

**The role of fructose -1, 6 - bisphosphate
aldolase (FBA) in the pathogenesis of
*Neisseria meningitidis***

Fariza Shams

B.Sc, M.Sc

Thesis submitted to the University of Nottingham for the
degree of Doctor of Philosophy

2015

Molecular Bacteriology and Immunology Group
School of Life Sciences
Centre for Bimolecular Sciences
University of Nottingham

Declaration

I declare that the work presented here is my own, unless otherwise stated. No part of this work has been submitted for another degree at The University of Nottingham or any other institute of learning.

Fariza Shams

Acknowledgements

First of all I express my deepest gratitude to almighty ALLAH for giving me the strength to complete this degree.

I am deeply indebted to my supervisors, Dr. David P. J. Turner, Dr. Karl G Wooldridge and Dr. Neil J. Oldfield for accepting me as a PhD student in Molecular Biology and Bacteriology Group (MBIG). Without their kind supervision, endless help, continuous support, advice and words of encouragement, I could not finish the study.

My sincerest gratitude goes to Dr. Aslam Akhmed for his tremendous help. I want to thank Prof. Alan Berry, University of Leeds, for allowing me work in his laboratory.

Special thanks to Hibah and Suzan. Sheyda, a friend in need is a friend indeed, thank you so much. Thanks to all past and present members of MBIG for their help and support. Special thanks to MSc student SiKei Lei.

Thanks to my parents for their unconditional love, support and patience.

I could not have achieved this without my husband Maqsud. Thank you for making the journey easier with your love, support and encouragement. This thesis is a result of your perseverance and understanding.

A special thanks to my little Nahwan for being my inspiration of life.

Finally, I would like to acknowledge the International Research Excellence Scholarship from University of Nottingham and financial support from School of Life Sciences.

Table of Contents

Declaration.....	i
Acknowledgements.....	ii
List of Figures.....	xii
List of Tables	xvi
Abbreviations.....	xvii
List of publications and presentations	xxi
Abstract.....	xxiii
Chapter One: General Introduction	1
1.1The genus <i>Neisseria</i>	2
1.2 <i>N. meningitidis</i>	2
1.3 Classification of <i>N. meningitidis</i>	3
1.4 Epidemiology.....	4
1.5 Clinical manifestations of invasive meningococcal disease.....	7
1.5.1 Sepsis	7
1.5.2 Meningitis	7
1.6 Diagnosis and treatment of meningococcal disease	8
1.7 Prevention of meningococcal diseases: vaccines against <i>N. meningitidis</i>	9
1.7.1 Polysaccharide vaccines	9
1.7.2 Conjugate vaccines	10

1.7.3 Meningococcal serogroup B vaccines	11
1.8 Virulence factors of <i>N. meningitidis</i>	12
1.8.1 Capsule	13
1.8.2 Lipooligosaccharide (LOS)	16
1.8.3 Pili.....	16
1.8.4 Opacity proteins.....	18
1.8.5 Factor H binding Protein (fHbp).....	19
1.8.6 Iron acquisition system	20
1.8.7 Secretion systems in <i>N. meningitidis</i> and autotransporter proteins	22
1.8.7.1 Type I secretion system	22
1.8.7.2 Two-partner secretion system (T5bSS)	23
1.8.7.3 Autotransporter proteins (T5aSS).....	24
Adhesion and penetration protein (App)	25
Meningococcal serine protease A (MspA)	25
Autotransported serine protease A (AspA)/ <i>Neisseria</i> autotransporter lipoprotein (NalP)	26
Auto-transporter A (AutA and Aut B).....	27
IgA protease.....	27
<i>Neisseria</i> Adhesin A (NadA).....	28
NhhA (<i>Neisseria</i> hia/hsf homologue)/ Msf (Meningococcal surface fibril)	28
1.9 Pathogenesis of <i>N. meningitidis</i>	29

1.9.1 Interaction of <i>N. meningitidis</i> with nasopharyngeal epithelial cells.....	29
1.9.2 Interaction of <i>N. meningitidis</i> with endothelial cells and invasion of brain endothelium	33
1.10 Moonlighting proteins: Characteristics and distribution in nature	36
1.11 Moonlighting proteins in <i>N. meningitidis</i>	45
1.12 Overview of Fructose-1, 6-bisphosphate aldolase (FBA)	47
1.13 Unusual location and non-glycolytic functions of FBA.....	49
1.14 Aims of the study.....	52
Chapter Two: Cloning, expression, purification and biochemical characterisation of recombinant FBA (rFBA)	53
2.1 Introduction.....	54
2.2 Materials and Methods	57
2.2.1 Bacterial strains and growth conditions.....	57
2.2.2 DNA techniques.....	57
2.2.2.1 Extraction of plasmid DNA	57
2.2.2.2 Primer design	57
2.2.2.3 Site directed mutagenesis and transformation of <i>E. coli</i> XL10-Gold ultracompetent cells	57
2.2.2.4 Sequencing of plasmid DNA and nucleic acid and amino acid sequence analysis	59
2.2.3 Protein techniques.....	59
2.2.3.1 Expression of rFBA using <i>E. coli</i> JM109 competent cells.....	59

2.2.3.2 Purification of rFBA by immobilised metal affinity chromatography (IMAC)	60
2.2.3.3 Buffer exchange.....	60
2.2.3.4 Concentration of purified protein samples	61
2.2.3.5 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).....	61
2.2.3.6 Immunoblotting	61
2.2.3.7 Protein assay	62
2.2.3.8 Enzyme assay.....	62
2.3 Results	64
2.3.1 Site directed mutagenesis in the active site of FBA	64
2.3.2 Correction of reading frame in the transcripts.....	66
2.3.3 Expression of rFBA by <i>E. coli</i> JM109 cells	69
2.3.4 Purification of rFBA (WT) by step elution and buffer exchange.....	69
2.3.5 Expression and purification of rFBA (D83A and H81/H84A).....	71
2.3.6 Purified rFBA (WT) is an active aldolase enzyme.....	77
2.4 Discussion.....	78
Chapter Three: FBA is a plasminogen binding protein of.....	84
<i>N. meningitidis</i>	84
3.1 Introduction.....	85
3.2 Materials and Methods	90

3.2.1 Bacterial techniques.....	90
3.2.1.1 Bacterial strains, growth conditions and media.....	90
3.2.1.2 Flow cytometry analysis	90
3.2.2 Protein techniques.....	93
3.2.2.1 Enzyme-linked immunosorbant assay (EIA).....	93
3.2.2.2 Plasminogen activation assay	94
3.3 Results	95
3.3.1 FBA is a plasminogen binding protein of <i>N. meningitidis</i>	95
3.3.2 Cofactor binding site residues of rFBA are not involved in plasminogen binding	96
3.3.3 Inhibition of FBA-plasminogen binding by the lysine analogue ϵ –amino caproic acid (EACA)	98
3.3.4 Effect of anti-FBA antiserum and NaCl on plasminogen binding	99
3.3.5 Plasminogen activation assay	100
3.3.6 Plasminogen binding is a common property of <i>Neisseria</i> spp.	101
3.4 Discussion.....	103
Chapter Four: The interaction between rFBA and plasminogen is mediated by lysine residue(s)	108
4.1 Introduction.....	109
4.2 Materials and Methods	112
4.2.1 Bacterial techniques.....	112

4.2.1.1 Bacterial strains and culture medium	112
4.2.2 DNA techniques.....	112
4.2.2.1 Extraction of DNA.....	112
4.2.2.2 Polymerase Chain Reaction (PCR) amplification of <i>cbbA</i>	112
4.2.2.3 Agarose gel electrophoresis	112
4.2.2.4 Purification of PCR products.....	114
4.2.2.5 Digestion of vectors and inserts.....	114
4.2.2.6 Dephosphorylation of digested vectors	114
4.2.2.7 Ligation.....	114
4.2.2.8 Site directed mutagenesis and transformation of <i>E.coli</i> XL10-gold ultracompetent cells and <i>E. coli</i> JM109 cells	114
4.2.2.9 Sequencing of plasmid DNA and nucleic acid and amino acid sequence analysis	114
4.2.3 Protein techniques.....	115
4.2.3.1 Expression and purification of tr-rFBA	115
4.2.3.2 SDS-PAGE and immunoblotting.....	115
4.2.3.3 Plasminogen binding assay.....	115
4.3 Results	116
4.3.1 Cloning of a 400bp fragment of <i>cbbA</i> into pQE-30 and pQE-70 vectors	116
4.3.2 Expression of truncated rFBA (tr-rFBA) by <i>E. coli</i> JM109 and purification of tr-rFBA	119

4.3.3 Interaction of lysine rich C-terminal fragment of FBA with human plasminogen.....	121
4.3.4 Effects of salt and anti-FBA antibody on tr-FBA-plasminogen interaction	121
4.3.5 Site directed mutagenesis	123
4.3.3 Expression and purification of mutated truncated rFBA1	124
4.3.4 Interaction of lysine rich C-terminal fragment of FBA with human plasminogen.....	126
4.3.5 Effects of salt and anti-FBA antiserum on tr-FBA-plasminogen interaction	127
4.5 Discussion.....	129
Chapter Five: Surface localisation of FBA on <i>N. meningitidis</i> is not affected by mutation(s) in the active site.....	137
5.1 Introduction.....	138
5.2 Materials and Methods	142
5.2.1 Bacterial techniques.....	142
5.2.1.1 Bacterial strains, growth conditions and media.....	142
5.2.1.2 Natural transformation of <i>N. meningitidis</i> MC58 Δ <i>cbbA</i>	143
5.2.1.3 Flow cytometry	144
5.2.1.4 Preparation of meningococci for cell culture.....	145
5.2.2 DNA techniques.....	145
5.2.2.1 Extraction of chromosomal and plasmid DNA	145

5.2.2.2 Polymerase Chain Reaction (PCR) amplification of <i>cbbA</i>	145
5.2.2.3 Agarose gel electrophoresis	146
5.2.2.4 Purification of PCR product	146
5.2.2.5 Digestion of vectors and inserts.....	146
5.2.2.6 Dephosphorylation of digested vector	147
5.2.2.7 Ligation.....	147
5.2.2.8 Site directed mutagenesis and transformation of XL10-gold ultracompetent cells and <i>E. coli</i> JM109 cells	147
5.2.2.9 Nucleic acid and amino acid sequence analysis	147
5.2.3 Cell culture techniques	147
5.2.3.1 Preparation of human cells	147
5.2.3.2 Association and invasion assay	148
5.2.3.3 Statistical analysis.....	149
5.3 Results	150
5.3.1 Complementation of <i>N. meningitidis</i> Δ <i>cbbA</i>	150
5.3.2 Construction of plasmid vector containing <i>cbbA</i> with various mutations	151
5.3.3 Complementation of <i>N. meningitidis</i> MC58 Δ <i>cbbA</i> with wild type or mutated <i>cbbA</i>	152
5.3.4 Expression of FBA by <i>N. meningitidis</i> MC58 Δ <i>cbbA</i> <i>cbbA</i> ^{Ect} strains ...	154

5.3.5 Mutation(s) in the cofactor binding site of FBA do not prevent its surface localisation.....	155
5.3.6 The Role of FBA in association and invasion of human epithelial and endothelial cells	156
5.4 Discussion.....	159
Chapter Six: General Discussion	165
6.1 General Discussion	166
6.2 Conclusion	176
6.3 Future Directions	177
Bibliography	181
Appendix A: Vectors and Plasmids.....	232
Appendix B: Standard curve.....	235

List of Figures

Figure 1.1: Prominent outer-membrane components of <i>N. meningitidis</i> that influence bacterial interactions with host cells.....	14
Figure 1.2: Stages in the pathogenesis of <i>N. meningitidis</i>	31
Figure 1.3: Examples of mechanisms for switching between usual and moonlighting functions:.....	38
Figure 1.4: EMP and ED pathways of glucose metabolism.	46
Figure 2.1: Optimal alignment of protein sequences of <i>E.coli</i> IIA FBA and <i>N. meningitidis</i> MC58 IIB FBA sequences	65
Figure 2.2: Multiple sequence alignment of <i>cbbA</i> and its translated product ..	66
Figure 2.3: SDS-PAGE analysis of <i>E. coli</i> JM109 cells for expression of rFBA.	67
Figure 2.4: Multiple sequence alignment of plasmid vector pQE-70, plasmid pSAT-9 and <i>cbbA</i> from <i>N. meningitidis</i> MC58.....	68
Figure 2.5: Multiple sequence alignment of the (A) DNA sequences and (B) the translated protein products derived from pSAT-9, pFS-1, pFS-2 and pFS-3	68
Figure 2.6: Expression of rFBA by <i>E. coli</i> JM109 cells.....	69
Figure 2.7: Purification of rFBA (WT) by step elution.	70
Figure 2.8: Purification of rFBA (WT) by buffer exchange.....	71
Figure 2.9: Expression of rFBA (D83A) and rFBA (H81A/ H84A).....	72
Figure 2.10: Purification of rFBA (D83A) by step elution.	73

Figure 2.11: Purification of rFBA (D83A) by buffer exchange.	74
Figure 2.12: Purification of rFBA (H81/84A) by step elution	75
Figure 2.13: Purification of rFBA (H81/84A) by buffer exchange.	76
Figure 2.14: Kinetic analysis of rFBA (WT).	77
Figure 3.1: Meningococcal FBA is a plasminogen-binding protein.	95
Figure 3.2: Mutations in the cofactor binding site of <i>N. meningitidis</i>	97
Figure 3.3: Binding of human plasminogen to rFBA was inhibited by the lysine analogue EACA.	98
Figure 3.4: Effect of NaCl on FBA-plasminogen interactions.	99
Figure 3.5: Effect of anti-FBA antiserum on rFBA-plasminogen interaction.	100
Figure 3.6: Flow cytometric analysis of plasminogen binding.	102
Figure 4.1: Nucleotide sequence of MC58 <i>cbbA</i> and the corresponding amino acid sequence of its encoded protein FBA.	117
Figure 4.2: Agarose gel showing amplification of <i>cbbA</i> and truncated <i>cbbA</i>	118
Figure 4.3: Multiple sequence alignment of translated products of truncated <i>cbbA</i> cloned into pQE-30 and pQE-70 showing the presence of the His-tag at desired position.	119
Figure 4.4: Immunoblot showing the expression of A) tr-rFBA1 and tr-rFBA2 in <i>E. coli</i> JM109 culture samples.	120
Figure 4.5: Immunoblots, probed with anti-His-tag antibody, showing tr-rFBA proteins purified under non-denaturing conditions.	120
Figure 4.6: A) tr-rFBA binds human plasminogen.	121

Figure 4.7: Effect of NaCl on tr-rFBA-plasminogen interaction	122
Figure 4.8: Effect of anti-FBA antiserum on tr-rFBA1-plasminogen interaction.	123
Figure 4.9: Multiple sequence alignment of amino acid sequences of tr-rFBA1 derivatives resulting from site-directed mutagenesis.	124
Figure 4.10: Immunoblot showing the expression of mutated N-His-tagged tr- rFBA1 in <i>E. coli</i> JM109 culture samples.	125
Figure 4.11: Immunoblots, probed with anti-His-tag antibody, showing mutated tr-rFBA1 proteins purified under non-denaturing conditions.....	125
Figure 4.12: plasminogen binding by mutant variants of tr-rFBA1 containing lysine to alanine mutations.	126
Figure 4.13: Effect of NaCl on tr-rFBA-plasminogen interaction	127
Figure 4.14: Effect of anti-FBA antiserum on tr-rFBA1-plasminogen interaction.	128
Figure 5.1: Amplification and ligation of <i>cbbA</i> with pCOMP-1 vector.....	150
Figure 5.2: Multiple sequence alignment of the A) DNA sequence and B) translated product of <i>cbbA</i> cloned in pFS-4 and pFS-5 and pFS-6 resulting from the desired site-directed mutagenesis.....	152
Figure 5.3: Confirmation of insertion of complementation cassette into the chromosomal DNA of MC58 Δ <i>cbbA</i>	153
Figure 5.4: Confirmation of presence of <i>cbbA</i> in the chromosomal DNA of complemented MC58 Δ <i>cbbA</i> strains.	153

Figure 5.5: Immunoblot probed with anti-FBA antibody shows the production of FBA by the complemented <i>N. meningitidis</i> $\Delta cbbA$ strains	154
Figure 5.6: Flow cytometry analysis for surface localisation of FBA.....	155
Figure 5.7: A reduction in association of MC58 $\Delta cbbA$ was observed with A) HBME cells but not with B) Detroit-562 epithelial cells	157
Figure 5.8: MC58 $\Delta cbbA$ has a reduced ability to invade A) HBME cells but not B) Detroit-562 epithelial cells	157

List of Tables

Table 1.1: Moonlighting proteins in eukaryotic organisms.....	39
Table1.2: Moonlighting proteins of bacteria.....	40
Table 2.1: Bacterial strains and plasmids used in work described in this chapter.....	58
Table 2.2: Primers used in work described in this chapter.....	59
Table 3.1: Bacterial strains used in work described in this chapter.....	92
Table 4.1: List of primers used in work described in this chapter.....	113
Table 5.1: Plasmids used in work described in this chapter.....	142
Table 5.2: Bacterial strains used in work described in this chapter.....	143
Table 5.3: List of primers used in work described in this chapter.....	146

Abbreviations

App	Adhesion and penetration protein
AspA	Autotransported serine protease A
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
AutA	Auto-transporter A
BBB	Blood brain barrier
BCIP/NBT	5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium
BglII	Restriction endonuclease enzyme from <i>Bacillus globigii</i>
BHI	Brain Heart Infusion
BM	Basal membrane
Bp	Base pair
BSA	Bovine serum albumin
CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1
Cfu	Colony forming unit
CREN	Contact regulatory element of <i>Neisseria</i>
CSF	Cerebrospinal fluid
dNTPs	De-oxy nuclotide triphosphate
DNA	Deoxy ribonucleic acid
EACA	ϵ -amino caproic acid
ECM	Endothelial Cell Medium
ECMs	Extracellular matrices
EHEC	Enterohaemorrhagic <i>Escherichia coli</i>
EIA	Enzyme-linked immunosorbant assay
EMMPRIN	Extracellular matrix metalloproteases inducer

EMP	Embden-Meyerhof- Parnas pathway
ED	Entner-Doudoroff pathway
EPEC	Enteropathogenic <i>Escherichia coli</i>
ET	Electrophoretic type
FACS	Fluorescence-activated cell sorter
FBA	Fructose bisphosphate aldolase
FCR	Flamingo cadherin receptor
FCS	Foetal Calf Serum
fHbp	Factor H binding Protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GBS	Group B Streptococcus
HBMECs	Human brain microvascular endothelial cells
His	Histidine
HRP	Horseradish peroxidase
HSPGs	Heparan sulfate proteoglycans
IMAC	Immobilised metal affinity chromatography
IPTG	Isopropyl- β -D-thiogalactopyranoside
kb	Kilo base pair
kDa	Kilo Dalton
LAP	Listeria adhesion protein
LB	Lysogeny Broth
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
MAC	Membrane attack complex
MEM	Minimum Essential Medium Eagle
MHC	Major Histocompatibility Complex

MLEE	Multilocus enzyme electrophoresis
MLST	Mult-ilocus sequence typing
MMPs	Matrix metalloproteinases
MspA	Meningococcal serine protease A
MWCO	Molecular weight cut-off
NadA	Neisseria adhesion A
NalP	Neisseria autotransporter lipoprotein
NCAM	Neural cell-adhesion molecule
NCTC	National Collection of Type Cultures
Nhha	Neisseria hia homologue
NspA	Neisserial surface protein A
OD	Optical density
OM	Outer membrane
OMP	Outer membrane protein
OMV	Outer membrane vesicle
Opa	Colony opacity factor
PAM	Plasminogen-binding group A Streptococcal M protein
PAP	Pre-activation peptide
PBS	Phosphate buffer saline
PBST	PBS with 0.05% Tween-20 (vol/vol)
PCR	Polymerase chain reaction
PE	Protein E
PP	Pentose Phosphate pathway
PGK	Phosphoglycerate kinase
Plg	Plasminogen
PorA	Porin A

PorB	Porin B
RT	Room temperature
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEN	Streptococcal enolase
SSM	Slipped strand mispairing
ST	Sequence type
<i>Taq</i>	<i>Thermus aquaticus</i>
tPA	Tissue-type plasminogen (tPA) activator
TPI	Triose phosphate isomerase
uPA	Urokinase plasminogen activator
V-ATPase	Vascular H ⁺ -ATPase
vol/vol	volume/volume
WT	Wild type
wt/vol	weight/volume

List of publications and presentations

Publication

Fariza Shams, Neil J. Oldfield, Si Kei Lai, Sarfraz A. Tunio, Karl G. Wooldridge and David P. J. Turner. Fructose-1,6-bisphosphate aldolase of *Neisseria meningitidis* binds human plasminogen via its C-terminal lysine residue. *Microbiologyopen*, 2016 Jan 5. doi: 10.1002/mbo3.331.

Poster Presentations

Fariza Shams, Neil J. Oldfield, SiKei Lai, Akhmed Aslam, Alan Berry, Karl G. Wooldridge & David P. J. Turner. Fructose 1,6-bisphosphate aldolase (FBA) of *Neisseria meningitidis* is a plasminogen-binding moonlighting protein. Presented at Society for General Microbiology Annual Conference, 13-17 April, 2014, Liverpool, UK.

Fariza Shams, Neil J. Oldfield, SiKei Lai, Akhmed Aslam, Alan Berry, Karl G. Wooldridge & David P. J. Turner. The moonlighting functions of meningococcal fructose 1,6-bisphosphate aldolase: adhesion and plasminogen binding. Presented at Nineteenth International Pathogenic *Neisseria* Conference, 12-17 October, 2014, Asheville, North Carolina, USA.

Fariza Shams, Neil J. Oldfield, SiKei Lai, Akhmed Aslam, Alan Berry, Karl G. Wooldridge & David P. J. Turner. The role of fructose-1, 6-bisphosphate aldolase (FBA) in the pathogenesis of *Neisseria meningitidis*. Presented at School of Life Sciences Postgraduate Symposium 2013, University of Nottingham, UK.

Oral Presentations

Fariza Shams, Neil J. Oldfield, SiKei Lai, Akhmed Aslam, Alan Berry, Karl G. Wooldridge & David P. J. Turner. The role of fructose-1, 6-bisphosphate aldolase (FBA) in the pathogenesis of *Neisseria meningitidis*. Presented at the 5th Annual CBS Research Symposium on 29th May 2014, University of Nottingham, UK.

Fariza Shams, Neil J. Oldfield, SiKei Lai, Akhmed Aslam, Alan Berry, Karl G. Wooldridge & David P. J. Turner. Aldolase activity is not required for the moonlighting functions of meningococcal fructose 1,6-bisphosphate aldolase. Presented at The Biological and Biomedical Consequences of Protein Moonlighting, 29—30 July 2014, London, UK.

Abstract

Neisseria meningitidis resides normally harmlessly in the human nasopharynx, but can cause fatal sepsis and meningitis worldwide.

Moonlighting proteins are a group of proteins which can perform several autonomous, unrelated functions when localised at different sites. They have been increasingly reported on the surface of both prokaryotic and eukaryotic organisms and shown to interact with a variety of host ligands and perform virulence-related functions.

Three enzymes of Embden-Meyerhof-Parnas (EMP) glycolytic pathway (an inactive pathway in *N. meningitidis*) namely enolase, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and fructose 1, 6 bisphosphate aldolase (FBA) have been shown to be localised to the meningococcal cell surface and to have non-glycolytic (moonlighting) functions related to interactions with host proteins or adhesion to host cells. This study further explores the moonlighting functions of FBA in the pathogenesis of meningococcal disease.

Recombinant wild-type FBA and FBA with mutations in the active cation-binding site (D83A and H81A/H84A) were cloned, overexpressed and purified under non-denaturing condition. A coupled enzyme assay confirmed the aldolase activity of wild-type rFBA. In contrast, rFBA with mutation(s) in the active (cation-binding) site (D83A and H81A/H84A) had no detectable enzymatic activity.

Employing flow cytometry, FBA could be detected on the surface of wild type *N. meningitidis* MC58 cells but not on MC58 Δ *cbbA* (FBA-deficient mutant). Complementation of MC58 Δ *cbbA* with an ectopic copy of *cbbA* (either wild

type or D83A and H81A/H84A variants) restored the ability to express FBA on the surface suggesting the lack of involvement of the active site in the transportation of FBA to the cell surface. Moreover, *N. meningitidis* MC58 Δ *cbbA* showed impaired adherence to human brain endothelial (HBME) cells compared with its wild type parent. Complementation of MC58 Δ *cbbA* with an ectopic copy of *cbbA* (either wild type, or D83A and H81A/H84A variants) restored the ability to adhere to HBME cells.

Furthermore, rFBA was shown to bind human plasminogen. No significant difference was observed between the plasminogen binding by wild type rFBA and rFBA lacking aldolase activity. Plasminogen binding was inhibited by the lysine analogue, ϵ -aminocaproic acid, indicating the involvement of lysine residue(s) in this interaction. A truncated rFBA comprising the C-terminal 134 amino acids and containing several lysine-rich motifs was shown to bind plasminogen as well as the wild type rFBA. Substitution of the terminal lysine residue of rFBA with alanine dramatically reduced the binding of plasminogen. Moreover, both pathogenic and non-pathogenic neisserial species were shown to bind human plasminogen.

Taken together, our data suggest that the moonlighting functions of meningococcal FBA on the bacterial surface include the binding of human plasminogen and the facilitation of optimal adhesion to host cells, and that both of these phenotypes are independent of its aldolase activity.

Chapter One: General Introduction

1.1 The genus *Neisseria*

The Genus *Neisseria* belongs to Kingdom Bacteria, Phylum Proteobacterium, Class β -Proteobacterium, Order *Neisseriales*, Family *Neisseriaceae*. Based on 16S rRNA gene sequence information the genus comprises of at least 25 species (Hung and Christodoulides, 2013). The two clinically relevant and most studied species are *Neisseria meningitidis* (the meningococcus), which is the causative agent of meningitis and sepsis and *Neisseria gonorrhoeae* (the gonococcus), the sole causative agent of gonorrhoea, a sexually transmitted infection (Rotman and Seifert, 2014). The other strains are either commensal organisms in humans and mammalian species. *Neisseria lactamica*, *Neisseria polysaccharea*, *Neisseria cinerea*, *Neisseria subflava*, *Neisseria flavescens*, *Neisseria perflava*, *Neisseria mucosa*, *Neisseria elongata*, and *Neisseria sicca* are commensal species of human (Hung and Christodoulides, 2013; Rotman and Seifert, 2014).

1.2 *N. meningitidis*

In 1887, Austrian pathologist and bacteriologist Anton Weichselbaum first isolated meningococci from the cerebrospinal fluid (CSF) of patients with “epidemic cerebrospinal meningitis” and designated the organism as *Diplococcus intracellularis meningitidis* (Weichselbaum, 1887). After the name of German physician Albert Neisser who discovered the diplococcus *N. gonorrhoeae*, the bacterium was later reclassified as a member of the genus *Neisseria* (Hung and Christodoulides, 2013).

N. meningitidis, (the meningococcus) is a Gram-negative, non-spore forming, non-motile non-acid-fast, diplococcus which appears a “kidney” or “coffee-bean” shape under the microscope. The bacterium may be encapsulated or

unencapsulated (Ala'Aldeen and Turner, 2006; Stephens *et al.*, 2007; Roupael and Stephens, 2012). *N. meningitidis* is fastidious in nature regarding its growth requirements. Smooth, convex, transparent, non-pigmented, non-haemolytic, moist, medium sized colonies with a clearly defined edge are produced on blood agar after overnight incubation. Meningococci optimally grow at 35-37°C, pH 7.0-7.4, aerobic humid environment with 5-10% CO₂ (Ala'Aldeen and Turner, 2006). Colonies are oxidase and catalase positive and able to oxidize glucose and usually maltose, but not sucrose or lactose (Roupael and Stephens, 2012).

1.3 Classification of *N. meningitidis*

Three surface structures of the bacteria, capsule, outer membrane proteins (OMP) and lipooligosaccharide (LOS) serve as basis for serological classification. Based on capsular polysaccharides, 12 meningococcal serogroups (A, B, C, E, H, I, K, L, W, X, Y and Z) have been described so far (Harrison *et al.*, 2013b). The outer membrane of meningococci contains a number of outer membrane proteins (OMPs). The OMPs PorB and PorA, which act as porins, are used to classify the meningococcal strains into serotypes and serosubtypes, respectively (Frasch and Chapman, 1972; Ala'Aldeen and Turner, 2006). An example of serological typing can be written as B:4:P1.15:L10 which denotes serogroup B serotype 4, serosubtype 15 and immunotype 10 (Ala'Aldeen and Turner, 2006).

N. meningitidis LOS can be divided into 12 immunotypes (L1-12). Immunotypes L1-L8 are primarily associated with *N. meningitidis* serogroups B and C while immunotypes L9-L12 are mainly associated with serogroup A (Zhu *et al.*, 2002). Previous studies showed that strains isolated from carriers

are mostly L1, L8 and L10 immunotypes while two-thirds of invasive strains isolated from CSF and blood belong to immunotypes L3, L7 and L9 (Scholten *et al.*, 1994; Yazdankhah and Caugant, 2004).

Amino acid substitutions in different isoforms of a selection of constitutive metabolic enzymes cause variation of electrophoretic mobilities and serve as the basis of multilocus enzyme electropherotyping (MLEE). A number score is assigned to each electrophoretic variant and each unique combination of scores is designated an electrophoretic type (ET) (Yakubu *et al.*, 1999; Ala'Aldeen and Turner, 2006). MLEE guided the discovery of clonal complexes, the identification of the disease associated hyperinvasive lineages and analysis of the global spread of hyperinvasive meningococci (Brehony *et al.*, 2007).

Multilocus sequence typing (MLST) is a generic typing scheme (Maiden *et al.*, 1998) where small fragments (433-501bp) from seven housekeeping genes (*abcZ*, *adk*, *aroE*, *fumC*, *gdh*, *pdhC*, *pgm*) are amplified and sequenced (Jolley *et al.*, 2007; Stephens, 2009). Each sequence is assigned an allele number, and the combination of alleles at each of the seven housekeeping loci is used to define each isolate's sequence type (ST) (Ala'Aldeen and Turner, 2006). MLST data are accessible via the Internet (<http://pubmlst.org/neisseria>) (Maiden *et al.*, 1998; Jolley *et al.*, 2004). MLST is a very useful tool for epidemiological studies and population diversity studies (Jolley *et al.*, 2007; Stephens, 2009).

1.4 Epidemiology

N. meningitidis colonises the human nasopharynx in 10–35% of healthy adults (Orr *et al.*, 2003; Cartwright *et al.*, 1987). In a small number of cases the bacterium crosses the nasopharyngeal epithelium and enters the bloodstream

(Jamet *et al.*, 2012). The incidence of meningococcal disease is highest in young children between 6 to 24 months old (Rosenstein *et al.*, 1999; Campagne *et al.*, 1999). Rates of meningococcal disease increase again for adolescents and young adults although people of all ages can be affected (Goldschneider *et al.*, 1969a; Peltola *et al.*, 1982). A number of factors have been implicated in the transmission of *N. meningitidis*, for example, close contact with an infected resident in military camps or university halls of residence, or during Hajj pilgrimage (Cartwright *et al.*, 1987; Cartwright, 1994; Yazdankhah and Caugant, 2004). Smoking, prior upper respiratory tract infection (e.g., mycoplasma, influenza, and other respiratory viral infections) causing damage to mucosal surfaces, and impairment of mucosal immunity, very low humidity, drying of mucosal surface, and trauma induced by dust, are predisposing factors for carriage and meningococcal disease. Meningococcal carriage has also been linked to secretion status of water soluble glycoprotein ABO blood group antigens and to ethnic background. (Moore *et al.*, 1990; Stephens *et al.*, 2007). Deficiencies in terminal complement components, asplenia, travel to epidemic areas (Zughaier *et al.*, 2004) and deficiencies in mannose binding lectin (Hibberd *et al.*, 1999) are also risk factors for invasive meningococcal disease.

N. meningitidis serogroups A, B, C, Y and W cause the majority of disease. (Ramsay *et al.*, 1997). Historically, in the first part of the 20th century, serogroup A strains (MenA) caused most meningococcal disease. MenA strains still cause epidemics in developing countries, particularly in the meningitis belt in sub-Saharan Africa. In these regions, case fatality rates reach up to 75% in children and adolescents (Hedari *et al.*, 2014). Serogroup B strains (MenB)

causes endemic disease in much of the developed world; 30%–40% of meningococcal disease in the USA and Canada and 80% in the European countries are caused by MenB. Over the past decade cases of MenB infections were reported in New Zealand, Taiwan, and Thailand(Hedari *et al.*, 2014). Serogroup C strains (MenC) are common in the developed world. They cause endemic disease (currently around 30% of disease in the USA and Europe), and localised epidemics. Introduction of vaccine against MenC fortunately has reduced its incidence (Hedari *et al.*, 2014).

In the past 20 years, serogroup W has emerged as a cause of epidemic disease during Hajj pilgrimage requiring the Hajj pilgrims to be immunised with the tetravalent meningococcal vaccines (A, C, W and Y). Infections caused by serogroup W have also been reported in Turkey, South Africa (with an outbreak from 2003–2007), Nigeria, Argentina, and, more recently, in Chile and Brazil (Lingappa *et al.*, 2000; Memish *et al.*, 2003; Borrow, 2009; Efron *et al.*, 2009) and in Burkina Faso (Koumaré *et al.*, 2007). Certain African countries such as Kenya (Materu *et al.*, 2007), Niger (Boisier *et al.*, 2007), and Ghana (Gagneux *et al.*, 2002) have recently seen small outbreaks caused by serogroup X strains. Serogroup Y strains (MenY) are emerging as an important cause of meningococcal disease in the USA, Canada and the UK over the past decade (Harrison *et al.*, 2009a). Older adults are mostly affected with MenY, causing meningococcal pneumonia, but infants less than 6 months of age can experience meningococcaemia and meningitis. Incidence of MenY disease has also risen recently in South Africa, South America, and Israel (Cohn *et al.*, 2010).

1.5 Clinical manifestations of invasive meningococcal disease

Meningococcal diseases include bacteraemia with or without sepsis, sepsis with or without meningitis, meningococcal meningitis, conjunctivitis, pneumonia, septic arthritis, pericarditis, myocarditis and endocarditis. Meningitis and sepsis are the most common clinical manifestations (Ala'Aldeen and Turner, 2006).

1.5.1 Sepsis

Acute sepsis is less common than meningitis but can become fatal. Young children are mostly affected. Clinical presentations include rapid onset of raised temperature, photophobia, convulsion, skin rash, irritability, drowsiness, diarrhoea, vomiting etc. Headache, restlessness, muscular pain and arthralgia have been noted in older children and adults. A classical sign of meningococcal sepsis is the appearance of a petechial or purpuric rash (Granier *et al.*, 1998; Thompson *et al.*, 2006). A maculopapular blanching rash is commonly present at the early stage of the disease (Marzouk *et al.*, 1991). Late signs of sepsis include confusion, delirium, hypotension and cerebral hypo-perfusion (Thompson *et al.*, 2006).

1.5.2 Meningitis

Meningitis is the most common presentation of invasive meningococcal disease which affects 30–60% of infected individuals (Nassif, 1999; Pace and Pollard, 2012). Typical symptoms of meningitis in children >5 years of age include fever, vomiting, headache, photophobia, irritability, agitation, drowsiness and neck stiffness (Wong *et al.*, 1989; Ragunathan *et al.*, 2000; Aponso and Bullen, 2001). Many cases are accompanied by a rash. Although irritability/lethargy is predominant (Ragunathan *et al.*, 2000), important clinical signs in infants and young children include neck stiffness, bulging fontanelle,

irritability and photophobia. Seizures occur in less than one third of affected children (Molyneux *et al.*, 2006). Meningococcal meningitis has a mortality of 5–18% (Pace and Pollard, 2012).

1.6 Diagnosis and treatment of meningococcal disease

Recognition of meningitis symptoms and diagnosis of meningococcal diseases are important for prompt management of invasive meningococcal infections and for prevention of secondary cases in close contacts (Cartwright, 1999). Petechial lesions are the most common characteristic of meningococemia. Other than that fever, meningeal signs, and altered mental status form the basis of clinical diagnosis of meningococcal meningitis. In children and adolescents, less specific early signs of sepsis such as leg pain, cold hands and feet and abnormal skin colour may be present but classical features of meningococcal disease develop 13-22 hours after the onset of symptoms (Thompson *et al.*, 2006).

Diagnosis by conventional techniques is based on Gram stain and culture of CSF, blood cultures or joint aspirates (Rosenstein *et al.*, 2001). Latex agglutination or chromatographic immunodetection have been used for rapid detection of *N. meningitidis* (Tunkel *et al.*, 2004). Molecular methods are advantageous over conventional techniques for decreasing the time in confirming meningococcal infection and to allow the detection of the organism in culture negative clinical samples. Polymerase chain-based methods are now widely used in the detection of bacterial DNA from CSF and whole blood samples and for genogrouping to identify serogroups of A, B, C, Y and W of *N. meningitidis* (Taha *et al.*, 2005).

Initial treatment with antibiotics must be started as soon as meningococcal disease is first suspected. Infected individuals can be treated with a third-generation cephalosporin, penicillin, or meropenem in early stages to stop proliferation of *N. meningitidis* (Tunkel *et al.*, 2004). Broad spectrum bactericidal antibiotics (such as cefotaxime or ceftriaxone) are administered empirically until the diagnosis is established (Peltola *et al.*, 1989). For individuals who come in close contact with patients, chemoprophylaxis with rifampicin, ciprofloxacin, ceftriaxone or azithromycin is recommended for eradication of nasopharyngeal carriage of the meningococcus and the prevention of further transmission (Bilukha and Rosenstein, 2005).

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

Vaccines against meningococci fall into three groups: polysaccharide vaccines, polysaccharide-protein conjugated vaccines and vaccines targeted against serogroup B strains using outer membrane proteins (Hedari *et al.*, 2014).

1.7.1 Polysaccharide vaccines

Emil C. Gotschlich and colleagues discovered and developed capsular polysaccharide as a vaccine antigen in the late 1960s (Goldschneider *et al.*, 1969b; Gotschlich *et al.*, 1969). This resulted in vaccines against serogroup A and C disease. Emergence of the W serogroup, as a potential epidemic strain in countries of the meningitis belt (Mayer *et al.*, 2002), necessitated the development of trivalent A/C/W and the increased use of tetravalent A/C/W/Y polysaccharide vaccines. In older children and adults, polysaccharide vaccines are safe and elicit bactericidal antibodies (Shao *et al.*, 2009). Plain polysaccharide vaccines have some disadvantages such as induction of low-affinity IgM and IgG2 which are poor activators of the complement system,

failure of subsequent immunisation to elicit secondary response, and hypo-responsiveness due to exhaustion of primary B cells with affinity to the polysaccharide. The lack of T-cell involvement in the immune response makes the polysaccharide a poor candidate for vaccines for young children. These vaccines were also shown to have a negligible effect on nasopharyngeal carriage and hence do not prevent transmission of the organism and do not confer herd immunity (Poolman and Borrow, 2011; Dbaibo *et al.*, 2013).

1.7.2 Conjugate vaccines

Meningococcal conjugate vaccines offer a number of advantages over the polysaccharide vaccines including strong B- and T-cell responses in children less than 2 years of age and the generation of immunologic memory in all ages (Harrison, 2006). They provide long-lasting protection and reduce nasopharyngeal colonization leading to herd immunity. Furthermore, hypo-responsiveness is not induced after repeated doses in contrast to polysaccharide vaccines (Baltimore, 2006; Nadel, 2012). Conjugate vaccines are currently available as either monovalent serogroup A or C vaccines, or tetravalent conjugate vaccines (serogroups A, C, W, and Y).

The monovalent MenA conjugate vaccine has been developed for mass vaccination of the population aged between 1 and 29 years, particularly in African countries (Hedari *et al.*, 2014). A quadrivalent conjugate vaccine MenACWY-DT containing meningococcal serogroups A, C, W and Y conjugated with diphtheria toxoid was used to vaccinate people aged 11 to 55 years (Croxtall and Dhillon, 2012). This vaccine was also shown to be safe and immunogenic in infants and toddlers (Pina *et al.*, 2012). US Food and Drug Administration (FDA) approved another quadrivalent conjugate vaccine

MenACWY-CRM197 (Menveo, Novartis Vaccine, Siena, Italy) containing a nontoxic mutant variant of *Corynebacterium diphtheriae* toxin (cross-reactive material 197; CRM197), as a carrier protein, to vaccinate people from 2 to 55 years of age. Later it was approved for use for infants and young toddlers (from the age of 2–23 months). (Stambouliau *et al.*, 2010; Cooper *et al.*, 2011). A third quadrivalent meningococcal vaccine (MenACWY-TT) with tetanus toxoid as a carrier protein, is licensed as Nimenrix (GlaxoSmithKline). This is generally well tolerated in all age groups (Croxtall and Dhillon, 2012).

1.7.3 Meningococcal serogroup B vaccines

N. meningitidis serogroup B causes about 50% of meningococcal disease cases worldwide (Granoff *et al.*, 2009). The capsular polysaccharide of meningococci serogroup B mimics a cell surface glycoprotein of human neurological tissue, which makes vaccine development against this organism a formidable challenge (Finne *et al.*, 1983; Griffiss *et al.*, 1991; Nair, 2012). Therefore outer membrane surface structures became the focus of vaccine research. Outer membrane vesicles (OMV) are proteoliposomes composed of outer membrane phospholipids, lipopolysaccharides and outer membrane and periplasmic proteins. (Kuehn and Kesty, 2005). Three different OMV vaccine formulations were developed to control regional outbreaks (Arnold *et al.*, 2011). OMV vaccines did not, however, provide broad protection, and because of this, other surface-exposed proteins have been explored as possible vaccine candidates (Caesar *et al.*, 2013).

The whole genome of a serogroup B strain (MC58) (Tettelin *et al.*, 2000) allowed the researchers to look for potential vaccine candidates using a technique called reverse vaccinology. This strategy identified factor H binding

protein (fHbp), an inhibitor of the alternative complement pathway (Madico *et al.*, 2006; Schneider *et al.*, 2006); neisserial heparin binding antigen (NHBA), involved in serum resistance (Serruto *et al.*, 2010); and the neisserial adhesin A (NadA), which is important for bacterial–host cell interactions (Comanducci *et al.*, 2002; Capecchi *et al.*, 2005).

A four-component MenB (4CMenB) vaccine consisting of NHBA (GNA2132) fused with GNA1030, fHbp (GNA 1870) fused with GNA2091, along with NadA (GNA1994) and outer membrane vesicles from the strain used for the New Zealand OMV vaccine (containing PorA 1.4) was formulated (Giuliani *et al.*, 2006). A robust immune response was induced against reference MenB strains containing the vaccine antigens by 4CMenB (commercial name Bexsero; Novartis Vaccines and Diagnostics, Siena, Italy) (Seib *et al.*, 2012). Bexsero is highly immunogenic in infants (from 2 months of age), toddlers, adolescents and adults. It is safe and well tolerated and can be co-administered with other routine vaccines (Vernikos and Medini, 2014).

1.8 Virulence factors of *N. meningitidis*

N. meningitidis expresses a range of virulence factors including capsular polysaccharide, lipopolysaccharide and a number of adhesive proteins located on the cell surface (Fig.1.1). To escape the host immune mechanisms, many of these structures undergo phase or antigenic variation. This can occur via a number of mechanisms including intragenomic recombination (Segal *et al.*, 1986; Stern *et al.*, 1986), slipped strand mispairing (SSM) and the reversible insertion of mobile elements (Segal *et al.*, 1986; Stern *et al.*, 1986; Frosch and Vogel, 2006; van der Woude and Baumler, 2004). Genetic islands present in the *N. meningitidis* genome contain genes that diverge in GC content and

codon usage from other surrounding sequences, suggesting they have been acquired from other mucosal bacteria by horizontal exchange (Tettelin *et al.*, 2000; Perrin *et al.*, 2002).

1.8.1 Capsule

N. meningitidis has a polysaccharide capsule which is the main virulence determinant of this bacterium. The hydrated nature of capsule protects meningococci during airborne transmission. Capsulated meningococci may lose the capsule by various genetic mechanisms while inside the respiratory tract (Hammerschmidt *et al.*, 1996; Deghmane *et al.*, 2002) but become encapsulated again during dissemination to new hosts. One of these mechanisms is the induction of *crgA*, which upon contact with target cells, is upregulated and produces CrgA, to transcriptionally regulate several genes, including genes involved in capsule biosynthesis (Deghmane *et al.*, 2002).

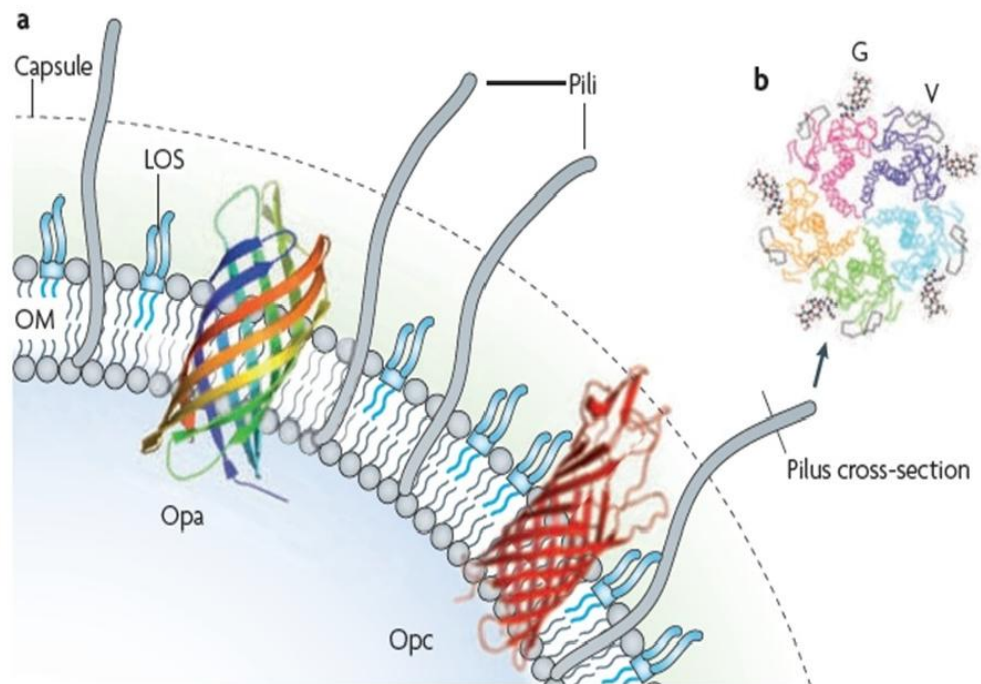


Figure 1.1: Prominent outer-membrane components of *N. meningitidis* that influence bacterial interactions with host cells. a) Pili traverse the capsule and are the most prominent adhesins of encapsulated *N. meningitidis*. In addition, the integral outer membrane (OM) adhesins, Opa and Opc, are known to mediate interactions with specific host-cell receptors in appropriate phenotypes. Lipooligosaccharide may interfere with the adhesion functions of OM proteins, but can also contribute to cellular interactions by interacting with various cellular receptors. b) A cross-section of a pilus fibre showing that variable domains (V) and glycans (G) as well as other substitutions (not shown) are located externally, whereas the constant domains are buried within the fibre, protected from the host environment (Modified from Virji, 2009).

The capsule of serogroup A is composed of (α 1 $\rightarrow$ 6)-linked *N*-acetylmannosamine-1-phosphate, while serogroup X expresses (α 1 $\rightarrow$ 4) linked *N*-acetyl-d-glucosamine 1-phosphate. Capsules containing sialic acid are expressed by serogroups B, C, W and Y. Serogroups B and C are homopolymers of *N*-acetylneuraminic acid in (α 2 $\rightarrow$ 8) or (α 2 $\rightarrow$ 9) linkage, respectively (Tzeng *et al.*, 2005). Serogroups W and Y express capsules composed of alternating disaccharide repeat units of sialic acid and D-galactose (W) or D-glucose (Y), respectively (Stephens, 2009).

Capsular polysaccharide (CPS) plays a crucial role in invasive meningococcal disease. Evasion of complement-mediated and phagocytic killing can be attributed to the capsule. (Stephens, 2009). Sialic acids help the bacteria to evade immune recognition by molecular mimicry. The most noted example is the (α 2 $\rightarrow$ 8) linked sialic acid homopolymer present in serogroup B capsule which is structurally identical to glycosyl residues of a component of human neural cell-adhesion molecule (NCAM), present in the central and peripheral nervous systems (Diaz Romero and Outschoorn, 1994).

A number of genetic events including SSM resulting in premature termination of translation of *siaD* can lead to on/off expression of capsule. Integration and excision of an insertion sequence element (IS1301) causes disruption of the sialic acid biosynthesis gene *siaA* (Frosch and Vogel, 2006). The genes involved in sialic acid biosynthesis for serogroups B, C, W and Y are similar; a fact which facilitates horizontal gene exchange and capsule switching between these serogroups (Swartley *et al.*, 1997).

1.8.2 Lipooligosaccharide (LOS)

The lipooligosaccharide (LOS) or endotoxin in *N. meningitidis* consists of an inner and outer core oligosaccharide attached to lipid A. The lack of a repeating polysaccharide O-side chain, commonly found in lipopolysaccharide (LPS) of most Gram-negative bacteria, distinguishes LOS from LPS (Kahler and Stephens, 1998).

The presence of sialic acids on the LOS makes *N. meningitidis* more resistant to phagocytosis and killing mediated by antibody and complement (Edwards and Apicella, 2004; Schneider *et al.*, 2007; van Putten and Robertson, 1995; Parsons *et al.*, 1988). LOS elicits inflammation during meningococcal sepsis (Brandtzaeg *et al.*, 2001). LOS also makes meningococci resistant to cationic antimicrobial peptides (CAMPs) produced by macrophages, neutrophils and mucosal epithelial cells (Brandtzaeg *et al.*, 2001; Stephens, 2009).

1.8.3 Pili

Neisserial pili are hair-like projections, flexible and helical, homopolymeric fibres, 6 nm in diameter and several microns in length and belong to the type IV pilus family (Tfp) (Hung and Christodoulides, 2013). Fifteen proteins (including PilC1/C2, PilD, PilE, PilF, PilG, PilH, PilI, PilJ, PilK, PilM, PilN, PilO, PilP, PilQ and PilW) are essential for Tfp biogenesis which consists of four steps: assembly; functional maturation; counter-retraction; and emergence onto the surface (Carbonnelle *et al.*, 2006). Seven additional proteins (ComP, PilT, PilT2, PilU, PilV, PilX and PilZ) are involved in Tfp function (Brown *et al.*, 2010).

The initial interaction between respiratory epithelial cells and *N. meningitidis* is mediated by Tfp which make contact with non-ciliated cells but not with

ciliated respiratory cells (Pinner *et al.*, 1991; Hardy *et al.*, 2000). Pili are thought to be the primary determinants of specificity for human epithelial and endothelial cells and are also known to mediate adhesion to the meninges (Virji *et al.*, 1993b; Hardy *et al.*, 2000; Kirchner and Meyer, 2005). PilQ has been shown to act as adhesin and targets laminin receptor (Orihuela *et al.*, 2009).

Recently, ComP, was reported to contribute in DNA uptake during transformation by binding to the DNA uptake sequence (DUS) (Cehovin *et al.*, 2013). Uptake of DNA from the environment facilitates natural competency of meningococci and maintains genetic diversity (Fussenegger *et al.*, 1997).

The rapid assembly and disassembly or retraction of Tfp, facilitated by PilC and the ATPase, PilT, result in ‘twitching motility’ (Maier *et al.*, 2002). PilG and PilX proteins play a role in pilus retraction and bacterial aggregation, respectively. (Helaine *et al.*, 2005; Carbonnelle *et al.*, 2006). Pilus retraction is responsible for numerous signal transduction events, including the formation of cortical plaque structures and the shedding of the complement regulatory factor CD46 (Maier *et al.*, 2002; Winther-Larsen *et al.*, 2005).

Neisserial pilin proteins can undergo several distinct and unusual post-translational modifications. Modifications to serine residues in PilE, including glycosylation at position 63 and addition of α -glycerophosphate at position 93 and replacement at position 68 by phosphate, phosphoethanolamine (PEA) or phosphorylcholine (ChoP) have been reported (Virji, 1997; Weiser *et al.*, 1998; Hegge *et al.*, 2004). It has been suggested that glycosylation could be involved in immune evasion and bacterial spread (Marceau and Nassif, 1999).

Binding of the pili and host cells may involve CD46, a transmembrane glycoprotein (Kallstrom *et al.*, 2001). Recently, CD147 (also known as extracellular matrix metalloproteases inducer or EMMPRIN), an immunoglobulin superfamily member, has been reported as a host receptor of major and minor pilins (PilE and PilV). This interaction plays a vital role in adhesion of meningococci to human endothelial cells and colonisation of blood vessels (Bernard *et al.*, 2014).

1.8.4 Opacity proteins

Opa and Opc are the most abundant outer membrane proteins of *N. meningitidis*. The term opacity was coined because expression of these proteins led to opaque colonies (Swanson, 1978; Virji *et al.*, 1992b).

Structurally Opa proteins (24–35 kDa), are composed of eight-stranded transmembrane beta-barrel structures with four surface-exposed loops. The beta barrel structures are highly conserved, the first loop (proximal to the N-terminus) is semi-variable, loops 2 and 3 are hypervariable (HV1 and HV2) and the fourth loop has no variation (Malorny *et al.*, 1998; Virji, 2009). A single meningococcal strain can harbour 3–4 *opa* genes (*opaA*, *opaB*, *opaD* and *opaJ*), which exhibit phase and antigenic variation. The Opa-encoding genes undergo high frequency phase variation (Belland *et al.*, 1989). Antigenic variation between Opa proteins can result from the shift of expression from one *opa* gene to the next, or by a variety of genetic events, for example point mutation, deletion, translocation and gene transfer from other members of the *Neisseriaceae* (Hill *et al.*, 2010).

Opc protein (24–35 kDa), has a 10-stranded β -barrel structure with five surface exposed loops (Prince *et al.*, 2002; Virji, 2009). Opc protein is encoded by a

single gene, *opcA*. Transcriptional regulation of a homopolymeric, variable-length and polycytidine (poly-C) tract, located in the promoter region of the *opc* gene, is responsible for phase variation in Opc protein (Sarkari *et al.*, 1994).

Heparan sulfate proteoglycans (HSPGs) and sialic acids are the common targets for both Opa and Opc (Moore *et al.*, 2005; Griffiths *et al.*, 2007). The human receptors of Opa proteins are members of the carcinoembryonic antigen-related cell-adhesion molecule (CEACAM) family. For example, CEACAM1, found on epithelial and endothelial cells, and cells of the immune system, CEA (epithelial cells), CEACAM3 (neutrophils), and CEACAM6 (epithelial cells and granulocytes) (Virji *et al.*, 1996b; Virji *et al.*, 1996a; Virji *et al.*, 1999; Hammarstrom, 1999). Opa–CEACAM interactions result in cellular invasion (Virji *et al.*, 1999; Griffiths *et al.*, 2007).

Opc is also able to mediate adhesion to and invasion of epithelial cells in the absence of serum proteins by binding to HSPGs (de Vries *et al.*, 1998). Meningococcal binding to endothelial $\alpha V\beta 3$ -integrin (the vitronectin receptor) and $\alpha 5\beta 1$ - integrin (the fibronectin receptor) is the result of interactions of Opc with vitronectin and fibronectin (Virji *et al.*, 1994; Virji *et al.*, 1995; Unkmeir *et al.*, 2002). While inside cells, *N. meningitidis* could escape the phagocytic vacuole leading to the possibility of Opc binding to the intracellular protein, alpha-actinin (Cunha *et al.*, 2009).

1.8.5 Factor H binding Protein (fHbp)

Factor H-binding protein (fHbp), previously referred to as genome-derived neisserial antigen 1870 (GNA1870) or lipoprotein 2086 (Pizza *et al.*, 2000; Maignani *et al.*, 2003; Fletcher *et al.*, 2004), is a 27-kDa lipoprotein present

on the surface of all strains of *N. meningitidis* (Masignani *et al.*, 2003; Fletcher *et al.*, 2004; Beernink *et al.*, 2007). Due to sequence variability, fHbp can be divided into two subfamilies (A and B) or three variant groups (1, 2 and 3). Subfamily B corresponds to variant group 1 (v1) whilst variant groups 2 and 3 (v2 and v3) are included in subfamily A (Masignani *et al.*, 2003; Fletcher *et al.*, 2004).

Factor H binding protein binds human factor H, which leads to down-regulation of the complement pathway. Also, bactericidal antibodies elicited by fHbp activate the classical complement pathway (Welsch *et al.*, 2008). Therefore, binding of factor H (fH) to the bacterial surface may help the bacteria to escape innate immune responses by inhibiting complement-mediated lysis in human plasma (Madico *et al.*, 2006). According to a recent report fHbp is able to bind siderophores, which have strong affinity for ferric iron. This suggests another important role of fHbp in iron uptake, which plays an important role in survival of bacteria in the human body (Veggi *et al.*, 2012).

1.8.6 Iron acquisition system

Pathogenic *Neisseria* species express surface receptors to extract and import iron from human iron binding proteins including haemoglobin, lactoferrin and transferrin (Cornelissen and Hollander, 2011).

The haemoglobin receptor HmbR specifically binds haemoglobin whilst haptoglobin–haemoglobin receptors (HpuAB) can bind both haemoglobin and haptoglobin–haemoglobin complexes (Stojiljkovic *et al.*, 1995; Lewis *et al.*, 1999). Meningococcal disease isolates overexpress HmbR and both receptors are present in high numbers in invasive isolates (Harrison *et al.*, 2009b;

Tauseef *et al.*, 2011) suggesting the possible involvement of haemoglobin receptors in invasion and dissemination of meningococci in the vascular system (Harrison *et al.*, 2013a).

Transferrin can be found in serum and on inflamed mucosal surfaces (Noinaj *et al.*, 2012). The transferrin–iron import system consists of two transferrin binding proteins: a TonB-dependent transporter (TbpA), and a lipoprotein co-receptor (TbpB). Both proteins work in concert to bind transferrin and then extract and import the iron across the outer membrane (Ronpirin *et al.*, 2001). TbpA is highly conserved among strains (Cornelissen *et al.*, 2000) whereas extensive variation in sequence and antigenic properties have been reported for TbpB (Cornelissen *et al.*, 1997) but neither protein undergoes phase or antigenic variation. Anti-Tbp antibody with opsonic activity can be detected in sera from carriers and in convalescent sera (Ala'Aldeen *et al.*, 1994).

Lactoferrin is found in secretions and in polymorphonuclear leucocytes (Noinaj *et al.*, 2012). The lactoferrin receptor is composed of two proteins, lactoferrin-binding protein A (LbpA) and lactoferrin-binding protein B (LbpB) (Prinz *et al.*, 1999). LbpA is an outer membrane iron transporter protein whereas LbpB is a lipoprotein exposed on the cell surface. In a recent study, LbpB has been shown to be released into the culture medium in a process mediated by the autotransporter protein, NalP. Released LbpB was responsible for the reduction in complement-mediated killing of bacteria in the presence of LbpB-specific bactericidal antiserum (Roussel-Jazédé *et al.*, 2010).

1.8.7 Secretion systems in *N. meningitidis* and autotransporter proteins

To allow the secretion of proteins in Gram-negative bacteria, proteins must transverse through three compartments: the inner membrane, the periplasm and the outer membrane (Lory, 1992). Secretion machineries in Gram-negative bacteria can be classified into two categories: those spanning both the inner and outer membrane and those that span the outer membrane only. Included in the first category are type 1, 2, 3, 4 and 6 secretion systems (T1SS, T2SS, T3SS, T4SS and T6SS). The type 5a and 5b secretion systems (T5aSS and T5bSS) are included in the second category (Gerlach and Hensel, 2007). Using a one-step mechanism, substrates from the bacterial cytoplasm are transported directly into the extracellular space or into a target cell in T1SS, T3SS, T4SS and T6SS. A two-step secretion mechanism is utilised by the T2SS and T5SS. In this mechanism substrates are first translocated into the periplasmic space by the SecYEG or twin-arginine translocation (Tat) system and are subsequently either transferred to the outer membrane or secreted into the extracellular space (Gerlach and Hensel, 2007).

Three secretion pathways have been described in *N. meningitidis*. These are the T1SS and the T5SS (comprising the autotransporter pathway and the two-partner secretion pathways) (Van Ulsen and Tommassen, 2006).

1.8.7.1 Type I secretion system

The T1SS is composed of three proteins: a channel-forming outer membrane protein, an inner membrane-embedded ABC transporter, and a large periplasmic domain-containing membrane-fusion protein (MFP) protein anchored in the inner membrane. Proteins synthesised in the cytoplasm are

secreted directly into the extracellular environment in a single step (Young and Holland, 1999; Delepelaire, 2004).

Many proteins, secreted by T1SS, are members of the repeat-in-toxin (RTX) family. The presence of 9 to 41 repeats of a glycine-rich motif (GGXGXD) and a secretion signal within the C-terminal domain that is essential for protein secretion are characteristic features of RTX family proteins. (Van Ulsen and Tommassen, 2006). In *E. coli*, the T1SS components are HlyB (ATP binding), HlyD (membrane fusion), and TolC (outer membrane channel) proteins. Meningococcal homologs are organised unusually because the *hlyD* and *tolC* genes are unlinked to *hlyB* and are expressed independently. Nevertheless, these genes encode components of a functional T1SS required for secretion of FrpC (Wooldridge *et al.*, 2005): a member of a family of iron-regulated secreted proteins with homology to the RTX family of toxins (Thompson *et al.*, 1993a; Thompson *et al.*, 1993b; Thompson *et al.*, 1993c).

