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The Role of Glutamine Synthetase in the Pathogenesis of *Neisseria meningitidis*

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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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September 2019

Abstract

Neisseria meningitidis is a human-restricted pathogen, residing harmlessly in the human nasopharynx, mostly without causing any disease. Nonetheless, the bacteria can invade into the bloodstream and cross the blood brain barrier causing fatal sepsis and meningitis.

The ability for a single protein to perform additional, yet unrelated functions to its canonical function when localised at different locations is termed moonlighting. These moonlighting proteins have been reported at the bacterial cell surface of both prokaryotic and eukaryotic organisms. Once translocated to the cell surface by an unknown mechanism, moonlighting proteins can interact with a variety of host proteins, and demonstrate virulence-related functions.

Meningococcal glutamine synthetase (GlnA) functions as the enzyme responsible for the reversible conversion of glutamate to glutamine, and thus is important for nitrogen metabolism within the cell. Glutamine synthetase has been identified on the surface of other bacteria, where it exhibits moonlighting functions, but its role in the pathogen *N. meningitidis* has not been studied. This study focused on establishing the moonlighting status of meningococcal GlnA, and identifying putative moonlighting functions of the protein.

Meningococcal GlnA was overexpressed in *E. coli*, and the corresponding recombinant protein affinity purified under non-denaturing conditions. The purified GlnA protein was then used to raise polyclonal monospecific rabbit antiserum (RαGlnA) for subsequent characterisation of the enzyme, with the aim to determine its subcellular localisation as well as potential roles in the pathogenesis of meningococcal disease.

Attempts were made to generate a *glnA*-knockout in *N. meningitidis* MC58 wild-type, and a mutant strain with *glnA* inserted in trans was generated in *N. meningitidis* MC58 wild-type.

Employing whole-cell ELISA, GlnA could be detected on the surface of wild-type encapsulated *N. meningitidis* MC58, as well as on the surface of the *glnA* in trans insertion mutant strain, suggesting that meningococcal GlnA may be a moonlighting protein carrying out possible moonlighting functions at the cell surface.

Pull down assays were employed to investigate possible moonlighting functions of GlnA and fructose bisphosphate aldolase (FBA) at the cell surface, specifically to identify human serum and cellular proteins which may interact with meningococcal rFBA and rGlnA, but no interacting partners were identified.

In summary, this study demonstrates that meningococcal GlnA is surface localised, and may be involved in moonlighting activities at the surface which may contribute to the pathogenesis of *N. meningitidis*.

List of Presentations

Poster/oral presentations

Iyelola O. Turner, Neil J. Oldfield, & David P. J. Turner. The role of glutamine synthetase in the pathogenesis of *Neisseria meningitidis*. Presented at the Microbiology Society Annual Conference, 8 – 12th April 2019, Belfast, Northern Ireland, UK.

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Iyelola O. Turner, Neil J. Oldfield, & David P. J. Turner. The role of glutamine synthetase in the pathogenesis of *Neisseria meningitidis*. Presented at the School of Life Sciences Symposium, 11 - 12th July 2018, Nottingham, UK.

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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	Qiagen
2	pIT1	NMB0359 gene from MC58 cloned into pQE30 vector	This study[a]
3	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	Promega
3	pIT2	NMB0359 gene plus 1kb up- and 1kb downstream flanking sequence from MC58 cloned into pGEM®-T Easy vector	This study
4	pIT3	Re-ligated pGUD1 after removing NMB0946 encoding gene by inverse PCR	This study
5	pIT4	Kanamycin resistance cassette and rpsL+ allele from MC58 cloned into pIT3 at the same NMB0946 deletion site	This study
6	pComp-1	A complementation cassette consisting of NMB0102, erythromycin resistance cassette and NMB0103, cloned into pGEM®-T Easy vector	Hiba Albasri
7	pIT5	NMB0359 gene from MC58 cloned into pComp-1	This study
8	pMOD2	Transposon Construction Vector with multiple cloning sites.	Ruth Griffin
9	pIT6	The 5' 688 bp portion of *glnA* cloned into pMOD2	This study
10	pIT7	The 3' 774 bp portion of *glnA* cloned into pIT6	This study

	Plasmid	Description	Source
11	pIT8	*KsnL* cloned into pIT7 to serve as mutagenic plasmid DNA for the inactivation of *glnA* in *N. meningitidis*	This Study
12	pSAT1	NMB1869 gene from MC58 cloned into pQE70 vector	Safraz Tunio[a]

[a]: Corresponding recombinant protein was successfully purified

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Acronyms and Abbreviations

ADP	Adenosine diphosphate
App	Adhesion and penetration protein
AspA	Autotransported serine protease A
ATP	Adenosine triphosphate
BamHI	*Bacillus amyloliquefaciens* strain H restriction endonuclease
BBB	Blood brain barrier
BCIP/NBT	5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium
BglII	*Bacillus globigii* 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
CPS	Capsular polysaccharide
CSF	Cerebrospinal fluid
dNTPs	Deoxy nucleotide triphosphate
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxy ribonucleic acid
E. coli	*Escherichia coli*
ECM	Extracellular matrix
EcoRI	*Escherichia coli* strain R restriction endonuclease
EDTA	Ethylenediaminetetraacetic acid
EIA	Enzyme linked immunosorbant assay
EPEC	Enteropathogenic *E. coli*
ET	Electrophoretic type
FBA	Fructose bisphosphate aldolase

fHBP	Factor H binding Protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GlnA	Glutamine synthetase
HBMECs	Human brain microvascular endothelial cells
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
LAMP	Lysosome-associated membrane proteins
LB	Lysogeny Broth
LOS	Lipooligosaccharide
LPS	Lipopolysaccharides
MBIG	Molecular Bacteriology and Immunology Group
MH	Mueller-Hinton agar
MHC	Major Histocompatibility
MLEE	Multi-locus enzyme electrophoresis
MLST	Multi-locus sequence typing
NAD	Nicotineamide adenine dinucleotide
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
PCR	Polymerase chain reaction
PL	Pellet lysate
Plg	Human glu-plasminogen
PMNs	Polymorphonuclear leukocytes
PorA	Porin A
PorB	Porin B
Prx	Peroxiredoxin
PTM	Posttranslational modifications
RBCs	Red Blood Cells
rFBA	Recombinant fructose bisphosphate aldolase
rGlnA	Recombinant glutamine synthetase
rPrx	Recombinant peroxiredoxin
rpm	Revolutions per minute
SAP	Shrimp alkaline phosphatase
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SE	Standard error
SL	Supernatant lysate
ST	Sequence type
T3SS	Type III secretion system
Taq	*Thermus aquaticus* thermostable polymerase
TCA	Tricarboxylic acid
tPA	Tissue plasminogen activator
TPS	Two partner secretion system
uPA	urokinase plasminogen activator
WCL	Whole cell lysate
WT	Wild type

X-GAL	5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside

CHAPTER 1: General Introduction

1.1. The genus *Neisseria*

The genus *Neisseria* is a member of Kingdom Bacteria, Phylum Proteobacterium, Class β-Proteobacterium, Order *Neisseriales*, Family *Neisseriaceae*. From 16S rRNA gene sequence analysis, at least 25 species form the genus *Neisseria,* of which two are well characterised human pathogens, namely *N. meningitidis* (meningococcus) *and N. gonorrhoeae* (gonococcus) which colonize the mucosal surfaces of the human nasopharynx and urogenital tract, respectively (Maiden, 2008, Hung and Christodoulides, 2013). The other species are commensal organisms in humans and other mammals, which may give rise to opportunistic infections under certain conditions (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. *Neisseria meningitidis*

Meningitis caused by *N. meningitidis* was first clinically described by the French Physician Gaspard Vieusseux, following an outbreak in Geneva, Switzerland in 1805 (Vieusseux, 1805).

Another breakthrough was in 1887 when Anton Weichselbaum first isolated the bacteria from cerebrospinal fluid (CSF) of a patient with epidemic cerebrospinal meningitis, and outlined the connection between the bacteria which he called *Diplococcus intracellularis meningitidis* and epidemic meningitis (Weichselbaum, 1887). The name *Diplococcus intracellularis meningitidis* was later changed to *Neisseria meningitidis,* after the German scientist Albert Neisser, who characterized the closely related *Neisseria gonorrhoeae* (Neisser, 1879). Furthermore, Marchiafava and Celli reported that oval micrococci were present within the leucocytes in the cerebrospinal

fluid (CSF) of patients dying with meningitis (Marchiafava and Celli, 1884), while Heubner and Kiefer were the first to isolate meningococci from the CSF and throat of living patients (Heubner, 1896, Kiefer, 1896).

Meningococci are gram negative, non-spore forming aerobes that lack flagella, and are often arranged in pairs (Ala'Aldeen and Turner, 2006).

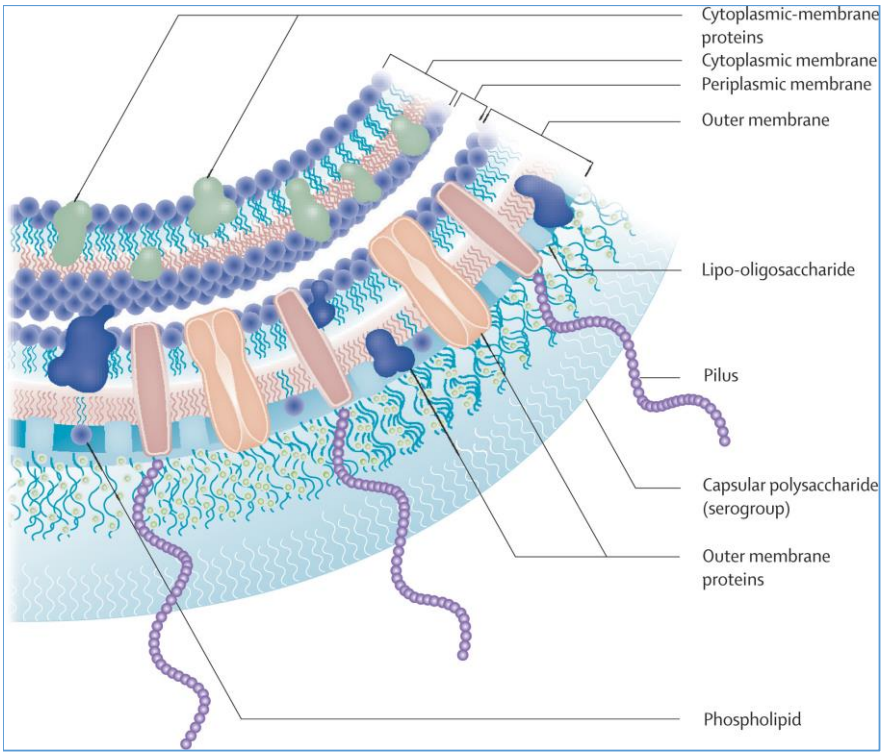


Figure 1.1: Cross-sectional view of the meningococcus (Stephens et al., 2007).

The bacteria are catalase- and oxidase-positive, are capable of fermenting both maltose and glucose (Wesley Catlin, 1973), and require special growth supplements and conditions to grow. On blood agar, and with overnight incubation, smooth, convex, transparent, non-pigmented, non-haemolytic, moist, medium sized colonies with a clearly defined edge are formed (Ala'Aldeen and Turner, 2006). Chocolate agar, modified New York City medium, and Muller-Hinton agar can also support the growth of *Neisseria*. Meningococci grow optimally at 35 - 37°C, pH 7.0 - 7.4 in a humid environment with 5 - 10% ($^{v}/_{v}$) CO_2 (Ala'Aldeen and Turner, 2006).

1.3. Classification of *N. meningitidis*

The meningococcal surface structures polysaccharide capsule, several outer membrane proteins (OMPs) and lipooligosaccharide (LOS) provide the basis for serological classification of *N. meningitidis* (Stephens et al., 2007). At present, there are twelve identified serogroups, based on the antigenic properties of the capsular polysaccharide, namely serogroups A, B, C, E, H, I, K, L, W, X, Y and Z, with serogroups A, B, C, W, X and Y linked with invasive meningococcal disease (Tzeng and Stephens, 2000, Stephens et al., 2007). Additionally, differences in the major OMP porin A (PorA) and porin B (PorB) are used to classify meningococci into sub-serotypes and serotypes (Frasch et al., 1985, Ala'Aldeen and Turner, 2006). Likewise, variation in the LOS structures distinguishes 12 immunotypes: L1 – L12, with immunotypes L1 – L8 principally associated with serogroups B and C, and immunotypes L9 –L12 principally associated with serogroup A (Zhu et al., 2002). An example of serological typing can be expressed as B:4:P1.15:L10, which denotes serogroup B, serotype 4, sub-serotype 15 and immunotype 10 (Ala'Aldeen and Turner, 2006).

Multilocus enzyme electrophoresis (MLEE) is a technique useful in the examination of the electrophoretic mobility of various isoforms of a collection of metabolic enzymes. A number score is assigned to each electrophoretic variant and each unique combination of scores is designated an electrophoretic type (ET) (Yakubu et al., 1999, Ala'Aldeen and Turner, 2006).

In the more recent past, multi-locus sequence typing (MLST) has been employed for genomic typing and serves as a very useful tool for epidemiological studies and population diversity studies (Jolley et al., 2007, Stephens, 2009). In MLST, small DNA fragments (433-501bp) from seven housekeeping genes (*abcZ, adk, aroE, fumC, gdh, pdhC, pgm*) are amplified

and sequenced, each sequence assigned an allele number, and the combination of alleles from the seven housekeeping loci used to define the sequence type (ST) (Ala'Aldeen and Turner, 2006, Jolley et al., 2007, Stephens, 2009). MLST has now been superseded by core genome analysis, which is essentially MLST but uses up to 1600 core genes rather than 7, providing a much improved resolution (Maiden and Harrison, 2016). 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 stored, not only for *Neisseria* but for a wide range of prokaryotic and eukaryotic genomes likewise.

1.4. Global Distribution and Epidemiology

N. meningitidis remains an important cause of mortality and morbidity worldwide (WHO, 2011). It is estimated that around the world, at least 1,000,000 new cases of invasive meningococcal disease (IMD), occur every year, resulting in more than 100,000 deaths (Rouphael and Stephens, 2012). Approximately 10%-20% of survivors have severe neurological, visual, or hearing impairments, rates of which are often higher in developing countries (Edmond et al., 2010).

Currently, in Europe, North America and Australia incidence ranges between 0.3 and 1 cases per 100,000 population with reported cases reaching up to 10 – 1,00 per 100,000 population in Africa during epidemics especially in the African Meningitis Belt (Jafri et al., 2013, Gabutti et al., 2015).

The six meningococcal serogroups which cause the majority of invasive disease vary considerably in their global distribution (Borrow et al., 2017).

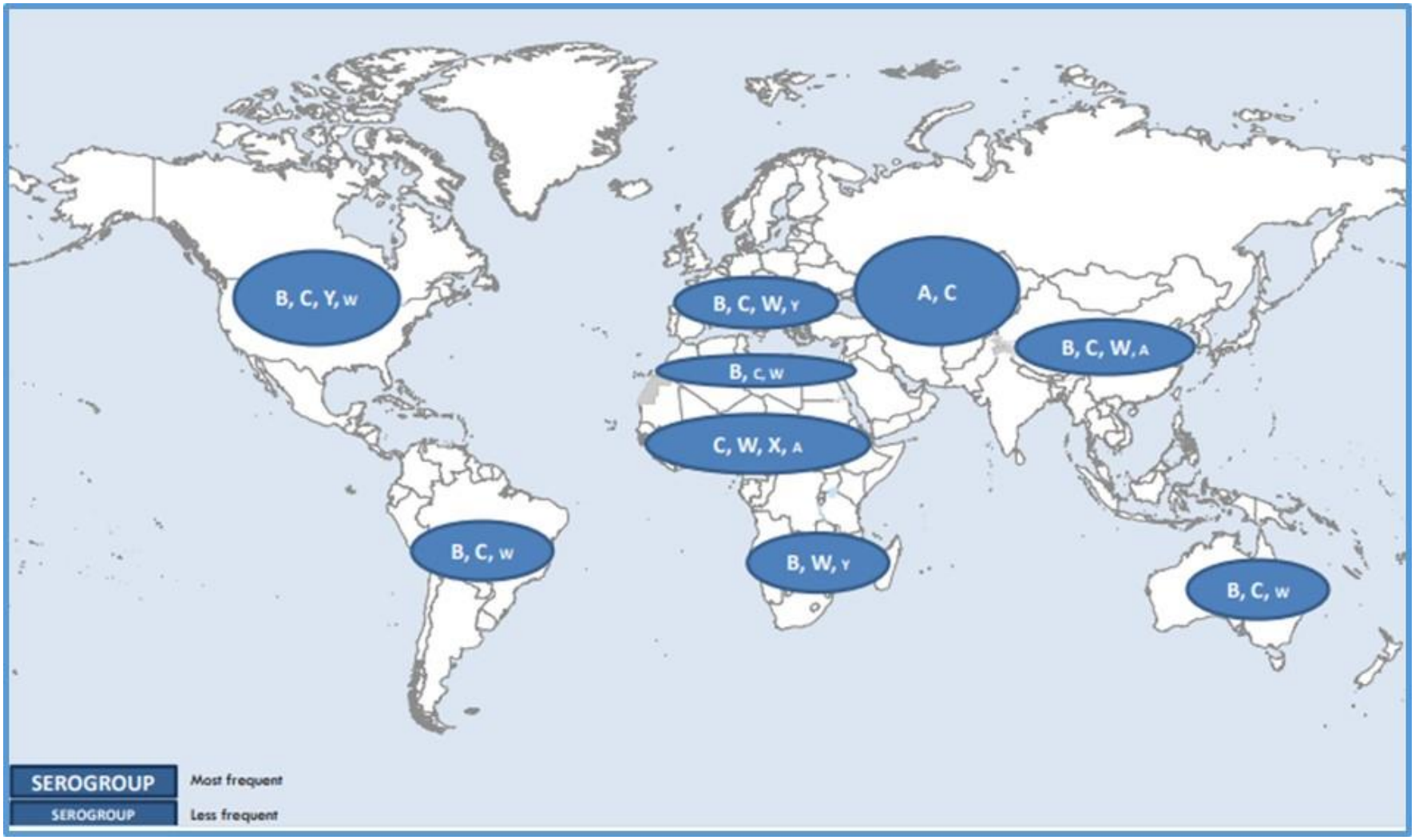


Figure 1.2: Serogroup distribution of invasive meningococcal disease worldwide. Source: WHO, 2018.

Serogroup A has resulted in major global epidemics with repeated outbreaks across the African Meningitis Belt (22 countries in sub-Saharan Africa and extends from Ethiopia in the east to Senegal in the west), and in south-eastern Asia (including India, Mongolia, the People's Republic of China, and the Philippines) with outbreaks occurring every 5 – 10 years (Wilder-Smith, 2008, Hedari et al., 2014, Koutangni et al., 2015).



Figure 1.3: Map showing the Africa meningitis belt: countries at high risk of meningitis epidemics, and countries at risk of meningitis epidemics. Source: WHO, 2015.

Serogroup B strains (MenB) cause endemic disease in much of the developed world; 30% –40% of meningococcal disease in the USA and Canada and 80% in the European countries are caused by MenB. These figures are expected to reduce with recent surges in MenW incidence occurring in South America, Europe, Australia and parts of sub-Saharan Africa, with hypervirulent strains being associated with severe IMD and higher fatality rates (Hedari et al., 2014, Booy et al., 2019). Over the past decade, clusters

of MenB infections were reported in New Zealand, Taiwan, and Thailand (Hedari et al., 2014).

MenC is prevalent in developed countries, has wavering rates of endemic disease, and also causes outbreaks. Fortunately, the MenC monovalent conjugate vaccine was introduced and this has led to a significant reduction in MenC disease where it has been implemented (Cohn et al., 2013). The year 2000 saw the emergence of MenW in Saudi Arabia and Hajj pilgrims returning from Saudi Arabia in countries throughout the world including Europe, the United States, Asia, Africa, and the Middle East leading to epidemics (Lingappa et al., 2003). Infections caused by MenW have also been reported in Turkey, South Africa (with an outbreak from 2003–2007), Nigeria, Argentina, Burkina Faso and, more recently, in Chile and Brazil (Lingappa et al., 2003, Memish et al., 2003, Koumaré et al., 2007, Borrow, 2009). More recently, since 2009, an increase in MenW IMD has also been observed in the UK, Europe, Australia, South America, and Africa (Booy et al., 2019). Serogroup X is becoming progressively frequent in parts of Africa, and causing small and moderate-sized outbreaks (Gagneux et al., 2002, Boisier et al., 2007, Materu et al., 2007).

In the past decade, MenY served as a significant cause of IMD in Canada, UK and the USA, and has also risen recently in South Africa, South America, and Israel (Harrison et al., 2009, Cohn et al., 2013).

1.5. Meningococcal Pathogenesis

N. meningitidis typically colonizes the nasopharynx of its natural human host without causing any evident symptoms, with this circumstance known as carriage, where it can spread from person to person by direct contact with nasal or oral secretions or via respiratory droplets (Caugant et al., 2007). Development of meningococcal disease is highly dependent on successful

colonization of the human upper respiratory tract mucosa/carriage state (Caugant et al., 2007, Stephens, 2009). The characteristics of each individual strain together with host-associated and environmental factors influence the outcome of infection. This means that the likelihood of meningococcal disease occurring is elevated when a person with predisposing factors for disease encounters a more virulent meningococcal strain (Tzeng and Stephens, 2000, Emonts et al., 2003). For meningococcus to survive, the pathogen requires adhesion to the host mucosal epithelial surfaces. Meningococci selectively bind non-ciliated columnar epithelial cells (Stephens et al., 1983). Adhesion is attained through a varied array of structures and components called adhesins, which can bind distinct ligands distributed on the surfaces of various human cells. Pili, Opa, Opc, LOS, fHbp, PorA, PorB, and NadA are some of the well-established meningococcal adhesins (Merz and So, 2000, Tettelin et al., 2000, Parkhill et al., 2000). Type IV pili are regarded as the major adhesins that promote the initial adhesion of meningococcal cells to host mucosal cells, while the capsule inhibits 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).

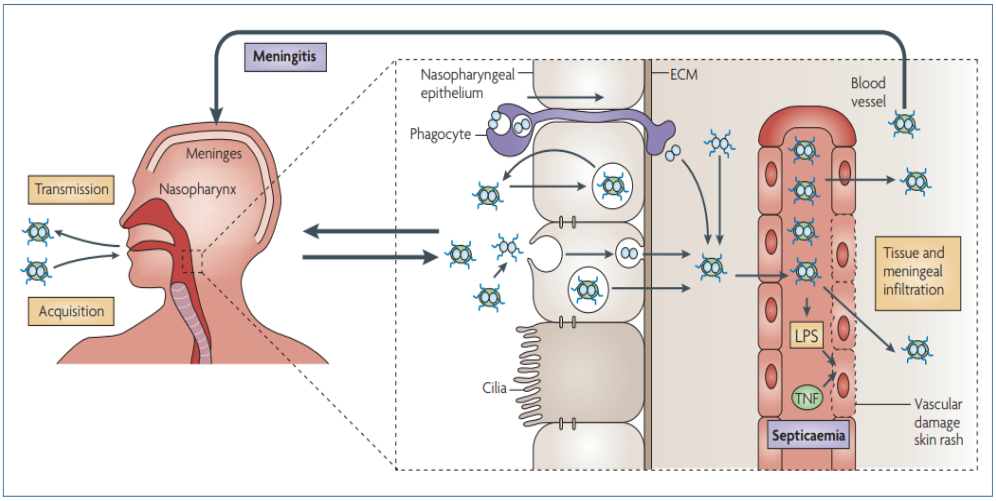


Figure 1.4: Steps of pathogenesis associated with *Neisseria meningitidis*. *N. meningitidis* may be acquired through the inhalation of respiratory droplets. The organism establishes intimate contact with non-ciliated mucosal epithelial cells of the upper respiratory tract, where it may enter the cells 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 (Virji, 2009).

Table 1.1: Some known *N. meningitidis* surface molecules and their human host cell binding ligands. Adapted from (Hung and Christodoulides, 2013).

Human Cell Ligand	***N. meningitidis* Surface Molecules**
CD46	PilE, PilQ, PilW
Integrin	NadA, Opa, Opc
Laminin (ECM)	NhhA, Msf, PilQ, PilC, PilW, PorA
Vitronectin/Fibronectin	NhhA, Msf, Opa, Opc
β2-adrenergic receptor	PilE, PilV
HSPG	NhhA, Msf, Opa, Opc
Toll Like Receptor 2	PorB
Toll Like Receptor 4	LOS
Complement Components	LOS
Plasminogen	DnaK, Peroxiredoxin, FBA, Enolase, GapA1
CAECAM's	Opa

Other surface structures together with the pili are also subject to phase variation, inclusive of 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). Propagation of meningococci on the non-ciliated epithelial cells progresses, resulting in the development of small micro-colonies, and eventually, cortical plaques on the apical surface (Merz et al., 1999, Nassif et al., 2002). At this stage, a close association mediated by the meningococcal opacity proteins Opa and Opc, resulting from the interactions of these proteins with CD66/CEACAMs and integrin receptors carried on the surface of the epithelial cells respectively is set, which generates specific signals and events, that ultimately cause the production and extension of

epithelial cell pseudopodia that envelope the meningococcus (Virji, 1996, Doulet et al., 2006). By yet to be determined mechanisms, meningococcal endotoxin and the NadA outer membrane protein also contribute to this close association between the bacteria and the host cell (Gray-Owen and Blumberg, 2006). After successful traversal of the nasopharyngeal mucosa, *N. meningitidis* circulates to sub-epithelial tissues from which it may gain access to the bloodstream.

Table 1.2: Steps of meningococcal pathogenesis and some known virulence factors linked.

Pathogenesis Step	Known Virulence Factors Involved
Carriage State	Type IV Pili
Epithelial Colonization	Type IV Pili, Opa, Opc, LOS, NadA, NhhA, App, HrpA-HrpB
Bloodstream Invasion	Capsule, LOS, OMV's, fHbp, Iron acquiring proteins
Endothelial Colonization	Type IV Pili, Opa, Opc

The bacterial virulence factors of meningococci that enter the blood stream, together with the host innate and acquired immune responses influence the establishment of meningococcal disease. When meningococci encounter an effective host immune response, brief bacteraemia is anticipated, with meningococcal septicaemia possible if the immune system is compromised (Hung and Christodoulides, 2013). For meningococci to survive in the blood, the expression of the capsule which provides protection against complement-mediated bacterial killing and phagocytosis is essential (Klein et al., 1996). Likewise, the diversion of antibodies and complement components away from the surfaces of intact bacteria can occur through the shedding of outer membrane blebs, which are rich in bacterial endotoxin and outer membrane proteins (Stephens et al., 1982). The bacteria also enhance their survival intracellularly by expressing an IgA1 protease that hampers

phagosome maturation via degrading lysosome-associated membrane proteins (LAMP1) (Hopper et al., 2000). As a result of the rapid replication of meningococci in the blood stream, vascular colonization in which meningococci adhere to endothelial cells ensues. As observed with nasopharyngeal mucosal colonization, the major adhesins that mediate bacterial adhesion to endothelial cells are type IV pili, Opa and Opc (Virji, 2009).

1.6. Clinical Manifestations of Invasive Meningococcal Disease

Invasive meningococcal disease (IMD) usually starts with a range of non-specific signs and symptoms identical to those occurring from other illnesses, making early stage diagnosis of IMD challenging (Theilen et al., 2008). The clinical classification of IMD include meningitis without shock, septicaemia without meningitis, septicaemia and meningitis, meningococcaemia without shock or meningitis, meningoencephalitis, conjunctivitis, pneumonia, septic arthritis, pericarditis, myocarditis and endocarditis (Brandtzaeg and van Deuren, 2012). The most common clinical manifestations of IMD are meningococcal meningitis and meningococcal septicaemia that may co-exist. (Ala'Aldeen and Turner, 2006).

1.6.1. Meningococcal Meningitis

Meningitis is the most prevalent presentation of IMD, with over 60% of patients with IMD in industrialized countries progressing to meningitis without septic shock, and the rate higher in developing countries (Stephens et al., 2007). Meningitis is an inflammation of the meninges: the membranes surrounding the brain and spinal cord. The inflammation is as a result of bacterial loads and elevated levels of endotoxin, consecutively resulting in

elevated levels of cytokines including tumour necrosis factor α (TNF-α) and the interleukins IL-1ß, IL-6, IL-8, and IL-I0, which direct the inflammatory response (Brandtzaeg et al., 1992, Øvstebø et al., 2004, Stephens et al., 2007). The inflammatory process extends throughout the subarachnoid space around the brain and spinal cord, and the ventricles (Mace, 2008).

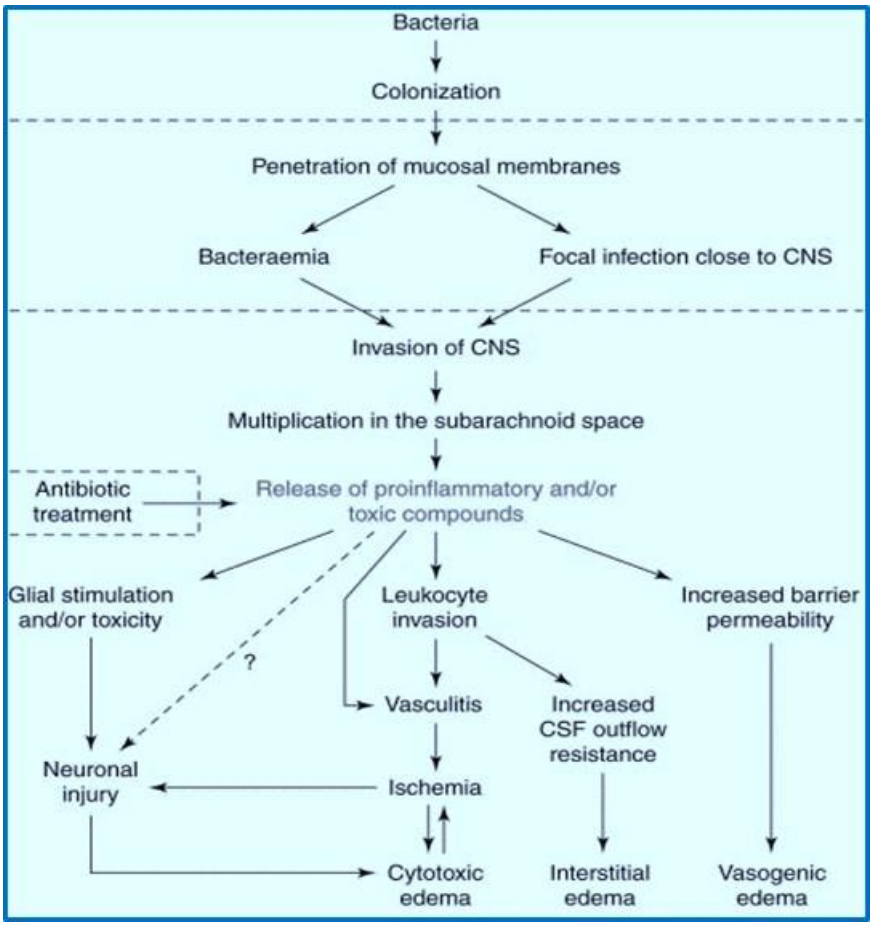


Figure 1.5: A schematic overview of pathophysiology of meningitis. Following colonization, bacteria can enter the central nervous system (CNS) through various means. Bacteria can invade the CNS and enter the cerebrospinal fluid (CSF). The bacteria multiply into the subarachnoid space and release proinflammatory and/or toxic compounds. Then the bacteria stimulate macrophages and glial cells, and cause leukocytes invasion leading to increased blood-CSF, blood-brain barrier permeability and vasculitis (Nau and Brück, 2002).

Symptoms of meningitis may vary with the age of sufferers. In infants and younger children, vague symptoms include poor feeding, irritability, a high pitched cry and a bulging fontanelle, whilst in adults, headache, fever, vomiting, photophobia, neck stiffness, positive Kernig's (inability to extend the knees more than 135 degrees) and Brudzinski's (severe neck stiffness causing a patient's hips and knees to flex when the neck is flexed) signs, altered mental status, seizures (may occur in 5% to 28% of cases) and lethargy are mostly experienced (Loring, 2004, Nadel and Kroll, 2007). Inflammation and bleeding in small blood vessels including those under the skin can result in a skin rash (Nielsen et al., 2001).

Early recognition of the symptoms, diagnosis and management of meningitis, including rapid administration of antibiotics, are critical towards a full recovery or else a mortality rate reaching as high as 70 – 90% is probable. Sadly, even with early management, up to 25% of meningococcal disease survivors develop permanent sequelae which includes loss of hearing, mental retardation, seizures, hydrocephalus, renal failure, skin scarring or limb(s) amputation (Rosenstein et al., 2001). Furthermore, 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).

1.6.2. Meningococcal Septicaemia

Meningococcal septicaemia is the most severe form of meningococcal infection, and is clinically classified as transient or fulminant. The transient form is identified by fever and non-specific rash, both of which can disappear within 2 – 5 days without any treatment (Stephens et al., 2007). Fulminant meningococcal septicaemia is represented by rapid proliferation of

meningococci 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}). These will activate the host innate and acquired immune systems resulting in generalized pathology of the microvascular endothelium, which then causes 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, Stephens et al., 2007). With fulminant septicaemia, severe and persistent shock lasting more than 24 h or until death may also be experienced. 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 suffered (Stephens et al., 2007).

1.7. Virulence factors of *N. meningitidis*

To maintain their survival, whether during the nasopharyngeal carriage stage or during the invasion and dissemination course in the human host, meningococci have developed a strategy which primarily involves 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). These virulence factors undergo phase and/or antigenic variation, which can occur via a number of mechanisms including intragenomic recombination, slipped strand mispairing and reversible insertion of mobile elements (Hammerschmidt et al., 1996a, Hammerschmidt et al., 1996b, Van Der Woude and Bäumler, 2004).

The polysaccharide capsule, lipooligosaccharide, outer membrane proteins (OMPs) and pili (Figure 1.2) represent major meningococcal virulence factors that play a crucial role in the bacterial pathogenesis (Hill et al., 2010). In addition to these, other important surface-exposed and secreted virulence-

associated proteins have been identified through complete genome projects of different pathogenic *Neisseria* strains (Bennett et al., 2012). Some virulence factors are described below.

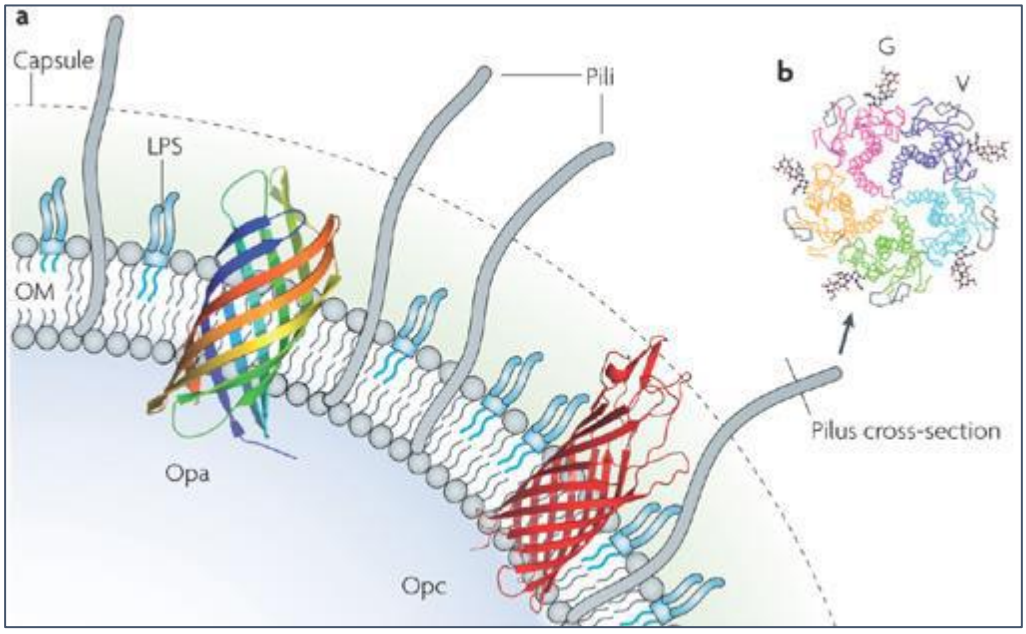


Figure 1.6: Major virulence factors of *Neisseria meningitidis*. (a) Pili traverse the capsule and are the most prominent adhesins of encapsulated *N. meningitidis*. In addition, the integral outer membrane (OM) adhesins, Opa and Opc, are known to mediate interactions with specific host-cell receptors 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).

1.7.1. The Capsule

The polysaccharide capsule is regarded as the major virulence factor and plays a decisive role in invasive meningococcal disease. Its hydrophilic nature has been suggested to enhance meningococcal survival in aerosol droplets, thus facilitating transmission from person to person (Swain and

Martin, 2007). While in the host, the capsule confers resistance to various arms of the immune system designed to eliminate foreign antigens, including phagocytic killing, opsonisation and complement-mediated bacterial lysis (Schneider et al., 2007). Phenotypic characterization of meningococci is based largely on the capsule present. A large number of colonizing meningococci are non-capsulated, but those associated with the invasive disease are invariably encapsulated (Claus et al., 2002, Frosch and Maiden, 2006). Each of the 12 classes of meningococci possesses a structurally distinct capsule, although the majority of IMD are linked to serogroups A, B, C, W, X, and Y (Stephens, 2007). Serogroup A capsule is composed of (α 1→6)-linked *N*-acetylmannosamine-1-phosphate, serogroup X expresses (α1→4) linked *N*-acetyl-d-glucosamine 1-phosphate, while serogroups B, C, W and Y are composed of derivatives of sialic acid (*N*-acetylneuraminic acid), a common component of human cells. Serogroups B and C are homopolymers of *N*-acetylneuraminic acid in α2→8 or α2→9 linkage respectively (Tzeng et al., 2005). Serogroups W and Y express capsules composed of alternating disaccharide repeat units of sialic acid and d-galactose or d-glucose respectively (Stephens, 2009). Sialic acids help the bacteria to evade immune recognition by molecular mimicry. 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 (NCAM), present in the central and peripheral nervous systems, hence, meningococci can utilize this structure similarity to evade recognition by the host immune system (Finne et al., 1983, Romero and Outschoorn, 1994). A gene complex spanning about 24 kb of the bacterial chromosome comprises the meningococcal capsule-synthesis (*cps*) locus, which in turn contains five distinct functional regions. Of these, the

sia operon and *ctr* operons were identified as encoding the products essential for the biogenesis of the sialic acid-containing capsule and polysaccharide transport respectively (Swartley et al., 1996, Harrison et al., 2013).

