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Bacterial moonlighting proteins of *N. meningitidis*: interaction with the host and role in pathogenesis

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Declaration

I do hereby declare that this thesis is my own work and has not been submitted elsewhere. Unless otherwise stated, all work contained here is my own.

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Abstract

Neisseria meningitidis is a human-restricted pathogen that colonizes the nasopharynx, mostly without causing any disease. However, bacteria can invade into the bloodstream and cross the blood brain barrier resulting in life threatening sepsis and meningitis.

Protein moonlighting is a concept established to describe the additional task(s) that a protein exhibits alongside its canonical function. After translocation to the cell surface by an unknown mechanism, moonlighting proteins may interact with various host proteins, and exhibit virulence-associated functions.

Enolase (Eno), peroxiredoxin (Prx) and DnaK are meningococcal housekeeping proteins that are functionally described as a glycolytic enzyme, an anti-oxidant protein and a molecular chaperon, respectively. They have been identified on the surface of meningococci achieving non-housekeeping (moonlighting) functions related to interactions with the human protein plasminogen. This study sheds light on the moonlighting properties of these three proteins in the pathogenesis of meningococcal disease.

Meningococcal Eno, Prx and DnaK were cloned, overexpressed in *E. coli* cells, and the corresponding wild type recombinant proteins were affinity purified under non-denaturing conditions. The oligomerization status of each recombinant protein was

determined by analytical gel filtration, in which rEno was identified as an octamer, rPrx as a hexamer and rDnaK was either in a dimeric or monomeric state. Polyclonal antiserum targeted against each recombinant protein was raised in rabbits.

A markerless *pxn*-knockout was generated in *N. meningitidis* MC58 *rpsL*⁻. The streptomycin resistance phenotype that the *rpsL*⁻ allele confers was the basis behind the adopted mutation strategy. To delete NMB0946 (encoding meningococcal Prx), two mutagenic plasmids (pGUD2 and pGUD3) were constructed to allow two homologous recombination events. *N. meningitidis* *rpsL*⁻ Δ *pxn* *Kan*^R *rpsL*⁺ was the resultant strain from the first event. The second homologous recombination event, facilitated by pGUD3, involved removing the selectable marker (*Kan*^R plus *rpsL*⁺ allele) to generate the markerless *N. meningitidis* *rpsL*⁻ Δ *pxn*. Given that NMB0946 is part of a two-gene operon necessitated the construction of a complementation strain, in which a wild type copy of NMB0946 was reintroduced at an ectopic site of *N. meningitidis* *rpsL*⁻ Δ *pxn* genome to complement that deletion.

Using equimolar amounts of the recombinant proteins in EIA assays, it was found that meningococcal rEno binds plasminogen (Plg) more strongly than rDnaK and rPrx. Plg binding was inhibited when the lysine analogue ϵ -aminocaproic

acid was added suggesting the potential involvement of lysine residues. Substitution of the C-terminal lysine residues of rEno, rPrx and rDnaK with alanine residues, significantly yet not completely, reduced binding to Plg. For rEno, lysine residues at positions 297 and 355 were identified for the first time as additional sites for Plg. rPrx with mutation in the active cysteine site (rPrx185CA), which is known to inactivate the protein, was able to bind Plg to the same level as the wild type strain. Unlike the C-terminal lysine residues, substituting the sub-terminal lysines of rPrx and rDnaK (at positions 230 and 641, respectively) did not alter binding to Plg.

Employing whole-cell EIA, Prx and DnaK could be detected on the surface of wild-type encapsulated *N. meningitidis* MC58, while surface localisation of Eno was not detected under these conditions.

Under hydrogen peroxide-mediated oxidative stress, the *N. meningitidis* Δpxn -mutant survived remarkably better than the parental strain. Using human whole blood as a model of meningococcal bacteraemia, it was found that *N. meningitidis* Δpxn -mutant has a survival defect compared with the wild-type strain. Preliminary data suggest that the survival of the Δpxn -mutant cells in the presence of polymorphonuclear leukocytes (PMNs) isolated from peripheral blood was similar to controls containing no PMNs. Moreover, the absence of Prx could not

significantly reduce the ability of whole meningococcal cells to bind Plg.

In summary, the data suggest that Eno, Prx and DnaK bind plasminogen mainly via the C-terminal lysine residues. Lysine 297 and 355 of rEno are required for optimal Plg binding. The canonical and the moonlighting functions of meningococcal Prx are independent. This study also proposes that the absence of Prx does not impair overall Plg binding, has a positive impact on the ability of meningococci to tolerate exogenous hydrogen peroxide, and finally, has a very significant effect on the survival of meningococcal cells in human whole blood.

List of Awards and Presentations

Awards

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Best poster prize at The Biological and Biomedical Consequences of protein Moonlighting Conference, 29-30 July 2014, London, UK; (Appendix 2).

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Mahab A. k. Aljannat, Neil J. Oldfield, Karl G. Wooldridge & David P. J. Turner. Investigating the moonlighting functions of meningococcal enolase, peroxiredoxin and DnaK. Presented at the Centre for Biomolecular Sciences Symposium, 17th September **2015**, Nottingham, UK.

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Mahab

List of Contents

Declaration-----	i
Abstract -----	ii
List of Awards and Presentations -----	vi
Awards -----	vi
Poster/oral presentations -----	vi
Acknowledgments -----	viii
List of Contents-----	x
List of plasmids used in this study -----	xviii
List of Figures-----	xxi
List of Tables-----	xxv
Acronyms and Abbreviations -----	xxvi

1. CHAPTER ONE: General introduction

1.1. The genus <i>Neisseria</i> -----	1
1.2. <i>N. meningitidis</i> -----	1
1.3. Classification of <i>N. meningitidis</i> -----	2
1.4. Meningococcal pathogenesis-----	4
1.5. Clinical picture of invasive meningococcal disease ----	8
1.5.1. Meningococcal Septicaemia -----	9
1.5.2. Meningococcal meningitis -----	10
1.6. Virulence factors of <i>N. meningitidis</i> -----	12
1.6.1. The capsule-----	13
1.6.2. Lipooligosaccharide -----	15

1.6.3.	Type IV pili -----	16
1.6.4.	Outer membrane proteins (OMPs) -----	18
1.6.5.	Neisseria hia homologue A (NhhA) -----	19
1.6.6.	Adhesion and penetration protein (App) -----	20
1.6.7.	HrpA-HrpB system -----	21
1.6.8.	Neisseria Adhesin A (NadA) -----	21
1.6.9.	Iron-acquiring proteins -----	22
1.7.	Moonlighting proteins: the concept -----	22
1.8.	Classification of moonlighting proteins-----	23
1.9.	Moonlighting proteins and secretion dilemma -----	26
1.10.	Meningococcal moonlighting proteins -----	29
1.10.1.	Enolase -----	31
1.10.2.	Peroxiredoxin-----	38
1.10.3.	DnaK -----	40
1.11.	The aim of the study -----	41

2. CHAPTER TWO: Cloning, overexpression and purification of meningococcal rEno, rPrx and rDnaK

2.1.	Introduction-----	42
2.2.	Materials and methods-----	45
2.2.1.	<i>In silico</i> sequence analysis -----	45
2.2.2.	Bacterial strains and growth conditions -----	45
2.2.3.	DNA extraction, purification and quantification --	46
2.2.4.	Polymerase chain reaction (PCR) -----	46
2.2.5.	Agarose gel electrophoresis -----	48

2.2.6.	Restriction digestion and de-phosphorylation ----	48
2.2.7.	Transformation procedure -----	49
2.2.8.	DNA sequencing-----	49
2.2.9.	Cloning of NMB1285, NMB0946 and NMB0455 into pQE30 -----	50
2.2.10.	Expression of recombinant enolase, peroxiredoxin and DnaK-----	50
2.2.11.	Purification of recombinant proteins by IMAC ----	51
2.2.12.	Desalting and concentration of proteins -----	52
2.2.13.	Purification by gel filtration -----	52
2.2.14.	SDS-PAGE -----	53
2.2.15.	Immunoblot analysis-----	53
2.2.16.	Protein quantification -----	54
2.2.17.	Raising polyclonal antisera against rEno, rPrx and rDnaK -----	54
2.3.	Results -----	55
2.3.1.	Meningococcal enolase, peroxiredoxin and DnaK lack signal peptide sequences -----	55
2.3.2.	Amplification and cloning of NMB1285, NMB0946 and NMB0455 into pQE30 -----	57
2.3.3.	Expression of recombinant enolase, peroxiredoxin and DnaK-----	61
2.3.4.	Purification of recombinant His-tagged enolase, peroxiredoxin and DnaK under native conditions-----	63

2.3.5.	Determining the oligomerization of rEno, rPrx and rDnaK by analytical gel filtration-----	68
2.4.	Discussion-----	72

3. CHAPTER THREE: Mutagenesis and complementation of the gene encoding peroxiredoxin in *N. meningitidis* MC58

3.1.	Introduction-----	77
3.2.	Materials and methods -----	80
3.2.1.	<i>In silico</i> sequence analysis -----	80
3.2.2.	Bacterial strains and growth conditions -----	80
3.2.3.	DNA techniques -----	81
3.2.4.	Cloning of NMB0946 and flanking DNA -----	81
3.2.5.	NMB0946 deletion by inverse-PCR-----	84
3.2.6.	Preparation of markers for selection and cloning to depxn -----	84
3.2.7.	Construction of pGUD3 -----	85
3.2.8.	Construction of ectopic complementation plasmid -----	85
3.2.9.	Natural transformation of <i>N. meningitidis</i> -----	86
3.3.	Results -----	88
3.3.1.	Sequence analysis of NMB0946 gene, flanking DNA and Prx protein -----	88
3.3.2.	Cloning of NMB0946 and flanking DNA -----	89
3.3.3.	NMB0946 deletion by inverse PCR -----	91

3.3.4.	Preparation of markers for selection and cloning to depxn -----	91
3.3.5.	Construction of pGUD3 vector-----	92
3.3.6.	NMB0946 mutagenesis of <i>N. meningitidis</i> -----	93
3.3.7.	Ectopic complementation plasmid construction --	97
3.3.8.	Complementation of NMB0946 -----	98
3.3.9.	Verification of NMB0946 mutagenesis and complementation -----	100
3.4.	Discussion-----	103

4. CHAPTER FOUR: Investigating the binding of meningococcal rEno, rPrx and rDnaK to human glu- plasminogen

4.1.	Introduction-----	108
4.2.	Materials and methods -----	113
4.2.1.	Enzyme-linked immunosorbant assay (EIA) ---	113
4.2.2.	Binding inhibition assay -----	114
4.2.3.	Bacterial strains and growth conditions -----	114
4.2.4.	Primer design-----	114
4.2.5.	DNA extraction, quantification and analysis----	117
4.2.6.	Site directed mutagenesis -----	117
4.2.6.1.	PCR-mediated mutagenesis-----	117
4.2.6.2.	Kinase-Ligase-DpnI (KLD) treatment -----	118
4.2.6.3.	Transformation -----	118
4.2.7.	DNA sequencing-----	118

4.2.8.	Protein techniques -----	119
4.2.9.	Protein structure analysis -----	119
4.2.10.	Statistical analysis -----	120
4.3.	Results -----	121
4.3.1.	Meningococcal rEno binds Plg more strongly than rDnaK and rPrx -----	121
4.3.2.	Site-directed mutagenesis of NMB1285, NMB0946 and NMB0554 -----	122
4.3.3.	Expression of recombinant mutated rEno, rPrx and rDnaK -----	126
4.3.4.	Purification of His-tagged mutated rEno, rPrx and rDnaK under native conditions-----	127
4.3.5.	The C-terminal lysine residue of rEno, rPrx and rDnaK contributes to plasminogen binding-----	129
4.3.6.	Conserved lysine residues of enolase and predicting their positions -----	132
4.3.7.	Pneumococcal enolase as a model to predict the 3D structure of meningococcal enolase -----	134
4.3.8.	Lysine residues 297 and 355 of meningococcal rEno are required for optimal plasminogen binding -----	136
4.3.9.	Mutation of the Grx catalytic cysteine residue of rPrx has no impact on Plg binding -----	139
4.3.10.	Cloning, overexpression and purification of Kringle domains of human glu-plasminogen-----	141

4.4.	Discussion-----	144
------	-----------------	-----

5. CHAPTER FIVE: Phenotypic characterisation of meningococcal peroxiredoxin

5.1.	Introduction-----	153
5.2.	Materials and methods -----	157
5.2.1.	Bacterial strains and growth conditions -----	157
5.2.2.	Investigating the <i>in vitro</i> growth of meningococcal cells -----	157
5.2.3.	Whole-cell EIA-----	158
5.2.3.1.	<i>Preparation and fixation of meningococcal cells -----</i>	<i>159</i>
5.2.3.2.	<i>Binding of anti-rEno, -rPrx and -rDnaK antibodies to whole meningococcal cells -----</i>	<i>159</i>
5.2.3.3.	<i>Binding of human glu-plasminogen to whole meningococcal cells -----</i>	<i>161</i>
5.2.4.	Hydrogen peroxide killing assay -----	162
5.2.5.	Human whole blood survival assay -----	163
5.2.6.	Investigating the survival of meningococcal cells in polymorphonuclear cells (PMNs)-----	164
5.2.6.1.	<i>Preparation of meningococcal cells -----</i>	<i>164</i>
5.2.6.2.	<i>Isolation of PMNs -----</i>	<i>165</i>
5.2.6.3.	<i>Infection of PMNs with meningococcal cells</i>	<i>166</i>
5.2.7.	Statistical analysis -----	167
5.3.	Results -----	168

5.3.1.	Determining the growth characteristics of meningococcal strains -----	168
5.3.2.	Unlike enolase, meningococcal DnaK and peroxiredoxin are surface localized -----	170
5.3.3.	Peroxiredoxin-null meningococcal cells bind human glu-plasminogen -----	173
5.3.4.	MC58 Δpxn -mutant tolerates hydrogen peroxide better than parental strains -----	175
5.3.5.	MC58 Δpxn -mutant has a survival defect in human whole blood -----	177
5.3.6.	Meningococcal cells killing by PMNs-----	179
5.4.	Discussion-----	180

6. CHAPTER SIX: General discussion

6.1.	General discussion-----	187
6.1.1.	Plasminogen binding -----	188
6.1.2.	The phenotype of Δpxn meningococcal cells ---	195
6.2.	Future directions-----	198
6.3.	Conclusion -----	202

Bibliography -----	204
---------------------------	------------

Appendix -----	240
-----------------------	------------

List of plasmids used in this study

Plasmid		Description	Source
1	pQE30	Cloning vector with ampicillin resistance cassette, and a His-tag encoding sequence to be incorporated at the N-terminus of any cloned gene product (Appendix 3)	Qiagen
2	pMAJ1	NMB1285 gene from MC58 cloned into pQE30 vector	This study ^a
3	pMAJ2	NMB0946 gene from MC58 cloned into pQE30 vector	This study ^a
4	pMAJ3	NMB0554 gene from MC58 cloned into pQE30 vector	This study ^a
5	pGEM [®] -T Easy	A linearized vector with a single 3'-terminal thymidine at both ends that facilitate TA cloning of any PCR product with 3' adenosine overhangs (Appendix 4)	Promega
6	pGUD1	NMB0946 gene plus 1kb up- and 1kb downstream flanking sequence from MC58 cloned into pGEM [®] -T Easy vector	This study
7	pGUD2	Kanamycin resistance cassette and <i>rpsL</i> ⁺ allele from MC58 cloned into pGUD3 at the same NMB0946 deletion site	This study
8	pGUD3	Re-ligated pGUD1 after removing NMB0946 encoding gene by inverse PCR	This study
9	pComp-1	A complementation cassette consisting of NMB0102, erythromycin resistance cassette and NMB0103, cloned into pGEM [®] -T Easy vector (Figure 3.1)	Hiba Albasri
10	pLD-1	NMB0946 gene from MC58 cloned into pComp-1	Louise Dorrington
11	pMAJ1-250253KKAA	Lysine residues (K) at amino acid sites 250 and 253 of NMB1285 gene substituted for two alanine residues (A)	This study ^a
12	pMAJ1-297KA	Lysine residue at amino acid site 297 of NMB1285 gene substituted for an alanine residue	This study ^a
13	pMAJ1-337KA	Lysine residue at amino acid site 337 of NMB1285 gene substituted for an alanine residue	This study ^a

	Plasmid	Description	Source
14	pMAJ1-355KA	Lysine residue at amino acid site 355 of NMB1285 gene substituted for an alanine residue	This study ^a
15	pMAJ1-388KA	Lysine residue at amino acid site 388 of NMB1285 gene substituted for an alanine residue	This study ^a
16	pMAJ1-400KA	Lysine residue at amino acid site 400 of NMB1285 gene substituted for an alanine residue	This study ^a
17	pMAJ1-428KA	Lysine residue at amino acid site 428 of NMB1285 gene substituted for an alanine residue	This study ^a
18	pMAJ2-185CA	Cysteine residue (C) at amino acid site 185 of NMB0946 gene substituted for an alanine residue	Radhica Ohri ^a
19	pMAJ2-230KA	Lysine residue at amino acid site 230 of NMB0946 gene substituted for an alanine residue	Radhica Ohri ^a
20	pMAJ2-244KA	Lysine residue at amino acid site 244 of NMB0946 gene substituted for an alanine residue	This study ^a
21	pMAJ2-230244KKAA	Lysine residues (K) at amino acid sites 230 and 244 of NMB0946 gene substituted for two alanine residues	This study ^a
22	pMAJ3-641KA	Lysine residue at amino acid site 641 of NMB0554 gene substituted for an alanine residue	This study ^a
23	pMAJ3-642KA	Lysine residue at amino acid site 642 of NMB0554 gene substituted for an alanine residue	This study ^a
24	pQE70	Cloning vector with ampicillin resistance cassette, and a His-tag encoding sequence to be incorporated at the C-terminus of any cloned gene product (Appendix 6)	Qiagen
25	pUC57-K1-5	Construct containing Kringle domains (KR) 1-5 of human plasminogen used to construct other plasmids	Dr Yihai Cao
26	pMK1-5	Kringle domains 1-5 of human plasminogen cloned into pQE70	Meena Ali-Naja ^b

	Plasmid	Description	Source
27	pMK1-2	Kringle domains 1 and 2 of human plasminogen cloned into pQE70	Meena Ali-Naja ^b
28	pMK3-5	Kringle domains 3, 4 and 5 of human plasminogen cloned into pQE70	Meena Ali-Naja ^b
29	pdeK-1	Kringle domains 2-5 of human plasminogen cloned into pQE70	This study ^b
30	pdeK-2	Kringle domains 1, 3, 4 and 5 of human plasminogen cloned into pQE70	This study ^b
31	pdeK-3	Kringle domains 1, 2, 4 and 5 of human plasminogen cloned into pQE70	This study ^b
32	pdeK-4	Kringle domains 1, 2, 3 and 5 of human plasminogen cloned into pQE70	This study ^b

^a: Corresponding recombinant protein was successfully purified

^b: Purifying the corresponding recombinant protein was attempted

List of Figures

Figure 1.1: Steps of pathogenesis associated with <i>Neisseria meningitidis</i> .-----	5
Figure 1.2: Major virulence factors of <i>Neisseria meningitidis</i> . -----	13
Figure 1.3: Cartoons illustrating how a protein can moonlight. ---	24
Figure 1.4: Plasminogen-bacterial cell interactions and its impact on pathogenesis. -----	36
Figure 2.1: Predicting putative signal peptide sequences of meningococcal enolase (A) and AspA (B), a known secreted protein that was used as a positive control.-----	56
Figure 2.2: Agarose gel analysis showing the PCR amplification of NMB1285 (A), NMB0946 (B) and NMB0554 (C).-----	58
Figure 2.3: Agarose gel analysis showing BamHI restriction digestion of plasmid DNA extracted from <i>E. coli</i> JM109 that harbour NMB1285, NMB0946 or NMB0554 in pQE30.-----	59
Figure 2.4: Agarose gel analysis showing EcoRI restriction digestion of plasmid DNA to verify the cloning of NMB1285, NMB0946 and NMB0455 into pQE30.-----	60
Figure 2.5: SDS-PAGE (left) or immunoblot analysis (right) with anti-penta-histidine antibodies demonstrating the overexpression of recombinant enolase (A, B), peroxiredoxin (C, D) and DnaK (E, F) in IPTG-induced <i>E. coli</i> JM109 cells containing pMAJ1, pMAJ2 or pMAJ3, respectively.-----	62
Figure 2.6: Representative chromatograms illustrate the steps of recombinant His-tagged protein purification using the AKTA prime plus system.-----	65
Figure 2.7: SDS-PAGE (left) and immunoblot analysis (right) demonstrating IMAC-purification steps of recombinant enolase (A, B), peroxiredoxin (C, D) and DnaK (E, F) by using 150 ml harvested cells	

pellet of the IPTG-induced <i>E. coli</i> JM109 containing pMAJ1, pMAJ2 or pMAJ3, respectively.-----	67
Figure 2.8: SDS-PAGE and immunoblot analysis demonstrating purification of rEno by gel filtration.-----	69
Figure 2.9: Chromatograms show the elution of the IMAC-purified rEno (A), rPrx (B) and rDnaK (C) during gel filtration. -----	71
Figure 3.1: A schematic representation of the complementation cassette of pComp-1 plasmid.-----	86
Figure 3.2: A schematic representation of NMB0946 in genomic context of <i>N. meningitidis</i> MC58, showing genes orientation, predicted termination sequence (in red) downstream of NMB0944, and approximate binding sites of primers used to facilitate NMB0946 deletion and complementation.-----	89
Figure 3.3: Agarose gel analysis showing; [A] PCR amplification of NMB0946 and 1 kb up- and 1 kb downstream flanking DNA (Up _{pxnD}) from <i>N. meningitidis</i> strain MC58.-----	90
Figure 3.4: Agarose gel analysis showing [A] dep _{pxn} and [B] KnsL.-----	92
Figure 3.5: Agarose gel analysis showing BamHI and EcoRI restriction digestion of putative pGUD3.-----	93
Figure 3.6: Schematic workflow of the mutagenesis strategy that was adopted to delete NMB0946.-----	95
Figure 3.7: Agarose gel analysis showing PCR-mediated verification of NMB0946 mutagenesis.-----	96
Figure 3.8: Agarose gel analysis showing PerComp amplification and restriction digestion.-----	98
Figure 3.9: Agarose gel analysis showing PCR to verify the complementation of NMB0946 in the genome of MC58 <i>rpsL</i> ⁻ Δ _{pxn} .-----	100
Figure 3.10: Immunoblot analysis verifying the successful mutagenesis and complementation of NMB0946.-----	102

- Figure 4.1:** The binding of meningococcal rEno, rPrx and rDnaK to human glu-plasminogen (Plg) in the presence or absence of ϵ -aminocaproic acid (EACA).----- **122**
- Figure 4.2:** Agarose gel analysis showing the PCR amplification of pMAJ1-428KA.----- **123**
- Figure 4.3:** Snapshots of DNA sequence chromatograms of wild type (WT) NMB1285 gene and NMB1285-428KA, showing substitution of the triple codon AAA, encoding the C-terminal lysine of meningococcal enolase, in the WT gene to GCA codon, encoding an alanine, to generate pMAJ1-428KA mutant.----- **124**
- Figure 4.4:** 10% SDS-PAGE (A) and immunoblot (B) probed with anti-penta-histidine antibodies demonstrating the rEno428KA overexpression in IPTG-induced *E. coli* JM109 (pMAJ1-428KA).--- **127**
- Figure 4.5:** SDS-PAGE and immunoblot analysis demonstrating the purification of His-tagged rEno428KA under native conditions.--- **128**
- Figure 4.6:** The C-terminal lysine residues of meningococcal rEno, rPrx and rDnaK contribute to plasminogen binding.----- **130**
- Figure 4.7:** The penultimate lysine residue of meningococcal rDnaK is not involved in plasminogen binding.----- **131**
- Figure 4.8:** Multiple amino acid sequence alignment of *N. meningitidis* MC58 enolase compared with that of *S. pneumoniae* R6, *S. pyogenes* M1 GAS, *Enterococcus hirae* and *Bacillus subtilis* subsp. *subtilis* str.168.----- **133**
- Figure 4.9:** Schematic representation of a monomeric pneumococcal enolase (PDB entry 1W6T) used to model positions of conserved internal lysine residues of meningococcal enolase.----- **135**
- Figure 4.10:** Lysine residues 297 and 355 of meningococcal rEno are required for optimal plasminogen binding.----- **137**

Figure 4.11: Binding of meningococcal rEno388KA and rEno400KA to human glu-plasminogen (Plg), compared with the wild type (WT).- -----	138
Figure 4.12: Mutation in the Grx catalytic cysteine residue of rPrx has no influence on plasminogen binding.-----	140
Figure 4.13: 10% SDS-PAGE (A) and immunoblot analysis (B) displaying the IMAC purification process of recombinant His-tagged kringle domain 1-5 under native conditions.-----	142
Figure 5.1: Growth characteristics of peroxiredoxin mutant and complemented strains compared to their <i>rpsL</i> ⁻ and wild-type parent strains -----	169
Figure 5.2: Binding of anti-rEno, -rPrx and -rDnaK antibodies (Abs) to intact <i>N. meningitidis</i> wild type MC58 cells examined via whole-cell EIA.-----	171
Figure 5.3: Binding of anti-rPrx antibodies to intact meningococcal cells from different MC58 strains, examined via whole-cell EIA.---	172
Figure 5.4: Binding of whole meningococcal cells to human glu-plasminogen (Plg) by EIA.-----	174
Figure 5.5: Hydrogen peroxide (H ₂ O ₂) killing of different <i>N. meningitidis</i> MC58 strains.-----	176
Figure 5.6: Pxn knockout strain has a survival defect in the <i>ex vivo</i> human whole blood model of meningococcal bacteraemia.-----	178
Figure 5.7: Survival of meningococcal cells in PMNs freshly isolated from human blood.-----	179

List of Tables

Table 2.1: List of the primers used in this chapter for amplification and/or sequencing of NMB1285, NMB0946 and NMB0554.-----	47
Table 2.2: Thermo-cycling conditions that were applied to amplify NMB1285, NMB0946 and NMB0554 utilizing genomic DNA of <i>N. meningitidis</i> strain MC58 as a template.-----	48
Table 2.3: Molecular weights and elution volume values of the proteins used as standards for the estimation of the molecular weight of rEno, rPrx and rDnaK purified under native conditions.-----	69
Table 3.1: PCR reactions used in the mutagenesis and complementation of NMB0946.-----	83
Table 3.2: Mutagenic plasmids used to mutate different meningococcal MC58 strains.-----	87
Table 4.1: Mutagenic primers and thermo-cycling conditions applied in PCR reactions to mutate NMB1285, NMB0946 and NMB0554.--	115
Table 4.2: List of plasmids that were used to express different recombinant meningococcal proteins.-----	125
Table 4.3: List of plasmids that were constructed to express different combinations of kringle domains from human plasminogen.-----	143
Table 5.1: Meningococcal strains used in work described in this chapter.-----	158

Acronyms and Abbreviations

App	Adhesion and penetration protein
AspA	Autotransported serine protease A
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BamHI	<i>Bacillus amyloliquefaciens</i> strain H restriction endonuclease
BBB	Blood brain barrier
BCIP/NBT	5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium
BglII	<i>Bacillus globigii</i> restriction endonuclease
BHI	Brain Heart Infusion
bp	base pair
BSA	Bovine serum albumin
CD	Cluster of differentiation
CEACAM	Carcinoembryonic antigen-related cell adhesion molecule
CFU	Colony-forming unit
CNS	Central nervous system
CSF	Cerebrospinal fluid
dNTPs	deoxy nucleotide triphosphate
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxy ribonucleic acid
EACA	ϵ -aminocaproic acid
<i>E. coli</i>	<i>Escherichia coli</i>
EcoRI	<i>Escherichia coli</i> strain R restriction endonuclease
EIA	Enzyme linked immunosorbant assay
EMP	Embden Meyerhof Parnas pathway
ET	Electrophoretic type
FBA	Fructose bisphosphate aldolase
fHBP	Factor H binding Protein
GAPDH-1	Glyceraldehyde 3-phosphate dehydrogenase
Gor	glutathione oxidoreductase
HBMECs	Human brain microvascular endothelial cells
HBSS	Hank's Balanced Salt Solution
His	Histidine
HrpA	Haemagglutinin/haemolysin-related protein A
HrpB	Haemagglutinin/haemolysin-related protein B

IMAC	Immobilized Metal Affinity Chromatography
IMD	Invasive meningococcal disease
IL	Interleukin
IPTG	Isopropyl-P-D-thiogalactopyranoside
Kb	Kilo base pair
kDa	Kilo Dalton
Lac	Lactose operator
LAMP	Lysosome-associated membrane proteins
LB	Lysogeny Broth
LBS	Lysine binding sites
LOS	Lipooligosaccharide
LPS	Lipopolysaccharides
Lys	Lysine
methH	5-methyltetrahydropteroyl-triglutamate
MH	Mueller-Hinton agar
MHC	Major Histocompatibility
MLEE	Multi-locus enzyme electrophoresis
MLST	Multi-locus sequence typing
MOI	Multiplicity of infection
NadA	Neisseria adhesion A
Nhha	Neisseria hia homologue
OD	Optical density
OMP	Outer membrane protein
Opa	Colony opacity factor
ORF	Open reading frame
OS	Oligosaccharide
PBS	Phosphate buffer saline
PBST	PBS with 0.05% Tween 20
PBMCs	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
Plg	Human glu-plasminogen
PMNs	Polymorphonuclear leukocytes
pxn	NMB0946 encodes meningococcal peroxiredoxin
PorA	Porin A
PorB	Porin B
Prx	Peroxiredoxin

PTM	Posttranslational modifications
RBCs	Red Blood Cells
rDnaK	recombinant DnaK
rEno	recombinant enolase
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
rPrx	recombinant peroxiredoxin
rpm	revolutions per minute
SAP	Shrimp alkaline phosphatase
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SE	standard error
ST	Sequence type
Taq	<i>Thermus aquaticus</i> thermostable polymerase
tPA	Tissue plasminogen activator
uPA	urokinase plasminogen activator
WT	Wild type
X-GAL	5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside

1. CHAPTER ONE: General introduction

1.1. The genus *Neisseria*

The genus *Neisseria* is a member of the family Neisseriaceae, which also comprises the genus *Kingella*. Based on 16S rRNA gene sequence analysis, the genus *Neisseria* includes 25 species in which two of them are well characterized human pathogens [Hung and Christodoulides, 2013], namely *N. meningitidis* (meningococci) and *N. gonorrhoeae* (gonococci), colonizing the mucosal surfaces of the human nasopharynx and urogenital tract, respectively [Maiden, 2008]. Other species are human and/or mammalian commensals, which might under certain conditions give rise to opportunistic infections [Hung and Christodoulides, 2013]. Human commensal *Neisseria* species include *N. cinerea*, *N. elongata*, *N. flavescens*, *N. lactamica*, *N. mucosa*, *N. perflava*, *N. polysaccharea*, *N. sicca* and *N. subflava* [Rotman and Seifert, 2014].

1.2. *N. meningitidis*

N. meningitidis is a human-restricted pathogen. The first time the bacteria isolated was in 1887 by Anton Weichselbaum from the cerebrospinal fluid (CSF) of a patient with epidemic cerebrospinal meningitis [Weichselbaum, 1887]. They are Gram-negative cocci, non-spore forming, aflagellate, strict aerobes that are often arranged in pairs (diplococci) [Ala'Aldeen and Turner, 2006]. Meningococci are reasonably fastidious *i.e.* special growth supplements and conditions are required to allow

bacterial growth. They are well propagated on blood agar where they form smooth, convex, non-pigmented, non-haemolytic, moist, medium sized colonies with well-defined edges. Chocolate agar, modified New York City medium, and Muller-Hinton Agar can also support the bacteria growth. Optimum growth conditions can be achieved at 35-37°C, pH 7.0-7.4 in a humid environment with 5-10% CO₂ [Ala'Aldeen and Turner, 2006].

1.3. Classification of *N. meningitidis*

N. meningitidis can be classified based on the serological or the genetic properties that the bacteria uniquely possess. The antigenic properties of several meningococcal surface structures including the polysaccharide capsule, several outer membrane proteins (OMPs) and lipooligosaccharide (LOS) serve as basis for serological classification [Stephens et al., 2007a]. Currently, twelve serogroups are recognized based on the antigenic properties of the capsule, namely serogroup A, B, C, E, H, I, K, L, W, X, Y and Z, with serogroups A, B, C, W, Y and X being frequently associated with invasive meningococcal disease [Tzeng and Stephens, 2000, Stephens et al., 2007a]. Likewise, variants of the OMP porin B (PorB) and porin A (PorA) are used to classify *N. meningitidis* into about 20 serotypes and 10 serosubtypes, respectively [Frasch et al., 1985, Ala'Aldeen and Turner, 2006]. Differences in the LOS structure also identifies

12 immunotypes (L1-L12), with L1-L8 largely correlated with serogroups B and C; the other three immunotypes being mostly seen in serogroup A strains [Zhu et al., 2002]. An example of serological typing can be expressed as B:4:P1.15:L10, which denotes serogroup B, serotype 4, serosubtype 15 and immunotype 10 [Ala'Aldeen and Turner, 2006]. The high frequency of phase and antigenic variation of surface antigens, however, reduces the reliability of the serological classification of meningococci.

To facilitate a better strains differentiation, multi-locus enzyme electrophoresis (MLEE) technique can be employed to investigate the electrophoretic mobilities of different isoforms of collection of metabolic enzymes. Each electrophoretic variant is allocated a number score and then the unique combination of scores is designated an electrophoretic type (ET) [Yakubu et al., 1999, Ala'Aldeen and Turner, 2006]. While some of these ETs are very discriminative, others are only marginally different [Yakubu et al., 1999].

More recently, multi-locus sequence typing (MLST) has been used for genomic typing and represents a very useful tool for epidemiological studies and population diversity studies [Jolley et al., 2007, Stephens, 2009]. In this technique, small DNA fragments (433-501bp) amplified from seven distinct

housekeeping genes (*abcZ*, *adk*, *aroE*, *fumC*, *gdh*, *pdhC*, *pgm*) are sequenced [Jolley et al., 2007, Stephens, 2009]. Each sequence is assigned an allele number, and the combination of alleles is used to define each isolate as sequence type (ST) [Ala'Aldeen and Turner, 2006]. Public databases for molecular typing and microbial genome diversity are now accessible via the Internet (<http://pubmlst.org/>) where MLST schemes and isolate data are hosted, not only for *Neisseria* but for a wide range of prokaryotic and eukaryotic genomes as well. In the last few years, whole-genome sequencing data is increasingly used for genomic comparison studies.

1.4. Meningococcal pathogenesis

N. meningitidis colonizes the human nasopharynx and spreads from one person to another by direct contact or via respiratory droplets [Caugant et al., 2007], (Figure 1.1). Colonizing the human upper respiratory tract mucosa is the first step in establishing a carrier state and meningococcal disease [Stephens, 2009]. The properties of individual strains of meningococci, plus host-associated factors mutually influence the outcome of infection [Tzeng and Stephens, 2000]. As a consequence, the incidence of meningococcal disease will increase when a more virulent meningococcal strain is encountered by a person with predisposing factors for disease.

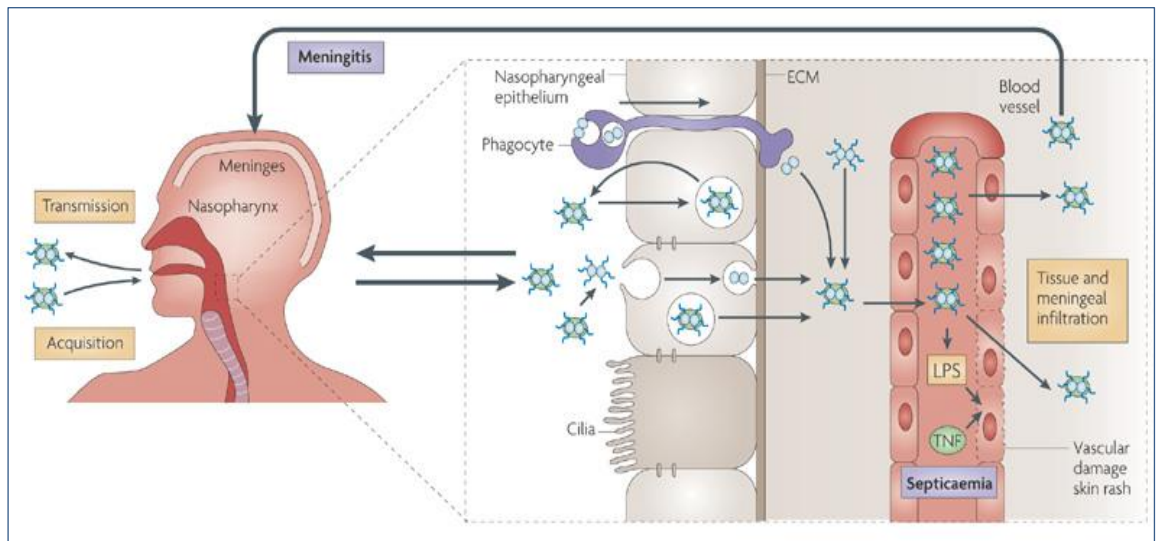


Figure 1.1: Steps of pathogenesis associated with *Neisseria meningitidis*. *N. meningitidis* may be acquired through the inhalation of respiratory droplets. The organism establishes intimate contact with non-ciliated mucosal epithelial cells of the upper respiratory tract, where it may enter the cells 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 in the bloodstream, *N. meningitidis* may survive, multiply rapidly and disseminate throughout the body and the brain. 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 cerebrospinal fluid. Adapted from: [Virji, 2009].

Adhesion to mucosal surfaces of the human host is essential for meningococcal survival. To achieve that, meningococci utilize a diverse array of structures and components called adhesins, which bind distinct ligands distributed on the surfaces of different human cells. Pili, Opa, Opc, LOS, factor H binding protein, Por A, HrpA, Por B, and NadA are some of the established adhesins [Merz and So, 2000]. Meningococci selectively bind non-ciliated columnar epithelial cells [Stephens et al., 1983]. While type IV Pili are the major adhesins that facilitate initial meningococcal adhesion to mucosal cells [Doulet et al., 2006], the capsule hampers adhesion, suggesting that it might aid in the transmission of meningococcal cells from one site to another via phase variation of capsule expression [Schembri et al., 2004]. Other surface structures including the pili are also subject to phase variation, as well as post translational modification, which will allow dissociation of those pili-phase variable meningococci from the bacterial aggregates, and hence dissemination to other sites [Chamot-Rooke et al., 2011]. Meningococci continue to proliferate on the non-ciliated epithelial cells, resulting in, first, the formation of small micro-colonies, and then cortical plaques formation on the apical surface. At this stage an intimate association is established, and it will generate specific signals and events, which eventually trigger the formation and extension of epithelial cell

pseudopodia that engulf the meningococcus [Doulet et al., 2006]. This intimate association is mediated by the meningococcal opacity proteins Opa and Opc, which interact with CD66/CEACAMs and integrin receptors carried on the surface of the epithelial cells respectively [Virji, 1996]. Additionally, meningococcal endotoxin and the NadA outer membrane protein also participate, albeit by unknown mechanisms, in bacterial-host cell close association [Gray-Owen and Blumberg, 2006].

Following the successful traversal of nasopharyngeal mucosa, *N. meningitidis* disseminates to sub-epithelial tissues from which it may gain access to the bloodstream. The fate of meningococci that reach the blood stream, and hence its ability to establish meningococcal disease, depends on the virulence factors that the bacteria possess and the host innate and acquired immune responses. Only a transient bacteraemia is expected when meningococci are faced with an effective immune response, however, if the latter is compromised then meningococcal septicaemia can be established [Hung and Christodoulides, 2013]. To maintain bacterial survival in the blood stream, meningococci require the expression of the capsule which provides protection against complement-mediated bacterial killing and phagocytosis [Klein et al., 1996]. Similarly, shedding of outer membrane blebs, which are rich in

bacterial endotoxin and outer membrane proteins, allows diversion of antibodies and complement components away from the surfaces of intact bacteria [Stephens et al., 1982]. Survival of intracellular *Neisseria* is influenced by expression of IgA1 protease that hampers phagosome maturation via degrading lysosome-associated membrane proteins (LAMP1) [Hopper et al., 2000]. Meningococci rapidly replicate in the blood leading to meningococcal vascular colonization in which meningococci adhere to endothelial cells, and it seems that type IV pili, Opa and Opc, similar to nasopharyngeal mucosal colonization, represent the major adhesins that mediate bacterial adhesion to endothelial cells [Virji, 2009].

1.5. Clinical picture of invasive meningococcal disease

Invasive meningococcal disease (IMD) usually starts with a range of non-specific signs and symptoms that are very similar to those resulted from many self-limiting viral illnesses *i.e.* fever, nausea and loss of appetite, making recognising IMD during its early stages very challenging [Theilen et al., 2008]. Soon after displaying these non-specific symptoms, around 60% of patients with IMD develop meningococcal meningitis [Stephens et al., 2007b], up to 20% progress to meningococcal septicaemia, or a combination of both clinical conditions can be experienced [Theilen et al., 2008].

1.5.1. Meningococcal Septicaemia

Meningococcal septicaemia is the most severe form of meningococcal infection. Clinically, it is classified into transient, chronic and fulminant forms. Transient meningococcaemia is characterized by fever and non-specific rash, which can both disappear within 2-5 days without treatment [Stephens, 2007]. The chronic form is rarely developed, and is clinically associated with intermittent fever, arthralgia, signs of vasculitis, and a non-specific maculopapular rash. These symptoms may disappear for days before being experienced again [Harwood et al., 2005, Stephens et al., 2007b]. Fulminant meningococcal septicaemia occurs when meningococci rapidly multiply in the circulation, and very high concentrations of bacteria (10^5 - 10^8 ml⁻¹) and its endotoxin (10^1 - 10^3 EU ml⁻¹) [Brandtzaeg et al., 1989, Stephens et al., 2007b] are reached. These will induce the host innate and acquired immune systems resulting in generalized pathology of the microvascular endothelium, which in turn leads to progressive circulatory collapse, severe coagulopathy and severe depression of myocardial function, all of which contribute towards multi-organ failure and may lead to death [Pathan et al., 2003]. Petechial or purpuric rash is usually seen in 40-80% of patients with meningococcal septicaemia [Thompson et al., 2006]. Patients with fulminant meningococcal septicaemia may present with severe, persistent shock, lasting

more than 24 h or until death. Defective renal, adrenal, and/or pulmonary function and thrombotic lesions in the skin, limbs, kidneys, adrenals, choroids plexus, and occasionally the lungs, can also be experienced [Stephens et al., 2007b, Hazelzet et al., 1996].

1.5.2. Meningococcal meningitis

Meningitis, as the name suggests, is an inflammation of the meninges, the membranes covering the brain and spinal cord. The inflammation occurs due to the bacterial loads and high levels of endotoxin [Brandtzaeg et al., 1992, Øvstebø et al., 2004], which in turn results in high levels of cytokines including tumour necrosis factor α (TNF- α) and the interleukins IL-1 β , IL-6, IL-8, and IL-10 [Stephens et al., 2007b], which direct the inflammatory response. The inflammatory process extends throughout the subarachnoid space around the brain and spinal cord, and the ventricles [Mace, 2008]. Developing meningitis without septic shock is the most common clinical presentation associated with IMD; more than 60% of patients in industrialised countries develop this condition, while in developing countries this percentage can be even higher [Stephens, 2007].

Symptoms of meningitis can vary based on age. In adults, meningitis is clinically characterized by a sudden fever

accompanied by headache and neck stiffness. Other symptoms that a patient can experience include nausea, vomiting, photophobia and altered mental status [Loring, 2004]. Additionally, inflammation and bleeding in small blood vessels including those under the skin can result in a skin rash [Nielsen et al., 2001]. On the other hand, non-specific symptoms including poor feeding, irritability, a high pitched cry and a bulging fontanelle are typical among infants and young children suffering from meningitis [Nadel and Kroll, 2007].

Early diagnosis and management, including rapid administration of antibiotics, are important for a full recovery. Otherwise, the mortality rate associated with meningococcal disease can be as high as 70–90%. Unfortunately, even with early management, around 25% of meningococcal disease survivors develop permanent sequelae. These include loss of hearing, mental retardation, seizures, hydrocephalus, renal failure, skin scarring or limb(s) amputation [Rosenstein et al., 2001]. Additionally, survivors without gross neurological damage can demonstrate more subtle neurodevelopmental sequelae such as defects in intellectual and behavioural abilities, which subsequently can influence their academic performance [Koomen et al., 2004].

1.6. Virulence factors of *N. meningitidis*

Meningococci have developed several strategies to maintain their survival inside the human host, whether during nasopharyngeal carriage stage or during the invasion and dissemination course. These strategies primarily involve expressing certain structures and components on the bacterial surface, secreting and/or releasing a range of molecules and outer membrane blebs that can modulate the host response, which are collectively referred to as virulence factors [Wall, 2001]. Many meningococcal virulence factors undergo phase and antigenic variation, by which the expression of the encoding gene(s) is/are switched on or off to increase the fitness of bacterium in its environment [Van Der Woude and Bäumler, 2004]. This can be accomplished by a variety of mechanisms including intragenic recombination [Hagblom et al., 1985], slipped strand mispairing [Hammerschmidt et al., 1996b] and reversible insertion of mobile elements [Hammerschmidt et al., 1996a, Van Der Woude and Bäumler, 2004]. Meningococcal surface structures including the polysaccharide capsule, lipooligosaccharide, several outer membrane proteins (OMPs) and pili (Figure 1.2) represent major virulence factors that play a crucial role in the bacterial pathogenesis [Hill et al., 2010]. In addition to these, complete genome projects of different pathogenic *Neisseria* strains enabled the identification of other

important surface-exposed and secreted virulence-associated proteins [Bennett et al., 2012]. Some of these virulence factors are briefly described below.

