Investigation of the distribution, antigenic variation and the biological role of phase variation of the haemoglobin receptors of *Neisseria meningitidis*

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By

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STATEMENT OF ORIGINALITY

This accompanying thesis submitted for the degree of PhD entitled: Investigation of the distribution, antigenic variation and the biological role of phase variation of the heamoglobin receptors of *Neisseria meningitidis*, is based on work conducted by the author in the Department of Genetics at University of Leicester mainly during the period between March 2008 and February 2012.

All of the work recorded in this thesis is original unless otherwise acknowledged in the text or by references. None of the work has been submitted for another degree in this or any other university

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ABSTRACT

Investigation of the distribution, antigenic variation and the biological role of phase variation of the haemoglobin receptors of *Neisseria meningitidis*

Isfahan Tauseef

Neisseria meningitidis is the major cause of bacterial meningitis worldwide. The genome of this pathogen contains >40 phase variable loci whose expression is regulated by tandem DNA repeat tracts. Majority of these loci encode OMPs, which are potential targets for host innate and adaptive immune responses. An analysis for the distribution, frequency and role of PV of these genes is relevant in determining their virulence association and suitability as a future vaccine candidate.

The project investigated the combined distribution, frequency and PV status of two important genes, *hpuAB* and *hmbR*, in disease (n=221) and carriage (n=305) isolates. Strains with both genes or only *hmbR* were present at similar frequencies among disease isolates as compared with carriage isolates. However, >90 % of isolates from CC5, CC8 and CC11 (CCs with the highest disease to carriage ratios) contained both genes. Strains with only *hpuAB* gene were under-represented among disease isolates, possibly due to the receptor having a high level of immunogenicity or being inefficient in iron acquisition during systemic spread. Absence of *hpuAB* resulted from either complete deletion or replacement by an insertion element. Further, one or both genes were found in an ON state in 96 % of disease and 66 % of carriage isolates. This suggests that expression of at least one Hb receptor is of major importance for disease, and that the presence of both receptors contributes to virulence in some strains.

The experimental findings also revealed the ability of strain 8047 to escape from MAb P1.2 mediated killing due to PV in *porA* promoter sequences. We conclude that incubation in the presence of antibody selected for phase variants (10C or 9C) with >3 fold lower PorA surface expression than wild type. This escape in the presence of a specific immune response is an elegant demonstration of the importance of PV for adaptation, and have implications in the development of PorA based vaccines. Finally, the bactericidal activity of HpuA polyclonal antisera generated during this project was evaluated and possible reasons for a lack of its activity are discussed.

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ISFAHAN TAUSEEF

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Chapter 1

INTRODUCTION

1.1 PHASE VARIATION IN BACTERIA

Multi-cellular organisms, such as human, provide environments to microbes for proliferation. The human body contains approximately 10 fold more bacterial cells than human cells (Berg, 1996). Many bacterial species colonise and replicate in an asymptomatic manner in multiple different niches either inside or on the surfaces of human hosts. However, some species have evolved factors, which may be detrimental to their human hosts. To cope with this situation, a very complex defence system has evolved in humans to control and eliminate these threats. In response, equally complex mechanisms have evolved in microbes that help bacterial cells, for example, to adjust and adapt to various niches. These complex mechanisms maintain the intricate balance of host-pathogen interactions, which we are only beginning to understand (Deitsch *et al.,* 2009).

Challenging conditions arise for bacterial species due to changes in the microenvironment and these changes are particularly frequent following transmission. Microbes need a comprehensive range of strategies that generate variation not only at the phenotypic level but also at the genotypic level. Bacteria have only limited pre-programmed responses to sense these changes and for adaptation to predictable stress conditions, so efficient localised hypermutable mechanisms have evolved for adaptation to unpredictable environmental challenges encountered during colonisation and persistence in human hosts (Bayliss, 2009; Jayaraman, 2011). These mechanisms promote either antigenic variation or modulate gene expression. Several bacteria from diverse taxonomic groups and lifestyles (commensal, pathogens, saprophytes,

symbionts) use phase variation to generate intra-population diversity and to increase bacterial fitness for niche adaptation and immune evasion (Wisniewski-Dye and Vial, 2008).

Phase variation is broadly defined as the ability of microorganisms to reversibly and rapidly alter the gene expression–state of various surface phenotypes, such as motility, pili synthesis, iron acquisition etc. This process usually involves switching between two phases (ON to OFF and vice versa) within cells of a population. Both phases differ from each other in such a way that the phase variant may express a particular phenotype, i.e. a surface structure, while the majority of the clonal population does not.

This change in phenotype is a stochastic event, usually independent of environmental signals and occurring without a sensing mechanism in contrast to normal two-component regulatory systems. The change is driven by random mutations of the special genetic loci or hypermutable DNA regions, which mediate high frequency, reversible and heritable switching in the absence of selection. To contrast with normal housekeeping genes, these loci have been called "contingency genes", by Moxon and co workers, to highlight their importance in adaptation of microbes to challenging and diverse environments of the host (Moxon *et al.,* 2006). Hyper mutation in contingency loci brings a change in the genetic material of a minority of cells resulting in a diverse population even in the absence of a selective pressure (Bayliss and Moxon, 2005; Moxon, *et al.,* 1994). The phase variants are recognized by a different ON-OFF status in gene expression or variations in the level of expression.

Phase variants present as a minority in the population may increase in proportion due to selection for that particular phenotype by exposure to the presence of a relevant selective pressure (Z) (Figure 1.1). This selection against parental phenotype selects the

phase variants and the cells with the altered phenotype may persist and accumulate as long as the selection pressure continues. The type of selection pressure can vary at different niches and the population may be switched back to the original phenotype if the selection pressure is lifted or if a different niche is occupied. The expression of a phenotype is regulated by a number of factors and is heritable.

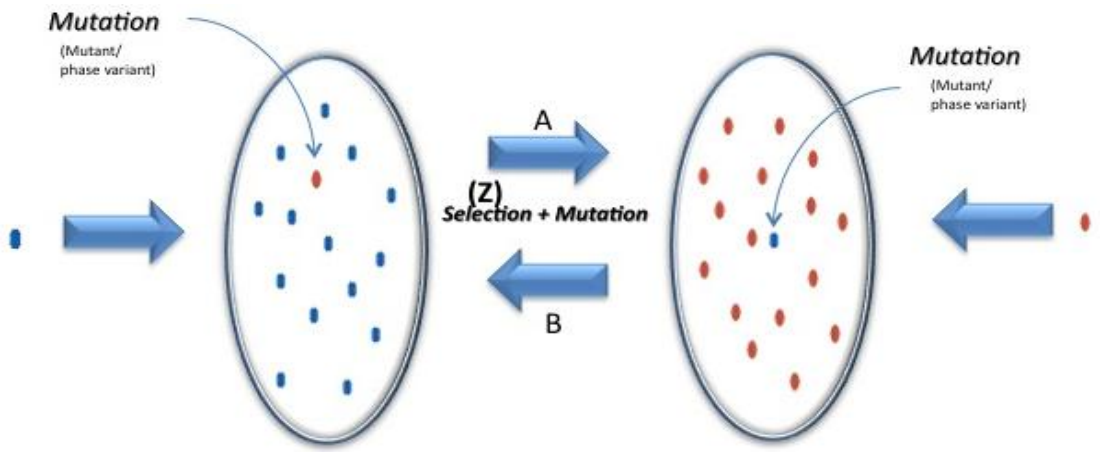

Figure 1.1: Phase variation mediates generation of heterogeneity in a population. (A) Mutant/and or phase variant (OFF - red colony) of a particular phenotype (ON expression – blue colonies) emerges by a stochastic event (e.g. a mutation in a repeat tract) and propagates in the presence of a selective pressure (Z). (B) A reversible switch to the original phenotype (ON expression – blue colonies) may occur when the ON variant out-competes the OFF variant either in the absence of a selective pressure or due to a different selective pressure. Selective pressure (Z) includes different environmental niches, immune responses or antibiotics.

Phase variation mediated phenotypic switches occur at a relatively high frequency (10^{-2} to 10^{-5} per cell/generation) as compared to spontaneous mutations (10^{-8} to 10^{-11} per cell/generation) and are reversible, but this rate may vary with the change of certain factors i.e. gene characteristics, bacterial species and the mechanism regulating the generation of variants (van der Woude and Baumler, 2004; Moxon *et al.,* 2006). Details of these factors are discussed in a later section (see section 1.3 and 1.4). Microorganisms exploit this strategy to avoid the host immune defence system and/or specific antibody responses and to generate diversity within a population, which is

helpful in niche adaptation (Moxon *et al.,* 2006). The ability to adapt is, however, largely dependent on the rate of switching for generation of variants (Bayliss and Moxon, 2005).

Bacteria can regulate multiple genes by phase variation to increase the number of variable phenotypes. If we assume that each gene is switched ON-OFF independently of others then 2^N phenotypes would be created by *N* independently phase-variable genes (Bayliss, 2009; Deitsch *et al.,* 2009). However, some phase variable genes are not regulated by only ON-OFF mechanism but phase vary between different levels of protein expression (Dixon *et al.,* 2007). In such a scenario, the possible outcomes and combination of phenotypes may increase many fold (Bayliss, 2009).

Thus, if we consider two genes and assume only ON-OFF expression for both, then 4 different combinations of phenotypes are possible (ON-OFF, ON-ON, OFF-ON and OFF-OFF). But if one or both phase variable genes have differential expression (high, medium and low), then 6 or 9 possible phenotypic combinations can be generated (Figure 1.2). If we consider a microorganism with ~50 phase variable genes, then due to rapid switching, phase variable genes can produce theoretically several thousand combinations of phenotypes, each differing from the other. This expected outcome of variants through PV of multiple loci may provide an indication for the relevance of these mechanisms in pathogenesis of bacteria (Bayliss, 2009).

Antigenic variation is similar to PV and is an important mechanism for the production of antigenically diverse variants in bacteria (van der Woude and Baumler, 2004). This type of variation is also responsible for adaptation, which is attained by evasion of immune surveillance due to a change in the amino acid sequence of surface antigens. This process is necessary for the production of various antigenically distinct phenotypes

to maintain chronic infection (Deitsch *et al.,* 1997). At a given time, one variant is expressed from a family of possible antigenic variants of the same gene while others remain silent.

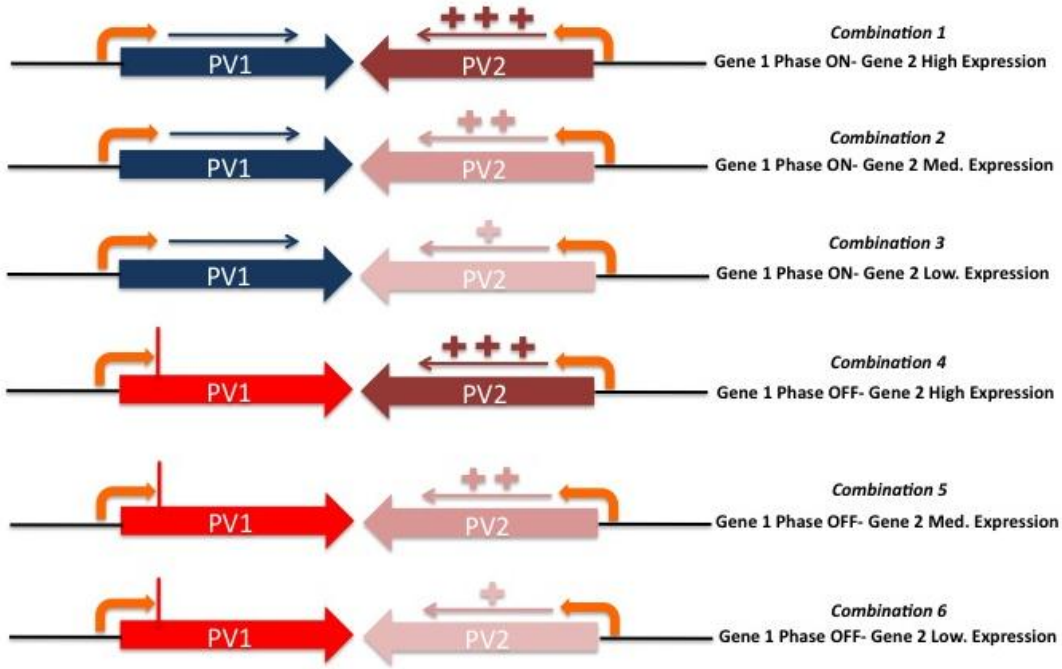

Figure 1.2: Possible theoretical combinations of phenotypes generated by two independently phase variable genes. Phase variable gene 1 is assumed to phase vary by ON-OFF switching while gene 2 phase vary by producing high, medium or a low level of expression. Six possible combinations of phenotypes are shown based on the phase variable status of one gene relative to the other.

However, the antigenic structure changes over the course of disease and a new variant may emerge from a previously silent copy of the gene. This systemic and continuous alteration displays a changed population to the immune system, which may not readily recognise and eradicate (Deitsch *et al.,* 2009). Although antigenic and phase variation share a common purpose of host immune evasion and also some common molecular features, yet they are distinct. In antigenic variation, antigens are always expressed but

with a different antigenic structure while phase variation is usually a "none or all" status.

The relationship between the phase variable change in phenotypes to that of virulence of a bacterial population is a focus of attention, and the interest is further fuelled by the observations that these variations affect the virulence of a strain (van der Woude and Baumler, 2004). One such example is the virulence of *Streptococcus pneumoniae*. Two colony phenotypes (opaque and translucent) of this bacterium are observed due to phase variation of genes encoding the surface structures. This variation leads to a six fold difference in the capsular expression and two fold in techoic acid between both phenotypes (Ring *et al.,* 1998). These phenotypes were tested for their ability to colonize or infect an animal model. The study indicated the association of an opaque colony morphology with virulence while the transluescent phenotype was favoured during colonization rather than systemic infection (Kim and Weiser, 1998; Weiser *et al.,* 1994).

1.2 PHASE AND ANTIGENIC VARIATION MEDIATED PHENOTYPES IN BACTERIA

Phase and antigenic variations are mainly reported to be associated with regulation of various surface exposed structures i.e. flagella, capsule and pili in bacteria (Wisniewski-Dye and Vial, 2008). This observation led to the classical view that these variations help bacteria to evade specific immune responses of the host, as the variable surface structures are directly exposed to the immune clearance mechanisms. A list of examples of phase and antigenic variable phenotypes is presented in Table 1.1. Random switching of these surface structures generates a diverse population containing variants resistant to immune mediated killing. However, studies have also identified non-surface structures

i.e. DNA modification, whose expression is controlled by phase variation (de Vries *et al.,* 2002). A brief overview of the various phenotypes controlled by phase or antigenic variations in bacteria is summarised in this section.

Table 1.1: Examples of phase variable genes associated with different microbial species and their assigned functions.

Bacterial species	Phase variable gene/ operon	Function or affected phenotype	Mechanism of PV	Reference
Phase variable surface structures				
Streptococcus pyogenes	*sclB*	Surface protein	SSM	Rasmussen and Bjorck, 2001
Moraxella catarrhalis	*uspA1*	Adhesin	SSM	Lafontaine *et al.,* 2001
Neisseria gonorrhoeae	*pgtA*	Type IV pilin modification	SSM	Banerjee *et al.,* 2002
Bordetella pertussis	*Fim3*	Fimbriae	SSM	Willems *et al.,* 1990
Salmonella enterica serotype	*pef* operon	Fimbriae	DNA methylation	Nicholson and Low, 2000
Staphylococcus epidermis	*Ica*	Adhesin (polysaccharide)	Transposition	Ziebuhr *et al.,* 1999
Phase variation in non-surface phenotypes				
Neisseria meningitidis	*modD*	DNA methyltransferase	SSM	Seib *et al.,* 2011
Haemophilus influenzae	*mod*	DNA modification	SSM	De Bolle *et al.,* 2000
Vibrio cholerae	*ToxR regulon*	Toxin genes regulation at transcriptional		Carroll *et al.,* 1997
Streptococcus pneumoniae	*spxB*	Metabolism	SSM	Pericone *et al.,* 2002

The impact of phase variable surface structures on the changes in colony morphology is perhaps the first characteristic, which laid the foundations for the description of various phenotypes i.e. smooth, transluescent etc. A well-studied example of colony morphology driven by phase variation is reported in *H. influenzae* type b strains. Three distinct colony variants produced by phase variation have been identified and include opaque, intermediate and translucent. These morphological variants differ from each other in terms of their virulence and other characteristics including resistance to serum and ability to colonize the host and may emerge due to combinatorial expression of more than one phase variable gene such as those involved in capsule production and cell envelope, thus leading to a difference in cell packaging for phase variants (Moxon *et al.,* 1996; Prasadarao *et al.,* 1999; Roche and Moxon, 1995; Weiser *et al.,* 1995).

Another phase variable phenotype and important virulence factor is the capsule structure in bacteria. This structure can directly influence the bacterial interaction with its host. Being the outermost envelope, capsule can influence adhesion and invasion of bacteria, and also protects them from phagocytic engulfment and complement during systemic spread (Romero and Outschoorn, 1994; Vogel *et al.,* 1997; Achtman, 1995). Classic on/off phase variable synthesis and variation of capsule is reported in a number of bacteria. One such example of phase variable variation is the expression of eight different capsule polysaccharides in *Bacteroides fragilis* generating a diverse clonal population (Krinos *et al.,* 2001).

In addition to the above described polysaccharide structures, expression of a variety of cell surface proteinaceous structures is also under the control of phase variation. Fimbriae are such important virulence factor. Fimbriae interact with specific receptors on host tissues and promote the adhesion of bacteria. Variable expression of fimbriae is

controlled at the transcriptional level and either whole fimbrial operons or some genes are affected by phase variation (Low *et al.,* 1996).

Phase variable flagellar expression is also observed in different bacteria. Expression of flagella leads to an increase in motility and adhesion, which ultimately results in an enhancement of virulence and pathogenesis (Ottemann and Miller, 1997). Phase variable expression of flagella in *Salmonella enterica* is a well studied example. The expression of flagellar subunits in this organism varies and is controlled at the transcriptional level (Bonifield *et al.,* 2003). Phase or antigenic variation of surface exposed proteins performing different functions, i.e. TonB dependent receptors, transporters and porins is also described in a number of species. The best-studied examples of phase variation in outer membrane proteins are discussed in the section 1.11.

Other surface epitopes and important virulence factors under the control of phase variable modifications include lipopolysaccharide in the OM of Gram-negative bacteria. LPS can be comprised of 3 components which include (i) lipid A (a potent endotoxin and strong immune stimulant) (ii) a polysaccharide core (R) and (iii) an O-antigen. The O-side chain (O-antigen) is directly exposed to external environment and contains repeating oligosaccharide units (may contain up to 40 subunits). Variations often occur in this antigen and contribute to antigenic variation (Lerouge and Vanderleyden, 2002). In addition the LPS of some Gram-negative bacteria lacks the O-antigen, often termed as LOS, (Preston, *et al.,* 1996). The side groups of LPS or LOS vary due to the activity of phase variable modifications i.e. glycosylation, sialylation or addition of phosphorylcholine (van der Woude and Baumler, 2004), influencing the bacterial sensitivity to serum, antigenic diversity and adhesion to host.

Phase variable associated expression is not only limited to surface expressed structures but influences the non-surface phenotypes as well. Phase variation in genes responsible for DNA restriction-modification systems (Phasevarions) and regulatory proteins acting as a repressor or activator are described (Moxon *et al.,* 2006; Bayliss *et al.,* 2006; Fox *et al.,* 2007). The RM systems recognize and restrict the entry of foreign DNA in to the bacterium on the basis of the different methylation patterns. Three types of R-M systems (designated as type I, II and type III) have been identified based on their subunit composition, specificity for sequence, restriction site and the cofactor required for their activity (Boyer, 1971). The components of these systems phase vary influencing their activity. A well-characterized example of such type of modification is observed for the *mod* gene of type III RM system in *H. influenzae*, encoding a DNA methyltransferase and acting as a component of this system.

The expression of *mod* system varies due to the presence of a tetranucleotide (AGTC or AGCC) repeat motif in its ORF (De Bolle *et al.,* 2000). Studies on these systems demonstrated that phase variable switching of type III R/M genes also regulate many unrelated genes and simultaneously influence a set of properties. This novel type of system, which controls the expression of more than one unlinked genes by a global regulator (phase variation in *mod* gene) is referred to as a phasevarion (Srikhanta *et al.,* 2005; Srikhanta *et al.,* 2010).

Recently, a novel phase variable regulon (Phasevarion) *modD* was reported in the ST41/44 clonal complex of *N. meningitidis* (Seib *et al.,* 2011). Phase variation of this gene regulates the expression of multiple virulence-associated genes, and its association with the hypervirulent clonal complex (ST41/44) suggest a role in pathogenesis and bacterial fitness. Similarly type I RM system enzymes also phase vary in *H. influenzae* (Zaleski *et al.,* 2005). The phase variation of this system determines the ability or

inability of a wild-type strain to restrict phage DNA. Phase variation of *hsdM* due to a change in length of a pentanucloetide (GACGA) repeat motif regulates this process, but unlike phase variation in type III systems, they do not influence the expression of other unlinked genes (Zaleski *et al.,* 2005; Jayaraman, 2011). Phase variable control of these RM systems also provides other advantages such as: - (i) allowing genetic exchange may benefit the bacteria by acquiring favourable characteristics; (ii) DNA release into the environment by degradation of self-DNA, thus allowing uptake by other cells.

Similarly other non-surface phase variable proteins are involved in DNA binding i.e. regulatory proteins. The phase variable status of a regulatory protein will determine the expression of a gene or genes, being controlled by that specific phase variable activator or repressor. One such example is the phase variable expression of TraE protein, a regulatory component controlling the expression of genes involved in the conjugation of *Enterococcus faecalis* (Heath *et al.,* 1995; Pontius and Clewell, 1991). The phase variation of a protein involved in metabolic pathways has also been recognized in *Streptococcus pneumoniae*. The expression of SpxB, a pyruvate reductase, was found to be differentially expressed between two colony variants and may provide a competitive advantage to $SpxB^{+}$ phase variants in the presence of hydrogen peroxide (Overweg *et al.,* 2000; Pericone *et al.,* 2002).

Keeping in view the importance of the above mentioned phenotypes, phase and antigenic variation play a significant biological role in generating population diversity, and combinations of all phase or antigenically variable phenotypes in a bacterium contribute to successful, persistent colonization and help in evading specific or cross immunity.

1.3 MOLECULAR MECHANISMS OF PHASE AND ANTIGENIC VARIATION IN PROKARYOTES

A variety of mechanisms mediate phase and antigenic variation in bacteria and involve both genetic and epigenetic regulation. A better understanding of these mechanisms is essential to study their impact on bacterial success. Genetic mechanisms include general recombination, site-specific inversion and slipped strand mispairing; epigenetic mechanisms include DNA methylation (reviewed by van der Woude and Baumler, 2004; Wisniewski-Dye and Vial, 2008; Bayliss, 2009), and each mechanism have differing influence of the PV rates.

1.3.1 Genetic mechanisms of phase variation

An overview of these mechanisms is described in this section.

1.3.1.1 Homologous recombination

Recombination between homologous DNA sequences in bacterial genomes is one of the major mechanisms for generating diversity in bacterial populations. Intra-genomic rearrangements mostly generate antigenic variants by a homologous recombination event between regions of homology mediated by proteins involved in recombination and/or repair of bacterial DNA. These recombination events may lead to gene conversion, duplication or deletion of genes. All these process can contribute to antigenic or phase variation of the respective genes.

Phase or antigenic variation mediated by gene conversion is one of the most common, wide spread recombination events and occurs due to unidirectional exchange of DNA between two alleles of a specific gene. The frequency ($>10^{-3}$) of DNA exchange is higher than other Rec-A dependent recombination events leading to phenotypic

variation due to movement of non-expressed copies of an entire gene or part of a gene in to a defined expression site of the same gene, resulting in chimeric sequences (van der Woude and Baumler, 2004; Deitsch *et al.,* 2009).

Gene conversion associated antigenic and/or phase variation is best exemplified by the type IV pilin of *Neisseria gonorrhoeae* (Criss *et al.,* 2005; Serkin and Seifert, 1998), and most of our knowledge about gene conversion associated antigenic or phase variation is based on studies conducted on this neisserial surface exposed structure. The pilus is composed of multiple subunits of the PilE pilin protein. Neisserial genomes have an expression locus called *pilE* and multiple loci containing one to six copies of the silent *pilS* loci.

The recombination process involves two steps, the first of which is RecA-independent while the second step requires the expression of RecA. Recombination between the sequences of *pilE* and *pilS* genes from a donor chromosome leads to a hybrid autonomous circular DNA (Howell-Adams and Seifert, 2000). This resulting hybrid molecule exists as an extra-chromosomal entity (Figure 1.3 A). In a second recombination step, the hybrid molecule recombines via a double cross over with *pilE* on the recipient chromosome leading to antigenic variation (Figure 1.3 B & C). This second recombination event requires the expression of genes such as *recA*, *recX* and *recF* (Kline *et al.,* 2003). This unidirectional exchange results in variations in the Carboxyl terminus of the pilin protein and many theoretical combinations of the chimeric protein can be generated.

Gene conversion in type IV pili may also result in switching of expression between ON-OFF phases. Phase variation mediated change in expression is primarily due to recombination in such a way that a non-functional combination is generated (van der

Woude and Baumler, 2004). This scenario of phase variation occurs due to gene conversion associated movement of a silent copy (*pilS*) containing a premature stop codon to the expression locus (*pilE*) resulting in a truncated protein (Howell-Adams and Seifert, 2000). However in some cases, recombination may also lead to irreversible and complete inactivation of the gene by deletion of the *pilE* locus (Manning *et al.,* 1991; Segal *et al.,* 1985).