1.7.2. Lipooligosaccharide (LOS)

Meningococcal lipooligosaccharide (LOS) or endotoxin is made up of an inner and outer core oligosaccharide attached to lipid A, but lacks the repeating polysaccharide O-antigen chain, commonly found in lipopolysaccharide (LPS) of most Gram-negative bacteria (Kahler and Stephens, 1998). The conserved inner core consists of phosphorylated diheptose sugars (HepI and HepII) linked to a lipid A moiety embedded in the outer membrane, whilst the outer core is heterogeneous with variable numbers of sugars conferring the molecule with the antigenic specificity used for classification of meningococci into the 12 known immunotypes L1 - L12 (Scholten et al., 1994, Tzeng and Stephens, 2000, Rouphael and Stephens, 2012). The lipid A moiety is chiefly responsible for the biological activities and toxicity of the meningococcal LOS (Zughaier et al., 2005). Meningococcal LOS is subject to phase variation of its terminal structures allowing switching between immunotypes, which is suggested to have functional significance in disease (Berrington et al., 2002). LOS shown to bind TLR4, host complement components and the mannose-binding lectin (MBL) is regarded as one of the most essential virulence factors that enables survival in the bloodstream, as well as avoidance of host mediated complement killing (Van Deuren et al., 2000, Devyatyarova-Johnson et al., 2000, Zughaier et al., 2004, Schneider et al., 2007, Kugelberg et al., 2008).

1.7.3. Pili

The pili, also known as fimbriae, are hair-like appendages formed by the helical assembly of pilin subunits. They extend out from the surface of most

bacterial types, and belong to the type IV pilus family (Tfp), which regularly facilitate attachment to various surfaces (Soto and Hultgren, 1999). A single meningococcal cell produces two types of pili, designated as class I and class II pili, which both belong to methyl-phenylalanine pili family (termed type IV pili), with each class purely identifiable by several serological and structural properties (Virji et al., 1989, Nassif, 1999, Tzeng and Stephens, 2000). The Tfp biosynthesis which consists of four steps: assembly; functional maturation; counter-retraction; and emergence onto the surface, is mediated by fifteen essential proteins PilC1/C2, PilD, PilE, PilF, PilG, PilH, PilI, PilJ, PilK, PilM, PilN, PilO, PilP, PilQ and PilW (Carbonnelle et al., 2005), with seven additional proteins ComP, PilT, PilT2, PilU, PilV, PilX and PilZ involved in Tfp function (Brown et al., 2010). Besides aiding meningococci-host cell adhesion, meningococcal micro-colony formation, and auto aggregation on mucosal surfaces, pili are also involved in several other functions (Carbonnelle et al., 2006, Pelicic, 2008, Hung and Christodoulides, 2013). For example, meningococcal pili are involved in twitching motility as well as promoting the uptake of foreign DNA from the extracellular milieu, thus increasing the transformation frequency of the bacteria (Mattick, 2002). Like LOS, type IV pili are also subject to phase and antigenic variation (Nassif, 1999). Although the nature of host receptor the pili adhere to are still obscure, it was proposed that a complement transmembrane glycoprotein CD46 may be acting as a possible ligand to meningococcal pili (Källström et al., 1997).

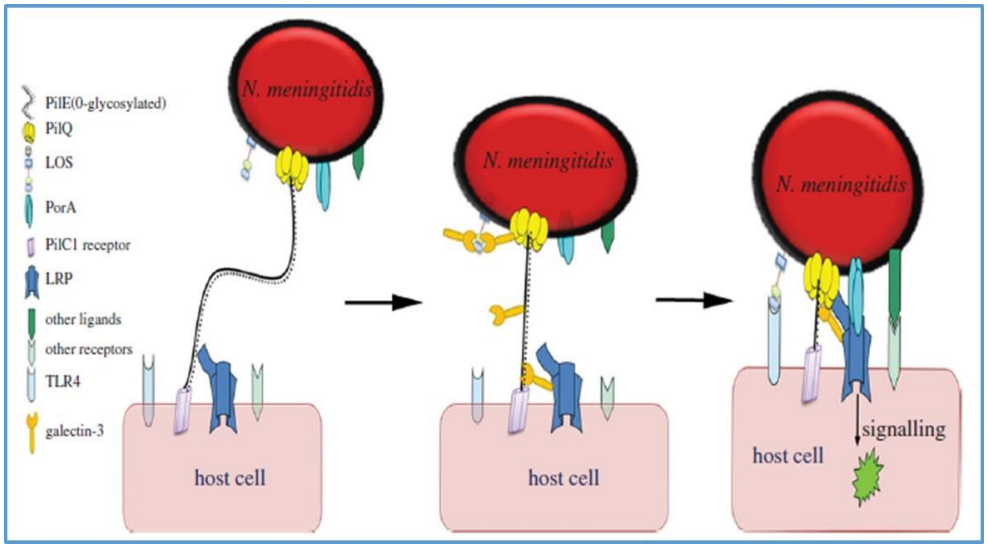


Figure 1.7: Illustration of meningococcal binding to host cells using surface ligands. Initial contact mediated by PilC1, which binds its putative receptor, followed by 37LRP/Gal-3-PilE binding, which strengthens the initial contact, and pilus retraction, which brings the two cells closer together. Decametric PilQ on the meningococci surface binds to both 37LRP and Gal-3, establishes intimate contact, strengthens interactions, and triggers host cell signalling events (Alqahtani et al., 2014).

Our research group: Molecular Bacteriology and Immunology Group (MBIG) also identified that PilQ binds to the laminin receptor on the BBB, thus promoting intimate contact with the BBB cells. Also, the presence of the laminin receptor on many cell types at many body sites also provides a means for the spread of the pathogen from site to site (Orihuela et al., 2009). PilE has also been shown to bind to galectin 3, thus promoting intimate contact and adhesion (Alqahtani et al., 2014). Of recent, an immunoglobulin superfamily member CD147 has been reported as a host receptor of meningococcal pilus components (PilE and PilV), promoting the adhesion of meningococci to human endothelial cells and colonisation of blood vessels (Bernard et al., 2014). The pili have also been proposed to aid the adhesion to host cells, by binding to CD46, a membrane cofactor human complement regulatory protein (Källström et al., 1997).

1.7.4. Major Outer Membrane Proteins (OMP's)

Meningococcal outer membrane proteins have been grouped into five major classes depending on their apparent molecular weights on sodium dodecyl sulphate (SDS) polyacrylamide gels and peptide analysis (Tsai et al., 1981). All meningococci express either a class 2 or class 3 outer membrane protein designated as PorB, with most strains also expressing a class 1 outer membrane protein designated PorA (Hitchcock, 1989). PorA has been predicted to have a two-dimensional secondary structure containing eight surface exposed loops (I-VIII) with variable regions 1 and 2 (VRI and VR2) deduced to be the most variable regions and coinciding with loops I and IV respectively (Maiden et al., 1991, Feavers et al., 1992). The PorA protein has been shown to selectively permit the passage of cations across the cell membrane (Tommassen et al., 1990). Our research group has also shown that PorA binds laminin receptor, suggesting a way by which the bacteria initiate contact with the vascular endothelium of the blood brain barrier (Orihuela et al., 2009).

Meningococcal PorB is a pore-forming protein involved in forming pores in eukaryotic cells, preventing apoptosis by modulating the mitochondrial membrane potential, and inducing the expression of the co-stimulatory molecule CD86 and of MHC class II, which are related to the porin's immune-potentiating ability (Massari et al., 2005). PorB also selectively permits the passage of anions across the cell membrane (Tommassen et al., 1990).

A class 4 OMP represented by the reduction modifiable protein M (RmpM) is also expressed by *N. meningitidis.* The protein is highly conserved and is closely related to the protein PIII of *Neisseria gonorrhoea* (Klugman et al., 1989). The RmpM protein is thought to connect the outer membrane to the peptidoglycan layer; form stable heterooligomeric complexes with meningococcal porins PorA and PorB and iron limitation-inducible OMPs (Koebnik, 1995, Jansen et al., 2000, Prinz and Tommassen, 2000).

N. meningitidis also expresses class 5 OMPs, represented by the opacity proteins Opa and Opc. The term opacity was coined because expression of these proteins led to opaque colonies (Swanson, 1978, Virji et al., 1992). The opacity proteins are highly immunogenic, trimeric or tetrameric basic outer membrane proteins with a similar molecular mass of *ca.* 30 kDa, but with other notable differences (Achtman et al., 1988, Olyhoek et al., 1991). The Opa and Opc proteins play an important role in adhesion and invasion of host epithelial, endothelial and phagocytic cells (De Jonge et al., 2003).

1.7.5. Neisseria hia homologue A (NhhA)

NhhA is a well-conserved outer membrane, autotransporter protein, identified as a homologue of the adhesin AIDA-I of *E. coli* in *N. meningitidis* (Peak et al., 2000), and named Neisseria hia homologue A owing to its close homology of 67% and 74% respectively to the Hia and Hsf adhesins of *Haemophilus influenzae* (Barenkamp and Geme 1996). Most meningococcal strains express NhhA on their surfaces, with NhhA able to adhere to human epithelial cells and components of the extra cellular matrix such as laminin and heparin sulphate (Peak et al., 2000, Scarselli et al., 2006). NhhA enables the bacteria resist phagocytosis, thus promoting the colonization of the nasopharyngeal mucosa by meningococci. Meningococcal NhhA has also been implicated in meningococcal resistance to complement attacks, as well as promoting host macrophage apoptosis (Sjölinder et al., 2008, Sjölinder et al., 2012).

1.7.6. Adhesion and penetration protein (App)

Adhesion and penetration protein (App) is a highly conserved, surface localised, and immunogenic autotransporter protein (Hadi et al., 2001). This protein was named so because of its homology to *Haemophilus* adherence and penetration protein (Hap), and it also shows a sequence similarity to

IgA protease (Turner et al., 2006). App is a strong stimulator for B cells and T cells and it can induce bactericidal antibodies (Hadi et al., 2001, van Ulsen et al., 2001). App has also been shown to function as an adhesin in *N. meningitidis*, and is able to bind to epithelial cells, but not endothelial cells, suggesting it might enhance bacterial detachment and subsequent spreading after auto-cleavage (Serruto et al., 2003). Work done by Khairalla and colleagues also showed that App interacts with the mannose receptor (MR) and transferrin receptor 1 (TfR1) leading to the blocking of dendritic cell (DC) uptake using mannan and transferrin, the specific physiological ligands for MR and TfR1. Their research also showed that App is capable of cleaving core histone H3, as well as inducing a dose-dependent increase in DC death via caspase-dependent apoptosis (Khairalla et al., 2015) .

1.7.7. Factor H binding Protein (fHbp)

The *fHbp* gene encodes a 27-kDa lipoprotein, which is present on the surface of nearly all strains of *N. meningitidis* (Masignani et al., 2003, Fletcher et al., 2004, Beernink et al., 2007).. The fHbp is one of the receptors for fH on the meningococcus, and recruitment of fH contributes to the ability of the meningococcus to avoid innate immune responses by inhibiting complement-mediated lysis in human plasma (Madico et al., 2006). The protein also appears to elicit protective bactericidal antibodies, which activate the classical complement pathway (Welsch et al., 2008). The fHbp is one of four antigenic proteins included in the formulation of the four component MenB (4CMenB) vaccine, and two fHbp variants are the component of the Trumenba vaccine both targeting *N. meningitidis* serogroup B (Su and Snape, 2011, Feavers and Maiden, 2017).

1.7.8. HrpA-HrpB System

A functional two-partner secretion system (TPS): haemagglutinin/haemolysin-related proteins (HrpA and HrpB, respectively) is present in all meningococcal strains. HrpA is the secreted effector protein and HrpB its 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). Talà and colleagues have also shown that HrpA-HrpB-null meningococci were less able to survive in Hela cells, and similarly less able to escape (Talà et al., 2008).

1.7.9. Neisseria Adhesin A (NadA)

Neisserial adhesion A (NadA) is a surface exposed trimeric autotransporter which has been identified as a homologue of UspA2 from *Moraxella catarrhalis* and of YadA from *Yersinia enterocolitica* (Van Ulsen and Tommassen, 2006, Linke et al., 2006). NadA is commonly associated with strains belonging to hypervirulent clusters. The *nadA* gene encodes a protein of *ca.* 360 amino-acid residues, which forms high molecular weight oligomers and appears to mediate cellular adhesion (Capecchi et al., 2005). NadA has also been shown to induce strong protective bactericidal antibodies and is one of four antigenic proteins included in the formulation of the four component MenB (4CMenB) vaccine targeting *N. meningitidis* serogroup B (Su and Snape, 2011).

1.7.10. Iron-acquiring Proteins

Neisseria require iron for survival and virulence (Grifantini et al., 2003). In *N. meningitidis*, iron acquisition is achieved by expressing surface receptors that extract and import iron from different sources. Extraction of iron from

human haemoglobin, transferrin and lactoferrin is accomplished by the haemoglobin-binding receptors (HmbR and HpuB/HpuA), transferrin-binding receptors (TbpA/TbpB), and lactoferrin-binding receptors (LbpA/LbpB) respectively (Noinaj et al., 2012).

1.8. Meningococcal Vaccines

Genomics and post-genomics, which have improved significantly the understanding of the biology and the pathogenesis of *N. meningitidis*, have also helped in identifying antigen candidates for vaccine development (Del Tordello and Serruto, 2013). The first major success towards vaccination against meningococcal disease was achieved in the 1960's by Goldschneider and co-workers, who successfully demonstrated the importance of anti-polysaccharide antibodies in the human immune response against serogroup A and C disease (Goldschneider et al., 1969a, Goldschneider et al., 1969b).

Table 1.3: Timeline of meningococcal vaccine development.

Year	**Vaccine Type**
1968/1969	Plain Capsular Polysaccharide (CPS) Vaccines
1970's	Outer membrane Vesicles (OMV)Vaccines
1999	CPS – Conjugate Vaccines
2015	Recombinant Protein Vaccines

Early meningococcal vaccines containing capsular polysaccharides (CPS) from MenA, MenC, MenW and MenY, were produced as monovalent, bivalent, trivalent and tetravalent vaccines. In Africa, the MenAfriVac campaign was implemented, and successfully minimized the MenA epidemics in the African Meningitis Belt. However, capsular polysaccharides are poorly immunogenic

in children under 2 years, and are predominantly T-cell independent antigens, making the use of this vaccine limited since infants and young children are the major group at risk for these infections (Gold et al., 1975, Hassan-King et al., 1988, Feavers and Maiden, 2017).

Vaccines from purified capsular polysaccharides alone have been subsequently replaced by capsular polysaccharides conjugated to carrier proteins such as tetanus toxoid, notably the MenC vaccine and quadrivalent vaccines against serogroups A, C, W and Y, that elicit potent antibody responses in all age groups (Girard et al., 2006). Polysaccharide conjugate vaccines are also thought to be highly advantageous in that they can decrease nasopharyngeal carriage and disrupt the transmission of meningococcus, thus effecting herd immunity, as well as overcoming the immunological hyporesponsiveness associated with some plain polysaccharide vaccines (Richmond et al., 2000, Maiden et al., 2008). Although polysaccharide conjugate vaccines arguably represent the most successful bacterial vaccine development in recent history, this approach cannot be used to formulate a vaccine against serogroup B, because there is a poor immunogenicity of N-propionylated serogroup B polysaccharide-tetanus toxoid conjugate. Furthermore, group B CPS is a polymer of α(2–8)- linked N-acetyl-neuraminic acid (polysialic acid), and might elicit a harmful autoimmune response as this polysialic acid is also present in human tissues such as the neural cellular adhesion molecule involved in cell-to-cell adhesion (Griffiss et al., 1991, Bruge et al., 2004). An alternative approach to resolving these challenges was to focus on other surface-exposed virulence factors such as lipo-oligosaccharide or outer membrane proteins, either in purified or recombinant form, or in outer membrane vesicles (OMV's).

Pioneering research on developing OMV component vaccines to control specific outbreaks in certain geographical regions was firstly coined

in 1970s – 1980s through research by Zollinger and colleagues, 1978; Helting and colleagues, 1981 and Frasch and Colleagues, 1985 (Zollinger et al., 1978, Helting et al., 1981, Frasch et al., 1985). Licenced MenB vaccines have been formulated from the OMV's containing the five major classes of OMP's (Detailed in Section 1.7.4). OMV vaccines: VA-MENGOCOC-BC™, MenBvac™, MeNZB™ developed from the homologous clonal strains of MenB at the Finlay Institute in Cuba; the Norwegian Institute of Public Health respectively and the five-tier consortium between NIPH, WHO, New Zealand Government, Auckland University, and Chiron respectively, were successful at controlling MenB epidemics in Cuba, Norway and New Zealand (Sierra et al., 1991, Rosenqvist et al., 1995, Arnold et al., 2011). OMV vaccines mostly induce strain-specific immune responses, especially in infants and children, directed predominantly against the immunodominant PorA protein (Tappero et al., 1999), and monovalent OMV vaccines do not provide cross-protection against strains expressing other serosubtype PorA (Christodoulides and Heckels, 2017).

Recently, two new recombinant protein based vaccines have been licensed, aimed at providing protection against serogroup B strains namely the four-component meningococcal serogroup B (4CMenB) vaccine, Bexsero (GSK) and the bivalent recombinant lipoprotein (rLP2086) vaccine, Trumenba (Feavers and Maiden, 2017). The Bexsero vaccine consists of outer membrane vesicles (OMV's) from the New Zealand Group B meningococcal outbreak strain (containing the PorA antigen: NZ PorA P1.4), with three other additional recombinant antigens: factor H binding protein (fHbp; variant 1), Neisseria adhesin A (NadA), and Neisseria heparin-binding antigen (NHBA). All four components of the vaccine elicited specific bactericidal antibody responses to a panel of reference isolates expressing antigens that were identical to those in the vaccine in a clinical study carried out in infants and children (Gossger et al., 2012, Vesikari et al., 2013). The

vaccine also proved to be safe, has been approved in many countries worldwide, and has subsequently been included in various national or regional recommendations. In September 2015, the UK included the vaccine in its publicly funded national infant immunization program. In 2014, the FDA approved for use the rLP2086 (Trumenba), which is a bivalent recombinant lipidated fHbp vaccine in people aged 10 to 25 years. In 2017, the European commission approved the Trumenba for the prevention of invasive meningococcal disease caused by MenB in individuals 10 years of age and older due to its toxicity in babies.

1.9. Moonlighting Proteins

Proteins are a group of macromolecules which perform a large variety of functions, with most of them having only a single function. However, a growing number of proteins have been identified as being multifunctional. Moonlighting proteins fall under a special class of multifunctional proteins. A standard definition of a moonlighting protein is a single protein with the ability to carry out multiple physiologically relevant biochemical or biophysical functions, which are not because of gene fusions, multiple RNA splice variants, or pleiotropic effects (Jeffery, 1999, Huberts and van der Klei, 2010). These proteins have been identified in both prokaryotic and eukaryotic cells and they represent a highly conserved subset of proteins (Henderson and Martin, 2011). Moonlighting proteins are of obvious benefits for the cells, as combining different functions or switching between functions can be considered as a cell responds to the changing environment (Jeffery, 2004). Proteins can employ a combination of mechanisms to deliver moonlighting functions in eukaryotic and prokaryotic organisms (Figure 1.3).

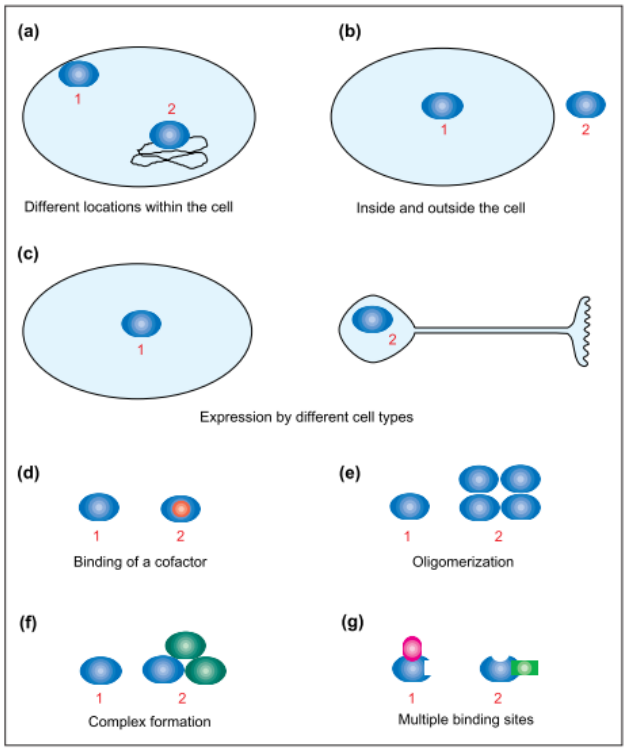


Figure 1.8: Examples of mechanisms for switching between usual and moonlighting functions: (a) Performing different functions at different locations within the same cell. (b) Having one function inside the cell but having a different one when secreted. (c) Same protein may have different functions in different cell types. (d) Altering activity when bound to substrate, product or a cofactor. (e) Forming multimeric structure or (f) complex with other proteins to switch between function performed by the monomeric one. (g) Binding with different substrates using different binding sites can also alter the functions. Adapted from: (Jeffery, 1999).

Moonlighting proteins have been classified according to their known or presumed cytosolic functions. In this context, moonlighting proteins are commonly metabolic pathway-associated enzymes, molecular chaperones, and transcription or translation factors (Henderson and Martin, 2011, Henderson and Martin, 2014). In both prokaryotic and eukaryotic organisms, enzymes involved in the glycolytic pathway for example GAPDH and enolase, have also been found to perform non-glycolytic functions especially when a particular enzyme is expressed on the bacterial cell surface or released into

the surrounding milieu. Also, enzymes such as alcohol acetaldehyde dehydrogenase, involved in other metabolic pathways also demonstrate moonlighting abilities.

Another group of proteins that carry out moonlighting functions are protein folding catalysts and molecular chaperones. These proteins, responsible for the proper folding/unfolding of different macromolecules, and transport of these molecules across the cell membrane, can also be found multi-tasking (Ellis, 2005). The surface localized Cpn60.2 in *Mycobacterium tuberculosis*, was reported to be essential to mediate competent bacterial adherence to host macrophages (Hickey et al., 2009). Likewise, the GroEL2 protein from *M. tuberculosis* was detected on the surface, serving as an inactivator for the expression of Rv2224c, a mycobacterial envelope-associated protease that mediates the release of GroEL2 out of the cell, thus significantly reducing the bacterial survival rate in macrophage culture, as well as the mycobacterial associated pathology within infected mice (Rengarajan et al., 2008). Many proteins that belong to either of these groups are known to mediate bacterial pathogenesis either via adhesion, interaction with host ligands, and/or modulating specific host elements. Many enzymes have also been found to be moonlighting as transcription and translation regulators. Notably, the tricarboxylic acid (TCA) cycle enzyme aconitase was shown to display RNA binding properties via interaction with sequences similar to iron responsive element (IRE) seen in eukaryotic and few prokaryotic cells (Beinert and Kennedy, 1993, Banerjee et al., 2007). It is still unclear how these moonlighting proteins reach the cell surface as they do not contain any known secretion signals or anchoring motifs and are thus known as anchorless proteins (Pancholi and Chhatwal, 2003). The possible explanation for surface localization of an originally cytosolic protein is passive release due to cell lysis. The protein can then be recruited to the surface of intact bacterial cells in the population. Following lysis, Glyceraldehyde 3-phosphate

dehydrogenase (GAPDH) from Group B streptococcus was shown to be expressed on the bacterial cell surface, binding non-specifically to the surfaces of other Gram-positive and Gram-negative bacteria (Oliveira et al., 2012). Moonlighting proteins can also be released due to stress conditions within the cell. For instance, GAPDH from *Streptococcus pyogenes* and DnaK from *Bifidobacterium animalis* subsp. *lactis* were observed to be released from the bacteria due to iron starvation and the presence of bile salts respectively (Eichenbaum et al., 1996, Oliveira et al., 2012). Likewise, 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). Cell-surface-associated GAPDH activity in *Lactobacillus plantarum* was shown to increase while in the stationary growth phase possibly due to increased permeability of plasma membrane, with five-fold more GAPDH on the bacterial surface observed in bacteria with impaired membrane permeability (Saad et al., 2009). However, in Gram-negative bacteria where there are two membrane barriers to cross, an alternative hypothesis of translocation is that moonlighting proteins can be secreted onto the cell surface by an undefined mechanism(s) (Henderson and Martin, 2011). Although it is believed that moonlighting proteins lack identifiable signal sequences, a study has suggested that fructose-1, 6-bisphosphate aldolase (FBA) from *N. meningitidis* has a 13-amino acid sequence signal peptide, with predicted cleavage site 11DHA-AE15 indicating the possibility of partial translocation across the cytoplasmic membrane (Tunio et al., 2010b). Additionally, Aguilera and colleagues established that enteropathogenic *E. coli* (EPEC) grown in Dulbecco's modified eagle's medium (DMEM) translocates the housekeeping protein GAPDH via the type III secretion system (T3SS) as

well as another unidentified secretion pathway that mediates GAPDH secretion in addition to T3SS (Aguilera et al., 2012). Although it can be said that the transport mechanism(s) of moonlighting proteins are largely unknown, a range of mechanisms depending on the bacterial species and/or the moonlighting protein being exported may be employed by bacteria for the surface localization of these proteins, which may include novel non-classical secretion pathways.

1.10. Meningococcal Moonlighting Proteins

The first evidence of moonlighting proteins in *N. meningitidis* was described in 2002 by Grifantini and colleagues (Grifantini et al., 2002). At present, up to five moonlighting proteins have been described in *N. meningitis*, which are discussed in this section.

1.10.1. Glyceraldehyde 3-phosphate Dehydrogenase (GAPDH)

Meningococcal GAPDH (locus tag NMB0207) is a glycolytic enzyme which catalyses the conversion of glyceraldehyde 3-phosphate to 1, 3-diphosphoglycerate. The most common form is the NAD+ dependent enzyme (EC 1.2.1.12) found in all organisms studied so far and which is usually located in the cytoplasm. Grifantini and colleagues using microarray analysis detected the up-regulation of the *gapA-1* gene following the interaction of bacterial cells with human epithelial cells encoding the GAPDH protein; the upregulation of GAPDH protein expression was confirmed by FACS analysis (Grifantini et al., 2002). Following the discovery of GAPDH also being localised at the cell surface in *N. meningitidis*, Tunio and colleagues also suggested likely functions of GAPDH at the cell surface. Their study showed that the expression of *gapA-1* was shown to be well conserved across diverse

isolates of *Neisseria*, with the cell surface expressed GAPDH enveloped by the capsule (Tunio et al., 2010a). Their study also showed that the ability of the bacteria to adhere to human epithelial and endothelial cells was significantly reduced in *gap-A1* deficient mutants compared with the wild type and complemented mutants, suggesting a role of the enzyme in the pathogenesis of meningococcal infection by contributing to the 'intimate adherence' that precedes invasion.

1.10.2. Fructose 1,6-bisphosphate Aldolase (FBA)

Meningococcal Fructose-1, 6-bisphosphate aldolase (locus tag NMB1869) catalyses the reversible cleavage of fructose-1, 6-bisphosphate into dihydroxyacetone phosphate and glyceraldehyde 3-phosphate. FBA in *N. meningitidis* belongs to the Class-II aldolases which are not found in animals, thus making them a possible therapeutic or vaccine target (Thomson et al., 1998, Ramsaywak et al., 2004). Through cell fractionation experiments, FBA was found to be localized to both the cytoplasm as well as the outer membrane, and was surface-accessible to FBA-specific antibodies (Tunio et al., 2010b). A likely moonlighting function of FBA at the cell surface would be promoting adhesion of meningococci to human cells as Tunio and colleagues discovered that an FBA null mutant although able to grow in vitro showed a significantly reduced ability to adhere to human brain microvascular endothelial and HEp-2 cells when compared to the wild type and complemented mutants (Tunio et al., 2010b). Another surface associated function of FBA was also established by Shams and colleagues, where they discovered that FBA binds human plasminogen at the bacterial cell surface (Shams et al., 2016).

1.10.3. DnaK

DnaK is a cytosolic molecular chaperone that plays an essential role in preserving the integrity of its client protein(s) by preventing potential aggregation and/or misfolding, as well as aiding in translocating the client proteins to their final destination (Kim et al., 2013). Research by Knaust and colleagues showed that DnaK, a usually intracellular protein can also be surface localised, where it acts as a plasminogen receptor (Knaust et al., 2007). Their research showed that as well as binding purified plasminogen, DnaK can also recruit plasminogen from human serum. The activation of *N. meningitidis*-associated plasminogen into plasmin by urokinase leads to functional activity and allows the bacteria to degrade fibrinogen which has linked to the invasiveness and pathogenicity of pathogens (Knaust et al., 2007).

1.10.4. Enolase

Enolase (2-phospho-d-glycerate hydrolase), is a glycolytic metalloenzyme that naturally catalyses the dehydration of 2-phosphoglycerate (2PG) to phosphoenolpyruvate (Pancholi, 2001). Along with its established cytoplasmic location, enolase has also been recognized on the surface of *N. meningitidis*, functioning as another plasminogen receptor. As observed with DnaK by flow cytometry and immunogold labelling, enolase can also bind purified plasminogen as well as recruit plasminogen from human serum (Knaust et al., 2007). As illustrated below (Figure 1.4), the binding of meningococci to plasminogen and its subsequent activation leads to a series of proteolytic events, which in addition to the binding itself contributes to pathogenesis, and are utilized in a variety of pathogenic processes (Sanderson-Smith et al., 2012).

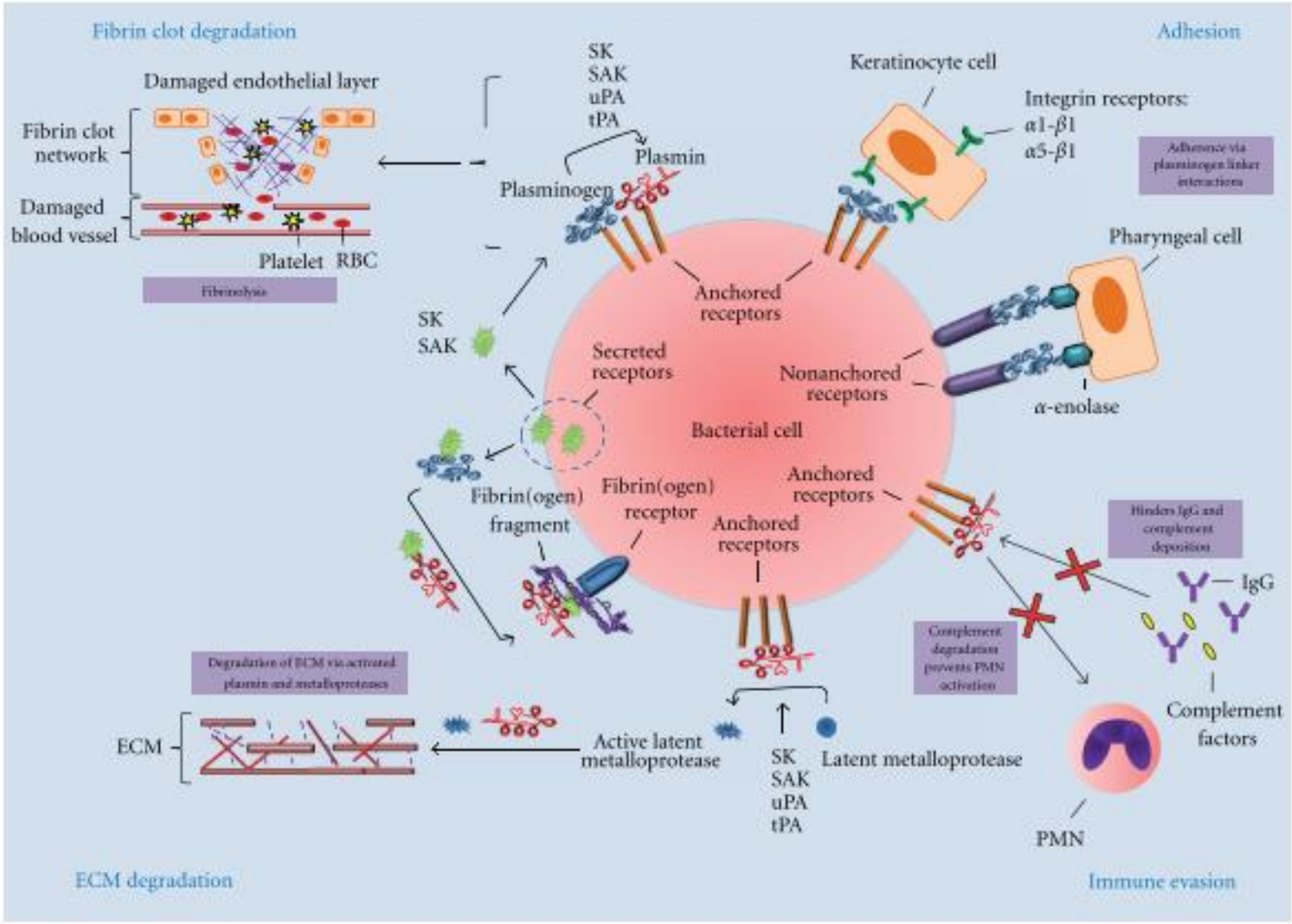

Fibrin clot degradation
Adhesion
Damaged endothelial layer
Fibrin clot network
Damaged blood vessel
Platelet
RBC
Fibrinolysis
SK
SAK
uPA
tPA
Plasmin
Plasminogen
Keratinocyte cell
Integrin receptors:
α1-β1
α5-β1
Adherence via plasminogen linker interactions
Pharyngeal cell
α-enolase
Anchored receptors
Secreted receptors
Nonanchored receptors
Bacterial cell
Fibrin(ogen) fragment
Fibrin(ogen) receptor
Anchored receptors
Anchored receptors
Hinders IgG and complement deposition
IgG
Complement degradation prevents PMN activation
Complement factors
Degradation of ECM via activated plasmin and metalloproteases
ECM
Active latent metalloprotease
SK
SAK
uPA
tPA
Latent metalloprotease
PMN
ECM degradation
Immune evasion

Figure 1.9: 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 (Sanderson-Smith et al., 2012).

1.10.5. Peroxiredoxin (Prx)

Meningococcal peroxiredoxin (NMB0946) like other Prx's possesses a highly conserved cysteine residue with an active thiol group, which in turn drives a peroxidase activity. Thus they possess antioxidant properties which help the bacterial cell circumvent the damaging effect of many free radicals such as reactive oxygen and nitrogen which are generated during different biological processes (Rhee and Woo, 2011). Meningococcal peroxiredoxin has been described as a moonlighting protein also localised to the cell surface where it functions as a plasminogen receptor (Knaust et al., 2007).

1.11. Glutamine Synthetase

Glutamine synthetase (GlnA) is an enzyme found in a wide variety of microorganisms, higher plants and animal tissues. The enzyme catalyses the ATP dependent and reversible formation of glutamine, ADP, and free phosphate from ammonia and glutamate (Meister, 1980).

$$\text{ATP} + \text{L-glutamate} + \text{NH3} \rightleftharpoons \text{ADP} + \text{phosphate} + \text{L-glutamine}$$

The glutamine produced by GlnA is important for the synthesis of proteins; it also acts as a nitrogen donor for many reactions in the cell, and its amide nitrogen is also donated to synthesize many essential metabolites (Kumada et al., 1993, Chandra et al., 2010).

Bacteria are known to synthesize more than one form of GlnA, but they fall into three distinct classes. GSI is the predominant form of glutamine synthetase in prokaryotes, and is encoded by the gene *glnA*, GSII encoded by *glnII* resembles the glutamine synthetase from eukaryotes and is found together with GSI in plant symbionts and Streptomyces, GSIII encoded by *glnN* has been found in two unrelated anaerobic rumen bacteria (Woods and Reid, 1993, Merrick and Edwards, 1995).

GSI encoded by *glnA,* has a quaternary structure consisting of 12 identical protein subunits of around *ca.* 52 kDa. Crystal structures by Yamashita and colleagues showed that the 12 identical subunits of GlnA are arranged as two superimposed hexagonal rings held together by hydrogen bonding between the subunits as well as hydrophobic interactions (Yamashita et al., 1989). Meningococcal GlnA (Locus tag NMB0359) belongs to the GSI class. The reaction occurs with either magnesium (Mg^{2+}) or manganese ions (Mn^{2+}) present. The term bifunnel is used to describe each of the 12 active sites formed between every two adjacent subunits within a ring. ATP enters the bifunnel from the exposed top surface of the dodecamer, near the 6-fold axis of symmetry. Glutamate enters the bifunnel at the bottom, the interface of the hexameric rings, near the 2-fold axes. At the joint of the bifunnel are the Mg^{2+} and the Mn^{2+} cation binding sites (Gill and Eisenberg, 2001).

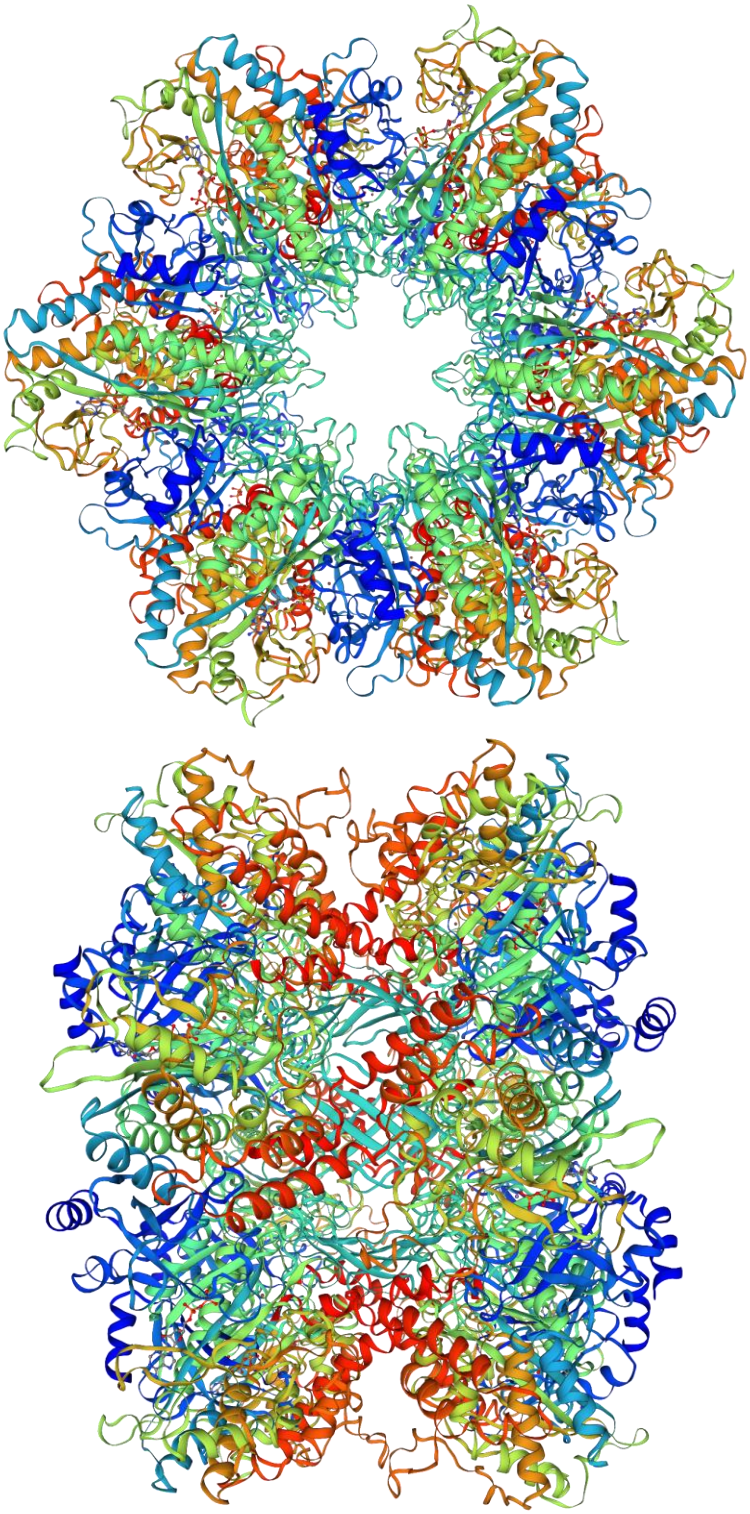


Figure 1.10: Crystal structure of glutamine synthetase from *Escherichia coli* (strain K12), showing hexagonal rings and protein subunits. Modelled using SWISS model online tool. Adapted from crystal structures from (Yamashita et al., 1989).