1.8.7.2 Two-partner secretion system (T5bSS)

The two-partner secretion (TPS) system employs a two-step mechanism to secrete a family of very large proteins (Jacob-Dubuisson *et al.*, 2001; Jacob-Dubuisson *et al.*, 2004). Genes, generically designated as *tpsA*, encode TpsA, secreted proteins with an N-terminal signal sequence for transportation across the inner membrane via the Sec machinery (Chevalier *et al.*, 2004) and *tpsB* encodes an outer membrane protein required to transport those proteins across the outer membrane (Jacob-Dubuisson *et al.*, 2001; Jacob-Dubuisson *et al.*, 2004).

At least five TpsA- and two TpsB-encoding genes (van Ulsen *et al.*, 2008) have been detected in the genome of *N. meningitidis*. (Schmitt *et al.*, 2007).

Due to homology with filamentous haemagglutinin (FHA) of *Bordetella pertussis*, TpsA and TpsB homologues in meningococci are designated as haemagglutinin/haemolysin-related proteins HrpA and HrpB, respectively (Schmitt *et al.*, 2007). Meningococci possess a functional HrpB–HrpA TPS system, which contributes to adhesion of un-encapsulated bacteria to epithelial cells (Schmitt *et al.*, 2007) and is essential for intracellular survival of meningococci (Tala *et al.*, 2008). This system also helps the bacteria to escape from infected cells possibly because the secreted HrpA proteins directly act as lysins or facilitate the action of bacterial phospholipases on the phagosomal membrane (Tala *et al.*, 2008).

1.8.7.3 Autotransporter proteins (T5aSS)

Autotransporter (or T5SS) proteins constitute an important class of virulence factors in *N. meningitidis*. Eight autotransporter proteins have been identified in *N. meningitidis*: IgA1 protease; NhhA; AutA; AutB; NadA; App; NalP (also known as AspA); and MspA (Turner *et al.*, 2009). Some structural features are shared by all autotransporter proteins such as N-terminal signal sequence to direct the transport of the protein across the inner membrane, a passenger domain, and a C terminal translocator domain assisting the secretion of the passenger domain across the outer membrane (Grijpstra *et al.*, 2013).

Six autotransporters, *i.e.* MspA, App, IgA protease, NalP, NhhA and NadA are expressed in *N. meningitidis* strain MC58 (Serruto *et al.*, 2003; Van Ulsen *et al.*, 2003; van Ulsen *et al.*, 2006; Capecchi *et al.*, 2005; Vidarsson *et al.*, 2005; Scarselli *et al.*, 2006; Van Ulsen and Tommassen, 2006). The former four proteins, which have proteolytic activities, constitute classical monomeric

autotransporters that are released into the extracellular environment, while trimeric autotransporters NhhA and NadA remain attached to the cell surface (Linke *et al.*, 2006).

Adhesion and penetration protein (App)

Adhesion and penetration protein (App) is a 160-kDa protein and is highly homologous to *Haemophilus* adhesion and penetration protein (Hap) in *Haemophilus influenzae* (Hadi *et al.*, 2001). App stimulates B-cells and T-cells and induces a bactericidal antibody response (Hadi *et al.*, 2001; van Ulsen *et al.*, 2001). Serruto *et al.* reported that App is able to mediate bacterial adhesion to human epithelial cells. It may have a role in detachment and dispersal of meningococci (Serruto *et al.*, 2003).

Meningococcal serine protease A (MspA)

Meningococcal Serine Protease A (MspA), also known as AusI (autotransporter serine protease I) (Turner *et al.*, 2006), is encoded by a phase-variable gene with high prevalence in invasive strains (van Ulsen *et al.*, 2006; Oldfield *et al.*, 2013). MspA can induce a bactericidal antibody response to a limited number of meningococcal strains (Turner *et al.*, 2006). *E.coli* expressing recombinant MspA were shown to have increased adherence to both human bronchial epithelial cells and human brain microvascular endothelial cells (HBMECs) suggesting a possible role in adherence and colonisation (Turner *et al.*, 2006). MspA expression increased significantly in an ex-vivo model of human whole blood infection indicating an important role for MspA during invasive disease (Oldfield *et al.*, 2013). Using a human CD46 transgenic mouse model, a recent report proved both App and MspA are virulence factors for meningococci (Khairalla *et al.*, 2015)._Also, the recombinant passenger

domains of these two proteins were shown to be internalised and partially localised in nuclei of human monocyte-derived dendritic cells and HBMECs. Moreover, both App and MspA bound histone proteins, cleaved histone H3 and induced cell death by caspase-dependent apoptosis (Khairalla *et al.*, 2015).

Autotransported serine protease A (AspA)/*Neisseria* autotransporter lipoprotein (NalP)

AspA has significant N-terminal homology to the autotransported serine proteases (Ssp-H1 and Ssp-H2) in *Serratia marcescens*. AspA contains a serine protease catalytic triad exhibiting autocatalytic activity. Although *aspA* is a phase-variable gene, the amino acid sequence of AspA is well-conserved in serogroup A, B, and C meningococci (Turner *et al.*, 2002). Van Ulssen *et al.* later re-designated AspA as NalP because of the presence of a lipidated cysteine residue at the N-terminal of the mature protein and a lipobox at the C-terminal end of the signal sequence (Van Ulsen *et al.*, 2003). Lipobox, [LVI][ASTVI][GAS]C, is a conserved sequence at the C terminal region of the signal peptides of lipoproteins in both Gram-negative and Gram-positive bacteria (Babu *et al.*, 2006). The presence of a lipobox at the C-terminal end of the signal sequence delays the autocatalytic release of the protein from the cell surface (Van Ulsen *et al.*, 2003). Proteolytic processing of autotransporters such as App, IgA1protease (Turner *et al.*, 2002; Van Ulsen *et al.*, 2003) and other surface proteins including LbpB (Roussel-Jazédé *et al.*, 2010) and NhbA (Serruto *et al.*, 2010) are mediated by NalP. Human whole blood mediated killing of a *nalP* knockout mutant suggested that NalP might be directly or indirectly involved in the survival of the meningococcus in blood (Echenique-Rivera *et al.*, 2011). DNA-dependent biofilm formation by meningococci has been reported to be affected by NalP activity. NalP-mediated proteolytic

cleavage releases DNA-binding fragments of NhbA and IgA1 protease, leading to a reduction in biofilm formation (Arenas *et al.*, 2013).

Auto-transporter A (AutA and Aut B)

AutA and AutB were first described by Peak and colleagues (Peak *et al.*, 1999) and Ait-Tahar and colleagues (Ait-Tahar *et al.*, 2000). According to a recent report, an auto-chaperone domain is present in the passenger domain (Arenas *et al.*, 2015). AutA was reported to be a potent CD4⁺ T-cell and B-cell stimulating antigen (Ait-Tahar *et al.*, 2000). AutA is expressed by a limited number of invasive meningococcal strains and also by commensal species, and it has been implicated in nasopharyngeal colonisation. AutA remains at the bacterial cell surface where it mediates autoaggregation which enhances microcolony formation and significantly reduces biofilm biomass (Arenas *et al.*, 2015).

IgA protease

IgA protease of *N. gonorrhoeae* was the first autotransporter protein to be described (Pohlner *et al.*, 1987). A T-cell response is elicited by the α -peptide of IgA protease which activates the immune system (Jose *et al.*, 2000). The DNA binding ability of both NhbA and the α -peptide of IgA protease contribute to biofilm formation by *N. meningitidis*. (Arenas *et al.*, 2013). Binding of heparin has recently been reported as a novel function of the α -peptide. Heparin, an immunomodulatory molecule found in the extracellular matrix, binds vitronectin and allows uptake of the bacteria into host cells expressing vitronectin receptors (Roussel-Jazede *et al.*, 2014).

***Neisseria* Adhesin A (NadA)**

NadA, a surface-exposed protein, is structurally similar to the ubiquitous surface protein A2 (UspA2) from *Moraxella catarrhalis* and of YadA from *Yersinia enterocolitica* (van Ulsen *et al.*, 2001). NadA is a trimeric autotransporter (Linke *et al.*, 2006). NadA is one of the antigenic proteins included in the formulation of 4CMenB, a multicomponent vaccine against *N. meningitidis* serogroup B (Su and Snape, 2011). Three variants, named NadA-1, NadA-2, and NadA-3, were originally identified in pathogenic strains (Comanducci *et al.*, 2002). NadA is expressed during invasive meningococcal disease and convalescent sera from children contain antibodies against this antigen (Litt *et al.*, 2004). According to recent studies, NadA interacts with and stimulates immune cells during infection, binds human chaperone Hsp90 and directly interacts with human β -1 integrin (Nägele *et al.*, 2011).

NhhA (*Neisseria* hia/hsf homologue)/ Msf (Meningococcal surface fibril)

Initially, in *N. meningitidis* strain MC58, NhhA (*Neisseria* hia homologue) was identified as a homologue of the adhesin AIDA-I of *E. coli* (Peak *et al.*, 2000). To describe the predicted fibrillar structure of this protein and to distinguish between the meningococcal protein from its homologue in *H. Influenzae* (*H. influenzae* surface fibril, Hsf and *H. influenzae* adhesin, Hia), Griffiths and colleagues used the term meningococcal surface fibril or Msf (Griffiths *et al.*, 2011). Msf belongs to the trimeric autotransporter family of proteins, and is encoded by *nhhA* (St Geme *et al.*, 1996). Reverse vaccinology also identified this protein (also called GNA0992) as a surface-exposed antigen able to induce a bactericidal antibody response (Pizza *et al.*, 2000). Also, the presence of anti-Msf antibodies in convalescent sera suggests its potential as a vaccine antigen

(Peak *et al.*, 2000; Litt *et al.*, 2004; Weynants *et al.*, 2007). NhhA mediates bacterial attachment to cells *in vitro*, which is cell type dependent (Scarselli *et al.*, 2006). Purified NhhA bound to the extracellular matrix components like laminin and heparan sulfate (Scarselli *et al.*, 2006). It inhibits membrane attack complex or MAC deposition on meningococci (Sjölander *et al.*, 2008). This is mediated by binding vitronectin (Griffiths *et al.*, 2011).

1.9 Pathogenesis of *N. meningitidis*

From initial nasopharyngeal colonisation, *N. meningitidis* progresses through several stages to cause sepsis or meningitis (Fig. 1.2). Although researchers have been able to reveal many secrets behind the pathogenesis of this organism, a whole range of virulence factors with roles in pathogenesis are still being discovered.

1.9.1 Interaction of *N. meningitidis* with nasopharyngeal epithelial cells

Initial adhesion is a crucial step in order for the bacteria to colonise the nasopharyngeal epithelial cells and also to survive the flushing action of mucus. Type IV pili are the primary mediators of binding to non-ciliated epithelial cells (Virji *et al.*, 1992a; Proft and Baker, 2009) using CD46 (Kallstrom *et al.*, 1997), although CD46-independent pilus-dependant adhesion has been reported (Kirchner *et al.*, 2005). Non-encapsulated phenotypes arising at this stage can engage more intimately with cells by means of LOS (Albiger *et al.*, 2003), and the outer membrane opacity proteins Opa and Opc (Virji *et al.*, 1993a; Virji *et al.*, 1994; Moore *et al.*, 2005). Opa interacts with carcinoembryonic antigen cell adhesion molecules CEACAM, high expression of which may be induced under certain circumstances (for example, inflammation provoked by preceding viral infection). This interaction may help

the meningococci to invade the epithelial barrier and enter the blood (Griffiths *et al.*, 2007; Rowe *et al.*, 2007). Through up regulation of endoglin (CD105) and with the help of β 1 integrins, Opa–CEACAM1 interactions open a way to overcome infected epithelial cell shedding mechanisms thus supporting epithelial cell attachment (Muenzner *et al.*, 2005). *Neisseria* Hia/Hsf homologue (NhhA) is also involved in colonisation and survival of meningococci in the bloodstream (Sjölinder *et al.*, 2008), the meningococcal HrpA–HrpB two-partner secretion system (TPS) aids in the adhesion of unencapsulated bacteria (Schmitt *et al.*, 2007).

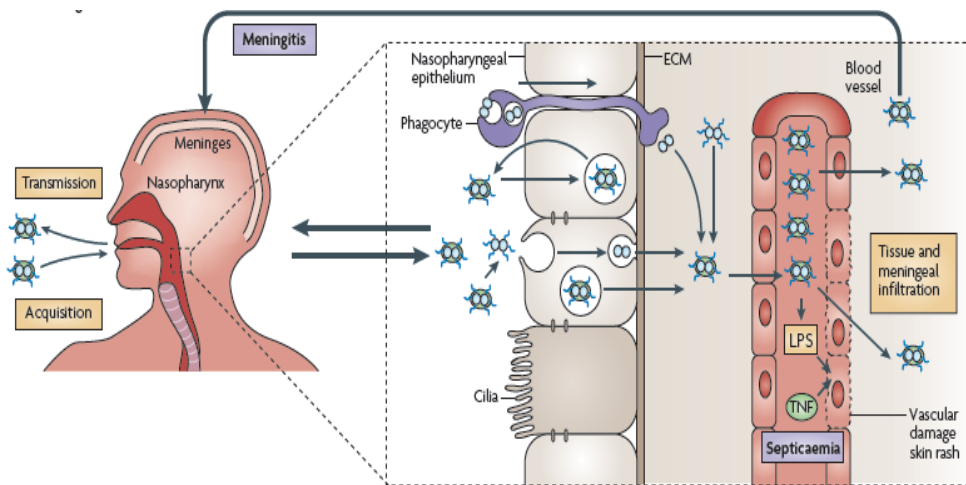


Figure 1.2: Stages in the pathogenesis of *N. meningitidis*. *N. meningitidis* may be acquired through the inhalation of respiratory droplets. The organism establishes intimate contact with non-ciliated mucosal epithelial cells of the upper respiratory tract, where it may enter the cells briefly before migrating back to the apical surfaces of the cells for transmission to a new host. Asymptomatic carriage is common in healthy adults in which bacteria that enter the body by crossing the epithelial barrier are eliminated. Besides transcytosis, *N. meningitidis* can cross the epithelium either directly following damage to the monolayer integrity or through phagocytes in a 'Trojan horse' manner. In susceptible individuals, once inside the blood, *N. meningitidis* may survive, multiply rapidly and disseminate throughout the body. Meningococcal passage across the brain vascular endothelium (or the epithelium of the choroid plexus) may then occur, resulting in infection of the meninges and the CSF (Virji, 2009).

N. meningitidis interacts with epithelial cells in a two-step process (Pujol *et al.*, 1997; Pujol *et al.*, 1999). After initial adhesion with the host cells, the bacteria form microcolonies on the surface of host cells via auto-agglutination caused by type IV pili (Trivedi *et al.*, 2011). This step has been referred to as 'localised adhesion'. The next step is diffuse adhesion where the bacteria down regulate expression of pili and spread on the apical surface to cover the cells as a monolayer (Eugene *et al.*, 2002). Pilus mediated adhesion also results in the formation of cellular protrusions within and surrounding these microcolonies (Eugene *et al.*, 2002; Nassif *et al.*, 2002). The minor pilin protein, PilV, is important for formation of this microvilli-like structure (Mikaty *et al.*, 2009). The actin-rich cell protrusions formed by epithelial cells are unable to protect the microcolonies from shear stresses. As a result, bacteria adhered to epithelial cells can spread *in vivo* to a new niche in the same host or between individuals (Lécuyer *et al.*, 2012). Chamot-Rooke *et al.* (Chamot-Rooke *et al.*, 2011) described that PptB (pilin phosphotransferase B) which was previously referred as one of the 16 genes regulated by CREN (contact regulatory element of *Neisseria*) (Morelle *et al.*, 2003; Deghmane *et al.*, 2000; Jamet *et al.*, 2009) and whose transcription increases after adhesion to epithelial cells (Morelle *et al.*, 2003), is responsible for addition of phosphoglycerol to the major pilin subunit PilE at Ser93 (Chamot-Rooke *et al.*, 2011). This unusual modification interferes with the formation of bundles of pili and aggregation of bacteria leading to detachment of some bacterial cells from the microcolonies. (Chamot-Rooke *et al.*, 2011).

Besides formation of cellular projections, PilV recruits cholesterol and thus is involved in the formation of the cortical plaque, a structure that resembles a

honeycomb. This cortical plaque, formed underneath the microcolonies, contains host proteins like actin, ezrin, ICAM 1, CD44, CD46 and Erb2 (Mikaty *et al.*, 2009; Merz *et al.*, 1999; Kallstrom *et al.*, 1997; Eugene *et al.*, 2002; Hoffmann *et al.*, 2001). After adhesion, *N. meningitidis* can be internalised (Pujol *et al.*, 1997) and is able to survive and multiply within epithelial cells (Spinosa *et al.*, 2007). Meningococci can also cross via transcellular passage through the epithelium, but for that transport route an intact microtubule network is needed and intercellular junctions are not disrupted (Sutherland *et al.*, 2010).

Biofilm formation by *N. meningitidis* has recently been studied in regards to pathogenesis. Encapsulated strains commonly found in carriage (Maiden *et al.*, 2008) form efficient biofilm on bronchial epithelium monolayers (Neil *et al.*, 2009), and the involvement of PilX, HrpA and HrpB in this process has been reported (Lappann *et al.*, 2006; Neil and Apicella, 2009). However the relevance of biofilm formation in the colonisation and pathogenesis of meningococcal infection needs further confirmation (Trivedi *et al.*, 2011). In addition nasopharyngeal colonisation has been strongly correlated with the generation of propionic acid by *Porphyromonas* inhabiting the same niche (human adult upper respiratory tract) by the fact that *N. meningitidis* has the ability to metabolise propionic acid due to presence of the *prp* gene cluster that encodes enzymes of methylcitrate cycle (Catenazzi *et al.*, 2014).

1.9.2 Interaction of *N. meningitidis* with endothelial cells and invasion of brain endothelium

Occasionally, hyper virulent strains of *N. meningitidis* translocate from the nasopharynx and reach the blood stream. Within the blood stream, the LOS, especially the lipid A moiety, induces the release of TNF- α , IL-6, chemokines,

reactive oxygen species (ROS) and nitric oxide (NO) (Chow *et al.*, 1999; Brandtzaeg *et al.*, 2001). As a result, the complement and the coagulation cascades are activated, leading to damage of endothelium (Brandtzaeg *et al.*, 1992). To promote survival, the meningococcus recruits negative regulators of complement onto the bacterial surface, for example, Factor H is recruited by factor H-binding protein (fHbp) and PorA binds C4-binding protein (Jarva *et al.*, 2005). Neisserial surface protein A (NspA), is able to bind human factor H (fH) (Lewis *et al.*, 2010). Also Neisserial heparin binding antigen (NHBA) can help meningococci to escape complement-mediated lysis (Serruto *et al.*, 2010). Opc can bind directly to vitronectin and makes the bacteria resistant to serum-mediated killing (Virji and Griffiths, 2008). Further, *N. meningitidis* secretes blebs containing outer membrane components and lipopolysaccharide, which assist in escaping the host defensive mechanisms, including the complement system (Schneider *et al.*, 2007).

Type IV pili enable the bacteria to preferentially adhere to vascular capillaries during a transient period of low shear stress (Mairey *et al.*, 2006) and start to proliferate in a way similar to that on epithelial cells. Cellular protrusions induced by pili help the bacteria to withstand the high shear stress present in the blood stream (Trivedi *et al.*, 2011). These structures are also involved in the internalisation of bacteria adhered to the apical surface of the cells. During this engulfment of bacteria, ezrin and meosin are first recruited beneath bacterial colonies followed by actin polymerisation. The first step is independent of Rho and GTPases, in contrast to the second step which requires Rho, Rho kinase, and Cdc42 (Eugene *et al.*, 2002). Cortical plaques formed at the site of interaction between bacteria and endothelial cells, may assist the bacteria to

invade the vascular endothelium by disrupting Cdc42-based signalling pathways and employing proteins from adherens junctions (Coureuil *et al.*, 2009). The laminin receptor was also described as a potential receptor for meningococcal PilQ and PorA to initiate contact with the vascular endothelium of blood brain barrier (BBB) (Orihuela *et al.*, 2009).

As a consequence of interaction between Opc and endothelial integrins, a number of signalling events occur. The outcome of these events are the release of the IL-6 and IL-8 (Sokolova *et al.*, 2004) and internalisation of *N. meningitidis* (Virji *et al.*, 1994; Virji *et al.*, 1995). A small number of piliated bacteria attached to endothelial cells are internalised which may reach the meninges by transcytosis or lead to disruption of tight intercellular junctions (Carbonnelle *et al.*, 2009). Indeed it has been documented that adherence via type IV pili induces a signalling cascade which recruits the polarity complex Par3/Par6/PKC ζ and several proteins like V-cadherin, ZO-1 and claudin-5, which comprise the tight junctions between endothelial cells. This leads to the formation of ectopic early junction-like domains (Coureuil *et al.*, 2009), thus compromising the tight cellular junctions and possibly opening a way to transverse the endothelial barrier for meningococci. Moreover, the requisition of β 2-adrenergic/ β -arrestin signalling pathway has been found to be responsible for the establishment of a stable host-bacteria interaction and subsequent penetration of host endothelia with involvement of activated Src tyrosine kinase and β -arrestin dependent delocalisation of junctional proteins, respectively. Thus, *N. meningitidis* is likely to hijack endothelial signalling pathways and open the paracellular pathway (Coureuil *et al.*, 2010).

In addition to all these mechanisms, matrix metalloprotease-8 (MMP-8) mediated proteolytic cleavage of the junction protein occludin has been reported (Schubert-Unkmeir *et al.*, 2010). Once they cross the blood-brain barrier, the bacteria have access to the CSF, an immunocomponent-depleted, but nutrient-rich medium, allowing the bacteria to proliferate in an unrestricted way and cause extensive inflammation of the meninges, leading to meningitis (Exley *et al.*, 2005).

1.10 Moonlighting proteins: Characteristics and distribution in nature

The first examples of moonlighting proteins, as described by Piatigorsky and Wistow (Piatigorsky and Wistow, 1989), were crystallins - structural proteins of the vertebrate eye lens. For example, duck ϵ -crystallin was found to be lactate dehydrogenase (Hendriks *et al.*, 1988), whereas turtle τ -crystallin acted as α -enolase (Wistow *et al.*, 1988). Though Piatigorsky proposed the term 'gene sharing' (Piatigorsky *et al.*, 1988), 'moonlighting' is now generally used to describe this phenomenon (Jeffery, 1999).

Moonlighting proteins are a group of fascinating proteins which can perform several autonomous, often dissimilar functions using a single polypeptide chain, rather than distinct protein domains (Jeffery, 1999; Huberts and van der Klei, 2010). This class of proteins excludes bifunctional proteins arising due to gene fusions or gene splicing, homologous protein families and different proteins performing the same function at different locations (Jeffery, 2009). Homologous proteins may not have moonlighting functions or even if they have, the functions may be different (Gancedo and Flores, 2008). Another characteristic of this group of proteins is that the different functions that a single protein can perform are independent of each other. Inactivation of one

function should not have any inhibitory effect on the other function. Thus moonlighting is not synonymous with pleiotropism which is the result of inactivation of single function of a protein interacting with multiple partners or an enzyme involved in several pathways (Huberts and van der Klei, 2010). Proteins can employ a combination of mechanisms to deliver moonlighting functions in eukaryotic and prokaryotic organisms (Fig. 1.3). For example, the presence of the same protein in different cellular organelles or inside and outside of the same cell, in different cells forming complexes with other proteins or binding with different substrates. Moonlighting proteins are of obvious benefits for the cells. Combining different functions or switching between functions can be considered as a cell's response towards the changing environment (Jeffery, 2004).

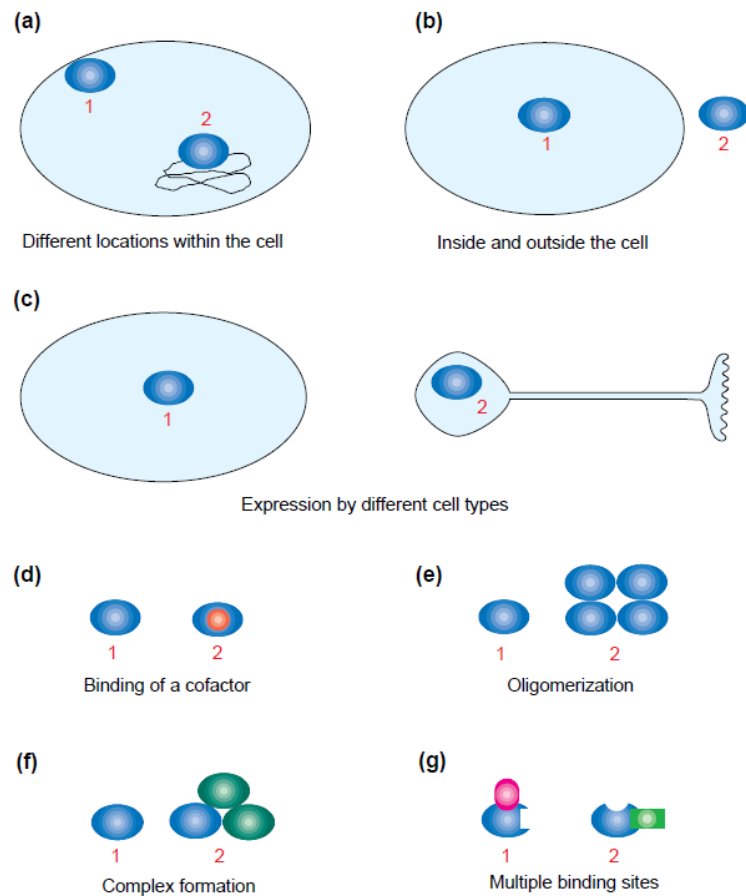


Figure 1.3: Examples of mechanisms for switching between usual and moonlighting functions: (a) Performing different functions at different locations within the same cell. (b) Having one function inside the cell but having a different one when secreted. (c) Same protein may have different functions in different cell types. (d) Altering activity when bound to substrate, product or a cofactor. (e) Forming multimeric structure or (f) complex with other proteins to switch between function performed by the monomeric one. (g) Binding with different substrates using different binding sites can also alter the functions (Jeffery, 1999).

Many of these proteins are conserved in both prokaryotic and eukaryotic organisms and are considered primarily as enzymes of metabolic pathways and molecular chaperones, but can be involved in other intracellular biochemical functions. There are examples of moonlighting proteins in yeast (Gancedo and Flores, 2008), plants (Moore, 2004), mammalian cells (Kim and Dang, 2005) and bacteria (Henderson and Martin, 2011); a few examples are listed in Tables 1.1 and 1.2.

Table 1.1: Moonlighting proteins in eukaryotic organisms (Huberts and van der Klei, 2010).

Organism	Protein	Regular function	Moonlighting Function	Reference
<i>Homo sapiens</i>	ERK2	MAP kinase	Transcriptional repressor	(Hu <i>et al.</i> , 2009)
<i>Homo sapiens</i>	Aconitase	TCA cycle enzyme	Iron homeostasis	(Philpott <i>et al.</i> , 1994)
<i>Arabidopsis thaliana</i>	Hexokinase	Glucose metabolism	Glucose signalling	(Moore <i>et al.</i> , 2003)
<i>Physcomitrella patens</i>	Presenilin	γ -secretase	Cytoskeletal function	(Khandelwal <i>et al.</i> , 2007)
<i>Hansenula polymorpha</i>	Pyruvate carboxylase	Anaplerotic enzyme	Assembly of alcohol oxidase	(Ozimek <i>et al.</i> , 2003)
<i>Saccharomyces cerevisiae</i>	Aldolase	Glycolytic enzyme	V-ATPase assembly	(Lu <i>et al.</i> , 2007)

Table1.2: Moonlighting proteins of bacteria (Adapted from Henderson and Martin, 2011)

Bacteria	Protein	Normal function	Moonlighting function	Reference
<i>N. meningitidis</i>	Enolase	Glycolytic enzyme	Plasminogen binding protein	(Knaust <i>et al.</i> , 2007)
	DnaK	Chaperone	Plasminogen binding protein	(Knaust <i>et al.</i> , 2007)
	Peroxired-oxin	Antioxidant	Plasminogen binding protein	(Knaust <i>et al.</i> , 2007)
	Aldolase	Glycolytic enzyme	Putative adhesin	(Tunio <i>et al.</i> , 2010a)
	GAPDH	Glycolytic enzyme	Putative adhesin	(Tunio <i>et al.</i> , 2010b)
<i>Streptococcus pneumoniae</i>	Aldolase	Glycolytic enzyme	Adhesion for Flamingo cadherin	(Blau <i>et al.</i> , 2007)
	GAPDH	Glycolytic enzyme	Plasminogen binding protein	(Bergmann <i>et al.</i> , 2004)
<i>Streptococcus pyogenes</i>	GAPDH	Glycolytic enzyme	Fibronectin binding protein, plasminogen binding protein, cell signalling kinase/ADP ribosylase	(Pancholi and Fischetti, 1992; Lottenberg <i>et al.</i> , 1992; Pancholi and Fischetti, 1993; Pancholi and Fischetti, 1997)
<i>Staphylococcus aureus</i>	GAPDH	Glycolytic enzyme	Transferrin receptor	(Modun <i>et al.</i> , 2000)
	Enolase	Glycolytic enzyme	Laminin binding protein	(Carneiro <i>et al.</i> , 2004)
Enterohemorrhagic <i>E.coli</i> (EHEC) Enteropathogenic <i>E.coli</i> (EPEC)	GAPDH	Glycolytic enzyme	Binding plasminogen and fibrinogen, NAD-ribosylating activity	(Egea <i>et al.</i> , 2007; Aguilera <i>et al.</i> , 2009)

<i>Listeria monocytogenes</i>	Alcohol acetaldehyde dehydrogenase	Metabolic enzyme	Binds to human Hsp60	(Kim <i>et al.</i> , 2006)
<i>Mycobacterium tuberculosis</i>	Glutamate racemase	Cell wall biosynthesis	DNA gyrase inhibition	(Sengupta <i>et al.</i> , 2008)
	Cpn60.2	Chaperone	Activates monocyte cytokine synthesis, macrophage binding	(Friedland <i>et al.</i> , 1993; Hickey <i>et al.</i> , 2009)
	Cpn60.1	Chaperone	Stimulates formation of granulomas	(Hu <i>et al.</i> , 2008)
	DnaK	Chaperone	Plasminogen binding protein	(Xolalpa <i>et al.</i> , 2007)
<i>Helicobacter pylori</i>	Cpn60	Chaperone	Surface location controls bacterial growth, stimulates monocyte cytokine synthesis by TLR2, adhesin for human epithelial cells	(Yamaguchi <i>et al.</i> , 1997; Takenaka <i>et al.</i> , 2004; Kamiya <i>et al.</i> , 1998)
	PPI		Induces apoptosis of gastric epithelial cells, activates monocyte IL-6 synthesis	(Basak <i>et al.</i> , 2005; Pathak <i>et al.</i> , 2006)

The first report of surface exposed GAPDH protein came from Pancholi and Fischetti. They observed GAPDH tightly bound on the surface of *S. pyogenes* M6 strain which could bind with lysozyme, cytoskeletal proteins (actin and myosin) and the extracellular matrix protein fibronectin (Pancholi and Fischetti, 1992). Surface exposed GAPDH of *S. pneumoniae*, and *Streptococcus agalactiae* binds to plasminogen to allow the bacteria to cross the epithelial and endothelial barrier, (Bergmann *et al.*, 2004; Atanassov *et al.*, 2002) and also has a B lymphocyte-modulatory activity (Madureira *et al.*, 2007). GAPDH (and enolase) of *Lactobacillus crispatus* can be found on the cell surface (in addition to the cytoplasm), where they can activate human plasminogen (Hurmalainen *et al.*, 2007). GAPDH proteins from both enterohemorrhagic (EHEC) and enteropathogenic (EPEC) strains of *Escherichia coli*, bind plasminogen and fibrinogen (Egea *et al.*, 2007) and have NAD-ribosylation activity (Allignet *et al.*, 2002). A number of bacteria possess enolase on their surface for various purposes. For example *Bacillus anthracis*, *Bifidobacterium* spp. and *S. pneumoniae* utilise enolase for plasminogen binding (Agarwal *et al.*, 2008; Candela *et al.*, 2010; Kolberg *et al.*, 2006). *S. aureus* use enolase as a laminin-binding protein (Carneiro *et al.*, 2004) and *Streptococcus gordonii* for salivary mucin (MUC7) binding (Kesimer *et al.*, 2009).

Enzymes from other metabolic pathways can also act as moonlighting proteins. For example, alcohol acetaldehyde dehydrogenase of *L. monocytogenes* acts as a main adhesin termed Listeria adhesion protein (LAP). On the surface of human cells, LAP interacts with chaperonin 60 (Cpn60) which in turn is a heat shock protein (Hsp60). This provides an example of interaction between

bacterial and human moonlighting proteins (Santiago *et al.*, 1999; Wampler *et al.*, 2004).

A number of protein folding catalysts or chaperones are well established moonlighting proteins (Henderson *et al.*, 2006). Cpn60 protein of *Francisella tularensis* can activate monocytes and macrophages alone or synergistically with LPS respectively (Noah *et al.*, 2010). *M. tuberculosis* has several genes coding for chaperonin 60 proteins or heat shock protein 60 (Hsp60) (Kong *et al.*, 1993). One of the chaperonin 60 proteins is Cpn60.1; another is Cpn60.2 or Hsp65 protein. Both of them have been suggested as virulence factors, interacting with monocytes and resulting in cytokine release (Friedland *et al.*, 1993; Lewthwaite *et al.*, 2001). Hsp65/Cpn60.2 is involved in the uptake of *M. tuberculosis* into macrophages (Hickey *et al.*, 2009) and Cpn60.1 can induce granuloma formation (Hu *et al.*, 2008). Cpn60 of *H. pylori* has been implicated in cytokine synthesis by monocytes (Takenaka *et al.*, 2004), epithelial cells (Zhao *et al.*, 2007), macrophages (Gobert *et al.*, 2004) and adhesion to human epithelial cells (Kamiya *et al.*, 1998). Peptidyl prolyl isomerase (PPI) of *H. pylori* induces apoptosis of gastric epithelial cells and IL-6 synthesis by monocytes (Basak *et al.*, 2005).

How these moonlighting proteins reach the bacterial cell surface is still unclear. Bacterial moonlighting proteins do not contain any recognised secretion signals or any anchoring motifs and thus are known as “anchorless proteins” (Pancholi and Chhatwal, 2003). One possibility may be that these proteins are released from the cytoplasm by cell lysis and nearby bacterial cells recruit them to their surfaces. Oliveira *et al* have recently showed that GAPDH, released after lysis

of group B streptococci, binds non-specifically to the surfaces of other Gram-positive and Gram-negative bacteria (Oliveira *et al.*, 2012). During the stationary growth phase of *L. plantarum*, cell-surface-associated GAPDH activity was shown to increase, possibly due to increased permeability of plasma membrane (Saad *et al.*, 2009). Another possibility might be the presence of yet unknown secretion mechanisms (Henderson and Martin, 2011). In support of this, genetically modified GAPDH and enolase were shown to be unable to translocate to the cell surface of *S. pyogenes* (Boel *et al.*, 2005) and *B. subtilis* (Yang *et al.*, 2011), respectively. Moreover an auxiliary secretion system, SecA2, has been reported to be present in *L. monocytogenes*. Seventeen proteins were dependent on this system for secretion. Among these secreted proteins, ten proteins were without any secretion signal and are regarded as moonlighting proteins including DnaK, GroEL, EF-Tu, enolase and others (Lenz *et al.*, 2003). One recent report identified a type III secretion system (T3SS) in enteropathogenic *E. coli* (EPEC) responsible for secretion of GAPDH. This was observed when the cells were grown in Delbecco's modified Eagle's medium (DMEM) medium, but not in LB medium (Aguilera *et al.*, 2012). All together it can be said that the transport mechanism(s) of the moonlighting proteins are largely unknown, especially in Gram-negative bacteria where there are two membrane barriers to negotiate, and may differ depending on the specific protein and bacterial species involved. Whether one or several mechanisms exist in bacteria for moonlighting protein export is a current focal point of research.

1.11 Moonlighting proteins in *N. meningitidis*

In *N. meningitidis*, glucose is mainly catabolized by the Entner-Doudoroff (ED) pathway (Fig. 1.4) and to a lesser extent by the Pentose Phosphate (PP) pathway. The absence of phosphofructokinase, one of the key enzymes of the Embden-Meyerhof-Parnas (EMP) glycolytic pathway (Fig. 1.4) renders this pathway inactive in *N. meningitidis* (Baart *et al.*, 2007). Nevertheless the meningococcal genome carries functional genes for the remaining glycolytic enzymes of this pathway and some of the enzymes are essential for meningococci. For example enolase which has been reported to be essential as an enolase knock out mutant was unable to survive (Knaust *et al.*, 2007). Three enzymes of this pathway have already been described as moonlighting proteins with additional non-glycolytic functions. One of them is enolase, present on the *N. meningitidis* surface which acts as plasminogen receptor (Knaust *et al.*, 2007). The second enzyme of the Neisserial glycolytic pathway to be assigned a moonlighting function was glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Tunio *et al.*, 2010b). Two predicted genes namely *gapA-1* and *gapA-2* are present in the genome of *N. meningitidis* MC58 which encode GAPDH enzymes. Only *gapA-1* expression is upregulated when the meningococci come into contact with human epithelial cells (Tettelin *et al.*, 2000; Grifantini *et al.*, 2002). Tunio *et al.* (Tunio *et al.*, 2010b) showed that GAPDH could be found on the surface of meningococci and contributes to adherence since mutation of *gapA-1* caused a significant reduction in the adherence capability of *N. meningitidis* to both epithelial and endothelial cells (Tunio *et al.*, 2010b).

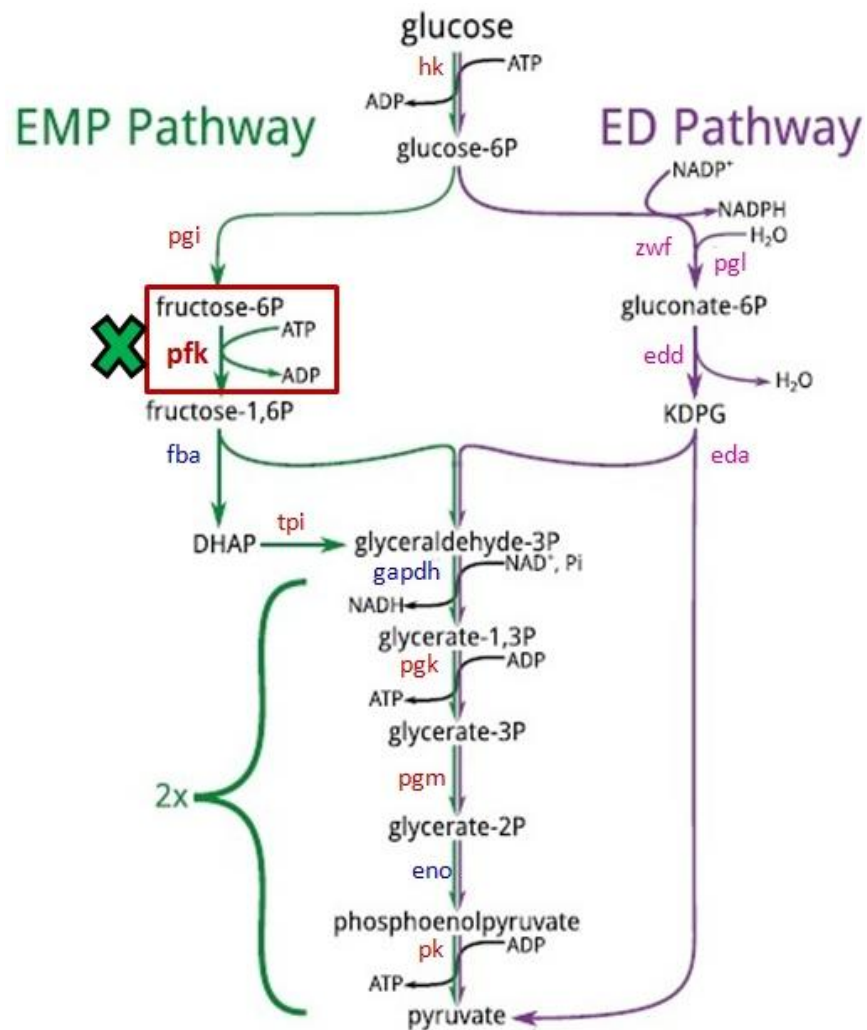


Figure 1.4: EMP and ED pathways of glucose metabolism. The red box indicates PFK of EMP pathway, which is missing in meningococci. Enzymes of EMP pathway with known moonlighting functions are highlighted in red while those having moonlighting functions in meningococci are highlighted in blue. Abbreviations: hk, hexokinase; pgi, phosphoglucose isomerase; pfk, 6-phosphofructokinase; fba, fructose bisphosphate aldolase; tpi, triose phosphate isomerase; gapdh, glyceraldehyde 3-phosphate dehydrogenase; pgk, phosphoglycerate kinase; pgm, phosphoglycerate mutase; eno, enolase; pk, pyruvate kinase; zwf, glucose 6-phosphate dehydrogenase. pgl, phosphogluconolactonase; edd, phosphogluconate dehydratase; eda, 2-keto-3-deoxy-6-phosphogluconate(kdp) aldolase (Modified from Flamholz *et al.*, 2013).

The third enzyme is fructose-1, 6- bisphosphate aldolase (FBA) (Tunio *et al.*, 2010a). FBA is a highly conserved protein in *Neisseria* spp. and although it is a cytoplasmic protein, it has also been reported to be localised to the outer

membrane of *N. meningitidis* and is accessible to anti-FBA antibody in flow cytometry experiments, thus confirming surface-exposure (Tunio *et al.*, 2010a). Loss of FBA made the FBA-deficient mutant strain less adherent to both human epithelial and endothelial cells (Tunio *et al.*, 2010a). The *in vivo* importance of FBA in *N. meningitidis* was underlined in an infant rat model study which identified 73 genes, including the gene encoding for FBA (*cbbA*) as being essential to cause sepsis (Sun *et al.*, 2000). This could lead to an assumption that FBA might have virulence-associated moonlighting function(s) although effects of a metabolic defect which is only apparent under *in vivo* conditions cannot be excluded.

Other than these glycolytic enzymes, DnaK, a chaperone and peroxiredoxin have been reported as plasminogen binding proteins present on the meningococcal surface (Knaust *et al.*, 2007).

1.12 Overview of Fructose-1, 6-bisphosphate aldolase (FBA)

FBA (EC 4.1.2.13) catalyses the reversible cleavage of fructose-1, 6-bisphosphate (FBP) into glyceraldehyde-3-phosphate (G3P) and dihydroxyacetone-phosphate (DHAP) or the reversible aldol condensation reaction. This is an indispensable enzyme for glycolysis, gluconeogenesis and the Calvin cycle (Rutter, 1964). According to the reaction mechanism employed, there are two classes of FBA. Class I FBAs act by forming a Schiff-base intermediate between the amino group of a lysine residue in the active site and the C-2 carbonyl group of the substrate (DHAP or FBP). The class I enzymes are mainly found in animals, plants, green algae and a few prokaryotes (Rutter, 1964; Sauve and Sygusch, 2001; Morse and Horecker, 1968). Class II FBAs require a divalent metal ion at the active site (Zgiby *et*

al., 2000; Zgiby *et al.*, 2002; Hall *et al.*, 1999). Bacteria, yeast, fungi and some green algae possess class II FBA (Rutter, 1964; Plaumann *et al.*, 1997). *M. tuberculosis* and *E. coli* are among the few organisms containing both classes of FBP aldolases (Bai *et al.*, 1974; Stribling and Perham, 1973). In such organisms one enzyme is constitutively expressed, whereas a change in growth conditions (aerobic/anaerobic, or autotrophic growth/heterotrophic growth) can induce the expression of the other (Willard and Gibbs, 1968; Stribling and Perham, 1973; Marsh and Lebherz, 1992; van den Bergh *et al.*, 1996). Based on the amino acid sequence, class II aldolases can be further divided into type A and type B (Plaumann *et al.*, 1997). Type A enzymes are dimeric in contrast to the dimeric, tetrameric or octameric structure of type B enzymes (Sauve and Sygusch, 2001; Nakahara *et al.*, 2003). It has been reported that group A enzymes are mostly involved in glycolysis and gluconeogenesis; while diverse metabolic roles and substrate specificities have been noted for group B aldolases (Sauve and Sygusch, 2001).

Although both classes of aldolase have the same overall $(\alpha/\beta)_8$ barrel fold (also known as TIM barrel), subunit molecular masses (around 40-kDa), and catalyze the same enzymatic reactions, there is no significant homology in their primary structures (Marsh and Lebherz, 1992; Imanaka *et al.*, 2002) or catalytic residues (Blom *et al.*, 1996). Moreover their active sites are located in different parts in the TIM barrel (Nagano *et al.*, 2002; Sanchez *et al.*, 2002). Archaeal aldolases belong to class I based on their reaction mechanism, but lack significant amino acid sequence homology with either class I or class II aldolases. The gene lineages of archaeal type class I aldolase and classical class I and class II aldolases presumably had common predecessor (Siebers *et al.*,

2001). Bifunctional fructose 1,6-bisphosphate aldolase/phosphatase enzymes, which are a unique class of proteins with both FBP aldolase and FBP phosphatase activity have also been found in archaea and some rare thermophilic and autotrophic bacteria (Say and Fuchs, 2010). Due to the absence of class II aldolases in animals, it has been suggested that antimicrobial drugs could be targeted to against these enzymes (Blom *et al.*, 1996).

1.13 Unusual location and non-glycolytic functions of FBA

Using cell fractionation, gel electrophoresis and the subsequent identification of proteins by mass spectrometry or amino-terminal amino acid sequencing, FBA (often with other cytosolic proteins) was initially identified as a surface-associated or secreted protein in several species of streptococci, including *S. pyogenes* (Lei *et al* 2000), *Streptococcus oralis* (Wilkins *et al* 2003), *S. pneumoniae* (Ling *et al* 2004), *S. agalactiae* (Fluegge *et al* 2004) and, more recently, *Streptococcus suis* (Wu *et al* 2008). Several proteins, including FBA, were present in mid-log-phase culture supernatants of *S. pyogenes*, suggesting specific and active secretion rather than just passive release (Lei *et al* 2000). Supernatants from stationary and logarithmic cultures of *S. agalactiae* contained FBA amongst the secreted proteins, many of which were immunogenic as they were recognised in immunoblots by sera from infected neonates or healthy adults (Fluegge *et al.*, 2004). Similarly in *S. suis* serotype 9, immunoblotting of extracted cell wall proteins with immune sera identified FBA as one of eight cell wall-associated immunogenic proteins (Wu *et al* 2008). In *S. pneumoniae*, FBA was identified in the cell wall enriched fraction and was found to be immunogenic when probed against paediatric and adult

sera. Moreover rFBA was immunogenic in mice and able to provide partial protection against a genetically different virulent *S. pneumoniae* strain (Ling *et al.*, 2004). Using the human lung carcinoma cell line (A549) it was shown that FBA functions as an adhesin for *S. pneumoniae*. The host molecule that *S. pneumoniae* FBA binds to was identified as Flamingo cadherin receptor (FCR) (Blau *et al.*, 2007).

Monroy and colleagues first reported the interaction between *M. tuberculosis* and plasminogen. ϵ -amino caproic acid (EACA), a lysine analogue, abolished the binding plasminogen binding (Monroy *et al.*, 2000). Xolalpa and colleagues studied the abundance of plasminogen receptors in *M. tuberculosis* H37Rv and some of them included the classical housekeeping enzymes including FBA (Xolalpa *et al.*, 2007). Santangelo and colleagues reported that apart from its usual location in the cytosol, FBA was also present in the culture filtrate, cell wall and cell membrane fractions and on the cell surface. They also reported that FBA-tb was a plasminogen binding protein. Plasminogen binding was inhibited by EACA but not TD3, a competitive inhibitor of aldolase activity, demonstrating that plasminogen binding was independent of aldolase enzyme activity (de la Paz Santangelo *et al.*, 2011).

FBA has recently been detected in the cell wall fraction of *Coxiella burnetii*, however, so far a definite role for FBA in pathogenesis *C. burnetii* has not been determined (Flores-Ramirez *et al.*, 2014).

Examples of non-glycolytic functions of FBA are not exclusive to bacteria. FBA is shared by yeast, fungi, and parasites as a moonlighting protein. Crowe *et al* reported that FBA was present in the cell wall fraction in *Candida*

albicans and was able to bind plasminogen which in turn is strongly dependent on lysine residues (Crowe *et al.*, 2003). Vacuolar proton-translocating ATPases (V-ATPases) are highly conserved proton pumps (Kane, 2006). In addition to its metabolic role in glycolysis in *S. cerevisiae*, aldolase is involved in the regulation of the activity and assembly of the V-ATPase. Deficiency in aldolase causes disruption of physical association between aldolase and V-ATPase resulting in malfunction of V-ATPase in yeast cells. Complementation of the aldolase-encoding gene in the mutant cells restored the ATP hydrolysis and proton transport activities of V-ATPase (Lu *et al.*, 2007). In addition to that, Fba1 of *S. cerevisiae* is involved in the control of RNA polymerase III (Pol III) transcription and physically interacts with the Pol III complex (Ciesla *et al.*, 2014). In *Aspergillus nidulans* FBA is essential for growth and at the same time is required for plasma membrane localisation of the AgtA acidic amino acid (L-glutamate/L-aspartate) transporter. Low expression of aldolase leads to impaired trafficking of AgtA (Roumelioti *et al.*, 2010). Plasminogen binding by *Cryptococcus neoformans* is mediated by cell wall localised glycolytic enzymes of cytosolic origin including FBA (Stie *et al.*, 2009). More recently FBA was reported as a plasminogen binding protein present in the secretome of the dimorphic pathogenic fungus *Paracoccidioides Pb01* (Chaves *et al.*, 2015).

FBA is a plasminogen binding protein in a number of parasites and helminths, for example *Schistosoma bovis* (Ramajo-Hernandez *et al.*, 2007) and *Dirofilaria immitis* (Gonzalez-Miguel *et al.*, 2013). Nandan and colleagues identified FBA of *Leishmania donovani*, as tyrosine phosphatase-1 binding and activating protein. (Nandan *et al.*, 2007). *Leishmania* infection attenuates

nitric-oxide production (Nandan *et al.*, 1999) which can be attributed to aldolase (Nandan *et al.*, 2007).

1.14 Aims of the study

The main objective of the current study was to determine the role of FBA in meningococcal pathogenesis. The initial aim was to express an enzymatically inactive variant of recombinant FBA by introducing mutations in its active site (Chapter Two). Having found such a mutation, a further aim was to test whether the enzymatic activity of FBA is required for its moonlighting function(s) or not. Also another objective of this project was to explore any additional moonlighting function of FBA. Having established that recombinant FBA is a human plasminogen binding protein (Chapter Three), a further aim was to characterise plasminogen-FBA interaction (Chapter Four). A further aim of the study was to explore the effect of inactivation of enzymatic activity of FBA on its surface localisation and involvement in adhesion to human cells (Chapter Five).

.

Chapter Two: Cloning, expression, purification and biochemical characterisation of recombinant FBA (rFBA)

2.1 Introduction

A growing number of proteins have been found in different cellular locations, performing two or more autonomous functions using a single polypeptide chain; these are referred to as moonlighting proteins (Jeffery, 1999). Moonlighting proteins have neither secretion signals nor anchoring motifs, and are therefore designated as anchorless proteins (Pancholi and Chhatwal, 2003). Moonlighting proteins are most commonly glycolytic enzymes, metabolic enzymes or molecular chaperones (Henderson and Martin, 2011).

Glycolytic enzymes are often considered to be housekeeping enzymes and are constitutively expressed in cells. These are highly conserved proteins which perform 'regular' metabolic functions (Pancholi and Chhatwal, 2003). The first report of a glycolytic enzyme being found on the surface of pathogenic bacteria was that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) on the surface of group A streptococci (Lottenberg *et al.*, 1992; Pancholi and Fischetti, 1992). Since then a growing number of reports have described the presence of these common cytoplasmic, metabolic proteins on the surface of bacteria (Henderson and Martin, 2011).

Due to the absence of the enzyme phosphofructokinase, *N. meningitidis* does not employ the EMP pathway for glycolysis. However, the organism does still encode for the remaining enzymes of this glycolytic pathway (Tettelin *et al.*, 2000; Baart *et al.*, 2007). Three enzymes of this pathway have already been described as moonlighting proteins with non-glycolytic functions. The first, enolase, was reported by Knaust *et al.* and was found to act as a plasminogen receptor (Knaust *et al.*, 2007). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is the second enzyme of the EMP pathway that has been assigned a

moonlighting function (Tunio *et al.*, 2010b). Tunio *et al.*, showed that GAPDH was found on the surface of meningococci and contributes to adherence since mutation of *gapA-1* caused a significant reduction in the capacity of *N. meningitidis* to bind both epithelial and endothelial cells (Tunio *et al.*, 2010b). The third enzyme of the EMP glycolytic pathway to be assigned a moonlighting function was fructose biphosphate aldolase (FBA). FBA is a highly conserved protein in *Neisseria* spp. and has been reported to be localised to the cytoplasm and outer membrane of *N. meningitidis* (Tunio *et al.*, 2010a)). An FBA-deficient mutant showed significantly reduced adhesion to both human epithelial and endothelial cells suggesting that FBA may act as an adhesin. (Tunio *et al.*, 2010a).

Fructose-1,6-bisphosphate aldolase (FBA / FBP aldolase) catalyses the reversible cleavage of Fructose-1,6-bisphosphate (FBP) into glyceraldehyde-3-phosphate (G3P) and dihydroxyacetone-phosphate (DHAP) in glycolysis or the reversible aldol condensation reaction in gluconeogenesis (Rutter, 1964). Besides performing its usual function in the EMP pathway, FBA has also been reported as a surface-exposed moonlighting protein in *S. pneumoniae* (Blau *et al.*, 2007), *M. tuberculosis* (de la Paz Santangelo *et al.*, 2011) and *N. meningitidis* (Tunio *et al.*, 2010a). In addition to this FBA has been reported to be surface exposed in *S. suis* SS9 (Wu *et al.*, 2008), *S. oralis* (Wilkins *et al.*, 2003) and *C. burnettii* (Flores-Ramirez *et al.*, 2014)

To further investigate the role of the active FBA enzyme in the pathogenesis of meningococci, site-directed mutagenesis was used to generate two constructs allowing the expression of recombinant FBA (rFBA) with mutations in the active (cation-binding) site (D83A and H81A/H84A, respectively). Purified

rFBA and its mutant derivatives were then used in enzyme activity assays and in further characterisation experiments. This chapter describes the cloning, expression, purification and enzymatic characterisation of rFBA (WT and mutants).

2.2 Materials and Methods

2.2.1 Bacterial strains and growth conditions

E. coli JM109 harbouring plasmids pSAT-9, pFS-1, pFS-2 or pFS-3 (Table 2.1) was routinely grown in Lysogeny Broth (LB, Oxoid Ltd) and on LB agar plates containing ampicillin ($100\text{ }\mu\text{g ml}^{-1}$) at 37°C .

2.2.2 DNA techniques

2.2.2.1 Extraction of plasmid DNA

Plasmid DNA was purified using the QIAGEN miniprep kit according to the manufacturer's instructions. The quality and concentration of extracted plasmid DNA was determined using a Nanodrop (ND-1000) spectrophotometer (Agilent Technologies).

2.2.2.2 Primer design

Three pairs of primers were designed to facilitate small deletions and introduction of mutations at specific sites (Table 2.2). These primers were supplied by Eurofins MWG.

2.2.2.3 Site directed mutagenesis and transformation of *E. coli* XL10-Gold ultracompetent cells

The QuikChange Lightning site-directed mutagenesis kit (Agilent Technologies) was used to introduce DNA mutation(s) into plasmids by following the manufacturer's instructions. XL10-Gold ultracompetent cells (Agilent Technologies) were transformed using the manufacturer's protocol with only one modification; instead of NZY+ broth, the ultracompetent cells were allowed to grow in LB broth for 1 h at 37°C .

Table 2.1: Bacterial strains and plasmids used in work described in this chapter

Bacterial strain	Use/Genotype	Source or reference
<i>E. coli</i> XL10-Gold ultracompetent cells	Cloning strain Tetr Δ (<i>mcrA</i>)183 Δ (<i>mcrCB-hsdSMR-mrr</i>)173 <i>endA1 supE44 thi-1 recA1 gyrA96 relA1 lac</i> Hte[F' <i>proAB lacIqZ</i> Δ M15 Tn10 (Tetr) Amy Camr]	Agilent Technologies
<i>E. coli</i> JM109	Cloning strain <i>endA1, recA1, gyrA96, thi, hsdR17</i> (rk ⁻ , mk ⁺), <i>relA1, supE44, Δ(lac-proAB), [F', traD36, proAB, laqIqZ</i> Δ M15]	Promega
Plasmids	Characteristics	Source or reference
pSAT- 9	MC58 <i>cbbA</i> gene cloned in pQE70	(Tunio <i>et al.</i> , 2010)
pFS-1	pSAT- FBA with a 4bp (GCAT) deletion at position 161-164	This study
pFS-2	pFS-1 with a single mutation at FBA codon 83 (GAC to GCC)	This study
pFS-3	pFS-1 with double mutations at codons 81/84 (CAC to GCC / CAC to GCC)	This study
pSC2	The passenger domain from the <i>Campylobacter jejuni</i> NCTC 11168 <i>capA</i> autotransporter cloned in pCold-TF plasmid, used as control for 6 $\times$ His-tagged protein expression	N. J. Oldfield (University of Nottingham)

Table 2.2: Primers used in work described in this chapter

Use of Primer	Primer	Sequence (5'→ 3')
GCAT 4bp deletion	FBA_repairF	AGGAGAAATTAAGCATGGCACTCGTATCCATGCG
	FBA_repairR	CGCATGGATACGAGTGCCATGCTTAATTTCTCCT
Introduction of mutation(s) at the active site of FBA	FBA_D83AF	CGTCATGCACCAAGCCACGGCGCATCAC
	FBA_D83AR	GTGATGCGCCGTGGGCTTGGTGCATGACG
	FBA_H8184AF	CCCCGTCGTCATGGCCCAAGACGCCGGCGCATCACCC
	FBA_H8184AR	GGGTGATGCGCCGGCGTCTTGGGCCATGACGACGGGG

2.2.2.4 Sequencing of plasmid DNA and nucleic acid and amino acid sequence analysis

Sequencing of purified plasmids was done using the Sanger sequencing method by Source Biosciences, UK. DNAMAN software package version 4.13 (Lynnon Biosoft) was used for the analysis of DNA and protein sequence.

2.2.3 Protein techniques

2.2.3.1 Expression of rFBA using *E. coli* JM109 competent cells

E. coli JM109 competent cells were transformed according to the manufacturer's instructions. The transformants were allowed to grow overnight at 37°C in 20 ml LB medium containing ampicillin (100 µg ml⁻¹).

1L fresh LB with ampicillin was inoculated with 20 ml overnight culture of *E. coli*. The culture was incubated at 37°C. When the optical density (OD₆₀₀) of the culture reached *ca.* 0.6, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1mM and the incubation was continued for 5 h.

Cells were then harvested by centrifugation ($4,200 \times g$ for 10 min) and stored at -80°C to be used for purification of rFBA.

2.2.3.2 Purification of rFBA by immobilised metal affinity chromatography (IMAC)

For the purification of rFBA (wild type and mutated), cell pellets harvested from *E. coli* cultures were resuspended in 20 ml lysis buffer (50mM NaH_2PO_4 , 300mM NaCl, 10mM imidazole, pH 7.4) followed by a 10 min cycle of 30 sec sonication (SoniPrep sonicator MSE 150)) and 30 sec of cooling, on ice. The cell lysate was centrifuged ($4,000 \times g$ for 10 min) and using a 50 ml super loop (GE Healthcare Biosciences, Sweden) the clarified lysate was loaded onto the His Trap^{FF} (GE Healthcare Biosciences) column prepacked with Ni Sepharose 6 Fast Flow (GE Healthcare Biosciences) connected to a ÄKTAprime plus liquid chromatography system (GE Healthcare Biosciences) and pre-equilibrated with 10 column volumes of wash buffer (50mM NaH_2PO_4 , 300mM NaCl, 15mM imidazole, pH 7.4). Step elution was done with elution buffer (50mM NaH_2PO_4 , 300mM NaCl, 300mM imidazole, pH 7.4). Each of ten eluted fractions (1 ml each) was collected in tubes already containing 3ml elution buffer. The whole chromatography process was graphically displayed, viewed and analysed using Prime view 5.0 software (Amersham Biosciences).

2.2.3.3 Buffer exchange

5.0 ml HiTrap column prepacked with SephadexTM G-25 Superfine (GE HealthCare) connected to a ÄKTAprime plus liquid chromatography system (GE Healthcare Biosciences, Sweden) and equilibrated with 5 column volumes of phosphate buffered saline (PBS, Oxoid) was used for buffer exchange. A mixture of diluted eluted fractions was loaded onto the column using a 50 ml super loop. Fractions, each of 1 ml, were collected in tubes containing PBS-

50% (vol/vol) glycerol. As before, the whole chromatography process was graphically displayed, viewed and analysed using Prime view 5.0 software (Amersham Biosciences).

2.2.3.4 Concentration of purified protein samples

Purified eluates, collected using the buffer exchange procedure, were concentrated into 1 ml using centrifugal force ($4,200 \times g$) and Vivaspin™ sample concentrators (Sartorius Stedim Biotech, Germany) with a 30,000 molecular weight cut-off (MWCO).