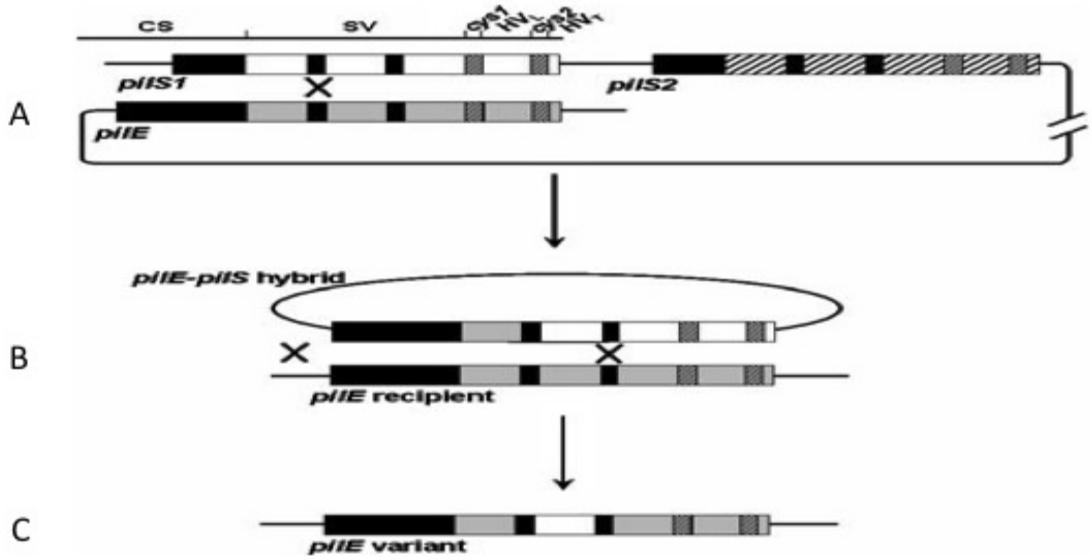

Figure1.3: Homologous recombination mediated phase and antigenic variation of Pilin protein in *N. gonorrhoeae*. (A) A silent *pilS* locus and donor *pilE* locus recombine on the same chromosome at regions of homology (small black boxes indicated by crosses). (B) A *pilE/pilS* hybrid is excised in a circular form and: (C) recombines with a recipient *pilE* via crossing over leading to *pilE* gene conversion. cs (5' constant region), SV (semi-variable), HV_L (Hyper variable loop), cys1 and cys2 (30 bp conserved sequences), HV_T (Hyper variable tail). (Adapted from Wisniewski-Dye and Vial, 2008).

Homologous recombination associated DNA duplication and excision is another mechanism of phase variation in certain bacteria. Capsule production and/ or its switching in various serotypes of *Streptococcus pneumoniae* is described under this mechanism. Tandem duplication and excision of 11-239 bp sequence in the capsule

production gene leads to disruption of the ORF, and subsequently an OFF phenotype (Waite *et al.,* 2001).

1.3.1.2 Site-specific recombination

Site-specific recombination is also termed conservative site-specific recombination when it mediates phase variation (Jayaraman, 2011). Specific enzymes belonging to families of recombinases mediate site-specific recombination. These enzymes ensure the sequence specificity of the recombination events, which occur between non-homologous regions. These recombination events may result in rearrangements such as inversion, insertion or excision of sequences, which ultimately lead to antigenic or phase variation (Komano, 1999; Johnson, 2002).

Inversion (flipping) of DNA mediated by serine or tyrosine recombinases is a well-studied phenomenon for phase and antigenic variation in certain bacteria. This specific recombination event results in either inversion of a regulatory sequence or a coding sequence, which leads to clonal antigenic diversity, and/or switching of phenotypes. The phenotypic switch mediated by flipping or inversion of small invertible DNA sequences (300 bp) found in *fim* and *fot* operons in *E. coli* and *mrp* operon in *P. mirabilis,* are well-studied examples (Abraham *et al.,* 1985; Honarvar *et al.,* 2003; Li *et al.,* 2002). These operons encode type 1, CS18 and MR/P pili respectively. The invertible sequence is found upstream of the gene encoding major subunit of pili, and contain the promoter sequence. A correct orientation of this small invertible sequence is essential for the transcription and production of pili (Figure 1.4).

Recombinases recognise inverted repeats located at either end of the invertible sequence and inversion of this DNA sequence leads to switching between two possible orientations of regulatory regions. In one of the two possible orientations, the promoter

is perfectly aligned leading to expression of the gene (Figure 1.4 A). However a change of orientation due to flipping of an invertible segment leads to the OFF state (Figure 1.4 B). This ON-OFF switching controls the expression of pili and plays an important role in attachment. Phase and antigenic variation of other phenotypes including flagella and capsular polysaccharides are also regulated by DNA inversion mechanisms.

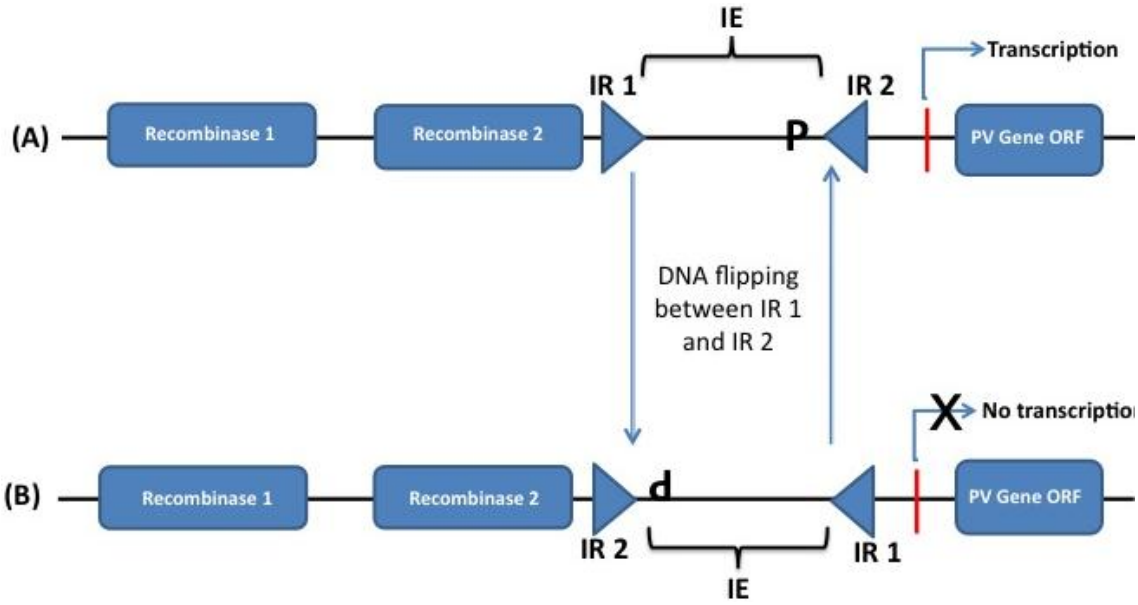

Figure 1.4: Phase variation driven by site-specific recombination. Phase variation relies on the recombinase-mediated reversible flipping of an invertible element (surrounded by two invert repeats) located upstream of the phase variable gene. The invertible element contains the promoter sequence (P) and a correct orientation (shown in A) of promoter is required for an ON phase of the gene. Flipping of the invertible element (shown in B) modifies the orientation of promoter and abolishes transcription leading to an OFF state. PV, Phase variable; P, Promoter sequence; IR, Invert repeat; IE, Invertible element (Modified from Woude and Baumler, 2004).

Another mechanism of site-specific recombination that leads to phase variation is by the precise insertion and excision of DNA sequences in bacterial genomes. This mechanism is limited and controls only few phenotypes. A well-studied example is the precise excision of IS*492* element from *eps* locus of *P. atlantica* (Perkins-Balding *et al.,* 1999). The process is mediated by a specific transposase MooV and differs from the classic method of transposition as it restores the original sequence of the recipient DNA

(Higgins *et al.,* 2007). The switch is reversible and affects biofilm formation by this bacterial species. A similar IS-like mediated reversible switching event occurs as a result of precise excision and insertion of IS*1301* in the *siaA* gene of *N. meningitidis* and this event controls the capsule production with a frequency of 1×10^{-4} (Hammerschmidt *et al.,* 1996a). Insertion and excision of extra-chromosomal DNA into a genome is another mechanism controlling reversible switching. One such example is the reversible insertion and excision of plasmid in to genomes of *Legionella pneumophila* (Luneberg *et al.,* 2001). However details and precise mechanisms regulating this process are still missing.

1.3.1.3 Slipped strand mispairing mediated phase variation

Repetitive DNA sequence units, sometimes referred to as short sequence repeats, microsatellites or variable number of tandem repeats are widely distributed in prokaryotic genomes (van Belkum *et al.,* 1998). Repeat units vary in length ranging from one to nine nucleotides and consist of a stretch of either identical nucleotides (poly purines or pyrimidines; termed a mononucleotide repeat or homopolymeric repeat tract) or repetition of contiguous units of nucleotides (di, tri, tetra, penta, or longer repeat units) (Power *et al.,* 2009). Homopolymeric stretches of purines or pyrimidines with varying lengths are a commonly observed repeat unit (microsatellites) in contingency genes of many prokaryotes (Jayaraman, 2011).

Microsatellites are unstable and are thus involved in mediation of phase variable ON/OFF expression by expansion or contraction of the repeat numbers in contingency genes due to slippage of nucleotides during DNA replication (Moxon *et al.,* 2006). The mechanism is a RecA independent stochastic genetic event referred to as slipped strand mispairing and results in mutations at a significantly rate higher than basal mutations

(De Bolle *et al.,* 2000). The slippage occurs due to misalignment between template and newly synthesized strand during replication, and unpaired nucleotides form a bulge like structure (Figure1.5). If this remains unrepaired by the DNA repair mechanism, the bulge results in a mutation of the DNA sequence.

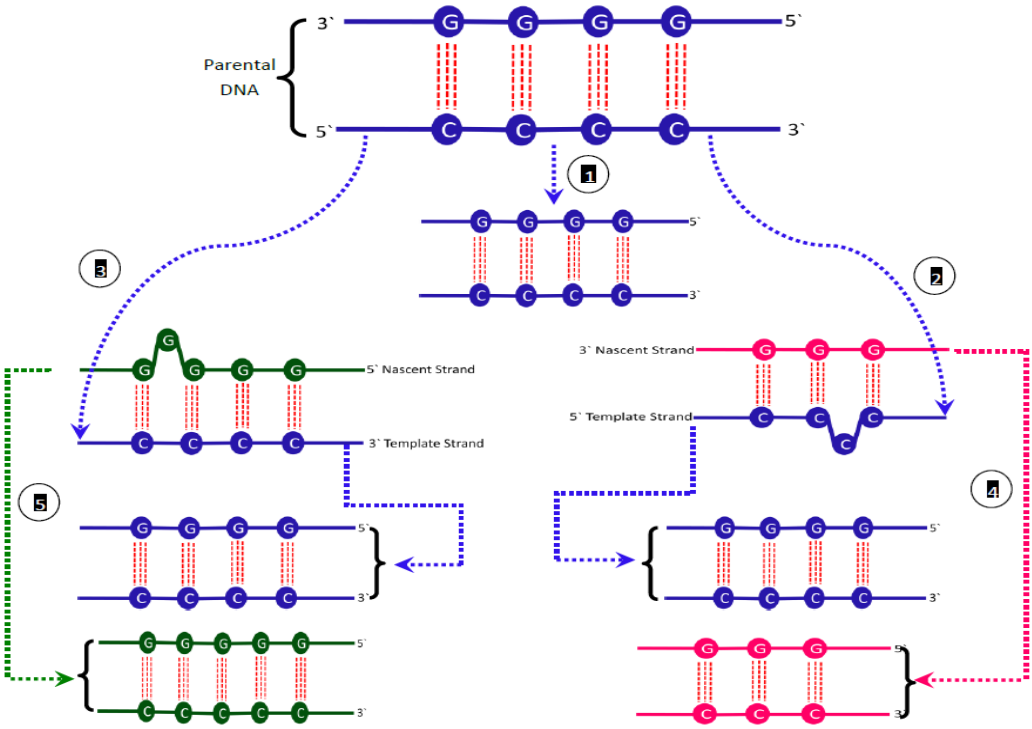

Figure 1.5: Mechanism of slipped strand mispairing in a homopolymeric repeat (poly-G) tract in bacteria. The tract may be present in an ORF or promoter sequence of a gene and mediates PV. (1) Normal replication of the parental DNA (2) Slippage (represented by a bulge) in the template strand results in shortening of the nascent strand by one nucleotide (3) Similarly, a bulge in the nascent strand leads to lengthening of this strand by one nucleotide. (4 & 5) Variants with altered poly(G) repeat tract arise in subsequent bacterial generations.

Misalignment in a repeat tract can occur on both strands resulting in a daughter strand which is shorter or longer than the parental strand by 1 repeat unit (Levinson and Gutman, 1987; van Belkum *et al.,* 1998). Repeat tracts of varying lengths as shown in Table 1.2 control expression of a wide variety of phenotypes in many bacteria. A study

in *E. coli* also indicated that the repeat units in DNA might form secondary structures i.e. hairpins, cruciforms etc, which may affect DNA polymerase III activity (reviewed by Bichara *et al.,* 2006). This disruption in polymerase activity may ultimately influence the separation of DNA strands and may lead to misalignment of repeats.

Table 1.2: Examples of microorganisms with varying number and size of repeat units along with the function of the genes containing these repeats.

Bacterial Species	P. Variable Gene	Function of Gene	Repeat unit	Position of repeat	Reference
Neisseria meningitidis	*modD*	DNA modification	5'-ACCGA-'3	ORF	Sieb *et al.,* 2011
Campylobacter jejuni	*wafN*	LOS synthesis	5'-C-'3	ORF	Linton *et al.,* 2000
Helicobacter pylori	HP722	Membrane protein	5'-CT-'3	ORF	Tomb *et al.,* 1997
Haemophilus influenzae	*hifA/B*	Synthesis of fimbriae	5'-TA-'3	Promoter	Van Ham *et al.,* 1993
Neisseria gonorrhoea	*opa*	Invasion	5'-CTCTT-'3	ORF	Stern *et al.,* 1986
Escherichia coli	*ahpC*	Stress response	5'-TCT-'3	ORF	Ritz *et al.,* 2001
Bordetella pertussis	*fimB*	Fimbriae synthesis	5'-C-'3	Promoter	Willems *et al.,* 1990
Yersinia pestis	*yadA*	Membrane protein	5'-A-'3	ORF	Rosqvist *et al.,* 1988

Depending upon the position of a repeat tract, hyper mutation of the repetitive DNA sequences and resulting changes in repeat number regulate the phase variation mediated protein expression either at the translational, or transcriptional levels (Figure 1.6). The hyper-mutability of DNA repeat tracts found within coding sequences (region 4 in figure) results in a translational frame shift by slipped strand mispairing which may

introduce a premature stop codon leading to the synthesis of a truncated or non functional protein (Moxon *et al.,* 2006).

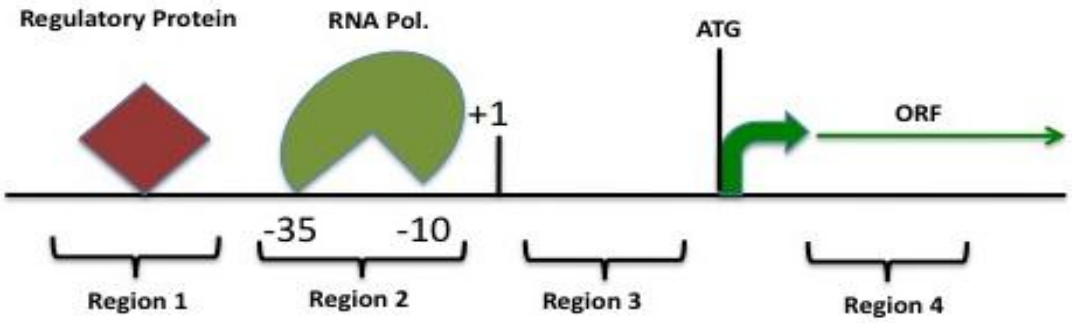

Figure 1.6: Mechanisms of phase variation and relative position of simple sequence repeats (1 through 4) causing PV in phase variable genes. Phase variation at position 1, 2 and 3 influence the levels of expression of the gene while PV at position 4 leads to ON-OFF switching. +1 (Transcription start site), RNA pol. (RNA polymerase), ATG (Start codon). (Modified from van der Woude and Baumler, 2004).

One such example of phase variation control is the expression of *mod* in *H. influenzae* (De Bolle *et al.,* 2000), which contains >30 repeats of a 5'-AGTC-'3 motif. An addition of one repeat motif by SSM introduces a premature stop codon in the coding sequence leading to the production of a non-functional protein, and ON-OFF phenotypic switching. However, the open reading frame may remain intact if the insertion or deletion is a multiple of three, but addition or deletion of an amino acid may alter or compromise the protein function due to a size and/or sequence polymorphism. One rare example of this type of polymorphism is described in *E. coli*. The AhpC protein functions as a peroxiredoxin enzyme in *E. coli* under normal conditions. However, under stress, a triplet expansion in the open reading frame reversibly changes the function of this enzyme to a disulfide reductase, perhaps giving it a growth advantage (Ritz *et al.,* 2001).

Repeat tracts are also present outside the open reading frame either in promoter or a region close to promoter (region 2 in figure) and regulate phase variation at the level of transcription. Changes in DNA repeat number in promoter sequences affect transcription by altering the binding position of RNA-polymerase and/or sigma factor (Moxon *et al.,* 2006), as a precise spacing between the -10 and -35 sequences is vital for an optimum level of transcription. A mutation leading to deletion or addition of even a single nucleotide in a polynucleotide sequence can affect the strength of the promoter.

In contrast to translational frame shift mutations, an alteration in a repeat tract found in the promoter does not necessarily turn a gene OFF or ON. It is likely that these changes in nucleotide sequence affect the level of expression in such a way that a clonal population has variants but with low or high levels of expression. Phase variation of fimbriae encoded by *hif* genes in *H. influenzae* is due to variation of a dinucleotide (TA) repeat tract in the promoter. The gene *hifA* (encoding the main fimbrial subunit) along with *hifB* (encoding a chaperon) have overlapping promoters and 17 nucleotides between the -10 and -35 regions for optimal transcription (Van Ham *et al.,* 1993). An alteration in repeat number results in variable levels of expression where high, medium or low levels of expression represent phase variants (Van Ham *et al.,* 1993).

Repeat tracts located close to, but outside of the promoter (region 1 in figure), can also regulate the phase variation at the transcriptional level. Slipped strand mispairing mediated expansion or contraction of these repeat sequences apparently does not directly affect the RNA polymerase binding to promoter, rather there is an influence on the interactions between regulatory proteins and RNA polymerase by a change in the spacing of regulatory sequences leading to differential expression (van der Woude and Baumler, 2004).

One example of this type is phase variation of *nadA* gene in *N. meningitidis* strain MC58. This gene encodes an adhesin and unlike most other phase variable loci of this organism, it has a tetranucleotide (TAAA) repeat tract distally located upstream of the core promoter sequence and a phase variation frequency of 4.4×10^{-4} has been estimated (Martin *et al.,* 2003). Martin *et al* also described that an alteration in repeat number affects the promoter strength and expression of *nadA*, with a high level at 13, 10 or 8 repeats, medium at 11 or 12 and low at 9 repeats. The transcription of *nadA* is regulated by the binding of both Fur and IHF protein to the promoter, while a loss or gain of tetranucleotide affects the IHF binding thus modulating the level of transcription (Martin et al., 2005; Metruccio *et al.,* 2009).

A *cis*-acting growth phase regulatory region (GPR) acting as a NadR binding sequence was identified between -108 and -170 bp upstream of the transcription start site. NadR binding to this region will repress gene expression. Contraction or expansion due to deletion or addition of a tetranucleotide repeat motif influences the interaction of NadR with the RNA polymerase, ultimately leading to high, medium or low expression levels. These authors also demonstrated that inhibition of NadR by 4-hydroxyphenylacetic acid prevented its binding within the identified operator region activating expression (Metruccio *et al.,* 2009).

Repeat DNA sequences found downstream of a promoter also influence the level of gene expression (region 3 in figure 1.6). In *Moraxella catarrhalis,* a stretch of poly(G) is located downstream of the promoter of the *uspA* gene but upstream of the start codon (Laity *et al.,* 1993). The author concluded that a change in length of the repeat tract is correlated with changes in the level of expression of this adhesin protein.

1.3.2 Epigenetic mechanism of Phase variation

Phase variation can also be mediated by mechanisms that do not involve a change in the DNA sequence. The term epigenetic is coined for this mechanism of phase variation, as the process occurs reversibly with out changing DNA sequence in a heritable manner. The mechanism relies on differential and reversible methylation of specific DNA sequences in phase variable genes by the activity of DNA adenine methylase, and influences the transcription by affecting the binding patterns of regulatory machinery. This phase variable methylation pattern may increase the pathogenicity and virulence of a strain by random expression of virulence factors (Srikhanta *et al.*, 2010). In *Salmonella,* Dam mediated methylation is important for its virulence while a *dam* mutant is attenuated and used as a live vaccine (Heithoff *et al.,* 1999; Chatti *et al.,* 2008).

Another example of epigenetic phase variation is reported in *E. coli* where phase variable expression of *pap* and *agn43* genes is controlled by *dam*-dependent DNA methylation of specific regulatory sequences (Hernday *et al.,* 2002). The phase variation mechanism of Ag43 protein is explained here as an example. This outer membrane protein is encoded by gene *agn*43 and promotes aggregation and influences biofilm formation in the absence of fimbrial expression (Wallecha *et al.,* 2002). In addition to Dam, a regulatory protein OxyR is required for the phase variation of the gene. Three GATC sequences are located upstream of the ORF in a regulatory region where both proteins, Dam and OxyR, bind depending upon the methylation status of the GATC sequence (Figure 1.7).

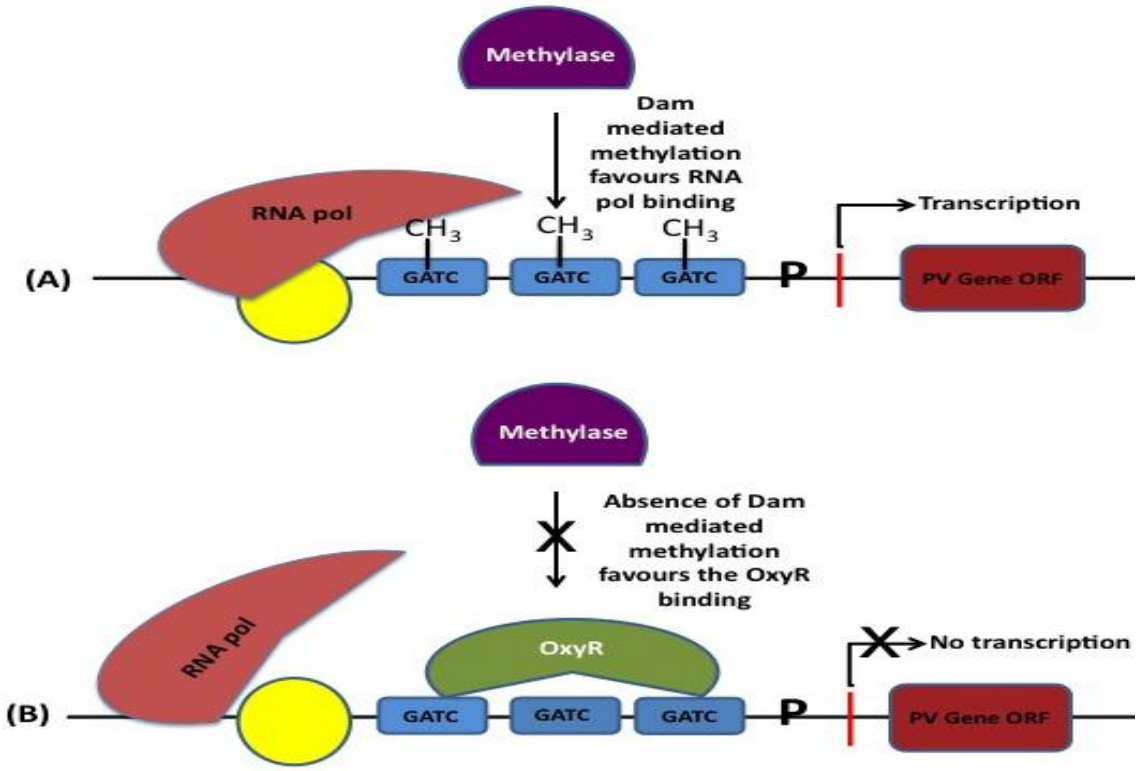

Figure 1.7: Phase variation regulated by epigenetic mechanism in *agn43* gene. The transcription of *agn43* gene is dependent on the methylation of specific repeat sequences (GATC) found in the upstream region of the gene. Methylation of these sequences by DNA adenine methylase (Dam) favours the binding of RNA polymerase, hence transcription of the gene (as shown in A), A lack of methylation of GATC sites by Dam leads to OxyR binding (a repressor protein) which ultimately abolishes the transcription by inhibiting RNA polymerase activity (as shown in B). Binding of both, Dam and OxyR, with GATC is reversible and determines the phase variable expression of the gene.

Methylation of these sequences by Dam favours the binding of RNA polymerase allowing the transcription and leads to an "ON" state for the gene. However, even a single un-methylated GATC motif favours the binding of OxyR, which acts as a repressor and blocks the access of Dam for sequence methylation and also subsequently inhibits binding of RNA polymerase generating the OFF state of the gene (Haagmans and van der woude, 2000; Waldron *et al.,* 2002). Thus both proteins compete for binding with the GATC sequences and one protein excludes the binding of the other

leading to phase variable expression. The transition between the two phases (ON-OFF) is governed by stochastic competition between both proteins, and depends upon the OxyR concentration in a cell and the level of DNA methylation determined by the Dam/DNA ratio (Correnti *et al.,* 2002; van der Woude, 2008).

For last couple of decades, researchers have investigated to answer the question that how epigenetic changes are inherited to the daughter progeny and yet remains reversible. All findings have concluded that differential methylation pattern of some target sequences in chromosomal DNA of microbes is responsible for this regulation (van der Woude, 2008). After cell division, the methylation state (i.e. methylated GATC sequences) is inherited by Dam-mediated methylation of nascent strand, thus maintaining the parental expression state of the gene (i.e. ON) in a majority of progeny cells (Figure 1.8 A).

However, as phase variation is a stochastic event, and in the absence of a known enzyme responsible for removing methyl group from methylated sequences (van der Woude, 2008), the switching between two states relies on this replication step. Thus, in a minority of daughter cells, the hemimethylated DNA sites may be occupied by OxyR and excludes the Dam-mediated complete methylation leading to OFF expression state (Figure 1.8 B). A second replication step in these phase variants may generate a completely unmethylated sequence from a hemimethylated DNA sequence (Figure 1.8 C). As both Dam and OxyR compete for same DNA sites, this expression state (OFF phase) may revert back to parental expression state (ON phase) if Dam completely methylates these DNA sites during a later DNA replication.