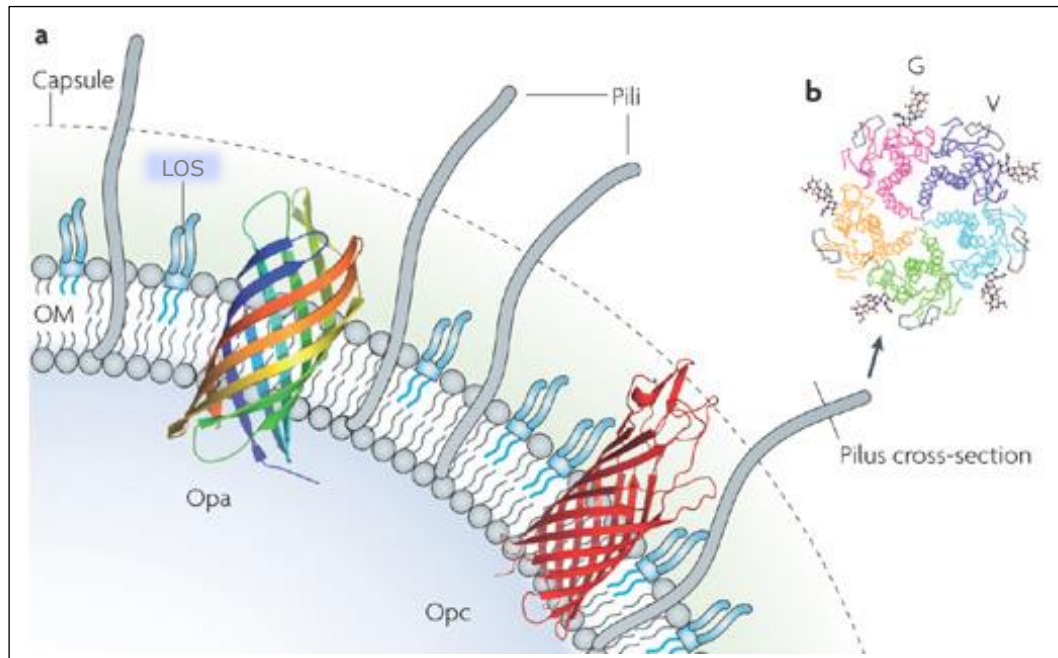


Figure 1.2: Major virulence factors of *Neisseria meningitidis*. (a) Pili traverse the capsule and are the most prominent adhesins of encapsulated *N. meningitidis*. In addition, the integral outer membrane (OM) adhesins, Opa and Opc, are known to mediate interactions with specific host-cell receptors in appropriate phenotypes. Lipooligosaccharide (LOS) 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 and are protected from the host environment. Adapted from: [Virji, 2009].

1.6.1. The capsule

The capsule is probably the main virulence factor in *N. meningitidis*, as it primarily extends meningococcal survival in

aerosol droplets, hence, facilitates transmission from one person to another [Swain and Martin, 2007]. While inside the host, it provides protection against several host-derived processes which are designed to eliminate foreign antigens, including phagocytic killing, opsonisation and complement-mediated bacterial lysis [Schneider et al., 2007].

Meningococci are primarily phenotypically characterized based on the presence of the capsule; while many colonizing meningococci are non-capsulated [Claus et al., 2002] those associated with invasive disease are almost always encapsulated [Frosch and Maiden, 2006]. Meningococci are classified into 12 serogroups, each with a structurally distinct capsule. Serogroups A, B, C, W, Y, and X are associated with the vast majority of invasive meningococcal disease [Stephens, 2007]. Unlike serogroup A, the capsules of virulence-associated serogroups B, C, Y, and W are composed of sialic acid derivatives. Sialic acid is a common component of human cells. While the sialic acid-derived capsule from serogroup C meningococci is sufficiently different from host structures for an efficient immune response to be raised, serogroup B meningococcal capsule, contains an alpha (2-8)-linked polysialic acid, which is structurally identical to glycosyl residues of human neural cell adhesion molecule, hence, meningococci can utilize this structure similarity to evade recognition by the

host immune system [Finne et al., 1983]. A gene complex spanning about 24 kb of the bacterial chromosome comprises the capsule biosynthesis locus (*cps*), which in turn contains distinct functional regions. Of these, *sia* operon and *ctr* operon were identified, and products essential for the biogenesis of the sialic acid-containing capsule and polysaccharide transport are encoded by these operons, respectively [Swartley et al., 1996].

1.6.2. Lipooligosaccharide

Meningococcal endotoxin, like some other respiratory Gram negative bacteria such as *Haemophilus influenzae*, lacks the repeating polysaccharide O-antigen portion, which characterize the lipopolysaccharide (LPS) of other enteric Gram negative bacilli. For this reason these shorter molecules are called lipooligosaccharide (LOS) [Kahler and Stephens, 1998]. Meningococcal LOS consists of an oligosaccharide (OS) portion attached to a phosphorylated diheptose (Hep) inner core linked to a lipid A moiety embedded in the outer membrane [Stephens, 2009]. The toxicity and the biological activities of meningococcal LOS are mostly due to the lipid A portion [Zughaier et al., 2005]. While all commensal *Neisseria* spp. Lack *lgt* gene, pathogenic *Neisseria* i.e. meningococci and gonococci each harbour several copies of this gene; all encode LOS but with a high genetic diversity [Arking et al., 2001, Zhu et al., 2002]. This diversity probably occurs due to phase variation

events [Berrington et al., 2002], which will eventually result in a variety of meningococcal immunotypes [Tzeng and Stephens, 2000]. Meningococcal LOS undergoes phase variation, hence, switching between immunotypes will occur, which is proposed to play a role in meningococcal disease [Berrington et al., 2002].

1.6.3. Type IV pili

Pili are hair-like structures that are formed by the helical assembly of pilin subunits, in the case of the meningococcal Type IV pilus, the product of *pilE* gene. Other minor subunits (PilC, PilV and PilX) can also be incorporated into the pilus and modulate its function [Craig and Li, 2008]. Meningococcal pili are mainly classified into two classes, designated class I and class II pili. While both classes belong to methyl-phenylalanine pili family, which is termed type IV pili, several serological and structural properties uniquely characterize each class [Virji et al., 1989, Parge et al., 1995, Tzeng and Stephens, 2000]. Pilus biogenesis can be categorized into four steps: assembly, functional maturation, counter-retraction, and emergence on the cell surface. Twenty three proteins were found associated to pili; 15 of them are essential for pili biogenesis [Carbonnelle et al., 2005, Carbonnelle et al., 2006].

In addition to its contribution to mediating meningococci-host cell adhesion [Hung and Christodoulides, 2013], meningococcal micro-colony formation and auto-aggregation on mucosal surfaces [Pelicic, 2008], pili also participate in other functions. First, meningococcal pili can generate a twitching motility, which will in turn increase the ability of bacteria to take up foreign DNA during transformation, a process that influences meningococcal virulence by promoting genetic adaptation [Mattick, 2002]. Similarly, pili-mediated motility can also produce a level of bacterial movement on cell surfaces, hence, the distance between the bacterial and eukaryotic membranes may be reduced, facilitating intimate cellular interactions through outer membrane adhesins [Winther-Larsen et al., 2005]. Type IV pili, like LOS, are subjected to phase and antigenic variation [Nassif, 1999]. While the role of pili as adhesins that facilitate meningococcal adherence to the host cell is well known, the nature of the host cell receptor is still unclear [Hung and Christodoulides, 2013]. One study suggested CD46, a membrane-located human complement regulatory protein, as a potential ligand to meningococcal pili [Johansson et al., 2003]. However, in another study it was shown that adherence of gonococci to urogenital mucosa can occur in a CD46-independent manner [Kirchner et al., 2005]. Recently, it has been demonstrated that CD147, an immunoglobulin

superfamily member, represents a critical host receptor for the meningococcal pilus components PilE and PilV, hence, mediating the bacterial vascular colonization [Bernard et al., 2014].

1.6.4. Outer membrane proteins (OMPs)

Molecular weight on sodium dodecyl sulphate polyacrylamide gel followed by peptide analysis techniques were used to classify the outer membrane proteins (OMPs) into five distinct classes [Tsai et al., 1981]. While most *N. meningitidis* strains express PorA, a class 1 OMP, all meningococci express either class 2 or 3 OMP, designated PorB [Hitchcock, 1989].

Meningococcal PorB is a pore-forming protein which is as the name suggests, form pores in the bacterial outer membrane. It has also been implicated in preventing apoptosis, and induces the expression of the co-stimulatory molecule CD86 and of MHC class II, which are related to the immune-potentiating ability of the porin [Massari et al., 2005].

Similarly, *N. meningitidis* express class 5 OMPs, which are represented by the opacity proteins Opa and Opc. The term opacity was coined because expression of these proteins led to opaque colonies. These proteins are highly immunogenic with a similar molecular weight of ca. 30-kDa [Achtman et al., 1988] although have dissimilar sequences [Olyhoek et al., 1991]. The meningococcal genome has up to five alleles; each encodes

distinct versions of the Opa proteins. Importantly, the same meningococcal cell can simultaneously express two Opa protein versions on its outer membrane [Estabrook et al., 2004]. The opaque appearance of meningococcal colonies is due to Opa proteins, which play, alongside type IV pili [Hung and Christodoulides, 2013], an essential role to mediate the adhesion and invasion of meningococci to the host epithelial, endothelial and phagocytic cells [De Jonge et al., 2003]. Opc also mediates meningococcal-eukaryotic cell interactions and adherence, and it was designated as a target of bactericidal antibodies [Tzeng and Stephens, 2000].

1.6.5. *Neisseria* hia homologue A (NhhA)

NhhA is an outer membrane protein that belongs to the family of trimeric autotransporter adhesins [Scarselli et al., 2006]. NhhA was first identified as a homologue of the adhesin AIDA-I of *E. coli* in *N. meningitidis* [Peak et al., 2000], and named due to sequencing similarity to Hsf and Hia adhesins of *Haemophilus influenzae* [Barenkamp and Geme, 1996] (74% and 67%, respectively). The vast majority of meningococcal strains, especially those associated with disease episode, are expressing NhhA on their surfaces [Peak et al., 2000].

In a mouse model, NhhA-deficient meningococci are unable to colonize the nasal mucosa. NhhA adheres to human epithelial cells and components of the extra cellular matrix such as

laminin and heparin sulphate [Scarselli et al., 2006]. In CD46 transgenic murine model, NhhA facilitated meningococcal colonization of the nasopharyngeal mucosa, via avoiding phagocytosis, which represents an important host innate immune defence mechanisms against infections. Not only that, NhhA was also required for meningococcal resistance to complement attacks. [Sjölander et al., 2008]. Recently, NhhA was described as the first meningococcal protein that promotes macrophages apoptosis [Sjölander et al., 2012].

1.6.6. Adhesion and penetration protein (App)

Adhesion and penetration protein (App) is a highly conserved, surface-localised and immunogenic protein, belongs to the autotransporter proteins family [Hadi et al., 2001]. Haemophilus adherence and penetration protein (Hap) from *Haemophilus influenzae* is a close homologous to App, which also exhibits a sequence similarity to IgA protease [Turner et al., 2006]. App is a strong stimulator for B cells and T cells [Hadi et al., 2001], and it can induce bactericidal antibodies [Hadi et al., 2001, van Ulsen et al., 2001]. App attached to the bacterial outer membrane mediates the adhesion to human epithelial cells [Serruto et al., 2003], which means it might enhance bacterial detachment and subsequent spreading after auto-cleavage [Serruto et al., 2003].

1.6.7. HrpA-HrpB system

Haemagglutinin/haemolysin-related proteins (HrpA and HrpB, respectively) are present in all meningococcal strains as a two-partner secretion system (TPS) where HrpA is the secreted effector protein and HrpB is the cognate transporter. While most of HrpA is secreted into the environment, a small percentage of the protein remains attached to the bacterial surface and contributes to the interaction of un-encapsulated *N. meningitidis* with some epithelial cell lines [Schmitt et al., 2007]. It has been demonstrated that HrpA-HrpB-null meningococci were less able to survive in Hela cells, and similarly less able to escape [Talà et al., 2008]

1.6.8. Neisseria Adhesin A (NadA)

NadA is a surface exposed trimeric autotransporter [Linke et al., 2006] that was identified as a homologue of UspA2 from *Moraxella catarrhalis* and of YadA from *Yersinia enterocolitica* [Van Ulsen and Tommassen, 2006]. NadA is more frequently associated with strains which belong to hypervirulent clusters, yet it appears that the gene encoding NadA is underrepresented in carrier strains [Comanducci et al., 2004]. NadA is one of four antigenic proteins included in the formulation of four components MenB (4CMenB) vaccine targeting *N. meningitidis* serogroup B [Su and Snape, 2011].

1.6.9. Iron-acquiring proteins

Neisseria require iron for survival and virulence [Grifantini et al., 2003]. In this microorganism, iron acquisition is accomplished via expressing surface receptors that are designated to extract and import iron from different sources. Haemoglobin-binding receptors (HmbR and HpuB/HpuA), transferrin-binding receptors (TbpA/TbpB), and lactoferrin-binding receptors (LbpA/LbpB), which are respectively assigned to extract iron from human haemoglobin, transferrin and lactoferrin, are of these [Noinaj et al., 2012].

1.7. Moonlighting proteins: the concept

Having more than one role or function is the idiomatic meaning of 'moonlighting', a new concept which was suggested in 1995 to describe the additional task that a certain protein (in the original case a neuropeptide) performs alongside its recognised function [Campbell and Scanes, 1995]. Introducing this concept refuted what was previously believed: that a protein translated from one gene achieves one, specific task. After an increasing number of proteins were reported to have more than one biological function, the generic term of 'moonlighting proteins' was introduced [Jeffery, 1999, Jeffery, 2009]. These proteins have been identified in both prokaryotic and eukaryotic cells representing a highly conserved subset of proteins that typically

are either metabolic pathway-associated enzymes or act as molecular chaperones [Henderson and Martin, 2011c].

1.8. Classification of moonlighting proteins

Moonlighting, or multi-tasking proteins, have been classified according to their known or presumed cytosolic functions. In this context, moonlighting proteins are typically metabolic pathway-associated enzymes, molecular chaperones, and transcription or translation factors [Henderson and Martin, 2011a, Henderson and Martin, 2014]. Figure 1.3 illustrates how a protein can moonlight [Jeffery, 2015].

Numerous enzymes that mediate glycolysis have been described in both prokaryotic and eukaryotic organisms, and found to perform non-glycolytic functions especially when a particular enzyme is expressed on the bacterial cell surface or released into the surrounding milieu. In addition, several enzymes that are involved in other non-glycolytic metabolic pathways also have moonlighting functions.

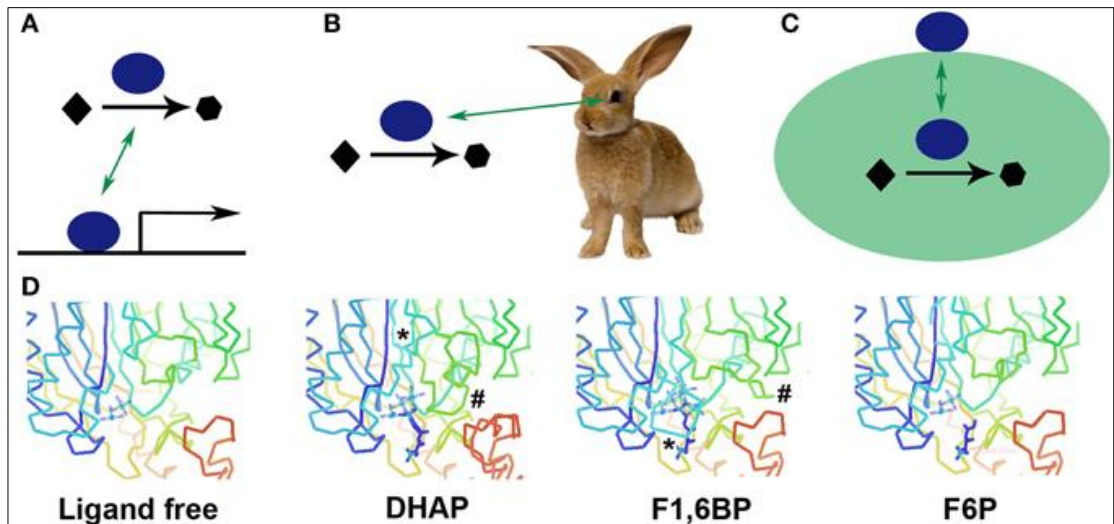


Figure 1.3: Cartoons illustrating how a protein can moonlight. A protein can catalyse an enzymatic reaction and also (A) bind to DNA and serve as a transcription factor, (B) have a non-catalytic role in the lens of the eye (crystallins), or (C) be placed on the surface of a cell to function as a receptor. Another type of moonlighting is illustrated in (D) where an active site undergoes significant remodelling, or movement of loops (marked by * and #) to bring in different catalytic amino acids to perform two different catalytic functions (D). Adapted from: [Jeffery, 2015].

Molecular chaperones and protein folding catalysts - a set of unrelated proteins that are required for the proper folding/unfolding of different macromolecules, and transport of these molecules across the cell membrane [Ellis, 2005], are another group of proteins that often multi-task. As an example, the GroEL2 protein from *Mycobacterium tuberculosis* was detected on the surface of *M. tuberculosis*. It was shown that inactivating the expression of Rv2224c, a mycobacterial envelope-associated protease that in turn mediates the release of GroEL2 out of the cell, significantly reduced the bacterial

survival rate in macrophage culture, as well as reduced the mycobacterial associated pathology within infected mice [Rengarajan et al., 2008]. Furthermore, the mycobacterial surface localized Cpn60.2 was shown to be required to mediate competent bacterial adherence to host macrophages [Hickey et al., 2009]. Many proteins that belong to either of these groups were shown to mediate bacterial pathogenesis either via adhesion, interaction with host ligands, and/or modulating specific host elements.

Many enzymes were found moonlighting as regulators of transcription or translation. For example, aconitase (Acn) from *M. tuberculosis*, which is primarily a tricarboxylic acid (TCA) cycle enzyme [Beinert and Kennedy, 1993], was shown to display RNA binding properties via interaction with sequences similar to iron responsive element (IRE) seen in eukaryotic and few prokaryotic cells [Banerjee et al., 2007]. IREs are defined as untranslated regions (UTRs) of mRNAs that can be recognised by iron responsive proteins (IRPs), which in turn act as an iron sensor; interaction of IRP with its IRE responsible for posttranscriptional regulation of the expression of proteins involved in iron homeostasis. Importantly the enzymatic activity of Acn was inactivated; the protein RNA-binding property remained preserved, suggesting that the enzymatic function

and the RNA-binding property of Acn are independent. [Banerjee et al., 2007].

1.9. Moonlighting proteins and secretion dilemma

Bacterial moonlighting proteins were initially described as being “anchorless” due to absence of a typical signal sequence or any known sequence motifs that is typically required to identify a given protein to be exported [Pancholi and Chhatwal, 2003]. In spite of the increasing number of studies that report the identification and/or participation of a particular protein in a moonlighting behaviour, there is no clear evidence for the mechanism of transport from the cytosolic compartment onto the surface of a cell. The simplest explanation for surface localization of an originally cytosolic protein is passive release due to cell lysis. The protein then can be recruited to the surface of intact bacterial cells in the population. For example, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) from Group B streptococcus (GBS) was shown to be expressed on the bacterial cell surface, or released into the buffer, following GBS lysis [Oliveira et al., 2012]. Cell stress conditions were also documented to increase the release of some moonlighting proteins. GAPDH from *Streptococcus pyogenes* due to iron starvation [Eichenbaum et al., 1996], and DnaK protein from *Bifidobacterium animalis* subsp. *lactis* in the presence of bile

salts [Candela et al., 2010], are examples of proteins being released from bacterial cells in response to stress. In *Lactobacillus plantarum* 299v, a stationary phase-associated increase in cell membrane permeability was reported to influence the concentration of cell wall GAPDH. Bacterial cells with impaired membrane permeability displayed five-fold more GAPDH on the bacterial surface [Saad et al., 2009]. However, provoked cell lysis did not lead to re-association of GAPDH onto the cell surface, suggesting that variations in plasma membrane permeability may only contribute to the movement of GAPDH through the plasma membrane. Interestingly, the surface-bound glutamine synthetase (GS), glucose-6-phosphate isomerase (GPI), enolase, and GAPDH from *L. crispatus* ST1 were shown to be released from the surface into the buffer under either alkaline stress or after treating the bacterial cells with LL-37 (a polypeptide with antimicrobial property, normally secreted by human epithelial cells) [Kainulainen et al., 2012]. This proposed mechanism does not, however, explain how a moonlighting protein can cross the outer membrane in Gram-negative bacteria. Another hypothesis of translocation is that moonlighting proteins can be secreted onto the cell surface by an undefined mechanism(s) [Henderson and Martin, 2011b]. For instance, secretion of enolase from *Listeria monocytogenes* was shown to be under the control of an accessory (*secA2*) gene

control [Lenz et al., 2003]. Several signal-free cytoplasmic proteins including DnaK, enolase, and pyruvate dehydrogenase subunits from *Bacillus subtilis* were detected extracellularly while constant cell density and viability counts were recorded. Moreover, the deletion of a central hydrophobic α -helical domain (residues 110 to 118) eliminated enolase surface translocation, suggesting a possible participation of this domain in the non-classical secretion mechanism being proposed [Yang et al., 2011]. Contrary to the idea that all moonlighting proteins lack identifiable signal sequences, a recent study suggested a 13-amino acid sequence of fructose-1, 6-bisphosphate aldolase (FBA) from *N. meningitidis* as a signal peptide with predicted cleavage site 11DHA-AE15 [Tunio et al., 2010b], suggesting another possibility for partial translocation across the cytoplasmic membrane. Furthermore, Aguilera and colleagues demonstrated type III secretion system (T3SS) as the mechanism by which enteropathogenic *E. coli* (EPEC), grown in Dulbecco's modified eagle's medium (DMEM), translocate the housekeeping protein GAPDH. They found that secretion of GAPDH was abolished in type III ATPase *escN*-mutant EPEC, and restored by *escN* gene complementation. Importantly, the GAPDH secretion was also detected in the mutant EPEC when the latter were propagated in LB media, suggesting another, yet

unidentified, secretion pathway that mediates GAPDH secretion in addition to T3SS [Aguilera et al., 2012]

Taken together, our knowledge of the mechanism(s) that permits secretion of moonlighting proteins, and their surface anchoring, remains enigmatic. A variety of mechanisms including novel non-classical secretion pathways may be utilized by bacteria to achieve surface localization of these proteins, depending on bacterial species and/or a moonlighting protein being exported.

1.10. Meningococcal moonlighting proteins

The first evidence of moonlighting proteins in *N. meningitidis* was reported in 2002 when Grifantini and colleagues used whole genome microarray analysis to monitor the changes in gene expression of human pathogenic *N. meningitidis* during initial contact with human epithelial cells. The results indicated the presence of GAPDH on the bacterial cell surface, following the up-regulation of the *gapA-1* gene encoding the GAPDH protein after cell contact [Grifantini et al., 2002]. However, the function of GAPDH surface localization was unknown. Later, Tunio and co-workers suggested that the presence of the capsule probably covers the GAPDH expressed on the cell surface of diverse isolates of *Neisseria spp.* While the absence of *gapA-1* gene that encodes GAPDH did not affect the growth rate or colony

morphology of *gap-A1* deficient mutants compared with the wild type and complemented mutants, it significantly reduced the capacity of bacteria to adhere to human epithelial and endothelial cells [Tunio et al., 2010a]. What is the relevance of this sub-capsule presence of GAPDH to adherence? A suggested explanation was that *gap-A1* may be exposed and then participates in adherence following the temporary down-regulation of *siaD* gene (required for capsule expression in meningococci), that occurs after the initial type IV pilus dependent-contact with host cells [Grifantini et al., 2002]. Thus, GAPDH may be involved in the development of 'intimate adherence' that precedes invasion. Similarly, adherence of meningococci to human cells was also found to be affected by FBA [Tunio et al., 2010b], a glycolytic enzyme reported to multi-task in different organisms [Pancholi and Chhatwal, 2003]. Unlike GAPDH, recombinant FBA was successfully purified in its enzymatically active form, and was reported to be localized on the outer membrane of the organism. In other words, while GAPDH might play an indirect capsule-dependent role in bacterial adherence, FBA seems to be required for optimal adhesion of meningococci to human cells. The precise mechanism(s) involved remains unidentified [Tunio et al., 2010b]. Recently, This was further confirmed by Shams and colleagues, who also identified FBA as a plasminogen binding

receptor on the surface of meningococci, and found that a non-functional copy of the protein *i.e.* lacking aldolase activity can recruit plasminogen as well as the wild type [Shams et al., 2016].

1.10.1. Enolase

Enolase (2-phospho-d-glycerate hydrolase), is a glycolytic metalloenzyme that naturally catalyzes the dehydration of 2-phosphoglycerate (2PG) to phosphoenolpyruvate [Pancholi, 2001]. Enolase binds divalent metal ions, with a preference for Mg^{2+} , in two distinct metal-binding sites [Faller et al., 1977]. The enolase- Mg^{2+} interaction at one site results in conformational changes that in turn will facilitate the binding of the substrate 2PG and the second catalytic Mg^{2+} on the other site, hence, the catalytic activity of enolase will take place [Zhang et al., 1997]. Along with its established cytoplasmic location, enolase has also been recognized on the surface of phylogenetically different species, exhibiting various non-glycolytic functions. Binding human extracellular matrix proteins (ECMs) in prokaryotes [Carneiro et al., 2004, Castaldo et al., 2009], contributing to myogenesis, cancer, and intracellular signalling in humans [Díaz-Ramos et al., 2012], are of the diverse functions that enolase can participate in.

Enolase from different streptococcal species was extensively investigated, and a variety of enolase-mediated moonlighting

activities has been recognized. One example is pneumococcal enolase, which was found to bind the major complement inhibitor C4b-binding protein (C4BP) [Agarwal et al., 2012]. As the name suggested, C4BP hinders both classical and lectin pathways of complement, via inhibiting the formation and/or degrading several components associated with these pathways [Fujita et al., 1978, Gigli et al., 1979]. Accordingly, it was suggested that a pneumococcal cell can utilize the C4BP bound to its surface to evade the complement-mediated host immune response [Agarwal et al., 2012]. Also, it has been established that, due to the involvement of different motifs on enolase, the ability of the latter to bind human C4BP has not been compromised by its ability to bind and activate plasmin(ogen), which was previously found to interact with pneumococcal enolase [Bergmann et al., 2001] and vice versa, suggesting a potential concurrent dual enolase-mediated action that pneumococci can use to facilitate host tissues invasion [Agarwal et al., 2012]. Interestingly, the capability of PorA from group B *N. meningitidis* to bind human C4BP was previously documented and explained as an approach that meningococci can use to avoid the constant complement components attacks [Jarva et al., 2005]. Does meningococcal enolase mimic its pneumococcal homologue in terms of binding to C4BP? This is a question that needs further exploration.

The presence of enolase was also identified on the surface of *Borrelia burgdorferi* [Nogueira et al., 2012, Floden et al., 2011], a tick-borne spirochetes that causes Lyme disease, which characterized by a disseminated course of infection that involves several organs of the mammalian host [Steere et al., 2004]. It has been shown that the binding of intact Borrelial cells to human plasminogen was significantly reduced when anti-enolase antibodies were added, suggesting enolase as a plasminogen ligand [Nogueira et al., 2012]. Similarly, the addition of EACA as a lysine analogue has significantly reduced this binding signifying that it can be lysine residues-mediated. While active immunization of mice with recombinant enolase from *B. burgdorferi* was unsuccessful in terms of elucidating protection against subsequent Borrelial infection [Nogueira et al., 2012], a total protection of mice against *Streptococcus suis* infection was achieved when *S. suis* enolase was used as an immunogenic antigen [Feng et al., 2009]. In *Streptococcus mutans*, which inhabit the oral cavity and cause dental caries and occasionally endocarditis [Nakano et al., 2006], both the surface localization of enolase and its ability to bind plasminogen were documented [Ge et al., 2004]. Besides, the capability of *S. mutans* enolase to interact with human saliva components was also examined, and resulted in identifying mucin MG2 (currently known as MUC7) protein as a ligand for

enolase [Ge et al., 2004]. Later, a similar result was obtained from *Streptococcus gordonii*, another oral microflora that also associates with dental plaque and endocarditis in susceptible individuals, in which MUC7 ability to bind enolase, in addition to three other bacterial proteins, was documented yet not explained [Kesimer et al., 2009]. As *N. meningitidis* colonises the nasopharyngeal epithelial cells, which are covered by various host cell secreted proteins, including mucin(s), it is worth trying to examine meningococcal enolase-mucin interaction, as if such interaction can be established, then more insight into the *in vivo* significance of this interaction especially during the early stages of meningococcal pathogenesis *i.e.* colonization and adhesion will be gained.

The ability of enolase to bind plasminogen has also characterized in *Listeria monocytogenes* [Schaumburg et al., 2004], *Lactobacillus crispatus* [Hurmala et al., 2007], *Bacillus anthracis* [Agarwal et al., 2008], *Streptococcus pyogenes* [Kornblatt et al., 2011], *Lactobacillus plantarum* [Glenting et al., 2013], *Vibrio parahaemolyticus* [Jiang et al., 2014], *Pseudomonas aeruginosa* [Ceremuga et al., 2014], and many others. It seems that the list of proteins (especially enolase) that are able to bind plasminogen is expanding, but it is unclear why this binding is so vital for different bacterial (especially pathogenic) species? Plasminogen is the proenzyme

of the fibrinolysis system. Lysine binding sites on the Kringle domain of plasminogen mediate its interaction with surfaces. Upon binding, and in the presence of serine proteases such as tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA), plasminogen becomes activated. The enzymatically active surface-bound plasmin exhibits a wide-ranging proteolytic activity. Plasmin itself can directly degrade vitronectin, laminin and fibronectin (Fn) components of the ECM and basal membrane [Plow et al., 1995]. In addition, plasmin can activate other enzymes, *i.e.* matrix metalloproteases and elastase, which in turn degrade collagen, elastin, and proteoglycan [Murphy et al., 1999]. The outcome is a series of sequential proteolytic events, which in addition to the binding itself contributes to the pathogenesis of several bacterial species, and are utilized in a variety of pathogenic processes (illustrated in Figure 1.4) [Sanderson-Smith et al., 2012].

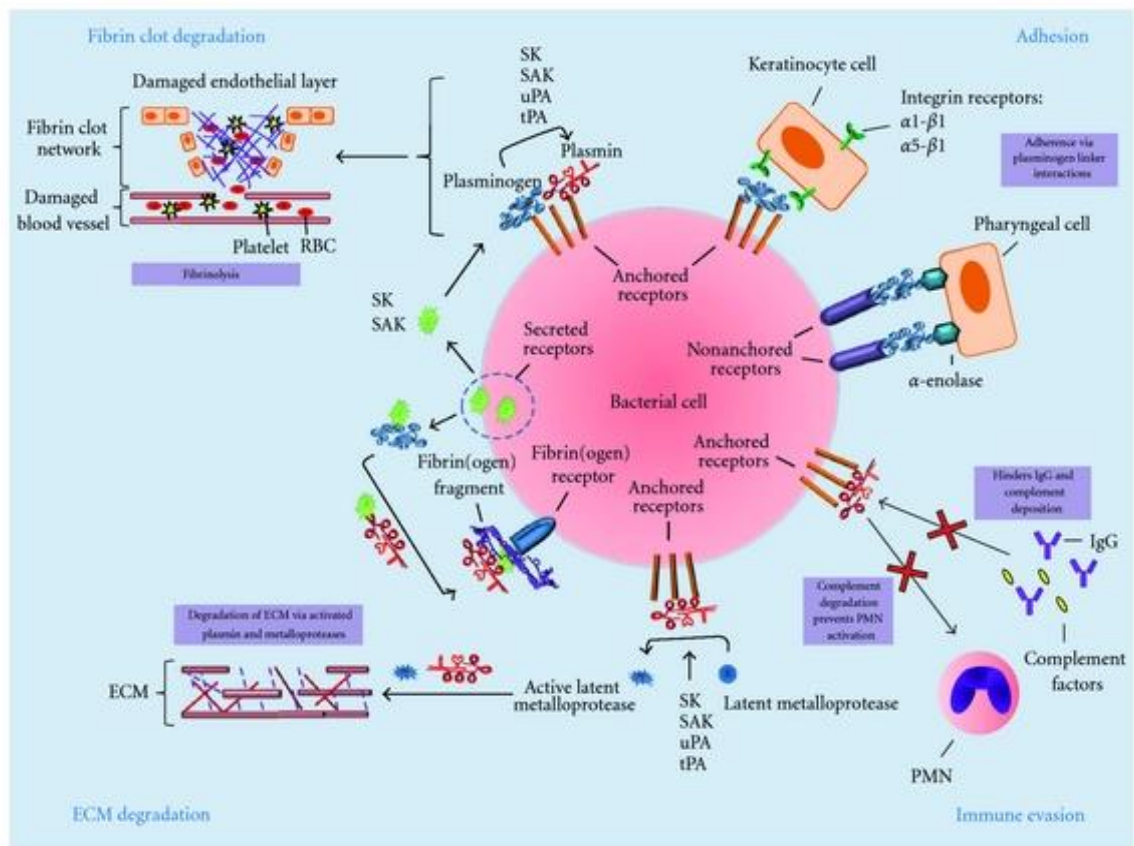


Figure 1.4: Plasminogen-bacterial cell interactions and its impact on pathogenesis. Plasmin(ogen) binding to the bacterial cell surface is accomplished through either anchored receptors, cell-surface-associated non-anchored receptors or secreted receptors that bind fibrin(ogen), which in turn interacts with bacterial cell-surface fibrin(ogen) receptors. Bound plasmin(ogen) leads to ECM degradation, fibrinolysis, immune evasion and adherence to host cells. ECM: extracellular matrix; IgG: immunoglobulin G; PMN: polymorphonuclear cells; RBC: red blood cell; SAK: staphylokinase; SEN: streptococcal α -enolase; Sk: streptokinase; tPA: tissue plasminogen activator; uPA; urokinase plasminogen activator. Adapted from: [Sanderson-Smith et al., 2012].

An early study established the binding of human plasminogen to the surface of 24 different *N. meningitidis* strains. However, in spite of determining ϵ -aminocaproic acid (EACA) as a binding inhibitor, suggesting the contribution of lysine binding sites of plasminogen in the binding, the plasminogen receptor(s) were unknown [Ullberg et al., 1992]. Later Knaust and co-workers identified enolase, peroxiredoxin and DnaK, which are primarily cytosolic proteins, as plasminogen binding ligands on the surface of the organism [Knaust et al., 2007]. Meningococcal cells with bound plasminogen, acquire the ability to directly (via plasmin) and indirectly (via plasmin-mediated enzymatic activation) degrade a wide range of proteins that represent parts of the host ECM and basal membrane elements [Sanderson-Smith et al., 2012]. This degradation may cause disruption of the ECM that covers the nasopharynx and other surfaces, and the basal lamina, which supports other tissues. The barriers that are designed (in terms of innate host defence mechanisms) to prevent colonization and further dissemination of invading pathogens, may thus be compromised. A similar proposed mechanism was also suggested in *Candida albicans* [Crowe et al., 2003]. Furthermore, it is known that bound plasmin also participates in the proteolytic cleavage of antibodies and complement components [Sanderson-Smith et al., 2012]. The resulting inactivation of these immune defence

tools, with their important roles in protection against meningococcal disease [Borrow et al., 2005], may represent another approach that *N. meningitidis* utilizes to survive in the host. It is noteworthy that, despite the presence of glycolytic pathway enzymes, this pathway is non-functional in *N. meningitidis* [Baart et al., 2007]. Nevertheless, the genes encoding the moonlighting glycolytic enzymes, GAPDH [Tunio et al., 2010a], enolase [Knaust et al., 2007], FBA [Tunio et al., 2010b] are highly conserved, suggesting alternative functions. While GAPDH and FBA were reasonably well investigated, more work is required to understand the potential non-glycolytic function(s) of enolase in *N. meningitidis*.

1.10.2. Peroxiredoxin

Peroxiredoxins (Prxs) is a large family of proteins (previously known as thiol-specific antioxidants [Chae et al., 1994]) that all share a highly conserved cysteine residue with an active thiol group, which in turn drives a peroxidase activity, a feature that characterize Prxs. Hence, they assist in avoiding the damaging effect of many free radicals such as reactive oxygen and nitrogen which are generated during different biological processes [Rhee and Woo, 2011]. Members belonging to this family were identified in human, animals, plants, protozoa and bacteria [Hofmann et al., 2002], in which several isoforms of the same protein were sometimes recognized, for example

three isoforms of peroxiredoxin were identified in *E. coli*, while six were recognized in some mammals [Zhou et al., 2000]. Prxs are primarily cytosolic proteins, however, several cellular organelles were also found to contain Prxs including mitochondria, chloroplasts, peroxisomes and the nucleus [Hofmann et al., 2002].

Prxs can be divided into six subfamilies (AhpC-Prx1, BCP-PrxQ, Tpx, Prx5, Prx6 and AhpE) with distinct amino acid sequences, but all contain an absolutely conserved active site cysteine in the motif P-XXX-T(S)-XX-C, which reacts with H₂O₂ to form a cysteine sulfenic acid [Nelson et al., 2011]. Prxs are described as either 1-Cys Prx or 2-Cys Prx, depending on whether a second cysteine residue participates in the enzymatic reaction [Nelson et al., 2011]. The 2-Cys Prxs are further subdivided into typical and atypical forms, depending on the location of second cysteine residue; in typical forms the second cysteine is found in the C-terminus of the partner subunit, whereas in atypical enzymes it is located in other positions [Nelson et al., 2011]. Glutaredoxins (Grxs) are small redox enzymes that use glutathione (GSH) as a cofactor [Fernandes and Holmgren, 2004]. Grxs possess an active site disulfide bond, which exists in either a reduced or an oxidized form. Grxs act as antioxidants by reducing dehydroascorbate, peroxiredoxins and methionine sulfoxide reductase. Some bacterial species contain tetrameric

Prx5-Grx hybrid enzymes in which the C-terminal Grx domain acts as an electron donor for the N-terminal Prx domain [Kim et al., 2003].

Meningococcal peroxiredoxin is an antioxidant protein with both peroxiredoxin and glutaredoxin activity sites [Rouhier and Jacquot, 2003]. Peroxiredoxin was previously designated as a moonlighting protein that expressed at the surface of un-encapsulated *N. meningitidis* where it can bind human plasminogen [Knaust et al., 2007]. Other moonlighting properties different bacterial Prxs possess are discussed in Chapter 5).

1.10.3. DnaK

DnaK is a cytosolic molecular chaperone that typically plays an essential role in preserving the integrity of its client protein(s) in terms of preventing potential aggregation and/or misfolding events, and also aids in translocating the client proteins to their final destination [Kim et al., 2013]. DnaK has been found to moonlight in several bacterial species. In *Listeria monocytogenes*, examining the cell wall subproteome revealed the presence of DnaK (in addition to enolase, GAPDH and elongation factor TU) in the cell wall of the pathogen, and all these proteins were able to bind plasminogen [Schaumburg et al., 2004]. Similar to meningococci [Knaust et al., 2007], DnaK of *Mycobacterium tuberculosis* was also identified as a

plasminogen binder, this binding was shown to be inhibited by EACA, suggesting lysine residues as putative binding mediators [Xolalpa et al., 2007]. Mycobacterial recombinant DnaK was also able to bind CD40 receptors on the surface of monocytes, resulting in stimulating the latter to produce CC chemokines [Wang et al., 2001]. Dendritic cell activation was also documented after interaction of mycobacterial recombinant DnaK with chemokine receptor CCR5 [Floto et al., 2006]. Importantly, HIV also uses CCR5 as a co-receptor, and hence DnaK can compete with HIV on the binding to CCR5 [Floto et al., 2006, Babaahmady et al., 2007]. It was shown that a C-terminal 407-426 DnaK peptide inhibited HIV infection of activated CD4⁺ T cells, this can have potential as a novel strategy to block the binding of HIV to its receptor [Babaahmady et al., 2007].

1.11. The aim of the study

The findings discussed earlier highlight the importance of moonlighting proteins as virulence factors that can escalate the capability of bacteria to establish an infection. The aim of this study is to further investigate the putative moonlighting activities of three meningococcal proteins: enolase, peroxiredoxin and DnaK, and assess their contribution to the pathogenesis of *N. meningitidis*.

2. CHAPTER TWO: Cloning, overexpression and purification of meningococcal rEno, rPrx and rDnaK

2.1. Introduction

Having more than one role or function is the idiomatic meaning of “moonlighting”, a new concept which was suggested to describe the additional task that a certain protein performs alongside its standard function. These proteins were identified both in prokaryotic and eukaryotic cells, where they primarily function as metabolic pathway-associated enzymes or as molecular chaperones [Henderson and Martin, 2011a]. Many studies have indicated that the moonlighting activity of a protein is most often evident when the protein is translocated, by an as yet unknown mechanism, to another aspect of the cell where the moonlighting activity then takes place.

Knaust and colleagues described three cytosolic proteins from *N. meningitidis*, namely enolase, peroxiredoxin and DnaK, as plasminogen (Plg) binding ligands on the surface of the organism [Knaust et al., 2007]. Enolase is a glycolytic enzyme of Embden–Meyerhof–Parnas (EMP) pathway. It naturally catalyses the dehydration of 2-phosphoglycerate to phosphoenolpyruvate [Pancholi, 2001]. As a moonlighting protein, enolase was reported on the surface of phylogenetically different species, exhibiting various non-glycolytic functions (some of these are discussed in Chapter 1). It is important to stress that while the gene encoding meningococcal enolase is highly conserved [Knaust et al., 2007], the principal enolase-

associated pathway (*i.e.* the EMP), is predicted to be non-functional in *N. meningitidis* due to the absence of *pfk* gene, which encodes phosphofructokinase that is required in the third step of the EMP pathway [Tettelin et al., 2000, Baart et al., 2007]. The burden associated with maintaining the gene in a functional state in the bacterial genome, and synthesising its product, tends to suggest that an alternative function is behind this conservation.

Peroxiredoxin (Prx) is an antioxidant protein with both peroxiredoxin and glutaredoxin activity sites [Rouhier and Jacquot, 2003]. Prx may assist in avoiding the damaging effect of many free radicals such as reactive oxygen and nitrogen, which are generated during different biological processes [Rhee and Woo, 2011].

DnaK is a cytosolic molecular chaperone that typically plays an essential role in preserving the integrity of its client protein(s) in terms of preventing potential aggregation and/or miss-folding events, and also aids in translocating the client protein to its final destination [Kim et al., 2013]. In addition to binding meningococcal plasminogen [Knaust et al., 2007], DnaK has been found to moonlight in other bacterial species. For instance, DnaK from *Francisella tularensis* was reported to bind to, and inhibit the major plasma tissue-nonspecific alkaline

phosphatase (TNAP) isoform [Arulanandam et al., 2012]. The latter represents an innate host defence device, which provides a degree of protection at the primary site of infection in the lung [Edelson et al., 1988], in addition to its neutralizing effect on bacterial endotoxin [Beumer et al., 2003]. The inhibitory effect of DnaK on TNAP could trigger an inflammatory response, which is advantageous to the organism. It is worth mentioning that exhibiting a moonlighting feature is sometimes a species-specific property, *i.e.* the additional task(s) that a particular protein displays may or may not exist in a related species. As a consequence, determining whether a protein multi-tasks or not is a *de novo* process that requires confirmation even in related organisms. What adds more complexity in terms of studying these proteins is the fact that on many occasions they represent essential housekeeping proteins, which means that the conventional strategy to study protein function by producing a knockout strain is not always possible.

This chapter describes the cloning, overexpression and purification of recombinant enolase, peroxiredoxin and DnaK, in order to facilitate the investigation of the putative moonlighting activities of these meningococcal proteins, and assess their contribution to the pathogenesis of *N. meningitidis*.

2.2. Materials and methods

2.2.1. *In silico* sequence analysis

The genome database of *Neisseria meningitidis* MC58 was interrogated at <http://www.ncbi.nlm.nih.gov/gene>. For secretion signal peptide prediction, meningococcal enolase (NP_274305.1), peroxiredoxin (NP_273984.1) and DnaK (NP_273598.1) were analysed using SignalP 4.1 server available at <http://www.cbs.dtu.dk/services/SignalP/>. Sequence of AspA (NP_274963.) was utilized as a positive control. DNAMAN software (Lynnon BioSoft) was employed to facilitate other DNA and protein sequence analyses such as restriction enzyme digestion, primer design and protein translation.

2.2.2. Bacterial strains and growth conditions

E. coli strain JM109 (Promega) was used for propagation of all plasmids constructed in this study. Similarly, the expression of recombinant proteins was performed utilizing the same strain. Bacteria were cultivated in Lysogeny broth (LB) and on LB agar plates supplemented with 100 $\mu\text{g ml}^{-1}$ ampicillin, unless otherwise mentioned. The plates were incubated at 37°C; broth cultures were incubated in a shaking incubator (200 rpm) at the same temperature.

2.2.3. DNA extraction, purification and quantification

Several kits including: GenElute PCR clean up kit; GenElute plasmid miniprep kit and GenElute Bacterial genomic DNA extraction kit (all from Sigma-Aldrich), were used to clean up both PCR products and products digested with restriction enzymes; plasmid DNA extraction; and bacterial genomic DNA extraction, respectively. In each instance, the manufacturer's instructions were followed. The concentration and the purity of any extracted DNA (genomic and plasmid) were determined using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific), at 260/280 nm wavelength.

