide gel electrophoresis .............. 41
2.3.2.5.2. Staining of Urea-PAGE .................................... 41

2.4. DNA cloning ............................................................. 42
2.4.1. Oligonucleotide primers ............................................ 42
2.4.2. Polymerase chain reaction (PCR) ............................... 42
2.4.3. Agarose gel electrophoresis and gel extraction of DNA .... 42
2.4.4. Gibson cloning reaction using PCR products ......................................................... 44
2.4.5. Plasmid propagation and plasmid purification .......................................................... 44
2.4.6. DNA concentration quantification ............................................................................. 44
2.4.7. DNA sequencing of plasmid constructs ................................................................. 44
2.4.8. Plasmid constructs .................................................................................................. 45
2.5. Protein expression and purification .............................................................................. 47
  2.5.1. Recombinant protein expression in E. coli ............................................................ 47
    2.5.1.1. Small scale recombinant protein expression trials in E. coli ....................... 47
  2.5.2. Recombinant protein expression of FemX, FemA, FemB, GlyRS and coexpressed full length PBP2 and PBP2a .................................................................................. 47
    2.5.2.1. Preparation of cell lysate for FemX, FemA, FemB, GlyRS expression ............. 48
    2.5.2.2. Preparation of cell lysate for coexpressed full length PBP2 and PBP2a .......... 48
  2.5.3. Recombinant protein expression of truncated PBP2-W59M and PBP2a-Y23M ....... 48
    2.5.3.1. Preparation of cell lysate ................................................................................. 49
  2.5.4. Affinity chromatography ......................................................................................... 49
    2.5.4.1. His tag cleavage with TEV protease and reverse affinity chromatography ..... 49
  2.5.5. Size exclusion chromatography ............................................................................. 50
    2.5.5.1. Preparative size exclusion chromatography .................................................. 50
  2.5.6. Ion exchange chromatography ............................................................................. 50
  2.5.7. Buffer exchange and protein concentration ......................................................... 51
  2.5.8. Specific protein purification procedures ............................................................... 51
    2.5.8.1 Fem X, FemA, FemB and GlyRS .................................................................. 51
    2.5.8.2. Coexpressed full length PBP2 and PBP2a .................................................. 52
    2.5.8.3. PBP2-W59M .......................................................................................... 52
    2.5.8.4. PBP2a-Y23M ...................................................................................... 53
2.6. Protein analysis ........................................................................................................... 53
  2.6.1. Protein quantification .............................................................................................. 53
  2.6.2. Protein visualisation by SDS-polyacrylamide gel electrophoresis ....................... 54
  2.6.3. Western blot .......................................................................................................... 54
    2.6.3.1. His-tagged protein antibody binding ........................................................... 55
    2.6.3.2. S-tagged protein antibody binding ............................................................... 55
    2.6.3.3. Western blot visualisation ............................................................................. 56
2.7. Synthesis of lipid-II intermediates .............................................................................. 56
  2.7.1. Synthesis of UDP-MurNAc pentapeptide ............................................................ 56
    2.7.1.1. Purification of UDP-MurNAc pentapeptide ............................................... 56
    2.7.1.2. Quantification of UDP-MurNAc pentapeptide ........................................... 57
    2.7.1.3. Purity of UDP-MurNAc pentapeptide ......................................................... 57
3. Optimising the yields of lipid-II during *in vitro* chemo-enzymatic synthesis ..... 71

3.1. Background ................................................................................. 71

3.2. Results ...................................................................................... 73

3.2.1. Comparison of Micrococcal and Staphylococcal growth media for high cell density ..... 73
3.2.2. Optimisation of bacterial cell lysis ................................................................. 75
3.2.3. Comparison of non-amidated lipid-II (Lys) yield from different bacterial membranes ... 78
  3.2.3.1. Small-scale analysis by TLC................................................................. 78
  3.2.3.2. Larger-scale analysis of lipid-II production by quantitative spectrophotometric assay ................................................................. 80
3.2.4. Comparison of amidated lipid-II (Lys) yield from different bacterial membranes........ 81
  3.2.4.1. Small-scale analysis by TLC................................................................. 81
  3.2.4.2. Larger-scale analysis by quantitative spectrophotometric assay .................... 82
3.3. Conclusions and further work ............................................................................ 83
  3.3.1. Enhancing bacterial cell lysis for membrane extraction ................................. 83
  3.3.2. Maximising yields of lipid-II ....................................................................... 84
  3.3.3. Improving membrane extraction from Staphylococcal spp. ......................... 85
  3.3.4. An alternative approach to synthesising amidated lipid-II .............................. 86

Chapter 4 Synthesis of S. aureus specific lipid-II intermediates ......................... 88

  4.1. Background ...................................................................................................... 88
  4.2. Chapter aims .................................................................................................. 92
  4.3. Results and discussion .................................................................................. 93
    4.3.1. Enzymatic synthesis of UDP-MurNAc pentapeptide (Lys) derivatives ........... 93
      4.3.1.1. Enzymatic synthesis of UDP-MurNAc pentapeptide (Lys) .................. 93
      4.3.1.2. Chemo-enzymatic synthesis of UDP-MurNAc-pentapeptide (Lys) mono-, tri- and penta-glycine ................................................................. 100
    4.4. Synthesis of lipid-II (Lys) species ................................................................ 104
      4.4.1. Enzymatic synthesis of lipid-II (Lys) ....................................................... 104
      4.4.2. Enzymatic synthesis of lipid-II (Lys) mono-, tri- and penta-glycine using branched UDP-MurNAc-pentapeptides ................................................. 109
      4.4.3. Enzymatic synthesis of lipid-II (Lys) mono-, tri- and penta-glycine using the Fem ligases – the ‘one-pot’ reaction ......................................................... 121
      4.4.4. Extraction of amidated lipid-II (Lys) pentaglycine from S. aureus culture .... 126
  4.5. Concluding remarks and further work ............................................................ 135
    4.5.1. Chemical or enzymatic synthesis of branched lipid intermediates? ............. 135
    4.5.2. Can the Walker method be optimised to extract larger yields of amidated lipid-II (Lys) Gly5? ................................................................. 136
Chapter 5 Enzymology of FemX, FemA and FemB; the characterisation of *S. aureus* Fem ligase activity, the formation of the lipid-II pentaglycine bridge. 139

5.1. Background .................................................................................................................................................. 139
5.1.1. Are the Fem ligases a valid therapeutic target? ....................................................................................... 140
5.2. Chapter aims .................................................................................................................................................. 141
5.3. Results and discussion ................................................................................................................................. 142
5.3.1. Expression and purification of *S. aureus* Mu50 FemX, FemA, FemB and GlyRS ................................. 142
5.3.2. Extraction of *S. aureus* ATCC 25923 tRNA .......................................................................................... 142
5.3.3. Investigating the activity of the Fem peptidyl-transferases ................................................................. 144
5.3.3.1. Investigating the ability of the Fem ligases to append the glycine cross-bridge to non-amidated lipid-II (Lys) via TLC and mass spectrometry analysis .................................................. 144
5.3.3.2. Investigating the ability of the Fem ligases to append the glycine cross-bridge to amidated lipid-II (Lys) via TLC ........................................................................................................... 148
5.3.3.3. Investigating the ability of the Fem ligases to append the [3H]-glycine cross-bridge to lipid-II (Lys) via radioactive assay ............................................................................................................. 149
5.3.3.3.1. Can FemX append [3H]-glycine to both amidated and non-amidated lipid-II (Lys)? ......................... 149
5.3.3.3.2. Can FemA append [3H]-glycine to both amidated and non-amidated lipid-II (Lys) Gly? .................. 153
5.3.3.3.3. Can FemB append [3H]-glycine to both amidated and non-amidated lipid-II (Lys) Gly? ............... 155
5.4. Discussion and future work ....................................................................................................................... 155
5.4.1. Microscale thermophoresis of the Fem ligases ....................................................................................... 157
5.4.2. Can FemB be crystallised to elucidate its 3D structure by X-ray crystallography? ............................... 168
5.4.3. What is the preferred substrate for the Fem ligases – amidated or non-amidated lipid-II (Lys)? ...... 168

Chapter 6 The ability of *S. aureus* PBP2 and PBP2a to form a protein complex.. 171

6.1. Background .................................................................................................................................................. 171
6.1.1. Localisation of PBPs to the cellular membrane ....................................................................................... 171
6.1.2. Are PBPs in close enough physical proximity to form protein-protein complexes? ............................ 172
6.1.3. Techniques used to study protein-protein interactions in this study .................................................. 174
6.1.3.1. Analytical ultracentrifugation ......................................................................................................... 174
6.1.3.2. Protein-protein crosslinking ................................................................. 174
6.1.3.4. Carbene footprinting ............................................................................. 178

6.2. Chapter aims ............................................................................................... 180

6.3. Results and discussion ................................................................................ 181

6.3.1. Are full-length PBP2 and PBP2a able to form a protein-protein complex following recombinant protein co-expression, and can they be successfully purified as a complex? .... 181

6.3.1.1. Cloning of full-length S. aureus Mu50 PBP2 and S. aureus Mu50 PBP2a into pPROEX and recombinant protein expression ......................................................... 181
6.3.1.2. Cloning of full-length S. aureus Mu50 pbp2 and S. aureus Mu50 pbp2a into pET-Duet for co-expression .................................................................................. 184
6.3.1.3. Protein co-expression trials of full-length S. aureus Mu50 PBP2 and PBP2a .............................................................. 184

6.3.2. Are the full-length PBP2 and PBP2a able to form a protein-protein complex that can be co-purified? ......................................................................................... 186

6.3.2.1. Purification of PBP2-PBP2a complex ....................................................... 186
6.3.2.2. Mass spectrometry of full-length PBP2-PBP2a visualized by SDS-PAGE .................................................................................. 187
6.3.2.3. Are the Western blot antibodies used to detect His-tagged PBP2 and S-tagged PBP2a specific only for their intended affinity tags? ......................................................... 189

6.3.3. Are the truncated PBP2-W59M and PBP2a-Y23M able to form a protein-protein complex in the absence of their TM domains? ................................................................. 190

6.3.3.1. Recombinant expression and purification of PBP2-W59M and PBP2a-Y23M...... 190
6.3.3.2. Assessing protein-protein interaction by Analytical ultracentrifugation .......... 193
6.3.3.3. Assessing weak protein-protein interaction by chemical protein crosslinking studies .................................................................................................................. 197
6.3.3.4. Assessing protein-protein interaction by carbene foot-printing .................. 199
6.3.3.5. Assessing protein-protein interaction by native MS .................................. 204

6.4. Concluding remarks and future work ......................................................... 205

6.4.1. Do full-length PBP2 and PBP2a form a protein-protein complex, and can this be purified for enzymatic and structural studies? ................................................................. 205
6.4.2. Do the truncated PBP2-W59M and PBP2a-Y23M proteins form a protein-protein complex in the absence of the transmembrane helix? ................................................................. 206
6.4.3. Can the interaction of full-length PBP2 and PBP2a be monitored by AUC? .......... 207
6.4.4. Techniques to visualise full-length PBP2-PBP2a complex formation in vivo .......... 208
6.4.5. Techniques to further stabilise the full-length PBP2-2a complex .......................... 209
6.4.6. Studying the transglycosylase and transpeptidase activity of PBP2-2a complex .......... 210
Chapter 7 Transglycosylation and transpeptidation activity of PBPs .......... 211

7.1. Background .................................................................................................................. 211

7.1.1. Techniques to measure transglycosylase activity of PBP2 .................................... 211
  7.1.1.1. Discontinuous Tricine-SDS-PAGE ................................................................. 211
  7.1.1.2. Continuous fluorescent assays for transglycosylase activity .......................... 213

7.1.2. Techniques to measure transpeptidation activity of PBPs .................................... 214

7.1.3. Inhibitors of transglycosylase activity ................................................................. 216

7.1.4. Inhibitors of transpeptidase activity ..................................................................... 217

7.1.5. Chapter aims .......................................................................................................... 218

7.2. Results and Discussion .............................................................................................. 220

7.2.1. Are the recombinantly expressed PBP2-W59M and full-length PBP2:PBP2a complex enzymatical active for transglycosylation? ................................................................. 220

7.2.2. Does PBP2-W59M have greater transglycosylase activity in the presence of PBP2a-Y23M? .......................................................................................................... 222

7.2.3. Can Tricine-SDS-PAGE be used to visualise transglycosylation and transpeptidation by PBP2-W59M using a mix of non-branched and pentaglycyl branched lipid-II substrate? ...... 226

7.2.4. Can Tricine-SDS-PAGE be used to visualise transglycosylation and transpeptidation by PBP2-W59M using a mix of non-branched and mono- or tri- glycyl branched lipid-II substrate? ................................................................................................ 230

7.2.4. Can transglycosylase activity of PBP2-W59M be followed by native mass spectrometry? ................................................................................................................. 233

  7.2.4.1. Is PBP2-W59M still enzymatically active as a transglycosylase in the presence of ammonium acetate? .................................................................................. 234

  7.2.4.2. Can transglycosylase activity of PBP2-W59M be slowed for real-time native mass spectrometry analysis? ................................................................. 234

  7.2.4.3. Can lipid-II be delivered to the PBP2-W59M transglycosylase assay in the absence of detergent? ................................................................................................. 237

  7.2.4.4. Transglycosylase activity of PBP2-W59M measured by native mass spectrometry ................................................................................................................. 238

............................................................................................................................................. 241

7.2.5. Optimisation of PBP2-W59M transglycosylase activity ........................................... 242

  7.2.5.1. Does the presence of S. aureus membranes affect the transglycosylase activity of PBP2-W59M? .................................................................................. 242

  7.2.5.2. Is nascent polymerised lipid-II degraded by S. aureus membranes? ............... 243

  7.2.5.3. Does the presence of S. aureus membrane component Cardiolipin affect the transglycosylase activity of PBP2-W59M? .................................................. 245
7.2.6. Assessing the interactions of fifth generation cephalosporins with PBP2-W59M and PBP2a-Y23M by intact and native mass spectrometry .......................................................... 247

7.3. Concluding remarks and further work .................................................................................. 255
    7.3.1 Can PBP2 transglycosylase activity be enhanced by PBP2a? .............................................. 255
    7.3.2. Is Tricine-SDS-PAGE an alternative way to visualise transpeptidase products? .......... 256
    7.3.3. What information does native mass spectrometry give about transglycosylation? ...... 257
    7.3.4. Can PBP2-W59M transglycosylase activity be optimised in the presence of S. aureus membranes or membrane constituents? .......................................................... 258
    7.3.5. Does PBP2-W59M have a transpeptidase allosteric site like that of PBP2a-Y23M? ...... 258

8. General discussion and concluding remarks ........................................................................... 260

   8.1. Optimising the yields of lipid-II (Lys) ............................................................................... 261
   8.2. Synthesising branched lipid-II intermediates ....................................................................... 262
   8.3. The substrate specificity of the Fem ligases ......................................................................... 263
   8.4. The ability of the Fem ligases to form a protein complex ..................................................... 264
   8.5. The PBP2 and PBP2a protein complex and the effect of PBP2a on PBP2 transglycosylase activity .................................................................................................................. 265
   8.6. The novel putative transpeptidase allosteric site of PBP2-W59M ........................................ 267
   8.7. Conclusion ........................................................................................................................ 268

9. References .................................................................................................................................. 269

10. Appendix. .................................................................................................................................. 297
## List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1.1</td>
<td>The common classes of antibiotics and their modes of action.</td>
<td>3</td>
</tr>
<tr>
<td>Table 1.2</td>
<td>Common variations in the UDP-MurNAc pentapeptide stem.</td>
<td>17</td>
</tr>
<tr>
<td>Table 1.3</td>
<td>An overview of the various peptide cross-bridges in peptidoglycan.</td>
<td>19</td>
</tr>
<tr>
<td>Table 1.4</td>
<td>An overview of the different penicillin binding proteins and their classes across bacteria.</td>
<td>26</td>
</tr>
<tr>
<td>Table 2.1</td>
<td><em>E. coli</em> strains used for DNA cloning and protein expression.</td>
<td>36</td>
</tr>
<tr>
<td>Table 2.2</td>
<td>The components of bacterial growth media used in this project.</td>
<td>37</td>
</tr>
<tr>
<td>Table 2.3</td>
<td>Primers used for DNA amplification.</td>
<td>43</td>
</tr>
<tr>
<td>Table 2.4</td>
<td>Sequences of primers used throughout this project.</td>
<td>45</td>
</tr>
<tr>
<td>Table 2.5</td>
<td>Plasmid constructs used throughout this project.</td>
<td>46</td>
</tr>
<tr>
<td>Table 3.1</td>
<td>Comparison of growth in different media.</td>
<td>73</td>
</tr>
<tr>
<td>Table 3.2</td>
<td>Concentration of non-amidated lipid-II (Lys) synthesis using different bacterial membranes.</td>
<td>81</td>
</tr>
<tr>
<td>Table 4.1</td>
<td>The substrates of <em>S. aureus</em> Fem ligases, PBP2 and PBP2a.</td>
<td>88</td>
</tr>
<tr>
<td>Table 4.2</td>
<td>Predicted m/z and observed m/z for negative mode TOF-MS analysis of UDP-MurNAc-pentapeptide (Lys) species.</td>
<td>97</td>
</tr>
<tr>
<td>Table 4.3</td>
<td>TOF-MS/MS analysis of amidated lipid-II (Lys) Gly₁.</td>
<td>110</td>
</tr>
<tr>
<td>Table 4.4</td>
<td>TOF-MS/MS analysis of non-amidated lipid-II (Lys) Gly₁.</td>
<td>111</td>
</tr>
<tr>
<td>Table 4.5</td>
<td>Predicted m/z of lipid-II species analysed by negative ion mode TOF-MS.</td>
<td>122</td>
</tr>
<tr>
<td>Table 4.6</td>
<td>TOF-MS analysis of reaction 1.</td>
<td>123</td>
</tr>
<tr>
<td>Table 4.7</td>
<td>TOF-MS analysis of reaction 2.</td>
<td>124</td>
</tr>
<tr>
<td>Table 4.8</td>
<td>TOF-MS analysis of reaction 3.</td>
<td>124</td>
</tr>
<tr>
<td>Table 4.9</td>
<td>TOF-MS analysis of reaction 3.</td>
<td>125</td>
</tr>
<tr>
<td>Table 4.10</td>
<td>TOF-MS/MS analysis of amidated lipid-II (Lys) Gly₅ extracted from <em>S. aureus</em> RN4220 culture – Walker method.</td>
<td>128</td>
</tr>
<tr>
<td>Table 4.11</td>
<td>The pros and cons of chemical or enzymatic syntheses.</td>
<td>136</td>
</tr>
<tr>
<td>Table 5.1</td>
<td>Overview of Fem ligase substrate and peptidyl-transferase activity.</td>
<td>149</td>
</tr>
<tr>
<td>Table 5.2</td>
<td>Binding affinities of the Fem ligases, as measured by Microscale thermophoresis.</td>
<td>159</td>
</tr>
</tbody>
</table>
Table 5.3. Previous successful crystal screens for *S. aureus* FemB..........................162
Table 5.4. Successful crystallisation screen conditions for *S. aureus* FemB.............163
Table 5.5. Successful crystallisation screen conditions for *S. aureus* FemB in Morpheus screen. .................................................................................................................................165

Table 6.1. The reactive groups of crosslinker molecules used for protein-protein crosslinking. ........................................................................................................................................176
Table 6.2. Characterisation of *S. aureus* PBP2-W59M and PBP2a-Y23M by analytical ultracentrifugation. ........................................................................................................................................196
Table 6.3. Important residues in PBP2a-Y23M structure. ...........................................203
List of Figures

Figure 1.1. An overview of the common modes of action of antibiotics. .........................4
Figure 1.2 The Gram-positive and Gram-negative bacterial cell wall.........................13
Figure 1.3. An overview on peptidoglycan biosynthesis in S. aureus. .........................15
Figure 1.4. Transglycosylation and transpeptidation in S. aureus. ..............................22
Figure 1.5. The classes of penicillin-binding proteins. ..............................................25
Figure 1.6. Crystal structures of PBP2a-Y23M and PBP2-W59M. .........................33
Figure 2.1. The aryl diazirine used for carbene footprinting. ....................................67
Figure 3.1. Growth curve and cfu/ml of M. flavus, S. epidermidis and S. aureus........74
Figure 3.2. Change in turbidity due to cell lysis of Staphylococci...............................77
Figure 3.3. Non-amidated lipid-II (Lys) synthesis with different bacterial membranes. ......................................................................................................................79
Figure 4.1. Lipid-II variants. ..........................................................................................91
Figure 4.2. The *in vitro* synthesis of UDP-MurNAc-pentapeptide (Lys).............95
Figure 4.3. Typical AKTA chromatogram following purification of UDP-MurNAc-pentapeptide (Lys) by anion exchange chromatography.................................96
Figure 4.4. Non-amidated UDP-MurNAc pentapeptide (Lys) analysed by TOF-MS...98
Figure 4.5. Amidated UDP-MurNAc pentapeptide (Lys) analysed by TOF-MS............99
Figure 4.6. TOF-MS analysis of non-amidated UDP-MurNAc pentapeptide Gly1.....102
Figure 4.7. TOF-MS analysis of non-amidated UDP-MurNAc pentapeptide Gly3.....103
Figure 4.8. The *in vitro* synthesis of lipid-II (Lys). ..................................................105
Figure 4.9. Thin layer chromatography analysis of lipid-II (Lys) purified by DEAE sephacel. ...................................................................................................................106
Figure 4.10. TOF-MS analysis of non-amidated lipid-II (Lys). .................................107
Figure 4.11. TOF-MS analysis of amidated lipid-II (Lys). ..........................................108
Figure 4.12. Thin layer chromatography analysis of amidated lipid-II (Lys) purified by DEAE sephacel ...........................................................................................................113
Figure 4.13. TOF-MS analysis of amidated lipid-II (Lys) Gly1. ...............................114
Figure 4.14. TOF-MS analysis of amidated lipid-II (Lys) Gly3. ...............................115
Figure 4.15. TOF-MS analysis of non-amidated lipid-II (Lys) Gly1. ......................116
Figure 4.16. TOF-MS analysis of non-amidated lipid-II (Lys) Gly3. ......................117
Figure 6.1. Overview of protein-protein crosslinking using SATA and sulfo-SMCC...177
Figure 6.2. An overview of carbene foot-printing. .................................................................179
Figure 6.3. Expression trials of S. aureus Mu50 PBP2. ..........................................................182
Figure 6.4. Expression trials of S. aureus Mu50 PBP2a. .........................................................183
Figure 6.5. Cloning of full-length PBP2 and PBP2a into the coexpression vector pET-Duet. ..........................................................................................................................184
Figure 6.6. Coexpression trials of S. aureus Mu50 PBP2a and PBP2. ..................................185
Figure 6.7. Purification of coexpressed S. aureus Mu50 PBP2a and PBP2.........................187
Figure 6.8. Final purity of S. aureus Mu50 PBP2:2a complex.............................................188
Figure 6.9. Western blot of protein standard ladders. ..............................................................189
Figure 6.10. A typical purification of PBP2-W59M using Ni-IMAC, size exclusion and anion exchange chromatography. ...........................................................................191
Figure 6.11. A typical purification of PBP2a-Y23M using anion exchange, cation exchange and size exclusion chromatography.................................................................192
Figure 6.12. Final purity of S. aureus PBP2a Y23M and S. aureus PBP2 W59M.................193
Figure 6.13. Characterisation of S. aureus PBP2-W59M and PBP2a-Y23M by analytical ultracentrifugation. ........................................................................................................195
Figure 6.14. Protein-protein crosslinking of PBP2-W59M and PBP2a-Y23M with sulfo-SMCC and SATA crosslinkers, respectively. ..............................................................198
Figure 6.15. Carbene labelling of PBP2a-Y23M in the presence of PBP2-W59M...........201
Figure 6.16. Peptides modified by carbene labelling of PBP2a-Y23M in the presence of PBP2-W59M ..................................................................................................................202
Figure 6.17. Native mass spectrometry of PBP2a-Y23M and PBP2-W59M...............204
Figure 7.1. Overview of transglycosylase assays by PAGE-based assay......................212
Figure 7.2. Overview of transglycosylase assays by continuous, coupled fluorometric assay. ..............................................................................................................................214
Figure 7.3. Transglycosylase activity of PBP2 and PBP2a. ..................................................221
Figure 7.4. Transglycosylase activity of PBP2 W59M in the presence or absence of PBP2a Y23M over time. ........................................................................................................224
Figure 7.5. Transglycosylase activity of PBP2-W59M, in the presence of PBP2a-Y23M at varying concentrations. ......................................................................................225
Figure 7.6. Transglycosylase and transpeptidase activity of PBP2-W59M in the presence of amidated lipid-II (Lys) Glys. .................................................................229
Figure 7.7. Transglycosylase activity of PBP2-W59M using mono- and tri- glycyll lipid-II. ...................................................................................................................232
Figure 7.8-A. Transglycosylase activity of PBP2-W59M – testing ammonium acetate as a reaction buffer. .................................................................236
Figure 7.8-B. Transglycosylase activity of PBP2-W59M – testing ammonium acetate as a reaction buffer and varying lipid-II substrate. .................................236
Figure 7.8-C. Transglycosylase activity of PBP2-W59M – screen for lipid-II delivery vehicle. .....................................................................................................................236
Figure 7.9. PBP2-W59M transglycosylase activity visualised by native mass spectrometry. .........................................................................................................................240
Figure 7.10. PBP2-W59M transglycosylase activity visualised by native MS/MS – Quadrupole isolation. .....................................................................................241
Figure 7.11. (A) TG activity of PBP2-W59M in the presence of S. aureus ATCC 25923 membranes.). (B) TG activity of PBP2-W59M in the presence of S. aureus ATCC 25923 membranes – degradation trial........................................244
Figure 7.12. PBP2-W59M transglycosylase activity in the presence of cardiolipin...246
Figure 7.13. Intact MS of PBP2-W59M with either ceftobiprole or ceftaroline.......248
Figure 7.14. Intact MS of PBP2a-Y23M with either ceftobiprole or ceftaroline......249
Figure 7.15. Native MS analysis of PBP2a-Y23M with ceftaroline........................252
Figure 7.16. Native MS analysis of PBP2a-Y23M with ceftobiprole....................253
Figure 7.17. Native MS analysis of PBP2-W59M with ceftaroline and ceftobiprole..254
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Declaration

I hereby declare that I personally have carried out the work presented in this thesis under the supervision of Professor David Roper and Dr Adrian Lloyd, at the School of Life Sciences, University of Warwick. Work contributed to by other individuals has been explicitly stated.

No part of this work has been previously submitted to be considered for any other degree or qualification. All sources of information and published work has been specifically acknowledged and cited.
Abstract

*Staphylococcus aureus* is one of the leading causes of infective endocarditis and skin and soft tissue infections in humans, and is also a serious problem in agricultural livestock, such as cattle, pigs and poultry, causing mastitis, lameness and septicaemia. The threat posed by *S. aureus* has been amplified by the acquisition of *mecA*, the resistance determinant for high level β-lactam resistance in methicillin-resistant *S. aureus* (MRSA). The *mecA* gene encodes for the penicillin-binding protein (PBP) PBP2a. PBPs are responsible for the polymerisation (transglycosylation) and cross-linking (transpeptidation) of lipid-II during the biosynthesis of peptidoglycan, an essential component in maintaining bacterial rigidity, cell shape and integrity. In methicillin sensitive strains, β-lactam antibiotics prevent transpeptidation. However, PBP2a has an extremely low affinity for β-lactams, allowing peptidoglycan synthesis to continue in the presence of β-lactam antibiotics that would otherwise be lethal.

The work presented in this thesis primarily focuses on the enzymatic activities of MRSA PBP2 and PBP2a, with the aim of determining whether these two PBPs act as part of a synergistic protein complex. This thesis provides, for the first time, *in vitro* evidence for the formation of a PBP2:PBP2a complex. The use of native mass spectrometry has also identified a previously unseen putative allosteric binding site in PBP2. The Fem ligase enzymes, responsible for peptidoglycan precursors branching have also been investigated, with new *in vitro* evidence for complex formation established. Furthermore, new methods for the chemical and enzymatic synthesis of various branched species of peptidoglycan precursor have been established, allowing for the discovery of the non-selective use of either amidated or non-amidated branched lipid-II (Lys) substrates by FemX and FemA, respectively.

This thesis provides a greater understanding of peptidoglycan synthesis in MRSA, which may provide a basis for the design of novel therapeutics towards this essential and conserved target in the future, aiding to reduce the threat of antimicrobial resistance.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Å</td>
<td>Angstrom</td>
</tr>
<tr>
<td>$A_{x\text{nm}}$</td>
<td>Absorbance at x wavelength</td>
</tr>
<tr>
<td>ABC</td>
<td>Ammonium bicarbonate</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine 5’-diphosphate</td>
</tr>
<tr>
<td>AMES</td>
<td>Aminoglycoside modifying enzymes</td>
</tr>
<tr>
<td>AMR</td>
<td>Antimicrobial resistance</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine 5’-tri-phosphate</td>
</tr>
<tr>
<td>AU</td>
<td>Absorbance units</td>
</tr>
<tr>
<td>BHI</td>
<td>Bovine heart infusion</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>Cfr</td>
<td>Chloramphenicol-florfenicol resistance</td>
</tr>
<tr>
<td>CHAPS</td>
<td>3-((3-chloramidopropyl)-dimethylammonio)-1-propanesulfonate</td>
</tr>
<tr>
<td>CMC</td>
<td>Critical micelle concentration</td>
</tr>
<tr>
<td>C-terminus</td>
<td>Carboxyl terminus</td>
</tr>
<tr>
<td>CV</td>
<td>Column volume</td>
</tr>
<tr>
<td>D-Ala</td>
<td>D-Alanine</td>
</tr>
<tr>
<td>D-Ala-D-Ala</td>
<td>D-Alanyl-D-Alanine</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DEAE</td>
<td>Diethylaminoethyl</td>
</tr>
<tr>
<td>D-iGln</td>
<td>D-isoGlutamine</td>
</tr>
<tr>
<td>D-Glu</td>
<td>D-Glutamate</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNase</td>
<td>Deoxyribonuclease</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EDC</td>
<td>1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>eDNA</td>
<td>Extracellular DNA</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ESI-MS</td>
<td>Electrospray ionization mass spectrometry</td>
</tr>
<tr>
<td>et al</td>
<td>et alia, and others</td>
</tr>
<tr>
<td>FAD</td>
<td>Flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FADH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Flavin adenine dinucleotide, reduced</td>
</tr>
<tr>
<td>Fmoc</td>
<td>Fluorenylmethyloxycarbonyl</td>
</tr>
<tr>
<td>g</td>
<td>Grams</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>N-acetyl glucosamine</td>
</tr>
<tr>
<td>Gly&lt;sub&gt;x&lt;/sub&gt;</td>
<td>Glycine, x number of repeating units</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HMM</td>
<td>High molecular mass</td>
</tr>
<tr>
<td>HMW</td>
<td>High molecular weight</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>IAA</td>
<td>Iodoacetamide</td>
</tr>
<tr>
<td>IMAC</td>
<td>Immobilised metal affinity chromatography</td>
</tr>
<tr>
<td>IPP</td>
<td>Inorganic pyrophosphatase</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1 thiogalactopyranoside</td>
</tr>
<tr>
<td>kb</td>
<td>Kilobases</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium chloride</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Daltons</td>
</tr>
<tr>
<td>K&lt;sub&gt;d&lt;/sub&gt;</td>
<td>Dissociation constant</td>
</tr>
<tr>
<td>Kpsi</td>
<td>Kilopound per square inch</td>
</tr>
<tr>
<td>L</td>
<td>Litre</td>
</tr>
<tr>
<td>L-Ala</td>
<td>L-Alanine</td>
</tr>
<tr>
<td>LA-MRSA</td>
<td>Livestock-associated MRSA</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>LDAO</td>
<td>Lauryldimethylamine oxide</td>
</tr>
</tbody>
</table>

Lipid-IV Undecaprenyl-pyrophosphoryl-(N-acetyl-muramyl-(N-acetyl-glucosamyl)-L-Ala-γ-D-Glu/D-iGln-L-Lys-D-Ala-D-Ala)_2

L-Lys L-Lysine
LMM Low molecular mass
LMW Low molecular weight
LPS Lipopolysaccharide
LTA Lipoteichoic acids
M Molar
mA Milliamperes
MATE Multi-drug and toxic compound extrusion
MBC Minimum bactericidal concentration
MESG 7-methyl-6-guanosine
M. flavus Micrococcus flavus
mg Milligrams
MgCl₂ Magnesium chloride
MGT Monofunctional transglycosylase
MIC Minimum inhibitory concentration
min Minute
mL Millilitres
MOPS 3-(N-morpholino)propanesulfonic acid
MRSA Methicillin-resistant Staphylococcus aureus
MSSA Methicillin-sensitive Staphylococcus aureus
MST Microscale thermophoresis
MurNAc N-acetyl-muramic acid
NB Nutrient broth
NAD⁺ Nicotinamide adenine dinucleotide (oxidized)
NADH Nicotinamide adenine dinucleotide (reduced)
NADP⁺ Nicotinamide adenine dinucleotide phosphate (oxidized)
NADPH Nicotinamide adenine dinucleotide phosphate (reduced)
Tris 2-amino-2-(hydroxymethyl)propanol-1,3-diol
TEMED N,N,N',N'-tetramethylethylenediamine
TEV Tobacco Etch virus
TLC Thin layer chromatography
TM Trans-membrane domain/helix
tRNA Transfer ribonucleic acid
TSB Tryptic soy broth
UDP Uridine 5'-diphosphate
UDP-GlcNAc Uridine diphosphate N-acetylglucosamine
UDP-MurNAc Uridine diphosphate N-acetylmuramic acid
UMP Uridine 5'-monophosphate
WTA Wall teichoic acid
w/v Weight to volume ratio
V Volts
VRE Vancomycin-resistant Enterococcus
v/v Volume to volume ratio
x g Centrifugal force

Other abbreviations are denoted in the main text, where appropriate.
1. Introduction

1.1. Antimicrobial resistance

Antimicrobial resistance (AMR) is defined as the ability of microorganisms, such as bacteria, viruses, fungi and parasites, to become resistant to the drugs used against them leading to a drastic reduction in the effectiveness of treatment. Antibiotics, however, refer to drugs used only to treat bacteria, and hence antibiotic resistance is the ability of bacteria to survive in the presence of therapeutic levels of antibiotics that should otherwise be lethal.

1.1.1. The discovery of the first antibiotics

The discovery of antibiotics was arguably led by Paul Ehrlich and Alexander Fleming – two scientists who paved the way for the ‘antibiotic era’. In 1904, Ehrlich began a large-scale, systematic screening to find a synthetic compound that targeted the bacterium Treponema pallidum, the cause of the venereal disease syphilis (Aminov 2010). Ehrlich was successful in finding a compound that cured both syphilis-infected rabbits, and showed huge promise in albeit limited human trials – this compound was named Salvarsan, and with its less toxic successor Neosalvarsan, was the most widely prescribed drug up until the 1940’s before its replacement with Penicillin (Ehrlich and Hata 1910, Aminov 2010).

The first clinically exploited naturally occurring antibiotic, Penicillin, was inadvertently discovered by Alexander Fleming in 1928 when a Penicillium sp. mold spore was introduced into a petri dish containing Staphylococcus aureus culture (Fleming 1929). Fleming noticed this mold caused Staphylococcal cell death, which we now know to be due to the secretion of Penicillin from the contaminating mold. It wasn’t until the 1940’s, however, that Howard Florey and Ernst Chain were able to purify Penicillin in large enough quantities to fully assess its chemotherapeutic abilities and medicinal uses (Chain et al, 1940).
These two landmark discoveries kickstarted what was known as ‘the Golden era’ of antibiotics, and in fact, between 1950-1960 over half of the antibiotics used today were discovered (Davies 2006).

1.1.2. Antibiotics mode of action

The most common antibiotics are selectively directed towards essential bacterial targets that are either absent from, or sufficiently different from, eukaryote, host mechanisms, and therefore are able to prevent bacterial growth with limited host toxicity. In general, the main targets of most common antibiotics are DNA replication and repair mechanisms, protein synthesis, cell wall biosynthesis and folate synthesis. Examples of common antibiotics and their modes of action are illustrated in Figure 1.1 and summarised in Table 1.1, however this is not an exhaustive list. Nonetheless, most of the clinically relevant antibiotics target three main areas – DNA replication/repair, protein translation and peptidoglycan synthesis (Ma et al, 2016).

1.1.2.1. DNA replication and repair

For bacterial replication to occur, the bacterial DNA of the parent cell must be replicated and passed to the newly dividing daughter cell. For this to happen, the bacterial DNA double helix must be unwound and complimentary strands must be separated to allow each strand to be transcribed by DNA replication machinery. DNA topoisomerases create temporary single or double stranded breaks in DNA allowing for a relaxed DNA topology (Champoux 2001). Whilst all topoisomerases can catalyse the breakage of DNA strands, the prokaryotic topoisomerase, DNA gyrase, has the ability to introduce negative supercoils into the DNA, thereby reducing DNA torsional stress and allowing replication machinery access to each DNA strand (Champoux 2001, Collin et al, 2011). Antibiotics, such as the quinolone and fluoroquinolone classes, act to stabilise DNA-topoisomerase complexes such that the number of
double stranded DNA breaks is vastly increased leading to chromosomal fragmentation and the inhibition of DNA replication (Van Eijk et al, 2017).

Table 1.1. The common classes of antibiotics and their modes of action.

(Davies and Davies 2010, Coates et al, 2011)

<table>
<thead>
<tr>
<th>Class of antibiotic</th>
<th>Example</th>
<th>Target</th>
</tr>
</thead>
</table>
| β-lactams           | - Penicillins (Ampicillin, methicillin)  
|                     | - Cephalosporins (Ceftriaxone, Ceftaroline)  
|                     | - Carbapenems (Meropenem)  
|                     | - Monobactams (Aztreonam) | Peptidoglycan synthesis |
| Glycopeptides       | Vancomycin, Teicoplanin | Peptidoglycan synthesis |
| Quinolones          | Ciprofloxacin | DNA (replication) |
| Rifamycins          | Rifampicin | DNA (transcription) |
| Aminoglycosides     | Gentamycin, Streptomycin, Spectinomycin | Protein translation |
| Macrolides          | Erythromycin, Azithromycin | Protein translation |
| Tetracyclines       | Tetracycline, Doxycycline, Minocycline | Protein translation |
| Phenicols           | Chloramphenicol | Protein translation |
| Oxazolidinones      | Linezolid | Protein translation |
| Lipopeptides        | Daptomycin | Cell membrane integrity |
| Cationic peptides   | Colistin | Cell membrane integrity |
| Sulphonamides       | Sulfamethoxazole | Folate synthesis |
| Diaminopyrimidines  | Trimethoprim | Folate synthesis |
Figure 1.1. An overview of the common modes of action of antibiotics. This is not an exhaustive list and does not include a consideration of the outer membrane of Gram-negative bacteria which presents an additional barrier to antibiotic entry. Red text indicates the antibiotic mode of action. Black text indicates classes of antibiotics for each mode of action.
1.1.2.2. Protein translation

The translation of bacterial proteins from nascent mRNA acts as an attractive target for drug development as toxicity is limited due to the distinctive translational processes in eukaryotes and prokaryotes. In bacterial translation, the 70S ribosome which is responsible for translation of the mRNA code into the amino acid sequence, is made up of two subunits – the large 50S and the small 30S ribosomal subunits, whilst in eukaryotes the 80S ribosome is made up of the 60S and 40S subunits (Berg et al, 2002). The complex nature of protein translation allows for multiple antibiotic targets – the main clinically relevant antibiotics target either the 30S entry site, the 50S peptidyl transferase centre, or the 50S peptide exit tunnel (McCoy et al, 2011). Aminoglycosides bind the 30S ribosomal subunit; for example, Streptomycin interferes with the 30S initiation complex (Luzzatto et al, 1968) whilst Spectinomycin binds to the 30S subunit to inhibit mRNA translocation along the ribosome (McCoy et al, 2011). Phenicols and Oxalodinones bind to the peptidyl transferase centre, preventing proper amino-acyl tRNA placement and hence peptide bond formation is unable to occur (McCoy et al, 2011, Marks et al, 2016). By contrast, macrolides bind to the 50S exit tunnel causing premature dissociation of peptidyl-tRNAs to prevent elongation of the amino acid chain (Tenson et al, 2003, McCoy et al, 2011).

1.1.2.3. Peptidoglycan biosynthesis

The bacterial cell wall surrounds the cytoplasmic membrane and is essential in maintaining bacterial cell shape and integrity, whilst acting as an anchor for outer membrane proteins (Vollmer et al, 2008). Both Gram-positive and Gram-negative bacterial cells contain peptidoglycan; in Gram-positive bacteria peptidoglycan accounts for up to 90% of the dry weight of the cell, whilst this value is only 10% in Gram-negative bacteria (Malanovic and Lohner 2016). Peptidoglycan, along with the outer membrane in Gram negative bacteria, are therefore prime targets for antibiotics. The synthesis of peptidoglycan and its precursors are the topic of this
thesis and will therefore be covered in more detail in Section 1.2, along with discussion of the antibiotics which inhibit peptidoglycan biosynthesis and integrity.

1.1.3. Mechanisms of antibiotic resistance

Bacteria have developed many sophisticated mechanisms of antibiotic resistance. Whilst most antibiotics are naturally produced by bacteria themselves, these organisms as well as other bacterial species that share the same environment have therefore co-evolved resistance mechanisms in order to survive, rendering them intrinsically resistant to many antibiotics (Munita and Arias 2015). In many cases of antibiotic producing bacteria, the genes involved in self-resistance are both clustered with, and co-regulated with, the biosynthetic genes involved in antibiotic synthesis (Mak et al, 2014).

Resistance genes in bacteria have evolved via two intrinsic resistance mechanisms – genetic mutation of genes under selection pressures, associated directly or indirectly with the antibiotic mechanism of action, and/or the acquisition of resistance genes by horizontal gene transfer from resistant bacteria (Munita and Arias 2015). In the case of genetic mutation, a subset of bacteria may evolve resistance allowing them to survive in the presence of the antibiotic. The sensitive population will be unable to survive in the presence of the antibiotic, and hence the resistant population will predominate and propagate. Conversely, resistance genes can be acquired by horizontal gene transfer via the following methods; bacterial conjugation (from bacterium to bacterium), bacteriophage induced transduction (from phage to bacteria) or transformation (external naked DNA to bacteria) (Frost et al, 2005, Munita and Arias 2015). Whilst only a small subset of bacteria are naturally transformable, and thus able to take up naked DNA, the transfer of bacterial resistance genes by conjugation is highly efficient and includes the transfer of mobile genetic elements such as transposons, plasmids and integrons (Frost et al, 2005, Munita and Arias 2015). The following sections will briefly discuss the plethora of antibiotic resistance mechanisms, both acquired and evolved.
1.1.3.1. Antibiotic modification and degradation

One mechanism of antibiotic resistance is the ability of bacteria to produce enzymes that are able to either modify the antibiotic molecule in such a way that renders the antibiotic inactive and/or unable to interact with its target molecule, or the complete degradation of the antibiotic molecule itself.

In the case of antibiotic alteration, the most common types of antibiotic modifications include acetylation of aminoglycosides, chloramphenicols and streptogramins, phosphorylation of aminoglycosides and chloramphenicols and adenylation of aminoglycosides and lincosamides, resulting in an increased steric hindrance, and hence a decreased affinity of the antibiotic molecule for its target (Munita and Arias 2015). The modification of aminoglycosides by aminoglycoside modifying enzymes (AMEs) has been stated as one of the most common mechanisms of aminoglycoside resistance in the clinical setting (Ramirez and Tolmasky 2010). AMEs are phosphotransferases, nucleotidyltransferases or acetyltransferases that modify aminoglycosides at the -O\text{H} or -NH\text{2} group of the 2-deoxystreptamine ring or the surrounding sugar moieties (Ramirez and Tolmasky 2010). AMEs, their mode of action and their vast nomenclatures have been extensively reviewed by Ramirez and Tolmasky (2010).

Perhaps the most well-known example of antibiotic degrading enzymes are the β-lactamases, discovered in *Escherichia coli* by Abraham and Chain (1940) and later in *S. aureus* by Segalove (1947). β-lactamases hydrolyse the amide bond of the β-lactam ring of the β-lactam antibiotics, leading to inactive β-lactams and are generally encoded by the *bla* genes which can be chromosomal or acquired on mobile genetic elements (Munita and Arias 2015).
1.1.3.2. Increased efflux of antibiotics

Bacterial efflux pumps allow for the efflux of molecules, including antibiotics, from the inside to the outside of the bacterial cell – efflux pumps are transmembrane proteins that can be specific to one, or many different structurally related molecules (Webber and Piddock 2003). These efflux pumps are classified into five different efflux pump families based on the number of their transmembrane spanning regions, substrate specificities and sources of energy – these families include the RND (resistance-nodulation-division) family, the ATP-binding cassette family, the small multi-drug resistance family, the major facilitator family, and finally, the multi-drug and toxic compound extrusion (MATE) family (Sun et al, 2014, Blanco et al, 2016). These efflux systems are heavily regulated - in the case of multi-drug resistance efflux pumps, resistance as a result of these efflux pumps may be transient or constitutive, as a result of inducers of efflux pump expression, or due to mutations of the molecules that downregulate their expression, respectively (Blanco et al, 2016).

1.1.3.3. Sequestering antibiotics

Many Gram-positive and Gram-negative bacteria are able to sequester antibiotic molecules in numerous ways, including via the release of membrane lipid decoys or sequestration with DNA, proteins or polysaccharides (Sabnis et al, 2018). In the case of membrane lipid decoys, *Staphylococcus aureus* and *Enterococcus faecalis* release *de novo* polysaccharides which are able to sequester daptomycin, preventing it from inserting into the bacterial membrane (Pader et al, 2016, Ledger et al, 2017, Sabnis et al, 2018). Gram-negative organisms, such as *E. coli* and *Pseudomonas aeruginosa*, are able to upregulate the release of decoy outer membrane vesicles (OMV) in response to exposure to antibiotics such as polymyxin B and colistin – however it is unknown whether this increase in OMV release is a direct, regulated response, or an artefact of membrane disruption (Manning and Kuehn 2011, Macdonald and Kuehn 2013, Kulkarni et al, 2015, Sabnis et al, 2018).
Antibiotics can also be sequestered following the release of DNA, proteins or polysaccharides. The release of extracellular DNA (eDNA) from *P. aeruginosa* and *S. aureus* biofilms allows eDNA to bind positively charged antimicrobials such as aminoglycosides and vancomycin, respectively, allowing these biofilms to evade antibiotic exposure (Chiang *et al*, 2013, Doroshenko *et al*, 2014, Sabnis *et al*, 2018). *Burkholderia spp.* have been shown to release the protein lipocalin which binds to a broad range of antibiotics such as rifampicin, polymyxin B, norfloxacin and ceftazidime, preventing their interaction with their target (El-Halfawy *et al*, 2017, Sabnis *et al*, 2018). Interestingly, a lipocalin homologue is conserved in *P. aeruginosa*, methicillin-resistant *S. aureus* and *Mycobacterium tuberculosis* and expression of this homologue in a lipocalin-inactive mutant (drug sensitive) *Burkholderia* strain, restored antibiotic resistance suggesting that the role of lipocalin in antibiotic sequestration may be conserved (El-Halfawy *et al*, 2017, Sabnis *et al*, 2018).

Finally, the release of polysaccharides from *Klebsiella pneumoniae*, *Streptococcus pneumoniae* and *P. aeruginosa*, either as result of membrane damage or a specific stress induced response, following exposure to polymyxin B has been shown to enhance bacterial survival as a result of antibiotic binding (Llobet *et al*, 2008, Sabnis *et al*, 2018).

### 1.1.3.4. Modification of antibiotic targets

Many bacteria have evolved mechanisms that act to modify the existing targets of antimicrobials, hence reducing, or completely inhibiting the interaction of the antibiotic with its target. Examples of target modifications include the methylation of the 16S rRNA of the 30S ribosome subunit binding site by the erythromycin ribosome methylases, sterically hindering the ability of lincosamines, macrolides and streptogramins, to bind to the 23S rRNA of the 50S ribosome subunit(Lai and Weisblum 1971, Maravic 2004, Morić *et al*, 2010, Blair *et al*, 2014). Methylation of the 23S rRNA by Cfr (chloramphenicol-florfenicol resistance) methyltransferases also prevents the binding of antibiotics, such as phenicols, lincosamides and streptogramins, to the 50S ribosome; this methylation has also been previously
shown to correlate to the amount of erythromycin exposure (Lai and Weisblum 1971, Long et al, 2006, Blair et al, 2014).

Another example of target modification is provided by the glycopeptide resistance mechanism wherein the D-Ala-D-Lactate ligases which substitute the terminal D-Ala-D-Ala of lipid-II, a component of the bacterial cell wall peptidoglycan, for D-Ala-D-Lac, such that glycopeptides, including vancomycin, can no longer bind to its target (D-Ala-D-Ala) (Healy et al, 2000). In vancomycin resistant Enterococci (VRE), vancomycin has a 1000-fold lower binding affinity to D-Ala-D-Lac than to D-Ala-D-Ala, therefore rendering vancomycin significantly less effective against VRE strains (Healy et al, 2000). However, perhaps most relevant to this thesis is the modification of the penicillin-binding proteins (PBPs) in S. aureus and S. pneumoniae. In S. pneumoniae, homologous recombination between S. pneumoniae and S. viridans PBP gene sequences has led to a series of point mutations in PBP2x, whilst S. aureus acquired an extra PBP from S. sciuri as a result of horizontal gene transfer of a cassette which included the pbp2a/mecA gene. (Chambers 1999, Fuda et al, 2007). The mutated PBP2x and the acquired PBP2a have an intrinsically low affinity for β-lactam antibiotics. The acquisition of PBP2a in S. aureus will be discussed further in Section 1.4.

1.1.3.5. **What can be done about antibiotic resistance?**

Antibiotic resistance is a natural event in bacteria and bacteria will always be evolving new mechanisms to evade antibiotics as a direct response to antibiotic exposure. Nevertheless, the rate at which bacteria are becoming resistant to antibiotic treatment is alarming and it will only continue as a result of our continuing, inappropriate overuse of antibiotics. Alarmingly, it is predicted that if the rising rates of antimicrobial resistance is not tackled, antimicrobial resistant infections will cause over 10 million deaths per year by 2050 - this is more than cancer alone (O’Neill 2014). We are therefore in desperate need of novel antibiotic treatment regimens, whether that comes from chemical derivatives of already established and successful
antibiotics, from the discovery of antibiotics secreted from bacteria in the natural environment, or from combination therapy where antibiotics that are becoming less potent as a result of antibiotic resistance are rendered more effective as a result of combination therapy with a drug adjuvant that, for example, may reduce bacterial fitness or directly targets the antibiotic resistance mechanism.

1.2. The bacterial cell wall

Whilst the cell wall of both Gram-positive and Gram-negative bacteria consist of peptidoglycan, their overall structures are vastly different, as can be highlighted by the Gram stain which exploits these distinct characteristics. As in Fig. 1.2, the Gram-negative cell wall consists of three major components; the inner cytoplasmic membrane, the thin peptidoglycan layer and the outer membrane. The latter outer membrane is not principally classed as a phospholipid bilayer, but is deemed a lipid bilayer due to all phospholipids being confined within the inner leaflet of the outer membrane, with glycolipids such as lipopolysaccharides (LPS) within the extracellular leaflet of the outer membrane (Silhavy et al, 2010). This outer membrane plays a crucial role in protecting Gram-negative bacteria from environmental stressors and in maintaining bacterial cell shape and integrity (Silhavy et al, 2010). Gram-positive organisms on the other hand, lack this outer membrane and as such, have a much thicker, multi-layered peptidoglycan ranging between 30-100 nm thick – much thicker in comparison to Gram-negative organisms where peptidoglycan is up to 15 nm thick (Vollmer et al, 2008, Silhavy et al, 2010).

The Gram-positive peptidoglycan layer is also highly dispersed with teichoic acids, including both lipoteichoic acids (LTA) which are anchored at the cellular membrane, as well as wall teichoic acids (WTA) which are covalently linked to the peptidoglycan (PG) layer, as shown in Fig. 1.2 (Silhavy et al, 2010, Brown et al, 2013). Teichoic acids are the most abundant lipid linked polymers in many Gram-positive organisms accounting for up to 50% of the cell wall mass – they are highly important, and for
example, are an essential requirement in the resistance to β-lactam antibiotics in methicillin resistant S. aureus (Vollmer et al, 2008, Brown et al, 2013).
1.2.1. The synthesis of peptidoglycan

The major constituent of the Gram-positive bacterial cell wall is peptidoglycan, which is synthesised in three different stages – the cytoplasmic stage, the lipid-linked stage and the extracellular stage (Scheffers and Pinho 2005, Jarick et al, 2018), as illustrated in Fig. 1.3.

1.2.1.1. The cytoplasmic stage

Firstly, in the cytoplasmic stage, the amidotransferase enzyme GlmS catalyses the conversion of D-fructose-6-phosphate to D-glucosamine-6-phosphate, using L-glutamine as its nitrogen donor (Badet et al, 1987, Barreteau et al, 2008). D-glucosamine-6-phosphate is then converted to D-glucosamine-1-phosphate by GlmM where the bifunctional enzyme GlmU forms UDP-GlcNAc (Mengin-Lecreulx and van Heijenoort 1996, Barreteau et al, 2008). First, GlmU catalyses the transfer of an acetyl group from acetyl-CoA to form N-acetylglucosamine-1-phosphate (GlcNAc-1-phosphate), and then transfers a uridyl group from UTP to form uridine-5’-diphosphate (UDP)-GlcNAc (Mengin-Lecreulx and van Heijenoort 1996, Barreteau et al, 2008).

