Investigating the Mechanisms of Secretion of the Moonlighting Proteins Enolase and Fructose-1, 6-Bisphosphate Aldolase in *Neisseria meningitidis*

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Declaration

I declare that this work was carried out by Banan Atwah and I take full responsibility of whatever has been reported. No previous submission for the award of higher degree has been made elsewhere and related work by others, which served as a source of knowledge has been duly acknowledged or referenced.

Banan Atwah

2019

Abstract

Neisseria meningitidis is an obligate commensal of humans that mainly colonises the nasopharynx. It is also an important cause of serious diseases, such as meningitis and sepsis. *N. meningitidis* uses several secretion systems (type I, type Va, type Vb, and type Vc) for secreting proteins, which enable the bacteria to modulate their cell surface and evade the human immune system.

The moonlighting proteins fructose bis-1, 6-phosphate aldolase (FBA) and enolase were previously shown to localise to the surface of *N. meningitidis.* The mechanisms of secretion of both proteins were investigated in this study. Specifically, lysine residue 337 of enolase was substituted to investigate its role in secretion since in *Escherichia coli* mutation of the corresponding residue has been reported to decrease export efficiency.

This study proposes that K337E substitution in meningococcal enolase causes a growth defect confirming an important functional role of this residue. However, its role in the localisation of the protein to the meningococcal cell surface is still not confirmed despite the several attempts to enhance enolase expression.

The alanine residue 14 in the predicted cleavage site of the N-terminal region of FBA was substituted to investigate the role of the putative signal peptide in the surface localisation. Immunoblot analysis showed a reduction in FBA expression compared to the MC58Δ*cbbA cbbA*^{Ect}. Sub-cellular fractionation suggested that this mutation affected protein expression rather than its localisation to the meningococcal cell surface.

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Table of Contents

Declaration i

Abstract ii

Acknowledgement iv

Table of Contents vi

List of Figures xiii

List of Tables xxii

Abbreviations xxiii

1 Chapter One: General Introduction 1

1.1 The genus *Neisseria* 2

1.2 Neisseria meningitidis 3

1.3 Classification of *N. meningitidis* 4

1.4 Epidemiology of invasive meningococcal disease 6

1.5 Prevention of meningococcal diseases: vaccines against *N. meningitidis* 8

1.5.1 Polysaccharide vaccines 8

1.5.2 Conjugate polysaccharide vaccines 9

1.5.3 Meningococcal group B vaccines 10

1.6 Meningococcal pathogenesis 13

1.6.1 Meningococcal Colonisation and Adhesion 13

1.6.2 Phase and antigenic variation 14

1.7 Virulence Factors of *N. meningitidis* 15

1.7.1 Polysaccharide Capsule 15

1.7.2 Type IV Pili (T4P) 16
1.7.3 Outer-Membrane Proteins (OMPs) 17
1.7.3.1 PorA porin 17
1.7.3.2 PorB porin 18
1.7.3.3 Neisseria hia homologue A (NhhA) 19
1.7.3.4 Opacity proteins or class V (Opa and Opc) 20
1.7.4 Lipooligosaccharide (LOS) 21
1.7.5 IgA1 Protease 22
1.7.6 Factor H Binding Protein (fHbp) 24
1.7.7 Minor Adhesins 25
1.8 Moonlighting proteins 26
1.8.1 Meningococcal moonlighting proteins 30
1.8.1.1 Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 30
1.8.1.2 Fructose-1, 6-bisphosphate aldolase (FBA) 31
1.8.1.3 Peroxiredoxin 32
1.8.1.4 DnaK 33
1.8.1.5 Enolase 34
1.8.1.5.1 Conserved residues of enolase 35
1.8.1.5.2 3D structure of enolase 38
1.9 Secretion pathways in diderms 40
1.9.1 Type I secretion pathway (ISP): ABC exporters 40
1.9.2 Type II secretion pathway (IISP) 43
1.9.3 Type III secretion pathway (IIISP): pathogenicity-related system 47
1.9.4 Type IV secretion pathway (IVSP): conjugation-related system 51
1.9.5 Type V secretion pathway (VSP) 55
1.9.5.1 Type Va secretion (classical autotransporters) 56

1.9.5.2 Type Vb secretion (Two-partner secretion system) 60
1.9.5.3 Type Vc secretion: trimeric autotransporter system 64
1.9.5.4 Type Vd Secretion: Fused Two-Partner Secretion System 67
1.9.5.5 Type Ve Secretion: Inverted Classical Autotransporter 68
1.9.6 Type VI secretion pathway (VISP) 71
1.9.7 Type VII secretion pathway (VIISP) 75
1.9.8 Other secretion systems 76

Aims of Study 78

2 Chapter Two: Materials and Methods 79

2.1 Bacterial strains, media, and growth conditions 80

2.2 Plasmids 87

2.3 Antibodies 90

2.3.1 Pre-adsorption of rabbit polyclonal anti-FBA (Acetone Powder) 91

2.4 Whole-cell ELISA 92

2.4.1 Preparation of meningococcal cells 92

2.4.2 The optimised protocol of whole-cell ELISA (Enzyme-linked immunosorbent assay) 92

2.5 Synthesis of oligonucleotide primers 93

2.6 DNA manipulation 95

2.6.1 Chromosomal DNA extraction 95
2.6.2 Plasmid DNA extraction 96
2.6.3 DNA clean up 96
2.6.4 Restriction endonuclease digestion 96
2.6.5 Ligation of DNA 96
2.6.6 Dephosphorylation of DNA fragments 96

2.6.7 A-tailing 97
2.6.8 Annealing oligonucleotides 97
2.6.9 DNA quantification 98
2.6.10 Sequencing 98
2.7 Polymerase Chain Reaction (PCR) 98
2.8 Agarose gel electrophoresis and gel extraction 99
2.9 Site-directed mutagenesis 100
2.10 Bacterial transformations 101
2.10.1 Heat shock transformation of JM109 competent *E. coli* 101
2.10.2 Natural transformation of *N. meningitidis* 101
2.11 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis 102
2.11.1 SDS-PAGE 102
2.11.2 Immunoblot analysis 103
2.11.3 Stripping of immunoblots 104
2.12 Sub-cellular fractionation of meningococcal cells 104
2.13 Bacterial growth curves 105
2.14 Binding of plasminogen to meningococcal cells 106
2.15 Web-based search (promoter prediction) 107
2.16 Statistical analysis 107
2.17 Protein structure analysis 107
3 Chapter Three: Identification of secretion-related or pathogenesis-related systems that could be involved in the secretion of enolase and FBA 108
3.1 Introduction 109

3.2 Results 113

3.2.1 Identification of surface-exposed FBA by whole-cell ELISA 113

3.2.2 FBA surface localisation was unaffected in mutants lacking known protein secretion-related or pathogenesis-related proteins 115

3.2.3 Enolase surface localisation was unaffected in the mutants lacking known protein secretion-related or pathogenesis-related proteins 118

3.2.4 Peroxiredoxin surface localisation was unaffected in the mutants lacking known protein secretion-related or pathogenesis-related proteins 120

3.3 Discussion 121

4 Chapter Four: Mutagenesis and complementation of the gene encoding enolase in *N. meningitidis* MC58 .. 125

4.1 Introduction 126

4.2 Results 129

4.2.1 Amplification and cloning of *NMB1285* (enolase gene) in pComp-1 vector 129

4.2.2 Mutagenesis of *NMB1285* 131

4.2.3 Transformation of the clones pComp1285 and pComp1285-K337E 132

4.2.4 Markerless mutation of enolase 135

4.2.4.1 TA cloning of *NMB1285* with the upstream and downstream flanking sequences 135

4.2.4.2 Inactivating *NMB1285* by inverse PCR 137

4.2.4.3 TA cloning of Kanamycin^{R} *rpsL*^{S} (*KnsL*) 138

4.2.4.4 Construction of pBA3 140

4.2.5 First double homologous recombination event 141

4.2.6 Verification of the first transformation using pBA3 construct 142
4.2.7 Second double homologous recombination event 144
4.2.8 Verification of the second transformation using pBA2 construct .. 145
4.2.9 Expression of ectopic enolase in the unmarked mutants 147
4.3 Discussion 148
5 Chapter Five: Investigating the role of a single lysine residue in the surface localisation of enolase 153
5.1 Introduction 154
5.2 Results 159
5.2.1 Identification of surface-exposed enolase by whole-cell ELISA 159
5.2.2 In vitro growth of MC58 wild type, MC58Δ*NMB1285 NMB1285*^{Ect}, and MC58Δ*NMB1285 NMB1285*^{EctK337E} strains 160
5.2.3 Reduction of enolase expression affects plasminogen binding 162
5.2.4 Attempts to enhance enolase expression 163
5.2.4.1 Incorporating Shine–Dalgarno-like sequence into pComp1285 vector 163
5.2.4.2 Replacing the promoter sequence (Opa-cbbA promoters) in pComp1285 with a stronger promoter (PorA promoter) 168
5.3 Discussion 173
6 Chapter Six: The role of the putative N-terminal signal peptide in the surface localisation of fructose-1,6-bisphosphate aldolase 177
6.1 Introduction 178
6.2 Results 181
6.2.1 Mutagenesis of A14 in the predicted cleavage site of the putative N-terminal signal peptide of *cbbA* 181

6.2.2 The effect of A14V mutation on FBA surface localisation 184
6.2.3 Expression of *cbbA* in MC58Δ*cbbA* $cbbA^{EctA14V}$ 185
6.2.4 Mutagenesis of A13, A13A14, A22, and D11 in the predicted cleavage site of the putative N-terminal signal peptide of *cbbA* 187
6.2.5 The effect of the putative N-terminal signal peptide mutations (A13V, A13VA14V, A22V, D11E) on FBA surface localisation 189
6.2.6 Expression of cbbA in MC58Δ*cbbA* $cbbA^{EctA13V}$, MC58Δ*cbbA* $cbbA^{EctA13VA14V}$, MC58Δ*cbbA* $cbbA^{EctA22V}$, and MC58Δ*cbbA* $cbbA^{EctD11E}$ 191
6.2.7 In vitro growth of MC58Δ*cbbA*, MC58Δ*cbbA* $cbbA^{EctA14V}$, MC58Δ*cbbA* $cbbA^{EctA13V}$, MC58Δ*cbbA* $cbbA^{EctA14VA13V}$, MC58Δ*cbbA* $cbbA^{EctA22V}$, and MC58Δ*cbbA* $cbbA^{EctD11E}$ 193
6.2.8 Identification of FBA sub-cellular localisation in MC58Δ*cbbA* $cbbA^{Ect}$ and MC58Δ*cbbA* $cbbA^{EctA14V}$ 195
6.2.9 Identification of FBA sub-cellular localisation in MC58Δ*cbbA* $cbbA^{Ect}$ and MC58Δ*cbbA* $cbbA^{EctD11E}$ 198
6.3 Discussion 201
7 Chapter Seven: General Discussion 205
7.1 General discussion 206
7.1.1 Enolase 208
7.1.2 FBA 212
7.2 Proposed future work 214
7.3 General conclusion 216
Bibliography 218
Appendix A: Buffers and Reagents 281

List of Figures

Figure 1.1: The glycolytic pathway displaying the moonlighting functions of glycolytic enzymes identified in a range of organisms **29**

Figure 1.2: Multiple sequence alignment of several enolase amino acid sequences from different bacteria, which are *N. meningitidis* MC58, *S. pneumoniae* R6, *S. pyogenes* serotype M1, and *E. coli* K12 ..... **36**

Figure 1.3: Optimal alignment of protein sequences of enolase from *N. meningitidis* MC58 and enolase from *E. coli* **37**

Figure 1.4: Pneumococcal enolase model used to determine the positions of the conserved lysine residues of meningococcal enolase; K355, K337, and K297 ..... **39**

Figure 1.5: ISP (ABC) complex in *E. coli* **41**

Figure 1.6:.IISP and T4P. **44**

Figure 1.7: Yersinia injectisome..... **48**

Figure 1.8: Agrobacterium VirB/D4 IVSP translocation system. **52**

Figure 1.9: The two-partner secretion pathway..... **61**

Figure 1.10: The chromosomal organization of the three TPS systems of *N. meningitidis* H44/76. ..... **62**

Figure 1.11: The full-length trimeric autotransporter adhesin. **64**

Figure 1.12: Comparison between type Va and type Ve autotransporters **69**

Figure 1.13: Va and Vc autotransporters final assembly **70**

Figure 1.14: The similarities between VISP components and the tail of T4 bacteriophage **73**

Figure 3.1: Whole-cell ELISA of MC58 wild type (WT) or MC58Δ*cbbA* for FBA surface localisation **114**

Figure 3.2: Whole-cell ELISA of MC58 wild type, MC58Δ*cbbA*, and other mutants (MC58Δ*cbbA* $cbbA^{EctK354A}$ and MC58Δ*pilQ*Δ*porA*) for FBA surface localisation **116**

Figure 3.3: Whole-cell ELISA of MC58 wild type, MC58Δ*cbbA,* and other mutants (MC58Δ*skp*, MC58Δ*surA*, MC58Δ*skp*Δ*surA*, and MC58Δ*hlyB*) for FBA surface localisation **117**

Figure 3.4: Whole-cell ELISA of MC58 wild type and mutants (MC58Δ*skp*, MC58Δ*surA*, MC58Δ*skp*Δ*surA*, and MC58Δ*hlyB*) for enolase surface localisation **119**

Figure 3.5: Whole-cell ELISA of MC58 wild type and mutants (MC58Δ*skp*, MC58Δ*surA*, MC58Δ*skp*Δ*surA*, MC58Δ*pilQ*Δ*porA,* and MC58Δ*hlyB*) for peroxiredoxin surface localisation. .. **120**

Figure 4.1: (A) Agarose gel image. Lane 1: 1 kb ladder. Lane 2: the amplified *NMB1285* (1.3 kb). **(B)** pComp-1 vector map. **129**

Figure 4.2: (A) pComp1285 map **(B)** Restriction digestion of pComp1285 construct using *BamH*I and *Sal*I..... **130**

Figure 4.3: A sequence from pComp1285 showing the mutated AAA to GAA (K337E)..... **131**

Figure 4.4: The two sets of primers used for confirming the complemented MC58 strains (pComp1285 + pComp1285-K337E) **132**

Figure 4.5: Agarose gel image showing **(A)** Confirmation of the insertion of pComp1285 into *rpsL*^{R} MC58 **(B)** Confirmation of the insertion of pComp1285-K337E into *rpsL*^{R} MC58..... **133**

Figure 4.6: Immunoblot analysis of the whole cell lysates of the complemented strains compared to the wild type using RαEnolase..... **134**

Figure 4.7: (A) Agarose gel image showing the amplified 3.3 kb *NMB1285* with the flanking sequences **(B)** pBA1 construct. **135**

Figure 4.8: Restriction digestion of pBA1 with *Sal*I and *BamH*I. **136**

Figure 4.9: Restriction digestion of pBA2 construct using *Bgl*II. **137**

Figure 4.10: (A) The construct formed from the assembly of *Kan*^{R} and *rpsL*^{S} fragments (*KnsL*), *NMB0342* upstream sequence, *NMB0342* downstream sequence, and the vector pUC19 using Gibson assembly reaction **(B)** *N. meningitidis* MC58Δ*NMB0342 Kan*^{R} *rpsL*^{S} strain. **138**

Figure 4.11: (A) Amplified *KnsL* cassette **(B)** *BamH*I-digested pGEM-*KsnL* **139**

Figure 4.12: (A) pBA3 map **(B)** Restriction digestion of pBA3 using *Sal*I and *EcoR*I **140**

Figure 4.13: First double recombination event in the process for construction of a markerless mutation in enolase using a construct containing a *knsL* cassette **141**

Figure 4.14: The three sets of primers used for verification of the first transformation of pBA3 into the two MC58 *rpsL*^{R} complemented strains..... **142**

Figure 4.15: (A) Verification of the 1st transformation of pBA3 construct into MC58 *rpsL*^{R} complemented strain (wild type ectopic enolase) **(B)** Verification of the 1st transformation of

the pBA3 construct into MC58 *rpsL*^{R} complemented strain (K337E mutant ectopic enolase)..... **143**

Figure 4.16: The second double recombination event in the process for construction of a markerless mutation in enolase using a construct containing a deleted enolase gene **144**

Figure 4.17: Primers used for verification of the second transformation of pBA2 into the two MC58 *rpsL*^{R} complemented strains..... **145**

Figure 4.18: (A) Verification of the 2nd transformation of pBA2 construct into MC58 *rpsL*^{R} complemented strain (wild type ectopic enolase) containing the *KnsL* cassette **(B)** Verification of the 2nd transformation of the pBA2 construct into MC58 *rpsL*^{R} complemented strain (K337E mutant ectopic enolase) containing the *KnsL* cassette..... **146**

Figure 4.19: Immunoblot analysis of the whole cell lysates of the complemented strains containing wild type or K337E ectopic enolase compared to the wild type MC58 using RαEnolase. **147**

Figure 5.1: Whole-cell ELISA of MC58 wild type (WT), MC58Δ*NMB1285* *NMB1285*^{Ect} and MC58Δ*NMB1285* *NMB1285*^{EctK337E} for enolase surface localisation.. ..... **159**

Figure 5.2: Growth profiles of MC58 wild type, MC58Δ*NMB1285 NMB1285*^{Ect}, and MC58Δ*NMB1285 NMB1285*^{Ectk337E} under laboratory conditions **161**

Figure 5.3: Whole-cell ELISA showing the binding of plasminogen to MC58 wild type, MC58Δ*NMB1285 NMB1285*^{Ect}, and MC58Δ*NMB1285 NMB1285*^{Ectk337E} with/without the addition of ACA (α-amino caproic acid) **162**

Figure 5.4: Optimal alignment of nucleotide sequences of a section of pComp1285 and pComp1285SD vectors..... **163**

Figure 5.5: Agarose gel image showing the correct insertion of pComp1285SD into *rpsL*^{R} MC58..... **164**

Figure 5.6: (A) Verification of the 1st transformation of pBA3 construct into MC58 *rpsL*^{R} complemented strain to produce MC58 *rpsL*^{R} Δ*NMB1285 NMB1285SD*^{Ect} *Kan*^{R} *rpsL*^{S} **(B)** Verification of the 2nd transformation of the pBA2 construct into MC58 *rpsL*^{R} complemented strain containing the *KnsL* cassette to produce MC58 *rpsL*^{R} Δ*NMB1285 NMB1285SD*^{Ect}. **166**

Figure 5.7: Immunoblot analysis of the whole cell lysates of the complemented strain (MC58 *rpsL*^{R} Δ*NMB1285 NMB1285SD*^{Ect}) containing wild type ectopic enolase compared to the wild type MC58 using RαEnolase **167**

Figure 5.8: Agarose gel image showing the annealed product of the two complementary DNA oligonucleotides, SynOligo F3 and SynOligo R3. ..... **169**

Figure 5.9: Agarose gel image showing the correct insertion of pComp1285porAp into MC58 *rpsL*^{R} **169**

Figure 5.10: (A) Verification of the 1st transformation of pBA3 construct into MC58 *rpsL*^{R} complemented strain to produce MC58 *rpsL*^{R} *ΔNMB1285 NMB1285PP*^{Ect} *Kan*^{R} *rpsL*^{S} **(B)** Verification of the 2nd transformation of the pBA2 construct into MC58 *rpsL*^{R} complemented strain containing the *KnsL* cassette to produce MC58 *rpsL*^{R} *ΔNMB1285 NMB1285PP*^{Ect}. **171**

Figure 5.11: Immunoblot analysis of the whole cell lysates of the complemented strain (MC58 *rpsL*^{R} *ΔNMB1285 NMB1285PP*^{Ect}) containing wild type ectopic enolase compared to the wild type MC58 using RαEnolase **172**

Figure 6.1: The protein sequence of *N. meningitidis* MC58 FBA. Highlighted in red is the predicted cleavage site. **182**

Figure 6.2: The two sets of primers used for confirming the complemented MC58 strain MC58Δ*cbbA cbbA*^{EctA14V} **182**

Figure 6.3: Verification of the transformation of pFS4-A14V construct into MC58Δ*cbbA* **183**

Figure 6.4: Whole-cell ELISA of MC58 wild type, MC58Δ*cbbA*, MC58Δ*cbbA* *cbbA*^{Ect} and MC58Δ*cbbA* *cbbA*^{EctA14V} for FBA surface exposure..... **184**

Figure 6.5: Immunoblot of the whole cell lysates of MC58 wild type, MC58Δ*cbbA*, MC58Δ*cbbA* *cbbA*^{Ect} and MC58Δ*cbbA* *cbbA*^{EctA14V} probed with anti-FBA..... **186**

Figure 6.6: Verification of the transformation of pFS4-A13V, pFS4-A13VA14V, pFS4-A22V, and pFS4-D11E constructs into MC58Δ*cbbA*..... **188**

Figure 6.7: Whole-cell ELISA of MC58 wild type, MC58Δ*cbbA*, MC58Δ*cbbA* *cbbA*^{Ect}, MC58Δ*cbbA* *cbbA*^{EctA14V}, MC58Δ*cbbA* *cbbA*^{EctA13V}, MC58Δ*cbbA* *cbbA*^{EctA14VA13V}, MC58Δ*cbbA* *cbbA*^{EctA22V}, and MC58Δ*cbbA* *cbbA*^{EctD11E} for FBA surface exposure **190**

Figure 6.8: (A) Immunoblot of the whole cell lysates of MC58 wild type, MC58Δ*cbbA*, MC58Δ*cbbA* *cbbA*^{Ect}, MC58Δ*cbbA* *cbbA*^{EctA14V}, MC58Δ*cbbA* *cbbA*^{EctA13V}, MC58Δ*cbbA* *cbbA*^{EctA13VA14V}, MC58Δ*cbbA* *cbbA*^{EctA22V}, and MC58Δ*cbbA* *cbbA*^{EctD11E} probed with anti-FBA **(B)** Protein densitometry of MC58Δ*cbbA* *cbbA*^{EctA14V}, MC58Δ*cbbA* *cbbA*^{EctA13V}, MC58Δ*cbbA* *cbbA*^{EctA13VA14V}, MC58Δ*cbbA* *cbbA*^{EctA22V}, and MC58Δ*cbbA*

cbbA^{EctD11E} using the control condition (MC58Δ*cbbA* *cbbA*^{Ect}). **192**

Figure 6.9: Growth profiles of MC58 wild type, MC58Δ*cbbA,* MC58Δ*cbbA* *cbbA*^{Ect} MC58Δ*cbbA* *cbbA*^{EctA14V,} MC58Δ*cbbA* *cbbA*^{EctA13V}, MC58Δ*cbbA* *cbbA*^{EctA14VA13V}, MC58Δ*cbbA* *cbbA*^{EctA22V}, and MC58Δ*cbbA* *cbbA*^{EctD11E} under laboratory conditions **194**

Figure 6.10: (A) Immunoblot of cellular fractions; periplasmic-cytosolic protein enriched (PCP), cytoplasmic membrane protein-enriched (CMP), and outer-membrane protein-enriched (OMP) fractions collected from MC58Δ*cbbA* *cbbA*^{Ect} and MC58Δ*cbbA* *cbbA*^{EctA14V} probed with anti-FBA antibody **(B)** Immunoblot of cellular fractions; PCP, CMP, and OMP fractions collected from MC58Δ*cbbA* *cbbA*^{Ect} and MC58Δ*cbbA* *cbbA*^{EctA14V} probed with anti-PorA antibody **196**

Figure 6.11: Protein densitometry of the outer-membrane protein-enriched (OMP) fraction collected from MC58Δ*cbbA* *cbbA*^{Ect} and MC58Δ*cbbA* *cbbA*^{EctA14V}. **197**

Figure 6.12: (A) Immunoblot of cellular fractions; periplasmic-cytosolic protein enriched (PCP), cytoplasmic membrane protein-enriched (CMP), and outer-membrane protein-enriched (OMP) fractions collected from MC58Δ*cbbA* *cbbA*^{Ect}

and MC58Δ*cbbA* *cbbA*^{EctD11E} strains probed with anti-FBA antibody **(B)** Immunoblot of cellular fractions; PCP, CMP, and OMP fractions collected from MC58Δ*cbbA* *cbbA*^{Ect} and MC58Δ*cbbA* *cbbA*^{EctD11E} probed with anti-PorA antibody... **199**

Figure 6.13: Protein densitometry of the outer-membrane protein-enriched (OMP) fraction collected from MC58Δ*cbbA* *cbbA*^{Ect} and MC58Δ*cbbA* *cbbA*^{EctD11E}. **200**

List of Tables

Table 2.1: Bacterial strains used in work described in this study **81**

Table 2.2: Plasmids used in this study **87**

Table 2.3: Antibodies used in this study **90**

Table 2.4: Primers used in this study **93**

Table 2.5: Standard PCR amplification program for Phusion High Fidelity DNA polymerase **99**

Table 2.6: Standard PCR amplification program for *Taq* DNA Polymerase **99**

Table 2.7: Q5 Site-directed mutagenesis cycling conditions . **100**

Abbreviations

ABC	ATP-binding cassette
AC	Autochaperone domain
App	Adhesion and penetration protein
AP	Alkaline phosphatase
AspA	Autotransported serine protease A
ATP	Adenosine triphsphate
Bam	β-barrel assembly machinery
BHI	Brian Heart Infusion
bp	Base pair
BSA	Bovine serum albumin
Cbp	Chitin-binding protein
CM-ABC	Cytoplasmic-membrane ABC transporters
CP	Calprotectin
DNA	Deoxy ribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
EU/EEA	European Union and European Economic Area
FACS	Fluorescent-activated cell sorter
FBA	Fructose-1, 6-bisphosphate aldolase
fHbp	Factor H binding Protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HRP	Horseradish peroxidase
IgA	Immunoglobulin A
IMD	Invasive meningococcal disease
Kan	Kanamycin
kb	Kilo base pair
kDa	Kilo Dalton
LamR	Laminin receptor

LB	Lysogeny Broth
LOS	Lipooligosaccharides
MCC	Meningococcal C conjugate
MenB	Serogroup B meningococci
MenC	Serogroup C meningococci
MenX	Serogroup X meningococci
MenZ	Serogroup Z meningococci
MFP	Membrane fusion proteins
MLEE	Multilocus enzyme electrophoresis
MspA	Meningococcal serine protease A
NadA	Neisseria adhesion A
NalP	Neisseria autotransporter lipoprotein
NCBI	National Center for Biotechnology Information
NHBA	Neisseria heparin binding antigen
Nhha	Neisseria hia homologue
OD	Optical density
OMP	Outer membrane protein
OMV	Outer membrane vesicle
Opa	Colony opacity factor
ORF	Open reading frame
PBS	Phosphate Buffered Saline
PBST	PBS with 0.05% Tween-20
PCR	Polymerase chain reaction
PorA	Porin A
PorB	Porin B
POTRA	Periplasmic polypeptide transport-associated
RTX	Repeat in toxin
SD	Shine-Dalgarno
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Sm	Streptomycin
SP	Signal peptide

TAAs	Trimeric autotransporter adhesins
Taq	*Thermus aquaticus*
TPI	Triose phosphate isomerase
T4P	Type IV Pili
TPS	Two-partner secretion system
ISP	Type I secretion pathway
IISP	Type II secretion pathway
IIISP	Type III secretion pathway
IVSP	Type IV secretion pathway
VSP	Type V secretion pathway
VISP	Type VI secretion pathway
VIISP	Type VII secretion system

1 Chapter One: General Introduction

1.1 The genus *Neisseria*

Neisseria are Gram-negative cocci that belong to the family Neisseriaceae, which also includes the *Kingella* and *Eikenella* genera. Based on 16S rRNA gene sequence, the genus *Neisseria* includes 25 species (Hung and Christodoulides 2013). Out of the 25 species, two are obligate human pathogens, namely *Neisseria meningitidis* (meningococci) and *Neisseria gonorrhoeae* (gonococci) (Tobiason and Seifert 2010). Both species share genetic and antigenic similarities, however, they are distinguished by their niche preference, the nature of the diseases they cause, and the expression of surface polysaccharide capsule (Caugant and Maiden 2009; Virji 2009). *N. meningitidis* colonises the mucosal membrane of the human nasopharynx and is the worldwide leading cause of bacterial meningitis and sepsis, whereas *N. gonorrhoeae* inhabits the mucosal surfaces of the urogenital tract and causes gonorrhoea - one of the most common sexually transmitted infections (Maiden 2008; Wetten *et al.* 2015).

Neisseria lactamica, closely related to *N. meningitidis,* is the best-studied commensal *Neisseria* species, which colonises the human nasopharynx and shares sequence homology with pathogenic *Neisseria* species (Maiden 2008; Tobiason and Seifert 2010). There has been some evidence that protection against

meningococcal infection can arise from the carriage of *N. lactamica,* which is even more potent than after the administration of the quadrivalent ACWY glycoconjugate vaccine (Evans *et al.* 2011; Read *et al.* 2014; Deasy *et al.* 2015). In 1906, von Lingelsheim described *Neisseria cinerea*, which was the first isolated commensal *Neisseria* colonising the human nasopharynx (Von Lingelsheim 1906). Other commensal, often opportunistic, *Neisseria* species were also described by von Lingelsheim namely *Neisseria sicca*, *Neisseria flava* and *Neisseria subflava* (Liu *et al.* 2015).

1.2 Neisseria meningitidis

N. meningitidis is a Gram-negative, aflagellate, non-sporing diplococcus (0.8 µm in diameter). It grows on blood, chocolate, or modified New York City agar as a grey, convex, transparent, non-pigmented, and non-haemolytic colony. Depending on the length of incubation, the colony size ranges from 0.5-5 mm in diameter. It grows optimally at 35-37 °C with 5-10% CO_2 in a moist environment, at pH 7.0-7.4. It is oxidase and catalase positive. It utilises glucose and maltose, but not lactose, sucrose, or fructose (Ala'aldeen and Turner 2006).

It is an obligate human pathogen and the main causative agent of two life-threatening diseases: meningitis and sepsis (Tzeng and Stephens 2000). The meningococcus is mainly transmitted from person to person by direct contact or aerosols and colonises the epithelial surfaces of the nasopharynx asymptomatically in around 10% of the population. The meningococcal envelope consists of the inner membrane, the periplasmic space, and the outer membrane, which can be surrounded by a polysaccharide capsule (Tzeng and Stephens 2000; Caugant and Maiden 2009). *N. meningitidis* uses several secretion systems (type I, type Va, type Vb, and type Vc) for secreting proteins, which enable the bacteria to modulate their cell surface and evade the human immune system (Woude and Bäumler 2004).

1.3 Classification of *N. meningitidis*

Based on the capsular polysaccharides, meningococci are primarily classified into twelve serogroups. Out of the twelve serogroups, six (A, B, C, W, X, and Y) cause the majority of meningococcal diseases (Harrison *et al.* 2009). *N. meningitidis* is further classified into 20 serotypes and 10 serosubtypes based on the major outer-membrane porins and into 12 immunotypes based on the LOS (lipooligosaccharide) (Frasch *et al.* 1985;

Ala'aldeen and Turner 2006). Based on the presence of the isoforms of cytosolic enzymes with variable molecular weights and their electrophoretic mobility, another method of classification, multilocus enzyme electrophoresis (MLEE) has also been used (Achtman 1994). The MLEE electrotyping scheme has established that there are subdivisions of genetically highly diverse but related strains of serogroup A to subgroups and serogroup B and C to lineages and clonal complexes. However, only a few clonal complexes cause the majority of cases of meningococcal disease. An additional typing scheme, multilocus sequence typing (MLST), based on the nucleotide sequence of seven genes encoding house-keeping enzymes, serves the same purpose but has a greater distinguished power and is technically less demanding to perform. Whole genome sequencing can also be performed to search genes that are specific for disease isolates, to analyse chromosomal structure variation and to define the evolutionary relationship between the sequenced carriage and disease strains (Schoen *et al.* 2008).

1.4 Epidemiology of invasive meningococcal disease

Invasive meningococcal disease (IMD) is caused by *N. meningitidis,* which is the worldwide leading cause of potentially life-threatening meningitis and sepsis (Rosenstein *et al.* 2001). It leads to death in about 50–80% of untreated cases (Manchanda *et al.* 2006; CDC 2017). Furthermore, 12–20% of IMD survivors may suffer from neurological sequelae, loss of vision, paralysis, deafness, amputations, lung or kidney damage, epilepsy, and memory loss (Pollard 2004; Al-Tawfiq *et al.* 2010; Abio *et al.* 2013). In 1661, Thomas Willis was the first to describe an epidemic of meningitis. In 1805, Gaspard Vieusseux was the first to report an epidemic meningitis in Geneva city, describing it as a non-contagious malignant cerebral fever. Anton Weichselbaum first isolated the causative agent of epidemic meningitis in 1887, which was named "*Diplococcus intracellularis meningitidis"* (Schmitz and Stratton 2015).

Six meningococcal serogroups (A, B, C, W, Y, and X) cause the majority of IMD cases worldwide. However, they display a variable geographical distribution, which may change over time. Epidemics of IMD in Europe are highly associated with serogroups A and B (Harrison *et al.* 2009; Kriz *et al.* 2011; Halperin *et al.* 2012). In the late 1990s, there was an increase

in the incidence of serogroup C in several European countries (Gray *et al.* 2006; Trotter and Ramsay 2007; Bijlsma *et al.* 2014). In 1999, meningococcal C conjugate (MCC) vaccine was introduced into the childhood immunisation programme in the United Kingdom followed by 13 countries of the European Union and European Economic Area (EU/EEA) (ECDP). In addition, another MCC programme was introduced in adolescents and young adults in some countries (ECDP ; EU-IBIS 2006; Trotter and Ramsay 2007). As a consequence, many European countries subsequently experienced a decline in serogroup C IMD cases (Larrauri *et al.* 2005; Campbell *et al.* 2010; Borrow *et al.* 2013; Bijlsma *et al.* 2014; Garrido-Estepa *et al.* 2014). Several reports over the last 10 years have shown an increase in serogroup Y and W cases and a decline in serogroup B cases (Hellenbrand *et al.* 2013; Bijlsma *et al.* 2014; Broker *et al.* 2015; Ladhani *et al.* 2015; Schurink-van 't and de Melker 2017). 4CMenB vaccine has been introduced into the routine childhood vaccinations in the United Kingdom in September 2015 and in Ireland in October 2016 (ECDP). Since 2011, Czech Republic, Greece, and Austria introduced a quadrivalent meningococcal conjugate vaccine (MenACWY) in adolescents. While in the United Kingdom, MenACWY vaccine has been offered routinely to adolescents since 2015 (ECDP ; Wise 2015).

1.5 Prevention of meningococcal diseases: vaccines against *N. meningitidis*

Three groups of vaccines against *N. meningitidis* have been identified: polysaccharide vaccines (MPSV); polysaccharide-protein conjugated vaccines; and relatively new vaccines against serogroup B based on recombinant outer membrane proteins (Hedari *et al.* 2014).

1.5.1 Polysaccharide vaccines

The polysaccharide vaccines (MPSV) were first developed against serogroups A and C in the 1970s and used among military recruits in the United States (Girard *et al.* 2006; Joshi *et al.* 2009). Subsequently, MPSV has been licensed for older children and adults as bivalent (serogroups A and C), trivalent (serogroups A, C, and W), or tetravalent (serogroups A, C, Y, and W) vaccines (Shao *et al.* 2009; Croxtall and Dhillon 2012; Granoff *et al.* 2013). Nevertheless, MPSVs were proven to have poor immunogenicity in infants providing short-lived protection. Therefore, especially with serogroup C, repeated vaccinations were required, which might lead to a reduced response to the vaccine (Tan *et al.* 2010). In addition, MPSVs have shown an insignificant impact on nasopharyngeal carriage. Therefore, they do not inhibit meningococcal transmission (Jodar *et al.* 2002;

Poolman and Borrow 2011; Dbaibo *et al.* 2013). No IMD outbreaks has been reported in the Middle East since 2002 during Hajj season, as a result of the quadrivalent polysaccharide meningococcal vaccine introduction as a visa requirement (Wilder-Smith 2008).

1.5.2 Conjugate polysaccharide vaccines

The polysaccharides of meningococci were covalently linked to a carrier protein to generate the meningococcal conjugate vaccines. These generate a T-cell dependent immune response, thus their immunogenicity has been improved over the polysaccharide vaccines (Lesinski and Westerink 2001a; Lesinski and Westerink 2001b). It has been suggested that the meningococcal C conjugate (MCC) vaccines are the preferred approach for providing optimal protection and for use in meningococcal C outbreak control (Richmond *et al.* 2000). The Meningitis Vaccine Project (MVP) was established in 2001 to eliminate epidemics of serogroup A meningitis in Africa through developing more effective and affordable vaccines than polysaccharide vaccines (LaForce *et al.* 2007). A PsA-TT (MenAfriVac, Serum Institute of India), cost effective serogroup A meningococcal conjugate vaccine, was tested to evaluate the immunogenicity of a single dose compared with a licensed polysaccharide vaccine (PsACWY, Mencevax ACWY, GlaxoSmith-

Kline) (Sow *et al.* 2011). Both vaccines had a similar safety profile, however, PsA-TT provided a stronger response against serogroup A meningococci. Since PsA-TT vaccine also had the ability to induce herd immunity, it could potentially decrease epidemics of serogroup A meningitis in Africa (Sow *et al.* 2011).

1.5.3 Meningococcal group B vaccines

Since the 1960s, there was no available vaccine for serogroup B. Despite the decades of research, the development of a global serogroup B vaccine has been a problem (Frasch and Robbins 1978; Frasch 1985; Bjune *et al.* 1991; Boslego *et al.* 1995; Bruge *et al.* 2004; Feiring *et al.* 2006; Sadarangani and Pollard 2010). The main reason for this is that the serogroup B polysaccharide cross reacts immunologically with human neurologic tissue (Wyle *et al.* 1972; Finne *et al.* 1983; Griffiss *et al.* 1991). Therefore, an alternative approach was evolved to develop a meningococcal serogroup B vaccine using outer membrane protein antigens.

PorA protein is considered as a good candidate for protein-based meningococcal serogroup B vaccine development (Peeters *et al.* 1996; van der Voort *et al.* 1996; S.L. Martin *et al.* 2000). This feature resulted from the fact that PorA-specific monoclonal antibodies are highly bactericidal *in vitro* (Saukkonen *et al.*

1987). Furthermore, the PorA mutant strains showed a reduction in the bactericidal antibodies level in mice. However, a full bactericidal response was retrieved in a heterologous strain containing one transferred *porA* gene. Van der Ley *et al.* indicated that there are some limitations in the efficiency of PorA-based vaccines due to PorA antigenic variations of its expression (random on/off switching) (van der Ley *et al.* 1993). Therefore, in the United States, at least twenty PorA serosubtypes would have to be included in a polyvalent PorA-based vaccine to achieve approximately 80% coverage of strains that cause sporadic meningococcal disease (Tondella *et al.* 2000).

Lipoprotein 2086 (LP2086), also identified as factor H-binding protein (fHbp), is a vaccine candidate that was first identified by detergent extraction, protein purification, and reverse vaccinology (Rappuoli 2000; Bernfield *et al.* 2002; Masignani *et al.* 2003; Fletcher *et al.* 2004). Bivalent rLP2086 (Trumenba®; MenB-FHbp) and 4CMenB (Bexsero®; MenB-4C) are two fHBP-based serogroup B vaccines (Gandhi *et al.* 2016). These vaccines differ in their composition. Bivalent rLP2086 vaccine is composed of fHBP A05 and B01 variants from each of the fHBP subfamily A and subfamily B. In 2014, it was licensed for meningococcal B disease prevention in the United States, the preferred age for

vaccination is 10–25 years (Package Insert - TRUMENBA 2014). Two-and-a-half years later, Bivalent rLP2086 vaccine was licensed in Europe then in the UK for meningococcal B disease prevention (Pfizer EU approval 2017; Trumenba UK approval 2017).

Three recombinant proteins identified by reverse vaccinology, NHBA (Neisseria heparin binding antigen) and fHbp (factor H binding protein) and Neisseria adhesin A (NadA) protein along with outer membrane vesicles (OMVs) are the components of the 4CMenB vaccine (Capecchi *et al.* 2005; Madico *et al.* 2006; Scarselli *et al.* 2006; Schneider *et al.* 2006). OMVs in this vaccine contain PorA (P1.4), and are derived from strain NZ98/254 used for meningococcal B clonal outbreak control in New Zealand (Arnold 2010). It has been proven that 4CMenB vaccine is safe for infants and adults and induces a strong immune response against strains expressing one or more of the vaccine antigens (Donnelly *et al.* 2010; Donnelly *et al.* 2011; Rappuoli 2011; Seib *et al.* 2012). 4CMenB vaccine was licensed in Europe, the UK, Australia, Canada, and the US (Novartis EU approval for Bexsero® 2013; MenB vaccine - NHS 2014; Australian public assessment report 2014; Canadian Immunization Guide 2014; U.S. (FDA) Approval Letter - BEXSERO 2015).

1.6 Meningococcal pathogenesis

1.6.1 Meningococcal Colonisation and Adhesion

Colonisation is the first step in the pathogenesis of an invasive organism in susceptible individuals, which contributes to its survival and propagation (Smith 1998; Corbett *et al.* 2004). In the natural life cycle, replication of a pathogen follows the colonisation of the host tissues, which allows it to disseminate to other tissues and cause disease. However, this event is accidental and irrelevant to the natural evolution of the organism (Bull 1994). Meningococcal interaction with host cells is mediated by the expression of adhesins, which include three major surface structures: type IV pili, Opc, and Opa. Several minor adhesins have also been identified (Corbett *et al.* 2004; Hill and Virji 2012). Adhesion of *N. meningitidis* to epithelial and endothelial cells occurs in two steps. The first step is initial adhesion, in which the bacteria attach to the host cell surface via type IV pili. Then, following pilus retraction and engagement of other surface receptors, a close contact of the attached bacteria with the surface of host cells takes place (intimate adhesion) (Deghmane *et al.* 2002). CrgA may play a major role in the regulation of the meningococcal intimate adhesion to the target cells (Deghmane *et al.* 2000).