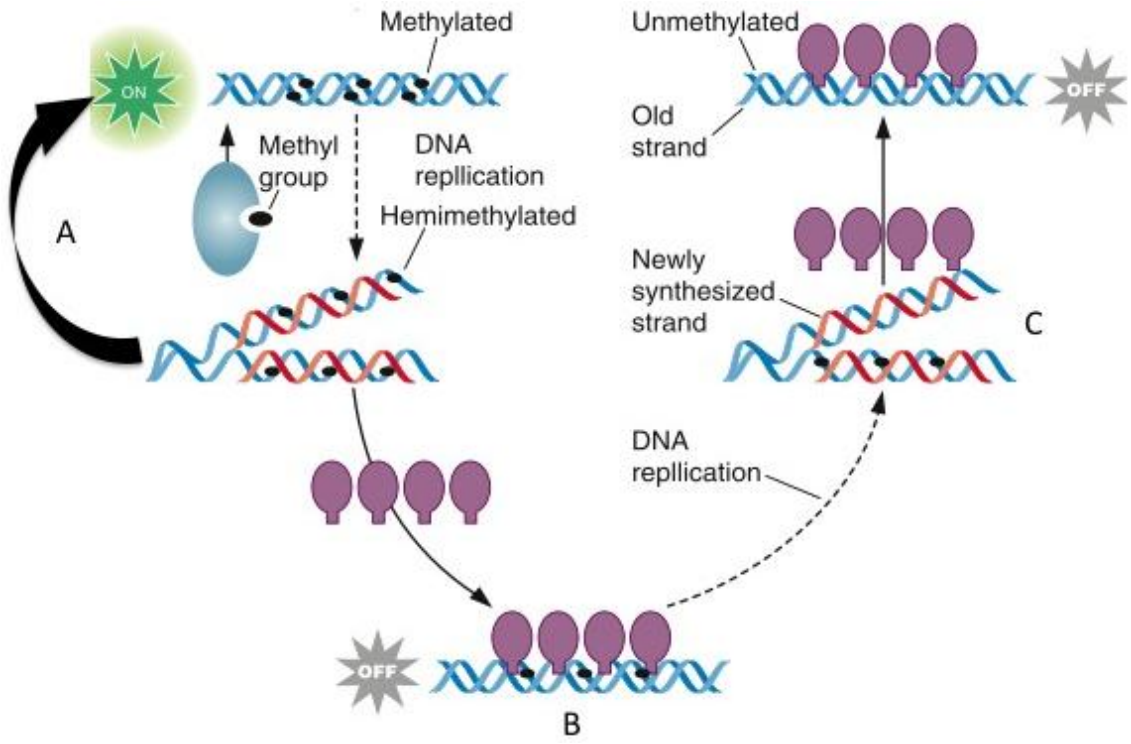

Figure 1.8: Replication of methylated DNA sequence and the events influencing the heritability of the parental expression state of the *agn43* gene. A competition based binding of either Dam or OxyR with the specific target DNA sequence during and/ or after replication is the key event that contributes in maintenance or a change in parental expression state, as a change in their relative ratio can bias the switch. (A) In higher Dam concentration, hemimethylated DNA sequence obtained after DNA replication is completely methylated by Dam resulting in inheritance of parental expression state in daughter cells. A stochastic event may alter the normal Dam-OxyR ratio, thus favouring the OxyR binding with hemimethylated DNA sequence leading to an alteration in expression state in daughter cells, as depicted in (B). Subsequent divisions of these daughter cells may lead to a completely unmethylated sequence (C), yet able to revert their expression state. Unmethylated nascent strand generated after replication is depicted in red. Filled magenta bulb represent OxyR. Other symbols are labelled within figure box. Adapted and modified from van der Woude (2008).

1.4 REGULATION OF SIMPLE SEQUENCE REPEAT-MEDIATED PHASE VARIATION

The genetic diversity of a bacterial population is partly influenced by the frequency of phase variation of contingency loci, which ultimately contributes to its fitness in an environment and virulence (Bayliss *et al.,* 2001; De Bolle *et al.,* 2000). Hence, the study

of factors involved in the instability of repeat tracts is important for understanding their role in bacterial biology, as these differing rates confer adaptive advantage (Bayliss, 2009). Phase variation rate is regulated by a number of environmental, *trans* and *cis*-acting factors, which influence the hypermutable loci either directly or indirectly. Trans acting factors are mostly comprised of genes and their molecular products that directly control the rate of mutations in the repeat tract. These factors may be heritable and stable or they may enable cells to respond only to those challenges, which bacteria occasionally face during colonization of a host.

The rate of phase variation is mainly controlled by the mismatch repair genes, though not all SSRs are MMR responsive. Mainly *mutS*, *mutL* and *uvrD* genes are involved in repairing of mismatch repair mutations and strains defective of either component show elevated phase variation rates. *mutS* and *mutL* are the most important factors influencing the phase variation rates of some contingency loci and a mutation of these genes also results in increased generalized mutation (Richardson *et al.,* 2002; Alexander *et al.,* 2004b). This increase in phase variation rate due to a mutation of *mutS*, *mutL* and *uvrD* was up to 100 fold for poly(C) or poly(G) containing loci encoding for example HmbR, and enzymes involved in capsular or LPS biosynthesis (Richardson and Stojiljkovic, 2001; Martin *et al.,* 2004).

Bayliss and co-workers studied the relative and combined role of various DNA stabilizing and repair genes including *mutS*, *mutL*, *mutH*, *dam*, *polI* and *uvrD* on phase variation regulation in *H. influenzae* (Bayliss *et al.,* 2002). These authors concluded that *polI* is involved in stabilizing the tetra nucleotide repeats whilst absence of this gene favours deletion rather than insertion. Conversely mutations in *mutS*, *mutL* and *mutH* lead to an increase in phase variation by mono or dinucleotide repeats but not tetra nucleotide repeats (Bayliss *et al.,* 2002).

Other *trans*-acting factors include *dinB* (DNA polymerase IV), which directly affects the stability of short homopolymeric tracts of *siaD* but had no effect on frequency of switching in longer homopolymeric tracts (Martin *et al.,* 2004). In addition, XseB (a component of exonuclease VII), involved in the methyl-directed mismatch repair pathway, increases the rate of phase variation of *N. meningitidis* genes during contacts with host cells (Morelle *et al.,* 2005). During a contact with its host, the expression of this component is reported to be up-regulated which ultimately increases the rate of phase variation. However, the details of this process are still incomplete and it is assumed that the up regulation of this mismatch repair system may facilitate adaptation to the host environment at the earliest contact.

Transformation and uptake of foreign DNA in *N. meningitidis* is also reported to increase the rate of phase variation (Alexender, *et al.,* 2004a). The author has suggested that heterologous DNA uptake by a serogroup B strain (IR3261) increased the instability of mononucleotide repeat tracts of the contingency loci and may increase the phase variation rate up to 73 fold. However the rate was reduced by over-expression of MutS and MutL. A few other iron transport and utilization genes i.e. *pilP*, *fbpA* and *fbpB* are believed to be involved in the phase variation regulation but their exact role is as yet unclear (Alexander *et al.,* 2004b).

A number of local factors (*cis*-acting factors) have been investigated which augment the frequency of phase variation mediated by repetitive DNA. One important and major *cis*-acting factor influencing the rate, and determining the capability of a gene for phase variation, is the length of repeat tract in contingency loci. Martin and co workers predicted a correlation between repeat numbers and phase variation in strain MC58 (Martin *et al.,* 2003). These authors indicated that a homopolymeric repeat tract containing more than 5 C or G residues, a stretch of 10 or more adenine or a tetra

nucleotide repeat tracts containing 4 or more repeat units are prone to phase variation. Using a *lacZ* reporter, DeBolle *et al* investigated the effect of tract length on the rates of mutation in *mod* gene of *H. influenzae* (DeBolle *et al.,* 2000), and reported a linear increase in rate of phase variation in correlation with the tract lengths. Richardson and co-workers have investigated the influence of poly(G) tract lengths on phase variation frequencies of HmbR receptor in serogroup A meningococcal strains (Richardson *et al.,* 2002). The Hb gene in wild type and mutator background was replaced with varying lengths of poly(G) tract. The phase variation frequencies in both, wild type and mutator phenotype, increased linearly with increase in tract lengths.

Composition of tracts also influence the rate of phase variation i.e. purines or pyrimidines. Studies indicated that due to a lower dissociation constant, poly(A) repeat tract are more stable and less prone to phase variation than a poly(G) repeat tract (Dornberger *et al.,* 1999). This is due to a difference in stacking energy of base pairs with their neighbours in both types of tracts, which determines the rate of base pairs opening during transcription and recombination (Dornberger *et al.,* 1999). In poly(A) tract sequences, a propeller twist between base pairs is found which is optimum for stacking while base pairs in a poly(G) tract are planer (Warmlander *et al.,* 2002). This difference in stacking lowers the dissociation constant for poly(A) tract, hence comparatively more stability and lower mutability for this tract is observed as compared to a poly(G) tract (Dornberger *et al.,* 1999).

Another *cis*-acting factor is the sequence of the repeat unit in a tract, which determines the orientation of the sequence relative to DNA replication (Bayliss *et al.,* 2004). In addition, *cis*-acting factors include the sequence of the region surrounding the repeats, location of repeat tract in genome and location of repeats in leading or lagging strand

during replication which also effect the stability of the repeat tracts hence influencing the rate of phase variation (Bayliss *et al.,* 2004).

1.5 *N. meningitidis*, THE BACTERIUM AND ITS CLASSIFICATION

N. meningitidis, the causative agent of meningitis and other forms of meningococcal disease, is an obligate and sole commensal of humans, which has not yet been isolated from elsewhere in nature. It is a frequent part of the normal microbiota of the human upper respiratory tract but is also one of the most common infectious causes of death in childhood and young adults worldwide (Stephens, 2007). The bacterium is a Gram-negative, oxidase positive diplococcus (Figure 1.9), and belongs to the family Neisseriaceae (Cimolai and Caugant, 2001). The majority of organisms of this family are mammalian commensals and form part of the normal microbial flora.

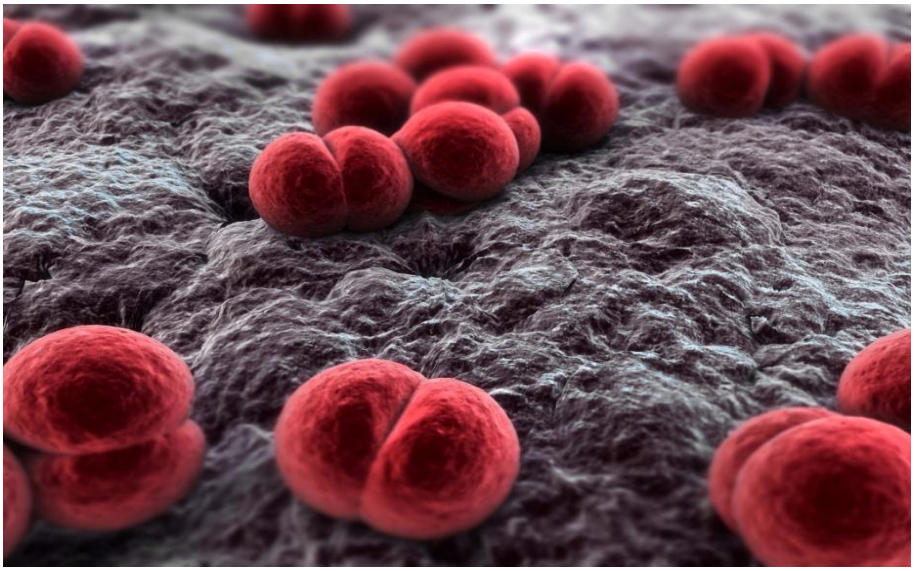

Figure 1.9: *Neisseria meningitidis* cells (A diplococcus bacterium). (http://www.bioquell.com/technology/microbiology/neisseria-meningitidis/).

A capsule of polysaccharide nature usually envelops clinically relevant strains of this microorganism, and protects the bacterium during systemic invasion. Although not common, recently clinical isolates without capsule have also been identified (Findlow *et*

al., 2007; Hoang *et al.,* 2005; Johswich *et al.,* 2012). The antigenic variation of the capsule is exploited for the serological classification of *N. meningitidis.* At least 13 groups have been distinguished and chemically defined so far. However, >90% of disease cases are associated with only six serogroups which include A, B, C, W135, X and Y (Stephens 2007; Tan *et al.,* 2010). The expression of capsule is reported to be absent in ~50% of carriage isolates either due to a lack of capsule synthesizing gene region, phase variation in *siaD* or inactivation of the *siaA* gene leaving them as nontypeable, which hinders their classification based on capsule antigenic characteristics (Claus *et al.,* 2002; Hammerschmidt *et al.,* 1996a & b). The capsule structures of serogroups B, C, W-135 and Y contain sialic acid, a component important for immune evasion (Schneider *et al.,* 2007).

Other classification methods include serotyping based on the antigenic differences of the PorB outer membrane protein, serosubtyping based on variation in PorA outer membrane protein and immunotyping based on lipopolysaccharide (Frasch *et al.,* 1985; Hitchcock, 1989). Monoclonal antibodies are used for the determination and characterization of these outer membrane targets and are being investigated widely with an aim to develop sub-capsular vaccines effective against serogroup B pathogens (Bethell and Pollard, 2002).

Other surface antigen genes have also been included in the typing regime. One such example is the *fetA* gene, which encodes for an outer membrane protein FetA, which is expressed under iron-limited conditions (Beucher and Sparling, 1995). Studies have demonstrated that FetA is useful as an epidemiological marker due to its conservation within hyper-invasive lineages (Urwin *et al.,* 2004; Thompson *et al.,* 2003). A total of 13 surface exposed loops have been identified for this protein. The immunodominant epitope of this receptor protein is present in a variable region of loop 7. This variable

region determines the antigenic diversity of this protein and its sequencing profile, along with the variable regions of *porA*, is used for genetic characterization as part of the neisseria database (http://neisseria.org/nm/typing/feta).

However due to limitations of the above mentioned typing scheme for epidemiological investigations and population genetics, the serological classification methods have now been replaced with more sophisticated and portable techniques for bacterial classification. Currently, MLST is the main scheme in use and this involves the identification of sequence variation at multiple loci (7 housekeeping genes) using specific primers. Based on this information, allele numbers are arbitrarily assigned (Maiden *et al.,* 1998). A sequence type (ST) represents the combination of all allele numbers assigned to multiple loci. Closely related ST types are grouped into clonal complexes which represent the combination of clones marginally different from one another, but similar enough to have a common origin (Caugant, 2008). Hyper-virulent lineages of meningococci with increased propensity to cause disease, and identified previously using other typing methods (MLEE), have now been assigned with ST types and clonal complexes using MLST. Only 11 of the 37 known clonal complexes are clearly identified as disease associated which include ST-1, ST-5, ST-4, ST-8, ST-174, ST-269, ST-334, ST-461, ST-32, ST-11, ST41/44 and ST-18 complexes (Caugant, 2008; http://pubmlst.org.neisseria).

1.6 HISTORICAL BACKGROUND OF MENINGOCOCCAL INFECTION

The history of meningococcal disease can be traced back to the 19th century when in 1806 Vieusseux described an outbreak in EauX vives near Geneva (Vieusseux, 1805). The majority of the cases (out of 33 documented by him) had meningitis. Following

that, a large number of cases were reported and recognized as meningococcal disease. In this pre-antibiotic period, the fatality rate reached more than 70%. It was 1887, when Anton Weichselbaum in Vienna was able to isolate the bacterium and recognized it as *"Diplococcus intracellularis meningitidis"* (Weichselbaum, 1887).

The most important development for the study of disease spread was the isolation of meningococci from throat swabs by Kiefer in 1896, the major advance in disease prevention was at the start of the 20th century when a German group led by Jochman and US researchers Flexner and Jobling were able to develop an antisera for meningococci, which dramatically reduced the mortality to 25%. The first effective treatment by sulfonamides started in 1937 and is considered a major landmark for the treatment of meningococcal disease. A number of serogroup/serotype specific vaccines were introduced throughout the previous century whilst efforts for the development of a safe and effective vaccine covering all serogroups still continue.

1.7 CARRIAGE RATES OF MENINGOCOCCI AND GLOBAL BURDEN OF DISEASE

A significant proportion of healthy individuals in a population may carry the bacterium at one time or other in their upper respiratory pathway, a state known as carriage (Broome, 1986). Transmission of the bacterium occurs occasionally from a diseased individual but most of the time from a symptomless individual to another host. This transmission is crucial for bacterial survival and spread. Respiratory droplets and secretions such as saliva mediate the spread of the bacterium (Yazdankhah and Caugant, 2004).

Meningococcal carriage rates in a healthy population of the industrialized world may vary from 10 to 50% at any given time and is highly age dependant (Yazdankhah and

Caugant, 2004; Claus *et al.,* 2005; Metruccio *et al.,* 2009). The rates of carriage increase in teenagers and reach a peak in young adults. The carrier state may persist for up to 6 months depending upon the colonizing strain (Bidmos *et al.,* 2011). Similarly, transmission rates, and subsequently prevalence of carriage, are also reported to increase in populations living in close contacts such as military recruits and university students (Claus *et al.,* 2005).

Carriage itself may lead to immunization and elicit specific bactericidal responses. Longitudinal carriage studies on meningococci have reported the induction of specific immune response against surface antigens (i.e. PorA) after nasopharyngeal colonization (Jones *et al.,* 1998; Jordens *et al.,* 2004). Bactericidal activity against the homologous PorA variant was detected at a high level throughout carriage in multiple carriers in one study, and these PorA antibodies also showed heterologous activity against other PorA variants (Jordens *et al.,* 2004). Such immune responses may drive the antigenic structuring of surface proteins and could influence the dynamics of transmission (Caugant and Maiden, 2009; Bidmos *et al.,* 2011).

Compared with its high carriage rate, *N. meningitidis* disease incidence rates are relatively low in the industrialized world with ~1-3 cases per 100000 population, with the majority of cases being found in children under 5 (Stephens, 2007). Adults are usually resistant to meningococcal infection due to acquired immunity (Virji, 2009). In the developing world, almost half of the cases occur in the "meningitis belt" comprising of 18 countries of sub-Saharan Africa extending from Ethiopia in the east to Senegal in the west, and meningococcal disease presents predominantly as meningitis alone (Lapeysonnie 1963; Molesworth *et al.,* 2002). The mortality rate is much higher in the explosive epidemics which occur approximately every 5-10 years in this belt during dry seasons, and the mortality rate may climb up to 1000 per 100000 (Stephens, 2007).

A non-uniform geographical distribution of the disease-associated serogroups is also evident. Serogroup A associated epidemics occur mainly in Africa while serogroup B is a major concern in Europe and the Americas (Stephens *et al.,* 2007; Hill *et al.,* 2010). On the other hand, serogroup C associated outbreaks occur worldwide while serogroups W135, X and Y have recently emerged with significant pathogenic potential (Stephens *et al.,* 2007).

The disease has raised significant public concerns due to rapid onset of disease symptoms and being unpredictable. It has also presented a challenge for its clinical management. Although a polyvalent vaccine targeting the serogroups A, C, Y and W135 is commercially available as a preventive measure (Pace and Pollard, 2007), an effective vaccine to protect children of less than two years against the serogroup B meningococci still needs to be developed. Despite the availability of effective chemotherapy, association of meningococci with significant mortality (10%) and an ability for epidemic spread, especially in the so-called meningitis belt, are major factors, which make this bacterial species an important, and serious health problem (Goldrace *et al.,* 2003).

1.8 GENETICS OF *N. meningitidis*

The complete genome sequences of five strains (serogroup A strain Z2491, serogroup B strain MC58, serogroup C strain Fam 18, un-encapsulated strain alpha14 and serogroup C strain 053442) were completed and published between 2000 and 2008 (Parkhill *et al.,* 2000; Tettelin *et al.,* 2000; Bentley *et al.,* 2007; Peng *et al.,* 2007; Schoen, *et al.,* 2008). These genome analyses revealed a single circular chromosome (see figure 1.10) of about 2.2 Mb in all strains with ~52% GC content. The size of the genome is

comparable to other respiratory tract pathogens such as *H. influenzae* (1.9 Mb) (Harrison *et al.,* 2005) and *S. pneumoniae* (2.2 Mb) (Tettelin *et al.,* 2001).

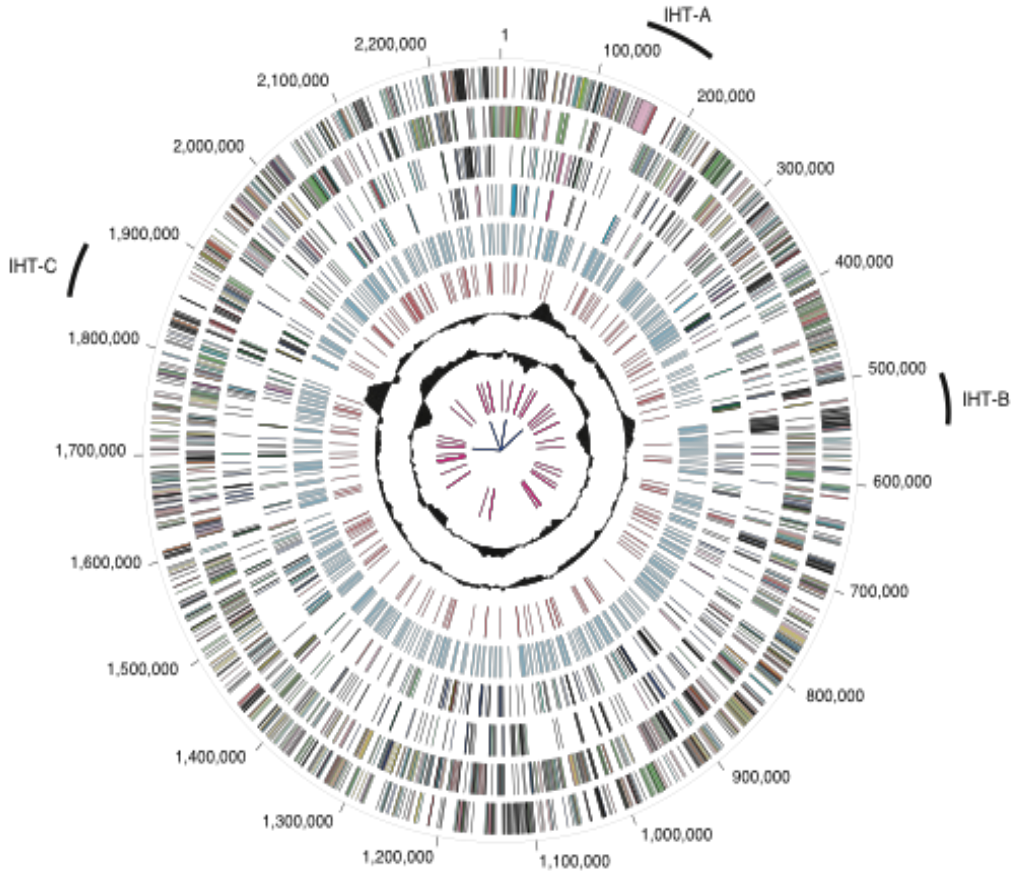

Figure 1.10: Circular representation of the *N. meningitidis* strain MC58 genome. Outer circle, predicted coding regions on the plus strand colour coded by role categories: - salmon, amino acid biosynthesis; light blue, biosynthesis of cofactors, prosthetic groups, and carriers; light green, cell envelope; red, cellular processes; brown, central intermediary metabolism; yellow, DNA metabolism; green, energy metabolism; purple, fatty acid and phospholipids metabolism; pink, protein fate and synthesis; orange, purines, pyrimidines, nucleosides, and nucleotides; blue, regulatory functions; gray, transcription; teal, transport and binding proteins; black, hypothetical and conserved hypothetical proteins (Adapted from Tettelin *et al.,* 2000).

More than 2000 ORFs have been identified which occupy ~83% of the sequenced genome. Biological roles for ~1200 genes have been assigned based on the similarities with other known proteins. Approximately 190 sequences did not show any similarity with other proteins and the majority of them are predicted as hypothetical surface

exposed structures and virulence determinants (Caugant, 2008). In a recent study, Joseph *et al.* have analysed the composition of 29 meningococcal strains belonging to 22 different clonal complexes using microarray comparative genome hybridization (mCGH) (Joseph *et al.,* 2011).

The analysis found 1,139 genes, out of 1,679 compared, were common in all strains (core genes) and suggested to be part of the core genome. For the remaining 540 genes, it was found that the gene was absent in at least one of the 29 strains tested, thus these genes constitute the accessory set of adaptive genes (accessory genome). In addition, at least 31 genes out of these 540 genes were designated as strain specific due to their presence in only 4 specific strains (Joseph *et al.,* 2011). These authors also observed that the core genome was enriched for genes encoding proteins for housekeeping functions localized in the cytoplasm while the accessory genome encoded proteins destined for the outer membrane.

A diversity of prophages is also found in different strains, which encode membrane associated antigenic proteins and thus contribute to meningococcal pathogenicity (Masignani *et al.,* 2001). These researchers revealed the presence of a 35 kb DNA fragment homologous to the sequence of *E. coli* MU phage. The sequence was shown to contain 46 ORFs and three of the ORFs encode surface antigens capable of eliciting bactericidal immune responses. Studies have also indicated that bacteriophages may contribute a role in the evolution of virulence of meningococci due to a frequent presence of Nf prophages in hyperinvasive lineages, and that Nf1 and MSD prophages may increase the fitness of the meningococci due to genetic variability by acting as mutators in bacterial chromosomes (Joseph *et al.,* 2011; Schoen *et al.,* 2008). In addition, some cryptic plasmids responsible for resistance against antibiotics are also occasionally present in the neisserial genome (Backman *et al.,* 2000).

A variety of IS elements have also been reported in the sequenced meningococcal genomes including IS*3*, IS*5*, IS*30*, and IS*110* (Tettelin *et al.,* 2000; Schoen *et al.,* 2007; Schoen *et al.,* 2008). This genetic variability complicates the production of a vaccine effective for all serogroups and also clinical management of meningococcal infections.

1.8.1 Repetitive DNA elements in *Neisseria meningitidis*

Another unique, striking and significant property of *N. meningitidis* genomes is the presence and diversity of a large number of intra and intergenic repetitive DNA sequences identified by genome sequencing projects (Parkhill *et al.,* 2000; Tettelin *et al.,* 2000). The length of these sequences ranges from short repeats of a few nucleotides to as long as hundreds of base pair. These repeat elements play an important role in the meningococcal genome dynamics and antigenic variability due to their capability of altering the genomes (Davidsen and Tonjum, 2006). These alterations are driven by repeat associated recombination, duplication or deletion of the genomic sequences.

The most abundant repeat sequences, present in ~2000 copies per genome, are the 10 bp long (GCCGTCTGAA) DNA uptake signal sequences (Davidsen *et al.,* 2004). These sequences may be present alone or in inverted pairs of repeats and play an important role in natural transformation of meningococci and gonococci (Smith *et al.,* 1999). Although its role in DNA binding and uptake is not well documented, Neisseria uptake DUS-containing DNA preferentially when exposed to a mixture of homologous and foreign DNA (Elkins *et al.,* 1991). However, the over-representation of these sequences in the genes encoding for DNA repair, recombination, replication and restriction/modification components indicates that their major biological significance is in recovery or replacement of these important systems in isolates where these genes have been subject to damage or a permanent loss (Davidsen and Tonjum, 2006).

