Molecular and immunological characterisation of glycolytic enzymes: FBA and GAPDH-1 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. All work contained within this report is my own unless otherwise acknowledged.

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ABBREVIATIONS

abcZ	Putative ABC transporter
adk	Adenylate kinase
App	Adhesion and penetration protein
aroE	Shikimate dehydrogenase
AspA	Autotransported serine protease A
AT	Autotransporter pathways
ATP	Adenosine triphsphate
AutA	Auto-transporter A
BBB	Blood brain barrier
BCIP/NBT	5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium
***Bgl*II**	Restriction endonuclease enzyme from *Bacillus globigii*
Bp	Base pair
BSA	Bovine serum albumin
C	Complement
CD	Cluster of differentiation
CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1
CNS	Central nervous system
CSF	Cerebrospinal fluid
dNTPs	De-oxy neuclotide triphosphate
DIC	Disseminated intravascular coagulation
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxy ribonucleic acid
E. coli	*Escherichia coli*
ED	Entner Douderoff
ELISA	Enzyme linked immunosorbant assay
EMP	Embden Meyerhof Parnas pathway
ET	Electrophoretic type
ET	Electrophoretic type
FACS	Fluorescent-activated cell sorter
FBA	Fructose bisphosphate aldolase

fHbp	Factor H binding Protein
fumC	Fumarate hydratase
GAPDH-1	Glyceraldehyde 3-phosphate dehydrogenase
gdh	Glucose-6-phosphate dehydrogenase
GNA	Genome- derived neisserial antigen
GP	Glycoprotein
GSP	General secretory pathway
HBMECs	Human brain microvascular endothelial cells
His	Histidine
HmbR	Haemoglobin binding receptor
HRP	Horseradish peroxidase
HrpA-HrpB	Haemagglutinin/haemolysin-related protein A
HUVEC	Human umbilical vein endothelial cells
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IM	Inner membrane
IPTG	Isopropyl-β-D-thiogalactopyranoside
IS	Insertion sequences
kb	Kilo base pair
kDa	Kilo Dalton
lac	Lactose operator
Lamp1	Lysosome-associated membrane protein 1
LB	Lysogeny Broth
LbpAB	Lactoferrin binding protein A and B
LOS	Lipooligosaccharides
LP	Lipoprotein
LPS	Lipopolysaccharides
mAb	Monoclonal antibody
MAC	Membrane attack complex
MCC	Meningococcal C conjugate vaccine
MCD	Meningococcal disease
MCV4	Quadrivalent meningococcal conjugate vaccine

MHC	Major histocompatibility
MLEE	Multi-locus enzyme electrophoresis
MLST	Mult-ilocus sequence typing
MspA	Meningococcal serine protease A
MSPs	Meningococcal secreted proteins
MWCO	Molecular weight cut-off
NadA	Neisseria adhesion A
NalP	Neisseria autotransporter lipoprotein
Nhha	Neisseria hia homologue
Ni-NTA	Nickel-Nitrilotriacetic acid
NspA	Neisserial surface protein A
Oca	Oligomeric coiled-coil adhesins
OD	Optical density
OM	Outer membrane
OMP	Outer membrane protein
OMV	Outer membrane vesicle
Opa	Colony opacity factor
ORF	Open reading frame
OS	Oligosaccharide
PAI-1	Plasminogen activator inhibitor
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
pdhC	Pyruvate dehydrogenase subunit C
Pfam	Protein families database
PFK	Phosphofructokinase
pgm	Phosphoglucomutase
PorA	Porin A
PorB	Porin B
PP	Pentose Phosphate pathways
PVDF	Polyvinylidene fluoride
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
***Sph*I**	Restriction endonuclease from *Streptomyces phaeochromogens*

SSC	Sodium citrate
ST	Sequence type
Taq	*Thermus aquaticus*
Tat	Twine arginine translocation
TbpAB	Transferring binding protein A and B
TfnR	Transferrin receptor
TLRs	Toll-like receptors
TNF	Tumour necrosis factor
TSP	Two- partner secretion pathway
TspA	T-cell stimulating protein A
VR	Variable region
X-gal	5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside

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PUBLICATIONS

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Summary

There is growing evidence that several glycolytic enzymes, so-called housekeeping enzymes, including fructose bisphosphate aldolase (FBA) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), despite being devoid of any apparent secretion signal, may be localised to the cell surface of several bacterial and fungal species, where they exhibit diverse non-glycolytic biological functions. However, the mechanism(s) of secretion of such signal-less proteins to the cell surface or to external environment is not well understood. Whilst their intracellular functions are well known, it is unclear whether they perform any additional functions, unconnected to their central role in glycolysis, on the bacterial surface. It is becoming apparent that such proteins may be immunogenic and they may be capable of eliciting protective immunity in animal models. As such, they represent potential vaccine candidates. In a search for novel surface-exposed proteins as potential vaccine candidates against *N. meningitidis* serogroup B, and in accordance with the fact that glycolytic enzymes are putative virulence factors in some bacterial species, it is hypothesised that meningococcal FBA and GAPDH-1, may be present on the cell surface and thus may contribute to the pathogenesis of disease.

In *N. meningitidis* serogroup B, there is a single gene *cbbA* (NMB1869) and two genes *gapA-1* and *gapA-2*, predicted to encode fructose bisphosphate aldolase and glyceradehyde 3-phosphate dehydrogenase (GAPDH) enzymes, respectively. Sequence analysis shows that FBA and GAPDH-1 are highly conserved at the amino acid level. The amino acid sequences of FBA from *N. meningitidis* and those from *Xanthobacter flavus* and *Synechocystis* sp. displayed high identities

(67 and 65%, respectively), which suggests that the meningococcal FBA (like those of *X. flavus* and *Synechocystis* sp.) belongs to bacterial Class-II FBP aldolases.

The *cbbA* and *gapA-1* genes were cloned and over-expressed in host *E. coli*. FBA was purified under non-denaturing and denaturing conditions, whilst GAPDH-1 was purified under denaturing conditions. Recombinant native FBA was used in a coupled enzymic assay confirming that it has fructose bisphosphate aldolase activity. The purified FBA and GAPDH-1 proteins were then used to raise polyclonal monospecific rabbit antiserum (RαFBA and RαGAPDH-1) for subsequent characterisation of enzymes with the aim to determine their sub-cellular localization as well as potential roles in pathogenesis of meningococcal disease. RαFBA and RαGAPDH-1 reacted with *ca.* 38-kDa and 37-kDa proteins, respectively, in immunoblot analysis against whole cell lysates from meningococcal strain MC58 but not the *cbbA* and *gapA-1* isogenic mutants, respectively, confirming that *cbbA* and *gapA-1* are naturally-expressed proteins in *N. meningitidis*. Furthermore, expression of *cbbA* was detected in 26/26 and GAPDH-1 in 17/17 diverse meningococcal strains.

Cell fractionation experiments showed that meningococcal FBA and GAPDH-1 are localized both to the cytoplasm and to the outer membrane. These results were validated by flow cytometry. The data demonstrated that outer membrane-localized FBA was surface-accessible to FBA-specific antibodies in encapsulated *N. meningitidis*, whereas flow cytometry analysis confirmed that GAPDH-1 could be detected on the cell surface, but only in a *siaD*-deficient background,

suggesting that GAPDH-1 is inaccessible to antibody in encapsulated meningococci. Mutational analysis and functional complementation was used to identify additional functions of FBA and GAPDH-1. The *cbbA* and *gapA-1* knock-out mutant strains were unaffected in their ability to grow *in vitro*, but showed a significant reduction in adhesion to HBME and HEp-2 cells compared to their isogenic parent and complemented derivatives. In a transgenic mouse model, *cbbA* mutant strains were shown to be less able to establish bacteraemia compared to their wild-type parent strains.

In summary, in this study, expression of FBA and GAPDH-1 was shown to be well conserved across diverse isolates of *Neisseria* species. This study also demonstrates for the first time that meningococcal glycolytic enzymes, FBA and GAPDH-1, are surface localised proteins and required for optimal adhesion of meningococci to host cells. Taken together, these results suggest that FBA and GAPDH-1 may be involved in the pathogenesis of meningococcal disease.

CHAPTER 1: General Introduction

1.1 Introduction

1.2 Historical background

Meningococcal disease (MCD), previously known as cerebrospinal fever, was first described by Vieusseux in the area around Geneva, Switzerland, in the spring of 1805. Just two years after this report, epidemics of what appeared to be the same disease were reported in New England and among the Prussian army. Following these outbreaks, the disease became widely recognised in Europe, in parts of Asia and in America (DeVoe, 1982). However, it was not until 1887 that an equally novel discovery was made by the Austrian pathologist Anton Weichselbaum, who identified the etiological agent of meningococcal disease by showing, for the first time, that there was a connection between *Neisseria meningitidis* (then known as *Diplococcus intracellularis meningitidis*) and 'epidemic cerebrospinal meningitidis' (de Souza & Seguro, 2008).

1.3 Cultural and biochemical characteristics of *N. meningitidis*

N. meningitidis, (the meningococcus) is a Gram-negative, oxidase and catalase positive, non-sporing, aflagellate, aerobic diplococcus of approximately 0.8 μm in diameter. The bacterium may be encapsulated or unencapsulated, and is a member of the bacterial family *Neisseriaceae* (Ala'Aldeen & Turner, 2006; Stephens *et al.*, 2007). This family includes the genera *Neisseria, Moraxella, Kingella*, and *acinetobacter.* The genus Neisseria includes two human pathogens, *N. meningitidis* and *N. gonorrhoeae. N. meningitidis* is relatively fastidious in growth requirements and grows reasonably well on blood, chocolate, Modified New York City medium, and on Muller-Hinton Agar. Optimum growth

conditions can be achieved at 35-37°C at pH 7.0-7.4 in a humid environment with 5-10% CO_2 (Ala'Aldeen & Turner, 2006).

1.4 Epidemiology of meningococcal disease

In spite of considerable success in the development of drugs and effective vaccines, the problem of disease due to *N. meningitidis* is far from solved (Peltola, 1983) and still remains a serious threat to global health (Fig 1.1) occurring sporadically throughout the world (Milonovich, 2007). Strains of *N. meningitidis* have been classified into 13 serogroups on the basis of the immunological specificity of capsular polysaccharides but invasive meningococcal infection is limited to the serogroups A, B, C, W-135, Y and more recently X (Stephens, 2007; Tzanakaki & Mastrantonio, 2007).

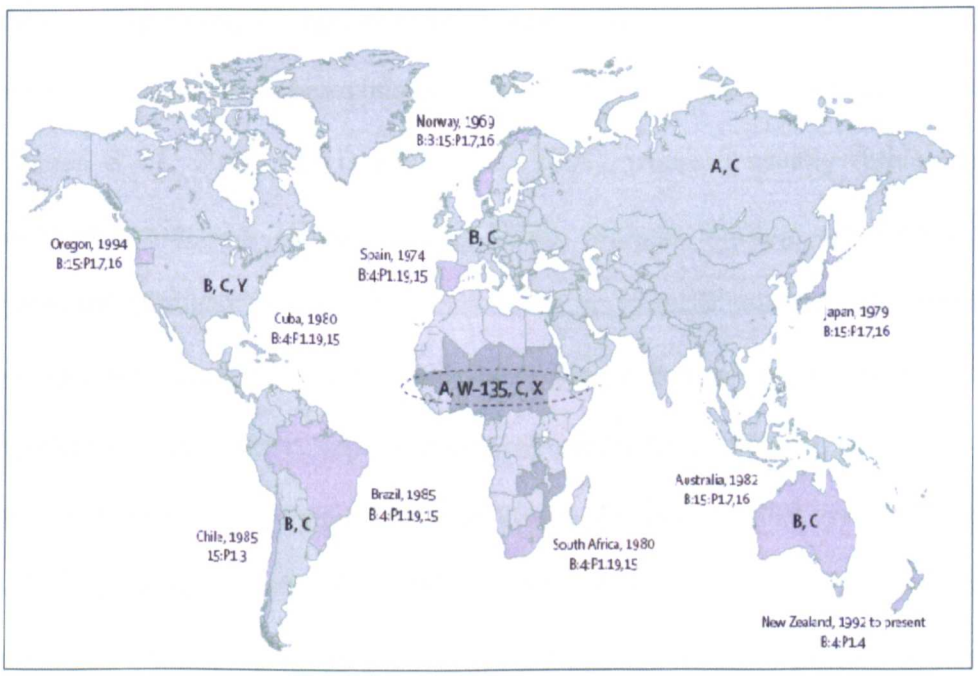


Figure 1. 1 Worldwide distribution of major meningococcal serogroups and outbreaks of serogroup B by serotype (shaded in purple). The meningitis belt (dotted line) of Sub-Saharan Africa and other areas of substantial meningococcal disease in Africa are shown (adapted from reference Stephens *et al.*, 2007).

The five common serogroups: A, B, C, Y and W-135 are responsible for more than 90% of the total cases of invasive meningococcal disease in the human population (Serruto *et al.*, 2004). However, serogroups A, B, and C account for most of the meningococcal disease throughout the world (Tzanakaki & Mastrantonio, 2007).

The bacterium is not only a common commensal of the human upper respiratory tract (nasopharynx) but also an important and devastating human pathogen that remains a leading cause of bacterial meningitis and septicaemia (Schneider *et al.*, 2007; Tzanakaki & Mastrantonio, 2007), affecting all age groups but primarily targeting children and young adults (Harrison, 2006; Soriano-Gabarro *et al.*, 2002). The only natural reservoir of *N. meningitidis* is the human nasopharyngeal mucosa. Depending on age, climate, country, socioeconomic status, and other factors, it is carried by approximately 10% of adult population in the nasopharynx (Schoen *et al.*, 2007; van Deuren *et al.*, 2000), where it usually resides as a harmless commensal (Girard *et al.*, 2006), without causing any detectable symptoms (Caugant *et al.*, 2007). However, in a small number of colonized persons, the organism can traverse the mucosal tissues to gain access into bloodstream to cause septicaemia/meningococcemia and/or subsequently progresses to the cerebrospinal fluid (CSF) by mechanisms that are not clearly understood, to cause meningitis (Nassif, 1999). The traversal of these barriers are essential steps in the pathogenesis of meningococcal meningitis (Sokolova *et al.*, 2004). The invasive disease is very rare and only occurs when the following conditions are fulfilled: (i) contact with a virulent strain, (ii) colonization by that strain, (iii) penetration of the bacterium through the mucosa, and (iv) survival and

eventually outgrowth of the meningococcus in the bloodstream (van Deuren *et al.*, 2000). The septicaemia / meningococcemia and meningitis caused by the meningococcus are collectively known as meningococcal disease.

Serogroup A meningococcus has remained a major cause of meningococcal disease in the so called "African Meningitis Belt", which spans several sub-saharan countries, and also in parts of Asia (Schoen *et al.*, 2007; Stephens, 2007). The African Meningitis Belt was first described by Lapyeysonnie in 1963, and initially comprised Burkina Faso, Ghana, Togo, Benin, Niger, Nigeria, Chad, Cameroon, Central African Republic, and the Sudan. Later it was extended to include Ethiopia, Mali, Guinea, Senegal and Gambia, which is now known as "The Expanded Meningitis Belt" (van Deuren *et al.*, 2000). Outbreaks within the African Meningitis Belt have been reported to prevail in the dry season and gradually reduce in the rainy season (Stephens, 2007).

Serogroup B disease accounts for a substantial portion of cases in the United States, with half of the invasive infections occurring in infants (Harrison, 2006), and also remains the major cause of sporadic or endemic disease in many industrialized countries, accounting for 30-70% of cases of disease (Schoen *et al.*, 2007). Prolonged outbreaks have been described in Europe, Cuba, Chile, and recently in New Zealand, where it has caused significant morbidity and mortality (Stephens, 2007).

Serogroup C strains cause small scale outbreaks worldwide. However, it has been associated with an endemic pattern of disease since the 1990s, causing multiple

outbreaks in schools and in the community in North America and Europe (Harrison, 2006).

Serogroup Y cases have substantially increased in number in recent years, accounting for 30% of the cases in United States (Schoen *et al.*, 2007). This serogroup has also classically been associated with cases of meningococcal pneumonia (Harrison, 2006).

Another relatively uncommon meningococcal serogroup is W-135, which currently accounts for fewer than five percent of all cases worldwide (Rosenstein *et al.*, 2001). The first international outbreak of serogroup W-135 meningococcal disease was reported among pilgrims to the Hajj in Saudi Arabia during 2000-2001, and in Burkina Faso in 2002 (Chiou *et al.*, 2006; Taha *et al.*, 2003). Serogroup W-135 causes a relatively small number of cases in the United States (Harrison, 2006), and is similarly uncommon in Europe.

1.5 Pathogenesis of meningococcal disease

There are a multitude of molecular/cellular receptors and mediators that are involved in the outcome of host-meningococcal interactions (Emonts *et al.*, 2003; van der Flier *et al.*, 2003). These factors work in concert with various strongly interacting pathways within the vascular tree and in the subarchnoid space (Van Amersfoort *et al.*, 2003). A key factor in meningococcal pathogenesis is the ability of bacteria to attach to host cell receptors, which is a complex phenomenon and involves different adhesive factors depending upon the environment that meningococci encounter with the host cell (Serruto *et al.*, 2003). The primary attachment is mediated by Type IV pili that act as 'meningococcal sensory organ' establishing the primary cellular communication between the organism and host target cells (Nassif, 2000).

1.5.1 Colonisation

N. meningitidis, an obligate human commensal, colonizes the nasopharynx (Fig. 1.2) and spreads from person to person by direct contact or via respiratory droplets (from a distance up to approximately 1 metre) from infected (with a potentially pathogenic strain) but asymptomatic carriers to other healthy individuals (Caugant *et al.*, 2007). The human naso-oropharyngeal mucosa serves as the reservoir for the spread of meningococci in the population. A number of molecules are required by the bacteria to enable them to colonise and/or infect the host, including adhesins, which are key factors that are required for initial colonisation of human mucosal sites (Virji, 2009).

Colonisation of the upper respiratory mucosal surfaces by *N. meningitidis* is the first step in the establishment of a human carrier state and invasive meningococcal disease (Stephens, 2009). Colonisation by the meningococcus occurs both at the exterior surface of the mucosal cell and intra- or subepithially (van Deuren *et al.*, 2000). Colonization of the oropharynx by meningococci produces an antibody response from the three major immunoglobulin classes within a few weeks after acquisition of the bacterium and may act as an immunizing event (Kremastinou *et al.*, 1999). Most oropharyngeal carriers not only develop homologous antibodies but also develop heterologous antibodies to other meningococcal strains (Goldschneider *et al.*, 1969). Damage to nasopharyngeal ciliated epithelium may be an important first step in meningococcal colonization of the human nasopharynx (Rayner *et al.*, 1995; Stephens *et al.*, 1986).

Active or passive tobacco smoke exposure independently increases the risk of carriage and developing meningococcal disease (Fischer *et al.*, 1997; Haneberg *et al.*, 1983; Stuart *et al.*, 1989).

1.5.2 Adhesion

Adhesion to human mucosal surfaces is essential for meningococcal survival. Stephens *et al.*, developed a human nasopharyngeal organ culture model and found that meningococci specifically bind non-ciliated columnar epithelial cells and induce pseudopodia that ultimately result in internalization of meningococci within these cells (Stephens *et al.*, 1983).

The identified meningococcal adhesins include Pili, PilC, PilQ, Opa, Opc, LOS, Factor H-binding protein, PorA, HrpA, PorB and NadA and their proposed host receptors include platelet activating factor, CD46, CEACAM1, vitronectin and α-actinin integrins, complement receptor 3, laminin and the CD scavenger receptor (reviewed in Stephens, 2009). Pili are major adhesins that contribute to meningocoocal attachment to mucosal cells. Stephens and McGee found that pilated meningococcal strains consistently attached to human nasopharyngeal cells in greater numbers than meningococci without pili suggesting that pili are important mediators of meningococcal attachment to host tissues. However, the number and distribution of receptor sites for pili or pili-associated meningococcal ligands differ among human cells and may determine sites of meningococcal colonization (Stephens & McGee, 1981). Pili have been shown to bind to receptors on nasopharyngeal cells, i.e. the membrane co-factor protein or CD46 (Kallstrom *et al.*, 1997) and this binding induces signal transduction pathways in host cells (Kallstrom *et al.*, 1998)

After attachment, meningococci continue to proliferate on the surface of human non-ciliated epithelial cells, resulting in the formation of small microcolonies at the site of initial attachment. Intimate adherence of meningococci to the host epithelial cells result in the formation of cortical plaques (Stephens, 2009) and leads to the recruitment of factors ultimately responsible for the formation and extension of epithelial cell pseudopodia that engulf the meningococcus (Doulet *et al.*, 2006). After initial binding, intimate association is mediated via class 5 outer membrane proteins (OMPs) such as opacity proteins, Opa and Opc with

CD66/CEACAMs and integrins, respectively, on the surface of the epithelial cells (Virji *et al.*, 1996).

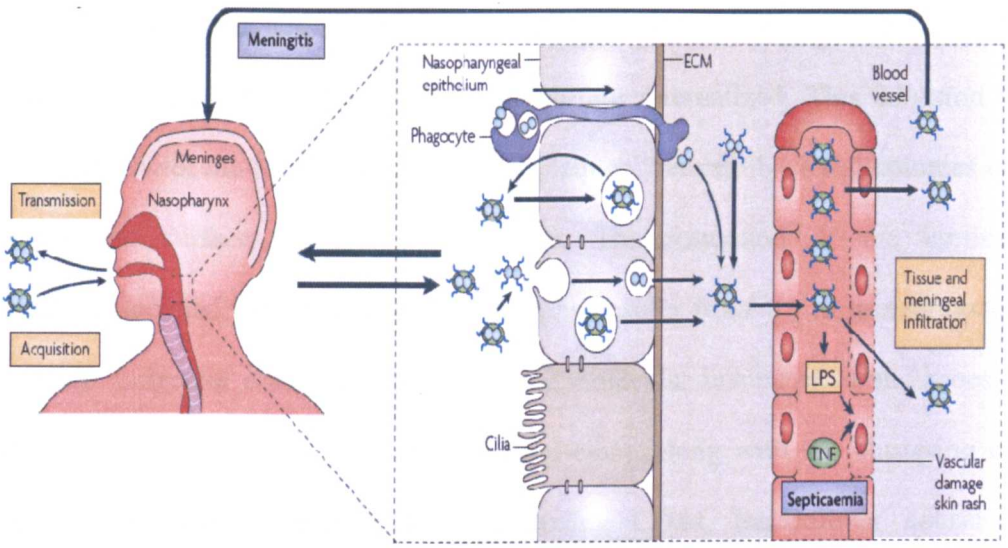


Figure 1. 2 Stages in the pathogenesis of *N. meningitidis*. *N. meningitidis* may be acquired through the inhalation of respiratory droplets. The organism establishes intimate contact with non-ciliated mucosal epithelial cells of the upper respiratory tract, where it may enter the cells briefly before migrating back to the apical surfaces of the cells for transmission to a new host. Asymptomatic carriage is common in healthy adults in which bacteria that enter the body by crossing the epithelial barrier are eliminated. Besides transcytosis, *N. meningitidis* can cross the epithelium either directly following damage to the monolayer integrity or through phagocytes in a 'Trojan horse' manner. In susceptible individuals, once inside the blood, *N. meningitidis* may survive, multiply rapidly and disseminate throughout the body. Meningococcal passage across the brain vascular endothelium (or the epithelium of the choroid plexus) may then occur, resulting in infection of the meninges and the cerebrospinal fluid (adapted from reference Virji, 2009).

1.5.3 Invasion

Meningococci traverse the mucosal epithelium via phagocytic vacuoles (Fig 1.2) as a result of endocytosis (McGee *et al.*, 1983; Nassif & So, 1995; Stephens *et al.*, 1983; Stephens & Farley, 1991). During invasion several bacterial factors have been demonstrated to modulate the metabolism of the mucosal cells (Virji, 1996). Two virulence factors appear to be essential for meningeal invasion by *N.*

meningitidis: the capsular polysaccharide, and type IV pili (Nassif *et al.*, 1994; Virji *et al.*, 1991). Type IV pili play a pivotal role in meningeal invasion, which is associated with an increase in the expression of PilC (Hardy *et al.*, 2000; Pron *et al.*, 1997). Capsulated piliated bacteria adhere to the apical surface of the cells and only a small proportion of the bacteria are internalized. This adhesion is associated with the formation of 'cortical plaques' beneath bacterial colonies on the apical surface (Merz *et al.*, 1999). The formation of the cortical plaque-associated cell membrane protrusions results from the organization of specific molecular complexes involving the molecular linkers ezrin and moesin (known as ERM [ezrin-radixin-moesin] proteins), along with the clustering of several membrane-integral proteins, including CD44, intracellular adhesion molecule (ICAM)1, and cortical actin polymerization (Eugene *et al.*, 2002; Hoffmann *et al.*, 2001; Lambotin *et al.*, 2005; Merz *et al.*, 1999). Some lipooligosaccharide (LOS) mutant meningococcal strains, show structurally altered actin polymerization and are defective in the recruitment and phosphorylation of cortactin, thus are poorly invasive (Hoffmann *et al.*, 2001).

1.5.4 Survival of meningococcus in bloodstream

Meningococci can survive and proliferate in the bloodstream through the expression of particular bacterial virulence factors or because of naivety or specific immune system defects in the host (van Deuren *et al.*, 2000). The steps of meningococcal intracellular survival and transcytosis through the basolateral tissues and dissemination into the bloodstream are less well studied. Following the successful traversal of nasopharyngeal mucosa, *N. meningitidis* progresses to sub-epithelial tissue and may gain access to the bloodstream. Once viable

meningococci have reached the bloodstream, the bacterium, in order to protect itself in such a hostile environment, requires the expression of the capsule which is known to protect against complement-mediated bacteriolysis and phagocytosis (Klein *et al.*, 1996). In addition some class 1 OMPs hamper ingestion of meningococcus by neutrophils via down-regulation of the Fcγ receptor and the C1 and C3 receptor (Bjerknes *et al.*, 1995). Meningococcal intracellular survival is determined by factors including IgA_1 protease, which degrades lysosome-associated membrane proteins (LAMPs), thus preventing phagosomal maturation, and up-regulation of expression of capsule (Hopper *et al.*, 2000). Furthermore, IgA1 protease appears to break IgA1, releasing monomeric Fabα fragments, that can block the interaction of IgG and IgM (Mulks & Plaut, 1978).

Intracellular meningococci reside within a membranous vacuole and are capable of translocating through the epithelial layers within 18–40 h (Stephens *et al.*, 1983; Stephens *et al.*, 2007). Following internalization *N. meningitidis* are capable of intracellular replication and this is due in part to the capacity of the organism to acquire iron through specialized transport systems, such as the hemoglobin-binding receptor (HmbR), transferring-binding protein (TbpAB) and lactoferrin-binding protein (LbpAB) (Perkins-Balding *et al.*, 2004).

1.5.5 Nasopharayngeal carriage and mucosal immunity

The meningococcus has its natural habitat in the mucus membranes of the oropharynx of the human host. Those harbouring the organism are usually asymptomatic and are commonly referred to as 'carriers'. The frequency of carriage in the normal population ranges from 10-30% during non-epidemic

periods but may approach 100% during epidemics (DeVoe, 1982). The duration of meningococcal carriage can vary from days to months (Cartwright *et al.*, 1987; Stephens, 1999). The probability of meningococcal disease after the acquisition of *N. meningitidis* declines very sharply, such that invasive disease becomes unlikely 10-14 days after acquisition (Stephens, 2009). Meningococcal carriage is affected by age, intimate personal contacts, crowding (eg, bars, dormitories) and smoking (Tzeng & Stephens, 2000; Yazdankhah & Caugant, 2004). Damage to upper respiratory tract by co-infections (e,g. mycoplasma, influenza, and other respiratory viral infections), smoking, very low humidity, drying of mucosal surfaces, and trauma induced by dust, predisposes to carriage and meningococcal disease (Artenstein *et al.*, 1967; Greenwood *et al.*, 1985; Moore *et al.*, 1990; Young *et al.*, 1972).

The first and most important line of defense against infection with *N. meningitidis* is the integrity of the mucosal membrane. The increased incidences at the end of the dry season in Africa have been attributed to the dryness of the air, which probably influences the integrity of the mucosal membranes of nasopharynx (Verheul *et al.*, 1993). Asymptomatic carriage by *N. lactamica*, a non-pathogenic species of *Neisseria*, occurs especially during early childhood and is associated with a rise in antibody titers to pathogenic meningococci, and these cross-reactive antibodies may be useful in the development of natural immunity to *N. meningitidis* (Gold *et al.*, 1978; Goldschneider *et al.*, 1969). Rates of carriage of *N. lactamica* increase from 3.8% in 3-month old infants to a peak of 21.0% at 18 months and then decline to 1.8% by 14-17 years of age. However,

in the same population, the prevalence of *N. meningitidis* average 0.71% during the first four years of life and increase to 5.4% in teenagers (Gold *et al.*, 1978).

1.5.6 Host susceptibility

The presence of sufficient pre-existing immunity or ability to rapidly generate a relevant immune response limits new acquisition to the mucosal surface, whereas immunological naivety is permissive for invasion (Wall, 2001). The most well known host factors that predispose invasive meningococcal disease include deficiencies in the terminal complement pathway (C5-C9) and in properdin. (Fijen *et al.*, 1999; Sjoholm *et al.*, 1982). Disappearance of maternally transferred antibodies increases the risk of invasive meningococcal disease in infants and young children (Stephens *et al.*, 2007).

A number of additional host factors have been recognised to be associated with increased risk of meningococcal disease. These include polymorphisms in genes encoding for Fcγ-receptor II (CD32), Fcγ-receptor III (CD16), mannose-binding lectin, TLR4, and plasminogen activator inhibitor (PAI-1) (Emonts *et al.*, 2003; Faber *et al.*, 2006; Fijen *et al.*, 2000; Hibberd *et al.*, 1999; Read *et al.*, 2001; Smirnova *et al.*, 2003; Tully *et al.*, 2006). Furthermore, viral (influenza) and mycoplasma respiratory infections can predispose to meningocoocal disease, perhaps by damaging mucosal surfaces, altering dynamics of adherence, and spread and impairing mucosal immunity (Moore *et al.*, 1990). Infectious agents such as enteric bacteria may induce cross-reacting IgA antibodies, which competitively inhibit the binding of bactericidal IgG and IgM antibodies to the meningococcus (Wenzel *et al.*, 1973).

1.6 Clinical manifestations of invasive meningococcal disease

N. meningitidis causes a spectrum of disease ranging from benign self-limited meningococcemia to fulminant septic shock, multiple organ failure, and death (can happen within 6 h after the symptoms). Among the wide range of clinical manifestations of meningococcal disease, the two most common are meningitis and septicaemia (Kirsch *et al.*, 1996), which may co-exist.

1.6.1 Meningococcal septicaemia

Meningococcal septicaemia is the most severe form of infection, characterized by wide spread haematogenous dissemination, but is less common than meningitis. Meningococcal septicaemia may be: transient, chronic and fulminant. The clinical course of severe meningococcaemia is rapidly progressive, with the time from onset of fever until death often as short as 12 h (Kirsch *et al.*, 1996).

Transient meningococcaemia is characterized by fever and non-specific rash, and is usually detected as an unexpected result from blood culture. Both fever and rash can disappear within 2-5 days without treatment (Stephens *et al.*, 2007).

Chronic meningococcaemia is rare infection that can lasts from weeks to months without meningeal symptoms. The symptoms include intermittent fever, arthralgia, signs of vasculitis, and a non-specific maculopapular rash. Symptoms may disappear for days and then re-appear (Harwood *et al.*, 2005; Stephens *et al.*, 2007).

Fulminant meningococcal septicaemia is characterised by a rapid proliferation of meningococci in the circulation, resulting in very high concentrations of bacteria (10^5–10^8 ml^{-1}) and meningococcal endotoxin (10^1–10^3 EU ml^{-1}) (Brandtzaeg *et al.*, 1989; Brandtzaeg *et al.*, 2001; Stephens *et al.*, 2007).

Patients suffering from fulminant meningococcal septicaemia may present with severe, persistent shock, lasting more than 24 h or until death, and no distinct clinical signs of meningitis. Due to the presence of few meningococci in the cerebrospinal fluid (CSF), pleocytosis is negligible (Brandtzaeg *et al.*, 1989; Brandtzaeg *et al.*, 1992; Stephens *et al.*, 2007). Other clinical presenting features include impaired renal, adrenal, and pulmonary function and disseminated intravascular coagulation with thrombotic lesions in the skin, limbs, kidneys, adrenals, choroids plexus, and occasionally the lungs (Hazelzet *et al.*, 1996; Stephens *et al.*, 2007). The septemic inflammatory response leads to progressive circulatory collapse and severe coagulopathy. Patients may suffer from vascular complications that can lead to loss of digits or limbs, and survivors can be severely handicapped (Brandtzaeg *et al.*, 1989; Hazelzet *et al.*, 1996; Hazelzet *et al.*, 1998).

1.6.2 Meningococcal meningitis

Meningitis is the most common clinical presentation of invasive meningococcal disease; more than 60% of patients in industrialized countries develop meningitis without shock. In developing countries, the proportion of patients with meningitis is much higher than that in developed countries (Stephens *et al.*, 2007). In meningococcal meningitis bacteria are localized primarily to the meningeal

compartment, resulting in a clinical picture indistinguishable from other forms of bacterial meningitis (Kirsch *et al.*, 1996).

Patients with meningitis usually have a low concentration of meningococci ($<10^3$ ml^{-1}) and endotoxin (<3 EU ml^{-1}) in plasma but high concentrations in CSF (Brandtzaeg *et al.*, 1989; Brandtzaeg *et al.*, 1992; Ovstebo *et al.*, 2004), leading to a compartmentalised inflammatory response in the subarchnoid space, with a pronounced increase in the concentrations of tumor necrosis factor α (TNF-α), interleukins (IL-1β, IL-6, IL-8, and IL-10), different chemokines, and other mediators (Stephens *et al.*, 2007).

In adult patients with meningococcal meningitis the following symptoms and signs predominate: headache, fever, vomiting, photophobia, neck stiffness, positive Kernig's and Brudzinski's signs, and lethargy. In infants and younger children, non-specific symptoms including poor feeding, irritability, a high pitched cry and a bulging fontanelle are typical findings. Seizures may occur in up to 20% of cases and meningitis is a cause of a first episode of status epilepticus in 12% of cases (Nadel & Kroll, 2007).

1.7 Diagnosis and chemotherapy of meningococcal disease

Clinical diagnosis of meningococcal disease largely relies on the recognition of symptoms such as fever, rash and meningeal signs and altered mental status, and is confirmed by pleocytosis, gram stain with and without culture of cerebrospinal fluid, or blood or skin lesions (Stephens *et al.*, 2007). In early stages of meningococcal disease diagnosis is, however, extremely difficult especially when there is no epidemic outbreak of the disease, and requires a high degree of suspicion, as symptoms may be similar to other conditions (e.g. Influenza and other viral infections), and specific signs may be absent. In a recent study on clinical recognition of meningococcal disease in children and adolescents it was shown that classical features developed later on in disease progression (median time of onset 13-22 h after symptoms began), where as early less-specific features of sepsis such as leg pain, cold hands and feet and abnormal skin colour first developed after a median period of 8 h in the majority of children (Thompson *et al.*, 2006).

Cultures of blood, or CSF, (in the absence of contraindications for lumber puncture), and skin lesion aspirates may confirm the diagnosis (Nadel & Kroll, 2007). Latex agglutination assays on blood, CSF, or urine have been used as adjunctive diagnostic tests but have a poor sensitivity and specificity (Perkins *et al.*, 1995). Meningococcal PCR is now widely used in developed countries for diagnosis.

As soon as the disease is first suspected based on suggestive clinical features, initial treatment with antimicrobials should not be delayed whilst waiting for the

results of laboratory investigations. Penicillin resistance is rare amongst clinical isolates of *N. meningitidis* in the UK. Benzylpenicillin or a third generation cephalosporin such as ceftriaxone is usually used for specific treatment. The recommended duration of antibiotic therapy for meningococcal disease is 7 days for both meningococcal meningitis and septicaemia (Nadel & Kroll, 2007). Until the diagnosis is established, the patients must be treated empirically with broad spectrum bactericidal antibiotics (such as cefotaxime or ceftriaxone and amoxicillin [for Listeria cover] depending on age) (Peltola *et al.*, 1989).

1.8 Classification of *N. meningitidis*

Several classification systems, to aid in epidemiological studies, have been developed for *N. meningitidis*. The most clinically relevant and well established is a serological typing method that divides strains immunologically into serogroups, serotypes, and serosubtypes, based on antigenic differences in their capsule, PorB protein, PorA protein, and LOS, respectively. For example, a serogroup B serotype 4, serosubtype 15 and immunotype 10 is written as B:4:P1.15:L10 (Ala'Aldeen & Turner, 2006).

Multi-locus enzyme electrophoresis (MLEE) uses the natural electrophoretic mobility of various cytoplasmic enzymes whose molecular weights vary between different strains of meningococci. Using MLEE it was possible to classify meningococci with similar characteristics into clonal families, designated electrophoretic type (ET) (Caugant *et al.*, 1986a; Caugant *et al.*, 1986b).

A genotyping method based on sequence analysis called multi-locus sequence typing (MLST) has also been developed for *N. meningitidis* (Maiden *et al.*, 1998). The method has been a valuable approach in revealing associations between isolates, showing the existence of distinct clones over-represented in certain communities (Chiou *et al.*, 2006; Feavers *et al.*, 1999; Maiden *et al.*, 1998; Murphy *et al.*, 2003; Nicolas *et al.*, 2001).

MLST, an adaptation of multi-locus enzyme electrophoresis, is fully portable and data stored in a single expanding central multi-locus sequence database can be interrogated electronically via the internet providing a powerful resource for global epidemiology (Maiden *et al.*, 1998).

MLST is based on DNA sequence variation in specific regions of seven housekeeping genes (*abcZ, adk, aroE, fumC, gdh, pdhC, pgm*) that range from 433 to 501 bp in length. For each gene fragment, different sequences are assigned as distinct alleles, and each isolate is defined by the combination of alleles at each of the seven housekeeping loci. This is known as the allelic profile or sequence type (ST). The STs are assigned to lineages using the BURST software (http://neisseria.mlst.net) (Baethgen *et al.*, 2008).

1.9 Virulence factors of *N. meningitidis*

The meningococcus can avoid the host defense mechanisms through the expression of several surface components and secretion of various molecules as well as shedding complex vesicle structures (blebs) that may modulate or deflect the immune system (Wall, 2001). It can also undergo phase and antigenic variation (Berrington *et al.*, 2002; Jennings *et al.*, 1999), and is able to use host factors for its own protection and growth. Some of the bacterial factors that may be employed by the meningococcus to evade immune killing and in establishment of infection are discussed below.

1.9.1 Capsule

The polysaccharide capsule is a major virulence factor and plays a crucial role in invasive meningococcal disease. The capsule confers resistance to a number of arms of the immune system (Schneider *et al.*, 2007). The capsule of the pathogenic meningococcal serogroups differs in both chemical structure and antigenenic properties (Morley & Pollard, 2001). Capsule is the basis for immunological serogrouping and confers resistance to the meningococcus against phagocytosis, complement-mediated lysis and offers protection to the cells against environmental insults (McNeil *et al.*, 1994; Morley & Pollard, 2001; Schoen *et al.*) Thirteen structurally distinct capsular serogroups have been described and six (A, B, C, W-135, Y, and X) of which cause the invasive meningococcal disease (Stephens *et al.*, 2007). Of six invasive meningoccoal serogroups, the capsule of four serpgroups (B, C, Y, and W-135) , except serogroup A, is composed of sialic acid derivatives, which bestows the organism

antiphagocytic properties enhancing its survival in the bloodstream or central nervous system (Stephens, 2009).

1.9.2 Lipooligosaccharide

Meningococcal lipopolysaccharide (LPS, endotoxin), also referred to as lipooligosaccharide (LOS), is composed of an oligosaccharide (OS) portion with a phosphorylated diheptose (Hep) core attached to the toxic lipid A moiety embedded in the outer membrane (Griffiss *et al.*, 1988; Stephens, 2009). Meningococcal lipid A is responsible for much of the biological activity and toxicity of meningococcal endotoxin. Neisserial lipopolysaccharide lacks repeating O-antigens and is thus often referred to as LOS. The core oligosaccharide of meningococcal LOS and the two short chains are attached to the two inner core heptose residues. The oligosaccharide chains vary in composition and are the basis for immuno-typing meningococcal strains (Tzeng & Stephens, 2000). Meningococcal LOS is subject to phase variation of its terminal structures allowing switching between immunotypes, which is proposed to have functional significance in disease (Berrington *et al.*, 2002).

1.9.3 Pili

Pili (hair-like projections; also known as fimbriae) (Fig 1.3) are thin filamentous protein structures on the surface of *N. meningitidis* and are composed of repeating identical subunits (pilins) of approximately 17 to 21 kDa. The meningococcal pili belong to the type IV family of pilins. Recent systematic genetic analysis has identified 15 proteins that are involved in the biogenesis, assembly and disassembly of pili (known as pil proteins) (Virji, 2009). The meningococcal pili

play a key role in mediating the interaction of the bacterial cell with host cellular membranes leading to the bloodstream invasion from the nasopharynx, and the traversal across the blood-brain barrier (BBB) (Nassif, 1999). In addition to adhesion, pili are involved in several other functions. For example, they facilitate uptake of foreign DNA from the extracellular milieu, thereby increasing the transformation frequency of bacteria, and are also responsible for twitching motility (Frye *et al.*, 2006; Fussenegger *et al.*, 1997). *N. meningitidis* is genetically capable of producing antigenically different pilins. There are two types of pilin designated class I and class II, produced by a single meningococcal cell (Nassif, 1999).

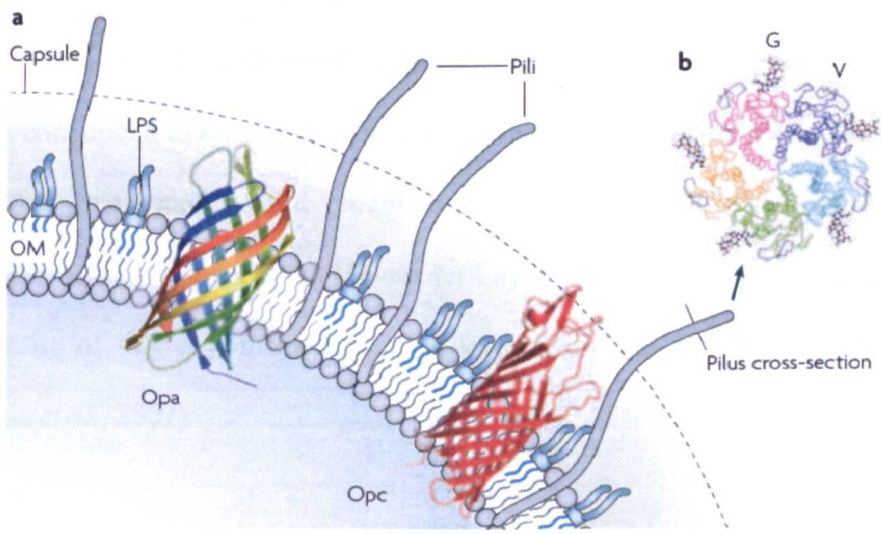


Figure 1. 3 Prominent outer membrane components of *N. meningitidis* that influence bacterial interaction with host cells, (a) Pili traverse the capsule and are the most prominent adhesins of encapsulated *N. meningitidis*. In addition, the integral outer membrane (OM) adhesins, Opa and Opc, are also known to mediate interactions with specific host-cell receptors. Lipopolysaccharide may interfere with the adhesion functions of OM proteins, but can also contribute to cellular interactions by interacting with various cellular receptors. (B) A cross-section of a pilus fibre showing that variable domains (V) and glycans (G) as well as other substitutions (not shown) are located externally, whereas the constant domains are buried within the fibre, protected from the host environment (adapted from reference Virji, 2009).

1.9.4 Major outer membrane proteins

Meningococcal outer membrane proteins have been grouped into five major classes based on their apparent molecular weights on sodium dodecyl sulphate (SDS) polyacrylamide gels and peptide analysis (Tsai *et al.*, 1981). All five classes of OMPs have been studied as potential vaccine candidates in *N. meningitidis* (Chiou *et al.*, 2006).

All meningococci express either a class 2 or class 3 outer membrane protein named PorB and most strains also express a class 1 outer membrane protein named PorA (Hitchcock, 1989). A two-dimensional secondary structure containing eight surface exposed loops (I-VIII) has been predicted for PorA and most variability is thought to occur in variable regions 1 and 2 (VR1 and VR2) which correspond to loops I and IV, respectively (Feavers *et al.*, 1992; Maiden *et al.*, 1991). In addition, a third variable region, designated VR3 has been reported to be present on the top of loop V (van der Ley *et al.*, 1991), however, the genetic variability of this region appears to be lower than that of VR1 and VR2 (de Filippis *et al.*, 2007).

The outer membrane protein PorB of *N. meningitidis* is a pore-forming protein which has various effects on eukaryotic cells. It has been shown to (1) up-regulate the surface expression of the co-stimulatory molecule CD86 and of MHC class II (which are TLR2/MyD88 dependent and related to the porin's immune-potentiating ability), (2) be involved in prevention of apoptosis by modulating the mitochondrial membrane potential, and (3) form pores in eukaryotic cells (Massari *et al.*, 2005).

The class1 PorA and Class 2/3 PorB proteins have been studied most because they are also used in the meningococcal typing scheme for the serosubtype and serotype, respectively. Both proteins contain variable and hyper variable regions (Diggle & Clarke, 2006).

Reduction modifiable protein M was formally known as the class 4 outer membrane protein of *N. meningitidis*. The protein shows 94.2% homology with protein III of *N. gonorrhoeae,* with both proteins having two potential disulfide loops. The protein also shares low-level homology with *Escherichia coli* OmpA (Klugman *et al.*, 1989).

The *N. meningitidis* opa (opacity) proteins and Opc (opacity protein 5c), (Fig 3.1) are highly immunogenic, trimeric or tetrameric basic outer membrane proteins with a similar molecular mass of *ca.* 30 kDa, which migrate aberrantly slowly on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) unless they have been fully denatured by boiling (Achtman *et al.*, 1988). Opa proteins are distinguished from the Opc protein because they have very little sequence similarity (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; Nassif, 1999; Prince *et al.*, 2001).

1.9.5 Non-capsular virulent factors of *N. meningitidis*

1.9.5.1 Neisseria hia homologue A (NhhA)

NhhA is a well-conserved outer membrane, autotransporter protein, which was identified as a homologue of the adhesin AIDA-I of *E. coli* in *N. meningitidis.*

This gene was designated *nhhA* (Neisseria hia homologue) due to its close homology to the Hia and Hsf adhesin of non-typeable *H. influenzae* (Peak *et al.*, 2000). Antibodies to NhhA can be detected from the sera of healthy carriers as well as patients suggesting that NhhA is expressed during colonization of humans.

1.9.5.2 Adhesion and penetration protein (App)

App stands for Adhesion and penetration protein (App). The gene was so named because of its homology to Hap (*Haemophilus* adherence and penetration protein). App is a highly conserved, surface-localised, immunogenic protein, which belongs to the autotransporter family of proteins (Hadi *et al.*, 2001). App was identified by genome analysis and by screening of a *N. meningitidis* expression library using polyclonal antisera raised against the OMPs from *N. meningitidis* (Hadi *et al.*, 2001). App appears to be expressed during invasive disease, as sera from convalescent patients contain antibodies that recognize recombinant App. App has also been demonstrated to function as adhesin in *N. meningitidis*. It binds to epithelial cells, but not endothelial cells (Serruto *et al.*, 2003).

1.9.5.3 Autotransported serine Protease A (AspA/NalP)

AspA is a 112-kDa phase variable classical autotransporter protein containing a subtilisin-type serine protease motif in the passenger domain. AspA was bioinformatically identified from the genome of *N. meningitidis* serogroup A. It shares significant homology with autotransported serine protease of *Serratia marcescens*. Although the *aspA* gene is phase variable, its amino acid sequence

appears to be well conserved in serogroup A, B, and C meningococci (Turner *et al.*, 2002).

As the signal peptide of AspA contains a lipoprotein motif, the protein was also designated NalP (Neisserial autotransporter lipoprotein) (van Ulsen *et al.*, 2003). The protease activity is required for release of the passenger domain of AspA from the cell surface. The autocatalytic release was abolished by substitution of alanine for active-site serine (S426 in AspA from strain MC58 was mutated to an alanine residue) (Turner *et al.*, 2002; van Ulsen *et al.*, 2003). The presence of anti-AspA antibodies in sera of convalescent patients suggest that AspA is expressed during invasive disease.

1.9.5.4 Factor H binding Protein (fHbp)

The factor H-binding protein (fHbp), previously referred as genome-derived neisserial antigen 1870 (GNA1870) (Masignani *et al.*, 2003) or lipoprotein 2086 (Fletcher *et al.*, 2004), was identified by recent genomic studies carried out to search for promising vaccine candidates for serogroup B disease (Masignani *et al.*, 2003; Pizza *et al.*, 2000). The fHbp gene encodes a 27-kDa surface lipoprotein, which is present on the surface of all strains of *N. meningitidis* (Beernink *et al.*, 2007; Fletcher *et al.*, 2004; Masignani *et al.*, 2003). The protein appears to elicit protective bactericidal antibodies that both activate classical complement pathway bacteriolysis and inhibit binding of the complement down-regulatory protein factor H (fH) to the bacterial surface (Welsch *et al.*, 2008). The fHbp is the sole receptor 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; Schneider *et al.*, 2006).

1.9.5.5 T-cell stimulating protein A (TspA)

TspA is an immunogenic, T-cell and B-cell-stimulating protein of *N. meningitidis* (Robinson *et al.*, 2005). TspA was identified by screening *N. meningitidis* using a polyclonal antisera raised against fractionated proteins of *N. meningitidis* (Kizil *et al.*, 1999). Homology searches revealed that TspA is a member of the FimV family of proteins. TspA is a highly conserved antigen that is universally expressed by meningococci and shown to mediate optimal adhesion of *N. meningitidis* to cultured epithelial and endothelial cells (Oldfield *et al.*, 2007).

1.9.5.6 HrpA-HrpB system

A functional two-partner secretion system (TPS), haemagglutinin/haemolysin-related protein A (HrpA)-HrpB, composed of the secreted effector protein HrpA and its cognate transporter HrpB, has recently been found in all *N. meningitidis* strains and shown to contribute to adhesion of un-encapsulated bacteria to epithelial cell lines. It also appears to be essential for intracellular survival of the *N. meningitidis* (Schmitt *et al.*, 2007; Tala *et al.*, 2008).

1.9.5.7 Neisseria Adhesin A (NadA)

Neisserial adhesion A (NadA), a surface-exposed protein, was identified as a homologue of UspA2 from *Moraxella catarrhalis* and of YadA from *Yersinia enterocolitica* (van Ulsen *et al.*, 2001; van Ulsen & Tommassen, 2006). NadA is present in 50% of meningococcal isolates obtained from patients (and is more

commonly associated with three hypervirulent clusters ET-37, ET-5 and cluster A4) than with carriage isolates (16%). 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 suggesting that this protein may represent a novel vaccine antigen for meningococcal disease caused by three hypervirulent lineages (Comanducci *et al.*, 2002).

1.9.5.8 Meningococcal Serine Protease A (MspA)

MspA, also described as AusI, is a phase-variable serine protease, which was identified by *in silico* genome analysis of *N. meningitidis* serogroup B strain MC58 (Turner *et al.*, 2006).

1.9.5.9 Iron-acquiring proteins

Transferrin binding proteins (TbpA & B) and lactoferin binding proteins (LbpA & LbpB) are outer membrane proteins that are expressed by meningococci in iron-restricted environments during infection for iron acquisition. Antibodies to Tbps can be detected in sera from carriers and in convalescent sera (Ala'Aldeen *et al.*, 1994; Gorringe *et al.*, 1995) and these antibodies were shown to possess opsonic activity (Lehmann *et al.*, 1999). In laboratory animals, antibodies to Tbps were shown to be bactericidal (Ala'Aldeen & Borriello, 1996; Danve *et al.*, 1993) and were able to block iron uptake by the organism (Pintor *et al.*, 1996). The vaccine potential of these proteins lies in their limited antigenic heterogeneity (Ala'Aldeen, 1996; Rokbi *et al.*, 1997). Preliminary data from adult studies suggest that they are safe and immunogenic (Pollard & Frasch, 2001).

Neisseria spp. may also express lactoferrin binding proteins: LbpA (Pettersson *et al.*, 1993; Pettersson *et al.*, 1994) and LbpB (Pettersson *et al.*, 1998). Lactoferrin, a major human iron carrier protein, is present on mucosal surfaces and is presumably an important source of iron for *N. meningitidis* during its colonisation of the nasopharynx (Johnson *et al.*, 1999). LbpA is highly conserved and essential for iron uptake from lactoferrin (Biswas & Sparling, 1995; Bonnah & Schryvers, 1998).

1.10 Blebing and autolysis

Meningococci are characterized by frequent vesiculation of the outer membrane and/or shedding of endotoxin (McAllister & Stephens, 1993). The shedding of endotoxin from *N. meningitidis* is a strain specific virulence factor and occurs by a specific process called blebing. These blebs contain outer membrane protein and LOS and the amount of blebing varies considerably between strains (Andersen *et al.*, 1981). Blebing appears to contribute to the rapid initiation of the inflammatory cascades of sepsis. Blebing may also be related to the natural autolysis of meningococci that occurs in stationary growth phase, resulting in release of DNA and facilitating genetic transformation (Tzeng & Stephens, 2000).

1.11 Animal models for meningococcal infection

Animal models are often required to understand the mechanisms of pathogenesis, and to develop novel therapies and prevention regimes. Thus, relevant models are key factors for deciphering microbial virulence (Bakaletz, 2004). Several animal models have been developed and used for studying the pathogenesis of

meningococcal disease. These include monkeys, guinea pigs, chicken embryos, adult and infant mice, and infant rats (Arko, 1989). Of these, the mouse and infant rat systems appears to be the most suitable for laboratory experiments (Nassif & So, 1995). None of the present models, however, completely satisfy all the criteria required for an ideal animal model. Harter and Petersdorf (Harter & Petersdorf, 1960) have described the criteria for an ideal animal model as follows:

(i) The portal of entry and route of dissemination of the organism must be similar to those in man, (ii) the bacterium must be pathogenic for man as well as experimental animals, (iii) the course of the disease must be relatively predictable and of sufficient duration to permit evaluation of therapy, (iv) the disease must be reproducible within the limits of biological variation, (v) the lesions in the experimental infection must be morphologically similar to those in man, and (vi) the technique must be relatively simple. Unfortunately, it is unlikely that any one animal model can meet all of these requirements for studies on meningococcal disease.

Recently, two transgenic mice models, one expressing human CD46 (Johansson *et al.*, 2003) and another expressing human transferrin (Zarantonelli *et al.*, 2007), have been developed. These are potentially useful tools for studying pathogenesis and evaluating vaccine candidates. The transgenic mice expressing CD46 were shown to be susceptible to meningococcal disease after intranasal, but not intraperitoneal challenge, suggesting that human CD46 facilitates pilus-dependent interactions at the epithelial mucosa (Johansson *et al.*, 2003). Since iron is

essential for meningococcal growth, the transgenic mouse model expressing human transferrin may represent an important advance for *in vivo* studies of meningococcal virulence and immunogenicity factors (Zarantonelli *et al.*, 2007).

1.12 Meningococcal vaccines

Prevention of meningococcal disease can effectively be accomplished by vaccination. The first major success towards vaccination against meningococcal disease was achieved in the 1960s by Gotschlich and co-workers, who successfully demonstrated the importance of anti-polysaccharide antibodies in the human immune response against serogroup A and C disease (Morley & Pollard, 2001). Meningococcal serogroup A, C, W-135 and Y infections can be prevented by vaccines based on the respective capsular polysaccharides.

1.12.1 Plain polysaccharide vaccines

Meningococcal purified polysaccharide vaccines have been used widely since the development of a highly immunogenic vaccine for serogroups A and C in 1969 by Gotshlich *et al.*, (Gotschlich *et al.*, 1969a). The vaccines have been shown to be safe and efficacious in preventing disease in US military recruits, and have also been used in an attempt to minimize the serogroup A epidemics in Africa. The efficacy has been estimated at 85 to 100 percent among older children and adults (Artenstein *et al.*, 1970; Peltola *et al.*, 1977; Wahdan *et al.*, 1977).