1.6.2 Phase and antigenic variation

The reversible phase switch between an "all-or-none" or "on/off" expression is referred to as phase variation, which results in variation in the expression of one or more proteins between individual cells of a clonal population. On the other hand, the change in the expression of antigenically distinct conserved moieties in a clonal population is referred to as antigenic variation. A classic example is the antigenic variation of pilin, which occurs as a results of non-reciprocal recombination between one of multiple *pilS* silent loci and *pilE* locus (Hagblom *et al.* 1985). Phase variable structures of the bacterium, including a variety of surface-exposed proteins and capsule, are commonly found on the meningococcal cell surface, where they would be exposed to the environment and the host immune system. The expression of the opacity proteins (Opa) of *N. gonorrhoeae* and *N. meningitidis* is subject to phase variation, as well as the expression of PorA. Since PorA is one of the protein-based vaccine components of 4CMenB, this variation in some cases affects vaccine efficacy (Stern and Meyer 1987; van der Ende *et al.* 2000; Clarke *et al.* 2003). In addition, the gonococcal siderophore receptor FetA, the two meningococcal hemoglobin receptors (HpuAB and HmbR), and the two meningococcal autotransporters (NalP and MspA), are subject to

phase variation (Lewis *et al.* 1999; Carson *et al.* 2000; Turner *et al.* 2002; van Ulsen *et al.* 2003; Turner *et al.* 2006; van Ulsen *et al.* 2006).

1.7 Virulence Factors of *N. meningitidis*

1.7.1 Polysaccharide Capsule

Meningococcal capsule is categorised as group II capsule (Roberts 1996; Whitfield and Roberts 1999). The capsule expressed by serogroup A, which is composed of (α1→6)-linked N-acetylmannosamine-6-phosphate, does not contain sialic acid (Liu *et al.* 1971b). On the other hand, the capsular polysaccharide of serogroups B, C, Y and W are composed of sialic acid. The capsule expressed by serogroups B and C is composed of (α2→8) or (α2→9) linkages of sialic acid entirely (Liu *et al.* 1971a). However, the capsule expressed by serogroups Y and W is composed of sialic acid and D-glucose (Y) or D-galactose (W) alternating units (Bhattacharjee *et al.* 1976). The host innate immune system is composed of cationic antimicrobial peptides (CAMPs) that are expressed by the epithelial cells upon exposure to bacteria in order to defend against microbes (Hancock 2001). Spinosa *et al.* suggested that the resistance to CAMPs may be promoted by the capsule to

prevent meningococcal degradation intracellularly. Furthermore, this mechanism involves two capsular genes (*siaD* and *lipA*) demonstrating up regulation following the exposure to CAMPs (Spinosa *et al.* 2007).

1.7.2 Type IV Pili (T4P)

Pili are hair-like filamentous surface structures, which are essential for bacterial attachment and biofilm formation on target cells. They are composed of long thin polymers of pilin proteins (Soto and Hultgren 1999; Bernard *et al.* 2014; Kolappan *et al.* 2016). Based on their morphology and assembly, they can be classified into several types, however, T4P are the best studied and the most widespread among both Gram-positive and Gram-negative bacteria (Pelicic 2008). T4P are thin (5–8 nm in width) filaments that interact with CD147 receptor to promote adhesion and with b2A receptor to enhance host cell signalling cascades then subsequent recruitment of GRK2 (Beta-adrenergic receptor kinase 1) and β-arrestins, in order to facilitate the crossing through the blood brain barrier (BBB) (Craig *et al.* 2004; Coureuil *et al.* 2010; Bernard *et al.* 2014). T4P also promote other phenotypes, such as twitching motility and DNA uptake (Mattick 2002). The assembly and disassembly of T4P filaments requires a traffic ATPase, which in *Neisseria* is PilF and PilT, respectively (Sakai *et al.* 2001; Crowther *et al.* 2004). Two

subtypes of T4P, T4PA and T4PB, have been defined based on lengths of the leader peptide and the mature protein (Pelicic 2008). The major pilin PilE of *N. meningitidis* share about 78% sequence resemblance with PilE from *N. gonorrhoeae* (Potts and Saunders 1988). The hypervariable region (residues ~ 127 to 146) amongst *N. meningitidis* type IV pilins is a highly variable region that represents the most distinguished segment of the D-region, the β-hairpin loop and β6 plus a part of the following loop (Miller *et al.* 2014).

1.7.3 Outer-Membrane Proteins (OMPs)

1.7.3.1 PorA porin

Class I OMP, named PorA, is expressed by most meningococcal isolates (Hitchcock 1989). PorA of *N. meningitidis* is an outer membrane trimeric porin. Abouseada *et al.* investigated the surface-exposed loop structures of PorA of *N. meningitidis* and OmpP2 of *Haemophilus influenzae* that are required for laminin receptor (LamR)-binding (Abouseada *et al.* 2012). Their data suggest that amino acids 171–240 of PorA and amino acids 91–99 of OmpP2 show significantly increased LamR-binding. Subsequently, the predicted surface-exposed loops were mapped to the LamR-binding regions for both PorA and OmpP2. Based on their sequences, synthetic peptides were used to

confirm the importance of these loops. It has been reported that the binding of LamR to full-length PorA could be inhibited by PorA loop 4. Peptides based on PorA loop 4 or OmpP2 loop 2 have been shown to bind LamR, however, peptides based on PorA loop 1 bound less well (Abouseada *et al.* 2012).

1.7.3.2 PorB porin

PorB is the second major antigenically variable porin that is expressed by all meningococci, either as class II or class III OMP (Tsai *et al.* 1981). Unlike PorA, PorB is essential for antimicrobial susceptibility since the lack of PorB expression decreases the sensitivity to several antibiotics, such as ceftazidime, ciprofloxacin, cefotaxime, cefsulodin, cephalothin, tetracycline, and doxycycline (Tommassen *et al.* 1990; Peak *et al.* 2014). In addition, a PorB mutant showed a slight growth impairment *in vitro* (Tommassen *et al.* 1990). Meningococcal PorB was shown to interact with mitochondria in host cells leading to an anti-apoptotic effect, which is dependent on the nitrosative stress (Massari *et al.* 2000; Massari *et al.* 2003; Tunbridge *et al.* 2006). Nevertheless, it was suggested that the role of porins is not significant for apoptosis inhibition considering the involvement of other bacterial factors in this process (Peak *et al.* 2014).

1.7.3.3 Neisseria hia homologue A (NhhA)

NhhA, a homologue of Hia and Hsf of *H. influenzae* and AIDA-I of *E. coli*, is a highly conserved meningococcal outer membrane autotransporter protein composed of 590 amino acids (Benz and Schmidt 1992; Barenkamp and St Geme 1996; Peak *et al.* 2000). Several studies have demonstrated that NhhA is involved in bacterial adhesion, nasopharyngeal colonisation, and disease progression (Scarselli *et al.* 2006; Sjolinder *et al.* 2008). In contrast, NhhA was shown to be essential neither for the meningococcal invasion nor for the induction of host inflammatory response. All the data confirm the pathological roles of NhhA demonstrating a major role in meningococcal protection against complement-mediated killing and phagocytosis (Sjolinder *et al.* 2008). Furthermore, it has been demonstrated that NhhA decreases the phagocytic activity of macrophages by promoting caspase-dependent apoptosis, which might contribute to the severity of meningococcal disease (Sjolinder *et al.* 2012). Wang *et al.* confirmed the ability of meningococcal NhhA to modulate the host immune response through macrophage (Mφ) and monocyte (Mo) differentiation in vivo using a murine model. Upon meningococcal stimulation, NhhA-derived Mφ phenotype was found to reduce inflammatory

response, however, it could be involved in engulfing and eliminating bacteria (Wang *et al.* 2016).

1.7.3.4 Opacity proteins or class V (Opa and Opc)

Colony-opacity-associated (Opa) outer membrane proteins are required for optimal Neisserial adhesion to host epithelial and endothelial cells, and polymorphonuclear neutrophils. CD66-epitope carcinoembryonic-antigen members of human cell adhesins' family (CEACAM) mediate this adhesion (Swanson 1978; Virji *et al.* 1993; Dehio *et al.* 1998). However, these are not the only human receptors for Opa proteins as has been suggested that they recognise other human receptors, such as surface-associated heparan sulfate proteoglycans (HSPGs) (Chen *et al.* 1995; van Putten and Paul 1995; Muenzner *et al.* 2000). There are multiple copies of *opa* genes in *N. meningitidis* and *N. gonorrhoeae*, up to 4 and 11, respectively (Aho *et al.* 1991; Bhat *et al.* 1991; Malorny *et al.* 1998). Strains of distinct serogroups of *N. meningitidis* may express variant Opa proteins. Antigenic variation of Opa proteins arises from the independent switching on and off of distinct genes (Stern *et al.* 1986). In addition, this shift in the expression between *opa* genes can also arise from deletions, point mutations, and translocation from other Neisseriaceae members (Callaghan *et al.* 2006). Opc, another meningococcal class V protein, shares similar size and

physico-chemical properties with Opa proteins, but has structural differences (Achtman *et al.* 1988; Olyhoek *et al.* 1991; Uberos *et al.* 2015). Both *N. meningitidis* and *N. gonorrhoeae* express Opa proteins, whilst Opc is only expressed by meningococci. Opa proteins are structurally composed of a β-barrel, which consists of four surface-exposed loops. Similarly, Opc is a β-barrel protein but with five surface-exposed loops (Aho *et al.* 1991; Prince *et al.* 2002).

1.7.4 Lipooligosaccharide (LOS)

Meningococcal LOS, is composed of Lipid A, two KDOs (2-keto-3-deoxy-d-manno-2-octulosonic acids) and two heptose residues (HepI and HepII), comprising the inner core. Extending from Hep, are a heterogeneous and variable number of sugars, comprising the outer core. Antigenic variation in the HepI α-chain and HepII β-chain extensions are the determining factors in the classification of meningococci into different immunotypes (Jennings *et al.* 1999). The lipid A component (endotoxin) is generally known to be the primary cause of the induction of cytokine synthesis and subsequent septic shock (Hurley 1995). Nevertheless, a study suggested that LOS is not the only sole element for cytokine induction since there was no effect on cytokine production and mortality in mice when infected with a *N. meningitidis* LOS mutant. This confirms that non-LOS

components of meningococci may have a role in sepsis by inducing the production of NF-alpha, IL-1beta, and IFN-gamma (Sprong *et al.* 2001).

1.7.5 IgA1 Protease

Immunoglobulin A (IgA) antibodies are locally produced to provide a first line of defence upon commensal or pathogen mucosal contact, preventing adherence and colonisation of bacteria. They are divided into IgA1 and IgA2 secretory antibodies. In humans, the majority of the IgA antibodies, approximately 90%, are IgA1 subclasses (Engstrom *et al.* 1990). IgA1 protease, encoded by the *iga* gene, is a metal chelator-sensitive serine-type endopeptidase, which specifically cleaves Pro-Ser or Pro-Thr single peptide bonds within the hinge region of human IgA1 (Plaut *et al.* 1975; Bachovchin *et al.* 1990). Vidarsson *et al.* suggested that this cleavage releases the effector Fc domain of IgA1 in a great proportion from the bacteria. This results in the reduction of the binding affinity of IgA1 antibodies, promoting neissaerial evasion of opsonization (Vidarsson *et al.* 2005). Other targets of this protease have been identified, such as LAMP1 (Lysosome-Associated Membrane Protein 1) promoting the survival of *Neisseria* within epithelial cells. In addition, *Neisseria* IgA1 protease was previously reported to cleave p65/RelA subunit of NF-κB within the host

nucleus inducing apoptosis (Hauck and Meyer 1997; Lin *et al.* 1997; Senior *et al.* 2001; Ayala *et al.* 2002; Besbes *et al.* 2015). *H. influenzae* and *Streptococcus pneumoniae*, which are also common causative agents of bacterial meningitis, have been shown to produce a homologous serine IgA1 protease (Kilian *et al.* 1996).

Meningococcal IgA1 protease is a classical monomeric autotransporter, which consist of N-terminal signal sequence, a secreted passenger domain, and a C-terminal translocator domain (TD). The TD, a 12-stranded β-barrel, facilitates the secretion of the passenger domain across the outer membrane (Roussel-Jazede *et al.* 2011; Sauri *et al.* 2011; Grijpstra *et al.* 2013; van Ulsen *et al.* 2014). The protease domain, the γ-peptide and the α-peptide are the three domains that form the passenger domain (Pohlner *et al.* 1987). Studies have shown that the α-peptide domain can either bind extracellular DNA or heparin promoting biofilm formation while covalently or non-covalently associated with cell surfaces (Arenas *et al.* 2013; Roussel-Jazede *et al.* 2014).

1.7.6 Factor H Binding Protein (fHbp)

Lipoprotein 2086 (LP2086), also identified as genome-derived neisserial antigen 1870 (GNA1870), was named factor H-binding protein (fHbp) later on due to its binding to the regulatory protein factor H (fH) in the alternative complement pathway (Madico *et al.* 2006). Factor H (150 kDa), composed of 20 short consensus repeats (SCRs), has an important role in inhibiting complement activation to maintain homeostasis of the complement system and attachment to host cell surface (Rodriguez de Cordoba *et al.* 2004). *N. meningitidis* has the ability to capture fH on its surface, which binds fHbp via SCR6 to mimic human cells avoiding the complement-mediated killing (Schneider *et al.* 2006; Schneider *et al.* 2009). FHbp consists of N-terminal domain arranged in long loops and C-terminal domain arranged in a well-defined β-barrel. The C-terminal domain resembles lipocalins that are found in bacteria, fungi, plants, vertebrates, and invertebrates (Bishop 2000; Grzyb *et al.* 2006; Esposito *et al.* 2011). Lipocalins have been shown to bind siderophores, which are considered as virulence factors due to their strong affinity for ferric iron. This metal is necessary for the growth of all microorganisms during infection (Schalk et al. 2011). Veggi et al. suggested that fHbp has the affinity to bind

enterobactin *in vitro* confirming its involvement in iron uptake and bacterial survival (Veggi *et al.* 2012).

1.7.7 Minor Adhesins

NadA (Neisserial adhesin A) was described as a novel surface oligomeric protein of *N. meningitidis* B strain MC58 (Tettelin *et al.* 2000; Comanducci *et al.* 2002). NadA was proposed to be a good vaccine candidate due to the possibility of its involvement in bacterial adhesion to host cells. In addition, it was expressed by approximately 50% of invasive meningococcal strains existing in three of the four hypervirulent lineages (ET37, ET5, and cluster A4) and induces strong antibactericidal activity (Comanducci *et al.* 2002; Comanducci *et al.* 2004).

Another protein, App (adhesion and penetration protein) was also confirmed to be surface localised, immunogenic, and conserved among meningococci (Hadi *et al.* 2001). It is homologous to Hap (Haemophilus adhesion and penetration) protein of *H. influenza*e, which suggests that App could be involved in the meningococcal-host cells interactions. Both App and Hap are belonging to autotransporters' family possessing all the domains (N-terminal, passenger, and C-terminal domains) but with an additional conserved serine protease motif (GDSGSP) (van Ulsen *et al.* 2001). This motif has been shown

to cleave human IgA by the serine-type IgA proteases. It is also responsible for autocatalytic cleavage of the Hap protein at several sites (Hendrixson *et al.* 1997).

MspA (meningococcal serine protease A) protein, homologous to App, may also be secreted and expressed by the majority of meningococcal group B isolates. However, disease development is not completely dependent on MspA since some invasive strains harbour *mspA* mutations or lack the gene. This perhaps indicates that the function of MspA in these strains could be replaced by another protein. MspA, like App, is immunogenic and capable of producing a bactericidal response in humans as a result of invasive infection (Turner *et al.* 2006; Oldfield *et al.* 2013).

1.8 Moonlighting proteins

Campbell and Scanes introduced the term "moonlight" in 1995 due to the ability of a protein or, in this case, "neuropeptide" to have an immunomodulatory activity beside its neuronal signalling function (Campbell and Scanes 1995). Subsequently, a growing number of proteins have been identified to moonlight; hence the generic term "moonlighting proteins" is used for such proteins (Jeffery 1999; Jeffery 2009). Moonlighting proteins are multifunctional proteins in both prokaryotic and eukaryotic

organisms (Henderson and Martin 2011). Many of them are highly conserved metabolic enzymes, protein-folding catalysts, or molecular chaperones, which perform multiple pathogenesis-related and often independent functions, excluding functions as a result of gene fusion. This independency means that the inactivation of one function does not affect the activity of the other function (Huberts and van der Klei 2010).

Glycolysis has evolved in all three domains of life; Bacteria, Archaea, and Eukarya (Fothergill-Gilmore and Michels 1993). The glycolytic pathway involves 10 enzymes and some have been found to act as moonlighting proteins, such as triose phosphate isomerase (TPI), enolase, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Figure 1.1) (Henderson and Martin 2011). Several reports suggested that most of the glycolytic enzymes of Gram-positive bacteria, such as streptococci and staphylococci, are found on their cell surface. (Modun and Williams 1999; Hughes *et al.* 2002; Ling *et al.* 2004).

Autocrine motility factor (AMF), also identified as PGI (phosphoglucose isomerase) and neuroleukin (NLK), is a multifunctional human/mammalian moonlighting protein, which acts as autocrine motility factor and contributes to tumour malignancy (Baumann and Brand 1988; Chaput *et al.* 1988; Faik

et al. 1988; Watanabe *et al.* 1991; Watanabe *et al.* 1996). It stimulates the conversion of glucose-6-phosphate to fructose-6-phosphate and vice versa (Harrison 1974). The secreted AMF binds to AMF receptor (AMFR) on the surface of B16-F1 melanoma cells, which stimulates cell motility (Silletti *et al.* 1991). This binding is followed by AMFR internalisation, phosphorylating the receptor and catalysing the production of inositol triphosphate (Kohn *et al.* 1990; Watanabe *et al.* 1991).

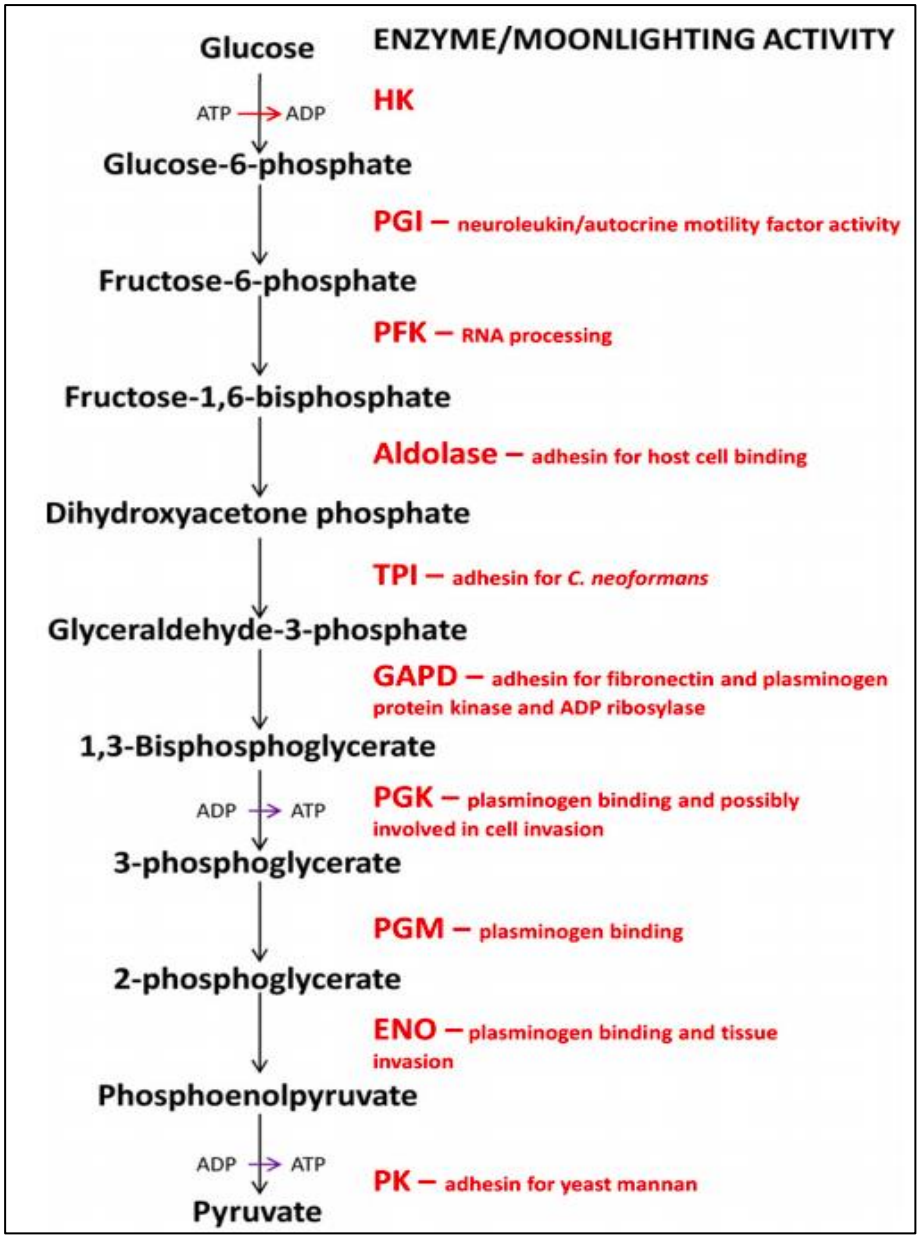


Figure 1.1: The glycolytic pathway displaying the moonlighting functions of glycolytic enzymes identified in a range of organisms. It starts with the conversion of glucose to glucose-6-phosphate by HK (hexokinase) and ends with the conversion of phosphoenolpyruvate into pyruvate by PK (pyruvate kinase)(Henderson and Martin 2011).

1.8.1 Meningococcal moonlighting proteins

1.8.1.1 Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was the first glycolytic moonlighting protein identified in bacteria (Pancholi and Fischetti 1992). It is a glycolytic enzyme, which catalyses the conversion of glyceraldehyde 3-phosphate to 1, 3-diphosphoglycerate. GAPDH has been identified on the surface of Gram-positive bacteria (*Staphylococcus* sp., *Streptococcus pyogenes, Streptococcus agalactiae, S. pneumoniae,* and *Listeria monocytogenes*) (Pancholi and Fischetti 1992; Modun and Williams 1999; Seifert *et al.* 2003; Ling *et al.* 2004; Schaumburg *et al.* 2004), as well as Gram-negative bacteria (*N. meningitidis*, and enterohemorrhagic (EHEC) and enteropathogenic (EPEC) *Escherichia coli*) (Egea *et al.* 2007; Tunio *et al.* 2010a), fungi (*Candida albicans*), and single-celled protozoan (*Trichomonas vaginalis*) (Gil-Navarro *et al.* 1997; Lama *et al.* 2009). Two putative genes, *gapA-1* and *gapA-2,* encode GAPDH, which share 50% nucleotide identity (Tettelin *et al.* 2000). Tunio *et al.* showed that meningococcal GapA-1 is expressed and exposed on the cell surface, but is only detectable using antibodies in the absence of capsule. There was no effect on the growth rate of *N. meningitidis* in a GapA-1 mutant;

however, there was a significant effect on the bacterial adhesion to human epithelial and endothelial cells in a capsule-independent process confirming the positive influence of GapA-1 on meningococcal-host cell interactions (Tunio *et al.* 2010a).

1.8.1.2 Fructose-1, 6-bisphosphate aldolase (FBA)

Meningococcal fructose-1, 6-bisphosphate aldolase (FBA) has been shown to have moonlighting roles (Tunio *et al.* 2010b). It catalyses the conversion of fructose-1,6-bisphosphate into glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. It has been previously reported that FBA is localised to the surface of some Gram-positive bacteria, such as *S. pneumoniae* (Blau et al. 2007). The FBA of *N. meningitidis* is a tetrameric enzyme belonging to subclass IIB, which confers thermal stability (Sauve and Sygusch 2001; Izard and Sygusch 2004). In the published meningococcal MC58 genome sequence, there is only one gene (*cbbA*) encoding an FBA enzyme (Tettelin *et al.* 2000). It has been shown that FBA exhibits non-glycolytic functions due to the localisation to the cell surface of *N. meningitidis*. This localisation of FBA plays a role in the optimal adhesion of meningococci to host cells, which is independent of its aldolase activity (Tunio *et al.* 2010b; Shams *et al.* 2016). Shams *et al.* demonstrated that FBA was found to be localised on the surface of both pathogenic and commensal *Neisseriae*. It

was also found to bind human plasminogen (Shams *et al.* 2016). It has been confirmed that FBA is also present in the cytoplasm suggesting a potential translocation across the cytoplasmic membrane to the surface. Tunio *et al*. suggested that FBA mutant had the same growth rate as the MC58 wild type strain, however, the adhesive activity was significantly reduced in the mutant (Tunio *et al.* 2010b).

1.8.1.3 Peroxiredoxin

Peroxiredoxins are the family of peroxidases that reduce peroxides by using a conserved cysteine residue (Rhee *et al.* 2001; Hall *et al.* 2011; Rhee *et al.* 2012). Oxidative stress can initiate apoptosis signalling leading to significant intracellular redox events. Peroxiredoxins may have a role in the regulation of redox changes during the initiation of apoptosis (Hampton and O'Connor 2016). It has been suggested that peroxiredoxin is localised to the cell surface of *N. meningitidis* strain MC58 where it binds plasminogen mainly via the C-terminal lysine residues (AlJannat 2016). Knaust *et al.* confirmed the peroxiredoxin surface-exposure by flow cytometry, immune electron microscopy, confocal microscopy, and ELISA (Knaust *et al.* 2007). Plasminogen binding to other meningococcal strains has been documented (Ullberg *et al.* 1992).

1.8.1.4 DnaK

Molecular cytosolic chaperones, such as DnaK, have a major role in facilitating the folding, assembly and transport of newly synthesized polypeptides (Hartl and Hayer-Hartl 2009). DnaK, the major bacterial Hsp70 in *E. coli*, is able to bind and release nonnative protein substrates upon ATP binding (Jordan and McMacken 1995). It was reported to act as a plasminogen receptor on the cell surface of *Mycoplasma pneumoniae, Bifidobacterium lactis, Bifidobacterium bifidum, Bifidobacterium longum, Mycobacterium tuberculosis,* and *N. meningitidis* (Candela *et al.* 2007; Knaust *et al.* 2007; Xolalpa *et al.* 2007; Candela *et al.* 2010; AlJannat 2016; Hagemann *et al.* 2017). Plasminogen binding to recombinant DnaK was further confirmed to be lysine mediated due to the use of ε-aminocaproic acid, which inhibited the binding (Xolalpa *et al.* 2007; AlJannat 2016). It has been confirmed that DnaK binds CD40 receptor stimulating chemokine synthesis and also binds chemokine receptor CCR5 resulting in the maturation of dendritic cells (Wang *et al.* 2001; Xolalpa *et al.* 2007). Furthermore, Hagemann *et al.* suggested that DnaK of *M. pneumoniae* is involved in the adhesion and dissemination processes during infections (Hagemann *et al.* 2017).

1.8.1.5 Enolase

In the late 1980s, the glycolytic enzyme α-enolase/ τ-Crystallin was one of the best-described multifunctional proteins playing two unrelated functions encoded by a single gene. τ-Crystallin is distributed in the lens of the embryonic turtle. Sequencing of τ-Crystallin revealed a remarkable resemblance to α-enolase (Wistow *et al.* 1988). Enolase is essential for glycolysis and gluconeogenesis. It stimulates the conversion of 2-phosphoglycerate into phosphoenolpyruvate and vice versa (Wold and Ballou 1957; Wold 1971). It has been reported that many bacteria and fungi retain enolase to the cell surface or export it into the external medium (Edwards *et al.* 1999; Hara *et al.* 2000). In *N. meningitidis,* cell surface enolase acts as a major plasminogen binding protein (Knaust *et al.* 2007). Enolase has been used by other pathogens; such as *S. pyogenes, Aeromonas hydrophila*, *S. pneumoniae*, *Staphylococcus aureus*, *Bacillus anthracis*, *Onchocerca volvulus*, *T. vaginalis*, and *Paracoccidioides brasiliensis;* to interact with the host (Pancholi and Fischetti 1998; Jolodar *et al.* 2003; Antikainen *et al.* 2007; Knaust *et al.* 2007; Agarwal *et al.* 2008; Mundodi *et al.* 2008; Donofrio *et al.* 2009; Sha *et al.* 2009).

1.8.1.5.1 Conserved residues of enolase

In order to predict the conserved lysine residues of meningococcal enolase, multiple enolase sequence alignments of *N. meningitidis* MC58 enolase compared with enolase from several strains; *S. pneumoniae* R6, *S. pyogenes* serotype M1, and *E. coli* K12. Based on the *N. meningitidis* MC58 enolase numbering, the multiple alignment revealed that there are eight conserved lysine residues (K) among these strains; K56, K66, K103, K191, K337, K355, K388, and K400 (Figure 1.2).

A sequence alignment of *E. coli* enolase and *N. meningitidis* MC58 enolase revealed a conserved lysine residue (lysine 337) of meningococcal enolase and its corresponding residue (lysine 341) of *E. coli* enolase (Figure 1.3). It has been demonstrated that the substitution of lysine 341 of *E. coli* enolase with other amino acids (alanine, glutamine, arginine, or glutamate) prevents the automodification and export of enolase to the cell surface (Boel *et al.* 2004). The correlation of the loss of enolase export with the loss of modification was established in K341E mutant enolase since it was almost as active as the wild type (Boel *et al.* 2004).

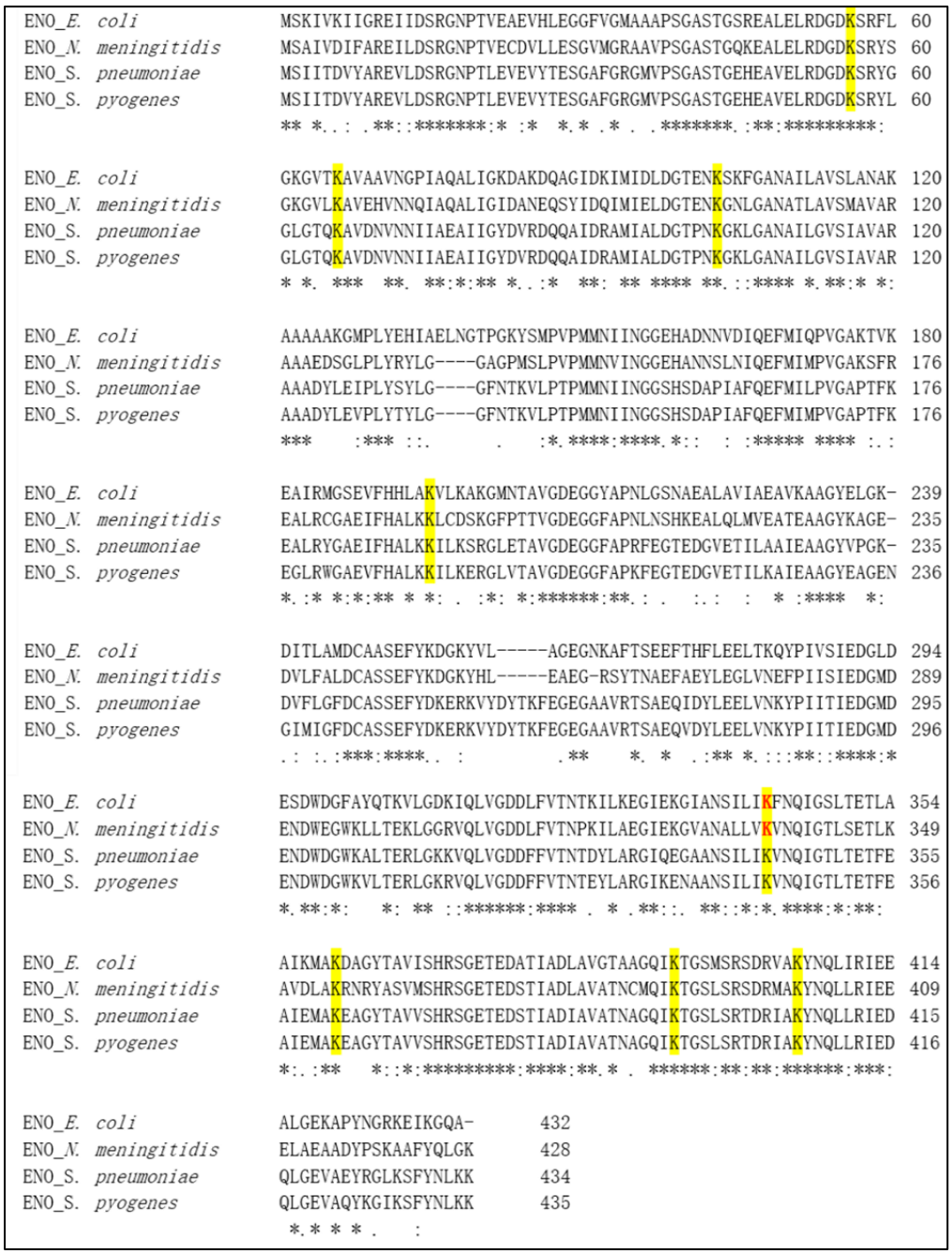


Figure 1.2: Multiple sequence alignment of several enolase amino acid sequences from different bacteria, which are *N. meningitidis* MC58, *S. pneumoniae* R6, *S. pyogenes* serotype M1, and *E. coli* K12. Based on meningococcal enolase numbering, the conserved lysine residues (K) among these strains; K56, K66, K103, K191, K337, K355, K388, and K400 are highlighted in yellow. Lysine residue 337 (K337) of meningococcal enolase and its corresponding residue (K341) of *E. coli* enolase are marked in bold red.

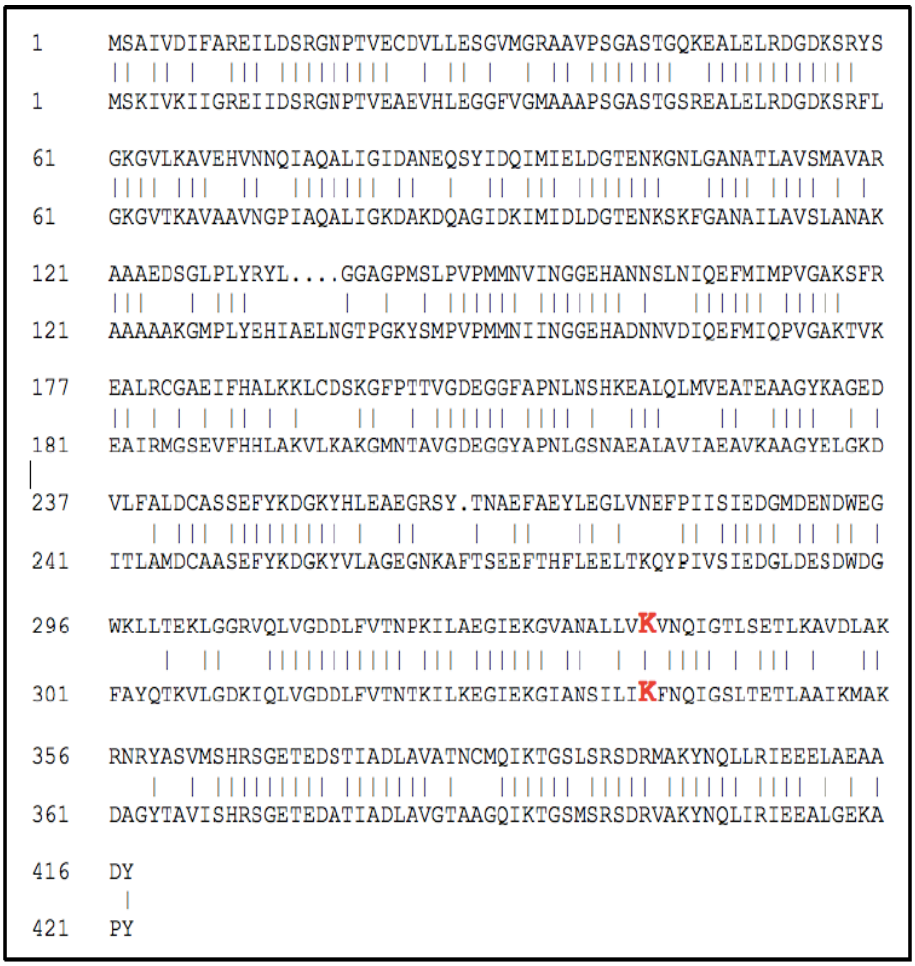


Figure 1.3: Optimal alignment of protein sequences of enolase from *N. meningitidis* MC58 and enolase from *E. coli*. Upper line is enolase from MC58, from residues 1 to 417. Lower line is enolase from *E. coli*, from residues 1 to 422. Marked in bold red are the conserved lysine residues (Lys^{337} and Lys^{341}).

1.8.1.5.2 3D structure of enolase

Enolase structure was adapted from Mahab AlJannat (AlJannat 2016) in agreement with Ehinger and co-workers who suggested that enolase of pneumococci is present as an octamer based on its crystal structure (Ehinger *et al.* 2004). Pneumococcal enolase was used as a model to predict the positions of the conserved residues in meningococcal enolase since they were both found to be localised to the cell surface where they play a major role in plasminogen binding (Bergmann *et al.* 2001; Knaust *et al.* 2007; AlJannat 2016). The predicted positions of the conserved lysine residues of meningococcal enolase are shown in Figure 1.4. AlJannat suggested that the mutation of lysine 297 and lysine 355 of meningococcal recombinant enolase affects plasminogen binding.

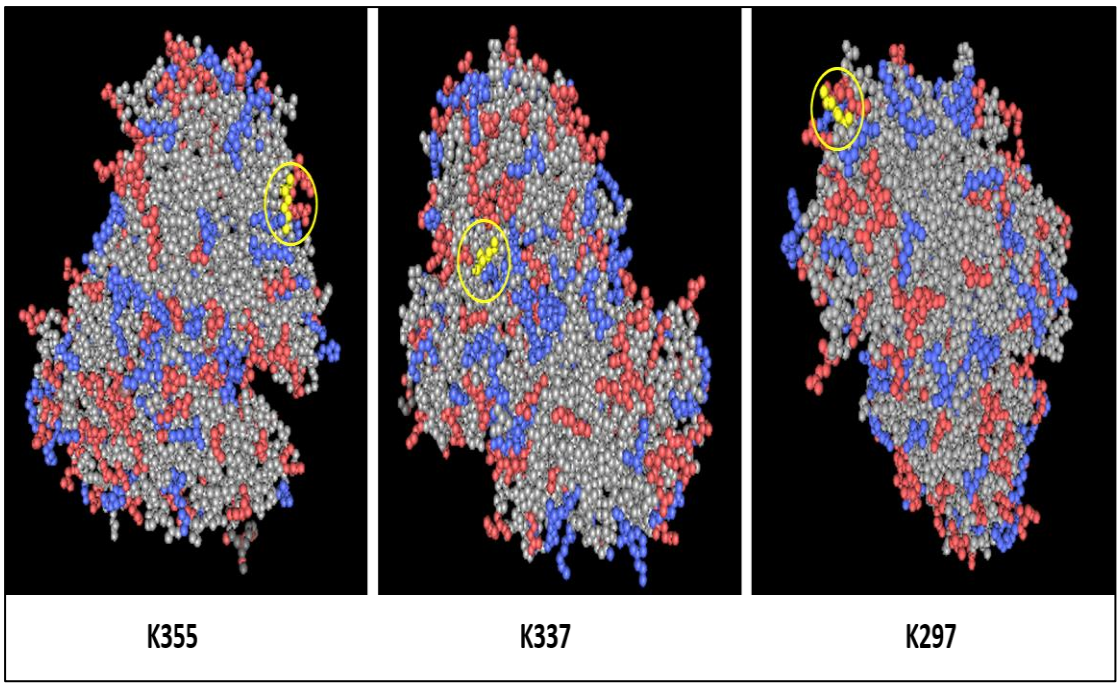


Figure 1.4: Pneumococcal enolase model used to determine the positions of the conserved lysine residues of meningococcal enolase; K355, K337, and K297, which are circled in yellow based on the numbering of meningococcal enolase. K337 was tested in this study for its role in enolase export. K355 and K297 were previously suggested to be involved in plasminogen binding. Cn3D4.3.1 structure viewer software was used to produce these figures, which were coloured based on charge. The negatively charged residues are in red, the positively charged are in blue, and the neutrally charged are in grey.

1.9 Secretion pathways in diderms

1.9.1 Type I secretion pathway (ISP): ABC exporters

The type I secretion pathway (ISP) allows proteins to be transported from the cytoplasm to the extracellular milieu in a single step (Delepelaire 2004). Two families within the ATP-binding cassette (ABC) superfamily export large proteins. In addition, four families export small proteins and peptides (Saier 2006). The ABC system complex is composed of membrane fusion proteins (MFP), pore-forming outer-membrane channel proteins (OMP) and cytoplasmic-membrane ABC transporters (CM-ABC), which are assembled to form a channel in the cell envelope. Therefore, they are very important to facilitate proteins' transport activity (van Ulsen and Tommassen 2006; Gerlach and Hensel 2007). The successive assembly of the ABC secretion complex is triggered by the interaction of the C-terminal portion of the secretion signal with the ABC protein producing further interactions between the MFP, OMP and CM-ABC (Delepelaire 2004). It has been shown that this assembly along with ATP hydrolysis is necessary for protein secretion (Morgan *et al.* 2017).

In *E. coli,* these proteins are called HlyD, TolC, and HlyB, respectively (Binet *et al.* 1997). Alpha hemolysin (HlyA) of some uropathogenic *E. coli* was the first protein found to be secreted

by ISP and remains the best described (Goebel and Hedgpeth 1982). HlyA is transported across the inner membrane via HlyB and then channeled through the periplasm by HlyD into the outer membrane before being secreted via TolC to the extracellular milieu (Schlor *et al.* 1997) (Figure 1.5).

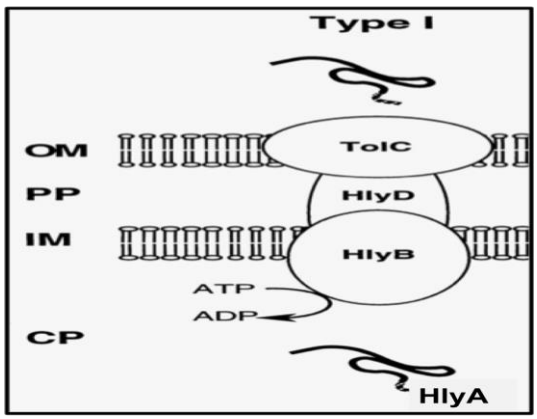


Figure 1.5: ISP (ABC) complex in *E. coli*. It is composed of three proteins, which are HlyB, HlyD, and TolC. HlyA protein secretion is an ATP-dependent process. HlyA is transported across the inner membrane by HlyB then channelled through periplasmic space by HlyD before being exported across the outer membrane via TolC (Hueck 1998).

