THE ROLE OF PHASE-VARIABLE EXPRESSION OF MENINGOCOCCAL SURFACE PROTEINS DURING CARRIAGE AND DISEASE

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ABSTRACT

Neisseria meningitidis is the most common cause of bacterial meningitis, a disease that kills thousands of people yearly. Asymptomatic colonisation of the oropharynx (i. e. carriage) occurs in 10% – 30% of humans. Significant features of meningococcal genomes are simple sequence repeats (SSR), which have been shown to control gene expression in a reversible process known as phase variation (PV). This study investigated the consequence of "switching-OFF" of two haemoglobin-acquisition systems (*hpuAB* and *hmbR*) during disease and also explored potential associations between PV state of the immunodominant meningococcal antigen, PorA, and the adaptive immune response during carriage. Using an *ex vivo* human whole blood model, *hmbR*-OFF mutants of strain MC58 (*hpuAB*-negative) exhibited a pattern of growth similar to wild-type. Conversely, an inability to utilise transferrin ($\Delta tbpBA$) significantly affected growth but not survival in blood. Five recombinant versions of these Hb receptors were prepared in this study and subsequently used as antigens for the generation of polyclonal and monoclonal antibodies in mice. The polyclonal anti-rHpuA, anti-rHpuB and anti-rHmbR antisera were reactive with homologous receptors in lysates of diverse meningococcal strains. Surface expression of HpuA and HmbR was detected by flow cytometry but all antisera were incapable of mediating killing of iron-restricted meningococci. An immunodetection assay employed in this study revealed the induction of variant-specific anti-PorA IgG antibodies following acquisition of carriage. These antibodies may have contributed to subsequent loss of carriage but a role for PV in immune escape *in vivo* was not established. This study posits that HmbR is less important than TbpBA and HpuAB during disease and that phase variable expression of surface receptors is irrelevant for immune evasion during carriage. Further studies are recommended to confirm the proposed importance of HpuAB over HmbR during invasive disease and to investigate the opsonophagocytic property of the anti-HpuA and anti-HmbR antisera.

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LIST OF ABBREVIATIONS

%	Percent
/	per
^{0}C	degree Centigrade
A	Adenine
aa	Amino acid
Amp	Amperes
APS	Ammonium persulphate
AU	Arbitrary units
BA	Blood agar
BHI	Brain Heart Infusion
bp	Base pairs
BSA	Bovine serum albumin
C	Cytosine
CaCl$_2$	Calcium chloride
CC	Clonal complex
CFU	Colony-forming units
CMP-NANA	Cytidine-5′-monophospho-N-acetylneuraminic acid sodium salt
CO$_2$	Carbon dioxide
CSF	Cerebrospinal fluid
Dam	DNA adenine methyltransferase
dATP	Deoxyadenosine triphosphate
DEAE-Sephacel	Diethylaminoethyl–Sephacel
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
dRS3	Duplicated repeat sequence 3
DTT	Dithiothreitol
DUS	DNA uptake sequence
eDNA	Extracellular DNA
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting

FeCl$_3$	Iron (III) chloride
FI	Fluorescence intensity
Fur	Ferric uptake regulator
G	Guanine
g	Grams
GCB	Gonococcal medium base
GuHCl	Guanidine hydrochloride
H$_2$O	Water
Hb	Haemoglobin
Hb$^-$	Meningococci incapable of utilising haemoglobin
HCl	Hydrogen chloride
Hp	Haptoglobin
Hp-Hb	Haptoglobin-Haemoglobin
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IMD	Invasive meningococcal disease
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kb	Kilobase pairs
KCl	Potassium chloride
kDa	Kilodaltons
l	Litres
LDAO	N,N-Dimethyldodecylamine N-oxide
Lf	Lactoferrin
LOS	Lipooligosaccharide
M	Molar
mAb	Monoclonal antibody
MFI	Mean fluorescence intensity
mg	Milligrams
MgCl$_2$	Magnesium chloride
MH	Mueller-Hinton
ml	Millilitres
mM	Millimolar
MPL	Monophosphoryl lipid A

Mw	Molecular weight
$Na_2B_4O_7$	Sodium borate
Na_2CO_3	Sodium carbonate
NaCl	Sodium chloride
NaH_2PO_4	Sodium dihydrogen phosphate
$NaHCO_3$	Sodium hydrogen carbonate
NaOH	Sodium hydroxide
ng	Nanograms
NIBSC	National Institute for Biological Standards and Control, Potters Bar
OD_{260}	Optical density at 260 nanometres
OD_{405}	Optical density at 405 nanometres
OD_{600}	Optical density at 600 nanometres
OMP	Outer membrane protein
ORF	Open reading frame
PBS	Phosphate buffered saline
PC	Pooled human complement
PCR	Polymerase chain reaction
pH	Decimal cologarithm of hydrogen
pI	Isoelectric point
PNACL	Protein Nucleic Acid Chemistry Laboratory, University of Leicester
PROTEX	Protein Expression Laboratory, University of Leicester
PV	Phase variation / phase-variable
PV-OFF	Phase variation state OFF
PV-ON	Phase variation state ON
REP2	Repetitive extragenic palindromic sequence 2
rHmbR	Recombinant HmbR
rHpuA	Recombinant HpuA
rHpuB	Recombinant HpuB
RNA	Ribonucleic acid
rpm	Revolutions per minute
RS	Repeat sequence
SBA	Serum bactericidal assay
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SNP	Single-nucleotide polymorphism
spp.	Species (plural)
SSR	Simple sequence repeats
ST	Sequence type
T	Thymine
TAE	Tris-acetate-EDTA buffer
TEMED	Tetramethylethylenediamine
TEV	Tobacco Etch Virus protease
Tf	Transferrin
Tris	Trisaminomethane
Tris.Cl	Trisaminomethane base adjusted with hydrogen chloride
U	Unit
V	Volts
v/v	Volume per volume
VR	Variable region
w/v	Weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
μg	Micrograms
μl	Microliters
μM	Micromolar

CHAPTER 1

1.0 Introduction

This report will present findings of investigations into the biological role of the phase variation of three meningococcal surface proteins, HpuAB, HmbR and PorA, during carriage and disease. A general overview of the research background is presented in the first chapter, providing a review of published literature relating to the biology of the meningococcus including phase variation in the species and other bacterial species. In the second chapter, the protocols employed in the studies that compose this project are described. Specific details regarding these protocols are further described in the relevant chapters. Each of the following four chapters i. e. Chapters 3 – 6, provide specific and concise background information relating to each component study, details of each experiment performed and results obtained before the potential implications of these results are discussed. An overall discussion of specific results obtained from this project within the context of previous research with recommendations for future studies are stated in the seventh chapter. Appendices and a bibliographic index are provided in the eighth and ninth chapters respectively.

1.1 *Neisseria meningitidis*

Neisseria meningitidis is a pathogenic member of the beta-proteobacteria class of gram-negative bacteria. It exists as a strictly aerobic diplococcus and is host-specific; found exclusively in humans (Stephens, 2009). Transmission from host-to-host is achieved by contact with bodily fluids such as saliva droplets and mucus (Swain and Martin, 2007). It is a frequent coloniser of the oropharyngeal mucosa of humans (Caugant *et al.*, 2006) but could also be isolated from the rectum and urogenital track (Faur *et al.*, 1975; Givan *et al.*, 1977). The meningococcus is non-motile and therefore, relies on an efficient adhesion system for survival in its ecological niche. Adhesion is often mediated by surface structures such as the pili (Stephens and McGee, 1981) and adhesins (Capecchi *et al.*, 2005; De Vries *et al.*, 1998; Sjölinder *et al.*, 2008).

Like other gram-negative bacteria, the meningococcus is commonly encased by different variants of a polysaccharide capsule (Harrison *et al.*, 2013). This capsule functions as a protective layer during inter-host transmission by preventing desiccation of the meningococcal cell (Weber *et al.,* 2006) and intra-host survival by mediating resistance to humoral components of the host immune system (Vogel and Frosch, 1999). In addition to the capsule, the cytoplasmic contents of the meningococcal cell are bound by two lipidated membranous layers, the outer and inner membranes, separated by a periplasmic space (Rosenstein *et al.*, 2001). These membranes embed proteins that are involved in transport of materials such as nutrients, metabolic products and toxins, into and out of the cytoplasm. In addition to the membrane proteins, a lipooligosaccharide, or LOS, primarily composed of sugars and a highly toxic Lipid A subunit is present in the outer membrane of the meningococcus. Like the capsule and many outer membrane proteins, this compound exhibits intra-specific structural variability with 11 immunotypes identified so far (Brandtzaeg *et al.*, 2001).
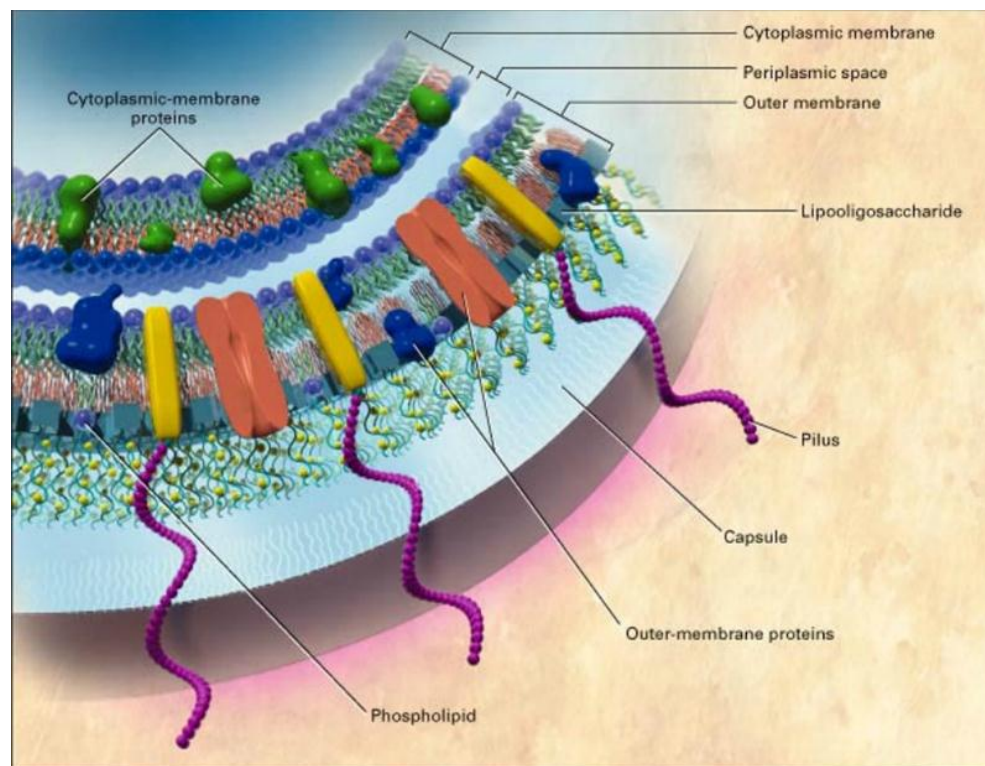
Figure 1.1: Schematic representation of a cross-section of the meningococcal membrane. Reproduced with permission from Rosenstein *et al*. (2001), Copyright Massachusetts Medical Society.

There is a significant amount of genetic and antigenic diversity exhibited by the meningococcus. Therefore, strains have been classified based on structural variations in cellular components such as the capsule and outer membrane proteins (OMP). The polysaccharide capsule (serogrouping) and three OMPs, the immunodominant PorA (serosubtype), PorB (serotype) and a ferric enterochelin protein, FrpB/FetA (Russell *et al.*, 2004; Thompson *et al.*, 2003; Urwin *et al.*, 1998), were selected as major components of a standard typing scheme approved at the 2009 European Monitoring Group for Meningococci (EMGM) conference. So far, thirteen capsular types have been identified, with the most clinically important being serogroups A, B, C, W, X and Y (Boisier *et al.,* 2007; Stephens, 2009; Harrison *et al.*, 2013). A significantly higher proportion of *porA* and *fetA* alleles have been discovered owing to the high level of antigenic diversity of the proteins encoded by these genes. The presence of a significant immune response to certain surface-exposed loops in these proteins is believed to be an underlying factor to this evident diversity (Gupta and Anderson, 1999). Seven housekeeping genes, which are not associated with frequent mutation events, complete the list of molecular tools employed in the meningococcal typing scheme. Based on the sequences of these housekeeping genes, meningococci are grouped into sequence types (ST) while related STs form a clonal complex (Maiden *et al.*, 1998). Thus, the meningococcal typing scheme has the following components: the capsular type or serogroup, the PorA type or serosubtype, the FetA type and the clonal complex (CC). A meningococcal strain could therefore be identified as follows:

$$\textbf{B : P1.22,14 : F 5-5 : CC-213}$$

Figure 1.2: Molecular typing of meningococcal isolates. Genetic and serological methods are used to determine the capsular type (red text), serosubtype or PorA type (green text), the FetA type (purple text) and the clonal complex (blue text) of meningococcal isolates. The meningococcal type shown above represents a strain that was isolated from a healthy individual (Bidmos *et al.*, 2011).

1.1.1   Asymptomatic meningococcal carriage

N. meningitidis is regarded as one of the most successful human pathogens as it is the most common cause of epidemic outbreaks of bacterial meningitis (World Health Organization, WHO, 2009). However, it can also exist in the host without clinical implications or

symptoms in a state commonly referred to as carriage (Broome, 1986). In the absence of epidemics, approximately 11% of the human population are asymptomatic carriers of the bacterium (Cartwright *et al.*, 1987) with young adults composing the larger proportion of carriers (Christensen *et al.*, 2010). Carriage rates increase drastically in semi-enclosed populations such as army training camps and university halls of residence and can reach up to 70% (Caugant *et al.*, 1992; Bidmos *et al.*, 2011).

Carriage studies are designed to reveal rates of meningococcal carriage, duration of meningococcal persistence within the host and circulating strains within the population. In these studies, carriage is assessed by culturing swabs of the nasopharyngeal mucosa or the posterior pharyngeal wall (Esposito *et al.*, 2013) on agar plates that are selective for meningococcal growth. Both serological and genetic methods are subsequently employed to characterise the meningococcal isolates obtained from colonised volunteers. In one of such studies, rapid meningococcal transmission in crowded settings was demonstrated using a cohort of 190 first-year university students at the University of Nottingham, UK (Bidmos *et al.*, 2011). Apart from the high carriage rate that was reported in the study, a shift in the type of circulating strains was revealed. While the endemic serogroup B strains were still in circulation, serogroup Y strains had assumed dominant status and composed more than 20% of the strains isolated from volunteers. Isolation of serogroup Y strains from the blood and cerebrospinal fluid of meningitis patients during the period of study highlighted the ability of some of these strains to cause disease, sometimes with fatal consequences, and the need for preventive measures against an epidemic due to serogroup Y strains (Ala'Aldeen *et al.*, 2011).

The duration of persistence of meningococci in the host has been observed at six months or more in certain individuals (de Wals and Bouckaert, 1985; Broome, 1986; Jones *et al.*, 1998). Factors that contribute to persistence of the meningococcus in the host have not been fully elucidated but social and genetic factors have been linked to the predisposition of individuals to meningococcal carriage (Blackwell *et al.*, 1990; Davies *et al.*, 1996; MacLennan *et al.*, 2006). The ability to utilize extracellular DNA, eDNA, in biofilm production was reported as a factor contributing to persistence of meningococci in the host. Lappann *et al.* (2010) propose that meningococci capable of utilising eDNA in biofilm

formation persist in the host for long periods and possess what is termed as the settler phenotype. Most meningococci including members of the disease-related CC-41/44 and CC-32 clonal complexes are believed to belong to this class. Conversely, biofilm formation was observed to be eDNA-independent in members of the spreader class. Poorer colonization of the host, brought about by an inability to utilize eDNA, is compensated for by rapid inter-host transmission in the spreader class. Neither class has been shown to have a higher propensity to cause disease as strains associated with both phenotypes have been implicated in epidemics of meningococcal disease (Dyet *et al*. 2005; Nicolas *et al*., 2005). Furthermore, the presence or absence of an immediate and active immune response, representing differences in host immunity, may be contributory towards the duration of carriage but experimental evidence for this theory is lacking.

1.1.2   Invasive meningococcal disease or meningitis

Invasion of the nasopharyngeal epithelium and subsequent presentation in the blood and cerebrospinal fluids occur occasionally, leading to severe sepsis and/or meningitis (Caugant, 2008). Onset of disease is rapid with the time-frame between the manifestation of specific meningitis symptoms (such as haemorrhagic rash, photophobia and neck stiffness) and mortality being less than 24 hours (Thompson *et al*., 2006). Misdiagnosis often results from the non-specificity of early symptoms of meningitis and/or septicaemia and sometimes leads to rapid progression of the disease and subsequent mortality (Thompson *et al*., 2006). The most affected individuals are children under 3 years of age and adolescents, with a morbidity rate of up to 15 cases per 100,000 in US infants (Schaffner *et al*., 2004).

Morbidity rates of meningococcal meningitis vary by region. Members of the Japanese population rarely suffer from the disease with morbidity rates as low as <0.02 per 100,000 (Takahashi *et al*., 2004). Conversely, meningococcal meningitis is a constant threat in the African meningitis belt with epidemics occurring annually. During epidemics, >1 in 100 people could be affected by IMD in this region of sub-Saharan Africa that spans at least 15 countries (Harrison *et al*., 2009). Localised epidemics are occasionally experienced in other parts of the world with overall morbidity rates ranging from 0.28 cases per 100,000 in the United States to 2.4 cases per 100,000 in New Zealand (Halperin *et al*., 2012).

Analyses of the causal strains of meningococcal meningitis isolated during localised epidemics in different geographical regions of the world reveal the predominance of certain serogroups in different regions. Serogroup A strains are still the primary cause of epidemics in the meningitis belt but hypervirulent serogroups W and X strains have recently being implicated as the causative pathogens in a few epidemics in the region (Boisier *et al.*, 2007; du Chatelet *et al.*, 2005). While robust epidemiological data emanating from Asia is lacking, studies reviewing the disease aetiology in different countries of the region reveal the frequent isolation of serogroup A strains during major disease outbreaks (Halperin *et al.*, 2012). In Europe, meningococcal disease is frequently caused by *N. meningitidis* strains that possess either a serogroup B or C capsule (Harrison *et al.*, 2009). In the 1990s, the UK witnessed a steady increase in disease rates due to a hyperinvasive clone that had been isolated in Canada, Spain and the Czech Republic (C : P1.5,2 : CC-11) (Campbell *et al.*, 2009; Whalen *et al.*, 1995). The introduction of a meningococcal serogroup C conjugate vaccine (MCC) in 1999, however, led to a drastic reduction in the number of cases due to this serogroup C strain (Campbell *et al.*, 2009). Consequently, 90% of disease cases in the UK are now due to serogroup B strains (Lucidarme *et al.*, 2010) and a significant proportion (66%) of these are due to members of clonal complexes 41/44 and 269 (Ladhani *et al.*, 2012).

Susceptibility to IMD has been attributed to factors including innate deficiencies in the host immune system (Picard *et al.*, 2011) and, more importantly, the lack of protective serum bactericidal antibodies in affected individuals (Frasch *et al.*, 2009). This explains the high mortality rate due to IMD in infants as antibodies to meningococcal surface proteins are absent in this age-group prior to immunisation with currently available meningococcal vaccines (Schaffner *et al.*, 2004).

Figure 1.3: Geographical distribution of meningococcal invasive isolates (Adapted from Halperin *et al.*, 2012).

A consequence of the host-specificity of the meningococcus manifests in the lack of appropriate models to study meningococcal infections *in vivo*. This lack of robust experimental disease models has limited knowledge of how disease progresses but recent advancements such as the use of transgenic mice (Gorringe *et al.*, 2005) and the isolation of related *Neisseria* spp. from rhesus macaques (Weyand *et al.*, 2013) may provide better tools for the study of the pathogen-host interactions, *in vivo*. In the absence of suitable *in vivo* experimental models, rodent models involving the provision of exogenous iron sources (Gorringe *et al.*, 2005) and *ex vivo* human whole blood models (Ison *et al.*, 1995; Seib *et al.*, 2009; Echenique-Rivera *et al.*, 2011) have been used in studies investigating the molecular and cellular interactions that occur during a meningococcal infection. Similarly, comparative genomics studies have been employed to reveal a divergence in gene content and transcriptome profile between carriage and disease isolates (Grifantini *et al.*, 2002; Joseph *et al.*, 2010); differences which elucidate on both essential (adhesion and nutrient acquisition) and non-essential factors (DNA synthesis) during the migration of a meningococcal population from the nasopharynx to the meninges via the bloodstream. These studies have shown modifications to cell surface structures accompanied by the

upregulation of virulence factors in disease isolates only that permit an increased capability to invade upon contact with epithelial cells (Joseph *et al.*, 2010). A major proportion of these virulence factors are composed of surface-exposed opacity-associated proteins, adhesins and iron-acquisition proteins (Grifantini *et al.*, 2002; Carbonelle *et al.*, 2009). Furthermore, the transition from carriage to disease states of the pathogen are characterised by substantial differences in the niches and these differences include quantitative and qualitative differences in nutrient availability. The meningococcus has been shown to utilise these differences as niche indicators, driving the up-regulation of virulence factors and concomitant down-regulation of non-essential factors. Jordan and Saunders (2009) theorise that significant exposure of the meningococcus to specific host iron-binding proteins (modelled by providing transferrin, lactoferrin and haemoglobin as sole sources of iron *in vitro*) signals a change in niche to the meningococcus and is accompanied by a significant transcriptional response.

1.1.3   Tackling disease: antibiotics and vaccines

The similarity of early symptoms of IMD to other microbial infections and the speed with which disease progresses make quick and efficient diagnosis and treatment difficult to achieve and substantially increase the frequency of mortality (Stephens, 2009). Previously, gram-staining, blood culture and cerebrospinal fluid (CSF) bacterial antigen tests were used to diagnose meningitis but these techniques were inadequate (Jordens *et al.*, 2002; Tunkel *et al.*, 2004). To counter the problem of false results in gram-staining and culture of CSF, PCR-based assays were developed and 50% or more of cases ordinarily missed by earlier diagnostic techniques are now confirmed as meningococcal meningitis (van Gastel et *al.*, 2007; Richardson *et al.*, 2003).

Following accurate diagnosis, sulphonamides were the preferred choice in the treatment of meningococcal disease (Vazquez, 2001). However, the rampant isolation of sulphonamide-resistant strains followed by the evolution of rifampicin- and penicillin-resistant strains led to the search for alternative prophylactic measures (Riley *et al.*, 1991; Nolte, 1997). The decrease in sensitivity of meningococci to penicillin is facilitated by DNA exchange with commensal neisserial species, *N. cinerea* and *N. flavescens*, at the *penA* locus, thereby, altering the structure of the penicillin binding proteins (Bowler *et al.*, 1994).

Increased resistance to chloramphenicol (Galimand *et al.*, 1998) and rifampin (Abadi *et al.*, 1996), which could be used in the chemoprophylaxis of IMD, has also been reported. These reports substantiated fears that the high transformability of the meningococcus will lead to the acquisition of antibiotic resistance genes from plasmids and commensal neisserial species. Currently, third-generation cephalosporins are employed in the treatment of meningococcal meningitis (Tunkel *et al.*, 2004) but more emphasis is being placed on the development of more effective preventive measures as a way of reducing mortality due to meningitis.

Since the first meningococcal vaccine was licensed in the 1970s, many others have been developed with the aim of conferring protection against either a specific hypervirulent strain (Balmer *et al.*, 2002; Oster *et al.*, 2005) or a broader panel of meningococcal strains (Vipond *et al.*, 2012). The main primary target of previously licensed meningococcal vaccines is the polysaccharide capsule. Antibodies raised in humans against the meningococcal capsules A and C were found to possess complement-mediated bactericidal activity (Gotschlich *et al.*, 1969) and often protect individuals against carriage of the organism (Maiden *et al.*, 2002). However, vaccines containing plain polysaccharides as the only antigen had severe limitations. The meningococcal polysaccharides were weakly immunogenic in infants and do not activate a T-cell response. Consequently, early polysaccharide vaccines, which were composed of two, three or four different meningococcal polysaccharides, were ineffective in infants and did not offer long-term protection in vaccinated adults owing to the very poor immunological memory response (reviewed in Vipond *et al.*, 2012). The efficacy of meningococcal vaccines has, however, been increased by conjugating the polysaccharide capsule(s) with carrier proteins, which elicit better immune responses in vaccinees by enhancing T-cell help (Dagan *et al.*, 2010). Carrier proteins that have been used successfully include diphtheria toxoid (DT), its non-toxic mutant form CRM197 and tetanus toxoid (TT) (Dagan *et al.*, 2010). The latest vaccines licensed for use in Europe and North America, Menactra® and Menveo®, are quadrivalent vaccines conjugated to DT and CRM197 respectively while a monovalent conjugate vaccine targeting serogroup A strains, MenAfriVac™, is currently being used to tackle the devastating epidemics characteristic of the African Meningitis belt (LaForce and Okwo-Bele, 2011).

While a considerable amount of success has been made in reducing the impact of IMD by vaccination, the high disease burden due to hypervirulent serogroup B meningococci still presents a huge challenge. The significant homology of the serogroup B meningococcal capsule to a polysialic acid expressed on human cells, α (2–8) *N*-acetylneuraminic acid, has hindered the development of vaccines targeting these strains as there are concerns over poor immunogenicity of the capsule or a potential generation of auto-immune responses (Finne *et al.*, 1983). Outer membrane proteins (OMPs) have been successfully employed as alternative vaccine targets to tackle different endemic serogroup B outbreaks of meningitis including those experienced in Chile (Boslego *et al.*, 1995), New Zealand (Galloway *et al.*, 2009) and Norway (Rosenqvist *et al.*, 1995). Buoyed by these successes, the main targets of vaccines currently being developed to tackle IMD due to serogroup B strains worldwide are OMP antigens that are highly-conserved and constitutively-expressed in abundant amounts on the meningococcal surface. One of these protein-based vaccines is currently awaiting introduction into the childhood immunisation schedules in Europe and the USA (Granoff, 2013 and see Chapter 6 for a more detailed review of protein-based vaccines).

1.1.4   The meningococcal genome

Prior to the advent of genome sequencing and the compilation of the first bacterial genome sequence in 1995, *Haemophilus influenzae* (Fleischmann *et al.*, 1995), genome-wide studies in meningococcal research were restricted to techniques such as pulse field gel electrophoresis (PFGE) (Schoen *et al.*, 2009). Advancements in sequencing technology has led to the publication of several complete genomes of both carriage and disease strains (Parkhill *et al.*, 2000; Tettelin *et al.*, 2000; Bentley *et al.*, 2007; Peng *et al.*, 2008; Schoen *et al.*, 2008). The availability of these sequences provides a valuable resource for the development of whole genome comparison assays, which will improve understanding of the highly flexible and diverse meningococcal genome.

The average size of the meningococcal genome is 2.2 million base pairs irrespective of the source - disease or carriage. The genome contains approximately 2000 coding sequences, 82% of which are shared by all strains sequenced to date (the core genome) with a G+C

content of >51.5%. Variations abound, however, in the arrangement of this core genome. Commonly found genes are those involved in DNA maintenance, capsule biosynthesis, the glycolytic pathway and synthesis of ribosomal proteins (Bentley *et al.*, 2007; Davidsen and Tonjum, 2006; Parkhill *et al.*, 2000; Peng *et al.*, 2008; Schoen *et al.*, 2008; Tettelin *et al.*, 2000).

A striking feature of the meningococcal genome is the large number of pseudogenes and repetitive DNA it possesses. A study comparing 53 bacterial genomes ranked the meningococcal genome as possessing the 4th highest number of repeat sequences (Achaz *et al.*, 2002). Most notable is the unique 12-bp Neisserial DNA uptake sequence (DUS), which plays an important role in the transformation of meningococci with DNA from plasmids, commensal Neisserial species and other bacterial species. There are approximately 1900 copies of this family of repetitive DNA in each genome with this sequence being over-represented in the core genome (Treangen *et al.*, 2008). This has led to the belief that the DUS, via transformation, may be involved in protecting the core genome and consequently, play an important role in the evolution of the species (Treangen *et al.*, 2008; Davidsen and Tonjum, 2006; Schoen *et al.*, 2009). Other families of repetitive DNA found in the meningococcal genome are the Neisserial intergenic mosaic elements (NIMES) composed by 20-bp dRS3 elements and 30-160 bp RS elements, simple sequence repeats (SSR), Correia and REP2 elements (Davidsen and Tonjum, 2006; Schoen *et al.*, 2009).

Since repeats are known to increase susceptibility to mutations, which could be deleterious, such a huge number of repeat elements present in the meningococcal genome would contribute to high levels of instability. This fluidity of the meningococcal genome was highlighted by a stability model, reported by Rocha (2006), which showed that the meningococcal genome was less stable than 120 out of 126 bacterial genomes analysed. However, some of these repetitive elements have known functions that are beneficial to the meningococcus ranging from genome organization to regulation of gene expression (Davidsen and Tonjum, 2006). The generation of phenotypic diversity, sometimes mediated by these repetitive elements, is also crucial to the meningococcus as it increases chances of survival in the host via the colonisation of different niches, utilisation of diverse sources of nutrients and the evasion of host immune responses.

1.2   Phase variation

The ability of a pathogen to evade host immune responses is embedded in a variety of processes. Some are adaptive responses to changing environmental conditions, a well-studied example of which is the two-component PhoP-PhoQ regulatory system of *Salmonella enterica* (Groisman, 2001). Other processes, which are not necessarily triggered by environmental signals, are inherent in the bacterial DNA and are controlled by high levels of mutation at regions of the DNA known as contingency loci (Moxon *et al.*, 2006). These loci, when present in the upstream region or within the open reading frame of a gene, affect expression in a reversible manner. This well-studied phenomenon occurs at a high frequency in many bacterial species and is known as phase variation (van der Woude, 2006).

Phase variable expression can be at a transcriptional or translational level. The presence of a hypermutable sequence upstream of a gene, either within or around the promoter region, affects expression at the transcriptional level. Conversely, translational phase variation provides a direct switch in gene expression from an 'ON' state where the phase variable gene is expressed, to an 'OFF' state where no functional gene products are produced. This mode of phase variation is obtained when the mutational mechanism is located within the reading frame (van der Woude and Baumler, 2004).
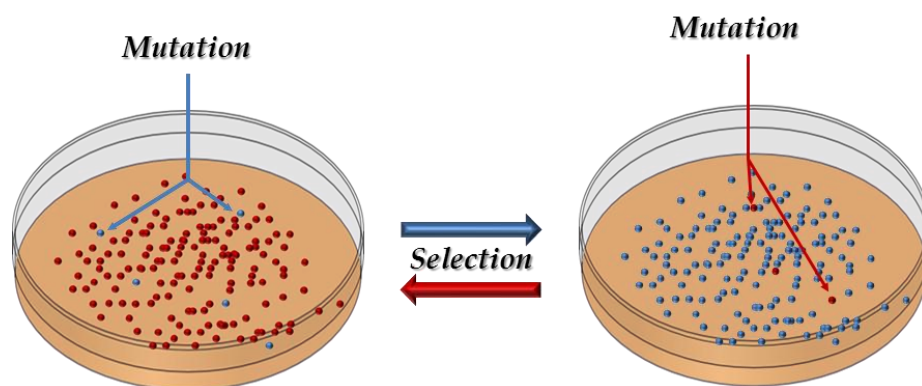
Figure 1.4: Schematic representation of phase variation. Phase variation is dependent on two factors: mutation at a contingency locus and selection for phase variants expressing a desirable phenotype. Reversion to a previous expression state is a major characteristic of phase variation. Blue colonies in left plate and red colonies in right plate represent variants of the prevailing phenotype.

1.2.1   Simple sequence repeat-mediated phase variation

Phase variable expression has been identified and proven experimentally in many genes encoding surface proteins, restriction-modification systems and regulatory proteins (Carroll *et al.*, 1997; Park *et al.*, 2000; Zaleski *et al.*, 2005). These cellular components exhibit different mechanisms of phase variable expression. Perhaps, the best studied mechanism of phenotypic switching via phase variation is simple sequence repeat (SSR)-mediated phase variation. Repeat tracts could be mononucleotide polyG/C tracts as seen in the adhesin, *uspA1*, in *Moraxella catarrhalis* (Lafontaine *et al.*, 2001) or composed of polynucleotide repeat units, evident in the reading frame of *mod* in *H. influenzae* (de Bolle *et al.*, 2000). The number of repeat units composing a tract is also variable; expression of *wlaN* in *Campylobacter jejuni* is dependent on the eight-unit polyG tract within its open reading frame (Linton *et al.*, 2000) while *lic2A* of *H. influenzae* has its expression controlled by up to fifty-seven units of a tetrameric repeat tract, CAAT (High *et al.*, 1996). Alterations in the length of these repeat tracts via slipped-strand mispairing during DNA replication cause a change in gene expression, by modifying the coding sequence of the reading frame or the spacer between the -35 and -10 elements of the promoter.

Studies on phase variation in *H. influenzae* have shown that phase variable expression of a gene could impact on the expression of multiple genes in what has been termed a phasevarion. This scenario was highlighted in a study conducted by Srikhanta and co-workers (2005) to elucidate the mechanism of phase variation in a type III restriction/modification system involving *mod*, a methyltransferase. This study revealed, while comparing the gene expression profiles of *mod* mutants and a wild-type *H. influenzae* strain, an association between the phase variable expression of *mod* and the genes in its regulon. The β-galactosidase assay employed in this study to further elucidate this association showed that PV-ON variants of the opacity-associated protein, Opa, had the *mod* gene in an OFF state while those expressing *mod* (PV-ON) lacked expression of Opa (Srikhanta *et al.*, 2005). This study was the first report of phase variation of a gene affecting expression of multiple genes.

1.2.2   Other mechanisms of phase variation

Recombination and epigenetic mechanisms of phase variation have been described in published literature. Homologous recombination at the *pilE* locus of *N. gonorrhoeae* is responsible for the production of phenotypic variants of pili (Segal *et al.* 1985). This recombination event, which occurs between an actively expressed *pilE* locus and a silent, truncated *pilS* locus, is mediated in part by the multifunctional recombinase, RecA (Cox, 2007; Koomey *et al.*, 1987). Gonococci that lose expression of *pilE* after recombination can, however, regain it if a second recombination event occurs between both loci. In *Escherichia coli*, the reversible site-specific inversion of the promoter region of *fimA* alters expression of the fimbrial subunit (Abraham *et al.*, 1985) and is mediated by two members of the tyrosine recombinase family, FimB and FimE (Klemm, 1986). Similarly, phase variation of the fimbriae in *Proteus mirabilis* is mediated by MrpI, a recombinase that facilitates the inversion of a 251-bp locus containing the promoter of the *mrp* operon (Li *et al.*, 2002).

More complex interactions between regulatory proteins and the DNA adenine methyltransferase (Dam) mediate the phase variable expression of certain bacterial genes (Haagmans and van der Woude, 2000) and operons (Hernday *et al.*, 2003) at the transcriptional level. In the PV-ON state of Agn43 in *E. coli*, this epigenetic mechanism requires the methylation of three GATC recognition sequences in the promoter region of *agn43* by Dam, thereby, protecting the locus from OxyR binding. Conversely, inhibition of *agn43* expression observed in the PV-OFF state is mediated by the prevention of Dam methylation by OxyR, which binds to the regulatory region containing the GATC recognition sequences of Dam and the *ag43* promoter (Wallecha *et al.*, 2002; Kaminska and van der Woude, 2010).
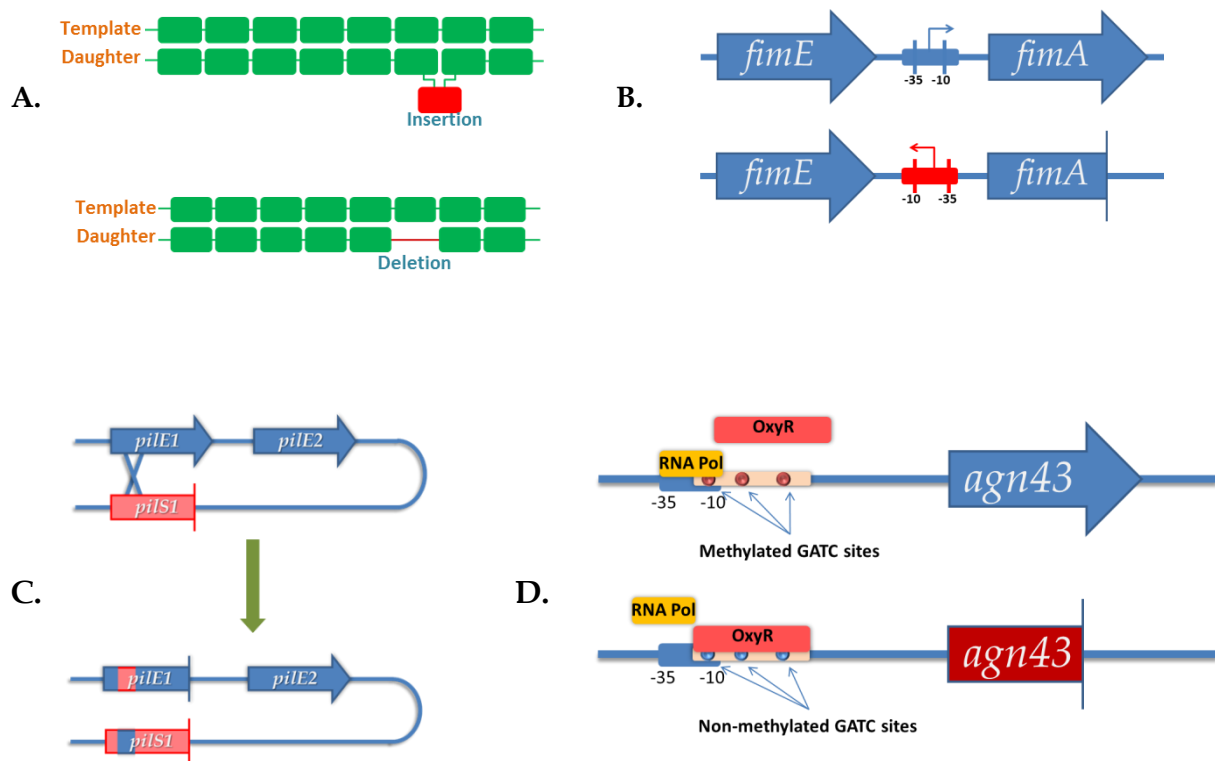
Figure 1.5: Mechanisms of phase variation. **(a)** Insertion (top) or deletion (bottom) of a repeat unit via slipped-strand mispairing during DNA replication. **(b)** Site-specific inversion of a 314-bp DNA element containing the promoter of *fimA* (rounded rectangle) either allows for (top) or prevents (bottom) transcription of the gene and subsequent production of type I fimbriae in *E. coli* (McClain *et al.*, 1991). **(c)** Homologous recombination between two *pil* loci, *pilE* and *pilS*, control production of type IV pilin in *N. gonorrhoeae* (Segal *et al.*, 1985). **(d)** Epigenetic control of *agn43* PV. Methylation of GATC target sites by Dam prevents OxyR binding and allows for *agn43* expression (top). Non-methylation of GATC sites allows for OxyR binding, which prevents RNA polymerase from initiating transcription of *agn43* (bottom) (Wallecha *et al.*, 2002). Solid line, DNA; arrow, expressed locus (PV-ON); blunt-ended arrow, silent locus (PV-OFF).

1.3   Phase variation in *N. meningitidis*

More than sixty genes in the meningococcal genome are predicted to be phase variable (Saunders *et al.*, 2000; Schoen *et al.*, 2009). However, only fifteen of these have been proven experimentally (Schoen *et al.*, 2009) with simple sequence repeats being the main mechanism controlling phase variation of these genes.

As in other bacterial species, both transcriptional and translational modes of phase variation are observed in the expression of these genes, which encode surface proteins (Kawula *et al.*, 1988) and type III restriction/modification systems (Srikhanta *et al.*, 2009). Insertion or deletion of 1 or more repeat units at a polyG tract in the core promoter of *opc*,

an opacity protein, affects the level of transcription effected by the promoter (Sarkari *et al.*, 1994). Similarly, translational phase variation is achieved when frameshifts caused by mutations at a polyG tract in the reading frame of *lgtG* switch the gene from ON to OFF and vice versa, and determine the final structure of the lipooligosaccharide (Mackinnon *et al.*, 2002).

A well-studied but complex example of phase variation in meningococci involves *nadA*, which encodes NadA, an important adhesin and vaccine component. This gene exhibits a transcriptional mode of phase variation that involves a tetrameric repeat tract, 5′ TAAA, located upstream of the core promoter (Comanducci *et al.*, 2002; Martin *et al.*, 2005). Previously, binding sites for the integration host factor, IHF, and the ferric uptake regulator, Fur, were mapped upstream of the gene suggesting that these factors co-regulated expression of *nadA* (Martin *et al.*, 2005). However, Metruccio and co-workers (2009) showed no role for Fur in regulating the expression of *nadA*. Instead, a transcriptional repressor, NadR, which binds at 2 operator sequences that span the repeat tract locus, was discovered. Alterations in the length of the tetrameric tract control the spacer region between the operator sequences, affect NadR binding and yield transcripts of different quantities. Absence of NadR, on the other hand, yields a similar level of transcripts for different tract lengths as shown in NadR knockout mutants. The study also identified a compound in human saliva, 4-hydroxyphenylacetic acid (4-HPA), which binds to NadR and prevents repression of NadA production (Metruccio *et al.*, 2009). Therefore, *nadA* expression is phase variable due to the tetrameric tract but heavily influenced by NadR and 4-HPA. This study also showed that cis-, trans-acting and environmental factors could affect phase variation.

1.4   Biological role of phase variation in *N. meningitidis*

The fact that many bacterial genes undergo phase variation implies an important biological function for the process. A sizeable proportion of phase variable genes are involved in the biosynthesis of surface structures suggesting that this biological function involves interactions between the bacterium and host cells. The meningococcus encounters disparate environmental conditions that require the expression and a concomitant repression of different factors for effective colonisation. The phenotypic diversity within a

meningococcal population would, therefore, enhance the probability of survival of that population in the host. For example, PV-ON variants of *nadA* will be favoured in the nasopharynx where efficient attachment to epithelial tissues is required, but not in the blood where bactericidal serum IgG antibodies against this adhesin may be present. A meningococcal population that contains both variants of *nadA* would, therefore, experience continuous presence in the host as selection against a disadvantageous phenotype will not lead to complete eradication of the population. The sub-population, depending on its fitness, could potentially continue to proliferate. Phase variation, brought about by mutation and selection, would therefore ensure that the bacterium can continue to persist in the host.

The biological role of phase variation in the meningococcus is often associated with the evasion of the host humoral immune response during carriage and disease. Evidence from *in vivo* experiments is lacking for this hypothesis, not least because of the unavailability of suitable models. However, a series of *in vitro* assays strongly suggest that a meningococcal population composed of phase variants of an immunogenic OMP will be able to survive in the presence of bactericidal antibodies. Structural alterations to the meningococcal LOS, for example, are associated with PV of *lgtG*, and these alterations affect binding specificities of the LOS to a bactericidal monoclonal antibody (Mackinnon *et al.*, 2002) and immune escape (Bayliss *et al.*, 2008). Tauseef *et al.* (2013) provided further evidence for PV-mediated immune escape when investigating expression levels of PorA from high, medium and low expressors of the protein in a meningococcal disease isolate, 8047 (polyG tracts of 11, 10 and 9 units respectively). The ability to evade complement-mediated killing in the presence of the bactericidal PorA P1.2 mAb was associated with lower levels of the surface-exposed target of the mAb (Tauseef *et al.*, 2013). These studies provide compelling evidence for an immune-evasion biological role of PV but further studies with appropriate *in vivo* models would be required to confirm this hypothesis.

1.5 Iron acquisition systems as virulence factors of the meningococcus

Iron is an essential co-factor of cellular metabolic processes and its non-availability is deleterious to both eukaryotic and prokaryotic cells. Similarly, significantly high intracellular iron levels contribute to an increase in oxidative stress via the production of

reactive oxygen species and subsequently lead to DNA damage (reviewed in Mello-Filho and Meneghini, 1991 and Meneghini, 1997). In many bacterial species, including *N. meningitidis*, iron homeostasis is achieved by the repression or activation of iron acquisition and uptake systems under iron-replete or iron-restricted conditions, respectively. The Ferric uptake regulator (Fur), encoded by *fur*, is widely reported, in various studies on different bacterial species, to act as a repressor of genes within its regulon, some of which are iron-acquisition proteins. Fur requires free iron molecules as a co-repressor to bind consensus sequences (Fur box) often overlapping the promoters of target genes to prevent transcription of these genes during iron-replete conditions (Escolar *et al.*, 1999). Conversely, during periods of iron starvation, Fur is unable to bind the Fur box owing to the intracellular paucity of its co-repressor leading to the activation of gene transcription by RNA polymerase. Expression of Fur-regulated iron-acquisition systems and the consequent scavenging and uptake of iron is thereby, enabled. While *fur* is non-essential to survival in the meningococcus, its experimentally-induced absence produces a significantly negative impact on the viability of the bacterium (Delany *et al.*, 2003). In addition to its gene repression function in the meningococcus, Fur also serves as a direct activator of respiratory genes utilised under anaerobic conditions (Delany *et al.*, 2004). Thus, iron is an important nutrient to living cells, intracellular amounts of which must be maintained in a relatively stable equilibrium.

Iron is abundant in the human host but is rarely freely available, complexed to ferritin or haemoglobin (Evans *et al.*, 1999, cited in Perkins-Balding *et al.*, 2004). Iron-binding proteins such as lactoferrin (Lönnerdal and Iyer, 1995), transferrin (Gomme and McCann, 2005) and hepcidin (Deschemin and Vaulont, 2013) are also involved in the sequestration of free extracellular iron, thereby creating an iron-limited environment in the human host. Some of these iron-binding proteins have also been implicated in the process of nutritional immunity, or hypoferremic response, during the early stages of infection (Johnson and Wessling-Resnick, 2012). Like other pathogens, the meningococcus has adapted to the iron-limited environment of the host and relies on its efficient iron acquisition and transport systems for the scavenging of iron complexed to the host glycoproteins and the subsequent transport of the iron molecules into the cell. One such method of acquiring iron from host glycoproteins is via the production of siderophores. Unlike many other

bacterial species that inhabit the human host, there are no established reports of siderophore production by the meningococcus but previous studies have hinted at an ability of the pathogen to utilise siderophores produced by other organisms, *in vitro*. Surface receptors such as the vaccine candidate, fHbp (Veggi *et al.*, 2012) and the less well-characterised TonB-dependent iron transporter, FrpB/FetA (Saleem *et al.*, 2013) interact with enterobactin produced by *E. coli*, suggesting this siderophore as a possible source of iron for the meningococcus. However, meningococci and *E. coli* occupy separate niches in the human host, therefore, the likelihood of enterobactin utilisation by meningococci *in vivo* is unlikely. Despite the inability to produce siderophores, meningococci may benefit from the production of metabolic intermediates or an increased solubility of iron via the activities of other commensal bacterial species, as was found with gonococcci inhabiting the genital tract of female mice (Jerse *et al.*, 2002). In general, the mechanisms and substrates involved in the meningococcal siderophore-utilisation system and their importance to virulence are still poorly understood.

1.5.1   Iron acquisition from transferrin and lactoferrin

Since the meningococcus can also be internalised by epithelial cells, intracellular replication and survival will be dependent on its ability to acquire iron. There is some evidence that suggests the presence of a novel TonB-dependent iron uptake system that is crucial to the proliferation of the meningococcus within human epithelial cells (Larson *et al.*, 2002). When meningococci are not internalised within epithelial cells and exist freely in extracellular spaces, iron complexed to lactoferrin and transferrin serve as valuable iron sources, which are utilised via the bipartite LbpBA and TbpBA systems, respectively (Lewis *et al.*, 1998; Renauld-Mongenie *et al.*, 2004). Each system is composed of a substrate-binding lipoprotein (LbpB and TbpB) and a transmembrane pore-forming protein (LbpA and TbpA). Expression of both systems is non-phase variable but transcriptionally regulated by Fur and has been shown experimentally to be up-regulated in human whole blood (Echenique-Rivera *et al.*, 2011), indicating the importance of these iron uptake systems to the meningococcus.

The importance of TbpBA, as a virulence factor, to both pathogenic *Neisseria* spp., has been investigated in previous studies involving both human and experimental mice models. In

a study reported by Cornelissen *et al*. (1998), a wild-type gonococcal strain FA1090 and its isogenic Δ*tbpB*Δ*tbpA* mutant were used to infect seven male volunteers intra-urethrally. Both strains were unable to utilise lactoferrin as an iron source while only the wild-type strain could utilise transferrin, *in vitro*. Symptoms of a gonococcal infection were apparent in volunteers infected with the wild-type strain but not the Δ*tbpB*Δ*tbpA* mutant supporting the theory that transferrin utilisation was crucial to growth and survival of pathogenic *Neisseria*, *in vivo*. Similar results were obtained when meningococci were used to challenge mice in a later study reported by Renauld-Mongenie and co-workers (2004). In the absence of exogenous human transferrin (holo-hTf), virulence of both wild-type and isogenic Δ*tbp* mutants (Δ*tbpB*, Δ*tbpA* and Δ*tbpB*Δ*tbpA*) was inconsequential and no mortality was recorded. Conversely, injection of 24 mg of holo-hTf into mice prior to challenge with meningococcal wild-type strain M982 or isogenic *tbp* mutants yielded detectable bacteraemia after three hours. This result showed the importance of transferrin utilisation to virulence in this model and highlighted the very high species-specificity of the meningococcal transferrin receptor.

Studies on the individual functions of the component parts of the Neisserial transferrin receptor, *tbp*, in both gonococci (Anderson *et al*., 1994) and meningococci (Renauld-Mongenie *et al*., 2004) show a more important role for TbpA than TbpB. Similar to results obtained in the gonococcal study, bacteraemia in mice infected with a meningococcal Δ*tbpB* mutant was sustained after 24 hours and led to mortality in five out of six mice that were challenged with the Δ*tbpB* mutant after 48 hours. In contrast to the Δ*tbpB* mutant, the Δ*tbpA* mutant was significantly less virulent, causing mortality in only one out of six mice infected with the mutant (Renauld-Mongenie *et al*., 2004). These studies show that while TbpB enhanced Tf utilisation by enhancing interactions between the receptor and the ligand, its absence was without severe consequences to Tf utilisation as TbpA was also capable of binding Tf. Conversely, absence of TbpA leads to a more deficient Tf-utilisation system as the first stage of transport of the scavenged iron molecules into the cell will be significantly impaired.

1.5.2 Iron acquisition from haemoglobin or haemoglobin complexes

As stated earlier, iron bound to haemoglobin (Hb) or haemoglobin complexes could provide a valuable resource for any pathogen during intravascular colonisation. In addition to the ability to utilise transferrin and lactoferrin, meningococci, like gonococci, possess two receptors that allow for the utilisation of Hb.

The first of these receptors, encoded by *hpuAB*, is a bipartite receptor like the Tf and Lf receptors with exceptions in the nomenclature of both components – *hpuA* is the substrate-binding lipoprotein while *hpuB* is the transmembrane protein (Lewis *et al.*, 1997). In addition to binding Hb, HpuAB can release haem stored in haemoglobin-haptoglobin (Hb-Hp) and apo-haptoglobin (apo-Hp) complexes (Lewis *et al.*, 1997) and transport intact haem into the cell (Lewis *et al.*, 1998). However, utilisation of free haem by gonococci is neither HpuAB nor TonB-dependent (Turner *et al.*, 1998). Expression of *hpuAB* is transcriptionally-regulated by Fur (Lewis *et al.*, 1997) and translationally-controlled by a polyG tract in the reading frame of *hpuA* (Lewis *et al.*, 1999). Like the TbpBA receptor, the lipoprotein component of HpuAB i. e. HpuA, is unable to mediate Hb or Hb-Hp utilisation independently of HpuB but experimental data suggests that it contributes significantly to optimal binding of HpuAB to Hb, Hb-Hp and apo-Hp. HpuB, on the other hand, can mediate Hb utilisation in the absence of HpuA, albeit at levels lower than the functional HpuAB receptor (Chen *et al.*, 2002; Rohde *et al.*, 2002; Rohde and Dyer, 2004).

The second Hb receptor, HmbR, is a TonB-dependent receptor of molecular mass ~89 kDa (Stojiljkovic *et al.*, 1996). Like *hpuAB*, expression of *hmbR* is phase-variable via a polyG tract within the reading frame (Lewis *et al.*, 1999; Richardson and Stojiljkovic, 1999) and down-regulated under iron-replete conditions. Several isotypes of the Hb receptors exist with antigenic variation in HmbR being primarily determined by sequences of three putative surface-exposed loops (Perkins-Balding *et al.*, 2003; Evans *et al.*, 2010; Tauseef *et al.*, 2011). The importance of these Hb receptors to meningococcal virulence have been demonstrated in an infant rat model where proliferation of a Δ*hmbR* mutant was attenuated in an infant rat model (Stojiljkovic *et al.*, 1995) and in an accidental human passage that revealed a difference in the *hpuAB* expression status of the inoculum (*hpu*-OFF) and output (*hpu*-ON) populations (Omer *et al.*, 2011). These experimental data coupled with genetic studies that

reported a bias for the presence and PV-ON status of one or both genes (*hpuAB* and *hmbR*) in disease isolates present strong indications that Hb-utilisation is crucial to meningococcal virulence (Harrison *et al.*, 2009; Tauseef *et al.*, 2011).

1.5.3   Transport of ferric iron and haem into the cytosol

Transport of ferric iron molecules or haem across the outer membrane and into the periplasmic space by the TbpBA / LbpBA or HpuAB / HmbR systems respectively, is a process that is dependent on conformational changes in the structures of these receptors. These conformational changes undergone by the receptors are energised by the TonB complex, comprised of the neisserial TonB and ExbBD proteins. Inactivation of TonB, especially, led to an inability to utilise Hb, Hb-Hp, Tf or Lf as sole sources of iron in one study conducted by Stojiljkovic and Srinivasan (1997). Evidence presented in a later study by Desai *et al.* (2000), however, strongly suggested the presence of a second but silent TonB system, which is activated in gonococcal Δ*tonB* and meningococcal Δ*tonB*Δ*exbB*Δ*exbD* mutants.

Once iron molecules have been transported into the periplasmic space by the neisserial Tf and Lf receptors, an iron-regulated ATP-binding cassette transporter shuttles the iron molecules from the periplasm into the meningococcal cytoplasm (Adhikari *et al.*, 1996; Khun *et al.*, 1998). Transcribed in an operon (Khun *et al.*, 2000), the tripartite transporter is composed of a well-characterised iron-binding protein, FbpA; a putative inner membrane-spanning permease, FbpB; and a putative ATPase that is believed to power the activity of the operon, FbpC. The functions of FbpB and FbpC in iron transport are still poorly understood; FbpC has been shown to be non-essential to the internalisation of ferric iron in *N. gonorrhoeae* (Sebastian and Genco, 1999).