Following the formation of UDP-GlcNAc, the enzyme MurA catalyses the attachment of enolpyruvate from phosphoenolpyruvate (PEP) to the 3’ hydroxyl group of UDP-GlcNAc, yielding UDP-GlcNAc-enolpyruvate and the release of inorganic phosphate (Marquardt et al, 1992, Brown et al, 1995, Barreteau et al, 2008). UDP-GlcNAc-enolpyruvate is then converted to UDP-N-acetyl-muramic acid (UDP-MurNAc) by MurB, a flavoprotein with a flavin adenine dinucleotide (FAD) as a cofactor (Nishida et al, 2006). Firstly, the FAD cofactor is reduced to FADH₂ by NADPH bound to MurB; the release of NADP⁺ induces the binding of UDP-GlcNAc-enolpyruvate to the MurB active site where electron transfer from FADH₂ to UDP-GlcNAc-enolpyruvate can occur, yielding UDP-MurNAc (Benson et al, 1993, Nishida et al, 2006, Barreteau et al, 2008).
Figure 1.3. An overview of peptidoglycan biosynthesis in *S. aureus*. Fructose-6-phosphate – F6P. Penicillin binding protein – PBP. Glycyl-tRNA synthetase – GlyRS. Figure adapted from Pinho *et al* (2013) and Jarick *et al* (2018).
The final steps in the cytoplasmic stage of peptidoglycan synthesis comprise of the production of UDP-N-acetylmuramyl pentapeptide (UDP-MurNAc pentapeptide) from UDP-MurNAc in a sequence of reactions catalysed by MurC-F. The pentapeptide stem of lipid-II, made up of both L- and D- amino acids, is attached via its N-terminus to the carboxyl group of MurNAc and in general consists of L-Ala-D-Glu-L-Lys(or meso-DAP)-D-Ala-D-Ala, where the third position amino acid is generally L-Lys in Gram-positive bacteria, and meso-DAP in Gram-negative bacteria and some Gram-positive bacteria such as Mycobacteria and Bacilli (Munch and Sahl 2015). The amino acids are added sequentially by MurC (L-Ala), MurD (D-Glu), MurE (L-Lys or meso-DAP), MurF (D-Ala-D-Ala), where the fourth and fifth position D-Ala-D-Ala is commonly added as a dipeptide, pre-formed by Ddl ligase (Munch and Sahl 2015).

1.2.1.2. The lipid-linked stage

The second stage of peptidoglycan synthesis occurs at the inner cytoplasmic membrane, where nascent UDP-MurNAc pentapeptide is attached to the membrane acceptor undecaprenyl phosphate (C55-P, also known as bactoprenol) by MraY leading to the concomitant release of UMP and the production of lipid-I which is now anchored to the bacterial membrane (Scheffers and Pinho 2005, Bouhss et al, 2007, Pinho et al, 2013). Lipid-II is subsequently produced at the inner-face of the cytoplasmic membrane by the addition of the GlcNAc moiety of UDP-GlcNAc to the C4 hydroxyl group of lipid-I by MurG, resulting in β-linked GlcNAc-MurNAc sugar residues (Scheffers and Pinho 2005, Bouhss et al, 2007, Pinho et al, 2013).

1.2.1.2.1. Variation in the pentapeptide stem

Whilst the general structure of the pentapeptide stem is discussed above, there are many species-specific modifications that occur to the pentapeptide stem, as in Table 1.2. Although in general, the third position amino acid is usually L-Lys or meso-DAP in Gram-positive and -negative bacteria, respectively, in some species other diamino
acids such as L-orthinine, D-Lys and L-2,4-diaminobutyricacid may be present instead (Schleifer and Kandler 1972, Munch and Sahl 2015).

Furthermore, vancomycin resistant bacterial strains can have either D-lactate or D-Ser at the fifth position of the pentapeptide stem, rather than the terminal D-Ala; this is due to the acquisition of transposable elements which encode for D-Ala-D-lactate or D-Ala-D-Ser ligases, respectively (Healy et al, 2000, Munch and Sahl 2015).

**Table 1.2. Common variations in the UDP-MurNAc pentapeptide stem.** This is not an exhaustive list of variations or organisms, but is included as an overview. Adapted from (Vollmer et al, 2008).

<table>
<thead>
<tr>
<th>Pentapeptide stem position</th>
<th>Amino acid variation</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>L-Ala</td>
<td>Most species</td>
</tr>
<tr>
<td></td>
<td>Gly</td>
<td><em>Mycobacterium leprae</em></td>
</tr>
<tr>
<td></td>
<td>L-Ser</td>
<td><em>Butyribacterium rettgeri</em></td>
</tr>
<tr>
<td>2</td>
<td>D-Glu</td>
<td>Most Gram-negative</td>
</tr>
<tr>
<td></td>
<td>D-Gln</td>
<td>Most Gram-positive, <em>Mycobacteria</em></td>
</tr>
<tr>
<td>3</td>
<td>meso-DAP</td>
<td>Most Gram-negative, <em>Bacilli, Mycobacteria</em></td>
</tr>
<tr>
<td></td>
<td>L-Lys</td>
<td>Most Gram-positive</td>
</tr>
<tr>
<td></td>
<td>L-Orthinine</td>
<td>Spirochetes, <em>Thermophilus thermophilus</em></td>
</tr>
<tr>
<td></td>
<td>Amidated meso-DAP</td>
<td><em>Bacillus subtilis</em></td>
</tr>
<tr>
<td>4</td>
<td>D-Ala</td>
<td>All bacteria</td>
</tr>
<tr>
<td>5</td>
<td>D-Ala</td>
<td>Most bacteria</td>
</tr>
<tr>
<td></td>
<td>D-Ser</td>
<td><em>Enterococcus gallinarum</em></td>
</tr>
<tr>
<td></td>
<td>D-lactate</td>
<td>Enterococci with acquired vancomycin resistant strains, <em>Lactobacillus casei</em></td>
</tr>
</tbody>
</table>
The pentapeptide stem can also be amidated in Gram-positive bacteria at the α-carboxyl group of the second position D-Glu to form D-isoGlu (D-Gln) and in Gram-negative bacteria at the ε-carboxyl group of meso-DAP (Vollmer et al, 2008). Glutamate amidation has shown to be performed by a heterodimer protein complex of GatD glutaminase and MurT synthetase which interact through the C-terminal domain of MurT - essential for the amidation of lipid-II and the formation of peptidoglycan in Gram-positive bacteria (Münch et al, 2012, Zapun et al, 2013, Gonçalves et al, 2019). The GatD-MurT complex uses L-glutamine as a nitrogen donor, allowing ammonia to be shuttled across to the MurT active site from the GatD active site where MurT can amidate lipid-II in an ATP-dependent manner (Münch et al, 2012). It has not yet been determined whether amidation of the lipid-II pentapeptide stem occurs before or after the formation of the inter-peptide bridge. However, amidation can occur once UDP-MurNAc pentapeptide is linked to undecaprenol pyrophosphate/bactoprenol, when lipid-I is formed (Münch et al, 2012).

Once lipid-II is formed a series of amino acids are appended to the third position L-Lys or meso-DAP of the pentapeptide stem – this is termed lipid-II ‘branching’. These amino acids will later serve to form an interpeptide bridge between stem peptides that are characteristic of the cross-linked peptidoglycan found in many Gram-positive organisms. These amino acids are appended to the pentapeptide stem by enzymes that generally fall into two classes; the Fem transferases which append glycine or L-amino acids to the pentapeptide stem via acylated-tRNA in a non-ribosomal dependent fashion, or ATP-grasp enzymes which append D-amino acids to the pentapeptide stem via ATP-dependent ligation (Vollmer et al, 2008). The amino acid precursors and the ligating enzymes vary between bacterial Genera, as in Table 1.3.

Following lipid-II branch formation, the nascent mature lipid-II is flipped across the cytoplasmic membrane by an enzyme – the identify of which is the topic of popular scientific debate. Recent evidence suggests that there are two main contenders – MurJ and/or FtsW/RodA. FtsW and its homologue RodA are members of the SEDS (Shape, Elongation, Division and Sporulation) proteins; whilst RodA is essential in
rod-shaped bacteria, its homologue FtsW remains essential for cell wall assembly at the septum during cell division, regardless of bacterial cell shape (Scheffers and Tol 2015, Taguchi et al, 2019). MurJ by contrast is conserved across all bacteria which contain peptidoglycan, and is an integral membrane protein that is part of the MOP (multidrug/oligosaccharidyl-lipid/polysaccharide) exporter family – a family of proteins which also contains transporters of other undecaprenyl-linked molecules (Scheffers and Tol 2015).

**Table 1.3. An overview of the various peptide cross-bridges in peptidoglycan.**

*FemX appends the first position cross-bridge L-Ala, the second and third amino acids are appended by an unknown enzyme. Adapted from (Vollmer et al, 2008)*

<table>
<thead>
<tr>
<th>Organism</th>
<th>Cross-bridge</th>
<th>Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Streptococcus pneumoniae</em></td>
<td>L-Ser-L-Ala or L-Ala-L-Ala</td>
<td>MurM, MurN</td>
</tr>
<tr>
<td><em>Weissella virens</em></td>
<td>L-Ala-L-Ser or L-Ala-L-Ser-L-Ala</td>
<td>FemX*</td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em></td>
<td>L-Ala-L-Ala</td>
<td>BppA1, BppA2</td>
</tr>
<tr>
<td><em>Enterococcus faecium</em></td>
<td>D-Asp</td>
<td>Asf_{im}</td>
</tr>
</tbody>
</table>

### 1.2.1.2.2. FtsW or MurJ as the lipid-II flippase?

Recent work from the Breukink group has shown that FtsW must be present for lipid-II flipping to occur. This was shown in a novel *in vitro* fluorescence resonance energy transfer (FRET)-based flipping assay in *E. coli* membrane vesicles – strains with overexpressed FtsW resulted in increased lipid-II flipping when compared to wild-type strains, whilst strains with depleted FtsW resulted in decreased lipid-II flipping (Mohammadi et al, 2011). To demonstrate that this lipid-II flipping was due directly to FtsW, this protein was reconstituted into model proteoliposome membranes and was shown to induce trans-bilayer movement of lipid-II, where control proteins did
not (Mohammadi et al, 2011). Interestingly, in the same FRET assay, overexpression of MurJ did not increase lipid-II transport in *E. coli* vesicles, and purified MurJ did not induce lipid-II translocation across the lipid bilayer in model membranes (Mohammadi et al, 2011).

Furthermore, FtsW and RodA have recently been shown to have transglycosylation activity, however, of course this does not mean they cannot be bifunctional enzymes and act as both a transglycosylase and a flippase enzyme (Cho et al, 2016, Meeske et al, 2016, Emami et al, 2017, Taguchi et al, 2019).

On the flip side, bioinformatic data is suggestive of MurJ being a likely lipid-II flippase owing to its conservation across peptidoglycan producing bacteria, and its absence in non-peptidoglycan producing bacteria (Ruiz 2008). Furthermore, FtsW was shown to be absent from some peptidoglycan producing bacteria, yet present in bacteria with no peptidoglycan (Ruiz 2008). The *murJ* gene has also been shown to be essential in temperate sensitive strains of *E. coli*, which when *murJ* is mutated, exhibit cell swelling and an increasing likelihood of lysis and ghost cell formation suggesting defective cell wall rigidity (Ruiz 2008). Contrary to MurJ essentiality in *E. coli*, it has been shown that *B. subtilis* lacking any of its four MurJ homologues have no defects in growth or cell shape (Fay and Dworkin 2009, Meeske et al, 2015). Depletion of Amj (alternate to MurJ), in the absence of MurJ, however, causes defects in cell shape and cell lysis in *B. subtilis* highlighting another potential flippase candidate (Meeske et al, 2015). Amj is a formerly uncharacterised protein, which bears no sequence similarity to either MurJ or FtsW (Meeske et al, 2015, Bolla et al, 2018).

However, perhaps the strongest piece of evidence for MurJ as a lipid-II flippase comes from the Ruiz and Bernhardt groups who designed an *in vivo* lipid-II flippase assay in which radiolabelled lipid-II is translocated across the membrane where it can be cleaved by colicin M – a secreted toxin from some *E. coli* strains that cleaves lipid-II once it has entered into the target cell periplasm (Sham et al, 2014, Ruiz 2015, Scheffers and Tol 2015). As lipid-II is radiolabelled, both the uncleaved intracellular lipid-II and the cleavage products generated from periplasmic lipid-II can be
detected. Those cells which expressed MurJ were able to transport lipid-II, whilst those cells with a MurJ mutant capable of inducible inactivation, were unable to transport lipid-II (Sham et al, 2014). Interestingly, lipid-II translocation was able to occur in the absence of the RodA/FtsW (Sham et al, 2014).

1.2.1.3. The extracellular stage

Once lipid-II has been flipped across the membrane by either MurJ or FtsW, the final extracellular stage of peptidoglycan synthesis occurs at the outer face of the cytoplasmic membrane. Firstly lipid-II is polymerised into glycan strands (transglycosylation, illustrated in Fig. 1.4) by penicillin-binding proteins (PBPs) or mono-functional glycosyltransferases (MGTs) – the sugar backbone of lipid-II is polymerised by the condensation of the C-4 hydroxy of GlcNAc of one lipid-II with the reducing end of the MurNAc of a second lipid-II, leading to the concomitant release of undecaprenyl pyrophosphate (van Heijenoort 2001, Scheffers and Pinho 2005). This newly released undecaprenyl pyrophosphate is then dephosphorylated to yield undecaprenyl phosphate/bactoprenol which can be recycled for use in the next round of lipid-II synthesis and translocation (van Heijenoort 2001, Scheffers and Pinho 2005). Glycan chains are then cross-linked via the peptide branch (transpeptidation, illustrated in Fig. 1.4) by PBPs. This transglycosylation and transpeptidation of lipid-II by PBPs results in a highly cross-linked, mesh like layer of peptidoglycan that serves as a rigid backbone to maintain cell shape and morphology.
Figure 1.4: Transglycosylation and transpeptidation in *S. aureus*. Lipid-II is flipped to the extracellular space where the disaccharide units (GlcNAc and MurNAc) are polymerised into glycan strands by transglycosylases. Glycan strands are cross-linked via the pentaglycine branch by transpeptidases. The lipid carrier is translocated back into the cytoplasmic space for further lipid-II synthesis.
1.3. **Penicillin-binding proteins**

The PBP forms a non-covalent complex with the donor glycan strand allowing the active site serine to attack the D-Ala-D-Ala peptide bond (Scheffers and Pinho 2005, Sauvage *et al*, 2008). This forms an acyl-enzyme complex and the concomitant cleavage and release of the terminal D-Ala from the donor pentapeptide stem (Scheffers and Pinho 2005, Sauvage *et al*, 2008). The cleavage reaction provides the energy required, in the absence of ATP, for a peptide bond to be formed between the fourth position D-Ala (donor) and the acceptor which is either the terminal residue of the pentapeptide branch, or the third position of the pentapeptide stem, if no pentapeptide branch exists (Scheffers and Pinho 2005, Sauvage *et al*, 2008).

PBPs are membrane associated proteins classified into two groups, the low molecular mass (LMM) and high molecular mass (HMM) PBPs, as illustrated in Fig. 1.5 (Sauvage *et al*, 2008). The HMM PBPs (~60-140 kDa) are further subdivided into Class A (bi-functional) and Class B (mono-functional) depending on their catalytic activity and structure (Waxman and Strominger 1983, Sauvage *et al*, 2008, Pinho *et al*, 2013). They consist of a transmembrane (TM) anchor and two extracellular domains which are joined by a β sheet rich linker (Sauvage *et al*, 2008). In both HMM classes, the C-terminal domain has transpeptidase activity, however, the N-terminal domain of the Class A PBPs is responsible for lipid-II transglycosylase activity and the N-terminal domain of Class B interacts with proteins involved in cell morphogenesis (Sauvage *et al*, 2008, Pinho *et al*, 2013).

In addition, the LMM PBPs (~40-50 kDa), referred to as Class C, generally only have N-terminal DD-carboxypeptidase activity (mono-functional) (Waxman and Strominger 1983, Sauvage *et al*, 2008, Pinho *et al*, 2013). Furthermore, the topology of LMM PBPs is unusual as the N-terminal penicillin binding domain is encoded for after a signal peptide domain and has a C-terminal trans-membrane or amphipathic helix (Macheboeuf *et al*, 2006).
Additionally, MGTs play a complimentary role in peptidoglycan synthesis due to their transglycosylase domain, similar to that of Class A HMM PBPs, however MGTs are not PBPs and belong to neither the HMM or LMM PBP classes (Spratt et al, 1996, Macheboeuf et al, 2006, Sauvage et al, 2008).

As briefly covered in Table 1.4, each bacterium has its own set of PBPs and due to the evolution of nomenclature and the timeline of PBP discovery, the specific name of each PBP as well as which class that PBP belongs to can be confusing. For example, PBP2 is a class B PBP in *E. coli*, yet is designated as a class A PBP in *S. aureus*.

1.3.1. Inhibitors of PBPs

PBPs can be inhibited either by direct inhibition of their transglycosylase or transpeptidase activity, or indirectly by sequestering the PBP transglycosylase substrate, lipid-II. For example, vancomycin binds to the terminal D-Ala-D-Ala of lipid-II, masking the D-Ala-D-Ala termini of the transpeptidase donor from PBPs preventing cross-linking (Galley et al, 2014). To date, the only antibiotic able to inhibit PBP transglycosylase activity is moenomycin; an antibiotic of the glycolipid family naturally produced by the organism *Streptomyces ghananensis* (Ostash and Walker 2005, Galley et al, 2014). The main family of transpeptidase inhibitor are the β-lactams which are structurally similar to the terminal D-Ala-D-Ala of lipid-II, such that the β-lactams bind to the active site serine in the transpeptidase domain of the PBP (Zapun et al, 2008). Inhibitors of transglycosylase and transpeptidase activity are discussed in further detail in Chapter 7.
Figure 1.5. The classes of penicillin-binding proteins. The penicillin-binding proteins (PBP) are broadly grouped into two classes, high molecular mass (HMM) and low molecular mass (LMM). Mono-functional glycosyltransferases (MGT) are not PBP, however play a complimentary role due to their trans-glycosylase domain. - β-rich linker, N: N-terminal, C: C-terminal, TM: trans-membrane domain, AH: amphipathic helix, SP: signal peptide domain. Figure adapted from Macheboeuf et al (2006).
Table 1.4. An overview of the different penicillin binding proteins and their classes across bacteria. This table includes two Gram negative (E. coli and N. gonorrhoeae) and two Gram positive (S. aureus and S. pneumoniae) bacteria, and is not intended as an exhaustive list. This table does not include MGTs. Common protein name – non italics. Gene name – italics. Adapted from Sauvage et al (2008).

<table>
<thead>
<tr>
<th>Organism</th>
<th>Class A</th>
<th>Class B</th>
<th>Class C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A1</td>
<td>A2</td>
<td>A3</td>
</tr>
<tr>
<td>Escherichia coli K12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neisseria gonorrhoeae FA1090</td>
<td>PBP1</td>
<td>PBP1b</td>
<td>PBP1c</td>
</tr>
<tr>
<td>Bacillus subtilis 168</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staphylococcus aureus MRSA252</td>
<td>PBP2</td>
<td>PBP2</td>
<td>PBP2</td>
</tr>
<tr>
<td>Streptococcus pneumoniae R5</td>
<td>PBP1a</td>
<td>PBP1a</td>
<td>PBP1b</td>
</tr>
</tbody>
</table>

Adapted from Sauvage et al (2008).
1.4. **S. aureus**

*S. aureus*, a Gram-positive bacterium, is both a commensal organism and a major causative agent of infectious disease in both humans and animals. *S. aureus* is a well-known leading cause of infective endocarditis and skin and soft tissue infections in humans, however it is also a serious problem in agricultural livestock, defined here as cattle, pigs and poultry (Fluit 2012, Peacock 2015, Smith 2015, Tong et al, 2015). Whilst pigs are rarely infected by *S. aureus*, and are usually carriers of the bacterium, *S. aureus* is known to cause systemic infections in farmed rabbits, as well as bumblefoot, lameness and septicaemia in poultry (Fluit 2012, Peacock 2015, Smith 2015). *S. aureus* is also one of the major aetiologicaal agents causing bovine mastitis in dairy cattle and other ruminants, majorly affecting animal health and causing estimated economical losses of approximately €300 per cow per year (Fluit 2012, Peacock 2015, Smith 2015).

1.4.1. **Methicillin-resistant S. aureus**

The threat posed by *S. aureus* has been amplified by the emergence of methicillin resistance following the acquisition of the mecA gene via horizontal gene transfer of the mobile genetic element SCCmec - the Staphylococcal cassette chromosome mec (Rolo et al, 2017). The mecA gene encodes for an extra class B HMM PBP, commonly known as MecA or, as will be referred to in this project, PBP2a (Sauvage et al, 2008, Rolo et al, 2017).

In methicillin-sensitive *S. aureus* (MSSA) which normally have four PBPs (One class A HMM PBP, two class B HMM PBP and one LMM PBP), β-lactam antibiotics such as methicillin prevent cross-linking of peptidoglycan strands of the bacterial cell wall, therefore leading to cell lysis (Sauvage et al, 2008). This occurs because β-lactam antibiotics are structural analogues of the terminal D-Ala-D-Ala residues of the lipid-II pentapeptide stem, which are recognised by PBPs during peptidoglycan cross-linking (Wilke et al, 2005). Thus, β-lactam antibiotics bind irreversibly to the active
site serine of the transpeptidase domain of PBPs, preventing peptidoglycan cross-linking, resulting in bacterial cell lysis and cell death (Chambers 1999, Wilke et al, 2005, Acebron et al, 2015).

However, methicillin-resistant *S. aureus* (MRSA) have acquired PBP2a which is responsible for high-level β-lactam resistance in *S. aureus* due to the exceptionally low affinity of its active site for β-lactam antibiotics, thus peptidoglycan cross-linking can proceed in the presence of methicillin at concentrations which would otherwise be lethal to MSSA strains (Chambers 1999, Sauvage et al, 2008).

1.4.1.1. The emergence of livestock-associated MRSA


Resistant strains of livestock-associated *S. aureus* have started to arise - partly due to common practices in farming and animal husbandry, such as the prophylactic treatment of livestock (Smith 2015). Antibiotics are often used as additives in livestock feed in order to promote livestock growth, prevent bacterial infection and to treat existing infection in a herd, and hence increase profit (Mole 2013, Smith 2015). Reviewing the antibiotic usage in the USA over the last decade highlights the scale of this antibiotic usage in farming. In 2009, 80% of the antibiotics sold in the USA were used in farming, with 61% (approximately 7,687 tonnes) of these being medically-important antibiotics (Mole 2013, FDA 2016). More shockingly, the Food and Drug Administration reported that in 2011, 93% of these medically-important
antibiotics used in farming were administered to either feed or water as prophylactic treatment and were therefore not use therapeutically (Smith 2015, FDA 2016). In addition, it was estimated that 75-90% of these antibiotics were excreted unmetabolised from livestock and therefore entered sewage and water sources (Marshall and Levy 2011, FDA 2014).

Most recent reports by the FDA show that whilst the proportion of medically-important antibiotics used in food-producing farming has remained the same, the total usage of antibiotics has increased to approximately 9,702 tonnes – a staggering increase in usage of 26% from 2009 to 2015 (FDA 2016).

Whilst this is by no means an in-depth study into the use of antibiotics in farming, it is a reflection on the state of usage of medically-important antibiotics in the USA. There is a growing problem with the widespread of use of antibiotics for prophylaxis in farming, and this is highlighted in the USA, arguably one of the largest and most influential countries in the world. This widespread and liberal use of antibiotics is not a problem confined to one country, and is being seen globally in both agricultural and healthcare settings. The overuse of medically important antibiotics increases the risk of the emergence of antibiotic resistance, and any resistant strains of \textit{S. aureus} arising from such usage of these antibiotics not only poses a threat to agricultural healthcare, but also to human healthcare. This is exemplified by new evidence which is now emerging that is highly suggestive of the direct passage of MRSA from livestock to farm worker, and \textit{vice versa}, and has been noted on cattle, poultry and swine farms across the world (van Rijen \textit{et al}, 2008, Dahms \textit{et al}, 2014, Unnerstad \textit{et al}, 2018).

1.4.2. Peptidoglycan synthesis in MRSA

1.4.2.1. MRSA and the Fem ligases
Peptidoglycan synthesis in S. aureus follows the same general pathway as outlined in Section 1.2, however with a few slight differences that yield S. aureus lipid-II consisting of an L-Ala-D-iGln-L-Lys-D-Ala-D-Ala pentapeptide stem, with a pentaglycyl branch position at the third position L-Lys – denoted amidated lipid-II (Lys) pentaglycine (Kuhner et al. 2014). The pentaglycyl branch is appended to lipid-II by the Fem peptidyl-transferases (or Fem ligases, as they will be referred to henceforth) at the ε-amino group of the third position lysyl residue of the stem peptide (Fig. 1.3) (Scheffers and Pinho 2005, Pinho et al, 2013). FemX is responsible for the addition of the first glycine residue, followed by FemA which adds the second and third glycine residues and finally, FemB which adds the fourth and fifth glycine residues. However, the co-ordination with lipid-II amidation has not yet been established and it is unknown whether amidation of lipid-II occurs before or after pentaglycyl branch formation. Chapter 5 of this thesis aims to elucidate this.

Unlike other bacteria, such as Lactobacillus viridescens (Weisella viridescens), the Fem ligases of S. aureus can only append the pentaglycyl branch to the membrane bound substrate lipid-II, whilst the aforementioned bacteria have the ability to append their respective peptide branches to UDP-MurNAc pentapeptide before conversion to lipid-II (Bouhss et al, 2001, Hegde and Shrader 2001, Schneider et al, 2004).

The pentaglycyl branch of S. aureus is crucial for the development of the highly cross-linked mature peptidoglycan, so much so that a separate pool of tRNA\textsuperscript{Gly} is dedicated to utilisation by the Fem ligases during peptidoglycan synthesis (Giannouli et al, 2009). This separate pool of tRNA\textsuperscript{Gly} is comprised of tRNA\textsuperscript{Gly} species lacking crucial recognition elements essential for binding to Ef-Tu, thereby reserving a pool of glycyl-tRNA\textsuperscript{Gly} exclusive to peptidoglycan synthesis that is incapable of supporting ribosomal protein synthesis (Giannouli et al, 2009). The essentiality of the Fem ligases and the result of their individual deletion is discussed in further detail Chapter 5.
1.4.2.2. MRSA and the penicillin binding proteins

MRSA has five PBPs – one class A bi-functional PBP with both transglycosylase and transpeptidase activity (PBP2), three class B PBPs with transpeptidase activity (PBP1, PBP3 and the acquired PBP2a) and one class C PBP with transpeptidase activity (PBP4). With regard to the class B PBPs in \textit{S. aureus}, RodA-PBP3 and FtsW-PBP1 form cognate pairs and mediate sidewall and septal peptidoglycan incorporation, respectively, and their activity is balanced to maintain coccoid morphology (Reichmann \textit{et al}, 2019). The main focus of this thesis will be PBP2, the only PBP with transglycosylase activity, and PBP2a, the resistance determinant of MRSA.

\textit{S. aureus} PBPs are membrane proteins tethered to the membrane by an N-terminal transmembrane (TM) helix. The truncation of these PBPs such that there is no TM helix aids in their ease of purification, and as such, both PBP2 (minus the TM region residues 1-58) and PBP2a (minus the TM region residues 1-22) are the most widely studied versions of these PBPs – from here, denoted as PBP2-W59M and PBP2a-Y23M, respectively.

Crystal structures of both PBP2-W59M and PBP2a-Y23M have been obtained, both in the apoenzyme form and in complex with various substrates (Lim and Strynadka 2002, Lovering \textit{et al}, 2007). The apoenzymes of each are shown in Fig. 1.6. PBP2-W59M consists of a transglycosylase region (Fig. 1.6, light purple, residues 82-257) with two active site glutamic acid residues at position E114 and E171 (Fig. 1.6, highlighted in orange). Linked to the transglycosylase domain, by a linker region shown in white, is the transpeptidase domain (Fig. 1.6, residues 361-631) which has an active site serine at residue S398.

PBP2a-Y23M on the other hand has only a transpeptidase domain, shown in Fig. 1.6 (Pink, residues 327-668), where the active site serine is residue S403 (Lim and Strynadka 2002). PBP2a-Y23M also has an allosteric domain (Fig. 1.6, cyan, residues 27-326), sometimes referred to as the non-penicillin binding domain (Lim and Strynadka 2002, Fuda \textit{et al}, 2005, Otero \textit{et al}, 2013, Fishovitz \textit{et al}, 2014). The
transpeptidase active site of PBP2a-Y23M is in a largely inaccessible narrow cleft which is under allosteric control (Lim and Strynadka 2002, Otero et al, 2013). When a substrate binds to the allosteric domain, approximately 60 Å from the transpeptidase active site serine, PBP2a-Y23M undergoes a conformation change involving modifications of salt bridge interactions, allowing the active site serine to change to its open conformation via conformational changes of the β3 sheet and the α2 helix (Lim and Strynadka 2002, Peacock 2015). In the apoenzyme conformation, the PBP2a-Y23M active site serine is in a poor position for nucleophilic attack (Lim and Strynadka 2002). Therefore, the twisting of the β3 sheet is required to prevent steric clash of Ser598 and Ser403 and is followed by the concomitant α2 helix movement such that Ser403 is now accessible (Lim and Strynadka 2002). The low affinity of β-lactams for PBP2a-Y23M is suggested to be due to their bulky side groups and hence are unable to fit into the narrow active site cleft of PBP2a-Y23M (Lim and Strynadka 2002). In the case of PBP2-W59M, the active site Ser398 is approximately 2 Å more exposed than the PBP2a-Y23M active site serine Ser403, and is therefore much more vulnerable to acylation by β-lactams (Lovering et al, 2007, Lovering et al, 2012).
Figure 1.6. Crystal structures of PBP2a-Y23M and PBP2-W59M.

PBP2a-Y23M crystal structure – PDB 1VQQ (Lim and Strynadka, 2002).
Highlighted in yellow is the transpeptidase active site serine, S403.

PBP2-W59M crystal structure – PDB 2OLU (Lovering et al, 2007).
Light purple – transglycosylase domain, residues 82-257.
Highlighted in orange are the two catalytic site glutamic acid residues E114, and E171.
Highlighted in yellow is the transpeptidase active site serine, residue S398.
1.4.3. **Is peptidoglycan synthesis a realistic target for antibiotic therapy in MRSA?**

Peptidoglycan synthesis remains an excellent target for antibiotics, largely due to the complex and conserved nature of its biosynthesis (Bugg *et al.*, 2011). There are many different enzymes and substrates within the peptidoglycan biosynthesis pathway, all making excellent targets to block the downstream synthesis of peptidoglycan. Furthermore, the inhibition of more than one part of the pathway may also act to reduce the emergence of resistance.

1.5. **Aims of thesis**

Antibiotic resistance is one of the biggest threats to both livestock and human health, and with very few antibiotics in the pipeline to tackle this problem, the outlook for future generations is daunting. This thesis aims to elucidate the mechanisms of resistance in the global pathogen MRSA through an understanding of the substrate specificities of the Fem ligases, PBP2 and PBP2a, and how these proteins work in synergy to synthesise peptidoglycan in the presence of β-lactam antibiotics.

Understanding the key role of these proteins will help us to understand how organisms evade antibiotic therapy, and may eventually lead to the design of novel antibiotics towards these targets. This may also allow for the design of novel combination therapies which may give new life to antibiotics with a reduced efficacy due to the evolution of resistance.
2. Materials and methods

2.1 Buffers and solutions

All analytical grade chemicals were purchased from the following suppliers, unless otherwise stated; Acros Organics (USA), Bachem (Germany), Calbiochem (USA), Oxoid (UK), Sigma-Alrich/Merck (UK), and Thermo Fischer Scientific (USA).

All buffers were made using MilliQ purified water and the pH of solutions measured using a Mettler Toledo SevenEasy pH machine with pH 4, pH 7 and pH 10 buffer standards. Before chromatography, all buffers were passed through a 0.2 µm filter.

2.2 Bacterial strains

2.2.1. *Escherichia coli* strains

The *Escherichia coli* strains used for DNA cloning and recombinant protein expression in this project are summarised in Table 2.1.

2.2.2. *Staphylococcal* strains

*Staphylococcus aureus* ATCC 25923 was used for tRNA, membrane and amidated lipid-II (Lys) pentaglycine extraction, whilst *S. epidermidis* ATCC 12228 was used as a source of membranes only.

2.2.3. Bacterial growth media

2.2.3.1. Luria-Bertani (LB)-agar plates

Luria-Bertani media (Table 2.2) plus 1.5% w/v bacto-agar was autoclaved and allowed to cool before the addition of appropriate antibiotics. To a sterile petri dish, 25 ml of
the appropriately supplemented media was poured and allowed to set before storage at 4°C.

Table 2.1. *E. coli* strains used for DNA cloning and protein expression.

<table>
<thead>
<tr>
<th><em>E. coli</em> strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TOP10</td>
<td>F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZ∆M15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str&lt;sup&gt;8&lt;/sup&gt;) endA1 λ&lt;sup&gt;-&lt;/sup&gt;</td>
<td>(Grant et al, 1990)</td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>F&lt;sup&gt;-&lt;/sup&gt; ompT gal dcm hsdSa(r&lt;sub&gt;ε&lt;/sub&gt; m&lt;sub&gt;ε&lt;/sub&gt;) λ(DE3)</td>
<td>(Studier 2005)</td>
</tr>
<tr>
<td>BL21(DE3)Star.pRosetta</td>
<td>F&lt;sup&gt;-&lt;/sup&gt; ompT gal dcm hsdSa(r&lt;sub&gt;ε&lt;/sub&gt; m&lt;sub&gt;ε&lt;/sub&gt;) λ(DE3) plus pRosetta plasmid for rare tRNA codon overexpression</td>
<td>(Studier 2005)</td>
</tr>
<tr>
<td>C41(DE3)</td>
<td>F&lt;sup&gt;-&lt;/sup&gt; ompT gal dcm hsdSa(r&lt;sub&gt;ε&lt;/sub&gt; m&lt;sub&gt;ε&lt;/sub&gt;) λ(DE3)pLysS (Cm&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>(Miroux and Walker 1996)</td>
</tr>
<tr>
<td>C43(DE3)</td>
<td>F&lt;sup&gt;-&lt;/sup&gt; ompT gal dcm hsdSa(r&lt;sub&gt;ε&lt;/sub&gt; m&lt;sub&gt;ε&lt;/sub&gt;) λ(DE3)pLysS (Cm&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>(Miroux and Walker 1996)</td>
</tr>
<tr>
<td>Lemo21(DE3)</td>
<td>fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS/ pLemo(CamR) λ DE3 = λ sBamHlo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5 pLemo = pACYC184-PrhaBAD-lysY</td>
<td>(Wagner et al, 2008)</td>
</tr>
</tbody>
</table>

2.2.3.2. Growth media

The following bacterial growth media (Table 2.2) were used throughout this project.
Table 2.2. The components of bacterial growth media used in this project.

<table>
<thead>
<tr>
<th>Media</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>Super Optimal Broth with Catabolite Repression (SOC) (Hanahan 1983)</td>
<td>2% (w/v) vegetable peptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄ and 20 mM glucose</td>
</tr>
<tr>
<td>Luria-Bertani (LB) (Bertani 1951)</td>
<td>1% (w/v) tryptone, 0.5% (w/v) NaCl, 0.5% (w/v) yeast extract</td>
</tr>
<tr>
<td>Nutrient Broth (NB)</td>
<td>0.% (w/v) D-glucose, 1.5% (w/v) peptone, 0.6% (w/v) NaCl, 0.3% (w/v) yeast extract</td>
</tr>
<tr>
<td>Tryptic Soy Broth (TSB)</td>
<td>1.7% (w/v) pancreatic casein peptone, 0.25% (w/v) disodium hydrogen phosphate, 0.25% (w/v) D-glucose, 0.5% (w/v) NaCl, 0.3% (w/v) soya peptone</td>
</tr>
<tr>
<td>Bovine Heart Infusion (BHI)</td>
<td>0.5% (w/v) beef heart, 1.25% (w/v) calf brain, 0.025% (w/v) disodium hydrogen phosphate, 0.2% (w/v) D-glucose, 1% (w/v) peptone, 0.5% (w/v) NaCl</td>
</tr>
</tbody>
</table>

2.2.4. Preparation of *E. coli* competent cells

*E. coli* strains were streaked onto LB-agar plates with appropriate antibiotics and incubated overnight at 37°C. One colony was inoculated into 2.5 ml LB and grown overnight at 37°C, with shaking at 180 rpm. The overnight culture (2.5 ml) was inoculated into 250 ml LB plus 20 mM MgCl₂, and grown to A₆₀₀ 0.5-0.6 at 37°C, 180 rpm. Cells were pelleted at 4,000 rpm at 4°C for 10 minutes using an Eppendorf 5810R centrifuge. Pellets were resuspended in 100 ml ice cold TFB1 (30 mM potassium acetate, 10 mM CaCl₂, 50 mM MnCl₂, 100 mM RbCl, 15% (v/v) glycerol, pH 5.8), incubated on ice for 5 minutes and centrifuged as previously. Pellets were resuspended in 10 ml ice cold TFB2 (10 mM MOPS (3-(N-morpholino)-propanesulfonic acid), 75 mM CaCl₂, 10 mM RbCl, 15% (v/v) glycerol, pH 6.5) and
incubated for 60 minutes on ice. Competent cells were aliquoted (50 µl), flash frozen with liquid nitrogen and stored at -80°C.

### 2.2.5. Transformation of *E. coli* competent cells

Plasmid DNA (2 µl) was incubated with 50 µl of the appropriate competent cell suspension on ice for 15 minutes. Cells were heat shocked in a 42°C water bath for 30 seconds and immediately incubated on ice for 2.5 minutes. SOC media (200 µl) was added and cells were incubated at 37°C, 180 rpm for 30-45 minutes before 150 µl of the cell suspension was spread onto LB-agar with appropriate antibiotic, and incubated overnight at 37°C.

### 2.2.6. Preparation of glycerol stocks

Following fresh transformation, one colony was inoculated into 10 ml LB and incubated overnight at 37°C, 180 rpm. A (500 µl) aliquot of the culture was mixed with 500 µl of 40% (v/v) sterile glycerol, and flash frozen with liquid nitrogen before storage at -80°C.

### 2.2.7. Determining bacterial cell count

To measure the bacterial cell count over time, samples of bacterial culture were taken at set hourly intervals. Samples were serially diluted (10⁻¹-10⁻⁹), and 20 µl of each dilution was spotted onto LB-agar. Plates were dried in the laminar flow hood before incubating overnight at 37°C.

### 2.2.8. Bacterial turbidity assays

Bacterial cultures grown overnight at 37°C, with shaking at 100 rpm, were centrifuged at 4000 rpm (Eppendorf 5810R) and resuspended in PBS. Turbidity assays were performed at 600 nm in a volume of 150 µl for 60 minutes at 37°C to measure
lysis of *S. aureus* ATCC 25923 and *S. epidermidis* ATCC 12228 in the presence of 0 - 6 μg/ml lysostaphin (Dr Stephene Mesnage, Sheffield), 0 - 2.5 mg/ml hen egg white lysozyme, combinations of both lysostaphin and lysozyme, and 3 - 50 μg/ml autolysin (Dr Christopher Thoroughgood, Warwick).

### 2.3. Preparation of Staphylococcal tRNA

#### 2.3.1. Growth of *Staphylococcus aureus* ATCC 25923

*S. aureus* ATCC 25923 was grown on BHI agar, overnight at 37°C. One colony was inoculated into 10 ml BHI, and grown overnight at 37°C, with shaking at 50 rpm. Following overnight growth, 10 ml of overnight culture was inoculated into 1 L of BHI, and grown at 37°C, with shaking at 50 rpm until the OD$_{600nm}$ of the culture reached 0.5-1.5. Cultures were centrifuged 6,000 xg, for 15 min at 4°C. Pellets were washed with 20 mM Tris (2-amino-2(hydroxymethyl)propane-1,3-diol), 1 mM MgCl$_2$, 2 mM β-mercaptoethanol, pH 7.5 and centrifuged again as previously. Pellets were stored at -80°C.

#### 2.3.2. Extraction of tRNA

Where possible, all buffers and equipment used for tRNA extraction were sterile.

##### 2.3.2.1. Initial tRNA extraction

Pellets were defrosted on ice and resuspended in 20 mM Tris, 20 mM magnesium acetate, pH 7.4 (tRNA buffer 1, TB1), supplemented with 5 μg/ml lysostaphin, for 1 hr at 37°C, 180 rpm. An equal volume of phenol was added, and sample was incubated for a further 20 min at room temperature (RT), on rollers. Sample was centrifuged at 5,000 xg for 20 min at 4°C and the aqueous phase retained. To the phenol phase, an equal volume of TB1 was added and the sample was incubated for a further 20 min at RT, on rollers before centrifuging at 5,000 xg for 20 min at 4°C.
The aqueous phase was retained. Aqueous phases were combined and 2x volume of phenol added, and mixed on rollers for 20 min at RT, before centrifuging at 5,000 xg for 20 min at 4°C. The aqueous phase was retained and centrifuged again to remove an residual precipitant from the phenol:aqueous phase boundary. To the resulting aqueous phases, 1/10th volume of 3 M sodium acetate, and 1x volume ice cold isopropanol was added, and tRNA was precipitated overnight at -20°C.

2.3.2.2. tRNA deacetylation

Following overnight precipitation, samples were centrifuged at 8,000 xg, for 20 min at 4°C. The tRNA pellet was washed in 13.5 ml ethanol, and 1.5 ml sterile water, and centrifugation repeated. Pellets were dried under vacuum. tRNA was deacetylated by the addition of 5 ml/pellet of 0.2 M Tris, pH 9 for 30 min, at 37°C. To this, 1/10th volume 3 M sodium acetate, pH 5 and 2x volumes ice cold ethanol was added. tRNA was reprecipitated at -20°C, for 20 min. Sample was centrifuged at 8,000 xg, for 20 min at 4°C, and the pellet was washed with 13.5 ml ethanol followed by 1.5 ml sterile water, and centrifugation was repeated. Supernatant was removed and pellet was dried under vacuum.

2.3.2.3. tRNA purification

tRNA was resuspended in sterile water (approx. 5 ml) and the volume was made up to 50 ml with 10 mM Tris, pH 7.5. tRNA was loaded onto a 15 ml DEAE sephacel column, equilibrated with 10 mM Tris, pH 7.5, and the flowthrough was collected as one fraction. The column was washed with 50 ml of 10 mM Tris, pH 7.5, followed by 50 ml of 10 mM Tris, 0.8 M NaCl, pH 7.5, collected as two separate 50 ml fractions. tRNA was quantified as in Section 2.3.2.4. Fractions containing tRNA were combined, and 5 ml of 3 M sodium acetate, pH 5 and 100 ml of ice-cold ethanol was added per 50 ml sample. tRNA was precipitated overnight at -20°C.
Precipitated tRNA was centrifuged at 8,000 xg, for 20 min at 4°C and the pelleted tRNA washed with 13.5 ml ethanol followed by 1.5 ml sterile water, before centrifugation was repeated. Pellets were resuspended in ethanol, vortexed and centrifuged at 8,000 xg, for 20 min at 4°C. Pellets were dried under vacuum and resuspended in sterile water.

2.3.2.4. Quantification of tRNA

tRNA (2 µl) was quantified at A_{260nm} using the NanoDrop ND60 (Agilent). Sterile water was used as a blank.

2.3.2.5. Visualisation of tRNA

2.3.2.5.1. Urea-Polyacrylamide gel electrophoresis

tRNA (5-15 µg) was visualised on a urea-Polyacrylamide gel electrophoresis (Urea-PAGE) gel (40% (v/v) 29:1 acrylamide:Bis-acrylamide, 42% (w/v) urea in 1xTBE, polymerised with 0.6% (v/v) 10% APS, and 0.06% (v/v) TEMED). tRNA was loaded in 1:1 ratio of urea loading dye (10 M urea, 0.2% bromophenol blue, 0.2% xylene cyanol in 1x TBE). Gels were run in 1x TBE, at 25 V until tRNA entered gel, and 120 V thereafter.

2.3.2.5.2. Staining of Urea-PAGE

All steps took place on a gel rocker at RT. Gel was soaked in fixer solution (49% (v/v) acetone, 1.2% (w/v) TCA, 0.015% (v/v) formaldehyde) for 15 min, then washed quickly 3x with water. The gel was soaked for a further 5 min in water on a gel rocker and again washed quickly 3x with water. The gel was soaked for 5 min in 50% (v/v) acetone, then for 5 min in 1 mM Na₂S₂O₃ before washing quickly 3x with water. The gel was stained for 8 min in 16 mM silver nitrate, 0.37% (v/v) formaldehyde before washing quickly 3x with water. The gel was developed for approximately 30 seconds.
in developer (188 mM Na$_2$CO$_3$, 0.26 mM Na$_2$S$_2$O$_3$, 0.015% (v/v) formaldehyde), or until bands appeared. As soon as bands appeared, the gel was soaked in quencher (1% (v/v) acetic acid) for 1-2 min. The gel was stored in distilled water.

2.4. DNA cloning

2.4.1. Oligonucleotide primers

Oligonucleotide primers were designed against the target gene or target plasmid using NEBuilder Assembly Tool (NEB), and ordered from Integrated DNA Technologies (UK). Primers used are detailed in Table 2.3.

2.4.2. Polymerase chain reaction (PCR)

In order to amplify DNA, Q5 DNA Polymerase (NEB) was used according to manufacturer’s protocol. Unless otherwise stated, PCR reactions were carried out in 50 µl volumes using an Agilent Technologies SureCycler 8800 thermocycler. Annealing temperatures were used according to the oligonucleotide manufacturer protocol (IDT).

2.4.3. Agarose gel electrophoresis and gel extraction of DNA

Agarose powder was added at 0.8% w/v to 1x Tris-acetate EDTA (Ethylenediaminetetraacetic acid) buffer (TAE; 40 mM Tris acetate, 1mM EDTA) by microwaving until fully dissolved. Once cooled to touch, GelRed Nucleic Acid Gel Stain (Biotum) was added (1 µl to 10 ml agarose) before pouring into a gel cast. Once set, the gel was submerged into a Fischer Scientific gel tank containing 1x TAE, and DNA samples were loaded with 1x DNA loading dye (Stock, 6x Purple DNA Loading Dye, NEB). DNA standard 1 kb ladder (5 µl, NEB) was loaded as a size reference. Electrophoresis was run at 100V for 50 minutes, and gels visualised with UV under a Vilber E-box illuminator.
<table>
<thead>
<tr>
<th>Number</th>
<th>Sequence (5’-3’)</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>acc tgt att ttc agg gcc ccA TGA AAA AAG ATA AAA ATT GTT CCA C</td>
<td>Amplification of full length PBP2a, with overlap specific for pPROEX. Forward (1). Reverse (2).</td>
</tr>
<tr>
<td>2</td>
<td>ttc tga ttt aat ctg tat cat tat tca TCT ATA TCG TAT TTT TTA TTA CC</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>acc tgt att ttc agg gcc ccA TGA CGG AAA ACA AAG GAT C</td>
<td>Amplification of full length PBP2, with overlap specific for pPROEX.</td>
</tr>
<tr>
<td>4</td>
<td>ttc tgc ttt aat ctg tat cat TAG TTG AAT ATA CCT GTT AAT CC</td>
<td>Forward (3). Reverse (4).</td>
</tr>
<tr>
<td>5</td>
<td>tga tac aga tta aat cag aac g</td>
<td>Amplification of pPROEX plasmid. Forward (5). Reverse (6).</td>
</tr>
<tr>
<td>6</td>
<td>ggc gcc ctg aaa ata cag</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>gcc atc acc atc atc acc ACG ATT ACG ATA TCC CAA CG</td>
<td>Amplification of full length PBP2 from pPROEX::pbp2 construct, including TEV cleavage site, with overlap specific for pET-Duet His&lt;sub&gt;6&lt;/sub&gt; site. Forward (7). Reverse (8).</td>
</tr>
<tr>
<td>8</td>
<td>gct cga att cgg atc ctg gct TAG TTG AAT ATA CCT GTT AAT CC</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>gcc agg atc cga att cga g</td>
<td>Amplification of pET-Duet plasmid from His&lt;sub&gt;6&lt;/sub&gt; site. Forward (9). Reverse (10).</td>
</tr>
<tr>
<td>10</td>
<td>gtc gtc atg atg gtc atg</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>AAC GCC AGC ACA TGG ACT CGG ATT ACG ATA TCC CAA CG</td>
<td>Amplification of full length PBP2a from pPROEX::pbp2a construct, including TEV cleavage site, with overlap specific for pET-Duet::pbp2 S-tag site. Forward (11). Reverse (12).</td>
</tr>
<tr>
<td>12</td>
<td>TAA TTA AGC TGCGCT AGT AGT TAT TCA TCT ATA TCG TAT TTT TTA TTA CC</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>cta cta gcc cag ctt aat ta acct agc c</td>
<td>Amplification of pET-Duet::pbp2a from S-tag site. Forward (13). Reverse (14).</td>
</tr>
<tr>
<td>14</td>
<td>cga gtc cat gtc ctg gcc</td>
<td></td>
</tr>
</tbody>
</table>
2.4.4. Gibson cloning reaction using PCR products

Gibson assembly was performed using PCR products from Section 2.4.2, as per manufacturers’ protocol (NEB) for a 2-3 fragment assembly, with DNA ratio of 1:2 vector:insert. Reactions were performed in 5 µl volumes. Following assembly, plasmid constructs were transformed into *E. coli* TOP10, as per Section 2.2.5.

2.4.5. Plasmid propagation and plasmid purification

Successfully transformed colonies from Section 2.4.4, were individually inoculated into 10 ml LB and incubated overnight at 37°C, 180 rpm. Plasmids were purified using a Monarch Plasmid Miniprep Kit (NEB) as per the manufacturer’s protocol, and eluted in 30 µl sterile water. Where plasmid constructs were approximately larger than 10 kb water was heated to 50°C before elution to aid DNA recovery.

2.4.6. DNA concentration quantification

The concentration of DNA (1-2 µl) was quantified using a NanoDrop ND60 (Agilent), with sterile water as a blank.

2.4.7. DNA sequencing of plasmid constructs

To ensure correct DNA sequences, and confirm successful cloning, plasmid DNA (500 ng) was sequenced by GATC Biotech (Germany), with 25 pmol primer in a final volume of 10 µl. Plasmid-specific primers used during this project are detailed in Table 2.4. All other sequencing primers were specific to the gene of interest. DNA sequences were translated to protein sequences using ExPASy Translate (Gasteiger *et al*, 2003) and aligned using EMBL-EBI ClustalW (*Sievers et al*, 2011).
Table 2.4. Sequences of primers used throughout this project.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Details</th>
<th>Primers Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T7 promoter</td>
<td>Forward primer, starting at T7 promoter</td>
<td>TAA TAC GAC TCA CTA TAG GG</td>
</tr>
<tr>
<td>T7 terminator</td>
<td>Reverse primer starting at T7 terminator</td>
<td>GCT AGT TAT TGC TCA GCG G</td>
</tr>
<tr>
<td>pPROEX promoter</td>
<td>Forward primer, starting at pPROEX promoter</td>
<td>AGC GGA TAA CAA TTT CAC ACA</td>
</tr>
<tr>
<td>pPROEX terminator</td>
<td>Reverse primer starting at pPROEX terminator</td>
<td>CTA CTC AGG AGA GCG</td>
</tr>
</tbody>
</table>

2.4.8. Plasmid constructs

The plasmid constructs used throughout this project are detailed in Table 2.5.
<table>
<thead>
<tr>
<th>Plasmid construct</th>
<th>Description</th>
<th>Antibiotic</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pET28::femX</td>
<td>Full length <em>S. aureus</em> Mu50 <em>femX</em>, with C-terminal His tag, TEV cleavage site</td>
<td>Kanamycin</td>
<td>J. Shephard (Warwick, Roper group)</td>
</tr>
<tr>
<td>pET28a::femB</td>
<td>Full length <em>S. aureus</em> Mu50 <em>femB</em> with C-terminal His tag, TEV cleavage site</td>
<td>Kanamycin</td>
<td>J. Shephard (Warwick, Roper group)</td>
</tr>
<tr>
<td>pET28b::glyRS</td>
<td>Full length <em>S. aureus</em> Mu50 <em>glyRS</em> with C-terminal His tag, TEV cleavage site</td>
<td>Kanamycin</td>
<td>J. Shephard (Warwick, Roper group)</td>
</tr>
<tr>
<td>pET22b::femA</td>
<td>Full length <em>S. aureus</em> Mu50 <em>femA</em> with C-terminal His tag, TEV cleavage site.</td>
<td>Ampicillin</td>
<td>J. Shephard (Warwick, Roper group)</td>
</tr>
<tr>
<td>pPROEX::pbp2</td>
<td>Full length <em>S. aureus</em> Mu50 <em>pbp2</em> with N-terminal His tag, TEV cleavage site.</td>
<td>Ampicillin</td>
<td>This study</td>
</tr>
<tr>
<td>pPROEX::pbp2a</td>
<td>Full length <em>S. aureus</em> Mu50 <em>pbp2a</em> with N-terminal His tag, TEV cleavage site.</td>
<td>Ampicillin</td>
<td>This study</td>
</tr>
<tr>
<td>pET-DUET::pbp2::pbp2a</td>
<td>Coexpression construct of full-length <em>S. aureus</em> Mu50 <em>pbp2</em> with N-terminal His tag, TEV cleavage site, and full-length <em>S. aureus</em> Mu50 <em>pbp2a</em> with N-terminal S-tag, TEV cleavage site.</td>
<td>Ampicillin</td>
<td>This study</td>
</tr>
<tr>
<td>pET-41a::pbp2W59M</td>
<td>Truncated <em>S. aureus</em> <em>pbp2</em> Δ1-58 <em>W59M</em>, untagged.</td>
<td>Kanamycin</td>
<td>(Lovering et al, 2007)</td>
</tr>
<tr>
<td>pET-41a::pbp2W59M ΔS403A</td>
<td>Truncated <em>S. aureus</em> <em>pbp2</em> Δ1-58 <em>W59M</em>, untagged, active site Ser-403 mutated to Ala.</td>
<td>Kanamycin</td>
<td>This study</td>
</tr>
<tr>
<td>pET-15b::pbp2aY23M</td>
<td>Truncated <em>S. aureus</em> <em>pbp2a</em> Δ1-22 <em>Y23M</em>, untagged.</td>
<td>Ampicillin</td>
<td>(Lim and Strynadka, 2002)</td>
</tr>
<tr>
<td>pET-15b::pbp2aY23M ΔS398A</td>
<td>Truncated <em>S. aureus</em> <em>pbp2a</em> Δ1-22 <em>Y23M</em>, untagged, active site Ser-398 mutated to Ala.</td>
<td>Ampicillin</td>
<td>This study</td>
</tr>
</tbody>
</table>
2.5. Protein expression and purification

2.5.1. Recombinant protein expression in *E. coli*

Various *E. coli* strains (Table 2.1 Section 2.2) were transformed (Section 2.2.5) with the appropriate plasmid constructs (Table 2.5 Section 2.3) and plated on to appropriate antibiotic LB-agar. One colony was inoculated into 10 ml LB and incubated overnight at 37°C, 180 rpm.