Most IPS secreted proteins have identifiable nonapeptide glycine-rich repeats (GGXGXDXXX; **X** can be any amino acid), which are located in the C-terminal portion of the protein and are also important in transport activity (Finnie *et al.* 1998;

Delepelaire and Wandersman 2002; Linhartová *et al.* 2010). The glycine repeats bind specifically to calcium ions, and the proteins containing these repeats are given the name repeat in toxin (RTX) toxins (Strathdee and Lo 1989). Because the concentration of calcium in the cytoplasm is quite low, the binding of the RTX repeats to calcium ions only occurs upon secretion (Gangola and Rosen 1987). Beside these glycine-rich repeats, many IPS proteins have other repeats, which are linked to adhesion (Delepelaire 2004).

Under iron limitation, meningococci have been shown to secrete Frp proteins, (Frp stands for Fe-regulated protein), for example FrpC, which has sequence similarity to RTX proteins (Thompson *et al.* 1993). Moreover, low iron levels may also lead to the production of FrpD as demonstrated by Prochazkova *et al.* The gene *frpD* was observed in 38 invasive isolates out of 65 examined isolates of *N. meningitidis,* as well as 24 of 27 noninvasive isolates. It has also been suggested that there was a strong interaction between FrpC and FrpD proteins leading to high affinity binding of FrpD to the amino-proximal end of FrpC (Prochazkova *et al.* 2005).

Wooldridge *et al.* screened the MC58 meningococcal serogroup B genome using ISP components of *E. coli*. It has been suggested that *hlyB, hlyD* and *tolC* of meningococci were unlinked to any

of the ISP substrates and may have been acquired independently. Mutation of either *hlyB* or *hlyD* resulted in the loss of secretion of FrpC and a closely related FrpC-like protein (FrpC2) (Wooldridge *et al.* 2005). Kamal *et al.* have also demonstrated that *hlyB* and *hlyD* genes of *N. meningitidis* are essential for the secretion of FrpC and FrpC2 due to the failure of their secretion when the two genes (*hlyB*, *hlyD*) were mutated (Kamal *et al.* 2007).

1.9.2 Type II secretion pathway (IISP)

The type II secretion pathway (IISP) is a two-step process, in which proteins are first transported across the inner membrane into the periplasm via either Sec or Tat pathways accompanied by N-terminal signal peptide cleavage, followed by their export across the outer membrane to the exterior. This export includes folded, partially folded or unfolded proteins, which may be energised by ATP or GTP (Cianciotto 2005; Saier 2006). The IISPs share structural similarities with type IV pili. They both have a secretin protein in the outer membrane (XcpQ/PilQ), an inner membrane protein (XcpS/PilC), and share the XcpA/PilD pseudopilin peptidase protein. In addition, the inner membrane associated proteins (XcpZ/PilO and XcpY/PilN, PilM) are structurally related (Figure 1.6) (Bally *et al.* 1992; Durand *et al.* 2003; Filloux 2004; Robert *et al.* 2005).

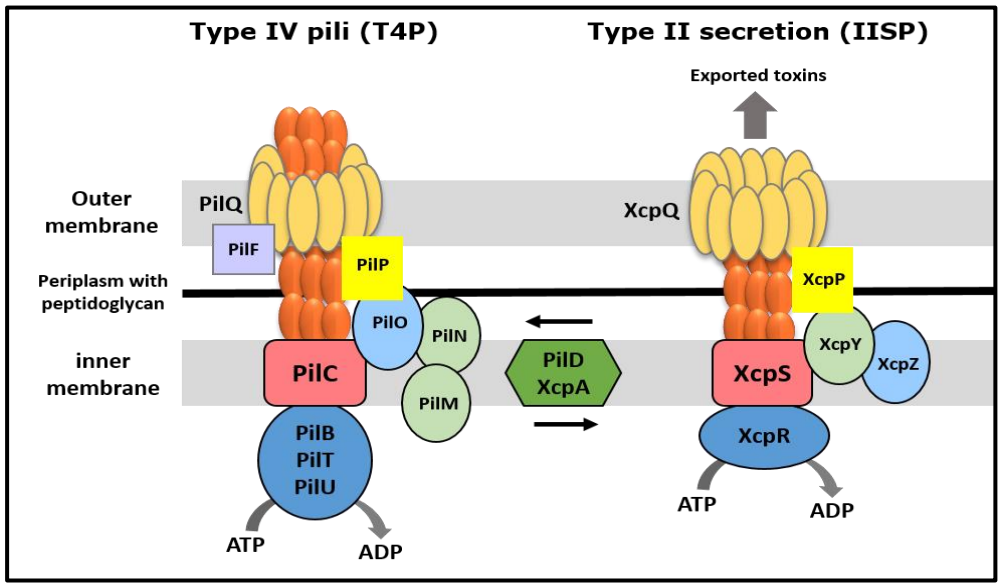


Figure 1.6:.IISP and T4P. They are structurally related. Both have a secretin protein in the outer membrane (XcpQ/PilQ), an inner membrane protein (XcpS/PilC), and share the XcpA/PilD pseudopilin peptidase protein. Other similarities include the inner membrane associated proteins (XcpZ/PilO and XcpY/PilN, PilM).

Several genera, such as *Vibrio, Klebsiella, Pseudomonas,* and *Aeromonas* have been studied for the mechanism of IISP, which has provided a clear understanding of IISP (Filloux 2004; Tosi *et al.* 2011; Dunstan *et al.* 2013; Putker *et al.* 2013; Cadoret *et al.* 2014; Vanderlinde *et al.* 2014). It has been demonstrated that components of IISP are highly conserved among most Gram-negative bacteria; they were first discovered in *Klebsiella*

oxytoca then subsequently in *Pseudomonas aeruginosa* (d'Enfert *et al.* 1987; Filloux *et al.* 1990).

Cadoret *et al.* characterized a new IISP (Txc, stands for third homolog to Xcp), which was the third to be identified and the best studied IISP in *P. aeruginosa.* Under phosphate-limiting conditions, another IISP (Hxc system) of *P. aeruginosa* strain PA7 works to secrete alkaline phosphatases. They identified a chitin-binding protein (CbpD), which is an exoprotein secreted by the Txc system. Txc-deficient strains were analysed for the secretion of CbpE. They concluded that the secretion of CbpE in strain PA7 is mediated by Txc system, which is regulated by the two-component system (TtsS/TtsR) (Cadoret *et al.* 2014).

Another *Pseudomonas* species (*Pseudomonas putida*) has been shown to possess Xcp IISP for the secretion of phosphatases (Putker *et al.* 2013). The inner membrane proteins (XcpS, XcpY, XcpZ, XcpR) are necessary for the assembly of pilus like structures. Initial studies suggested that the Xcp system might be inactive in *P. putida* because the inactivation of *xcp* genes led to no alteration being detected in the extracellular protein profile (de Groot *et al.* 1996). Putker *et al.* re-invesigated whether *P. putida* has a complete and active Xcp IISP for protein secretion under phosphate starving conditions. The reason for the previous failure to determine the activity of the Xcp system in *P.*

putida is that high levels of phosphate might lead to the suppression of system expression. Another reason is that the substrates for the Xcp system (lipoproteins), as shown for UxpA and UxpB (alkaline phosphatases), were still attached to the cell surface when the cultures were in aerobic conditions. Therefore, no activity was detected in the extracellular medium (Putker *et al.* 2013). It has been suggested that UxpA might be involved in the acquisition of phosphate based on a conserved-domain search at NCBI (National Center for Biotechnology Information). They found that UxpA belongs to the metallophosphatase superfamily (Putker *et al.* 2013). An additional IISP was also found in *P. putida* (Xcm system), which is involved in the secretion of a manganese oxidase (De Vrind *et al.* 2003).

Secretins are membrane-channels that form large pores in the outer membrane for the passage of folded proteins into the surrounding milieu or into other cells. They are not only found in IISPs but also IIISPs, type IV pili and filamentous bacteriophages (Allaoui *et al.* 1993; Linderoth *et al.* 1996; Craig *et al.* 2004; Collins *et al.* 2005; Ayers *et al.* 2009; Hodgkinson *et al.* 2009). Secretin domains may interact with other proteins, such as pilotins (Korotkov *et al.* 2011). Pilotins are considered to be chaperones that target the monomers of secretin to the outer membrane. PulS is a pilotin, which targets the monomers of PulD

secretin in IISP of *K. oxytoca* (Collin et al. 2011). Tosi *et al.* performed many substitutions on PulS residues including valine replacement at position 42 (V42), which completely abolished the interaction of PulS with the S-domain of PulD *in vitro* and *in vivo*. Subsequently, substitutions of other residues also minimized the interaction *in vitro* but with less effects *in vivo* (Tosi *et al.* 2011).

1.9.3 Type III secretion pathway (IIISP): pathogenicity-related system

Many pathogenic Gram-negative bacteria use a type III secretion pathway (IIISP) to directly inject virulence factors into the cytosol of eukaryotic cells (Buttner *et al.* 2008). The secretion apparatus is a complex that extends from the inner membrane through the peptidoglycan layer and the outer membrane then ends like a hypodermic needle-structure to secrete proteins directly into the cytosol of the host cell. Therefore, the secreted proteins are transported to host cells without being exposed to the external milieu (Saier 2006). The IIISP is also called an injectisome, which resembles the bacterial flagellum to a high degree (Dewoody *et al.* 2013) (Figure 1.7).

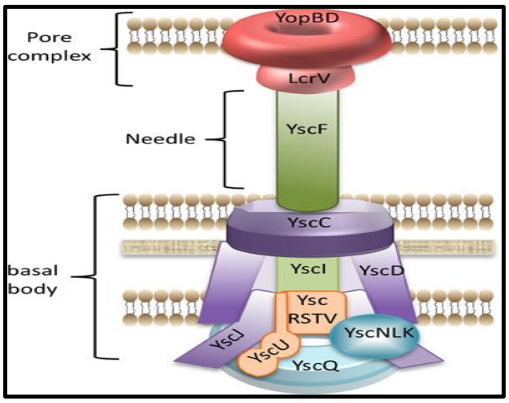


Figure 1.7: Yersinia injectisome, which is composed of the basal body, the needle, and the pore complex. YscI allows the passage of substrates across the bacterial membranes. YscF is secreted through the YscI rod and polymerises to form the needle. LcrV localises on the host membrane to transport Yop proteins into the cytoplasm of host cells. YopBD comprises the pore complex (Dewoody *et al.* 2013).

The IIISPs have been studied in many Gram-negative genera, such as *Yersinia*, *Shigella, Salmonella, Escherichia, Chlamydia, Bordetella,* and *Pseudomonas* (Fennelly *et al.* 2008; Picking and Picking 2009; Ramos-Morales 2012; Dewoody *et al.* 2013; Dai and Li 2014; Neeld *et al.* 2014; Zhou *et al.* 2014). Nevertheless, this system was first discovered and is best characterized in *Yersinia.* The machinery of *Yersinia* secretion consists of three components, which are Yersinia secretion apparatus (Ysc), Yersinia outer proteins (Yops), and specific Yop chaperones (Syc) (Lawton *et al.* 2002). V antigen or LcrV is the key component of the *Yersinia* IIISP, which facilitates the transfer of Yop proteins into the cytosol of eukaryotes by extracellular localisation of LcrV on the bacterial membrane (Pettersson *et al.* 1999).

Dewoody *et al.* reviewed the injectisome assembly and the roles of YopN, YopK, YopE, and YopT during translocation and IIISP regulation (Dewoody *et al.* 2013). In the early stage of needle assembly, YscI allows the passage of substrates across the bacterial membranes by forming a rod, through which the needle protein YscF is secreted. Subsequently, YscF polymerises to form the needle. Journet *et al*. suggested that YscP regulates the needle length, and therefore is called a molecular ruler (Journet *et al.* 2003). Once the needle is built, IIISP secretes the middle

and the late substrates. The middle substrates, such as YopBD, comprise the pore complex. The late substrates, such as effector Yops, are then inserted into host cells. It has been suggested that the secretion of late substrates is inhibited in calcium-containing medium due to the formation of a calcium plug (YopN-TyeA-YscB-SycN complex) (Dewoody *et al.* 2013). The deletion of any of the genes of this complex results in the secretion of high levels of the middle and the late substrates (Sundberg and Forsberg 2003). Zhang *et al.* suggested that the arrangement of the effector proteins, in a large and mostly immobile complexes, occur in a great proportion within the cytoplasm, however, this is not associated with the injectisome (Zhang *et al.* 2017).

Salmonella typhimurium uses two IIISPs upon ingestion. IIISP-1 is activated first to facilitate the entry to epithelial cells in the ileum and colon by injecting dozens of effector proteins, which attract host proteins to trigger cytoskeletal rearrangements. Subsequently, the pathogen utilises a second IIISP (IIISP-2) to escape phagocytic contact via injecting effectors into host tissues altering vesicular trafficking (Galán and Curtiss 1989; Hensel *et al.* 1995; LaRock *et al.* 2015).

1.9.4 Type IV secretion pathway (IVSP): conjugation-related system

Type IV secretion pathway (IVSP) can be sub-divided into three groups based on function. The first group mediates the process of conjugation to deliver plasmid or transposon DNA from one cell to another (Lawley *et al.* 2003). Conjugation promotes bacterial adaptation to environmental conditions. Furthermore, it has a major importance in spreading antibiotic resistance genes among pathogens contributing to multidrug resistance in health care facilities and in other environments. An important environment where this is likely to happen is amongst farmed animals where 'growth promoters' – essentially antibiotics – are used (Fronzes *et al.* 2009; Zechner *et al.* 2012). The *Agrobacterium tumefaciens* VirB/VirD4 system is a type IV conjugation system (Zechner *et al.* 2012) (Figure 1.8). In some organisms, such as *Bordetella pertussis*, *N. gonorrhoeae*, and *Helicobacter pylori,* a second group of IVSPs mediate a contact-independent secretion, in which the pathogen secretes DNA into the extracellular milieu for genetic incorporation into the genome of other pathogens by homologous recombination (Smeets and Kusters 2002; Backert and Meyer 2006; Hamilton and Dillard 2006; Locht *et al.* 2011; Ramsey *et al.* 2014). The third pilus-based group of IVSPs mediate the contact-dependent injection

of effector macromolecules into the host cytoplasm. *H. pylori, Brucella suis,* and *Legionella pneumophila* can deliver virulence proteins into eukaryotic cells (Ninio and Roy 2007; Fronzes *et al.* 2009).

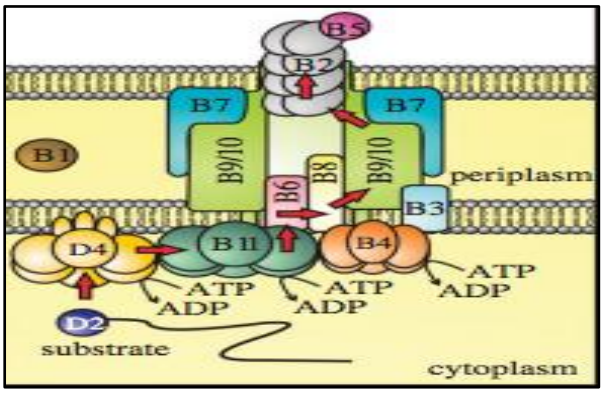


Figure 1.8: Agrobacterium VirB/D4 IVSP translocation system. It encodes VirB1 through VirB11. VirB4 and VirB11 are ATPases, which energise the assembly and activate IVSP secretion. The substrate binds to the VirD4 receptor, which transfers it to VirB11. Then VirB11 transfers the substrate to VirB6 and VirB8 for transporting the substrate across the inner membrane. Inside the channel, the substrate binds to VirB9 and VirB2 for transportation across the outer membrane (Zechner *et al.* 2012)

H. pylori strains contain a region called the *cag* pathogenicity island (PAI), which is associated with gastric cancer or ulcer disease. The *cag* IVSP enhances the translocation of CagA effector protein (Blaser 2005; Bourzac and Guillemin 2005). In 2011, many studies demonstrated the role of *cag* IVSP in *H. pylori* pathogenesis (Fischer 2011; Tegtmeyer *et al.* 2011; Terradot and Waksman 2011). Johnson *et al.* mutated seven genes in the *cag* PAI that are involved in IVSP of *H. pylori,* and investigated whether these genes are required for pilus formation. It has been demonstrated that mutants of *cagV*, *cagM, cagT, cagX,* and *cag3* suppressed the activity of IVSP and pilus formation, however, IVSP function and pilus formation activity were regained in the complemented mutant strains. Mutants of *cagC* and *cagY* were defective in IVSP function but kept the activity for pilus formation (Johnson *et al.* 2014). Several studies suggested that mutant strains of *cag* IVSP were defective in pilus formation (Tanaka *et al.* 2003; Shaffer *et al.* 2011; Barrozo *et al.* 2013; McClain *et al.* 2013).

The Johnson *et al.* study, along with the other previous studies, has shown that pili of *H. pylori* are components of the *cag* IVSP and are encoded by the *cag* PAI. IVSP activity is increased under iron starvation conditions, which is associated with increased pilus formation (Noto *et al.* 2013). A host protein, calprotectin

or CP, plays an important role in the host responses to *H. pylori* (Gaddy et al. 2014). Previous studies have shown that a functional *cag* IVSP results in IL-8 production and the recruitment of neutrophils to the site of *H. pylori* infection (Noto and Peek 2012; Sanchez-Zauco *et al.* 2014). Gaddy *et al.* studied the role of CP in the colonisation and pathogenesis of *H. pylori*. Their data indicated that the proinflammatory activity of the *cag* IVSP is regulated by zinc homeostasis. The gastric CP levels are elevated following *H. pylori* infection, subsequently suppressing the translocation of cagA by *cag* IVSP into epithelial cells and inhibiting IVSP-associated pili formation via zinc sequestration, which ultimately decreases IL-8 secretion and increases the bacterial persistence (Gaddy *et al.* 2014).

Most strains of *N. gonorrhoeae* secrete DNA into the extracellular medium using IVSP (Hamilton *et al.* 2005). *N. gonorrhoeae* harbours a gonococcal genetic island (GGI), which encodes IVSP genes (Dillard and Seifert 2001). Ramsey *et al.* studied the level of expression and localisation of three highly conserved OMPs of IVSP, which are TraK, TraV and TraB (Ramsey *et al.* 2014). They demonstrated that TraK interacts with the TraV lipoprotein and localises on the outer membrane of gonococci. Similarly, TraB was also localised to the outer membrane. However, neither TraK nor TraB was detectable by western blotting in wild-type

cells unless overexpressed or tagged. Their results suggested that low levels of IVSP proteins are adequate for DNA secretion (Ramsey *et al.* 2014).

Chronic persistence of *Brucella abortus* is highly related to IVSP in mice, which is encoded by the *virB*1-12 operon (Hong *et al.* 2000). It also causes a mild inflammatory response (Roux *et al.* 2007). This inflammatory response can be induced by IVSP effector protein VceC, which triggers endoplasmic reticulum (ER) stress via binding the chaperone BIP (binding immunoglobulin protein) (de Jong *et al.* 2013). Subsequently, the unfolded protein response (UPR) is induced by the ER stress in macrophages. This results in the activation of inositol-requiring enzyme 1a (IRE1a), which leads to the activation of nuclear factor kappa B (NF-kB) and the expression of pro-inflammatory genes as a result of the recruitment of NOD-like receptors (NLRs), NOD1 and NOD2 (Keestra-Gounder *et al.* 2016).

1.9.5 Type V secretion pathway (VSP)

Type V secretion pathway (VSP) is considered as the simplest protein secretion pathway. This pathway is classified into subclasses: Va system (classical or monomeric autotransporter or also termed as AT-1), Vb system (two-partner secretion pathway), and Vc system (AT-2) (Henderson *et al.* 2004).

However, in 2014, Van Ulsen *et al.* stated that VSP proteins are also secreted by an additional two subclasses Vd system (patatin-like autotransporter) and Ve system (classical autotransporter with a reversed domain). These five subclasses share a β-barrel domain that is integrated into the outer membrane. The VSP protein transport across the inner membrane to the periplasmic space occurs through the Sec complex, while the translocation of proteins across the outer membrane to the cell surface depends on the Bam (β-barrel assembly machinery) complex. The subclasses Va and Vb were the first and the best studied of the type V subclasses (van Ulsen *et al.* 2014).

1.9.5.1 Type Va secretion (classical autotransporters)

The classical autotransporter pathway was the first intensively studied VSP. The term "autotransporters" refers to proteins with unique properties that facilitate their independent transport across the membrane to the cell surface. However, studies have shown that a group of periplasmic molecular chaperones, such as Skp, SurA, and DegP, play a critical role in the periplasmic folding and insertion of proteins into the outer membrane of *E. coli*. It has been proposed that there are two overlapping chaperone pathways for OMPs in *E. coli*: one consisting of DegP and Skp and the other of SurA (Behrens *et al.* 2001; Rizzitello *et*

al. 2001; Purdy *et al.* 2007). Although *N. meningitidis* does not possess DegP, it does contain a DegQ functional homolog. However, there was no evidence for the presence of a general role for Skp, SurA, or DegQ as OMP chaperones in *N. meningitidis* (Volokhina *et al.* 2011).

Several important autotransporter proteins have been identified in *N. meningitidis*, such as IgA protease, NalP (Neisseria autotransporter lipoprotein); formerly known as AspA (Autotransported serine protease A), App (Adhesion and penetration protein), and MspA (Meningococcal serine protease A) (Hadi *et al.* 2001; Turner *et al.* 2002; van Ulsen *et al.* 2003; Turner *et al.* 2006; Roussel-Jazede *et al.* 2014).

Hadi *et al.* confirmed the localisation of App to *N. meningitidis* surface by electron microscopy of immunogold-labelled whole cells of strain MC58 meningococci. Subsequently, App was cleaved and secreted by VSP. It has been demonstrated that App is highly conserved among meningococcal isolates. It stimulates T cells and B cells to produce antibodies (Hadi *et al.* 2001).

NalP (AspA) is another autotransporter protein with well-conserved amino acid sequence in diverse meningococcal strains (Turner *et al.* 2002). AspA, like App, is an immunogenic protein due to the detection of cross-reactive antibodies in patients' sera. It has been confirmed that AspA is translocated to the outer

membrane of *N. meningitidis.* Western blotting has shown that smaller components of AspA (AspA68 and AspA70) are cleaved and secreted after becoming surface-exposed. S426 *aspA* gene was cloned and then expressed in *E. coli*. S426A mutant *aspA* gene abolished the secretion of both rAspA68 and rAspA70 in *E. coli* (Turner et al. 2002). Van Ulsen *et al.* favoured the use of acronym NalP instead of AspA because the acronym AspA was already used in meningococci for the unrelated enzyme aspartate ammonia-lyase (van Ulsen *et al.* 2003).

The sequence of the NalP protein, from *N. meningitidis* strain H44/76 was analysed and the presence of lipoprotein motifs was detected at the signal-sequence cleavage site, however, the lack of the lipid moiety from the N-terminal domain was observed in the secreted form of NalP. Therefore, no lipidation could be detected in the NalP wild type. The processing of the autotransporters App and IgA protease was affected by mutation of *nalP*, which resulted in altered profiles of protein secretion (van Ulsen *et al.* 2003).

The role of MspA in the pathogenesis of *N. meningitidis* has been demonstrated by Turner *et al.* They showed that MspA is secreted in meningococcal B strain MC58 into the extracellular milieu and that recombinant MspA expressed in *E. coli* could mediate meningococcal adherence to human bronchial epithelial

and human brain microvascular endothelial cells (Turner *et al.* 2006). It has been suggested that MspA is a substrate for AspA/NalP due to MspA homology with App and IgA1 protease. A smaller (ca. 45 kDa) anti-MspA-reactive protein was detected in the NalP mutant after being probed with MspA-specific antiserum. However, this anti-MspA reactive protein was absent from the wild type. This suggested that NalP uses MspA as an additional substrate (Turner *et al.* 2006).

An autochaperone domain (AC) is present in several autotransporters at the C-terminal region of the passenger and upstream of the translocator. Recently, there was an increased conservation of this structural element (AC) in the VSPa. It has been shown that the AC domain is required for proper secretion and folding of BrkA (Bordetella resistance to killing), AIDA-I (Escherichia coli Adhesin Involved in Diffuse Adherence I), Hbp (Hemoglobin-binding protease), and IcsA (Intra-cellular spread protein A) (Oliver *et al.* 2003; Berthiaume *et al.* 2007; May and Morona 2008; Soprova *et al.* 2010). Three-dimensional structures of the AC from IcsA were investigated to determine its prevalence in the VSPa. Phylogenetic and proteogenomic analysis revealed the presence of AC as a conserved domain in autotransporters, which is exclusively associated with passengers as a single-stranded right-handed parallel β-helix

(Rojas-Lopez *et al.* 2017). The C-terminal transmembrane β-barrel has a role in the folding and secretion, and in preventing the unfolding of the N-terminal virulence region (passenger) (Drobnak *et al.* 2015; Baclayon *et al.* 2016).

1.9.5.2 Type Vb secretion (Two-partner secretion system)

The two-partner secretion system (TPS), in Gram-negative bacteria, exports large exoproteins (TpsA), which are analogous to the passenger domains of classical (type Va) autotransporter proteins across the outer membrane via a second protein (TspB), which is analogous to the pore-forming domain of classical autotransporters. Both TpsA and TpsB have an N-terminal signal sequence to facilitate their transport through the inner membrane into the periplasm via the Sec-machinery. Thereafter, the TpsB transporter is embedded into the outer membrane where it helps to mediate the secretion of TpsA across the outer membrane (Figure 1.9).

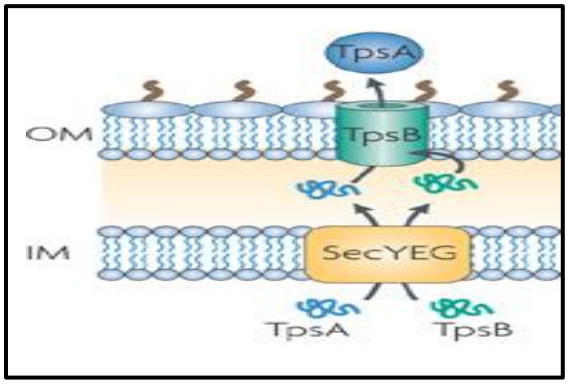


Figure 1.9: The two-partner secretion pathway. TpsA and TpsB proteins are transported across the inner membrane in a Sec-dependent manner. Subsequently, the translocator domain of the protein (TpsB) is inserted into the outer membrane to mediate TpsA secretion into the extracellular medium (Fronzes *et al.* 2009).

The TpsA exoprotein targets this transporter via the TPS domain, which is located at the N-terminus of TpsA; this interacts with TpsB leading to secretion of TpsA. The secreted TpsA is either localised to the cell surface or is released into the external medium. TpsA proteins typically function as adhesins or toxins (Henderson *et al.* 2004; Jacob-Dubuisson *et al.* 2004; Fronzes *et al.* 2009; ur Rahman and van Ulsen 2013).

The genome of *N. meningitidis* includes up to two TpsB- and five TpsA-encoding genes. Analysing the genomes of *N. meningitidis* strains Z2491, FAM18, H44/76 (Figure 1.10), and MC58 – identified the open reading frames (ORFs) of TpsA and TpsB

(Parkhill *et al.* 2000; Tettelin *et al.* 2000; Bentley *et al.* 2007; Piet *et al.* 2011).

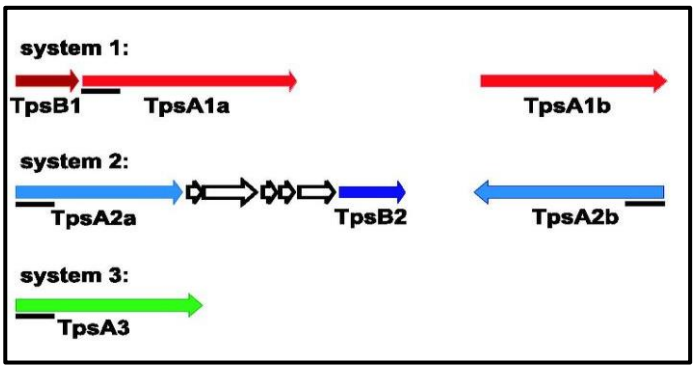


Figure 1.10: The chromosomal organization of the three TPS systems of *N. meningitidis* H44/76. The white arrows between TpsA2a and TpsB2, represent ORFs that do not encode TPS proteins (ur Rahman and van Ulsen 2013).

Three separate TPS systems were identified by van Ulsen *et al*., who studied the distribution of TPS systems among 92 meningococcal isolates including 88 disease isolates representing 13 clonal complexes (van Ulsen *et al.* 2008). It was observed that system 2 and system 3 were more prevalent among hyperinvasive isolates and that antibodies against TPS domains of system 2 and system 3 could be detected in patients' sera. Despite the wide distribution of system 1 among *N. meningitidis* isolates, antibodies against system 1 TPS domains were not detected in the tested sera. All 92 tested strains were *tpsB1* gene positive, however, only 79 strains were positive for the *tpsA1* gene. The *tpsB2* and *tpsA2a* genes were detected in 44 isolates, nevertheless, *tpsA2b* and *tpsA3* were found in 43 and 26 strains, respectively. Their results also suggested that TpsA2 is secreted in a TpsB1-independent way (van Ulsen *et al.* 2008). A further study by ur Rahman and van Ulsen investigated the transport of neisserial TPS domains by TpsBs. Their results showed that the TpsB2 transporter has the ability to secrete all meningococcal TPS domains. In contrast, TpsB1 was only shown to secrete its cognate meningococcal TPS domains and not other domains (ur Rahman and van Ulsen 2013).

1.9.5.3 Type Vc secretion: trimeric autotransporter system

Trimeric autotransporter adhesins (TAAs) are composed of three identical polypeptide chains, each with a signal peptide that leads them to the Sec-machinery. TAAs can be divided into two-distinct domains: N-terminal passenger domain, which contains a trimeric head, a neck, and a coiled stalk domain, followed by a C-terminal anchor domain for translocation of the N-terminal domain across the outer membrane to the extracellular milieu through a pore formed by the anchor domain. This pore is made from three polypeptide chains, which form the trimeric 12-stranded β-barrel (Figure 1.11) (Hoiczyk *et al.* 2000; Linke *et al.* 2006; Leo *et al.* 2012).

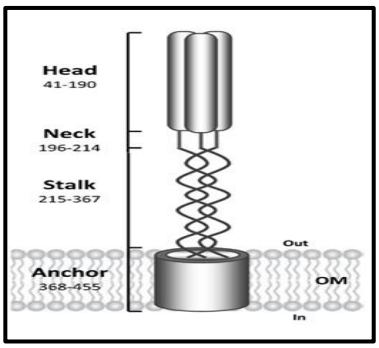


Figure 1.11: The full-length trimeric autotransporter adhesin, showing the N-terminal passenger domain, which is composed of a trimeric head, a neck, and a coiled stalk domain, followed by the C-terminal domain, which is important for translocation of the passenger domain to the external medium (Mil-Homens and Fialho 2011).

The trimer of the passenger domain is translocated through the pore as unfolded or partially unfolded polypeptides. The folded trimer is unable to cross this pore; thus, trimerization of the passenger domain cannot take place in the periplasmic space (Cotter *et al.* 2005a). On the other hand, trimerization of the translocation domain occurs before or during the insertion into the membrane as suggested by Mikula *et al.* In order to keep the passenger domain in the unfolded state in the periplasm, a chaperone might be required for autotransportation control. The TAAs autotransportation mechanism has been less studied than the classical autotransportation mechanism, however, both share similarities. Well-studied members of the subfamily TAAs include *Yersinia enterocolitica* YadA adhesin, the *H. influenzae* Hia adhesin and Hsf, *Bartonella henselae* BadA adhesin, and *Moraxella* ubiquitous surface proteins UspA1 and UspA2 (St Geme and Cutter 2000; Nummelin *et al.* 2004; Riess *et al.* 2004; Yeo *et al.* 2004; Cotter *et al.* 2005b). NadA (Neisserial Adhesin A) and NhhA (Neisseria hia/hsf homologue) belong to TAA family, which have been identified in *Neisseria* (Comanducci *et al.* 2002; Scarselli *et al.* 2006).

Grosskinsky *et al.* demonstrated a role for the conserved glycine residue (G389) of *Y. enterocolitica* YadA anchor domain in the autotransport function. This residue was replaced by alanine

(G389A), threonine (G389T), serine (G389S), histidine (G389H) or asparagine (G389N): amino acids with an increasing size of side chains in the order G < A < S < T < N < H and an increasing polarity in the order A < G < S = T < N = H. The mutation of G389 has led to reduced expression levels and export of YadA, nevertheless, there was no effect on YadA-mediated adhesion to collagen. Furthermore, the periplasmic protease DegP has been shown to degrade YadA with high capacity, which is triggered by the accumulation of the mutant protein in the periplasm (Grosskinsky *et al.* 2007).

Mikula *et al.* further investigated the phenomenon of hetero-trimerization, which is formed by *E. coli* immunoglobulin binding (Eib) TAA proteins (Mikula *et al.* 2012). It has been demonstrated that the co-expression of the two distinctly unrelated proteins EibA and YadA was lethal for the cells. YadA and EibA do not produce viable heterotrimerization, while the two closely related proteins EibA and EibD or EibC and EibD do (Mikula *et al.* 2012). As suggested in previous studies, the passenger domain of the *Y. enterocolitica* YadA is important for the adhesive function but not for trimerization and transport because there was no effect on TAA expression after disrupting the passenger domain (Roggenkamp *et al.* 2003; Cotter *et al.* 2006). Due to this conclusion, the translocation domain would

be more involved in transport and trimerization functions than the passenger domain would. Mikula *et al.* suggested that substituting the barrel of either protein (YadA or EibA) with the barrel of the other to be identical would result in a viable heterotrimerization. It has been demonstrated that a functional β-barrel is essential for autotransportation, while the passenger domain is not involved (Mikula *et al.* 2012).

1.9.5.4 Type Vd Secretion: Fused Two-Partner Secretion System

Salacha *et al.* demonstrated that PlpD of *P. aeruginosa* is secreted via a novel secretion pathway, which is related to the type Vb secretion pathway but with major differences. This pathway is named type Vd secretion pathway (Salacha *et al.* 2010). PlpD belongs to the patatin-like protein family (PLPs) (reviewed by (Banerji and Flieger 2004)). PlpD has a passenger domain (lipolytic enzyme), which is attached by a POTRA (periplasmic polypeptide transport-associated) domain to a β-barrel domain for translocation. The lipolytic passenger domain is immediately dislocated after the autotransportation (Leo *et al.* 2012). It has been suggested that the PlpD passenger domain is secreted by PAO1 and PA14 strains into the extracellular milieu. However, the C-terminal anchor domain remains folded and

incorporated into the outer membrane even after autotransportation is completed.

In order to determine by which novel secretion system PlpD is exported, Salacha *et al.* investigated the autotransporter pathways (type Va/Vc) and TPS (type Vb) to differentiate them from type Vd pathway (Salacha *et al.* 2010). In some cases, the protease activity of one autotransporter is essential for maturation of another autotransporter, such as the NalP (*N. meningitidis*) serine protease activity and IgA protease maturation (van Ulsen *et al.* 2003). Salacha *et al.* investigated the POTRA domain for its role in PlpD secretion. POTRA domains have chaperone functions that prevent PlpD folding in the periplasm and thus promote effective translocation. The patatin domain of PlpD is fused with a TpsB-like domain. This fusion might facilitate the export into the extracellular milieu (Salacha *et al.* 2010).

1.9.5.5 Type Ve Secretion: Inverted Classical Autotransporter

The inverse autotransporter family is a large family of virulence-related adhesins, which include intimin and invasin. Both adhesins share an N-terminal domain containing a signal peptide (SP), a β-barrel anchor domain, and a C-terminal adhesive domain (Oberhettinger *et al.* 2012). Invasin is a virulence factor

of *Yersinia pseudotuberculosis* and *Y. enterocolitica*, which enhances the binding to β1-integrins of host epithelial cells (Grassl *et al.* 2003). Intimin of enteropathogenic *E. coli* enhances the adhesion to epithelial cells of the intestine (Wong *et al.* 2011). It has been suggested that this system is similar to the classical type Va secretion pathway but in an inverted domain order (Figure 1.12, Figure 1.13).

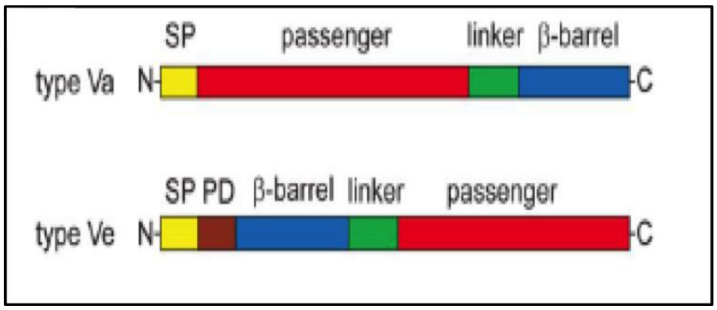


Figure 1.12: Comparison between type Va and type Ve autotransporters. The classical Va autotransporter has three regions; N-terminal signal peptide (SP; in yellow), passenger domain (in red), and C-terminal β-barrel (in blue) that is attached to the passenger domain by a linker region (in green). The inverted Ve autotransporter has the same regions but in a reverse manner. The passenger domain is in the C-terminal position and there is an additional periplasmic domain (PD; in brown) in the N-terminus (Leo *et al.* 2015).

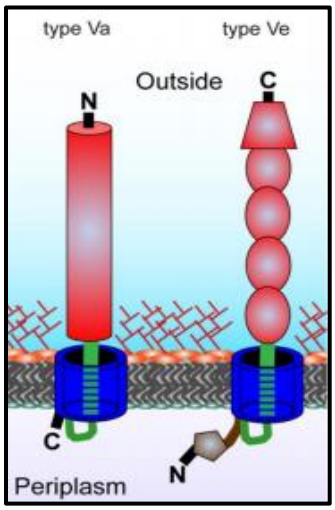


Figure 1.13: Va and Vc autotransporters final assembly. The passenger domain of the Va autotransporter is cylindrical in shape with the N-terminus extending from the outer membrane to the extracellular medium. However, the passenger domain of the Ve autotransporter forms oval-shaped structures, which are connected to a lectin-like domain in the C-terminus. Ve autotransporters have an additional periplasmic LysM domain in the N-terminus, which binds peptidoglycan (Leo *et al.* 2015).

In type Ve secretion system, unlike the classical type Va system, folding and insertion of the passenger domain is induced from N- to C-terminus. This finding prompted several researchers to classify it as a new VSP (Tsai *et al.* 2010; Leo *et al.* 2012; Oberhettinger *et al.* 2012). G260 is a highly conserved glycine residue, which has been proposed to be important in the translocation of the passenger domain. In order to investigate this role, G260 was substituted with amino acids of increasing size of side chains (G260A, G260S, G260T, G260N and G260H) (Oberhettinger *et al.* 2012). The interference of the G260 mutation in β-barrel formation and in passenger domain translocation was tested. Their results showed that there was no effect on β-barrel folding and membrane insertion in all mutants. Using flow cytometry, these researchers showed that substitution of G260 with amino acids with increasingly large side chains resulted in a corresponding decrease in the passenger domain translocation.

1.9.6 Type VI secretion pathway (VISP)

Type VI secretion pathway (VISP) was first described in *Vibrio cholerae* in 2006 (Pukatzki *et al.* 2006). VAS (virulence-associated secretion) genes of *V. cholera* encode the VISP. VISP was also described in *P. aeruginosa* (Mougous *et al.* 2006; Filloux 2011). It has been demonstrated that VISPs have either an

antibacterial or anti-eukaryotic activity. The antibacterial activity makes VISP a good candidate for vaccine development (Hood *et al.* 2010; Coulthurst 2013; Sana *et al.* 2016). VISP is encoded by gene clusters, containing about 13 conserved and essential genes (named TssA-TssM) (Shalom *et al.* 2007; Blondel *et al.* 2009; Boyer *et al.* 2009). There are several components that are conserved among many VISPs, such as TagF and PpkA (post-translational regulators), Fha (structural protein), and VasH (alternative activator of sigma factor 54) (Kitaoka *et al.* 2011; Lin *et al.* 2014). In addition, some VISP-related genes are shared with other systems, such as chaperones, regulators, or substrates. The VISP machinery mediates a one-step process, in which the effector proteins are secreted in Sec- or Tat-independent manner. Hcp tube and VgrG spike are components of VISP, which form a structure resembling the T4 bacteriophage tail (gp19 tube/ gp273–gp53 spike) but in an inverted orientation. VgrG/Hcp structure forms a puncturing device injecting the effector proteins directly into the target cell in a single-step (Figure 1.14) (Basler *et al.* 2012).

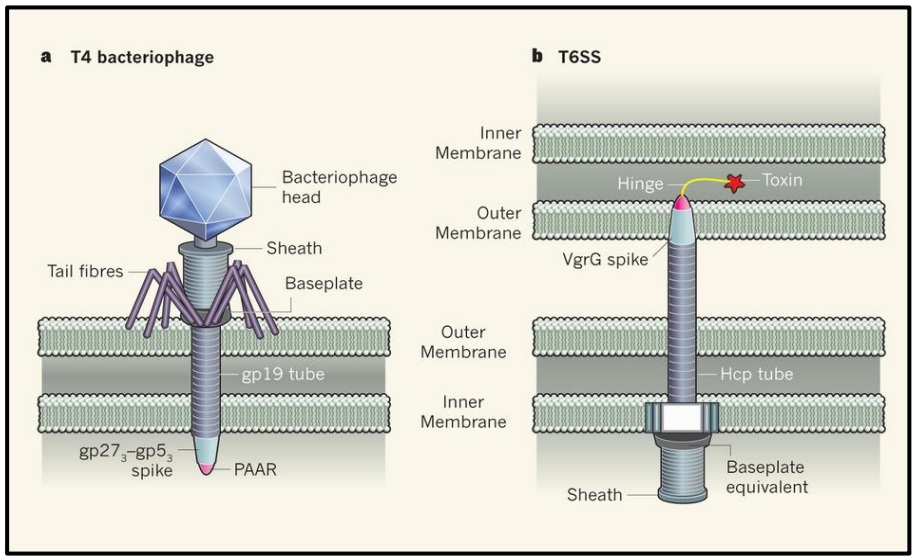


Figure 1.14: The similarities between VISP components (Hcp tube/ VgrG spike) and the tail of T4 bacteriophage (gp19 tube/ gp273–gp53 spike). The VgrG/Hcp structure is used by VISP to inject the effector proteins into the target cell with the help of a sheath structure, which contracts to force the VgrG spike out through the bacterial membrane. The same mechanism is used by the bacteriophages for DNA transfer to infect cells (Shneider *et al.* 2013).