There are, however, some important limitations associated with meningococcal polysaccharide vaccines: (1) the serogroup C polysaccharide is poorly immunogenic in young children (King *et al.*, 1996; Maslanka *et al.*, 1998), with

little or no efficacy among children younger than 24 months of age (Gold *et al.*, 1977; MacLennan *et al.*, 1999), (2) they are unable to induce long-acting T cell-dependent immunologic memory (Reingold *et al.*, 1985) and, hence, offer only a relatively short duration of protection, especially in young children, (3) the repeated doses of polysaccharide vaccine may induce a degree of hyporesponsiveness. However, the clinical relevance of this finding is unclear (King *et al.*, 1996; Maslanka *et al.*, 1998), (4) some studies conducted among military recruits suggested that the meningococcal polysaccharide vaccines may decrease short-term acquisition of carriage (Gotschlich *et al.*, 1969b), however, these vaccines failed to produce long-term protection against carriage and, are thus unable to induce herd immunity (Hassan-King *et al.*, 1988; Moore *et al.*, 1988). In addition to bivalent polysaccharide vaccines, an outbreak in the meningitis belt during 2003 with serogroup W-135 prompted the development of a trivalent A/C/W-135 and tetravalent A/C/W-135/Y polysaccharide vaccines (Girard *et al.*, 2006).

1.12.2 Meningococcal conjugate vaccines

Efforts to overcome the poor immunogenicity of capsular polysaccharide vaccines led to development of conjugate vaccines. The premise for the development of new conjugated meningococcal vaccines was based on the experience gained with Hib (*H. influaenzae* type b) conjugated vaccine, which has succeeded in virtually eliminating Hib disease in young children in many countries (Adams *et al.*, 1993; Peltola *et al.*, 1992). Conjugation of polysaccharides to protein carriers appears to change the nature of the antibody response from a T-cell-independent to a T-cell-dependent response. The

conjugate vaccines are, therefore, typically T-cell dependant and confer major immunologic improvements over plain polysaccharide vaccines (Lesinski & Westerink, 2001a; Lesinski & Westerink, 2001b). Meningococcal serogroup C conjugate vaccines have been developed using a genetically detoxified diptheria toxin (CMR197) or tetanus toxoid as the protein-carrier (Anderson *et al.*, 1994; Safadi & Barros, 2006). In November 1999, the United Kingdom became the first country to introduce a conjugate vaccine against serogroup C meningococcal disease into the childhood immunization program (Miller *et al.*, 2001). Later on, in January 2005, a quadrivalent meningococcal conjugate vaccine (MCV4) was developed and licensed (Harrison, 2006). Meningococcal conjugate vaccines have been demonstrated to be safe, and shown to elicit an improved immune response in infants (Anderson *et al.*, 1994; Campagne *et al.*, 2000; Fairley *et al.*, 1996; Lieberman *et al.*, 1996; Richmond *et al.*, 1999). They also possess prime immunologic memory, and lead to a booster response to subsequent doses (Borrow *et al.*, 2000; Twumasi *et al.*, 1995).

1.12.3 Meningococcal serogroup B vaccine

The capsular polysaccharide of meningococcal serogroup B differs from the others because of its structural homology with human embryonal neural tissue (Wyle *et al.*, 1972). This mimicry rules out the use of polysaccharide–protein conjugate vaccines as an effective strategy to combat serogroup B disease because of the theoretical danger of induction of auto-antibodies that cross-react with glycosylated host antigens (Jodar *et al.*, 2002). Therefore, currently there is no universal vaccine available against serogroup B disease. Instead vaccine research has focused on protein-based vaccines composed of outer membrane

vesicles (OMVs) purified from the bacterium. Due to the high sequence and antigenic variability of the antigens present in the OMVs, these vaccines mostly induce immunity against the highly variable PorA membrane protein, and efficacy trials have shown serosubtype-specific protection (Serruto *et al.*, 2003). However, the OMV-based vaccine produced by the Finlay Institute in Cuba (commercially marketed at VA-MENGOC-BC), which contains outer membrane proteins and capsular polysaccharide of meningococcal serogroup C, is believed to have contributed to the rapid decline of the epidemic in Cuba (Isabel *et al.*, 1999), and has also been shown to be efficacious in subjects of more than 4 years of age in Brazil where heterologous strains were circulating (de Moraes *et al.*, 1992). In addition, a recently introduced bivalent meningococcal OMV vaccine has offered a wide vaccine coverage particularly of the circulating strains in Europe and is able to induce bactericidal antibodies not only against the vaccine-homologous/PorA-related strains but also against heterologous strains (Boutriau *et al.*, 2007). Although currently available vaccines based on OMVs may induce a functional immune response against serogroup B, none of these vaccines are universally protective due to the great heterogeneity of the surface-exposed regions of many of the outer membrane proteins. An alternative approach to OMV vaccines has been to the use of 'reverse vaccinology' to identify conserved, immunogenic OMPs, which can be developed as protein subunit vaccines (Rappuoli, 2000; Serruto *et al.*, 2004).

Two promising vaccines are currently being assessed in phase II and III clinical trials. One of these vaccines (developed by Novartis) is a multi-component vaccine, which includes a combination of five antigens and OMVs from the

NZ98/254 strain of *N. meningitidis* serogroup B (Giuliani *et al.*, 2006). The other (produced by Wyeth) contains two variants of a single lipoprotein (rLP2086 also known to as GNA1870/fHbp) (Richmond P, 2008). In addition, numerous other outer membrane proteins such as OpcA, NspA, and iron regulated proteins (TbpA and B, FbpA, FetA) are being evaluated and explored for use as potential meningococcal vaccine candidates.

1.13 Genome sequence of meningococci

The meningococci genome consists of about 2.1-2.2 million bases encoding approximately 2000 genes (Parkhill *et al.*, 2000; Tettelin *et al.*, 2000). The genome sequences of *N. meningitidis* strain MC58 (serogroup B) (Tettelin *et al.*, 2000), strain Z2491 (serogroup A) (Parkhill *et al.*, 2000) and strain FAM18 (serogroup C) (Bentley *et al.*, 2007) have been published. In addition, the annotated genome sequence of obligate commensal species *N. lactamica* has also been made publicly available. The three sequenced meningococcal genomes differ from each other by approximately 9-10% and from the *N. gonorrhoeae* genome by roughly the same amount (Stephens *et al.*, 2007). The chromosome of *N. meningitidis* strain MC58 is almost 100 kb larger than the other two sequenced strains mainly due to large duplication of about 30 kb comprising 36 coding sequences (NMB1124-NMB1159 duplicated in NMB1162-NMB1197) and the acquisition of two additional islands of horizontally transferred DNA (IHT-B 17.1 kb and IHT-C 32.6kb). Both of these islands are absent in the genomes of strains Z2491 and FAM18 (Schoen *et al.*, 2007).

1.14 Protein secretion pathways in *N. meningitidis*

Protein secretion in bacteria plays an essential role in nutrient acquisition, survival in and adaptation to different environments, inter- and intraspecies communication, and virulence. For example, in *N. meningitids,* secreted proteins are involved in adherence of the bacteria to host cells or required to suppress the host's defense mechanisms (Dautin & Bernstein, 2007; van Ulsen & Tommassen, 2006). Gram-negative bacteria use several different pathways for secretion of the exoproteins. These pathways are categorized as Type-I, II, III, IV, autotransporter pathway and two-partner secretion (TSP) pathway (type-V) (Henderson *et al.*, 2004; van Ulsen & Tommassen, 2006). *N. meningitidis* uses three of the six known protein secretion pathways: the autotransporter pathway (AT) and the two partner secretion pathway (collectively known as Type V secretion pathway) and the type one secretion pathway (van Ulsen & Tommassen, 2006). Like all Gram-negative bacteria, meningococci are bounded by two hydrophobic barriers, the inner membrane (IM) and the outer membrane (OM), which are separated by the periplasmic space containing peptidoglycan (Morley & Pollard, 2001). Thus, the pathways allowing the extracellular secretion of proteins from Gram-negative bacteria must traverse three distinct compartments: the inner membrane, the periplasm and the outer membrane, which separate the cytoplasm (the site of synthesis) from the exterior of the cell (Lory, 1992). However, an increasing number of cytoplasmic proteins (including some that are normally considered to be house-keeping enzymes with a primary biological role in the cytoplasmic compartment) have been reported to be secreted either to the cell surface or into the extracellular environment independently of any recognized classical secretion pathway.

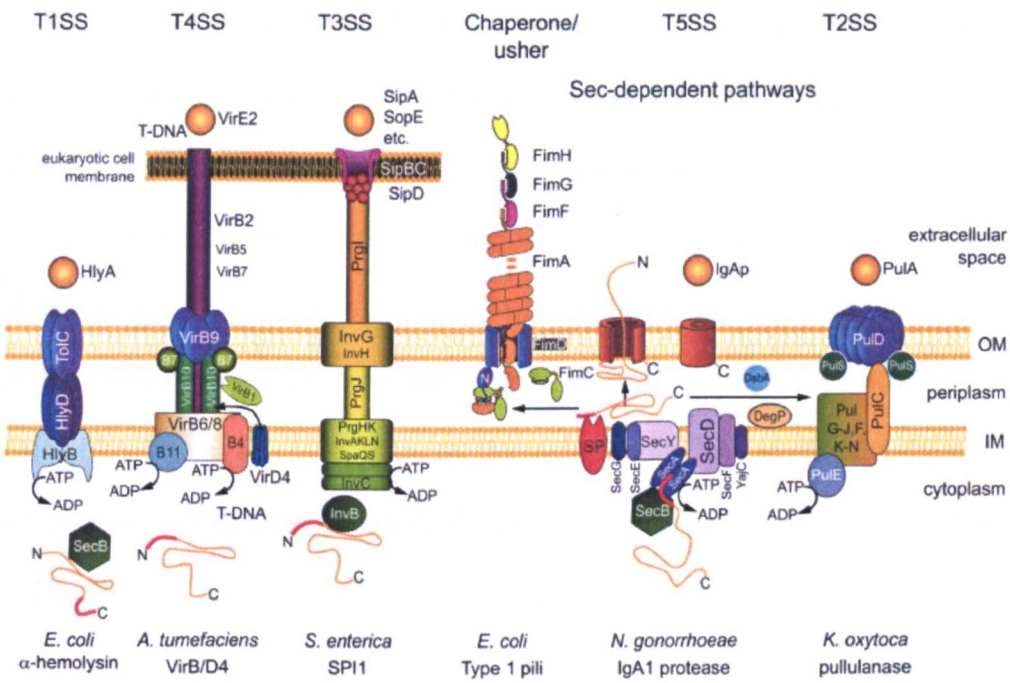


Figure 1. 4 Overview of secretion systems in Gram-negative bacteria. Protein transport across the outer membrane of Gram-negative bacteria can be subdivided into Sec-independent and Sec-dependent pathways. The three main terminal branches allowing further transport of Sec-transported periplasmic intermediates are: the chaperone/usher (CU) pathway for the synthesis of fimbrial adhesins, the autotransporter pathway and the complex type II secretion system. No stable periplasmic intermediates are found in type I, type III and type IV secretion systems. Each of the three export mechanisms features a protein-conducting channel able to span the two bacterial membranes and, in the case of type III and type IV, one additional membrane of the host cell. Examples for pathogens utilizing the various secretion systems are indicated at the bottom of the figure (adapted from reference Gerlach & Hensel, 2007).

1.14.1 Type 1 secretion system

The type one secretion system (or ATP-binding cassette [ABC] transporters) is hetrotrimeric complex composed of three proteins: an cytoplasmic membrane-embedded ABC transporter, a pore-forming outer membrane-embedded channel protein, and an adapter or membrane-fusion protein (Gerlach & Hensel, 2007; van Ulsen & Tommassen, 2006). These proteins are represented in *E. coli* HlyB, TolC, and HlyD, respectively (Binet *et al.*, 1997). A well-known protein secreted via type 1 is the α-haemolysin (HlyA)

of *E. coli* (Goebel & Hedgpeth, 1982). Secretion of HlyA, and similar effector molecules occurs in a Sec-independent, continuous process across both the cytoplasmic and outer membrane (Henderson *et al.*, 2004). These proteins completely bypass the periplasm and are directly secreted from the cytoplasm (Binet *et al.*, 1997). A wide range of substrates (proteinaceous and non-proteinaceous) varying in size between 78 to 8682 residues are secreted in a single step from the cytoplasm to the extracellular space, without a stable periplasmic intermediate (Gerlach & Hensel, 2007; van Ulsen & Tommassen, 2006).

1.14.2 Autotransporter secretion system

The autotransporters, a family of secreted proteins from Gram-negative bacteria, are synthesized as a single polypeptide containing all the information required for their export and secretion (Henderson *et al.*, 1998). The primary structure of these proteins is basically modular and composed of three functional domains: the amino-terminal leader sequence, the secreted mature protein (passenger domain) and translocation unit that forms a β-barrel pore to allow secretion of the passenger protein (Desvaux *et al.*, 2004). Autotransporter secretion was first described for the IgA1 proteases of *N. gonorrhoeae* (Pohlner *et al.*, 1987). The signal sequence present at the end of N-terminus directs the autotransporter to the Sec machinery for the transport across the cytoplasmic membrane and its further export into the periplasm (Brandon *et al.*, 2003; Henderson *et al.*, 1998). The passenger domain of the autotransporters has been shown to confer various phenotypes and the last domain is the translocation unit that facilitates translocation of the passenger domain through the outer membrane (Henderson *et*

al., 2004). The term 'autotransporter' was coined because of the apparent absence of a dedicated secretion machine (van Ulsen & Tommassen, 2006). However, recent work has shown accessory periplasmic proteins are important (Ruiz-Perez *et al.*, 2009).

1.14.3 Two-Partner secretion system

Two-partner secretion (TPS) systems export large 'exoproteins' rich in beta-helical structure (TpsA family members) across the outer membranes of Gram-negative bacteria using channel-forming β-barrel proteins (TpsB family members). Like autotransporter proteins, most of these proteins are associated with virulence traits and their characterized diverse functions include autoaggregation, biofilm formation, iron acquisition, adherence to host tissues, cytolysis, immunomodulation, binding of heme and/or hemopexin, and contact-dependent inhibition (Aoki *et al.*, 2005; Henderson *et al.*, 2004; Jacob-Dubuisson *et al.*, 2004). The first member of the TPS family to be characterized was the ShlB (HlyB) protein of *Serratia marcescens*, which exports the ShlA hemolysin from the periplasm of the Gram-negative bacterial envelope into the external medium (Poole *et al.*, 1988).

The hallmarks of TPS systems are the presence of (1) an N-proximal 250-residue-long 'TPS' domain where specific secretion signals in the substrate protein are found and (2) a β-barrel channel (TpsB) homologue (Clantin *et al.*, 2004; Jacob-Dubuisson *et al.*, 2001). Usually, the genes encoding these two proteins occur within an operon. The TPS domain is essential for secretion and mediates interactions between the TpsA protein and its TpsB partner (Hodak *et*

al., 2006). The TpsA protein is exported across the cytoplasmic membrane by the Sec machinery (Chevalier *et al.*, 2004; Grass & St Geme, 2000). Its TPS domain then interacts with the periplasmic domain of the TpsB partner, which triggers channel opening and initiates the translocation of the TpsA protein, probably in an extended conformation through the TpsB pore (Clantin *et al.*, 2007; Guedin *et al.*, 2000; Meli *et al.*, 2006).

In silico analyses have revealed that a large number of TspA proteins are encoded in various bacterial genomes, ranging from those of plant pathogens such as *Xylella fastidiosa* and *Ralstonia solanacearum* to those of human pathogens such as *N. meningitidis* and *Y. pestis* (Jacob-Dubuisson *et al.*, 2004; Locht *et al.*, 2001) Taken together, these findings demonstrate that TPS systems are present in nearly all groups of Gram-negative bacteria (hence, they probably participate in processes other than virulence) and that TpsB proteins belong to a large family of outer membrane protein-translocating porin type proteins with members in the animal, plant and fungal kingdoms, making this general secretion mechanism the most widely distributed in nature (Yen *et al.*, 2002).

1.15 Non-Classical secretion of proteins in bacteria

Most proteins destined to be released in the extracellular media contain an N-terminal or C-terminal classical signal peptide, which mediates the export of envelope-associated as well as some secreted, proteins, across the inner membrane via the Sec-dependant pathway (van Ulsen & Tommassen, 2006). However, almost two decades ago, a small number of eukaryotic proteins lacking N-terminal signal peptide or other identifiable targeting peptides were shown to

be secreted and thus localised extracellularly. This phenomenon, termed leaderless secretion and later non-classical secretion was initially identified in eukaryotes (Bendtsen & Wooldridge, 2009). For some years only eukaryotes were known to secrete proteins via alternative or non-classical secretory routes. Strikingly, a similar phenomenon was later observed in the bacterial kingdom. It is apparent that non-classical secretion does not follow a simple route of secretion but that proteins can be secreted via different rather undefined secretion systems in prokaryotic as well as eukaryotic organisms (Bendtsen & Wooldridge, 2009).

The GlnA glutamine synthetase of *Mycobacterium tuberculosis* was the first published example of apparent bacterial non-classical protein secretion, whilst in *Mycobacterium smegmatis*, GlnA is localized solely to the cytoplasm of the bacterium (Harth & Horwitz, 1997). The proteomic research on *Bacillus subtilus* has revealed that twenty four proteins with highly divergent functions are secreted to the extracellular environment without having classical Sec-signal peptides (Bendtsen & Wooldridge, 2009). Furthermore, an alternative secretion system has also been identified in *Listeria monocytogenes* (Lenz *et al.*, 2003). Recently, proteins lacking known secretion signals have also been shown to be secreted by yeast cells via alternative/non-conventional secretion mechanisms (Nombela *et al.*, 2006).

A growing number of diverse proteins have been identified which are multifunctional. Such proteins have been named 'moon-lighting proteins'. (Jeffery, 1999; Jeffery, 2003; Jeffery, 2009). Moonlighting proteins are a diverse set of proteins that include glycolytic enzymes, chaperones, transcription factors,

and proteins with many other types of functions (Nombela *et al.*, 2006). An interesting example is phosphoglucose isomerise (PGI), a cytosolic glycolytic enzyme that catalyses the second step of glycolysis. A secreted form of this protein has been shown to act as cytokine in eukaryotes (Amraei & Nabi, 2002). Thus, some of the proteins found to be secreted via non-classical system may play a different role in the unexpected location than that of their role in the cytoplasm (Schaumburg *et al.*, 2004).

A less well-defined method of protein secretion may be mediated by OMVs released from the outer cellular membrane of Gram-negative bacteria. This phenomenon, also called membrane blebbing (or vesicle-mediated secretion) has now been found in a range of Gram-negative bacteria (Kuehn & Kesty, 2005). The OMVs from pathogenic bacteria appears to contain adhesins, toxins, and immunomodulatory compounds, and they have been shown to directly mediate bacterial binding and invasion, cause cytotoxicity, and modulate the host immune response (Bendtsen & Wooldridge, 2009; Kuehn & Kesty, 2005). Two of the well characterised examples of vesicle-mediated secretion include ClyA of *E. coli* (Wai *et al.*, 2003) and VacA of *Helicobacter pylori* (Keenan *et al.*, 2000). Furthermore, *Actinobacillus actimycocetemcomitans* has also been shown to secrete leukotoxin, a membranolytic cytotoxin, via membrane blebs and these toxins are likely to be incorporated into host cell membranes (Demuth *et al.*, 2003; Kato *et al.*, 2002).

1.16 Multi-faceted (moon-lighting) roles of glycolytic enzymes

Several studies have provided intriguing evidence of the unexpected nature and location of glycolytic enzymes on the surface of numerous microbial pathogens (Bergmann *et al.*, 2004; Ling *et al.*, 2004; Lottenberg *et al.*, 1992; Pancholi & Fischetti, 1992; Pancholi & Fischetti, 1998; Pancholi V, 2003). However, little is known about the molecular mechanisms of the multi-faceted roles of glycolytic enzymes, specifically regarding the relationship between their glycolytic and non-glycolytic functions (Kim & Dang, 2005). Glycolytic enzymes, such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH), enolase and fructose bisphosphate aldolase (FBA) have been reported as potential virulence factors in a variety of organisms (Pancholi & Chhatwal, 2003). Although long recognized for their cytosolic role in glycolysis and gluconeogenesis, dual (or 'moon-lighting') functions have been increasingly recognized. In particular, glycolytic enzymes have been found on the bacterial cell surface (despite lacking identifiable secretion signals) where they interact directly with host soluble proteins and surface ligands. However, the mechanisms by which these cytosolic enzymes are translocated and retained on the cell surface of pathogens are not yet understood.

1.16.1 The glycolytic pathway is non-functional in *N. meningitidis*

Glycolysis is the main pathway for carbohydrate degradation (glucose) in nearly all organisms. The end product of glycolysis is pyruvate. The conversion of glucose into pyruvate requires a cascade of nine enzymatic reactions and most of these reactions are reversible during the gluconeogenic cycle (Fourrat *et al.*, 2007). Glycolysis can be generally separated into two phases, the priming phase

and the energy-yielding phase. The priming phase uses two moles of ATP to convert glucose to fructose- 1, 6-bisphosphate. In the second phase, fructose-1,6-bisphosphate is further converted stepwise into pyruvate with the production of four moles of ATP and two moles of NADH (Fig 1.6) (Kim & Dang, 2005).

Genome-based studies on meningococcal strain MC58 revealed that glycolysis (also known as the Embden Meyerhof Parnas [EMP] pathway) is not involved in pyruvate synthesis in *N. meningitidis*. This is because of the absence of one essential enzyme in the pathway: phosphofructokinase (EC 2.7.1.11) (Fig 1.5). The generation of pyruvate from glucose has, instead, been shown to occur through the Entner Douderoff (ED) and the Pentose Phosphate (PP) pathways (Fig 1.5), confirming that EMP (glycolysis) is non-functional in *N. meningitidis* (Baart *et al.*, 2007).

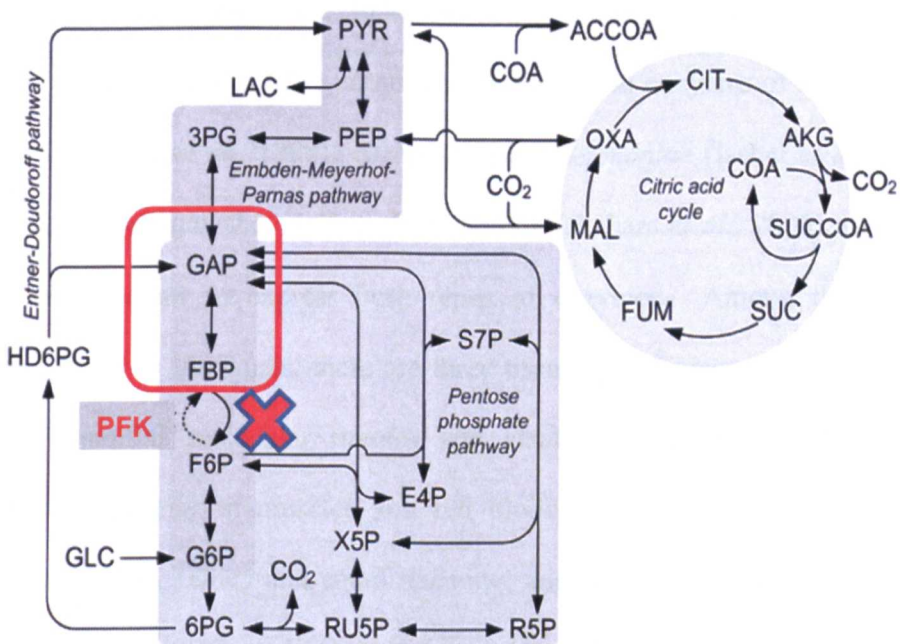


Figure 1. 5 Central metabolism of *N. meningitidis*. The dashed arrow indicates phosphofructokinase (PFK), which is not present in *N. meningitidis*. The red box area indicates the site of enzymes (FBA and GAPDH-1) under study. (adapted from reference Baart *et al.*, 2007).

1.16.2 Fructose 1, 6-bisphosphate aldolase (FBA)

Fructose 1, 6-bisphosphate aldolase (E.C.4.1.2.13) is the best studied glycolytic enzyme in the aldolases family. FBA catalyses the reversible cleavage of fructose 1,6 bisphosphate into two triose sugars: dihydroxyacetone phosphate (glycerine-P) and glyceraldehyde 3-phosphate (Fig 1.6) (Ramsaywak *et al.*, 2004; Wehmeier, 2001; Zgiby *et al.*, 2000). Aldolases can be broadly divided into two groups with different catalytic mechanisms, designated Class-I and Class-II (Arakaki *et al.*, 2004; Thomson *et al.*, 1998). The Class-I enzymes utilize an active site lysine to stabilize a reaction intermediate via schiff-base formation, and are usually found in higher eukaryotic organisms. The Class-II enzymes, however, have an absolute requirement for a divalent ion, usually zinc (Zgiby *et al.*, 2000) and are usually found in prokaryotic organisms and lower eukaryotes such as fungi and some green algae grown under heterotrophic conditions (Plater *et al.*, 1999; Ramsaywak *et al.*, 2004; Sauve & Sygusch, 2001). Most organisms contain only one class of FBA, although a few possess enzymes of both classes. *E. coli* (Thomson *et al.*, 1998)., *Streptococcus pneumoniae* (Isabel *et al.*, 1999) *Corynebacterium synechocystis* sp. PCC 6803 (Nakahara *et al.*, 2003) and some others are reported to express both types of enzymes. Among the Class-I enzymes found in mammals, there are three tissue-specific isozymes of aldolase that have similar molecular masses and catalytic mechanisms: aldolase A (expressed primarily in muscles and red blood cells), aldolase B (expressed primarily in liver, kidney and small intestine) and aldolase C (expressed mainly in brain, smooth muscle, and neuronal tissues (Arakaki *et al.*, 2004).

The class-II family has been sub-divided, in this case into two groups depending on their sequences; they are known as Type A and Type B (Nakahara *et al.*, 2003; Sauve & Sygusch, 2001). Since the Class-II aldolases are not found in animals, it has been suggested that they could provide a possible therapeutic and vaccine targets (Blom *et al.*, 1996; Ramsaywak *et al.*, 2004).

In addition to its metabolic function, studies have demonstrated that FBA is present on the surface of several microbial pathogens and may facilitate their adhesion to host tissues by interacting directly with host surface ligands. For example, in *S. pneumoniae*, surface-associated FBA was shown to bind to a large 7-transpass transmembrane receptor belonging to the cadherin superfamily (Blau *et al.*, 2007) and also shown to be immunogenic in humans and capable of inducing a protective immune response against *S. pneumoniae* in mice (Ling *et al.*, 2004). In addition, FBA was found to be a surface-localized immunogenic protein in *S. suis* (Zongfu *et al.*, 2008) and a possible role for FBA in immunity to a nematode parasite *Onchocerca volvulus* has also been reported (McCarthy *et al.*, 2002).

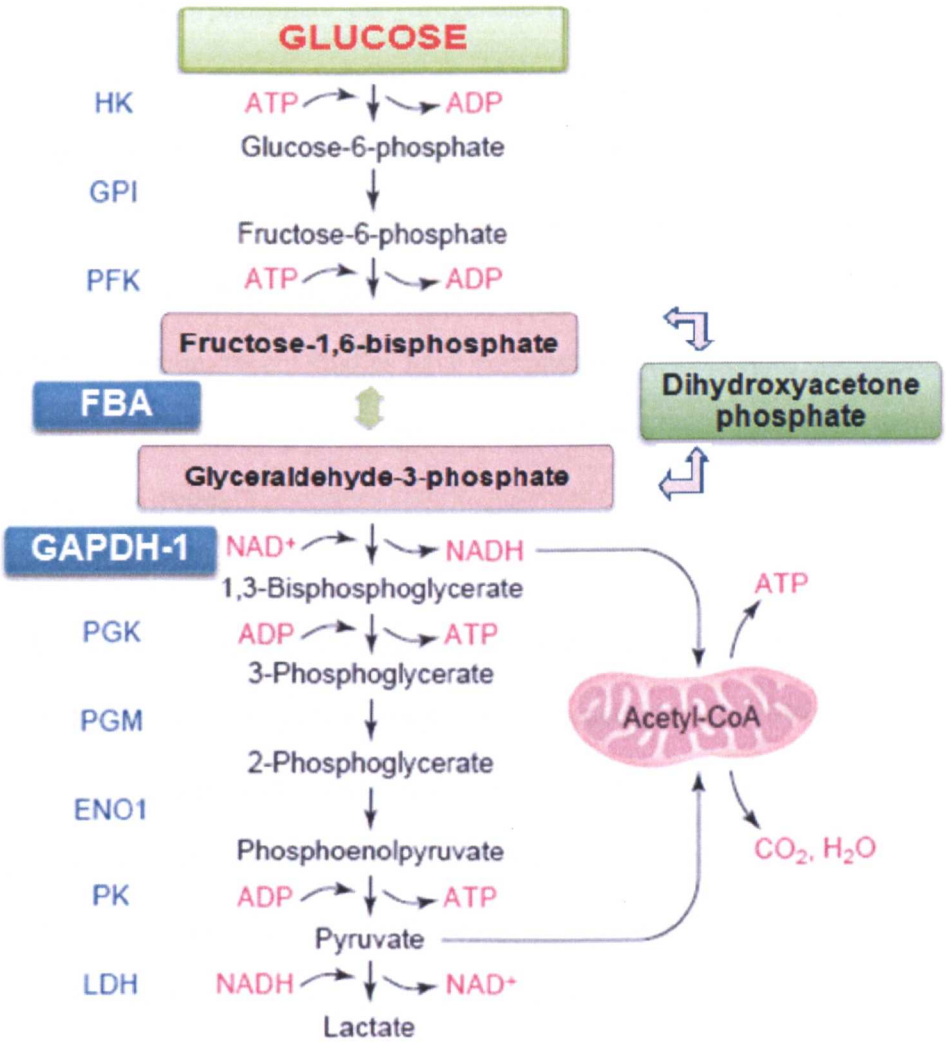


Figure 1.6 Schematic diagram shwoing glycolytic pathway in a eukaryotic cell. Glycolysis is an ancient metabolic pathway in which one mole of glucose is catabolised to two moles of each pyruvate, NADH and ATP. Under aerobic conditions, pyruvate is further oxidized by mitochondrial enzymes to carbon dioxide and water. (Abbreviations: ENO1, enolase1; FBA, Fructose bisphosphate aldolase, GAPDH-1, glyceraldehyde-3-phosphate dehydrogenase; GPI, glucose-6-phosphate isomerase; HK, hexokinase; LDH, lactate dehydrogenase; PFK, phosphofructokinase; PGK, phosphoglycerate kinase; PGM, phosphoglycerate mutase; PK, pyruvate kinase; TPI, triose phosphate isomerase) (adapted from reference Kim & Dang, 2005) .

1.16.3 Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is a glycolytic enzyme which catalyzes 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 (Kim & Dang, 2005). In addition to its well-established metabolic function, recent studies have demonstrated unexpected, non-glycolytic functions of GAPDH in physiological and pathological processes (Kim & Dang, 2005; Sirover, 1999).

The localisation of GAPDH on the surface of a pathogen was first described in the Gram-positive pathogen, *S. pyogenes* (Lottenberg *et al.*, 1992; Pancholi V, 2003). In this organism, surface-exposed GAPDH binds several mammalian proteins including the uPAR/CD87 membrane protein on pharyngeal cells (Jin *et al.*, 2005; Lottenberg *et al.*, 1992; Pancholi & Fischetti, 1992; Winram & Lottenberg, 1996), regulates intracellular host cell signaling events (Pancholi & Fischetti, 1997) and contributes to host immune evasion (Terao *et al.*, 2006). GAPDH was subsequently identified on the surface of other Gram-positive bacteria including staphylococci (Modun & Williams, 1999; Modun *et al.*, 2000), *S. agalactiae* (Seifert *et al.*, 2003) and *Listeria monocytogenes* (Schaumburg *et al.*, 2004). In addition, surface localization of GAPDH has been reported in enterohemorrhagic (EHEC) and enteropathogenic (EPEC) *Escherichia coli*; the protein of these pathogens has been observed to bind to human plasminogen and fibrinogen, suggesting a role in pathogenesis (Egea *et al.*, 2007). Similar to the surface localized-GAPDHs from other species, the EHEC and EPEC GAPDH

proteins possess NAD-ribosylating activity (Aguilera *et al.*, 2009). In *Mycoplasma genitalium*, surface-associated GAPDH is important for adhesion to human mucin (Alvarez *et al.*, 2003), and in *Lactobacillus plantarum*, a normal inhabitant of the human gastrointestinal tract, GAPDH was shown to be involved in adherence to gastric mucin and Caco-2 cells (Kinoshita *et al.*, 2008; Ramiah *et al.*, 2008). Interestingly, the major fimbriae of *Porphyromonas gingivalis* bind to GAPDH on the surface of several oral streptococci, and this interaction is important for colonization of the oral cavity (Nagata *et al.*, 2009). Fungi also express GAPDH on their cell surface, for example, the GAPDH of *Candida albicans* was shown to be associated with the cell wall and involved in mediating adhesion to fibronectin, laminin and plasminogen (Gil-Navarro *et al.*, 1997; Gozalbo *et al.*, 1998; Jonathan *et al.*, 2003). GAPDH has also been found on the surface of the single-celled protozoan, *Trichomonas vaginalis*, and shown to bind extracellular matrix components, including fibronectin (Lama *et al.*, 2009). Thus, GAPDH is a multi-functional protein displayed on the surface of several fungi and Gram-positive pathogens, which contributes to their adhesion to host cells and may act as virulence factor (Egea *et al.*, 2007).

1.17 Aims of the Project

The aim of this study was to investigate the molecular, immunological, and functional attributes of two of the glycolytic pathway enzymes, namely FBA and GAPDH of *N. meningitidis*.

CHAPTER 2: Cloning and expression of *cbbA* and *gapA-1* and purification of their products

2.1 Introduction

Glycolytic enzymes, which along with other enzymes involved in central metabolism, are sometimes called 'housekeeping enzymes' are classical cytoplasmic proteins that are found in all cell types. These enzymes are known to be constitutively expressed and localised mainly to the cytoplasm of the cell. There is growing evidence that several so-called housekeeping enzymes, including fructose bisphosphate aldolase (FBA) and glyceraldehydes-3-phosphate dehydrogenase (GAPDH), are often localised to the surface of bacterial pathogens. Whilst their intracellular functions are well known, it is unclear whether they perform any additional functions on the bacterial surface.

Fructose 1, 6-bisphosphate aldolase catalyses the reversible cleavage of fructose 1,6-bisphosphate into two triose sugars: dihydroxyacetone phosphate (glycerine-P) and glyceraldehyde 3-phosphate in the glycolysis cycle (Ramsaywak *et al.*, 2004; Wehmeier, 2001; Zgiby *et al.*, 2000). Besides performing a key role in glycolytic cycle, aldolases have also been shown to be localised to the surface of numerous bacterial and fungal pathogens. In *S. pneumoniae* surface-localized FBA was shown to bind the host cell ligand Flamingo cadherin (Blau *et al.*, 2007), and was also found to be a surface-localized immunogenic protein in *S. suis* (Zongfu *et al.*, 2008).

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is a cytoplasmic glycolytic enzyme that converts glyceraldehyde 3-phosphate to 1, 3-bisphosphoglycerate coupled with the reduction of NAD^+ to NADH. Recent studies have demonstrated unexpected, non-glycolytic, functions of GAPDH in

physiological and pathological processes (Kim & Dang, 2005). Several reports have shown that in some Gram-positive bacteria and fungi extracellular GAPDH interacts with various host components, including plasminogen, the cytoskeletal proteins actin and myosin, and extracellular matrix proteins including fibronectin (Gozalbo *et al.*, 1998; Pancholi & Fischetti, 1992; Schaumburg *et al.*, 2004; Seifert *et al.*, 2003; Winram & Lottenberg, 1996). The GAPDH of *Mycoplasma bovis* has been shown to induce an immune response in infected cattle (Perez-Casal & Prysliak, 2007). Moreover, other cytosolic proteins: Enolase, DnaK, and peroxiredoxin, of *N. meningitidis* serogroup B, have recently been reported localised to the outer membrane, where they can act as plasminogen receptors (Knaust *et al.*, 2007). Thus, FBA and GAPDH-1 may be multifunctional proteins with a role in central metabolism and an additional role unconnected to their primary role which is associated with an alternative sub-cellular localisation. Such proteins have been described as 'moonlighting proteins' (Jeffery, 1999; Jeffery, 2003; Jeffery, 2009).

The aims of this chapter were to clone and over-express *cbbA* and *gapA*-1, to affinity purify full length recombinant FBA and GAPDH-1, and to raise polyclonal antisera against the purified proteins to facilitate subsequent characterisation studies.

2. 2 Materials and Methods

2.2.1 Bacterial strains, growth conditions, and media

E. coli strains and plasmids used in this chapter are described in Table 2.2. *E. coli* strains were routinely grown at 37°C in Lysogeny Broth (LB) (Bertani, 2004) or on LB agar. Where appropriate, antibiotics were used at the following concentrations: ampicillin (100 μg ml^{-1}), kanamycin (30 μg ml^{-1}). Where appropriate, blue/white selection of transformants in cloning experiments was achieved using IPTG (Roche) and X-gal at 0.5 mM and 80 μg ml^{-1}, respectively. All liquid cultures were aerated by agitation at 200 revolutions per minute (rpm) in a shaking incubator.

2.2.2 Extraction of chromosomal DNA

Chromosomal DNA was prepared by using a DNeasy Blood & Tissue kit (Qiagen) using the protocol for bacterial cells recommended by the manufacturer. Briefly, overnight culture of *N. meningitidis* grown on chocolate agar plates was scraped off the plate with a sterile loop and re-suspended in 180 μl of ATL buffer (provided in kit) and 20 μl of Proteinase K to disrupt the cells. The sample was then heated at 56°C until the complete lysis of cells was achieved. The lysate was applied to DNeasy spin column. The bound DNA was washed twice using the buffers provided in the kit. The bound DNA was eluted in 200 μl of elution buffer.

2.2.3 Extraction of plasmid DNA

E. coli JM109, harboring the desired plasmid, were streaked out from -80 glycerol stock on to LB agar plates containing appropriate antibiotic and

incubated overnight at 37°C . The following day, a single colony was used to inoculate a 10 ml of LB broth supplemented with 100 μg ml^{-1} ampicillin. The broth culture was incubated at 37°C overnight with shaking at 200 rpm. Small-scale purification of high copy number plasmids was achieved using 5 ml overnight cultures using a QIAprep spin kit (Qiagen) according to the manufacturer's instructions. In case of low copy number plasmids or large-scale (70-100 μg) plasmid purification, GeneElute the Midi Prep kit (Sigma) was used according to manufacturer's recommendation.

2.2.4 Quantification of DNA and protein

The concentration of purified PCR products, plasmid, genomic DNA and purified proteins was quantified using a NanoDrop (ND-1000) spectrophotometer (Agilent Technologies).

2.2.5 Polymerase chain reaction (PCR)

All PCR reactions were performed in a 25 μl final volume using sterile 0.2 ml thin-walled PCR tubes and cycled using a C1000 model Thermal Cycler (BIO-RAD). A master mix containing all of the PCR components except template DNA was prepared in a pre-chilled, sterile microcentrifuge tube and thoroughly mixed by vortex. Following a brief centrifugation, a 24 μl aliquot of master mix was dispensed into chilled PCR tubes and 1 μl appropriate template DNA. The solution was mixed by gently stirring with a pipette tip and kept on ice prior to placement in the thermal cycler. Unless otherwise stated, all PCR mixtures contained: 100 ng of chromosomal DNA or 1-10 ng of Plasmid DNA; each of the respective primers to a final concentration of 200 nM; 2.5 μl 10 ×

Expand buffer (Roche); dNTPs (Roche or Promega) to a final concentration of 200 μM, Expand Hi Fidelity DNA polymerase 0.2 μl (3.5 U μl^{-1}) (Roche) and the reaction mixture was made up to a final volume of 25 μl with dH_2O. The PCR conditions were: initial template denaturation step of 3 min at 95°C, followed by 30 cycles of incubation, annealing at 48°C for 1 min, primer extension at 68°C for 3.5 min, and 94°C for 45s, with final incubation at 48°C for 1 min and 68°C for 10 min.

2.2.6 Agarose gel electrophoresis

An agarose gel (0.8-1%) was prepared according to the standard protocol. DNA ladders 2-Log (New England BioLabs) and 0.5 kb (Fermentas) were run alongside the samples to enable analysis of DNA fragment size in the samples. Gels were viewed using an Uvitec gel documentation system.

2.2.7 Purification of PCR and Gel-extracted DNA products

DNA fragments generated by PCR or restriction endonuclease digestion were purified using a QIAquick® PCR purification kit (Qiagen) according to manufacturer's recommendations. For gel extraction of DNA fragments amplified by PCR or generated by restriction digestion, samples were separated by agarose gel electrophoresis and the band of interest was excised with Gene catcher Tips (Web Scientific) and purified using a Gel extraction Kit (Qiagen) according to manufacturer's instructions.

2.2.8 Cloning of *cbbA* in N-terminal Histidine-tag vector

A 1,065-bp DNA fragment corresponding to the *cbbA* coding sequences was amplified by PCR using genomic DNA of *N. meningitidis* strain MC58 and the primer pair FBA_E1 and FBA_E2 incorporating *Bam*H1 and *Kpn*I sites, respectively (Table 2.1). The PCR reaction was performed as described in Section 2.5. The DNA fragment was purified and ligated to the pEXPNT-TOPO expression vector (Invitrogen) according to the manufacturer's instructions. Screening for a successful clone was performed by colony PCR amplification of the desired DNA fragment, restriction digestion with *Bam*H1 and *Kpn*I and subsequent DNA sequencing. A construct in which *cbbA* was correctly oriented for translation was chosen and used to transform *E. coli* BL21 (DE3) pLysS according to the manufacturer's instructions. The pEXP/NT-TOPO-based constructs drive expression of the recombinant protein with N-terminal 6-histidine tag under the control of phage T7 promoter.

2.2.9 Cloning of *cbbA* in C-terminal Histidine-tag vector

A DNA fragment corresponding to the *cbbA* coding sequence was amplified by PCR using genomic DNA of *N. meningitidis* strain MC58 and the primers FBA_pQE70 (F) and FBA_pQE70(R) incorporating *Sph*I and *Bgl*II sites, respectively (Table 2.1). The PCR reaction was performed as described in Section 2.5. The PCR conditions used to amplify this insert were: initial template denaturation step of 3 min at 95°C, followed by 30 cycles of incubation, annealing at 51°C for 1 min, primer extension at 68°C for 2.5 min, and 94°C for 45s, with final incubation at 51°C for 1 min and 68°C for 10 min. The pQE70 vector was linearized by digestion (section 2.10) with *Sph*I and *Bgl*II. The

plasmid pSAT-FBA was constructed by ligating *Sph*I and *Bgl*II-digested, gel-purified *cbbA* PCR product with pQE70 (Appendix B) according to the manufacturer's instructions (Qiagen). Positive clones were screened for the successful ligation of c*bbA* by colony PCR, restriction digestion with *Sph*I and *Bgl*II and DNA sequencing to determine in frame ligation. A clone that contained *cbbA* in the correct orientation for translation and in frame with the start codon of the vector was selected and used to transform *E. coli* BL21 (DE3) pLysS (Invitrogen) according to the manufacturer's instructions.

2.2.10 Restriction endonuclease digestion

Restriction digestion reactions were routinely carried out in a 10 µl final volume. Unless otherwise stated, 100-200 ng of DNA (for screening purpose) and 1 µg of DNA (for using in sub-cloning), was digested either as a single digest or double digest with appropriate enzymes and compatible buffers. Restriction endonuclease enzymes and buffers were purchased from New England Biolabs or Roche and used according to the directions of the manufacturer.

Table 2. 1 List of primers used in work described in this chapter

Primer	DNA sequence [a]	Restriction site
	Expression of *cbbA*	
FBA_E1(F)	5'-CGC**GGATCC**ATGGCACTCGTATCCATGCG- 3'	*Bam*HI
FBA_E2(R)	5'-CGC**GGTACC**GTCGTCCGAACGGCGG- 3'	*Kpn*I
FBA_pQE70 (F)	5'-CGC**GGATCC**ATGGCACTCGTATCCATGCG-3'	*Sph*I
FBA_pQE70 (R)	5'-CGC**GGTACC**GTCGTCCGAACGGCGG-3'	*Bgl*II
	Expression of *gapA-1*	
NMB0207(F)	5'-CGC**GGATCC**ATGGGCATCAAAGTCGCCATC-3'	*Bam*HI
NMB0207(R)	5'-CGC**GTCGAC**TTATTTGAGCGGGCGCACTTC-3'	*Sal*I

[a]Sequences in bold identify restriction enzyme sites.

Table 2. 2 Bacterial strains and plasmids used in work described in this chapter

Strains/plasmids	Description	Source or reference
	***E. coli* strains**	
JM109	*end*A1 *rec*A1 *gyr*A96 *thi hsd*R17 (r_K^- r_K^-) *rel*A1 *sup*E44 Δ(*lac-pro*AB) [F' *tra*D36 *pro*AB *laq*I^{q}ZΔM15]	Promega
TOP10F'	F'*lac*Iq*Tn*10(TetR) *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L *end*A1 *nup*G	Invitrogen
BL21(DE3)pLysS	F- *ompT hsd*SB (rB-mB-) *gal dcm* (DE3) pLysS (CamR)	Invitrogen
	Plasmids	
pQE70	Cloning vector encoding resistance to ampicillin	Qiagen
pEXP/NT-TOPO	Expression vector encoding resistance to ampicillin	Invitrogen
pCR/NT-TOPO	Expression vector encoding resistance to ampicillin	Invitrogen
pSAT-FBA	MC58 *cbbA* gene cloned in pQE70	This study
pDT-GapA1	MC58 *gapA-1* gene cloned in pCR/NT-TOPO	This study
pSAT-1	MC58 *cbbA* gene cloned in pEXP/NT-TOPO	This study

2.2.11 Ligation Reaction

Appropriate amounts of vector and insert DNA were combined in a 1:1 or 1:3 ratios in a sterile microcentrifuge tube and the reaction was assembled in 10µl final volume. A typical ligation reaction contained; 100 ng insert, 50-100 ng plasmid DNA, 1µl T4 DNA ligase (Roche or Promega), 1 µl salt solution in case of TOPO cloning, 1 µl 10 × ligation buffer (Roche), and where required dH_2O was added to make a final volume of 10 µl. The ligation reaction was incubated at 16°C for 1h and then overnight at 4°C. An aliquot of 1.5 µl was used to transform *E. coli* JM109 competent cells.

Where appropriate, Antarctic phosphatase ([AP] New England BioLabs) was used to catalyze the removal of the 5′ phosphate groups from linear DNA fragments to prevent self-ligation. The procedure was carried out according to the manufacturer's recommendations. Typical reactions contained 1 µl [≡ 10 U] of AP, approximately 1 µg of vector DNA, 5 µl of 10× AP buffer and nuclease-free deionized water to a final volume of 50 µl. This was incubated at 37°C for 15 min followed by purification using the QIAquick PCR purification kit (Qiagen)

2.2.12 Transformation

Briefly, an aliquot of 100 µl of frozen competent *E. coli* cells JM109 (Promega), One shot Top 10 and BL21 (DE3) pLysS (Invitrogen) was thawed on ice 5 min prior to transformation. 100 ng of plasmid DNA was added to cells and mixed gently by stirring with a pipette tip. The cells were incubated on ice for 20 min followed by heat shock at 42°C in water bath for 50 s without shaking. Immediately after heat treatment, the cells were transferred on to ice for 2 min.

To the cells was added a 450 µl of room temperature (RT) S.O.C medium (Invitrogen) and the cell suspension was incubated at 37°C for 1.5 hr with shaking at 250 rpm. The transformation reaction was plated out as 100µl, 150 µl, and 200 µl, on LB agar containing appropriate antibiotic and incubated overnight at 37°C.

2.2.13 DNA sequencing

DNA constructs were sequenced in both directions using specific primers at the School of Biomedical Sciences (University of Nottingham) on an ABI 377 automated DNA sequencer.

2.2.14 Pilot expression of the recombinant rFBA and rGAPDH-1

E. coli strain BL21 (DE3) pLysS harboring appropriate expression plasmid were grown overnight in LB broth containing 100 µg ml^{-1} ampicillin. The following day, 500 µl of the overnight culture was inoculated into fresh 10 ml LB/ampicillin broth. The cultures were allowed to grow to mid log phase (OD_{600} = 0.6). The culture tube was split into two 5 ml culture aliquots and IPTG was added to a final concentration of 1 mM to one of the cultures. The culture tubes were then incubated at 37°C for 4 h with shaking and 500 µl aliquot was removed from each culture at times 0, 1, 2, 3, and 4 h, and cell were harvested by centrifugation at 13,000 × *g* . The cell pellets were either stored at -20°C or resuspended in 100 µl of 1× SDS-PAGE sample Buffer (Appendix A). The samples were heated to 100°C for 5 min and subsequently resolved by SDS-PAGE followed by immunoblot analysis.

2.2.15 Sodium dodecyl sulfate polyacrylamide gel electrophoresis

The recombinant proteins were electrophretically separated by 10-12% SDS-PAGE (Appendix A) using Mini- Protean III equipment (BIO-RAD) following the method of Laemmli (Laemmli, 1970). The protein samples were prepared by mixing bacterial cell pellets with 1 × SDS-PAGE sample buffer (Appendix A) and heated at 100°C for 5 min. An aliquot of 10µl of sample was loaded into each well of the gel and 10µl of pre-stained protein markers (New England Biolabs) were loaded into an adjacent lane. Proteins separated by SDS-PAGE were visualized by staining with SimplyBlue SafeStain (Invitrogen) or PageBlue^{TM} Protein Stain Solution (Fermentas) according to the manufacturer's instructions. Gels were scanned using a GS-800 calibrated densitometer (BIO-RAD).

2.2.16 Immunoblot analysis

For Immunoblot analysis, the protein mixtures separated by 10% SDS-PAGE were transferred onto a nitrocellulose membrane (Amersham Biosciences) in semi-dry blotting buffer (Appendix A) using a Trans-Blot SD semidry transfer cell (BIO-RAD) at a constant currant of 14 mA for approximately 30 min. The membranes were incubated in blocking solution (Appendix A) or 1% Bovine serum albumin (BSA) (Sigma) for 30 min at RT. The membranes were then probed with mouse anti-pentahistidine antibody (Qiagen) or rabbit primary antibody diluted 1:10,000 and 1:1000, respectively, in blocking buffer and incubated overnight with shaking at 4°C. The following day, the membrane was washed three times 15 min each with PBS containing 0.05% Tween-20 (PBS-Tween), and incubated into secondary antibody (anti-mouse IgG

conjugated to alkaline-phosphtase or anti-rabbit conjugated to alkaline phosphtase) (Sigma) at the concentration of 1:30,000 for 2 h at RT. After 2 h the membrane was washed thrice with PBS-Tween for 15 min each time. The membrane was then developed using the BCIP (5-bromo-4- chloro-3-indolylphosphate)–Nitro Blue Tetrazolium liquid substrate (PerkinElmer^{TM}). The membrane was finally washed with dH_2O and a digital image of the membrane was taken using a GS-800 calibrated densitometer (BIO-RAD).

2.2.17 Protein purification under denaturing conditions

The large-scale expression of recombinant His-tagged proteins for purification was performed using the common expression protocol. Briefly, a single colony of *E. coli* BL21 (DE3) pLysS harboring the expression plasmid was inoculated into 10 ml LB containing ampicillin (100 $\mu g\ ml^{-1}$) and grown at 37°C overnight. One litre of LB broth containing ampicillin was then inoculated with 10 ml of an overnight starter culture and grown to mid log phase ($OD_{600} = 0.6$), and induced by addition of IPTG to a final concentration of 1 mM. After 3h of induction the cells were harvested by centrifugation (8,000 × *g* for 10 min at 4°C) using an Allegra^{TM} X-22R centrifuge (Beckman Coulter) and the cell pellet was then either stored at -20°C or used for purification. Protein purification was achieved by re-suspending the culture pellet from 20 ml culture in 1 ml lysis buffer (B) (Appendix A) and sonicated (10 s on, 20 s off pulses for 5 min on ice) using a (SoniPrep sonicater MSE 150) to disrupt the cells. The lysate from disrupted cells was centrifuged at 8,000 × *g* for 30 min at 4°C. The supernatant was collected in a sterile Eppendorf tube for purification of the recombinant protein. Purification was carried out using a Ni-NTA spin kit (Qiagen). Firstly, Ni-NTA spin columns

were equilibrated with 600 µl of buffer B, and centrifuged at 700 × *g* for 2 min to drain the buffer and then 600 µl of supernatant collected from the lysate of disrupted cells was loaded into pre-equilibrated columns and centrifuged at 700 × *g* for 2 min. The flow through was collected and saved in a separate Eppendorf tube for SDS-PAGE analysis. Secondly, the spin columns were washed twice with approximately 600 µl of buffer C (Appendix A) to release non-specific proteins. The third and last step was elution of pure proteins bound to spin column with buffer E (Appendix A). The protein elutes were stored at -20°C

2.2.18 Protein purification under native conditions

Briefly, the culture pellet from a 1-litre culture of *E. coli* BL21 (DE3) pLysS cells containing pSAT-FBA and induced with IPTG for 3 h, was resuspended in the lysis buffer (Appendix A) and the cells were then disrupted by sonication (10s on, 20s off pulses for 10 min on ice) in ice water. Soluble and insoluble fractions were separated by centrifugation (8,000 × *g* for 30 min at 4°C). Five hundred µl of Hispure™ cobalt resin (Thermo) was centrifuged at 2,000 × *g* for 1 min to drain the storage buffer and then the resin was added to the soluble fraction containing the recombinant FBA. The supernatant-resin mixture was then incubated overnight at 4°C to allow binding of the tagged protein to the resin. The following day, the mixture was passed through gravity purification columns (Sigma). The flow through was collected and re-applied to the column to maximize protein binding. The resin was then extensively washed with approximately 10 column volumes of wash buffer (Appendix A) to minimize the yield of contaminating proteins in the eluted fraction. The protein bound to the

resin was then eluted in elution buffer (Appendix A). The purity of the protein was examined by SDS-PAGE.

2.2.19 Protein purification by gel electro-elution

The partially purified samples of rGAPDH-1 were resolved in a single lane on 10% SDS-PAGE. The gel was stained briefly with SimplyBlue SafeStain (Invitrogen). The band corresponding to the recombinant protein of interest was identified according to its size estimated by comparing with the molecular weight markers. The protein band with an apparent molecular mass of *ca.* 37-kDa demonstrating rGAPDH-1 was excised out from gel and further cut into small slices. For electro-elution, the gel slices were transferred to a D-tube (D-Tube Dialyzer midi, MWCO 6-8 kDa, Novagen) containing filter sterile 1× SDS-running buffer (Appendix A) and electro-elution was performed at constant voltage of 110 volts for 2 h in a agarose gel casting tank filled with 1 × SDS running buffer. The eluted protein was aspirated from the D-Tubes and dialysed in clean D-tubes against PBS for 48 h at 4°C and then concentrated using centrifugal filter device (Microcon, Amicon Bioseparation, 5,000 Da MWCO).

2.2.20 Raising polyclonal antiserum against FBA and GAPDH-1

Rabbit polyclonal antibodies against the denatured purified recombinant FBA and GAPDH-1 and non-denatured purified FBA proteins were raised in New Zealand white female rabbits. Typically, rabbits were immunized three times at 2-week intervals with 30 μg of protein emulsified in Freud's Complete (first immunization) or incomplete adjuvant. After two doses, the rabbits were test bled, boosted once more, and sacrificed 10 days later.

2.2.21 Protein and nucleic acid sequence analysis

The genome database of *N. meningitidis* MC58 was interrogated at http://cmr.jcvi.org/cgi-bin/CMR/GenomePage.cgi?org=gnm. Sequence homology data were obtained using the CLUSTALX software (http://www-igbmc.u-strasbg.fr/BioInfo/ClustalX). Protein secretion signals were analyzed using the SignalP 3.0 server available at http://www.cbs.dtu.dk/services/SignalP/ (Emanuelsson *et al.*, 2007). The SoftBerry Bacterial Promoter Prediction algorithm- (http://linux1.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb) was used to examine DNA segments for potential promoter sequences. Other DNA and protein sequence analyses, and primers designing, were carried out using the DNAman package of programs (Lynnon BioSoft).