2.5.1.1. Small scale recombinant protein expression trials in *E. coli*

Overnight culture (50 µl, Section 2.5.1) of *E. coli* BL21(DE3)Star.pRosetta, C41(DE3), C43(DE3) or Lemo21(DE3) was inoculated into 10 ml LB, with appropriate antibiotics and grown at 37°C, with shaking at 180 rpm until reaching OD₆₀₀ 0.6. For *E. coli* Lemo21(DE3), media were supplemented with 1mM L-Rhamnose. A 100 µl sample was taken of culture before the induction of protein expression. Protein expression was induced with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) at 25°C, with shaking at 180 rpm for 4 hours or overnight. Cultures were centrifuged at 4000 rpm at 4°C for 20 min and pellets retained. Protein expression was visualised by SDS-PAGE (Section 2.6.2).

2.5.2. Recombinant protein expression of FemX, FemA, FemB, GlyRS and coexpressed full length PBP2 and PBP2a

An overnight culture (Section 2.5.1) of *E. coli* BL21(DE3)Star.pRosetta transformed with either pET28::femX, pET28a::femB, pET28b::glyRS, pET22b::femA or pET-Duet::pbp2::pbp2a was inoculated into 1 L of LB, with appropriate antibiotics and grown at 37°C, with shaking at 180 rpm until reaching OD₆₀₀ 0.6. Protein expression was induced with 1 mM IPTG at 25°C, with shaking at 180 rpm for 4 hours. Cultures were centrifuged at 6,000 xg for 20 min at 4°C and pellets stored at -80°C.
2.5.2.1. Preparation of cell lysate for FemX, FemA, FemB, GlyRS expression

Cell pellets were defrosted on ice, resuspended in 3 ml/g of 50 mM sodium phosphate, 1M NaCl pH 7 plus protease inhibitors (1 µM leupeptin and 1 µM pepstatin, 0.2 mM PMSF (Phenylmethylsulfonyl fluoride)), supplemented with 2.5 mg/ml lysozyme from hen egg white and 20 µg/ml DNase, and incubated at 4°C on rollers for 1 hour. The resuspension was sonicated on ice at 70% power, 10 times for 30 seconds each (with 30 seconds of cooling on ice between each) and centrifuged at 50,000 xg for 1 hour at 4°C. Supernatant was retained for protein purification.

2.5.2.2. Preparation of cell lysate for coexpressed full length PBP2 and PBP2a

Cell pellets were defrosted on ice, resuspended in 100 mM Tris, 500 mM NaCl, 20 mM imidazole, 10 mM MgCl₂, 2% (v/v) glycerol, pH 8, supplemented with 2.5 mg/ml lysozyme from hen egg white and 20 µg/ml DNase, and incubated at 4°C on rollers for 30 min. The cells were lysed by duplicate passage through a cell disruptor at 25 kpsi. Lysed cells were supplemented with 3.25% (w/v) CHAPS (3-((3-chloramidopropyl)-dimethylammonio)-1-propanesulfonate) and were incubated at 4°C for 4 hours before centrifugation at 16,000 rpm for 30 min, 4°C. Supernatant was retained for protein purification.

2.5.3. Recombinant protein expression of truncated PBP2-W59M and PBP2a-Y23M

As adapted from Lovering et al. (2007), Roychoudhury et al, (1994) and Lim and Strynadka (2002), overnight culture (Section 2.5.1) of E. coli BL21(DE3)Star.pRosetta transformed with either pET-41a::pbp2W59M or pET-15b::pbp2aY23M was inoculated into 1 L of LB, with appropriate antibiotics and grown at 37°C with shaking at 180 rpm until an OD₆₀₀ 0.6 was attained. Culture was heated shocked for 1 hour at 42°C, 180 rpm followed by a further hour on ice. Protein expression was induced with 1 mM IPTG at 20°C, 180 rpm overnight. Cultures were centrifuged at 6,000 xg for 20 min, 4°C and pellets stored at -80°C.
2.5.3.1. Preparation of cell lysate

Cell pellets were defrosted on ice, resuspended in 50 mM potassium phosphate, 1 mM EDTA, pH 8 plus protease inhibitors (1 µM leupeptin and 1 µM pepstatin, 0.2 mM PMSF), supplemented with 2.5 mg/ml lysozyme from hen egg white and 20 µg/ml DNase, and incubated at 4°C on rollers for 20 min. The resuspension was then sonicated on ice at 70% power, 10 times for 30 seconds each (with 30 seconds cooling on ice between each) and centrifuged at 20,000 rpm (Beckman JA25.5) for 40 min at 4°C.

2.5.4. Affinity chromatography

Proteins with a His6 affinity tag were purified by nickel immobilised metal affinity chromatography (IMAC) using a 5 ml HP HiTrap column (GE Healthcare) on an AKTA Pure system (GE Healthcare). Columns were washed with 10 column volumes (CV) water and equilibrated with 10 CV of purification buffer, typically 50 mM sodium phosphate, 500 mM NaCl, 20% (v/v) glycerol plus 1 µm leupeptin and 1 µM pepstatin, 0.2 mM PMSF (Buffer A) supplemented with 10 mM imidazole, unless otherwise stated. Supernatant was loaded onto the column using a bench top peristaltic pump, flow through was collected and the column washed with a further 10 CV of buffer. Using the AKTA Pure, proteins were eluted over a gradient of 0-100% buffer A plus 200 mM imidazole, at 1 ml/min over 50 min. Fractions were collected in 2.5 ml, and protein elution monitored at A280.

2.5.4.1. His6 tag cleavage with TEV protease and reverse affinity chromatography

Fractions from Section 2.5.4. containing the required protein were pooled together and the protein concentration was measured using BioRad reagent. The protein was supplemented with TEV protease to a ratio of 1:50 mg (TEV protease:protein), and dialysed overnight at 4°C into buffer A. Protein was loaded onto a second nickel IMAC column as in Section 2.5.4, and the flow through was collected. The column was
washed with 5 CV buffer A, buffer A plus 10 mM imidazole and buffer A plus 20 mM imidazole using a peristaltic pump, each collected as separate fractions. Proteins with a cleaved His$_6$ were typically found in the flowthrough, buffer A wash or buffer A plus 10 mM imidazole washes.

2.5.5. Size exclusion chromatography

2.5.5.1. Preparative size exclusion chromatography

Proteins were separated according to molecular size using a HiLoad Superdex 200 (16/60), capable of separating proteins between 10-600 kDa, on an AKTA Pure system. Columns were washed with 1 CV water and 1.5 CV buffer B, typically 20 mM Tris, 1 mM MgCl$_2$, 100 mM NaCl, pH 8.2 plus 1 μm leupeptin and 1 μM pepstatin, 0.2 mM PMSF and 2 mM β-mercaptoethanol. Protein samples were concentrated to <4% of CV to aid in high resolution and loaded onto the column using an injection loop. Proteins were eluted with 1 CV of buffer B at 1 ml/min, and fraction volumes of 5 ml were collected. Protein elution was monitored at $A_{280}$.

2.5.6. Ion exchange chromatography

Anion exchange chromatography was performed with a 1 ml HiTrap Q HP strong anion exchange column (GE Healthcare) or a 5 ml DEAE sephacel weak anion exchange column, using an AKTA Pure system or by gravity flow, respectively. Columns were washed with 5 CV of water, and equilibrated in 5 CV of buffer. For the HiTrap column, protein was eluted over a gradient between 0 – 100 mM NaCl in 10 mM Tris, pH 8, over 25 min at 2 ml/min. For the DEAE column, protein was incubated with resin for 1 hour at 4°C and resin was subsequently loaded into a gravity flow column. Flowthrough was collected and column washed using two 40 ml washes of 50 mM potassium phosphate, pH 8. Protein typically eluted from the DEAE resin in the flowthrough and two buffer washes.
2.5.7. Buffer exchange and protein concentration

Proteins were concentrated using a Vivaspin centrifugal concentrator (Sartorius) with a molecular weight cut off of 10 kDa at 4000 rpm at 4°C (Eppendorf 5810R). Buffer exchange was performed by replacement of buffer with exchange buffer until a sufficient dilution was reached. Proteins were concentrated until the desired concentration was attained.

2.5.8. Specific protein purification procedures

Where significant changes to published protein purification procedures were made, the protocols are detailed here.

2.5.8.1 Fem X, FemA, FemB and GlyRS

Initial protein purification was carried out using nickel IMAC, as in Section 2.5.4. Where His\textsubscript{6} affinity tag cleavage was required, proteins were treated with TEV protease as in Section 2.5.4.1, during overnight dialysis into buffer A. The proteins (minus His\textsubscript{6} affinity tag) were purified away from the cleaved His\textsubscript{6} affinity tag and the TEV protease via a second nickel IMAC purification.

Proteins (with or without His\textsubscript{6} affinity tag) were concentrated to approximately 5 ml and were further purified by size exclusion chromatography (Superdex 200 16/60, GE Healthcare), as in Section 2.5.5. Fractions containing the desired protein were concentrated and dialysed overnight into storage buffer (30 mM HEPES (2-[4-2-(hydroxyethyl)piperazine-1-ethanesulfonic acid), 50 mM KCl, 1 mM MgCl\textsubscript{2}, pH 7.6, 50% (v/v) glycerol, plus 1 \mu M leupeptin and 1 \mu M pepstatin, 0.2 mM PMSF and 3 mM DTT).
2.5.8.2. Coexpressed full length PBP2 and PBP2a

Cell lysate from Section 2.5.2.2. was loaded onto a nickel IMAC column (as in Section 2.5.4) equilibrated in 100 mM Tris pH 8, 500 mM NaCl, 20 mM imidazole, 2% (v/v) glycerol, 1% (w/v) CHAPS. Proteins were eluted with a 0-100% equilibration buffer plus 500 mM imidazole. Proteins were dialysed into 100 mM Tris, 500 mM NaCl, pH 8, 50% (v/v) glycerol, 1% (w/v) CHAPS and stored at -80°C.

2.5.8.3. PBP2-W59M

Cell lysate from Section 2.5.3.1. was loaded onto a nickel IMAC column (as in Section 2.5.4) equilibrated in 10 mM Tris pH 8, 0.2M NaCl and 0.28 mM LDAO. Proteins were eluted with 0-50% equilibration buffer containing 200 mM imidazole over 50 ml, followed by 50-100% equilibration buffer plus 200 mM imidazole over 10 ml. The flow rate was 1 ml/min and 2.5 ml fractions were collected. Fractions containing the desired protein were concentrated and further purified by size exclusion chromatography (As in Section 2.5.5), using a column equilibrated in 10 mM Tris pH 8, 0.2 M NaCl and 0.28 mM LDAO. The flow rate was 1 ml/min, and 5 ml fractions were collected.

Fractions containing the desired protein were dialysed overnight at 4°C in 10 mM Tris pH 8, 0.28 mM LDAO. Proteins were further purified by anion exchange chromatography (as in Section 2.5.6), using a column equilibrated in 10 mM Tris pH 8 and 0.28 mM LDAO. Proteins were eluted with a 0-10% gradient of 10 mM Tris pH 8, 1 M NaCl and 0.28 mM LDAO over 25 min, at 2 ml/min. Fractions (2.5ml) containing the desired proteins were concentrated and dialysed overnight at 4°C into 10 mM Tris pH 8 and 0.28 mM LDAO. Proteins were stored at -80°C.

Where proteins were required to be detergent-free, LDAO was not included in all buffers following nickel IMAC.
2.5.8.4. PBP2a-Y23M

Lysate was applied to DEAE sephacel (As in Section 2.5.6), pre-equilibrated with 50 mM potassium phosphate, pH 8. Proteins were eluted with two 40 ml washes of 50 mM potassium phosphate, pH 8, collected as one fraction. Proteins were dialysed overnight at 4°C into 50 mM potassium phosphate, 1 M NaCl, pH 8, and subsequently centrifuged 15,000 rpm for 30 min at 4°C. Supernatant was further purified by anion exchange chromatography (As in Section 2.5.6) using a 5 ml SP Sepharose column, pre-equilibrated with 50 mM potassium phosphate, pH 8. Proteins were eluted with a gradient of 0-100% 50 mM potassium phosphate and 1 M NaCl, pH 8, over 50 min at 1 ml/min. Fractions (2.5 ml) containing the desired proteins were concentrated and dialysed overnight at 4°C into 50 mM potassium phosphate and 150 mM NaCl, pH 7. Final purification was performed by size exclusion chromatography (As in Section 2.5.5) on a column pre-equilibrated with 50 mM potassium phosphate and 150 mM NaCl, pH 7. Protein were eluted at 1 ml/min. Fractions (5 ml) containing the desired proteins were concentrated and stored at -80°C.

2.6. Protein analysis

2.6.1. Protein quantification

Proteins purified in the absence of detergent were quantified using the BioRad colorimetric assay. In a 1 ml plastic cuvette, 790 µl sterile water and 200 µl BioRad reagent was added to 10 µl protein solution. The solution was mixed and incubated at RT for 5 min. Absorbance was measured at 595nm in a Jenway 7305 UV-Visible spectrophotometer. Measurements were taken in triplicate, and protein solutions diluted if absorbance readings were outside of the linear 0.1-0.6 range.

The protein concentration was calculated using the following formula,

\[ \text{[Protein], } \mu\text{g/ml} = \left( \frac{A_{595\text{nm}}}{0.1} \right) \times 1.95 \times \text{dilution factor} \times 100 \]
Proteins purified in the presence of detergent were quantified using the Pierce BCA protein assay kit. The assay was carried out according to manufacturer’s protocol using either a Jenway 7305 UV-Visible spectrophotometer at 562nm or a Varioskan Flash (Thermo Scientific) at 562nm.

2.6.2. Protein visualisation by SDS-polyacrylamide gel electrophoresis

Proteins were visualised and separated using discontinuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE), using a Tris-glycine buffer system as described by Laemmli (1970). A 5 ml resolving gel (366 mM Tris, pH 8.8 containing 0.4% (w/v) SDS, 12% (v/v) acrylamide:bis-acrylamide (29.1) was cast using the BioRad Mini-PROTEAN system, and was polymerised with 58 µl 10% (w/v) ammonium persulphate (APS) and 5 µl N,N,N',N'-Tetramethylethylenediamine (TEMED). The resolving gel was overlaid with ethanol to ensure the meniscus of the resolving gel was perfectly flat on setting. Once polymerised, the ethanol was removed from the top of the resolving gel, and a 2 ml stacking gel (125 mM Tris, pH 6.5, 0.4% (w/v) SDS, 4% (v/v) acrylamide:bis-acrylamide (29.1)) set with 50 µl 10% (w/v) APS and 10 µl TEMED was overlaid on top. A 10-well or 15-well comb was inserted and the stacking gel allowed to polymerise.

Protein samples (10 µg, where possible) were loaded into the wells with 1x loading dye (2x loading dye, 58 mM Tris-HCl, pH 6.8, 1.9% (w/v) SDS, 19% (v/v) glycerol, 0.7 M β-mercaptoethanol). Protein ladder (10 µl, Amersham LMW protein ladder) was loaded into one well for molecular weight determination. Where SDS-PAGE was subsequently used for Western blot detection (Section 2.6.3), specific tagged ladders were also loaded, as per manufacturer’s instructions. These were either Benchmark His-tagged markers (Invitrogen) or Perfect Protein Western S-tagged markers (Novagen).

SDS-PAGE was run using 25 mM Tris, 0.19 mM glycine, pH 8, 0.1% (w/v) SDS buffer at 180V, until the dye front reached the bottom of the gel. SDS-PAGE gels were
stained with InstantBlue Coomassie Protein Stain (Expedeon) and rinsed with distilled water to remove excess stain before visualisation using the SynGene GeneSNap G:Box Gel Doc or Vilber E-box.

2.6.3. Western blot

Proteins were separated by SDS-PAGE, as in Section 2.6.2., however using a Pre-cast Mini-PROTEAN TGX Stain Free gel. Gels were not stained with InstantBlue, but were imaged using the ChemiDoc XRS+ (BioRad). Proteins were transferred to TransBlot Turbo 0.2 μm PVDF membranes (BioRad) using the TransBlot Turbo Transfer System (BioRad), as per manufacturers protocol.

2.6.3.1. His-tagged protein antibody binding

All steps were performed at RT, on a gel rocker. Membranes were blocked overnight in 10% (w/v) milk powder in 1x TBS-Tween, and then washed 3x for 20 min each in 1x TBS-Tween to remove excess blocking solution. Membranes were washed with primary antibody (Mouse Anti-His Ab, Roche) in 0.1% (w/v) milk powder in TBS for 2 hours, and then washed 3x for 20 min each in 1x TBS-Tween to remove excess primary antibody. Membranes were washed with secondary antibody (Anti-mouse IgG (H+L) HRP conjugate, Promega) in 0.1% milk in TBS for 4 hours, and then washed again 3x for 20 min each in 1x TBS-Tween to remove excess secondary antibody.

2.6.3.2. S-tagged protein antibody binding

All steps were performed at RT, on a gel rocker. Membranes were blocked overnight in 10% (w/v) milk powder in 1x TBS-Tween, and then washed 3x for 20 min each in 1x TBS-Tween to remove excess blocking solution. Membranes were washed with primary antibody (1:5000 Monoclonal mouse S-tag Ab, Novagen) in 0.1% (w/v) milk powder in TBS for 2 hours, and then washed 3x for 20 min each in 1x TBS-Tween to remove excess primary antibody. Membranes were washed with secondary antibody
(Anti-mouse IgG (H+L) HRP conjugate, Promega) in 0.1% milk in TBS for 4 hours, and then washed again 3x for 20 min each in 1x TBS-Tween to remove excess secondary antibody.

2.6.3.3. Western blot visualisation

Following the antibody binding procedure, membranes were washed in 15 ml per membrane of BioRad Clarity ECL Western Substrate (1:1, Peroxide solution: enhancer solution), for 5 min at RT on a gel rocker. Proteins were visualised using the ImageQuant (GE Healthcare).

2.7. Synthesis of lipid-II intermediates

2.7.1. Synthesis of UDP-MurNAc pentapeptide

The method was adapted from Lloyd et al. (2008). Reactions (2 ml) were performed at 37°C overnight, containing 50 mM HEPES, pH 7.5, 10 mM MgCl₂, 200 mM PEP, 1 mM DTT, 8.2 mM UDP-GlcNAc (Sigma), 35 mM L-Ala, 35 mM D-Glu or 75 mM D-IsoGln, 35 mM L-Lys, 35 mM D-Ala-D-Ala, 50 mM KCl, 2.8 units IDH (units defined by manufacturers), 26 mM DL-isocitrate, 6 mM ATP, 500 units pyruvate kinase (Sigma, units defined by manufacturers), and approximately 200-2000 µg MurA-F (Dependent upon protein concentration and activity, supplied by BACWAN, University of Warwick).

2.7.1.1. Purification of UDP-MurNAc pentapeptide

Overnight reactions were diluted to 5 ml with water before filtration using a Vivaspin centrifugal concentrator (10 kDa MWCO). Anion exchange chromatography was used to purify UDP-MurNAc pentapeptides using a Source 30Q column (75 ml CV) on an AKTA purifier. Samples were loaded in batches of two in 100ml of water onto the column, and eluted with an ammonium acetate gradient of 10 mM – 1 M ammonium
acetate (0-100% in 120 min) at 10 ml/min. The presence of UDP-MurNAc pentapeptide was detected at 254 and 280 nm; these fractions were freeze dried to remove salts and resuspended in sterile water.

### 2.7.1.2. Quantification of UDP-MurNAc pentapeptide

Absorbance of UDP-MurNAc pentapeptide was measured at 260nm in a quartz cuvette using a Jenway 7305 UV-visible spectrophotometer. The extinction coefficient (10,000 M\(^{-1}\)cm\(^{-1}\)) of the uracil of the UDP group was used to determine final UDP-MurNAc pentapeptide concentration.

### 2.7.1.3. Purity of UDP-MurNAc pentapeptide

Anion exchange chromatography of UDP-MurNAc pentapeptides using a MonoQ HR 5/5 column (GE Healthcare) was used to assess purity. Samples were diluted 700-fold in a final volume of 700 ml 10 mM ammonium acetate pH 7.5, loaded onto the column and eluted with a gradient of 10 mM-1M ammonium acetate pH 7.5 (0-100% in 10 min) at 1 ml/min. Eluate was monitored at 280 nm and 254 nm, and UNICORN analysis software was used to integrate peaks at 254 nm. Purity of amidated UDP-MurNAc pentapeptide (Lys) at 254nm was 95%. Non-amidated UDP-MurNAc pentapeptide was provided by Dr Ricky Cain, Warwick.

### 2.7.2. Synthesis of branched UDP-MurNAc pentapeptide derivatives

#### 2.7.2.1. Desalting of UDP-MurNAc pentapeptide (Lys)

UDP-MurNAc pentapeptide (Lys) was desalted on a Biogel P2 (BioRad), equilibrated in water. Samples were loaded (35-40 mg) onto the column at 1 ml/min and elution was measured at 280 nm, 254 nm and 218 nm. Fractions (10 mls) were collected and those containing desalted sample were freeze dried overnight, before resuspending in sterile water and subsequent quantification as in Section 2.7.1.2.
2.7.2.2. Synthesis of UDP-MurNAc pentapeptide monoglycine and triglycerine (Lys)

Fmoc-Gly-OH (Sigma), or Fmoc-Gly-Gly-Gly-OH (Dr Ricky Cain, Warwick) (12 equiv.), EDC (55 equiv.) and N-hydroxy succinimide (NHS, 25 equiv.) were dissolved in 2 ml of 80% (v/v) acetonitrile in water and stirred at RT for 30 mins. The pH was then adjusted to pH 10 by the addition of 500 mM NaHCO$_3$. Desalted UDP-MurNAc pentapeptide (Lys) was added to the reaction (1 equiv.) which was then stirred overnight, RT. The reaction yielded a white precipitate. Piperidine (100 µl) was added and the reaction was stirred at RT for 30 min. The precipitate disappeared and 18 ml of sterile water was added to the reaction, again forming a white precipitate. The reaction mixture was filtered (0.2 µm), and lyophilised until dry.

2.7.2.3. Purification of UDP-MurNAc pentapeptide monoglycine and triglycerine (Lys)

The lyophilised product was purified by anion exchange chromatography on an AKTA Pure system (MonoQ 10/10, GE Healthcare) with a gradient of 10 mM- 1 M ammonium acetate over 1 hr, with a flow rate of 4 ml/min. Absorbance was measured at 254, 280 and 218 nm and fractions containing UDP-MurNAc pentapeptide mono-Gly (Lys), or tri-Gly (Lys) were combined and freeze dried overnight.

2.7.2.4. Quantification of UDP-MurNAc pentapeptide monoglycine and triglycerine (Lys)

As in Section 2.7.1.2 for non-branched UDP-MurNAc pentapeptide (Lys).

2.7.3. Preparation of bacterial membranes for lipid-II synthesis

2.7.3.1. Growth of bacterial culture
S. aureus ATCC 25923 and S. epidermidis ATCC 12228 were grown as in Section 2.3.1, to an OD$_{600nm}$ of between 0.5-2, and an OD$_{600nm}$ of between 3-5, respectively. *Micrococcus flavus* was grown in 600 ml of TSB growth media and cultures were grown to OD$_{600nm}$ of between 3-5 at 37°C with shaking at 180 rpm.

2.7.3.2. Membrane extraction

Pellets were defrosted on ice and resuspended in 3 ml/g of 20 mM Tris, 1 mM MgCl$_2$, 2 mM β-mercaptoethanol, pH 7.5 (resuspension buffer), supplemented with 2.5 mg/ml lysozyme. Suspended cells were incubated at 4°C for 15 min with stirring, before 2x passage through a cell disruption at 30 kpsi. Lysed cells were centrifuged at 10,000 xg at 4°C for 1 hr and the supernatant was centrifuged at 50,000 xg at 4°C for 1 hr. The supernatant was centrifuged again at 75,000 xg at 4°C for 1 hr. The 50,000 xg and 75,000 xg pellets were homogenised in resuspension buffer and stored at -80°C.

2.7.3.3. Membrane quantification

Membrane concentrations were quantified as in Section 2.6.1.

2.8. Preparation of lipid-II

2.8.1. Synthesis of lipid-II

Lipid-II (Lys) (Adapted from Breukink *et al.*, (2003)) was synthesised in a 3.5ml final volume at 37°C overnight, containing 4 mg undecaprenyl phosphate (Laradon), 2 mM non-amidated UDP-MurNac pentapeptide (Lys) or 4 mM amidated UDP-MurNac pentapeptide (Lys), 6 mM UDP-GlcNAc, 5-10 mg *M. flavus* membranes, 200 µg MurG and 100 mM Tris, 1% (v/v) Triton X-100, 5 mM MgCl$_2$, pH 8. Following overnight incubation samples were extracted with 1x vol 6M pyridium acetate, pH 4.5 and 2x volume n-butanol, centrifuged for 10 min at 3000 rpm, 4°C and the top aqueous layer
extracted again with equal volume of water. Centrifugation was repeated, and the top aqueous layer dried under vacuum.

2.8.2. Synthesis of lipid- II pentaglycine using the Fem ligases

The method was adapted from Schneider et al, (2004). Analytical scale reactions (per 100 µl) were performed at 30°C with shaking at 100 rpm for 3 hr and contained 25 µg tRNA, 10 µg GlyRS, 2.7 µg of each FemX, FemA and FemB, 2 mM ATP, 5 nmol lipid- II, 50 nmol glycine in 100 mM Tris-HCl, 20 mM MgCl₂, pH 7.5 and 0.8% (v/v) Triton X-100. Reactions were made up to 100 µl with 100 mM Tris-HCl, 20 mM MgCl₂, pH 7.5 and 0.8% (v/v) Triton X-100. Lipids were purified as in Section 2.8.4.

2.8.2.1. Synthesis of lipid- II pentaglycine using the Fem ligases – the ‘one-pot’ reaction

Reactions (1750 µl) contained 100 mM Tris, pH 8, 5 mM MgCl₂, 1% (v/v) Triton X-100, 2 mg undecaprenyl phosphate, 2 mM or 4 mM non-amidated or amidated UDP-MurNAc-pentapeptide (Lys), respectively, 6 mM UDP-GlcNAc, 100 µg MurG, 438 µg S. aureus ATCC 25923 tRNA, 175 µg GlyRS, 47 µg FemX, 47 µg FemA, 47 µg FemB, 2 mM ATP, 3.3 µg myokinase, 175 µg pyruvate kinase, 2 mM PEP and 50 nmol glycine. Reactions were topped up to 1750 µl with S. aureus ATCC 25923 membranes. Where L-glutamine (50 mM) was added as required. Reactions were incubated at 37°C for 3 hr, with frequent mixing. Lipids were purified as in Section 2.8.4.

2.8.3. Extraction of amidated lipid- II pentaglycine from culture

This method was performed as in Qiao et al, (2017), however the 20 min incubation following moenomycin treatment was performed at RT with shaking, as opposed to 37°C to avoid cell lysis. To ensure maximum lipid purity, lipid- II was purified as in Section 2.8.4.
2.8.4. Purification of lipid-II

Lipid-II was purified via anion exchange using a 4 ml DEAE sephacel column in a glass burette, pre-equilibrated with 10 CV of 1 M ammonium acetate, 12 CV of water and 10 CV of solvent A (Chloroform:methanol:water, 2:3:1). Lipid-II was resuspended in 6 ml solvent A, loaded onto the column and washed with 3 CV of solvent A. Lipid II was eluted from the column with 3 CV of chloroform:methanol:ammonium bicarbonate (2:3:1) in a step-wise manner where the concentration of the ammonium bicarbonate component was increased between 50mM – 1 M. Non-amidated lipid-II usually separates well from the undecaprenyl phosphate, however, amidated lipid-II often overlapped with elution of undecaprenyl phosphate, and therefore these fractions required further purification before use.

2.8.5. Analysis of lipid-II by thin layer chromatography (TLC)

Fractions from Section 2.8.4 (400 µl) were dried under vacuum, resuspended in 25 µl of solvent A and loaded onto a Silica TLC plate (Machery-Nagel), 2 cm from bottom and 1.5 cm apart. TLC running buffer (chloroform:methanol:water:0.88 ammonia 88:48:10:1) at a depth of 1 cm was used to separate sample components via TLC at RT. Lipids were visualised by iodine vapour staining of TLC plates. Fractions containing lipid-II were pooled and dried under vacuum.

2.8.6. Quantification of lipid-II

Lipid-II samples (50 µl) were dried under vacuum in duplicate, with a solvent A control. Once dried, they were resuspended in 50 µl of 50 mM HEPES pH 7.5, 10 mM MgCl₂, 30 mM KCl and 1.5% (w/v) CHAPS. The control and one of the lipid samples were acid-hydrolysed with 50 µl of 1M HCl at 95°C for 30 min, before neutralisation to pH 7.6 with 1M NaOH. A spectrophotometric assay that detects the release of phosphate at 360 nm was used to quantify the samples. Reactions (200 µl) contained 50 mM HEPES, pH 7.6, 10 mM MgCl₂, 200 µM MESG, 2.5 µMol/min PNP, 5 µMol/min
IPP, and 10 µl of sample. Lipid-II could be quantified following the hydrolysis of the lipid-II, releasing inorganic pyrophosphate and phosphate, and hence the phosphate detected by the coupled enzymatic assay allowed for quantification of the lipid-II. The extinction coefficient of phosphate (10,000 M⁻¹cm⁻¹) is used to calculate the amount of lipid-II per sample, given that every molecule of lipid-II released two molecules of phosphate following acid hydrolysis.

2.9. Mass spectrometry

2.9.1. TOF-MS of lipid-II intermediates

UDP-MurNAc-pentapeptides and lipid-II species were analysed via negative or positive ion electrospray ionisation mass spectrometry (ESI-MS) using a Waters Synapt G2Si quadrupole-time of flight instrument at the University of Warwick by Dr Adrian Lloyd, Julie Tod or Anita Catherwood, or by Proteomics Research Technology Platform, University of Warwick.

The nanospray source was calibrated with an error of less than 1 ppm with sodium iodide over a 200-2500 m/z range. Samples were introduced into the instrument using Waters thin wall nanoflow capillaries. Capillary voltage - 2.0 kV, cone voltage - 100 V and source offset - 41 V. Source and desolvation temperature - 80°C and 150°C respectively. Desolvation and purge gas flow rates were both 400 litres.min⁻¹. Scan time – 1 second. Interscan time - 0.014 seconds. Scans were combined into centred mass spectra by Waters Mass Lynx software. Resolution (m/z/half-height spectral peak width) was measured as 1 in 20,100.

2.9.2. Proteomic mass spectrometric identification of proteins

Proteins were visualised by SDS-PAGE, and bands of interest cut out using a scalpel. Gel fragments were cut into small cubes of 2-4 mm, and de-stained with 50% (v/v)
ethanol, 50 mM ammonium bicarbonate (ABC) for 20 min at RT with shaking at 650 rpm. This step was repeated, removing the liquid each time, until the stain was fully removed. For each subsequent step, liquid was removed between steps, unless otherwise stated, and volumes used were enough to cover the gel.

The gel fragments were dehydrated with 100% (v/v) ethanol for 5 min, at RT with shaking at 650 rpm. Disulphide bonds were reduced with 10 mM DTT in 50 mM ABC for 30 min, 56°C with shaking at 650 rpm. Cysteine residues were alkylated with 55 mM iodoacetamide (IAA) in 50 mM ABC for 20 min at RT in the dark. The gel fragments were then washed twice with 50% (v/v) ethanol and 50 mM ABC for 20 min at RT with shaking at 650 rpm, before dehydration with 100% (v/v) ethanol for 5 min at RT with shaking at 650 rpm. The gel fragments were rehydrated with 2.5 ng/µl trypsin in 50 mM ABC and digested overnight at 4°C. Peptides were extracted from the gel by incubation in 25% (v/v) acetonitrile for 5-10 min at RT in a sonicator bath. Sonication was repeated twice. Acetonitrile containing extracted peptides was concentrated using a Speed-Vac to approx. 20 µl before resuspension with 30 µl of 2.5% (v/v) acetonitrile and 0.05% (v/v) trifluoroacetic acid. Insoluble particles were removed with a 0.22 µm cellulose acetate spin column, at 16,000 xg for 5 min.

Following peptide extraction, samples were processed by the Proteomics Research Technology Platform, University of Warwick, as follows. Tryptic peptides were separated by reversed phase chromatography (Acclaim PepMap µ-precolumn cartridge 300 µm i.d. x 5 mm 5 µm 100 Å and an Acclaim PepMap RSLC 75 µm x 25 cm 2 µm 100 Å (Thermo Scientific)) prior to mass spectrometric analysis. The columns were installed on an Ultimate 3000 RSLCnano system (Dionex). Mobile phase buffer A was composed of 0.1% (v/v) formic acid in water and mobile phase B 0.1% (v/v) formic acid in acetonitrile.

Samples were loaded onto the µ-precolumn equilibrated in 2% (v/v) aqueous acetonitrile containing 0.1% (v/v) trifluoroacetic acid for 5 min at 10 µL min⁻¹. Peptides were subsequently eluted onto the analytical column at 250 nL min⁻¹ by increasing the mobile phase B concentration in a step wise manner as follows; i) 4%
Elut ed peptides were converted to gas-phase ions by means of ESI and analysed on a Thermo Orbitrap Fusion (Q-OT-qIT, Thermo Scientific). Survey scans of peptide precursors from 375 to 1575 m/z were performed at 120K resolution (at 200 m/z) with a $2 \times 10^5$ ion count target. Tandem MS was performed by isolation at 1.2 Th using the quadrupole, HCD fragmentation with normalized collision energy of 33, and rapid scan MS analysis in the ion trap. MS$^2$ ion count target - $1 \times 10^4$. Max injection time - 200 ms. Precursors with charge state 2–6 were selected and sampled for MS$^2$. Dynamic exclusion duration - 25 s with a 10 ppm tolerance. Monoisotopic precursor selection was turned on. The instrument was run in top speed mode with 2 s cycles.

Results were compared against the recombinant protein sequence of interest, the Uniprot E. coli database and the common Repository of Adventitious Proteins (cRAP) using the MaxQuant software. Scaffold software was used to analyse and visualise the results given by MaxQuant.

2.9.3. Intact mass spectrometry analysis of protein-drug interaction

Intact mass spectrometry was performed by Dr Matthew Jenner, Department of Chemistry (University of Warwick), at the University of Nottingham. Intact mass spectrometry of PBP2a or PBP2 (50 μM) with ceftobiprole or ceftaroline (500 μM) was performed on a Bruker MaXis II ESI-Q-TOF-MS connected to a Dionex 3000 RS UHPLC fitted with an ACE C4-300 RP column (100 x 2.1 mm, 5 μm, 30 °C). Proteins were eluted from the RP column with a linear gradient of 5-100% (v/v) MeCN containing 1% (v/v) formic acid over 30 min. Mass spectrometry was performed in positive ion mode, with a scan range of 200-3000 m/z.

2.9.4. Native mass spectrometry

All native mass spectrometry was performed by Dr Matthew Jenner, Department of Chemistry (University of Warwick), at the University of Nottingham. Samples were
analysed using a Waters SYNAPT HDMS, a hybrid quadrupole-ion mobility-orthogonal acceleration TOF instrument (oa-TOF). nano-ESI was used for ionisation, utilising home-made nanospray emitters with a platinum wire insert. Typical capillary voltages were in the range of 1.5 - 2.0 kV. Sample cone voltage was held at 40 V. The spectrometer was operated in TOF-mode using parameters optimised for unmodified PBP2-W59M and PBP2a-Y23M. Trap and transfer voltages were 20 V and 10 V, respectively; backing pressure 3.0 mbar; trap pressure, 2.0 x 10^{-2} mbar; TOF region pressure 1.6 x 10^{-6} mbar; bias voltage, 15 V for declustering. Bound ceftaroline or ceftobiprole were ejected from PBP2-W59M and PBP2a-Y23M with a collision energy in the range of 25-30 V.

2.10. Protein interaction studies

2.10.1. Microscale thermophoresis (MST)

Proteins with a His6 affinity tag were labelled using the Protein Labelling Kit RED-tris-NTA (NanoTemper Technologies). The labelling reaction was performed according to manufacturer’s instructions; however, PBS was used instead of PBS-T due to aggregation of proteins in the presence of PBS-T. Protein (100 nM) was labelled for 30 min at RT with 100 nM RED-tris-NTA dye in a 1:1 volume ratio. Labelled proteins were centrifuged at 13,000 rpm for 10 min at 4°C (Eppendorf 5418R) before use.

To PCR tubes, protein buffer (30 mM HEPES, 50 mM KCl, 1 mM MgCl2, pH 7.6, 50% (v/v) glycerol, 1 µM leupeptin, 1 µM pepstatin and 0.2 mM PMSF) was used to serially dilute the ligand protein (minus His6 affinity tag) as appropriate. To each ligand protein dilution, one volume of labelled protein was added which led to a final concentration of labelled protein of 50 nM. Proteins were incubated together at RT for 15 min before MST measurement. The samples were loaded into Monolith NT.115 Standard Capillaries (NanoTemper Technologies), and MST measured at RT, 40% LED power and medium MST power with a laser-on time of 20 s and laser-off
time of 10 s. Data was analysed using MO Affinity Analysys Software (NanoTemper Technologies).

2.10.2. Analytical ultracentrifugation (AUC)

All AUC was performed by Dr Gemma Harris, at the Harwell Research Complex. AUC was performed at a range of concentrations of both PBP2-W59M alone and PBP2a-Y23M alone, or a mixture of the two, where PBP2-W59M concentration was constant, and PBP2a-Y23M concentration was varied. All AUC experiments were performed at 50000 rpm, at 20°C (Beckman Optima analytical ultracentrifuge, An-50Ti rotor). Data was recorded using the absorbance (280 nm) optical detection system. Both buffer density and viscosity was measured experimentally using a DMA 5000M densitometer equipped with a Lovis 200ME viscometer module. The partial specific volume for each protein was calculated using Sednterp from the amino acid sequences. Data were processed using SEDFIT, fitting to the c(s) model. Figures were made using GUSSI.

2.10.3. Carbene footprinting

Carbene footprinting was performed by Dr Matthew Jenner, Department of Chemistry (University of Warwick). Carbene footprinting photolysis was performed (in triplicate) in a total volume of 20 μl using 50 μM PBP2a and/or 50 μM PBP2 in 50 mM potassium phosphate, 150 mM NaCl, pH 7 with 10 mM aryl diazirine, pH 7.4 (Fig. 2.1). Reactions were left to equilibrate at RT, for 5 min before 6 μl aliquots were flash frozen in liquid nitrogen in crystal clear vials (Fisher Scientific UK). Carbene labelling was initiated by photolysis of reaction mixtures using the third harmonic wavelength of 347 nm. Frozen samples were irradiated for 30 sec. Following irradiation, each reaction was reduced by the addition of 10 mM DTT in 10 mM ammonium bicarbonate, alkylated with 55 mM iodoacetamide in 10 mM ammonium bicarbonate and finally, digested overnight at 37°C with a 1:20 trypsin:protein ratio in 10 mM ammonium bicarbonate.
The trypsin digest was quenched by the addition of formic acid, adjusting the pH to below 6 with a final formic acid concentration of <2%. Digested proteins were analysed on a Bruker MaXis II ESI-Q-TOF-MS connected to a Dionex 3000 RS UHPLC fitted with an ACE C18 RP column (100 x 2.1 mm, 5 µM, 30°C). A linear gradient of 5-100% (v/v) MeCN containing 0.1% (v/v) formic acid over 40 min was used to elute peptides from the C18 column. Mass spectrometry was performed in positive ion mode, with a scan range of 200-3000 m/z. Sodium formate (1 mM) was used for calibration via a 20 µl loop injection at the beginning of each run.

Quantitation of each peptide fraction was performed using previously described methods (Manzi et al, 2016). The chromatograms for each singly- and unlabelled-peptides were extracted within a window of ± 0.1 m/z. Each spectrum was manually inspected to ensure the correct ion sampling. The peptide fractional modification was calculated using Eq. 1,

\[
\text{Equation 1 } f_{\text{mod}} (n) = \frac{I(n_{\text{labelled}})}{I(n_{\text{labelled}}) + I(n_{\text{unlabelled}})}
\]

Where \( A_{\text{labelled}} \) and \( A_{\text{unlabelled}} \) correspond respectively to the peak area of each labelled and unlabelled peptide. Differences in the extent of labelling between peptides were

![Figure 2.1. The aryl diazirine used for carbene footprinting.](image_url)
considered as significant only when the p-value obtained from a Student T-test was <0.05.

2.10.4. Native-PAGE

A native-PAGE gel composed of 5 ml resolving gel (245 mM Tris, pH 8.8, 12% (v/v) 29:1 acrylamide:bis-acrylamide) was cast using the BioRad Mini-PROTEAN system, and was polymerised with 100 µl 10% (w/v) APS and 10 µl TEMED. The resolving gel was overlaid with ethanol to ensure a flat interface between the resolving and stacking gel. Once polymerised, the ethanol was removed from the top of the resolving gel, and a 2 ml stacking gel (321 mM Tris, pH 8.8, 4% (v/v) 29:1 acrylamide:bis-acrylamide) set with 50 µl 10% (w/v) APS and 5 µl TEMED was overlaid on top. A 10-well or 15-well comb was inserted and the stacking gel allowed to polymerise. Native-PAGE gels were loaded with 10 µg protein per well, using loading dye minus SDS. Gels were run at 100 V, 400 mA with the buffer tank on ice (approx. 4 hours) using 25 mM Tris and 0.19 mM glycine, pH 8. Gels were stained with InstantBlue Coomassie Protein Stain (Expedeon) and rinsed with distilled water to remove excess stain before visualisation using the SynGene GeneSNap G:Box Gel Doc or Vilber E-box.

2.11. Assays for the ligation of [3H]-glycine to lipid-II by the Fem ligases

2.11.1. Synthesis of [3H]-glycyl-tRNA

*S. aureus* ATCC 25923 tRNA was labelled with [3H]-glycine via the incubation of a final concentration of 0.5 mg/ml tRNA, 37.6 µM [3H]-glycine at 500 cpm/pmol, 5 µM DTT and 0.3 mg/ml GlyRS, plus 100 µl of 2x assay buffer 60 mM HEPES, pH 7.5, 30 mM MgCl2, 50 mM KCl, 10 mM DTT and 4 mM ATP in a final volume of 200 µl. In minus tRNA controls, tRNA was replaced with water. Each reaction was pre-incubated at 37°C for 10 min, before the addition of GlyRS. Reactions were incubated for a further 60 min at 37°C. Before purification of [3H]-glycyl-tRNA, 20 µl was spotted onto 3MM
Whatman filter paper (1.5 cm x 1.5 cm) and submerged into ice cold 10% (w/v) TCA. Total counts (5 µl of 1 in 10 dilution) were spotted in duplicate onto filter paper and added straight to 10 ml scintillant fluid (Optiphase HiSafe 3, PerkinElmer). The reactions were supplemented with 20 µl of 3M sodium acetate, pH 5 and 205 µl buffer saturated phenol, vortexted and centrifuged 13,000 x g for 3 min. The aqueous phase was removed and supplemented with 520 µl ethanol and tRNA was precipitated at -20°C for 15 min. Ethanol was removed and pellets washed with 70% (v/v) ethanol before centrifugation. Ethanol was removed and pellets dried under vacuum before being resuspended in 50 µl of 3 mM sodium acetate, pH 5. In duplicate, 2 µl of tRNA was spotted onto filter paper and submerged into 10% (w/v) ice-cold TCA. Filter papers were washed for 15 min in 10% (w/v) TCA, three times and once with ethanol for 15 min, before being air dried. Filter papers were added to 10 ml scintillant each and radioactivity detected using the Tri-Carb 2800TR liquid scintillation analyser, with a count time of 5 min. Purified [³H]-glycyl-tRNA was stored at -80°C.

2.11.2. Ligation of [³H]-glycine to lipid-II by the Fem ligases – time course

The ability of the Fem ligases to transfer [³H]-glycine from [³H]-glycyl-tRNA to non-amidated or amidated lipid-II (Lys) was assessed under the following conditions. Each reaction contained 1 mM DTT, 10 µM non-amidated or amidated lipid-II (Lys), 0.27 µM (or 0.18 µM) [³H]-tRNA at 281 cpm/pmol (or 648 cpm/pmol), 65 nM Fem ligase, 1 mM glycine and 50 mM HEPES, 10 mM MgCl₂, 30 mM KCl, 0.3 % (v/v) Triton X-100 pH 7.6 (assay buffer), to a final volume of 40 µl. Controls were performed in the absence of FemX or lipid-II (replaced with assay buffer) or in the presence of 0.25 mg/ml RNase. Reactions were pre-warmed at 37°C in the absence of [³H]-glycyl-tRNA, and initiated via the addition of Fem ligase and [³H]-glycyl-tRNA. At proscribed times, 40 µl of ice-cold pyridinium acetate was added to 40 µl of reaction, vortexted and then kept on ice. When the final time point of the reaction was quenched with pyridinium acetate, reactions were centrifuged at 13,000 x g for 3 min. The n-butanol (top-phase) was retained and supplemented with 80 µl water, vortexted and
centrifuged again. The n-butanol phase (top-phase) was added straight into 10 ml scintillant. From the remaining unquenched reaction mixture, 5 µl was added straight into scintillant to measure total counts. Radioactivity was detected using the Tri-Carb 2800TR liquid scintillation analyser, using a count time of 5 min.

### 2.12. Analysis of transglycosylase and transpeptidase activity

PBP (5 µM) were incubated with 10 µM dansylated lipid-II (Lys), with 20% (v/v) DMSO, 50 mM HEPES pH 7.5, 100 mM NaCl and 10 mM MgCl₂, up to a total volume of 15 µl with HPLC grade water, unless otherwise stated. Reactions were incubated at 30°C for 180 min, unless otherwise stated, after which time they were quenched with 1 µl of 0.5 M EDTA, pH 8 and kept on ice.

Loading dye (3µl, 6x loading dye - 100 mM Tris-HCl pH 8.8, 4% (w/v) SDS, 40% (v/v) glycerol, 0.05% bromophenol blue) was added to each sample. Samples were loaded onto a Tricine-SDS-PAGE gel (16.5% Criterion, BioRad) and gels were run at 100V, 100 mA until dye front reached end of gel (approximately 2.5 hours). Gel running buffers were 0.1 M Tris-HCl pH 8.8 (Anode) and 0.1 M Tris-HCl pH 8.25, 0.1 M Tricine, 0.1% (w/v) SDS (Cathode). Gels were visualised on the ethidium bromide setting of the ImageQuant (GE Healthcare) GelDoc, using a 5 sec exposure time.
3. Optimising the yields of lipid-II during in vitro chemo-enzymatic synthesis

3.1. Background

The current method for the in vitro chemo-enzymatic production of lipid-II employs Micrococcus flavus membranes as a source highly rich in MraY and MurG, the enzymes responsible for the conversion of UDP-MurNAc-pentapeptide into lipid-II (Breukink et al, 2003, Lloyd et al, 2008). However, this process has proven to be highly time consuming and costly, with unreliable and variable yields, especially in the case of amidated lipid-II (Lys). Increasing the overall yields of lipid-II from these syntheses would not only reduce the overall cost per mg of synthesising lipid-II, but it would also make downstream lipid-II modifications easier and more accessible, as the inevitable losses of lipid-II material during the sequential purification steps are high (Dr Adrian Lloyd, Personal communication). Hence, having larger amounts of starting material (i.e. lipid-II) for modification reactions (i.e. penta-glycyl bridge synthesis by the Fem ligases) would hopefully yield larger amounts of product (i.e. lipid-II pentaglycine).

In order to study the in vitro enzymatic activities and substrate specificities of various S. aureus enzymes, such as PBP2, PBP2a and the Fem ligases, the synthesis of a wide range of lipid-II substrates was required. These include lipid-II substrates with species specific modifications and fluorescently labelled lipid-II for use in transglycosylase assays.

One potential mechanism to yield larger amounts of lipid-II during syntheses is the use of a different bacterial membrane as the source of MraY and MurG. Previous contamination of M. flavus cultures with Staphylococcal sp. within the laboratory (Julie Tod, Personal communication) have led to the use of a mixed membrane populations during lipid-II synthesis. Unexpectedly, this led to unusually high yields.
of lipid-II when compared to the standard use of *M. flavus* membranes for lipid-II synthesis.

Therefore, this chapter aims to compare the use of different bacterial membranes, instead of *M. flavus*, in the synthesis of lipid-II in order to maximise lipid-II yields. The yield of both amidated and non-amidated lipid-II, synthesised from either *M. flavus* membranes, *S. aureus* ATCC 25923 (CAT2) membranes with or without *M. flavus* membranes, and *S. epidermidis* ATCC 12228 (CAT1) membranes with or without *M. flavus* membranes was compared. In addition, this chapter describes the optimal growth conditions that were needed to enable Staphylococcal cultures to reach a high cell density, as well as optimal cell lysis methods to ensure efficient membrane extraction.
3.2. Results

3.2.1. Comparison of Micrococal and Staphylococcal growth media for high cell density

Cultures of *M. flavus* grown for the isolation of membranes are grown to high cell densities (Optical density, OD\textsubscript{600nm} approx. 9, in TSB). However, the cfu/ml that this OD corresponds to was unknown, as well as how these OD values relate to OD and cfu/ml of *S. aureus* and *S. epidermidis*. Knowledge of these parameters was essential for comparison of the efficiency of lipid-II synthesis achieved with the membranes of these organisms.

As shown in Fig. 3.1, *M. flavus* growth in TSB, *S. epidermidis* growth in NB and BHI, and *S. aureus* growth in BHI were compared. Cultures (4L) were grown to late exponential phase at 37 °C, 180 rpm – OD\textsubscript{600nm} and cfu/ml for each organism are summarized in Table 3.1.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Media</th>
<th>OD\textsubscript{600nm}</th>
<th>Log(cfu/ml)</th>
<th>Cf u/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. flavus</em></td>
<td>TSB</td>
<td>5.2</td>
<td>9.1</td>
<td>$1.3 \times 10^9$</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>BHI</td>
<td>2.2</td>
<td>9.4</td>
<td>$2.5 \times 10^9$</td>
</tr>
<tr>
<td><em>S. epidermidis</em></td>
<td>BHI</td>
<td>5.2</td>
<td>9.2</td>
<td>$2.5 \times 10^9$</td>
</tr>
<tr>
<td></td>
<td>NB</td>
<td>2.5</td>
<td>8.4</td>
<td>$2.5 \times 10^8$</td>
</tr>
</tbody>
</table>

Membranes isolated for use in lipid-II synthesis were therefore harvested from *M. flavus* grown in TSB at late exponential phase, *S. epidermidis* grown in BHI and *S. aureus* grown in BHI at late exponential phase of approx. OD\textsubscript{600nm} >8, 6-7 and 1.5-2, respectively. These cultures were harvested at late exponential phase to allow for maximal cell density to be achieved, hence allowing for maximal membrane extraction.
Figure 3.1. Growth curve and cfu/ml of *M. flavus*, *S. epidermidis* and *S. aureus*. A. *M. flavus* grown in TSB at 37°C, 180 rpm (cfu/ml n=2, OD n=1), B. *S. aureus* grown in BHI (n=3) at 37°C, 180 rpm and C. *S. epidermidis* grown in BHI (squares, n=3) or NB (triangles, cfu/ml n=2, OD n=3) at 37°C, 180 rpm. Log(cfu/ml), open symbols. OD<sub>600nm</sub>, closed symbols. Where error bars cannot be seen, error bars are smaller than the data symbol. Where n=3, error bars are standard deviation. Where n=2, error bars are the range.
3.2.2. Optimisation of bacterial cell lysis

The lysis of *M. flavus* using lysozyme in order to isolate bacterial membranes is reasonably straightforward. Lysozyme is a muramidase that cleaves the β1,4 linkage between the GlcNAc and MurNac residues of peptidoglycan, and therefore efficiently causes cell lysis in *M. flavus* (Bera *et al*, 2005). However, Staphylococci are one of the few bacterial Genera that are completely resistant to degradation of the cell wall by lysozyme (Blake *et al*, 1967, Bera *et al*, 2005). This high-level lysozyme resistance is due to three inherent features of Staphylococcal peptidoglycan; the high level of peptidoglycan cross-linking, and the steric hindrance caused by the O-acetylation of the C6-hydroxyl group of MurNac by OatA, a MurNAC specific O-acetyltransferase and the phosphoester-linked cell wall techoic acid at the C6 position of MurNac (Bera *et al*, 2005, Bera *et al*, 2006, Bera *et al*, 2007). Therefore, an alternative enzyme that was able to cleave the cell wall of *S. aureus* and *S. epidermidis* was sought.

Turbidity assays were performed at 600 nm (60 minutes, 37°C) to measure lysis of *S. aureus* and *S. epidermidis* in the presence of 0 - 6 μg/ml lysostaphin (Dr Stephane Mesnag, Sheffield), 0 - 2.5 mg/ml lysozyme, combinations of both lysostaphin and lysozyme, and 3 - 50 μg/ml autolysin (AtlA). These were chosen due to their specific modes of action; autolysin cleaves the β1,4 bond between GlcNAc and MurNAc whilst lysostaphin cleaves the penta-glycyl bridge of peptidoglycan between the third and fourth glycyl residue (Bravetti *et al*, 2009, Bastos *et al*, 2010).