Other repeat types are largely found within an intergenic repeat array of variable length (200-2700bp) and the most abundant among those are the "neisserial intergenic mosaic elements" (Parkhill *et al,* 2000). These NIMEs are composed of repeat sequence (RS) elements of 50-150 bp lengths, which contain unique sequences flanked by duplicate repeat sequence 3 (dRS3) elements ($ATTCCN_8GGGAAT$). These names are given due to their repetitive presence within genome. The "Correia element" is another repetitive sequence found within the repeat arrays (Correia *et al.,* 1988). These elements have an inverted repeat of 26 bp on both terminals. The length of these units varies and are found either in (i) full length (150-159 bp), (ii) with a deletion of 50 bp without affecting its mobilizing ability or (iii) with a deletion resulting in loss of both or one terminal inverted repeat (Parkhill *et al.,* 2000). An absolute function of Correia elements is unknown. However, studies identified that these elements contain transcription initiation signals and also a binding site for IHF (integration host factor) whose deletion led to a suggested role in modulation of virulence gene expression (Buisine *et al.,* 2002; Rouquette *et al.,* 2004).

Multiple copies of a number of other "repetitive extragenic palindromic sequences" (REP) of variable length are also reported in the extragenic repeat arrays. Most significant among those are REP2 with an average length of 59-154 bp, REP3 (60 bp), REP4 (26 bp) and a 20 bp long REP5 element (Parkhill *et al.,* 2000). These repetitive sequences in repeat arrays are considered as major organizational components of the genome and involved in repeat mediated recombination, duplication or deletion of regions of the genome and also targets for insertion and mobile genetic elements (Shapiro, 2002).

The genome sequence of *Neisseria meningitidis* also reveals the presence of a number of tandem repeats which range from homopolymeric tracts of G or C nucleotides or di,

tetra and penta nucleotide repeats (Parkhill *et al.,* 2000). Tandem repeats are mostly concentrated within genes producing surface exposed proteins or associated with modification and biosynthesis of these surface structures.

1.9 PHASE VARIATION IN *N. meningitidis*

Phase variation was first identified in *Salmonella* and reported to control the expression of flagella by DNA inversion (Zeig *et al.,* 1977). Phase variation has been extensively studied in *N. meningitidis* and *N. gonorrhoeae,* and there is a good understanding of the role of this mechanism in persistence in the host. The success of both organisms for colonization and infection of human hosts is based in part on their ability to modulate their surface structure profile constantly and rapidly, which in combination with other point mutations leads to immune evasion (Virji, 2009).

Due to its small genome (2.2 Mb), *Neisseria* has a low number of classical gene regulatory mechanisms. To cope with this situation, a variety of other mechanisms have evolved in this pathogen for adaptation. These mechanisms mostly rely on mutations in short motifs and repeat sequences, which results in variation in the genes of these pathogens and commensals. In *N. meningitidis*, although the number of phase variable loci varies between different serotypes, more than 40 genes undergo phase variation due to changes in repeat tracts mediated by SSM (Moxon *et al.,* 2006).

The majority of these proteins are either surface proteins such as opacity proteins (Opa), Pili (Pil), adhesins (NadA), iron acquisition receptors (HpuA and HmbR) and porins (PorA), or the enzymes controlling the biosynthesis of surface structures, including those involved in the biosynthesis of LPS and capsule. In *N. meningitidis*, the synthesis of capsule is mediated by a gene cluster *cps*, which is comprised of five regions designated as regions A-E (Frosch *et al.,* 1989). However, the genes responsible for

synthesis of polysaccharide, its modifications and transport are mainly located in regions A, B and C. Region A of the *cpl* locus in serogroups B, C, W-135 and Y consists of *siaA*, *siaB*, *siaC*, *siaD* genes, of which *siaA*, *siaB* and *siaC* are highly conserved (Claus *et al.,* 1997). The genes contained in region B and C are required for transportation of polysaccharides to the bacterial surface and a CtrABCD complex modulates this transport. One of the several reported mechanisms of phase variation is already described in the general phase variation section.

The lipopolysaccharide of meningococci is considered as the most abundant antigenic structure on the bacterial surface and consists of two distinct structural regions termed as lipid A and core region. The biosynthesis of lipid A components of LPS is coordinated by a group of genes i.e. *lpxA*, *lpxC* etc (Kahler *et al.,* 1998). The oligosaccharide core region of LPS contains a stable and conserved inner core region and a heterogeneous outer region. The genes responsible for polysaccharide modifications are clustered in three regions termed as *lgt1*, *lgt2* and *lgt3* (Zhu *et al.,* 2002).

A number of *lgt* genes (up to 5) designated as (*lgtA*, *lgtB*, *lgtC*, *lgtD* and *lgtE*) are present in *lgt1* region and express glycosyltransferases which are required for extension of outer core regions. The *lgt2* and *lgt3* regions also encode the glycosyl transferases required for chain elongation and addition of Glc or PEtn, respectively (Kahler *et al.,* 1996; van der Ley *et al.,* 1997; Banerjee *et al.,* 1998). Variation of both chains defines the 14 known immunotypes of meningococci (Kahler *et al.,* 1998; Scholten *et al.,* 1994). Immunotypes L3, L7 and L9 can be sialylated while L8 does not harbour terminal sialic acid due to the lack of a terminal component required for this sialylation (Jennings *et al.,* 1999).

The core region varies greatly among different strains and also through phase variation. Expression of LgtA, LgtC, LgtD and LgtG glycosyltransferases is phase variable due to presence of a homopolymeric guanine/cytosine tracts which introduces these variations to the LPS, resulting in a subset of the population differing in antigenic and functional properties. The functional aspects of LgtG phase variation have been studied by Bayliss and co workers and provided experimental evidence for the role of repeat mediated PV in evasion from specific immune responses (Bayliss *et al.,* 2008). This transferase adds glucose residue to the 3-position of Hep-II while PEtn is added at the same position, if *lgtG* gene is out of frame (Mackinnon *et al.,* 2002). MAb B5 requires PEtn for its binding to inner core of LPS , which may be lost in the presence of LgtG expression (Plested *et al.,* 1999; Mackinnon *et al.,* 2002). A phase variable switch due to a frame shift mutation in poly(G) tract and with a frequency of 1×10^{-5} resulted in resistance to immune clearance by monoclonal antibody mediated killing (Bayliss *et al.,* 2008).

As with other bacteria, phase variation in non-surface exposed systems of meningococci is also reported, which includes phase variable type III restriction-modification systems controlling the expression of a whole set of genes (Jennings *et al.,* 1999; Saunders *et al.,* 2000; Fox *et al.,* 2007). Thus, many phenotypes in *N. meningitidis* are controlled by phase variation and switching between two states of these genes gives an adaptive advantage in the environment and plays a significant role in iron acquisition, resistance to adaptive immunity, and adhesion to host surface (Bayliss *et al.,* 2001; Moxon *et al.,* 2006; van der Woude and Baumler, 2004). Meningococcal disease strains with high frequency switching have been identified in epidemics, which suggest the importance of variability in surface structures in transmission (Richardson and Stojiljkovic, 2001; Richardson *et al.,* 2002). A constant and rapid modulation of antigenic surface structures of meningococci not only facilitates evasion of defence mechanism, but also

poses a considerable challenge for the development of an effective vaccine covering all virulent strains (Virji, 2009). A better understanding of the strategies employed by this pathogen to evade the human defence system is a key goal for understanding its pathogenesis.

1.10 MECHANISM OF PATHOGENESIS, DISEASE CYCLE AND HOST SUSCEPTIBILITY

Once meningococci have successfully colonized a human host, occasionally and due to unknown reasons, it can invade mucosal surfaces and enter the bloodstream (see figure 1.11). Prior to invasion, contact between bacterial cells and host mucosal surfaces is established by type IV pili and human CD46 receptor proteins (Kallstorm *et al.,* 2001).

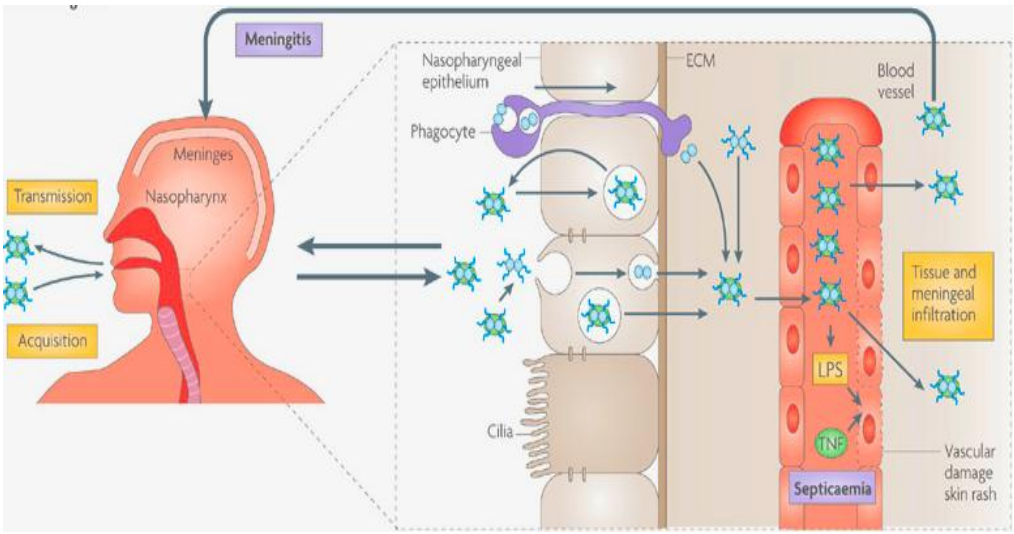

Figure 1.11: Meningococcal pathogenic route leading to septicaemia and meningitis. Bacterium is transmitted from a carrier by respiratory aerosols and acquired through inhalation. After colonization, bacteria cross the epithelial layer by: (i) direct penetration from a damaged part of the epithelial layer; (ii) traversal by transcytosis; (iii) phagocytic mediated. In blood, bacteria survive, multiply and spread leading to a high level bacteraemia while LPS release may result in septicaemia. Infection of meninges and cerebrospinal fluid is the ultimate manifestation if bacterium crosses the blood brain barrier (BBB). (Adapted from Virji, 2009).

Following adhesion, the uptake and invasion of a bacterium takes place due to an intimate contact between bacterial membrane components and host cell surface receptors. This involves the binding of Opa and Opc proteins (a family of invasion associated outer membrane proteins) to CEACAM and heparin sulphate proteoglycan receptors (HSPG) on the surface of host cells (Moore *et al.,* 2005; Virji, 2009). This specific event activates the signalling pathway in host tissues resulting in the release of chemokines, followed by endocytosis of meningococci and dissemination (Griffiths *et al.,* 2007; Muenzner *et al.,* 2000; Nassif *et al.,* 1999). Its worth noting that the expression of many bacterial structures controlling adhesion and invasion is phase variable.

Once the epithelial layer has been invaded, bacteria face multiple immune clearance strategies of the host, which need to be neutralized by virulence factors. Capsule is one of the first lines of defence against the human innate arm of immunity and it protects the invading bacteria from engulfment by neutrophils and macrophages (Achtman, 1995). Another factor that helps bacterial survival in blood is the production of factor H binding proteins. One protein, fHBP, binds the Factor H in circulating blood and the resulting complex inactivates the alternate complement system by blocking C3b component deposition on bacterial surfaces. This component (C3b) is formed by the cleavage of C3 by the activity of C3 convertase in the alternative pathway of complement.

The deposition of the C3b component on the bacterial surface is required for the formation of membrane attack complexes (MAC) in a cascade reaction, which subsequently lyses the bacteria (Walport, 2001). The inhibition of C3b conversion leads to the interruption of the cascade reaction of complement system and protect against cell lysis. After evading the immune system, bacteria can multiply and remain in blood

leading to septicaemia, a dangerous clinical manifestation that may lead to septic shock and needs urgent treatment due to its rapid onset (Thomson and Riordan, 2000).

Survival of bacteria in blood causes a high level of bacteraemia and bacterial load may reach up to 10^6/ml of blood (Hackett *et al.,* 2002). Subsequently, the bacteria may cross the blood brain barrier and enter into cerebrospinal fluid. Meningococci can also induce an increased permeability of the blood–brain barrier (BBB) and pleocytosis, which ultimately leads to oedema and increased intracranial pressure (Kim, 2003). This results in release of pro-inflammatory compounds from infiltrated white blood cells and other host cells leading to neuronal injury. Although meningitis also needs rapid treatment, it is more likely to lead to neurodevelopment sequelae.

The current knowledge about factors involved in host susceptibility to invasion is largely based on assumptions due to lack of a good model. One major factor involved in systemic spread of meningococci, and described for many decades as the single most important factor in invasion, is the absence of an effective bactericidal activity against the invasive isolates (Goldschneider *et al.,* 1969). However with the advent of more sophisticated techniques for genetic study of host and bacterium, factors including the defects in complement mediated killing and mutations in genes involved in the production of various components of the immune system have been reported to be associated with heightened susceptibility and severity of disease (Pollard and Nadel, 2007).

1.11 *N. meningitidis* VIRULENCE FACTORS

The most important and significant virulence factors identified for meningococcal infection can be broadly categorized in to 5 major classes:- (i) Attachment/adhesins; (ii) antiphagocytic and serum resistance factors i.e. capsule; (iii) extracellular proteins; (iv)

surface proteins; (v) iron uptake. An overview of each class is covered in this section and depicted in figure 1.12.

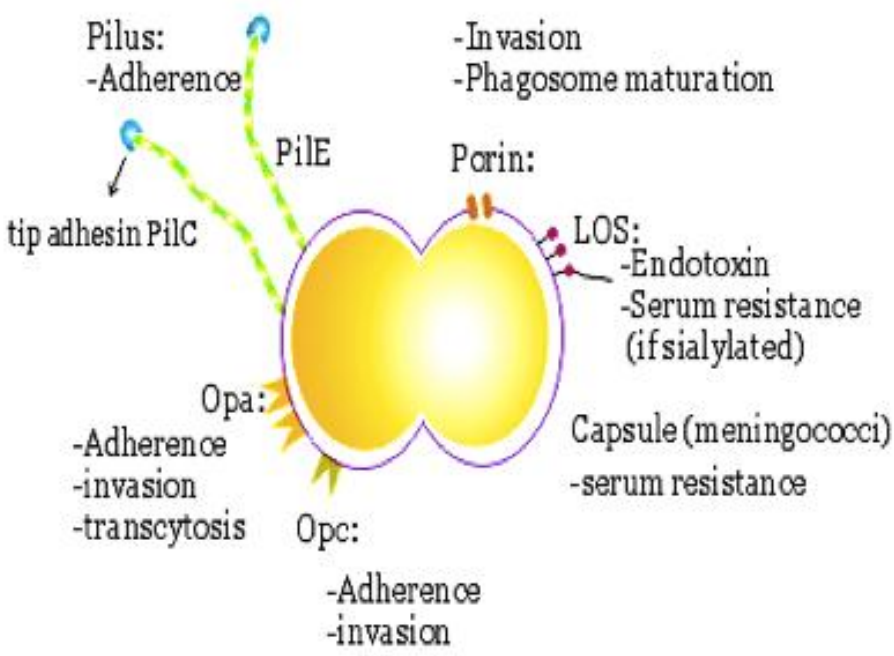

Figure 1.12: Major virulence factors identified in *N. meningitidis* with assigned role in host-pathogen interactions. Five known groups of virulence factors and their role in host-pathogen interaction is depicted in this figure. (Adapted from http://www.mgc.ac.cn/cgi-bin/VFs/genus.cgi?Genus=Neisseria).

1.11.1 Attachment/ adhesion to host epithelial surface

Adhesion to host tissues is a prerequisite for successful colonization and subsequent invasion. The most important virulence factor involved in adhesion/attachment is type IV pili and along with LPS, plays a key role in determining the host and tissue specificity and play important role in virulence of many pathogens including meningococci (Virji, 2009; Mattick, 2002). Other adhesin proteins i.e. Opa, Opc and NadA are also reported which in addition to adhesion, facilitate the invasion and discussed below in a separate section.

The type IV pilus is an important virulence factor that traverses the capsule and is involved in the attachment of meningococci to host CD46 cellular receptors leading to firm adhesion (Kallstrom *et al.,* 2001). However, CD46 independent attachment is also observed in later studies. This implicates the involvement of other unidentified receptors as well (Kirchner *et al.,* 2005). Type IV pili also influence the level and dynamics of adhesion by twitching motility and auto agglutination (Merz and So, 2000). In addition, bacterial competence for DNA-uptake is also powered by the retraction of pili such that these structures also contribute to the virulence of bacteria by genetic adaptability (Merz and So 2000).

The correct assembly and function of this surface structure is driven by a set of genes i.e. *pilD*, *pilT* and *pilC* etc. At least 15 Pil proteins have been identified and the precise functions of majority of them are recently reported (Carbonnelle *et al.,* 2009; Carbonnelle *et al.,* 2006; Carbonnelle *et al.,* 2005). The mechanisms involved in initial attachment are unclear. However, PilC1 protein is thought to be mainly involved in adhesion, while despite a similar contribution to pilus expression, its second locus (PilC2) does not seem to promote adhesion (Morand *et al.,* 2001). Both genes are subjected to phase variation by frame shift mutations caused by SSM in a poly(G) tract and reported in meningococcal clinical samples (Jonsson *et al.,* 1991; Rytkonen *et al.,* 2004). Later studies have also included PilX as an important protein involved in aggregation and adhesion of meningococci (Helaine *et al.,* 2005).

1.11.2 Antiphagocytic and serum resistance factors

Being the outermost covering, capsule is constantly exposed to both innate and specific immunity and almost all clinically significant strains of meningococci are encapsulated. Together with LPS, the capsule acts as a protective shield from the immune system. The

capsule's main function as a virulence factor involves resistance to desiccation during transmission and the protection of bacteria from the phagocytic activity of professional macrophages and neutrophils, as the mutants lacking the ability to synthesize capsular polysaccharide are serum sensitive (Romero and Outschoorn, 1994; Vogel *et al.,* 1997; Achtman, 1995).

The capsule may also block IgG deposition consequently mediating resistance to complement-mediated killing (reviewed by Virji, 2009). A higher-level expression of capsule is reported in some strains to inhibit the PorA specific antibody mediated killing by the complement system (Uria *et al.,* 2008). The authors reported that the observed higher level was due to the presence of an IS element (IS*1301*) in the intergenic region between the *sia* and *ctr* operons which are involved in biosynthesis and export of capsule, respectively. This insertion led to an enhanced transcription and expression levels of both operons, which subsequently reduced the complement-mediated killing. The increased level of expression was associated with a likely presence of promoter elements on the IS*1301*, or due to the fact that the insertion of this element inhibits the binding of negatively acting regulators (Mitchell and Demuth, 2003).

Lipopolysaccharide is considered as the most abundant antigenic component of the meningococcal cell envelope. Sialylation of neisserial LPS may play a role in the serum resistance during infection by protecting the bacteria from complement mediated destruction in non capsulated meningococci due to presence of lacto-N-tetroase, an inhibitor of C3b component (Wright *et al.,* 2006). It is also considered to be the most important virulence factor due to release of its lipid A moiety followed by activation of a large number of cytokines and chemokines which mediate septic shock, a dangerous manifestation of the meningococcal disease (Brandtzaeg, 2003; Brandtzaeg, 2002). The plasma concentration of meningococcal LPS is important for the disease outcome and

the patients with persistent septic shock or organ failure have high endotoxin concentrations (Brandtzaeg, 2003).

Also in a concerted role with pili, OMPs and capsule, LPS mediates attachment/adhesion of meningococci to human epithelial layers and subsequent invasion. However the pili are essential for sialylated LPS mediated adherence and apparently occurs without twitching motility (Virji *et al.,* 1995; Albiger *et al.,* 2003). Similarly, LPS is essential for defensin-like mediated adhesion and invasion is impaired in LPS-deficient mutants (Brandtzaeg, 2003; Albiger *et al.,* 2003). In addition to LPS, Factor H binding protein (fHBP or GNA1870) makes a major contribution to serum resistance and is reported to inhibit the complement mediated killing by binding with factor H1 of the regulators of the alternative complement pathway (Madico *et al.,* 2006; Schneider *et al.,* 2006).

1.11.3 Extracellular secreted proteins as virulence factors

The neisserial *iga* gene encodes an extracellular sequence specific endopeptidase, IgA1 protease, which cleaves the human immunoglobulin IgA1 found in mucosal linings. IgA1 protease is a putative virulence determinant secreted by the type V secretion system (also called two-partner secretion pathway) and belongs to a family of serine proteases (Henderson *et al.,* 2004). The protease is reported in pathogenic neisserial species only and in addition to auto-cleavage, it cleaves the mucosal IgA1 immunoglobulin at the hinge region, separating the F(ab) and Fc fragments (Plaut *et al.,* 1975; Jose *et al.,* 2003). In the absence of Fc, the functions of the IgA antibodies are inhibited whilst the F(ab) fragment may remain attached to the bacteria. This ability of released F(ab) to remain attached with antigen masks the bacteria from intact

immunoglobulin and may contribute to long-term bacterial survival on mucosal surface (Mansa and Kilian, 1986).

This protease is also involved in phagosomal escape due to the degradation of host lysosomal LAMP-1 (lysosomal-associated membrane protein1), a major lysosomal membrane protein component that normally protects the membrane from degradative enzymes enclosed within it (Lin *et al.,* 1997). IgA1 protease mediated cleavage of LAMP1 protein enables bacteria to survive intra-cellularily (van ulsen, 2006). A family of Frp proteins in *N. meningitidis* is also secreted and shares homology to RTX (repeat-in-toxin) proteases of other Gram-negative bacteria (Osicka *et al.,* 2001). However, their exact role in virulence is not well documented in meningococci but they may be involved in dissemination.

1.11.4 Surface proteins

This group includes the virulence factors Opa, Opc and porins. These proteins have been identified to express at higher levels during infection and to be involved in bacterial invasion. As described in earlier sections, the opacity-associated proteins (Opa) are encoded by the *opa* gene and 3-4 *opa* genes are present in each meningococcal genome. The difference between *opa* genes is mainly found within a short hyper-variable region surrounded by a highly conserved region and the genes (*opaA, opaB, opaD* and *opaJ*) are located on a wide distance from each other (Reviewed by Hobbs *et al.,* 1998). The term Opa was coined due to their ability to change the opacity of colonies on agar plates (Swanson, 1978). The aggregation or self-agglutination by these opacity proteins may influence adhesion of bacteria (Virji, 2009). Opa proteins mediate the interaction of meningococci with human epithelial receptors, and can bind to either heparansulphate proteoglycan (HSPG) or in >90% cases

Carcinoembryonic antigen-related cell adhesion molecules (CEACAMs) (Chen *et al.,* 1995; Hauck and Thomas, 2003). Expression of Opc is also associated with the binding of bacteria with that of HSPG receptors on human epithelial surfaces (Prince *et al.,* 2002).

Recently several new minor adhesins have also been identified and these include NhhA, App, HrpA-HrpB system and NadA. Neisseria hia homologue A (NhhA) mediates bacterial adhesion to endothelial cells (via heparin sulphate proteoglycans and laminin) and is present in the majority of meningococcal disease isolates (Scarselli *et al.,* 2006). Adhesion penetration protein (App) is found in all sequenced genomes and mediates the adhesion in early stages of colonization, but is autocleaved at a later stage allowing the release of bacteria for spread (Serruto *et al.,* 2003).

Similarly, HrpA-HrpB system (haemagglutinin/haemolysin-related protein A) is found in all strains, and this system acts as a two-partner secretion system. Adhesion is mediated by HrpA secretory protein, a small proportion of which remains on the outer membrane (Schmitt *et al.,* 2007). Neisserial adhesin A (NadA) can also mediate adhesion to epithelial cells, and is mainly found in hyper-virulent lineages and is being investigated as a serogroup B vaccine target due to induction of protective immune responses (Comanducci *et al.,* 2002; Ciabattini *et al.,* 2008).

In meningococci, two types of trimeric porin proteins, PorA and PorB, have been identified. Porins are widely distributed in pathogenic neisserial species and considered as most abundantly represented OMPs in meningococci (Blake and Gotschlich, 1986). Porins antigenic variability is exploited as serological markers for classification. Porins perform essential functions by the formation of nutrient and ion channels/pores in the outer membrane which is crucial for the modulation of ion exchange between bacteria

and their surrounding (Massari *et al.,* 2003b; Virji, 2009). Porins may also trigger the immune response via TLR2 by acting as adjuvant leading to the B cells activation (Massari *et al.,* 2002).

The virulence characteristics of PorB have been extensively studied in gonococci, a closely related pathogen in the genus *Neisseria* (PorA is not expressed in gonococci due to a mutation). Porin's translocation induce a temporary change in the host cell membrane potential and also influence its cell signalling pathways (Ayala *et al.,* 2005; Massari *et al.,* 2003a). Massari and co-workers have determined that purified PorB or intact meningococci can translocate in to host mitochondria and interact with it to prevent apoptosis by controlling the release of cytochrome c in to the cytoplasm. This cytochrome is required for the apoptosis cascade. Similarly, the translocation of PorB in to eukaryotic membranes triggers a calcium influx by forming a gated-channel, which leads to endosome exocytosis (Muller *et al.,* 1999). The calcium ion influx may alter the endocytic trafficking and may redistribute the Lamp1 protein to plasma membrane where it is cleaved by IgA1 protease (Ayala, *et al.,* 2001; Ayala, *et al.,* 2002). This translocation of PorB to infected cells followed by Lamp1 degradation reduces the number of lysosomes. Studies on gonococci have also demonstrated that porins (PorB) enhance invasion without the aid of opacity protein or invasins (van Putten *et al.,* 1998).

PorA is a surface exposed outer membrane component involved in the formation of cation selective pores (van der Ende *et al.,* 1995). It is a putative porin but its exact function is unknown. Commensal neisserial strains harbour only PorB like structures indicating a potential fitness advantage is associated with PorA in virulent meningococci (Feavers and Pizza, 2009). Studies have also indicated that meningococcal PorA can bind with C4BP, a complement inhibitor, thus helping to evade immune surveillance (Merianos, 2007; Jarva *et al.,* 2005). This antigen is being

investigated as a potential vaccine candidate, as it elicits strong immune responses during natural infection or immunization and immune surveillance drives the sequence variation of *porA* gene (Peeters *et al.,* 1996; Mandrell and Zollinger, 1989; Idanpaan-Heikkila *et al.,* 1995).

Bactericidal antibodies raised against this antigen suggested that variable loops are the potential targets for these protective antibodies (Martin *et al.,* 2000). The expression of this important antigen is phase variable due to presence of a poly(C) repeat tract in its promoter and a poly(A) repeat tract in the ORF (van der Ende *et al.,* 2000; Alcala *et al.,* 2004). Shortening or lengthening of the repeat tract may result in variable or complete absence of expression. The understanding for the biological role of this variable expression in immune evasion still continues.