2.2.4. Polymerase chain reaction (PCR)

A master mix containing all of the PCR components was prepared. The master mix contained 1 μ L of the appropriate template DNA; each of the respective primers (Table 2.1) to a final concentration of 0.5 μ M; 60 μ L 5 $\times$ Phusion Buffer; dNTPs to a final concentration of 0.2 mM, 3 μ L of Phusion High Fidelity DNA polymerase. The mixture was made up to a final volume of 300 μ L with dH₂O. Aliquots of 50 μ L of the master mix were dispensed into 6 PCR tubes. The PCR conditions were initial template denaturation step at 98°C for 30 s, followed by 30 cycles of incubation including: denaturation at 98°C for 10 s, annealing for 30 s, and extension at 72°C. The temperature

used for annealing differed depending on the primer pair used; the extension time used depended on the expected size of the amplicon, but was generally 30 s/1 kb. A final extension step at 72°C for 5 min was also included (Table 2.2).

Table 2.1: List of the primers used in this chapter for amplification and/or sequencing of NMB1285, NMB0946 and NMB0554.

Primer	Primer sequence* (5'-3')	Purpose
1285-F1	CGC GGATCC ATGAGCGCAATCGTTGAT ATTTTCGC	Amplification of NMB1285 encoding meningococcal enolase
1285-R1	CGC GGATCC TTATTTGCCAGTTGGTA GAATGCG	
NMB0946F1	CGC GGATCC ATGGCTTTGAAGATCGTA CCGG	Amplification of NMB0946 encoding meningococcal peroxiredoxin
NMB0946R1	CGC GGATCC TCAGTTTTTAGCCAAGTA AGCTTCC	
DnaK-F1	CGC GGATCC ATGGCAAAAGTAATCGGT ATCG	Amplification of NMB0554 encoding meningococcal DnaK
DnaK-R1	CGC GGATCC CAGACGGTATTAATTATT TTTTGTCG	
Eno-F2	GCTACAAAGCGGGCGAAGACG	Internal primer for NMB1285 sequencing
DnaK-F2	AGCCAACGTCAAGCCACCAAAGACG	Internal primer for NMB0554 sequencing
DnaK-F3	CGAAGTAACCTTCGACATCGACG	Internal primer for NMB0554 sequencing

* Sequence in bold indicates BamHI recognition sequence.

Table 2.2: Thermo-cycling conditions that were applied to amplify NMB1285, NMB0946 and NMB0554 utilizing genomic DNA of *N. meningitidis* strain MC58 as a template.

Target gene	Thermo-cycling conditions	
	Annealing temperature (°C)	Extension time (s)
NMB1285	60	30
NMB0946	67	15
NMB0554	67	60

2.2.5. Agarose gel electrophoresis

Agarose gel (1% w/v) containing 10 µg ml⁻¹ ethidium bromide (Sigma-Aldrich) was poured into a casting tray (Bio-Rad). After immersing the gel with 1% Tris-acetate-EDTA (TAE) buffer; samples containing 1× DNA loading dye were loaded alongside 1 kb DNA Ladder (N3232S; New England Biolabs), unless otherwise mentioned. The gel was run at 80 V for 45 min. Finally, DNA bands were visualized using UVitec scanner (Bio-Rad).

2.2.6. Restriction digestion and de-phosphorylation

Digestion of DNA samples was carried out using different restriction enzymes (New England Biolabs), following the manufacturer's instructions. Digestion reactions were incubated in a water bath at 37°C for ≥3 h. Before ligation, and to increase cloning efficiency, vector DNA digested with a single restriction enzyme was dephosphorylated with Shrimp Alkaline

Phosphatase (SAP) (New England Biolabs), following the manufacturer's instructions.

2.2.7. Transformation procedure

Briefly, an aliquot of ≥ 50 μL of frozen *E. coli* JM109 competent cells (Promega) was thawed on ice 5 min prior to transformation. As required, five microliters of a recombinant plasmid or PCR product was added to the cells and mixed gently. The cells were incubated on ice for 10 min. The cells were heat shocked at 42°C in water bath for 45 s without shaking, and then immediately transferred on to ice for 2 min. Then, 950 μL of S.O.C medium (New England BioLabs) were added and the cell suspension was incubated at 37°C for 1 h with shaking. The transformation reaction was plated out on LB agar containing ampicillin (100 $\mu\text{g ml}^{-1}$) and incubated at 37° for > 16 h.

2.2.8. DNA sequencing

All cloned DNA fragments were checked by DNA sequencing to confirm orientation and lack of PCR-generated errors. Sequencing was performed by Source Bioscience utilizing Sanger chain-termination method. PCR primers and internal primers, which were used for sequencing, are shown in (Table 2.1).

2.2.9. Cloning of NMB1285, NMB0946 and NMB0455 into pQE30

The pQE30 vector (Qiagen; Appendix 3) was linearized by digestion with BamHI and dephosphorylated with SAP. Plasmids pMAJ1, pMAJ2 and pMAJ3 were constructed by ligating BamHI-digested PCR fragment corresponding to the NMB1285, NMB0946 and NMB0554 coding sequence to the pre-treated pQE30 according to the manufacturer's instructions. Resulting clones were screened by restriction digestion with BamHI and DNA sequencing.

2.2.10. Expression of recombinant enolase, peroxiredoxin and DnaK

E. coli JM109 carrying the appropriate plasmid, pMAJ1, pMAJ2 or pMAJ3, were grown overnight in LB broth containing 100 µg ml⁻¹ ampicillin. The overnight culture was inoculated into fresh 500 ml LB/ampicillin broth (1:50 dilution). The cultures were allowed to grow to mid log phase ($OD_{600nm} \geq 0.5$). IPTG was added to a final concentration of 0.5 mM. The culture was then incubated at 37°C for 3 h with shaking. One-millilitre aliquots were taken before and after induction with IPTG to be used for SDS-PAGE analysis, and cells were harvested by centrifugation at 2087 x *g* for 15 min at 4°C, using Heraeus LABOFUGE 400R centrifuge (Thermo Scientific). The cell pellets were then stored at -80°C.

2.2.11. Purification of recombinant proteins by IMAC

Immobilized metal ion affinity chromatography (IMAC) was used to purify the His-tagged recombinant proteins, using an AKTA PrimePlus system (GE Healthcare) and following the manufacturer's instructions. The stored cell pellets from 150 ml culture of *E. coli* JM109 (pMAJ1); (pMAJ2) and (pMAJ3) were re-suspended with 30 ml lysis/binding buffer (50mM NaHPO₄; 300mM NaCl; 40mM imidazole, pH 7.4). Sonication on ice was carried out to disrupt the cells for 8 min in short bursts (30 sec ON and OFF), using an MSE SoniPrep 150 sonicator. The lysate was centrifuged at 4500 × *g* for 15 min at 4°C. The supernatant was collected for purification of recombinant enolase (rEno), peroxiredoxin (rPrx) and rDnaK, when *E. coli* JM109 (pMAJ1), (pMAJ2) and (pMAJ3) were used, respectively. Briefly, a HisTrap FF crude column was mounted to the AKTA PrimePlus system and equilibrated with the lysis/binding buffer. The sample soluble lysate was then injected into the column at a flow rate of 1 ml/min. Finally, elution buffer (50mM NaHPO₄; 300mM NaCl; 300mM imidazole, pH 7.4) was applied to the column to elute the bound His-tagged recombinant protein. The purified protein was then collected from the corresponding fraction(s) by monitoring the purification chromatogram. When required, glycerol at a final concentration of 10-25% was added to the

eluted protein to prevent aggregation. Throughout the purification process, aliquots were taken and subjected to SDS-PAGE and immunoblot analysis.

2.2.12. Desalting and concentration of proteins

After mounting on the AKTA PrimePlus system, a HiTrap desalting column was equilibrated with desalting buffer (phosphate buffer saline; PBS). The purified protein was then injected into the column, where it was buffer exchanged from elution buffer into the desalting buffer. Desalted protein fractions were combined and concentrated using 10-kDa or 30-kDa (based on the molecular weight of the purified protein) membrane cut-off Vivaspins tubes (Sartorius) by centrifugation at $4500 \times g$ at 4°C.

2.2.13. Purification by gel filtration

Gel filtration was performed as a final step in the purification scheme. A pre-packed Superdex column (GE Healthcare) mounted on the AKTA PrimePlus system was used to carry out the process, following the manufacturer's instructions. After equilibration with 150 ml PBS or PBS containing 10% glycerol, the IMAC-purified protein sample was injected into the column. Fractions containing the purified protein were collected, and then concentrated as previously described (section 2.2.12.).

2.2.14. SDS-PAGE

Proteins were electrophoretically separated using 10% SDS-PAGE gels following the method of Laemmli [Laemmli, 1970], using mini-protein III apparatus (Bio-Rad). Samples to be analysed were mixed with 5 × SDS-PAGE loading dye (620 mM Tris-HCl [pH 6.8], 5% SDS, 25% Glycerol, 12.5% β -mercaptoethanol, 0.002% Bromophenol blue brought up to 25 ml with dH₂O) and heated at 100°C for 5 min. Unless otherwise stated, ColorPlus™ Prestained Protein Ladder (P7709S; New England Biolabs) was used to estimate the molecular weight of proteins. Proteins were visualized by staining with Simply Blue Safe stain (Invitrogen) according to the manufacturer's instructions. Gels were scanned using a GS-800 densitometer.

2.2.15. Immunoblot analysis

The protein mixtures separated by SDS-PAGE were transferred onto a nitrocellulose membrane in semi-dry blotting buffer using a Trans-Blot SD semi-dry transfer cell (Bio-Rad) at 15 V for approximately 20 min. The membrane was incubated in 5% skimmed milk in PBS-Tween (PBS containing 0.1% Tween-20) for 1 h at room temperature (RT) or overnight at 4°C. The membrane was probed with mouse anti-penta-histidine antibodies (Qiagen; 1:2000 dilution), and incubated for 2 h with shaking at RT or overnight at 4°C. The membrane was washed three times for 5 min each with PBS-Tween, and then incubated

in secondary anti-mouse IgG antibodies conjugated to alkaline-phosphatase (Sigma-Aldrich; 1:10000 diluted in 5% skimmed milk-PBS-Tween) for 2 h at RT or overnight at 4°C. The membrane was washed again thrice with PBS-Tween for 5 min each time. The membrane was then developed using 5-Bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium liquid substrate system (BCIP/NBT, Sigma-Aldrich), and finally washed briefly with dH₂O and visualized with GS-800 densitometer.

2.2.16. Protein quantification

To estimate the concentration of purified recombinant proteins, the Pierce™ BCA Protein Assay kit (Thermo Scientific) was used, following the manufacturer's instructions.

2.2.17. Raising polyclonal antisera against rEno, rPrx and rDnaK

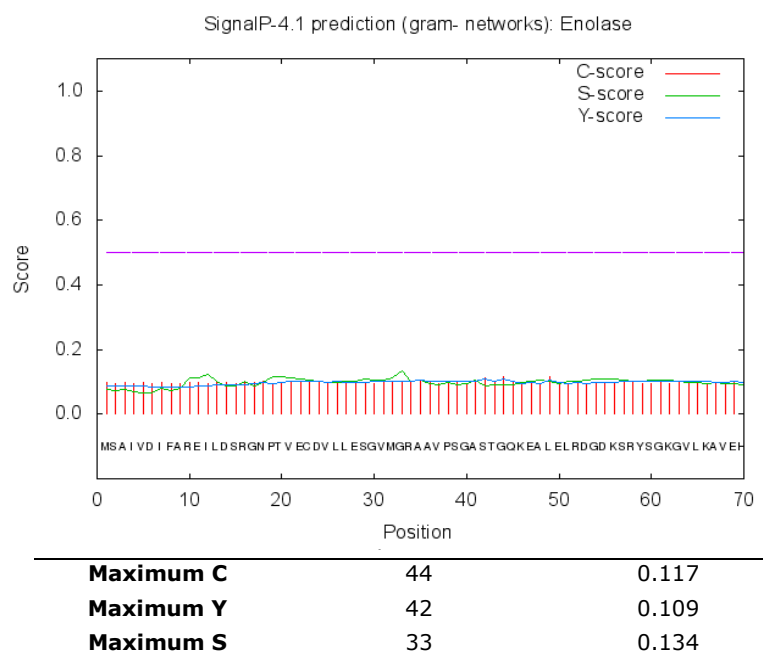
New Zealand white female rabbits were used to raise polyclonal antibodies against the purified rEno, rPrx and rDnaK. Pre-immune sera were first collected from each animal. The immunization scheme included injecting the animal subcutaneously, three times at two week-intervals, with 30 µg of the appropriate protein emulsified in Freud's Complete (first immunization) or incomplete adjuvant. After two doses, rabbits were test bled, boosted once more, and sacrificed 10 days later.

2.3. Results

2.3.1. Meningococcal enolase, peroxiredoxin and DnaK lack signal peptide sequences

In order to examine whether or not any of meningococcal enolase, peroxiredoxin or DnaK possesses a signal peptide sequence, which is required to warrant protein translocation to the outer aspects of the cell, SignalP 4.1 prediction tool was employed. Protein sequence of AspA, a known outer membrane protein of *N. meningitidis* MC58, was used as a positive control. It has been confirmed that, unlike AspA, none of the investigated proteins has a predicted signal peptide sequence, a feature that is common among moonlighting proteins [Henderson and Martin, 2011a], (Figure 2.1 for enolase (A) and AspA (B); Appendix 5 for other proteins).

[A]



[B]

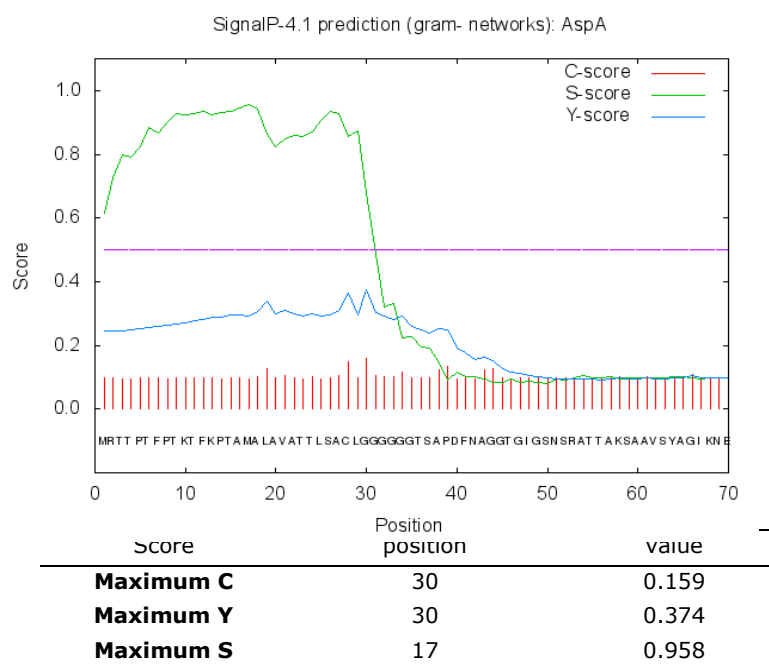


Figure 2.1: Predicting putative signal peptide sequences of meningococcal enolase (A) and AspA (B), a known secreted protein that was used as a positive control. SignalP 4.1 server was utilized to perform the prediction. C-score distinguishes putative signal peptide cleavage sites. S-score distinguishes positions within signal peptides from positions in the mature part of the proteins and from proteins without signal peptides. Y-score (geometric average), provides a better cleavage site prediction than the raw C-score alone. Unlike

AspA, enolase-associated scores were very low (close to 0.1), suggesting that it probably lacks any signal peptide sequence.

2.3.2. Amplification and cloning of NMB1285, NMB0946 and NMB0455 into pQE30

NMB1285, NMB0946 and NMB0554 comprise 1287, 738 and 1929 bp, and encode meningococcal enolase, peroxiredoxin and DnaK, respectively. The entire ORF of each was amplified by PCR to yield single bands of the expected sizes (Figure 2.2). Each PCR product was digested with BamHI (a restriction enzyme recognition site was included in the primers used to amplify each product), ligated to BamHI-digested and dephosphorylated pQE30 vector, and then transformed into *E. coli* strain JMI09. Plasmids extracted from the resultant clones were analysed by restriction digestion and DNA sequencing. In terms of NMB1285, digestion of the extracted plasmid DNA with BamHI was expected to produce two fragments of *ca.* 1.3 kb and 3.4 kb corresponding to NMB1285 and pQE30, respectively (Figure 2.3, A). Similarly, digestion of NMB0946-containing plasmids were expected to yield two fragments of *ca.* 0.7 kb and 3.4 kb corresponding NMB0946 and pQE30, respectively, when BamHI-digested (Figure 2.3, B). Finally, digesting plasmid DNA containing NMB0554 was expected to produce two fragments of *ca.* 1.9 kb and 3.4 kb corresponding to NMB0554 and pQE30, respectively (Figure 2.3, C). Several clones were

analysed by restriction digestion; in each case, some were identified to contain fragments of the expected size.

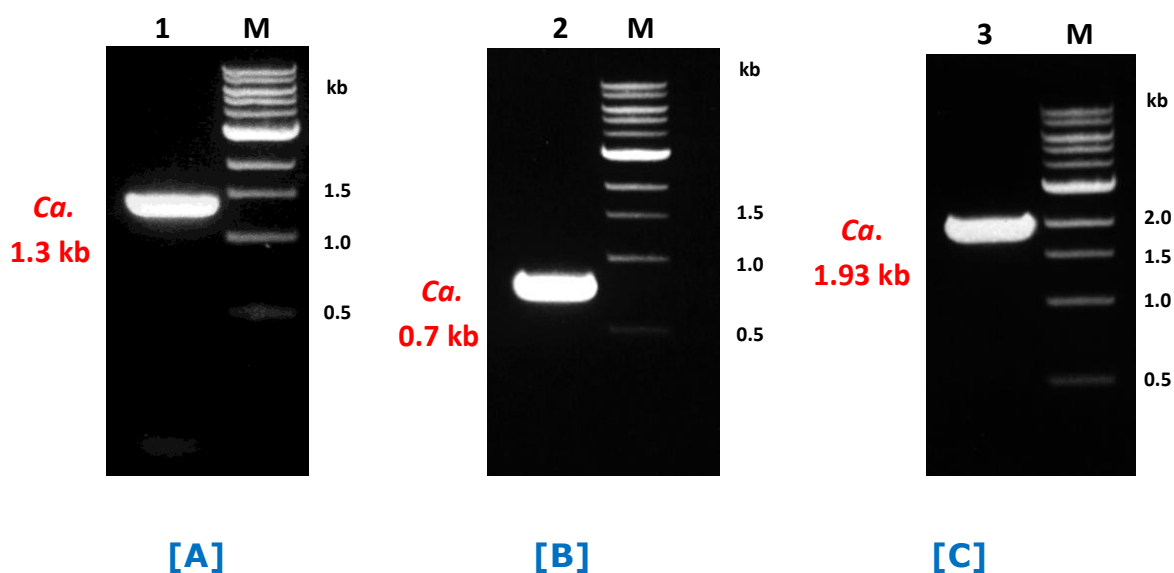


Figure 2.2: Agarose gel analysis showing the PCR amplification of NMB1285 (A), NMB0946 (B) and NMB0554 (C). Chromosomal DNA was prepared from *N. meningitidis* MCS8 and used as a template to amplify the genes by PCR. Lane M, 1 kb DNA ladder. Lanes 1, 2 and 3: PCR products corresponding to NMB1285, NMB0946 and NMB0554, respectively.

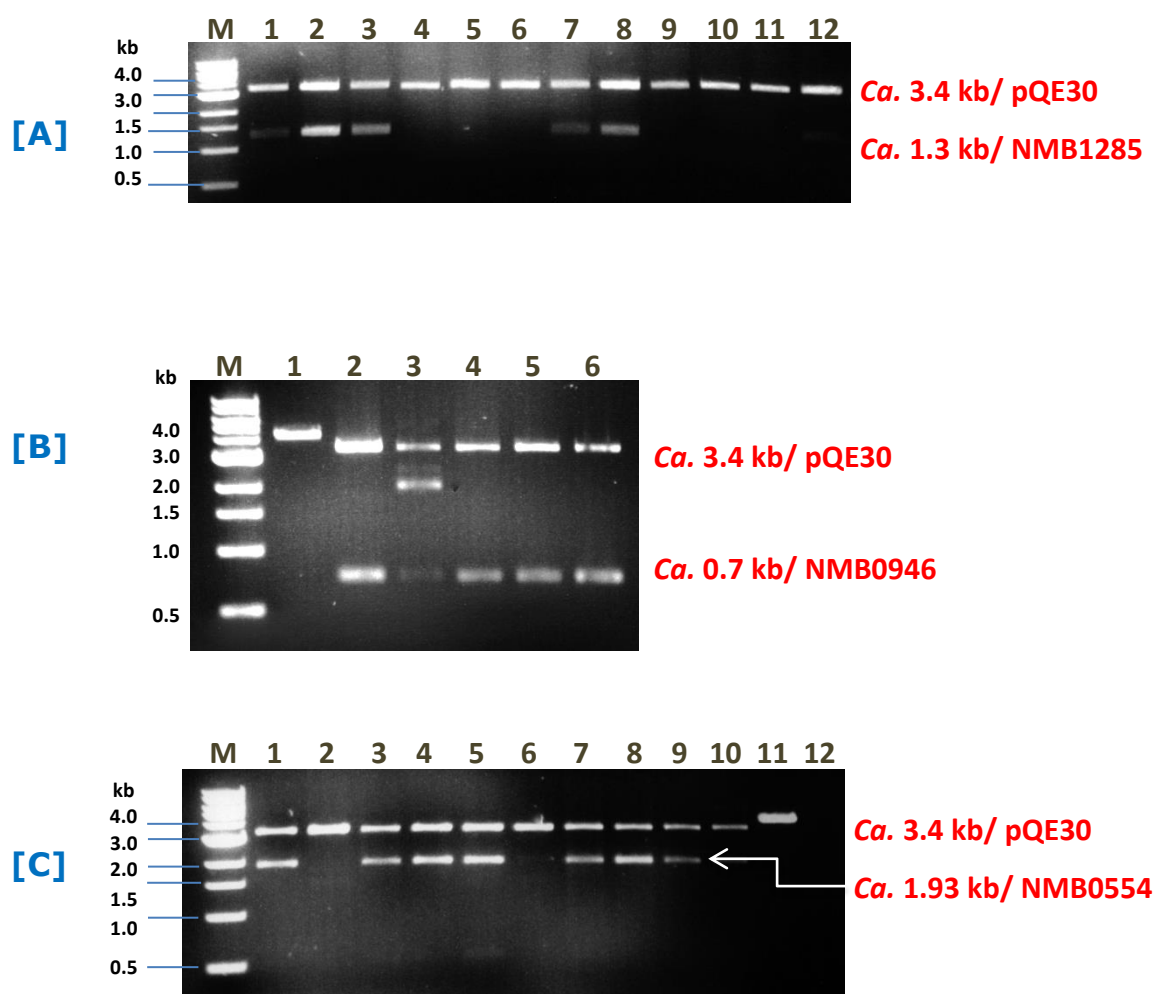


Figure 2.3: Agarose gel analysis showing BamHI restriction digestion of plasmid DNA extracted from *E. coli* JM109 that harbour NMB1285, NMB0946 or NMB0554 in pQE30. Lane M, 1 kb DNA ladder; lanes 1-3, 6, 7, and 12 (A), putative pMAJ1; lanes 2 and 4-6 (B), putative pMAJ2; lanes 1, 3-5, and 7-10 (C), putative pMAJ3.

Furthermore, and due to the non-directional cloning strategy that was adopted, putative plasmids were also digested with EcoRI, which was found, based on an *in silico* restriction enzymes digestion analysis, to cut pQE30 once without having any recognition sequences in NMB1285, NMB0946 or NMB0554. Hence, it was used to linearize each putative construct to confirm its identity and to exclude the possibility of

incorporating more than one copy of any cloned fragment (Figure 2.4). Finally, the sequence and orientation of the cloned NMB1285, NMB0946 and NMB0554 PCR products were confirmed by DNA sequencing. Sequencing demonstrated that in each instance, the extracted plasmid contained the appropriate insert DNA fragment in frame with the His-tag of the vector and in the correct orientation to the T5 promoter. pQE30 containing NMB1285, NMB0946, or NMB0554 were designated pMAJ1, pMAJ2 and pMAJ3, respectively.

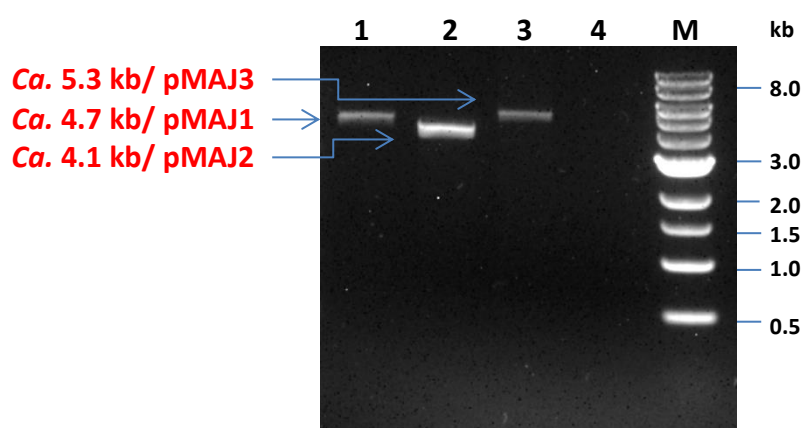


Figure 2.4: Agarose gel analysis showing EcoRI-restriction digestion of plasmid DNA to verify the cloning of NMB1285, NMB0946 and NMB0455 into pQE30. Lane M, 1kb DNA ladder, lane 1: putative pMAJ1 digested with EcoRI, lane 2: putative pMAJ2 digested with the same restriction enzyme, lane 3: EcoRI-digested pMAJ3, lane 4: negative control (H₂O was added to the digestion reaction instead of any DNA template). The size of each band reflects the size of pQE30 (3.4 kb) plus a single copy of the cloned gene *i.e.* 4.7 kb when NMB1285 (1.3 kb) encoding meningococcal enolase incorporated to produce pMAJ1, 4.1 kb when NMB0946 (0.7 kb) incorporated to produce pMAJ2, and 5.3 kb due to the insertion of NMB0455 (1.93 kb) to construct pMAJ3.

2.3.3. Expression of recombinant enolase, peroxiredoxin and DnaK

E. coli JM109 cells containing pMAJ1, pMAJ2 or pMAJ3 were used to express recombinant enolase (rEno), peroxiredoxin (rPrx) and DnaK (rDnaK), respectively. In each instance, after 3 h induction with 0.5mM IPTG, optimal expression was obtained. This was determined by SDS-PAGE which revealed a band of either *ca.* 48-kDa, 27-kDa or 69-kDa, which is the expected size for rEno, rPrx and rDnaK, respectively (Figure 2.5 A, C, E). The identity of each His-tagged fusion protein was confirmed by immunoblot analysis using anti-penta-histidine antibodies, in which a highly immuno-reactive band of the expected size was detected when IPTG-induced samples were examined (Figure 2.5 B, D, F) compared to less intense bands of the same size from un-induced samples.

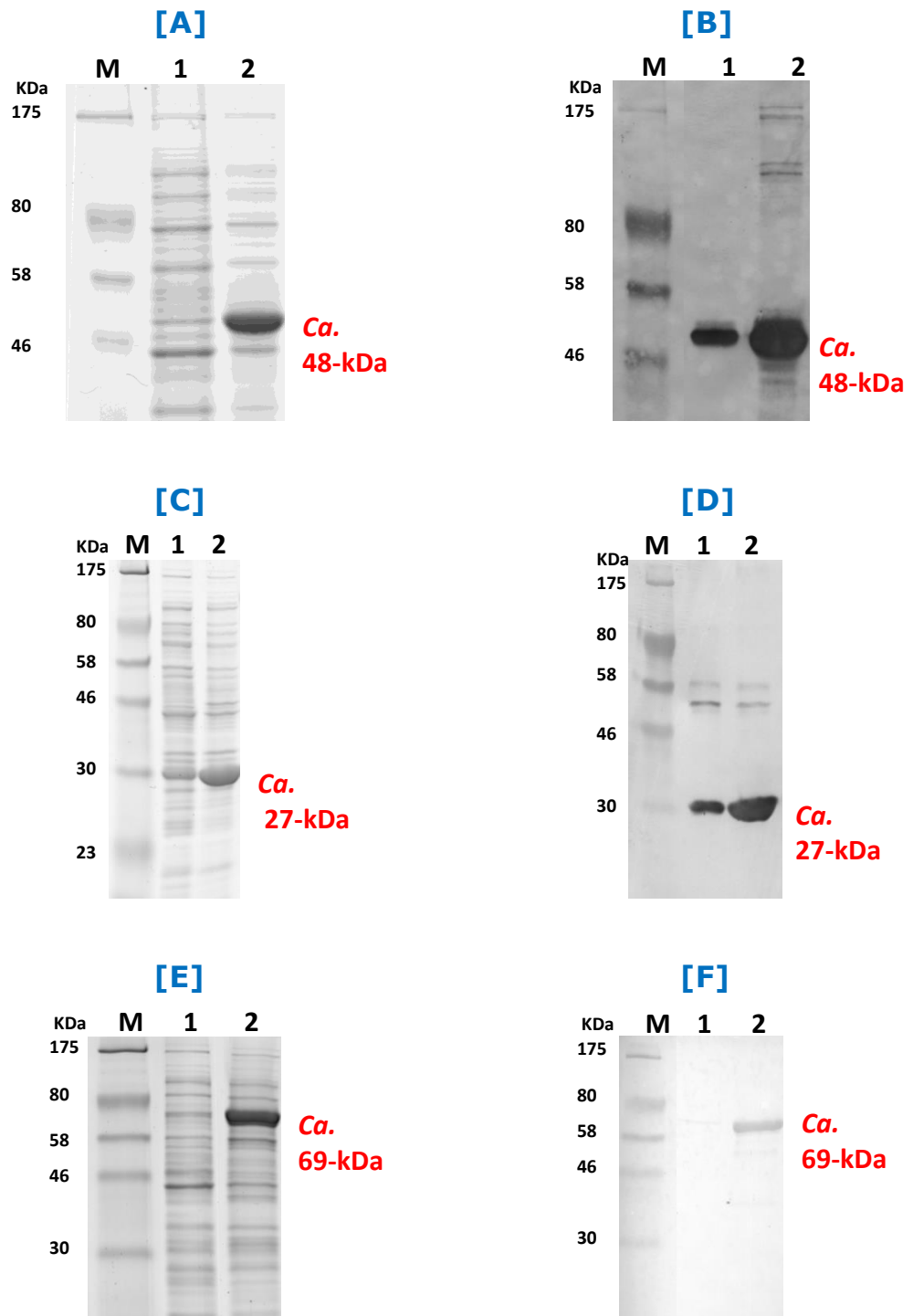


Figure 2.5: SDS-PAGE (left) or immunoblot analysis (right) with anti-penta-histidine antibodies demonstrating the overexpression of recombinant enolase (A, B), peroxiredoxin (C, D) and DnaK (E, F) in IPTG-induced *E. coli* JM109 cells containing pMAJ1, pMAJ2 or pMAJ3, respectively [lane 2] compared to non-induced cells [lane 1]; Lane M: pre-stained protein marker.

2.3.4. Purification of recombinant His-tagged enolase, peroxiredoxin and DnaK under native conditions

Recombinant His-tagged enolase, peroxiredoxin and DnaK were purified under native conditions, utilizing immobilized nickel affinity chromatography. The purification process was performed in three stages (Figure 2.6). The first stage included binding of the His-tagged protein to nickel ions immobilized on the purification column (Figure 2.6, A). After washing to remove non-specifically bound proteins, the recombinant protein was eluted using a high concentration of imidazole (Figure 2.6, B). A desalting step with PBS was then performed to exchange the high-imidazole containing elution buffer with PBS (Figure 2.6, C). Finally, fractions that containing the desired protein (according to the purification chromatogram) were combined and the protein concentrated. For rEno, aggregation was noted after initial attempts at protein purification. In subsequent purifications, glycerol (as a protein stabilizer), at 25% final concentration in PBS was added directly to the eluted protein. In terms of peroxiredoxin and DnaK, freezing and thawing when the protein was highly concentrated was found to enhance aggregation, and as a result, glycerol to a final concentration of 10% was added to any desalted protein subsequently purified.

Immunoblot analysis, using anti-penta-histidine antibodies, confirmed the identity of the bands as His-tagged rEno, rPrx and rDnaK, respectively (Figure 2.7 B, D and F). However, while the IMAC-purified rPrx was pure (Figure 2.7 C & D, lane 8 & 9), some additional faint bands were apparent in the gels but not in the blots corresponding to rEno (Figure 2.7 A & B, lane 8) and rDnaK (Figure 2.7 E & F, lane 8), suggesting the presence of additional co-purified *E. coli* proteins.

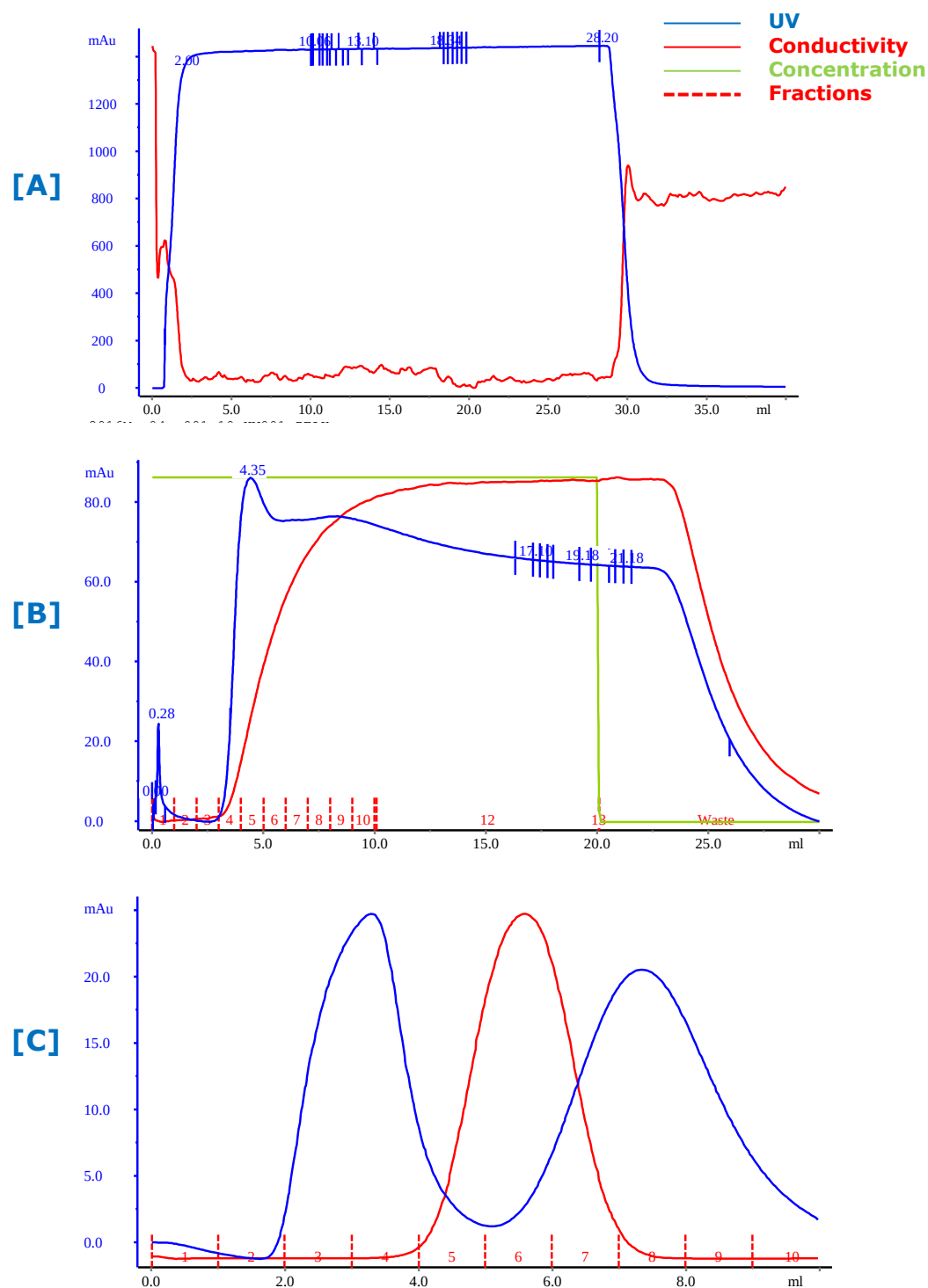


Figure 2.6: Representative chromatograms illustrate the steps of recombinant His-tagged protein purification using the AKTA prime plus system. (A) During the binding step the His-tagged protein was loaded into the purification column, where it bound strongly to immobilized Ni^{2+} attached to the column. The high UV peak verified the high protein concentration present in the sample. (B) As a high concentration (300 mM) of competitive imidazole was pumped during

the elution step (increased conductivity), His-tagged protein eluted away from the HisTrap column (increased absorbance). Eluted protein collected from fractions 4-10 that associated with a high UV peak, was then combined. (C) To exchange the imidazole with PBS, the combined protein was loaded in to a Hi-Trap column where the high molecular weight protein eluted (fractions 3-4, high UV peak) before the low molecular weight imidazole , which required a longer time to escape the pores of the column beads (fractions 5-7, high conductivity peak).

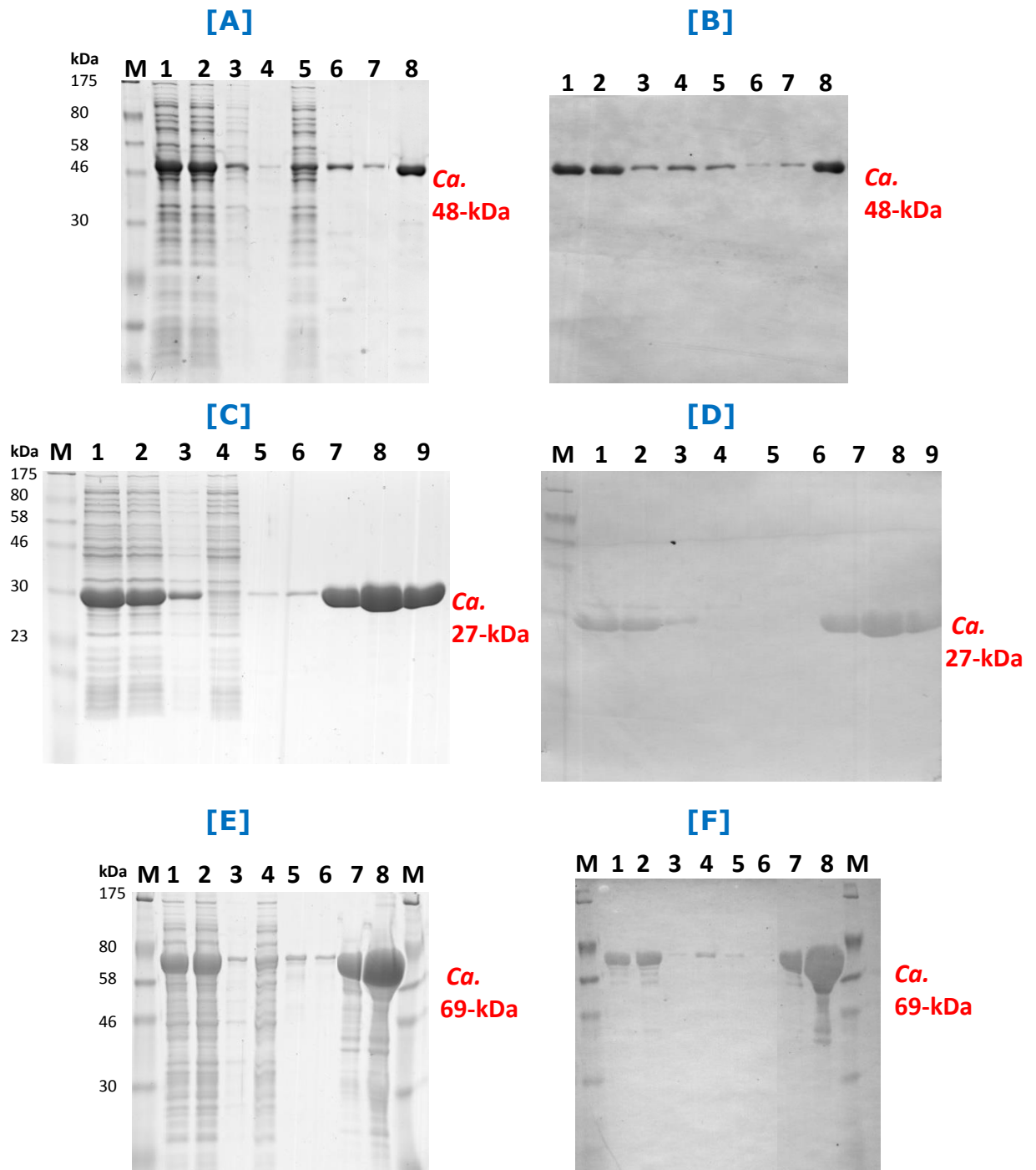


Figure 2.7: SDS-PAGE (left) and immunoblot analysis (right) demonstrating IMAC-purification steps of recombinant enolase (A, B), peroxiredoxin (C, D) and DnaK (E, F) by using 150 ml harvested cells pellet of the IPTG-induced *E. coli* JM109 containing pMAJ1, pMAJ2 or pMAJ3, respectively. The fate of each recombinant protein was determined throughout the various purification steps. Lane M: pre-stained protein marker, lane 1: whole cell lysate, lane 2: soluble lysate, lane 3: pellet, lane 4: flow through, lane 5: wash, lane 6:

fraction 3, lane 7: combined fractions 4-8, lane 8: desalted and concentrated protein, lane 9: concentrated gel filtered protein (C, D only). SDS-PAGE analysis demonstrates the level of purity of each purified recombinant protein. Immunoblot analysis shows that recombinant His-tagged proteins are recognized by anti-pentahistidine antibodies.

2.3.5. Determining the oligomerization of rEno, rPrx and rDnaK by analytical gel filtration

Gel filtration was primarily performed to increase the purity of the IMAC-purified rEno and rDnaK. However, subsequent SDS-PAGE and immunoblot analysis suggested that nearly the same level of purity was obtained when the IMAC-purified rEno (Figure 2.8) and rDnaK were gel-filtered. Furthermore, gel filtration allowed a determination of the oligomerization status of purified rEno, rPrx and rDnaK. The molecular mass of each protein (purified under native conditions) was estimated by comparing the volume at which each protein eluted with the elution volume values obtained when proteins of known molecular weights (standards) were run through the gel filtration column (Table 2.3).

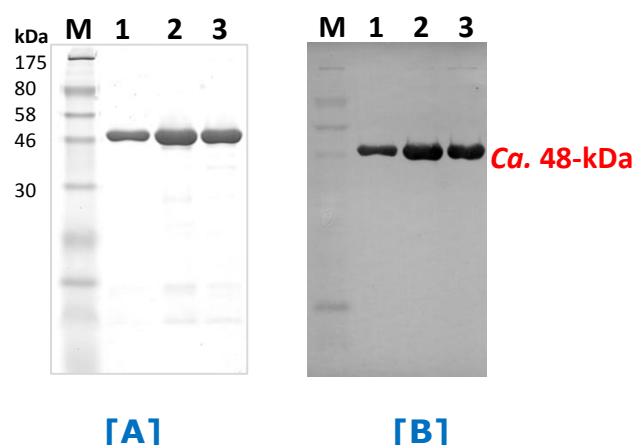


Figure 2.8: SDS-PAGE and immunoblot analysis demonstrating purification of rEno by gel filtration. (A) SDS-PAGE analysis confirms almost the same level of purity seen with IMAC-only purified rEno. (B) Immunoblot analysis shows that recombinant His-tagged protein was recognized by anti-penta-histidine antibodies. (Lane M: pre-stained protein marker, lane 1: IMAC-only purified enolase, lane 2: desalted and concentrated IMAC-only purified enolase, lane 3: gel filtration of IMAC-purified enolase).

Table 2.3: Molecular weights and elution volume values of the proteins used as standards for the estimation of the molecular weight of rEno, rPrx and rDnaK purified under native conditions.

Protein	Molecular weight (kDa)	Elution volume (ml)
Bovine thyroglobulin	670	48.19
Bovine gamma globulin	158	62.46
Albumin	44	78.04
Myoglobin	17	91.56
Vitamin B12	1.35	110.38

In terms of rEno, most of the protein was eluted at 54.02 ml volume (Figure 2.9, A). Elution at this volume suggests that the molecular mass of the protein was approximately ≤ 400 -kDa. As the molecular weight of monomeric meningococcal enolase is *ca.* 48-kDa, it can be inferred that the purified rEno was mostly present as octamers; with monomers probably eluted at 61.29 ml (Figure 2.9, A). Similarly, most of the gel-filtered rPrx was eluted at 67.53 ml volume (Figure 2.9, B), suggesting that the molecular mass of the eluted protein was *ca.* 140-kDa. Since the monomeric peroxiredoxin molecular weight is *ca.* 27-kDa, it can be inferred that peroxiredoxin was mostly present as hexamers. Regarding rDnaK, the molecular mass of the eluted protein at the 42.23 l ml, 60.35 ml and 72.86 ml elution volume values (Figure 2.9, C) were predicted to be >670 -kDa, *ca.* 140-kDa and 69-kDa, respectively. Based on this, it appears that DnaK is present in several oligomerization states. Since monomeric DnaK is *ca.* 69-kDa in molecular weight, it is apparent that DnaK is present either as large multimers, dimers or monomers, and that the dimeric species of DnaK is the most abundant.