The exact mechanism with which haem is transported into the cytosol is still unknown as homologues of haem transporters present in other gram-negative bacteria, such as the *hemTUV* transporter of *Yersinia enterolitica* (Stojiljkovic and Hantke, 1994), have not been identified in the meningococcal or gonococcal genomes. The mechanism, however, does not involve *fbpABC* as inactivation of the transporter is inconsequential to haem uptake in the meningococcus (Khun *et al.*, 1998). Notwithstanding the paucity of experimental data

elucidating haem transport in the meningococcus, its utilisation in the meningococcus is directly linked to the presence of a heme oxygenase, HemO, which is co-transcribed with *hmbR* (Zhu *et al.*, 2000). HemO is responsible for the degradation of haem in the cytosol and the prevention of haem toxicity in the meningococcus. While *hemO* mutants also failed to utilise Hb and Hb-Hp, transport of haem into the cytoplasm or the utilisation of non-haem iron sources were not impaired in these mutants (Zhu *et al.*, 2000).
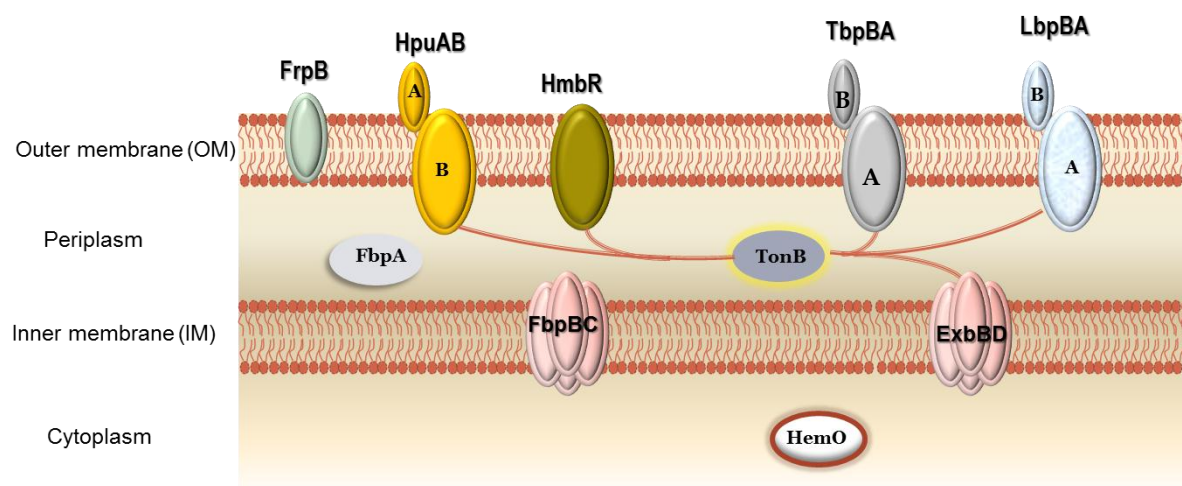
Figure 1.6: Meningococcal receptors involved in the acquisition of iron in the human host. HpuAB and HmbR sequester iron from haemoglobin and haemoglobin complexes while TbpBA and LbpBA enable the utilisation of iron bound to transferrin and lactoferrin, respectively. The FbpABC, TonB and ExbBD receptor complexes are involved in the transport of ferric iron across the membrane and into the cytoplasm. The function(s) of FrpB have not been fully characterised but it may be involved in siderophore utilisation and ferric iron transport. Transport of haem into the cytoplasm has not been elucidated in the meningococcus but its utilisation is dependent on the activity of a cytoplasmic haem oxygenase, HemO.
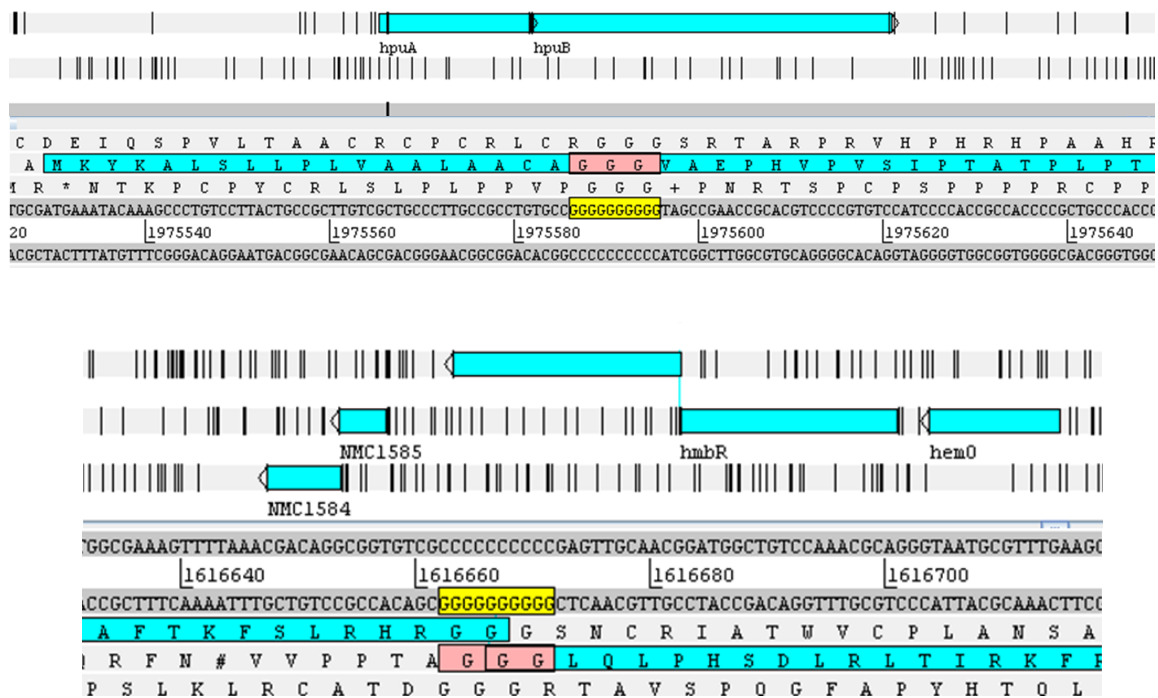
Figure 1.7: Genomic context of *hpuAB* and *hmbR*. The polyG repeat tracts that govern the phase variable expression of both genes (*hpuAB* – top; *hmbR* - bottom) are shown. In the case of *hmbR*, a tract length of 10 units will lead to the production of a truncated gene product, represented above as a split in the *hmbR* coding region. *hemO*, whose gene product is a haem oxygenase crucial to the utilisation of haem by the meningococcus, lies adjacent to *hmbR*. Expression of *hemO* and *hmbR* is transcriptionally linked.

1.6 Aims of the project

The relative abundance of Hb and Hb complexes in the blood and the theory that posits a consequent importance of the meningococcal Hb receptors to blood-borne meningococci is supported by genetic studies of both receptors in separate collections of disease isolates. These studies report that more than 90% of carriage and disease isolates contain one or both receptors in a PV-ON state (Harrison *et al.*, 2009; Tauseef *et al.*, 2011). Further evidence for a crucial role played by one or both receptors was presented in a report detailing the genotypic differences between inoculum and output populations of a meningococcal strain after an accidental infection of a laboratory worker (Omer *et al.*, 2011). It follows, therefore, that meningococcal strains lacking expression of both isolates would be significantly disadvantaged during passage in human blood. One aim of this study is to test the hypothesis that strains incapable of utilising Hb or Hb-complexes via

hpuAB and/or *hmbR* (PV-OFF for both genes) will be unable to proliferate in human whole blood.

Experimental evidence for the importance of the Hb receptors during carriage and disease, despite their phase variable nature, could have important ramifications for meningococcal vaccine development. Expression of these receptors on the meningococcal surface could induce a humoral immune response that may possess bactericidal or opsonic properties. Since a potential biological role for phase variation *in vivo* is to assist the pathogen in the escape of the bactericidal immune response, it would be interesting to explore an association between phase variable expression of these Hb receptors and levels of specific serum IgG antibodies, if present, in human sera. This study will develop an assay, based on previously established protocols, to measure specific serum IgG levels against HpuA in healthy carriers. Preliminary data generated from assay development in this study will serve as background for future studies into the immunogenicity of the Hb receptors and reveal potential associations between PV and specific serum IgG levels.

This study also aims to produce antibodies against recombinant protein preparations of HpuA, HpuB and HmbR, which could be used in downstream applications. For example, a variety of immunological assays that could be used to validate data originating from the aforementioned genetic studies require antibodies to these receptors. Another aim of this study is to confirm surface protein expression in selected isolates and show differences between PV-ON and PV-OFF phenotypes of these receptors using the antigen-specific antibodies generated herein. Finally, the ability of these antibodies to protect against meningococcal bacteraemia will be investigated in *in vitro* serum bactericidal assays.

CHAPTER 2
MATERIALS AND METHODS

2.1   Bacterial strains and growth conditions

Meningococcal carriage strains used in this study were obtained from a carriage study conducted from November 2008 to June 2009 at the University of Nottingham (Bidmos *et al.*, 2011). Disease isolates, MC58 (B:P1.7,16-2:F1-5:CC-32), 8047 (B:P1.5-1,2-2:F3-6:ST-8) and H44/76 (B:P1.7,16:F3-3:CC-32) were obtained from meningococcal disease patients in the UK (McGuinness *et al.*, 1991), US (Weslch and Granoff, 2007) and Norway (Piet *et al.*, 2011), respectively. Strains MC58 and 8047 were provided by Chris Bayliss (Laboratory 121, Department of Genetics, University of Leicester, UK) while strain H44/76 was provided by Hannah Chan (National Institute for Biological Standards and Control, NIBSC, UK).

All strains were grown at the same conditions (37^0C, 5% CO_2, overnight) on Brain Heart Infusion (BHI) agar plates (Oxoid), except otherwise stated. Strains were also grown in brain heart infusion (BHI) broth (Oxoid) at 37^0C with shaking at 200 rpm (overnight). Live cells were handled in a class II microbiological safety cabinet. Before use of whole cells in assays, meningococci were killed by incubation at 56^OC for 18 hours. Mueller-Hinton (MH) agar and broth (Oxoid) were used for specialised assays.

E. coli strains DH5α and BL21 (DE3) were obtained from laboratory stocks (Laboratory 121, Department of Genetics, University of Leicester). *E. coli* strains were grown in Luria broth (LB) (Oxoid) supplemented with appropriate antibiotics at 37^0C under aerobic conditions. *E. coli* cells were also cultured on Luria Agar (LA) (Oxoid).

Selection for antibiotic resistance was achieved by supplementing media with antibiotics at the following final concentrations: ampicillin (100 µg/ml); kanamycin (50 µg/ml); and erythromycin (5 µg/ml for *N. meningitidis* and 50 µg/ml for *E. coli*). For blue-white screening, an X-gal-IPTG mixture (10 mg/ml; Melford) was added to media at a working concentration of 10 µg/ml.

Induction of the expression of iron-regulated meningococcal proteins was achieved by growing cells in iron-chelated media. Dilutions (1:20) of overnight broth culture was grown until an OD_{600} of ~0.5 was reached. Desferal (EMD Chemicals Inc.) was then added at a final concentration of 30 μM (1:1000 of a 30 mM stock preparation) and the cultures allowed to grow, at conditions stated above, for 3 hours.

2.2   Plasma and serum samples

Whole human blood was collected by qualified phlebotomists from healthy volunteers in either sterile heparin-coated vacutainers for plasma or plain vacutainers for sera (BD Vacutainer Systems, UK). For the preparation of sera, whole blood samples were coagulated on ice for 7 hours. A centrifugation step of 4000 rpm at 4^0C for 10 minutes was used to separate the serum from cellular material. Similarly, plasma was obtained from non-coagulated blood via centrifugation (4000 rpm at 4^OC for 10 minutes). Plasma and serum samples were stored at -80^OC. Samples were always thawed on ice for ~ 3 hours prior to use in assays.

2.3   Plasmids and primers

Plasmids were propagated in *E. coli* DH5α and extracted from cells using the EZNA® Plasmid Mini Kit I (Omega Bio-Tek) as per the manufacturer's protocol. For a complete list of plasmids constructed in this study, see Table 2.1.

Primers were designed using Artemis for Windows Release 15.0.0 software (Rutherford *et al*., 2000) hosted on the Wellcome Trust Sanger Institute website (http://www.sanger.ac.uk/resources/software/artemis) and synthesised by Sigma-Aldrich. Stock and working primer solutions were prepared in distilled water. For a complete list of primers used in this study, see Table 2.2.

2.4   DNA analysis methods

Concentration of purified DNA samples was measured using a Nanodrop 2000c spectrophotometer (Thermo Scientific).

2.4.1   Polymerase chain reaction (PCR)

Amplification of DNA was done in a 10 μl reaction volume composed of the following: 1 μl of a 1:10 dilution of DNA as template, 0.2 μM of the required primers, 2.5 mM of $MgCl_2$, 0.25 mM dNTPs; 1x PCR buffer (Kapa Biosystems) and 0.5 U of Kapa *Taq* DNA polymerase (Kapa Biosystems). Each cycle of the PCR was composed of a denaturing step (95^0C for 30 seconds) and extension at 72^0C. The annealing temperature was primer-dependent and the length of the desired product determined the extension time (1 minute for every kilobase). Each PCR was done for 25 cycles.

High-fidelity PCR amplification was used to generate amplicons for cloning purposes using the Phusion® polymerase and buffers, as per the manufacturer's protocol (New England BioLabs® Inc. or NEB).

2.4.2   Restriction endonuclease digestion and DNA ligation

For sub-cloning and plasmid analysis, appropriate restriction endonucleases at a working concentration of 1 U / μg of DNA (NEB) were incubated with plasmid DNA for 90 minutes at 37^0C. Relevant buffers, as per the manufacturer's recommendations, were included in the reactions at a final concentration of 10% v/v of reaction mixture.

Ligation of DNA fragments was achieved using 1 μl of T4 DNA Ligase (NEB - 400 U/μl or Promega – 3 U/μl) in a mixture volume of 20 μl. Vectors and inserts were ligated in a 1:1 ratio while supplied buffers were added to the mixture as follows: 2 μl of the 10x NEB ligase buffer or 10 μl of the 2x Promega ligase buffer. Ligation mixtures were incubated at 16^0C for 18 hours.

Table 2.1: List of plasmids

Plasmid	Description	Source
pUC4kan	Source of kanamycin resistance cassette (*kan*)	Chris Bayliss (Leicester)
pDH20	Source of erythromycin resistance cassette (*ermC*)	Richard Haigh (Leicester)
pUC19porAkan	pUC19, *kan*, *porA* promoter locus (fixed 17-bp spacer i. e. non-phase variable)	Rory Care (NIBSC)
pGEM®-T Easy	Cloning vector, Amp^r	Promega
pLEICS-03	Expression vector, *kan*, N-His$_6$, TEV cleavage site-1	PROTEX (Leicester)
pFAB-20	pGEM®-T Easy, *ermC* flanked by 12-bp Neisserial DNA uptake sequence (*ermC*-DUS)	This study
pFAB-21	pGEM®-T Easy, *kan*, *porA* promoter locus (fixed 17-bp spacer)	This study
pFAB-37	pGEM®-T Easy, *kan*, *porA* promoter locus (fixed 17-bp spacer), Neisserial DUS (2 copies)	This study
pFAB-40	pGEM®-T Easy, *kan*, *porA* promoter locus (fixed 17-bp spacer), Neisserial DUS (1 copy)	This study
pIT–Δ*tbpBA*	pGEM®-T Easy, *kan*, Δ*tbpBA* (880 bp of MC58-*tbpA* fused with 962 bp of MC58-*tbpB*)	Isfahan Tauseef*
pFAB-7	pGEM®-T Easy, Δ*hmbR* (deletion of ~1.3 kb from middle of *hmbR*)	This study
pFAB-9	pGEM®-T Easy, *kan*, Δ*hmbR*	This study
pFAB-22	pGEM®-T Easy, *ermC*-DUS, Δ*tbpBA*	This study
pFAB-2	pLEICS-03, *hpuA* cloned from carriage isolate N88	This study
pFAB-3	pLEICS-03, *hpuA* cloned from disease isolate 8047	This study

Plasmid	Description	Source
pFAB-4	pLEICS-03, *hpuB* cloned from disease isolate 8047	This study
pFAB-5	pLEICS-03, *hmbR* cloned from disease isolate MC58	This study
pFAB-11	pGEM®-T Easy, *hmbR* from strain 8047 with mutated polyG tract (locked to PV-ON)	This study
pFAB-12	pLEICS-03, *hmbR* cloned from pFAB-11	This study
pFAB-17	pGEM®-T Easy, ~1 kb of 8047-*hpuA* from start codon	This study
pFAB-18	pGEM®-T Easy, 1.1 kb of upstream region of 8047-*hpuA* including 456 bp of NMBG2136_1862	This study
pFAB-27	Derivative of pFAB-18 to include 8047-*hpuA* from pFAB-17	This study
pFAB-28	Derivative of pFAB-27, *kan, porA* promoter locus (fixed 17-bp spacer)	This study
pFAB-38	Derivative of pFAB-27, *kan, porA* promoter locus (fixed 17-bp spacer), Neisserial DUS (2 copies)	This study
pFAB-41	Derivative of pFAB-27, *kan, porA* promoter locus (fixed 17-bp spacer), Neisserial DUS (1 copy)	This study
pFAB-29	pGEM®-T Easy, ~1 kb of MC58-*hmbR* from start codon	This study
pFAB-30	pGEM®-T Easy, 815 bp of upstream region of MC58-*hmbR* including 629 bp of NMB_1669	This study
pFAB-31	Derivative of pFAB-30 to include MC58-*hmbR* from pFAB-29	This study

* Source was a PhD student at Laboratory 121, Department of Genetics, University of Leicester at time of plasmid construction.

Table 2.2: List of primers

Primer	Sequence	Notes
T7-pro	taatacgactcactataggg	Vector-specific primer - used to amplify / sequence insert
SP6-rev	atttaggtgacactatag	Vector-specific primer - used to amplify / sequence insert
T7-term	tatgctagttattgctcag	Vector-specific primer - used to amplify / sequence insert
over-for	ccggatccacatggcgatagc	Used to amplify *kan-porA* promoter fragment from pUC19porAkan
over-rev	gcatatggcttcctttgt	Used to amplify *kan-porA* promoter fragment from pUC19porAkan
overDUS-for	ccggtacc**ATGCCGTCTGAA**acatggcgatagc	Variant of over-for - used to introduce DUS
overDUS-rev	gcatatgatgccgtctgaagcttcctttgt	Variant of over-rev - used to introduce DUS
kanR-for	tcgtgaagaaggtgttgctg	Used to amplify ~600 bp region from *kan*
kanR-rev	ggttgcattcgattcctgtt	Used to amplify ~600 bp region from *kan*
ermDUS-for	ttt**ggatcc**ATGCCGTCTGAAcatgcaagctagctttggc	Used to introduce *BamHI* (bolded) and DUS to *ermC* amplicon
ermDUS-rev	ttt**ggatcc**TTCAGACGGCATcttacttattaaataattatagctattg	Used to introduce *BamHI* (bolded) and DUS to *ermC* amplicon
ctrA-for	gctgcggtaggtggttcaa	Used to amplify short region from *ctrA*
ctrA-rev	ttgtcgcggatttgcaacta	Used to amplify short region from *ctrA*
crgA-for	gctggcgccgctggcaacaaattc	Used to amplify short region from *crgA*
crgA-rev	cttctgcagattgcggcgtgccgt	Used to amplify short region from *crgA*

Primer	Sequence	Notes
HpuA-N-Pleic	tacttccaatccatggccgaaccgcacgtcccgtgt	Cloning primer for recombinant *hpuA* expression
HpuA-C-Pleic	tatccacctttactgtcaggggaaacgcttgggcgat	Cloning primer for recombinant *hpuA* expression
hpuA-rev-672	ataaccactgttttcggc	Sequencing primer
1945-for-NcoI	gtcCCATGGggaaatcgtcaaactc	Cloning primer for *hpuAB* over-expression– contains *Nco*I site
Hpu-up-rev	CATATGtctGGTACCaaagaaaccgttttacccg	Cloning primer for *hpuAB* over-expression – contains *Kpn*I and *Nde*I sites
HpuA-for-Nde	gcgCATATGaaatacaaagcc	Cloning primer for *hpuAB* over-expression – contains *Nde*I site
HpuA-rev-Nsi	agATGCATggtggtacttgtatcag	Cloning primer for *hpuAB* over-expression – contains *Nsi*I site
1945F2-seq	gcaccagtcattcctacg	Sequencing primer
1945R2-seq	gcctgcacttttggatgac	Sequencing primer
hpuF-seq	gcgaaatcatcggcaacagc	Sequencing primer
hpuR-seq	gctgaatggaaatgccgga	Sequencing primer
HpuB-N-Pleic	tacttccaatccatggcgtttccgcctttgcggcaga	Cloning primer for recombinant *hpuB* expression
HpuB-C-Pleic	tatccacctttactgtcattagaacttcgcttcgatggt	Cloning primer for recombinant *hpuB* expression
hpuB-1659F	caggctctttggtttggatg	Sequencing primer
HPB-Bam	ccattggatccggcatatcc	Sequencing primer
hpuB-1850rev	gccaaacgggcgagctgacc	Sequencing primer
hpuB-2356R	cggtggtgtatttcggatcg	Sequencing primer

Primer	Sequence	Notes
HmbR-N-Pleic	tacttccaatccatggcagatgaagctgcaactga	Cloning primer for recombinant *hmbR* expression
HmbR-C-Pleic	tatccacctttactgtcattaaaacttccattccagcgat	Cloning primer for recombinant *hmbR* expression
HmbR-lck-for	cgttgcaactcggAggggggcgacacc	Used to lock *hmbR* repeat tract – substitutes an adenine for a guanine in the 5′ – 3′ direction
HmbR-lck-rev	ggtgtgcgcccccTccgagttgcaacg	Used to lock *hmbR* repeat tract – substitutes a thymine for a cytosine in the 3′ – 5′ direction
hmbRF-Nde	aatCATATGaaaccattacaaatgc	Cloning primer for *hmbR* over-expression – contains *Nde*I site
hmbRR-Nsi	ttgATGCATtgcgccgtctgtttacgt	Cloning primer for *hmbR* over-expression – contains *Nsi*I site
1669F-NcoI	aatCCATGGatgagtgaaccg	Cloning primer for *hmbR* over-expression – contains *Nco*I site
1669R-Nde-Kpn	CATATGcaaGGTACCctcaagatgtatggattg	Cloning primer for *hmbR* over-expression – contains *Kpn*I and *Nde*I sites
1669R2	cgtgttcgccgttgtaatcg	Sequencing primer
1669F2	gcatagctgtttgcagggac	Sequencing primer
hmbR-seqR	gcaccaagtctttattgtcgc	Sequencing primer
hmbR-seqF	gcgaatatccgtggttcggc	Sequencing primer
HmbR-Nterm	caccatgaaaccattacaaatgctccc	Used to amplify region of *hmbR*
HmbR-Cterm	ttaaaacttccattccagcg	Used to amplify region of *hmbR*
RF3	tgccaacctctttacgaatgg	Used to amplify region of *hmbR*

Primer	Sequence	Notes
RF4	gctactgaacacgtcgttcc	Used to amplify region of *hmbR*
dhmbR-for	gctGGATCCcaatctgtatc	Cloning primer for *hmbR* deletion – contains *BamHI* site
dhmbR-rev	tgGGATCCattcacggttacgc	Cloning primer for *hmbR* deletion – contains *BamHI* site
TbpAF-Bam	cctGGATCCccggcatcgtc	Cloning primer for *tbpBA* deletion - contains *BamHI* site
TbpAR	ggcatttgcggcgtttggac	Cloning primer for *tbpBA* deletion
TbpF	ggtgaatcaggctgctatgg	Cloning primer for *tbpBA* deletion
TbpR-Bam	agGGATCCtccttggtttcg	Cloning primer for *tbpBA* deletion - contains *BamHI* site
hpuAC-6FAM	6FAM-atgcgatgaaatacaaagccc	Genescan primer – fluorescently-labelled at 5′ with 6FAM
hpuA350 rev	ggatgaaagggcgtattgcgc	Genescan primer
RF3-6FAM	6FAM-tgccaacctcttttacgaatgg	Genescan primer – fluorescently-labelled at 5′ with 6FAM

* Upper case letters represent sequences introduced by the primer into amplicons.

2.4.3   Agarose gel electrophoresis

PCR amplicons and restriction digests were analysed on 1% agarose gels prepared in 1x TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.2). Ethidium bromide was added to gels, at a working concentration of 0.5 µg/ml, to facilitate visualisation of samples on exposure of the gel to ultraviolet light. Sample sizes were measured against a DNA size standard, HyperLadder™ 1kb (Bioline Reagents Ltd.). Gels were visualised using the Syngene Bioimaging system.

2.4.4   Gel purification of DNA fragments

Desired DNA fragments were carefully excised from agarose gels before isolation using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research), as per the manufacturer's protocol. DNA was eluted in 10 µl of pure distilled water.

2.4.5   Genescan analysis

Amplicons were generated using appropriate fluorescently-labelled primers and cycling conditions. To ensure that all amplicons contained the extra adenine residue non-specifically incorporated by *Taq* polymerase at the 3′ end, a further extension step, known as A-tailing, was performed. To 10 µl of the PCR reaction, 4 µl of the A-tailing mix (0.4 µl 10x PCR buffer, 0.05 µl *Taq* DNA polymerase and 3.55 µl distilled water) was added and the mixture incubated for 45 minutes at 72^0C. Each sample was subsequently diluted 10-fold before 0.5 µl of the diluted samples were transferred onto separate wells of semi-skirted microplates. Formamide (9.25 µl) and 0.25 µl of the DNA size standard, GeneScan™ 500 LIZ™ Size Standard (Life Technologies™) were added to each well before plates were loaded onto the ABI 3730 DNA Sequencer for analysis. Output data generated by the sequencer were analysed using the Peak Scanner software (Applied Biosystems) and Microsoft Excel.

2.4.6   DNA sequencing

DNA fragments were sequenced using Big Dye v3.1 provided by the Protein and Nucleic Acids laboratory (PNACL). Each sequencing reaction mixture was composed of 1 µl template DNA, 1 µl of the required primer, 4 µl of the Big Dye v3.1 mix (0.5 µl Big Dye v3.1, 0.8 µl 5x sequencing buffer, 2.7 µl distilled water) and 4 µl distilled water

to give a reaction mixture volume of 10 µl. Each cycle in the sequencing reaction was composed of a DNA denaturing step of 96^0C for 20 seconds before primers were allowed to anneal at 50^0C. An extension step that lasted for 4 minutes at 60^0C completed each cycle. Products from the sequencing reaction were purified using Performa® DTR gel filtration cartridges (EdgeBio) and analysed using an automated sequencer, ABI 3730 DNA Sequencer (ABI, Applied Biosystems) at PNACL.

Consensus DNA sequences were generated using the Pregap4 and Gap4 applications in the Staden software package (Sourceforge) on the SPECTRE Linux platform of the University of Leicester. Sequence alignment was performed with Clone Manager 9 (Sci-Ed software). Complete meningococcal genomes were obtained from the NCBI website (http://www.ncbi.nlm.nih.gov/genome/browse/) and analysed using Artemis for Windows Release 15.0.0 (Rutherford *et al.*, 2000) hosted on the Wellcome Trust Sanger Institute website.

2.4.7 TA-Cloning

Amplicons generated from high-fidelity PCRs (see section 2.4.1) were blunt-ended as the Phusion® polymerase does not incorporate an extra adenine residue at the 3' end of amplicons, unlike *Taq* polymerase. Thus, amplicons from these PCRs were A-tailed prior to cloning into the pGEM-T® Easy vector. Completed PCR reactions were first analysed on agarose gels and the desired amplicons gel-purified and eluted in 10 µl of pure distilled water. To 7 µl of this purified amplicon, 1µl of the following were added to give a final volume of 10 µl: 10x Kapa PCR buffer, 2 mM dATP and Kapa *Taq* polymerase. The A-tailing process was completed by incubating the mixture at 70^0C for 30 minutes. A-tailed amplicons were subsequently TA-cloned using the manufacturer's protocol (Promega).

2.4.8 Transformation of *E. coli* DH5α cells (heat shock method)

Briefly, 50 µl of DH5α cells were mixed with the plasmid and incubated on ice for 25 minutes. The mixture was transferred to a 37^0C water bath for 5 minutes followed immediately by a 5-minute incubation on ice. The transformation mixture was supplemented with 900 µl of LB and incubated in a 37^0C water bath for 1 hour.

Transformed cells were grown overnight in LA supplemented with appropriate antibiotics and X-gal, where blue-white discrimination of transformants was required.

2.5 Protein analysis methods

2.5.1 Cell lysate preparation

Bacterial cells were grown overnight as stated in section 2.1. Cultures were killed by incubation at 56^0C for 18 hours before the OD_{600} of each culture was measured and fixed at ~0.5 by diluting cells in PBS. A 1 ml aliquot of the fixed OD cell suspension was centrifuged at 13000 rpm for 1 minute before re-suspending the cell pellets in 100 µl of 1x Laemmli buffer (1M Tris pH 6.8, 4% SDS, 0.1% bromophenol blue, 20% glycerol, 200mM DTT). The suspension was subsequently boiled for 5 minutes at 98^OC.

2.5.2 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Cell lysates and protein samples were analysed on 10% SDS-PAGE gels as described by Laemmli (1970). The separating gel was prepared with the following components: 2.7 ml buffer A (750 mM Tris, 0.2% SDS, pH 8.8), 1.83 ml UltraPure Protogel® (Geneflow), 2.3 ml distilled water, 190 µl 1% APS and 15 µl TEMED (Sigma-Aldrich®). The stacking gel was composed of: 1 ml buffer B (250 mM Tris, 0.2% SDS, pH 6.8), 330 µl UltraPure Protogel® (Geneflow), 1.9 ml distilled water, 50 µl 1% APS and 4 µl TEMED (Sigma-Aldrich®). Electrophoresis was performed at constant voltage of 80 V for 3 hours.

2.5.3 SDS-PAGE gel staining

SDS-polyacrylamide gels were stained with staining solution (10% glacial acetic acid, 45% methanol, 0.25% w/v Brilliant Blue R – Sigma-Aldrich®) for 30 minutes at room temperature with gentle shaking (40 rpm). Stained gels were subsequently bleached with three changes of de-staining solution (7.5% glacial acetic acid, 20% methanol).

2.5.4 Western blotting

Electrophoresed samples were transferred from SDS-PAGE gels onto pre-activated polyvinylidene fluoride (PVDF) membrane (pre-activated with methanol) using ice-cold Towbin transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol). Transfer

was done for 1 hour at constant ampere of 150 Amp before membranes were blocked overnight at 4^0C with blocking buffer (PBS, 0.5% Tween-20, 5% Milk). Following blocking, membranes were incubated with an appropriate concentration of primary antibodies in blocking buffer for 1 hour at room temperature with gentle shaking. Unbound primary antibodies were removed by incubating membranes with wash buffer (PBS, 0.5% Tween-20) for 5 minutes at room temperature with gentle shaking. The wash step was repeated at least thrice. Membranes were subsequently incubated in a 1:2000 dilution of anti-mouse horseradish peroxidise (secondary antibody), prepared in blocking buffer, for 1 hour at room temperature. Unbound secondary antibodies were removed as previously described with wash buffer before signals were developed with an EZ-ECL Chemiluminescence kit for HRP (Geneflow). Blots were quantified with ImageJ software, as prescribed by Miller (2010).

2.5.5   Enzyme-linked immunosorbent assay (ELISA)

Inactivated meningococcal whole cell suspensions were prepared and normalised to an OD_{600} of ~0.5 as described in section 2.5.1. Whole cells (100 µl of cell suspensions) or purified proteins (1 – 5 µg) in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$) were transferred into designated wells of flat-bottom polystyrene 96-well plates and incubated at 4^0C overnight. Plates were coated in either duplicates or triplicates. The supernatants were discarded leaving the whole cell or protein antigen bound to the walls of the wells. Whole cell or protein antigens were subsequently incubated with 150 µl of blocking buffer (PBS, 0.05% Tween-20, 1% BSA) for 1 hour at room temperature prior to addition of 100 µl of an appropriate dilution of the primary antibody (prepared in blocking buffer). Incubation with the primary antibody was done for 1 hour at room temperature. A no-primary antibody control was included in which wells were incubated with blocking buffer only. After three washes with the wash buffer (PBS, 0.05% Tween-20) to remove unbound antibodies, a 1:2000 dilution of anti-mouse alkaline phosphatase (prepared in blocking buffer), was used to probe wells for 1 hour at room temperature. A set of wells were excluded from this step and blocking buffer only was added to this wells (no secondary antibody control). Unbound antibodies were removed with >3 wash cycles as described previously before plates were developed using a phosphatase substrate kit (Thermo-Fisher Scientific) as per the manufacturer's instructions. Colour development was measured at OD_{405} using

a microplate reader. Background values (negative controls) were subtracted from the mean of values obtained for a sample to obtain the final value for the sample.

2.5.6 Flow cytometry

From a meningococcal culture, grown in iron-replete or iron-restricted conditions (see section 2.1), 150 μl of cells were transferred to a clean, sterile 1.5 ml Eppendorf microcentrifuge tube. Cells were pelleted by centrifugation at 8000 rpm for 1 minute and washed with assay buffer twice (50 mM Tris.Cl pH 7.5, 150 mM NaCl, 5 mM CaCl$_2$, 0.05% Tween-20). Cells were subsequently treated with a 1:20 dilution of antigen-specific antibodies prepared in 150 μl assay buffer. The antibody-cell suspension was incubated at room temperature for 1 hour after which unbound antibodies were removed in 3 wash cycles. Following the removal of unbound primary antibodies, 20 μg/ml of the secondary antibody, Alexa Fluor® 488 Goat Anti-Mouse IgG (H+L) (Life Technologies™), was added to cells and the suspension incubated for 1 hour at room temperature. Three wash cycles were performed to remove unbound secondary antibodies before cells were inactivated in 1 ml fixer solution (PBS, 0.5% formalin). Samples were analysed on a FACS analyser with the help of Dr. Mohammed Ali (Department of Infection, Immunity and Inflammation, University of Leicester).

2.5.7 Bioinformatic analysis and mass spectroscopy of recombinant proteins

Molecular weight and isoelectric point (pI) estimations were made using an online computation tool hosted on the ExPASy Bioinformatics Resource Portal (http://web.expasy.org/compute_pi/). A beta-barrel OMP prediction tool based on an Hidden Markov model (Bagos *et al.*, 2004) was used to predict the topologies of HpuA and HmbR (http://biophysics.biol.uoa.gr/PRED-TMBB/input.jsp). The identity of recombinant proteins were confirmed with a mass spectrometry service provided at PNACL.

2.6 Gene knockouts, transformation of meningococci and whole blood assay

2.6.1 Deletion of *hmbR* in strains 8047 and MC58

Primers, HmbR-Nterm and dhmbR-rev, were used to amplify a 542 bp region from the N-terminus of *hmbR*. Similarly, HmbR-Cterm and dhmbR-for amplified 676 bp from

the C-terminus of *hmbR*. Genomic DNA from strain 8047 was used as template in these PCRs. These amplicons contained *BamH*I restriction sites, introduced via dhmbR-for and dhmbR-rev, which would facilitate downstream ligation and sub-cloning steps.
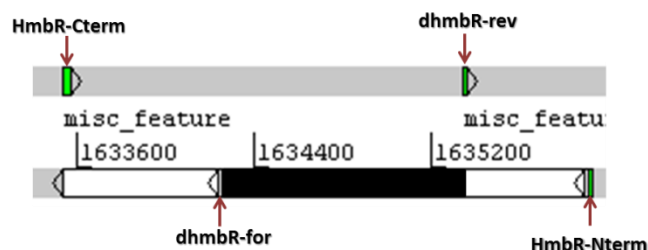
Figure 2.1: Primer sites relative to *hmbR*. The area shaded in black was the deleted region.

Both amplicons were subjected to *BamH*I restriction digests and ligated together as previously described. The ligation reaction was analysed on a 1% agarose gel and the desired 1.3 kb fragment was gel-purified. This fragment was TA-cloned into the pGEM-T® Easy plasmid vector using a kit supplied by the manufacturer (Promega). The plasmid was subsequently used to transform chemically-competent *E. coli* DH5α cells using a heat-shock protocol. Transformed cells were grown overnight in LA supplemented with ampicillin (100 μg/ml) and X-gal (30 mg/ml). Plasmids were extracted from white colonies (indication of successful insertion of 1.3 kb fragment into pGEM-T® Easy vector) and analysed via an *EcoR*I digest and DNA sequencing. To complete the construction of the *hmbR* deletion construct, a kanamycin resistance cassette was sub-cloned from pUC4kan into the *hmbR* deletion plasmid via the unique *BamH*I site. The resulting plasmid, extracted from kanamycin-resistant colonies, was analysed via *BamH*I and *EcoR*I restriction digests.

2.6.2 Natural transformation of meningococcal cells

Natural transformation of meningococci was successfully achieved using either method below:

a. The *hmbR* deletion plasmid construct was transformed into strain 8047 as follows: 500 ng of purified plasmid DNA, in ultra-pure water, was mixed with cells of strain 8047 on BHI agar with Levinthal's supplement. Genomic DNA from strain IT-8047Δ*hmbR* (insertional inactivation of *hmbR* with a kanamycin resistance cassette made by Isfahan Tauseef) was also used to transform cells

(positive control). Incubation was done for 5 hours to allow for DNA uptake. Cells were subsequently harvested and plated on BHI agar supplemented with kanamycin (80 µg/ml). Plates were incubated for 48 hours at 37^0C with 5% CO_2. Kanamycin-resistant colonies were sub-cultured and DNA preparations made from these cultures were used as templates in PCRs (see section 3.2). The inability to utilise Hb as a sole iron source was confirmed in a disc diffusion assay.

b. Strain MC58 was transformed with the *hmbR* deletion construct as follows: a 1:10 dilution of cells grown overnight in supplemented BHI medium was incubated for an hour with shaking at 37^0C. 200 µl of the culture was added to supplemented BHI agar in 24-well plates. The culture was incubated for 5 hours at 37^0C with 5% CO_2 before 1.5 µg of the *hmbR* deletion construct, linearized with *Nde*I, was added. The transformation mixture was incubated overnight at 37^0C with 5% CO_2. Cells were harvested from the mixture and plated on selective BHI agar plates infused with 80 µg/ml of kanamycin. Kanamycin-resistant colonies that were visible on agar plates after 24 or 48 hours were processed as previously described. As with the 8047Δ*hmbR* mutants, inability of the MC58Δ*hmbR* mutants to utilise Hb as a sole iron source was confirmed in a disc diffusion assay.

2.6.3 Deletion of *tbpBA* in MC58 and 8047 wild-type and mutant strains

A *tbpBA* deletion construct (pIT-Δ*tbpBA*) was constructed by Isfahan Tauseef with kanamycin resistance as the selectable marker. Like the *hmbR* deletion construct, 2 regions of the *tbpBA* locus corresponding to the 5' region of *tbpB* or *tbp2*, the 3' region of *tbpA* or *tbp1* and a kanamycin resistance cassette compose pIT-Δ*tbpBA* (see Figure 2.2 for relative positions of the primers used to amplify the *tbpBA* regions).
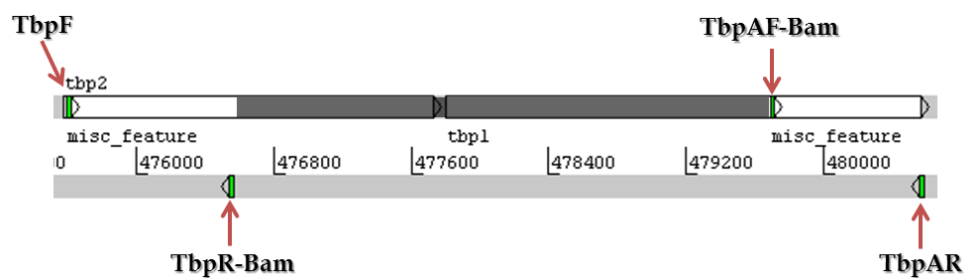
Figure 2.2: Primer sites relative to *tbpBA*. The transparent area shaded in grey was the deleted region.

For the generation of double knockout strains (i. e. $\Delta hmbR\Delta tbpAB$ or $\Delta hpu\Delta tbp$), a different selectable marker for the deletion of *tbpBA* in these strains was required as both $\Delta hmbR$ and $\Delta hpuAB$ mutant strains (either made in this study or obtained from other sources) were kanamycin resistant. Incorporation of the 12-bp Neisserial DUS into the plasmid was also desired as this short sequence significantly enhances uptake of exogenous DNA by the meningococcus (Ambur *et al.*, 2007). Primers, ermDUS-for and ermDUS-rev, were designed to incorporate the 12-bp Neisserial DUS at both ends of the *ermC* gene amplified from pDH20. These primers also contained *BamH*I restriction sites that would facilitate sub-cloning of the *ermC*-DUS fragment into pIT-Δtbp. The amplicon was TA-cloned into the pGEM-T® Easy vector and transformed into *E. coli* DH5α cells. Erythromycin-resistant colonies were sub-cultured in LB supplemented with 50 µg/ml erythromycin and plasmids were extracted from these cultures. The kanamycin resistance cassette was excised from pIT-$\Delta tbpBA$ in a *BamH*I digest leaving sticky ends, to which the *BamH*I-excised *ermC*-DUS fragment was ligated. This plasmid was propagated in *E. coli* DH5α and transformed cells were selected on LA supplemented with erythromycin at 50 µg/ml. Extracted plasmids were subsequently analysed in an *EcoR*I-*BamH*I digest before meningococcal cells were transformed, as outlined in section 2.6.2b.

2.6.4   Disc diffusion assay

From an overnight growth on agar plates, a loopful was taken and re-suspended in 500 µl PBS before 20 µl of the suspension was taken and added to 980 µl of lysis buffer (0.1 M NaOH, 1% SDS). The DNA concentration of the lysate (OD_{260}) was measured using a Nanodrop spectrophotometer and the volume of the cell suspension containing 10^9 CFU was deduced using the following formula:

$$\frac{1.8}{OD_{260} \times \text{Dilution factor}}$$

This volume of the cell suspension containing 10^9 cells was subsequently spread evenly on iron-chelated Mueller-Hinton (MH) agar (MH agar with 40 µg/ml desferal). Plates were allowed to dry before sterile filter discs (5 mm in diameter) infused with an iron source were carefully placed on different sections of the plate. Alternatively, 5 µl of the exogenous iron sources were dropped on different sections of the plate. Exogenous

iron sources were 500 μg of human holo-transferrin (Sigma-Aldrich®), 100 μg of ferrous-stabilised human haemoglobin A0 (Sigma-Aldrich®) or 100 mM $FeCl_3$. Sterile PBS buffer was also included as a negative control. Plates were incubated overnight for 24 to 48 hours until rings of growth were clearly visible around the iron sources.

2.6.5   Meningococcal growth assay

Growth of wild-type and mutant strains was compared in MH broth. Overnight cultures in MH broth were diluted to an OD_{600} of 0.1 in fresh MH broth and 200 μl of these dilutions were transferred to 96-well flat-bottom polystyrene plates. Plates were sealed with perforated films before loading onto a multifunctional FLUOstar Omega microplate reader (BMG Labtech). The reader, with the aid of an in-built atmospheric control unit, provided the ideal growth conditions for the meningococcal cultures: 37^0C, 5% CO_2 with gentle shaking. Six replicate cultures of each strain were analysed on each plate and the assay was repeated on two separate days. The mean of OD_{600} readings taken at hourly intervals for each strain were plotted on a chart.

2.6.6   Whole blood assay

The protocol for the whole blood assay used in this study was adapted from similar studies that employed the model (Ison *et al.*, 1995; Seib *et al.*, 2009). Two healthy volunteers deficient in the Mannose-binding lectin (MBL) were recruited for the provision of whole blood. A 1:20 dilution of an overnight culture in MH broth supplemented with 20 μM CMP-N-acetylneuraminic acid sodium salt (CMP-NANA) and 0.25% glucose was grown to an OD_{600} of ~0.5. Approximately $10^6 – 10^8$ cells were taken from this mid-logarithmic culture and used to inoculate 500 μl of freshly-collected heparinized human blood (0.01 U of heparin/μl of blood) in 24-well plates. Duplicate blood cultures of each strain were prepared. The 24-well plates were subsequently incubated at 37^OC, 5% CO_2 for 4 – 8 hours (see section 3.10 – 3.11). Dilutions of each strain culture pre-inoculation into blood were plated on Brain-Heart Infusion (BHI) Agar supplemented with Levinthal's supplement and grown overnight. This was recorded as the actual inoculum CFU population. Samples were also taken from the blood culture immediately after inoculation (T_0) and at hourly intervals afterwards. Each sample was serially diluted before plating, in duplicates. The assay was done at least 5 times with each volunteer.

2.7 Generation of rHpuA, rHpuB and rHmbR antisera

2.7.1 Expression and purification of recombinant proteins rHpuA, rHpuB and rHmbR

Amplification of *hpuA*, *hpuB* and *hmbR* from strains N88, 8047 and MC58 respectively was done using primers listed in Table 2.2. A cloning service provided by the PROTEX laboratory was employed to clone the amplicons into a pLEICS-03 vector (Table 2.1). Confirmation of cloning was done by sequencing the insert using vector-specific primers and DNA sequencing of the insert (Table 2.2). To express the desired genes, clones were transformed into an *E. coli* expression strain BL21 and grown overnight in 10 ml of LB supplemented with kanamycin (50 µg/ml) at conditions outlined in 2.1. Overnight cultures were used to inoculate 100 ml of LB supplemented with kanamycin (50 µg/ml) and grown till an OD_{600} of ~0.5 was reached. Expression of recombinant protein was subsequently induced in these cultures by adding IPTG at a final concentration of 1 mM. Cultures were allowed to grow for a further 3 hours; 500µl of the cultures were taken every hour (including pre-induction) and boilates prepared for analysis on SDS-PAGE as stated previously in sections 2.5.2 – 2.5.3.

2.7.2 Purification of rHpuA

Cultures containing expressed proteins were pelleted and re-suspended in lysis buffer (20 mM NaH_2PO_4, 500 mM NaCl, 100 mM KCl, 10% glycerol, 0.5% Triton X-100, 10 mM imidazole). Lysozyme (7.5 µg/ml) was added to the cell suspension and mixed thoroughly by vortexing. The suspension was incubated in a 37^0C water bath for 30 minutes before sonication in an ice bath (15 seconds on, 15 seconds off for 30 minutes). Soluble and insoluble fractions of the lysate were separated by centrifugation at 13000 rpm for 10 minutes. Recombinant HpuA was purified from contaminating *E. coli* proteins in the soluble fraction using a Ni-Sepfast™ gravity column (Flowgen Bioscience). The slurry was first equilibrated with 6x volume of lysis buffer before wash steps with buffer (20mM NaH_2PO_4, 500mM NaCl, 100mM KCl, 10% glycerol, 0.5% Triton X-100) containing 20mM, 50mM, 80mM and 100mM of imidazole. Elution of the desired his-tagged protein was done with the same buffer containing 120mM, 150mM, 200mM, 250mM and 500mM of imidazole. After analysis on SDS-PAGE gels,

purified proteins were dialysed into PBS using Millipore™ centrifugal filter units. Proteins were quantified using the Quickstart™ Bradford kit (Biorad Laboratories Inc.), as per the manufacturer's instructions.

2.7.3 Purification of rHpuB and rHmbR

A modified version of the protocol provided by Jeremy Derrick (previously used to purify rHmbR) was employed in the isolation of inclusion bodies, refolding and purification of rHpuB and rHmbR. Cells containing expressed rHpuB or rHmbR (from a 150 ml culture) were pelleted by centrifugation (10 min, 3500 rpm) before resuspension in 15ml of 50mM Tris.Cl pH 8.0. Cells were subsequently lysed by sonication in an ice-cold water bath for 30 minutes (15 seconds intervals). Lysates were separated into soluble and insoluble fractions by centrifugation at 4^0C, 15 minutes, 3500 rpm. The insoluble fraction was resuspended in 40 ml of 50mM Tris.Cl pH 8.0, 5% v/v LDAO and the suspension stirred for 2 hours at 4^0C. Pellets (i. e. inclusion bodies) were washed thrice in 10 ml of 50mM Tris.Cl pH 8.0 before solubilisation and refolding of the recombinant proteins. These inclusion bodies were briefly solubilised in 4 ml of solubilisation buffer (20 mM Tris.Cl pH 8.0, 6M GuHCl, 1mM EDTA) and applied in a drop-wise manner to 36 ml of refolding buffer (20 mM Tris.Cl pH 8.0, 5% v/v LDAO, 250mM NaCl, 50µM Hemin) on a magnetic stirrer. The protein was allowed to refold for 4 hours at room temperature. Haem-bound proteins were dialysed overnight at 4^0C against 500 ml of dialysis buffer (20 mM Tris.Cl pH 8.0, 0.1% v/v LDAO). Insoluble material, after dialysis, was removed by centrifugation at 13000 rpm for 10 minutes in chilled conditions (4^0C). The supernatant was loaded onto 5 ml of pre-equilibrated DEAE-Sephacel column (pre-equilibrated with 30 ml of dialysis buffer) and the flow-through collected. The column was subsequently washed with dialysis buffer containing 150mM NaCl and eluted with 300mM, 450mM, 600mM and 1M NaCl.

2.7.4 Immunisation of mice and analysis of antisera

Immunisations of mice were performed at NIBSC, UK. Six groups of 10 female BALB/c mice (6 – 7 weeks old) were subcutaneously injected with 200 µl of antigen preparations (20 µg antigen + 10 µg Monophosphoryl Lipid A) as follows:

- Group 1: r8047-HmbR + MPL adjuvant
- Group 2: rMC58-HmbR + MPL adjuvant
- Group 3: r8047-HpuA + MPL adjuvant
- Group 4: rN88-HpuA + MPL adjuvant
- Group 5: r8047-HpuB + MPL adjuvant
- Group 6: MPL adjuvant only

Boosters i. e. 200 µl of the antigen preparations as above, were administered at weeks 3 and 5. Tail bleeds were obtained at week 6 for initial screening of antibody production and to identify suitable sources of spleen for monoclonal antibody production. Terminal bleeds were obtained at week 7 and spleens were removed from 4 mice per group, excluding Group 6.

Polyclonal antisera were tested by western blotting, ELISA and flow cytometry, as described in sections 2.5.4 - 2.5.6. Antisera from each mouse were tested against all antigens for reactivity against homologous proteins and non-specific cross-reactivity against unrelated proteins. Antigens used in these assays were the recombinant protein preparations, *E. coli* and meningococcal cell lysates.

2.8   Multiplex immunodetection (Liquichip) assay

Fluorescently-labelled beads coupled to 7 purified PorA variants and 1 PorA protein lacking loops corresponding to hypervariable regions of *porA*, VR1 and VR2, were obtained from Hannah Chan and Hema Patel (Division of Bacteriology, National Institute of Biological Standards and Control – NIBSC - UK). All experiments were performed at NIBSC, UK. Serial dilutions of test sera (25 µl), prepared in assay buffer (PBS, 0.1% BSA, 0.05% Triton X-100), were incubated with beads (25 µl) in 96-well filter plates (room temperature, 30 minutes with gentle shaking). Pooled sera from vaccinees were used as control for the assay. Unbound antibodies in sera were removed by washing the microspheres thrice with 50 µl assay buffer using a vacuum pump. The protein-antibody complex on beads was subsequently incubated with 50 µl anti-human R-phycoerythrin (reporter molecule) at same conditions as before. Prior to loading onto the Liquichip workstation for analysis, the plate was washed thrice with 50 µl of assay buffer and the microspheres re-suspended in 150 µl of the same buffer. The mean fluorescence intensity (MFI) of the dilution series of the control sera were used to

generate a standard curve, from which antibody amounts (in arbitrary units) for test sera were derived. See section 5.1 for a more detailed outline of the experiments performed.

2.9 Generation of meningococcal strains over-expressing *hpuAB* and *hmbR*

The same cloning strategy was used to construct *hpuAB* and *hmbR* over-expression plasmids. A plasmid, pUC19porAKan, containing a fusion of the 17-bp *porA* promoter and kanamycin resistance cassette (*kan*) was kindly provided by Rory Care (NIBSC, London). The *kan – porA* promoter fusion was amplified from this plasmid using specific primers and TA-cloned into the pGEM-T® Easy vector to give pFAB-21. Similarly, approximately 1 kb each of the genes (*hpuAB* and *hmbR*) and their upstream regions were separately TA-cloned into pGEM-T® Easy vectors. After determining the orientation of the inserts by *Nde*I-*Nsi*I digests, an excised insert from either plasmid was ligated into a copy of the other plasmid that was linearised. The resulting over-expression plasmid containing both gene and upstream fragments were subsequently analysed via restriction digests and DNA sequencing. Finally, the *kan – porA* promoter fusion was sub-cloned from pFAB-21 into the over-expression plasmids to complete their construction. This plasmid was also sequenced to confirm the sequences of both the gene and *porA* promoter locus were unaltered.
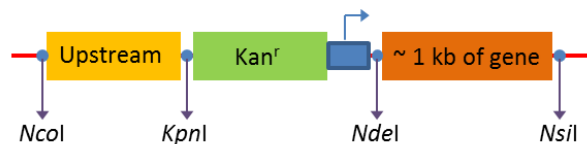
Figure 2.3: Arrangement of the different constituent fragments in the over-expression construct. The restriction endonucleases used in the construction of the plasmids and their respective sites are shown. The blue box represents the *porA* promoter locus.

2.10 Serum sensitivity and bactericidal assays

A modified protocol based on the methods outlined in Mountzouros and Howell (2000) and Borrow *et* al. (2005) were used to assess serum sensitivity of meningococcal strains and the bactericidal activity of monoclonal and polyclonal antibodies. Cells, from an overnight culture on agar or in broth, were used to inoculate blood agar and grown for a further four hours or grown until an OD_{600} of ~0.5 was reached. Where iron restriction conditions were required, 30 μM desferal was added to liquid cultures

and grown for a further 3 hours. Cultures were subsequently diluted to an OD_{600} of 0.1 in PBSB (PBS, 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$). For the assay, the following components were transferred to specified wells of a 96-well plate in the following order in duplicate wells: an appropriate volume of the PBSB buffer (usually to make the assay total volume to 50 µl) to all wells; antibodies into the first column and 2-fold serial dilutions were performed as required (polyclonal antisera were first incubated in a 56^0C water bath to inactivate the mouse complement); 10 µl of a 1:2500 dilution of the PBSB cell suspension (expected to contain ~10^4 CFU); and the exogenous complement source (human complement). Three control assays were setup on each plate to assess intra-assay serum sensitivity to the exogenous complement source in the absence of bactericidal antibodies (designated AC wells), sensitivity to antisera or antibodies in the absence of complement (cells + heat-inactivated complement, designated CC wells) and the fitness of cells used in the assay (cells in buffer and heat-inactivated complement, designated BC wells). Plates were subsequently incubated at 37^0C for 1 hour. Prior to incubation, a 10 µl sample from BC wells was plated in duplicate on BHI agar supplemented with Levinthal's supplement and served as T_0 counts. Similarly, samples from all assay wells were plated at the end of the experiment to provide T_{60} counts. The mean of 4 counts (each duplicate well provides 2 counts) multiplied by 100 was taken as the CFU/ml while percentage survival was measured by dividing the T_{60} counts of test wells by those of the corresponding BC well multiplied by 100. Percentage killing was, therefore, 100 minus percentage survival.

CHAPTER 3

3.0 *Ex vivo* blood assay to study growth of mutants and phase variants of *hpuAB* and *hmbR* in human blood

The use of the human whole blood model in investigating meningococcal behaviour during disease was first described in a report by Ison and co-workers (1995). The report detailed results of experiments that investigated the effect of blood sampling methods, pre-inoculation growth conditions and release of host factors on survival of different meningococcal strains in whole blood. Other workers have employed this model to study the bactericidal activity of whole blood obtained from different age groups (Ison *et al.*, 2003), to assess the immune response to meningococcal disease in convalescent children (Ison *et al.*, 1999) and to measure the efficacy of a serogroup B vaccine in infants (Morley *et al.*, 2001).