2.2.3.5 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples collected from the chromatographic purification process, were mixed with SDS-PAGE sample buffer (0.62 M Tris-Cl (pH 6.8), 5% (wt/vol) SDS, 25% (vol/vol) glycerol, 12.5% (vol/vol) β -mercaptoethanol, 0.002% (wt/vol) Bromophenol blue) and heated at 100° C for 5 minutes. The proteins in the samples were separated on 10% (wt/vol) polyacrylamide gels stained by Simply Blue Safe Stain (Invitrogen), destained with dH₂O and scanned using a GS-800 calibrated densitometer (BIO-RAD). A Prestained protein marker (New England Biolabs) was run alongside the samples for determination of molecular weight of the purified protein.

2.2.3.6 Immunoblotting

The proteins, separated by SDS-PAGE, were transferred to a nitrocellulose membrane (BIO-RAD) using a Trans-Blot semidry transfer cell (BIO-RAD) at constant voltage of 15 V for 15 min. The membrane was incubated overnight at 4°C in blocking buffer [5% (wt/vol) nonfat dry milk, 0.1% (vol/vol) Tween 20 in 1 × phosphate-buffered saline (PBS)]. Mouse anti-pentahistidine antibody (Qiagen, 1:10,000 diluted) or rabbit anti-FBA antiserum ((Tunio *et al.*, 2010a)

1:1000 diluted) was added to the membrane and incubated for 2 h at room temperature (RT). After that the membrane was washed three times (5 min each) with PBS-T (PBS containing 0.05% (vol/vol) Tween-20 , and incubated for 2 h at RT with secondary antibody (1:10,000 diluted goat anti-mouse IgG or 1:1000 diluted goat anti-rabbit IgG) conjugated to alkaline phosphatase (Sigma-Aldrich). After washing with PBST, the membrane was developed using BCIP (5-bromo- 4- chloro-3-indolylphosphate)–Nitro Blue Tetrazolium liquid substrate (PerkinElmer). The membrane was washed with dH₂O and a scanned using a GS-800 calibrated densitometer (BIO-RAD).

2.2.3.7 Protein assay

Protein concentrations of purified rFBA samples were assayed using the Pierce BCA Protein Assay Reagent Kit (Thermo Scientific) following the manufacturer's instructions for the microplate procedure using Bovine serum albumin (BSA) as standard.

2.2.3.8 Enzyme assay

A coupled assay (Berry and Marshall, 1993) was carried out to determine the enzymatic activity and kinetic parameters of the purified rFBA. Briefly, a 5 µl aliquot of rFBA (0.29 mg/ml) was added to a final volume of 1 ml containing the following reagents: 50 mM Tris-HCl buffer with 0.1 M potassium acetate buffer (pH 8.0), coupling enzyme (2 µl of a 10 mg ml⁻¹ mixture of glycerol phosphate dehydrogenase/ triose phosphate isomerase, Fig.1.4), fructose 1, 6 bisphosphate (concentrations ranging from 0 to 0.5mM), NADH (20mM). The assay was performed at 30°C. Using a Jasco V-560 UV/Vis spectrophotometer and built-in Spectra Manager software, a decrease in absorbance at 340 nm was detected. One unit of aldolase activity was defined as the amount of purified

fructose 1, 6 bisphosphate aldolase enzyme which catalysed the oxidation of 2 mmol NADH min⁻¹ considering the molar extinction coefficient for NADH as 6220 M⁻¹ cm⁻¹. Kinetic parameters were determined using Origin Pro 7.5 software.

2.3 Results

2.3.1 Site directed mutagenesis in the active site of FBA

In a previous study, the FBA-encoding gene of *N. meningitidis* MC58 (known as *cbbA*; locus tag NMB1869) was cloned into the plasmid vector pQE-70 to form pSAT-9 to facilitate the expression and subsequent purification of 6 × histidine tagged rFBA (Tunio *et al.*, 2010a).

A His-X-X-His motif, a highly conserved zinc cofactor binding site, is present in class II aldolases. In *E.coli* this motif is present as His-Thr-Asp-His (H-T-D-H). It has already been published that these two histidine residues are directly responsible for zinc binding (Berry and Marshall, 1993) and the aspartic acid residue plays a critical role in the catalytic mechanism of FBA (Plater *et al.*, 1999). An alignment of primary sequences of the class II FBA from *E. coli* and *N. meningitidis* MC58 (Fig. 2.1) showed that in meningococcal FBA, this highly conserved motif is present as His-Gln-Asp-His (⁸¹H-⁸²Q-⁸³D-⁸⁴H). Therefore in order to generate recombinant FBA lacking aldolase activity, these highly-conserved aspartate (⁸³D) or histidine (⁸¹H and ⁸⁴H) residues were targeted for site directed mutagenesis and replaced (Fig.2.1) with alanine. These two different mutant derivatives were generated by site directed mutagenesis using pSAT-9 as template.

Upper line: *E.coli* FBA IIA protein sequence, from 24 to 355
Lower line: *N. meningitidis* MC58 FBA IIB, from 13 to 352

E.coli FBA IIA protein sequence: *N. meningitidis* MC58 FBA IIB
identity= 27.30%(83/304) gap=17.39%(64/368)

```

24  AKENNEFALPAVNCVGTDSINAVLETAAKVKAPVIVQFSNGGASFAGKGVKSDVPQGAAI
    | ||::|||. | . : ::::|. |. | |||| | |. : :: ||::
13  AAENSYGLPAFNVNNLEQMRAIMEAADQVDAPVIVQASAGARKYA.....GAPF

84  LGAISGAHHVHQMAEHYGVFVILHTDHC AKKLLPWIDGLLDAGEKHFAATGKPLFS SHMI
    | : | . : | : ||::| ||. |. : . | : |||. | :
62  LRHLILAA...VEEFPHIPVVMHQDHGASPDVCQRSIQLG.....FSSWM

144 DLSEE.....SLQENIEICSKYLERMSKIGMTLEIELGCTGGEEDGVDNSHMDASALY
    | | . : | : | : . : . : | : : | | : | . | . : : | :
105 DGSLMEDGKTPSSYEYNVNATRTVVNFSSHACGVSVEGEIGVLGNLETGEAGEEDGVGAVG

197 TQPEDVDYAYTELSKISPRFTIAASFGNVHGVYKPGNVVLTPTILRDSQEYVSKKHNLPH
    . : | . : | . : . : | : : : . : | . : . | . : :
165 KLSHDQMLTSVEDAVCFVKDTGVDALAIAGTSHGAYKFTRPPTGDVLRIDRIKEIHQAL

257 NSLNFVVFHGG.....GSTAQEIKDSVSYGVVKMNIDTDTQWAT
    . : | . | : | | . : | . : | : : | | | : | : |
225 PNTHIVMHGSSSVPQEWLKVINEYGGNIGETYGVPVEEIVEG IKHGVRKVNIDTDLRIAS

296 WEGVINYYKANEAYLQGQLGNPKGED...QPNKKYYDPRVWLRAGQTSMIARLEK....
    : : | . | . : : . : | . : | . : . : . : | : |
285 TGAVRRYLAENPSDFDPRKYL SKTIEAMKQICLDRYLAFGCEGQAGKIKFVSLEKMASRY

348 AFQEINAI
    | . | | . |
345 AKGEINQI

```

Figure 2.1: Optimal alignment of protein sequences of *E.coli* IIA FBA and *N. meningitidis* MC58 IIB FBA sequences. The amino acid residues highlighted in red are the conserved amino acid residues in zinc cofactor binding site.

DNA sequencing data (Fig. 2.2) revealed that in one mutant (pSAT-9_ D83A) the codon encoding for aspartic acid was correctly replaced by one encoding alanine at position 83 (D83A mutation) and in the other mutant plasmid (pSAT-9_H81/84A) two codons encoding for histidine residues were correctly replaced by codons for alanine at positions 81 and 84 (H81/H84A mutation).

NMB1869 DNA	CACACATCCCGTCGTCATGCA	CCAAGACC	ACGGCGCATCACCCGAC
pSAT-9 DNA	CACACATCCCGTCGTCATGCA	CCAAGACC	ACGGCGCATCACCCGAC
pSAT-9 (D83A) DNA	CACACATCCCGTCGTCATGCA	CCAAGACC	ACGGCGCATCACCCGAC
pSAT-9 (H81/H84A) DNA	CACACATCCCGTCGTCATGCA	CCAAGACC	ACGGCGCATCACCCGAC
Consensus	cacacatcccgtcgtcatg	ccaag c	cggcgcgcatcacccgac

FBA_Protein	HLILAAVEEFPHIPVVMHQDE	GASPDVCQRSIQLGFSS
pSAT-9 Protein	HLILAAVEEFPHIPVVMHQDE	GASPDVCQRSIQLGFSS
pSAT-9 (D83A) Protein	HLILAAVEEFPHIPVVMHQDE	GASPDVCQRSIQLGFSS
pSAT-9 (H81/H84A) Protein	HLILAAVEEFPHIPVVMHQDE	GASPDVCQRSIQLGFSS
Consensus	hlilaaveefphipvvm q	gaspdvcqrsiqlgfss

Figure 2.2: Multiple sequence alignment of *cbbA* and its translated product: multiple sequence alignments of (A) *cbbA* sequences including NMB1869 and those cloned into pSAT-9 and (B) the translated products of the wild-type and cloned *cbbA* genes showing changes due to the site directed mutations.

2.3.2 Correction of reading frame in the transcripts

pSAT-9 plasmid was used for expression of wild type rFBA in *E.coli* JM 109 cells. However, contrary to expectations, no difference was observed between bacterial culture samples collected before and after induction with IPTG. Furthermore, no protein band specific for rFBA was observed by immunoblotting using rabbit anti-FBA or mouse anti-pentahistidine antibodies (Fig. 2.3).

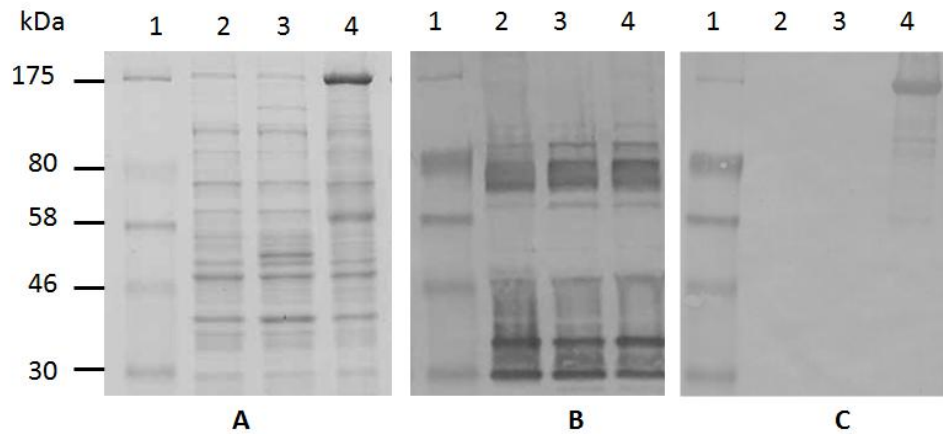


Figure 2.3: SDS-PAGE analysis of *E. coli* JM109 cells for expression of rFBA. Whole cell lysates look identical for uninduced and induced samples and no clear band corresponding to rFBA of ca. 38 kDa was observed in A) SDS-PAGE or B) Immunoblot with anti-FBA antibody or C) Immunoblot with anti-His-tag antibody. Lane 1: Prestained Protein marker; Lane 2: Uninduced *E. coli* JM 109 cells containing pSAT-9; Lane 3: induced *E. coli* JM 109 cells containing pSAT-9; Lane 4: *E. coli* JM109 cells containing plasmid pSC2 as a positive control for expression of a 6 × His-tagged protein.

Additional DNA sequencing data revealed the presence of two ATG start codons upstream of *cbbA* (Fig. 2.4). The first was the optimal, pQE70-encoded, ATG start codon, whilst the second ATG start codon (located 4bp downstream of the first) was derived from the cloned meningococcal *cbbA* sequence (Fig. 2.4). The lack of rFBA expression may have resulted from the sub-optimal distance of the second start codon from the vector-encoded ribosome-binding site.

```

pQE-70:                GAGGAGAAATTAAGCATGCGAGGATCCAGATCTCATCACCATC
pSAT-9 consensus DNA:  GAGGAGAAATTAAGCATGCGACTCGTATCCATGCGCCAA
FBA_NMB1869 (DNA_SEQ): .....ATGCGACTCGTATCCATGCGCCAA

```

Figure 2.4: Multiple sequence alignment of plasmid vector pQE-70, plasmid pSAT-9 and *cbbA* from *N. meningitidis* MC58. pSAT-9 has two start codons, the first highlighted in yellow, is from derived from pQE-70 and the other one highlighted in blue, comes from the cloned *cbbA*.

In order to optimise the distance between the RBS and the start codon, a 4 bp deletion mutation was introduced into pSAT-9 (Fig.2.5). The plasmid pSAT-9 with the 4 bp deletion was named pFS-1. Using this pFS-1 plasmid as template, two mutated plasmids were created which had specific mutation(s) in the active site of the FBA protein product: pFS-2 (containing the D83A mutation) and pFS-3 (containing the H81/H84A mutation).

A	pSAT-9 consensus DNA pFS-1 consensus DNA pFS-2 consensus DNA pFS-3 consensus DNA Consensus	CATTAAAGAGGAGAAATTAAGCATGCATGGCACTCGTATC CATTAAAGAGGAGAGAAATTAA....GCATGGCACTCGTATC CATTAAAGAGGAGAGAAATTAA....GCATGGCACTCGTATC CATTAAAGAGGAGAGAAATTAA....GCATGGCACTCGTATC cattaaagaggagaaattaa gcatggcactcgtatc
B	pSAT-9 protein pFS-1 protein pFS-2 protein pFS-3 protein Consensus	MALVSMRQLLDHAAENSYGLPAFNVNNLEQMRA MALVSMRQLLDHAAENSYGLPAFNVNNLEQMRA MALVSMRQLLDHAAENSYGLPAFNVNNLEQMRA MALVSMRQLLDHAAENSYGLPAFNVNNLEQMRA malvsmrqllldhaaensyglpafnvnnleqmra

Figure 2.5: Multiple sequence alignment of the (A) DNA sequences and (B) the translated protein products derived from pSAT-9, pFS-1, pFS-2 and pFS-3.

2.3.3 Expression of rFBA by *E. coli* JM109 cells

E. coli JM109 cells, transformed with pFS-1, pFS-2 or pFS-3, were grown in order to express rFBA (WT and mutants). Bands corresponding to rFBA were clearly observed from the IPTG induced culture samples in polyacrylamide gels and immunoblots probed with anti-pentahistidine antibody (Fig. 2.6).

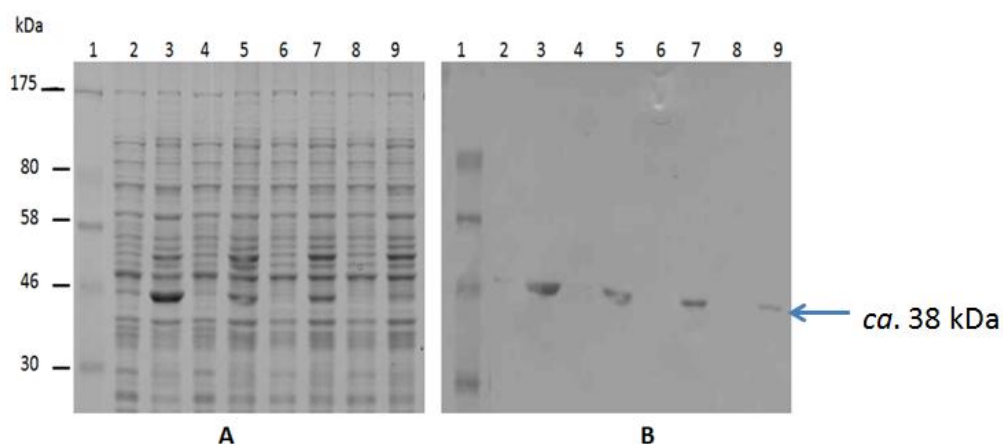


Figure 2.6: Expression of rFBA by *E. coli* JM109 cells. A) SDS-PAGE gel and B) Immunoblot with anti-His-tag antibody showing presence of bands corresponding to rFBA protein (ca. 38-kDa) in IPTG induced *E. coli* JM109 culture samples. Lane 1: Prestained Protein marker; Lane 2-9: uninduced culture and culture induced to produce rFBA (wild type) from pSAT-9,rFBA (WT) from pFS-1, rFBA (D83A) from pFS-2, rFBA (H81A/H84A) from pFS-3 respectively. The blue arrow indicates presumed rFBA bands of ca. 38-kDa.

2.3.4 Purification of rFBA (WT) by step elution and buffer exchange

In order to purify rFBA, *E.coli* JM109 (pFS-1) cells were harvested after IPTG induction. The harvested cells were sonicated and centrifuged to obtain a soluble lysate. This was loaded onto a HisTrap crude column and recombinant rFBA (WT) recovered in elution buffer containing imidazole from the column by immobilised metal affinity chromatography (IMAC).

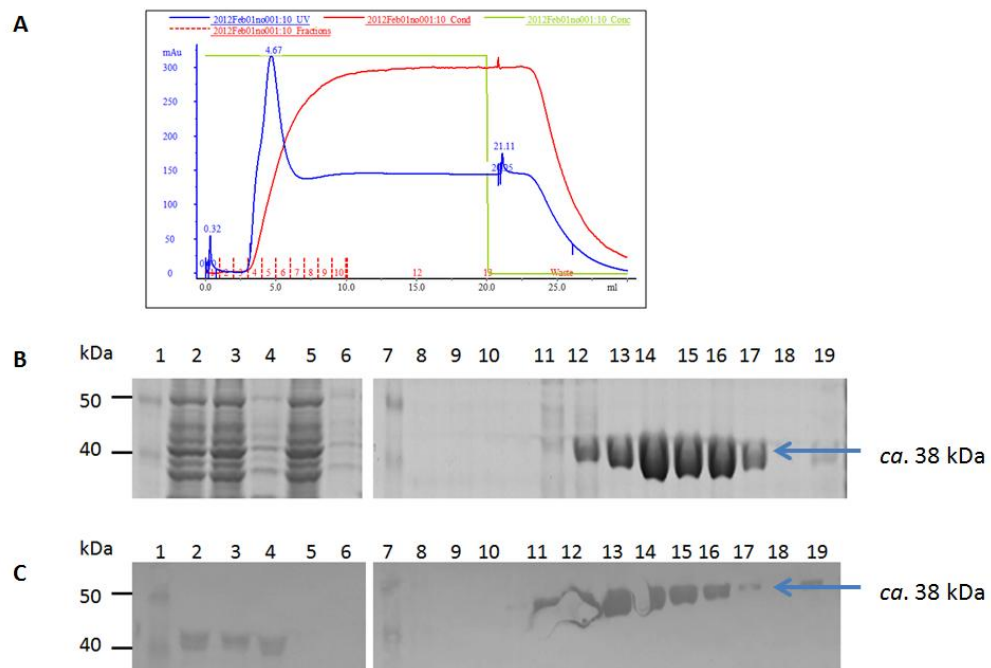


Figure 2.7: Purification of rFBA (WT) by step elution. (A) Representative chromatogram displaying the typical purification profile for rFBA (WT). The Y-axis shows absorbance at 280nm and X-axis displays elution volume. rFBA bound inside the column, was displaced by imidazole concentration (green line) and conductivity (red line) gradually increased with elution of protein (blue line). (B) SDS-PAGE and (C) immunoblot probed with anti-His-tag antibody, showing fractions containing purified rFBA (WT). Lane 1: Prestained protein ladder; Lane 2: whole cell lysate; Lane 3: soluble lysate; Lane 4: insoluble pellet; Lane 5: flow through; Lane 6: washing from column; Lane 7: Prestained protein ladder; Lane 8-17: eluted fractions 1-10 in panel A; Lane 18: after elution wash; Lane 19: *E. coli* JM109 cells induced to produce rFBA (WT) was used as positive control. The blue arrow indicates purified rFBA.

The purity of the fractions were analysed by SDS-PAGE and immunoblotting with anti-His-tag antibody (Fig. 2.7B and 2.7C). The fractions containing rFBA(WT) (lanes 11-17, Fig.2.7 B and C) were pooled together, subjected to buffer exchange to remove the imidazole from the purified protein preparation and collected in PBS-50% glycerol (vol/vol) (Fig. 2.8A-C).

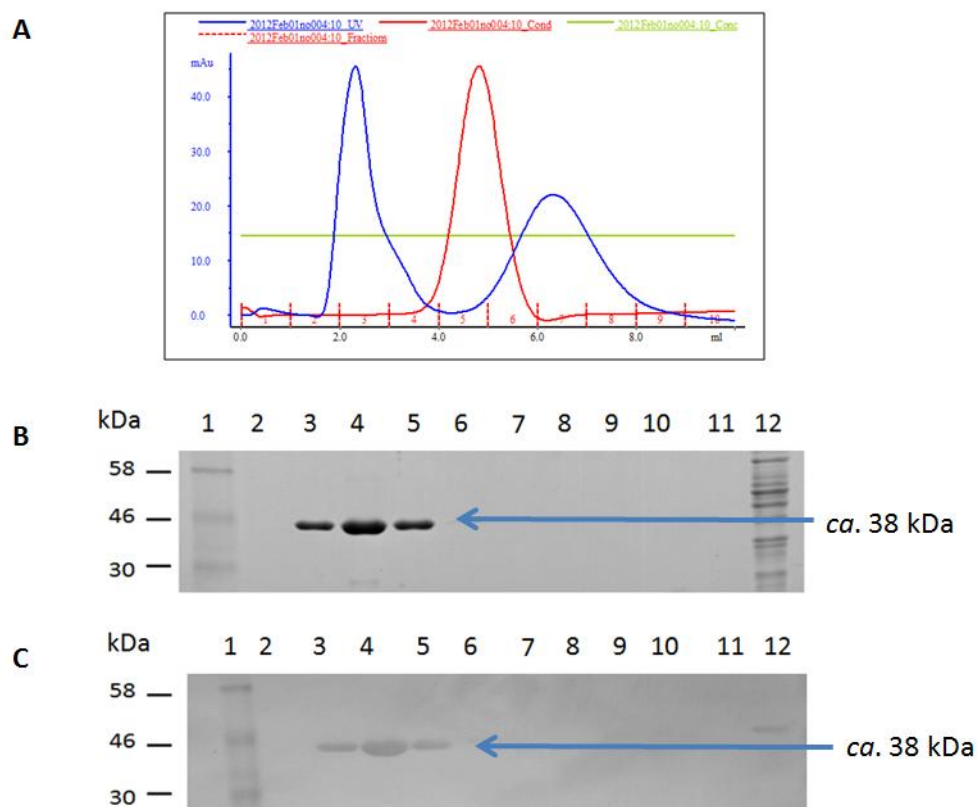


Figure 2.8: Purification of rFBA (WT) by buffer exchange. (A) Representative chromatogram displaying the typical purification profile for rFBA(WT). The Y-axis shows absorbance at 280nm and X-axis displays elution volume.. UV absorbance, concentration and conductivity are indicated by blue, green and red lines respectively. Purified rFBA (WT) samples collected from buffer exchange were separated by (B) SDS-PAGE and rFBA was identified by (C) immunoblot with anti-His-tag antibody. Lane 1: Prestained Protein marker; Lane 2-11: eluted fractions 1-10 in panel A; Lane 12: rFBA purified from IMAC was used as positive control. The blue arrow indicates purified rFBA.

2.3.5 Expression and purification of rFBA (D83A and H81/H84A)

E. coli JM109 (pFS-2) and *E. coli* JM109 (pFS-3) were induced to express rFBA (D83A) and rFBA (H81/H84A) respectively. Whole cell lysates from uninduced and induced cultures were run out on SDS-PAGE gels. The level of expression of rFBA (D83A) was found to be similar to rFBA (WT) (Fig. 2.9). However, it was observed that the level of expression of rFBA (H81/H84A) was lower than rFBA (D83A) or WT rFBA (Fig. 2.9). As expected, this

resulted in a lower yield of protein upon subsequent purification (Fig. 2.12-2.13).

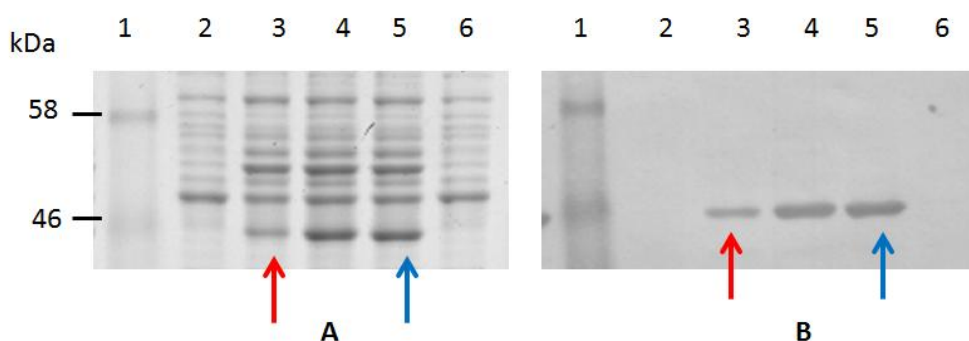


Figure 2.9: Expression of rFBA (D83A) and rFBA (H81A/ H84A). A) SDS-PAGE gel and B) immunoblot with anti-His-tag antibody showed that expression of rFBA (H81A/ H84A) was lower than rFBA (D83A) or rFBA (WT). Lane 1: Prestained protein ladder; Lane 2: uninduced *E. coli* JM109 (pFS-3); Lane 3: *E. coli* JM109 (pFS-3) induced to produce rFBA (H81A/ H84A); Lane 4: *E. coli* JM109 (pFS-1) culture induced to produce rFBA (WT); Lane 5: induced *E. coli* JM109 (pFS-2) culture producing rFBA (D83A); Lane 6: uninduced JM109 (pFS-2) culture. The red and blue arrows highlight the bands corresponding to rFBA (H81A/ H84A) and rFBA (D83A) respectively.

rFBA (D83A and H81/84A) were purified by IMAC followed by buffer exchange, as described for the purification of rFBA (WT). rFBA (D83A) was present in the soluble lysate and the purification pattern was similar to rFBA (WT) (Fig. 2.10). In several trials, it was found that the concentration of purified rFBA H81/H84A (Fig. 2.12-2.13) obtained after purification was much lower compared to that of rFBA WT (Fig. 2.7-2.8) and rFBA D83A (Fig. 2.10-2.11). From SDS-PAGE gel and immunoblot analysis, it was evident that the majority of rFBA (H81/H84A) was present in the insoluble pellet, rather than the soluble lysate (Fig. 2.12B), suggesting that rFBA H81/H84A was being localised to inclusion bodies in the expression strain. This observation also correlates with low expression level of rFBA H81/H84A (Fig. 2.9).

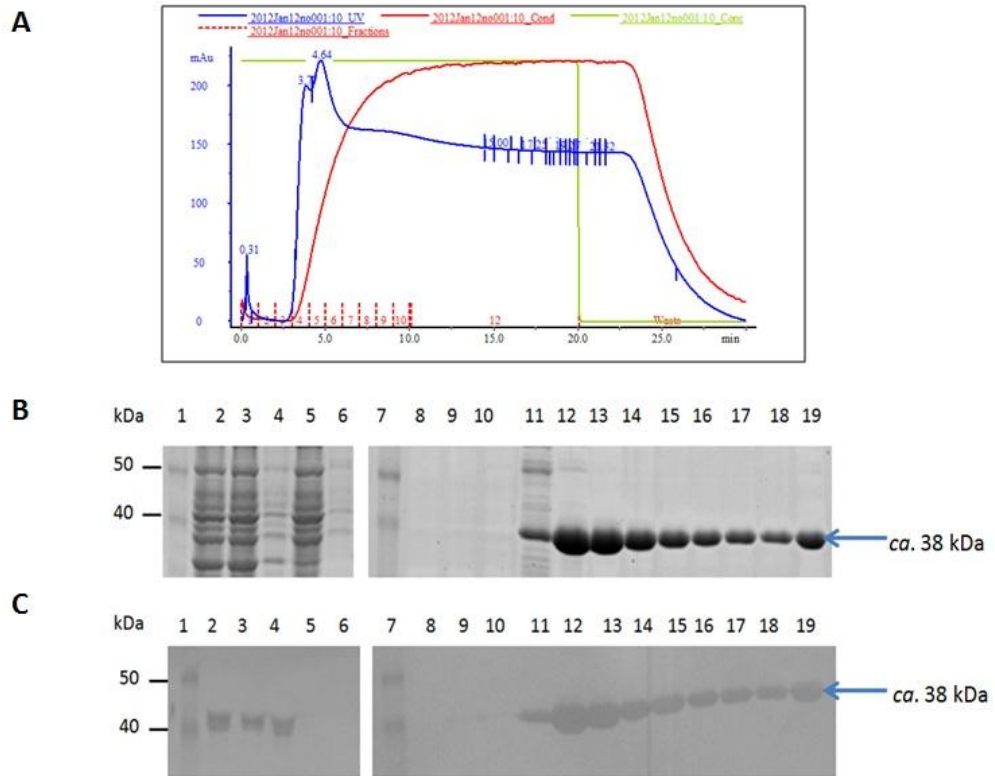


Figure 2.10: Purification of rFBA (D83A) by step elution. (A) Representative chromatogram displaying the typical purification profile for rFBA (D83A). The Y-axis shows absorbance at 280nm and X-axis displays elution volume. rFBA bound inside the column, was displaced by imidazole concentration (green line) and conductivity (red line) gradually increased with elution of protein (blue line). (B) SDS-PAGE and (C) immunoblot probed with anti-His-tag antibody, showing fractions containing purified rFBA (D83A). Lane 1: Prestained protein ladder; Lane 2: whole cell lysate; Lane 3: soluble lysate; Lane 4: insoluble pellet; Lane 5: flow through; Lane 6: washing from column; Lane 7: Prestained protein ladder; Lane 8-17: eluted fractions 1-10 in panel A; Lane 18: after elution wash; Lane 19: purified rFBA (WT) was used as positive control. The blue arrow indicates purified rFBA.

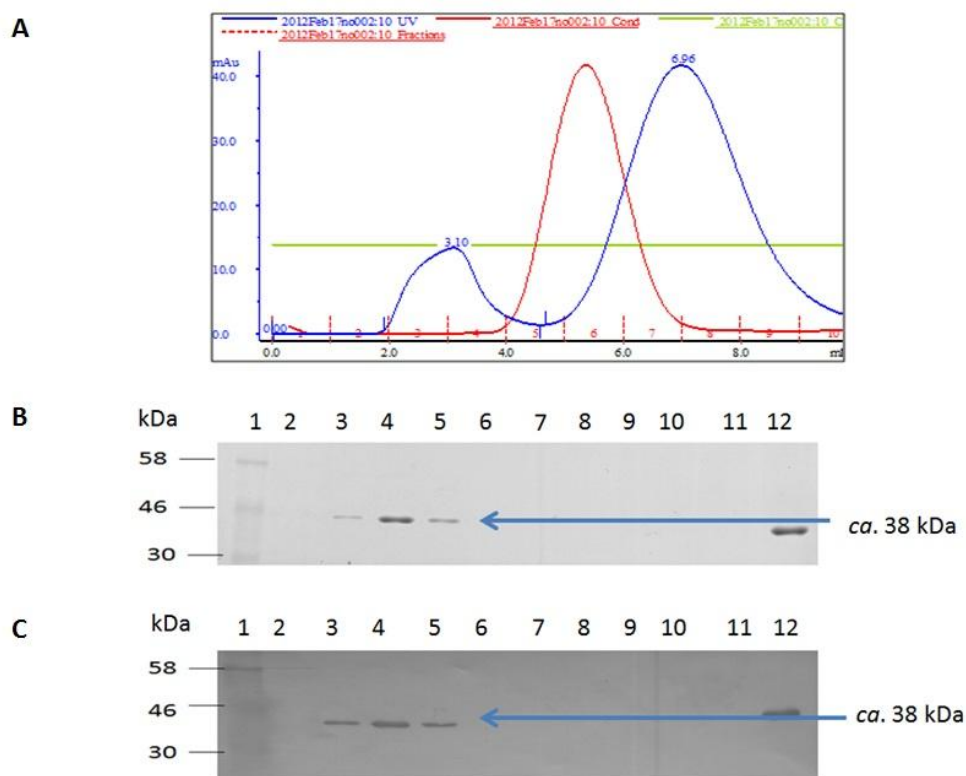


Figure 2.11: Purification of rFBA (D83A) by buffer exchange. (A) Representative chromatogram displaying the typical purification profile for rFBA(D83A). The Y-axis shows absorbance at 280nm and X-axis displays elution volume. UV absorbance, concentration and conductivity are indicated by blue, green and red lines respectively. Purified rFBA (D83A) samples collected from buffer exchange were separated by (B) SDS-PAGE and rFBA was identified by (C) immunoblot with anti-His-tag antibody. Lane 1: Prestained Protein marker; Lane 2-11: eluted fractions 1-10 in panel A; Lane 12: Purified rFBA was used as positive control. The blue arrow indicates purified rFBA.

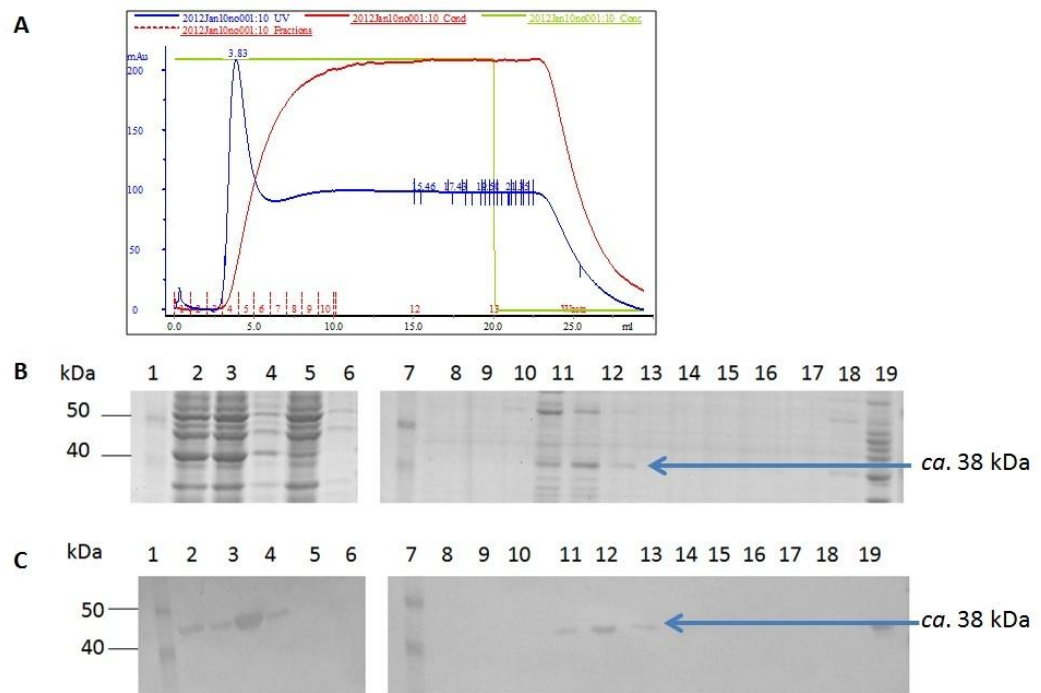


Figure 2.12: Purification of rFBA (H81/84A) by step elution. (A) Representative chromatogram displaying the typical purification profile for rFBA (H81/84A). The Y-axis shows absorbance at 280nm and X-axis displays elution volume. . rFBA bound inside the column, was displaced by imidazole concentration (green line) and conductivity (red line) gradually increased with elution of protein (blue line). (B) SDS-PAGE and (C) immunoblot probed with anti-His-tag antibody, showing fractions containing purified rFBA (H81/84A). Lane 1: Prestained protein ladder; Lane 2: whole cell lysate; Lane 3: soluble lysate; Lane 4: insoluble pellet; Lane 5: flow through; Lane 6: washing from column; Lane 7: Prestained protein ladder; Lane 8-17: eluted fractions 1-10 in panel A; Lane 18: after elution wash; Lane 19: purified rFBA (WT) was used as positive control. The blue arrow indicates purified rFBA.

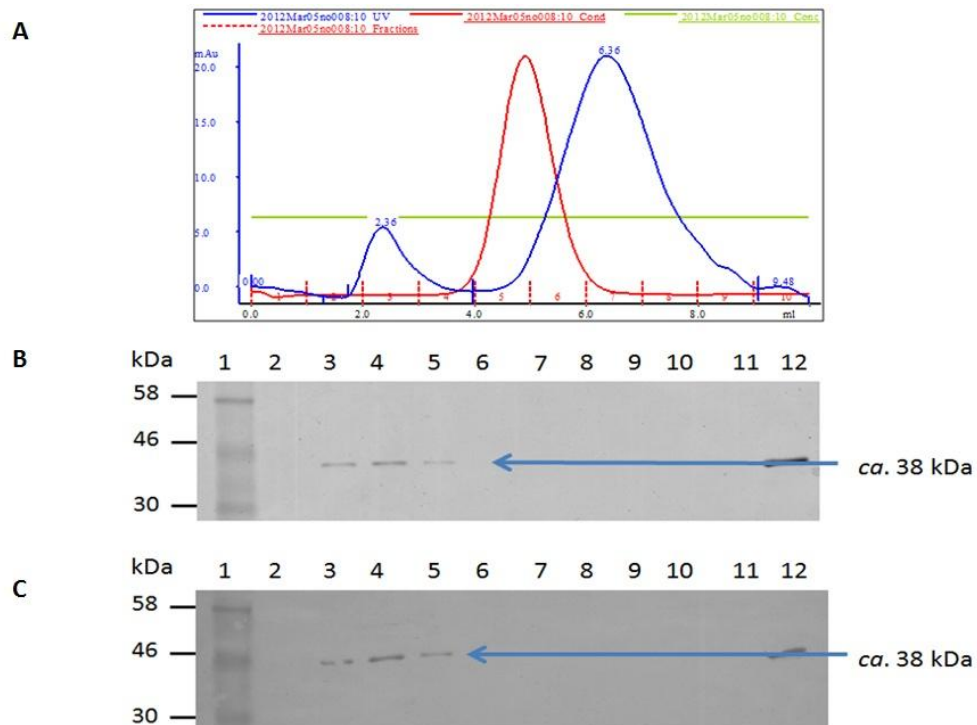


Figure 2.13: Purification of rFBA (H81/84A) by buffer exchange. (A) Representative chromatogram displaying the typical purification profile for rFBA(H81/84A). The Y-axis shows absorbance at 280nm and X-axis displays elution volume. UV absorbance, concentration and conductivity are indicated by blue, green and red lines respectively. Purified rFBA (H81/84A) samples collected from buffer exchange were separated by (B) SDS-PAGE and rFBA was identified by (C) immunoblot with anti-His-tag antibody. Lane 1: Prestained Protein marker; Lane 2-11: eluted fractions 1-10 in panel A; Lane 12: Purified rFBA was used as positive control. The blue arrow indicates purified rFBA.

2.3.6 Purified rFBA (WT) is an active aldolase enzyme

A coupled assay showed that the purified rFBA (WT) had aldolase enzymic activity (Fig. 2.14). The kinetic parameters of rFBA (WT) for the cleavage of FBP were calculated as $K_m=0.027$ mM and $k_{cat}=420$ min⁻¹.

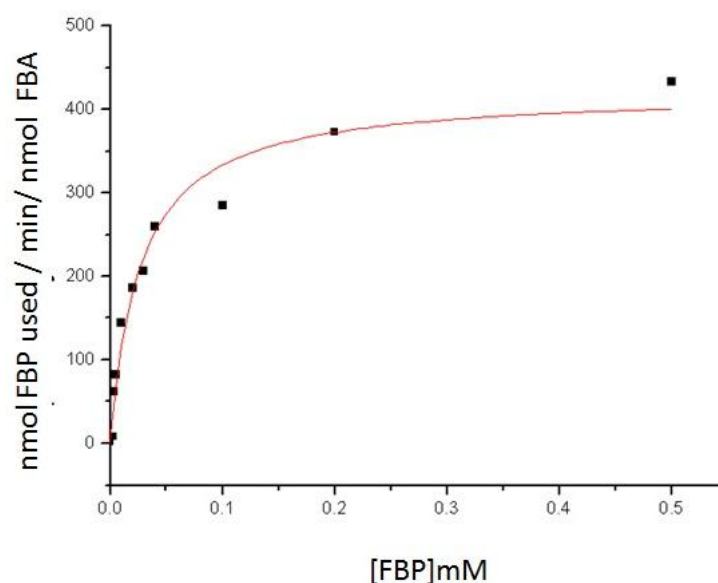


Figure 2.14: Kinetic analysis of rFBA (WT). The reaction was carried out for one min with a range of FBP concentrations (0-0.5 mM). Enzyme activity was measured by the decreasing absorbance at 340 nm and using 6220 M⁻¹ cm⁻¹ as the molar extinction coefficient for NADH. Kinetic parameters were estimated using the Origin Pro 7.5 software.

In contrast, the two mutated rFBA's were virtually inactive. Addition of excess enzyme (0.5 mg/ml) did not result in a measurable activity (data not shown). This loss of aldolase activity suggested that mutation of either D83A or H81/H84A was sufficient to abolish enzymic activity, possibly due to the absence of zinc co-factor binding.

2.4 Discussion

The *N. meningitidis* MC58 genome contains only one *cbbA* gene (NMB1869) to encode FBA (Tettelin *et al.*, 2000). Tunio *et al* reported that, although found in the cytoplasm, FBA could also be found localised to the outer membrane of *N. meningitidis* and was surface accessible to anti-FBA antibody. Furthermore, an FBA-deficient mutant was less adherent to human epithelial and endothelial cells (Tunio *et al.*, 2010) and the FBA deletion mutant was unable to produce sustained bacteraemia in a transgenic mouse model of infection (D. Turner, personal communication) confirming the *in vivo* significance of this protein in meningococcal pathogenesis.

For the biochemical and functional analysis of a protein, it is common practise to express, in *E. coli*, a recombinant version and then to purify that protein (Lebendiker and Danieli, 2014). In a previous study, Tunio *et al.*, successfully cloned *cbbA* into pQE-70 to yield pSAT-9. Based on this plasmid, in this study, several other plasmids were generated to express rFBA having mutation(s) in the co-factor binding site of FBA. This chapter detailed the expression, purification of rFBA (WT and mutants) and their biochemical characterisation.

Site directed mutagenesis was used to introduce mutations into the cofactor binding site of FBA - namely D83A and H81A/H84A. In a pilot expression experiment, no expression of rFBA was observed. One reason for this was thought to be the distance between ribosome binding site (RBS) and start codon. Therefore another 4bp deletion mutation was carried out to bring the RBS and start codon together. After that had been achieved, the new construct (pFS-1), was used as template to introduce the mutation(s) in the same cofactor

binding site as described above. After confirmation of correct mutation(s) by sequencing, a pilot expression experiment was carried out. *E. coli* JM 109 cells transformed with these vectors, produced rFBA after induction with IPTG. Therefore *E.coli* JM109 was used to over express rFBA WT, D83A and H81A/H84A.

The next step was the purification process. Use of His-tag at the C-terminal end facilitated the purification of rFBA using His Trap^{FF} column prepacked with Ni Sepharose 6 Fast Flow connected to a ÄKTAprime plus liquid chromatography system. All the steps in the purification process were monitored by collecting fractions and running SDS-PAGE gel and immunoblot probed with anti-His-tag antibody.

One observation from this study was that following whole cell lysis, rFBA (H81A/H84A) was found to be associated with cellular material rather than in the soluble fraction.

Human copper/zinc superoxide dismutase (SOD1) does not form intracellular aggregates but a mutant variant of SOD1 containing alanine to valine mutation at position 4 (SOD1A4V) greatly increases the aggregation-propensity. (Gregoire *et al.*, 2014). In another study, mutations in either two (H46R/H48Q) or four copper binding sites (H46R/H48Q/H63G/H120G or QUAD) cause formation of aggregates of SOD1 (Wang *et al.*, 2002; Wang *et al.*, 2003). According to a recent report, another human protein α -synuclein (α -Syn) aggregates faster than the wild-type when it carries a single mutation of H50Q, (Porcari *et al.*, 2015). A number of variants of human aldolase B (A174D, L256P, and N334K) were difficult to recover when recombinantly expressed in *E. coli* at 37 °C. The authors suggested possible proteolysis of the recombinant

proteins during expression resulted in denaturation *in situ*, and precluded the formation of stable tetramers (Rellos *et al.*, 2000). GST tagged wild type N-terminal domain of *E.coli* hydrogenase maturation factor (HypF-N) remains soluble after recombinant expression. In contrast some mutant variants (F22A, L29A, L33A, L58A, and F88A) appear to aggregate completely after expression *in vivo* (Calloni *et al.*, 2005).

In zinc metalloenzymes, the zinc ion can perform catalytic, cocatalytic or structural functions. Apart from involvement in the catalytic process, zinc can confer structural stability, therefore abolishing zinc binding can be considered as a cause of protein misfolding or aggregation (McCall *et al.*, 2000). The possible involvement of the double mutation introduced into the zinc binding site, in the formation of inclusion bodies, cannot be excluded.

Expression of recombinant proteins often causes the formation of insoluble aggregates or inclusion bodies in host *E. coli* cells (Kane and Hartley, 1988). There are numerous methods available for the purification of proteins from inclusion bodies in a denatured state which can be refolded again (Singh and Panda, 2005). Variation in expression conditions like changing host *E.coli*, growing the bacterial cells at lower temperature, longer induction period, using lower concentration of IPTG could have been followed for soluble expression of recombinant protein (Rosano and Ceccarelli, 2014). However, the whole process needed to be optimised according to the protein, which would be time consuming, so it was decided to purify the small amount of soluble rFBA (H81A/H84A), rather than try to trouble-shoot alternative methodologies to purify the protein from the insoluble material.

If the conditions for expression, purification, storage of proteins are not maintained at optimal level, proteins tend to aggregate with a loss of activity (Kerwin *et al.*, 1998; Lu *et al.*, 2001; Wilson *et al.*, 1996; Kreilgaard *et al.*, 1998). A problem with precipitation of purified rFBA was encountered in this study when ice cold buffers were used or the purified fractions were stored at -20°C or -80°C. Occasionally the purified rFBA started to precipitate immediately after purification even before storage at -20°C. Several approaches were taken to prevent precipitation of the purified protein, for example using storage at ambient temperatures, collecting the eluted proteins into elution buffer in order to dilute down the protein, and after buffer exchange collecting purified protein in PBS - glycerol. Co-solvents such as, amino acids, salts and polyols (Timasheff, 1998; Collins, 2004) improve stability of proteins. Glycerol is one of the most extensively used polyols (Gekko and Timasheff, 1981) in experiments involving protein refolding and crystallization (Sedgwick *et al.*, 2007; Mishra *et al.*, 2005). Glycerol induces a compact conformation of proteins (Priev *et al.*, 1996), stabilizes partially unfolded intermediates, and thus inhibits progression to an aggregated state (Mishra *et al.*, 2007). Use of glycerol in this study successfully prevented the aggregation of purified rFBA.

In a previous study, Tunio *et al.*, successfully cloned *cbbA* in the pQE-70 vector to produce C-terminally His-tagged rFBA which was purified and enzymatic activity was determined (Tunio *et al.*, 2010a) but failed to purify rFBA with N-His-tag under native conditions. A similar situation arose during purification of rFBA of *M. tuberculosis* by Ramsaywak *et al.* This was possibly due to interference by the N-His-tag in the oligomerisation of aldolase, resulting in an inactive enzyme (Ramsaywak *et al.*, 2004). Rukseree *et al.*,

purified rFBA with a C-terminal His-tag and determined its enzymatic activity and compared this to the biochemical characteristics of a non-His-tagged FBA. Both of them were found to be similar (Rukseree *et al.*, 2008). Therefore it can be assumed that addition of His-tag to the C-terminal end would not have any adverse effect on the enzymatic activity of FBA purified in this study. Moreover FBA purified from *M. tuberculosis* had lower specific activity than the same FBA recombinantly expressed in *E.coli* (Bai *et al.*, 1975). Therefore it is not unlikely that meningococcal FBA expressed recombinantly in *E.coli* and FBA purified from *N. meningitidis* might have different activity. But this could only be checked if FBA could be purified in its native form from *N. meningitidis* which in turn might impose technical difficulty, therefore was not attempted in this study.

All the purified rFBA (WT and MT) proteins were subjected to enzymatic activity measurements in order to see the effect of the site-directed mutations on aldolase activity. The WT rFBA was confirmed to be a highly active aldolase with the kinetic parameters $K_m=0.027$ mM and $k_{cat}=420$ min⁻¹. These values are similar to that of the corresponding class IIB protein of *E. coli* $k_{cat}=490$ min⁻¹ and $K_m=0.19$ mM (Plater *et al.*, 1999) and was broadly in agreement with the previously determined values for the *N. meningitidis* FBA protein, $K_m=0.05$ mM and $k_{cat}=126$ min⁻¹, which was purified using a different methodology (Tunio *et al.*, 2010a). Neither of the two mutant rFBA exhibited detectable aldolase enzyme activity meaning that the mutations in the predicted cation binding site abolished the aldolase activity of rFBA. These results were also in agreement with those found in *E. coli*. In *E. coli*, mutation at zinc binding histidine residues His-108 and His-111 resulted aldolase

enzyme with no detectable catalytic activity (Berry and Marshall, 1993). In contrast mutations at aspartic acid residue (Asp-109) caused a dramatic decrease in k_{cat} but not in K_m , whereas aspartic acid residues at other positions changed the steady state kinetic parameters to a lesser extent (Plater *et al.*, 1999).

As previously stated, FBA is involved in the pathogenesis of meningococcal infection; the question arises whether or not it is the aldolase activity of the protein which is required for optimal pathogenesis. To address this, recombinant wild type and mutant derivatives of FBA were expressed and purified under non-denaturing conditions and their respective aldolase activities determined. As specific amino acids which could be altered to abolish aldolase activity, were identified, the same point mutation(s) could be introduced into the meningococcus to assess the effects of this mutation on pathogenesis.

Chapter Three: FBA is a plasminogen binding protein of
N. meningitidis

3.1 Introduction

Moonlighting proteins are a group of proteins which can perform several autonomous, unrelated functions when localised at different sites within a cell. These proteins do not employ multiple protein domains to perform different functions (Jeffery, 1999; Huberts and van der Klei, 2010). Several reports have been published demonstrating the presence of housekeeping enzymes or molecular chaperones on the surface of prokaryotic and eukaryotic cells, despite lacking anchoring motifs or secretion signals, and imparting virulence properties to pathogenic bacteria (Pancholi and Chhatwal, 2003; Henderson and Martin, 2011). A number of glycolytic enzymes have been reported to act as moonlighting proteins in bacteria, for example, triose phosphate isomerase (TPI), aldolase, enolase and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Henderson and Martin, 2011). Research has established that bacterial moonlighting proteins impart virulence properties to many pathogens such as *M. tuberculosis*, *S. pyogenes*, *S. pneumoniae*, *S. aureus* and *H. pylori*. Adhesion to host epithelia and extracellular matrices (ECMs), recruitment and activation of plasminogen (Plg) and the modulation of host immune responses are some of the non-glycolytic moonlighting functions which have been reported (Henderson and Martin, 2011).

Fructose 1, 6-bisphosphate aldolases (FBA) are glycolytic enzymes of the EMP pathway and are commonly reported as moonlighting proteins (Henderson and Martin, 2011). The different cellular localisation of FBA and its possible alternate functions have already been addressed. There are reports regarding the subcellular localisation of FBA in mammalian cells (Mamczur and Dzugaj,

2004; Mamczur and Dzugaj, 2008; Saez and Slebe, 2000) and its various roles, such as modulation of signal transduction pathways (Kim *et al.*, 2002) and interaction with the cytoskeleton by binding with actin (Benziane *et al.*, 2007). In human and yeast cells, membrane assembly of vascular H⁺-ATPase has been induced by fructose 1,6-biphosphate aldolase (Lu *et al.*, 2007). Roumelioti *et al.*, reported the possible involvement of FBA from *A. nidulans* in the turnover of the L-glutamate/L-aspartate transporter (AgtA) by acting as a link between the vesicle containing amino acid transporters with actin and the cytoskeleton (Roumelioti *et al.*, 2010). In *Toxoplasma* and *Plasmodium*, a bridge is formed between F-actin and extracellular adhesion molecules by aldolase to facilitate motility and invasion of host cells (Jewett and Sibley, 2003). Much less work has been done with bacterial FBA. It has been shown that FBA functions as an adhesin for *S. pneumoniae* to bind epithelial cells and the host molecule that binds FBA was putatively identified as Flamingo cadherin receptor (FCR) (Blau *et al.*, 2007). In addition, Class II FBA was also found to be localised to the surface of *M. tuberculosis* where it binds human plasminogen and protects FBA-tb bound plasmin from the regulatory effect of α 2-antiplasmin (de la Paz Santangelo *et al.*, 2011).

Plasminogen is a 92-kDa glycoprotein proenzyme which can be proteolytically activated to plasmin. Bacteria can interfere with the plasminogen system by two general mechanisms: firstly by producing activators of plasminogen and secondly by producing receptors to immobilise plasminogen on the cell surface which can then be activated to plasmin by host activators such as tissue-type plasminogen (tPA) activators and urokinase plasminogen activator (uPA) (Bergmann and Hammerschmidt, 2007). Streptokinase, staphylokinase and PIa

produced by Group A, C and G streptococci, *S. aureus* and *Yersinia pestis*, respectively, are some well-established examples of plasminogen activators of bacterial origin (Lahteenmaki *et al.*, 2001b). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, SDH, Pir) produced by Group A and C streptococci (Broder *et al.*, 1991; Lottenberg *et al.*, 1992; Winram and Lottenberg, 1996; Winram and Lottenberg, 1998; Gase *et al.*, 1996; Pancholi and Fischetti, 1992), enolase of Group A streptococci (Pancholi and Fischetti, 1998), flagella of *E. coli* (Lahteenmaki *et al.*, 1993), aspartase and protein E (PE) of *H. influenzae* (Sjostrom *et al.*, 1997; Barthel *et al.*, 2012), ErpA / ErpC / ErpP, OspA, OspC, and enolase of *Borrelia burgdorferi* (Brissette *et al.*, 2009; Fuchs *et al.*, 1994; Lagal *et al.*, 2006; Floden *et al.*, 2011) are a few examples of plasminogen receptors on the bacterial cell surface. Once activated, plasminogen can be converted to the serine protease, plasmin, which can degrade extracellular matrix and basal membrane (BM) proteins such as fibronectin, vitronectin and laminin (Plow *et al.*, 1995) and activate other proteolytic enzymes including matrix metalloproteinases (MMPs) and latent macrophage elastase, resulting in the cleavage of elastin, collagen, and proteoglycans (Murphy *et al.*, 1999). The destruction of matrix proteins facilitates dissemination of bacteria, and thus contributes to the pathogenicity and invasive nature of disease-causing bacteria (Lahteenmaki *et al.*, 2005; Lahteenmaki *et al.*, 2001b). Binding and activation of plasminogen in order to degrade ECM or BM during bacterial infection is similar to that observed in tumour cell metastasis (Liotta *et al.*, 1986), thus the phenomenon of degradation of basal membrane for bacterial translocation has been termed 'bacterial metastasis' (Korhonen *et al.*, 1992). Enhanced fibrinolytic activity associated with meningococcal meningitis has raised

suggestions that plasminogen activation might be involved in systemic infections (Brandtzaeg *et al.*, 1990). Since many pathogenic bacteria produce activators or receptors for plasminogen, the formation and retention of plasmin on the bacterial surface may play an important role in invasive infections (Korhonen *et al.*, 1992; Lottenberg *et al.*, 1994; Boyle and Lottenberg, 1997; Coleman and Benach, 1999). Examples include: *Borrelia burgdorferi* (Coleman *et al.*, 1997; Coleman *et al.*, 1995); *Y. pestis* (Lahteenmaki *et al.*, 2001a; Sodeinde *et al.*, 1992); *S. pyogenes* (Sun *et al.*, 2004); *H. influenzae* (Ullberg *et al.*, 1990; Virkola *et al.*, 1996) and *S. pneumoniae* (Bergmann *et al.*, 2005; Eberhard *et al.*, 1999). Moreover, according to recent reports, activation of surface bound plasminogen to plasmin imparts anti-phagocytic and anti-opsonisation properties to *B. anthracis* (Chung *et al.*, 2011) and *B. burgdorferi* (Grosskinsky *et al.*, 2009).

Ullberg *et al.*, first reported that *N. meningitidis* and *N. gonorrhoeae* could bind plasminogen which could then be activated to plasmin by uPA or tPA, although no receptors were identified (Ullberg *et al.*, 1992). In a later study, enolase, DnaK and peroxiredoxin were identified as putative plasminogen-binding proteins on the surface of *N. meningitidis* (Knaust *et al.*, 2007). However, they failed to identify three additional plasminogen-binding proteins that were found during the same study. One of these unidentified proteins had a molecular weight of approximately 35-kDa, which is close to that predicted for FBA (38-kDa), indicating the possibility that FBA may be an additional plasminogen binding protein in the meningococcus. Furthermore, FBA has a lysine-rich region at the C-terminal end of the protein, which is often found in plasminogen binding proteins. Moreover, in preliminary studies in our

laboratory, rFBA was shown to bind human plasminogen in a dose dependent manner, but rFBA did not bind laminin, collagen or fibronectin. FBA-plasminogen interaction was inhibited by EACA. This study aimed to confirm these initial observations and determine whether meningococcal FBA can bind plasminogen. For this purpose, EIA assays using rFBA and flow cytometric analysis using whole meningococcal cells were employed.

3.2 Materials and Methods

3.2.1 Bacterial techniques

3.2.1.1 Bacterial strains, growth conditions and media

Neisserial strains (Table 3.1) were grown on chocolated horse blood agar (Oxoid), Brain Heart Infusion (BHI) agar (Oxoid), or in Brain Heart Infusion broth (Oxoid), containing kanamycin ($50 \mu\text{g ml}^{-1}$; Sigma-Aldrich) or erythromycin ($5 \mu\text{g ml}^{-1}$; Sigma-Aldrich) as appropriate, and incubated at 37°C (with or without shaking) in 5% CO_2 .

3.2.1.2 Flow cytometry analysis

The ability of whole cells of *Neisseria* spp. to bind plasminogen was determined by flow cytometry. Samples were prepared for analysis by flow cytometry as described previously (Tunio *et al.*, 2010a; Barthel *et al.*, 2012). Briefly, bacteria were grown to mid-log phase (OD 0.7 at 600nm). Aliquots containing *ca.* 1×10^7 cfu were washed by re-suspending in sterile filtered PBS followed by centrifugation at $5000 \times g$ for 5 min. Human glu-plasminogen ($3 \mu\text{g ml}^{-1}$; Calbiochem) was added to the resuspended cells and incubated for 1 h at 37°C . After incubation the cells were washed twice with sterile filtered PBS to remove any unbound plasminogen. Goat anti-plasminogen antibody (1:100; Rockland Immunochemicals) and donkey anti-goat IgG-Alexa Fluor 488 conjugate (1:50; Invitrogen), both diluted in PBS, 0.1% (wt/vol) BSA, 0.1% (wt/vol) sodium azide and 2% (vol/vol) foetal calf serum were used as primary and secondary antibodies, respectively. After addition of antibodies, cells were incubated for 2 h in ice followed by washing three times with PBS. Samples treated with secondary antibody were kept in the dark. After final washing, the cells were fixed by resuspending in 1 ml PBS containing 0.5% (wt/vol)

formaldehyde. For each strain, four samples were prepared: untreated, treated with primary antibody only, treated with secondary antibody only or treated with both primary and secondary antibodies. Analysis of all samples was performed using BD FACS Aria™ flow cytometer and BD FACS Diva software (BD Biosciences). The cells were detected using forward and side scatter dot plot and bacterial aggregates and cell debris were excluded by defining a gating region. For each sample, a total of 20,000 events were analysed for fluorescence signals. Subsequent analysis of data was performed using Weasel software.