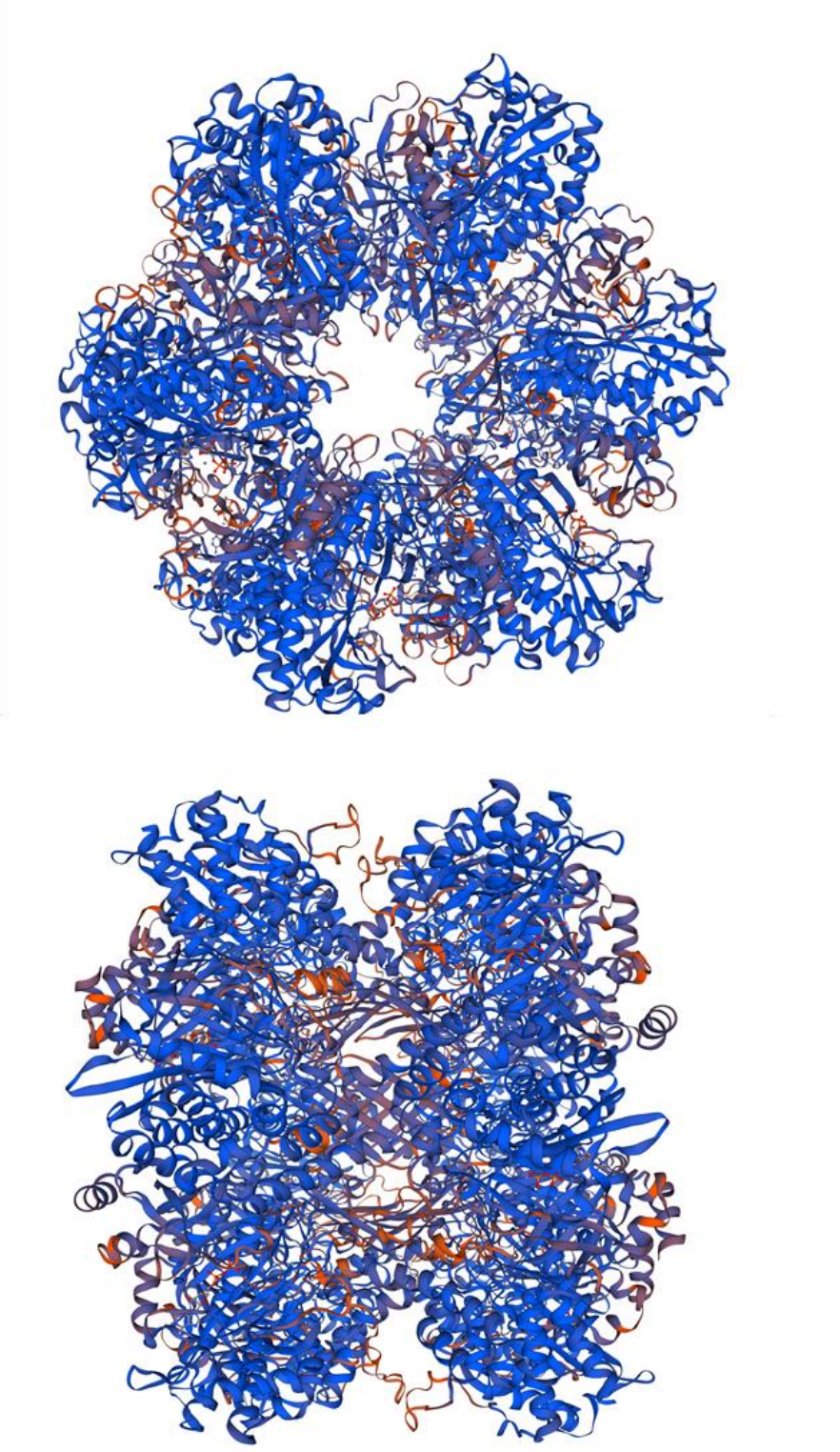


Figure 1.11: Model of the crystal structure of glutamine synthetase from *Neisseria meningitidis* showing the two superimposed hexagonal rings, the 12 active sites between each monomer and the protein subunits. Generated using SWISS MODEL online tool with *E. coli* structure (Figure 1.5) as template.

Aside its role as the enzyme for glutamine synthesis and its responsibility for nitrogen assimilation for the cell during low nitrogen conditions, GlnA has also been implicated in carrying out other moonlighting functions in different bacteria. Many studies have shown that nitrogen metabolism; in particular, the metabolism of glutamine which is regulated by glutamine synthetase is necessary for the virulence of a great number of bacterial pathogens (Kloosterman et al., 2006).

Klose and Mekalanos have previously shown that simultaneous prevention of the synthesis of glutamine as well as its transport weakens virulence in *Salmonella typhimurium* (Klose and Mekalanos, 1997). The role of GlnA in the pathogenicity of *Mycobacterium tuberculosis* was assessed by Tullius and colleagues, and they discovered that a *glnA* mutant which had no detectable level of GlnA protein had its activity weakened for intracellular growth in human THP-1 macrophages and showed no virulence in the highly susceptible guinea pig model of pulmonary tuberculosis (Tullius et al., 2003). Likewise, research by Chandra and colleagues showed that knocking out the *glnA* gene in *Mycobacterium bovis* weakens as well as alters the cell wall chemistry of the pathogen (Chandra et al., 2010). *Streptococcus suis* serotype 2 which is known for causing a wide variety of diseases in humans and swine also requires the gene *glnA* for full virulence. Si and colleagues were able to show that a *glnA* knockout strain was less virulent and could reduce the rate of sickness and death in infected mice; and organ cultures also showed GlnA is necessary for the colonization of the specific organs involved in *S. suis* infection (Si et al., 2009).

Research has shown that bacteria and other pathogens can use the host plasminogen system to increase their invasiveness as well as expedite the breakdown of the host tissue barriers. These bacteria have plasminogen-binding proteins which enables them bind to plasminogen. DnaK, GlnA1, and Ag85 complex were shown to be plasminogen-binding proteins in *M.*

tuberculosis (Xolalpa et al., 2007). Their study proved that these bacterial proteins can bind plasminogen to the bacteria, which is then activated to plasmin, thus providing the bacteria with the ability to break down host extracellular membrane and membrane proteins, increasing tissue injury in tuberculosis. In meningococcus, DnaK, enolase, FBA, and peroxiredoxin have been shown to be plasminogen binding proteins (Knaust et al., 2007, Shams et al., 2016). GlnA moonlights as a plasminogen binding protein in other organisms such as in *Bifidobacterium lactis* (Candela *et al*, 2007) and *Lactobacillus crispatus* (Kainulainen *et al*, 2012).

As highlighted earlier, GlnA is essential for full virulence in various organisms such as *S. suis*, *M. tuberculosis*, *M. bovis* amongst others, and *glnA* knockout strains have a reduced virulence potential. GlnA also functions as a plasminogen receptor in other pathogens, bestowing the bacteria with the ability to break down host extracellular membrane and membrane proteins, but its role in the bacterial pathogen *N. meningitidis* has not been studied.

1.12. The Aim of the Study

The findings discussed earlier highlight the importance of moonlighting proteins as virulence factors that improve the ability of bacteria to cause infection. The aims of this study were to test the hypothesis that GlnA is a moonlighting protein in *N. meningitidis*, investigate its putative moonlighting activities and in doing so, assess its contribution to the pathogenesis of *N. meningitidis*.

CHAPTER 2: Cloning, Overexpression, and Purification of meningococcal recombinant GlnA (rGlnA)

2.1. Introduction

As mentioned previously, proteins are a group of macromolecules which perform a large variety of functions, with most of them having only a single function. However, a growing number of proteins have been identified as being multifunctional. Moonlighting proteins fall under a special class of multifunctional proteins. A standard definition of a moonlighting protein therefore is a single protein with the ability to carry out multiple physiologically relevant biochemical or biophysical functions which is not because of gene fusions, multiple RNA splice variants, or pleiotropic effects (Jeffery, 1999, Huberts and van der Klei, 2010).

Meningococcal proteins GAPDH, enolase, peroxiredoxin, FBA, and DnaK have been shown to be surface exposed and carrying out moonlighting functions at the bacterial cell surface.

The number of moonlighting proteins is steadily increasing as more studies are being carried out, but the focus of this study is on the enzyme glutamine synthetase (GlnA). Asides its role as the enzyme for glutamine synthesis as well as its responsibility for nitrogen assimilation for the cell during low nitrogen conditions, GlnA has also been implicated in carrying out other moonlighting functions in different bacteria. In *N. meningitidis*, the gene encoding a putative GlnA enzyme is present in published genomes (designated NMB0359 in the meningococcal strain MC58) but has not been studied extensively.

This chapter describes the cloning, overexpression, purification of recombinant glutamine synthetase, and the raising of polyclonal antisera against the purified protein to facilitate subsequent characterisation studies.

2.2. Materials and Methods

2.2.1. *In* silico sequence analysis

The whole genome database of *N. meningitidis* MC58 was extensively studied at http://www.ncbi.nim.nih.gov/gene. The SignalP 4.1 server available at http://www.cbs.dtu.dk/services/SignalP/ was used to predict the secretion signal peptide of meningococcal glutamine synthetase (NP_273408.1). Meningococcal AspA (NP_274963.1) which has a known secretion signal peptide was used as a positive control. DNAMAN software (Lynnon Biosoft) was used for other sequence analyses such as primer design, restriction digestion, protein translation and sequence alignment/assembly.

2.2.2. Bacterial strains, growth conditions and media

E. coli strain JM109 (Promega) was used for plasmid transformation and recombinant protein expression. Bacteria were grown in Lysogeny broth (LB) or on LB agar plates (See appendix for recipes) supplemented with 100 µg/ml ampicillin (Sigma-Aldrich), unless mentioned otherwise. LB agar plates were incubated at 37°C; broth cultures were incubated in a shaking incubator (200 rpm) at the same temperature.

2.2.3. Polymerase Chain Reaction (PCR)

PCR was performed using a C1000 thermal cycler (Bio-Rad) to amplify the 1.4 kb fragment encoding *glnA* from the chromosomal DNA of *N. meningitidis* serogroup B strain MC58 using primer pairs GlnAF1 and GlnAR1 (Table 2.1). PCR master mix contained 30 µl of each primer at a final concentration of 5 pmol/µl; deoxyribonucleotide mix (dNTPs; Roche) to a

final concentration of 0.2 mM; 3 µl Phusion-high fidelity DNA polymerase (New England Biolabs); 1 µl of the appropriate template DNA and 60 µl of 5X buffer. The mixture was made up to a final volume of 300 µl with dH_2O. 50 µl aliquots of the master mix were added into six PCR tubes. The PCR conditions used were initial denaturation at 98°C for 30 s, followed by 30 cycles of denaturation at 98°C for 10 s; annealing at 60°C for 30 s then at 72°C for 1 min; followed by extension at 72°C for 5 min and final incubation at 4°C.

Table 2.1: List of the primers used for the amplification and/or sequencing of glutamine synthetase (NMB0359).

Primer	Primer Sequence* (5' – 3')	Purpose
GlnAF1	GCG**GGATCC**ATGTCTATTAAAAA CGCCG	Amplification of NMB0359 encoding Meningococcal glutamine synthetase
GlnAR1	GCG**GGATCC**AAACCAGACGTGCTTA CAGGC	
PQE30F	CCCGAAAAGTGCCACCTG	PQE30 sequencing Primers
PQE30R	GTTCTGAGGTCATTACTGG	

* Sequence in bold indicates *BamHI* recognition sequence.

2.2.4. Agarose Gel Electrophoresis

Agarose gels (1% $^{w}/_{v}$) pre-stained with 5 µl Syber safe gel stain (Sigma) were run in gel tanks (Bio-Rad) using 5x Tris-acetate-EDTA (TAE) buffer as running buffer; the setup was connected to power pack (Bio-Rad) at 120 V for 40 min for DNA separation. DNA bands were visualised using Uvitec scanner (Bio-Rad). 1kb DNA ladder (N3232S; New England Biolabs) unless otherwise stated was included to help estimate the size of the DNA.

2.2.5. DNA Extraction, Purification and Quantification

Purification of DNA products resulting from PCR amplifications or restriction digestion was performed using the GenElute PCR clean-Up Kit (Sigma), following manufacturer's instructions. Extraction of DNA from agarose gels was performed using GenElute gel extraction kit (Sigma, Aldrich), as described by the manufacturer. The GenElute Plasmid Miniprep Kit (Sigma, Aldrich) was used for plasmid DNA extraction, following manufacturer's instructions.

Prior to subsequent experiments, the concentration and purity of isolated DNA was determined using Nanodrop ND-1000 Spectrophotometer (Thermo Scientific).

2.2.6. Restriction Digestion

Restriction enzymes used in this study were obtained from New England Biolabs or Thermo Scientific. Digestion was performed following manufacturer's instructions. All the reaction mixtures were incubated in a water bath at 37°C for ≥3 h. The digested product was purified using the GenElute PCR clean-up kit (Sigma) and confirmed by agarose gel electrophoresis.

2.2.7. Dephosphorylation

Dephosphorylation of vector DNA digested with one restriction enzyme was carried out as per manufacturer's instructions using shrimp alkaline phosphatase (SAP; New England Biolabs) and was performed before ligation to increase cloning efficiency.

2.2.8. DNA Sequencing

All cloned DNA fragments used in this study were sequenced to confirm orientation and lack of PCR-generated errors prior to protein expression. PCR primers and internal primers used for sequencing are shown in Table 2.1 DNA sequencing was performed by Source Bioscience, Nottingham, UK utilizing the Sanger chain-termination method. DNAMAN software package version 4.13 (Lynnon Biosoft) was used for the analysis of DNA and protein sequence.

2.2.9. *E. coli* transformation

An aliquot of frozen *E. coli* JM109 competent cells (Promega) was thawed on ice 5 min prior to transformation. As required, 5 µl of plasmid (20 ng) was added to 50 µl cells and mixed gently. The cells were kept 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 to ice for 2 min. Then, 950 µl of S.O.C medium (New England BioLabs) was added and the cell suspension was incubated at 37°C for 1 h with shaking (200 rpm). The transformation reaction was plated out on LB agar containing ampicillin (100 µg ml^{-1}) and incubated at 37° for > 16 h.

2.2.10. Expression of recombinant protein

E. coli JM109 (pIT1) was cultured in LB + Ampicillin (100 µg ml^{-1}) and incubated overnight at 37°C with shaking (200 rpm). The overnight culture (20 ml) was inoculated into fresh LB broth (1 L) with ampicillin (1/50 dilution) and grown to a mid-log phase optical density (OD_{600}) ~0.5. At this OD, 1 ml of un-induced culture was removed for SDS–PAGE analysis, before the rest of the culture was induced with 1mM isopropyl β–D 1-thiogalactopyranoside (IPTG) and incubated at 37°C for 4 h with shaking at

200 rpm. Samples were taken at hourly time points for SDS-PAGE analysis. Cells were harvested by centrifuging 150 ml aliquots of the culture at 4200 × *g* for 10 min at 4°C using an Allegra X-22R centrifuge (Beckman Coulter). The harvested cell pellets were frozen at -80°C until use.

2.2.11. Protein Purification by immobilised metal affinity chromatography (IMAC)

IMAC was used to purify His-tagged protein under native conditions using AKTA prime plus analyser (GE Healthcare) and following the manufacturer's instructions. Harvested cells were suspended in 30 ml of lysis buffer [50mM sodium phosphate (pH 7.4), 300mM NaCl, 10mM Imidazole, pH 7.4]. Sonication was performed on ice in short bursts for 4 min (15s on and 15s off) using model 705 sonic dismembrator (Fisher Scientific) at amplitude 15 microns.

The lysate was centrifuged at 4200 x *g* at 4°C for 15 min on an Allegra X -22 centrifuge (Beckman Coulter). The supernatant lysate loaded onto the 5ml HisTrap FF crude column using a peristaltic pump at a flow rate of 1ml/min. The column was mounted onto the AKTA PrimePlus system and washed with wash buffer [50mM sodium phosphate (pH 7.4), 300mM NaCl, 50mM Imidazole, pH 7.4] to remove loosely bound proteins.

Elution of the strongly bound his-tagged protein was carried out using elution buffer [50mM sodium phosphate (pH 7.4), 300mM NaCl, 500mM Imidazole, pH 7.4. Eluates were collected over a gradient elution monitored via the chromatogram. The eluates were stored at 4°C for further analysis. Throughout the purification process, aliquots were taken and subjected to SDS-PAGE and immunoblot analysis.

2.2.12. Desalting and Concentration of Purified Protein

Fractions corresponding to purified protein were pooled together and transferred into a Spectra/Por dialysis membrane (spectrum labs) and dialysed at 4°C into 1 L of 20% ($^{v}/_{v}$) glycerol in PBS over 48 h. Dialysis buffer was refreshed every 24 h. The desalted recombinant protein was concentrated by centrifugation at 4500 × *g* at 4°C to 5 ml using membrane cut-off (30 kDa) Vivaspin tubes (Sartorious).

2.2.13. SDS-PAGE Analysis

Protein samples were resolved by 10% ($^{w}/_{v}$) SDS-PAGE gels based on Laemmlis' method (See appendix for recipe) using mini–protein III apparatus (Bio-Rad). Samples were mixed with 5 X SDS sample loading dye [620 mM Tris-HCl (pH 6.8), 5% ($^{w}/_{v}$) SDS, 25% ($^{v}/_{v}$) Glycerol, 12.5% ($^{v}/_{w}$) β-mercaptoethanol, 0.002% ($^{w}/_{v}$) Bromophenol blue brought up to 25 ml with dH2O] and heated at 100°C for 5 min to denature the protein. Colour Plus pre-stained protein ladder (P7709S; New England Biolab) was loaded alongside the sample to help estimate the molecular weights of proteins.

Proteins were visualized by staining with Simply Blue Safe stain (Invitrogen) according to the manufacturer's instructions. Gels were scanned using the Uvitec scanner (Bio-Rad).

2.2.14. Immunoblot Analysis

After resolving protein samples by SDS-PAGE, proteins were transferred in semi–dry blotting buffer [10% ($^{v}/_{v}$) methanol, 10% ($^{w}/_{v}$) SDS, 2.92g glycine and 5.8g Tris base] to nitrocellulose membrane using a trans–blot SD Semidry transfer cell (Bio-Rad) at 20 V for approximately 20 min. The

membrane was blocked using 5% ($^{w}/_{v}$) skimmed milk powder dissolved in 20ml PBS with 1% ($^{w}/_{v}$) bovine serum from albumin (BSA; Sigma) to prevent non-specific antibody binding and incubated overnight at 4°C. The membrane was probed with mouse primary anti - penta-histidine monoclonal antibody [Qiagen; 1:2000 diluted in 5% ($^{w}/_{v}$) skimmed milk - 1% ($^{w}/_{v}$) BSA - PBS] for 2 h at room temperature on an orbital table then washed 3 × using PBS-0.1%Tween for 5 min each. After washing, the membrane was again probed with anti-mouse IgG alkaline phosphate conjugate [Sigma; 1:10000 diluted in 5% ($^{w}/_{v}$) skimmed milk - 1% ($^{w}/_{v}$) BSA - PBS] for 2 h and developed using the 5-Bromo-4-chloro-3-indolyl phosphate / nitro blue tetrazolium liquid substrate system (BCIP/NBT; Sigma), and finally washed briefly with dH_2O and visualized with Uvitec scanner (Bio-Rad).

2.2.15. Protein Quantification

Protein concentration was measured by Pierce™ BCA Protein Assay kit (Thermo Scientific) according to the manufacturer's instructions. The microplate method was followed using 20% ($^{v}/_{v}$) glycerol in PBS as diluent.

2.2.16. Raising of polyclonal antisera against rGlnA

Purified rGlnA was provided to Antibody Production Services (APS, U.K). Three White New Zealand rabbit were pre-screened for background antibody levels against rGlnA, and the rabbit with the least background antibody levels against rGlnA was selected. Pre-immune serum was collected. The immunization scheme included injecting the animal subcutaneously, three times at two week intervals, with 30 µg of rGlnA emulsified in Freud's Complete (first immunization) or incomplete adjuvant. Test bleeds were collected at regular intervals to monitor the effectiveness of the protocol

being followed and the immunogenicity of the antigen. Exsanguination was performed on the 49th day and final bleed was collected.

2.3. Results

2.3.1. Bioinformatics

In *N. meningitidis* MC58, the 1419 bp *glnA* (NMB0359) is translated into a protein with 472 amino acids, with an estimated molecular weight of 52,137 Da. *glnA* is located at position 365978 – 367396 in the MC58 genome with 54.97% GC content. The gene *glnA* is upstream of, and in the same orientation to, NMB0360, predicted to encode an AmpG-like protein which is a transmembrane protein with permease activity; and downstream of, and in the opposite orientation to, NMB0358 (*aroE*) which encodes the shikimate dehydrogenase protein that catalyses one step of the shikimate pathway, leading to the biosynthesis of aromatic amino acids.

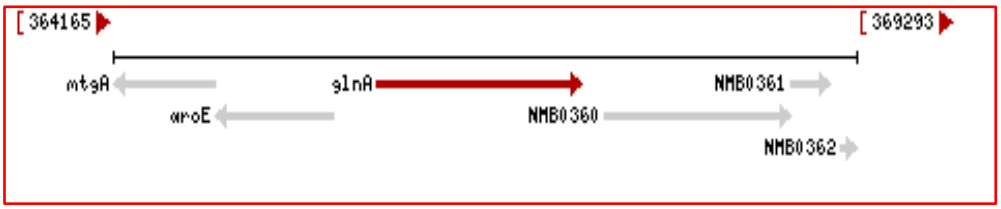


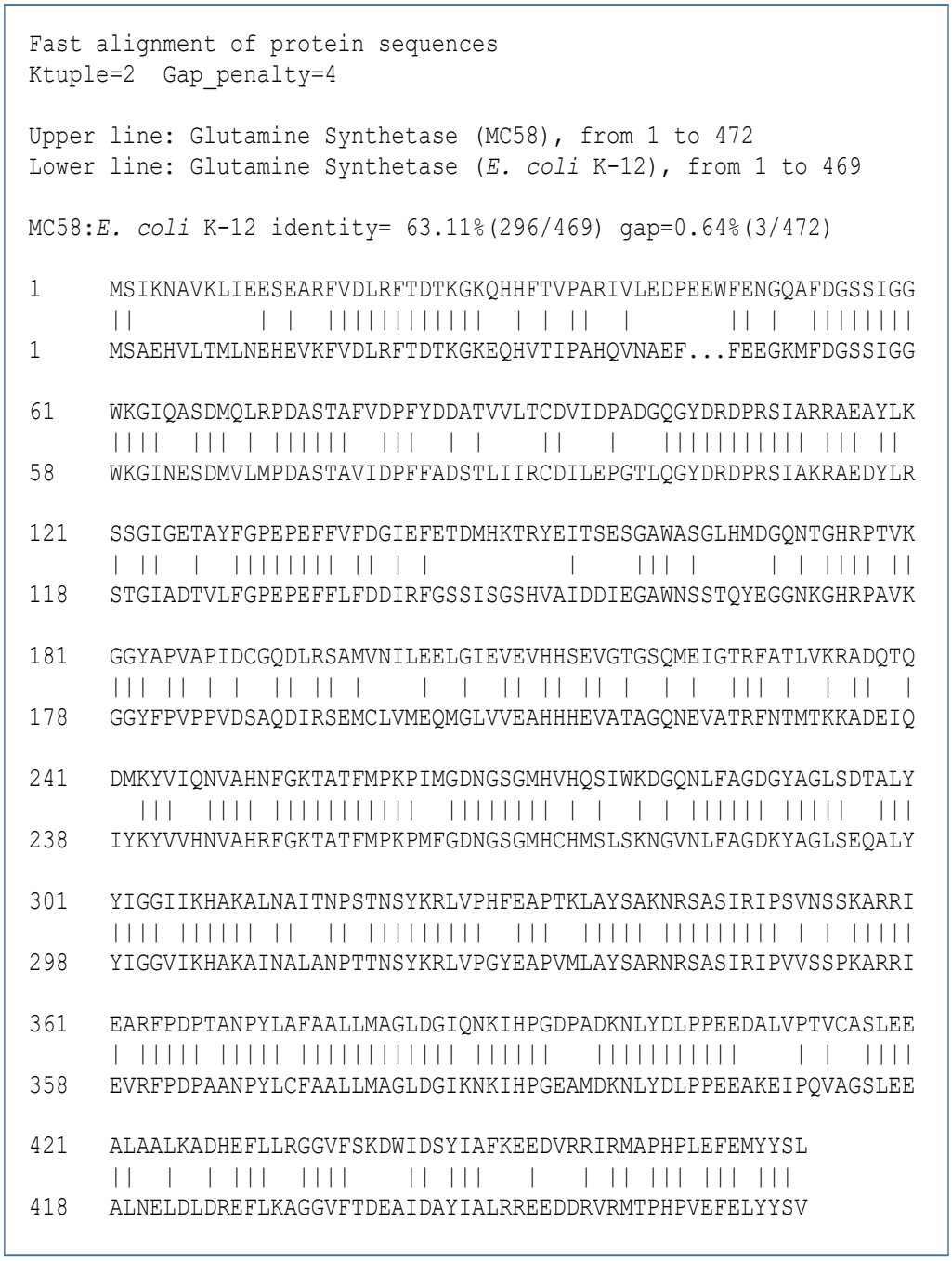
Figure: 2.1: Position and arrangement of NMB0359 and adjacent genes in the *Neisseria meningitidis* MC58 genome.

A similar genomic arrangement is present in the serogroup C meningococcal strain 8013 (NMV0398, NMV0400 and NMV0401 encoding shikimate dehydrogenase, GlnA and a putative AmpG-like protein respectively). In the serogroup C strain FAM18 (NMC1808, NMC1809 and NMC1810 encoding a putative AmpG-like protein, GlnA and shikimate dehydrogenase respectively) and the serogroup A strain Z2491 (NMA2127, NMA2128 and NMA2129 encoding a putative AmpG-like protein, GlnA and shikimate dehydrogenase respectively) the genomic arrangement of the gene *glnA* is upstream of, and in a different orientation to shikimate dehydrogenase; and

downstream of, and in the same orientation to a putative AmpG-like protein. In these three genomes, the *glnA* sequences are > 95% identical to the MC58 *glnA* gene, indicating a high level of conservation across *N. meningitidis*.

Additionally, sequences > 90% identical to MC58 *glnA* are found in the gonococcal strain FA1090 (similar genomic arrangement with 93% identity) and *N. lactamica* strain 020-06 (similar genomic arrangement with 95% identity) suggesting that *glnA* is highly conserved across *Neisseria* species. At the amino acid level, GlnA sequences from meningococcal strains MC58, FAM18, 053442, Z2491, *lactamica* strain 020-06, and the gonococcal strain FA1090 are > 98% identical.

By alignment using DNAMAN software, the *Neisseria* GlnA protein (NMB0359) was around 63% identical at amino acid level to a well characterized GlnA from *E. coli* strain K-12, thus confirming that GlnA is correctly annotated in the MC58 genome as the start of GlnA (Methionine) in MC58 matches the start of that in *E. coli* (Figure 2.2).



```
Fast alignment of protein sequences
Ktuple=2  Gap_penalty=4

Upper line: Glutamine Synthetase (MC58), from 1 to 472
Lower line: Glutamine Synthetase (E. coli K-12), from 1 to 469

MC58:E. coli K-12 identity= 63.11%(296/469) gap=0.64%(3/472)

1     MSIKNAVKLIEESEARFVDLRFTDTKGKQHHFTVPARIVLEDPEEWFENGQAFDGSSIGG
      ||         | |  ||||||||||||  | | ||  |       || |  ||||||||
1     MSAEHVLTMLNEHEVKFVDLRFTDTKGKEQHVTIPAHQVNAEF...FEEGKMFDGSSIGG

61    WKGIQASDMQLRPDASTAFVDPFYDDATVVLTCDVIDPADGQGYDRDPRSIARRAEAYLK
      ||||  ||| | ||||||  |||  | |    ||   |   |||||||||| ||| ||
58    WKGINESDMVLMPDASTAVIDPFFADSTLIIRCDILEPGTLQGYDRDPRSIAKRAEDYLR

121   SSGIGETAYFGPEPEFFVFDGIEFETDMHKTRYEITSESGAWASGLHMDGQNTGHRPTVK
      | ||  |  |||||||| || | |          |    ||| |     | | |||| ||
118   STGIADTVLFGPEPEFFLFDDIRFGSSISGSHVAIDDIEGAWNSSTQYEGGNKGHRPAVK

181   GGYAPVAPIDCGQDLRSAMVNILEELGIEVEVHHSEVGTGSQMEIGTRFATLVKRADQTQ
      ||| || | |  || || |    |  |  || || || |  | |  ||| |  | ||  |
178   GGYFPVPPVDSAQDIRSEMCLVMEQMGLVVEAHHHEVATAGQNEVATRFNTMTKKADEIQ

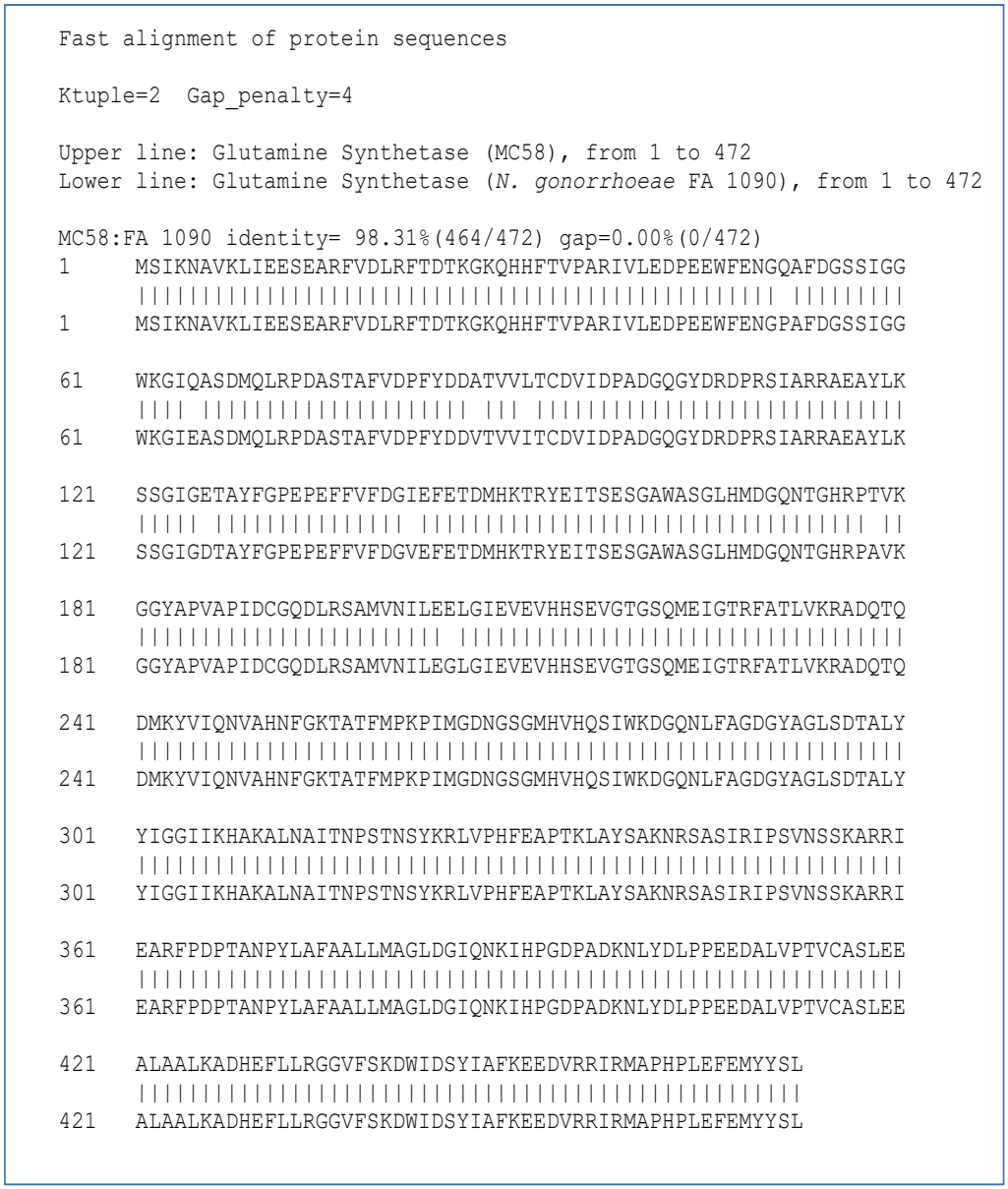
241   DMKYVIQNVAHNFGKTATFMPKPIMGDNGSGMHVHQSIWKDGQNLFAGDGYAGLSDTALY
        |||  |||| ||||||||||  |||||||| | |  | | |||||| |||||  |||
238   IYKYVVHNVAHRFGKTATFMPKPMFGDNGSGMHCHMSLSKNGVNLFAGDKYAGLSEQALY

301   YIGGIIKHAKALNAITNPSTNSYKRLVPHFEAPTKLAYSAKNRSASIRIPSVNSSKARRI
      |||| |||||| ||  || |||||||||  |||  ||||| ||||||||| | | |||||
298   YIGGVIKHAKAINALANPTTNSYKRLVPGYEAPVMLAYSARNRSASIRIPVVSSPKARRI

361   EARFPDPTANPYLAFAALLMAGLDGIQNKIHPGDPADKNLYDLPPEEDALVPTVCASLEE
      | ||||| ||||| |||||||||||| ||||||   ||||||||||    | |  ||||
358   EVRFPDPAANPYLCFAALLMAGLDGIKNKIHPGEAMDKNLYDLPPEEAKEIPQVAGSLEE

421   ALAALKADHEFLLRGGVFSKDWIDSYIAFKEEDVRRIRMAPHPLEFEMYYSL
      ||  |  | |||  ||||    || |||   |   | || ||| ||| |||
418   ALNELDLDREFLKAGGVFTDEAIDAYIALRREEDDRVRMTPHPVEFELYYSV
```