2.3 Results

2.3.1 Sequence analysis of the *cbbA* and flanking DNA and FBA protein

In *N. meningitidis* strain MC58, the *cbbA* gene (NMB1869) is 1,065 bp long encoding a predicted protein of 354 amino acids (Fig 2.1) and has a G+C content of 56.18%. The *cbbA* gene is downstream of *xerC* (NMB1868) predicted to encode the XerC integrase/recombinase and upstream of NMB1870, which encodes factor H-binding protein, fHbp (Madico *et al.*, 2006). This genetic organization appears to be conserved in published genomes of *N. meningitidis* Z2491 (serogroup A), *N. meningitidis* FAM18 (serogroup C) and *N. gonorrhoeae* FA1090 strains. However, the gene encoding for fHbp is annotated as putative lipoprotein and/or hypothetical open-reading frame. Sequences >94% identical to the MC58 *cbbA* gene are found in the genomic sequences of the serogroup C meningococcal strain FAM18 (GenBank accession no. YP_974462; (Bentley *et al.*, 2007)), the ST-4821 strain 053442 (GenBank accession no. YP_001598513; (Peng *et al.*, 2008)) and the group A strain Z2491 (GenBank accession no. YP_002342063; (Parkhill *et al.*, 2000). Additionally sequences >93% identical to MC58 *cbbA* are found in the gonococcal strain FA1090 (94% identical; GenBank accession no. YP_207215), and *N. lactamica* strain ATCC 23970 (93% identical; GenBank accession no. ZP_03723075) sequences confirming that *cbbA* is highly conserved across *Neisseria* species. At the amino acid level, FBA sequences from meningococcal strains Z2491, MC58, FAM18 and 053442, and the gonococcal strain FA1090, are > 99% identical. By alignment, the neisserial FBA (NMB1869) was 67 and 65% identical to class-IIB FBA enzymes from *Xanthobacter flavus* and *Synechocystis* sp., respectively, but was only 21% identical to the *E. coli* class-IIA FBA, confirming that the neisserial FBA is a

class-IIB enzyme. As expected for a class-II FBA, a conserved putative zinc-binding site (Berry & Marshall, 1993) was also identified (H^{81}-XX- H^{84}). Using the SignalP software, FBA was predicted to be a non-secreted protein by the SignalP-HMM program

2.3.2 Cloning and expression of *cbbA* in pEXP/NT-TOPO

The *cbbA* gene comprises of 1,065 bp, which encodes a protein of a 354 amino acids (a.a). The entire ORF was successfully amplified by PCR (Fig 2.2). A single band with the expected molecular weight of *ca.* 1-kb was observed by agarose gel electrophoresis. The PCR product was ligated to the pEXP-NT/TOPO expression vector followed by transformation of *E. coli* JM109 cells for plasmid proliferation and subsequent screening for the successful clones.

The pEXP-NT/TOPO expression vector, unlike pGEM-T Easy, lacks the white/blue phenotype based screening system; therefore, the screening for the successful clones was initially attempted by colony PCR (Fig 2.3 A). Several recombinant clones were identified to contain the *cbbA* insert by observing a DNA fragment corresponding to *cbbA* alongside a band amplified with the same primers using MC58 DNA as a positive control. In order to confirm the colony PCR results and to ascertain the orientation of *cbbA*, recombinant clones were selected and used to extract plasmid DNA for restriction analysis and subsequent partial sequencing.

Six clones were subjected to further verification by restriction digestion analysis. The restriction sites *Bam*HI and *Kpn*I (present in FBA_E1 and E2 primers respectively) were used to ascertain the orientation of the desired insert. Insertion

of *cbbA* in the same orientation as the T7 promoter of the vector was expected to produce single linear fragments of *ca* 3.7 kb in a double digest with *Bam*HI and *Kpn*I. In contrast, insertion of *cbbA* in a reverse orientation to the vector yielded the expected two DNA fragments of *ca.* 1 kb and 2.7 kb. Out of six clones analysed by colony PCR, three were identified to contain *cbbA* in the desired orientation whilst the remaining three produced two DNA bands consistent with the reverse orientation (Fig 2.3 B).

One plasmid containing *cbbA* in correct orientation was designated pSAT-1. Finally, the sequence and orientation of the cloned *cbbA* was confirmed by DNA sequencing. The plasmid pSAT-1 was partially sequenced using the T7 forward and reverse primers. Sequencing demonstrated that pSAT-1 contained the *cbbA* in frame with the His-tag of the vector and in correct orientation under the control of phage T7 promoter for expression of the recombinant protein.

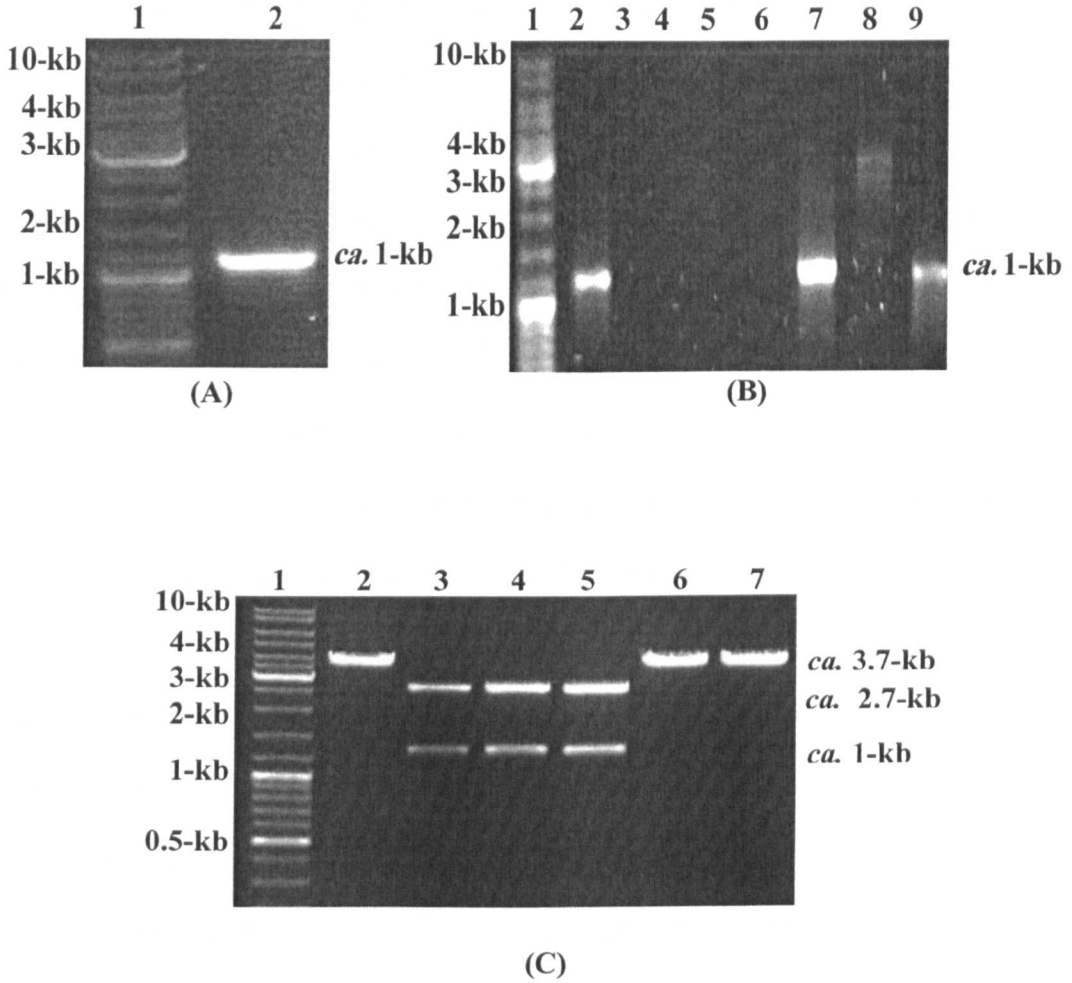


Figure 2. 1 Agarose gel analysis showing (A) the PCR amplification of *cbbA*. Chromosomal DNA was prepared from *N. meningitidis* MC58 and used to amplify out *cbbA* by PCR. Lane 1, DNA ladder, lane 2, PCR product corresponding to *cbbA,* (B) colony PCR amplification of *cbbA,* putative transformant colonies were used for PCR amplification of *cbbA,* lane 1, DNA markers, lane 2, PCR product of *cbbA* from MC58 chromosomal DNA (Control), lanes, 3-9, putative transformant colonies. The DNA product in lanes 7 and 9 was of the expected size suggesting successful cloning of *cbbA* (C) restriction digestion of pSAT-1, plasmid DNA was used to verify the cloning of *cbbA* using the *Bam*HI and *Kpn*I. Lane 1, DNA markers, lanes 2-7, putative pSAT-1 digested with *Bam*HI and *Kpn*I.

Expression of rFBA was successfully achieved in *E. coli* BL21 (DE3) pLysS cells harboring the plasmid pSAT-1 following induction with IPTG. Expression was checked by SDS-PAGE followed by either staining or immunoblot analysis. A recombinant protein band with apparent molecular weight of *ca.* 38-kDa corresponding to the rFBA was observed in induced samples on the SDS gel after staining with SimplyBlue SafeStain (Fig 2.4). Immunoblot analysis also detected the tagged recombinant protein corresponding to the full length rFBA. A faint protein band was also observed in un-induced samples. This was probably due to leaky expression of the gene without induction as it was absent in negative control cells containing the same vector without the insert (Fig 3.5).

The solubility of the expressed rFBA was determined by resuspending the cell pellet in PBS. Following 30 min incubation at room RT, cells were disrupted by sonication and centrifuged to separate soluble and insoluble fractions. Both fractions were collected separately and analysed by SDS-PAGE (Fig 2.6 A) followed by immunoblot analysis using anti-penta histidine antibodies (Fig 2.6 B). A strong protein band corresponding to FBA was detected in insoluble fraction whereas a weak band was present in soluble fraction of expressed protein suggesting that the rFBA protein was expressed mostly as insoluble protein.

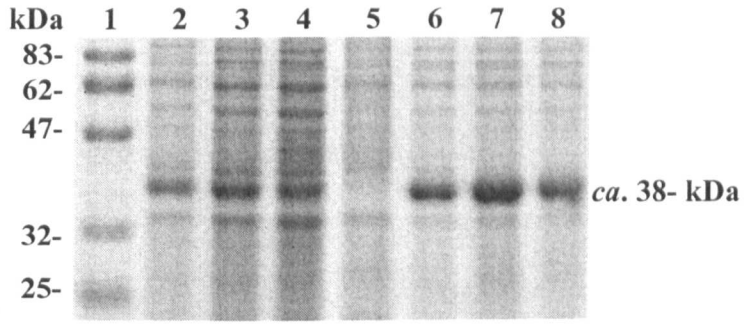


Figure 2. 2 SDS-polyacrylamide gel showing pilot expression of FBA. Whole cell lysate of *E. coli* strain BL21(DE3) pLysS containing the plasmid pSAT-1, non-induced and IPTG-induced samples were separated by 10% SDS-PAGE. Lane 1,protein markers; lanes 2-4, whole cell lysate of non-induced cultures after , 1, 2, and 3 h, respectively; lane 5, lysate from *E. coli* containing empty vector, lanes 6-8 whole cell lysate of IPTG-induced cultures after 1, 2, and 3 h, respectively. The protein was expressed within 1 h after induction and the expression reached maximum level at 3 h.

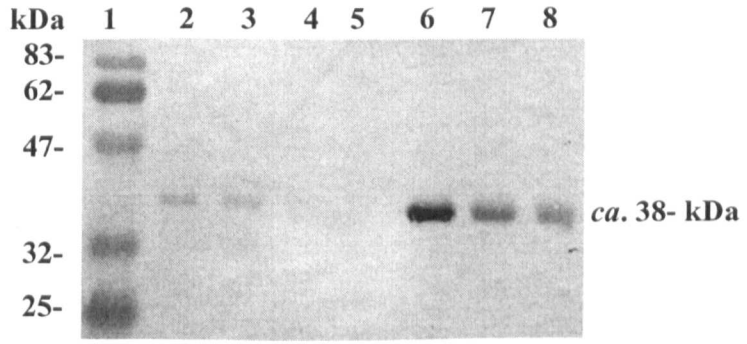


Figure 2. 3 Immunoblot analysis showing the pilot expression of FBA. Whole cell proteins were separated by 10% SDS-PAGE and then transferred on to nitrocellulose membrane followed by probing with anti-penta histidine antibodies. Lane 1, protein markers; lane 2-4, un-induced samples lane 5, lysate from *E. coli* containing empty vector, lanes 6-8 induced samples.

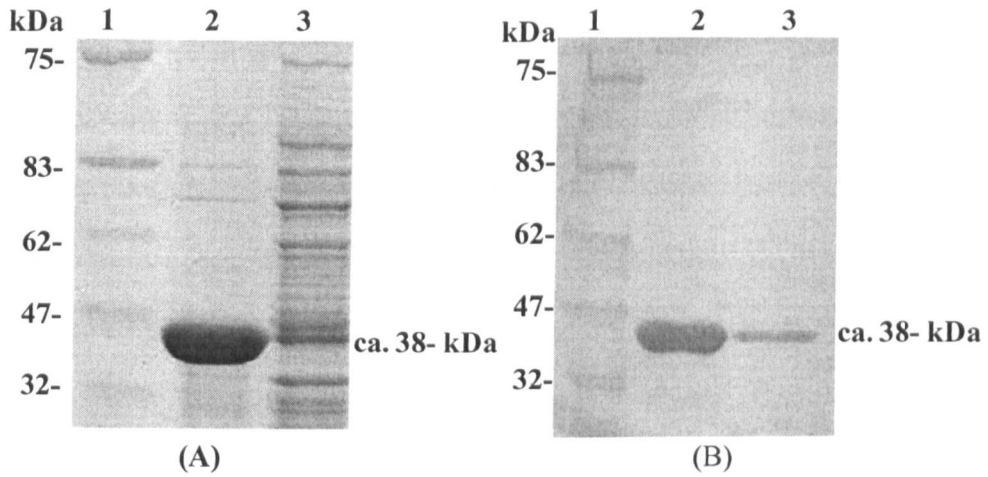


Figure 2. 4 Solubility of the rFBA *E. coli* BL21(DE3) pLysS harboring pSAT-1 were induced with 1 mM IPTG and incubated at 37°C for 3 h. (A) Protein were resolved by 10% SDS-PAGE Lane 1, protein markers; lanes 2 pellet (insoluble fraction; inclusion bodies); lane 3, supernatant fraction. (soluble fraction), (B) Immunoblot analysis of protein resolved by SDS-PAGE. Lane 1, protein markers; lane 2, pellet (insoluble fraction; inclusion bodies); lane 3, supernatant fraction. This is showing that FBA was expressed as insoluble protein.

2.3.3 Purification of rFBA under denaturing conditions

Due to the insoluble nature of recombinant protein, purification was performed under denaturing conditions. The 20 ml culture pellet of *E. coli* harbouring pSAT-1 was induced with IPTG in a mid log phase and further grown for 3h. Following sonication of the harvested cells, purification of the recombinant protein was successfully achieved under denaturing conditions using affinity chromatography (Fig 2.7). The protein was eluted in elution buffer containing urea. After purification urea was removed by dialysis against PBS using D-tube-dialyzer. Purified protein was analysed by SDS-PAGE to confirm the purity and integrity of the purified protein.

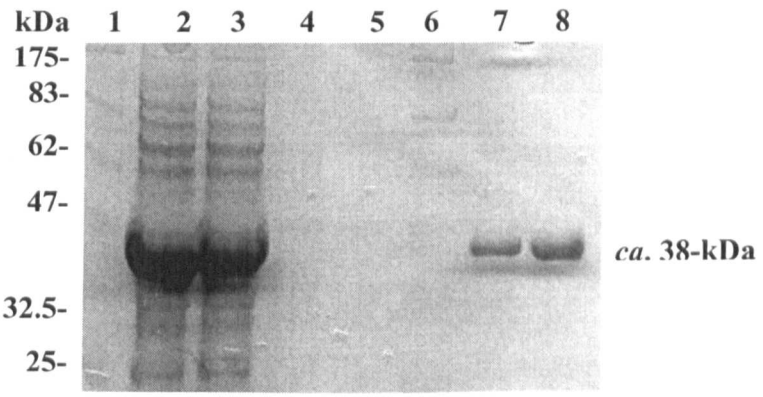


Figure 2. 5 SDS-polyacrylamide gel demonstrating affinity purification of rFBA. *E. coli* strain BL21 containing plasmid pSAT-1 were grown until the exponential phase and induced with 1 mM IPTG. After 3 h of induction, cells were harvested by centrifugation and purified using a Qiagen Ni-NTA spin kit under denaturing conditions and the samples were separated by 10% SDS-PAGE. Lane 1, protein markers; lane 2, whole cell lysate of *E. coli* harboring pSAT-1; lane 3, flow through; lane 4, first wash; lane 5 second wash; lane 6, protein markers, lane 7 first eluate; and lane 8 second eluate. The eluate showed the single protein band representing the full sized rFBA

2.3.4 PCR amplification and cloning of *cbbA* in pQE70

Although N-terminally His-tagged rFBA was successfully expressed and subsequently purified under denaturing conditions, in order to examine the functional activity of the recombinant protein and to generate polyclonal antibodies against conformational epitopes of the protein, *cbbA* was re-cloned with C-terminal 6 × His-tag in an endeavour to express recombinant proteins in soluble form to facilitate subsequent purification under native conditions. The *cbbA* gene was amplified by PCR using primers incorporating *Sph*I and *Bgl*II restriction sites to facilitate the ligation with pQE70. The purified, digested *cbbA* product was successfully ligated to pQE70 after both molecules were digested with *Sph*I and *Bgl*II followed by transformation of *E. coli* JM109 cells. A number of recombinant clones were selected and screened for the presence of *cbbA* in order to confirm successful cloning. Screening was initially performed by colony PCR of putative transformants colonies. Several recombinant clones produced an expected band of *ca.* 1-kb were then selected and propagated for purification of plasmid DNA. The plasmid DNA was subjected to further confirmation by restriction digestion analysis using *Sph*I and *Bgl*II. As expected two DNA bands corresponding to *cbbA* and vector backbone were observed by agarose gel analysis demonstrating the successful ligation. Finally, one of the positive clones was sequenced. The sequencing results confirmed that the ligation was in frame with the start codon of the vector and the sequences were identical to that of the *cbbA*. This clone was selected for expression of recombinant protein and subsequent purification under non-denaturing conditions.

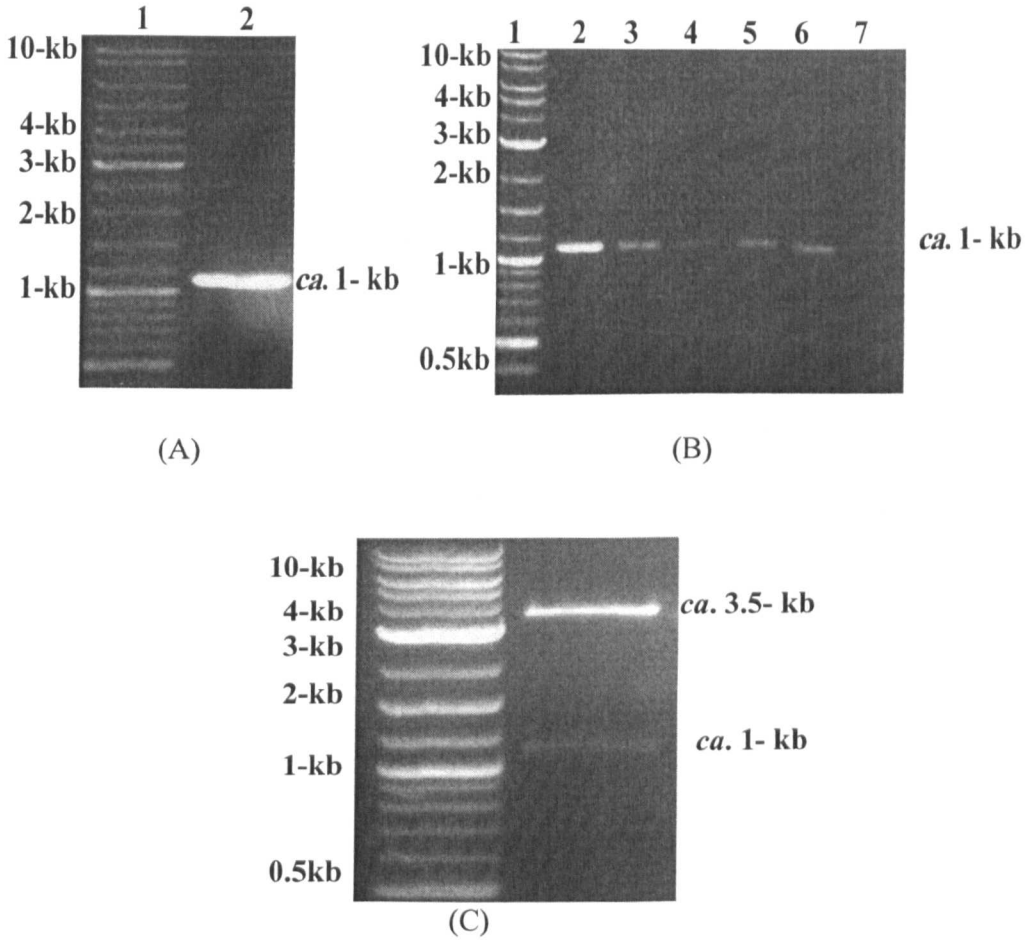


Figure 2. 6 Agarose gel analysis demonstrating the (A) PCR amplification of *cbbA,* Chromosomal DNA was prepared from *N. meningitidis* MC58 and used to amplify the entire open reading frame of *cbbA* for cloning into pQE70. Lane 1, DNA markers, lane 2, PCR product of *cbbA*. (B) colony PCR amplification of *cbbA*. Screening for *cbbA* cloning was performed by colony PCR amplification of *cbbA* from putative transformants colonies. Lane 1, DNA markers, lane 2-7, *cbbA* amplified by colony PCR, (C) the restriction digestion of pSAT-9. Plasmid DNA was isolated from *E. coli* harboring pSAT-9 and used to confirm the cloning of *cbbA* in pQE70. Lane 1, DNA markers, Lane 2, *Sph*I & *Bgl*II-digested pSAT-9. Lane 2 shows the expected two bands confirming the successful ligation of *cbbA* with pQE70

2.3.5 Expression and purification of rFBA under native conditions

E. coli BL21 (DE3) pLysS cells harboring the plasmid pSAT-FBA were used to express rFBA. Optimal expression was achieved after 3 h induction with IPTG. Expression was confirmed by SDS-polyacrylamide gel followed by immunoblot analysis. Despite the high levels of expression, no formation of inclusion bodies was detected (data not shown).

The Recombinant FBA from a 1-litre culture of *E. coli* harboring pSAT-FBA was purified under native conditions. After sonication of the harvested cells, purification of the recombinant protein was performed by affinity chromatography. The protein was eluted in elution buffer containing imidazole. After purification the imidazole was removed by dialysis against the same buffer (minus imidazole) using BD-dialyzer tubes. Purified protein was analysed by SDS-PAGE to confirm the purity and predicted molecular weight. SDS-PAGE analysis demonstrated a strong recombinant protein band with an apparent molecular weight of *ca.* 38-kDa corresponding to the FBA (Fig 2.9A). Immunoblot analysis using anti-penta histidine and RαFBA antisera also detected the protein band representing rFBA (Fig 2.9B & C respectively). The purified protein was used to examine enzymic activity of natively purified protein and to raise polyclonal antisera to facilitate subsequent characterisation.

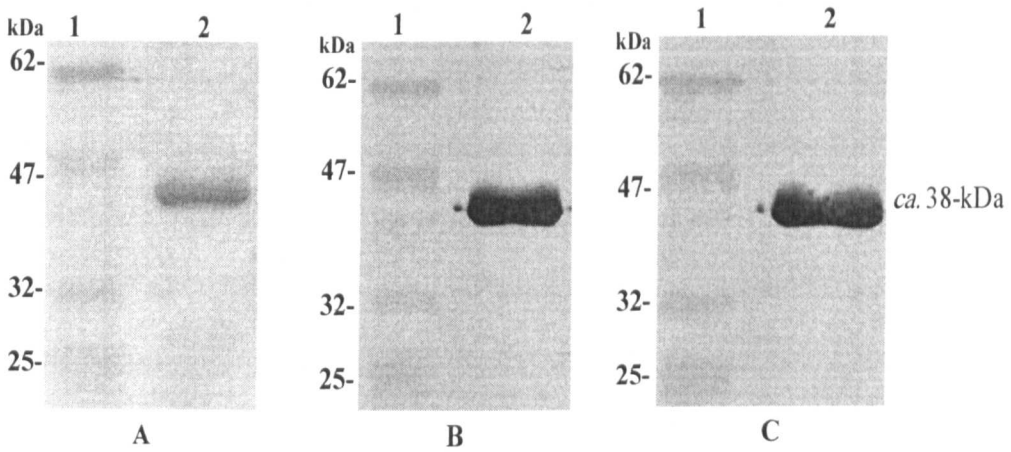


Figure 2. 7 SDS-PAGE and immunoblot analysis demonstrating the rFBA purified under native conditions. (A) SDS-PAGE (10%) analysis confirms the purity of the recombinant FBA purified under native conditions. (B) Immunoblot analysis shows that recombinant FBA is recognized by RαFBA (C) and anti-penta histidine antibodies.

2.3.6 Sequence analysis of *gapA-1*, flanking DNA and GAPDH-1 protein

In *N. meningitidis* strain MC58; the 1,032-bp *gapA-1* gene (Fig 2.10) (NMB0207) has a G+C content of 58 % and encodes a predicted protein of 343 amino acids (estimated molecular weight 37.0 kDa). The *gapA-1* gene is downstream of, and in the opposite orientation to, *aat* (NMB0206) predicted to encode the leucyl/phenylalanyl-tRNA-protein transferase and upstream of, and in the same orientation as, NMB0208, which is predicted to encode an electron transport protein, ferredoxin (4Fe-4S-type). The same genetic organization occurs in *N. meningitidis* Z2491 (serogroup A), *N. meningitidis* FAM18 (serogroup C). Sequences >98% identical to the MC58 *gapA-1* gene are found in the genomic sequences of the group A strain Z2491 (Parkhill *et al.*, 2000), the serogroup C meningococcal strain FAM18 (Bentley *et al.*, 2007), and the ST-4821 strain 053442 (Peng *et al.*, 2008). Additionally, *gapA-1* orthologues are found in the

gonococcal strain FA1090 (98% identical) and *N. lactamica* strain ATCC 23970 (90% identical) confirming that *gapA-1* is highly conserved across *Neisseria* species. At the amino acid level, GAPDH-1 sequences from meningococcal strains MC58, Z2491, FAM18, 053442, and the gonococcal strain FA1090 are >97% identical. As expected, the highly conserved GAPDH active site was identified (^{153}ASCTTNCL^{160}), and GAPDH-1 shows significant homology to GAPDH enzymes from higher organisms, including the human GAPDH enzyme (45% identity). Despite its demonstrated presence on the bacterial surface, the GAPDH-1 of *N. meningitidis* was not predicted to possess a classical secretion signal by the SignalP-HMM and -NN programs. GenBank accession numbers for the *gapA-1* sequences analyzed in this study are as follows: YP_97432562 (FAM18), YP_00160027 (ST-4821 strain 053442), YP_002341615 (Z2491), YP_208807 (gonococcal strain FA1090) and ZP_03723143 (*N. lactamica* ATCC 23970).

2.3.7 Cloning and expression of *gapA-1* of *N. meningitidis* strain MC58

The entire open reading frame of *gapA*-1 was amplified by PCR using the primers NMB0207 (F) & NMB0207 (R) primers (Table 2.1) and cloned into the pCR-T7/NT-TOPO expression vector to yield pDT-GapA1. The plasmid pDT-GapA1 was constructed by Dr. David P Turner (unpublished).

In order to express N-terminally 6 × His-tagged recombinant proteins, overnight culture of *E. coli* BL21 (DE3) harboring pDT-GapA1 were grown to mid log phase followed by induction with 1mM IPTG and further grown for 3 h. Samples were taken from induced and un-induced cultures at an hourly interval to monitor

the optimal expression. Pre-induced and post-induced samples were then analysed by SDS-polyacrylamide gel including negative control sample (*E. coli* BL21 (DE3) pLysS containing pCRT7/NT-TOPO vector without insert, treated under same expression conditions). A protein band of apparent molecular weight of *ca.* 37-kDa demonstrating rGAPDH-1 was observed in induced samples on SDS- gel (Fig 2.11) and also a weak band of the same size was present in uninduced samples suggesting the leaky expression. This band was absent in the negative control.

The resolved protein samples were subsequently transferred onto nitrocellulose membrane followed by probing with anti-penta antibodies. A strong immune-reactive band of predicted molecular mass of ca. 37-kDa corresponding to rGAPDH was detected by immunoblot analysis.

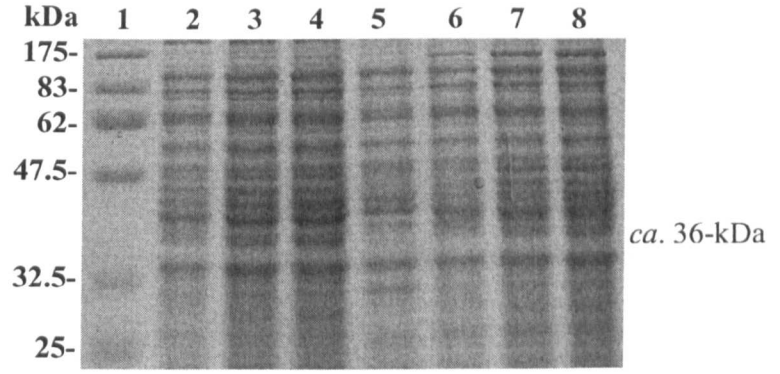


Figure 2. 8 SDS-PAGE (10%) analysis showing the expression of GAPDH-1 in induced and un-induced samples, Lane 1, protein markers, lane 2- 4, un-induced 0-3 h sample, respectively, lane 5, control (*E. coli* harboring empty vector), and lane 6-8, induced 0-3 h sample, respectively.

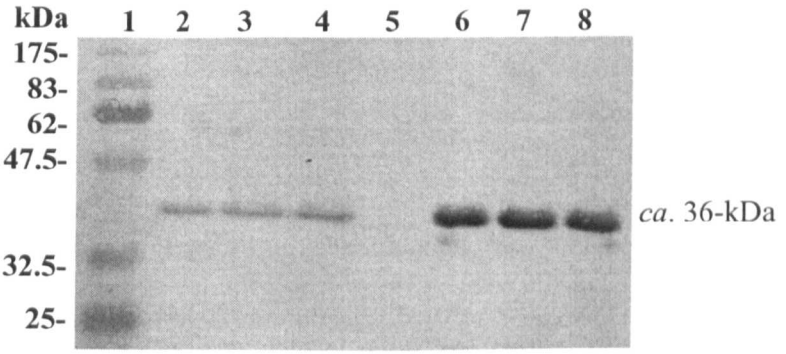
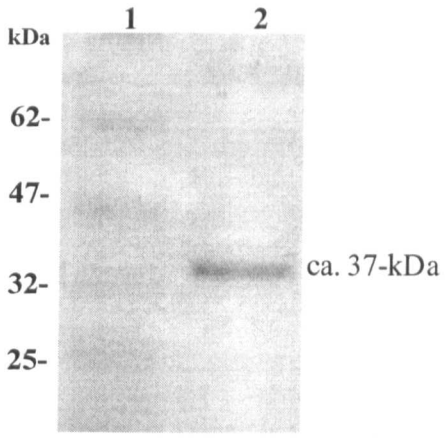


Figure 2. 9 Immunoblot analysis confirming expression of GAPDH-1in induced and un-induced samples, Lane 1, protein markers, lane 2- 4, un-induced whole cell proteins, lane 6, control (E. coli containing empty vector), lanes 6-8 whole cell proteins from induced samples.

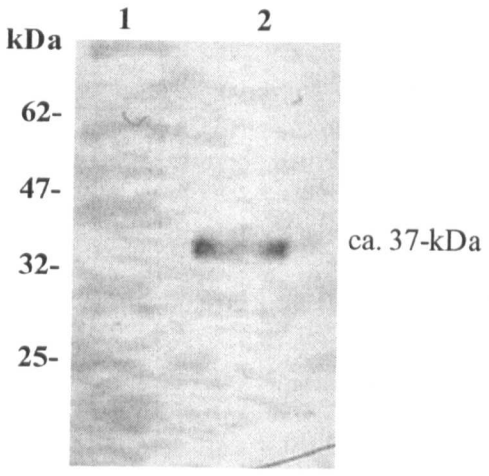
2.3.8 Purification of rGAPDH-1 by D-tube Dialyzer

The purification of the rGAPDH-1 was performed by using metal affinity chromatography. The rGAPDH-1 was resolved on SDS-PAGE to confirm the purity and expected size based on predicted molecular weight. A *ca.*37-kDa band corresponding to rGAPDH was observed together with several additional protein bands, which were assumed to be non-specific contaminating proteins. Therefore, in order to obtain a purified rGAPDH-1 for raising polyclonal antiserum the partially purified elutes containing rGAPDH-1were resolved on SDS-PAGE and the protein band corresponding to GAPDH-1 was excised from the SDS-polyacrylamide gel, and the gel pieces were used for purification by electro-eluation using D-tube dialyzers.

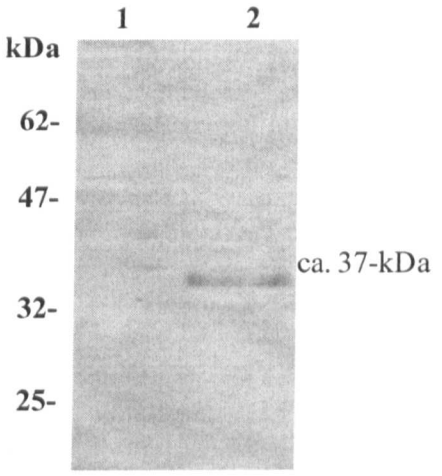
The purified protein was analysed by SDS-PAGE and immunoblot analysis. One gel was stained with SimplyBlue SafeStain and subsequently destained with dH_2O whilst another gel containing the same profile of proteins was used to transfer the resolved protein onto the nitrocellulose membrane for probing with either anti-penta histidine and/or Rα-GAPDH-1 antibodies. The SDS-PAGE demonstrated a single band with an apparent molecular weight of 37-kDa corresponding to rGAPDH-1. The immunoblot analysis demonstrated that anti-penta histidine recognised the purified protein as rGAPDH.



(A)



(B)



(C)

Figure 2. 10 SDS-PAGE and immunoblot analysis demonstrating the rGAPDH-1 purified under denaturing conditions. (A) 10% SDS-PAGE analysis confirms the purity of rGAPDH-1 (B) Immunoblot analysis shows that rGAPDH-1 is recognized by anti-penta histidine antibodies (C) and by RαGAPDH-1

2.4 Discussion

This chapter details the cloning of *cbbA* and *gapA-1* of *N. meningitidis* strain MC58 and the expression and subsequent purification of their products: FBA and GAPDH-1. A preliminary strategy adopted towards characterising both FBA and GAPDH-1 proteins was to clone and over express both proteins and subsequently to raise polyclonal antisera against both proteins. The antisera were then used in a number of experiments to facilitate identification of the putative roles of both enzymes in pathogenesis of meningococcal disease. In *N. meningitidis* strain MC58 genome there is a single copy of *cbbA* (NMB1869) predicted to encode FBA protein. The entire open reading frame encoding full length FBA was successfully cloned in N-terminally histidine-tagged vector to express the recombinant protein. The recombinant protein was shown to be optimally expressed in host *E. coli* using 1mM IPTG for 3 h. Expression was detected by immunoblot analysis using anti-penta histidine antibodies. As a negative control, vector without insert was used to transform *E. coli* and treated in parallel. Recombinant protein with apparent molecular weight of 38-kDa corresponding to FBA was obtained in induced samples. FBA was affinity purified under denaturing conditions and used to raise polyclonal antiserum in rabbit. The antiserum was shown to strongly react against purified FBA protein.

Following several unsuccessful attempts to purify under native conditions, the rFBA was successfully purified under denaturing conditions. Failure to purify protein under native conditions may possibly be due to the insoluble form of the protein, as confirmed by finding that majority of the proteins were in the pellet (inclusion bodies) fraction rather than in supernatant of expressed samples.

Despite optimisation of expression parameters such as lowering the growth temperature (20, 25, 30°C), adding less concentration of IPTG (0.5mM) at late exponential phase which is believed to improve the solubility of recombinant proteins, several attempts to express the protein in a more soluble form were unsuccessful. This insolubility issue was not just the case with meningococcal FBA, but this enzyme expressed with an N-terminal His-tag from *Mycobacterium tubeculosis* was also shown to be functionally inactive and insoluble protein (Ramsaywak *et al.*, 2004). In that study, to improve the yield of soluble protein efforts were made to express the tagged FBA at low temperature (15°C) and also by co-expression with different chaperones (GroES and GroEL; or DnaJ, Dnak, and grpE). These parameters although were shown to improve a little the yield of soluble protein but failed to yield an active enzyme (Ramsaywak *et al.*, 2004). Another possible explanation for inability to purify the protein under native conditions might also be due to the manner in which the protein is folded in its native form, such that the histidine-tag is buried within the protein and may not be exposed sufficiently to be purified by affinity chromatography.

Alternatively, to express recombinant FBA protein in a soluble form to achieve subsequent purification under native conditions, a DNA fragment corresponding to full length *cbbA* was successfully cloned in pQE70. This vector carries 6 × histidine-tag at C-terminal of the recombinant protein. Recombinant protein expressed with C-terminal histidine-tag was successfully purified under native conditions. SDS-PAGE analysis confirmed the expected size and purity by demonstrating a single band of apparent molecular mass of *ca.* 38-kDa corresponding to the recombinant FBA.

In the published meningococcal genome sequences, there are two genes (*gapA-1*; NMB0207 and *gapA-2*; NMB2159).encoding putative GAPDH enzymes. We began our investigations by focusing on *gapA-1* (NMB0207) rather than *gapA-2,* because the *gapA-1* has been shown to be up-regulated in *N. meningitidis* upon the contact with human epithelial cell whereas *gapA*-2 was not found to be similarly regulated. The *gapA-1* was successfully cloned in pCR-NT/TOPO and optimal expression of the recombinant protein was achieved by induction with 1mM IPTG for 3 h. The expression was shown to be not under tight IPTG control as non-induced samples contained a weakly expressed protein band of apparent molecular weight of *ca.* 37-kDa. Initial attempts to purify rGAPDH-1 under native conditions were not successful suggesting that the expressed protein was not folded correctly and appeared to be in insoluble form. Since previous optimisation attempts to improve expression of the FBA protein in more soluble form were unsuccessful, protein was therefore purified under denaturing conditions. The SDS-PAGE analysis of the affinity purified protein demonstrated several protein bands including the protein band with apparent molecular weight of *ca.* 37-kDa corresponding to rGAPDH-1. In order to obtain pure protein for raising polyclonal antiserum, the purification was carried out by electro-elution using D-tube dialyzers. The partially purified GAPDH-1 was first separated by polyacrylamide gel from SDS- polyacrylamide gels and then the protein bands corresponding to the GAPDH-1 were excised and eluted. The eluted proteins were dialyzed against PBS for 48 h. The immunoblot analysis using anti-penta histidine antibodies recognized a protein bands with apparent molecular weight of *ca.* 37-kDa.

In conclusion, expression plasmids for rFBA and rGAPDH-1 were successfully constructed. The rFBA was over expressed with N-terminal and C-terminal histidine tags and the recombinant proteins were successfully purified by affinity chromatography. The purification of N-terminal His-tagged rFBA under native conditions was unsuccessful, which may be due to the insoluble nature of the recombinant protein or in accessibility of His-tag to nickel column, however, the protein was successfully purified under denaturing conditions and used to raise polyclonal antisera. The rFBA was successfully purified under native conditions from C-terminally His-tagged vector. This natively purified rFBA was then used to study the functional/enzymic activity of the protein and used for generating polyclonal antiserum for using in subsequent characterisation experiments. In addition, the rGAPDH-1 was also over expressed with an N-terminal histidine-tag, again this protein was found to be in insoluble (inclusion bodies) fraction, and the purification was performed under denaturing conditions as insoluble proteins that were subsequently purified by gel electro-elution. The purified rGAPDH-1 was used for raising polyclonal antiserum to facilitate the characterisation experiments. Taken together, the work described in this chapter, resulted in the successful production of reagents that would facilitate further studies into the role of these proteins.

CHAPTER 3: Mutagenesis and complementation of *cbbA* and *gapA-1of N. meningitidis*

3.1 Introduction

In this chapter the generation of *cbbA* and *gapA-1* null mutants and subsequent complementation of *cbbA* and *gapA-1* mutations at an ectopic site in respective isogenic null mutants is described. The mutant and complemented strains will be utilized in subsequent characterization experiments to facilitate a study of the potential role of FBA and GAPDH-1 in pathogenesis of meningococcal disease. In addition to mutating *cbbA* in MC58, the *cbbA* isogenic mutants were created in four meningococcal strains of different genetic backgrounds (Z4181, Z4667, Z4673, and ST-18). The mutated strains were used as negative controls in immunoblot experiments to assess the expression of *cbbA* and *gapA-1* in meningococcal strains, and in subsequent molecular and immunological characterisation experiments (Chapter 4 & 5). Whilst the *cbbA*-isogenic mutants generated in additional four known MLST-type strains (Z4181Δ*cbbA*, Z4667Δ*cbbA*, Z4673Δ*cbbA*, and ST-18Δ*cbbA*) were used in challenge experiments using a transgenic mouse model to assess the potential role of *cbbA* in development of bacteraemia. The transgenic mice express human transferrin, which is an important source of iron during the course of meningococcal infection: a process requiring the binding of human transferrin by the meningococcal transferrin receptor, which is unable to efficiently bind transferrin of non-human origin (Zarantonelli *et al.*, 2007).

Using the *cbbA* and *gapA-1* mutant strains, a potential *in vitro* role of FBA and GAPDH-1 in adhesion of *N. meningitidis* strain MC58 to cultured meningothelial and epithelial cell lines to host cells was investigated. Both mutants exhibited a reduced capacity to adhere to these cell lines compared to their isogenic wild-

type parents. To exclude the possibility that the impaired adhesion was not due to either polar effects or undetected secondary mutations, both *cbbA* and *gapA-1* mutations were complemented at an ectopic site in isogenic-null mutants to determine whether the replacement of a wild-type copy of each of these genes could result in restoration of the wild-type phenotype.

The aims of this part of study were to create *cbbA* and *gapA-1* mutants in *N. meningitidis* and to subsequently complement the mutations in isogenic null mutant strains and to determine the growth characteristics between wild-type MC58, mutants and complemented strains.

3.2 Materials and methods

3.2.1 Bacterial strains, growth condition, and media

Bacterial strains and plasmids described in this chapter are listed in (Table 3.1 and 3.3). Growth conditions and media for culturing *E. coli* are described in Chapter 2. Meningococcal strains (Table 3.3, stored at -80 in a mixture of 50% MHB and sterilized glycerol) were grown on Brain Heart Infusion (BHI) agar and/or Brain Heart Infusion Broth or Muller-Hinton Agar (MHA) or Muller-Hinton Broth (MHB) or Dulbecco's Modified Eagle Medium (DMEM) supplemented with Vitox at the concentration suggested by the manufacturer (Oxoid) or 2% Fetal calf serum (Invitrogen) and where appropriate kanamycin (50 $\mu g\ ml^{-1}$) or erythromycin (5 $\mu g\ ml^{-1}$) and incubated in an atmosphere of 5% CO_2 at 37 °C with or without shaking.

3.2.2 Cloning of *cbbA* plus flanking DNA

A *ca.* 2.3-kb fragment of DNA (Fig 3.1 A) consisting of the *cbbA* gene, 1 kb of upstream and *ca.*300 bp of downstream flanking DNA was amplified by PCR using the primer pair FBA_M1 (F) and FBA_M2(R) (Table 3.2) and *N. meningitidis* strain MC58 chromosomal DNA. The PCR was performed as described in section 2.5 with the following modifications, annealing at 48°C for 1 min, primer extension at 68°C for 4.5 min, and 94°C for 45s. The PCR product was purified using the QIAquick PCR purification kit (Qiagen) according to manufacturer's instructions (Section 2.7). The PCR product was used to ligate with the pGEM-T Easy vector according to the manufacturer's instructions. Recombinant clones were identified as white colonies on selective agar plates containing 100 $\mu g\ ml^{-1}$ ampicillin, 0.5 mM IPTG and 80 $\mu g\ ml^{-1}$ X-gal. A number

of clones were selected and confirmed by restriction digestion (Section 2.2.10) and PCR using the primers FBA_M1 (F) and FBA_M2(R) (Table 3.2). The resulting plasmid was designated pSAT-2. The plasmid pSAT-2 (Fig 3.1B) was then used to transform *E. coli* JM109 competent cells (Promega) according to the manufacturer's instructions (Section 2.2.12).

3.2.3 Cloning of *gapA-1* plus flanking DNA

A *ca.* 3-kb region of DNA encompassing *gapA-1* and 1-kb flanking DNA from either side of the gene and containing a neisserial uptake sequence was amplified by PCR using the primer pair NMB0207 (F) FL and NMB0207 (R) FL (Table 3.2) and chromosomal DNA prepared from *N. meningitidis* strain MC58. The PCR was performed as described in section 2.5 with modifications: annealing at 48°C for 1 min, primer extension at 68°C for 3.5 min, and 94°C for 45s, with final incubation at 48°C for 1 min and 68°C for 10 min. The PCR product was gel-purified using a Gel-extraction kit (Qiagen) according to manufacturer's instructions. The PCR product was then cloned into the pGEM-T Easy vector (Promega). Putative transformants were identified as white colonies on selective agar plates (section 3.2.2). Recombinant clones were verified by restriction digestion analysis and PCR using the primer pair NMB0207 (F) FL and gapA-1 (R) FL (Table 3.2).

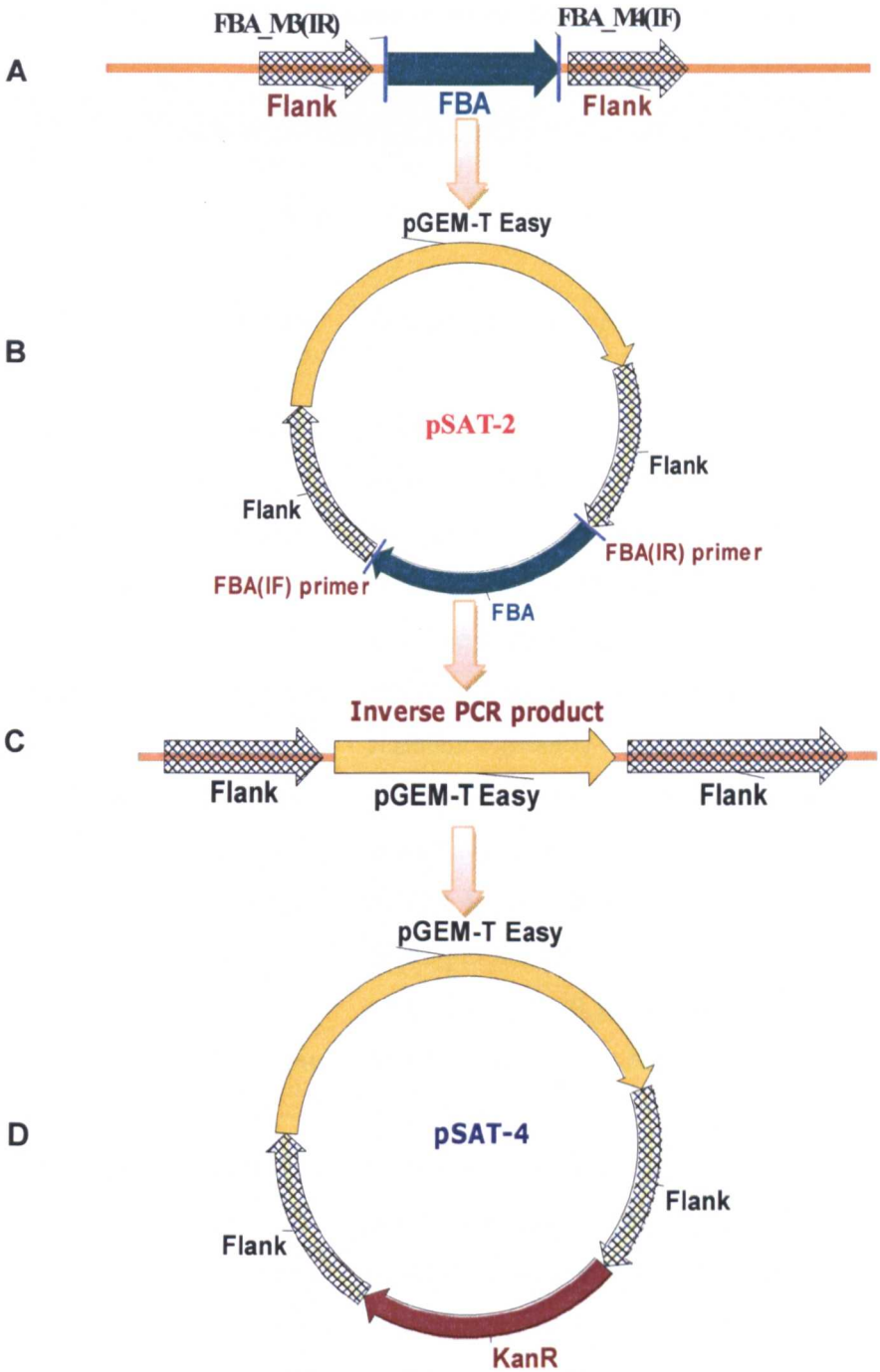


Figure 3. 1 Schematic diagram representing the mutagenesis strategy for *cbbA* in *N. meningitidis* A) A 2.3-kb DNA fragment encompassing *cbbA* and flanking DNA B) cloning of *cbbA* and flanking DNA in pGEM-T Easy to generate pSAT-2 C) Inverse PCR product containing the flanking DNA and pGEM-T easy vector for ligation of Kan^R resistance marker d) Mutagenic plasmid designated pSAT-4 containing Kan^R and inverse PCR product.

Table 3. 1 List of Plasmids used in work described in this chapter.

Plasmids	Resistance	Description	Source/ Reference
pGEM-T Easy	Amp	Cloning vector	Promega
pJMK30	Amp/kan	Source of kanamycin resistance cassette	(van Vliet *et al.*, 1998)
pHYS25	Erym	Ectopic complementation vector	(Winzer et al., 2002)
pSAT-2	Amp	pGEM-T Easy vector cloned with 2.3 kb insert of FBA plus flanking DNA	This study
pSAT-3	Amp	Inverse PCR product of *ca.* 5kb, digested with *Bgl*II and self-ligated by both ends to make it circularize as plasmid	This study
pSAT-4	Amp/kan	pSAT3 cloned with kanamycin resistance cassette of 1.5 kb	This study
pSAT-6	Amp	pGEM-T Easy cloned with *gapA-1* plus 2-kb flanking DNA	This study
pSAT-7	Amp	pGEM-T Easy vector with 2 kb DNA flaking either side of *gapA-1* gene amplified by PCR and self-ligated	This study
pSAT-8	Amp/kan	pSAT-7 ligated with Kan^R gene digested with *Bam*HI	This study
pSAT-11	Amp/ermy	pGEM-T Easy cloned with complementation cassette of *ca.* 2.7 kb	This study
pSAT-12	Amp/ermy	pSAT-11 cloned with *cbbA* along with its native promoter sequences	This study
pSAT-13	Amp/ermy	pSAT-11 containing *cbbA* promoter and *Bgl*II site for cloning of inserts for expression under *cbbA* promoter	This study
pSAT-14	Amp/ermy	pSAT-13 cloned with *gapA-1* within unique *Bgl*II site present downstream the *cbbA* promoter sequences	This study

3.2.4 Inverse-PCR mutagenesis of *cbbA*

The plasmid pSAT-2 was subjected to inverse-PCR using the primer pair FBA_M3 (IR) and FBA_M4 (IF) (Table 3.2, Fig 3.1 C). The PCR was performed as described in section 2.2.5 with the following modifications: annealing at 48°C for 1 min, primer extension at 68°C for 5 min, and 94°C for 45 s. The PCR product was digested overnight at 25°C with *Bgl*II before being self-ligated. The resulting plasmid designated pSAT-3 was used to transform *E. coli* for propagation and subsequent purification. The purified pSAT-3 was subsequently digested with *Bgl*II to enable insertion of resistance cassette. The kanamycin resistance cassette was prepared from the plasmid pJMK30 (van Vliet *et al.*, 1998) by digestion with *Bam*HI (Section 3.2.6). The *Bgl*II-digested and purified i-PCR product was ligated to *Bam*HI-digested Kanamycin resistance cassette. The resulting plasmid was designated pSAT-4 (Fig 3.1 D)

3.2.5 Inverse-PCR mutagenesis of *gapA-1*

The plasmid pSAT-6 was used as a template DNA in inverse PCR using the primer pair gapA1_M1 (IF) and gapA1_M2 (IR) (Table 3.2) and chromosomal DNA of *N. meningitidis* strain MC58. This PCR was carried out as described in section 2.5 with modifications: annealing at 48°C for 1 min, primer extension at 68°C for 5 min, and 94°C for 45 s. The inverse PCR product was purified and digested with *Bgl*II for 1h at 37°C and then overnight at 25°C. The *Bgl*II-digested i-PCR product was then self-ligated using T4 DNA ligase (Roche) to generate pSAT-7. The resulting plasmid pSAT-7 was purified and subsequently digested with *Bgl*II and ligated to 1.5-kb antibiotic cassette (encoding resistance to kanamycin). The resulting plasmid was designated pSAT-8.

3.2.6 Preparation of selectable marker

The antibiotic cassette encoding resistance to kanamycin (1.5 kb) was obtained by restriction digestion of 2 μg of pJMK30 plasmid (Appendix B) with *Bam*HI followed by preparative gel electrophoresis and gel extraction. The purified kanamycin resistance cassette (Kan^R) was then ligated to linearized pSAT-3 and pSAT-7 to yield pSAT-4 and pSAT-8, respectively.

3.2.7 Construction of ectopic complementation vector

A DNA fragment of *ca.* 2.7-kb (Fig 3.1) consisting of *opa* promoter and erythromycin resistance cassette flanked by two meningococcal genes (NMB0102 and NMB0103) was amplified by PCR using the primer pair CompCass_(F) and CompCass_(R) (Table 3.2) and plasmid DNA pYHS25 (Winzer et al., 2002) . The PCR was performed as described in section 2.5 with the following modifications, annealing at 51°C for 1 min, primer extension at 68°C for 4 min, and 94°C for 45s. Prior to cloning into pGEM-T Easy (Promega), the PCR product was fully sequenced using the primers comcass_F1 and comcass_R1 (Table 3.2). The gel purified PCR product was ligated to pGEM-T Easy vector (Promega). Screening for successful clones was performed on the basis of blue/white phenotype. Plasmid DNA from putative transformants was analysed by restriction digestion and DNA sequencing to confirm the successful cloning. The resulting plasmid was designated pSAT-11(Table 3.1, Fig 3.2).