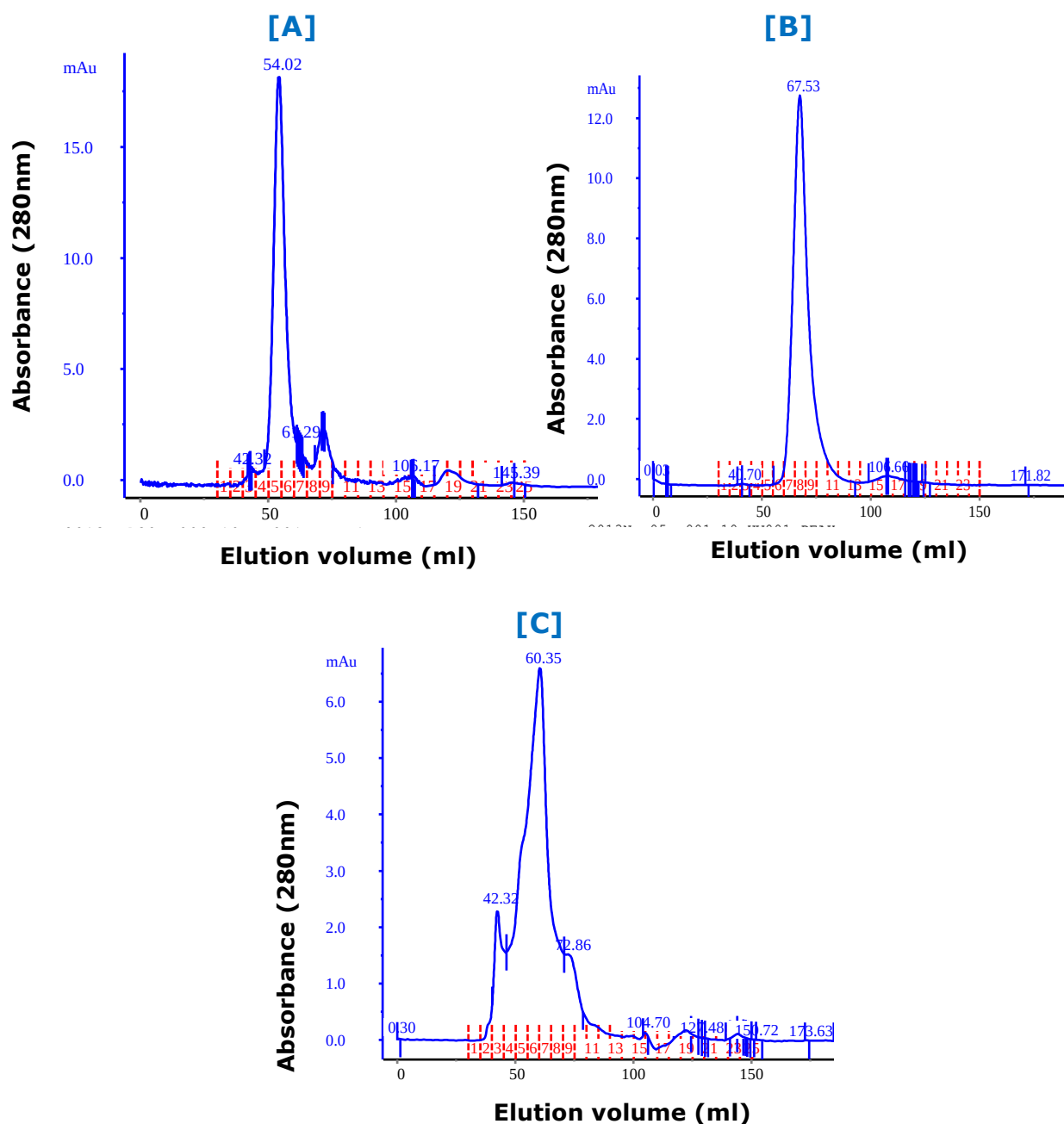


Figure 2.9: Chromatograms show the elution of the IMAC-purified rEno (A), rPrx (B) and rDnaK (C) during gel filtration. As each sample is pumped into the gel filtration column, molecules of higher molecular weight elute earlier than low molecular weight molecules, a feature that was utilized both to remove *E. coli* contaminant proteins and to estimate the molecular weight of the recombinant proteins. In each case, the corresponding fractions were collected, combined and analysed on the denaturing SDS-gel and immunoblot to investigate both the identity and the purity of the eluted protein.

2.4. Discussion

The genome of *N. meningitidis* strain MC58 carries single copies of NMB1285, NMB0946 and NMB0554 encoding meningococcal enolase, peroxiredoxin and DnaK, respectively. Designating these as moonlighting proteins was first suggested by Knaust and co-workers [Knaust et al., 2007]. It is important to mention that although those researchers accomplished the cloning and expression of enolase, peroxiredoxin and DnaK from *N. meningitidis* MC58, they were unable to provide the relevant materials when we requested them. As a result, it was essential to repeat these steps in order to explore the moonlighting activities of these three proteins. NMB1285, NMB0946 and NMB0554 were successfully cloned into the expression vector pQE30, and transformed into *E. coli* JM109. Screening for successful clones was performed by restriction digestion and DNA sequencing. IPTG was added to the mid log phase-growing *E. coli* containing pMAJ1, pMAJ2 or pMAJ3 to induce the overexpression of rEno, rPrx and rDnaK, respectively. To purify these N-terminal His-tagged recombinant proteins, immobilized Nickel affinity chromatography was utilized, exploiting the fact that the His-tag binds strongly, nonetheless reversibly, to immobilized Ni²⁺ ions within the purification column [Porath et al., 1975]. It is noteworthy that other amino acids with imidazole and thiol groups such as tryptophan and cysteine

possess affinity to imidazole that is not dissimilar to that of histidine [Porath et al., 1975, Hemdan and Porath, 1985]. Therefore, to prevent the non-specific binding of any histidine, tryptophan and/or cysteine containing-proteins to Ni^{2+} , the sample protein was prepared and pumped into the purification column using 40mM imidazole containing buffer. As imidazole has a high affinity to bind to Ni^{2+} , it will act as a competitor that will block non-specific binding. Applying a high concentration of imidazole in the elution buffer (300mM) resulted in the release of all the His-tagged protein molecules away from the immobilized Ni^{2+} . One impediment behind the use of imidazole is that it can interfere with downstream protein-mediated applications such as competition assays, crystallography and importantly, it can enhance protein aggregation [Sharma et al., 1992, Hefti et al., 2001]. To avoid such a situation, a desalting step with PBS was performed to exchange the high-imidazole containing elution buffer with PBS. In terms of Eno, aggregates of precipitated protein molecules were immediately seen after the desalting procedure. There are a variety of possible causes for such aggregation, including non-optimal temperature, pH, and/or buffer composition during protein overexpression or the purification procedure [Cleland et al., 1992, Krielgaard et al., 1998, Carpenter et al., 1999, Lu et al., 2001]. Macromolecular crowding due to high protein concentrations, as SDS gels

suggest, may also induce aggregation [Shire et al., 2004]. In terms of peroxiredoxin and DnaK, freezing and thawing when the protein was highly concentrated was also found to enhance aggregation. To circumvent protein precipitation, glycerol, as a protein stabilizer [Gekko and Timasheff, 1981] at 10-25% final concentration in PBS was added directly to each eluted protein. A slight alteration in protein conformation into a more compact, and hence more stable, form is one proposed mechanism to explain the glycerol-mediated protein stability [Priev et al., 1996, Chanasattru et al., 2008]. In addition, the antifreeze property that glycerol lends when added to water [Shepard et al., 1976], via reducing its freezing point, allows the prolonged storage of purified proteins, and minimizes the hostile effect of freezing-thawing cycles on protein quality.

While the results of SDS-PAGE and immunoblot analysis confirmed both the identity and the purity of rPrx, some additional *E. coli* proteins were present (albeit in trace amounts) alongside the purified rEno and rDnaK. In an attempt to remove these and to determine the oligomerization status of each recombinant protein, gel filtration was performed. However, the latter was not efficient in totally removing the contaminant proteins as almost the same level of purity was observed when each protein sample was analysed by SDS-PAGE and immunoblot before and after gel filtration. The molecular mass

of each protein (purified under native conditions) was relatively predicted by comparing the volume at which the recombinant protein eluted with the elution volume values of the standards. Based on this technique, the purified rEno was mostly present as an octamer; this agrees with the oligomerization state of this protein in various *Streptococcal spp.* [Ehinger et al., 2004, Feng et al., 2009, Lu et al., 2012], *Bacillus subtilis* [Brown et al., 1998], and *Lactobacillus gasseri* [Raghunathan et al., 2014]. To our knowledge, this is the first Gram-negative bacterial enolase shown to arrange in octameric masses of the protein. Similarly, rPrx was mostly present as a hexamer, in which the same property was previously suggested to describe the oligomerization of mitochondrial PrxII from *Pisum sativum* [Barranco-Medina et al., 2008]. The dimeric *Toxoplasma gondii* Prx2 can also form non-covalently bound tetramers and hexamers [Deponte and Becker, 2005]. Regarding DnaK, gel filtration analysis revealed that the protein molecules were arranged either as multimers, dimers and monomers, in which the dimeric species of the protein was the most prevalent. These three forms were similarly described in DnaK from *E. coli* using several approaches including gel filtration chromatography, dynamic light scattering, analytical ultracentrifugation, and native gel electrophoresis [Schönfeld et al., 1995]. Although results of estimating the molecular mass of each recombinant

protein were consistent with the literature, more sophisticated methods, such as those mentioned above could be used for a more accurate estimation of these molecular masses.

The natively purified meningococcal rEno, rPrx and rDnaK were then used to raise rabbit polyclonal antibodies targeted against each protein. The ability of each to recognise its protein target was examined by SDS-PAGE and immunoblot analysis.

In summary, the recombinant proteins and specific anti-sera described in this chapter were subsequently utilized to facilitate the investigation of the moonlighting properties of meningococcal enolase, peroxiredoxin and DnaK, and examine the localization of these proteins on the surface of *N. meningitidis*.

3. CHAPTER THREE: Mutagenesis and complementation of the gene encoding peroxiredoxin in *N. meningitidis* MC58

3.1. Introduction

Mutagenesis and complementation is a strategy widely used to investigate the function(s) of the gene product through comparing the phenotype of the resultant mutant strain with that of the original and complemented mutant strains. In *S. pneumoniae*, attempts to delete the gene encoding enolase from the bacterial genome were unsuccessful, and as a result, it was suggested that enolase is probably essential for cell viability [Bergmann et al., 2001]. Similar results were obtained when Knaust and co-workers tried to generate an enolase- or DnaK-deficient meningococcal strain, suggesting that these proteins are essential for bacterial survival under the *in vitro* growth conditions used [Knaust et al., 2007]. On the other hand, several studies have utilized the mutagenesis strategy to investigate the function of peroxiredoxin in *N. meningitidis* [Knaust et al., 2007] and *L. monocytogenes* [Dons et al., 2014]. Unlike enolase and DnaK, any putative moonlighting activity that meningococcal peroxiredoxin may exhibit, can therefore be examined by mutating the gene encoding peroxiredoxin and then characterizing the phenotype of the mutant strain.

Whilst introduction of an antibiotic resistance cassette is the most commonly used method to mutate a gene, it has some limitations. If a strain with several mutated genes is required, the number of mutations will be limited by the number of

different selectable markers available. *Mycobacterium tuberculosis* is a good example to illustrate such a limitation. The bacterial genome carries a minimum of 79 toxin-antitoxin (TA) encoding gene pairs [Sala et al., 2014]. Products of some of these genes sometimes share a similar function. As a consequence, trying to delete one gene may also require deleting other gene(s) that otherwise will compensate for the lost function of the deleted gene. Therefore, if the traditional selectable marker-mediated mutagenesis strategy is to be adopted, then several antibiotic genes need to be introduced, which is not always feasible due to the limited number of resistance genes that are suitable for Mycobacteria [Mao et al., 2016]. Additionally, generating a bacterial strain, especially if pathogenic, with multiple antibiotic resistance properties may not be desirable as it may lead to spread of multi-drug resistant pathogens [O'Brien, 2002]. To avoid this, a markerless mutation can be introduced. One advantage associated with a markerless mutation, is that it allows serial deletions or modification of genes and hence facilitates the evaluation of the relative contribution of each gene product [Banerjee and Biswas, 2008]. This benefit could be utilized in the future since it is already known that, for example, multiple proteins in meningococci contribute to plasminogen binding. In addition, the potential for polar effects that may result from the insertion

of a DNA fragment (*i.e.* the antibiotic selectable marker that replaces the gene to be mutated) is avoided.

This chapter describes the process by which the gene encoding peroxiredoxin from *N. meningitidis* MC58 was deleted from the bacterial genome using a markerless mutation strategy. Complementation of the null mutant strain is also described.

3.2. Materials and methods

3.2.1. *In silico* sequence analysis

UCSC Microbial Genome Browser <http://microbes.ucsc.edu/> (03/10/2000 Assembly) was utilized to investigate the gene encoding peroxiredoxin (designated NMB0946) from *N. meningitidis* MC58 and the upstream and downstream sequences. ARnold online tool <http://rna.igmors.u-psud.fr/toolbox/arnold/index.php> was used to predict the transcription terminator sequence upstream to NMB0946. To facilitate DNA sequences analyses, such as restriction enzyme digestion and primer design, DNAMAN software (Lynnon BioSoft) was employed.

3.2.2. Bacterial strains and growth conditions

E. coli strain JM109 was utilized for propagation of all plasmids described in this chapter. The propagation was performed under the same growth conditions described in section 2.2.2. Where appropriate, antibiotic and chemical agents were used at the following concentrations: ampicillin ($100\ \mu\text{g ml}^{-1}$), kanamycin ($50\ \mu\text{g ml}^{-1}$), IPTG ($0.5\ \text{mM}$) and X-gal ($80\ \mu\text{g ml}^{-1}$). Meningococcal strains (wild type MC58, MC58 *rpsL*⁻ and MC58 *rpsL*⁻ *Kan*^R *rpsL*⁺, the latter provided by H. Albasri; University of Nottingham) were grown on chocolate agar, Brain Heart Infusion (BHI) agar or BHI broth. Where appropriate, kanamycin ($50\ \mu\text{g ml}^{-1}$), streptomycin ($100\ \mu\text{g ml}^{-1}$) and

erythromycin (5 µg ml⁻¹) were used for selection. Cultures were incubated at 37°C in an atmosphere of air plus 5% CO₂ with or without shaking at 200 rpm.

3.2.3. DNA techniques

All techniques involving DNA, including extraction, purification and quantification of genomic and plasmid DNA, amplification by PCR, agarose gel electrophoresis, digestion with restriction enzymes, vector de-phosphorylation, transformation and DNA sequencing were performed as described in chapter two (materials and methods section), unless otherwise stated.

3.2.4. Cloning of NMB0946 and flanking DNA

A DNA fragment of *ca.* 2.7 kb (designated UpxnD) corresponding to NMB0946 (738 bp) flanked by 1 kb up- and 1 kb downstream DNA, was amplified by PCR using UpxnDF1 and UpxnDR1 primers (Table 3.1) and genomic DNA of *N. meningitidis* MC58 as template. This fragment contained a single copy of the DNA uptake sequence (DUS; 5'-GCCGTCTGAA-3'), which is required for any DNA fragment to be recognized and taken up by *Neisseria spp.* [Hamilton and Dillard, 2006], present in the downstream flanking sequence of NMB0946. PCR was performed as previously described under the thermo-cycling conditions given in Table 3.1. The amplicon was cleaned using the GenElute PCR clean up kit (Sigma-Aldrich) following the manufacturer's instructions. Next, an A-

tailing reaction was performed by mixing: 15 μL of the cleaned UpxnD; 6 μL of 5x GoTaq Buffer; dATP to a final concentration of 0.2 mM; and 3 μL of GoTaq polymerase made up to a final volume of 30 μL with dH_2O . The mixture was incubated at 70°C for 30 min. A recombinant plasmid was constructed by ligating A-tailed UpxnD into pGEM[®]-T Easy (Promega; Appendix 4) following the manufacturer's instructions. The recombinant plasmids were used to transform *E. coli* strain JM109 (as previously described in section 2.2.7), and potential clones were cultivated on selective agar plates containing 100 $\mu\text{g ml}^{-1}$ ampicillin, 0.5 mM IPTG and 80 $\mu\text{g ml}^{-1}$ X-gal. Plasmid extraction was performed using the GenElute plasmid miniprep kit (Sigma-Aldrich) following the manufacturer's instructions. Digestion of the resulting plasmid DNA with EcoRI (as described in section 2.2.6) was used to screen for potential desired clones. After confirming with DNA sequencing, a pGEM[®]-T Easy clone containing the UpxnD PCR product was designated pGUD1.

Table 3.1: PCR reactions used in the mutagenesis and complementation of NMB0946.

PCR product	DNA template	Primer sequence* (5'-3')	Thermo-cycling conditions		Amplicon description
			Annealing temperature	Extension time (s)	
UpxnD	NMB MC58	UpxnDF1: CGC AATTGT GAAACCACCGATTATCTTTGGC UpxnDR1: CGC AATTGT CCCAATGGACTTCTACATTGC	64°C	75	NMB0946 coding sequence plus 1 kb up and 1 kb downstream flanking DNA
depxn	pGUD1 plasmid	depxnF1: CGC GGATCC GGCTAAAACTGATAGCTGTTTGC depxnR1: CGC GGATCC ACCGGTACGATCTTGCAAAGC	68°C	180	pGUD1 construct minus NMB0946 coding sequence
KnsL	NMB MC58 <i>rpsL⁻ Kan^R rpsL⁺</i>	KnsLF1: GCG AGATCT CAGCTTATCATCGATAAACCCAGCG KnsLR1: GCG AGATCT GTAATTATTTAGGACGCTTAGCACCG	70°C	60	Kanamycin resistance cassette and <i>rpsL⁺</i> gene (each preceded by its promoter)
PerComp	NMB MC58	PerCompF1: GGAAGAT CTTTGCGGCGCAAGGGCGCAGTTGC PerCompR1: GGAAGAT CTTCAGTTTTTAGCCAAGTAAGCTTCC	60°C	30	NMB0946 coding sequence plus ca. 219 bp upstream flanking DNA

* Sequences in bold indicate recognition sequences of different restriction enzymes.

3.2.5. NMB0946 deletion by inverse-PCR

NMB0946 was removed from pGUD1 by inverse PCR using the primers depxnF1 and depxnR1 (Table 3.1). The PCR was performed as described in section 2.2.4, under the thermo-cycling conditions shown in table 3.1. The PCR product was purified using the GenElute PCR clean up kit (Sigma-Aldrich), and then digested with BamHI at 37°C for 3 h. The resultant product was designated depxn.

3.2.6. Preparation of markers for selection and cloning to depxn

Chromosomal DNA from *N. meningitidis* MC58 *rpsL*⁻ *Kan*^R *rpsL*⁺ was extracted using the GenElute Bacterial genomic DNA extraction kit (Sigma-Aldrich) and used to amplify a DNA fragment spanning the kanamycin resistance cassette (*Kan*^R) and *rpsL*⁺ gene utilizing the primers KnsLF1 and KnsLR1. The PCR was performed as described in section 2.2.4 under the thermo-cycling conditions shown in Table 3.1. The purified amplicon was digested with BglII and designated KnsL. BglII-digested KnsL was then ligated to BamHI-digested depxn in the presence of T4 DNA ligase. The ligated construct was used to transform *E. coli* strain JM109 to propagate and select successful clones. Finally, the sequence of the cloned KnsL was confirmed by DNA sequencing. The resultant construct containing depxn and KnsL was designated pGUD2.

3.2.7. Construction of pGUD3

This plasmid was constructed by the self-ligation of BamHI-digested depxn PCR product (see section 3.2.5), in the presence of T4 DNA ligase. Transformation of *E. coli* strain JM109 was then performed to allow plasmid propagation and selection of successful clones. Finally, the sequence of the cloned NMB0946 flanking DNA (present within depxn) was confirmed by DNA sequencing. The flanking DNA, 1 kb up- and 1 kb downstream of NMB0946, cloned in pGEM®-T Easy was designated pGUD3.

3.2.8. Construction of ectopic complementation plasmid

A DNA fragment, designated PerComp, of 957 bp spanning NMB0946 (738 bp) was amplified by PCR. Primers PerCompF1 and PerCompR1 containing BglII recognition sequences, together with the genomic DNA of *N. meningitidis* MC58 were used with the thermo-cycling conditions described in Table 3.1. After cleaning with GenElute PCR clean up kit, the amplicon was digested with BglII, and ligated into BglII-digested and dephosphorylated pComp-1. The latter was provided by H. Albasri, after being modified from pSAT-12 [Tunio et al., 2010b]. pComp-1 consists of a complementation cassette cloned into pGEM®-T Easy vector. The cassette includes two meningococcal genes, NMB0102 and NM0103, in opposing orientation. The genetic content in between these two genes is

annotated in Figure 3.1. The ligation reaction was then used to transform *E. coli* strain JM109, and plasmid DNA extracted from putative clones, was analysed by BglII-digestion and DNA sequencing. A construct containing PerComp and pComp-1 was designated pLD1.

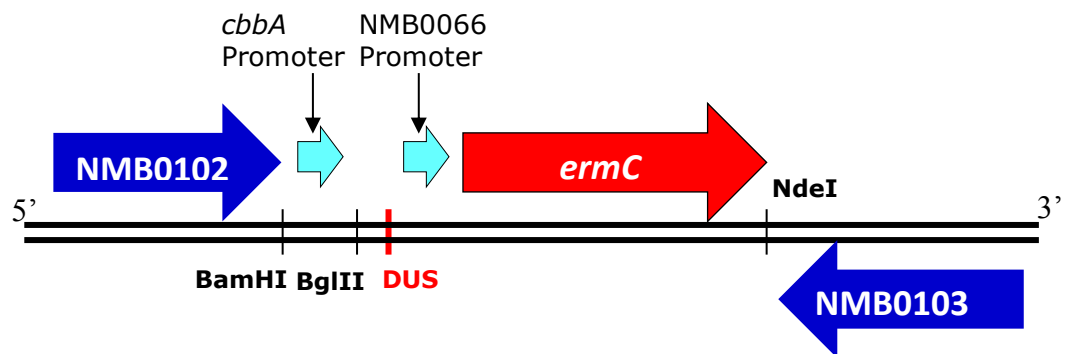


Figure 3.1: A schematic representation of the complementation cassette of pComp-1 plasmid. The cassette consists of two meningococcal genes NMB0102 and NMB0103 in opposing orientation. In between these two genes are: a BamHI recognition sequence, *cbbA* promoter, BglII recognition sequence, DNA uptake sequence (DUS), NMB0066 promoter, NMB0066 gene (*ermC*; confers resistance to erythromycin), and NdeI recognition sequence in a 5'-3' direction.

3.2.9. Natural transformation of *N. meningitidis*

The appropriate *N. meningitidis* strain (Table 3.2) was streaked onto chocolate agar and incubated overnight at 37°C in an air plus 5% CO₂ atmosphere. BHI broth was inoculated with a single colony from the overnight culture, and incubated overnight at 37°C with shaking. The overnight broth culture of *N. meningitidis* was used to inoculate 5 ml of fresh BHI broth to obtain a final optical density (OD_{600nm}) of 0.05, and the latter

broth was further grown to an OD of 0.5. Aliquots of 20 µl of the culture were spotted on BHI agar plates, and left to dry before incubating the plate for 100 min. Two micrograms (in a final volume of 20 µl) of the appropriate mutagenic plasmid (Table 3.2) were added to each spot on the BHI plates, which were then further incubated for 4 h. Selection of the putative transformants was performed by transferring the bacterial growth onto fresh BHI agar plates supplemented with the appropriate antibiotic (Table 3.2). The resulting colonies were analysed by PCR, SDS-PAGE and immunoblot analysis, detailed in sections 2.2.4, 2.2.14 and 2.2.15, respectively.

Table 3.2: Mutagenic plasmids used to mutate different meningococcal MC58 strains.

Mutagenic plasmid	Plasmid description	Targeted strain	Antibiotic used for selection of transformants
pGUD2	Kanamycin resistance cassette and <i>rpsL</i> ⁺ allele from MC58 cloned into pGUD3 at the same NMB0946 deletion site	MC58 <i>rpsL</i> ⁻	Kanamycin (50 µg ml ⁻¹)
pGUD3	Re-ligated pGUD1 after removing NMB0946 encoding gene by inverse PCR	MC58 <i>rpsL</i> ⁻ <i>Δpxn</i> <i>Kan</i> ^R <i>rpsL</i> ⁺	Streptomycin (100 µg ml ⁻¹)
pLD1	NMB0946 gene from MC58 cloned into pComp-1	MC58 <i>rpsL</i> ⁻ <i>Δpxn</i>	Erythromycin (5 µg ml ⁻¹)

3.3. Results

3.3.1. Sequence analysis of NMB0946 gene, flanking DNA and Prx protein

Using UCSC Microbial Genome Browser, NMB0946, encoding peroxiredoxin from *N. meningitidis* MC58, was examined *in silico*. It was found that *methH* (NMB0944), which encodes a putative 5-methyltetrahydropteroyl-triglutamate, is located 277 bp upstream of NMB0946. Similarly, NMB0947, which encodes glutathione oxidoreductase (Gor), is 247 bp downstream of NMB0946. NMB0946 and NMB0947 form a two-gene OxyR regulon, which was previously described in *N. gonorrhoeae* [Seib et al., 2007, Barth et al., 2009]. It should be noted that NMB0947 is incorrectly annotated as dihydrolipoamide dehydrogenase in the *N. meningitidis* MC58 genome [Tettelin et al., 2000]. A transcription termination sequence, predicted by ARNOLD online tool and located downstream of NMB0944 (Figure 3.2), predicted that NMB0944 and NMB0946 genes are independent.

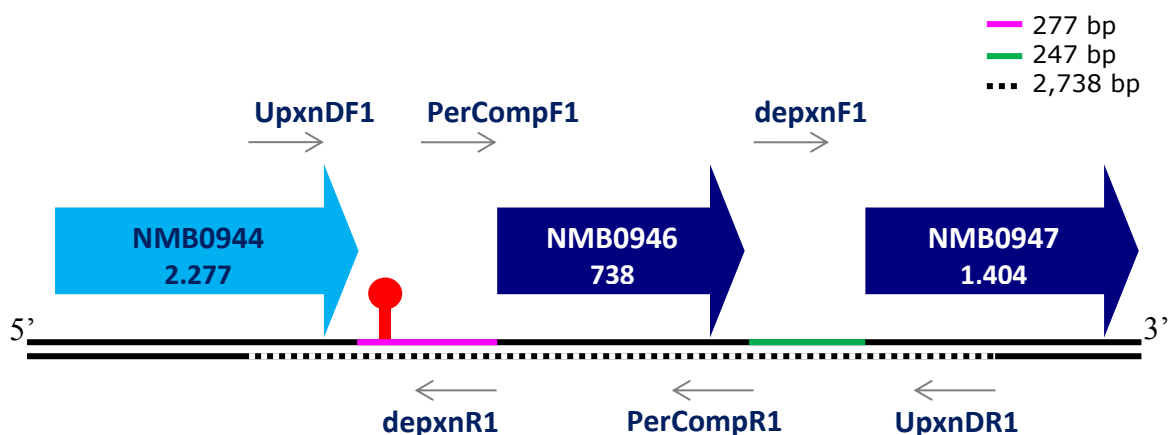


Figure 3.2: A schematic representation of NMB0946 in genomic context of *N. meningitidis* MC58, showing genes orientation, predicted termination sequence (in red) downstream of NMB0944, and approximate binding sites of primers used to facilitate NMB0946 deletion and complementation.

3.3.2. Cloning of NMB0946 and flanking DNA

NMB0946, which comprises 738 bp, plus 1 kb up- and 1 kb downstream flanking DNA (UpxnD) from *N. meningitidis* MC58 was successfully amplified by PCR (Figure 3.3, A). The fragment was then ligated into pGEM®-T Easy. The resulting construct was then used to transform *E. coli* strain JM109. Using blue/white selection, putative white colonies were further examined by extraction of plasmid DNA, followed by restriction digestion and DNA sequencing. Digestion of the extracted plasmid DNA with EcoRI was predicted to produce three fragments of *ca.* 0.5 kb, 2.2 kb (corresponding the insert UpxnD *ca.* 2.7 kb) and 3 kb corresponding to pGEM®-T Easy. All of the six plasmids that were analysed by restriction digestion were

identified as harbouring pGEM®-T Easy containing an insert of the correct size (Figure 3.3, B). Finally, the sequence of the cloned UpxnD was confirmed by DNA sequencing in one of these clones. The sequence was identical to that in the MC58 genome, and so contained no PCR-generated errors. A pGEM®-T Easy containing UpxnD PCR product was designated pGUD1 (lane 1 in Figure 3.3, B).

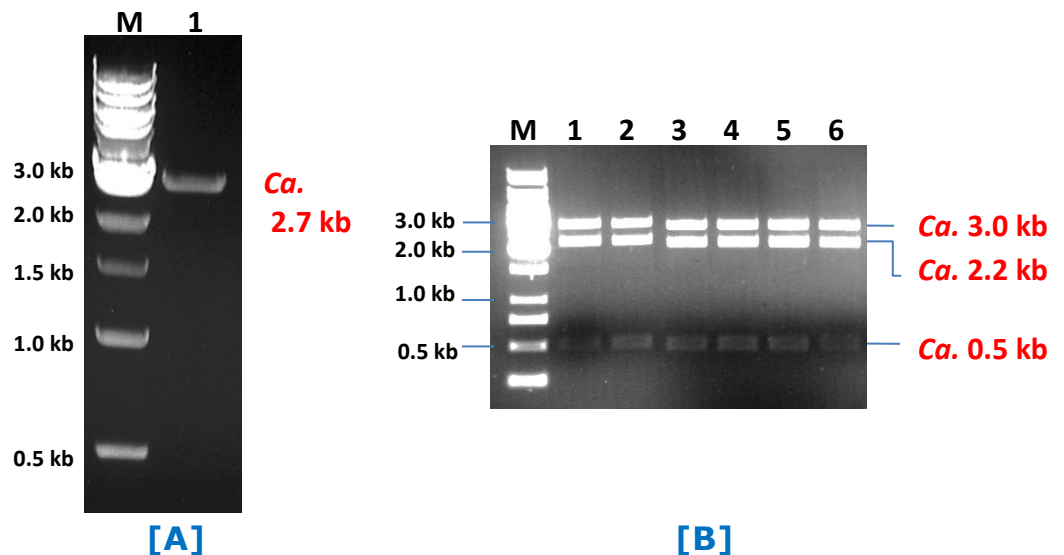


Figure 3.3: Agarose gel analysis showing; [A] PCR amplification of NMB0946 and 1 kb up- and 1 kb downstream flanking DNA (UpxnD) from *N. meningitidis* strain MC58. Lane M: 1 kb DNA ladder, lane 1: PCR product corresponding to UpxnD. [B] Restriction digestion using EcoRI of plasmid DNA to verify the cloning of UpxnD into pGEM®-T Easy. Lane M, 2-log DNA ladder, lanes 1-6: putative pGUD1 clones digested with EcoRI.

3.3.3. NMB0946 deletion by inverse PCR

The recombinant plasmid (pGUD1) was used as a template to amplify the whole construct apart from NMB0946. This was accomplished by inverse PCR using the primers depxnF1 and depxnR1. A PCR product of *ca.* 5 kb, which is of the expected size of the combined NMB0946 flanking sequence (2 kb) and the pGEM®-T Easy vector (3 kb), was generated (Figure 3.4, A). This product was designated depxn.

3.3.4. Preparation of markers for selection and cloning to depxn

Chromosomal DNA was prepared from *N. meningitidis* MC58 *rpsL*⁻ *Kan*^R *rpsL*⁺ and used to amplify a DNA fragment spanning the kanamycin resistance cassette (*Kan*^R) and *rpsL*⁺ genes (each preceded by its own promoter) by PCR. A PCR product of *ca.* 2 kb was obtained (Figure 3.4, B) corresponding to *Kan*^R (1.5 kb) and *rpsL*⁺ (0.5 kb). This amplicon, designated KnsL, was ligated (after digestion with BglII) to BamHI-digested depxn. The ligated construct was utilized to transform *E. coli* JM109 to allow plasmid propagation and selection of successful clones. Finally, the sequence of the cloned KnsL was confirmed by DNA sequencing. The construct containing depxn and KnsL was designated pGUD2.

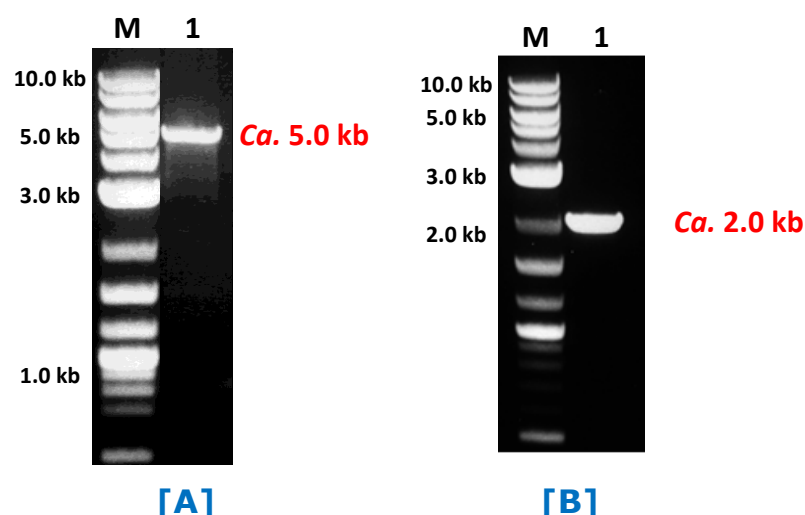


Figure 3.4: Agarose gel analysis showing [A] depxn and [B] KnsL. [A] Inverse PCR was performed to remove NMB0946 DNA sequence from pGUD1. Lane M: GeneRuler™ 1 kb DNA Ladder, lane 1: PCR product depxn. [B] PCR amplification of kanamycin resistance cassette and *rpsL*⁺ gene (collectively designated KnsL) from *N. meningitidis* MC58 *rpsL*⁻ *Kan*^R *rpsL*⁺. Lane M: GeneRuler™ 1 kb DNA Ladder, lane 1: PCR product KnsL.

3.3.5. Construction of pGUD3 vector

A plasmid was constructed by the self-ligation of BamHI-digested depxn PCR product. *E. coli* strain JMI09 was then transformed with this construct. Restriction digestion and DNA sequencing were used to screen potential clones. Digestion of the extracted plasmid DNA with BamHI and EcoRI was predicted to produce four fragments of *ca.* 0.5 kb, 0.5 kb, 1.0 kb (collectively corresponding to the insert *i.e.* NMB0946 flanking DNA; *ca.* 2.0 kb) and 3 kb corresponding to pGEM®-T Easy. Six of the analysed clones were identified to contain pGEM®-T Easy containing an insert of the correct size (Figure 3.5). Finally, the sequence of the cloned NMB0946 flanking DNA was confirmed

by DNA sequencing. A pGEM®-T Easy containing NMB0946 flanking DNA was designated pGUD3 (lane 5 shown in Figure 3.5).

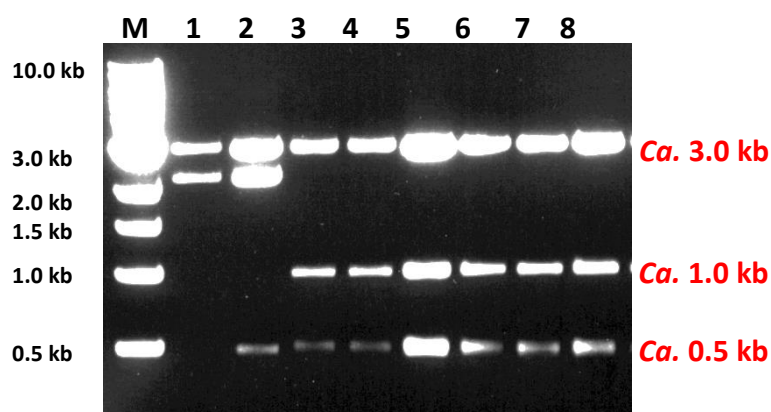


Figure 3.5: Agarose gel analysis showing BamHI and EcoRI restriction digestion of putative pGUD3. Plasmid DNA extracted from each clone was digested with BamHI and EcoRI to verify the cloning of NMB0946 flanking DNA into pGEM®-T Easy. Eight clones were examined Lane M: 1 kb DNA ladder; lanes 3-8: putative pGUD3.

3.3.6. NMB0946 mutagenesis of *N. meningitidis*

Mutagenesis of NMB0946 was accomplished in two stages, schematically represented in Figure 3.6. Firstly, pGUD2 was utilized to transform *N. meningitidis* MC58 *rpsL*⁻. Following homologous recombination, NMB0946 was replaced by the kanamycin resistance cassette and *rpsL*⁺ gene (KnsL). Selection of the successful transformants was made by screening for resistance to kanamycin. This crossover event was verified by PCR by determining the amplicon size of the product generated using the primers UpxnDF1 and UpxnDR1, and KnsLF1 and KnsLR1, respectively. The former primers were designed to

anneal 1 kb up- and 1 kb downstream of NMB0946, respectively. Hence, a product of *ca.* 4 kb corresponding to KnsL flanked by the 2 kb NMB00946 flanking DNA was obtained (Figure 3.7, A, lane 1), while the latter primers resulted in a PCR product of *ca.* 2 kb corresponding to KnsL only (Figure 3.7, A, lane 2). The resultant strain was designated *N. meningitidis rpsL⁻ Δpxn Kan^R rpsL⁺*. In order to remove KnsL from the genome, *N. meningitidis rpsL⁻ Δpxn Kan^R rpsL⁺* was subsequently transformed using pGUD3. This resulted in the replacement of KnsL by the NMB0946 flanking DNA in pGUD3. Resistance to streptomycin was used to screen for a successful recombinant strain, which was verified by PCR using the primers UpxnDF1 and UpxnDR1, which resulted in an amplicon of *ca.* 2 kb corresponding to the NMB0946 flanking DNA only (Figure 3.7, B, lane 3), whereas no PCR product was obtained with the primers KnsLF1 & KnsLR1 (Figure 3.7, B, lane 4), confirming the absence of KnsL. The verified strain was designated *N. meningitidis rpsL⁻ Δpxn*.

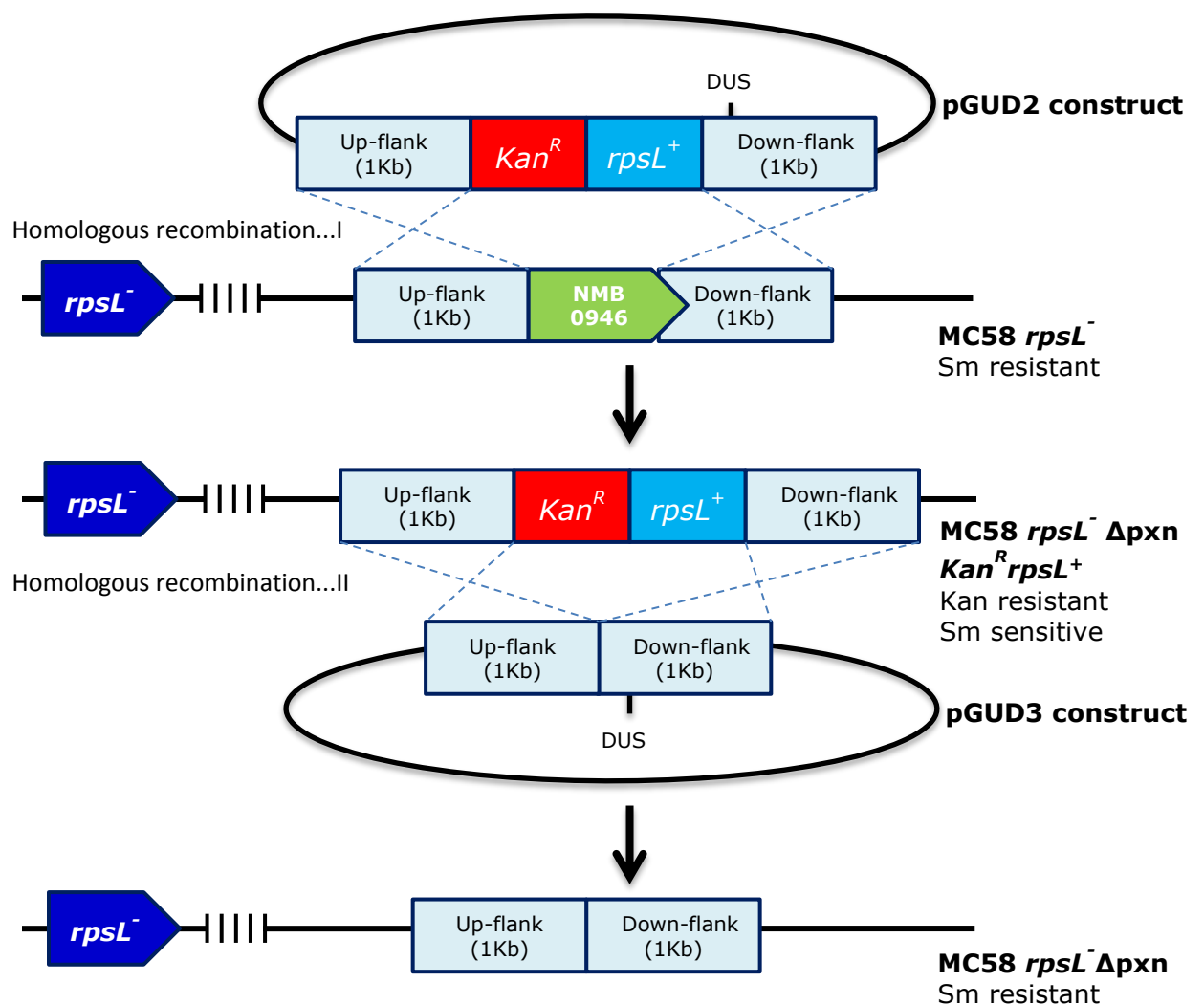


Figure 3.6: Schematic workflow of the mutagenesis strategy that was adopted to delete *NMB0946*. A cassette consisting of a *Kan^R* gene plus an *rpsL⁺* allele was cloned between the 1 kb up- and 1 kb downstream flanking DNA of *NMB0946* to generate pGUD2, which was used to transform the streptomycin (Sm) resistant *MC58 rpsL⁻* strain. At this stage, a homologous recombination event occurred, resulting in the replacement of *NMB0946* carried in the genome by the *Kan^R rpsL⁺* cassette, and the resultant strain was designated *MC58 rpsL⁻ Δpxn Kan^R rpsL⁺*, which was phenotypically resistant to kanamycin (Kan) and sensitive to Sm by virtue of the presence of *Kan^R* and *rpsL⁺* genes, respectively. The latter strain was transformed again by pGUD3, and another recombination event was facilitated. This time, removal of

the *Kan^R rpsL⁺* cassette generated the strain MC58 *rpsL⁻ Δpxn*, thus restoring Sm resistance due to the absence of the *rpsL⁺* dominant allele. DUS denotes DNA uptake sequence. Adapted from: [van Dam and Bos, 2012].

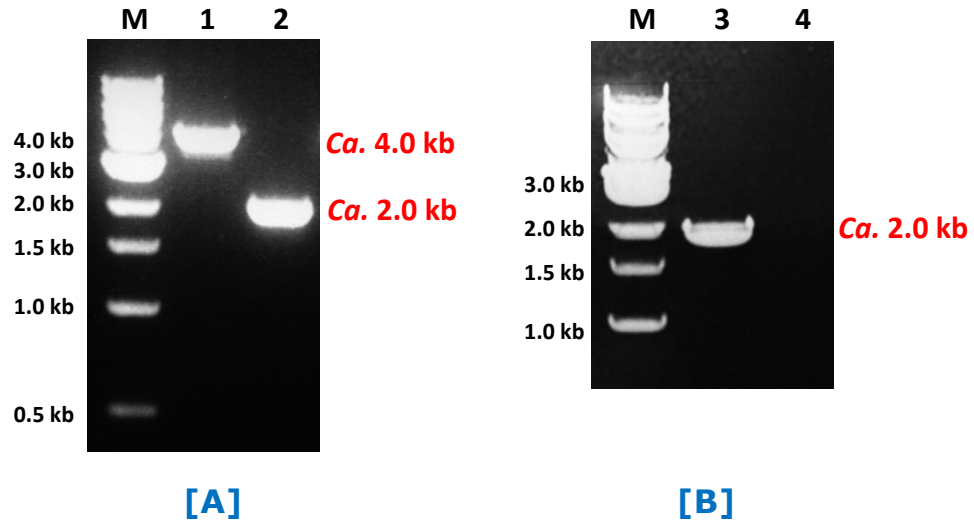


Figure 3.7: Agarose gel analysis showing PCR-mediated verification of NMB0946 mutagenesis. [A] Chromosomal DNA from *N. meningitidis rpsL⁻ Δpxn Kan^R rpsL⁺* was used to confirm NMB0946 mutagenesis by PCR amplification of NMB0946 flanking DNA and KnsL (lane 1) or KnsL only (lane 2) using primers UpxnDF1 and UpxnDR1, and KnsLF1 and KnsLR1, respectively. [B] Chromosomal DNA from putative *N. meningitidis rpsL⁻ Δpxn* was used as a DNA template to amplify NMB0946 flanking DNA (lane 3) using primers UpxnDF1 and UpxnDR1, to confirm the removal of the *Kan^R rpsL⁺* cassette. The use of primers KnsLF1 and KnsLR1 with the same template resulted in no PCR product (lane 4). Lane M: 1 kb DNA ladder.