Murdoch *et al.* revealed that *Serratia marcescens* strain Db10 has an active VISP. Essential components of the genome were deleted and the mutants, Δ*ClpV*, Δ*Lip*, Δ*IcmH*, Δ*TssE*, and Δ*SMA2254-2274*, were constructed (Murdoch *et al.* 2011). Immunoblotting using anti-Hcp showed that the secreted proteins were produced by the wild type but not the deletion mutants. To assess the secretion of Hcp even further, the Hcp levels were measured in the supernatant and within the cells using antibodies. Similar levels of Hcp were detected in the supernatant and inside the cells of the wild type. However, Hcp of the deletion mutants (ΔClpV, ΔLip, ΔIcmH, ΔTssE) were not detected in the supernatant but was still present within the cells in large amounts. Therefore, the secretion of Hcp is dependent on an active VISP and not on cell lysis. It has also been demonstrated that the VISP of *S. marcescens* strain Db10 has an antibacterial activity rather than, or in addition to an antieukaryotic activity. This was determined by competition assay, in which the wild type of *S. marcescens* Db10 was mixed with *E. coli* strain MC4100 at 5:1 ratio then co-cultured on an agar plate for 4 h. A 10^7 × drop was observed in the number of the recovered *E. coli* viable cells. The same procedure was repeated by co-culturing the deletion mutants of *S. marcescens* Db10 with *E. coli* MC4100. The number of the recovered cells of

E. coli was increased 10^3 to 10^4 × compared to that with *S. marcescens* wild type. Murdoch *et al.* also concluded that VISP of *S. marcescens* Dp10 has an antibacterial killing activity against closely related strains, such as *S. marcescens* ATCC 274. The two strains were co-cultured, resulting in a 10^4 × decline in the number of the recovered viable cells of *S. marcescens* ATCC 274 (Murdoch *et al.* 2011).

VISP contributes to metal ion uptake, such as iron, manganese, and zinc (Chen *et al.* 2016; Lin *et al.* 2017; Si *et al.* 2017). Gallique *et al.* suggested that VISP might have a similarity to the QS pathway due to cell-to-cell signal between sister cells, which depends on cell density. They confirmed that VgrG, Hcp, and effectors of VISP are transferred to adjacent cells and subsequently reused for a new VISP assembly (Gallique *et al.* 2017).

1.9.7 Type VII secretion pathway (VIISP)

Type VII secretion system (VIISP) is restricted to Mycobacterium species, which have a lipid-rich cell wall. VIISP is not found in Gram-negative bacteria. The first VIISP was identified in 2003 and called ESX. Mycobacteria utilize up to five ESX secretion systems (ESX-1 to ESX-5) to export virulence proteins (Champion and Cox 2007). ESX-1 secretes proteins that are not

encoded by ESX-2 through ESX-5, such as EspA, EspB, EspC, EspE, EspF, EspK, and EspJ (Fortune *et al.* 2005; Sani *et al.* 2010; Millington *et al.* 2011; Champion *et al.* 2014). The mechanism of secretion of Esp proteins by ESX-1 is still not completely clear. EsxA and EsxB substrates of WXG100 family have been studied for the structure of the secretion signal (Poulsen *et al.* 2014). Solomonson *et al.* were the first who described EspB crystal structure from *Mycobacterium smegmatis,* which revealed that EspB belongs to WXG100 superfamily. It has also been confirmed that EspB has a conserved secretion signal, which is essential for its localisation to *M. tuberculosis* surface (Solomonson *et al.* 2015). Daleke *et al.* suggested that YxxxD/E motif is required for EspB secretion. This motif constitutes a signal that is conserved among all VIISP substrates representing a general secretion signal (Daleke *et al.* 2012).

1.9.8 Other secretion systems

Although six different types of systems have been studied for the secretion of many proteins of Gram-negative bacteria, some proteins are still secreted via unknown or ill-defined secretion mechanisms. Obvious examples are the moonlighting proteins, which are proteins that generally lack recognisable sequence motifs for secretion or anchoring. Thus, these proteins have been

called “anchorless surface proteins” or “unconventional secreted proteins” (Henderson and Martin 2011; Copley 2012). It has been suggested that moonlighting proteins might be secreted from dead cells and subsequently bind to the surface of other cells; alternatively they might translocate to the cell surface by an unknown mechanism (Kainulainen and Korhonen 2014).

Boel *et al.* also introduced a hydrophobic tail of 12 amino acids to the C-terminal end of the anchorless protein SDH (surface dehydrogenase) of *S. pyogenes*. This resulted in the abolition of SDH export to the cell surface. The created SDH mutant showed similar GAPDH activity to that of the wild type, however, there was a drop in plasminogen binding and anti-phagocytic activity (Boel *et al.* 2005). Yang *et al.* demonstrated that the hydrophobic α-helical domain (residue 110 to 118) of *Bacillus subtilis* enolase is necessary for its translocation to the cell surface (Yang *et al.* 2011).

Aims of Study

The main focus of this study was to investigate the mechanisms of secretion of the meningococcal moonlighting proteins, enolase and FBA. The initial aim was to test the hypothesis that the conserved lysine residue 337 may influence enolase export and to further characterise any additional functional roles of this residue by generating unmarked mutants lacking the native enolase gene, but containing ectopic copies of wild type or mutant enolase. A further aim was to investigate the role of the putative N-terminal signal peptide of FBA in its surface localisation.

2 Chapter Two: Materials and Methods

2.1 Bacterial strains, media, and growth conditions

N. meningitidis strains used in this study (Table 2.1) were grown on chocolate agar (Oxoid Ltd, Thermo Fisher Scientific) or Brain Heart Infusion (BHI) (Sigma) and incubated at 37°C for 24 h in atmosphere of air with 5 % CO_2. Suspensions of meningocccal strains were grown in BHI broth at 37°C with shaking 200 rpm. *E. coli* strains were used for transformation of plasmids. They were grown on Lysogeny Broth (LB) agar and LB broth at 37°C. Antibiotics (Sigma Aldrich) were used where appropriate at the following concentrations: ampicillin (100 µg ml^{-1}), kanamycin (50 µg ml^{-1}), streptomycin (100 µg ml^{-1}), and erythromycin (5 µg ml^{-1}).

Table 2.1: Bacterial strains used in work described in this study

Bacterial strain	**Use/Genotype** **Relevant characteristics**	**Antibiotic resistance**	**Source or reference**
E. coli			
E. coli JM109 competent cells	Cloning strain endA1, recA1, gyrA96, thi, hsdR17 (r_k^-, m_k^+), relA1, supE44, Δ(lac-proAB), [F´ traD36, proAB, laqI^{q}ZΔM15].		Promega
NEB 5-alpha Competent *E. coli* cells	Cloning strain fhuA2 (argF-lacZ)U169 phoA glnV44 80(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17		New England BioLabs

***N. meningitidis* MC58**			
N. meningitidis MC58Δ*cbbA*	Unmarked deletion of *NMB1869* (*cbbA* or *fba* gene)	Streptomycin	(Tunio *et al.* 2010b)
N. meningitidis MC58Δ*NMB0342 Kan*^{R} *rpsL*^{S}	The *NMB0342* gene in the *rpsL*^{R} strain replaced by kanamycin cassette and *rpsL*^{S} allele	Kanamycin Erythromycin	(Albasri 2014)
N. meningitidis MC58*ΔcbbA cbbA*^{EctK354A}	A copy of *NMB1869* carrying a point mutation (K354A) and erythromycin cassette inserted at an ectopic site of the markerless MC58Δ*cbbA*	Streptomycin Erythromycin	Fariza Shams University of Nottingham
N. meningitidis MC58Δ*pilQΔporA*	*NMB1812* (*pilQ*) replaced by kanamycin cassette and *NMB1429* (*porA*) replaced by Omega	Kanamycin Spectinomycin	(Orihuela *et al.* 2009)
N. meningitidis MC58Δ*skp*	*NMB0181* replaced by a kanamycin cassette	Kanamycin	(Albasri 2014)
N. meningitidis MC58Δ*surA*	*NMB0182* replaced by an Omega cassette	Spectinomycin Streptomycin	(Albasri 2014)
N. meningitidis MC58Δ*skpΔsurA*	*NMB0182* replaced by an Omega cassette and NMB0181 replaced by an kanamycin cassette	Spectinomycin Kanamycin	Hibah Albasri, University of Nottingham

N. meningitidis MC58Δ*hlyB*	*hlyB* replaced by an Omega cassette	Spectinomycin Streptomycin	(Wooldridge *et al.* 2005)
N. meningitidis MC58	Wild type serogroup B isolate		(Tettelin *et al.* 2000)
N. meningitidis MC58 *rpsL*^{R}	*NMB0136* (*rpsL*) carries a point mutation leading to lysine 43 being substituted with threonine	Streptomycin	(Albasri 2014)
N. meningitidis MC58 *rpsL*^{R} pComp1285	Another copy of the *NMB1285* (encoding enolase) and erythromycin cassette inserted at ectopic site	Streptomycin Erythromycin	This study
N. meningitidis MC58 *rpsL*^{R} pComp1285-K337E	Another copy of the *NMB1285*, which carries a point mutation leading to lysine 337 being substituted with glutamate, and erythromycin cassette inserted at ectopic site	Streptomycin Erythromycin	This study
N. meningitidis MC58 *rpsL*^{R} Δ*NMB1285 NMB1285*^{Ect} *Kan*^{R} *rpsL*^{S}	The native *NMB1285* gene in the complemented strain with ectopic enolase replaced by kanamycin cassette and *rpsL*^{S} allele (*knsL*)	Kanamycin Erythromycin	This study
N. meningitidis MC58 *rpsL*^{R} Δ*NMB1285 NMB1285*^{EctK337E} *Kan*^{R} *rpsL*^{S}	The native *NMB1285* gene in the complemented strain with ectopic K337E enolase replaced by kanamycin cassette and *rpsL*^{S} allele (*KnsL*)	Kanamycin Erythromycin	This study

N. meningitidis MC58 *rpsL*^{R} Δ*NMB1285* *NMB1285*^{Ect}	The selective marker (*KnsL*) excised from the *NMB1285* locus of *N. meningitidis* MC58 *rpsL*^{R} Δ*NMB1285 NMB1285*^{Ect} *Kan*^{R} *rpsL*^{S}	Streptomycin	This study
N. meningitidis MC58 *rpsL*^{R} Δ*NMB1285* *NMB1285*^{EctK337E}	The selective marker (*KnsL*) excised from the *NMB1285* locus of *N. meningitidis* MC58 *rpsL*^{R} Δ*NMB1285 NMB1285*^{EctK337E} *Kan*^{R} *rpsL*^{S}	Streptomycin	This study
N. meningitidis MC58 *rpsL*^{R} pComp1285SD	Another copy of the *NMB1285* (encoding enolase) with the incorporation of SD-like sequence upstream and erythromycin cassette inserted at ectopic site	Streptomycin Erythromycin	This study
N. meningitidis MC58 *rpsL*^{R} Δ*NMB1285* *NMB1285SD*^{Ect} *Kan*^{R} *rpsL*^{S}	The native *NMB1285* gene in the complemented strain with ectopic enolase, which includes the upstream SD-like sequence, replaced by kanamycin cassette and *rpsL*^{S} allele (*knsL*)	Kanamycin Erythromycin	This study
N. meningitidis MC58 *rpsL*^{R} Δ*NMB1285* *NMB1285SD*^{Ect}	The selective marker (*KnsL*) excised from the *NMB1285* locus of *N. meningitidis* MC58 *rpsL*^{R} Δ*NMB1285 NMB1285SD*^{Ect} *Kan*^{R} *rpsL*^{S}	Streptomycin	This study
N. meningitidis MC58 *rpsL*^{R} pComp1285porAp	Another copy of the *NMB1285* (encoding enolase), after the replacement of Opa-cbbA promoters in pComp1285 by PorA promoter, and erythromycin cassette inserted at ectopic site	Streptomycin Erythromycin	This study

N. meningitidis MC58 *rpsL*^{R} Δ*NMB1285 NMB1285PP*^{Ect} *Kan*^{R} *rpsL*^{S}	The native *NMB1285* gene in the complemented strain with ectopic enolase, which includes the upstream porA promoter, replaced by kanamycin cassette and *rpsL*^{S} allele (*knsL*)	Kanamycin Erythromycin	This study
N. meningitidis MC58 *rpsL*^{R} Δ*NMB1285 NMB1285PP*^{Ect}	The selective marker (*KnsL*) excised from the *NMB1285* locus of *N. meningitidis* MC58 *rpsL*^{R} Δ*NMB1285 NMB1285PP*^{Ect} *Kan*^{R} *rpsL*^{S}	Streptomycin	This study
N. meningitidis MC58Δ*cbbA cbbA*^{EctA14V}	A copy of *NMB1869* carrying a point mutation (A14V) and erythromycin cassette inserted at an ectopic site of the markerless MC58Δ*cbbA*	Streptomycin Erythromycin	This study
N. meningitidis MC58Δ*cbbA cbbA*^{EctA13V}	A copy of *NMB1869* carrying a point mutation (A13V) and erythromycin cassette inserted at an ectopic site of the markerless MC58Δ*cbbA*	Streptomycin Erythromycin	This study
N. meningitidis MC58Δ*cbbA cbbA*^{EctA13VA14V}	A copy of *NMB1869* carrying a double mutation (A13VA14V) and erythromycin cassette inserted at an ectopic site of the markerless MC58Δ*cbbA*	Streptomycin Erythromycin	This study
N. meningitidis MC58Δ*cbbA cbbA*^{EctA22V}	A copy of *NMB1869* carrying a point mutation (A22V) and erythromycin cassette inserted at an ectopic site of the markerless MC58Δ*cbbA*	Streptomycin Erythromycin	This study

N. meningitidis MC58Δ*cbbA cbbA*^{EctD11E}	A copy of *NMB1869* carrying a point mutation (D11E) and erythromycin cassette inserted at an ectopic site of the markerless MC58Δ*cbbA*	Streptomycin Erythromycin	This study

2.2 Plasmids

Plasmids used in this study are listed in Table 2.2.

Table 2.2: Plasmids used in this study

Plasmids	**Relevant characteristics**	**Antibiotic resistance**	**Source**
pComp-1	Complementation vector consisting of; an Opa promoter, *BamH*I site, cbbA promoter, *Bgl*II site, erythromycin cassette flanked by two meningococcal (NMB0102 and NMB0103); cloned into pGEM®-T Easy vector	Erythromycin Ampicillin	(Albasri 2014)
pComp1285	*NMB1285* (enolase) gene cloned into pComp-1 vector	Erythromycin Ampicillin	This study
pComp1285-K337E	lysine residue (K) at amino acid sites 337 of enolase gene in pComp1285 substituted for glutamate residue (E)	Erythromycin Ampicillin	This study
pComp1285SD	pComp1285 vector with incorporated Shine-Dalgarno like-sequence upstream *NMB1285* gene	Ampicillin	This study

pComp1285proDel	pComp1285 vector after removing Opa-cbbA promoters by inverse PCR	Ampicillin	This study
pComp1285porAp	PorA promoter cloned into pComp1285proDel at the same Opa-cbbA promoter deletion site	Ampicillin	This study
pGEM® -T Easy	Linearized cloning vector with a single 3´-terminal thymidine at both ends	Ampicillin	Promega
pUC19	Cloning vector	Ampicillin	New England Biolabs
pGEM-*ksnL*	Kanamycin resistance cassette and *rpsL*^{S} allele (*KnsL*) cloned into pGEM®-T Easy vector	Kanamycin Ampicillin	This study
pBA1	*NMB1285* gene plus 1kb upstream and 1kb downstream flanking sequence from MC58 cloned into pGEM®-T Easy vector	Ampicillin	This study
pBA2	Re-ligated pBA1 after removing *NMB1285* gene by inverse PCR	Ampicillin	This study
pBA3	Kanamycin resistance cassette and *rpsL*^{S} allele (*KnsL*), which was gel extracted from pGEM-*ksnL* vector, cloned into pBA2 at the same NMB1285 deletion site	Ampicillin Kanamycin	This study

pFS-4	NMB1869 (*cbbA or fba)* gene cloned into pComp-1 vector	Ampicillin	(Shams 2016)
pFS4-A14V	Alanine residue (A) at amino acid site 14 of *cbbA* gene in pFS-4 substituted for valine residue (V)	Ampicillin	This study
pFS4-A13V	Alanine residue (A) at amino acid site 13 of *cbbA* gene in pFS-4 substituted for valine residue (V)	Ampicillin	This study
pFS4-A13VA14V	Alanine residues (A) at amino acid sites 13 and 14 of *cbbA* gene in pFS-4 substituted for two valine residues (V)	Ampicillin	This study
pFS4-D11E	Aspartate residue (D) at the amino acid site 11 of *cbbA* gene in pFS-4 substituted for glutamate residue (E)	Ampicillin	This study
pFS4-A22V	Alanine residue (A) at amino acid site 22 of *cbbA* gene in pFS-4 substituted for valine residue (V)	Ampicillin	This study

2.3 Antibodies

Antibodies used in this study are listed in Table 2.3.

Table 2.3: Antibodies used in this study

Target/ method	Primary antibodies	Concentration	Secondary antibodies	Concentration	Source/ Reference
FBA/ ELISA	Rabbit polyclonal antibody	1:100	Anti-rabbit conjugated to alkaline phosphatase (AP)	1:30000	Provided by Fariza Shams
FBA/ Blotting	Rabbit polyclonal antibody	1:500	Anti-rabbit conjugated to AP	1:30000	Provided by Fariza Shams
FBA/ Blotting	Rabbit polyclonal antibody	1:500	Anti-rabbit Horseradish peroxidase linked F $(ab')_2$	1:30000	GE Healthcare Lifescience
Enolase/ ELISA	Rabbit polyclonal antibody	1:100	Anti-rabbit conjugated to AP	1:30000	Provided by Mahab Al-Jannat
Enolase/ Blotting	Rabbit polyclonal antibody	1:1000	Anti-rabbit conjugated to AP	1:30000	Provided by Mahab Al-Jannat
Pxn/ ELISA	Rabbit polyclonal antibody	1:100	Anti-rabbit conjugated to AP	1:30000	Provided by Mahab Al-Jannat
PorA/ ELISA	Rabbit polyclonal antibody	1:100	Anti-rabbit conjugated to AP	1:30000	(Orihuela *et al.* 2009)
Plg/ ELISA	Goat polyclonal antibody	1:100000	Donkey anti-goat conjugated to-AP	1:5000	Invitrogen

2.3.1 Pre-adsorption of rabbit polyclonal anti-FBA (Acetone Powder)

This method was adopted from (*Antibodies: A Laboratory Manual*). *N. meningitidis* MC58Δ*cbbA* was cultured overnight on chocolate agar then grown in BHI broth at 37°C (5% CO_2). The next day, cells were pelleted by centrifugation (20 min at 3000 × *g*). After determining the weight of the pellet, 1 ml of 0.9% [w/v] NaCl (Sigma) was added per gram of pellet. Following incubation on ice for 5 min, 8 ml of ice-cold acetone was added per 2 ml of saline. This was then incubated at 4°C for 30 min and centrifuged (10 min at 3000 × *g*) then the precipitate was collected. The pellet was re-suspended in fresh acetone, mixed and left for 10 min prior to a further centrifugation (20 min at 3000 × *g*). The acetone-pellet was then left to air dry. Then, 0.5 g of acetone powder was re-suspended in 1 ml of native rabbit anti-FBA antisera and agitated overnight at 4°C. The following day, the supernatant was used as pre-adsorbed sera after spinning out the acetone powder (10 min at 11600 × *g*) and stored at -20°C.

2.4 Whole-cell ELISA

2.4.1 Preparation of meningococcal cells

Meningococci were cultured overnight on chocolate agar then grown in BHI broth at 37°C (5% CO_2) with shaking overnight. The next day, the bacteria were inoculated in 5 ml of fresh BHI broth then grown to OD_{600} ≥ 0.5 (mid-log phase). After centrifuging the broth at 1900 × *g* for 10 min, the supernatant was discarded and the pellet was washed twice with phosphate buffered saline (PBS) (Appendix A) then centrifuged (10 min at 1900 × *g*). Next, the pellet was re-suspended in 5 ml of 0.5% formaldehyde in PBS. After incubating the cells for 30 min with gentle shaking, they were centrifuged at 1900 × *g* for 10 min then washed twice with PBS. Finally, the pellet was re-suspended in 5 ml of sodium carbonate buffer (Appendix A).

2.4.2 The optimised protocol of whole-cell ELISA (Enzyme-linked immunosorbent assay)

Wells (Thermo Fisher Scientific; Nunc immobilizer amino 12×8 strips) were homogeneously coated overnight at 4°C with 100 µl of formaldehyde-fixed whole meningococcal cells in carbonate buffer reproducing the same well-to-well optical densities of several strains. Then 100 µl of 1% BSA/PBS was added to each well to reduce non-specific binding and incubated overnight at

4°C. After that, the plate was washed three times with 0.05% PBST (PBS + 0.05% Tween 20) then 100 µl of the appropriate primary antibody (Table 2.3) in 1% BSA/PBS was added. This step was followed washing the plate three times then adding 100 µl of the appropriate secondary antibody (Table 2.3) in 1% BSA/PBS. Finally, colour was developed by the addition of 200 µl of phosphatase substrate (Sigma; each tablet dissolved in 5 ml of glycine buffer) (Appendix A). A Tecan spark 10M was used for reading the plates at an absorbance of 405 nm.

2.5 Synthesis of oligonucleotide primers

In this study, several pairs of primers were designed (listed in Table 2.4). They were supplied by Sigma (UK).

Table 2.4: Primers used in this study

Primers	Sequences (5'-3')	Restriction sites
Complementation of *NMB1285*		
NMB1285F1	CGCGGATCCATGAGCGCAATCGTTGATATTTTCGC	*BamH*I
NMB1285R1	CGCGGATCCTTATTTGCCCAGTTGGTAGAATGCG	*BamH*I
NMB102F	GTTCGGCGGCTTGCGTGGTCG	
NMB103F	TACGGAGTTGGAAAGCACGGC	

Mutagenesis of *NMB1285*		
enoK337E_F	ATTGCTGGTCGAAGTCAATCAAATC	
enoK337E_R	GCGTTTGCTACGCCTTTTTC	
enomutF1	CCGCCTTTCTTGCGCGGCAGACCG	
enomutR1	TGTCGTCCGACAACGTACAGGTCG	
enomutF2	GGAAGATCTCTGCGTATCGAGGAAGAATTGGC	*Bgl*II
enomutR2	GGAAGATCTACTGTGGGGTTGCCGCGTGAGTCC	*Bgl*II
KnsLF2	GCGGGATCCCAGCTTATCATCGATAAACCCAGCG	*BamH*I
KnsLR2	GCGAGATCTGTAATTATTTAGGACGCTTAGCACCG	*Bgl*II
NMB1284F	GCGCATTGGTACGAATAAGGG	
NMB1285F	GCCGGTATGTCGCAACCTTGTG	
Sequencing		
enoF2	GCTACAAAGCGGGCGAAGACG	
enoRR	CGTGTTCGACCGCCTTCAATACGC	
pBA2seq	GGCGGCTTTCCGGCAAAGATGCCG	
FBAseq	GCCTTATTTACCTATTGCCCAAGGAGACAC	
Shine-Dalgarno like-sequence		
SDseqF1	CAAGGAGACATGGAGAAGATCCATGAGCGC	
SDseqR1	GGCAATAGGTAAATAAGG	
PorA promoter		
proDel F1	GGAAAGATCTATGAGCGCAATCGTTGATATTTTC	*Bgl*II
proDel R1	GGAAAGATCTGGATCCACCGCCATTAGC	*Bgl*II
SynOligo F3	[phosph]GATCCGTGTTTGCCCGATGTTTTTAGGTTTTTATCAAATTTACAAAAGGAAGCCGAT	*BamH*I
SynOligo R3	[phosph]GATCCATCGGCTTCCTTTTGTAAATTTGATAAAAACCTAAAAACATCGGGCAAACAC	*BamH*I

Putative signal peptide of FBA (site-directed mutagenesis)		
A14V FBA-F1	TGATCATGCTGTCGAAAACAGCTAC	
A14V FBA-R1	AGCAGTTGGCGCATGGAT	
A13V FBA-F1	GCTTGATCATGTTGCCGAAAACAG	
A13V FBA-R1	GTTGGCGCATGGATACG	
A13VA14V FBA-F1	GCTTGATCATGTTGTCGAAAACAGCTAC	
A13VA14V FBA-R1	AGTTGGCGCATGGATACG	
D11E FBA-F1	CCAACTGCTTGAACATGCTGCCG	
D11E FBA-R1	CGCATGGATACGAGTGCC	
A22V FBA-F1	CGGCCTGCCGGTGTTCAACGTCA	
A22V FBA-R1	TAGCTGTTTTCGGCAGCATGATCAAGC	

2.6 DNA manipulation

2.6.1 Chromosomal DNA extraction

Genomic DNA was extracted from *N. meningitidis* using the GenElute™ Bacterial Genomic DNA kit (Sigma- Aldrich) according to manufacturer's instructions.

2.6.2 Plasmid DNA extraction

Plasmid DNA was extracted using the GenElute™ Plasmid Miniprep kit (Sigma- Aldrich) according to manufacturer's instructions.

2.6.3 DNA clean up

The PCR products were cleaned using GenElute™ PCR Clean-Up kit (Sigma-Aldrich) according to manufacturer's instructions.

2.6.4 Restriction endonuclease digestion

Restriction enzymes *BamH*I, *Bgl*II, *EcoR*I, and *Sal*I (New England Biolabs) were used to cleave DNA at specific recognition sites following the manufacturer's protocol.

2.6.5 Ligation of DNA

DNA fragments were ligated using T4 DNA ligase (New England Biolabs) according to the manufacturer's instructions.

2.6.6 Dephosphorylation of DNA fragments

To ensure that the vector does not re-circularize during ligation, Antarctic Phosphatase (New England Biolabs) was used following the manufacturer's protocol. 1 µg of plasmid was incubated with 5 units of Antarctic Phosphatase and Antarctic Phosphatase

Reaction Buffer (10X) at 37°C for 30 min then the reaction was stopped by heat-inactivation at 65°C for 5 minutes.

2.6.7 A-tailing

A-tailing was performed following the manufacturer's protocol (pGEM®-T Vector System, Promega) to increase the number of A-overhangs on the 3' end of the PCR products. This procedure started with adding; 1µl *Taq* DNA Polymerase 10X Reaction Buffer, 5 units of *Taq* DNA polymerase with $MgCl_2$, and dATP to a final concentration of 0.2mM; to 7 µl of the PCR product. Then deionized water was added to a final volume of 10µl. This reaction mix was incubated at 70°C for 15–30 minutes.

2.6.8 Annealing oligonucleotides

Two oligonucleotides were re-suspended to the same molar concentration using sterilised distilled water. Then, equal volumes of both complementary oligonucleotides were mixed in 1.5 ml microfuge tube with 10X annealing buffer (Appendix A). The thermal cycler was used for the annealing procedure, 100 µl aliquots was dispensed into PCR tube then placed in a thermal cycler and the program was set as follows:

a. Heat to 95 °C for 2 min.
b. Cool to 25 °C gradually over a period of 45 min.
c. Spin the tube briefly and store at 4 °C until ready to use.

2.6.9 DNA quantification

NanoDrop ND-1000 Spectrophotometer (Thermo Scientific) was used to determine the concentration of the extracted DNA by measuring the absorbance at 260 nm. The purity of DNA was determined by the ratio of the readings at 260 and 280 nm.

2.6.10 Sequencing

Sequencing was performed on Applied Biosystems 3730 DNA Analyser by Source Biosciences. Sequence data was analysed using ApE Plasmid Editor Software.

2.7 Polymerase Chain Reaction (PCR)

Phusion High Fidelity DNA Polymerase (New England BioLabs) was used in PCRs for cloning. In other PCRs, such as verification of transformations, *Taq* DNA Polymerase was used. All PCRs were performed following manufacturer's protocols using a C1000 Touch™ thermal cycler (Bio Rad) using the cycling parameters shown in Table 2.5. and Table 2.6.

Table 2.5: Standard PCR amplification program for Phusion High Fidelity DNA polymerase

Segment	Cycles	Temperature	Time
1	1	98°C	30 s
2	30	98°C	5-10 s
		45-72°C	10-30 s
		72°C	15-30 s/kb
3	1	72°C	5-10 min
Hold		4°C	

Table 2.6: Standard PCR amplification program for *Taq* DNA Polymerase

Segment	Cycles	Temperature	Time
1	1	95°C	30 s
2	30	95°C	15-30 s
		45-68°C	15-60 s
		68°C	1 min/kb
3	1	68°C	5 min
Hold		4-10°C	

2.8 Agarose gel electrophoresis and gel extraction

1× SYBR® Safe DNA Gel Stain (Invitrogen, 0.1 µl ml^{-1}) was used to pre-stain the agarose gels (1%). After loading the samples alongside 1 kb DNA ladder (New England BioLabs), the gels were run in a gel tank (Bio Rad) loaded with 1× Tris-Acetate-EDTA buffer (TAE) at 100V for 60 min. The DNA bands were visualized

using Molecular Imager Gel DocTM XR (Bio Rad) and the sizes were estimated by comparison to the 1-kb ladder. Bands of interest were excised from agarose gels and DNA extracted and purified using GenElute™ Gel Extraction Kit (Sigma-Aldrich).

2.9 Site-directed mutagenesis

Primers for site-directed mutagenesis were designed using the NEBaseChanger™ software (www.NEBaseChanger.neb.com). Mutagenesis was performed according to the manufacturer's instructions (Q5 Site-Directed Mutagenesis kit, New England BioLabs) using the cycling parameters shown in Table 2.7. The KLD-digested PCR product was transformed into NEB 5-alpha Competent *E. coli* cells.

Table 2.7: Q5 Site-directed mutagenesis cycling conditions

Segment	Cycles	Temperature	Time
1	1	98°C	30 sec
2	25	98°C	10 sec
		50-72°C	10-30 sec
		72°C	30 sec/kb
3	1	72°C	2 min

2.10 Bacterial transformations

2.10.1 Heat shock transformation of JM109 competent *E. coli*

Eppendorf tubes were pre-chilled on ice for the experimental transformation and for the control (pUC18 control plasmid). SOC medium was preheated to 42°C. 100 µl aliquots of the JM109 competent *E. coli* cells (Promega) were thawed on ice for 5 min. 0.1–50 ng of DNA in a final volume of 5 µl of dH_2O was added to one aliquot of cells and 1 µl of the pUC18 control DNA was added to the other aliquot, which were returned to ice for a further 10 min. The tubes were heat shocked at 42°C for 45 sec then incubated on ice for 2 min. 0.9 ml of preheated SOC medium was added to the tubes then incubated at 37°C for 1 h with shaking at 200 rpm. The transformation and the control mixtures were plated on LB agar supplemented with the appropriate antibiotic, followed by an overnight incubation at 37°C.

2.10.2 Natural transformation of *N. meningitidis*

N. meningitidis MC58 (*rpsL*^{R}, confers streptomycin resistance, SmR) was plated on chocolate agar then incubated overnight at 37°C (5% CO_2). The next day, meningococci were grown in BHI broth to mid-log phase (OD_{600} approximately 0.5). 20 µl volume of the culture was spotted onto a BHI agar plate then grown for

100 min at 37°C (5% CO_2). After incubation, DNA was spotted (2 µg) on MC58 *rpsL*^{R} then grown for 4 h at 37°C (5% CO_2). The bacterial growth was removed using a sterile loop and re-suspended in 1 ml BHI broth. Aliquots were plated on BHI agar supplemented with the appropriate antibiotic then incubated at 37°C (5% CO_2). The BHI cultures were observed for up to 48 h and further analysed by PCR and sequencing.

2.11 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis

2.11.1 SDS-PAGE

N. meningitidis MC58 strains were plated on chocolate agar then incubated overnight at 37°C (5% CO_2). The next day, meningococci were grown in BHI broth to mid-log phase (OD_{600} approximately 0.5). Whole cell lysates were prepared by re-suspending the cell pellet in 5 × SDS sample buffer (Appendix A) then boiling at 100°C for 5 min. The lysates were loaded onto 10% polyacrylamide gels for electrophoretic separation of proteins alongside ColorPlus™ prestained protein ladder (New England BioLabs). Gels were stained with SDS stain (Appendix

A) and scanned using a GS-800 densitometer to visualise the proteins.

2.11.2 Immunoblot analysis

Proteins were transferred onto nitrocellulose membrane (Amersham™ Protran™ 0.2 µm NC) in semi-dry transfer buffer (Appendix A) using Trans-Blot SD Semi-Dry Transfer Cell at 20V for 20 min. Membranes were blocked in 5% semi-skimmed dry milk (in 0.1% Phosphate Buffered Saline-Tween 20, PBST). After blocking for 1 h, membranes were incubated for 1 h at room temperature with appropriate primary antibodies diluted in the blocking buffer (Table 2.3). Membranes were washed 3 times with PBST then incubated for 1 h with the appropriate concentration of secondary antibodies (Table 2.3) at room temperature. After washing the membranes, nitro-blue tetrazolium and 5-bromo-4-chloro-3'-indolyphosphate substrate (NBT/BCIP, Sigma) was used to produce sharp protein band resolution with little background staining of the membranes, which was visualised using GS-800 densitometer (BIO-RAD). The C-DiGit® Blot Scanner was also used for blot imaging, however, the bands were produced using horseradish peroxidase (HRP) substrate (Bio-Rad).

2.11.3 Stripping of immunoblots

Nitrocellulose membranes were washed three times with PBS then stripped with Restore Western Blot Stripping Buffer (Thermo Scientific) for 15 to 30 min. This was followed by washing the membranes 3 times with PBS then blocking for 1 hr. Next, the membranes were probed with the appropriate primary then secondary antibodies as described in section 2.11.2.

2.12 Sub-cellular fractionation of meningococcal cells

This method was undertaken based on a modification of a previously described method (Nossal and Heppel 1966; Filip *et al.* 1973; Tunio *et al.* 2010b). *N. meningitidis* strains was grown overnight in 20 ml BHI broth at 37°C with shaking 200 rpm. Following harvesting the cells (13 000 × *g*, 2 min), the pellet was re-suspended in 1 ml of elution buffer (Appendix A). To release the cytoplasmic contents, the re-suspended pellet was treated with intermittent sonication using MSE SoniPrep 150 sonicator for 10 cycles (each cycle, 10 sec burst followed by a 10 sec cooling). This was followed by centrifugation at 5000 × *g* for 1 min to remove non-disturbed cells. The clear supernatant was centrifuged at 17 000 × *g* for 30 min then the supernatant

(representing the periplasmic and cytosolic fractions) was removed and stored at −20°C. 0.4 ml of 10 mM Tris-HCl pH 7.5, 10mM $MgCl_2$, 2% Triton X-100 was used to re-suspend the remaining pellet, which was incubated 30 min 37°C then centrifuged 17 000 × *g* for 30 min. The supernatant (representing the cytoplasmic membrane fraction) was removed and stored at −20°C. The final pellet (representing the outer membrane fraction) was re-suspended in 1 ml of 10 mM Tris-HCl pH 7.5, 10mM $MgCl_2$, 1% Triton X-100 by a brief sonication, which was incubated for 30 min at 37°C and then centrifuged at 17 000 × *g* for 30 min. The pellet was re-suspended in 5 × SDS sample buffer and stored at −20°C.

2.13 Bacterial growth curves

Bacterial cultures were incubated overnight at 37°C, with shaking at 250 rpm. The next day, 20 ml of fresh BHI broth was used to dilute the overnight cultures and adjusted to obtain OD 0.1 at 600 nm. The cultures were incubated at 37°C, with shaking at 250 rpm. The OD_{600} was measured every hour for 8 h and three independent experiments were performed.

2.14 Binding of plasminogen to meningococcal cells

Wells (Thermo Fisher Scientific; Nunc immobilizer amino 12×8 strips) were homogeneously coated overnight at 4°C with 100 µl of formaldehyde-fixed whole meningococcal cells in carbonate buffer reproducing the same well to well optical densities of several strains. The next day, the plate was washed three times with 0.05% PBST then 100 µl of 1% BSA/PBS was added to each well to reduce non-specific binding and incubated for 2 h at room temperature with gentle shaking. Following another washing step, 100 µl of 5 µg glu-plasminogen (diluted in 1% BSA/PBS) was added then the plate was incubated for 2 hr at room temperature with shaking. After a washing step, 100 µl of goat anti-plasminogen antibody (1:100000 diluted in 1% BSA/PBS) was added to the wells then incubated overnight at 4°C with shaking. This was followed by washing three times with PBST. Then, 100 µl of donkey anti-goat IgG-alkaline phosphatase conjugate (Sigma) diluted (1:5000) in 1% BSA/PBS was added to each well then followed by a final washing step after incubating for 1 hr. Finally, colour developed by the addition of 200 µl of phosphatase substrate to each well. A Tecan spark 10M was used for reading the plates at an absorbance of 405 nm.

2.15 Web-based search (promoter prediction)

http://www.fruitfly.org/seq_tools/promoter.html.

2.16 Statistical analysis

Statistical analysis was performed using GraphPadPrism 7 and Microsoft® Office Excel 2010 software programmes. All whole-cell ELISA experiments were done in triplicate wells and repeated at least three times unless otherwise stated. Statistical significance was measured using two-tailed t test. A *p* value of <0.05 was considered significant. Some blot images were analysed by ImageJ densitometry software.

2.17 Protein structure analysis

ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) was used for multiple sequence alignments of meningococcal enolase compared with enolase from several strains. Cn3D4.3.1 structure viewer software was utilised to predict the positions of conserved lysine residues of meningococcal enolase based on pneumococcal enolase crystal structure (PDB entry 1W6T), which was used as a model.

3 Chapter Three: Identification of secretion-related or pathogenesis-related systems that could be involved in the secretion of enolase and FBA

3.1 Introduction

Bacteria secrete proteins across their cell envelope either into the extracellular milieu or to the cell surface (van Ulsen and Tommassen 2006; Gerlach and Hensel 2007). The secreted proteins from saprophytic bacteria are involved in nutrient acquisition; however, in pathogenic bacteria they also play important roles as virulence factors, for example in adherence to host cells. Furthermore, they are required to modulate host-cell responses. The cell envelope of Gram-negative bacteria consists of two hydrophobic membranes, the outer membrane and the inner membrane, separated by the periplasmic space, which contains peptidoglycan (Morley and Pollard 2001; Desvaux *et al.* 2009). Proteins have to be transported across this barrier from the cytosolic compartment to reach the cell surface (van Ulsen and Tommassen 2006; Kudva *et al.* 2013). However, this process requires hydrophilic proteins to traverse the two hydrophobic barriers. In prokaryotic cells, protein translocation takes place at the plasma membrane, while in eukaryotic cells, it occurs in the endoplasmic reticulum (Ito and Mori 2009).

Gram-negative bacteria have evolved several different systems for protein secretion, which can be subdivided into Sec-dependent and Sec-independent pathways (Koster *et al.* 2000).

There is a remarkable difference between these systems in their mechanisms, complexity and the sources of energy used to export unfolded, partially folded, or fully folded proteins. Sec-dependent pathways mediate protein transport by two distinct steps. The first step allows the transport of proteins into the periplasmic space, which is followed by a second step for translocation across the outer membrane to the cell surface or extracellular milieu. By contrast, Sec-independent systems mediate the export of proteins in a single step (Saier 2006). Four of the secretory pathways – type I (ISP), type III (IISP), type IV (IVSP), and type VI (VISP) pathways – are Sec-independent; whilst the type II (IISP) and type V secretory pathways use either Sec (general secretory) or Tat (twin-arginine targeting translocase) pathways to export proteins across the inner membrane. Periplasmic proteins are then directed to the extracellular milieu via any of the other systems for exporting proteins across the outer membrane (Saier 2006).

Proteins can also be exported via the OMVs (Bonnington and Kuehn 2014; Kuhn 2014). Some authors have classified OMVs as secretion system type zero (Thay *et al.* 2014; Kieselbach *et al.* 2015; Guerrero-Mandujano *et al.* 2017). OMVs are released from all Gram-negative bacteria and can be observed on the outer membrane in the process of budding by electron

microscopy. The OMV pathway results in the export of more proteins in comparison with the other methods of secretion and vesicles may be capable of long distance transport of proteins. Furthermore, OMV-mediated secretion facilitates the export of membrane-embedded or membrane-associated proteins, which can be delivered directly into the membranes of host cells via fusion of the vesicles with target membranes (Kato *et al.* 2002; Bonnington and Kuehn 2014). These vesicle-associated proteins play major roles in adherence, invasion, antibiotic resistance, and modulation of host immune responses. The vast majority of extracellular proteins are found associated with OMVs (Kesty and Kuehn 2004).

The moonlighting proteins have been referred to as "anchorless surface proteins" or "unconventional secreted proteins", which suggests that they do not have known secretion sequence motifs on the bacterial cell surface. They might be secreted from stressed cells and subsequently bind to the surface of the same or other cells as reported by Oliveira *et al.* Enhancing cell lysis by several factors, such as Triton X-100 and penicillin, increased the amount of GAPDH at the cell surface as well as in the medium (Oliveira *et al.* 2012). At stationary growth phase, the activity of *B. subtilis* isocitrate dehydrogenase, considered to be a cytoplasmic enzyme marker, was increased by 5% in the culture

medium due to a spontaneous lysis (Stephenson *et al.* 1999). Enolase of *E. coli* can be covalently modified (Boel *et al.* 2004). It has been suggested that posttranslational modifications might have a role in the surface localisation of some moonlighting proteins. Despite these findings, a generalised mechanism(s) for translocation of moonlighting proteins to the cell exterior still remains unknown (Oliveira *et al.* 2012).

This chapter describes the whole-cell ELISA method, which was used to identify the secretion-related or pathogenesis related systems that could be involved in enolase, FBA, and peroxiredoxin export by testing a panel of mutants.

3.2 Results

3.2.1 Identification of surface-exposed FBA by whole-cell ELISA

Three independent whole-cell ELISAs were performed (each using triplicate wells per experimental condition) to investigate the surface accessibility of FBA to antibodies. The binding of wild type FBA to antibodies was significantly higher compared to the null mutant MC58Δ*cbbA*, as well as to the negative control (BSA) (p value < 0.0001; based on un-paired two-tailed T-test), confirming the surface localisation of FBA. PorA is a major outer-membrane protein of *N. meningitidis*, therefore, the wild type was treated with anti-PorA antibody to be used as a positive control (Figure 3.1).