The model has also been used to identify virulence factors of *N. meningitidis*. In a study reported by Seib and co-workers (2009), the importance of a meningococcal serogroup B vaccine component, factor-H binding protein encoded by fHbp, to survival of blood-borne meningococci was investigated. The fHbp knockout strain, constructed in 4 different meningococcal backgrounds, was incapable of surviving in whole blood converse to the isogenic wild-type and fHbp complement strains (Seib *et al.*, 2009). Observations made by Seib *et al*. were corroborated in a later study where a genome-wide analysis of the meningococcal transcriptome was conducted on cells previously grown in human whole blood. Results from the transcriptome analysis suggest crucial roles for fHbp and the global regulator of iron-acquisition systems in the meningococcus, Fur (Echenique-Rivera *et al.*, 2011). A third of ORFs in the meningococcal genome were found to be up-regulated in response to the presence of host defence factors and nutritional limitations. These up-regulated ORFs included genes involved in the acquisition of iron from transferrin (Tf) and haemoglobin (Hb). The whole blood assay, used in the study by Echenique-Rivera and co-workers, produced results that confirmed suggestions from experiments conducted previously in rodent infection models. For example, inactivation of genes involved in iron uptake and transport, *tbpBA, tonB*, *exbB* and *exbD*, led to attenuated virulence in rat and murine infection models (Sun et al., 2000; Renauld-Mongenie *et al.*, 2004) suggesting

crucial roles for these meningococcal iron acquisition systems during systemic infection of humans. The whole blood assay, therefore, is a useful model in the study of virulence factors for growth and survival of the host-specific meningococcus in humans.

While the constitutively-expressed meningococcal Tf receptor, TbpBA, has been established as an important virulence factor (Gorringe *et al.*, 1995), the importance of other iron acquisition systems, encoded by genes subject to SSR-mediated phase variation, to survival and growth of the meningococcus in human whole blood is yet to be elucidated. *hpuAB* and *hmbR* are transcriptionally-regulated by Fur but also contain polyG tracts within the reading frame that contribute to ON/OFF switching of these genes (Lewis *et al.*, 1999). Both genes, when in phase-ON, enable the meningococcus to utilize iron bound to haemoglobin and haemoglobin complexes, increasing the repertoire of available iron sources during infection. This ability to utilize Hb and Hb-complexes was implied to be key to meningococcal survival and proliferation in the blood in a comparison between carriage and disease strains (Tauseef *et al.*, 2011). A strong association between the presence of one or both genes as well as PV status (ON/OFF) and disease was made in the study by Tauseef and co-workers supporting data from an earlier study that discovered an over-representation of *hmbR* in disease isolates (Harrison *et al.*, 2009). Similar to observations made with a *tbpBA*-null mutant in a mouse infection model (Renauld-Mongenie *et al.*, 2004), a *hmbR*-null mutant was less virulent than the wild-type strain (Stojiljkovic *et al.*, 1995) indicating that phase-OFF variants of *hmbR* will be less able to proliferate in the blood. However, there has been no experimental evidence to show the inability of phase-OFF variants of either *hpuAB*, *hmbR* or both systems to grow in human blood.

Using an *ex vivo* human whole blood model, this study aimed to examine if phase-OFF variants (or their equivalent i. e. mutants) of *hmbR* and *hpuAB* are capable of causing disease by proliferating in human blood. To achieve this aim, strains incapable of utilising Hb were generated in two different strain backgrounds: MC58 (*hmbR*-ON; *hpuAB*-null; *tbpBA*) and 8047 (*hmbR*-OFF; *hpuAB*-ON; *tbpBA*). The importance of Hb utilisation in the absence of Tf utilisation was also investigated by generating mutants incapable of utilising Hb and Tf i. e. MC58 Δ*hmbR*Δ*tbpBA*, 8047Δ*hmbR*Δ*tbpBA* and

8047Δ*hpuAB*Δ*tbpBA*. These mutants were subsequently tested for survival in freshly-collected heparinised human whole blood.

3.1 Construction of *hmbR* deletion plasmid

Phase-OFF variants of *hmbR* and *hpuAB* are incapable of utilizing haemoglobin or haemoglobin-haptoglobin (Lewis *et al.*, 1999) and are similar to deletion mutants in this respect. For the purpose of the whole blood assay, deletion mutants were preferred as the generation of phase-ON variants that could utilize available haemoglobin in the blood would have been impossible. False negative results would, therefore, be avoided.

Different versions of the meningococcal strain 8047 with mutations in *hpuAB* (IT-8047Δ*hpuAB*) or *hmbR* (IT-8047Δ*hmbR*) were constructed by Isfahan Tauseef (as part of his PhD project). A double mutant incapable of utilizing Hb as a sole iron source was also constructed (IT-8047Δ*hmbR*Δ*hpuAB*). Deletion of *hmbR* in IT-8047Δ*hmbR* was achieved by disrupting the reading frame with the insertion of a kanamycin resistance cassette. These mutant strains generated by Isfahan Tauseef were employed for different purposes during the course of this study.

In this study, a similar mutant in a different meningococcal background, MC58 (*hpuAB* negative), was required. Concerns over the possible expression of the *hmbR* region upstream of the resistance cassette in IT-8047Δ*hmbR* led to the construction of a plasmid carrying an allele of *hmbR* with a ~1.1 kb deletion. To obtain this plasmid, primers HmbR-Nterm and dhmbR-rev (included *BamH*I site) were used to amplify 542 bp of the 5′ region of *hmbR* while primers dhmbR-for (included *BamH*I site) and HmbR-Cterm amplified 676 bp of the 3′ region of the gene from strain 8047 (see organisation of *hmbR* locus and primer sites in section 2.6.1, Figure 2.1). The resulting amplicons (Figure 3.1) were digested with *BamH*I and ligated together to create a ~1.3 kb fragment.
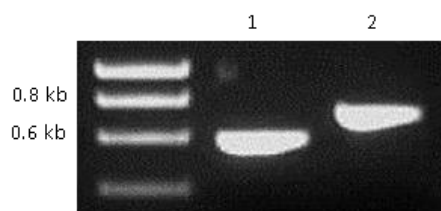
Figure 3.1: Amplification of the 5′ (lane 1) and 3′ (lane 2) regions of the *hmbR* gene from strain 8047.

Since *Taq* non-specifically incorporates an extra adenine residue at the ends of amplicons, the ~1.3 kb fragment could be ligated into a linearized pGEM-T Easy vector that had thymine residue overhangs. The resulting plasmid, pFAB-7, was transformed into *E. coli* DH5α cells and grown on LA plates infused with ampicillin (100 μg/ml) and X-gal (10 μg/ml). Successful ligation of the ~1.3 kb fragment of *hmbR* with the vector would produce white colonies as the insert would have disrupted the *lacZ* gene on the pGEM-T Easy vector. pFAB-7 was retrieved from white colonies and tested in an *EcoR*I restriction digest where the ~1.3 kb *hmbR* deletion fragment was excised from the 3 kb pGEM-T vector (Figure 3.2).

The plasmids were subsequently sequenced to confirm deletion of a 1.1 kb region from *hmbR*. Alignment of the full sequence of the gene from strain 8047 with the sequence of pFAB-7 shows the absence of an approx. 1.1 kb (from position 578 to 1673) in pFAB-7 (Figure 3.3). The consequence of using a low-fidelity polymerase, such as *Taq*, was also observed as errors made during the PCR yielded amplicons that contained missense mutations at different positions.
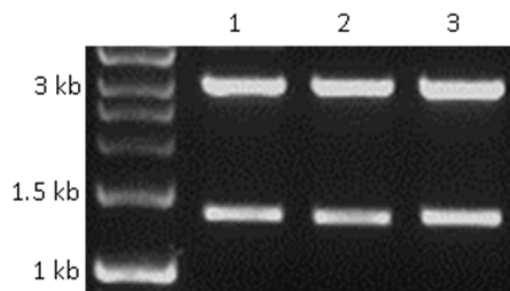
Figure 3.2: *EcoR*I digest of three copies of pFAB-7 isolated from different colonies.

Figure 3.3: Confirmation of a ~1.1 kb deletion in *hmbR* in pFAB-7 by sequencing. A consensus DNA sequence was obtained from two DNA sequencing reactions with primers spanning *hmbR*. The consensus sequence was aligned to a previously-derived sequence (provided by Chris Bayliss) of the wild-type *hmbR* gene of strain 8047 cloned into a plasmid vector. Areas shaded in orange indicate differences between both sequences (hyphens represent deletions) while sequence homology is represented by dots.

Kanamycin resistance was chosen as the selectable marker for successful deletion of *hmbR* in the meningococcal background. A kanamycin resistance cassette (termed *kan*) was sub-cloned into pFAB-7 via a *BamH*I site in the *hmbR* deletion fragment (introduced by primers dhmbR-for and dhmbR-rev). *kan* was excised from pUC4kan and ligated into the sticky ends produced by a *BamH*I digest of pFAB-7 (Figure 3.4) to produce pFAB-9.
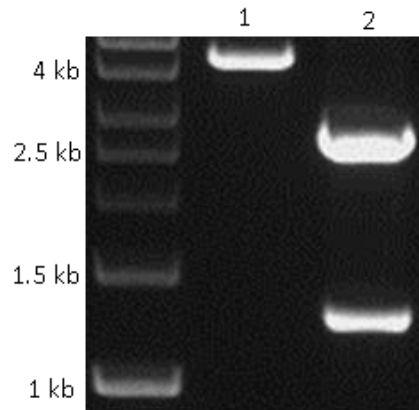
Figure 3.4: Construction of pFAB-9. *BamH*I digests of pFAB-7 (lane 1) and puc4kan (lane 2) were analysed on a 1% agarose gel.

pFAB-9 was subsequently analysed in restriction digests. A *BamH*I digest of pFAB-9 released *kan* while an *EcoR*I digest excised the ~2.5 kb insert (Δ*hmbR-kan*) from the 3 kb pGEM-T vector (Figure 3.5). Subjecting pFAB-9 to an *EcoR*I-*Sma*I sequential digest showed the orientation of *kan* with respect to the *hmbR* locus. *Sma*I divided *kan* into 807 bp and 434 bp fragments. Possible results from the digest were (excluding the 3.0 kb pGEM-T Easy vector excised by *EcoR*I): fragments of 1.35 kb and 1.11 kb if *kan* was in a forward orientation with respect to *hmbR*; or 1.48 kb and 0.98 kb fragments in the event of a reverse-oriented *kan* fragment. Resolution of the *EcoR*I-*Sma*I sequential digest on a 1% agarose gel confirmed the forward orientation of *kan* (Figure 3.6). These digests confirmed successful construction of the *hmbR* deletion plasmid, pFAB-9.
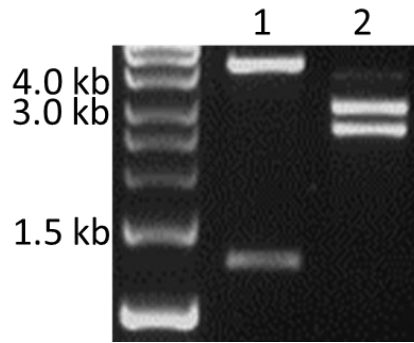
Figure 3.5: Restriction digests of pFAB-9. *BamH*I digest of pFAB-9 was analysed in lane 1 while an *EcoR*I digest of the plasmid was analysed in lane 2.
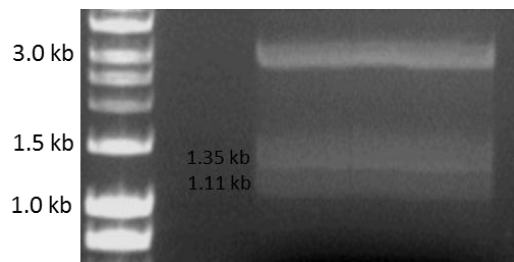
Figure 3.6: Orientation of kanamycin resistance cassette in pFAB-9. An *EcoR*I-*Sma*I sequential digest was performed to determine the context of the *kan* fragment.

3.2   Transformation of pFAB-9 into *N. meningitidis* strain 8047

Transformation of strain 8047 cells with pFAB-9 were done using methods outlined in section 2.6.2a. All transformants were confirmed to be meningococci in a species-specific PCR that involves amplification of 81 bp and 210 bp from the *ctrA* and *crgA* genes respectively (Figure 3.7a). Confirmation of *hmbR* deletion was achieved with a PCR using primers, RF3 and RF4, which anneal within the deleted region of *hmbR* (Figure 3.7b). 8047 wild-type DNA produced the expected 400 bp amplicon while IT-8047Δ*hmbR* produced a ~1.7 kb amplicon, illustrating the insertion of the ~1.3 kb *kan* fragment within the 400 bp locus. An amplicon was absent from the pFAB-9 transformants of strain 8047, indicating that *hmbR* had been deleted. The context of *kan* within the genome in addition to its orientation, with respect to the *hmbR* locus, were also determined using primers HmbR-Cterm and kan-for. *kan* was shown to be located within the *hmbR* locus and transcribed in the same orientation as *hmbR* (Figure 3.7c).
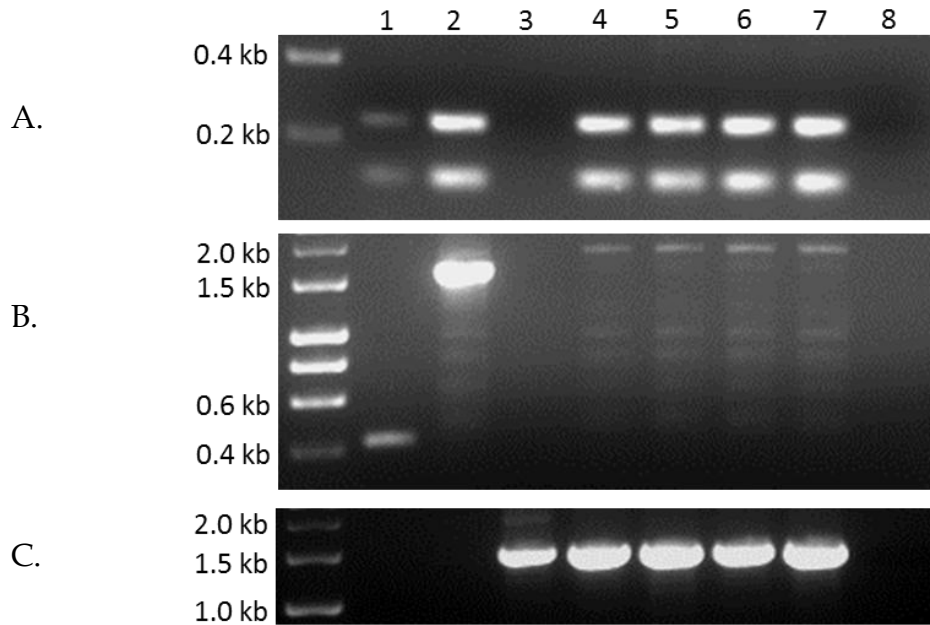
Figure 3.7: Confirmation of *hmbR* deletion in strain 8047 by PCR. **(A)** Meningococci-specific PCR – amplicons of *ctrA* (81 bp) and *crgA* (210 bp) are observed in the wild-type 8047 (lane 1), IT-8047Δ*hmbR* genomic DNA (lane 2) and Δ*hmbR* transformants (lanes 4 -7 respectively) but not in pFAB-9 (lane 3) or no DNA control (lane 8). **(B)** Primers RF3 and RF4 were used to confirm deletion of *hmbR*. The presence/absence of a 400 bp amplicon differentiates wild-type from mutant DNA. **(C)** The orientation of the kanamycin resistance cassette, with respect to the *hmbR* locus, was determined using primers HmbR-Cterm and kan-for.

3.3 Transformation of pFAB-9 into *N. meningitidis* strain MC58

Attempts to transform MC58 cells with pFAB-9, using the protocol employed in the transformation of 8047 cells, were unsuccessful. Several modifications were made to the protocol before successful transformation was achieved. Natural transformation of MC58 cells was achieved with a biphasic method, which induces expression of type IV pili. Type IV pili are actively involved in the uptake of exogenous DNA and consequently play a crucial role in the natural transformation of *N. meningitidis* (Fussenegger *et al.*, 1997). See section 2.6.2b for details of the method employed.

Colonies obtained, after 48 hours of incubation, were first confirmed to be meningococci by PCR then assessed for presence of the kanamycin cassette. Amplicons of *ctrA* (81 bp) and *crgA* (210 bp) were obtained from wild-type MC58 and 8047 DNA as well as the MC58Δ*hmbR* mutants but not in pFAB-9. All transformants (MC58Δ*hmbR*) tested positive for the presence of *kan* by producing a 600 bp amplicon that was absent in both 8047 and MC58 wild-type DNA (Figure 3.8).
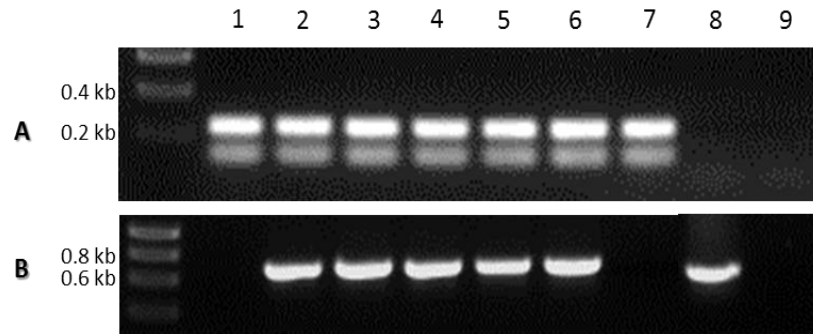
Figure 3.8: Presence of kanamycin resistance cassette in MC58Δ*hmbR* transformants. **(A)** A duplex PCR involving primers that anneal within meningococcal-specific genes *ctrA* and *crgA* was performed on samples analysed on a 1% agarose gel as follows: wild-type MC58 (lane 1), MC58Δ*hmbR* (lanes 2 – 6) wild-type 8047 (lane 7), pFAB-9 (lane 8) and no-DNA control (lane 9). **(B)** Presence of the kanamycin resistance cassette was tested in a PCR using primers kan-for and kan-rev. Samples were analysed in the same order as in (A) above.

The context of *kan* within the genome of the transformants was determined in a PCR using primers HmbR-Cterm and kan-for. Amplicons of the expected size, ~1.5 kb, were obtained from all transformants but not from wild-type MC58. This PCR showed that the antibiotic marker was successfully used to replace a segment of *hmbR* and was oriented in an identical direction to *hmbR*. Another PCR using primers, RF3 and RF4 that anneal within the deleted segment of *hmbR*, was used as further confirmation of the deletion. No amplicons were obtained with any of the transformants. A 400-bp amplicon was obtained in this PCR from the wild-type DNA template (Figure 3.9).
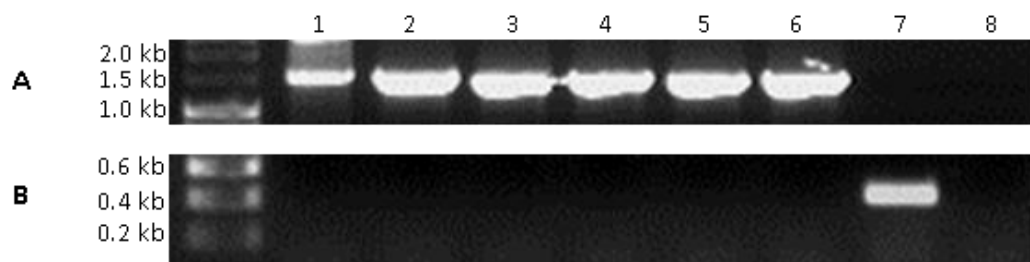
Figure 3.9: Deletion of *hmbR* in strain MC58. **(A)** Primers annealing to *hmbR* (HmbR-Cterm) and the kanamycin resistance cassette (kan-rev) were used to assess the orientation and location of the cassette. **(B)** Deletion of ~1.1 kb from *hmbR* was confirmed with primers RF3 and RF4. Samples were analysed as follows: pFAB-9 (lane 1), MC58Δ*hmbR* mutants (lanes 2 - 6), MC58 wild-type (lane 7) and no-DNA control (lane 8).

3.4   Construction of a *tbpBA* deletion plasmid

Virulence is also associated with the ability to acquire iron from Tf; therefore, meningococci incapable of utilizing Hb (Hb⁻) are expected to grow in human blood. These Hb⁻ meningococci will rely on their ability to utilize Tf once iron stores have been

depleted. To enable an accurate assessment of the impact of PV of *hmbR* and *hpuAB* on growth in human blood, both phases (i. e. PV-ON and PV-OFF) should be assessed in the absence of Tf utilization since Tf has been identified as a major iron source in blood (Letendre *et al.*, 1987). A clear distinction between both phases of expression should be detected as Hb utilization will be a major determinant of survival in the blood in a Δ*tbpBA* background.

A *tbpBA* deletion plasmid, pIT-Δ*tbpBA*, containing 962 bp of the 5′ region of MC58-*tbpB* ligated with 880 bp of the 3′ region of MC58-*tbpA* was previously made by Isfahan Tauseef. This plasmid also contained a kanamycin resistance cassette that separated both *tbpBA* fragments. For the generation of mutants incapable of utilizing Tf and Hb (MC58Δ*hmbR*Δ*tbpBA*, 8047Δ*hmbR*Δ*tbpBA* and 8047Δ*hpuAB*Δ*tbpBA*), the antibiotic selection marker had to be substituted since a kanamycin resistance cassette was already present in the Δ*hmbR* (both 8047 and MC58) and IT-8047Δ*hpuAB* mutants. An erythromycin resistance cassette, *ermC,* was chosen for this purpose. Considering the problems encountered during transformation of MC58 with pFAB-9, a 12-bp version of the meningococcal DNA uptake sequence (DUS) was added to both ends of *ermC* (Figure 3.10) in order to enhance the natural transformation of the *tbpBA* deletion plasmid. This 12-bp DUS has been shown to increase transformation efficiencies in both meningococci and gonococci by up to 150-fold (Ambur *et al.,* 2007; Duffin and Seifert, 2010). Primers used to introduce the DUS also contained *BamH*I to facilitate sub-cloning of the *ermC*-DUS into pIT-Δ*tbpBA*.
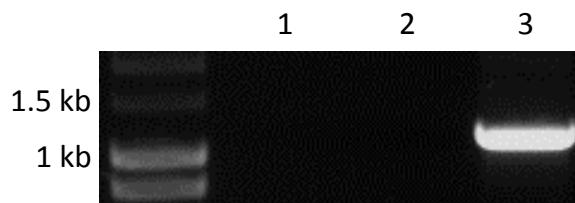
Figure 3.10: PCR to add meningococcal DNA uptake sequences (DUS) and *BamH*I to both ends of *ermC*. Primers including the uptake sequences were used to amplify the erythromycin-resistance cassette from pDH20 (lane 3). No DNA (lane 1) and negative DNA (lane 2) controls were included in the PCR.

The *ermC*-DUS amplicon was TA-cloned into the pGEM-T Easy vector to produce pFAB-20. Confirmation of successful ligation was done by subjecting pFAB-20 to an *EcoR*I digest. The digest yielded 2 fragments: 3 kb and 1.2 kb fragments corresponding to the pGEM-T Easy vector and the *ermC*-DUS amplicon (Figure 3.11).
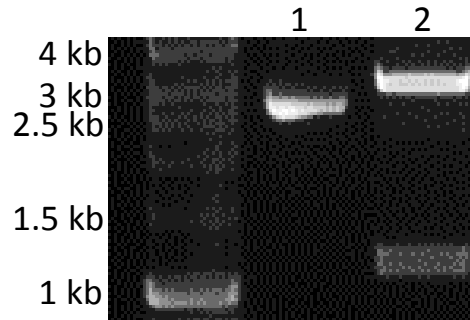
Figure 3.11: *EcoR*I digest of pFAB-20. Undigested pFAB-20 was analysed in lane 1 while *EcoR*I-digested plasmid was analysed in lane 2.

The antibiotic selection marker on pIT-Δ*tbpBA* was subsequently substituted with the erythromycin resistance marker. pIT-Δ*tbpBA* and pFAB-20 were digested with *BamH*I to excise both antibiotic cassettes (Figure 3.12). The *ermC*-DUS fragment was ligated with the pIT-Δ*tbpBA* vector to give pFAB-22. An *EcoR*I-*BamH*I double digest produced four fragments (two are indistinguishable on the agarose gel) as follows: 3 kb pGEM-T Easy vector, 1.2 kb *ermC* fragment and fragments obtained from the 5′ and 3′ regions of the *tbpBA* locus (Figure 3.13), thus, confirming successful construction of the *tbpBA* deletion plasmid, pFAB-22.
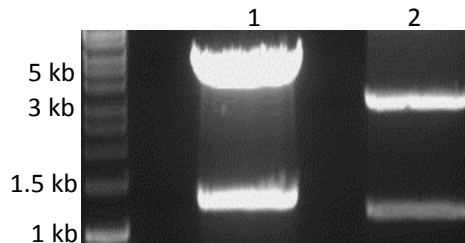
Figure 3.12: Substitution of *kan* with *ermC*-DUS in construction of pFAB-22 construction. pIT-Δ*tbpBA* and pFAB-20 were subjected to *BamH*I digests to release the kanamycin-resistance cassette from pIT-Δ*tbpBA* (lane 1) and the *ermC*-DUS fragment from pFAB-20 (lane 2).
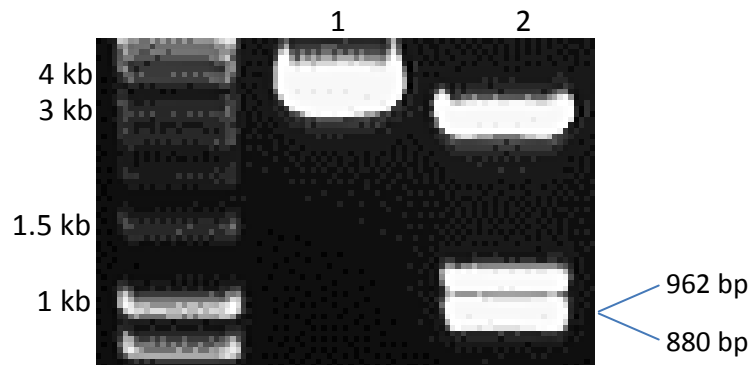
Figure 3.13: *EcoR*I and *BamH*I double digest of pFAB-22. Undigested pFAB-22 was analysed in lane 1 while the digest was analysed in lane 2.

3.5 Deletion of *tbpBA* in MC58 and MC58Δ*hmbR*

*Nde*I-digested pFAB-22 was used to transform MC58 and MC58Δ*hmbR* cells using methods outlined in section 2.6.2b. Transformants were selected on supplemented BHI agar plates infused with 6 µg/ml of erythromycin. Growth of individual colonies was observed on agar plates after 24 hours. Multiple colonies were selected and all were confirmed as meningococci in a PCR (*ctrA* and *crgA*) prior to assessing deletion of *tbpBA*. As expected, only double mutants and the isogenic Δ*hmbR* mutant strain produced an amplicon from *kan* when primers, kan-for and kan-rev were employed. PCR primers, ermDUS-for and ermDUS-rev, produced amplicons from the *ermC*-DUS fragment in all single (MC58Δ*tbpBA*) and double (MC58Δ*hmbR*Δ*tbpBA*) mutants but not from parental wild-type and MC58Δ*hmbR* mutant strains. The context of the erythromycin-resistance cassette was confirmed by using primers that annealed to both *tbpBA* and *ermC* loci. A similar orientation of the erythromycin-resistance cassette was observed with MC58Δ*tbpBA* and MC58Δ*hmbR*Δ*tbpBA* mutants as well as pFAB-22. Wild-type and Δ*hmbR* DNA yielded no products (Figure 3.14).
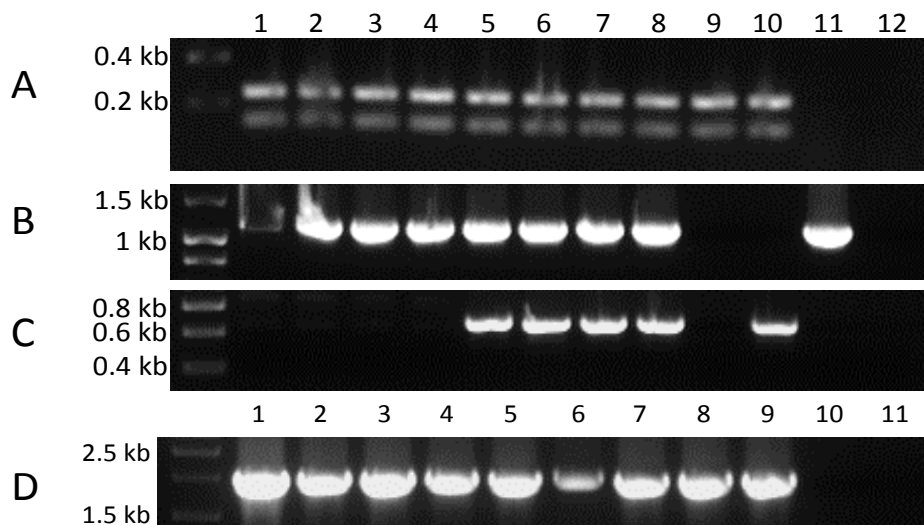
Figure 3.14: Confirmation of *tbpBA* deletion in MC58 and MC58Δ*hmbR* by PCR. PCRs were done to confirm transformants as meningococci by amplifying 81 bp of *ctrA* and 200 bp of *crgA* **(A)** and to assess the presence of erythromycin **(B)** and kanamycin **(C)** resistance cassettes. check the orientation and location of the *ermC*-DUS fragment. Samples were analysed on a 1% agarose gel as follows: MC58Δ*tbpBA* mutants (lanes 1 - 4), MC58Δ*hmbR*Δ*tbpBA* mutants (lanes 5 - 8), MC58 wild-type (lane 9), MC58Δ*hmbR* (lane 10), pFAB-22 (lane 11) and no-DNA control (lane 12). **(D)** The orientation and location of the *ermC*-DUS fragment was also determined in a PCR using primers TbpAR and ermDUS-rev. Samples were analysed as follows: pFAB-22 (lane 1), MC58Δ*tbpBA* mutants (lanes 2 - 5), MC58Δ*hmbR*Δ*tbpBA* mutants (lanes 6 - 9), MC58 wild-type (lane 10) and no-DNA control (lane 11).

3.6   **Utilization of Hb and Tf by MC58 wild-type and mutant strains**

Phenotypic characterization of MC58 wild-type and mutant strains was performed by employing a disk diffusion assay (see section 2.6.4). Tf (500 µg) or Hb (100 µg) served as sole sources of iron to cells plated on MH agar infused with 40 µg/ml of desferal. After 24 hours of incubation, rings of growth were observed around discs where cells could utilize the source of iron provided. MC58Δ*hmbR* utilized Tf but not Hb whilst the converse was observed for MC58Δ*tbpBA*. The double mutant was unable to utilize Tf or Hb whilst the wild-type strain utilised both. All strains utilized free iron provided in the form of 0.1 M FeCl$_3$ (Figure 3.15).

Figure 3.15: Hb/Tf utilization assay of strain MC58 and mutants. Desferal (40 µg/ml) was added to molten MH Agar to chelate available iron in the agar. 10^8 cells of each strain were spread onto each plate before sterile filter discs infused with either 100 µg of Hb or 500 µg of Tf were placed on the agar. Plates were incubated at 37ºC, 5% CO$_2$ for 24 hours. Discs infused with FeCl$_3$ and PBS were used as positive and negative controls.

3.7   **Growth rates of MC58 wild-type and mutant strains in MH broth**

An assessment of the impact of natural transformation on viability of the mutant strains was performed by measuring the growth rates of wild-type and mutant strains in MH broth. Overnight cultures of each strain were diluted to an OD$_{600}$ of 0.1 in fresh

MH broth, in sextuplicate, and incubated on a rocking platform for 24 hours at 37⁰C with 5% CO_2. The OD_{600} of each culture was measured hourly.

Although the wild-type strain had a longer lag phase (Figure 3.16), similar doubling times were recorded for all strains between the early and late logarithmic phases of growth (2 – 5 hours) (Table 3.1). Growth of all mutants reached a plateau after 5 hours but the wild-type strain showed continuous growth for a further 3 hours.

Table 3.1: Doubling times of MC58 wild-type and mutant strains

Strain	Doubling time (minutes)
Wild-type	68
Δ*hmbR*	73
Δ*tbpBA*	65
Δ*hmbR*Δ*tbpBA*	68

Note: Log_2 values of the absorbance readings (OD_{600}), taken for the duration of exponential growth (2 – 5 hours), were plotted on a graph and the slope calculated (see Figure 3.16). The inverse of the slope was taken as the doubling time.
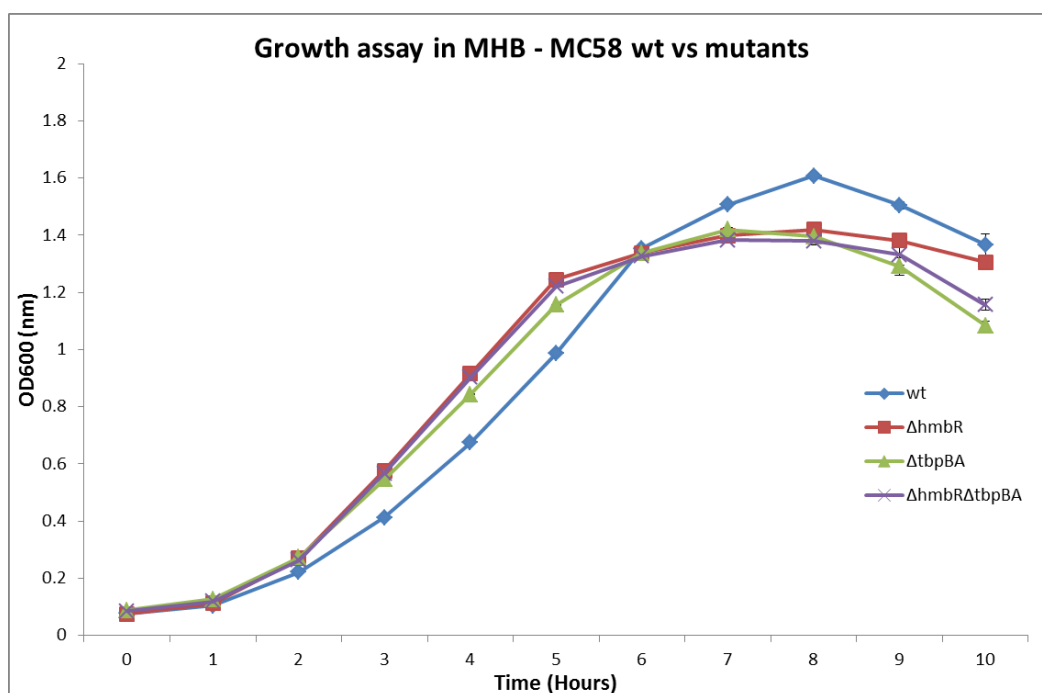
Figure 3.16: Growth curves of MC58 wild-type and mutants grown in MH broth. Overnight cultures were diluted to a starting OD_{600} of 0.1. Readings were taken hourly for 24 hours (only first 10 hours shown above). Two biological replicates of the assay were performed on separate days and each assay contained 6 replicates of each strain. Error bars indicate the standard error of the mean of all 12 replicates for each strain.

3.8   Deletion of *tbpBA* in 8047, 8047*ΔhmbR* and IT-8047*ΔhpuAB*

Genomic DNA extracted from MC58*ΔtbpBA* cells was used to transform 8047 wild-type, 8047*ΔhmbR* and IT-8047*ΔhpuAB* strains. Transformants were obtained after 24 hours incubation at 37°C, 5% CO_2. A species-specific PCR was used to confirm transformants as meningococci prior to assessing deletion of *tbpBA*. All transformants gave positive results for the meningococci-specific PCR while the presence of the kanamycin resistance cassette differentiated the 8047*ΔhmbRΔtbpBA* and 8047*ΔhpuABΔtbpBA* mutants from 8047 wild-type and 8047*ΔtbpBA* mutant strains. The genomic context of the erythromycin-resistance cassette was assessed by using primers that annealed to both *tbpBA* and *ermC* loci. The *ermC*-DUS fragment was found to be in an opposite orientation relative to the *tbpBA* locus in all strains (Figure 3.17).
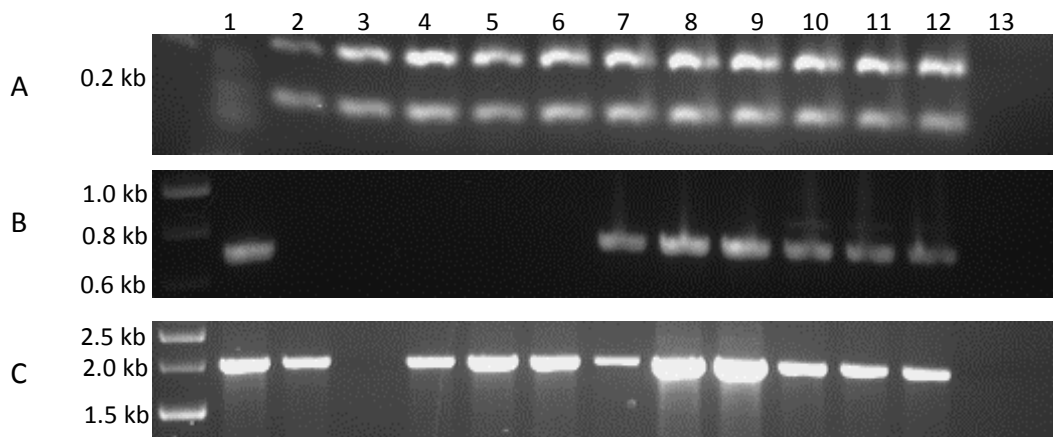
Figure 3.17: Confirmation of *tbpBA* deletion in 8047, 8047 *ΔhmbR* and 8047 *ΔhpuAB* by PCR. Primers that anneal to *ctrA* and *crgA* (A) and the kanamycin resistance cassette (B) were used in the PCRs analysed on 1% agarose gels above. The context of the *ermC*-DUS fragment was assessed using primers that amplify from the 3' regions of the *tbpBA* locus and *ermC*- DUS fragment (C). The following templates were used in the PCRs: MC58*ΔhmbRΔtbpBA* (lane 1), MC58*ΔtbpBA* genomic DNA (lane 2), wild-type 8047 strain (lane 3), 8047*ΔtbpBA* (lanes 4-6), 8047*ΔhmbRΔtbpBA* (lanes 7-9) and 8047*ΔhpuABΔtbpBA* mutants (lanes 10-12). A no-DNA control was analysed in lane 13.

3.9   Growth rates of 8047 wild-type and mutants in MH broth

Growth of the 8047 wild-type strain and 6 mutants – *ΔhmbR*, *ΔhpuAB*, *ΔtbpBA*, *ΔhmbRΔhpuAB*, *ΔhmbRΔtbpBA* and *ΔhpuABΔtbpBA* – was compared in MH broth. A starting OD_{600} of 0.1 was used and growth was followed over a time course of 24 hours. Although, differences in the duration of the lag phase were observed (Figure 3.18), similar doubling times were recorded for all strains (Table 3.2). Growth of the 8047*ΔhmbR* and 8047*ΔhmbRΔtbpBA* mutants plateaued at a lower OD_{600} than other strains. This could be due to the formation of cellular aggregates when the population of the culture exceeded certain limits.

Table 3.2: Doubling times of 8047 wild-type and mutant strains.

Strain	Doubling time (minutes)
Wild-type	68
ΔhmbR	73
ΔhpuAB	68
ΔtbpBA	65
ΔhmbRΔhpuAB	68
ΔhmbRΔtbpBA	68
ΔhpuABΔtbpBA	64

Note: Log_2 values of the absorbance readings (OD_{600}), taken for the duration of exponential growth (2 – 6 hours), were plotted on a graph and the slope calculated (see Figure 3.18). The inverse of the slope was taken as the doubling time.
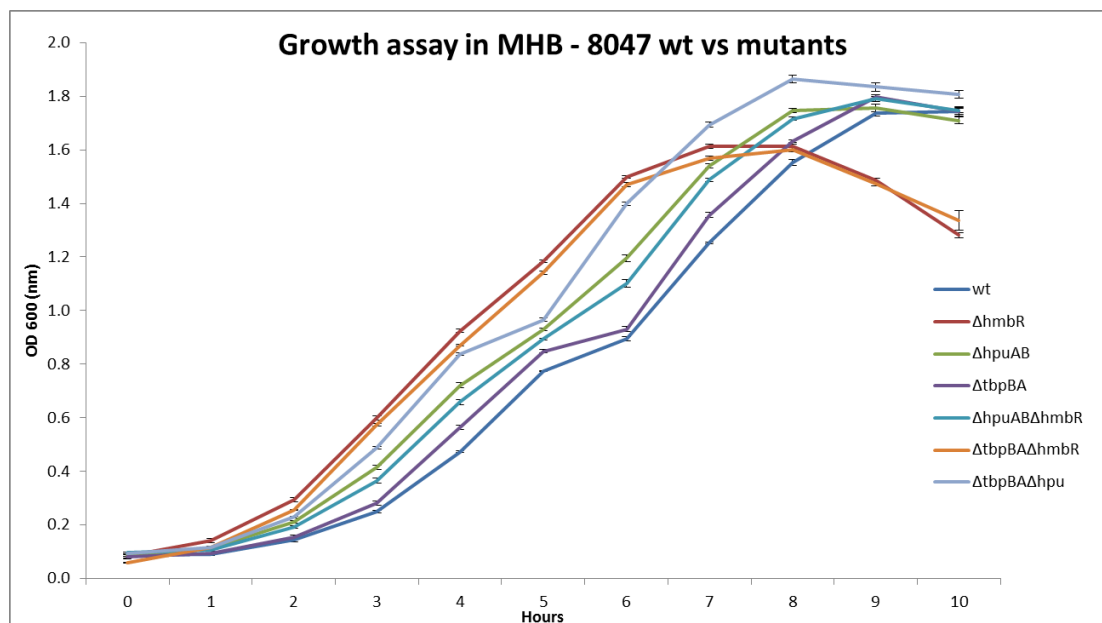
Figure 3.18: Growth curves of 8047 wild-type and mutants grown in MH broth. Overnight cultures were diluted to a starting OD_{600} of 0.1. Readings were taken hourly for 24 hours (only first 10 hours shown above). Two biological replicates of the assay were performed on separate days and each assay contained 6 replicates of each strain. Error bars indicate the standard error of the mean of all 12 replicates, from both biological replicates, for each strain.

3.10   Utilization of Hb and Tf by 8047 wild-type and mutant strains

Approximately 10^8 CFU of the wild-type and mutant strains were spread on MH agar plates infused with 40 μg/ml desferal. Exogenous sources of iron were provided to cells by spotting 5 μl of each iron source on different regions of the plate. After 24

hours of incubation, growth was observed in regions where cells could utilize the source of iron provided. Only the 8047Δ*hmbR*Δ*hpuAB* mutant was unable to utilise Hb while all strains carrying a *tbpBA* deletion failed to grow when Tf was the sole iron source. The ability of Δ*hpuAB* mutants to utilise Hb could be due to the presence of *hmbR*-ON phase variants in the inoculum. All strains utilized free iron provided in the form of 0.1 M FeCl$_3$ (Figure 3.19).
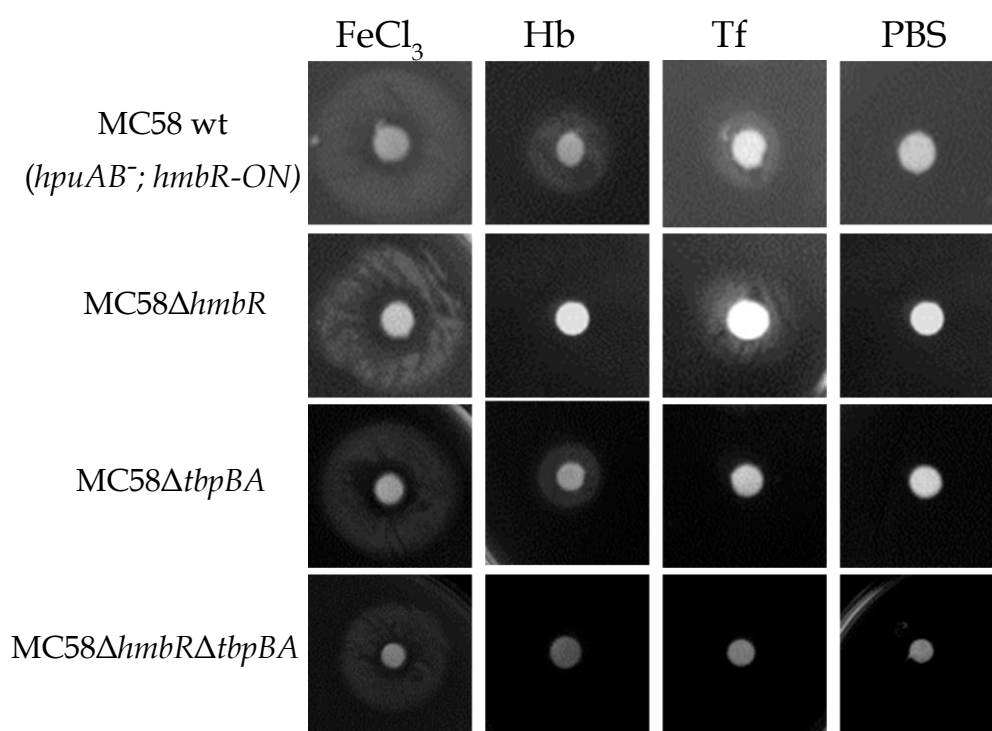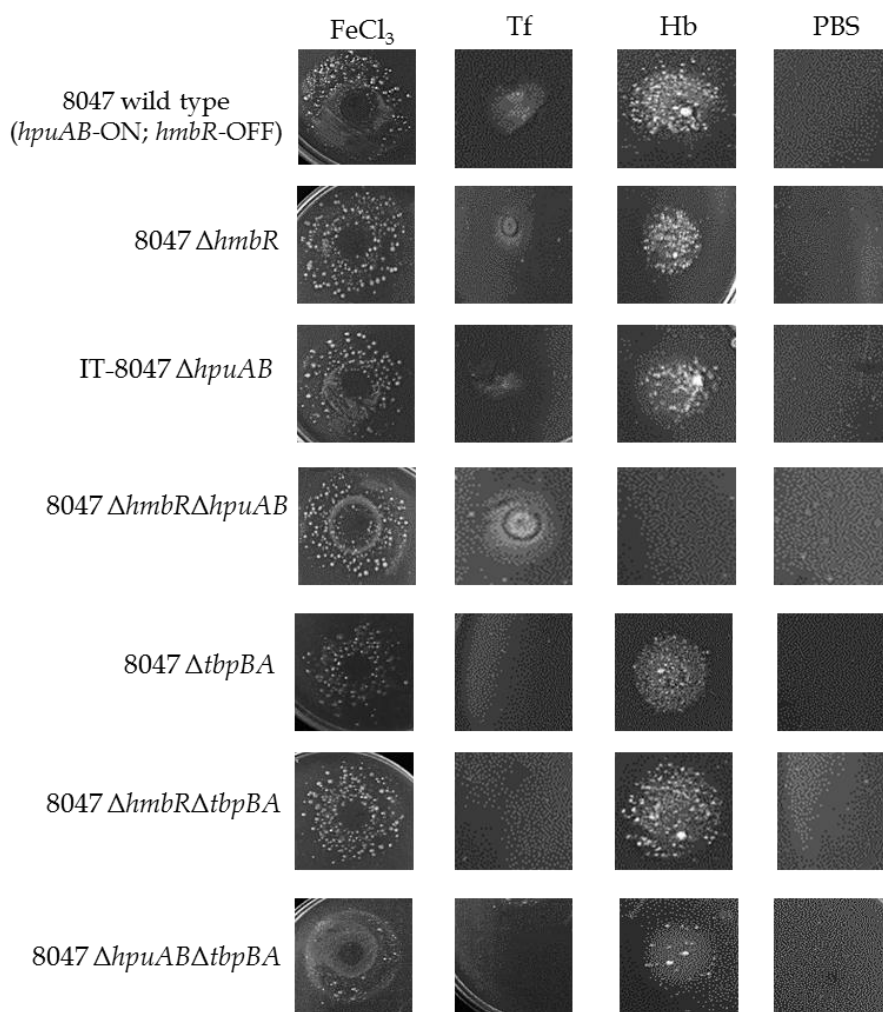

Figure 3.19: Hb/Tf utilization assay of strain 8047 wild-type and mutants. Desferal (40 µg/ml) was added to molten MH Agar to chelate available iron in the agar. Approximately 10^8 cells of each strain were spread onto each plate before 100 µg of Hb or 500 µg of Tf were spotted on the agar. Plates were incubated at 37^{0}C, 5% CO$_2$ for 24 hours. 0.1 M FeCl$_3$ and PBS were used as positive and negative controls.

3.11   Growth of MC58 wild-type and mutant strains in human whole blood

A "mock" blood assay was conducted in MH broth with MC58 wild-type and mutant strains to assess growth of the strains under experimental conditions. A 1:20 dilution of overnight culture of each strain was grown to mid-log (OD$_{600}$ = 0.4 - 0.6) in MH broth supplemented with 0.25% glucose. 20 µM CMP-NANA was also added to the culture

to induce the sialylation of the LOS and reduce the effect, if any, of complement-mediated bactericidal activity of the whole blood samples (Estabrook *et al.*, 1997). A sample of 10 μl of these mid-log cultures, estimated to contain ~10^7 CFU, was used to inoculate 490 μl of MH broth in duplicate. The CFU count of the inoculum (T_0) was estimated prior to incubation at 37^0C, 5% CO_2 by plating serial dilutions of the samples on supplemented BHI agar. Growth of all strains was monitored over a 4-hour period by plating dilutions of samples taken at 30, 60, 120 and 240 minutes.

All strains grew exponentially during the 4-hour period with doubling times of 47.7, 44.01, 44.5 and 48.18 minutes recorded for MC58 wild-type, MC58Δ*hmbR*, MC58Δ*tbpBA* and MC58Δ*hmbR*Δ*tbpBA* respectively (Figure 3.20).
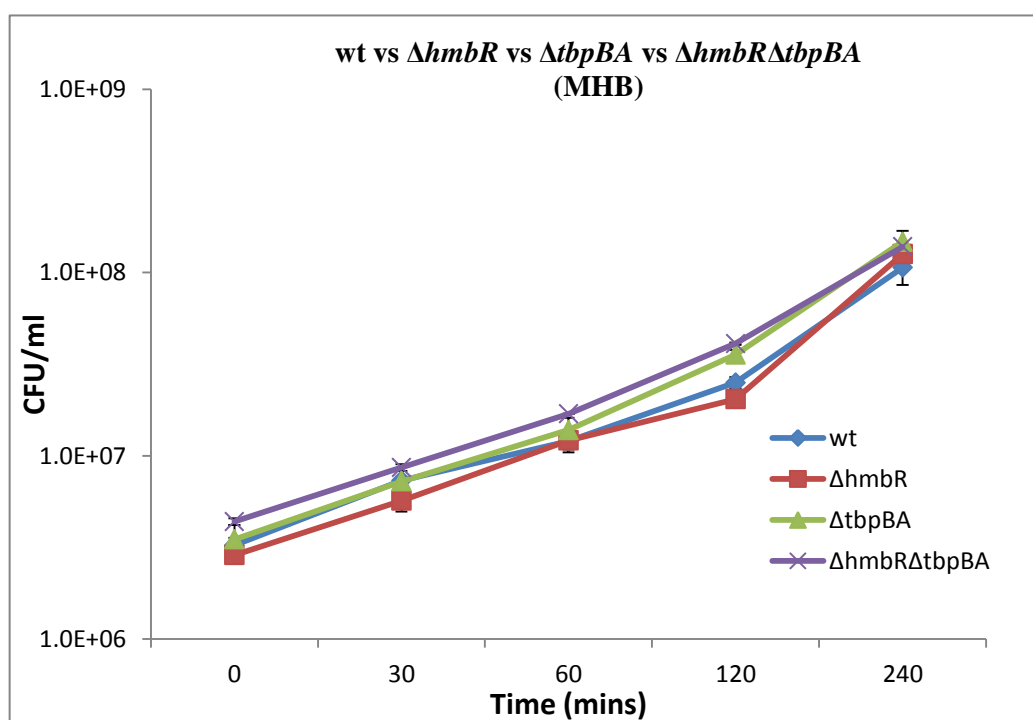
Figure 3.20: Mock blood assay in MHB. The wild-type (MC58), MC58Δ*hmbR*, MC58Δ*tbpBA* and MC58Δ*hmbR*Δ*tbpBA* strains were grown to mid-log (OD600 = 0.4 - 0.6) in MHB supplemented with 20 μM CMP-NANA. Samples from these cultures were used to inoculate the human whole blood substitute (MHB). Cultures were incubated at 37^0C, in 5% CO_2 for 4 hours. Dilutions of samples, taken at 0, 30, 60, 120 and 240 minutes, were plated onto supplemented BHI agar, in duplicate. The experiment was performed in duplicate for each culture and repeated twice on separate days (2 biological replicates). The mean of 8 counts (2 counts x 2 cultures x 2 biological replicates) was recorded as the CFU/ml for each strain at each timepoint. Error bars indicate the standard error of the mean of all 8 counts.

A similar experimental protocol used in the "mock" blood assay was employed in assessing the growth of wild-type MC58 cells in whole blood. The 30-minute timepoint was replaced with a 180-minute timepoint because of technical difficulties involving

plating samples within a 30-minute period. Thus, samples were taken at hourly intervals. Blood samples were obtained from 2 healthy volunteers, V1 and V2, who have deficiencies in the lectin pathway. Hence, blood samples obtained from these volunteers were expected to show little or no complement-mediated bactericidal activity due to the lack of the mannose-binding protein. This was true of V2 but not V1 as a degree of bactericidal activity was observed with V1 whole blood (Figure 3.21). This bactericidal activity was, however, not sustained during the course of the experiment as exponential growth was recorded for wild-type cells after 60 minutes. Mean doubling times calculated for wild-type MC58 were 56 and 60 minutes in V1 and V2 blood, respectively.
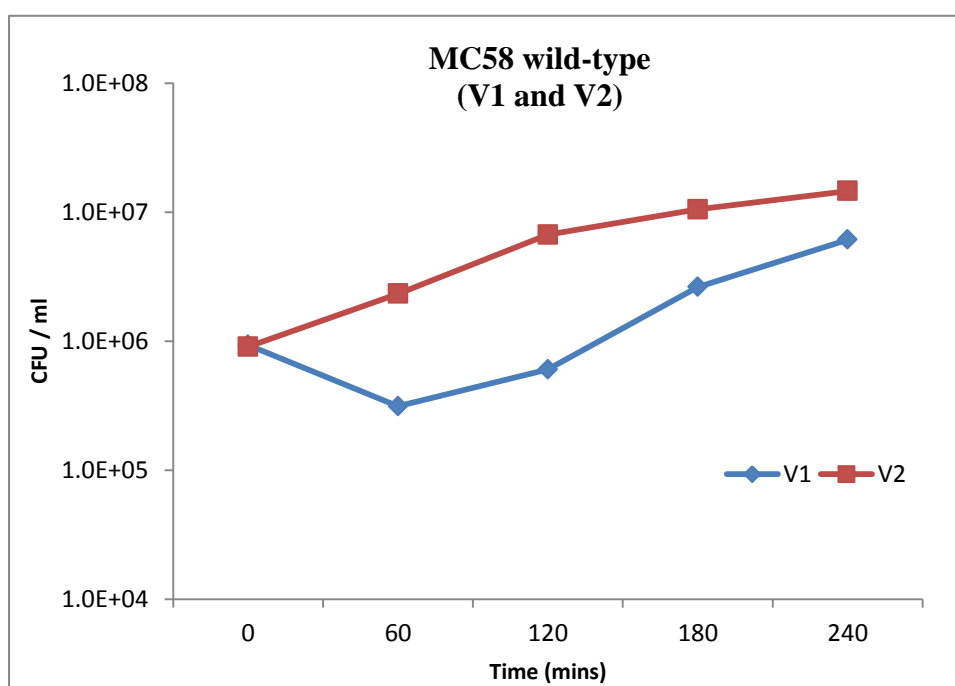
Figure 3.21: MC58 growth assay in human whole blood. 10 µl of wild-type (MC58), grown to mid-log (OD600 = 0.4 - 0.6) in MHB supplemented with 20 µM CMP-NANA, were added to freshly-collected heparinised human blood. Blood samples were collected from two healthy MBL-deficient volunteers; V1 and V2.Cultures were incubated at 37^0C, w5% CO_2 for 4 hours. Dilutions of samples, taken at 0, 60, 120, 180 and 240 minutes, were plated onto supplemented BHI agar, in duplicates. The experiment was performed once.