It is worth noting that whilst the peptidoglycan of *S. aureus* contains repeating units of lipid-II linked by a pentaglycyl cross bridge, *S. epidermidis* peptidoglycan can either contain lipid-II cross-linked by a pentaglycyl cross bridge, or by a non-random mix of L-serine and glycine, most notably the first and third residues being L-serine (Tipper and Berman 1969, Hilderman and Riggs 1973).

As can be seen in Fig. 3.2, lysis of *S. aureus* and *S. epidermidis* was most effective in the presence of 5 μg/ml lysostaphin (approx. 80% cell lysis), and 2 mg/ml lysozyme
plus 5 μg/ml lysostaphin (25% cell lysis), respectively. These concentrations of lysostaphin and lysozyme plus lysostaphin were therefore used for all lysis of *S. aureus* and *S. epidermidis*, respectively, to ensure greatest bacterial cell lysis for membrane extraction.
Figure 3.2. Change in turbidity due to cell lysis of Staphylococi. *S. aureus* (dark grey) and *S. epidermidis* (light grey) in the presence of A. 0 – 6 μg/ml lysostaphin, B. 0 – 2.5 mg/ml lysozyme, C. 3 – 50 μg/ml autolysin and D. 3 – 6 μg/ml lysostaphin and 1 – 2.5 mg/ml lysozyme, following 60 minute incubation at 37°C. n=2, except for 50 μg/ml autolysin, where n=1. Where n = 2 , error bars represent the range.
3.2.3. Comparison of non-amidated lipid-II (Lys) yield from different bacterial membranes

3.2.3.1. Small-scale analysis by TLC

In order to ascertain whether Staphylococcal membranes were able to produce non-amidated lipid-II (Lys), a small-scale non-amidated lipid-II synthesis (200 μl) was performed using bacterial membranes at a final concentration of 0.25 mg/ml. Controls were also performed where undecaprenyl phosphate (11P), UDP-MurNAc (Lys) pentapeptide (5P), MurG and UDP-GlcNAc were omitted.

Reactions were analysed by TLC to visualize the products of the lipid-II synthesis. As seen in Fig. 3.3, non-amidated lipid-II (Lys) was produced in the full reaction (Fig. 3.3, lane 1) where membranes from *M. flavus*, *S. epidermidis*, *S. aureus*, *M. flavus* plus *S. epidermidis (1:1)* and *M. flavus* plus *S. aureus (1:1)* were used. The production of non-amidated lipid-II (Lys) was also confirmed by mass spectrometric analysis.

As expected in the minus 11P control lane (Fig. 3.3, lane 2), there was no non-amidated lipid-II (Lys) production seen, as 11P is the lipid carrier to which the phospho-MurNAc moiety of MurNAc (Lys) pentapeptide is attached to create lipid-I. There was also no 11P band seen on the TLC, as expected.

In the minus UDP-MurNAc pentapeptide control lanes (Fig. 3.3, lane 3), there was also no non-amidated lipid-II (Lys) production seen due to the inability to form lipid-I via the attachment of 5P to 11P. There was an 11P band seen as expected. The minus UDP-GlcNAc (Fig. 3.3, lane 5) control lane showed the production of lipid-I only, as there was no UDP-GlcNAc present to attach to lipid-I, and hence lipid-II synthesis could not occur. However, in the absence of MurG (Fig. 3.3, lane 4) there was still a small amount of lipid production. This was expected as the production of bacterial membranes was a crude method and hence there may be MurG already present in the isolated bacterial membranes.
**Figure 3.3. Non-amidated lipid-II (Lys) synthesis with different bacterial membranes.** Lipid-II was synthesized using *M. flavus* (MF), *S. epidermidis* (SE), *S. aureus* (SA), or a 1:1 mix of MF and SE or MF and SA. Full reactions (200 µl) were analysed by TLC (1), as well as controls minus 11P (2), UDP-MurNAc pentapeptide (3), MurG (4) and UDP-GlcNac (5). 11P – undecaprenyl phosphate. LII – non-amidated lipid-II (Lys).
3.2.3.2. Larger-scale analysis of lipid-II production by quantitative spectrophotometric assay

As non-amidated lipid-II (Lys) production was successful on a small scale using various bacterial membranes, the synthesis was scaled up (875 μl) to allow for lipid-II quantification by spectrophotometric assay (Section 2.8.6). Bacterial membranes were used at a total final concentration of 5.7 mg/ml in a non-amidated lipid-II (Lys) synthesis and lipid-II was purified via anion exchange on a DEAE sephacel column using an ammonium bicarbonate gradient (Section 2.8.4). Fractions containing lipid-II were identified via TLC and combined. Solvents were removed from samples using a RotaVap and ammonium bicarbonate removed via multiple rounds of freeze-drying. Each lipid-II sample was resuspended in a final volume of 400 μl solvent A (2:3:1, chloroform:methanol:water). Successful non-amidated lipid-II (Lys) synthesis was confirmed by mass spectrometry (Fig. 3.3 and was quantified via phosphate release assay at 360 nm (Table 3.2).

The greatest yield of non-amidated lipid-II (Lys) was synthesised using S. aureus ATCC 25923 membranes; greater than 2x the yield of lipid-II was produced when compared to a lipid-II synthesis using M. flavus membranes (Table 3.2). The lowest yields of non-amidated lipid-II (Lys) occurred from syntheses using either S. epidermidis membranes, or S. aureus plus M. flavus (1:1) – approximately one seventh, and one fifteenth of the largest lipid-II yield when compared to using S. aureus membranes, respectively.
3.2.4. Comparison of amidated lipid-II (Lys) yield from different bacterial membranes

3.2.4.1. Small-scale analysis by TLC

As yields for amidated lipid-II (Lys) were much lower than that for non-amidated lipid-II (Lys), the same investigation to ascertain whether *Staphylococcal* membranes were able to produce greater yields of amidated lipid-II (Lys) were repeated. Again, a small-scale amidated lipid-II (Lys) synthesis (200 μl) was performed using bacterial membranes at a final concentration of 0.25 mg/ml. Controls were also performed where undecaprenyl (11P), UDP-MurNAc-pentapeptide (5P), MurG and UDP-GlcNAc where omitted. Unfortunately, no production of amidated lipid-II (Lys) was seen on the TLC for these small-scale reactions. However, this is most probably due to the inherently low yields for amidated lipid-II (Lys) syntheses, such that a small-scale 200 μl synthesis is too small to be detected visually by TLC. This inherently low yield of amidated lipid-II (Lys) could indicate that amidated UDP-MurNAc-pentapeptide (Lys) is a poor substrate for MraY conversion to lipid-I, which fits with the dogma that amidation occurs at the lipid-linked stage of peptidoglycan synthesis, rather than the cytoplasmic stage.

### Table 3.2. Concentration of non-amidated lipid-II (Lys) synthesis using different bacterial membranes.

<table>
<thead>
<tr>
<th>Membrane used for non-amidated lipid-II (Lys) synthesis</th>
<th>Concentration of non-amidated lipid-II (Lys), μM</th>
<th>Amount of non-amidated lipid-II (Lys), mg</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. flavus</em></td>
<td>293.8</td>
<td>0.22</td>
</tr>
<tr>
<td><em>S. aureus ATCC 25923</em></td>
<td>595.6</td>
<td>0.45</td>
</tr>
<tr>
<td><em>S. epidermidis ATCC 12228</em></td>
<td>87.6</td>
<td>0.066</td>
</tr>
<tr>
<td><em>M. flavus</em> plus <em>S. aureus ATCC 25923</em></td>
<td>40.9</td>
<td>0.031</td>
</tr>
</tbody>
</table>
3.2.4.2. Larger-scale analysis by quantitative spectrophotometric assay

As small-scale syntheses of amidated lipid-II (Lys) could not be seen via TLC, a half-scale synthesis was therefore performed (1750 μl) and purified as per for the non-amidated lipid-II (Lys). During purification via DEAE sephacel, amidated lipid-II (Lys) elutes off the sephacel column at much lower ammonium bicarbonate concentrations than non-amidated lipid-II (Lys), meaning that fractions containing amidated lipid-II (Lys) are often unfortunately cross-contaminated with 11P which also elutes off the sephacel column a low ammonium bicarbonate concentration. Unfortunately, this cross contamination occurred such that a second round of DEAE sephacel purification was necessary. This meant ultimately that yields were too low for quantification via phosphate release assay. Amidated lipid-II (Lys) was also not detected via mass spectrometry.
3.3. Conclusions and further work

The need to increase the yields of lipid-II synthesised from in vitro chemo-enzymatic assays is imperative – not only due to the relatively complex and time consuming nature of lipid-II synthesis and purification, but also due to the high costs associated with the reagents used for these syntheses (most notably undecaprenyl phosphate which costs in excess of €2000 for 50 mg).

3.3.1. Enhancing bacterial cell lysis for membrane extraction

Turbidity assays were undertaken to find the best method to ensure the greatest cell lysis of Staphylococcal cells in order to maximise membrane extraction. Staphylococcal sp. are intrinsically resistant to lysozyme due to their high level of peptidoglycan cross-linking, and the steric hindrance caused by the O-acetylation of the C6-hydroxyl group of MurNac by OatA, a MurNAC specific O-acetyltransferase and the phosphoester-linked cell wall techoic acid at the C6 position of MurNac (Bera et al, 2005, Bera et al, 2006, Bera et al, 2007). Therefore, lysozyme cannot cleave the β1,4 linked MurNAc and GlcNAc backbone of Staphylococcal peptidoglycan.

For this reason, the ability of autolysin and lysostaphin to lyse S. aureus and S. epidermidis was also investigated. Autolysin also cleaves the β1,4 bond between GlcNAc and MurNAc whilst lysostaphin cleaves the penta-glycyl bridge of peptidoglycan between the third and fourth glycyl residue (Bravetti et al, 2009, Bastos et al, 2010). As discussed in Section 3.2.2, the peptidoglycan of S. aureus is linked by a pentaglycyl cross bridge, whilst S. epidermidis peptidoglycan contains lipid-II cross-linked by L-serine and glycine (Tipper and Berman 1969, Hilderman and Riggs 1973). In S. epidermidis, the cell wall peptidoglycan consists of 20% pentaglycyl cross-bridges, 55% cross-bridges with a third position serine, 15% cross-bridges with a first and third position serine, and 10% cross-bridges with a second position serine from the N-terminus of the peptide cross-bridge (Tipper 1969, Tipper and Berman
In *S. aureus*, it is also thought that the latter condition occurs in 7% of cells (Tipper 1969, Tipper and Berman 1969).

Lysostaphin was able to lyse *S. aureus* across all concentrations tested causing approximately 80% cell lysis when compared to no treatment. However, when in combination with lysozyme, this lytic activity was greatly reduced with increasing lysozyme concentration. As for *S. epidermidis*, lysostaphin lysed less than 20% of *S. epidermidis* across all concentrations tested. This is probably due to the variation in the composition of the pentapeptide cross-bridge between peptidoglycan units, thus as pentaglycyl cross-bridges are present at 20%, the ability of lysostaphin to cleave at glycyl residues 3-4 is greatly reduced. This is illustrated by the <20% cell lysis in the presence of lysostaphin. In general, this lysis was only slightly increased in the presence of lysozyme, where greatest cell lysis was with 2 mg/ml lysozyme plus 5 μg/ml lysostaphin.

### 3.3.2. Maximising yields of lipid-II

The yields of non-amidated lipid-II (Lys) were doubled when using *S. aureus* membranes, instead of *M. flavus* membranes in an *in vitro* chemo-enzymatic lipid-II synthesis. Bacterial membranes are used in an *in vitro* lipid-II synthesis as they deliver a pool of naturally occurring MraY – the enzyme responsible for conversion of UDP-MurNAc-pentapeptide into lipid-I. Therefore, perhaps *S. aureus* membranes naturally contain more of the membrane-bound MraY than *M. flavus* membranes, and hence there is more MraY available to convert UDP-MurNAc-pentapeptide into lipid-I.

It is also interesting to note the lowest yield of lipid-II (Table 3.1) came from the mixed species of *M. flavus* with *S. aureus* membranes. It was hypothesised that a mixed species of membranes may increase lipid-II yield following observations made by colleagues in the laboratory (Julie Tod, Personal Communication C10) where greater lipid-II yields were accomplished when *M. flavus* membrane preparations were contaminated with *S. aureus* membranes. However, it is impossible to know
the extent of this contamination and therefore the ratio of *M. flavus* to *S. aureus* membranes. For the purpose of this investigation a ratio of 1:1 *M. flavus* to *S. aureus* membranes were used. It is possible that varying yields of lipid-II would be seen for varying ratios of *M. flavus* to *S. aureus* membranes.

In the case of amidated lipid-II (Lys), yields of lipid-II following syntheses with the different membranes were all too low to be visualised via TLC. This finding, taken with the on-going poor yields of amidated lipid-II during full-scale lipid-II syntheses within the laboratory, may be reflective of amidated UDP-MurNAc-pentapeptide being an less suitable substrate for MraY and whilst it can still be converted to lipid-I, the activity of MraY is reduced when compared to non-amidated UDP-MurNAc-pentapeptide.

When scaled up to a larger amidated lipid-II synthesis, the purification of lipid-II via DEAE sephacel did not adequately remove contaminating 11P from lipid-II containing fractions. It is important to remove this contaminating 11P as the quantification of lipid-II relies on spectrophotometric assay of free phosphates hydrolysed from the lipid-II molecule, hence any contaminating sources of 11P may decrease the accuracy of the spectrophotometric assay.

The low yields of amidated lipid-II (Lys) and the ease with which lipid-II could be lost during the purification process further highlights the urgent need for a more robust method of *in vitro* chemo-enzymatic lipid-II synthesis with greater yields of lipid-II, especially in the case of amidated lipid-II where yields are inherently low and variable.

### 3.3.3. Improving membrane extraction from *Staphylococcal spp.*

Unfortunately, it could not be determined whether Staphylococcal membranes can increase the yield of amidated lipid-II (Lys) due to the nature of the low yielding syntheses and difficult purification method. However, it was shown that *S. aureus* membranes produce a 2x greater yield of non-amidated lipid-II (Lys) compared to *M.*
flavus membranes. A caveat to the use of S. aureus membranes is the poor yield of membranes that can be extracted when compared to M. flavus which is utilized readily due to its ease of lysis and the ability to subsequently isolate large volumes of membrane. For example, one batch of S. aureus membranes would allow for one lipid-II synthesis, whilst one batch of M. flavus membranes would be likely to allow for several lipid-II syntheses. For these reasons it would be sensible to continue lipid-II synthesis with M. flavus membranes, until yields of Staphylococcal membranes has been improved.

Due to the difficulty of extracting membranes from Staphylococcal species, it may be beneficial to increase the amounts of membrane that can be extracted from Staphylococci. This may therefore mean that more lipid-II syntheses can be performed for every batch of membrane extraction, instead of the current situation where one batch of S. aureus membrane can be used for only one lipid-II synthesis. A strain of vancomycin-intermediate resistant S. aureus (VISA), such as Mu50, which exhibits cell wall thickening may be more suitable – hence giving greater membrane yields and allowing for multiple lipid-II syntheses (Cui et al, 2003). However, the use of VISA strains presents additional biohazards, and is not guaranteed to increase lipid-II yields.

3.3.4. An alternative approach to synthesising amidated lipid-II

As the yields of non-amidated lipid-II (Lys) are much more robust, an alternative method to synthesising amidated lipid-II (Lys) could be presented via the amidation of non-amidated lipid-II through the use of the GatD/MurT amidases. The GatD/MurT enzyme complex has already been shown to successfully amidate the second pentapeptide stem position (D-Glu) of non-amidated lipid-II (Lys) in in vitro assay, in the presence of glutamine and ATP (Münch et al, 2012). The method consists of incubating 2 nmol non-amidated lipid-II (Lys) with 2 µg GatD/MurT complex, 6 mM ATP, 7 mM glutamine, in 160 mM Tris-HCl, 0.7% Triton X-100, 5 mM KCl, and 40 mM MgCl₂, pH 7.5, in a total volume of 30 µl for 2 hr at 30°C (Münch et al, 2012). This
method could of course be scaled up to allow amidation of larger scale amounts of non-amidated lipid-II (Lys) with minimal usage of reagents and time; the GatD/MurT complex could also be purified by following the published protocol by Münch et al, (2012).
Chapter 4 Synthesis of *S. aureus* specific lipid-II intermediates

4.1. Background

In order to study the *in vitro* enzymatic activities and substrate specificities of the *S. aureus* Fem ligases, PBP2 and PBP2a, a range of substrates are required (Summarised in Table 4.1). For example, each of the FemX, FemA and FemB ligases have different branched lipid-II (Lys) substrates; lipid-II (Fig. 4.1, A1), lipid-II mono-glycine (Fig. 4.1, A3) and lipid-II tri-glycine (Fig. 4.1, A4), respectively. Whilst the substrate for PBP2 and PBP2a is amidated lipid-II (Lys) pentaglycine (Gly$_5$) (Fig. 4.1, A5). It is worth noting that the point at which lipid-II amidation occurs in the cytoplasmic stage of lipid-II synthesis is currently unknown, hence the specificity of the Fem ligases for non-amidated (Fig. 4.1, B1) or amidated lipid-II (Fig. 4.1, B2) substrates is also unknown and will be investigated in Chapter 5. Additional modifications to lipid-II include fluorescent labelling with a dansyl group attached to the ε-amino group of the third position L-Lysine via a sulphonamide linkage (Fig. 4.1, A2) allowing for the detection of transglycosylation of lipid-II via continuous, or gel-based stopped assays (Schwartz *et al*, 2002, Galley *et al*, 2014).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Non-amidated</th>
<th>Amidated</th>
</tr>
</thead>
<tbody>
<tr>
<td>FemX</td>
<td>Lipid-II (Lys)</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>FemA</td>
<td>Lipid-II (Lys) Gly$_1$</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>FemB</td>
<td>Lipid-II (Lys) Gly$_3$</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>PBP2</td>
<td>Lipid-II (Lys) Gly$_5$</td>
<td>-</td>
<td>✓</td>
</tr>
<tr>
<td>PBP2a</td>
<td>Lipid-II (Lys) Gly$_5$</td>
<td>-</td>
<td>✓</td>
</tr>
</tbody>
</table>

The study of the enzymatic activities of the aforementioned enzymes has been obstructed, largely due to the laborious and complex synthesis of lipid-II, yielding small amounts of lipid-II. Despite this, several groups have developed methods for
the chemical, chemo-enzymatic or biosynthetic synthesis of lipid-II and its associated variants (Schwartz et al, 2001, VanNieuwenhze et al, 2002, Breukink et al, 2003, Schneider et al, 2004, Lloyd et al, 2008). Until recently, attempts to extract lipid-II directly from bacterial cultures were particularly poor (Guan et al, 2005), most probably due to the low natural abundance of lipid-II within the bacterial cell (van Heijenoort et al, 1992). However, recent publications from the Walker group (Harvard University) have yielded substantial amounts of amidated lipid-II (Lys) pentaglycine from S. aureus culture following lipid-II accumulation in the presence of moenomycin or vancomycin (Qiao et al, 2017). Whilst this method would allow for the extraction of amidated lipid-II (Lys) pentaglycine from S. aureus, as well as mono-glycine and tri-glycine variants from S. aureus ΔfemA or ΔfemB mutants, this method does not yet allow for the synthesis of non-amidated lipid-II variants due to the lethality of ΔfemX mutants (Tschierske et al, 1999). The method published by the Walker group has been trialled within our laboratory, however yields were considerably lower than published yields and therefore this method is not currently in use within our laboratory (Qiao et al, 2017).

Thus far in Sections 4.1-4.2, work to produce branched lipid-II has been performed post-lipid-II production, however here instead of branching lipid-II using the Fem ligases (post-lipid-II production), the UDP-MurNAc-pentapeptide is branched chemically and then subsequently converted enzymatically into branched lipid-II as usual. Unlike species such as Weisella that utilise a UDP-MurNAc pentapeptide L-alanine ligase to branch UDP-MurNAc pentapeptide (i.e. before lipid-II formation), the S. aureus Fem ligases are unable to use UDP-MurNAc pentapeptide as a substrate (Bouhss et al, 2001). In W. viridescens, the residues important for substrate binding in domain two of FemX are found in the α7-α8 loop; interestingly, when compared to S. aureus FemA, this loop is not seen (Biarrotte-Sorin et al, 2004). Furthermore, S. aureus FemA has an additional crevice formed by the β5-β6 loop and the α13 helix which is absent in W. viridescens FemX, and may explain the altered substrate specificities (Biarrotte-Sorin et al, 2004). The Fem ligases cannot therefore be utilised
to branch UDP-MurNAc pentapeptide, and this must be synthesised chemically, rather than enzymatically.

Previous attempts at synthesizing branched UDP-MurNAc-pentapeptides (L-Ala/L-Ser) specific to *Streptococcus pneumoniae* have been reasonably successful within our laboratory, however the conversion to lipid-II by MraY/MurG has proven more difficult (Catherine Rowland, Dowson group, University of Warwick). However, this has not yet been trialed for *S. aureus* specific UDP-MurNAc-pentapeptides, where appendage of a glycyl cross-bridge and subsequent conversion to lipid-II may have greater yields.
Figure 4.1. Lipid-II variants. The basic structure of lipid-II (Lys) variants are synthesised to be modified at two positions (A and B). The third position lysine can either be unchanged (A1), fluorescently labelled with a dansyl group (A2), or with a mono-, tri- and penta-glycyl branch (A3, 4 or 5, respectively). Lipid-II is either non-amidated (D-Glu, B1), or amidated (D-iGln, B2).
4.2. Chapter aims

- To synthesise amidated and non-amidated UDP-MurNAc-pentapeptide (Lys).

- To chemically synthesise mono-, tri- or penta-glycyl branched UDP-MurNAc-pentapeptide (Lys), both amidated and non-amidated.

- To enzymatically convert the aforementioned lipid intermediates into lipid-II.

- To synthesise mono-, tri- and penta-glycyl branched, non-amidated and amidated lipid-II using the Fem ligases.

NB. The fluorescent dansylated lipid-II (Lys) was given as a kind gift, as synthesised by BACWAN, University of Warwick (Julie Tod and Anita Catherwood).
4.3. Results and discussion

4.3.1. Enzymatic synthesis of UDP-MurNAc pentapeptide (Lys) derivatives

UDP-MurNAc-pentapeptide (Lys) was enzymatically synthesised in a one-step reaction using the enzymes MurA-F and their substrates, as illustrated in Fig. 4.2. Non-amidated and amidated UDP-MurNAc-pentapeptide (Lys) could both be synthesised using this method (Lloyd et al., 2008), where MurD was used to incorporate either D-Glu or D-iGln, respectively, into the second position pentapeptide stem.

4.3.1.1. Enzymatic synthesis of UDP-MurNAc pentapeptide (Lys)

Both amidated and non-amidated UDP-MurNAc-pentapeptide (Lys) were synthesised in a one-step reaction (Section 2.7) and purified via anion exchange on a Source 30Q column (Fig. 4.3). Elution of non-amidated UDP-MurNAc pentapeptide (Lys) and amidated UDP-MurNAc pentapeptide (Lys) was monitored by absorbance at 218, 254 and 280 nm, indicated by AI and BI on Fig. 4.3 Panel A and B, respectively. The amidated UDP-MurNAc-pentapeptide (Lys) has a lower net negative charge compared to non-amidated UDP-MurNAc-pentapeptide (Lys), therefore has a lower affinity for the anion exchange resin and an earlier elution time, as indicated by the conductivity at which each pentapeptide elutes from the column; conductivity at peak of elution was 6 mS/cm and 12 mS/cm, respectively. UDP-MurNAc pentapeptide (Lys) elution was well separated from the elution of ATP, added to the one-pot reaction as a substrate for MurC-MurF, which can be seen to elute much later than UDP-MurNAc-pentapeptide (Lys), as indicated in Fig. 4.3 Panel A and B at AII and BII.

Eluted UDP-MurNAc pentapeptide (Lys) species (Fig. 4.3, AI and BI) were pooled and freeze-dried multiple times to remove residual ammonium acetate from anion exchange. UDP-MurNAc-pentapeptide (Lys) was quantified at 260 nm, using the
molar extinction coefficient of 10,000 M$^{-1}$cm$^{-1}$. Average typical yields of amidated and non-amidated UDP-MurNAc pentapeptide (Lys) were 12 mg and 8 mg per 2 ml reaction, respectively per synthesis.
Figure 4.2. The *in vitro* synthesis of UDP-MurNAc-pentapeptide (Lys). The enzymes MurA-F are added to a one-pot synthesis with appropriate substrates and cofactors to synthesise UDP-MurNAc-pentapeptide from UDP-GlcNAc. \( X = \text{NH}_2 \) or \( \text{OH} \) for D-iGln and D-Glu, respectively. PEP – phosphoenolpyruvate. IDH – Isocitrate dehydrogenase. PK – Pyruvate kinase.
Figure 4.3. Typical AKTA chromatogram following purification of UDP-MurNAc-pentapeptide (Lys) by anion exchange chromatography. Non-amidated (A) and amidated (B) UDP-MurNAc-pentapeptide (Lys) were purified by anion exchange on a Source 30Q column, with an elution gradient of 10 mM-1M ammonium acetate (0-100% buffer B (Conc B) over 120 min) at 10 ml/min. Once UDP-MurNAc-pentapeptide was eluted, the gradient increased to 100% B over 0 min to remove remaining contaminants from column. Absorbance measured at 280 nm, 254 nm and 218 nm. AI and BI indicate elution of non-amidated and amidated UDP-MurNAc-pentapeptide (Lys), respectively. AII and BII indicate elution of ATP, resynthesised by pyruvate kinase and phosphoenol pyruvate following MurC-MurF catalysis.
Final purity of amidated and non-amidated UDP-MurNAc pentapeptide (Lys) was determined by analytical size exclusion chromatography (Section 2.7.1.3). Integration of the subsequent elution peaks using the UNICORN software (GE Healthcare) established the purity of amidated and non-amidated UDP-MurNAc pentapeptide (Lys) as >95% purity at 254 nm. The synthesis of both was also confirmed by negative ion mode LC-MS (Fig. 4.4-4.5), performed by Dr Adrian Lloyd, Julie Tod or Anita Catherwood – predicted m/z and observed m/z are summarised in Table 4.2.

**Table 4.2. Predicted m/z and observed m/z for negative mode TOF-MS analysis of UDP-MurNAc-pentapeptide (Lys) species.**

<table>
<thead>
<tr>
<th>Species</th>
<th>Charge state</th>
<th>Predicted m/z</th>
<th>Charge state</th>
<th>Observed m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Non-amidated UDP-MurNAc-pentapeptide (Lys)</strong></td>
<td>Single</td>
<td>1148.35</td>
<td>Single</td>
<td>1148.345 (plus isotopes 1149.347 and 1150.349)</td>
</tr>
<tr>
<td>Exact mass – 1149.35 Da</td>
<td>Double</td>
<td>573.675</td>
<td>Double</td>
<td>573.673 (plus isotopes 574.172 and 574.672)</td>
</tr>
<tr>
<td></td>
<td>Triple</td>
<td>382.117</td>
<td>Triple</td>
<td>382.110</td>
</tr>
<tr>
<td><strong>Amidated UDP-MurNAc-pentapeptide (Lys)</strong></td>
<td>Single</td>
<td>1147.37</td>
<td>Single</td>
<td>1147.363 (plus isotopes 1148.362 and 1149.364)</td>
</tr>
<tr>
<td>Exact mass – 1148.35</td>
<td>Double</td>
<td>573.185</td>
<td>Double</td>
<td>573.185 (plus isotopes 573.684, 574.169 and 574.682).</td>
</tr>
<tr>
<td></td>
<td>Triple</td>
<td>381.79</td>
<td>Triple</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 4.4. Non-amidated UDP-MurNAc pentapeptide (Lys) analysed by TOF-MS. TOF-MS in negative electrospray ionization mode. Non-amidated UDP-MurNAc pentapeptide (Lys). Predicted exact mass 1149.35 Da. Predicted m/z - singly charged 1148.35, doubly charged 573.675 and triply charged 382.117. Observed m/z – singly charged species m/z 1148.345 (plus singly charged isotopes 1149.347 and 1150.349), doubly charged species m/z 573.673 (plus doubly charged isotopes 574.172 and 574.672) and triply charged species m/z 382.11.
Figure 4.5. Amidated UDP-MurNAc pentapeptide (Lys) analysed by TOF-MS. TOF-MS in negative electrospray ionization mode. Amidated UDP-MurNAc pentapeptide (Lys). Predicted exact mass 1148.37 Da. Predicted m/z - singly charged 1147.37, doubly charged 573.185 and triply charged 381.79. Observed m/z – singly charged species m/z 1147.363 (plus singly charged isotopes 1148.362 and 1149.364), and doubly charged species m/z 573.185 (plus doubly charged isotopes 573.684, 574.169 and 574.682).
4.3.1.2. Chemo-enzymatic synthesis of UDP-MurNAc-pentapeptide (Lys) mono-, tri- and penta-glycine

Where UDP-MurNAc pentapeptide (Lys) was to be used for carbodiimide coupling to create branched UDP-MurNAc pentapeptides, residual ammonium acetate that may cause inhibition of coupling reactions was removed by size exclusion chromatography using a BioGel P2 column (Section 2.7.2.1). Desalting was performed on approximately 40 mg of pentapeptide. Desalting of amidated UDP-MurNAc-pentapeptide (Lys) resulted in an 88% recovery (35 mg), whilst desalting of non-amidated UDP-MurNAc-pentapeptide resulted in full recovery.

Using carbodiimide coupling, Gly and Gly-Gly-Gly were attached to the ε-carbon of the third position L-Lys of UDP-MurNAc pentapeptide. Fmoc-Gly (Sigma) and Fmoc-Gly-Gly-Gly (Synthesised by Dr Ricky Cain, Roper group) were activated at the free carboxyl group yielding an O-aclysisourea conjugated Fmoc-Gly or Fmoc-Gly-Gly-Gly. NHS (N-hydroxysuccinimide) was then used to displace the EDC (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide) urea derivative, forming an Fmoc-Gly or Fmoc-Gly-Gly-Gly NHS ester. This was then attacked by the ε-amino group nucleophile of the third position L-Lys of UDP-MurNAc-pentapeptide forming a peptide bond, subsequently removing the NHS. UDP-MurNAc-Ala-Glu/Gln-Lys(Gly/Gly-Gly-Gly-Fmoc)-Ala-Ala was then deprotected with piperidine to yield the final product UDP-MurNAc-Ala-Glu/Gln-Lys(Gly/Gly-Gly-Gly)-Ala-Ala (herein denoted amidated or non-amidated UDP-MurNAc-pentapeptide (Lys) Gly1 or Gly3), with an Fmoc piperidine derivative as a by-product.

The branched UDP-MurNAc-pentapeptides were subsequently purified on a MonoQ 10/10 (8 ml column volume) and eluted with a gradient of 10 mM – 1M ammonium acetate (0-100%). Fractions were analysed by TOF-MS to confirm successful synthesis (Fig. 4.6-4.7) – however it should be noted that non-amidated UDP-MurNAc-pentapeptide (Lys) Gly3 (Fig. 4.6) contained some contamination with the Gly1 variant (m/z 602.185 doubly charged), and is therefore not 100% pure, suggesting
incomplete conversion to Gly3 during chemical synthesis. Final yields of non-amidated UDP-MurNAc-pentapeptide Gly1 and Gly3 were 2.1 mg and 7.4 mg, respectively. Amidated UDP-MurNAc-pentapeptide (Lys) Gly1, amidated UDP-MurNAc-pentapeptide (Lys) Gly3 and UDP-MurNAc-Ala-Gln-Lys(Gly-Gly-Gly-Gly-Gly)-Ala-Ala (Amidated UDP-MurNAc-pentapeptide (Lys) Gly5) was previously synthesised by Dr Ricky Cain, Roper group. TOF-MS analysis given in Appendix (Fig. 8.1-8.3)
Figure 4.6. TOF-MS analysis of non-amidated UDP-MurNAc-pentapeptide (Lys) Gly$_1$. Negative ion mode TOF-MS. A. Non-amidated UDP-MurNAc-pentapeptide (Lys) Gly$_1$, predicted exact mass 1206.37 Da. Predicted m/z 1205.37 singly charged, 602.185 doubly charged and 401.12 triply charged.
Figure 4.7. TOF-MS analysis of non-amidated UDP-MurNAc-pentapeptide (Lys) Gly₃. Negative ion mode TOF-MS. Non-amidated UDP-MurNAc-pentapeptide (Lys) Gly₃ predicted exact mass 1320.42 Da. Predicted m/z 1319.42 singly charged, 659.21 doubly charged and 439.14 triply charged.
4.4. Synthesis of lipid-II (Lys) species

UDP-MurNAc pentapeptide (Lys), including branched and fluorescent species, can be converted to lipid-II enzymatically (Section 2.8), using the enzymes MraY and MurG which convert UDP-MurNAc pentapeptide (Lys) into lipid-I and lipid-II, respectively. Due to MraY having ten transmembrane helices, the recombinant expression and purification of such membrane proteins is difficult, hence lipid-II was synthesised from UDP-MurNAc pentapeptide (Lys) in an *in vitro* reaction using *M. flavus* membranes containing membrane associated MraY and supplemented with undecaprenyl phosphate and UDP-GlcNAc, as in Fig. 4.8 (Breukink et al, 2003, Bouhss *et al*, 2004). This method had been shown within the laboratory to be robust enough to synthesise fluorescent dansylated lipid-II, but had not yet been trialled for glyceryl-branched lipid-II.

4.4.1. Enzymatic synthesis of lipid-II (Lys)

Amidated and non-amidated lipid-II (Lys) were synthesised as in Section 2.8. Lipid-II was purified via anion exchange chromatography (DEAE sephacel) and fractions analysed by TLC. Fig. 4.9, Panel A and B shows TLC purification of amidated and non-amidated lipid-II, respectively – as shown, amidated lipid-II (Lys) elutes from the anion exchange column at lower ammonium bicarbonate concentrations (95 mM – 200 mM) than non-amidated lipid-II (Lys) (250 mM – 1M) due to its more positive charge. In some cases, this can result in contamination of lipid-II with undecaprenyl phosphate – this lipid-II would therefore need further purification which often results in major product losses.

Typical yields were approximately 0.3 mg per 3750 µl synthesis and 0.22 mg per 3750 µl synthesis for non-amidated lipid-II (Lys) and amidated lipid-II (Lys), respectively. Successful synthesis of non-amidated and amidated lipid-II (Lys) was confirmed by negative ion mode TOF-MS (Fig. 4.10 and Fig. 4.11, respectively).
Figure 4.8. The in vitro synthesis of lipid-II (Lys). Lipid-II was synthesised from UDP-MurNAc-pentapeptide in the presence of UDP-GlcNAc, undecaprenyl phosphate and *M. flavus* membranes. UDP-MurNAc-pentapeptide is attached to the membrane acceptor, undecaprenyl phosphate, by MraY (a membrane bound protein extracted with *M. flavus* membranes) forming lipid-I and UMP. MurG is responsible for the addition of UDP-GlcNAc to lipid-I, forming lipid-II and UDP. X = NH$_2$ or OH for D-iGln (amidated lipid-II (Lys)) and D-Glu (non-amidated lipid—II (Lys)), respectively.
Figure 4.9. Thin layer chromatography analysis of lipid-II (Lys) purified by DEAE sephacel. (A) Amidated or (B) non-amidated lipid-II (Lys) purified by anion exchange on DEAE sephacel (4 ml), eluted with 12 ml washes of increasing concentrations of ammonium bicarbonate (2 ml of 0 mM-1M) in chloroform (4 ml) and methanol (6 ml). FT – flowthrough. Sol – Solvent A wash. Blue arrow – undecaprenyl phosphate. Grey arrow and underlined – lipid-II (Lys).
Figure 4.10. TOF-MS analysis of non-amidated lipid-II (Lys). TOF-MS in negative electrospray ionization mode. Non-amidated lipid-II (Lys) predicted exact mass 1875.06 Da. Predicted m/z –1874.06 singly charged, 936.53 doubly charged and 624.02 triply charged.
Figure 4.11. TOF-MS analysis of amidated lipid-II (Lys). TOF-MS in negative electrospray ionization mode. Amidated lipid-II (Lys) Predicted exact mass – 1874.08 Da. Predicted m/z – 1873.08 singly charged, 936.04 doubly charged and 623.69 triply charged.
4.4.2. Enzymatic synthesis of lipid-II (Lys) mono-, tri- and penta-glycine using branched UDP-MurNAc-pentapeptides

The synthesis of *S. aureus* branched lipid-II (Lys) species has not yet been investigated within our laboratory, and to date, there have been no published robust methods where branched UDP-MurNAc-pentapeptides have been successfully converted into branched lipid-II in quantities that may be sufficient for quantification and future enzymatic studies with PBPs.

Both amidated UDP-MurNAc-pentapeptide-Gly1, -Gly3 and -Gly5 (4 mM) and non-amidated UDP-MurNAc-pentapeptide-Gly1 and Gly3 (2 mM) were added separately into the one-pot lipid-II synthesis reaction (3.5 ml volume, except for non-amidated UDP-MurNAc-pentapeptide-Gly1 which was performed in 1.5 ml due to availability of smaller quantities of pentapeptide). Syntheses were incubated overnight at 37°C before extraction, as in Section 2.8.1. Non-amidated UDP-MurNAc-pentapeptide Gly5 was not converted to lipid-II as non-amidated lipid-II (Lys) Gly5 is not the natural substrate for PBP2 or PBP2a, and was therefore not necessary for this study. Each branched lipid-II was purified by anion exchange chromatography and fractions containing branched lipid-II were visualised by TLC (Section 2.8.4-2.8.5).

Non-amidated lipid-II (Lys) Gly1 and Gly3 were eluted in fractions 1M, and 500 mM-1M, respectively (Fig. 4.12 Panel A and B, respectively). Final yields were 89 µg and 172 µg per 3.5 ml reaction, respectively. Amidated lipid-II (Lys) Gly1 and Gly3 were eluted in fractions 300 mM-1M, and 500 mM-1 M, respectively (Fig. 4.12 Panel C and D, respectively). Final yields were 312 µg per 1.5 ml reaction, and 845 µg per 3.5 ml reaction, respectively. Successful synthesis of each lipid-II was confirmed by negative ion mode TOF-MS (Fig. 4.12-4.16), and positive ion mode TOF-MS/MS (Table 4.3 and Table 4.4, respectively and Fig. 4.17-4.19). It should be noted that for the lipid-II (Lys) Gly3 species (both amidated and non-amidated) there was some contamination with lipid-II (Lys) Gly1. However, this is not surprising as this contamination was seen at the pentapeptide stage, and is an artefact of incomplete turnover during the
chemical synthesis of Fmoc-Gly-Gly-Gly. For this reason, the tri-glycine variants were not assessed by positive ion mode TOF-MS/MS.

Table 4.3. TOF-MS/MS analysis of amidated lipid-II (Lys) Gly1. Positive ion mode TOF-MS/MS. Denoted fragment number refers to ion peak in TOF-MS trace.

<table>
<thead>
<tr>
<th>Fragment product (Denoted fragment number)</th>
<th>Predicted exact mass</th>
<th>Charge state</th>
<th>Expected m/z</th>
<th>Observed m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlcNAc (1)</td>
<td>220.1</td>
<td>Singly</td>
<td>222.1</td>
<td>222.1</td>
</tr>
<tr>
<td>GlcNAc minus water (2)</td>
<td>204.09</td>
<td>Singly</td>
<td>204.09</td>
<td>204.09</td>
</tr>
<tr>
<td>D-Gln-L-Lys-(Gly1) (3)</td>
<td>313.18</td>
<td>Singly</td>
<td>314.18</td>
<td>314.18</td>
</tr>
<tr>
<td>D-Gln-L-Lys-(Gly1)-D-Ala (4)</td>
<td>384.21</td>
<td>Singly</td>
<td>385.21</td>
<td>385.22</td>
</tr>
<tr>
<td>L-Lys-(Gly1)-D-Ala-D-Ala (5)</td>
<td>344.19</td>
<td>Singly</td>
<td>346.19</td>
<td>346.21</td>
</tr>
<tr>
<td>D-Gln-L-Lys-(Gly1) (7)</td>
<td>313.18</td>
<td>Singly</td>
<td>314.18</td>
<td>314.18</td>
</tr>
<tr>
<td>D-Gln-L-Lys-(Gly1)-D-Ala-D-Ala (8)</td>
<td>472.25</td>
<td>Singly</td>
<td>474.25</td>
<td>474.27</td>
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<tr>
<td>Lactyl-L-Ala-D-Gln-L-Lys-(Gly1)-L-Ala (9)</td>
<td>527.27</td>
<td>Singly</td>
<td>528.28</td>
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<tr>
<td>Lactyl-L-Ala-D-Gln-L-Lys-(Gly1)-L-Ala (10)</td>
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<td>617.32</td>
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<td>MurNAc-L-Ala-D-Gln-L-Lys-(Gly1)-D-Ala (11)</td>
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<td>Singly</td>
<td>802.39</td>
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<tr>
<td>GlcNAc-ppMurNAc-L-Ala-D-Gln-L-Lys-(Gly1)-D-Ala (12)</td>
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<td>Singly</td>
<td>1183.4</td>
<td>1183.4</td>
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<tr>
<td>GlcNAc-MurNAc-L-Ala-D-Gln-L-Lys-(Gly1)-D-Ala-D-Ala (13)</td>
<td>1005.47</td>
<td>Singly</td>
<td>1005.47</td>
<td>1005.47</td>
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<tr>
<td>Amidated lipid-II (Lys) Gly1 (14)</td>
<td>1931.1</td>
<td>Singly</td>
<td>1932.1</td>
<td>1931.1</td>
</tr>
<tr>
<td>Amidated lipid-II (Lys) Gly1 (15)</td>
<td>1931.1</td>
<td>Doubly</td>
<td>966.55</td>
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</tbody>
</table>
Table 4.4. TOF-MS/MS analysis of non-amidated lipid-II (Lys) Gly\(_1\). Positive ion mode TOF-MS/MS. Denoted fragment number refers to ion peak in TOF-MS trace.

<table>
<thead>
<tr>
<th>Fragment product (Denoted fragment number)</th>
<th>Predicted exact mass</th>
<th>Charge state</th>
<th>Expected m/z</th>
<th>Observed m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlcNAc (1)</td>
<td>220.1</td>
<td>Singly</td>
<td>222.1</td>
<td>-</td>
</tr>
<tr>
<td>GlcNAc minus water (2)</td>
<td>204.09</td>
<td>Singly</td>
<td>204.09</td>
<td>-</td>
</tr>
<tr>
<td>L-Lys-(Gly(_1)) (3)</td>
<td>185.12</td>
<td>Singly</td>
<td>186.12</td>
<td>-</td>
</tr>
<tr>
<td>D-Glu-L-Lys-(Gly(_1)) (4)</td>
<td>314.16</td>
<td>Singly</td>
<td>315.16</td>
<td>315.17</td>
</tr>
<tr>
<td>D-Glu-L-Lys-(Gly(_1))-D-Ala (5)</td>
<td>385.19</td>
<td>Singly</td>
<td>386.19</td>
<td>-</td>
</tr>
<tr>
<td>L-Lys-(Gly(_1))-D-Ala-D-Ala (6)</td>
<td>344.19</td>
<td>Singly</td>
<td>346.19</td>
<td>346.21</td>
</tr>
<tr>
<td>Lactyl-L-Ala-D-Glu-L-Lys-(Gly(_1)) (7)</td>
<td>457.21</td>
<td>Singly</td>
<td>458.21</td>
<td>458.22</td>
</tr>
<tr>
<td>D-Glu-L-Lys-(Gly(_1))-D-Ala-D-Ala (8)</td>
<td>473.23</td>
<td>Singly</td>
<td>475.23</td>
<td>-</td>
</tr>
<tr>
<td>Lactyl-L-Ala-D-Gln-L-Lys-(Gly(_1))-L-Ala (9)</td>
<td>528.25</td>
<td>Singly</td>
<td>529.26</td>
<td>529.26</td>
</tr>
<tr>
<td>Lactyl-L-Ala-D-Glu-L-Lys-(Gly(_1))-L-Ala-L-Ala (10)</td>
<td>616.29</td>
<td>Singly</td>
<td>618.3</td>
<td>618.31</td>
</tr>
<tr>
<td>MurNAc-L-Ala-D-Glu-L-Lys-(Gly(_1))-D-Ala-D-Ala (11)</td>
<td>802.37</td>
<td>Singly</td>
<td>803.37</td>
<td>803.38</td>
</tr>
<tr>
<td>GlcNAc-ppMurNAc-L-Ala-D-Glu-L-Lys-(Gly(_1))-D-Ala-D-Ala (12)</td>
<td>1182.38</td>
<td>Singly</td>
<td>1184.38</td>
<td>1184.38</td>
</tr>
<tr>
<td>GlcNAc-MurNAc-L-Ala-D-Glu-L-Lys-(Gly(_1))-D-Ala-D-Ala (13)</td>
<td>1006.45</td>
<td>Singly</td>
<td>1006.45</td>
<td>-</td>
</tr>
<tr>
<td>Non-amidated lipid-II (Lys) Gly(_1) (14)</td>
<td>1932.08</td>
<td>Doubly</td>
<td>967.53</td>
<td>967.55</td>
</tr>
<tr>
<td>Non-amidated lipid-II (Lys) Gly(_1) (15)</td>
<td>1932.08</td>
<td>Singly</td>
<td>1933.08</td>
<td>-</td>
</tr>
</tbody>
</table>
It is worth noting that these yields are much lower than for standard non-branched lipid-II (Lys), which on average yielded quantities of lipid-II in the 0.2-1 mg range, suggesting that branching of UDP-MurNAc-pentapeptides is detrimental to final lipid-II yields and it may be of more benefit to consider branching of lipid-II directly, rather than branching of the precursors, which may therefore enhance yields of branched lipid-II.

Furthermore, amidated UDP-MurNAc-pentapeptide-Gly5 did not successfully convert to lipid-II and could not be seen via TLC following anion exchange purification (Data not shown). This again highlights the detrimental effect of the branched precursors of pentapeptide turnover to lipid-I by MraY.

The branching of UDP-MurNAc-pentapeptides has a clear detrimental effect on the ability of MraY or MurG to convert pentapeptides to lipid-I and lipid-II, respectively. Whilst the addition of the glycyl cross-bridge does not affect the overall charge of the lipid-II molecule, it could however increase steric hindrance leading to a much slower interaction of enzyme-substrate. In the case of the pentaglycyl pentapeptide, the pentaglycyl cross-bridge appears to prevent any turnover of pentapeptide to lipid-II, perhaps suggesting that the pentaglycyl cross-bridge may not only increase steric hindrance, but also prevents any interaction of MraY and/or MurG to the branched pentapeptide. Possibly the pentaglycyl branch is mis-recognised by MraY leading to misorientation of the UDP-MurNAc moiety within the active site of the enzyme.

As previously discussed, in S. aureus branching occurs at the lipid-II level and not the UDP-MurNAc pentapeptide level, which is seen in species such as Weissella viridescens (Hegde and Shrader 2001). Hence, it is not surprising that the pentaglycyl pentapeptide cannot be turned over into lipid-II as it is not a natural phenomenon in vivo for S. aureus, most probably due to the inability of the enzymes to access the pentapeptide with a large pentaglycyl branch. In Weissella viridescens, the cross-bridge consists of L-Ala-L-Ser or L-Ala-L-Ser-L-Ala, and so perhaps MraY and/or MurG can still get access to its appropriate substrate (Hegde and Shrader 2001, Biarrotte-Sorin et al, 2004, Maillard et al, 2005, Vollmer et al, 2008).
Figure 4.12. Thin layer chromatography analysis of branched lipid-II (Lys) purified by DEAE sephacel. Non-amidated lipid-II (Lys) Gly\textsubscript{1} (A) and Gly\textsubscript{3} (B), and amidated lipid-II (Lys) Gly\textsubscript{1} (C) and Gly\textsubscript{3} (D), purified by anion exchange on DEAE sephacel (4 ml), eluted with 12 ml washes of increasing concentrations of ammonium bicarbonate (2 ml of 0 mM-1M) in chloroform (4 ml) and methanol (6 ml). Blue arrow – undecaprenyl phosphate. Grey arrow and underlined – Branched lipid-II (Lys).
Figure 4.13. TOF-MS analysis of amidated lipid-II (Lys) Gly₁ – Negative ion mode TOF-MS

A. Full spectra. B. MS quadrupole isolation of m/z 964.55. Amidated lipid-II (Lys) Gly₁. Predicted mass - 1931.10 Da. Predicted m/z – 1930.10 singly charged, 964.55 doubly charged and 642.7 triply charged.
Figure 4.14. TOF-MS analysis of amidated lipid-II (Lys) Gly\textsubscript{3}. Negative mode ion TOF-MS. A-B Amidated lipid-II (Lys) Gly\textsubscript{3}. A. Full spectra. Amidated lipid-II (Lys)-Gly\textsubscript{3} Predicted mass - 2045.14 Da. Predicted m/z – 2044.14 singly charged, 1021.57 double charged and 680.71 triply charged. B. m/z 948-978 zoom. * indicates contaminating amidated lipid-II (Lys) Gly\textsubscript{1} species. Amidated lipid-II (Lys)-Gly\textsubscript{1} Predicted mass - 1931.10 Da. Predicted m/z – 1930.10 singly charged, 964.55 doubly charged and 642.7 triply charged.
Figure 4.15. TOF-MS analysis of non-amidated lipid-II (Lys) Gly$_1$. Negative mode ion MS. A-B. Non-amidated lipid-II (Lys) Gly$_1$. A. Full spectra. B. m/z 958-984 zoom. Non-amidated lipid-II (Lys) Gly$_1$ predicted exact mass 1932.08 Da. Predicted m/z – 1931.08 singly charged, 965.04 doubly charged and 643.027 triply charged.
Non-amidated lipid-II (Lys) Gly$_3$ – Negative ion mode TOF-MS

**Figure 4.16.** TOF-MS analysis of branched non-amidated lipid-II (Lys) Gly$_3$. Negative mode ion MS. A. Full spectra. B. m/z 1016-1032 zoom. Non-amidated lipid-II (Lys) Gly$_3$ predicted exact mass 2046.125 Da. Predicted m/z – 2045.125 singly charged, 1022.06 doubly charged and 681.04 triply charged. Contaminating non-amidated lipid-II (Lys) Gly$_1$ denoted with * - predicted exact mass 1932.08 Da. Predicted m/z – 1931.08 singly charged, 965.04 doubly charged and 643.027 triply charged.
Figure 4.17. TOF-MS/MS analysis of amidated lipid-II (Lys) Gly\textsubscript{1} – Part 1. Positive ion mode TOF-MS/MS and TOF-MS\textsuperscript{n}. A-B. 0 collision energy. C. Quadrupole isolation of m/z 1932.1, HCD 30.
Figure 4.18. TOF-MS/MS analysis of amidated lipid-II (Lys) Gly₁ – Part 2. Positive ion mode TOF-MS/MS and TOF-MSⁿ. D. Quadrupole isolation of m/z 1932.1, HCD 30, then quadrupole isolation of m/z 617.31, HCD 30. E. Quadrupole isolation of m/z 966.55, HCD 30.
Non-amidated lipid-II (Lys) Gly₁ – Positive ion mode TOF-MS/MS

A. No collision energy. B. Quadrupole isolation of m/z 1933.08, HCD 30. C. Quadrupole isolation of m/z 1933.08, HCD 30, then quadrupole isolation of 618.31, HCD 30. D. Quadrupole isolation of m/z 1933.08, HCD 30, then quadrupole isolation of 803.37, HCD 30.

Figure 4.19. LC-MS/MS analysis of non-amidated lipid-II (Lys) Gly₁. Positive ion mode LC-MS/MS, and LC-MSⁿ. A. No collision energy. B. Quadrupole isolation of m/z 1933.08, HCD 30. C. Quadrupole isolation of m/z 1933.08, HCD 30, then quadrupole isolation of 618.31, HCD 30. D. Quadrupole isolation of m/z 1933.08, HCD 30, then quadrupole isolation of 803.37, HCD 30.
4.4.3. Enzymatic synthesis of lipid-II (Lys) mono-, tri- and penta-glycine using the Fem ligases – the ‘one-pot’ reaction

As previously discussed, the yields associated with converting branched pentapeptides into lipid-II are reasonably low when compared to non-branched lipid-II. Hence, we wanted to design a lipid-II synthesis in the presence of the Fem ligases – in essence almost replicating the natural in vivo synthesis of branched lipid-II, whereby lipid-II is synthesised from UDP-MurNac-pentapeptide and then the Fem ligases append the pentaglycyl cross-bridge.

Whilst it was shown in Section 5.3.3, that the Fem ligases could append the pentaglycyl branch to lipid-II in a small-scale reaction containing pre-synthesised and purified non-amidated lipid-II (Lys) (and in theory this reaction could be scaled up to create much larger yields of branched lipid-II that could be purified for further use), designing a synthesis reaction which was able to not only synthesise lipid-II and branch the lipid-II simultaneously, would reduce the number of purification steps and also hopefully reduce the losses of lipid-II associated with these purification steps.

This was termed the ‘one-pot’ reaction. Three reactions were set up – one containing non-amidated UDP-MurNAc-pentapeptide (Reaction 1) to yield branched non-amidated lipid-II (Lys), one containing amidated UDP-MurNAc-pentapeptide (Reaction 2) to yield branched amidated lipid-II (Lys), and one containing non-amidated UDP-MurNAc-pentapeptide plus L-glutamine (Reaction 3) to yield branched amidated lipid-II (Lys). For reaction three, it was hoped that any GatD/MurT still associated to the S. aureus ATCC 25923 membranes supplemented into the reaction may induce amidation of in-vivo non-amidated lipid-II (Lys) during the lipid-II synthesis (Münch et al, 2012). This would eliminate the need to synthesise amidated UDP-MurNAc-pentapeptide and also increase the poor yields associated with the synthesis of amidated lipid-II (Lys).