[A]



```
Fast alignment of protein sequences

Ktuple=2  Gap_penalty=4

Upper line: Glutamine Synthetase (MC58), from 1 to 472
Lower line: Glutamine Synthetase (N. gonorrhoeae FA 1090), from 1 to 472

MC58:FA 1090 identity= 98.31%(464/472) gap=0.00%(0/472)
1     MSIKNAVKLIEESEARFVDLRFTDTKGKQHHFTVPARIVLEDPEEWFENGQAFDGSSIGG
      |||||||||||||||||||||||||||||||||||||||||||||||||| |||||||||
1     MSIKNAVKLIEESEARFVDLRFTDTKGKQHHFTVPARIVLEDPEEWFENGPAFDGSSIGG

61    WKGIQASDMQLRPDASTAFVDPFYDDATVVLTCDVIDPADGQGYDRDPRSIARRAEAYLK
      |||| |||||||||||||||||| ||| ||||||||||||||||||||||||||||||
61    WKGIEASDMQLRPDASTAFVDPFYDDVTVVITCDVIDPADGQGYDRDPRSIARRAEAYLK

121   SSGIGETAYFGPEPEFFVFDGIEFETDMHKTRYEITSESGAWASGLHMDGQNTGHRPTVK
      ||||| ||||||||||||||| |||||||||||||||||||||||||||||||||| ||
121   SSGIGDTAYFGPEPEFFVFDGVEFETDMHKTRYEITSESGAWASGLHMDGQNTGHRPAVK

181   GGYAPVAPIDCGQDLRSAMVNILEELGIEVEVHHSEVGTGSQMEIGTRFATLVKRADQTQ
      ||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
181   GGYAPVAPIDCGQDLRSAMVNILEGLGIEVEVHHSEVGTGSQMEIGTRFATLVKRADQTQ

241   DMKYVIQNVAHNFGKTATFMPKPIMGDNGSGMHVHQSIWKDGQNLFAGDGYAGLSDTALY
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
241   DMKYVIQNVAHNFGKTATFMPKPIMGDNGSGMHVHQSIWKDGQNLFAGDGYAGLSDTALY

301   YIGGIIKHAKALNAITNPSTNSYKRLVPHFEAPTKLAYSAKNRSASIRIPSVNSSKARRI
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
301   YIGGIIKHAKALNAITNPSTNSYKRLVPHFEAPTKLAYSAKNRSASIRIPSVNSSKARRI

361   EARFPDPTANPYLAFAALLMAGLDGIQNKIHPGDPADKNLYDLPPEEDALVPTVCASLEE
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
361   EARFPDPTANPYLAFAALLMAGLDGIQNKIHPGDPADKNLYDLPPEEDALVPTVCASLEE

421   ALAALKADHEFLLRGGVFSKDWIDSYIAFKEEDVRRIRMAPHPLEFEMYYSL
      ||||||||||||||||||||||||||||||||||||||||||||||||||||
421   ALAALKADHEFLLRGGVFSKDWIDSYIAFKEEDVRRIRMAPHPLEFEMYYSL
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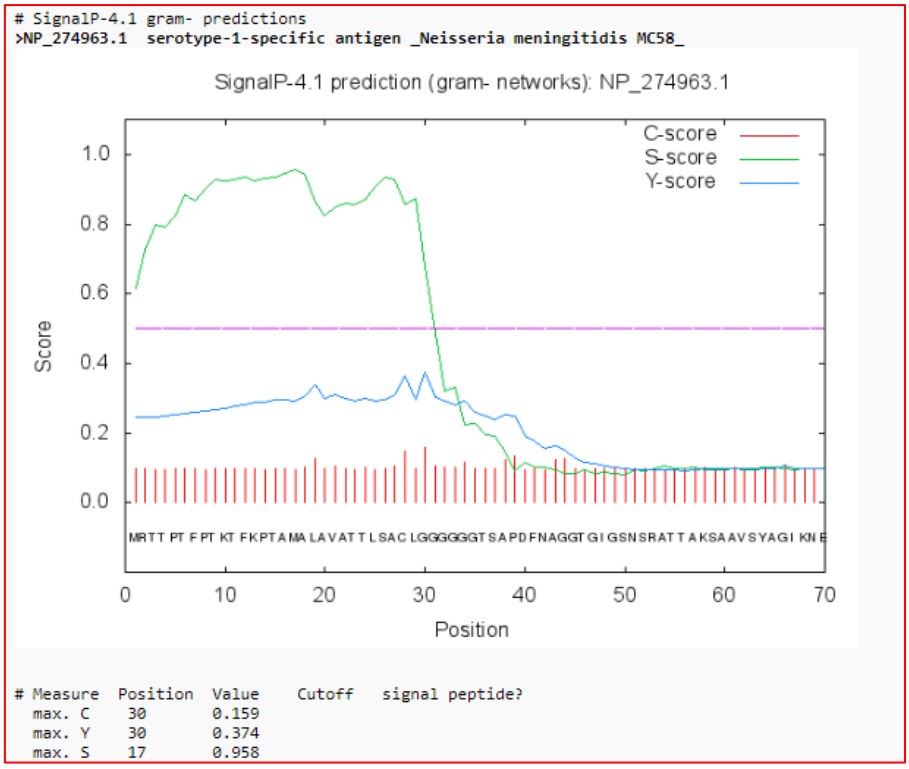
[B]



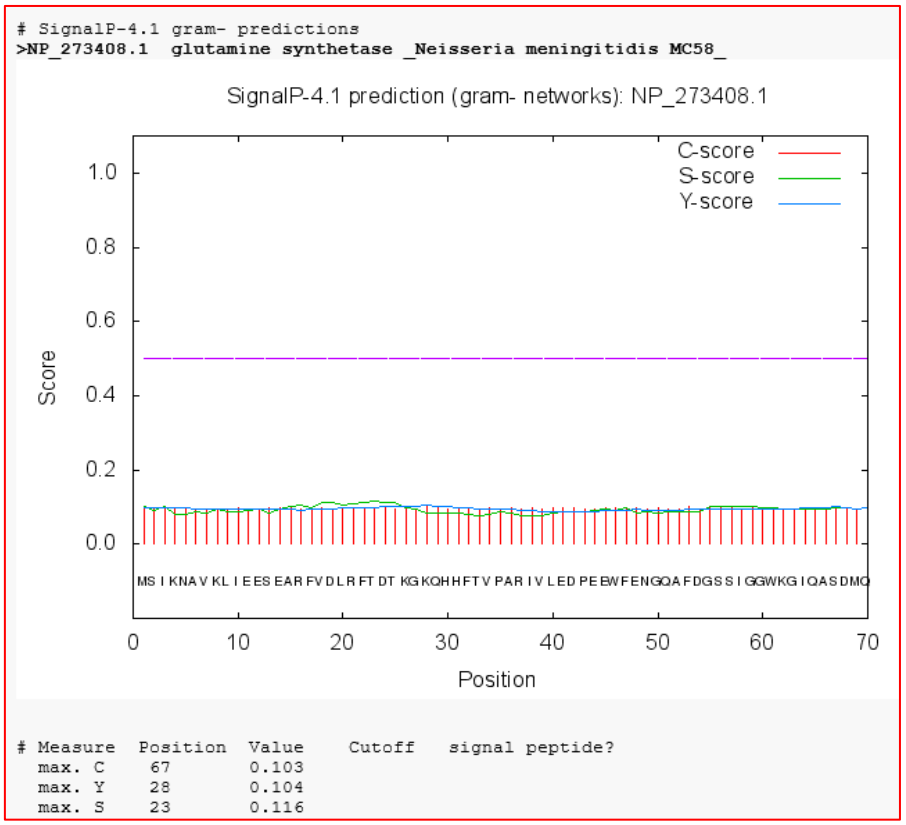
[C]

Figure 2.2: Alignment of the MC58 GlnA to (A) *E. coli* GlnA at amino acid level to confirm correct annotation of GlnA start in MC58, (B) *N. gonorrhoeae* FA 1090 GlnA, (C) Eukaryotic GlnA from *Homo sapiens*.

The SignalP 4.1 prediction tool was used to determine if meningococcal GlnA possesses a signal peptide sequence for translocation of the protein from the cytosol to the outer parts of the cell. The peptide sequence of a known meningococcal outer membrane AspA/NalP was used as a positive control. Comparing the score for both proteins, it would suggest that glutamine synthetase does not possess a predicted signal peptide.



[A]



[B]

Figure 2.3: Prediction of putative signal peptide sequences using SignalP 4.1 server of (A) AspA/NalP (B) GlnA.

The C score represents putative signal peptide cleavage sites, while the Y score represents a better cleavage site than the C score on its own, and the S score differentiates positions in the signal peptide from positions in the mature proteins, and from proteins without signal peptides. All scores associated with glutamine synthetase were much lower (close to 0.1) in comparison to scores associated with AspA/NalP, indicating that a signal peptide sequence is lacking in GlnA.

2.3.2. Amplification and cloning of NMB0359 into pQE30

NMB0359 encodes the meningococcal glutamine synthetase. The entire ORF was amplified by PCR to yield a single band of the expected size (Figure 2.4 A). The PCR product was digested with *BamHI* (a restriction enzyme recognition site was included in the primers used to amplify each product), ligated into *BamHI*-digested and dephosphorylated pQE30 vector (Figure 2.4 B), and then transformed into *E. coli* strain JM109. Plasmids extracted from the resultant clones were analysed by restriction digestion and DNA sequencing.

Digestion of plasmid DNA containing NMB0359 with *BamHI* was expected to produce two fragments of *ca.* 1.4 kb and 3.4 kb corresponding to NMB0359 and pQE30 respectively (Figure 2.5).

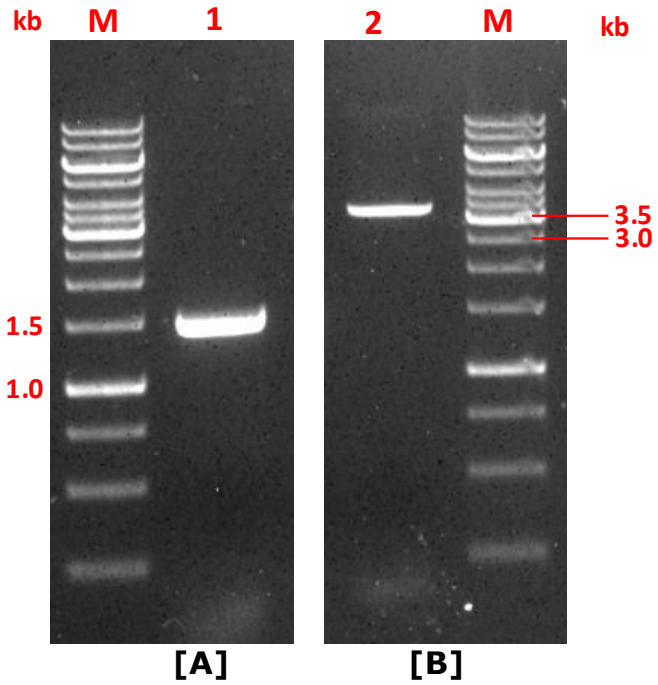


Figure 2.4: Agarose gel analysis showing the PCR amplification of NMB0359 (A). Chromosomal DNA was prepared from *N. meningitidis* MC58 and used as a template to amplify the gene by PCR. Lane M, 1 kb DNA ladder. Lane 1: PCR products corresponding to NMB0359. (B) *BamHI*-digested and dephosphorylated pQE30 vector (Lane 2).

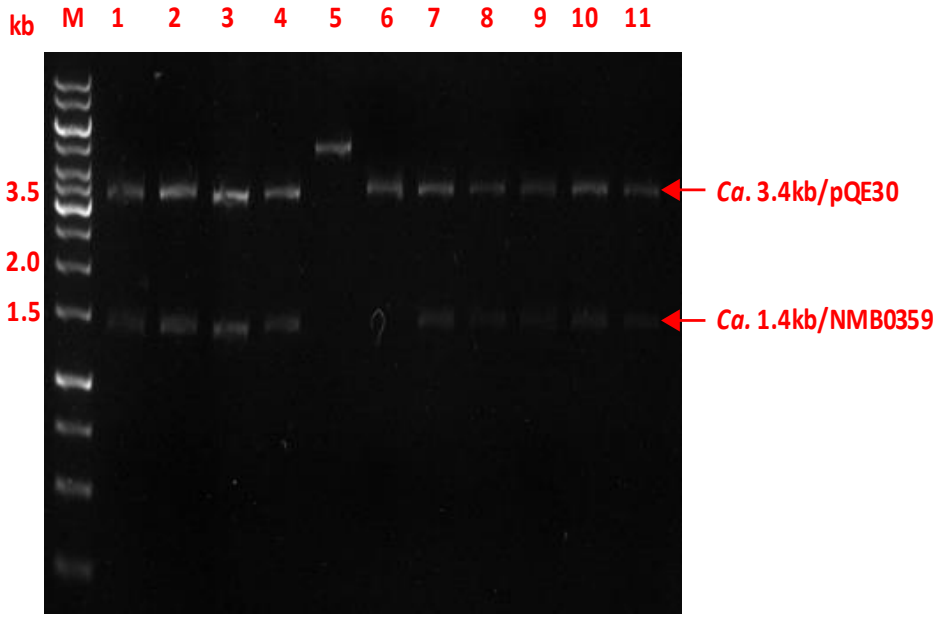


Figure 2.5: Agarose gel analysis showing *BamHI* restriction digestion of plasmid DNA extracted from *E. coli* JM109 potentially harbouring NMB0359 in pQE30. Lane M, 1 kb DNA ladder; lanes 1 – 4; 7 - 11, putative pIT1.

Nine of the eleven colonies analysed by restriction digestion with *BamHI* showed the expected band size of 1.4kb and 3.4kb (Figure 2.5). DNA sequence analysis of one of these nine plasmids showed that the *glnA* fragment was present in the correct orientation to the vector encoded promoter, and in frame with the 6-his tag. DNA sequence analysis also confirmed the absence of any PCR-generated errors. This plasmid was designated as pIT1.

2.3.3. Expression of recombinant glutamine synthetase

E. coli JM109 (pIT1) was used to express recombinant glutamine synthetase (rGlnA). After 4 h induction with 1 mM IPTG, expression was obtained. This was determined by SDS-PAGE which revealed a band of *ca.* 52-kDa, which is the expected size for rGlnA (Figure 2.6 A). The identity of the 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 2.6 B) compared to less intense band of the same size from un-induced samples.

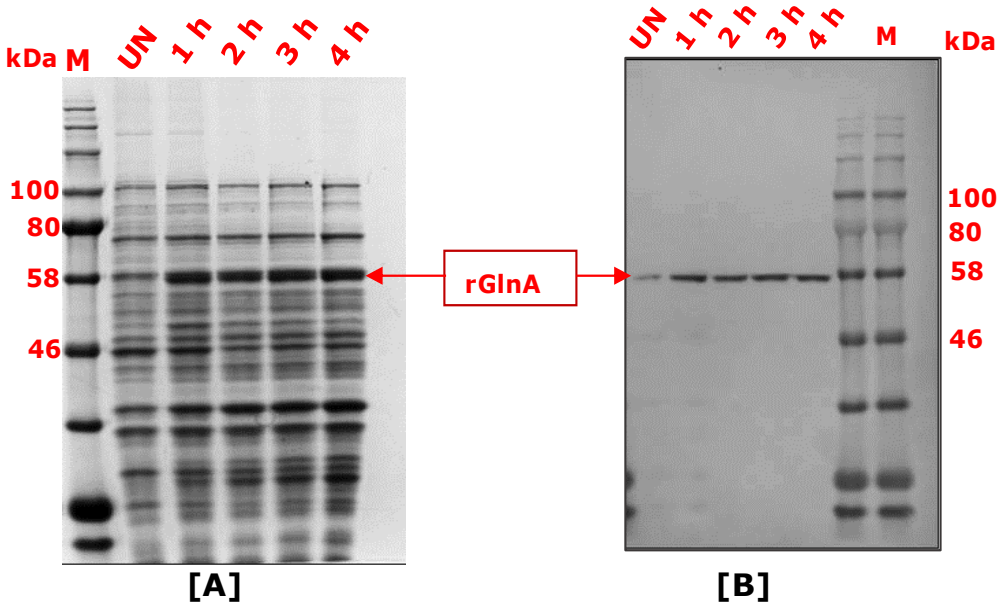


Figure 2.6: SDS-PAGE (A) or immunoblot analysis (B) with anti-penta-histidine antibodies demonstrating the 4 h overexpression of rGlnA in IPTG-induced *E. coli* JM109 cells containing pIT1 [lanes 1 h – 4 h] compared to non-induced cells [lane UN]; Lane M: pre-stained protein marker.

2.3.4. Purification of His-tagged rGlnA under native conditions

Recombinant His-tagged glutamine synthetase was purified under native conditions, utilizing immobilized nickel affinity chromatography. The purification process was performed in three stages. The first stage involved the binding of the His-tagged protein to nickel ions immobilized on the purification column. After washing with low concentration imidazole to remove non-specifically bound proteins, the recombinant protein was eluted using a high concentration of imidazole. To generate the clean and purified protein obtained in this study, several optimisation steps where included during the purification process. These optimisation steps include adding imidazole (10 mM into the lysis buffer), varying the imidazole content of the wash buffer (between 20 mM - 50 mM) as well as the imidazole content of

the elution buffer (200 mM – 500 mM). Cleaner purification preparations were observed in the higher imidazole content buffers (50 mM and 500 mM for the wash and lysis buffer respectively). Likewise, cleaner purification fractions were observed when elution was performed over a gradient rather than a single step elution, with the His-tagged protein eluting from the column at around 85% imidazole content (425 mM imidazole).

Finally, fractions containing the desired protein (according to the purification chromatogram, SDS-PAGE, and immunoblot analysis) were combined. A desalting step with PBS containing 20% $^{v}/_{v}$ glycerol (for protein stability) was then performed to exchange the high-imidazole containing elution buffer with PBS. Finally, the protein was concentrated, quantified and stored at -80^{o}C till further use. The protein concentration, measured by Pierce^{TM} BCA Protein Assay kit (Thermo Scientific) was elucidated to be 1.33 mg m^{l-1}.

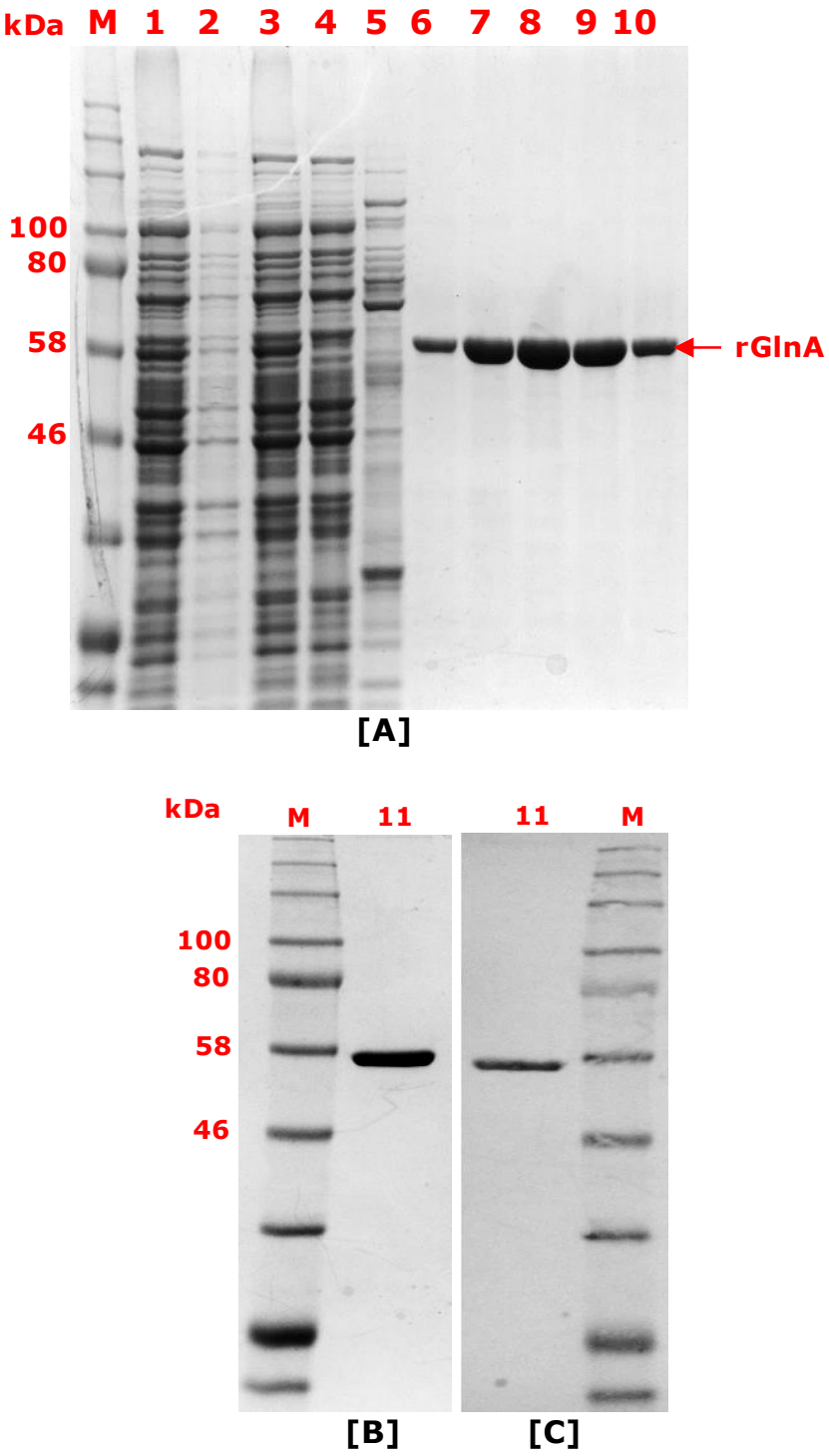
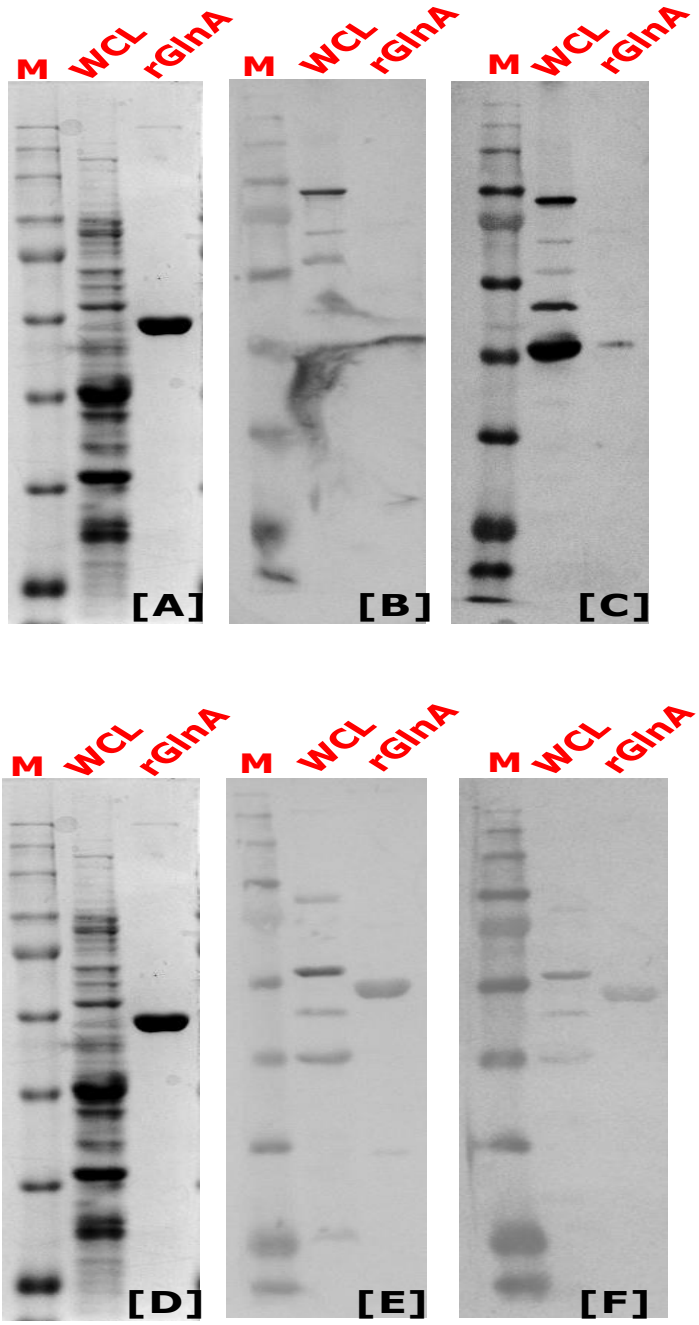


Figure 2.7: SDS-PAGE showing the IMAC-purification steps of recombinant glutamine synthetase from 150 ml harvested cells pellet of the IPTG-induced *E. coli* JM109 containing pIT1 (A). SDS-PAGE (B), Immunoblot analysis (C) of desalted and concentrated protein. Lane M: pre-stained protein marker lane 1: whole cell lysate, lane 2: pellet lysate, lane 3: supernatant lysate, lane 4: flow through, lane 5: wash, lane 6-10: elution fractions, lane 11: desalted and concentrated protein.

2.3.5. Production of Polyclonal Antibodies

Three White New Zealand were pre-screened for background antibody levels against rGlnA. Although meningococcal whole cell lysates and rGlnA showed some reactivity to the pre-immunization sera from the three rabbits, the rabbit with the least reactivity to rGlnA (Rabbit 1, Figure 2.8 B/C) was selected to raise the polyclonal antibodies in (See Figure 2.8 A – I).



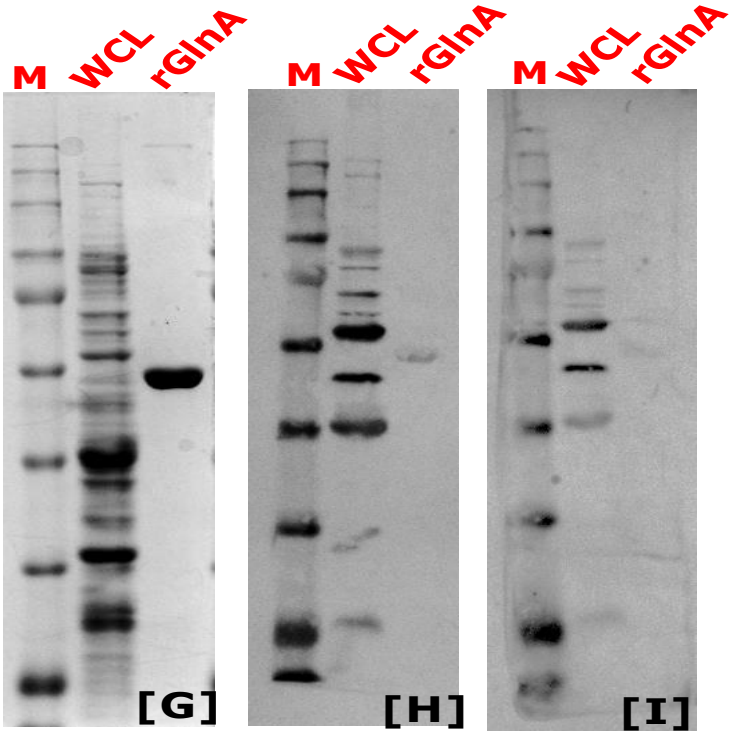


Figure 2.8: Pre-screening of rabbits for selection for the production of anti-GlnA antibodies. SDS PAGE (A, D, G) or immunoblot analysis (B, C, E, F, H, I) of all three rabbits pre-screened. For immunoblot analysis, sera was diluted 1/2000 (B, E, H) or 1/5000 (C, F, I) to determine the best concentration for subsequent experiments, with dilution of 1/5000 selected.

To test the effectiveness of the protocol as well as the immunogenicity of the antigen over the 49-days period, rabbit pre-immunization bleed as well as test bleeds were immunoblotted against rGlnA and meningococcal whole cell lysates, before the rabbit was finally exsanguinated and final bleed collected.

A highly reactive band of *ca.* 52 kDa was observed when rGlnA was probed with the final bleed from the rabbit, and a weak band of *ca.* 52 kDa corresponding to GlnA observed when MC58-WT whole cell lysates (WCL) were probed with the final bleed from the rabbit. Bleeds were also observed to react with two other proteins in the meningococcal WCL although very faintly.

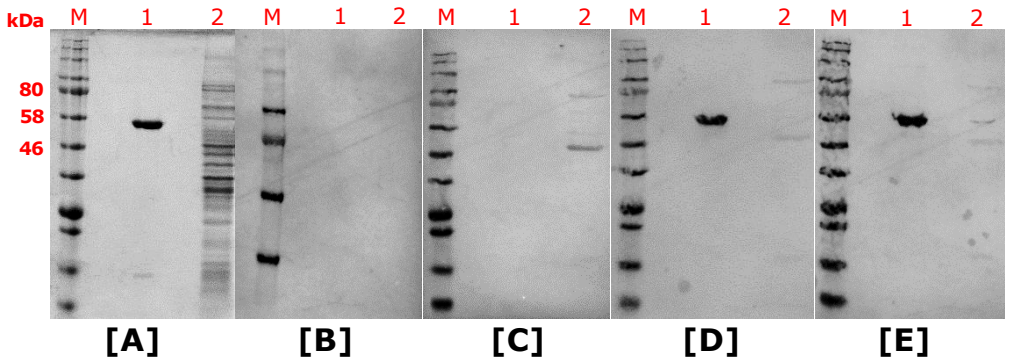


Figure 2.9: SDS PAGE (A) or immunoblot analysis (B-E). (B) pre-immunization serum, (C) test serum 1, (D) test serum 2, (E) final serum all diluted to concentration 1:5000. All membranes probed with anti-rabbit IgG-alkaline phosphatase as secondary antibody, diluted 1:30,000. Colour developed by adding BCIP/NBT alkaline phosphatase substrate. lane M: colour pre-stained protein marker, lane 1: rGlnA, lane 2: MC58-WT whole cell lysate.

2.4. Discussion

The genome of *N. meningitidis* carries a single copy of NMB0359 encoding the meningococcal glutamine synthetase. The contribution of GlnA to pathogenesis in several other pathogens has been documented but its role (if any) in *N. meningitidis* is poorly understood.

In order to study this protein, determine if it is a moonlighting protein and eventually explore some of the moonlighting activities, it was essential to clone and express GlnA in *E. coli*.

NMB0359 was successfully ligated unto the expression vector pQE30 which adds 6-Histidine tag to the N terminus of the protein to be expressed. Competent *E. coli* JM109 cells were transformed with the ligation mixture. Successful clones were screened by restriction digestion as well as DNA sequencing.

A successful clone which had *glnA* in the right orientation as well as had no PCR generated errors was designated as pIT1. IPTG was added to *E. coli* cells containing pIT1, grown to mid-log phase to induce the overexpression of rGlnA.

The method of IMAC was employed to purify the N terminus His-tagged protein, using nickel ions as the metal of choice. This is based on the principle that Histidine molecules bind strongly although reversibly to the Ni^{2+} ions immobilized to the purification column (Porath et al., 1975
, Knaust et al., 2007). It is important to note that other amino acids such as cysteine and tryptophan also contain imidazole and thiol groups that can bind to imidazole in the manner similar to histidine (Porath et al., 1975
, Hemdan and Porath, 1985). To help negate this, the sample was lysed in buffer containing low concentration imidazole (20 mM) and loaded onto the purification column. The imidazole which also has a high affinity for Ni^{2+} would act as a competitor and help prevent non-specific binding. The release

of the His-tagged protein from the purification column was achieved by applying high imidazole (500 mM) elution buffer.

Research by Sharma *et al* (2001) and Hefti *et al* (2001) has shown that high imidazole content can interfere with applications such as crystallography, competition assays as well as increase chances of protein aggregation (Sharma et al., 1992, Hefti et al., 2001). To prevent this, desalting of the high imidazole elution buffer into PBS was performed. It was observed after desalting and concentrating, that the protein formed aggregates, possibly from macromolecular crowding due to high protein concentration (Shire et al., 2004). To avoid protein precipitation, glycerol was added into PBS at a final concentration of 20% ($^{v}/_{v}$) to act as protein stabilizer; possibly by slightly altering the protein conformation to a more compact form (Gekko and Timasheff, 1981, Priev et al., 1996, Chanasattru et al., 2008). Glycerol was also confirmed by (Shepard et al., 1976) to provide antifreeze properties when added to water by reducing the freezing point of water, thus promoting longer storage of purified proteins as well as reducing the effects of freezing-thawing on protein quality.

The results of SDS-PAGE and immunoblot analysis using anti-penta his antibodies confirmed the identity as well as quality of the purified rGlnA. The purified recombinant protein molecular mass was estimated to be around 53 kDa (including 6 His-tag) which is similar to the estimated molecular mass of native GlnA of around 52 kDa.

As the protein was successfully purified under native conditions, there was no requirement for a re-folding step after purification. Rabbit polyclonal antibodies against rGlnA (RɑGlnA) were generated, and the ability for the antibodies to recognise its target protein rGlnA was investigated by SDS-PAGE and immunoblotting.

In conclusion, an expression plasmid for rGlnA was successfully constructed. The rGlnA was over expressed with an N-terminal histidine tag and the recombinant protein was successfully purified by affinity chromatography under native conditions. The purified rGlnA was used to raise rabbit polyclonal antiserum.

Finally, the recombinant GlnA and RαGlnA detailed in this chapter were employed in subsequent characterisation experiments including the detection of glutamine synthetase on the meningococcal cell surface as well as its moonlighting properties.

CHAPTER 3: Mutagenesis of the Gene Encoding GlnA in *Neisseria meningitidis*

3.1. Introduction

The use of mutagenesis and complementation as a means for exploring the functions of a gene product by comparing the phenotype of the resultant mutant strain with that of the original and complemented mutant strains is a widely explored concept. Specifically, the mutant and complemented strains expected to be generated in this study would be employed in experiments to determine the moonlighting properties of glutamine synthetase and the potential role of any moonlighting activities in the pathogenesis of meningococcal disease.

N. meningitidis is naturally competent for transformation throughout its life cycle and is able to distinguish between self and non-self DNA, in that DNA containing a 10-bp DNA uptake sequence (DUS) is taken up with much higher efficiency than DNA lacking this sequence (Jyssum and Lie, 1965). This DUS (5' GCCGTCTGAA 3') occurs around 2,000 times in the average 2.3 Mbp genomes of *N. meningitidis*, occupying as much as 1% of the chromosome, with an increase in transformation observed in some strains containing the DUS with the addition of two semiconserved residues to the 5' region, yielding the 12-mer DUS 5 'ATGCCGTCTGAA 3' (Ambur et al., 2007). Transformation in *N. meningitidis* is thought to match the process in *N. gonorrhoeae*, which involves the process of DNA uptake, processing, and chromosomal integration (Lorenz and Wackernagel, 1994, Alexander et al., 2004). The uptake of genus-specific DNA has been shown to be mediated by type IV pili at the bacterial surface (Mattick, 2002).

It is common practice to create a mutant by replacing the relevant gene with an antibiotic marker which allows for easy selection, but for safety reasons and the development of multi-drug resistant pathogens, it is recommended to limit the number of different antibiotic resistance markers in a meningococcal strain which is quite challenging especially if a strain with

several mutated genes is required. To avoid this, a markerless mutation can be introduced as 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).

A markerless mutant can be created by exploiting the counter-selective properties of the *rpsL* gene encoding ribosomal protein 12. This strategy involves the use of a two-gene cassette that contains both a selectable marker (*ermC*, conferring erythromycin resistance) and a counter-selectable marker (*rpsL*). Streptomycin resistance can be mediated by a particular allele of the *rpsL* gene, $rpsL^R$. The sensitive *rpsL* allele, $rpsL^S$, is dominant over the resistant one (Lederberg, 1951). Firstly, $rpsL^S$ is introduced together with *ermC* in a double gene cassette into the gene of interest in a streptomycin-resistant strain, causing the strain to become sensitive for streptomycin but resistant towards erythromycin. In a second step, the *ermC*- $rpsL^S$ cassette is removed by transformation with DNA containing only flanking regions of the gene of interest and selecting for streptomycin resistance and erythromycin sensitivity.

This chapter describes the attempts at generating a *glnA* null mutant, complementation of *glnA* at an ectopic site in the *N. meningitidis* MC58 WT strain, and whole cell ELISA experiments to investigate surface localisation of glutamine synthetase.

3.2. Materials and Methods

3.2.1. *In Silico* Sequence Analysis

GenomeNet Database Resources https://www.genome.jp/ was employed to analyse the gene encoding glutamine synthetase (NMB0359) from *N. meningitidis* MC58, together with the upstream and downstream sequences. Arnold online tool http://rssf.i2bc.paris-saclay.fr/toolbox/arnold/index.php was utilized to predict transcription termination sequences downstream of NMB0359. Finally, DNAMAN software (Lynnon BioSoft) was used in DNA and protein sequence analyses, restriction enzyme digestion, and primer design.

3.2.2. Bacterial Strains, Media and Growth Conditions

E. coli strain JM109 was utilized for propagation of all plasmids described in this chapter, under the same growth conditions described in section 2.2.2. When required, antibiotics and chemical agents were used at the following final concentrations: ampicillin (100 µg/ml), kanamycin (50 µg/ml), erythromycin (5 µg/ml), IPTG (0.5 mM) and X-gal (80 µg/ml). Meningococcal strains (wild type MC58 and MC58 *rpsL*$^{-}$ *Kan*^{R} *rpsL*$^{+}$, provided by Dr N. Oldfield and H. Albasri; University of Nottingham were grown on chocolate agar, Brain Heart infusion (BHI) agar or in BHI broth. Cultures were incubated at 37°C with 5% ($^{v}/_{v}$) CO_2 with or without shaking at 200 rpm.

3.2.3. DNA Techniques

DNA techniques such as 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 NMB0359 and Flanking DNA

The NMB0359 fragment (*glnA*) together with its flanking sequence: 1kb upstream and downstream of the gene was PCR amplified as described in the previous chapter using primer pairs GlnAF2/GlnAR2 (Table 3.1). Chromosomal DNA from WT *N. meningitidis* MC58 was used as template DNA with appropriate thermocycling conditions (Table 3.1). The resulting PCR product (UglnA) was cleaned using the GenElute PCR clean up kit (Sigma) and thereafter, 48 µl of the product was incubated with 6.6 µl GoTaq polymerase, 14 µl of of 5x GoTaq buffer, and 10mM dATP to a final concentration of 0.2 mM at 72˚C for 45 min to add A-tail.

To construct a recombinant plasmid, the A-tailed PCR product was ligated into the pGEM-T Easy vector (Promega). Chemically competent *E. coli* JM109 cells were transformed with the resulting recombinant plasmids by heat shock according to manufacturer's instructions (described in section 2.2.10). Positive transformants were identified by blue and white screening on indicator plates (containing 100 µg/ml ampicillin, 0.5 mM IPTG and 80 µg/ml X-Gal).

Potential clones were screened for by restriction digestion with *EcoRI* and by DNA sequencing to confirm insert orientation and to check for PCR generated errors (Source Bioscience). A correct clone containing *glnA* together with the upstream and downstream flanking sequence in pGEM – T_Easy was designated as pIT2.

Table 3.1: Mutagenic primers and PCR thermo-cycling conditions used in the mutagenesis by deletion of NMB0359

PCR Product	DNA Template	Primer Sequence 5′ – 3′	Thermo-cycling conditions		Amplicon Description
			Annealing Temperature (°C)	Extension time (s)	
UglnA	NMB MC58	glnAF2A: GCGCAGCCGTACACCATATCG glnAR2A: GCATCCACACACCGCCCAAGA	62	120	NMB0359 coding sequence with 1 kb upstream and 1 kb downstream flanking DNA
deglnA	pIT2	glnAF3: GCG**GGATCC**ACTGGATCGACAGCTACATCG glnAR3: GCG**GGATCC**TGCTGCTTGCCTTTGGTATCG	62	150	pIT1 construct with NMB0359 deleted by Inverse PCR
KnsL	NMB MC58 $rpsL^{-}$ Kan^{R} $rpsL^{+}$	KnsLF1: GCG**GGATCCC**AGCTTATCATCGATAAACCCAGCG KnsL R1: GCG**GGATCC**GTAATTATTTAGGACGCTTAGCACCG	60	80	Kanamycin resistance cassette and *rpsL*$^{+}$ gene (each gene preceded by its own promoter)
glnA	NMB MC58	GlnAF5: GCG**AGATCT**ATGTCTATTAAAAACGCCG GlnAR5: GCG**AGATCT**AAACCAGACGTGCTTACAGGC	60	60	NMB0359