3.3.7. Ectopic complementation plasmid construction

Construction of the complementation plasmid, pLD1, was achieved in collaboration with Louise Dorrington (M.Sc. dissertation; University of Nottingham). After predicting the transcription terminator sequence of *methH* gene, which is located 277 bp upstream to NMB0946, amplification of NMB0946 (738 bp) plus the 219 bp upstream sequence, potentially carrying the NMB0946 promoter sequence, was performed by PCR, and an amplicon, designated PerComp, of ca. 0.96 kb was obtained (Figure 3.8, A). After cleaning, the amplicon was digested with BglII, and then ligated into BglII-digested and de-phosphorylated pComp-1. *E. coli* strain JM109 was transformed using the ligated construct. Selection of recombinants was based on their resistance to both ampicillin and erythromycin. Restriction digestion and DNA sequencing were used to screen potential clones. Digestion of the extracted plasmid DNA with BglII was predicted to produce two fragments of ca. 0.96 kb and 5.3 kb corresponding to PerComp and pComp-1, respectively. Three of the analysed clones were identified to contain pComp-1 containing an insert of the correct size (Figure 3.8, B). Finally, the sequence of the cloned PerComp was confirmed by DNA sequencing. A pComp-1

containing PerComp was designated pLD1 (lane 1 in Figure 3.8, B).

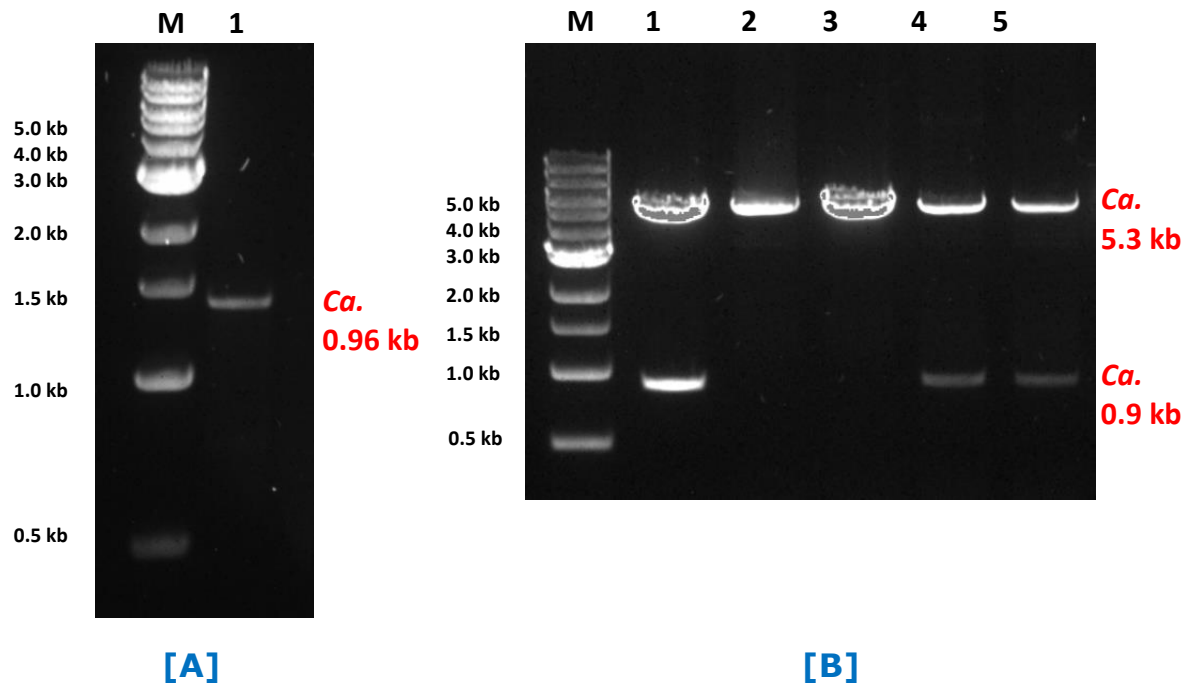


Figure 3.8: Agarose gel analysis showing PerComp amplification and restriction digestion. [A] PCR amplification of NMB0946 coding sequence plus *ca.* 219 bp upstream flanking DNA (PerComp) from *N. meningitidis* strain MC58. Lane M: 1 kb DNA ladder, lane 1: PCR product corresponding to PerComp. [B] BglII restriction digestion of plasmid DNA from 5 different clones (Lanes 1-5), to verify the cloning of PerComp into pComp-1. Lane M: 1 kb DNA ladder; lanes 1, 4 and 5: putative pLD1.

3.3.8. Complementation of NMB0946

The construct pLD1 was utilized to transform the markerless peroxiredoxin knockout strain *i.e.* *N. meningitidis* MC58 *rpsL*⁻ Δ *pxn*, via facilitating a double recombination event. NMB0102 and NMB0103, carried on pLD1, were the two homologous regions that underwent the recombination, which resulted in the

introduction of NMB0946 (preceded by its promoter) plus the *Erm^R* cassette between NMB0102 and NMB0103 in the bacterial genome. Resistance to erythromycin was used to select putative successful transformants. The correct integration was verified by PCR using the primers NMB0102F1 (GTTCGGCGGCTTGCGTGGTCG) and NMB0103F1 (TACGGAGTTGGAAAGCACGGC), which were designed to anneal to the 5' ends of NMB0102 and NMB0103, respectively. As these two genes are in opposing orientation in the bacterial genome, the size of the resultant amplicon would determine whether the desired fragment was present. The amplicon size was *ca.* 1.4 kb when genomic DNA from MC58 *rpsL⁻ Δpxn* was used as a template (Figure 3.9, lane 2), compared to amplicons of *ca.* 3.4 kb, when genomic DNA from erythromycin resistant transformants were used (Figure 3.9, lanes 3 & 4). The resultant strain was designated *N. meningitidis rpsL⁻ Δpxn pxn^{Ect} Erm^R* (Figure 3.9, strain 1 in lane 4).

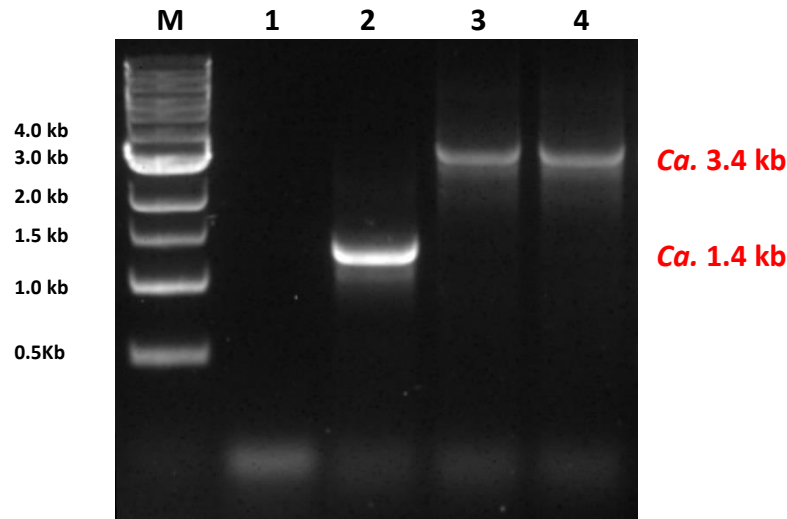


Figure 3.9: Agarose gel analysis showing PCR to verify the complementation of NMB0946 in the genome of MC58 *rpsL*⁻ Δ *pxn*. PCR was performed using primers NMB0102F1 and NMB0103F1 with genomic DNA from: MC58 *rpsL*⁻ Δ *pxn* (lane 2) or putative MC58 *rpsL*⁻ Δ *pxn* *pxn*^{Ect} *Erm*^R strain 1 or 2 (lane 3 and 4, respectively), H₂O was used as a negative control (lane 1). Lane M: 1 kb DNA ladder.

3.3.9. Verification of NMB0946 mutagenesis and complementation

SDS-PAGE and immunoblot analysis were employed to verify both NMB0946 mutagenesis and complementation. The expression of peroxiredoxin (Prx), the gene product of NMB0946, was examined via resolving a whole cell lysate sample prepared from each strain *i.e.* WT MC58, MC58 *rpsL*⁻, MC58 *rpsL*⁻ Δ *pxn* *Kan*^R *rpsL*⁺, MC58 *rpsL*⁻ Δ *pxn* and MC58 *rpsL*⁻ Δ *pxn* *pxn*^{Ect} *Erm*^R by SDS-PAGE. An immunoblot was then probed with the rabbit polyclonal anti-rPrx antiserum. While highly immuno-reactive bands of the expected size, *ca.* 27-kDa, were detected in whole cell lysate samples from WT MC58 and

MC58 *rpsL*⁻ (Figure 3.10, lane 1 & 2, respectively), no band was seen in either of the two NMB0946-null strains (Figure 3.10, lanes 3 & 4). Furthermore, a band of the same size was seen in the whole cell lysate of the complemented strain (Figure 3.10, lane 5). This demonstrated that the expression of NMB0946 was successfully abolished by the mutation, and restored by complementation. Due to the polyclonal nature of anti-peroxiredoxin antibodies that were used, other bands were consistently detected across all strains, suggestive of non-specific binding.

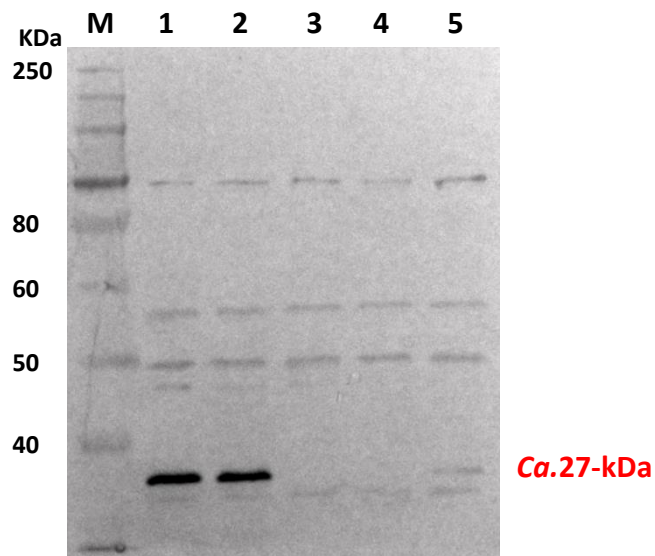


Figure 3.10: Immunoblot analysis verifying the successful mutagenesis and complementation of NMB0946. A whole cell lysate sample was prepared from each analysed strain, resolved on 10% SDS-polyacrylamide gel, blotted onto a membrane and the latter was then probed with polyclonal rabbit anti-rPrx antibodies. The antibodies recognized bands of apparent molecular weight of *ca.* 27-kDa corresponding to peroxiredoxin in WT MC58 (lane 1) and MC58 *rpsL*⁻ (lane 2), while no band of an equivalent molecular weight was seen in the lysate samples of MC58 *rpsL*⁻ Δ *pxn* *Kan*^R *rpsL*⁺ (lane 3) and MC58 *rpsL*⁻ Δ *pxn* (lane 4). A band of the same molecular weight was detected in the lysate of MC58 *rpsL*⁻ Δ *pxn* *pxn*^{Ect} *Erm*^R (lane 5). Lane M: pre-stained protein marker.

3.4. Discussion

Before commencing any genetic manipulation to mutate NMB0946 from *N. meningitidis* MC58, it was important to examine NMB0946 within its genomic context to determine whether or not mutating this gene would affect neighbouring genes. It was found that *methH* gene (NMB0944), which was predicted to encode 5-methyltetrahydropteroyl-triglutamate that catalyses the transfer of a methyl group from 5-methyltetrahydrofolate to homocysteine to form methionine [Ferla and Patrick, 2014], is located 277 bp upstream to NMB0946. While these two genes are both encoded on the same (sense) strand, it is unlikely that they are arranged within an operon or controlled by the same promoter, as they are: (i) reasonably distant from each other, and (ii) unlike many genes organised within an operon, performing distinct and apparently unrelated functions [Salgado et al., 2000]. Identifying the transcription termination sequence of *methH* further confirmed that *methH* and NMB0946 are independent. On the contrary, NMB0946 and NMB0947, which encodes glutathione oxidoreductase (Gor) and is located 247 bp downstream of NMB0946, form a two-gene OxyR regulon, which was previously described in *N. gonorrhoeae* [Seib et al., 2007, Barth et al., 2009]. The fact that these two genes are organized within an

operon makes constructing a complemented strain alongside any peroxiredoxin mutant strain, a necessity.

The streptomycin resistance phenotype that the *rpsL*⁻ allele confers was the basis behind the markerless mutation strategy that was adopted to generate the NMB0946-knockout in *N. meningitidis* MC58 *rpsL*⁻ strain. The wild type *rpsL* gene (*i.e.* *rpsL*⁺) encodes ribosomal protein S12, which interacts with 16S rRNA that in turns mediates protein translation [Sparling et al., 1968]. In susceptible cells, streptomycin can interfere with 16S rRNA, and inhibit protein synthesis. It has been established in many organisms, including *N. meningitidis* [Johnston and Cannon, 1999], that mutations in *rpsL*⁺ (or in *rrs* that encodes 16S rRNA), affects the ability of streptomycin to interfere with 16S rRNA, and as a consequence, blocks its inhibitory effect on protein synthesis [Sparling et al., 1968]. Importantly, in an organism that is an *rpsL*-merodiploid *i.e.* carries the WT allele (*rpsL*⁺; which confers sensitivity to streptomycin) and the mutated allele (*rpsL*⁻ ; which confers resistance to streptomycin) in the same genome, *rpsL*⁺ is dominant, and hence the organism remains sensitive to streptomycin [Breckenridge and Gorini, 1970].

To facilitate the markerless deletion of NMB0946, two mutagenic plasmids, pGUD2 and pGUD3, each carrying two

homologous regions of the NMB0946 flanking DNA sequence, were constructed to allow two homologous recombination events. In the first recombination event, NMB0946 from the streptomycin resistant *N. meningitidis* MC58 *rpsL*⁻ strain was replaced by the kanamycin cassette and *rpsL*⁺ gene (KnsL) carried on pGUD2. At this stage, the insertion of the wild type dominant *rpsL*⁺ allele left transformed cells sensitive to streptomycin, so selection of the successful transformants was made by screening for resistance to kanamycin. The resultant strain was designated *N. meningitidis rpsL*⁻ Δ *pxn* *Kan*^R *rpsL*⁺. Although obtaining a NMB0946-null strain had been accomplished, the goal was to remove the selectable marker and generate a markerless NMB0946 mutant. To achieve this, *N. meningitidis rpsL*⁻ Δ *pxn* *Kan*^R *rpsL*⁺ was transformed using pGUD3, and the second homologous recombination event was facilitated, thereby replacing both the *Kan*^R and the *rpsL*⁺ genes with the NMB0946 flanking DNA. As both the kanamycin resistance cassette and *rpsL*⁺ were removed, screening for resistance to streptomycin could be used to select for successful transformants. The final markerless NMB0946 null strain was designated *N. meningitidis rpsL*⁻ Δ *pxn*. It can be expected that having no DNA fragment inserted at the NMB0946 deletion locus in this strain, may diminish, if not abolish, potential polar effects on the transcription of genes located downstream to NMB0946.

However, and due to multiple genetic manipulations, deleting a gene can be accompanied by secondary undetected mutation(s), which can consequently alter the phenotype of the mutant, making interpretation of the impact of deleting the gene more complicated. For this reason, it was necessary to generate a complemented strain for use in functional assays, in which a wild type copy of NMB0946 was successfully reintroduced into the genome of MC58 *rpsL*⁻ Δ *pxn* at an ectopic site. The complemented strain was used later as a control to rule out those polar effects during the phenotypic characterization of NMB0946 mutant strain.

To confirm the insertion or deletion of different DNA fragments into the genome of the targeted strain during NMB0946 mutation and complementation, PCR was first employed. This was further verified by immunoblot analysis, which revealed that while protein bands corresponding to peroxiredoxin were apparent in the whole cell lysate of WT MC58 and MC58 *rpsL*⁻ strains, they were absent in both NMB0946-null strains. Interestingly, a less intense band was seen in the lysate sample of the complemented strain. Although detecting such a band confirmed that peroxiredoxin expression, due to NMB0946 complementation, had successfully been restored, it suggests a lower level of protein expression. Any potential impact of such a reduction in peroxiredoxin expression in the complemented

strain compared to the wild type MC58 was examined later (section 5.3.1) by monitoring the growth rate of all generated strains.

To summarize, a markerless NMB0946-knockout was successfully generated in *N. meningitidis* MC58 *rpsL*⁻, and a WT copy of NMB0946 was reintroduced into the bacterial genome to complement that deletion. The generated strains will be utilized to characterize the function(s) of peroxiredoxin.

4. CHAPTER FOUR: Investigating the binding of meningococcal rEno, rPrx and rDnaK to human glu-plasminogen

4.1. Introduction

Human plasminogen is produced in the liver as a preproenzyme consisting of 810 amino acids. Upon secretion from the liver, the amino terminal 19-amino-acid signal peptide is cleaved generating a 90-kDa proenzyme, glu-plasminogen, which has, as the name suggests, a glutamic acid residue at the N-terminus [Raum et al., 1980, Forsgren et al., 1987]. Glu-plasminogen (Plg) forms a closed diamond shaped structure that consists of seven domains including a pre-activation peptide (PAP), followed by five kringle domains (KR, 1-5) and a serine protease domain (SP) at the C-terminal end of the protein [Ponting et al., 1992]. Releasing the 8-kDa pre-activation peptide, via breaking the peptide bond between either Arg⁶⁷-Met⁶⁸, Lys⁷⁶-Lys⁷⁷ or Lys⁷⁷-Val⁷⁸, results in a truncated protein termed lys-plasminogen, which displays a more open U-shaped structure that makes the molecule more accessible to activation by plasminogen activators, compared to glu-plasminogen [Claeys and Vermylen, 1974, Weisel et al., 1994]. KR domains are repeating triple-disulfide-bonded units, each around 80 amino acids in length. Interaction of different ligands with Plg is thought to be mediated via KR domains. In most cases, the lysine residue (Lys) at the C-terminal end and/or internal Lys residues of these ligands bind to aromatic residues that form hydrophobic clefts called lysine binding sites (LBS) carried on

KR domains [Sanderson-Smith et al., 2012]. Differences in the composition of these LBS result in a disparity in the binding affinity of each KR to Lys (or similar amino acids), with KR1 exhibiting the highest and KR2 the weakest, affinity to Lys, respectively [Marti et al., 1997]. KR3 lacks such an affinity [Söhndel et al., 1996]. Importantly, maintaining the closed structure, and hence the less-reactive form, of Plg is achieved by the intramolecular interaction of these LBS with lysine residues [Ponting et al., 1992, Law et al., 2012]. However, such an interaction is weakened when Plg is in contact with a Lys-containing ligand, which competes for LBS binding. As a consequence, conformational changes in the structure of the bound Plg occur, resulting in the opening of the closed diamond structure exposing the Arg⁵⁶¹-Val⁵⁶² activation loop. The latter can then be readily attacked by plasminogen activators (PA) such as tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA) that activate Plg to plasmin [Ponting et al., 1992, Ranson and Andronicos, 2003].

The enzymatically active surface-bound plasmin (Plm) exhibits wide-ranging proteolytic activity. Plm can directly degrade components of ECM and basal membrane [Plow et al., 1995], and also can activate other proteolytic enzymes [Murphy et al., 1999]. The outcome is a series of sequential proteolytic events,

which further contributes to the pathogenesis of several bacterial species [Sanderson-Smith et al., 2012].

An early study established the binding of Plg to the surface of 24 different *N. meningitidis* strains. However, in spite of demonstrating that the lysine analogue ϵ -aminocaproic acid (EACA) is a binding inhibitor, and thus suggesting the contribution of lysine binding sites of Plg in the interaction, the Plg receptor(s) were not identified [Ullberg et al., 1992]. Later, Knaust and co-workers confirmed, using flow cytometry and immuno-gold labelling, the ability of meningococcal strain MC58 to recruit plasminogen on the bacterial cell surface. Furthermore, they identified enolase, peroxiredoxin and DnaK as putative Plg binding ligands on the surface of the organism, although the presence of additional ligands was suspected. They also suggested that internal Lys residues rather than the C-terminal Lys of each of the proposed meningococcal Plg ligands are probably involved in the interaction [Knaust et al., 2007]. However, the importance of the C-terminal Lys residue in the binding of different ligands to Plg has been well documented [Christensen, 1985, Derbise et al., 2004, Grundy et al., 2007]. In *Streptococcus pneumoniae*, Bergmann and colleagues first identified enolase as a Plg ligand on the surface of bacteria, and they described the two C-terminal Lys residues of pneumococcal enolase as the pivotal site for the protein-Plg interaction

[Bergmann et al., 2001]. Later, an internal nine amino acid Lys-rich motif (FYDKERKVY) was identified as a second binding site (BS2) for the pneumococcal enolase-Plg interaction [Bergmann et al., 2003]. Conversely, Ehinger and colleagues, who solved the crystal structure of enolase and suggested that it was an octamer, predicted that while the C-terminal Lys residues of the protein are at inter-dimer position, and hence less likely to be involved in Plg binding, the nonameric peptide (BS2) is well exposed, and hence more likely to be involved in Plg binding [Ehinger et al., 2004]. Recently, Fructose-1,6-bisphosphate aldolase (FBA), a glycolytic enzyme that was earlier shown to be surface-localized and required for optimal adhesion of meningococci to human cells [Tunio et al., 2010b], was also found to recruit Plg in a dose-dependent manner [Shams et al., 2016]. The contribution of the C-terminal Lys, rather than other sub-terminal Lys residues, in mediating FBA-Plg interaction was also reported [Shams et al., 2016]. As it is presently uncertain whether or not the C-terminal Lys is important in Plg binding, further investigation is required to answer this question.

Meningococcal peroxiredoxin is a hybrid protein consisting of a Prx domain and a glutaredoxin (Grx) domain that respectively constitute the N-terminal and the C-terminal regions of the protein [Rouhier and Jacquot, 2003]. While this natural fusion is unusual among peroxiredoxins, it has also been described in

other bacterial species including *Chromatium gracile* [Vergauwen et al., 2001] and *Haemophilus influenzae* [Pauwels et al., 2003]. The Grx domain acts as an electron donor for the oxidised Prx, restoring it to the reduced form. It was earlier shown that a mutation in the second cysteine active site of the Grx (*i.e.* C¹⁸⁵) resulted in an inactive hybrid protein that was unable to reduce different hydroperoxides [Rouhier and Jacquot, 2003]. Whether or not enzyme activity is required for Plg binding is unclear.

The aim of work described in this chapter was to investigate the binding of the previously purified meningococcal recombinant enolase, peroxiredoxin and DnaK to human glu-plasminogen, and also to identify Lys residues responsible for these interactions.

4.2. Materials and methods

4.2.1. Enzyme-linked immunosorbant assay (EIA)

EIA was utilized to assess the binding of recombinant proteins to human glu-plasminogen (Plg; Callibiochem). Microplate wells (ThermoFisher Scientific; Nunc immobilizer amino 12×8 strips) were first coated with 100 µl aliquots of 5 pmol of the appropriate recombinant protein in sodium carbonate buffer (10 mM sodium carbonate Na_2CO_3 ; 90 mM sodium bicarbonate NaHCO_3 ; pH 9.4). As a positive control, 5 pmol of Plg in the same buffer was used in the coating step. Similarly, in order to measure non-specific binding, 1% bovine serum albumin (BSA; Sigma-Aldrich; A3059) in PBS (1%BSA/PBS) was added in the coating step. After overnight incubation at 4°C with gentle agitation, the plate was washed with PBS containing 0.05% Tween 20 (PBST0.05%) three times, and then 100 µl of 1%BSA/PBS were added for blocking at room temperature (RT) for 2 h. The washing step was repeated and then 100 µl of either 5 pmol Plg in 1% BSA/PBS (binding step) or 1% BSA/PBS alone was added, and the plate was incubated at RT for 2 h with gentle shaking. After removing the unbound proteins by washing, 100 µl of goat anti-plasminogen antibody (Rockland Immunochemicals; 1:10000 diluted in 1% BSA/PBS) was added to each well and the plate was incubated overnight at 4°C with gentle agitation. After repeat washing, 100 µl of 1:5000-diluted

donkey anti-goat IgG-alkaline phosphatase conjugate (Promega) was added to each well. After a final washing step, 200 μ l of 1 mg ml⁻¹ phosphatase substrate (Sigma-Aldrich) in glycine buffer (99 mM glycine; 0.99 mM ZnCl₂; 0.99 mM MgCl₂; pH 10.4) were added to each well, and the absorbance was measured at 405 nm using a Biotek EL 800 reader.

4.2.2. Binding inhibition assay

For binding inhibition, the EIA protocol, described in section 4.2.1, was performed. However, this time 50 mM of ϵ -aminocaproic acid (EACA; Sigma-Aldrich) was added to Plg prior to its addition to the EIA plate.

4.2.3. Bacterial strains and growth conditions

E. coli strain JM109 (Promega) was the host strain used for propagation of all plasmids that were constructed in this chapter. Similarly, the expression of recombinant proteins was performed utilizing the same strain. Bacteria were cultivated as previously described in section 2.2.2.

4.2.4. Primer design

Mutagenic primers were designed using a NEBaseChanger online tool (<http://nebasechanger.neb.com/>), following their instructions. In each primer, a nucleotide triplet that encodes the targeted residue was replaced by (GCA) encoding the amino acid alanine (Table 4.1).

Table 4.1: Mutagenic primers and thermo-cycling conditions applied in PCR reactions to mutate NMB1285, NMB0946 and NMB0554.

Primer	Primer sequence* (5'-3')	Targeted residue(s)	Annealing temperature /Extension time
Primers used to mutate NMB1285 cloned into pMAJ1			
Q5enokk250253AA-F	GGC GC ATACCACTTGGAAGCCGAAG	Lysine (K) residues 250 and 253	66°C / 140 s
Q5enokk250253AA-R	GTCT GC GTAGAACTCGCTGGAGGC		
Q5enoK297A-F	GGAAGGCTGG GC ACTGCTGACCG	K297	65°C / 140 s
Q5enoK297A-R	CAGTCGTTTTTCATCCATGCC		
Q5enoK337A-F	ATTGCTGGTC GC AGTCAATCAAATCGG	K337	62°C / 140 s
Q5enoK337A-R	GCGTTTGCTACGCCTTTTTTC		
Q5enoK355A-F	CGACTTAGCC GC ACGCAACCGCTA	K355	67°C / 140 s
Q5enoK355A-R	ACGGCTTTCAGGGTCTCG		
Q5enoK388A-F	TATGCAGATC GC AACCGGTTCTTTGAGCC	K388	67°C / 140 s
Q5enoK388A-R	CAGTTGGTGGCGACTGCC		
Q5enoK400A-F	CCGCATGGCG GC ATACAACCAAC	K400	65°C / 140 s
Q5enoK400A-R	TCGGAACGGCTCAAAGAAC		
Q5enoK428A-F	CCAACTGGGCG GC ATAAGGATCCG	K428 (C-terminal)	62°C / 140 s
Q5enoK428A-R	TAGAATGCGGCTTTGCTG		

Primer	Primer sequence* (5'-3')	Targeted residue(s)	Annealing temperature /Extension time
Primers used to mutate NMB0946 cloned into pMAJ2			
Q5pxnC185A-F	TTGCCAATTC G CAGCTAAAGCCAAAC	Cysteine (C) residue 185	58°C / 120 s
Q5pxnC185A-R	CCTGGTTTtagTGAAAATTGC		
Q5pxnK230A-F	CATCGGCGGT G CATACATCGGCG	K230	64°C / 120 s
Q5pxnK230A-R	AAGACTTGAGGGGCAGTC		
Q5pxnK244A-F	TTACTTGGCT G CAAACTGAGGATC	K244 (penultimate)	59°C / 120 s
Q5pxnK244A-R	GCTTCCAAATCTTCGCTG		
Primers used to mutate NMB0554 cloned into pMAJ3			
Q5DnaK641KA-F	AAAAGACGAC G CAAAATAATACCGTCTG	K641(penultimate)	59°C / 170 s
Q5DnaK641KA-R	ACTTCTTCAAAGTCGGCATC		
Q5DnaK642KA-F	AGACGACAA G CATAATACCGTCTG	K642 (C-terminal)	58°C / 170 s
Q5DnaK642KA-R	TTTACTTCTTCAAAGTCGGC		

* Letters in bold indicate mutagenic sequence.

4.2.5. DNA extraction, quantification and analysis

The GenElute plasmid miniprep kit (Sigma-Aldrich) was used for plasmid DNA extraction, following the manufacturer's instructions. DNA quantification and agarose gel electrophoresis were performed as detailed in sections 2.2.3 and 2.2.5, respectively.

4.2.6. Site directed mutagenesis

Q5 Site-Directed Mutagenesis Kit (New England BioLabs) was used in all mutagenesis reactions, following the manufacturer's instructions. Constructing each mutation was achieved in three stages as detailed below.

4.2.6.1. PCR-mediated mutagenesis

Briefly, a mixture of PCR components was prepared including: 2 × Q5-Hot Start High-Fidelity master mix to a final 1 × concentration; plasmid DNA to a final concentration of 15-20 ng per reaction; each of the respective primers (Table 4.1) to a final concentration of 0.5 μM. The reaction mixture was made up to a final volume of 25 μl with dH₂O. The PCR conditions were initial template denaturation step at 98°C for 30 s, followed by 25 cycles of incubation including: denaturation at 98°C for 10 s, annealing for 30 s, and extension at 72°C. The temperature used for annealing differed depending on the primer pair used; the extension time used depended on the expected size of the

amplicon, but was generally 30 s/1Kb. A final extension step at 72°C for 2 min was also included (Table 4.1).

4.2.6.2. Kinase-Ligase-DpnI (KLD) treatment

One-microliter PCR product was mixed thoroughly with an equal volume of 10 × KLD enzyme mix (provided in the Q5 Site-Directed Mutagenesis Kit), in the presence of 5 × KLD reaction buffer. The reaction mixture was made up to 10 µl with dH₂O, and incubated at 37°C for 8 min.

4.2.6.3. Transformation

Instead of using NEB 5-alpha competent *E. coli* recommended by the mutagenesis kit, *E. coli* JM109 competent cells were used as a host to facilitate the overexpression of recombinant proteins. Transformation was performed as previously detailed in section 2.2.7.

4.2.7. DNA sequencing

Sequencing was performed by Source BioScience utilizing the Sanger chain-termination method. Primers pQE30-F and pQE30-R were used to confirm the site-directed mutagenesis in NMB1285, NMB0946 and NMB0554, cloned into pMAJ1, pMAJ2 and pMAJ3, respectively. In terms of NMB1285 and NMB0554, primers eno-F2, DnaK-F2 and DnaK-F3 (Table 2.1), were also used to facilitate sequencing.

4.2.8. Protein techniques

All protein analysis techniques used in this chapter have been described previously. They include expression of recombinant proteins (2.2.10), IMAC-mediated protein purification (2.2.11), desalting and concentration of proteins (2.2.12), SDS-PAGE analysis (2.2.14), immunoblot analysis (2.2.15) and protein quantification (2.2.16).

4.2.9. Protein structure analysis

ClustalW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>) was utilized for the multiple amino acid sequence alignment of enolase from *N. meningitidis* MC58 (NP_274305.1), *S. pneumoniae* R6 (NP_358630.1), *S. pyogenes* M1 GAS (NP_268959.1), *Enterococcus hirae* ATCC 9790 (WP_010718611.1) and *Bacillus subtilis* subsp. *subtilis* str.168 (NP_391270.1).

In addition, pneumococcal enolase (PDB entry 1W6T) was used as a model to predict the positions of Lys residues in meningococcal enolase. Cn3D4.3.1 structure viewer software was employed to serve this purpose.

4.2.10. Statistical analysis

GraphPad Prism (v.6.05) was utilized to perform statistical analyses. All samples were analysed in triplicate and each experiment was repeated no less than three times. Statistical significance was measured using a two-tailed Student's t-test. A *p*-value of > 0.05 was considered non-significant (ns), while values ≤ 0.05 , ≤ 0.01 , ≤ 0.001 or ≤ 0.0001 were considered significant (*), very significant (**), highly significant (***) and very highly significant (****), respectively.

4.3. Results

4.3.1. Meningococcal rEno binds Plg more strongly than rDnaK and rPrx

The binding of wild type (WT) rEno, rPrx and rDnaK to human glu-plasminogen (Plg), compared with BSA, which was used as a negative control, was examined. All recombinant proteins were able to bind Plg significantly (Figure 4.1). The minimal BSA-Plg binding confirmed that the binding of each tested recombinant protein is unlikely to be due to a non-specific interaction. While rEno demonstrated the highest binding affinity, rPrx and rDnaK showed lower, though significant, binding to Plg. Furthermore, rDnaK was shown to bind Plg significantly better than rPrx (Figure 4.1).

Using the same approach, the potential contribution of Lys residues in mediating the binding of each recombinant protein to Plg was also examined by adding EACA to Plg before adding the latter to each recombinant protein. In all cases, the binding of Plg to each protein was significantly reduced, suggesting that the interaction of each recombinant protein with Plg can be Lys-mediated (Figure 4.1).

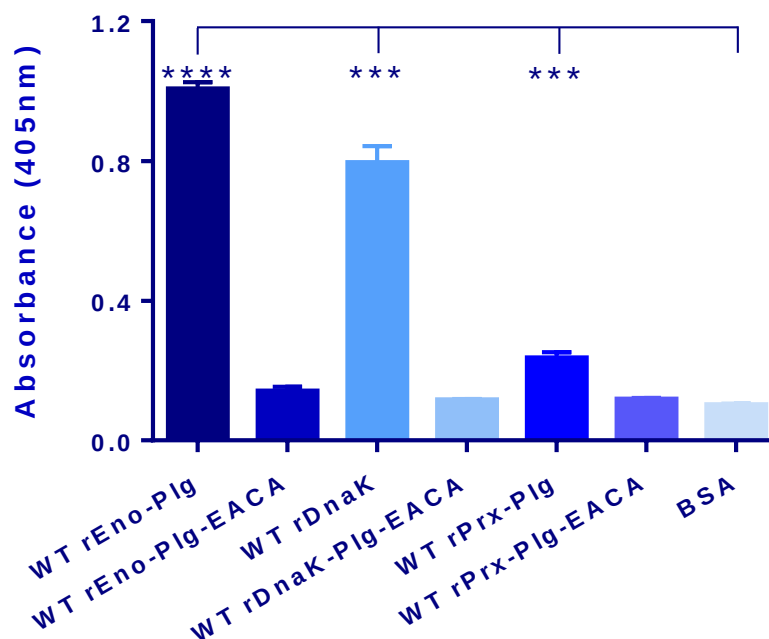


Figure 4.1: The binding of meningococcal rEno, rPrx and rDnaK to human glu-plasminogen (Plg) in the presence or absence of ϵ -aminocaproic acid (EACA). Equal numbers of molecules (5 pmol/well) of each purified protein were used to coat EIA wells, and then the same number of Plg molecules, with/out 50 mM EACA, was added. Plg binding was detected by adding goat anti-Plg primary antibody and then donkey anti-goat IgG-alkaline phosphatase conjugate. Bovine serum albumin (BSA) was used as a negative control. Meningococcal rEno, rPrx and rDnaK were significantly able to bind Plg with a p -value of < 0.0001 , 0.0008 and 0.0001 , respectively. In each case, the addition of EACA reduced the binding. Six independent experiments, each in triplicate, were performed. Error bars reflect SE.

4.3.2. Site-directed mutagenesis of NMB1285, NMB0946 and NMB0554

To facilitate mutagenesis, the appropriate construct *i.e.* pMAJ1, pMAJ2 or pMAJ3, together with the desired mutagenic primers pair (Table 4.1) were used to substitute nucleotides that encode either a Lys residue (AAA) or a cysteine residue (TGC) at the desired position(s) with an alanine residue (GCA) by PCR. A

representative example is shown in Figure 4.2, lane 1. After PCR, the DNA was treated with KLD (Figure 4.2, lane 2), a mixture of three enzymes including kinase, ligase and DpnI. This mixture generates and ligates the blunt ends of the amplified PCR product and digests the methylated *E. coli* JM109-generated DNA template. The KLD-treated DNA was then used to transform *E. coli* JM109 cells. After overnight incubation, several colonies were observed. DNA sequence analysis of extracted plasmids revealed that in each case, desired substitution(s) (e.g. 428KA in rEno; Figure 4.3) had been successfully obtained. Moreover, sequencing of each clone also demonstrated the absence of other mutations. The verified constructs are shown in Table 4.2.

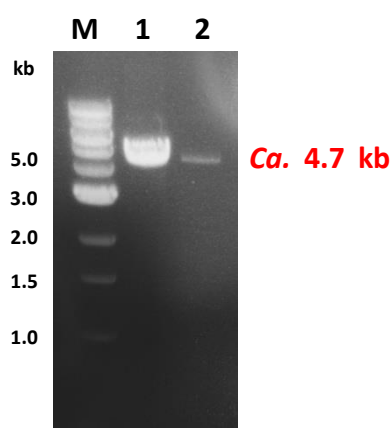
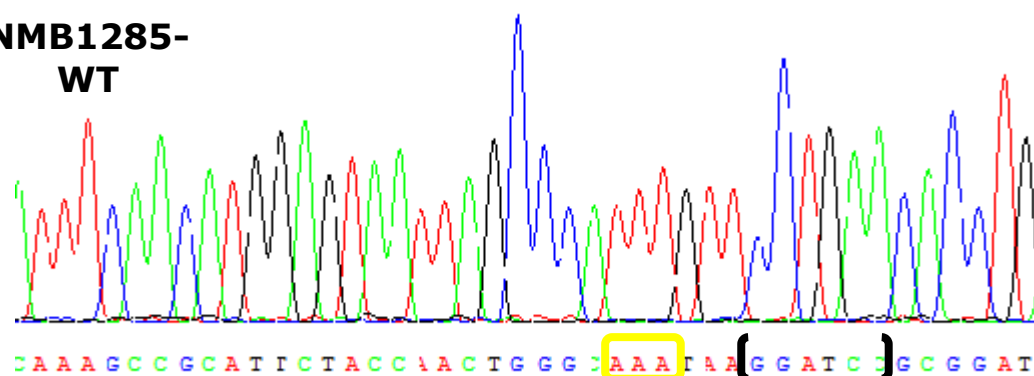


Figure 4.2: Agarose gel analysis showing the PCR amplification of pMAJ1-428KA. pMAJ1 plasmid DNA was used as a template with Q5enoK428A-F and Q5enoK428A-R mutagenic primers to amplify the whole plasmid while generating a single lysine to alanine substitution at position 428 of enolase by PCR. Lane M: 1kb DNA ladder, lane 1: PCR product corresponding to putative pMAJ1-428KA, lane 2: KLD-treated PCR product.

To construct pMAJ2-230244KKAA, pMAJ2-244KA plasmid DNA (Table 4.2), together with Q5pxnK230A-F and Q5pxnK230A-R primers set (Table 4.1) were used to introduce the desired substitution. Steps of site-directed mutagenesis procedure that was earlier described were similarly performed.

**NMB1285-
WT**



**NMB1285-
428KA**

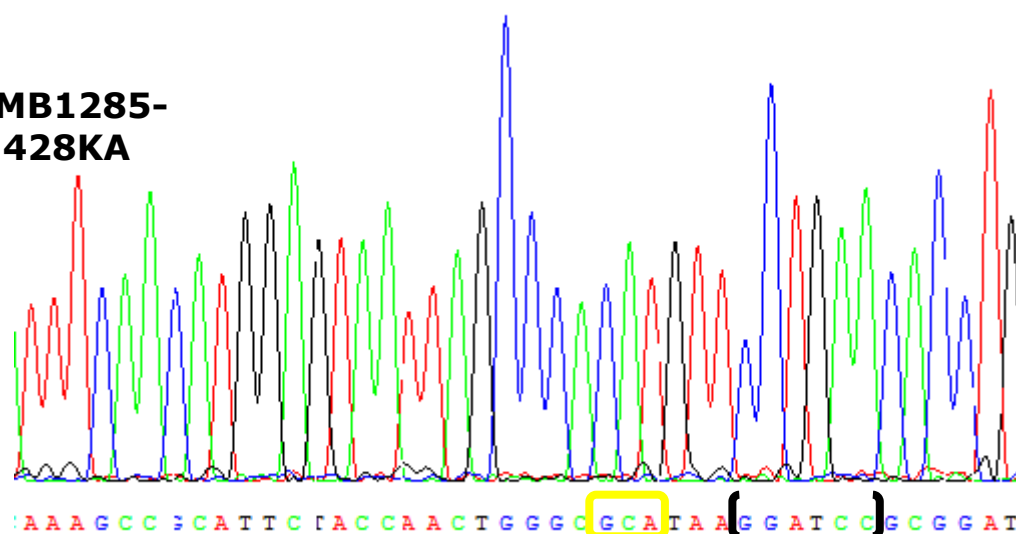


Figure 4.3: Snapshots of DNA sequence chromatograms of wild type (WT) NMB1285 gene and NMB1285-428KA, showing substitution of the triple codon AAA, encoding the C-terminal lysine of meningococcal enolase, in the WT gene to GCA codon, encoding an alanine, to generate pMAJ1-428KA mutant. A BamHI recognition sequence (GGATCC, in black brackets) is also seen directly after the stop codon (TAA) that follows the C-terminal encoding triple codon (encircled in yellow).

Table 4.2: List of plasmids that were used to express different recombinant meningococcal proteins.

Plasmid	Expected protein after expression in <i>E. coli</i> JM109	Source
pMAJ1-250253KKAA	rEno250253KKAA with two lysine (K) to alanine (A) amino acid substitutions at positions 250 and 253	This study
pMAJ1-297KA	rEno297KA with single K to A amino acid substitution at position 297	This study
pMAJ1-337KA	rEno337KA with single K to A amino acid substitution at position 337	This study
pMAJ1-355KA	rEno-355KA with single K to A amino acid substitution at position 355	This study
pMAJ1-388KA	rEno388KA with single K to A amino acid substitution at position 388	This study
pMAJ1-400KA	rEno400KA with single K to A amino acid substitution at position 400	This study
pMAJ1-428KA	rEno428KA with the C-terminal K to A amino acid substitution at position 428	This study
pMAJ2-185CA	rPrx185CA with single cysteine (C) to A amino acid substitution at position 185	Collaboration with R. Ohri
pMAJ2-230KA	rPrx230KA with single K to A amino acid substitution at position 230	Collaboration with R. Ohri
pMAJ2-244KA	rPrx244KA with the C-terminal K to A amino acid substitution at position 244	This study
pMAJ2-230244KKAA	rPrx230244KKAA with two K to A amino acid substitutions at positions 230 and 244	This study
pMAJ3-641KA	rDnaK641KA with single K to A amino acid substitution at position 641	This study
pMAJ3-642KA	rDnaK642KA with the C-terminal K to A amino acid substitution at position 642	This study

4.3.3. Expression of recombinant mutated rEno, rPrx and rDnaK

E. coli JM109 cells harbouring one of the plasmids outlined in Table 4.2 were used to express the corresponding recombinant protein. In each instance, after 3 h induction with 0.5 mM IPTG, optimal expression was obtained. This was determined by SDS-PAGE, which revealed bands of *ca.* 48-kDa, 27-kDa and 69-kDa, which are the expected sizes of rEno, rPrx and rDnaK, respectively. The identity of each His-tagged fusion protein was confirmed by immunoblot analysis using anti-penta-histidine antibodies. As a representative example, the overexpression of rEno428KA, analysed by SDS-PAGE and immunoblot, is shown in Figure 4.4.

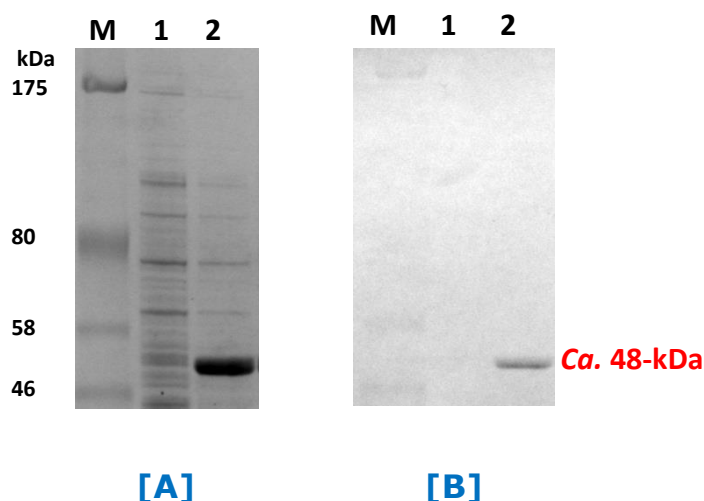


Figure 4.4: 10% SDS-PAGE (A) and immunoblot (B) probed with anti-penta-histidine antibodies demonstrating the rEno428KA overexpression in IPTG-induced *E. coli* JM109 (pMAJ1-428KA). Lane M: pre-stained protein marker, lane 1: whole cell lysate of un-induced cells, lane 2: whole cell lysate of 0.5 mM IPTG-induced cells.