Reactions (1750 µl) contained 100 mM Tris, pH 8, 5 mM MgCl₂, 1% (v/v) Triton X-100, 2 mg 11P, 2 mM or 4 mM non-amidated or amidated UDP-MurNAc-pentapeptide...
(Lys), respectively, 6 mM UDP-GlcNAc, 100 µg MurG, 438 µg S. aureus ATCC 25923 tRNA, 175 µg GlyRS, 47 µg FemX, FemA and FemB, 2 mM ATP, 3.3 µg myokinase, 175 µg pyruvate kinase, 2 mM PEP and 50 nmol glycine. Reactions were topped up to 1750 µl with S. aureus ATCC 25923 membranes. For reaction 3, 50 mM L-glutamine was also added. Reactions were incubated for 3 hours at 37°C with frequent mixing and were then extracted, purified via DEAE sephacel and visualized via TLC. DEAE sephacel fractions were kept separate, and the 300 mM and 1M fractions were analysed by negative ion mode TOF-MS (predicted m/z summarized in Table 4.5).

Table 4.5. Predicted m/z of lipid-II species analysed by negative ion mode TOF-MS.

<table>
<thead>
<tr>
<th>Lipid-II species</th>
<th>Branch Length</th>
<th>Exact mass, Da</th>
<th>Predicted m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Singly charged</td>
</tr>
<tr>
<td>Non-amidated</td>
<td>None</td>
<td>1875.06</td>
<td>1874.06</td>
</tr>
<tr>
<td></td>
<td>Mono</td>
<td>1932.08</td>
<td>1931.08</td>
</tr>
<tr>
<td></td>
<td>Tri</td>
<td>2046.125</td>
<td>2045.125</td>
</tr>
<tr>
<td></td>
<td>Penta</td>
<td>2161.487</td>
<td>2060.487</td>
</tr>
<tr>
<td>Amidated</td>
<td>None</td>
<td>1875.06</td>
<td>1873.06</td>
</tr>
<tr>
<td></td>
<td>Mono</td>
<td>1931.10</td>
<td>1930.10</td>
</tr>
<tr>
<td></td>
<td>Tri</td>
<td>2045.14</td>
<td>2044.14</td>
</tr>
<tr>
<td></td>
<td>Penta</td>
<td>2159.18</td>
<td>2158.18</td>
</tr>
</tbody>
</table>

TOF-MS observed m/z is summarized in Table 4.5 (See Appendix Fig. 8.4-8.24 for TOF-MS spectra). TOF-MS analysis of reaction 1 (Table 4.6) and reaction 2 (Table 4.7) both detected successful synthesis of non-amidated lipid-II (Lys) and amidated lipid-II (Lys), respectively. Both also showed conversion of lipid-II to the pentaglycyl branch by the Fem ligases. In the case of non-amidated lipid-II (Lys) (Reaction 1), the presence of tri-glycyl lipid-II was also detected.

In the case of reaction 3 (Table 4.8-4.9), TOF-MS analysis detected the presence of non-amidated lipid-II (Lys), and amidated lipid-II (Lys) suggesting that the GatD/MurT enzymes were able to amidadate the non-amidated lipid-II (Lys) under these conditions.
Furthermore, mono-, tri- and penta-glycyl versions of non-amidated lipid-II (Lys) were detected. However, unfortunately no branching of amidated lipid-II (Lys) was detected in these fractions.

**Table 4.6. TOF-MS analysis of reaction 1.** Reaction contained non-amidated UDP-MurNAc-pentapeptide, FemX, FemA and FemB. TOF-MS to detected non-amidated lipid-II (Lys) branched species. – indicates m/z not detected.

<table>
<thead>
<tr>
<th>Branch length</th>
<th>300 mM fraction</th>
<th>1 M fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Observed m/z</td>
<td>Observed m/z</td>
</tr>
<tr>
<td></td>
<td>Single</td>
<td>Double</td>
</tr>
<tr>
<td>None</td>
<td>1874.06, plus sodiated</td>
<td>936.52, plus sodiated</td>
</tr>
<tr>
<td>Mono</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tri</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Penta</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

123
Table 4.7. TOF-MS analysis of reaction 2. Reactions contained amidated UDP-MurNAc-pentapeptide (Lys), FemX, FemA and FemB. TOF-MS to detected amidated lipid-II (Lys) branched species. – indicates m/z not detected.

<table>
<thead>
<tr>
<th>Branch length</th>
<th>300 mM fraction</th>
<th>1 M fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Observed m/z</td>
<td>Observed m/z</td>
</tr>
<tr>
<td></td>
<td>Single</td>
<td>Double</td>
</tr>
<tr>
<td>None</td>
<td>-</td>
<td>936.02</td>
</tr>
<tr>
<td>Mono</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tri</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Penta</td>
<td>-</td>
<td>1078.582</td>
</tr>
</tbody>
</table>

Table 4.8. TOF-MS analysis of reaction 3 – Non-amidated UDP-MurNAc-pentapeptide (Lys) plus L-glutamine, FemX, FemA and FemB. TOF-MS to detect non-amidated lipid-II (Lys) branched species. - indicates m/z not detected.

<table>
<thead>
<tr>
<th>Branch length</th>
<th>300 mM fraction</th>
<th>1 M fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Observed m/z</td>
<td>Observed m/z</td>
</tr>
<tr>
<td></td>
<td>Single</td>
<td>Double</td>
</tr>
<tr>
<td>None</td>
<td>1874.06</td>
<td>936.53</td>
</tr>
<tr>
<td>Mono</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tri</td>
<td>-</td>
<td>1022.0675</td>
</tr>
<tr>
<td>Penta</td>
<td>2159.1509</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 4.9. TOF-MS analysis of reaction 3 – Non-amidated UDP-MurNAc-pentapeptide (Lys) plus L-glutamine. TOF-MS to detect amidated lipid-II (Lys) branched species. - indicates m/z not detected.

<table>
<thead>
<tr>
<th>Branch length</th>
<th>300 mM fraction</th>
<th>1 M fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Observed m/z</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Single</td>
<td>Double</td>
</tr>
<tr>
<td>None</td>
<td>-</td>
<td>936.0192</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>Very low</td>
</tr>
<tr>
<td>Mono</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tri</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Penta</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

These results suggest that the ‘one-pot’ simultaneous synthesis of lipid-II and branched lipid-II has potential to synthesise both non-amidated and amidated lipid-II (Lys). However, in both cases there appeared to be a mixture of either non-branched lipid-II or various subspecies of branched lipid-II, in the 300 mM and 1M fractions. This suggests that the incubation time for the synthesis was not long enough (3 hr), such that the lipid-II synthesis did not go to completion. Allowing the ‘one-pot’ synthesis to be incubated overnight, would allow for the lipid-II synthesis to go to completion, and hopefully therefore mean that the Fem ligases can fully branch all nascent lipid-II. Furthermore, if the GatD/MurT enzymes are also present, this overnight incubation would allow for the conversion of all species to amidated lipid-II (Lys).

Whilst not all of the fractions obtained from the DEAE sephacel purification were analysed by TOF-MS, the analysis of the 300 mM and 1M washes provided enough evidence to suggest that the ‘one-pot’ reaction, whilst not perfect, could be a valid way of efficiently synthesizing amidated pentaglycyl lipid-II (Lys), alleviating the need for multiple reactions and purifications. Further optimization of incubation conditions is required to avoid mixed species of lipid-II.
4.4.4. Extraction of amidated lipid-II (Lys) pentaglycine from *S. aureus* culture

Previous approaches to directly obtain nascent lipid-II from bacterial cultures yielded only minute quantities of lipid-II from *E. coli*, most probably due to the very small amounts of available undecaprenyl phosphate to make lipid-II in *E. coli* (Guan *et al.*, 2005). However, a recent publication by the Walker group at Harvard University (Qiao *et al.*, 2017) has introduced a new method for the extraction of amidated lipid-II (Lys) Gly$_5$ directly from *S. aureus* culture. Once at a high cell density, *S. aureus* cultures are treated with twice the MIC of moenomycin or vancomycin. Moenomycin acts to inhibit transglycosylase activity of PBPs whilst vancomycin acts to sequester lipid-II preventing the access of PBPs to lipid-II (van Heijenoort 2001).

As such, the normally diminutive pool of amidated lipid-II (Lys) Gly$_5$ is accumulated and can be readily purified in a two-step extraction. The first extraction encompasses bacterial cell lysis and lipid extraction using chloroform and methanol, which is then separated into the aqueous and organic phases, with a thick lipid-II containing interphase. In the second extraction stage, the interphase is treated with pyridinium acetate and n-butanol to remove lipid-II from any of the water-soluble lipid-II precursors, such as the Park nucleotide. This published method yields approximately 0.5 mg of amidated lipid-II (Lys) Gly$_5$ from 1L of *S. aureus* RN4220 culture. However, following multiple trials of this published method using moenomycin to drive lipid-II accumulation, purification of amidated lipid-II (Lys) Gly$_5$ was unsuccessful. However, following personal communication with Dr Michael Welsh (Walker group, Harvard University) led to some deviations from the published protocol which led to successful purification of small amounts of amidated lipid-II (Lys) Gly$_5$. *S. aureus* RN4220 was grown to OD$_{600nm}$ 0.5 in BHI, at which point cultures were treated with 5 μg/ml vancomycin for 20 min at RT (not 37°C as per protocol as this can lead to cell lysis), before harvesting by centrifugation.

Vancomycin was used at 5 μg/ml following suggestion from Dr Michael Welsh, and after investigation of MIC and MBC of *S. aureus* RN4220 at OD$_{600nm}$ 0.5 (Appendix Fig. 8.25 Panel A and B, respectively). MIC assays performed at OD$_{600nm}$ 0.5 indicated an
MIC value of approx. 0.5 - 1 µg/ml vancomycin, giving an MBC value of 16 µg/ml vancomycin. A vancomycin concentration of 5 µg/ml was chosen as it was well below the MBC value for vancomycin, and it was the suggested optimal concentration to gain maximal yields of amidated lipid-II (Lys) Gly\textsubscript{5} from \textit{S. aureus} RN4220 culture. Using this altered method, a very small amount of amidated lipid-II (Lys) Gly\textsubscript{5} was successfully extracted from the \textit{S. aureus} cells, giving a yield of 2 µg per L of culture. This is a reduction of 250-fold from the published yields of 500 µg per L of culture (Qiao et al, 2017).

The extraction of amidated lipid-II (Lys) Gly\textsubscript{5} was confirmed by positive ion mode TOF-MS/MS. Predicted and observed m/z of fragmented products are detailed in Table 4.10 and TOF-MS/MS traces shown in Fig. 4.20-4.25.
Table 4.10. TOF-MS/MS analysis of amidated lipid-II (Lys) Gly<sub>5</sub> extracted from <i>S. aureus</i> RN4220 culture – Walker method. Positive ion mode TOF-MS/MS.

<table>
<thead>
<tr>
<th>Fragment product (Denoted fragment number)</th>
<th>Predicted exact mass</th>
<th>Charge state</th>
<th>Expected m/z</th>
<th>Observed m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gly&lt;sub&gt;5&lt;/sub&gt; (1)</td>
<td>285.11514</td>
<td>Singly</td>
<td>286.11514</td>
<td>286.117</td>
</tr>
<tr>
<td>MurNAc-L-Ala-H&lt;sub&gt;2&lt;/sub&gt;O (2)</td>
<td>328.1349</td>
<td>Singly</td>
<td>329.1349</td>
<td>329.142</td>
</tr>
<tr>
<td>L-Lys-(Gly&lt;sub&gt;5&lt;/sub&gt;) (3)</td>
<td>413.21011</td>
<td>Singly</td>
<td>414.21011</td>
<td>414.211</td>
</tr>
<tr>
<td>D-Gln-L-Lys-(Gly&lt;sub&gt;5&lt;/sub&gt;) (4)</td>
<td>541.2687</td>
<td>Singly</td>
<td>542.2687</td>
<td>542.269</td>
</tr>
<tr>
<td>L-Lys-(Gly&lt;sub&gt;5&lt;/sub&gt;)-D-Ala-D-Ala (5)</td>
<td>573.2949</td>
<td>Singly</td>
<td>574.2949</td>
<td>574.295</td>
</tr>
<tr>
<td>D-Gln-L-Lys-(Gly&lt;sub&gt;5&lt;/sub&gt;)-L-Ala (6)</td>
<td>612.3058</td>
<td>Singly</td>
<td>613.3058</td>
<td>613.309</td>
</tr>
<tr>
<td>L-Ala-D-Gln-L-Lys-(Gly&lt;sub&gt;5&lt;/sub&gt;) (7)</td>
<td>612.3058</td>
<td>Singly</td>
<td>613.3058</td>
<td>613.309</td>
</tr>
<tr>
<td>D-Gln-L-Lys-(Gly&lt;sub&gt;5&lt;/sub&gt;)-D-Ala-D-Ala (8)</td>
<td>701.3535</td>
<td>Singly</td>
<td>702.3535</td>
<td>702.354</td>
</tr>
<tr>
<td>Lactyl-L-Ala-D-Gln-L-Lys-(Gly&lt;sub&gt;5&lt;/sub&gt;)-L-Ala (9)</td>
<td>755.3641</td>
<td>Singly</td>
<td>756.3641</td>
<td>756.364</td>
</tr>
<tr>
<td>Lactyl-L-Ala-D-Gln-L-Lys-(Gly&lt;sub&gt;5&lt;/sub&gt;)-L-Ala-L-Ala (11)</td>
<td>844.4118</td>
<td>Singly</td>
<td>845.4118</td>
<td>845.411</td>
</tr>
<tr>
<td>MurNAc-L-Ala-D-Gln-L-Lys-(Gly&lt;sub&gt;5&lt;/sub&gt;)-D-Ala-D-Ala (12)</td>
<td>2058.952</td>
<td>Doubly</td>
<td>1030.476</td>
<td>1030.476</td>
</tr>
<tr>
<td>GlcNAc-MurNAc-L-Ala-D-Gln-L-Lys-(Gly&lt;sub&gt;5&lt;/sub&gt;)-D-Ala-D-Ala (13)</td>
<td>1232.5599</td>
<td>Singly</td>
<td>1233.5599</td>
<td>1233.549</td>
</tr>
<tr>
<td>Amidated lipid-II (Lys) Gly&lt;sub&gt;5&lt;/sub&gt; (14)</td>
<td>2159.1838</td>
<td>Doubly</td>
<td>1080.5919</td>
<td>1080.59</td>
</tr>
</tbody>
</table>
Figure 4.20. TOF-MS/MS analysis of amidated lipid-II (Lys) Gly$_5$ – Walker method (Trace 1). Positive ion mode TOF-MS/MS. Full spectrum at 0 CE. See Table 4.10 for predicted m/z.
Figure 4.21. TOF-MS/MS analysis of amidated lipid-II (Lys) Gly₅ (Walker) method (Trace 2). Positive ion mode TOF-MS/MS. Trace range of 200-550 m/z at 40 CE. See Table 4.10 for predicted m/z.
Figure 4.22. TOF-MS/MS analysis of amidated lipid-II (Lys) Gly₅ – Walker method (Trace 3). Positive ion mode TOF-MS/MS. Trace range of 500-750 m/z at 40 CE. See Table 4.10 for predicted m/z.
Figure 4.23. TOF-MS/MS analysis of amidated lipid-II (Lys) Gly₅ (Walker method) (Trace 4). Positive ion mode TOF-MS/MS. Trace range of 700-815 m/z at 40 CE. See Table 4.10 for predicted m/z.
Figure 4.24. TOF-MS/MS analysis of amidated lipid-II (Lys) Gly₅ – Walker method (Trace 5). Positive ion mode TOF-MS/MS. Trace range of 800-900 m/z at 40 CE. See Table 4.10 for predicted m/z.
Figure 4.25. TOF-MS/MS analysis of amidated lipid-II (Lys) Gly<sub>5</sub> – Walker method (Trace 6). Positive ion mode TOF-MS/MS. Trace range of 1000-1500 m/z at 40 CE. See Table 4.10 for predicted m/z.
4.5. Concluding remarks and further work

Within this chapter multiple variants of lipid-II substrates have been successfully synthesised, whether that be from an already existing protocol as part of the Roper group, or a new protocol that has been trialled as part of this thesis. As a result, a toolkit of lipid-II intermediates is now available, from the UDP-MurNAc-pentapeptide all the way through to lipid-II. Each of these intermediates is also available in mono-, tri- and penta-glycyl branched form, which has opened up the possibility of studying not only the activity and substrate preferences of the Fem ligases, but also the transpeptidase activity of PBP2 and PBP2a in *S. aureus*. Without the pentaglycyl branched version of lipid-II, transpeptidase activity of *S. aureus* PBPs could not be elucidated.

4.5.1. Chemical or enzymatic synthesis of branched lipid intermediates?

Whilst both the chemical synthesis of branched UDP-MurNAc-pentapeptides and the enzymatic branching of lipid-II using the Fem ligases have proven somewhat successful, they both have their pros and cons, as summarised in Table 4.11.

The chemical branching of UDP-MurNAc-pentapeptide (Lys) (Pre-lipid-II synthesis) yields mono-glycyl variants which have a high level of purity, whilst the tri-glycyl variants unfortunately contained some mono-glycyl species. This unfortunately carried over into the lipid-II synthesis leading to a mixed species of mono- and tri-glycyl lipid-II. Furthermore, the synthesis of penta-glycyl UDP-MurNAc-pentapeptide species is futile as MraY/MurG cannot utilise it as a substrate and therefore it cannot be converted to lipid-II, again adding a further constraint to the uses of this system.

Enzymatic branching of lipid-II using the Fem ligases (post-lipid-II synthesis) however does allow for the synthesis of penta-glycyl lipid-II (Lys), and assuming reaction conditions could be optimised, may also have minimal contaminating mono- or tri-glycyl subspecies. Though, this optimisation still needs to be performed.
Furthermore, the enzymatic branching can be performed simultaneously with lipid-II synthesis, and therefore reduces time and costs. However, the recombinant expression of the Fem ligases and GlyRS would also have to be factored in.

**Table 4.11. The pros and cons of chemical or enzymatic syntheses.**

<table>
<thead>
<tr>
<th>Question</th>
<th>Chemical synthesis</th>
<th>Enzymatic synthesis using Fem ligases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Can be performed on UDP-MurNAc-pentapeptides?</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Can be performed on both amidated and non-amidated lipid-II?</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Can result in mixed species of branched lipid-II using current conditions?</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Requires multiple rounds of synthesis and purification?</td>
<td>Yes</td>
<td>Yes, but less than chemical synthesis</td>
</tr>
<tr>
<td>Can produce penta-glycyl lipid-II?</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>

**4.5.2. Can the Walker method be optimised to extract larger yields of amidated lipid-II (Lys) Gly₅?**

The recent paper published by the Walker group represents a fantastic opportunity to be able to extract nascent lipid-II direct from *S. aureus* cells, and if optimised could yield large quantities of lipid-II. It also represents a chance to utilise *femA* and *femB* knockout mutants to yield mono- and tri-glycyl amidated lipid-II (Lys). However, this method does have one caveat in that it cannot solely be used to extract non-amidated lipid-II (Lys) (branched or non-branched), as *S. aureus* cannot survive in the absence of stem peptide amidation. A reduction in amidation results in *S. aureus* cells which are less viable, have a reduced growth rate, and are more susceptible to β-lactam antibiotics, and lysozyme (Figueiredo *et al*, 2014, Noldeke *et al*, 2018).
Therefore, if non-amidated branched lipid-II is needed, such as for the study of the substrate preferences of the Fem ligases, then the ‘one-pot’ enzymatic synthesis using the Fem ligases should be used.

In hindsight, the addition of vancomycin at OD$_{600nm}$ of 0.5 may well be too late. If lipid-II is almost instantly flipped to the outer membrane for transglycosylation and transpeptidation to form peptidoglycan, and if this synthesis of peptidoglycan is hugely retarded or almost halted during cells that have reached the end of their exponential phase or are at stationary phase, then perhaps inducing lipid-II accumulation at early exponential phase could be beneficial. In the Qiao et al, (2017) paper, lipid-II accumulation using vancomycin is induced anywhere between OD$_{600nm}$ 0.4-0.6, so it may be worth repeating this closer to OD$_{600nm}$ 0.4 rather than OD$_{600nm}$ 0.5-0.6.

The addition of a β-lactam as well as vancomycin or moenomycin may also be beneficial. The S. aureus mono-functional PBP4 is the first PBP shown to have the capacity to cross-link both glycan strands and none polymerised lipid-II (Qiao et al, 2014). Whilst vancomycin and moenomycin can inhibit the transglycosylase activity of other PBPs in S. aureus, they cannot inhibit the transpeptidation activity of PBP4, and hence PBP4 may indeed be catalysing lipid-II cross-linking in the absence of glycan strands. Therefore, the pool of amidated lipid-II (Lys) Gly$_5$ available for accumulation and subsequent extraction will be vastly reduced.

Furthermore, ensuring that all S. aureus cells within the culture are at the same growth phase may be crucial to achieving optimal yields of lipid-II. This could be achieved by treatment of cultures with D,L serine hydroxamate (SHX). SHX, an inhibitor of the seryl-tRNA synthetase, has previously been used to synchronise growth of E. coli culture via amino acid starvation, such that when treated with SHX, asynchronous cultures enter a stringent response phase leading to the arrest of DNA replication at the point of initiation (Ferullo et al, 2009). Cultures were grown to a mid-logarithmic growth phase (OD$_{600nm}$ 0.2-0.4) at 37°C at which point they were treated with a final concentration of 1 mg/ml SHX (Ferullo et al, 2009).
treatment, cells were grown for a further 90 minutes to allow sufficient time for cells to finish their current round of replication – at this point cells cannot initiate a new round of replication, but they can all finish their current round of replication (Ferullo et al, 2009). To release *E. coli* cells from their arrested growth phases, cells are pelleted and washed with SHX-free media to remove residual SHX - cell pellets are then resuspended in warm growth media, and growth recommenced at 37°C (Ferullo et al, 2009).

Whilst this does not achieve complete bacterial cell synchronisation, this method achieved approximately 73% synchrony in *E. coli* cells (Ferullo et al, 2009). Similar methods have also been shown to be applicable in *S. aureus* cell culture, where treatment with 1.5 mg/ml SHX lead to serine starvation and the induction of a stringent response, suggesting that this same method could be applied to achieve bacterial cell synchrony in *S. aureus* (Gieger et al, 2012).
Chapter 5 Enzymology of FemX, FemA and FemB; the characterisation of \textit{S. aureus} Fem ligase activity, the formation of the lipid-II pentaglycine bridge.

5.1. Background

In \textit{S. aureus}, three main modifications to mature peptidoglycan have been identified. Firstly, the O-acetylation of the C6 hydroxyl group of MurNAc by the \textit{oatA} gene product in pathogenic \textit{S. aureus}, a modification that sterically inhibits the action of lysozymes leading to a high lysozyme resistance phenotype (Bera et al, 2005, Bera et al, 2006, Sychantha et al, 2018). Secondly, the amidation of the second position stem peptide, D-glutamate, to D-isoglutamine by the MurT/GatD complex (Figueiredo \textit{et al}, 2012, Münch \textit{et al}, 2012), and finally, the formation of a pentaglycyl interpeptide bridge at the third position L-lysine by the FemX, FemA and FemB ligase enzymes (Rohrer \textit{et al}, 1999, Rohrer and Berger-Bäch, 2003b, Schneider \textit{et al}, 2004). A central question in the biology of this process relates to the order of amidation and glycylation with respect to lipid-II biosynthesis \textit{S. aureus}, especially as these reactions occur following the synthesis of lipid-II itself at the cytoplasmic membrane surface, and occur at adjacent amino acid positions on the peptide stem of lipid-II.

Both the amidation of lipid-II and the formation of the interpeptide pentaglycyl bridge are essential for high level \(\beta\)-lactam resistance in MRSA strains. In the case of amidation, inhibition of the MurT/GatD complex results in reduced growth rate and greatly reduced resistance to \(\beta\)-lactams, highly suggestive that this is an essential modification of the lipid-II peptidoglycan precursor (Figueiredo \textit{et al}, 2012). In addition, an increased sensitivity to lysozyme was observed, leading to inhibited growth and the degradation of peptidoglycan (Figueiredo \textit{et al}, 2012).

Inhibition of the formation of a complete pentaglycyl bridge also causes drastic consequences for MRSA strains. \(\Delta f_{\text{emAB}}\) and \(\Delta f_{\text{emA}}\), and \(\Delta f_{\text{emB}}\), null mutants
producing only mono- or tri- glycyl cross-bridges, respectively, are viable, yet have significant cellular defects including strongly reduced growth rates, methicillin hypersensitivity and a reduced rate of peptidoglycan cross-linking (Maidhof et al, 1991, Henze et al, 1993, Strandén et al, 1997). This viability in the presence of such shortened cross-bridges has been postulated by Ling and Berger-Bächi (1998) to be due to compensatory mutations in the \textit{femAB} null mutants. Without these compensatory mutations, deletion of the \textit{femAB} operon would be lethal (Ling and Berger-Bächi 1998). In addition, \textit{femX} has been shown to be essential for growth, and \textit{femX} mutants are not viable (Rohrer et al, 1999, Tschierske et al, 1999).

5.1.1. Are the Fem ligases a valid therapeutic target?

Scrutiny of current literature suggests that Cyslabdan is the only published inhibitor of FemA. Cyslabdan, a non-antibiotic small molecule, produced by \textit{Streptomyces} sp. K04-114 isolated from soil in Okinawa, Japan specifically targets FemA and has been shown to act synergistically with \textit{\beta}-lactams, increasing the susceptibility of MRSA to \textit{\beta}-lactams (Fukumoto \textit{et al}, 2008, Koyama \textit{et al}, 2012).

Unfortunately, there have been no new inhibitors of \textit{S. aureus} FemX, FemA or FemB discovered since Cyslabdan. However, the Fem ligases share sequence similarity, with FemX sharing 36\% homology and 23\% identity to FemA, and FemB sharing 52\% homology and 39\% identity to FemA (Benson \textit{et al}, 2002). It has been suggested that the Fem ligases are highly likely to therefore share the same protein folds (Benson \textit{et al}, 2002) making them ideal targets for inhibitor design, as one molecule could potentially target all three Fem ligases (Benson \textit{et al}, 2002). The Fem ligases represent a valuable target for antibiotic development and therefore understanding their enzymatic activities and structures is crucial in the targeted design of potent novel antibiotics. The specific targeting of novel antimicrobials towards the Fem ligases, specifically FemX and FemA, may potentiate the drugs already used to treat MRSA infections, or alternatively, may restore sensitivity to those drugs rendered ineffective by resistance mechanisms, when used in combination therapy. It is
therefore critical that the activities of the Fem ligases are understood – from their substrate specificities to their 3D structures. This chapter aims to contribute to this knowledge.

5.2. Chapter aims

This chapter aims to investigate the role and activity of the Fem ligases in the resistance phenotype of MRSA. The aims of this chapter are;

- To recombinantly express and purify *S. aureus* Mu50 (methicillin resistant) FemX, FemA and FemB, and the glycyl-tRNA synthetase (GlyRS)

- To extract and purify tRNA from *S. aureus* ATCC 25923

- To assess the ability of FemX, FemA and FemB to append the pentaglycyl bridge to non-amidated and amidated lipid-II (Lys) mono-, tri- and penta-glycine, respectively

- To investigate the structure of FemX, FemA and FemB
  - Do FemX, FemA and FemB form a protein complex?
  - To perform crystallisation screens for FemB
5.3. Results and discussion

5.3.1. Expression and purification of *S. aureus Mu50* FemX, FemA, FemB and GlyRS

In order to investigate the activity of the Fem peptidyl transferases, FemX, FemA, FemB and GlyRS from *S. aureus Mu50* were expressed as full-length proteins, each with a TEV protease cleavable C-terminal His\(_6\) affinity tag (IPTG induction, in LB). His\(_6\) affinity tagged proteins were purified via nickel IMAC and subsequent size exclusion chromatography where necessary, as described in Methods Section 2.5. Where His\(_6\) affinity tags were not required, the His\(_6\) affinity tag was cleaved with TEV protease (1:50 protein:TEV protease) and proteins were further purified via reverse nickel IMAC. Proteins were stored at -80°C in 30 mM HEPES, 50 mM KCl, 1 mM MgCl\(_2\), pH 7.6, 50% (v/v) glycerol, plus 1 µm leupeptin, 1 µm pepstatin, 0.2 mM PMSF and 3 mM DTT. A high level of purity was achieved for each protein, as shown in Fig. 5.1. with typical yields as follows; FemX – 6 mg per L culture, FemA – 3.5 mg per L culture, FemB – 2 mg per L culture and GlyRS – 90 mg per L culture.

5.3.2. Extraction of *S. aureus ATCC 25923* tRNA

tRNA was purified from 4 L of *S. aureus ATCC 25923* culture using a phenol:chloroform extraction method, as described in Section 2.3. tRNA purity was high, when compared to commercial *E. coli* tRNA (Sigma), as seen in Fig. 5.2. Typical yields were approximately 1 mg per L of culture.

![Figure 5.2. *S. aureus ATCC 25923* and *E. coli* crude tRNA](image)
5.3.3. Investigating the activity of the Fem peptidyl-transferases

5.3.3.1. Investigating the ability of the Fem ligases to append the glycine cross-bridge to non-amidated lipid-II (Lys) via TLC and mass spectrometry analysis

To assess the ability of the Fem ligases to append the pentaglycyl cross-bridge to lipid-II, and hence confirm their activity before further radioactive studies, small scale Fem ligase reactions were set up, as in Section 2.8.2, to contain either FemX alone (FemX), FemX and FemA (FemXA), or FemX, FemA and FemB (FemXAB). Each 100 µl reaction contained 2.7 µg of each Fem ligase (where appropriate) and 5 nmol non-amidated lipid-II (Lys). Reactions were purified by DEAE sephacel, fractions visualised by TLC and the presence of each branched lipid-II variant was confirmed by time of flight mass spectrometry (TOF-MS).

Each Fem ligase reaction product (branched lipid-II) was eluted from the DEAE sephacel column in the 500 mM – 1M ammonium bicarbonate washes. Fractions were pooled and dried under vacuum for resuspension in 100 µl 70% (v/v) MeOH:30% (v/v) 25 mM ammonium acetate for TOF-MS. As shown by TOF-MS (Fig. 5.3-5.5), non-amidated lipid-II (Lys) Gly₁, Gly₃ and Gly₅ variants were successfully synthesised in the FemX, FemXA and FemXAB reactions, respectively, indicating that all three Fem ligase enzymes are active and able to synthesise the pentaglycyl cross-bridge.

To further confirm that the Fem ligases had indeed appended the pentaglycyl cross-bridge to the third position L-Lys of lipid-II, samples were analysed by TOF-MS/MS (positive ion mode) to fragment the molecule in order to characterise its structure. Unfortunately, due to small-scale nature of the samples, there was not enough lipid-II material present in the samples to be visualised by TOF-MS/MS once fragmented.
Figure 5.3. FemX ligase activity assessed by TOF-MS – appending the glycine branch to non-amidated lipid-II (Lys). TOF-MS in negative electrospray ionization mode. Non-amidated lipid-II (Lys) Gly\textsubscript{1} predicted exact mass 1932.08 Da. Predicted m/z – 1931.08 singly charged, 965.04 doubly charged and 643.027 triply charged.
Figure 5.4. FemXA ligase activity assessed by TOF-MS – appending the glycine branch to non-amidated lipid-II (Lys). Non-amidated lipid-II (Lys) Gly₃ predicted exact mass 2046.125 Da. Predicted m/z – 2045.125 singly charged, 1022.06 doubly charged and 681.04 triply charged.
Figure 5.5. FemXAB ligase activity assessed by TOF-MS – appending the glycine branch to non-amidated lipid-II (Lys). Non-amidated lipid-II (Lys) Gly5 predicted exact mass 2161.487 Da. Predicted m/z – 2060.487 singly charged, 1079.74 doubly charged and 719.496 triply charged.
5.3.3.2. Investigating the ability of the Fem ligases to append the glycine cross-bridge to amidated lipid-II (Lys) via TLC

As in Section 5.3.3.1, small-scale Fem ligase reactions (100 µl) were performed – this time to assess the ability of the Fem ligases to append glycine to the third position L-Lys of amidated lipid-II (Lys). Reactions were performed identically to Section 5.3.3.1, where non-amidated lipid-II (Lys) was exchanged for amidated lipid-II (Lys). Again, reactions were purified by DEAE sephacel and fractions visualised by TLC. Unfortunately, there were no visible bands in the expected eluent fractions for any of the FemX, FemXA or FemXAB reactions, and therefore all the fractions which had potential to contain branched or non-branched lipid-II were pooled, dried under vacuum and resuspended in 100 µl 70% MeOH:30% 25 mM ammonium acetate for TOF-MS analysis.

None of the predicted m/z ratios for either the amidated lipid-II (Lys) Gly$_3$, Gly$_3$ or Gly$_5$ variants were detected by TOF-MS from the FemX, FemXA or FemXAB reactions, respectively. Therefore, each reaction was analysed by TOF-MS with respect to detecting the predicted m/z for non-reacted amidated lipid-II (Lys). Unfortunately, this was also not detected, suggesting that the amidated lipid-II (Lys) (branched or non-branched) may have been lost during DEAE sephacel purification methods.

This method was therefore unable to confirm whether the Fem ligases are able to append the pentaglycyl cross-bridge to amidated lipid-II (Lys), however, as in Section 5.3.3.1., this method has proven that the Fem ligases are active in the presence of non-amidated lipid-II (Lys), and therefore further study into the activity of the Fem ligase via radioactive ligation assays can be undertaken.
5.3.3.3. Investigating the ability of the Fem ligases to append the $[^3\text{H}]-\text{glycine}$ cross-bridge to lipid-II (Lys) via radioactive assay

Radiochemical assays were employed to follow the transfer of $[^3\text{H}]-\text{glycine}$ from $[^3\text{H}]-\text{glycyl-tRNA}^{\text{Gly}}$ onto the third position L-Lys of the lipid-II pentapeptide stem, as catalysed by the Fem ligases. Methods were adapted from Lloyd et al., (2008), and assays were performed for both amidated and non-amidated lipid-II. Where appropriate, previously synthesised branched lipid-II (Section 2.8) was used as the substrate for the Fem ligase, as in Table 5.6. These assays were performed to determine whether each Fem ligase has a preference of either amidated or non-amidated substrate, and hence to elucidate the point of amidation within the lipid-linked stage of peptidoglycan synthesis.

Table 5.1. Overview of Fem ligase substrate and peptidyl-transferase activity.

<table>
<thead>
<tr>
<th>Fem ligase</th>
<th>Lipid-II substrate</th>
<th>Number of glycine residues appended to lipid-II substrate by this enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>FemX</td>
<td>Lipid-II (Lys)</td>
<td>1</td>
</tr>
<tr>
<td>FemA</td>
<td>Lipid-II (Lys) Gly&lt;sub&gt;1&lt;/sub&gt;</td>
<td>2</td>
</tr>
<tr>
<td>FemB</td>
<td>Lipid-II (Lys) Gly&lt;sub&gt;3&lt;/sub&gt;</td>
<td>2</td>
</tr>
</tbody>
</table>

5.3.3.3.1. Can FemX append $[^3\text{H}]-\text{glycine}$ to both amidated and non-amidated lipid-II (Lys)?

Assays were set up to contain a final concentration of 133 nM FemX, 0.27 µM $[^3\text{H}]-\text{glycyl-tRNA}$ (at 281 cpm/pmol) and 9.9 µM non-amidated or amidated lipid-II (Lys) – see Section 2.11 for details of full reaction components. Assays were performed in parallel with controls in the presence of RNase, or the absence of FemX or lipid-II. Each reaction was pre-warmed (minus FemX and $[^3\text{H}]-\text{glycyl-tRNA}$) for 3 min at 37°C. FemX and $[^3\text{H}]-\text{glycyl-tRNA}$ were added and reactions incubated at 37°C for a further 10 min; samples were quenched at proscribed times in 6M pyridinium acetate. Lipid-
II (Lys) \[^{3}\text{H}]-\text{Gly}_1\] was extracted via n-butanol extraction, with non-reacted water soluble \[^{3}\text{H}]-\text{glycyl-tRNA}\) removed in the water phase. cpm for each sample determined.

Controls were performed in the absence of lipid-II or FemX and in the presence of RNase. In the absence of lipid-II or FemX, \[^{3}\text{H}]-\text{glycine}\) could not be appended to the stem peptide of lipid-II, and hence cpm were equivalent to that of background. RNase is an enzyme which catalyses the breakdown of RNA into oligonucleotides – consequently, in the presence of RNase, any tRNA within the reaction was degraded and therefore \[^{3}\text{H}]-\text{glycine}\) could not be presented as acyl-tRNA to FemX for ligation to lipid-II. As in Fig. 5.6 Panel B, for the +RNase reaction, the cpm detected was slightly greater than background levels, indicating that not all tRNA was degraded by the RNase. Furthermore, \[^{3}\text{H}]-\text{glycyl-tRNA}\) was not pre-incubated with RNase, therefore some \[^{3}\text{H}]-\text{glycine}\) could have been appended to lipid-II by FemX before the RNase had fully degraded any remaining \[^{3}\text{H}]-\text{glycyl-tRNA}\). These controls prove that the ligation of \[^{3}\text{H}]-\text{glycine}\) to the stem peptide of lipid-II are dependent on the presence of lipid-II, FemX and \[^{3}\text{H}]-\text{glycyl-tRNA}\).

It should be noted that time-courses were only performed over ten minutes, due to the instability of \[^{3}\text{H}]-\text{glycyl-tRNA}\) at 37°C. At this temperature, the tRNA may become deacylated and therefore \[^{3}\text{H}]-\text{glycine}\) will be unable to be appended to the stem peptide of lipid-II by FemX, therefore making cpm readings past this time point highly unreliable. Furthermore, the time zero samples take into account the finite time taken to set up the reaction and the time taken to remove and quench the time zero sample, and therefore the time course does not start at zero pmol \[^{3}\text{H}]-\text{glycine}\) per µmol of lipid-II. However, no radioactivity is detected in the control reactions indicating that any detectable radioactivity in the test samples is indeed due to FemX appending \[^{3}\text{H}]-\text{glycine}\) to lipid-II.

FemX was shown to be able to append \[^{3}\text{H}]-\text{glycine}\) to both amidated and non-amidated lipid-II (Lys) (Fig. 5.6). A sharp increase, approximately 40%, in glycine ligation to the pentapeptide stem is apparent within the first two minutes of the
assay for both lipid-II variants (Fig. 5.6 Panel A). However, this activity plateaus after the first two minutes, with the ligation of [³H]-glycine to amidated lipid-II exhibiting a small decrease over time. Moreover, there appears to be a slight preference for non-amidated lipid-II (Lys) with a greater number of pmol of [³H]-glycine per µM of lipid-II (Lys).

Although there was a small increase in substrate preference for non-amidated lipid-II (Lys) compared to amidated lipid-II (Lys) by FemX, from time 0 min to 10 min, this difference was not significant (Unpaired, Mann-Whitney).
Figure 5.6. The ability of FemX to append $[^3]$H-glycine to amidated or non-amidated lipid-II (Lys). FemX (65 nM) was incubated at 37°C with 0.27 µM $[^3]$H-glycyl-tRNA at 281 cpm/pmol for 10 min, and samples were quenched at proscribed times. n=3 for all time points, bar non-amidated time 0, where n=2. A. FemX appending $[^3]$H-glycine to the third position L-Lys of amidated (red) or non-amidated (blue) lipid-II (Lys) over time. B. Time point comparisons and controls of FemX appending $[^3]$H-glycine to the third position L-Lys of amidated (warm tone reds) or non-amidated (cool tone blues) lipid-II (Lys). Controls, in the presence of 0.25 mg/ml RNase (Sigma), or absence of lipid-II or FemX, were performed for 10 min before quenching. Non-paired, Mann-Whitney (Two-tailed) test of amidated vs non-amidated 0 and 10 min, not significant (n.s). Time points 2-8 min n.s (data not shown).
5.3.3.3.2. Can FemA append \([^3H]\)-glycine to both amidated and non-amidated lipid-II (Lys) Gly₁?

Assays were set up to contain a final concentration of 130 nM FemA, 0.18 µM \([^3H]\)-glycyl-tRNA (at 648 cpm/pmol) and 9.9 µM non-amidated or amidated lipid-II (Lys) Gly₁ – see Section 2.11 for details of full reaction components. Assays were performed in parallel with controls in the presence of RNase, or the absence of FemA or lipid-II. Each reaction was pre-warmed (minus FemA and \([^3H]\)-glycyl-tRNA) for 3 min at 37°C. FemA and \([^3H]\)-glycyl-tRNA were added and reactions incubated at 37°C for a further 10 min; samples were quenched at proscribed times in 6M pyridinium acetate. Lipid-II (Lys) \([^3H]\)-Gly₁ was extracted via n-butanol extraction, with non-reacted water soluble \([^3H]\)-glycyl-tRNA removed in the water phase. cpm for each sample determined.

FemA was shown to be able to append \([^3H]\)-glycine to both amidated and non-amidated lipid-II (Lys) Gly₁, at the first glycine branch of the third position L-Lys of the pentapeptide stem, leading to the formation of lipid-II (Lys) Gly₂ and Gly₃ (Fig. 5.7 Panel A). Although there was a small increase in substrate preference for non-amidated lipid-II (Lys) Gly₁ compared to amidated lipid-II (Lys) Gly₁ by FemA from time 0 min to 10 min, this difference was not significant (Unpaired, Mann-Whitney). Again, controls were performed in the absence of lipid-II or FemA and in the presence of RNase (Fig 5.7, Panel B). In the absence of lipid-II or FemA, \([^3H]\)-glycine could not be appended to the first position glycine branch of the stem peptide of lipid-II (Lys) Gly₁, and hence cpm was equivalent to that of background. In the presence of RNase, tRNA within the reaction was degraded and therefore \([^3H]\)-glycine could not be presented to FemA for ligation to lipid-II (Lys) Gly₁. These controls prove that the ligation of \([^3H]\)-glycine to the stem peptide of lipid-II by FemA are dependent on the presence of lipid-II, FemA and \([^3H]\)-glycyl-tRNA.

The calculation of pmol \([^3H]\)-glycine per µmol lipid-II at each time point is based on the assumption that FemA is appending two \([^3H]\)-glycine residues to the lipid-II (Lys)
Gly1, however it is unknown whether full conversion to lipid-II (Lys) Gly3 has been completed. Hence there may be some species of lipid-II that have only had the second position glycine branch added by FemA.

Figure 5.7. The ability of FemA to append [3H]-glycine to amidated or non-amidated lipid-II (Lys) Gly1. FemA (65 nM) was incubated at 37°C with 0.18 µM [3H]-glycyl-tRNA at 348 cpm/pmol for 10 min, and samples were quenched at proscribed times. A. FemA appending [3H]-glycine to the third position L-Lys of amidated (red) or non-amidated (blue) lipid-II (Lys) Gly1 over time. n = 3 for time points 0-8 min (amidated), 0-2 min and 10 min (non-amidated). n = 2 for time point 10 min (amidated). N = 1 for time point 4-8 min (non-amidated). B. Time point comparisons and controls of FemA appending [3H]-glycine to the third position L-Lys of amidated (warm tone reds) or non-amidated (cool tone blues) lipid-II (Lys) Gly1. Controls, in the presence of 0.25 mg/ml RNase (Sigma), or absence of lipid-II or FemA, were performed for 10 min before quenching. Non-paired, Mann-Whitney (Two-tailed) test of amidated vs non-amidated 0 and 10 min, not significant (n.s).
5.3.3.3. Can FemB append $[^3]H$-glycine to both amidated and non-amidated lipid-II (Lys) Gly$_3$?

Both non-amidated and amidated lipid-II (Lys) Gly$_3$ variants were synthesised as discussed in Chapter 4. However, as a result of the synthesis process, there was some contamination of tri-glycyl lipid-II with the mono-glycyl lipid-II variant. For this reason, FemB ligation assays of $[^3]H$-glycine to lipid-II were not performed as the substrate was not pure and this may have complicated the datasets obtained. The mixed species lipid-II Gly$_{1/3}$ could however be converted to complete tri-glycyl species by incubating the lipid with FemA – this does come with some caveats however, including the potential loss of lipid-II material and the potential of mono-glycyl lipid-II to not fully convert to tri-glycyl lipid-II.

5.3.4. Investigating the ability of FemX, FemA and FemB to form a protein complex

In order for the Fem ligases to efficiently catalyse the synthesis of the pentaglycyl branch of lipid-II, it would be catalytically beneficial for the three enzymes to form a complex, such that the lipid-II substrate could be channelled along the complex once each Fem ligase has appended the appropriate number of glycine residues, as illustrated in Fig. 5.8. For example, FemX appends the first glycine to lipid-II, FemA then accepts lipid-II mono-glycine and appends the subsequent two glycines to form lipid-II tri-glycine. Finally, lipid-II tri-glycine is channelled to FemB where the final two glycines are appended to yield lipid-II penta-glycine, which is subsequently released from the Fem ligase complex. Without this formation of a complex, the probability that each Fem ligase would couple with its cognate substrate is low and the efficiency of pentaglycyl branch formation would surely be greatly reduced.

Furthermore, given that there is a conserved pool of glycyl-tRNA$^{Gly}$ reserved for peptidoglycan synthesis (Lloyd et al, 2008), it makes sense for there to not only be a Fem ligase complex, but also a Fem ligase:GlyRS:tRNA$^{Gly}$ complex in existence with a proportion of GlyRS reserved for peptidoglycan synthesis.
Figure 5.8. Proposed mechanism of lipid-II pentaglycyl branching by the fem ligase complex in *S. aureus*. The FemX:A:B complex associates to lipid-II allowing FemX to attach the first glycine to L-Lys. Lipid-II-Gly₁ is then funneled along the complex to FemA, where the second and third glycine are attached to form lipid-II-Gly₃, and again to FemX where the fourth and fifth glycine are attached to form lipid-II-Gly₅. Branched lipid-II is now ready for translocation across the membrane by FtsW/MurJ.
Previously, evidence for the formation of a Fem ligase protein complex was shown using the \textit{in vivo} bacterial two-hybrid (BTH) system by Rohrer and Berger-Bächi (2003a). Each Fem ligase was labelled as both ‘bait’ and ‘prey’ and all nine combinations of Fem ligase pair was assessed by BTH - homodimerization of FemA-FemA, FemA-FemB, FemB-FemA and FemB-FemB were seen (Prey-Bait) (Rohrer and Berger-Bächi 2003a). No evidence of homodimerization of any combinations containing FemX was seen. The combinations of Fem ligase that appeared positive for protein-protein interaction shown by BTH were further confirmed by glutathione S-transferase (GST)-pulldown assay. FemA and FemB were labelled with either a GST- or His\textsubscript{6} - affinity tag, such that when a GST-tagged protein is tethered to an immobilised affinity ligand specific for the GST-tag, any interacting His\textsubscript{6}-tagged proteins can be identified via Western blot using antibodies specific for the His\textsubscript{6}-tag.

Whilst these techniques are useful for investigating strongly interacting proteins, weak interactions may be more difficult to determine, prompting us to investigate this possibility further using the purified proteins made in this study.

\textbf{5.3.4.1. Microscale thermophoresis of the Fem ligases}

The potential binding interactions of each Fem ligase to another Fem ligase were assessed by microscale thermophoresis using the NanoTemper Monolith NT.115. The target Fem ligase with a His\textsubscript{6} affinity tag was labelled with the fluorescent RED-tris-NTA dye whilst the His\textsubscript{6} affinity tag of the ligand Fem ligase was cleaved with TEV protease.

Both target and ligand were incubated at room temperature, for 15 min before MST analysis. The ligand Fem ligase was titrated in storage buffer across a broad range of concentrations against 50 nM target Fem ligase (diluted and labelled in PBS) to ascertain a binding affinity. The two manufacturers recommended dilution buffers (PBS-Tween, or MST optimised buffer) used for protein labelling were not used for the Fem ligases as it caused protein precipitation. Instead the Fem ligases were diluted in PBS only for protein labelling; this prevented precipitation allowing MST to be performed without aggregation in the capillaries. Each Fem ligase was evaluated
as a target and ligand, with and without the His$_6$ affinity tag, respectively. MST analysis was performed at RT, 40% LED power, medium MST power with a 20s on time.

Each labelled-protein and non-labelled protein pair were subjected to a ‘binding check’ before protein-protein affinity analysis and appropriate controls were performed to ensure there was no ligand or buffer fluorescence, or ligand-induced fluorescence. The latter can occur when, in the absence of the MST laser being turned on, the fluorescence of the target and ligand is greater than the target alone. This can be due to interactions of the ligand with the fluorescent dye itself, or due to site of protein-protein interaction being in close proximity to the dye, causing it to fluoresce. Affinity analysis can determine a dissociation constant (Kd) for each protein-protein interaction and is a measure of the strength of an interaction between two molecules, where the smaller the Kd value, the stronger the interaction between the two molecules, and vice versa.

Kd affinity analysis was performed for FemX (target) vs FemA (ligand), FemA (target) vs FemX (ligand) and FemB (target) vs FemA (ligand) – Kd values are summarised in Table 5.2 and affinity analysis curves shown in Fig. 5.9. Each Kd value was determined to be within the low µM range, indicating that each protein-protein interaction has a weak interaction. This is consistent with the affinity analysis curves shown in Fig. 5.9, which indicate a slow on/off rate. The Kd values determined for FemX (target) vs FemA (ligand) (7.481 ± 5.219 µM) and FemA (target) vs FemX (4.62 ± 1.107 µM) are both very similar within the low µM range. These similar Kd values enhances the reliability of this dataset as these two analyses are essentially measuring the same protein-protein interaction (FemX and FemA), but alternating which protein is labelled each time. Hence, the Kd values obtained should in theory be extremely similar, assuming the fluorescent dye does not interfere with protein-protein interaction.
It should be noted that the affinity analysis of FemB (target) vs FemA (ligand) exhibits a large error associated with the Kd (21.79 ± 21.27 µM) - this again could be attributed to the extremely slow on/off rate. However, regardless of this large error, this data does indicate that FemB and FemA have the ability to form a protein-protein complex. Interestingly, there was no protein interaction detected for FemA (target) vs FemB (ligand). However, this could be due to the positioning of the fluorescent dye bound to FemA, and could indicate that the site of interaction of FemA to FemB occurs around the site of the FemA His6 affinity tag, such that when labelled with the fluorescent dye, the label prevents FemA (target) and FemB (ligand) interaction.

Whilst initial binding checks for FemB (target) vs FemX (ligand) detected a protein-protein interaction, a Kd value could not be determined, suggestive that this interaction is extremely weak or transient (See Appendix Fig. 8.26). It is worth noting that Fnorm does not pass through zero as Fnorm is a ratio of the movement of a protein in a ligand bound or unbound form before and after laser treatment. A plot of the ΔFnorm would however pass through zero when normalized against the no ligand control.

### Table 5.2. Binding affinities of the Fem ligases, as measured by Microscale thermophoresis. NanoTemper Monolith NT.115, 40% LED power, medium MST power with a 20s on time.

<table>
<thead>
<tr>
<th>Target (His-tag labelled)</th>
<th>Ligand (His-tag cleaved)</th>
<th>Evidence of protein:protein interaction?</th>
<th>Kd value, µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>FemX</td>
<td>FemA</td>
<td>✓</td>
<td>7.481 ± 5.219</td>
</tr>
<tr>
<td>FemX</td>
<td>FemB</td>
<td>X</td>
<td>-</td>
</tr>
<tr>
<td>FemA</td>
<td>FemB</td>
<td>X</td>
<td>-</td>
</tr>
<tr>
<td>FemA</td>
<td>FemX</td>
<td>✓</td>
<td>4.62 ± 1.107</td>
</tr>
<tr>
<td>FemB</td>
<td>FemA</td>
<td>✓</td>
<td>21.79 ± 21.27</td>
</tr>
<tr>
<td>FemB</td>
<td>FemX</td>
<td>✓</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 5.9. Microscale thermophoresis binding analysis of the Fem ligases. The binding affinity of FemA, FemX or FemB with a His$_6$ affinity tag labelled with RED-tris-NTA fluorescent dye (50 nM) was assessed against FemX, FemA or FemB minus a His$_6$ affinity tag. MST was performed at 40% LED power, medium MST power and an on time of 19 sec. n=3. A. FemX (target) vs FemA. Kd value = 7.48 µM +/- 5.2 µM. B. FemA (target) vs FemX. Kd value = 4.62 µM +/- 1.107 µM. C. FemB (target) vs FemA. Kd value = 21.79 µM +/- 21.27 µM. Data plotted as normalized fluorescence (Fnorm), where Fnorm = F(hot)/F(cold).
5.3.5. Crystallography trials for FemB

Crystal structures exist for both FemX (PDB 6SNR) and FemA (PDB 1LRZ, 2.1 Å), however there is currently no available, complete crystal structure for FemB. Previous 96-well crystallisation screens for *S. aureus* FemB were trialled by Jennifer Shephard (Roper group, University of Warwick) where protein was dialysed into 50 mM ethanolamine, 100 mM NaCl, 20% (v/v) glycerol, pH 10 and screens set up at a final concentration of 6.5 mg/ml FemB. Crystals were successfully grown in conditions described in Table 5.3, at 18°C after two weeks. Crystals were soaked in mother liquor supplemented with 30% (v/v) glycerol prior to flash freezing in liquid nitrogen, and were diffracted at Diamond Light Source (Oxfordshire, UK). Unfortunately, crystals obtained in the Clear Strategy screen were likely to be salt. Crystals obtained from the JCSG+ and PACT screen both diffracted well (3.5 Å and 3 Å, respectively), however due to weakening as a result of X-ray exposure, only a 60% complete dataset could be obtained.