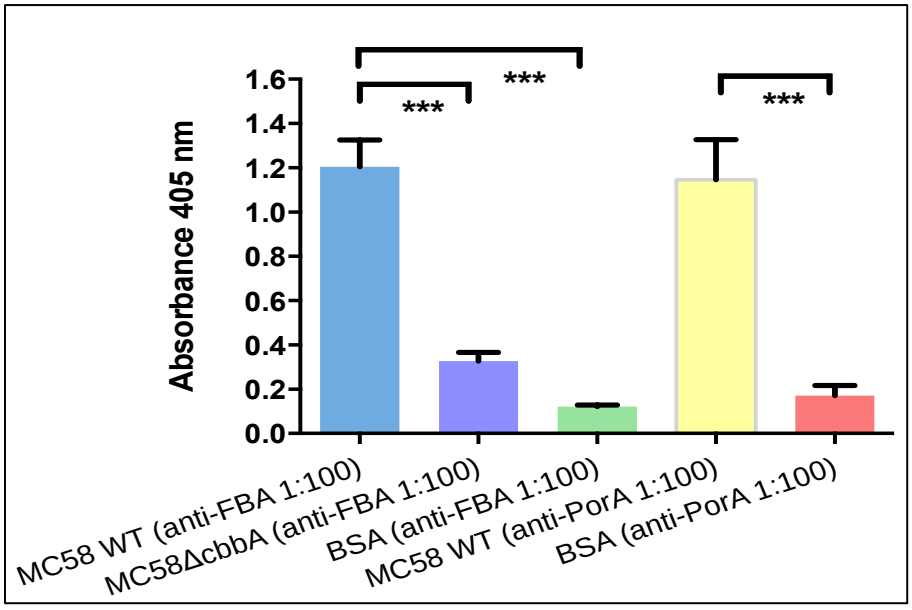


Figure 3.1: Whole-cell ELISA of MC58 wild type (WT) or MC58Δ*cbbA* for FBA surface localisation. The significant difference between the wild type and the FBA-deficient meningococci suggests that FBA is surface exposed to antibodies. Anti-PorA (an outer membrane protein) was used as positive control. The bars indicate the standard error of means. Values shown are means of three independent experiments; on each occasion each sample was tested in triplicate. Significant difference between the means is indicated with *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3.2.2 FBA surface localisation was unaffected in mutants lacking known protein secretion-related or pathogenesis-related proteins

Several mutants were tested using whole-cell ELISA (Table 2.1). MC58Δ*cbbA cbbA*$^{Ect\text{K354A}}$ is a complemented strain containing K354A mutation in the C-terminal lysine residue of meningococcal FBA, which was previously reported to be required for optimal binding to plasminogen (Shams *et al.* 2016). Nevertheless, FBA secretion was unaffected in MC58Δ*cbbA cbbA*$^{Ect\text{K354A}}$, as well as in MC58Δ*pilQ*Δ*porA,* which has a *pilQ* mutation affecting the translocation of type IV pili across the outer membrane (Figure 3.2) (Collins *et al.* 2005).

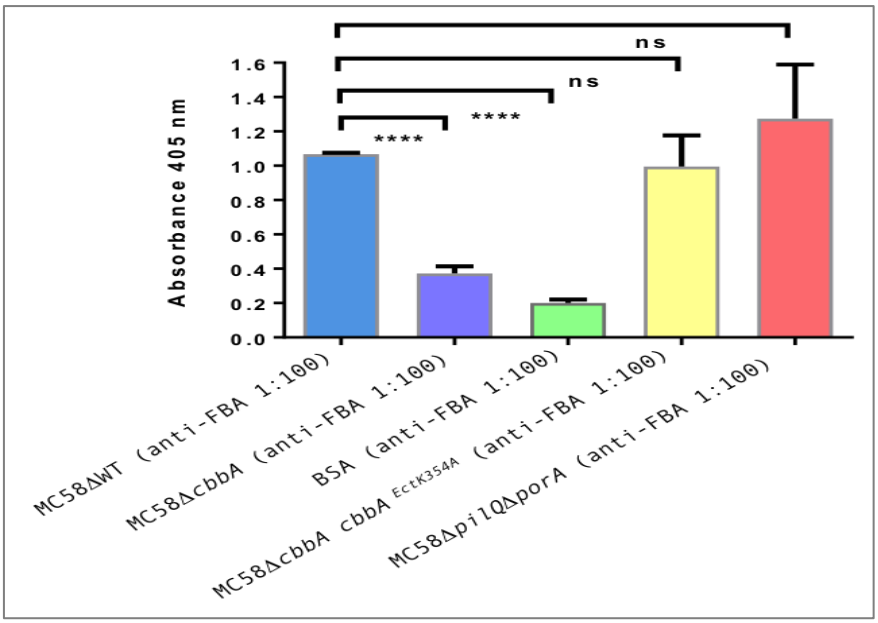


Figure 3.2: Whole-cell ELISA of MC58 wild type, MC58Δ*cbbA*, and other mutants for FBA surface localisation. MC58Δ*cbbA* *cbbA*^{EctK354A} and MC58Δ*pilQΔporA* showed no defect in FBA surface exposure. The bars indicate the standard error of means. Values shown are means of three independent experiments; on each occasion each sample was tested in triplicate. Significant difference between means is indicated with *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

MC58Δ*surA*, MC58Δ*skp,* and MC58Δ*surA*Δ*skp*, lacking the periplasmic chaperones which aid in the biogenesis of autotransporters in some Gram-negative bacteria; as well as MC58Δ*hlyB* (type I secretion system mutant), showed no significant difference compared to the wild type (Figure 3.3).

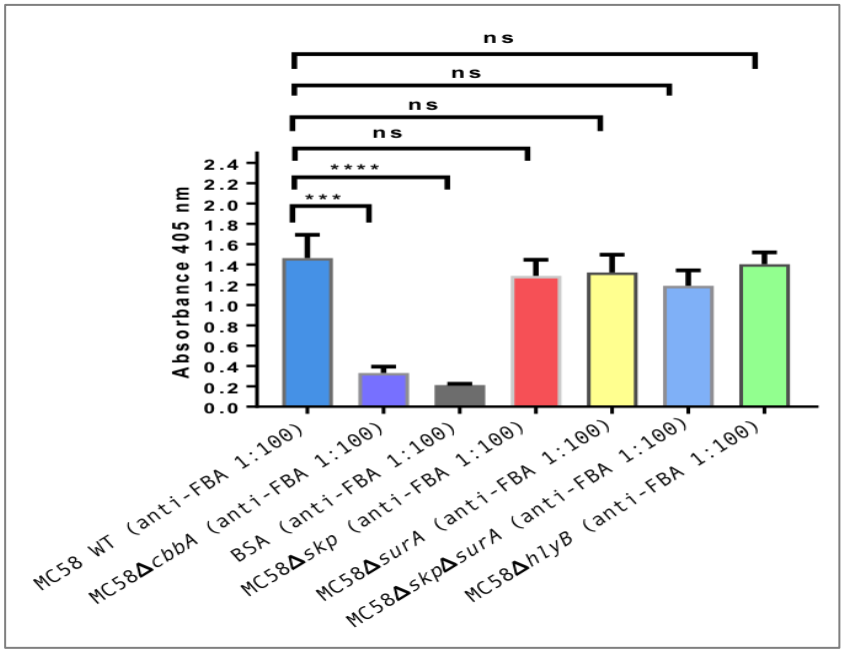


Figure 3.3: Whole-cell ELISA of MC58 wild type, MC58Δ*cbbA,* and other mutants for FBA surface localisation. MC58Δ*skp* and MC58Δ*surA* showed no significant effect in FBA surface exposure compared to MC58 wild type, as well as MC58Δ*skp*Δ*surA* and MC58Δ*hlyB*. The bars indicate the standard error of means. Values shown are means of three independent experiments; on each occasion each sample was tested in triplicate. Significant difference between means is indicated with *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3.2.3 Enolase surface localisation was unaffected in the mutants lacking known protein secretion-related or pathogenesis-related proteins

Several mutants were tested using whole-cell ELISA. Since there is no enolase knockout mutant as it is lethal to meningococci, only BSA was used as a negative control in this experiment. Enolase secretion was unaffected in MC58Δ*surA,* MC58Δ*skp*, MC58Δ*skp*Δ*surA,* and MC58Δ*hlyB* showing no difference compared to MC58 wild type (Figure 3.4).

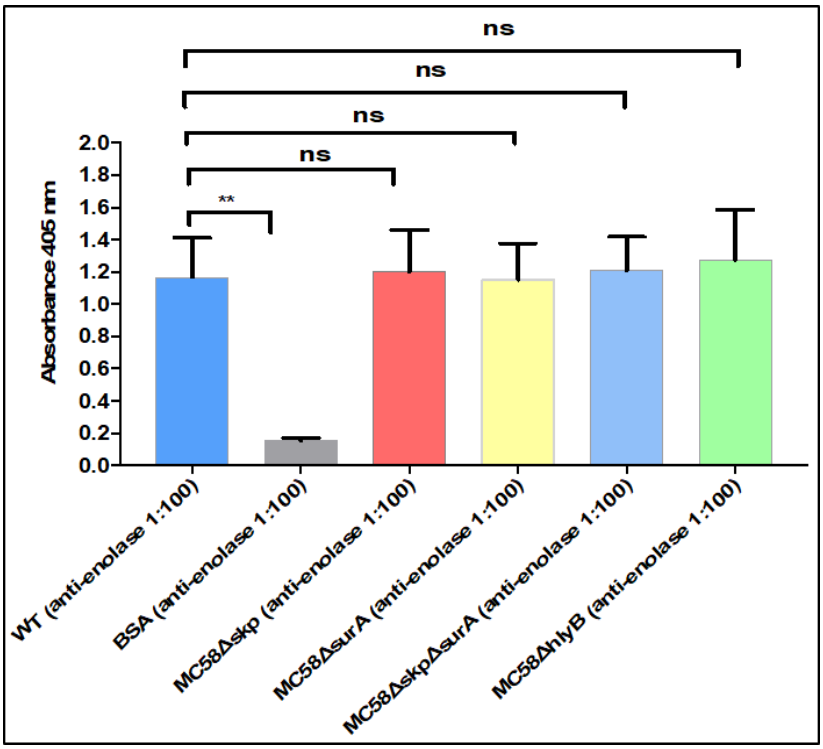


Figure 3.4: Whole-cell ELISA of MC58 wild type and mutants for enolase surface localisation. MC58Δ*skp* and MC58Δ*surA* showed no significant effect in enolase surface exposure compared to MC58 wild type, as well as MC58Δ*skpΔsurA* and MC58Δ*hlyB*. The bars indicate the standard error of means. Values shown are means of three independent experiments; on each occasion each sample was tested in triplicate. Significant difference between means is indicated with *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3.2.4 Peroxiredoxin surface localisation was unaffected in the mutants lacking known protein secretion-related or pathogenesis-related proteins

Several mutants were tested using whole-cell ELISA. Peroxiredoxin secretion was unaffected in MC58Δ*surA*, MC58Δ*skp*, MC58Δ*skp*Δ*surA,* MC58Δ*hlyB*, and MC58Δ*pilQ*Δ*porA* showing no difference compared to the wild type (Figure 3.5).

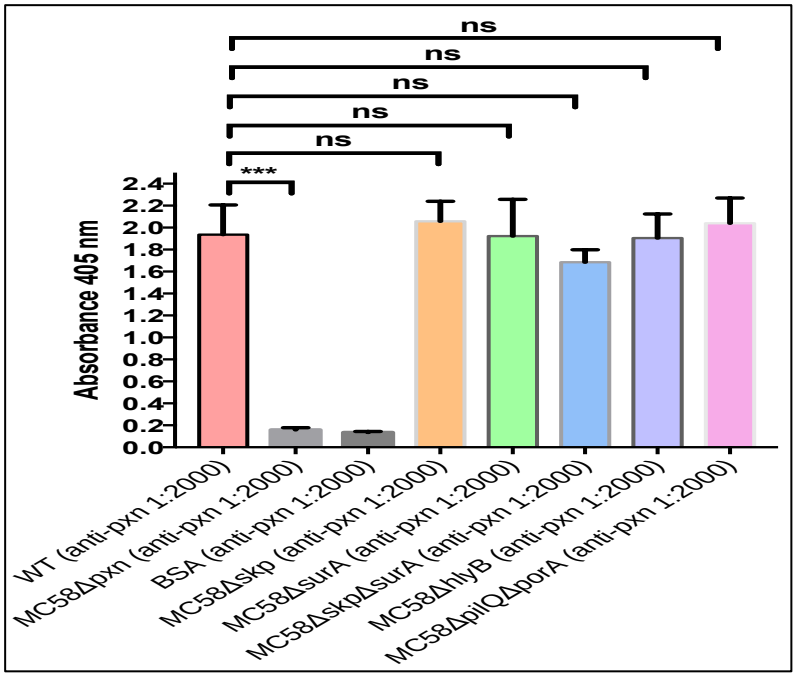


Figure 3.5: Whole-cell ELISA of MC58 wild type and mutants for peroxiredoxin surface localisation. MC58Δ*skp,* MC58Δ*surA,* and MC58Δ*skp*Δ*surA* showed no significant effect in peroxiredoxin surface exposure compared to MC58 wild type, as well as MC58Δ*pilQ*Δ*porA* and MC58Δ*hlyB*. The bars indicate the standard error of means. Values shown are means of three independent experiments; on each occasion each sample was tested in triplicate. Significant difference between means is indicated with *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3.3 Discussion

Whole-cell ELISA screening method was successfully optimised. This method was performed to investigate the localisation of FBA to the surface, which revealed a significantly high signal when probing MC58 wild type cells with both primary and secondary antibodies. This agrees with Tunio *et al.* FACS (fluorescence-activated cell sorting) results confirming that FBA is exposed at the cell surface to exert virulence functions despite the lack of identifiable secretion signal. It has been demonstrated that FBA is not required for growth of *N. meningitidis,* as the knockout mutant has the same growth rate as the wild type (Tunio *et al.* 2010b). It has been shown that FBA is expressed by *N. gonorrhoeae* FA1090, *N. lactamica* ATCC 23970, *Neisseria polysaccharea*, and several meningococcal strains: two MenB; two serogroup C (MenC); one serogroup X (MenX); and one serogroup Z (MenZ). Flow cytometry analysis also confirmed the localisation of FBA to the surface of all strains (Shams *et al.* 2016). A study suggested that FBA is localised to the surface of *S. pneumoniae*, where it acts as an adhesion factor and could possibly be a potential vaccine candidate (Blau *et al.* 2007). Another study also suggested that FBA is exposed at the cell surface of *M. tuberculosis* by measuring the enzymatic activity and labelling the cell surface. Knockout experiments at the *fba*-

tb locus revealed that FBA is essential for growth *of M. tuberculosis* (de la Paz Santangelo *et al.* 2011).

Previous studies have shown that FBA, enolase, and peroxiredoxin are localised to the bacterial cell surface (Knaust *et al.* 2007; Tunio *et al.* 2010b; AlJannat 2016). Nevertheless, the mechanism of their translocation is still not understood. Mutants lacking known protein secretion-related or pathogenesis-related systems were tested for FBA, enolase, and peroxiredoxin surface exposure using whole-cell ELISA, such as MC58Δ*PilQ*Δ*PorA*, MC58Δ*skp*, MC58Δ*surA*, MC58Δ*skp*Δ*surA*, MC58Δ*hlyB* (type I secretion system), and MC58Δ*cbbA* *cbbA*$^{Ect\text{k}354\text{A}}$. Whole-cell ELISA of these mutants probed with both primary and secondary antibodies was employed to investigate surface exposure. It was previously shown that Skp and SurA periplasmic chaperones play a major role in the biogenesis of the serine protease autotransporters of Enterobacteriaceae (Ruiz-Perez *et al.* 2009). In *E. coli*, Skp and SurA chaperones have been shown to play a role in the periplasmic folding and insertion of proteins into the outer membrane (Behrens *et al.* 2001; Rizzitello *et al.* 2001). It is thus likely that the SurA and Skp homologues in *Neisseria* may play a similar role in the secretion of autotransporters or other secreted proteins. A potential role of Skp and SurA chaperones in the secretion of moonlighting

proteins, FBA, enolase, and peroxiredoxin, was investigated in our study, which revealed that there was no effect of Skp and SurA mutation on their surface localisation. Lysine residue at position 354 (C-terminal lysine residue) of FBA was previously reported to be required for optimal binding to human plasminogen (Shams *et al.* 2016). A complemented strain containing this mutation (MC58Δ*cbbA cbbA*$^{Ect\text{k}354\text{A}}$) was made to rule out the possibility that this residue also affect the secretion. However, FBA secretion was unaffected in MC58Δ*cbbA cbbA*^{EctK354A} compared to MC58 wild type. PilQ encodes a secretin, which is a member of a family of proteins required for a number of secretion systems, and in meningococci, it is an essential component of type IV pili and forms the channel through which the pilus translocate across the outer membrane (Collins *et al.* 2005). Therefore, a double mutant containing PilQ mutation (MC58Δ*PilQ*Δ*PorA*) was tested, in case it showed an effect then each mutant will be tested separately, however, it resulted in no effect on FBA and peroxiredoxin secretion.

To summarise, a whole-cell ELISA method for investigating the surface exposure of meningococcal moonlighting proteins was used to suggest that type I secretion pathway is not involved in FBA, enolase, and peroxiredoxin surface localisation. It was also suggested that Skp and SurA molecular chaperones have no role

in the translocation of FBA, enolase, and peroxiredoxin to the cell surface.

4 Chapter Four: Mutagenesis and complementation of the gene encoding enolase in *N. meningitidis* MC58

4.1 Introduction

Mutagenesis and complementation is a strategy used to determine whether two mutations leading to the same phenotype are in the same or different genes, and define the ability of the gene of interest to restore wild-type function by comparing the phenotype of the mutant strain with that of the original and complemented strains. Many studies suggested that enolase might be essential for cell viability due to several unsuccessful attempts to delete the enolase-encoding genes from the bacterial genome of *S. pneumoniae* and *N. meningitidis* (Bergmann *et al.* 2001; Knaust *et al.* 2007). Similar to enolase, Knaust *et al.* suggested that DnaK might also be essential for bacterial survival due to failed attempts to generate a DnaK-deficient meningococcal strain (Knaust *et al.* 2007). In contrast, the mutagenesis strategy has been successful in deleting the gene encoding peroxiredoxin and FBA to investigate their moonlighting functions in *N. meningitidis* (Knaust *et al.* 2007; Tunio *et al.* 2010b; AlJannat 2016).

Two strategies of mutagenesis, insertion duplication and marked allelic replacement, are highly effective and simple to engineer for genetic studies of *Streptococcus mutans.* However, unwanted artefacts can be created affecting the outcome of both

approaches (Russell 1994; Kuramitsu 2003). In addition, the use of insertion duplication and marked allelic replacement mutagenesis within operons can be limited due the polar effects downstream of the mutation site. This limitation, in some instances, has been resolved by the use of antibiotic resistance cassettes, which lack endogenous terminators (Merritt *et al.* 2005; Ahn *et al.* 2006; Zeng *et al.* 2006). Such constructs contain upstream promoters and are referred to as non-polar. They permit a read-through from the upstream promoter and the antibiotic cassette promoter since this cassette has its own promoter. This can potentially result in a differential regulation of downstream genes affecting phenotypic analysis. To overcome such limitations, a markerless mutation can be introduced. This approach is typically a two-step integration and excision process, which uses either a replication vector or a counterselectable suicide vector containing both positive- and negative-selection (Wu and Kaiser 1996; Tatum and Briggs 2005; Atlagic *et al.* 2006). To ease the identification of the markerless mutant clones, the negative-selection marker of the suicide vector removes clones that have not yet finished the excision step (Merritt *et al.* 2007). Nevertheless, it is essential to screen the resultant strains to differentiate the mutant strains from the wild-type strains since the yield of the

counterselectable constructs can be a maximum 50% of the recombinants containing a mutant genotype.

This chapter describes the complementation-first strategy by which the wild type or mutant (K337E) gene encoding enolase in *N. meningitidis* MC58 was inserted at an ectopic site of the bacterial genome. This was followed by markerless mutation strategy to delete the native enolase gene from the genome. The phenotypes of the resulting strains will be subsequently described in Chapter 5.

4.2 Results

4.2.1 Amplification and cloning of *NMB1285* (enolase gene) in pComp-1 vector

The *NMB1285* gene was amplified from *N. meningitidis* MC58 genomic DNA using NMB1285F1 and NMB1285R1 primers (Table 2.4). The PCR product was loaded onto the agarose gel to reveal a fragment of the expected size (1.3 kb) (Figure 4.1A). *BamH*I digestion of the PCR product was performed to obtain restriction sites at the ends of the amplified gene. The vector pComp-1 (Figure 4.1B) was *Bgl*II-digested then de-phosphorylated to prevent re-ligation.

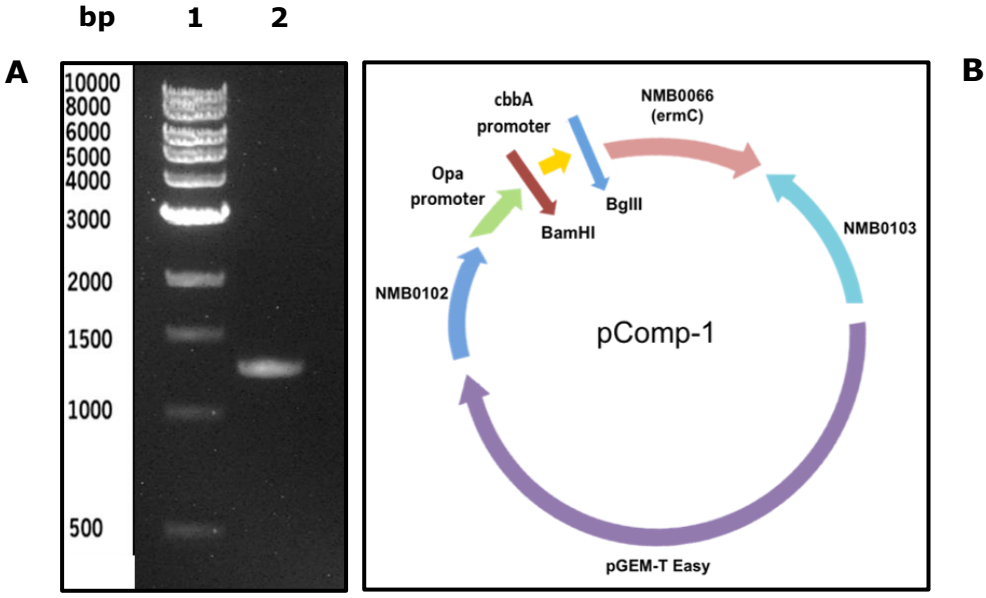


Figure 4.1: (A) Agarose gel image. Lane 1: 1 kb ladder. Lane 2: the amplified *NMB1285* (1.3 kb). (B) pComp-1 vector map.

The *BamH*I-digested amplicon was ligated to the de-phosphorylated pComp-1 vector (Figure 4.2A) then transformed into *E. coli* JM109 competent cells as described in section 2.10.1. The confirmation of the presence of the desired clone (pComp1285) was achieved by restriction digestion using *BamH*I, which yielded a 6.7 kb fragment of the expected size and *Sal*I, which yielded two bands (2.1 kb + 4.6 kb) of the expected sizes (Figure 4.2B). Sequencing using NMB1285F1 and enoF2 primers (Table 2.4) confirmed that the gene was in the right orientation with respect to the promoter and contained no PCR-generated errors.

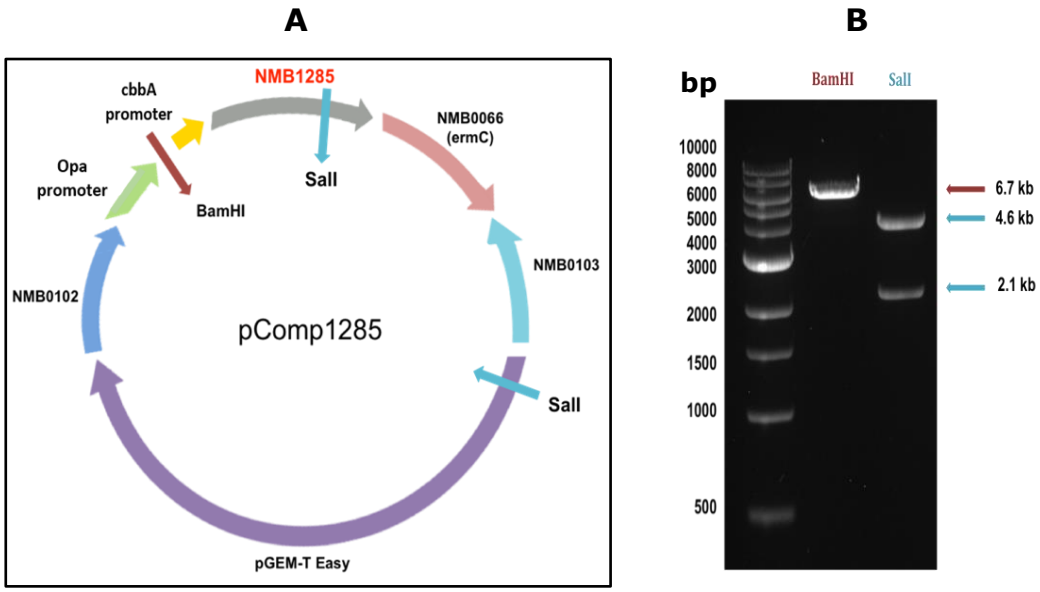


Figure 4.2: (A) pComp1285 map. Highlighted in red is the inserted NMB1285 gene into pComp-1 vector. (B) Restriction digestion of pComp1285 construct using *BamH*I and *Sal*I. The product of the *BamH*I digestion was a 6.7 kb fragment of the expected size. *Sal*I digestion yielded two fragments (2.1 kb + 4.6 kb) of the expected sizes.

4.2.2 Mutagenesis of *NMB1285*

Primers were designed to facilitate the mutation of the codon encoding enolase lysine residue (K337) to glutamate or glutamic acid (E) in pComp1285 (Table 2.4). Mutagenesis was carried out according to the manufacturer's instructions as described in section 2.9. The resulting plasmid was named pComp1285-K337E. Sequencing confirmed the desired mutation (K337E) with NMB1285F1, enoF2, and enoRR primers (Table 2.4) (Figure 4.3).

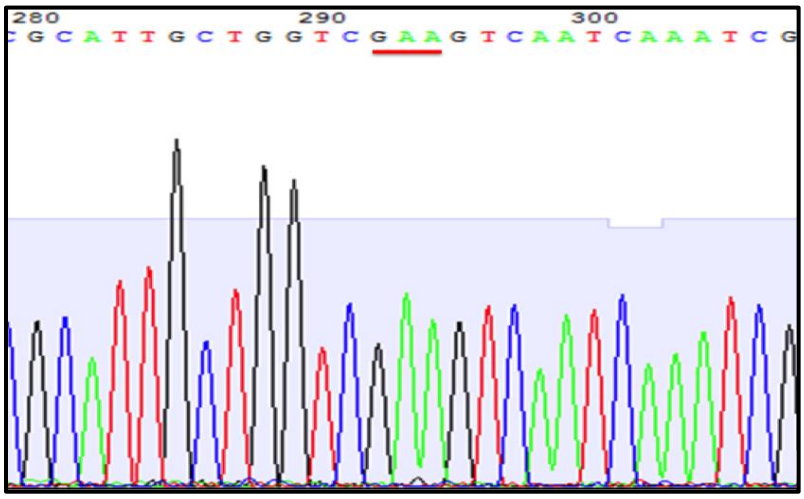


Figure 4.3: A sequence from pComp1285 showing the mutated AAA to GAA (K337E)

4.2.3 Transformation of the clones pComp1285 and pComp1285-K337E

After the confirmation of the desired clones (pComp1285 and pComp1285-K337E), these constructs were transformed into *N. meningitidis* MC58 *rpsL*^{R} as described in section 2.10.2. The constructs used contain an erythromycin resistance cassette flanked by the *NMB0103* and *NMB0102* genes. PCR, and gel electrophoresis were performed to confirm the correct insertion of the enolase gene - erythromycin resistance cassette region of the pComp-based plasmid between the *NMB0103* and *NMB0102* genes of the meningococcal genome using two sets of primers (set1: NMB102F and NMB103F, set2: NMB1285F1 and NMB103F) (Figure 4.4, Figure 4.5).

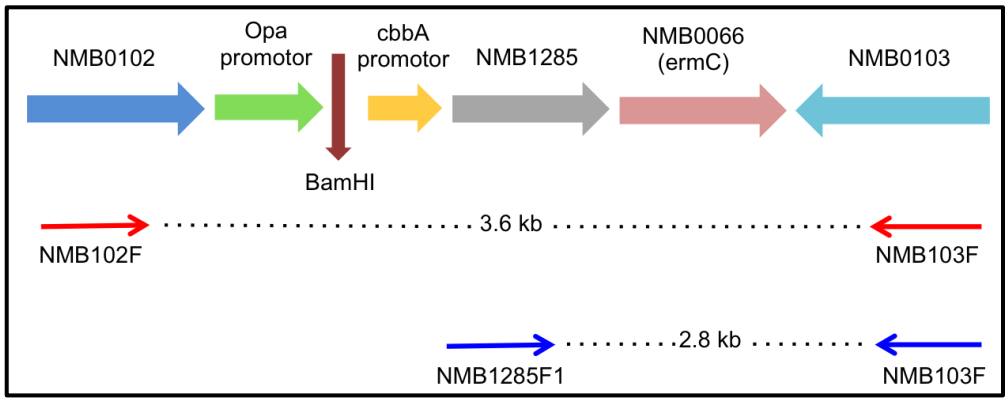


Figure 4.4: The two sets of primers used for confirming the complemented MC58 strains (pComp1285 + pComp1285-K337E). Red arrows represent the 1st set of primers (NMB102F and NMB103F) amplifying a 3.6 kb fragment. Blue arrows represent the 2nd set of primers (NMB1285F1 and NMB103F) amplifying 2.8 kb fragment.

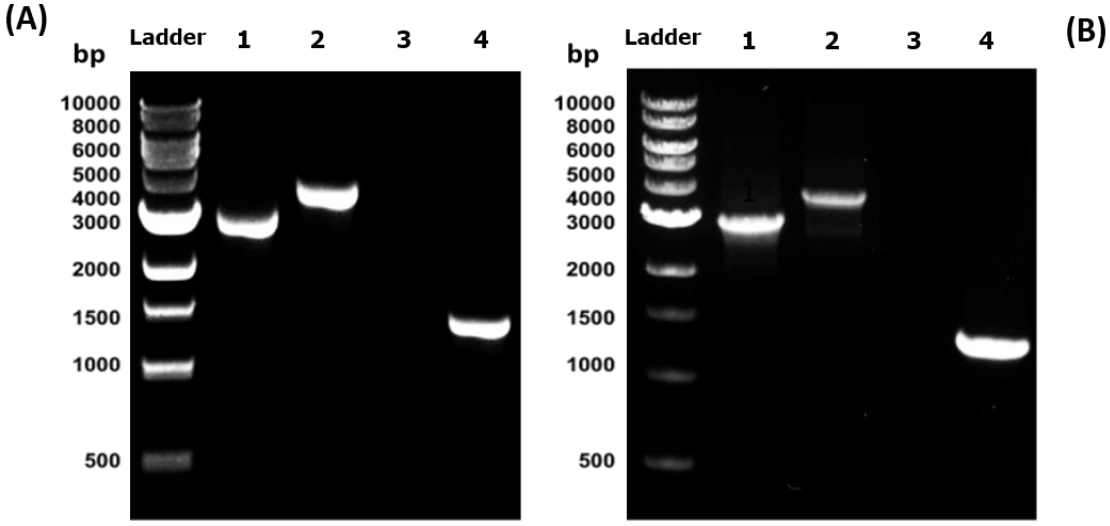


Figure 4.5: Agarose gel image showing (A) The ladder is 1 kb DNA ladder. Lane 1 represents the amplified region of pComp1285 inserted into *rpsL*^{R} MC58 with NMB1285F1 and NMB103F primers showing the expected size of 2.8 kb; Lane 2 represents the amplified region with NMB102F and NMB103F primers showing the expected size band of 3.6 kb; Lane 3 represents the negative control; Lane 4 represents the amplified MC58 wild type using NMB102F and NMB103F primers showing a 1.1 kb band. (B) The ladder is 1 kb DNA ladder. Lane 1 represents the amplified region of pComp1285-K337E inserted into *rpsL*^{R} MC58 with NMB1285F1 and NMB103F primers showing the expected size of 2.8 kb; Lane 2 represents the amplified region with NMB102F and NMB103F primers showing the expected 3.6 kb band. Lane 3 is the negative control and Lane 4 is the amplified wild type with NMB102F and NMB103F primers showing a 1.1 kb band.

SDS-PAGE and immunoblotting experiments were also carried out to determine enolase protein expression as described in section 2.11.1 and 2.11.2. The immunoblot analysis, using rabbit anti-enolase-specific polyclonal antiserum (RαEnolase) as a primary antibody (Table 2.3), showed no difference in enolase (*ca.* 48 kDa) expression levels between the complemented strains and the wild type (Figure 4.6).

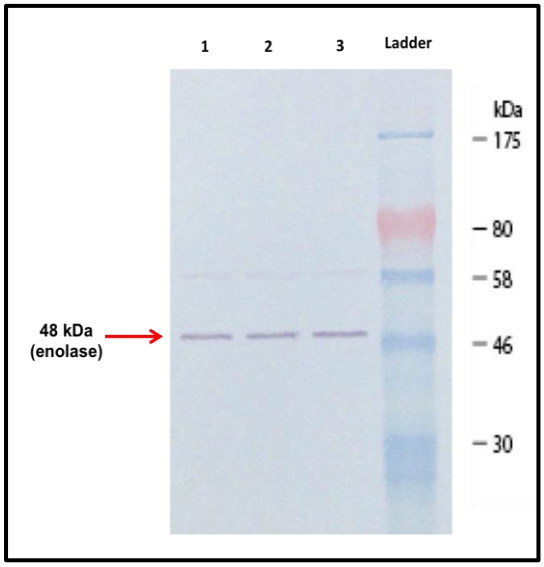


Figure 4.6: Immunoblot analysis of the whole cell lysates of the complemented strains compared to the wild type using RαEnolase. Lane 1 represents the wild type. Lane 2 is *N. meningitidis* MC58 *rpsL*^{R} pComp1285. Lane 3 is *N. meningitidis* MC58 *rpsL*^{R} pComp1285-K337E. This indicates that there was no apparent difference in the expression levels of enolase between the complemented strains (containing two apparently functional enolase genes) and the wild type (only containing one enolase gene).

4.2.4 Markerless mutation of enolase

4.2.4.1 TA cloning of *NMB1285* with the upstream and downstream flanking sequences

The *NMB1285* gene with upstream and downstream flanking regions (1 kb ← *NMB1285* → 1 kb) was amplified using enomutF1 and enomutR1 primers (Table 2.4). A PCR product corresponding to the size 3.3 kb was obtained (Figure 4.7A). This amplicon was purified and ligated to pGEM-T easy vector as described in section 2.6.7, then transformed into *E. coli* JM109 competent cells as described in section 2.10.1 (Figure 4.7B).

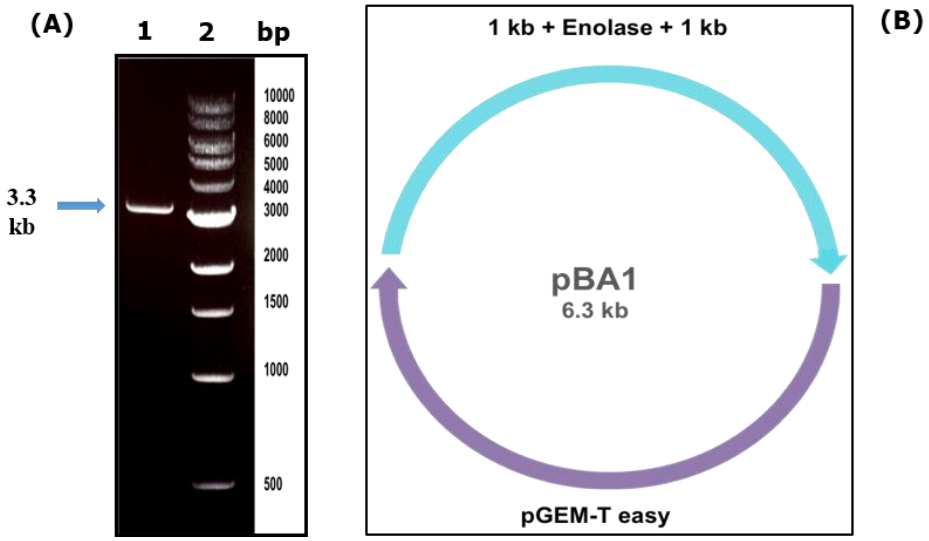


Figure 4.7: (A) Agarose gel image. Lane 1 shows the amplified 3.3 kb *NMB1285* with the flanking sequences. Lane 2 is the 1 kb ladder. (B) pBA1 construct.

The confirmation of a successful TA cloning was carried out by restriction digestion using *Sal*I, which yielded two bands of the expected sizes (5.1 kb + 1.2 kb) and *BamH*I, which yielded one linear fragment of the expected size (6.3 kb) (Figure 4.8). DNA sequencing confirmed the presence of the gene with the flanking sequences.

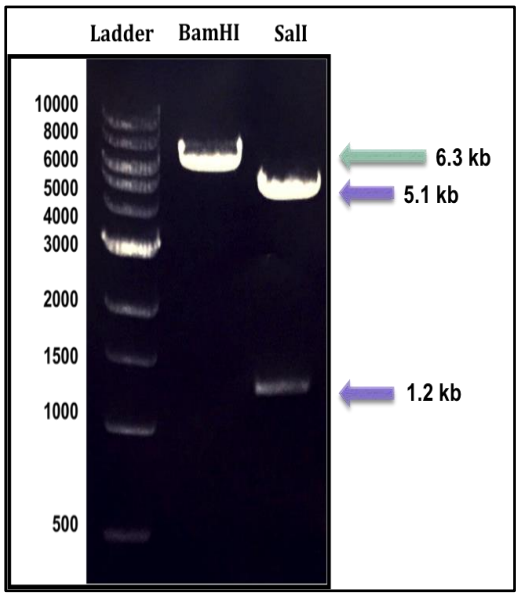


Figure 4.8: Restriction digestion of pBA1 with *Sal*I and *BamH*I. *BamH*I restriction yielded one band of the expected size (6.3 kb). *Sal*I digestion yielded two DNA fragments (5.1 kb + 1.2 kb).

4.2.4.2 Inactivating *NMB1285* by inverse PCR

Following inverse PCR using primers enomutF2 and enomutR2 (Table 2.4) and digestion with *Bgl*II, the product was circularised by ligation then transformed into *E. coli* JM109 competent cells as described in section 2.10.1. The resulting plasmid pBA2 was digested using *Bgl*II, which yielded a 5 kb fragment of the expected size (Figure 4.9). DNA sequencing was also carried out, which confirmed the desired construct containing the deleted gene was obtained.

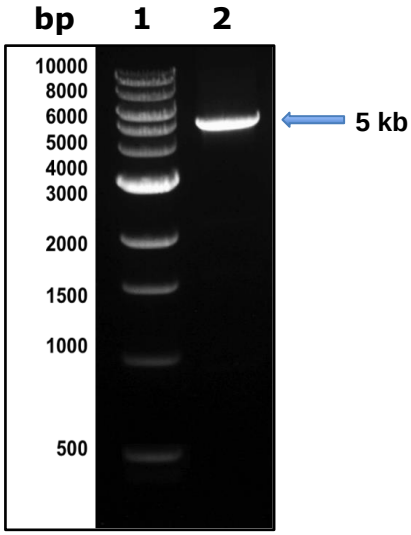


Figure 4.9: Restriction digestion of pBA2 construct using *Bgl*II. Lane 1: 1 kb ladder. Lane 2: linearised pBA2 digested with *Bgl*II showing a 5 kb band.

4.2.4.3 TA cloning of Kanamycin^R *rpsL*^{S} (*KnsL*)

Two primers, KnsLF2 and KnsLR2 (Table 2.4), were designed to amplify *KnsL* cassette from an MC58 strain already containing this cassette (*N. meningitidis* MC58Δ*NMB0342 Kan*^{R} *rpsL*^{S}). This cassette was originally made by Hibah Albasri, University of Nottingham. The amplified *Kan*^{R} and *rpsL*^{S} fragments (*KnsL*), *NMB0342* upstream sequence, *NMB0342* downstream sequence, and the vector pUC19 were assembled by Gibson assembly reaction to build the construct required for the first mutagenesis of *NMB0342* (Gibson 2011; Albasri 2014) (Figure 4.10 A, B).

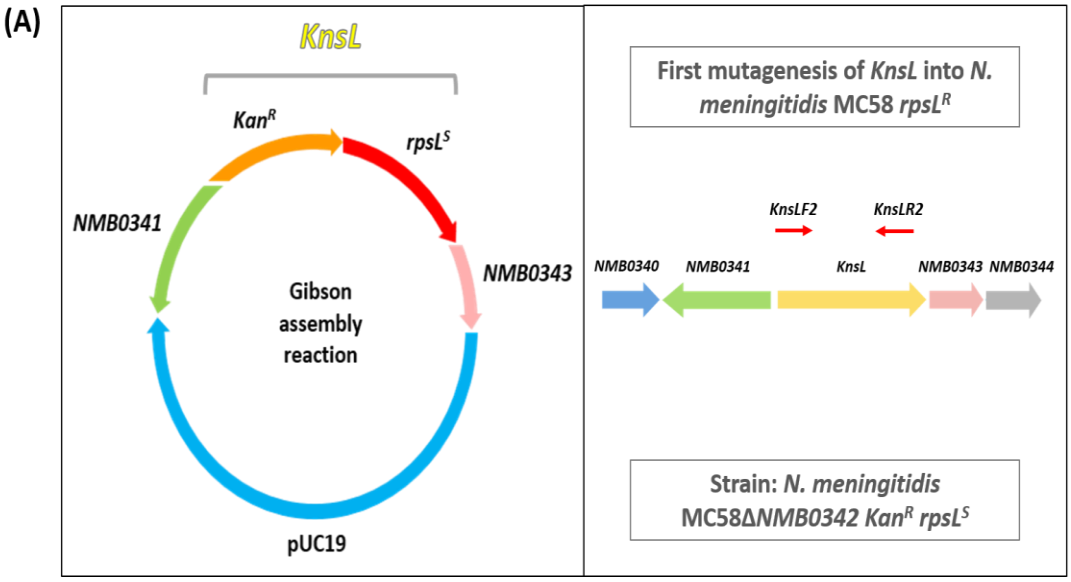


Figure 4.10: (A) The construct formed from the assembly of *Kan*^{R} and *rpsL*^{S} fragments (*KnsL*), *NMB0342* upstream sequence, *NMB0342* downstream sequence, and the vector pUC19 using Gibson assembly reaction (Albasri 2014). (B) *N. meningitidis* MC58Δ*NMB0342 Kan*^{R} *rpsL*^{S} strain generated from the first mutagenesis of *KnsL* into *N. meningitidis* MC58 *rpsL*^{R}. The red arrows represent the primers used to amplify the *KnsL* cassette.