1.12 IRON AVAILABILITY, ITS PROTEIN COMPLEXES IN HUMAN HOST AND UPTAKE BY MENINGOCOCCI

Iron concentrations are critical for both host and pathogen and iron acquisition is an important virulence factor. Bacterial survival, once inside the host, largely depends upon its ability to accumulate essential nutrient elements, especially iron and its complexes. Iron is known to be a major pathogenic determinant in bacterial infections. Previous studies in animal models demonstrated that the virulence of pathogenic microbes increases significantly when bacteria are injected in combination with iron complexes (Perkins-Balding *et al.,* 2004; Holbein *et al.,* 1981). Inside the human host, iron is usually complexed with iron-binding proteins such as lactoferrin, transferrin and haemoglobin (Otto *et al.,* 1992). Transferrin and lactoferrin are the most common iron binding proteins in extracellular body fluids, such as serum and milk respectively. Both iron-sequestering proteins have high binding constants for iron and they are usually

unsaturated which results in virtually zero free iron for bacterial growth (Griffiths and Williams, 1999). On the other hand, ferritin and haemoglobin molecules store the majority of intracellular iron (Schryvers *et al.,* 1999).

Research on *N. meningitidis* has established the fact of bacterial dependence on various iron sources for growth and propagation. Meningococci can utilize a variety of host iron sources including ferric and ferrous salts, heme (Hm), transferrin, lactoferrin, haemoglobin and its complexes but not heme-hemopexin or heme-albumin (Rohde and Dyer, 2003; Dyer *et al.,* 1987).

1.12.1 Transferrin

Human transferrin is a well-characterized 80 kDa bilobed glycoprotein, which binds the iron found in serum and lymph (Schryvers *et al.,* 1999). After synthesis in the liver, transferrin circulates in serum and sequesters the iron with high affinity. This iron attachment is beneficial in two ways (i) it avoids the damaging effects of iron and (ii) results in non-availability of the iron for microbes (Evans *et al.,* 1999). Each lobe of a monomeric transferrin molecule has a deep cleft that contains a ferric ion binding site and a bicarbonate anion-binding site which is believed to be responsible for neutralization of charges around the binding site (Schryvers *et al.,* 1999 and references within the review). Iron bound to transferrin is transported from the site of absorption to the location of utilization and released by a conformational change of the two domains of each lobe.

As transferrin is found most abundantly in serum, there is a strong potential of Tf association with the invasion and establishment of early stage of infection. This is due to the fact that Tf is the major source of iron available for bacterial growth during early phase of infection and invasion (Otto *et al.,* 1992). However, the availability of this iron

source during late phase of septicaemic spread is restricted due to hypoferremic response, as suggested by the Holbein *et al* (1981). At this stage, alternative Fe acquisition systems (described in Hb acquisition section) my be required to sustain the septicaemia.

1.12.2 Lactoferrin

Lactoferrin is a member of the transferrin family sharing many structural and functional features, such as the presence of a conserved three dimensional structure, reversible binding to ferric iron, an identical iron binding site with higher affinity than transferrin and about 60% sequence identity with transferrin (Baker *et al.,* 2002). It is found most abundantly in secretions such as mucus, milk and tears. Lactoferrin has been shown to cross the blood brain barrier during acute inflammation and may play an important role in iron acquisition during the course of disease (Pettersson *et al.,* 1998). It is also secreted by phagocytic cells i.e. neutrophils at the site of clearance (Schryvers *et al.,* 1998). *N. meningitidis* can differentiate human transferrin and lactoferrin from those derived from other sources, such as bovine and sheep. This provides one reason why meningococci are strict human pathogens (Schryvers and Gonzalez, 1990).

1.12.3 Haemoglobin

Haemoglobin is another important and well-characterized human iron-binding protein. The most important function of haemoglobin is the transport of oxygen bound to its heme moiety. Bound oxygen is released at the areas of higher carbon dioxide concentration, which lowers the pH by producing carbonic acid. Iron bound within the heme moiety of haemoglobin is not readily available for acquisition, as the heme moiety is not surface exposed. Under normal conditions, the concentration of free haemoglobin in human serum is very low and produced as a result of spontaneous haemolysis. This

free haemoglobin quickly dissociates into dimers and subsequently binds with circulating haptoglobin and the complex is transported to the liver for metabolism (Perkins-Balding *et al.,* 2004). In blood, haemoglobin bound with haptoglobin is the predominant iron complex compared to free haemoglobin (Hershko, 1975).

Keeping in view the physiological importance of haptoglobin, a brief discussion is relevant at this point. In humans, the protein is encoded by two (1 and 2) alleles, generating 2 homozygous phenotypes (1-1 or 2-2) and a heterozygous phenotype (2-1) of haptoglobin (Langlois and Delanghe, 1996). The same authors identified that type 1-1 exists as an 86 kDa monomer but 2-2 or 2-1 my reach up to 900 kDa by polymerization. Studies on *E. coli* have suggested that haptoglobin acts as a bacteriostatic agent due to its binding with Hb and the bacterial inability to utilize Hb-Hp complexes (Eaton *et al.,* 1982).

Although like transferrin, the availability of haemoglobin and its haptoglobin complex at mucosal surface is limited, Schryvers and co-workers have established that available heme is enough for the colonization and survival of heme-requiring microbes in the oropharynx (Schryvers and Stojiljkovic, 1999). The authors also concluded that the concentration of free haemoglobin could also fluctuate due to a local haemolysis, which may increase its availability to acquisition by meningococci and other pathogens using their haemoglobin receptors.

1.13 MECHANISMS OF IRON ACQUISITION

Generally, bacteria acquire iron by two mechanisms (i) Siderophores mediated iron acquisition (ii) Receptor mediated iron acquisition from host-iron binding proteins (Braun, 2001). Siderophores are low molecular weight iron sequestering compounds secreted by many environmental, commensal and pathogenic bacterial species whose

uptake as iron bound complexes is mediated by specific receptors. In *N. meningitidis*, no siderophores have been detected. However, some studies have suggested that these pathogens might use siderophores secreted by resident microbiota in their surrounding environment. Enterobactin, an *E. coli* siderophore can enhance gonococcal growth in iron-starved conditions (Carson *et al.,* 1999). These siderophores are transported by the ferric enterobactin receptor (FetA), but enterobactin binds to FetA with a relatively low affinity (K_d = 5 μM). This low affinity for this ligand may be due to the fact that enterobactin is not a natural substrate of this receptor. A homologue of this protein with 90% identity to that of gonococcal FetA has been identified in meningococci and presumably performs a similar function (Pettersson *et al.,* 1995).

During colonization and subsequent infection of a host, *N. meningitidis* comes across a variety of environments i.e. nasopharynx, bloodstream and finally cerebrospinal fluids. The concentration, availability and source of iron in each environment vary and may require a different mechanism for acquisition. Meningococci have evolved several TonB dependent iron acquisition systems for receptor-mediated uptake, which enable it to strip and acquire iron or heme under iron limited conditions from iron complexes such as transferrin, lactoferrin, haemoglobin and its haptoglobin complex (Schryvers and Stojiljkovic, 1999). These receptors are designated as TbpAB, LbpAB, HmbR and HpuAB, respectively. Apart from HmbR, all receptors contain a separate surface exposed substrate binding protein component. However a membrane-spanning protein component forming a pore is common to all the receptors including HmbR (Perkins-Balding *et al.,* 2004). The structure and mechanism of iron acquisition by each system is described here.

1.13.1 Transferrin and lactoferrin transport systems

In *N. meningitidis*, iron acquisition from transferrin is mediated by transferrin binding receptor proteins TbpA and TbpB, as shown in figure 1.13 (Cornelissen, 2003; Gray-Owen and Schryvers, 1996). Like other iron responsive outer membrane proteins, expression of both transferrin receptor proteins is induced in iron-limited conditions (Legrain *et al.,* 1993). The molecular weight of transferrin binding proteins was determined by affinity isolation method. TbpA is approximately 98 kDa trans-membrane protein while surface exposed TbpB is 68 kDa, although the molecular mass of both proteins vary among different meningococcal isolates (Ferreiros *et al.,* 1991; Griffiths *et al.,* 1990; Oakhill *et al.,* 2002).

Lactoferrin binding protein of *N. meningitidis* is also considered as a potential virulence factor due to its ability to strip iron from lactoferrin at the first entry and colonization point. As described earlier in the iron availability section, lactoferrin may be the predominant iron source on nasopharyngeal surfaces and iron acquisition from this source is likely to be important. The lactoferrin receptor is comprised of two proteins (see figure 1.13), LbpA and LbpB with molecular masses of 98 kDa and 84 kDa respectively (Schryvers *et al.,* 1998).

LbpA, like its transferrin counterpart component TbpA, is also a membrane spanning protein while LbpB is predicted to be a surface exposed lipoprotein. The stripping of iron by lactoferrin and transferrin receptors from their respective iron sources is thought to be initiated by high affinity binding to the substrate, followed by stripping and subsequent transport of iron to the periplasm (Perkins-Balding *et al.,* 2004). The translocation across the membrane and transport of ferric iron through the periplasm is mediated by the ferric binding protein system (Fbp) containing three components and

this system is employed for both TbpAB and LbpAB mediated iron acquisition (Khun *et al.*, 1998). Phase variation is not reported in these receptors and hence they will likely to be expressed under iron-limited conditions.

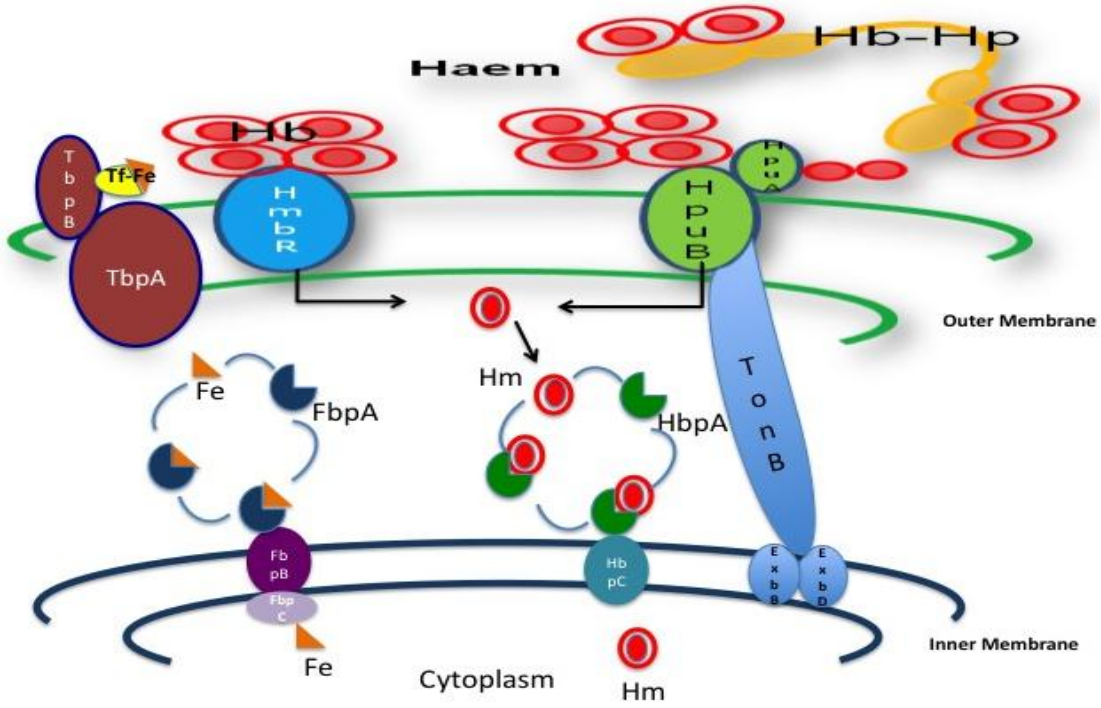

Figure 1.13: Model of iron acquisition systems expressed under iron-limited conditions and embedded in the outer membrane of *N. meningitidis*. The iron complexes are depicted along with their respective receptors. The transmembrane components of all receptors (shown for HpuB in this diagram) have a Ton Box, which interacts with a TonB system (coloured blue) located in inner membrane. This three-component system (TonB) provides the energy and its interaction with iron receptor complex results in formation of a gated pore for the transport of iron or heme into periplasm. In the periplasm FbpABC system transport iron from transferrin to the cytoplasm while a putative Hem TUV like system transports the heme moiety. The LbpAB system (Not shown) follows the same pattern as described for TbpAB. Hb, Haemoglobin; Tf, Transferrin; Hm, Haem; Hb-Hp; Haemoglobin-haptoglobin complex; Fe, Iron. (Modified from Perkins-Balding, 2004).

1.13.2 Haemoglobin transport systems; HmbR and HpuAB

In *N. meningitidis*, two different and independent heme acquisition systems (HpuAB and HmbR) have been described, which can recognize heme-containing proteins i.e.

Haptoglobin-haemoglobin or haemoglobin (Lewis and Dyer, 1995; Stojiljkovic *et al.,* 1995). HmbR was the first haemoglobin receptor identified in *N. meningitidis*, which preferentially, but not necessarily, binds to human haemoglobin (Stojiljkovic *et al.,* 1995; Stojiljkovic *et al.,* 1996). Insertional inactivation of *hmbR* by these authors suggested that prevention of haemoglobin uptake by meningococci can occur without affecting the uptake of transferrin and lactoferrin. Its association with virulence is also evident from studies conducted in the infant rat model, which showed that *hmbR* mutants were attenuated (Stojiljkovic *et al.,* 1995).

HmbR is an ~89 kDa Ton-B dependant outer membrane localized receptor (Figure 1.13) that specifically binds haemoglobin, strips out the heme and forms a gated pore for transportation of heme to the periplasm (Stojiljkovic *et al.,* 1995; Perkin-Balding *et al.,* 2004). The HmbR amino terminal region contains a signal peptide sequence and mature protein is 23 amino acids shorter than the nascent protein (Perkins-Balding *et al.,* 2003). A weak homology (21 and 22%) of the amino acids sequence with those of Tbp and Lbp is observed (Stojiljkovic *et al.,* 1995).

The structural model of HmbR protein is yet to be elucidated. However, a two dimensional model on the basis of its homology with other TonB-transport systems was proposed previously (Perkins-Balding *et al.,* 2003). The model suggested that HmbR consists of 22 putative membrane spanning beta-sheets, 11 surface exposed loops and an N-terminal “cork” region. A series of experiments based on deletions, insertions and site directed mutagenesis have identified loops L2 and L3 as putative Hb-binding regions. Similarly, mutations localized to loops L6, L7 and L10 identified their importance in haemoglobin utilization, without affecting its binding (Perkins-Balding *et al.,* 2003). Evans and co-workers have recently complemented this 2D model by using hydrophobicity plots to predict the topology for HmbR (Evans *et al.,* 2010). The

deduced model was similar to the 2D model except with different lengths of beta strands and confirming the presence of 22 membrane spanned beta sheets, 10 periplasmic loops and 11 surface-exposed loops.

Once heme is transported to the periplasm by HmbR, the transport through the inner membrane is not well known. However, like other Gram-negative bacteria, an ABC transporter system is likely to be involved in heme transport (Perkins-Balding *et al.,* 2004). The utilization of iron from transported heme in the cytoplasm is also unclear.

HpuAB, a second haemoglobin receptor in meningococci and constitute a bipartite haemoglobin binding protein complex (Figure 1.13). Both the HpuA and HpuB components are required for binding to haemoglobin. In contrast with HmbR, this receptor can also bind to other iron complexes such as haptoglobin-haemoglobin and apo-haptoglobin (Lewis and Dyer, 1995; Rhode and Dyer, 2004). Growth assays performed previously to analyse the preferred iron sources by this receptor complex and the binding affinities for these sources in strain DNM140 have suggested that HpuAB utilizes Hb-Hp complex more efficiently (up to 14 folds) than Hb alone (K_d = 555 nM), and more importantly a preference for 2-2 (K_d = 84 nM) and 2-1 (K_d = 39 nM) Hb-Hp phenotype was observed (Rhode and Dyer, 2004). However, studies have indicated no significant difference in recognition of haemoglobin from different sources by the meningococcal HpuAB (Rohde *et al.,* 2002). This author has also indicated a preference for binding to oxidized methaemoglobin, possibly due to the abundance of this iron source compared to haemoglobin during erythrocyte lysis.

In HpuAB receptor complex, HpuA is a 37 kDa surface-exposed lipoprotein while an 85 kDa HpuB transmembrane component likely to act as a gated pore. A bicistronic operon encodes the HpuAB complex and insertional inactivation of *hpuA* leads to the

inactivation of *hpuB*, as it lacks its own promoter (Lewis *et al.,* 1997). HpuA is attached to the outer membrane by its lipid component, however structural predictions are inconclusive due to the fact that it does not have significant sequence similarity to any other known structure (Perkins-Balding *et al.,* 2004; Lewis *et al.,* 1997). The protein's predicted pI is 6.51 and it is fairly uncharged due to a similar distribution of positive and negative amino acids.

Although structural data for HpuB is also limited and a structural model has not been published, a beta-barrel structure forming a pore is predicted due to its similarity (although weak) with other TonB-dependent systems including 25% and 40% sequence identity with TbpA and LbpA respectively (Rohde *et al.,* 2002). Protease susceptibility assays performed by this author have suggested that both components, HpuA and HpuB, have physical interactions and both proteins have surface accessibility. This assay identified a 21 kDa HpuA domain containing protease restriction sites when digested alone, but protected from cleavage in the presence of HpuB.

Interestingly, the distribution of HpuAB and HmbR in meningococci varies among different stains. Some strains contain both systems, while other strains appear to encode either HpuAB or HmbR suggesting a role in meningococcal disease (Lewis *et al.,* 1999; Richardson and Stojiljkovic, 1999). A significantly higher frequency of *hmbR* has been detected in disease isolates compared to carriage isolates, and suggested a link between haemoglobin acquisition and meningococcal pathogenesis (Harrison *et al.,* 2009). A significant level of antigenic diversity in HmbR is also observed which supports the idea that HmbR variations may also facilitate immune evasion (Evans *et al.,* 2010). A recent study using human whole blood as an *ex vivo* model of infection also reported up-regulation of *hmbR* in a time-course transcriptome analysis of strain MC58

(Echenique-Rivera *et al.,* 2011). This observation further highlights the importance of Hb receptors in blood adaptation and survival.

An absolute reason for the redundancy in receptors specific for haemoglobin and its complexes is not clear. A possible explanation is that it may enable meningococci to acquire heme from a variety of haemoglobin complexes i.e. haemoglobin, haemoglobin-haptoglobin and haptoglobin, or a variety of niches and also may facilitate immune evasion.

Like many other surface exposed and virulence related proteins, the expression of both haemoglobin-binding systems, HpuAB and HmbR, is phase variable due to presence of a poly(G) repeat tract in their respective open reading frames (Lewis *et al.,* 1999; Richardson and Stojiljkovic, 1999). Slipped strand associated mutations in these repeat tracts during DNA replication results in switches between ON to OFF states of expression and vice versa. The switching frequency of Hb receptors varies between 10^{-2} to ~10^{-6} in isolates of different serogroups, and the rate of phase variation within a specific strain is reported to remain similar if the length of homopolymeric repeat tracts remains constant (Richardson and Stojiljkovic, 2001). However, the phase variation rates of these genes were increased up to >100 fold higher in epidemic serogroup A strains defective in mismatch repair system than the wild type strains (Richardson and Stojiljkovic, 2001; Richardson *et al.,* 2002). As the HpuA and HpuB components are co-transcribed, the frame-shift mutations due to slippage in poly(G) tract of *hpuA* also leads to lack of HpuB expression (Lewis *et al.,* 1999).

However, in addition to scavenging iron, adaptation of *Neisseria meningitidis* to a dynamic and highly selective host environment is a key attribute and may need for example a balance between iron acquisition and immune evasion. Meningococci face

this situation by switching phenotypes to accommodate the changing environment and to survive the selective pressure. A recent publication identified that after accidental human passage of a serogroup A *mutS* mutator strain, the blood isolate showed modifications in these phase variable genes compared to the infecting/un-passaged strain allowing a different phenotypic expression (Omer *et al.,* 2011).

The authors suggested that haemoglobin acquisition from haemoglobin-haptoglobin is important for *in vivo* growth of meningococci based on their observation of the preferential switching of *hpuAB* from OFF to ON compared with *hmbR,* which surprisingly switched OFF. They also hypothesized that this may be due to the presence of HmbR antibodies (although they did not test for them) facilitating the immune escape, and that even a passage of a few generations can lead to genomic modifications which may assist the bacterial survival and invasion.

1.14 IRON REGULATION IN MENINGOCOCCI

N. meningitidis and other bacteria require iron as an essential element for their growth where it performs a variety of essential metabolic functions by serving as a cofactor for a number of enzymes. These iron dependent enzymes include those involved in DNA replication, electron transport and oxygen metabolism (Griffiths, 1999). However excess iron may have serious detrimental effects on microorganisms due to its ability to react with free oxygen forming toxic radicals. These harmful effects are reduced by strictly regulating iron uptake to avoid overload inside the cell.

Regulation of uptake is the only known option, as no system to export excess iron from the cells has yet been identified. In most of the Gram-negative microbes, iron-uptake regulation is achieved by regulating the level of transcription through the action of the ferric uptake regulatory (Fur) protein (Escolar *et al.,* 1999). When iron is available in

higher concentration, Fur recognizes and binds to a consensus sequence found inside or close to the promoter sequence of iron-uptake genes. This binding with the consensus sequence, also called the Fur box or iron box, repress and blocks the binding of RNA polymerase and ultimately transcription. However, Fur does not repress the expression of all genes but also causes activation of some genes (Delany *et al.,* 2004). Fur is also associated with the controlling expression of genes involved in iron storage and expression is enhanced many fold when intracellular iron levels are high. A Fur homolog is present in *N. meningitidis* and Fur boxes have been found in many iron acquisition genes including *hpuA* and *hmbR* (Lewis *et al,* 1997; Stojiljkovic *et al.,* 1995).

Project background

Capsule based vaccines including those specific for serogroups A, C, Y and W135 have been successfully introduced and used for preventing meningococcal infection. However, this type of vaccine is ineffective against serogroup B strains due to structural resemblance between the capsular polysaccharides and the human glycans of neuronal-cell adhesion molecules (Finne *et al.,* 1987). Similarly, multi-component vaccines have been reported for *N. meningitidis* with comparatively broad coverage, but efforts to develop a comprehensive vaccine development are still continuing due to limitations of coverage against all hypervirulent strains (Weynants *et al.,* 2007; Giuliani *et al.,* 2006).

Outer membrane proteins (OMPs) of *N. meningitidis* are potential targets for immune clearance mechanisms and are the new options being explored for development of vaccines. However, the expression of the majority of these surface components varies whilst the impact of phase variation on host-microbe interactions is largely unknown. Specific antibodies for phase variable surface exposed antigens have been detected and

are associated with bactericidal activity against *N. meningitidis* (Borrow and Miller, 2005; Heyderman *et al.,* 2006).

Previously, phase variable surface structures were considered unsuitable as vaccine targets due to fear of the constant variations and immune escape. Detailed and in depth studies of these surface exposed structures and exploration of their structure-function relationships is needed and may lead to selection of an appropriate vaccine candidate(s). It is predicted that a future broad coverage vaccine will not be comprised of only one antigen but a combination of more than one antigen. Such vaccines have been developed for protection against the circulating virulent strains but the potential for protection against new resistant strains in future is still unclear (Giuliani *et al.,* 2006).

Haemoglobin binding proteins were identified as important surface components but not yet included in a vaccine regime. As described in earlier sections, epidemiological studies conducted on one of the haemoglobin receptors and analysis of *in vivo* passaged strains shed light on the importance of haemoglobin acquisition by HmbR for invasion and virulence of meningococci (Harrison *et al.,* 2009; Omer *et al.,* 2011). However the combined influence of both receptors on meningococcal virulence is not well documented. In particular, the lack of a comprehensive study on distribution and phase variation (PV) status of haemoglobin receptors, and the impact of immune responses on these surface proteins in epidemiological samples or infection models have restricted our current knowledge. Thus, a detailed study of distribution, antigenic and PV status of receptors in diverse meningococcal samples from disease and carriage may contribute to our understanding of the disease. As the bacterium is a strict human commensal and a good animal model has not been developed so far, this restricts *in vivo* analysis of virulence characteristics. In such a scenario, data obtained from collections of carriage and virulent strains and *in vitro* studies are of great significance.

In addition, as described earlier, the expression of both haemoglobin receptors is phase variable due to presence of a stretch of poly(G) residues in their open reading frames. Previous research by Bayliss and colleagues has demonstrated the association of phase variation to escape from MAb B5 specific antibodies through high frequency switches in expression of *lgtG* gene (Bayliss *et al.,* 2008). Both, *in vivo* and *in vitro,* assays demonstrated that a change in length of tandem repeats (12C to 11C) present in open reading frame of the gene influence the antigenic structure of the component and responsible for the production of variants associated with immune evasion of specific antibody. However, a similar role of phase variation of haemoglobin receptors in immune evasion is largely unexplored. The generation of antibodies against the haemoglobin receptors and subsequent tests for bactericidal activity against meningococci might be useful to understand the biological role of phase variation of these receptors. An understanding of the capacity for phase variation to mediate immune evasion by *N. meningitidis* is relevant to an understanding of virulence, progression and pathogenesis of meningococcal disease.

This study focussed on two phase variable genes, *hmbR* and *hpuAB,* of *N. meningitidis* for an epidemiological survey of their presence or absence and PV status in various isolate collections. The study of variability of both *hmbR* and *hpuAB* revealed a complex relationship between presence of these genes and heme acquisition. As the biological significance of PV of these genes in immune evasion is not previously published, the study also investigated such role of phase variation of both *hpuAB* and *hmbR*. For such analysis, phase variation of *porA* was used as a model to study PV mediated immune escape by meningococcal strain 8047. PorA was selected as a model keeping in view its importance as a major epitope on bacterial surface and a potential vaccine candidate. However, as the impact of PV on escape by *porA* is not described

previously and the validity of immune evasion assay was needed, the PV mediated escape by *porA* was established firstly to validate the modified bactericidal assay. The data generated was then used as a rationale to study the role of PV of haemoglobin receptor encoding genes in immune evasion. The analysis of distribution of haemoglobin receptor encoding genes and subsequent investigation of immune evasion by phase variation of these genes is relevant to test the potential of these genes as a future vaccine candidate(s).

Aims:

The study has the following aims:

(i) Investigate the frequency, distribution and variations in the repeat tracts of *hpuAB* and *hmbR* in diverse collections of disease and carriage isolates in order to test whether presence of these genes is associated with meningococcal virulence?

(ii) Assess the potential for PV mediated immune escape for *porA* using a modified *in vitro* serum bactericidal assay.