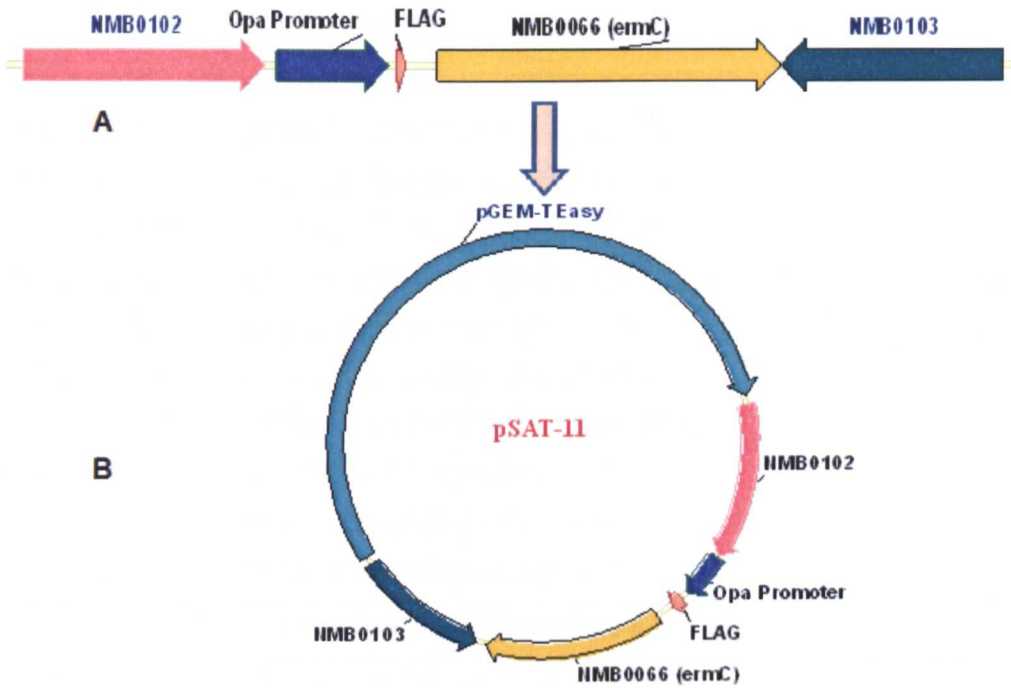


Figure 3. 2 Schematic diagram representing A) the gene organization of complementation cassette B) and subsequent cloning of this cassette into the pGEM-T Easy vector to generate pSAT-11

Table 3. 2 List of primers used in work described in this chapter

Primer	DNA sequence[a]	Restriction site
Mutagenesis of *cbbA* and *gapA-1*		
FBA_M1(F)	CTGCTGTGCCCGAGC	
FBA_M2(R)	CCGCTGCTGCAGGCG	
FBA_M3(IR)	GCG**AGATCT**TGTGTCTCCTTGGGCAATAGG	***Bgl*II**
FBA_M4 (IF)	GCG**AGATCT**GCTCCATCCAACTGGG	***Bgl*II**
FBA_SAT (F)	GCGCGCGGGTGGGCTTCGTCATAC	
FBA_SAT(R)	GTCTTTATGGTCGAGCGGTGCGG	
gapA1_M1 (IF)	GCG**AGATCT**GCAACAAACCGTC	***Bgl*II**
gapA1_M1(IR)	GCG**AGATCT**GGTTTGTTCCTTTGTTGAGGG	***Bgl*II**
NMB0207(R)FL	GAGAACTGTCATGCGTATTCC	
NMB0207(F)FL	CCAAACCCAATGCCGCGAATG	
gapA-1_C1 (F)	GCATACAATTCTGCTAAAATACGC	
CFR (R)	CAGCGACTGTCAGGCC	
Kan-CTR	GACAACGCAGACCGTTCCG	
Kan-NTR	TCGCGGCCTCGAGCAAGACG	
Complementation of *cbbA* and *gapA-1*		
CompCass_F	ATGTGGCGGGTTTTGAGTGC	
CompCass_R	GATTTTTCTTGCGGCGCGGC	
FBA_COM(F)	CGC**GGATCC**ATGAGCTGTTTATGGTTTTTTGCTG	***Bam*HI**
FBA_COM(R)	CGC**GGATCC**GGCATTTTGTTTACAGGCAACCTG	***Bam*HI**
pSAT-12iPCR(IF)	CGC**AGATCT**GATACCCCCGATGAC	***Bgl*II**
pSAT-12iPCR(IR)	CGC**AGATCT**CATTTGTGTC TCCTTGG	***Bgl*II**
gapA1_Comp(F)2	CGC**GGATCC**ATGGGCATCAAAGTC	***Bam*HI**
gapA1_Comp(R)2	CGC**GGATCC**TTTGTTTGACGGTTTGTTG	***Bam*HI**
Comcass_F	TCAGCGGGTGCGTCGAGAAGC	
Comcass_R	GCAAATCACAATTCTTGAGCG	
NMB0102(F)2	ATGTGGCGGGTTTTGAGTGC	
NMB0103(R)	TTTGGATTTTTCTTGCGGCGC	

[a]Sequences in bold identify restriction enzyme sites.

3.2.8 Natural transformation in *N. meningitidis* strain MC58

N. meningitidis strain MC58 was streaked on chocolate plates and incubated overnight as described in (section 3.2.1). A single colony was sub-cultured into fresh Brain-Heart Infusion (BHI) broth supplemented with Vitox (Oxoid) and incubated overnight in 5% CO_2 at 37°C. The overnight broth culture of *N. meningitidis* strain MC58 was diluted 1:20 in fresh BHI broth and further grown to an optical density of 0.2. An aliquot of 0.2 ml of the culture was transferred to a 15-ml tube with conical bottom containing 1.5 ml of BHI agar supplemented with Vitox. After incubation for 5-6 hrs at 37°C in 5% CO_2 without shaking, 10 µl (*ca.* 1 µg) of the mutagenic plasmid DNA was added to the tubes and incubation was continued for 16 h. The putative transformants were selected on BHI containing kanamycin 50 µg ml^{-1}. Kanamycin-resistant *N. meningitidis* colonies were obtained and further analysed by PCR and immunoblotting of whole cell extracts of the putative mutants.

3.2.9 Complementation of *cbbA*

To complement the *cbbA* mutation, *cbbA,* together with its predicted promoter sequences, was amplified by PCR from genomic DNA of *N. meningitidis* strain MC58 using the primers FBA_COM (F) and FBA_COM (R) (Table 3.2) incorporating *Bam*HI restriction sites. The PCR was performed as described in section 2.5 with the following modifications: annealing at 55°C for 1 min, primer extension at 70°C for 2.5 min, and 94°C for 45s. The PCR product was ligated to pGEM-T Easy (Promega), excised and then cloned into *Bam*HI-digested pSAT-11. Positive clones were analysed by colony PCR, restriction digestion analysis and DNA sequencing to confirm in frame ligation. The resulting plasmid was

designated as pSAT-12 and used to transform MC58Δ*cbbA* (Section 3.2.8) introducing a single chromosomal copy of the complementing gene in the intergenic region between NMB0102 and NMB0103 ORFs, which are orientated in a tail-to-tail fashion. Insertion of the gene at this ectopic site was confirmed by PCR analysis with the primer pair NMB0102 (F) and NMB0103 (R) (Table 3.2) and immunoblotting for the expression of the gene using RαFBA antisera.

3.2.10 Complementation of *gapA-1*

The plasmid pSAT-12 was subjected to inverse PCR using the primer pair pSAT-12iPCR (IF) and pSAT-12iPCR (IR) (Table 3.2). The PCR was performed as described in section 2.5 with the following modifications, annealing at 52°C for 1 min, primer extension at 68°C for 6.5 min, and 94°C for 45s. The PCR resulted in deletion of *cbbA* and introduction of a unique *Bgl*II site to facilitate the cloning of *gapA-1* under the promoter of *cbbA*. The amplicon was digested with *Bgl*II before being self-ligated. This plasmid was designated as pSAT-13. The *gapA-1* gene was amplified by PCR from the genomic DNA of *N.meningitidis* strain MC58 using primers gapA1_comp (F) 2 and gapA1_comp (R) 2 (Table 3.2). The PCR was performed as described in section 2.5 with the following modifications: annealing at 52°C for 45s min, primer extension at 68°C for 1.5 min, and 94°C for 45s. The PCR product was purified and digested with *Bam*HI to enable ligation to *Bgl*II-digested and dephosphorylated linear pSAT-13. The resulting construct was designated pSAT-14 and used to complement MC58Δ*gapA-1*

3.2.11 Determining *in vitro* meningococcal growth

Meningococcal growth characteristics in liquid cultures were conducted by re-suspending 1:100 overnight cultures of *N. meningitidis* in 10 ml of MH broth. The following day, overnight broth culture was diluted in fresh BHI or DMEM and adjusted to a starting OD_{600} of 0.06. The cultures were incubated with shaking at 200 rpm as described in section 3.2.1. The OD_{600} reading was taken at hourly intervals for 8h and then at 24h h and experiments were performed in triplicate.

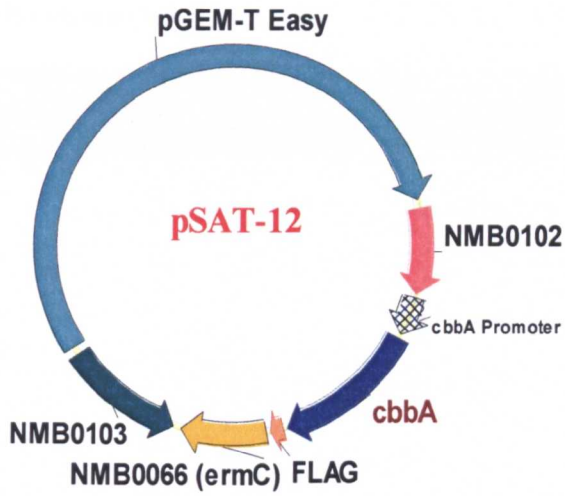


Figure 3. 3 A map of the pSAT-12 constructed by cloning *cbbA* into pSAT-11 and used to complement *cbbA* mutation in isogenic-mutants. The plasmid contains ampicillin and erythromycin resistance genes and *cbbA* promoter for driving the expression of *cbbA* gene.

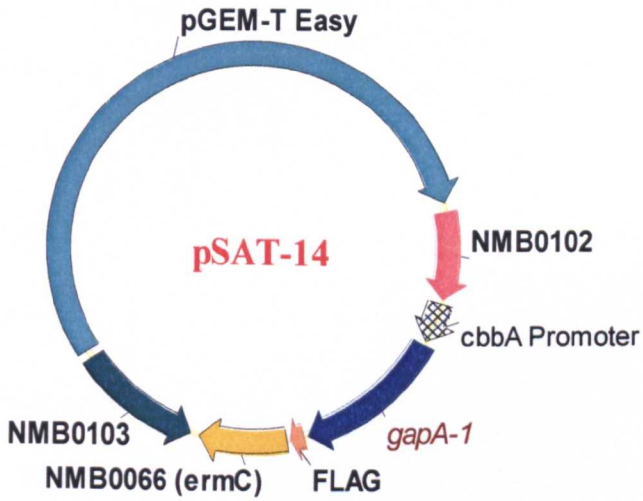


Figure 3. 4 A map of the pSAT-14 constructed by inverse PCR using pSAT-12 as template to substitute the *cbbA* with *gapA*-1 under the *cbbA* promoter. The resulting plasmid containing *gapA*-1 was designated pSAT-14 and used to complement *gapA*-1 mutation in isogenic-mutants. The plasmid contains ampicillin and erythromycin resistance genes and *cbbA* promoter for driving the expression of *gapA*-1.

Table 3. 3 Bacterial strains used in work described in this chapter

Strain	Description	Source or reference
E. coli		
JM109	*end*A1 *rec*A1 *gyr*A96 *thi hsd*R17 ($r_K^- r_K^-$) *rel*A1 *sup*E44 Δ(*lac-pro*AB) [F′ *tra*D36 *pro*AB *laq*I^{q}ZΔM15]	Promega
N. meningitidis		
MC58	wild-type serogroup B strain	(Tettelin *et al.*, 2000)
Z4181	Clinical isolate	
Z4667	Clinical isolate	
Z4673	Clinical isolate	
ST-18	Clinical isolate	
MC58□*siaD*	*siaD* deletion and replacement with erythromycin cassette	C. Tang Imperial College
MC58Δ*cbbA*	*cbbA* deletion and replacement with kanamycin cassette	This study
Z4181Δ*cbbA*	*cbbA* deletion and replacement with kanamycin cassette	This study
Z4667Δ*cbbA*	*cbbA* deletion and replacement with kanamycin cassette	This study
Z4673Δ*cbbA*	*cbbA* deletion and replacement with kanamycin cassette	This study
ST18Δ*cbbA*	*cbbA* deletion and replacement with kanamycin cassette	This study
MC58Δ*gapA-1*	*gaA-1* deletion and replacement with kanamycin cassette	This study
MC58Δ*cbbA cbbA*^{Ect}	MC58Δ*cbbA* complemented with an ectopic copy of *cbbA*	This study
Z4181Δ*cbbA cbbA*^{Ect}	Z4181Δ*cbbA* complemented with an ectopic copy of *cbbA*	This study
ST18Δ*cbbA cbbA*^{Ect}	ST18Δ*cbbA* complemented with an ectopic copy of *cbbA*	This study
MC58Δ*gapA-1 gapA-1*^{Ect}	MC58Δ*gapA-1* complemented with an ectopic copy of *gapA-1*	This study

3.3 Results

3.3.1 Cloning of *cbbA* plus flanking DNA

In order to create a *cbbA* knock-out mutant in *N. meningitidis* strain MC58, a region of DNA containing 1-kb upstream and *ca.*300 bp downstream of the start codon of *cbbA* was successfully amplified by PCR (Fig 3.5A). The fragment contained one copy of the neisserial DNA uptake sequence (5'-GCCGTCTGAA-3') downstream of the *cbbA* gene, which is required for efficient DNA uptake by natural transformation of *N. meningitidis* (Elkins *et al.*, 1991). The amplicon was gel-purified and used to ligate to the pGEM-T Easy vector followed by transformation of *E. coli* JM109 to yield pSAT-2 (Fig 3.1 B).

The positive clones were identified by blue/white screening. The plasmid DNA was prepared from resulting putative transformants and used to confirm the cloning of the DNA fragment (*cbbA* plus flanking DNA) by restriction digestion and PCR analysis. The cloned DNA fragment was subsequently extracted from pSAT-2 after digestion with *Not*I (Fig 3.5C). This digestion produced the expected two bands of *ca.*3 kb and 2.3 kb. In addition, the DNA fragment was amplified by PCR (Fig 3.5B) using the plasmid pSAT-2 as template and the original primer pair used to amplify the product. Both approaches confirmed the successful ligation of the desired insert to pGEM-T Easy.

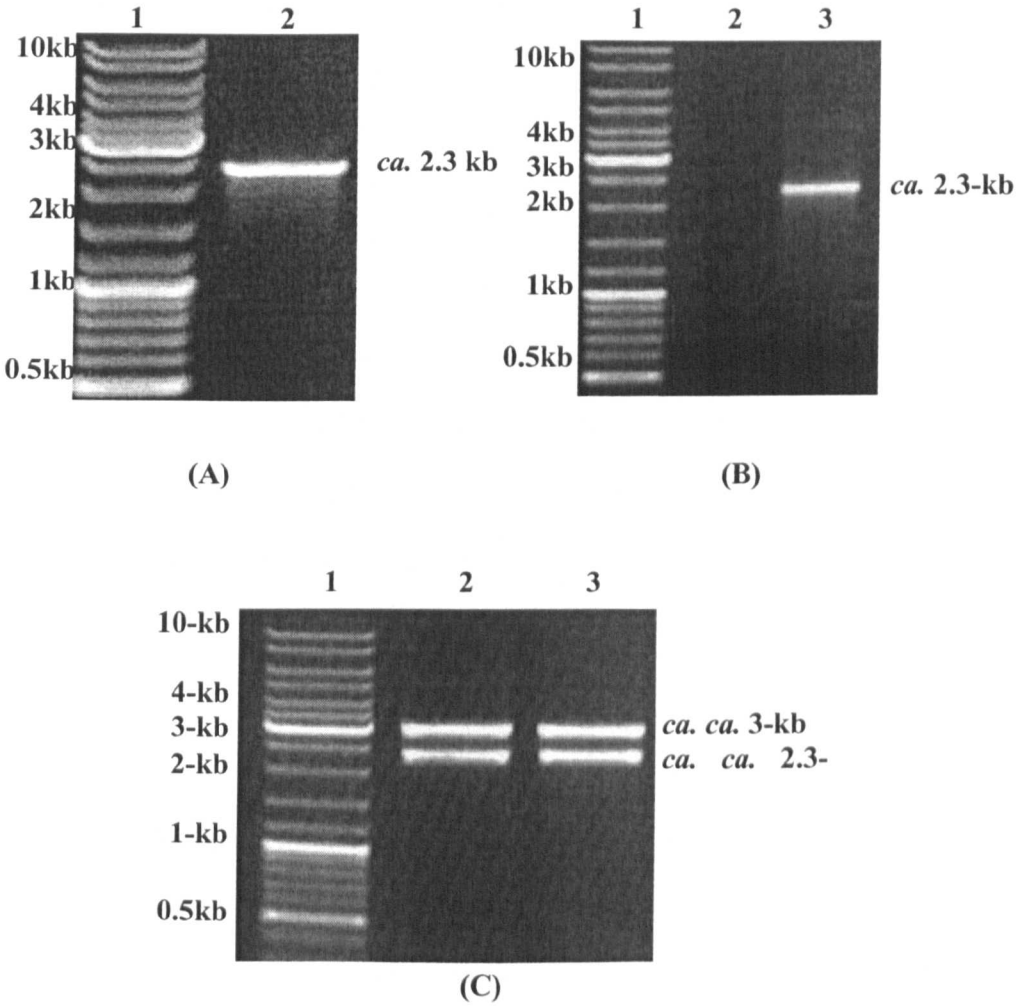
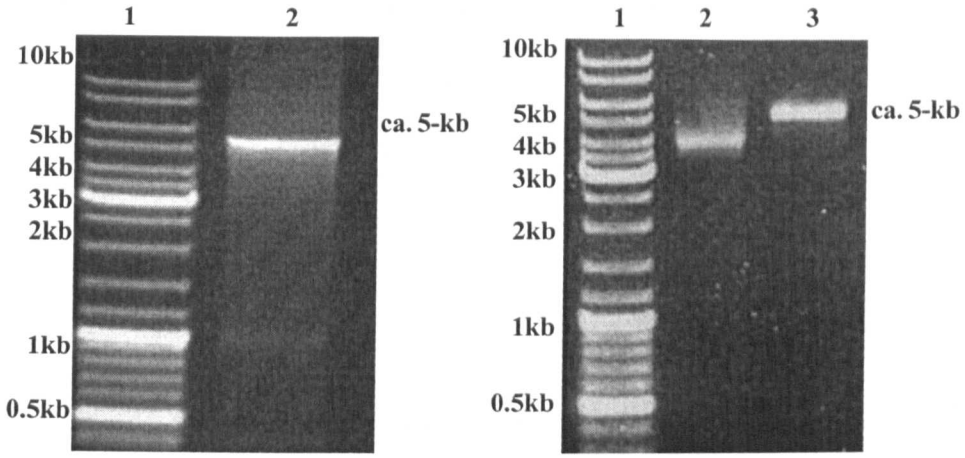


Figure 3. 5 Agarose gel analysis (A) showing the PCR amplification of a DNA fragment consisting of *cbbA* and flanking DNA. Lane 1, DNA markers, lane 2, PCR product of *cbbA* and flanking DNA. (B) PCR amplification of DNA fragment from pSAT-2 to confirm ligation, plasmid DNA from putative transformants was used as a template for amplification of DNA fragment consisting *cbbA* and flanking DNA. Lane 1, DNA markers, lane 2 and 3 test samples. A band representing the desired amplicon was observed in lane 3 indicating successful ligation in this clone. (C) The restriction digestion analysis of pSAT-2, Plasmid DNA was prepared from *E. coli* harboring putative pSAT-2 and used to verify the cloning of DNA fragment by digestion with *Not*I. Lane 1, DNA markers, lanes 2 and 3, putative pSAT-2 digested with *Not*I. As expected two bands of expected size were observed in both samples, confirming the successful ligation of DNA fragment with pGEM-T Easy.

3.3.2 Mutagenesis of *cbbA* by inverse PCR

The plasmid pSAT-2 was used as template for inverse PCR. This PCR resulted in deletion of approximately 300bp of the *cbbA* gene from start codon and amplification of a 5-kb amplicon (Fig 3.6 A) and the introducing a unique *Bgl*II site. The product was gel-extracted and digested with *Bgl*II to facilitate self-ligation followed by transformation of *E. coli* JM109 cells. Putative transformants were selected and analysed by restriction digestion with *Bgl*II. Clones that produced bands corresponding to the expected size of *ca.*5-kb were designated as pSAT-3 and were chosen for insertion of a selectable marker. The pSAT-3 plasmid was subsequently purified and digested with *Bgl*II (Fig 3.6 B) to enable the insertion of selectable marker encoding resistance to kanamycin.

The selectable marker encoding resistance to kanamycin was isolated from the vector pJMK30 by digestion with *Bam*HI. However, one difficulty encountered was obtaining sufficient amounts of the kanamycin cassette, as the vector containing this cassette appeared to be at a very low copy number. In an attempt to increase the yield of the vector, 10 ml cultures of *E. coli* JM109 competent cells containing the pJMK30 vector were harvested and used to purify the plasmid followed by elution in 30 µl rather than 50 µl.



(A) (B)

Figure 3. 6 Agarose gel analysis (A) demonstrating the amplification of a 5-kb PCR product. The plasmid pSAT-2 was used as template DNA for inverse PCR. Lane 1, DNA markers, lane 2, inverse PCR product representing pGEM-T Easy vector (3-kb) and 2-kb DNA flanking *cbbA* (B) the restriction digestion analysis of pSAT-3. Plasmid DNA was prepared from putative pSAT-3 transformants and linearized by digestion with *Bgl*II. Lane 1, DNA markers, lane 2, uncut pSAT-3 and lane 3, *Bgl*II-digested pSAT-2. As expected a *ca.* 5-kb band corresponding to linear pSAT-2 was observed in lane 3.

In order to introduce the marker into the unique *Bam*HI site in the intermediate vector pSAT-3, the Kan^R cassette was prepared by digestion of the vector pJMK30 and purified by gel electrophoresis followed by gel extraction. The kanamycin cassette was obtained with the expected size of 1.5 kb (Fig 3.7A). The *Bam*HI-digested, purified antibiotic resistance cassette (encoding resistance to kanamycin) was ligated to the intermediate vector pSAT-3 linearized by digestion with *Bgl*II followed by transformation of *E. coli* JM109 competent cells to yield pSAT-4. Putative transformants were selected on kanamycin plates. Successful ligation of Kan^R cassette to *Bgl*II-digested linear pSAT-3 was confirmed by restriction digestion with *Sma*I. The *Sma*I site is available in the region of the Kan^R cassette, therefore an

expected *ca.* 6.5 kb single linear band was observed on an agarose gel (Fig 3.7B). The resulting plasmid was designated pSAT-4. Plasmid DNA was purified from *E. coli* harbouring pSAT-4 for further confirmation by PCR analysis.

Using the primer pair FBA_M1 (F) and FBA_M2 (R) (Table 3.2) the cloning of Kan^R resistance cassette in pSAT-4 was further confirmed by PCR amplification of a *ca.*3.5 kb band consisting of 1.5 kb representing the Kan^R cassette and 2-kb of flanking DNA. A negative control of plasmid (pSAT-2) without the Kan^R cassette was also used in the same PCR reaction. The expected 3.5 kb band was obtained in all five samples tested whereas the negative control produced the expected 2.0 kb band representing flanking DNA only (Fig 3.7C). This confirmed that, the Kan^R cassette was successfully cloned in the pSAT-4. The pSAT-4 was then chosen to mutate the *cbbA* by natural transformation in *N. meningitidis* strain MC58.

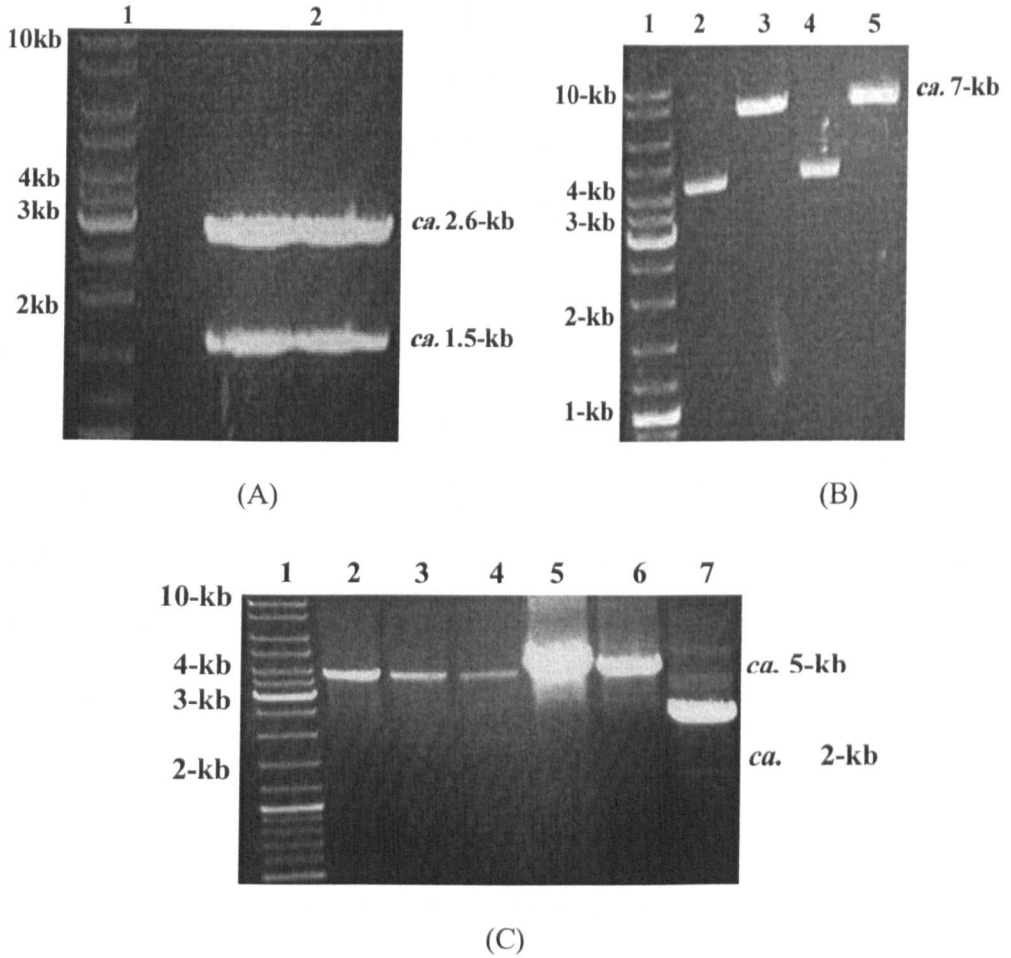


Figure 3. 7 Agarose gel analysis demonstrating (A) restriction digest of pJMK30 vector with *Bam*HI to obtain kanamycin resistance cassette, pJMK30 vector was prepared and digested with *Bam*HI. *BamH*1 sites are present at both ends of the kanamycin cassette in pJMK30 vector and could be used to isolate resistance cassette. Lane 1, DNA markers and lane 2, *Bam*HI-digested pJMK30. The lower band of *ca.*1.5 kb represents Kan^R cassette, whereas the upper band corresponds to the vector backbone (B) Restriction digest analysis of pSAT-4 to confirm the presence of Kan^R cassette Lane 1, DNA markers, lanes 2 and 4, uncut pSAT-4, lanes 3 & 5, *Sma*I-digested pSAT-4. An expected *ca.* 6.5 kb band was observed in lanes 3&5 indicating the successful ligation of Kan^R cassette in pSAT-4 (C) PCR amplification of Kan^R cassette and flanking DNA. Lane 1, DNA markers, lanes 2-6 pSAT-4 as template DNA and lane 6, pSAT-3 as template DNA (negative control). Lanes 2-6 demonstrate an expected 3.5 kb band representing Kan resistance cassette and flanking DNA, whereas, lane 7 produced 2-kb band.

3.3.4 Verification of *cbbA* mutagenesis

The mutation of *cbbA* was confirmed by the PCR analysis and immunoblotting by probing the whole cell lysate from MC58-WT and MC58Δ*cbbA* using RαFBA antisera. Initially, putative *cbbA*-null mutants were verified for the presence of the Kan^R cassette at proper location and orientation of the cloned cassette in the chromosome by PCR analysis using the of *cbbA*-specific primer pair FBA_E1 (F) and FBA_E2 (R) (Table 3.2) to amplify *cbbA* from the chromosomal DNA of the putative mutant clones. Chromosomal DNA of *N. meningitidis* strain MC58 was also amplified with the same primer pair as a positive control. The PCR resulted in amplification of an expected *ca.* 1-kb band from chromosomal DNA of the MC58-WT strain whilst no band was observed in the mutant samples (Fig 3.8A) suggesting the successful mutation of *cbbA* in the putative mutants, which were designated as MC58Δ*cbbA*. It was still not clear that the inserted Kan^R cassette was in the same orientation as *cbbA* in the chromosome of the putative mutants. To determine the orientation of the Kan^R cassette another PCR using the primer pair Kan-CTR and FBA_M2 (R) (Table 3.2) was performed. This PCR would be expected to produce a 1-kb product where the orientation of the Kan^R cassette was the same as the deleted *cbbA*. This confirmation was important in order to minimize the impact of the mutation on the downstream genes (if the Kan^R was cloned in same orientation) by facilitating expression of downstream genes in the same operon. An expected 1-kb band was observed in 9 out of 10 DNA samples from putative mutants (Fig. 3.8B) confirming the presence and the correct orientation of the cloned selectable marker in putative mutants. Taken together, these results appear to have

genotypically confirmed that *cbbA* has been substituted in putative mutant genome with Kan^R cassette.

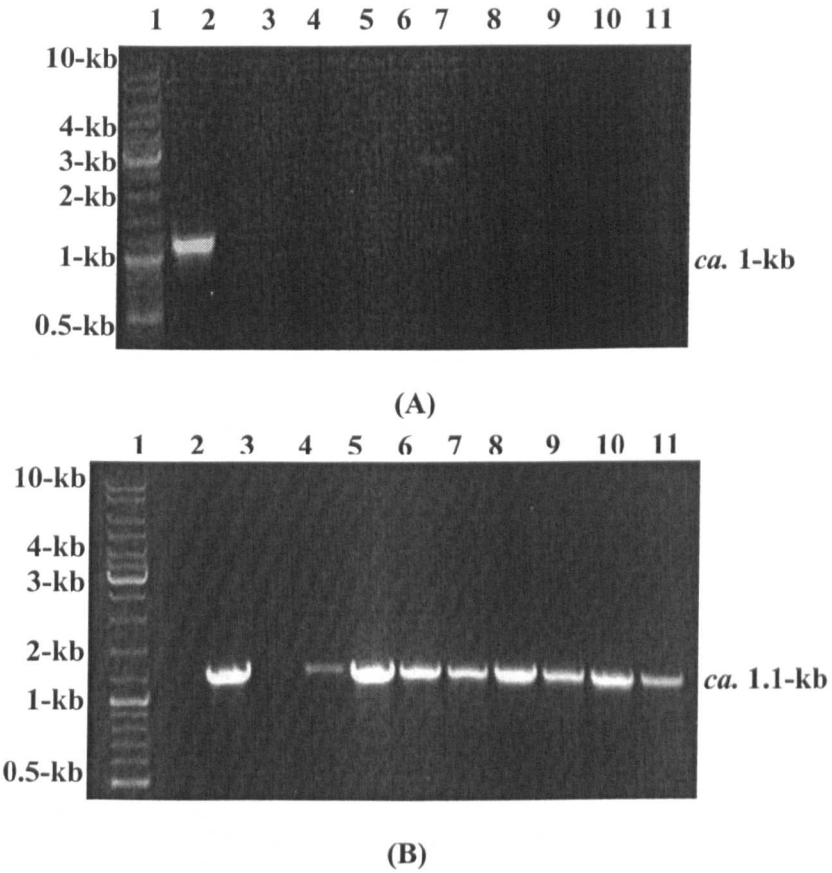


Figure 3. 8 Agarose gel demonstrating the (A) PCR amplification of *cbbA* from the putative mutants. Chromosomal DNA was extracted from putative *cbbA* mutants and used to confirm the mutation by PCR amplification of *cbb*A. Lane 1 DNA markers, lane 2, MC58-WT DNA, lanes 3-11 putative *cbbA* mutant DNA. The PCR failed to produce a band corresponding to *cbbA* size from putative mutants, except the positive control of MC58 chromosomal DNA. (B) orientation and presence of Kan^R cassette in putative *cbbA* knock-out mutants. Chromosomal DNA was prepared from putative mutants and used to verify the orientation and presence of Kan^R cassette by PCR using primers Kan-CTER and FBA_M1(R). This PCR was expected to produce *ca.*1-kb product in case of presence of Kan^R cassette in same orientation as of *cbbA* in putative mutants. Lane 1, DNA markers, lane 2, MC58-WT DNA (control), lanes 3-12, putative *cbbA* mutant DNA. lane 2, failed to produce any band due to absence of the cassette in the WT, whereas a band of expected size was observed in 3, 5-12 lanes, confirming the successful replacement of *cbbA* with the Kan^R in the same orientation. Lane 4 could not produce any band suggesting that the Kan^R cassette was in opposite orientation in this mutant.

To confirm that the *cbbA* gene is not expressed in the isogenic-null mutant strain, whole cell lysate of MC58-WT and MC58Δ*cbbA* were resolved by SDS-PAGE followed by immunoblot analysis by probing the membranes with RαFBA antisera. A strongly reactive protein band with an apparent molecular weight of *ca.* 38-k-Da was detected in the lysate of the MC58-WT sample which was absent from the lysate of the MC58Δ*cbbA* extract (Fig 3.9). This demonstrated that the expression of *cbbA* has been abolished by the mutation. The RαFBA also detected another protein band of higher molecular weight than FBA from the lysate of both MC58-WT and MC58Δ*cbbA*, which was presumably non-specific band due to the polyclonal nature of antisera

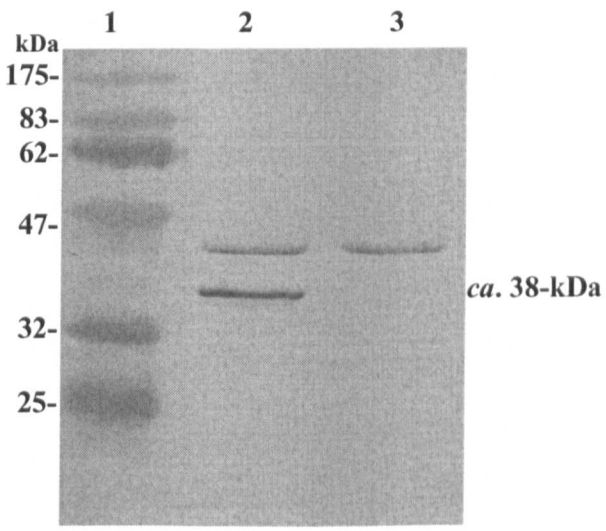


Figure 3. 9 Immunoblot analysis demonstrating the successful deletion of *cbbA* in putative mutants Whole cell extracts were prepared from MC58-WT and MC58-putative *cbbA* mutants and resolved on 10% SDS-PAGE followed by immunoblotting with RαFBA to verify the *cbbA* deletion. Lanel, protein markers, lane 2, whole cell extracts from MC58-WT, and lane 3, whole cell extracts from MC58 *cbbA* mutant. RαFBA recognized a band of apparent molecular weight of *ca.* 38-kDa corresponding to FBA in lane 2, whereas the same band was absent in lane 3, confirming that the *cbbA* is not expressed in *cbbA* mutant cells.

3.3.6 Mutagenesis of *cbbA* in clinical isolates of *N. meningitidis*

In addition to mutating *cbbA* in *N. meningitidis* strain MC58, attempts were made to mutate *cbbA* in four additional clinical isolates of *N. meningitidis*. The plasmid pSAT-4 was used to mutate *N. meningitidis* strains Z4181, Z4667, Z4673, and ST18 by natural transformation and allelic exchange. The putative transformants were analysed for *cbbA* mutation and for the presence of Kan^R resistance marker in the chromosome by PCR and immunoblot analysis as described above. PCR analysis (Fig 3.10) showed that *cbbA* was successfully replaced by kanamycin resistance marker in all four strains. Furthermore, a band corresponding to the apparent molecular weight of FBA was observed in all wild-type strains but absent from the respective isogenic mutants in immunoblots (Fig 3.11) confirming the deletion of *cbbA* in these mutants.

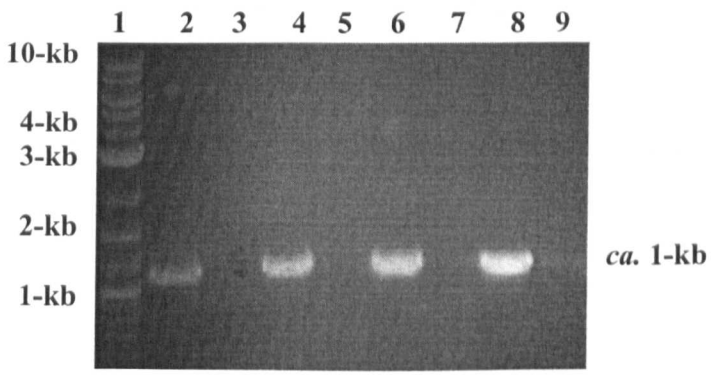


Figure 3. 10 Agarose gel analysis demonstrating the PCR amplification of *cbb*A from clinical isolates of *N. meningitidis*-WT and absent in their respective mutants, Chromosomal DNA was prepared from putative mutant strains and used to verify the mutation by PCR amplification of *cbb*A. Lanes 1, DNA markers, lanes 2,4,6,8 chromosomal DNA from Z4181, Z4667, Z4673, ST-18 WT strains, respectively, lanes 3,5,7,9, Z4181, Z4667, Z4673, ST-18 putative *cbb*A mutant stains, respectively. The DNA band of *ca.* 1-kb representing *cbbA* was observed in all Wwild-type strains but absent in isogenic mutant strains indicating that *cbb*A was successfully deleted.

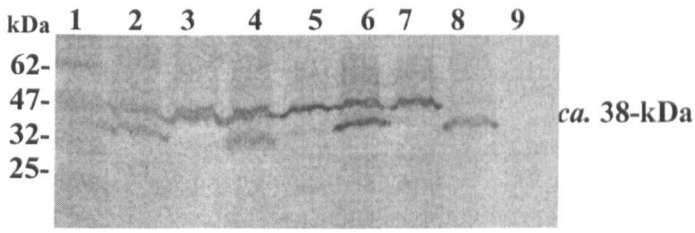


Figure 3. 11 Immunoblot analysis demonstrating the loss of expression of *cbb*A in mutant strains. Whole cell extracts from putative *cbbA* mutants created in different clinical isolates of *N. meningitidis* were resolved by 10% SDS-PAGE followed by immunoblot analysis. Lanes 1, protein markers, lanes 2,4,6,8 Z4181, Z4667, Z4673, ST-18 WT strains, respectively, lanes 3,5,7,9, putative *cbbA* mutants in: Z4181, Z4667, Z4673, ST-18 strains. The lower band of *ca.* 38-kDa represents FBA, which is present in all WT strains but absent in all respective mutant strains indicating that *cbbA* is not expressed in mutant strains.

3.3.7 Cloning of *gapA*-1 gene plus flanking DNA

In order to create a *gapA*-1-null mutant in *N. meningitidis* strain MC58, a fragment of DNA encompassing *gapA-1* and *ca.*1-kb of DNA both upstream and downstream of the start codon of *gapA-1* was amplified by PCR (Fig 3.12A). This amplicon was purified and ligated with pGEM-T Easy to yield pSAT-6. The plasmid pSAT-6 was then used to transform *E. coli* JM109 cells and the successful clones were identified by blue/while selection. Plasmid DNA was prepared from putative transformant clones and analysed by restriction digestion with *Sma*I. Two *Sma*I sites are present in the cloned DNA fragment, thus, as expected two bands of 3.0 and 3.2 kb were obtained (Fig 3.12B). PCR analysis of pSAT-6 also confirmed the successful ligation (Fig 3.12C).

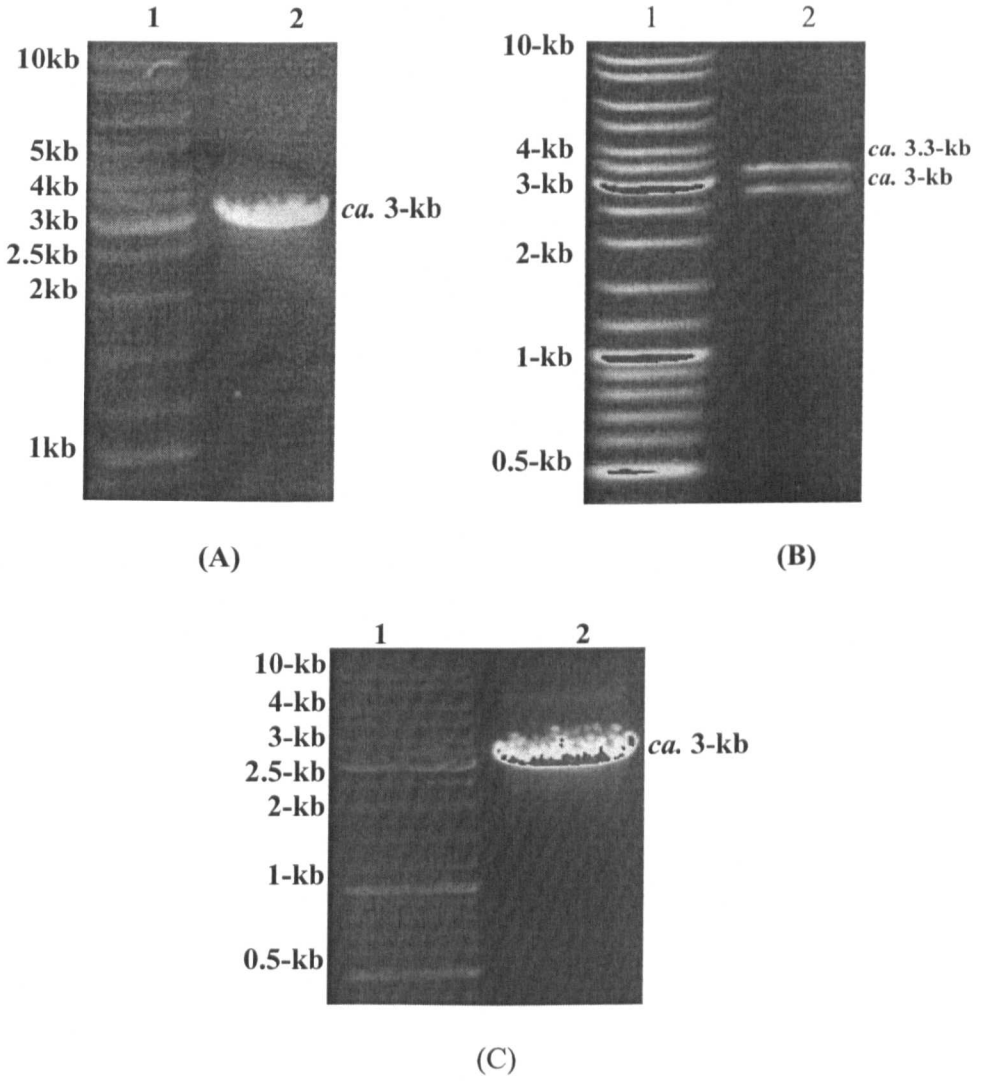


Figure 3. 12 Agarose gel showing (A) PCR amplification of *gapA-1* plus flanking DNA. Lane 1, DNA markers, lane 2, PCR product consisting of *gapA-1* and flanking DNA. (B) showing the restriction digestion of pSAT-6. Plasmid DNA was prepared from putative pSAT-6 transformants and used to verify the cloning by restriction digestion with *Sma*I. Lane 1, DNA markers, lane 2, pSAT-6 plasmid DNA. As expected two bands (3 and 3.3-kb) were observed in lane 2 confirming the successful ligation of DNA fragment in pSAT-6 (C) showing the PCR amplification of *gapA-1* plus flanking DNA from the plasmid construct pSAT-6. Lane 1, DNA markers, lane 2, PCR product of *gapA-1* plus flanking DNA.

3.3.8 Mutagenesis of *gapA-1* by inverse PCR

The plasmid pSAT-6 was used as template DNA for inverse PCR. This resulted in amplification of a 5-kb band (Fig 3.13A), deletion of the *gapA-1* open reading frame (ORF), and introduction of a unique *Bgl*II site to facilitate the introduction of selectable marker encoding resistance to kanamycin. The i-PCR product was digested with *Bgl*II and self-ligated generating a pSAT-7. The pSAT-7 plasmid was subsequently purified from a transformant clone and digested with *Bgl*II to facilitate the insertion of the Kan^R cassette (Fig 3.13B). The *Bgl*II digested plasmid was purified and analysed by agarose gel electrophoresis. The kanamycin resistance marker was prepared as described previously (Section 3.3.2) and ligated to the plasmid pSAT-7 digested with *Bgl*II to yield pSAT-8. Plasmid DNA was prepared from putative pSAT-8 transformants and used to verify the cloning of Kan^R cassette by restriction digestion with *Sma*I. As expected, three DNA bands were obtained by this digestion confirming the successful cloning of the kanamycin resistance marker (Fig 3.13C). The plasmid pSAT-8 was then used to mutate *gapA*-1 in *N. meningitidis* strain MC58.

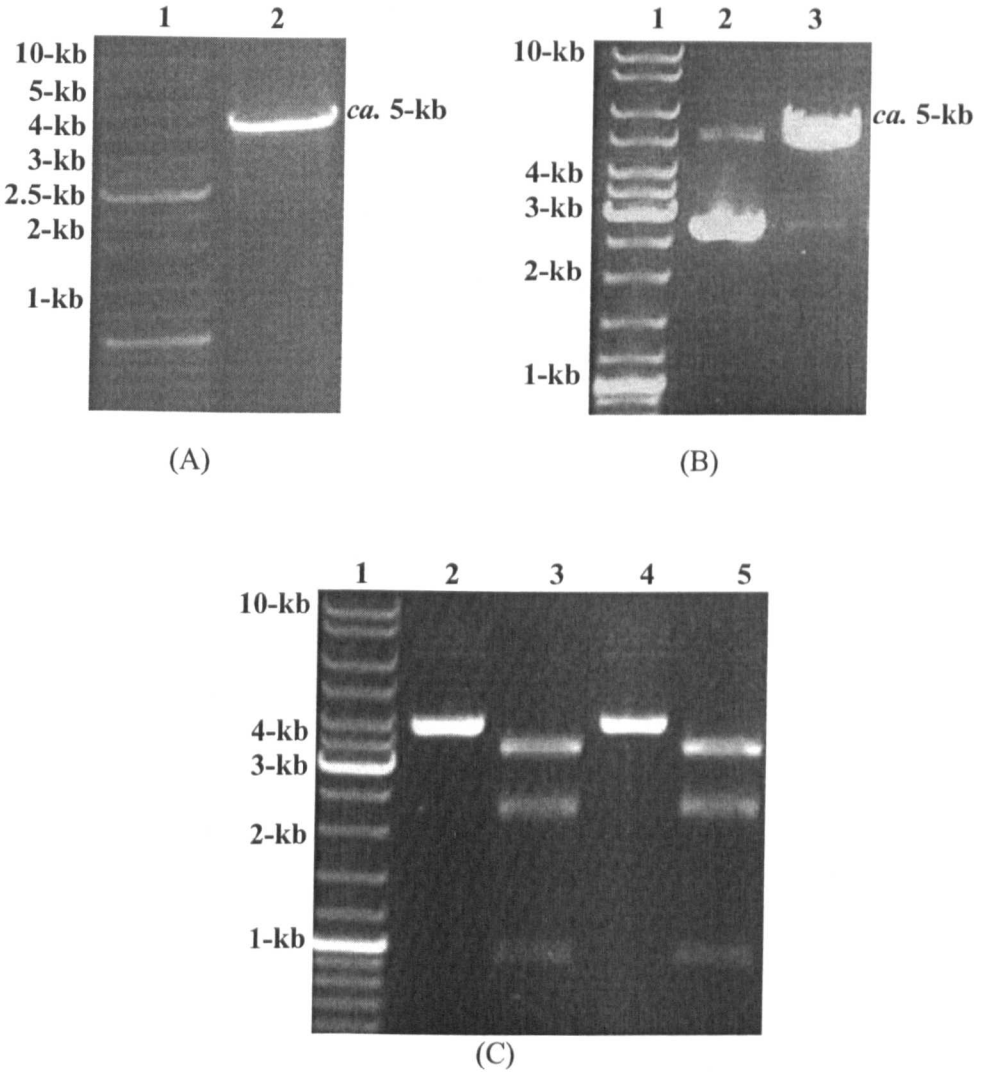


Figure 3. 13 Agarose gel analysis demonstrating (A) the successful amplification of inverse-PCR product for *gapA-1* mutagenesis. Plasmid DNA prepared from pSAT-6 was subjected to inverse-PCR to delete *gapA-1* and amplification of 5-kb amplicon consisting of DNA flanking the *gapA-1* and pGEM-T easy vector, (B) the restriction digestion of pSAT-7. Plasmid DNA was prepared from putative pSAT-7 transformnts and digested with *Bgl*II to enable the insertion of Kan^R cassette. Lane 1, DNA markers, lane 2, uncut pSAT-7, lane 3, pSAT-7 digested with *Bgl*II, lane 3 produced an expected 5-kb band, (C) the restriction digestion analysis of pSAT-8, The plasmid pSAT-8 DNA was prepared and verified by restriction digestion with *Sma*I for the presence of Kan^R cassette, lane 1, DNA markers, lanes 2 and 4, undigested pSAT-8, lanes 3 and 5, *Sma*I digested pSAT-8. As expected three products were observed in digested samples confirming the ligation of Kan^R.

3.3.9 Verification of *gapA-1* mutagenesis

Initially, verification of *gapA-1* null-mutants was accomplished by PCR analysis. Chromosomal DNA was prepared from putative mutants and used to confirm deletion of *gapA*-1 by amplifying a band corresponding to *gapA*-1. As expected this PCR failed to produce a band of the expected size for *gapA*-1 from the putative mutants, indicating that the *gapA*-1 ORF has been successfully substituted with the kanamycin resistance marker, whereas the positive control of MC58-WT produced an expected *ca.* 1kb band representing *gapA*-1(Fig 3.24). In addition, PCR analysis was performed to determine the orientation of the kanamycin resistance marker in the chromosome of putative mutants using the primers Kan_CTR and gapA-1 (R) FL (Table 2.3). Where necessary, MC58 wild-type DNA was used as a control (Fig 3.25). This PCR resulted in amplification of expected 1-kb band from the putative mutants cloned with the appropriately oriented Kan^R marker whereas the MC58-WT negative control did not produce a band. The *gapA*-1 null mutants were designated MC58Δ*gapA*-1.

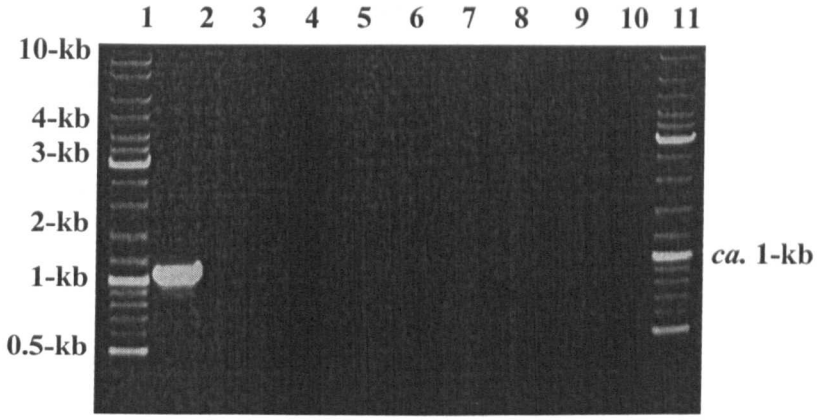


Figure 3. 14 Agarose gel analysis demonstrating the verification of *gapA-1* deletion. Chromosomal DNA was prepared from putative mutant strains and used to verify the deletion of *gapA-1* by PCR. Lane 1, DNA markers, lane 2, MC58-WT DNA (positive control), lanes 3-10, putative *gapA-1* mutant DNA. Lane 2, shows the expected band corresponding to *gapA-1*, which is absent in lanes 3-10, confirming the successful deletion of *gapA-1* in the mutant DNA.

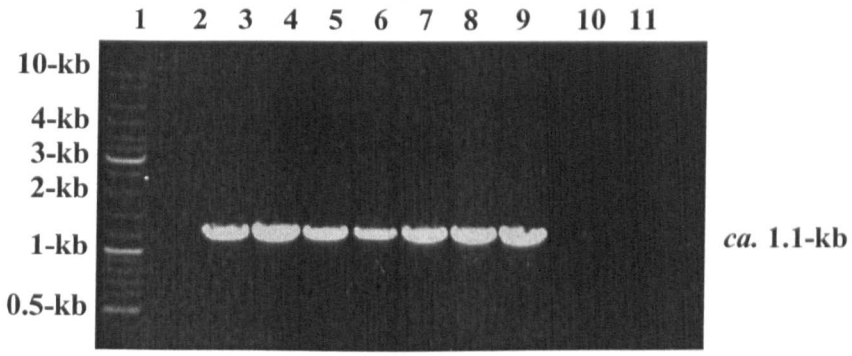


Figure 3. 15 Agarose gel analysis demonstrating the presence and orientation of Kan^R cassette. Chromosomal DNA was prepared from putative mutant strains and used to confirm the orientation and presence of Kan^R cassette by PCR using primers Kan-CTER and *gapA-1*_(R) FL. This PCR was expected to produce *ca.* 1-kb product in case of presence of Kan^R cassette in same orientation as of *gapA-1* in putative mutant strains. Lane 1, DNA markers, lane 2, MC58-WT DNA (control), lanes 3-11, DNA from putative *gapA-1* mutant. Lane 2, failed to produce any band due to absence of the cassette in the WT, whereas a band of expected size was observed in 2-9 lanes, confirming the successful replacement of *gapA-1* by the Kan^R in the same orientation. Lanes 10 and 11 could not produce any band suggesting that the Kan^R cassette was in wrong orientation.

In order to confirm that *gapA-1* is not expressed in the isogenic-null mutant strain, whole cell lysates of MC58-WT and MC58Δ*gapA*-1 were resolved on SDS-PAGE followed by immunoblot analysis by probing the membranes with RαGAPDH-1 antiserum. A protein band of apparent molecular weight of *ca.* 37-k-Da was detected in the lysate of the MC58-WT sample, which was absent from the lysate of MC58Δ*gaA*-1 (Fig 3.16). This confirmed that the expression of *gapA*-1 has been abolished by the mutation. The RαGAPDH-1 also detected a number of additional protein bands, including one immediately above the *gapA-1* band in lysates of both MC58-WT and MC58Δ*cbbA*, which were assumed to be non-specific due to the polyclonal nature of antiserum.

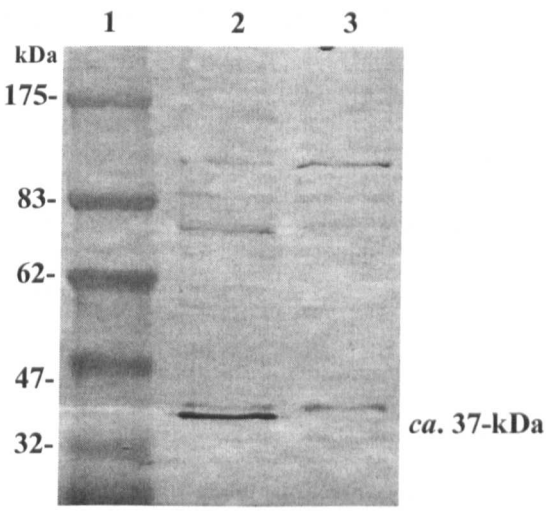


Figure 3. 16 Immunoblot analysis demonstrating the successful deletion of *gapA-1* in putative mutant strain, Whole cell extracts were prepared from MC58-WT and *gapA-1* mutant and resolved on 10% SDS-PAGE followed by immunoblotting with RαGAPDH-1. Lane 1, protein markers, lane 2, whole cell extracts from MC58-WT, and lane 3, whole cell extracts from MC58Δ*gapA-1*. RαGAPDH-1 recognized a band of apparent molecular weight of *ca.* 37-kDa representing GAPDH-1 in lane 2, whereas the same band was absent in lane 3, confirming that the *gapA-1* is not expressed in *gapA-1* mutant cells.

3.3.11 Construction of ectopic complementation vector

In order to rule out polar effects on neighbouring genes during phenotypic analysis of the *cbbA* and *gapA-1* mutant strains, the *cbbA* and *gapA-1* mutations were complemented using an ectopic complementation vector (Fig 3.3 and Fig 3.4) in which the gene of interest may be cloned downstream of the *cbbA* promoter and upstream of the *ermy* gene (encoding resistance to erythromycin). These sequences are flanked by the genes NMB102 and NMB103, which are in a tail-to-tail configuration in both the vector and the meningococcal genome. The construct facilitates insertion of genes of interest, under the control of the *cbbA* promoter, into the meningococcal genome at a site that is unlikely to effect the expression of any other genes. The complementation cassette was amplified by PCR from the pYHS25. The 2.7 kb PCR product representing the complementation cassette (Fig. 3.17A, Fig 3.2 A) was gel-purified for cloning into pGEM-T Easy vector followed by transformation of *E. coli* JM109 cells. Positive clones were identified by blue/white phenotype of the transformants colonies. The resulting ectopic complementation plasmid was designated as pSAT-11 (Fig 3.2 B).

One successful clone was chosen for plasmid propagation and purification. The complementation cassette was fully sequenced by using universal M13 (F and R) and a pair of internal primers (Table 3.2) designed from sequences within the complementation cassette to confirm the predicted promoter sequences and other flanking genes. The sequencing results, when aligned with available sequences of the promoter, revealed that there were differences in the predicted promoter region.