Table 3.1: Bacterial strains used in work described in this chapter

Bacterial strain	Characteristics	Source or reference
<i>N. meningitidis</i>		
MC58	Wild type serogroup B isolate	(Tettelin <i>et al.</i> , 2000)
Z4181	Serogroup C clinical isolate (carrier)	Mali, 1989
Z4667	Serogroup B clinical isolate (invasive)	Netherlands, 1963
Z4673	Serogroup B clinical isolate (invasive)	Netherlands, 1986
8013	Serogroup C clinical isolate (invasive)	(Rusniok <i>et al.</i> , 2009)
MC58 Δ <i>cbbA</i>	<i>cbbA</i> deleted and replaced with kanamycin cassette	(Tunio <i>et al.</i> , 2010a)
Z4181 Δ <i>cbbA</i>	<i>cbbA</i> deleted and replaced with kanamycin cassette	S. Tunio
Z4667 Δ <i>cbbA</i>	<i>cbbA</i> deleted and replaced with kanamycin cassette	S. Tunio
Z4673 Δ <i>cbbA</i>	<i>cbbA</i> deleted and replaced with kanamycin cassette	S. Tunio
8013 Δ <i>cbbA</i>	<i>cbbA</i> deleted and replaced with kanamycin cassette	S. Tunio
Other <i>Neisseria</i> spp.		
<i>Neisseria gonorrhoeae</i>	ATCC 700825 (FA1090)	NCTC
<i>Neisseria lactamica</i>	ATCC strain 23970	(Minogue <i>et al.</i> , 2014)
<i>Neisseria polysacchareae</i>	Clinical isolate	(Oldfield <i>et al.</i> , 2007)

3.2.2 Protein techniques

3.2.2.1 Enzyme-linked immunosorbant assay (EIA)

The binding of rFBA (WT) to glu-plasminogen (Calbiochem), laminin (human laminin, Merck Millipore), fibronectin (fibronectin from human plasma, Sigma-Aldrich) and collagen (human collagen I, BDBioscience) was done by Enzyme-linked immunosorbant assay (EIA). Microplate wells were coated with 100 μ l aliquots of 0.5-5 μ g ml⁻¹ protein in sodium carbonate buffer (0.1M sodium carbonate- sodium bicarbonate buffer pH 9.6) and incubated at room temperature (RT) for an hour. The plate was washed five times with PBS-T and blocked with 100 μ l of 1% (wt/vol) BSA in PBS for 1 h at RT. After washing, 100 μ l of 5 μ g ml⁻¹ rFBA in 1% (wt/vol)BSA in PBS was added and incubated at RT for 1h or at 4°C overnight. As a positive control, 100 μ l of 5 μ g ml⁻¹ recombinant His-tagged human laminin receptor in 1% (wt/vol) BSA in PBS was added and incubated at 4°C overnight. Following vigorous washing with PBST, 100 μ l of anti-penta His antibody (Qiagen; diluted 1:2000 in 1% (wt/vol) BSA in PBS) was added for incubation for 1 h at RT. Plates were again washed, before the addition of 100 μ l goat anti-mouse IgG-HRP conjugate (Sigma-Aldrich; diluted 1:10,000 in 1% (wt/vol) BSA in PBS) was added and incubated for 1 h. After washing 100 μ l ABTS substrate (Roche) was added Absorbance at 405 nm was measured (Biotek EL800).

rFBA (10 μ g ml⁻¹) (WT, D83A, H81A/84A), diluted in coating buffer (0.1M sodium carbonate- sodium bicarbonate buffer pH 9.6), was immobilised onto a microtitre plate (Nunc 96-well plates, PolySorp) for 1 h at RT . The plate was washed five times with PBS-T and blocked with 100 μ l 1% (wt/vol) BSA in PBS for 1 h at RT. 100 μ l glu-plasminogen in 1% (wt/vol) BSA in PBS (10 μ g

ml⁻¹) was added and incubated for 1 h. Goat anti-human plasminogen antibody (1:10,000; Rockland Immunochemicals) was incubated for 1 h at RT followed by donkey anti-goat IgG-alkaline phosphatase (1:5,000; Promega). 200 µl of phosphatase substrate (Sigma-Aldrich), dissolved in buffer containing 0.1M glycine, 1mM ZnCl₂ and 1mM MgCl₂, pH 10.4, was added into each well and readings were taken at 405 nm using an EIA plate reader (BioTek).

The same EIA procedure was used in the presence of either NaCl (0-500mM), rabbit anti-FBA antiserum (0, 1:100, 1:250, 1:500 or 1:1000 dilutions) and ε-aminocaproic acid (EACA; 5mM; Sigma-Aldrich) to evaluate their effect on plasminogen binding by rFBA.

Absorbance values shown in the graphs are minus the values obtained from control wells coated with 1% (wt/vol) BSA in PBS. All experiments were repeated at least three times unless otherwise stated. Statistical analysis was performed using GraphPad Prism6. Student's t-test was performed to calculate significant differences. *p* value of <0.05 was considered significant.

3.2.2.2 Plasminogen activation assay

A microtiter plate coated with 10 µg ml⁻¹ rFBA (WT, D83A or H81A/84A) was incubated with plasminogen and washed as described in section 3.2.2.1. Four ng well⁻¹ uPA (derived from human kidney cells; Sigma-Aldrich) and 50 µg well⁻¹ chromogenic substrate (D-Valyl-leucyl-lysine p-nitroanilide dihydrochloride, Sigma-Aldrich) diluted in PBS, were added. The cleavage of the substrate was followed by measuring the absorbance at 405 nm for 24 h at 37°C.

3.3 Results

3.3.1 FBA is a plasminogen binding protein of *N. meningitidis*

In this preliminary study, a number of human proteins were examined for their ability to bind rFBA. As shown in Figure 3.1, rFBA was able to bind plasminogen but not fibronectin, laminin or collagen. Binding of plasminogen was increased in the presence of a higher concentration of rFBA ($5 \mu\text{g ml}^{-1}$ compared to $0.5 \mu\text{g ml}^{-1}$). Binding of rFBA to 1% BSA was used as the negative control to determine non-specific binding/absorbance

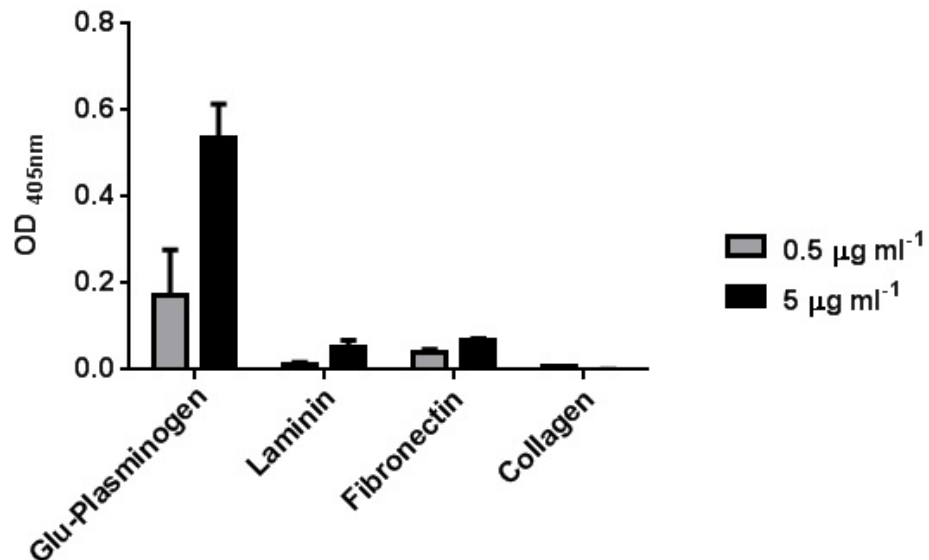


Figure 3.1: Meningococcal FBA is a plasminogen-binding protein. Glu-plasminogen, laminin, fibronectin and collagen were coated to EIA wells and then incubated with purified rFBA ($5 \mu\text{g ml}^{-1}$). Binding was detected by anti-penta-histidine antibody and anti-mouse IgG conjugate. The data represents the means of three independent experiments carried out in triplicate wells. Error bars indicate SE.

3.3.2 Cofactor binding site residues of rFBA are not involved in plasminogen binding

In Chapter Two, it was described that whereas the wild type rFBA had aldolase enzyme activity, the two mutated rFBA proteins (with mutation(s) in the cofactor binding site of FBA, D83A and H81/84A, respectively) were enzymatically inactive. Therefore all purified rFBA (WT, D83A and H81/84A) were tested for their ability to bind glu-plasminogen by EIA. No significant difference was observed in the plasminogen binding ability of rFBA WT, D83A and H81A/H84A, which indicates that mutation(s) in the cofactor binding site of FBA has no significant effect on plasminogen binding (Figure 3.2).

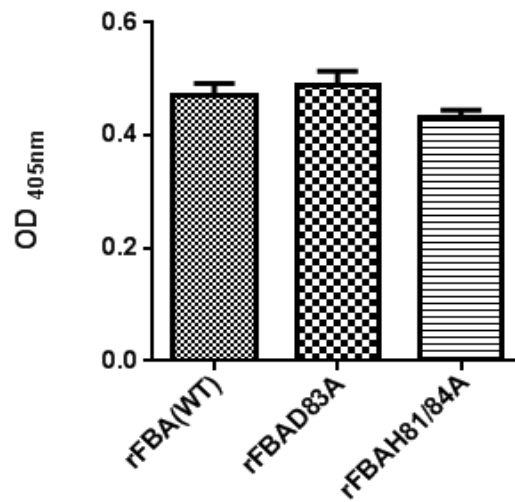


Figure 3.2: Mutations in the cofactor binding site of *N. meningitidis* FBA has no significant effect on plasminogen binding. rFBA (WT, D83A and H81/84A), immobilised on microtiter plate wells, was incubated with human glu-plasminogen. Bound plasminogen was detected by addition of goat anti-plasminogen antibody and donkey anti-goat IgG-AP conjugate. rFBA (D83A) and rFBA (H81/84A), which are enzymatically inactive, were also able to bind plasminogen similar to enzymatically active rFBA(WT) indicating an important characteristic of FBA as a moonlighting protein. Six separate experiments were performed in triplicate wells. Error bars indicate SE.

3.3.3 Inhibition of FBA-plasminogen binding by the lysine analogue ϵ -amino caproic acid (EACA)

To determine whether lysine residues in FBA were important for plasminogen binding, EIA assays were performed in presence of EACA - a lysine analogue known to inhibit the lysine-mediated binding of partners to plasminogen. It was found that in presence of 5 mM EACA, plasminogen binding was reduced significantly (Figure 3.3). This indicates that lysine residue(s) play a critical role in plasminogen binding. As expected, EACA inhibited plasminogen binding by the wild type rFBA and also the two mutant rFBAs (D83A and H81/84A).

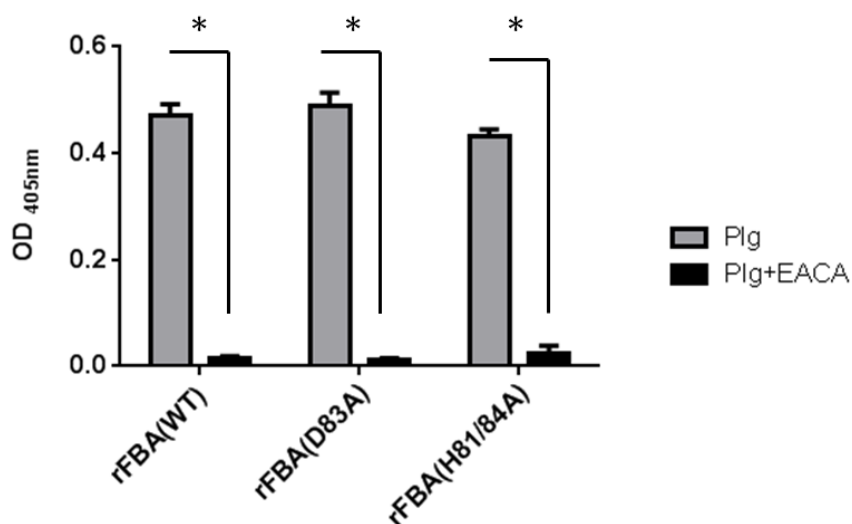


Figure 3.3: Binding of human plasminogen to rFBA was inhibited by the lysine analogue EACA. Human Plg with (black bars) or without EACA (dark grey bars) was added to rFBA immobilised on microtiter plate wells. Bound plasminogen was detected by addition of anti-plasminogen antibody and donkey anti-goat IgG-AP conjugate. EACA inhibited plasminogen binding by rFBA irrespective of its enzymatic activity indicating the involvement of lysine residues in this interaction. $n=6$. * denotes p value <0.05 .

3.3.4 Effect of anti-FBA antiserum and NaCl on plasminogen binding

To determine whether the interaction between plasminogen and FBA is ionic or hydrophobic in nature, the plasminogen binding assay was performed in the presence of an increasing concentration of NaCl (0-500mM). The binding of rFBA to plasminogen was found to decrease in the presence of an increasing concentration of NaCl, with a statistically significant reduction in binding occurring at 500mM NaCl (Figure 3.4). As expected, this effect was not altered by mutation of the rFBA active site.

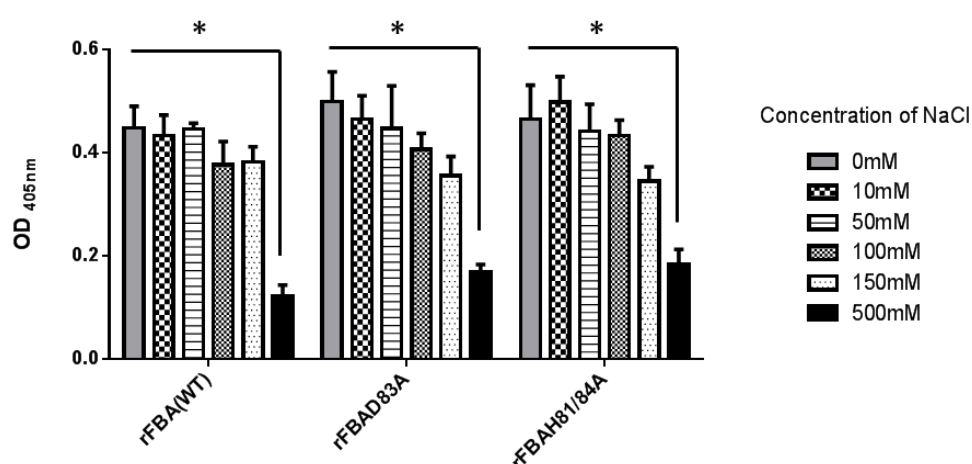


Figure 3.4: Effect of NaCl on FBA-plasminogen interactions. Binding of human plasminogen to rFBA was inhibited by an increasing concentration of NaCl. Statistically significant inhibition was observed at 500mM NaCl (black bars) compared to 0mM NaCl (dark grey bars). n=4. * denotes p value <0.05.

Similarly, the effect of anti-FBA antibody on the ability of FBA to bind plasminogen was examined by EIA. Four different dilutions of anti-FBA antibody were used in this experiment. At a dilution of $\leq 1:250$ of rabbit anti-FBA antiserum, the binding of plasminogen by rFBA was significantly inhibited (Figure 3.5). This suggests competition between rabbit anti-FBA

antiserum and plasminogen to bind to FBA. As expected, this effect was not altered by mutation of the rFBA active site.

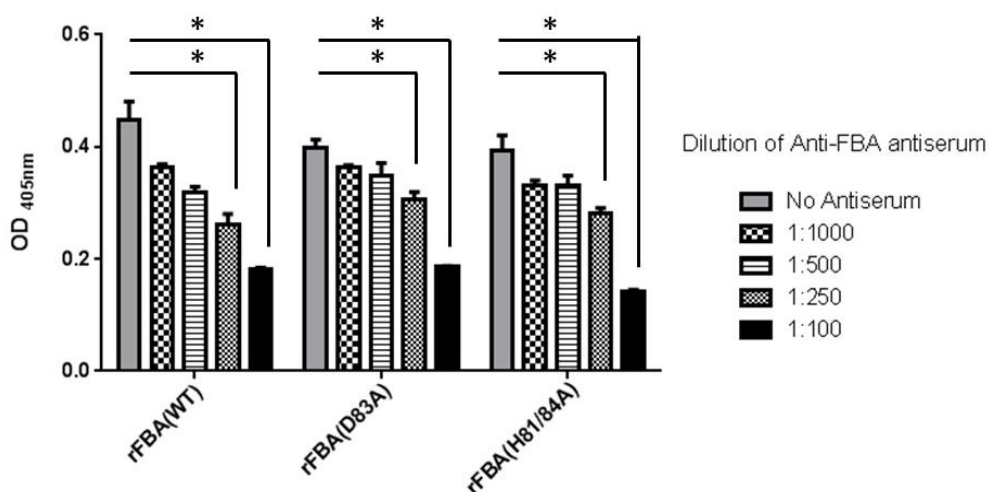


Figure 3.5: Effect of anti-FBA antiserum on rFBA-plasminogen interactions. Binding of human plasminogen to rFBA was inhibited by an increasing concentration of anti-FBA antiserum. 1:250 and higher dilutions of anti-FBA antiserum were able to significantly inhibit the binding of plasminogen to rFBA. $n=3$. * denotes p value <0.05 .

3.3.5 Plasminogen activation assay

In order to determine whether rFBA-bound plasminogen was able to become activated to plasmin by uPA, a plasminogen activation assay was performed. rFBA was incubated with plasminogen and uPA. Time dependent cleavage of a chromogenic substrate by the generated plasmin was used to detect the activation of plasminogen to plasmin. In absence of rFBA, cleavage of chromogenic substrate by plasmin generated from plasminogen was observed but no cleavage of the substrate was observed in presence of rFBA even after 24 h (result not shown). Therefore, it can be concluded that although rFBA may sequester plasminogen, it prevents its activation to plasmin.

3.3.6 Plasminogen binding is a common property of *Neisseria* spp.

MC58 WT and MC58 Δ *cbbA* were incubated with plasminogen, then goat anti-plasminogen antibody followed by anti-goat IgG-Alexa Fluor 488 conjugate. Both the strains (94.6% for MC58; 92.5% for MC58 Δ *cbbA*, respectively), were able to bind plasminogen suggesting that plasminogen binding is not affected by the absence of FBA on the meningococcal cell surface (Figure 3.6, rows A and B). It was also observed that >90% cells of the wild type *N. meningitidis* strains Z4667 and Z4673 (Figure 3.6A) and their corresponding *cbbA* null mutant derivatives (Figure 3.6B) were able to recruit plasminogen to their surface. Therefore the strains, irrespective of FBA expression status, were able to bind plasminogen, presumably reflecting redundancy due to other plasminogen binding proteins on the cell surface. Interestingly the binding pattern of two serogroup C strains, Z4181 and 8013, appeared consistently different than that observed with the serogroup B strains MC58, Z4667 and Z4673 (Figure 3.6, rows A and B). In case of wild type *N. meningitidis* strains Z4181 and 8013 and their corresponding *cbbA* null mutant strains, >70% population were able to bind plasminogen (Figure 3.6, rows A and B, respectively). Flow cytometric analysis was also performed to investigate the ability of other *Neisseria* spp. to bind human plasminogen. Similar results were observed with representative *N. gonorrhoeae*, *N. polysacchareae* and *N. lactamica* strains (Figure 3.6, row C).

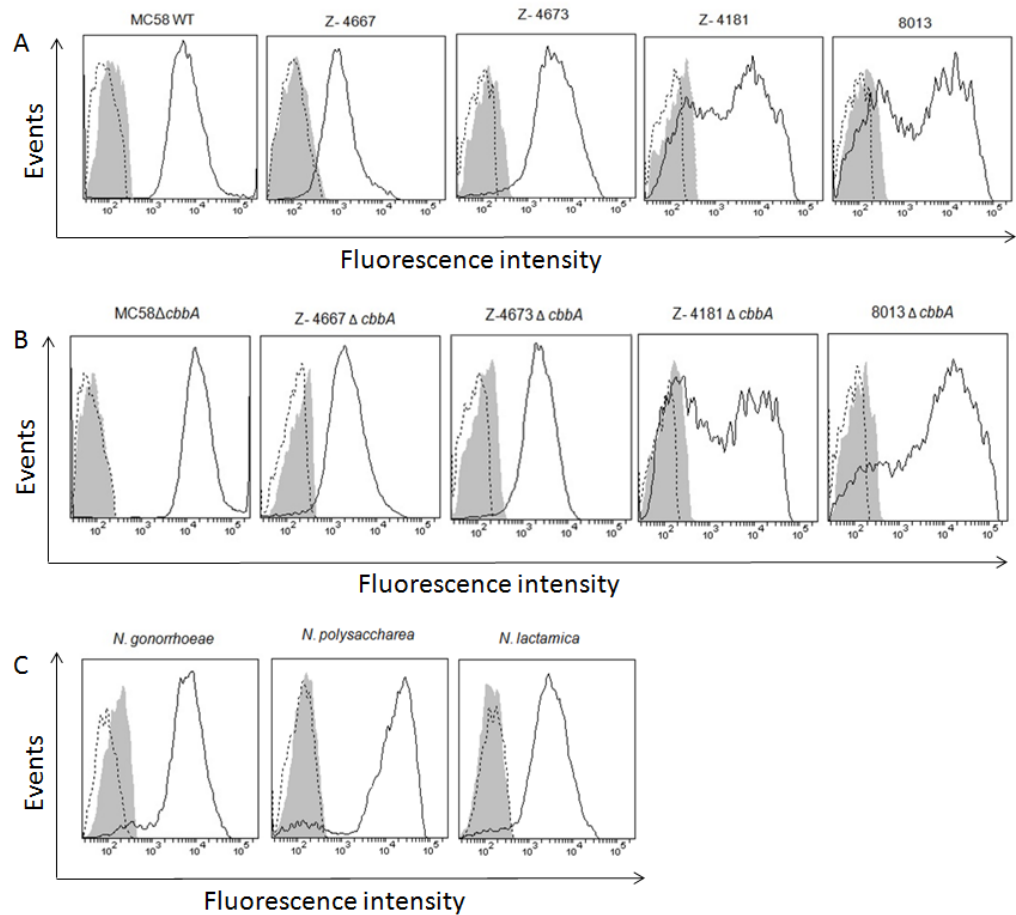


Figure 3.6: Flow cytometric analysis of plasminogen binding. Binding of plasminogen to the surface of neisserial strains was detected by anti-plasminogen antibody and anti-goat IgG-Alexa Fluor488. Plasminogen binds equally to the surface of wild type meningococcal strains (row A) and their corresponding *cbbA* mutant strains (row B). Other *Neisseria* spp. *N. gonorrhoeae* FA1090, a clinical isolate of *N. polysaccharea* and *N. lactamica* ATCC 23970 can also bind plasminogen (row C). Fluorescence is displayed as a histogram: area enclosed by the dashed line indicates bacteria treated with primary antibody only; area shaded in grey indicates bacteria treated with secondary only; area enclosed by the solid line indicates bacteria treated with both antibodies.

3.4 Discussion

FBA is a glycolytic enzyme in the EMP pathway – a pathway that is not functional in *N. meningitidis*. For the first time, Tunio *et al* reported that although found in the cytoplasm, FBA is also localised to the outer membrane of *N. meningitidis* and was surface accessible to anti-FBA antibodies (Tunio *et al.*, 2010a). Furthermore, an FBA-deficient mutant was less adherent to human epithelial and endothelial cells suggesting a moonlighting role for FBA. It is not surprising that a protein might have more than one moonlighting function on the bacterial surface.

Interaction with a variety of extracellular matrix (ECM) proteins contributes to colonisation, dissemination and virulence of microorganisms within their host organisms. Numerous examples of moonlighting proteins interacting with components of ECM and serum proteins such as fibronectin, laminin, collagen and plasminogen, have already been reported (Henderson and Martin, 2011; Kainulainen and Korhonen, 2014). Therefore an initial screening assay was carried out to identify human protein ligands for FBA. From that screening EIA assay, it was found that rFBA was able to bind plasminogen but not laminin, collagen or fibronectin. Further characterisation of the rFBA-plasminogen interaction was carried out using a series of additional EIA assays described in this chapter.

To confirm the status of FBA as a moonlighting protein, it was necessary to examine whether aldolase activity was required for FBA to be able to bind plasminogen. To address this, recombinant wild type and mutant derivatives of FBA were expressed and purified under non-denaturing conditions and their respective aldolase activities determined. In this study, the wild-type rFBA was

confirmed to be an active aldolase. Neither of the two mutant rFBA exhibited detectable aldolase enzyme activity. These experiments are discussed in chapter 2.

Binding of plasminogen to rFBA was examined by EIA assays. It was found that WT rFBA was able to bind plasminogen as well as the two mutated rFBA proteins (rFBA D83A and rFBA H81/H84A). These data indicate that amino acid residues in the active site are not required for plasminogen binding. Therefore, it may be concluded that FBA, irrespective of its enzymatic activity, is able to bind human glu-plasminogen.

The interaction between plasminogen and its receptors is mediated by kringle domains which are triple-disulfide-bonded regions of plasminogen. These kringle domains have lysine binding sites. Inhibition of plasminogen binding by rFBA by EACA confirms the involvement of lysine residue(s) in this interaction. Previously, Ullberg *et al.*, reported for the first time that *N. meningitidis* and *N. gonorrhoeae* could bind plasminogen, which was inhibited by EACA (Ullberg *et al.*, 1992). Binding of plasminogen to other proteins has been reported to involve C-terminal lysine residues or internal lysine rich motifs (Plow *et al.*, 1995; Wistedt *et al.*, 1998; Bergmann *et al.*, 2003; Ehinger *et al.*, 2004). It was also observed in this study that anti-FBA inhibited plasminogen binding by rFBA. Song *et al.*, reported that enolase is a major plasminogen binding protein of *Mycoplasma bovis*; plasminogen binding by recombinant enolase (rMbEno) was decreased in a dose-dependent manner by anti-rMbEno antiserum (Song *et al.*, 2012).

NaCl was able to reduce plasminogen binding by rFBA in a dose-dependent manner. High concentrations of NaCl (350 mM) affected plasminogen binding

by the LipL32 and rLICi2238 proteins of *Leptospira interrogans* (Vieira *et al.*, 2010). Additionally, plasminogen binding by Protein E (PE) of *H. influenzae* was reported to be ionic strength dependent - 25% and 75% inhibition of plasminogen binding by PE was observed at physiological level of 150 mM and 1 M NaCl, respectively (Barthel *et al.*, 2012). Similarly 800 mM NaCl caused a 40% reduction in plasminogen binding by *S. pneumoniae* endopeptidase O (PepO) compared to that of 150 mM (Agarwal *et al.*, 2013). NaBr, but not NaCl, reduced plasminogen binding by BBA70 of *B. burgdorferi* (Koenigs *et al.*, 2013). A possible explanation for this observation was also provided. As plasminogen binds chloride ions and the concentration of NaCl used in the experiment (0.15 mM – 1 M) was not rigorous enough, NaCl did not exert any effect on plasminogen binding by BBA70. In contrast, NaBr, even at 0.25 M, significantly inhibited plasminogen binding. Considering all these, it was suggested that the BBA70-plasminogen interaction depends on ionic strength (Koenigs *et al.*, 2013).

Binding of plasminogen could contribute to bacterial virulence. Once activated into plasmin, bacteria become capable of degrading fibrin clots and extracellular matrix proteins (Lahteenmaki *et al.*, 2001b). In this study, rFBA-bound plasminogen was not activated to plasmin. Sijbrandi *et al.* stated that Pbp, a protein exposed on the surface of *Bacteroides fragilis*, can bind plasminogen which is not activated, suggesting that sequestering plasminogen is the principal function of Pbp (Sijbrandi *et al.*, 2008). Also triose phosphate isomerase (TPI) on the *S. aureus* cell surface decreases activation of plasminogen bound by enolase (Furuya and Ikeda, 2011).

Flow cytometry was performed with purified human plasminogen. Whole cells of *N. meningitidis* MC58 were able to bind plasminogen irrespective of the presence or absence of FBA on their surface. Greater than 90% of the population of wild type MC58 and other wild type strains and their corresponding *cbbA* mutant strains were able to bind plasminogen, indicating that, as expected, there is a significant degree of redundancy in surface binding of plasminogen. Knaust *et al.* similarly reported that deletion of peroxiredoxin did not have a detectable effect on plasminogen binding and its activation to plasmin by MC58 cells. In the same study, despite several attempts, they failed to generate enolase or DnaK knock out strains, suggesting that these proteins are essential for cell viability, in addition to their putative role on the cell surface as plasminogen receptors. Therefore, FBA might be an additional receptor for plasminogen, indicating considerable redundancy in the process. Moreover, due to high concentration of plasminogen in human serum, all the plasminogen receptors on meningococcal cells are likely to be saturated with plasminogen. Consequently, the number of surface-exposed receptors, and their available binding epitope(s), are important for this interaction, whilst the binding affinity is less important (Knaust *et al.*, 2007). Ulberg *et al.*, estimated that the total number of plasminogen receptors per meningococcal cell was between 13,000-26,000 (Ullberg *et al.*, 1992). Knaust *et al.*, estimated that the three putative receptors they identified could only account for approximately 10,000 receptors (Knaust *et al.*, 2007), further suggesting the presence of other plasminogen binding proteins on the bacterial surface. The presence of such high number of plasminogen receptors indicates that plasminogen binding has a very significant role in neisserial biology.

Flow cytometry data also show that *N. gonorrhoeae* can bind plasminogen as previously reported (Ullberg *et al.*, 1990). In addition to the pathogenic *Neisseria* spp., the commensal species, *N. lactamica* and *N. polysacchareae* were also found able to bind plasminogen in this study. It is worth mentioning that only one isolate from each of *N. gonorrhoeae*, *N. lactamica* and *N. polysacchareae* and a few meningococcal isolates were tested in this study. It is possible that there might be strains that do not bind plasminogen or the ability to bind plasminogen might not be similar for all strains. Therefore screening a large number of strains is required. Occurrence of anchorless glycolytic enzymes on the surface of pathogenic bacteria and their interaction with plasminogen and other human proteins are now well established, but only recently it has become evident that similar processes are adopted by commensal bacteria. For example, Candela *et al.*, demonstrated that enolase serves as a surface receptor for human plasminogen for four *Bifidobacterium* species, *Bifidobacterium longum*, *Bifidobacterium bifidum*, *Bifidobacterium breve* and *Bifidobacterium lactis* (Candela *et al.*, 2009). In addition, α -enolase on the surface of *Lactobacillus plantarum* binds fibronectin and plasminogen (Vastano *et al.*, 2013; Castaldo *et al.*, 2009). Ramiah *et al.*, also reported that *Lactobacillus plantarum* 423 expressing surface bound GAPDH, TPI and EF-Tu, could compete strongly with *Clostridium sporogenes* and *Enterococcus faecalis* for binding sites on Caco-2 cells and prevent their adhesion (Ramiah *et al.*, 2008).

In conclusion, the results described in this chapter strongly suggest that FBA contributes to plasminogen binding on the cell-surface of *N. meningitidis*, and characterise the FBA-plasminogen interaction.

**Chapter Four: The interaction between rFBA and
plasminogen is mediated by lysine residue(s)**

4.1 Introduction

Human plasminogen (Plg) is a glycoprotein synthesised as a 90-kDa zymogen, consisting of 810 amino acids. During secretion from the liver, a leader peptide of 19 amino acids is cleaved off to generate the mature form of this protein comprising of 791 amino acids (Forsgren *et al.*, 1987; Raum *et al.*, 1980). This native form of Plg contains a glutamic acid residue at the amino terminal end, and is therefore called glu-plasminogen (glu-Plg) (Lahteenmaki *et al.*, 2001b). A pre-activation peptide (PAP) in the N-terminal region of the plasminogen molecule is followed by five triple-disulfide-linked peptide regions of approximately 80 amino acids in length (the kringle domains K1–K5) and a catalytic C-terminal serine proteinase domain. (Sottrup-Jensen *et al.*, 1975; Castellino and McCance, 1997). Cleavage of the peptide bond between Arg67-Met68, Lys76-Lys77, or Lys77-Val78, releases an 8-kDa activation peptide leading to lys-plasminogen, which is more readily activated to plasmin than the glu-form (Claeys and Vermynen, 1974).

The human plasminogen activators urokinase-type (uPA) and tissue-type plasminogen activator (tPA) or bacterial activators, such as staphylokinase and streptokinase (Lahteenmaki *et al.*, 2005) cleave the peptide bond between Arg561-Val562 in Plg leading to the formation of plasmin. Plasmin contains a 65-kDa heavy chain of 561 amino acid residues comprising PAP, the five kringle domains (K1–K5) and a 25-kDa light chain of 230 amino acid residues harbouring the catalytic triad consisting of His602, Asp645 and Ser740 (Castellino and Powell, 1981; Parry *et al.*, 2000).

Plasmin is a broad spectrum serine protease and its functions include regulation of fibrinolysis and homeostasis, degradation of fibrin clots and components of the extracellular matrix and the basal membrane, including laminin, fibrin, and fibrinogen (Lahteenmaki *et al.*, 2000), and activation of matrix metalloproteases and elastase (Myohanen and Vaheri, 2004).

While in circulation, plasminogen has a 'closed' conformation which upon binding to a surface, adopts an extended, 'open' conformation that is more rapidly activated to plasmin (Parry *et al.*, 2000). In its closed conformation plasminogen is known to contain high-affinity lysine binding sites in the kringle domains, which interact with C-terminal lysine residues in fibrin clots or cell-surface plasminogen receptors (Ponting *et al.*, 1992). All the kringle domains are able to bind lysine except KR3 (Christen *et al.*, 2010). Among these, the lysine binding sites of KR1, KR4 and KR5 have highest affinity for a C-terminal lysine moiety while KR2 has the lowest affinity (Menhart *et al.*, 1991; Menhart *et al.*, 1993; Menhart and Castellino, 1995; Nilsen *et al.*, 1999).

Plasminogen receptors on mammalian cells include proteins with C-terminal lysine residues, such as enolase, as well as proteins rich in internal lysines, such as amphoterin, and also non-protein receptors, such as gangliosides and glycosaminoglycans (Plow *et al.*, 1995). Lysine residues are also involved in the interaction of plasminogen with its receptors on bacterial surfaces. Examples include enolase in *S. pneumoniae* (Bergmann *et al.*, 2003; Ehinger *et al.*, 2004) and *S. pyogenes* (Derbise *et al.*, 2004), enolase, DnaK and peroxiredoxin in *N. meningitidis* (Knaust *et al.*, 2007) and FBA in *M. tuberculosis* (de la Paz Santangelo *et al.*, 2011). However there are exceptions, such as the plasminogen-binding group A streptococcal M-like protein

(Sanderson-Smith *et al.*, 2006) and M protein (PAM)-related protein (Prp) from *S. pyogenes* which use arginine and histidine residues to bind plasminogen (Sanderson-Smith *et al.*, 2007).

In Chapter Three, FBA was determined to be a plasminogen binding protein. It was also demonstrated that the binding of plasminogen was inhibited by the lysine analogue EACA, indicating the involvement of lysine residues in this interaction. In this chapter further investigations were carried out to determine which lysine residue(s) of meningococcal FBA are involved in this interaction.

4.2 Materials and Methods

4.2.1 Bacterial techniques

4.2.1.1 Bacterial strains and culture medium

E. coli JM109 harbouring plasmids, pFS-7, pFS-71, pFS-72, pFS-73 or pFS-8 was routinely grown in Lysogeny Broth (LB) and on LB agar plates containing ampicillin ($100\text{ }\mu\text{g ml}^{-1}$) at 37°C .

4.2.2 DNA techniques

4.2.2.1 Extraction of DNA

Procedures were followed as described in Chapter Two, section 2.2.2.1.

4.2.2.2 Polymerase Chain Reaction (PCR) amplification of *cbbA*

PCR was performed to amplify the 400 bp C-terminal fragment of *cbbA* from meningococcal DNA using the primers shown in Table 4.1 and Phusion High-Fidelity DNA polymerase (New England Biolabs). Amplification conditions were as follows: initial denaturation at 98°C for 30s, denaturation at 98°C for 10s, annealing at 65°C for 30 s, extension at 72°C for 30 s, and final extension at 72°C for 5 min.

4.2.2.3 Agarose gel electrophoresis

PCR products were analysed in 1% agarose gels containing ethidium bromide ($0.5\text{ }\mu\text{g/ml}$; Sigma-Aldrich). Electrophoresis was conducted at 100 V for 60 min in $1\times$ Tris Acetate EDTA buffer (TAE buffer, Sigma-Aldrich) buffer and visualised using an ultraviolet trans-illuminator. PCR product size was estimated by comparison with molecular mass standards (New England Biolabs or Promega).

Table 4.1: List of primers used in work described in this chapter

Primer	Sequence ^a	Restriction site
Amplification of C-terminal fragment of <i>cbbA</i>		
C ter FBA F1	CGC GGATCCC ACGGCTCCAGCTCCGTTCCG (30bp)	<i>Bam</i> HI
C ter FBA R1	CGC GGATCCTT ATTTGACGATTTGGTTCAATTC G (34bp)	<i>Bam</i> HI
C ter FBA R2	CGC GGATCCTT TGACGATTTGGTTCAATTCG (31bp)	<i>Bam</i> HI
Site directed mutagenesis		
FBA_K331 F	CTGCGAAGGTCAGGCAGGCGCAATCAAACCTGTTTCGTT G (40bp)	
FBA_K331 R	CAACGAAACAGGTTTGATTGCGCCTGCCTGACCTTCGCA G (40bp)	
FBA_K333 F	GAAGGTCAGGCAGGCAAAATCGCACCTGTTTCGTTGGAA AAAA (43bp)	
FBA_K333 R	TTTTTTCCAACGAAACAGGTGCGATTTTGCCTGCCTGACC TTC (43bp)	
FBA_K339 F	CAAACCTGTTTCGTTGGAAGCAATGGCAAGCCGTTATGC C (40bp)	
FBA_K339 R	GGCATAACGGCTTGCCATTGCTTCCAACGAAACAGGTTT G (40bp)	
FBA_K346 F	AATGGCAAGCCGTTATGCCGCGGGCGAATTGAACCAAAT C (40bp)	
FBA_K346 R	GATTTGGTTCAATTCGCCCCGCGGCATAACGGCTTGCCAT T (40bp)	
FBA_K354 F	CGAATTGAACCAAATCGTCGCATAAGGATCCGCATGCGA G (40bp)	
FBA_K354 R	CTCGCATGCGGATCCTTATGCGACGATTTGGTTCAATTCG (40bp)	

^a Restriction sites are highlighted in bold

4.2.2.4 Purification of PCR products

PCR products or digested vectors were purified using the QIAquick PCR purification kit (Qiagen) following the manufacturer's protocol.

4.2.2.5 Digestion of vectors and inserts

DNA was digested by *Bam*HI (New England Biolabs) according to the manufacturer's instruction.

4.2.2.6 Dephosphorylation of digested vectors

After digestion, vectors were dephosphorylated with Antarctic Phosphatase (New England Biolabs) according to the manufacturer's protocol. After purification, the digested product was quantified using a NanoDrop 1000 Spectrophotometer (NanoDrop Technologies).

4.2.2.7 Ligation

A Rapid DNA Ligation kit (Fermentas Life Sciences) was used to ligate DNA fragments following the manufacturer's instructions.

4.2.2.8 Site directed mutagenesis and transformation of *E.coli* XL10-gold ultracompetent cells and *E. coli* JM109 cells

Mutation(s) at specific site(s) of *cbbA* were introduced; primers used for mutagenesis are shown in the Table 4.1. Procedures described in Chapter Two, section 2.2.2.3 were followed for these mutagenesis reactions. The resulting plasmids were renamed pFS-71, pFS-72 and pFS-73, respectively. *E. coli* JM109 competent cells were transformed following the procedures described in Chapter Two, section 2.2.3.1.

4.2.2.9 Sequencing of plasmid DNA and nucleic acid and amino acid sequence analysis

Procedures were followed as described in Chapter Two, section 2.2.2.4.

4.2.3 Protein techniques

4.2.3.1 Expression and purification of tr-rFBA

E. coli JM109 competent cells were transformed according to the manufacturer's instructions. Expression of truncated rFBA was confirmed by procedures described in Chapter Two, section 2.2.3.1. All the truncated rFBA proteins were purified according to the procedures described in Chapter Two, sections 2.2.3.2, 2.2.3.3 and 2.2.3.4 respectively. The only exception was that after purification of the recombinant proteins by IMAC and desalting, fractions containing tr-FBA were concentrated using Vivaspin™ sample concentrators (Sartorius Stedim Biotech, Germany) with a 10,000 molecular weight cut-off.

4.2.3.2 SDS-PAGE and immunoblotting

Purified truncated rFBA proteins were identified by SDS-PAGE electrophoresis followed by immunoblot using mouse anti-His-tag antibody and goat anti-mouse-IgG-AP secondary antibody. These procedures are described in Chapter Two, sections 2.2.3.5 and 2.2.3.6.

4.2.3.3 Plasminogen binding assay

Plasminogen binding by the purified proteins, and effects of EACA, NaCl and anti-FBA antiserum on plasminogen-FBA interactions were determined by enzyme immune assay (EIA). The protocol was as described in Chapter Three, section 3.2.2.1. Negative controls were prepared following the same procedure with the exception that BSA was used in place of plasminogen. The data are presented as the mean $\pm$ S.E. All experiments were repeated at least three times unless otherwise stated. Statistical analysis was performed using GraphPad Prism6. Student's t-test was performed to calculate significant differences. A *p* value of <0.05 was considered significant.

4.3 Results

4.3.1 Cloning of a 400bp fragment of *cbbA* into pQE-30 and pQE-70 vectors

In Chapter Three, it was shown that rFBA binds human glu-plasminogen and the interaction could be inhibited by the lysine analogue EACA. The amino acid sequence of FBA contains 17 lysine residues of which 11 are present towards the C-terminal end of FBA (Fig.4.1).

Translation of gb|AE002098.2|_1974134-1975198_Neisseria_meningitidis_MC58 comp(RF+1)(1-1063)
 Universal code
 Total amino acid number: 354, MW=38275
 Max ORF: 1-1062, 354 AA, MW=38275

```

1      ATGGCACTCGTATCCATGCGCCAACTGCTTGATCATGCTGCCGAAAACAGCTACGGCCTG
1      M A L V S M R Q L L D H A A E N S Y G L

61     CCGGCGTTCAACGTCAACAACCTCGAACAGATGCGCGCCATCATGGAGGCTGCAGACCAA
21     P A F N V N N L E Q M R A I M E A A D Q

121    GTGACGCCCCCGTCATCGTACAGGCGAGTGCCGGTGCGCGCAAATATGCGGGTGCGCGG
41     V D A P V I V Q A S A G A R K Y A G A P

181    TTTTACGCCACCTGATTTTGGCGGCTGTGGAAGAATTTCCACACATCCCCGTCGTCATG
61     F L R H L I L A A V E E F P H I P V V M

241    CACCAAGACCACGGCGCATCACCCGACGTGTGCCAACGCTCCATCCAACTGGGCTTCTCC
81     H Q D H G A S P D V C Q R S I Q L G F S

301    TCTGTAATGATGGACGGCTCGCTGATGGAAGACGGCAAAACCCCTTCTTCTTACGAATAC
101    S V M M D G S L M E D G K T P S S Y E Y

361    AACGTCAACGCCACACGTACCGTGGTTAACTTCTCCACGCTTGCGGCGTATCCGTTGAA
121    N V N A T R T V V N F S H A C G V S V E

421    GGCGAAATCGGCGTATTGGGCAACCTCGAAACCGGCGAAGCAGGCGAAGAAGACGGTGTA
141    G E I G V L G N L E T G E A G E E D G V

481    GGCGCAGTGGGCAAACTTTCCACGACCAAAATGCTGACCAGCGTCGAAGATGCCGTATGT
161    G A V G K L S H D Q M L T S V E D A V C

541    TTCGTTAAAGATACCGGCGTTGACGCAATTGGCTATTGCCGTCGGCACCGCCACGGCGCA
181    F V K D T G V D A L A I A V G T S H G A

601    TACAAATTACCCGTCGCGCCACAGGCGATGTATTACGTATCGACCGCATCAAAGAATC
201    Y K F T R P P T G D V L R I D R I K E I

661    CACCAAGCCCTGCCCAATACACACATCGTGATGCACGGCTCCAGCTCCGTTCCGCAAGAA
221    H Q A L P N T H I V M H G S S S V P Q E

721    TGGCTGAAAGTCATCAACGAATACGGCGGCAATATCGGCGAAACCTACGGCGTGCCGGTT
241    W L K V I N E Y G G N I G E T Y G V P V

781    GAAGAAATCGTCGAAGGCATCAACACCGGCGTGCGCAAGTCAACATCGATACCGACTTG
261    E E I V E G I K H G V R K V N I D T D L
      Motif 5

841    CGCCTTGCTTCTACCGGCGCGGTACGCCGCTACCTTGCCGAAAATCCGTCGCACTTTGAC
281    R L A S T G A V R R Y L A E N P S D F D
      Motif 4

901    CCGCGCAAATACCTGAGCAAAACCATGAGGCCATGAAGCAAATCTGCCTCGACCGTTAT
301    P R K Y L S K T I E A M K Q I C L D R Y
      Motif 3

961    CTTGCGTTTGGCTGCGAAGGTCAGGCAGGCAAAATCAAACCTGTTTCGTTGGAAAAATG
321    L A F G C E G Q A G K I K P V S L E K M
      Motif 2

1021   GCAAGCCGTTATGCCAAGGGCGAATTGAACCAATCGTCAAATAA
341    A S R Y A K G E L N Q I V K
      Motif 2                      Motif 1

```

Figure 4.1: Nucleotide sequence of MC58 *cbbA* and the corresponding amino acid sequence of its encoded protein FBA. A 400 bp region of *cbbA*, encoding the C-terminal region of FBA (highlighted in red), was cloned into pQE-30 and pQE-70 vectors. The underlined lysine residues were targeted for site directed mutagenesis.

To further investigate the involvement of lysine residues, the 400 bp fragment of *cbbA* (Fig. 4.1), coding for the C-terminal region of FBA, was amplified, digested and ligated with digested pQE-30 and pQE-70 vectors (Fig. 4.2A) for the expression of truncated rFBA with N and C terminal His-tag, respectively. Two different reverse primers (Table 4) were designed to facilitate the cloning of the 400 bp fragment of *cbbA* into two different vectors. The ligated products were used to transform *E. coli* JM109 cells. Restriction digestion using *Bam*HI was used to confirm the presence of the desired insert in those ligated products (Fig. 4.2B and C).

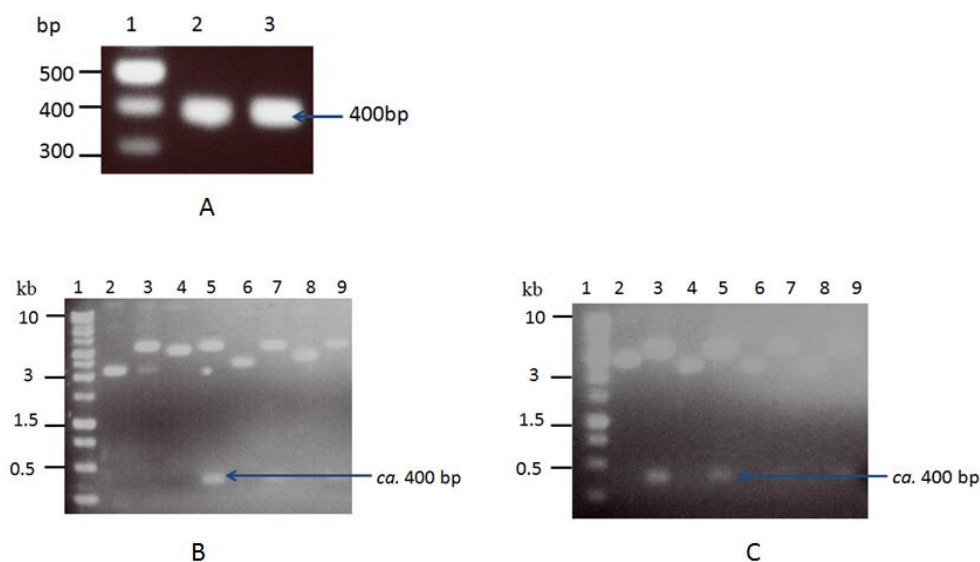


Figure 4.2: Agarose gel showing amplification of *cbbA* and truncated *cbbA*. Upper panel: A) Agarose gel showing amplification of *cbbA*. Lane 1: 100 bp DNA ladder; Lane 2-3: amplified truncated *cbbA* (400 bp). Lower panel: Agarose gel showing presence of C-terminal fragment of *cbbA* after *Bam*HI digestion of ligated products. Lane 1: 1Kb DNA ladder; Lane 2-9: In alternate order, undigested and *Bam*HI-digested pQE-30- (B) or pQE-70-based (C) plasmids showing the presence of a 400 bp fragment of *cbbA*.

Clones containing the correctly sized insert were sent for DNA sequencing. One clone from each set, containing the correct fragment of *cbbA* in-frame with the His-tag at the desired position, were subsequently re-named pFS-7 (pQE-30 based vector for N-His-tagged tr-FBA1) and pFS-8 (pQE-70 based vector for C-His-tagged tr-FBA2), respectively (Fig. 4.3).

```

FBA_PROTEIN      IHQALPNTTHIVMHGSSSSVPQEWLKVINEYGGNIGETYGVPVEEIVEGIKHGVRKVNIDTDLR
tr-rFBA1         MRGSHHHHHHSHGSSSSVPQEWLKVINEYGGNIGETYGVPVEEIVEGIKHGVRKVNIDTDLR
tr-rFBA2         .....MRGSHGSSSSVPQEWLKVINEYGGNIGETYGVPVEEIVEGIKHGVRKVNIDTDLR
rFBA (WT)        IHQALPNTTHIVMHGSSSSVPQEWLKVINEYGGNIGETYGVPVEEIVEGIKHGVRKVNIDTDLR
Consensus                hgsssvpqewlkvineyggnigetygvpveeivegikhgvrkvnidtdlr

FBA_PROTEIN      LASTGAVRRYLAENPSDFDPRKYLSTIEAMKQICLDRYLAFGCEGQAGKIKPVS
tr-rFBA1         LASTGAVRRYLAENPSDFDPRKYLSTIEAMKQICLDRYLAFGCEGQAGKIKPVS
tr-rFBA2         LASTGAVRRYLAENPSDFDPRKYLSTIEAMKQICLDRYLAFGCEGQAGKIKPVS
rFBA (WT)        LASTGAVRRYLAENPSDFDPRKYLSTIEAMKQICLDRYLAFGCEGQAGKIKPVS
Consensus                lastgavrrylaenpsdfdprkylsktieamkqicldrylafgcegqagkikpvs

FBA_PROTEIN      LEKMASRYAKGELNQIVK.....
tr-rFBA1         LEKMASRYAKGELNQIVK.....
tr-rFBA2         LEKMASRYAKGELNQIVKSSRSHHHHHH
rFBA (WT)        LEKMASRYAKGELNQIVKSSHHHHHH..
Consensus                lekmasryakgelinqivk

```

Figure 4.3: Multiple sequence alignment of translated products of truncated *cbbA* cloned into pQE-30 and pQE-70 showing the presence of the His-tag at desired position.

4.3.2 Expression of truncated rFBA (tr-rFBA) by *E. coli* JM109 and purification of tr-rFBA

E. coli JM109 cells, transformed with either pFS-7, or pFS-8, were grown to obtain tr-rFBA1 (tr-rFBA with N-His-tag) or tr-rFBA2 (tr-rFBA with C-His-tag) respectively. Immunoblotting using anti His-tag antibody followed by rabbit anti-mouse IgG-Alkaline phosphatase, was used to detect the tr-FBA in both uninduced and induced culture samples (Fig. 4.4).

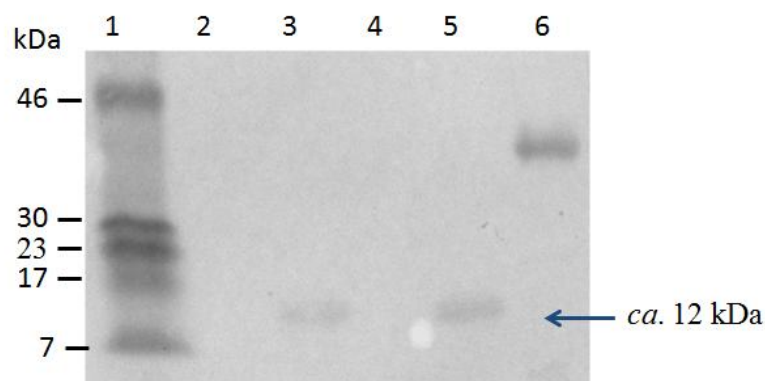


Figure 4.4: Immunoblot showing the expression of A) tr-rFBA1 and tr-rFBA2 in *E. coli* JM109 culture samples. Lane1: Protein marker; Lane 2-5: in alternate order, uninduced and induced culture sample of *E. coli* JM109 containing pFS-7 and pFS-8, respectively; Lane 6: purified rFBA (WT) as positive control.

All the tr-rFBA proteins were purified by IMAC followed by desalting as described in Chapter Two. Purity of the tr-rFBA samples was checked by SDS-PAGE followed by staining (not shown) and immunoblotting using anti-His-tag antibody (Fig.4.5).

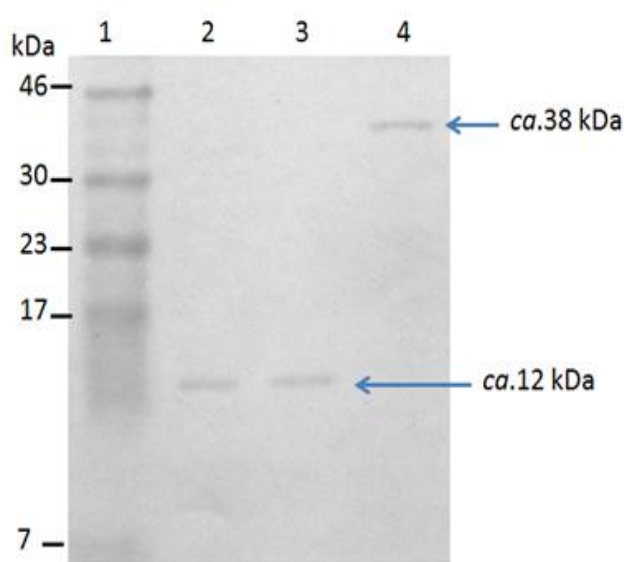


Figure 4.5: Immunoblots, probed with anti-His-tag antibody, showing tr-rFBA proteins purified under non-denaturing conditions. Lane 1: Prestained protein ladder; lane 2: tr-rFBA1; lane 3: tr-rFBA2; lane 4: rFBA (WT) used as positive control.

4.3.3 Interaction of lysine rich C-terminal fragment of FBA with human plasminogen

Full length rFBA (WT) was used as a control. Although both tr-FBA1 and tr-FBA2 were able to bind plasminogen, binding was significantly higher with tr-FBA1 compared to tr-FBA2 and rFBA (WT). This interaction was inhibited by EACA confirming the involvement of lysine residues (Fig. 4.6).

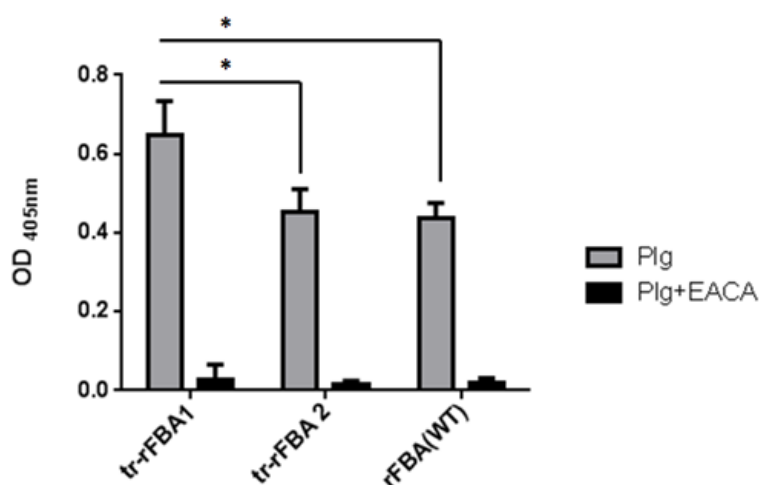


Figure 4.6: A) tr-rFBA binds human plasminogen. Binding of tr-rFBA1 was significantly higher than tr-rFBA2 and rFBA (WT) indicating possible involvement of terminal lysine residue(s) which might be partially masked by the His-tag present at the C-terminal end of tr-rFBA2 and rFBA (WT). EACA (5mM, black bars) inhibited this interaction. n=3. * denotes p value <0.05.

4.3.4 Effects of salt and anti-FBA antibody on tr-FBA-plasminogen interaction

As tr-FBA1 showed higher binding to plasminogen than tr-FBA2, the interaction between tr-FBA1 and plasminogen was further characterised. To verify whether plasminogen binding by tr-FBA1 is affected by NaCl and anti-FBA antibody, EIA was performed in the presence of different concentrations of NaCl and rabbit anti-FBA antiserum.

As shown in Fig. 4.7 NaCl at physiological concentrations did not affect binding and interference was only observed at higher salt concentration. In the presence of 500mM NaCl, the binding of plasminogen by tr-FBA1 (Fig.4.7) was significantly reduced compared to that without NaCl.

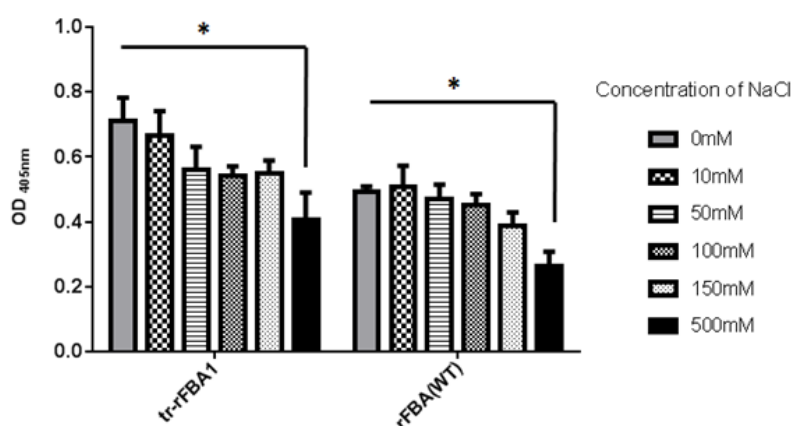


Figure 4.7: Effect of NaCl on tr-rFBA-plasminogen interaction. Binding of human plasminogen to tr-rFBA1 was inhibited by increasing concentration of NaCl. Significant inhibition was observed at 500mM NaCl (black bars) compared to 0mM NaCl (dark grey bars). n=4. * denotes p value <0.05 .

Similarly, the effect of anti-FBA antibody on the ability of FBA to bind plasminogen was examined by EIA. Four different dilutions of anti-FBA antiserum were used in these experiments. At a dilution of $\leq 1:250$ of anti-FBA antiserum the binding of plasminogen by tr-rFBA1 (Fig 4.8) was significantly inhibited in a dose-dependent manner. This suggests competition between rabbit anti-FBA antiserum and plasminogen to bind to FBA.

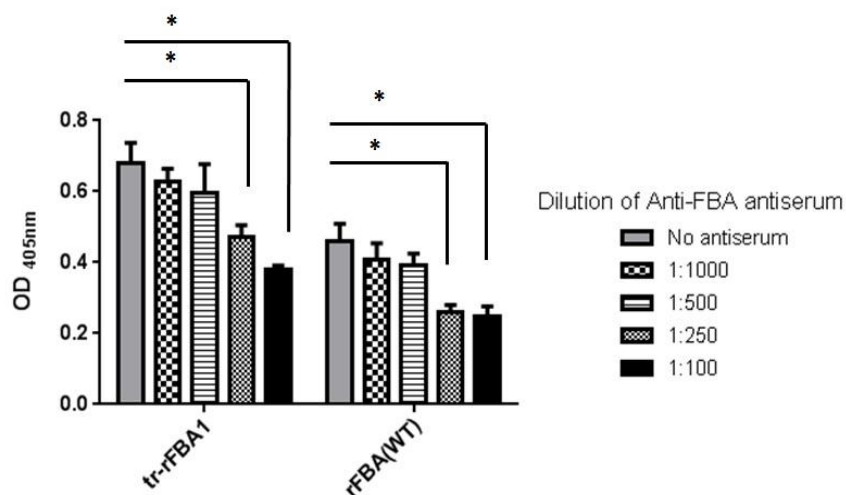


Figure 4.8: Effect of anti-FBA antiserum on tr-rFBA1-plasminogen interaction. Binding of human plasminogen to tr-rFBA1 was inhibited by increasing concentration of rabbit anti-FBA antiserum. 1:250 and lower dilutions of anti-FBA antibody were able to significantly inhibit the binding of plasminogen to rFBA. n=3. * denotes p value <0.05.

4.3.5 Site directed mutagenesis

As described in section 4.3.3, binding of human plasminogen was higher with tr-FBA1 which has a His-tag at the N-terminal end. This observation prompted the investigation of lysine residue(s) that might be involved in plasminogen binding.

pFS-7 was used as template in order to replace lysine residues at positions 331, 333, 339, 346 and 354 (numbers refer to MC58 FBA amino acid sequence) individually with alanine in tr-FBA1 (Fig 4.9). Sequencing data revealed that lysine residues at positions 339, 346 and 354 were correctly replaced by alanine. Attempts to replace lysine residue at positions 331 and 333 were unsuccessful despite several attempts. After confirmation, the newly constructed plasmids were renamed pFS-71, pFS-72, and FS-73 to encode tr-rFBA1 (K339A), tr-rFBA1 (K346A) and tr-rFBA1 (K354A), respectively (Fig.4.9).

```

FBA_Protein      IHQALPNTHIVMHGSSSVPPQEWLKVINEYGCNIGETYGVPVEEIVEGIKHGVRKVNIDTDLR
tr-rFBA1         MRGSHHHHHHSGSHGSSSVPPQEWLKVINEYGCNIGETYGVPVEEIVEGIKHGVRKVNIDTDLR
tr-rFBA1 (K339A) MRGSHHHHHHSGSHGSSSVPPQEWLKVINEYGCNIGETYGVPVEEIVEGIKHGVRKVNIDTDLR
tr-rFBA1 (K346A) MRGSHHHHHHSGSHGSSSVPPQEWLKVINEYGCNIGETYGVPVEEIVEGIKHGVRKVNIDTDLR
tr-rFBA1 (K354A) MRGSHHHHHHSGSHGSSSVPPQEWLKVINEYGCNIGETYGVPVEEIVEGIKHGVRKVNIDTDLR
rFBA (WT)        IHQALPNTHIVMHGSSSVPPQEWLKVINEYGCNIGETYGVPVEEIVEGIKHGVRKVNIDTDLR
Consensus        h  hgsssvppqewlkvineygcnigetygvpveeivegikhgvrkvnidtdlr

FBA_Protein      LASTGAVRRYLAENPSDFDPRKYLSTIEAMFQICLDRYLAFGCEGQAGKIKPVSLEFMMAS
tr-rFBA1         LASTGAVRRYLAENPSDFDPRKYLSTIEAMFQICLDRYLAFGCEGQAGKIKPVSLEFMMAS
tr-rFBA1 (K339A) LASTGAVRRYLAENPSDFDPRKYLSTIEAMFQICLDRYLAFGCEGQAGKIKPVSLEFMMAS
tr-rFBA1 (K346A) LASTGAVRRYLAENPSDFDPRKYLSTIEAMFQICLDRYLAFGCEGQAGKIKPVSLEFMMAS
tr-rFBA1 (K354A) LASTGAVRRYLAENPSDFDPRKYLSTIEAMFQICLDRYLAFGCEGQAGKIKPVSLEFMMAS
rFBA (WT)        LASTGAVRRYLAENPSDFDPRKYLSTIEAMFQICLDRYLAFGCEGQAGKIKPVSLEFMMAS
Consensus        lastgavrrylaenpsdfdprkylsktieamkqicldrylafgceggagkikpvsle mas

FBA_Protein      RYAFGELNQIVK.....
tr-rFBA1         RYAFGELNQIVK.....
tr-rFBA1 (K339A) RYAFGELNQIVK.....
tr-rFBA1 (K346A) RYAFGELNQIVK.....
tr-rFBA1 (K354A) RYAFGELNQIVK.....
rFBA (WT)        RYAFGELNQIVKRSHHHHHH
Consensus        rya gelnqiv

```

Figure 4.9: Multiple sequence alignment of amino acid sequences of tr-rFBA1 derivatives resulting from site-directed mutagenesis.