4.3.4. Purification of His-tagged mutated rEno, rPrx and rDnaK under native conditions

All recombinant proteins, described in Table 4.2, were purified under native conditions, utilizing IMAC. The purification process was performed in three stages similar to those described in section 2.3.4. A desalting step with PBS was then performed to exchange the imidazole-containing elution buffer with PBS. Finally, fractions containing the desired protein were combined together and the protein concentrated. Glycerol, at 10-25% final concentration in PBS was added directly to the desalted protein. Immunoblot analysis, using anti-penta-histidine antibodies, confirmed that each purified protein was His-tagged (Figure 4.5 for rEno428KA; data not shown for other purified proteins).

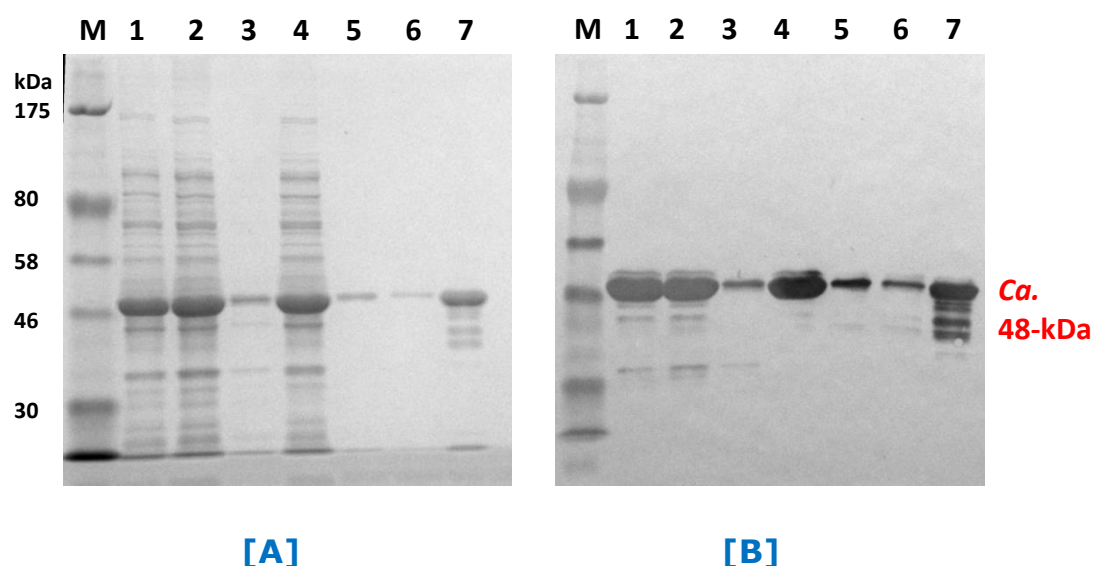


Figure 4.5: SDS-PAGE and immunoblot analysis demonstrating the purification of His-tagged rEno428KA under native conditions. SDS-PAGE (10%; A) confirmed the purity of the recombinant protein. Immunoblot analysis (B) showed that recombinant His-tagged protein is recognized by anti-penta-histidine antibodies. The protein was traced throughout purification steps. Lane M: pre-stained protein marker, lane 1: whole cell lysate, lane 2: soluble lysate, lane 3: pellet, lane 4: flow through, lane 5: wash, lane 6: fraction 2, lane 7: desalted and concentrated fractions 3-10, corresponding to the purified protein.

4.3.5. The C-terminal lysine residue of rEno, rPrx and rDnaK contributes to plasminogen binding

Initially, the contribution of the C-terminal Lys residue of meningococcal rEno (K428), rDnaK (K642) and also rPrx, which has a Lys residue in the penultimate position of the amino acid sequence of peroxiredoxin, to plasminogen binding was assessed. Recombinant proteins, each with the desired substitution, were successfully generated (section 4.3.4). The binding of each of the mutated proteins to Plg was then examined. Compared with the wild type rEno, rDnaK and rPrx, the binding of rEno428KA, rDnaK642KA and rPrx244KA, respectively, to Plg was significantly reduced (Figure 4.6). Importantly, the addition of EACA to Plg resulted in a further significant reduction in the binding, suggesting that in addition to the C-terminal Lys; other Lys residue(s) may be involved in the interaction with Plg.

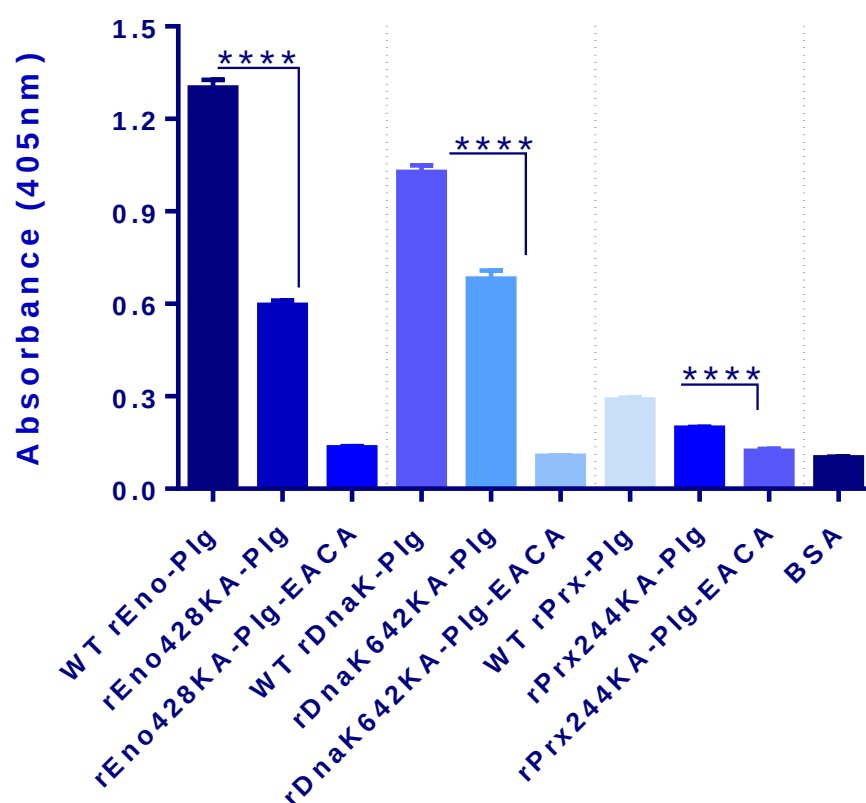


Figure 4.6: The C-terminal lysine residues of meningococcal rEno, rPrx and rDnaK contribute to plasminogen binding. Binding of meningococcal rEno428KA, rDnaK642KA and rPrx244KA to human glu-plasminogen (Plg) was examined by EIA, which showed that rEno428KA, rDnaK642KA and rPrx244KA were significantly reduced in Plg binding compared with the wild type version of each protein. The addition of EACA further reduced the binding affinity to Plg. **** denotes a p -value < 0.0001 . Six independent experiments, each in triplicate, were performed. Error bars reflect SE.

In terms of meningococcal DnaK, the C-terminal Lys (*i.e.* K642) is preceded by another Lys residue at position 641. The potential involvement of this penultimate Lys in mediating DnaK-Plg interaction was also investigated.

Figure 4.7 shows that, unlike rDnaK642KA, rDnaK641KA exhibited a very similar Plg binding pattern to that of the wild type, suggesting that the penultimate Lys residue of DnaK has no impact on the interaction with Plg.

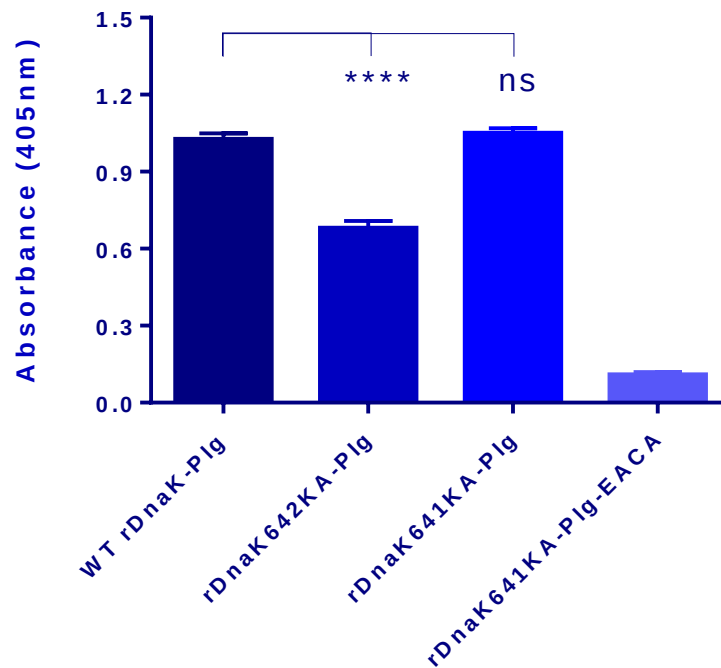


Figure 4.7: The penultimate lysine residue of meningococcal rDnaK is not involved in plasminogen binding. The binding of meningococcal WT rDnaK, rDnaK642KA and rDnaK641KA to human glu-plasminogen (Plg) was examined by EIA. Whilst substituting K642 of rDnaK significantly reduced the ability of the protein to bind Plg compared with the wild type (WT), substituting the penultimate lysine (K641) of the same protein did not significantly alter Plg binding. The addition of EACA further reduced the binding to Plg. **** and ns denote < 0.0001 and non-significant p -value, respectively. Four independent experiments, each in triplicate, were performed. Error bars reflect SE.

4.3.6. Conserved lysine residues of enolase and predicting their positions

While substituting the C-terminal Lys of rEno, rDnaK and rPrx significantly reduced the binding affinity to Plg, it did not abolish it, suggesting the involvement of other Lys residue(s) in the interaction. As meningococcal rEno was the strongest Plg-binder, compared with rDnaK and rPrx, it was initially chosen to further investigate the putative contributions of these other Lys residues. In addition to 428K, meningococcal rEno contains 23 Lys residues; because it was not feasible to substitute each of these residues to examine their roles in the interaction, it was necessary to apply certain criteria to narrow down the number of residues to be substituted. Lys residues that are highly conserved among different species, was the main feature used to select candidate residues. To identify highly conserved Lys residues within bacterial enolases, an amino acid sequence alignment of several enolase sequences from different bacteria was performed (Figure 4.8). The alignment revealed that only seven Lys residues are fully conserved among all sequences examined. Based on meningococcal MC58 enolase numbering, these residues are K56, K103, K297, K337, K355, K388 and K400.

<i>S. pneumoniae</i>	MSIITDVYAREVLDSRGNPTLEVEVYTESGAFGRGMVPSGASTGEHEAVELRDGDKSRYG	60
<i>S. pyogenes</i>	MSIITDVYAREVLDSRGNPTLEVEVYTESGAFGRGMVPSGASTGEHEAVELRDGDKSRYL	60
<i>E. hirae</i>	MSIITDVYAREILDSRGNPTIEVEVYTESGAFGRGMVPSGASTGEYEAVELRDGDKARYG	60
<i>B. subtilis</i>	MPYIVDVYAREVLDSRGNPTVEVEVYTTETGAFGRALVPSGASTGEYEAVELRDGDKDRYL	60
<i>N. meningitidis</i>	MSAIVDIFAREILDSRGNPTVECDVLLESVGMRAAVPSGASTGQKEALELRDGDKSRYL	60
	* . * . : : *	
<i>S. pneumoniae</i>	GLGTQKAVDNVNNIIAEAIIGYDVRDQQAIDRAMIALDGTPTNKGKLGANAILGVSIIVAR	120
<i>S. pyogenes</i>	GLGTQKAVDNVNNIIAEAIIGYDVRDQQAIDRAMIALDGTPTNKGKLGANAILGVSIIVAR	120
<i>E. hirae</i>	GKGVTKAVDNVNNIIAEAIIGYDVRDQQAIDKAMIALDGTPTNKGKLGANAILGVSIIVAR	120
<i>B. subtilis</i>	GKGVLTAVNNVNEIIAPELLGFDVTEQNAIDQLLIELDGTENKGKLGANAILGVSMACAR	120
<i>N. meningitidis</i>	GKGVLKAVEHVNNQIAQALIGIDANEQSYIDQIMIELDGTENKGNLGANATLAVSMAVAR	120
	* . * . : : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : *	
<i>S. pneumoniae</i>	AAADYLEIPLYSYLGGFNTKVLPTPMMNIIINGGSHSDAPIAQEFMILPVGAPTFFKEALR	180
<i>S. pyogenes</i>	AAADYLEVPLYTYLGGFNTKVLPTPMMNIIINGGSHSDAPIAQEFMIMPVGAPTFFKEGLR	180
<i>E. hirae</i>	AAADYLEVPLYHYLGGFNTKVLPTPMMNIIINGGSHADNSIDFQEFMIMPVGAPTFFKEALR	180
<i>B. subtilis</i>	AAADFLQIPLYQYLGGFNSKTLVPVPMNIVNGGEHADNNVDIQEFMIMPVGAPNFREALR	180
<i>N. meningitidis</i>	AAAEDSGLPLYRYLGGAGPMSLPVPMNIVNGGEHANNLSNIQEFMIMPVGAKSFREALR	180
	*** : : *	
<i>S. pneumoniae</i>	YGAEIFHALKKILKSRGLETAVGDEGGFAPRFEGTEDGVETILAAIEAAGYVPGKD-VFL	239
<i>S. pyogenes</i>	WGAEVFHALKKILKERGLTVAVGDEGGFAPKFEGTEDGVETILKAIEAAGYEAGENGIMI	240
<i>E. hirae</i>	MGAEVFHALAAILKSRGLATSVGDEGGFAPNLGSNEEGFEVIIEAIEKAGYVPGKD-VVL	239
<i>B. subtilis</i>	MGAQIFHSLKSVLSAKGLNTAVGDEGGFAPNLGSNEEALQTIVEAIEKAGFKPGEE-VKL	239
<i>N. meningitidis</i>	CGAEIFHALKKLDCSKGFPTTVGDEGGFAPNLNSHKEALQLMVEATEAAGYKAGED-VLF	239
	** : : *	
<i>S. pneumoniae</i>	GFDCASSEFYDKERKVVYDYTKFEGEGAAVRTSAEQIDYLEELVNKYPIITIEDGMDENDW	299
<i>S. pyogenes</i>	GFDCASSEFYDKERKVVYDYTKFEGEGAAVRTSAEQVDYLEELVNKYPIITIEDGMDENDW	300
<i>E. hirae</i>	AMDAASSEFYDKERKVVY---VLADSGEGEKTDEMIFYEELVSKYPIISIEDGLDENDW	296
<i>B. subtilis</i>	AMDAASSEFYNKEDGKY---HLSGEG-VVKTSDEMVDWYEELVSKYPIISIEDGLDENDW	295
<i>N. meningitidis</i>	ALDCASSEFYK---DGY---HLEAEG-RSYTNAEFAEYLEGLVNEFPIISIEDGMDENDW	293
	. : * . * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : *	
<i>S. pneumoniae</i>	DGWKALTERLGKKVQLVGDDFFVTNTDYLRARGIQEGAANSILIKVNQIGTLTETFEAIEM	359
<i>S. pyogenes</i>	DGWKVLTERRLGKRVQLVGDDFFVTNTTEYLARGIKENAANSILIKVNQIGTLTETFEAIEM	360
<i>E. hirae</i>	DGFEKLTDLVGDKVQLVGDDLFVTNTQKLSEGIEKGIANSILIKVNQIGTLTETFEAIEM	356
<i>B. subtilis</i>	EGHKLLTERLGKKVQLVGDDLFVTNTKKLSEGIKNGVGSILIKVNQIGTLTETFDIAIEM	355
<i>N. meningitidis</i>	EGWKLLTEKLGRVQLVGDDLFVTNPKILAEIEKGVANALLKVNQIGTLSETLKAVDL	353
	: * * * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : *	
<i>S. pneumoniae</i>	AKEAGYTAVVSHRSGETEDSTIADIATNAGQIKTGSLSRDTRIAKYNQLLRIEDQLGE	419
<i>S. pyogenes</i>	AKEAGYTAVVSHRSGETEDSTIADIATNAGQIKTGSLSRDTRIAKYNQLLRIEDQLGE	420
<i>E. hirae</i>	AKEAGYTAVVSHRSGETEDSTISDIATNAGQIKTGSLSRDTRIAKYNQLLRIEDQLGE	416
<i>B. subtilis</i>	AKRAGYTAVISHRSGETEDSTIADIATNAGQIKTGAPSRDTRIAKYNQLLRIEDQLAE	415
<i>N. meningitidis</i>	AKRNRYASVMSHRSGETEDSTIADLAVATNCMQIKTGSLSRSDRMAKYNQLLRIEELAE	413
	** . : : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : *	
<i>S. pneumoniae</i>	VAEYRGLKSFYNLKK- 434	
<i>S. pyogenes</i>	VAQYKGIKSFYNLKK- 435	
<i>E. hirae</i>	VAEYKGLKSFYNLKNK- 432	
<i>B. subtilis</i>	TAQYHGINSFYNLKNK- 430	
<i>N. meningitidis</i>	AADYPSKAAFYQLGK- 428	
	. : * * . : * : * : *	

Figure 4.8: Multiple amino acid sequence alignment of *N. meningitidis* MC58 enolase compared with that of *S. pneumoniae* R6, *S. pyogenes* M1 GAS, *Enterococcus hirae* and *Bacillus subtilis* subsp. *subtilis* str.168. Seven internal lysine residues (K; highlighted in yellow) are universally conserved among these sequences, including K56, K103, K297, K337, K355, K388 and K400 based on

meningococcal MC58 enolase numbering. Lysine residues at positions 250 and 253, highlighted in green, are equivalents to K251 and K254 within the internal motif FYDKERKVY (highlighted in red), which was previously nominated as a second binding site that mediates the interaction of pneumococcal enolase with plasminogen [Bergmann et al., 2003]. Positions that are fully, strongly or weakly conserved are denoted by an asterisk, a colon and a full-stop, respectively.

4.3.7. Pneumococcal enolase as a model to predict the 3D structure of meningococcal enolase

Both pneumococcal [Bergmann et al., 2001] and meningococcal enolases [Knaust et al., 2007] were found to be localized at the surface of bacteria where they can bind human Plg. The solved crystal structure of pneumococcal enolase suggests that the protein is present as an octamer [Ehinger et al., 2004], as described in this study for meningococcal enolase (see section 2.3.5). For the above reasons, and others detailed later, the pneumococcal enolase crystal structure was used as a model to predict the 3D position of conserved residues, highlighted in Figure 4.8, in relation to the C-terminal end of the protein. Lys residues at position 297, 337 and 335 are close to the C-terminal Lys residue (K428), and are also predicted to be surface-exposed, making them the most likely candidates for mediating the rEno-Plg interaction (Figure 4.9).

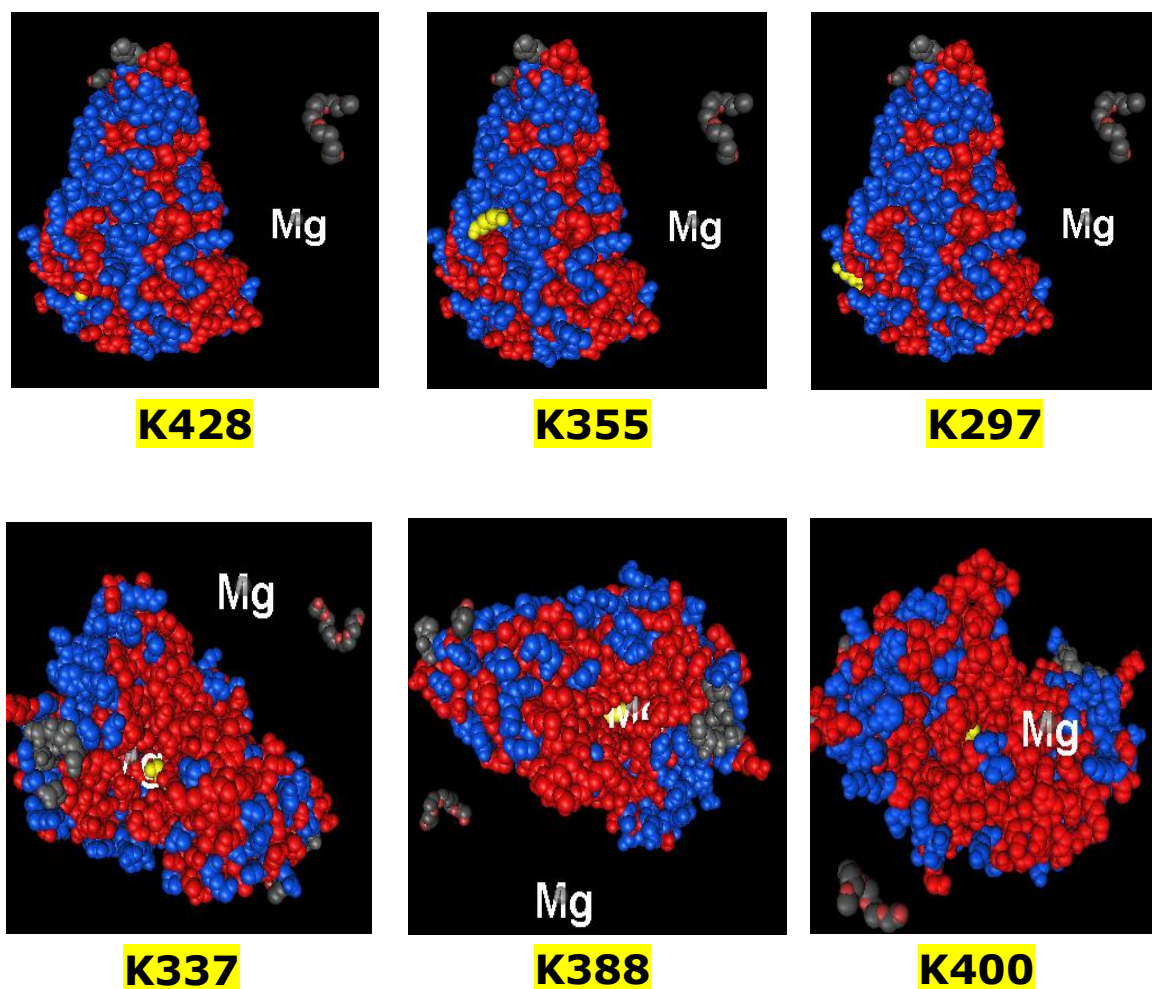


Figure 4.9: Schematic representation of a monomeric pneumococcal enolase (PDB entry 1W6T) used to model positions of conserved internal lysine residues of meningococcal enolase. The identification number of each lysine residue, highlighted in yellow, is based on meningococcal numbering. Each of these figures was prepared using Cn3D4.3.1 structure viewer software. Positively charged residues are in blue, negative in red and neutral in grey. Mg refers to one of two Mg²⁺ metal binding sites in pneumococcal enolase, which requires two divalent cations for the protein activity [Faller et al., 1977].

4.3.8. Lysine residues 297 and 355 of meningococcal rEno are required for optimal plasminogen binding

As shown earlier, Lys residues at positions 297, 337 and 355 of meningococcal enolase were initially nominated as possible candidates for mediating the rEno-Plg interaction. As a consequence, each of these residues was individually substituted with an alanine, and corresponding recombinant enolases were successfully expressed and purified (Table 4.2). The binding of each to Plg was then examined. Similarly, an earlier study identified the internal motif FYDKERKVY, as a second Plg binding site for pneumococcal enolase [Bergmann et al., 2003]. The two Lys residues within this nonameric sequence correspond to Lys 250 and 253 in meningococcal enolase, and due to the fact that both pneumococcal and meningococcal enolases share several features, the concurrent significance of these two Lys residues in mediating meningococcal rEno-Plg interaction was also examined. While rEno250253KKAA and rEno337KA adopted a similar binding pattern as that of the WT, and no significant differences in binding affinities were recorded, rEno297KA and rEno355KA bound Plg significantly less efficiently than WT rEno, suggesting that K297 and K355 are required for optimal interaction with Plg. As expected, the

addition of EACA significantly reduced the binding of each protein to Plg (Figure 4.10).

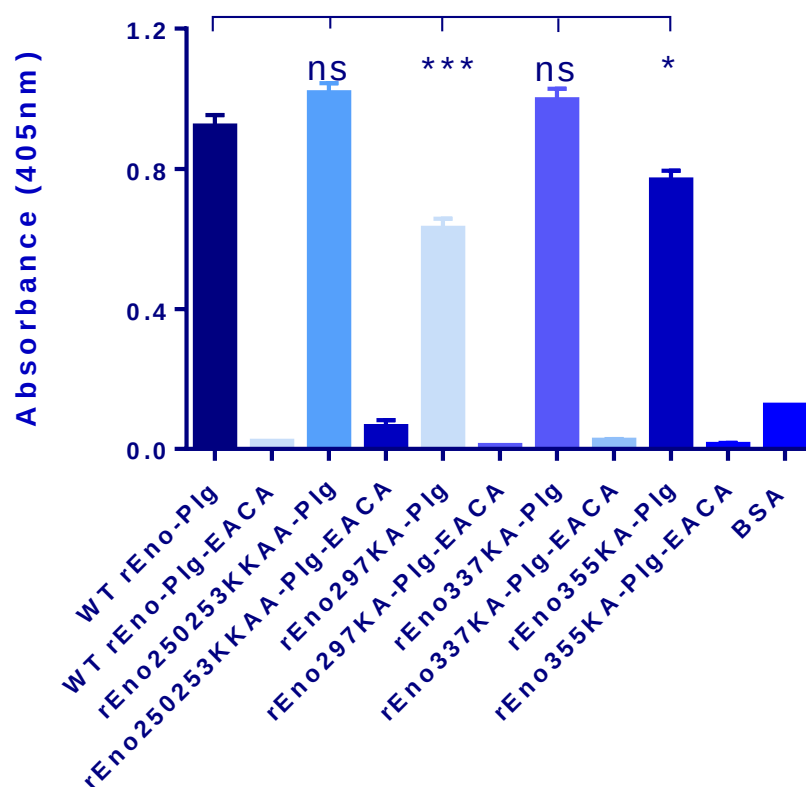


Figure 4.10: Lysine residues 297 and 355 of meningococcal rEno are required for optimal plasminogen binding. The binding of meningococcal rEno250253KKAA, rEno297KA, rEno337KA and rEno355KA to human glu-plasminogen (Plg) was investigated by EIA. Unlike rEno250253KKAA and rEno337KA, rEno297KA and rEno355KA were significantly less able to bind Plg, compared to the wild type (WT). In each instance, the addition of EACA further reduced the Plg binding affinity. ***, * and ns denote a *p*-value of 0.0005, 0.0116 and non-significant value, respectively. Four independent experiments, each in triplicate, were performed. Error bars reflect SE.

Other conserved internal lysine residues including K388 and K400 were also tested for their contribution to rEno-Plg interactions. After expressing and purifying rEno388KA and

rEno400KA, the binding affinity of each to Plg was compared with the WT. Neither of these introduced substitutions had a significant effect on the binding of rEno to Plg (Figure 4.11). Again, the addition of EACA massively reduced the binding of each protein to Plg (Figure 4.11).

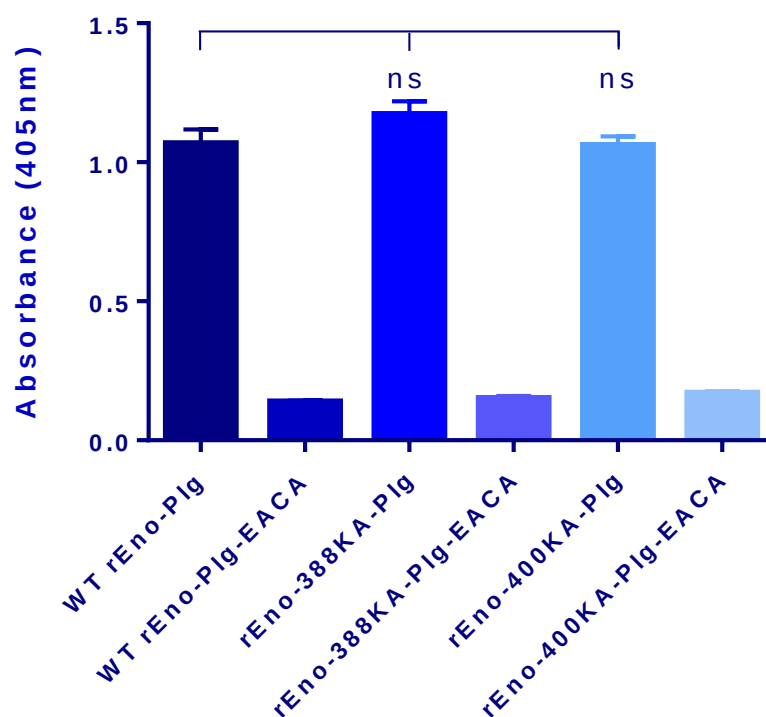


Figure 4.11: Binding of meningococcal rEno388KA and rEno400KA to human glu-plasminogen (Plg), compared with the wild type (WT). Substituting each of the tested lysine residues of rEno did not significantly alter the binding affinity to Plg, compared to that of the WT. In each case, the addition of EACA reduced the binding to a negligible level. ns denotes a non-significant *p*-value. Four independent experiments, each in triplicate, were performed. Error bars reflect SE.

4.3.9. Mutation of the Grx catalytic cysteine residue of rPrx has no impact on Plg binding

For meningococcal peroxiredoxin, in addition to the C-terminal lysine, two other residues were also examined for their putative contribution to Plg binding. The first was K230: a sub C-terminal Lys before K244. The second was cysteine (C185), which is the second cysteine of the glutaredoxin active site of meningococcal peroxiredoxin. Recombinant proteins with the respective substitutions were successfully prepared including rPrx230KA and rPrx185CA. Likewise, rPrx230244KKAA with substitutions at the C-terminal and sub-terminal Lys residues was also generated. rPrx230KA and rPrx185CA were shown to bind Plg in a similar manner to WT. Conversely, a significant reduction in binding was observed for rPrx230244KKAA. The level of this reduction was comparable to that of rPrx244KA, suggesting that the observed effect is due to the substitution of K244 rather than K230 (Figure 4.12).

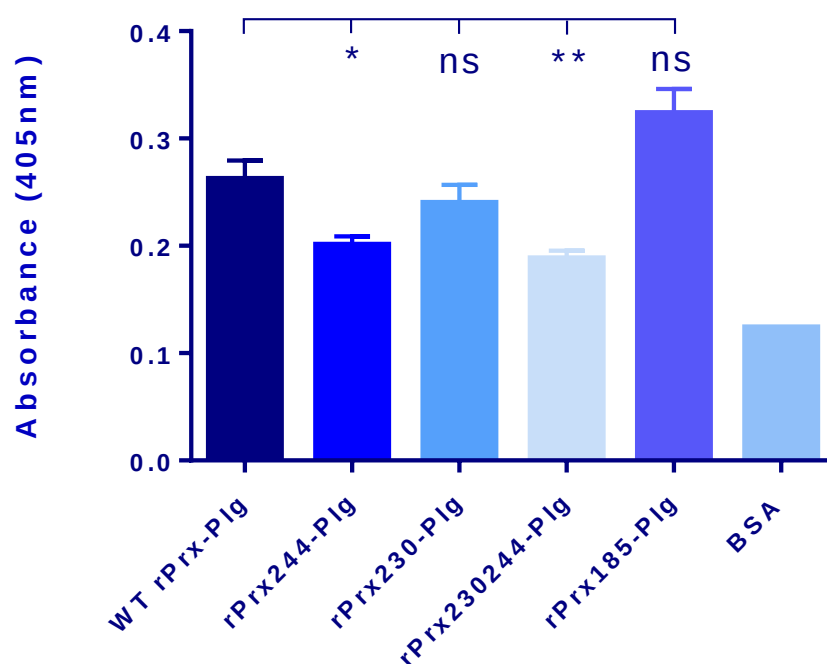


Figure 4.12: Mutation in the Grx catalytic cysteine residue of rPrx has no influence on plasminogen binding. Binding of meningococcal rPrx230KA, rPrx185CA and rPrx230244KKAA to human glu-plasminogen (Plg) was tested by EIA. rPrx230KA and rPrx185CA bound Plg in a similar manner to wild type (WT). The significant reduction in binding affinity of rPrx230244KKAA, which was similar to that of rPrx244KA, suggests that K230 is not involved in Plg binding. *, ** and ns denote 0.0186, 0.0076 and a non-significant p -value, respectively. Four independent experiments, each in triplicate, were performed. Error bars reflect SE.

4.3.10. Cloning, overexpression and purification of Kringle domains of human glu-plasminogen

As described earlier, while Plg-binding by different receptors, and the involvement of Lys residues carried on these receptors, in mediating the binding have been extensively studied, little is known about the motif(s) in the kringle domains of plasminogen to which these receptors bind. Therefore, an attempt was made to investigate these kringle domain-contained motifs.

In collaboration with Meena Ali-Naja (BMedSci student, University of Nottingham), cloning and expression of recombinant kringle domains (KR) 1-5 was achieved; the desired clone was designated *E. coli* JM109 (pMK1-5). By using inverse PCR, clones encoding KR1-2 and KR3-5 were obtained (Table 4.3), and expression of the encoded C-terminally 6 × His-tagged recombinant proteins performed. Trials to natively purify these recombinant proteins, however, failed due to persistent aggregation of the recombinant proteins (Figure 4.13 for rKR1-5; data not shown for other proteins). To avoid protein aggregation, several approaches were adopted including various modifications to expression conditions. Purification of KR1-5 under denaturing conditions, using urea as a denaturing agent, followed by either rapid desalting or overnight dialysis to remove the denaturing agent and facilitate native protein refolding, was also performed. Unfortunately, these

modifications were largely unsuccessful. Construction and expression of KR1-5 minus a single domain at a time, designated pdeK1, pdeK2, pdeK3, and pdeK4, was also performed (Table 4.3). Attempts to purify some of these expressed proteins also led to protein aggregation. Reconstructing KR1-5 encoding sequence into another vector and/or trying to express the recombinant protein in different host cells (perhaps in a mammalian cell), might assist in obtaining soluble proteins but was not attempted as part of this study. While these suggestions are time consuming, they also not guaranteed to yield a properly folded recombinant protein. As a result, and for the above-explained reasons, this aspect of the project has been discontinued.

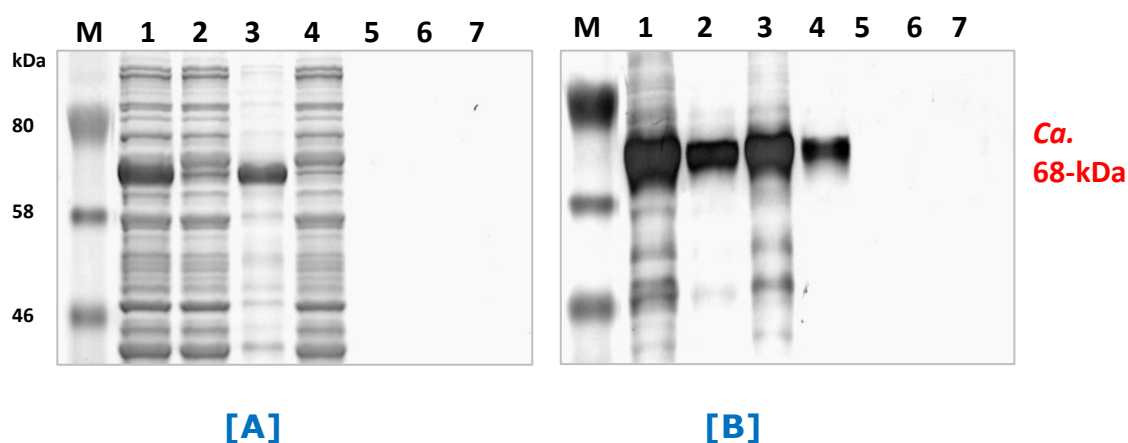


Figure 4.13: 10% SDS-PAGE (A) and immunoblot analysis (B) displaying the IMAC purification process of recombinant His-tagged kringle domain 1-5 under native conditions. The protein was traced throughout purification steps, and confirmed as a His-tagged protein by immunoblot analysis using anti-penta-histidine antibodies. Most of the protein detected in the whole cell lysate fraction as an intense band of *ca.* 68-kDa (lane 1), was then identified in the insoluble lysate

fraction (lane 3), indicating that the expressed protein had been aggregated into inclusion bodies by *E. coli* host cells. Lane M: pre-stained protein marker, lane 2: soluble lysate, lane 4: flow through, lane 5: wash, lane 6: fraction 2, lane 7: desalted and concentrated fractions 3-10.

Table 4.3: List of plasmids that were constructed to express different combinations of kringle domains from human plasminogen.

Plasmid	Description	Source
pUC57-K1-5	Construct containing Kringle domains (KR) 1-5 of human plasminogen used to construct other plasmids	Dr Yihai Cao, Karolinska Institute, Stockholm, Sweden
pMK1-5	KR1-5 of human plasminogen cloned in to pQE70 (Qiagen; Appendix 6)	M. Ali-Naja
pMK1-2	KR 1 and 2 of human plasminogen cloned in to pQE70	M. Ali-Naja
pMK3-5	KR 3, 4 and 5 of human plasminogen cloned in to pQE70	M. Ali-Naja
pdeK-1	KR 2-5 of human plasminogen cloned in to pQE70	This study
pdeK-2	KR 1, 3, 4 and 5 of human plasminogen cloned in to pQE70	This study
pdeK-3	KR 1, 2, 4 and 5 of human plasminogen cloned in to pQE70	This study
pdeK-4	KR 1, 2, 3 and 5 of human plasminogen cloned in to pQE70	This study

4.4. Discussion

In this chapter, the binding of meningococcal enolase, peroxiredoxin and DnaK, to human glu-plasminogen was investigated. Using equimolar amounts of each recombinant protein in EIA assays, it was found that rEno consistently bound Plg at higher levels than rDnaK, which in turn, bound Plg at higher levels than rPrx. Knaust and colleagues had previously identified these three proteins as meningococcal plasminogen-binding ligands using an overlay plasminogen assay, where natively purified proteins were directly blotted on to a nitrocellulose membrane and then probed with Plg [Knaust et al., 2007]. They found that the binding affinity of all the proteins for Plg was similar, which was probably due to the use of a non-quantitative method to visually quantify differences in binding. Moreover, while the binding of rPrx to Plg appeared relatively weak (compared to enolase), it cannot be ignored, because the number of peroxiredoxin molecules expressed on the bacterial surface rather than the strength of the binding itself could influence the biological significance of this interaction. In this context, it is noteworthy that peroxiredoxins are generally produced in large amounts. For example, *E. coli* peroxiredoxin is among the most ten abundant proteins in the cell [Link et al., 1997]. This is further supported as a significant amount of

peroxiredoxin was detected on the surface of MC58 meningococcal cells (section 5.3.2).

Lys-dependent binding to Plg has been documented previously for proteins of pneumococci [Bergmann et al., 2001], *M. tuberculosis* [Xolalpa et al., 2007], meningococci and gonococci [Ullberg et al., 1992], and many others. Consequently, the lysine analogue EACA was used to examine the contribution of Lys residues in mediating the interaction of meningococcal rEno, rPrx and rDnaK with Plg. As expected, the addition of EACA significantly reduced the binding of each recombinant protein to Plg to a negligible level, presumably due to competition with Lys-binding sites, suggesting the involvement of Lys residues.

The contribution of the C-terminal Lys of meningococcal enolase, peroxiredoxin and DnaK in mediating the interaction of each to Plg was investigated earlier [Knaust et al., 2007]. In that study, C-terminal Lys 428 of enolase, Lys 641 and 642 of DnaK, and the penultimate Lys of peroxiredoxin (K244) together with asparagine at the C-terminal end (245) were each replaced by alanine, and equivalent recombinant proteins were examined by the overlay plasminogen assay. This showed that the binding affinity of each mutated protein was similar to that of the wild type, hence, the contribution of the C-terminal Lys in Plg binding was excluded [Knaust et al., 2007]. To confirm

these results, the involvement of the C-terminal lysine was re-examined in the current study. The use of a non-quantitative technique *i.e.* the overlay assay to assess the binding of each mutated protein to Plg, was mainly behind this choice. The expanding number of studies that describe the C-terminal Lys residues of different receptors as the major binding site for Plg [Christensen, 1985, Derbise et al., 2004, Grundy et al., 2007, Shams et al., 2016], was another reason to re-assess the contribution of this residue in Plg binding. Site-directed mutagenesis was utilized to introduce the desired mutations in to the gene encoding meningococcal enolase, peroxiredoxin and DnaK, cloned into pMAJ1, pMAJ2 and pMAJ3, respectively. The desired substitutions were all introduced and confirmed by DNA sequencing, which also confirmed the absence of any other PCR-generated mutations. Corresponding mutant proteins *i.e.* rEno428KA, rPrx244KA and rDnaK642KA were successfully expressed, and purified using IMAC. Compared with the wild type, the protein clearly exhibited reduced binding to Plg. In each case, the reduction was statistically highly significant. Importantly, when EACA was used; a further reduction in the binding capacity of rEno428KA, rPrx244KA and rDnaK642KA was detected, suggesting that one or more Lys residues, other than the C-terminal residue, might also be involved in the interaction.

Turning to meningococcal DnaK, the individual rather than the dual contribution of the two C-terminal Lys residues (641 and 642) to recruit Plg was also assessed by EIA, which showed that unlike rDnaK642KA, rDnaK641KA exhibited a very similar Plg binding to that of WT rDnaK, suggesting that the penultimate Lys residue of DnaK is probably not involved in the interaction with Plg. This finding differs from that shown by Bergmann and colleagues, who suggested the two C-terminal Lys residues of pneumococcal enolase as the pivotal site for Plg binding [Bergmann et al., 2001]. Although these two residues were examined together and separately, the use of an overlay assay to compare the capacity of each mutated protein to bind Plg was perhaps insufficiently sensitive to provide a reliable discrimination. The results presented here have possible implications for the re-examination of Plg binding by pneumococcal enolase using a more sensitive assay.

Having confirmed that meningococcal rEno bound Plg, the involvement of Plg binding site(s) was further investigated. First, highly conserved Lys residues in meningococcal enolase were further examined. Only seven Lys residues were found universally conserved among proteins from the species examined. While these residues are well conserved, it was still unknown whether each of them is likely to be exposed on the surface of the native protein, or partially or totally embedded

within the protein structure. Thus, pneumococcal enolase was used as a model to examine the predicted position of the conserved Lys residues.

Pneumococci and meningococci colonize the mucosal surfaces of human upper respiratory tract, from where they can invade mucosal tissue, reach the blood stream and cause life-threatening meningitis. Importantly pneumococcal [Bergmann et al., 2001] and meningococcal [Knaust et al., 2007] enolases were both found expressed on the surface of bacteria where they could potentially interact with human Plg. Moreover, the present study strongly suggests the C-terminal lysine as the main site through which rEno and Plg bound, a finding that was first assumed regarding pneumococcal enolase when a C-terminal Lys mutated protein was examined under reducing conditions [Bergmann et al., 2001]. However, when the same protein purified under relatively non-denaturing conditions was examined, the mutated protein exhibited similar Plg binding as the wild type. Furthermore, an internal nine amino acids Lys-rich motif ($_{248}\text{FYDKERKVY}_{256}$) was identified as a second binding site (BS2) for pneumococcal enolase-Plg interaction, in which substituting three critical residues within this motif, to yield $_{248}\text{FYDLGRLVY}_{256}$, reduced plasminogen-binding to *ca.* 44% of wild type values [Bergmann et al., 2003]. Later, the crystal structure of pneumococcal enolase was solved by Ehinger and

colleagues, who suggested that it was an octamer [Ehinger et al., 2004], resembling that of GAS streptococci [Cork et al., 2015] and *B. subtilis* [Brown et al., 1998]. This octameric structure forms a ring with a circa 19-Å-wide central pore, and importantly, the N-terminal part of each monomer is oriented towards the centre, while the C-terminus is more accessible and facing the outer ring [Ehinger et al., 2004]. The Plg binding sites were also annotated on this model, and predicted that while the C-terminal Lys residues of the protein are at the inter-dimer position, and hence less likely to be involved in Plg binding, the nonameric peptide (BS2) is well exposed, and hence more likely to participate in Plg binding (Ehinger et al., 2004). Analytical gel filtration results in the current study have also identified meningococcal rEno as an octamer. Due to the previously discussed similarity between pneumococcal and meningococcal enolase, the former was chosen as a model to predict the position of conserved meningococcal Lys residues, focusing on residues which are close to the more accessible C-terminus of the octamer. Similarly, the significance of the two Lys residues carried on meningococcal enolase (at position 250 and 253) that correspond to those within the internal nonameric sequence of pneumococcal enolase in mediating meningococcal rEno-Plg interaction was also examined.

Based on the pneumococcal enolase crystal structure (PDB entry 1W6T), while Lys 388 and 400 were predicted to be unexposed, residues at positions 297, 337 and 355 were each close to the C-terminal end and predicted to be in an exposed position within the molecule. Site-directed mutagenesis was utilized to introduce the desired mutations, and corresponding mutant proteins *i.e.* rEno297KA, rEno337KA, rEno355KA and rEno250253KKAA were successfully expressed, purified and examined in terms of their ability to bind Plg. It was found that while the binding of rEno337KA to Plg was very similar to that of the wild type, a significant reduction was detected when Lys 297 and 355 of meningococcal rEno were individually substituted and the Plg binding capacity of each corresponding protein was assessed. When the Plg binding activity of rEno388KA and rEno400KA was assessed, it was clear that each of these mutated proteins was able to bind a similar amount of Plg as the WT, suggesting that these residues *i.e.* Lys 388 and 400 are probably not involved in the Plg interaction. The uncertainty regarding the position and the exposure of Lys 388 and 400 of meningococcal enolase based on the pneumococcal model further supports the resemblance of the 3D structure of pneumococcal and meningococcal enolases. These findings circuitously support our previous suggestion that meningococcal enolase is mainly arranged as an octameric protein. Further

examination of these residues *i.e* K297 and K355, and generating double or even triple (together with K428) mutant enolases with different combinations of Lys to alanine substitutions, might be necessary to validate the obtained results.