(iii) Generation of HpuA polyclonal antisera, and development of an escape assay for one of the Hb receptors.

Chapter 2

MATERIALS AND METHODS

2.1 BACTERIAL ISOLATES AND CULTURE CONDITIONS

For the epidemiological study, four different, unrelated and separate groups of strains were analysed. First group, provided by Martin Maiden, comprised of a stock of 107 isolates was assembled globally between 1937 and 1996 and has already been typed by MLST (Maiden *et al.*, 1998). This group was extended by inclusion of nine separate isolates (MC58, 8047, Fam18, Z2491, 4664B, 4464, 44/76, 8047 *ΔmutS*, and 5824) during this study (MLST 107 + 9 = 116 isolates). The second group contained 88 carriage isolates collected from first year students of Nottingham University in a study conducted by Bayliss and co-workers (Bidmos *et al.*, 2011). The third collection includes 153 isolates collected in the Czech Republic in 1993 (Jolley *et al.*, 2000).

The fourth group comprised of 159 strains (77 disease and 82 carriage isolates) was collected in a study conducted between 1999 and 2000 in England and Wales (Health protection agency meningococcal reference unit and the UK carriage study). Twelve additional isolates were also included and were entirely comprised of strains from the ST41/44 complex, provided by Ian Feavers (National Institute for Biological Standards, UK). The classification of isolates in to disease and carriage was based on whether they have been isolated from a patient or a healthy carrier.

Where appropriate, the bacteria were grown either on chocolate agar plates (Oxoid) or brain heart infusion agar (Oxoid) supplemented with Levinthals (40ml per 400ml BHIA) overnight at 37°C in the presence of 5% CO_2. For Levinthals supplement, 500 ml of defibrinated horse blood (Oxoid) was added to 1 L (autoclaved) of brain heart

infusion medium (Oxoid) and heated at 95°C for 40 min. After cooling to below 50°C, the mixture was centrifuged at 2000 g for 25 min at 4°C. The supernatant was aliquoted into 40 ml lots and frozen at –20°C.

Frozen cultures of bacteria were stored at –80°C in brain heart infusion broth (BHI) containing 20% v/v glycerol. For recovery of cultures, a small amount from frozen stock was scraped by using a 10 µl loop and streaked on agar plates followed by an overnight incubation at 37°C in the presence of 5% CO_2. For each assay, fresh cultures from frozen stocks were used to maintain reproducibility of assay. Stocks of *E. coli* strains, were also stored under similar conditions but in LB (1% w/v tryptone, 0.5% w/v yeast extract and 0.5% w/v sodium chloride) containing 15% glycerol and recovered on LA (1.5% w/v agar) plates whenever necessary. All media were purchased from Oxoid and autoclaved at 121°C for 15 min for sterilization. Antibiotic solutions were added as appropriate.

2.2 DNA MANIPULATION METHODS AND ANALYSIS

Chromosomal DNA extraction was done either by the CTAB method or using a DNeasy Blood & Tissue Kit (Qiagen) and the recommended protocol of the manufacturer. In the CTAB method for chromosomal DNA extraction, a loop full of bacteria from an overnight culture on a plate was re-suspended in 576 µl of TE buffer. To this suspension was added 30 µl of 10% SDS and 3 µl of proteinase K (15 mg/ml) followed by incubation at 37°C for 1 h. After incubation, 100 µl NaCl (5 M) and 80 µl of CTAB solution {(25 ml Tris HCl (pH 8), 5 M NaCl (70 ml), 0.5 M EDTA (10 ml), 20 g cetyltrimethyl ammonium bromide (CTAB), distilled water to make 250 ml)} was added and again incubated at 65°C for 10 min. An approximately equal volume of chloroform:isoamyl alcohol (24:1) was added, mixed thoroughly and centrifuged

(12100 g for 5 min at room temperature). The supernatant was transferred to a fresh tube, the above extraction step was repeated and again supernatant was recovered in a fresh tube. Isopropanol (0.6 volume) was added (to precipitate the DNA) followed by centrifugation (12100 g) for 20 min. The pellet was recovered, washed with 70% ethanol and, after 15 min centrifugation, the supernatant was discarded. The pellet was dried in speed vacuum (Fischer scientific) for 2 min and resuspended in 50 µl TE with added RNase and stored at 4°C for further use.

2.2.1 Plasmid extraction

Plasmid isolation from *E. coli* was achieved using E.Z.N.A Plasmid Mini Prep Kit (Omega) and the instructions within it.

2.2.2 Gel extraction

Where necessary, the fragments from agarose gels were identified and excised under the UV visualization. The recovery and cleaning up of DNA fragments from agarose gels was achieved by spin columns and buffers provided in a Zymoclean Gel DNA Recovery Kit (Zymo Research). The fragments were re-loaded on 1% agarose gel to confirm the recovery and to determine the concentration, and subsequently used for ligation and cloning.

2.2.3 Restriction enzyme digestion

All enzymes used were purchased from New England Biolabs Ltd and used for digests in the presence of buffer provided by the supplier. Approximately 10 units of enzyme were used to digest every ~5 µg of plasmid DNA or PCR products.

2.2.4 Estimation of DNA concentration using a NanoDrop spectrophotometer.

DNA was quantified using a NanoDrop spectrophotometer (ND 1000, Thermoscientific), which was calibrated against the distilled water used as diluent. 1 µl of sample DNA was loaded onto the pedestal and the concentration and purity of sample was measured.

2.3 PCR AMPLIFICATION FOR DETECTION OF *hpuA* AND *hmbR*

PCR amplification and sequencing of fragments of the *hpuA* and *hmbR* genes was performed using the oligonucleotide primers listed in Table 2.1 which were supplied desalted by Sigma, UK. Primers were designed on the basis of analysis of published genome sequence data available at NCBI and by using the software Clone Manager (Sci-Ed Software). The PCR were performed using Taq DNA Polymerase (ABgene) unless mentioned otherwise.

The presence or absence of *hpuAB* was detected with primers HpuAC and HpuA350-Rev and a DNA segment of *hpuA* gene (spanning the repeat tract at 5' end of the gene) was amplified. Genomic DNA (1 µl) of appropriate dilution was used as template in a PCR mixture having 2 µl of a PCR buffer, 0.4 µl of 25 mM $MgCl_2$, 0.5 µl of 10 mM dNTPs, 0.2 µl of a 10 µM stock of each primer and 0.1 µl of Taq DNA polymerase (5U/µl). The final volume of the reaction mixture was 20 µl, after adding the required volume of water. Thermocycler conditions used for this PCR are listed in Table 2.2.

The presence of the *hpuA* gene was further confirmed by amplifying the whole *hpuA* locus using primers HpuAC and p26.85. The concentration of PCR ingredients and the thermocycler conditions were kept same as described for above PCR. *hpuAB* negative

Table 2.1: List and sequences of oligonucleotides used for PCR amplification in the study.

Primer	Sequence (5' to 3')
HpuA-NTERM	5'-CACCGCCGAACCGCACGTCCCCG-3'
HmbR-NTERM	5'-CACCATGAAACCATTACAAATGCTCCC-3'
HmbR-STOP	5'-TCATTGGGCCCCGAGTTGCAACGGATGG-3'
HmbR-CTERM	5'-TTAAAACTTCCATTCCAGCG-3'
HpuB-CTERM	5'-TTAGAACTTCGCTTCGATGG-3'
hmbr-RF3	5'-TGCCAACCTCTTTTACGAATGG-3'
hmbr-RF4	5'-GCTACTGAACACGTCGTTCC-3'
HpuA350-Rev	5'-GGATGAAAGGGCGTATTGCGC-3'
HpuAC	5'-ATGCGATGAAATACAAAGCCC-3'
p26.85	5'-GGGAAACGCTTGGGCGATGG-3'
HpuF-Seq	5'-GGC AAC TTT TCC ACC GTC ATT C-3'
HpuR-Seq	5'-TGG TCT GGA TTC CCG CCT GC-3'
Hpu-for1	5'-GCAACAATGCCTTGTCATCC-3'
Hpu-Rev13	5'-TGATCGAAATGGGCGTACTC-3'
HmbR-for2	5'-CGGCATTCAGGTCAAAATCCC-3'
HmbR-Rev7	5'-GCCGAAGGATCCAATTTGGG-3'
HYP-HP F	5'-CCTGCCGCACACCAAACAAC-3'
GR-HP R	5'-TGATATGGGCGGCATGGGTG-3'
HpuB-1850 Rev	5'-GCC AAA CGG GCG AGC TGA CC-3'
HpuA-end-For	5'-GCT CCC TCG ATA CCG TAT TC-3'
HA-Inv 938	5'-CTGATATCAGTGTCCCGGTAGCCG-3'
HB-Inv1	5'-CGGATATCGACTGGCGGTTTACCAAG-3'
FAM-HpuA350Rev	FAM-5'-GGATGAAAGGGCGTATTGCGC-3'
FAM-hmbr-RF3	FAM-5'-TGCCAACCTCTTTTACGAATGG-3'

isolates were further screened with primers Hpu-for1 and Hpu-Rev13, which flank the *hpuAB* locus. The concentration of PCR ingredients was kept same as described for the previous PCR. However, the extension time was increased up to 5 min for this PCR. Similarly the presence of *hmbR* gene was detected with primers hmbR-RF3 and hmbR-RF4. The cycling program and concentration of the chemicals required for PCR mix were similar to that described for *hpuA* detection PCR (using HpuAC and HpuA350-Rev primers).

Table 2.2: Thermocycler conditions for PCR amplification

No.	PCR step	Temperature	Time
1	First denaturation	94°C	5 min
2	Denaturation	94°C	60 s
3	Annealing	55°C	1 min
4	Elongation	72°C	60 s
5	Go to 2, repeat 30 cycles		
6	Final elongation	72°C	10 min

2.3.1 Agarose gel electrophoresis of PCR products

Amplified fragments were separated and detected by using agarose gel electrophoresis. A 1% agarose (Seakem LE agarose, Cambrex) gel in 1× TAE (9.68 g/L Tris base, 0.74 g/L EDTA, pH 7.7 adjusted by glacial acetic acid) was prepared by heating in a microwave oven. The solution was cooled to ~65°C and ethidium bromide was added with a final concentration of 0.5 μg/ml. The prepared solution was poured in to a gel casting tray containing a comb. Once the gel is set, the comb was removed to create wells and DNA samples mixed with loading buffer (1× TAE , 1.5% glycerol and 0.5% Orange G) were loaded and subjected to electrophoresis in 1× TAE buffer. The gels were visualized under UV transilluminator in a gel documentation system (Syngene). DNA marker (Hyperladder1, Bioline) was used for determination of size of the products by visually comparing the sample to the known sizes of the marker.

2.4 SEQUENCE ANALYSIS FOR ON-OFF STATUS AND TRACT LENGTHS DETERMINATION

Sequence analyses for ON-OFF status and tract length distribution of isolates from MLST (Maiden *et al.,* 1998) and carriage groups (Bidmos *et al.,* 2011) were determined

by a combination of GeneScan and sequencing. For *hmbR* and *hpuAB* sequence analysis, amplicons were sequenced using the primers hmbR-RF3 and HpuA350-Rev primers, respectively. The DNA sequencing reaction mixture (10 µl), consisted of 0.5 µl PCR product as a template, 0.5 µl Big Dye 3.1 terminator mix, 1 µl primer (10 µM), 0.8 µl sequencing buffer (Applied Biosciences) and the appropriate amount of water. Sequencing reactions were done in a G-storm GS1 thermocycler with an initial denaturation step of 96°C for 5 min followed by 24 cycles of denaturation at 96°C for 20 s, annealing at 50°C for 15 s and primer extension step at 60°C for 4 minutes.

Subsequently, 10 µl of dH_2O and 2 µl of 2.2% SDS was added and boiled in the thermocycler for 5 min at 96°C. The mixture was cooled to room temperature followed by a brief spin to collect the liquid. Samples were purified using gel filtration cartridges (Edge Bio) for the extraction of unincorporated nucleotides of terminator and excess of primers. The purified reactions were dispatched for sequencing to the Protein and Nucleic Acid Chemistry laboratory (PNACL), using an applied Biosystems 3730 sequencer. Traces were analysed using Gap4 in STADEN package and G residues in the repeat tracts of *hpuA* and *hmbR* were enumerated.

The ON-OFF status and tract length distribution of isolates from 2008 carriage group were determined by a combination of GeneScan (details below) and sequencing. The isolates were sub-grouped on the basis of identical *fetA, porA* and MLST types. One/two representative from each group were sequenced for *hpuA* and *hmbR* genes with primers HpuA350-Rev and hmbR-RF3, respectively. For GeneScan, all isolates from this group (carriage) were amplified with FAM-labelled HpuA350-Rev (for *hpuA*) or hmbR-RF3 (for *hmbR*) primers with their appropriate matching primers, as described in section 2.3.

2.4.1 A-tailing of the PCR products

After PCR, the samples (10 µl) were subjected to A-tailing by adding 4 µl reaction mix containing 0.4 µl reaction buffer (10X), 0.4 µl of $MgCl_2$ (25mM), 0.05 µl Taq and 3.15 µl dH_2O, followed by incubation at 72°C for 45 minutes. PCR products were diluted 10 folds in dH_2O and a mix was prepared containing 0.5 µl diluted PCR sample, 0.5 µl GeneScan 500LIZ as size standard (Applied Biosystems), and 9 µl formamide. The samples were denatured at 95°C for 3 min followed by electrophoresis on an autosequencer (Applied Biosystems, USA). GeneScan data was analysed using Peak Scanner software V1.0 software (Applied Biosytems).

2.5 STATISTICAL ANALYSIS

The statistical analysis for epidemiological studies was done by using Graphpad Prism version 5. A Chi-square test was used to derive odds ratio (OR) and 95% confidence intervals (CI) and *p* values were derived with a Fisher's exact test.

2.6 HAEMOGLOBIN DISC ASSAY FOR ANALYSIS OF PHASE VARIANTS

Phenotypic analysis of phase variants was done by using sterile filter discs impregnated with or without an added iron source. Bacteria were grown overnight on BHI plates with added Leventhals supplement. A single colony was re-streaked on BHIA and again incubated overnight. A suspension of 1×10^8 bacterial cells were prepared from the overnight culture and spread onto MHA plates with and/ or with out desferal (30-40 µM). 10 µl of human haemoglobin (100 µM, Sigma), transferrin (50 mg/ml, Sigma) or iron (II) sulphate heptahydrate (40 mM) were applied to sterile filter discs and left for 5 min to dry. The discs were placed on plates previously inoculated with bacteria. A third

disc without any added iron source was also placed as a control and the plates were incubated overnight at 37°C. The growth on plain MHA plates and those with desferal were compared.

2.7 *hpuAB* FLANKING PCR AND SEQUENCE ANALYSIS FOR DETERMINATION OF MECHANISM OF DELETION AND REPLACEMENT OF LOCUS

The deletion or replacement of this locus was confirmed by amplification with Hpu-for1 and Hpu-Rev13 primers. *hpuAB* negative isolates were subjected to PCR and the products were analysed by electrophoreses. The PCR products were sequenced in both directions using primers Hpu-for1/ Hpu-Rev13, followed by amplification and sequencing with HpuF-Seq and HpuR-Seq to reveal the replaced sequence of *hpuAB*. The upstream and downstream sequences obtained were aligned with the homologous sequence from Z2491 genome sequence. Similarly a blast search was done to identify the sequences replacing the *hpuAB* locus.

2.8 *hpuA* SEQUENCE VARIATION ANALYSIS

The sequence variation of complete *hpuA* sequences among different isolates was determined by PCR amplification of the complete *hpuA* locus with primers HpuAC and p26.85, The amplicons generated were sequenced with both primers and a set of internal primers, HpuR-seq2 and Hpu-seq3. The sequences were aligned using Clone Manager (Sci-Ed Software) to identify polymorphisms or indels. Amino acid sequences were also determined by translating the DNA sequences in ClustalW (http://www.genome.jp/tools/clustalw/) for each strain and subsequently aligned to identify the regions of potential variation among the HpuA sequence of isolates from different *porA* types and serogroups.

2.9 *IN VITRO* SERUM BACTERICIDAL STUDIES FOR 8047 PorA BY MONOCLONAL ANTIBODY P1.2

The bacterial escape and selection assay utilized in this study was developed and described by Bayliss and co-workers (Bayliss *et al.*, 2008). Monoclonal antibody P1.2 was used in this assay, which recognizes an outer membrane PorA protein epitope in strain 8047. Bacterial cultures were grown overnight on BHI agar plates supplemented with Leventhals. The bacterial culture was re-suspended in 1.5-2 ml of phosphate buffered saline B {PBS supplemented with $MgCl_2$ (0.5 mM) and $CaCl_2$ (0.9 mM) at pH 7.4}. Known dilutions of cells (1:50) were lysed in 1% sodium dodecyl sulfate-0.1 M NaOH, and the concentration of cells was estimated by measuring optical densities at 260 nm (OD260) on a NanoDrop spectrophotometer (Thermoscientific) with PBSB as a blank. Using this reading, following formula was used to calculate number of cells. As OD260 of 1.8 = 1×10^9 cells, thus volume containing 1×10^9 cells/ml in sample can be calculated as given;

Volume (ml) containing 1×10^9 cells = 1.8/OD260 × dilution of sample.

2.9.1 Preparation of inoculum for passage in the presence of monoclonal antibody

An aliquot of 1×10^9 cells per 300 µl was prepared by calculating the amount of cells required from the OD results and then diluted serially. Variable sizes of inoculum were prepared by mixing 50 µl of bacteria from the appropriate serial dilution with 450 µl of PBSB containing 0.1% glucose and pipetted separately in to wells of a 24 well tissue culture plate. Appropriate volumes of serial dilutions of the suspensions were also plated on solid media to obtain CFU count of the inoculum used.

2.9.2 Passage of inoculum in presence of MAb P1.2 and human serum

The inoculum in each well was mixed with 500 µl of 10% human pooled serum collected from healthy individuals and prepared in PBSB-0.1% glucose. Finally, a suitable concentration of antibody was added to all wells except those being used as a control. The culture plate was incubated at 37°C in the presence of 5% carbon dioxide for two hours. After first passage, 500 µl culture was removed from each well including the control, and mixed again with 500 µl of fresh 10% human serum (5% final concentration in wells after adding 500 µl culture) and antibody to continue for a second passage.

The remaining passaged cells from the first incubation were diluted serially and an appropriate dilution was plated overnight on BHI agar. The subsequent passages (3rd and 4th) were performed as described for the second passage. The number of CFU of each inoculum and passage were estimated from incubated plates. The CFU count from samples incubated without antibody was compared with samples of the same size incubated with antibody to observe any change in cell number in the presence of antibody.

2.9.3 Immunoblotting of passaged and un-passaged population and detection of phase variants

The detection of phase variants was done by immunoblotting in the presence of antibody as described by Martin and co-workers (Martin *et al.*, 2003). The overnight grown colonies were transferred to nitrocellulose membrane filters (Whatman) and dried for ten min, followed by 1 h incubation in blocking buffer (2% BSA in PBS + 0.01% sodium azide) with gentle shaking. Plates were transferred to an incubator for re-incubation (~6-8 h) after transferring the colonies on to nitrocellulose filters.

The filters were washed three times with PBS and incubated with 1:2000 dilution of antibody diluted in PBST-BSA (PBS containing 0.1% Tween-20 and 2% BSA). The filters were washed three times prior to incubation with a suitable dilution (1:3000) of secondary antibody (Anti-mouse IgG alkaline phosphatase-conjugated, Sigma) in PBST-BSA and left for 1 h on a shaker. The filters were washed with PBS and developed with alkaline phosphatase substrate solution containing 5-bromo-4-chloro-3-inodylphosphate-nitroblue tetrazolium supplied by Perkin Elmer Life Sciences.

Phase variant colonies on developed filters were detected by difference in staining of colonies. The identified variants on filters were matched with those on representative re-incubated plates and each variant colony was re-streaked on to a fresh BHI agar plate followed by an overnight incubation and used for preparation of a boiled lysate for genetic analysis.

2.9.4 *porA* repeat tract lengths determination

Genomic DNA preparation was performed by a modified protocol. The sweeps from these plates were re-suspended in 150 µl of water containing 0.01% sodium azide and incubated for 1 h in hood. Following this, bacteria were incubated at 50°C for 1 h, then at 98°C for 3 min and centrifuged (12100 g) to collect the supernatant. *porA* repeat tract was amplified by PCR using the primer porA-p21 and porA-72. The repeat tract number was determined by a combination of sequencing with primer porA-72 and GeneScan analysis as described above.

2.10 ASSAYS FOR CONFIRMATION OF DIFFERENCE OF LEVEL OF EXPRESSION OF PorA ANTIGEN IN PHASE VARIANTS

Three different assays were used to compare levels of expression of an antigen in phase variants from the selection assay. Phase variants collected above after selection assay were used for this.

2.10.1 ELISA of the PorA phase variants of strain 8047

2.10.1.1 formalin fixation

N.meningitidis strain 8047 was grown overnight in 10 ml of BHIB supplemented with Leventhals. Next day, the bacteria were harvested by centrifugation at 2000 g and cell pellet was re-suspended in PBS and the step was repeated. The above step was repeated and the pellet was re-suspended in 0.05% formalin in PBS. After 1 h fixation, the cell pellet was washed two times with PBS and the bacteria were re-suspended in coating buffer. The concentration was determined by determining OD550 of the sample and then adjusted to ~0.5.

2.10.1.2 ELISA of fixed cells

ELISA plates were coated with 100 µl/well of fixed cells (OD550 = 0.5) in coating buffer and incubated overnight at 4°C. The blocking of the binding sites was performed as described in section 2.15.1. After blocking and washing three times with washing buffer (TBS with 0.05% Tween-20), 100 µl of *porA* antibody dilutions (starting from 1:100) were added to duplicate wells and incubated the plate for 1 h at room temperature. Wells containing buffer only were kept as negative controls. Plates were washed and 100 µl of a 1:3000 dilution of anti-mouse alkaline phosphatase conjugated antibody (Sigma) in washing buffer was added and incubated for another hour at room

temperature. The plate was washed and developed by a developer solution and OD405 was measured for each sample and the control.

2.10.2 Western blotting of lysates from wild type and phase variants

Phase variants collected were grown overnight on BHI agar plates. The cells were re-suspended in PBS and adjusted to a concentration of 1×10^9 cells/ml. The cells were centrifuged and then the cell lysate was prepared. An equal volume (10 µl) of each sample was loaded on to two different gels and subjected to electrophoresis. Western blot was performed for one gel using MAb P1.2 (1:2000 dilution) to detect PorA protein and examine the difference in level of expression between phase variants. The second gel was stained with Coomassie blue to confirm that equal amounts of protein were loaded for each sample.

2.10.3 Flow cytometry analysis for detection of surface expression of PorA in phase variants

An additional method to assess the difference and level of expression in phase variants through flow cytometry was also used. An over night culture of strains in liquid medium (10 ml) was prepared and 200 µl was aliquoted into a 1.5 ml tube. The samples were centrifuged and washed twice with ELISA wash buffer (TBS with 0.05% Tween-20). To this was added 150 µl of a 1:50 dilution of MAb P1.2 in ELISA buffer and the sample was incubated on ice for 1 h. Samples were then washed twice with wash buffer and 150 µl of 1:100 dilution of secondary antibody, anti-mouse IgG-FITC conjugate, was added and incubated on ice for another hour. Bacterial cells were washed twice and re-suspended 750 µl of fixer (ELISA buffer containing 0.05% formalin). A negative control of strain 8047 without primary antibody but treated with secondary antibody was used for comparison. The mean fluorescent intensity (MFI) for

each sample was measured using FACS machine (Becton Dickinson FACS Calibur) and analysed.

2.11 CLONING, EXPRESSION AND PURIFICATION OF HpuA PROTEIN

2.11.1 *hpuA* amplification for cloning into expression vector

For *hpuA* cloning, the pET Directional TOPO Expression Kit (Invitrogen) was utilised. A PCR was performed using Phusion High-Fidelity PCR Kit (New England Biolabs) and a pair of primers (HpuA-NTERM and p26.85) to amplify the *hpuA* gene. A 40 µl reaction mixture was prepared using the ingredients supplied and included 8 µl Phusion buffer (HF), 0.8 µl dNTPs (10 mM), 1 µl of each primer from a 10 µM stock, 0.4 µl Phusion High-Fidelity DNA polymerase and 27.8 µl of distilled water. Thermocycler conditions and PCR steps are described in Table 2.3.

Table 2.3: Thermocycler conditions for Phusion PCR amplification of *hpuA* gene.

No.	PCR step	Temperature	Time
1	First denaturation	98°C	30 s
2	Denaturation	98°C	10 s
3	Annealing	58°C	15 s
4	Elongation	72°C	45 s
5		Go to 2, repeat 30 cycles	
6	Final elongation	72°C	10 min

2.11.2 PCR clean up

The PCR product was purified using E.Z.N.A. Cycle Pure Kit (Omega) to remove the contaminants including salts, unused nucleotides and enzymes to avoid any interference in cloning of the fragment in the expression vector.

2.11.3 Cloning of *hpuA* fragment

The size of product was determined and the fragment was cloned in to pET200 TOPO vector for a high level T7 regulated expression of recombinant protein with fused N-terminal peptide tag (6xHis-tag) for detection and purification, and a kanamycin resistance marker for selection of transformants. The molar concentrations of PCR product, vector and other conditions for cloning were followed as recommended by the supplier.

2.11.4 Preparation of chemically competent *E.coli*

For the competent cells preparation, 5 ml of an overnight culture of *E. coli* DH5-alpha strain was set up. 1 ml of this overnight culture was used to inoculate 100 ml of 2× LB containing 10 mM $MgSo_4$. The cells were grown until the OD600 was ~0.4 and then incubated on ice for 5 minutes. The pellet was obtained by centrifugation at 2000 g for 15 min and at 4°C temperature. The pellet was re-suspended in 20 ml of filter sterilized TFB 1 (30 mM KOAC, 50 mM $MnCl_2.4H_2O$, 100 mM KCl, 10 mM $CaCl_2.6H_2O$, 15% v/v glycerol). The cells were centrifuged again at 2000 g for 15 min and re-suspended in 4 ml of filter sterilized TFB 2 {(10mM Na-MOPS, (pH 7.0), 10mM KCl, 75 mM $CaCl_2$. $6H_2O$, 15% v/v glycerol)}. The competent bacteria were aliquoted and stored at −80°C.

2.11.5 Transformation *E. coli* DH5-alpha with recombinant construct

Chemically competent *E. coli* were transformed with 6 µl of cloning reaction and incubated on ice for 20 min, followed by a heat-shock for 30 s at 42°C without shaking. Immediately, the tube was transferred to ice and incubated for 5 min prior to adding 250 µl of SOC medium. The tube was incubated at 37°C on a shaker for 1 h and two different volumes of bacterial culture were then spread on pre-warmed selective LB plates containing kanamycin for overnight growth.