*Sequences in bold represent recognition sites of different restriction enzymes.

3.2.5. Deletion of NMB0359 by Inverse PCR

Inverse PCR was carried out on plasmid pIT2 as template DNA, to delete NMB0359 using primer pairs GlnAF3 and GlnAR3 (Table 3.1). PCR was performed as described in the previous chapter using the thermos cycling conditions outlined in Table 3.1. Agarose gel analysis was used to confirm the size of the PCR product (deglnA) which was then digested with *BamHI*. The digested PCR product was then cleaned up, re-ligated and used to transform competent *E. coli* JM109 cells. Cells were grown overnight at 37˚C on LB agar with ampicillin (100 µg/ml). Single colonies were selected and re-grown on LB agar with ampicillin overnight and then cultured in LB broth with ampicillin (100 µg/ml). Confirmation of the deletion of *glnA* was carried out by restriction digestion and DNA sequencing. A correct clone was designated as pIT3.

3.2.6. Preparation of Markers for Selection and Cloning into pIT3

The DNA fragment spanning the kanamycin resistant cassette (Kan^R) and the $rpsL^+$ gene was PCR amplified using primer pairs KsnLF1/KsnLR1 (see table 3.1) and chromosomal DNA from *N. meningitidis* MC58 $rpsL^- Kan^R rpsL^+$ extracted using GenElute bacterial genomic DNA extraction kit (Sigma-Aldrich). PCR was performed as described in the previous chapter using thermo-cycling conditions outlined in Table 3.1. The amplicon designated as KsnL was purified, digested with *BamHI*, and ligated into *BamHI* digested pIT3. Competent *E. coli* JM109 cells were transformed with the resulting construct. Positive transformants were screened by resistance to kanamycin, restriction digestion with *BamHI*, and DNA sequencing. A correct clone was designated as pIT4.

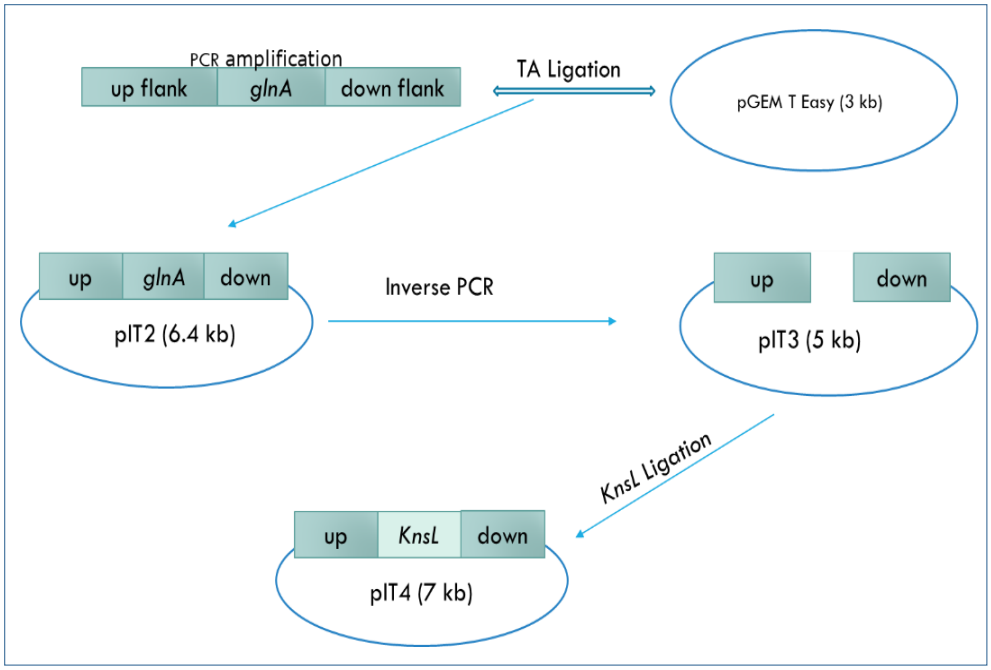


Figure 3.1: Schematic workflow of the deletion of NMB0359 and the cloning of constructs in *E. coli*.

3.2.7. Creation of a *glnA* inactivation construct in *E. coli*

The 5' 688 bp region of *glnA* was PCR amplified by PCR from the MC58 genome using primer pairs GlnAF1A/GlnAR1A (see table 3.2 for reaction conditions). The amplicon was cleaned up, double digested with restriction enzymes *SacI/KpnI* and ligated to the *SacI/KpnI* digested pMOD-2 vector provided by Dr Ruth Griffin (University of Nottingham). Competent *E. coli* cells were transformed with the resulting ligation mixture. Potential clones were screened by ampicillin resistance, restriction digestion with *SacI/KpnI* and DNA sequencing. A correct clone was designated as pIT6.

The 3' 774 bp portion of *glnA* (including a *Neisseria* DUS) was PCR amplified using primer pairs GlnAF1B/GlnAR1B (See Table 3.2 for PCR conditions), cleaned up, double digested with *XbaI/SalI* and then ligated to the *XbaI/SalI* digested pIT6 vector. Competent *E. coli* cells were transformed

with the resulting construct. Potential clones were screened by ampicillin resistance, restriction digestion with *XbaI/SalI* and DNA sequencing. A correct clone was designated as pIT7.

Lastly, the *KsnL* was PCR amplified using primer pairs KsnLF1A/KsnLR1 (See table 3.2 for PCR conditions), double digested with *XmaI/BamHI* and ligated to the *XmaI/BamHI* digested pIT7. Competent *E. coli* cells were transformed with the resulting construct, and potential clones screened by kanamycin resistance, colony PCR using primer pairs KsnLF1A/GlnAR1B, restriction digestion with *XmaI/BamHI* and DNA sequencing. A correct clone was designated as pIT8.

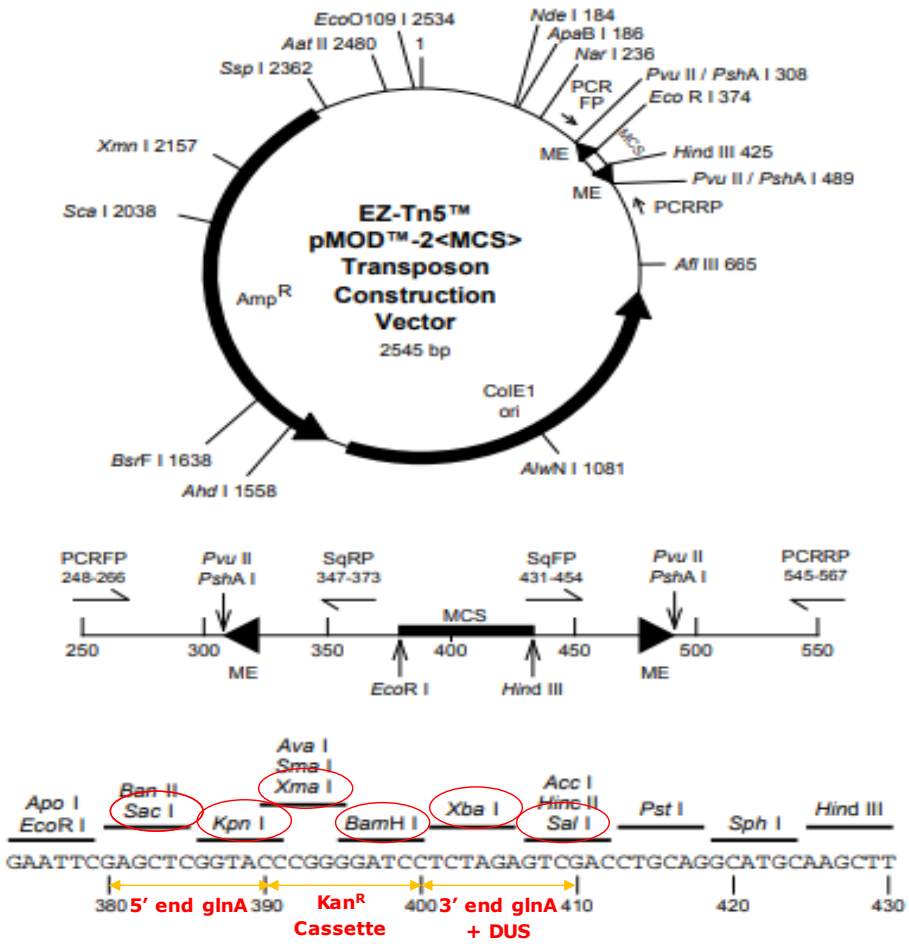


Figure 3.2: Schematic showing the points of insertion of fragments into pMOD-2 for the mutation of NMB0359 by gene inactivation.

Table 3.2: Mutagenic primers and PCR thermo-cycling conditions used in the mutagenesis by inactivation of NMB0359

PCR Product	DNA Template	Primer Sequence 5′ – 3′	Thermo-cycling conditions		Amplicon Description
			Annealing Temperature (°C)	Extension time (s)	
5′ region of *glnA*	NMB MC58	glnAF1A: GCG**GAGCTC**ATGTCTATTAAAAACGCCG glnAR1A: GCG**GGTACC**GCAAAGCGCGTGCCGATTTC	62	30	5′ region of NMB0359 coding sequence (688 bp)
3′ region of *glnA*	NMB MC58	glnAF1B: GCG**TCTAGA**TACTTTGGTCAAACGCGCCG glnAR1B: GCG**GTCGAC**CGTCTGAAACTGTGTTCAGACGGCAT	62	30	3′ region of NMB0359 coding sequence with *Neisseria* DNA uptake sequence at the end (774 bp)
KnsL	NMB MC58 $rpsL^{-}$ Kan^{R} $rpsL^{+}$	KnsLF1A: GCG**CCCGGG**CAGCTTATCATCCGATAAACCCAGCG KnsL R1: GCG**GGATCC**GTAATTATTTAGGACGCTTAGCACCG	60	80	Kanamycin resistance cassette and *rpsL*+ gene (each gene preceded by its own promoter)

*Sequences in bold represent recognition sites of different restriction enzymes.

3.2.8. Construction of Ectopic Complementation Plasmid

NMB0359 was PCR amplified using primer pairs GlnAF5/GlnAR5 (see Table 3.1), with chromosomal DNA from *N. meningitidis* MC58 serving as template DNA. The amplicon was cleaned using the GenElute PCR clean up kit, digested with *BglII*, and ligated unto *BglII* digested and dephosphorylated pComp-1 plasmid (obtained from Dr H. Albasri). pComp-1 comprises a pGEM -T- Easy backbone containing two meningococcal genes (NMB-0102 and NMB-0103) in opposite orientation to each other separated by a unique *BglII* site, promoters to drive expression of a cloned gene, and an erythromycin cassette to facilitate selection. Competent *E. coli* JM109 cells were transformed with the resulting construct, and prospective clones were screened by restriction digestion with *BglII* and DNA sequencing. A correct clone was designated as pIT5.

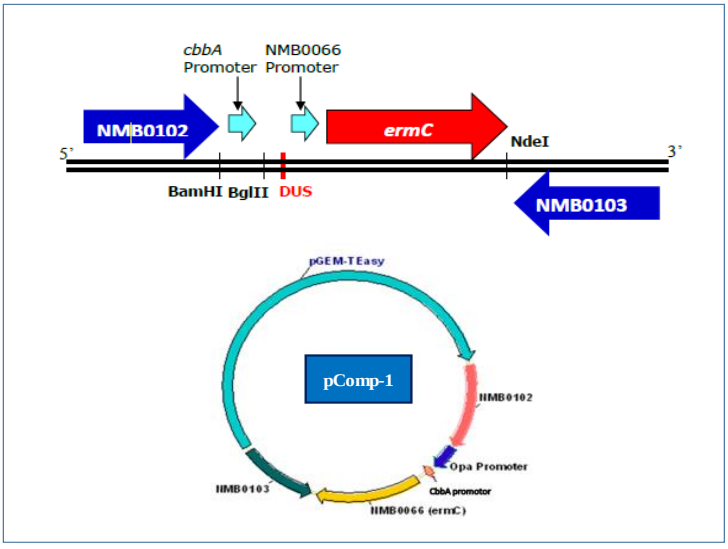


Figure 3.3: A schematic representation of the pComp-1 plasmid. The complementation cassette contains 2 meningococcal genes NMB0102 and NMB0103 in opposite orientation. In between these two genes are: a *BamHI* recognition sequence, *cbbA* promoter, *BglII* recognition sequence, DNA uptake sequence (DUS), 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 3.3) was streaked onto, and grown overnight at 37°C in 5% ($^{v}/_{v}$) CO_2 on chocolate blood agar plates. A single colony from the overnight growth was inoculated into 5 ml brain heart infusion broth (BHIB) and grown at 37°C with shaking to an OD_{600} of 0.5. Aliquots of 20 µl of culture were spotted on BHI agar, allowed to air dry and grown for 100 min at 37°C in 5% ($^{v}/_{v}$) CO_2. Two micrograms of appropriate mutagenic plasmid DNA (in a final volume of 20 µl) was added to each culture spot on the BHI plates, allowed to absorb and grown for 4 h at 37°C in 5% ($^{v}/_{v}$) CO_2. Control plates received no DNA.

Bacterial growth was re-suspended in 1 ml BHIB with appropriate antibiotics (Table 3.3), and aliquots of either control or test culture were inoculated on BHI agar plates containing appropriate antibiotic and supplement (where appropriate). Plates were grown at 37°C in 5% ($^{v}/_{v}$) CO_2 for up to 48 h. Resulting colonies were screened by PCR.

Table 3.3: Mutagenic plasmids and antibiotics/supplement concentrations used in the mutagenesis of NMB0359 in *N. meningitidis*.

Mutagenic Plasmid	Plasmid Description	Target Strain	Antibiotic used for selection	Supplement
pIT4	Kanamycin resistance cassette and *rpsL*$^{+}$ allele from MC58 cloned into pIT3 at the NMB0359 deletion site	MC58 – WT	Kanamycin 50 µg/ml	Glutamine and/or Glutamate (5 mM – 100 mM).
pIT5	*glnA* from MC58 cloned into pComp-1	MC58 – WT	Erythromycin 5 µg/ml	N/A
pIT8	Kanamycin resistance cassette and *rpsL*$^{+}$ allele from MC58 inserted in NMB0359, cloned into pMOD-2	MC58 – WT	Kanamycin 50 µg/ml	Glutamine and/or Glutamate (5 mM – 100 mM).
pIT4	Kanamycin resistance cassette and rpsL+ allele from MC58 cloned into pIT3 at the NMB0359 deletion site	MC58 – WT - GlnA^{ect}	Kanamycin 50 µg/ml	Glutamine and/or Glutamate (5 mM – 100 mM).

3.2.10. Whole Cell Enzyme Linked Immununosorbent Assay (ELISA)

3.2.10.1. Preparation and Fixation of Whole Meningococcal Cells

N. meningitidis strains to be analysed were grown overnight on chocolate blood agar plates. A single colony of each strain was inoculated into 5 ml brain heart infusion broth and grown at 37°C with shaking to an OD_{600} of ≥0.5. The culture was centrifuged at 2087 *x g* for 10 min at 4°C. Supernatants were discarded and bacterial pellets were suspended in 10 ml of filtered PBS and washed. This washing step was repeated twice, before pellets were suspended in 10 ml of 0.5% ($^{v}/_{v}$) formaldehyde (EMS; 15710-S) in PBS and kept at 4°C for 30 min with gentle agitation.

Fixed bacteria were centrifuged at 2087 *x g* for 10 min at 4°C, washed with PBS and pellets suspended in 10 ml of sodium carbonate buffer (See Appendix). The $O.D_{600}$ of strains were checked and adjusted to 0.2 to ensure uniformity between strains.

3.2.10.2. Binding of Anti-rGlnA Antisera to Whole Meningococcal Cells

Microplate wells (Thermo Fisher Scientific, Nunc immobilizer amino 12 x 8 strips) were homogenously coated with 100 µl of the appropriate formaldehyde-fixed whole meningococcal cells in carbonate buffer. After overnight incubation with gentle agitation, wells were washed three times with 0.05% ($^{v}/_{v}$) Tween 20 in PBS and then 100 µl of 1% ($^{w}/_{v}$) BSA/PBS was added to each well to reduce non-specific binding and incubated for 2 h at room temperature. The washing step was repeated and 100 µl of rabbit anti-rGlnA antisera (diluted 1/2000 in 1% ($^{w}/_{v}$) BSA/PBS) was added and kept overnight at 4°C. Washing step was repeated, followed by the incubation with 100 µl of anti-rabbit IgG-alkaline phosphatase conjugate [Sigma;

diluted 1/30,000 in 1% ($^{w}/_{v}$) BSA/PBS] for 2h at room temperature, with gentle shaking. After a final washing step, 200 µl of 1 mg/ml phosphatase substrate (Sigma) in glycine buffer (See Appendix) was added to each well and absorbance was read at 405 nm using a Tecan spark 10M for > 2 h at constant temperature. Rabbit anti-rPorA antisera to detect PorA and rabbit anti-rPrx antisera to detect peroxiredoxin [(Orihuela et al., 2009) and (Aljannat et al., 2020) respectively] were used as positive controls. Likewise, rabbit antisera were replaced with 1% ($^{w}/_{v}$) BSA/PBS in order to measure non-specific binding.

3.2.11. Statistical Analysis

GraphPad Prism 7.04 was employed for statistical analysis. Samples were analysed in triplicates, and each experiment was repeated at least thrice. 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.

3.3. Results

3.3.1. Sequence Analysis of NMB0359 and Flanking DNA

Examination *in silico* was performed using the GenomeNet Database Resource. It was revealed that NMB0358 encoding shikimate dehydrogenase is located 300 bp upstream of NMB0359, and NMB0360 encoding an AmpG-like protein is located 156 bp downstream of NMB0359.

Using the Arnold online tool, a transcription termination sequence was predicted to be upstream of NMB0358, as well as downstream NMB0359, indicating that these three genes (NMB0358, NMB0359, and NMB0360) are likely to be independent.

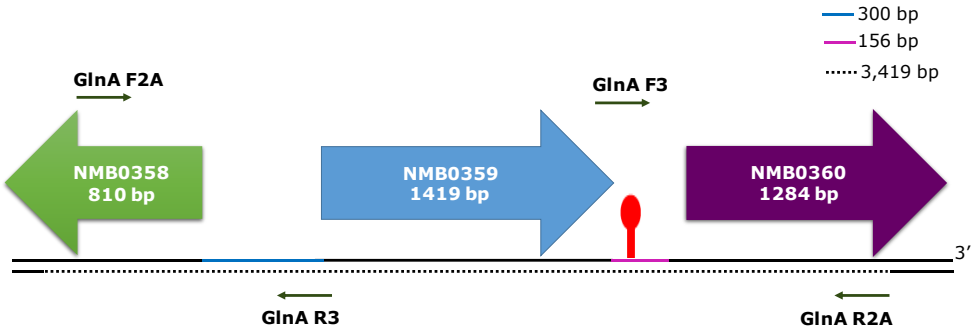


Figure 3.4: A schematic representation of NMB0359 in genomic context of *N. meningitidis* MC58, showing orientation of genes, predicted termination sequence (in red) downstream of NMB0359, and approximate binding sites of primers used to aid NMB0359 deletion.

3.3.2. Cloning of NMB0359 and Flanking DNA

NMB0359 (1419 bp), with 1 kb upstream and 1 kb downstream flanking DNA (UglnA) from *N. meningitidis* MC58 was successfully amplified by PCR (Figure 3.5 A). The PCR fragment was then ligated into the pGEM - T Easy vector

and *E. coli* JM109 strain was transformed with the resulting construct. Prospective clones were screened by blue/white screening.

Plasmid DNA from white colonies were further examined by restriction digestion and DNA sequencing. Digestion of the extracted plasmid DNA with *EcoRI* was predicted to produce three fragments of *ca.* 1.1 kb, 2.3 kb (corresponding the insert *ca.* 3.4 kb) and 3 kb corresponding to pGEM - T Easy.

Finally, the sequence of the cloned fragment was confirmed by DNA sequencing of one of these clones. The sequence was identical to that in the MC58 genome, and hence contained no PCR-generated errors. A pGEM–T-Easy containing UglnA PCR product was designated pIT2 (Figure 3.5 B, Lane 1).

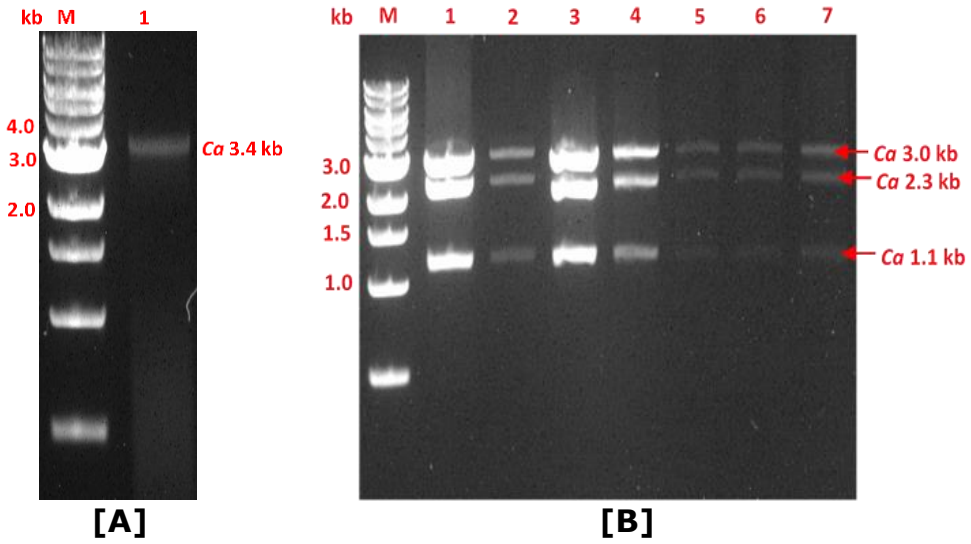


Figure 3.5: Agarose gel analysis showing; [A] PCR amplification of NMB0359 and 1 kb upstream and 1 kb downstream flanking DNA (*UglnA*) from *N. meningitidis* strain MC58. Lane M: 1 kb DNA ladder, lane 1: PCR product corresponding to *UglnA*. [B] Restriction digestion of plasmid DNA using *EcoRI* to verify the cloning of *UglnA* into pGEM - T Easy. Lane M: 1 kb DNA ladder, lane 1 - 7: Prospective pIT2 clones digested with *EcoRI.*

3.3.3. Deletion of NMB0359 by Inverse PCR

Serving as template DNA, pIT2 was amplified by inverse PCR to amplify the whole construct but excluding NMB0359, using primer pair GlnAF3/GlnAR3. A PCR product of *ca.* 5.1 kb, which is of the expected size of the combined NMB0359 flanking sequences (2.1 kb) and the pGEM–T-Easy vector (3 kb), was generated (Figure 3.6, A). This product designated *deglnA* was digested with *BamHI,* re-ligated and transformed into *E. coli* JM109 cells. Potential clones were screened by restriction digestion with *EcoRI* (Figure 3.6 B) and DNA sequencing. A correct clone was designated as pIT3.

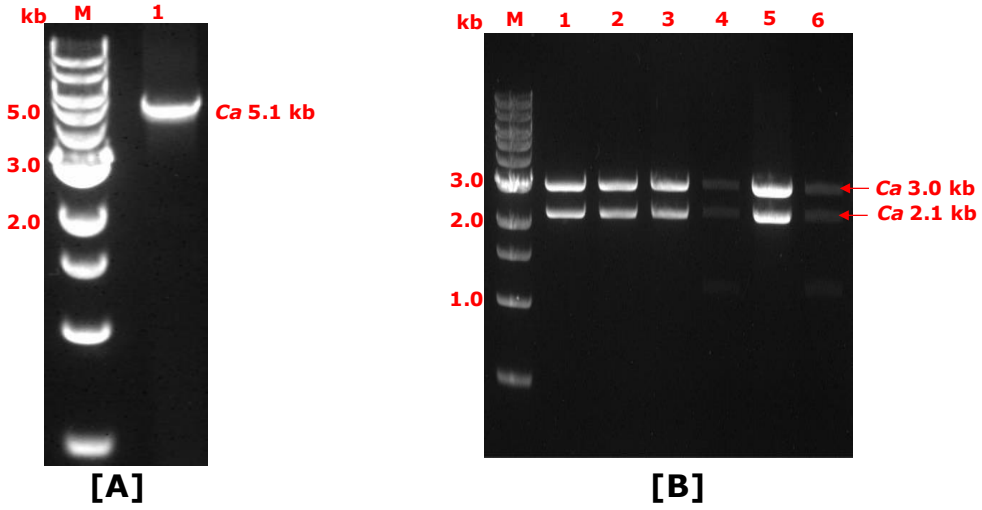


Figure 3.6: Agarose gel analysis showing (A) Inverse PCR was performed to delete NMB0359 DNA sequence from pIT2 to give product: deglnA. (B) [B] *EcoRI* digestion of pIT3 potential clones. Lane M: 1 kb DNA Ladder.

3.3.4. Preparation of Markers for Selection and Cloning into pIT3

Chromosomal DNA was extracted from *N. meningitidis* MC58 *rpsL*$^{-}$ *Kan*^{R} *rpsL*$^{+}$ and used to PCR amplify a DNA fragment spanning the kanamycin resistance cassette (*Kan*^{R}) and *rpsL*$^{+}$ genes (each preceded by its own

promoter). A PCR product of *ca.* 2 kb designated as *KsnL* was obtained (Figure 3.7) corresponding to *Kan*^{R} (1.5 kb) and *rpsL*$^{+}$ (0.5 kb). *KnsL* was digested with *BamHI* and ligated to *BamHI* digested pIT3.

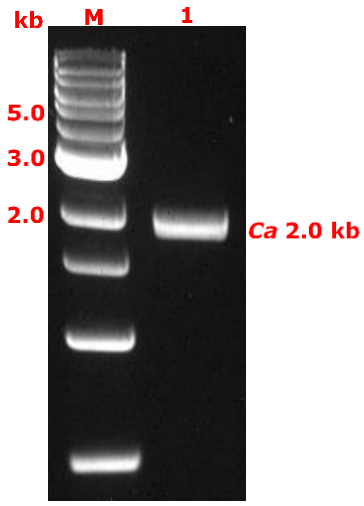


Figure 3.7: Agarose gel analysis showing *KnsL*. The kanamycin resistance cassette and *rpsL*$^{+}$ gene (collectively designated *KnsL*) from *N. meningitidis* MC58 *rpsL*$^{-}$ *Kan*^{R} *rpsL*$^{+}$ was PCR amplified. Lane M: 1 kb DNA Ladder.

3.3.5. Construction of pIT4 vector

The plasmid pIT4 was constructed by ligating *BamHI* digested pIT3 with *BamHI* digested *KsnL* and *E. coli* JM109 transformed with the ligation reaction to allow plasmid propagation. Potential cones were screened by kanamycin resistance and restriction digestion. Digestion of the extracted plasmid DNA with *EcoRI* was predicted to produce two fragments of *ca.* 4.1 kb, corresponding to the insert *i.e.* NMB0359 flanking DNA (2.1 kb) and *KsnL* (2 kb); *ca.* 3 kb corresponding to pGEM–T-Easy. Six of the seven analysed clones were identified to contain pGEM–T-Easy containing an insert of the correct size (Figure 3.8 A). Further confirmation was carried out by restriction digestion with *BamHI*, predicted to produce two fragments of *ca.* 5.1 kb, corresponding to the insert NMB0359 flanking DNA (2.1 kb) and

pGEM –T- Easy (3 kb); *ca.* 2 kb corresponding to the insert *KsnL*. The sequence of the cloned NMB0359 flanking DNA was confirmed by DNA sequencing. Plasmid DNA containing pGEM–T-Easy, *KsnL,* and NMB0359 flanking DNA was designated pIT4 (shown in Figure 3.8 B, lane 1).

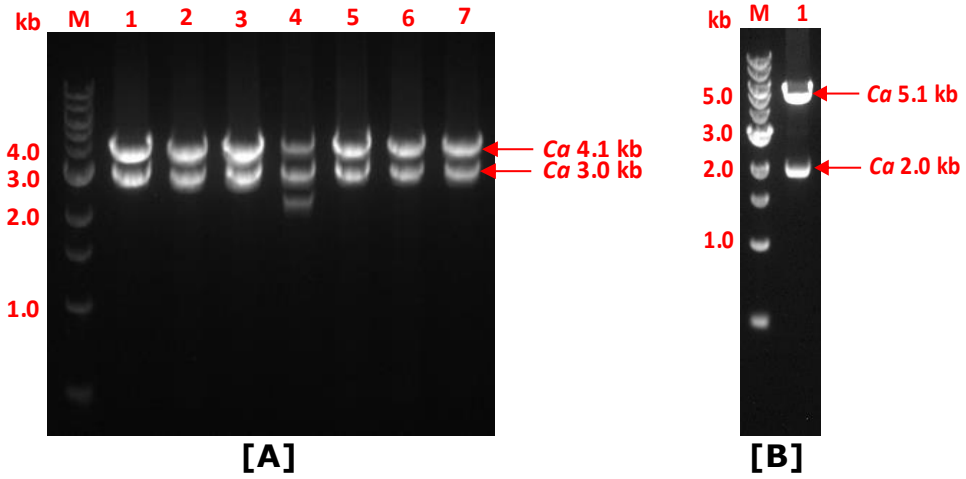


Figure 3.8: Agarose gel analysis showing (A) *EcoRI* restriction digestion of putative pIT4 clones. (B) *BamHI* restriction digestion of a correct pIT4 clone. Plasmid DNA extracted from each clone was digested with *EcoRI* to verify the cloning of NMB0946 flanking DNA and *KnsL* into pGEM–T-Easy. Seven clones were examined Lane M: 1 kb DNA ladder; lanes 1-7: putative pIT4. Further confirmation was carried out by DNA sequencing restriction digestion with *BamHI.*

3.3.6. Mutagenesis of NMB0359 in *Neisseria meningitidis* Wild-type (WT) with pIT4 Construct

The mutagenesis of NMB0359 in *Neisseria meningitidis* WT was firstly attempted by naturally transforming plasmid DNA of pIT4. It was expected that after transformation with pIT4 there would be recombination, and the double cross over recombinants would lose *glnA* (NMB_0359) which would be replaced by the 2 kb kanamycin resistance cassette, thus conferring kanamycin resistance on the deletion mutant.

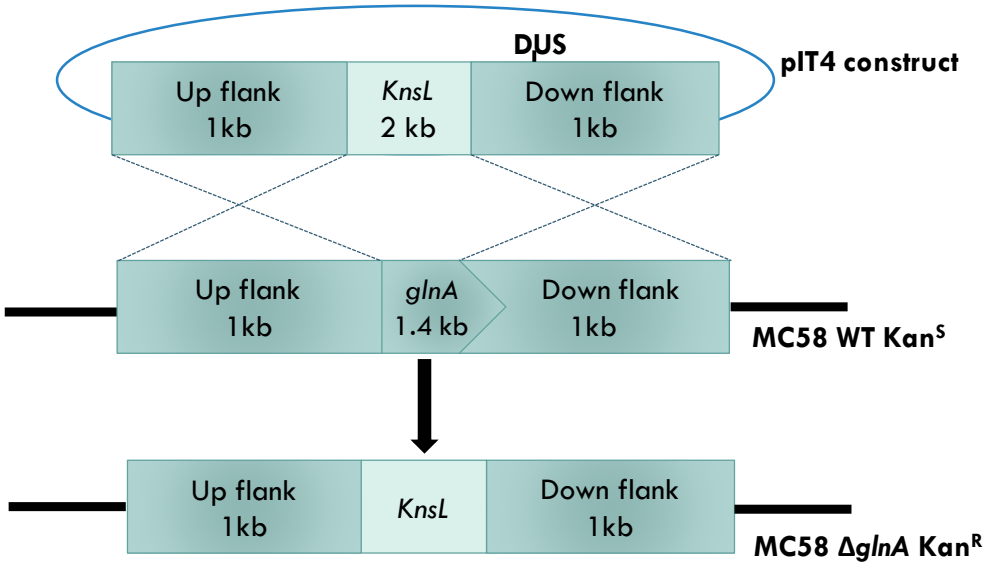
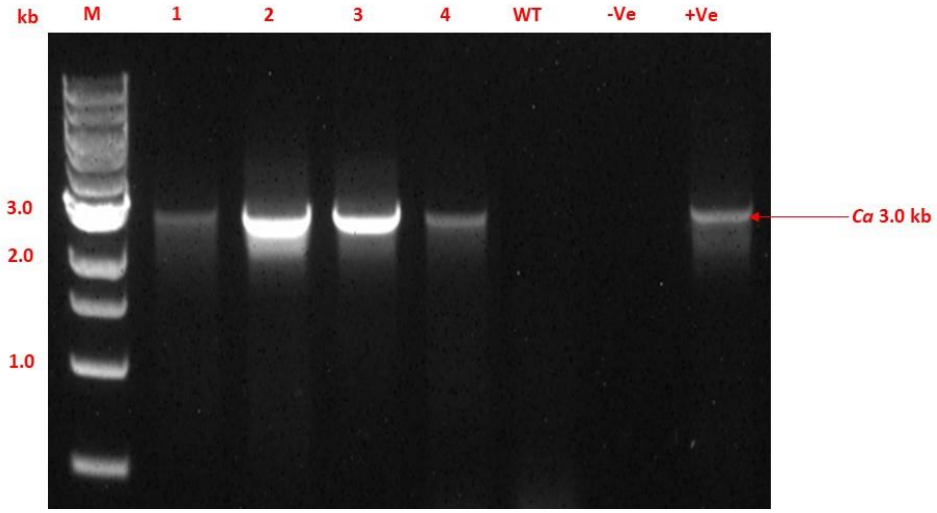


Figure 3.9: Schematic workflow of the first mutagenesis strategy that was adopted to delete NMB0359. A cassette consisting of a *KanR* gene plus an *rpsL*+ allele was cloned between the 1 kb up- and 1 kb downstream flanking DNA of NMB0359 to generate pIT4, which was used to transform the MC58 WT Kan^S strain. At this stage, it was expected that homologous recombination event would occur, resulting in the replacement of NMB0359 carried in the genome by the *KanR rpsL*+ cassette, and the resulting strain would be designated MC58 Δ*glnA* Kan^R which would be phenotypically resistant to kanamycin (Kan) by virtue of the presence of *Kan^R* gene.

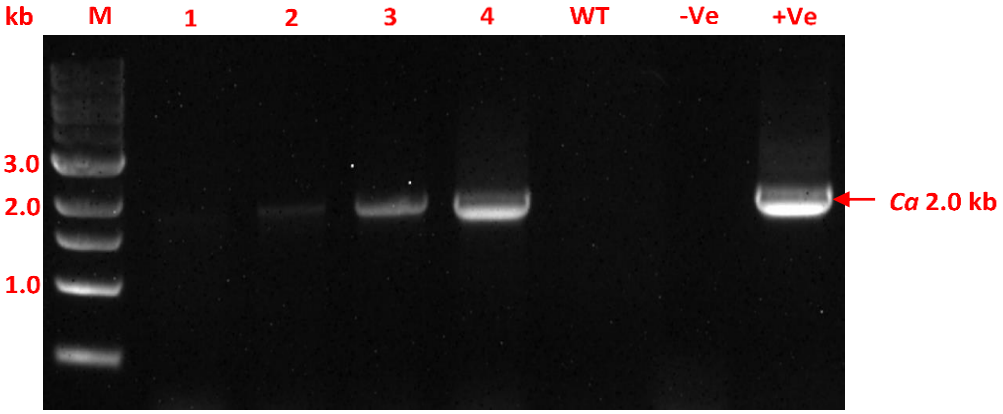
To confirm the success of this mutagenesis strategy, colony PCR using DNA from prospective mutants as template DNA was performed using four different primer pairs. Screening with primer pair GlnAF2A/KsnLR1 which would amplify the forward up flank to the reverse kanamycin resistance gene, it was expected to produce a fragment of *ca.* 3.0 kb (1.0 kb upstream flanking sequence with 2.0 kb kanamycin cassette). This was observed as shown in Figure 3.10 A. Screening with primer pair KsnLF1/KsnLR1 was expected to produce a fragment of *ca.* 2.0 kb corresponding to the kanamycin resistace gene with the *rpsl*$^{+}$ gene. This was observed in the clones analysed (Figure 3.10 B).

Further screening with primer pair GlnAF2A/GlnR2A to amplify forward up flank to reverse down flank. It was expected to produce a fragment of *ca.* 4.0 kb (1.0 kb up flank, 1 kb down flank and 2.0 kb kanamycin cassette), but upon screening, a band size of *ca* 3.4 kb was observed which would correspond to 2 kb of the upstream and downstream flank and 1.4 kb of *glnA,* indicating that although the clones had taken up the kanamycin resistant cassette (Figure 3.10 C), it had not replaced NMB0359 as expected.

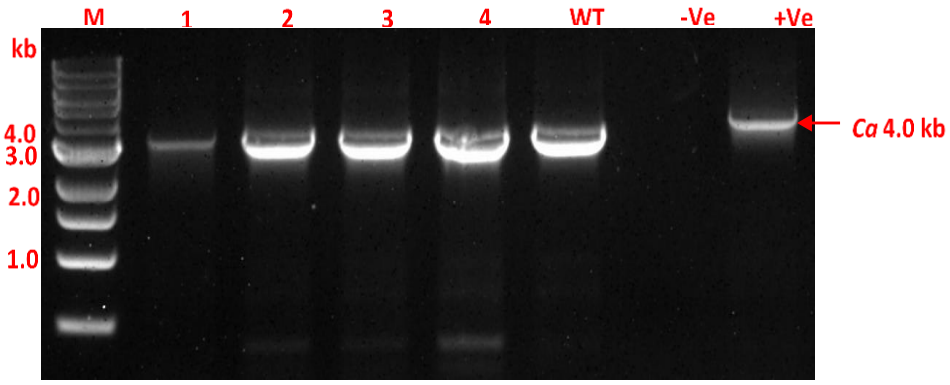
A final screening was performed using primer pair GlnAF1A/GlnAR1A which was expected to amplify the region containing *glnA* that should have been replaced by *KsnL* and was expected to produce a fragment of *ca.* 2.0 kb. Rather, a fragment of 1.4 kb corresponding to the size of *glnA* was observed (Figure 3.10 D), confirming the earlier result that clones contained the kanamycin cassette but NMB0359 had not been deleted from the genome.


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Ca 3.0 kb

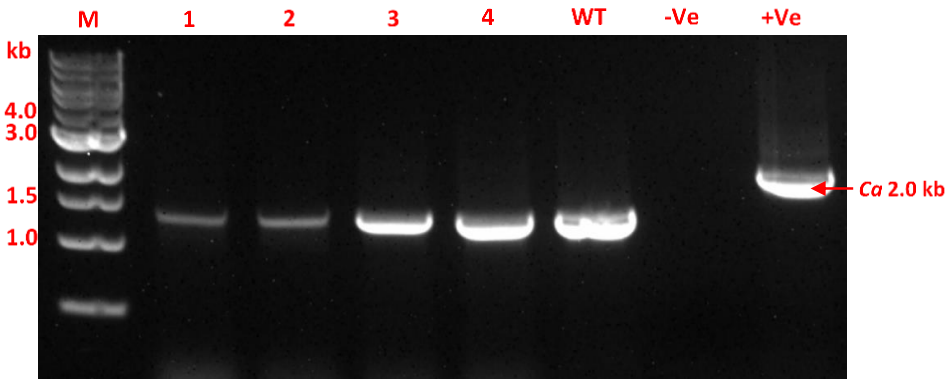
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[D]

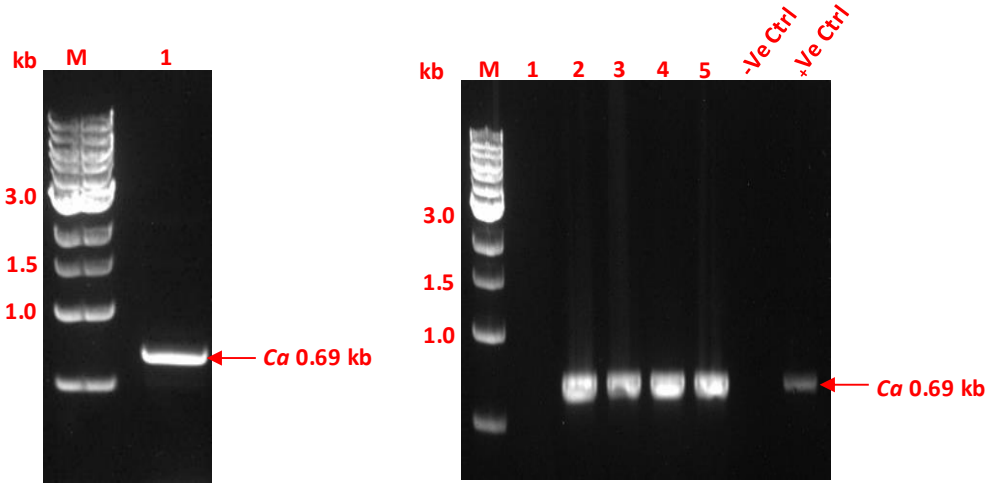
Figure 3.10: Screening of prospective clones by colony PCR using four different primer pairs. (A). GlnAF2A/KsnLR1. (B). KsnLF1/KsnLR1. (C). GlnAF2A/GlnR2A. (D). GlnAF1A/GlnAR1A. The PCR-mediated verification of NMB0359 mutagenesis using primer pairs GlnAF2A/KsnLR1 and KsnLF1/KsnLR1 confirmed the incorporation of the *KsnL* gene into the mutant strain genome, but the verification with primer pairs GlnAF2A/GlnR2A and GlnAF1A/GlnAR1A produced lower size bands of *ca.* 3.0 kb and 1.4 kb respectively, rather than the expected sizes of 4.0 kb and 2.0 kb respectively, indicating that although the mutants had incorporated the *KsnL* gene into the genome, it was not in place of *glnA* as intended.

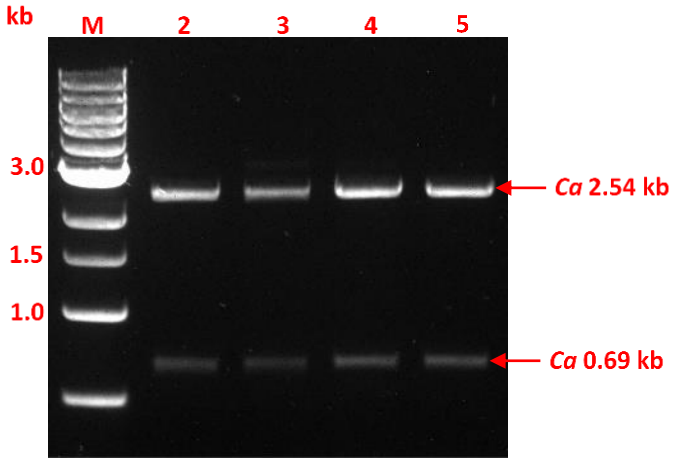
This strategy was repeated several times to delete NMB0359 from the MC58 WT-*Neisseria* genome but the same result was achieved, with the bacteria taking up the *KsnL* gene, but not in place of *glnA*. As a positive control in this study, the transformation protocol being employed was used to successfully delete a gene (peroxiredoxin) that has been shown to be knocked in MC58 (Aljannat et al., 2020) to confirm that the transformation system works.

3.3.7. Creation of inactivation of *glnA* construct in *E. coli*

Rather than gene deletion, another strategy to mutate a gene is by gene inactivation. This can be achieved by insertion of a fragment in the open-reading frame of gene to be mutated which prevents translation of the encoded protein. This method was employed as it provides a means for mutagenesis of a specific gene, without polar effects on downstream genes

To inactivate *glnA*, a kanamycin resistance cassette was inserted in the open-reading frame of the gene. The 5' region of the gene (nucleotides 1 - 690) was PCR amplified (Figure 3.11 A), digested with *SacI/KpnI,* ligated into the pMOD2 vector and transformed into *E. coli* JM109 cells. Prospective clones were screened by colony PCR using primer pair GlnAF1A/GlnAR1A which would amplify the *ca* 690 bp insert (Figure 3.11 B), restriction digestion with *SacI/KpnI,* predicted to produce two fragments of *ca.* 2.5 kb (corresponding to pMOD2 vector) and 690 bp (corresponding to the insert) as shown in Figure 3.11 C, and by DNA sequencing. A correct clone was designated as pIT6.





[C]