Whilst the crystals obtained by Jennifer Shephard were large and diffracted well, they were not stable enough to withstand X-ray exposure long enough to retrieve a complete dataset. Therefore, crystallization trials were set up under different storage buffer conditions (30 mM HEPES, 50 mM KCl, 1 mM MgCl₂, pH 7.6, 50% (v/v) glycerol, plus 1 µm leupeptin, 1 µm pepstatin, 0.2 mM PMSF and 3 mM DTT) and also different crystallization screen conditions. Crystallisation screens were set up for Wizard 1-2 and Morpheus, at RT using the Mosquito LCP crystallisation Robot (200 nL buffer, 200 nL protein). Morpheus was chosen as each condition contains cryo-protectant which reduces stress to any crystals grown, reduces the need to soaking in mother liquor, and hopefully would result in more stable crystals that could withstand X-ray diffraction.
Table 5.3. Previous successful crystal screens for *S. aureus* FemB. Crystals obtained in the described conditions by Jennifer Shephard (Roper group, University of Warwick) using 6.5 mg/ml FemB.

<table>
<thead>
<tr>
<th>Crystal Screen</th>
<th>Well descriptor</th>
<th>Buffer condition</th>
<th>Maximum crystal length obtained</th>
</tr>
</thead>
<tbody>
<tr>
<td>JCSG+ A5</td>
<td>2M magnesium formate, 20% (w/v) PEG 3350</td>
<td>50 µm</td>
<td></td>
</tr>
<tr>
<td>Clear Strategy, G6</td>
<td>0.1M Tris, 0.2 M calcium acetate, 15% (w/v) PEG 4K, pH 8.5</td>
<td>5 µm</td>
<td></td>
</tr>
<tr>
<td>PACT D10</td>
<td>0.1M Tris, 0.2M magnesium chloride, 20% (w/v) PEG 6K, pH 8</td>
<td>50 µm</td>
<td></td>
</tr>
</tbody>
</table>

Crystals were seen in the conditions summarised in Table 5.4 after one day of growth at room temperature, as are shown in Fig. 5.10 Panel A-C. Unfortunately, it was decided that crystals were too small to pick for X-ray diffraction and crystallisation screens were repeated at higher FemB concentrations (8.5 mg/ml and 17 mg/ml) with the hope that this would increase crystal size and allow crystals for be picked for X-ray diffraction. As seen in Fig. 5.10 Panel D-I, potential FemB crystals appeared much larger when this higher concentration of FemB was used. Furthermore, there was also a greater number of crystallisation conditions which gave rise to crystal growth at these concentrations (Summarised in Table 5.5).

The large crystal grown in Morpheus condition B11, was picked, flash frozen in liquid nitrogen and was diffracted at Diamond Light Source (Oxfordshire, UK) by Dr Allister Crow (University of Warwick). Unfortunately, following X-ray diffraction this crystal was shown to more than likely be a salt crystal.
Table 5.4. Successful crystallisation screen conditions for *S. aureus* FemB. Crystals grown at RT, at 0.4-0.8 mg/ml FemB.

<table>
<thead>
<tr>
<th>Crystal Screen</th>
<th>FemB concentration</th>
<th>Well</th>
<th>Buffer condition</th>
<th>Crystals shown in Figure 5.10.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wizard 1-2</td>
<td>0.4 mg/ml</td>
<td>H10</td>
<td>1000 mM Ammonium phosphate dibasic, 100 mM Imidazole/Hydrochloric acid pH 8.0, 200 mM Sodium chloride</td>
<td>Panel A</td>
</tr>
<tr>
<td>Morpheus</td>
<td>0.8 mg/ml</td>
<td>C9</td>
<td>0.09 M NPS (0.3M sodium nitrate, 0.3 sodium phosphate dibasic, 0.3M ammonium sulphate), 0.1 M Buffer system 3 (Tris/Bicine, pH 8.5), 30% v/v precipitant mix 1 (40% v/v PEG 500 MME, 20% w/v PEG 20000)</td>
<td>Panel B</td>
</tr>
<tr>
<td>Morpheus</td>
<td>0.8 mg/ml</td>
<td>C12</td>
<td>0.09 M NPS (0.3M sodium nitrate, 0.3 sodium phosphate dibasic, 0.3M ammonium sulphate), 0.1 M Buffer system 3 (Tris/Bicine, pH 8.5), 37.5% precipitant mix 4 (25% v/v MPD, 25% PEG 1000, 25% w/v PEG 3350)</td>
<td>Panel C</td>
</tr>
</tbody>
</table>
Figure 5.10. *S. aureus* FemB crystallisation trials. Wizard ½ screen – (A) H10 - 0.4 mg/ml. Growth at day 2. Morpheus screen - (B) C9 - 0.8 mg/ml. Growth at day 1. (C) C12 – 0.8 mg/ml. Growth at day 1. (D) A5 - 17 mg/ml. (E) A9 - 17 mg/ml. (F) A10 - 8.5 mg/ml. (G) D12 - 8.5 mg/ml. (H-I) B11 - 8.5 mg/ml. Growth at day 4. All crystals grown at RT.
Table 5.5. Successful crystallisation screen conditions for *S. aureus* FemB in Morpheus screen. Crystals grown at RT, at 8.5 or 17 mg/ml FemB.

<table>
<thead>
<tr>
<th>FemB concentration</th>
<th>Well</th>
<th>Buffer condition</th>
<th>Crystals shown in Figure 5.10.</th>
</tr>
</thead>
<tbody>
<tr>
<td>17 mg/ml</td>
<td>A5</td>
<td>0.06 M divalents (0.3M Magnesium chloride hexahydrate; 0.3M Calcium chloride dihydrate), 0.1M buffer system 2 (1M HEPES/MOPS, pH 7.5), 30% v/v precipitant mix 1 (40% v/v PEG 500* MME; 20 % w/v PEG 20000)</td>
<td>Panel D</td>
</tr>
<tr>
<td>8.5 mg/ml</td>
<td>A9</td>
<td>0.06 M divalents (0.3M Magnesium chloride hexahydrate; 0.3M Calcium chloride dihydrate), 0.1 M buffer system 3 (1M Tris/Bicine, pH 8.5), 30% v/v precipitant mix 1 (40% v/v PEG 500* MME; 20 % w/v PEG 20000)</td>
<td>Panel E</td>
</tr>
<tr>
<td>17 mg/ml</td>
<td>A9</td>
<td>Not shown</td>
<td>Not shown</td>
</tr>
<tr>
<td>8.5 mg/ml</td>
<td>A10</td>
<td>0.06 M divalents (0.3M Magnesium chloride hexahydrate; 0.3M Calcium chloride dihydrate), 0.1 M buffer system 3 (1M Tris/Bicine, pH 8.5), precipitant mix 2 (40% v/v Ethylene glycol; 20 % w/v PEG 8000)</td>
<td>Panel F</td>
</tr>
<tr>
<td>8.5 mg/ml</td>
<td>B11</td>
<td>0.09 M Halogens (0.3M Sodium fluoride; 0.3M Sodium bromide; 0.3M Sodium iodide), 0.1M buffer system 3 (1M Tris/Bicine, pH 8.5), 30% precipitant mix 3 (40% v/v Glycerol; 20% w/v PEG 4000)</td>
<td>Panel H-I</td>
</tr>
<tr>
<td>17 mg/ml</td>
<td>B11</td>
<td>Not shown</td>
<td>Not shown</td>
</tr>
<tr>
<td>8.5 mg/ml</td>
<td>D12</td>
<td>0.12 M alcohols (0.2M 1,6-Hexanediol; 0.2M 1-Butanol 0.2M 1,2-Propanediol; 0.2M 2-Propanol; 0.2M 1,4-Butanediol; 0.2M 1,3-Propanediol), 0.1M buffer system 3 (1M Tris/Bicine, pH 8.5), 37.5% v/v precipitant mix 4 (25% v/v MPD; 25% PEG 1000; 25% w/v PEG 3350)</td>
<td>Panel G</td>
</tr>
<tr>
<td>17 mg/ml</td>
<td>E10</td>
<td>0.12 M ethylene glycols (0.3M Diethylene glycol; 0.3M Triethylene glycol; 0.3M Tetraethylene glycol; 0.3M Pentaethylene glycol), 0.1M buffer system 3 (1M Tris/Bicine, pH 8.5), 30% v/v precipitant mix 2 (40% v/v Ethylene glycol; 20 % w/v PEG 8000)</td>
<td>Not shown</td>
</tr>
</tbody>
</table>
5.4 Discussion and future work

The Fem ligases and GlyRS are almost an ideal set of proteins to study as they are proteins which are soluble, recombinantly co-express in large amounts and purify to a high level of purity. They are also reasonably stable and can tolerate freeze-thaw well. This has yielded large amounts of protein, with which I have been able to employ various different biochemical and biophysical techniques to study them with. However, the hardest part of studying the Fem ligases has been synthesising their substrates – branched lipid-II. This is discussed further in Chapter 4.

5.4.1. Do the Fem ligases form a protein complex?

One of the main aims of this chapter was to elucidate whether the Fem ligases can truly form a protein complex (trimer). NanoTemper MST has provided further evidence that FemX-FemA and FemA-FemB have the ability to form heterodimers together, which therefore provides in vitro evidence that these three proteins could potentially form a trimer of proteins.

However, there is a potential that the presence of lipid-II, glycyl-tRNA Gly or GlyRS may stabilise the Fem ligase trimer, hence causing tighter binding and acting to reduce their respective Kd values. Therefore, future work should include the repetition of MST analysis of these protein-protein interactions, with the addition of lipid-II, glycyl-tRNA Gly or GlyRS within the buffer system. Binding of lipid-II, glycyl-tRNA Gly or GlyRS to each of the Fem ligases independently would also need to be investigated.

Whilst the notion of the Fem ligase complex is supported by previously published work (Rohrer and Berger-Bächi 2003a), other similar techniques such as Isothermal calorimetry (ITC) or Surface plasmon resonance (SPR) could also be performed. ITC involves the measurement of the binding equilibrium of a target and its ligand, by measuring the heat produced or absorbed during a target-ligand association event (Pierce et al, 1999). The heat produced or absorbed by this event is a direct measurement of the fraction of ligand bound to the target, and therefore ITC allows
measurement of target-ligand affinity, stoichiometry and enthalpy (Pierce et al, 1999). In SPR on the other hand, the target protein is immobilised to chip covered in a thin-film of gold whilst the ligand is washed over the tether target in increasing concentrations; if the target and ligand interact, there is a change in the refractive index of the gold chip which can be assessed to calculate association, dissociation and affinity constants (Patching 2014). These techniques could therefore provide further information on the interaction of the Fem ligases as a trimer, and could support the affinity constants elucidated in this chapter. One caveat to this study is that the affinities were measured in the absence of substrate, which may be required for strong protein-protein interaction. Furthermore, since lipid-II will be available only at a membrane surface, this is another variable that has not been explored in this study.

Additional techniques that may be pertinent in this study include the investigation of the surface interface of these interactions which could be mapped using methods such as carbene labelling/footprinting where a diazirine molecule is irradiated to produce a highly reactive carbene, which labels the surface of the target proteins (Manzi et al, 2016). The labelling of this target is compared by tryptic digest and electrospray ionisation mass spectrometry, in the presence and absence of the ligand, such that any areas of carbene masking (carbene can no longer bind) indicates protein-ligand interaction, and any areas of carbene unmasking (carbene can now bind) indicates areas of target conformational change. Other methods of protein-protein surface interface mapping include nuclear magnetic resonance (NMR) which, as well as carbene footprinting, is a highly sensitive technique and allows mapping of weak protein-protein interactions (Zuiderweg 2001).

Crystallisation of the protein complex would also allow mapping of the protein interfaces – however this would be a huge undertaking and even if crystallisation conditions produce crystals, it may not be a complete crystal containing all three Fem ligases. Furthermore, as the crystal structures for FemX (PDB 6SNR) and FemA (PDB 1LRZ, 2.1 Å) are available, but FemB is not, this could indicate that crystallising FemB may be difficult.
If the hypothesis of a Fem ligase protein complex were true, this would aid in enhancing the efficiency of lipid-II branching, therefore ultimately aiding in enhancing the efficiency of peptidoglycan synthesis. A mechanism where a substrate is funneled down a protein complex would be the simplest system for lipid-II branching, especially in the case of *S. aureus* lipid-II branching where there are multiple proteins and substrates.

5.4.2. Can FemB be crystallised to elucidate its 3D structure by X-ray crystallography?

Crystallisation trials of FemB yielded many crystals, however when one of these was sent for X-ray diffraction it was unfortunately found to be a salt crystal. Previous attempts at crystallising FemB were successful, however crystals appeared unstable and degraded during X-ray diffraction. The crystals grown in Section 5.3.5 should be regrown and a larger number of crystals sent for X-ray diffraction. Additionally, it may be worthwhile screening for crystallisation conditions for a FemA-FemB homodimer or FemB-tRNA homodimer, as this may act to stabilise the FemB crystal such that it may be able to withstand X-ray diffraction, and would also be a step forward in mapping the interaction of the Fem ligase complex.

5.4.3. What is the preferred substrate for the Fem ligases – amidated or non-amidated lipid-II (Lys)?

As in Section 5.3.3, whilst there is a small difference in FemX preference for non-amidated lipid-II (Lys) across the ten minute time course, there appears to be no significant difference in catalysis of FemX incorporating [*3H*]-glycine onto the third position stem peptide (L-Lys) of either amidated or non-amidated lipid-II (Lys).

Given that there is no distinct significant difference in the initial rates, perhaps the Fem ligase complex can bind to both amidated or non-amidated lipid-II allowing
branching to occur whether the GatD/MurT complex has amidated the second position D-Glu or not. If non-amidated lipid-II (Lys) encounters the Fem ligases before the GatD/MurT complex, then it is branched and then amidated, whilst if non-amidated lipid-II (Lys) encounters the GatD-MurT complex first it is amidated and then branched by the Fem ligases. As published by Münch et al, (2012), the GatD/MurT complex does not have a substrate preference between lipid-II (Lys) and lipid-II (Lys) Gly5. Therefore, the hypothesis that the Fem ligases can branch both amidated and non-amidated lipid-II is in keeping with this notion.

However, the question that then arises is does only amidated lipid-II (Lys) Gly5 get flipped to the outer membrane? Does FtsW/MurJ have a substrate preference for only amidated versions of lipid-II, or can S. aureus survive with a certain amount of non-amidated lipid-II (Lys) Gly5? We know that non-amidated lipid-II is a poor substrate for PBP2 transglycosylation and transpeptidation activity (unpublished), however could still be polymerised and cross-linked in theory. However, it is known that the amidase complex is considered to be essential in S. pneumoniae and S. aureus, and depletion in the latter leads to increased susceptibility of β-lactams and lysozyme, including in MRSA strains (Figueiredo et al, 2012, Figueiredo et al, 2014). Thus, it would appear likely given the evidence presented here that there are cellular mechanisms to ensure both the addition of the pentaglycyl side chain and amidation at position 2 of the lipid-II pentapeptide stem to ensure the fidelity of peptidoglycan biosynthesis in S. aureus.

It has been reported that certain strains of vancomycin-resistant S. aureus such as Mu50, have been shown to be able to survive with increased incorporation of non-amidated muropeptides into the peptidoglycan, although exact amounts are not stated, and therefore the incorporation of some non-amidated lipid-II (Lys) Gly5 into the peptidoglycan is apparently tolerated (Hanaki et al, 1998). In Streptococcus pneumoniae, a MurT/GatD depletion strain showed an increase in non-amidated muropeptides from 18% to 42%, indicating that a minimum of 58% amidated lipid-II is essential for growth (Morlot et al, 2018). The morphology of these strains is highly deformed however suggesting that depletion of the MurT/GatD amidase has severe
effects on overall peptidoglycan synthesis, and that the amidase may be a suitable target for antibiotics. A mechanism that maintains the incorporation of a majority of amidated lipid-II (Lys) Gly5 into *S. aureus* peptidoglycan is highly likely to be present.
Chapter 6 The ability of \textit{S. aureus} PBP2 and PBP2a to form a protein complex

6.1. Background

6.1.1. Localisation of PBPs to the cellular membrane

The \textit{S. aureus} membrane acts as a platform for the localisation of specific sets of membrane or membrane-associated proteins which are involved in cellular processes, such as cell signalling cascades and specific multi-protein reactions (Lopez and Koch 2017). Within the bacterial membrane, nanoscale membrane segments called functional membrane microdomains (FMMs) exist, supported by specific proteins (García-Fernández et al, 2017, Lopez and Koch 2017). In MRSA, these FMMs are formed from aggregated isoprenoid membrane lipids which co-localise with the membrane bound chaperone flotillin commonly referred to as a scaffold protein (Bramkamp and Lopez 2015, García-Fernández et al, 2017). In \textit{S. aureus}, FloA is the only flotillin protein and acts to recruit proteins to the lipid raft; in certain membrane environments, these FMMs are said to resemble eukaryotic lipid rafts - however, the full organisation of these bacterial FMMs is not yet fully understood (Bramkamp and Lopez 2015, García-Fernández et al, 2017, Koch et al, 2017).

Recent studies by García-Fernández et al, (2017) have highlighted one role of FMMs in determining β-lactam resistance in MRSA. In MRSA, the FMMs were shown to serve as a platform for the oligomerisation of various protein complexes, including those involved in membrane lipid metabolism, membrane transport, protein quality control, virulence and cell wall synthesis - notably, PBP2a and PBP2 in the latter category (García-Fernández et al, 2017). In both \textit{ΔfloA} mutants and MRSA cultures treated with statins, PBP2a oligomerisation was reduced – highlighting that FMM disruption inhibits PBP2a oligomerisation (García-Fernández et al, 2017). Furthermore, the antibiotic resistance profile of statin-treated MRSA and \textit{ΔfloA}
mutants appeared comparable, indicating that the disruption of FMM organisation, either from flotillin knockout, or statin treatment, resensitises MRSA to penicillin treatment (García-Fernández et al, 2017). The nature of the described PBP2a oligomerisation has not yet been elaborated however.

Many studies have also provided further evidence to suggest that membrane lipid perturbation by epicatechin gallate (ECg), an abundant polyphenol extract found in green tea, sensitises MRSA to many β-lactams (Stapleton et al, 2004, Taylor et al, 2005, Bernal et al, 2010). ECg has been shown to alter MRSA cell membrane fluidity – initially causing a decrease in membrane fluidity followed by an attempt to repair the cell membrane resulting in an increase in lysyl-phosphatidylglycerol, a decrease in phosphatidylglycerol and an increase in the overall proportion of fatty acids (Bernal et al, 2010). This vast membrane reorganisation leads to an overall increase in membrane fluidity (Bernal et al, 2010). As a result of this membrane reorganisation, PBP2 is delocalised from the septum as shown by GFP-PBP2 fusion studies; this contrasts with the FtsZ cell wall machinery which remained localised to the cell membrane in the presence of ECg (Bernal et al, 2010). In addition, studies have shown that even in strains which constitutively express PBP2a, MRSA is rendered sensitive to the β-lactam oxacillin by ECg, suggesting that PBP2a is also delocalised from the cell membrane in the presence of ECg, or that any potential protein-protein complexes formed at the septum are disrupted such that efficient PBP2a transpeptidation activity is disturbed (Stapleton et al, 2004, Bernal et al, 2010).

6.1.2. Are PBPs in close enough physical proximity to form protein-protein complexes?

Whilst there has been in-depth work to suggest that both PBP2 and PBP2a localise to the cellular membrane during peptidoglycan synthesis, this does not provide evidence to prove that these two PBPs are able to form an active protein-protein complex. Due to their complimentary enzymatic activities, it would be reasonable to
propose that these proteins form a protein-protein complex during MRSA treatment with β-lactams; lipid-II could be transglycosylated by PBP2, and efficiently funnelled straight to PBP2a for transpeptidation, in the absence of PBP2 transpeptidase activity as a result of β-lactams exposure.

However, recent work by Paulin et al, (2014) has demonstrated the close physical proximity of PBP2 and PBP2a following recruitment by FtsZ to the cellular membrane. Styrene-co-maleic acid (SMA) nanoparticles were used to solubilise and extract FtsZ, PBP2 and PBP2a from intact MRSA cells, demonstrating their proximity within the lipid bilayer (Paulin et al, 2014). Furthermore, treatment with ECg disrupted this spatial relationship such that only PBP2 and FtsZ were extracted from intact cells treated with ECg, again supporting the hypothesis that PBP2 and PBP2a form a protein-protein complex sufficient to facilitate β-lactam resistance (Paulin et al, 2014).

If S. aureus membrane organisation and the localisation of PBPs to the cell membrane is important in the MRSA phenotype, then could PBP2 and PBP2a also be forming protein complexes, not only with themselves, but with other binding partners within the FMMs, localising and/or enhancing their enzymatic activity and efficiency as a result of this interaction? Could the close proximity of these PBPs allow them to form protein complexes, that in turn enhances their activity? Would a PBP2-PBP2a complex have greater transglycosylase (PBP2) or transpeptidase activity (PBP2 and PBP2a) as a result of their interaction? In the presence of β-lactams, the transpeptidase activity of PBP2 is inhibited, but if PBP2 and PBP2a interact, perhaps the transglycosylase activity of PBP2, and/or the transpeptidase activity of PBP2a is enhanced as a direct result of this complex formation, allowing MRSA to survive in otherwise lethal concentrations of β-lactams.
6.1.3. Techniques used to study protein-protein interactions in this study

6.1.3.1. Analytical ultracentrifugation

Analytical ultracentrifugation (AUC) was selected to characterise the stoichiometry of the association of PBP2-W59M and PBP2a-Y23M, and to assess their interaction. AUC studies the behaviours of proteins in solution by measuring their change in concentration distribution and sedimentation under gravitational force (Ghirlando 2010). Macromolecules vary in their density, and therefore studying their sedimentation behaviour by AUC can provide information about a protein's size, shape, density and stoichiometric composition (Cole et al, 2008). Furthermore, AUC analyses macromolecules in free solution, such that molecules do not need to be tethered or immobilised, affinity tagged or fluorescently labelled in any way, meaning that molecule interactions are unlikely to be blocked or interfered with (Lebowitz et al, 2002, Scott and Shuck 2014).

Two methods exist for AUC, sedimentation velocity (SV) and sedimentation equilibrium (SE). SV assesses the movement of proteins under high centrifugal force over time in order to define molecular mass, shape and molecule interactions (Ghirlando 2010, Shuck 2013). SE uses a thermodynamic method where sedimentation is balanced by diffusion at low centrifugal force, providing information on molecular mass, potential molecule interaction and stoichiometry (Ghirlando 2010, Shuck 2013). Here, SV-AUC was used to assess the native molecular weights of PBP2 and PBP2a, as well as to assess their ability to form protein-protein interactions with one another.

6.1.3.2. Protein-protein crosslinking

The cross-linking of proteins with a covalent bond during protein-protein interactions can provide a means of studying interactions which may be transient, or inherently weak. Cross-linking reagents are designed to contain terminii which are reactive to
specific functional groups on a protein's surface, as detailed in Table 6.1. These chemical crosslinkers are classed as either homo- or hetero-bifunctional depending on whether they have identical or different functional groups at either end of the carbon chain spacer arm, respectively (Hermanson 2008b, Hermanson 2008a, Thermo-Scientific 2012). Homo-bifunctional crosslinkers are used to polymerise proteins containing like functional groups in a single-step reaction; whilst these crosslinkers can provide the freedom to assess protein-protein interactions when protein interaction partners are unknown, they are therefore not highly selective, and can also result in protein homo-dimerisation and insoluble high molecular weight multi-protein polymers (Hermanson 2008b, Thermo-Scientific 2012).

Hetero-bifunctional crosslinkers on the other hand allow for precise, directed protein conjugation, minimising self dimerisation and undesirable crosslinking to proteins that are not of interest (Hermanson 2008a, Thermo-Scientific 2012). Heterobifunctional crosslinkers can be used in a one-step reaction, or used in a sequential two-step reaction. In the former case, the heterobifunctional crosslinker (such as sulfo-SMCC, Fig. 6.1, A) reacts with protein 1 using the most labile functional group first; excess unreacted crosslinker is then removed, and modified protein 1 is incubated with protein 2 allowing the second reactive group of the crosslinker to bind protein 2 (Hermanson 2008a, Thermo-Scientific 2012). The most widely used heterobifunctional crosslinkers have an amine-reactive succinimidyl ester (NHS ester) at one end, and a sulfhydryl-reactive (maleimide) at the other (Thermo-Scientific 2012). If, however, protein 2 does not contain a reactive sulfhydryl group, this can be added to the protein via sulfhydryl addition reactions (SATA followed by SATA deprotection, Fig 6.1, B-C). Sulfhydryl-reactive maleimide modified protein 1 and sulfhydryl-modified protein 2 can then be incubated together to form crosslinked protein (Fig 6.1, D). These types of cross-linking reactions are therefore highly specific and flexible.

To assess the ability of truncated PBP2-W59M and PBP2a-Y23M to form a protein-protein complex, a two-step conjugation reaction using a heterobifunctional crosslinker was performed, as illustrated in Fig 6.1.
Table 6.1. The reactive groups of crosslinker molecules used for protein-protein crosslinking. Adapted from (Thermo-Scientific 2012).

<table>
<thead>
<tr>
<th>Reactivity Class</th>
<th>Target Amino Acid</th>
<th>Cross-linker Chemical Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amine-reactive (-NH₂)</td>
<td>Lysine (or N-terminus of polypeptide chain)</td>
<td>NHS Ester, Imidoester, Pentafluorophenyl ester, Hydroxymethyl phosphine</td>
</tr>
<tr>
<td>Carboxyl-to-amine reactive (-COOH)</td>
<td>Aspartic acid, Glutamic acid (or C-terminus of polypeptide chain)</td>
<td>Carbodiimide</td>
</tr>
<tr>
<td>Sulphydryl-reactive (-SH)</td>
<td>Cysteine</td>
<td>Maleimide, Haloacetyl, Pyridylsulfide, Thiosulfonate, Vinylsulfonate</td>
</tr>
<tr>
<td>Photo-reactive</td>
<td>Non-specific</td>
<td>Diazirine, Aryl azide</td>
</tr>
<tr>
<td>Azide-reactive (-N₃)</td>
<td>-</td>
<td>Phosphine</td>
</tr>
</tbody>
</table>
Figure 6.1. Overview of protein-protein crosslinking using SATA and sulfo-SMCC. The sulfo-NHS ester of sulfo-SMCC is reacted with the primary amine of lysine residues of protein 1, resulting in protein 1 plus an available sulfahydryl-reactive maleimide group (A). The NHS ester of SATA is reacted with the primary amine of lysine residues of protein 2 to create a SATA-modified protein intermediate (B). Hydroxylamine is used to deprotect the sulfhydryl group of the SATA-modified protein 2 to yield the sulfhydryl-modified protein 2 (C). The sulfhydryl-reactive maleimide modified protein 1 is incubated with the sulfhydryl-modified protein 2 to yield cross-linked protein 1 and protein 2 (D). Adapted from Thermo Scientific (2012).
6.1.3.4. Carbene footprinting

Carbene footprinting is a technique used to chemically label the surface of a protein to allow the mapping of potential non-covalent protein-ligand interactions, where ligands can range from proteins, nucleic acids, lipids, carbohydrates and other small molecules (Ge et al, 2018). A chemical probe, such as a photoreactive diazirine or diazo compound is irradiated with high energy light (350-380 nm), forming a highly reactive carbene molecule (Fig. 6.2) - other popular photoreactive probes for protein labelling, including aryl azides, benzophenones and 2-aryl-5-carboxytetrazoles, are classified by their photochemically generated reactive species; nitrenes, diaradicals and carboxy-nitrile imines, respectively (Smith and Collins 2015, Ge et al, 2018).

In the case of diazirines, the photochemically generated carbene molecule has an extremely short lifespan and spontaneously labels the surface amino acids of the protein, forming covalent bonds via insertion into C-H, O-H, N-H or C-C protein bonds (Ge et al, 2018). The labelled proteins are subsequently enzymatically digested for mass spectrometry analysis yielding a carbene “footprint” of peptides successfully labelled with the carbene molecule. This carbene footprint is compared to the footprint of the protein in the presence of a potential binding partner. If peptides are no longer seen to be labelled with the carbene molecule in the presence of the binding partner, these are areas of carbene masking i.e. areas of protein-partner interaction which are no longer exposed to carbene labelling. Conversely, peptides which now become labelled with carbene molecules in the presence of the binding partner, are areas of carbene unmasking i.e. areas which are now exposed to carbene labelling as a result of protein conformational change in the presence of the binding partner.
Figure 6.2. An overview of carbene foot-printing. A chemical probe is irradiated with high energy light, such as UV, forming a carbene with two shared, and two unshared valencies. This highly reactive carbene spontaneously covalently binds to protein molecules.
6.2. Chapter aims

This chapter aims to identify whether *S. aureus* PBP2 and PBP2a have the ability to form a protein-protein complex. In order to assess this, the specific aims of this chapter are;

- To clone full-length *S. aureus* pbp2 and full-length *S. aureus* pbp2a into separate vectors, and subsequently clone both together into the co-expression vector, pET-Duet, under the control of separate promoters
  - Perform expression trials to determine optimal expression strains

- To perform large scale co-expressions of full-length PBP2 and full-length PBP2a

- To assess the ability of full-length PBP2 and full-length PBP2a to form a protein-protein complex and subsequently co-purify the protein complex via nickel IMAC following large-scale co-expression

- To assess the ability of truncated (minus TM domain) PBP2-W59M and PBP2a-Y23M to form a protein-protein complex via;
  - Carbene foot printing
  - Analytical ultracentrifugation
  - Protein crosslinking
  - Native MS
6.3. Results and discussion

6.3.1. Are full-length PBP2 and PBP2a able to form a protein-protein complex following recombinant protein co-expression, and can they be successfully purified as a complex?

In order to investigate the role of *S. aureus* PBP2 and PBP2a in the methicillin resistance phenotype of MRSA strains, it was necessary to design and clone expression constructs containing the full-length PBP2 and PBP2a genes. Constructs for the truncated PBPs, minus their trans-membrane (TM) helix, were already available and therefore did not need to be cloned.

6.3.1.1. Cloning of full-length *S. aureus* Mu50 PBP2 and *S. aureus* Mu50 PBP2a into pPROEX and recombinant protein expression

Full-length *S. aureus* Mu50 *pbp2* and *pbp2a* were each cloned separately into pPROEX with an N-terminal His₆ tag, cleavable with TEV protease. Successful cloning was confirmed by Sanger Sequencing (Eurofins, GATC). Protein expression trials were performed in 10 ml LB, using *E. coli* BL21(DE3)Star.pRosetta, C41(DE3), C43(DE3) and Lemo21(DE3) grown to an OD₆₀₀ of 0.6 at 37°C, 180 rpm. Protein expression was induced with 1 mM IPTG for 4 hours at 25°C before cell harvest.

Soluble and insoluble fractions were analysed by SDS-PAGE (Fig. 6.3-6.4, Left). The presence of PBP2 or PBP2a was confirmed by Western blot using an anti-His antibody (Fig. 6.3-6.4, Right). PBP2a was shown (Fig. 6.4) to have been expressed only from *E. coli* Lemo21(DE3) cells, and as expected was located in the insoluble fraction due to the presence of the transmembrane helix. By contrast, PBP2 was shown (Fig. 6.3) to express well in all four cell lines, and again as expected was located in the insoluble fraction. As can be seen from the Western blot (Fig. 6.3, Right), there appears to be some degradation of PBP2 when expressed from *E. coli* C43(DE3) and Lemo21(DE3)
cell lines. From this point, full-length PBP2a was expressed in *E. coli Lemo21(DE3)* cells, whilst full-length PBP2 was expressed in *E. coli BL21(DE3)Star.pRosetta* cells.

**Figure 6.3. Expression trials of *S. aureus* Mu50 PBP2.** PBP2 was expressed from pPROEX::pbp2 in *E. coli* BL21(DE3)*Rosetta* (denoted B*R), Lemo21(DE3) (denoted L), C41(DE3) (denoted C41) and C43(DE3) (denoted C43). Protein expression was induced with 1 mM IPTG for 4 hours at 25°C before harvest. Protein samples (10ug) were analysed by SDS-PAGE (left) and Western blot (right). M – Amersham LMW protein ladder. H – Benchmark His-tagged protein ladder. Lane 1 uninduced soluble, lane 2 uninduced insoluble, lane 3 induced soluble, lane 4 induced insoluble. PBP2 = 83 kDa.
Figure 6.4. Expression trials of *S. aureus* Mu50 PBP2a. PBP2a was expressed from pPROEX::pbp2a in E. coli BL21(DE3)*Rosetta* (denoted B*R), Lemo21(DE3) (denoted L), C41(DE3) (denoted C41) and C43(DE3) (denoted C43). Protein expression was induced with 1 mM IPTG for 4 hours at 25°C before harvest. Protein samples (10ug) were analysed by SDS-PAGE (left) and Western blot (right). M – Amersham LMW protein ladder. H – Benchmark His-tagged protein ladder. Lane 1 uninduced soluble, lane 2 uninduced insoluble, lane 3 induced soluble, lane 4 induced insoluble. PBP2a = 82 kDa.
6.3.1.2. Cloning of full-length *S. aureus Mu50* pbp2 and *S. aureus Mu50* pbp2a into pET-Duet for co-expression

*pPROEX::pbp2* and *pPROEX::pbp2a* constructs were used as template DNA to synthesise the co-expression vector *pETDUET::pbp2:pbp2a*. The construct was designed such that PBP2 has an N-terminal His<sub>6</sub> tag, and PBP2a has an N-terminal S-tag, both cleavable with TEV protease, as illustrated in Figure 6.5. Successful cloning was confirmed by Sanger Sequencing (Eurofins, GATC).

![Figure 6.5. Cloning of full-length PBP2 and PBP2a into the coexpression vector pET-Duet.](image)

Figure 6.5. Cloning of full-length PBP2 and PBP2a into the coexpression vector pET-Duet. nt denotes nucleotide position.

6.3.1.3. Protein co-expression trials of full-length *S. aureus Mu50* PBP2 and PBP2a

Protein co-expression trials were performed in 10 ml LB, using *E. coli* BL21(DE3)Star.pRosetta, C41(DE3), C43(DE3) and Lemo21(DE3) grown to an OD<sub>600</sub> of 0.6 at 37°C, 180 rpm. Protein expression was induced with 1 mM IPTG for 4 hours at 25°C before cell harvest. Soluble and insoluble fractions were analysed by SDS-PAGE (Fig. 6.6, Left) and the presence of PBP2 and PBP2a confirmed by Western blot using an anti-His antibody, or anti S-tag antibody, respectively (Fig. 6.6, Right). PBP2 and PBP2a were shown to have co-expressed in all four cell lines to varying extents (Fig. 6.6.), however, the greatest expression of both PBP2 and PBP2a in the same cell line was from *E. coli* BL21(DE3)Star.pRosetta, therefore this cell line was chosen for large scale protein expression. Again, it is worth noting that there appeared to be a level of PBP2 degradation (Fig. 6.6 Panel B), and therefore the addition of protease inhibitors to protein purification buffers would be necessary.
Figure 6.6. Coexpression trials of *S. aureus* Mu50 PBP2a and PBP2. PBP2a (Panel A) and PBP2 (Panel B) were coexpressed from pET-Duet::pbp2::pbp2a, in *E. coli* BL21(DE3)Star.pRosetta (denoted B*R), Lemo21(DE3) (denoted L), C41(DE3) (denoted C41) and C43(DE3) (denoted C43). Protein expression was induced with 1 mM IPTG for 4 hours at 25°C before harvest. Protein samples (10ug) were analysed by SDS-PAGE (Left of Panel A and B) and Western blot (Right of Panel A and B). M – Amersham LMW protein ladder. H – Benchmark His-tagged protein ladder. S – Merck Perfect Protein S-tagged ladder. Lane 1 uninduced soluble, lane 2 uninduced insoluble, lane 3 induced soluble, lane 4 induced insoluble. PBP2a = 82 kDa, PBP2 = 83 kDa.
6.3.2. Are the full-length PBP2 and PBP2a able to form a protein-protein complex that can be co-purified?

6.3.2.1. Purification of PBP2-PBP2a complex

Full-length PBP2-PBP2a were co-expressed in 2L LB from *E. coli* BL21(DE3)Star.pRosetta, as per expression trials. Insoluble PBP2-PBP2a was extracted from the cell pellet using cell disruption, CHAPS solubilization and centrifugation to remove cell debris (Section 2.5.8.2). Proteins were loaded onto a nickel IMAC column and PBP2-PBP2a eluted off with increasing gradient of imidazole. Fractions that appeared to contain His<sub>6</sub> tagged PBP2 (83 kDa) or S-tagged PBP2a (82 kDa) were visualized via SDS-PAGE, and Western blot performed using anti-His Ab and anti-S tag Ab, respectively.

As shown in Fig. 6.7., both PBP2a (Fig. 6.7. Top panels) and PBP2 (Fig. 6.7. Bottom panels) eluted from the nickel IMAC column in the same fractions, as confirmed by Western blot (Fig. 6.7. Right panels). The co-purification of PBP2-PBP2a is suggestive of complex formation. If PBP2-PBP2a did not form a complex, it would be expected that only the His-tagged PBP2 would be selectively purified by Nickel IMAC, and that the S-tagged PBP2a would have eluted on initial low imidazole concentration column washes due to the S-tag having no affinity for the immobilized nickel ion.

Fractions containing full-length PBP2-PBP2a were pooled and concentrated, giving a final yield of approximately 0.15 mg per litre.
Figure 6.7. Purification of coexpressed *S. aureus* Mu50 PBP2a and PBP2. PBP2a (Top) and PBP2 (Bottom) were coexpressed from pET-Duet::pbp2::pbp2a, in *E. coli* BL21(DE3)Star.pRosetta for 4 hrs at 25°C before harvest. PBP2:2a were purified via nickel IMAC, 10µg protein samples were loaded onto SDS-PAGE (Left) and analysed by Western blot (Right). M – Amersham LMW protein ladder. H – Benchmark His-tagged protein ladder. S – Merck Perfect Protein S-tagged ladder. Sup – supernatant. P – pellet. FT – flowthrough. A12, B1, C2, C4 and C6 – IMAC washes. PBP2a = 82 kDa, PBP2 = 83 kDa

6.3.2.2. Mass spectrometry of full-length PBP2-PBP2a visualized by SDS-PAGE

Co-expressed and purified PBP2-PBP2a (10 µg) was visualized by SDS-PAGE (Fig. 6.8. Left) and extracted for analysis by mass spectrometry (Fig. 6.8. Right). Full-length PBP2 (83 kDa) and PBP2a (82 kDa) were detected in the protein sample with 81% and 78% peptide coverage, respectively, again suggestive that full-length PBP2-PBP2a co-purified as a protein-protein complex. To our knowledge this has not been documented in the literature before.
Figure 6.8. Final purity of *S. aureus* Mu50 PBP2a complex. Protein fractions were pooled and concentrated to 0.7 mg/ml, of which 3 ug was analysed by SDS-PAGE (Left). 1 – NEB Prestained coloured markers. 2 – PBP2a complex. The protein band (arrow head) was excised and analysed by mass spectrometry for the presence of PBP2 and PBP2a (Right). Amino acid sequences detected by proteomic analysis are shown (yellow).
6.3.2.3. Are the Western blot antibodies used to detect His-tagged PBP2 and S-tagged PBP2a specific only for their intended affinity tags?

In order to confirm that the antibodies used in this experiment were mutually exclusive, i.e. anti-His Ab was specific for only His-tagged proteins (and not S-tagged), and that the Anti-S Ab was specific for only S-tagged proteins (and not His-tagged), two western blots were performed against a standard protein ladder with no tag, His-tagged protein ladder and S-tagged protein ladder using both anti-His Ab (Fig. 6.9. Left) and anti-S Ab (Fig. 6.9. Right). No antibody cross reactivity was seen supporting the conclusion that PBP2-PBP2a were purified as a complex.

Figure 6.9. Western blot of protein standard ladders. Western blot using Anti-His Ab (Left) and Anti-S-tag Ab (Right). M – Amersham LMW protein ladder. H – Benchmark His-tagged protein ladder. S – Merck Perfect Protein S-tagged ladder.
6.3.3. Are the truncated PBP2-W59M and PBP2a-Y23M able to form a protein-protein complex in the absence of their TM domains?

Protein-protein interactions can be studied by carbene footprinting (Manzi et al, 2016, Jenner et al, 2018) if an X-ray crystal structure of each protein is available and the protein sample is detergent free. The insoluble full-length PBP2 and PBP2a are solubilized and purified in the presence of detergent, and a full-length x-ray crystal structure is also not currently available. Therefore, the full-length PBP2 and PBP2a are not suitable for analysis by carbene footprinting. However, X-ray crystal structures are available for PBP2-W59M (PDB 2OLU (Lovering et al, 2007)) and PBP2a-Y23M (PDB 1VQQ (Lim and Strynadka 2002)) minus their TM domains, and their purification is detergent free such that their interaction could be analysed by carbene footprinting.

6.3.3.1. Recombinant expression and purification of PBP2-W59M and PBP2a-Y23M

*pET-41a::pbp2-W59M* and *pET-15b::pbp2a-Y23M* were transformed into *E. coli* BL21(DE3)Star.pRosetta and were grown (4L in LB) as per Lovering et al (2007) and Lim and Strynadka (2002). PBP2-W59M was purified by nickel IMAC, size exclusion and anion exchange chromatography (Fig. 6.10), as in Section 2.5.8.3. Half the protein was purified in the presence of detergent according to Lovering et al, (2007), and half in the absence of detergent. PBP2a-Y23M was purified by anion exchange, cation exchange and size exclusion chromatography in the absence of detergent, Fig. 6.11, as in Section 2.5.8.4. Both proteins were expressed at high levels, and final preparations were >90% pure (Fig. 6.12), with yields of 3 mg PBP2-W59M per L of culture and 10 mg PBP2a-Y23M per L of culture.
Figure 6.10. A typical purification of PBP2-W59M using Ni-IMAC, size exclusion and anion exchange chromatography. 10 µg protein visualized by 12% SDS-PAGE. (A). Ni-IMAC purification of PBP2-W59M. Ai. SDS-PAGE. Lane 1 – Amersham LMW ladder, 2 - pellet, 3 – supernatant, 4 – flowthrough, 5 – buffer wash, 6-10 - fractions 6-10, respectively. Aii. AKTA chromatogram. * indicates PBP2-W59M peak. (B). Size exclusion purification (Superdex 200 16/600) of PBP2-W59M. Bi. SDS-PAGE. Lane 1 - Amersham LMW ladder, 2 - before SEC, 3 - 10 - fractions 1C4-5, 2A1-5 and 2B5, respectively. Bii. SDS-PAGE continued. Lane 1 - Amersham LMW ladder, 2 - before SEC, 3 – fractions 2B4-2B1, 2C1-2C2. Biii. AKTA chromatogram. * indicates PBP2-W59M peak. Ci. Anion exchange purification (Q Sepharose) of PBP2-W59M. Lane 1 - Amersham LMW ladder, 2 – empty, 3 – flowthrough, 4-9 – fractions 1B3-1B1, 1C—1C3, respectively. Blue line denoted ‘Pooled’ indicates fractions that were pooled together and taken forward to the next round of purification.
Figure 6.11. A typical purification of PBP2a-Y23M using anion exchange, cation exchange and size exclusion chromatography. 10 µg protein visualized by 12% SDS-PAGE. (Ai). Anion exchange purification of PBP2a-Y23M. Lane 1 – Amersham LMW ladder, 2 – after sonication, 3 – pellet, 4 – supernatant, 5 – FT, 6 – Wash 1 and 7 – wash 2. (B) Cation exchange (SP Sepharose). (Bi.) SDS-PAGE. Lane 1 – Amersham LMW ladder, 2 – before cation exchange, 3 – FT, 4 – Buffer wash, 5 – 6 – fractions 1A5-1C3. (Bii). AKTA chromatogram. * denotes PBP2a-Y23M peak. (C) Size exclusion chromatography (Superdex 200 16/600) of PBP2a-Y23M. (Ci) SDS-PAGE. Lane 1 – Amersham LMW ladder, 2 – before SEC, 3-10 – fractions 1B4-1C4. (Cii). AKTA chromatogram. * denotes PBP2a-Y23M peak. Blue line denoted ‘Pooled’ indicates fractions that were pooled together and taken forward to the next round of purification.
6.3.3.2. Assessing protein-protein interaction by Analytical ultracentrifugation

All AUC protein-protein interaction analysis was performed by Dr Gemma Harris, Harwell Research Complex. AUC was carried out on *S. aureus* PBP2-W59M and PBP2a-Y23M to determine their molecular weight, oligomeric states, and whether they have the ability to form a protein-protein complex. The full-length PBPs were not used for AUC as this would require the presence of detergent. The use of detergents during AUC can complicate data analysis as different detergents can affect protein migration during sedimentation and hence obscure molecular weight determination; however this can be overcome if the detergent binding ratio is known (Le Maire *et al*, 2000). Detergents can also interfere with protein absorbance at

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Figure 6.12. Final purity of *S. aureus* PBP2a Y23M and *S. aureus* PBP2 W59M. A 12% SDS-PAGE showing final purify of PBP2a-Y23M (Panel A, 3 ug), PBP2-W59M - 0.28 mM LDAO (Panel B, 2.5 ug) and PBP2-W59M + 0.28 mM LDAO (Panel C, 12 ug). M – Amersham LMW protein ladder.
certain wavelengths. For example, non-ionic polyoxyethylene detergents such as Triton X-100 absorb within the UV-range and hence are not suitable for UV monitoring at 280 nm.

All samples behaved with ideal sedimentation as no concentration-dependent repulsive interactions were seen, characterised by decreasing sedimentation coefficient (Sed. Co) with increasing protein concentration, Table 6.22 (Scott and Shuck 2014). Both proteins were found to be homogenous with a high purity (Fig. 6.13). The frictional ratio (f/fo) represents the deviation of asymmetry of a protein, where an f/fo of 1.0 represents a completely symmetrical non-hydrated sphere (Unzai 2018). For well-folded globular proteins, an f/fo ratio of 1.05-1.3 is expected whilst for more asymmetric or glycosylated proteins, an f/fo ratio of up to 1.5-1.8 is not unusual (Balbo and Shuck 2005, Zhao et al., 2013, Unzai 2018). Both PBP2-W59M and PBP2a-Y23M have an f/fo of between 1.34-1.37 (Table 6.2), indicating that both proteins are well folded.

Both PBP2-W59M and PBP2a-Y23M were shown by AUC sedimentation coefficient analysis to be primarily monomeric, with some dimerization existing as concentration is increased; the observed AUC molecular weights for both PBP2-W59M and PBP2a-Y23M were very close to their theoretical values (Table 6.2, and Fig. 6.13). For PBP2-W59M the monomer-dimer equilibrium is pronounced occurring at all ranges from 1.2 μM – 5.9 μM, whilst PBP2a-Y23M is predominantly monomeric with dimerization not occurring below 5.5 μM. The ability of PBP2-W59M and PBP2a-Y23M to form a protein-protein complex was also assessed by AUC. Interpretation of data was complicated by the existence of a dominant PBP2-W59M monomer-dimer equilibrium with the protein concentration used. However, analysis of sedimentation coefficients (Table 6.2, Fig. 6.13) is not suggestive of a protein-protein interaction in this scenario (i.e. in the absence of the TM-helices). If this were the case, a decrease in the monomer peak or increase in the dimeric species should be seen in a concentration dependent manner, as PBP2a-Y23M concentration is increased. Here, the dimeric species seen are likely to be due to PBP2-W59M dimerization, or PBP2a-Y23M dimerization, and not due to PBP2-W59M-PBP2a-Y23M complex formation.
Figure 6.13. Characterisation of *S. aureus* PBP2-W59M and PBP2a-Y23M by analytical ultracentrifugation. A. 1.2 µM – 5.9 µM PBP2-W59M. B. 1.1 µM – 5.5 µM PBP2a-Y23M. C. Mixture 1.2 µM PBP2-W59M and 1.2 µM – 5.7 µM PBP2a-Y23M. Sedimentation velocity analysis by AUC of *S. aureus* PBP2-W59M, PBP2a-Y23M and a mixture of both proteins, at varying concentrations. Buffer conditions were 50 mM potassium phosphate, 150 mM NaCl, pH 7. Data was recorded using the absorbance optical detection system (280 nm). Data were processed using SEDFIT, fitting to the c(s) model. Figures were made using GUSSI.
Table 6.2. Characterisation of *S. aureus* PBP2-W59M and PBP2a-Y23M by analytical ultracentrifugation. Sedimentation coefficient (Sed. Co (S)), molecular weight (MW), Frictional ratio (f/f₀). Sedimentation velocity analysis by AUC of *S. aureus* PBP2-W59M, PBP2a-Y23M and a mixture of both proteins, at varying concentrations. Buffer conditions were 50 mM potassium phosphate, 150 mM NaCl, pH 7. Data was recorded using the absorbance (optical detection system (280 nm)).

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6.3.3.3. Assessing weak protein-protein interaction by chemical protein crosslinking studies

To assess the ability of PBP2-W59M and PBP2a-Y23M to form a protein-protein interaction, and to thus investigate potentially weak protein-protein interactions in the absence of the TM domains, PBP2-W59M and PBP2a-Y23M were chemically cross-linked together for future use in enzymatic activity assays. PBP2-W59M was chemically labelled with sulfo-SMCC at the primary amine of lysine residues to create a sulphydryl-reactive maleimide group. PBP2a-Y23M was chemically labelled with SATA at the primary amine of lysine residues to create a SATA-modified protein intermediate. Hydroxyl amine was used to deprotect the sulphydryl group of the SATA-modified PBP2a-Y23M to give a sulphydryl-modified PBP2a-Y23M. If the two PBPs have a tendency to form protein-protein interaction partners with each other, then they will be chemically cross-linked together via a sulphydryl Michael addition reaction.

As PBP2a-Y23M does not contain any cysteine residues the introduction of a sulphydryl-reactive group via SATA modification was essential for cross-linking in the manner described. PBP2-W59M does however contain a cysteine residue at position 222, and therefore, the addition of a sulphydryl-reactive maleimide group at lysine residues could have led to homodimerization of PBP2-W59M. However, PBP2-W59M and PBP2a-Y23M did not show any self-cross-linking, as shown in Fig. 6.14 lane 2 and 3, respectively. It is likely that homodimerization of sulfo-SMCC modified PBP2-W59M did not occur as the Cys-222 is buried deep within the protein structure and is therefore not accessible.

Fig. 6.14 Lanes 4-8 show successful cross-linking of PBP2-W59M and PBP2a-Y23M, where cross-linking appears complete within 5 min (Lane 4); a heterodimer of PBP2-PBP2a is illustrated at approx. 130 kDa (Fig. 6.14, 1:1).

Although PBP2-W59M and PBP2a-Y23M appear to successfully chemically cross-link to form a protein-protein cross-link consistent with a weak interaction, we cannot be
unequivocally sure that this cross-linking has occurred because of the formation of a naturally occurring PBP2-PBP2a complex or because the chemical cross-linkers have trapped an interaction at non-biologically relevant sites. In hindsight, the latter is more likely as the chemical cross-linkers attach spontaneously to free amine groups, and are not specific to the site of protein-protein interaction. However, if the two PBPs do form a complex at a biologically relevant site of interaction, then the two chemical cross-linkers will be able to spontaneously cross-link the two PBPs together via the free amine at the non-biological relevant site of interaction. This may still act to stabilize the PBP complex interaction, albeit not via the natural site of interaction.

The fact that there is still some residual non-cross-linked PBP present in the samples even after 60 minutes (Fig. 6.14, NC bands), could suggest that chemical cross-linking only occurs when a specific protein-protein interaction has occurred, and not as a result of random chemical cross-linking. However, this could also reflect the instability of the linker modifications under the prevailing conditions leaving a proportion of the PBPs unable to cross-link.

Figure 6.14. Protein-protein crosslinking of PBP2-W59M and PBP2a-Y23M with sulfo-SMCC and SATA crosslinkers, respectively. Lane 1 - Amersham MW protein ladder, 2 – Sulfo-SMCC modified PBP2-W59M, 3 – SATA modified PBP2a-Y23M, 4-8 – Both PBPs incubated together for 5, 10, 20, 30, and 60 min, respectively. Samples run on 4% SDS-PAGE. Not crosslinked (NC). Crosslinked in 1:1 ratio (1:1). Crosslinked in 1:2 ratio (1:2).
6.3.3.4. Assessing protein-protein interaction by carbene foot-printing

All carbene foot-printing protein-protein interaction analysis was performed by Dr Matthew Jenner, University of Warwick (Chemistry). Carbene footprinting of 50 μM PBP2-W59M and 50 μM PBP2a-Y23M by photoactivation of 10 mM aryl diazirine, pH 7.4, was performed individually, and following incubation of both proteins at a 1:1 Molar ratio, as in Section 2.10.3. Following tryptic digest, peptide fragments were analysed by electrospray ionisation-quadrupole time of flight mass spectrometry (ESI-Q-TOF-MS) to quantify the degree of carbene labelling of PBP2-W59M and PBP2a-Y23M, separately, in comparison to the PBP2-W59M and PBP2a-Y23M mixture.

In the presence of PBP2-W59M, carbene labelling of PBP2a-Y23M was masked at peptide positions 55-68, 103-124, 158-176, 189-198, 220-229, 231-241, 248-265, 274-280, 470-477 and 487-506 (Fig. 6.15, Panel A, red circles), and was unmasked at peptide positions 77-83, 323—331 and 445-456 (Fig. 6.15, Panel A, blue circles), when compared to PBP2a-Y23M alone. Peptides that were unaffected by carbene labelling are indicated by yellow circles. These none affected areas, and the areas of peptide masking (potential protein-protein interaction) and unmasking (conformational change) were mapped onto the PBP2a-Y23M (1VQQ) crystal structure and the crystal structure of PBP2-W59M (2OLU) overlaid for visual representation (Fig. 6.15, Panel B). The exact peptide sequences are annotated in Fig. 6.16.