2.11.6 Analysis of positive clones

Five colonies were cultured overnight in 5 ml LB containing kanamycin. The plasmid DNA was isolated using E.Z.N.A Plasmid Miniprep Kit-1 (Omega). The plasmid was analysed by restriction analysis to confirm the presence and correct orientation of the insert. A sequencing reaction using 5' end and 3' end primers was also performed to confirm that gene was in frame and no mutation were introduced during PCR and cloning.

2.11.7 HpuA pilot expression in BL21 (DE3)

Once the correct clones were identified and after plasmid isolation, *E. coli* BL21 Star (DE3) were transformed as the host for a pilot expression of HpuA recombinant protein. As per manufacturer's guidelines, one vial of BL21 cells were gently mixed with 10 ng of plasmid DNA and incubated on ice for 30 minutes. A heat shock of the cells was performed followed by transfer to ice and subsequent mixing with 250 µl of SOC medium. After 30 min incubation on a shaker at 37°C, the entire transformation reaction was added to 10 ml of LB containing kanamycin and incubated overnight at 37°C with shaking.

Another 10 ml of LB containing antibiotic was inoculated with 500 µl of the overnight culture and incubated under the same conditions until the culture reached mid log phase i.e. OD600 = 0.6. The culture was split in to duplicates of 5 ml and one of the cultures was induced with 1 mM IPTG. An aliquot of 500 µl from each culture (induced and un-induced) was removed and centrifuged at 12100 g in a microcentrifuge for 30 s to collect the pellet. These pellets were zero time point samples and were frozen at –20°C. The remaining culture was incubated for 5 h and 500 µl samples were collected for each time point from induced and un-induced cultures, as described for the zero time point.

2.11.8 Analysis of samples by polyacrylamide gel electrophoresis

The samples were separated and analysed on a SDS-polyacrylamide gel by electrophoresis (Geneflow). The ingredients needed for the 10% separating gel were prepared by mixing 8.1 ml of buffer A (9.1% Tris base, 0.2% SDS in water, pH 8.8), 5.5 ml of acrylamide (37.5:1 acrylamide:bisacrylamide), 570 µl of 1% ammonium persulphate (APS), 2.3 ml of distilled water and 45 µl of N,N,N',N'-tetramethylenediamine (TEMED). The solution was poured quickly into gel casting form after adding TEMED to avoid localized solidification, leaving enough room for the stacking gel (about 2 centimetres below the bottom of the comb).

A thin layer of isopropanol was applied at top of the gel to remove bubbles and to ensure that this part does not dry out. After 30 min, washed out the isopropanol completely and dried with filter paper. The stacking gel was prepared from 3 ml of buffer B (3% Tris base and 0.2% SDS in water, pH 6.8), 990 µl of acrylamide mix, 150 µl of 1% APS, 1.9 ml of distilled water and 12 µl of TEMED. After pouring the 5% stacking gel on top of the running gel, the comb was inserted and allowed another 45

min for complete polymerization. The wells were washed again with distilled water and inserted in the gel tank.

The pellets collected previously from both induced and un-induced culture for each time point were re-suspended in 80 µl of 2× SDS-PAGE sample buffer {(1 ml of 1M Tris (pH 6.8), 10% SDS (4 ml), 1% bromophenol blue (1 ml), glycerol (2 ml) and 200 mM DTT in sodium phosphate (pH 5.2)} and boiled for 5 minutes. The protein samples and marker were loaded (10 µl) and the gel was run in 1× SDS-PAGE running buffer (0.025 M Tris, 0.192 M glycine, 0.19% SDS prepared in distilled water) to analyse the recombinant protein. The gel was run at 150 volts until the blue dye front reached the bottom. The gel was removed from the power supply and soaked in 0.25% Coomassie Brilliant Blue R250 (Sigma) solution with shaking. The gel was destained after 75 min by incubating in destaining solution (45% v/v methanol, 10% v/v glacial acetic acid prepared in distilled water) to remove nonspecific staining until the protein bands were completely visible above the background.

2.11.9 Western blot analysis of recombinant protein

The samples were subjected to Western blotting in order to confirm the identity of the over expressed band. Recombinant protein contains a 6xHis-tag in the above described expression vector. This N-terminal tag not only permits purification of recombinant protein on a metal chelating resin but also allows its detection with anti His-tag antibodies.

The proteins were first separated by SDS-PAGE electrophoresis (Geneflow) and then the resolving gel was placed in cold Western blot buffer (0.24% Tris, 1.14% glycine and 20% methanol). Electroblotting apparatus was set up in following order, black plastic cassette, soaked sponge in Western blot buffer, 3 sheets soaked 3M filter paper,

gel, prewetted nitrocellulose, 3 sheets soaked 3M filter paper, soaked sponge and lastly the red plastic of cassette.

The apparatus was assembled and filled with Western buffer/transfer buffer (0.24% Tris, 1.14% Glycine and 20% Methanol) and allowed to transfer for a minimum of 1 h at 150 mAmps. Following transfer, the membrane was removed with forceps and submerged in Ponceau S (Sigma) for 1-2 min to verify the transfer of proteins. Following that, the membrane was incubated in PBST-milk (PBS, 0.5% Tween 20, 5% dried skimmed milk powder) for 60 min with continuous shaking. This blocking solution was discarded and the membrane was incubated in 1:10,000 dilution of anti-His antibody in PBST-milk. The membrane was washed 2-3 times with PBST and incubated for 60 min in an appropriate dilution (1:3000) of suitable secondary antibody (horse reddish peroxidase or alkaline phosphatase conjugated) and the blot was developed by incubating membrane either in ECL solutions (Amersham) and exposed to an autoradiography film (Fujifilm) for 1-15 min depending upon the signal strength, or alternatively using substrate solution containing 5-bromo-4-chloro-3-inodylphosphate-nitroblue tetrazolium supplied by Perkin Elmer Life Sciences.

2.11.10 Large scale production of recombinant protein

Once the optimum time for the maximum expression of recombinant protein had been determined, 500 ml of LB in a 2 L flask with kanamycin was inoculated with 10 ml of overnight culture and grown to reach the OD600 = 0.6. The culture was induced with IPTG (1 mM) and incubated for a further 4 h (the optimum time determined in a pilot expression). The pellet was collected by centrifugation for 10 min in batches of 50 ml at 3220 g in a centrifuge (eppendorf).

The pellet was re-suspended in a lysis buffer (50 mM Potassium phosphate, 0.5 M NaCl, 100 mM KCl, 10% glycerol, 0.5% Triton X-100 and 10 mM imidazole) to disrupt the cell membrane and release the recombinant protein. Lysozyme was also added to a final concentration of 200 µg/ml to facilitate the lysis and the mixture was incubated for 30 min at 37°C. After this incubation, sonication (Soniprep 150, MSE, UK) was also done four times (20 s each with 10 s pause) on ice using a sonicator equipped with a microtip to release the maximum amount of recombinant protein from the lysed cells. The suspension was centrifuged at 3220 g for 30 min to remove insoluble material from the solution.

2.11.11 Purification of expressed HpuA protein by gravity flow column

The recombinant protein was purified by using IMAC Ni-SepFast BG Resin in a gravity flow column (Flowgen). The amount of slurry required was determined (660 µl binds 5-10 mg of protein) and poured in to a column, as described by the manufacturer. The lysate was poured on top of the column and allowed to flow through the column by gravity. After that, the column was subjected to gradient washes with an increasing concentration of imidazole (20-500 mM) in sodium phosphate buffer (pH 7.4) containing 0.5 M NaCl. The proteins were analysed on SDS-PAGE gels and the optimum imidazole concentration required for elution was determined.

2.12 MATRIX-ASSISTED LASER DESORPTION/IONIZATION MASS SPECTROMETRY FOR IDENTIFICATION OF RECOMBINANT PROTEIN

The purified recombinant protein was run by electrophoresis on a 10% SDS-PAGE gel. The gel was stained and then destained using the solutions as described in section 2.11.8. Destained gel was taken to PNACL, Department of Biochemistry, University of

Leicester. The bands of interest were pinched off using a fine micropipette tip. The spectra were collected by the PNACL staff and the identification of protein was made based on MALDI–TOF spectral analysis.

2.13 DETERMINATION OF PROTEIN CONCENTRATION

The concentration of protein was measured by using Bradfords Assay Kit (Bio-Rad) and according to instruction manual inside. Different concentrations of standard albumin (BSA) were supplied with Kit ranging from 2 mg/ml to 125 µg/ml. Twenty microlitres of each standard and samples were pipetted in to cuvettes and mixed with 980 µl of reagent solution. The cuvettes were incubated at room temperature for 5 min and the absorbance of each sample was read at 595 nm using spectrophotometer (eppendorf). Plotting the OD595 of each standard against the concentration drew a standard curve. The concentration of the unknown sample was estimated by using the standard curve.

2.14 POLYCLONAL ANTIBODY PRODUCTION FROM PURIFIED HpuA ANTIGEN AND ITS IDENTIFICATION

The HpuA protein was concentrated using centrifugal concentrators (Millipore) to a final concentration of 1 mg/ml in Tris HCl (50 mM, pH 7.4). The polyclonal antisera was raised against concentrated antigen and collected by Dr Roger James, Department of Infection, Immunity and Inflammation, University of Leicester. Briefly, a pre-bleed was collected and then three different mice were immunized simultaneously with 0.3 ml (1 mg/ml) of antigen and 0.3 ml (100 µg) of Freund's complete adjuvant (FCA). Two booster dosages of adjuvant and protein were given after first and second week of immunization, respectively. First test bleed was collected after three weeks and its reactivity was tested by ELISA against purified protein, as described below. Once

confirmed by ELISA, a third booster dose was given after the 4th week of immunization followed by a terminal bleed in the following week. The collected antisera were aliquoted and frozen at –80°C for further use.

2.14.1 ELISA with polyclonal antibodies for the specificity of antisera against purified HpuA protein

An ELISA protocol was used to detect the binding of polyclonal antisera with purified protein. HpuA protein (100 µl with 5 µg/ml final concentration) in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) was coated on micro-titer ELISA plates (Nunc, Denmark) and incubated overnight at 4°C. A control without protein was also set up. The binding sites were blocked for one hour at room temperature with 250 µl of 1% (w/v) BSA (Sigma) prepared in TBS buffer (140 mM NaCl, 10 mM Tris, 1.5 mM NaN_3, pH 7.4). Micro-titer plates were then washed twice with ELISA washing buffer (TBS with 5mM $CaCl_2$) without Tween-20.

Serial dilutions of polyclonal antibody in blocking buffer (TBS + 1% BSA) were added to the plates in duplicate starting from 1:100 dilution. A negative control with only blocking buffer was also used. Following one hour incubation at room temperature, plates were washed three times with 250 µl of ELISA wash buffer. The binding of polyclonal antisera was detected by addition of 100 µl of alkaline phosphatase conjugated goat anti-mouse (Sigma) antibody diluted 1:2500 in ELISA washing buffer. The plate was incubated for 1 h at room temperature. A negative control without this secondary antibody was also used, in addition to above mentioned controls. Plates were washed three times with 250 µl of wash buffer and the presence of alkaline phosphatase conjugate was detected by adding 100 µl of substrate solution (PNP tablets, Sigma).

The plate was incubated at room temperature for 10-15 min and the absorbance was measured at 405 nm on a Bio-Rad model 608, ELISA micro-titer plate reader.

2.14.2 Western blot against purified protein and meningococcal cell lysates

The polyclonal antibody was also tested against purified protein and bacterial cell lysate to further confirm that it was raised against the desired protein. For purified protein Western blot analysis, 5 µl of protein from a stock of 300 µg/ml was loaded and separated by electrophoresis. The protein bands were transferred to membrane and the blotting was done.

Similarly, for Western blot analysis of cell lysates, the bacterial cells were grown overnight and 1×10^9 cells as determined by measuring OD260 (see section 2.9 for quantification method) were pelleted by centrifugation at 12100 g in a microcentrifuge. The pellet was re-suspended with 100 µl of SDS-PAGE buffer and the samples were boiled for 10 minutes. A 15 µl sample from boiled lysates was pipetted and loaded on to the gel for electrophoresis followed by Western blot. Purified protein was also loaded with lysates to compare and locate the position of the correct band.

2.14.3 Immunoblotting of 8047 wild type and mutant strains by polyclonal antibody

The ability of binding of polyclonal antibody with cells was also tested by immunoblotting. Both wild type and a knockout mutant were streaked side by side on a Mueller Hinton agar plates containing 15 µM desferal. After overnight growth colonies were transferred to nitrocellulose membrane and immunoblot with polyclonal antisera, as described in section 2.9.3.

2.15 MODIFIED *IN VITRO* SERUM BACTERICIDAL ASSAYS BY HpuA AND HmbR POLYCLONAL ANTIBODIES

The selection assays using HpuA and HmbR polyclonal antisera were performed on strains 8047 and MC58, respectively. HmbR rabbit polyclonal antiserum was gifted by Ian Feavers, National Institute for Biological Standards, UK. In the first assay, the non-induced strains 8047 and MC58 with an ON state for HpuA and HmbR respectively, were used as inoculum and passage experiments were performed separately. The passaged cells were serially diluted to obtain a CFU count. The control (without antibody) was compared with that of treated samples to observe the bactericidal activity.

In a second phase of experiments, the bacteria were grown in the presence of desferal (30 μM) and transferrin (100 μg/ml) to induce the expression of the receptors. These cells grown in an iron-limited environment were then used as inoculum to passage in the presence of polyclonal antibodies and human serum as described for the PorA serum bactericidal assay. The numbers of CFU were counted for each passage including the control containing human serum but no antibody.

2.16 DETECTION OF HpuA SPECIFIC ANTIBODIES IN HUMAN SERA

Selected human sera collected from healthy carriers in the 2008 carriage study (Bidmos *et al.,* 2011) were tested by Bioplex xMAP assay for the presence of HpuA antigen specific antibody. The process involved the following steps.

2.16.1 Activation of carboxylated microspheres

The magnetic beads (Bio-Rad) were vortexed for 30 s followed by sonication. The process was repeated for two times and 100 µl microspheres were transferred to a 1.5 ml tube. The beads were mixed with 150 µl of PBS (pH 7.2) by vortex. After repeating the above step twice, the beads were collected by centrifugation at 13,000 g for 4 minutes. The pellet was re-suspended in 80 µl of PBS and mixed with 10 µl (50 mg/ml) of 1-Ethyl-3-3-dimethylaminopropyl (EDC) and 10 µl (50 mg/ml) N-hydroxysulfosuccinimide (S-NHS) supplied by Pierce.

After a vigorous vortex for 30 s, the tube was covered with foil and gently mixed for 20 min on a rocker. 150 µl of PBS was added and vortex again for 10 s. The pellet was collected after two PBS wash and the activated microspheres were resuspended in 100 µl of PBS.

2.16.2 Coupling of protein with activated microspheres

Purified HpuA antigen was coupled with microspheres using a standard protocol. Activated microspheres were mixed with 5 µg of protein with a final volume of 500 µl and incubated overnight at 4°C on a rocker. The pellet was collected after centrifugation at 13,000 g for 4 min and re-suspended in blocking buffer followed by 30 min mixing on a rocker. Centrifugation and washing with 500 µl blocking buffer was repeated and finally the beads were re-suspended in 150 µl of blocking buffer. The coupled microspheres were counted on a hemocytometer to adjust to the required concentration of 500 microspheres/µl.

2.16.3 Bioplex immunodetection assay

Bioplex assay for detection of HpuA specific antibody was performed in City Hospital, Nottingham and the process is summarised in figure 2.1. Previously coupled

microspheres were pipetted (25 µl) into pre wet wells of a filter plate (Millipore) in duplicates and mixed with serial dilutions of test sera (25 µl) collected from healthy carriers and diluted in liquichip buffer (PBS containing 0.1% BSA and 0.05% Triton X-100). A convalescent serum was also used along with the serum of a non-carrier as controls. Additional controls using a mouse polyclonal antibody and also wells either without serum (primary antibody) or reporter molecule (secondary antibody) were set up.

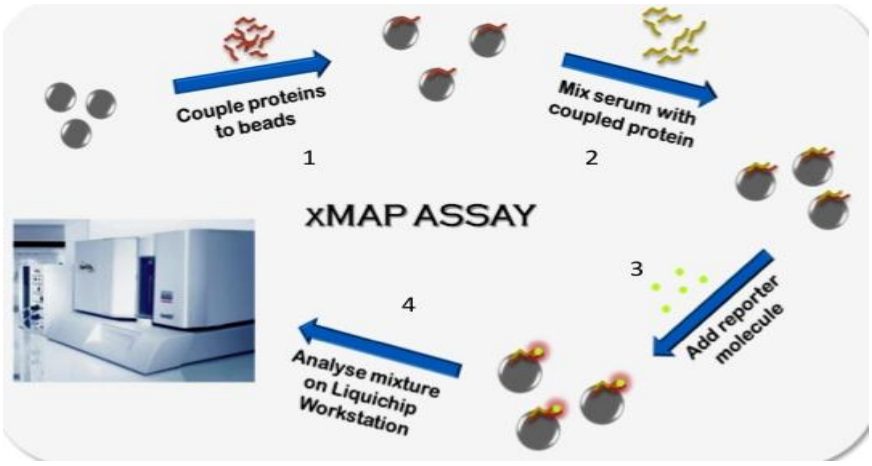

Figure 2.1: Bioplex assay for the detection of HpuA antigen specific antibodies in human serum. HpuA purified protein was coated on magnetic beads and incubated with human serum. A FITC-labelled human specific secondary antibody detected the binding of human antibody with HpuA and MFI values were recorded. (1) Pre-activated antigen was coupled with the magnetic beads and (2) mixed with the human serum. The presence or absence of the antigen specific antibody was detected by a secondary antibody (3) using a liquichip workstation for the measurement of mean flourescent index of the sample. (The diagram was drawn by the collective effort of author and Fadil Bidmos, a fellow student).

Following 25 min incubation with gentle shaking, the wells were washed thrice with liquichip buffer using a vacuum pump to remove excess liquid. The samples were incubated with 50 µl (100 ng) of reporter antibody (anti-human R-phycoerythrin and anti-mouse R-phycoerythrin, as appropriate) and left on a shaker for 25 min at room temperature. The plate was washed again three times with 50 µl of liquichip buffer per

well and the beads were re-suspended in 150 µl of liquichip buffer before the plate was loaded onto a liquichip work station for analysis. The mean fluorescence intensity (MFI) for each sample including negative control was measured. The average value of background MFI obtained from the negative control (without primary antibody) was subtracted to get the MFI of each sample and values derived were used for graphical analysis.

2.17 MUTAGENESIS OF *hpuAB* AND *hmbR* IN STRAINS 8047 AND MC58

hpuAB and *hmbR* mutants were constructed by the insertion of either a kanamycin or an erythromycin cassette into these genes. For *hpuAB* mutagenesis, the whole gene amplified with HpuA-NTERM and HpuB-CTERM primer was cloned into a pGem-Teasy vector system (Promega). An inverse PCR was performed using the *hpuAB*-pGEMT construct as a template and HA-Inv938/ HB-Inv1 oligonucleotides, which had an EcoRV restriction sites on their 5' ends for the production of blunt ends after amplification. This inverse PCR ensured a deletion of ~120 bp at 3' end of *hpuA,* and of ~1.5 kb in the 5' end of the *hpuB* gene so that the gene remains non-functional even if the antibiotic cassette is lost.

2.17.1 Digestion and recovery of inverse PCR product

The amplified DNA fragments were digested with EcoRV in a total volume of 40 µl containing 15 µl DNA, 1 µl enzyme, 4 µl buffer and 20 µl distilled water. The digested fragments were cleaned up again by using PCR clean up method, which inactivates the enzyme due to ethanol treatments and subsequently removes by the column.

2.17.2 Re-ligation of the purified PCR product

Purified and EcoRV treated inverse PCR products were re-ligated in a 20 µl total reaction volume which contained 1 µl T4 DNA ligase (New England Biolabs), 2 µl of ligase buffer, 9 µl distilled water and appropriate amounts of the PCR product. The amount of PCR fragments used for ligation was calculated using a NanoDrop. The ligation reaction was incubated overnight at room temperature and used for transformation.

2.17.3 Transformation of *E. coli* strain DH5-alpha.

For transformation, the ligation reaction was mixed with *E. coli* competent cells and transformed as mentioned in section 2.11.5. Transformed cells were plated on LA plates with ampicillin (50 µg/ml), X-Gal (30 µg/ml) and incubated overnight. Approximately 6 to 8 white colonies were individually resuspended in LB containing ampicillin (50 µg/ml) and incubated on shaker overnight.

2.17.4 Isolation of *hpuAB*-pGEMT plasmid construct and insertion of antibiotic cassette

E.Z.N.A Plasmid Miniprep Kit 1 was used according to the protocol supplied by manufacturer (Omega) to prepare plasmid DNA. The plasmid construct was digested with EcoRV which had a restriction site in the middle of the ligated product and ligated with a kanamycin cassette (1.2 kb), purified after digestion of puc4-Kan plasmid with HincII (Biolabs). The ligated product was transformed again in to *E. coli* competent cells and plated on LA with kanamycin (50 µg/ml), in addition to ampicillin (100 µg/ml). Selected colonies were subcultured and a plasmid extraction was performed. Restriction analysis using EcoRI confirmed the insertion of the cassette based on the

size of the fragments obtained. An additional double digest using XmnI and SpeI (Biolabs) was also performed for further confirmation.

2.17.5 Construction of *hmbR*-pGEMT plasmid construct and insertion of antibiotic cassettes

For *hmbR* mutagenesis, whole *hmbR* gene (~2.4 kb) was amplified by using 5' and 3' termini primers and cloned into pGEM-Teasy vector system. An EcoRV restriction site was identified in middle of the cloned gene and used for the insertion of antibiotic cassette. After digestion with EcoRV, a kanamycin cassette with blunt ends was inserted and the constructs were used to transform *E. coli*. Recombinants were selected on appropriate media and subsequently confirmed by the restriction analysis using EcoRI enzyme. Similarly a p*hmbR*-Ery construct was obtained in the same way. Briefly, pER2 plasmid was digested with HaeIII enzyme and an erythromycin cassette was purified and ligated to the p*hmbR*-GEMT plasmid. The ligated products were used to transform *E. coli* and selected on appropriate combination of antibiotics. Further confirmation was done by restriction analysis.

2.17.6 Transformation of *Neisseria meningitidis* strains 8047 and MC58

Neisseria meningitidis 8047 strain was transformed with p*hpuAB*-Kan plasmid, p*hmbR*-Kan and p*hmbR*-Ery constructs, separately, by a standard protocol. Each plasmid construct was linearized by digestion with SpeI which had a unique restriction site in the poly linker region. A loopful of bacteria from an overnight growth of 8047 strain and resuspended in 100 µl PBS. On a fresh plate with no antibiotic, 5 µl of bacterial suspension was mixed with 3 µl of linearized plasmid DNA and 7 µl of water. Solutions were mixed with a 10 µl loop and left uncovered to dry.

The plates were incubated overnight and the growth was re-suspended into 150 µl of PBS. Aliquots of 10 and 90 µl of suspension were plated out on two fresh plates containing appropriate antibiotics and incubated overnight. A negative control with no DNA was included to check the specificity of the transformation. Chromosomal DNA was extracted using the CTAB method and amplified with an appropriate set of oligonucletides to confirm the mutants were produced as a result of a homologous recombination event.

Strain MC58 was also transformed with the *hmbR*-resistance gene constructs but with a slightly different protocol. A 10 ml culture was grown overnight in BHIB in a 50 ml falcon tube and diluted 1/10 the next morning prior to incubation for one more hour. Following that, 200 µl of this culture were added to 15 ml culture tubes, containing 1.5 ml of BHIA and incubated for 4-6 h in the presence of CO_2. After this, the appropriate amount of DNA was added to tubes without mixing and incubated overnight. A 1/10 dilution of the total volume was plated on appropriate selective media and again incubated for 1-2 days. The single colonies were re-streaked on fresh plates and chromosomal DNA was prepared by using the CTAB method. Presence of antibiotic cassettes in the mutants was confirmed by PCR.

A double knockout mutant (8047 *ΔhmbR*::*ΔhpuAB*) was also generated. An *ΔhmbR*-Ery 8047 mutant strain was transformed with *ΔhpuAB*-Kan construct and the transformants were selected on BHI agar plates containing kanamycin (75 µg/ml) and erythromycin (6 µg/ml). The mutants were re-streaked to obtain genomic DNA for using in PCR analysis to confirm the mutation of both haemoglobin genes.

Chapter 3[1]

EPIDEMIOLOGICAL DISTRIBUTION OF HAEMOGLOBIN RECEPTOR ENCODING GENES, *hmbR* AND *hpuAB*, BETWEEN VARIOUS GROUPS OF MENINGOCOCCAL ISOLATES

Neisseria meningitidis can utilize haemoglobin and its complexes as sources of iron via two phase variable Hb receptors, HmbR and HpuAB, though their distribution among meningococcal isolates may vary. The importance of both genes in invasion was first suggested by Richardson and Stojiljkovic (1999) when they analysed the presence/absence of both genes in a limited number (twenty five) of disease isolates belonging to four different serogroups (A, B, C and Y). Sixteen isolates out of 25 consisted of both genes while remaining isolates had either *hmbR* or *hpuAB* gene. A subsequent study by Harrison *et al* (2009) reported an over-representation of *hmbR* in disease isolates compared to carriage isolates. This suggested a possible association of haemoglobin receptors with virulence of meningococci. However, the above studies had the limitations either due to investigation of only one gene (Harrison *et al.,* 2009), or due to low number of samples in the study (Richardson and Stojiljkovic, 1999). In addition, the phase variable status of these genes in disease and carriage isolates is not well documented. Hence, a comprehensive epidemiological study was required to investigate the PV status and combined role of both haemoglobin genes in virulence of meningococci.

[1]A part of this chapter is modified from the results published by author in Microbiology, SGM, UK.

This epidemiological study aimed to investigate and assess the distribution, antigenic variation, and phase variation status of both genes, *hmbR* and *hpuAB*, in diverse meningococcal disease and carriage isolates collections. All clinically important serogroups and a diverse range of strains collected from different regions were investigated and results were published (Tauseef *et al.,* 2011). A total of 507 strains belonging to four distinct groups and 21 additional strains were screened by PCR amplification for the absence or presence of the two genes. Among these isolates, 305 were from carriers and 221 were from disease background, while the disease or carriage association of 2 isolates was unclear. Nine serogroups were represented (A, B, C, 29E, H, W-135, X, Y, and Z) by 429 strains while 99 were non-serogroupable strains. About 33 different clonal complexes were represented with the majority of isolates belonging to 16 clonal complexes. The association of one or both receptors with disease/carriage, serogroups and clonal complex was determined to reveal the relationship between presence/absence of gene and meningococcal disease. The mechanism of *hpuAB* deletion in *hpu*$^-$ isolates and the PV status of both genes was also revealed in this part of the project.