However, unlike pneumococcal enolase, it seems that substitution of Lys 250 and 253 of meningococcal enolase did not significantly alter the binding of the latter to Plg. The difference in findings may be explained by our use of an EIA-based assay to detect Plg binding rather than a dot blot assay or possibly the replacement of three residues *i.e.* Lys 251, glutamic acid 252 and Lys 254 with leucine, glycine and leucine, in the respective positions of pneumococcal enolase in the study by Bergmann *et al.* [Bergmann et al., 2003].

As mentioned, meningococcal peroxiredoxin is a natural hybrid protein consisting of a Prx domain and a Grx domain, in which a mutation in the second cysteine active site of the Grx *i.e.* C185 was found to inactivate enzymatic activity [Rouhier and Jacquot, 2003]. The current study aimed to examine whether enzyme activity was a requirement for Plg binding. Site-directed mutagenesis targeting C185, followed by expression and purification of the corresponding protein were performed. EIA results revealed no significant difference in the binding of the

cysteine mutant protein, compared with that of the wild type. From this, it is apparent that the canonical and the moonlighting functions of meningococcal Prx are clearly independent. This finding also agrees with Shams and colleagues, who have recently demonstrated that a non-functional copy of meningococcal FBA was able to recruit Plg [Shams et al., 2016]. Finally, in addition to Lys 244 and cysteine 185, Lys 230 of meningococcal Prx has also been substituted to examine its putative enrolment in Plg binding, which was similar to that of the wild type.

In summary, meningococcal rEno, rPrx and rDnaK bind Plg predominantly, yet not exclusively, via C-terminal lysine residues. For rEno, Lys residues at positions 297 and 355 represent additional sites for Plg binding. Finally, a non-functional copy of rPrx recruits a similar amount of Plg as the wild type.

5. CHAPTER FIVE: Phenotypic characterisation of meningococcal peroxiredoxin

5.1. Introduction

Neisseria meningitidis is an important cause of sepsis and meningitis in susceptible individuals with the majority of cases occurring in children and young adults. The organism frequently colonizes the nasopharynx asymptomatically and is transmitted from person-to-person by close contact. Rarely, however, hyper virulent strains may invade the mucosal epithelial barrier to reach subcutaneous tissues and subsequently enter the bloodstream [Coureuil et al., 2013]. The ability of the meningococcus to survive in subcutaneous tissues and blood is therefore a prerequisite for invasive meningococcal disease. Several bacterial components are known to be essential for, or enhance, survival in non-immune blood, including the sialic acid (groups B, C, W and Y) and nonsialic acid (group A) containing capsular polysaccharide [Jarvis and Vedros, 1987], the factor H-binding proteins (fHbp and NspA), NalP, Mip, Fur, TbpB and the L-lactate permease, LctP [Seib et al., 2009, Echenique-Rivera et al., 2011]. Likewise, various detoxifying enzymes are used by *N. meningitidis* to protect against reactive oxygen and nitrogen species (ROS and RNS, respectively) produced by polymorphonuclear (PMN) cells and monocytes/macrophages [Anjum et al., 2002, Seib et al., 2004]. Up-regulation of catalase (Kat), superoxide dismutase (Sod) and nitrite reductase (AniA) have been demonstrated following incubation

in human blood [Echenique-Rivera et al., 2011]. SodC has previously been shown to protect *N. meningitidis* from phagocytosis [Dunn et al., 2003] and AniA protects *N. gonorrhoeae* in human serum [Cardinale and Clark, 2000], however Kat was shown not to be required for meningococcal survival in human blood [Echenique-Rivera et al., 2011].

Peroxiredoxins (Prxs) are a large family of cysteine-based peroxidases, present in all kingdoms of life, that catalyse the reaction $\text{ROOH} + 2\text{e}^- \rightarrow \text{ROH} + \text{H}_2\text{O}$ and reduce hydrogen peroxide (H_2O_2), peroxynitrite and a wide range of organic hydroperoxide compounds [Christman et al., 1985, Netto et al., 1996, Bryk et al., 2000]. Prxs are ubiquitous and abundant proteins that are important for antioxidant defence and the regulation of cell signalling pathways [Poole, 2005, Rhee and Woo, 2011]. Prxs are increasingly being recognized as multifunctional or moonlighting enzymes, which are involved in various aspects of bacterial survival and pathogenesis. In *Helicobacter pylori*, a BCP-PrxQ subfamily Prx was found to contribute to resistance to oxidative stress and host colonization [Wang et al., 2005] and an AhpC-Prx1 subfamily Prx was shown to oligomerise and acquire protein chaperone activity in oxidative stress conditions [Chuang et al., 2006]. In *Haemophilus influenzae*, a Prx-Grx hybrid enzyme was found to be expressed preferentially during growth in biofilms [Murphy et al., 2005] and a mutant strain

was more susceptible to organic hydroperoxides [Pauwels et al., 2004] and showed persistence defects in a chinchilla model of otitis media and a murine model of chronic obstructive pulmonary disease [Juneau et al., 2015]. In *Streptococcus agalactiae* a similar AhpC-Prx1 enzyme was shown to bind haem and participate in its intracellular availability [Lechardeur et al., 2010]. In *Coxiella burnetii*, a BCP-PrxQ Prx was shown to bind DNA and protect the organism from oxidative stress during the exponential phase of growth [Hicks et al., 2010]. A Prx6 subfamily protein, LsfA, in *Pseudomonas aeruginosa*, was found to reduce the oxidative burst in macrophages and be important for virulence in a murine model of pneumonia [Kaihami et al., 2014]. In *Vibrio vulnificus*, Prx3, a Grx3/GSH-dependent 1-Cys Prx, was shown to be required for optimal growth in medium containing peroxides and the Prx3 mutant strain was less virulent in mice [Lim et al., 2014]. Recently, a 1-Cys Prx6 enzyme from *Thermococcus kodakarensis*, a thermophilic anaerobic archaeon, was found to act as a molecular chaperone that blocked oxidative stress-mediated aggregation of proteins and DNA [Lee et al., 2015].

In this chapter, the role of meningococcal peroxiredoxin during H_2O_2 -mediated oxidative stress was characterized. Peroxiredoxin was confirmed to be present on the bacterial surface, and importantly, it was shown that Prx is required for meningococcal survival in human non-immune whole blood.

5.2. Materials and methods

5.2.1. Bacterial strains and growth conditions

Meningococcal strains described in Table 5.1 were grown on GC agar, chocolate agar, Mueller-Hinton (MH) agar or in BHI broth. Where appropriate, antibiotics at the following final concentrations were used for selection: kanamycin ($50 \mu\text{g ml}^{-1}$), streptomycin ($100 \mu\text{g ml}^{-1}$) and erythromycin ($5 \mu\text{g ml}^{-1}$). Cultures were incubated at 37°C in an atmosphere of air plus 5% CO_2 with or without shaking at 200 rpm. Gentle agitation was applied when required.

5.2.2. Investigating the *in vitro* growth of meningococcal cells

The growth characteristics of meningococcal strains (Table 5.1) growing in liquid culture was investigated. For each strain, a single colony cultured on chocolate agar was used to inoculate 10 ml of BHI broth, which was then incubated overnight at 37°C with shaking. The overnight broth culture was sub-cultured in fresh BHI broth and adjusted to a starting OD_{600} of 0.06. Under the same incubation conditions, the culture was allowed to grow, and the OD_{600} was measured at hourly intervals for 7 h and then at 24 h. Each sample was examined in triplicate, and three independent experiments were performed.

Table 5.1: Meningococcal strains used in work described in this chapter.

Strain	Description	Source
MC58	Wild type (WT) serogroup B	[Tettelin et al., 2000]
MC58 <i>rpsL</i> ⁻	NMB0136 carries a point mutation to substitute k43 of the resultant protein by threonine.	H. Albasri, University of Nottingham
MC58 <i>rpsL</i> ⁻ Δ <i>pxn</i> <i>Kan</i> ^R <i>rpsL</i> ⁺	NMB0946 gene replaced by kanamycin cassette and <i>rpsL</i> ⁺ allele	This study
MC58 <i>rpsL</i> ⁻ Δ <i>pxn</i>	NMB0946 deletion (markerless)	This study
MC58 <i>rpsL</i> ⁻ Δ <i>pxn</i> <i>pxn</i> ^{Ect} <i>Erm</i> ^R	NMB0946 copy and erythromycin cassette inserted at ectopic site of the markerless Δ <i>pxn</i> strain	This study
MC58 Δ <i>siaD</i>	<i>siaD</i> gene replaced by erythromycin cassette	C. Tang, Imperial College
MC58 Δ <i>cbbA</i>	<i>cbbA</i> gene replaced by kanamycin cassette	[Tunio et al., 2010b]

5.2.3. Whole-cell EIA

EIA was employed to examine the ability of polyclonal monospecific antisera raised against meningococcal rEno, rPrx and rDnaK to bind to intact meningococcal cells. All buffers and solutions used in this assay were prepared as described in section 4.2.1. Preparation of meningococcal cells, their binding to anti-rEno, -rPrx and -rDnaK antibodies and binding to glu-plasminogen are detailed below.

5.2.3.1. Preparation and fixation of meningococcal cells

Several strains of *N. meningitidis* were examined in this assay. In each case, the bacteria was first grown overnight on a chocolate agar plate. A single colony was used to inoculate 10 ml of BHI broth, which was then incubated overnight at 37°C with shaking. The following day, the bacteria was sub-cultured in 10 ml of fresh BHI broth and allowed to grow to mid-log phase ($OD_{600} \geq 0.5$). Next, the broth was centrifuged at $2087 \times g$ at 4°C for 10 min. After discarding the supernatant, the bacterial pellet was washed twice with sterile filtered PBS, centrifuged as previously described, and then re-suspended in 10 ml of 0.5% formaldehyde (EMS; 15710-S) in PBS. The cells were incubated at 4°C for 30 min with gentle agitation. After fixation, cells were centrifuged at $2087 \times g$ for 10 min at 4°C, washed one more time with PBS, and then re-suspended in sodium carbonate buffer. Finally, the OD_{600} was adjusted to 0.2.

5.2.3.2. Binding of anti-rEno, -rPrx and -rDnaK antibodies to whole meningococcal cells

Microplate wells (Thermo Fisher Scientific; Nunc immobilizer amino 12×8 strips) were first coated with 100 µl aliquots of the appropriate formaldehyde-fixed meningococcal cells in sodium carbonate buffer. After overnight incubation at 4°C with gentle agitation, the plate was washed three times with PBST 0.05%,

and then 100 μ l of 1%BSA/PBS were added to each well for blocking for 2 h at RT. Another washing step was then followed by adding 100 μ l of polyclonal primary anti-rEno, -rPrx or -rDnaK antibodies that were respectively raised in rabbits against meningococcal rEno, rPrx and rDnaK, each diluted in 1%BSA/PBS, and the plate was incubated for 2 h at RT with gentle shaking. The anti-rPrx antiserum was pre-adsorbed with the peroxiredoxin mutant strain before use in the assay. The washing step was repeated, and 100 μ l of anti-rabbit IgG-alkaline phosphatase conjugate (Sigma-Aldrich) 1:30000 diluted in 1% BSA/PBS were added, and the plate was then incubated for 2 h at RT with gentle shaking. Finally, removing the unbound secondary antibodies by washing was followed by adding 200 μ l of 1 mg ml⁻¹ phosphatase substrate (Sigma-Aldrich) in glycine buffer, and the absorbance was measured at 405 nm for > 3 h using Tecan spark10M reader, which was also employed as an incubator that facilitated a constant temperature (25°C) throughout the measurement process. Rabbit anti-rPorA polyclonal antiserum [Orihuela et al., 2009] was used as a positive control. Similarly, in order to measure non-specific binding, the rabbit antisera were replaced by 1%BSA/PBS.

5.2.3.3. Binding of human glu-plasminogen to whole meningococcal cells

Microplate wells (Thermo Fisher Scientific; Nunc immobilizer amino 12×8 strips) were first coated with 100 µl aliquots of formaldehyde-fixed meningococcal cells in sodium carbonate buffer (described in section 5.2.3.1). After overnight incubation at 4°C with gentle agitation, the plate was washed three times with PBST 0.05%, and then 100 µl of 1%BSA/PBS was added to each well for blocking for 2 h at RT. Another washing step was then followed by adding different concentrations of human glu-plasminogen (with or without 50 Mm EACA) in a final volume of 100 µl diluted in 1%BSA/PBS, and the plate was incubated for 2 h at RT with gentle shaking. Goat anti-plasminogen antibody (Rockland Immunochemicals; 1:10000 diluted in 1% BSA/PBS) was added to each well and the plate was incubated overnight at 4°C with gentle agitation. After washing, 100 µl of 1:5000-diluted donkey anti-goat IgG-alkaline phosphatase conjugate (Promega) was added to each well. After a final washing step, 200 µl of 1 mg ml⁻¹ phosphatase substrate in glycine buffer was added to each well, and the absorbance was measured at 405 nm using Tecan spark10M reader, at a constant temperature (25°C). In order to measure non-specific binding, Plg was replaced by 1%BSA/PBS.

5.2.4. Hydrogen peroxide killing assay

Hydrogen peroxide (H₂O₂) killing assay was performed to investigate the ability of the Δpxn -mutant strain to survive in H₂O₂-containing environment compared with the wild type strain. Meningococcal cells were first grown overnight on a chocolate agar plate. A single colony was used to inoculate 10 ml of BHI broth, which was then incubated overnight at 37°C with shaking. The following day, the bacteria was sub-cultured in 10 ml of fresh BHI broth and allowed to grow to mid-log phase (OD₆₀₀ ≥ 0.5). The bacterial suspension was then adjusted to *ca.* 10⁷ colony-forming units [CFU] per millilitre for all examined strains (confirmed retrospectively by plating out serially-diluted aliquots). A sterile 96-well polystyrene round bottom microwell plate (ThermoFisher Scientific) was used. Fifty microliters of H₂O₂ (Fisher Scientific) at concentrations of 10, 12.5, 15, 17.5 or 20 mM was added to 100 µl of the meningococcal cell suspension (containing *ca.* 10⁶ CFU) in each well, and the volume was adjusted to 300 µl with BHI broth. The plate was incubated at 37°C in 5% CO₂ atmosphere with gentle shaking for 1 h. At 20 min intervals, 10 µl aliquots were taken, serially diluted, and plated onto MH agar plates, which were incubated overnight at 37°C in 5% CO₂. Finally, the number of colonies was counted and expressed as CFU/ml.

5.2.5. Human whole blood survival assay

Human whole blood was used as a model of meningococcal bacteraemia to study host-bacterial interactions. Whole venous blood, collected from two healthy adult males and anticoagulated with heparin (ca. 15 IU/ml) was used in the assay. None of the donors had been vaccinated against *N. meningitidis*. For each donor, five different strains of meningococci were assessed including MC58 WT, MC58 *rpsL*⁻, MC58 *rpsL*⁻ Δ *pxn*, MC58 *rpsL*⁻ Δ *pxn* *pxn*^{Ect} *Erm*^R and MC58 Δ *siaD*. Meningococcal cells were first propagated overnight on GC agar plates. BHI broth was inoculated with a single colony from the overnight culture, and incubated at 37°C with shaking. The broth culture of *N. meningitidis* was used to inoculate 5 ml of fresh BHI broth to obtain a final OD₆₀₀ of 0.05, and the latter broth was further grown to an OD $\geq$ 0.5. The bacterial suspension was adjusted to ca. 10⁸ CFU/ml (confirmed retrospectively by culture of serially-diluted aliquots). The assay was started by the addition of 190 μ l of 100% whole human blood, from a single donor, to 10 μ l of the bacterial suspension (containing ca. 10⁶ CFU) in a sterile 96-well plate. The plate was incubated at 37°C in 5% CO₂ atmosphere with gentle shaking for 2 h, with sampling of aliquots of 10 μ l at various time points (30, 60, 90 and 120 min). The number of viable bacteria was determined by culture of serial dilutions onto MH agar followed

by overnight incubation at 37°C in 5% CO₂. For each sample, two repeats were prepared, and the experiment was performed twice for donor A and once for donor B. Survival of different meningococcal strains in human whole blood was determined by comparison of the viable count at each time point with time zero.

5.2.6. Investigating the survival of meningococcal cells in polymorphonuclear cells (PMNs)

In this assay the survival of different meningococcal strains in the presence of PMNs isolated from human whole blood was assessed. Before commencing the assay, meningococci and PMNs were first prepared as described below.

5.2.6.1. Preparation of meningococcal cells

Three different strains of meningococci were assessed in terms of the ability of each to survive in the presence of PMNs, including MC58 WT, MC58 *rpsL*⁻ Δ *pxn* and MC58 *rpsL*⁻ Δ *pxn* *pxn*^{Ect} *Erm*^R. Meningococcal cells were first propagated on a GC agar. A single colony was used to inoculate 10 ml of BHI broth and incubated overnight at 37°C with shaking at 200 rpm. The following day, bacteria were transferred in to 10 ml of fresh BHI broth and allowed to grow at 37°C to mid-log phase. Next, the broth was centrifuged at 2087 x *g* at 4°C for 10 min. The bacterial pellet was washed twice with cold Dulbecco's PBS supplemented with Ca²⁺ and Mg²⁺, centrifuged as previously

described, and re-suspended in the same solution. In each strain, the bacterial count, determined retrospectively, was adjusted to 4×10^6 CFU/ml.

5.2.6.2. Isolation of PMNs

A density gradient centrifugation-based protocol was applied to harvest PMNs from 50 ml of heparinised whole blood collected from a healthy adult female donor. The gradient was prepared by adding 12 ml of Histopaque®-1077 (density of 1.077 g/ml; Sigma-Aldrich) to a 50 ml falcon tube. Through a sterile glass Pasteur pipette immersed to the bottom of the tube, 12 ml of Histopaque®-1119 (density of 1.1119 g/ml; Sigma-Aldrich) was then added with care to avoid disrupting the forming gradient. Once the two Histopaque layers had distinctly formed, 12 ml of the blood sample diluted in equal volume of Hank's Balanced Salt Solution (HBSS; ThermoFischer Scientific) was gently added without disturbing the underlying Histopaque gradient. Next, the tube was centrifuged at $700 \times g$ for 30 min at RT (one acceleration and one deceleration that respectively allow soft start and stop of the centrifugation run to preserve the sample and avoid haemolysis). Two distinct opaque layers were seen after the centrifugation: the upper layer comprising peripheral blood mononuclear cells (PBMCs) and the lower denser layer consisted of mainly PMNs. After removing the PBMCs by aspiration, the PMN cell layer was collected in a fresh 15 ml

tube, washed with the same volume of HBSS to remove any remaining Histopaque, and then the tube was centrifuged at 350 x *g* for 7 min at RT (3 accelerations and 3 decelerations). After that, the supernatant was discarded and 2 ml of 2% sterile saline was added to the cells to lyse any contaminating red blood cells. After 1-2 min incubation at RT, 2 ml of 1.6% sterile saline was added, and the tube was filled with HBSS before centrifuging again at 250 x *g* for 5 min at RT (3 accelerations and 3 decelerations). Under the same centrifugation conditions, washing with HBSS was repeated one more time before using HBSS supplemented with Ca²⁺ and Mg²⁺ in two washing cycles. Finally, the cells were re-suspended in HBSS with Ca²⁺ and Mg²⁺ plus 5% human serum albumin (HSA; Seralab). Using the trypan blue exclusion method, the cells were counted and then adjusted to a concentration of 8 x 10⁶ PMNs per millilitre.

5.2.6.3. Infection of PMNs with meningococcal cells

The assay was performed in sterile 1.5 ml Eppendorf tubes, to which the assay components were added. Each tube contained 100 µl of the appropriate meningococcal cells (4 x 10⁶ CFU/ml) mixed with 100 µl of freshly isolated PMNs (8 x 10⁶ CFU/ml) to obtain a final multiplicity of infection (MOI) of 2. Tubes contained meningococcal cells without any PMNs added (100 µl of HBSS supplemented with Ca²⁺ and Mg²⁺ plus 5% HSA was added instead) were also prepared as a control. A reaction

consisted of PMNs cells mixed with equal volume of HBSS supplemented with Ca^{2+} and Mg^{2+} plus 5% HSA was also prepared to address any potential contamination. All tubes were placed on a tube rotator that achieves 18 rpm at 360° , and incubated at 37°C in 5% CO_2 atmosphere for 30 min. At the end of the assay aliquots were taken, serially diluted and inoculated on MH agar plates, which were incubated as previously described (Section 5.2.1). After overnight incubation, the number of colonies at each dilution was counted and reported as the number of CFU per millilitre. Each reaction was performed in duplicate and the experiment was performed once.

5.2.7. Statistical analysis

GraphPad Prism 6.05 was utilized to perform statistical analyses. Unless otherwise stated, samples were analysed in triplicate and each experiment was repeated at least three times. Statistical significance was measured as in section (4.2.10).

5.3. Results

5.3.1. Determining the growth characteristics of meningococcal strains

The purpose behind examining observable growth characteristics *i.e.* colonial morphology and the growth rate of the peroxiredoxin mutant strains was to inspect whether or not the absence of peroxiredoxin would have an impact on the maximal growth of *N. meningitidis*, a situation that would affect the interpretation of any peroxiredoxin-associated phenotype. On chocolate agar, the peroxiredoxin mutant strains formed colonies that were undistinguishable in terms of the morphology, size, colour and texture from those of the parent MC58 *rpsL*⁻ and wild type strains (data not shown). Equally, when meningococcal cells were grown in BHI broth, growth rates, expressed as the absorbance measured at 600 nm, of both Δpxn -mutant strains were similar to those observed in the wild type and complemented strains. By the time points of 7 and 24 h, all strains achieved a similar OD₆₀₀ (Figure 5.1). It can be inferred from such findings that the absence of peroxiredoxin did not have a substantial effect on the *in vitro* growth of *N. meningitidis* on solid media or in broth culture. Likewise, the mutation in the *rpsL* gene has no impact on the *in vitro* growth characteristics of *N. meningitidis* strain MC58 *rpsL*⁻,

the parental strain for both Δpxn -mutants and complemented strains (Figure 5.1).

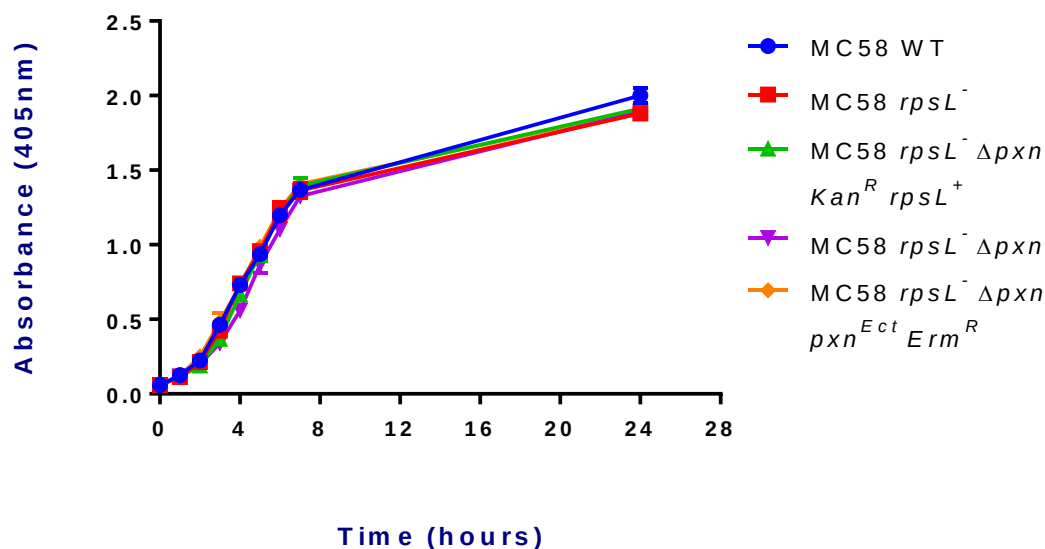


Figure 5.1: Growth characteristics of peroxiredoxin mutant and complemented strains compared to their *rpsL*⁻ and wild-type parent strains. *N meningitidis* strain MC58 WT, MC58 *rpsL*⁻, MC58 *rpsL*⁻ Δpxn *Kan*^R *rpsL*⁺, MC58 *rpsL*⁻ Δpxn and MC58 *rpsL*⁻ Δpxn *pxn*^{Ect} *Erm*^R were grown in BHI broth and OD₆₀₀ was measured at hourly intervals for 24 h. All strains adopted a very similar growth pattern, demonstrating that neither the absence of peroxiredoxin nor the mutation in the *rpsL*⁻ gene has any significant effect on *in vitro* growth characteristics. Three repeats were prepared for each sample, and three independent experiments were performed. Error bars denote SE of the mean.

5.3.2. Unlike enolase, meningococcal DnaK and peroxiredoxin are surface localized

Localization of enolase, peroxiredoxin and DnaK on the surface of intact *N. meningitidis* MC58 WT cells was investigated using EIA. Meningococcal cells grown to mid-log phase were first coated into wells of a microtiter plate, and then probed with anti-rEno, -rPrx or -rDnaK antibodies. Meningococcal cells treated with anti-rPorA antibodies were also prepared to be used as a positive control to detect PorA, a well-characterised outer membrane porin of *N. meningitidis*, and also to provide evidence that intact rather than lysed meningococcal cells were likely employed in the assay. The results indicate that compared with BSA, anti-rPrx antiserum detected a significant amount (p value = 0.0128) of peroxiredoxin on the surface of intact WT meningococcal cells (strain MC58) (Figure 5.2). Likewise, a highly significant amount of DnaK (p value <0.001), resembling that of PorA, was recognized respectively by anti-rDnaK and anti-rPorA antibodies on the bacterial surface (Figure 5.2). Conversely, while anti-rEno antiserum showed insignificant binding to intact meningococcal cells, suggesting that enolase may be expressed on the bacterial surface, this binding did not reach statistical significance (Figure 5.2).

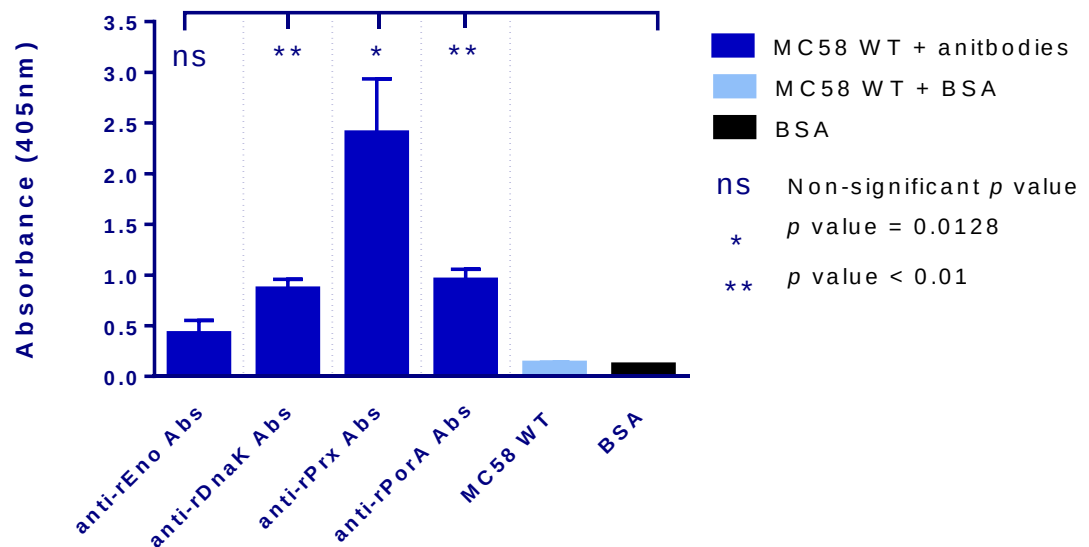


Figure 5.2: Binding of anti-rEno, -rPrx and -rDnaK antibodies (Abs) to intact *N. meningitidis* wild type MC58 cells examined via whole-cell EIA. EIA plates were coated with formaldehyde-treated whole meningococcal cells, probed with rabbit anti-rEno (1:500 diluted in 1%BSA/PBS), -rPrx (1:2000), -rDnaK (1:500) or -rPorA (1:50) Abs, and then stained with anti-rabbit IgG alkaline phosphatase conjugate antibodies before adding the phosphatase substrate and measuring the absorbance at 405nm. Unlike anti-rEno Abs, a significant amount of anti-rDnaK and -rPrx Abs bound whole meningococcal cells, suggesting that significant amount of corresponding target antigens *i.e.* DnaK and Prx are expressed on the bacterial surface. The same applies to anti-rPorA Abs, which were used as a positive control. Error bars denote SE of the mean. Each sample was tested in triplicate, and the experiment was repeated four times, with consistent results.

The specificity of the adsorbed anti-rPrx antiserum to recognize meningococcal peroxiredoxin was confirmed when the serum was used to probe WT MC58 as well as the two Δpxn -mutant strains; binding of anti-rPrx to the latter strains was negligible (Figure 5.3). The binding was partially restored in a strain

complemented with an ectopic copy of the gene encoding Prx (Figure 5.3). This further validates the previous finding of the surface localization of Prx, which is unlikely be due to non-specific binding.

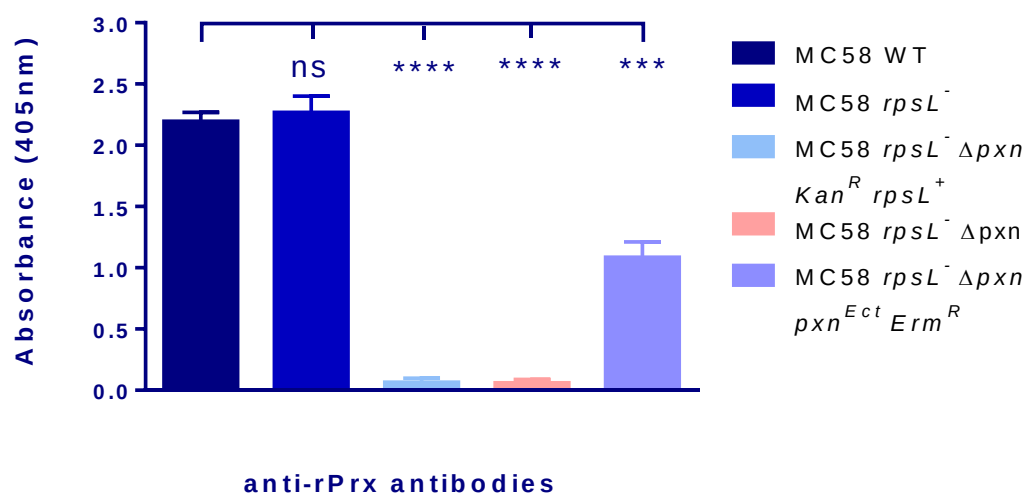


Figure 5.3: Binding of anti-rPrx antibodies to intact meningococcal cells from different MC58 strains, examined via whole-cell EIA. EIA plates were coated with formaldehyde-treated whole meningococcal cells before being probed with rabbit anti-rPrx antiserum 1:2000 diluted in 1% BSA/PBS. Binding of the antiserum with whole meningococcal cells was similar in WT MC58 as well as MC58 *rpsL*⁻. This binding was reduced to a negligible level, and restored when Δ *pxn*-mutants and complemented strains were examined, respectively. ****, *** and ns denote < 0.0001, <0.001 and non-significant *p*-value, respectively. Four independent experiments, each in triplicate, were performed. Error bars denote SE of the mean.

5.3.3. Peroxiredoxin-null meningococcal cells bind human glu-plasminogen

The ability of intact meningococcal cells to immobilize Plg was investigated. Formaldehyde-fixed cells from different meningococcal strains were coated on wells of a microtiter plate, to which 5 $\mu\text{g ml}^{-1}$ Plg was then added. It was found that the binding of whole mid-log phase meningococcal cells to Plg compared with BSA, which was used as a negative control, was highly significant (p value < 0.0001) in all tested strains (Figure 5.4). This finding firstly confirms the ability of meningococci to sequester Plg. The negligible level of Plg binding to BSA provides evidence that none of these interactions could be due to non-specific interactions (Figure 5.4). Importantly, there was no significant difference in the Plg-binding affinity of Δpxn -mutants when compared with that of the WT (Figure 5.4). Likewise, the amount of Plg sequestered by MC58 $\Delta cbbA$ meningococcal cells was similar to that seen in the WT (Figure 5.4). In all cases, the use of EACA in binding reactions has almost abolished the ability of meningococci to bind Plg (Figure 5.4). Applying 2.5, 1 or 0.5 $\mu\text{g ml}^{-1}$ of Plg in the binding assay produced similar results (data not shown).

It is worth mentioning that levels of Plg recruitment on the surface of both *N. meningitidis* Δpxn -mutant strains were significantly lower than that detected in MC58 $\Delta cbbA$ strain,

which lacks *cbbA* gene that encodes meningococcal FBA, a glycolytic enzyme recently recognised as a Plg-binding ligand on the bacterial surface [Shams et al., 2016]. However, having the binding of MC58 WT was also lower than that of the $\Delta cbbA$ -mutant suggesting significant inter-assay variation (Figure 5.4; data not highlighted).

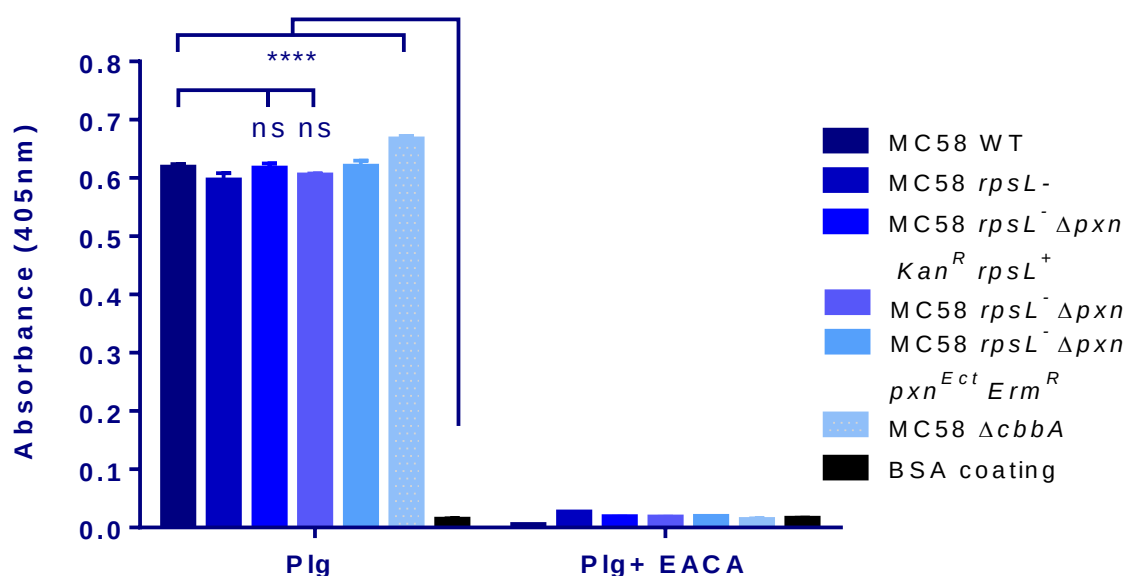


Figure 5.4: Binding of whole meningococcal cells to human plasminogen (Plg) by EIA. Compared with BSA, all tested MC58 strains were significantly able to bind Plg. The minimal BSA-Plg binding confirmed that none of these interactions is likely to be non-specific. There was no significant (ns) difference in the Plg-binding of the Δpxn -mutant meningococcal cells and that of the wild-type (WT). In each case, the addition of ϵ -aminocaproic acid (EACA) nearly abolished the interaction, providing evidence that all Plg-involved interactions on the surface of meningococci are lysine-mediated. **** denotes p value < 0.0001. Error bars denote SE of the mean. Three experiments, each in triplicate, were performed.

5.3.4. MC58 Δpxn -mutant tolerates hydrogen peroxide better than parental strains

To understand the behaviour of *N. meningitidis* grown under H₂O₂-mediated oxidative stress, meningococcal cells at mid-log phase of growth were re-suspended in BHI broth and exposed to various concentrations of H₂O₂. At 10 mM H₂O₂, there was no significant difference in the number of surviving WT and Δpxn -mutant meningococcal cells at all time points (Figure 5.5, A). This finding highlights that although Prx is absent, MC58 *rpsL*⁻ Δpxn was able to withstand H₂O₂ at a level similar to that observed in the WT. Conversely, at 12.5 mM H₂O₂, the number of Δpxn -mutant cells recovered after the first 20 min of exposure was significantly higher than that obtained for other meningococcal strains (Figure 5.5, B). The complemented strain, MC58 *rpsL*⁻ Δpxn *pxn*^{Ect} *Erm*^R, behaved in the same way as the wild type strain. At later time points, the survival rate of MC58 *rpsL*⁻ Δpxn (expressed as the number of CFU obtained) was nearly stationary (Figure 5.5, B). For all other strains, however, it remained below the level of detection. Prx-deficient meningococci were able to tolerate higher concentrations of H₂O₂ (up to 20 mM), while no growth was detected for other strains at all time points of exposure (data not shown).

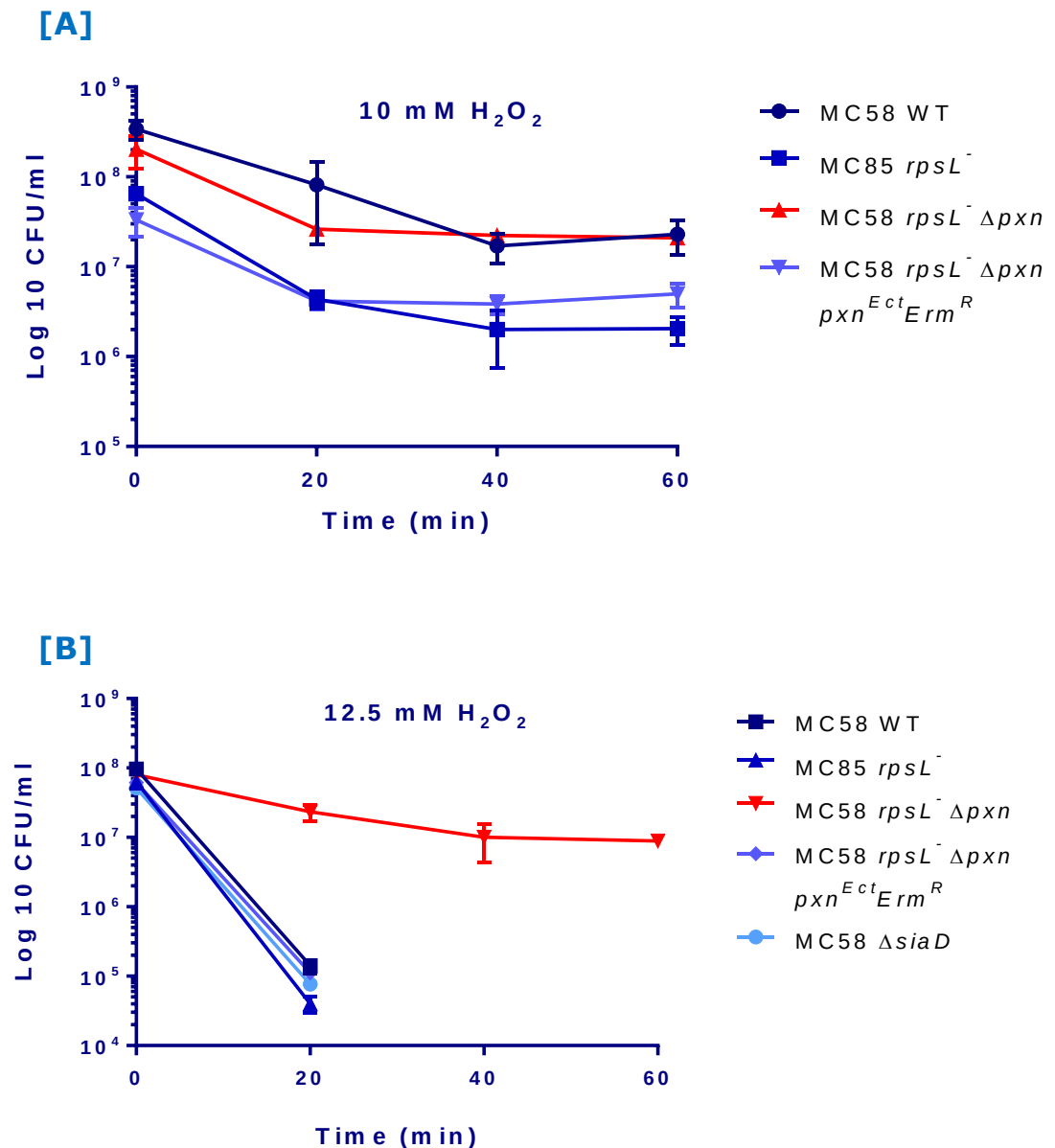
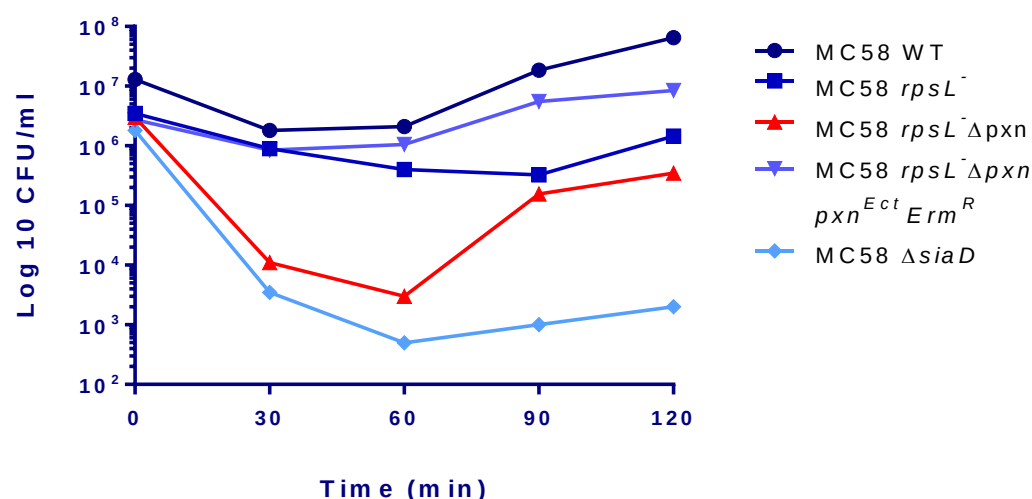


Figure 5.5: Hydrogen peroxide (H₂O₂) killing of different *N. meningitidis* MC58 strains. Meningococcal cells were re-suspended in BHI broth and exposed to a final concentration of 10 or 12.5 mM H₂O₂. [A] At all time points; there was no significant difference in the number of surviving wild type (WT) and Δ*pxn*-mutant meningococcal cells. [B] After 20 min of exposure to 12.5 mM H₂O₂, the survival of MC58 *rpsL*⁻ Δ*pxn* was significantly higher (*p* value < 0.05) than other strains, which were undetectable at later time points. Three experiments, each in triplicate, were performed. Error bars indicate SE of the mean.

5.3.5. MC58 Δpxn -mutant has a survival defect in human whole blood

The survival of *N. meningitidis* in an *ex vivo* human whole blood model of meningococcal bacteraemia was investigated. Non-immune blood samples collected from two donors were individually inoculated with meningococci, incubated at 37°C for 2 h, and numbers of cells recovered at different time points were counted. Δpxn -mutant meningococcal cells were less able to survive in whole blood, compared with the wild type (Figure 5.6). Restoring the expression of Prx in the complemented strain, MC58 *rpsL*⁻ Δpxn *pxn*^{Ect} *Erm*^R, clearly improved the survival rate to near WT levels (Figure 5.6). These data suggest that Prx could be important for survival of *N. meningitidis* in human blood.

[Donor A]



[Donor B]

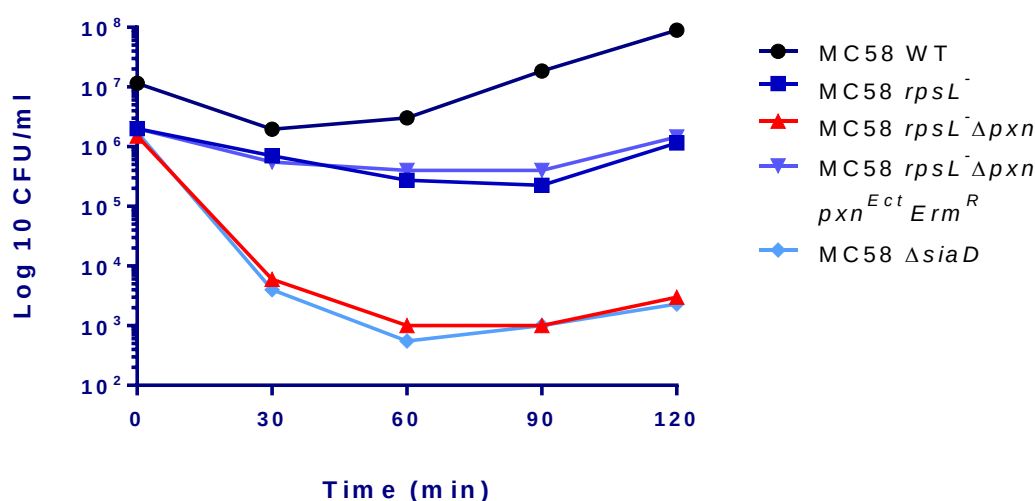


Figure 5.6: Pxn knockout strain has a survival defect in the *ex vivo* human whole blood model of meningococcal bacteraemia. Non-immune heparinized blood samples collected from donor A and donor B were inoculated with different strains of meningococci, and the number of CFU recovered at each time point was calculated. In both cases, the survival of MC58 *rpsL*⁻ Δ *pxn* strain was clearly lower than that observed in the WT, yet close to that measured in the capsule-deficient strain, MC58 Δ *siaD*.