Unfortunately, labelling of PBP2-W59M with the aryl diazirine probe was not successful. The aryl diazirine probe is inherently negatively charged. This therefore favours the labelling of positively charged residues such as arginine and lysine. However, labelling of aromatic residues such as tyrosine and phenylalanine can also occur. The inability of the aryl diazirine probe to label surface residues of PBP2-W59M may suggest that PBP2-W59M is also inherently negatively charged – the isoelectric points (pI) of PBP2-W59M and PBP2a-Y23M are 6.48 and 8.27, respectively, which is in agreement with this suggestion. Therefore, a new generation
of probe is currently being pursued, which is hoped will specifically target the labelling of the negatively charged residues on the surface of PBP2-W59M. The labelling of PBP2-W59M alone, and PBP2-W59M in the presence of PBP2a-Y23M with the new generation diazirine probe is ongoing work and will be completed in the near future.

Whilst the complete interaction interface of PBP2-W59M:PBP2a-Y23M has not fully been mapped, the information collected for PBP2a-Y23M in the presence of PBP2-W59M is valuable. The masking of carbene labelling of PBP2a-Y23M indicates areas of protein-protein interaction, which all occur towards the N-terminus. The unmasking of carbene labelling of PBP2a-Y23M indicates areas of conformational change, which occurs towards the C-terminus of the protein. Interestingly, the transpeptidase active site of PBP2a-Y23M is positioned at residue Ser-403, towards the C-terminus of the protein, whilst the allosteric site is located towards the N-terminus of the protein (Summarised in Table 6.3). This, taken with the sites of masking and unmasking, could suggest that PBP2-W59M interacts with PBP2a-Y23M within the allosteric domain, allowing for conformational change within the transpeptidase domain, which could allow the pentaglycine branch of polymerised glycan chains access to the active site Ser-403 allowing for transpeptidation to occur.
Figure 6.15. Carbene labelling of PBP2a-Y23M in the presence of PBP2-W59M.  

A. Overview of carbene labelling of peptides from PBP2a alone (Black) and PBP2a in the presence of PBP2 (Grey). Peptide sequences were either detected to be masked from carbene labelling (red), unmasked from carbene labelling (blue), not affected by carbene labelling (brown) or not detected (black). Fractional modifications were considered significant only when the \( p \)-value obtained from a Student T-test was <0.05.  

B. Model of potential protein-protein interaction of PBP2a-PBP2, indicating masking (red highlight), unmasking (blue highlight) and unaffected (brown). PDB structures used; PBP2 (2OLU) and PBP2a (1VQQ). TM helix – trans-membrane helix, residues 1-22. NB. PBP2a-Y23M is truncated and does not have TM helix.
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NKKYDIDE

Figure 6.16. Peptides modified by carbene labelling of PBP2a-Y23M in the presence of PBP2-W59M.

Peptide sequence for *S. aureus* Mu50 PBP2a shown. Peptide position 1-23 denotes the transmembrane helix. PBP2a-Y23M is a truncated form and does not include the peptide sequence 1-22 (underlined); the lysine, Y at position 23 (Bold) is mutated to Methionine, M in PBP2a-Y23M. Peptide sequences were either detected to be masked from carbene labelling (red), unmasked from carbene labelling (blue), not affected by carbene labelling (brown) or not detected (black).
Table 6.3. Important residues in PBP2a-Y23M structure.

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<th>Residue</th>
<th>Role</th>
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<tr>
<td>Ser-403</td>
<td>Transpeptidase active site, located at the N-terminus of the α2 helix in an extended groove</td>
<td>Lim and Strynadka (2002)</td>
</tr>
<tr>
<td>Ser-403 and Thr-600</td>
<td>Backbones of these residues form the oxyanion hole</td>
<td></td>
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<tr>
<td>Ser-403 and Lys-406</td>
<td>Ωγ of Ser-403 and Nζ form a hydrogen bond, most likely activating the Ωγ of Ser-403 for reaction with the peptidoglycan donor</td>
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<tr>
<td>Residues 327-668</td>
<td>Transpeptidase domain</td>
<td>Otero et al, (2013)</td>
</tr>
<tr>
<td>Residues 27-326</td>
<td>AllostERIC domain, located between intersecting sections of lobe 1 (residues 166–240), lobe 2 (residues 258–277) and lobe 3 (residues 364–390)</td>
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<td>Ser-598, Gly-599, and Thr-600</td>
<td>Following acylation, the β3 sheet is twisted to change the position and orientation of Ser-598, Gly-599, and Thr-600 to avoid steric clashes with bound β-lactams</td>
<td>Lovering et al, (2012)</td>
</tr>
<tr>
<td>Ala-601, Glu-602, Arg-612 and Gln-613</td>
<td>Following β3 sheet twisting, neighbouring Ala-601, Glu-602, Arg-612 and Gln-613 are repositioned to allow the exposure of Ser-403</td>
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<tr>
<td>Tyr-446, Met-641, and Thr-600</td>
<td>These residues are within the active site groove, and fit closely to the pentaglycyl bridge of <em>S. aureus</em> lipid-II or the R2 group of β-lactams</td>
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6.3.3.5. Assessing protein-protein interaction by native MS

The interaction of PBP2-W59M and PBP2-Y23M was assessed via native MS. Here both proteins were first flown separately in the gas phase in 150 mM ammonium acetate buffer. Both PBP2-W59M and PBP2a-Y23M were shown to be monomers, which is in agreement with the primarily monomeric species seen by AUC (Section 6.3.2.2). Whilst proteins flew well in the native mass spectrometry gas phase and there appeared to be no apparent degradation of either protein within this buffer system, there appeared to be no detectable interaction of the two proteins, such that when incubated together at 1:1 ratio for 30 min, RT, there was no detectable protein-protein interaction seen (Fig. 6.17).

**Figure 6.17. Native mass spectrometric analysis of PBP2a-Y23M and PBP2-W59M.** PBP2s in 150 mM ammonium acetate incubated together in a 1:1 ratio, for 30 min at RT before analysis by MS. Red – PBP2a-Y23M. Predicted mass 73670.36 Da. Blue – PBP2-W59M. Predicted mass 72824.46 Da. n\(^{\pm}\) denotes charge state.
6.4. Concluding remarks and future work

6.4.1. Do full-length PBP2 and PBP2a form a protein-protein complex, and can this be purified for enzymatic and structural studies?

Recombinant co-expression of both full-length PBP2 (with an N-terminal His\textsubscript{6}) and PBP2a (with an N-terminal S-tag) has shown that both proteins can successfully be purified together via nickel IMAC.

The ability to successfully purify both full-length PBP2 and PBP2a as a protein-protein complex has not been observed before, and could be suggestive that they work in partnership during MRSA challenge with β-lactam antibiotics, using their complimentary activities for transglycosylase and transpeptidase activity, respectively, when PBP2 transpeptidase activity is inhibited. Hence, allowing MRSA to continue with peptidoglycan synthesis in the presence of β-lactams. PBP2-PBP2a cooperativity has also been displayed in MRSA strains which carry the Enterococcal \textit{vanA} gene and exhibit high level vancomycin resistance; PBP2a cannot utilise peptidoglycan precursors containing terminal D-lactate residues, but PBP2 can (Severin \textit{et al}, 2003, Severin \textit{et al}, 2004). Whilst dual vancomycin and β-lactam resistance cannot occur simultaneously, PBP2 activity allows for vancomycin resistance and PBP2a allows for β-lactam resistance (Severin \textit{et al}, 2003, Severin \textit{et al}, 2004).

It is also worth highlighting that the full-length PBP2 and PBP2a cannot be purified separately following separate recombinant expression. Both proteins express well, however degrade rapidly following extraction from the cell membrane. The ability to successfully co-express and subsequently purify full-length PBP2-PBP2a as a protein complex, that was shown to maintain transglycosylase activity (Chapter 7), indicates that this protein complex formation could also serve to maintain protein stability.
6.4.2. Do the truncated PBP2-W59M and PBP2a-Y23M proteins form a protein-protein complex in the absence of the transmembrane helix?

Due to the full-length PBP2 and 2a proteins containing a trans-membrane helix, and therefore being insoluble membrane proteins, their purification involves the use of the detergent CHAPS. However, many of the techniques used to study protein-protein interactions require the absence of detergent. For this reason, the truncated versions of PBP2 and PBP2a were also purified without their transmembrane helix and therefore without detergent; denoted PBP2-W59M and PBP2a-Y23M, respectively.

When the ability of PBP2-W59M and PBP2a-Y23M to form a protein-protein complex was assessed via carbene footprinting, carbene labelling of PBP2a-Y23M was altered in the presence of PBP2-W59M, suggestive of protein-protein interaction. This data allowed for mapping of specific peptide residues involved in protein-protein interaction. In general, protein-protein interaction was conserved to the N-terminus of PBP2a-Y23M, whilst conformational change was seen in the C-terminus, transpeptidase domain of PBP2a-Y23M. The mapping of specific peptide residues involved in protein-protein interaction on PBP2-W59M is to be completed with a new aryl diazirine probe, with a more positive charge, in the near future and is not included in this thesis. However, as PBP2-W59M has a transpeptidase and a transglycosylase active site, it will be interesting to see which specific residues are involved in protein-protein interaction, and if there is any conformational change associated with this interaction.

Recombinant full-length PBP2 and PBP2a have been shown to successfully purify from *E. coli* overexpression cultures as a protein-protein complex. Furthermore, their truncated versions, PBP2-W59M and PBP2a-Y23M, respectively, have been shown to form a protein-protein complex via carbene labelling, and this is supported by the protein crosslinking studies described. However, this interaction has not been seen via techniques such as native mass spectrometry or AUC and thus must be a relatively weak interaction which needs further investigation. Given the evidence for a
A functional complex between PBP2 and PBP2a, this suggests that in the absence of the TM helix, the interaction of PBP2-W59M and PBP2a-Y23M is either weakened or transient, such that this interaction cannot withstand great changes in environmental conditions. For example, the protein-protein interaction may not be stable in the gas phase (Native mass spectrometry), or under high centrifugal forces (AUC). Carbene labelling is a highly sensitive technique in which labelling of proteins using a highly reactive carbene molecule occurs extremely rapidly, and hence, may be quick enough to capture the transient protein-protein interaction of the truncated PBPs.

The transmembrane helix present in the full-length PBPs whilst acting to anchor the PBPs to the cell membrane may also act to stabilise the weak protein-protein interaction of both PBPs. It could also be true that there may be additional sites of protein-protein interaction within the TM helix of each PBP which was not studied here.

### 6.4.3. Can the interaction of full-length PBP2 and PBP2a be monitored by AUC?

Whilst the study of protein-protein interactions by AUC in absence of detergents is relatively straightforward, the standard detergents used for the solubilisation of membrane proteins such as full-length PBP2 and PBP2a are generally not compatible due to their impact on the sedimentation of proteins during AUC analysis. However, certain detergents such as octyl-pentaethylene glycol ($C_8E_5$) can be used with AUC as they have a neutral buoyancy of 0.993 g/ml which is similar to that of water, 0.999 g/ml (Fleming et al, 1997). Any bound detergent will not contribute to buoyant molecular weight of the target protein due to its neutral buoyancy, and therefore data can be analysed as for soluble proteins with no detergent. Furthermore, any unbound neutrally buoyant detergent, or detergent less dense than water or buffer, is unlikely to experience sedimentation and therefore unlikely to affect protein sedimentation (Fleming 2016).
However, this is assuming that C₈E₅ is compatible with the PBP2-PBP2a protein complex and does not cause any protein degradation or aggregation. A further caveat to the use of this method, is that proteins must be assessed alone, and in complex. This is problematic as currently, whilst the two full-length PBP2 and PBP2a proteins express well separately in recombinant expression systems, they are not stable following cell lysis and exhibit rapid degradation during purification techniques. For AUC of the full-length PBP2-PBP2a interaction to be feasible, the separate purification of each PBP must be optimised to prevent rapid degradation, if at all possible. This phenomenon however does perhaps suggest that both full-length PBP2 and PBP2a act to stabilise each other from degradation and therefore allow subsequent purification following recombinant co-expression.

6.4.4. Techniques to visualise full-length PBP2-PBP2a complex formation in vivo

Whilst this chapter includes in vitro evidence for the formation of a PBP2-PBP2a complex, any in vivo evidence of this complex formation would complement this work. For example, visualisation of the protein-protein complex using fluorescently labelled PBPs. The Pinho group (ITQB-UNL) uses Photoactivation localisation microscopy (PALM) to study the localisation of proteins involved in cell division and peptidoglycan synthesis in S. aureus, and for example, have used this technique to show that PBP4-mCherry localises to the division septum (Atilano et al, 2010). PALM could therefore be used to study the PBP2-PBP2a protein complex in vitro. PBP2 and PBP2a could be tagged with separate photoactivable fluorescent dyes and live S. aureus cells could then be visualised by PALM. This would therefore give an indication as to whether these two PBPs are likely to localise to the same sites in vivo.

Other research groups, such as the Foster group (Sheffield) use super resolution and atomic force microscopy to study the structure of peptidoglycan across a variety of organisms such as S. aureus, Enterococcus faecalis and E. coli. This technique involves the use of fluorescently labelled antibiotics, such as vancomycin which binds to the terminal D-Ala of lipid-II, to visualise sites of peptidoglycan synthesis. Instead, fluorescent probes specific to either PBP2 or PBP2a could be used to visualise PBP2-
PBP2a complex formation. Moenomycin could be used for PBP2, as it binds the TG active site and would therefore be specific only to PBP2, whilst quinazolinones could be used to label PBP2a as they specifically target the allosteric site of PBP2a (Ostash and Walker 2005, Janardhanan et al, 2019).

Other in vivo techniques to measure protein-protein interactions include Forster resonance energy transfer (FRET) with multiphoton fluorescence lifetime imaging technology (FLIM). FRET-FLIM allows for not only the measurement of the distances between molecules, but also direct visualisation of the proximity of both molecules.

6.4.5. Techniques to further stabilise the full-length PBP2-2a complex

The ability to recombinantly co-express and purify full-length PBP2-PBP2a as a protein complex has been illustrated in this chapter. However, the long-term stability of this protein could be an issue, with the potential for these membrane proteins to dissociate and degrade once they are extracted from their membrane environment. Recently, Bayburt et al, (2002) have developed a technique in which membrane proteins are encapsulated into protein nanodiscs, in which membrane proteins are incubated with detergent solubilised phospholipids and membrane scaffold proteins (MSP). Once detergent is removed, nanodisk particles are formed containing the membrane protein at the centre, surrounded by phospholipids and MSPs around the outside (Bayburt et al, 2002). The advantages of nanodisc encapsulation include the mimicking of a near-native environment, the preservation of membrane protein oligomeric state, as well as long term stability (Rouck et al, 2017).

Once the PBP2-PB2a protein complex has been purified it should be encapsulated into a nanodisc for long-term storage, which may also reduce the degradation and loss of activity seen in the PBP2-PBP2a complex seen over long-term storage at minus 80°C.
6.4.6. Studying the transglycosylase and transpeptidase activity of PBP2-2a complex

The transglycosylase activity of the PBP2-PBP2a protein complex will be assessed in Chapter 7.
Chapter 7 Transglycosylation and transpeptidation activity of PBPs

7.1. Background

7.1.1. Techniques to measure transglycosylase activity of PBP2

7.1.1.1. Discontinuous Tricine-SDS-PAGE

Tricine-SDS-PAGE is a technique that can be used to assess transglycosylase (TG) activity, and is a variation of the standard discontinuous glycine-SDS-PAGE technique that allows for the separation of very low molecular weight material; the separation of molecules of 1-100 kDa, and 5-20 kDa are possible when using 10% or 16.5% acrylamide gels, respectively (Schagger and von Jagow 1987). Glycan products formed during transglycosylation reactions are composed of repeating units of lipid-II which have an overall net negative charge allowing them to be separated via gel electrophoresis with shorter chain lengths migrating further down the gel, as illustrated in Fig. 7.1 (Schagger and von Jagow 1987, Galley et al, 2014). In order to visualise the products of the TG reaction on the PAGE gel, usually a fluorescently or radioactively labelled version of lipid-II can be used; for example, lipid-II with a dansyl group at the third position lysine of the pentapeptide stem (Chapter 4, Fig. 4.1) allows for direct visualisation of the glycan strands on the PAGE gel when exposed to UV light (Barrett et al, 2007, Helassa et al, 2012).

In general, any unreacted lipid-II migrates furthest down the gel (Fig. 7.1, A), whilst polymerised lipid-II migrates slower depending on chain length (Fig. 7.1, B) and any cross-linked (transpeptidation, TP) glycan strands are unable to migrate down the gel due to their high molecular weight, and therefore their migration is vastly retarded (Fig. 7.1, C). This gel-based system provides resolution of glycan chains that differ by single lipid-II units, and reports on the length of glycan chains produced in any reaction. Since the amount of fluorescent lipid-II added to the reaction is finite and
quantifiable, analysis of the remaining lipid-II after the reaction provides a semi-quantitative analysis of substrate utilisation and product length.

**Figure 7.1. Overview of transglycosylase assays by PAGE-based assay.** Following incubation of dansylated lipid-II with PBPs, reactions are quenched with EDTA and glycan products are separated by Tris-Tricine SDS-PAGE. Gels are visualized with UV light. The mock visualized gel illustrates unreacted lipid-II (A), transglycosylation forming glycan chains (B) and transpeptidation forming high molecular weight cross-linked glycan chains (C).
Due to the position of the fluorescent dansyl group at the third position L-Lys of the pentapeptide stem of lipid-II, PBP-mediated transpeptidation is blocked and therefore these reactions report on transglycosylation only. However, if unlabelled lipid-II, with no dansyl group is mixed in the reaction along with labelled lipid-II, then a mixture of both labelled and non-labelled lipid-II will be polymerised and cross-linked, and fluorescence could be visualised. The caveat to this is that the cross-linked products of the transpeptidation reactions are of a high molecular weight, beyond the resolution of the Tricine-SDS-PAGE gel and therefore can migrate only into the top of the gel.

As discussed above, whilst Tricine-SDS-PAGE is primarily used to visualise polymerisation of lipid-II transglycosylation reactions, it can also be used to identify transpeptidation, cross-linking of glycan strands. However, whilst transglycosylation activity can be measured using non-branched lipid-II, the measurement of transpeptidation activity of Staphylococcal enzymes requires the availability of branched lipid-II, for example lipid-II (Lys) pentaglycine for S. aureus PBP transpeptidation activity. Assessing TG activity by Tricine-SDS-PAGE allows for a crude estimate of reaction rates via time course experiments, and also an estimation of fluorescent signals from polymerised and unpolymerized products. However, it is not a quantitative technique for measuring TG rate of reaction or specific activities; for this, a continuous fluorescent assay must be used.

7.1.1.2. Continuous fluorescent assays for transglycosylase activity

The first successful quantitative, continuous coupled assay to measure transglycosylase activity was reported by Schwartz et al, (2002). The assay involves delivery of dansylated lipid-II in detergent micelles to PBPs, allowing subsequent polymerisation of glycan chains, as illustrated in Fig. 7.2. Also present in this assay is N-acetylmuramidase which hydrolyses nascent glycan chains into soluble monomers leading to a measurable decrease in fluorescence signal. This assay measures the changes in fluorescence properties between fluorescent monomeric lipid-II and lipid-II polymers in a detergent micelle, and the soluble sugar-peptide liberated during the
assay as a result of muramidase cleavage. Specifically, this assay exploits the intrinsic fluorescent properties of dansylated lipid-II such that the fluorescent properties of dansylated lipid-II are reduced once the dansylated muropeptide is cleaved from the hydrophobic lipid micelle (Schwartz et al, 2002, Galley et al, 2014, Egan et al, 2015). Transglycosylase activity can therefore be continuously measured via a loss of fluorescent signal over time. This assay has been adapted into a 96-well format, allowing for high throughput screening of transglycosylase assay conditions (Offant et al, 2010).

Figure 7.2. Overview of transglycosylase assays by continuous, coupled fluorometric assay. Dansylated lipid-II in detergent micelles is delivered to PBPs which polymerise the glycan strands. The glycan chains associated with the micelle can be cleaved with a muramidase to yield a dansylated muropeptide which intrinsically has a lower fluorescence emission than dansylated lipid-II in a hydrophobic micellar environment. Therefore, transglycosylation activity of PBPs can be measured via a loss of fluorescence. Adapted from Schwartz et al (2002).

7.1.2. Techniques to measure transpeptidation activity of PBPs

Whilst there are several methods available to measure transpeptidase activity in the literature, these methods are either not continuous, use radiolabelled substrates and/or are unable to distinguish between the transpeptidation of glycan strands or the cleavage of the terminal D-Ala from the pentapeptide stem (carboxypeptidation).
The assay used by Bertsche et al, (2005) measures *E. coli* PBP1b transglycosylation and transpeptidation of \(^{14}\text{C}\)-labelled lipid-II, where after 60 min, reactions are quenched and products are analysed by HPLC. Lupoli et al, (2014) on the other hand, follow transpeptidation via the incorporation of radiolabelled D-Ala to nascent peptidoglycan, synthesised using lipid-II (Lys), acetylated at the lysyl-\(\varepsilon\)-amine. During transpeptidation, the transpeptidase domain of PBPs catalyses the attack of the carbonyl group of the terminal D-Ala-D-Ala amide bond by an active site serine (Macheboeuf et al, 2006, Lupoli et al, 2014). This displaces the C-terminal donor peptide D-alanine forming a PBP-acylated by a tetrapeptide which can be attacked by water or by an amine from an adjacent acceptor, constituting D,D-carboxypeptidase or transpeptidase activity (Macheboeuf et al, 2006, Lupoli et al, 2014). Hence, in the presence of donor only lipid-II and radiolabelled D-Ala, transpeptidation activity can be directly inferred from the rate of incorporation of radiolabelled D-Ala as an acceptor into peptidoglycan. Acetylation of lipid molecules, or the presence of L-Lys at the third position, renders them as substrates such that they can only be used for transglycosylation or as exclusive transpeptidase donors, allowing radiolabelled D-Ala to be incorporated into the glycan strand (Lupoli et al, 2014).

Within our laboratory, Dr Adrian Lloyd (Principal Research Fellow, University of Warwick) has recently developed a continuous spectrophotometric assay which is able to distinguish between both carboxypeptidase and transpeptidase activity of *E. coli* PBP1b, and furthermore, does not use labelled substrates (Manuscript in preparation). In this assay, acetylated lipid-II (Lys) is used exclusively as a donor - as *E. coli* is DAP specific, lipid-II can only be polymerised during transglycosylation. Furthermore, residual carboxypeptidation can be measured via the release of D-Ala, and as no transpeptidation activity can occur due to the use of acetylated lipid-II, D-Ala release is solely due to carboxypeptidase activity. Transpeptidation and carboxypeptidation activity can be measured via the addition of MurNAc-pentapeptide which is an exclusive acceptor due to the presence of a third position DAP (not Lys), and hence can only be used for transpeptidation due to the lack of the GlcNAc sugar residue. The rate of transpeptidation is therefore calculated as the rate
of D-Ala release as a result of transpeptidation and carboxypeptidation, minus the rate of D-Ala release due solely to carboxypeptidation.

7.1.3. Inhibitors of transglycosylase activity

Despite their key role in peptidoglycan synthesis, the transglycosylase activity of PBPs are an under exploited area. To date, the only antibiotic to target the transglycosylase activity of PBPs is moenomycin; an antibiotic of the glycolipid family naturally produced by the organism *Streptomyces ghananensis* (Ostash and Walker 2005, Galley *et al*, 2014). The C25 chain of moenomycin is essential for its antimicrobial activity, and is similar in structure to the C55 chain of lipid-II, such that moenomycin has a similar structure to that of the substrate for the transglycosylase active site - the growing glycan chain (Ostash and Walker 2005, Galley *et al*, 2014). Unfortunately, moenomycin cannot be used for human administration due to its unfavourable pharmacokinetic properties, however it has been used prophylactically in the past in animal feed for livestock growth promotion (Butaye *et al*, 2003, Ostash *et al*, 2009). Its chemical properties mean that it is effective on a wide range, but not all Gram-positive bacteria (Ostash and Walker 2010).

Other natural product inhibitors of transglycosylation include vancomycin, which inhibits transglycosylation activity by binding to the lipid-II substrate via the terminal D-Ala-D-Ala moiety of the lipid-II pentapeptide (Healy *et al*, 2000, Galley *et al*, 2014). Hence, vancomycin acts to sequester lipid-II and prevent the polymerisation of lipid-II into glycan strands. However, hydrophobic derivatives of vancomycin, such as chlorobiphenyl vancomycin, have been shown to inhibit transglycosylase activity most likely through direct binding to the transglycosylase active site of PBPs, much like moenomycin (Ge *et al*, 1999, Sinha Roy *et al*, 2001, Chen *et al*, 2003, Leimkuhler *et al*, 2005, Galley *et al*, 2014).
7.1.4. Inhibitors of transpeptidase activity

The transpeptidase activity of PBPs can be inhibited by β-lactams, however many organisms, including MRSA, are now resistant to many of the most commonly used β-lactams via the use of a number of different resistance mechanisms. Nevertheless, novel β-lactams, such as the fifth-generation cephalosporin, ceftaroline, has been shown to have broad-spectrum activity against MRSA and vancomycin-intermediate resistant S. aureus (VISA), as well as other community-acquired Gram-positive and Gram-negative organisms (Duplessis and Crum-Cianflone 2011). Ceftaroline was approved by the FDA in 2010, and is the only PBP2a targeting cephalosporin approved for use by the FDA (Duplessis and Crum-Cianflone 2011, Fernandez et al, 2014).

Similarly, ceftabiprole is another fifth-generation cephalosporin, with similar broad-spectrum activity to ceftaroline, and is approved for clinical use in the EU (Kisgen and Whitney 2008, Duplessis and Crum-Cianflone 2011, Liapikou et al, 2015).

Ceftaroline has been shown to bind to the allosteric site of PBP2a causing a conformational change in the PBP2a structure, whilst an earlier structure of PBP2a showed ceftabiprole bound to the active site serine only (Lovering et al, 2012, Otero et al, 2013, Acebron et al, 2015). The ceftaroline induced conformational change of PBP2a leads to the opening of the active site cleft from its intrinsic closed conformation, allowing a second molecule of ceftaroline to bind directly into the active site, acylating the Ser-403 active site residue (Lovering et al, 2012, Otero et al, 2013, Acebron et al, 2015). This second binding event renders PBP2a inactive, ceasing transpeptidation by PBP2a.

The crystal structure of the methicillin-sensitive PBP2, however, does not show an allosteric binding site, only a transglycosylase active site at residue Ser-398 (Lovering et al, 2007). Therefore, β-lactams must bind directly to the Ser-398 active site residue. Whilst ceftaroline and ceftobiprole had both previously been shown to have
a good affinity for PBP2 (Davies et al, 2007, Kosowska-Shick et al, 2010), increased resistance has now been documented in both passaged mutants of S. aureus and from clinical S. aureus isolates (Long et al, 2014, Chan et al, 2015, Argudin et al, 2017, Morroni et al, 2018). Resistance in both cases was shown to be due to direct amino acid substitutions in PBP2, or compensatory mutations in PBP2a (Chan et al, 2015, Argudin et al, 2017, Morroni et al, 2018).

7.1.5. Chapter aims

- To assess the transglycosylase activity of PBP2-W59M (i.e. without the N-terminal TM helix).
  - To assess the ability of PBP2a-Y23M to enhance PBP2-W59M transglycosylase activity.
  - To assess the ability of exogenous S. aureus membrane lipids, such as cardiolipin, and purified endogenous S. aureus membranes to enhance PBP2-W59M transglycosylase activity.
  - To assess the transpeptidation activity of PBP2a-Y23M, following transglycosylation of lipid-II by PBP2-W59M and PBP2a-Y23M – can this be measured by discontinuous Tricine-SDS-PAGE?

- To assess the transglycosylase activity of full-length PBP2
  - To assess the ability of full-length PBP2a to enhance full-length PBP2 transglycosylase activity.
  - To perform a detergent screen for optimal full-length PBP2 TG activity

- To assess transglycosylase activity of PBP2-W59M via native mass spectrometry
  - To assess PBP2-W59M conformation during lipid-II polymerisation
  - Does lipid-II polymerise to become lipid-IV, lipid-VII, lipid-IX etc and is this consistent with the fluorescent banding of polymerised lipid-II we see via discontinuous Tricine-SDS-PAGE?
To explore the binding of two fifth generation cephalosporins, ceftobiprole and ceftaroline, to PBP2-W59M and PBP2a-Y23M via native mass spectrometry

- Do we see binding to the active sites of PBP2-W59M, and can we detect conformational change in PBP2-W59M? Could there be an undiscovered allosteric site in PBP2-W59M?
- Do we see binding to the active and allosteric sites of PBP2a-Y23M, and can we detect conformational change in PBP2a-Y23M?
7.2. Results and Discussion

7.2.1. Are the recombinantly expressed PBP2-W59M and full-length PBP2:PBP2a complex enzymatical active for transglycosylation?

To test the transglycosylase activity of recombinantly expressed and purified PBP2-W59M and full-length PBP2:PBP2a using dansylated amidated lipid-II (Lys), a Tris-Tricine-PAGE transglycosylase assay was performed over an hour incubation, as in Section 2.12. As seen in Fig. 7.3, lane 1 shows transglycosylase activity of a C-terminally biotinylated form of PBP2-W59M (Dr Chris Thoroughgood, Roper group), an enzyme known to be enzymatically active for transglycosylation (positive control). The negative control (Fig. 7.3 Lane 3), as expected, showed no transglycosylase activity when using PBP2a-Y23M, a class B PBP with transpeptidase activity only. Interestingly, 5 µM PBP2-W59M in the presence of 5 µM PBP2a-Y23M (Fig. 7.3 Lane 2) appears to have increased transglycosylase activity than 5 µM PBP2-W59M alone (Fig. 7.3 Lane 4) – higher molecular weight bands can be seen for PBP2-W59M in the presence of PBP2a-Y23M and the unused lipid-II band also appears smaller, indicating that more lipid-II was polymerised.

Furthermore, the full-length PBP2:PBP2a complex appears to have greater transglycosylase activity (Fig. 7.3 lane 5) when compared to both PBP2-W59M alone, or PBP2-W59M in the presence of PBP2a-Y23M. The apparent retardation in migration of the unpolymerized lipid-II band is due to the presence of the detergent CHAPS in the full-length PBP2:PBP2a complex sample. These results may be suggestive that PBP2 transglycosylase activity is not only greater in the presence of PBP2a, but also when the TM helix of both PBP2 and PBP2a is present. However, the behaviour demonstrated in lane 5 may also be due to enhanced PBP2 activity caused solely by the presence of an intact TM helix.

It should be noted that due to the rapid degradation of full-length PBP2 and full-length PBP2a when purified separately, the transglycosylase activity of full-length
PBP2 could not be tested alone, or in the presence of PBP2a-Y23M. Therefore, the transglycosylase activity of the full-length PBP2:2a complex could only be compared against PBP2-W59M.

Figure 7.3. Transglycosylase activity of PBP2 and PBP2a. TG activity assessed using dansylated amidated lipid-II (Lys) at 30°C for 1 hr. Lane 1 - 5 µM Biotinylated PBP2-W59M, 2 – 5 µM PBP2-W59M plus 5 µM PBP2a-Y23M, 3 – 5 µM PBP2a-Y23M, 4 – 5 µM PBP2-W59M and 5 – 0.18 µM FL PBP2:2a complex. LII – unused dansylated amidated lipid-II (Lys).
7.2.2. Does PBP2-W59M have greater transglycosylase activity in the presence of PBP2a-Y23M?

The data in Figure 7.3 only relates to transglycosylase product accumulation at a single time point, and therefore to investigate the observation that transglycosylase activity of PBP2-W59M is enhanced in the presence of PBP2a-Y23M (as suggested in Section 7.2.1.) time course experiments were performed over 90 minutes at 30°C, comparing the transglycosylase activity of 5 μM PBP2-W59M (Fig. 7.4, Panel A) against 5 μM PBP2-W59M in the presence of 5 μM PBP2a-Y23M (Fig. 7.4, Panel B). Reactions appeared to have reached completion by approximately 32-60 minutes, and there appeared to be no observable difference in rate of transglycosylation by PBP2-W59M in absence or presence of PBP2a-Y23M at 1:1 ratio. Controls were also performed in the presence of PBP2-W59M and absence of lipid-II or PBP2a-Y23M (Fig. 7.4-A Lane 1), in the absence of PBP2-W59M and PBP2a-Y23M and the presence of lipid-II (Fig. 7.4-A Lane 2), in the presence of PBP2-W59M and lipid-II and the absence of PBP2a-Y23M (Fig. 7.4-B Lane 1), the presence of PBP2a-Y23M and lipid-II, and the absence of PBP2-W59M (Fig. 7.4-B Lane 2), in the presence of PBP2-W59M and absence of lipid-II and PBP2a-Y23M (Fig. 7.4-B Lane 3) and finally in the presence of PBP2a-Y23M and absence of PBP2-W59M and lipid-II (Fig. 7.4-B Lane 4). Controls were tested at a fixed concentration of 5 μM of PBP2-W59M and PBP2a-Y23M, as appropriate.

As the time-course experiments were performed at a 1:1 Molar ratio of PBP2-W59M to PBP2a-Y23M, PBP2a-Y23M was titrated against 5 μM PBP2-W59M (15 minutes at 30°C) to find an optimal ratio that may enhance PBP2-W59M transglycosylase activity. Reactions were performed at Molar ratios of PBP2-W59M:PBP2a-Y23M of 1:0.25 up to 1:3, however there was no apparent difference in the transglycosylase activity of PBP2-W59M at the ratios of PBP2-W59M:PBP2a-Y23M studied here (Fig. 7.5). Controls were performed in the absence of lipid-II (PBP2-W59M, or PBP2a-Y23M alone) and in the presence of lipid-II (PBP2-W59M or PBP2a-Y23M plus lipid-II).
It is interesting that whilst initial experiments of transglycosylation activity indicated that PBP2-W59M transglycosylase activity was enhanced in the presence of PBP2a-Y23M, this result was not replicated during a transglycosylation time course and titration of PBP2a-Y23M against PBP2-W59M, indicating that this initial observation may have been in error. However, this does prompt an important question – does the full-length PBP2:PBP2a complex have greater transglycosylase activity than PBP2-W59M incubated with PBP2a-Y23M because of the presence of the transmembrane helix? Furthermore, given that PBP2a-Y23M has only transpeptidase activity, and also has an allosteric domain, does the formation of this PBP2:PBP2a protein complex serve to enhance transpeptidation activity, rather than transglycosylation activity which was studied here? The study of transpeptidation for *S. aureus* PBP2 and PBP2a does not only require lipid-II, it requires amidated lipid-II (Lys) penta-glycine - a precious and limiting substrate which our laboratory can currently only make in µg quantities, as in Chapter 4.
Figure 7.4. Transglycosylase activity of PBP2 W59M in the presence or absence of PBP2a Y23M over time. TG activity assessed using dansylated amidated lipid-II (Lys) at 30°C over time. (A) 5 µM PBP2 W59M alone. Controls, 1 - No lipid-II. 2 - No PBP2. (B) 5 µM PBP2 W59M alone plus 5 µM PBP2a Y23M. Controls. 1 – PBP2 alone plus lipid-II. 2 – PBP2a alone plus lipid-II. 3 – PBP2 alone minus lipid-II. 4 – PBP2a alone minus lipid-II.
Figure 7.5. Transglycosylase activity of PBP2-W59M, in the presence of PBP2a-Y23M at varying concentrations. TG activity assessed using 10 µM dansylated amidated lipid-II (Lys) at 30°C, 15 min. Controls. 1 – 5 µM PBP2 alone plus lipid-II. 2 – 5 µM PBP2a alone plus lipid-II. 3 – 5 µM PBP2 alone minus lipid-II. 4 – 5 µM PBP2a alone minus lipid-II. Where PBP2a-Y23M was titrated against PBP2-W59M, a ratio of 1:1 was 5 µM: 5 µM.
7.2.3. Can Tricine-SDS-PAGE be used to visualise transglycosylation and transpeptidation by PBP2-W59M using a mix of non-branched and pentaglycyl branched lipid-II substrate?

As discussed in Section 7.1.2, there are continuous assays available within our laboratory to measure the continuous transpeptidation activity of PBPs, however it requires the availability of amidated lipid-II (Lys) pentaglycine, which is not readily available in large quantities as of yet in our laboratory. An alternative way to assess transpeptidation activity could be via the Tricine-SDS-PAGE system. However, due to the presence of the fluorescent dansyl group attached to the third position L-Lys of lipid-II, transpeptidation appears not to occur if this is present. Similarly, transpeptidation cannot occur if there is no pentaglycyl species of lipid-II available since this is required for S. aureus cell wall formation. One way around this could be to use both dansylated lipid-II, and non-dansylated lipid-II pentaglycine as a mixture. Assuming the lipid-II species are both used with an equal preference by PBP2-W59M, the dansylated species would be polymerised by the transglycosylase activity as normal and allow for fluorescent visualisation of products, whilst the pentaglycine species could also be transglycosylated into the fluorescent glycan chains, and subsequently allow for cross-linking to occur. Helassa et al, (2012) used this technique to assess the transpeptidation activity of E. coli PBP1b, however it is important to remember that E. coli lipid-II is not branched and therefore this strategy has not been used with pentaglycine branched lipid-II and S. aureus enzymes before.

To assess the use of the Tricine-SDS-PAGE system to measure transglycosylation and transpeptidation, 5 µM PBP2-W59M was incubated with 10 µM dansylated amidated lipid-II (Lys) alone (Fig. 7.6, Panel A Lane 1), or a 1:2, 1:1 or 2:1 ratio of dansylated amidated lipid-II (Lys) : amidated lipid-II (Lys) Gly₅ (Lanes 2, 3 and 4, respectively), for 180 min at 30°C. Controls were also performed in the absence of PBP2-W59M (lane 5), the absence of dansylated amidated lipid-II (Lys) (lane 6) or in the presence of 133 µM ampicillin (lane 7), an inhibitor of transpeptidase activity. These reactions were performed by John Deering (iCASE PhD student, Roper group) on my behalf, however
all reagents, unless stated elsewhere, were synthesised and assays were designed by myself.

The dark, high molecular weight bands shown in Fig. 7.6-A, lanes 2-3 are suggestive that PBP2-W59M was able to perform transglycosylation and transpeptidation at all three ratios of dansylated:pentaglycyl lipid-II used, with the most prominent high molecular weight banding of potential peptidoglycan occurring at the 1:1 ratio. However, there is also a high molecular weight band produced in the absence of pentaglycyl lipid-II (Fig. 7.6-A, lane 1) which migrates slightly lower than the bands shown in lanes 2-3. Therefore, these potential transpeptidation products are not easily distinguishable from transglycosylation products, which if transglycosylation occurs quickly, may result in long, high molecular weight glycan strands which could easily be confused for transpeptidation products.

The ampicillin control (Fig. 7.6-A Lane 7) appeared successful in preventing very high molecular weight products – however we do not know if modifications to the transpeptidase active site by ampicillin results in a loss of transglycosylase activity, and indeed, this loss of high molecular weight banding seen could be due to a loss of very high molecular weight glycan strands.

To confirm that the dark, high molecular weight bands seen in Fig. 7.6, Panel A, lanes 2-4 were indeed peptidoglycan, a degradation experiment of the transpeptidation products was performed (Fig. 7.6, Panel B). A 3x volume reaction was set up to contain a 1:1 ratio of dansylated and pentaglycyl amidated lipid-II (Lys) at 10 μM each. The reaction was incubated with 5 μM PBP2-W59M for 180 min, at 30°C, before heat denaturation at 95°C. The reaction was then split into three and each treated with either water (control), 2.3 μM lysozyme or 6.7 μM lysostaphin. The control reaction was left on ice, whilst the two enzyme treated reactions were incubated for a further 30 min at 30°C, before all three were visualised by Tricine-SDS-PAGE.
As shown in Fig. 7.6, Panel B, the control reaction (lane 1) shows both the transglycosylase and potential transpeptidase reaction products - glycan chains and peptidoglycan, respectively. The treatment of these reaction products with lysozyme (lane 2) lead to the loss of both transpeptidation and transglycosylation products, as lysozyme specifically cleaves the GlcNAc-MurNAc bond, degrading any glycan chains and thus breaking down peptidoglycan. Furthermore, the unpolymerized band of lipid-II at the bottom of the gel appears darker and thicker in lane 2 than in control lane 1, suggesting that lysozyme treatment has cleaved glycan chains into monomeric glycan-peptide units. By contrast, in the presence of lysostaphin (lane 3), only the high molecular weight transpeptidation products were degraded, leaving only the shorter non-cross-linked glycan polymers. Lysostaphin specifically cleaves the pentaglycyl cross-bridge, thus glycan chains are undisrupted and can still be seen on the PAGE gel. This is highly suggestive that the high molecular weight product seen at the top of lane 1 is indeed peptidoglycan formed following transglycosylation and transpeptidation of dansylated and pentaglycyl species of lipid-II by PBP2-W59M.
Figure 7.6. Transglycosylase and transpeptidase activity of PBP2-W59M in the presence of amidated lipid-II (Lys) Gly5. Assays performed using 5 µM PBP2-W59M, for 180 min at 30°C.

A. Assessing PBP2-W59M TG and TP activity. PBP2-W59M activity assessed using 10 µM dansylated amidated lipid-II (Lys) alone (Lane 1), or a 1:2, 1:1 or 2:1 ratio of dansylated amidated lipid-II (Lys) : amidated lipid-II (Lys) Gly5 (Lanes 2, 3 and 4, respectively). Minus PBP2-W59M (Lane 5) and minus dansylated amidated lipid-II (Lys) (Lane 6) controls were performed. PBP2-W59M activity was assessed in the presence of 143 µM ampicillin, using both dansylated amidated lipid-II (Lys) : amidated lipid-II (Lys) Gly5 (Lane 7).

B. Degradation of PBP2-W59M products. All reactions contained 10 µM dansylated amidated lipid-II (Lys) and 10 µM amidated lipid-II (Lys) Gly5 alone (lane 1), or in the presence of 2.3 µM lysozyme (lane 2) or 6.7 µM lysostaphin (lane 3).
7.2.4. Can Tricine-SDS-PAGE be used to visualise transglycosylation and transpeptidation by PBP2-W59M using a mix of non-branched and mono- or triglycyl branched lipid-II substrate?

It is likely that the Tricine-SDS-PAGE method for visualisation of transglycosylase products is also suitable for the visualisation of the high molecular weight transpeptidase product, peptidoglycan. As is well known, and has been discussed as part of this thesis, *S. aureus* peptidoglycan is made up of pentaglycyl cross-linked peptidoglycan. Nonetheless, *S. aureus* can tolerate the incorporation of mono- or triglycyl crosslinks into the peptidoglycan following *femA* or *femB* knockout – albeit with significant deficiencies in cellular fitness and increase antibiotic sensitivities (Maidhof *et al*, 1991, Henze *et al*, 1993). However, *femX* knockout is lethal due to the inability to cross-link unbranched peptidoglycan precursors (Rohrer *et al*, 1999, Tschierske *et al*, 1999). This is discussed in more detail in Chapter 5.

To assess whether Tricine-SDS-PAGE is a suitable method to visualise the incorporation of mono- and triglycyl lipid-II species into peptidoglycan, the same method was employed as in Section 7.2.4. The effect of amidation on this incorporation was also studied. PBP2-W59M (5 µM) was incubated with either 10 µM dansylated amidated lipid-II (Lys) alone (Fig. 7.7, lane 1), dansylated non-amidated lipid-II (Lys) alone (Fig. 7.7, lane 5), a 1:1 ratio of dansylated amidated : amidated lipid-II (Lys) Gly₁ or Gly₃ (Fig. 7.7, lane 2 and 3, respectively) or a 1:1 ratio of dansylated non-amidated : non-amidated lipid-II (Lys) Gly₁ or Gly₃ (Fig. 7.7, lane 6 and 7, respectively). Controls were performed in the presence of 667 µM moenomycin, for the non-branched lipid-II reactions. All reactions were incubated for 180 min at 30°C.

As expected, non-branched amidated lipid-II (Lys) was successfully polymerised by PBP2-W59M, but was unable to be cross-linked into peptidoglycan by PBP2-W59M due to the absence of a glycyl branch (Fig. 7.7, lane 1). However, the mono- and triglycyl lipid-II (Lys) species were both polymerised and faint bands were present at
the top of the gel which could correspond to the formation of high molecular weight cross-linked species (Fig. 7.7, lane 2 and 3, respectively). In hindsight, a control lane containing pentaglycyl lipid-II and hence cross-linked peptidoglycan would have been advantageous in determining the ability of PBP2-W59M to cross-link the mono- and tri- glycyll lipid-II species.

Interestingly, whilst each species of non-amidated lipid-II (Lys) was polymerised by PBP2-W59M, none of these species were shown to be cross-linked by PBP2-W59M (Fig. 7.7, lane 1-3). Whilst this was expected for the non-branched species, this could in part be due to the shorter glycan chain lengths, and hence perhaps PBP2-W59M does not recognise these shorter chain lengths as a substrate for transpeptidation? As has been shown by Dr Ricky Cain (Roper group), PBP2-W59M has a strong preference during transglycosylation for amidated lipid-II (Lys) over non-amidated lipid-II (Lys) (Manuscript in preparation).
Figure 7.7. Transglycosylase activity of PBP2-W59M using mono- and tri- glyeryl lipid-II.

Transglycosylase activity of 5 μM PBP2-W59M assessed using 5 μM dansylated amidated (1) or non-amidated (5) lipid-II (Lys) at 30°C for 180 min, in the presence of 5 μM amidated lipid-II (Lys) Gly₁ (2) and Gly₃ (3), or 5 μM non-amidated lipid-II (Lys) Gly₁ (6) and Gly₃ (7). Controls were performed in the presence of 667 μM moenomycin, with amidated lipid-II (Lys) (4) or non-amidated lipid-II (Lys) (8).
7.2.4. Can transglycosylase activity of PBP2-W59M be followed by native mass spectrometry?

Whilst transglycosylase activity and the apparent polymerisation of lipid-II can be visualised via Tris-Tricine-PAGE due to the natural fluorescent properties of the dansyl-group attached to lipid-II, it has not been independently shown that each dark band migrating through the gel corresponds to differentially polymerised lipid-II (i.e. lipid-II, lipid-IV, lipid-VI, lipid-VIII etc.). Furthermore, Tris-Tricine-PAGE analysis of transglycosylase activity requires the reactions to be quenched before samples are loaded onto a gel, hence transglycosylase activity is measured at fixed, stopped time points. However, if transglycosylase reactions were analysed by native mass spectrometry, activity could be measured without the need to quench the reaction. Thus, the interaction of lipid-II, and the growing polymerised chains of lipid-II, with PBP2 could be investigated, and potentially could elucidate how substrate interactions with the PBP active and/or allosteric site occurs.

Native mass spectrometry uses electrospray ionisation whereby a high voltage is applied to a liquid sample in a non-denaturing solvent, creating desolvated ions of the protein sample within the gas-phase vacuum (Leney and Heck 2017). Whilst ‘native’ in this context does not mean that the protein is in its natural cellular environment, the term ‘native’ describes the set of conditions that maintains the protein in a state approximately to that it possessed prior to ionisation process – in this case, protein samples are kept in solution in their non-denatured conformation in optimal buffer conditions to retain the native folded state of the protein (Leney and Heck 2017). This is contrary to denaturing mass spectrometry, where proteins are denatured before ionisation.

The standard protocol for native mass spectrometry (Performed by Dr Matthew Jenner, Department of Chemistry, University of Warwick) was to provide protein samples in 150 mM ammonium acetate as the non-denaturing solvent, where samples were ionised in the mass spectrometer and data collected over a 30-minute period. Therefore, PBP2-W59M had to be stable in the gas phase in ammonium
acetate and be enzymatically active in ammonium acetate. In addition, the transglycosylation reaction had to span the 30-minute sampling time – if the reaction occurred too fast then there will be too many different species of polymerised lipid-II making the data analysis complicated and limiting the chance of gathering meaningful data. If the reaction occurred too slowly then there may not be sufficient lipid-II chains of sufficient length to access both the allosteric and active sites of PBP2-W59M. Furthermore, the lipid-II needed to be delivered in a suitable non-denaturing solvent. Currently lipid-II is resuspended in chloroform:methanol:water (2:3:1) or buffer with detergent, both of which are problematic for native mass spectrometry – the former may cause protein denaturation whilst the latter contains detergent which causes severe interference or noise on the mass spectrometer.

7.2.4.1. Is PBP2-W59M still enzymatically active as a transglycosylase in the presence of ammonium acetate?

The transglycosylase activity of PBP2-W59M using 10 µM dansylated amidated lipid-II (Lys) was assessed in both standard transglycosylase reaction buffer and 150 mM ammonium acetate, as would be used for native mass spectrometry. As shown in Fig. 7.8-A, the use of 150mM-300mM ammonium acetate as the reaction buffer (Fig. 7.8-A lane 2-3) supported the transglycosylase activity of PBP2-W59M when compared to the transglycosylase activity of PBP2-W59M in standard reaction buffer (Fig. 7.8-A lane 1), thus suggesting that ammonium acetate is a suitable buffer to use for assessing the transglycosylase activity of PBP2-W59M by native mass spectrometry.

7.2.4.2. Can transglycosylase activity of PBP2-W59M be slowed for real-time native mass spectrometry analysis?

As shown in Fig. 7.4, the transglycosylase reaction using 5 µM PBP2-W59M and 10 µM dansylated amidated lipid-II (Lys) at 30°C reaches completion within 32-60 minutes. To slow this reaction down such that lipid-II polymerisation could be analysed by native mass spectrometry, reactions were also performed with 10 µM
dansylated amidated lipid-II (Lys) at room temperature in 150 mM ammonium acetate (Fig. 7.8-B lane 2) and performed with 10 µM dansylated non-amidated lipid-II (Lys) at room temperature in 150 mM ammonium acetate (Fig. 7.8-B lane 3). Non-amidated lipid-II (Lys) was used as it is known to be turned over by PBP2-W59M at a much slower rate than amidated lipid-II (Lys), yet is not its natural substrate (Dr Ricky Cain, University of Warwick, Roper Group, unpublished). When compared to the transglycosylase reaction using 5 µM PBP2-W59M and 10 µM dansylated amidated lipid-II (Lys) at 30°C in standard reaction buffer (Fig. 7.8-B lane 1), performing the reactions at room temperature and in ammonium acetate buffer did not significantly reduce the rate of PBP2-W59M transglycosylase activity (Fig. 7.8-B lane 2). However, using 10 µM non-amidated lipid-II (Lys) significantly reduced the rate of PBP2-W59M transglycosylase activity (Fig. 7.8-B lane 3) such that these conditions could be used for real-time native mass spectrometry.
Figure 7.8-A. Transglycosylase activity of PBP2-W59M – testing ammonium acetate as a reaction buffer. Transglycosylase activity assessed using 10 µM dansylated amidated lipid-II (Lys) and 5 µM PBP2-W59M at 30°C for 120 min in standard reaction buffer (1), at 30°C for 30 min in 150 mM ammonium acetate (2) or at 30°C for 120 min in 300 mM ammonium acetate (3).

Figure 7.8-B. Transglycosylase activity of PBP2-W59M – testing ammonium acetate as a reaction buffer and varying lipid-II substrate. Transglycosylase activity assessed using 10 µM dansylated amidated lipid-II (Lys) at 30°C, 30 min (Reaction 1, in standard reaction buffer), 10 µM dansylated amidated lipid-II (Lys) at RT, 30 min (Reaction 2, in 150 mM ammonium acetate) and 10 µM dansylated non-amidated lipid-II (Lys) at RT, 30 min (Reaction 3, in 150 mM ammonium acetate).

Figure 7.8-C. Transglycosylase activity of PBP2-W59M – screen for lipid-II delivery vehicle. Transglycosylase activity assessed using 10 µM dansylated non-amidated lipid-II (Lys) and 5 µM PBP2-W59M, at room temperature for 30 minutes. Lipid-II was delivered into the assay in either 10 mM Tris, 0.28 mM LDAO, pH 8 (lane 1), 150 mM ammonium acetate (lane 2), 10% DMSO (lane 3), 10% methanol:90% 25 mM ammonium acetate (lane 4) or dried lipid-II (lane 5)
7.2.4.3. Can lipid-II be delivered to the PBP2-W59M transglycosylase assay in the absence of detergent?

As in Section 7.2.4.1-2, suitable buffer and lipid-II species conditions to measure transglycosylation by real time native mass spectrometry had been established. However, the lipid-II could not be delivered into the system for native mass spectrometry in the currently used standard solvents – lipid-II is generally stored in chloroform:methanol:water (2:3:1) or 10 mM Tris, 0.28 mM LDAO, pH 8 when used for gel-based transglycosylase assays, as previously discussed. Therefore, to test whether lipid-II can successfully be delivered into the transglycosylase assay and be accessible to PBP2-W59M for polymerisation, transglycosylase assays were set up using 10 µM dansylated non-amidated lipid-II (Lys) resuspended in either 10 mM Tris, 0.28 mM LDAO, pH 8 (Fig. 7.8-C lane 1), 150 mM ammonium acetate (Fig. 7.8-C lane 2), 10% (v/v) DMSO (Fig. 7.8-C lane 3), 10% methanol:90% (v/v) 25 mM ammonium acetate (Fig. 7.8-C lane 4) or dried into the reaction vessel (Fig. 7.8-C lane 5). Transglycosylase activity was measured at room temperature for 30 minutes using 5 µM PBP2-W59M.

Unexpectedly, PBP2-W59M was able to polymerise lipid-II when delivered to the assay system in all conditions. In fact, the transglycosylase activity appeared to be increased when lipid-II was given to the system in either 150 mM ammonium acetate (Fig. 7.8-C lane 2), 10% DMSO (Fig. 7.8-C lane 3) or 10% methanol:90% 25 mM ammonium acetate (Fig. 7.8-C lane 4) when compared to the standard delivery buffer 10 mM Tris, 0.28 mM LDAO, pH 8 (Fig. 7.8-C lane 1). PBP2-W59M was also able to polymerise lipid-II when lipid-II was dried into the reaction vessel before the addition of the remaining assay components – however, there did appear to be less lipid-II visualised on the gel (Fig. 7.8-C lane 5) in comparison to all other four conditions (Fig. 7.8-C lane 1-4), as seen by the lighter appearance of the lipid-II bands. This suggests that not all the lipid-II that is dried into the reaction vessel is successfully resuspended into the final reaction volume following addition of the remaining assay components (Fig. 7.8-C lane 5). Whilst this is not a major concern if the purpose of
an assay is to confirm or deny activity, it would be a problem if a reaction depended upon the final concentration of lipid-II in the system, and should be taken into consideration.