4.3.3 Expression and purification of mutated truncated rFBA1

E. coli JM109 cells, transformed with either pFS-71, pFS-72 or pFS-73 were grown to obtain tr-rFBA1 (K339A), tr-rFBA1 (K346A) or tr-rFBA1 (K354A) respectively. Anti His-tag antibody followed by rabbit anti-mouse IgG-Alkaline phosphatase were used to detect the tr-FBA in both uninduced and induced culture samples (Fig. 4.10).

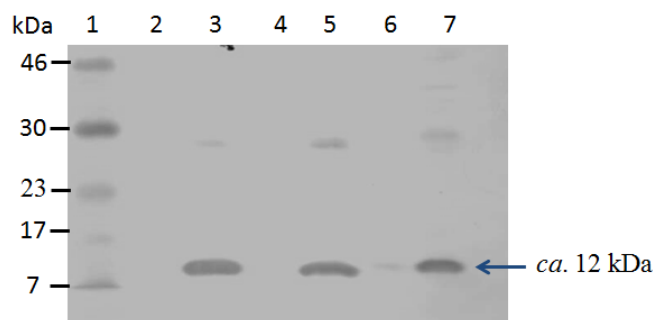


Figure 4.10: Immunoblot showing the expression of mutated N-His-tagged tr-rFBA1 in *E. coli* JM109 culture samples. Lane1: Protein marker; Lane 2-7: in alternate order, uninduced and induced culture sample of *E. coli* JM109 containing pFS-71, pFS-72 and pFS-73, respectively.

All the tr-rFBA proteins were purified by IMAC followed by desalting as described in Chapter Two. The mutant derivatives of tr-rFBA1 were confirmed by immunoblotting using anti-His-tag antibody (Fig.4.11).

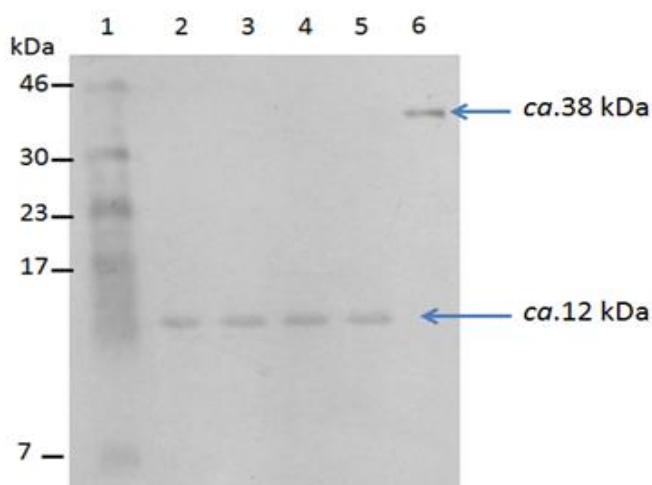


Figure 4.11: Immunoblots, probed with anti-His-tag antibody, showing mutated tr-rFBA1 proteins purified under non-denaturing conditions. Lane 1: Prestained protein ladder; lane 2: tr-rFBA1(K339A); lane 3: tr-rFBA1(K346A); lane 4: tr-rFBA1(K354A); lane 5: tr-rFBA1; lane 6: rFBA(WT) as positive control.

4.3.4 Interaction of lysine rich C-terminal fragment of FBA with human plasminogen

All mutant derivatives of tr-rFBA1 proteins were tested for their ability to bind glu-plasminogen using EIA. Replacement of lysine at positions 339 or 346 did not have any statistically significant effect on plasminogen binding by tr-rFBA1 (Fig.4.12). However, replacement of the terminal lysine at position 354 by alanine significantly reduced plasminogen binding. These results indicated that the lysine residue at position 354 is mainly responsible for plasminogen binding (Fig.4.12).

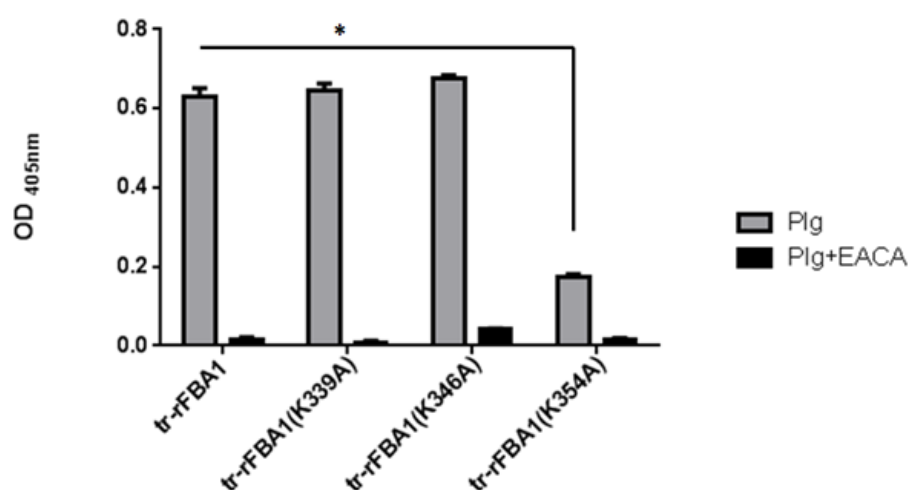


Figure 4.12: plasminogen binding by mutant variants of tr-rFBA1 containing lysine to alanine mutations. Replacement of the terminal lysine at position 354 in tr-rFBA1 (K354A) caused a significant reduction in plasminogen binding while lysine replacements at positions 339 or 346 had no effect on plasminogen binding. EACA (5mM, black bars) inhibited this interaction. n=3. * denotes p value <0.05.

4.3.5 Effects of salt and anti-FBA antiserum on tr-FBA-plasminogen interaction

The interaction between mutant derivatives of tr-FBA1 and plasminogen was further characterised. To verify whether plasminogen binding is affected by NaCl and anti-FBA antiserum, EIA was performed in presence of different concentrations of NaCl and anti-FBA antiserum.

As shown in Fig. 4.13 NaCl at physiological concentrations did not affect binding and interference was only observed at higher salt concentration. In the presence of 500mM NaCl, the binding of plasminogen by mutant variants of tr-rFBA1 (Fig.4.13) was significantly reduced compared to that of without NaCl.

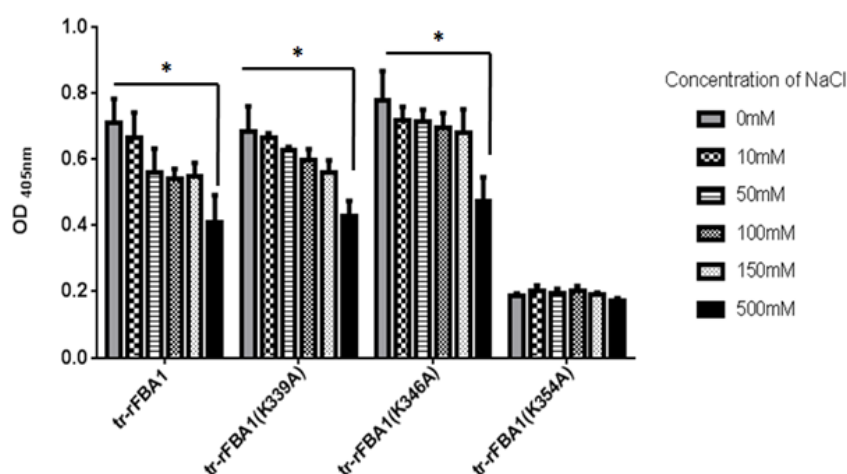


Figure 4.13: Effect of NaCl on tr-rFBA-plasminogen interaction. Binding of human plasminogen mutant variants of tr-rFBA1, was inhibited by increasing concentration of NaCl. Significant inhibition was observed at 500mM NaCl (black bars) compared to 0mM NaCl (dark grey bars). $n=4$. * denotes p value <0.05 .

Similarly, the effect of anti-FBA antiserum on the ability of FBA to bind plasminogen was examined by EIA. Four different dilutions of anti-FBA antiserum were used in these experiments. At a dilution of $\leq 1:250$ of anti-FBA

antiserum the binding of plasminogen by mutant variants of tr-rFBA1 (Fig.4.14) was significantly inhibited in a dose-dependent manner. This suggests competition between anti-FBA antiserum and plasminogen to bind to FBA.

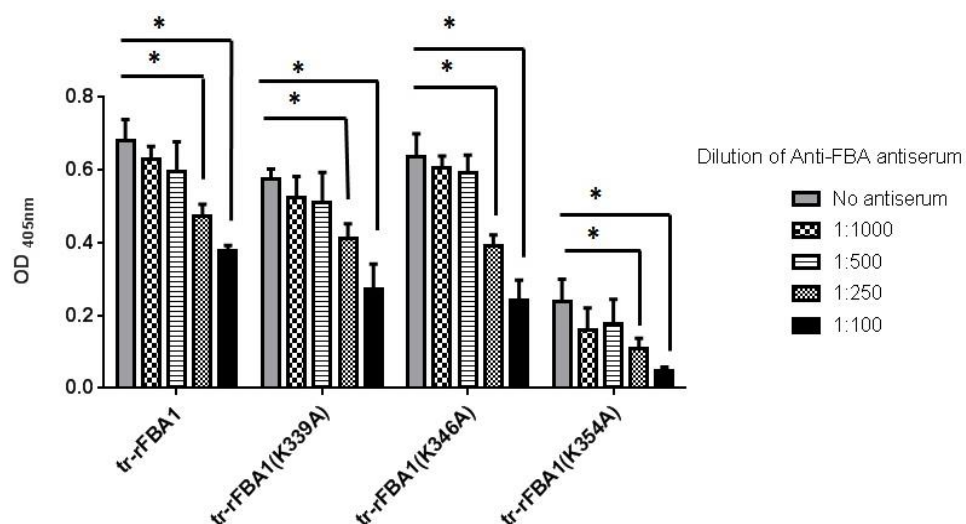


Figure 4.14: Effect of anti-FBA antiserum on tr-rFBA1-plasminogen interaction. Binding of human plasminogen to mutant variants of tr-rFBA1 was inhibited by increasing concentration of anti-FBA antiserum. 1:250 and lower dilutions of anti-FBA antibody were able to significantly inhibit the binding of plasminogen to rFBA. $n=3$. * denotes p value <0.05 .

4.5 Discussion

Bacteria can interfere with the plasminogen system by two general mechanisms: firstly by producing activators of plasminogen and secondly by producing receptors to immobilise plasminogen on the cell surface which can then be activated to plasmin by host activators such as tissue-type plasminogen (tPA) activators and urokinase plasminogen activator (uPA) (Bergmann and Hammerschmidt, 2007). The fact that many pathogenic bacteria produce activators or receptors for plasminogen suggests that plasmin formation has an important role in invasive infections (Korhonen *et al.*, 1992; Lottenberg *et al.*, 1994; Boyle and Lottenberg, 1997; Coleman and Benach, 1999). Examples include: *B. burgdorferi* (Coleman *et al.*, 1997; Coleman *et al.*, 1995), *Y. pestis* (Lahteenmaki *et al.*, 2001a; Sodeinde *et al.*, 1992), *S. pyogenes* (Sun *et al.*, 2004), *H. influenzae* (Ullberg *et al.*, 1990; Virkola *et al.*, 1996) and *S. pneumoniae* (Bergmann *et al.*, 2005; Eberhard *et al.*, 1999). Moreover according to recent reports, activation of surface bound plasminogen to plasmin imparts anti-phagocytic and anti-opsonisation properties to *B. anthracis* (Chung *et al.*, 2011) and *B. burgdorferi* (Grosskinsky *et al.*, 2009).

Enhanced fibrinolytic activity associated with meningococcal meningitis initially suggested that plasminogen activation might be involved in systemic infections caused by bacteria (Brandtzaeg *et al.*, 1990). Ullberg *et al.* reported for the first time that *N. meningitidis* and *N. gonorrhoeae* could bind plasminogen and this was inhibited by EACA (Ullberg *et al.*, 1992). The presence of plasminogen-binding proteins, identified as enolase, DnaK and peroxiredoxin on the surface of *N. meningitidis* has been reported by Knaust *et*

al. (Knaust *et al.*, 2007). Until now plasminogen binding by *N. meningitidis* and its physiological significance have not been studied in detail.

Tunio *et al.* reported that FBA was dispensable for meningococcal growth but required for optimal adhesion to human cells (Tunio *et al.*, 2010a). This study was undertaken to further investigate non-glycolytic functions of meningococcal FBA. In Chapter Three, FBA was shown to be a novel plasminogen binding protein of *N. meningitidis*. The possible involvement of lysine residues was also shown by the inhibition of plasminogen binding by the lysine analogue EACA. Therefore further experiments were carried out to determine which lysine residues were involved in this interaction.

A 400 bp fragment of *cbbA* encoding a lysine-rich C-terminal region of 123 amino acids in length, was cloned into two different vectors namely pQE-30 and pQE-70 to express two different truncated versions of rFBA: tr-rFBA1 with N-His-tag and tr-rFBA2 with C-His-tag, respectively. These truncated proteins were expressed and purified under non-denaturing conditions, and confirmed by anti-His-tag antibody. The full-length and truncated rFBA molecules were then examined for their ability to bind plasminogen.

N-His-tagged tr-rFBA1 (amino acids 232-354) was able to bind plasminogen better than its C-His-tagged counterpart and also full length rFBA (WT). EACA inhibited this interaction indicating that lysine residues in this region, probably in the terminal or sub-terminal positions might be involved in plasminogen binding. Lysine residues at the terminal or sub-terminal regions of FBA may be partially masked by the His-tag in full length rFBA and tr-rFBA2 both of which have the His-tag at their C-terminal ends. Based on this result, further experiments were focused on tr-rFBA1.

To further identify the lysine residue(s) involved in plasminogen binding, several lysine residues at the C-terminal end of FBA were replaced with alanine using site directed mutagenesis.

Substitution of lysines at position 331 and 333 was unsuccessful. The failure of site directed mutagenesis has been attributed to a number of factors such as poor primer design, length of final extension step in mutagenesis PCR, low PCR amplification efficiency, additional template leading to formation of hemimethylated DNA molecules resistant to *DpnI* digestion, incomplete *DpnI* digestion leading to recovery of non-mutant DNA (Zheng *et al.*, 2004; Liu and Naismith, 2008).

In contrast, three lysine residues at positions at position 339, 346 and 354 were successfully replaced individually by alanine. These three mutant variants of tr-rFBA1, all with an N-His-tag, were expressed and purified. When subjected to EIA, two of the mutated proteins rFBA1 (K339A) and rFBA1 (K346A) were able to bind plasminogen to a level similar to tr-rFBA1. For the other protein, tr-FBA1 (K354A), a significant decrease in plasminogen binding was observed.

One important characteristic of the interaction between plasminogen and its receptor(s) is the involvement of lysine residues in the ligand which bind to binding sites in the kringle domains of plasminogen (Redlitz and Plow, 1995). Examples of receptors with lysine residue(s) at the C-terminal end include enolase of *S. pneumoniae* (Bergmann *et al.*, 2001), streptococcal surface enolase (SEN) (Derbise *et al.*, 2004) and FBA of *M. tuberculosis* (de la Paz Santangelo *et al.*, 2011).

Previously, the plasmin-binding protein, Plr, initially isolated from *S.pyogenes* strain 64/14 (Broder *et al.*, 1991) was found to be a functional GAPDH (Winram and Lottenberg, 1996). Later Winram and Lottenberg undertook another study to identify unique amino acid residues that are not required for GAPDH activity but are important for plasmin binding (Winram and Lottenberg, 1998). Plr was able to bind Lys-plasmin but failed to bind significant amounts of Glu-plasminogen. Streptococcal Plr has a lysine residue at the C-terminus (position 334) (Lottenberg *et al.*, 1992). To assess the contribution of this residue to the binding of plasmin, a mutant protein (PIr-45) containing leucine at 334 position instead of lysine, was created. Another Plr molecule, Plr-63, which possessed the C-terminus of the *Bacillus stearothermophilus* GAPDH (Branlant *et al.*, 1983) was also examined. This protein had glycine and leucine downstream of the terminal the lysine. Both these proteins had reduced ability to bind plasmin compared to the wild type PIr protein. In contrast to the terminal lysine at position 334, substitution of the penultimate lysine residue at amino acid position 330 of Plr had no effect on plasmin binding. Winram and Lottenberg suggested that the penultimate lysine of Plr may not interact with the low-affinity lysine binding site of Glu-plasminogen (Winram and Lottenberg, 1998). Interestingly, equivalent amounts of plasmin was bound by wild type and isogenic streptococcal strains expressing Plr containing the terminal lysine mutation, suggesting PIr was not a unique plasmin receptor (Winram and Lottenberg, 1998).

Streptococcal surface enolase (SEN) is a ubiquitously expressed surface protein and major plasminogen-binding protein of *S. pyogenes*. Derbise *et al.* reported that the two terminal lysine residues located at the C-terminal end of

SEN are the major contributors to the plasminogen-binding activity (Derbise *et al.*, 2004). In this study SEN variants with mutations in the C-terminal region were created by either deleting one or more terminal lysine residues or substituting with leucine. All these proteins were enzymatically active. Interestingly the study reported that wild type SEN molecules had three times the affinity towards Lys-plasminogen than to Glu-plasminogen. When the mutated recombinant SEN molecules were tested for their ability to bind plasminogen, it was found that the recombinant mutant SEN proteins with deletion of two terminal lysine residues (Lys434-435) or with a single mutation replacing the terminal lysine with leucine (K435L) or double mutations replacing two lysines to leucine (K434-435L) showed significantly reduced binding of both Glu- and Lys-plasminogen (Derbise *et al.*, 2004).

Knaust *et al.* reported enolase, DnaK and peroxiredoxin as plasminogen binding proteins on the meningococcal surface (Knaust *et al.*, 2007). The study also investigated the possible role of the C-terminal lysine residues of these proteins in plasminogen binding. Mutant variants of recombinant enolase with the C-terminal lysine residue replaced by alanine, DnaK with the two C-terminal lysine residues replaced by two alanines, and peroxiredoxin with the C-terminal Lys-Asn dipeptide replaced by two alanines were expressed. Using plasminogen overlay assay and denatured proteins, mutant DnaK and peroxiredoxin but not enolase, showed a decreased capacity to bind plasminogen compared to the wild type variants. In contrast to the overlay assay, dot blot assay using non-denatured wild type and mutant proteins showed similar levels of plasminogen binding. These results suggested that

internal lysine rich motifs, rather than the C-terminal lysine residues might be responsible for plasminogen binding (Knaust *et al.*, 2007).

Agarwal *et al.* reported alpha-enolase as a plasminogen binding protein on the surface of *B. anthracis*. Alpha-enolase has two lysine residues at its C-terminal end (Agarwal *et al.*, 2008). In a later study Matta *et al.*, reported GAPDH (GapA isoform) as another plasminogen binding protein (Matta *et al.*, 2010). Whereas pneumococcal GAPDH has two terminal lysine residues (Bergmann *et al.*, 2004), GAPDH of *B. anthracis* has one sub-terminal lysine at position 332. The authors reported similar K_D values for both pneumococcal and *B. anthracis* GAPDH. The authors also suggested a single surface exposed C-terminal lysine could be important for plasminogen binding although the possibility of a second plasminogen binding site within GAPDH molecules could not be excluded (Matta *et al.*, 2010). Moreover the GapB isoform of GAPDH differed from the GapA isoform with aspartate at position 332 instead of lysine, leading to poor affinity of GapB for plasminogen (Matta *et al.*, 2010).

In an earlier report Boone *et al.* demonstrated phosphoglycerate kinase (PGK), present on the surface of group B streptococcus (GBS), as a plasminogen and actin binding protein (Boone *et al.*, 2011). Later, Boone and Tyrrell identified two actin and plasminogen binding sites (amino acids 126-134 and 204-208) of GBS-PGK using a number of truncated GBS-PGK variants and peptide mapping (Boone and Tyrrell, 2012). They also identified lysine residues at positions 126, 127, 130, 204, and 208, and the glutamic acid residue at position 133, were necessary for plasminogen binding (Boone and Tyrrell, 2012).

BBA 70 is a member of pFam54 family of proteins and is a plasminogen binding protein of *B. burgdorferi* (Koenigs *et al.*, 2013). Lysine residues have been demonstrated to be involved in the interaction with plasminogen. The predicted secondary structure has seven putative α -helices. A number of C-terminally truncated proteins lacking putative α -helices were constructed and tested for their ability to bind plasminogen. Deletion of one putative α -helix, containing 9 lysine residues (BBA701-166), eliminated plasminogen binding indicating this predicted α -helix of BBA70 is crucial for the interaction with plasminogen (Koenigs *et al.*, 2013).

L. plantarum enolase (EnoA1) exhibits binding activity for human plasminogen with two plasminogen binding domains. Binding site 1 was characterised by a C-terminal lysine at position 422, and binding site 2 consisting of 12 amino acids of which lysine 259 was shown to be the most important residue with a minor contribution from lysine 255 in plasminogen binding. A predicted structure of EnoA1 shows surface exposure of these residues (Vastano *et al.*, 2013).

Oliveira *et al.* identified the regions of leptospiral adhesin Lsa66 that are responsible for binding extracellular matrix components (Oliveira *et al.*, 2014). They generated three recombinant fragments of Lsa66 proteins: N-terminal (amino acids 30-219), central (amino acids 170-483) and C-terminal fragments (amino acids 420-590). Along with full length Lsa66 (containing amino acids 30-590), these fragments were evaluated for their ability to bind to immobilised laminin, fibronectin and plasminogen. The intermediate region of leptospiral adhesin Lsa66 was involved in binding fibronectin and plasminogen while

laminin bound to both intermediate and C-terminal region of the protein (Oliveira *et al.*, 2014).

Apart from lysine, arginine and histidine have been reported in plasminogen binding M-like protein (PAM) plasminogen-binding and Prp from *S. pyogenes* (Sanderson-Smith *et al.*, 2006; Sanderson-Smith *et al.*, 2007). The residues arginine 107 and histidine 108 were shown to be indispensable for plasminogen binding by Prp (Sanderson-Smith *et al.*, 2007). High affinity plasminogen binding by the PAM protein was shown to be mediated by arginine 101 and 114 together with histidine residues at positions 102 and 115 (Sanderson-Smith *et al.*, 2006).

Protein E (PE) of *H. influenzae* has been reported to be a multifunctional protein capable of interacting with vitronectin, laminin and plasminogen. Previously lysine 85 and arginine 86 has been reported to be involved in vitronectin binding (Singh *et al.*, 2011). By peptide mapping, the region of PE consisting of amino acids 41-68 was later shown to be the region responsible for binding to laminin and plasminogen (Barthel *et al.*, 2012). In a more recent report, the crystal structure of PE revealed that the vitronectin binding residues (lysine 85 and arginine 86) are clearly exposed on the surface of PE and the laminin/plasminogen binding region is partially exposed at the surface (Singh *et al.*, 2013). Further mutagenesis experiments with full length rFBA replacing terminal or sub-terminal lysine residues will be necessary to fully characterise the plasminogen binding region of FBA.

**Chapter Five: Surface localisation of FBA on
N. meningitidis is not affected by mutation(s) in the
active site**

5.1 Introduction

N. meningitidis colonises the nasopharynx of 10% to 30% of the human population (Stephens *et al.*, 2007). Therefore the intimate relationship between *N. meningitidis* and the human host begins with the attachment to the nasopharyngeal mucosa. On rare occasions, an invasive strain of *N. meningitidis* crosses the nasopharyngeal epithelium and enters the bloodstream to cause bacteraemia and sepsis (Stephens and Farley, 1991). During bacteremia, *N. meningitidis* may adhere to the endothelial cells of brain microvessels, cross the blood–brain barrier to cause meningitis (Taha *et al.*, 2002). Therefore, adhesion to epithelial and endothelial cells are two central stages of infection, even though these complex interactions are not fully understood. Type IV pili are the primary mediators of binding to non-ciliated epithelial cells (Stephens and Farley, 1991; Proft and Baker, 2009; Virji *et al.*, 1992a). The receptor CD46 has been implicated in Type IV pilus-mediated adhesion (Kallstrom *et al.*, 1997), although CD46-independent pilus-dependant adhesion has also been reported (Kirchner *et al.*, 2005). After initial adhesion to host cells, the bacteria form microcolonies on the surface of host cells via auto agglutination caused by type IV pili (Trivedi *et al.*, 2011). Pilus mediated adhesion also results in the formation of cellular protrusions within and surrounding these microcolonies (Eugene *et al.*, 2002; Nassif *et al.*, 2002). The initial type IV pili-mediated contact is followed by contact-dependent down regulation of pili and capsule regulated by the CrgA protein resulting in spreading of bacteria on the cell surface as monolayers (Pujol *et al.*, 1999; Deghmane *et al.*, 2002).

Non-encapsulated phenotypes arising at this stage can engage more intimately with cells by means of lipopolysaccharide (LPS) (Albiger *et al.*, 2003), and the outer membrane opacity proteins Opa and Opc (Virji *et al.*, 1993a; Virji *et al.*, 1994; Moore *et al.*, 2005), which subsequently lead to uptake of the bacterial cells (Virji, 2009).

Proteins performing functions in addition to their recognised functions are termed as moonlighting proteins (Jeffery, 1999). These proteins perform their functions in separate cellular locations e.g. cytoplasm and cell surface; thus a dual cellular localization of a protein suggests moonlighting activities (Kainulainen and Korhonen, 2014). Moonlighting proteins are often termed as anchorless surface proteins, referring to the absence of known secretion signal or anchoring motifs in their amino acid sequences (Pancholi and Chhatwal, 2003). The mechanism(s) of translocation of moonlighting proteins are yet to be determined (Kainulainen and Korhonen, 2014).

Bacterial moonlighting proteins impart virulence properties to many pathogens including *M. tuberculosis*, *S. pyogenes*, *S. pneumoniae*, *S. aureus* and *H. pylori*. Adhesion to host epithelia and extracellular matrices (ECMs), recruitment and activation of plasminogen (Plg), and the modulation of host immune responses are some of the non-cytoplasmic moonlighting functions which have been reported (Henderson and Martin, 2011). The mounting list of proteins with moonlighting activities includes metabolic enzymes, chaperones and translational elongation factors (Kainulainen and Korhonen, 2014).

Almost all enzymes in the Embden-Meyerhof-Parnas (EMP) glycolytic pathway of bacteria have been assigned adhesive moonlighting functions. The

two best-characterized bacterial moonlighting enzymes are GAPDH and enolase (Henderson and Martin, 2011).

Fructose-1,6-bisphosphate aldolase (FBA) is another enzyme in the EMP glycolytic pathway. Increasingly, it is also recognised that FBA has additional functions beyond its housekeeping role in central metabolism. Blau *et al* in 2007 showed that FBA functions as an adhesin for *S. pneumoniae* strain WU2 . Flamingo cadherin receptor (FCR), a human membrane protein of the cell adhesion molecules (CAM) family, was found to be the putative host receptor of pneumococcal FBA (Blau *et al.*, 2007). Santangelo and colleagues reported that FBA from *M. tuberculosis* is also a plasminogen-binding protein (de la Paz Santangelo *et al.*, 2011). In *S. suis* SS9, immunoproteomic analysis revealed the presence of FBA on the bacterial cell surface and found it to be immunogenic (Wu *et al.*, 2008). Twenty seven surface-associated proteins from *S. oralis*, including FBA, were identified by Wilkins and colleagues (Wilkins *et al.*, 2003). Meningococcal FBA (FBA-nm) has been reported to be localised to the outer membrane of *N. meningitidis*, in addition to its usual location in cytoplasm. An FBA-deficient mutant strain was less adherent to both human epithelial and endothelial cells, but its ability to invade these cells was unaffected. Complementation with an ectopic copy of the gene encoding FBA-nm restored the adherent phenotype (Tunio *et al.*, 2010a). Recently, Flores-Ramirez and colleagues reported the presence of FBA in the cell wall fraction of *C. burnetii*, suggesting the possibility that translocation of FBA to the Gram-negative cell envelope might be a more generalised phenomenon (Flores-Ramirez *et al.*, 2014).

The aim of the work described in this chapter was to explore the effect of aldolase enzymatic activity of FBA on surface localisation and its involvement in adhesion of meningococci to human cells.

5.2 Materials and Methods

5.2.1 Bacterial techniques

5.2.1.1 Bacterial strains, growth conditions and media

All the plasmids and bacterial strains used are listed in Table 5.1 and Table 5.2 respectively. *E. coli* JM109 harbouring plasmids pCOMP-1, pFS-4, pFS-5 or pFS-6 was routinely grown in Lysogeny Broth (LB) and on LB agar plates containing ampicillin (100 µg/ml) and erythromycin (2 µg/ml) at 37°C. Meningococcal strains (Table 5.2) were grown on chocolate agar, Brain Heart Infusion (BHI) agar or in Brain Heart Infusion Broth containing kanamycin (50 µg/ml) and/or erythromycin (2 µg/ml) and incubated at 37°C with or without shaking and/or 5% CO₂.

Table 5.1: Plasmids used in work described in this chapter

Plasmid	Characteristics	Source or reference
pCOMP-1	pGEM-T Easy with a complementation cassette consisting of a <i>Bam</i> HI site, <i>cbbA</i> promoter, <i>Bgl</i> III site, erythromycin resistance cassette flanked by two meningococcal genes (NMB0102 and NMB0103)	H. Albasri (University of Nottingham) modified from (Tunio <i>et al.</i> , 2010a)
pFS-4	MC58 <i>cbbA</i> gene cloned in pCOMP-1	This study
pFS-5	pFS-4 with single nucleotide mutation at codon 83 (GAC to GCC) of <i>cbbA</i>	This study
pFS-6	pFS-4 with nucleotide mutations at codons 81/84 (CAC to GCC / CAC to GCC) of <i>cbbA</i>	This study

Table 5.2: Bacterial strains used in work described in this chapter

Bacterial strain	Characteristics	Source or reference
<i>E. coli</i>		
JM109	<i>endA1 recA1 gyrA96 thi hsdR17 (r_K⁻ r_K⁻) relA1 supE44 Δ(lac-proAB) [F' traD36 proAB laqI^qΔM15]</i>	Promega
<i>N. meningitidis</i>		
MC58	Wild type serogroup B isolate	(Tettelin <i>et al.</i> , 2000)
MC58Δ <i>cbbA</i>	<i>cbbA</i> deleted and replaced with kanamycin cassette	(Tunio <i>et al.</i> , 2010a)
MC58Δ <i>cbbA cbbA</i> ^{Ect}	MC58Δ <i>cbbA</i> complemented with an ectopic copy of <i>cbbA</i>	This study
MC58Δ <i>cbbA cbbA-m1</i> ^{Ect}	MC58Δ <i>cbbA</i> complemented with an ectopic copy of <i>cbbA</i> (mutation at codon 83 (GAC to GCC))	This study
MC58Δ <i>cbbA cbbA-m2</i> ^{Ect}	MC58Δ <i>cbbA</i> complemented with an ectopic copy of <i>cbbA</i> (mutations at codons 81/84 CAC to GCC / CAC to GCC)	This study

5.2.1.2 Natural transformation of *N. meningitidis* MC58Δ*cbbA*

N. meningitidis MC58Δ*cbbA* was grown overnight on chocolate blood agar plates. A single colony was inoculated into brain heart infusion broth (BHIB) and grown at 37°C with shaking to an OD₆₀₀ of 0.2. After that 200 µl of the culture was transferred to a 15 ml tube containing 1.5 ml of brain heart infusion agar (BHI) (Oxoid) and incubated for 2-4 h at 37°C in 5% CO₂ without

shaking. After incubation, 1µg of plasmid pFS-4, pFS-5 or pFS-6, was added to the culture and incubation was continued overnight. The following day, the culture was inoculated onto BHI agar plates containing kanamycin (50 µg/ml), and erythromycin (2 µg/ml). Putative transformant colonies were selected after overnight incubation at 37°C with 5% CO₂. The clones were checked for presence of *cbbA* and complementation cassette by PCR using primers in Table 5.3.

5.2.1.3 Flow cytometry

The presence of FBA on the surface of *N. meningitidis* strains was determined by flow cytometry. Samples were prepared for flow cytometry analysis as described previously (Tunio *et al.*, 2010a). Briefly, bacteria were grown to mid-log phase (OD 0.7 at 600nm). Aliquots containing 1×10^7 cfu were washed by resuspending in sterile filtered PBS followed by centrifugation at $5000 \times g$ for 5 min. For each strain, four samples were prepared: untreated, treated with primary antibody only, treated with secondary antibody only or treated with both primary and secondary antibodies. Anti-rFBA antiserum (1:500) and goat anti-rabbit IgG-Alexa Flour 488 conjugate (Invitrogen; 1:50), both diluted in PBS containing 0.1% (wt/vol), BSA 0.1% (wt/vol sodium azide) and 2% (vol/vol foetal calf serum), were used as primary and secondary antibodies, respectively. After addition of antibodies, cells were incubated for 2 h in ice followed by washing three times with PBS. Samples treated with secondary antibody were kept in the dark. After final washing, the cells were fixed by resuspending in 1 ml PBS containing 0.5% (wt/vol formaldehyde). Analysis of all samples was performed using BD FACSAria™ flow cytometer and BD FACS Diva software (BD Biosciences). The cells were detected using

forward and side scatter dot plot and bacterial aggregates and cell debris were excluded by defining a gating region. For each sample, a total of 20,000 events were analysed for fluorescence signals. Subsequent analysis of data was done using Weasel software.

5.2.1.4 Preparation of meningococci for cell culture

N. meningitidis MC58, MC58 Δ *cbbA* and MC58 Δ *cbbA* *cbbA*^{Ect} strains were streaked onto chocolate agar plates and incubated at 37°C. The following day a single colony was inoculated into 10 ml Endothelial Cell Medium (ECM, ScienCell) or Minimum Essential Medium Eagle (MEM, Sigma-Aldrich) containing 10% (vol/vol) heat inactivated foetal calf serum (FCS) and incubated overnight at 37°C with shaking. The following day 1 ml of overnight culture was inoculated in 10 ml ECM or MEM containing 10% FCS (vol/vol) and grown for further 2 hours at 37°C with shaking.

5.2.2 DNA techniques

5.2.2.1 Extraction of chromosomal and plasmid DNA

DNA from *N. meningitidis* MC58 and plasmids pCOMP-1, pFS-4, pFS-5 or pFS-6 from *E. coli* JM109 were extracted using DNeasy Blood and Tissue extraction kit (Qiagen) and QIAGEN miniprep kit respectively, according to the manufacturer's instructions.

5.2.2.2 Polymerase Chain Reaction (PCR) amplification of *cbbA*

PCR was performed to amplify the *cbbA* from the meningococcal DNA using the primers listed in the Table 5.3 and Phusion High-Fidelity DNA polymerase (New England Biolabs). Amplification conditions were as follows: initial denaturation at 98°C for 30s, denaturation at 98°C for 10s, annealing at 65°C for 30 sec, extension at 72°C for 30 sec, final extension at 72°C for 5 mins.

Table 5.3: List of primers used in work described in this chapter

Primer	Sequence ^a	Restriction site
Amplification of <i>cbbA</i>		
FBA comp F1	GGA A GATCTCAAATAAGCGTCCAAGTA TGCCGC (33-bp)	<i>Bgl</i> II
FBA comp R1	CGC G ATCCTTACAGGCAACCTGTTAT TTGACG (33-bp)	<i>Bam</i> HI
Site directed mutagenesis		
FBA_D83AF	CGTCATGCACCAAGCCACGGCGCATCAC (29-bp)	
FBA_D83AR	GTGATGCGCCGTGGGCTTGGTGCATGACG (29-bp)	
FBA_H8184 AF	CCCCGTCGTCATGGCCCAAGACGCCGGCGCATCAC CC (37-bp)	
FBA_H8184 AR	GGGTGATGCGCCGGCGTCTTGGGCCATGACGACGG GG (37-bp)	
Amplification of complementation cassette		
NMB102F	GTTCGGCGGCTTGCGTGGTCG (21-bp)	
NMB103F	TACGGAGTTGGAAAGCACGGC (21-bp)	

^a Sequences in bold identify restriction enzyme sites

5.2.2.3 Agarose gel electrophoresis

Procedures were followed as described in Chapter Four, section 4.2.2.3.

5.2.2.4 Purification of PCR product

Procedures were followed as described in Chapter Four, section 4.2.2.4.

5.2.2.5 Digestion of vectors and inserts

Amplified *cbbA* and pCOMP-1vector were digested by *Bgl*II and/or *Bam*HI (New England Biolabs) according to the manufacturer's instruction.

5.2.2.6 Dephosphorylation of digested vector

Procedures were followed as described in Chapter Four, section 4.2.2.6.

5.2.2.7 Ligation

A Rapid DNA Ligation kit (Fermentas Life Sciences) was used to ligate digested pCOMP-1 and *cbbA* following the manufacturer's instructions. After ligation with the insert, the plasmid was renamed as pFS-4.

5.2.2.8 Site directed mutagenesis and transformation of XL10-gold ultracompetent cells and *E. coli* JM109 cells

Mutation(s) at specific site(s) of *cbbA* in pFS-4 plasmid were introduced using the primers listed in the Table 5.3 and procedures described in Chapter Two, section 2.2.2.3. Resulting plasmids were renamed pFS-5 and pFS-6, respectively. *E. coli* JM109 competent cells were transformed following the procedures described in Chapter Two, section 2.2.3.1.

5.2.2.9 Nucleic acid and amino acid sequence analysis

Procedures were followed as described in Chapter Two, section 2.2.2.4.

5.2.3 Cell culture techniques

5.2.3.1 Preparation of human cells

Human brain microvascular endothelial (HBME) cells and human pharyngeal carcinoma cells (Detroit-562) were grown to confluence in 24-well tissue culture plates (Nunc) at 37°C in presence of 5% CO₂. Prior to use, 24-well plates were coated with 0.3% bovine fibronectin (from bovine plasma; Sigma-Aldrich) in PBS. To grow HBME cells, Endothelial Cell Medium (ECM, ScienceCell) supplemented with 5% (vol/vol) foetal bovine serum (FBS, ScienCell), 1% (vol/vol) endothelial cell growth supplement (ECGS, ScienCell) and 1% (vol/vol) penicillin/streptomycin solution (10,000 units/mL of penicillin and 10 mg/mL of streptomycin, P/S, ScienCell) were used. For Detroit-562 epithelial cells, Minimum Essential Medium Eagle (MEM, Sigma-

Aldrich) supplemented with 10% (vol/vol) foetal bovine serum (FBS, Sigma-Aldrich), 1% (vol/vol) non-essential amino acids (NEAA, Sigma-Aldrich) and 1% (vol/vol) penicillin/streptomycin solution (10,000 units/mL of penicillin and 10 mg/mL of streptomycin, P/S, Sigma-Aldrich) were used. Prior to all association and invasion experiments, the cell monolayers were washed with ECM or MEM without any antibiotics.

5.2.3.2 Association and invasion assay

HBME cells or Detroit-562 monolayers were infected with 1×10^7 cfu/ml meningococci. The size of inoculum was confirmed retrospectively by plating out serial dilutions of the inoculum. The monolayers were left in the incubator for 2 h. After that the monolayers were washed three times with 1 ml PBS per well. The monolayers were disrupted with 0.1% (wt/vol) saponin in PBS. The homogenised suspension was serially diluted and 10 μ l aliquots of the dilutions were plated on chocolate agar.

For invasion assay the duration of infection was extended to 4 h. After three washes with PBS, 1ml ECM or MEM containing gentamicin (150 μ g/ml) was added to each well. After 1 h, cell monolayers were washed three times with PBS (1ml per well). Prior to washing, aliquots from the wells were plated out to confirm the killing of meningococci. 0.1% (wt/vol) saponin in PBS was used to disrupt the monolayers. One hundred microlitres of the homogenised suspension was spread on chocolate agar. The chocolate agar plates were incubated overnight at 37°C with 5% CO₂ and colony forming unit (cfu) per ml was recorded.

5.2.3.3 Statistical analysis

Statistical analysis was performed using GraphPadPrism 6 (GraphPad Software, Inc.). All the association and invasion assay experiments were done in quadruplet 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.

5.3 Results

5.3.1 Complementation of *N. meningitidis* $\Delta cbbA$

In order to complement the FBA knock out *N.meningitidis* strain MC58 $\Delta cbbA$, the meningococcal FBA-encoding gene (*cbbA*) was first amplified by PCR (Figure 5.1A). Following restriction digestion, the resulting amplicon and complementation plasmid vector pCOMP-1 were ligated.

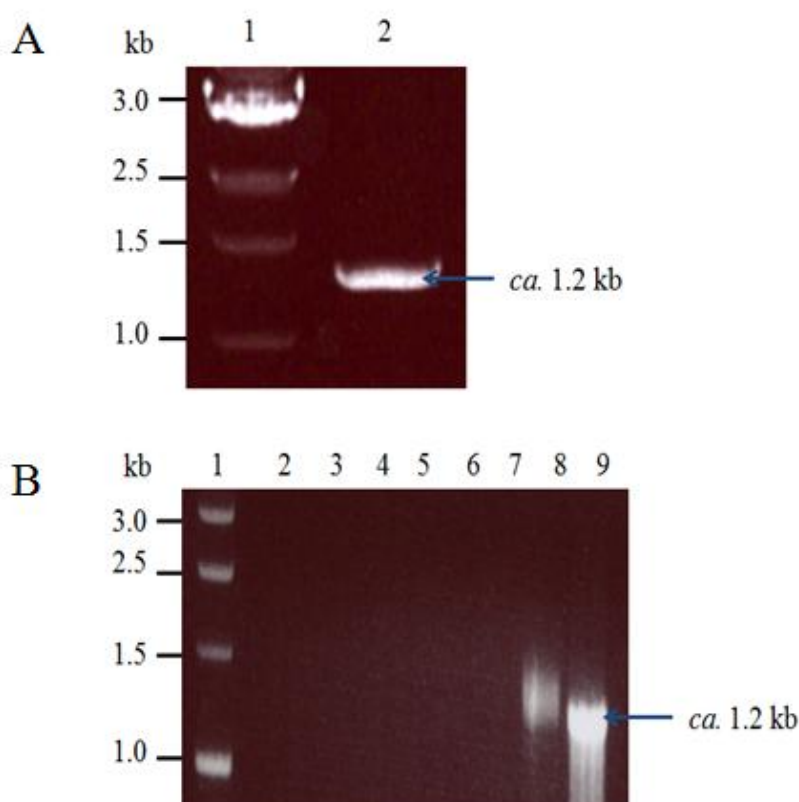


Figure 5.1: Amplification and ligation of *cbbA* with pCOMP-1 vector. Agarose gel electrophoresis showing A) amplification of *cbbA*. Lane 1: 1 kb DNA ladder; Lane 2: amplified *cbbA* of 1.2 kb length. B) Successful ligation of *cbbA* in pCOMP-1 in one transformant (Lane 8). Lane 1: 1 kb DNA ladder; Lane 2-8: plasmid DNA extracted from different transformant colonies of *E. coli* JM109 cells; Lane 9: *N. meningitidis* MC58 chromosomal DNA (positive control).

Colonies of *E. coli* JM109 cells, transformed with pCOMP-1 ligated to *cbbA* were selected and plasmid DNA was purified from these clones. PCR amplification using the primer pair FBA comp F1 and FBA comp R1 and subsequent agarose gel electrophoresis showed one clone contained the ligated product (Figure 5.1 B). The presence of *cbbA* was confirmed by sequencing (Figure 5.2). The new construct, pCOMP-1 containing *cbbA*, was renamed pFS-4 and was used for site-directed mutagenesis as described in the section 5.3.2.

5.3.2 Construction of plasmid vector containing *cbbA* with various mutations

The aldolase active site (divalent cation-binding site) in FBA contains highly-conserved histidine (H81 and H84) and aspartic acid (D83) residues. In order to generate enzymatically inactive FBA, primers were designed for site-directed mutagenesis reactions to replace both H81 and H84 with alanine and, separately, D83 with alanine. Sequencing confirmed that GAC to GCC (D83A) and CAC to GCC / CAC to GCC (H81A/H84A) mutations were successfully introduced into *cbbA* in pFS-4. Newly generated plasmids harbouring the mutation(s) D83A and H81A/H84A were renamed pFS-5 and pFS-6, respectively (Fig. 5.2).

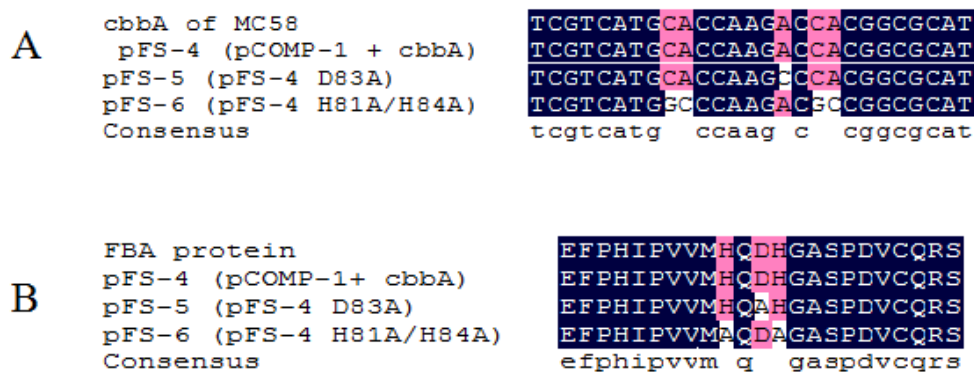


Figure 5.2: Multiple sequence alignment of the A) DNA sequence and B) translated product of *cbbA* cloned in pFS-4 and pFS-5 and pFS-6 resulting from the desired site-directed mutagenesis.

5.3.3 Complementation of *N. meningitidis* MC58Δ*cbbA* with wild type or mutated *cbbA*

The plasmids pFS-4, pFS-5 and pFS-6 were used for natural transformation of MC58Δ*cbbA*, thereby introducing a single chromosomal copy of the complementing gene in the intergenic region between the genes NMB0102 and NMB0103 (which are orientated in a tail-to-tail fashion and have been previously used to complement mutations in *N. meningitidis*). Chromosomal DNA was extracted from putative transformant colonies for further confirmation of complementation. Using the primer pair NMB 102F and NMB 103F, PCR was performed to amplify the complementation cassette from the chromosomal DNA of the complemented strains. An expected *ca.* 3.2-kb band indicated correct insertion of the complementation cassette (Figure 5.3) whereas wild type *N. meningitidis* MC58 and *N. meningitidis* MC58Δ*cbbA* were expected to produce a band of *ca.* 1.3 kb, representing the amplified NMB0102F and NM0103F. Presence of nonspecific bands in the agarose gel indicated non-optimal primer design or reaction conditions.

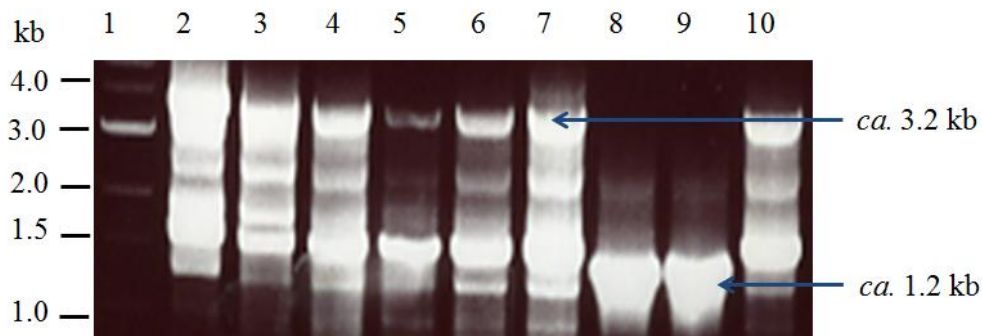


Figure 5.3: Confirmation of insertion of complementation cassette into the chromosomal DNA of MC58 Δ *cbbA*. The presence of complementation cassette of *ca.* 3.2 kb in the MC58 Δ *cbbA* strains complemented with either WT or mutated copy of *cbbA* was confirmed by agarose gel electrophoresis. Some non-specific bands were also present. Lane 1: 1kb DNA ladder; lane 2-7: complemented MC58 Δ *cbbA* strains; lane 8: MC58 WT; lane 9: MC58 Δ *cbbA*; lane 10: Previously constructed MC58 Δ *cbbA* *cbbA*^{Ect}.

Using the primer pair FBA compF1 and FBA compR1 (Table 5.2), an expected band of *ca.* 1.2 kb consistent with the expected size of *cbbA* was amplified from the chromosomal DNA from MC58 WT and all the complemented mutants, but not from MC58 Δ *cbbA* (Figure 5.4), which confirmed the successful complementation of the *cbbA* mutation.

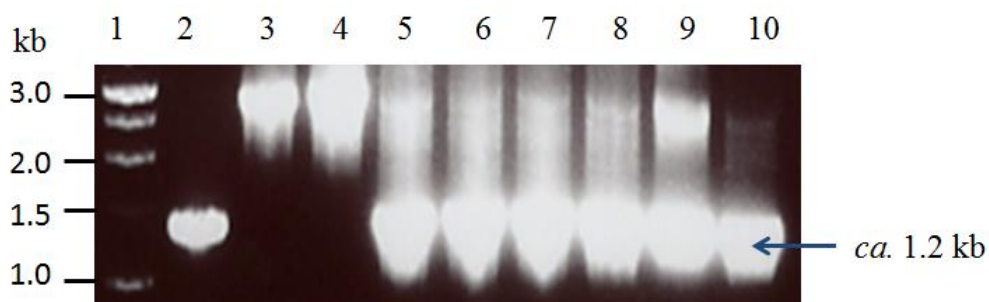


Figure 5.4: Confirmation of presence of *cbbA* in the chromosomal DNA of complemented MC58 Δ *cbbA* strains. Agarose gel showing the presence of *cbbA* (*ca.* 1.2-kb) in the MC58 Δ *cbbA* strains complemented with either a WT or mutated copy of *cbbA*. Lane 1: 1-kb DNA ladder; lane 2: MC58 WT; lane 3: MC58 Δ *cbbA*; lane 4: previously constructed MC58 Δ *cbbA* *cbbA*^{Ect} strain; lane 5-10: newly constructed MC58 Δ *cbbA* *cbbA*^{Ect} strains.

5.3.4 Expression of FBA by *N. meningitidis* MC58 $\Delta cbbA$ $cbbA^{Ect}$ strains

Proteins from the whole cell lysates of wild type MC58, MC58 $\Delta cbbA$ and MC58 $\Delta cbbA$ strains complemented with pFS-4, pFS-5 or pFS-6 were separated by SDS-PAGE and probed with anti-FBA serum followed by anti-rabbit conjugated with alkaline phosphatase. The blot confirmed the production of FBA by the *N. meningitidis* $\Delta cbbA$ strains complemented with pFS-4 or pFS-5 but little expression was observed from the strain complemented with pFS-6 (Figure 5.5). The newly constructed MC58 $\Delta cbbA$ strains complemented with pFS-4, pFS-5 and pFS-6 plasmids were designated as MC58 $\Delta cbbA$ $cbbA^{Ect}$, MC58 $\Delta cbbA$ $cbbA-m1^{Ect}$ and MC58 $\Delta cbbA$ -m2 $cbbA^{Ect}$, respectively.

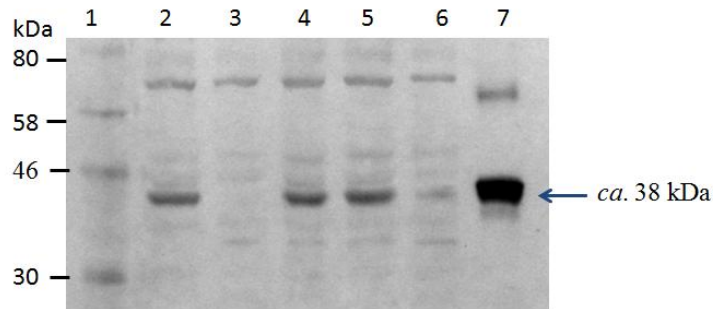


Figure 5.5: Immunoblot probed with anti-FBA antibody shows the production of FBA by the complemented *N. meningitidis* $\Delta cbbA$ strains. Lane 1: Prestained protein marker; lane 2: MC58 WT; lane 3: MC58 $\Delta cbbA$; lane 4: MC58 $\Delta cbbA$ $cbbA^{Ect}$; lane 5: MC58 $\Delta cbbA$ $cbbA-m1^{Ect}$; lane 6: MC58 $\Delta cbbA$ $cbbA-m2^{Ect}$; lane 7: purified rFBA (WT) used as positive control.

5.3.5 Mutation(s) in the cofactor binding site of FBA do not prevent its surface localisation

N.meningitidis MC58, MC58 $\Delta cbbA$, or MC58 $\Delta cbbA$ complemented with either wild type or a mutated copy of *cbbA*, were subjected to flow cytometry analysis. Wild type MC58 cells, treated with both primary and secondary antibody, demonstrated a significant shift in fluorescence signal. 84% of cells were found to be fluorescently labelled (Fig. 5.6). No corresponding shift signal was observed for MC58 $\Delta cbbA$ indicating an absence of FBA on the surface of the cells (Fig. 5.6). MC58 $\Delta cbbA$ *cbbA*^{Ect}, MC58 $\Delta cbbA$ *cbbA*-m1^{Ect} and MC58 $\Delta cbbA$ -m2*cbbA*^{Ect} strains all showed clear evidence of surface-localised FBA with more than 70% cells (Fig. 5.6) found to have FBA on their surface.

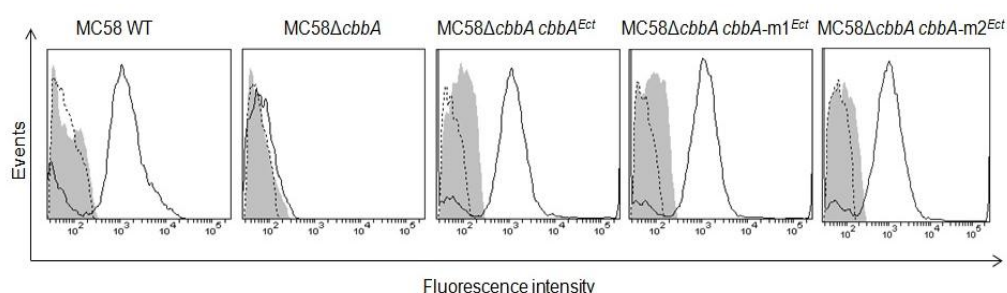


Figure 5.6: Flow cytometry analysis for surface localisation of FBA. Cells were treated with rabbit anti-FBA antiserum followed by anti-rabbit IgG-AlexaFluor 488 conjugate. Fluorescence is displayed as a histogram: area enclosed by the dashed line indicates bacteria treated with primary antibody only; area shaded in grey indicates bacteria treated with secondary only; area enclosed by the solid line indicates bacteria treated with both antibodies.

5.3.6 The Role of FBA in association and invasion of human epithelial and endothelial cells

Association assays were performed to investigate a possible role for FBA as an adhesin for epithelial and endothelial cells by viable counting of bacteria associated with the infected monolayers. In HBME cells, significantly lower levels of adherence were observed in MC58 $\Delta cbbA$ compared to the MC58 wild type (Fig. 5.7A). In complemented strains, levels of adhesion were restored to levels that were not significantly different to the wild type strain.

For Detroit-562 epithelial cells, the difference(s) in the level of adherence did not reach the level of significance between the MC58 wild type and MC58 $\Delta cbbA$, indicating that the effect of FBA might be cell type-specific (Fig. 5.7B). It is also possible that a significant difference between these two strains could have been observed with more repeats. The level of adherence of MC58 $\Delta cbbA$ *cbbA*^{Ect}, MC58 $\Delta cbbA$ *cbbA-m1*^{Ect} strains was similar to the MC58 wild type strain (Fig. 5.7B). Although MC58 $\Delta cbbA$ -m2*cbbA*^{Ect} adhered slightly less than the wild type, it was not statistically significant (Fig. 5.7B). Taken together, these results suggest that the mutation(s) in the cofactor binding site of FBA has no effect on the association of *N. meningitidis* MC58 with at least HBME cells. This strongly suggests that the aldolase enzymatic activity of FBA is not involved in the association related phenotype of FBA to HBME cells.

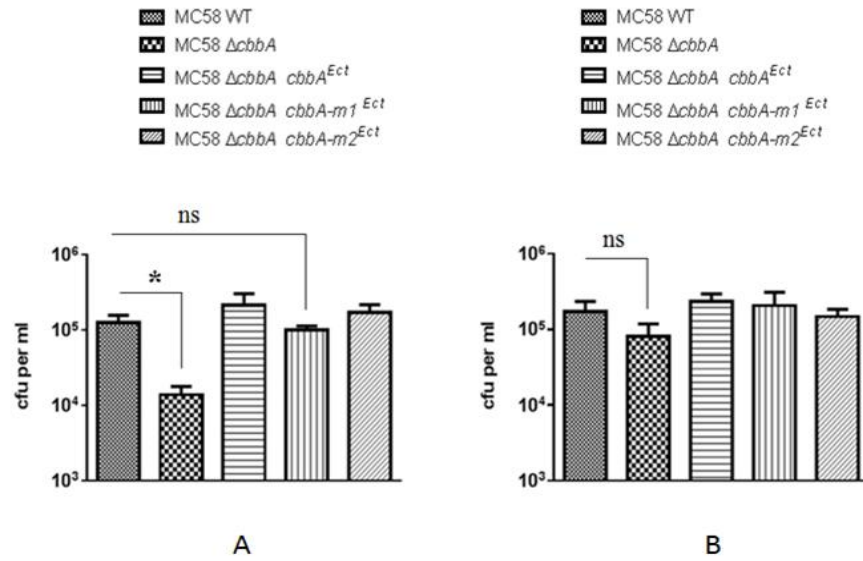


Figure 5.7: A reduction in association of MC58 $\Delta cbbA$ was observed with A) HBME cells but not with B) Detroit-562 epithelial cells compared to MC58 wild type strain. Complementation of MC58 $\Delta cbbA$ strains with either a wild type or a cofactor binding site mutant copy of *cbbA* restored the ability to adhere with HBME cells. Data represent mean values ($\pm$ SE) based on five independent assays carried out in triplicates. cfu denotes colony forming units; ns denotes non significant; * denotes $p < 0.05$.

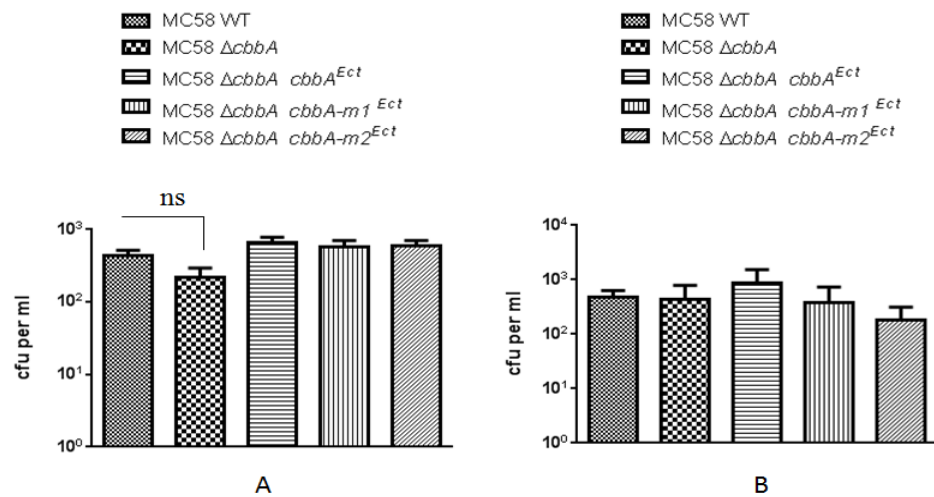


Figure 5.8: MC58 $\Delta cbbA$ has a reduced ability to invade A) HBME cells but not B) Detroit-562 epithelial cells compared to MC58 wild type strain, although the difference between the MC58 wild type and MC58 $\Delta cbbA$ strains is not statistically significant. The level of cellular invasion of MC58 $\Delta cbbA$ strains complemented with either a wild type or a cofactor binding site mutant copy of *cbbA* was similar to the wild type strain. cfu denotes colony forming units; ns denotes non significant.

To evaluate the role of FBA in the internalisation process of *N. meningitidis* into epithelial and endothelial cells, D562 and HBME cell monolayers were infected with wild type MC58, MC58 $\Delta cbbA$ and MC58 $\Delta cbbA$ *cbbA*^{Ect} cells. After 4 h co-incubation of human cell lines with meningococcal cells, gentamicin was used to kill unattached or surface attached bacterial cells. Cell monolayers were disrupted with saponin to release internalised bacteria which were enumerated by spreading the lysate onto chocolate agar. In four experiments with HBME cells, a small difference was observed between MC58 wild type and MC58 $\Delta cbbA$ but this did not reach statistical significance (Fig. 5.8A). No difference was observed for Detroit-562 epithelial cells (Fig. 5.8B).