Therefore, an alternative strategy was devised in which the native promoter of *cbbA* was utilised. The plasmid pSAT-11 was digested at the *Bam*HI site immediately downstream of the predicted *opa* promoter in the multiple cloning site and an expected band of a *ca.*6.5 kb corresponding to the linear pSAT-11was observed (Fig 3.17B). The clone was further confirmed by partial DNA sequencing using a M13 (F and R) primers. This pYHS25-based newly constructed plasmid pSAT-11 was used as basis to clone complementing genes *cbbA* and *gapA-1* gene under the *cbbA* promoter present immediate downstream of NMB0102 for driving the expression of complementing genes.

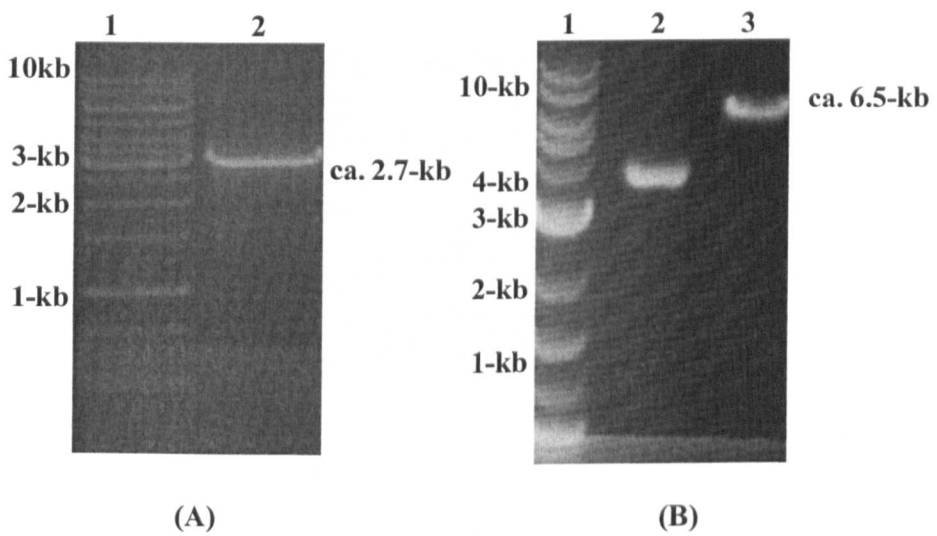


Figure 3. 17 Agarose gel analysis (A) showing the PCR product of complementation cassette Plasmid DNA was prepared from pYHS25 and used as template for amplification of a *ca.*2.7-kb DNA fragment corresponding to complementation cassette. Lane 1, DNA markers, lane 2, PCR product representing complementation cassette, (B) restriction digestion analysis of pSAT-11 with *Bam*HI showing the successful ligation of complementation cassette in pGEM-T Easy, lane 1, DNA markers, lane 2, uncut pSAT-11 DNA, and lane 3, *Bam*HI-digested linear pSAT-11 (*ca.*6.2 kb).

3.3.12 PCR amplification and cloning of *cbbA* in pSAT-11

A *ca.* 1.1 kb product, consisting of the *cbbA* and its predicted promoter sequences, was amplified by PCR (Fig 3.18). The amplicon was purified and ligated into pGEM-T Easy, excised with *Bam*HI and then introduced into *Bam*HI-digested linearized pSAT-11. Transformants were selected on agar plates containing appropriate antibiotics and verified by restriction digestion analysis and subsequent sequencing of the putative clone to confirm the in-frame ligation. The resulting plasmid was designated pSAT-12 (Fig 3.3) and used to complement *cbbA* mutation in isogenic null mutant by natural transformation and allelic exchange.

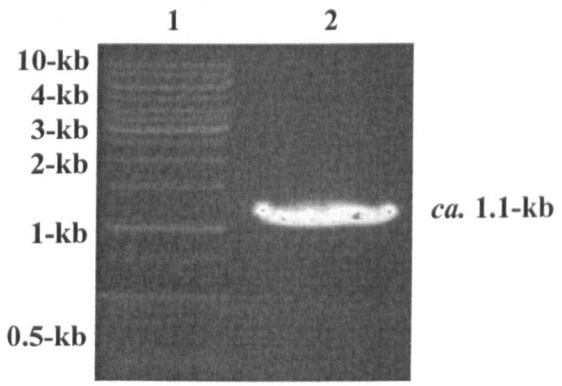


Figure 3. 18 Agarose gel analysis demonstrating the successful PCR amplification of *cbbA* from chromosomal DNA of *N. meningitidis.* Chromosomal DNA was prepared from MC58-WT and used to amplify the *cbbA* for cloning into pSAT-11 to generate pSAT-12. Lane 1, DNA markers, lane 2, PCR product of *cbbA* gene.

Prior to introduction of pSAT-12 into MC58Δ*cbbA* by natural transformation, pSAT 12-harboring *E. coli* JM109 cells were grown overnight and the culture pellet was used to determine the expression of cloned *cbbA* by immunoblot analysis using RαFBA antisera. Six different clones were selected for preparation of whole cell

extracts. The proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane for immunoblot analysis with RαFBA antisera. All six clones were shown to express a protein band with an apparent molecular mass similar to the expected size of FBA, which was absent in the pSAT-11 (negative control) suggesting that the FBA is expressed in *E. coli* from this plasmid without any induction (Fig 3.19).

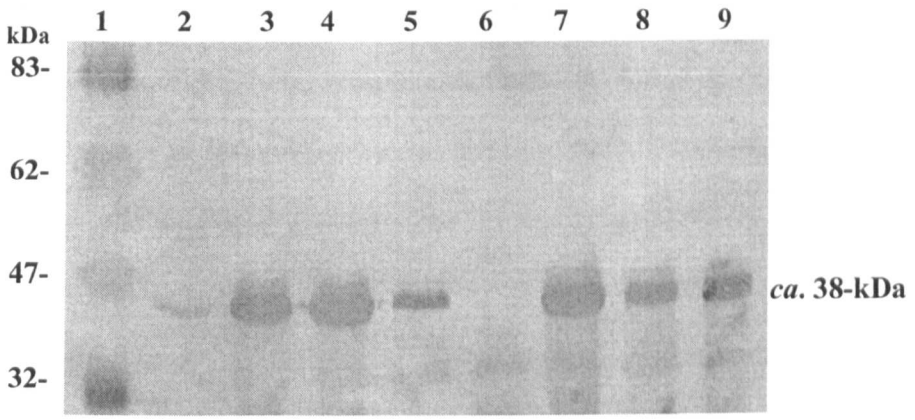


Figure 3. 19 Immunoblot analysis of whole cell lysates from *E. coli* harbouring pSAT-12. Whole cell lysates of *E. coli* were resolved by 10% SDS-PAGE followed by iimmunoblot analysis to determine the expression of FBA. Lane 1, DNA markers, lane 2, MC58-WT whole cell lysate, lanes 3-5, pSAT-12, 1-3 samples, respectively, lane 6, pSAT-11 (negative control), lanes 7-9, pSAT-12, 4-6 samples, respectively. As expected all pSAT-12 constructs represented a band equivalent to FBA.

The plasmid pSAT-12 was subsequently introduced into MC58Δ*cbbA* by natural transformation and allelic exchange (Section 3.2.8), introducing a single chromosomal copy of the complementing gene in the intergenic region between NMB0102 and NMB0103, open reading frames orientated in a head-to-head fashion. In addition, pSAT-12 was used to transform in two other mutants in clinical isolates of meningococci: Z4181Δ*cbbA* and ST18Δ*cbbA*. These mutants were created to act as background in challenging transgenic mice to assess a potential role of FBA in

development of bacteraemia in these mice. Transformants were selected on selective BHI agar plates. Successful transformants were sub-cultured and used for DNA extraction and preparation of whole cell extracts for PCR and immunoblot analysis, respectively. The resulting complemented mutant strains were designated MC58Δ*cbbA* $cbbA^{Ect}$, Z4181Δ*cbbA* $cbbA^{Ect}$ and ST18Δ*cbbA* $cbbA^{Ect}$.

3.3.13 PCR analysis of putative *N. meningitidis*-Δ*cbbA* $cbbA^{Ect}$ strains

To confirm the successful complementation of *cbbA* at ectopic site in isogenic-null mutants, chromosomal DNA was prepared from the putative complemented strains. The presence of *cbbA* was identified by PCR amplification of a DNA band corresponding to *cbbA* from chromosomal DNA of the complemented mutant strains using the FBA_pQE70 (F&R) primers and genomic DNA from MC58-WT and putative complemented strains and their isogenic-null mutant strains. Agarose gel analysis demonstrated an expected band of *ca.* 1-kb representing *cbbA* from MC58-WT and complemented mutants, but absent in isogenic-mutant strain (Fig 3.20) confirming the successful complementation of *cbbA* mutation.

In addition, another PCR using the primers NMB0102 (F) 2 & NMB0103(R) was performed to verify the insertion of complementation cassette at the proper location in the chromosome of complemented strains. Insertion of the complementation cassette at the proper location was identified by obtaining a *ca.* 4-kb band in complemented mutant strains whereas WT and mutant strains were expected to produce a band of *ca.* 1.3 kb representing only NMB0102 & NM0103 PCR product.

As expected all three complemented strains produced an expected ca. 4-kb band whereas WT and mutant strains produced 1.3 kb band further confirming the insertion of *cbbA* at desired location in complemented strains (Fig 3.21).

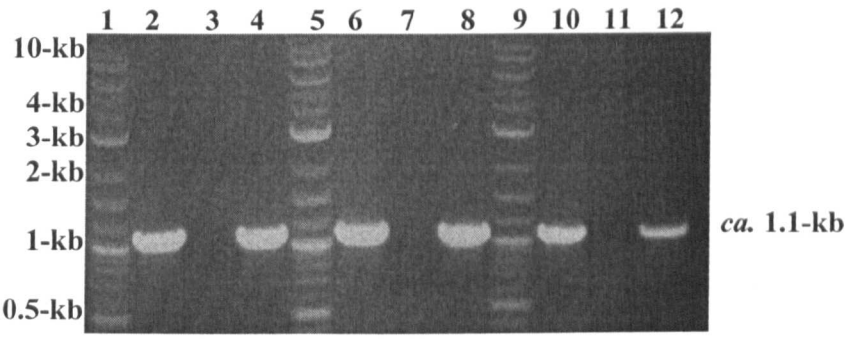


Figure 3. 20 Agarose gel analysis demonstrating the amplification of *cbbA* from *cbbA* mutant and their complemented strains. Chromosomal DNA was prepared and used to amplify *cbbA* using the primer pair FBA_pQE70 (F and R) and genomic DNA from *N. meningitidis*-WT (positive control) and Δ*cbbA* (negative control). Lanes 1, 5, 9, DNA markers, lanes 2,6,10, MC58-WT, Z4181-WT, and ST-18-WT genomic DNA, respectively, lanes 3,7,11, MC58Δ*cbbA*, Z4181Δ*cbbA*, and ST-18Δ*cbbA* , lanes 4, 8, 12, MC58Δ*cbbA* $cbbA^{Ect}$, Z4181Δ*cbbA* $cbbA^{Ect}$ and ST18Δ*cbbA* $cbbA^{Ect}$, respectively.

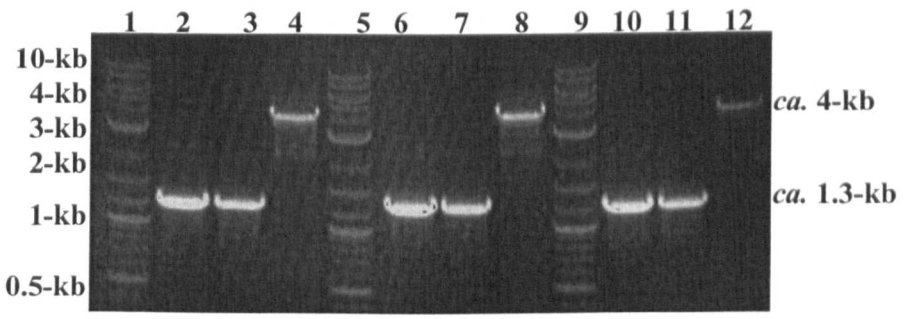


Figure 3. 21 Agarose gel analysis confirming the insertion of *cbbA* at desired location in complemented strains. Chromosomal DNA was prepared and used to amplify the complementation cassette using the primer pair NMB0102 (F) 2 and NMB0103 (R) and genomic DNA from complemented mutants strains and *N. meningitidis*-WT and Δ*cbbA*. Lanes 1, 5, 9, DNA markers, lanes 2,6,10, MC58-WT, Z4181-WT, and ST-18-WT genomic DNA, respectively, lanes 3,7,11, MC58Δ*cbbA*, Z4181Δ*cbbA*, and ST-18Δ*cbbA* , lanes 4,8,12, MC58Δ*cbbA* $cbbA^{Ect}$, Z4181Δ*cbbA* $cbbA^{Ect}$ and ST18Δ*cbbA* $cbbA^{Ect}$, respectively.

3.3.14 Immunoblot analysis of *N. meningitidis*-Δ*cbbA cbbA*^{Ect} strains

The whole cell extracts were prepared from *N. meningitidis*-MC58 WT, Z4181, ST18 wild-type, their respective null-mutants and putative complemented strains and resolved on 12% SDS-PAGE followed by immunoblot analysis using RαFBA antisera. All three complemented strains (MC58Δ*cbbA cbbA*^{Ect}, Z4181Δ*cbbA cbbA*^{Ect} and ST18Δ*cbbA cbbA*^{Ect}) tested were shown to express a protein band corresponding to apparent molecular weight of ca. 38-kDa representing FBA (Fig 3.22 A,B,C). These complemented strains will be used to confirm the previously identified phenotype of *cbbA* mutant in various characterisation experiments.

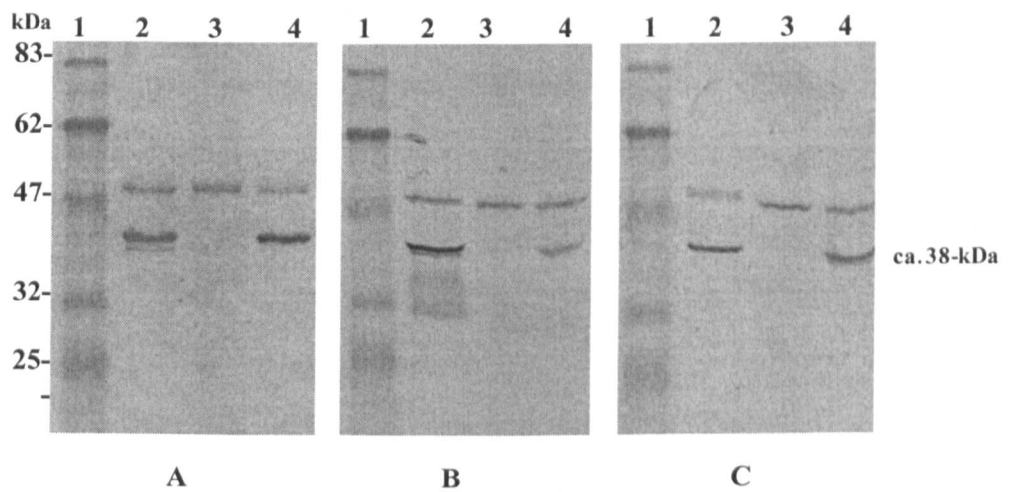


Figure 3. 22 Immunoblot analysis demonstrating the expression of *cbbA* in complemented mutant strains, MC58Δ*cbbA cbbA*^{Ect}, Z4181Δ*cbbA cbbA*^{Ect} and ST18Δ*cbbA cbbA*^{Ect}. Whole cell extracts were resolved by 10% SDS-PAGE followed by immunoblot analysis using RαFBA. (A) Lane 1, protein marker, lane 2, MC58-WT, lane 3, MC58Δ*cbbA*, lane 4, MC58Δ*cbbA cbbA*^{Ect}, (B) lane 1, protein marker, lane 2, Z4181-WT, lane 3, Z4181Δ*cbbA*, lane 4, Z4181Δ*cbbA cbbA*^{Ect},(C) Lane 1, protein markers, lane 2, ST-18-WT, lane 3, ST-18Δ*cbbA*, Lane 4, ST-18Δ*cbbA cbbA*^{Ect}.

3.3.15 Mutagenesis of pSAT-12 by inverse-PCR

Sequencing of the complementation construct revealed that there were differences in promoter region compared to the expected sequence. In order to ensure expression of the *cbbA* gene, therefore, the *cbbA* was cloned together with its predicted promoter sequences. Again, in order to ensure expression of an ectopic copy of *gapA*-1 inserted into the genome via the complementation vector, the *gapA-1* locus was analysed on the MC58 chromosome in an attempt to identify the native promoter sequences of *gapA-1*. Visual inspection revealed that in *N. meningitidis* strain MC58 the *gapA-1* gene is downstream to NMB0208 (predicted to encode ferridoxin), and bioinformatics analysis could not identify a strong promoter sequences upstream of *gapA-1*. Therefore, the promoter of *cbbA* was employed to drive expression of *gapA*-1 at the ectopic site. Inverse-PCR was performed of the pSAT-12. This resulted in deletion of *cbbA* from the ATG start codon to the stop codon and introduction of unique *Bgl*II site. The i-PCR product was digested with *Bgl*II and self-ligated to yield pSAT-13 intermediate vector. The pSAT-13 was transformed in *E. coli* JM109 for plasmid propagation and subsequent purification. A successful clone was identified by restriction digestion with *Bgl*II of plasmid DNA prepared from putative transformants. As expected a band of *ca.* 6-kb representing linear pSAT-13 was observed in all five samples tested (Fig 3.23) confirming the deletion of *cbbA*.

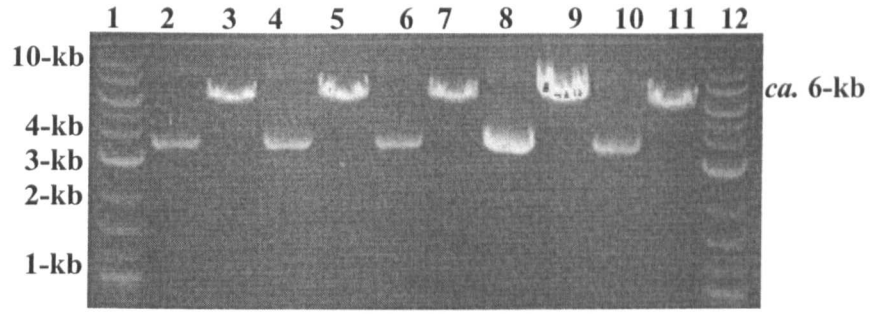
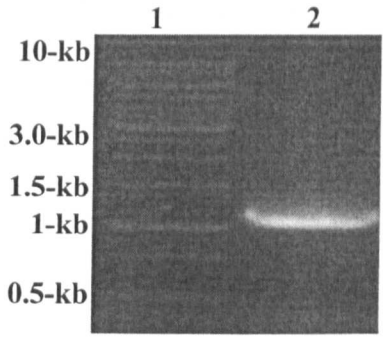


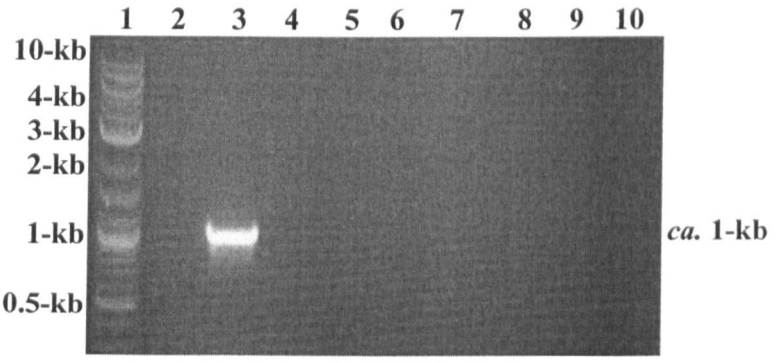
Figure 3. 23 Agarose gel analysis showing the restriction digestion analysis of pSAT-13 using *Bgl*II. Plasmid DNA was purified from *E. coli* harboring putative pSAT-13 and used to confirm the deletion of *cbbA* and presence of *Bgl*II site to facilitate ligation of *gapA-1*. Lane 1, DNA markers, lanes 2,4,6,8,10 uncut pSAT-13, lanes 3,5,7,9, and 11, pSAT-13 digested with *Bgl*II. All lanes containing *Bgl*II digested pSAT-13 produced a plasmid of correct size based on predicted molecular weight of linear construct. One of the correct clones was chosen and used for plasmid propagation and purification for cloning of *gapA*-1 in *Bgl*II site.

3.3.16 PCR amplification and cloning of *gapA-1* in pSAT-13

The promoter-less *gapA-1* consisting of 1,032 bp was PCR amplified (Fig 3.36). This PCR resulted in amplification of an expected *ca.* 1-kb band representing *gapA-1* and introduction of a unique *Bam*HI site to facilitate the ligation into pSAT-13 (Fig 3.24A). The pSAT-13 plasmid was prepared by digestion with *Bgl*II (site present downstream of *cbbA* promoter) and gel-purified, followed by dephosphorylation to prevent self-ligation. The *Bam*HI-digested and purified *gapA-1* product was then ligated to linearized pSAT-13 to yield pSAT-14, followed by transformation of *E. coli* JM109 cells. The positive clones were identified by colony PCR (Fig 3.24B), and partial sequencing using a primer FBA_Com (F) (Table 3.2) to confirm in frame ligation. This primer anneals at the start of *cbbA* promoter available upstream of *gapA-1* to drive the expression of this gene.



(A)



(B)

Figure 3. 24 Agarose gel analysis demonstrating ((A) the successful amplification of *gapA-1* from chromosomal DNA of *N. meningitidis* for ligation into pSAT-13 to generate pSAT-14. Lane 1, DNA markers, lane 2, PCR product of *gapA-1* (B) colony PCR amplification of *gapA-1* from putative transformants. Lane 1, DNA marker, lanes 2-10, PCR product representing the *gapA*-1. Several clones were identified and further confirmed by sequencing.

After verification of *gapA*-1 cloning in pSAT-14, the expression of *gapA-1* under the control of the *cbbA*-promoter was determined in *E. coli* JM109 cells. Whole cell proteins were prepared from *E. coli* JM109 cells harboring pSAT-14 and resolved on 10% SDS-PAGE and transferred to nitrocellulose membrane followed by immunoblotting by probing with RαGAPDH-1 antiserum. The pSAT-14 clone was shown to express a protein band with an apparent molecular mass corresponding to

the expected size of GAPDH-1, which was absent in the pSAT-12 (negative control) indicating that the GAPDH-1 is expressed in *E. coli* under the *cbbA* promoter (Fig 3.25). The pSAT-14 was then used to complement the *gapA*-1 mutation in MC58Δ*gapA-1* by natural transformation and allelic exchange. The putative complemented transformants were selected on selective BHI agar plates. The chromosomal DNA was prepared from putative complemented mutants for PCR analysis.

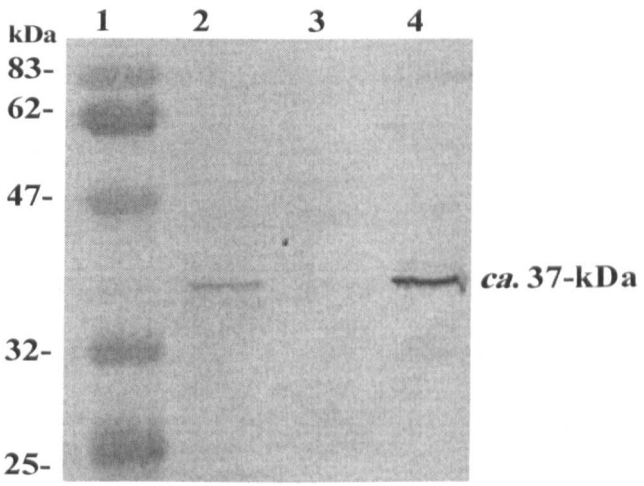


Figure 3. 25 Immunoblot analysis demonstrating the expression of *gapA*-1 in *E. coli* harboring pSAT-14, Whole cell extracts were prepared from *E. coli* containing pSAT-14 and reolved by 10% SDS-PAGE to verify the expression of *gapA*-1 by immunoblot analysis using RαGAPDH-1. Lane 1, DNA markers, Lane 2, whole cell extracts from MC58-WT, lane 3, whole cell extracts from *E. coli* harboring pSAT-12 (Negative control), lane 4, and whole cell extracts from *E. coli* harboring pSAT-14.

3.3.17 PCR analysis of putative MC58Δ*gapA-1 gapA-1*^{Ect}

In order to verify the successful complementation of *gapA-1* at ectopic site in isogenic-null mutants, chromosomal DNA was prepared from the putative complemented and isogenic-null mutant strains and used to amplify the DNA band corresponding to *gapA*-1 using a pair of *gapA*-1-specific primers. Agarose gel analysis demonstrated an expected band of *ca.* 1-kb representing the *gapA-1* from complemented mutants that was absent in the isogenic-mutant strain (Fig 3.26A) confirming the successful complementation of *gapA-1* mutation.

In addition, another PCR using the primers NMB0102 (F) 2 & NMB0103(R) was performed to verify the insertion of the complementation cassette at the proper location in the chromosome of complemented strains. Insertion of the complementation cassette at the proper location was expected to produce *ca* 4-kb band in complemented mutant strains whereas mutant strain was expected to produce a band of *ca* 1.3 kb representing only NMB0102 & NM0103 PCR product. As expected the complemented strain produced an expected *ca.* 4-kb band whereas the mutant strains produced a 1.3-kb band (Fig 3.26B) further confirming the insertion of *gapA-1* at the desired location in the chromosome of complemented strains.

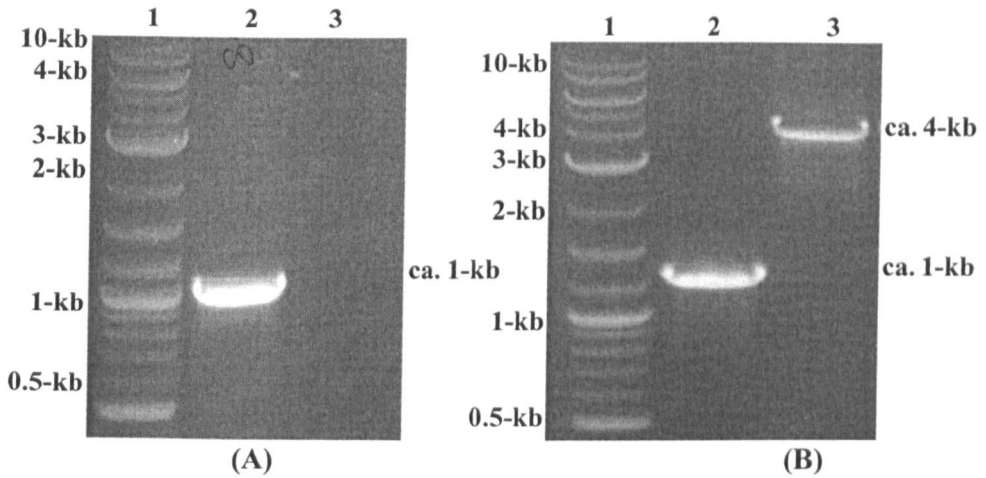
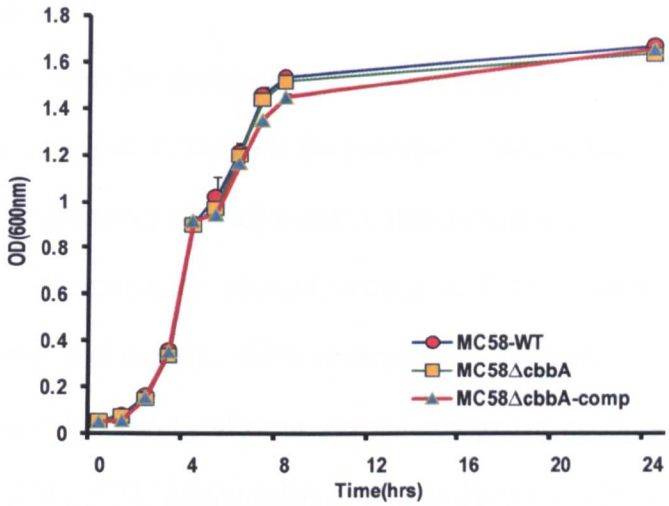


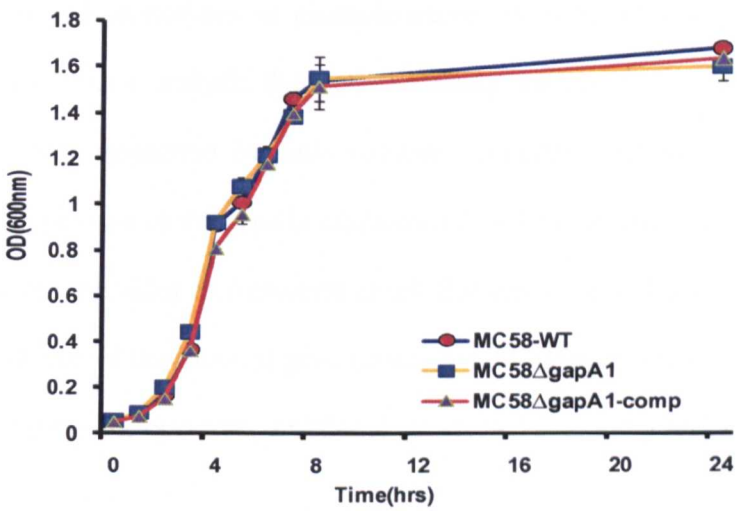
Figure 3. 26 Agarose gel analysis demonstrating (A) the PCR amplification from the *gapA-1* mutant and complemented strain. Genomic DNA was extracted from putative *gapA-1* complemented strains to confirm the complementation of *gapA-1* in PCR amplification of *gapA-1*. Lane 1, DNA markers, Lane 2, MC58Δ*gapA-1* *gapA-1*$^{-Ect,}$ and Lane 3, MC58Δ*gapA-1*. Lane 2 shows a band of apparent molecular weight of *gapA-1* confirming the successful complementation, whereas no band was observed in the mutant strain (B) Confirmation of the insertion of *gapA*-1 at desired location in the chromosome of complemented strain. Genomic DNA was extracted from putative *gapA-1* complemented strain for PCR amplification of *gapA-1*. Lane 1, DNA markers, lane 2, MC58Δ*gapA-1,* and lane 3, MC58Δ*gapA-1* *gapA-1*^{Ect},. Lane 3 shows a band corresponding to complementation cassette, whereas an expected 1.3-kb DNA fragment corresponding to NMB0102 & NMB0103 was observed in Lane 2.

3.3.18 Determining the growth characteristics of meningococcal strains

Before undertaking phenotypic analysis of the *cbbA* and *gapA-1* mutants, it was first essential to demonstrate that mutations of these genes did not significantly affect the growth rate of *N. meningitidis*. Growth and colonial characteristics of mutant strains were assessed by visual inspection of colony morphology on agar plates and monitoring the rate of growth in liquid culture. The mutant strains demonstrated similar colony morphology and colour to that of wild-type strain MC58 (data not shown). The wild-type MC58, isogenic *cbbA* and *gapA-1* null mutant and complemented strains were then grown in BHI broth or DMEM with a starting OD_{600} adjusted to 0.06. Growth rate was assessed by measurement of OD_{600} of samples that were removed from the culture at hourly intervals. The growth rate of the strains with deletion of *cbbA* and *gapA-1* was not substantially different to that observed for wild-type MC58 and complemented strains. All strains were observed to grow to a similar OD_{600} by the final time point of eight and 24 h. These experiments were performed in triplicate and the results are presented in (Figure 3.27). In order to further explore whether the addition of normal human serum may have any effect on the growth of wild-type and mutant strains, meningococcal growth was monitored in DMEM or DMEM supplemented with 10% human serum. Again, no substantial differences between the growth rates of wild-type MC58 and either *cbbA* and *gapA-1* mutants were observed (data not shown).



(A)



(B)

Figure 3. 27 Growth characteristics of meningococcal mutant and complemented strains compared to their wild-type parent strain. (A) *N. meningitidis* strain MC58-WT, MC58Δ*cbbA*, MC58Δ*cbbA cbbA*^{Ect} (B) *N. meningitidis* strain MC58-WT, MC58Δ*gapA-1*, MC58Δ*gapA-1 gapA-1*^{Ect} were grown in BHI broth in triplicate and OD reading was taken at hourly intervals for 24 hrs. No substantial differences were observed demonstrating that the *cbbA* and *gapA-1* mutation has no effect on *in vitro* maximal growth of *N. meningitidis* strain.

3.4 Discussion:

This chapter describes the mutagenesis of *cbbA* and *gapA-1* in *N. meningitidis*. The mutant strains were used to facilitate the functional characterisation of the products of these genes in a number of *in vitro* and *in vivo* experiments (described in detail in Chapter 4 & 5). The mutagenic plasmids were generated by cloning DNA fragments flanking the *cbbA* and *gapA-1*, which contained one naturally-occurring Neisserial uptake sequences. The *cbbA* and *gapA-1* mutants were successfully generated in *N. meningitidis* strain MC58. Additionally, *cbbA* was disrupted in four clinical isolates of *N. meningitidis*. Importantly, both *cbbA* and *gapA-1* mutant strains were shown to exhibit unexpected phenotypes in characterisation experiments (Chapter 4 & 5), which initiated further analysis that led to subsequent complementation of both mutations in their respective isogenic mutants. Complementation of mutants, in which a wild-type copy of the gene is reintroduced in the respective isogenic-mutant at an ectopic site, provides an important check that any observed phenotype can be ascribed to the loss of the mutated gene rather than to polar effects of the mutation on adjacent genes in the operon or additional undetected secondary mutations.

The complementation of *cbbA* and *gapA-1* mutations in *N. meningitidis* strain MC58 was initially attempted using the pAP2-1 based plasmid pNJO95 and pNJO96, respectively. The plasmid pAP2-1 is based on a gonococcal cryptic plasmid; it has a porA promoter and a spectinomycin resistance cassette. The plasmid pNJO95 and pNJO96 constructs were created by Dr Neil Oldfield and shown to express desired proteins, FBA and GAPDH-1 by immunoblot analysis. These plasmid borne genes

were assumed to be expressed independently on the plasmid rather than integrating into the chromosome of the bacteria. Several attempts to introduce these plasmid constructs by either natural transformation or electroporation in respective mutants were unsuccessful. Alternatively, the complementation strategy based on the allelic exchange was adopted. Under this strategy, the complementation was performed using a pYHS25-based ectopic complementation vector in which the expression of genes of interest is driven under the control of the *cbbA* promoter. Moreover, these constructs facilitate insertion of genes of interest into the meningococcal genome at a site that is unlikely to effect the expression of any other genes.

A pYHS25-based complementation plasmid was generated by cloning a *ca.*2.7 DNA fragment, which contained two neisserial DNA uptake sequences and the erythromycin resistance cassette flanked by the two meningococcal genes: NMB0102 & NMB0103. Although the complementation plasmid contained promoter sequences upstream of erythromycin resistance cassette, the sequences were found to differ from that which was expected. Therefore, the *cbbA* and *gapA-1* genes were cloned under the predicted promoter sequence upstream of *cbbA*. In an attempt to identify promoter sequences upstream of FBA, analysis of the organization of genes at the *cbbA* locus (NMB1869) was performed. This analysis demonstrated that *cbbA* is the first gene in its orientation, so must have its own promoter. The predicted *cbbA* gene (based on gene-finding algorithms used at the Comprehensive microbial resource [CMR] database) starts from an internal ATG start codon, meaning that the first 25 predicted amino acids in the ORF are not

predicted to be part of the gene. This is in agreement with a visual inspection, which shows that there is a good ribososmal binding site (RBS) upstream of the internal ATG but not at the first ATG. The *cbbA* was amplified from a position upstream of the predicted promoter to a position downstream of its stop codon and cloned in a complementation vector. The resulting complementation plasmid was used to transform *N. meningitidis* strain MC58Δ*cbbA*, Z4181Δ*cbbA* and ST18Δ*cbbA*, returning wild-type copies of *cbbA* to these mutants. Successful complementation was confirmed by PCR analysis and immunoblot analysis using RαFBA antiserum.

For complementation of *gapA-1* mutation, examination of the orientation of the open reading frame around the *gapA-1* gene indicated that polar effect could not be responsible for the attenuation of the *gapA*-1because *gapA-1* is the second and last gene in the operon and that the immediately downstream gene is in the opposite orientation. However, this mutant was also complemented *in trans* with a single copy of the wild gene in an ectopic chromosomal location to generate MC58Δ*gapA-1* *gapA-1*^{Ect}. The genetic organization at the *gapA-1* locus (NMB0207), which is downstream of NMB0208 (predicted to encode ferrodoxin) was analysed to identify potential promoter sequences. This showed that the two genes might be cistronic (i.e. they may share a promoter). Bioinformatics prediction identified a possible promoter upstream of *gapA-1* (NMB0207), but with only a weakly identified -35 region. The *gapA-1* gene was, therefore, cloned downstream of the *cbbA* promoter. The *gapA-1* gene was amplified from a position upstream of its ribosome binding site to a position downstream of its termination codon to generate a complementation

plasmid. The resulting plasmid designated pSAT-14 was introduced into *N. meningitidis* strain MC58Δ*gapA-1* by natural transformation to reintroduce the wild-type copy of *gapA-1*. Complementation of *gapA-1* at ectopic site was successfully achieved in the *gapA-1* isogenic null mutant. Prior to employing the mutants in characterisation experiments, *in vitro* assessment of growth rate was undertaken to determine whether mutation of *cbbA* or *gapA-1* had any overt influence on meningococcal growth. The wild-type MC58, *cbbA* and *gapA-1* mutant and their complemented strains were shown to grow at a similar rate without any noticeable differences either in colonial morphology or by growth rate in broth culture. Moreover, the addition of normal human serum to DMEM or BHI had no effect on the growth of *N. meningitidis* strain MC58 and both mutants. The pattern of growth remained the same in the presence or absence of human serum in the culture medium. These findings are consistent with the findings of Baart *et al.*, who showed that the glycolytic pathway was non-functional in the meningococcus due to absence of one of the important enzymes in this pathway: phosphofructokinase (Baart *et al.*, 2007).

In conclusion, *cbbA* isogenic mutants were generated in five meningococcal strains, and a *gapA-1* isogenic mutant was generated in *N. meningitidis* strain MC58. In addition, the *cbbA* mutation was complemented in three of the isogenic mutants and the *gapA-1* mutant was also complemented. Additionally, the growth of wild-type MC58 was shown to be unaffected by the mutation of either *cbbA* or *gapA-1*,

demonstrating that the products of these genes were not required for optimal growth of *N. meningitidis*.

CHAPTER 4: Molecular and immunological characterisation of FBA and GAPDH-1

4.1 Introduction

The functional, molecular, and immunological characterisation of two of the glycolytic enzymes namely FBA and GAPDH-1 of *N. meningitidis* is described in this chapter. It is important to note that recent genome-based studies have revealed that one of the glycolytic (EMP) pathway enzymes, namely phosphofructokinase (EC 2.7.1.11) is absent from *N. meningitidis* strain MC85, rendering this pathway non-functional (Baart *et al.*, 2007). Despite the inability to utilize the glycolysis pathway, FBA, GAPDH-1 and other enzymes required for glycolysis are maintained in the meningococcal genome, presumably for other roles. In the absence of alternative roles for these enzymes, spontaneous mutations would be expected to accumulate in the encoding sequences. This chapter will focus on the characterisation of both of enzymes with the aim of determining their conservation, expression, cellular localization, and additional non-glycolytic role(s).

An important property for a putative vaccine antigen is that it should be well-conserved across divergent isolates of meningococci and expressed naturally. To confirm that the *cbbA* and *gapA-1* genes were conserved across, and expressed in, diverse clinical isolates of menigococci, whole cell proteins from a panel of meningococcal strains and one strain each *of N. gonorrhoeae, N. polysacchareae,* and *N. lactamica* were screened by PCR and subsequently by immunoblot analysis using RαFBA and RαGAPDH-1, respectively. To determine the enzymic activity, rFBA was successfully purified under native conditions, which was then used to confirm the functional activity of this enzyme. In keeping with the fact that

glycolytic enzymes may reach the surface of numerous bacterial and fungal pathogens, in this study surface-location of FBA and GAPDH-1 meningococcal glycolytic enzymes was investigated using a combination of molecular and immunological techniques, including sub-cellular fractionation of meningococcal cells, surface-biotinylation of intact meningococcal cells, enzyme-linked immunosorbant assay (ELISA) and flow cytometry.

In an attempt to determine the surface location and evaluate the vaccine potential of FBA in meningococci, whole cell ELISA was performed with polyclonal antiserum raised against the denatured purified rFBA protein. Initial results were promising and demonstrated that FBA was present in the outer membrane fraction of meningococcal cells. To confirm these results, and assess surface-exposure we used a technique that involves the biotinylation of surface-exposed proteins. Although considered to be an invaluable approach in the identification of surface exposed proteins, it failed to demonstrate surface-exposure of FBA and GAPDH-1. To further investigate the surface location of FBA, the polyclonal antiserum raised against natively purified FBA was used to evaluate the accessibility of FBA epitopes on the surface of live encapsulated *N. meningitidis* serogroup B bacteria using flow cytometry analysis. Using the polyclonal antisera, raised against denatured purified FBA and GAPDH-1 protein, the susceptibility of meningococci to antibody-dependent, complement-mediated bacteriolysis using *in vitro* serum bactericidal assay was also investigated.

In addition, to assess whether FBA is essential for bacteraemia in a novel transgenic mouse model of infection, isogenic *cbbA* mutants created in five different genetic backgrounds of *N. meningitidis*, were used by our collaborators at the Pasteur Institute in Paris. The results of the animal studies have shown that all *cbbA* mutant strains were significantly less able to cause bacteraemia compared to their wild-type parent strains indicating that FBA may play a role in the pathogenesis of meningococcal disease.

The aim of this chapter was to characterize the FBA and GAPDH-1 enzymes of *N. meningitidis* using molecular and immunological techniques. The key aims were to determine their surface localisation and evaluate the vaccine potential of both proteins. This chapter also aimed to explore the potential role of FBA in meningococcal pathogenesis.

4.2 Materials and methods

4.2.1 Bacterial strains, growth conditions and media

The bacterial strains and plasmids used in this chapter are described in chapter 2 and 3. Culture conditions for the growth of *E. coli* and *N. meningitidis* were as described in sections 2.2.1 and 3.2.1, respectively. All meningococcal strains were clinical isolates belonging to different serogroups and types (Table 4.1) and included representative isolates of recognized hypervirulent lineages. The antibiotics were used as appropriate at the following concentrations: kanamycin 50 $\mu g\ ml^{-1}$ and erythromycin 5 $\mu g\ ml^{-1}$.

4.2.2 Preparation of whole cell proteins

Meningococcal cells (Table 4.1) were grown overnight in BHI supplemented with Vitox (Oxoid) at 37°C in a shaking incubator (250 rpm). The cultures were harvested by centrifugation at 13000 × *g* for 10 min. The pelleted cells were re-suspended in sterile PBS and 5× SDS-PAGE sample buffer (Appendix A) to achieve 1× final concentration of whole cell protein extracts. The whole cell lysate was briefly sonicated using a 10 s pulse to obtain a homogenous suspension, boiled for 5 min and then mixed thoroughly before SDS-PAGE analysis.

Table 4. 1 Clinical isolates of *N. meningitidis*.

Strain	Country of origin	Serogroup	Sequence type	Clonal complex
Z1001	USA	A	4	ST-4 complex/subgroup IV
Z1035	Pakistan	A	1	ST-1 complex/subgroup I/II
Z1054	Finland	A	5	ST-5 complex/subgroup III
Z1213	Ghana	A	4	ST-4 complex/subgroup IV
Z1269	Burkina Faso	A	4	ST-4 complex/subgroup IV
Z1503	China	A	5	ST-5 complex/subgroup III
Z3771	UK	A	5	ST-5 complex/subgroup III
Z3842	Norway	B	32	ST-32 complex/ET-5 complex
Z4181	Mali	C	11	ST-11 complex/ET-37 complex
Z4323	Israel	C	11	ST-11 complex/ET-37 complex
Z4662	Netherlands	B	8	ST-8 complex/Cluster A4
Z4667	Netherlands	B	48	ST-41/44 complex/Lineage 3
Z4673	Netherlands	B	41	ST-41/44 complex/Lineage 3
Z4676	Denmark	B	37	ST-37 complex
Z4678	Germany	B	19	ST-18 complex
Z5826	China	A	7	ST-5 complex/subgroup III
Z6413	South Africa	C	8	ST-8 complex/Cluster A4
Z6414	New Zealand	C	66	ST-8 complex/Cluster A4
Z6418	Cuba	B	33	ST-32 complex/ET-5 complex
Z6419	Austria	B	40	ST-41/44 complex/Lineage 3
Z6420	Greece	B	41	ST-41/44 complex/Lineage 3
Z6417	England	C	11	ST-11 complex/ET-37 complex
Z4684	Norway	B	13	ST-269 complex
Z4685	Norway	B	14	ST-269 complex
Z4701	Norway	B	11	ST-11 complex/ET-37 complex

4.2.3 Strain distribution of *cbbA* and *gapA-1* in *N. meningitidis*

A panel of 26 strains of *N. meningitidis* of known MLST-type (Table 4.1) was selected to determine the expression of *cbbA* and *gapA-1*. The whole cell proteins from these strains were analysed by immunoblotting and probing with anti-FBA or anti-GAPDH-1 serum, respectively.

4.2.4 Kinetic analysis of the fructose bisphosphate aldolase activity

Kinetic analysis studies of FBA protein were performed at the University of Leeds under the supervision of Dr Alan Berry. A coupled enzymic assay was used to determine FBP-aldolase activity. The assay was performed in 1 ml of 50 mM Tris-HCl supplemented with 0.1 M potassium acetate buffer, pH 8.0 containing 0.1-5 mM fructose 1, 6-bisphosphate, 0.2 mM NADH and 2 µl of 10 mg ml^{-1} mixture of glycerol-phosphate dehydrogenase-triosephosphate isomerase at 30°C. The reagents were added in the following order: buffer, FBP, NADH and coupling enzymes. The reaction was then started by adding purified FBA enzyme. The reaction mixture was mixed well before recording the decrease in absorbance at 340 nm as the measure of enzyme activity on an Uvikon 930 spectrophotometer. Activities were calculated using the molar extinction coefficient for NADH as 6220 $M^{-1}cm^{-1}$. One unit of aldolase activity was defined as the amount of enzyme which catalyses the oxidation of 2 µmol NADH per min in the assay system. Kinetic parameters were estimated using the Origin Pro 7.5 software program.

4.2.5 Sub-cellular fractionation of meningococcal cells

To investigate the sub-cellular localisation of FBA and GAPDH-1 in *N. meningitidis* MC58, a traditional method of cell fractionation was used to prepare cytoplasmic, periplasmic, inner membrane and outer membrane fractions of the cell and each fraction was probed with the anti-FBA or anti-GAPDH-1 antiserum in order to identify the cellular localisation of FBA and GAPDH-1, respectively, in *N. meningitidis*. Meningococcal cells were grown overnight at 37°C in BHI broth with or without supplement and DMEM supplemented with 10% human serum.

Periplasmic proteins were prepared by a modification of the method of Nossal and Heppel (Nossal & Heppel, 1966) or a chloroform extraction method (Ames *et al.*, 1984). Briefly, cells from 100 ml overnight cultures were harvested at 13000 × *g* for 2 min and the pellet was re-suspended in 1 ml of EB buffer (10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 25% sucrose), and washed twice in the same buffer. Finally, the pellet was re-suspended in EB buffer and incubated for 10 min on ice. The preparation was centrifuged at 13000 × *g* for 4 min, following rapid re-suspension in 0.4 ml of ice cold water and incubation on ice for a further 10 min, followed by centrifugation at 13000 × *g* for 2 min. The upper fraction of the supernatant consisting of periplasmic proteins was transferred to a fresh eppendorf and stored at -20°C. After collection of the periplasmic fraction, the cell pellets (spheroplasts) were re-suspended into 0.4 ml Tris-HCl (pH 7.5) and sonicated to release the cytoplasmic contents. Non-disrupted cells were removed by centrifugation at 5000 × *g* for 1 min. The upper clear supernatant was transferred to a fresh eppendorf and centrifuged at

17000 × *g* for 30 min. The supernatant was collected as the cytoplasmic protein fraction and stored at -20°C. The remaining pellet was re-suspended in 0.2 ml of 10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$ and 0.2 ml of 10 mM Tris-HCl pH7.5, 10 mM $MgCl_2$ and 4% Triton X-100. The sample was incubated at 37°C for 30 min and then centrifuged at 17000 × *g* for 30 min. The supernatant was collected as the cytoplasmic membrane fraction and stored at -20°C. The final pellet (yielded after collection of the cytoplasmic membrane fraction) was deemed the outer membrane protein-enriched fraction. This pellet was re-suspended by brief sonication in 10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$ and then 0.5 ml of 10mM Tris-HCl pH7.5, 10 mM $MgCl_2$ and 2% Triton X-100 was added. The suspension was incubated at 37°C for 30 min and then centrifuged at 17000 × *g* for 30 min. This step was repeated to remove any remaining soluble proteins to ensure the purity of fraction. Final pellet was re-suspended in 0.2 ml 10 mM Tris-HCl pH 7.5 and stored at -20°C.

4.2.6 Methodologies to assess surface exposure of FBA and GAPDH-1

4.2.6.1 Cell surface biotinylation of meningococci

Cells of *N. meningitidis* strain MC58 were grown overnight on chocolate agar. Colonies were collected and placed into 1 ml of sterile PBS in an eppendorf tube (1.5 ml). Cells were harvested by centrifugation at 13000 × *g* for 5 min. Before being re-suspended in carbonate buffer (Appendix A), cells were washed three times with fresh ice-cold PBS. The optical density (OD_{600}) was adjusted to 0.2 and biotin-streptavidin (Sigma) was added to a final concentration of 0.5 mg ml^{-1}. The cells were incubated at RT for 30 min and then harvested by centrifugation at 13000 × *g*

for 5 min. In order to remove excess unbound biotin, the cells were washed two times with sterile PBS. The cell pellet was briefly sonicated (10 s pulses). The biotinylated samples were analysed by SDS-PAGE followed by immunoblotting using anti-goat anti-biotin conjugated to alkaline phosphatase at a dilution of 1:30000. Membranes were developed using BCIP substrate (Section 2.2.15 and 2.2.16).

4.2.6.2 Enzyme linked immunosorbant assay (ELISA)

An ELISA was used to analyze the ability of polyclonal antisera raised against recombinant proteins to bind to intact meningococcal cells. The antiserum was pre-adsorbed with the mutant strain and serially diluted (1:10, 1:100, and 1:1000) in coating (carbonate) buffer (Appendix A), before being added in 100 µl aliqoutes to plate wells (Nunc 96-well plates, PolySorp) for 1 h at RT. Control wells were coated with PBS containing 1% BSA (PBS-BSA) (negative control). Plate was washed three times in PBS containing 0.05% Tween 20 (PBS-Tween) buffer. To block the remaining binding sites 100 µl of PBS-BSA was added to each well and incubated for 1 h at RT. Microtiter plate wells were again washed three times with PBS-Tween before adding the digoxygenin-labeled bacterial cells. For labeling, *N. meningitidis* strains were grown in liquid culture and washed 3 times in PBS-Tween before being resuspended in carbonate buffer to an OD of 0.1 at 600 nm. Bacteria were labeled by adding 10 µg of digoxygenin (Roche) per 1 ml bacterial suspensions for 2 h at RT. One hundred microlitres labeled cells were added to each well, and plates were incubated for 2 h at RT. Plates were washed 5 times with PBS-Tween and incubated

with anti-digoxigenin Fab fragment–conjugated antibody (1:5,000; Roche) in 1% BSA in PBS-Tween (blocking buffer) at 100 µl per well . Plates were incubated at RT for an additional 1 hour and washed 5 times. One hundred microliters of 2, 2'-azino-bis; 3-ethylbenzthiazoline-6-sulphonic acid (ABTS) (Chemicon international ES004) substrate (5 mg ml^{-1}; Roche) were added to each well, and the absorbance was measured at 405 nm after 30 min using an ELISA plate reader (BioTek).

4.2.6.3 Flow cytometry analysis

The ability of antisera elicited by the recombinant FBA and GAPDH-1 to bind to the surface of pathogenic strains of *N. meningitidis* group B was determined by flow cytometry using an indirect fluorescence assay. *N. meningitidis* strain MC58 wild-type and MC58Δ*cbb*A cells were grown to OD_{600} <0.7 to obtain mid-log phase bacteria. Four samples per strain, each containing 1×10^7 CFU ml^{-1} in PBS, were prepared for the flow cytometry analysis. In each case one sample was: untreated; treated with primary antibody only; treated with secondary antibody only; or treated with both primary and secondary antibodies. The cells were washed twice in sterile filtered PBS by centrifugation at 5000 × *g* for 5 min. The test sample cells were treated with either RαFBA or RαGPADH-1 polyclonal antiserum (1:500) diluted in PBS containing 0.1% BSA, 0.1% sodium azide and 2% foetal calf serum (PBS-BSA-FCS buffer), whereas the control cells were re-suspended without antibody, and incubated on ice for 2 h. The samples were washed 3 times in PBS and the test sample pellets were mixed with secondary antibody, goat anti-rabbit IgG conjugated-Alexa Flour 488 (Invitrogen) diluted 1:50 in PBS-BSA-FCS buffer. The control

sample pellets were re-suspended in PBS-BSA-FCS buffer, and all samples were incubated on ice for 2 h in the dark. Finally, the samples were washed in PBS twice before being re-suspended in 1 ml PBS containing 0.5% formaldehyde to fix the cells. The samples were then analysed for fluorescence using Coulter Altra Flow Cytometer. The cells were detected using forward and log-side scatter dot plots, and a gating region was set to exclude cell debris and aggregates of bacteria. A total of 50,000 bacteria (events) were analyzed for fluorescence signals. All buffers and solutions were filtered using 0.2 μm filter to eliminate any small particles. As negative controls, one sample was untreated, one sample was treated with primary antibody only and one sample was treated with secondary antibody only.

4.2.7 Serum bactericidal assay

N. meningitidis strain MC58 was grown on chocolate agar overnight. Ten colonies were inoculated into 5 ml MHB (without supplement) and incubated for two hours at 37°C in a shaking incubator. The OD_{600} nm was measured and cells were harvested (8000 × *g* for 5 min) from a 500 μl aliquot of the culture. The cells were re-suspended in PBS/bovine serum albumin (BSA) to achieve an OD_{600} of 0.1.

The assay was performed in a sterile 96-well tissue culture plate, to which the assay components (antibody, complement, and bacteria) were added sequentially. Each well contained: 20 μl of RαFBA or RαGAPDH-1 antiserum (or pre-immune serum taken from the same rabbit) pre-adsorbed with the cells of strain MC58Δ*cbbA* or MC58Δ*gapA-1*, respectively (and de-complemented by heating to 56°C for 30 min),

10 µl of bacteria (diluted to *ca.* 800 colony-forming-unites [CFU] per well), and 10 µl of sterile baby rabbit serum (Pel-Freeze) as a source of complement. Sera were used at the final dilution of 1:2, 1:4, 1:8, and 1:16. After the addition of all components to the wells, the plates were covered and incubated for 60 min at 37°C on a microplate shaker (150 rpm). Ten microliters from control wells were inoculated on to chocolate agar at time zero. After 60 min incubation serial dilutions were performed and 10 µl aliquots were inoculated onto chocolate agar. The chocolate agar plates were incubated as previously described (Section 3.2.1).

After overnight incubation, the number of colonies at each dilution of RαFBA and RαGAPDH-1 were counted, and the serum bactericidal titer was reported as the reciprocal of the serum dilution yielding ≥ 50% killing of the bacteria. Control wells included: a serum with known bactericidal activity (anti-meningococcal whole cell); a complement control containing PBS-BSA, complement and bacteria; an inactive complement control containing RαFBA or RαGAPDH-1 serum, heat inactivated complement, and bacteria; an antibody control containing PBS-BSA, RαFBA or RαGAPDH-1 serum and the bacteria to determine that the organisms were viable in antibody in the absence of complement).

4.2.8 Transgenic mouse model of meningococcal bacteraemia

Murine bacteraemia studies were performed at the Pasteur institute by Dr M. K. Taha as previously described (Zarantonelli *et al.*, 2007). Briefly, prior to challenging with meningococcal isolates, mice were kept in a biosafety containment facility in cages with sterile litter, water, and food according to institutional guidelines. The experimental design was approved by the Institut Pasteur Review Board (France). Mice were infected at 6 weeks of age by intraperitoneal challenge with standardized inocula (5×10^6 CFU). Bacterial counts in the blood were determined 2, 6, and 24 h, after meningococcal challenge by plating serial dilutions of blood samples on GCB medium and were expressed in log10 CFU ml^{-1} of blood. Student's *t-test* and analysis of variance were used to examine the data. A P value of 0.05 was considered statistically significant.