7.2.4.4. Transglycosylase activity of PBP2-W59M measured by native mass spectrometry

All native mass spectrometry was performed by Dr Matthew Jenner, Department of Chemistry, University of Warwick, using the native mass spectrometer at the School of Chemistry, University of Nottingham.

Transglycosylase reactions were initially set up as per the conditions set down in Sections 7.2.4. However, when PBP-W59M (5 µM) and non-amidated lipid-II (Lys) (10 µM) were combined in 150 mM ammonium acetate the reaction mixture was too thick to spray through the mass spectrometer needle. Therefore, the concentration of non-amidated lipid-II (Lys) was halved to 5 µM. PBP2-W59M and lipid-II were combined in 150 mM ammonium acetate and were immediately loaded into the mass spectrometer, at room temperature. Reactions were sprayed and data collected over 30 minutes.

The native mass spectrometry traces for PBP2-W59M alone (Fig. 7.9, A) and PBP2-W59M plus non-amidated lipid-II (Lys) (Fig. 7.9, B) were compared; PBP2-W59M was detected at different charge states alone (indicated by blue circles) and with a bound molecule (indicated by red circles). Ions with an m/z in the eighteenth charge state were isolated by wide quadrupole isolation (Fig. 7.9, Panel A). The detected ions had m/z values consistent with the expected values for PBP2-W59M alone (Observed eighteenth charge state m/z 4064.64), PBP2-W59M with one copy of GlcNAc-MurNAc-pentapeptide bound (Observed eighteenth charge state m/z 4109.90) and either two copies of GlcNAc-MurNAc-pentapeptide bound or GlcNAc-MurNAc-pentapeptide-GlcNAc-MurNAc-pentapeptide bound (Observed eighteenth charge state m/z 4160.33). GlcNAc-MurNAc-pentapeptide is formed following the
concomitant release of the prenyl chain from lipid-II following transglycosylation, however, the loss of the prenyl chain from lipid-II could also be an artefact of mass spectrometry analysis as the prenyl chain-MurNAc bond is labile.

The ions in the eighteenth charge state isolated by wide quadrupole isolation were treated with a high collision energy, such that an ion consistent with the expected m/z of GlcNAc-MurNAc-pentapeptide (Observed eighteenth charge state m/z 949.50) was ejected. This ejection event also created an ion peak (denoted X) consistent with the expected m/z as a result of loss of mass associated with the loss of a GlcNAc-MurNAc-pentapeptide molecule from PBP2-W59M.

Tight quadrupole isolation of ions in the eighteenth charge state were also isolated (Fig. 7.9, Panel B). The detected ions had m/z values consistent with the expected values for PBP2-W59M alone (m/z 4058.91) and PBP2-W59M with one copy of GlcNAc-MurNAc-pentapeptide bound (m/z peak masked by PBP2-W59M peak). Again, ions were treated with a high collision energy, such that an ion consistent with the expected m/z of GlcNAc-MurNAc-pentapeptide (m/z 949.50) was ejected, creating the ion peak denoted X, as previously seen. The detected m/z values for each detected ion peak are more accurate in tight quad isolation due to less interference of the distribution of mass peak centroids by other ion peaks, hence the detected m/z values differ slightly from wide and tight quadrupole isolation.

This data is highly suggestive that we have successfully shown the binding of lipid-II to PBP2-W59M, and potentially the transglycosylation product formed from two units of lipid-II by PBP2-W59M by native mass spectrometry. The loss of the undecaprenyl chain is also consistent with the concomitant release of the prenyl chain from lipid-II during transglycosylation, and is suggestive that this may occur upon PBP2-W59M binding.
Figure 7.9. PBP2-W59M transglycosylase activity visualised by native mass spectrometry. (A) 50 µM PBP2–W59M alone in 150 mM ammonium acetate. (B) 50 µM PBP2–W59M with 50 µM non-amidated lipid-II (Lys) in 150 mM ammonium acetate. Blue circle – Different charges states of PBP2-W59M alone. Red circle – Different charges states of PBP2-W59M with bound molecule. Exact mass – PBP2-W59M (72,834), non-amidated lipid-II (Lys) (1875.06), GlcNAc-MurNAc-5P (950.44), GlcNAc-MurNAc-5P-GlcNAc-MurNAc-5P (1898.97). Charge states in red text. NB. Values for the detected mass of an ion may vary due to reduced resolving power when Gaussian peaks from multiple ions are closely-spaced or overlap.
Figure 7.10. PBP2-W59M TG activity visualised by native MS/MS – Quadrupole isolation. 50 µM PBP2-W59M with 50 µM non-amidated lipid-II (Lys) in 150 mM ammonium acetate. Quadrupole isolation of 18⁺ charge state ion and collision induced dissociation (CID) at 25-30 V energy. Blue circle - PBP2-W59M alone. Red circle - PBP2-W59M plus bound molecule. Exact mass – PBP2-W59M (72,834), non-amidated lipid-II (Lys) (1875.06), GlcNAc-MurNAc-5P (950.44), GlcNAc-MurNAc-5P-GlcNAc-MurNAc-5P (1898.97). Charge states in red text. NB. Values for the detected mass of an ion may vary due to reduced resolving power when Gaussian peaks from multiple ions are closely-spaced or overlap.
7.2.5. Optimisation of PBP2-W59M transglycosylase activity

7.2.5.1. Does the presence of *S. aureus* membranes affect the transglycosylase activity of PBP2-W59M?

Recombinant PBP2-W59M is truncated such that it does not have its TM domain, and is therefore not membrane bound anchored (by virtue of the single N-terminal TM helix). However, the effects of removing this TM domain and the lack of association to the membrane have not been fully studied, in the most part due to the difficulty in the expression and purification of recombinant membrane proteins. To date, full-length PBP2 from *S. aureus* has not been shown to have transglycosylase activity and so any comparison of full-length vs truncated PBP2 transglycosylase activity has not been explored in this study, or to my knowledge, within the published literature. Therefore, the transglycosylase activity of PBP2-W59M was assessed in this study in the presence of membranes extracted from *S. aureus* ATCC 25923 to investigate whether the presence of membrane lipids within the transglycosylase reaction may allow for a more physiological environment for transglycosylase activity to occur in.

Transglycosylase activity of PBP2-W59M was assessed using dansylated amidated lipid-II (Lys) over 90 min at 30°C in presence of a final concentration of 1 mg/ml or 2 mg/ml *S. aureus* ATCC 25923 membranes (Fig. 7.11-A Lane 1 and 2, respectively) or in the absence of membranes (Fig. 7.11-A Lane 3). Controls were also performed in the presence of 1 mg/ml membrane in the absence of lipid-II or PBP (Fig. 7.11-A Lane 4 and 5, respectively) and in the absence of 1 mg/ml membrane in the absence of lipid-II or PBP (Fig. 7.11-A Lane 6 and 7, respectively).

Both 1-2 mg/ml *S. aureus* membranes were shown to inhibit polymerisation of lipid-II by PBP2-W59M when compared to polymerisation of lipid-II in the absence of membranes (Fig. 7.11-A Lanes 1-3) – this could be due to a true inhibition of PBP2-W59M, or could be due to degradation of nascent polymerised lipid-II, by components of the membranes themselves (e.g. hydrolytic proteins such as MltG).
(Yunck et al, 2016)) or by a component of the membrane resuspension buffer. Controls showed that S. aureus membranes were insufficient to catalyse transglycosylation in the absence of PBP (Fig. 7.11-A Lane 5) indicating that extracted membranes did not contain active membrane associated PBPs. Alternatively, if the S. aureus membranes contained sufficient amounts of free amidated lipid-II (Lys) Gly5 this could compete against the provided dansylated substrate, effectively quenching the fluorescent signal.

7.2.5.2. Is nascent polymerised lipid-II degraded by S. aureus membranes?

The potential inhibition of transglycosylase activity of PBP2-W59M (5 µM) or degradation of nascent polymerised lipid-II was assessed using dansylated amidated lipid-II (Lys) over 90 min at 30°C in the absence of S. aureus ATCC 25923 membranes (Fig. 7.11-B Lane 1), or in the presence of a final concentration of 1 mg/ml S. aureus ATCC 25923 membranes (Fig. 7.11-B Lane 2). After 90 min, samples were heat denatured at 95°C for 15 min, before a final concentration of 1 mg/ml membrane was added to a third fully completed transglycosylase reaction sample that had no previous incubation with membranes (Fig. 7.11 Lane 3). Samples were incubated for a further 30 min at 30°C before visualisation by Tricine-SDS-PAGE.

Fig. 7.11-B indicates that S. aureus membranes are indeed inhibiting polymerisation of lipid-II by PBP2-W59M, and not degrading nascently polymerised lipid-II. If the latter were the case, Fig. 7.11-B Lane 3 would resemble that of Lane 2. It is worth noting however, that the presence of extracted S. aureus membranes in transglycosylase assays does affect the resolution of the Tricine-SDS-PAGE, causing a ‘jump’ or an almost wave-like effect in the polymerised lipid-II bands which is often seen when samples contain detergents. However, the membrane resuspension buffer contained only 20 mM Tris, 1 mM MgCl2, 2 mM β-mercaptoethanol, pH 7.5, and no detergents, therefore this phenomenon may be due to an excess of membrane proteins within the sample.
Figure 7.11. (A) TG activity of PBP2-W59M in the presence of \textit{S. aureus} ATCC 25923 membranes.
Reactions were performed with 5 µM PBP2-W59M and 10 µM dansylated amidated lipid-II (Lys) at 30°C for 120 min. \textit{S. aureus} ATCC 25923 membranes were added to the reaction to a final concentration 1 mg/ml and 2 mg/ml (Lanes 1 and 2, respectively) or were not included (Lane 3). Controls were performed containing 1 mg/ml membrane in the absence of lipid-II (lane 4) or PBP (lane 5) and without membrane in the absence of lipid-II (lane 6) or PBP (lane 7). (B) TG activity of PBP2-W59M in the presence of \textit{S. aureus} ATCC 25923 membranes – degradation trial.
Reactions were performed with 5 µM PBP2-W59M and 10 µM dansylated amidated lipid-II (Lys) at 30°C for 90 min. \textit{S. aureus} ATCC 25923 membranes were either not added to the reaction (Lane 1), or added to the reaction to a final concentration 1 mg/ml before incubation (Lane 2) or after incubation (Lane 3). Following the 90 min TG reaction, samples were heated at 95°C for 10 min, before the addition of a final concentration of 1 mg/ml membranes to the ‘minus membrane’ sample (Lane 3) and incubated at 30°C for 15 min.
7.2.5.3. Does the presence of *S. aureus* membrane component Cardiolipin affect the transglycosylase activity of PBP2-W59M?

Cardiolipin, phosphatidylylglycerol, lysyl-phosphatidylylglycerol (LPG) account for 95-98% of the *S. aureus* cell membrane, with each component accounting for an estimated 18-23%, 43% and 30-35% of it, respectively (White and Freman 1967, Hayami *et al*, 1979, Sohlenkamp and Geiger 2016). Whilst it was shown in Section 7.2.5.1, that crude membranes extracted from *S. aureus* did not enhance the transglycosylase activity of PBP2-W59M, this may have been due factors such as the components of the membrane buffer or proteins associated to the extracted membranes. Therefore, the effect of cardiolipin on PBP2-W59M transglycosylase activity was assessed here by Tricine-SDS-PAGE.

PBP2-W59M (50 µM) was incubated with 10 µM dansylated amidated lipid-II (Lys) at 30°C for 60 min. Reactions were run in the absence of cardiolipin or detergent (Fig. 7.12 Lane 1), 0 µM, 1 µM, 10 µM and 100 µM cardiolipin resuspended in 2x CMC of LDAO (Fig. 7.12 Lane 2-5, respectively), 2x CMC Triton X-100 (Fig. 7.12 Lanes 6-9, respectively) or 2x CMC E6C12 (Fig. 7.12 Lanes 10-13, respectively). Overall, there was no significant increase in PBP2-W59M transglycosylase activity in the presence of up to 100 µM cardiolipin, in any of the three detergents. However, as this assay was quenched after 60 min, any impact on initial rate of reaction would have been missed. Interestingly cardiolipin resuspended in 2x CMC of E6C12 appeared to have a detrimental to PBP2-W59M transglycosylase activity and should be avoided for future work.
Figure 7.12. PBP2-W59M transglycosylase activity in the presence of cardiolipin. PBP2-W59M (50 µM) incubated with dansylated amidated lipid-II (Lys) (10 µM) at 30°C for 60 min. Reactions were run in the absence of cardiolipin or detergent (Lane 1), 0 µM, 1 µM, 10 µM and 100 µM cardiolipin resuspended in 2x CMC of LDAO (Lane 2-5, respectively) 2x CMC Triton X-100 (Lanes 6-9, respectively) and 2x CMC E6C12 (Lanes 10-13, respectively).
7.2.6. Assessing the interactions of fifth generation cephalosporins with PBP2-W59M and PBP2a-Y23M by intact and native mass spectrometry

All intact and native mass spectrometry was performed by Dr Matthew Jenner, Department of Chemistry, University of Warwick, using the native mass spectrometer at the School of Chemistry, University of Nottingham. The interactions of PBP2-W59M, and PBP2a-Y23M with both ceftaroline and ceftobiprole were assessed by intact mass spectrometry. As discussed in Section 7.1.4., the crystal structure of PBP2a-Y23M is illustrative of the presence of both an allosteric site that binds ceftaroline (the structure of which is a peptidoglycan peptide stem mimetic), and a transpeptidase active site. However, whilst the crystal structure of PBP2-W59M illustrates an active site for both the transglycosylase and transpeptidase sites, it does not provide evidence so far for an allosteric site for either catalytic domains of PBP2 or the linker in between (Lovering et al, 2007). This is intriguing as both PBP2-W59M and PBP2a-Y23M both have transpeptidase activity, it would be entirely plausible if they both worked via the same mechanism – allosteric activation followed by conformational change and ligand binding to the active site. That said, the activation of the PBP2-W59M transpeptidase site may occur allosterically via an interaction of the substrate bound at the transglycosylase active site. This native mass spectrometry work will also further confirm that allosteric binding occurs before active site binding in PBP2a-Y23M.

Controls were performed with 50 µM PBP2-W59M alone (Fig. 7.13, CTL), or PBP2a-Y23M alone (Fig. 7.14, CTL) and the predicted masses of 72.8 kDa and 73.5 kDa, respectively, were observed. PBP2-W59M, and PBP2a-Y23M were both incubated with 500 µM of ceftaroline or ceftobiprole for 20 min at room temperature and then analysed. The measured masses for both PBP2-W59M and PBP2a-Y23M shifted by 605 Da and 534 Da in the presence of ceftaroline and ceftobiprole, respectively, confirming that both ceftaroline and ceftobiprole bound to both PBP2-W59M and PBP2a-Y23M, presumably by forming an acyl-enzyme intermediate with the transpeptidase active site serine.
Figure 7.13. Intact MS of PBP2-W59M with either ceftobiprole or ceftaroline. 50 μM PBP2-W59M alone (CTL), with 500 μM ceftaroline (RXN 1) or 500 μM ceftobiprole (RXN 2). Incubated at RT, 15 min before MS analysis. Samples flown in 0.1% formic acid in acetonitrile. CTL – control, PBP only. RXN – reaction. Predicted expected mass 72,824 Da. See Appendix Fig. 9.27 for raw mass spectra.
Figure 7.14. Intact MS of PBP2α-Y23M with either ceftobiprole or ceftaroline. 50 μM PBP2α-Y23M alone (CTL), with 500 μM ceftaroline (RXN 1) or 500 μM ceftabiprole in DMSO (RXN 2). incubated at RT, 15 min before MS analysis. Samples flown in 0.1% formic acid in acetonitrile. CTL – control, PBP only. RXN – reaction. Predicted expected mass 73,539 Da. See Appendix Fig. 9.27 for raw mass spectra.
The interaction of 50 µM PBP2a-Y23M in 150 mM ammonium acetate with 100 µM ceftaroline or ceftobiprole in DMSO was then investigated by native mass spectrometry. Fig. 7.15 Panel A and Fig. 7.16 Panel A shows the charge states associated with PBP2a-Y23M alone. When incubated with either ceftaroline, or ceftobiprole, the expected m/z values consistent with PBP2a-Y23M occupied at either one or two different sites by ceftaroline (Fig. 7.15, Panel B) or ceftobiprole (Fig. 7.15 Panel B) providing direct evidence for both drugs binding to an allosteric site, as well as an active site serine. Also shown is a shift in charge states, which can be associated with a conformational change in the protein structure. However, this shift in charge state was later shown to occur in the presence of DMSO, which is used as a vehicle for ceftaroline and ceftobiprole (data not shown), and therefore this shift in charge state cannot be fully attributed to a conformational change of PBP2a-Y23M as a result of ceftaroline or ceftobiprole binding. This would need to be repeated with a different antibiotic vehicle that does not cause a shift in charge state as any change in charge state as a result of true conformational change as a result of drug binding may well be masked by the effects of DMSO.

To confirm that one binding site is the transpeptidase active site, and the other is the transpeptidase allosteric site, the ion peak associated with the expected m/z value for PBP with two bound molecules was isolated using a quadrupole ion trap. This ion peak was treated with a high collision energy, such that any molecules that are not covalently bound, such as those at the allosteric site, will be propelled out of the allosteric site, whilst those that are covalently bound at the active site would be unaffected.

As in Fig. 7.15 Panel C, and Fig. 7.16 Panel C, the ion associated with PBP2a-Y23M bound to two molecules of ceftaroline (m/z 4985.36) and the ion associated with PBP2a-Y23M bound to two molecules of ceftobiprole (m/z 4940.60) were isolated using the quadrupole ion trap and hit with high energy. This led to the expulsion of one molecule of ceftaroline (m/z 605.12), or ceftobiprole (m/z 535.19) from the allosteric site leaving PBP2a-Y23M with one molecule of either ceftaroline (m/z 5298.26) or ceftobiprole (m/z 4975.75) bound in the transpeptidase active site.
The interaction of PBP2-W59M with ceftaroline or ceftobiprole was also investigated by native mass spectrometry. Fig. 7.17 Panel A shows the m/z charge states associated with PBP2-W59M alone. Interestingly, when incubated with either ceftaroline, or ceftobiprole, PBP2-W59M is occupied at either one or two different sites by ceftaroline (Fig. 7.17, Panel B) or ceftobiprole (Fig. 7.17 Panel C). Given that the crystal structure of PBP2-W59M was not shown to contain a transpeptidase allosteric site this data is highly suggestive that PBP2-W59M has a second binding site available for ceftaroline and ceftobiprole. Unfortunately, quadrupole isolation of PBP2-W59M bound with two molecules of antibiotic was not performed and therefore it has not yet been proven as to whether PBP2-W59M does also contain an allosteric binding site. However, nevertheless this data is highly suggestive of the presence of an allosteric site in PBP2-W59M.
Figure 7.15. Native MS analysis of PBP2a-Y23M with ceftaroline. PBP2a-Y23M (50 µM) analysed alone (A.) or with 100 µM ceftaroline in DMSO (B). Peak with m/z of 4985.36 isolated with the Quadrupole Orbitrap and treated with 25-30 V collision energy (C). Samples flown in 150 mM ammonium acetate. Green sphere – PBP. Purple tear – antibiotic molecule.
Figure 7.16. Native MS analysis of PBP2a-Y23M with ceftobiprole. PBP2a-Y23M (50 µM) analysed alone (A.) or with 100 µM ceftobiprole in DMSO (B). Peak with m/z of 4940.26 isolated with the Quadrupole OrbiTrap and treated with 25-30 V collision energy (C). Samples flown in 150 mM ammonium acetate. Green sphere – PBP. Purple tear – antibiotic molecule.
Figure 7.17. Native MS analysis of PBP2-W59M with ceftaroline and ceftobiprole. PBP2-W59M (50 µM) analysed alone (A.) or with 100 µM ceftaroline in DMSO (B) or with 100 µM ceftobiprole in DMSO (C). Samples flown in 150 mM ammonium acetate. Green sphere – PBP. Purple tear – antibiotic molecule.
7.3. Concluding remarks and further work

7.3.1 Can PBP2 transglycosylase activity be enhanced by PBP2a?

Initial experiments suggested that both the full-length PBP2:PBP2a complex, and PBP2-W59M incubated with PBP2a-Y23M had greater transglycosylase activity than that of PBP2-W59M alone, with the full-length PBP2:PBP2a complex polymerising the longest glycan chains. However, unfortunately due to the inability to purify full-length PBP2 and full-length PBP2a membrane proteins in isolation, the exact stoichiometry of this relationship could not be investigated further. Therefore, the ability of the truncated PBP2a-Y23M to enhance transglycosylase activity of PBP2-W59M was investigated – however unfortunately the initial observation could not be replicated, and no increase in transglycosylase activity was seen even when PBP2a-Y23M was titrated against PBP2-W59M.

We therefore postulate that perhaps the increase in transglycosylase activity seen for the full-length PBP2:PBP2a complex could be due to the presence of the transmembrane helices, which acts to stabilise the two proteins as a complex, and also aids in the purification of the two proteins as a stable complex. As discussed in Chapter 6, the truncated proteins may be unable to form a stable protein complex, and may only interact transiently due to the absence of the transmembrane region. The co-localisation of these proteins to the membrane via the TM helices makes logical sense since the lipid-II substrate of PBP2 and PBP2a is membrane anchored, and is polymerised into a glycan strand whilst still anchored at one end by the C55 lipid tail. Whilst PBP2a is a transpeptidase only enzyme, it must interact with the glycan polymer emanating from the cell surface. Moreover, for PBP2a to be able to functionally compliment the lack of transpeptidase activity of PBP2 upon β-lactam inhibition, a complex between PBP2 and PBP2a is a logical consequence.

Furthermore, this complex formation may serve to enhance transpeptidation activity, rather than transglycosylase activity, or may not directly enhance either
transglycosylase and transpeptidase activity directly, but actually facilitate the efficient transport of glycan chains from PBP2 to PBP2a during β-lactam exposure. Further work should include the investigation of the transpeptidation activity of PBP2-W59M, in the presence of PBP2a-Y23M, and vice versa.

7.3.2. Is Tricine-SDS-PAGE an alternative way to visualise transpeptidase products?

This chapter has highlighted the use of Tricine-SDS-PAGE as a means to not only visualise transglycosylase products, but also for the visualisation of transpeptidase products which cannot migrate through the PAGE gel. The cross-linking of not only amidated pentaglycyl lipid-II species has been shown, but also the cross-linking of mono- and tri-glycyl lipid-II species. Interestingly, neither of the non-amidated mono- or tri-glycyl lipid-II species were detectably cross-linked into peptidoglycan, but they were polymerised into glycan chains. Whilst in theory, one unit of lipid-II could be cross-linked to another unit of lipid-II, this is unlikely to occur in vivo. Glycan chains are generally much longer than the peptide chains in peptidoglycan, and only few lipid-II branches are involved in creating a peptide cross-bridge, unlike each sugar disaccharide of lipid-II which must be polymerised to create the glycan chain (van Heijenoort et al., 2001). Therefore, it is highly unlikely that one lipid-II unit alone would be incorporated by transpeptidation into a pre-existing glycan chain (van Heijenoort et al., 2001). This hypothesis is further exemplified by the S. aureus moenomycin resistant mutant, PBP2-Y196D, which can only make short glycan chains due to the amino acid substitution in the catalytic cleft of PBP2, causing the premature release of the glycan chain. (Rebets et al., 2014) Whilst this does not cause any phenotypic abnormalities in the absence of moenomycin, most likely due to the compensatory mechanisms of the transglycosylase SgtA, it does however, cause severe cell division defects in the presence of moenomycin suggesting that there is a minimum glycan chain length that can be tolerated for transpeptidation (Rebets et al., 2014). Therefore, the inability of PBP2-W59M to cross-link non-amidated mono- or tri-glycyl lipid-II (Lys) may be due to the shortened glycan chain lengths.
7.3.3. What information does native mass spectrometry give about transglycosylation?

Native mass spectrometry of PBP2-W59M transglycosylase activity has allowed us to study transglycosylation. Not only has the binding of lipid-II, and potentially two polymerised units of lipid-II, minus their prenyl chains, to PBP2-W59M been shown, but the preliminary work to optimise the transglycosylase assay for analysis by native mass spectrometry has enhanced knowledge into this area. This work has highlighted that the transglycosylase activity of PBP2-W59M can be studied in ammonium acetate buffer and also whilst in the gas phase of a mass spectrometer, highlighting how robust PBP2-W59M is in comparison to other purified PBPs. Furthermore, previous resuspension buffers for lipid-II were limited to detergent based buffers or a harsh solvent. Here we showed that lipid-II can be provided to the transglycosylase reaction in a wide range of buffer systems, such as ammonium acetate, DMSO or low concentrations of methanol with ammonium acetate, without compromising bioavailability within the assay or PBP2-W59M transglycosylase activity. Lipid-II can even be dried down into the reaction vessel and the remaining reaction components added to the reaction vessel to be used as the resuspension buffer, with minimal loss to transglycosylase activity.

Whilst lipid-II, glycan chains and peptidoglycan have been detected by mass spectrometry methods including LC-MS/MS, to my knowledge, the continuous detection of the transglycosylation of lipid-II into glycan chains by bacterial PBPs via native mass spectrometry has not been shown. If transglycosylation could be followed by native mass spectrometry up to higher glycan chain lengths, this could be directly related back to the fluorescent glycan chain bands seen by Tricine-SDS-PAGE, and hence would be further evidence that the characteristic banding seen by Tricine-SDS-PAGE correlates to production of glycan chains increasing by one unit at time. If branched lipid-II was used as a substrate, transpeptidation may also be successfully analysed, and this may provide information as to the optimal glycan chain length required for transpeptidation to occur.
7.3.4. Can PBP2-W59M transglycosylase activity be optimised in the presence of S. aureus membranes or membrane constituents?

The incorporation of extracted S. aureus membranes, or the membrane component cardiolipin, did not enhance transglycosylase activity of PBP2-W59M. However, it is important to note that this investigation was performed on the truncated PBP2, without the TM helix. As it is the TM domain that interacts with the cardiolipin of the cellular membrane, or indeed the S. aureus membrane itself as a whole, the absence of the TM domain may have rendered PBP2-W59M unable to physically interact with these components. If full-length PBP2 could be successfully purified it would be interesting to assess whether these components are able to enhance transglycosylase activity.

7.3.5. Does PBP2-W59M have a transpeptidase allosteric site like that of PBP2a-Y23M?

The analysis of the interaction of PBP2a-Y23M with either ceftobiprole or ceftaroline by native mass spectrometry, as expected, has shown that both antibiotics are able to bind putatively to two binding sites in PBP2a-Y23M – the transpeptidase allosteric site, and the transpeptidase active site. Even more interestingly, this native mass spectrometry work has shown the same binding of ceftobiprole and ceftaroline to two binding sites in PBP2-W59M. This is remarkable, as according to the PBP2-W59M crystal structure there is only a transpeptidase active site present in PBP2-W59M.

Whilst PBP2-W59M is a bifunctional PBP with both transglycosylase and transpeptidase activity, and PBP2a-Y23M is a mono-functional PBP with only transpeptidase activity, it would be logical for the same mechanism of activation to be conserved amongst these two PBPs. However, to my knowledge, there is only one other PBP with a transpeptidase allosteric site. S. pneumoniae PBP2x contains a transpeptidase active site within the PASTA (penicillin-binding protein and serine-threonine kinase associated) domain – binding of the first pentapeptide stem unit of a growing glycan chain to the allosteric site causes a conformational change, allowing
for the pentapeptide stem of the third unit of the growing glycan chain to enter the transpeptidase active site, hence causing initiation of cross-linking of glycan chains to form peptidoglycan (Bernardo-García et al, 2018). That said, the sequence alignments of \textit{S. pneumoniae} PBP2x and \textit{S. aureus} PBP2a showed very little similarity in amino acid sequence.

To confirm whether the secondary binding event of ceftaroline or ceftabiprole to PBP2-W59M was indeed at an allosteric site, as well as at the active site, further confirmation is needed. The PBP2-W59M:antibiotic complex should be reanalysed by native mass spectrometry, and treated with high energy to expel the second molecule from the proposed allosteric site. Furthermore, binding of control molecules to PBP2-W59M which should either not bind, or bind in one event, should be assessed to rule out anomalies during mass spectrometry analysis. Crystallography trials should also be set up with the hope that the binding of two molecules of antibiotic could be shown, and compared with the apo-structure.
8. General discussion and concluding remarks

Antibiotic resistance poses a huge threat to both livestock and human healthcare, and the combination of increasing rates of resistance emergence and the lack of new antibiotic discovery threatens a worrying future if current trends are not curbed. Whilst it is important to prevent the wide prescribing and misuse of antibiotics through new public and professional education schemes and legally enforced access schemes, it is also vital that new antibiotics are not only discovered, but make it successfully through clinical trials and into clinic, where their usage can be monitored to prevent overuse, misuse, and inappropriate prescribing. We should not forget however that whilst antibiotic resistance is an issue in many countries and societies, lack of access to antibiotics has a major source of morbidity and mortality in many other countries. Thus, antibiotic resistance and access to antibiotics is a complex socioeconomic problem that requires world-wide action.

Whilst novel drug discovery and drug derivative design is a crucial part of fighting antimicrobial resistance, understanding the fundamental basis of the biochemical mechanisms behind these bacterial resistance determinants is also required if we are to target resistant bacteria successfully and efficiently to prevent the unnecessary reoccurrence of resistance to these new drug regimes. Whilst the emergence of resistance is somewhat inevitable as a natural part of bacterial evolution, with appropriate prescribing and treatment, the emergence of resistance could be reduced to much slower rates than are currently being exhibited.

This thesis has focused upon bacterial cell wall synthesis in MRSA, which is clearly a fantastic target for antibiotics due to its conserved, and essential nature. Key in the synthesis of peptidoglycan in MRSA are the Fem ligases, which act to append the pentaglycine branch to the peptidoglycan precursor lipid-II, and the PBPs, which are responsible for the high level of cross-linking of branched lipid-II and the formation of peptidoglycan as we know it. Both the Fem ligases and the PBPs are valid targets for antibiotic discovery, and thus this thesis aids in the knowledge of the fundamental
biophysical processes underlying resistance in MRSA, and could potentially aid in future drug design.

8.1. Optimising the yields of lipid-II (Lys)

The study of PBP enzymology has been largely hindered by the complex synthesis of lipid-II. In our laboratory, we are able to chemo-enzymatically synthesise lipid-II using a step-wise procedure which involves the synthesis of the pentapeptide precursor before conversion to lipid-II. Whilst the synthesis of non-amidated lipid-II species has always been relatively fruitful, this is not always the case for amidated lipid-II species which has always required a greater input of precursor starting material and yields much lower quantities of lipid-II in comparison to non-amidated lipid-II. This is most likely due to the MraY and/or MurG used in the conversion of pentapeptide to lipid-II having a lower affinity for the amidated pentapeptide species.

As the amidated lipid-II species are the natural substrates for PBPs in most Gram-positive bacterial species, we aimed to optimise the conversion of pentapeptide to lipid-II via the use of an alternative bacterial membrane. This stemmed from the observation that *M. flavus* membranes contaminated with *S. aureus* yielded a larger quantity of amidated lipid-II (Lys). We therefore investigated the yields of non-amidated and amidated lipid-II (Lys) when *M. flavus, S. aureus, S. epidermidis* and a combination of *M. flavus* and *S. aureus or S. epidermidis* membranes were used in the lipid-II synthesis.

The greatest yields for non-amidated lipid-II (Lys) were from syntheses containing *S. aureus* membranes alone; twice that of the lipid-II yield when *M. flavus* membranes alone were used. However, unfortunately, the yields of amidated lipid-II (Lys) could not be assessed due to large losses of lipid-II during the purification procedure. Whilst the outcome of the non-amidated lipid-II (Lys) investigation is promising, the extraction of *S. aureus* membranes from *S. aureus* culture is difficult, and yields much smaller quantities of membrane than an *M. flavus* membrane extraction. This would mean having to vastly increase the scale of *S. aureus* membrane extraction to yield
membrane quantities anywhere near that of the *M. flavus* membrane extractions. It would therefore be more sensible and less time consuming to continue with the current procedure of using *M. flavus* membrane for non-amidated lipid-II (Lys) synthesis, unless the extraction of *S. aureus* membranes could be optimised to warrant its usage for lipid-II syntheses. The results of this investigation are promising however, and with some further optimisation, could allow for greater amounts of both non-amidated and amidated lipid-II to be synthesised, and hence, provide the laboratory with more lipid-II reagent for use in enzymatic PBP assay.

8.2. Synthesising branched lipid-II intermediates

Whilst the laboratory is able to synthesise non-branched lipid-II, the synthesis of branched lipid-II is more complex. In the case of *S. aureus*, lipid-II with a pentaglycine branch must be used in order to measure the transpeptidase activity of PBPs. Without this branched lipid species, only transglycosylation can be measured, and therefore the availability of pentaglycyl lipid-II is crucial for the investigation of transpeptidation activity. Furthermore, whilst non-branched lipid-II can be used to assess the activity of FemX, the mono- and tri-glycyl lipid-II species are needed to assess the activity of FemA and FemB, respectively. Therefore, the synthesis of these complex peptidoglycan intermediates is key to our ability to study peptidoglycan biosynthesis *in vivo*.

This thesis has successfully implemented the chemical synthesis of mono-, tri- and penta-glycyl pentapeptide species, to both reasonably high yields and purity. This had not yet been attempted in our laboratory for *S. aureus* lipid-II intermediates. Furthermore, the mono- and tri- pentapeptide species, both amidated and non-amidated, were successfully converted to lipid-II. However, interestingly the pentaglycyl pentapeptide species was unable to be converted to lipid-II via the usual enzymatic synthesis using MraY and MurG. This suggests that pentaglycyl branch may inhibit the access of MraY or MurG to the pentapeptide, and therefore, the pentapeptide is unable to be converted to lipid-II.
Enzymatic synthesis of branched non-amidated lipid-II (Lys) species using the Fem ligases has also been successful, albeit on a small scale. Incubating purified non-amidated lipid-II with either FemX alone, FemX and FemA or FemX, FemA and FemB enabled the synthesis of mono-, tri- and penta- glycyll lipid-II (Lys), respectively. This could easily be scaled up to yield quantities of branched lipid-II for use in enzymatic assay. Furthermore, the Walker group method for the extraction of amidated lipid-II (Lys) pentaglycene was trialled within our laboratory (Qiao et al, 2017). However, disappointingly the yields of extracted lipid-II were up to 1000-fold less than the expected published values. Therefore, until this method is optimised within our laboratory to yield the milligram quantities of lipid-II as published, it may be wiser to continue with the enzymatic synthesis of pentaglycyll lipid-II using the Fem ligases.

8.3. The substrate specificity of the Fem ligases

The point at which lipid-II amidation occurs during peptidoglycan biosynthesis is currently still unknown - we do not know whether it occurs before, during or after the pentaglycene branching of lipid-II by the Fem ligases. Previous work as part of this thesis has shown that the Fem ligases have the ability to branch both amidated and non-amidated lipid-II, however this was not quantitative. We therefore investigated whether the Fem ligases have a preference for either amidated or non-amidated lipid-II species using a radioactive assay, in which $[^3]$H-glycine was appended to lipid-II by FemX, or to lipid-II monoglycine by FemX, over time.

Interestingly, both FemX and FemA showed no preference for their respective amidated or non-amidated lipid-II species, suggesting that the first, second and third glycine resides can be appended to lipid-II in its amidated or non-amidated form. Unfortunately, the substrate specificity of FemB was not investigated as non-amidated lipid-II (Lys) pentaglycene was unavailable, and the amidated version was in very short supply. However, it would be reasonable be assume that FemB also has the same lack of substrate preference.
As the vast majority of *S. aureus* peptidoglycan is made up from amidated lipid-II (Lys) pentaglycine, therefore there must be a mechanism to maintain this fidelity. If the Fem ligases do not have a preference for amidated or non-amidated species, and therefore do not actively prevent non-amidated lipid-II species from continuing into peptidoglycan synthesis, then there must be a more downstream mechanism to filter out non-amidated species. Could it therefore be that the enzyme responsible for lipid-II flipping to the outer membrane (MurJ or FtsW) has a preference for amidated lipid-II (Lys) pentaglycine? Previous work within our laboratory has shown that PBP2-W59M has a large preference for amidated lipid-II over non-amidated lipid-II (Cain *et al.*, manuscript in prep) – however it would seem energetically wasteful to synthesise and flip non-amidated lipid-II (Lys) pentaglycine to the outer membrane when it will not be used preferentially by PBPs, and is also potentially damaging if it enters the cell wall in large quantities. Therefore, it seems more likely that the lipid-II flippase acts to prevent non-amidated lipid-II (Lys) pentaglycine from flipping to the outer membrane, and therefore maintain the fidelity of peptidoglycan synthesis in this manner.

8.4. The ability of the Fem ligases to form a protein complex

The ability of the Fem ligases to form a heterotrimer protein complex is a logical mechanism to allow for the efficient transport of each branched lipid-II species to the next Fem ligase for the addition of the next glycine residue. For example, once FemX has appended the first glycine, it would be logical for the mono-glycyl lipid-II species to be shuttled directly to FemA for the addition of the second and third glycine residues, and so on to FemB.

Previous publications using bacterial two-hybrid screens have shown successful homodimerization of FemA-FemA, FemA-FemB, FemB-FemA and FemB-FemB, however no interactions of any combinations of Fem ligase with FemX were seen (Rohrer and Berger-Bächi 2003a). We therefore investigated the interaction of all six combinations of Fem ligase using microscale thermophoresis (MST), using each Fem ligase with and without its His$_6$ affinity tag. MST analysis indicated protein-protein
interactions for FemX-FemA, FemA-FemX and FemB-FemA (With His$_6$-without His$_6$), with measurable Kd values for each within the micromolar range. An interaction of FemB-FemX was also indicated, however a Kd value could not be accurately determined, suggesting that any interactions here may be extremely weak.

This data is in agreement with the work performed by Rohrer and Berger-Bächli (2003a), however homodimerization was not investigated here, and we also include evidence for the interaction of FemX with FemA, which was not previously seen by Rohrer and Berger-Bächli. These two independent characterisations of Fem ligases complex formation are highly suggestive that the Fem ligases do indeed form a protein complex. Interestingly, we have strong evidence for FemX binding to FemA, and FemA binding to FemB, but not FemX binding to FemB, which is logical when you imagine lipid-II is shuttled across from FemX to FemA to FemB. If FemX were able to interact with FemB, perhaps the Fem ligase complex formation and the lipid-II shuttling effect would be disrupted.

Whilst crystallisation trials of FemB was not entirely successful as part of this thesis, the idea of the Fem ligase complex formation may be useful in obtaining stable crystal forms. Perhaps Fem ligase trimer complex crystal trials should be performed in the published crystal conditions for FemA, with a view to obtaining a stable Fem ligase trimer.

Furthermore, the disruption of this Fem ligase complex could act as a novel therapeutic target for antibiotic drug design in the future. However, more investigation is needed to elucidate their protein-protein interface.

8.5. The PBP2 and PBP2a protein complex and the effect of PBP2a on PBP2 transglycosylase activity

As part of this thesis, we have discovered a novel PBP2:PBP2a protein complex. Recombinantly coexpressed full-length PBP2 with an N-terminal His$_6$ affinity tag and full-length PBP2a with an N-terminal S-tag affinity tag were discovered to copurify
via nickel IMAC, a resin with high specificity for histidine affinity tagged proteins. This is highly suggestive of the formation of a stable full-length PBP2:PBP2a protein complex. Even more interestingly, the full-length PBP2, and full-length PBP2a proteins, whilst they can be recombinantly expressed, they cannot be successfully purified without substantial degradation. The full-length PBP2:PBP2a complex, on the other hand, appears to withstand both recombinant expression and purification, suggestive of a stabilising effect of the protein complex.

Unfortunately, the presence of detergent in the full-length PBP2:PBP2a complex sample limits the techniques available to study the protein-protein interaction, and as stated previously, the full-length PBPs do not purify well alone. Therefore, the truncated versions of each PBP, without their TM helix (PBP2-W59M, and PBP2a-Y23M) were purified in order to further study this protein-protein interaction.

The potential PBP2-W59M and PBP2a-Y23M protein complex was assessed using various biophysical techniques. Unfortunately, native mass spectrometry and AUC were both unable to detect a protein-protein interaction between PBP2-W59M and PBP2a-Y23M, whilst the highly sensitive technique of carbene labelling successfully detected a PBP2-W59M:PBP2a-Y23M interaction and also allowed for the mapping of the surface residues involved in the protein-protein interaction of PBP2a-Y23M, in the presence of PBP2-W59M. The ability to detect a protein-protein interaction between PBP2-W59M and PBP2a-Y23M using this highly sensitive technique is suggestive of a weak, transient interaction, which is unable to withstand the harsh environmental conditions of AUC or native mass spectrometry. This could be due to the absence of the TM helices, which may be responsible for anchoring the two PBPs in a stable protein conformation.

In order to further understand the role of the PBP2-W59M:PBP2a-Y23M complex, the transglycosylase activity of PBP2-W59M in the presence of PBP2a-Y23M was investigated. The presence of PBP2a-Y23M appeared to have no effect on PBP2-W59M transglycosylase activity. However, interestingly the carbene footprinting analysis of the residues involved in PBP2-W59M:PBP2a-Y23M indicates a potential
conformational change in the transpeptidase domain of PBP2a-Y23M in the presence of PBP2-W59M. Therefore, perhaps the PBP2-W59M:PBP2a-Y23M complex acts to enhance the transpeptidase activity of PBP2a-Y23M. This has not been investigated as part of this thesis.

The concept of the formation of complexes between PBPs with other proteins that act to enhance their enzymatic activity is now established in the literature. For example, in *E. coli* the outer membrane proteins LpoA and LpoB bind directly to their cognate PBPs (PBP1a and PBP1b, respectively) and act to enhance transpeptidase activity (Typas *et al*, 2010). However, the direct interaction of different PBPs is less well explored at present. Therefore, perhaps the same general mechanism of transpeptidase stimulation is true for PBP2a-Y23M in the presence of PBP2-W59M, acting to enhance transpeptidation by PBP2a-Y23M in the presence of β-lactam antibiotics.

Furthermore, with further investigation and the completion of the mapping of the residues involved in the formation of the PBP2-W59M:PBP2a-Y23M complex, the formation of the PBP2/PBP2a complex, or indeed, the prevention of this complex formation could pose as a valuable antibiotic target. Examples of current early-stage antibiotics shown to prevent protein-protein interactions include inhibitors of FtsZ/ZipA, with activity against a broad range of pathogens including *E. coli*, *S. pneumoniae*, *B. subtilis*, *E. faecalis* and *S. aureus* (Kenny *et al*, 2003, Sutherland *et al*, 2003, Jennings *et al*, 2004, Carro 2018) and the NusB/NusE inhibitors, with activity against MSSA and MRSA (Yang *et al*, 2017).

### 8.6. The novel putative transpeptidase allosteric site of PBP2-W59M

The investigation of ceftaroline or ceftobiprole binding to PBP2-W59M by native mass spectrometry has revealed a previously undiscovered potential allosteric transpeptidase binding site of PBP2-W59M, previously seen only to possess a transpeptidase active site at Ser-398 (Lovering *et al*, 2007). Whilst further
investigation must be performed to confirm whether binding to this putative allosteric site causes the conformational changes usually associated to an allosteric binding event, this finding is undoubtably exciting and may elucidate currently unknown information about the biophysical properties of PBP2-W59M. Proof of an allosteric site interaction would require detailed enzymological characterisation with the pentaglycyl lipid-II substrate of PBP2-W59M and possible other supporting structural data. If the exact residue positions of a PBP2-W59M allosteric site could be determined through further structural investigation, this could allow for the future design of antibiotics targeted towards both the allosteric and active sites with the hope

8.7. Conclusion

The work presented in this thesis has contributed to the greater understanding of the enzymology involved in peptidoglycan synthesis in the important global pathogen, MRSA. This work will allow for the future in-depth study of the enzymatic processes involved in peptidoglycan synthesis, and hopefully this greater understanding of such fundamental bacterial processes will allow for the discovery and development of crucial, novel antibiotics.
9. References


FDA (2014). FDA 2011 Summary report on Antimicrobials sold or distributed for use in Food producing animals.

FDA (2016). 2015 Summary report on antimicrobials sold or distributed for use in food-producing animals.


Figure 10.1. TOF-MS analysis of amidated UDP-MurNAc-pentapeptide Gly₁. Negative ion mode TOF-MS. Predicted exact mass 1205.389 Da. Predicted m/z 1204.389 singly charged, m/z 601.6945 doubly charged (Observed Panel A) and m/z 400.796 triply charged. Singly, doubly and triply sodiated forms observed in Panel B. Synthesised by Dr Ricky Cain, Roper group.
Figure 10.2. TOF-MS analysis of amidated UDP-MurNAc-pentapeptide Gly₃. Negative ion mode TOF-MS. Predicted exact mass 1319.43198 Da. Predicted m/z 1318.43198 singly charged (observed Panel B), 658.71599 doubly charged (Observed Panel A) and 438.81 triply charged (Observed Panel C). Singly, doubly and triply sodiated forms observed in Panel B. Synthesised by Dr Ricky Cain, Roper group.
Figure 10.3. TOF-MS analysis of amidated UDP-MurNAc-pentapeptide Gly₅. Positive ion mode TOF-MS. Predicted exact mass 1433.47491 Da. Predicted m/z 1432.47491 singly charged, 715.7375 doubly charged (Observed Panel A) and 476.825 triply charged (Observed Panel B). Singly and doubly sodiated forms observed in Panel A. Synthesised by Dr Ricky Cain, Roper group.
Table 10.1. Predicted m/z of lipid-II species analysed by negative ion mode TOF-MS. Denoted fragment number (n) refers to ion peak in TOF-MS trace.

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<th>Lipid-II species</th>
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<th>Predicted m/z</th>
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Figure 10.4. The synthesis of lipid-II variants using non-amidated UDP-MurNAc-pentapeptide (Lys) plus FemXAB ligases – 300 mM DEAE sephacel purification fractions (Trace 1). TOF-MS in negative electrospray ionization mode. See Appendix Table XX for predicted masses and m/z values.
Figure 10.5. The synthesis of lipid-II variants using non-amidated UDP-MurNAc-pentapeptide (Lys) plus FemXAB ligases – 300 mM DEAE sephacel purification fractions (Trace 2). TOF-MS in negative electrospray ionization mode. See Appendix Table XX for predicted masses and m/z values.
Figure 10.6. The synthesis of lipid-II variants using non-amidated UDP-MurNAc-pentapeptide (Lys) plus FemXAB ligases – 1M DEAE sephacel purification fraction (Trace 1). TOF-MS in negative electrospray ionization mode. See Appendix Table XX for predicted masses and m/z values.
Figure 10.7. The synthesis of lipid-II variants using non-amidated UDP-MurNAc-pentapeptide (Lys) plus FemXAB ligases – 1M DEAE sephacel purification fraction (Trace 2). TOF-MS in negative electrospray ionization mode. See Appendix Table XX for predicted masses and m/z values.
Figure 10.8. The synthesis of lipid-II variants using amidated UDP-MurNAc-pentapeptide (Lys) plus FemXAB ligases (Reaction 2) – 300 mM purification fraction (Trace 1). TOF-MS in negative electrospray ionization mode. See Appendix Table XX for predicted masses and m/z values.
Figure 10.9. The synthesis of lipid-II variants using amidated UDP-MurNAc-pentapeptide (Lys) plus FemXAB ligases (Reaction 2) – 300 mM purification fraction (Trace 2). TOF-MS in negative electrospray ionization mode. See Appendix Table XX for predicted masses and m/z values.
Figure 10.10. The synthesis of lipid-II variants using amidated UDP-MurNAC-pentapeptide (Lys) plus FemXAB ligases (Reaction 2) – 1M DEAE sephacel purification fraction (Trace 1). TOF-MS in negative electrospray ionization mode. See Appendix Table XX for predicted masses and m/z values.
Figure 10.11. The synthesis of lipid-II variants using amidated UDP-MurNAc-pentapeptide (Lys) plus FemXAB ligases (Reaction 2) – 1M DEAE sephacel purification fraction (Trace 2). TOF-MS in negative electrospray ionization mode. See Appendix Table XX for predicted masses and m/z values.
Figure 10.12. The synthesis of lipid-II variants using non-amidated UDP-MurNAc-pentapeptide (Lys) plus FemXAB ligases and glutamine (Reaction 3) – 300 mM DEAE sephacel purification fractions (Trace 1). TOF-MS in negative electrospray ionization mode. See Appendix Table XX for predicted masses and m/z values.
Figure 10.13. The synthesis of lipid-II variants using non-amidated UDP-MurNAc-pentapeptide (Lys) plus FemXAB ligases and glutamine (Reaction 3) – 300 mM DEAE sephacel purification fractions (Trace 2). TOF-MS in negative electrospray ionization mode. See Appendix Table XX for predicted masses and m/z values.
Figure 10.14. The synthesis of lipid-II variants using non-amidated UDP-MurNAc-pentapeptide (Lys) plus FemXAB ligases and glutamine (Reaction 3) – 300 mM DEAE sephacel purification fractions (Trace 3). TOF-MS in negative electrospray ionization mode. See Appendix Table XX for predicted masses and m/z values.
Figure 10.15. The synthesis of lipid-II variants using non-amidated UDP-MurNAc-pentapeptide (Lys) plus FemXAB ligases and glutamine (Reaction 3) – 300 mM DEAE sephacel purification fractions (Trace 4). TOF-MS in negative electrospray ionization mode. See Appendix Table XX for predicted masses and m/z values.
Figure 10.16. The synthesis of lipid-II variants using non-amidated UDP-MurNAc-pentapeptide (Lys) plus FemXAB ligases and glutamine (Reaction 3) – 300 mM DEAE sephacel purification fractions (Trace 5). TOF-MS in negative electrospray ionization mode. See Appendix Table XX for predicted masses and m/z values.
Figure 10.17. The synthesis of lipid-II variants using non-amidated UDP-MurNAc-pentapeptide (Lys) plus FemXAB ligases and glutamine (Reaction 3) – 300 mM DEAE sephacel purification fractions (Trace 6). TOF-MS in negative electrospray ionization mode. See Appendix Table XX for predicted masses and m/z values.
Figure 10.18. The synthesis of lipid-II variants using non-amidated UDP-MurNAc-pentapeptide (Lys) plus FemXAB ligases and glutamine (Reaction 3) – 1 M DEAE sephacel purification fractions (Trace 1). TOF-MS in negative electrospray ionization mode. See Appendix Table XX for predicted masses and m/z values.
Figure 10.19. The synthesis of lipid-II variants using non-amidated UDP-MurNAc-pentapeptide (Lys) plus FemXAB ligases and glutamine (Reaction 3) – 1 M DEAE sephacel purification fractions (Trace 2). TOF-MS in negative electrospray ionization mode. See Appendix Table XX for predicted masses and m/z values.
Figure 10.20. The synthesis of lipid-II variants using non-amidated UDP-MurNAc-pentapeptide (Lys) plus FemXAB ligases and glutamine (Reaction 3) – 1 M DEAE sephacel purification fractions (Trace 3). TOF-MS in negative electrospray ionization mode. See Appendix Table XX for predicted masses and m/z values.
Figure 10.21. The synthesis of lipid-II variants using non-amidated UDP-MurNAc-pentapeptide (Lys) plus FemXAB ligases and glutamine (Reaction 3) – 1 M DEAE sephacel purification fractions (Trace 4). TOF-MS in negative electrospray ionization mode. See Appendix Table XX for predicted masses and m/z values.
Figure 10.22. The synthesis of lipid-II variants using non-amidated UDP-MurNAc-pentapeptide (Lys) plus FemXAB ligases and glutamine (Reaction 3) – 1 M DEAE sephacel purification fractions (Trace 5). TOF-MS in negative electrospray ionization mode. See Appendix Table XX for predicted masses and m/z values.
Figure 10.23. The synthesis of lipid-II variants using non-amidated UDP-MurNAc-pentapeptide (Lys) plus FemXAB ligases and glutamine (Reaction 3) – 1 M DEAE sephacel purification fractions (Trace 6). TOF-MS in negative electrospray ionization mode. See Appendix Table XX for predicted masses and m/z values.
Figure 10.24. The synthesis of lipid-II variants using non-amidated UDP-MurNAc-pentapeptide (Lys) plus FemXAB ligases and glutamine (Reaction 3) – 1 M DEAE sephacel purification fractions (Trace 7). TOF-MS in negative electrospray ionization mode. See Appendix Table XX for predicted masses and m/z values.
Figure 10.25. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assay for *S. aureus* RN4220 in the presence of vancomycin. MIC and MBC performed at *S. aureus* OD$_{600}$ 0.5, using vancomycin at final concentration from 65 mg/ml - 0.05 µg/ml. i. 65 mg/ml – 32 µg/ml. ii. 16 – 0.05 µg/ml. Positive control (no vancomycin, +). Negative control (No *S. aureus*, -). MIC value 0.5-1 µg/ml, MBC 16 µg/ml.

Figure 10.26. Microscale thermophoresis binding analysis of the FemB (target) vs FemX (ligand). The binding affinity of FemB with a His$_6$ affinity tag labelled with RED-tris-NTA fluorescent dye (50 nM) was assessed against FemX minus a His$_6$ affinity tag. MST was performed at 40% LED power, medium MST power and an on time of 20 sec. n=3.
Figure 10.27. Raw intact mass spectra for PBP2-W59M and PBP2a-Y23M, in the presence or absence of either ceftaroline or ceftobiprole. See Chapter 7, Section 7.2.6 for deconvoluted intact mass spectra. 50 µM PBP2-W59M, 50 µM PBP2a-Y23M, 500 µM ceftaroline and 500 µM ceftobiprole.