Figure 3.11: Cloning of the 5′ region (nucleotides 1 – 690) of *N. meningitidis glnA* into *E.coli.* (A) PCR amplification of glnA 1st half using primer pairs GlnAF1A/GlnAR1A (B) Screening of prospective clones by colony PCR using primer pairs GlnAF1A/GlnAR1A. (C) Restriction digestion of prospective clones with *SacI/KpnI*.

The 3′ region (nucleotides 691 – 1419) of *glnA* with the *Neisseria* DNA uptake sequence, was PCR amplified using primer pairs GlnAF1B/GlnAR1B (Figure 3.12 A), digested with *XbaI/SalI,* ligated into the pIT6 vector and transformed into *E. coli* JM109 cells. Prospective clones were screened by colony PCR using primer pair GlnAF1A/GlnAR1B which would amplify the whole *glnA ca* 1.4 kb (Figure 3.12 B), restriction digestion with *XbaI/SalI* predicted to produce two fragments of *ca.* 3.2 kb (corresponding to pMOD2 vector with nucleotides 1 – 690 of *glnA*) and 729 bp (corresponding to the insert) as shown in Figure 3.12 C, and by DNA sequencing. A correct clone was designated as pIT7.

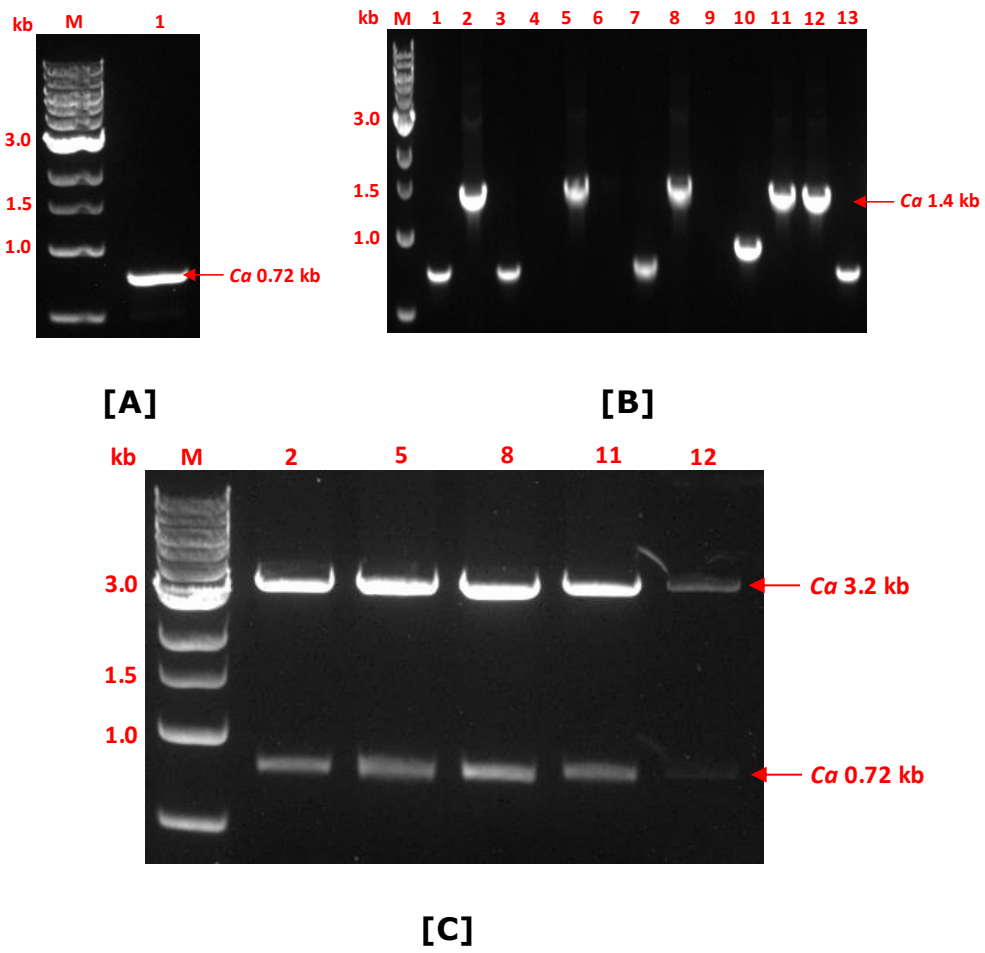


Figure 3.12: Cloning the 3' region (nucleotides 691 – 1419) of *glnA* with the Neisseria DNA uptake sequence into *E.coli.* (A) PCR amplification of nucleotides 691 – 1419 of *glnA* using primer pairs GlnAF1A/GlnAR1A (B) Screening of prospective clones by colony PCR using primer pairs GlnAF1A/GlnAR1B. (C) Restriction digestion of prospective clones with *XbaI/SalI*.

The *KsnL* cassette was PCR amplified (Figure 3.13 A), digested with *XmaI/BamHI,* ligated into the *XmaI/BamHI* digested pIT7 vector and transformed into *E. coli* cells. Prospective clones were screened by colony PCR using primer pair KsnLF1A/GlnAR1B which would amplify the *KsnL* with the nucleotides 691 – 1419 of *glnA ca* 2.8 kb (Figure 3.13 B) and restriction digestion with *XmaI/BamHI* predicted to produce two fragments of *ca.* 4.0 kb (corresponding to pMOD2 vector with *glnA*) and 2 kb (corresponding to

the insert *KsnL*) as shown in Figure 3.13 C, and by DNA sequencing. A correct clone was designated as pIT8.

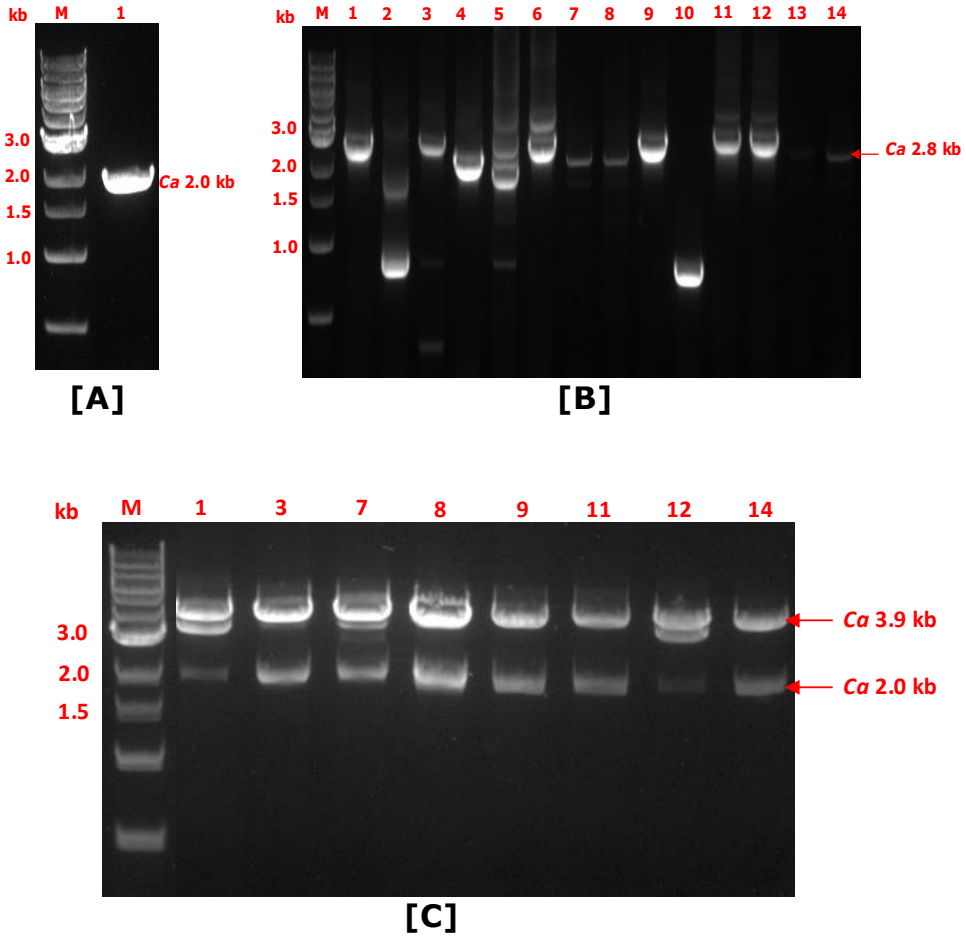


Figure 3.13: Cloning of *KsnL* of *N. meningitidis* into *E.coli.* (A) PCR amplification of *KsnL* using primer pairs KsnLF1A/KsnLR1 (B). Screening of prospective clones by colony PCR using primer pairs KsnLF1A/GlnAR1B. (C) Restriction digestion of prospective clones with *XmaI/BamHI*.

3.3.8. Mutagenesis of NMB0359 in *N. meningitidis* WT with pIT8 Construct

The second strategy to mutate NMB0358 was to naturally transform *Neisseria* with the gene inactivation construct pIT8. It was expected that after transformation with pIT8 there would be recombination, and the double cross over recombinants would have *KsnL* inserted in the open reading frame of *glnA*, thus conferring kanamycin resistance to the mutant and rendering *glnA* inactive.

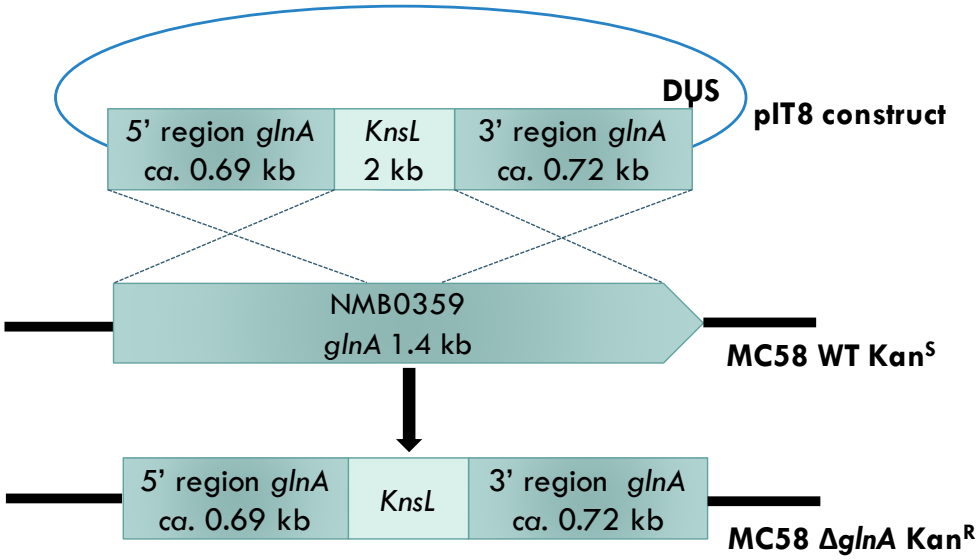
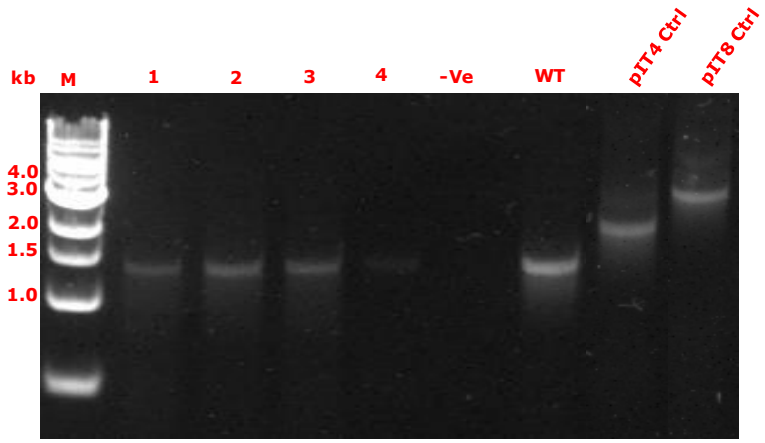


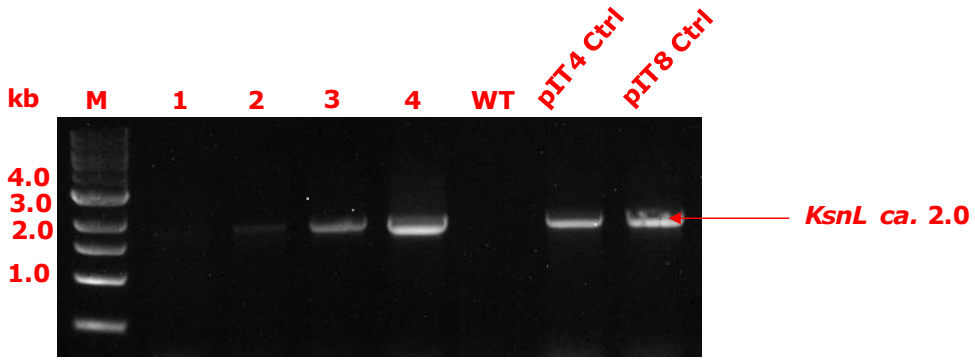
Figure 3.14: Schematic workflow of the second mutagenesis strategy that was adopted to delete NMB0359. A cassette consisting of a *KanR* gene plus an *rpsL*+ allele was cloned between the NMB0359 to generate pIT8, which was used to transform the MC58 WT Kan^S strain. At this stage, it was expected that homologous recombination event would occur, resulting in the insertion of the *KanR rpsL*$^+$ cassette in the *glnA* open reading frame. The resultant strain would be designated MC58 Δ*glnA* Kan^R which would be phenotypically resistant to kanamycin (Kan) by virtue of the presence of the *Kan*^{R} gene.

To confirm the success of this mutagenesis strategy, colony PCR with DNA from prospective mutants as template DNA was performed using two different primer pairs.

Screening with primer pair GlnAF1/GlnAR1 which would amplify the *glnA* open reading frame containing *KsnL*, and expected to produce a fragment of *ca.* 3.4 kb. Rather, a fragment of *ca.* 1.4 kb corresponding to the wild-type *glnA* was observed (Figure 3.15 A). Further screening of the colonies with KsnLF1A/KsnLR1 was expected to produce a fragment of *ca.* 2.0 kb corresponding to *KsnL*, this band was observed, indicating that the clones had incorporated the *KsnL* within the genome, but not within the open reading frame of *glnA* (Figure 3.15 B).



[A]



[B]

Figure 3.15: Screening of prospective clones by colony PCR using two different primer pairs. (A). GlnAF1/GlnAR1. (B). KsnLF1/KsnLR1. The PCR-mediated verification of *glnA* inactivation using primer pairs GlnAF1/GlnAR1 showed a smaller band size of *ca.* 1.4 kb corresponding to the WT *glnA* rather than the expected size of *ca.* 3.4 kb corresponding to the *KsnL* gene inserted in the open reading frame of *glnA.* Using primer pairs KsnLF1/KsnLR1, it was confirmed that the *ca.* 2.0kb *KsnL* gene been incorporated into the genome.

This strategy was repeated several times to delete NMB0359 from the MC58 WT-*Neisseria* genome but as with the initial approach using the pIT4 construct, the bacterial clones analysed did not lose *glnA*.

3.3.9. Creation of an Ectopic Complementation Plasmid

Due to the inability to mutate NMB0359 in *Neisseria,* the strategy of complementation first was adopted. That is, a second copy of *glnA* was PCR amplified (Figure 3.16 A), ligated into the complementation vector, and transformed into *E.coli* JM109 cells ready for subsequent transformation into MC58 WT. Prospective clones were screened by restriction digestion and DNA sequencing. A correct clone was designated as pIT5 (Figure 3.16 B).

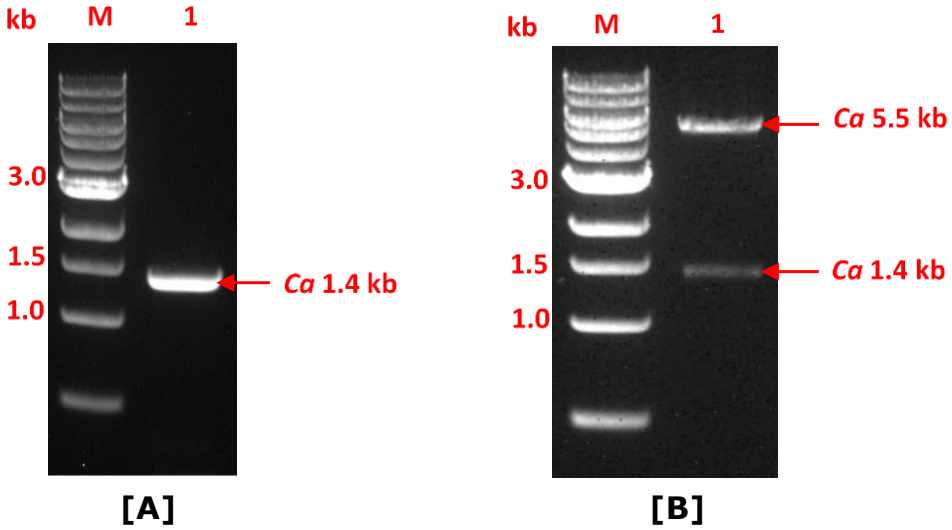
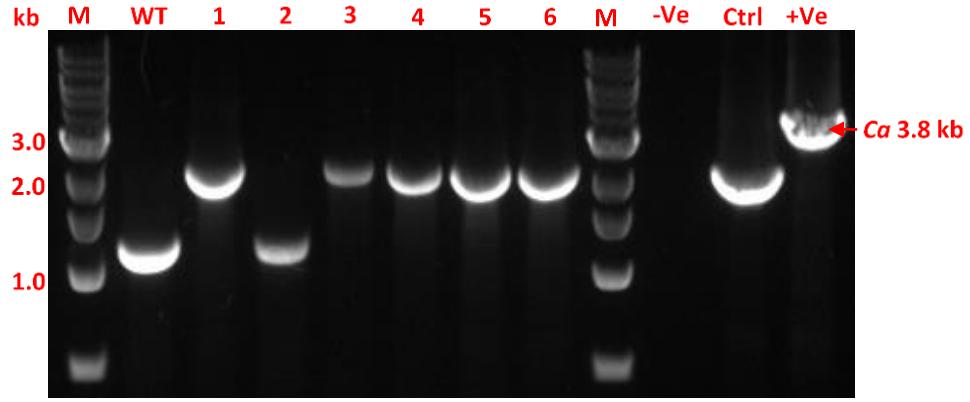


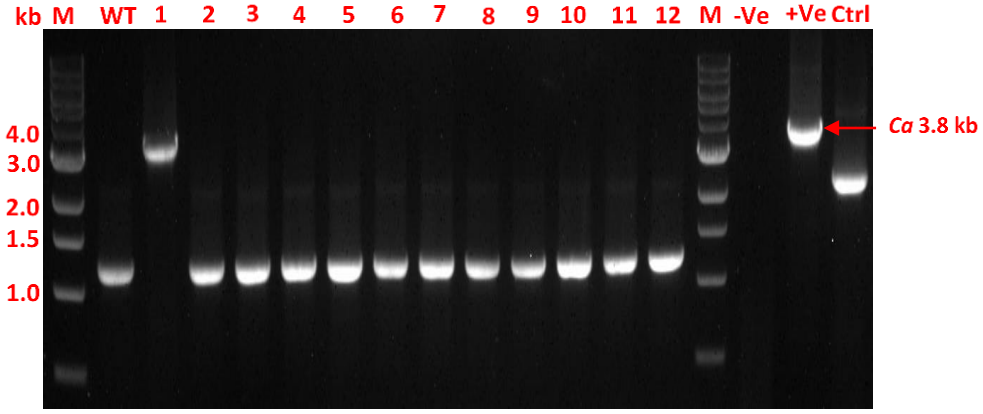
Figure 3.16: Agarose gel analysis showing (A) PCR amplification of *glnA* from the *Neisseria* genome using primer pair GlnAF5/GlnAR5. The amplicon was ligated into pCOMP-1 and transformed into *E. coli.* A correct clone was designated as pIT5 (B) *BglII* restriction digestion of correct pIT5. Plasmid DNA extracted from a correct clone was digested with *BglII* to verify the cloning of *glnA* into pCOMP-1. Further confirmation was carried out by DNA sequencing. Lane M: 1 kb DNA ladder; lane 1A: NMB0359, lane 1B: pIT5 digested with *BglII.*

3.3.10. Complementation of NMB0359 in *Neisseria meningitidis* WT

To introduce a second copy of *glnA* into the *Neisseria* genome at an ectopic site (in between meningococcal genes NMB0102 and NMB0103), plasmid DNA from construct pIT5 was naturally transformed into MC58 WT as described previously (section 3.2.9). Positive transformants were screened by erythromycin resistance and colony PCR with primer pairs NMB0102F1/NMB0103R1, which would amplify the 5' region of NMB0102 to the 3' region of NMB0103. A band size of *ca.* 3.8 kb was expected, but on analysis of several colonies, only one colony showed this (Figure 3.17 B, lane 1), with other colonies still showing either the WT *glnA* size (*ca.* 1.4 kb) or pIT5 construct size (*ca.* 2.4 kb), see figure 3.17 A. Finally, the sequence of the clone was confirmed by DNA sequencing, and designated as MC58 WT-GlnA^{ect}.



[A]



[B]

Figure 3.17: Agarose gel analysis showing colony PCR to amplify from NMB0102 – NMB103 using primer pair NMB0102F1/NMB0103R1. Only one colony (Figure B, lane 1 showed expected band size of *ca.* 3.8 kb. The DNA sequence of strain at the complementation locus was further confirmed by DNA sequencing and the strain designated as MC58 WT-GlnA^{ect}.

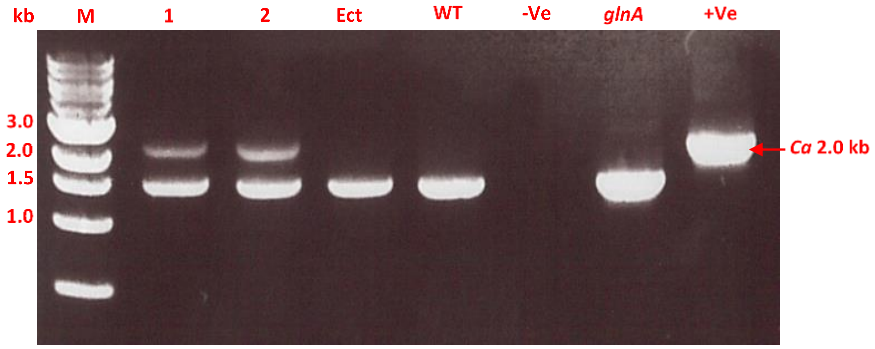
3.3.10.1. Deletion of NMB0359 in MC58 WT-GlnA^{ect}.

Due to the inability to mutate NMB0359 in the WT strain, the strategy to insert a second copy of *glnA* in trans, to create the MC58 WT-GlnA^{ect} strain and then proceed to delete *glnA* in cis, was adopted. The MC58 WT-GlnA^{ect} strain was naturally transformed with plasmid DNA from the deletion mutant plasmid (pIT4) as described in previous sections. Prospective mutants were screened by resistance to kanamycin, as well as colony PCR using four

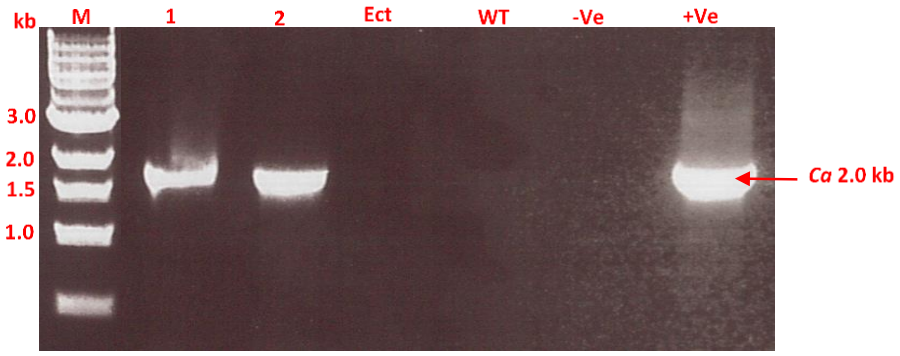
different primer pairs. PCR using primer pair GlnAF1/GlnAR1 was expected to produce two fragments of size *ca.* 1.4 kb and 2.0 kb corresponding to the primers amplifying *glnA* in trans and *glnA* in cis replaced by the *KsnL* gene respectively. The colonies analysed showed this expected result (Figure 3.18 A).

Further analysis of these colonies using primer pair KsnLF1/KsnLR1 which would amplify the *KsnL* cassette that should have replaced *glnA* in cis showed the expected fragment size of *ca.* 2.0 kb (Figure 3.18 B). *KsnL* would confer kanamycin resistance to the mutants and is absent in the WT and WT-GlnA^{ect} strains. Screening with primer pair GlnAF2A/KsnLR1 which would amplify the downstream flanking DNA of *glnA* in cis to the *KsnL* gene was expected to produce a fragment of size *ca.* 3.0 kb was also observed in the mutants (Figure 3.18 C).

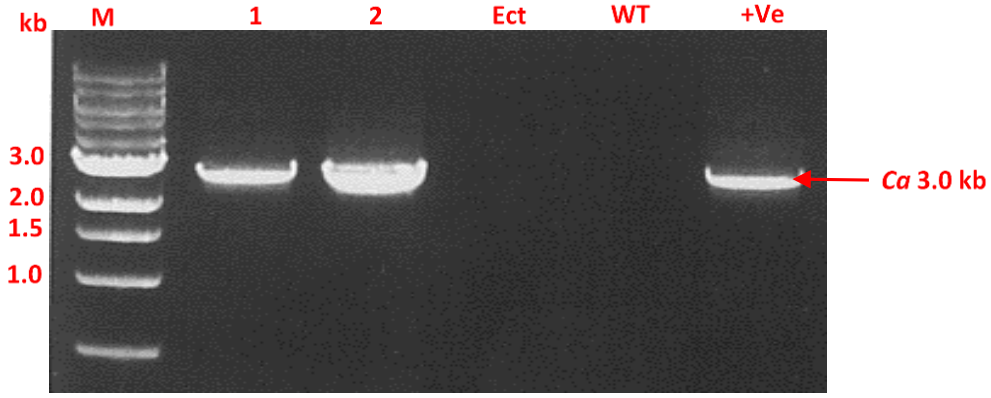
A final colony PCR screening with primer pairs GlnAF2A/GlnAR2A which would amplify the *glnA* in cis downstream flanking sequence to the upstream flanking sequence was expected to produce a fragment of size *ca.* 4.0 kb but rather, a fragment size of *ca.* 3.4 kb was observed. This size corresponds to the WT and WT-GlnA^{ect} strains (Figure 3.18 D). This result is consistent with results in the earlier section which showed that although the putative mutant strains incorporate the kanamycin resistance cassette, deletion of *glnA* was not successful. Several attempts were made to delete *glnA*, but all were not successful.


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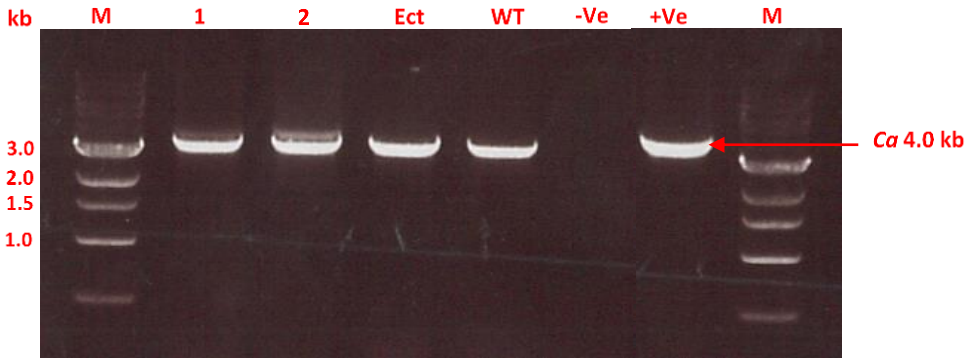
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[D]

Figure 3.18: Screening of putative clones by colony PCR using four different primer pairs. (A). GlnAF1/GlnAR1. (B). KsnLF1/KsnLR1. (C). GlnAF2A/KsnLR1. (D). GlnAF2A/GlnAR2A. The PCR-mediated verification of the in cis deletion of *glnA* using primer pairs GlnAF2A/KsnLR1 and KsnLF1/KsnLR1 confirmed the incorporation of the *KsnL* gene into the mutant strain genome. The verification with primer pair GlnAF1A/GlnAR1A produced the expected two bands of *ca.* 1.4 kb and 2.0 kb corresponding to *glnA* in trans and *glnA* in cis replaced by the *KsnL* gene respectively. The final verification with primer pair GlnAF2A/GlnAR2A produced a band size of *ca.* 3.4 kb, rather than the expected size of 4.0 kb, indicating that although the mutants had incorporated the *KsnL* gene into the genome, it was not in place of *glnA* as intended.

3.3.11. Investigation of GlnA Surface Localization in MC58 WT and WT - GlnA^{Ect}

Whole cell ELISA was performed to determine if GlnA was exposed on the surface of intact *N. meningitidis* MC58 WT and WT-GlnA^{ect} cells. Meningococcal cells were grown to mid log phase and the wells of a microtiter plate were coated with either WT or WT-GlnA^{ect} meningococcal whole cells, and incubated with rabbit anti-GlnA polyclonal antibodies (rαGlnA).

As a positive control, meningococcal cells were also treated with rabbit anti-rPorA polyclonal antisera to detect PorA, a well characterised outer membrane porin of *N. meningitidis*, and to also provide evidence that intact rather than lysed meningococcal cells were present in the assay. As an additional positive control, meningococcal cells were treated with rabbit anti-rPrx antisera to detect peroxiredoxin, a cytosolic protein shown to also be localised in the outer membrane of *N. meningitidis*.

As a control, meningococcal cells were treated with BSA rather than polyclonal anti-sera to measure non-specific binding.

All wells were incubated with secondary antibody (anti rabbit IgG) linked to alkaline phosphatase. Colour was developed by adding substrate for alkaline phosphatase, and absorbance read at 405 nm for 30 cycles at 15 min intervals. Data collected at cycle 7 (time point 105 minutes) and analysed using GraphPad Prism 7 application.

The results obtained (Figure 3.19) show that compared with BSA, rαGlnA serum detected a significant amount of glutamine synthetase (*p* value < 0.0001) on the surface of whole WT and WT-GlnA^{ect} meningococcal cells (strain MC58). The positive control assays also showed that significant amount of PorA and peroxiredoxin were bound significantly by rαPorA and rαPrx respectively.

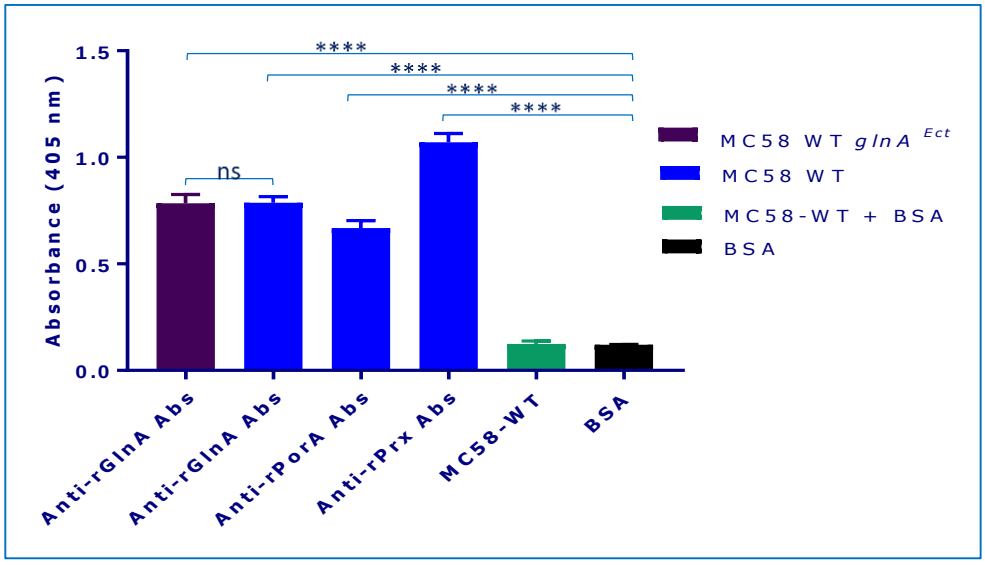


Figure 3.19: Data representation the binding of rαGlnA (1:2000 diluted in 1% BSA/PBS), rPorA (1:50), and rPrx (1:2000) to intact meningococcal cells (WT and WT-GlnA^{ect}). A significant amount of rαGlnA antibodies bound whole meningococcal cells, suggesting that significant amount of corresponding target antigen *i.e.* GlnA may be exposed on the bacterial surface. Error bars denote SE of the mean. Experiments were repeated three independent times and run in triplicate. Unpaired t-test analysis between either WT or WT-GlnA^{ect} and the control well (BSA) showed significant difference ****$p<0.0001$. Unpaired t-test analysis between WT and WT-GlnA^{ect} wells showed no significant difference.

3.4. Discussion

This chapter describes the different attempts at the mutagenesis of *glnA* in *N. meningitidis*. Mutation of the gene of interest was important as the mutant strains were to be used in functional characterisation of GlnA in a number of *in vitro* and *in vivo* experiments. The enzyme glutamine synthetase has not been extensively studied in *N. meningitidis,* thus the majority of the methods and procedures employed in its investigation in this study were based on research on GlnA in other pathogens, and/or research on other meningococcal enzymes/proteins.

Conclusive evidence on whether GlnA is an essential or non-essential enzyme in *N. meningitidis* was unavailable at the time of this study, thus the mutagenesis strategies were designed on the assumption of both instances.

On the assumption that meningococcal GlnA was not an essential protein, the methods of mutagenesis by gene deletion and gene activation were employed. Chandra and colleagues investigated the contribution of *glnA* to cell wall resistance and pathogenicity in *Mycobacterium bovis*, using the direct deletion strategy (Chandra et al., 2010). The mutagenic plasmid was created by inverse PCR and *M. bovis* was transformed with the plasmid DNA, resulting in the successful deletion of *glnA* from the genome.

Similarly, to investigate the contribution of glutamine synthetase to virulence, a mutant strain with *glnA* deleted was constructed by homologous replacement with a spectinomycin resistance cassette in the pathogen *Streptococcus suis* serotype 2 (Si et al., 2009). Kloosterman and colleagues successfully mutated *glnA* in *Streptococcus pneumoniae* by deleting the open reading frame of *glnA* (Kloosterman et al., 2006). An in-frame deletion (90 bp) within *glnA* mutant was successfully created by Klose and colleague in the pathogen *Salmonella typhimurium* when investigating the contribution of GlnA to virulence (Klose and Mekalanos, 1997).

Alternatively, to mutate *glnA*, rather than deleting the gene, the method of insertion in the open reading frame of the gene can be employed to render the gene inactive. This disruption method was adopted by Tullius and colleagues where a kanamycin resistance cassette was inserted at a unique site in the open reading frame and in the same orientation to *glnA*, to inactivate the gene in the pathogen *Mycobacterium tuberculosis* (Tullius et al., 2003).

As with research on mutagenesis of *glnA* in other pathogens, these strategies of either deletion or disruption were employed to mutate *glnA* in *N. meningitidis*. Several attempts were made to create double cross-over mutants in a single selection, but all proved unsuccessful. Rather than replacement of *glnA* with the kanamycin resistance cassette, the mutants obtained in this study incorporated the kanamycin resistance cassette but retained the gene, indicating that these mutants were possibly single cross-over recombinants rather than the double cross-over recombinants expected. Tullius and colleagues were also unable to generate a double cross-over recombinant in a single selection in *M. tuberculosis*. They employed a two-step protocol where the initial transformants were selected at a temperature and were re-plated on selective agar plates and grown for up to three weeks at a higher temperature than the initial selection to facilitate the second homologous cross over event (Tullius et al., 2003).

Glutamine synthetase is responsible for the reversible formation of glutamine from glutamate. The glutamine produced by GlnA is important for the synthesis of proteins; it also acts as a nitrogen donor for many reactions in the cell, and its amide nitrogen is also donated to synthesize many essential metabolites (Kumada et al., 1993, Chandra et al., 2010, Capel et al., 2016). Glutamine synthetase is clearly important to these organisms and thus deletion of this gene from the genome would make the organism

auxotrophic. As expected, researchers who have successfully mutated *glnA* have supplemented the agar/broth used for growing the mutants with glutamine. Most studies supplemented agar/broth with glutamine between 2 mM – 20 mM. Likewise in this study, growth media were supplemented with glutamine up to 100 mM. With our knowledge that GlnA catalyses a reversible reaction (conversion of glutamine to glutamate), growth media were supplemented with up to 100 mM glutamine, glutamate, or both. Regardless of the concentration of supplement(s) added to the growth media, attempts to achieve double cross-over recombinants were still unsuccessful.

Due to our inability to successfully mutate *glnA* by deletion or inactivation, we attempted mutation on the assumption that *glnA* is an essential enzyme to the pathogen. Reports by Capel and colleagues have shown that the involvement of GlnA in glutamate metabolism which is directly linked to the tricarboxylic acid (TCA) cycle make this enzyme possibly essential (Capel et al., 2016).

To mutate a gene that could be essential for growth, the strategy of complementation first is usually employed. In this study, a second copy of *glnA* was introduced at an ectopic site (between meningococcal genes NMB102 and NMB103). On successful creation of the WT-GlnA^{ect} strain containing two copies of *glnA*, attempts were then made to delete the cis copy of *glnA* (NMB0359). As with the initial deletion strategy, double cross over recombinants could not be obtained perhaps indicating that the ectopic copy of *glnA* was not being expressed at high enough levels (or at all) to enable mutagenesis of the native locus.

The surface localisation of MC58 glutamine synthetase was also examined. Moonlighting proteins have been described as proteins with the ability to perform other functions asides its known primary function, which

are usually unrelated. Also, these additional or supplementary functions usually occur in a different location from that which the protein is normally found (Jeffery, 1999). Functions of a moonlighting protein also differ as a result of changes in cell type, oligomeric state, or the cellular concentration of a ligand, substrate, cofactor or product (Jeffery, 1999).

Cytoplasmic enzymes play essential roles in DNA replication, metabolism, and protein biosynthesis and folding amongst other functions which occur within the cell. But studies have shown that these proteins can also be released into the supernatant in both Gram-positive and Gram-negative bacteria as well as in eukaryotes (Jeffery, 2015, Ebner and Götz, 2019).

Many cytoplasmic enzymes have been described as moonlighting proteins such as phosphoglucose isomerase, fructose bisphosphate aldolase, and glyceraldehyde-3-phosphate dehydrogenase (Jeffery, 1999, Pancholi and Chhatwal, 2003). Meningococcal moonlighting proteins identified to date include GAPDH, enolase, FBA, peroxiredoxin, and DnaK (Grifantini et al., 2002, Knaust et al., 2007, Tunio et al., 2010a, Tunio et al., 2010b, Shams et al., 2016, Aljannat et al., 2020).

Studies by Knaust and colleagues showed that whole meningococcal cells were able to bind surface associated plasminogen using flow cytometry and immunogold labelling (Knaust et al., 2007). Their study showed that whole meningococcal cells were able to bind plasminogen at the cell surface via 6 different meningococcal proteins; three of which were identified as DnaK, enolase, and peroxiredoxin with the other three being unidentified. GAPDH, encoded by *GapA-1,* was shown to be accessible to specific antibodies on the surface on whole meningococcal cells using flow cytometry (Grifantini et al., 2002). Similarly, Tunio and colleagues showed that GapA-

1 is also surface accessible to specific antibodies on the surface on un-encapsulated whole meningococcal cells (Tunio et al., 2010a).

We undertook to investigate if the cytoplasmic enzyme GlnA was accessible to specific antibodies at the cell surface in *N. meningitidis* by the whole cell ELISA method due to its high specificity and sensitivity, using the rabbit anti-rGlnA antiserum described in chapter 2. Intact whole meningococcal cells (MC58 WT and WT-GlnA^{ect}) were investigated for GlnA surface exposure with BSA acting as a control. The results showed that GlnA may be present at the surface of MC58 WT and WT-GlnA^{ect} cells, suggesting that GlnA like some other meningococcal cytosolic enzymes, may possibly be surface localised and could be a moonlighting protein carrying out other functions at the cell surface. Due to the inability to create the deletion mutant which would serve as a definitive negative control, this hypothesis could not be validated. But if the mechanism leading to the export of GlnA to the cell surface can be identified, that system could be knocked out and ELISA performed to confirm GlnA absence at the cell surface. As GlnA would be expressed within the cytoplasm, the strain may be viable, but GlnA would be unable to get to the bacterial surface, which could be confirmed by ELISA using anti-GlnA.