Comparisons of growth in human blood between wild-type MC58, MC58Δ*hmbR* and MC58Δ*hmbR*Δ*tbpBA* were performed initially for 240 minutes. No growth defects were observed for the MC58Δ*hmbR* mutant when compared to wild-type in both V1 (Figure 3.22) and V2 (Figure 3.23). In the V1 experiment, a rapid increase in amount of CFU was observed for wild-type and MC58Δ*hmbR*Δ*tbpBA* strains between T_0 and T_{60} (>10-

fold increase in CFU count) in V1. This was possibly caused by an under-estimation of T_0 counts or alternatively, an aberrant measurement at the 1 hour time point. This increase was not consistent during the experiment for both strains but the doubling time of the wild-type strain was approximately twice the doubling time of the MC58Δ*hmbR*Δ*tbpBA* from T_{60} to T_{240} – 155 minutes for wild-type as opposed to 305 minutes for MC58Δ*hmbR*Δ*tbpBA*.
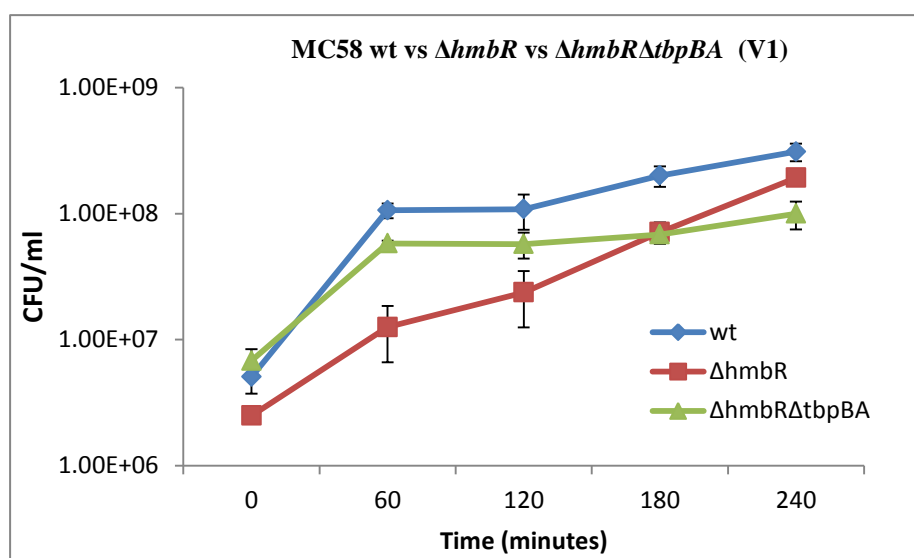
Figure 3.22: Growth assay for *hmbR* mutants of MC58 in human whole blood (V1). 10 µl of wild-type (MC58), MC58Δ*hmbR* and MC58Δ*hmbR*Δ*tbpBA* grown to mid-log (OD600 = 0.4 - 0.6) in MHB supplemented with 20 µM CMP-NANA, were added to freshly-collected heparinised human blood. The experiment was performed with duplicates of each strain. Serial dilutions of samples, taken at 0, 60, 120, 180 and 240 minutes, were plated onto supplemented BHI agar, in duplicates. The experiment was performed once. The mean of four counts was plotted above and the error bars indicate the standard error of the mean.
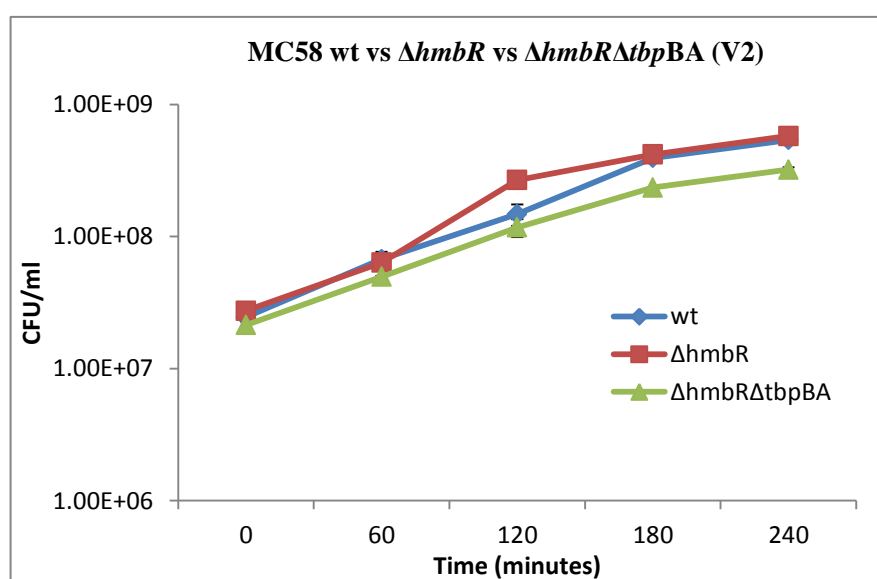
Figure 3.23: Growth assay for *hmbR* mutants of MC58 in human whole blood (V2). The experiment was performed as previously outlined in Figure 3.22. The mean of four counts was plotted above and the error bars indicate the standard error of the mean.

For a more accurate measurement of the inoculum size in later experiments, the CFU of bacteria was measured prior to inoculating blood in addition to measurement of the T_0 CFU count. The inoculum size was also reduced from ~10^7 to ~10^6 and the experiment extended for a further 120 minutes to allow for a better assessment of the growth of all strains.

It was observed that the inocula of wild-type and MC58Δ*hmbR* strains were 3-fold and 5-fold higher than T_0 counts, respectively, in V1 blood. Further decreases in bacterial counts stretching to 77-fold and 281-fold differences were discerned at T_{60}. Conversely, an insignificant 1.2-fold difference was observed between inoculum and T_0 CFU counts for the MC58Δ*hmbR*Δ*tbpBA* strain but a reduction in CFU counts (up to 18-fold) was observed between T_0 and T_{120} for this strain. Following the reduction in CFU counts, the MC58Δ*hmbR*Δ*tbpBA* mutant grew less vigorously than wild-type and MC58Δ*hmbR* strains. Exponential growth was recorded for MC58Δ*hmbR*Δ*tbpBA* between T_{120} and T_{180} only, with cells of this strain remaining at stationary phase from T_{180} to T_{360}, while wild-type and MC58Δ*hmbR* cells grew exponentially from T_{120} and T_{180}, respectively, till the end of the experiment at T_{360} (Figure 3.24).

Similar observations were made in the experiment with V2 blood. Bactericidal activity of the V2 blood sample, not seen in previous experiments, was evident in this assay. Consequently, 3.4-fold, 2.8-fold and 2.9-fold differences between the inoculum and T_0 CFU counts were recorded for wild-type, MC58Δ*hmbR* and MC58Δ*hmbR*Δ*tbpBA* strains respectively. Further reductions in CFU counts were observed for wild-type and MC58Δ*hmbR* at T_{60} but not MC58Δ*hmbR*Δ*tbpBA*. As in V1 blood, exponential growth was recorded for MC58Δ*hmbR*Δ*tbpBA* between T_{120} and T_{180} only, while wild-type and MC58Δ*hmbR* cells grew exponentially from T_{60} to T_{360} (Figure 3.25). These results strongly suggested an inability of the MC58Δ*hmbR*Δ*tbpBA* strain to grow in blood, thereby hinting at a more important role for *tbpBA* than *hmbR* in this model.
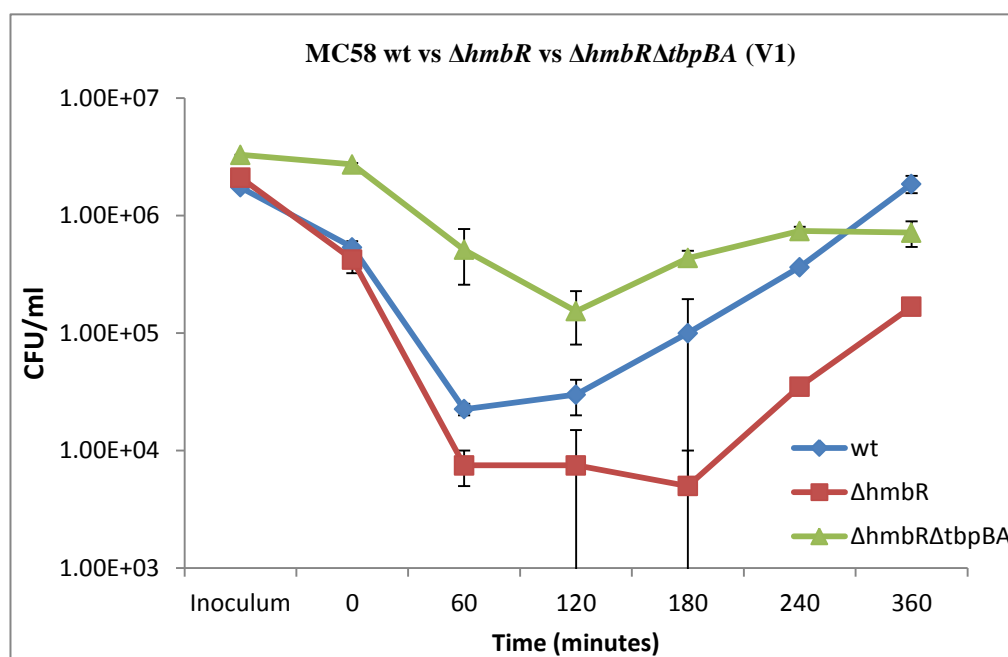
Figure 3.24: Extended **g**rowth assay for *hmbR* mutants of MC58 in human whole blood (V1). The experiment was performed as previously described in Figure 3.21. CFU counts of the inocula were determined, prior to inoculation of blood samples, in order to understand reasons for the discrepancies observed between the T_0 counts of all strains. The experiment was conducted for 360 minutes. The mean of four counts was plotted above and the error bars indicate the standard error of the mean.
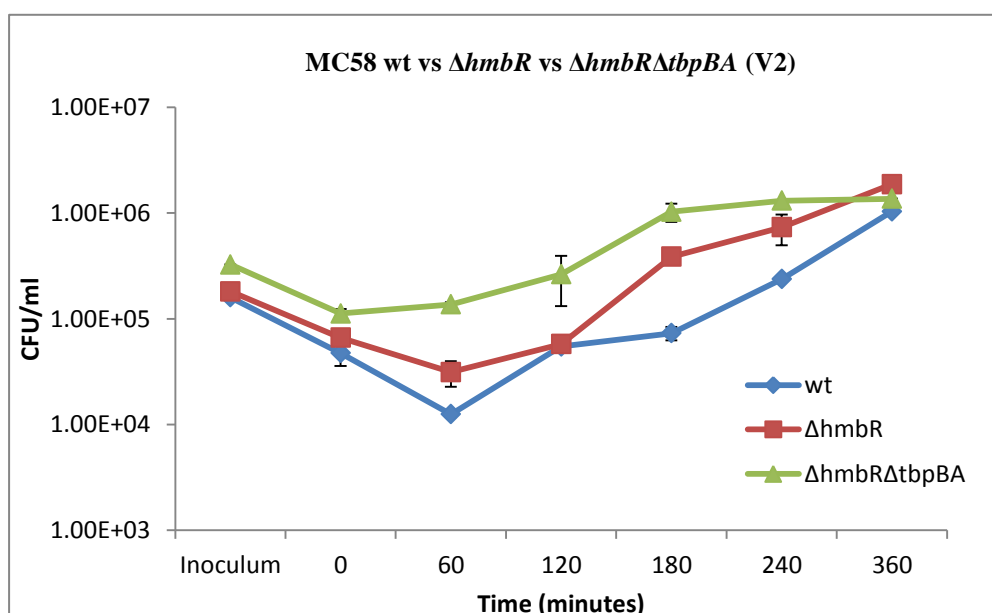
Figure 3.25: Extended **g**rowth assay for *hmbR* mutants of MC58 in human whole blood (V2). The experiment was performed as previously described in Figure 3.21 with modifications as mentioned in Figure 3.24. The mean of four counts was plotted above and the error bars indicate the standard error of the mean.

A further investigation of the cause of the dissimilar growth rate of the double mutant was done by including the Δ*tbpBA* strain in the assay. The duration of the assay was also extended for another 120 minutes to provide a clearer and more distinct pattern between strains. Three biological replicates of the assay were performed in blood from each volunteer on separate days to check for reproducibility and validity of the results obtained.

Results from only one assay done in V1 was analysed as medications taken by the volunteer at the time of the other 2 experiments (non-antibiotics) significantly affected growth of all strains in the blood. Results from the lone experiment were consistent with previous assays. All strains showed similar growth patterns to those seen in previous experiments. Wild-type and MC58Δ*hmbR* cells continued to grow exponentially after a population size of ~10^6 CFU was reached. Conversely, no doubling of population size was observed for both MC58Δ*tbpBA* and MC58Δ*hmbR*Δ*tbpBA* (Figure 3.26)

Three biological assay replicates were successfully performed in V2 blood. An average doubling time of 36 minutes was calculated for the MC58Δ*hmbR* strain between T_{60} and T_{360}, a growth rate which was 1.4 times faster than the wild-type strain. In comparison to the wild-type and MC58Δ*hmbR* strains, doubling of MC58Δ*tbpBA* and MC58Δ*hmbR*Δ*tbpBA* populations in V2 blood occurred at a slower rate; 80 minutes and 93 minutes respectively (Figure 3.27).
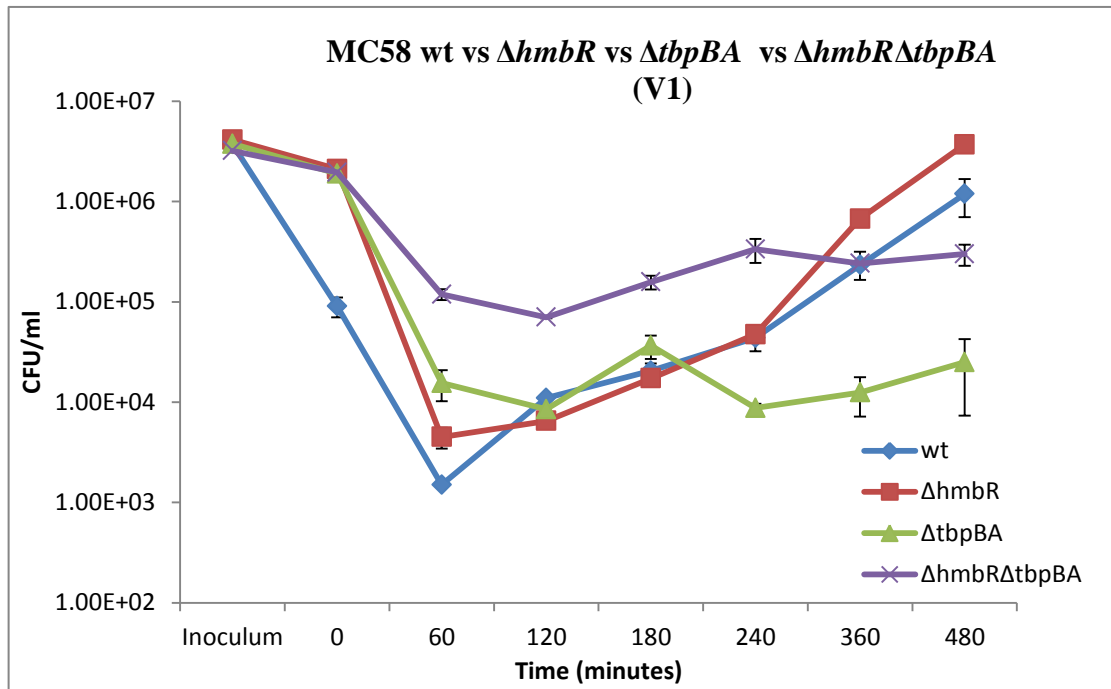
Figure 3.26: Extended **g**rowth assay for *hmbR* and *tbpBA* mutants of MC58 in human whole blood (V1). The experiment was performed as previously described in Figure 3.25. The MC58Δ*tbpBA* mutant was included in the assay and the experiment was conducted for 480 minutes. Results from one (out of three biological assay replicates) were analysed above. The mean of four counts (each strain was used to inoculate blood in duplicates) was plotted and the error bars indicate the standard error of the mean.
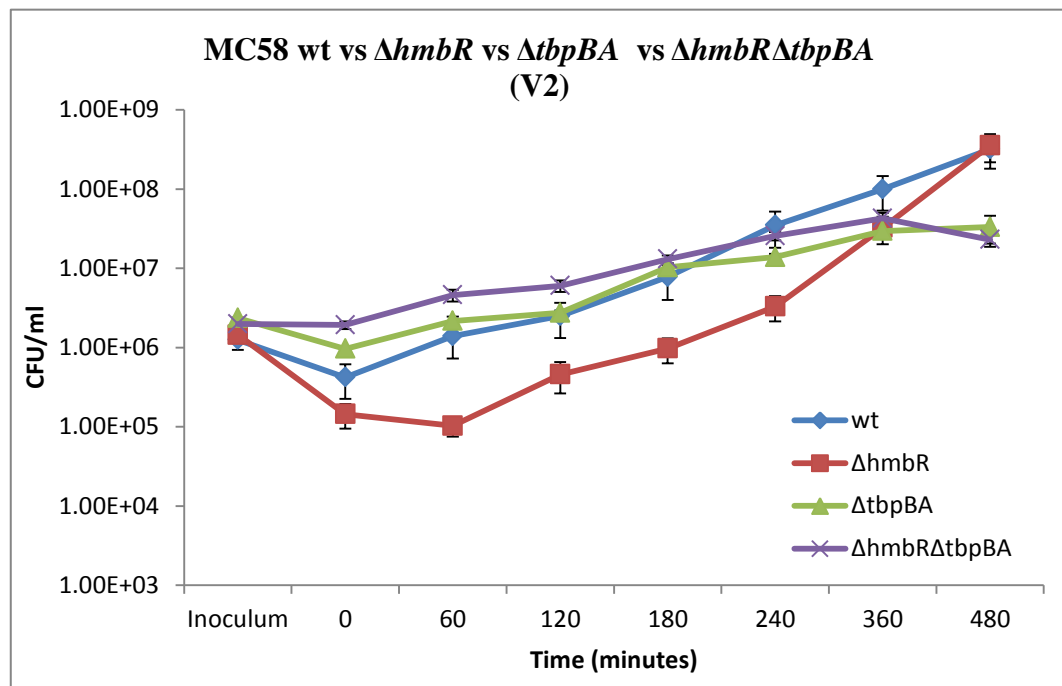
Figure 3.27: Extended **g**rowth assay for *hmbR* and *tbpBA* mutants of MC58 in human whole blood (V2). The experiment was performed as previously described in Figure 3.25. Results from three biological assay replicates (performed on different days) were analysed above. The mean of 12 counts (2 counts x 2 cultures x 3 biological replicates) was recorded as the CFU/ml for each strain at each timepoint. Error bars indicate the standard error of the mean of all 12 counts.

3.12   Growth of 8047 wild-type and mutant strains in human whole blood

To assess the importance of *hpuAB* to survival of meningococci in blood, strain 8047 (*hpuAB*-ON) was passaged in human whole blood. Experimental protocols utilised during the assays performed with strain MC58 were employed. Some modifications were, however, made in an attempt to increase resistance to killing by whole blood. In addition to the exogenous sialylation of the LOS achieved by the provision of 20 μM CMP-NANA, whole blood samples were treated with either 5 mM or 10 mM of EDTA to inactivate the complement system. A third volunteer was also recruited for the provision of blood samples as V1 blood showed significant bactericidal activity in previous experiments conducted in this study. Approximately 10^6 CFU of the wild-type strain 8047 was used to inoculate pre-treated blood samples, duplicate. Growth of the strain was monitored over a 4-hour period by plating samples from each culture at hourly intervals.

There was no increase in population size recorded for strain 8047 in human whole blood pre-treated with either 5 mM or 10 mM of EDTA. In fact, reductions in CFU were observed at T_0, similar to observations previously made in experiments using strain MC58. 3 – 4 fold decreases in population were calculated for blood cultures pre-treated with 10 mM EDTA. 5 mM EDTA appeared to have a lesser effect on complement sensitivity as reductions of 6- and 19-fold were observed for V2 and V3 cultures, respectively. Continuous reductions in CFU were observed at T_{60} and by T_{120}, the population size of strain 8047 in V2 or V3 blood crossed the threshold of undetectable limits. No recovery was discerned at any of the latter sampling points in the experiment (Figure 3.28).
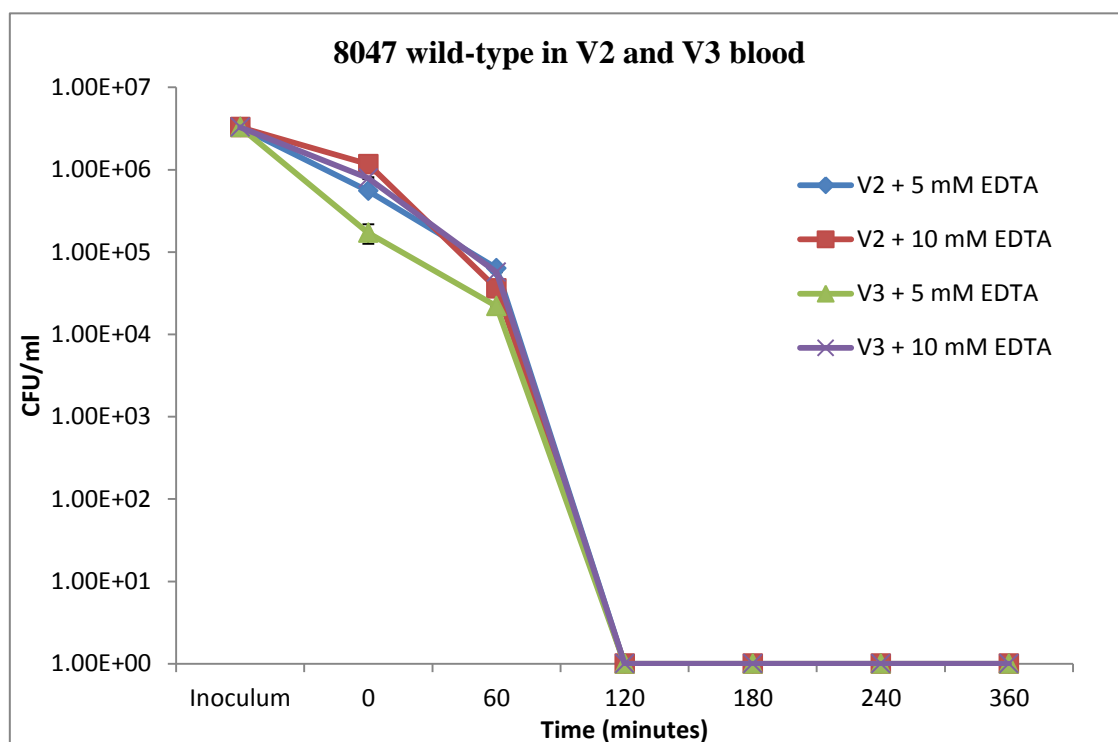
Figure 3.28: 8047 growth assay in human whole blood. 10 µl of wild-type 8047, grown to mid-log (OD600 = ~ 0.6) in MHB supplemented with 20 µM CMP-NANA, was added to freshly-collected human blood, treated with either 5 mM or 10 mM EDTA. Blood samples were collected from one healthy MBL-deficient volunteer; V2 and an uncharacterised volunteer, V3. Cultures were incubated at 37⁰C, w5% CO_2 for 4 hours. Dilutions of samples, taken at 0, 60, 120, 180 and 240 minutes, were plated onto supplemented BHI agar, in duplicate. The experiment was performed once. Intra-assay variation was measured by calculating the standard error of the mean of 6 CFU counts obtained at each timepoint.

3.13   Discussion

Haemoglobin utilization is believed to be crucial to survival of any blood-borne pathogen as Hb is one of the most abundant sources of iron in the blood (Evans *et al.*, 1999, cited in Perkins-Balding *et al.*, 2004). This is important as the amount of iron-bound Tf molecules can be significantly reduced in the early stages of infection. Two phase-variable Hb receptors have been described in the meningococcus and previous studies investigating the importance of these proposed virulence factors suggest an impairment to the survival and/or growth of meningococci in the absence of an ability to utilise Hb. The study conducted by Stojiljkovic *et al.* (1995) showed that a meningococcal strain (*hpuAB*-negative) that was incapable of Hb utilisation via the loss of *hmbR* was less virulent as compared to the wild-type *hmbR*-positive strain in a rat infection model. Later genetic epidemiology studies revealed a bias towards the presence of *hmbR* in disease isolates and for PV-ON status for one or both genes during infection in >95% of this collection of disease isolates (Harrison *et al.*, 2009; Tauseef *et al.*, 2011).

Considering that the meningococcus is strictly host-specific, infecting only humans, the exact nature of the involvement of these Hb receptors in meningococcal virulence was sought in this study, using an *ex vivo* human whole blood model. A comparison between the importance of Tf-utilisation and Hb-utilisation to growth of the meningococcus was also performed. Unlike the impaired survival and growth of a Δ*hmbR* mutant in the rat infection model of Stojiljkovic *et al.* (1995), this study showed that a similar mutant incapable of utilising Hb via the loss of *hmbR* (*hpuAB*-negative; Δ*hmbR*) was not disadvantaged when grown in human whole blood. Instead, growth of a Δ*tbpBA* mutant was impaired in human whole blood. The reason for the difference between results obtained in this study and those of Stojiljkovic *et al.* lies in the models employed. It is believed that the human whole blood model employed in this study mimics more closely the intravascular environment encountered by the meningococcus during systemic disease. Therefore, it is possible that the attenuated virulence of the Δ*hmbR* mutant in the rat infection model is not solely due to the loss of Hb-utilisation but to other factors, which are connected to the host specificity of the pathogen (Schryvers and Gonzalez, 1989). For example, meningococcal TbpBA preferentially binds human Tf (Schryvers and Gonzalez, 1989; Schryvers and Gonzalez, 1990);

consequently, utilisation of iron bound to rat Tf would have been impossible in the Stojiljkovic *et al*. study and could have affected growth of the pathogen in the rat.

Furthermore, the fact that a meningococcal strain isolated from a convalescent patient differed from its pre-infection parental strain in *hpuAB* and *hmbR* expression status (inoculum – *hmbR*-ON, *hpuAB*-OFF; isolate - *hmbR*-OFF, *hpuAB*-ON) shows that loss of *hmbR* expression is not detrimental to meningococcal virulence in its human host (Omer *et al*., 2011). Perhaps, the loss of expression of *hmbR* was compensated for by the gain of expression of *hpuAB* suggesting *hpuAB* as a more important receptor than *hmbR*. This is supported by the fact that HpuAB interacts and releases iron from more substrates than HmbR (Perkings-Balding *et al*., 2004). Observations in this study where growth of Δ*hmbR* mutants in either a *tbpBA*-positive or *tbpBA*-null background were not dissimilar to their *hmbR*-positive counterparts also show that utilisation of Hb via *hmbR* was non-existent in this model. This could be as a result of the total sequestration of Hb molecules by Hp; a scenario in which HpuAB would be the preferred receptor. However, an attempt to assess the importance of Hb-utilisation via HpuAB, in this study, was not successful as the *hpuAB*-positive strain employed (8047 – *hpuAB*-ON; *hmbR*-OFF) was found to be highly sensitive to human whole blood.

Despite the advantages of using this model over non-human animal infection models, some drawbacks exist. The most significant limitation to the use of this model to study meningococcal virulence factors is the inherent bactericidal activity of human blood. This bactericidal activity, also reported in several studies in which the model has been employed (Ison *et al*., 1995; Morley *et al*., 2001; Seib *et al*., 2009), is likely due to the activity of both humoral (complement pathway and anti-meningococcal antibodies) and cellular factors (neutrophils and macrophages) present in blood of donors. In this study, steps taken to reduce the effect of this bactericidal activity include the procurement of blood samples from volunteers with deficiencies in the classical complement pathway and the pre-culture of inocula in the presence of CMP-NANA to induce sialylation of the LOS. This exogenous sialylation of LOS in serogroup C meningococci was shown to suppress the expression of lacto-N-neotetraose (LNnT), amounts of which were directly proportional to serum sensitivity (Estabrook *et al*., 1997). It is evident, however, that bactericidal activity of whole blood in this model is mainly due to the activity of neutrophils and macrophages since serum obtained from

one volunteer was incapable of killing both the wild-type MC58 and MC58Δ*tbpBA* mutant strains (data not shown).

Another limitation to the use of this model is that the model mimics only the initial stages of an infection since a response to the infection from the human immune system via nutritional immunity (reviewed in Cassat and Skaar, 2013) will be absent in the *ex vivo* model. It would be difficult using this model, therefore, to ascertain the importance of virulence factors that are utilised at later stages of an infection. Hb-utilisation, via *hpuAB* or *hmbR*, is believed to be one of such virulence factors and as such its importance during the entire course of an infection may be underestimated using this model.

In summary, no Hb-utilisation via *hmbR* was observed using the whole blood model, thus, negating a crucial role for *hmbR* expression during the early stages of a meningococcal infection.

CHAPTER 4

4.0   Generation of polyclonal antibodies against rHpuA, rHpuB and rHmbR in mice

The surface expression profiles of the phase-variable meningococcal Hb receptors during different stages of infection, from nasopharynx to the meninges, are relatively unknown. Previous studies have shown that *hpuAB* and/or *hmbR* were in a PV-ON state in more than 90% of one collection of disease isolates (Tauseef *et al*., 2011). The study by Tauseef and co-workers also included analysis of 305 carriage isolates obtained from first-year University of Nottingham students (Bidmos *et al*., 2011). Seventy-one per cent of these 305 isolates had either or both Hb receptors in an ON-state suggesting surface expression of these receptors and implying a crucial role for these receptors during colonization of different niches in the human host by the meningococcus.

Deductions from these studies were made by measuring the repeat tract lengths of *hpuA* and *hmbR* by a PCR-based method. Despite limiting the *in vitro* passaging of isolates to a minimum and analyses of several isolates from individual volunteers (in the case of carriage isolates), only strong suggestions could be made with regards to the surface expression of these receptors. To validate data generated from these assays, experiments that show surface expression are required. These methods utilize the binding of labelled ligands (Rohde and Dyer, 2004) or IgG antibodies, produced in mice or rabbits, to surface-exposed epitopes of the target antigen.

In biophysical cell sorting assays where fluorescence probes are employed, the expression level of the target antigen is taken as a function of the relative intensity of the signal obtained when compared to cells lacking the antigen. Immunocytochemical assays, on the other hand, allow for the visualizing of all or parts of the surface-expressed antigen within the context of the membrane using advanced microscopy. Different versions of these methods have been used successfully to show surface expression (Comanducci *et al.,* 2002; Jiang *et al*., 2010; Moe *et al*., 1999) and topology (Frye *et al*., 2006; van der Ley *et al*., 1991) of meningococcal virulence factors and vaccine candidate proteins. For example, a microscopy-based assay showed that gold-

labelled transferrin bound to an iron-inducible protein on the surface of live meningococcal cells. Rabbit antisera recognising the meningococcal transferrin-binding proteins, TbpBA, interfered with this interaction providing proof that the iron-inducible receptor was TbpBA and it was localised to the meningococcal surface (Ala'aldeen *et al.*, 1993). Therefore, antibody-utilizing methods such as flow cytometry would be useful in measuring expression of these meningococcal Hb receptors during carriage and disease. These antibodies would also be useful in measuring amounts of protein in cell lysates using conventional methods such as Western blotting and enzyme-linked immunosorbent assay (ELISA).

Since antibodies are required to measure surface expression of HpuA, HpuB and HmbR, this study aimed to generate polyclonal antibodies to these Hb receptors in female BALB/c mice. An attempt to produce monoclonal antibodies from spleen cells of immunized mice was also made.

4.1   Expression and purification of rHpuA

Two alleles of *hpuA* were amplified from a carriage strain, N88 (Y:P1.21,16:F3-7:CC-174) and a disease isolate 8047 (B:P1.5-1,2-2:F3-6:ST-8). The region of the gene containing the repeat locus was omitted to eliminate the possibility of phase variation of the expression construct. Cloning into a pLEICS-03 vector (see Chapter 2, Table 2.1 for description) was done by employing a service at the Protein Expression laboratory (PROTEX) at the Department of Biochemistry, University of Leicester. Sequences obtained for rN88-*hpuA* (pFAB-2) and r8047-*hpuA* (pFAB-3) were confirmed as meningococcal *hpuA* when compared with published meningococcal genome sequences on the National Centre for Biotechnology Information (NCBI) website. Both sequences were also shown to be antigenic variants of HpuA using the sequence alignment facility of Clone Manager 9 (Figure 4.1). Using a beta-barrel OMP topology prediction tool (Bagos *et al.*, 2004), the surface-exposed loops of HpuA were determined and these corresponded with regions of variation seen in the alignment between antigenic variants (Figure 4.1).

```
Assembled Protein alignment.    Mol 1 pFAB-3.txt (1 to 340)    Mol 2 pFAB-2.txt (1 to 340)
Method: FastScan - Max Qual (Cons N)

Sequence View: Difference Format, Color behind non-matches

                          6xHis                    hpuA
pFAB-3.txt      1 hhhhhhssgvdlgtenlyfqsmaephvpvsiptatpltgevkltddnski
pFAB-2.txt      1 ...................................p...t.s..svn.

pFAB-3.txt     51 enintantgnt-sgisiqqreykvnnygvestakafifktpggaqytlss
pFAB-2.txt     51 v.....g..s.s.......k.n.ts..ftree...............

pFAB-3.txt    100 yadpivpsysspdfkipdrhagqrladgsrificcsdsgatnqaeitkqd
pFAB-2.txt    101 ..............................................

pFAB-3.txt    150 ymkfgawigpngeidlfaggfpvgktpasssyygsstpetqgkgkityqv
pFAB-2.txt    151 ...............................-...l..a.........

pFAB-3.txt    200 wgirvrngqfvtssytppknsgyysstptntpvlsfitanfnthklsgei
pFAB-2.txt    200 .....kd.............ss.lyk...............sn..a...

pFAB-3.txt    250 ignsdygpnvkienatidglsfsgdatsggkngklegkffgkfngyrdte
pFAB-2.txt    250 ........d...............n......t...d.......dst.ssd

pFAB-3.txt    300 tsiggkitfdgdrsldtvfggvsykkelenntdtstthltk
pFAB-2.txt    300 .g.......kd...........i.e.k.ddk.sqd......
```

Figure 4.1: Alignment of r8047-HpuA versus rN88-HpuA. DNA sequences were obtained for *hpuA* from pFAB-2 (N88-*hpuA*) and pFAB-3 (8047-*hpuA*). An alignment of the amino acid sequences shows regions of variation between both sequences (shaded orange).

For expression of recombinant HpuA, pFAB-2 and pFAB-3 were first transformed into the *E. coli* expression vector BL21 (DE3). Expression was performed as outlined in section 2.7.1. Optimum expression was obtained 2 hours post-induction with 1 mM IPTG (Figures 4.2 and 4.3).

Figure 4.2: Expression of pFAB-2. 1 mM IPTG was added to *E. coli* BL21 cells containing pFAB-2 that were grown to mid-log (OD_{600} = ~0.5). Induction of expression was done for 3 hours and samples were taken at hourly intervals (lanes 3, 5 and 7). Samples were also taken from cultures without IPTG (lanes 2, 4 and 6). The zero timepoint sample was analysed in lane 1.
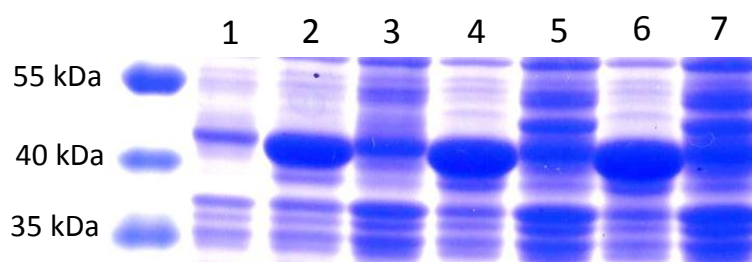
Figure 4.3: Expression of pFAB-3. 1 mM IPTG was added to *E. coli* BL21 cells containing pFAB-3 that were grown to mid-log (OD$_{600}$ = ~0.5). Induction of expression was done for 3 hours and samples were taken at hourly intervals (lanes 2, 4 and 6). Samples were also taken from cultures without IPTG (lanes 3, 5 and 7). The zero timepoint sample was analysed in lane 1.

Over-expressed rHpuA was purified from BL21 cells using methods outlined in section 2.7.2. Electrophoresis of the purified fractions showed the presence of another protein, slightly larger than the expected size of rHpuA, but in smaller quantities. Mass spectrometry of this protein by Isfahan Tauseef revealed that it was identical to rHpuA and is probably a lipidated version of the protein. A slight difference in size between both variants of rHpuA was discerned on a denaturing polyacrylamide gel (Figure 4.5). While the predicted molecular weight of both proteins are similar (rN88-HpuA: 36.53 kDa; r8047-HpuA: 36.63 kDa), the theoretical isoelectric points are 6.54 and 6.38 respectively. A possibility stemming from this isoelectric point differential is that r8047-HpuA will migrate faster than rN88-HpuA in a SDS-running buffer of pH 8.3 – 8.6.
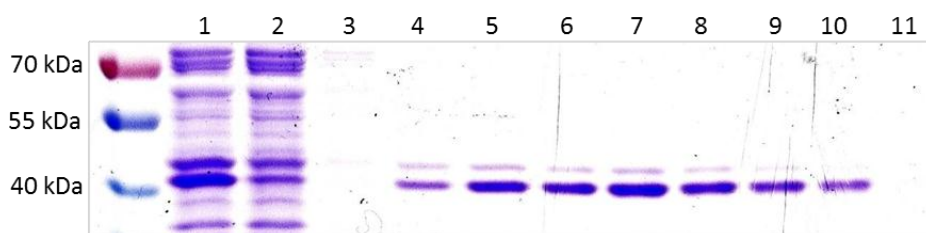
Figure 4.4: Purification of rHpuA using a nickel-affinity column. After equilibration of the column with 5x volume of the lysis buffer, *E. coli* lysate containing expressed rHpuA was loaded onto the column (lane 1). rHpuA bound to the column while unbound *E. coli* proteins flowed through (lane 2). The column was subsequently washed with 20mM imidazole (lane 3) before bound rHpuA was eluted with higher concentrations of imidazole: 50 mM (lane 4), 80 mM (lane 5), 100 mM (lane 6), 120 mM (lane 7), 150 mM (lane 8), 200 mM (lane 9) and 250 mM (lane 10).
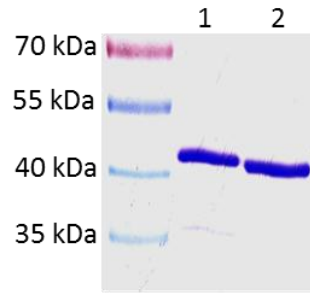
Figure 4.5: Purified rHpuA. 1 µg of rHpuA cloned from carriage strain N88 (lane 1) and disease isolate 8047 (lane 2) were analysed on a 8% SDS-PAGE gel.

4.2   Expression and purification of rHpuB

hpuB was cloned from disease isolate 8047 into the pLEICS-03 vector to give an expression vector labelled as pFAB-4. The first 16 aa of the 22 aa signal peptide of the gene (Oliver, 1987 cited in Lewis *et al.*, 1997) were omitted. Sequencing of pFAB-4 showed an intact *hpuB* sequence that was identified as a haemoglobin receptor found in meningococci by the Basic Local Alignment Search Tool (BLAST) on the NCBI website. Based on this sequence, the recombinant 8047-HpuB protein was computed to have a molecular weight of 91.5 kDa and a theoretical isoelectric point (pI) of 9.35 using the web-based pI/Mw tool on the ExPASy resource portal website. Alignment of the translated amino acid sequence with the sequence from strain G2136 showed a 100% match (Figure 4.7).

Recombinant HpuB was expressed in *E. coli* BL21 (DE3) cells as previously outlined in section 2.7.1. Expression of rHpuB was optimum after 2 hours of induction with 1 mM IPTG (Figure 4.7).
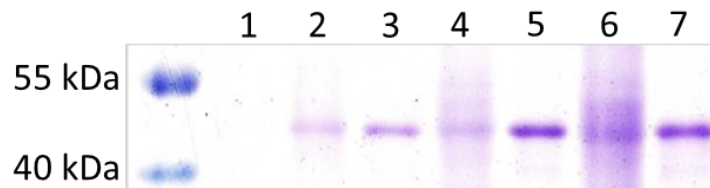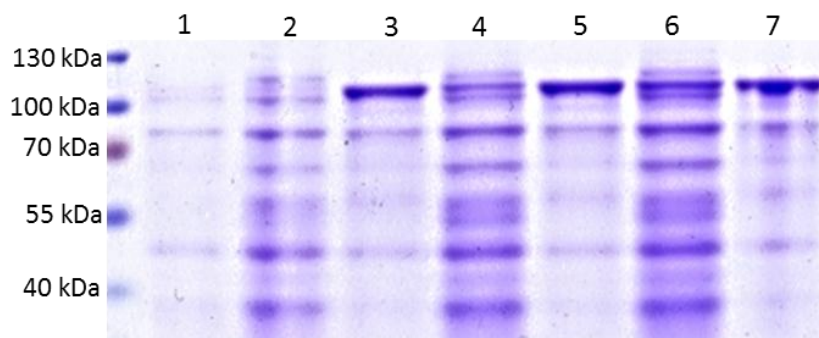

Figure 4.6: Expression of pFAB-4. IPTG (1 mM) was added to *E. coli* BL21 cells harbouring pFAB-4 that were grown to mid-log (OD$_{600}$ = ~0.5). Induction of expression was done for 3 hours and samples were taken at hourly intervals (lanes 3, 5 and 7). Samples were also taken from cultures without IPTG (lanes 2, 4 and 6). The zero timepoint sample was analysed in lane 1.

```
Assembled Protein alignment.    Mol 1 pFAB-4.txt (1 to 816)    Mol 2 G2136 HpuB.txt (1 to 810)
Method: FastScan - Max Qual (Cons N)

 Sequence View: Difference Format, Color behind non-matches


pFAB-4.txt          1 hhhhhhssgvdlgtenlyfqsm---afpafaadpapqsaqtlneitvtgthktqklgeek
G2136 HpuB.txt      1 ---------mpipfkpvlaaaaiaq.................................

pFAB-4.txt         58 irrktldkllvndehdlvrydpgisvveggragsngftirgvdkdrvainvdglaqaesr
G2136 HpuB.txt     52 .........................................................

pFAB-4.txt        118 sseafqelfgaygnfnanrntsepenfsevtitkgadslksgsgalggavnyqtksasdy
G2136 HpuB.txt    112 .........................................................

pFAB-4.txt        178 vsedkpyhlgikggsvgknsqkfssitaagrlfgldallvytrrfgketknrstegdvei
G2136 HpuB.txt    172 .........................................................

pFAB-4.txt        238 kndgyvynptdtggpskyltyvatgvarsqpdpqewvnkstlfklgynfndqnrigwife
G2136 HpuB.txt    232 .........................................................

pFAB-4.txt        298 dsrtdrftnelsnlwtgtttsaatgdyrhrqdvsyrrrsgveyknelehgpwdslklryd
G2136 HpuB.txt    292 .........................................................

pFAB-4.txt        358 kqridmntwtwdipknydkrgingevyhsfrhirqntaqwtadfekqldfskavwaaqyg
G2136 HpuB.txt    352 .........................................................

pFAB-4.txt        418 lgggkgdnansdysyfaklydpkilasnqakitmlienrskykfaywnnafhlggndrfr
G2136 HpuB.txt    412 .........................................................

pFAB-4.txt        478 lnagirydknsssakddpkyttairgqiphlgserahagfsygtgfdwrftkhlhllaky
G2136 HpuB.txt    472 .........................................................

pFAB-4.txt        538 stgfraptsdetwllfphpdfylkanpnlkaekaknwelglagsgkagnfklsgfktkyr
G2136 HpuB.txt    532 .........................................................

pFAB-4.txt        598 dfieltymgvssddknnpryaplsdgtalvsspvwqnqnrsaawvkgiefngtwnldsig
G2136 HpuB.txt    592 .........................................................

pFAB-4.txt        658 lpkglhtglnvsyikgkatqnngketpinalspwtavyslgydapskrwginayatrtaa
G2136 HpuB.txt    652 .........................................................

pFAB-4.txt        718 kkpsdtvhsnddlnnpwpyakhskaytlfdlsaylnigkqvtlraaaynitnkqyytwes
G2136 HpuB.txt    712 .........................................................

pFAB-4.txt        778 lrsirefgtvnrvdnkthagiqrftspgrsynftieakf
G2136 HpuB.txt    772 .......................................
```

Figure 4.7: Alignment of r8047-HpuB versus G2136 HpuB.

The presence of large amounts of the recombinant protein in the insoluble fraction of *E. coli* cell lysates was indicative of its expression as inclusion bodies (Figure 4.8). The identity of the recombinant protein was subsequently determined using the Mass Spectrometry service at PNACL. Significant similarities of the protein to the ~91 kDa HpuB of meningococcal serogroups A and C strains were discerned with Mowse scores of 363 and 325 respectively.
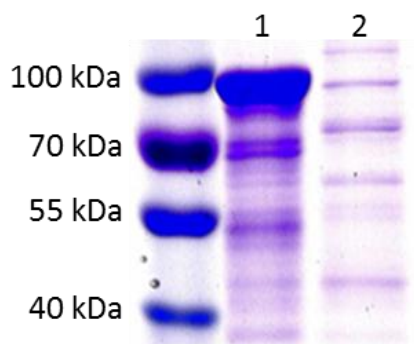
Figure 4.8: Expression of r8047-HpuB as inclusion bodies. The insoluble fraction after lysis of IPTG-induced *E. coli* cells containing pFAB-4 was analysed in lane 1 while the supernatant containing soluble proteins was analysed in lane 2.

Purification of rHpuB was attempted using a protocol employed to purify rHmbR by colleagues at the University of Manchester (Jeremy Derrick, Manchester). The protocol was based on the ability of the denatured protein to recognize and bind haem, thereby regaining its native conformation. Impurities were removed from the purified protein preparation via ion exchange chromatography. It is worth noting at this juncture that rHpuB and rHmbR were purified concurrently since both proteins were expressed as inclusion bodies. Similar problems were encountered during the purification of both proteins and modifications to protocols employed applied to both proteins. Results from the purification of rHmbR are shown later in this chapter.

Initial attempts to re-fold rHpuB in the presence of haem proved abortive as a few problems were encountered. The first of these problems was the precipitation of Na salts in the solubilisation and refolding buffers. Both buffers contained 50 mM $Na_2B_4O_7$ pH 9.5, which at a stock concentration of 0.1 M was insoluble in water at room temperature. Using a new batch of $Na_2B_4O_7.10H_2O$ did not solve this problem. Since the protocol recommended solubilisation at 4^0C followed by refolding at room temperature, purification of rHpuB was impossible using buffers containing 50 mM $Na_2B_4O_7$ pH 9.5. A comparison was made between buffers made with 50 mM $Na_2B_4O_7$ pH 9.5 and 20 mM Tris.Cl pH 9.5. On addition of the neat solubilisation buffer (no solubilised protein) to the refolding buffer, a colourless precipitate was obtained in the $Na_2B_4O_7$ buffer but not the Tris buffer.

The next problem faced was the instantaneous precipitation of the protein on addition to the refolding buffer. Proceeding with the latter stages of the purification process by

removing relatively large amounts of insoluble material at each stage, as outlined in section 2.7.3, yielded *pure* but insoluble rHpuB (Figure 4.9). Since the main constituent of the refolding buffer was 50 µM hemin, it was assumed that the preparation of the stock hemin (50 mM hemin in 0.1 M NaOH) was inadequate. Substituting the NaOH solvent with dimethyl sulphoxide (DMSO) did not reduce the level of precipitation of rHpuB during the refolding process.
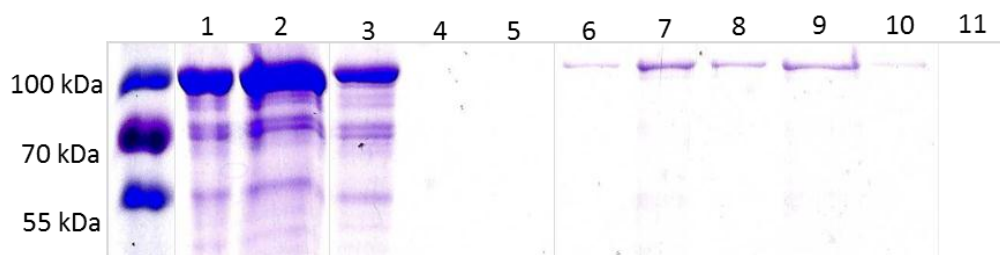
Figure 4.9: Optimization of r8047-HpuB purification. Insoluble material was removed by centrifugation at 4000 rpm after refolding (lane 1) and dialysis (lane 2) before loading the pre-equilibrated DEAE-Sephacel column with soluble material (lane 3). Unbound proteins in the flow-through were analysed in lane 4. The 50mM NaCl wash fraction was analysed in lane 5 while eluates in 100 mM, 150 mM, 300 mM, 450 mM and 600 mM NaCl were analysed in lanes 6 – 10 respectively. The 1M NaCl fraction was analysed in lane 11.

A new batch of hemin was obtained and prepared in DMSO at a stock concentration of 1 mg/ml. The refolding buffer was subsequently made with hemin at a final concentration of 1 µg/ml (1.5 µM). Solubilised protein was also added in a drop-wise fashion to the refolding buffer (10 times volume of the solubilised protein) on a magnetic stirrer. Removal of insoluble protein after refolding was also done at a higher centrifugal speed (13000 rpm as opposed to 4000 rpm previously used) to counter the problem of carry-over of insoluble material into downstream stages of the purification process. The volumes of the DEAE Sephacel resin and equilibration buffer (20 mM Tris.Cl pH 9.5, 0.1% v/v LDAO) were also increased 5-fold and 10-fold respectively – from 1 ml to 5 ml of resin and from 3 ml to 30 ml of equilibration buffer. These modifications led to the isolation of soluble rHpuB with different concentrations of NaCl. rHpuB constituted 59% and 77% of 0.3 M and 0.45 M eluates respectively with contaminating *E. coli* proteins constituting the remainder portions. No contaminants were found in the 0.6 M and 1 M fractions (Figure 4.10). However, amounts of the purified protein in these fractions were very low - a combined total of 0.1 mg was obtained.
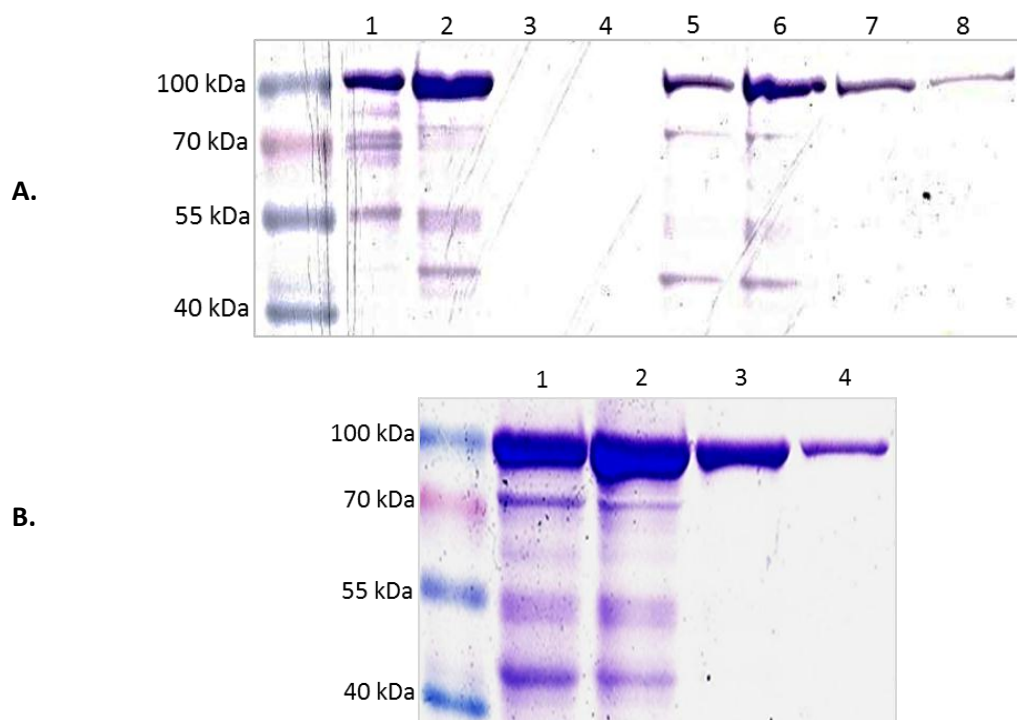
Figure 4.10: (A) Purification of r8047-HpuB. After dialysis, insoluble protein was removed by centrifugation at 13000 rpm (lane 1). Soluble protein (lane 2) was loaded onto pre-equilibrated DEAE Sephacel. Unbound proteins in the flow-through were analysed in lane 3 while the 150 mM NaCl wash fraction was analysed in lane 4. 300 mM (lane 5), 450 mM (lane 6), 600 mM (lane 7) and 1 M NaCl (lane 8) fractions contained r8047-HpuB with varying degrees of purity. **(B)** 10-fold concentrates of 300 mM (lane 1), 450 mM (lane 2), 600 mM (lane 3) and 1 M (lane 4) NaCl eluates.

4.3 Expression and purification of rHmbR

For the purpose of this study, 2 alleles of *hmbR* were cloned from disease isolates, MC58 and 8047. The *hmbR* allele from strain 8047 has a polyG repeat tract that contains 8 units leading to a premature stop codon just downstream of the tract. To obtain the full length protein from this allele, the repeat tract was mutagenized to include an extra adenine residue ensuring that the gene is in-frame (Figure 4.11). Insertion of an adenine instead of a guanine into the repeat tract interrupted the run of guanines leading to the inability of the tract to cause phase variation to an OFF state in the expression vector.

The first 18 aa of the 23 aa signal peptide of *hmbR* was excluded from the expression clones. Both *hmbR* alleles were cloned into the pLEICS-03 vector to give pFAB-5 (rMC58-*hmbR*) and pFAB-12 (r8047-*hmbR*). Sequencing of both plasmids showed intact translated HmbR sequences that match to the respective genome-derived sequences

(Figures 4.11 and 4.13). Based on these sequences, recombinant 8047-HmbR and MC58-HmbR were computed to have molecular weights of 89.4 kDa and 89.41 kDa, respectively. Theoretical isoelectric points (pI) of 9.22 and 9.26 respectively were also calculated using the web-based pI/Mw tool on the ExPASy resource portal website.

One SNP observed in the sequence of pFAB-12 (Figure 4.12) was introduced by the cloning primer, which was designed based on the genome sequence of the MC58-*hmbR* allele. Prediction of the structure of HmbR, using a beta-barrel OMP topology prediction tool that utilizes the more accurate Hidden Markov model (Bagos *et al.*, 2004), shows the irrelevance of this SNP as it is not present in any of the predicted surface-exposed loops; antibodies to which are required for this study (Figure 4.11). Differences in the protein sequences of both antigenic variants are also evident and these variations are mainly in the predicted surface-exposed loops of the protein (Figure 4.14).