5.4 Discussion

This chapter describes the effect of mutation(s) in the co-factor binding site of FBA on surface localisation and its role in the adhesion to and invasion of human cells.

The enzymatic assay described in Chapter Two confirmed that mutation(s) in the zinc cofactor binding site abolished the aldolase activity of FBA. Based on these findings, the same mutations (D83A and H81/84A) were introduced into *cbbA* of meningococcal strain MC58. In order to do that, three different constructs were generated with plasmid pCOMP-1 containing *cbbA* (WT, D83A or H81/84A). These constructs were introduced into MC58Δ*cbbA* by natural transformation. These complemented mutant cells were subsequently utilised to characterise the surface localisation of FBA and the involvement of FBA in adhesion to and invasion into human cells.

The presence of *cbbA* in the complemented mutant strains was confirmed by PCR. Immunoblots, probed with anti-FBA antiserum, also confirmed that FBA was produced by the complemented mutant strains. One interesting observation was that introduction of the double mutations (H81A/84A) caused low level expression of FBA in meningococcal cells. This was also apparent in *E. coli* during the expression of rFBA with the same mutations (described in chapter two). It is possible that the introduction of the double substitution in the mutant protein resulted in protein instability and thus either degradation or the formation of inclusion bodies but this was not examined further.

To check the effect of mutation(s) in the cofactor binding site, on the surface localisation of FBA, flow cytometry analysis was performed. The presence of FBA could be detected on the surface of more than 80% of the population of

wild type MC58 cells. In contrast, as expected, and previously described by Tunio *et al* (Tunio *et al.*, 2010a), FBA was absent on the surface of MC58 Δ *cbbA*. Complementation of the mutant strain with an ectopic copy of either wild-type or mutated *cbbA* restored the ability of the strains to express FBA on their surface (>70% of the cell population was found to have FBA on the surface). Introduction of double mutations in the active site (H81A/84A) resulted in expression of reduced levels of the doubly-substituted FBA. Nevertheless, expression of this protein on the surface was indistinguishable from that of the wild type protein. Therefore, it can be concluded that mutations in the cofactor binding site, leading to the abolition of aldolase activity, have no effect on the transportation of FBA to the surface of meningococcal cells

Bacterial moonlighting proteins are termed as “anchorless proteins” as they do not contain any recognised secretion signal or any anchoring motifs (Pancholi and Chhatwal, 2003). The mechanism of translocation of moonlighting proteins to the cell surface remains an enigma. One hypothesis that has been mooted is that these proteins are released from the cytoplasm by cell lysis and neighbouring bacterial cells recruit them to their surfaces. This has been observed for GAPDH of group B streptococci (Oliveira *et al.*, 2012). In *L. plantarum*, levels of surface-associated GAPDH are related to increased permeability of the plasma membrane during the stationary phase of growth (Saad *et al.*, 2009), whereas iron starvation increases release of GAPDH from *S. pyogenes* (Eichenbaum *et al.*, 1996). Moreover an auxiliary secretion system, SecA2, was reported to be present in *L. monocytogenes*. Seventeen proteins were dependent on this system for secretion, of which ten proteins

contained no apparent secretion signals and were regarded as moonlighting proteins; these included DnaK, GroEL, EF-Tu and enolase (Lenz *et al.*, 2003). However, the SecA2 system is only found in certain pathogenic Gram-positive bacteria, including *S. aureus*, virulent strains of *S. pneumoniae*, *M. tuberculosis*, *Bacillus anthracis*, and, *Clostridium difficile* (Lenz *et al.*, 2003; Feltcher and Braunstein, 2012). One recent report identified a type III secretion system (T3SS) in enteropathogenic *E. coli* (EPEC) which was responsible for the secretion of some, but not all, GAPDH (Aguilera *et al.*, 2012). As no T3SSs are encoded by the *N. meningitidis* genome (Van Ulsen and Tommassen, 2006), this cannot be the route for meningococcal FBA to reach the surface of the cell. Finding the export machinery for meningococcal FBA would be an interesting area for investigation.

There might be some yet unknown secretion mechanisms for the moonlighting proteins (Henderson and Martin, 2011). The addition of a 12 amino acid hydrophobic tail at the C-terminus of GAPDH and deletion of 19 amino acids from a central hydrophobic α -helical domain of enolase, prevented their export to the cell surface of *S. pyogenes* (Boel *et al.*, 2005) and *B. subtilis* (Yang *et al.*, 2011), respectively. Taking these observations into consideration, future experiments could be designed to look for the regions of meningococcal FBA which are required for secretion. Tunio *et al* previously noted that FBA was predicted to be a non-secreted protein by the SignalP-HMM algorithm. Analysis with the SignalP-NN algorithm, by contrast predicted a 13-amino acid signal peptide with a predicted cleavage site between 11DHA-AE15 (Tunio *et al.*, 2010a). This could be investigated by targeted substitution of residues within this region, specifically the alanines at positions 13 and 14.

Tunio *et al* reported that FBA is involved in the adhesion process to epithelial and endothelial cells (Tunio *et al.*, 2010a). This study was undertaken to further confirm whether or not FBA needs to be enzymatically active to exert this moonlighting function. Therefore the complemented mutant strains were employed in adhesion and invasion assays. The association assay performed in this study further confirmed that MC58 Δ *cbbA* strain had reduced ability to adhere to HBME cells. The MC58 derived strains expressing enzymatically inactive FBA were able to associate with HBME cells as well as the wild type MC58 strain. Complementation of the mutant strain with either wild type or aldolase-inactive *cbbA*, restored the capability to adhere with HBME cells. Although MC58 Δ *cbbA* *cbbA-m1^{Ect}* appeared to associate less than the other two complemented mutant strains but this was not statistically significant. From this part of the study, it can be concluded that FBA does not have to be enzymatically active to exert its activity in the adhesion process to HBME cells.

Moonlighting functions may involve any part or parts of a protein, including the region responsible for the canonical function. An example of this is the ribosomal protein S10 in *E. coli*. This protein is a structural component of ribosomal RNA and can also form the NusB-S10 transcription antitermination complex (Luo *et al.*, 2008). Both interactions involve overlapping regions of S10. More commonly, however, moonlighting proteins employ separate regions or motifs for moonlighting functions than that used for their canonical functions. For example, in *Hansenula polymorpha*, pyruvate carboxylase, alongside its established role to catalyse carboxylation of pyruvate into oxaloacetate, is also essential for the assembly of the peroxisomal protein

alcohol oxidase (AO). Amino acid substitutions inactivating the enzyme activity of pyruvate carboxylase, has no effect on its function in AO assembly and import. Likewise, mutations that inhibit the function of pyruvate carboxylase in import and assembly of AO, have no effect on its enzyme activity (Ozimek *et al.*, 2007).

Antlions are larvae of the Myrmeleontidae family of insects. They paralyse their insect prey by injecting a paralyzing toxin produced by the salivary bacteria *Enterobacter aerogenes*. This paralyzing toxin is a homologue of GroEL which is a molecular chaperone (Braig *et al.*, 1994; Yoshida *et al.*, 2001). Only four amino acids Val 100, Asn 101, Asp 338 and Ala 471 are critical for toxicity, as shown by mutational analysis. These residues are not involved in the chaperone function of GroEL. Interestingly, when these residues are introduced in place of their corresponding residues in the GroEL homologue in *E.coli*, which differs from *E. aerogenes* GroEL by only 11 amino acid residues, it too becomes toxic. (Yoshida *et al.*, 2001).

In *Kluyveromyces lactis*, galactokinase is involved in galactose utilization and acts as a transcriptional activator for itself and the other proteins of the galactose pathway (Meyer *et al.*, 1991; Hittinger and Carroll, 2007). Mutational analysis reveals that its function as a transcriptional activator is independent of its enzymatic function and *vice versa*.

Dihydrolipoamide dehydrogenase (DLD) is a mitochondrial enzyme (Babady *et al.*, 2007) predominantly present as active homodimers. Under certain conditions, such as acidification of the mitochondrial matrix, the protein is mostly found in the monomeric form which is inactive as a dehydrogenase but gains proteolytic activity due to exposure of a catalytic dyad at the dimer

interface. Replacement of a single amino acid in the catalytic dyad, S456A, impairs proteolytic activity only (Babady *et al.*, 2007).

M. tuberculosis glutamate racemase (MurI) is essential for cell wall biosynthesis. It can also reduce binding of gyrase to DNA thus acting as a DNA gyrase inhibitor. Both functions are independent of each other (Sengupta *et al.*, 2008). Moreover, overproduction of either enzymatically active or inactive MurI, makes *M. tuberculosis* resistant to ciprofloxacin (Sengupta *et al.*, 2008), an antibiotic that binds DNA gyrase to stimulate introduction of double-strand DNA breaks (Drlica and Zhao, 1997).

In addition to HBME cells, Detroit-562 epithelial cells were also employed to examine involvement of FBA in the adhesion process of meningococci to epithelial cells. Detroit-562 epithelial cells presented a different picture in contrast to what was observed with HBME cells. The level of adherence of MC58 wild type and MC58 Δ *cbbA* strains to Detroit-562 epithelial cells was similar. This result appears to be in contrast to the previous report by Tunio *et al* (Tunio *et al.*, 2010a). However, in the previous study Hep-2 epithelial cells were used. This might indicate that the involvement of FBA in the adhesion process might be cell type specific. Further study should be undertaken to understand the exact nature of interaction of FBA and HBME cells. To identify the cell receptor on HBME cells, it would be necessary to design future experiments using, for example, immunoprecipitation, re-tagging or the two-hybrid system.

Chapter Six: General Discussion

6.1 General Discussion

Moonlighting proteins have been reported in all three domains of life. To date more than 300 moonlighting proteins have been reported (Hernandez *et al.*, 2014; Mani *et al.*, 2015). Pathogenic Gram positive bacteria were the first reported to have moonlighting proteins as putative virulence factors (Pancholi and Fischetti, 1992). Now it is well established that moonlighting proteins are also displayed by the Gram-negative bacteria and also by weakly pathogenic or commensal bacteria. The bacterial moonlighting proteins (90 referenced so far) are predominantly conserved metabolic enzymes of glycolysis and the tricarboxylic acid cycle, molecular chaperones, or transcription and translation factors (Henderson, 2014).

FBA is the enzyme responsible the cleavage of fructose 1,6-bisphosphate (FBP) into glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (DHAP) or the reverse aldol condensation reaction in glycolysis and gluconeogenesis, the two vital metabolic pathways vital for cellular function (Rutter, 1964). FBA is a highly conserved enzyme found in all forms of life. Based on catalytic mechanisms and structural characteristics, there are class I and class II FBAs (Rutter, 1964). In addition to the usual metabolic role in cytoplasm, the unexpected presence of FBA on the surface of various microbial species performing non-glycolytic functions, have been demonstrated by an increasing number of reports. The most common moonlighting function of FBA is plasminogen binding, as observed in various organisms: *M. tuberculosis* (de la Paz Santangelo *et al.*, 2011) , *Candida albicans* (Crowe *et al.*, 2003), *Cryptococcus neoformans* (Stie *et al.*, 2009), *Paracoccidioides Pb01* (Chaves *et al.*, 2015), *Streptococcus bovis* (Ramajo-Hernandez *et al.*, 2007) and

Dirofilaria immitis (Gonzalez-Miguel *et al.*, 2013). Other moonlighting functions include adhesion to human epithelial cells (Blau *et al.*, 2007; Tunio *et al.*, 2010a) and control of RNA polymerase III mediated transcription (Ciesla *et al.*, 2014).

N. meningitidis is a major cause of bacteraemia and meningitis worldwide (Tan *et al.*, 2010). Understanding meningococcal pathogenesis may facilitate the discovery of novel treatments or preventative vaccines. There have been an increasing number of reports about moonlighting proteins acting as virulence factors (Henderson and Martin, 2011). Tunio *et al* conducted studies to characterise two glycolytic enzymes, GAPDH and FBA in *N. meningitidis*. It is worth mentioning that *N. meningitidis* does not employ the EMP pathway due to absence of phosphofructokinase but retains *cbbA*, the gene that encodes FBA. It has been reported that FBA is constitutively expressed by the bacterium, which is exported to the surface where it performs function(s) unrelated to enzymatic activity. Specifically, FBA was shown to be required for optimal adhesion to epithelial and endothelial cells (ref). However, several fundamental questions remained unanswered. How is FBA translocated to the cell surface? What is the ligand(s) that FBA interacts with? What is the exact role played by FBA in the pathogenesis of meningococci (and other *Neisseria* species)? The studies undertaken in this work aimed to investigate possible host ligand(s) that FBA might interact with, and whether the enzymatic activity of FBA is required for its non-glycolytic functions.

The first experiment undertaken was the cloning and purification of recombinant FBA. The FBA-encoding gene (*cbbA*) in *N. meningitidis*, was cloned into pQE-70 to express C-terminally His-tagged rFBA (WT).

Mutation(s) in the predicted cofactor binding site of FBA were then introduced by site-directed mutagenesis in order to express rFBA lacking aldolase activity. Two mutagenesis reactions were carried out to introduce mutations D83A or H81A/84A in *cbbA* cloned in pQE-70. All the proteins were purified by IMAC under non-denaturing conditions. All rFBA reacted similarly with monoclonal anti-His-tag antibody.

A coupled enzymic assay confirmed that the aldolase activity of FBA was abolished by mutation(s) in the zinc cofactor binding site (either D83A or H81/84A). As rFBA (WT) was enzymatically active thus it can be suggested that the protein folding needed for enzymatic reactions was not disturbed by the presence of the His-tag.

The extracellular matrix (ECM) is the non-cellular component present within all tissues and organs. ECM provides physical scaffolding for the cellular constituents and plays crucial roles in the initiation of tissue morphogenesis, differentiation and homeostasis. The ECM is composed of two proteoglycans (PGs) and fibrous proteins (Jarvelainen *et al.*, 2009; Schaefer and Schaefer, 2010). Collagens, elastins, fibronectins and laminins are the main fibrous ECM proteins (Frantz *et al.*, 2010). Binding human ECM proteins is one of the most common functions of moonlighting proteins (Henderson and Martin, 2011; Wang *et al.*, 2013).

Plasminogen is a key component of the host fibrinolytic system. This is produced as a zymogen which upon activation by host activators (like uPA or tPA) forms plasmin. Plasmin is a serine protease that has the ability to degrade a broad spectrum of substrates, including fibrin clots, connective tissue, and components of extracellular matrices (Plow *et al.*, 1995; Bergmann and

Hammerschmidt, 2007). Ulberg *et al.*, first reported that *N. meningitidis* and *N. gonorrhoeae* could bind plasminogen, which could then be activated to plasmin by uPA or tPA, although no receptors were identified (Ullberg *et al.*, 1992). Subsequently, three plasminogen-binding proteins, identified as enolase, DnaK and peroxiredoxin, have been identified on the surface of *N. meningitidis* (Knaust *et al.*, 2007). Three additional proteins were unidentified in that study. One of the unidentified plasminogen-binding proteins had an apparent molecular weight of *ca.* 35-kDa, consistent with the predicted molecular weight of 38-kDa for FBA (Knaust *et al.*, 2007). In addition to that, FBAs from a number of pathogenic organisms including *C. albicans* (Crowe *et al.*, 2003), *C. neoformans* (Stie *et al.*, 2009), *M. tuberculosis* (de la Paz Santangelo *et al.*, 2011) and *Paracoccidioides* (Chaves *et al.*, 2015) have been shown to bind plasminogen. Taken together it was hypothesized that meningococcal FBA would also bind human plasminogen. Therefore experiments were carried out to find out to find out if FBA binds plasminogen and any ECM proteins.

rFBA was found to be a plasminogen binding protein. rFBA (WT) was able to bind plasminogen as well as rFBA (D83A) and rFBA (H81A/84A) confirming that the co-factor binding site is not involved in plasminogen binding activity. Next plasminogen binding was further characterised; increasing concentration of NaCl inhibited plasminogen binding confirming the ionic nature of the interaction, which could also be inhibited using polyclonal anti-FBA antiserum.

Furthermore, inhibition by EACA suggested that lysine residues are important for this interaction. Similar interactions exist between plasminogen with other pathogen proteins such as pneumococcal enolase (Bergmann *et al.*, 2005) and

PepO (Agarwal *et al.*, 2013), *H. influenzae* Protein E (Barthel *et al.*, 2012), *Pseudomonas aeruginosa* Tuf (Kunert *et al.*, 2007), *C. albicans* Pra1 and Gmp1 (Poltermann *et al.*, 2007; Luo *et al.*, 2009) and *B. burgdorferi* CRASP (Hallstrom *et al.*, 2010). Binding of plasminogen to other proteins has been reported to involve C-terminal lysine residues or internal lysine rich motifs (Plow *et al.*, 1995; Wistedt *et al.*, 1998; Bergmann *et al.*, 2003; Ehinger *et al.*, 2004). To gain additional information on the region of FBA involved in binding human plasminogen, two truncated rFBA proteins containing the lysine-rich C-terminal region of 123 amino acids, were expressed and purified. These two proteins, tr-rFBA1 with N-His-tag and tr-rFBA2 with C-His-tag, were tested for their capacity to interact with plasminogen. Plasminogen binding was higher with N-His-tagged tr-FBA1 compared to its C-His-tagged counterpart and also full length rFBA (WT). Inhibition of plasminogen binding by EACA indicated that probably lysine residues in the terminal or sub-terminal positions might be involved in plasminogen binding which might be otherwise partially masked by the His-tag in full length rFBA and tr-rFBA2; both of which have the His-tag at their C-terminal ends.

To identify the amino acid residues responsible for the specific interaction of FBA with human plasminogen, further studies were necessary. Although bioinformatic approaches might help to identify some residues as possible binding sites, the construction of mutant proteins is essential for confirmation. Based on EIA results, three lysine residues of tr-rFBA1 were successfully replaced individually by alanine. Of these three, a significant decrease in plasminogen binding was observed with tr-FBA1 (K354A), whereas the other two proteins, tr-rFBA1 (K339A) and tr-rFBA1 (K346A) were able to bind

plasminogen to a level similar to tr-rFBA1. Notably, plasminogen binding by tr-FBA1 (K354A) was not completely abolished indicating the involvement of other internal lysine residue(s).

C-terminal lysine residues have been pointed out as a major binding site for plasminogen in numerous proteins. Examples include enolase of *S. pneumoniae* (Bergmann *et al.*, 2001), streptococcal surface enolase (SEN) (Derbise *et al.*, 2004) and FBA of *M. tuberculosis* (de la Paz Santangelo *et al.*, 2011). Similarly FBA in *N.meningitidis* also utilises its C-terminal lysine residue to serve the purpose. This is in contrast with other meningococcal proteins reported as plasminogen receptors, such as enolase, DnaK and peroxiredoxin (Knaust *et al.*, 2007).

Only very low levels of binding to fibronectin, laminin or collagen were observed in this study. Further work would be required to determine whether meningococcal FBA binds to additional host molecules.

Several pathogenic bacteria have been described to bind plasminogen and plasmin on their cell surface (Lahteenmaki *et al.*, 2001b; Lahteenmaki *et al.*, 2005; Bergmann and Hammerschmidt, 2007). Activation of surface bound plasminogen to plasmin by host activators turn the bacteria into proteolytic organisms (Lahteenmaki *et al.*, 2005). Enhanced fibrinolytic activity associated with meningococcal meningitis has raised suggestions that plasminogen activation might be involved in systemic infections (Brandtzaeg *et al.*, 1990). Plasminogen binding by whole cells of *N.meningitidis* and its activation to plasmin has previously been reported (Knaust *et al.*, 2007).

FACS analysis confirmed the ability of *N.meningitidis* to bind plasminogen. Deletion of FBA did not affect the plasminogen binding ability of *N. meningitidis* presumably due to the presence of other receptors on the cell surface (Knaust *et al.*, 2007). Due to the high concentration of plasminogen in human plasma, the number of plasminogen receptors on bacterial cell surface and their affinity towards plasminogen can be considered as negligible (Knaust *et al.*, 2007).

To analyse whether interactions with the host plasminogen system are restricted to *N.meningitidis* only, flow cytometry experiments were performed with other neisserial species. This is the first report to state that plasminogen binding is a property shared by pathogenic species *N.meningitidis* and *N. gonorrhoeae* and commensal species like *N.lactamica* and *N. polysacchareae*. This finding complements other studies showing surface localisation and additional functions of moonlighting proteins in other commensal bacteria. For example enolase of several commensal bacterial species are known to bind to extracellular matrix proteins such as collagen, fibronectin, and laminin (Antikainen *et al.*, 2007; Castaldo *et al.*, 2009; Candela *et al.*, 2009; Sanchez *et al.*, 2009; Glenting *et al.*, 2013). The presence of moonlighting proteins on the surface of resident microbiota and pathogenic species which compete for binding to the same ECM proteins may contribute to host protection (Spurbeck *et al.*, 2015).

Since many pathogenic bacteria produce activators or receptors for plasminogen, the formation and retention of plasmin on the bacterial surface may play an important role in invasive infections. Examples include: *B. burgdorferi* (Coleman *et al.*, 1997; Coleman *et al.*, 1995); *Y. pestis*

(Lahteenmaki *et al.*, 2001a; Sodeinde *et al.*, 1992); *S. pyogenes* (Sun *et al.*, 2004); *H. influenzae* (Ullberg *et al.*, 1990; Virkola *et al.*, 1996) and *S. pneumoniae* (Bergmann *et al.*, 2005; Eberhard *et al.*, 1999). Moreover, according to recent reports, activation of surface bound plasminogen to plasmin imparts anti-phagocytic and anti-opsonisation properties to *B. anthracis* (Chung *et al.*, 2011) and *B. burgdorferi* (Grosskinsky *et al.*, 2009).

Apart from that plasminogen may act as molecular bridge between bacteria and human cells as observed in the case of *S. pyogenes* whereby adherence of bacteria increased in the presence of plasminogen either bound to bacteria or epithelial cells (Pancholi *et al.*, 2003). Plasminogen-mediated increased adherence to enterocytes has also observed in *Bifidobacterium animalis* subsp.*lactis* BI07 (Centanni *et al.*, 2012).

The interaction of the plasminogen system and meningococci may have a significant impact on its colonisation and ability to produce invasive disease. Depending on the surrounding host environment, the surface bound plasminogen may be more or less activated to plasmin which in turn has consequences on pathogenesis. Therefore, more intensive studies are required for a deeper characterisation of the role of plasminogen in the *N. meningitidis* - host interplay.

FBA is expressed under standard growth conditions. Deletion of *cbbA* did not affect the growth of *N.meningitidis* meaning it is not essential for growth under the *in vitro* conditions tested (Tunio *et al.*, 2010a). Based on the results of enzymatic assay described in chapter two of this thesis, the same mutations (D83A and H81/84A) in the co-factor binding site, were introduced into the FBA-encoding gene (*cbbA*) of meningococcal strain MC58. Plasmid vector

pCOMP-1 containing *cbbA* (WT, D83A or H81/84A), were introduced into MC58 Δ *cbbA* by natural transformation and the engineered FBA alleles inserted into the genome via homologous recombination. To characterise the surface localisation of FBA and the involvement of FBA in adhesion and invasion into human cells, these complemented mutant cells were employed. Upon confirmation of presence of *cbbA* and expression of FBA by these complemented cells, flow cytometry experiments were performed which clearly demonstrated the presence of FBA on the surface of all three complemented mutant strains. This clearly demonstrated that despite mutation in the cofactor binding site, FBA could be transported to the surface where it could be detected by anti-FBA antibody, thus enzymatic activity does not affect the surface localisation of FBA.

Human brain microvascular endothelial (HBME) cells are the major element of the blood-brain barrier. These cells have been used to study the meningitis-causing pathogens such as *E. coli*, *S. agalactiae*, *S. pneumoniae* and *N. meningitidis* (Prasadarao *et al.*, 1996; Nizet *et al.*, 1997; Zysk *et al.*, 2001; Orihuela *et al.*, 2009).

Tunio *et al* reported that FBA is involved in the optimal adhesion of meningococci to human cells (Tunio *et al.*, 2010a). This study confirmed that FBA is indeed required for the optimal adhesion to HBME cells. Next, adhesion and invasion assays were undertaken to examine whether or not enzymatically-active FBA was required to exert this moonlighting function. In comparison to wild type MC58 strain, MC58 Δ *cbbA* strain had reduced ability to adhere with HBME cells however this could be restored by complementation of the mutant strain with either wild type or aldolase-inactive

FBA. Although MC58 Δ *cbbA cbbA-m1^{Ect}* exhibited less association compared to the other two complemented mutant strains, this was not statistically significant. Thus this study concludes that to exert its activity in the adhesion process to HBME cells, FBA does not have to be enzymatically active.

Flamingo cadherin receptor (FCR), a cadherin superfamily receptor present on the surface of host epithelial cells, was reported to be the receptor of *S. pneumoniae* FBA (Blau *et al.*, 2007) but the exact nature of this interaction has not been characterised. It cannot be clearly stated if meningococcal FBA binds the same receptor as meningococcal FBA has only 40% identity to the pneumococcal FBA at the amino acid level. The exact nature of interaction between FBA and HBME cells is not clear. Hence, it remains to be determined whether FBA directly binds to certain receptors on HBME cells or is indirectly involved in the adhesion process.

Commercially available human pharyngeal epithelial cell lines include the Detroit-562 and Fadu cell lines. Both have been used to characterise *N. meningitidis* colonisation and invasion (Bradley *et al.*, 2005; Griffiths *et al.*, 2007; Sa E Cunha *et al.*, 2009; Sutherland *et al.*, 2010). Here Detroit-526 cells were used for meningococcal association and invasion assays. As opposed to HBME cells, the absence of surface FBA did not have a significant effect on adhesion of meningococci to Detroit-562 epithelial cells. This is in contrast to previous report by Tunio *et al* (Tunio *et al.*, 2010a), which utilized Hep-2 epithelial cells rather than Detroit-562 cells. Therefore the possibility that the role of FBA in the adhesion process might be cell type-specific cannot be excluded. Differential association of *N. meningitidis* with epithelial and endothelial cells has been identified by Virji *et al.*(Virji *et al.*, 1992a) and

utilisation of different mechanisms of host cell signalling have been found more recently (Lécuyer *et al.*, 2012).

In summary, the results presented herein identify meningococcal FBA as a plasminogen binding protein. This study also confirms that FBA is involved in the adhesion of *N. meningitidis* to human endothelial cells. Both of these non-glycolytic activities are independent of aldolase activity thus providing confirmatory evidence that FBA is a moonlighting protein.

6.2 Conclusion

The ability of a protein to perform biological functions distinct to its canonical function is common in nature. Originally considered as a virulence trait, it has become evident that same protein with moonlighting functions can be present in both pathogens and in members of the normal flora as part of their communication with their environment and as a response to environmental stress. The importance of moonlighting proteins in physiological and pathological processes is becoming increasingly clear. Extracellular export and attachment to the cell surface remain areas to be investigated.

N. meningitidis possesses several plasminogen binding proteins on its surface; detailed molecular and biochemical characterisation of neisserial surface plasminogen receptors has not been carried out. *In vitro* binding experiments with eukaryotic cell proteins, presented in this thesis identified FBA as a human plasminogen binding protein and show that this interaction is mediated mainly via the C-terminal lysine residue. FBA is a surface exposed protein in *N. meningitidis*. Despite being a glycolytic enzyme, enzymatic activity is not required for cell surface localization of FBA and its role in optimal adhesion to human endothelial cells. The findings of this study reveal the impact of

glycolytic moonlighting enzymes, such as FBA, in the biology of the *Neisseria*–host interaction.

At present, *N. meningitidis* are the only Gram-negative bacteria in which surface-localised FBA has been described. However, FBA homologues are present in other pathogenic Gram-negative bacteria including *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. The presence of class II FBAs in many pathogens (and the absence of a structurally or antigenically similar protein in humans) makes FBA a potential target for antimicrobial therapy (Daher *et al.*, 2010) or a possible vaccine candidate. Therefore further characterising the role of meningococcal FBA in pathogenesis may shed light into the possible involvement of surface-localised FBA in the pathogenesis of other pathogens as well. Successfully identifying the host molecule(s) with which meningococcal FBA interacts and determining the importance of such interaction(s) in the pathogenesis of meningococcal disease, will be major objectives for future research. Insights into the whole process may lead to novel strategies for developing preventive and therapeutic approaches to combat meningococcal disease.

6.3 Future Directions

A potentially useful approach may be to compare the gene expression profiles of the *cbbA* mutant strains with the wild-type using microarray technology (Chan *et al.*, 2005; Liu *et al.*, 2005). Alternatively, microarrays could be utilised to compare the gene expression profile of human cells co-cultured with wild type and *cbbA* mutant strains of *N. meningitidis*.

It has been shown that transcription of *cbbA* is dependent on oxygen (Oriente *et al.*, 2010) and iron availability (Sanders *et al.*, 2012). The level of surface-

exposed FBA under different conditions mimicking the host environment can be examined, and its impact on adhesion to human cells tested.

Involvement of FBA in the adhesion was observed with HBME cells but not with Detroit-562 cells. Differences between primary cells and cell lines have previously been reported (Rydberg *et al.*, 2009). Therefore different nasopharyngeal epithelial cell line such as Fadu (Rangan, 1972; Sutherland *et al.*, 2010) can be used for association assay to investigate whether or not FBA on the meningococcal surface is required for adhesion to epithelial cells.

To demonstrate the validity of the results from the association assay, different independent approaches such as addition of microbeads coated with the purified rFBA (WT and MT) to cultured human cells, immunofluorescence microscopy, and environmental scanning electron microscopy can be used (Oldfield *et al.*, 2007).

Pull down assay using whole cell lysate could be was used to find out a receptor of FBA. Different independent approaches such as the re-tagging, cross linking and MALDI-TOF or immunoprecipitation could also be employed.

Furthermore, an FBA-deficient mutant was unable to produce sustained bacteraemia in a transgenic mouse model of infection (D. Turner, personal communication) confirming the *in vivo* significance of this protein in meningococcal pathogenesis. The question arises whether or not it is the enzymatic activity of the protein or just the presence of the protein on the cell surface which is required for optimal pathogenesis. To address this issue, the

complemented mutant strains expressing enzymatically inactive FBA on their surface could be tested in such a mouse model.

cbbA null mutants of *N. gonorrhoeae* and commensal neisseriae species can be created. These strains could be used for association and invasion assays to determine whether the involvement of FBA in adhesion is widespread in neisseriae.

This study identified plasminogen as a ligand for meningococcal FBA, but FBA may have multiple host ligands. Therefore future work should be aimed to find out additional protein(s) that might interact with FBA. Proteomic techniques like 2D gel electrophoresis and/or MS analysis can be employed to uncover other possible plasminogen receptors, apart from those already reported, in *N. meningitidis*.

Sequestration of human plasminogen is vital for pathogenesis of many pathogenic organisms. Plasminogen binding may increase adhesion of meningococcal cells or activation of plasminogen to plasmin may lead to increased level of invasion. Binding of plasminogen by non pathogenic *Neisseria* spp. reported here raise the possibility that interaction with the plasminogen system might not be only a virulence characteristic of *Neisseria* rather it is a common property utilised for competition for the available ecological niches. Overall, the *in vivo* importance of plasminogen binding function in neisseriae remains to be elucidated. Therefore the exploitation of plasminogen system by pathogenic and non-pathogenic neisseriae, offers an interesting field of research.

Finding the export machinery for meningococcal FBA would be another interesting area for investigation. There might be some yet unknown secretion mechanisms for FBA or the moonlighting proteins in general. Experiments could be designed to look for the regions of meningococcal FBA which are required for surface transportation and anchorage. All this information will provide more insights about the pathogenic mechanism(s) employed by meningococci.

Bibliography

- AGARWAL, S., KULSHRESHTHA, P., BAMBHA MUKKU, D. & BHATNAGAR, R. 2008. alpha-Enolase binds to human plasminogen on the surface of *Bacillus anthracis*. *Biochimica et Biophysica Acta*, 1784, 986-94.
- AGARWAL, V., KUCHIPUDI, A., FULDE, M., RIESBECK, K., BERGMANN, S. & BLOM, A. M. 2013. *Streptococcus pneumoniae* endopeptidase O (PepO) is a multifunctional plasminogen- and fibronectin-binding protein, facilitating evasion of innate immunity and invasion of host cells. *The Journal of Biological Chemistry*, 288, 6849-63.
- AGUILERA, L., FERREIRA, E., GIMENEZ, R., FERNANDEZ, F. J., TAULES, M., AGUILAR, J., VEGA, M. C., BADIA, J. & BALDOMA, L. 2012. Secretion of the housekeeping protein glyceraldehyde-3-phosphate dehydrogenase by the LEE-encoded type III secretion system in enteropathogenic *Escherichia coli*. *The International Journal of Biochemistry & Cell Biology*, 44, 955-62.
- AGUILERA, L., GIMENEZ, R., BADIA, J., AGUILAR, J. & BALDOMA, L. 2009. NAD⁺-dependent post-translational modification of *Escherichia coli* glyceraldehyde-3-phosphate dehydrogenase. *International Microbiology*, 12, 187-92.
- AIT-TAHAR, K., WOOLDRIDGE, K. G., TURNER, D. P., ATTA, M., TODD, I. & ALA'ALDEEN, D. A. 2000. Auto-transporter A protein of *Neisseria meningitidis*: a potent CD4⁺ T-cell and B-cell stimulating antigen detected by expression cloning. *Molecular Microbiology*, 37, 1094-105.
- ALA'ALDEEN, D. A., STEVENSON, P., GRIFFITHS, E., GORRINGE, A. R., IRONS, L. I., ROBINSON, A., HYDE, S. & BORRIELLO, S. P. 1994. Immune responses in humans and animals to meningococcal transferrin-binding proteins: implications for vaccine design. *Infection and Immunity*, 62, 2984-90.
- ALA'ALDEEN, D. A. A. & TURNER, D. P. J. 2006. *Neisseria meningitidis. Principles and Practice of Clinical Bacteriology*. John Wiley & Sons, Ltd.
- ALBIGER, B., JOHANSSON, L. & JONSSON, A. B. 2003. Lipooligosaccharide-deficient *Neisseria meningitidis* shows altered pilus-associated characteristics. *Infection and Immunity*, 71, 155-62.
- ALLIGNET, J., ENGLAND, P., OLD, I. & EL SOLH, N. 2002. Several regions of the repeat domain of the *Staphylococcus caprae* autolysin, AtlC, are involved in fibronectin binding. *FEMS Microbiology Letters*, 213, 193-7.

- ANTIKAINEN, J., KUPARINEN, V., LAHTEENMAKI, K. & KORHONEN, T. 2007. Enolases from Gram-positive bacterial pathogens and commensal lactobacilli share functional similarity in virulence-associated traits. *FEMS Immunol Med Microbiol*, 51, 526 - 534.
- APONSO, D. & BULLEN, C. 2001. Presenting features of meningococcal disease, public health messages and media publicity: are they consistent? *The New Zealand Medical Journal* 114, 83-85.
- ARENAS, J., CANO, S., NIJLAND, R., VAN DONGEN, V., RUTTEN, L., VAN DER ENDE, A. & TOMMASSEN, J. 2015. The meningococcal autotransporter AutA is implicated in autoaggregation and biofilm formation. *Environmental Microbiology*, 17, 1321-37.
- ARENAS, J., NIJLAND, R., RODRIGUEZ, F. J., BOSMA, T. N. & TOMMASSEN, J. 2013. Involvement of three meningococcal surface-exposed proteins, the heparin-binding protein NhbA, the alpha-peptide of IgA protease and the autotransporter protease NalP, in initiation of biofilm formation. *Molecular Microbiology*, 87, 254-68.
- ARNOLD, R., GALLOWAY, Y., MCNICHOLAS, A. & O'HALLAHAN, J. 2011. Effectiveness of a vaccination programme for an epidemic of meningococcal B in New Zealand. *Vaccine*, 29, 7100-7106.
- ATANASSOV, C., PEZENNEC, L., D'ALAYER, J., GROLLIER, G., PICARD, B. & FAUCHERE, J. L. 2002. Novel antigens of *Helicobacter pylori* correspond to ulcer-related antibody pattern of sera from infected patients. *Journal of Clinical Microbiology*, 40, 547-52.
- BAART, G. J., ZOMER, B., DE HAAN, A., VAN DER POL, L. A., BEUVERY, E. C., TRAMPER, J. & MARTENS, D. E. 2007. Modeling *Neisseria meningitidis* metabolism: from genome to metabolic fluxes. *Genome Biology*, 8, R136.
- BABADY, N. E., PANG, Y. P., ELPELEG, O. & ISAYA, G. 2007. Cryptic proteolytic activity of dihydrolipoamide dehydrogenase. *Proceedings of the National Academy of Sciences of the United States of America*, 104, 6158-63.
- BABU, M. M., PRIYA, M. L., SELVAN, A. T., MADERA, M., GOUGH, J., ARAVIND, L. & SANKARAN, K. 2006. A database of bacterial lipoproteins (DOLOP) with functional assignments to predicted lipoproteins. *Journal of Bacteriology*, 188, 2761-73.
- BAI, N. J., PAI, M. R., MURTHY, P. S. & VENKITASUBRAMANIAN, T. A. 1974. Effect of oxygen tension on the aldolases of *Mycobacterium tuberculosis* H37Rv. *FEBS Letters*, 45, 68-70.
- BAI, N. J., PAI, M. R., MURTHY, P. S. & VENKITASUBRAMANIAN, T. A. 1975. Fructose-1,6-diphosphate aldolase of *Mycobacterium*

tuberculosis H37Rv. *Indian Journal of Biochemistry & Biophysics*, 12, 181-3.

BALTIMORE, R. S. 2006. Recent trends in meningococcal epidemiology and current vaccine recommendations. *Current Opinion in Pediatrics*, 18, 58-63.

BARTHEL, D., SINGH, B., RIESBECK, K. & ZIPFEL, P. F. 2012. *Haemophilus influenzae* uses the surface protein E to acquire human plasminogen and to evade innate immunity. *Journal of Immunology*, 188, 379-85.

BASAK, C., PATHAK, S. K., BHATTACHARYYA, A., PATHAK, S., BASU, J. & KUNDU, M. 2005. The secreted peptidyl prolyl cis,trans-isomerase HP0175 of *Helicobacter pylori* induces apoptosis of gastric epithelial cells in a TLR4- and apoptosis signal-regulating kinase 1-dependent manner. *Journal of Immunology*, 174, 5672-80.

BEERNINK, P. T., WELSCH, J. A., HARRISON, L. H., LEIPUS, A., KAPLAN, S. L. & GRANOFF, D. M. 2007. Prevalence of factor H-binding protein variants and NadA among meningococcal group B isolates from the United States: implications for the development of a multicomponent group B vaccine. *The Journal of Infectious Diseases*, 195, 1472-9.

BELLAND, R. J., MORRISON, S. G., VAN DER LEY, P. & SWANSON, J. 1989. Expression and phase variation of gonococcal P.11 genes in *Escherichia coli* involves ribosomal frameshifting and slipped-strand mispairing. *Molecular Microbiology*, 3, 777-786.

BENZIANE, B., DEMARETZ, S., DEFONTAINE, N., ZAAROUR, N., CHEVAL, L., BOURGEOIS, S., KLEIN, C., FROISSART, M., BLANCHARD, A., PAILLARD, M., GAMBA, G., HOUILLIER, P. & LAGHMANI, K. 2007. NKCC2 surface expression in mammalian cells: down-regulation by novel interaction with aldolase B. *The Journal of Biological Chemistry*, 282, 33817-33830.

BERGMANN, S. & HAMMERSCHMIDT, S. 2007. Fibrinolysis and host response in bacterial infections. *Thrombosis and Haemostasis*, 98, 512-20.

BERGMANN, S., ROHDE, M., CHHATWAL, G. S. & HAMMERSCHMIDT, S. 2001. alpha-Enolase of *Streptococcus pneumoniae* is a plasmin(ogen)-binding protein displayed on the bacterial cell surface. *Molecular Microbiology*, 40, 1273-87.

BERGMANN, S., ROHDE, M. & HAMMERSCHMIDT, S. 2004. Glyceraldehyde-3-phosphate dehydrogenase of *Streptococcus pneumoniae* is a surface-displayed plasminogen-binding protein. *Infection and Immunity*, 72, 2416-9.

- BERGMANN, S., ROHDE, M., PREISSNER, K. T. & HAMMERSCHMIDT, S. 2005. The nine residue plasminogen-binding motif of the pneumococcal enolase is the major cofactor of plasmin-mediated degradation of extracellular matrix, dissolution of fibrin and transmigration. *Thrombosis and Haemostasis*, 94, 304-11.
- BERGMANN, S., WILD, D., DIEKMANN, O., FRANK, R., BRACHT, D., CHHATWAL, G. S. & HAMMERSCHMIDT, S. 2003. Identification of a novel plasmin(ogen)-binding motif in surface displayed alpha-enolase of *Streptococcus pneumoniae*. *Molecular Microbiology*, 49, 411-23.
- BERNARD, S. C., SIMPSON, N., JOIN-LAMBERT, O., FEDERICI, C., LARAN-CHICH, M. P., MAISSA, N., BOUZINBA-SEGARD, H., MORAND, P. C., CHRETIEN, F., TAOUI, S., CHEVET, E., JANEL, S., LAFONT, F., COUREUIL, M., SEGURA, A., NIEDERGANG, F., MARULLO, S., COURAUD, P. O., NASSIF, X. & BOURDOULOUS, S. 2014. Pathogenic *Neisseria meningitidis* utilizes CD147 for vascular colonization. *Nature Medicine*, 20, 725-31.
- BERRY, A. & MARSHALL, K. E. 1993. Identification of zinc-binding ligands in the class II fructose-1,6-bisphosphate aldolase of *Escherichia coli*. *FEBS Letters*, 318, 11-6.
- BILUKHA, O. O. & ROSENSTEIN, N. 2005. Prevention and control of meningococcal disease. Recommendations of the Advisory Committee on Immunization Practices (ACIP). *Morbidity and Mortality Weekly Report. Recommendations and Reports / Centers for Disease Control*, 54, 1-21.
- BLAU, K., PORTNOI, M., SHAGAN, M., KAGANOVICH, A., ROM, S., KAFKA, D., CHALIFA CASPI, V., PORGADOR, A., GIVON-LAVI, N., GERSHONI, J. M., DAGAN, R. & MIZRACHI NEBENZAHL, Y. 2007. Flamingo cadherin: a putative host receptor for *Streptococcus pneumoniae*. *The Journal of Infectious Diseases*, 195, 1828-37.
- BLOM, N. S., TETREAULT, S., COULOMBE, R. & SYGUSCH, J. 1996. Novel active site in *Escherichia coli* fructose 1,6-bisphosphate aldolase. *Nature Structural Biology*, 3, 856-62.
- BOEL, G., JIN, H. & PANCHOLI, V. 2005. Inhibition of cell surface export of group A streptococcal anchorless surface dehydrogenase affects bacterial adherence and antiphagocytic properties. *Infection and Immunity*, 73, 6237-48.
- BOISIER, P., NICOLAS, P., DJIBO, S., TAHA, M.-K., JEANNE, I., MAÍNASSARA, H. B., TENEBRAY, B., KAIRO, K. K., GIORGINI, D. & CHANTEAU, S. 2007. Meningococcal Meningitis: Unprecedented Incidence of Serogroup X—Related Cases in 2006 in Niger. *Clinical Infectious Diseases*, 44, 657-663.

- BOONE, T. J., BURNHAM, C. A. & TYRRELL, G. J. 2011. Binding of group B streptococcal phosphoglycerate kinase to plasminogen and actin. *Microbial Pathogenesis*, 51, 255-61.
- BOONE, T. J. & TYRRELL, G. J. 2012. Identification of the actin and plasminogen binding regions of group B streptococcal phosphoglycerate kinase. *The Journal of Biological Chemistry*, 287, 29035-44.
- BORROW, R. 2009. Meningococcal disease and prevention at the Hajj. *Travel Medicine and Infectious Disease*, 7, 219-225.
- BOYLE, M. D. & LOTTENBERG, R. 1997. Plasminogen activation by invasive human pathogens. *Thrombosis and Haemostasis*, 77, 1-10.
- BRADLEY, C. J., GRIFFITHS, N. J., ROWE, H. A., HEYDERMAN, R. S. & VIRJI, M. 2005. Critical determinants of the interactions of capsule-expressing *Neisseria meningitidis* with host cells: the role of receptor density in increased cellular targeting via the outer membrane Opa proteins. *Cellular Microbiology*, 7, 1490-503.
- BRAIG, K., OTWINOWSKI, Z., HEGDE, R., BOISVERT, D. C., JOACHIMIAK, A., HORWICH, A. L. & SIGLER, P. B. 1994. The crystal structure of the bacterial chaperonin GroEL at 2.8 Å. *Nature*, 371, 578-86.
- BRANDTZAEG, P., BJERRE, A., OVSTEBO, R., BRUSLETTO, B., JOO, G. B. & KIERULF, P. 2001. *Neisseria meningitidis* lipopolysaccharides in human pathology. *Journal of Endotoxin Research*, 7, 401-20.
- BRANDTZAEG, P., BRYN, K., KIERULF, P., OVSTEBO, R., NAMORK, E., AASE, B. & JANTZEN, E. 1992. Meningococcal endotoxin in lethal septic shock plasma studied by gas chromatography, mass-spectrometry, ultracentrifugation, and electron microscopy. *The Journal of Clinical Investigation*, 89, 816-23.
- BRANDTZAEG, P., JOO, G. B., BRUSLETTO, B. & KIERULF, P. 1990. Plasminogen activator inhibitor 1 and 2, alpha-2-antiplasmin, plasminogen, and endotoxin levels in systemic meningococcal disease. *Thrombosis Research*, 57, 271-8.
- BRANLANT, G., FLESCHE, G. & BRANLANT, C. 1983. Molecular cloning of the glyceraldehyde-3-phosphate dehydrogenase genes of *Bacillus stearothermophilus* and *Escherichia coli*, and their expression in *Escherichia coli*. *Gene* 75, 145-155.
- BREHONY, C., JOLLEY, K. A. & MAIDEN, M. C. 2007. Multilocus sequence typing for global surveillance of meningococcal disease. *FEMS Microbiology Reviews*, 31, 15-26.

- BRISSETTE, C. A., HAUPT, K., BARTHEL, D., COOLEY, A. E., BOWMAN, A., SKERKA, C., WALLICH, R., ZIPFEL, P. F., KRAICZY, P. & STEVENSON, B. 2009. *Borrelia burgdorferi* infection-associated surface proteins ErpP, ErpA, and ErpC bind human plasminogen. *Infection and Immunity*, 77, 300-6.
- BRODER, C. C., LOTTENBERG, R., VON MERING, G. O., JOHNSTON, K. H. & BOYLE, M. D. 1991. Isolation of a prokaryotic plasmin receptor. Relationship to a plasminogen activator produced by the same microorganism. *The Journal of Biological Chemistry*, 266, 4922-8.
- BROWN, D. R., HELAINE, S., CARBONNELLE, E. & PELICIC, V. 2010. Systematic functional analysis reveals that a set of seven genes is involved in fine-tuning of the multiple functions mediated by type IV pili in *Neisseria meningitidis*. *Infection and Immunity*, 78, 3053-63.
- CAESAR, N. M., MYERS, K. A. & FAN, X. 2013. *Neisseria meningitidis* serogroup B vaccine development. *Microbial Pathogenesis*, 57, 33-40.
- CALLONI, G., ZOFFOLI, S., STEFANI, M., DOBSON, C. M. & CHITI, F. 2005. Investigating the Effects of Mutations on Protein Aggregation in the Cell. *The Journal of Biological Chemistry*, 280, 10607-10613.
- CAMPAGNE, G., SCHUCHAT, A., DJIBO, S., OUSSEINI, A., CISSE, L. & CHIPPAUX, J. P. 1999. Epidemiology of bacterial meningitis in Niamey, Niger, 1981-96. *Bulletin of the World Health Organization*, 77, 499-508.
- CANDELA, M., BIAGI, E., CENTANNI, M., TURRONI, S., VICI, M., MUSIANI, F., VITALI, B., BERGMANN, S., HAMMERSCHMIDT, S. & BRIGIDI, P. 2009. Bifidobacterial enolase, a cell surface receptor for human plasminogen involved in the interaction with the host. *Microbiology*, 155, 3294-3303.
- CANDELA, M., CENTANNI, M., FIORI, J., BIAGI, E., TURRONI, S., ORRICO, C., BERGMANN, S., HAMMERSCHMIDT, S. & BRIGIDI, P. 2010. DnaK from *Bifidobacterium animalis* subsp. lactis is a surface-exposed human plasminogen receptor upregulated in response to bile salts. *Microbiology*, 156, 1609-18.
- CAPECCHI, B., ADU-BOBIE, J., DI MARCELLO, F., CIUCCHI, L., MASIGNANI, V., TADDEI, A., RAPPUOLI, R., PIZZA, M. & ARICO, B. 2005. *Neisseria meningitidis* NadA is a new invasins which promotes bacterial adhesion to and penetration into human epithelial cells. *Molecular Microbiology*, 55, 687-98.
- CARBONNELLE, E., HELAINE, S., NASSIF, X. & PELICIC, V. 2006. A systematic genetic analysis in *Neisseria meningitidis* defines the Pil proteins required for assembly, functionality, stabilization and export of type IV pili. *Molecular Microbiology*, 61, 1510-22.

- CARBONNELLE, E., HILL, D. J., MORAND, P., GRIFFITHS, N. J., BOURDOULOUS, S., MURILLO, I., NASSIF, X. & VIRJI, M. 2009. Meningococcal interactions with the host. *Vaccine*, 27 Suppl 2, B78-89.
- CARNEIRO, C. R., POSTOL, E., NOMIZO, R., REIS, L. F. & BRENTANI, R. R. 2004. Identification of enolase as a laminin-binding protein on the surface of *Staphylococcus aureus*. *Microbes and Infection* 6, 604-8.
- CARTWRIGHT, K. 1994. Meningococcal carriage and disease. In: CARTWRIGHT, K. (ed.) *Meningococcal Disease*. Chichester: Wiley & Sons.
- CARTWRIGHT, K. A. 1999. Early management of meningococcal disease. *Infectious Disease clinics of North America*, 13, 661-84, viii.
- CARTWRIGHT, K. A., STUART, J. M., JONES, D. M. & NOAH, N. D. 1987. The Stonehouse survey: nasopharyngeal carriage of meningococci and *Neisseria lactamica*. *Epidemiology and Infection*, 99, 591-601.
- CASTALDO, C., VASTANO, V., SICILIANO, R., CANDELA, M., VICI, M., MUSCARIELLO, L., MARASCO, R. & SACCO, M. 2009. Surface displaced alfa-enolase of *Lactobacillus plantarum* is a fibronectin binding protein. *Microbial Cell Factories*, 8, 14.
- CASTELLINO, F. J. & MCCANCE, S. G. 1997. The kringle domains of human plasminogen. *Ciba Foundation Symposium*, 212, 46-60; discussion 60-5.
- CASTELLINO, F. J. & POWELL, J. R. 1981. Human plasminogen. *Methods in Enzymology*, 80 Pt C, 365-78.
- CATENAZZI, M. C., JONES, H., WALLACE, I., CLIFTON, J., CHONG, J. P., JACKSON, M. A., MACDONALD, S., EDWARDS, J. & MOIR, J. W. 2014. A large genomic island allows *Neisseria meningitidis* to utilize propionic acid, with implications for colonization of the human nasopharynx. *Molecular Microbiology*, 93, 346-55.
- CEHOVIN, A., SIMPSON, P. J., MCDOWELL, M. A., BROWN, D. R., NOSCHESSE, R., PALLETT, M., BRADY, J., BALDWIN, G. S., LEA, S. M., MATTHEWS, S. J. & PELICIC, V. 2013. Specific DNA recognition mediated by a type IV pilin. *Proceedings of the National Academy of Sciences of the United States of America*, 110, 3065-70.
- CENTANNI, M., BERGMANN, S., TURRONI, S., HAMMERSCHMIDT, S., CHHATWAL, G. S., BRIGIDI, P. & CANDELA, M. 2012. Tumor necrosis factor alpha modulates the dynamics of the plasminogen-mediated early interaction between *Bifidobacterium animalis* subsp. lactis and human enterocytes. *Applied and Environmental Microbiology*, 78, 2465-9.

- CHAMOT-ROOKE, J., MIKATY, G., MALOSSE, C., SOYER, M., DUMONT, A., GAULT, J., IMHAUS, A. F., MARTIN, P., TRELLET, M., CLARY, G., CHAFEY, P., CAMOIN, L., NILGES, M., NASSIF, X. & DUMENIL, G. 2011. Posttranslational modification of pili upon cell contact triggers *N. meningitidis* dissemination. *Science*, 331, 778-82.
- CHAVES, E. G., WEBER, S. S., BAO, S. N., PEREIRA, L. A., BAILAO, A. M., BORGES, C. L. & SOARES, C. M. 2015. Analysis of *Paracoccidioides* secreted proteins reveals fructose 1,6-bisphosphate aldolase as a plasminogen-binding protein. *BMC Microbiology*, 15, 53.
- CHOW, J. C., YOUNG, D. W., GOLENBOCK, D. T., CHRIST, W. J. & GUSOVSKY, F. 1999. Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. *The Journal of Biological Chemistry*, 274, 10689-92.
- CHRISTEN, M. T., FRANK, P., SCHALLER, J. & LLINAS, M. 2010. Human plasminogen kringle 3: solution structure, functional insights, phylogenetic landscape. *Biochemistry*, 49, 7131-50.
- CHUNG, M. C., TONRY, J. H., NARAYANAN, A., MANES, N. P., MACKIE, R. S., GUTTING, B., MUKHERJEE, D. V., POPOVA, T. G., KASHANCHI, F., BAILEY, C. L. & POPOV, S. G. 2011. *Bacillus anthracis* interacts with plasmin(ogen) to evade C3b-dependent innate immunity. *PloS ONE*, 6, e18119.
- CIESLA, M., MIERZEJEWSKA, J., ADAMCZYK, M., FARRANTS, A. K. & BOGUTA, M. 2014. Fructose bisphosphate aldolase is involved in the control of RNA polymerase III-directed transcription. *Biochimica et Biophysica Acta*, 1843, 1103-10.
- CLAEYS, H. & VERMYLEN, J. 1974. Physico-chemical and proenzyme properties of NH₂-terminal glutamic acid and NH₂-terminal lysine human plasminogen. Influence of 6-aminohexanoic acid. *Biochimica et Biophysica Acta*, 342, 351-9.
- COHN, A. C., MACNEIL, J. R., HARRISON, L. H., HATCHER, C., THEODORE, J., SCHMIDT, M., PONDO, T., ARNOLD, K. E., BAUMBACH, J., BENNETT, N., CRAIG, A. S., FARLEY, M., GERSHMAN, K., PETIT, S., LYNFIELD, R., REINGOLD, A., SCHAFFNER, W., SHUTT, K. A., ZELL, E. R., MAYER, L. W., CLARK, T., STEPHENS, D. & MESSONNIER, N. E. 2010. Changes in *Neisseria meningitidis* Disease Epidemiology in the United States, 1998–2007: Implications for Prevention of Meningococcal Disease. *Clinical Infectious Diseases*, 50, 184-191.
- COLEMAN, J. L. & BENACH, J. L. 1999. Use of the plasminogen activation system by microorganisms. *The Journal of Laboratory and Clinical Medicine*, 134, 567-76.

- COLEMAN, J. L., GEBBIA, J. A., PIESMAN, J., DEGEN, J. L., BUGGE, T. H. & BENACH, J. L. 1997. Plasminogen is required for efficient dissemination of *B. burgdorferi* in ticks and for enhancement of spirochetemia in mice. *Cell*, 89, 1111-9.
- COLEMAN, J. L., SELLATI, T. J., TESTA, J. E., KEW, R. R., FURIE, M. B. & BENACH, J. L. 1995. *Borrelia burgdorferi* binds plasminogen, resulting in enhanced penetration of endothelial monolayers. *Infection and Immunity*, 63, 2478-84.
- COLLINS, K. D. 2004. Ions from the Hofmeister series and osmolytes: effects on proteins in solution and in the crystallization process. *Methods*, 34, 300-11.
- COMANDUCCI, M., BAMBINI, S., BRUNELLI, B., ADU-BOBIE, J., ARICÒ, B., CAPECCHI, B., GIULIANI, M. M., MASIGNANI, V., SANTINI, L., SAVINO, S., GRANOFF, D. M., CAUGANT, D. A., PIZZA, M., RAPPUOLI, R. & MORA, M. 2002. NadA, a Novel Vaccine Candidate of *Neisseria meningitidis*. *The Journal of Experimental Medicine*, 195, 1445-1454.
- COOPER, B., DETORA, L. & STODDARD, J. 2011. Menveo(R)): a novel quadrivalent meningococcal CRM197 conjugate vaccine against serogroups A, C, W-135 and Y. *Expert Review of Vaccines*, 10, 21-33.
- CORNELISSEN, C. N., ANDERSON, J. E., BOULTON, I. C. & SPARLING, P. F. 2000. Antigenic and sequence diversity in gonococcal transferrin-binding protein A. *Infection and Immunity*, 68, 4725-35.
- CORNELISSEN, C. N., ANDERSON, J. E. & SPARLING, P. F. 1997. Characterization of the diversity and the transferrin-binding domain of gonococcal transferrin-binding protein 2. *Infection and Immunity*, 65, 822-8.
- CORNELISSEN, C. N. & HOLLANDER, A. 2011. TonB-Dependent Transporters Expressed by *Neisseria gonorrhoeae*. *Frontiers in Microbiology*, 2, 117.
- COUREUIL, M., LECUYER, H., SCOTT, M. G., BOULARAN, C., ENSLEN, H., SOYER, M., MIKATY, G., BOURDOULOUS, S., NASSIF, X. & MARULLO, S. 2010. Meningococcus Hijacks a beta2-adrenoceptor/beta-Arrestin pathway to cross brain microvasculature endothelium. *Cell*, 143, 1149-60.
- COUREUIL, M., MIKATY, G., MILLER, F., LECUYER, H., BERNARD, C., BOURDOULOUS, S., DUMENIL, G., MEGE, R. M., WEKSLER, B. B., ROMERO, I. A., COURAUD, P. O. & NASSIF, X. 2009. Meningococcal Type IV Pili Recruit the Polarity Complex to Cross the Brain Endothelium. *Science*, 325, 83-87.

- CROWE, J. D., SIEVWRIGHT, I. K., AULD, G. C., MOORE, N. R., GOW, N. A. & BOOTH, N. A. 2003. *Candida albicans* binds human plasminogen: identification of eight plasminogen-binding proteins. *Molecular Microbiology*, 47, 1637-51.
- CROXTALL, J. D. & DHILLON, S. 2012. Meningococcal quadrivalent (serogroups A, C, W135 and Y) tetanus toxoid conjugate vaccine (Nimenrix). *Drugs*, 72, 2407-30.
- CUNHA, C. S. E., GRIFFITHS, N. J., MURILLO, I. & VIRJI, M. 2009. *Neisseria meningitidis* Opc invasin binds to the cytoskeletal protein α -actinin. *Cellular Microbiology*, 11, 389-405.
- DAHER, R., COINCON, M., FONVIELLE, M., GEST, P. M., GUERIN, M. E., JACKSON, M., SYGUSCH, J. & THERISOD, M. 2010. Rational design, synthesis, and evaluation of new selective inhibitors of microbial class II (zinc dependent) fructose bis-phosphate aldolases. *Journal of Medicinal Chemistry*, 53, 7836-42.
- DBAIBO, G., EL-AYOUBI, N., GHANEM, S., HAJAR, F., BIANCO, V., MILLER, J. M. & MESAROS, N. 2013. Immunogenicity and Safety of a Quadrivalent Meningococcal Serogroups A, C, W-135 and Y Tetanus Toxoid Conjugate Vaccine (MenACWY-TT) Administered to Adults Aged 56 Years and Older: Results of an Open-Label, Randomized, Controlled Trial. *Drugs & Aging*, 30, 309-319.
- DE LA PAZ SANTANGELO, M., GEST, P. M., GUERIN, M. E., COINCON, M., PHAM, H., RYAN, G., PUCKETT, S. E., SPENCER, J. S., GONZALEZ-JUARRERO, M., DAHER, R., LENAERTS, A. J., SCHNAPPINGER, D., THERISOD, M., EHRT, S., SYGUSCH, J. & JACKSON, M. 2011. Glycolytic and non-glycolytic functions of *Mycobacterium tuberculosis* fructose-1,6-bisphosphate aldolase, an essential enzyme produced by replicating and non-replicating bacilli. *The Journal of Biological Chemistry*, 286, 40219-31.
- DE VRIES, F. P., COLE, R., DANKERT, J., FROSCH, M. & VAN PUTTEN, J. P. 1998. *Neisseria meningitidis* producing the Opc adhesin binds epithelial cell proteoglycan receptors. *Molecular Microbiology*, 27, 1203-12.
- DEGHMANE, A. E., GIORGINI, D., LARRIBE, M., ALONSO, J. M. & TAHA, M. K. 2002. Down-regulation of pili and capsule of *Neisseria meningitidis* upon contact with epithelial cells is mediated by CrgA regulatory protein. *Molecular Microbiology*, 43, 1555-64.
- DEGHMANE, A. E., PETIT, S., TOPILKO, A., PEREIRA, Y., GIORGINI, D., LARRIBE, M. & TAHA, M. K. 2000. Intimate adhesion of *Neisseria meningitidis* to human epithelial cells is under the control of the *crgA* gene, a novel LysR-type transcriptional regulator. *The EMBO Journal*, 19, 1068-78.