4.3 Results

4.3.1 Fructose bisphosphate aldolase activity

A coupled enzymic assay using native recombinant FBA was used to confirm that the purified meningococcal FBA was active as a fructose bisphosphate aldolase (Fig. 4.1), despite the apparent lack of an intact EMP pathway in this organism. The kinetic parameters for the purified enzyme for the cleavage of FBP were estimated as K_m (FBP) = 0.05 mM and k_{cat} = 126 min^{-1}. These values are similar to those found for FBA enzymes from a variety of sources such as *E. coli* (K_m (FBP) ~ 0.19 mM and k_{cat} ~ 490 min^{-1}) (Plater *et al.*, 1999).

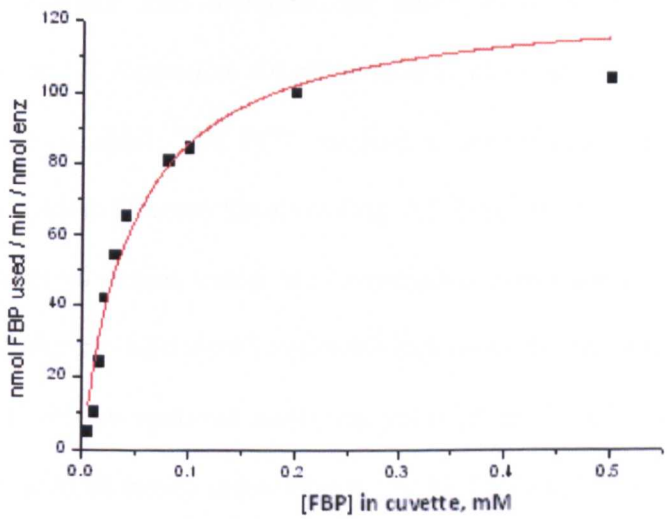
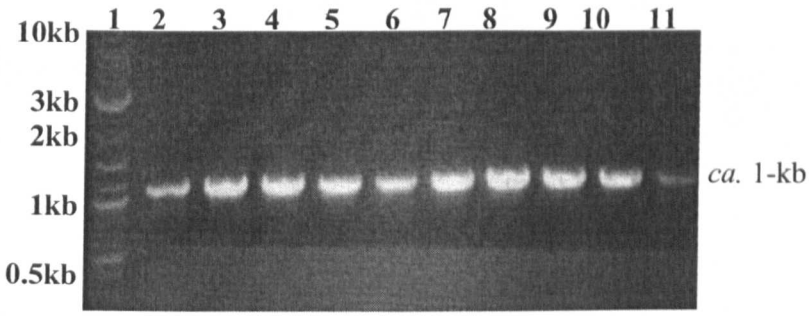


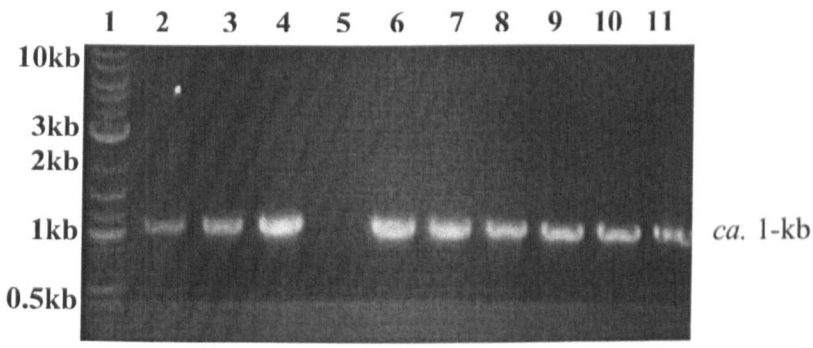
Figure 4. 1 Kinetic analysis of the rFBA The enzyme activity was measured using different substrate (FBP) concentrations (0.1 mM–5.0 mM) under standard reaction conditions for one min. A decrease in absorbance at 340 nm was recorded as the measure of enzyme activity. Activities were calculated using the molar extinction coefficient for NADH as 6220 M^{-1} cm^{-1}. Kinetic parameters were estimated using the Origin Pro 7.5 software program.

4.3.2 Distribution and expression of *cbbA* in *N. meningitidis*

Due to the accessible outer membrane location, immunogenic nature and potential roles in pathogenesis, glycolytic enzymes have been suggested as possible candidate vaccine antigens against several infections caused by Gram-positive bacteria and fungi (Gil-Navarro *et al.*, 1997; Kim & Dang, 2005; Ling *et al.*, 2004; McCarthy *et al.*, 2002). One important prerequisite for a vaccine candidate is that a large proportion of target strains should possess and express the relevant antigen. To assess the presence of the *cbbA* gene and FBA expression in the *N. meningitidis* population, 26 clinical isolates of *N. meningitidis*, representative of the three main disease-associated serogroups (A, B, C,), were screened by PCR and/or immunoblot analysis. The analysis also included one strain each *of N. gonorrhoeae, N. polysacchareae* and *N. lactamica.* All of the isolates examined were positive by PCR for the presence of *cbbA.* The PCR resulted in amplification of a single DNA fragment of *ca.* 1-kb in all assayed strains (Fig. 4.2 A and B). Having established that *cbbA* is present in all strains tested, we investigated expression of its gene product FBA. Total cellular proteins were immunoblotted using the anti-FBA rabbit serum. A protein band with an apparent molecular mass of *ca.* 38-kDa corresponding to FBA was detected in all strains tested (Fig 4.3 A, B, C). In addition, *N. lactamica, N. gonorrhoeae,* and *N. polysacchareae* were also positive for FBA expression (data not shown).



(A)



(B)

Figure 4. 2 Distribution of the *cbbA* gene and prevalence of *cbbA* in different clinical isolates of *N. meningitidis* of known-MLST type (A) Lane 1, DNA markers, lane 2, MC58-WT, lanes 3-11 Z1035, Z1054, Z1213, Z1503, Z4676, Z1534, Z3771, Z3842, Z4662 respectively (B) Lane 1, DNA markers, lanes 2-4,- Z4678, Z6413, Z6419, Lane 5, MC58Δ*cbbA*, lanes 6-11, Z6420, *N. gonorrhoeae*, Z4181, Z4667, Z4673, Clone 12 (ST-11), respectively. PCR-amplified products comprising the complete *cbbA* (1065 bp) of 17 meningococcal strains with known MLST-type were observed in all strains examined.

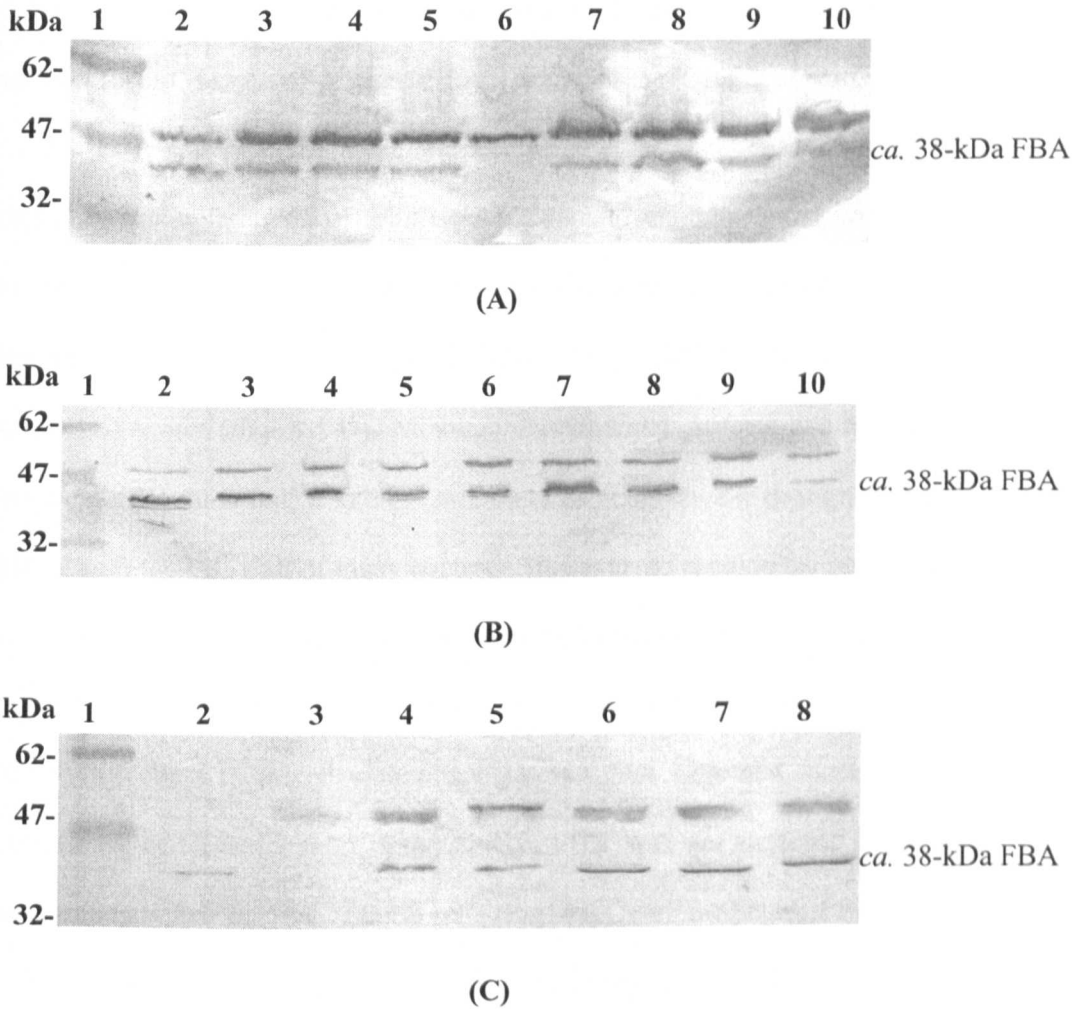


Figure 4. 3 FBA expression in divergent clinical isolates of *N. meningitidis.* Whole cell proteins were resolved by 10% SDS-PAGE followed by immunoblot analysis using RαFBA. The FBA is present in whole-cell lysates of all strains examined (A) Lane 1, protein markers, lanes 2-5, Z1035, Z1054, Z1213, Z1269 respectively, lane 6, MC58Δ*cbbA*, lanes 7-10, Z1503, Z3771, Z3842, Z4181, respectively (B) Lane 1, protein markers, lane 2, MC58-WT, lanes 3-10, Z4662, Z4667, Z4673, Z4676, Z4678, Z5826, Z6413, Z6414, respectively (C) Lane 1, protein markers, lane 2, MC58-WT, Lane 3, MC58Δ*cbbA*, Lanes 4-8, Z4684, Z4685, Z4701, Z6417, Z4323, respectively. A band corresponding to FBA was observed in all strains examined except the *cbbA* null mutant.

4.3.3 Sub-cellular location of *cbbA* in *N. meningitidis*

To localise *cbbA* in the meningococcus, and to determine whether FBA may be exported to the surface of *N. meningitidis*, sub-cellular fractions were probed with RαFBA. FBA was predominately detected in the outer membrane-enriched and the cytosolic protein fractions of wild-type MC58 and complemented *cbbA* isogenic mutant strain. (Fig. 4.4 A and B), but was absent from the concentrated culture supernatant (secreted protein fraction). FBA was not detected in fractions derived from MC58Δ*cbbA* (Fig. 4.4 D). Although experimental artifacts due to partial cell lysis cannot be ruled out, it is clear that these proteins have a strong propensity to interact with the cell wall of many bacteria. However, to exclude the possibility that the presence of FBA in outer membrane-enriched fraction was due to autolysis with the attachment of released FBA to the surface of unbroken cells, whole cell lysate of MC58-WT cells was mixed with intact MC58Δ*cbbA* cells and incubated at RT followed by cell fractionation. Interestingly, FBA was not detected in the outer membrane-enriched fraction of these cells (Fig. 4.4 C) confirming that the FBA does not bind to the cell surface as a consequence of autolysis/cell leakage. Additionally, immunoblotting experiments with antiserum against PorA, a known outer membrane protein of *N. meningitidis*, gave an identical profile except that PorA was absent from the cytosolic fraction (data not shown). These results confirm that meningococcal FBA is a cytosolic protein, but is also translocated to the outer membrane.

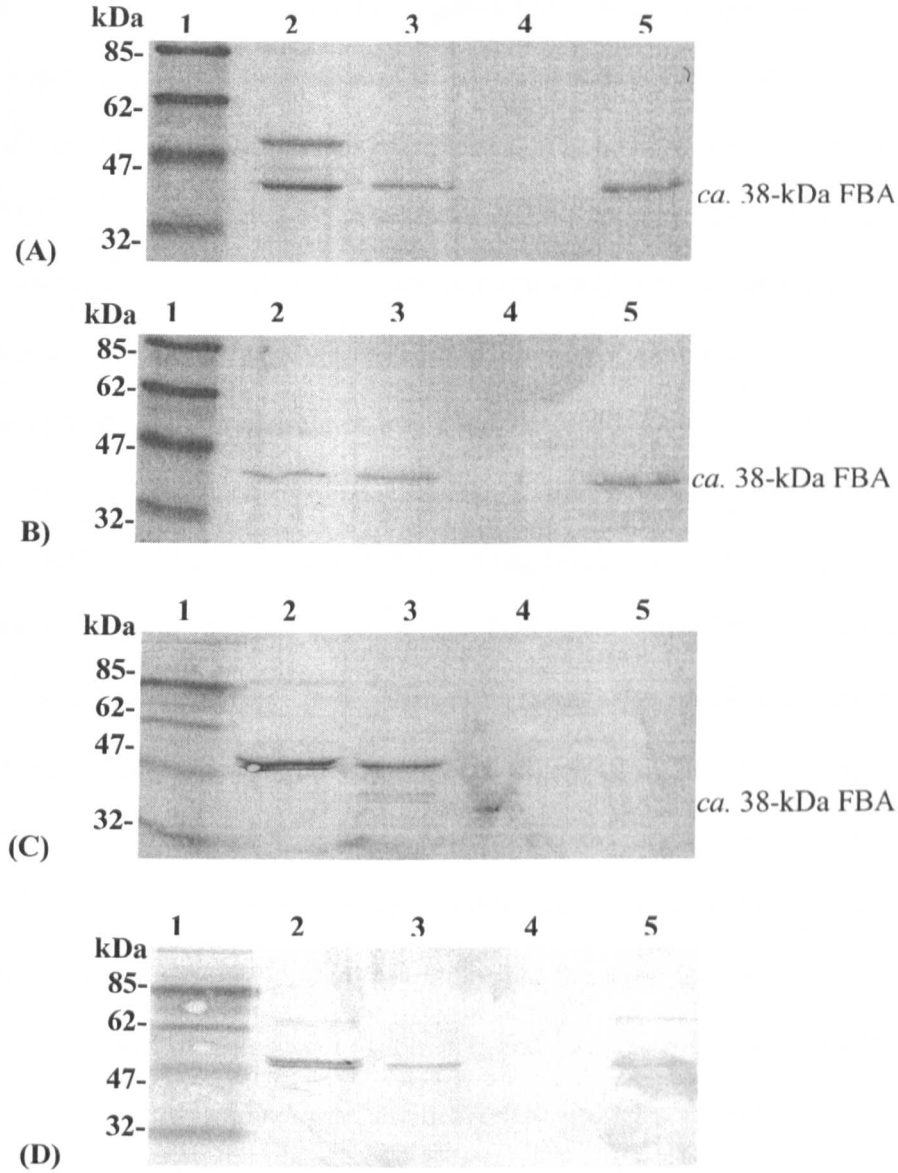
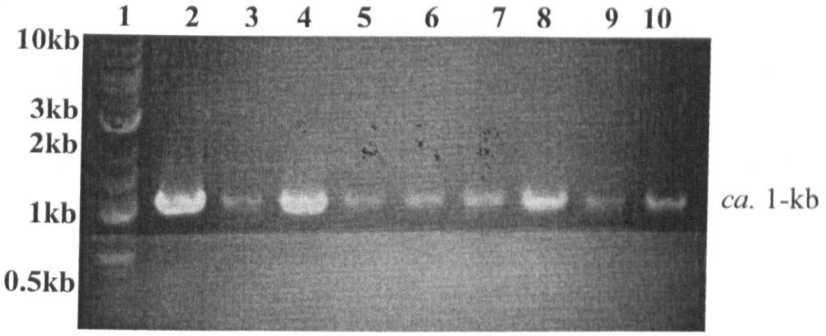


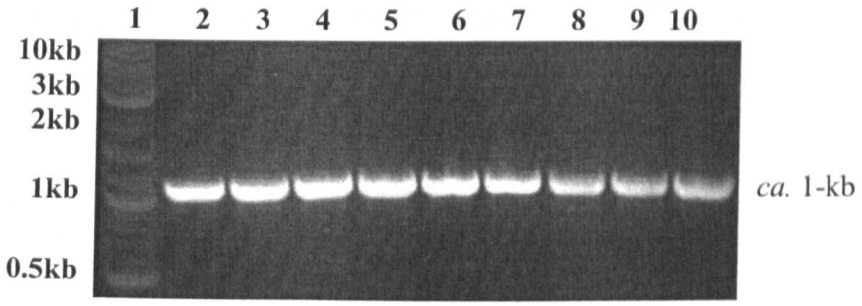
Figure 4. 4 Subcellular localization of FBA (A) MC-WT (B) MC58Δ*cbbA* Δ*cbbA*^{Ect} (C) MC58Δ*cbbA* mixed with MC58-WT sonicated lysate (D) MC58Δ*cbbA* cell fractionation. In all Figs, lane 1, protein markers, lanes 2, periplasmic proteins, lane 3, cytoplasmic protein-enriched fractions, lane 4, cytoplasmic membrane protein-enriched fractions, and lane 5, outer membrane protein-enriched fractions (adjusted to ensure equal protein loading). All samples were separated on a 10% acrylamide gel and probed in immunoblotting experiments with RαFBA. A band corresponding to FBA was observed in outer membrane enriched fractions of WT-MC58 and the complemented strain and was absent from the isogenic null mutant.

4.3.4 Distribution and expression of *gapA-1* in *N. meningitidis* strains

The *gapA-1* gene has been shown to be up-regulated following meningococcal interaction with host cells demonstrating that it may be involved in a critical step of pathogenesis. We examined the presence of *gapA-1* and its expression in population of *N. meningitidis*. A total of 17 strains of known MLST-type representing most of the known virulent lineages were screened by PCR for the presence of a *gapA-1* homologue. All neisserial isolates tested by this PCR screening method yielded a single amplified product of the expected size *ca.* 1-kb corresponding to *gapA-1* (Fig. 4.5 A and B). Additionally, after demonstrating that *gapA*-1 was present across all the meningococcal strains screened by PCR, we extended this analysis to demonstrate that *gapA-1* is expressed *in vitro* in the same panel of strains. Whole-cell lysates of the 17 neisserial isolates were examined by immunoblot analysis to detect the presence of a GAPDH-1 protein using RαGAPDH-1 antiserum. All neisserial isolates expressed an immunoreactive GAPDH-1 protein, with the exception of MC58Δ*gapA-1*, demonstrating that the gene is expressed in diverse clinical isolates of meningococci (Table 4.1, and Fig.4.6 A, B and C). In addition, single isolates of *N. gonorrhoeae* strain FA1090 was also examined. The protein band corresponding to the GAPDH-1 was detected in this isolate (Fig. 4.6 C).



(A)



(B)

Figure 4. 5 Distribution of the *gapA-1* gene in diverse clinical isolates of *N. meningitidis* (A) Lane 1, DNA markers, lane 2, MC58-WT, lanes 3-10, Z1035, Z1054, Z1213, Z1503,Z4676, Z1534, Z3771, Z3842, respectively. (B) Lane 1, DNA markers, lane 2-10, Z4678, Z6413, Z6414, Z6420, *N. gonorrhoeae* FA1090, Z4181, Z4667, Z4673, Clone 12 (ST-11), respectively. PCR-amplified products of the expected size of *gapA-1* (1032 bp) were observed in all 17 meningococcal strains examined.

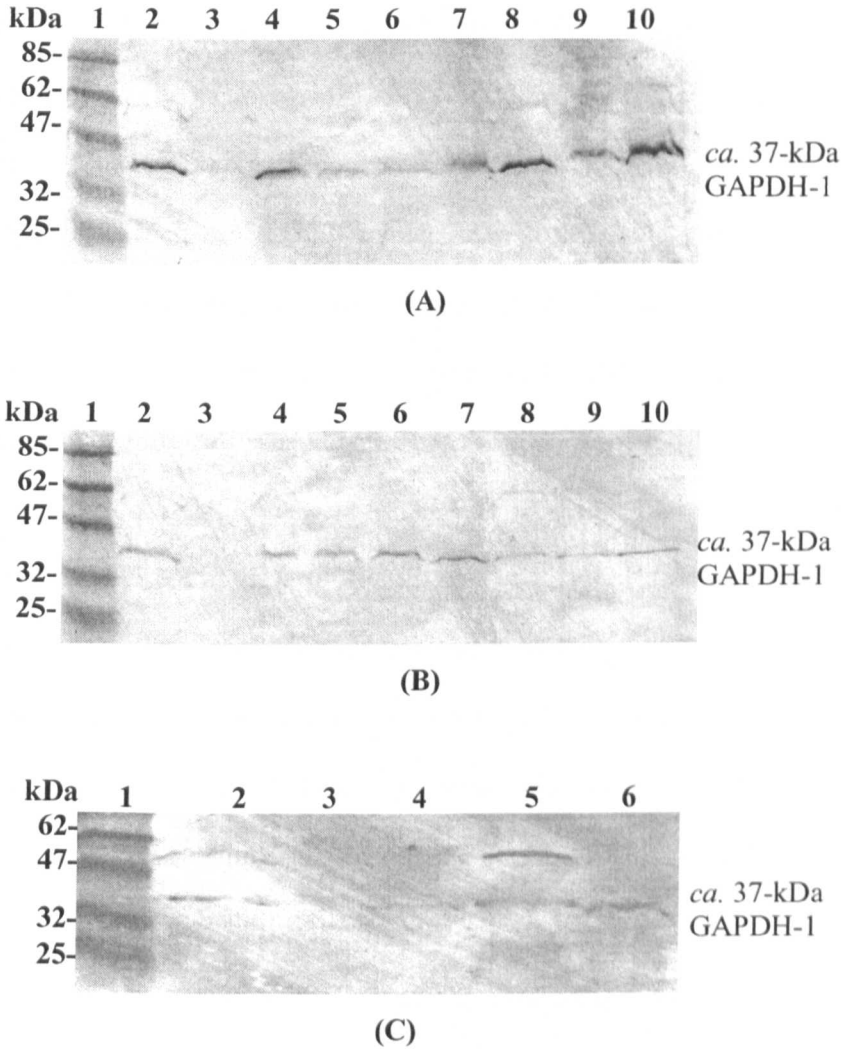


Figure 4.6. The *gapA-1* gene is expressed across divergent clinical isolates of *N. meningitidis*. Whole cell extracts were separated by 10% SDS-PAGE followed by immunoblot analysis using RαGAPDH-1. The GAPDH-1 is detected in whole-cell lysates of all strains tested. (A) Lane 1, protein markers, lane 2, MC58-WT , lane 3, MC58Δ*gapA-1,* lanes 4-10, Z1035, Z1054, Z1213, Z1503, Z4676, Z1534, Z3771, respectively (B) Lane 1, protein markers, lane 2, MC58-WT , lane 3, MC58Δ*gapA-1,* lanes 4-10 Z4678, Z6413, Z6414, Z6420, *N. gonorrhoeae strain* FA1090, Z4181, Z4667, respectively (C) Lane 1, protein markers, lane 2, MC58-WT, lane 3, MC58Δ*gapA-1,* lanes 4-6, Z4673, Z3842, Clone 12 (ST-11), respectively. A protein band corresponding to GAPDH-1 was observed in all strains examined except MC58Δ*gapA-1* mutant strain.

4.3.5 Sub-cellular localization of *gapA-1* in *N. meningitidis*

Since the GAPDH has been described to be present on the surface of cells of several microbial species (Fernandes *et al.*, 1992; Goudot-Crozel *et al.*, 1989; Pancholi & Fischetti, 1992) and recently three proteins from *N. meningitidis* (enolase, DnaK, and peroxiredoxin), which are usually intracellular proteins, have been shown to be located in the outer membrane of *N. meningitidis* (Knaust *et al.*, 2007) , we undertook to investigate the presence of a cell wall-associated form of GAPDH in outer membrane-enriched fractions obtained from *N. meningitidis*. The sub-cellular fractions of wild-type MC58 were probed using RαGAPDH-1. GAPDH-1 was predominately detected in the cytosolic fraction and a proportion was also present in the outer membrane-enriched fraction, but was absent from the concentrated culture supernatant (secreted protein fraction) (Fig. 4.7). A trace amount, possibly representing transient GAPDH-1 during translocation across the inner membrane, can also be seen in the cytoplasmic-membrane enriched fraction. GAPDH-1 was not detected in fractions derived from MC58Δ*gapA-1* (data not shown). These results confirm that meningococcal GAPDH-1 is a cytosolic protein, but is also found in the outer membrane.

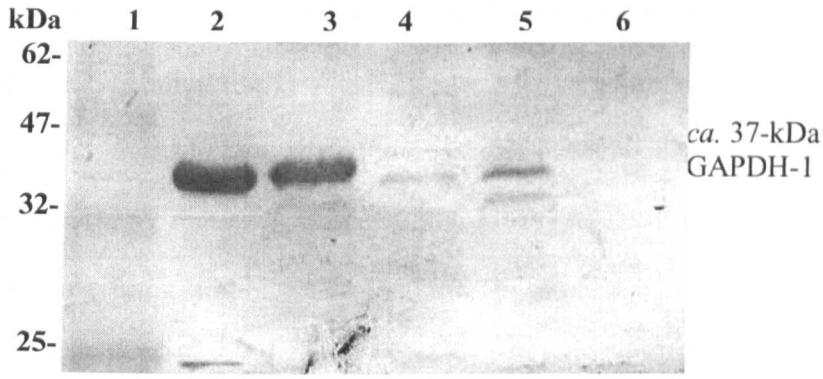


Figure 4. 7 Sub-cellular localization of GAPDH-1 in *N. meningitidis* MC58-WT cell fractionation Lane 1, protein markers, lanes 2-6, periplasmic protein-enriched fraction, cytoplasmic membrane protein-enriched fractions, cytoplasmic membrane protein-enriched fractions, outer membrane protein-enriched fractions, and secreted proteins (and adjusted to ensure equal protein loading) respectively. Proteins were separated on a 10% acrylamide gel and probed in immunoblotting experiments with RαGAPDH-1. A protein band corresponding to GAPDH-1 was observed in outer membrane-enriched fractions.

4.3.6 Determining the surface exposure of FBA and GAPDH-1

4.3.6.1 Biotinylation of surface-exposed proteins

As a complementary approach to investigate the surface exposure of FBA and GAPDH-1 in *N. meningitidis*, a novel biotinylation method was employed. This method has been shown to discriminate truly surface-accessible proteins from cytoplasmic and periplasmically oriented proteins (Myers-Morales *et al.*, 2007). This experiment was aimed to biotinylate the surface of intact *N. meningitidis*-WT, *cbbA* and *gapA-1* null mutant strains followed by SDS-PAGE analysis and immunoblotting using anti-biotin antibodies. The protein bands corresponding to FBA and GAPDH-1 were anticipated to be present only in MC58-WT samples but should be absent in the respective isogenic null mutants. As can be seen in Fig 4.8 due to either high background or a large amount of biotin-labeled proteins, no clear difference in the profiles of the three samples was observed on immunoblotting.

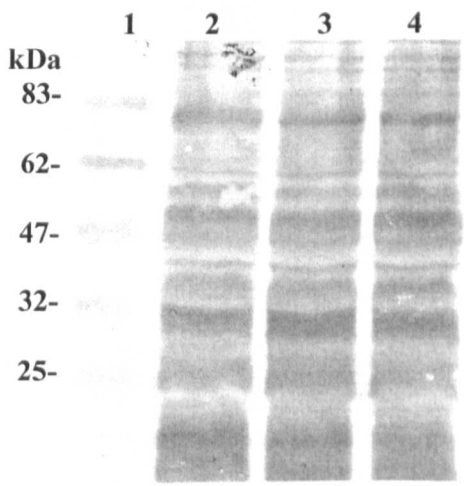


Figure 4. 8 Immunoblot analysis to investigate the surface-exposed biontinylated proteins of *N. meningitidis* Lane 1, protein markers, lane 2, MC58-WT, lane 3, MC58Δ*cbbA*, lane 4, MC58Δ*gapA-1*

4.3.6.2 Whole cell ELISA

To investigate the presence of FBA on the surface of *N. meningitidis,* an FBA ELISA was performed using whole cells of *N. meningitidis* followed by probing with different concentrations of anti-FBA antibodies. As the target antigen, the ELISA included encapsulated *N. meningitidis* cells from strain MC58 or the same strain in which the *cbbA* gene had been deleted (MC58Δ*cbbA*). With anti-FBA antiserum, strain MC58 showed significantly higher binding (*P* value 0.015) in the whole-cell ELISA compared to that of its isogenic *cbbA* mutant strain (Fig. 4.9). The ELISA demonstrated that the FBA antiserum recognized antigen(s) on the meningococcal cell surface, indicating that the protein was present and in a conformation that could be recognized by the serum raised against the recombinant protein.

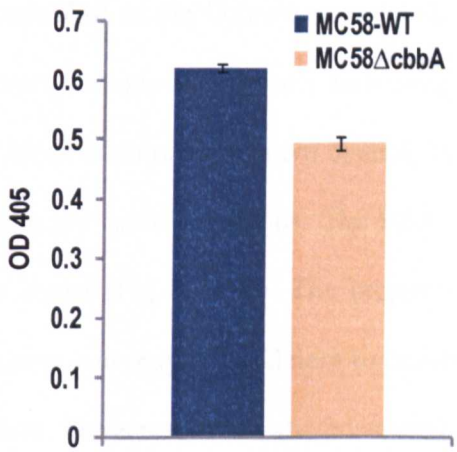
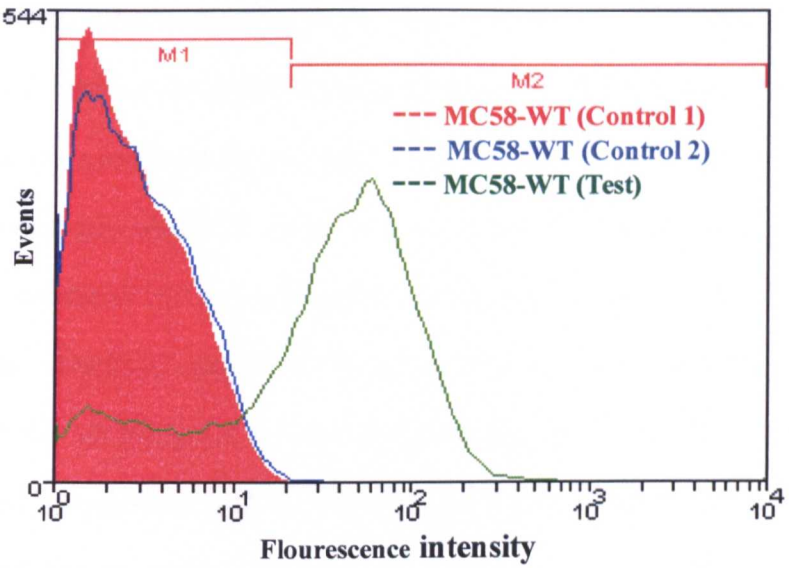


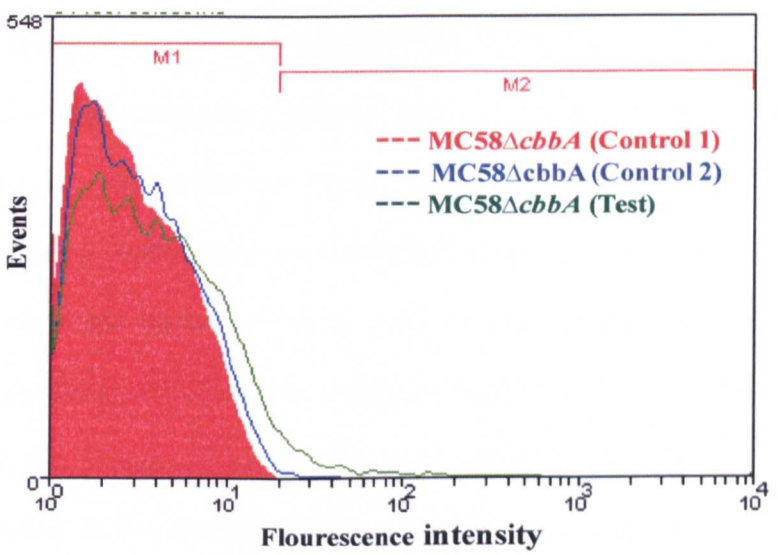
Figure 4. 9 Whole bacterial cell ELISA to determine the binding of anti-FBA antibodies to intact *N. meningitidis* cells. ELISA plates were coated with various dilutions of rabbit anti- FBA antibodies. The plate contents were reacted with labeled intact *N. meningitidis* cells followed by anti-digoxygenin antibodies. ABTS substrate was added and absorbance was measured at 405 nm in a microplate ELISA reader (BioTek). Bars denote standard error of the mean. Experiments were repeated three times, with consistent results.

4.3.6.3 Flow cytometry analysis

In order to provide confirmatory evidence for the localization of FBA on the bacterial cell surface, and to substantiate the sub-cellular fractionation results showing the outer membrane localisation of FBA, the polyclonal mono-specific antiserum raised against the natively purified rFBA (nRαFBA) was used to probe intact *N. meningitidis* strain MC58 cells followed by treatment with secondary antibody conjugated with Alexa Flour 488. The stained cells were visualized by flow cytometry. Control cell samples that were either treated with nRαFBA polyclonal antiserum alone or with Alexa Flour conjugated secondary antibody alone did not produce significant fluorescence signals. In comparison, the cells stained with both nRαFBA antibody followed by Alexa Flour 488 conjugated secondary antibody, demonstrated a significant shift in the fluorescence signals (approximately 79% of cells in the population were strongly fluorescent), indicating cell surface localization of FBA (Fig. 4.10 A). As an additional negative control, MC58Δ*cbbA* mutant cells were also examined using the same conditions. The FBA mutant cells showed no significant fluorescence signal (Fig. 4.10 B). The proportion of surface expressed FBA seems to be promising and may be sufficient to endow a biologically distinct phenotype to the bacterium. These results are also consistent with data obtained from the association assays, which showed that FBA is required for optimal *in-vitro* interactions with cultured epithelial and meningothelial cells.



(A)



(B)

Figure 4. 10 Flow cytometry analysis of MC58 wild-type (A) or MC58Δ*cbbA* cells (B) for FBA surface localization. Cells were stained with nRαFBA alone, (control 1) anti-rabbit IgG-Alexa Flour 488 conjugate alone (control 2) or both (test). Flourescence was displayed as a histogram. The histogram area in M2 represents the population of fluorescently labeled meningococci.

To investigate the surface exposure of GAPDH-1, flow cytometry analysis was performed on live bacteria during the logarithmic phase of growth, using GAPDH-1 antiserum. The encapsulated wild-type MC58 and isogenic *gapA-1* mutant strains were stained with anti-GAPDH-1 followed by Alexa Flour 488. The fluorescence intensity was measured by flow cytometry. As shown in Fig. 4.11 A and B, polyclonal anti- GAPDH-1 antibodies failed to bind to either the encapsulated MC58-WT or the MC58Δ*gapA-1* strain demonstrating that GAPDH-1 was not surface exposed in capsulated strains. There can be several reasons for failure of GAPDH-1 to bind to the surface of encapsulated meningococci. RαGAPDH-1 was raised against the denatured purified protein and linear proteins may lack important conformational epitopes present in the naturally folded protein, or the protein may reach to the surface transiently and at specific phases of growth. Additionally, it is also known that the polysaccharide capsule can functionally mask membrane proteins. We, therefore, used a capsule mutant MC58 strain and followed the binding of the antibodies to the surface of intact cells by flow cytometry. The data demonstrated a significant shift in the fluorescence signal (Fig. 4.12) (approximately 25% of cells in the population were fluorescent) indicating that GAPDH-1 was available for binding in the absence of polysaccharide capsule suggesting that the presence of the capsule may hinder the binding of the antibody. In summary, GAPDH-1 is an outer membrane localized protein which is only surface exposed in the non-capsulated strains. In contrast, in a capsulated background the GAPDH-1 could be masked by the capsule.

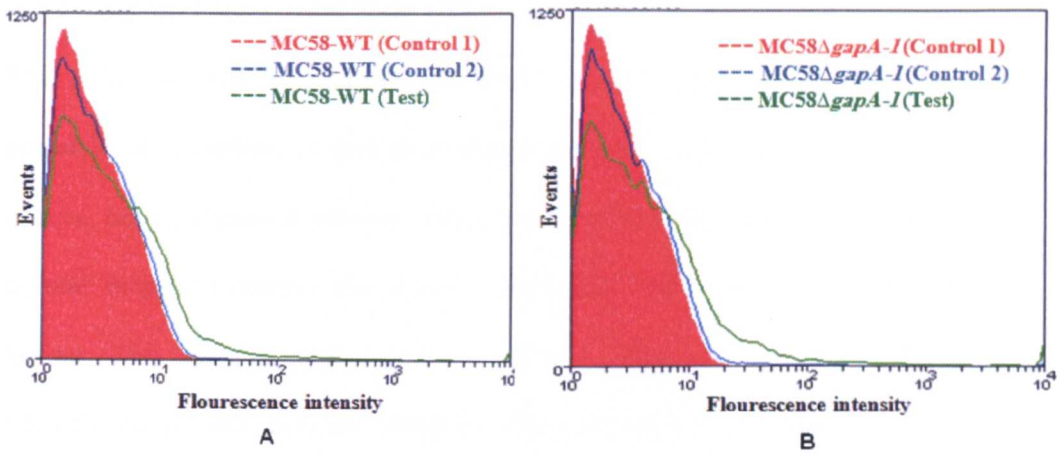


Figure 4. 11 Flow cytometry analysis of MC58 wild-type (A) or MC58Δ*gapA-1* cells (B) for GAPDH-1 surface localization. Cells were stained with RαGAPDH-1 alone, (control 1) anti-rabbit IgG-Alexa Flour 488 conjugate alone (control 2) or both (test). Flourescence was displayed as a histogram.

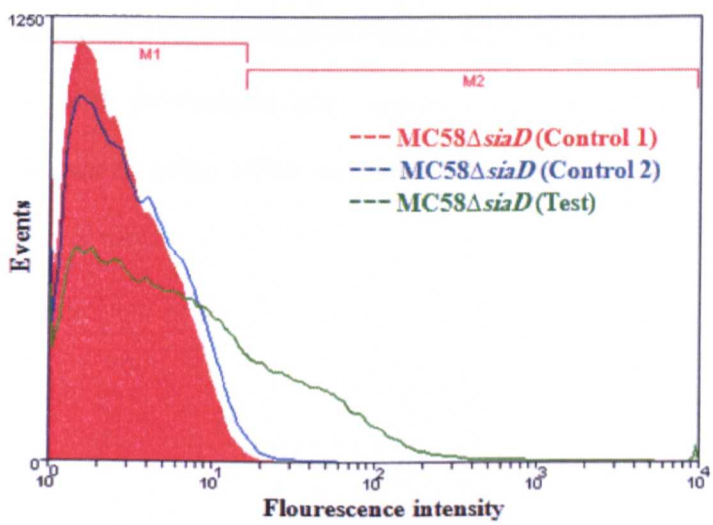


Figure 4. 12 Flow cytometry analysis showing binding of mouse polyclonal anti-rGAPDH-1 antiserum to live nonencapsulated *N. meningitidis* strains Cells were stained with RαGAPDH-1 alone, (control 1) anti-rabbit IgG-Alexa Flour 488 conjugate alone (control 2) or both (test). Flourescence was displayed as a histogram. The histogram area in M2 represents the population of fluorescently labeled meningococci.

4.3.7 Serum bactericidal assay (SBA)

The ability of RαFBA and RαGAPDH-1 antisera to kill meningococcal cells in presence of complement was determined. Prior to using the sera in bactericidal assays, both polyclonal antisera were pre-adsorbed with their respective isogenic mutant strains to remove non-specific antibodies that might result in non-specific killing. The antisera were used at various dilutions: 1:10, 1:50, and 1:100. Bactericidal activity was not observed with antisera raised against either FBA or GAPDH-1 (data not shown). Positive control assays using anti-whole meningococcal serum consistently gave 100% killing. No killing was seen in control wells lacking active complement, serum, or both. To exclude the possibility that capsule may limit the accessibility of the proteins on the surface, a *siaD* (encoding capsule) mutant *N. meningitidis* strain MC58 was used in the assay. Unfortunately, this strain seemed to be sensitive to both pre-immune and immune sera, thus, it was not possible to interpret the impact of either of the antisera on the killing of *N. meningitidis siaD* mutant strain.

4.3.8 Evaluation of *cbbA* mutant strains using a transgenic mouse model of infection

A transgenic mouse model expressing human transferrin (hTf) bred under specific-pathogen-free conditions was established from line 803 C57B6/SJLJ mice at the Institut Pasteur (Zarantonelli *et al.*, 2007) to use as a model of meningococcal bacteraemia. To provide further insights into the potential role of FBA in the development of bacteraemia, six-week-old female B6/SJL mice expressing (hTf) were infected by intraperitoneal injection of *N. meningitidis*-WT, Z4181-WT, Clone 12-WT (ST-18), 24198-WT (ST-11) (Zarantonelli *et al.*, 2007), and their isogenic *cbbA*-mutant strains at a dose (5×10^6 CFU) in groups of five mice for each wild-type, and compared to a group of five mice that were administered *cbbA* mutant strain. Mice were able to survive with the 5×10^6 CFU dose, and bacteraemia could therefore be assessed by blood cultures at 2, 6, and 24 h post inoculation. All meningococcal wild-type isolates examined were shown to induce bacteraemia in the transgenic mouse model whereas the isogenic mutant strains showed a reduced capacity to establish bacteraemia. The number of bacteria recovered at different time points is shown in (Fig. 4.13 and Table 4.2) and demonstrate that there were significant differences in the level of bacteraemia established by the mutant strains compared to the wild-type.

Table 4.2 Meningococcal strains used for mouse model of meningococcal bacteraemia. The data denote bacteria per milliliter of blood using means of 5 mice per time point and per strain.

Strains	t=0	2h	6h	24h
MC58-wt	3.45×10^7	2.82×10^6	2.54×10^6	2.60×10^6
MC58-ΔFBA	1.25×10^7	1.46×10^5	1.05×10^5	1.78×10^2
Z4181-wt	1.10×10^7	2.38×10^6	3.50×10^6	2.51×10^6
Z4181-ΔFBA	2.40×10^6	1.20×10^4	2.72×10^3	1.00×10^1
Clone 12 –wt (ST-18)	5.50×10^6	2.45×10^6	1.31×10^6	2.21×10^5
Clone12-ΔFBA	2.60×10^6	2.10×10^4	1.60×10^4	4.20×10^1
24198wt (ST-11)	5.75×10^6	2.16×10^6	4.03×10^6	3.50×10^5
24198ΔFBA	1.90×10^6	3.60×10^6	2.90×10^4	1.00×10^1

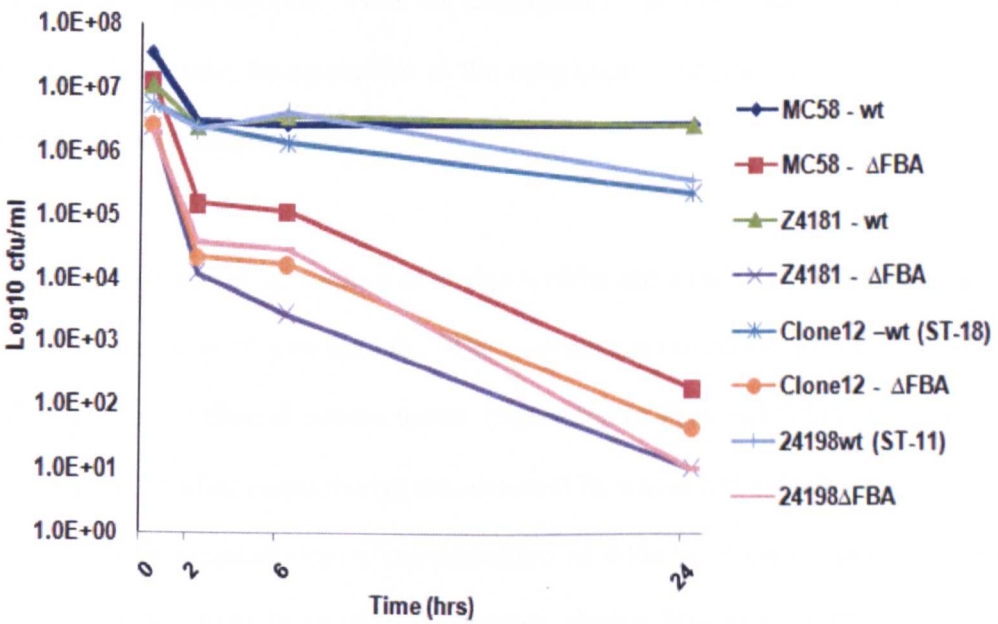


Figure 4. 13 Bacteraemia in hTf transgenic mice after intraperitoneal meningococcal challenge. Transgenic mice expressing hTf (B6/SJL hTf) were challenged intraperitoneally with 5×10^6 CFU of *N. meningitidis*-WT, Z4181-WT, Clone 12-WT (ST-18), 24198-WT (ST-11) and their isogenic *cbbA*-mutant strains. The data are the means and standard deviations from five independent experiments with groups of five mice per time point in each experiment.

4.4 Discussion

This chapter describes the molecular and functional characterisation of FBA and GAPDH-1 of *N. meningitidis*, with the aim of identifying their additional non-glycolytic roles. Recent qualitative proteomic studies of *N. meningitidis* have shown that OMV vaccines also contain a high number of periplasmic and cytoplasmic proteins (Ferrari *et al.*, 2006; Jun *et al.*, 2007; Uli *et al.*, 2006; Vipond *et al.*, 2006). For example in OMVs from serogroup B *N. meningitidis*Δgna33, although the vast majority of proteins belonged to the outer membrane compartment, the minority of cytoplasmic proteins were experimentally shown to be surface-associated and exposed in *N. meningitidis*. Here we demonstrate that FBA and GAPDH-1 of *N. meningitidis*, besides being present in the cytoplasmic compartment, are also outer membrane localised proteins.

According to our *in silico* sequences analysis, *cbbA* and *gapA-1* appears to be highly conserved genes in *N. meningitidis*. PCR analysis confirmed the presence of *cbbA* and *gapA-1* in all clinical isolates tested. Expression of FBA and GAPDH-1 (*ca.* 38-kDa and *ca.* 37-kDa, respectively) was detected in whole cell proteins preparations from all meningococcal strains, representative of different hyper-virulent lineages, examined in this study. In an effort to establish whether FBA and GAPDH-1 localise on the surface of meningococci, a cell fractionation approach was employed (Nossal & Heppel, 1966). The fractionation results demonstrated a protein band of apparent molecular mass of *ca.* 38-kDa corresponding to FBA in the cytosolic and outer membrane fractions. This indicates that FBA is localised to the outer membrane as

well as the cytoplasm of *N. meningitidis*. Similarly, GAPDH-1 was also found localised to the outer membrane of *N. meningitidis*. In spite of a growing list of cytoplasmic proteins identified on the bacterial surface, the mechanism(s) of their surface localization and attachment to the bacterial envelope remain unclear. These proteins do not appear to possess signal peptides that direct proteins into secretory pathways, and nor do they appear to have any known cell wall-anchoring motifs. Further experiments are needed to elucidate the mechanism(s) of delivery and attachment of these proteins to the bacterial surface.

Based on the sub-cellular fractionation results indicating the outer membrane localisation of FBA and GAPDH, surface expression of FBA and GAPDH-1 proteins was further investigated by using a combination of immunological and molecular techniques. Initially, a biotin labelling technique was applied to intact bacterial cells (Myers-Morales *et al.*, 2007). The data from immunoblot analysis profile showed a large number of biotinylated proteins which masked the protein bands corresponding to FBA or GAPDH-1. Subsequently, a whole-cell ELISA was used to determine the accessibility of polyclonal antiserum to bind to the surface of encapsulated meningococci. The data provided evidence that the binding of polyclonal anti-FBA serum to the intact MC58 wild-type cells was significantly higher compared to the isogenic null mutant indicating the surface expression of FBA in *N. meningitidis*. To further support these findings, flow cytometry analysis was performed. The data presented here demonstrates that FBA protein was abundantly associated with the external side of the bacterial membrane and accessible to antibodies as indicated by

the fluorescence intensity shift observed upon antibody binding. FBA was available at the cell surface with levels of fluorescence comparable to those found with recognised surface antigens such as NMB1468 (Chi-An *et al.*, 2008), NMB2132 and NMB2091 (Giuliani *et al.*, 2006), although considerably lower than other surface antigen such as fHbp (Giuliani *et al.*, 2006). Despite being found localised to the outer membrane by the cell fractionation method, the results of flow cytometry indicated that the GAPDH-1 was not accessible to antibodies on the surface of encapsulated wild-type cells. A possible explanation of the failure of GAPDH-1 antiserum to bind to the bacterial surface could be that surface exposure of this protein may be transient or only occurring during specific phase of the meningococcal cell cycle, or it may indeed be located on the outer surface of the membrane, but inaccessible from the antibodies due to the polysaccharide capsule. In a previous study, Grifantini *et al.* used microarrays to show that expression of *gapA-1* was up regulated in meningococcal strain MC58 (4.8-fold) following contact with human 16HBE14 epithelial cells (Grifantini *et al.*, 2002a; Grifantini *et al.*, 2002b). Subsequent flow cytometry experiments showed that GAPDH-1 could be detected on the cell surface of free grown and adherent meningococci (Grifantini *et al.*, 2002b). However, the methodology used involved a pre-treatment of cells with 70% ethanol to permeabilize the capsule layer, thus making it unclear if GAPDH-1 is antibody-accessible in encapsulated meningococci. To address this issue, a *siaD* (encoding capsule) mutant meningococcal strain was probed with GAPDH-1 antiserum followed by flow cytometry analysis. The data showed a significant shift in the intensity of fluorescence indicating that GAPDH-1 could only be detected on

the meningococcal cell surface in mutants lacking capsule, suggesting that GAPDH-1 is usually masked.

The induction of functional antibodies after immunization with recombinant proteins has been a tremendous challenge during the evaluation and development of meningococcal vaccine candidates. Anti-FBA and anti-GAPDH-1 were tested for their ability to promote *in vitro* complement-mediated killing of *N. meningitidis* MC58. Unfortunately, neither of the antibodies was found to exhibit bactericidal activity against *N. meningitidis*. The reason for lack of bactericidal activity could be due to the fact that the FBA and GAPDH-1 antisera used in the serum bactericidal assay were raised against denatured purified recombinant proteins. Several studies with recombinant meningococcal antigens, including PorA (Christodoulides *et al.*, 1998; Niebla *et al.*, 2001) and PorB (Wright *et al.*, 2002), have clearly demonstrated that the production of bactericidal antibodies is dependent on refolding of the protein to produce a native conformation. Hence, these antibodies were unlikely to recognise surface-exposed, conformational epitopes, which may be important *in vivo*. Antibodies raised against histidine-tagged NspA were shown to recognise epitopes that are present in denatured NspA but not in native NspA on the surface of live meningococcal cells (Moe *et al.*, 1999). In future work, the bactericidal activity of anti-FBA serum raised against natively purified rFBA will be determined.

This study demonstrates that both FBA and GAPDH-1, well-known cytoplasmic proteins, are present on the surface of neisserial cells and so may be involved in the

normal *in vivo* immune response to live meningococcal cells. The presence of neisserial "cytoplasmic" proteins on the surface has been reported previously. For example, *Ferrari et al.* (Ferrari *et al.*, 2006) found enolase, Hsp60, TufA and glyceraldehyde 3-phosphate dehydrogenase (gapA-2 not gapA-1) on the neisserial surface by flow cytometry while other researchers have identified RplL (Spence & Clark, 2000; Spence *et al.*, 2002), and DnaK, (Knaust *et al.*, 2007) as being surface exposed.

In addition, FBA was shown to be essential for bacteraemia in a novel transgenic ('humanised') mouse model of infection. All five isogenic *cbbA* mutants created in five different genetic backgrounds of *N. meningitidis* were impaired in their ability to sustain bacteraemia compared to their wild-type parent strains indicating that FBA may play a potential role in the pathogenesis of meningococcal disease. This result suggests that the mutants were less able to access the bloodstream and/or to survive in the bloodstream after intraperitonial inoculation compared to wild-type. In future work, the antiserum raised against purified FBA in native form (retaining aldolase activity) will be used in a passive protection experiment using the transgenic mouse model to determine whether anti-FBA antibodies elicit a protective response against *N. meningitidis*.

In summary, purified recombinant FBA protein was found to be ezymatically active. The data represented in this chapter indicates that FBA and GAPDH-1 are both highly conserved in pathogenic *N. meningitidis* strains and that FBA and GAPDH-1

are constitutively expressed in all *N. meningitidis* strains examined. Besides their cytosolic location they are also localised to the outer membrane of *N. meningitidis*. Additionally, FBA was shown to be available to antibodies on the surface of intact capsulated *N.* meningitidis cells. This is the first time that FBA has been demonstrated on the surface of a Gram-negative bacterium. However, GAPDH-1 was only accessible to antibodies on the surface of non-capsulated meningococci. Furthermore, *in vivo* studies demonstrated the potential role of FBA in the pathogenesis of the meningococcal disease.

CHAPTER 5: Determining the roles of FBA and GAPDH-1 in meningococcal association to human cells

5.1 Introduction

In this chapter the potential roles of FBA and GAPDH-1 in meningococcal adhesion to, and invasion of, cultured human epithelial and endothelial cells are discussed. Adhesion of pathogens to host tissue has gained increasing attention as an important initial event in the pathogenesis of bacterial infections before colonization and eventual invasion of these surfaces (Beachey, 1981). The meningococcus is a common commensal of the human nasopharynx, which in a small percentage of carriers, crosses the epithelial barrier and gains access to the bloodstream. Colonization of the nasopharynx is a complex and incompletely understood process, which involves long-range attachment to host epithelial cells via type IV pili, remodeling of the meningococcal outer membrane, and interactions between several additional bacterial adhesins including Opa, Opc, LOS, fHbp, PorA, HrpA, PorB and NadA and proposed or demonstrated host cell receptors including platelet activating factor, CD46, CEACAM1, vitronectin and α–actinin integrins, complement receptor 3, laminin and the GP96 scavenger receptor (reviewed in Stephens, 2009). Following bacteraemia, *N. meningitidis* may bind to and subsequently cross the blood-cerebrospinal fluid (B-CSF) barrier to enter the sub-arachnoid space, resulting in acute and purulent meningitis (Nassif *et al.*, 2002).

Glycolytic enzymes, such as GAPDH, enolase and FBA have been reported as potential virulence factors in a variety of organisms (Pancholi & Chhatwal, 2003). Although long recognized for their cytosolic role in glycolysis and gluconeogenesis, additional or 'moon-lighting' functions have been increasingly recognized. In

particular, despite lacking identifiable secretion signals, glycolytic enzymes have been found on the bacterial cell surface where they interact directly with host soluble proteins and surface ligands. In *Mycoplasma genitalium*, surface-associated GAPDH was shown to be important for adhesion to human mucin (Alvarez *et al.*, 2003). In *S. pyogenes* and *C. albicans* surface-associated GAPDH was shown to bind to fibronectin (Gozalbo *et al.*, 1998; Pancholi & Fischetti, 1992) In *S. pneumoniae*, surface-associated FBA was shown to bind to transmembrane receptor belonging to the cadherin superfamily (Blau *et al.*, 2007). FBA and GAPDH were also shown to be immunogenic in humans and capable of inducing a protective immune response against *S. pneumoniae* in mice (Ling *et al.*, 2004). In addition, FBA was found to be a surface-localized immunogenic protein in *S. suis* (Zongfu *et al.*, 2008) and a potential role for FBA in immunity to nematode parasite *Onchocerca volvulus* has also been described (McCarthy *et al.*, 2002).

The aim of this chapter was to explore whether, like these other examples, meningococcal FBA and GAPDH-1 are involved in adhesion to, and invasion of, host cells.

5.2 Materials and methods

5.2.1 Bacterial strains, growth conditions and media

Bacterial strains described in this chapter are listed in (Table 3.1 and 3.3). Growth conditions and media for culturing meningococci and *E. coli* are described in Section 2.2.1. Mutant and complemented strains were created as described in Chapter 3. Antibiotics were used at the following concentration: kanamycin (50 μg ml^{-1}) or erythromycin (5 μg ml^{-1}).