Figure 4.11: Predicted topology of r8047-HmbR. A web-based OMP topology prediction software that utilizes the Hidden Markov model, PRED-TMBB, was used to predict the context of HmbR epitopes within the membrane. Regions highlighted in green correspond to predicted inner-membrane epitopes (**in**); red represent transmembrane epitopes (**tm**); and blue represent outer membrane epitopes (**out**). The region including the amino acid substitution (encircled) shown in Fig. 4.11 above is predicted to be located within the inner membrane.

```
                        6
p8047-HmbR.txt      1  ------------npvla--adeaasettpvkaeikavrvkgqrnvpaavervnlnrikqemirdnkdlvrystdvglsdsgrhqkgf
G2136 HmbR (stop)   1  mlpiaalvgsifg--npvla--adeaasettpvkaeikavrvkgqrnvpaavervnlnrikqemirdnkdlvrystdvglsdsgrhqkgf
pFAB-12.txt         1  hhhhhhssgvdlgtenlyfqsmdeaatettpvkaeikavrvkgqrnvpaavervnlnrikqemirdnkdlvrystdvglsdsgrhqkgf

p8047-HmbR.txt     74  airgvegnrvgvsidgvnlpdseenslyarygnfnssrlsidpelvrnidivkgadsfntgsgalgggvnyqtlqgrdlllperqfgvmm
G2136 HmbR (stop)  87  airgvegnrvgvsidgvnlpdseenslyarygnfnssrlsidpelvrnidivkgadsfntgsgalgggvnyqtlqgrdlllperqfgvmm
pFAB-12.txt        91  airgvegnrvgvsidgvnlpdseenslyarygnfnssrlsidpelvrnidivkgadsfntgsgalgggvnyqtlqgrdlllperqfgvmm

p8047-HmbR.txt    164  kngystrnrewtntlgfgvsndrvdaallysqrrghetesagnrgypvegagketnirgsargipdpskhkyhsflgkiayqindnhrig
G2136 HmbR (stop) 177  kngystrnrewtntlgfgvsndrvdaallysqrrghetesagnrgypvegagketnirgsargipdpskhkyhsflgkiayqindnhrig
pFAB-12.txt       181  kngystrnrewtntlgfgvsndrvdaallysqrrghetesagnrgypvegagketnirgsargipdpskhkyhsflgkiayqindnhrig

p8047-HmbR.txt    254  aslngqqhnytieesynllasywreaddvnrrrntnlfyewtpesdrlsmvkadvdyqktkvsavnykgsfpiedsstltnynqkdld
G2136 HmbR (stop) 267  aslngqqhnytieesynllasywreaddvnrrrntnlfyewtpesdrlsmvkadvdyqktkvsavnykgsfpiedsstltnynqkdld
pFAB-12.txt       271  aslngqqhnytieesynllasywreaddvnrrrntnlfyewtpesdrlsmvkadvdyqktkvsavnykgsfpiedsstltnynqkdld
                                                                                *
p8047-HmbR.txt    344  eiynrsmdtrfkritlrldshplqlggg-----------------------------------dt-----
G2136 HmbR (stop) 357  eiynrsmdtrfkritlrldshplqlggg-----------------------------------dt-----
pFAB-12.txt       361  eiynrsmdtrfkritlrldshplqlgggrhrlsfktfasrdfenlnrddyyfsgqisrtssiqhpvkttnvgfslsdqiqwndvfssr

p8047-HmbR.txt    374  -------------------------------------------------------------ac-----
G2136 HmbR (stop) 387  -------------------------------------------------------------ac-----
pFAB-12.txt       451  agirydhtkmtpqelnadchacdktppapntysgwsfaglaaqlnqawrvgyditsgyrvpnasevyftynhgagtwlpspnlkaerst

p8047-HmbR.txt         ---------------------------------------------------------------
G2136 HmbR (stop)      ---------------------------------------------------------------
pFAB-12.txt       541  thtlslqgrgekgtldanlyqsnyrnflseeqklttsgtpgcteenayygicsdpykekldwqmknidkarirgieltgrlnvdkvasfv

p8047-HmbR.txt    376  -------------------------rlklspaavilkt-----
G2136 HmbR (stop) 389  -------------------------rlklspaavilkt-----
pFAB-12.txt       631  pegwklfgslgyaksklsgdnsllstqplkviagidyespsekwgvfsrltylgakkvdaqtvvyenkgwgtplqkkvkdypwlnksay

p8047-HmbR.txt         ---------------------------------------------------------------
G2136 HmbR (stop)      ---------------------------------------------------------------
pFAB-12.txt       721  vfdmygfykpakmltlragvnlfnrkyttwdslrglysystnavdrdgkgldryrapgrnyavslewkf
```

Figure 4.12: Protein sequence alignment of *hmbR* from plasmids and genomic DNA. A consensus nucleotide sequence for the *hmbR* insert in pFAB-12 was determined from 4 sequence reactions. Using the Clone Manager software, this sequence was aligned with the sequence of the *hmbR* insert used as template for the mutagenesis of the repeat tract (p8047-HmbR provided by Chris Bayliss) and *hmbR* from the genome sequence of strain 8047 (G2136). Expression of the full-length protein was made possible by altering the polyG repeat tract in the p8047-HmbR from 8 to 9 units. A single nucleotide substitution leading to a change in the amino acid sequence at position 6 (indicated in red font) from serine to threonine is also shown above. The green background represents sequence homology.

```
Assembled Protein alignment.    Mol 1 pFAB-5.txt (1 to 790)    Mol 2 MC58 HmbR protein seq.txt (1 to 791)
Method: FastScan - Max Qual (Cons N)

  Sequence View: Difference Format, Color behind non-matches
                                    6xHis
pFAB-5.txt            1 hhhhhhssgvdlgtenlyfqsm--adeaatettpvkaeikavrvkgqrnapaavervnln
MC58 HmbR protei      1 -mkplqmlpiaalvgsifgnpvla....................................

pFAB-5.txt           59 rikqemirdnkdlvrystdvglsdsgrhqkgfavrgvegnrvgvsidgvnlpdseensly
MC58 HmbR protei     60 ............................................................

pFAB-5.txt          119 arygnfnssrlsidpelvrnieivkgadsfntgsgalgggvnyqtlqgrdlllddrqfgv
MC58 HmbR protei    120 ............................................................

pFAB-5.txt          179 mmkngystrnrewtntlgfgvsndrvdaallysqrrghetesagnrgyavegegsganir
MC58 HmbR protei    180 ............................................................

pFAB-5.txt          239 gsargipdsskhkyhsflgkiayqindnhrigaslngqqghnytveesynltasswread
MC58 HmbR protei    240 ............................................................

pFAB-5.txt          299 dvnrrrnanlfyewmpdsnwlsslkadfdyqktkvaavnnkgsfpmdystwtrnynqkdl
MC58 HmbR protei    300 ............................................................

pFAB-5.txt          359 deiynrsmdtrfkrftlrldshplqlgggrhrlsfktfvsrrdfenlnrddyyfsgrvvr
MC58 HmbR protei    360 ............................................................

pFAB-5.txt          419 ttssiqhpvkttnygfslsdqiqwndvfssragirydhtkmtpqelnaechacdktppaa
MC58 HmbR protei    420 ............................................................

pFAB-5.txt          479 ntykgwsgfvglaaqlnqawrvgyditsgyrvpnasevyftynhgsgnwlpnpnlkaers
MC58 HmbR protei    480 ............................................................

pFAB-5.txt          539 tthtlslqgrsekgmldanlyqsnyrnflseeqklttsgtpgcteenayygicsdpykek
MC58 HmbR protei    540 ............................................................

pFAB-5.txt          599 ldwqmknidkarirgieltgrlnvdkvasfvpegwklfgslgyaksklsgdnsllstqpl
MC58 HmbR protei    600 ............................................................

pFAB-5.txt          659 kviagidyespsekwgvfsrltylgakkvkdaqytvyenkgwgtplqkkvkdypwlnksa
MC58 HmbR protei    660 ............................................................

pFAB-5.txt          719 yvfdmygfykpaknltlragvynlfnrkyttwdslrglysysttnavdrdgkgldryrap
MC58 HmbR protei    720 ............................................................

pFAB-5.txt          779 grnyavslewkf
MC58 HmbR protei    780 ............
```

Figure 4.13: Protein sequence alignment of the sequence of the hmbR insert in pFAB-5 versus the hmbR gene from the MC58 genome sequence.

```
                    6xHis
pFAB-5.txt     1  hhhhhhssgvdlgtenlyfqsmadeaatettpvkaeikavrvkggrnapaavervnlnrikqemirdnkdlvrystdvglsdsgrhqkgf
pFAB-12.txt    1  hhhhhh...........................................v.......................................

pFAB-5.txt    91  avrgvegnrvgvsidgvnlpdseenslyarygnfnssrlsidpelvrnieivkgadsfntgsgalgggvnqtlqgrdllddrqfgvmm
pFAB-12.txt   91  .i.................................d...........................................pe
                                                                              Loop 2

pFAB-5.txt   181  kngystrnrewtntlgfgvsndrvdaallsgrrghetesagnrgyavegegsganirgsargipdsskhkyhsflgkiavqindnhrig
pFAB-12.txt  181  .........................p..a.ket.............................p
                           Loop 3                               Loop 4

pFAB-5.txt   271  aslnggghnytveesynltasswreaddvnrrnanlfyewmpdsnwlsslkadfdyqktkvaavnnkgsfp-mdystwtrnynqkld
pFAB-12.txt  271  ...............g...t...i...l.y...........t.......t.e.dr..mv..v....s..y...ie.s..l
                              Loop 6

pFAB-5.txt   360  eiynrsmdtrfkrftlrldshplqlgggrhrlsfktfvsrrdfenlnrddyyfsgrvvrttssiqhpvkttnygfslsdqiqwndvfssr
pFAB-12.txt  361  ..................i...........................a...........qis
                                                                                        Loop 7

pFAB-5.txt   450  agirydhtkmtpqelnaechacdktppaantykgwsgfvglaaqlnqawrvgydvtsgyrvpnasevyftynhgsgnwlpnpnlkaerst
pFAB-12.txt  451  ...............d...........p..s............a........a.t...s
                                    Loop 8

pFAB-5.txt   540  thtlslqgrsekgmldanlygsnyrnflseeqklttsgtpgcteenavygicsdpykekldwqmknidkarirgheltgrlnvdkvasfv
pFAB-12.txt  541  .................g...t
                                                                  Loop 10

pFAB-5.txt   630  peqwklfgslgyaksklsgdnsllstqplkviagidyespsekwgvfsrltylgakkvkdaqytvyenkgwgtplqkkvkdypwlnksay
pFAB-12.txt  631  ...........................
                                                                        Loop 11

pFAB-5.txt   720  vfdmygfykpaknltlragvynlfnrkytwtdslrglysvsttnavdrdgkgldryrapgrnyavslewkf
pFAB-12.txt  721  
```

Figure 4.14: Alignment of rMC58-HmbR (pFAB-5) versus r8047-HmbR (pFAB-12). Regions of variation between both sequences are highlighted in an orange background. Putative surface-exposed loops that are either positively-selected (Evans *et al.*, 2010) and/or involved in Hb utilization (Perkings-Balding *et al.*, 2003) are highlighted.

As observed for rHpuB, rHmbR formed insoluble inclusion bodies when expressed from the recombinant plasmid. An optimum level of expression was obtained after 1 hour of induction with 1 mM IPTG for both HmbR variants (Figure 4.15). The identity of the recombinant protein was determined using the Mass Spectrometry service at PNACL. The protein was highly homologous to the ~87 kDa HmbR of strain MC58 strain with a Mowse score of 297.

r8047-HmbR　　　　　　　　　　**rMC58-HmbR**
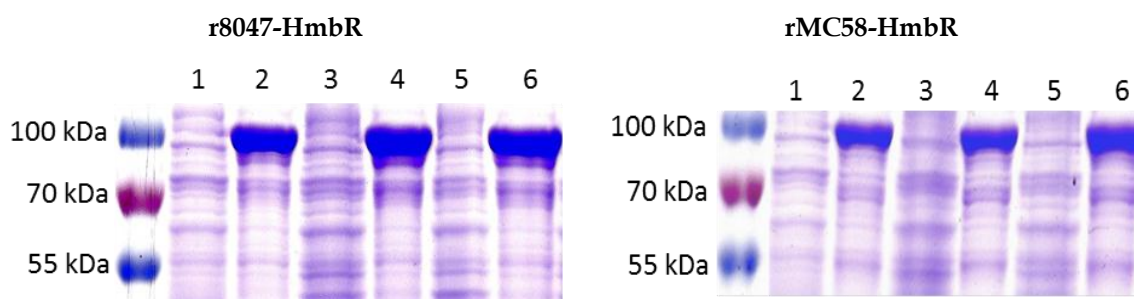
Figure 4.15: Expression of r8047-HmbR and rMC58-HmbR. Induction with 1 mM IPTG was performed for 3 hours. Samples were taken hourly from cultures with IPTG (lanes 2, 4 and 6) and without IPTG (lanes 1, 3 and 5). Lysates were prepared from these samples and electrophoresed on an 8% SDS-PAGE gel before staining with Coomassie dye.

An attempt was made to purify rHmbR before refolding the purified protein in the presence of hemin. A nickel-affinity method of purifying his-tagged proteins, with modifications, was initially employed. rHmbR inclusion bodies were solubilised using 8 M urea and then applied to pre-equilibrated nickel-affinity columns at room temperature. Weakly-bound impurities were removed by washing the column with imidazole concentrations of 20 mM and 50 mM. In order to elute bound proteins, the column was treated with increasing concentrations of imidazole ranging from 80 mM to 500 mM. Similar amounts of protein, believed to be rHmbR, were recovered in the wash fractions but no proteins were obtained with higher concentrations of imidazole. rHmbR may not have bound effectively to the nickel affinity column due to the ineffective denaturation of rHmbR leading to the inaccessibility of the his-tag for binding to the resin.

Urea was replaced with 6 M GuHCl as the denaturant and binding to the column was done for 18 hours at 4°C. These modifications made no difference and the majority of the rHmbR protein was visible in flow-through and wash fractions when analysed with SDS-PAGE (Figure 4.16).
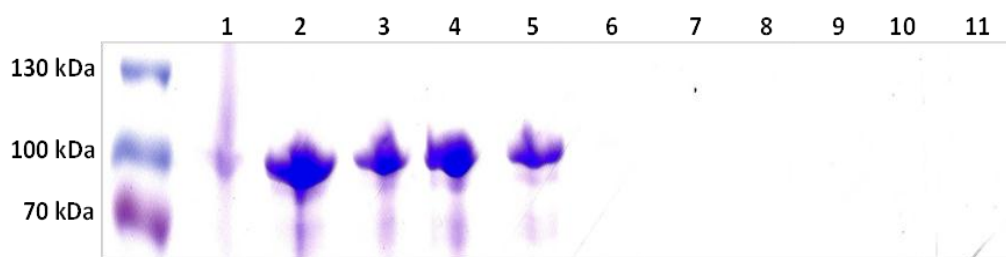
Figure 4.16: Attempted purification of rHmbR using a nickel affinity column. rHmbR inclusion bodies were solubilised in a buffer containing 6 M GuHCl, 500 mM NaCl and 50 mM Tris.Cl 8.0. Solubilised inclusion bodies were bound to a pre-equilibrated nickel affinity column for 18 hours at 4°C. The flow-through fraction was analysed in lane 1. Unbound proteins were removed by washing with 20 mM (lane 2), 50 mM (lane 3) and 80 mM imidazole (lane 4). The column was subsequently eluted with 120 mM (lane 5), 150 mM (lane 6), 200 mM (lane 7), 250 mM (lane 8) and 500 mM imidazole (lane 9).

As stated previously, purification of rHmbR and rHpuB was done concurrently. Hence, similar problems listed in section 4.2 during the purification of rHpuB were encountered. Following the unsuccessful attempt at purifying rHmbR with a nickel affinity column, ion exchange chromatography was utilised. Solubilisation and re-folding buffers containing 50 mM $Na_2B_4O_7$ pH 9.5 were used to solubilise and re-fold rHmbR. Large amounts of precipitates, believed to be rHmbR and the sodium borate salt were obtained at each step and were removed by centrifugation. The supernatant obtained after refolding of the protein was dialysed against 50 mM $Na_2B_4O_7$ pH 9.5, 0.1% v/v LDAO overnight at 4°C. Insoluble material after dialysis was also removed before the supernatant was applied to a pre-equilibrated DEAE-Sephacel column. The column was washed with 150 mM NaCl before elution with 1 M NaCl. As expected, a significant proportion of the starting amount of rHmbR was lost at different stages of the process and this was reflected in the amount of protein observed in the flow-through and wash fractions. No purified rHmbR was discernible in the 1 M eluate (Figure 4.17).
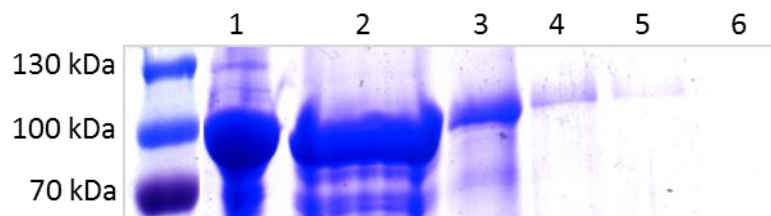
Figure 4.17: Purification of rHmbR with DEAE-Sephacel. Inclusion bodies (lane 1) were solubilized in 6 M GuHCl (lane 2) and refolded in the presence of hemin. Precipitates after refolding were removed by centrifugation before dialysis against 50 mM sodium borate pH 9.5, 0.1% v/v LDAO. A further centrifugation step was introduced to remove precipitates after dialysis before the soluble fraction (lane 3) was loaded onto a pre-equilibrated DEAE-Sephacel column. Small quantities of purified protein were recovered in the flow-through and 150 mM NaCl wash fractions (lanes 4 and 5 respectively) but no discernible protein was found in the 1 M NaCl eluate (lane 6).

A more stable buffer at temperatures below 25^{0}C was substituted for sodium borate. The use of Tris-based solubilisation and re-folding buffers eliminated the level of salt precipitation. The refolding buffer was also made with hemin prepared in DMSO and solubilised protein was added in a drop-wise fashion to this buffer on a magnetic stirrer. Precipitates, believed to be mainly denatured rHmbR, were removed prior to the commencement of each step in the purification process. The supernatant obtained after dialysis was subsequently loaded onto a column equilibrated with 6x resin volume of equilibration buffer (20 mM Tris.Cl 9.5, 0.1% v/v LDAO). After collecting the flow-through, the column was washed with 150 mM NaCl before eluting rHmbR with 300 mM, 450 mM, 600 mM and 1 M NaCl. No proteins were obtained in flow-through or wash fractions but 300 mM and 450 mM NaCl fractions contained rHmbR and an ~70 kDa protein, believed to be an *E. coli* protein. A minute amount of rHmbR was discerned in the 600 mM eluate while no proteins were recovered with 1 M NaCl (Figure 4.18).
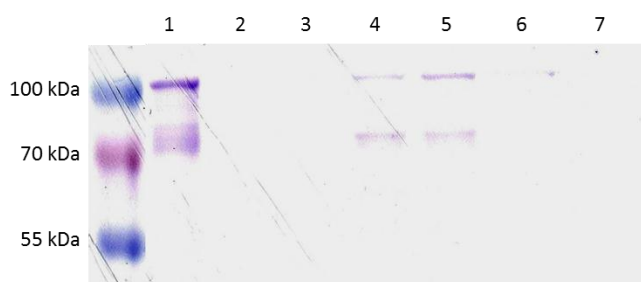
Figure 4.18: Substitution of sodium borate with Tris.Cl 9.5. Solubilisation, refolding and dialysis buffers were prepared with Tris.Cl 9.5 instead of sodium borate 9.5 to counter the problem of salt precipitation in the sodium borate buffer. Equilibration of the DEAE-Sephacel column (volume increased from 1 ml to 5 ml) was also done with 30 ml of buffer instead of 5 ml previously used. Soluble protein after dialysis was loaded onto the column (lane 1); the column was washed with 150 mM NaCl to remove unbound proteins (lane 3). rHmbR was eluted with 300 mM (lane 4), 450 mM (lane 5), 600 mM (lane 6) and 1 M NaCl (lane 7). Unbound proteins in the flow-through were analysed in lane 2.

To increase the efficiency of re-folding, a new stock of hemin was obtained, prepared in DMSO at a stock concentration of 1 mg/ml and used at a final concentration of 1 μg/ml (1.5 μM). Precipitation of large amounts of protein during the refolding process was significantly reduced using this new stock of hemin. Precipitates were, however, still obtained after dialysis. Purification with a DEAE Sephacel column produced elution fractions that contained small amounts of rHmbR (Figure 4.19a). Concentration of these fractions using centrifugal filters confirmed the presence of rHmbR in these fractions. Proteins of smaller sizes were also obtained and these were believed to be either degradation products of rHmbR or contaminant *E. coli* proteins (Figure 4.19b).
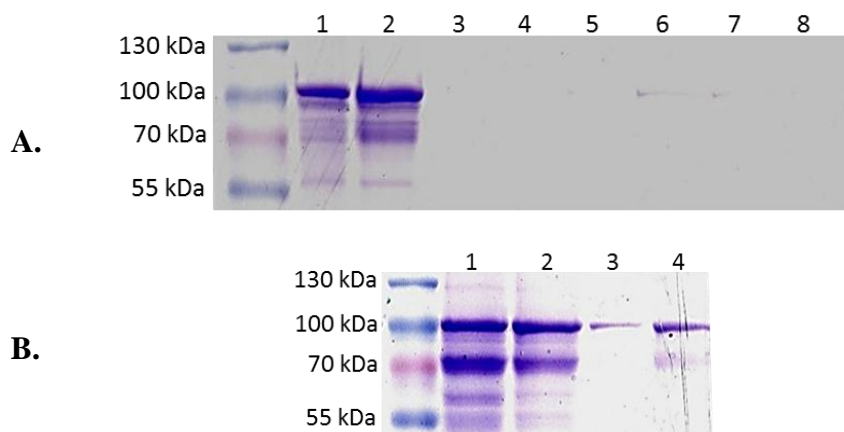
Figure 4.19: Purification of rHmbR. **(A)** After dialysis, insoluble protein was removed by centrifugation at 13000 rpm (lane 1). Soluble protein (lane 2) was loaded onto pre-equilibrated DEAE Sephacel. Unbound proteins in the flow-through were analysed in lane 3 while the 150 mM wash fraction was analysed in lane 4. 300 mM, 450 mM, 600 mM and 1 M NaCl fractions were analysed in lanes 5 – 8 respectively. **(B)** 10-fold concentrates of 300 mM (lane 1), 450 mM (lane 2), 600 mM (lane 3) and 1 M (lane 4) NaCl fractions were analysed with SDS-PAGE.

A total of 600 μg of recombinant protein was required for immunisation of mice. This amount was obtained for both rHpuA variants but not rHpuB and rHmbR. Protein quantities of ~4 mg/ml were consistently obtained for rHmbR and rHpuB after dialysis but the amount of purified protein obtained as at the time of immunisations was low (<0.2 mg per protein). It appeared that the majority of the protein was lost during the purification with the ion exchange column. Assessment of the dialysed protein on a SDS-PAGE gel showed that the recombinant protein constituted more than 90% of the preparation suggesting that purification with the ion exchange column had a negligible effect on purity. Therefore, the purification step was omitted and refolded proteins dialysed into 20 mM Tris.Cl pH 9.5, 0.1% v/v LDAO were used to immunize mice (Figure 4.20).
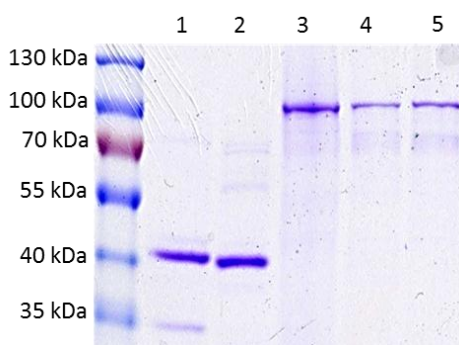
Figure 4.20: Antigens used to immunize mice. 1μg of antigens were analysed on an 8% SDS-PAGE gel in the following order: rN88-HpuA (lane 1), r8047-HpuA (lane 2), r8047-HpuB (lane 3), r8047-HmbR (lane 4) and rMC58-HmbR (lane 5).

4.4   Reactivity of mice antisera to homologous and unrelated antigens in ELISAs

Five groups of 10 mice (6 – 7 weeks old female BALB/c) were immunized subcutaneously with 20 µg of each purified antigen: groups 1 and 2 were immunized with r8047-HmbR and rMC58-HmbR respectively. Groups 3, 4 and 5 were immunized with r8047-HpuA, rN88-HpuA and r8047-HpuB respectively. A control group (group 6) were immunised with the adjuvant, Monophosphoryl lipid A (MPL). Terminal bleeds were obtained from each mouse at week 7. Immunization and bleeding of mice were performed by collaborators at the National Institute of Biological Standards and Control (NIBSC).

To measure the presence and specificity of antibodies, sera diluted to a 1:2000 concentration were used to probe 5 µg of the protein preparations used previously to immunise mice (antigens), by ELISA. The control group sera were not included in the analysis as each immune serum sample was used to probe homologous and unrelated proteins. For example, anti-rHpuA sera were used to probe rHpuA, rHpuB and rHmbR. In this example, the anti-rHpuA sera would serve as the negative control, like the group 6 sera, in the rHpuB and rHmbR assays.

Anti-rHpuA antibodies reacted with both antigenic variants of rHpuA but not with the rHpuB or rHmbR antigens in ELISAs. Minor differences were observed in the reactivity of anti-rHpuA antisera to the homologous rHpuA and variant rHpuA preparations (Figure 4.21). In contrast, a degree of cross-reactivity was observed between the anti-rHmbR antisera and the rHpuB antigen. Reactivity to the homologous protein was 3-fold higher than reactivity to the rHpuB antigen. Conversely, signals detected when the anti-rHpuB antisera were used to probe the rHmbR antigens were similar to signals obtained with homologous rHpuB antigen. No signals were detected when the anti-rHmbR antisera were used to probe both rHpuA variants (Figures 4.22 and 4.23). These results suggest that antibodies recognising epitopes in both antigen preparations are present in both anti-rHpuB and anti-rHmbR antisera. It is more probable that these epitopes originated from the smaller *E. coli* proteins present in the rHpuB and rHmbR antigen preparations.

Figure 4.21: Reactivity and cross-reactivity of anti-rHpuA against antigenic variants and unrelated proteins. Sera diluted to a 1:2000 concentration were used to probe 5 µg of each antigen preparation. Fluorescence readings of antisera from all 10 mice in each group, adjusted by subtraction of a background reading (no primary antibody value), were averaged and plotted above. Error bars represent the standard error of the mean of FI for all 10 mice antisera in a group. FIs for unrelated proteins that were >30% of FIs for homologous antigen were taken as considerably cross-reactive.
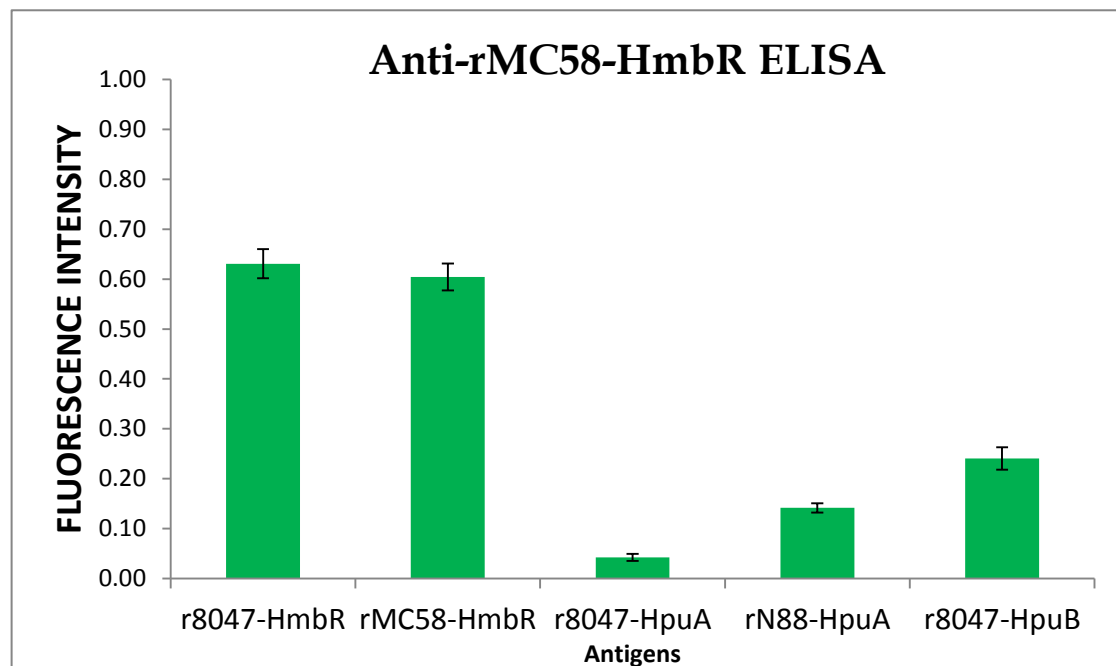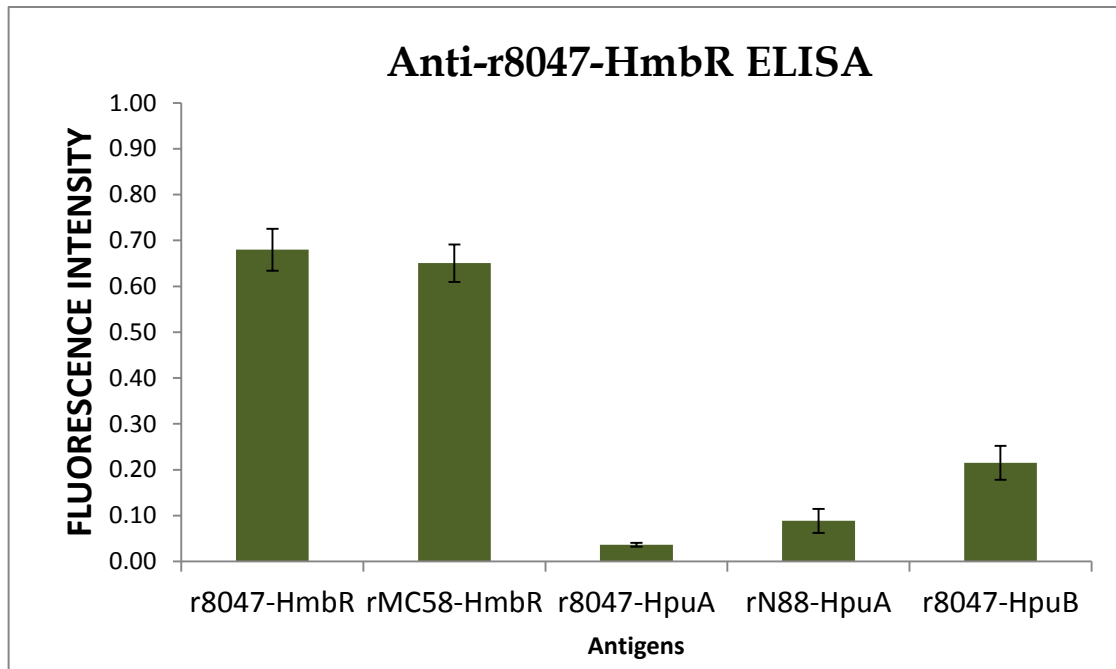
Figure 4.22: Reactivity and cross-reactivity of anti-rHmbR against antigenic variants and unrelated proteins. Sera diluted to a 1:2000 concentration were used to probe 5 µg of each antigen preparation. Fluorescence readings of antisera from all 10 mice in each group, adjusted by subtraction of a background reading (no primary antibody value), were averaged and plotted above. Error bars represent the standard error of the mean of FI for all 10 mice antisera in a group. FIs for unrelated proteins that were >30% of FIs for homologous antigen were taken as considerably cross-reactive.
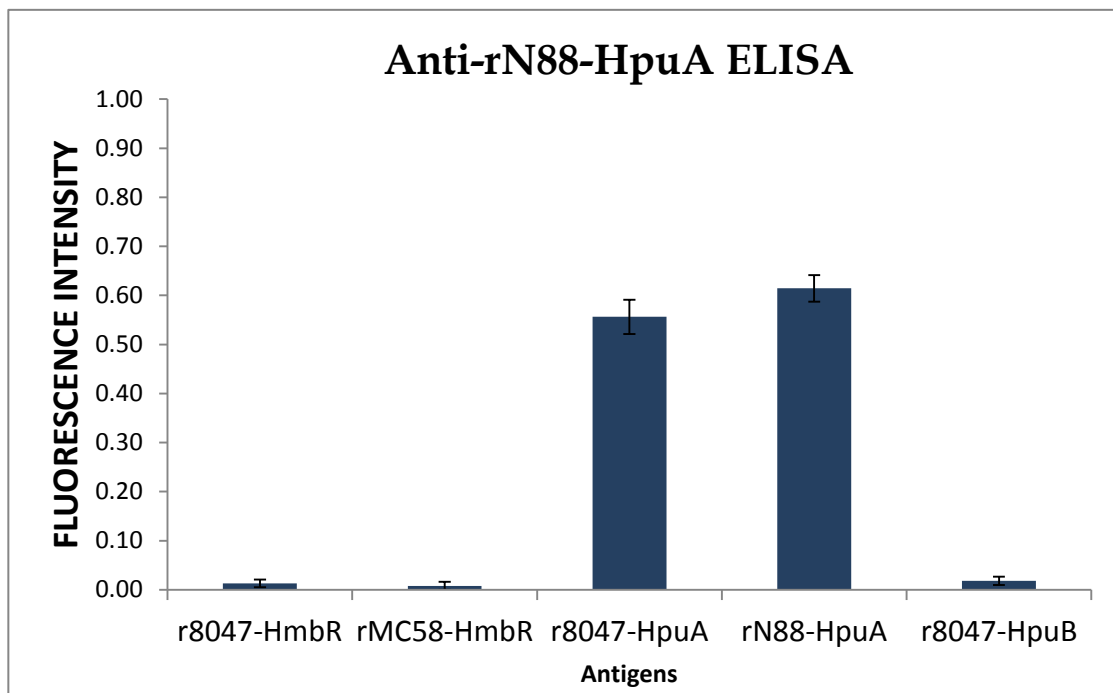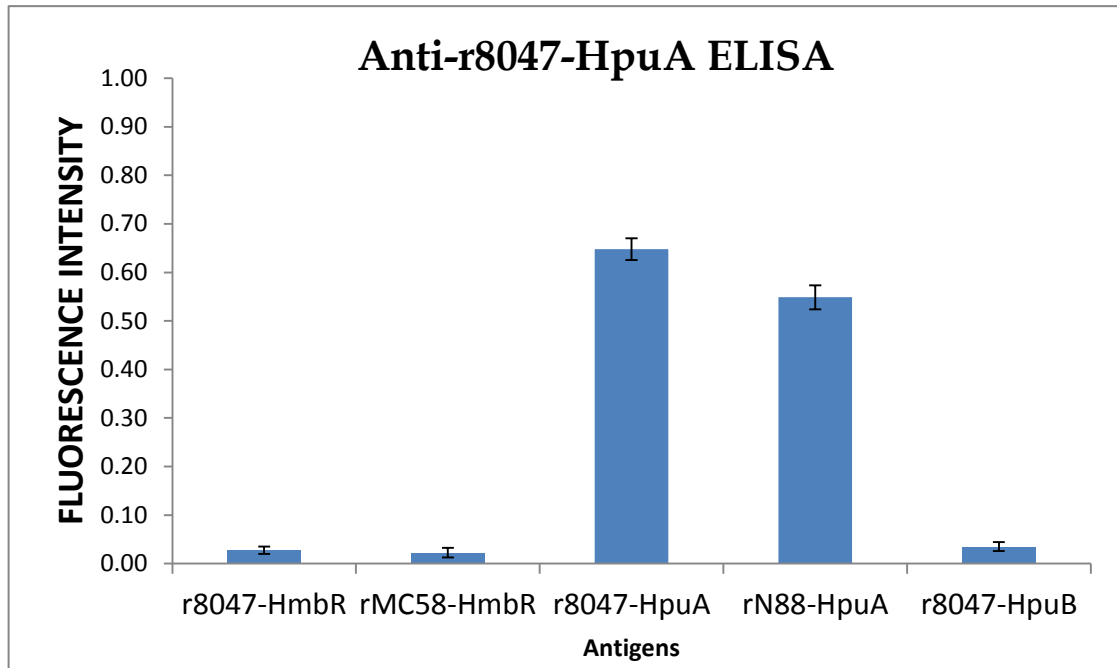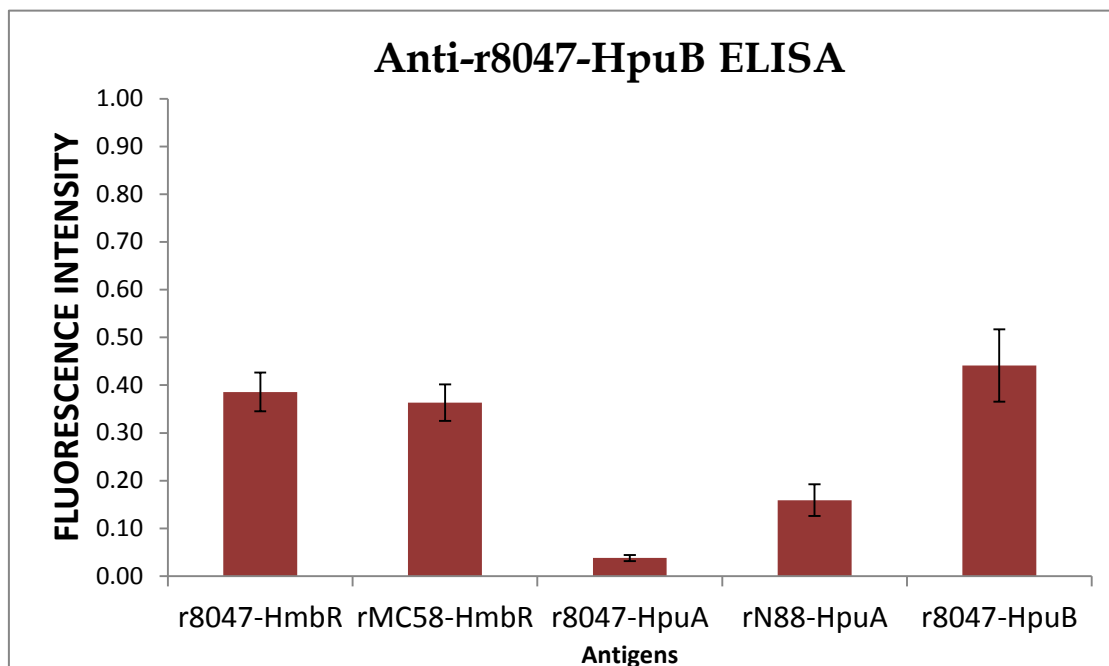
Figure 4.23: Reactivity and cross-reactivity of anti-r8047-HpuB against rHpuB and unrelated proteins. Sera diluted to a 1:2000 concentration were used to probe 5 µg of each antigen preparation. Fluorescence readings of antisera from all 10 mice in each group, adjusted by subtraction of a background reading (no primary antibody value), were averaged and plotted above. Error bars represent the standard error of the mean of FI for all 10 mice antisera in a group. FIs for unrelated proteins that were >30% of FIs for homologous antigen were taken as considerably cross-reactive.

4.5   Reactivity of mice antisera to *E. coli* BL21 lysates in Western blots

To investigate the cross-reactivity observed with anti-rHpuB and anti-rHmbR antibodies to the rHmbR and rHpuB antigen preparations respectively, western blots using 1:500 dilutions of the antisera were performed. Reactivity of anti-rHpuA antibodies against antigenic variants of rHpuA was also measured. Antigens used in these blots were lysates of uninduced and induced *E. coli* BL21 cells harbouring the expression plasmids and lysates of untransformed *E. coli* BL21 cells.

Both anti-r8047-HpuA and anti-rN88-HpuA recognised rHpuA expressed from homologous sources. Variation was observed in the reactivity of these antibodies against antigenic variants of rHpuA as stronger signals were obtained on blots where antigens were probed with homologous antisera. This variation was more apparent with the anti-rN88-HpuA antisera where a 3.4- to 5-fold difference in signal strength was detected when low (uninduced cell lysates) and high (induced cell lysates) amounts, respectively, of both rN88-HpuA and r8047-HpuA were probed. Reactivity of anti-r8047-HpuA with r8047-HpuA was only 1.2-fold higher than its reactivity with

rN88-HpuA when lysates from induced BL21 cells were used as template. No signals were obtained with lower amounts of rN88-HpuA compared to a faint signal obtained with a similar amount of r8047-HpuA when anti-r8047-HpuA antisera were used as probe. Neither antisera reacted with *E. coli* proteins present in the untransformed BL21 lysates (Figure 4.24).
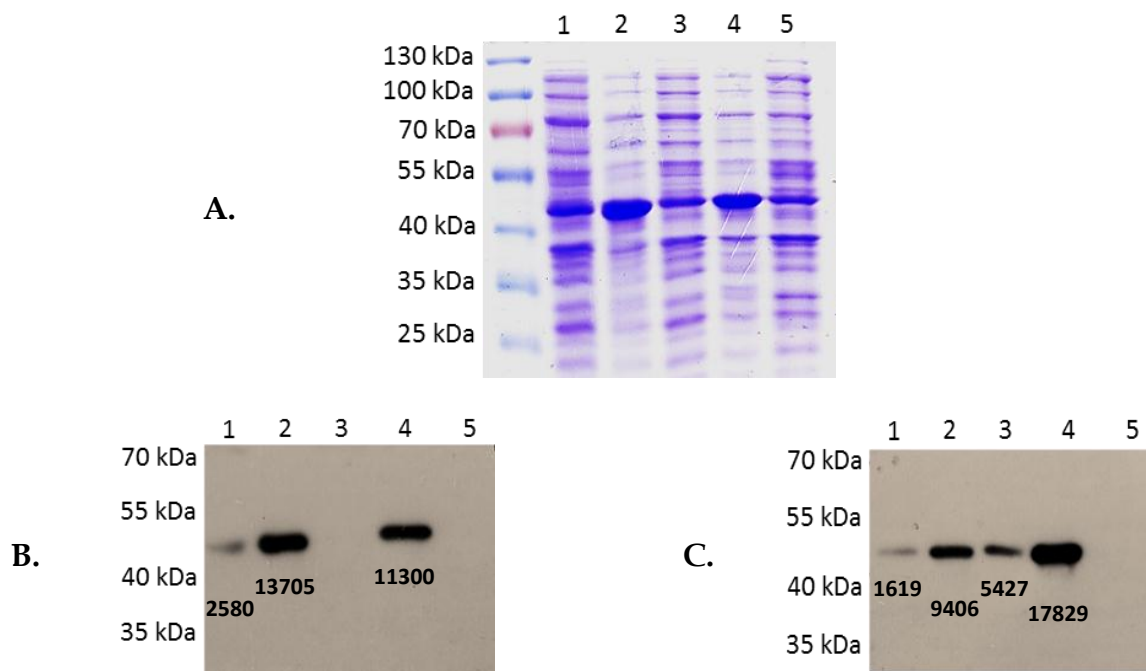
Figure 4.24: Reactivity of anti-rHpuA antisera against rHpuA in *E. coli* lysates. Induction of rHpuA expression was achieved with the addition of 1 mM IPTG to *E. coli* BL21 cells grown to mid-log (OD_{600} = 0.5). For the preparation of cell lysates, each culture was adjusted to an OD_{600} of 0.5. 1 ml of each adjusted culture was subsequently pelleted before cells were re-suspended in 1x Laemmli buffer and boiled for 5 minutes at 98^0C. Proteins were separated on an 8% SDS-PAGE gel followed by coomassie staining **(A)**. Proteins were transferred to PVDF membranes and probed with a 1:500 dilution of either anti-r8047-HpuA **(B)** or anti-rN88-HpuA **(C)**. Samples were loaded in the following order: uninduced r8047-HpuA cells (lane 1); induced r8047-HpuA cells (lane 2); uninduced rN88-HpuA cells (lane 3); induced rN88-HpuA cells (lane 4); and untransformed BL21 cells (lane 5).). A 1:2000 dilution of an anti-Mouse IgG HRP-conjugate was used to generate signals. Fold differences in signal strength (values below signals indicate signal strength) were measured using Image J software.

Anti-rHpuB antisera recognised rHpuB in western blots but not rHmbR (lanes 1 and 2 of Figure 4.25b) while both variants of rHmbR were recognised by anti-rHmbR antisera only (lanes 3 – 6 of Figures 4.25c and 4.25d). A low level of reactivity to unrelated *E. coli* proteins was also observed in these blots (lane 7) where anti-rHpuB and anti-rHmbR antisera were used to probe lysates of untransformed *E. coli* BL21 cells (Figure 4.25). This observed background reactivity offered a plausible explanation for the cross-reactivity previously seen in the ELISAs and negates the possibility of antisera recognising shared epitopes between HpuB and HmbR in this instance. Reactivity of

antisera with many smaller proteins in the induced cell lysates of both rHpuB and rHmbR was also observed. These proteins were absent in uninduced cell lysates, suggesting that these proteins were products of the degradation of the over-expressed recombinant proteins.

With regards to reactivity of anti-rHmbR antisera to antigenic variants of rHmbR, no discernible differences were observed in reactivity when anti-r8047-HmbR antisera were used to probe uninduced cell lysates containing variants of rHmbR (Figure 4.25c). Conversely, anti-rMC58-HmbR antisera was 2.3-fold more reactive with uninduced cell lysates containing r8047-HmbR than those containing rMC58-HmbR. However, this antiserum recognised more proteins believed to be degenerated rMC58-HmbR than similar proteins in the r8047-HmbR lysates when recombinant proteins were over-expressed (induced) (Figure 4.25d). A plausible explanation of this variation could be unequal loading of the antigens used in this western blot.

A.

B.

C.

D.
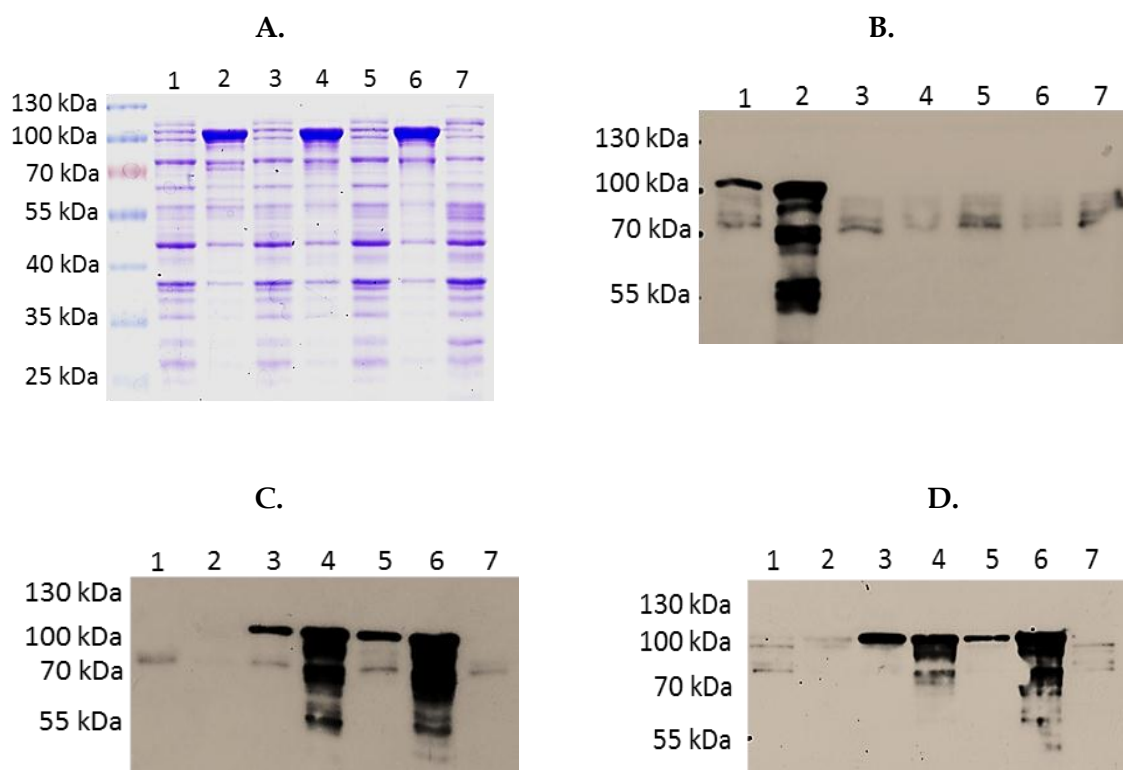
Figure 4.25: Reactivity of anti-rHpuB and anti-rHmbR antisera against rHpuB, rHmbR and unrelated *E. coli* proteins. Induction of protein expression and preparation of lysates was performed as previously outlined in Figure 4.24. Proteins were separated on an 8% SDS-PAGE gel followed by coomassie staining **(A)**. Proteins were transferred to PVDF membranes and probed with a 1:500 dilution of either anti-r8047-HpuB **(B)**, anti-r8047-HmbR **(C)** or anti-rMC58-HmbR **(D).** Samples were loaded in the following order: uninduced r8047-HpuB cells (lane 1); induced r8047-HpuB cells (lane 2); uninduced r8047-HmbR cells (lane 3); induced r8047-HmbR cells (lane 4); uninduced rMC58-HmbR cells (lane 5); induced rMC58-HmbR cells (lane 6); and untransformed BL21 cells (lane 7). A 1:2000 dilution of an anti-Mouse IgG HRP-conjugate was used to generate signals.

4.6   Preparation of mouse monoclonal anti-HmbR antibodies

Non-specific reactivity of polyclonal α-rHmbR antisera is undesirable for downstream applications of these antisera. The isolation of monoclonal antibodies (mAbs) that would bind specifically to certain HmbR epitopes was, therefore, necessary. These mAbs were produced using a service provided by Dr. Uta Praekelt at the Department of Genetics, University of Leicester. Epitopes corresponding to predicted surface-exposed loops 2 ($HmbR_{193-229}$), 8 ($HmbR_{550-577}$) and 11 ($HmbR_{739-750}$) of HmbR were selected as targets. Loop 2 was selected as it was predicted to correspond to one of three variable regions of HmbR (Evans *et* al., 2010). Variation at this loop could be inferred to be a consequence of the immunogenicity of this loop. Loops 8 and 11 were

chosen because of their relatively high conservation between HmbR variants. Antibodies to these loops will, therefore, recognise the majority of HmbR variants. All three HmbR loops were cloned and expressed as part of a recombinant carrier protein, EspA, from *E. coli*. Pure preparations of these recombinant protein fusions were used to immunise mice and mAb-producing cells were generated. Three clones were successfully obtained: 1 clone recognising the loop 2 epitope (designated anti-HmbR-L2) and 2 clones recognising loop 11 (anti-HmbR-L11). No anti-HmbR-L8 clones were obtained. These mAbs were shown to recognise the purified homologous recombinant EspA protein and rHmbR expressed in *E. coli* lysates in Western blots (Figure 4.26).
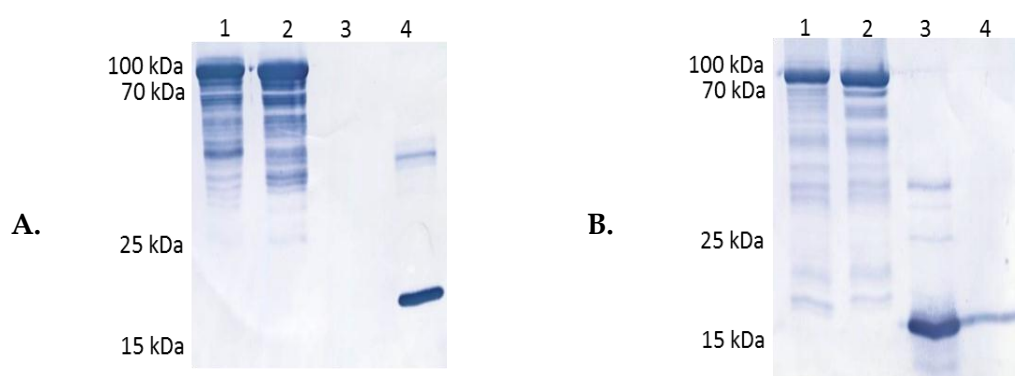
Figure 4.26: Screening of anti-HmbR mAbs. Induction of rHmbR expression in *E. coli* cells was performed as previously outlined. Supernatants from cultures of anti-HmbR-L2 (A) and anti-HmbR-L11 (B) mAb-producing cells were used to probe the following: rMC58-HmbR in *E. coli* (lane 1), r8047-HmbR in *E. coli* (lane 2), purified rEspA containing HmbR loop 11 (lane 3) and loop 2 (lane 4). This western blot was performed by Dr. Uta Praekelt. Expressed rHmbR in *E. coli* was provided by Fadil Bidmos.

* Note: There was spillage of the sample analysed in lane 3 into lane 4.

4.7 Reactivity of mice antisera and monoclonal α-HmbR antibodies to meningococcal lysates in Western blots

Expression of *hpuAB* and *hmbR* is transcriptionally regulated by Fur (Stojiljkovic *et al*., 1996; Lewis *et al*., 1997). Growth of meningococci in iron-replete conditions, found in meningococcal culture media, would lead to expression levels that may be difficult to detect in Western blots. To measure reactivity of immune mice antisera against these Hb receptors, induction of expression was performed by growing meningococci in iron-limited conditions. This was achieved by following methods outlined in section 2.1. Each culture was normalised to an OD$_{600}$ of ~0.5 before lysates were prepared. The following antisera - anti-r8047-HpuA, anti-rN88-HpuA and anti-r8047-HpuB - were

used to probe lysates of 8047 wild-type, IT-8047Δ*hpu*, N88 (*hpu*-OFF) and N272 (*hpu*-ON) strains. Both strains N88 and N272 were isolated from the same healthy volunteer in a carriage study (Bidmos *et al.*, 2011) at 2 different timepoints and were shown to be phase variants of the same strain (Y:P1.21,16:F3-7:CC-174) (Alamro and Bayliss, personal communication). Since strain 8047 is *hmbR*-OFF, lysates from disease isolates, H44/76 (*hmbR*-ON) and MC58 were used as antigens to measure cross-reactivity of anti-r8047-HmbR and anti-rMC58-HmbR with multiple HmbR antigens. Lysates from the 8047 wild-type strain were also included to confirm the *hmbR*-OFF genotype.

4.7.1   Reactivity to HpuA and HpuB

1:500 dilutions of anti-r8047-HpuA and anti-r8047-HpuB antisera consistently detected increases in HpuA and HpuB expression respectively (up to 4-fold in multiple experiments) when cells of strain 8047 and N272 were grown in iron-limited conditions. Reactivity of these antisera was shown to be specific as no signals were observed when the induced Δ*hpu* mutant or N88 strain (*hpu*-OFF) was used as antigen.

The presence of antibodies that bind invariable regions of HpuA and HpuB in these antisera was evident as both anti-r8047-HpuA and anti-r8047-HpuB antisera showed reactivity against the corresponding antigenic variants in carriage strain N272 (Figure 4.27). Similarly, anti-rN88-HpuA antisera recognized its corresponding antigen in the carriage strain N272 and in strain 8047.