Agarose gel analysis revealed a band of the expected size (2 kb) (Figure 4.11A). The *KnsL* cassette was cloned in pGEM-T easy vector as described in section 2.6.7, then transformed into JM109 competent cells as described in section 2.10.1. The resulting plasmid pGEM-*KnsL* was *BamH*I digested, loaded onto a gel then the *KnsL* cassette was gel-extracted and purified (Figure 4.11B).

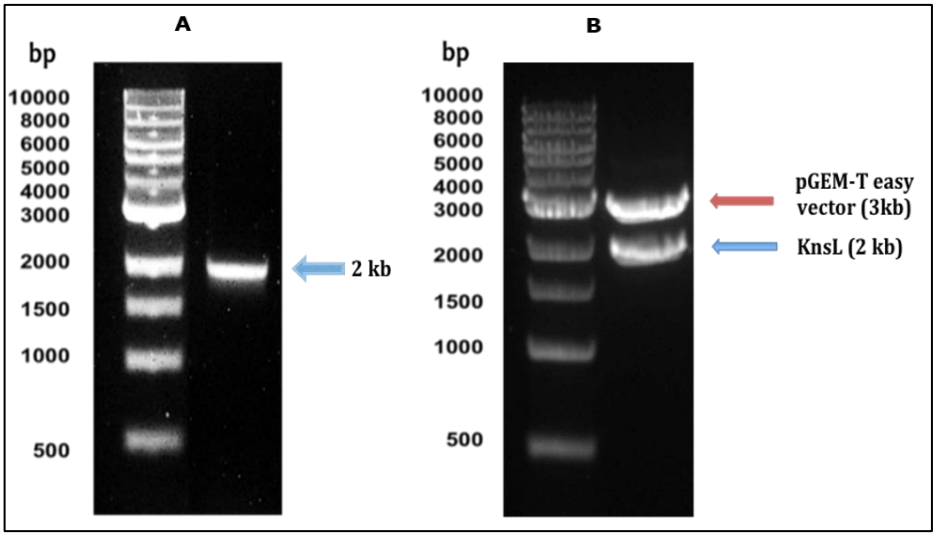


Figure 4.11: (A) Amplified *KnsL* cassette (2 kb). (B) *BamH*I-digested pGEM-*KsnL*. The red arrow represents pGEM-T easy vector. The blue arrow shows the *KnsL* cassette that was gel-extracted and purified.

4.2.4.4 Construction of pBA3

The purified *KnsL* was cloned in *Bgl*II-digested and dephosphorylated pBA2 vector then transformed into JM109 competent cells as described in section 2.10.1 (Figure 4.12A). Restriction digestion using *Sal*I and *EcoR*I and DNA sequencing were carried out to confirm the successful production of pBA3 construct. *Sal*I digestion yielded 1.6 kb and 5.3 kb fragments. While *EcoR*I digestion yielded 3 kb and 3.9 kb expected DNA fragments (Figure 4.12B).

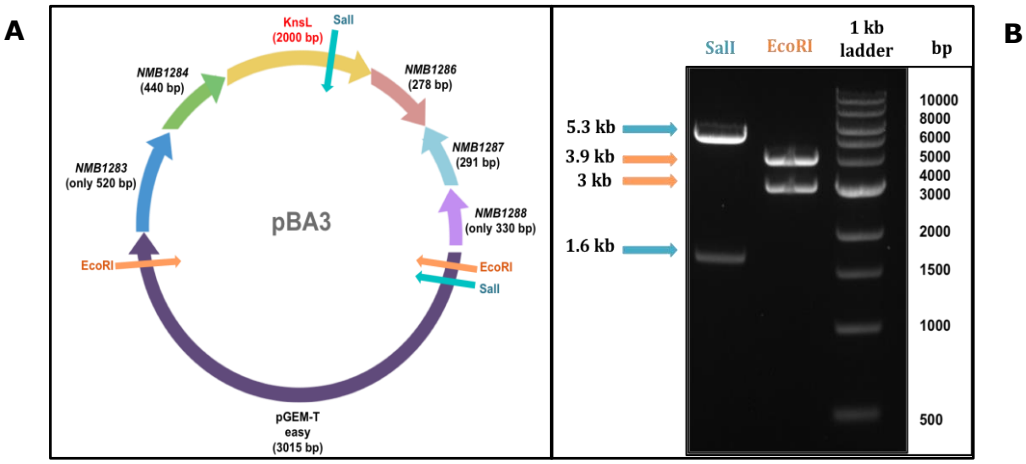


Figure 4.12: (A) pBA3 map. Highlighted in red is the inserted *KnsL* cassette into pBA2 vector. (B) Restriction digestion of pBA3 using *Sal*I and *EcoR*I. SalI digestion yielded 1.6 kb and 5.3 kb expected DNA fragments. While *EcoR*I digestion yielded 3 kb and 3.9 kb fragments of the expected sizes.

4.2.5 First double homologous recombination event

A positive marked selection (KanR SmS) was used for the natural transformation of the construct containing *KnsL* into the native enolase gene in the two previously generated MC58 *rpsL*^{R} strains containing either the wild type or the mutated (K337E) ectopic versions of *NMB1285* (Figure 4.13). The transformants were selected on kanamycin BHI agar (50 µg ml^{-1}) and screened for streptomycin sensitivity.

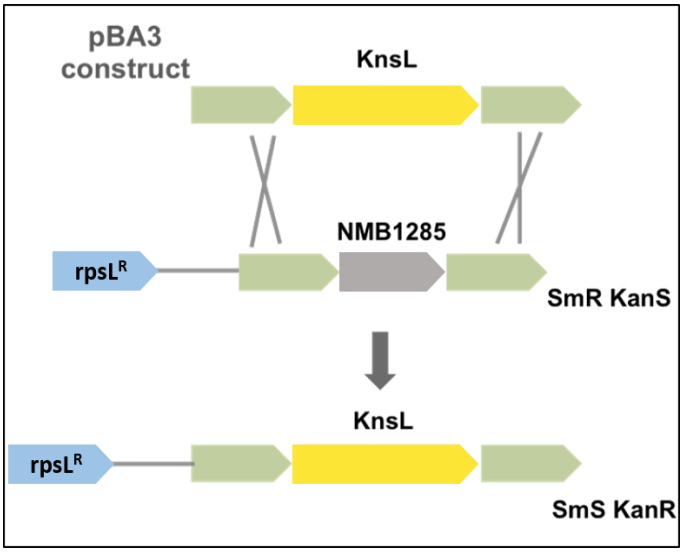


Figure 4.13: First double recombination event in the process for construction of a markerless mutation in enolase using a construct containing a *knsL* cassette. This cassette was transformed into *rpsL*^{R} MC58 complemented strains to replace the *NMB1285* gene. The generated strains were selected on kanamycin (Kan) and tested for streptomycin (Sm) sensitivity.

4.2.6 Verification of the first transformation using pBA3 construct

Genomic DNA of the resulting transformants was used as a template for PCR to confirm the replacement of *NMB1285* gene with *KnsL* cassette in the MC58 *rpsL*^{R} complemented strains (wild type or mutant K337E ectopic enolase). Different sets of primers were designed. DNA sequencing was also performed and the transformation was successful (Figure 4.14, Figure 4.15).

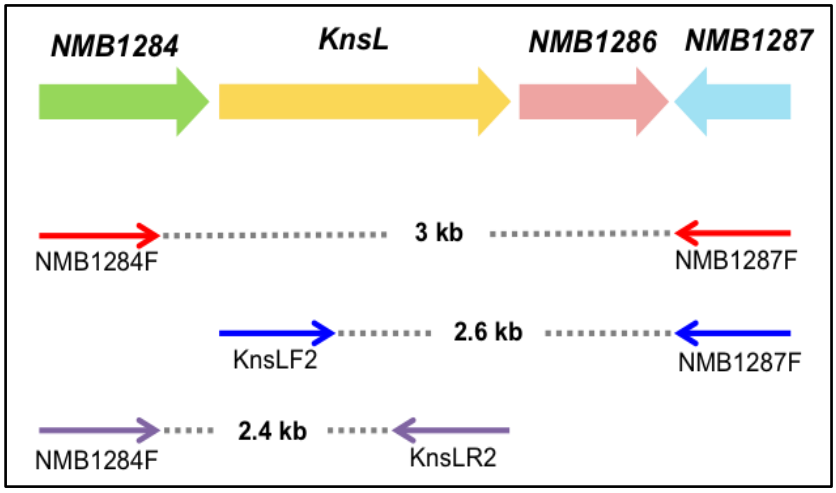


Figure 4.14: The three sets of primers used for verification of the first transformation of pBA3 into the two MC58 *rpsL*^{R} complemented strains. The red arrows represent the first sets of primers (NMB1284F and NMB1287F) amplifying a 3 kb region. The blue arrows represent the second combination of primers (NMB1287F) amplifying a 2.6 kb region. The last combination of primers is represented by purple arrows, which amplify a 2.4 kb region.

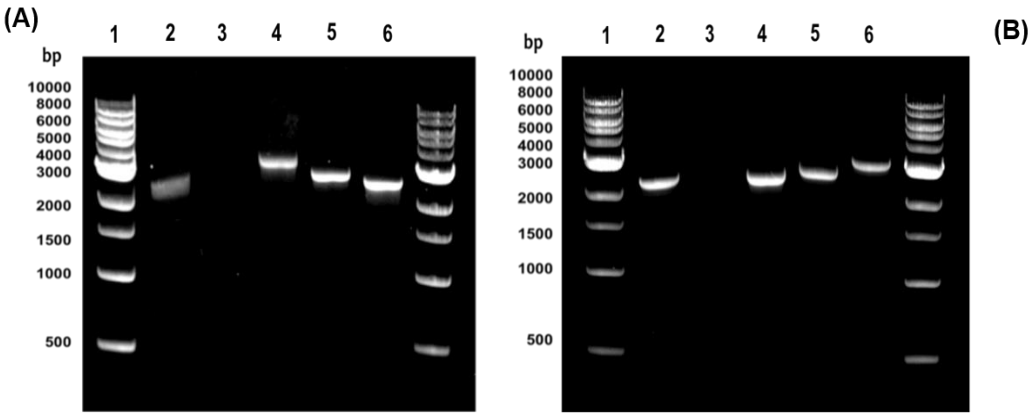


Figure 4.15: **(A)** Verification of the 1st transformation of pBA3 construct into MC58 *rpsL*^{R} complemented strain (wild type ectopic enolase). Lane 1:1 kb ladder. Lane 2: 2.3 kb band of the expected size representing the amplified region of MC58 wild type as a positive control using NMB1284F and NMB1287F primers. Lane 3: the negative control. Lane 4: 3 kb band of the expected size representing the amplified region (from *NMB1284* to *NMB1287*) of *NMB1285* mutant after the insertion of *KnsL* cassette using NMB1284F and NMB1287F primers. Lane 5: 2.6 kb band of the expected size representing the amplified region of NMB1285 mutant (KanR SmS) using NMB1287F and KnsLF2 primers. Lane 6: 2.4 kb band of the expected size representing the amplified region of *NMB1285* mutant (KanR SmS) using NMB1284F and KnsLR2 primers. **(B)** Verification of the 1st transformation of the pBA3 construct into MC58 *rpsL*^{R} complemented strain (K337E mutant ectopic enolase). Lane 1:1 kb ladder. Lane 2: 2.3 kb band of the expected size representing the amplified region of MC58 wild type as a positive control using NMB1284F and NMB1287F primers. Lane 3: the negative control. Lane 4: 2.4 kb band of the expected size representing the amplified region of *NMB1285* mutant (KanR SmS) using NMB1284F and KnsLR2 primers. Lane 5: 2.6 kb band of the expected size representing the amplified region using NMB1287F and KnsLF2 primers. Lane 6: 3 kb band of the expected size representing the amplified region (from *NMB1284* to *NMB1287*) of *NMB1285* mutant after the insertion of *KnsL* cassette using NMB1284F and NMB1287F primers.

4.2.7 Second double homologous recombination event

A negative selection was used to remove the *KnsL* cassette from the two generated MC58 *rpsL*^{R} strains by a second transformation with the construct pBA2 containing an unmarked and inactivated *NMB1285* gene (Figure 4.16). The transformants were selected on streptomycin BHI agar (100 µg ml^{-1}) and screened for kanamycin sensitivity.

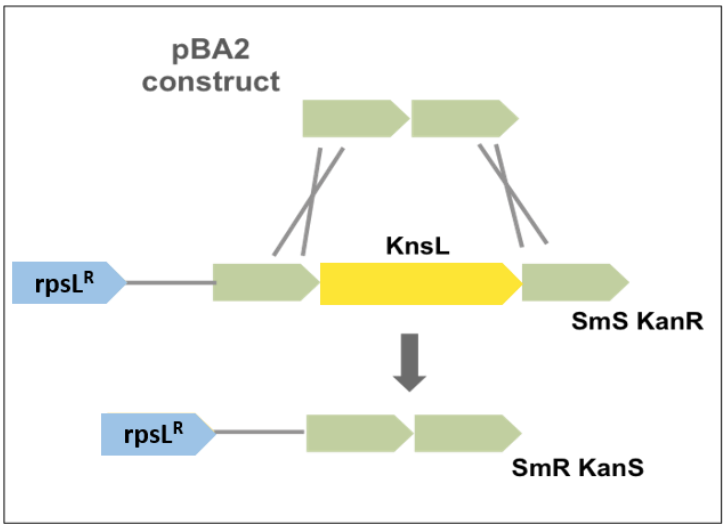


Figure 4.16: The second double recombination event in the process for construction of a markerless mutation in enolase using a construct containing a deleted enolase gene. This construct was transformed into the generated MC58 strains (SmS KanR) to replace the *KnsL* cassette with the unmarked and deleted gene. The final transformants were selected on streptomycin.

4.2.8 Verification of the second transformation using pBA2 construct

The unmarked pBA2 construct containing the inactivated and deleted version of the enolase gene was transformed into the generated kanamycin-resistant strains from the first transformation to replace the *KnsL* cassette. Using different sets of primers, the genomic DNA was amplified by PCR. Successful transformation was also confirmed by DNA sequencing (Figure 4.17, Figure 4.18).

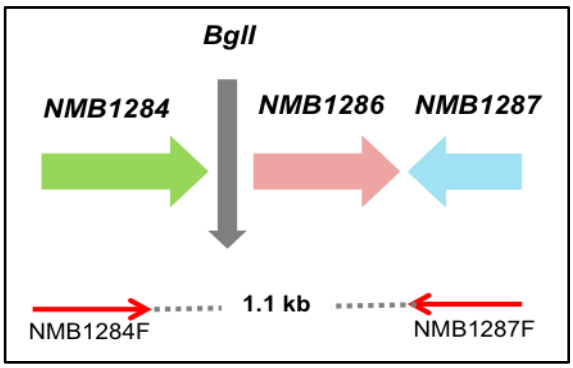
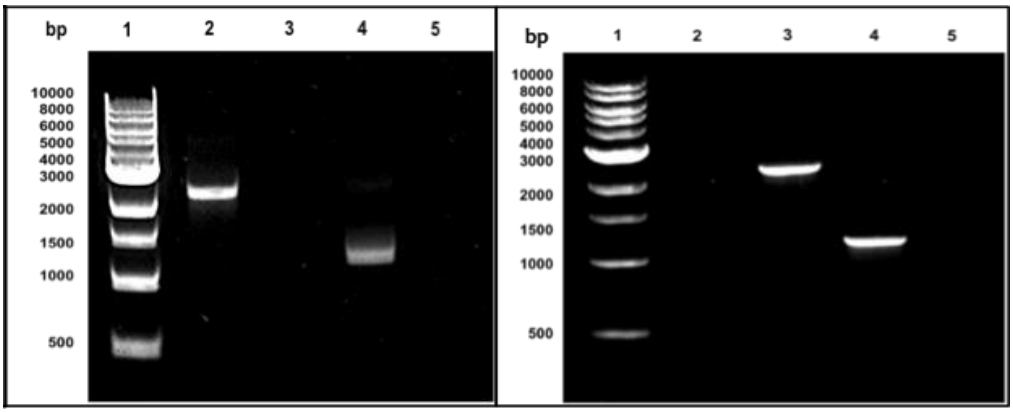


Figure 4.17: Primers used for verification of the second transformation of pBA2 into the two MC58 *rpsL*^{R} complemented strains. The red arrows represent primers (NMB1284F and NMB1287F) amplifying a 1.1 kb region after the replacement of the *KnsL* cassette with the construct containing the inactivated and deleted gene.

A



B

Figure 4.18: **(A)** Verification of the 2nd transformation of pBA2 construct into MC58 *rpsL*^{R} complemented strain (wild type ectopic enolase) containing the *KnsL* cassette. Lane 1:1 kb ladder. Lane 2: 2.3 kb band of the expected size representing the amplified region of MC58 wild type as a positive control using NMB1284F and NMB1287F primers. Lane 3: the negative control. Lane 4: 1.1 kb band of the expected size representing the amplified region (from *NMB1284* to *NMB1287*) of *NMB1285* mutant using NMB1284F and NMB1287F primers after the replacement of *KnsL* cassette with the unmarked construct containing the inactivated and deleted gene. Lane 5: No band representing the amplification using NMB1284F and KnsLR2 primers due to the removal of the *KnsL* cassette. **(B)** Verification of the 2nd transformation of the pBA2 construct into MC58 *rpsL*^{R} complemented strain (K337E mutant ectopic enolase) containing the *KnsL* cassette. Lane 1:1 kb ladder. Lane 2: the negative control. Lane 3: 2.3 kb band of the expected size representing the amplified region of MC58 wild type as a positive control using NMB1284F and NMB1287F primers. Lane 4: 1.1 kb band of the expected size representing the amplified region (from *NMB1284* to *NMB1287*) of *NMB1285* mutant using NMB1284F and NMB1287F primers after the replacement of *KnsL* cassette with the unmarked construct containing the inactivated and deleted gene. Lane 5:.No band representing the amplification using NMB1284F and KnsLR2 primers due to the removal of the *KnsL* cassette.

4.2.9 Expression of ectopic enolase in the unmarked mutants

SDS-PAGE and immunoblotting experiments were carried out to determine enolase expression in the unmarked mutants. The immunoblot analysis was performed using rabbit anti-enolase-specific polyclonal antiserum (RαEnolase) as a primary antibody (Table 2.3), showed a considerable reduction of enolase expression in the two unmarked mutants containing wild type or K337E ectopic enolase (Figure 4.19).

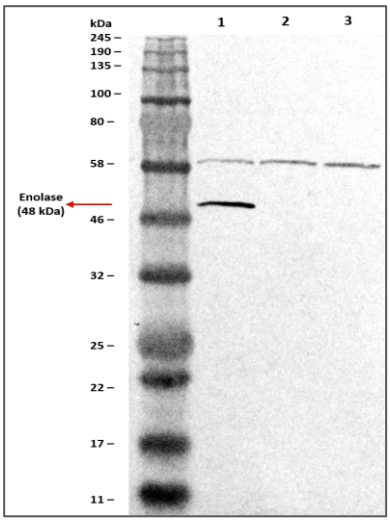


Figure 4.19: Immunoblot analysis of the whole cell lysates of the complemented strains containing wild type or K337E ectopic enolase compared to the wild type MC58 using RαEnolase. Lane 1 represents the wild type. Lane 2 is *N. meningitidis* MC58 *rpsL*^{R} Δ*NMB1285* *NMB1285*^{Ect}. Lane 3 *N. meningitidis* MC58 *rpsL*^{R} Δ*NMB1285* *NMB1285*^{EctK337E}. This indicates that there is a reduction of enolase expression in the complemented strains.

4.3 Discussion

Studies have shown that it is not possible to obtain enolase mutants of *A. hydrophila, C. albicans*, *S. pneumoniae* and *N. meningitidis* (Bergmann *et al.* 2001; Knaust *et al.* 2007; Sha *et al.* 2009; Ko *et al.* 2013). For this reason, MC58 strains were complemented in this study with another copy of the enolase gene before knocking out the original gene. Lysine residue 337 (Lys^{337}) of meningococcal enolase was mutated to glutamate. Then the *N. meningitidis* MC58 $rpsL^R$ pComp1285 and *N. meningitidis* MC58 $rpsL^R$ pComp1285-K337E complemented strains were successfully produced. In this strategy, the enolase gene was inserted alongside an erythromycin resistance cassette at an ectopic site, which confers the complemented strains an erythromycin resistance. Although another copy of gene had been inserted into the genome of the complemented strains, immunoblot analysis showed no difference in the protein expression between the complemented strains and the wild type perhaps suggesting that the amount of enolase expressed is tightly regulated in the cell. Alternatively, expression of enolase from the ectopic site was low (or absent), hence the lack of apparent difference in expression levels between strains harbouring one or two apparently functional copies of the gene.

An unmarked mutation strategy, also called a null or in-frame mutation, was successfully used to knockout the original copy of the enolase gene, and to study the effect of K337E mutagenesis on enolase surface exposure. This strategy was preferable for gene disruption since there would be no selection marker and no concern regarding polar effects (Ishikawa and Hori 2013). Ishikawa and Hori have successfully developed a new methodology for introducing an unmarked mutant into non-competent Gram-negative bacteria using FLP/FRT recombination system. The linear DNA double-crossover events using FLP-FRT recombination based method was also developed for *Cytophaga hutchinsonii* to generate an unmarked deletion of *porU* gene. It is often difficult to introduce an unmarked deletion into a chromosome by simple transformation, therefore, a cassette with a selectable and a counterselectable markers could be used.

The markerless mutation was based on the streptomycin resistance phenotype, which is conferred by *rpsL*^{R} allele. The ribosomal protein S12, encoded by *rpsL*^{R}, interacts with 16S rRNA mediating protein translation (Sparling *et al.* 1968). In susceptible cells, streptomycin can inhibit 16S rRNA mediated protein synthesis. Johnston and Cannon suggested that mutations in *rpsL*^{S} could block the inhibitory effect of streptomycin on protein synthesis, which was implemented in

many organisms, including *N. meningitidis* (Sparling *et al.* 1968; Johnston and Cannon 1999). It has been demonstrated that streptomycin sensitivity (*rpsL*^{S}, wild type allele) is dominant in a streptomycin resistant strain (*rpsL*^{R}, mutant allele) when both alleles are expressed (Breckenridge and Gorini 1970).

This strategy was also adopted in *N. gonorrhoeae* to improve the efficiency of the counterselection. The *rpsL* gene of *E. coli* has been cloned and incorporated into a cassette containing a selectable marker (ermC'). The ermC^{R}-*rpsL*^{S} cassette has been cloned into the gene of interest and then used to replace the wild-type gene on the genome by allelic exchange. Subsequently, this cassette is replaced with an unmarked deletion or other mutations (Johnston and Cannon 1999; Cloud and Dillard 2002). Other cassettes have been constructed implicating a selective kanamycin resistance marker (*Kan*^{R}) and a counterselective streptomycin sensitivity marker (*rpsL*^{S}*),* referred to as *KnsL* in this study. This was demonstrated in *S. pneumoniae* by homologous recombination in a two-step transformation event to replace the gene of interest in order to avoid introducing new antibiotic resistance markers (Sung *et al.* 2001; Li *et al.* 2014).

Two plasmids, pBA2 and pBA3, were constructed in this study for the markerless mutation of *NMB1285*. Both contain the

flanking regions of *NMB1285* to facilitate the two homologous recombination events. In the first transformation, the *NMB1285* gene was replaced with *KnsL* (*Kan*^{R}/*rpsL*^{S}) cassette in the two *N. meningitidis* MC58 *rpsL*^{R} complemented strains, one contains the wild type ectopic enolase and the other contains K337E mutated ectopic enolase. As a consequence, the resultant transformants were sensitive to streptomycin, therefore, the successful insertion of the dominant *rpsL*^{S} allele was identified by screening for kanamycin resistance. The resultant strains were specified as *N. meningitidis* MC58 *rpsL*^{R} Δ*NMB1285 NMB1285*^{Ect} *Kan*^{R} *rpsL*^{S} and *N. meningitidis* MC58 *rpsL*^{R} Δ*NMB1285 NMB1285*^{EctK337E} *Kan*^{R} *rpsL*^{S}*.* The next step was to remove the selective marker from these strains in a second homologous recombination event, which was successfully accomplished by transforming pBA2 into *N. meningitidis* MC58 *rpsL*^{R} Δ*NMB1285 NMB1285*^{Ect} *Kan*^{R} *rpsL*^{S} and *N. meningitidis* MC58 *rpsL*^{R} Δ*NMB1285 NMB1285*^{EctK337E} *Kan*^{R} *rpsL*^{S}*.* Subsequently, *KnsL* cassette (*Kan*^{R}/*rpsL*^{S}*)* in both strains was replaced with the *NMB1285* flanking DNA. As a result, the successful transformants were screened for streptomycin resistance. The final markerless strains were specified as *N. meningitidis* MC58 *rpsL*^{R} Δ*NMB1285 NMB1285*^{Ect} and *N. meningitidis* MC58 *rpsL*^{R} Δ*NMB1285 NMB1285*^{EctK337E}.

PCR was first utilized to confirm the insertion of various DNA fragments into the genome of MC58 *rpsL*^{R} during complementation or deletion of *NMB1285* during markerless mutation. In addition, further verification was implemented by immunoblot analysis, which indicated that there is a reduction of enolase expression in the two strains, *N. meningitidis* MC58 *rpsL*^{R} *ΔNMB1285 NMB1285*^{Ect} and *N. meningitidis* MC58 *rpsL*^{R} Δ*NMB1285 NMB1285*^{EctK337E}, compared to MC58 wild type. Despite the fact that it was difficult to detect apparent bands of enolase in the lysate samples of the complemented strains, the expression of enolase had been restored since previous studies have shown that enolase is essential for meningococcal survival.

To conclude, mutants lacking the native enolase gene, but harbouring ectopic copies of wild type or mutant (K337E) enolase were successfully generated in *N. meningitidis* MC58 *rpsL*^{R}*.* However, this method caused a considerable reduction of enolase expression. The growth rates of all strains were subsequently monitored (Chapter 5) to investigate the impact of such a reduction in the expression of enolase.

5 Chapter Five: Investigating the role of a single lysine residue in the surface localisation of enolase

5.1 Introduction

Enolase, also known as phosphopyruvate hydratase, is one of the key catalytic enzymes of a number of phylogenetically distinct organisms from Archaea, Bacteria, and Eukarya (Wold and Ballou 1956; Brewer 1981; Reed *et al.* 1996; Zhang *et al.* 1997). It catalyses the conversion of 2-phosphoglycerate (2-PG) into phosphoenolpyruvate and vice versa. For this reason, it is essential for glucose synthesis and carbohydrates' degradation during gluconeogenesis and glycolysis, respectively (Wold and Ballou 1957; Wold 1971). It was first discovered by Lohman and Meyerhof in 1934 (Lohman and Meyerhof 1934). Enolases are highly conserved in bacteria with molecular weights ranging from 80 to 100 kDa (Zadvornyy *et al.* 2015). Studies have reported that enolase, which is purified from hyperthermophilic bacteria, *Thermus aquaticus* and *Thermotoga maritime,* has an octameric structure (Stellwagen *et al.* 1973; Schurig *et al.* 1995). In addition, a homodimeric structure of enolase has been reported in the anaerobic archaeon *Pyrococcus furiosus* (Peak *et al.* 1994). In vertebrates, enolase exists in three isoforms: γ-enolase (Eno2) or the neuron specific enolase is localised only in neurons and neuroendocrine tissues, and β-enolase (Eno3) or the non-neuronal enolase is localised in glial cells, whereas α-

enolase (Eno1) is present in almost all human cells (Marangos *et al.* 1978).

It has been suggested that α-enolase acts as a heat-shock protein (HSP48), a neurotrophic factor, and a hypoxic stress protein (Iida and Yahara 1985; Takei *et al.* 1991; Aaronson *et al.* 1995). In addition, α-enolase appears to be a part of the crystallin lens of vertebrates (Mathur *et al.* 1992). Interestingly, high affinity binding of enolase to adjacent enzymes in the glycolytic pathway, such as pyruvate kinase and phosphoglycerate mutase, has been reported (Figure 1.1) (Merkulova *et al.* 1997). Furthermore, the upregulation of α-enolase has been suggested to be involved in non-glycolytic mechanisms, which contributes to hypoxia tolerance (Subramanian and Miller 2000). A correlation of the increased expression of α-enolase with cancer progression has also been reported, therefore, enolase could potentially be considered as a marker for many tumours, such as neuroblastoma and lung cancer (Niklinski and Furman 1995; Baudin *et al.* 1998; Eriksson *et al.* 2000; Takashima *et al.* 2005).

The list of confirmed "moonlight proteins" (Piatigorsky 1998; Jeffery 1999), that are proteins demonstrating multiple unrelated functions at distinct cellular sites, is growing. Ullberg *et al.* were the first to describe plasminogen binding on the cell

surface of *N. meningitidis* and *N. gonorrhoeae* (Ullberg *et al.* 1992). This phenomenon has been also recognised in other bacterial pathogens (Lahteenmaki *et al.* 2005; Sanderson-Smith *et al.* 2012). α-Enolase has been previously identified as a plasminogen receptor on the surface of carcinoma cells; pathogenic bacteria, such as *Streptococcus* sp.; and non-pathogenic bacteria, such as lactobacilli (Lopez-Alemany *et al.* 1994; Pancholi 2001; Hurmalainen *et al.* 2007; Glenting *et al.* 2013). For this reason, α-enolase has a substantial role in the regulation of extracellular and intravascular fibrinolytic systems facilitating disease development and progression (Ji *et al.* 2016). The interaction of α-enolase with plasminogen is mediated by high affinity binding of C-terminal lysine residues of α-enolase to the kringle domains of plasminogen facilitating its cleavage to plasmin by plasminogen activators (Miles *et al.* 1991; Redlitz *et al.* 1995; Lahteenmaki *et al.* 2000). Candela *et al.* were the first to report enolase localisation on the cell surface of four species of Bifidobacterium: *B. longum*, *B. bifidum*, *B. breve* and *B. lactis*. They demonstrated the localisation of enolase on the surface of *B. lactis* BI07 by immunoelectron microscopy (Candela *et al.* 2009). Their data suggest that the internal plasminogen binding site, involved in the interaction between the model strain *B. lactis* BI07 enolase and human plasminogen, is homologous to

the BS2 site of pneumococcal enolase (Candela *et al.* 2009). It has been demonstrated that despite the low abundance of surface-exposed pneumococcal enolase, plasminogen binding is high and contributes to the pathogenesis of *S. pneumoniae* (Bergmann *et al.* 2003; Kolberg *et al.* 2006).

Enolase is considered to be a cytosolic enzyme, however, it is also associated with the cell surface of some pathogenic and commensal bacteria as an "anchorless'' protein, however, the mechanism of its secretion is still under debate as it lacks a typical signal peptide (Chhatwal 2002; Huberts and van der Klei 2010; Henderson and Martin 2011; Kainulainen and Korhonen 2014). On the other hand, it has been demonstrated that a hydrophobic membrane-embedded domain of *B. subtilis* enolase has an important role in its secretion (Hu *et al.* 2004; Hu *et al.* 2007; Yang *et al.* 2011). This domain was substituted with a β-galactosidase sequence, which decreased the hydrophobicity of the α-helix structure and completely abolished the translocation of the modified enolase. Another α-helix domain (Q132P) was also mutated, however, this substitution had no effect on the secretion of enolase (Yang *et al.* 2011).

This chapter describes the role of Lys^{337} in enolase surface localisation. In the previous chapter, a reduced expression of enolase was observed following the unmarked mutation of

enolase, therefore, several attempts to enhance the expression of enolase were made before making the final conclusion. The effect of this reduction on plasminogen binding to whole meningococcal cells and on growth rates was also assessed.

5.2 Results

5.2.1 Identification of surface-exposed enolase by whole-cell ELISA

MC58Δ*NMB1285* *NMB1285*^{Ect} and MC58Δ*NMB1285* *NMB1285*^{EctK337E} strains were tested for enolase surface localisation using whole-cell ELISA. Both strains showed a significant reduction on enolase surface exposure compared to MC58 wild type (Figure 5.1).

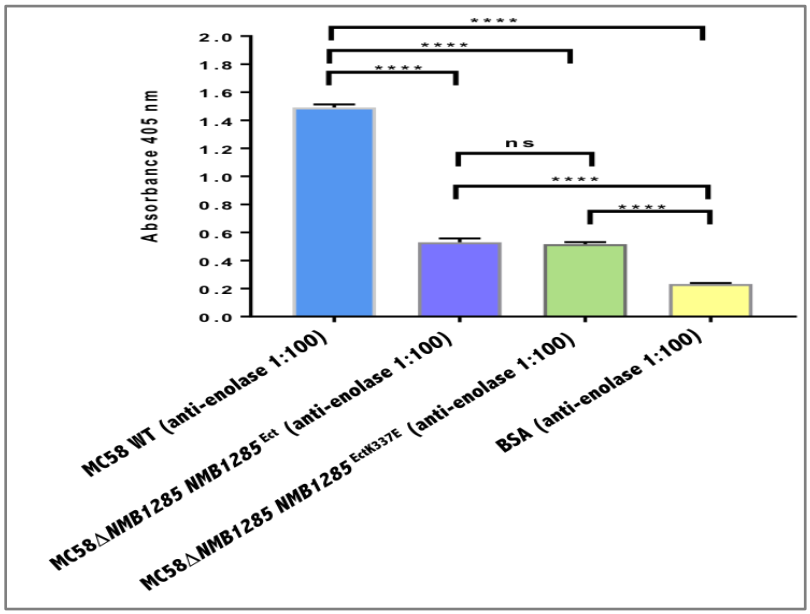


Figure 5.1: Whole-cell ELISA of MC58 wild type (WT), MC58Δ*NMB1285* *NMB1285*^{Ect} and MC58Δ*NMB1285* *NMB1285*^{EctK337E} for enolase surface localisation. Enolase secretion was affected in MC58Δ*NMB1285* *NMB1285*^{Ect,} as well as MC58Δ*NMB1285* *NMB1285*^{EctK337E}. The bars indicate the standard error of means. Values shown are means of three independent experiments; on each occasion each sample was tested in triplicate. Significant difference between means is indicated with *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

5.2.2 In vitro growth of MC58 wild type, MC58Δ*NMB1285 NMB1285*^{Ect}, and MC58Δ*NMB1285 NMB1285*^{EctK337E} strains

In vitro growth profiles of MC58 wild type, MC58Δ*NMB1285 NMB1285*^{Ect}, and MC58Δ*NMB1285 NMB1285*^{Ectk337E} strains were assessed to determine the effect of K337E enolase mutation on the growth under laboratory conditions as described in section 2.13. BHI broth was used to grow the strains with an equivalent starting OD_{600} of 0.1 then measured at hourly intervals. Through the entire period of observation, MC58Δ*NMB1285 NMB1285*^{Ect} strain showed a very similar growth profile to MC58 wild type. However, MC58Δ*NMB1285 NMB1285*^{Ectk337E} strain showed a diminished growth rate as presented in Figure 5.2. Values shown are means of three independent experiments.

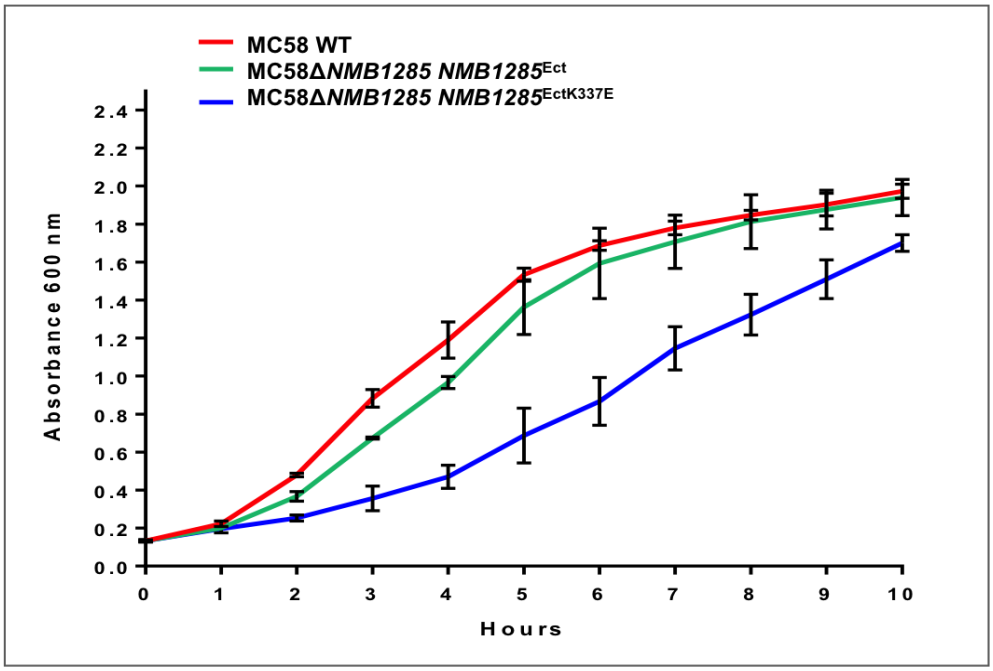


Figure 5.2: Growth profiles of MC58 wild type, MC58Δ*NMB1285 NMB1285*^{Ect}, and MC58Δ*NMB1285 NMB1285*^{Ectk337E} under laboratory conditions. The OD_{600} was measured at the indicated time points during shaking. The wild type and MC58Δ*NMB1285 NMB1285*^{Ect} strains had a normal growth profiles through the entire period of observation. While the MC58Δ*NMB1285 NMB1285*^{Ectk337E} strain had a lower than the normal growth rate. Values shown are means of three independent experiments.

5.2.3 Reduction of enolase expression affects plasminogen binding

Plasminogen binding to MC58 wild type, MC58Δ*NMB1285 NMB1285*^{Ect}, and MC58Δ*NMB1285 NMB1285*^{EctK337E} was assessed using whole-cell ELISA to demonstrate that both mutants showed a significant reduction on the binding of plasminogen to the whole meningococcal cells as presented in Figure 5.3.

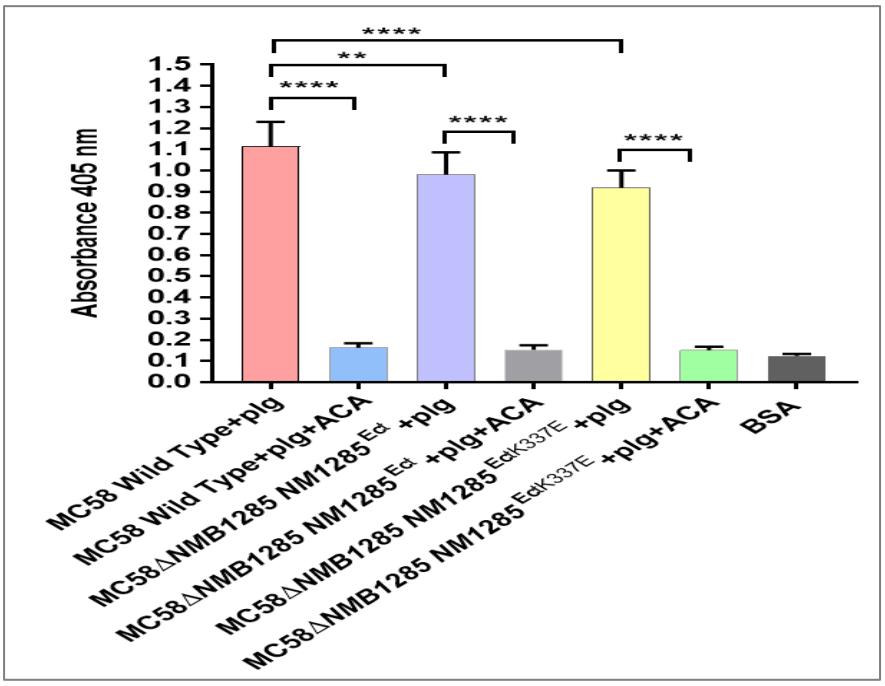


Figure 5.3: Whole-cell ELISA showing the binding of plasminogen to MC58 wild type, MC58Δ*NMB1285 NMB1285*^{Ect}, and MC58Δ*NMB1285 NMB1285*^{Ectk337E} with/without the addition of ACA (α-amino caproic acid). The reduced enolase expression in the mutants significantly affected the plasminogen binding to the whole meningococcal cell. ACA addition led to a significant inhibition in the plasminogen binding. The bars indicate the standard error of means. Values shown are means of three independent experiments; on each occasion each sample was tested in triplicate.

5.2.4 Attempts to enhance enolase expression

5.2.4.1 Incorporating Shine–Dalgarno-like sequence into pComp1285 vector

Mutations were introduced at specific sites of pComp1285 vector (Figure 4.2A) by designing primers, SDseqF1 and SDseqR1 (Table 2.4) to incorporate Shine-Dalgarno (SD)-like sequence. Mutagenesis reactions were performed as described in section 2.9. The resulting plasmid was renamed pComp1285SD (Figure 5.4) (Table 2.2). The transformation of JM109 competent *E.coli* was followed as described in section 2.10.1. DNA sequencing confirmed the successful incorporation of the SD-like sequence into pComp1285 vector. Then, pComp1285SD construct was transformed into *N. meningitidis* MC58 *rpsL*^{R} as described in section 4.2.3, which was verified by PCR (Figure 5.5).