- DELEPELAIRE, P. 2004. Type I secretion in gram-negative bacteria. *Biochimica et Biophysica Acta*, 1694, 149-61.
- DERBISE, A., SONG, Y. P., PARIKH, S., FISCHETTI, V. A. & PANCHOLI, V. 2004. Role of the C-terminal lysine residues of streptococcal surface enolase in Glu- and Lys-plasminogen-binding activities of group A streptococci. *Infection and Immunity*, 72, 94-105.
- DIAZ ROMERO, J. & OUTSCHOORN, I. M. 1994. Current status of meningococcal group B vaccine candidates: capsular or noncapsular? *Clinical Microbiology Reviews*, 7, 559-75.
- DRLICA, K. & ZHAO, X. 1997. DNA gyrase, topoisomerase IV, and the 4-quinolones. *Microbiology and Molecular Biology Reviews*, 61, 377-92.
- EBERHARD, T., KRONVALL, G. & ULLBERG, M. 1999. Surface bound plasmin promotes migration of *Streptococcus pneumoniae* through reconstituted basement membranes. *Microbial Pathogenesis*, 26, 175-81.
- ECHENIQUE-RIVERA, H., MUZZI, A., DEL TORDELLO, E., SEIB, K. L., FRANCOIS, P., RAPPUOLI, R., PIZZA, M. & SERRUTO, D. 2011. Transcriptome analysis of *Neisseria meningitidis* in human whole blood and mutagenesis studies identify virulence factors involved in blood survival. *PLoS Pathogens*, 7, e1002027.
- EDWARDS, J. L. & APICELLA, M. A. 2004. The molecular mechanisms used by *Neisseria gonorrhoeae* to initiate infection differ between men and women. *Clinical Microbiology Reviews*, 17, 965-981.
- EFRON, A. M., SORHOUE, C., SALCEDO, C., ABAD, R., REGUEIRA, M. & VÁZQUEZ, J. A. 2009. W135 Invasive Meningococcal Strains Spreading in South America: Significant Increase in Incidence Rate in Argentina. *Journal of Clinical Microbiology*, 47, 1979-1980.
- EGEA, L., AGUILERA, L., GIMENEZ, R., SOROLLA, M. A., AGUILAR, J., BADIA, J. & BALDOMA, L. 2007. Role of secreted glyceraldehyde-3-phosphate dehydrogenase in the infection mechanism of enterohemorrhagic and enteropathogenic *Escherichia coli*: interaction of the extracellular enzyme with human plasminogen and fibrinogen. *The International Journal of Biochemistry & Cell Biology*, 39, 1190-203.
- EHINGER, S., SCHUBERT, W. D., BERGMANN, S., HAMMERSCHMIDT, S. & HEINZ, D. W. 2004. Plasmin(ogen)-binding alpha-enolase from *Streptococcus pneumoniae*: crystal structure and evaluation of plasmin(ogen)-binding sites. *Journal of Molecular Biology*, 343, 997-1005.
- EICHENBAUM, Z., GREEN, B. D. & SCOTT, J. R. 1996. Iron starvation causes release from the group A streptococcus of the ADP-ribosylating

protein called plasmin receptor or surface glyceraldehyde-3-phosphate-dehydrogenase. *Infection and Immunity*, 64, 1956-60.

- EUGENE, E., HOFFMANN, I., PUJOL, C., COURAUD, P. O., BOURDOULOUS, S. & NASSIF, X. 2002. Microvilli-like structures are associated with the internalization of virulent capsulated *Neisseria meningitidis* into vascular endothelial cells. *Journal of Cell Science*, 115, 1231-41.
- EXLEY, R. M., SHAW, J., MOWE, E., SUN, Y. H., WEST, N. P., WILLIAMSON, M., BOTTO, M., SMITH, H. & TANG, C. M. 2005. Available carbon source influences the resistance of *Neisseria meningitidis* against complement. *The Journal of Experimental Medicine*, 201, 1637-45.
- FELTCHER, M. E. & BRAUNSTEIN, M. 2012. Emerging themes in SecA2-mediated protein export. *Nature Reviews. Microbiology*, 10, 779-89.
- FINNE, J., LEINONEN, M. & PH., M. 1983. Antigenic similarities between brain components and bacteria causing meningitis: implications for vaccine development and pathogenesis. *The Lancet* 322, 355e7.
- FLAMHOLZ, A., NOOR, E., BAR-EVEN, A., LIEBERMEISTER, W. & MILO, R. 2013. Glycolytic strategy as a tradeoff between energy yield and protein cost. *Proceedings of the National Academy of Sciences of the United States of America*, 110, 10039-44.
- FLETCHER, L. D., BERNFIELD, L., BARNIAK, V., FARLEY, J. E., HOWELL, A., KNAUF, M., OOI, P., SMITH, R. P., WEISE, P., WETHERELL, M., XIE, X., ZAGURSKY, R., ZHANG, Y. & ZLOTNICK, G. W. 2004. Vaccine potential of the *Neisseria meningitidis* 2086 lipoprotein. *Infection and Immunity*, 72, 2088-100.
- FLODEN, A. M., WATT, J. A. & BRISSETTE, C. A. 2011. *Borrelia burgdorferi* enolase is a surface-exposed plasminogen binding protein. *PloS ONE*, 6, e27502.
- FLORES-RAMIREZ, G., JANKOVICOVA, B., BILKOVA, Z., MIERNYK, J. A. & SKULTETY, L. 2014. Identification of *Coxiella burnetii* surface-exposed and cell envelope associated proteins using a combined bioinformatics plus proteomics strategy. *Proteomics*, 14, 1868-81.
- FLUEGGE, K., SCHWEIER, O., SCHILTZ, E., BATSFORD, S. & BERNER, R. 2004. Identification and immunoreactivity of proteins released from *Streptococcus agalactiae*. *European Journal of Clinical Microbiology & Infectious diseases*, 23, 818-24.
- FORSGREN, M., RADEN, B., ISRAELSSON, M., LARSSON, K. & HEDEN, L. O. 1987. Molecular cloning and characterization of a full-length cDNA clone for human plasminogen. *FEBS Letters*, 213, 254-60.

- FRANTZ, C., STEWART, K. M. & WEAVER, V. M. 2010. The extracellular matrix at a glance. *Journal of Cell Science*, 123, 4195-200.
- FRASCH, C. E. & CHAPMAN, S. S. 1972. Classification of *Neisseria meningitidis* Group B into Distinct Serotypes IV. Preliminary Chemical Studies on the Nature of the Serotype Antigen. *Infection and Immunity*, 6, 674-681.
- FRIEDLAND, J. S., SHATTOCK, R., REMICK, D. G. & GRIFFIN, G. E. 1993. Mycobacterial 65-kD heat shock protein induces release of proinflammatory cytokines from human monocytic cells. *Clinical and Experimental Immunology*, 91, 58-62.
- FROSCH, M. & VOGEL, U. 2006. *Structure and genetics of the meningococcal capsule*, Weinheim, Wiley-VCH
- FUCHS, H., WALLICH, R., SIMON, M. M. & KRAMER, M. D. 1994. The outer surface protein A of the spirochete *Borrelia burgdorferi* is a plasmin(ogen) receptor. *Proceedings of the National Academy of Sciences of the United States of America*, 91, 12594-8.
- FURUYA, H. & IKEDA, R. 2011. Interaction of triosephosphate isomerase from *Staphylococcus aureus* with plasminogen. *Microbiology and Immunology*, 55, 855-862.
- FUSSENEGGER, M., RUDEL, T., BARTEN, R., RYLL, R. & MEYER, T. F. 1997. Transformation competence and type-4 pilus biogenesis in *Neisseria gonorrhoeae*--a review. *Gene*, 192, 125-34.
- GAGNEUX, S. P., HODGSON, A., SMITH, T. A., WIRTH, T., EHRHARD, I., MORELLI, G., GENTON, B., BINKA, F. N., ACHTMAN, M. & PLUSCHKE, G. 2002. Prospective Study of a Serogroup X *Neisseria meningitidis* Outbreak in Northern Ghana. *Journal of Infectious Diseases*, 185, 618-626.
- GANCEDO, C. & FLORES, C. L. 2008. Moonlighting proteins in yeasts. *Microbiology and Molecular Biology Reviews* 72, 197-210, table of contents.
- GASE, K., GASE, A., SCHIRMER, H. & MALKE, H. 1996. Cloning, sequencing and functional overexpression of the *Streptococcus equisimilis* H46A gapC gene encoding a glyceraldehyde-3-phosphate dehydrogenase that also functions as a plasmin(ogen)-binding protein. Purification and biochemical characterization of the protein. *European Journal of Biochemistry* 239, 42-51.
- GEKKO, K. & TIMASHEFF, S. N. 1981. Mechanism of protein stabilization by glycerol: preferential hydration in glycerol-water mixtures. *Biochemistry*, 20, 4667-76.

- GERLACH, R. G. & HENSEL, M. 2007. Protein secretion systems and adhesins: the molecular armory of Gram-negative pathogens. *International Journal of Medical Microbiology*, 297, 401-15.
- GIULIANI, M. M., ADU-BOBIE, J., COMANDUCCI, M., ARICO, B., SAVINO, S., SANTINI, L., BRUNELLI, B., BAMBINI, S., BIOLCHI, A., CAPECCHI, B., CARTOCCI, E., CIUCCHI, L., DI MARCELLO, F., FERLICCA, F., GALLI, B., LUZZI, E., MASIGNANI, V., SERRUTO, D., VEGGI, D., CONTORNI, M., MORANDI, M., BARTALESI, A., CINOTTI, V., MANNUCCI, D., TITTA, F., OVIDI, E., WELSCH, J. A., GRANOFF, D., RAPPUOLI, R. & PIZZA, M. 2006. A universal vaccine for serogroup B meningococcus. *Proceedings of the National Academy of Sciences of the United States of America*, 103, 10834-9.
- GLENTING, J., BECK, H. C., VRANG, A., RIEMANN, H., RAVN, P., HANSEN, A. M., ANTONSSON, M., AHRNE, S., ISRAELSEN, H. & MADSEN, S. 2013. Anchorless surface associated glycolytic enzymes from *Lactobacillus plantarum* 299v bind to epithelial cells and extracellular matrix proteins. *Microbiological research*, 168, 245-53.
- GOBERT, A. P., BAMBOU, J. C., WERTS, C., BALLOY, V., CHIGNARD, M., MORAN, A. P. & FERRERO, R. L. 2004. *Helicobacter pylori* heat shock protein 60 mediates interleukin-6 production by macrophages via a toll-like receptor (TLR)-2-, TLR-4-, and myeloid differentiation factor 88-independent mechanism. *The Journal of Biological Chemistry*, 279, 245-50.
- GOLDSCHNEIDER, I., GOTSCHLICH, E. C. & ARTENSTEIN, M. S. 1969a. Human immunity to the meningococcus. II. Development of natural immunity. *The Journal of Experimental Medicine*, 129, 1327-48.
- GOLDSCHNEIDER, I., GOTSCHLICH, E. C. & ARTENSTEIN, M. S. 1969b. Human Immunity to the Meningococcus: I. The Role of Humoral Antibodies *The Journal of Experimental Medicine*, 129, 1307-1326.
- GONZALEZ-MIGUEL, J., MORCHON, R., CARRETON, E., MONTOYA-ALONSO, J. A. & SIMON, F. 2013. Surface associated antigens of *Dirofilaria immitis* adult worms activate the host fibrinolytic system. *Veterinary parasitology*, 196, 235-40.
- GOTSCHLICH, E. C., GOLDSCHNEIDER, I. & ARTENSTEIN, M. S. 1969. Human Immunity to the Meningococcus: : IV. Immunogenicity of Group A and Group C Meningococcal Polysaccharides in Human Volunteers. *The Journal of Experimental Medicine*, 129, 1367-1384.
- GRANIER, S., OWEN, P. & STOTT, N. C. 1998. Recognizing meningococcal disease: the case for further research in primary care. *The British Journal of General Practice*, 48, 1167-1171.

- GRANOFF, D. M., WELSCH, J. A. & RAM, S. 2009. Binding of Complement Factor H (fH) to *Neisseria meningitidis* Is Specific for Human fH and Inhibits Complement Activation by Rat and Rabbit Sera. *Infection and Immunity*, 77, 764-769.
- GREGOIRE, S., ZHANG, S., COSTANZO, J., WILSON, K., FERNANDEZ, E. J. & KWON, I. 2014. Cis-Suppression to Arrest Protein Aggregation in Mammalian Cells. *Biotechnology and bioengineering*, 111, 462-474.
- GRIFANTINI, R., BARTOLINI, E., MUZZI, A., DRAGHI, M., FRIGIMELICA, E., BERGER, J., RATTI, G., PETRACCA, R., GALLI, G., AGNUSDEI, M., GIULIANI, M. M., SANTINI, L., BRUNELLI, B., TETTELIN, H., RAPPUOLI, R., RANDAZZO, F. & GRANDI, G. 2002. Previously unrecognized vaccine candidates against group B meningococcus identified by DNA microarrays. *Nature Biotechnology*, 20, 914-21.
- GRIFFISS, J. M., YAMASAKI, R., ESTABROOK, M. & KIM, J. J. 1991. Meningococcal molecular mimicry and the search for an ideal vaccine. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 85 Suppl 1, 32-6.
- GRIFFITHS, N. J., BRADLEY, C. J., HEYDERMAN, R. S. & VIRJI, M. 2007. IFN-gamma amplifies NFkappaB-dependent *Neisseria meningitidis* invasion of epithelial cells via specific upregulation of CEA-related cell adhesion molecule 1. *Cellular Microbiology*, 9, 2968-83.
- GRIFFITHS, N. J., HILL, D. J., BORODINA, E., SESSIONS, R. B., DEVOS, N. I., FERON, C. M., POOLMAN, J. T. & VIRJI, M. 2011. Meningococcal surface fibril (Msf) binds to activated vitronectin and inhibits the terminal complement pathway to increase serum resistance. *Molecular Microbiology*, 82, 1129-1149.
- GRIJPSTRA, J., ARENAS, J., RUTTEN, L. & TOMMASSEN, J. 2013. Autotransporter secretion: varying on a theme. *Research in Microbiology*, 164, 562-82.
- GROSSKINSKY, S., SCHOTT, M., BRENNER, C., CUTLER, S. J., KRAICZY, P., ZIPFEL, P. F., SIMON, M. M. & WALLICH, R. 2009. *Borrelia recurrentis* employs a novel multifunctional surface protein with anti-complement, anti-opsonic and invasive potential to escape innate immunity. *PloS ONE*, 4, e4858.
- HADI, H. A., WOOLDRIDGE, K. G., ROBINSON, K. & ALA'ALDEEN, D. A. 2001. Identification and characterization of App: an immunogenic autotransporter protein of *Neisseria meningitidis*. *Molecular Microbiology*, 41, 611-23.
- HALL, D. R., LEONARD, G. A., REED, C. D., WATT, C. I., BERRY, A. & HUNTER, W. N. 1999. The crystal structure of *Escherichia coli* class

- II fructose-1,6-bisphosphate aldolase in complex with phosphoglycolohydroxamate reveals details of mechanism and specificity. *Journal of Molecular Biology*, 287, 383-394.
- HALLSTROM, T., HAUPT, K., KRAICZY, P., HORTSCHANSKY, P., WALLICH, R., SKERKA, C. & ZIPFEL, P. F. 2010. Complement regulator-acquiring surface protein 1 of *Borrelia burgdorferi* binds to human bone morphogenic protein 2, several extracellular matrix proteins, and plasminogen. *The Journal of Infectious Diseases*, 202, 490-8.
- HAMMARSTROM, S. 1999. The carcinoembryonic antigen (CEA) family: structures, suggested functions and expression in normal and malignant tissues. *Seminars in Cancer Biology*, 9, 67-81.
- HAMMERSCHMIDT, S., MULLER, A., SILLMANN, H., MUHLENHOFF, M., BORROW, R., FOX, A., VAN PUTTEN, J., ZOLLINGER, W. D., GERARDY-SCHAHN, R. & FROSCH, M. 1996. Capsule phase variation in *Neisseria meningitidis* serogroup B by slipped-strand mispairing in the polysialyltransferase gene (siaD): correlation with bacterial invasion and the outbreak of meningococcal disease. *Molecular Microbiology*, 20, 1211-20.
- HARDY, S. J., CHRISTODOULIDES, M., WELLER, R. O. & HECKELS, J. E. 2000. Interactions of *Neisseria meningitidis* with cells of the human meninges. *Molecular Microbiology*, 36, 817-29.
- HARRISON, L. H. 2006. Prospects for Vaccine Prevention of Meningococcal Infection. *Clinical Microbiology Reviews*, 19, 142-164.
- HARRISON, L. H., TROTTER, C. L. & RAMSAY, M. E. 2009a. Global epidemiology of meningococcal disease. *Vaccine*, 27, Supplement 2, B51-B63.
- HARRISON, O. B., BENNETT, J. S., DERRICK, J. P., MAIDEN, M. C. & BAYLISS, C. D. 2013a. Distribution and diversity of the haemoglobin-haptoglobin iron-acquisition systems in pathogenic and non-pathogenic *Neisseria*. *Microbiology*, 159, 1920-30.
- HARRISON, O. B., CLAUS, H., JIANG, Y., BENNETT, J. S., BRATCHER, H. B., JOLLEY, K. A., CORTON, C., CARE, R., POOLMAN, J. T., ZOLLINGER, W. D., FRASCH, C. E., STEPHENS, D. S., FEAVERS, I., FROSCH, M., PARKHILL, J., VOGEL, U., QUAIL, M. A., BENTLEY, S. D. & MAIDEN, M. C. 2013b. Description and nomenclature of *Neisseria meningitidis* capsule locus. *Emerging Infectious Diseases*, 19, 566-73.
- HARRISON, O. B., EVANS, N. J., BLAIR, J. M., GRIMES, H. S., TINSLEY, C. R., NASSIF, X., KRIZ, P., URE, R., GRAY, S. J., DERRICK, J. P., MAIDEN, M. C. & FEAVERS, I. M. 2009b. Epidemiological evidence

for the role of the hemoglobin receptor, hmbR, in meningococcal virulence. *The Journal of Infectious Diseases*, 200, 94-8.

- HEDARI, C. P., KHINKARLY, R. W. & DBAIBO, G. S. 2014. Meningococcal serogroups A, C, W-135, and Y tetanus toxoid conjugate vaccine: a new conjugate vaccine against invasive meningococcal disease. *Infection and Drug Resistance*, 7, 85-99.
- HEGGE, F. T., HITCHEN, P. G., AAS, F. E., KRISTIANSEN, H., LOVOLD, C., EGGE-JACOBSEN, W., PANICO, M., LEONG, W. Y., BULL, V., VIRJI, M., MORRIS, H. R., DELL, A. & KOOMEY, M. 2004. Unique modifications with phosphocholine and phosphoethanolamine define alternate antigenic forms of *Neisseria gonorrhoeae* type IV pili. *Proceedings of the National Academy of Sciences of the United States of America*, 101, 10798-803.
- HELAINÉ, S., CARBONNELLE, E., PROUVENSIER, L., BERETTI, J. L., NASSIF, X. & PELICIC, V. 2005. PilX, a pilus-associated protein essential for bacterial aggregation, is a key to pilus-facilitated attachment of *Neisseria meningitidis* to human cells. *Molecular Microbiology*, 55, 65-77.
- HENDERSON, B. 2014. An overview of protein moonlighting in bacterial infection. *Biochemical Society Transactions*, 42, 1720-7.
- HENDERSON, B., ALLAN, E. & COATES, A. R. 2006. Stress wars: the direct role of host and bacterial molecular chaperones in bacterial infection. *Infection and Immunity*, 74, 3693-706.
- HENDERSON, B. & MARTIN, A. 2011. Bacterial virulence in the moonlight: multitasking bacterial moonlighting proteins are virulence determinants in infectious disease. *Infection and Immunity*, 79, 3476-91.
- HENDRIKS, W., MULDER, J. W., BIBBY, M. A., SLINGSBY, C., BLOEMENDAL, H. & DE JONG, W. W. 1988. Duck lens epsilon-crystallin and lactate dehydrogenase B4 are identical: a single-copy gene product with two distinct functions. *Proceedings of the National Academy of Sciences of the United States of America*, 85, 7114-8.
- HERNANDEZ, S., FERRAGUT, G., AMELA, I., PEREZ-PONS, J., PINOL, J., MOZO-VILLARIAS, A., CEDANO, J. & QUEROL, E. 2014. MultitaskProtDB: a database of multitasking proteins. *Nucleic Acids Research*, 42, D517-20.
- HIBBERD, M. L., SUMIYA, M., SUMMERFIELD, J. A., BOOY, R. & LEVIN, M. 1999. Association of variants of the gene for mannose-binding lectin with susceptibility to meningococcal disease. *The Lancet*, 353, 1049-1053.
- HICKEY, T. B., THORSON, L. M., SPEERT, D. P., DAFÉ, M. & STOKES, R. W. 2009. *Mycobacterium tuberculosis* Cpn60.2 and DnaK are

- located on the bacterial surface, where Cpn60.2 facilitates efficient bacterial association with macrophages. *Infection and Immunity*, 77, 3389-401.
- HILL, D. J., GRIFFITHS, N. J., BORODINA, E. & VIRJI, M. 2010. Cellular and molecular biology of *Neisseria meningitidis* colonization and invasive disease. *Clinical Science*, 118, 547-64.
- HITTINGER, C. T. & CARROLL, S. B. 2007. Gene duplication and the adaptive evolution of a classic genetic switch. *Nature*, 449, 677-81.
- HOFFMANN, I., EUGENE, E., NASSIF, X., COURAUD, P. O. & BOURDOULOUS, S. 2001. Activation of ErbB2 receptor tyrosine kinase supports invasion of endothelial cells by *Neisseria meningitidis*. *The Journal of Cell Biology*, 155, 133-43.
- HU, S., XIE, Z., ONISHI, A., YU, X., JIANG, L., LIN, J., RHO, H. S., WOODARD, C., WANG, H., JEONG, J. S., LONG, S., HE, X., WADE, H., BLACKSHAW, S., QIAN, J. & ZHU, H. 2009. Profiling the human protein-DNA interactome reveals ERK2 as a transcriptional repressor of interferon signaling. *Cell*, 139, 610-22.
- HU, Y., HENDERSON, B., LUND, P. A., TORMAY, P., AHMED, M. T., GURCHA, S. S., BESRA, G. S. & COATES, A. R. 2008. A *Mycobacterium tuberculosis* mutant lacking the groEL homologue cpn60.1 is viable but fails to induce an inflammatory response in animal models of infection. *Infection and Immunity*, 76, 1535-46.
- HUBERTS, D. H. & VAN DER KLEI, I. J. 2010. Moonlighting proteins: an intriguing mode of multitasking. *Biochimica et Biophysica Acta*, 1803, 520-5.
- HUNG, M.-C. & CHRISTODOULIDES, M. 2013. The Biology of *Neisseria* Adhesins. *Biology*, 2, 1054-1109.
- HURMALAINEN, V., EDELMAN, S., ANTIKAINEN, J., BAUMANN, M., LAHTENMAKI, K. & KORHONEN, T. K. 2007. Extracellular proteins of *Lactobacillus crispatus* enhance activation of human plasminogen. *Microbiology*, 153, 1112-22.
- IMANAKA, H., FUKUI, T., ATOMI, H. & IMANAKA, T. 2002. Gene cloning and characterization of fructose-1,6-bisphosphate aldolase from the hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1. *Journal of Bioscience and Bioengineering*, 94, 237-43.
- JACOB-DUBUISSON, F., FERNANDEZ, R. & COUTTE, L. 2004. Protein secretion through autotransporter and two-partner pathways. *Biochimica et Biophysica Acta*, 1694, 235-57.

- JACOB-DUBUISSON, F., LOCHT, C. & ANTOINE, R. 2001. Two-partner secretion in Gram-negative bacteria: a thrifty, specific pathway for large virulence proteins. *Molecular Microbiology*, 40, 306-13.
- JAMET, A., FRAPY, E., SCHNEIDER, D., NASSIF, X. & MARTIN, P. 2012. A cell-contact-regulated operon is involved in genetic variability in *Neisseria meningitidis*. *Research in Microbiology*, 163, 192-9.
- JAMET, A., ROUSSEAU, C., MONFORT, J. B., FRAPY, E., NASSIF, X. & MARTIN, P. 2009. A two-component system is required for colonization of host cells by meningococcus. *Microbiology*, 155, 2288-95.
- JARVA, H., RAM, S., VOGEL, U., BLOM, A. M. & MERI, S. 2005. Binding of the complement inhibitor C4bp to serogroup B *Neisseria meningitidis*. *Journal of Immunology*, 174, 6299-307.
- JARVELAINEN, H., SAINIO, A., KOULU, M., WIGHT, T. N. & PENTTINEN, R. 2009. Extracellular matrix molecules: potential targets in pharmacotherapy. *Pharmacological Reviews*, 61, 198-223.
- JEFFERY, C. J. 1999. Moonlighting proteins. *Trends in Biochemical Sciences*, 24, 8-11.
- JEFFERY, C. J. 2004. Molecular mechanisms for multitasking: recent crystal structures of moonlighting proteins. *Current Opinion in Structural Biology*, 14, 663-8.
- JEFFERY, C. J. 2009. Moonlighting proteins--an update. *Molecular BioSystems*, 5, 345-50.
- JEWETT, T. J. & SIBLEY, L. D. 2003. Aldolase forms a bridge between cell surface adhesins and the actin cytoskeleton in apicomplexan parasites. *Molecular Cell*, 11, 885-94.
- JOLLEY, K. A., BREHONY, C. & MAIDEN, M. C. 2007. Molecular typing of meningococci: recommendations for target choice and nomenclature. *FEMS Microbiology Reviews*, 31, 89-96.
- JOLLEY, K. A., CHAN, M.-S. & MAIDEN, M. C. J. 2004. mlstdbNet – distributed multi-locus sequence typing (MLST) databases. *BMC Bioinformatics*, 5, 86-86.
- JOSE, J., WOLK, U., LORENZEN, D., WENSCHUH, H. & MEYER, T. F. 2000. Human T-cell response to meningococcal immunoglobulin A1 protease associated alpha-proteins. *Scandinavian Journal of Immunology*, 51, 176-85.
- KAHLER, C. M. & STEPHENS, D. S. 1998. Genetic basis for biosynthesis, structure, and function of meningococcal lipooligosaccharide (endotoxin). *Critical Reviews in Microbiology*, 24, 281-334.

- KAINULAINEN, V. & KORHONEN, T. K. 2014. Dancing to another tune-adhesive moonlighting proteins in bacteria. *Biology*, 3, 178-204.
- KALLSTROM, H., BLACKMER GILL, D., ALBIGER, B., LISZEWSKI, M. K., ATKINSON, J. P. & JONSSON, A. B. 2001. Attachment of *Neisseria gonorrhoeae* to the cellular pilus receptor CD46: identification of domains important for bacterial adherence. *Cellular Microbiology*, 3, 133-43.
- KALLSTROM, H., LISZEWSKI, M. K., ATKINSON, J. P. & JONSSON, A. B. 1997. Membrane cofactor protein (MCP or CD46) is a cellular pilus receptor for pathogenic *Neisseria*. *Molecular Microbiology*, 25, 639-47.
- KAMIYA, S., YAMAGUCHI, H., OSAKI, T. & TAGUCHI, H. 1998. A virulence factor of *Helicobacter pylori*: role of heat shock protein in mucosal inflammation after *H. pylori* infection. *Journal of Clinical Gastroenterology*, 27 Suppl 1, S35-9.
- KANE, J. F. & HARTLEY, D. L. 1988. Formation of recombinant protein inclusion bodies in *Escherichia coli*. *Trends in Biotechnology*, 6, 95-101.
- KANE, P. M. 2006. The Where, When, and How of Organelle Acidification by the Yeast Vacuolar H(+)-ATPase. *Microbiology and Molecular Biology Reviews*, 70, 177-191.
- KERWIN, B. A., HELLER, M. C., LEVIN, S. H. & RANDOLPH, T. W. 1998. Effects of Tween 80 and sucrose on acute short-term stability and long-term storage at -20 degrees C of a recombinant hemoglobin. *Journal of Pharmaceutical Sciences*, 87, 1062-8.
- KESIMER, M., KILIC, N., MEHROTRA, R., THORNTON, D. J. & SHEEHAN, J. K. 2009. Identification of salivary mucin MUC7 binding proteins from *Streptococcus gordonii*. *BMC Microbiology*, 9, 163.
- KHAIRALLA, A. S., OMER, S. A., MAHDAVI, J., ASLAM, A., DUFAILU, O. A., SELF, T., JONSSON, A. B., GEORG, M., SJOLINDER, H., ROYER, P. J., MARTINEZ-POMARES, L., GHAEMMAGHAMI, A. M., WOOLDRIDGE, K. G., OLDFIELD, N. J. & ALA'ALDEEN, D. A. 2015. Nuclear trafficking, histone cleavage and induction of apoptosis by the meningococcal App and MspA autotransporters. *Cellular Microbiology*, 17, 1008-20.
- KHANDELWAL, A., CHANDU, D., ROE, C. M., KOPAN, R. & QUATRANO, R. S. 2007. Moonlighting activity of presenilin in plants is independent of gamma-secretase and evolutionarily conserved. *Proceedings of the National Academy of Sciences of the United States of America*, 104, 13337-42.
- KIM, J.-W. & DANG, C. V. 2005. Multifaceted roles of glycolytic enzymes. *Trends in Biochemical Sciences*, 30, 142-150.

- KIM, J. H., LEE, S., KIM, J. H., LEE, T. G., HIRATA, M., SUH, P. G. & RYU, S. H. 2002. Phospholipase D2 directly interacts with aldolase via Its PH domain. *Biochemistry*, 41, 3414-21.
- KIM, K. P., JAGADEESAN, B., BURKHOLDER, K. M., JARADAT, Z. W., WAMPLER, J. L., LATHROP, A. A., MORGAN, M. T. & BHUNIA, A. K. 2006. Adhesion characteristics of *Listeria* adhesion protein (LAP)-expressing *Escherichia coli* to Caco-2 cells and of recombinant LAP to eukaryotic receptor Hsp60 as examined in a surface plasmon resonance sensor. *FEMS Microbiology Letters*, 256, 324-32.
- KIRCHNER, M., HEUER, D. & MEYER, T. F. 2005. CD46-independent binding of neisserial type IV pili and the major pilus adhesin, PilC, to human epithelial cells. *Infection and Immunity*, 73, 3072-82.
- KIRCHNER, M. & MEYER, T. F. 2005. The PilC adhesin of the *Neisseria* type IV pilus-binding specificities and new insights into the nature of the host cell receptor. *Molecular Microbiology*, 56, 945-57.
- KNAUST, A., WEBER, M. V., HAMMERSCHMIDT, S., BERGMANN, S., FROSCH, M. & KURZAI, O. 2007. Cytosolic proteins contribute to surface plasminogen recruitment of *Neisseria meningitidis*. *Journal of Bacteriology*, 189, 3246-55.
- KOENIGS, A., HAMMERSCHMIDT, C., JUTRAS, B. L., POGORYELOV, D., BARTHEL, D., SKERKA, C., KUGELSTADT, D., WALLICH, R., STEVENSON, B., ZIPFEL, P. F. & KRAICZY, P. 2013. BBA70 of *Borrelia burgdorferi* is a novel plasminogen-binding protein. *The Journal of Biological Chemistry*, 288, 25229-43.
- KOLBERG, J., AASE, A., BERGMANN, S., HERSTAD, T. K., RODAL, G., FRANK, R., ROHDE, M. & HAMMERSCHMIDT, S. 2006. *Streptococcus pneumoniae* enolase is important for plasminogen binding despite low abundance of enolase protein on the bacterial cell surface. *Microbiology*, 152, 1307-17.
- KONG, T. H., COATES, A. R., BUTCHER, P. D., HICKMAN, C. J. & SHINNICK, T. M. 1993. *Mycobacterium tuberculosis* expresses two chaperonin-60 homologs. *Proceedings of the National Academy of Sciences of the United States of America*, 90, 2608-12.
- KORHONEN, T. K., VIRKOLA, R., LAHTENMAKI, K., BJORKMAN, Y., KUKKONEN, M., RAUNIO, T., TARKKANEN, A. M. & WESTERLUND, B. 1992. Penetration of fimbriate enteric bacteria through basement membranes: a hypothesis. *FEMS Microbiology Letters*, 79, 307-12.
- KOUMARÉ, B., OUEDRAOGO-TRAORÉ, R., SANOU, I., YADA, A. A., SOW, I., LUSAMBA, P.-S., TRAORÉ, E., DABAL, M., SANTAMARIA, M., HACEN, M.-M., KABORÉ, A. B. & CAUGANT, D. A. 2007. The first large epidemic of meningococcal

- disease caused by serogroup W135, Burkina Faso, 2002. *Vaccine*, 25, Supplement 1, A37-A41.
- KREILGAARD, L., JONES, L. S., RANDOLPH, T. W., FROKJAER, S., FLINK, J. M., MANNING, M. C. & CARPENTER, J. F. 1998. Effect of Tween 20 on freeze-thawing- and agitation-induced aggregation of recombinant human factor XIII. *Journal of Pharmaceutical Sciences*, 87, 1597-603.
- KUEHN, M. J. & KESTY, N. C. 2005. Bacterial outer membrane vesicles and the host-pathogen interaction. *Genes & Development*, 19, 2645-55.
- KUNERT, A., LOSSE, J., GRUSZIN, C., HUHN, M., KAENDLER, K., MIKKAT, S., VOLKE, D., HOFFMANN, R., JOKIRANTA, T. S., SEEBERGER, H., MOELLMANN, U., HELLWAGE, J. & ZIPFEL, P. F. 2007. Immune evasion of the human pathogen *Pseudomonas aeruginosa*: elongation factor Tuf is a factor H and plasminogen binding protein. *Journal of Immunology*, 179, 2979-88.
- LAGAL, V., PORTNOI, D., FAURE, G., POSTIC, D. & BARANTON, G. 2006. *Borrelia burgdorferi* sensu stricto invasiveness is correlated with OspC-plasminogen affinity. *Microbes and Infection*, 8, 645-52.
- LAHTEENMAKI, K., EDELMAN, S. & KORHONEN, T. K. 2005. Bacterial metastasis: the host plasminogen system in bacterial invasion. *Trends in Microbiology*, 13, 79-85.
- LAHTEENMAKI, K., KUKKONEN, M. & KORHONEN, T. K. 2001a. The Pla surface protease/adhesin of *Yersinia pestis* mediates bacterial invasion into human endothelial cells. *FEBS Letters*, 504, 69-72.
- LAHTEENMAKI, K., KUUSELA, P. & KORHONEN, T. K. 2000. Plasminogen activation in degradation and penetration of extracellular matrices and basement membranes by invasive bacteria. *Methods*, 21, 125-32.
- LAHTEENMAKI, K., KUUSELA, P. & KORHONEN, T. K. 2001b. Bacterial plasminogen activators and receptors. *FEMS Microbiology Reviews*, 25, 531-52.
- LAHTEENMAKI, K., WESTERLUND, B., KUUSELA, P. & KORHONEN, T. K. 1993. Immobilization of plasminogen on *Escherichia coli* flagella. *FEMS Microbiology Letters*, 106, 309-14.
- LAPPANN, M., HAAGENSEN, J. A., CLAUS, H., VOGEL, U. & MOLIN, S. 2006. Meningococcal biofilm formation: structure, development and phenotypes in a standardized continuous flow system. *Molecular Microbiology*, 62, 1292-309.
- LEBENDIKER, M. & DANIELI, T. 2014. Production of prone-to-aggregate proteins. *FEBS Letters*, 588, 236-46.

- LÉCUYER, H., NASSIF, X. & COUREUIL, M. 2012. Two Strikingly Different Signaling Pathways Are Induced by Meningococcal Type IV Pili on Endothelial and Epithelial Cells. *Infection and Immunity*, 80, 175-186.
- LENZ, L. L., MOHAMMADI, S., GEISLER, A. & PORTNOY, D. A. 2003. SecA2-dependent secretion of autolytic enzymes promotes *Listeria monocytogenes* pathogenesis. *Proceedings of the National Academy of Sciences of the United States of America*, 100, 12432-7.
- LEWIS, L. A., GIPSON, M., HARTMAN, K., OWNBEY, T., VAUGHN, J. & DYER, D. W. 1999. Phase variation of HpuAB and HmbR, two distinct haemoglobin receptors of *Neisseria meningitidis* DNM2. *Molecular Microbiology*, 32, 977-89.
- LEWIS, L. A., NGAMPASUTADOL, J., WALLACE, R., REID, J. E., VOGEL, U. & RAM, S. 2010. The meningococcal vaccine candidate neisserial surface protein A (NspA) binds to factor H and enhances meningococcal resistance to complement. *PLoS Pathogens*, 6, e1001027.
- LEWTHWAITE, J. C., COATES, A. R., TORMAY, P., SINGH, M., MASCAGNI, P., POOLE, S., ROBERTS, M., SHARP, L. & HENDERSON, B. 2001. *Mycobacterium tuberculosis* chaperonin 60.1 is a more potent cytokine stimulator than chaperonin 60.2 (Hsp 65) and contains a CD14-binding domain. *Infection and Immunity*, 69, 7349-55.
- LING, E., FELDMAN, G., PORTNOI, M., DAGAN, R., OVERWEG, K., MULHOLLAND, F., CHALIFA-CASPI, V., WELLS, J. & MIZRACHI-NEBENZAHL, Y. 2004. Glycolytic enzymes associated with the cell surface of *Streptococcus pneumoniae* are antigenic in humans and elicit protective immune responses in the mouse. *Clinical and Experimental Immunology*, 138, 290-8.
- LINGAPPA, J. R., AL-RABEAH, A. M., HAJJEH, R., MUSTAFA, T., FATANI, A., AL-BASSAM, T., BADUKHAN, A., TURKISTANI, A., AL-HAMDAN, N., AL-JEFFRI, M., AL MAZROU, Y., PERKINS, B. A., POPOVIC, T., MAYER, L. W. & ROSENSTEIN, N. E. 2000. Serogroup W-135 Meningococcal Disease during the Hajj, 2000. *Emerging Infectious Diseases*, 9, 665-671.
- LINKE, D., RIESS, T., AUTENRIETH, I. B., LUPAS, A. & KEMPF, V. A. 2006. Trimeric autotransporter adhesins: variable structure, common function. *Trends in Microbiology*, 14, 264-70.
- LIOTTA, L. A., RAO, C. N. & WEWER, U. M. 1986. Biochemical interactions of tumor cells with the basement membrane. *Annual Review of Biochemistry*, 55, 1037-57.
- LITT, D. J., SAVINO, S., BEDDEK, A., COMANDUCCI, M., SANDIFORD, C., STEVENS, J., LEVIN, M., ISON, C., PIZZA, M., RAPPUOLI, R.

- & KROLL, J. S. 2004. Putative Vaccine Antigens from *Neisseria meningitidis* Recognized by Serum Antibodies of Young Children Convalescing after Meningococcal Disease. *Journal of Infectious Diseases*, 190, 1488-1497.
- LIU, H. & NAISMITH, J. 2008. An efficient one-step site-directed deletion, insertion, single and multiple-site plasmid mutagenesis protocol. *BMC Biotechnology*, 8, 91.
- LORY, S. 1992. Determinants of extracellular protein secretion in gram-negative bacteria. *Journal of Bacteriology*, 174, 3423-8.
- LOTTENBERG, R., BRODER, C. C., BOYLE, M. D., KAIN, S. J., SCHROEDER, B. L. & CURTISS, R., 3RD 1992. Cloning, sequence analysis, and expression in *Escherichia coli* of a streptococcal plasmin receptor. *Journal of Bacteriology*, 174, 5204-10.
- LOTTENBERG, R., MINNING-WENZ, D. & BOYLE, M. D. 1994. Capturing host plasmin(ogen): a common mechanism for invasive pathogens? *Trends in Microbiology*, 2, 20-4.
- LU, H., ZHANG, H., WANG, Q., YUAN, H., HE, W., ZHAO, Z. & LI, Y. 2001. Purification, refolding of hybrid hIFN γ -kringle 5 expressed in *Escherichia coli*. *Current Microbiology*, 42, 211-6.
- LU, M., AMMAR, D., IVES, H., ALBRECHT, F. & GLUCK, S. L. 2007. Physical interaction between aldolase and vacuolar H⁺-ATPase is essential for the assembly and activity of the proton pump. *The Journal of Biological Chemistry*, 282, 24495-503.
- LUO, S., POLTERMANN, S., KUNERT, A., RUPP, S. & ZIPFEL, P. F. 2009. Immune evasion of the human pathogenic yeast *Candida albicans*: Pra1 is a Factor H, FHL-1 and plasminogen binding surface protein. *Molecular Immunology*, 47, 541-50.
- LUO, X., HSIAO, H.-H., BUBUNENKO, M., WEBER, G., COURT, D. L., GOTTESMAN, M. E., URLAUB, H. & WAHL, M. C. 2008. Structural and Functional Analysis of the *E. coli* NusB-S10 Transcription Antitermination Complex. *Molecular Cell*, 32, 791-802.
- MADICO, G., WELSCH, J. A., LEWIS, L. A., MCNAUGHTON, A., PERLMAN, D. H., COSTELLO, C. E., NGAMPASUTADOL, J., VOGEL, U., GRANOFF, D. M. & RAM, S. 2006. The meningococcal vaccine candidate GNA1870 binds the complement regulatory protein factor H and enhances serum resistance. *Journal of Immunology*, 177, 501-10.
- MADUREIRA, P., BAPTISTA, M., VIEIRA, M., MAGALHAES, V., CAMELO, A., OLIVEIRA, L., RIBEIRO, A., TAVARES, D., TRIEU-CUOT, P., VILANOVA, M. & FERREIRA, P. 2007. *Streptococcus*

agalactiae GAPDH is a virulence-associated immunomodulatory protein. *Journal of Immunology*, 178, 1379-87.

MAIDEN, M. C., BYGRAVES, J. A., FEIL, E., MORELLI, G., RUSSELL, J. E., URWIN, R., ZHANG, Q., ZHOU, J., ZURTH, K., CAUGANT, D. A., FEAVERS, I. M., ACHTMAN, M. & SPRATT, B. G. 1998. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. *Proceedings of the National Academy of Sciences of the United States of America*, 95, 3140-5.

MAIDEN, M. C., IBARZ-PAVON, A. B., URWIN, R., GRAY, S. J., ANDREWS, N. J., CLARKE, S. C., WALKER, A. M., EVANS, M. R., KROLL, J. S., NEAL, K. R., ALA'ALDEEN, D. A., CROOK, D. W., CANN, K., HARRISON, S., CUNNINGHAM, R., BAXTER, D., KACZMARSKI, E., MACLENNAN, J., CAMERON, J. C. & STUART, J. M. 2008. Impact of meningococcal serogroup C conjugate vaccines on carriage and herd immunity. *The Journal of Infectious Diseases*, 197, 737-43.

MAIER, B., POTTER, L., SO, M., LONG, C. D., SEIFERT, H. S. & SHEETZ, M. P. 2002. Single pilus motor forces exceed 100 pN. *Proceedings of the National Academy of Sciences of the United States of America*, 99, 16012-7.

MAIREY, E., GENOVESIO, A., DONNADIEU, E., BERNARD, C., JAUBERT, F., PINARD, E., SEYLAZ, J., OLIVO-MARIN, J. C., NASSIF, X. & DUMENIL, G. 2006. Cerebral microcirculation shear stress levels determine *Neisseria meningitidis* attachment sites along the blood-brain barrier. *The Journal of Experimental Medicine*, 203, 1939-50.

MALORNY, B., MORELLI, G., KUSECEK, B., KOLBERG, J. & ACHTMAN, M. 1998. Sequence diversity, predicted two-dimensional protein structure, and epitope mapping of neisserial Opa proteins. *Journal of Bacteriology*, 180, 1323-30.

MAMCZUR, P. & DZUGAJ, A. 2004. Nuclear localization of aldolase A in pig cardiomyocytes. *Histology and Histopathology*, 19, 753-8.

MAMCZUR, P. & DZUGAJ, A. 2008. Aldolase A is present in smooth muscle cell nuclei. *Acta Biochimica Polonica*, 55, 799-805.

MANI, M., CHEN, C., AMBLEE, V., LIU, H., MATHUR, T., ZWICKE, G., ZABAD, S., PATEL, B., THAKKAR, J. & JEFFERY, C. J. 2015. MoonProt: a database for proteins that are known to moonlight. *Nucleic Acids Research*, 43, D277-82.

MARCEAU, M. & NASSIF, X. 1999. Role of glycosylation at Ser63 in production of soluble pilin in pathogenic *Neisseria*. *Journal of Bacteriology*, 181, 656-61.

- MARSH, J. J. & LEBHERZ, H. G. 1992. Fructose-bisphosphate aldolases: an evolutionary history. *Trends in Biochemical Sciences*, 17, 110-3.
- MARZOUK, O., THOMSON, A. P., SILLS, J. A., HART, C. A. & HARRIS, F. 1991. Features and outcome in meningococcal disease presenting with maculopapular rash. *Archives of Disease in Childhood*, 66, 485-487.
- MASIGNANI, V., COMANDUCCI, M., GIULIANI, M. M., BAMBINI, S., ADU-BOBIE, J., ARICO, B., BRUNELLI, B., PIERI, A., SANTINI, L., SAVINO, S., SERRUTO, D., LITT, D., KROLL, S., WELSCH, J. A., GRANOFF, D. M., RAPPUOLI, R. & PIZZA, M. 2003. Vaccination against *Neisseria meningitidis* using three variants of the lipoprotein GNA1870. *The Journal of Experimental Medicine*, 197, 789-99.
- MATERU, S., COX, H. S., ISAAKIDIS, P., BARUANI, B., OGARO, T. & CAUGANT, D. A. 2007. Serogroup X in Meningococcal Disease, Western Kenya. *Emerging Infectious Diseases*, 13, 944-945.
- MATTA, S. K., AGARWAL, S. & BHATNAGAR, R. 2010. Surface localized and extracellular Glyceraldehyde-3-phosphate dehydrogenase of *Bacillus anthracis* is a plasminogen binding protein. *Biochimica et Biophysica Acta*, 1804, 2111-20.
- MAYER, L. W., REEVES, M. W., AL-HAMDAN, N., SACCHI, C. T., TAHA, M. K., AJELLO, G. W., SCHMINK, S. E., NOBLE, C. A., TONDELLA, M. L., WHITNEY, A. M., AL-MAZROU, Y., AL-JEFRI, M., MISHKHIS, A., SABBAN, S., CAUGANT, D. A., LINGAPPA, J., ROSENSTEIN, N. E. & POPOVIC, T. 2002. Outbreak of W135 meningococcal disease in 2000: not emergence of a new W135 strain but clonal expansion within the electrophoretic type-37 complex. *The Journal of Infectious Diseases*, 185, 1596-605.
- MCCALL, K. A., HUANG, C.-C. & FIERKE, C. A. 2000. Function and Mechanism of Zinc Metalloenzymes. *The Journal of Nutrition*, 130, 1437S-1446S.
- MEMISH, Z. A., VENKATESH, S. & AHMED, Q. A. 2003. Travel epidemiology: the Saudi perspective. *International Journal of Antimicrobial Agents*, 21, 96-101.
- MENHART, N. & CASTELLINO, F. J. 1995. The importance of the hydrophobic components of the binding energies in the interaction of ω -amino acid ligands with isolated kringle polypeptide domains of human plasminogen. *International Journal of Peptide and Protein Research*, 46, 464-70.
- MENHART, N., MCCANCE, S. G., SEHL, L. C. & CASTELLINO, F. J. 1993. Functional independence of the kringle 4 and kringle 5 regions of human plasminogen. *Biochemistry*, 32, 8799-806.

- MENHART, N., SEHL, L. C., KELLEY, R. F. & CASTELLINO, F. J. 1991. Construction, expression, and purification of recombinant kringle 1 of human plasminogen and analysis of its interaction with ω -amino acids. *Biochemistry*, 30, 1948-57.
- MERZ, A. J., ENNS, C. A. & SO, M. 1999. Type IV pili of pathogenic *Neisseriae* elicit cortical plaque formation in epithelial cells. *Molecular Microbiology*, 32, 1316-32.
- MEYER, J., WALKER-JONAH, A. & HOLLENBERG, C. P. 1991. Galactokinase encoded by GAL1 is a bifunctional protein required for induction of the GAL genes in *Kluyveromyces lactis* and is able to suppress the gal3 phenotype in *Saccharomyces cerevisiae*. *Molecular and Cellular Biology*, 11, 5454-61.
- MIKATY, G., SOYER, M., MAIREY, E., HENRY, N., DYER, D., FOREST, K. T., MORAND, P., GUADAGNINI, S., PREVOST, M. C., NASSIF, X. & DUMENIL, G. 2009. Extracellular bacterial pathogen induces host cell surface reorganization to resist shear stress. *PLoS Pathogens*, 5, e1000314.
- MINOGUE, T. D., DALIGAULT, H. A., DAVENPORT, K. W., BISHOP-LILLY, K. A., BRUCE, D. C., CHAIN, P. S., CHERTKOV, O., COYNE, S. R., FREITAS, T., FREY, K. G., JAISSE, J., KOROLEVA, G. I., LADNER, J. T., PALACIOS, G. F., REDDEN, C. L., XU, Y. & JOHNSON, S. L. 2014. Draft Genome Assembly of *Neisseria lactamica* Type Strain A7515. *Genome Announcements*, 2.
- MISHRA, R., BHAT, R. & SECKLER, R. 2007. Chemical chaperone-mediated protein folding: stabilization of P22 tailspike folding intermediates by glycerol. *Biological Chemistry*, 388, 797-804.
- MISHRA, R., SECKLER, R. & BHAT, R. 2005. Efficient refolding of aggregation-prone citrate synthase by polyol osmolytes: how well are protein folding and stability aspects coupled? *The Journal of Biological Chemistry*, 280, 15553-60.
- MODUN, B., MORRISSEY, J. & WILLIAMS, P. 2000. The staphylococcal transferrin receptor: a glycolytic enzyme with novel functions. *Trends in Microbiology*, 8, 231-7.
- MOLYNEUX, E., RIORDAN, F. A. & WALSH, A. 2006. Acute bacterial meningitis in children presenting to the Royal Liverpool Children's Hospital, Liverpool, UK and the Queen Elizabeth Central Hospital in Blantyre, Malawi: a world of difference. *Annals of Tropical Paediatrics*, 26, 29-37.
- MONROY, V., AMADOR, A., RUIZ, B., ESPINOZA-CUETO, P., XOLALPA, W., MANCILLA, R. & ESPITIA, C. 2000. Binding and activation of human plasminogen by *Mycobacterium tuberculosis*. *Infection and Immunity*, 68, 4327-30.

- MOORE, B. 2004. Bifunctional and moonlighting enzymes: lighting the way to regulatory control. *Trends in Plant Science*, 9, 221-8.
- MOORE, B., ZHOU, L., ROLLAND, F., HALL, Q., CHENG, W. H., LIU, Y. X., HWANG, I., JONES, T. & SHEEN, J. 2003. Role of the *Arabidopsis* glucose sensor HXK1 in nutrient, light, and hormonal signaling. *Science*, 300, 332-6.
- MOORE, J., BAILEY, S. E., BENMECHERNENE, Z., TZITZILONIS, C., GRIFFITHS, N. J., VIRJI, M. & DERRICK, J. P. 2005. Recognition of saccharides by the OpcA, OpaD, and OpaB outer membrane proteins from *Neisseria meningitidis*. *The Journal of Biological Chemistry*, 280, 31489-97.
- MOORE, P. S., HIERHOLZER, J., DEWITT, W., GOUAN, K., DJORE, D., LIPPEVELD, T., PLIKAYTIS, B. & BROOME, C. V. 1990. Respiratory viruses and mycoplasma as cofactors for epidemic group A meningococcal meningitis. *The Journal of the American Medical Association*, 264, 1271-5.
- MORELLE, S., CARBONNELLE, E. & NASSIF, X. 2003. The REP2 repeats of the genome of *Neisseria meningitidis* are associated with genes coordinately regulated during bacterial cell interaction. *Journal of Bacteriology*, 185, 2618-27.
- MORSE, D. E. & HORECKER, B. L. 1968. The mechanism of action of aldolases. *Advances in Enzymology and Related Areas of Molecular Biology*, 31, 125-81.
- MUENZNER, P., ROHDE, M., KNEITZ, S. & HAUCK, C. R. 2005. CEACAM engagement by human pathogens enhances cell adhesion and counteracts bacteria-induced detachment of epithelial cells. *The Journal of Cell Biology*, 170, 825-36.
- MURPHY, G., STANTON, H., COWELL, S., BUTLER, G., KNAUPER, V., ATKINSON, S. & GAVRILOVIC, J. 1999. Mechanisms for pro matrix metalloproteinase activation. *Acta Pathologica, Microbiologica, Et Immunologica Scandinavica*, 107, 38-44.
- MYOHANEN, H. & VAHERI, A. 2004. Regulation and interactions in the activation of cell-associated plasminogen. *Cellular and Molecular Life Sciences*, 61, 2840-58.
- NADEL, S. 2012. Prospects for eradication of meningococcal disease. *Archives of Disease in Childhood*, 97, 993-998.
- NAGANO, N., ORENGO, C. A. & THORNTON, J. M. 2002. One fold with many functions: the evolutionary relationships between TIM barrel families based on their sequences, structures and functions. *Journal of Molecular Biology*, 321, 741-65.

- NÄGELE, V., HEESEMANN, J., SCHIELKE, S., JIMÉNEZ-SOTO, L. F., KURZAI, O. & ACKERMANN, N. 2011. *Neisseria meningitidis* Adhesin NadA Targets β 1 Integrins: Functional similarity to Yesrsinia invasin. *The Journal of Biological Chemistry*, 286, 20536-20546.
- NAIR, M. 2012. Protein conjugate polysaccharide vaccines: challenges in development and global implementation. *Indian Journal of Community Medicine*, 37, 79-82.
- NAKAHARA, K., YAMAMOTO, H., MIYAKE, C. & YOKOTA, A. 2003. Purification and characterization of class-I and class-II fructose-1,6-bisphosphate aldolases from the cyanobacterium *Synechocystis* sp. PCC 6803. *Plant & Cell Physiology*, 44, 326-33.
- NANDAN, D., LO, R. & REINER, N. E. 1999. Activation of phosphotyrosine phosphatase activity attenuates mitogen-activated protein kinase signaling and inhibits c-FOS and nitric oxide synthase expression in macrophages infected with *Leishmania donovani*. *Infection and Immunity*, 67, 4055-63.
- NANDAN, D., TRAN, T., TRINH, E., SILVERMAN, J. M. & LOPEZ, M. 2007. Identification of leishmania fructose-1,6-bisphosphate aldolase as a novel activator of host macrophage Src homology 2 domain containing protein tyrosine phosphatase SHP-1. *Biochemical and Biophysical Research Communications*, 364, 601-7.
- NASSIF, X. 1999. Interaction mechanisms of encapsulated meningococci with eucaryotic cells: what does this tell us about the crossing of the blood-brain barrier by *Neisseria meningitidis*? *Current Opinion in Microbiology*, 2, 71-77.
- NASSIF, X., BOURDOULOUS, S., EUGENE, E. & COURAUD, P. O. 2002. How do extracellular pathogens cross the blood-brain barrier? *Trends in Microbiology*, 10, 227-32.
- NEIL, R. B. & APICELLA, M. A. 2009. Role of HrpA in biofilm formation of *Neisseria meningitidis* and regulation of the hrpBAS transcripts. *Infection and Immunity*, 77, 2285-93.
- NEIL, R. B., SHAO, J. Q. & APICELLA, M. A. 2009. Biofilm formation on human airway epithelia by encapsulated *Neisseria meningitidis* serogroup B. *Microbes and Infection*, 11, 281-7.
- NILSEN, S. L., PROROK, M. & CASTELLINO, F. J. 1999. Enhancement through mutagenesis of the binding of the isolated kringle 2 domain of human plasminogen to ω -amino acid ligands and to an internal sequence of a Streptococcal surface protein. *The Journal of Biological Chemistry*, 274, 22380-6.

- NIZET, V., KIM, K. S., STINS, M., JONAS, M., CHI, E. Y., NGUYEN, D. & RUBENS, C. E. 1997. Invasion of brain microvascular endothelial cells by group B streptococci. *Infection and Immunity*, 65, 5074-81.
- NOAH, C. E., MALIK, M., BUBLITZ, D. C., CAMENARES, D., SELLATI, T. J., BENACH, J. L. & FURIE, M. B. 2010. GroEL and lipopolysaccharide from *Francisella tularensis* live vaccine strain synergistically activate human macrophages. *Infection and Immunity*, 78, 1797-806.
- NOINAJ, N., BUCHANAN, S. K. & CORNELISSEN, C. N. 2012. The transferrin-iron import system from pathogenic *Neisseria* species. *Molecular Microbiology*, 86, 246-57.
- OLDFIELD, N. J., BLAND, S. J., TARAKTSOGLU, M., DOS RAMOS, F. J., ROBINSON, K., WOOLDRIDGE, K. G. & ALA'ALDEEN, D. A. 2007. T-cell stimulating protein A (TspA) of *Neisseria meningitidis* is required for optimal adhesion to human cells. *Cellular Microbiology*, 9, 463-78.
- OLDFIELD, N. J., MATAR, S., BIDMOS, F. A., ALAMRO, M., NEAL, K. R., TURNER, D. P., BAYLISS, C. D. & ALA'ALDEEN, D. A. 2013. Prevalence and phase variable expression status of two autotransporters, NalP and MspA, in carriage and disease isolates of *Neisseria meningitidis*. *PloS ONE*, 8, e69746.
- OLIVEIRA, L., MADUREIRA, P., ANDRADE, E. B., BOUABOUD, A., MORELLO, E., FERREIRA, P., POYART, C., TRIEU-CUOT, P. & DRAMSI, S. 2012. Group B Streptococcus GAPDH Is Released upon Cell Lysis, Associates with Bacterial Surface, and Induces Apoptosis in Murine Macrophages. *PloS ONE*, 7, e29963.
- OLIVEIRA, R., DOMINGOS, R. F., DE MORAIS, Z. M., VASCONCELLOS, S. A., ALVES, I. J., ROMERO, E. C. & NASCIMENTO, A. L. 2014. Intermediate and C-terminal regions of leptospiral adhesin Lsa66 are responsible for binding with plasminogen and extracellular matrix components. *Journal of Medical Microbiology*, 63, 1119-30.
- ORIENTE, F., SCARLATO, V. & DELANY, I. 2010. Expression of factor H binding protein of meningococcus responds to oxygen limitation through a dedicated FNR-regulated promoter. *Journal of Bacteriology*, 192, 691-701.
- ORIHUELA, C. J., MAHDAVI, J., THORNTON, J., MANN, B., WOOLDRIDGE, K. G., ABOUSEADA, N., OLDFIELD, N. J., SELF, T., ALA'ALDEEN, D. A. & TUOMANEN, E. I. 2009. Laminin receptor initiates bacterial contact with the blood brain barrier in experimental meningitis models. *The Journal of Clinical Investigation*, 119, 1638-46.

- ORR, H. J., GRAY, S. J., MACDONALD, M. & STUART, J. M. 2003. Saliva and meningococcal transmission. *Emerging Infectious Diseases*, 9, 1314-5.
- OZIMEK, P., VAN DIJK, R., LATCHEV, K., GANCEDO, C., WANG, D. Y., VAN DER KLEI, I. J. & VEENHUIS, M. 2003. Pyruvate carboxylase is an essential protein in the assembly of yeast peroxisomal oligomeric alcohol oxidase. *Molecular Biology of the Cell*, 14, 786-97.
- OZIMEK, P. Z., KLOMPMAKER, S. H., VISSER, N., VEENHUIS, M. & VAN DER KLEI, I. J. 2007. The transcarboxylase domain of pyruvate carboxylase is essential for assembly of the peroxisomal flavoenzyme alcohol oxidase. *FEMS Yeast Research*, 7, 1082-1092.
- PACE, D. & POLLARD, A. J. 2012. Meningococcal disease: clinical presentation and sequelae. *Vaccine*, 30 Suppl 2, B3-9.
- PANCHOLI, V. & CHHATWAL, G. S. 2003. Housekeeping enzymes as virulence factors for pathogens. . *International Journal of Medical Microbiology*, 293, 391– 401.
- PANCHOLI, V. & FISCHETTI, V. A. 1992. A major surface protein on group A streptococci is a glyceraldehyde-3-phosphate-dehydrogenase with multiple binding activity. *The Journal of Experimental Medicine*, 176, 415-26.
- PANCHOLI, V. & FISCHETTI, V. A. 1993. Glyceraldehyde-3-phosphate dehydrogenase on the surface of group A streptococci is also an ADP-ribosylating enzyme. *Proceedings of the National Academy of Sciences of the United States of America*, 90, 8154-8.
- PANCHOLI, V. & FISCHETTI, V. A. 1997. Regulation of the phosphorylation of human pharyngeal cell proteins by group A streptococcal surface dehydrogenase: signal transduction between streptococci and pharyngeal cells. *The Journal of Experimental Medicine*, 186, 1633-43.
- PANCHOLI, V. & FISCHETTI, V. A. 1998. alpha-enolase, a novel strong plasmin(ogen) binding protein on the surface of pathogenic streptococci. *The Journal of Biological Chemistry*, 273, 14503-15.
- PANCHOLI, V., FONTAN, P. & JIN, H. 2003. Plasminogen-mediated group A streptococcal adherence to and pericellular invasion of human pharyngeal cells. *Microbial Pathogenesis*, 35, 293-303.
- PARRY, M. A., ZHANG, X. C. & BODE, I. 2000. Molecular mechanisms of plasminogen activation: bacterial cofactors provide clues. *Trends in Biochemical Sciences*, 25, 53-9.
- PARSONS, N. J., PATEL, P. V., TAN, E. L., ANDRADE, J. R., NAIRN, C. A., GOLDNER, M., COLE, J. A. & SMITH, H. 1988. Cytidine 5'-

monophospho-N-acetyl neuraminic acid and a low molecular weight factor from human blood cells induce lipopolysaccharide alteration in gonococci when conferring resistance to killing by human serum. *Microbial Pathogenesis*, 5, 303-9.