Antigenic variants of HpuA were better recognised by homologous antisera. Anti-r8047-HpuA was 8 times more reactive with the 8047-HpuA protein than anti-rN88-HpuA. Conversely, anti-rN88-HpuA produced a higher signal intensity (~2-fold) with N88-HpuA than anti-r8047-HpuA. Interestingly, signal strengths obtained with both anti-rHpuA antisera were 2.6-fold (anti-r8047-HpuA) to 40-fold (anti-rN88-HpuA) higher for the N272 template than the 8047 template. Reactivity of anti-r8047-HpuB to N272-HpuB was 3-fold stronger than to 8047-HpuB. These results will be discussed in detail later in the chapter.
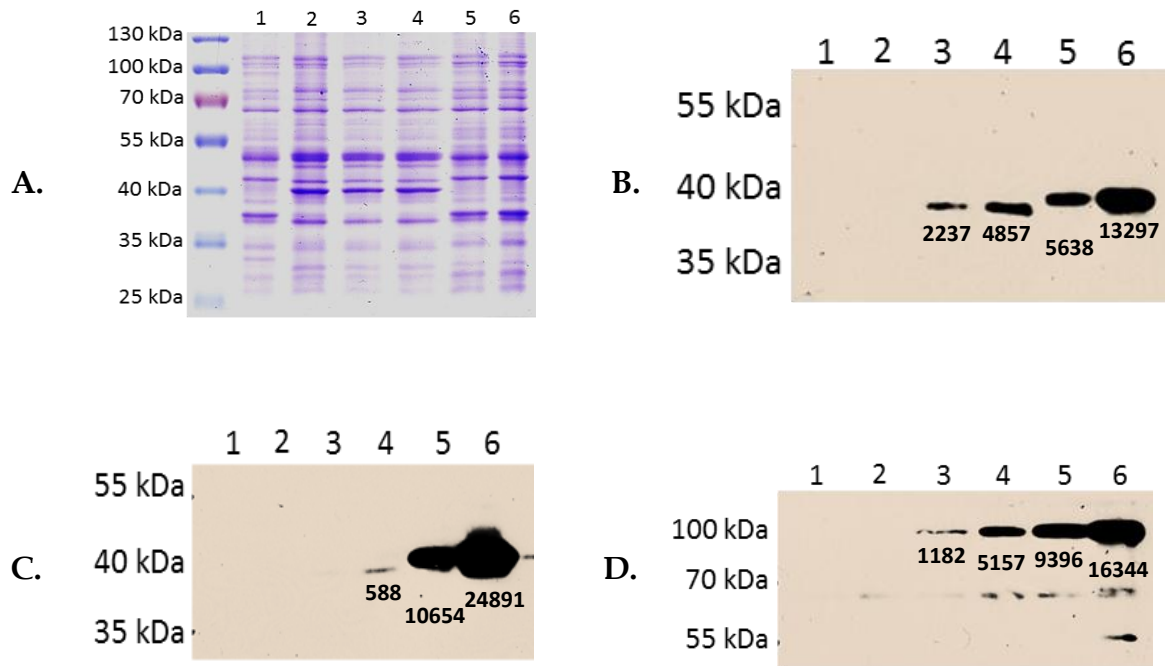
Figure 4.27: Reactivity of anti-rHpuA and anti-rHpuB antisera with HpuAB in meningococcal lysates. Meningococcal cells of strain 8047, its isogenic Δ*hpu* mutant and carriage strains N88 (*hpu*-OFF) and N272 (*hpu*-ON) were grown to mid-log (OD$_{600}$ = ~0.5) before 30 µM of desferal was added to produce iron-limited conditions. Cultures were also grown concurrently in iron-replete conditions. All cultures were incubated for two hours before heat-inactivation at 56^{0}C overnight. After normalizing number of cells used to prepare lysates **(A)**, 1:500 dilutions of anti-r8047-HpuA **(B)**, anti-rN88-HpuA **(C)** and anti-r8047-HpuB **(D)** were used to probe lysates of induced strain N88 (lane 1), IT-8047Δ*hpuAB* (lane 2), wild-type 8047 (uninduced – lane 3; induced – lane 4) and N272 strains (uninduced – lane 5; induced – lane 6). A 1:2000 dilution of an anti-Mouse IgG HRP-conjugate was used to generate signals. Fold differences in signal strength (values below signals indicate signal strength) were measured using Image J software.

4.7.2   Reactivity to HmbR

Anti-r8047-HmbR, anti-rMC58-HmbR and pooled mAbs (anti-HmbR-L2 and anti-HmbR-L11) detected a 3-fold increase in HmbR expression when cells of strains MC58 and H44/76 were grown in iron-limited conditions. No differences were found in the ability of the pooled mAbs to recognise both variants of HmbR, which indicated that HmbR was expressed to similar levels in MC58 and H44/76. In contrast, both polyclonal antisera selectively recognised HmbR variants. Anti-r8047-HmbR showed a 2.2-fold higher reactivity for MC58-HmbR than H44/76-HmbR. This indicates that anti-r8047-HmbR was more able to recognise HmbR epitopes in the MC58 strain than in the H44/76 background. The same reactivity pattern was observed with anti-rMC58-HmbR. The *hmbR*-OFF genotype in strain 8047 was confirmed by both monoclonal and polyclonal antibodies as no reactivity to a protein of the correct size in the lysates from this strain was observed (Figure 4.28).

Cross-reactivity to other meningococcal proteins present in the lysates was also observed with the polyclonal antisera. An iron-inducible protein of size 70 kDa was detected by anti-r8047-HmbR and anti-rMC58-HmbR as reactivity to this protein was 2-fold less in uninduced meningococcal cells. This protein was also found in the MC58Δ*hmbR* mutant, thus, negating the possibility of being a breakdown product of HmbR in cell lysates.
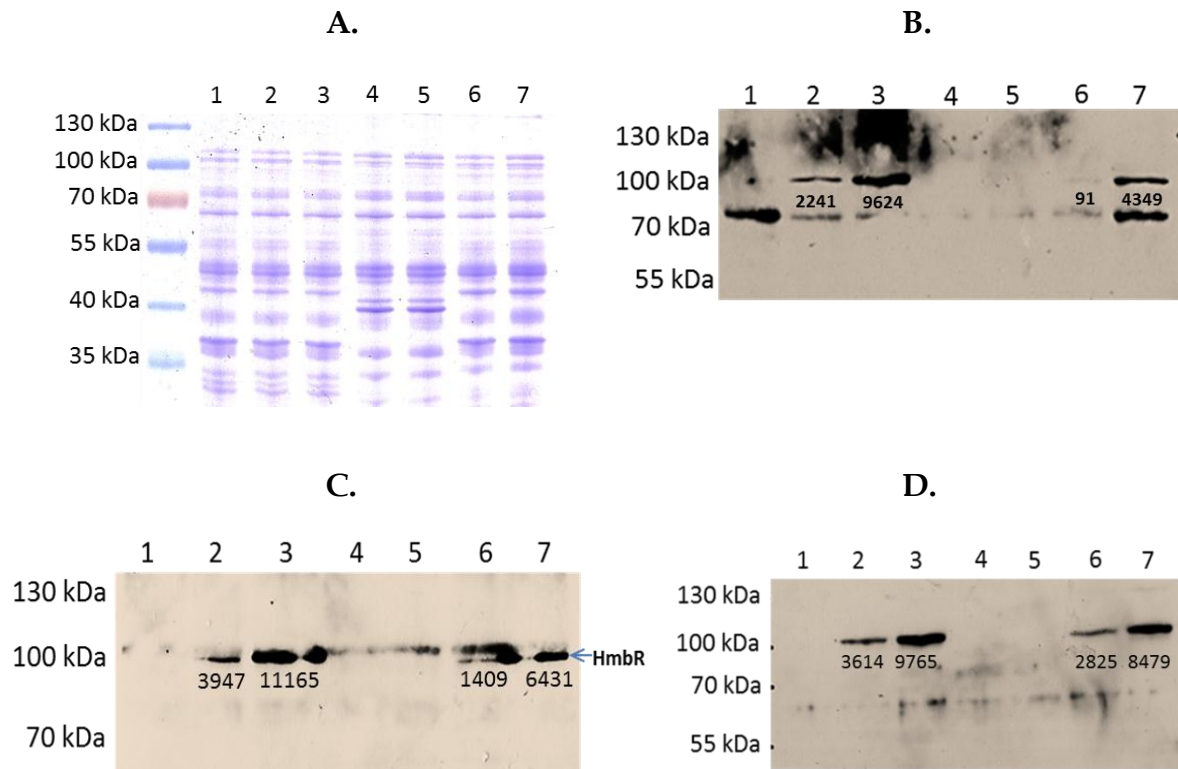
Figure 4.28: Reactivity of monoclonal and polyclonal antisera with HmbR in meningococcal lysates. Induction of *hmbR* expression was performed as previously outlined in Figure 4.27. After normalizing the amount of samples analysed **(A)**, 1:500 dilutions of anti-r8047-HmbR **(B)**, anti-rMC58-HmbR **(C)** and pooled mAbs **(D)** were used to probe lysates of induced strain MC58Δ*hmbR* (lane 1), wild-type MC58 (uninduced – lane 2; induced – lane 3), wild-type 8047 (uninduced – lane 4; induced – lane 5) and wild-type H44/76 (uninduced – lane 6; induced – lane 7). A 1:2000 dilution of an anti-Mouse IgG HRP-conjugate was used to generate signals. Fold differences in signal strength (values below signals indicate signal strength) were measured using Image J software.

4.8 Measurement of surface expression of HpuA, HpuB and HmbR by flow cytometry

Meningococcal whole cell preparations were probed with 1:20 dilutions of mAbs and polyclonal sera to assess the ability of these antibodies to recognise surfaced-exposed epitopes of HpuA, HpuB and HmbR. Induction of iron-regulated protein expression in the meningococcal culture was performed, as previously outlined. Flow cytometry (see section 2.5.5) was utilised to detect surface expression as whole cell ELISAs produced inconclusive results (data not shown).

4.8.1 Assessment of HpuA and HpuB surface expression

Meningococcal cells of strains IT-8047Δ*hpu*, 8047 wild-type, N88 and N272 were grown in the presence of 30 μM desferal to induce expression of HpuAB. Expression was subsequently measured using a 1:20 dilution anti-r8047-HpuA, anti-rN88-HpuA and anti-r8047-HpuB. Both anti-HpuA antibodies bound meningococcal cells of strain 8047 and N272 but not the IT-8047Δ*hpu* mutant or the *hpu*-OFF strain, N88. This showed that the antibodies were interacting specifically with HpuA on the meningococcal surface. No difference was observed in binding of anti-r8047-HpuA to N272 or 8047 cells converse to anti-rN88-HpuA, which was significantly less reactive with 8047-HpuA compared to N88-HpuA. This may be an indication of the presence of more VR-specific antibodies in the anti-rN88-HpuA antiserum than in the anti-r8047-HpuA antiserum. No discernible reactivity by anti-r8047-HpuB to surface-exposed HpuB regions in any of the four strains analysed was observed (Figure 4.29).

4.8.2 Assessment of HmbR surface expression

Meningococcal whole cells of strains MC58 and its Δ*hmbR* mutant, 8047 and H44/76 grown in iron-restricted conditions were successively probed with the following antibodies: anti-HmbR-L2, anti-HmbR-L11, anti-r8047-HmbR and anti-rMC58-HmbR. Neither mAb recognised surface epitopes of HmbR in any of the strains tested. Both polyclonal antisera bound to iron-induced MC58 cells but not to the MC58Δ*hmbR* mutant or H44/76 cells. This suggests that the cross-reactive protein detected in western blots was not surface-exposed and validates the specificity of the reactivity observed in the assay. A degree of reactivity to iron-starved 8047 cells was also observed with the homologous anti-r8047-HmbR antibody (Figure 4.30).
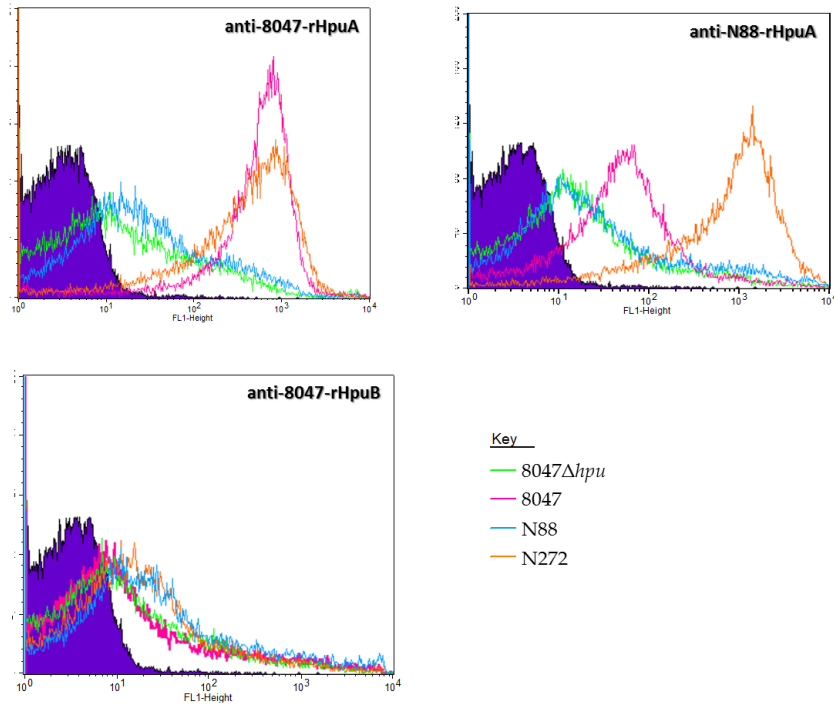
Figure 4.29: Evaluation of HpuAB surface expression by flow cytometry. Cultures of meningococcal strains IT-8047Δ*hpu*, 8047 wild-type, N88 and N272 were subjected to iron-limited conditions using methods previously outlined. Cells were subsequently assessed for HpuAB surface expression in a FACS assay using a 1:20 dilution of the following antibodies generated in this study: anti-r8047-HpuA, anti-rN88-HpuA and anti-r8047-HpuB. A 1:100 dilution of the secondary antibody, Alexa Fluor® anti-Mouse IgG, was used to generate reporter signals, which were measured by a Flow cytometer. 25000 events (corresponding to cells) were used for analysis in each assay.
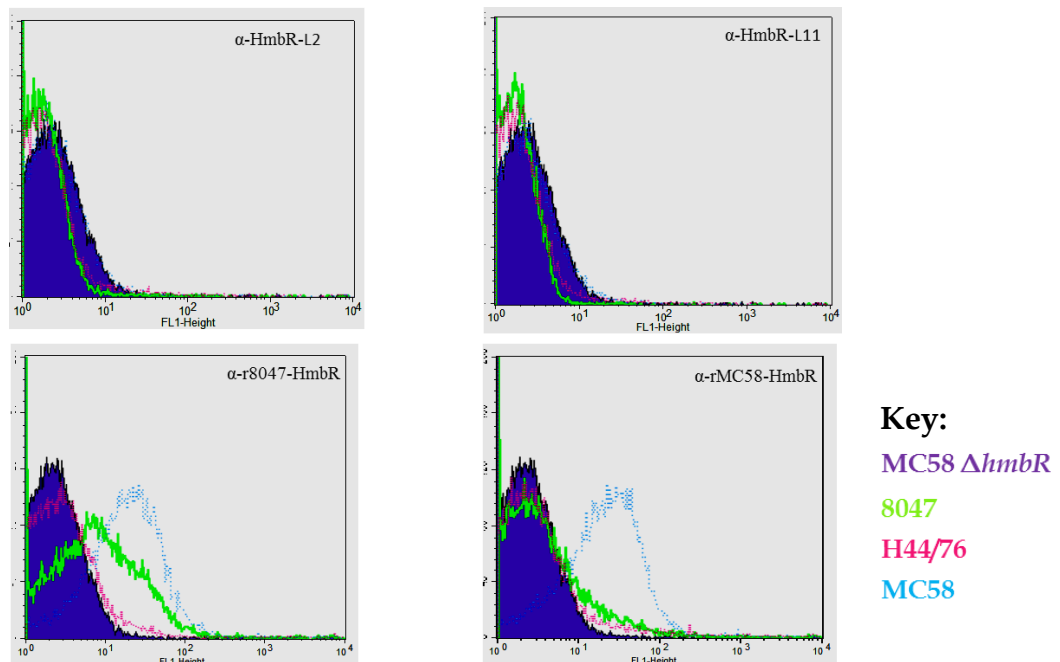
Figure 4.30: Evaluation of HmbR surface expression by flow cytometry. Cultures of meningococcal strains MC58Δ*hmbR*, MC58 wild-type, 8047 and H44/76 were subjected to iron-limited conditions using methods previously outlined. Cells were subsequently assessed for HmbR surface expression in a FACS assay using a 1:20 dilution of the following antibodies generated in this study: anti-HmbR-L2, anti-HmbR-L11, anti-r8047-HmbR and anti-rMC58-HmbR.

4.9   Discussion

The meningococcal Hb receptors are known to be part of a number of iron-regulated proteins that are mainly over-expressed during periods of iron starvation. Using antisera generated in mice with recombinant HpuA, HpuB and HmbR as antigens, increases in gene expression were detected for proteins measuring ~35kDa, ~85 kDa and ~89 kDa when meningococci were grown in iron-limited conditions. These proteins were identified as HpuA, HpuB and HmbR respectively as reactivity to these proteins were undetectable in deletion mutants and PV-OFF variants of *hpuAB* and *hmbR*. This data is consistent with findings from earlier studies that showed the iron-regulated and phase-variable expression of these Hb receptors (Lewis *et al.*, 1997; Lewis *et al.*, 1999; Richardson and Stojiljkovic, 1999; Echenique-Rivera *et al.*, 2011).

Preliminary data gathered in this study showed specific reactivity between anti-rHpuA and anti-rHmbR antisera and homologous antigens expressed on the meningococcal surface. This specific reactivity could be attributed to the interactions between VR-specific antibodies and the target surface-exposed loops. These results are consistent with data obtained from sequence typing and structural model predictions of HpuAB and HmbR that reveal the presence of hyper-variable surface-exposed loops in the Hb receptors of gonococcal and meningococcal strains (Perkings-Balding *et al.*, 2003; Evans *et al.*, 2010; Harrison *et al.*, 2013). These hyper-variable regions are believed to be under strong immune selection, like those found in other OMPs such as PorA and fHbp, leading to the structuring of meningococcal populations into non-overlapping antigenic repertoires (Gupta and Anderson, 1999). It will be interesting to explore the extent of this reactivity and the cross-reactivity, if any exists, between heterologous HpuA variants by screening more strains as defined in the phylogenetic studies of Evans *et al.* (2010) and Harrison *et al.* (2013).

Unlike the anti-rHpuA and anti-rHmbR antisera, interactions between anti-rHpuB antibodies present in polyclonal antisera did not interact with whole meningococcal cells despite recognising the HpuB target in whole cell lysates. This non-interaction between anti-rHpuB antibodies and surface-expressed HpuB could be due to conformational changes to HpuB that occur in the process of forming a receptor complex with HpuA (Rohde *et al.*, 2002). These structural changes would have obscured the accessibility of epitopes recognised by the anti-rHpuB antibodies. This

theory is further supported by the fact that rHmbR was purified with the same method as rHpuB in this study and antibodies recognising surface-expressed HmbR were generated. This suggests that both rHmbR and rHpuB regained their native conformations in the presence of haem; a conformation, which was similar to the cell surface-expressed molecule in the case of HmbR but different to the cell surface-expressed protein in the case of HpuB because of its interactions with HpuA.

Previous studies have reported similar findings with antigens whose structures were different from their native conformations. Moe *et al.* (1999) reported an inability of anti-NspA polyclonal antisera, generated from mis-folded rNspA, to recognise NspA expressed on the surface of live meningococcal cells. This anti-rNspA antiserum also lacked bactericidal activity owing to its inability to interact with the NspA target but recognised linear epitopes present in both denatured and native rNspA in western blots. Future studies involving the generation of antisera targeting surface-exposed loops of HpuB must, therefore, consider an affinity purification method in the presence of HpuA. This will ensure that only functional rHpuB, with a structure that more closely resembles its conformation as a component of the Hb receptor complex, is purified. This method has been successfully used to purify the similar TonB-dependent receptor, TbpA, which was subsequently used as an antigen that induced bactericidal antibodies in rabbits and as a vaccine that protected mice against meningococcal disease (West *et al.*, 2001).

Monoclonal antibodies targeting putative surface-exposed loops 2 and 11 of HmbR (from the MC58 background) detected HmbR of strains MC58 and H44/76 in western blots but failed to bind to whole meningococcal cells. A plausible reason for the lack of interactions between the anti-HmbR mAbs and the meningococcal surface is the surface exposure of the regions targeted by the mAbs. Ala'aldeen *et al.* (1994) reported a similar finding with a murine anti-FrpB mAb that recognised its FrpB target in western blots but did not react with native crude OMPs in dot blotting experiments. This anti-FrpB mAb also lacked bactericidal activity due to its inability to interact with its target region in FrpB.

In conclusion, anti-rHpuA and anti-rHmbR antisera generated in this study interacted with their homologous cell surface-expressed antigens and would be useful in determining the vaccine potential of these receptors.

CHAPTER 5

5.0   Assessment of the immunogenicity of meningococcal OMPs during carriage

The OMPs of *N. meningitidis* are targets for host immune responses and some OMPs are known to elicit bactericidal antibodies during carriage and disease. Thus, a potential consequence of the expression of *hpuAB* and *hmbR* is the presentation of these receptors as targets for bactericidal and/or opsonic antibodies. The meningococcus can, however, avoid killing by these antibodies by "switching" to an OFF-expression state of both receptors. A bias for PV-ON variants of one or both receptors, reported in Tauseef *et al*. (2011), could imply weak immunogenicity of these receptors during carriage and disease or the lack of bactericidal activity of antibodies produced against HpuAB and HmbR.

An assessment of the immunogenicity of HpuAB and HmbR could be performed by measuring antibody levels of these receptors in sera obtained from convalescent patients and healthy carriers using immunodetection assays. The Liquichip assay, allows for the screening of antibody levels to multiple variants of a selected OMP. Multiplexing in this assay is made possible by the utilization of microspheres (beads) labelled internally with different combinations of red and infra-red fluorescent dyes. Antigenic variants of an OMP coupled to specific beadsets are subsequently used as antigens to which antibodies, if present in test sera, bind. Fluorescently-labelled antibodies that bind to the antigen-antibody complex are used to generate reporter signals and the intensity of these signals is measured with a Liquichip workstation. Advantages of using this technique over conventional immunodetection methods, such as ELISA, include the very low amounts of antigen and antisera required and the sensitivity of the assay.

The Liquichip assay was designed and used extensively to measure the presence of antibodies to variants of the immunodominant meningococcal OMP, PorA, by collaborators at NIBSC, Potters Bar, UK. Purified recombinant versions of seven variants of PorA and a PorA VR-negative allele (termed loopless) were coupled to beads and the specificity of the assay was validated using mouse monoclonal antibodies that targeted specific VRs. Cross-reactivity between the mAbs and unrelated

VRs was non-existent and multiplexing had no effects on antibody binding (Poster presented at the European Monitoring Group for Meningococci meeting, June 2009 by Hema Patel).

This study aimed to design and validate a similar assay to evaluate the levels of antibodies to HpuAB and HmbR in sera obtained from healthy volunteers during the 24-week 2008/2009 carriage study (see section 2.8 and Bidmos *et al.*, 2011). Isolation of meningococcal strains from some volunteers would allow for associations to be made between the ON/OFF phase variation status of *hpuAB* and *hmbR* in the carried strain and the level of antibodies present in the serum sample at each timepoint. In this study, prior to assessing test sera for the presence of HpuAB and HmbR antibodies, an evaluation of the levels of PorA antibodies was performed.

PorA is the immunodominant meningococcal antigen and previous studies have reported the presence of anti-PorA antibodies in sera of healthy carriers. PorA exhibits phase and antigenic variation; a polyG tract in the core promoter controls transcription of *porA* (van der Ende et al., 1995) while two hypervariable regions corresponding to surface-exposed loops of PorA, are responsible for the production of antigenic variants of the protein (Russell et al., 2004). This antigenic variation of *porA* is beneficial to the meningococcus as antibodies raised against these hypervariable surface-exposed loops are not cross-reactive. The presence of PorA antibodies in test sera would show that immune responses were mounted against meningococcal OMPs during carriage. The absence of HpuAB or HmbR antibodies would subsequently not be misconstrued as false negative results.

Since PorA is transcriptionally-controlled by a phase-variable promoter (van der Ende *et al.*, 2000), an attempt was also made to correlate changes in repeat number at the promoter, leading to differing levels of surface-expressed PorA, to antibody amounts in longitudinal sera samples. If such a correlation could be made with PorA, a similar association between ON-OFF status and antibody amount could be pursued with HpuAB and HmbR.

5.1   Meningococcal carriage elicits PorA-specific antibodies

Serum samples obtained at 3 or 4 timepoints from volunteers in the carriage study were assessed for the presence of PorA antibodies using methods outlined in section

2.8. 110 serum samples from 32 volunteers were screened using this assay and were composed of samples from:

- volunteers naïve to meningococcal carriage during the study (persistent non-carriers);
- volunteers colonized by the same strain (as determined by molecular typing of longitudinal isolates) throughout the study (persistent carriers);
- volunteers that acquired carriage during the study (non-carrier at week 0) ; and
- volunteers subject to clearance or strain replacement during the study.

The selection of samples was dependent on the availability of the PorA variants that had been coupled to beads at NIBSC. These variants were:

i.   P1.7,16 (later replaced with P1.21,16);
ii. P1.7-2,4;
iii. P1.5-2,10;
iv. P1.5,2;
v.   P1.5-1,2-2;
vi. P1.19-1,15-11
vii. P1.19,15; and
viii. P1.-,- (loopless PorA protein).

Hence, samples from volunteers that had carried isolates of the following PorA types were selected (PorA types were determined by methods outlined in Bidmos *et al.*, 2011):

i.   P1.21,16;
ii. P1.21-7,16;
iii. P1.5-1,10-1;
iv. P1.5,2;
v.   P1.5-1,2-2;
vi. P1.19-1,15-11; and
vii. P1.19,15.

Samples from volunteers where phase variation of *porA* had been confirmed in the corresponding isolates were also chosen for analysis to facilitate comparisons between *porA* PV and immune response (Table 5.1). The immune response in carriers subject to

antigenic variation of PorA during carriage as well as strain replacement (Bidmos *et al.,* 2011) was also investigated.

To determine the parallelism of data obtained from different assays (20 test sera were analysed per plate, therefore multiple plates were involved), data parameters – correlation coefficient (r^2), lower and upper limits of the calibration curve, the midpoint and the slope – were obtained from the workstation software and recorded for each plate. Comparisons between the parameters obtained for homologous beadsets in multiple plates were made and plates with outlier values were excluded from further analyses. The upper and lower limits of quantitation were also determined from these assays and values of test sera dilutions outside these limits (outliers) were excluded (these analyses were performed by Hannah Chan, NIBSC). Antibody amounts (in AU) or the mean fluorescence intensity (MFI) were derived by calculating the mean of values obtained for each test serum dilution, excluding outliers.

5.1.1 Specificity of the Liquichip assay using sera from carriers

Results from assays performed with carrier sera provided a basis for the classification of sera into 3 broad categories: sera with specific reactivity to the homologous or related PorA allele only (group 1); sera with reactivity to the homologous PorA variant and/or other unrelated PorA proteins (group 2); and sera with no reactivity to any of the PorA variants including the homologous PorA protein (group 3) (see Figure 5.1 for representative examples of each group). The reactivity of group 2 sera to unrelated heterologous PorA VRs was found to be variable ranging from antibody amounts that were lower than those for homologous PorA VRs to amounts calculated to be at higher levels. A very low level of reactivity to the loopless protein (<100 AU) was observed suggesting that antibodies were predominantly induced against the VRs. Since cross-reactivity between unrelated VRs was not evident in previous assays performed by Hema Patel at NIBSC, reactivity to multiple variants was not anticipated. In addition, only antibodies to the VR of the carried strain were expected in sera. Thus, cross-reactivity between unrelated VRs could be an indication of non-specific binding of antibodies to the PorA VRs. Other factors, which could be responsible for the cross-reactivity are discussed later in the chapter.

Table 5.1: PorA type and phase variation status in carriers utilised for analysis of PorA antibodies.

Volunteer	PorA type of isolate	Repeat tract length (No. of guanine residues)[1]			
		Week 0	Week 4	Week 12	Week 24
V43	P1.21,16	12	12	12	Cleared
V50	P1.5,2	ND[2]	ND	ND	Replaced
V51	P1.21,16	12	12	11	11
V52	P1.21,16	12	11	12	Cleared
V54	P1.21,16	14	17	17	Replaced
V58	P1.21,16	11	12	Cleared	11
V64	P1.5-1,10-1	11	11	11	Replaced
V69	P1.5-1,10-1	ND	11	ND	11
V70	P1.21-7,16	ND	ND	ND	Replaced
V88	P1.21,16	11	11	11	11
V93	P1.5-1,10-1	ND	ND	ND	ND
V96	P1.5-1,10-1	ND	ND	ND	ND
V100	P1.21-7,16	ND	ND	11	11
V114	P1.5,2	12	14	13	ND
V115	P1.5,2	13	ND	Replaced	ND
V117[3]	P1.5-1,10-1	12	12	ND	ND
V124	P1.5-1,10-1	11	11	11	Replaced
V128	P1.5-1,10-1	12	Replaced	ND	ND
V131	P1.19-1,15-11	13	11	11	11
V134	P1.5,2	13	13	13	ND
V138	P1.21,16	12	12	12	Replaced
V176	P1.19,15	11	11	11	12
V188	P1.5-1,2-2	12	ND	12	12
V208	P1.5-1,2-2	11	11	ND	Cleared
V222	P1.5-1,10-1	11	11	10	10

[1]   Up to 20 isolates from each timepoint were analysed using a PCR-based method and the modal repeat length is shown below. Timepoints where PV occurred are highlighted in red text. (This data was collated by Fadil Bidmos and other members of the Bayliss' research group).

[2]   ND (no data) indicates either the isolates have not been analysed or no samples were collected.

[3]   Antigenic variation of PorA from P1.5-1,**10-1** to p1.5-1,**10-29** was observed in this volunteer at week 12.

To confirm the specificity of interactions between serum antibodies and the PorA VR epitopes, inhibition assays with PorA monoclonal antibodies were performed. A 1:900 dilution of monoclonal antibodies of PorA VR epitopes P1.2, P1.5, P1.16 and P1.19 were mixed with all PorA beadsets before different sera titres were incubated with the pre-treated beadsets. Eight serum samples from volunteers colonised with meningococcal isolates of the following PorA types were analysed: P1.21,16; P1.5,2; P1.5-1,10-1; P1.19,15 and P1.19-1,15-11.

MFIs of pre-treated beads were 5 to 10-fold lower than untreated beads when mAbs to homologous VRs were used (Figure 5.2). Blocking with unrelated mAbs had no effect on binding of antibodies as similar MFIs were recorded for treated and untreated beads. Blocking with the homologous P1.2 mAb had an inhibitory effect that was up to 4-times greater than that of the P1.5 mAb on the P1.5,2 carrier sera. This indicates that the P1.2 VR2 epitope was more immunogenic than the P1.5 VR1 epitope.
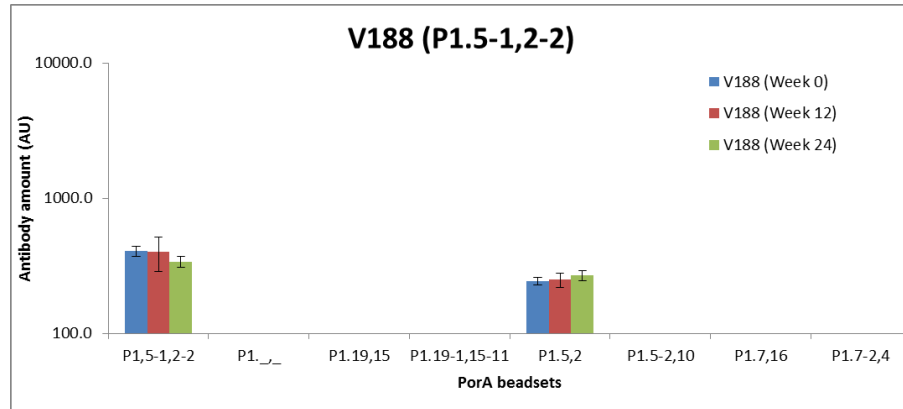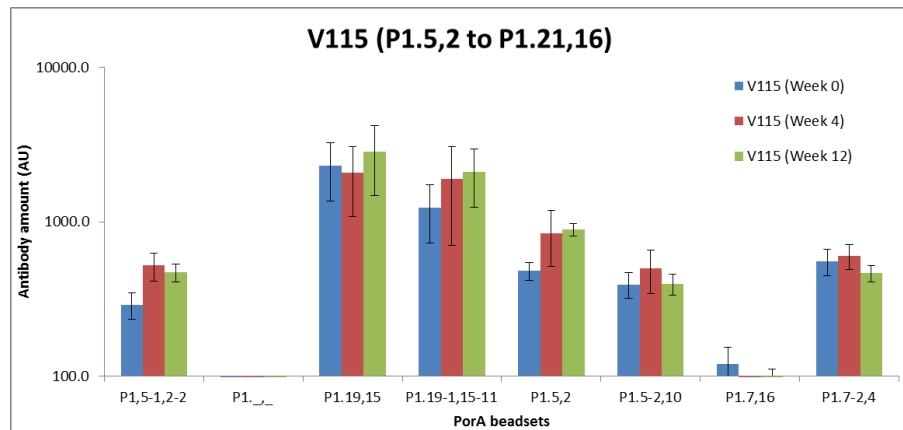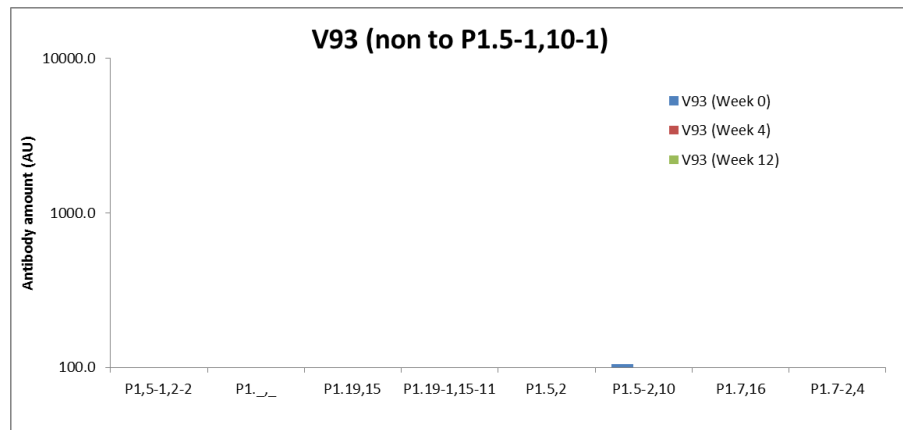
Figure 5.1: Serum antibody reactivity to homologous and heterologous PorA variants. Four dilutions, ranging from 1:100 to 1:8100, prepared from 2 µl of each test serum sample were mixed with a panel of beadsets coupled to the different PorA variants listed above in 96-well filter plates, in singlicate. A 1:25 dilution of an anti-IgG R-Phycoerythrin conjugate was used to generate fluorescent signals, which were subsequently measured with a Liquichip workstation. Beadsets were sorted by the workstation based on the combinations of internal red and infra-red dye labels. Antibody amounts (in arbitrary units) were obtained by plotting the MFIs of test sera against a standard curve generated by MFIs of pooled convalescent sera. Values displayed are the mean of readings of 4 dilutions of each serum sample and error bars represent the standard error of the mean. V in V188 means Volunteer 188; PorA types of carried strain for volunteers above are indicated in brackets. In the examples above, V188 represents volunteers in which reactivity was detected against only 1 PorA type (Group 1), V115 represents volunteers where significant reactivity to heterologous PorA proteins was observed (Group 2) and V93 represents carriers where little or no reactivity to the PorA protein was observed (Group 3).

Analysis of sera in which reactivity to other VR epitopes was observed showed significant reductions in ability of serum antibodies to bind such VR epitopes on mAb-treated beads. Cross-reactivity of antibodies in sera obtained from V114 (P1.5,2 carrier) with P1.19,15 was up to 5-fold less when P1.19 mAbs were used to treat beads but was uninhibited if P1.5 or P1.2 mAbs were used for blocking (Figure 5.3). Conversely, binding to the P1.5,2 beads was blocked by homologous P1.5 and P1.2 mAbs but not the PorA P1.19 mAb. Similarly, inhibition of antibody binding was recorded for V176 (P1.19,15 carrier) and V131 (P1.19-1,15-11 carrier) sera when P1.2 and P1.5 mAbs were used to block beads. These sera had shown lower amounts of specific antibodies to the P1.19 or P1.15 epitope families and were more reactive to the P1.5 and P1.2 families. Reactivity to the P1.5-1,2-2 protein seen in V131 and V176 sera was down by 7- and 3-fold respectively when either P1.2 or P1.5 mAbs were used to block proteins. Only the V131 serum showed a similar reduction in antibody binding in the presence of the P1.19 mAb suggesting the presence of specific antibodies to the carried P1.19-1,15-11 isolate.

Overall, these results indicate that interactions between serum antibodies and the PorA VRs are specific and also suggest that antibodies to VRs unrelated to the carried PorA strain could be induced in an individual during carriage. The possible mechanisms that could be responsible for this are discussed later in this chapter.

An overview of the reactivity profile for sera obtained from each volunteer is presented below (see Appendix I for a pictorial representation of the profile of each volunteer). Since this study aimed to examine reactivity of carrier sera to the PorA protein of the carried strain and make possible deductions regarding the impact of phase variation of PorA on the immune response (antibody levels), the figures shown in sections 5.1.2 – 5.1.5 below focus on the reactivity of carrier sera to the homologous PorA beadset only.
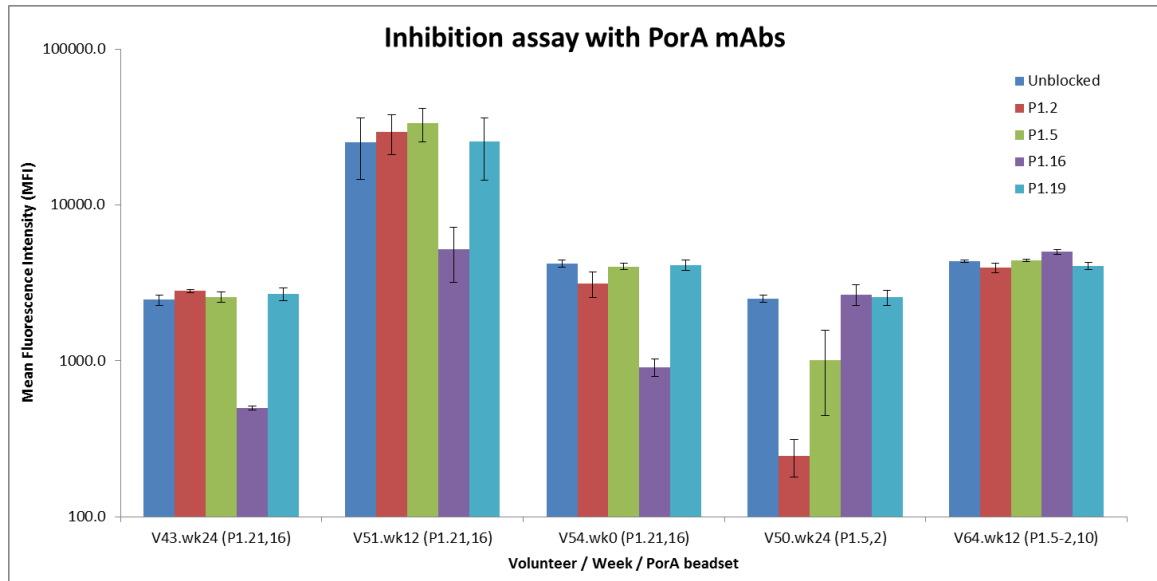
Figure 5.2: Inhibition assay to validate reactivity between serum antibodies and PorA. The assay was performed as previously outlined in Figure 5.1. 1:900 dilutions of monoclonal antibodies of P1.2, P1.5, P1.16 and P1.19 were incubated with each PorA beadset before adding sera. Values displayed are the mean of readings of 4 dilutions of each serum sample and error bars represent the standard error of the mean.
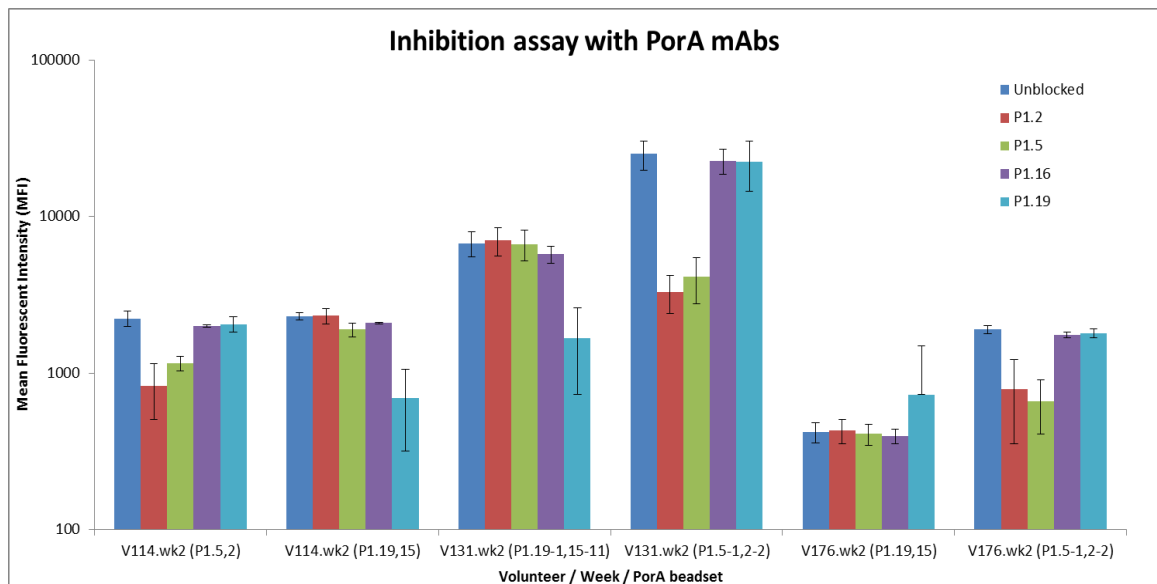

Figure 5.3: Inhibition assay to validate cross-reactivity between serum antibodies and unrelated PorA alleles. The assay was performed as previously outlined. Values displayed are the mean of readings of 4 dilutions of each serum sample and error bars represent the standard error of the mean.

5.1.2 Non-carrier sera lack PorA antibodies

The level of PorA antibodies detected in the sera obtained from 6 out of 7 non-carriers was below 100 AU; the average level was 38 AU (Figure 5.4). This antibody level i. e. 38 AU was set as the benchmark to which antibody levels in carriage positive volunteers were compared. The level of PorA antibodies in one of the volunteers (V87) at week 0 was 4-fold higher than levels seen in other non-carriers but decreased at week 12 by 1.7-fold. This may be indicative of recent carriage, prior to the carriage study, in this carrier (See Appendix I).
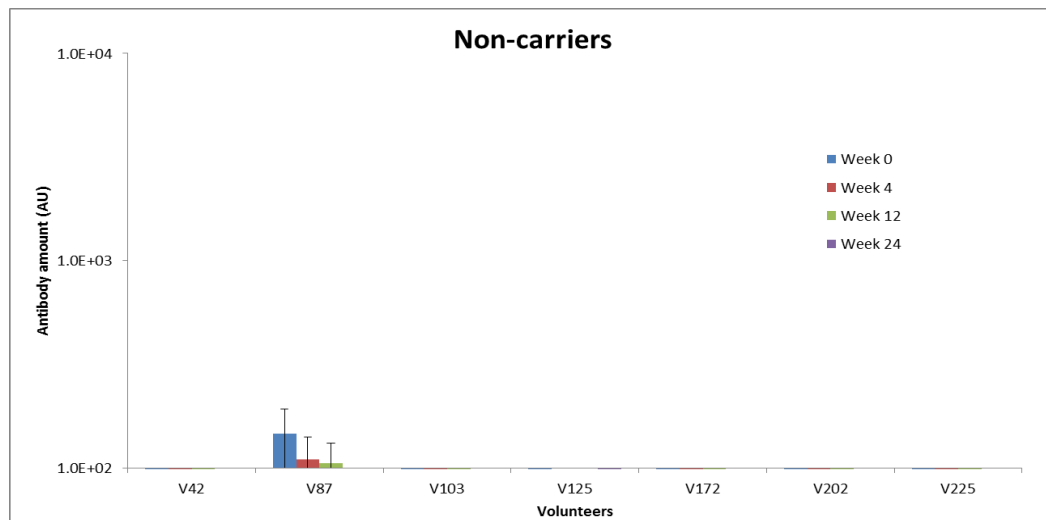
Figure 5.4: Levels of antibody in sera obtained from persistent non-carriers. The assay was performed as previously outlined in Figure 5.1. Antibody amounts (in arbitrary units) were obtained by plotting the MFIs of test sera against a standard curve generated by MFIs of pooled convalescent sera. Values at each timepoint are a mean of the values obtained for all beadsets (i.e. against the seven PorA variants) and error bars represent the standard error of the mean.

5.1.3 Acquisition of carriage is associated with induction of antibodies

PorA-specific antibodies were assessed for 6 volunteers subject to acquisition of a meningococcal strain during the carriage study. In each case, these volunteers were non-carriers at week 0 and the amount of PorA-specific antibodies at week 0 was similar to that found in non-carriers (mean antibody amount was 58.6 AU; 1-tailed t-test produced a p-value of 0.33). In 5 out of 6 of these volunteers, significant induction of antibodies was observed against the PorA type of the carried strain (Figure 5.5). A significant 7-fold difference was found between pre- and post-colonisation mean antibody amounts of 53.6 AU and 375.4 AU, respectively (p<0.01). In V50, a 4-fold increase in PorA antibody levels is discernible between weeks 4 and 12, which coincided with acquisition of a CC-60 strain with a PorA type, P1.5,2. Continued

induction of antibodies, up to 28-fold is evident at week 24 and could have contributed to clearance of the CC-60 strain (29E:P1.5,2:F1-7:CC-60). The lack of cross-protection offered by VR-specific antibodies may have allowed for the replacement of the CC-60 strain with a CC23 strain in this volunteer (Y:P1.5-1,10-10:F4-1:CC-23) (Bidmos *et al.*, 2011). In volunteers V69, V70, V96 and V100, similar levels of induction of specific PorA antibodies were observed with 4- to 18-fold increases in antibody amounts from week 0 to week 24. As in V50, a 19-fold increase in antibodies (from 20.2 AU to 390.6 AU) to the P1.16 VR2 epitope between weeks 4 and 12 may have contributed to the replacement of a CC-1157 strain in V70 with a P1.7,30 strain. The induction of antibodies to other PorA proteins in this volunteer (V70), notably P1.5-2,10 (from 91.9 AU to 781.9 AU) and P1.19-1,15-11 (from 39.1 AU to 403.4 AU), was also evident. No induction of PorA antibodies was discernible in V93 sera samples with antibody amounts reducing from 104.7 AU at week 0 to 53.4 AU at week 12.

Cross-reactivity between antibodies in particular sera and other PorA variants was minimal in most of these sera from the carriers subject to acquisition (See Appendix I). Exceptions were in carriers of a P1.21-7,16 strain, V70 and V100, where significant levels of antibodies were detected to the P1.5-2,10 and P1.19-1,15-11 proteins.

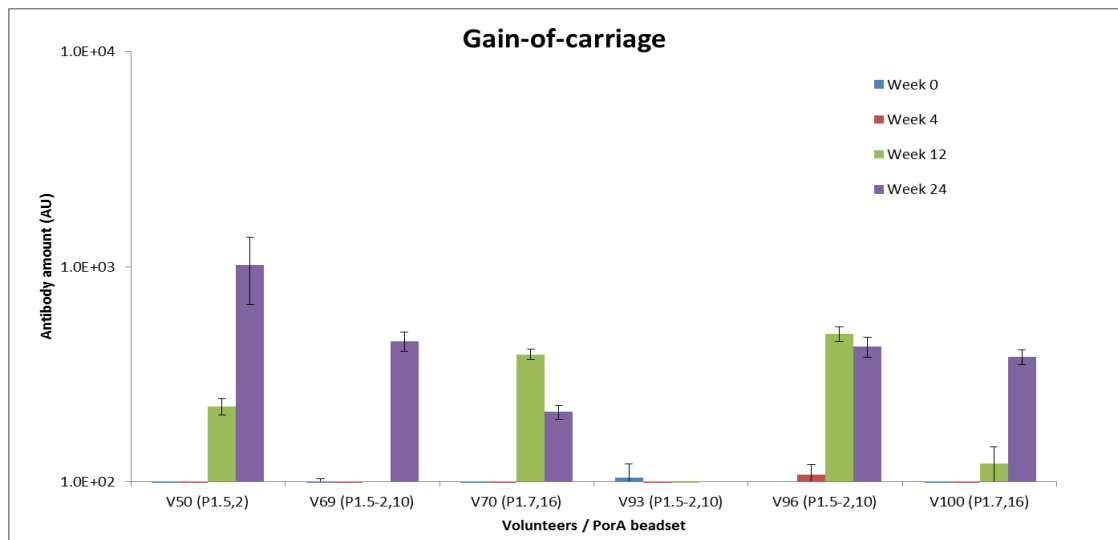**Figure 5.5**: Levels of antibody in sera obtained from gain-of-carriage volunteers. Antibody amounts (in arbitrary units) were obtained as previously outlined in Figure 5.1.Only data obtained from beadsets corresponding to the PorA type of the carried strain are shown. Values displayed are the mean of readings of 4 dilutions of each serum sample and error bars represent the standard error of the mean.

5.1.4 Persistent carriage is associated with high PorA antibody levels

Sera from 9 persistent carriers were analysed for presence and levels of specific PorA antibodies. The mean level of antibodies in these carriers at week 0 was 226.7 AU with statistically insignificant increases of 1.5- to 2-fold from weeks 4 to 24 ($p = 0.34$). This indicates that, in contrast to gain-of-carriage volunteers, there was no rise in the level of specific antibodies. The mean level of antibodies was 8-fold higher than in non-carriers ($p = 0.004$) indicating a clear induction of PorA antibodies in the sera of these carriers. Thus, after the initial induction of antibodies, possibly associated with acquisition of carriage, antibody levels in sera either remained at similar levels or decreased with time (Figure 5.6).

There was no evidence for *porA* phase variation in isolates obtained from four persistent carriers (V117, V88, V188 and V134). V117 showed antigenic variation of *porA* at week 12, from P1.5-1,10-1 to P1.5-1,10-29 (Bidmos *et al.*, 2011), which was preceded by a 1.4-fold induction of specific antibodies between weeks 4 and 8. Antibody amounts in V117 subsequently increased by another 1.3-fold at week 24. A more significant increase in antibody levels was seen in V88 where a 4-fold increase in antibody amounts was observed. This could indicate that acquisition of the CC-174 strain occurred just before the carriage study as this induction level is similar to those of the gain-of-carriage volunteers. This increase was, however, not sustained and antibody levels remained the same for the remaining 20 weeks. Significant cross-reactivity with the 'loopless' PorA was also observed with sera from V88. Meningococci (Y:P1.5-1,2-2:F5-8:CC-23) with an interrupted polyG tract in the *porA* promoter (with no evidence of phase variation) were isolated from V188 at all timepoints. Antibody levels in V188 remained unchanged from week 0 to week 24 with <10% variation in antibody amounts at weeks 0, 12 and 24 (405.7 AU, 400.0 AU and 340.4 AU respectively). Amounts of specific PorA antibodies in V134 sera were not dissimilar to those found in non-carriers ($p=0.19$). [N. B.: For carriers in which isolates exhibited PV of *porA*, see section 5.1.6].
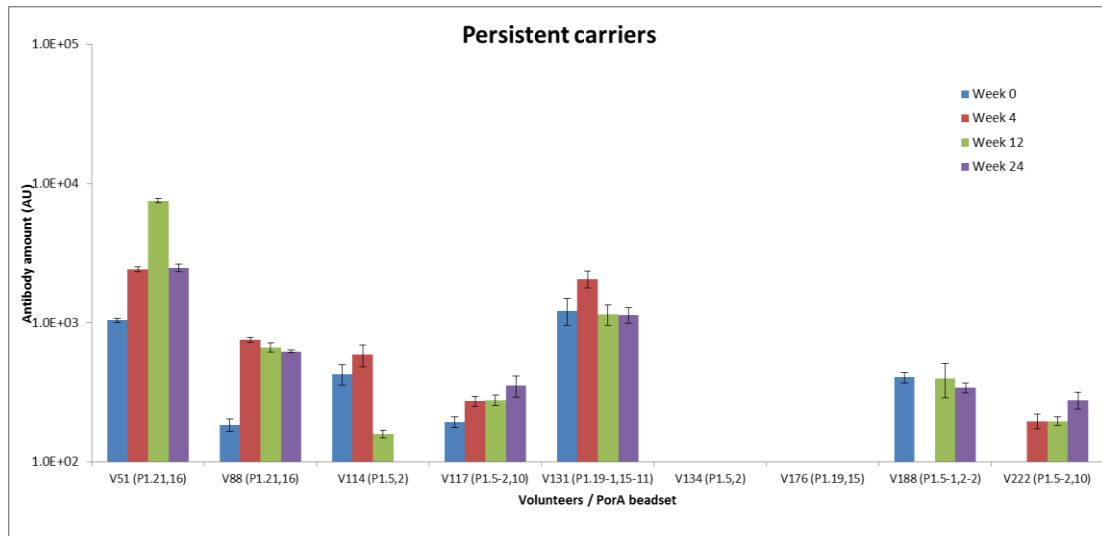
Figure 5.6: Levels of antibody in sera obtained from persistent carriers. The assay was performed and antibody amounts (in arbitrary units) were obtained as previously outlined in Figure 5.1.Only data obtained from beadsets corresponding to the PorA type of the carried strain are shown. Values displayed are the mean of readings of 4 dilutions of each serum sample and error bars represent the standard error of the mean.
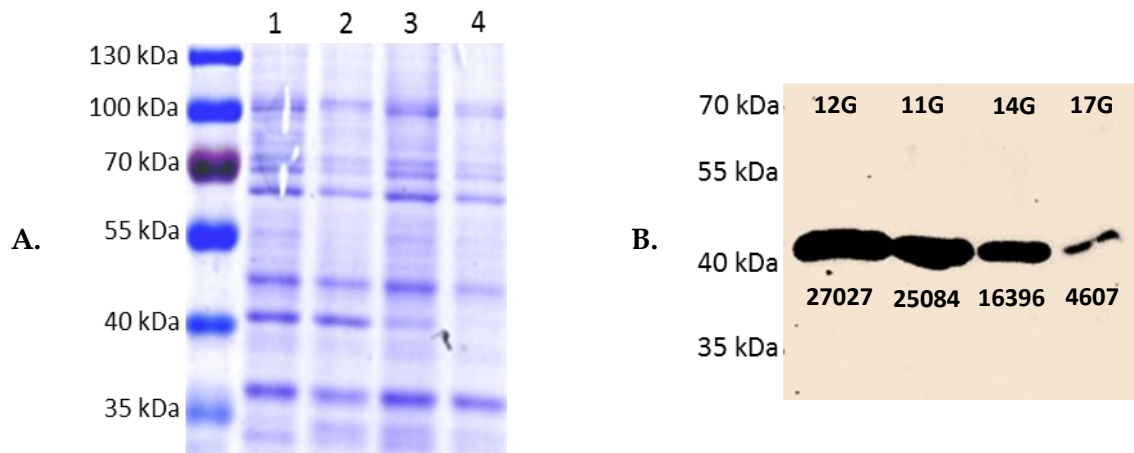
Figure 5.7: Expression of PorA from promoters with different repeat lengths in CC-174. The repeat tract lengths of the *porA* promoter in carriage strains isolated from V43 and V54 were first determined using a PCR-based method. V43 strains contained 12G (lane 1) and 11G (lane 2) tracts while V54 strains contained 14G (lane 3) and 17G (lane 4) tracts. For the preparation of cell lysates, cultures of each strain were adjusted to an OD_{600} of 0.5. 1 ml of each adjusted culture was subsequently pelleted before cells were re-suspended in 1x Laemmli buffer and boiled for 5 minutes at 98⁰C. Proteins were separated on an 8% SDS-PAGE gel followed by coomassie staining **(A)**. Proteins were transferred to PVDF membranes and probed with a 1:2500 dilution of the P1.16 antibody (VR2) in a western blot. A 1:2000 dilution of an anti-Mouse IgG HRP-conjugate was used to generate signals. Signals on blots were quantified using the ImageJ software (values below signals indicate signal strength).
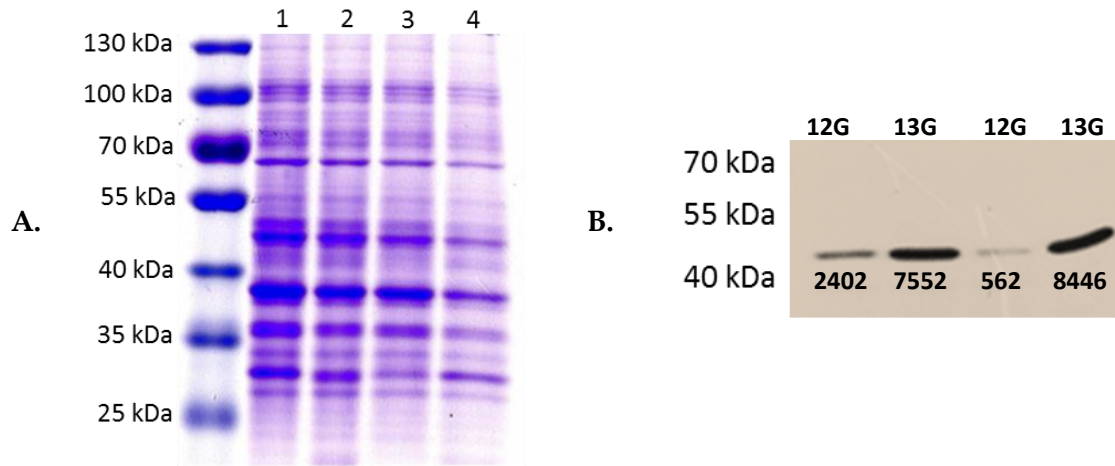
Figure 5.8: Expression of PorA from promoters with different repeat lengths in CC-60. The repeat tract lengths of the *porA* promoter in carriage strains isolated from V114 and V134 were first determined using a PCR-based method. V114 strains analysed in lanes 1 and 2 contained 12G and 13G tracts, respectively. V134 strains with the same repeat tracts were analysed in the same order (12G in lane 3 and 13G in lane 4). Preparation of lysates was performed as previously outlined in Figure 5.4. Proteins were separated on an 8% SDS-PAGE gel followed by coomassie staining **(A)**. Proteins were transferred to PVDF membranes and probed with a 1:2500 dilution of the P1.2 antibody (VR2) in a western blot. A 1:2000 dilution of an anti-Mouse IgG HRP-conjugate was used to generate signals. Signals on blots were quantified using the ImageJ software (values below signals indicate signal strength).
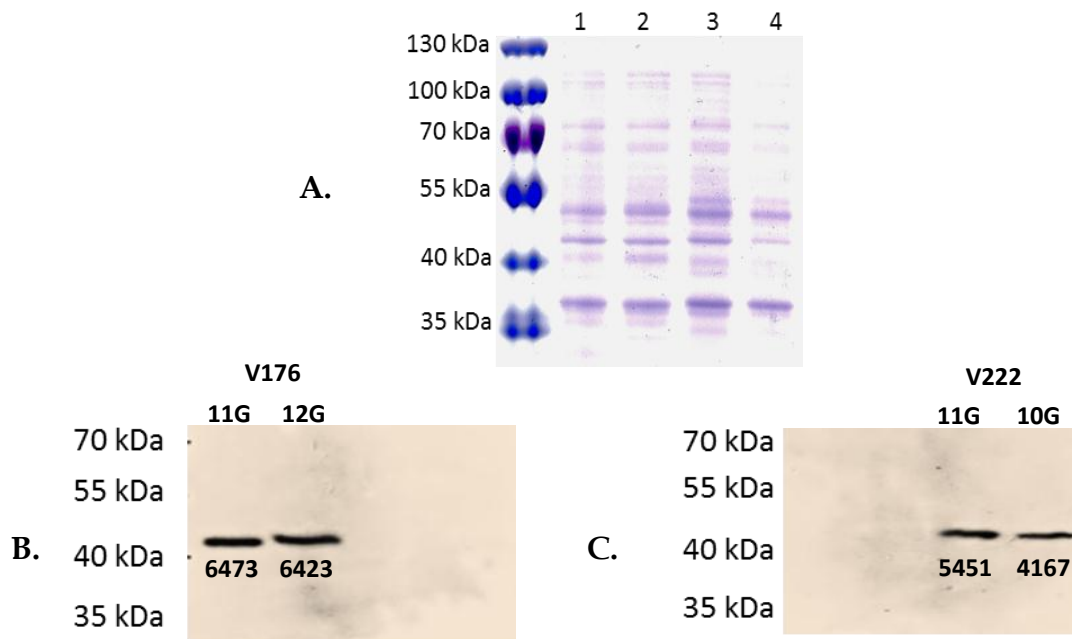
Figure 5.9: Expression of PorA from promoters with different repeat lengths in CC-32 and CC-23. The repeat tract lengths of the *porA* promoter in carriage strains isolated from V176 (CC-32) and V222 (CC-23) were first determined using a PCR-based method. V176 strains contained 11G (lane 1) and 12G (lane 2) tracts while V222 strains contained 11G (lane 3) and 10G (lane 4) tracts. Preparation of lysates was performed as previously outlined in Figure 5.4. Proteins were separated on a 12.5% SDS-PAGE gel followed by coomassie staining **(A)**. Proteins were transferred to PVDF membranes and probed with a 1:2500 dilution of the P1.19 **(B)** and the P1.5 **(C)** in western blots. A 1:2000 dilution of an anti-Mouse IgG HRP-conjugate was used to generate signals. Signals on blots were quantified using the ImageJ software (values below signals indicate signal strength).