5.2.2 Preparation of meningococci

N. meningitidis strains, MC58-WT, MC58Δ*cbbA*, MC58Δ*gapA-1*, MC58Δ*cbbA* *cbbA*^{Ect} and MC58Δ*gapA-1* *gapA-1*^{Ect} were streaked onto chocolate agar plates and incubated as described in section 3.2.1. The following day, a single colony was inoculated into 10 ml Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) containing 2% heat-inactivated fetal calf serum (FCS; Invitrogen) and incubated overnight at 37°C with shaking (200 rpm). The following day, overnight broth culture was diluted 1:10 in DMEM containing 2% FCS and further grown for 2 h.

5.2.3 Preparation of human cells

Human brain microvascular endothelial (HBME) cells or human larynx carcinoma (HEp-2) cells were grown to confluence in DMEM supplemented with 10% FCS and 1% antibiotic antimycotic solution (Sigma) in 24-well tissue culture plates (Costar) at 37°C in an atmosphere of 5% CO_2. Prior to all experiments, mono-layers were transferred to DMEM supplemented with 2% FCS to remove the antibiotics.

5.2.4 Association assay

Association assays were performed essentially as previously described (Oldfield *et al.*, 2007). Briefly, HBME or HEp-2 monolayers were infected with 1×10^6 CFU ml^{-1} of meningococci (confirmed retrospectively by plating out aliquots of serially diluted inoculums) and left to associate for 2 h in 5% CO_2 at 37°C. To assess total cell association, monolayers were washed four times with 1 ml 1 × PBS per well. The monolayers were then disrupted and homogenized in 1 ml 0.1% saponin in PBS. Meningococci were enumerated by serial dilution of the homogenized suspensions and subsequent determination of colony-forming units by plating 10 µl spots from appropriate dilutions of the lysates on agar.

5.2.5 Adhesion inhibition assay

To block bacterial adhesion, meningococci were incubated for 1 h at RT with polyclonal anti-FBA sera at different dilutions ranging from 1:100 to 1:500 in PBS. The bacteria were pelleted by centrifugation (17,000 × *g* for 1 min) and washed twice with PBS before being used to infect human cell monolayers as described in section 5.1.4. Alternatively, monolayers were treated with 25 µg ml^{-1} of natively purified rFBA or PBS only (negative control) for 1 h and the assay continued as mentioned above (Section 5.2.4)

5.2.6 Invasion assay

HBME or HEp-2 monolayers were infected as described in section 5.2.4, but were left to associate for 4 h in 5% CO_2 at 37°C. The media was then removed and 2 ml DMEM containing gentamicin (100 μg ml^{-1}) added per well to kill the extra-cellular bacteria. Prior to further steps, aliquots of the gentamicin-containing supernatants were plated out to confirm killing of extra-cellular bacteria. Furthermore, the susceptibility of all meningococcal strains to gentamicin at 100 μg ml^{-1} was confirmed prior to testing. To assess cell invasion, monolayers were washed three times with 1 ml 1 × PBS per well. The monolayers were then disrupted and homogenized in 1 ml 0.1% saponin in PBS. Meningococci were enumerated by serial dilution of the homogenized suspensions and subsequent determination of colony-forming units by plating 10 μl aliquots from appropriate dilutions of the lysates on agar.

5.2.7 Statistical methods

Statistical analysis was performed with Microsoft Excel. All experiments were performed in triplicate unless otherwise stated and were repeated at least three times. Statistical significance was measured using a two-tailed Student *t*-test.

5.3 Results

5.3.1 Association assay of MC58Δ*cbbA* using HBME and HEp-2 cells

To determine a potential role of FBA in adhesion to epithelial and endothelial cells, viable counts of bacteria associated with homogenized infected monolayers were used to compare the capacity of the wild-type and *cbbA* mutant strain to associate with either HBME or HEp-2 cells. These experiments showed that *cbbA*-deficient meningococci had a significantly reduced capacity to adhere to monolayers of HBME cells and HEp-2. Experiments were repeated on three separate occasions, with both cell lines, with consistent results. To confirm that this effect was not due to reduced growth, the growth rate of both strains was compared by measuring the optical density at 600 nm (OD_{600}) in triplicate on three separate occasions. No substantial differences between strains were observed (section 3.3.18 and Fig. 3.27 A).

To exclude the possibility that the impaired adherence was due to a polar effect or undetected secondary mutation in MC58Δ*cbbA*, a wild-type copy of the gene was introduced *in trans* into MC58Δ*cbbA* using plasmid pSAT-12 (Table 3.1). Introduction of pSAT-12 led to similar levels of FBA expression to the MC58-WT (section 3.3.12 and Fig. 3.19) and restored levels of adhesion to both human cell types to approximately wild-type levels (Fig. 5.1 A and 5.1 B). In summary, these experiments show that FBA plays a role in the adherence of *N. meningitidis* with human cells.

In addition to complementation, to further rule out the possibility that the impaired adherence was due to undetected secondary mutation in the original *cbbA* mutant, the *cbbA* mutation was introduced by natural transformation into *N. meningitidis* strain Z4673 to create an independent *cbbA*-deficient derivative. Adherence assays with meningothelial cells showed that the Z4673 *cbbA* mutant exhibited a similar reduction in the number of adherent bacteria compared with its parental strain as did the MC58Δ*cbbA* mutant (Fig. 5.2).

To study whether the impaired adhesion was due to the direct involvement of FBA *i.e.* that FBA is an adhesin and binds a specific host cell receptor(s), cultured HBME cells were incubated with or without rFBA before infection with *N. meningitidis*. The adhesion to HBME cells by *N. meningitidis* MC58 could not be inhibited by rFBA (data not shown). Alternatively, to further assess the involvement of FBA in bacterial adhesion, the wild-type strain MC58 was treated with polyclonal RαFBA serum, pre-immune serum or not treated (negative control) to investigate whether this would block adhesion. Treatment with either immune or pre-immune serum demonstrated no substantial effect on bacterial adhesion (Fig 5.3). A possible reason for increased number of adherent bacteria observed in the inhibition assay result was that the bacteria used for infecting the monolayers were twice in number (2×10^6) as compared to previous adhesion assays

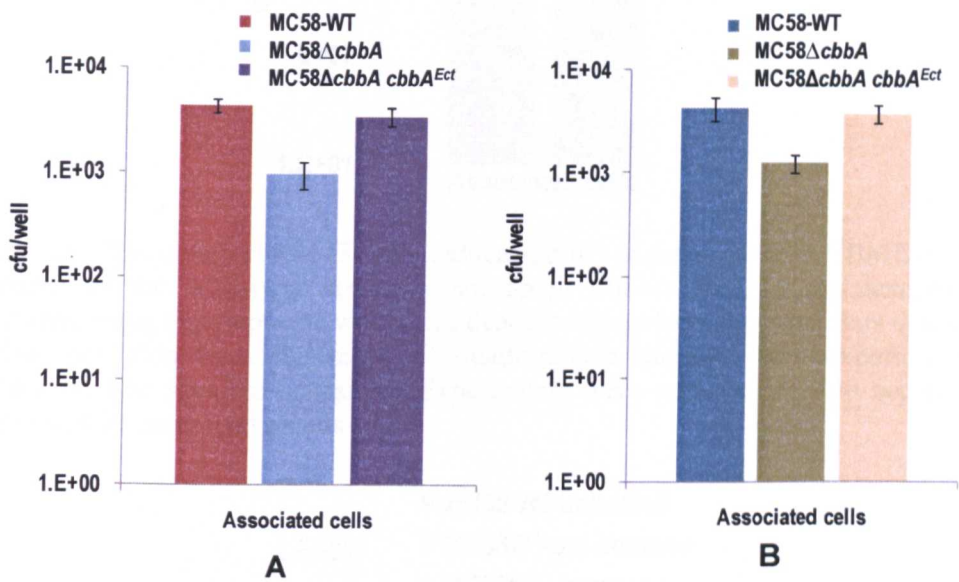


Figure 5.1. *cbbA*-deficient meningococci have a reduced ability to associate with (A) HBME or (B) HEp-2 cells compared to the wild-type or complemented strains. The number of *cbbA*-deficient meningococci associating to HBME cells was significantly lower than the wild-type (P = 0.0011). Bars denote standard deviation. Cfu denotes colony forming units. In (B), experiments were repeated on two separate occasions with consistent results, but only one representative experiment is shown. Bars denote deviation from the mean of quadruplicate samples from experiments repeated on two separate occasions.

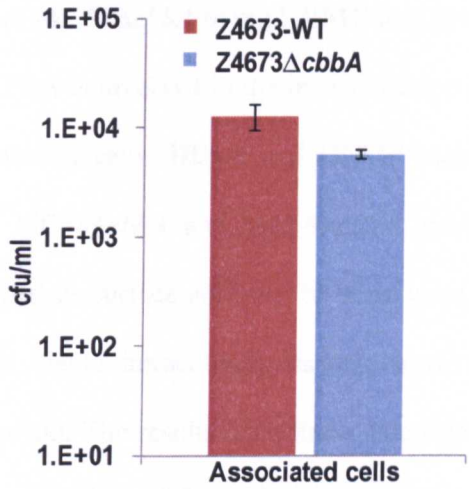


Figure 5.2. *cbbA*-deficient Z4673 has a reduced ability to associate with HBME cells compared to the wild-type strain. Mean levels shown from a representative experiment using quadruplicate wells. Cfu denotes colony forming units. Bars denote standard deviation from the mean of quadruplicate samples from experiments repeated on two separate occasions. Experiments were repeated on two separate occasions, with consistent results

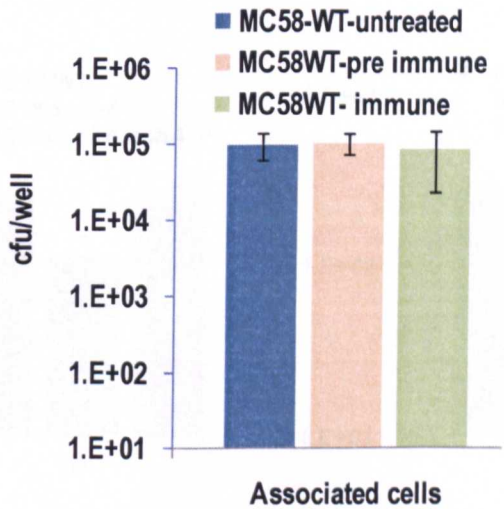


Figure 5.3. Inhibition of meningococcal association to HBME cells using RαFBA. Wild-type MC58 was treated with RαFBA sera or pre-immune sera before infection of HBME cells. Mean levels shown from a representative experiment using quadruplicate wells. Cfu denotes colony forming units. Bars denote standard deviation from the mean of quadruplicate samples Experiments were repeated on two separate occasions, with consistent results.

5.3.2 Invasion assay of MC58Δ*cbbA* using HBME and HEp-2 cells

To investigate whether FBA is involved in the internalization of *N. meningitidis* into epithelial and meningothelial cells, HEp-2 and HBME monolayers were infected with wild-type MC58, MC58Δ*cbbA* and MC58Δ*cbbA* *cbbA*^{Ect}. After 4 h of co-incubation, any unattached or surface-adherent bacteria were killed by exposure to gentamicin, after which viable intracellular bacteria were enumerated following release by lysis with saponin. The results from three independent experiments using the HBME cell line (Fig. 5.4 A) and from one representative experiment using the HEp-2 cell line (Fig. 5.4 B), demonstrated that the *cbbA*-deficient MC58 had reduced capacity to invade the monolayers, but this difference was not statistically significant and is likely to be a consequence of the reduced adherence of the mutants to the monolayer.

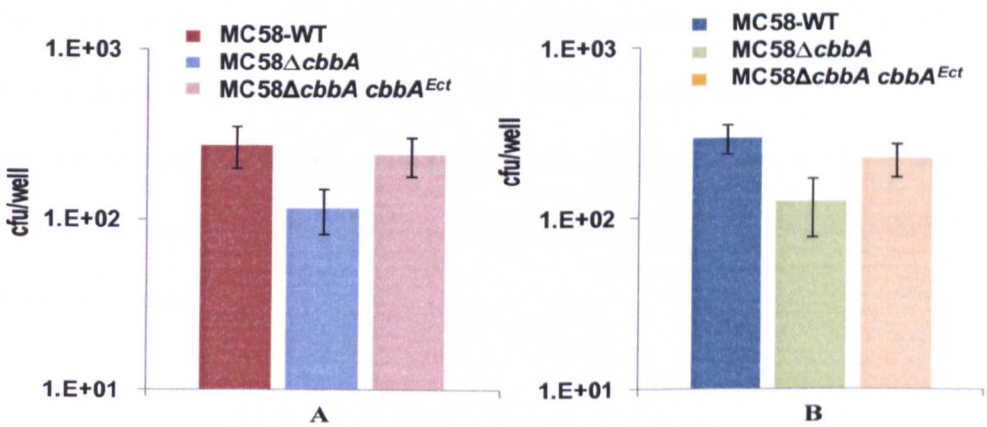


Figure 5.4. *cbbA*-deficient meningococci have a reduced ability to invade into (A) HBME cells or (B) HEp-2 cells compared to the wild-type or complemented strains. Numbers of mutant cells invading HBME cells were lower than the wild-type strain, but this difference was not statistically significant (P = 0.13). Mean levels shown from three independent experiments, each using triplicate wells. Cfu denotes colony forming units. Bars denote standard deviation. In (B), experiments were repeated on two separate occasions with consistent results, but only one representative experiment is shown. Bars denote standard deviation from the mean of quadruplicate samples.

5.3.3 Association assay of MC58Δ*gapA-1* using HBME and HEp-2 cells

Viable counts of bacteria associated with homogenized infected monolayers were also used to compare the capacity of the wild-type and *gapA-1* mutant strain to associate with either HBME or HEp-2 cells. These experiments showed that GAPDH-deficient meningococci had a significantly reduced capacity to adhere with monolayers of HBME (Fig. 5.4 A) and HEp-2 cells (Fig 5.4 B). Experiments were repeated on more than three separate occasions, with both cell lines, with consistent results. Once again, to confirm that this effect was not due to reduced growth, the growth rate of both strains was compared by measuring the optical density at 600 nm (OD_{600}) in triplicate on three separate occasions. No substantial differences between strains were observed (section 3.3.18 and Fig. 3.27 B).

The ability of the complemented strain to adhere to HEp-2 and epithelial cells was then analysed. As shown in Fig. 5.5 A and B; complementation reversed the adhesion defect of the mutant to wild-type levels. Taken together, the results strongly suggest that *gapA-1* is involved in adhesion of meningococci to both epithelial and endothelial cells.

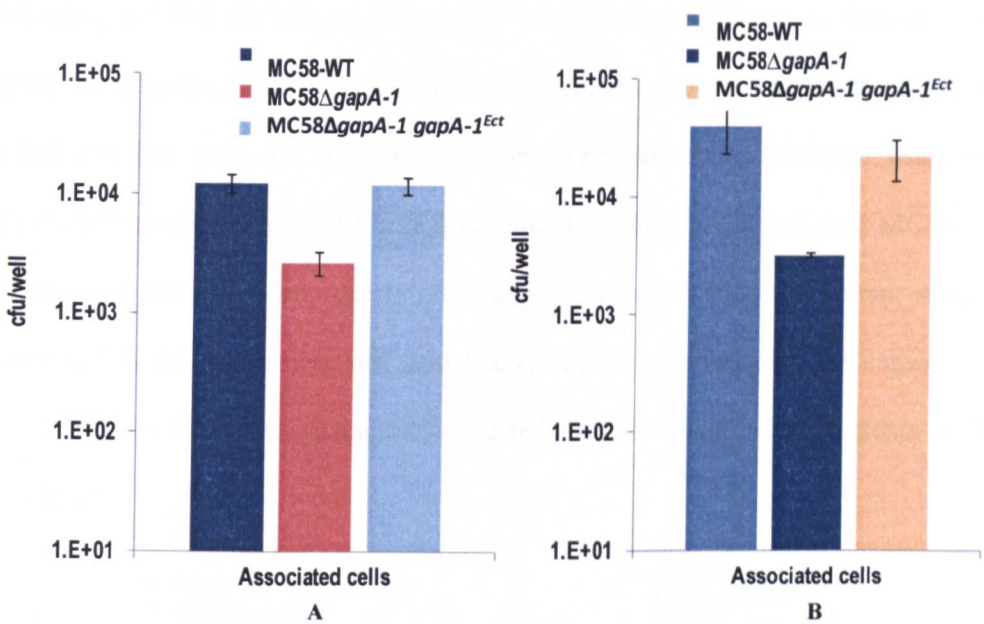


Figure 5.5. GAPDH-1-deficient meningococci have a reduced ability to associate with (A) HBME and (B) HEp-2 cells compared to the wild-type or complemented strains. The number of GAPDH-1-deficient meningococci associating was significantly lower than the wild-type (HBME cells $P = 0.0018$, HEp-2 cells 0.017). Mean levels shown from three independent experiments using quadruplicate wells. Bars denote standard deviation. Cfu denotes colony forming units.

5.3.4 Invasion assay of MC58Δ*gapA-1* using HBME and HEp-2 cell line

To evaluate the role of *gapA-1* in the invasion of meningococcus into epithelial and meningothelial cells, viable counts of bacteria released from homogenized infected monolayers were used to compare the capacity of the MC58 wild-type, *MC58ΔgapA-1* and MC58Δ*gapA-1gapA-1*^{Ect} complemented strains to invade either HBME or HEp-2 cells. The results from three independent experiments using the HBME cell line (Fig. 5.6 A), and from one representative experiment using the HEp-2 cell line represented in Fig. 5.6 B, show that the *gapA-1*-deficient MC58 are less invasive than the parent strain and the complemented isogenic mutant strains, however, this difference was not statistically significant. The reduced capacity to invade is likely to be a consequence of the reduced adherence of the mutants to the monolayer.

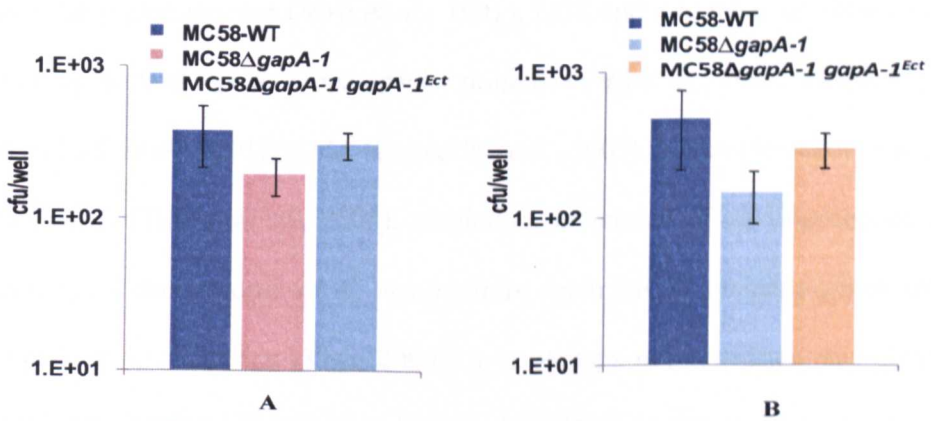


Figure 5.6. GAPDH-1-deficient meningococci have a reduced ability to invade to (A) HBME or (B) HEp-2 cells than the wild-type or complemented strains. The number of GAPDH-1-deficient meningococci invading HBME cells compared to the wild-type was not significantly different ($P = 0.1338$). Mean levels shown from three independent experiments, each using triplicate wells. Bars denote standard deviation. Cfu denotes colony forming units. In (B) mean levels shown from a representative experiment using quadruplicate wells. Bars denote standard deviation from the mean of quadruplicate samples. Experiments were repeated on two separate occasions, with consistent results.

5.4 Discussion

This chapter describes the characterisation of the potential roles of meningococcal FBA and GAPDH-1 in adhesion to, and invasion into, host cells *in vitro* using the well-established HBME cells and HEp-2 cell models. Although regarded as an extracellular pathogen generally, *N. meningitidis* has the capacity to enter eukaryotic cells by interacting with several distinct cellular receptors. This has been demonstrated in numerous *in vitro* studies (Claudia Sa *et al.*, 2009). The ability of bacteria to colonize their hosts and cause infection is often linked to their ability to express several different adhesins with different receptor specificities (Scarselli *et al.*, 2006). *N. meningitidis* has evolved a diverse array of surface structures to interact with host cells. These include Type IV pili, which are considered to be the prime attachment-promoting factor for capsulated meningococci to the nasopharyngeal mucosa (Virji *et al.*, 1991), LOS and a number of outer membrane proteins, such as Opc and Opa opacity proteins (Virji *et al.*, 1992; Virji *et al.*, 1993), App (Hadi *et al.*, 2001), NadA (Capecchi *et al.*, 2005), NhhA (Scarselli *et al.*, 2006), and MspA (Turner *et al.*, 2006), mediating interaction of meningococcus to host cells. Since the genome of *N. meningitidis* strain MC58 contains genes encoding several known adhesins (Virji, 2009), it is perhaps not surprising that ablation of additional putative adhesins (*i.e.* FBA and GAPDH-1) did not dramatically reduce adhesion in this model. Nevertheless, there was a significant reduction in adhesion for the *cbbA* and *gapA-1* mutants compared to their wild-type parent strain, indicating that *cbbA* and *gapA-1* play a role in association with human endothelial and epithelial cells.

Multifunctional, extra-cytoplasmic glycolytic enzymes acting as adhesins is not unprecedented. For example, in *Streptococcus spp.* an extra-cytoplasmic GAPDH has been described to mediate binding to plasmin and shown to possess an ADP-ribosylating activity (Pancholi & Fischetti, 1992), while in *C. albicans* the extracellularly localized GAPDH has been shown to bind fibronectin and laminin (Gil-Navarro *et al.*, 1997; Gozalbo *et al.*, 1998). Likewise, FBA of *S. suis* (Zongfu *et al.*, 2008) and *S. pneumoniae* (Ling *et al.*, 2004) has been shown to be immunogenic and surface-localized proteins. In addition, *S. pneumoniae* FBA has, recently, been demonstrated to act as adhesin mediating *in vitro* adhesion to host receptor Flamingo cadherin on A549 type II lung carcinoma epithelial cells (Blau *et al.*, 2007).

In the present study, a potential role of *cbbA* and *gapA-1* in mediating interaction with host cells was investigated using a mutational analysis and functional complementation of both genes in *N. meningitidis* strain MC58. To assess the adhesive properties of FBA and GAPDH-1, *cbbA* and *gapA-1* knock-out mutants were created in *N. meningitidis* strain MC58 and *cbbA* was also mutated in four additional genetic backgrounds representative of clinical isolates of *N. meningitidis* (see Chapter 3). Before using in association assays, mutant strains were assessed for the attenuation of growth in liquid culture and colony morphology on solid agar. The *cbbA* and *gapA-1* deficient strains appeared to grow at the same rate in liquid culture as well as showed the same colony morphology, as determined by measuring the optical density OD_{600} of broth culture and visual inspection of colony morphology after 24, 48 and 72 h respectively. This indicated that both of these enzymes are

unlikely to contribute to the glycolysis in *N. meningitidis*, consistent with the finding by Baart *et al.*, (Baart *et al.*, 2007), and that the mutation of both enzymes individually do not affect *in vitro* maximal growth of *N. meningitidis*.

In previous chapters, FBA and GAPDH-1 have been identified as outer membrane localized proteins. Additionally, flow cytometry analysis confirmed that FBA was available to antibodies on the surface of encapsulated *N. meningitidis*, whilst GAPDH-1 was also accessible to antibodies, but only in the absence of capsule. It was speculated that these proteins may play a role in adhesion to host cells. Association assay were then carried out with different cell lines, including HBME and HEp-2 cell lines. In adhesion experiments, nonetheless, both mutants demonstrated a significantly reduced capacity to adhere to HBME and HEp-2 cells. Although results of invasion experiments using *cbbA* and *gapA-1* mutants demonstrated a reduced capacity in both cell lines, this difference was not statistically significant. The reduced capacity to invade is likely to be a consequence of the reduced adherence of the mutants to the monolayer. Due to time constraints, experiments using HEp-2 cells were carried out only two times and ideally they should be repeated at least three to five times at different occasions to allow statistical significance to be assessed. It was hypothesized that if FBA has a direct role in adherence, exogenously added purified rFBA or anti-FBA may competitively inhibit FBA-mediated meningococcal adherence. To investigate this, adherence to HBME cells was determined in the presence of free rFBA and anti-FBA antiserum. Both (rFBA and anti-FBA) failed to interfere *in vitro* with meningococcal adhesion

to HBME cells. One possible explanation to the inability of either rFBA or anti-rFBA antiserum to inhibit the adherence process could be that the FBA may have indirect role in adhesion. This can be addressed by determining the ability of meningococci expressing a non-enzymatically functional, but structurally identical FBA to adhere to host cells. To exclude the possibility that the defective adhesion was due to polar effects of both mutations, functional complementation of both mutations was performed to prove unequivocally the involvement of FBA and GAPDH-1 in binding to host cells and to verify that the observed phenotypic effect was not due to an unanticipated effect on another gene. Single wild-copies of desired genes were reintroduced in respective mutants at an ectopic site. The complemented strains were then analyzed to determine their adhesive properties. The results demonstrated a restoration of adhesion levels to those observed for the wild-type strains indicating that the reduced capacity in adherence of mutants with both cell lines was the outcome of the loss of the expression of *cbbA* and *gapA-1*. The impaired adhesion of *cbbA* knock-out strain to host cells also correlate to our previous finding of animal studies showing that the *cbbA* mutants were impaired in their ability to sustain bacteraemia compared to their wild-type parent strains. Together, these results indicate that FBA may play a potential role in the pathogenesis of meningococcal disease.

In addition to the viable count methodology used in this study, which measure levels of cell-associated (i.e. adherent and internalized) bacteria, several other methods can be employed to investigate adhesive properties of *N. meningitidis*. Future

experiments could be performed using different approaches such as immuno-fluorescence microscopy, assay using micro-beads coated with purified protein, and environmental scanning electron microscopy (ESEM), which can be used to visualize surface-associated bacteria alone, to validate the results independently.

In summary, FBA and GAPDH-1 are highly conserved proteins and universally expressed by meningococci, and are required for optimal adhesion to human cells. To the best of our knowledge, this is the first study demonstrating the contribution of FBA and GAPDH-1 of *N. meningitidis* in adhesion to the host cells. Future studies are required to determine the mechanism(s) by which these proteins modulate adhesion to host cells.

CHAPTER 6: Genaral Discussion

6.1 General discussion

There are an increasing number of reports showing that classical cytoplasmic house-keeping enzymes, without any identifiable secretion signals, are often localized to the surface of microbial pathogens, where they exhibit various functions, unrelated to glycolysis, such as surface localization and adhesion to host cell receptors, thus, contributing to the pathogenesis of disease (Pancholi & Chhatwal, 2003). In order to gain a deeper understanding of microbial pathogenesis, there is considerable interest in identifying the additional roles of these glycolytic enzymes. Given their surface localisation and immunogenicity, these enzymes may represent potential vaccine candidates. The work presented in this thesis details molecular, functional and immunological characterisation of two of the meningococcal glycolytic enzymes namely, fructose 1,6-bisphosphate aldolase (FBA) and glyceraldehydes-3-phosphate dehydrogenase (GAPDH-1).

The discussion of the results of this study will include the validity of the approach of selecting both genes under analysis, the strategies used to achieve the aims/goals and discussion of the finding achieved. The choice of genes for investigation in this project was based on the premise that glycolytic enzymes are often found localized to the surface of variety of bacterial and fungal pathogens and shown to have potential roles in adhesion to various host molecules such as fibronectin (Esgleas *et al.*, 2008), laminin (Gozalbo *et al.*, 1998; Pancholi & Fischetti, 1992), plasminogen (Agarwal *et al.*, 2008), mucin (Alvarez *et al.*, 2003) and cadherin (Blau *et al.*, 2007). It was hypothesized that similar to glycolytic enzymes from other pathogens,

meningococcal FBA and GAPDH-1 may be multi-functional proteins and localized to the outer membrane of *N. meningitidis*. The strategy that was adopted towards characterizing both enzymes included cloning the respective genes, *cbbA* and *gapA-1*, and subsequent purification of recombinant FBA (rFBA) and rGAPDH-1 proteins to generate polyclonal rabbit antiserum. In addition, knock-out mutants of *cbbA* and *gapA-1* were created in *N. meningitidis*. The antiserum and mutant strains were then employed in undertaking phenotypic analysis to facilitate the characterisation of both proteins. The following sections will explore the subsequent characterisation of these proteins, and, in particular, describe the evidence for their surface localisation, and the potential roles of FBA and GAPDH-1 in the pathogenesis of meningococcal disease.

6.1.1 Fructose bisphosphate aldolase (FBA)

There have now been a number of studies showing that certain glycolytic proteins, such as GAPDH, enolase, and FBA, which can be usually found in the cytoplasm of the cell, are involved in various other functions unrelated to their primary function in glycolysis (Agarwal *et al.*, 2008; Alvarez *et al.*, 2003; Bergmann *et al.*, 2001; Bergmann *et al.*, 2004; Ling *et al.*, 2004; Pancholi & Chhatwal, 2003). One such protein, fructose-1, 6-bisphosphate aldolase (FBA) has been previously reported to be localized to the surface of some Gram-positive bacteria. In *S. pneumoniae*, for example, surface-exposed FBA (Class IIB) was demonstrated to act as an adhesin, specifically binding to a large 7-transpass transmembrane receptor belonging to the cadherin superfamily (Blau *et al.*, 2007) . In *N. meningitidis*, two cytoplasmic house-

keeping enzymes, GAPDH and enolase (Grifantini *et al.*, 2002a; Knaust *et al.*, 2007) have been shown to be surface-localized, but their non-glycolytic roles are undefined. We undertook to investigate whether FBA was also surface-localized in *N. meningitidis*, as has been described for GAPDH and enolase, and to determine whether FBA plays a role in meningococcal pathogenesis.

In the published meningococcal and gonococcal genome sequences, there is only one gene *cbbA*, predicted to encode a putative FBA enzyme. Despite being predicted to be part of a non-functional metabolic pathway, *cbbA* has not acquired spontaneous mutations in any of the Neisserial genomes examined, which suggests that the gene was acquired recently, that the glycolytic pathway became non-functional recently, or that the protein has one or more additional functions. Sequence analysis shows that FBA is highly-conserved at the amino acid level. The deduced protein sequence exhibited high identities to Class-II FBA from *Xanthobacter flavus* and *Synechocystis* sp (67 and 65%, respectively) suggesting that the meningococcal FBA belongs to bacterial Class-II FBP aldolases. The enzyme was highly related to FBP aldolases from other meningococcal strains (in the range of 93-98% identity at the amino acid level). In addition, a 21-amino acid insertion sequence, which is present in two subclasses of the Class IIB FBA enzymes, was also present in the Neisserial sequences. The presence of this sequence suggests that the neisserial FBA enzyme is a tetrameric, rather than a dimeric enzyme; a feature which is present in extremophiles, and which has been suggested to confer thermal stability (Izard & Sygusch, 2004; Sauve & Sygusch, 2001).

In this study, rFBA was successfully expressed with an N-terminal histidine-tag in host *E. coli*; however, the expression resulted in insoluble proteins (inclusion bodies). Modification of the expression parameters including induction temperature, time, and IPTG concentration failed to result in the production of soluble proteins. The reason for this insolubility is unclear; however, it might be related to the nature of the vector promoter (T7 promoter) or be intrinsic to the FBA protein. Attempts to purify rFBA under native conditions were unsuccessful. Therefore, the expressed protein was purified under denaturing conditions and used to raise rabbit polyclonal anti-FBA serum (RαFBA).

To examine the aldolase activity and generate polyclonal antiserum against native purified FBA, the protein was expressed with a histidine-tag at the C-terminal and purified under non-denaturing conditions. The purified rFBA was shown to have aldolase activity confirming that the enzyme was in native conformation after purification. In accordance with the *in silico* prediction of tetrameric structure of FBA, the purified native protein was used to investigate the tetrameric nature of meningococcal FBA using native gel analysis. However, due to the either aggregation of native proteins or other unknown reasons, the results did not show an intact protein band. The purified protein was also used to generate rabbit polyclonal anti-FBA antiserum (nRαFBA), which was used to confirm that FBA is expressed *in vitro* in each of a range of Neisserial strains tested including commensal species. This suggests that FBA plays an important role which is required by both non-pathogenic and pathogenic species.

Many studies have now reported that, in addition to the cytosol, FBA is localized to the outer membrane of the some Gram-positive bacterial pathogens, but no such evidence is available for Gram-negative bacteria. This study thus aimed to investigate sub-cellular localisation of FBA in *N. meningitidis*. Cell fractionation studies demonstrated that FBA was present in both the cytosol and in an outer membrane-enriched fraction. Although the cell fractionation results indicated that FBA was localized to the outer membrane of *N. meningitidis*, we further investigated the possibility that cytosolic enzyme released following cell lysis may adhere to intact *N. meningitidis* cells. Incubation of *cbbA*-deficient mutant cells with either whole cell lysates of wild-type MC58 or soluble rFBA protein prior to fractionation failed to show any reactivity, suggesting that FBA is not recruited onto the surface of intact bacteria. The cell fractionation results showing outer membrane localisation were substantiated by whole cell ELISA and flow cytometry analysis. Flow cytometry and ELISA data indicate that FBA is surface exposed in *N. meningitidis* and present in a form that was accessible to antibodies, suggesting that, similar to GAPDH and enolase, FBA is translocated (or diverted) to the outer membrane. To our knowledge, this is the first report demonstrating that a proportion of FBA is found on the cell surface of any Gram-negative bacterium.

In *N. meningitidis* strain MC58, *cbbA* is the first of four genes that could be part of an operon, and thus, polar effects may account for the observed phenotypic characteristics of mutation of *cbbA*. To exclude this possibility, the *cbbA* mutation was complemented in three isogenic *cbbA* mutants created in *N. meningitidis* (MC58,

Z4181, and ST-18) by reintroducing a wild copy of *cbbA* at an ectopic site on the chromosome of respective mutant strains. As a complementary experiment, to confirm that *cbbA* mutation does not affect the expression of downstream genes in the operon, we determined the expression of the immediately down-stream gene which encodes fHbp using immunoblot analysis. The expression of fHbp was similar in both wild-type MC58 and *cbbA* mutant strains.

Phenotypic analysis of mutant strains was undertaken to determine the role of FBA in neisserial biology. In initial growth profiling experiments, the *cbbA*-deficient mutant grew at the same rate (in broth culture and on solid media) as the wild-type and the complemented mutant strains, demonstrating that FBA is not required for optimal growth of the meningococcus under the *in vitro* conditions tested. No differences in either colony or bacterial cell morphology (using light microscopy) were observed. In adhesion experiments, however, the *cbbA*-deficient mutant strain exhibited a significantly reduced capacity to adhere to both HBME and HEp-2 cells. This phenotype was completely restored in a complemented strain. Although *cbbA* mutant strain showed a reduced capacity to invade both monolayers, this difference was not statistically significant. It is hypothesized that the observed defect in invasion was likely to have been a consequence of the reduced ability of the mutant strain to initially adhere to the cell monolayers. The observation that FBA is involved in adhesion to both epithelial and endothelial cells, and that FBA expression is conserved in non-pathogenic strains (such as *N. polysaccharea*e) may suggest a role for FBA during colonization of the nasopharyngeal mucosa by

commensal Neisserial species. To determine whether the observations of impaired adhesion of *cbbA* mutants to host cells were also reflected *in vivo*, the *cbbA* knock-mutant strains created in five different genetic backgrounds (MC58, Z4181, Z4667, Z4673, and ST-18) were assessed to determine the potential role of FBA in establishing bacteraemia in a transgenic (expressing human transferrin) mouse model of meningococcal disease. The mutant strains were impaired in their ability to sustain bacteraemia compared to their wild-type parent strains suggesting that the mutants were less able to access the bloodstream and/or to survive in the bloodstream after intraperitonial inoculation compared to wild-type parent strains. Together, these results indicate a potential role for FBA in the pathogenesis of meningococcal disease. In future work, the antiserum raised against natively purified rFBA will be used in passive protection experiments using the transgenic mouse model to determine whether anti-FBA antibodies elicit a protective response against *N. meningitidis*. Active protection experiments using mice vaccinated with natively purified rFBA will also be performed.

It is theoretically possible that the *cbbA* deletion may affect the expression of meningococcal genes determining piliation or other factors important for adhesion. Variation in the expression of these genes, between wild-type and mutant strains, may be responsible for differences in observed phenotypes. Unfortunately, due to unavailability of antiserum and time constraints, the expression of these various genes was not assessed. However, given that type IV pili mediate natural transformation and that this methodology was successfully used to transform the

cbbA knock-out strain with the complementation construct, it is assumed that piliation is not grossly affected by deletion of *cbbA*. Although this study demonstrates that FBA is present on the meningococcal cell surface and is required for optimal adhesion, the role of FBA in this process is unknown. It is possible that the enzymatic activity of FBA plays an indirect role that is required for optimal adhesion. However, given that other FBA homologues (such as FBA in *S. pneumoniae*) have been shown to directly bind to host cell ligands it is also possible that the meningococcal protein has direct host receptor binding activity. Determining the ability of meningococci expressing a non-functional FBA to adhere to host cells would address this. *S. pneumoniae* FBA was putatively shown to bind to a cadherin superfamily receptor (Flamingo cadherin receptor, FCR) on the surface of host epithelial cells, but it is unknown which FBA residues participate in this interaction (Blau *et al.*, 2007). Meningococcal FBA is only 40% identical to the pneumococcal enzyme at the amino acid level, so it is unclear whether meningococcal FBA binds the same receptor. The preliminary data of serum bactericidal experiments using antiserum raised against denatured purified FBA showed no bactericidal activity against *N. meningitidis* strain MC58. Future work will be performed with the antiserum raised against natively purified proteins and bacteria grown under conditions that mimic *in vivo* conditions. It is possible that FBA may be up-regulated *in vivo*, leading to great level of surface expression, which may be of importance in relation to bactericidal activity of specific antibodies.

Pneumococcal FBA has been shown to be immunogenic in humans and capable of eliciting a partially protective immune response against lethal *S. pneumoniae* intranasal challenge in mice (Ling *et al.*, 2004). Given that meningococcal FBA is: highly conserved, expressed by a wide range of isolates, surface-accessible to antibodies and structurally and antigenically unrelated to the human (Class I) FBA protein, meningococcal FBA is worthy of future study as a possible candidate vaccine component against this important human pathogen.

6.1.2 Glyceraldehyde 3-phosphate dehydrogenase (GAPDH-1)

During last two decades a number of reports have indicated that GAPDH, a classical cytoplasmic glycolytic enzyme is often present at bacterial surfaces and performs a variety of non-glycolytic functions (Pancholi, 2001; Pancholi & Chhatwal, 2003; Sirover, 1996; Sirover, 1999). Surface localization of GAPDH was first demonstrated in the Gram-positive pathogen, *S. pyogenes*. In this organism, surface-exposed GAPDH binds several mammalian proteins including the uPAR/CD87 membrane protein on pharyngeal cells (Jin *et al.*, 2005; Lottenberg *et al.*, 1992; Pancholi & Fischetti, 1992; Winram & Lottenberg, 1996), regulates intracellular host cell signaling events (Pancholi & Fischetti, 1997) and contributes to host immune evasion (Terao *et al.*, 2006). GAPDH was subsequently identified on the surface of other Gram-positive bacteria including staphylococci (Modun & Williams, 1999; Modun *et al.*, 2000), *S. agalactiae* (Seifert *et al.*, 2003) and *Listeria monocytogenes* (Schaumburg *et al.*, 2004). However, the prerequisites for secretion of GAPDH are not yet understood. The surface localised GAPDH appears to contribute to the

virulence of pathogens by means of binding to host proteins including plasminogen/plasmin, and fibronectin, thus facilitating their colonization and invasion of host tissue (Bergmann *et al.*, 2004; Egea *et al.*, 2007; Gozalbo *et al.*, 1998; Pancholi & Fischetti, 1992). In *M. genitalium*, for example, only a small proportion of the total cellular GAPDH is trafficked to the bacterial surface, however, this is sufficient to impart a biologically-significant phenotype (mucin-binding) on this organism (Alvarez *et al.*, 2003). For organisms with relatively small genomes, multi-functional proteins may be advantageous to optimize the potential of the genome. However, non-glycolytic roles of surface-localised GAPDH are poorly studied in Gram-negative pathogens. Although Grifantini *et al.* for the first time demonstreted that GAPDH expression was up-regulated on the meningococcal cell surface following contact with human epithelial cells; no biological function has so far been ascribed to this observation. Furthermore, another recent study has shown that surface-localised GAPDH of a Gram-negative *E. coli* mediates binding to human plasminogen and fibrinogen (Egea *et al.*, 2007). It was, thus, speculated that similar to surface localized-GAPDH enzymes from other microbial pathogens, surface-exposed meningococcal GAPDH-1 may also contribute to pathogenesis of meningococcal disease due to its functional diversity.

In *N. meningitidis*, *gapA-1* is one of the two genes coding for the metabolic enzyme GAPDH. Although predicted to be part of a non-functional metabolic pathway, the genes encoding GAPDH and other enzymes required for glycolysis are maintained in the meningococcal genome, presumably for other roles. Sequence analysis shows

that GAPDH-1 is highly-conserved at the amino acid level. In this study, recombinant GAPDH-1 (rGAPDH-1) was expressed and purified under denaturing conditions. Purified protein was used to generate rabbit polyclonal antiserum (RαGAPDH-1). Anti-GAPDH-1 was then used to investigate the strain distribution and sub-cellular localization of the enzyme. A strain survey for distribution and expression of *gapA-1* was carried out by PCR and immunoblot analysis of whole cell proteins from different clinical isolates of known MLST-type. Immunoblot analysis of these strains showed that they all express GAPDH-1, suggesting that GAPDH-1 is constitutively-expressed in *N. meningitidis*.

To facilitate studies of the potential role of *gapA-1* in the pathogenesis of meningococcal disease, a *gapA-1* knock-out mutant was created in *N. meningitidis* strain MC58. The mutant strain was evaluated by screening for defects in growth, on agar and in broth culture. The *gapA-1* mutant grew at the same rate (in both broth culture and on solid media) as the wild-type strain demonstrating that *gapA-1* is not required for maximal growth of the meningococcus under *in vitro* conditions. Moreover, the colony or bacterial cell morphology of mutant strain (using light microscopy) was indistinguishable compared to wild-type parent strain.

In accordance with the previous observations that GAPDH is a multi-functional protein and appears to be translocated to the surface of numerous bacterial and fungal pathogens (including *N. meningitidis*), surface-location of meningococcal GAPDH-1 was confirmed using sub-cellular fraction and flow cytometry analysis.

Sub-cellular fractions were probed using anti-GAPDH-1 in immunoblotting experiments. A band corresponding to GAPDH-1 was demonstrated to be present in both the cytosolic and the outer membrane protein-enriched fraction of wild-type meningococci, suggesting that, similar to enolase and FBA, a proportion of GAPDH-1 is translocated to the outer membrane. To exclude the possibility that cytosolic GAPDH-1 released from lysed cells is recruited back onto the surface of intact meningococci prior to fractionation, *gapA-1*-deficient meningococci were incubated with lysates of wild-type MC58 cells and rGAPDH-1. The fractionated cells were then probed with RαGAPDH-1 in immunoblot analysis and demonstrated no reactivity. This is in agreement with the recent report by *Saad et al.*, who showed that provoked cell lysis of *Lactobacillus plantarum* did not lead to re-association of GAPDH onto the cell surface (Saad *et al.*, 2009). Instead, Saad *et al.*, suggested that changes in plasma membrane permeability during the growth cycle may facilitate the movement of GAPDH onto the external surface of the plasma membrane in this Gram-positive organism (Saad *et al.*, 2009). Clearly, however, such a mechanism would only permit periplasmic localization in a Gram-negative organism. The results of sub-cellular fractionation were further validated by flow cytometry analysis. The data demonstrate that GAPDH-1 was not accessible by antibodies on the surface of capsulated MC58; however, GAPDH-1 was shown to be exposed on the surface and accessible by antibodies in non-capsulated MC58. It is important to note that in the previous report by Grifantini *et al.*, GAPDH-1 was shown to be accessible to antibodies following permeabilisation of the capsule with 70% alcohol and using meningococci grown under the conditions mimicking *in vivo* conditions (i.e. co-

incubated with host cells). Our flow cytometry data independently confirm that in capsulated meningococci, GAPDH-1 appears to be functionally masked by the capsule and is only exposed on the surface of non-capsulated *N. meningitidis*. However, capsule expression in meningococci is known to be down-regulated following the initial type IV pilus dependant-contact with host cells in order to facilitate intimate adherence (Deghmane *et al.*, 2002; Virji *et al.*, 1995). Thus, it is possible that surface-localised GAPDH-1 may be unmasked following this change allowing it to influence subsequent steps in adhesion. This speculation was supported by our observation that in adhesion experiments the GAPDH-1 mutant strain exhibited a significantly reduced capacity to adhere to both HBME and HEp-2 cells compared to the wild-type strain. This phenotype is unlikely to be due to polar effects arising from the interruption of transcription of genes that are downstream from *gapA-1* in *N. meningitidis;* given the opposite orientation of immediately downstream genes and the observation that complementation completely restores the wild-type phenotype.

In serum bactericidal assays (SBAs), antibodies raised against denatured GAPDH-1 failed to kill meningococci. It is likely that the absence of SBA activity is due to lack of accessibility of antibody to the antigen owing to the masking of GAPDH-1 by the capsule. Additionally, for the proteins with either low or transient surface expression, it seems likely that either the level of antigen on the surface is insufficient to promote bactericidal killing or only a fraction of the population may be killed. In addition, bactericidal assays in this study were carried out by incubating the immune serum

and complement with meningococcal cells grown in an enriched broth. Under these conditions, the bactericidal activity of antibodies induced by antigens that are specifically up-regulated during adhesion to host cells is expected to be underestimated. Moreover, antigens appearing on the bacterial surface only after adhesion or during a later phase of infection may give a negative result in SBAs even though, *in vivo*, they may play an important role in protection. In future work, the bactericidal assays should be performed using bacteria grown under conditions that mimic *in vivo* conditions and antibodies raised against the native GAPDH-1 protein will be assessed for serum bactericidal activity.

In conclusion, meningococcal *gapA-1* is a constitutively-expressed, highly-conserved protein, which appears to be partially localized to the outer membrane. Loss of *gapA-1* does not affect the *in vitro* maximal growth rate of *N. meningitidis*, but significantly affects the ability of the organism to adhere to human epithelial and meningothelial cells. Given the surface localisation and a potential role in adhesion to host cells, GAPDH-1 may be involved in the pathogenesis of meningococcal disease.

6.2 Conclusion

In the present study, *cbbA* and *gapA-1* (encoding FBA and GAPDH-1 proteins, respectively) were cloned and the recombinant proteins were subsequently purified either under denaturing or non-denaturing conditions. Polyclonal antisera were raised in rabbit against native or denatured rFBA and rGAPDH-1. Both antisera were

shown to react against the cognate purified proteins in immunoblot experiments. PCR and immunoblot analysis demonstrated that the *cbbA* and *gapA-1* genes were well conserved and expressed in divergence isolates of *N. meningitidis*, which is an important property required of a prospective vaccine candidate. Sub-cellular fractionation results indicated that both FBA and GAPDH-1 were localized to the outer membrane and cytosol of *N. meningitidis*. The *cbbA* and *gapA-1* knock-out mutants were successfully created in *N. meningitidis* strain MC58. Both mutants grew normally when cultured on solid media as well as in broth culture compared to the wild-type strain. Both mutants, however, demonstrated a clear phenotype of reduced capacity to adhere to, and invasion, cultured epithelial and meningothelial cells.

The *cbbA* gene was also mutated in four other clinical isolates of *N. meningitidis* to act as additional controls in assessing a potential role of FBA in the development of bacteraemia in a novel transgenic mouse model of meningococcal disease. The *cbbA* knock-out mutant in all four genetic backgrounds demonstrated reduced capacity to establish bacteraemia compared to the parent wild-type strains in the mouse model. Flow cytometry and ELISA data demonstrated that FBA was surface exposed and accessible by antibodies on the surface of intact cells. GAPDH-1 was shown to be surface-exposed on the non-capsulated MC58 strain and was inaccessible to antibodies on the surface of a capsulated strain. Although insufficient to promote detectable complement mediated killing, given their surface localisation and potential role in adhesion, immune responses against FBA and GAPDH-1 may still

be involved in immunity to meningococcal disease. In summary, both *cbbA* and *gapA-1* are constitutively expressed and partially localized to outer membrane of *N. meningitidis*. The mutation of both genes (*cbbA* and *gapA-1*) did not affect the growth rate of bacteria, however, the mutant strains showed a significantly reduced ability to adhere to cultured epithelial and meningothelial cells. Moreover, the *cbbA* mutation was associated with attenuation in the establishment of bacteraemia in a transgenic mouse model of meningococcal infection.

6.3 Future directions

Although this study has demonstrated that both FBA and GAPDH-1 are required for optimal *in vitro* adhesion of *N. meningitidis* to epithelial and meningothelial cells, future experiments, using different independent approaches such as immunoflourescence microscopy and ESEM (Oldfield *et al.*, 2007), should be performed to show the validity of the results. Future work should also aim to identify FBA and GAPDH-1 interacting host proteins and clarify the mechanism(s) that are responsible for the defect in adherence to host cells. A potentially useful approach may be to compare the gene expression profiles of the *cbbA* and *gapA-1* mutant strains with the wild-type using microarray technology (Chan *et al.*, 2005; Liu *et al.*, 2005). Disruption of a single gene may result in changes in gene expression that arise as a direct consequence of the disruption or as a result of compensatory changes.

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7.1 References

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APPENDIX- A: Buffers and reagents

TAE buffer (50 × stock)

242 g Tris base (life technologies), 57.1 ml glacial acetic acid (Fisher chemicals) 100 ml 500 mM EDTA (pH 8.0) made up to 1 L with dH_2O

Agarose gel

0.8-1.0 % agarose was prepared by dissolving 0.8-1 g of agarose powder (sigma), 2 ml 50 × TAE buffer and made up to 100 ml with dH_2O; melt and add 5 µl ethidium bromide (10 mg ml^{-1}).

Antibiotics

Antibiotics were purchased from Sigma-Aldrich UK, prepared according to the manufacturer's recommendations, sterilized by filtration and store 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)
Streptomycin	(50 mg ml^{-1} stock solution prepared in dH_2O)
Erythromycin	(100 mg ml^{-1} stock solution prepared in ethanol)

ELISA coating (carbonate) buffer

142 mM sodium bicarbonate, 8 mM sodium carbonate [pH 9.4]) to 5µg ml^{-1})

Sodium dodecyl sulfate (SDS)-resolving buffer

(36.34 g Tris base, 8 ml 10% SDS add de-ionised water (dH_2O) up to 200 ml, pH at 8.8) or 1.5 M Tris-Chloride (FW 121.1), pH 8.8, 0.4% SDS.

SDS-staking buffer

12.114 g Tris base, 8 ml 10% SDS) add dH2O up to 200 ml, pH at 6.8 or 0.5 M Tris-Chloride (FW 121.1), pH 8.8, 0.4% SDS.

SDS running buffer (10×)

(30.3 g (0.25 M) Tris base, 187.7 g (2.5 M) Glycine, 950 ml dH2O, 10 g SDS (1%), made up to 1000 ml with dH_2O, mix well.

SDS-sample buffer (5×)

0.62 M Tris-Cl (pH 6.8), 5% SDS, 25% glycerol, 12.5% β- meracaptoethanol, 0.002% Bromophenol blue, or 7.8125 ml (2 M) Tris-Cl, 1.25 g SDS, 6.25 ml glycerol, 3.125 ml β-meracaptoethanol, traces of bromophenol blue, made up to 25 ml with dH_2O.

Resolving (separating) gel

1.7 ml SDS-resolving buffer, 2.33 ml Acrylamide/Bis-Acrylamide (30%), 2.88 ml dH_2O, 30 µl 10% ammonium persulfate (APS) and 30 µl Tetramethyl ethylenediamine (TEMED).

Staking gel

1 ml SDS-stacking buffer, 0.8 ml Acrylamide/Bis-Acrylamide (30%), 2.18 ml dH_2O, 30 µl 10% APS and 30 µl TEMED.

Semi-dry blotting buffer

5.82 g Tris base, 2.93 g Glycine, 3.75ml 10% SDS, 200 ml methanol and make up to 1000 ml with dH_2O.

Phosphate buffered saline solution (PBS)

1 × PBS was prepared by dissolving 1 tablet of Phosphate buffered saline (Dulbecco, Oxoid) in 100 ml dH_2O and autoclave, this gives sodium chloride 0.16 mol, Potassium chloride 0.003 mol, Disodium hydrogen phosphate 0.008 mol and Potassium dihydrogen phosphate 0.001 mol with a pH value of 7.3

BSA (Albumin from bovine serum, Sigma A3912) as lyophilized powder (M W c. 66 kDa) was prepared in sterile PBS according to the concentration needed.

Blocking buffer

2.52 g skimmed milk powder (Marvel), 50 ml PBS/0.05% Tween-20 (400 ml PBS with 200 µl Tween20).

Washing buffer

400 ml PBS/200 µl Tween-20

Lysogeny broth (LB)

Tryptone 10 g, Yeast extracts 5 g and sodium chloride 10 g for 1000 ml dH_2O.

LB agar

Tryptone 10 g, Yeast extract 5 g, Sodium chloride 10 g, Microbial tested agar 15 g for 1000 ml of dH_2O, pH 7.0 ± 0.2 at 25°C.

IPTG (Isopropyl-β-D-1 thiogalactopyranoside [FW 238.8])

1 M solution stock solution was prepared by dissolving 0.23 g of IPTG in 1 ml dH_2O, sterilized by filtration and stored at -20°C.

LB ampicillin/IPTG/X-gal agar

LB agar cooled at 50°C, 100 µg ml^{-1} Ampicillin, 0.5 mM IPTG (dissolved in dH_2O) and 80 µg ml^{-1} X-gal (dissolved in dimethylformamide), mix and pour into plates

DNA loading dye (10×)

10 mM Tris-HCl (pH 7.6), 0.03% Bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol, 60 mM Ethylenediamine tetraacetic acid (EDTA)

Buffers used for protein purification under denaturing condition

Lysis Buffer (buffer B)

100 mM NaH_2PO_4, 10 mM Tris-Cl, and 8 M Urea, pH 8.0

Wash Buffer (buffer C)

100 mM NaH_2PO_4, 10 mM Tris-Cl, and 8 M Urea, pH 6.3

Elution Buffer (buffer E)

100 mM NaH_2PO_4, 10 mM Tris-Cl, and 8 M Urea, pH 4.5

Buffers for protein purification under native condition

Lysis Buffer

50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, and pH adjusted to 7.4 using NaOH

Wash buffer

50 mM NaH_2PO_4, 300 mM NaCl, 15 mM imidazole, and pH adjusted to 7.4 using NaOH.

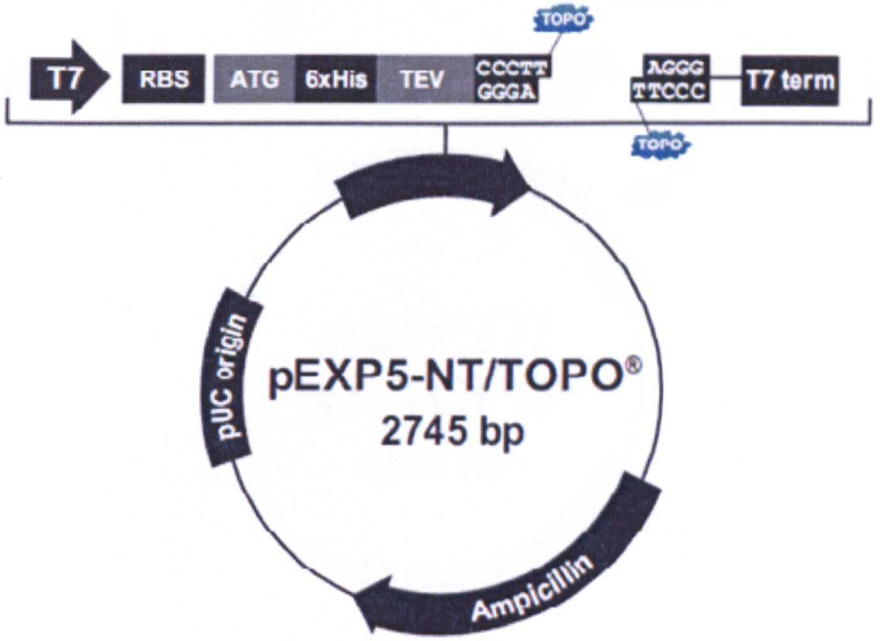
Elution buffer

50 mM NaH_2PO_4, 300 mM NaCl, 300 mM imidazole, and pH adjusted to 7.4 using NaOH.