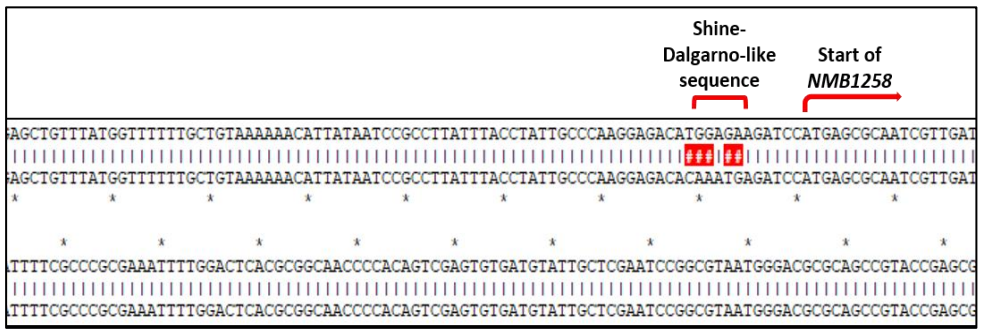


Figure 5.4: Optimal alignment of nucleotide sequences of a section of pComp1285 and pComp1285SD vectors. Lower line is enolase and upstream sequence from pComp1285 vector. Upper line is enolase and upstream sequence from pComp1285SD vector, which shows the incorporation of the SD-like sequence.

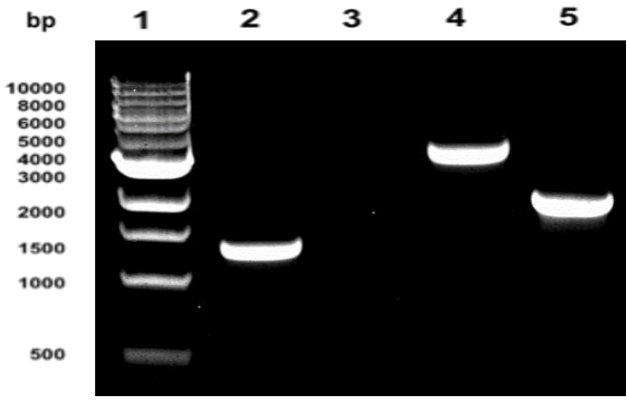


Figure 5.5: Agarose gel image. Lane 1: 1 kb DNA ladder. Lane 2: the amplified wild type (MC58) using NMB102F and NMB103F primers showing a 1.1 kb band; Lane 3: the negative control; Lane 4: the amplified region of pComp1285SD inserted into *rpsL*^{R} MC58 with NMB102F and NMB103F primers showing the expected size band of 3.6 kb; lane 5 represents the amplified region with NMB1285F1 and NMB103F primers showing the expected size of 2.8 kb.

Two double homologous recombination event were initiated using pBA3 then pBA2 vectors as described in sections 4.2.5 and 4.2.7. Successful transformations were verified as described in sections 4.2.6 and 4.2.8 (Figure 5.6A, B), which was followed by DNA sequencing for further confirmation. SDS-PAGE and immunoblotting experiments were carried out to determine enolase expression using rabbit anti-enolase-specific polyclonal antiserum (RαEnolase) as a primary antibody (Table 2.3), which showed a considerable reduction of enolase expression in the complemented mutant (MC58 *rpsL*^{R} Δ*NMB1285 NMB1285SD*^{Ect}) containing the wild type ectopic enolase (Figure 5.7).

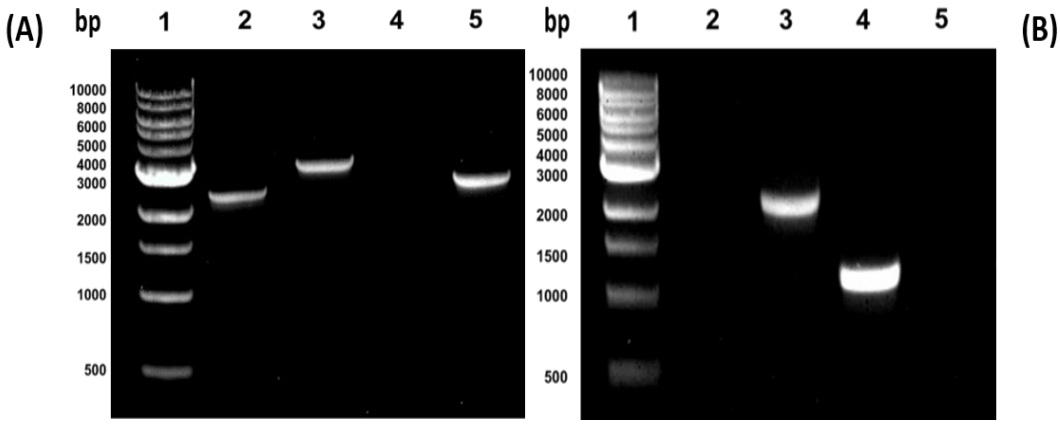


Figure 5.6: **(A)** Verification of the 1st transformation of pBA3 construct into MC58 *rpsL*^{R} complemented strain to produce MC58 *rpsL*^{R} Δ*NMB1285 NMB1285SD*^{Ect} *Kan*^{R} *rpsL*^{S}. Lane 1:1 kb ladder. Lane 2: 2.3 kb band of the expected size representing the amplified region of MC58 wild type as a positive control using NMB1284F and NMB1287F primers. Lane 3: 3 kb band of the expected size representing the amplified region (from *NMB1284* to *NMB1287*) after the insertion of *KnsL* cassette using NMB1284F and NMB1287F primers. Lane 4: the negative control. Lane 5: 2.6 kb band of the expected size representing the amplified region using NMB1287F and KnsLF2 primers. **(B)** Verification of the 2nd transformation of the pBA2 construct into MC58 *rpsL*^{R} complemented strain containing the *KnsL* cassette to produce MC58 *rpsL*^{R} Δ*NMB1285 NMB1285SD*^{Ect}. Lane 1:1 kb ladder. Lane 2: the negative control. Lane 3: 2.3 kb band of the expected size representing the amplified region of MC58 wild type as a positive control using NMB1284F and NMB1287F primers. Lane 4: 1.1 kb band of the expected size representing the amplified region (from *NMB1284* to *NMB1287*) using NMB1284F and NMB1287F primers after the replacement of *KnsL* cassette with the unmarked construct containing the inactivated and deleted gene. Lane 5: No band representing the amplification using NMB1284F and KnsLR2 primers due to the removal of the *KnsL* cassette.

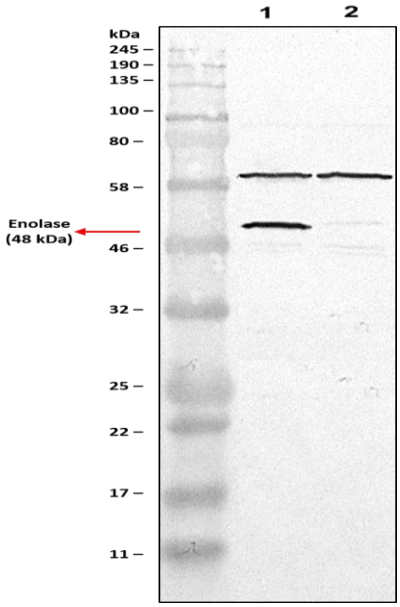


Figure 5.7: Immunoblot analysis of the whole cell lysates of the complemented strain (MC58 *rpsL*^{R} Δ*NMB1285 NMB1285SD*^{Ect}) containing wild type ectopic enolase compared to the wild type MC58 using RαEnolase. Lane 1 represents the wild type. Lane 2 is the complemented strain (MC58 *rpsL*^{R} Δ*NMB1285 NMB1285SD*^{Ect}) containing wild type ectopic enolase. This indicates that there is a reduction of enolase expression in the complemented strain.

5.2.4.2 Replacing the promoter sequence (Opa-cbbA promoters) in pComp1285 with a stronger promoter (PorA promoter)

Primers, proDel F1 and proDel R1 (Table 2.4), were designed to delete Opa-cbbA promoters from pComp1285 vector (Figure 4.2A) by inverse PCR. The resulting vector was renamed pComp1285proDel (Table 2.2). The vector pComp1285proDel was digested with *BamH*I (New England BioLabs). Two complementary DNA oligonucleotides, SynOligo F3 and SynOligo R3 (Table 2.4), were designed for PorA promoter synthesis. They were annealed as described in section 2.6.8. Successful annealing was verified by loading the product (57 bp) onto the agarose gel (Figure 5.9). The annealed product, with incorporated *Bgl*II site (5'), was ligated to *BamH*I-digested amplicon. This product was digested using *BamH*I, to remove any intact vector, before transforming it into *E. coli* JM109 competent cells as described in section 2.10.1. The resulting plasmid was renamed pComp1285porAp. DNA sequencing confirmed the successful promoter replacement. Then, pComp1285porAp construct was transformed into *N. meningitidis* MC58 *rpsL*^{R} as described in section 4.2.3 which was verified by PCR (Figure 5.8).

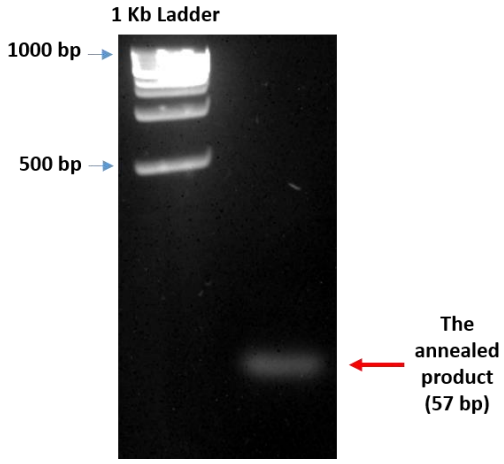


Figure 5.9: Agarose gel image showing the annealed product of the two complementary DNA oligonucleotides, SynOligo F3 and SynOligo R3.

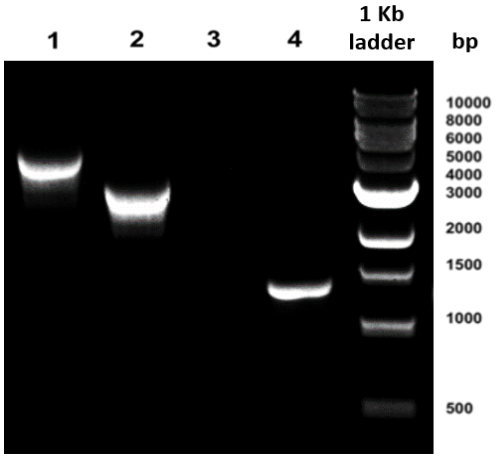


Figure 5.8: Agarose gel image. Lane 1: the amplified region of pComp1285porAp inserted into MC58 *rpsL*^{R} with NMB102F and NMB103F primers showing the expected size band of 3.6 kb. Lane 2: the amplified region with NMB1285F1 and NMB103F primers showing the expected size of 2.8 kb. Lane 3: the negative control. Lane 4: the amplified wild type (MC58) using NMB102F and NMB103F primers showing a 1.1 kb band.

Two double homologous recombination event were initiated using pBA3 then pBA2 vectors as described in sections 4.2.5 and 4.2.7. Successful transformations were verified as described in sections 4.2.6 and 4.2.8 (Figure 5.10A, B), which was followed by DNA sequencing for further confirmation. SDS-PAGE and immunoblotting experiments were carried out to determine enolase expression using rabbit anti-enolase-specific polyclonal antiserum (RαEnolase) as a primary antibody (Table 2.3), which showed a considerable reduction of enolase expression in the complemented mutant (MC58 *rpsL*^{R} *ΔNMB1285 NMB1285PP*^{Ect}) containing the wild type ectopic enolase (Figure 5.11).

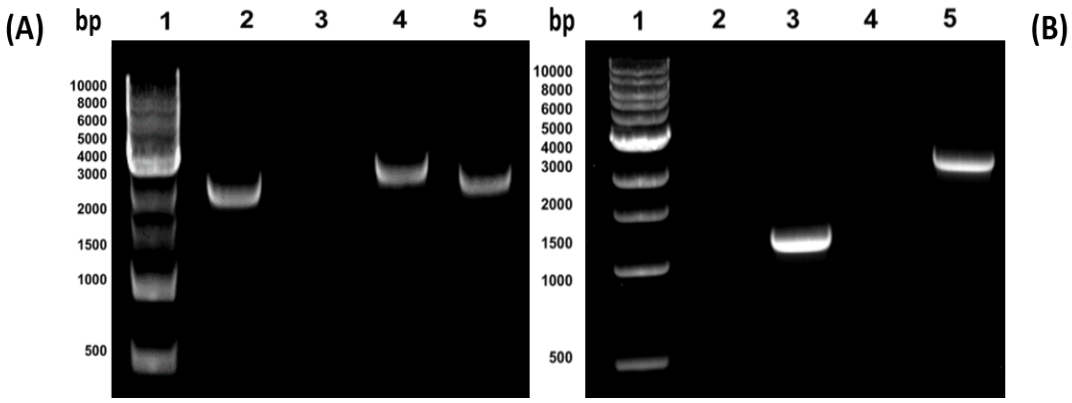


Figure 5.10: **(A)** Verification of the 1st transformation of pBA3 construct into MC58 *rpsL*^{R} complemented strain to produce MC58 *rpsL*^{R} *ΔNMB1285 NMB1285PP*^{Ect} *Kan*^{R} *rpsL*^{S}. Lane 1:1 kb ladder. Lane 2: 2.3 kb band of the expected size representing the amplified region of MC58 wild type as a positive control using NMB1284F and NMB1287F primers. Lane 3: the negative control. Lane 4: 3 kb band of the expected size representing the amplified region (from *NMB1284* to *NMB1287*) after the insertion of *KnsL* cassette using NMB1284F and NMB1287F primers: Lane 5: 2.6 kb band of the expected size representing the amplified region using NMB1287F and KnsLF2 primers. **(B)** Verification of the 2nd transformation of the pBA2 construct into MC58 *rpsL*^{R} complemented strain containing the *KnsL* cassette to produce MC58 *rpsL*^{R} *ΔNMB1285 NMB1285PP*^{Ect}. Lane 1:1 kb ladder. Lane 2: No band representing the amplification using NMB1284F and KnsLR2 primers due to the removal of the *KnsL* cassette. Lane 3: 1.1 kb band of the expected size representing the amplified region (from *NMB1284* to *NMB1287*) using NMB1284F and NMB1287F primers after the replacement of *KnsL* cassette with the unmarked construct containing the inactivated and deleted gene. Lane 4:.The negative control. Lane 5:.2.3 kb band of the expected size representing the amplified region of MC58 wild type as a positive control using NMB1284F and NMB1287F primers.

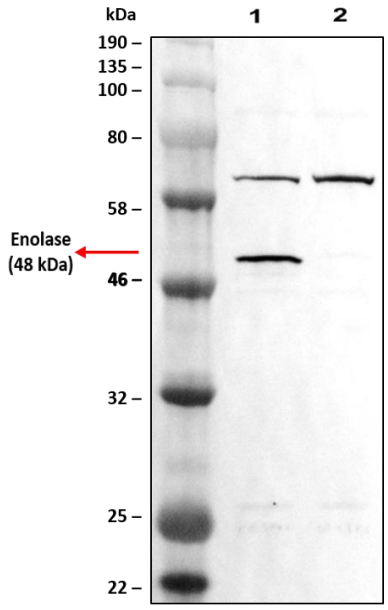


Figure 5.11: Immunoblot analysis of the whole cell lysates of the complemented strain (MC58 *rpsL*^{R} *ΔNMB1285 NMB1285PP*^{Ect}) containing wild type ectopic enolase compared to the wild type MC58 using RαEnolase. Lane 1 represents the wild type. Lane 2 is the complemented strain (MC58 *rpsL*^{R} *ΔNMB1285 NMB1285PP*^{Ect}) containing wild type ectopic enolase. This indicates that there is a reduction of enolase expression in the complemented strain.

5.3 Discussion

Many cytoplasmic proteins have been found to lack identifiable secretion signals, which suggests that they could be secreted via non-conventional or non-classical secretion systems. They demonstrate multiple functions in other cellular compartments. These proteins, including enolase, are termed as "moonlighting" proteins (Jeffery 2003; Bendtsen *et al.* 2005; Lopez-Villar *et al.* 2006; Nombela *et al.* 2006; Pal-Bhowmick *et al.* 2007). The potential role of a single lysine residue in meningococcal enolase secretion has been investigated in this study. We predicted a conserved lysine residue (Lys^{337}) in meningococcal enolase as a possible signal for enolase translocation to the cell surface, which was revealed by aligning protein sequences of enolase from *N. meningitidis* MC58 and enolase from *E. coli* (Lys^{341}) (Figure 1.3). Boel *et al* suggested that automodification of *E. coli* enolase is dependent on covalent binding of 2-Phosphoglycerate to Lys^{341}. Furthermore, this modification has been found to be important for enolase export, as a result of the substitution of Lys^{341} with other amino acids, which prevented automodification, as well as the export of enolase into the medium (Boel *et al.* 2004).

In chapter four, the K337 mutation of enolase was constructed to determine the significance of this residue in enolase secretion. However, the markerless mutation had an impact on enolase

expression and growth of *N. meningitidis* MC58. Whole-cell ELISA was used to test MC58Δ*NMB1285 NMB1285*^{Ect} and MC58Δ*NMB1285 NMB1285*^{EctK337E} mutants for enolase surface localisation. The results showed that both complemented mutants, containing the wild type and K337E-mutated enolase, had an effect on enolase export to the cell surface compared to the wild type MC58. Nevertheless, this could be due to the considerable reduction of enolase expression (Figure 4.19). To solve this problem, several attempts to enhance enolase expression were made.

In *E.coli,* the positive effect of an upstream Shine-Delgarno (SD) sequence (5' AGGAGG 3') on translation initiation has been confirmed (Shine and Dalgarno 1974). However, it is still unclear how this initiation is promoted. At the translational level in *E. coli*, the effect of the SD-like sequence on gene expression can be either positive or negative depending on its location (Jin *et al.* 2006). In this study, the incorporation of the SD-like sequence (5' GGAGAA 3', positions -11 to -6 from the start codon) into pComp1285 vector by introducing mutations at specific sites was successfully produced. This construct was transformed into MC58 *rpsL*^{R}. Then, the markerless mutation strategy was successfully used to knockout the original copy of the enolase gene (as described in chapter four), and to study the

effect of SD-like sequence incorporation on enhancing enolase expression. We have shown that this incorporation of the SD-like sequence had no effect on enhancing enolase expression.

We further examined another approach for enhancing enolase expression by placing it under the control of a strong promoter (the PorA promoter) upstream of the gene of interest. This promoter was proposed to have a great impact on the overexpression of genes encoding Omp85, Hsf/NhhA, and NspA (D. Martin *et al.* 2000; Scarselli *et al.* 2001; Voulhoux and Tommassen 2004; Norheim *et al.* 2005). In this work, Opa - cbbA promoters in pComp1285 vector were deleted then replaced with the PorA promoter. A plasmid pComp1285proDel harbouring the PorA promoter was constructed, resulting in the plasmid pComp1285porAp. This construct was transformed into MC58 *rpsL*^{R}. Then, a mutant lacking the native enolase gene, but containing an ectopic copy of wild type was successfully generated. We have found that the replacement of the promoters had an insignificant effect on the expression efficiency.

To better understand the function of Lys^{337}, growth profiles of MC58Δ*NMB1285 NMB1285*^{Ect} and MC58Δ*NMB1285 NMB1285*^{EctK337E} mutants were assessed under laboratory conditions, which revealed an impaired growth rate in

MC58ΔN*MB1285 NMB1285*^{EctK337E} mutant. This suggests that Lys^{337} of enolase might be required for optimal growth of *N. meningitidis MC58*. It has been confirmed that the high affinity binding of enolase to human plasminogen is lysine-mediated (Miles *et al.* 1991; Redlitz *et al.* 1995; Lahteenmaki *et al.* 2000). In this study, we assessed the plasminogen binding to meningococcal cells via whole-cell ELISA. The data indicate that the reduction of enolase expression, which resulted from the unmarked mutation, significantly affected the plasminogen binding to the whole meningococcal cells in the two complemented mutants compared to MC58 wild type.

Taken together, it is not considered possible to construct a knockout complemented mutant with the enolase gene of *N. meningitidis* without leading to a decrease or complete loss of its expression making it difficult to further examine any additional roles of Lys^{337}, especially in enolase secretion. We have shown that Lys^{337} mutation affects meningococcal growth. We have also found that the reduction of enolase expression caused a significant effect on plasminogen binding to meningococcal cells.

6 Chapter Six: The role of the putative N-terminal signal peptide in the surface localisation of fructose-1,6-bisphosphate aldolase

6.1 Introduction

Fructose-1, 6-bisphosphate aldolase (FBA) is an essential cytoplasmic enzyme in the glycolytic pathway. It catalyses the reversible cleavage of fructose-1, 6-bisphosphate into dihydroxyacetone phosphate (ketose), and glyceraldehyde-3-phophate (aldose) in glycolysis and gluconeogenesis (Rutter 1964; Zgiby *et al.* 2000; Wehmeier 2001; Ramsaywak *et al.* 2004). Based on the reaction mechanism, aldolases are divided into two classes: Class I and Class II (Thomson *et al.* 1998; Arakaki *et al.* 2004). Class I FBA stabilises a reaction intermediate by using an active-site lysine, forming a Schiff base. It mainly exists in eukaryotes; animals, plants, and algae (Wong and Whitesides 1994; Katebi and Jernigan 2015). Class II FBA is mainly present in bacteria and shows 15% sequence identity with the class I FBA (Naismith *et al.* 1992; Katebi and Jernigan 2015). Depending on amino acid sequence, Class II can be subdivided into groups, A and B (Plaumann *et al.* 1997). Group A enzymes are dimeric, while group B enzymes can be dimeric, tetrameric or octameric (Sauve and Sygusch 2001; Nakahara *et al.* 2003). Class II has been considered as a potential vaccine antigen and target for new antibiotics (Fonvielle *et al.* 2008; Daher *et al.* 2010; Elhaik Goldman *et al.*

2016). It was also identified as a potential immunodiagnostic tools for *Listeria* species detection (Mendonca *et al.* 2016).

Although most organisms possess only one class of FBA, some have been reported to contain both classes of the enzyme, such as *E. coli*, *S. pneumoniae*, and *Synechocystis* sp. PCC 6803 (Alefounder *et al.* 1989; Thomson *et al.* 1998; Jado *et al.* 1999; Nakahara *et al.* 2003). In the available meningococcal MC58 genome sequence, there is only one gene (*cbbA*) encoding a single, putative Class IIB FBA enzyme (Tettelin *et al.* 2000). It has been previously reported that FBA expression is highly conserved in both pathogenic and non-pathogenic *Neisseriae* sp. (Tunio *et al.* 2010b).

It has been demonstrated that FBA is essential for the survival of *M. tuberculosis* (de la Paz Santangelo *et al.* 2011; Puckett *et al.* 2014). In contrast, FBA was identified to be a dispensable protein for the viability of *N. meningitidis* and *Francisella* (Tunio *et al.* 2010b; Ziveri *et al.* 2017). It was shown to be located on the cell surface, performing non-glycolytic "moonlighting" functions, which are unrelated to its cytoplasmic roles and independent on its aldolase activity (Tunio *et al.* 2010b). Shams *et al.* demonstrated that FBA is also present on the cell surface of *N. gonorrhoeae,* and other pathogenic and commensal *Neisseriae* (Shams *et al.* 2016). Tunio *et al.* previously reported

that meningococcal FBA is required for optimal adhesion to host epithelial and endothelial cells (Tunio *et al.* 2010b).

It has been confirmed that FBA binds human plasminogen in a dose dependant manner. The C-terminal lysine residue was found to be responsible for this interaction (Shams *et al.* 2016). FBA binding to plasminogen was also described in other pathogens, such as *N. gonorrhoeae, M. tuberculosis, and Paracoccidioides,* (Xolalpa *et al.* 2007; Chaves *et al.* 2015; Shams *et al.* 2016). This strategy was also adopted by the commensal *Neisseria* sp., *N. lactamica*, and *N. polysaccharea* (Shams *et al.* 2016). It has been previously predicted that meningococcal FBA is a non-secreted protein by the program SignalP-HMM, However, another program (SignalP-NN) identified a 13 amino acid N-terminal putative signal peptide with a predicted cleavage site (^{11}DHA-AE^{15}) (Tunio *et al.* 2010b).

The aim of this chapter is to describe the role of the putative signal peptide of FBA in surface localisation by mutating residues within the predicted cleavage site (^{11}DHA.AE^{15}). Other residues in the N-terminal peptide were also mutated for further confirmation. Following these mutations, the sub-cellular localisation of FBA was investigated by sub-cellular fractionation to study the cellular locations of FBA.

6.2 Results

6.2.1 Mutagenesis of A14 in the predicted cleavage site of the putative N-terminal signal peptide of *cbbA*

In a previous study, the FBA-encoding gene of *N. meningitidis* MC58 (known as *cbbA*) was cloned into the vector pComp-1. The resulting plasmid was renamed pFS-4 (Fariza Shams, University of Nottingham) (Table 2.2). It is preferable for the substitution of the desired residue to use a residue that has a minor difference in size and chemistry, as this should not disrupt the overall 3D-fold of the protein. In addition, the change with amino acids having different physical and chemical properties might lead to phenotypical differences due to the major structural changes. Therefore, valine was chosen because it has similar properties to alanine. A14V FBA-F1 and A14V FBA-R1 primers (Table 2.4) were designed to facilitate the mutation of the codon encoding FBA alanine residue (A14) to valine in pFS-4 to obtain pFS4-A14V (Figure 6.2). Mutagenesis was carried out according to the manufacturer's instructions as described in section 2.9. Then, pFS4-A14V construct was transformed into *N. meningitidis* MC58Δ*cbbA* as described in section 2.10.2. The construct used contains an erythromycin resistance cassette flanked by the *NMB0103* and *NMB0102* genes. The correct insertion of the A14V mutated *cbbA* gene - erythromycin resistance cassette region of

the pComp-based plasmid between the *NMB0103* and *NMB0102* genes of the meningococcal genome was confirmed by PCR, and gel electrophoresis (Figure 6.1, Figure 6.3). Successful transformation was also confirmed by DNA sequencing.

MALVSMRQLL[11]DHA.AE[15]NSYGLPAFNVNNLEQMRAIMEAADQVDA
PVIVQASAGARKYAGAPFLRHLILAAVEEFPHIPVVMHQDHGASPDV
CQRSIQLGFSSVMMDGSLMEDGKTPSSYEYNVNATRTVVNFSHACGV
SVEGEIGVLGNLETGEAGEEDGVGAVGKLSHDQMLTSVEDAVCFVKD
TGVDALAIAVGTSHGAYKFTRPPTGDVLRIDRIKEIHQALPNTHIVM
HGSSSVPQEWLKVINEYGGNIGETYGVPVEEIVEGIKHGVRKVNIDT
DLRLASTGAVRRYLAENPSDFDPRKYLSKTIEAMKQICLDRYLAFGC
EGQAGKIKPVSLEKMASRYAKGELNQIVK

Figure 6.2: The protein sequence of *N. meningitidis* MC58 FBA. Highlighted in red is the predicted cleavage site.

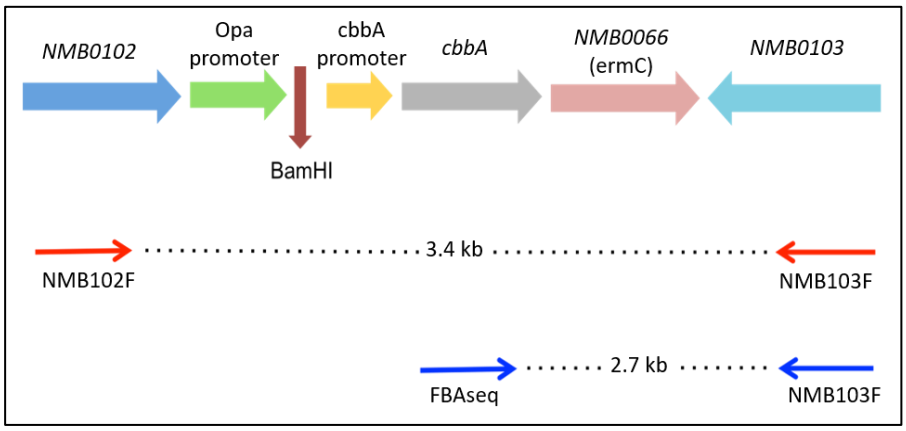


Figure 6.1: The two sets of primers used for confirming the complemented MC58 strain MC58Δ*cbbA* *cbbA*^{EctA14V}. Red arrows represent the 1st set of primers (NMB102F and NMB103F) amplifying a 3.4 kb fragment. Blue arrows represent the 2nd set of primers (FBAseq and NMB103F) amplifying 2.7 kb fragment.

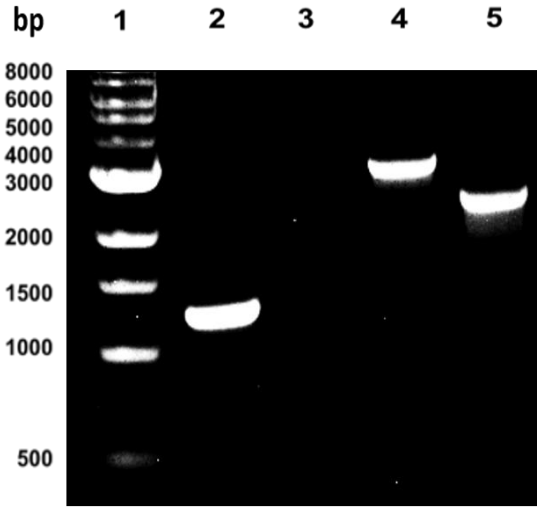


Figure 6.3: Verification of the transformation of pFS4-A14V construct into MC58Δ*cbbA*. Lane 1: 1kb ladder. Lane 2: the amplified MC58 wild type MC58 using NMB102F and NMB103F primers showing a 1.1 kb band. Lane 3: the negative control. Lane 4 represents the amplified region of pFS4-A14V inserted into MC58Δ*cbbA* with NMB102F and NMB103F primers showing the expected size band of 3.4 kb; lane 5 represents the amplified region with FBAseq and NMB103F primers showing the expected size of 2.7 kb.

6.2.2 The effect of A14V mutation on FBA surface localisation

Three independent whole-cell ELISAs were performed to investigate the effect of A14V mutation on the surface localisation of FBA. MC58Δ*cbbA cbbA*^{EctA14V} showed a significant effect on FBA surface exposure compared to the complemented strain harbouring the ectopic copy of intact *cbbA* gene (MC58Δ*cbbA cbbA*^{Ect}) (Figure 6.4).

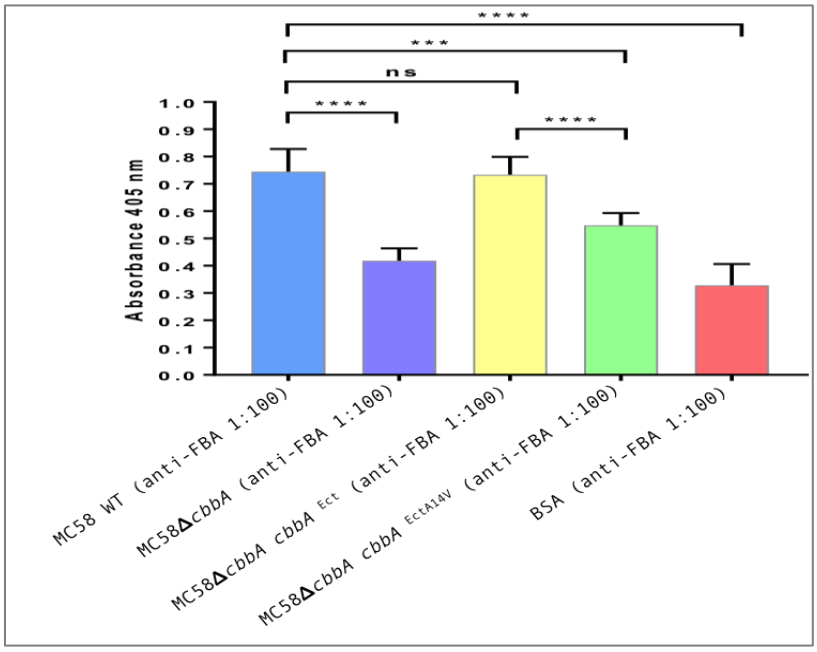


Figure 6.4: Whole-cell ELISA of MC58 wild type, MC58Δ*cbbA*, MC58Δ*cbbA cbbA*^{Ect} and MC58Δ*cbbA cbbA*^{EctA14V} for FBA surface exposure. FBA secretion was significantly affected in MC58Δ*cbbA cbbA*^{EctA14V,} compared to MC58Δ*cbbA cbbA*^{Ect}. The bars indicate the standard error of means. Values shown are means of three independent experiments; on each occasion each sample was tested in triplicate. Significant difference between means is indicated with *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

6.2.3 Expression of *cbbA* in MC58Δ*cbbA cbbA*^{EctA14V}

To determine FBA expression in MC58Δ*cbbA cbbA*^{EctA14V}, SDS-PAGE and immunoblotting experiments were carried out. A pre-adsorbed rabbit anti-FBA-specific polyclonal antiserum (RαFBA) primary antibody (Table 2.3) was used for the immunoblot analysis. The expected *ca.* 38 kDa band corresponding to FBA was observed in MC58 wild type, MC58Δ*cbbA cbbA*^{Ect}, and MC58Δ*cbbA cbbA*^{EctA14V}, whereas no band was detected in the null *cbbA* mutant strain MC58Δ*cbbA*. A reduction of FBA expression was shown in MC58Δ*cbbA cbbA*^{EctA14V} compared to MC58Δ*cbbA cbbA*^{Ect} (Figure 6.5).

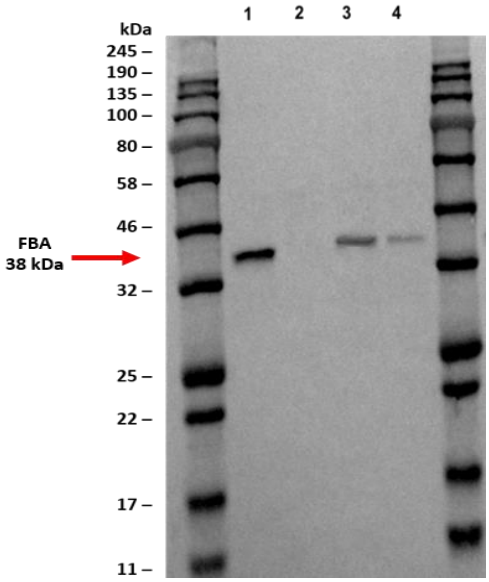


Figure 6.5: Immunoblot of the whole cell lysates of MC58 wild type, MC58Δ*cbbA*, MC58Δ*cbbA* *cbbA*^{Ect} and MC58Δ*cbbA* *cbbA*^{EctA14V} probed with anti-FBA. Lane 1: MC58 wild type, lane 2: MC58Δ*cbbA,* lane 3: MC58Δ*cbbA* *cbbA*^{Ect}, lane 4: MC58Δ*cbbA* *cbbA*^{EctA14V}. A band at *ca*. 38 kDa corresponding to FBA was observed in MC58 wild type, MC58Δ*cbbA* *cbbA*^{Ect}, and MC58Δ*cbbA* *cbbA*^{EctA14V}, but MC58Δ*cbbA* showed no band as expected. It is indicated that there is a reduction of FBA expression in MC58Δ*cbbA* *cbbA*^{EctA14V} compared to MC58Δ*cbbA* *cbbA*^{Ect}.

6.2.4 Mutagenesis of A13, A13A14, A22, and D11 in the predicted cleavage site of the putative N-terminal signal peptide of *cbbA*

Primers (Table 2.4) were designed to facilitate the mutation of the codon encoding FBA alanine residues (A13, A13A14, A22) to valine residue (V) and aspartate residue (D11) to glutamic acid (E) in the plasmid vector pFS-4 to obtain pFS4-A13V, pFS4-A13VA14V, pFS4-A22V, and pFS4-D11E (Table 2.2). Mutagenesis was carried out according to the manufacturer's instructions as described in section 2.9. Then, these constructs were transformed into *N. meningitidis* MC58Δ*cbbA* as described in section 2.10.2. PCR, and gel electrophoresis were used to confirm the correct insertion of the (A13V, A13VA14V, A22V, D11E) mutated *cbbA* gene - erythromycin resistance cassette region of the pComp-based plasmid between the *NMB0103* and *NMB0102* genes of the meningococcal genome (Figure 6.1, Figure 6.6). DNA sequencing was also used to confirm the identity of the complemented strains, MC58Δ*cbbA* $cbbA^{EctA13V}$, MC58Δ*cbbA* $cbbA^{EctA14VA13V}$, *MC58ΔcbbA* $cbbA^{EctA22V}$, and MC58Δ*cbbA* $cbbA^{EctD11E}$.

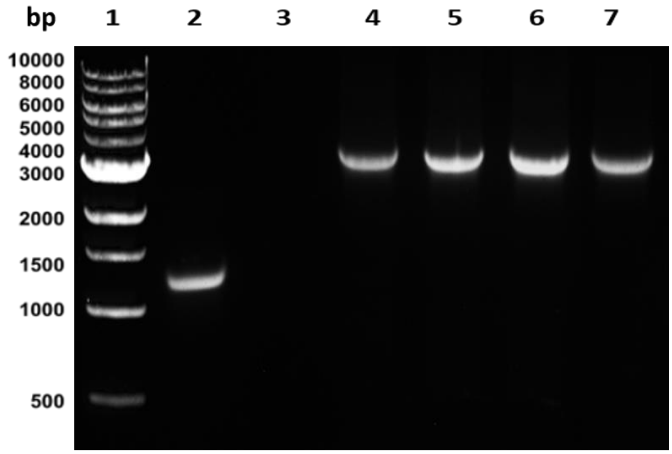


Figure 6.6: Verification of the transformation of pFS4-A13V, pFS4-A13VA14V, pFS4-A22V, and pFS4-D11E constructs into MC58Δ*cbbA*. Lane 1: 1kb ladder. Lane 2: the amplified wild type (MC58) using NMB102F and NMB103F primers showing a 1.1 kb band. Lane 3: the negative control; Lane 4 represents the amplified region of pFS4-A13V inserted into MC58Δ*cbbA* with NMB102F and NMB103F primers showing the expected size band of 3.4 kb. Lane 5 represents the amplified region of pFS4-A13VA14V inserted into MC58Δ*cbbA* with NMB102F and NMB103F primers showing the expected size band of 3.4 kb. Lane 6 represents the amplified region of pFS4-A22V inserted into MC58Δ*cbbA* with NMB102F and NMB103F primers showing the expected size band of 3.4 kb. Lane 7 represents the amplified region of pFS4-D11E inserted into MC58Δ*cbbA* with NMB102F and NMB103F primers showing the expected size band of 3.4 kb.

6.2.5 The effect of the putative N-terminal signal peptide mutations (A13V, A13VA14V, A22V, D11E) on FBA surface localisation

Several complemented derivatives; MC58Δ*cbbA* *cbbA*^{EctA13V}, MC58Δ*cbbA* *cbbA*^{EctA14VA13V}, MC58Δ*cbbA* *cbbA*^{EctA22V}, and MC58Δ*cbbA* *cbbA*^{EctD11E}; were tested using whole-cell ELISA to investigate the effect of the mutations of the codon encoding residues (A13V, A13VA14V, and D11E) within the predicted cleavage site and A22V mutation in another site of the putative signal peptide on the FBA surface exposure. FBA secretion was significantly affected in all complemented strains compared to MC58Δ*cbbA* *cbbA*^{Ect} (Figure 6.7).

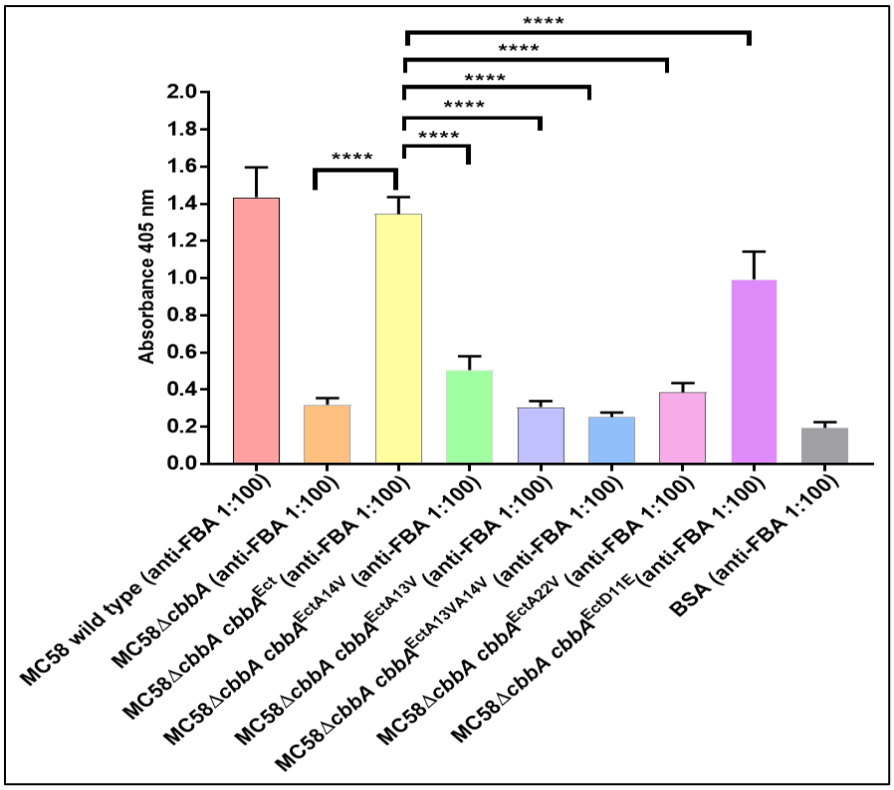


Figure 6.7: Whole-cell ELISA of MC58 wild type, MC58Δ*cbbA*, MC58Δ*cbbA* *cbbA*^{Ect}, MC58Δ*cbbA* *cbbA*^{EctA14V}, MC58Δ*cbbA* *cbbA*^{EctA13V}, MC58Δ*cbbA* *cbbA*^{EctA14VA13V}, MC58Δ*cbbA* *cbbA*^{EctA22V}, and MC58Δ*cbbA* *cbbA*^{EctD11E} for FBA surface exposure. All complemented strains showed a significant effect on FBA surface localisation compared to MC58Δ*cbbA* *cbbA*^{Ect}. The bars indicate the standard error of means. Values shown are means of three independent experiments; on each occasion each sample was tested in triplicate. Significant difference between means is indicated with *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

6.2.6 Expression of cbbA in MC58Δ*cbbA* *cbbA*^{EctA13V}, MC58Δ*cbbA* *cbbA*^{EctA13VA14V}, MC58Δ*cbbA* *cbbA*^{EctA22V}, and MC58Δ*cbbA* *cbbA*^{EctD11E}

The immunoblot analysis was performed to confirm the effect of these mutations on the expression of FBA. Whole cell lysates of MC58 wild type, MC58Δ*cbbA*, MC58Δ*cbbA* *cbbA*^{EctA13V}, MC58Δ*cbbA* *cbbA*^{EctA14VA13V}, MC58Δ*cbbA* *cbbA*^{EctA22V}, and MC58Δ*cbbA* *cbbA*^{EctD11E} were separated by SDS-PAGE then probed with rabbit anti-FBA-specific polyclonal antiserum (RαFBA) as a primary antibody (Table 2.3). Faint bands were observed in MC58Δ*cbbA* *cbbA*^{EctA13V}, MC58Δ*cbbA* *cbbA*^{EctA13VA14V}, and MC58Δ*cbbA* *cbbA*^{EctA22V} suggesting a reduction of FBA expression compared to the complemented strain MC58Δ*cbbA* *cbbA*^{Ect}, whereas the expression of FBA in MC58Δ*cbbA* *cbbA*^{EctD11E} was unaffected (Figure 6.8 A). This was supported by analysing the blot using imageJ software to calculate the relative density of each band of the complemented mutants compared with the control (MC58Δ*cbbA* *cbbA*^{Ect}) (Figure 6.8 B).