5.1.5   **Loss of carriage or strain replacement in healthy carriers (sequential carriers)**

Sera from 10 volunteers, who were subject to strain replacement or loss-of-carriage, were analysed. The mean level of specific antibodies across all timepoints in these carriers was 746.9 AU, which is significantly higher than levels found in non-carriers ($p < 0.05$). In addition, antibody levels were 2-fold higher in these carriers than mean levels found in persistent carriers ($p = 0.058$). This shows that high levels of specific antibodies were generated in these carriers over the 24-week study.

Increases in specific antibody amounts (2- to 18-fold) to pre-existing PorA antigens were observed in sera from five volunteers subject to strain replacement or loss-of-carriage (V43, V52, V64, V115 and V138 – see Table 5.1 for differentiation between strain replacement and clearance) (Figure 5.10). Little or no antibodies were detected against the PorA variant of the replacement strain. The repeat length of the *porA* promoters in strains carried by all five volunteers are known to produce the highest levels of PorA. This suggests that continuous expression of the PorA antigen at high levels triggered a sustained increase in antibody amounts, which may have contributed to the clearance of the pre-existing strains.

Conversely, a reduction in antibody levels at week 24 when compared to the high levels seen in week 0 was observed in V54, V58, V124 and V208 (Figure 5.10). All these volunteers had lost carriage by week 24 except V58 where carriage of the same strain was regained after week 12. Sampling sensitivity at week 12 could be responsible for the inability to isolate meningococci from V58 at this timepoint. Like the latter four volunteers mentioned above, a reduction in the levels of specific antibodies in V43, V52 and V64 were observed after clearance. This suggests that loss of the strain reduces stimulation of the immune response and hence, a discernible decrease in antibody levels.

Significant amounts of cross-reactivity to other PorA variants and the loopless protein were observed with V52, V124 and V138 sera. Similarly, cross-reactivity to other PorA variants was observed with sera from V58, V115 and V208; however, reactivity to the loopless protein was negligible. Specifically, serum antibodies from V115, as with samples from other P1.5,2 carriers (V114 and V134), were reactive with the P1.19,15 and P1.19-1,15-11 proteins while V208 sera were highly reactive with the P1.7-2,4

variant (carried strain was P1.5-1,2-2). A 6.6-fold decrease in the amount of this P1.7-2,4 antibody at week 24 probably illustrates recent exposure of V208 to the P1.7-2 and/or P1.4 epitopes. No cross-reactivity to other PorA variants or the loopless protein was observed with sera from V54 and V64 (see Appendix I). [For carriers in which isolates exhibited PV of *porA*, see section 5.1.6].
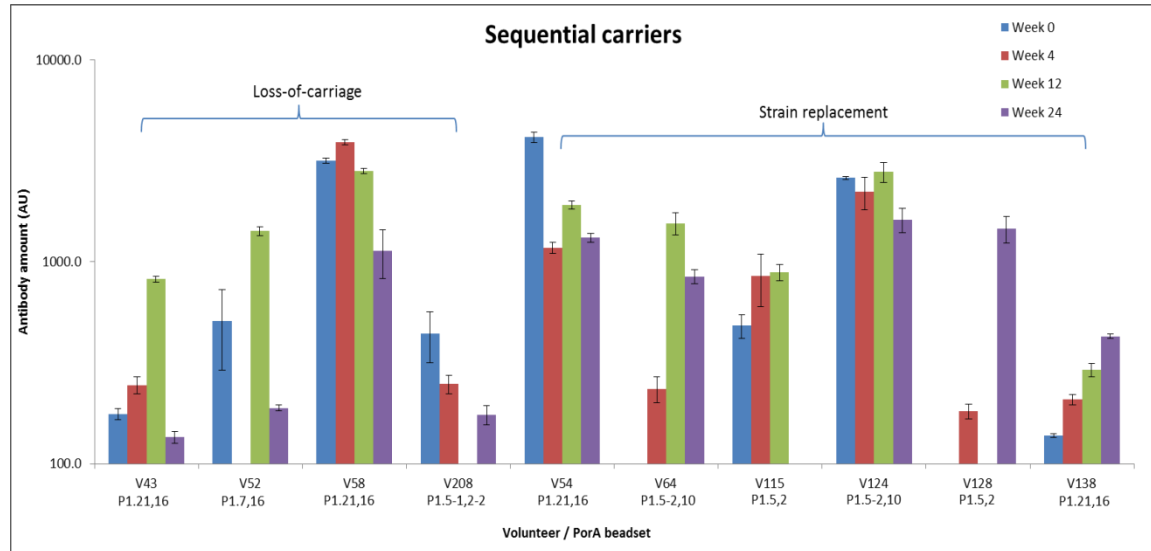
Figure 5.10: Levels of antibody in sera obtained from carriers subject to strain replacement or clearance. Antibody amounts (in arbitrary units) were obtained as previously outlined in Figure 5.1.Only data obtained from beadsets corresponding to the PorA type of the cleared strain are shown. Values displayed are the mean of readings of 4 dilutions of each serum sample and error bars represent the standard error of the mean. (Note: The y-axis starts with a value of 100 as opposed to 10 in Figures 5.1 and 5.2).

5.1.6 Association between *porA* PV and antibody amount in persistent and sequential carriers

Isolates from five persistent carriers had undergone phase variation of *porA* during the study (Table 5.1). A significant increase (3.1-fold; p=0.01) in antibody amount between week 4 and week 12 was observed in V51 and was concomitant with phase variation of *porA* (from 12G to 11G). No differences in expression were observed between 12G and 11G *porA* promoters of CC-174 strains in western blots (Figure 5.7). V114 carried a strain, which increased PorA expression via phase variation from 12G at week 0 to 14G at week 4 to 13G at week 12 (see Figure 5.8) without a corresponding increase in antibody amount (note: 14G and 13G produce similar PorA amounts; Alamro, personal communication). The levels of specific antibodies found in sera obtained from V176 were similar to those found in non-carriers (p>0.05 in both cases) indicating a lack of induction of antibodies in this volunteer. Despite the absence of antibodies, *porA* phase

variation was evident in the CC-32 strain isolated from V176 (11G to 12G - see Figure 5.9b). In V222, no change in antibody amount from week 4 to week 12 was observed while *porA* phase variation from 11G to 10G occurred (Figure 5.9c). Although considerable levels of specific antibodies were detected in sera obtained from V131 (PV from 13G at week 0 to 11G at week 4 – no change expected in PorA expression because both promoter spacers produce similar amounts of PorA), significant cross-reactivity to other PorA variants was also observed. There was, however, no reactivity to the loopless protein, which indicates that antibodies were binding to the VR epitopes only. Serum obtained from V131 at week 4 was more reactive to the P1.19-1,15-11 protein than sera obtained at weeks 0, 12 and 24, where antibody levels were similar.

With regards to *porA* phase variation in carriers subject to strain replacement, V52, V54 and V58 isolates exhibited *porA* phase variation between samplings but consequential changes to PorA expression are only expected with V54 isolates. Alteration of the repeat tract length from 14G to 17G in V54 isolates led to a detectable decrease in PorA expression subsequently accompanied by a corresponding 3.5-fold decrease in antibody levels (see Figure 5.7). As with other CC-174 strains, the alternation of the *porA* repeat tract length in isolates of V52 and V58 between 11G and 12G did not correlate to detectable changes in PorA expression as 11G and 12G-containing promoters yield similar levels of expression (see Figure 5.7). In both carriers, antibody levels were observed to decrease by week 24, a possible consequence of the loss of carriage at weeks 12 and 24 in these volunteers.

Overall, there was no significant difference in the relative occurrence of *porA* PV in persistent carriers (5 out of 9 carriers) or sequential carriers (3 out of 10 carriers) (p = 0.29). Out of 41 inter-sampling periods in 17 carriers (see Tables 5.2 and 5.3 below), 7 involved PV of *porA*. A subset of 5 PV-associated events were characterised by stable levels of PorA expression with no bias towards an increase or decrease in antibody levels. A similar pattern was observed when all 41 events were analysed i. e. equal proportions of antibody increases and decreases were observed.

Table 5.2: **Association between *porA* PV and antibody amounts in persistent and sequential carriers.** Only volunteers with detectable levels of anti-PorA antibodies are shown below (i. e. excludes V134 and V176).

Volunteer	Antibody amount / PorA tract length			
	Week 0	Week 4	Week 12	Week 24
V43	176.3 / 12	245.0 / 12	820.5 / 12	135.5 / 12
V51	1040.3 / 12	2423 / 12	7537.5 / 11	2483.3 / 11
V52*	510.2 / 12	ND[1] / 11	1415.6 / 12	189.1 / -[2]
V54	4142.5 / 14	1172.7 / 17	1908.0 / 17	1313.0 / -
V58	3155.7 / 11	3910.0 / 12	2815.0 / -	1129.5 / 11
V64	82.8 / 11	235.5 / 11	1552.9 / 11	845.3 / -
V88	184.3 / 11	757.5 / 11	667.3 / 11	622.0 / 11
V114	427.9 / 12	588.5 / 14	159.2 / 13	ND / ND
V115	481.8 / 13	847.4 / ND	890.2 / -	ND / ND
V117	193.9 / 12	273.3 / 12	278.5 / -	352.6 / -
V124	2601.0 / 11	2219.2 / 11	2779.3 / 11	1612.8 / -
V128	57.9 / 12	182.4 / -	ND / -	1460.6 / -
V131	1223.3 / 13	2059.8 / 11	1150.1 / 11	1136.4 / 11
V138	137.9 / 12	207.8 / 12	291.8 / 12	427.8 / -
V188	405.7 / 12	ND / ND	400 / 12	340.4 / 12
V208	441.7 / 11	248.8 / 11	ND / ND	175.0 / -
V222	90.2 / 11	196.5 / 11	196.3 / 10	277.8 / 10

* Both PV events for this volunteer were excluded from analysis as antibody amounts for week 4 were not determined.

[1] ND (no data) indicates that no serum sample or isolate was collected at this timepoint.

[2] Hyphen (-) indicates clearance, strain replacement or antigenic variation at this timepoint.

Table 5.3: **Breakdown of PV and non-PV events with respect to antibody levels.**

Antibody levels	PV events (change in PorA expression)			Non-PV events
	Increase (> 1.5-fold)	No change	Decrease (<1.5-fold)	
Increase (>1.5-fold)	0	2	0	11
Stable	1	2	0	11
Decrease (<1.5-fold)	1	1	0	9

5.2   **Cross-reactivity between P1.5,2 carrier sera and P1.19,15 / P1.19-1,15-11**

A high amount of cross-reactivity was observed with antibodies in the sera of some carriers. Notably, sera of three P1.5,2 carriers had a high amount of reactivity with P1.19,15 and its variant, P1.19-1,15-11. Similarly, sera from a P1.19-1,15-11 carrier showed significant reactivity to P1.5 and P1.2 epitope families.

To determine whether antibodies to the P1.19, P1.19-1, P1.15, P1.15-11 or other epitopes shared by the P1.5,2 and P1.19,15 proteins were present in sera of P1.5,2 carriers, an inhibition assay with different amounts of purified P1.19,15 protein was performed. Antibodies to different epitopes of the P1.19,15 protein were expected to be sequestered by the purified protein, thereby promoting the availability of only P1.5 and P1.2 antibodies for interactions with PorA. Consequently, specific reactivity to P1.5,2 and its variant, P1.5-1,2-2, will be obtained. Serum antibodies were previously non-reactive to the loopless, P1.7-2,4 and the P1.7,16 proteins, therefore, no novel effects would be derived by inhibition with P1.19,15.

Inhibition with 1, 2, 4, 6, 8 or 10 ng/µl of P1.19,15 protein significantly hindered interactions between serum antibodies of carriers of P1.5,2 strains and all proteins in the test panel. A reduction in signal strength, which was a consequence of the reduced amount of free antibodies, was also associated with an increase in rP1.19,15 concentration (Figure 5.11). As shown earlier, blocking of binding to P1.19,15 beads could also be inhibited by mAbs indicating that some of these antibodies are VR-specific. The nature of this cross-reactivity is discussed in section 5.4.
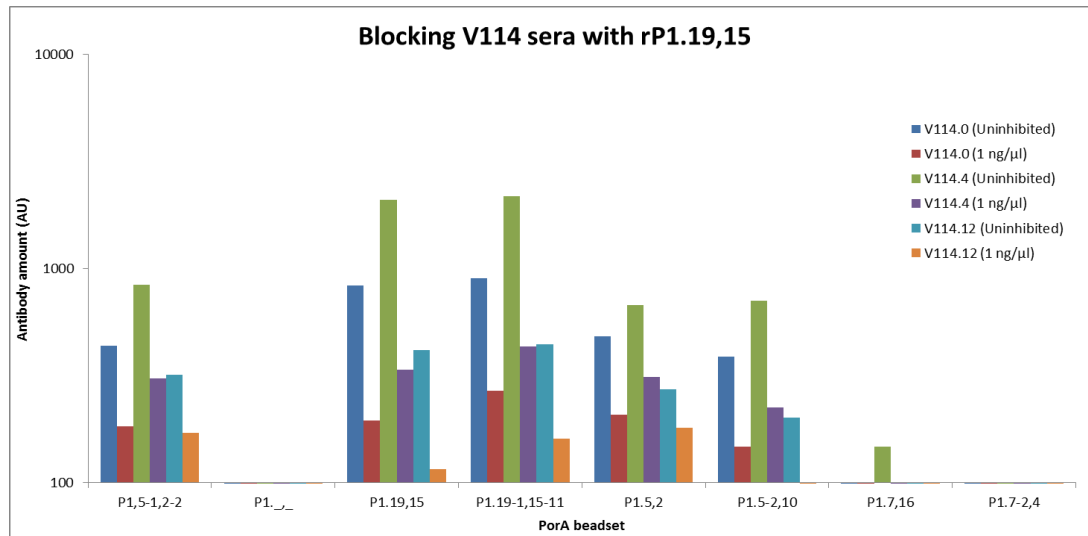
Figure 5.11: Inhibition assay to test cross-reactivity between P1.5,2 carrier sera and P1.19,15. Concentrations of 1, 2, 4, 6, 8 and 10 ng/µl of purified P1.19,15 (only inhibition with 1 ng/µl is shown above but is representative of all the data) were incubated with longitudinal sera samples obtained from V114, V115 and V134 (only V114 data shown above) for 1 hour. Pre-treated sera were subsequently added to the PorA beadsets and the assay was progressed as previously described.

5.3   Development of a Liquichip assay to evaluate levels of HpuA antibodies during carriage

Purified recombinant HpuA proteins from strains N88 and 8047 were coupled to non-magnetic carboxylated beads using an amine coupling kit, as per manufacturer's instructions. Bead counts were measured using a flow cytometer. To validate coupling of beads with rHpuA, sera from non-vaccinated and vaccinated mice (generated in Chapter 4) were used to probe each beadset. Antisera from each group of 10 mice immunized with rHpuA were pooled into 3 groups (labelled P1, P2 and P3). The assay was performed as previously outlined in section 2.8.

Results from the assay showed that both rHpuA variants were successfully coupled to beads but in dissimilar amounts. MFI values recorded for the rN88-HpuA beadset, when anti-rN88-HpuA and anti-r8047-HpuA antisera were used as probes, were 6-fold higher than values returned for beads coated with r8047-HpuA ($p < 0.05$). Reactivity of sera from non-vaccinated mice to both beadsets was, however, significantly lower than sera from immunised mice (Figure 5.12).

The presence of VR-specific antibodies in anti-rN88-HpuA was not easily discernible as MFI values recorded for these antisera with the rN88-HpuA beads were similar to

those observed for anti-r8047-HpuA antisera. In contrast, a 2-fold difference (p = 0.1) in MFI values returned for both antisera (anti-r8047-HpuA > anti-rN88-HpuA) when probed against r8047-HpuA beads could be due to the induction of VR-specific antibodies in mice immunised with r8047-HpuA.
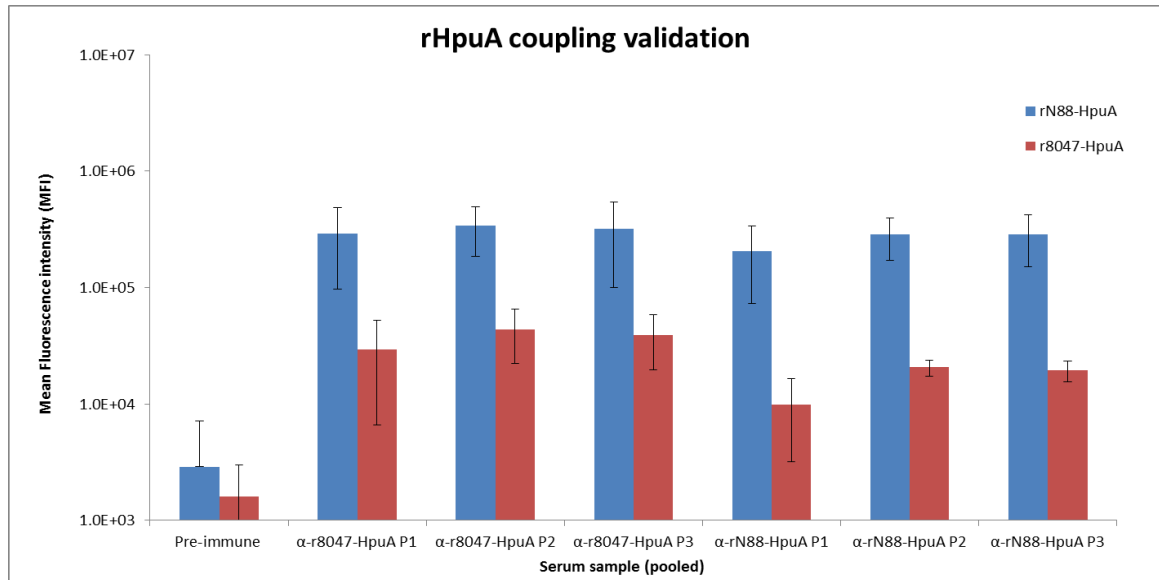

Figure 5.12: rHpuA protein coupling validation. Serial dilutions of pre-immune and post-immune antisera (pooled from 3 or 4 mice) were used to probe microspheres coated with either rN88-HpuA or r8047-HpuA. A Phycoerythrin-labelled antibody was used to generate a reporter signal that was measured and reported as the Mean Fluorescence Intensity (MFI). MFIs for each of four dilutions of a pooled serum sample (P1, P2 or P3) were averaged and plotted in the chart above. Error bars represent the standard error of the mean.

Following the verification of protein coupling, carrier sera were used to probe both rHpuA beadsets. Sera from carriers of CC-174 strains, including V88, collected concurrently to the isolation of *hpuAB* phase variants were selected for analysis. Samples from two non-carriers were also included in the analysis. Anti-r8047-HpuA antisera, which had previously produced positive reactivity with both beadsets was used as a positive control while sera from the non-vaccinated mice served as background control for the assay.

Consistent with results from the coupling validation assay, high signal intensities were obtained for anti-r8047-HpuA antisera but not non-vaccinated mice sera. While insignificant differences were observed between the MFI values of longitudinal V88 sera, significantly higher MFI values in comparison to non-carrier sera were obtained with the r8047-HpuA and rN88-HpuA beadsets (p = 0.03 and 0.02 respectively) (Figure 5.13). Reactivity of V88 sera to the rN88-HpuA antigen was also significantly higher

than reactivity to the r8047-HpuA antigen (p = 0.006). This is probably due to unequal amounts of the proteins coupled to beads. MFIs recorded for other carrier sera were significantly lower than the positive control (anti-r8047-HpuA; p<0.05) and were not dissimilar to non-carrier sera values.
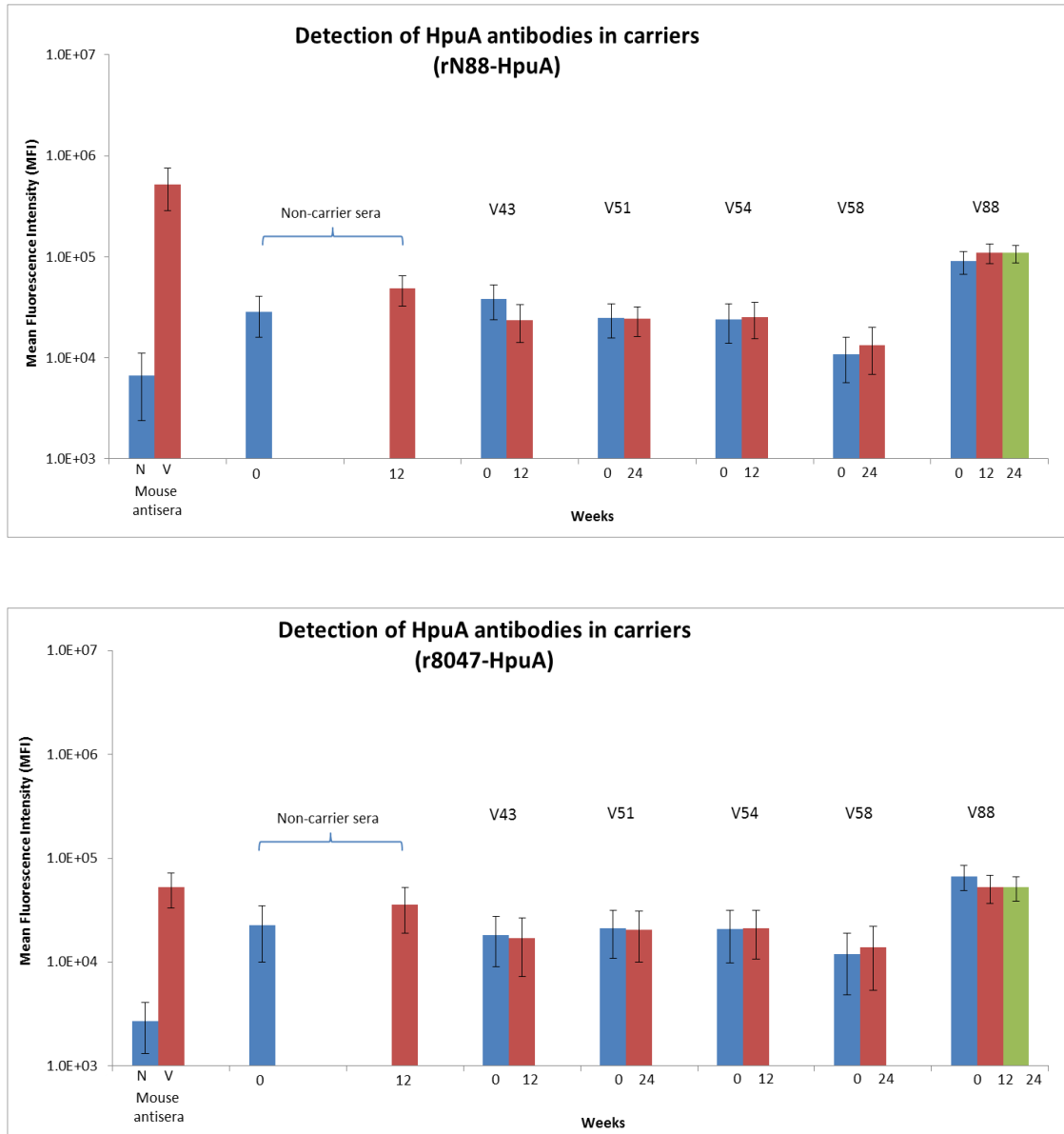

Figure 5.13: Detection of HpuA antibodies in carriers using rHpuA coupled to beads. Serial dilutions of carrier and non-carrier sera were used to probe microspheres coated with rN88-HpuA (top) and r8047-HpuA (bottom). MFIs were determined as previously outlined in Figure 5.11. Error bars represent the standard error of the mean.

5.4   **Discussion**

This study aimed to examine the presence of an adaptive immune response to phase-variable meningococcal OMPs during asymptomatic carriage of the bacterium. In agreement with similar studies conducted previously (Jones *et al*., 1998; Ala'aldeen *et al*., 2010), acquisition of meningococcal carriage was found to elicit the production of specific PorA IgG antibodies. A significant 7-fold increase in antibody levels was observed in volunteers previously naïve to carriage that were colonised during the 24-week carriage study (p = 0.003).

In the present study, a sustained increment in specific PorA antibody levels was associated with clearance of the acquired strain in certain individuals in this study (Table 5.2). Since serum IgG, following passive diffusion into mucosal surfaces (Murphy, 2005), can interact with antigens present on the nasopharynx, these antibodies may have contributed to clearance in certain volunteers. Data obtained from this study also showed that a consequence of clearance is the cessation of the stimulation of the immune system; evident in the gradual reduction of specific antibody amounts following clearance. Despite the reduction in antibody amounts, high levels of specific PorA antibodies were found in some carriers following clearance. The ability to acquire another meningococcal strain with an unrelated PorA VR repertoire (Bidmos *et al*., 2011), in the presence of PorA VR-specific antibodies, shows the specificity of interactions between the antibodies and the PorA VR targets. Therefore, PorA-specific antibodies are not cross-protective and the induction of specific PorA antibodies to match the newly-acquired strain may be required for strain clearance.

Since high levels of PorA-specific antibodies were associated with strain clearance, it was expected, therefore, that persistent carriers would present with low levels of specific PorA antibodies in their sera. Results from this study showed the contrary as significantly higher antibody levels were found in these carriers in comparison to non-carriers (p = 0.004). Compared with carriers subject to clearance or strain replacement, an insignificant 2-fold lower amount of specific antibodies was observed (p=0.06). The inability of similar anti-PorA IgG antibody levels to facilitate clearance in some volunteers (some of which were colonised by the same strain cleared in other volunteers) suggests the requirement of other factors, working in synergy, in the

temporary elimination of meningococcal carriage. Experimental evidence from *in vitro* bactericidal assays reported by Weynants and co-workers (2007) showed that expression of multiple OMPs on the meningococcal surface is required to elicit a bactericidal response. In the study, mice immunised with meningococcal OMVs that contained over-expressed TbpA and Hsf produced sera that had significantly higher bactericidal titres than mice immunised with OMVs containing either over-expressed TbpA or Hsf only. Similarly, the bactericidal activity of two monoclonal antibodies targeting non-overlapping epitopes of fHbp was demonstrated when both mAbs were combined in a bactericidal assay. Neither one of the mAbs, JAR3 and JAR4, were capable of inducing complement-mediated bactericidal activity when used individually against strains 4243 and NZ98/254 (Welsch *et al.*, 2008). Therefore, the absence of antibodies to other OMPs, which may be important to produce a synergistic bactericidal effect, could contribute to persistence of meningococcal carriage despite significant levels of specific anti-PorA IgG antibodies in the serum.

One must also consider that this study measured serum IgG levels only. It is possible that these anti-PorA IgG molecules play only a minor role in nasopharyngeal clearance. The mucosal response to meningococcal carriage, as represented in salivary secretions, should also be investigated to elucidate the roles of further factors that are key to clearance. One such factor is the dominant Ig isotype present on nasopharyngeal surfaces and in secretions, IgA (Kerr, 1991). Recent evidence has shown that anti-PorA IgA antibodies found in the saliva of healthy carriers (Horton *et al.*, 2005), impact on the interactions of non-encapsulated meningococci with epithelial cells, *in vitro* (Horton *et al.*, 2009). Furthermore, *N. meningitidis* possesses 2 classes of an IgA1 protease (Mulks *et al.*, 1980); an immunogenic autotransporter that may serve in the defence of the meningococcus via the inactivation of IgA antibodies or its involvement in eDNA-dependent biofilm formation (Morelli *et al.*, 1994; Arenas *et al.*, 2013). Expression of this autotransporter in carriage strains of the meningococcus (Mulks and Plaut, 1978) suggests an important role for IgA antibodies with respect to the limitation of meningococcal colonisation. Although, saliva samples were collected from the volunteers recruited in the carriage study (Bidmos *et al.*, 2011), levels of anti-PorA IgA1 have not been measured. Comparisons between measured IgA and IgG levels in both serum and saliva samples of these volunteers could provide explanations regarding persistence of colonisation in the presence of anti-PorA serum IgG. However,

conclusions cannot be drawn from the measurement of Ig antibody amounts only as other non-antibody factors could be involved. For example, it could be argued that continued colonisation by a meningococcal strain is a consequence of the inaccessibility of the bacterial cells to the antibodies. This could be made possible via the formation of host immunity-resistant biofilms (Lappann *et al.*, 2010) or the internalisation of the meningococci by the nasopharyngeal epithelial cells (de Vries *et al.*, 1996).

Evidence for a *porA* PV-mediated immune escape of the meningococcus was reported in a study where phase variants expressing lower amounts of PorA were resistant to antibody-mediated bactericidal activity (Tauseef *et al.*, 2013). On the other hand, Ala'aldeen *et al.* (2010) reported that the immune response to fHbp during infection was not associated with surface expression. Data obtained in this present study did not unravel a positive correlation between *porA* PV and immune response. While alterations in the repeat length of the *porA* promoter was evident in longitudinal isolates obtained from volunteers, some of these changes were inconsequential to PorA expression as determined by western blotting (Figures 5.7 – 5.9) and ELISAs (data not shown). Use of more sensitive laboratory methods, such as qPCR and FACS, performed by researchers working independently in separate laboratories on these carriage isolates did not disprove findings obtained from western blots and ELISAs (Alamro, Leicester; and Care, NIBSC, personal communication). Since no differences were observed in *porA* expression, despite PV of the OMP, no PV-associated changes in immune response were discernible. However, the use of *in vivo* surface expression, driven by PV, as a correlate of immune induction may not be viable if the results from Ala'aldeen *et al.* are considered.

The question that arises, thus, relates to the possible reason(s) for the selection of a phase variant over its isogenic counterpart when both variants express PorA at similar levels but from promoters with different tract lengths. This could be explained by considering the fact that other immunogenic OMPs will be targeted by the immune system and selective advantage could be obtained via any one of these OMPs. If this is the case, selection for strains will be independent of the *porA* promoter length and bi-directional switching will be observed between 11G and 12G promoters without any bias towards either tract length. This was evident in isolates obtained from V51 (12G to 11G), V52 (12G to 11G to 12G), V58 (11G to 12G), V176 (11G to 12G) and V222 (11G to

10G) where the frequencies of switching to either 11G or 12G tracts were not significantly different. Furthermore, at least four other phase-variable OMPs (NadA, FetA, Opc and HpuA) have been analysed for changes in their repeat loci during longitudinal carriage. All these OMPs were found to exhibit repeat tract alterations that correlated with significant changes in protein expression levels, as measured by western blotting (to measure total protein expression) and colony immunoblotting (to measure surface expression). For example, expression of NadA was down-regulated during longitudinal carriage and this may be associated with immune selection against the immunogenic OMP (Bayliss, personal communication). Therefore, selection driven by the immune response against any other meningococcal OMP and not PorA could be responsible for the apparent "switch" between *porA* promoters with similar strengths.

Cross-reactivity between PorA-specific antibodies and unrelated VRs was previously shown to be minimal in assays performed by collaborators at NIBSC. In this study, several serum samples contained antibodies that bound to multiple PorA variants. Using mAbs, the specificity of the interactions between serum anti-PorA IgG antibodies and the PorA VRs in the test panel was validated. Concurrent carriage of multiple strains could be taken as a possible explanation for the induction of antibodies against the different PorA antigens presented to the host immune system. While this is not a common phenomenon, it has been reported in a previous carriage study where a volunteer was found to carry two strains that differed in the PorA type (Jordens *et al.*, 2002). Therefore, the adaptive immune system of such an individual could be stimulated to produce antibodies targeting both PorA variants. There was no evidence of concurrent carriage of multiple strains in volunteers recruited to the 2008/09 carriage study (Bidmos *et al.*, 2011). Hence, simultaneous carriage of multiple strains is unlikely to be responsible for the production of antibodies to unrelated PorA variants in this study.

A more plausible explanation for the presence of antibodies targeting disparate PorA VRs involves the concept of an immunological memory of the adaptive immune system. It is widely believed that sub-populations of antigen-specific B-cell lymphocytes that have undergone clonal expansion are long-lived and remain in a resting state in lymphoid tissues (Janeway *et al.*, 2001). These memory B-cells, in secondary or subsequent immune responses, can be stimulated to differentiate into

plasma cells that would produce antibodies, which recognise the antigen that elicited the primary response, in the phenomenon known as the *original antigenic sin* (Frank, 2002). It can be deduced, therefore, with regards to the PorA antigen that antibodies to different PorA variants can be detected in sera of volunteers subject to multiple strain replacement events owing to the stimulation of a pool of heterogeneous memory B-cells. It is more likely that the immune systems of the CC-174 carriers would have been challenged with dissimilar PorA variants in their recent histories. Thus, the disparate immunological memories of these carriers will be manifested in the types of PorA-specific antibodies in their sera.

The induction of an immune response against other meningococcal OMPs was also investigated in this study. Preliminary data suggest the presence of anti-HpuA antibodies in sera of healthy carriers at levels significantly higher than non-carriers. The specificity of interactions between these antibodies and the HpuA antigen are yet to be determined.

In summary, this study reproduced earlier findings of an immune response to the immunodominant OMP, PorA, during carriage. The study also showed that the presence of antibodies targeting multiple PorA variants in sera of carriers is most likely due to the excitation of the immunological memory of carriers and this may unravel the history of meningococcal carriage or infections. Relatively high levels of anti-PorA IgG antibodies seen during the course of persistent carriage indicate that these IgG antibodies are not solely responsible for clearance of meningococcal carriage. Instead, a mucosal humoral response, a synergistic action of antibodies targeted against multiple OMPs or the Ig isotype may be crucial for clearance.

CHAPTER 6

6.0   Evaluation of bactericidal activity of α-HpuA and α-HmbR antisera

The standard assay employed in the measurement of the bactericidal property and vaccine candidacy of a chosen OMP is the serum bactericidal assay (SBA). The assay was described in a series of studies by Goldschneider *et al*. (1969) that showed experimental proof of a correlation between the absence of protective anti-meningococcal antibodies and increased susceptibility to meningococcal infection and disease. In contrast to newborn infants and individuals of >2 years of age, less than 30% of sera obtained from subjects within the 6 – 24 months age group were bactericidal against three disease isolates. Interestingly, the highest incidence of meningococcal disease was reported to occur within this age group i. e. 6 – 24 months, highlighting the lack of serum bactericidal activity as a potent risk factor in the development of meningitis and septicaemia. Furthermore, army training recruits that presented with non-bactericidal sera at the start of the training camp, as determined by the SBA, were more susceptible to the development of systemic meningococcal disease than recruits that presented with bactericidal sera at the start of training. Thus, bactericidal activity of sera, as determined by the SBA, could serve as a correlate of protection in individuals. The antigen used to derive the sera could subsequently be employed in vaccine preparations, as it would have been shown to be capable of inducing the production of protective antibodies against a meningococcal strain or a diverse panel of strains.

Protection from serogroup B meningococcal disease is currently sought from vaccines, whose main components are recombinant surface proteins and OMVs. Candidate OMPs must satisfy certain criteria before inclusion into potential vaccines; constitutive and abundant surface expression in all meningococcal strains in addition to high sequence conservation are key properties required of candidate OMPs. More importantly, the candidate OMP must be highly immunogenic, possessing the ability to elicit protective bactericidal antibodies in the human host.

A breakthrough in the identification of vaccine candidate OMPs was first presented by Pizza and co-workers (2000) in a process known as reverse vaccinology. This process

involved scanning the meningococcal genome for putative ORFs encoding OMPs before cloning and expressing these proteins using the *E. coli* machinery. Recombinant proteins were subsequently used to immunize mice for the production of antibodies, which were screened for reactivity to homologous antigens in a diverse panel of meningococcal strains. The bactericidal activity of these antibodies was subsequently explored. More than 500 putative ORFs were identified in the study, five of which encoded proteins that were highly conserved in different meningococcal strains (Pizza *et al*., 2000). While some of these OMPs were progressively employed in the development of a vaccine as a result of further comprehensive studies (Serruto *et al*., 2012), others were excluded from further use for reasons such as epitope mimicry (Granoff *et al*., 2001).

Studies stemming from reverse vaccinology led to the development of a multicomponent vaccine (Bexsero® by Novartis), whose 4 major OMP constituents are: the factor H binding protein, fHbp; *Neisseria* adhesin A, NadA; Neisserial Heparin Binding Antigen, NHBA; and an OMV preparation containing the PorA epitope P1.4 (Serruto *et al*., 2012). When individually measured against the vaccine candidacy criteria set, each of these proteins does not qualify as an ideal vaccine candidate. For example, NadA, a surface-exposed adhesin, is present in less than 60% of all meningococcal strains (Martin *et al*., 2003) and exhibits a novel form of phase-variable expression (Metruccio *et al*., 2009). Three variants of the factor-H binding protein, fHbp, sharing only 63% homology, exist and a sub-section of meningococcal strains express the protein at very low levels (Masignani *et al*., 2003). The third OMP component of the vaccine, the Neisseria heparin binding antigen NHBA is composed of several hypervariable regions leading to high levels of antigenic variation (Pizza *et al.,* 2000). Notwithstanding, the combination of these 3 OMPs, plus the OMV from a strain expressing the PorA P1.7-2,4 protein, is expected to compensate for the individual deficiencies of the vaccine constituents leading to the provision of protection against a wide variety of strains. This vaccine is reported to offer protection against at least 70% of serogroup B meningococci in western Europe (Frosi *et al.,* 2013). Improvements on this vaccine to provide better coverage against possibly all serogroup B strains will be sought by researching other OMPs that could, at least, satisfy some of the criteria listed above.

In the present study, the potential vaccine candidacy of the Hb receptors was explored. Antisera recognising these receptors were produced (see Chapter 4) and the ability of these antisera to mediate killing of meningococci and thus, protect against meningococcal infection using the SBA as a correlate of protection, was investigated.

6.1 Construction of over-expression plasmids

The cloning strategy outlined in section 2.9 was employed in the construction of plasmids containing approximately 1 kb of *hpuA* or *hmbR* fused to the altered *porA* promoter. The resulting plasmids, from several cloning attempts, always contained nonsense mutations in the sequences of the *hpuA* and *hmbR* fragments. The over-expression of *hpuA* or *hmbR* from the strong *porA* promoter may have been toxic to *E. coli* cells and led to selection against cells that harboured plasmids with intact sequences of the gene fragments. Nonetheless, strains MC58 and 8047 were transformed with the *hmbR* and *hpuA* over-expression plasmids, respectively, with the aim of selecting for transformants over-expressing *hpuA* or *hmbR*. While transformants with intact polyG tracts in *hpuAB* and *hmbR* were obtained from these transformations (as determined by methods outlined in section 2.4.5) (Tables 6.1 and 6.2; Figure 6.3), no *hpuA* or *hmbR* over-expressing strains were obtained despite the successful replacement of the native promoters with the constitutive *porA* promoter (Figures 6.1 and 6.2).
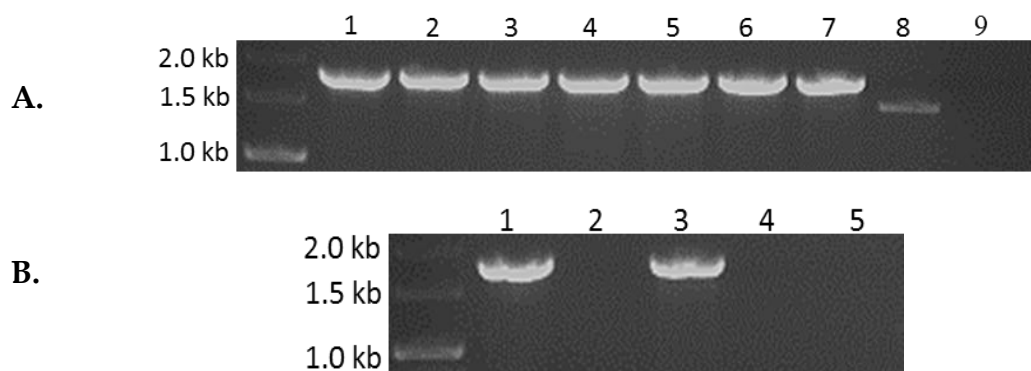

Figure 6.1: PCR screening of over-expression transformants, 8047-*hpuAB*+ and MC58-*hmbR*+. **(A)** The *hpuAB* over-expression plasmid, pFAB-38, was used to transform strain 8047. Boiled lysates from six transformants (lanes 2 - 7) were used as templates in a PCR in which primers overDUS-for and hpuR-seq were used. pFAB-38 was used as a positive control for the PCR and analysed in lane 1. Negative DNA, pFAB-39, and no-DNA controls were analysed in lanes 8 and 9 respectively. **(B)** Primers overDUS-for and hmbR-seqR were used to generate amplicons from pFAB-39 (lane 1) and both MC58-*hmbR*+ transformants (lanes 2 and 3). pFAB-38 (lane 4) and distilled water (lane 5) served as negative controls.
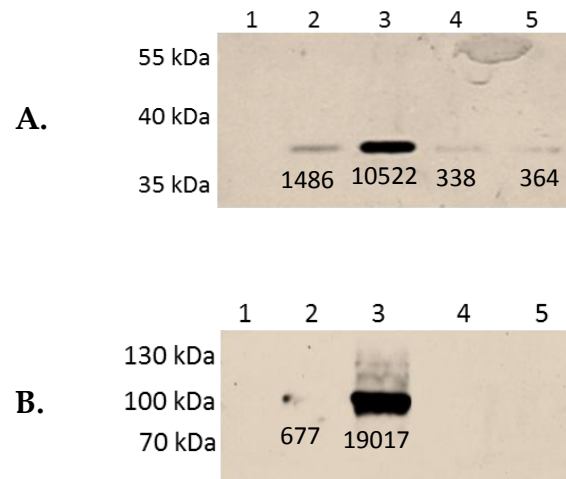
Figure 6.2: Expression of *hpuA* and *hmbR* in wild-type and over-expression strains. **(A)** A 1:500 dilution of anti-r8047-HpuA antisera was used to probe lysates of induced IT-8047-Δ*hpuAB* cells (lane 1), wild-type 8047 cells grown in iron-replete (lane 2) or iron-limited conditions (lane 3) and 8047-*hpuAB+* cells grown in the absence (lane 4) or presence (lane 5) of 30 μM desferal. **(B)** A 1:100 dilution of pooled anti-rHmbR mAbs was used to probe lysates of induced MC58-Δ*hmbR* cells (lane 1), wild-type MC58 cells grown in iron-replete (lane 2) or iron-limited conditions (lane 3) and MC58-*hmbR+* cells grown in the absence (lane 4) or presence (lane 5) of 30 μM desferal. A 1:2000 dilution of an anti-Mouse IgG HRP-conjugate was used to generate signals. Fold differences in signal strength (values below signals indicate signal strength) were measured using Image J software.

Table 6.1: PCR fragment lengths of 8047 wild-type and *hpuAB* over-expression strains. Measurement of the amplicon lengths was performed, as previously described. Peak heights correspond to the fluorescence intensity recorded for each amplicon.

| Sample	Fragment length	Peak height
8047 wild-type	308.2	1785
	307.2	1504
8047-*hpuAB*+ 4.1	305.3	5969
8047-*hpuAB*+ 4.2	305.3	8605
8047-*hpuAB*+ 4.3	305.4	7795
8047-*hpuAB*+ 4.4	305.2	10253
8047-*hpuAB*+ 4.5	305.4	10374
8047-*hpuAB*+ 4.6	305.3	14204
8047-*hpuAB*+ 4.7	305.4	5579
8047-*hpuAB*+ 4.8	305.3	11715
8047-*hpuAB*+ 4.9	305.4	8974
8047-*hpuAB*+ 4.10	305.2	5499
8047-*hpuAB*+ 4.11	305.3	8513
8047-*hpuAB*+ 4.12	305.3	7668
8047-*hpuAB*+ 4.13	305.3	6180
8047-*hpuAB*+ 4.14	305.2	7044
8047-*hpuAB*+ 4.15	305.4	4443
8047-*hpuAB*+ 4.16	305.3	9112
8047-*hpuAB*+ 4.17	305.4	10388
8047-*hpuAB*+ 4.18	305.3	12605
8047-*hpuAB*+ 4.19	305.4	10431
8047-*hpuAB*+ 4.20	305.2	9585
8047-*hpuAB*+ 4.21	305.4	8223
8047-*hpuAB*+ 4.22	305.3	11075
8047-*hpuAB*+ 4.23	305.4	6895
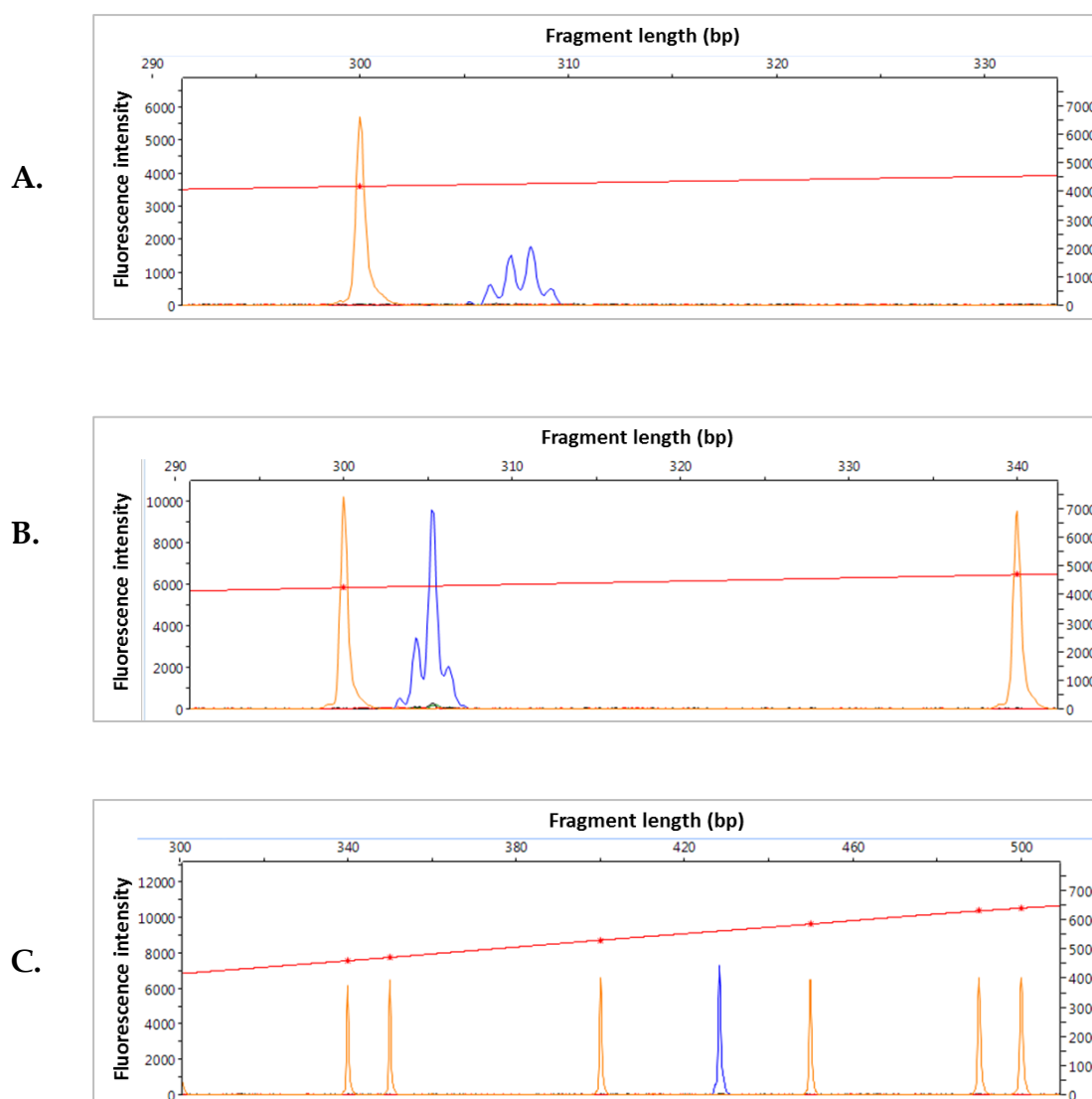
Figure 6.3: Measurement of polyG tract lengths in wild-type and over-expression strains. Fluorescently-labelled primers (hpuAC-VIC for *hpuA* and RF3-6FAM for *hmbR*) were used to generate fluorescently-tagged amplicons of *hpuA* and *hmbR* from wild-type and over-expression strains of 8047 and MC58 respectively. After A-tailing, to ensure all amplicons contain the extra adenine residue incorporated by *Taq* polymerase, samples were loaded in individual wells of a 96-well plate containing a size standard and formamide. Measurement of the PCR fragment lengths was done on a sequencer and data were analysed on the Peak Scanner software. Screenshots from the data analysis software shown above were taken from the analysis of the **(A)** 8047 wild-type and **(B)** 8047-*hpuAB*+ 4.20 for *hpuA* analysis and the MC58 wild-type **(C)** for *hmbR* analysis.

Table 6.2: PCR fragment lengths of MC58 wild-type and *hmbR* over-expression strains. Measurement of the amplicon lengths was performed, as previously described. Peak heights correspond to the fluorescence intensity recorded for each amplicon.