CHAPTER 4: Pilot Studies to Try and Identify Human Serum and Cellular Proteins Interacting with Meningococcal rFBA and rGlnA.

4.1. Introduction

As highlighted previously, a moonlighting protein has more than one function/role, with these multiple function(s) generally occurring in a different location from that in which the protein is usually found.

From section 3.3.10 of this study, it was indicated by the ELISA data that GlnA could possibly be found at the bacterial cell surface. The primary function of GlnA is within the cytoplasm where it is involved in nitrogen metabolism, as such it was important to investigate possible moonlighting functions the enzyme may be carrying out at the bacterial cell surface.

During infection, bacteria are exposed to proteins/cells in the host such as plasma and serum proteins, as well as endothelial and epithelial cells, and this has been shown to be a way by which adherence, colonization and invasion may be achieved.

N. meningitidis has been shown to bind plasminogen through plasminogen receptors. Plasminogen (plg) is a 92- kDa plasma glycoprotein proenzyme that undergoes posttranslational modifications resulting in several different forms, but the circulating mature form is known as glu-plasminogen (glu- plg), reflecting the presence of an N- terminal glutamic acid residue. Glu- plg is able to interact competitively with binding partners such as fibrin and cell surface receptors. This binding enables glu- plg to assume an open conformation, exposing an activation loop to cleavage by activators, such as tissue- type plasminogen activator (tPA) or urokinase-type plasminogen activator (uPA), to form plasmin (Lijnen, 2001). Plasmin is a serine protease which plays a crucial role in homeostasis through its degradation of fibrin clots; cleaving of extracellular matrix proteins such as fibronectin, laminin and vitronectin (Plow et al., 1995, Lijnen, 2001). Plasmin can also activate collagenases, matrix metalloproteinases and latent macrophage elastase, resulting in the cleavage of collagen, proteoglycans,

and elastin respectively (Murphy et al. 1999). Plasmin has also been shown to be a strong inhibitor of the complement components C3 and C5 (Barthel et al., 2012). There are two ways by which bacteria can interfere with and manipulate the plg system. The first is by producing activators of plg, and secondly by immobilizing plg (either directly or indirectly) on the bacterial cell surface, which may then be activated to plasmin by activators (Sanderson-Smith et al., 2012). The sequestering of plg by bacteria contributes to processes such as the ECM degradation, fibrinolysis, degradation of immune effectors and adherence, thus elevating host colonization and dissemination (Bhattacharya et al., 2012).

Previously studied meningococcal plasminogen receptors include DnaK, enolase, peroxiredoxin, and FBA (Knaust *et al*, 2007, Shams *et al*, 2016). Once *N. meningitidis*-associated plasminogen becomes activated by urokinase, it becomes functional and the bacteria are bestowed with the ability to degrade fibrinogen and other host proteins, thus enhancing virulence properties such as adhesion or invasion.

It has also been shown that bound plasmin is involved in the proteolytic cleavage of antibodies and complement components (Sanderson-Smith *et al*, 2012). Inactivation of immune defence tools via surface bound plasmin may contribute to the survival of *N. meningitidis* in its human host.

GAPDH is present on the surface of several microbial pathogens and may facilitate their colonization and invasion of host tissues by interacting directly with host soluble proteins and surface ligands. During meningococcal infection, Tunio and colleagues also demonstrated that GapA-1 can also be surface localised, and plays a vital role in the ability of *N. meningitidis* to adhere to human epithelial and endothelial cells (Tunio et al., 2010a). Their study showed that a GapA1 deficient mutant exhibited a significant reduction

in the ability to adhere to monolayers of HBME cells compared to the parent strain, in a capsule-independent process.

For IMD to occur, meningococci adhere to host epithelial cell, invade the sub-epithelial tissues and may gain access to the bloodstream where they may survive, multiply rapidly and adhere to host endothelial cells, leading to vascular colonization. Therefore, this chapter focuses on identifying human proteins from blood plasma, epithelial and endothelial cells that interact with recombinant GlnA or FBA in order to determine possible moonlighting functions of these proteins that may play a role in the pathogenesis of meningococcal infection

4.2. Materials and Methods

4.2.1. Expression of rFBA, and rGlnA

E. coli carrying the appropriate plasmid either pSAT1 (provided by Dr S. Tunio) for the expression of rFBA or pIT1 (this study) for the expression of rGlnA were cultured as described in chapter 2.2.11. Cells were induced with 1 mM IPTG and grown for 4 h, harvested by centrifugation and stored at -80°C until further use.

4.2.2. Collection of Blood and Separation of Plasma from Whole Blood

Whole blood samples (10 – 12 ml) were collected from five consenting adults into EDTA treated vacutainers. Cells were removed from plasma by centrifugation for 10 min at 1500 x *g* at 4°C. The plasma (supernatant) was aliquoted as 500 µl volumes into cryogenic tubes, labelled appropriately and stored at -80°C until further use.

4.2.3. Purification of rFBA and rGlnA Under Native Conditions

Recombinant His-tagged rFBA and rGlnA were purified under native conditions as described in section 2.2.12. Fractions containing the respective purified proteins were pooled together and either desalted into 20% $^{v}/_{v}$ glycerol in PBS and stored for future pull down assay experiments, or His-tag tethered onto the purification column and used for pull down assays.

4.2.4. Recombinant Proteins Pull Down Assays with Blood Plasma under Native Conditions

Blood plasma (1 ml) was passed slowly into the purification column containing non-eluted purified protein (from section 4.2.3), or purified and desalted proteins were re-tethered onto the purification column using a peristaltic pump, and 1 ml of blood plasma passed slowly through the column using a peristaltic pump at a flow rate of 0.1 ml/min to allow enough time for plasma proteins to interact with the either rFBA or rGlnA in the column. The column was transferred onto the AKTA prime machine, and 50 ml of non-denaturing buffer containing high salt and imidazole (see section 2.2.12) was passed through the column to release bound proteins from the column via a gradient elution method. Fractions corresponding to peaks observed in the chromatograph were collected in 0.5 ml volumes, and analysed using SDS-PAGE and western blot, with the focus on fractions containing the His-tagged recombinant proteins.

As a control, 1 ml of plasma was passed into the purification column without any recombinant protein tethered, elution was performed over a gradient using 50 ml of buffer. All fractions were collected and analysed by SDS-PAGE.

4.2.5. Denaturation of purified rGlnA

Either non-eluted recombinant GlnA purified under native conditions or previously purified rGlnA was re-tethered to the purification column, and 10 ml of 8M Urea solution was passed through the column at a flow rate of 1 ml/min to denature the recombinant protein. The column containing the denatured protein was washed extensively with distilled water to remove the denaturant from the column before being used for subsequent pull down experiments

4.2.5.1. Denatured rGlnA Pull Down Assay with Blood Plasma

Pull down experiments with denatured rGlnA were performed as described in section 4.2.4. All fractions were eluted over a 50 ml volume gradient, collected and analysed by SDS-PAGE.

4.2.6. Mass Spectrometry to Identify Potential Human Interacting Proteins

Proteins observed by SDS-PAGE to co-elute with recombinant proteins were excised and sent to the University of York for identification performed by matrix assisted laser desorption ionization tandem time-of-flight mass spectrometry (MALDI-TOF/TOF) using a Bruker ultrafleX-III. Typically, proteins are reduced, alkylated and digested in-gel with trypsin. The resulting peptides are analysed by MALDI-TOF/TOF. A mass spectrum was acquired between 800-5000 m/z and the ten strongest peaks, above a signal to noise threshold, were selected for MS/MS fragmentation. The tandem mass spectral data produced were searched against the NCBI database using the Mascot search program to generate peptide matches with associated expect values. Protein identifications were inferred from peptide matches.

4.3. Results

4.3.1. Expression of Recombinant Proteins

Recombinant protein rFBA was expressed for 4 h after induction with 1 mM IPTG in *E. coli* carrying plasmid construct pSAT1. Samples were taken post induction, and at hourly time points to confirm and monitor protein expression using SDS-PAGE and western blot. Recombinant GlnA was confirmed previously (See Chapter 2, Figure 2.6).

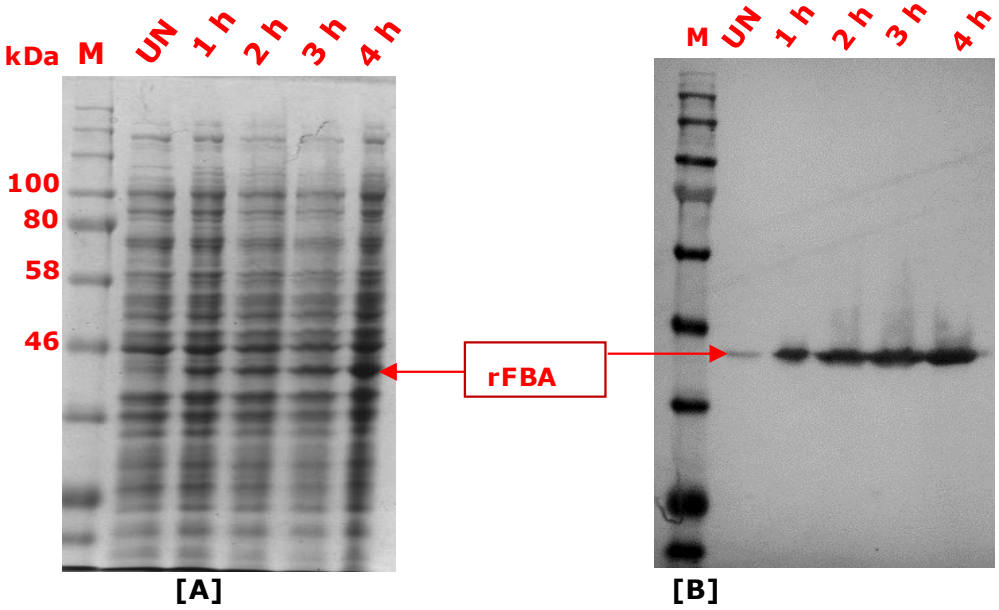


Figure 4.1: SDS-PAGE (A) or immunoblot analysis (B) with anti-penta-histidine antibodies demonstrating the expression of rFBA in IPTG-induced *E. coli* JM109 cells containing pSAT1 [lanes 1 h – 4 h] compared to non-induced cells [lane UN]; Lane M: pre-stained protein marker.

4.3.2. Analysis of Blood Plasma

Plasma collected from two volunteers (Plasma A and Plasma B) were run out on SDS-PAGE gel to compare and check for uniformity between observed plasma proteins, as well as to check that plasma samples were not degraded during preparation or storage, before subsequent use in pull down assays. Plasma proteins observed in both plasma samples appeared similar, with similar concentrations for each protein.

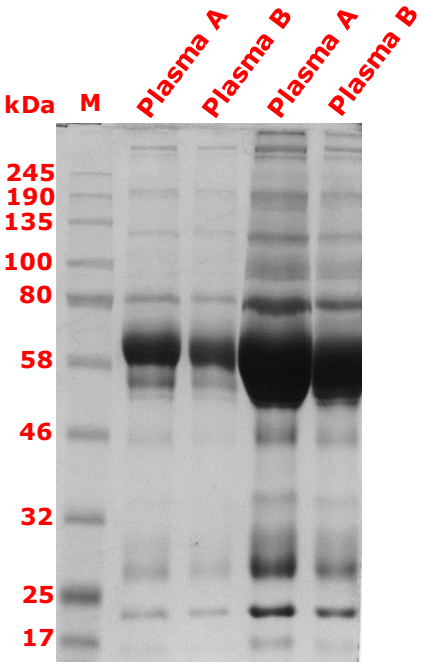


Figure 4.2: Plasma A or B run out on 10% ($^{w}/_{v}$) SDS-PAGE gel (10 µl or 20 µl)

4.3.3. Purification of rFBA and rGlnA under native conditions

Recombinant FBA and GlnA were purified under native conditions using the AKTA prime machine. Elution was performed over a gradient and elution fractions of 0.5 ml each were collected. Fractions corresponding to peaks in the chromatogram were analysed by SDS-PAGE. Both rGlnA and rFBA purification were successful (Figure 4.3 A and B respectively). Fractions

containing respective purified proteins were pooled together and stored till further use.

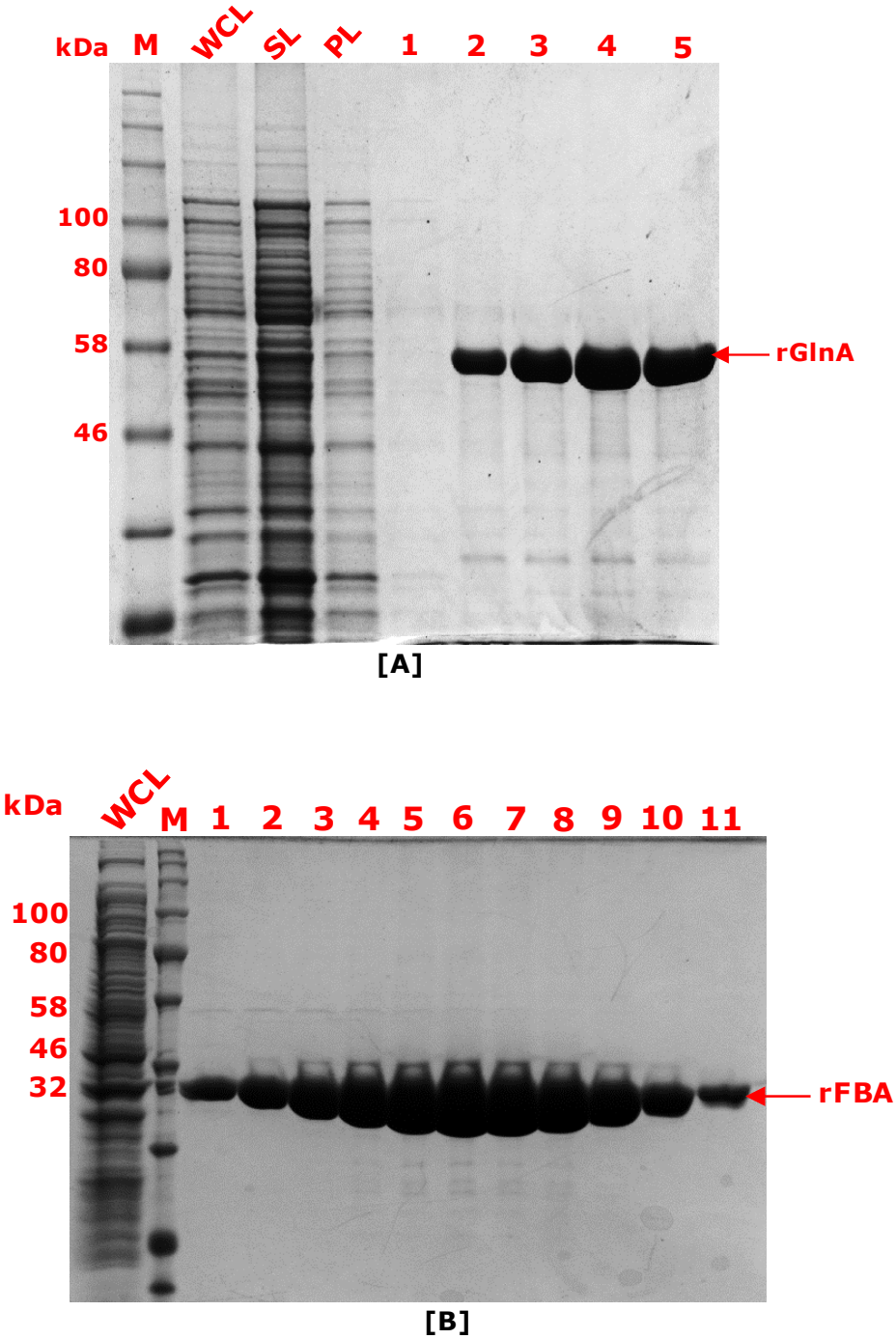


Figure 4.3: Purification of rGlnA (A) and rFBA (B) under native conditions. WCL: whole cell lysate, SL: supernatant lysate, PL: pellet lysate, fractions 1A – 4A: eluates of purified rGlnA, fractions 1B – 11B: eluates of purified rFBA.

4.3.4. Recombinant Proteins Pull Down Assays

Supernatant cell lysates of *E. coli* expressing either rFBA or rGlnA or previously purified recombinant proteins were passed through the nickel packed purification column, washed thoroughly with low imidazole buffer, and 1 ml of human plasma passed through to allow for interaction between plasma proteins and recombinant proteins. Elution of bound recombinant proteins was achieved by elution over a gradient using high imidazole buffer. All fractions were collected and analysed by SDS-PAGE.

4.3.4.1. Human plasma pull down with rGlnA

Fractions corresponding to peaks in the chromatograph were analysed by SDS-PAGE, and fractions containing His-tagged recombinant proteins were analysed by western blot. Assays were performed in triplicates using different plasma samples. In the fractions corresponding to eluted rGlnA (Fractions 14 – 26; Figure 4.4), one protein of *ca.* 52 kDa was observed to co-elute with rGlnA.

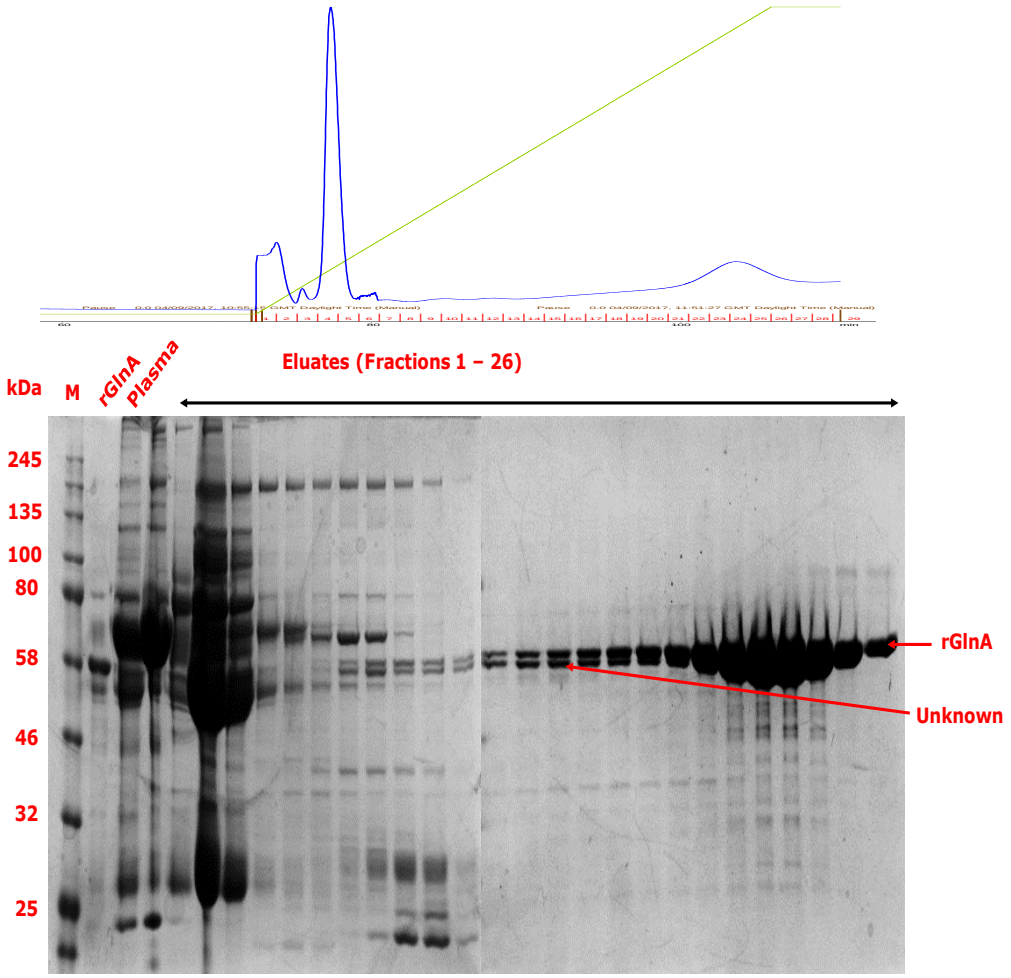


Figure 4.4: SDS-PAGE analysis on fractions corresponding to peaks in the chromatograph of human plasma pull down with recombinant glutamine synthetase. Experiment was repeated three times with consistent results generated.

These fractions (14 -21) were incubated with anti-his monoclonal antibodies and the unknown protein was not observed in the western blot imaging (Figure 4.5).

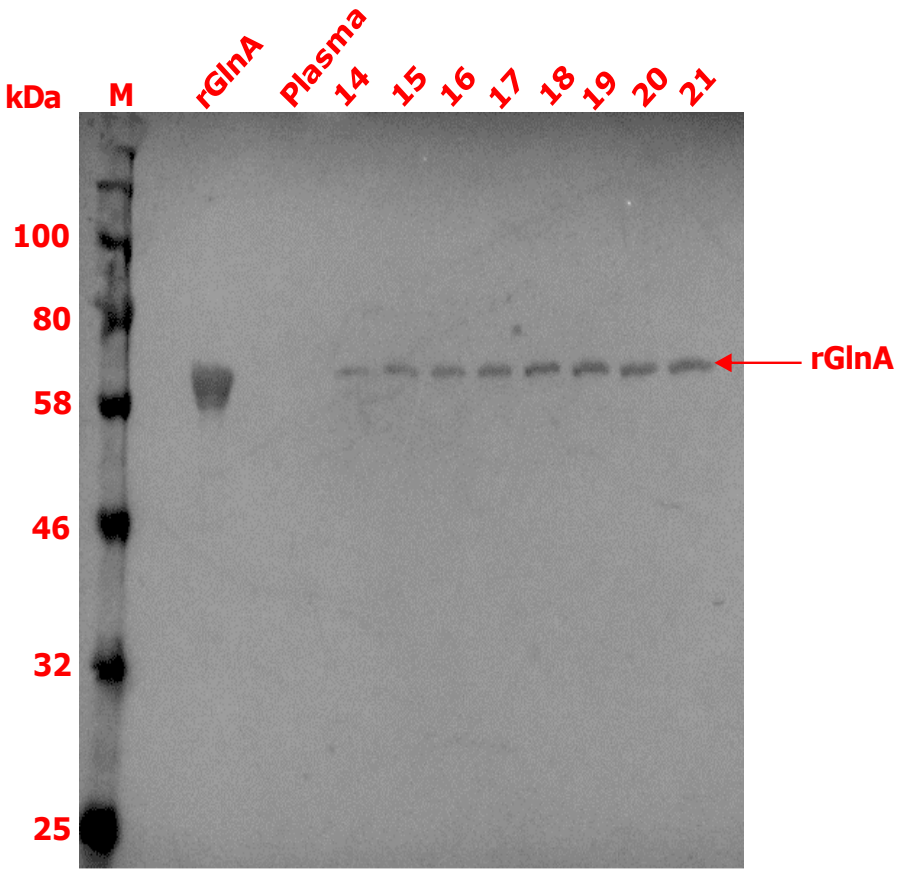


Figure 4.5: Western blot using anti-his monoclonal antibodies on fractions containing rGlnA co-eluting with an unknown protein.

Alternatively, pull down assay with human plasma was performed by re-tethering previously purified rGlnA to the purification column, followed by passing 1 ml of human plasma through and then elution via a gradient with all elution fractions collected and analysed by SDS-PAGE. Fractions containing rGlnA were also analysed by western blot. In this instance, four

proteins of size *ca.* 85, 90, 100, and 190 kDa respectively were observed to co-elute with rGlnA (Fractions 14 – 23; Figure 4.6).

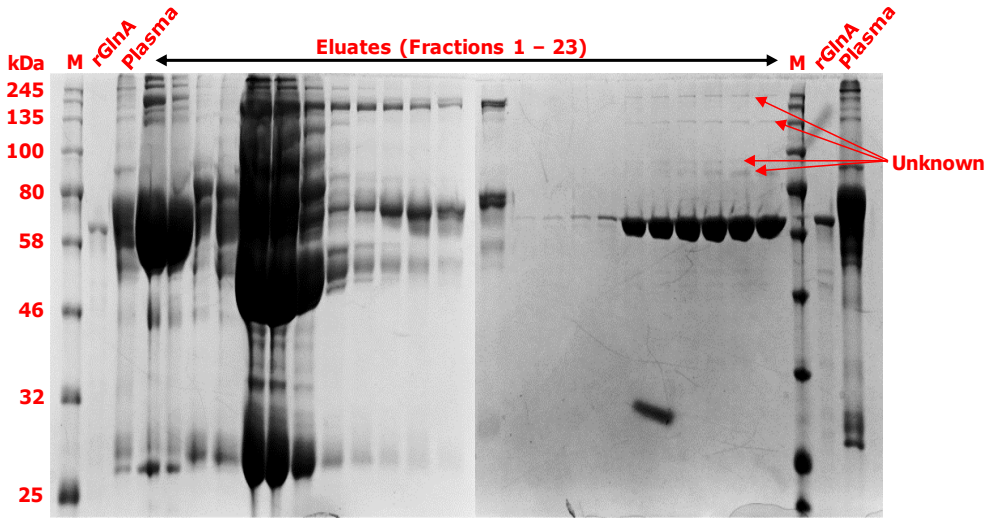


Figure 4.6: SDS-PAGE analysis on fractions corresponding to peaks in the chromatograph of human plasma pull down with previously purified recombinant glutamine synthetase. Experiment was repeated simulating the same experimental conditions as possible, but result was inconsistent between runs.

These proteins were not observed in the western blot incubated with RαGlnA antisera antibodies (Figure 4.7)

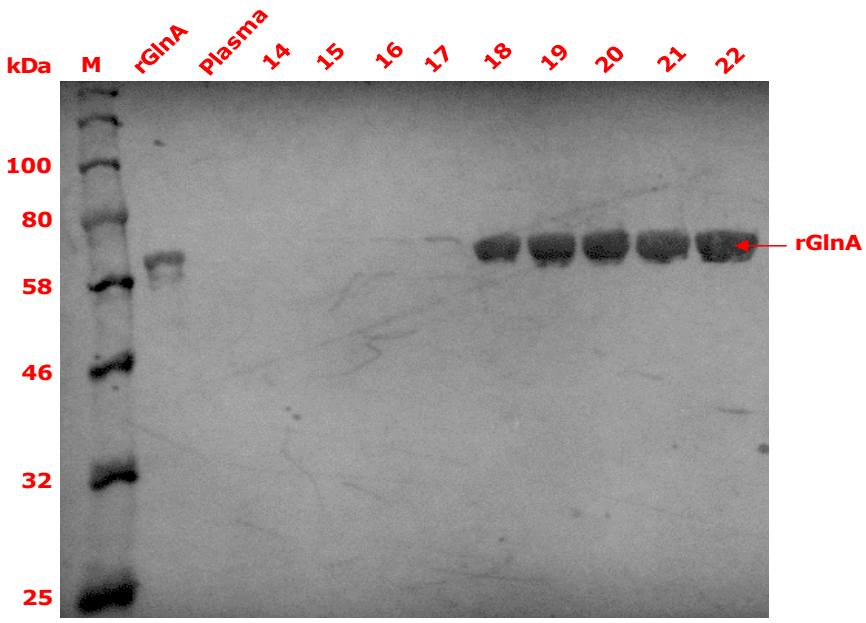


Figure 4.7: Western blot using RαGlnA polyclonal antibodies on fractions containing rGlnA co-eluting with four unknown proteins of size *ca.* 85, 90, 100, and 190 kDa respectively.

For control assays, plasma was passed through the column without any recombinant protein tethered, and elution was performed via a gradient. As in test assays, it was observed that all plasma proteins were eluted in the first four fractions and no protein bands observed in the remaining 19 fractions collected.

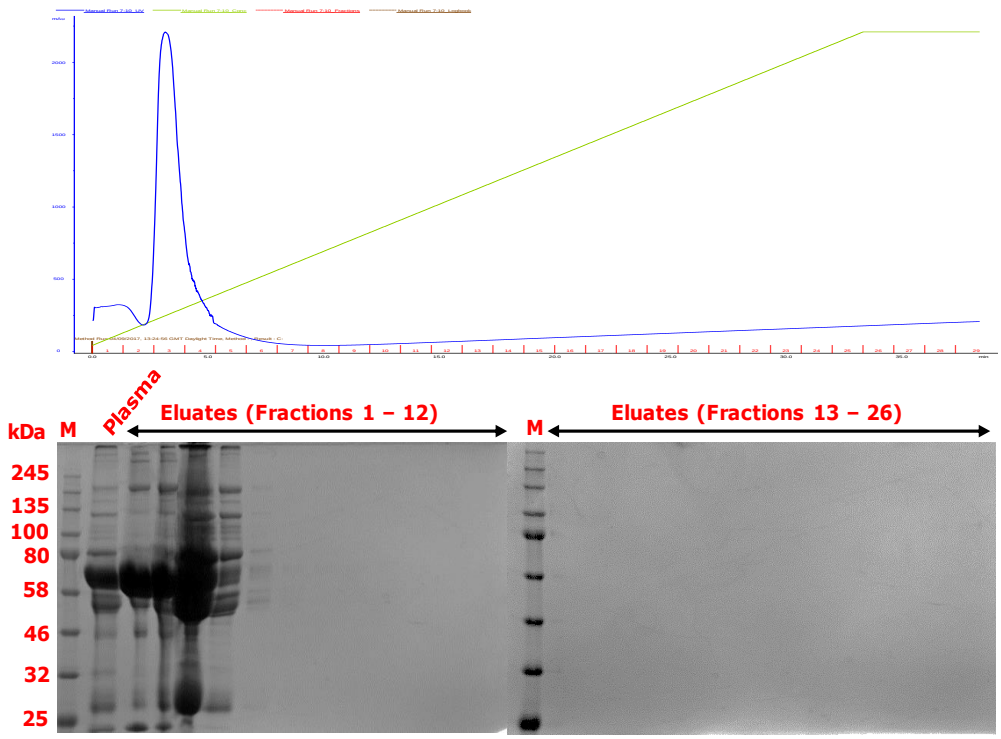


Figure 4.8: Human plasma control pull down assays without bait recombinant proteins.

4.3.4.2. Human plasma pull down with rFBA

As with rGlnA in section 4.3.4.1, fractions corresponding to peaks in the chromatograph were analysed by SDS-PAGE, and fractions containing rFBA were analysed by western blot. In the fractions corresponding to eluted rFBA (fractions 6 – 23), one protein of *ca.* 80 kDa was observed to co-elute with rFBA (Figure 4.9 A). Repeat of the assay produced a band co-eluting with rFBA of size *ca.* 90 kDa (Figure 4.9 B, fractions 17 - 23).

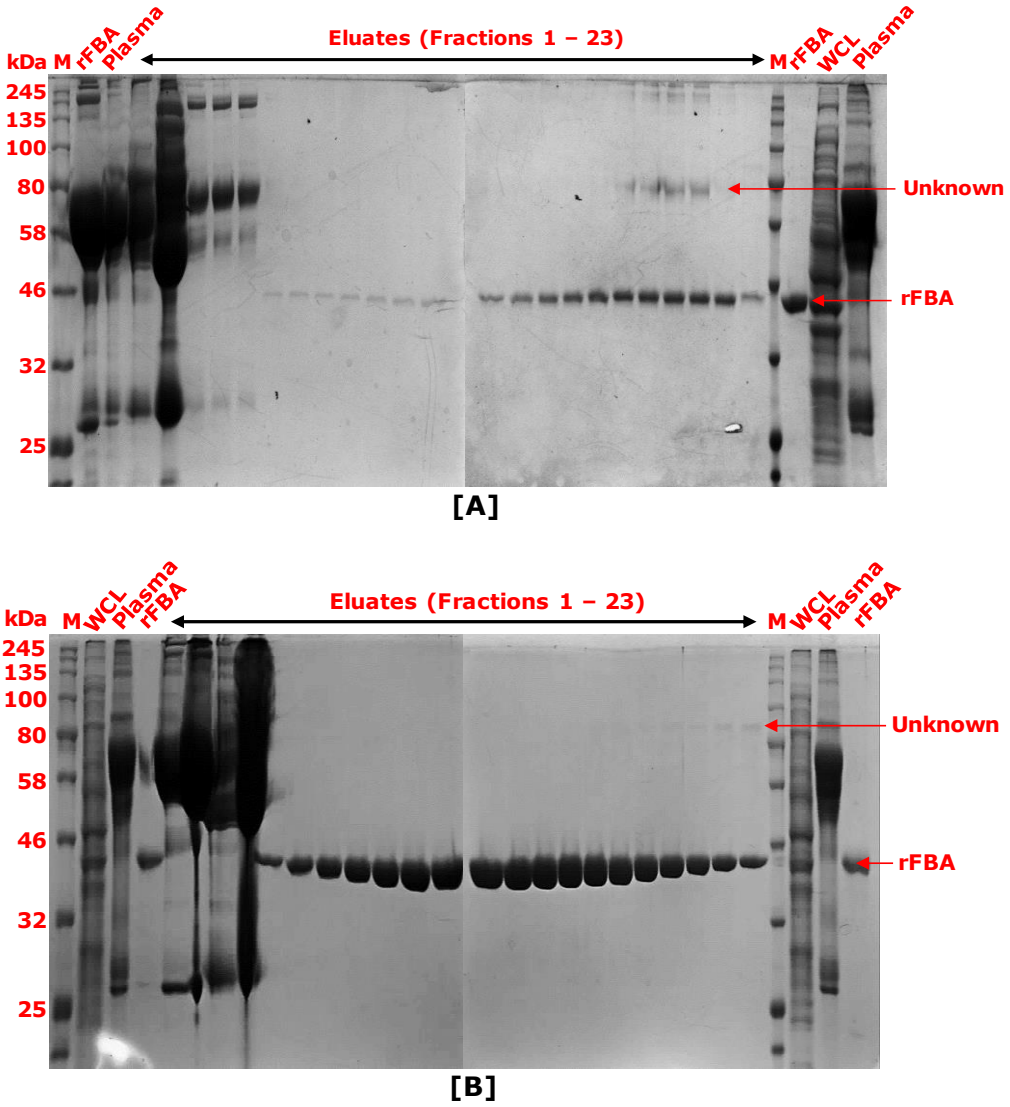


Figure 4.9: SDS-PAGE analysis of fractions corresponding to peaks in the chromatograph of human plasma pull down with (A): previously purified rFBA, (B): rFBA. Experiments were repeated in triplicate with observed co-eluting proteins in Figure 4.9 not observed in other attempts, thus inconsistent results.

Western blot analysis of these fractions in Figure 4.9 B with anti-plasminogen antibodies indicated that this unknown protein was not plasminogen which is a known plasma protein of around 90 kDa in size (Figure 4.10).

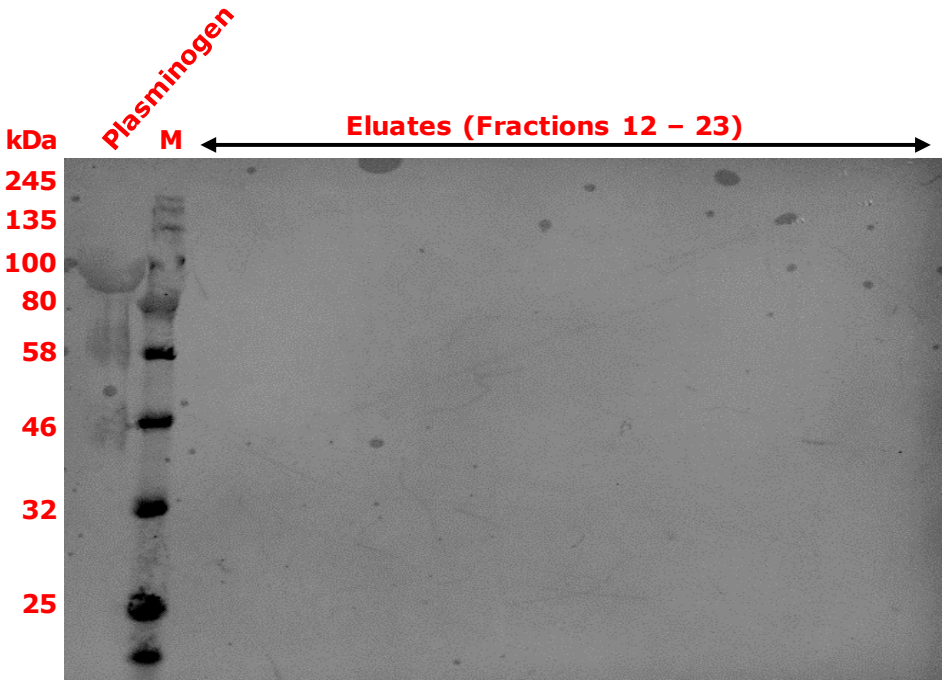
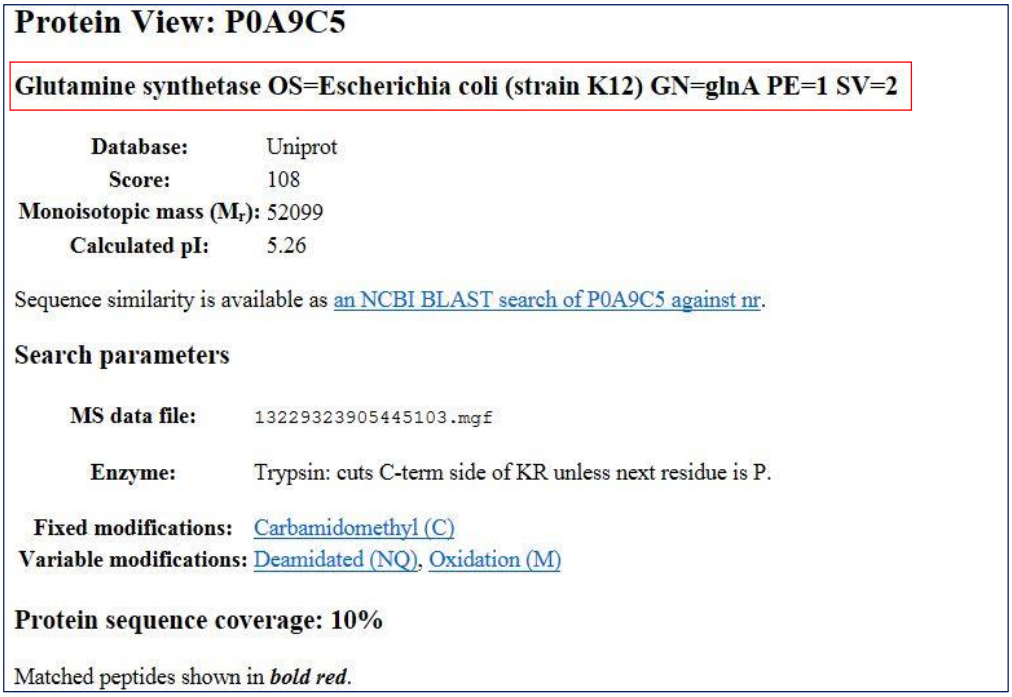


Figure 4.10: Western blot using anti-plasminogen monoclonal antibodies on fractions 17 – 23 from 4.9 B.

4.3.5. Mass Spectrometry to Identify Potential Human Interacting Proteins

The band observed to co-elute with rGlnA from figure 4.4 was excised from a coomassie stained SDS-PAGE gel, and sent to the Department of Biology, University of York for mass spectroscopy analysis to identify this protein. Mass spec data identified this unknown protein to be glutamine synthetase from *E. coli* strain K-12 with average mass (M_r) *ca.* 52kDa.



Protein View: P0A9C5

Glutamine synthetase OS=Escherichia coli (strain K12) GN=glnA PE=1 SV=2

Database: Uniprot
Score: 108
Monoisotopic mass (M_r): 52099
Calculated pI: 5.26

Sequence similarity is available as an NCBI BLAST search of P0A9C5 against nr.

Search parameters

MS data file: 13229323905445103.mgf

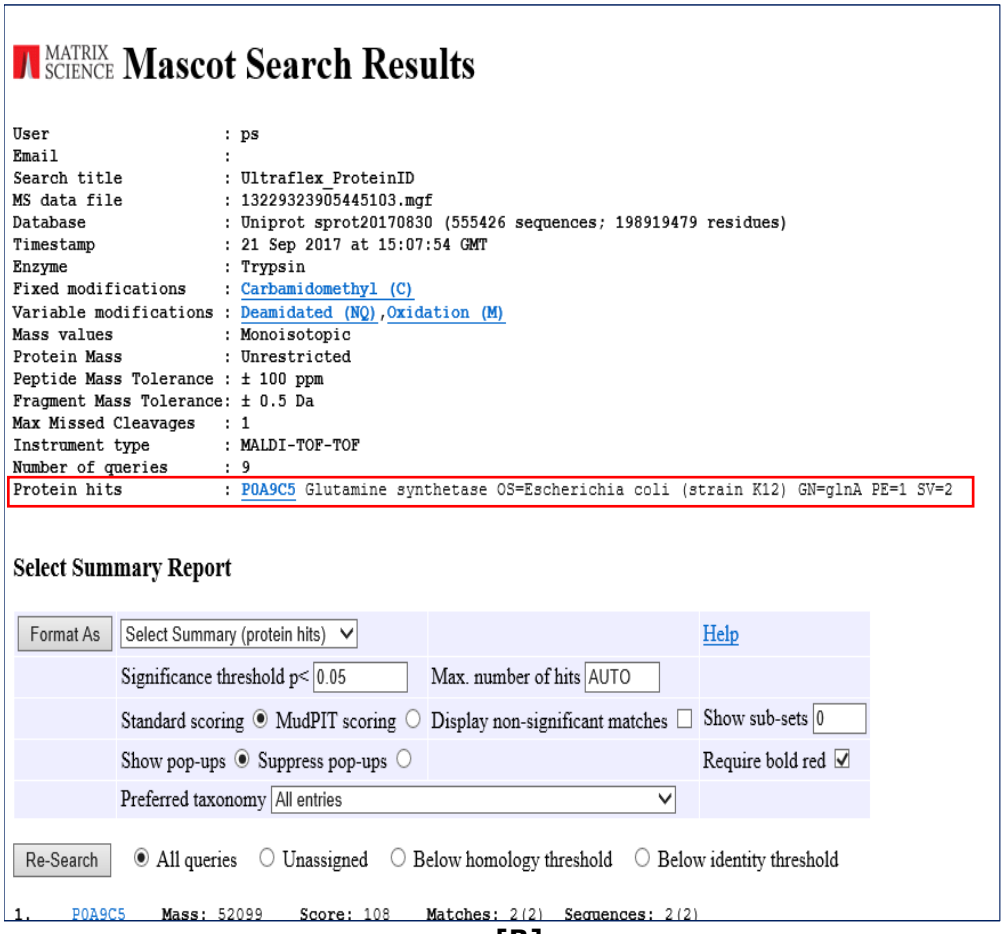
Enzyme: Trypsin: cuts C-term side of KR unless next residue is P.

Fixed modifications: Carbamidomethyl (C)
Variable modifications: Deamidated (NQ), Oxidation (M)

Protein sequence coverage: 10%

Matched peptides shown in ***bold red***.

[A]



MATRIX SCIENCE Mascot Search Results

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User                  : ps
Email                 :
Search title          : Ultraflex_ProteinID
MS data file          : 13229323905445103.mgf
Database              : Uniprot sprot20170830 (555426 sequences; 198919479 residues)
Timestamp             : 21 Sep 2017 at 15:07:54 GMT
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Fixed modifications   : Carbamidomethyl (C)
Variable modifications : Deamidated (NQ),Oxidation (M)
Mass values           : Monoisotopic
Protein Mass          : Unrestricted
Peptide Mass Tolerance : ± 100 ppm
Fragment Mass Tolerance: ± 0.5 Da
Max Missed Cleavages  : 1
Instrument type       : MALDI-TOF-TOF
Number of queries     : 9
Protein hits          : P0A9C5 Glutamine synthetase OS=Escherichia coli (strain K12) GN=glnA PE=1 SV=2
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Select Summary Report

Format As [Select Summary (protein hits)] Help

Significance threshold p< 0.05 Max. number of hits AUTO

Standard scoring ◉ MudPIT scoring ○ Display non-significant matches ☐ Show sub-sets 0

Show pop-ups ◉ Suppress pop-ups ○ Require bold red ☑

Preferred taxonomy [All entries]

Re-Search ◉ All queries ○ Unassigned ○ Below homology threshold ○ Below identity threshold

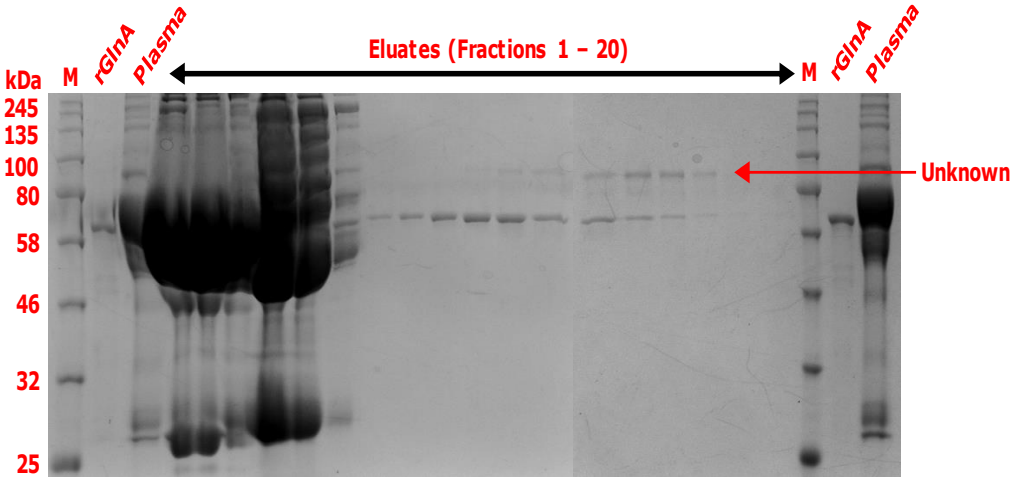
1. P0A9C5 Mass: 52099 Score: 108 Matches: 2(2) Sequences: 2(2)

[B]

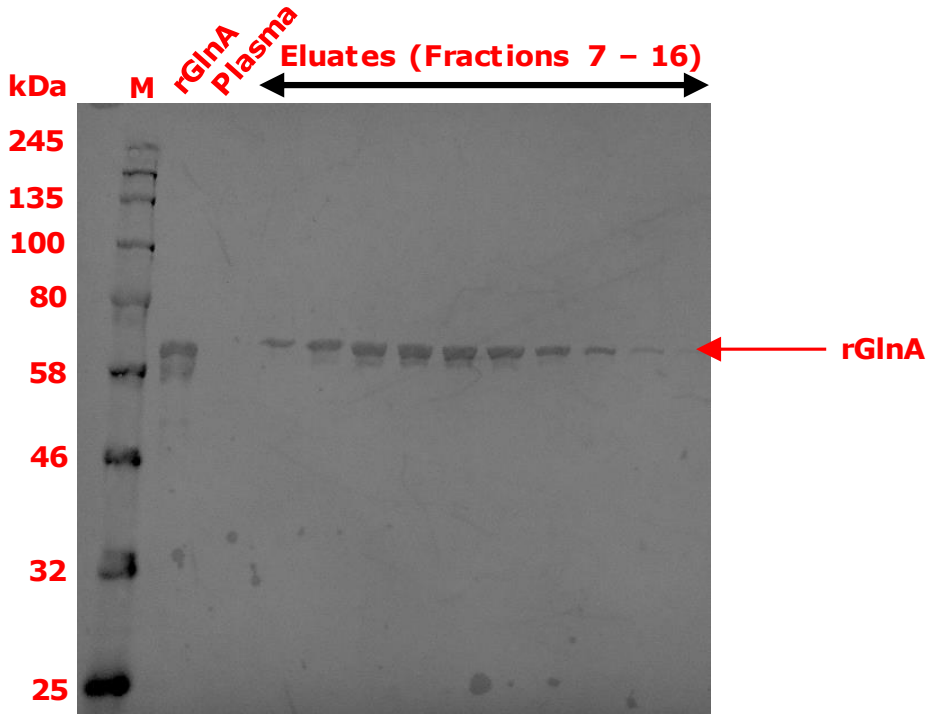
Figure 4.11: Protein view (A) and mascot search results (B) of mass spectrometry data to identify unknown protein co-eluting with rGlnA.

4.3.6. Denatured rGlnA Pull Down Assay with Blood Plasma

In order to separate the two species of glutamine synthetase (from *E. coli* and *N. meningitidis*), pull down assays were performed using denatured rGlnA. Fractions corresponding to peaks in the chromatograph were analysed by SDS-PAGE, and fractions containing rGlnA were analysed by western blot. In the fractions corresponding to eluted rGlnA (Fractions 7 – 16), one protein of *ca.* 90 kDa was observed to co-elute with rGlnA (Figure 4.11 A). This protein was not observed in the western blot analysis of these specific fractions using RαGlnA polyclonal antisera (Figure 4.11 B.). Although not visible in the coomassie stained gel, the band corresponding to GlnA from *E. coli* was still present in the fractions containing rGlnA when immunoblotted with RαGlnA antisera antibodies (Figure 4.11 B), indicating that the denaturant had not successfully separated both species GlnA.



[A]



[B]

Figure 4.12: Human plasma pull down with denatured recombinant glutamine synthetase. (A): SDS-PAGE analysis of fractions corresponding to peaks in the chromatograph, (B): western blot using RαGlnA polyclonal antibodies of fractions containing rGlnA co-eluting with an unknown protein.

4.4. Discussion

Neisseria meningitidis exists in humans in a harmless commensal relationship, with 8-25% of the human population serving as carriers of the organism (de Souza and Seguro, 2008). On occasion, in susceptible individuals, encapsulated bacteria can cause invasive meningococcal disease, most commonly meningococcal meningitis and sepsis (Nassif, 1999, Jodar et al., 2002, Arigita et al., 2004). During infection, the pathogen is able to interact with human epithelial and endothelial cells, cross the barrier, interact with the extracellular matrix, and distribute itself in human tissue (Dehio et al., 1998, Merz and So, 2000, Nassif et al., 2002). Pili, outer membrane proteins Opa, Opc, fHbp, NspA, App, MspA, NalP, HrpAHrpB, NhhA, NHBA, NadA, and the porin proteins, PorA and PorB are some of the major meningococcal surface receptors that contribute to the adhesion to and invasion of host cells (Virji, 2009, Hill and Virji, 2012, Pizza and Rappuoli, 2015).

Cytoplasmic proteins have also been shown to be surface localized and carrying out moonlighting functions at the bacterial cell surface. A wide variety of pathogenic bacteria have been shown to recruit the host plasminogen to their surface, thus acquiring a broad spectrum of proteolytic activity. Knaust and colleagues were able to show that *N. meningitidis* are able to bind purified human plasminogen as well as acquire plasminogen from human plasma. On acquiring plasminogen through plasminogen receptors, *N. meningitidis* associated plasminogen can become activated by urokinase thus allowing the bacteria to degrade fibrinogen (Knaust et al., 2007).

Noted plasminogen receptors in *N. meningitidis* include DnaK, Enolase, FBA, and peroxiredoxin. Using flow cytometry, Shams and colleagues (Shams et al., 2016) showed that *Neisseria* FBA is able to bind