APPENDIX- B: Vectors and plasmids

Vectors

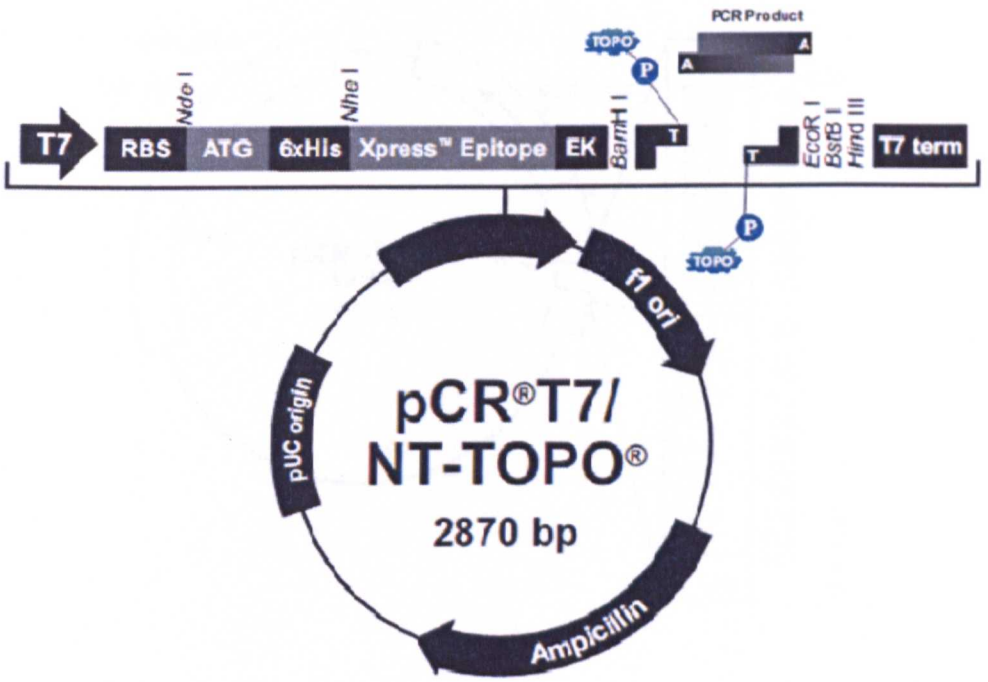
A- Schematic diagram of pEXP-NT/TOPO (Invitrogen)



Comments for pEXP5-NT/TOPO®
2745 nucleotides

T7 promoter: bases 1-17
T7 forward priming site: bases 1-20
Ribosome binding site (RBS): bases 68-73
Initiation ATG: bases 80-82
Polyhistidine (6xHis) region: bases 92-109
HisG epitope: bases 92-112
TEV recognition site: bases 122-142
TOPO® recognition site 1: bases 141-145
TOPO® recognition site 2: bases 146-150
T7 reverse priming site: bases 198-217
T7 transcription terminator: bases 159-287
bla promoter: bases 399-497
Ampicillin resistance gene: bases 498-1358
pUC origin: 1503-2176

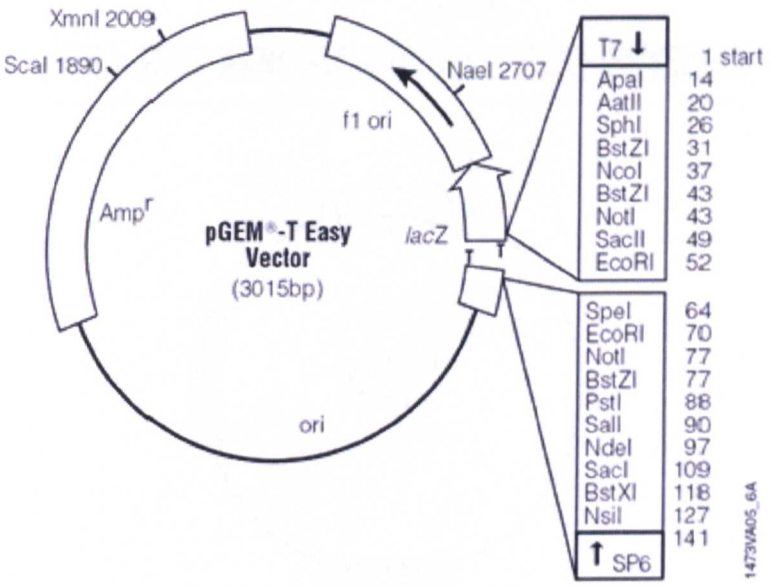
B- **Schematic diagram of pCR® T7/NT-TOPO (invitrogen)**



Comments for pCR®T7/NT-TOPO®
2870 nucleotides

T7 promoter: bases 20-36
T7 promoter priming site: bases 20-39
Ribosome binding site: bases 87-90
Initiation ATG: bases 100-102
Polyhistidine (6xHis) region: bases 112-129
Xpress™ epitope: bases 169-192
EK recognition site: bases 178-192
TOPO® Cloning site: bases 204-205
T7 reverse priming site: bases 270-289
T7 transcription termination region: bases 231-360
f1 origin: 431-886
Ampicillin resistance gene (ORF): bases 1017-1877
pUC origin: 2022-2695

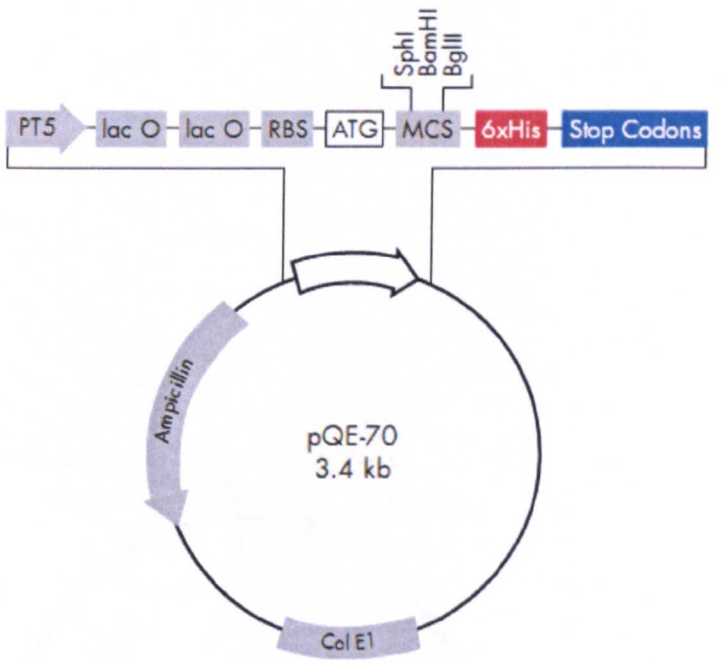
C- Schematic diagram of pGEM-T Easy vector (Promega)



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

D- Schematic diagram of pQE-70 (Qiagen)

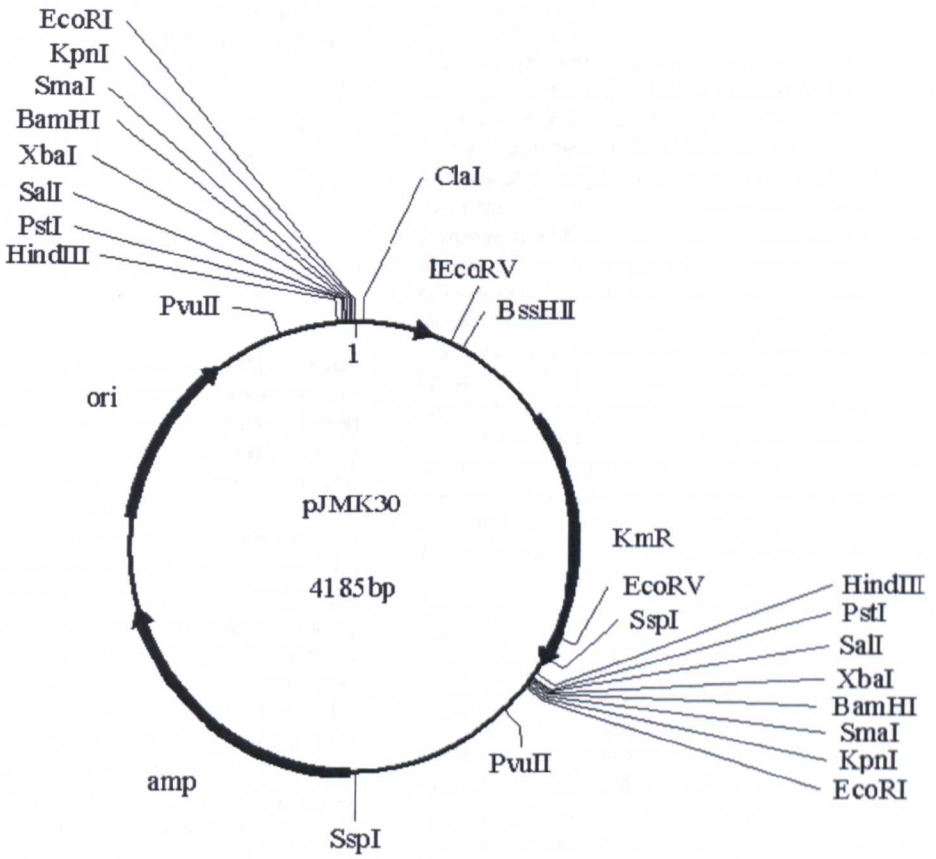


pQE-70

pQE-70 Vector

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

E- Schematic diagram of pJMK-30 (source of Kan^R cassette)



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The moonlighting protein fructose-1, 6-bisphosphate aldolase of *Neisseria meningitidis*: surface localization and role in host cell adhesion

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Summary

Fructose-1, 6-bisphosphate aldolases (FBA) are cytoplasmic glycolytic enzymes, which despite lacking identifiable secretion signals, have also been found localized to the surface of several bacteria where they bind host molecules and exhibit non-glycolytic functions. *Neisseria meningitidis* is an obligate human nasopharyngeal commensal, which has the capacity to cause life-threatening meningitis and septicemia. Recombinant native *N. meningitidis* FBA was purified and used in a coupled enzymic assay confirming that it has fructose bisphosphate aldolase activity. Cell fractionation experiments showed that meningococcal FBA is localized both to the cytoplasm and the outer membrane. Flow cytometry demonstrated that outer membrane-localized FBA was surface-accessible to FBA-specific antibodies. Mutational analysis and functional complementation was used to identify additional functions of FBA. An FBA-deficient mutant was not affected in its ability to grow *in vitro*, but showed a significant reduction in adhesion to human brain microvascular endothelial and HEp-2 cells compared to its isogenic parent and its complemented derivative. In summary, FBA is a highly conserved, surface exposed protein that is required for optimal adhesion of meningococci to human cells.

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Introduction

Neisseria meningitidis remains an important cause of septicemia and meningitis and is associated with high morbidity and mortality (Stephens *et al.*, 2007). As an obligate human commensal, it colonizes the nasopharyngeal mucosa of a substantial proportion of the population in an asymptomatic manner. In susceptible hosts, hyperinvasive strains of meningococci possess the ability to invade the nasopharyngeal sub-mucosa and enter the bloodstream, where they can multiply rapidly to high levels. Meningococci may also translocate across the brain vascular endothelium, proliferate in the cerebralspinal fluid (CSF) and cause meningitis (Stephens, 2009). To reach the meninges, *N. meningitidis* must therefore interact with two cellular barriers and adhesion to both epithelial and endothelial cells are crucial stages of infection. Various bacterial factors including lipooligosaccharide (LOS), capsule, type IV pili and outer membrane adhesins such as Opa, Opc, NhhA, App, NadA and MspA have been shown to have a role in meningococcal adhesion and invasion of epithelial and/or endothelial cells (reviewed in Virji, 2009).

Glycolytic enzymes, such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH), enolase and fructose bisphosphate aldolase (FBA) have been reported as potential virulence factors in a variety of organisms (Pancholi and Chhatwal, 2003). Although long recognized for their cytosolic role in glycolysis and gluconeogenesis, additional or 'moon-lighting' functions have been increasingly recognized. In particular, despite lacking identifiable secretion signals, glycolytic enzymes have been found on the bacterial cell surface where they interact directly with host soluble proteins and surface ligands. In *Mycoplasma genitalium*, surface-associated GAPDH was shown to be important for adhesion to human mucin (Alvarez *et al.*, 2003). In *Streptococcus pyogenes* and *Candida albicans* surface-associated GAPDH was shown to bind to fibronectin (Pancholi and Fischetti, 1992; Gozalbo *et al.*, 1998) and in *Staphylococcus aureus* the cell wall transferrin-binding protein was found to be GAPDH (Modun and Williams, 1999). GAPDH was also reported to be a virulence-associated immunomodulatory protein in

Streptococcus agalactiae (Madureira *et al.*, 2007). Surface-associated enolase has been reported as a plasminogen-binding protein in *S. pyogenes* (Pancholi and Fischetti, 1998), a fibronectin and plasminogen-binding protein in *Streptococcus suis* (Esgleas *et al.*, 2008; Tian *et al.*, 2009), and a plasminogen and laminin-binding protein in *Bacillus anthracis* (Agarwal *et al.*, 2008). In *Streptococcus pneumoniae*, surface-associated FBA was shown to bind to a large 7-transpass transmembrane receptor belonging to the cadherin superfamily (Blau *et al.*, 2007). FBA and GAPDH were also shown to be immunogenic in humans and capable of inducing a protective immune response against *S. pneumoniae* in mice (Ling *et al.*, 2004). In addition, FBA was found to be a surface-localized immunogenic protein in *S. suis* (Zongfu *et al.*, 2008) and a possible role for FBA in immunity to *Onchocerca volvulus* has also been reported (Mccarthy *et al.*, 2002).

Fructose-1, 6-bisphosphate aldolase catalyses the reversible cleavage of fructose-1, 6-bisphosphate into dihydroxyacetone phosphate and glyceraldehyde 3-phosphate (Zgiby *et al.*, 2000; Wehmeier, 2001; Ramsaywak *et al.*, 2004). Aldolases can be divided into two groups with different catalytic mechanisms, designated Class-I and Class-II respectively (Thomson *et al.*, 1998; Arakaki *et al.*, 2004). Class-I FBAs utilize an active site lysine residue to stabilize a reaction intermediate via Schiff-base formation, and are usually found in higher eukaryotic organisms (animals and plants). Class-II FBAs have an absolute requirement for a divalent ion, usually zinc (Zgiby *et al.*, 2000) and are commonly found in bacteria, archae and lower eukaryotes including fungi and some green algae grown under heterotrophic conditions (Plater *et al.*, 1999; Sauvé and Sygusch, 2001; Ramsaywak *et al.*, 2004). Most organisms contain only one class of FBA, although a few possess enzymes of both classes. *Escherichia coli* (Alefounder *et al.*, 1989; Thomson *et al.*, 1998), *S. pneumoniae* (Isabel *et al.*, 1999) and *Synechocystis* sp. PCC 6803 (Nakahara *et al.*, 2003) among others have been reported to express both types of the enzyme. The Class-II FBAs can be subdivided into two groups, Type A and B, depending on their amino acid sequences (Sauvé and Sygusch, 2001; Nakahara *et al.*, 2003). Because Class-II FBAs are not found in animals, it has been suggested that they could provide a possible therapeutic or vaccine target (Blom *et al.*, 1996; Ramsaywak *et al.*, 2004).

In *N. meningitidis*, it is noteworthy that, due to the absence of the enzyme phosphofructokinase, the Embden-Meyerhof-Parnas (EMP) glycolytic pathway is rendered non-functional (Baart *et al.*, 2007). Instead, the catabolism of glucose has been shown to be carried out through the Entner Douderoff (ED) and Pentose Phosphate pathways (PP) (Baart *et al.*, 2007). Nevertheless, the meningococcal genome retains functional genes for other glycolytic pathway enzymes, presumably for alternative (non-EMP pathway) functions. Furthermore, GAPDH expression (GapA1, but not GapA2) was found to be up-regulated on the meningococcal cell surface following contact with human epithelial cells (Grifantini *et al.*, 2002), although no biological function has so far been ascribed to this observation. In addition, enolase has recently been shown to be a surface-localized protein in *N. meningitidis*, where it acts to recruit plasminogen onto the bacterial surface (Knaust *et al.*, 2007). The available *N. meningitidis* genome sequences contain a single, putative Class II FBA-encoding gene (*cbbA*), which has not previously been characterized. The aim of this study was to characterize the enzymatic function, sub-cellular localization and putative role of FBA in the pathogenesis of meningococcal infection.

Results

Sequence analysis of the cbbA *gene, flanking DNA and FBA protein*

In *N. meningitidis* strain MC58, the 1065 bp *cbbA* gene (locus tag NMB1869) has a G+C content of 55.18% and encodes a predicted protein of 354 amino acids (estimated molecular weight 38.3 kDa). The *cbbA* gene is downstream of, and in the opposite orientation to, *xerC* (NMB1868) encoding the XerC integrase/recombinase and upstream of, and in the same orientation as, NMB1870, which encodes factor H-binding protein, fHbp (Madico *et al.*, 2006). A similar genomic arrangement is present in the serogroup A meningococcal strain Z2491 (NMA0588, NMA0587 and NMA0586 encoding XerC, FBA and fHbp respectively; Parkhill *et al.*, 2000), the serogroup C strain FAM18 (Bentley *et al.*, 2007) and the ST-4821 strain 053442 (Peng *et al.*, 2008), suggesting that this is a conserved arrangement. In these three genomes, the *cbbA* sequences are > 94% identical to the MC58 *cbbA* gene. Additionally, sequences > 92% identical to MC58 *cbbA* are found in the gonococcal strain FA1090 (94% identical) and *N. lactamica* strain ATCC 23970 (93% identical) confirming that *cbbA* is highly conserved across *Neisseria* species. At the amino acid level, FBA sequences from meningococcal strains MC58, FAM18, 053442, Z2491 and the gonococcal strain FA1090 are > 99% identical. By alignment, the neisserial FBA protein (NMB1869) was 70, 67, 65 and 40% identical to Class-IIB FBA enzymes from *Cupriavidus metallidurans*, *Xanthobacter flavus*, *Synechocystis* sp. and *S. pneumoniae*, respectively, but was only 21 and 29% identical to the *E. coli* and *Haemophilus influenzae* Class-IIA FBA enzymes, respectively, indicating

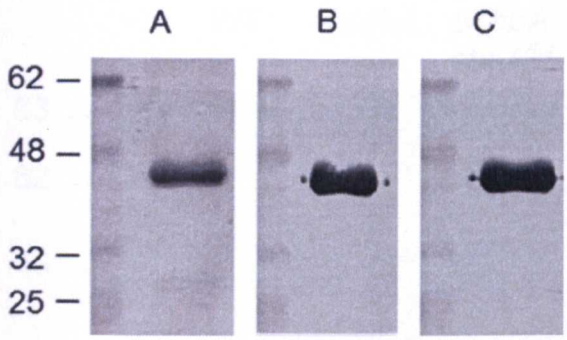

Fig. 1. SDS-PAGE analysis confirms the purity of the recombinant FBA purified under native conditions (A). Immunoblot analysis shows that recombinant FBA is recognized by RαFBA (B) and anti-pentahistidine antibodies (C).

that the neisserial FBA belongs to Class-IIB. Furthermore, the neisserial FBA enzyme contains a 21-amino acid insertion sequence (S^{236}-Y^{256}), which is unique to two subclasses of the Class IIB enzymes. The presence of this insertion sequence suggests that the neisserial FBA enzyme may have a tetrameric quaternary structure, rather than the dimeric structure, which is typical of Class II FBA enzymes that lack this sequence (Sauvé and Sygusch, 2001; Izard and Sygusch, 2004). As expected for a Class-II FBA, a conserved putative zinc/cobalt-binding site (Berry and Marshall, 1993) was also identified (H^{81}-XX-H^{84}). FBA of *N. meningitidis* was predicted to be a non-secreted protein by the SignalP-HMM program; although a possible 13-amino acid signal peptide (predicted cleavage site ^{11}DHA-AE^{15}) was identified by SignalP-NN. A signal peptide was similarly predicted for the Class-IIB FBA homologue in *X. flavus*, but not for the homologue in *Synechocystis* sp. or the *E. coli* Class-IIA FBA.

Cloning, expression and purification of recombinant FBA

To examine the aldolase function of meningococcal FBA, and to raise FBA-specific antibodies, the *cbbA* gene from MC58 was cloned into the expression vector pQE70 to facilitate the expression and subsequent purification of 6× histidine-tagged recombinant FBA (rFBA). After induction of *E. coli* cells harbouring the FBA expression plasmid, a recombinant protein with an apparent molecular mass consistent with the predicted mass of the tagged protein was strongly expressed, affinity-purified under non-denaturing conditions (Fig. 1A) and used to generate rabbit anti-FBA-specific polyclonal antiserum (RαFBA). Immunoblot analysis confirmed that RαFBA and anti-pentahistidine antibodies both reacted to the purified rFBA (Fig. 1B and C).

Meningococcal FBA has fructose bisphosphate aldolase activity

A previously described coupled enzymic assay (Berry and Marshall, 1993) was used to confirm that the purified native meningococcal FBA was active as an FBA (Fig. 2). Kinetic parameters of the purified enzyme for cleavage of fructose bisphosphate (FBP) were estimated as K_m (FBP) = 0.05 mM and k_{cat} = 126 min^{-1}. These values are similar to those found for Class-II FBA enzymes from a variety of sources such as *E. coli* (K_m (FBP) ~ 0.19 mM and k_{cat} ~ 490 min^{-1}) (Plater *et al.*, 1999).

Mutagenesis of cbbA *and strain survey*

To examine any additional roles of FBA, a *cbbA* knockout derivative of *N. meningitidis* MC58 was generated. To achieve this, the *cbbA* gene plus flanking DNA was amplified and cloned, and inverse PCR was employed to remove the open reading frame. The product was then ligated to a kanamycin resistance marker and the resulting plasmid used to transform *N. meningitidis* MC58. Using this strategy, the *cbbA* gene was successfully mutated to yield MC58Δ*cbbA*. The genotype of this mutant was confirmed by PCR and sequencing (data not shown). Immunoblotting using RαFBA showed that a *c.* 38 kDa protein corresponding to FBA could be detected in whole cell lysates of wild-type but not MC58Δ*cbbA* (Fig. 3, lanes 1 & 2) confirming that FBA is expressed under the

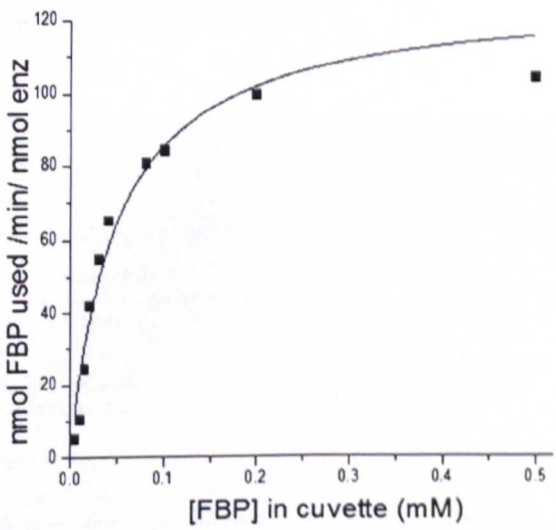

Fig. 2. Coupled enzymic assay to measure the activity of meningococcal fructose 1, 6-bisphosphate aldolase. Cleavage of fructose 1, 6-bisphosphate (FBP) was coupled to α-glycerophosphate dehydrogenase and NAD oxidation. One unit of aldolase activity was defined as the amount of enzyme which catalysed the oxidation of 2 μmol NADH min^{-1}.

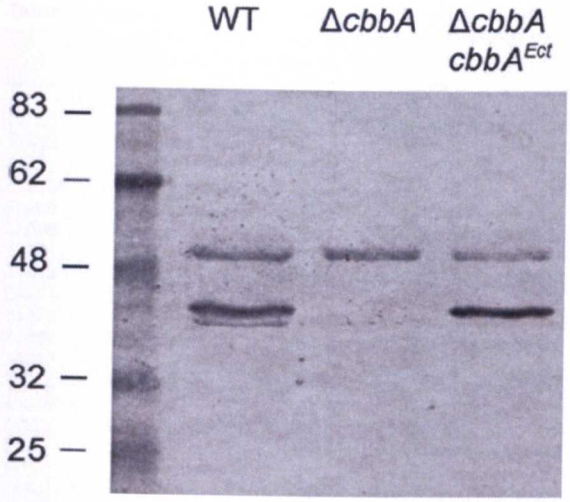

Fig. 3. Immunoblot analysis of whole cell proteins from the *N. meningitidis* MC58 wild-type, Δ*cbbA* mutant derivative and complemented mutant reveals the absence of FBA in the Δ*cbbA* mutant preparation.

conditions used and that expression had been abolished in the mutant. In addition to the strongly reactive FBA band, immunoblot analysis showed an additional cross-reactive band at *c.* 50 kDa (Fig. 3). However, this band was also present in preparations of the Δ*cbbA* mutant demonstrating that this protein was not FBA. To further confirm that the *c.* 38 kDa immuno-reactive protein was FBA, a wild-type copy of *cbbA* was introduced *in trans* into MC58Δ*cbbA* using the pYHS25-based plasmid pSAT-12 (Table 1). Introduction of *cbbA* at an ectopic site restored FBA expression (Fig. 3, lane 3). Further immunoblot analyses using a panel of 25 *N. meningitidis* strains (Table 2) including representatives of differing serogroups and sequence types showed that FBA expression was conserved across all strains (data not shown). Expression was also conserved in representative examples of *N. lactamica, N. polysacchareae* and *N. gonorrhoeae* examined (data not shown). These data complement *in silico* predictions that *cbbA* is universally present and constitutively expressed across *Neisseria* strains including commensal species.

Meningococcal FBA is localized to the cytoplasm and outer membrane

The sub-cellular localization of FBA was investigated by sub-cellular fractionation followed by immunoblot analysis of the fractions. FBA was predominately detected in outer membrane and cytosolic protein-enriched fractions, but was absent from the cytoplasmic membrane-enriched fraction (Fig. 4). FBA could also be detected in the periplasmic protein-enriched fraction, possibly representing transient FBA during translocation to the outer membrane (Fig. 4). FBA was not detected in concentrated culture supernatants (data not shown). Immunoblotting experiments with antisera against PorA, a known outer membrane protein of *N. meningitidis*, gave an identical profile except that PorA was absent in the cytosolic fraction (data not shown). These results demonstrate that meningococcal FBA is predominantly a cytosolic protein that is also found in the outer membrane.

Table 1. Bacterial strains and plasmids.

Strain or plasmid	Description	Source or reference
Strains		
E. coli		
JM109	*end*A1 *rec*A1 *gyr*A96 *thi hsd*R17 ($r_K^-m_K^-$) *rel*A1 *sup*E44 Δ(*lac-pro*AB) [F′ *tra*D36 *pro*AB *laq*I^{q}ZΔM15]	Promega
TOP10F′	F′*lac*I^q*Tn*10(TetR) *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L *end*A1 *nup*G	Invitrogen
BL21(DE3)pLysS	F- *ompT hsd*S$_B$ (r$_B$-m$_B$-) *gal dcm* (DE3) pLysS (CamR)	Invitrogen
N. meningitidis		
MC58	wild-type serogroup B strain	Tettelin *et al.*, 2000
MC58Δ*cbbA*	*cbbA* deletion and replacement with kanamycin cassette	This study
MC58Δ*cbbA cbbA*^{Ect}	MC58Δ*cbbA* complemented with an ectopic copy of *cbbA*	This study
Plasmids		
pQE70	Cloning vector encoding resistance to ampicillin	Qiagen
pSAT-FBA	MC58 *cbbA* gene cloned in pQE70	This study
pGEM-T Easy	Cloning vector encoding resistance to ampicillin	Promega
pSAT-2	2.3 kb fragment spanning the MC58 *cbbA* region cloned in pGEM-T Easy	This study
pJMK30	Source of kanamycin resistance cassette	van Vliet *et al.*, 1998
pSAT-4	pSAT-2 containing the kanamycin resistance cassette in the same orientation as the deleted *cbbA* gene	This study
pYHS25	Ectopic complementation vector encoding resistance to erythromycin	Winzer *et al.*, 2002
pSAT-12	pYHS25 containing *cbbA*	This study

Table 2. Isolates of *N. meningitidis* examined for the expression of FBA.

Strain[a]	Country of origin	Date of isolation	Disease	Serogroup	Sequence type	Clonal complex
Z1001	USA	1937	Invasive (unspecified)	A	4	ST-4 complex/subgroup IV
Z1035	Pakistan	1967	Meningitis and septicaemia	A	1	ST-1 complex/subgroup I/II
Z1054	Finland	1975	Invasive (unspecified)	A	5	ST-5 complex/subgroup III
Z1213	Ghana	1973	Invasive (unspecified)	A	4	ST-4 complex/subgroup IV
Z1269	Burkina Faso	1963	Invasive (unspecified)	A	4	ST-4 complex/subgroup IV
Z1503	China	1984	Invasive (unspecified)	A	5	ST-5 complex/subgroup III
Z3771	UK	1987	Invasive (unspecified)	A	5	ST-5 complex/subgroup III
Z3842	Norway	1976	Invasive (unspecified)	B	32	ST-32 complex/ET-5 complex
Z4181	Mali	1989	Carrier	C	11	ST-11 complex/ET-37 complex
Z4323	Israel	1988	Invasive (unspecified)	C	11	ST-11 complex/ET-37 complex
Z4662	Netherlands	1967	Invasive (unspecified)	B	8	ST-8 complex/Cluster A4
Z4667	Netherlands	1963	Invasive (unspecified)	B	48	ST-41/44 complex/Lineage 3
Z4673	Netherlands	1986	Invasive (unspecified)	B	41	ST-41/44 complex/Lineage 3
Z4676	Denmark	1962	Invasive (unspecified)	B	37	ST-37 complex
Z4678	East Germany	1985	Invasive (unspecified)	B	19	ST-18 complex
Z4684	Norway	1988	Invasive (unspecified)	B	13	ST-269 complex
Z4685	Norway	1988	Carrier	B	14	ST-269 complex
Z4701	Norway	1969	Invasive (unspecified)	B	11	ST-11 complex/ET-37 complex
Z5826	China	1992	Invasive (unspecified)	A	7	ST-5 complex/subgroup III
Z6413	South Africa	1990	Invasive (unspecified)	C	8	ST-8 complex/Cluster A4
Z6414	New Zealand	1994	Invasive (unspecified)	C	66	ST-8 complex/Cluster A4
Z6417	UK	1993	Invasive (unspecified)	C	11	ST-11 complex/ET-37 complex
Z6418	Cuba	1992	Invasive (unspecified)	B	33	ST-32 complex/ET-5 complex
Z6419	Austria	1991	Invasive (unspecified)	B	40	ST-41/44 complex/Lineage 3
Z6420	Greece	1992	Invasive (unspecified)	B	41	ST-41/44 complex/Lineage 3

a. Further details of strains are available at http://pubmlst.org/

Meningococcal FBA is surface accessible to antibodies

In order to investigate whether the outer membrane-localized FBA was accessible on the bacterial cell surface, RαFBA antibodies were used to probe intact meningococcal cells which were then analysed by flow cytometry. MC58 cells treated with RαFBA alone or secondary antibody alone did not produce high fluorescence signals (3.4 and 4.4 mean fluorescence intensities, respectively), while cells treated with RαFBA followed by anti-rabbit IgG-Alexa Flour 488 conjugate demonstrated a clear shift in fluorescence signal (55.2 mean fluorescence intensity) confirming the cell surface localization of FBA (Fig. 5A). No shift in fluorescence signal was observed when MC58Δ*cbbA* cells were examined under identical conditions [Fig. 5B; mean fluorescence intensity of 12.1 compared to samples treated with primary or secondary alone (3.6 and 6.7, respectively)]. From the wild-type cells probed with both antibodies, 79.05% were found in the M1 region (Fig. 5A), suggesting that the majority of the population had FBA present on the cell surface. Pre-immune serum showed no reactivity against wild-type MC58 in immunoblot experiments confirming that the binding of RαFBA to wild-type MC58 observed by flow cytometry was FBA-specific.

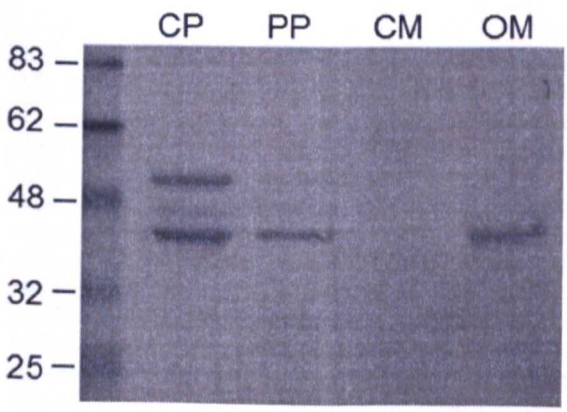

Fig. 4. Sub-cellular localization of FBA. Cytosolic protein-enriched (CP), periplasmic protein-enriched (PP), cytoplasmic membrane protein-enriched (CM) and outer membrane protein-enriched (OM) fractions of MC58 were separated on a 10% acrylamide gel and probed in immunoblotting experiments with RαFBA.

FBA is required for efficient adhesion to host cells

Viable counts of bacteria associated with homogenized infected monolayers were used to compare the capacity of the wild-type, FBA mutant and complemented mutant strains to associate with, and invade human brain microvascular endothelial (HBME) cells. FBA-deficient meningococci had a significantly reduced capacity to adhere to monolayers of HBME cells (Fig. 6A). No statistically significant reduction was observed in the ability

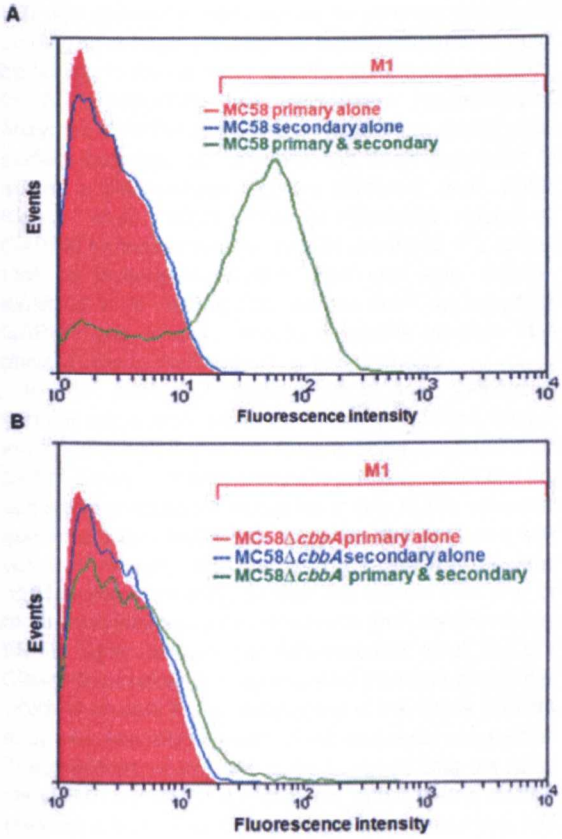

Fig. 5. Flow cytometric analysis of MC58 wild-type (A) or MC58Δ*cbbA* cells (B) for FBA surface localization. Cells were stained with RαFBA (primary alone), anti-rabbit IgG-Alexa Flour 488 conjugate (secondary alone) or both. Fluorescence was displayed as a histogram. The histogram area in M1 represents the population of fluorescently labelled meningococci.

of the FBA mutant to invade monolayers of HBME cells (Fig. 6B). Similar results were also obtained using HEp-2 (human larynx carcinoma) cells, confirming that the effect was not cell-type specific. To confirm that the observed effects were not due to an impairment in *in vitro* growth, the growth rate of the strains was compared by measuring the optical density at 600 nm (OD_{600}) and determining the viable counts of broth cultures sampled during exponential growth over 24 h in triplicate on three separate occasions. No significant difference between strains was observed (data not shown). To further exclude the possibility that mutation of *cbbA* affected expression of the downstream gene encoding factor H binding protein, whole cell lysates of MC58, MC58Δ*cbbA* and MC58Δ*cbbA* *cbbA*^{Ect} were probed with anti-fHbp. Expression levels of this protein were similar in the three strains. In summary, these experiments show that FBA plays a role in the adherence of *N. meningitidis* to human cells.

Discussion

An increasing number of reports show that classical cytoplasmic house-keeping enzymes without identifiable secretion signals may be localized to the surface of microbial pathogens, where they exhibit various functions, unrelated to glycolysis (Pancholi and Chhatwal, 2003). One such protein, fructose-1, 6-bisphosphate aldolase (FBA) has been previously reported to be localized to the surface of some Gram-positive bacteria. In *S. pneumoniae*, for example, surface-exposed FBA (Class

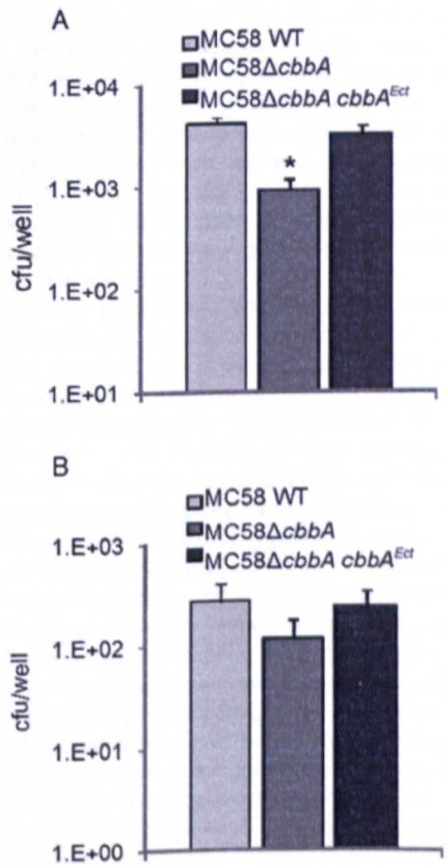

Fig. 6. FBA-deficient meningococci have a reduced ability to associate with (A) but not invade into (B) HBME cells compared with the wild-type or complemented strains. The number of FBA-deficient meningococci associating was significantly lower than the wild-type (*$P = 0.0011$). Numbers of mutant cells invading were not significantly lower compared with the wild-type ($P = 0.13$). Similar experiments were also carried out using HEp-2 cells with consistent results. Mean levels shown from three independent experiments, each using triplicate wells. Bars denote standard deviation. Cfu denotes colony forming units.

IIB) was demonstrated to act as an adhesin, specifically binding to a large 7-transpass transmembrane receptor belonging to the cadherin superfamily (Blau *et al.*, 2007). In *N. meningitidis*, two cytoplasmic house-keeping enzymes, GAPDH and enolase, have been shown to be surface-localized, and enolase has been suggested to act as a plasminogen receptor (Grifantini *et al.*, 2002; Knaust *et al.*, 2007). The non-glycolytic role(s) of GAPDH on the bacterial surface is undefined. We undertook to investigate whether FBA was also surface-localized in *N. meningitidis*, as has been described for GAPDH and enolase, and to determine whether FBA plays a role in meningococcal pathogenesis.

In the published meningococcal and gonococcal genome sequences, there is only one gene, *cbbA*, encoding a putative FBA enzyme. Despite being predicted to be part of a non-functional metabolic pathway, *cbbA* has not acquired spontaneous mutations in any of the neisserial genomes examined, which suggests that the gene was acquired recently, that the glycolytic pathway became non-functional recently, or that the protein has one or more additional functions. Sequence analysis shows that FBA is highly conserved at the amino acid level, and is a Class-IIB enzyme. A 21-amino acid insertion sequence, which is present in two subclasses of the Class IIB FBA enzymes, was also present in the neisserial sequences. The presence of this sequence suggests that the neisserial FBA enzyme is a tetrameric, rather than a dimeric enzyme; a feature which is present in extremophiles, and which has been suggested to confer thermal stability (Sauvé and Sygusch, 2001; Izard and Sygusch, 2004). Unexpectedly for a presumed cytosolic protein, a possible signal sequence was predicted for the neisserial FBA enzyme, suggesting a possible means of translocation across the cytoplasmic membrane.

In this study, rFBA was expressed and purified under non-denaturing conditions. Purified rFBA was shown to have aldolase activity confirming that the enzyme was in native conformation after purification. The purified protein was also used to generate rabbit polyclonal anti-FBA antiserum (RαFBA), which was used to confirm that FBA was expressed *in vitro* in each of a range of neisserial strains tested including commensal species. This suggests that FBA plays an important role which is required by both non-pathogenic and pathogenic lineages. FBA was shown to be present in both the cytosol and to be exposed at the cell surface of wild-type meningococci in a form that was accessible to antibodies, suggesting that, similar to GAPDH and enolase, FBA is translocated (or diverted) to the outer membrane. An alternative hypothesis is that FBA is released from lysed cells and recruited back onto the surface of intact meningococci; however, we have probed lysates of *cbbA*-deficient meningococci following co-incubation with rFBA and found no reactivity with RαFBA (data not shown). In *M. genitalium*, only a small proportion of the total cellular GAPDH is trafficked to the bacterial surface; however, this is sufficient to impart a biologically significant phenotype (mucin-binding) on this organism (Alvarez *et al.*, 2003). For organisms with relatively small genomes, multi-functional proteins may be advantageous to optimize the potential of the genome. To our knowledge, this is the first report demonstrating that a proportion of FBA is found on the cell surface of meningococci.

An FBA-deficient mutant grew at the same rate (in broth culture and on solid media) as the wild-type and the complemented mutant strains, demonstrating that FBA is not required for growth of the meningococcus under the *in vitro* conditions used. No differences in either colony or bacterial cell morphology (using light microscopy) were observed. The FBA-deficient mutant strain exhibited a significantly reduced capacity to adhere to both HBME and HEp-2 cells. This phenotype was completely restored in a complemented strain. Our observation that FBA is involved in adhesion to both epithelial and endothelial cells, and that FBA expression is conserved in non-pathogenic strains (such as *N. polysaccharea*e) may suggest a role for FBA during colonization of the nasopharyngeal mucosa by commensal neisserial species.

Although we have shown that FBA is present on the meningococcal cell surface and is required for optimal adhesion, the role of FBA in this process is unknown. It is possible that the enzymatic activity of FBA plays an indirect role that is required for optimal adhesion. However, given that other FBA homologues (such as FBA in *S. pneumoniae*) have been shown to directly bind to host cell ligands it is also possible that the meningococcal protein has a direct host receptor-binding activity. Determining the ability of meningococci expressing a non-enzymatically functional FBA to adhere to host cells would address this. *S. pneumoniae* FBA was shown to bind to a cadherin superfamily receptor (Flamingo cadherin receptor, FCR) on the surface of host epithelial cells, but it is unknown which FBA residues participate in this interaction (Blau *et al.*, 2007). Meningococcal FBA is only 40% identical to the pneumococcal enzyme at the amino acid level, so it is unclear whether meningococcal FBA binds the same receptor. Work is currently in progress to determine whether meningococcal FBA binds to FCR or a different host cell receptor.

Pneumococcal FBA has been shown to be immunogenic in humans and capable of eliciting a partially protective immune response against lethal *S. pneumoniae* intranasal challenge in mice (Ling *et al.*, 2004). Given that meningococcal FBA is highly conserved, expressed by a wide range of isolates, surface-accessible to antibodies and structurally and antigenically unrelated to the human

Table 3. List of primers used in this study.

Primer	DNA sequence[a]	Restriction site
Expression		
FBA_pQE70 (F)	CGC**GGATCC**ATGGCACTCGTATCCATGCG	SphI
FBA_pQE70 (R)	CGC**GGTACC**GTCGTCCGAACGGCGG	BglII
Mutagenesis		
FBA_M1(F)	CTGCTGTGCCCGAGC	
FBA_M2(R)	CCGCTGCTGCAGGCG	
FBA_M3(IR)	GCG**AGATCT**TGTGTCTCCTTGGGCAATAGG	BglII
FBA_M4(IF)	GCG**AGATCT**GCTCCATCCAACTGGG	BglII
Complementation		
FBA_COM(F)	CGC**GGATCC**ATGAGCTGTTTATGGTTTTTTGCTG	BamHI
FBA_COM(R)	CGC**GGATCC**GGCATTTTGTTTACAGGCAACCTG	BamHI

a. All primers were designed from the *N. meningitidis* MC58 genome sequence. Sequences in bold identify restriction enzyme sites.

(Class I) FBA protein, meningococcal FBA is worthy of future study as a possible candidate vaccine component against this important human pathogen.

Experimental procedures

Bacterial strains and growth conditions

Escherichia coli TOP10F′ and BL21(DE3) pLysS (Table 1) were used for the expression of 6× histidine-tagged rFBA encoded by plasmid pSAT-FBA (Table 1). *E. coli* JM109 was used as a host strain for the construction of mutagenic and complementation plasmids, pSAT-4 and pSAT-12 respectively. *E. coli* strains were grown at 37°C in Luria-Bertani (LB) broth or on LB agar supplemented, where appropriate, with ampicillin (100 μg ml^{-1}), kanamycin (30 μg ml^{-1}) or erythromycin (200 μg ml^{-1}). Strains of *Neisseria* (Tables 1 and 2) were grown at 37°C, air plus 5% CO_2, on Brain Heart Infusion (BHI) agar supplemented with 1% Vitox (Oxoid) and kanamycin (50 μg ml^{-1}) or erythromycin (5 μg ml^{-1}) where appropriate.

DNA manipulation

Genomic DNA was extracted from *N. meningitidis* using the DNeasy Tissue kit (Qiagen). Plasmid DNA was prepared by using the QIAprep Spin kit (Qiagen). Restriction enzymes and T4 DNA ligase were purchased from Roche. All enzymatic reactions were carried out according to the manufacturer's instructions. DNA sequencing was carried out at the School of Biomedical Sciences (University of Nottingham) on an ABI 377 automated DNA sequencer.

Preparation of recombinant FBA

The *cbbA* gene was amplified from *N. meningitidis* MC58 using oligonucleotide primers FBA_pQE70 (F) and FBA_pQE70 (R) (Table 3) using the Expand High Fidelity PCR system (Roche). The resulting amplicon was digested with SphI and BglII, before being ligated into similarly treated pQE70, and the resulting plasmid, pSAT-FBA, used to transform *E. coli* BL21 (DE3) pLysS. Transformants were grown to log phase, induced with 1 mM IPTG for 3 h and harvested by centrifugation. Recombinant 6× histidine-tagged FBA was then affinity-purified under native conditions. Briefly, the culture pellet from an IPTG-induced culture of *E. coli* BL21 (DE3) pLysS (pSAT-FBA) was dissolved in 20 ml of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 7.4) and disrupted by sonication using a MSE Soniprep 150 for 10 cycles (each cycle consisting of a 10 s burst followed by a 10 s cooling period). The cell lysate was then mixed with 1 ml of HisPur™ Cobalt Resin (Pierce) and incubated overnight at 4°C. The lysate-resin mixture was then applied to a column, and washed with 50 mM NaH_2PO_4, 300 mM NaCl, 15 mM imidazole, pH 7.4. Bound protein was then eluted in elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 300 mM imidazole, pH 7.4).

Production of a rabbit antiserum against purified recombinant FBA

New Zealand White female rabbits were immunized subcutaneously four times at 2 week intervals with 30 μg of rFBA protein emulsified in Freud's complete (first immunization only) or incomplete adjuvant. After three injections, the animals were test bled, boosted once more and sacrificed 10 days later.

SDS-PAGE and immunoblotting

Proteins were electrophoretically separated using 10% polyacrylamide gels (Mini-Protean III; Bio-Rad) and were stained using SimplyBlue Safestain™ (Invitrogen) or transferred to nitrocellulose membranes as previously described (Kizil *et al.*, 1999). Membranes were probed with mouse anti-pentahistidine antibody (Qiagen) or rabbit primary antibody diluted 1:10 000 or 1:1000, respectively, in blocking buffer [5% (wt/vol) nonfat dry milk, 0.1% (vol/vol) Tween 20 in 1 × phosphate-buffered saline (PBS)] and incubated for 2 h. After being washed in PBS with 0.1% Tween 20 (PBST), membranes were incubated for 2 h with 1:30 000-diluted goat anti-mouse (or anti-rabbit) IgG-alkaline phosphatase conjugate (Sigma). After washing with PBST, blots were developed using BCIP/NBT-Blue liquid substrate (Sigma).

Kinetic analysis of fructose bisphosphate aldolase activity

This was done using a previously described methodology (Berry and Marshall, 1993). Briefly, the assay was performed at 30°C in 1 ml of 50 mM Tris-HCl supplemented with 0.1 M potassium acetate buffer (pH 8.0) containing 0.1–5 mM fructose 1, 6-bisphosphate (FBP), 0.2 mM NADH and 2 µl of a 10 mg ml^{-1} mixture of glycerol phosphate dehydrogenase/triose phosphate isomerase (coupling enzymes). The reagents were added in the order: buffer; FBP; NADH; coupling enzymes. Finally, the reaction was started by adding 0.26 nmol of purified native FBA. A decrease in absorbance at 340 nm was recorded as the measure of enzyme activity on an Uvikon 930 spectrophotometer. Activities were calculated using the molar extinction coefficient for NADH as 6220 M^{-1} cm^{-1}. One unit of aldolase activity was defined as the amount of enzyme which catalysed the oxidation of 2 µmol NADH min^{-1}. Kinetic parameters were determined using Origin Pro 7.5 software.

*Construction of MC58Δ*cbbA

A 2.3 kb fragment of DNA consisting of the *cbbA* gene and flanking DNA was amplified using FBA_M1(F) and FBA_M2(R) (Table 3) from *N. meningitidis* MC58 chromosomal DNA. The amplified DNA was TA cloned into the pGEM-T Easy vector to generate pSAT-2. This was then subject to inverse PCR using primers FBA_M3(IR) and FBA_M4(IF) (Table 3) resulting in the amplification of a 5 kb amplicon in which the *cbbA* coding sequence was deleted and a unique BglII site had been introduced. The BglII site was used to introduce a kanamycin resistance cassette, BamHI-digested from pJMK30 (Table 1), in place of *cbbA*. One of the resulting plasmids, pSAT-4, containing the resistance cassette in the same orientation as the deleted gene, was confirmed by restriction digestion and sequencing and subsequently used to mutate the meningococcal strain MC58 by natural transformation and allelic exchange as previously described (Hadi *et al.*, 2001). The deletion in the resulting mutant (MC58Δ*cbbA*) was confirmed by PCR analysis and immunoblotting. Growth curve assays carried out using liquid cultures showed no significant differences between MC58Δ*cbbA* and the wild-type strain (data not shown).

Complementation of cbbA

A fragment corresponding to the *cbbA* coding sequence and upstream promoter was amplified from chromosomal DNA of strain MC58 with High Fidelity Expand *Taq* (Roche) using the primers FBA_COM(F) and FBA_COM(R) (Table 3) incorporating BamHI sites into the amplified fragment. The BamHI-digested fragment was then introduced into a unique BamHI site in pYHS25. This vector contains an erythromycin resistance gene flanked by the MC58 genes NMB0102 and NMB0103 (Winzer *et al.*, 2002). The resulting plasmid pSAT-12 was used to transform MC58Δ*cbbA* by natural transformation, thus introducing a single chromosomal copy of *cbbA* and the downstream erythromycin resistance cassette in the intergenic region between NMB0102 and NMB0103. Insertion of the *cbbA* gene and erythromycin resistance cassette at the ectopic site was confirmed by PCR analysis and sequencing. Expression of FBA was confirmed by immunoblotting.

Sub-cellular localization of FBA

Cells from 100 ml of overnight BHI broth cultures were harvested at 13 000 *g* for 2 min and the pellet re-suspended in 1 ml of EB buffer (10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 25% sucrose), and washed twice in the same buffer. Finally, the pellet was re-suspended in EB buffer and incubated for 10 min on ice. The preparation was centrifuged at 13 000 *g* for 4 min; following re-suspension in 0.4 ml of ice cold water, the mixture was incubated on ice for a further 10 min, followed by centrifugation at 13 000 *g* for 2 min. The supernatant, containing periplasmic proteins, was removed and stored at –20°C. The remaining cell pellet (spheroplasts) were re-suspended into 0.4 ml of Tris-HCl (pH 7.5) and sonicated using a MSE Soniprep 150 for 10 cycles (each cycle consisting of a 10 s burst followed by a 10 s cooling period) to release the cytoplasmic contents. Non-disrupted cells were removed by centrifugation at 5000 *g* for 1 min. The upper clear supernatant was transferred to a fresh tube and centrifuged at 17 000 *g* for 30 min. The supernatant (representing the cytosolic fraction) was removed and stored at –20°C. The remaining pellet was re-suspended in 0.4 ml of 10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 2% Triton X-100. The sample was incubated at 37°C for 30 min and then centrifuged at 17 000 *g* for 30 min. The supernatant, enriched for cytoplasmic membrane proteins, was removed and stored at –20°C. The final pellet (enriched for outer membrane proteins) was re-suspended by brief sonication in 1 ml of 10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1% Triton X-100, incubated at 37°C for 30 min and then centrifuged at 17 000 *g* for 30 min. The pellet was re-suspended in 0.2 ml of 10 mM Tris-HCl pH 7.5 and stored at –20°C.

Flow cytometry

Neisseria meningitidis strains were grown to mid-log phase (OD_{600} approximately 0.7). 1×10^7 cfu aliquots were centrifuged at 5000 *g* for 5 min and resuspended in 0.2 µm-filtered PBS. The cells were incubated for 2 h with αR-FBA [1:500 diluted in PBS containing 0.1% BSA, 0.1% sodium azide and 2% fetal calf serum (FCS)] and untreated cells were used as a control. Cells were washed three times with PBS and incubated for 2 h in the dark with goat anti-rabbit IgG-Alexa Flour 488 conjugate (Invitrogen; diluted 1:50 in PBS containing 0.1% BSA, 0.1% sodium azide and 2% FCS). Again, untreated cells were used as a control. Finally, the samples were washed in PBS twice before being re-suspended in 1 ml of PBS containing 0.5% formaldehyde to fix the cells. Samples were analysed for fluorescence using a Coulter Altra Flow Cytometer. Cells were detected using forward and log-side scatter dot plots, and a gating region was set to exclude cell debris and aggregates of bacteria. A total of 50 000 bacteria (events) were analysed.

Association and invasion assays

Association and invasion assays were performed essentially as previously described (Oldfield *et al.*, 2007). Briefly, HBME

or larynx carcinoma (HEp-2) cells were grown to confluence in DMEM supplemented with 10% heat-inactivated FCS (Invitrogen) and 2% antibiotic anti-mycotic solution (Invitrogen) in 24-well tissue culture plates (Costar) at 37°C in an atmosphere of 5% CO_2. Prior to all experiments, mono-layers were transferred to DMEM supplemented with 2% FCS to remove the antibiotics. Meningococci were cultured in MHB for 2 h and monolayers were infected with 1×10^6 cfu of meningococci and incubated for 2 h (association) or 4 h (invasion) in 5% CO_2 at 37°C. To assess total cell association, monolayers were washed four times with 1 ml of PBS per well. To assess invasion, monolayers were further incubated in DMEM containing gentamicin (100 μg ml^{-1}) for 2 h. Prior to further steps, aliquots of the gentamicin-containing supernatants were plated out to confirm killing of extra-cellular bacteria. Furthermore, the susceptibility of all meningococcal strains to gentamicin at 100 μg ml^{-1} was confirmed prior to testing. Monolayers were then washed four times with 1 ml of PBS. In both association and invasion experiments, monolayers were then disrupted and homogenized in 1 ml of 0.1% saponin in PBS. Meningococci were enumerated by serial dilution of the homogenized suspensions and subsequent determination of colony-forming units by plating 50 μl aliquots from appropriate dilutions of the lysates on agar. All association and invasion assays were repeated at least three times. Statistical significance was measured using a two-tailed Student's *t*-test.

Protein and nucleic acid sequence analysis

Public databases containing previously published protein and DNA sequences were searched using the BLAST and PSI-BLAST algorithms available at http://blast.ncbi.nlm.nih.gov/Blast.cgi. The genome database of *N. meningitidis* MC58 was interrogated at http://cmr.jcvi.org/cgi-bin/CMR/GenomePage.cgi?org=gnm. Sequence homology data were obtained using the CLUSTALX software (http://www.clustal.org/). Protein secretion signals were analysed using the SignalP 3.0 server available at http://www.cbs.dtu.dk/services/SignalP/ (Emanuelsson *et al.*, 2007). GenBank accession numbers for the *cbbA* sequences analysed in this study are as follows: YP_974462 (FAM18), YP_001598513 (ST-4821 strain 053442), YP_002342063 (Z2491), YP_207215 (gonococcal strain FA1090) and ZP_03723075 (*N. lactamica* ATCC 23970).

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