5.3.6. Meningococcal cells killing by PMNs

To investigate the impact of Prx on the survival of meningococci in the presence of PMNs freshly isolated from human blood, mid-log-phase meningococcal cells were incubated with PMNs at 37°C for 30 min. Controls containing only the bacteria were used for comparison. Likewise a PMN cell contamination control was also included. After the incubation period, numbers of recovered meningococcal cells in the presence or absence of PMNs and from one strain to another were very similar (Figure 5.7). No growth was detected in the PMN cell control, suggesting that the isolated PMNs were free of bacterial contamination.

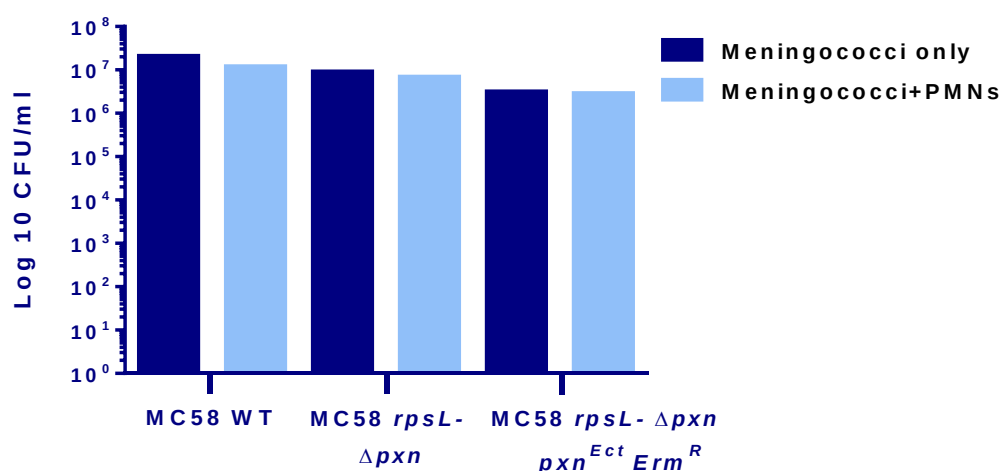


Figure 5.7: Survival of meningococcal cells in PMNs freshly isolated from human blood. Meningococcal cells were incubated for 30 min at 37°C with or without PMNs, and the number of meningococcal cells (expressed as the CFU) recovered after the incubation period was calculated. The assay was performed once in duplicate.

5.4. Discussion

Evaluating the growth characteristics of the *N. meningitidis* Δpxn mutant strain, which was earlier described in chapter 3, was crucial before commencing any phenotypic characterization, particularly as the encoding gene is part of a two-gene operon and Prx expression was lower level in the complemented strain compared to the wild-type strain. Likewise, as the Prx-mutant was generated in an *rpsL*⁻ MC58 genetic background, it was important to verify that the mutation in the *rpsL* gene did not interfere with the growth of *N. meningitidis*. The growth was investigated both on solid media and in liquid broth, and in both cases, the growth pattern and cell-density of both Δpxn -mutant strains were similar to these observed in the WT, which were in turn were similar to those seen in the *rpsL*⁻ genomic background. These data collectively indicate that neither deletion of Prx-encoding gene nor the mutation in the *rpsL*⁻ gene have any observable effect on the maximal growth of *N. meningitidis* under *in vitro* conditions.

The surface accessibility of meningococcal enolase, peroxiredoxin and DnaK was first examined by Knaust and co-workers, who suggested, using flow cytometry, that low but significant levels of each of these proteins could be detected on the surface of meningococcal cells [Knaust et al., 2007]. In that report, however, it was not clear which meningococcal strain

i.e. WT MC58 or MC58 $\Delta siaD$ (an isogenic capsule deletion mutant) was actually examined. This was further established using immuno-gold labelling, which provided further evidence that enolase, Prx and DnaK were accessible on the surface of un-encapsulated meningococcal cells [Knaust et al., 2007]. To confirm these findings, the current study utilized the polyclonal monospecific antisera raised against rEno, rPrx and rDnaK, respectively, to detect the target proteins on the surface of intact meningococcal cells using EIA. The results indicated that significant amounts of DnaK and Prx were present on the surface of wild type MC58 cells, providing further evidence that these two cytosolic proteins could be localized on the meningococcal cell surface. However, the presence of enolase on the meningococcal surface could not be confirmed. This may be explained by the presence of the polysaccharide capsule that might have masked enolase of the bacterial surface. In this context it was suggested that the absence of the capsule improved the binding affinity of intact meningococci to Plg by $146 \pm 21\%$ compared with the WT [Knaust et al., 2007]. In addition, GAPDH from *N. meningitidis* MC58 is a moonlighting protein that was found surface-accessible only in the absence of capsule [Tunio et al., 2010a]. Utilizing purified IgG fractions of the rabbit antisera to investigate the surface localization [Knaust et al., 2007], may also explain the difference in the

results obtained regarding the surface accessibility of meningococcal enolase.

Binding of intact meningococcal cells to Plg is a well-established property of *N. meningitidis* [Ullberg et al., 1992]. As a significant amount of Prx was detected on the bacterial surface, it was important to examine whether or not the absence of peroxiredoxin would alter this phenotype. However, while the ability of meningococci to sequester Plg was confirmed, the results clearly demonstrated that the absence of Prx from Δprx -mutant strains did not affect the binding of Plg. The same applies to FBA-deficient meningococci, *i.e.* the MC58 $\Delta cbbA$ -mutant, that was able to bind a similar amount of Plg as the WT. It seems that the absence of Prx or FBA may be compensated by other Plg-surface receptors. In this context, it was earlier suggested that there are at least six receptors on the surface of *N. meningitidis* for Plg [Knaust et al., 2007]. Furthermore, the massive reduction in the binding affinity to Plg that was observed after the addition of EACA, suggests that the lysine binding sites (LBS) of Plg are largely involved in perhaps all Plg interactions on the surface of meningococci.

Prxs, as discussed earlier, are a large family of cysteine-based peroxidases that mainly catalyse the reduction of H_2O_2 , peroxynitrite and a wide range of organic hydroperoxide

compounds [Christman et al., 1985, Netto et al., 1996, Bryk et al., 2000], and hence are significant players during oxidative stress [Poole, 2005, Rhee and Woo, 2011]. With that in mind, we aimed to investigate whether the absence of Prx would impact on the survival of meningococci under H₂O₂-induced oxidative stress. Challenge of the Δpxn -mutant with different concentrations of H₂O₂ clearly demonstrated that Prx-deficient meningococci were able to tolerate H₂O₂ far better than the WT strain. Restoring the expression of Prx in the complemented strain resulted in a reduction in the tolerance to H₂O₂, and hence the survival of the cells, to the WT level. These findings can be explained by the presence of one or more enzymes that may be up-regulated in response to H₂O₂ to compensate for the absence of Prx. *Kat*, which encodes catalase, is a gene that is known to be up-regulated in response to H₂O₂ in *N. meningitidis* [Grifantini et al., 2004, Stohl et al., 2005]. The expression of *Kat* is tightly regulated by OxyR, which is a redox-sensitive transcription factor that binds promoter sequences of different antioxidant-encoding genes [Ieva et al., 2008], collectively called the OxyR regulon. In *N. gonorrhoeae*, it was earlier found that in addition to *Kat*, a single locus comprising a two-gene operon was also upregulated in response to H₂O₂-mediated oxidative stress [Seib et al., 2007]. These two genes encode peroxiredoxin and glutaredoxin oxidoreductase (Gor), in which

the gonococcal Prx is 98% identical to its homologue in meningococci [Seib et al., 2007]. Importantly, when the Prx-null *N. gonorrhoeae* was challenged with H₂O₂, the strain exhibited increased resistance compared with the WT [Seib et al., 2007]; which is very similar to what was observed in the current study regarding Prx-null *N. meningitidis*. This can be explained by the dual role that OxyR plays in response to different oxidative stress signals; when the signal is absent, reduced OxyR represses OxyR regulated genes, while when an oxidative stress signal is sensed, oxidized OxyR activates the genes of the regulon [Ieva et al., 2008]. Similar findings were observed in non-typeable *Haemophilus influenzae*, which produce a peroxiredoxin/glutaredoxin (PGdx) hybrid protein that is similar to Prx from *N. meningitidis* [Rouhier and Jacquot, 2003]. In that study it was demonstrated that catalase-deficient and catalase/PGdx-deficient strains were significantly more susceptible to H₂O₂ (0.5 mM) than the parent strain, while the effect of H₂O₂ on a PGdx-null *H. influenzae* was less severe [Juneau et al., 2015].

The results discussed above suggest that in terms of H₂O₂ detoxification, meningococcal Prx may be dispensable. However, in the blood model of infection, Prx-deficient meningococci were clearly less able to survive, compared with the WT. Indeed, the survival pattern these cells adopted was

very similar to that observed in the capsule-deficient strain, suggesting that the absence of Prx could have serious consequences, similar to those found with the absence of the capsule, on the fitness of *N. meningitidis*. Failure to detoxify other reactive species might be the reason why Prx-null strain was less tolerant in non-immune blood. As Prxs in general play an important role in the regulation of cell signalling pathways [Rhee and Woo, 2011], interference in one or more of these pathways could be another reason why meningococcal Prx is important for survival in human blood. Last but not least, the moonlighting function of meningococcal Prx to recruit the plasma protein Plg might be important for bacterial survival in blood. In addition to Plg recruitment, meningococcal Prx may possess other, yet undiscovered, moonlighting functions that may in turn contribute to survival in blood.

PMNs generate, as part of their O₂-dependent bactericidal mechanisms, a range of reactive oxygen species, principally superoxide anions and H₂O₂. Inside these phagocytes, the local H₂O₂ concentrations may be as high as 100 µM [Park et al., 2005]. As the current study demonstrated that Δpxn -meningococci, unlike the WT, can tolerate H₂O₂ even at reasonably high levels (20 mM H₂O₂); it was anticipated that Prx-null meningococcal cells might show a differential survival phenotype in the presence of PMNs compared with the WT.

However, numbers of meningococcal cells incubated in the presence or absence of PMNs and from either the Prx-null mutant or WT strain were surprisingly very similar. Having this experiment performed only once, in addition to the lack of a PMN cell positive control, which would have addressed the activity of the isolated PMNs, reduce the significance of these results. In this context, the difficulty in isolating sufficient PMNs from blood to facilitate examining different conditions was a limitation. Moreover, in addition to the fact that PMNs are fragile cells and are prone to apoptosis on removal from plasma, it was previously shown that non-isolated PMNs were significantly more efficient at phagocytosis than the isolated PMNs, emphasizing that the isolation itself, regardless of the technique adopted, significantly impairs PMN cell activity [Zhou et al., 2012].

In conclusion, the work described here has shown that Prx-deficient meningococcal cells tolerate H_2O_2 better than the WT, but have a survival defect in human whole blood.

6. CHAPTER SIX: General discussion

6.1. General discussion

Protein moonlighting describes the additional task(s) that a protein exhibits alongside its canonical function. Interactions with various host proteins after being translocated to the cell surface via an unknown mechanism are biologically important moonlighting roles for some proteins. Moonlighting proteins have been identified in both prokaryotic and eukaryotic cells; they are typically metabolic pathway-associated enzymes, molecular chaperones, and transcription or translation factors [Henderson and Martin, 2014].

Enolase, peroxiredoxin and DnaK are meningococcal housekeeping proteins that are functionally described as a glycolytic enzyme, an antioxidant protein and a molecular chaperone, respectively. They have been identified on the surface of meningococci and are associated with non-housekeeping functions relating to interaction with the human protein plasminogen [Knaust et al., 2007]. The work presented in this thesis details molecular and functional characterization of these three housekeeping proteins from *N. meningitidis*.

The classical approach to investigate the role of a certain protein is to delete the encoding gene, and then examining the resulting phenotype of the mutant. However, this approach is not always feasible or appropriate. As meningococcal enolase and DnaK are essential proteins that are required for *N. meningitidis* viability

[Knaust et al., 2007], a different approach was required to investigate function. The approach used was to generate recombinant proteins which could be used in functional studies and also to raise monospecific polyclonal antibodies to examine the potential role of these meningococcal proteins in pathogenesis. Conversely, as meningococcal Prx is not essential in *N. meningitidis* the classical approach to generate a mutant strain followed by phenotypic analysis was followed.

6.1.1. Plasminogen binding

Employing receptors and/or manufacturing Plg activators are two mechanisms by which bacteria acquire and activate plasminogen on the bacterial surface. This acquisition in turn boosts the ability of bacteria to invade different tissues and to evade components of the host immune system [Bergmann and Hammerschmidt, 2007]. Tissue invasion occurs when the immobilized Plg on the cell surface of bacteria becomes activated, and hence acquires the ability to directly (via plasmin) and indirectly (via plasmin-mediated enzymatic activation) degrade components of the host ECM and basal membrane elements [Sanderson-Smith et al., 2012]. Streptokinase and staphylokinase that are respectively produced by Group A, C and G streptococci and *S. aureus*, are examples of plasminogen activators of bacterial origin [Lähteenmäki et al., 2001].

Designating cytosolic enolase, peroxiredoxin and DnaK from *N. meningitidis* MC58 as moonlighting proteins was first suggested by Knaust and co-workers, who demonstrated that human glu-plasminogen was the host ligand to which these proteins bind [Knaust et al., 2007]. The mode by which each of these proteins interacts with Plg is not, however, fully understood. Therefore, meticulous examination of these interactions considering both plasminogen and its putative receptors *i.e.* meningococcal enolase, Prx and DnaK, was one of the aims of the current study.

In order to further explore the moonlighting activities of meningococcal enolase, peroxiredoxin and DnaK, the encoding genes were individually cloned into the expression vector pQE30 to facilitate the overexpression of N-terminal His-tagged rEno, rPrx and rDnaK, which were then purified by IMAC under non-denaturing conditions. After confirming the identity and the purity of each recombinant protein by SDS-PAGE and immunoblot analysis, the oligomerization status of each was then investigated. Natively purified rEno was found mostly present as an octamer. To our knowledge, this is the first Gram-negative bacterial enolase shown to form octamers. Similarly, rPrx and rDnaK were mostly present as hexamers and dimers, respectively. Rabbit polyclonal monospecific antibodies targeted

against each recombinant protein were raised to examine the surface localisation and function of the respective proteins.

Examining the *in vitro* interaction of rEno, rPrx and rDnaK with Plg suggested that among the three, rEno was consistently the strongest binder to Plg followed by rDnaK and then rPrx, which was the weakest in terms of its affinity for Plg. The question arises here is that do these differences in affinity to Plg have any biological significance in meningococci? We hypothesize that the different stages of meningococcal pathogenesis inside the human body might require different levels of Plg recruitment and/or activation. So, having receptors with different affinities to Plg, expressed on the bacterial surface only when necessary, may serve that goal.

Binding of rEno, rPx and rDnaK to Plg was inhibited by the lysine analogue EACA, suggesting that lysine residues might mediate these interactions. It is important to stress, however, that abolishing Plg binding in the presence of EACA is not always a reflection of the involvement of lysine residues in the interaction, as it is actually reflecting the involvement of lysine binding sites (LBS) of the Plg molecule in that interaction [Sanderson-Smith et al., 2012]. Group A streptococcal plasminogen-binding M protein is one example to illustrate this point. Whilst the addition of EACA was shown to readily abolish

the interaction of M protein to Plg [Berge and Sjöbring, 1993], hence suggesting the contribution of lysine residues to the process, it was later demonstrated that substituting two arginine and two histidine residues (Arg¹⁰¹, His¹⁰², Arg¹¹⁴ and His¹¹⁵) of the protein entirely abolished binding to Plg [Sanderson-Smith et al., 2006]. This is however, not to say that lysine residues are not vital for Plg binding, on the contrary, it seems that the contribution of lysine residues in mediating Plg interactions is very common. Enolase and elongation factor from *Bacillus anthracis* [Chung et al., 2011], PepO from *S. pneumoniae* [Agarwal et al., 2013], EF-Tu from *Leptospira interrogans* [Wolff et al., 2013], GAPDH from *Dirofilaria immitis* [González-Miguel et al., 2015] and PbsP from *S. agalactiae* [Buscetta et al., 2016] are examples of proteins which were shown to interact with Plg (as part of their canonical or moonlighting functions) in a lysine-dependent manner.

Despite excluding the contribution of the C-terminal lysine of meningococcal enolase, peroxiredoxin and DnaK in mediating the interaction of each to Plg [Knaust et al., 2007], the current study demonstrated that mutated proteins lacking the C-terminal lysine residue, *i.e.* rEno428KA, rPrx244KA and rDnaK642KA, were significantly less able to bind Plg, compared with the corresponding wild type proteins. A further reduction in binding of Plg occurred when EACA was added, indicating that

while the C-terminal lysine is pivotal in each interaction, it is not exclusive. In terms of rDnaK, and unlike K642, the penultimate lysine (*i.e.* K641) did not have any detectable effect on Plg binding. Substituting the sub-terminal lysine of rPrx *i.e.* K 230 produced a similar result.

Employing pneumococcal enolase (PDB entry 1W6T) as a model to the predict position of lysine residues that are likely to be involved in the binding of meningococcal enolase to Plg, was informative. Based on this model, three lysine residues at positions 297, 337 and 355 were initially predicted to be important for Plg binding. The current study showed for the first time that single amino acid substitutions of K297 or K355 significantly decreased the ability of enolase to bind Plg. Examining other Lys residues including K337, K388 and K400 clearly indicated that none of these residues are likely to be involved in the enolase-Plg interaction. Likewise, when lysine 250 and 253, which correspond the two Lys residues within the internal motif (FYDKERKVY) that was suggested as a second Plg binding site for pneumococcal enolase [Bergmann et al., 2003], were simultaneously substituted, meningococcal enolase was able to bind a similar amount of Plg as the wild type protein. It can be concluded that these two residues, if not the entire motif, are not involved in Plg binding in meningococcal enolase. A similar finding was suggested regarding enolases from *S.*

salivarius strains SV17 and SV110, which are genetically related to *S. pneumoniae*. Each of these enolases has a variant internal motif (FYENGVY) that naturally lacks lysine residues. Despite that, both enolases were able to bind and activate a similar amount of plasmin(ogen) as those holding the FYDKERKVV motif, clearly indicating that the absence of lysine residues did not impact the ability to bind Plg [Itzek et al., 2010].

As mentioned earlier, meningococcal peroxiredoxin is a natural hybrid protein consisting of a Prx domain and a Grx domain, in which a mutation in the second cysteine active site of the Grx (*i.e.* C185) was found to inactive enzymatic activity [Rouhier and Jacquot, 2003]. The current study identified no significant difference in the binding of the cysteine mutant protein, compared with that of the wild type. From this, it is apparent that the canonical and the moonlighting functions of meningococcal Prx are clearly independent. A similar conclusion was drawn by Shams and colleagues, who have recently demonstrated that a non-functional copy of meningococcal FBA was able to recruit a similar amount of Plg as the functional wild type FBA [Shams et al., 2016]. This finding leaves us wondering whether or not the surface localized meningococcal Prx can actually perform its canonical function as an antioxidant alongside the moonlighting one.

Interaction of different ligands with Plg is thought to be mediated by kringle domains (KR), which are described as repeating triple-disulfide-bonded units. In most cases, the C-terminal lysine and/or internal lysine residues of these ligands bind to LBS carried on KR domains [Sanderson-Smith et al., 2012]. The composition of these LBS determines the binding affinity of each KR to lysine residues (or similar amino acids) [Marti et al., 1997]. Whilst the interaction with Plg by meningococcal rEno, rPrx and rDnaK has been examined, and the lysine residues that are predominantly responsible were identified, little is known about the motif(s) in the kringle domains (KR) of plasminogen to which these proteins bind. In an attempt to investigate these KR motifs, construction and expression of C-terminal 6× His-tagged recombinant KR1-5, KR1-2, KR3-5, and KR1-5 lacking individual domains were undertaken. Unfortunately, trials to purify these recombinant proteins under different conditions were unsuccessful. As a result, this aspect of the project was discontinued.

In summary, the current study demonstrates that meningococcal rEno, rPrx and rDnaK bind Plg predominantly, yet not exclusively, via C-terminal lysine residues. For rEno, lysine residues at positions 297 and 355 are proposed as additional sites for Plg binding. Finally, a non-functional copy of

rPrx recruits a similar amount of Plg as the wild type confirming that Plg binding activity is independent of enzymatic function.

6.1.2. The phenotype of Δpxn meningococcal cells

In silico analysis of sequences upstream and downstream of NMB0946, which encodes meningococcal Prx, confirmed that the upstream gene (NMB0944) and NMB0946 are independent. It also demonstrated that NMB0946 and NMB0947, which encodes Gor, form a two-gene OxyR regulon, which was previously described in *N. gonorrhoeae* [Seib et al., 2007]. In order to delete NMB0946 without inserting an antibiotic selectable marker, which may interfere with the expression of Gor, a markerless mutation strategy was adopted. To facilitate the mutation, two mutagenic plasmids, pGUD2 and pGUD3, were constructed to allow two homologous recombination events. In the first recombination event, Prx from the streptomycin-resistant *N. meningitidis* MC58 *rpsL*⁻ strain was replaced by the kanamycin cassette and *rpsL*⁺ gene (KnsL) carried on pGUD2. The resultant strain was designated *N. meningitidis rpsL*⁻ Δ *pxn Kan*^R *rpsL*⁺. To remove the selectable marker (*Kan*^R *rpsL*⁺) and generate a markerless Prx mutant, pGUD3 was used to transform the latter strain. The final markerless Prx null strain was designated *N. meningitidis rpsL*⁻ Δ *pxn*. Complementation of this null mutant strain was successfully achieved by reintroducing the gene encoding Prx

into the genome of MC58 *rpsL* Δ pxn at an ectopic site. The complemented strain was used as a control to exclude putative polar effects during the phenotypic characterization of the Prx mutant strain. Immunoblot analysis confirmed that Prx was absent in the mutant strain but restored in the whole cell lysate of the complemented strain. The current study showed that neither the deletion of Prx nor the mutation in the *rpsL* gene had any substantial effect on the *in vitro* growth of meningococci.

Knaust and co-workers demonstrated that meningococcal enolase, Prx and DnaK were significantly, yet not abundantly, localized at the surface of the capsule-deficient MC58 [Knaust et al., 2007]. The current study identified DnaK and Prx on the surface of encapsulated wild type MC58; meningococcal enolase, however, could not be detected, suggesting that the presence of the polysaccharide capsule may mask the presence of enolase. Factors such as the titre of the antiserum used for detection might also be important, especially if the target antigen (in this case enolase) is not abundant [Knaust et al., 2007]. In this context, the amount of enolase detected on the surface of un-encapsulated *S. pneumoniae* was relatively low [Kolberg et al., 2006], but was nevertheless biologically important.

While the presence of Prx on the surface of wild type *N. meningitidis* was significant, the absence of Prx did not alter the ability of cells to bind Plg, presumably due to the availability of other Plg surface receptors that could compensate for the absence of Prx. Likewise, the current study found that the Prx-deficient meningococcal cells were able to tolerate higher concentrations of exogenous hydrogen peroxide (H_2O_2) than wild type cells. This tolerance, however, was no longer detected when the expression of Prx was restored in the complemented strain. The overexpression of other H_2O_2 -induced enzyme(s), including catalase, that can detoxify H_2O_2 , was probably the cause of this phenotype. The presence of other Plg receptors and H_2O_2 -detoxifying enzymes that can respectively accomplish the canonical and the moonlighting functions of meningococcal Prx may give the impression that Prx is dispensable for the survival of meningococcal cells. However, in an *ex vivo* human whole blood model of meningococcal bacteraemia, Prx-deficient meningococcal cells were clearly less able to survive compared with wild type cells. Failure to detoxify other reactive species or anions might be the reason why the Prx-null strain was less able to survive in non-immune blood. For example, peroxynitrite is a strong and unusually stable anion that is generated from the interaction of nitric oxide with superoxide [Beckman et al., 1994]. Peroxynitrite is one of the anions that Prxs can, in

general, degrade [Bryk et al., 2000]. Activated macrophages and neutrophils produce nitric oxide and superoxide abundantly, and hence generate peroxynitrite, which can diffuse, due to its stability, far from the site of formation while being selectively reactive with cellular targets [Beckman and Crow, 1993, Beckman et al., 1994]. Examining the ability of meningococcal Prx to detoxify peroxynitrite and investigating the survival of meningococci in macrophages and neutrophils may be an interesting area for future work. Finally, it has been demonstrated that in the blood model of infection, expression of *ca.* 30% of open-reading frames in the *N. meningitidis* genome was altered [Echenique-Rivera et al., 2011], reflecting the complexity of cellular changes that occur to rapidly adapt to that niche.

In conclusion, this study suggests that the absence of Prx has no impact on overall Plg binding on the bacterial surface, enhances the ability of meningococcal cells to tolerate exogenous H₂O₂, and finally, significantly impaired survival of meningococcal cells in human non-immune whole blood.

6.2. Future directions

While the current study has shown that meningococcal enolase may not be surface localized, further examination is necessary to validate this finding. An un-encapsulated strain could be used

to examine if the capsule masks the protein on the bacterial surface. Techniques such as flow cytometry and immuno-gold labelling can also be employed to detect surface-localised enolase.

It is becoming clear that moonlighting proteins can actually play an important role in the virulence of bacteria, and hence finding the machinery by which these proteins are exported would be an interesting area to investigate. Initially, detailed *in silico* analysis of the amino acid sequence of the protein is required to look for putative regions or motifs that might be vital for surface transportation and anchorage. Importantly, posttranslational modifications (PTMs; e.g. acetylation) that some proteins undergo can sometimes impact localization. Examining the potential impact of these PTMs may offer more insights regarding the translocation of moonlighting proteins.

Deleting the *cbbA* gene, which encodes FBA, could be accomplished in the markerless Δpxn -mutant strain. This would facilitate study of the binding of Plg to meningococcal cells in the absence of Prx and FBA, providing a better estimation of the total number of Prx and FBA receptors that are expressed on the surface of meningococcal cells.

Identifying other moonlighting properties that these meningococcal proteins may accomplish is worth considering.

For instance, it has been reported that DnaK from *Francisella tularensis* was able to inhibit TNAP, the major plasma tissue-nonspecific alkaline phosphatase isoform. This DnaK-mediated inhibitory effect could trigger an inflammatory response, which is advantageous to the organism [Arulanandam et al., 2012]. It would be interesting to investigate the ability of DnaK from *N. meningitidis* to inhibit TNAP. Likewise, pneumococcal enolase was found to bind the C4b-binding protein (C4BP), which hinders both the classical and lectin pathways of complement. Such binding could enable pneumococcal cells to evade the complement-mediated host immune response [Agarwal et al., 2012]. Does meningococcal enolase mimic its pneumococcal homologue in terms of binding to C4BP? Answering this question will enable us to better understand the pathogenesis of *N. meningitidis*. The capability of meningococcal enolase to bind other host proteins such as MUC7, a mucin present in human saliva and previously shown to bind to enolase from *S. gordonii* [Kesimer et al., 2009], could also be examined.

Meningococcal enolase, Prx and DnaK were shown to sequester Plg *in vitro*. However, the *in vivo* significance of such sequestration is not yet established. To do that, it is crucial to first determine whether any of these interactions, or host plasminogen activators, can activate Plg into the proteolytic enzyme plasmin. Experiments could also be designed to

examine the ability of meningococcal cells pre-treated with Plg to lyse fibrin (a component of the ECM that can be degraded by plasmin), and invade nasopharyngeal and/or endothelial cells.

In terms of meningococcal enolase, while the contribution of lysine residues 428, 297 and 355 in mediating the binding to Plg, has already been individually addressed, the effect of substituting these three residues simultaneously on Plg binding could also be addressed.

To examine the hypothesis that Δpxn -mutant meningococcal cells grown under oxidative stress overexpress catalase, the transcription of *Kat*, which encodes catalase, could be investigated by quantitative reverse transcription PCR. Alternatively, microarray could be employed to investigate the gene expression profiles of the Δpxn -mutant strain compared to the wild type strain.

Given that Δpxn meningococcal cells were defective in their ability to survive in human whole blood, the putative role of PMNs, macrophages and other blood components on the survival of cells lacking Prx could be addressed. Examining the ability of meningococcal Prx to detoxify peroxynitrite may also be an interesting area for future work.

Attempts to purify C-terminal His-tagged kringle domains of human Plg were unsuccessful. As the C-terminal His-tag can

occasionally interfere with protein structure, and hence compromise solubility, re-cloning using a different vector that adds an N-terminal His-tag could be tried. Alternatively, use of a mammalian expression system to express the recombinant kringle domains may overcome protein aggregation as mammalian-specific PTMs, absent from bacterial expression systems, are sometimes essential to maintain protein structure, solubility and/or functionality.

6.3. Conclusion

Meningococcal enolase, Prx and DnaK can recruit Plg onto the bacterial surface. This recruitment is predominantly, yet not exclusively, mediated via the C-terminal lysine residues of the meningococcal receptors. In terms of enolase, two novel lysine residues (K297 and K355) are also required for optimal interactions with Plg. The contribution of K642, but not K641, of rDnaK in the interaction with Plg reflects the specificity of the C-terminal lysine in Plg binding for this protein at least.

Unlike Prx and DnaK, the abundance of enolase receptors on the surface of *N. meningitidis* can be low and/or masked by the polysaccharide capsule.

Despite the absence of Prx or FBA and the low abundance of enolase, the ability of meningococcal cells to sequester Plg was not impaired, presumably due to the involvement of the other

known and probably as yet unknown Plg receptors. This level of redundancy may reflect the significance of Plg acquisition for meningococcal survival and pathogenesis.

The features that meningococcal and pneumococcal enolases share *i.e.* the octameric conformation, the affinity for Plg, the involvement of the C-terminal lysine residues in the Plg interaction and the low abundance on the bacterial surface, collectively suggest that they are structurally and functionally alike, and may exhibit similar moonlighting properties.

The ability of a non-functional copy of rPrx to recruit a similar amount of Plg as the wild type indicates that the canonical and the moonlighting functions of meningococcal Prx are independent.

The ability of the Prx-deficient meningococcal cells to tolerate exogenous H₂O₂ suggests that under this kind of oxidative stress, Prx may be dispensable. In human whole blood, however, the absence of Prx resulting in impaired survival, suggests that Prx may be involved in processes that collectively aid in maintaining bacterial fitness.

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Appendix

Appendix 1



The University of
Nottingham

UNITED KINGDOM • CHINA • MALAYSIA

Certificate of Achievement

Mahab Al Jannat

School of Life Sciences
Early Researcher of Excellence Award

October 2014

Dr Ian Kerr, Director of Postgraduate Research Studies

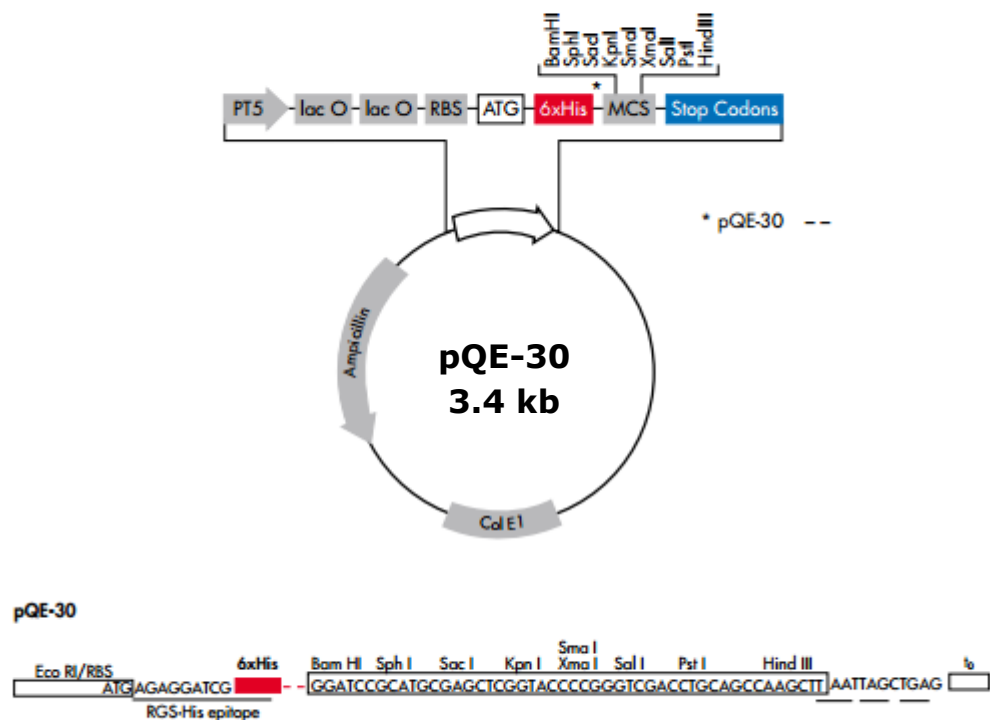
Professor Ian Macdonald, Head of School of Life Sciences

Appendix 2

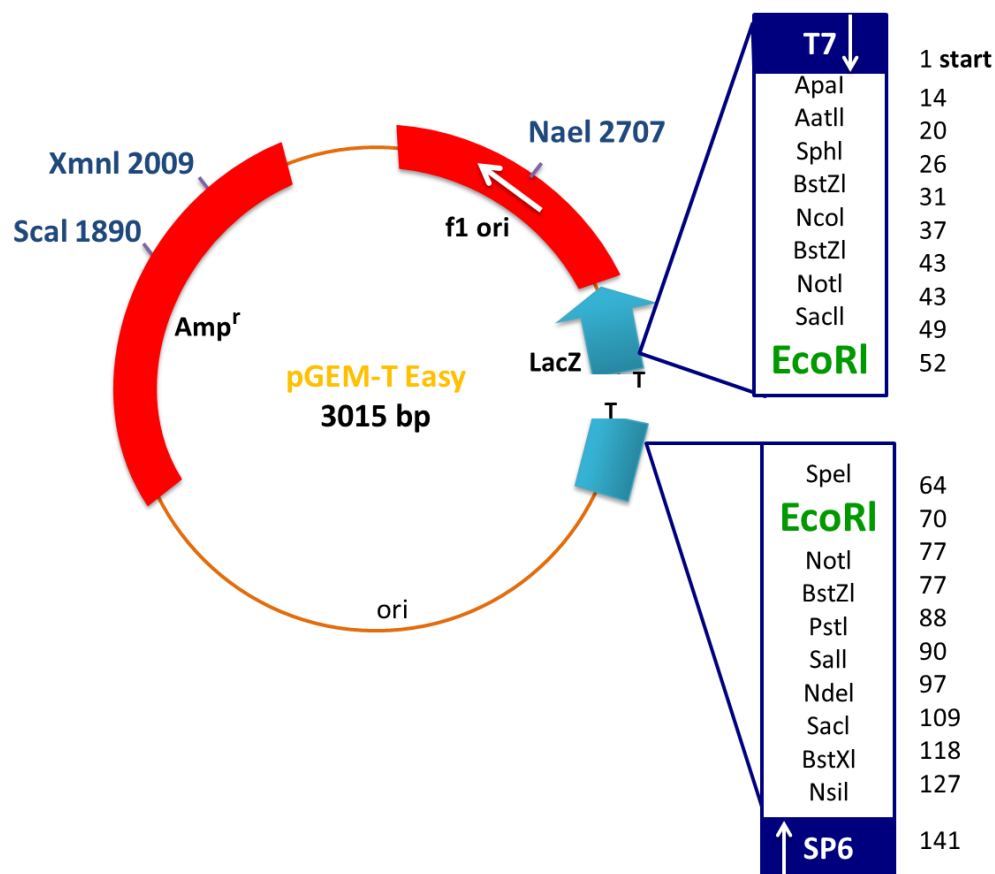


pQE-30 vector

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>rrnB</i> T1 transcriptional termination region	1064–1162
ColE1 origin of replication	1638
β -lactamase coding sequence	3256–2396



Appendix 3: Map of pQE30 vector (Qiagen), which was employed to clone the genes encoding meningococcal enolase, peroxiredoxin and DnaK. Adapted from http://www.helmholtz-muenchen.de/fileadmin/PEPF/pQEVectors/pQE-30_map.pdf.

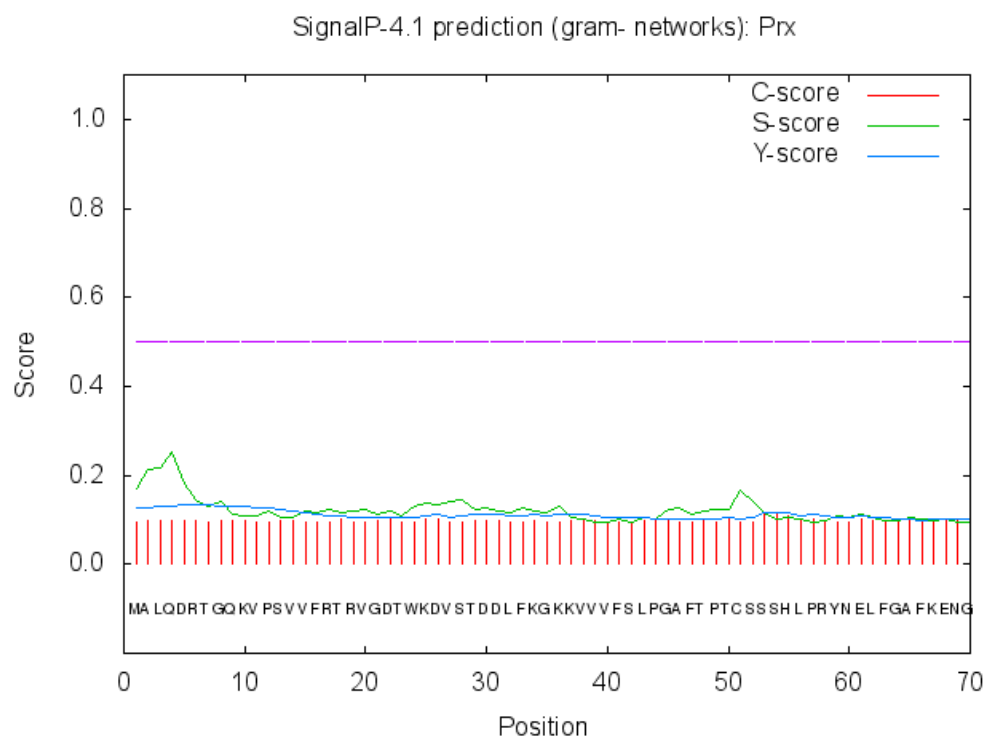


pGEM[®]-T Easy Vector sequence reference points:

T7 RNA polymerase transcription initiation site	1
Multiple cloning region	10–128
SP6 RNA polymerase promoter (–17 to +3)	139–158
SP6 RNA polymerase transcription initiation site	141
pUC/M13 Reverse Sequencing Primer binding site	176–197
<i>lacZ</i> start codon	180
<i>lac</i> operator	200–216
β-lactamase coding region	1337–2197
phage f1 region	2380–2835
<i>lac</i> operon sequences	2836–2996, 166–395
PUC/M13 Forward Sequencing Primer binding site	2949–2972
T7 RNA polymerase promoter (–17 to +3)	2999–3

Appendix 4: Map of the pGEM[®]-T Easy vector, which was employed to construct pGUD1 and pComp-1. Adapted from pGEM-T and pGEM-T Easy vectors systems-Promega Corporation.

[A] Prx (NP_273984.1)



Measure Position Value

max. C 53 0.113

max. Y 11 0.126

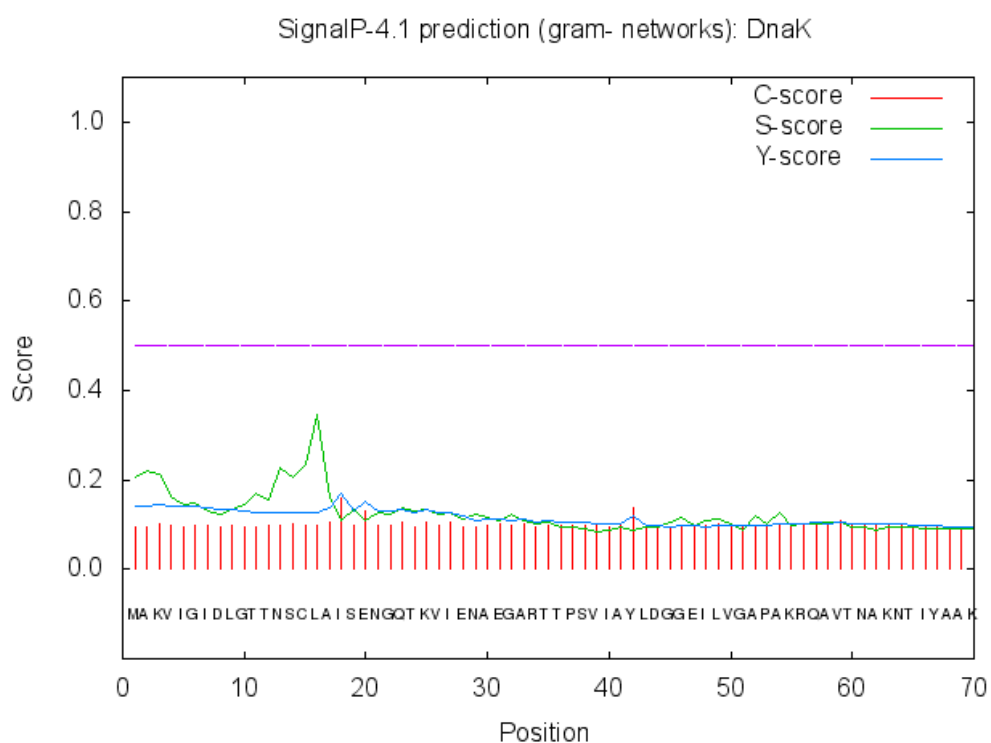
max. S 4 0.251

mean S 1-10 0.167

D 1-10 0.145 0.570 NO

Name=Prx **SP='NO'** D=0.145 D-cut off=0.570

[B] DnaK (NP_273598.1)



Measure	Position	Value
---------	----------	-------

max. C	18	0.160
--------	----	-------

max. Y	18	0.170
--------	----	-------

max. S	16	0.346
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mean S	1-17	0.184
--------	------	-------

D	1-17	0.177	0.570	NO
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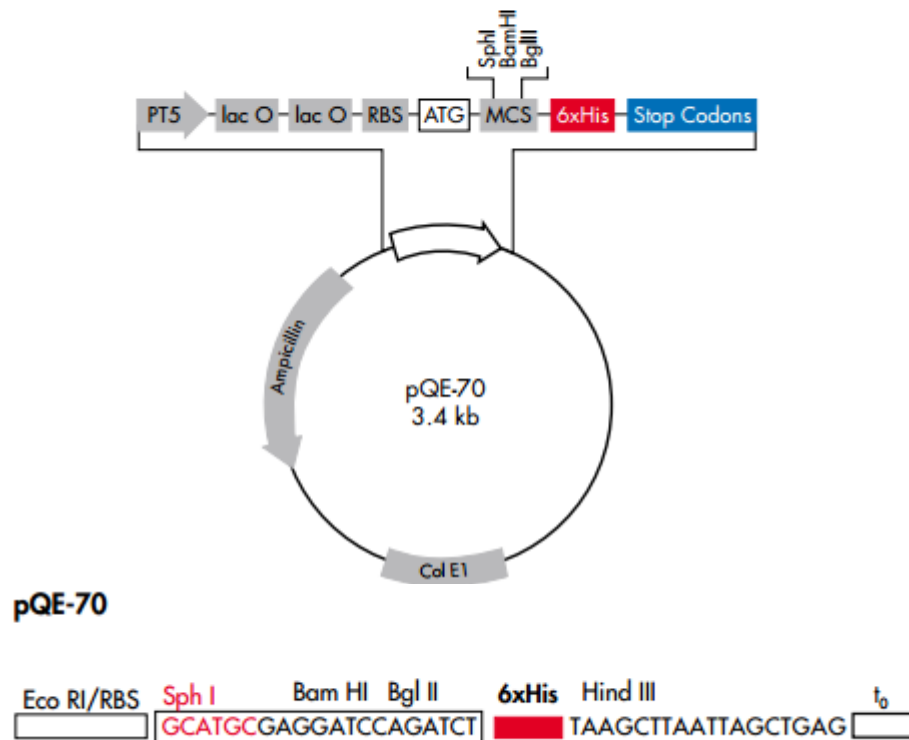
Name=DnaK **SP='NO'** D=0.177 D-cut off=0.570

Appendix 5: Predicting putative signal peptide (SP) sequences of meningococcal peroxiredoxin (A) and DnaK (B). SignalP 4.1 server was utilized to perform the prediction. According to this prediction tool, it seems that neither meningococcal Prx nor DnaK possess SP.

pQE-70 vector

Positions of elements in bases

Vector size (bp)	3426
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	133–150
Multiple cloning site	113–132
Lambda <i>t</i> 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



Appendix 6: Map of pQE70 vector (Qiagen), which was employed to express different kringle domains (KR; KR1-5, KR1-2, KR3-5, pdeK1, pdeK2, pdeK3, and pdeK4). Adapted from http://www.helmholtz-muenchen.de/fileadmin/PEPF/pQE_vectors/pQE-70_map.pdf.