human plasminogen via its C-terminal lysine residue. Similarly, by flow cytometry and immunogold labelling, (Knaust et al., 2007) also showed that *N. meningitidis* are able to bind plasminogen. Employing plasminogen overlay assays, they were able to show that bacterial binding to plasminogen was achieved through six plasminogen binding proteins. Three of these plasminogen binding proteins were identified to be DnaK, enolase, and peroxiredoxin.

We investigated the binding of recombinant protein to plasma proteins using an *in vitro* pull down assay. This method is widely used in identifying/screening for potential binding partners in which one protein/molecule is baited to a stationary surface and a solution containing prey protein(s)/molecule(s) are incubated with the bait protein in order for the bait to pull down its prey. It is important to note that a pull down assay offers an unbiased approach to investigating potential binding to human plasma proteins. Wysocka (2006) employed a peptide pull down assay to identify proteins recognizing histone modifications (Wysocka, 2006). Likewise, Ren and Colleagues (2003) used a modified glutathione S-transferase pull-down assay to study protein–protein interactions (Ren et al., 2003).

For our pull down assay using rGlnA as bait protein and human plasma as prey to identify potential interactions we observed that either three proteins of size *ca.* 90, 100, and 190 kDa respectively co-eluted with rGlnA or one protein of size *ca.* 52 kDa co-eluted with rGlnA. Mass spectrometry to identify this unknown protein of size *ca.* 52 kDa revealed that it was glutamine synthetase from *E. coli* strain K-12. Expression of rGlnA was performed in *E. coli* JM109 competent cells which is a K strain, thus confirming that this unknown protein was glutamine synthetase from *E. coli* GlnA rather than a human plasma protein.

Through electron microscopy, it was discovered that GlnA from *E. coli* exists as a dodecamer made up of 12 identical subunits arranged in two hexagonal layers (Valentine et al., 1968, Almassy et al., 1986). This 12 subunit glutamine synthetase (GS I) is reported to be expressed by all bacteria, with the active site residues conserved across all species and likely, a similar mechanism of action (Kumada et al., 1993, Eisenberg et al., 2000). Therefore, it is possible that glutamine synthetase from *E. coli* and *N. meningitidis* are able to form inter-species dodecamers, and a likely explanation for *E. coli* GlnA being present in the native purified GlnA from *N. meningitidis.* To separate both species of glutamine synthetases, we purified rGlnA and washed with 8 M urea before subsequent pull down assays, but GlnA from *E. coli* was still present in eluted rGlnA fractions when immunoblotted with RαGlnA polyclonal antibodies.

For pull down assays using rFBA from *N. meningitidis* as bait and human plasma as prey, we observed that two proteins *ca.* 80 kDa or 90 kDa co-eluted with the recombinant protein. It is known that FBA is able to bind plasminogen (Shams et al., 2016) which is 90 kDa protein. To determine if this unknown protein was plasminogen, we immunoblotted the fractions containing rFBA and the unknown protein with anti-plasminogen monoclonal antibodies, but no bands were detected.

As the results from the pull down assays were not definitive and inconsistent between repeated runs, more samples of proteins observed to co-elute with bait recombinant proteins were not sent for mass spectrometry analysis, neither were pull down assays with human endothelial and epithelial cells performed. Possibly the conditions of the pull down assays can be changed or optimized to improve binding before subsequent pull down assays to ensure more reliable samples are sent for mass spectrometry analysis. For instance, rGlnA or rFBA could be incubated with

human plasma or a mixture of solubilized proteins from human epithelial or endothelial cells overnight at 4°C or at room temperature for up to 2 hours, the solution tethered to the column, and elution performed, to optimize the binding of the target to its prey. Also, the buffer pH could be varied over the course of different experiments to determine the pH most favourable for the binding of the prey protein(s) to GlnA.

Alternatively, other techniques such as EIA and flow cytometry could have been employed to assess the binding of recombinant proteins to human proteins, where recombinant proteins are coated onto a flat surface, human plasma or a solution of human endothelial/epithelial cells added and incubated to allow for binding. Binding with known human proteins would be investigated through monospecific antibodies that bind specifically to these proteins, for instance, anti-plasminogen or anti-fibronectin antibodies.

Likewise, the solution of the binding of human proteins to recombinant proteins could be analysed by far-western blot or overlay assays, and any host proteins observed to bind to recombinant proteins identified by mass spectrometry.

CHAPTER 5: General discussion

5.1. General Discussion

The number of cytoplasmic enzymes shown to also be localized to the surface of microbial pathogens are steadily increasing. These proteins lack identifiable secretion signals, and exhibit various functions unrelated to their canonical functions, which contribute to the pathogenesis of disease (Pancholi and Chhatwal, 2003). To further understand microbial pathogenesis, it is essential to identify the additional moonlighting roles of these proteins, as they may represent potential vaccine candidates due to their surface localization and immunogenicity. The work presented in this thesis details the molecular and functional characterization of meningococcal glutamine synthetase (GlnA).

The discussion of the results of this study will include the validity of selecting GlnA for analysis, the strategies used to achieve the project aims, and analysis of the findings achieved.

GlnA was selected for investigation because the protein has been shown to be localized to the bacterial surface in a variety of pathogens and non-pathogenic organisms, playing key roles in adhesion to host proteins such as plasminogen (Candela et al., 2007, Kainulainen et al., 2012), as well as in virulence (Klose and Mekalanos, 1997, Tullius et al., 2003, Si et al., 2009, Chandra et al., 2010). As its role in the bacterial pathogen *N. meningitidis* has not been studied, it was hypothesized that similar to GlnA in other pathogens, meningococcal GlnA may be a multi-functional protein, also localized to the outer membrane.

The strategy adopted to characterize meningococcal GlnA included cloning the encoding open reading frame, subsequent purification of recombinant GlnA (rGlnA) and the generation of polyclonal rabbit antiserum (rαGlnA). Furthermore, the creation of a *glnA* knock-out mutant was attempted, and a mutant strain with *glnA* inserted in trans created. The

antiserum and mutant strain were used to establish the surface localization of meningococcal GlnA. Purified meningococcal rGlnA and rFBA were also employed to investigate potential moonlighting roles at the bacterial cell surface, including interaction with human cell ligands, which may contribute to the pathogenesis of meningococcal disease.

GlnA has been previously reported to be localized at the surface of some Gram-positive bacteria. For example, in *L. crispatus*, GlnA was identified to be surface-exposed, and functioning as an adhesin to extracellular matrix proteins (Kainulainen et al., 2012). Likewise, GlnA was shown to be surface-localized in *Bifidobacterium*, acting as a host protein receptor (Candela et al., 2007). We undertook to investigate whether GlnA was also surface-localized in *N. meningitidis*.

In the published meningococcal genome sequences, there is a single gene *glnA*, which encodes a GlnA enzyme. Sequence analysis indicated that GlnA is highly conserved at the amino acid level across the *N. meningitidis* strains. Sequence analysis also revealed that the enzyme was highly related to GlnA from other *Neisseria* species with conservation at the amino acid level across *Neisseria* species.

In this study, rGlnA was successfully expressed with an N-terminal histidine-tag in host *E. coli* cells, with a high level of expression of soluble recombinant protein. The expressed protein was purified under native conditions and used to raise rabbit polyclonal anti-GlnA serum (rαGlnA).

To study the phenotypic characteristics of *glnA,* attempts were made to create a *glnA* knockout mutant strain. Two different approaches were used: replacing *glnA* with a kanamycin cassette or inactivating *glnA* by inserting a kanamycin resistant cassette in the open-reading frame of *glnA*. Unfortunately, the attempts at creating a *glnA* knockout mutant strain using these different approaches were not successful. Rather than knockout

mutants, the mutants generated had incorporated the kanamycin resistant cassette into the genome but not in place of, or within the open reading frame of *glnA*. A reason for this could be because Δ*glnA* mutants are expected to be auxotrophic and can only grow when the growth medium is supplied with glutamine, it is possible that the double crossover recombinant auxotrophic mutants in this study where unable to uptake glutamine from the media and thus were unable to grow, while the single crossover mutants that still contained GlnA obtained in this study were able to grow.

The natural transformation of *N. meningitidis* was performed using plasmid DNA extracted from the deletion/inactivation constructs created in *E.coli*; future work may be to linearize the plasmid DNA prior to transformation into meningococci. It is known that plasmid DNA is linearized into double-stranded DNA during DNA uptake in gonococci, and cleaved in a non-site specific manner (Biswas et al., 1986). As researchers have also achieved successful transformation of gonococci as well as meningococci using linearized plasmid DNA or circular DNA (van Dam and Bos, 2012, Callaghan and Dillard, 2019), this approach could be used in further attempts to generate double cross-over recombinant knock out mutants.

This study also aimed to establish whether GlnA is surface-localized in *N. meningitidis.* Using whole cell ELISA and the rabbit polyclonal anti-GlnA serum, it was shown that GlnA is possibly surface-exposed in *N. meningitidis*, and present in a form accessible to antibodies, suggesting that, similar to meningococcal Prx, DnaK, FBA, GAPDH, and enolase (Knaust et al., 2007, Tunio et al., 2010a, Tunio et al., 2010b, Shams et al., 2016), GlnA is translocated to the outer membrane. As mentioned earlier, in other organisms, GlnA is transported to the outer membrane and surface-localized, by an unknown mechanism. If the mechanism leading to the export of GlnA to the cell surface could be identified, that system could be

knocked out and ELISA performed to confirm the absence of GlnA on the bacterial surface. As GlnA would continue to be expressed in the cytoplasm, the strain may be viable, but GlnA would be unable to reach the bacterial surface, which could be confirmed by ELISA using anti-GlnA.

Following the establishment of the surface localization of meningococcal GlnA in this study, as well as the previous knowledge of the surface localization of meningococcal FBA, this study undertook to investigate putative moonlighting functions of these enzymes at the bacterial cell surface. The close proximity of these surface exposed meningococcal proteins to host cell proteins at the bacterial cell surface increases the likelihood of adhesion to host cells. It is known that through adhesins, meningococci interact with proteins and receptors present on the surface of epithelial cells, before invading to sub-epithelial tissues from which it may gain access to the bloodstream (Merz and So, 2000, Tettelin et al., 2000, Parkhill et al., 2000).. In the bloodstream, meningococcal surface proteins also aid the binding to host plasma proteins which may hinder the host innate and acquired immune responses, thus influencing the establishment of meningococcal disease (Hung and Christodoulides, 2013). Meningococci replicate rapidly in the bloodstream and adhesins promote the adherence to endothelial cells, leading to vascular colonization (Virji, 2009).

Using pull down assays, we aimed to identify whether any human plasma or cellular proteins interact with meningococcal rFBA and rGlnA. It has been shown that meningococcal FBA binds to human plasminogen, a human plasma protein, thus bestowing the bacteria with the ability to break down host proteins (Shams et al., 2016). Thus, it is important to investigate if meningococcal FBA and GlnA can bind other plasma proteins, for instance fibronectin and vitronectin which regulate the proteolysis initiated by the activation of plasminogen, or haemoglobin and transferrin which may act as

important sources of iron for the bacteria, or the globulins and the host complement components which may serve to hinder the host immune responses during infections.

Several bands corresponding to possible proteins co-eluting with either rGlnA or rFBA were observed in the pull down assays. Only one protein was observed to consistently co-elute with rGlnA, and no protein consistently co-eluted with rFBA. The protein observed with rGlnA was identified by mass spectrometry to be GlnA from *E. coli* strain K-12.

This study proposed that glutamine synthetase from *E. coli* and *N. meningitidis* are possibly forming inter-species dodecamers, as it is known that that GlnA from *E. coli* exists as a dodecamer made up of 12 identical subunits arranged in two hexagonal layers (Valentine et al., 1968, Almassy et al., 1986). Although this theory provided a likely explanation for *E. coli* GlnA being present in the natively purified GlnA from *N. meningitidis*, attempts to separate species-specific GlnA using denaturants were not successful. Since it is possible to knock-out *E. coli* GlnA (Varricchio and Holzer, 1969, Rothstein et al., 1980, DasSarma et al., 1986), this could be done prior to cloning in meningococcal GlnA which would prevent the formation of the interspecies GlnA dodecamers.

Since the results from the pull down assays were not definitive, more samples of proteins observed to co-elute with bait recombinant proteins were not sent for mass spectrometry analysis, neither were pull down assays with human endothelial and epithelial cells performed. As described previously, the conditions need to be further optimized to improve binding before performing further pull down assays, to ensure more reliable samples for mass spectrometry analysis. Alternatively, the binding of meningococcal recombinant proteins to human proteins could be assessed with other techniques such as EIA and flow cytometry where recombinant proteins are

coated onto a flat surface, human plasma or a mixture of solubilized proteins from human endothelial/epithelial cells added and incubated to allow for binding. Binding with known human proteins could be investigated by using monospecific antibodies that bind to these proteins, for instance, anti-plasminogen or anti-fibronectin antibodies. Binding could also be studied by far-western blot, and host proteins observed to bind to the recombinant proteins identified by mass spectrometry.

5.2. Conclusions

In this study, *glnA* encoding GlnA was cloned into *E. coli*, and the recombinant protein subsequently purified under native conditions. Polyclonal monospecific antiserum was raised in rabbit against native rGlnA. ELISA data demonstrated that GlnA was surface exposed and accessible to antibodies on the surface of intact cells, indicating meningococcal GlnA is a putative moonlighting protein, which may perform different function(s) at the bacterial cell surface.

5.3. Future Directions

While this study has shown that meningococcal GlnA may be surface-localized, further examination using other techniques such as flow cytometry, sub-cellular fractionation and immuno-gold labelling can be employed to further validate this finding. Using sub-cellular fractionation, GlnA should be detected in the cytosolic protein-enriched fractions as well as in the outer membrane fractions thus confirming that predominantly cytosolic GlnA can also found at the cell surface. Likewise by flow cytometry, whole meningococcal cells treated with RαGlnA followed by treatment with

a suitable fluorescence anti-rabbit IgG secondary antibody should show a clear shift and increase in fluorescence signal when compared to meningococcal cells treated with RαGlnA alone or secondary antibody that would not produce high fluorescence signal, also confirming the cell surface localization of meningococcal GlnA.

On creation of a *glnA* knockout mutant, the in vitro adhesion of wild-type, knockout mutant, and complemented strains to human cells such as HBME and HEp-2 cells, via GlnA may be investigated by association and invasion assays.

The investigation of the mechanism of transport of meningococcal moonlighting proteins to the bacterial cell surface represents an important means to understanding the role these proteins play in pathogenesis. A vital step towards identifying putative regions or motifs involved in surface transportation hinges on the thorough *in silico* analysis of the protein sequence, which may provide significant insight into the translocation of these moonlighting proteins.

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Appendix

Appendix I – Commonly used buffers and solutions

Sodium dodecyl sulphate (SDS)-separating (resolving) buffer (Buffer A): 181.7 g Tris base, 40 ml 10% ($^{w}/_{v}$) SDS, made up to 1L with dH_2O, pH adjusted at 8.8.

SDS-stacking buffer (Buffer B): 60.6 g Tris base, 40 ml 10% ($^{w}/_{v}$) SDS, made up to 1L with dH_2O, pH adjusted at 6.8.

SDS running buffer (10×): 30.3 g Tris base, 144 g Glycine, 10 g SDS made up to 1L with dH_2O.

SDS-sample buffer (5×): 1 g SDS, 5 ml Glycerol, Bromophenol Blue 25 mg, Tris base 150 mg, 1 ml 2-mercaptoethanol, made up to 10 ml with dH_2O, pH adjusted at 6.8.

10% Acrylamide mini gels

- **Separating (resolving) gel master mix:** 2.5 ml Buffer A, 3.3 ml 30% ($^{w}/_{v}$) Acrylamide/Bis-acrylamide, 4.1 ml dH_2O, 30 µl 10% (w/v) Ammonium persulfate (APS), 30 µl Tetramethylethylenediamine (TEMED).
- **Stacking gel master mix:** 1.3 ml Buffer B, 1 ml Acrylamide/ Bis-acrylamide (30%, w/v), 2.7 ml dH_2O, 10 µl 10% (w/v) APS, 10 µl TEMED.

SDS-PAGE Coomassie Staining Solution: 0.6 g Coomassie Blue (Sigma-Aldrich), 250 ml methanol, 51 ml acetic acid, made up to 500 ml with dH_2O.

SDS-PAGE De-staining Solution: 250 ml methanol, 104.2 ml acetic acid, made up to 1L with dH_2O.

Semi-dry transfer buffer: 5.82 g Tris, 2.93 g Glycine, 3.75 ml 10% ($^{w}/_{v}$) SDS, 200 ml methanol, made up to 1L with dH_2O, pH adjusted at 9.

Phosphate buffered saline solution (PBS): prepared by dissolving 1 tablet of phosphate buffered saline (Dulbecco A, Oxoid) in 100 ml dH_2O

and autoclave. (NaCl 0.116 mol, KCl 0.003 mol, Na_2HPO_4 0.008 mol, K_2HPO_4 0.001 mol, pH 7.3).

Blocking buffer (BSA): 1% ($^{w}/_{v}$) Bovine serum albumin (Sigma-Aldrich A3912) in PBS-T, sterilize by filtration (0.22 µm filter).

Blocking buffer (Milk): 5% ($^{w}/_{v}$) skimmed milk powder in PBS-T.

Washing buffer (PBS-T): 0.05% ($^{v}/_{v}$) Tween20 in PBS.

Sodium carbonate buffer: 10 mM sodium carbonate Na2CO3, 90 mM sodium bicarbonate NaHCO3; pH 7.4.

Glycine buffer: 100 mM glycine, 1 mM zinc-chloride, 1 mM magnesium chloride; pH 10.4.

Antibiotics: All antibiotics were purchased from Sigma-Aldrich, UK, prepared according to the recommended concentration, sterilised by filtration, and stored at 4°C.

Ampicillin: (100 mg ml^{-1} stock solution prepared in dH_2O).
Kanamycin: (50 mg ml^{-1} stock solution prepared in dH_2O).
Erythromycin: (5mg ml^{-1} stock solution prepared in dH_2O)

Working concentrations were 1000x dilution from the prepared stock.

Lysogeny broth (LB): 10g Tryptone, 5g Yeast extract, 10g NaCl made up to 1 L with dH_2O.

LB agar: 10g Tryptone, 5g Yeast extract, 10g NaCl, 15g Microbial tested agar made up to 1 L with dH_2O, pH 7.0 ± 0.2 at 25°C.

Brain heart infusion broth (BHI):

BHI agar:

Isopropyl-β-D-1-thiogalactopyranoside (IPTG): to prepare 1 M stock solution, 0.23g of IPTG was dissolved in 1 ml dH_2O, sterilized by filtration and stored at -20°C.

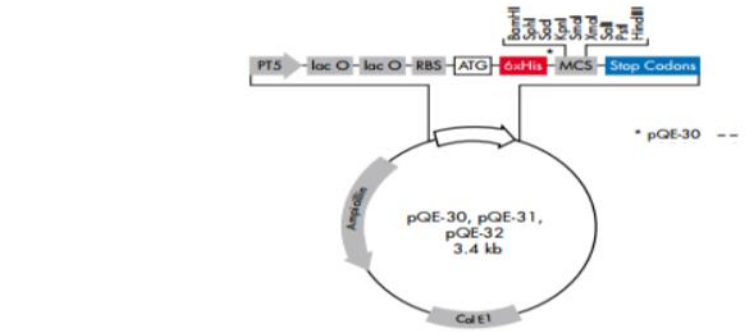
Buffers for protein purification under native condition:

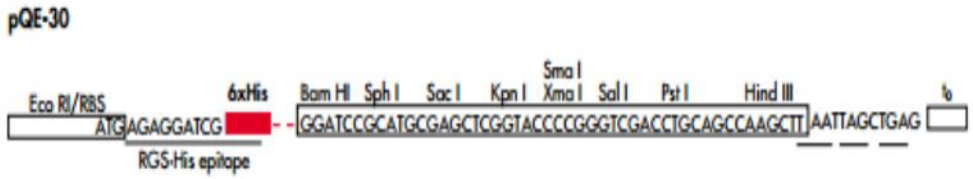
Lysis Buffer: 50mM sodium phosphate (pH 7.4), 300mM NaCl, 10mM Imidazole, pH 7.4.

Wash buffer: 50mM sodium phosphate (pH 7.4), 300mM NaCl, 50mM Imidazole, pH 7.4.

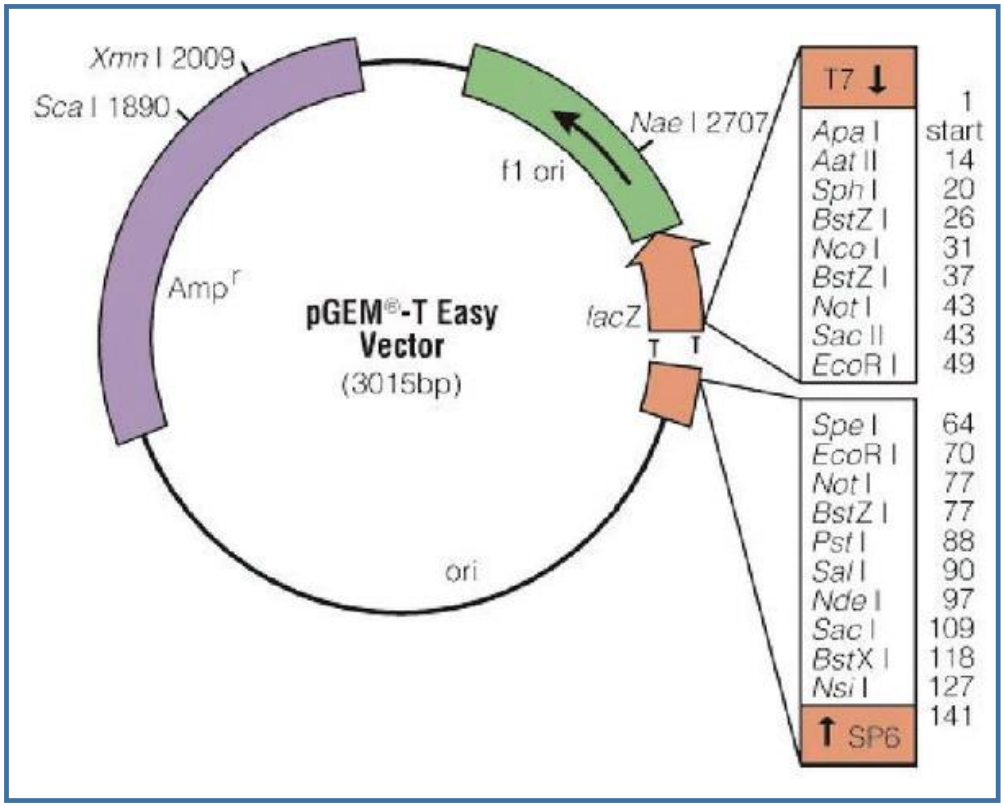
Elution buffer: 50mM sodium phosphate (pH 7.4), 300mM NaCl, 500mM Imidazole, pH 7.4.

Positions of elements in bases	pQE-30
Vector size (bp)	3461
Start of numbering at XhoI (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 t_0 transcriptional termination region	208–302
rrnB T1 transcriptional termination region	1064–1162
ColE1 origin of replication	1638
β-lactamase coding sequence	3256–2396





Appendix 2: Map of pQE30 vector (Qiagen), which was employed to clone the gene encoding meningococcal glutamine synthetase. 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
*lac*Z start codon	180
lac operator	200–216
β-lactamase coding region	1337–2197
phage f1 region	2380–2835
lac 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 3: Map of the pGEM®-T Easy vector, which was employed to construct PIT2 and pComp-1. Adapted from pGEM-T and pGEM-T Easy vectors systems-Promega Corporation.