Sample	Fragment length	Peak height
MC58 wild-type	428.3	7288
MC58-*hmbR*+ 1.1	428.2	9432
MC58-*hmbR*+ 1.6	428.3	15966
MC58-*hmbR*+ 2.1	428.1	8324
MC58-*hmbR*+ 2.2	428.2	18033
MC58-*hmbR*+ 2.3	428.2	24809
MC58-*hmbR*+ 2.4	428.4	1082
MC58-*hmbR*+ 2.5	428.1	25457
MC58-*hmbR*+ 3.1	428.2	4878
MC58-*hmbR*+ 3.2	428.4	1361
MC58-*hmbR*+ 3.3	428.2	2646
MC58-*hmbR*+ 3.5	428.3	2904
MC58-*hmbR*+ 3.6	428.3	1207
MC58-*hmbR*+ 3.7	428.3	1891
MC58-*hmbR*+ 3.8	428.4	2084
MC58-*hmbR*+ 3.9	428.2	3081
MC58-*hmbR*+ 3.10	428.4	1839
MC58-*hmbR*+ 4.1	428.1	5665
MC58-*hmbR*+ 4.2	428.3	6791
MC58-*hmbR*+ 4.3	428.3	2347
MC58-*hmbR*+ 4.4	428.4	6882
MC58-*hmbR*+ 4.5	428.3	4732
MC58-*hmbR*+ 4.6	428.3	4661
MC58-*hmbR*+ 4.7	428.2	6563
MC58-*hmbR*+ 4.8	428.4	8531
MC58-*hmbR*+ 4.9	428.1	866
MC58-*hmbR*+ 4.10	428.3	5043
MC58-*hmbR*+ 4.11	428.2	3512
MC58-*hmbR*+ 4.12	428.3	3895
MC58-*hmbR*+ 4.13	428.2	4318
MC58-*hmbR*+ 4.14	428.3	3350
MC58-*hmbR*+ 4.15	428.3	3743
MC58-*hmbR*+ 4.16	428.3	6029
MC58-*hmbR*+ 4.17	428.2	6689

6.2 Use of iron-starved meningococcal cells in serum bactericidal assays

The expression of *hpuA* and *hmbR* from the *porA* promoter appeared to be detrimental to the *E. coli* cells harbouring the over-expression plasmids. Consequently, only cells with mutated *hpuA* or *hmbR* sequences were retrieved. In the absence of over-expression plasmid constructs containing intact *hpuA* or *hmbR* sequences, this study proceeded with the use of iron-starved meningococcal cells in serum bactericidal assays. This method was employed in a host of vaccine candidacy studies where the TbpA and TbpB proteins were assessed for inclusion in a meningococcal vaccine (Danve *et al.*, 1993; Rokbi *et al.*, 1997; West *et al.*, 2001).

Iron starvation was achieved using 30 μM desferal in either MH broth or BHI broth. Active human complement used in the assay was derived from volunteers whose sera had been used previously in immune escape assays as complement source (Tauseef *et al.,* 2013). Sera from these volunteers lacked bactericidal activity in the absence of antibodies to meningococcal proteins, thus were chosen for use in this study. Recruitment of blood donors and the subsequent preparation of the serum samples were done by Isfahan Tauseef.

6.2.1 Sensitivity of meningococcal strain 8047 to human complement

The background bactericidal activity of the human complement (PC) in the absence of antibodies was first assessed using strain 8047 (see section 2.10 for experimental method). This strain was incapable of survival in human blood, thus, it was important to test its serum sensitivity levels prior to performing the SBA. In the immune escape assay of Tauseef *et al*. (2013), the final concentration of the exogenous complement source (serum obtained from the same cohort of volunteers that provided PC) was 5% as opposed to the 20% or 25% used in the SBAs of Mountzouros and Howell (2000) and Borrow *et al*. (2005). The 5% concentration of the complement source appeared to be an optimum level as it was incapable of causing bacteriolysis of strain 8047 in the absence of a bactericidal antibody. In addition, efficient killing of strain 8047 was observed when anti-PorA P1.2 mAb was combined with 5% complement (Tauseef *et al.*, 2013).

To show that a population of 8047 cells was not affected by 5% complement but killed by 20% complement, a comparison between both concentrations of PC was performed

in a serum sensitivity assay. Cells grown in both iron-replete (BHI broth only) and iron-restricted conditions (BHI broth + 30 μM desferal) were used in the assay. As expected, a population of strain 8047 cells was reduced to undetectable levels in the presence of 20% complement. On the other hand, a similar decrease in CFU counts was not discovered in the presence of 5% complement indicating an inability of this amount of complement to cause bacteriolysis of 8047 cells (Figure 6.4).
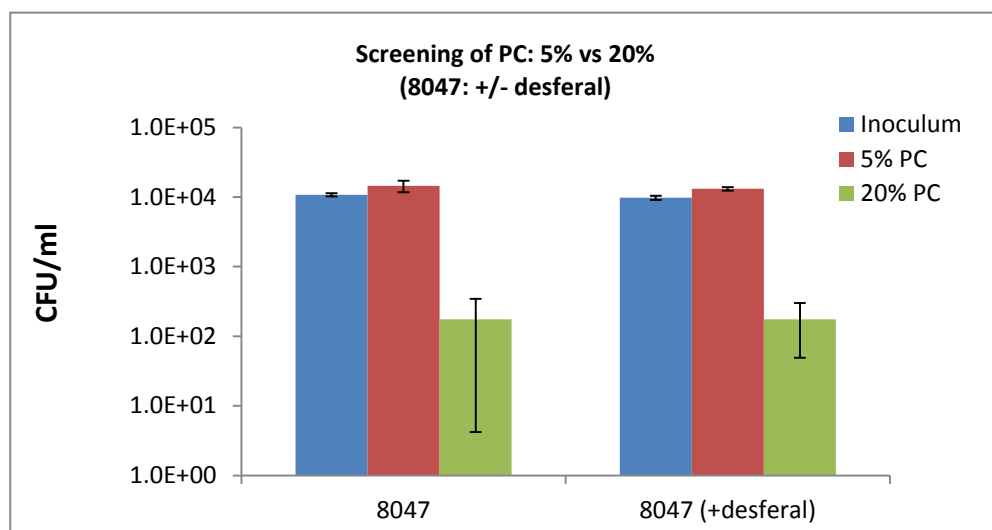

Figure 6.4: Effect of complement concentration on sensitivity of strain 8047 to pooled human complement (PC). Cell suspensions of strain 8047 were prepared from iron-replete or iron-restricted conditions. Approximately 10^4 cells of each preparation was mixed with either 5% or 20% of PC. 10 μl of each assay mixture was plated on blood agar plates, in duplicate, before incubation at 37^0C with 5% CO_2 for 60 minutes. Plating was repeated for all samples after the 60-minute incubation. The mean of 4 CFU counts are plotted above and the standard error of the mean of all 4 counts (SEM) is represented by the error bars.

6.2.2   Bactericidal activity of anti-rHpuA antisera on strain 8047

To study the bactericidal activity of anti-rHpuA antisera using wild-type 8047 and IT-8047Δ*hpuAB* as the target strains, SBAs were performed with 5% PC as exogenous complement source. The bactericidal anti-PorA P1.2 mAb was also used in the assay to show that 5% PC was sufficient to kill cells in the presence of bactericidal antibodies.

Consistent with results from the serum sensitivity assay, the population size of both 8047 and IT-8047Δ*hpuAB* was not negatively affected by 5% PC in the absence of bactericidal antibodies. Addition of varying concentrations of anti-PorA P1.2 mAb produced a bactericidal effect that was equal for all concentrations. 1:40 to 1:640 dilutions of the mAb completely killed both strains in the presence of 5% PC (Figure

6.5). This bactericidal effect was not replicated when heat-inactivated PC was provided as complement source or when cells were incubated with the P1.2 mAb without human complement.

The bactericidal effect produced by the synergy between anti-PorA P1.2 mAb and 5% PC was not reproduced with anti-rHpuA antisera. Instead of a decrease in CFU counts relative to the inoculum, the populations of both 8047 wild-type and IT-8047Δ*hpuAB* cells increased 1.5 to 2 fold at the end of the experiment (Figure 6.5). These results suggest that the anti-rHpuA antisera are incapable of mediating complement-dependent bacteriolysis of strain 8047 cells.
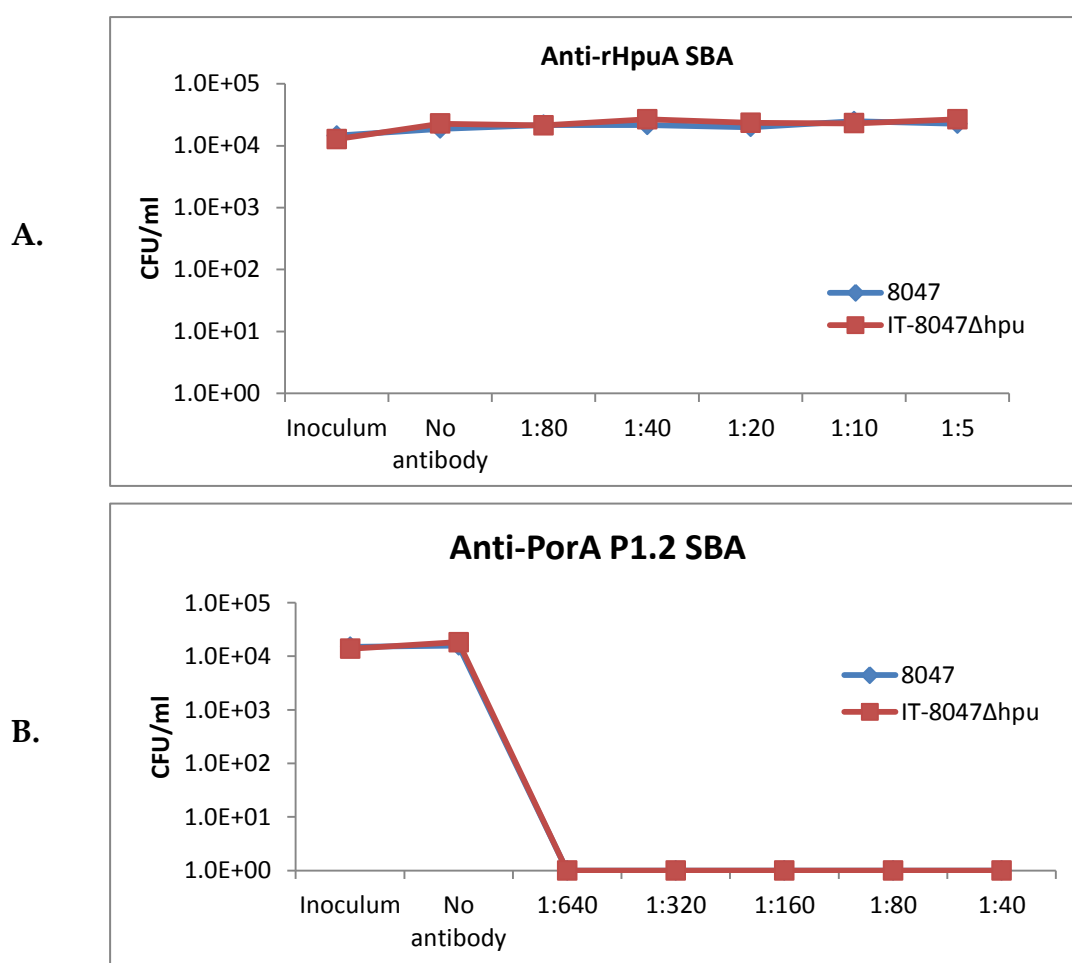
A.

B.

Figure 6.5: Serum bactericidal assay of pooled anti-rHpuA antisera and PorA P1.2 mAb. Cell suspensions were prepared as previously described. 10^4 CFU from these cell suspensions were mixed with pooled human complement (final concentration of 5%) and dilutions of anti-rHpuA antiserum ranging from 1:5 to 1:80 in a final assay volume of 50μl, in duplicate **(A)** or 1:40 to 1:640 of the anti-PorA P1.2 mAb **(B)**. The assay mixtures were subsequently incubated at 37^0C for 60 minutes, without shaking Control experiments were set-up as follows: cells + complement; cells + inactive complement; and cells + antibody + inactive complement.. CFU measurements of inoculum (T_0) and output (T_{60}) cultures were performed by plating 10μl of each mixture on BHI agar supplemented with Levinthal's supplement, in duplicate. The mean of 4 counts (2 counts per assay) was plotted in the figure above.

6.2.3 Bactericidal activity of monoclonal and polyclonal anti-HmbR antibodies

Anti-rHmbR antisera from 10 mice were first tested for reactivity to HmbR in the MC58 wild type and Δ*hmbR* mutant backgrounds. Sera were pooled in groups of two and western blotting was performed as outlined in section 2.5.3. Sera from 3 pools (Pools P1 to P3) were non-reactive to the Δ*hmbR* mutant and showed little reactivity to the uninduced cell lysates. As expected, an increase in *hmbR* expression was observed in lysates of iron-starved cells. Sera from the remaining 2 pools (P4 and P5) produced signals that were stronger than P1, P2 and P3 but also reacted with a protein, similar in size to HmbR, in the Δ*hmbR* mutant (Figure 6.6).

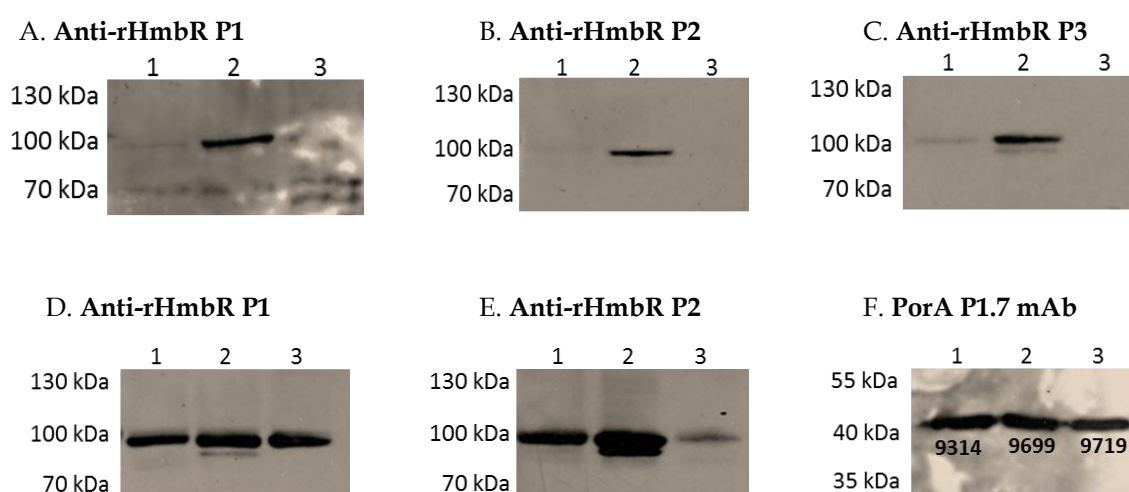**Figure 6.6**: Anti-rHmbR antisera reactivity to MC58 and MC58Δ*hmbR* lysates. Cells of strain MC58 and MC58Δ*hmbR* were grown in iron-limited conditions to induce expression of *hmbR*. Wild-type MC58 cells were also grown in iron-replete conditions. Lysates of 3 cultures: uninduced MC58 wild-type (lane 1), induced MC58 wild-type (lane 2) and induced MC58Δ*hmbR* (lane 3) were used as templates in western blots. Western blots were performed as previously described. Anti-rHmbR antisera from 10 mice were pooled into groups of two and a 1:500 dilution was used to probe meningococcal lysates as follows: **(A)** Mouse 215 and Mouse 222; **(B)** Mouse 211 + Mouse 225; **(C)** Mouse 212 + Mouse 221; **(D)** Mouse 213 + Mouse 223; and **(E)** Mouse 214 + Mouse 224. A western blot was also performed with a 1:2500 dilution of the PorA P1.7 mAb to compare sample amounts (signal intensities were measured using the ImageJ software and the values recorded are indicated below each signal on the blot). Note that all blots were exposed for the same duration of 5 minutes.

To study the bactericidal activity of anti-HmbR antibodies (monoclonal and polyclonal) using strain MC58 and MC58Δ*mbR* as the target strains, SBAs were performed with 20% PC as exogenous complement source. The bactericidal anti-PorA P1.7 mAb was also used in the assay to show sensitivity of the target strains to antibody-mediated complement-dependent bacteriolysis. Sera from P1, P2 and P3 mice (see Figure 6.6 above) were subsequently pooled and dilutions ranging from 1:20 to 1:320 were tested for bactericidal activity on uninduced and induced cells of strain

MC58. Sera from P4 and P5 mice were excluded because of the background reactivity to a protein in the Δ*hmbR* mutant.

No significant bactericidal activity (more than 50% reduction in CFU) was observed for neither pooled anti-HmbR mAbs nor anti-rHmbR antisera on induced cells. (Figure 6.7). Significant reductions in CFU counts were, however, observed when the PorA P1.7 mAb was added to cell suspensions in the presence of complement. 50% killing was obtained with the 1:320 dilution and percentage survival of strain MC58 decreased substantially in the presence of higher concentrations of the P1.7 mAb i. e. 1:40 – 1:160.
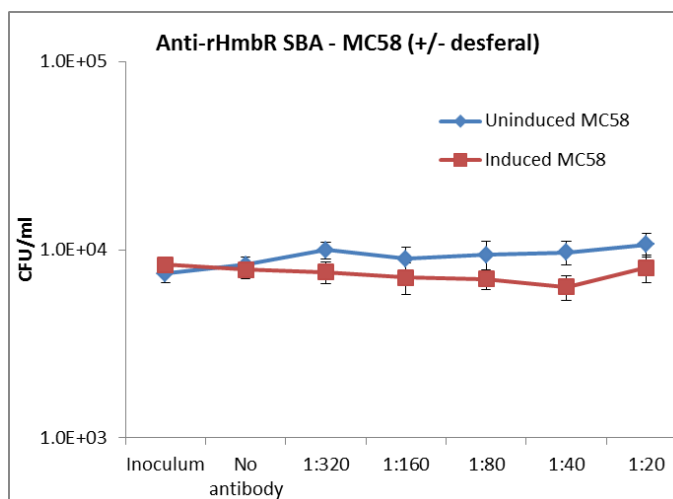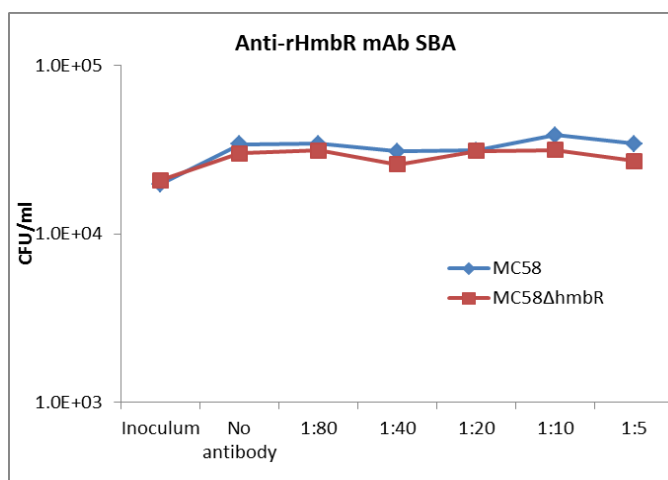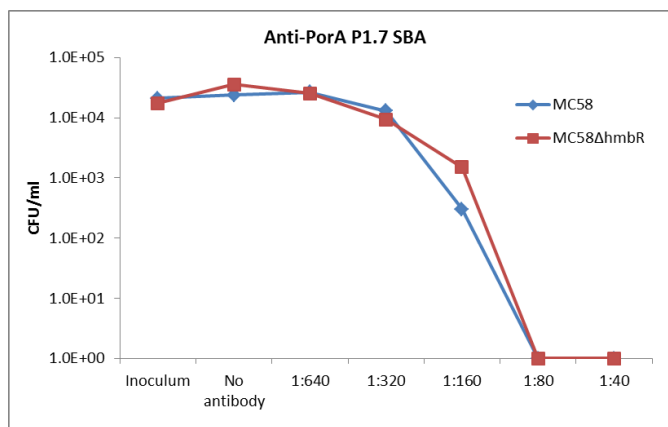
Figure 6.7: Serum bactericidal assay of anti-HmbR mAb and polyclonal antisera. The experiment was performed as previously described using MC58 wild-type and Δ*hmbR* strains as target. Antibody dilutions employed were 1:20 - 1:320 (polyclonal anti-rHmbR antisera – **panel A**), 1:5 – 1:80 (anti-HmbR mAbs – **panel B**) and 1:40 - :640 (PorA P1.7 mAbs – **panel C**). CFU measurements were calculated as described in Figure 6.7. Two independent experiments were performed on separate days for the pooled anti-rHmbR antisera but once for the PorA P1.7 and anti-HmbR mAbs. The mean of 8 counts (2 counts per assay per day) or 4 counts (2 counts per assay) was plotted in the figure above and error bars represent the standard error of the mean.

6.3   **Discussion**

In this study, antisera generated in mice against HpuA and HmbR were assessed for an ability to induce complement-mediated killing in an *in vitro* serum bactericidal assay. These antisera had been shown previously to interact with wild-type meningococcal 8047 and MC58 strains, respectively but not the mutants or PV-OFF variants of both genes (see section 4.8). Using iron starved cells, neither the polyclonal antisera of rHpuA and rHmbR nor the anti-HmbR mAbs were capable of recruiting complement to kill the meningococcal cells. These iron-starved 8047 and MC58 cells were, however, sensitive to killing by the PorA P1.2 and P1.7 mAbs respectively.

While it was unsurprising that the anti-HmbR mAbs failed to mediate killing of MC58 cells due to lack of interactions between the mAbs and target surface epitopes of HmbR, the apparent inability of the anti-rHpuA and anti-rHmbR antisera to mediate killing of the meningococcal cells could be a consequence of the relatively low levels of the target surface-exposed epitopes, despite increased expression levels following iron starvation. The effect of a paucity of surface-expressed targets on discernible bactericidal activity was shown in a study by Giuntini *et al*. (2012) where up to 10-fold higher amounts of anti-fHbp antibodies were required to elicit complement-mediated meningococcal bacteriolysis of wild-type cells when compared to a mutant strain over-expressing fHbp. Replacing the Fur-regulated *hpuAB* and *hmbR* promoters with an engineered version of the strong constitutive promoter of *porA* was unsuccessful possibly due to the toxicity of the gene products to the *E. coli* vector. An inducible promoter that will drive the expression of *hpuA* and *hmbR* could be considered as a replacement for the constitutive *porA* promoter in the construction of the over-expression plasmid vectors. A further consideration, though, is the effect of the selected inducer on the sensitivity or resistance of the meningococcal cells to the complement complex.

Qualitative assessments of the different IgG subclasses in the antisera may also elucidate the reasons for the non-bactericidal properties of these antisera. It is anticipated that such assessments would reveal low levels of the IgG1 and IgG3 antibody isotypes in these antisera, especially IgG1, as these isotypes are associated with an increased induction of complement-mediated bacteriolysis, in contrast to the low SBA activity associated with IgG2 antibodies (Aase *et* al., 1998; Giuntini *et al.*,

2012). It would also be interesting to investigate whether these antibodies are capable of inducing bacteriolysis when combined with other non-bactericidal antibodies targeting different surface proteins; the backdrop for this investigation being the study reported by Weynants *et al*. (2007) (see Chapter 5, pp. 148-149).

In conclusion, the inability of antibodies to mediate killing in an SBA does not translate into an overall inability to provide protection against meningococci. West *et al*. (2001) reported an incongruity between results obtained from SBAs and passive protection assays in mice. Antibodies produced in mice using an rTbpA immunogen were non-bactericidal in SBAs but the same rTbpA antigen, when used as a vaccine, protected mice from meningococcal infection. Against this backdrop, the anti-rHpuA / anti-rHmbR antisera may still be valuable in limiting meningococcal infection. This could be via the promotion of opsonophagocytosis, which has been described for a non-bactericidal IgG3 antibody preparation induced in recipients of the Norwegian serogroup B meningococcal vaccine (Aase *et al*., 1998), or via the inhibition of Hb utilisation by preventing interactions between HpuA / HmbR and Hb or Hb complexes.

CHAPTER 7

CONCLUSION AND RECOMMENDATIONS

The pathogenic Neisseria spp., *N. meningitidis* and *N. gonorrhoeae*, attract considerable research attention because of the significant threat they pose to human health and well-being. *N. meningitidis* is responsible for a large proportion of bacterial meningitis cases and limiting the impact of the disease via preventive measures requires a thorough understanding of the factors that aid survival and proliferation of the pathogen in the human host. In a bid to tackle the devastating disease caused by the meningococcus, surface proteins have been utilised as the major constituents of a vaccine that could provide protection against a wide variety of strains, including serogroup B meningococci (Gorringe and Pajon, 2012). This vaccine, which is yet to be included in routine immunisation schedules in the UK, Europe and other parts of the western world, has an estimated strain coverage of 73% - 91% in Europe and the US (Frosi *et* al., 2013; Granoff, 2013). There is, therefore, a need to identify other OMP targets which, could be included in future vaccines that would aid in the total eradication of invasive meningococcal disease.

The ability of the meningococcus to utilise human haemoglobin, in both simple and complex forms, via its phase-variable Hb receptors HpuAB and HmbR is widely believed to be a crucial virulence factor. Previous genetic studies (Harrison *et* al., 2009; Tauseef *et al*., 2011), the attenuated virulence of a Δ*hmbR* mutant in a rat infection model (Stojiljkovic *et al*., 1995) and an accidental *in vivo* human passage (Omer *et al*., 2011) provide a backdrop for this hypothesis and also imply that loss of the expression of both receptors, potentially via phase variation, will pose a severe disadvantage to virulence of the pathogen. However, there is no published experimental evidence of the implication of the non-expression of *hpuAB* and/or *hmbR* via PV during systemic infection in humans.

In contrast to the rat infection model employed by Stojiljkovic *et al*. (1995), findings from an experimental *ex vivo* human whole blood model employed in this study did not show a significant role for HmbR during growth in human whole blood. This disparity between results reported by Stojiljkovic *et al*. study and those obtained in this

study could have stemmed from the type of meningococcal strain used but more importantly, the infection models employed in both studies. Since meningococci are strictly host-specific, the behaviour of the pathogen in the rat infection model will be markedly different from its behaviour in human whole blood. One factor that would mitigate against the viability of the pathogen in the rat model is the unavailability of several iron sources, including Tf and Lf, to the pathogen (Schryvers and Gonzalez, 1989; Schryvers and Gonzalez, 1990).

On the other hand, the fact that the *hmbR*-OFF (or Δ*hmbR* phenotype as utilised in this study) did not pose any measurable impact on growth of the meningococcus in this study corroborates data produced from the *in vivo* passage study where a switch in *hmbR* PV status from ON in the inoculum strain to OFF in the disease isolate was discovered (Omer *et al.*, 2011). One theory that stems from this study suggests, therefore, that free haemoglobin was in minute quantities in the blood samples used in this whole blood model and may have been available only in complexed forms making it unavailable to strain MC58 owing to a lack of the HpuAB receptor. If this is the case, data from this study and the *in vivo* passage study strongly imply a more important role for HpuAB than the HmbR receptor in Hb utilisation during disease. Experimental evidence for this hypothesis was not obtainable in this study as the *hpuAB*-expressing strain 8047 was highly sensitive to killing in the human blood samples. Future studies would involve the generation of a *hpu*-expressing strain in the MC58 background (both with and without *tbp*). This strain could subsequently be tested in whole blood assays. Another alternative is to screen other *hpuAB*-expressing strains for sensitivity in human blood. Strains that are able to proliferate in the blood may be subsequently employed in assessing the importance of *hpuAB* to blood-bound meningococcus.

The results from this whole blood model, with regards to the importance of HmbR, still require validation with improved infection models. A limitation to the use of this whole blood model was that the model simulated only the early stages of an infection where iron-loaded Tf is more available than at later stages (Letendre, 1987; Cassat and Skaar, 2013). This could explain why presence of the TbpBA receptor significantly contributed to growth of meningococci during the entire duration of the assay. One would expect in an ideal infection model that a decrease of Tf levels over time would have occurred concomitant with increase in Hb levels caused by cytotoxin-mediated

haemolytic anaemia. Total sequestration of available haptoglobin molecules would also have contributed to this increase in free Hb levels. In such a model, an important role for HmbR, if existent, could have been shown.

This study also elucidated the reason for the apparent over-representation and expression of the Hb receptors in meningococcal disease isolates. These receptors were immunogenic in mice by virtue of the production of specific antibodies that recognised both membrane-bound and surface-exposed regions of the proteins. There were also strong indications from an immunodetection assay performed in this study that antibodies targeting HpuA were induced during carriage in healthy humans. A severe consequence for this immunogenicity to the meningococcus was not observed as antibodies targeting these receptors were non-bactericidal. One is tempted to presume that since the serum bactericidal assay is an immunological correlate of protection, an immune selection, even in the presence of an immune response, against the expression of these Hb receptors during carriage or disease is non-existent. This would subsequently explain why these Hb receptors are commonly found in a PV-ON state in a highly substantial proportion of carriage and disease isolates (Tauseef *et al*., 2011). Selection for phase variants of these Hb receptors could then be attributed to nutrient requirements rather than evasion of the immune response. Further studies are needed to assess if a synergistic bactericidal activity between these antibodies and other meningococcal antibodies is existent. In addition, an evaluation of the ability of these antibodies to either mediate opsonophagocytosis of the meningococcus or inhibit Hb utilisation are required.
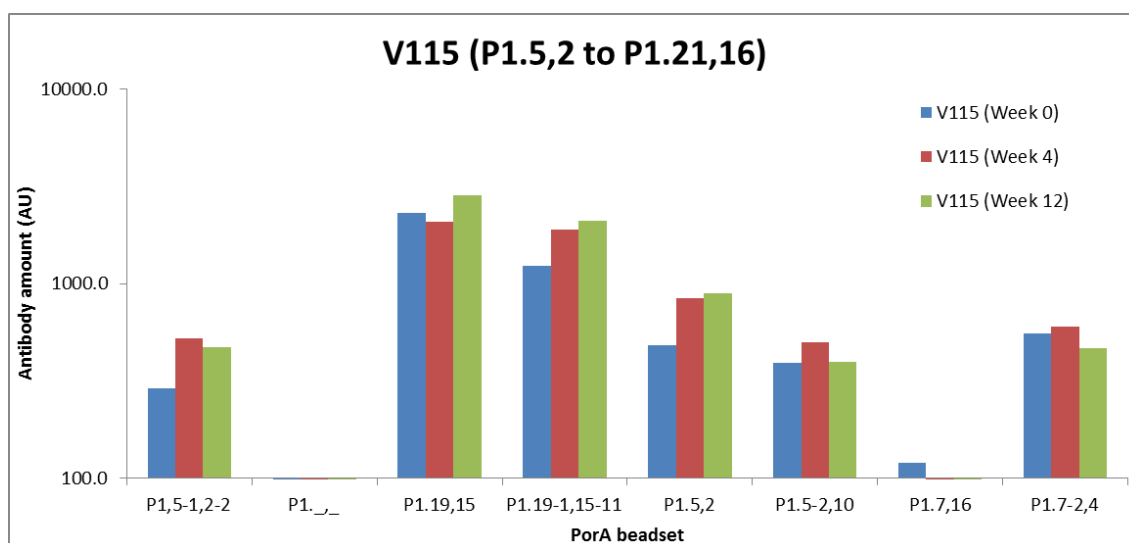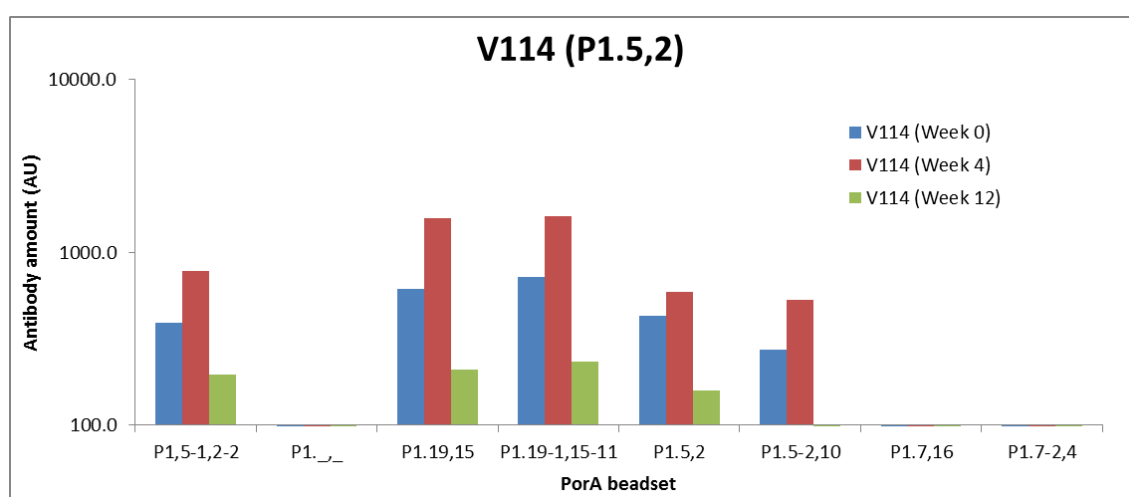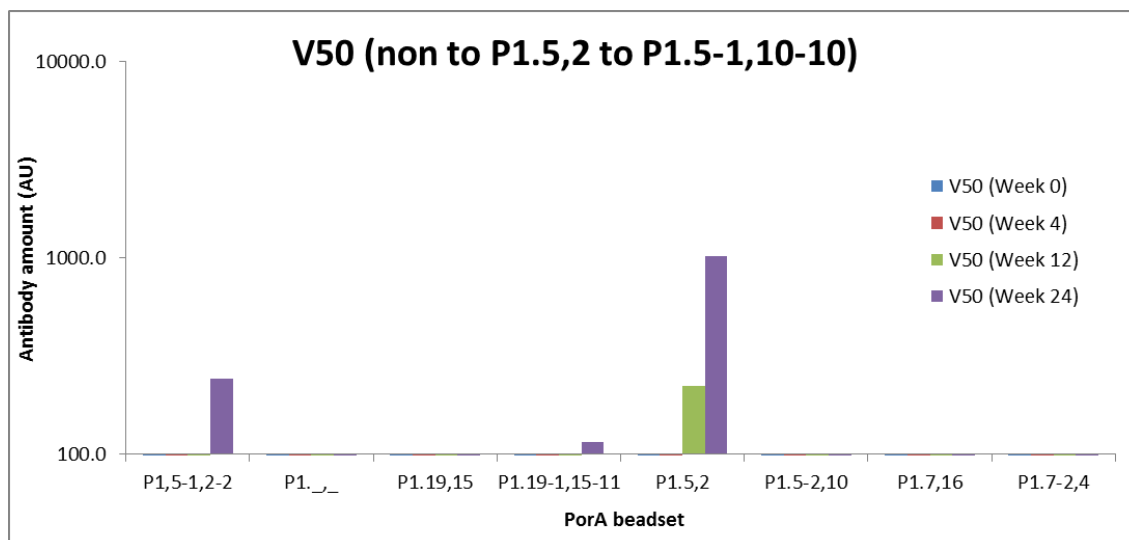
As stated earlier, the generation of phase variants could contribute to the persistence of a bacterial population in the host environment by mediating escape of the host immune system. Experimental evidence for this hypothesis abound in studies that have employed *in vitro* immune escape assays (Bayliss *et al*., 2008; Tauseef *et al*., 2013). Using the phase-variable PorA protein as subject, a similar PV-mediated immune escape during long-term meningococcal carriage was not found. The apparent deduction, however, was that *in vivo* selection was polygenic i. e. several OMPs are targeted by the immune system and selection could occur against one or more genes concurrently. A multi-gene approach rather than the single PorA protein used in this
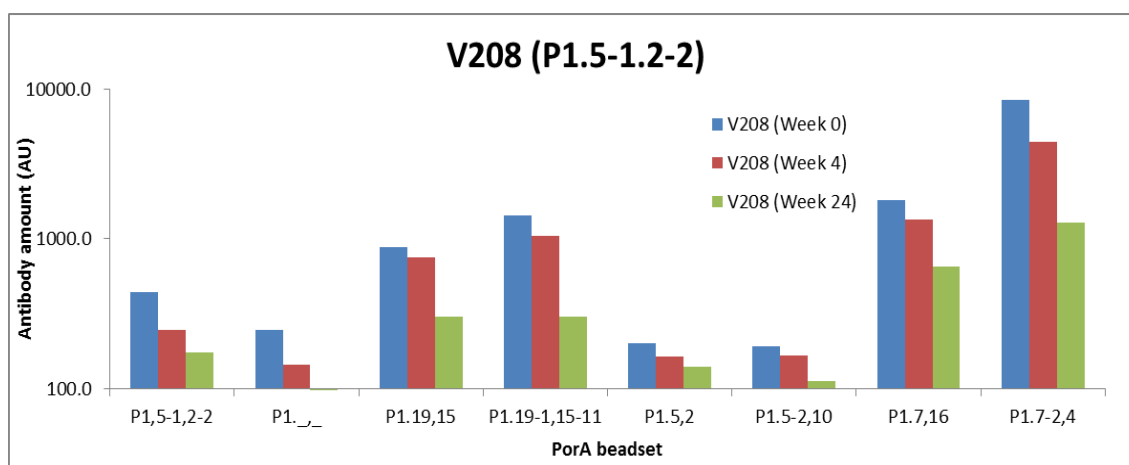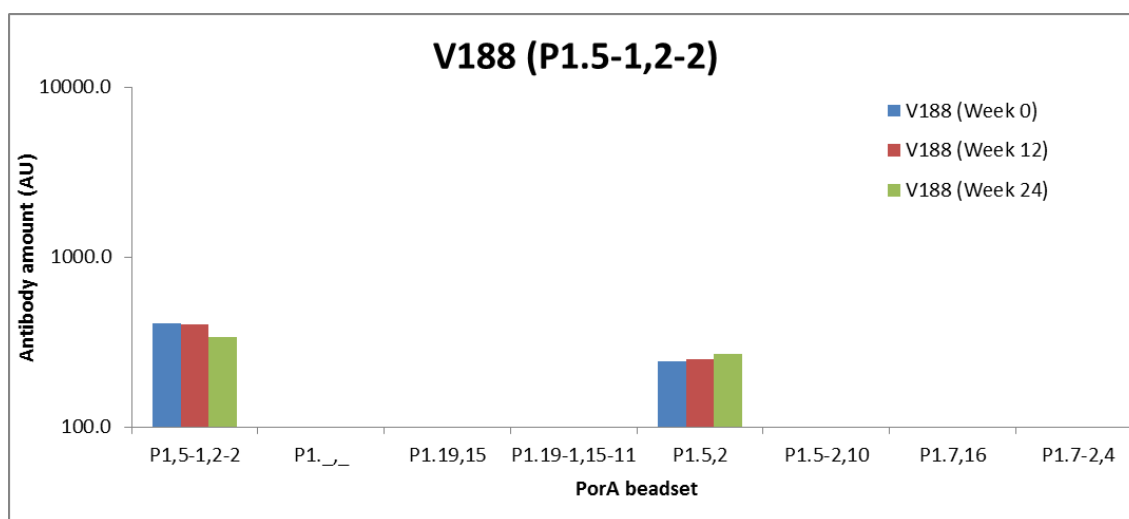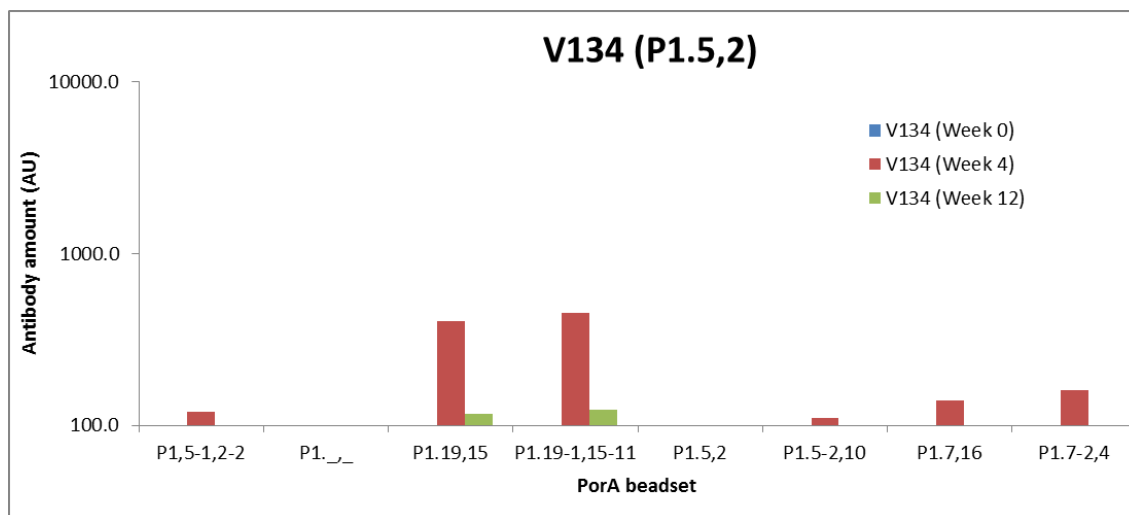
study may be more useful in studying associations between PV expression and the induction of specific antibodies.

In summary, this study reports for the first time that phase variation of *hmbR* is inconsequential to meningococcal virulence. This study also posits that *hpuAB* may be more beneficial to the meningococcus during the later stages of an infection because of its interactions with a more diverse panel of haemoglobin complexes. By virtue of the lack of bactericidal activity and the high level of antigenic specificity of polyclonal mouse anti-rHpuA and anti-rHmbR antisera reported in this study, the meningococcal Hb receptors are not believed to be viable vaccine candidates. More studies are required to ascertain the exact nature of the biological role of phase variation of *hmbR* and *hpuAB* during carriage and disease. This role is, however, not expected to be related to immune evasion as the immune response to both receptors are individually non-bactericidal.

APPENDIX

PorA LIQUICHIP DATA

V50 (non to P1.5,2 to P1.5-1,10-10)

Legend: V50 (Week 0), V50 (Week 4), V50 (Week 12), V50 (Week 24)

X-axis (PorA beadset): P1,5-1,2-2 · P1._,_ · P1.19,15 · P1.19-1,15-11 · P1.5,2 · P1.5-2,10 · P1.7,16 · P1.7-2,4

Y-axis: Antibody amount (AU)

V114 (P1.5,2)

Legend: V114 (Week 0), V114 (Week 4), V114 (Week 12)

X-axis (PorA beadset): P1,5-1,2-2 · P1._,_ · P1.19,15 · P1.19-1,15-11 · P1.5,2 · P1.5-2,10 · P1.7,16 · P1.7-2,4

Y-axis: Antibody amount (AU)

V115 (P1.5,2 to P1.21,16)

Legend: V115 (Week 0), V115 (Week 4), V115 (Week 12)

X-axis (PorA beadset): P1,5-1,2-2 · P1._,_ · P1.19,15 · P1.19-1,15-11 · P1.5,2 · P1.5-2,10 · P1.7,16 · P1.7-2,4

Y-axis: Antibody amount (AU)

V134 (P1.5,2)

V188 (P1.5-1,2-2)

V208 (P1.5-1.2-2)

V64 (P1.5-1,10-1 to P1.17,9)

V93 (non to P1.5-1,10-1)

V96 (non to P1.5-1,10-1)

V117 (P1.5-1,10-1)

V124 (P1.5-1,10-1 to P1.7,30)

V222 (P1.5-1,10-1)

V100 (non to P1.21-7,16)

V176 (P1.19,15)

V131 (P1.19-1,15-11)

V43 (P1.21,16)

V51 (P1.21,16)

V54 (P1.21,16)

V58 (P1.21,16)

V88 (P1.21,16)

V138 (P1.21,16)

V70 (non to P1.21-7,16 to P1.7,30)

V128 (P1.5-1,10-1 to P1.5,2-12 to P1.5-1,10-1)

52 (P1.21,16; cleared at 423)

Non-carrier data: Note scale is from **10 AU** to 10000 AU

V103 (non-carrier)

V172 (non-carrier)

V202 (persistent non-carrier)

V225 (Non-carrier)

V125 (Non-carrier)
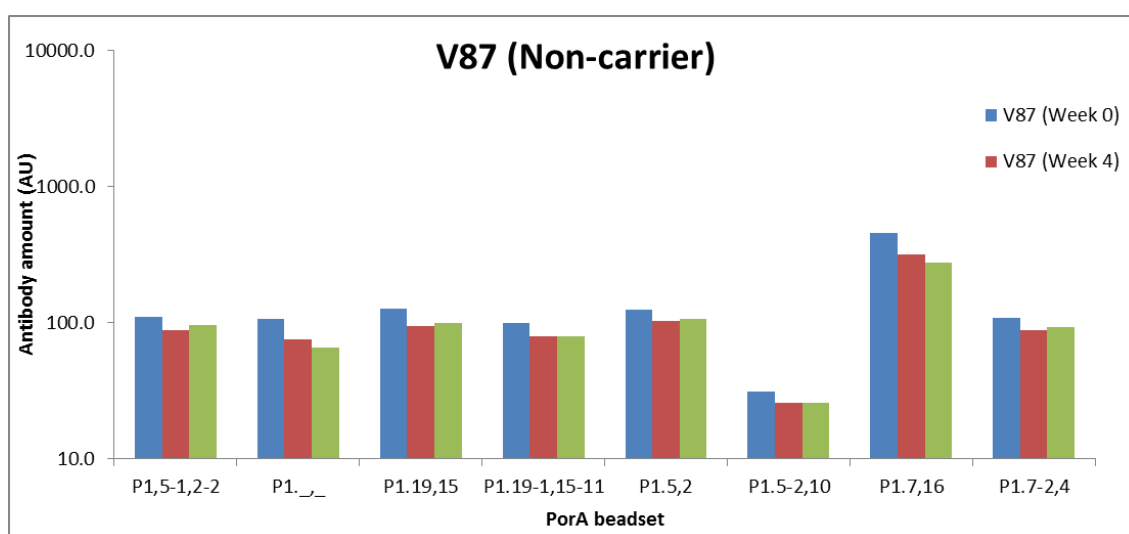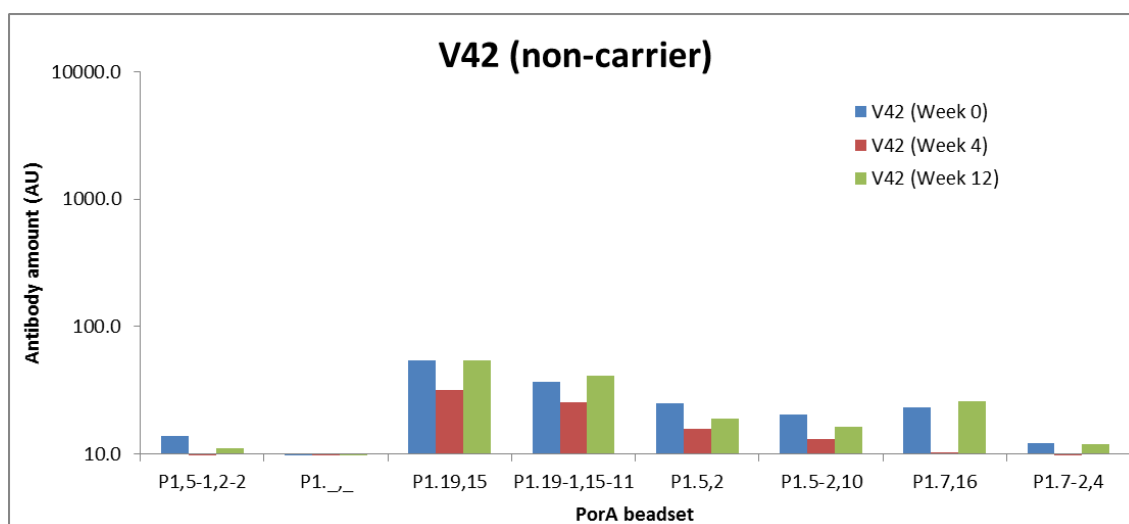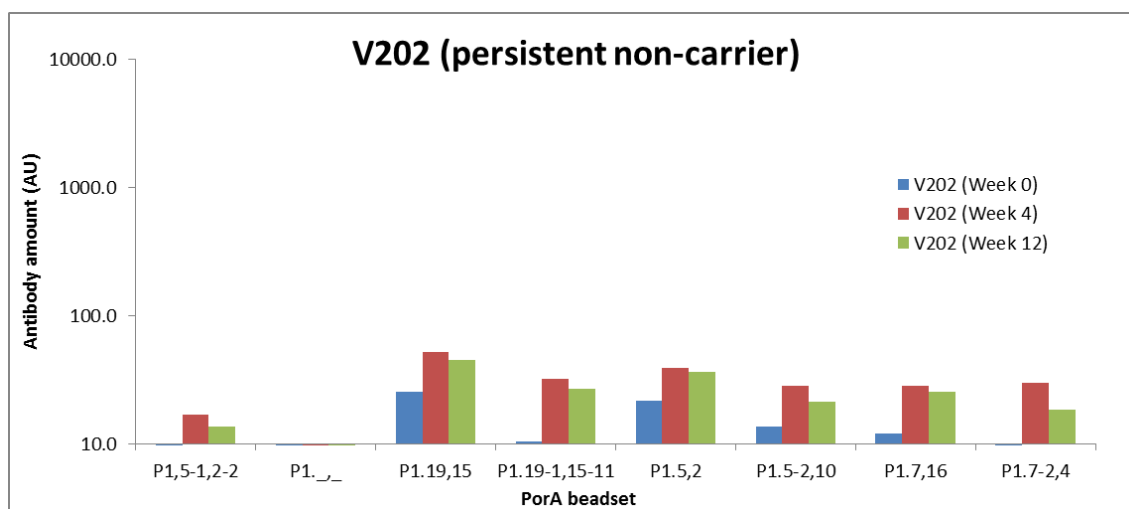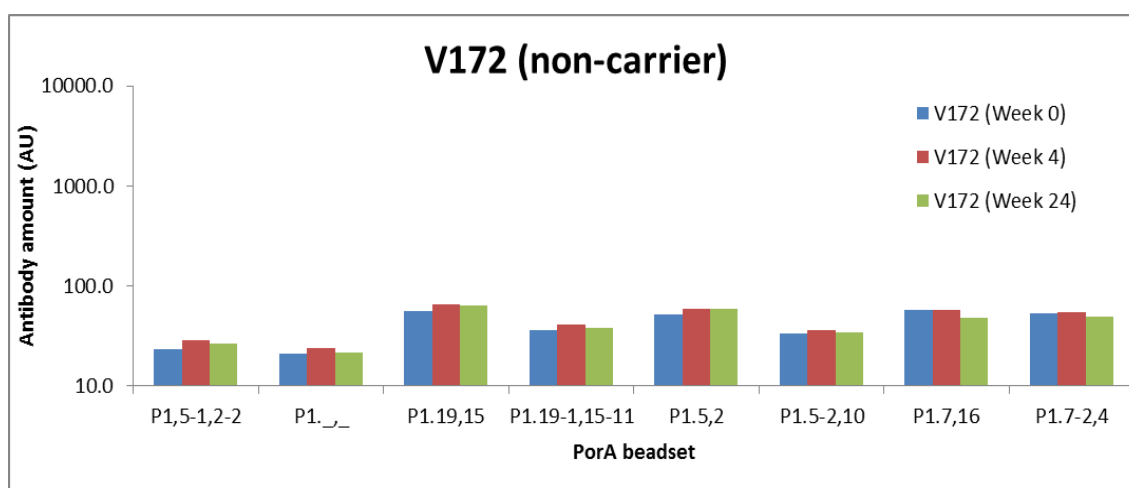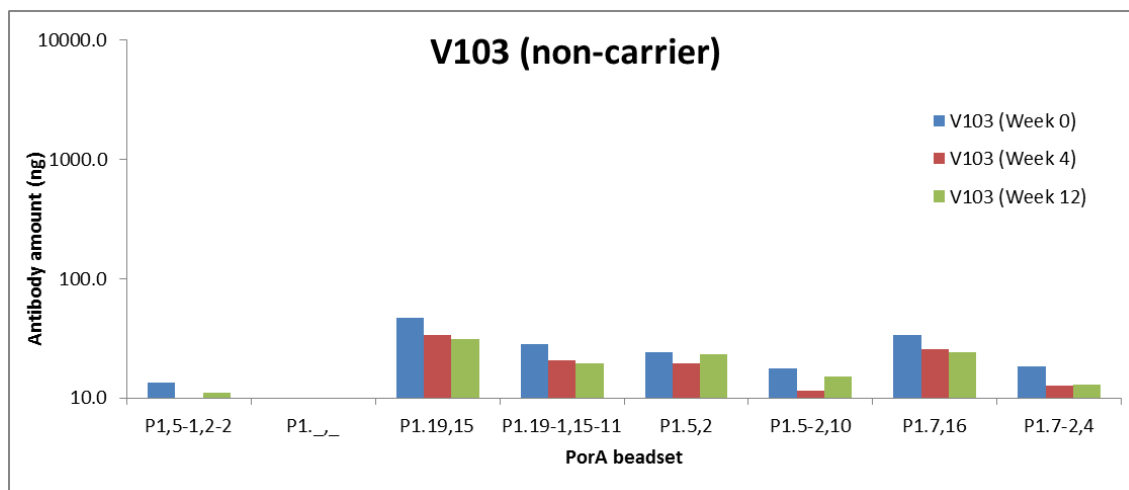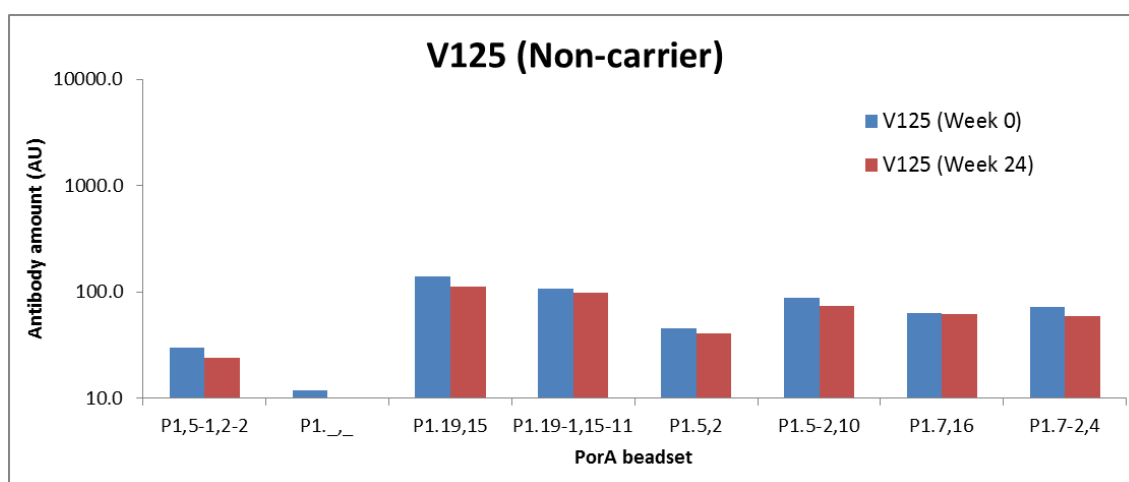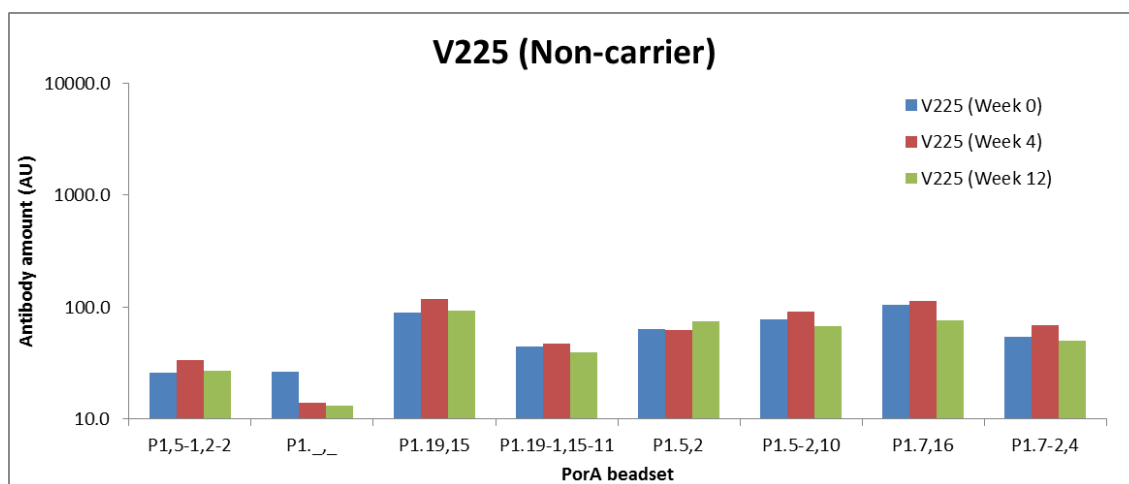
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