- PATHAK, S. K., BASU, S., BHATTACHARYYA, A., PATHAK, S., BANERJEE, A., BASU, J. & KUNDU, M. 2006. TLR4-dependent NF-kappaB activation and mitogen- and stress-activated protein kinase 1-triggered phosphorylation events are central to *Helicobacter pylori* peptidyl prolyl cis-, trans-isomerase (HP0175)-mediated induction of IL-6 release from macrophages. *Journal of Immunology*, 177, 7950-8.
- PEAK, I. R., JENNINGS, M. P., HOOD, D. W. & MOXON, E. R. 1999. Tetranucleotide repeats identify novel virulence determinant homologues in *Neisseria meningitidis*. *Microbial Pathogenesis*, 26, 13-23.
- PEAK, I. R., SRIKHANTA, Y., DIECKELMANN, M., MOXON, E. R. & JENNINGS, M. P. 2000. Identification and characterisation of a novel conserved outer membrane protein from *Neisseria meningitidis*. *FEMS Immunology and Medical Microbiology*, 28, 329-34.
- PELTOLA, H., ANTTILA, M. & RENKONEN, O. V. 1989. Randomised comparison of chloramphenicol, ampicillin, cefotaxime, and ceftriaxone for childhood bacterial meningitis. Finnish Study Group. *The Lancet*, 1, 1281-7.
- PELTOLA, H., KATAJA, J. M. & MAKELA, P. H. 1982. Shift in the age-distribution of meningococcal disease as predictor of an epidemic? *The Lancet*, 2, 595-7.
- PERRIN, A., BONACORSI, S., CARBONNELLE, E., TALIBI, D., DESSEN, P., NASSIF, X. & TINSLEY, C. 2002. Comparative genomics identifies the genetic islands that distinguish *Neisseria meningitidis*, the agent of cerebrospinal meningitis, from other *Neisseria* species. *Infection and Immunity*, 70, 7063-72.
- PHILPOTT, C. C., KLAUSNER, R. D. & ROUAULT, T. A. 1994. The bifunctional iron-responsive element binding protein/cytosolic aconitase: the role of active-site residues in ligand binding and regulation. *Proceedings of the National Academy of Sciences of the United States of America*, 91, 7321-5.
- PIATIGORSKY, J., O'BRIEN, W. E., NORMAN, B. L., KALUMUCK, K., WISTOW, G. J., BORRAS, T., NICKERSON, J. M. & WAWROUSEK, E. F. 1988. Gene sharing by delta-crystallin and argininosuccinate lyase. *Proceedings of the National Academy of Sciences of the United States of America*, 85, 3479-83.
- PIATIGORSKY, J. & WISTOW, G. J. 1989. Enzyme/crystallins: gene sharing as an evolutionary strategy. *Cell*, 57, 197-9.

- PINA, L. M., BASSILY, E., MACHMER, A., HOU, V. & REINHARDT, A. 2012. Safety and immunogenicity of a quadrivalent meningococcal polysaccharide diphtheria toxoid conjugate vaccine in infants and toddlers: three multicenter phase III studies. *The Pediatric Infectious Disease Journal*, 31, 1173-83.
- PINNER, R. W., SPELLMAN, P. A. & STEPHENS, D. S. 1991. Evidence for functionally distinct pili expressed by *Neisseria meningitidis*. *Infection and Immunity*, 59, 3169-75.
- PIZZA, M., SCARLATO, V., MASIGNANI, V., GIULIANI, M. M., ARICO, B., COMANDUCCI, M., JENNINGS, G. T., BALDI, L., BARTOLINI, E., CAPECCHI, B., GALEOTTI, C. L., LUZZI, E., MANETTI, R., MARCHETTI, E., MORA, M., NUTI, S., RATTI, G., SANTINI, L., SAVINO, S., SCARSELLI, M., STORNI, E., ZUO, P., BROEKER, M., HUNDT, E., KNAPP, B., BLAIR, E., MASON, T., TETTELIN, H., HOOD, D. W., JEFFRIES, A. C., SAUNDERS, N. J., GRANOFF, D. M., VENTER, J. C., MOXON, E. R., GRANDI, G. & RAPPUOLI, R. 2000. Identification of vaccine candidates against serogroup B meningococcus by whole-genome sequencing. *Science*, 287, 1816-20.
- PLATER, A. R., ZGIBY, S. M., THOMSON, G. J., QAMAR, S., WHARTON, C. W. & BERRY, A. 1999. Conserved residues in the mechanism of the *E. coli* Class II FBP-aldolase. *Journal of Molecular Biology*, 285, 843-55.
- PLAUMANN, M., PELZER-REITH, B., MARTIN, W. F. & SCHNARRENBURGER, C. 1997. Multiple recruitment of class-I aldolase to chloroplasts and eubacterial origin of eukaryotic class-II aldolases revealed by cDNAs from *Euglena gracilis*. *Current Genetics*, 31, 430-8.
- PLOW, E. F., HERREN, T., REDLITZ, A., MILES, L. A. & HOOVER-PLOW, J. L. 1995. The cell biology of the plasminogen system. *The FASEB Journal*, 9, 939-45.
- POHLNER, J., HALTER, R., BEYREUTHER, K. & MEYER, T. F. 1987. Gene structure and extracellular secretion of *Neisseria gonorrhoeae* IgA protease. *Nature*, 325, 458-62.
- POLTERMANN, S., KUNERT, A., VON DER HEIDE, M., ECK, R., HARTMANN, A. & ZIPFEL, P. F. 2007. Gpm1p is a factor H-, FHL-1-, and plasminogen-binding surface protein of *Candida albicans*. *The Journal of Biological Chemistry*, 282, 37537-44.
- PONTING, C. P., MARSHALL, J. M. & CEDERHOLM-WILLIAMS, S. A. 1992. Plasminogen: a structural review. *Blood Coagulation & Fibrinolysis* 3, 605-14.

- POOLMAN, J. & BORROW, R. 2011. Hyporesponsiveness and its clinical implications after vaccination with polysaccharide or glycoconjugate vaccines. *Expert Review of Vaccines*, 10, 307-322.
- PORCARI, R., PROUKAKIS, C., WAUDBY, C. A., BOLOGNESI, B., MANGIONE, P. P., PATON, J. F. S., MULLIN, S., CABRITA, L. D., PENCO, A., RELINI, A., VERONA, G., VENDRUSCOLO, M., STOPPINI, M., TARTAGLIA, G. G., CAMILLONI, C., CHRISTODOULOU, J., SCHAPIRA, A. H. V. & BELLOTTI, V. 2015. The H50Q Mutation Induces a 10-fold Decrease in the Solubility of α -Synuclein. *The Journal of Biological Chemistry*, 290, 2395-2404.
- PRASADARAO, N. V., WASS, C. A., WEISER, J. N., STINS, M. F., HUANG, S. H. & KIM, K. S. 1996. Outer membrane protein A of *Escherichia coli* contributes to invasion of brain microvascular endothelial cells. *Infection and Immunity*, 64, 146-53.
- PRIEV, A., ALMAGOR, A., YEDGAR, S. & GAVISH, B. 1996. Glycerol decreases the volume and compressibility of protein interior. *Biochemistry*, 35, 2061-6.
- PRINCE, S. M., ACHTMAN, M. & DERRICK, J. P. 2002. Crystal structure of the OpcA integral membrane adhesin from *Neisseria meningitidis*. *Proceedings of the National Academy of Sciences of the United States of America*, 99, 3417-21.
- PRINZ, T., MEYER, M., PETTERSSON, A. & TOMMASSEN, J. 1999. Structural characterization of the lactoferrin receptor from *Neisseria meningitidis*. *Journal of Bacteriology*, 181, 4417-9.
- PROFT, T. & BAKER, E. N. 2009. Pili in Gram-negative and Gram-positive bacteria - structure, assembly and their role in disease. *Cellular and Molecular Life Sciences* 66, 613-35.
- PUJOL, C., EUGENE, E., DE SAINT MARTIN, L. & NASSIF, X. 1997. Interaction of *Neisseria meningitidis* with a polarized monolayer of epithelial cells. *Infection and Immunity*, 65, 4836-42.
- PUJOL, C., EUGENE, E., MARCEAU, M. & NASSIF, X. 1999. The meningococcal PilT protein is required for induction of intimate attachment to epithelial cells following pilus-mediated adhesion. *Proceedings of the National Academy of Sciences of the United States of America*, 96, 4017-22.
- RAGUNATHAN, L., RAMSAY, M., BORROW, R., GUIVER, M., GRAY, S. & KACZMARSKI, E. B. 2000. Clinical Features, Laboratory Findings and Management of Meningococcal Meningitis in England and Wales: Report of a 1997 Survey. *Journal of Infection*, 40, 74-79.
- RAMAJO-HERNANDEZ, A., PEREZ-SANCHEZ, R., RAMAJO-MARTIN, V. & OLEAGA, A. 2007. *Schistosoma bovis*: plasminogen binding in

adults and the identification of plasminogen-binding proteins from the worm tegument. *Experimental Parasitology*, 115, 83-91.

RAMIAH, K., VAN REENEN, C. A. & DICKS, L. M. T. 2008. Surface-bound proteins of *Lactobacillus plantarum* 423 that contribute to adhesion of Caco-2 cells and their role in competitive exclusion and displacement of *Clostridium sporogenes* and *Enterococcus faecalis*. *Research in Microbiology*, 159, 470-475.

RAMSAY, M., KACZMARSKI, E., RUSH, M., MALLARD, R., FARRINGTON, P. & WHITE, J. 1997. Changing patterns of case ascertainment and trends in meningococcal disease in England and Wales. *Commun Disease Report. CDR Review*, 7, R49-R54.

RAMSAYWAK, P. C., LABBE, G., SIEMANN, S., DMITRIENKO, G. I. & GUILLEMETTE, J. G. 2004. Molecular cloning, expression, purification, and characterization of fructose 1,6-bisphosphate aldolase from *Mycobacterium tuberculosis*--a novel Class II A tetramer. *Protein Expression and Purification*, 37, 220-8.

RAUM, D., MARCUS, D., ALPER, C. A., LEVEY, R., TAYLOR, P. D. & STARZL, T. E. 1980. Synthesis of human plasminogen by the liver. *Science*, 208, 1036-7.

REDLITZ, A. & PLOW, E. F. 1995. Receptors for plasminogen and t-PA: an update. *Bailliere's Clinical Haematology*, 8, 313-27.

RELLOS, P., SYGUSCH, J. & COX, T. M. 2000. Expression, Purification, and Characterization of Natural Mutants of Human Aldolase B: role of quaternary structure in catalysis. *The Journal of Biological Chemistry*, 275, 1145-1151.

RONPIRIN, C., JERSE, A. E. & CORNELISSEN, C. N. 2001. Gonococcal genes encoding transferrin-binding proteins A and B are arranged in a bicistronic operon but are subject to differential expression. *Infection and Immunity*, 69, 6336-47.

ROSANO, G. L. & CECCARELLI, E. A. 2014. Recombinant protein expression in *Escherichia coli*: advances and challenges. *Frontiers in Microbiology*, 5.

ROSENSTEIN, N. E., PERKINS, B. A., STEPHENS, D. S., LEFKOWITZ, L., CARTTER, M. L., DANILA, R., CIESLAK, P., SHUTT, K. A., POPOVIC, T., SCHUCHAT, A., HARRISON, L. H. & REINGOLD, A. L. 1999. The changing epidemiology of meningococcal disease in the United States, 1992-1996. *The Journal of Infectious Diseases*, 180, 1894-901.

ROSENSTEIN, N. E., PERKINS, B. A., STEPHENS, D. S., POPOVIC, T. & HUGHES, J. M. 2001. Meningococcal disease. *The New England Journal of Medicine*, 344, 1378-88.

- ROTMAN, E. & SEIFERT, H. S. 2014. The Genetics of *Neisseria* Species. *Annual Review of Genetics*, 48, 405-431.
- ROUMELIOTI, K., VANGELATOS, I. & SOPHIANOPOULOU, V. 2010. A cryptic role of a glycolytic–gluconeogenic enzyme (aldolase) in amino acid transporter turnover in *Aspergillus nidulans*. *Fungal Genetics and Biology*, 47, 254-267.
- ROUPHAEL, N. G. & STEPHENS, D. S. 2012. *Neisseria meningitidis*: biology, microbiology, and epidemiology. *Methods in Molecular Biology*, 799, 1-20.
- ROUSSEL-JAZEDE, V., ARENAS, J., LANGEREIS, J. D., TOMMASSEN, J. & VAN ULSEN, P. 2014. Variable processing of the IgA protease autotransporter at the cell surface of *Neisseria meningitidis*. *Microbiology*, 160, 2421-31.
- ROUSSEL-JAZÉDÉ, V., JONGERIUS, I., BOS, M. P., TOMMASSEN, J. & VAN ULSEN, P. 2010. NalP-Mediated Proteolytic Release of Lactoferrin-Binding Protein B from the Meningococcal Cell Surface. *Infection and Immunity*, 78, 3083-3089.
- ROWE, H. A., GRIFFITHS, N. J., HILL, D. J. & VIRJI, M. 2007. Co-ordinate action of bacterial adhesins and human carcinoembryonic antigen receptors in enhanced cellular invasion by capsulate serum resistant *Neisseria meningitidis*. *Cellular Microbiology*, 9, 154-68.
- RUKSEREE, K., THAMMARONGTHAM, C. & PALITTAPONGARNPIM, P. 2008. One-step purification and characterization of a fully active histidine-tagged Class II fructose-1,6-bisphosphate aldolase from *Mycobacterium tuberculosis*. *Enzyme and Microbial Technology*, 43, 500-506.
- RUSNIOK, C., VALLENET, D., FLOQUET, S., EWLES, H., MOUZE-SOULAMA, C., BROWN, D., LAJUS, A., BUCHRIESER, C., MEDIGUE, C., GLASER, P. & PELICIC, V. 2009. NeMeSys: a biological resource for narrowing the gap between sequence and function in the human pathogen *Neisseria meningitidis*. *Genome Biology*, 10, R110.
- RUTTER, W. J. 1964. Evolution of Aldolase. *Federation Proceedings*, 23, 1248-57.
- SA E CUNHA, C., GRIFFITHS, N. J., MURILLO, I. & VIRJI, M. 2009. *Neisseria meningitidis* Opc invasin binds to the cytoskeletal protein α -actinin. *Cellular Microbiology*, 11, 389-405.
- SAAD, N., URDACI, M., VIGNOLES, C., CHAIGNEPAIN, S., TALLON, R., SCHMITTER, J. M. & BRESSOLLIER, P. 2009. *Lactobacillus plantarum* 299v surface-bound GAPDH: a new insight into enzyme cell

- walls location. *Journal of Microbiology and Biotechnology*, 19, 1635-43.
- SAEZ, D. E. & SLEBE, J. C. 2000. Subcellular localization of aldolase B. *Journal of Cellular Biochemistry*, 78, 62-72.
- SANCHEZ, B., SCHMITTER, J. M. & URDACI, M. C. 2009. Identification of novel proteins secreted by *Lactobacillus plantarum* that bind to mucin and fibronectin. *Journal of Molecular Microbiology and Biotechnology*, 17, 158-62.
- SANCHEZ, L., HORNER, D., MOORE, D., HENZE, K., EMBLEY, T. & MULLER, M. 2002. Fructose-1,6-bisphosphate aldolases in amitochondriate protists constitute a single protein subfamily with eubacterial relationships. *Gene*, 295, 51-9.
- SANDERS, H., BREHONY, C., MAIDEN, M. C., VIPOND, C. & FEAVERS, I. M. 2012. The effect of iron availability on transcription of the *Neisseria meningitidis* fHbp gene varies among clonal complexes. *Microbiology*, 158, 869-76.
- SANDERSON-SMITH, M. L., DOWTON, M., RANSON, M. & WALKER, M. J. 2007. The plasminogen-binding group A streptococcal M protein-related protein Prp binds plasminogen via arginine and histidine residues. *Journal of Bacteriology*, 189, 1435-40.
- SANDERSON-SMITH, M. L., WALKER, M. J. & RANSON, M. 2006. The maintenance of high affinity plasminogen binding by group A streptococcal plasminogen-binding M-like protein is mediated by arginine and histidine residues within the $\alpha 1$ and $\alpha 2$ repeat domains. *The Journal of Biological Chemistry*, 281, 25965-71.
- SANTIAGO, N. I., ZIPF, A. & BHUNIA, A. K. 1999. Influence of temperature and growth phase on expression of a 104-kilodalton *Listeria* adhesion protein in *Listeria monocytogenes*. *Applied and Environmental Microbiology*, 65, 2765-9.
- SARKARI, J., PANDIT, N., MOXON, E. R. & ACHTMAN, M. 1994. Variable expression of the Opc outer membrane protein in *Neisseria meningitidis* is caused by size variation of a promoter containing polycytidine. *Molecular Microbiology*, 13, 207-17.
- SAUVE, V. & SYGUSCH, J. 2001. Molecular cloning, expression, purification, and characterization of fructose-1,6-bisphosphate aldolase from *Thermus aquaticus*. *Protein Expression and Purification*, 21, 293-302.
- SAY, R. F. & FUCHS, G. 2010. Fructose 1,6-bisphosphate aldolase/phosphatase may be an ancestral gluconeogenic enzyme. *Nature*, 464, 1077-81.

- SCARSELLI, M., SERRUTO, D., MONTANARI, P., CAPECCHI, B., ADU-BOBIE, J., VEGGI, D., RAPPUOLI, R., PIZZA, M. & ARICÒ, B. 2006. *Neisseria meningitidis* NhhA is a multifunctional trimeric autotransporter adhesin. *Molecular Microbiology*, 61, 631-644.
- SCHAEFER, L. & SCHAEFER, R. M. 2010. Proteoglycans: from structural compounds to signaling molecules. *Cell and Tissue Research*, 339, 237-46.
- SCHMITT, C., TURNER, D., BOESL, M., ABELE, M., FROSCH, M. & KURZAI, O. 2007. A functional two-partner secretion system contributes to adhesion of *Neisseria meningitidis* to epithelial cells. *Journal of Bacteriology*, 189, 7968-76.
- SCHNEIDER, M. C., EXLEY, R. M., CHAN, H., FEAVERS, I., KANG, Y. H., SIM, R. B. & TANG, C. M. 2006. Functional significance of factor H binding to *Neisseria meningitidis*. *Journal of Immunology*, 176, 7566-75.
- SCHNEIDER, M. C., EXLEY, R. M., RAM, S., SIM, R. B. & TANG, C. M. 2007. Interactions between *Neisseria meningitidis* and the complement system. *Trends in Microbiology*, 15, 233-40.
- SCHOLTEN, R. J., KUIPERS, B., VALKENBURG, H. A., DANKERT, J., ZOLLINGER, W. D. & POOLMAN, J. T. 1994. Lipo-oligosaccharide immunotyping of *Neisseria meningitidis* by a whole-cell ELISA with monoclonal antibodies. *Journal of Medical Microbiology*, 41, 236-43.
- SCHUBERT-UNKMEIR, A., KONRAD, C., SLANINA, H., CZAPEK, F., HEBLING, S. & FROSCH, M. 2010. *Neisseria meningitidis* induces brain microvascular endothelial cell detachment from the matrix and cleavage of occludin: a role for MMP-8. *PLoS Pathogens*, 6, e1000874.
- SEDGWICK, H., CAMERON, J. E., POON, W. C. & EGELHAAF, S. U. 2007. Protein phase behavior and crystallization: effect of glycerol. *The Journal of Chemical Physics*, 127, 125102.
- SEGAL, E., HAGBLOM, P., SEIFERT, H. S. & SO, M. 1986. Antigenic variation of gonococcal pilus involves assembly of separated silent gene segments. *Proceedings of the National Academy of Sciences of the United States of America*, 83, 2177-81.
- SEIB, K. L., ZHAO, X. & RAPPUOLI, R. 2012. Developing vaccines in the era of genomics: a decade of reverse vaccinology. *Clinical Microbiology and Infection*, 18 Suppl 5, 109-16.
- SENGUPTA, S., GHOSH, S. & NAGARAJA, V. 2008. Moonlighting function of glutamate racemase from *Mycobacterium tuberculosis*: racemization and DNA gyrase inhibition are two independent activities of the enzyme. *Microbiology*, 154, 2796-2803.

- SERRUTO, D., ADU-BOBIE, J., SCARSELLI, M., VEGGI, D., PIZZA, M., RAPPUOLI, R. & ARICO, B. 2003. *Neisseria meningitidis* App, a new adhesin with autocatalytic serine protease activity. *Molecular Microbiology*, 48, 323-34.
- SERRUTO, D., SPADAFINA, T., CIUCCHI, L., LEWIS, L. A., RAM, S., TONTINI, M., SANTINI, L., BIOLCHI, A., SEIB, K. L., GIULIANI, M. M., DONNELLY, J. J., BERTI, F., SAVINO, S., SCARSELLI, M., COSTANTINO, P., KROLL, J. S., O'DWYER, C., QIU, J., PLAUT, A. G., MOXON, R., RAPPUOLI, R., PIZZA, M. & ARICO, B. 2010. *Neisseria meningitidis* GNA2132, a heparin-binding protein that induces protective immunity in humans. *Proceedings of the National Academy of Sciences of the United States of America*, 107, 3770-5.
- SHAO, P. L., CHANG, L. Y., HSIEH, S. M., CHANG, S. C., PAN, S. C., LU, C. Y., HSIEH, Y. C., LEE, C. Y., DOBBELAERE, K., BOUTRIAU, D., TANG, H., BOCK, H. L. & HUANG, L. M. 2009. Safety and immunogenicity of a tetravalent polysaccharide vaccine against meningococcal disease. *Journal of the Formosan Medical Association* 108, 539-47.
- SIEBERS, B., BRINKMANN, H., DÖRR, C., TJADEN, B., LILIE, H., VAN DER OOST, J. & VERHEES, C. H. 2001. Archaeal fructose-1,6-bisphosphate aldolases constitute a new family of archaeal type class I aldolase. *Journal of Biological Chemistry*, 276, 28710-28718.
- SIJBRANDI, R., STORK, M., LUIRINK, J. & OTTO, B. R. 2008. Pbp, a cell-surface exposed plasminogen binding protein of *Bacteroides fragilis*. *Microbes and Infection*, 10, 514-521.
- SINGH, B., AL-JUBAIR, T., MORGELIN, M., THUNNISSEN, M. M. & RIESBECK, K. 2013. The unique structure of *Haemophilus influenzae* protein E reveals multiple binding sites for host factors. *Infection and Immunity*, 81, 801-14.
- SINGH, B., JALALVAND, F., MORGELIN, M., ZIPFEL, P., BLOM, A. M. & RIESBECK, K. 2011. *Haemophilus influenzae* protein E recognizes the C-terminal domain of vitronectin and modulates the membrane attack complex. *Molecular Microbiology*, 81, 80-98.
- SINGH, S. M. & PANDA, A. K. 2005. Solubilization and refolding of bacterial inclusion body proteins. *Journal of Bioscience and Bioengineering*, 99, 303-310.
- SJÖLINDER, H., ERIKSSON, J., MAUDSDOTTER, L., ARO, H. & JONSSON, A.-B. 2008. Meningococcal Outer Membrane Protein NhhA Is Essential for Colonization and Disease by Preventing Phagocytosis and Complement Attack. *Infection and immunity*, 76, 5412-5420.

- SJOSTROM, I., GRONDAHL, H., FALK, G., KRONVALL, G. & ULLBERG, M. 1997. Purification and characterisation of a plasminogen-binding protein from *Haemophilus influenzae*. Sequence determination reveals identity with aspartase. *Biochimica et Biophysica Acta*, 1324, 182-90.
- SODEINDE, O. A., SUBRAHMANYAM, Y. V., STARK, K., QUAN, T., BAO, Y. & GOGUEN, J. D. 1992. A surface protease and the invasive character of plague. *Science*, 258, 1004-7.
- SOKOLOVA, O., HEPPEL, N., JAGERHUBER, R., KIM, K. S., FROSCH, M., EIGENTHALER, M. & SCHUBERT-UNKMEIR, A. 2004. Interaction of *Neisseria meningitidis* with human brain microvascular endothelial cells: role of MAP- and tyrosine kinases in invasion and inflammatory cytokine release. *Cellular Microbiology*, 6, 1153-66.
- SONG, Z., LI, Y., LIU, Y., XIN, J., ZOU, X. & SUN, W. 2012. alpha-Enolase, an adhesion-related factor of *Mycoplasma bovis*. *PloS ONE*, 7, e38836.
- SOTTRUP-JENSEN, L., ZAJDEL, M., CLAEYS, H., PETERSEN, T. E. & MAGNUSSON, S. 1975. Amino-acid sequence of activation cleavage site in plasminogen: homology with "pro" part of prothrombin. *Proceedings of the National Academy of Sciences of the United States of America*, 72, 2577-81.
- SPINOSA, M. R., PROGIDA, C., TALA, A., COGLI, L., ALIFANO, P. & BUCCI, C. 2007. The *Neisseria meningitidis* capsule is important for intracellular survival in human cells. *Infection and Immunity*, 75, 3594-603.
- SPURBECK, R. R., HARRIS, P. T., RAGHUNATHAN, K., ARVIDSON, D. N. & ARVIDSON, C. G. 2015. A Moonlighting Enolase from *Lactobacillus gasseri* does not Require Enzymatic Activity to Inhibit *Neisseria gonorrhoeae* Adherence to Epithelial Cells. *Probiotics and Antimicrobial Proteins*, 7, 193-202.
- ST GEME, J. W., CUTTER, D. & BARENKAMP, S. J. 1996. Characterization of the genetic locus encoding *Haemophilus influenzae* type b surface fibrils. *Journal of Bacteriology*, 178, 6281-6287.
- STAMBOULIAN, D., LOPARDO, G., LOPEZ, P., CORTES-BARBOSA, C., VALENCIA, A., BEDELL, L., KARSTEN, A. & DULL, P. M. 2010. Safety and immunogenicity of an investigational quadrivalent meningococcal CRM(197) conjugate vaccine, MenACWY-CRM, compared with licensed vaccines in adults in Latin America. *International Journal of Infectious Diseases*, 14, e868-75.
- STEPHENS, D. S. 2009. Biology and pathogenesis of the evolutionarily successful, obligate human bacterium *Neisseria meningitidis*. *Vaccine*, 27 Suppl 2, B71-7.

- STEPHENS, D. S. & FARLEY, M. M. 1991. Pathogenic events during infection of the human nasopharynx with *Neisseria meningitidis* and *Haemophilus influenzae*. *Reviews of Infectious Diseases*, 13, 22-33.
- STEPHENS, D. S., GREENWOOD, B. & BRANDTZAEG, P. 2007. Epidemic meningitis, meningococcaemia, and *Neisseria meningitidis*. *The Lancet*, 369, 2196-210.
- STERN, A., BROWN, M., NICKEL, P. & MEYER, T. F. 1986. Opacity genes in *Neisseria gonorrhoeae*: control of phase and antigenic variation. *Cell*, 47, 61-71.
- STIE, J., BRUNI, G. & FOX, D. 2009. Surface-associated plasminogen binding of *Cryptococcus neoformans* promotes extracellular matrix invasion. *PloS ONE*, 4, e5780.
- STOJILJKOVIC, I., HWA, V., DE SAINT MARTIN, L., O'GAORA, P., NASSIF, X., HEFFRON, F. & SO, M. 1995. The *Neisseria meningitidis* haemoglobin receptor: its role in iron utilization and virulence. *Molecular Microbiology*, 15, 531-41.
- STRIBLING, D. & PERHAM, R. N. 1973. Purification and characterization of two fructose diphosphate aldolases from *Escherichia coli* (Crookes' strain). *The Biochemical Journal*, 131, 833-41.
- SU, E. L. & SNAPE, M. D. 2011. A combination recombinant protein and outer membrane vesicle vaccine against serogroup B meningococcal disease. *Expert Review of Vaccines*, 10, 575-588.
- SUN, H., RINGDAHL, U., HOMEISTER, J. W., FAY, W. P., ENGLEBERG, N. C., YANG, A. Y., ROZEK, L. S., WANG, X., SJOBRING, U. & GINSBURG, D. 2004. Plasminogen is a critical host pathogenicity factor for group A streptococcal infection. *Science*, 305, 1283-6.
- SUN, Y.-H., BAKSHI, S., CHALMERS, R. & TANG, C. M. 2000. Functional genomics of *Neisseria meningitidis* pathogenesis. *Nature Medicine*, 6, 1269-1273.
- SUTHERLAND, T. C., QUATTRONI, P., EXLEY, R. M. & TANG, C. M. 2010. Transcellular passage of *Neisseria meningitidis* across a polarized respiratory epithelium. *Infection and Immunity*, 78, 3832-47.
- SWANSON, J. 1978. Studies on gonococcus infection. XIV. Cell wall protein differences among color/opacity colony variants of *Neisseria gonorrhoeae*. *Infection and Immunity*, 21, 292-302.
- TAHA, M. K., ALONSO, J. M., CAFFERKEY, M., CAUGANT, D. A., CLARKE, S. C., DIGGLE, M. A., FOX, A., FROSC, M., GRAY, S. J., GUIVER, M., HEUBERGER, S., KALMUSOVA, J., KESANOPOULOS, K., KLEM, A. M., KRIZ, P., MARSH, J., MOLLING, P., MURPHY, K., OLCEN, P., SANOU, O.,

- TZANAKAKI, G. & VOGEL, U. 2005. Interlaboratory comparison of PCR-based identification and genogrouping of *Neisseria meningitidis*. *Journal of Clinical Microbiology*, 43, 144-9.
- TAHA, M. K., DEGHMANE, A. E., ANTIGNAC, A., ZARANTONELLI, M. L., LARRIBE, M. & ALONSO, J. M. 2002. The duality of virulence and transmissibility in *Neisseria meningitidis*. *Trends in Microbiology*, 10, 376-82.
- TAKENAKA, R., YOKOTA, K., AYADA, K., MIZUNO, M., ZHAO, Y., FUJINAMI, Y., LIN, S. N., TOYOKAWA, T., OKADA, H., SHIRATORI, Y. & OGUMA, K. 2004. *Helicobacter pylori* heat-shock protein 60 induces inflammatory responses through the Toll-like receptor-triggered pathway in cultured human gastric epithelial cells. *Microbiology*, 150, 3913-22.
- TALA, A., PROGIDA, C., DE STEFANO, M., COGLI, L., SPINOSA, M. R., BUCCI, C. & ALIFANO, P. 2008. The HrpB-HrpA two-partner secretion system is essential for intracellular survival of *Neisseria meningitidis*. *Cellular Microbiology*, 10, 2461-82.
- TAN, L. K., CARLONE, G. M. & BORROW, R. 2010. Advances in the development of vaccines against *Neisseria meningitidis*. *The New England Journal of Medicine*, 362, 1511-20.
- TAUSEEF, I., HARRISON, O. B., WOOLDRIDGE, K. G., FEAVERS, I. M., NEAL, K. R., GRAY, S. J., KRIZ, P., TURNER, D. P., ALA'ALDEEN, D. A., MAIDEN, M. C. & BAYLISS, C. D. 2011. Influence of the combination and phase variation status of the haemoglobin receptors HmbR and HpuAB on meningococcal virulence. *Microbiology*, 157, 1446-56.
- TETTELIN, H., SAUNDERS, N. J., HEIDELBERG, J., JEFFRIES, A. C., NELSON, K. E., EISEN, J. A., KETCHUM, K. A., HOOD, D. W., PEDEN, J. F., DODSON, R. J., NELSON, W. C., GWINN, M. L., DEBOY, R., PETERSON, J. D., HICKEY, E. K., HAFT, D. H., SALZBERG, S. L., WHITE, O., FLEISCHMANN, R. D., DOUGHERTY, B. A., MASON, T., CIECKO, A., PARKSEY, D. S., BLAIR, E., CITTONE, H., CLARK, E. B., COTTON, M. D., UTTERBACK, T. R., KHOURI, H., QIN, H., VAMATHEVAN, J., GILL, J., SCARLATO, V., MASIGNANI, V., PIZZA, M., GRANDI, G., SUN, L., SMITH, H. O., FRASER, C. M., MOXON, E. R., RAPPUOLI, R. & VENTER, J. C. 2000. Complete genome sequence of *Neisseria meningitidis* serogroup B strain MC58. *Science*, 287, 1809-15.
- THOMPSON, M. J., NINIS, N., PERERA, R., MAYON-WHITE, R., PHILLIPS, C., BAILEY, L., HARNDEN, A., MANT, D. & LEVIN, M. 2006. Clinical recognition of meningococcal disease in children and adolescents. *The Lancet*, 367, 397-403.

- THOMPSON, S. A., WANG, L. L. & SPARLING, P. F. 1993a. Cloning and nucleotide sequence of frpC, a second gene from *Neisseria meningitidis* encoding a protein similar to RTX cytotoxins. *Molecular microbiology*, 9, 85-96.
- THOMPSON, S. A., WANG, L. L. & SPARLING, P. F. 1993b. Cloning and nucleotide sequence of frpC, a second gene from *Neisseria meningitidis* encoding a protein similar to RTX cytotoxins. *Molecular microbiology*, 9, 85-96.
- THOMPSON, S. A., WANG, L. L., WEST, A. & SPARLING, P. F. 1993c. *Neisseria meningitidis* produces iron-regulated proteins related to the RTX family of exoproteins. *Journal of Bacteriology*, 175, 811-8.
- TIMASHEFF, S. N. 1998. Control of protein stability and reactions by weakly interacting cosolvents: the simplicity of the complicated. *Advances in Protein Chemistry*, 51, 355-432.
- TRIVEDI, K., TANG, C. M. & EXLEY, R. M. 2011. Mechanisms of meningococcal colonisation. *Trends in Microbiology*, 19, 456-463.
- TUNIO, S. A., OLDFIELD, N. J., ALA'ALDEEN, D. A., WOOLDRIDGE, K. G. & TURNER, D. P. 2010b. The role of glyceraldehyde 3-phosphate dehydrogenase (GapA-1) in *Neisseria meningitidis* adherence to human cells. *BMC Microbiology*, 10, 280.
- TUNIO, S. A., OLDFIELD, N. J., BERRY, A., ALA'ALDEEN, D. A., WOOLDRIDGE, K. G. & TURNER, D. P. 2010. The moonlighting protein fructose-1, 6-bisphosphate aldolase of *Neisseria meningitidis*: surface localization and role in host cell adhesion. *Molecular microbiology*, 76, 605-15.
- TUNIO, S. A., OLDFIELD, N. J., BERRY, A., ALA'ALDEEN, D. A., WOOLDRIDGE, K. G. & TURNER, D. P. 2010a. The moonlighting protein fructose-1, 6-bisphosphate aldolase of *Neisseria meningitidis*: surface localization and role in host cell adhesion. *Molecular microbiology*, 76, 605-15.
- TUNKEL, A. R., HARTMAN, B. J., KAPLAN, S. L., KAUFMAN, B. A., ROOS, K. L., SCHELD, W. M. & WHITLEY, R. J. 2004. Practice guidelines for the management of bacterial meningitis. *Clinical Infectious Diseases* 39, 1267-84.
- TURNER, D., WOOLDRIDGE, K. & ALA'ALDEEN, D. 2009. Protein secretion and pathogenesis in *Neisseria meningitidis*. . In: KG, W. (ed.) *Bacterial secreted proteins*. Caister Academic Press.
- TURNER, D. P., MARIETOU, A. G., JOHNSTON, L., HO, K. K., ROGERS, A. J., WOOLDRIDGE, K. G. & ALA'ALDEEN, D. A. 2006. Characterization of MspA, an immunogenic autotransporter protein that

- mediates adhesion to epithelial and endothelial cells in *Neisseria meningitidis*. *Infection and Immunity*, 74, 2957-64.
- TURNER, D. P., WOOLDRIDGE, K. G. & ALA'ALDEEN, D. A. 2002. Autotransported serine protease A of *Neisseria meningitidis*: an immunogenic, surface-exposed outer membrane, and secreted protein. *Infection and Immunity*, 70, 4447-61.
- TZENG, Y. L., DATTA, A. K., STROLE, C. A., LOBRITZ, M. A., CARLSON, R. W. & STEPHENS, D. S. 2005. Translocation and surface expression of lipidated serogroup B capsular Polysaccharide in *Neisseria meningitidis*. *Infection and Immunity*, 73, 1491-505.
- ULLBERG, M., KRONVALL, G., KARLSSON, I. & WIMAN, B. 1990. Receptors for human plasminogen on gram-negative bacteria. *Infection and Immunity*, 58, 21-5.
- ULLBERG, M., KUUSELA, P., KRISTIANSEN, B.-E. & KRONVALL, G. 1992. Binding of Plasminogen to *Neisseria meningitidis* and *Neisseria gonorrhoeae* and Formation of Surface-Associated Plasmin. *Journal of Infectious Diseases*, 166, 1329-1334.
- UNKMEIR, A., LATSCH, K., DIETRICH, G., WINTERMEYER, E., SCHINKE, B., SCHWENDER, S., KIM, K. S., EIGENTHALER, M. & FROSCH, M. 2002. Fibronectin mediates Opc-dependent internalization of *Neisseria meningitidis* in human brain microvascular endothelial cells. *Molecular Microbiology*, 46, 933-46.
- VAN DEN BERGH, E. R., BAKER, S. C., RAGGERS, R. J., TERPSTRA, P., WOODSTRA, E. C., DIJKHUIZEN, L. & MEIJER, W. G. 1996. Primary structure and phylogeny of the Calvin cycle enzymes transketolase and fructosebiphosphate aldolase of *Xanthobacter flavus*. *Journal of Bacteriology*, 178, 888-93.
- VAN DER WOUDE, M. W. & BAUMLER, A. J. 2004. Phase and antigenic variation in bacteria. *Clinical Microbiology Reviews* 17, 581-611.
- VAN PUTTEN, J. P. & ROBERTSON, B. D. 1995. Molecular mechanisms and implications for infection of lipopolysaccharide variation in *Neisseria*. *Molecular Microbiology*, 16, 847-53.
- VAN ULSEN, P., ADLER, B., FASSLER, P., GILBERT, M., VAN SCHILFGAARDE, M., VAN DER LEY, P., VAN ALPHEN, L. & TOMMASSEN, J. 2006. A novel phase-variable autotransporter serine protease, AusI, of *Neisseria meningitidis*. *Microbes and Infection*, 8, 2088-2097.
- VAN ULSEN, P., RUTTEN, L., FELLER, M., TOMMASSEN, J. & VAN DER ENDE, A. 2008. Two-Partner Secretion Systems of *Neisseria meningitidis* Associated with Invasive Clonal Complexes. *Infection and Immunity*, 76, 4649-4658.

- VAN ULSEN, P. & TOMMASSEN, J. 2006. Protein secretion and secreted proteins in pathogenic Neisseriaceae. *FEMS Microbiology Reviews*, 30, 292-319.
- VAN ULSEN, P., VAN ALPHEN, L., HOPMAN, C. T. P., VAN DER ENDE, A. & TOMMASSEN, J. 2001. *In vivo expression of Neisseria meningitidis proteins homologous to the Haemophilus influenzae Hap and Hia autotransporters.*
- VAN ULSEN, P., VAN ALPHEN, L., TEN HOVE, J., FRANSEN, F., VAN DER LEY, P. & TOMMASSEN, J. 2003. A Neisserial autotransporter NalP modulating the processing of other autotransporters. *Molecular Microbiology*, 50, 1017-1030.
- VASTANO, V., CAPRI, U., CANDELA, M., SICILIANO, R. A., RUSSO, L., RENDA, M. & SACCO, M. 2013. Identification of binding sites of *Lactobacillus plantarum* enolase involved in the interaction with human plasminogen. *Microbiological Research*, 168, 65-72.
- VEGGI, D., GENTILE, M. A., CANTINI, F., LO SURDO, P., NARDI-DEI, V., SEIB, K. L., PIZZA, M., RAPPUOLI, R., BANCI, L., SAVINO, S. & SCARSELLI, M. 2012. The factor H binding protein of *Neisseria meningitidis* interacts with xenosiderophores in vitro. *Biochemistry*, 51, 9384-93.
- VERNIKOS, G. & MEDINI, D. 2014. Bexsero® chronicle. *Pathogens and Global Health*, 108, 305-316.
- VIDARSSON, G., OVERBEEKE, N., STEMERDING, A. M., VAN DEN DOBBELSTEEN, G., VAN ULSEN, P., VAN DER LEY, P., KILIAN, M. & VAN DE WINKEL, J. G. 2005. Working mechanism of immunoglobulin A1 (IgA1) protease: cleavage of IgA1 antibody to *Neisseria meningitidis* PorA requires de novo synthesis of IgA1 Protease. *Infection and Immunity*, 73, 6721-6.
- VIEIRA, M. L., ATZINGEN, M. V., OLIVEIRA, T. R., OLIVEIRA, R., ANDRADE, D. M., VASCONCELLOS, S. A. & NASCIMENTO, A. L. 2010. In vitro identification of novel plasminogen-binding receptors of the pathogen *Leptospira interrogans*. *PloS ONE*, 5, e11259.
- VIRJI, M. 1997. Post-translational modifications of meningococcal pili. Identification of common substituents: glycans and alpha-glycerophosphate--a review. *Gene*, 192, 141-7.
- VIRJI, M. 2009. Pathogenic neisseriae: surface modulation, pathogenesis and infection control. *Nature Reviews Microbiology*, 7, 274-86.
- VIRJI, M., ALEXANDRESCU, C., FERGUSON, D. J., SAUNDERS, J. R. & MOXON, E. R. 1992a. Variations in the expression of pili: the effect on adherence of *Neisseria meningitidis* to human epithelial and endothelial cells. *Molecular Microbiology*, 6, 1271-9.

- VIRJI, M., EVANS, D., HADFIELD, A., GRUNERT, F., TEIXEIRA, A. M. & WATT, S. M. 1999. Critical determinants of host receptor targeting by *Neisseria meningitidis* and *Neisseria gonorrhoeae*: identification of Opa adhesiotopes on the N-domain of CD66 molecules. *Molecular Microbiology*, 34, 538-51.
- VIRJI, M. & GRIFFITHS, N. J. 2008. Binding of Opc to vitronectin contributes to increased serum resistance of *Neisseria meningitidis* isolates. *Sixteenth International Pathogenic Neisseria Conference 2008, Rotterdam, The Netherlands, P096* (http://neisseria.org/ipnc/2008/Abstracts_poster_presentations_IPNC_2008.pdf).
- VIRJI, M., MAKEPEACE, K., FERGUSON, D. J., ACHTMAN, M. & MOXON, E. R. 1993a. Meningococcal Opa and Opc proteins: their role in colonization and invasion of human epithelial and endothelial cells. *Molecular Microbiology*, 10, 499-510.
- VIRJI, M., MAKEPEACE, K., FERGUSON, D. J., ACHTMAN, M., SARKARI, J. & MOXON, E. R. 1992b. Expression of the Opc protein correlates with invasion of epithelial and endothelial cells by *Neisseria meningitidis*. *Molecular Microbiology*, 6, 2785-95.
- VIRJI, M., MAKEPEACE, K., FERGUSON, D. J. & WATT, S. M. 1996a. Carcinoembryonic antigens (CD66) on epithelial cells and neutrophils are receptors for Opa proteins of pathogenic neisseriae. *Molecular Microbiology*, 22, 941-50.
- VIRJI, M., MAKEPEACE, K. & MOXON, E. R. 1994. Distinct mechanisms of interactions of Opc-expressing meningococci at apical and basolateral surfaces of human endothelial cells; the role of integrins in apical interactions. *Molecular Microbiology*, 14, 173-84.
- VIRJI, M., MAKEPEACE, K., PEAK, I. R., FERGUSON, D. J., JENNINGS, M. P. & MOXON, E. R. 1995. Opc- and pilus-dependent interactions of meningococci with human endothelial cells: molecular mechanisms and modulation by surface polysaccharides. *Molecular Microbiology*, 18, 741-54.
- VIRJI, M., SAUNDERS, J. R., SIMS, G., MAKEPEACE, K., MASKELL, D. & FERGUSON, D. J. 1993b. Pilus-facilitated adherence of *Neisseria meningitidis* to human epithelial and endothelial cells: modulation of adherence phenotype occurs concurrently with changes in primary amino acid sequence and the glycosylation status of pilin. *Molecular Microbiology*, 10, 1013-28.
- VIRJI, M., WATT, S. M., BARKER, S., MAKEPEACE, K. & DOYONNAS, R. 1996b. The N-domain of the human CD66a adhesion molecule is a target for Opa proteins of *Neisseria meningitidis* and *Neisseria gonorrhoeae*. *Molecular Microbiology*, 22, 929-39.

- VIRKOLA, R., LAHTEENMAKI, K., EBERHARD, T., KUUSELA, P., VAN ALPHEN, L., ULLBERG, M. & KORHONEN, T. K. 1996. Interaction of *Haemophilus influenzae* with the mammalian extracellular matrix. *The Journal of Infectious Diseases*, 173, 1137-47.
- WAMPLER, J. L., KIM, K. P., JARADAT, Z. & BHUNIA, A. K. 2004. Heat shock protein 60 acts as a receptor for the *Listeria* adhesion protein in Caco-2 cells. *Infection and Immunity*, 72, 931-6.
- WANG, G., XIA, Y., CUI, J., GU, Z., SONG, Y., CHEN, Y. Q., CHEN, H., ZHANG, H. & CHEN, W. 2013. The roles of moonlighting proteins in bacteria. *Current Issues in Molecular Biology*, 16, 15-22.
- WANG, J., SLUNT, H., GONZALES, V., FROMHOLT, D., COONFIELD, M., COPELAND, N. G., JENKINS, N. A. & BORCHELT, D. R. 2003. Copper-binding-site-null SOD1 causes ALS in transgenic mice: aggregates of non-native SOD1 delineate a common feature. *Human Molecular Genetics*, 12, 2753-2764.
- WANG, J., XU, G., GONZALES, V., COONFIELD, M., FROMHOLT, D., COPELAND, N. G., JENKINS, N. A. & BORCHELT, D. R. 2002. Fibrillar Inclusions and Motor Neuron Degeneration in Transgenic Mice Expressing Superoxide Dismutase 1 with a Disrupted Copper-Binding Site. *Neurobiology of Disease*, 10, 128-138.
- WEICHSELBAUM, A. 1887. Ueber die Aetiologie der akuten meningitis cerebro-spinalis. . *Fortschritte der Medizin*, 5, 573–583; 620–626.
- WEISER, J. N., GOLDBERG, J. B., PAN, N., WILSON, L. & VIRJI, M. 1998. The phosphorylcholine epitope undergoes phase variation on a 43-kilodalton protein in *Pseudomonas aeruginosa* and on pili of *Neisseria meningitidis* and *Neisseria gonorrhoeae*. *Infection and Immunity*, 66, 4263-7.
- WELSCH, J. A., RAM, S., KOEBERLING, O. & GRANOFF, D. M. 2008. Complement-dependent synergistic bactericidal activity of antibodies against factor H-binding protein, a sparsely distributed meningococcal vaccine antigen. *The Journal of Infectious Diseases*, 197, 1053-61.
- WEYNANTS, V. E., FERON, C. M., GORAJ, K. K., BOS, M. P., DENOEL, P. A., VERLANT, V. G., TOMMASSEN, J., PEAK, I. R., JUDD, R. C., JENNINGS, M. P. & POOLMAN, J. T. 2007. Additive and synergistic bactericidal activity of antibodies directed against minor outer membrane proteins of *Neisseria meningitidis*. *Infection and Immunity*, 75, 5434-42.
- WILKINS, J. C., BEIGHTON, D. & HOMER, K. A. 2003. Effect of acidic pH on expression of surface-associated proteins of *Streptococcus oralis*. *Applied and Environmental Microbiology*, 69, 5290-6.

- WILLARD, J. M. & GIBBS, M. 1968. Role of Aldolase in Photosynthesis. II Demonstration of Aldolase Types in Photosynthetic Organisms. *Plant Physiology*, 43, 793-8.
- WILSON, J. E., WRIGHT, L. L., MARTIN, J. L., HAIRE, S. E., RAY, P. H., PAINTER, G. R. & FURMAN, P. A. 1996. Recombinant human immunodeficiency virus type 1 reverse transcriptase is heterogeneous. *Journal of Acquired Immune Deficiency Syndromes and Human Retrovirology*, 11, 20-30.
- WINRAM, S. B. & LOTTENBERG, R. 1996. The plasmin-binding protein Plr of group A streptococci is identified as glyceraldehyde-3-phosphate dehydrogenase. *Microbiology*, 142 2311-20.
- WINRAM, S. B. & LOTTENBERG, R. 1998. Site-directed mutagenesis of streptococcal plasmin receptor protein (Plr) identifies the C-terminal Lys334 as essential for plasmin binding, but mutation of the plr gene does not reduce plasmin binding to group A streptococci. *Microbiology*, 144 2025-35.
- WINTHER-LARSEN, H. C., WOLFGANG, M., DUNHAM, S., VAN PUTTEN, J. P., DORWARD, D., LOVOLD, C., AAS, F. E. & KOOMEY, M. 2005. A conserved set of pilin-like molecules controls type IV pilus dynamics and organelle-associated functions in *Neisseria gonorrhoeae*. *Molecular Microbiology*, 56, 903-17.
- WISTEDT, A. C., KOTARSKY, H., MARTI, D., RINGDAHL, U., CASTELLINO, F. J., SCHALLER, J. & SJOBRING, U. 1998. Kringle 2 mediates high affinity binding of plasminogen to an internal sequence in streptococcal surface protein PAM. *The Journal of Biological Chemistry*, 273, 24420-4.
- WISTOW, G. J., LIETMAN, T., WILLIAMS, L. A., STAPEL, S. O., DE JONG, W. W., HORWITZ, J. & PIATIGORSKY, J. 1988. Tau-crystallin/alpha-enolase: one gene encodes both an enzyme and a lens structural protein. *The Journal of Cell Biology*, 107, 2729-36.
- WONG, V. K., HITCHCOCK, W. & MASON, W. H. 1989. Meningococcal infections in children: a review of 100 cases. . *The Pediatric Infectious Disease Journal*, 8, 224-227.
- WOOLDRIDGE, K. G., KIZIL, M., WELLS, D. B. & ALA'ALDEEN, D. A. 2005. Unusual genetic organization of a functional type I protein secretion system in *Neisseria meningitidis*. *Infection and Immunity*, 73, 5554-67.
- WU, Z., ZHANG, W. & LU, C. 2008. Immunoproteomic assay of surface proteins of *Streptococcus suis* serotype 9. *FEMS Immunology and Medical Microbiology*, 53, 52-9.

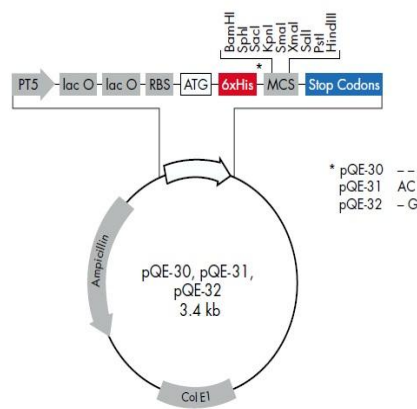
- XOLALPA, W., VALLECILLO, A. J., LARA, M., MENDOZA-HERNANDEZ, G., COMINI, M., SPALLEK, R., SINGH, M. & ESPITIA, C. 2007. Identification of novel bacterial plasminogen-binding proteins in the human pathogen *Mycobacterium tuberculosis*. *Proteomics*, 7, 3332-41.
- YAKUBU, D. E., ABADI, F. J. & PENNINGTON, T. H. 1999. Molecular typing methods for *Neisseria meningitidis*. *Journal of Medical Microbiology*, 48, 1055-64.
- YAMAGUCHI, H., OSAKI, T., TAGUCHI, H., HANAWA, T., YAMAMOTO, T., FUKUDA, M., KAWAKAMI, H., HIRANO, H. & KAMIYA, S. 1997. Growth inhibition of *Helicobacter pylori* by monoclonal antibody to heat-shock protein 60. *Microbiology and Immunology*, 41, 909-16.
- YANG, C. K., EWIS, H. E., ZHANG, X., LU, C. D., HU, H. J., PAN, Y., ABDELAL, A. T. & TAI, P. C. 2011. Nonclassical protein secretion by *Bacillus subtilis* in the stationary phase is not due to cell lysis. *Journal of Bacteriology*, 193, 5607-15.
- YAZDANKHAH, S. P. & CAUGANT, D. A. 2004. *Neisseria meningitidis*: an overview of the carriage state. *Journal of Medical Microbiology*, 53, 821-32.
- YOSHIDA, N., OEDA, K., WATANABE, E., MIKAMI, T., FUKITA, Y., NISHIMURA, K., KOMAI, K. & MATSUDA, K. 2001. Protein function. Chaperonin turned insect toxin. *Nature*, 411, 44.
- YOUNG, J. & HOLLAND, I. B. 1999. ABC transporters: bacterial exporters-revisited five years on. *Biochimica et Biophysica Acta*, 1461, 177-200.
- ZGIBY, S., PLATER, A. R., BATES, M. A., THOMSON, G. J. & BERRY, A. 2002. A functional role for a flexible loop containing Glu182 in the class II fructose-1,6-bisphosphate aldolase from *Escherichia coli*. *Journal of Molecular Biology*, 315, 131-140.
- ZGIBY, S. M., THOMSON, G. J., QAMAR, S. & BERRY, A. 2000. Exploring substrate binding and discrimination in fructose 1,6-bisphosphate and tagatose 1,6-bisphosphate aldolases. *European Journal of Biochemistry* 267, 1858–1868.
- ZHAO, Y., YOKOTA, K., AYADA, K., YAMAMOTO, Y., OKADA, T., SHEN, L. & OGUMA, K. 2007. *Helicobacter pylori* heat-shock protein 60 induces interleukin-8 via a Toll-like receptor (TLR)2 and mitogen-activated protein (MAP) kinase pathway in human monocytes. *Journal of Medical Microbiology*, 56, 154-64.
- ZHENG, L., BAUMANN, U. & REYMOND, J.-L. 2004. An efficient one-step site-directed and site-saturation mutagenesis protocol. *Nucleic Acids Research*, 32, e115-e115.

- ZHU, P., KLUTCH, M. J., BASH, M. C., TSANG, R. S., NG, L. K. & TSAI, C. M. 2002. Genetic diversity of three lgt loci for biosynthesis of lipooligosaccharide (LOS) in *Neisseria* species. *Microbiology*, 148, 1833-44.
- ZUGHAIER, S. M., TZENG, Y.-L., ZIMMER, S. M., DATTA, A., CARLSON, R. W. & STEPHENS, D. S. 2004. *Neisseria meningitidis* Lipooligosaccharide Structure-Dependent Activation of the Macrophage CD14/Toll-Like Receptor 4 Pathway. *Infection and Immunity*, 72, 371-380.
- ZYSK, G., SCHNEIDER-WALD, B. K., HWANG, J. H., BEJO, L., KIM, K. S., MITCHELL, T. J., HAKENBECK, R. & HEINZ, H. P. 2001. Pneumolysin is the main inducer of cytotoxicity to brain microvascular endothelial cells caused by *Streptococcus pneumoniae*. *Infection and Immunity*, 69, 845-52.

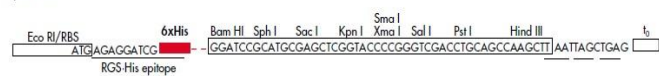
Appendix A: Vectors and Plasmids

pQE-30 Vector

Positions of elements in bases	pQE-30
Vector size (bp)	3461
Start of numbering at <i>Xho</i> I (CTCGAG)	1–6
T5 promoter/lac operator element	7–87
T5 transcription start	61
6xHis-tag coding sequence	127–144
Multiple cloning site	145–192
Lambda <i>t</i> ₀ transcriptional termination region	208–302
<i>rnnB</i> T1 transcriptional termination region	1064–1162
ColE1 origin of replication	1638
β -lactamase coding sequence	3256–2396



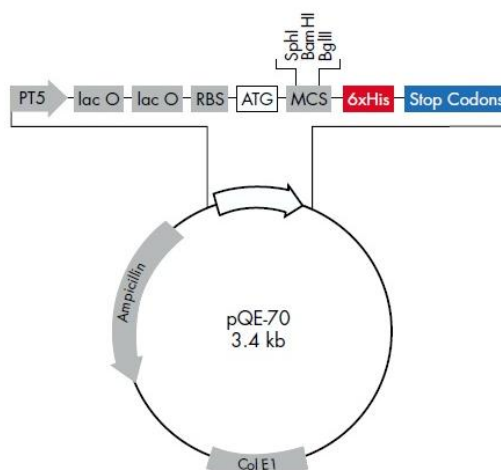
pQE-30



pQE-70 Vector

Positions of elements in bases

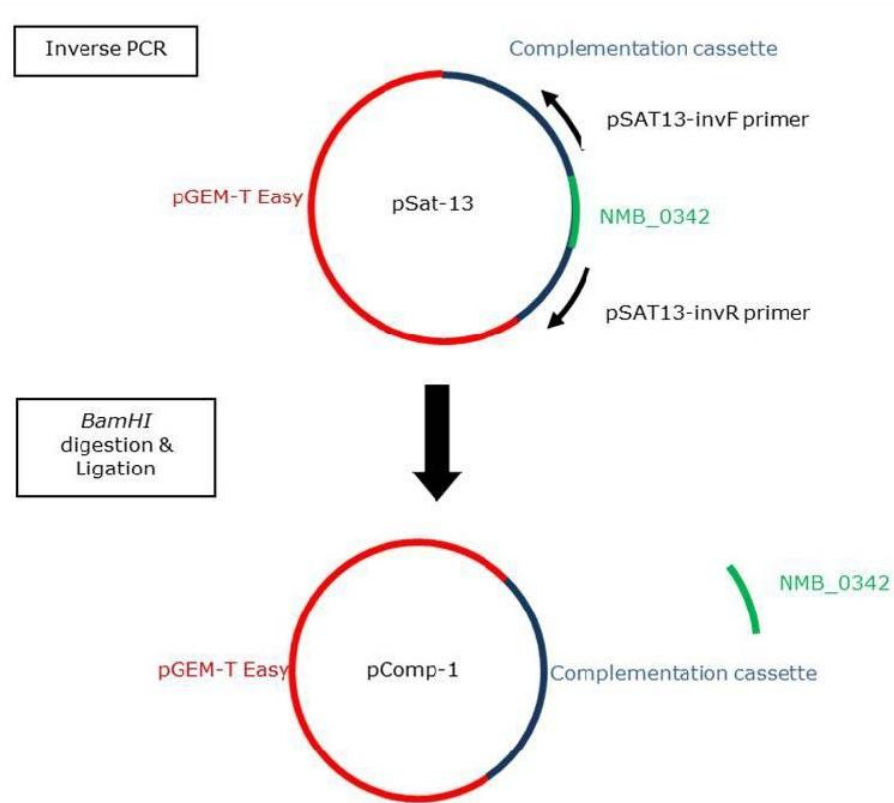
Vector size (bp)	3426
Start of numbering at XhoI (CTCGAG)	1–6
T5 promoter/lac operator element	7–87
T5 transcription start	61
6xHis-tag coding sequence	133–150
Multiple cloning site	113–132
Lambda t_0 transcriptional termination region	173–267
<i>rrnB</i> T1 transcriptional termination region	1029–1127
ColE1 origin of replication	1603
β -lactamase coding sequence	3221–2361



pQE-70

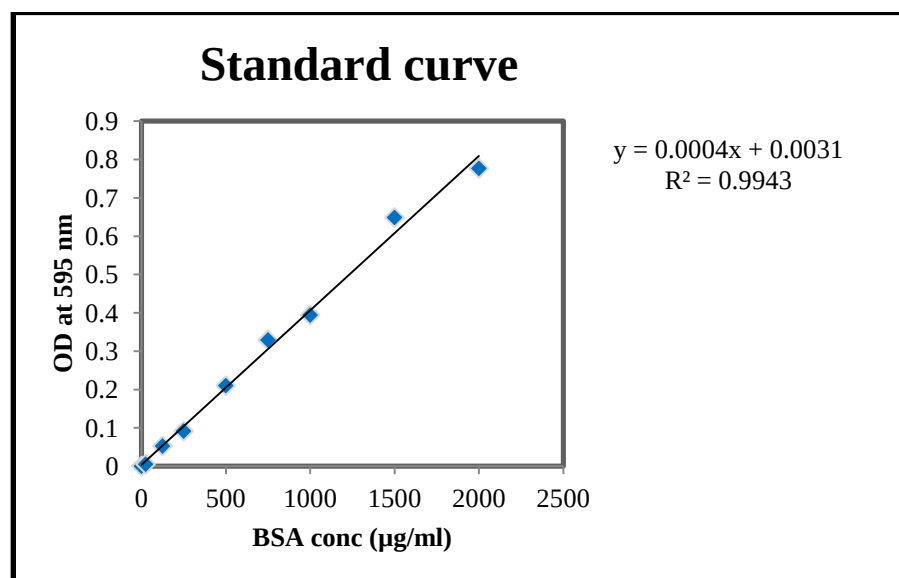


pCOMP-1 vector



Schematic diagram representing inverse PCR of pSAT-13 using primers with a *Bam*HI restriction site to exclude *NMB0342*. The resulting vector was designated as pComp-1 (pCOMP-1 vector was constructed and kindly provided by Hibah Albasri, University of Nottingham)

Appendix B: Standard curve



Standard curve used to calculate rFBA concentration in purified samples