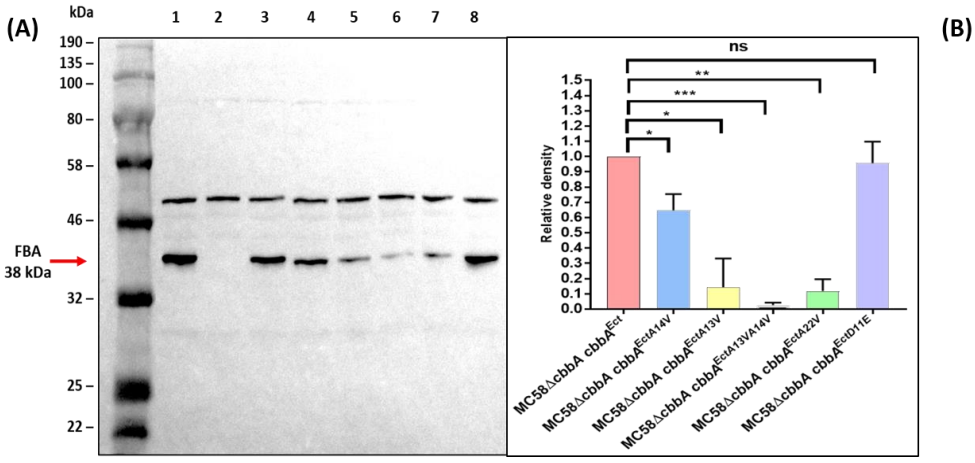


Figure 6.8: (A) Immunoblot of the whole cell lysates of MC58 wild type, MC58Δ*cbbA*, MC58Δ*cbbA* $cbbA^{Ect}$, MC58Δ*cbbA* $cbbA^{EctA14V}$, MC58Δ*cbbA* $cbbA^{EctA13V}$, MC58Δ*cbbA* $cbbA^{EctA13VA14V}$, MC58Δ*cbbA* $cbbA^{EctA22V}$, and MC58Δ*cbbA* $cbbA^{EctD11E}$ probed with anti-FBA. Lane 1: MC58 wild type, lane 2: MC58Δ*cbbA,* lane 3: MC58Δ*cbbA* $cbbA^{Ect}$, lane 4: MC58Δ*cbbA* $cbbA^{EctA14V}$, lane 5: MC58Δ*cbbA* $cbbA^{EctA13V}$, lane 6: MC58Δ*cbbA* $cbbA^{EctA13VA14V}$, lane 7: MC58Δ*cbbA* $cbbA^{EctA22V}$, and lane 8: MC58Δ*cbbA* $cbbA^{EctD11E}$. The complemented strains; MC58Δ*cbbA* $cbbA^{A13V}$, MC58Δ*cbbA* $cbbA^{A14VA13V}$, and MC58Δ*cbbA* $cbbA^{A22V}$; showed a reduction of FBA expression compared to MC58Δ*cbbA* $cbbA^{Ect}$. However, FBA expression was unaffected in MC58Δ*cbbA* $cbbA^{EctD11E}$. (B) Protein densitometry. The bars indicate the relative density of each band (MC58Δ*cbbA* $cbbA^{EctA14V}$, MC58Δ*cbbA* $cbbA^{EctA13V}$, MC58Δ*cbbA* $cbbA^{EctA13VA14V}$, MC58Δ*cbbA* $cbbA^{EctA22V}$, and MC58Δ*cbbA* $cbbA^{EctD11E}$) in the blot, using the control condition (MC58Δ*cbbA* $cbbA^{Ect}$). Values shown are from two independent experiments.

6.2.7 In vitro growth of MC58Δ*cbbA*, MC58Δ*cbbA* *cbbA*^{EctA14V}, MC58Δ*cbbA* *cbbA*^{EctA13V}, MC58Δ*cbbA* *cbbA*^{EctA14VA13V}, MC58Δ*cbbA* *cbbA*^{EctA22V}, and MC58Δ*cbbA* *cbbA*^{EctD11E}

In vitro growth profiles of MC58 wild type, MC58Δ*cbbA,* MC58Δ*cbbA* *cbbA*^{Ect}, MC58ΔcbbA *cbbA*^{EctA14V,} MC58Δ*cbbA* *cbbA*^{EctA13V}, MC58Δ*cbbA* *cbbA*^{EctA14VA13V}, MC58Δ*cbbA* *cbbA*^{EctA22V}, and MC58Δ*cbbA* *cbbA*^{EctD11E} were assessed to determine the effect of A14V, A13V, A13VA14V, A22V, and D11E mutations on the growth under laboratory conditions as described in section 2.13. BHI broth was used to grow the strains with an equivalent starting OD_{600} of 0.1 then measured at hourly intervals. The mutant and all complemented derivatives showed a very similar growth profile to MC58 wild type through the entire period of observation (Figure 6.9).

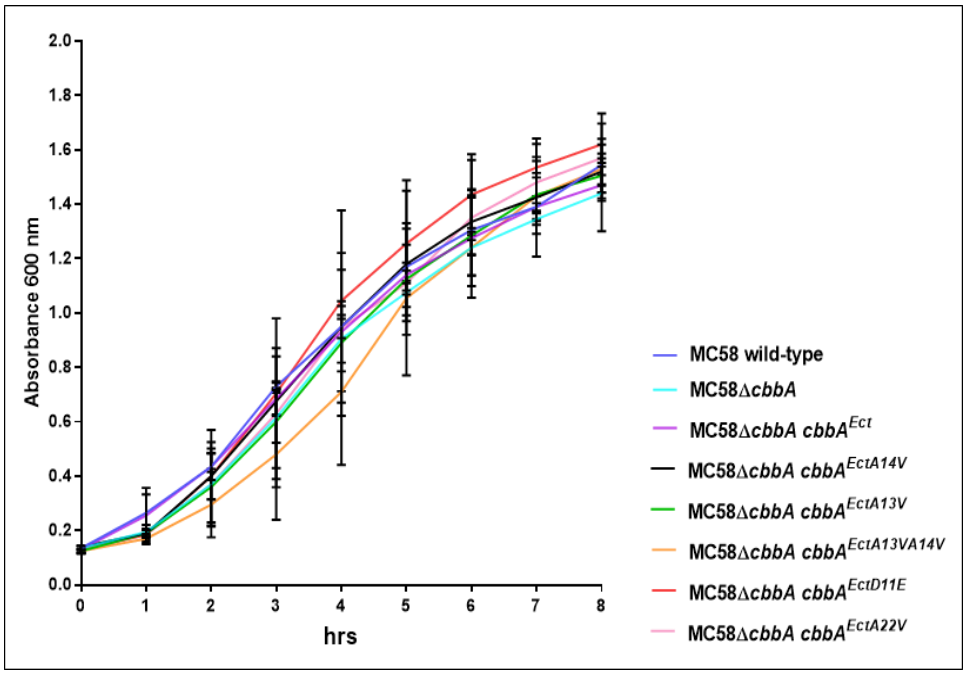


Figure 6.9: Growth profiles of MC58 wild type, MC58Δ*cbbA,* MC58Δ*cbbA* *cbbA*^{Ect} MC58Δ*cbbA* *cbbA*^{EctA14V,} MC58Δ*cbbA* *cbbA*^{EctA13V}, MC58Δ*cbbA* *cbbA*^{EctA14VA13V}, MC58Δ*cbbA* *cbbA*^{EctA22V}, and MC58Δ*cbbA* *cbbA*^{EctD11E} under laboratory conditions. The OD_{600} was measured at the indicated time points during shaking. Through the entire period of observation, the wild type, the mutant, and the complemented derivatives; MC58Δ*cbbA* *cbbA*^{EctA14V,} MC58Δ*cbbA* *cbbA*^{EctA13V}, MC58Δ*cbbA* *cbbA*^{EctA14VA13V}, MC58Δ*cbbA* *cbbA*^{EctA22V}, and MC58Δ*cbbA* *cbbA*^{EctD11E}; had normal growth profiles.

6.2.8 Identification of FBA sub-cellular localisation in MC58Δ*cbbA cbbA*^{Ect} and MC58Δ*cbbA cbbA*^{EctA14V}

Sub-cellular fractionation was used to investigate sub-cellular locations of FBA in MC58Δ*cbbA cbbA*^{Ect} and MC58Δ*cbbA cbbA*^{EctA14V} strains as described in section 2.12. The fractions were analysed by immunoblotting with anti-FBA antibody. FBA expression level was determined in the periplasmic-cytosolic protein enriched (PCP) fraction, cytoplasmic membrane protein-enriched (CMP) fraction, and outer-membrane protein-enriched (OMP) fraction collected from MC58Δ*cbbA cbbA*^{Ect} and MC58Δ*cbbA cbbA*^{EctA14V}. The expected *ca.* 38 kDa band corresponding to FBA was detected in the PCP and OMP fractions, however, FBA was absent in the CMP fraction from both complemented mutants (Figure 6.10). The membrane was stripped (as described in section 2.11.3) and re-probed with anti-PorA antibody to confirm the consistent sample loading for all fractions. A band at a molecular weight of *ca.* 45 kDa corresponding to PorA was observed in the PCP and OMP fractions from both strains with no apparent intensity difference, whereas no band was seen in the CMP fraction. The blot images were later analysed by ImageJ densitometry software to compare the density of the bands of the OMP fraction and to confirm the effect of A14V mutation on FBA secretion to the outer

membrane (Figure 6.11).The data suggest that this mutation has not prevented FBA translocation to the outer membrane. It was also suggested that FBA was more abundant in the OMP fraction than in the PCP fraction of MC58Δ*cbbA* *cbbA*^{Ect} and MC58Δ*cbbA* *cbbA*^{EctA14V}.

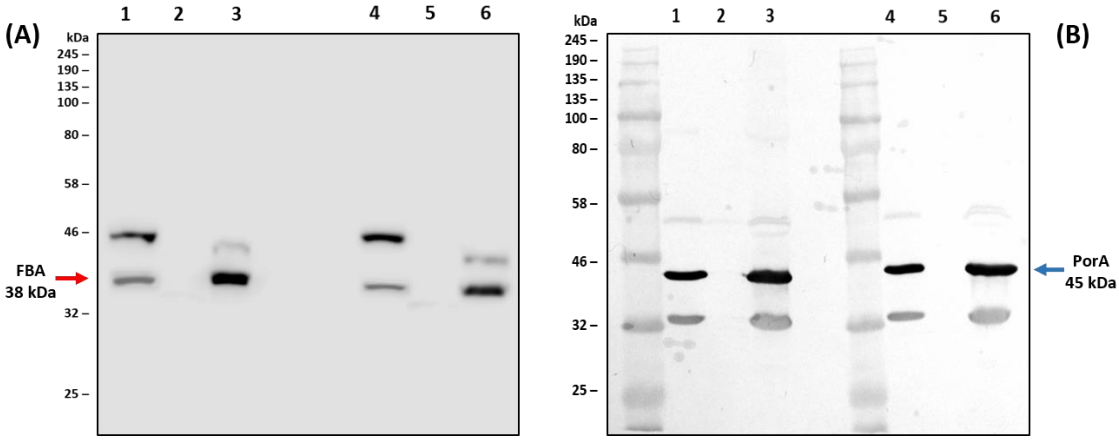


Figure 6.10: Immunoblot of cellular fractions; periplasmic-cytosolic protein enriched (PCP), cytoplasmic membrane protein-enriched (CMP), and outer-membrane protein-enriched (OMP) fractions collected from MC58Δ*cbbA* *cbbA*^{Ect} (lane 1 to 3) and MC58Δ*cbbA* *cbbA*^{EctA14V} (lane 4 to 6) strains (A) probed with anti-FBA antibody (B) probed with anti-PorA antibody. Lane 1: PCP fraction of MC58Δ*cbbA* *cbbA*^{Ect}. Lane 2: CMP fraction of MC58Δ*cbbA* *cbbA*^{Ect}. Lane 3: OMP fraction of MC58Δ*cbbA* *cbbA*^{Ect}. Lane 4: PCP fraction of MC58Δ*cbbA* *cbbA*^{EctA14V}. Lane 5: CMP fraction of MC58Δ*cbbA* *cbbA*^{EctA14V}. Lane 6: OMP fraction of MC58Δ*cbbA* *cbbA*^{EctA14V}. (A) A band corresponding to FBA (*ca.* 38 kDa) was observed in the PCP and OMP fractions from both strains, whereas this band was absent in the CMP fraction. (B) A band corresponding to PorA (*ca.* 45 kDa) was observed in the PCP and OMP fractions from both strains representing the loading controls with no apparent difference in the intensity, whereas no band was seen in the CMP fraction.

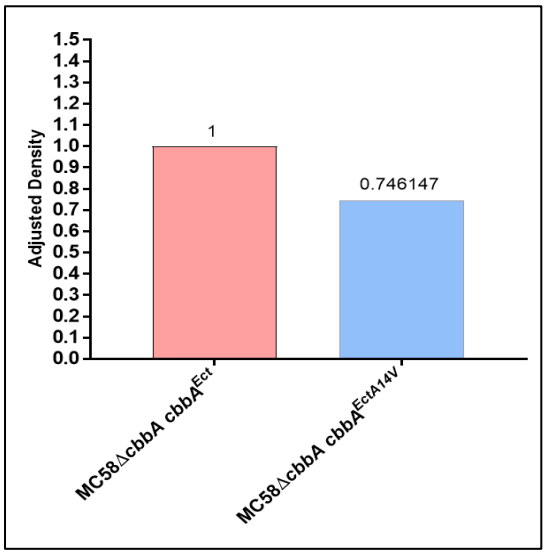


Figure 6.11: Protein densitometry of the outer-membrane protein-enriched (OMP) fraction collected from MC58Δ*cbbA* *cbbA*^{Ect} and MC58Δ*cbbA* *cbbA*^{EctA14V}. The bars indicate the adjusted relative density of the OMP fraction using the two blot images (The same membrane was probed with anti-FBA antibody and then re-probed with anti-PorA antibody). Values shown are from one experiment.

6.2.9 Identification of FBA sub-cellular localisation in MC58Δ*cbbA cbbA*^{Ect} and MC58Δ*cbbA cbbA*^{EctD11E}

Sub-cellular locations of FBA, PCP, CMP, and OMP were also investigated in MC58Δ*cbbA cbbA*^{EctD11E} compared with MC58Δ*cbbA cbbA*^{Ect}. Immunoblotting with anti-FBA antibody was used to analyse these fractions. A *ca.* 38 kDa band corresponding to FBA was seen in the PCP and OMP fractions, whereas no band was apparent in the CMP fraction from both complemented mutants (Figure 6.12). To confirm the consistent sample loading for all fractions, the membrane was stripped (as described in section 2.11.3) and re-probed with anti-PorA antibody. A band corresponding to PorA (*ca.* 45 kDa) was seen in the PCP and OMP fractions from both strains with no apparent difference in the intensity, whereas no band was observed in the CMP fraction. ImageJ densitometry software was used to analyse the blot images to compare the density of the bands of the OMP fraction and to confirm the effect of D11E mutation on FBA secretion to the outer membrane (Figure 6.13). It was suggested that FBA translocation to the outer membrane was unaffected. The data also suggest that FBA was more abundant in the OMP than in the PCP preparations of MC58Δ*cbbA cbbA*^{Ect} and MC58Δ*cbbA cbbA*^{EctD11E}.

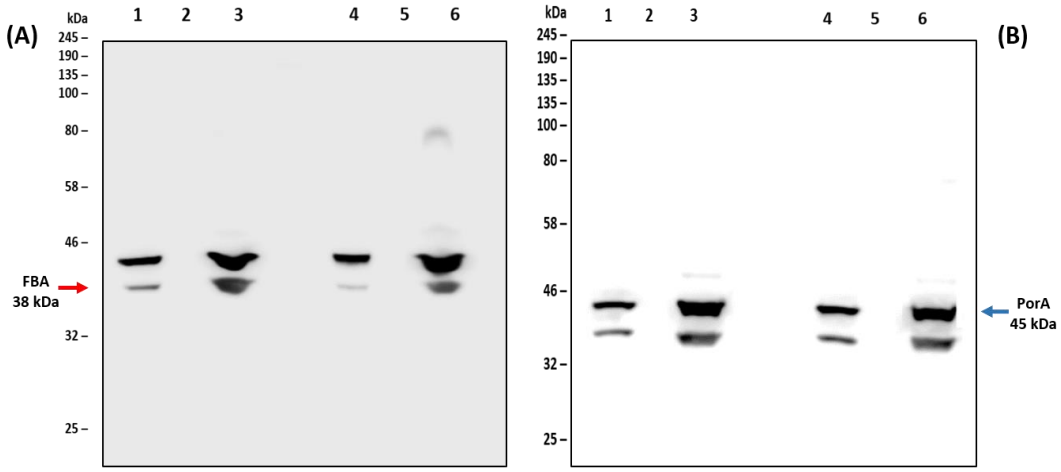


Figure 6.12: Immunoblot of cellular fractions; periplasmic-cytosolic protein enriched (PCP), cytoplasmic membrane protein-enriched (CMP), and outer-membrane protein-enriched (OMP) fractions collected from MC58Δ*cbbA cbbA*^{Ect} (lane 1 to 3) and MC58Δ*cbbA cbbA*^{EctD11E} (lane 4 to 6) strains (A) probed with anti-FBA antibody (B) probed with anti-PorA antibody. Lane 1: PCP fraction of MC58Δ*cbbA cbbA*^{Ect}. Lane 2: CMP fraction of MC58Δ*cbbA cbbA*^{Ect}. Lane 3: OMP fraction of MC58Δ*cbbA cbbA*^{Ect}. Lane 4: PCP fraction of MC58Δ*cbbA cbbA*^{EctD11E}. Lane 5: CMP fraction of MC58Δ*cbbA cbbA*^{EctD11E}. Lane 6: OMP fraction of MC58Δ*cbbA cbbA*^{EctD11E}. (A) A band corresponding to FBA (*ca.* 38 kDa) was observed in the PCP and OMP fractions from both strains, whereas this band was absent in the CMP fraction. (B) A band corresponding to PorA (*ca.* 45 kDa) was observed in the PCP and OMP fractions from both strains representing the loading controls with no apparent difference in the intensity, whereas no band was seen in the CMP fraction.

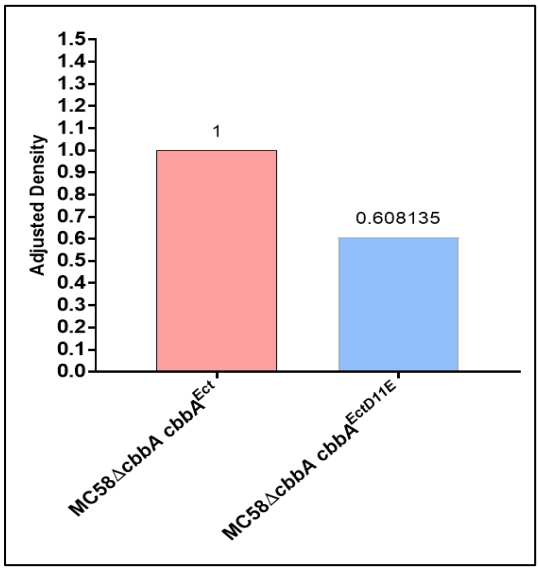


Figure 6.13: Protein densitometry of the outer-membrane protein-enriched (OMP) fraction collected from MC58Δ*cbbA* *cbbA*^{Ect} and MC58Δ*cbbA* *cbbA*^{EctD11E}. The bars indicate the adjusted relative density of the OMP fraction using the two blot images (The same membrane was probed with anti-FBA antibody and then re-probed with anti-PorA antibody). Values shown are from one experiment.

6.3 Discussion

It has been previously reported that FBA was found on the cell surface of *N. meningitidis*, other pathogenic and non-pathogenic species of *Neisseria* (Tunio *et al.* 2010b; Shams *et al.* 2016). This result is in agreement with our whole-cell ELISA data, which showed that there is a significant difference between MC58 wild type and a *cbbA* null mutant. Although it has been reported that FBA alongside other cytoplasmic house-keeping enzymes, such as enolase and GAPDH lack identifiable secretion signals, Tunio *et al.* predicted a 13 amino acid N-terminal putative signal peptide with a predicted cleavage site (^{11}DHA-AE^{15}) (Tunio *et al.* 2010b).

In an attempt to confirm this prediction, we mutated an alanine residue (A14) to valine in the predicted cleavage site. Then, we introduced an ectopic copy of a *cbbA* gene derivative containing this mutation into the *cbbA* null mutant (MC58Δ*cbbA*) to produce MC58Δ*cbbA* *cbbA*^{EctA14V} complemented derivative. Significantly, FBA surface localisation was affected in MC58Δ*cbbA* *cbbA*^{EctA14V} compared to the complemented strain harbouring the ectopic copy of intact *cbbA* gene (MC58Δ*cbbA* *cbbA*^{Ect}). By immunoblotting the whole cell lysates of these strains, we have shown that this mutation also had an impact on FBA expression, which could be the cause of its abolished translocation to the cell

surface. Subsequently, additional mutations of residues (A13, A13A14, and D11) within the predicted cleavage site and in another site (A22) of the putative N-terminal signal peptide were introduced into the *cbbA* null mutant to investigate their effect on FBA surface exposure. Our data demonstrate that all complemented derivatives harbouring these mutations had a significant effect on FBA surface localisation. In fact, the A14A13 double mutation within the predicted cleavage site showed the most significant impact, which was similar to that of the null mutant. To determine the effect of each mutation on the expression, immunoblot analysis was performed showing a reduction of FBA expression in MC58Δ*cbbA* $cbbA^{EctA13V}$, MC58Δ*cbbA* $cbbA^{EctA13VA14V}$, and MC58Δ*cbbA* $cbbA^{EctA22V}$, however, no effect was seen in MC58Δ*cbbA* $cbbA^{EctD11E}$. *In vitro*, Tunio *et al.* demonstrated that the *cbbA* null mutant had a very similar growth profile to MC58 wild type and the complemented mutant derivative (Tunio *et al.* 2010b). We have also shown a similar growth profile of the complemented derivatives harbouring mutations in the putative N-terminal signal peptide. This confirms that FBA is not required for meningococcal growth.

To ensure that the protein has not been degraded during the secretory pathway due to these mutations giving false positive results, FBA cellular fractions were investigated in the two

complemented derivatives harbouring A14V and D11E mutations using sub-cellular fractionation followed by immunoblot analysis. The results show that the expressed FBA was detected in the PCP and OMP enriched fractions of MC58Δ*cbbA* *cbbA*^{EctA14V} and MC58Δ*cbbA* *cbbA*^{EctD11E}, whereas no band was observed in the CMP enriched fraction of either strain. An identical profile was found by re-probing the same blot with anti-PorA antibody. This result is in agreement with Tunio *et al.* study, which showed that FBA was detected in the cytosolic, periplasmic, and outer membrane protein-enriched fractions, but was lost from cytoplasmic membrane-enriched fraction (Tunio *et al.* 2010b). These results suggest that FBA is not only a cytoplasmic protein, but is also present in the outer membrane. However, FBA was more abundant in the OMP fraction than in the PCP fraction. A similar profile has been shown by Voss *et al.,* who revealed that the four Cag proteins (Cag3, CagM, CagT, and CagX) of *H. pylori* were more abundant in the OMP fraction than in the PCP fraction (Voss *et al.* 2014). Our sub-cellular fractionation results suggest that the mutations in the predicted cleavage site in the putative N-terminal signal peptide did not prevent FBA localisation to the outer membrane confirming that FBA surface translocation was independent of its N-terminal peptide sequence.

In conclusion, introducing an ectopic copy a *cbbA* gene containing mutations in the putative N-terminal signal peptide within the cleavage site and in another site significantly affected FBA localisation to the cell surface. However, immunoblot analysis and sub-cellular fractionation experiment showed that this effect could be caused by reduced FBA expression due to these mutations confirming that the N-terminal peptide is not involved in the unknown FBA secretion pathway. This also showed that FBA is more abundant in the OMP fraction than in the PCP fraction. It was shown that the *in vitro* growth profiles of the complemented derivatives containing these mutations were identical to the wild type and the complemented mutant strain.

7 Chapter Seven: General Discussion

7.1 General discussion

Besides their intracellular functions, some bacterial glycolytic enzymes exhibit moonlighting activities, which are related to their surface location. Important biological roles of moonlighting proteins after being translocated to the cell surface involve interactions with many host components, such as plasminogen and extracellular matrices (Henderson and Martin 2014; Wang *et al.* 2014). Moonlighting proteins have been identified in all three domains of life: Bacteria, Archaea, and Eukarya as either molecular chaperone or metabolic enzymes (Henderson and Martin 2014). FBA and enolase are meningococcal glycolytic proteins that are found to be on the cell surface where they perform other functions unrelated to their cytoplasmic roles. The work presented in this thesis investigates the mechanisms of secretion of FBA and enolase to the meningococcal cell surface.

It has been demonstrated that FBA is localised to the cell surface of several meningococcal strains: two MenB; two MenC; one MenX; and one MenZ, which was confirmed by flow cytometry (Shams *et al.* 2016). This result is in agreement with our whole-cell ELISA data, which revealed a significant binding of FBA wild type to antibodies compared to the null mutant MC58Δ*cbbA*. This also agrees with Tunio and colleagues FACS data, which

suggested that FBA is exposed at meningococcal cell surface (Tunio *et al.* 2010b).

Although previous studies demonstrated that FBA, enolase, and peroxiredoxin are exposed at the meningococcal cell surface, the mechanism of their secretion is still not understood (Shams *et al.* 2016). In this study, whole-cell ELISA method was used to screen mutants lacking known protein secretion-related or pathogenesis-related systems, such as MC58Δ*PilQ*Δ*PorA*, MC58Δ*skp*, MC58Δ*surA*, MC58Δ*skp*Δ*surA*, MC58Δ*hlyB* (type I secretion system), and MC58Δ*cbbA* $cbbA^{Ectk354A}$, to test their effect on FBA, enolase, and peroxiredoxin surface exposure. Ruiz-Perez and co-workers reported that Skp and SurA periplasmic chaperones play a major role in the biogenesis of the serine protease autotransporters of Enterobacteriaceae (Ruiz-Perez *et al.* 2009). In *E. coli*, Skp and SurA aid in the folding and targeting of proteins to the outer membrane (Behrens *et al.* 2001; Rizzitello *et al.* 2001). It is thus likely that the SurA and Skp homologues in *Neisseria* may play a similar role in the export of autotransporters or other secreted proteins. In this study, a potential role of Skp and SurA in the translocation of FBA, enolase, and peroxiredoxin to the cell surface was investigated. The results suggest that the mutation of both chaperones had no effect on their secretion. Shams and colleagues reported that

the optimal binding of FBA to human plasminogen is dependant on its C-terminal lysine residue (Lys^{354}). A complemented strain harbouring Lys^{354} mutation (MC58Δ*cbbA* $cbbA^{Ectk354A}$) was made to investigate the involvement of this residue in FBA secretion. However, MC58Δ*cbbA* $cbbA^{Ectk354A}$ showed no defect in FBA surface exposure. It has been previously reported that meningococcal PilQ is an essential component of type IV pili and forms the channel through which the pilus traverses the outer membrane (Collins *et al.* 2005). Therefore, MC58Δ*PilQ*Δ*PorA,* a double mutant harbouring PilQ mutation, was tested. Nevertheless, no effect was shown on the secretion of FBA and peroxiredoxin. These results led to the conclusion that there is no involvement of type I secretion system and Skp and SurA chaperones in the surface localisation of FBA, enolase, and peroxiredoxin.

7.1.1 Enolase

Aligning protein sequences of enolase from *N. meningitidis* MC58 and enolase from *E. coli* (Lys^{341}) revealed a conserved lysine residue (Lys^{337}) on meningococcal enolase, which could be identified as a possible signal for enolase secretion to the cell surface. Boel and co-workers suggested that Lys^{341} of *E. coli* enolase is important for its export (Boel *et al.* 2004). As meningococcal enolase is an essential protein for the survival of

N. meningitidis, a different approach 'complementation-first strategy' was evolved to generate a complemented derivative harbouring ectopic copies of either an intact enolase gene or a derivative gene containing Lys^{337} mutation without affecting meningococcal viability. In order to facilitate this strategy, two plasmids, pComp1285 and pComp1285-K337E, were introduced into MC58 *rpsL*^{R} to insert another copy of enolase gene, alongside an erythromycin resistance cassette, into the genome at an ectopic site before knocking out the original copy. These two complemented strains showed no difference in enolase expression compared to the wild type although they have an additional enolase gene copy at an ectopic site. This could be an indication that enolase expression might be tightly regulated in the cell or the expression of ectopic enolase is very low (or absent), which resulted in no difference in the expression between strains harbouring one or two apparently functional copies of enolase gene. Then, a markerless mutation strategy was adopted to delete the original enolase gene without inserting an antibiotic marker to eliminate any concerns regarding polar effects (Ishikawa and Hori 2013). It was based on *rpsL*^{R} allele, which confers the streptomycin resistance phenotype. It has been demonstrated that streptomycin sensitivity (*rpsL*^{S}) is dominant in a *rpsL*^{R} strains when both alleles are expressed

(Breckenridge and Gorini 1970). In the first recombination event, the *NMB1285* gene from the two MC58 *rpsL*^{R} complemented strains, one contains the ectopic copy of an intact enolase gene and the other contains a gene derivative harbouring K337E mutation, was replaced with *Kan*^{R}*/rpsL*^{S} (*KnsL*) cassette. In the second recombination, *KnsL* cassette in both strains was removed and then replaced with the *NMB1285* flanking DNA. Immunoblot analysis indicated that there is a considerable reduction of enolase expression in the two strains, *N. meningitidis* MC58 *rpsL*^{R} Δ*NMB1285 NMB1285*^{Ect} and *N. meningitidis* MC58 *rpsL*^{R} Δ*NMB1285 NMB1285*^{EctK337E}, compared to the wild type. This indicated that the expression of enolase has been restored despite the fact that it was difficult to detect apparent bands of enolase in the whole cell lysates of the complemented strains since enolase has been shown to be essential for meningococcal survival in previous studies (Bergmann *et al.* 2001; Knaust *et al.* 2007; Sha *et al.* 2009; Ko *et al.* 2013). The growth rates of *N. meningitidis* MC58 *rpsL*^{R} Δ*NMB1285 NMB1285*^{Ect} and *N. meningitidis* MC58 *rpsL*^{R} Δ*NMB1285 NMB1285*^{EctK337E} strains were monitored to investigate the impact of such a reduction in enolase expression, which revealed a diminished growth rate in *N. meningitidis* MC58 *rpsL*^{R} Δ*NMB1285 NMB1285*^{EctK337E} strain, which suggests that

Lys^{337} mutation affects meningococcal growth. The two complemented derivatives were also examined for the surface localisation of enolase using whole-cell ELISA. Enolase export was affected in both complemented derivatives, harbouring ectopic copies of an intact enolase and K337E mutated enolase. However, this could be as a result of the reduced enolase expression. This reduction has also led to a significant decrease in plasminogen binding efficiency to whole meningococcal cells indicating that the presence of highly expressed enolase is essential for optimal binding to plasminogen.

Several attempts to enhance the expression of enolase have been made to solve this problem. In the first attempt, mutations have been introduced at specific sites of pComp1285 vector to incorporate a SD-like sequence. The resulting plasmid pComp1285SD was introduced into MC58 *rpsL*^{S}. Then the original copy of enolase gene was knocked out by markerless mutation to study the effect of the incorporation of SD-like sequence on enhancing enolase expression. The results showed that there was no effect on enhancing the efficiency of expression. In the second attempt, enolase was placed under the control of a strong promoter, PorA promoter, which was previously proposed to have a significant impact on the overexpression of genes encoding Hsf/NhhA, Omp85, and NspA

(D. Martin *et al.* 2000; Scarselli *et al.* 2001; Voulhoux and Tommassen 2004; Norheim *et al.* 2005). In our study, a plasmid harbouring PorA promoter was constructed by deleting Opa-cbbA promoters in pComp1285 then replacing them with PorA promoter. The resulting plasmid pComp1285porAp was introduced into MC58 *rpsL*^{R} then the unmarked mutation was performed. The immunoblot analysis results revealed that the effect of PorA promoter on enolase expression was insignificant. These results led to a conclusion that it is not considered possible to generate a complemented derivative with the enolase gene of meningococci without leading to a reduction or loss of its expression, which makes it extremely difficult to identify the actual role of Lys^{337} in enolase secretion.

7.1.2 FBA

Despite the lack of identifiable secretion signal in meningococcal FBA, Tunio and co-workers predicted a 13 amino acid putative N-terminal signal peptide with a predicted cleavage site (^{11}DHA-AE^{15}) (Tunio *et al.* 2010b). In our study, several mutations were introduced at specific sites of the putative signal peptide, in the cleavage site and in another site, in order to confirm this prediction. Ectopic copies of a *cbbA* gene derivative harbouring these mutations (A13V, A14V, A13VA14V, A22V, and D11E) were introduced into MC58Δ*cbbA* to generate these strains

(MC58Δ*cbbA* *cbbA*^{EctA13V}, MC58Δ*cbbA* *cbbA*^{EctA14V} MC58Δ*cbbA* *cbbA*^{EctA13VA14V}, MC58Δ*cbbA* *cbbA*^{EctA22V}, and MC58Δ*cbbA* *cbbA*^{EctD11E}). FBA surface localisation was affected in all strains compared to MC58Δ*cbbA* *cbbA*^{Ect}. Immunoblot analysis of all strains showed a reduction of FBA expression compared to MC58Δ*cbbA* *cbbA*^{Ect} except MC58Δ*cbbA* *cbbA*^{EctD11E,} which showed no effect on expression. The growth rates of these strains were also evaluated under laboratory condition, which revealed that *cbbA* null mutant and all complemented derivatives displayed a normal growth profile. This result is in agreement with the data of Tunio and colleagues, which suggested that FBA is not required for the growth of *N. meningitidis* (Tunio *et al.* 2010b). Subcellular fractionation followed by immunoblot analysis were performed to confirm that the protein has not been degraded during the secretory pathway, which could give false positive results. Cellular fractions collected from MC58Δ*cbbA* *cbbA*^{EctA14V} and MC58Δ*cbbA* *cbbA*^{EctD11E} were tested. The results showed a band corresponding to FBA in the PCP and OMP enriched fractions from both strains. Re-probing the same blot with anti-PorA antisera gave an identical profile. This finding agrees with Tunio and co-workers, who have demonstrated that meningococcal FBA was found in the cytosolic, periplasmic, and outer membrane protein-enriched fractions, but was not

detected in the cytoplasmic membrane fraction (Tunio *et al.* 2010b). Similarly, Voss *et al.* revealed that the Cag proteins (Cag3, CagM, CagT, and CagX), which are outer membrane proteins required for activity of the *cag* type IV secretion system of *H. pylori,* were more abundant in the OMP fraction than in the PCP fraction (Voss *et al.* 2014).

To conclude, this study suggests that introducing mutations into the putative N-terminal signal peptide of meningococcal FBA had an impact on its surface localisation. Nevertheless, this impact was due to a reduction of FBA expression, which was revealed by immunoblot analysis and cellular fractionation experiment. Finally, the null mutant and all complemented derivatives displayed a similar growth profile to the MC58 wild type.

7.2 Proposed future work

Our data demonstrated that mutations in the N-terminal sequence of meningococcal FBA had a significant impact on the expression of *cbbA* gene. RNA sequencing technology could be a useful tool to compare gene expression profiles of *cbbA* null mutant*, cbbA* complemented derivatives, and the wild type.

Additional genes that may be required for the surface exposure of enolase and FBA may also be identified by screening a

meningococcal transposon mutagenesis library, which contains a collection of mutants in most non-essential genes.

It has been previously reported that FBA and *NMB0345* (encoding a predicted cell binding factor) are required for the optimal adhesion of meningococci to host cells (Bakshi 2004; Tunio *et al.* 2010b; Hey *et al.* 2013). *NMB0345* was shown to have motifs' homology to Skp and SurA chaperones, which were previously demonstrated to have an important role in the biogenesis of the serine protease autotransporters of Enterobacteriaceae (Ruiz-Perez *et al.* 2009). In our work, *skp* and *surA* null mutants were tested for their effect on FBA secretion, which resulted in no significant difference between these mutants and MC58 wild type. Therefore, using the same approach for the identification of the potential role of *NMB0345* in the surface localisation of FBA is worth considering.

Given that the deficient plasminogen binding to meningococci was as a result of the considerable reduction of enolase expression in the two complemented enolase derivatives, other mutants of moonlighting proteins, such as FBA and peroxiredoxin null mutants, which were previously reported to bind plasminogen (Shams *et al.* 2016), could also be examined.

Other conserved lysine residues of enolase and FBA, which were previously shown to have a major role in mediating

meningococcal-host interactions, could be examined for their effect on the secretion.

7.3 General conclusion

Screening mutants lacking known protein secretion-related or pathogenesis-related systems using whole-cell ELISA led to the conclusion that type I secretion pathway is not involved in the surface localisation of FBA, enolase, and peroxiredoxin. It was also suggested that Skp and SurA molecular chaperones have no role in the periplasmic translocation of FBA, enolase, and peroxiredoxin across the outer membrane.

The generation of mutants lacking the native enolase gene, but harbouring ectopic copies of wild type or mutant (K337E) enolase gene was successful. However, this unmarked mutation resulted in a considerable reduction of enolase expression. Previous studies have shown that enolase is essential for meningococcal viability, which suggests that enolase expression had been restored in the unmarked mutants. This reduction of expression resulted in a significant effect on enolase surface translocation. This reduction also resulted in a deficient plasminogen binding to whole meningococcal cells.

Several attempts to enhance enolase expression have been made. Nevertheless, they were unsuccessful. Taken together, it is not considered possible to construct a complemented derivative with the enolase gene of *N. meningitidis* without causing a considerable reduction or complete loss of its expression. This resulted in a difficulty identifying the potential roles of Lys^{337} in enolase secretion. Our data suggested that Lys^{337} mutation affects meningococcal growth confirming an important functional role of this residue.

In this study, mutations were introduced into the putative N-terminal signal peptide, within the predicted cleavage site and in another site of the peptide, to confirm Tunio and co-workers prediction (Tunio *et al.* 2010b). Our results demonstrated that this predicted signal peptide is not involved in the unknown FBA secretion pathway.

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Appendix A: Buffers and Reagents

Carbonate buffer: 0.1 M sodium carbonate (Na_2CO_3), 0.1 M sodium bicarbonate ($NaHCO_3$), adjust the pH to 9.4

Glycine buffer: 1 ml 0.1 M $MgCl_2$, 100 ml glycine zinc chloride [0.1 M glycine, 1mM zinc-chloride], adjust pH to 10.4.

TAE buffer (50 × stock): 242 g Tris base, 57.1 ml glacial acetic acid, 100 ml 500 mM EDTA (pH 8.0) made up to 1000 ml with dH_2O.

Agarose gel (1 %): 1 g agarose powder (Sigma) dissolved in 100 ml 1× TAE buffer. After dissolving, add 5 µl SYBR™ Safe DNA Gel Stain (Invitrogen™).

SDS gel stain: 50% MeOH, 10% acetate, and 1.2g/L coomassie.

10 % SDS solution: 1 g of SDS in 100 ml of dH_2O.

SDS Separating or resolving buffer (Buffer A): 181.7 g Tris, 40 ml 10 % SDS, made up to 1000 ml with dH_2O, adjust pH to 8.8.

Resolving (separating) gel (10 %): 2.5 ml SDS-resolving buffer, 3.3 ml Acrylamide/Bis-Acrylamide (30 %), 4.1 ml dH_2O,

30 µl 10% ammonium persulfate (APS) and 30 µl Tetramethyl ethylenediamine (TEMED).

SDS Stacking buffer (Buffer B): 60.6 g Tris, 40 ml 10 % SDS, made up to 1000 ml with dH_2O, adjust pH to 6.8.

Stacking gel: 1.3 ml SDS-resolving buffer, 1 ml Acrylamide/Bis-Acrylamide (30 %), 2.7 ml dH_2O, 10 µl 10% ammonium persulfate (APS) and 10 µl Tetramethyl ethylenediamine (TEMED).

Semi dry transfer buffer: 5.8 g Tris, 3.75 ml of 10 % SDS, 2.9 g of glycine, 200 ml of methanol, made up to 1000 ml with dH_2O.

5 × SDS sample buffer: 620 mM Tris-HCl, 25% Glycerol, 5% SDS, 0.002% Bromophenol blue, and 12.5% β-mercaptoethanol.

Phosphate-buffered saline (PBS): 1 × PBS, 1 tablet of phosphate buffered saline (Sigma) dissolved in 200 ml dH_2O, autoclave. This gives 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4.

PBST: PBS with 0.05% Tween-20.

Bovine serum albumin (BSA): Albumin from bovine serum, Sigma.

Blocking buffer for immunoblotting: 5 % semi-skimmed milk, 0.1 % PBST.

10X annealing buffer: 10 mM Tris (PH 7.5), 10 mM NaCl, 1 mM EDTA.

Elution buffer for sub-cellular fractionation: 10mM Tris-HCl pH 7.5, 25% sucrose, 10mM $MgCl_2$.