3.1 DETECTION OF *hmbR* AND *hpuAB* IN MENINGOCOCCAL ISOLATES

The presence or absence of *hpuAB* system was detected by the amplification of *hpuA* using the primers HpuA350-Rev/ HpuAC. These primers surround the repeat tract of *hpuA* and generate a fragment of either 348 bp or 310 bp in *hpu*$^+$ isolates (Figure 3.1 A). The presence or absence of the system was further confirmed by a PCR of a complete *hpuA* gene using primers HpuAC/p26.85 generating ~1.1 kb fragments from hpu$^+$ isolates (Figure 3.1 B). This PCR also ensured that the negative PCR results

obtained from HpuA350-Rev/ HpuAC amplification were not due to the polymorphism in primer binding sites. A third PCR was also performed using flanking primers (Hpu-for1 and Hpu-Rev13) to screen all *hpu*$^-$ isolates (see section 3.3).

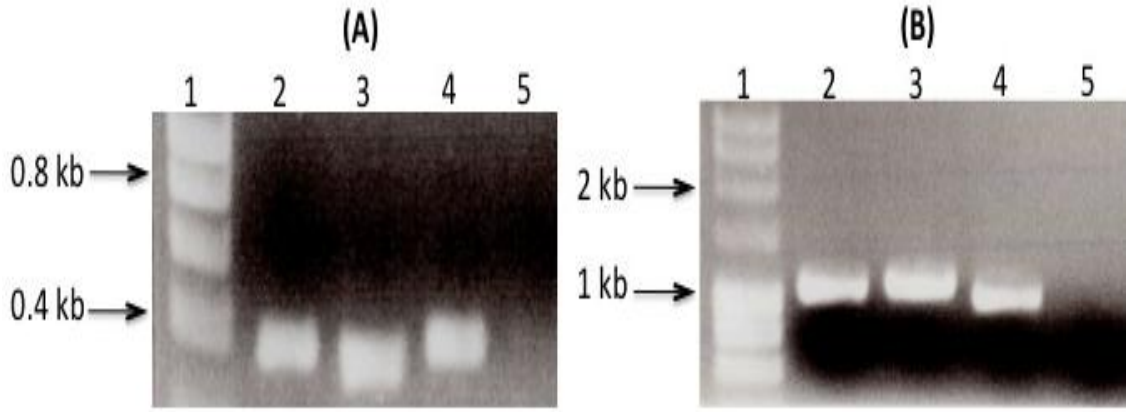

Figure 3.1. Agarose gel electrophoresis (1% agarose) loaded with the amplified PCR products for the detection of the presence of *hpuAB* using HpuA 350-Rev/ HpuAC or HpuAC/p26.5 primers. A fragment of *hpuA* encompassing the repeat tract, or the whole *hpuA* locus was amplified by PCR using two different set of primers. 5 µl of sample from PCR reaction was loaded into each gel and electrophoresed. Ethidium bromide was used to stain the DNA fragments and analysed under UV to detect the presence of *hpuA* gene. (A) PCR amplification of partial *hpuA* DNA sequence using HpuA 350-Rev/ HpuAC primers. Lane 1: standard molecular weight marker, Lanes 2-4: DNA fragments of the *hpuA* gene from *hpuAB*$^+$ isolates (88, 8047 and 119), Lane 5: No DNA PCR negative control (B) PCR amplification of complete *hpuA* gene obtained from the 88 (Lane 2), 119 (Lane 3) and 8047 (Lane 4) strains.

Similarly, the presence of an *hmbR* system in a strain was analysed by the amplification of a 430 bp fragment encompassing the poly(G) tract with primers hmbR-RF3 and hmbR-RF4 (Figure 3.2). The isolates with negative PCR result with this set of primer (hmbR-RF3 and hmbR-RF4) were tested with an additional PCR (HmbR-NTERM/HmbR-CTERM, HmbR-STOP/hmbR-RF3 and HmbR-NTERM/hmbR-RF4) to reconfirm the absence of *hmbR* in these isolates (Not shown).

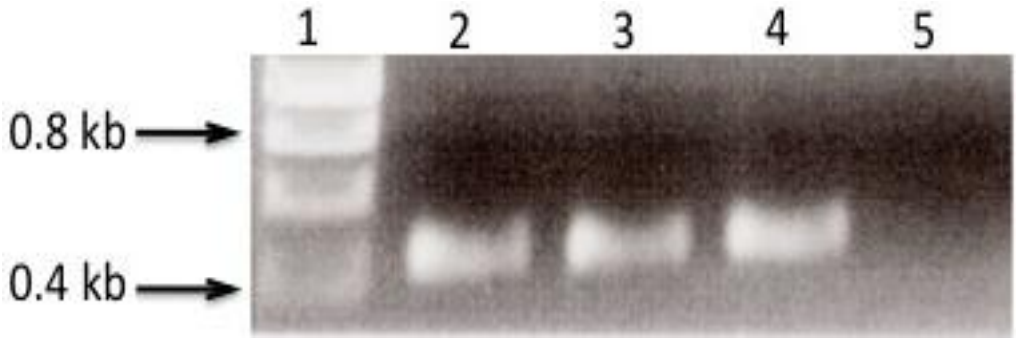

Figure 3.2. Agarose gel electrophoresis (1% agarose) loaded with the amplified PCR products for the detection of the presence of *hmbR*. A fragment of *hmbR* gene from meningococcal isolates was amplified using hmbR-RF3 and hmbR-RF4 primers. A 5 µl of sample from PCR reaction was loaded into the gel containing ethidium bromide and electrophoresed. The gel was visualized under UV to detect the presence of *hmbR* gene. Lane 1, standard molecular weight marker; Lanes 2-4, DNA fragments of the *hmbR* gene from *hmbR*$^{+}$ isolates (8047, MC58 and Z4685); Lane 5, No DNA PCR negative control.

3.2 DISTRIBUTION OF *hpuAB* AND *hmbR* IN MENINGOCOCCAL ISOLATES

The distribution of haemoglobin receptor encoding genes was investigated for the presence of a gene alone or in combination with the other gene. Among all 528 isolates tested, 48% of isolates possessed both genes while 27% of the isolates had *hmbR* as their sole haemoglobin binding receptor. The analysis also revealed that 21% of isolates had *hpuAB* alone and a minority (4%) of isolates were lacking both genes. The majority (88%) of the isolates lacking both systems were obtained from carriers. These isolates (lacking both genes) were further confirmed by more than one set of primers to address the annealing site polymorphism (see section 3.1).

The isolates were further split into disease and carriage groups to compare the percentage distribution of both genes in respective backgrounds (Figure 3.3). The presence of both genes simultaneously in disease and carriage isolates was 55% and

43%, respectively. This demonstrates a very slight difference (12%) between both groups for the presence of both systems. However, a noticeably lower percentage (5%) of an *hpuAB* only genotype in disease compared to carriage (31%) was observed. Contrastingly, a higher percentage (38%) of an *hmbR* only genotype in disease isolates compared to carriage (19%) was evident, which is similar to the results published by Harrison and co-authors describing a significantly higher frequency of this system in disease isolates (Harrison *et al.*, 2009).

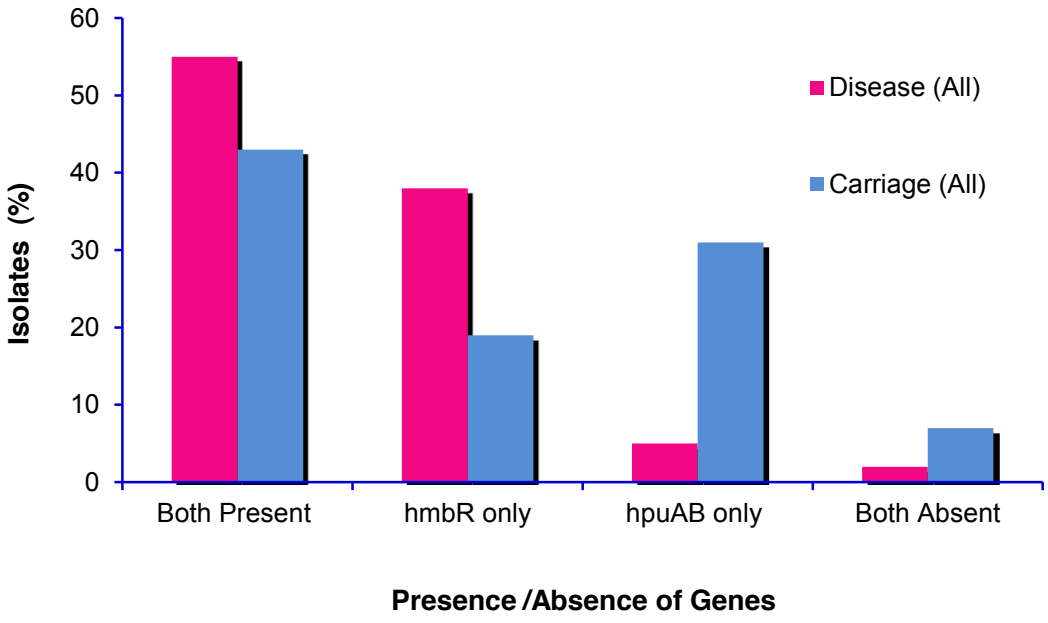

Figure 3.3. Percentage distribution of haemoglobin/haemoglobin-haptoglobin receptor encoding genes in disease and carriage isolates. Isolates were grouped in to disease (*n*=221) and carriage (*n*=305) and the percentage distribution of Hb receptor encoding genes in each group was plotted. Strains within each group were further separated in to four groups on the basis of the presence of both genes in combination, either one or both absent.

Statistical comparisons were made between the presence of both genes versus one gene and between *hpuAB* only and *hmbR* only isolates of both groups. The difference in the disease association odds ratio (OR, 95% CI) was obtained for all isolates (all disease vs

all carriage) as well as for each strain collection separately (Table 3.1). Analysis revealed a slightly significant (p = 0.0359) difference in the frequency of both genes in combination compared with *hmbR* only in disease versus carriage isolates. However this result was only significant on inclusion of the 2008 carriage study, which includes multiple isolates resulting from clonal expansion of a few strains (Bidmos *et al.*, 2011).

The direct comparison between both genes and *hmbR*-only versus *hpuAB*-only revealed a significantly higher frequency of both genes or *hmbR*-only in disease isolates but a significant under-representation of *hpuAB*-only phenotype in clinical isolates. The data indicates a significant selection against the presence of *hpuAB*-only in invasive isolates. These differences were obtained for each collection of strains as well as the combined strain collections. However due to a low number of isolates with an *hpuAB*-only genotype within some groups i.e. CZECH isolates, the significance was reduced. The carriage association odd ratio was also determined for *hpuAB*-only by a comparison of carriage versus disease isolates (Table 3.2). An over-representation of this gene was evident in carriage isolates (p = 0.0009). However, the significance was lost on exclusion of 2008/ 2009 carriage studies that includes clonal expansion of some strains.

3.2.1 Frequency of haemoglobin receptor encoding genes in serogroups and clonal complex association

The distribution of both haemoglobin genes, *hpuAB* and *hmbR*, was also biased between serogroups and clonal complexes. A majority of isolates from serogroups A, C and 29E exhibited a high prevalence of these genes with 76%, 87%, 57% of strains from these groups had both genes respectively. In addition, 51% strains harbouring both systems were non-serogroupable. A similar pattern was evident for serogroup Z isolate where

Table 3.1. Distribution of *hpuAB* and *hmbR* genes in meningococcal disease and carriage isolates

Collection of Strains	Group	Both genes	*hmbR* only	*hpuAB* only	Both absent	Total	Disease association OR (95% confidence interval)		
							Both vs *hmbR*	Both vs *hpuAB*	*hmbR* vs *hpuAB*
All	Disease	121(55%)	85(38%)	12(5%)	3(2%)	221	0.6 (0.4 to 0.9)[ф]	7.3 (3.8 to 14)***	11.6 (5.8 to 23)***
All	Carriage	131(43%)	58(19%)	95(31%)	21(7%)	305			
All	Disease	121(55%)	85(38%)	12(5%)	3(2%)	221	0.7 (0.5 to 1)[II]	5.2(2.6 to 10.)***	7.2 (3.5 to 14.9)***
All minus 2008	Carriage	97(45%)	49(22%)	50(23%)	21(10%)	217			
CZECH	Disease	26(68%)	9(24%)	1(3%)	2(5%)	38	1.5 (0.6 to 3.6)[II]	10.2 (1.3 to 80) [II]	6.9 (0.8 to 59) [II]
	Carriage	51(44%)	26(23%)	20(17%)	18(16%)	115			
MLST107+9 Group	Disease	55(57%)	31(32%)	10(10%)	1(1%)	97	1.2 (0.3 to 4.5)[II]	4.6 (1.2 to 18) [II]	3.9 (0.9 to 17) [II]
	Carriage	6(35%)	4(24%)	5(29%)	2(12%)	17			
UK 1999	Disease	39(51%)	37(48%)	1(1%)	0(0%)	77	0.5 (0.2 to 1)[II]	25 (3.2 to 194) ***	51.4 (6.4 to 410)***
	Carriage	39(48%)	18(22%)	25(30%)	0(0%)	82			
ST-41/44	Disease	5(11%)	40(87%)	0(0%)	1(2%)	46	0.5 (0.1 to 2.3)[II]	-	-
	Carriage	3(9%)	13(38%)	0(0%)	18(53%)	34			

*** $p < 0.0001$

[ф] $p = 0.0359$

[II] p Not significant

73% had both genes, although this percentage is representative of a limited number (11 isolates) tested for this group. Some serogroups exhibited under-representation of *hmbR* only and over representation of *hpuAB* only genotypes e.g. 85% of serogroup Y and 41% isolates of serogroup 29E isolates harbour *hpuAB*-only while none of the isolates from serogroup Y and 2% isolates from 29E had *hmbR* only genotype.

Contrastingly, an *hmbR* only genotype was over-represented in 58% of serogroup B isolates with a major under-representation of the *hpuAB* only genotype as only 12% of strains had this system. A statistical analysis between the presences of *hmbR* only versus both genes in disease and carriage isolates of serogroup B was also performed and demonstrated no significant difference in their presence/absence distribution (Table 3.3). Surprisingly a similar result was obtained when the frequency of both genes or *hmbR*-only versus *hpuA*-only was tested, but this was probably due to the fact that the number of *hpuA*-only genotype was too small for a statistical comparison (only 3 disease isolates had *hpuAB*-only genotype out of 115 isolates) resulting in no significant difference. However, apparently both genes and *hmbR*-only are over-represented as compared to *hpuAB*-only genotype or no gene (Table 3.3).

A bias between the clonal complexes for the distribution of both genes was also observed. Four groups (arbitrarily named 1-4) were recognised and defined on the basis of high or low prevalences of both genes in combination and/or alone (Table 3.4). The first group comprised of eight CCs, included the CCs with high prevalence of an *hmbR*-only genotype, such as ST-18 (92%), ST-32 (90%), ST41/44 (66%) and ST-269 (63%). Disease:carriage ratio of six CCs of this group are known (Caugant and Maiden, 2009). Two CCs of this group (ST-18 and ST-32) with highest prevalence (92% and 90%, respectively) of *hmbR*-only also possess a higher disease/carriage ratio among the

Table 3.2. Statistical analysis for *hpuAB* distribution and its carriage association

Strain collection	Group	*hpuAB* positive isolates (n)	*hpuAB* negative isolates (n)	Total	Disease association odd ratio (95% CI)	Carriage association odd ratio (95% CI)
All	Disease	133	88(40%)	221	0.5 (0.4 to 0.8)	1.9 (1.3 to 2.7) *
All	Carriage	226	79(26%)	305		
All	Disease	133	88(40%)	221	0.7 (0.5 to 1.1	1.4 (.9 to 2)[II]
All minus 2008	Carriage	147	70(32%)	217		

* $p = 0.0009$

[II] p = Not significant

Table 3.3. Statistical analysis for distribution of haemoglobin genes, *hmbR* and *hpuAB*, in serogroup B isolates

Group	Both present	*hmbR only*	*hpuAB* Only	Both absent	OR (95% confidence interval)		
					Both vs *hmbR*	Both vs *hpuAB*	*hmbR* vs *hpuAB*
Disease	34	77	3	1	0.6(0.3 to 1.2) 丌	2.9 (0.7 to 13)[II]	4.7(1.1 to 19.8)[II]
Carriage	23	33	6	13			

[II] p = Not significant

Table 3.4: Clonal complex representation of *hpuAB* and *hmbR* genes and the distribution of *hpuAB* deletion mechanisms

CC[a]		Disease	Carriage	Both Present[b]	*hmbR* only	*hpuAB* Only	Both absent	*hpu*AB$^+$ (Both + alone)[c]	IS element[d]	Complete deletion[e]	Dis./carr. ratio*
Group 1 (group of CCs with high prevalence of *hmbR*- only genotype)											
ST-41/44	80	46	34	8	53	0	19	8	69	2	1.2
ST-18	13	10	3	1	12	0	0	1	12	0	5.5
ST-213	8	1	7	2	6	0	0	2	6	0	0.6
ST-461	1	0	1	0	1	0	0	0	1	0	-
ST-334	2	0	2	1	1	0	0	1	1	0	-
ST-32	21	16	5	2	19	0	0	2	0	19	3.5
ST-269	19	13	6	7	12	0	0	7	0	12	2.8
ST-35	4	1	3	1	3	0	0	1	0	3	0.5
UNSPEC	38	-	-	0	34	0	4	0	33	4	
Group 2 (group of CCs with high prevalence of both receptors in combination)											
ST-11	60	47	13	59	1	0	0	59	1	0	6.6
ST-5	12	10	2	12	0	0	0	12	0	0	19.5
ST-4	12	12	0	12	0	0	0	12	0	0	-
ST-92	15	0	15	15	0	0	0	15	0	0	-
ST-8	11	11	0	11	0	0	0	11	0	0	24.5
ST-116	6	0	6	6	0	0	0	6	0	0	-
ST-231	3	1	2	3	0	0	0	3	0	0	-
ST-37	2	2	0	2	0	0	0	2	0	0	-
ST-53	1	0	1	1	0	0	0	1	0	0	-
ST-162	1	1	0	1	0	0	0	1	0	0	-
ST-292	1	1	0	1	0	0	0	1	0	0	-
ST-364	1	0	1	1	0	0	0	1	0	0	-
ST-750	1	1	0	1	0	0	0	1	0	0	-
UNSPEC	63	-	-	63	0	0	0	63	0	0	-
Group 3 (group of CCs with high prevalence of either both or *hpu*-only genotype)											
ST-60	29	2	27	18	1	10	0	28	0	1	0.7
ST-1157	10	0	10	8	0	2	0	10	0	0	-
ST-198	5	0	5	2	0	3	0	5	0	0	<0.1
ST-1	14	13	1	4	1	8	1	12	0	1	5.5
ST-22	18	4	14	7	0	11	0	18	0	0	0.6
ST-103	5	1	4	3	0	2	0	5	0	0	1.2
Group 4 (group of CCs exclusively containing *hpu*-only genotype)											
ST-174	15	0	15	0	0	15	0	15	0	0	-
ST-106	11	0	11	0	0	11	0	11	0	0	-
ST-23	13	1	12	0	0	13	0	13	0	0	0.8
ST-167	11	2	9	1	0	10	0	11	0	0	0.5
ST-254	1	0	1	0	0	1	0	1	0	0	-
ST-549	1	0	1	0	0	1	0	1	0	0	-
UNSPEC	20	-	-	0	0	20	0	20	0	0	

[a] Clonal complex , number of isolates of each CC analysed and the presence or absence of genes in particular CC.
[b] Isolates harbouring both, *hpuAB* and *hmbR*, genes simultaneously.
[c] Isolates containing *hpuAB* gene (in combination with *hmbR* plus *hpuAB*-only).
[d] *hpu*$^-$ isolates with IS element mediated type of *hpuAB* deletion/replacement.
[e] *hpu*$^-$ isolates with complete *hpuAB* deletion.* Disease/carriage ratios were taken from Caugant and Maiden (2009).

CCs of this group (shaded yellow in group 1). None of the CCs of this group contained the *hpuAB*-only genotype. Also, the majority (53%) of isolates in ST-41/44 CC lacking both genes were belong to carriage. The second group was comprised of 13 CCs exhibiting both genes in >90% of isolates. The group exclusively contained both genes simultaneously (except for one isolate). Three CCs (ST-11, ST-5 and ST-8) with the highest disease/carriage ratios (shaded red), among all CCs with known ratios, belong to this group (Table 3.4).

In the third group were six CCs with a high level of *hpuAB* only or both genes but a low level of *hmbR* only isolates e.g. ST-1 (64%), ST-22 (61%) and ST-60 (34%). The lower percentage of *hpuAB* only genotype in ST-60 is due to the presence of more strains with both genes (62%). However a very low percentage of *hmbR* only genotype (2%) was maintained. So the third group is an intermediate group comprised of CCs with lower levels of *hmbR* only genotype. The CCs of the fourth group were >90% *hpuAB* only isolates as evident from ST-174 (100%), ST-106 (100%), ST-23 (100%) and ST-167 (91%).

It is evident from the distribution of the CCs that as we move down from group 1 to group 4 (arbitrarily named), the distribution shifts from CCs (group 1) having an *hmbR* only genotype in the majority while both genes in minority, to group 2 containing CCs with both genes in the majority. Both groups (1 and 2) have no representation of *hpuAB* only genotype which appears in third group of CCs along with presence of both genes and then leads to group 4 where all isolates harbour *hpuAB* exclusively (except one isolate). In this scenario, group 3 is in between the group of CCs with *hpuAB* only genotype (group 4) and the groups without this genotype (group 1 and 2).

3.3 *hpuAB* LOCUS ARRANGEMENT, AMPLICON SIZE VARIATION AND DELETION MECHANISMS

The analysis of strains from all groups identified a number of isolates in which the *hpuAB* system has been deleted. The deletion mechanisms have been described and published (Tauseef *et al*., 2011). In order to characterise and elucidate the mechanism involved in deletion, the regions flanking this locus were amplified using primers specific for adjacent genes. Primers Hpu-for1 and Hpu-Rev13 were designed from the published sequence of three strains Z2491 (Serogroup A), MC58 (Serogroup B) and FAM18 (Serogroup C). These primers reside outside the *hpuAB* locus in adjacent genes that encode a GroEL chaperone protein and a hypothetical perisplasmic protein. With these primers, a PCR of meningococcal strains with a full-length gene system is predicted to produce an approximately 5.6 kb fragment.

The amplicons generated for strains previously tested positive for this system, revealed a significant variation in the size of PCR products (Figure, 3.4, lanes 2 and 3). For *hpuAB* negative isolates the PCR amplicons generated (lanes 4, 5 and 6), were significantly smaller than those of the reference strain Z2491 (lane 2). A total of 264 isolates (*hpuAB* positive and *hpuAB* deleted) were tested and were divided into 5 groups (Hpu1, Hpu2, ΔHpu3, ΔHpu4 and ΔHpu5) based on the size of the amplicon generated. Strains positive for *hpuAB* system were designated as Hpu1 and Hpu2 (Lanes 2 and 3), while *hpuAB* negative isolates (Lanes 4, 5 and 6) were designated as ΔHpu3, ΔHpu4, and ΔHpu5, respectively.

3.3.1 Amplicon size variation among *hpuAB* positive isolates

Strain Z2491 is a representative of the Hpu1 group generating full-length product (5.6 kb) with flanking primers. The sequence analysis has revealed that a cluster of 9 dRS3

{duplicate repetitive palindromic sequence (ATTCCCN8-GGGAAT) is present upstream of *hpuA* in Z2491. A pair of Correia elements flanks this cluster of repetitive elements and the region can be further subdivided in to two direct repeats of 118 bp. In addition to dRS3, REP elements (repetitive extragenic palindrome) of the lengths 18 bp, 22 bp and 25 bp are also present.

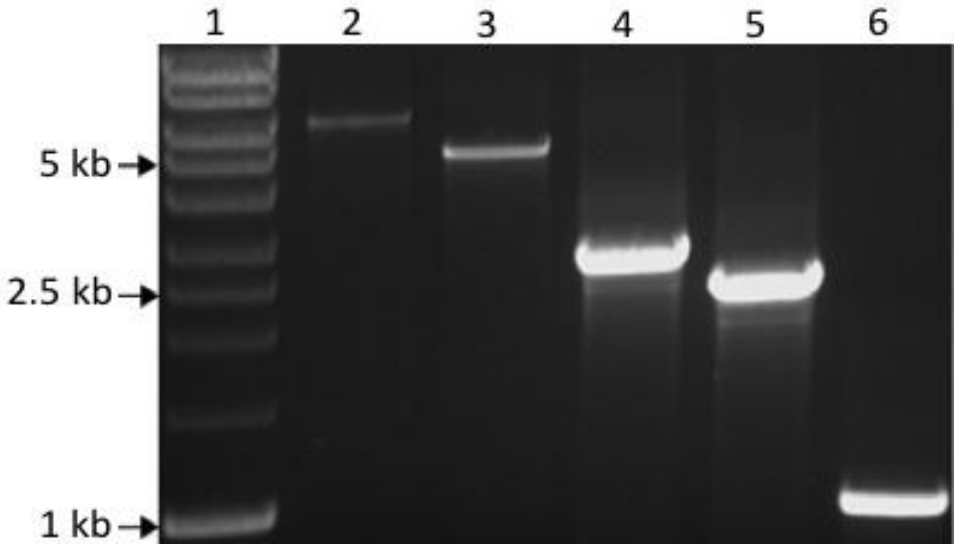

Figure 3.4: Variations in the size of the *hpuAB* locus among *N. meningitidis* isolates. The *hpuAB* locus was amplified with primers specific for flanking genes. Five groups were designated based on amplicon size generated, and representative samples are shown. Lane 1, size standards; Lane 2, Hpu1, *hpu*$^{+}$ strain (Z2491); Lane 3, Hpu2, *hpu*$^{+}$ strain (Z6414); Lane 4, ΔHpu3, *hpu*$^{-}$ strain (Z4686); Lane 5, ΔHpu4, *hpu*$^{-}$ strain (Z6427); Lane 6, ΔHpu5, *hpu*$^{-}$ strain (Z4685).

To identify the mechanism responsible for the PCR product size variation for those from the group having the *hpuAB* system but with a PCR product size of 5 kb (Hpu2), two representative isolates (4323 and 6414) were sequenced with upstream and downstream primers and compared with the sequence of reference strain Z2491. A comparison of sequences from strains positive (Hpu2) for *hpuAB* (4323 and 6414) but with smaller amplicon than Z2491, demonstrated a deletion of 0.6 kb in an upstream sequence flanked by Correia elements (Figure 3.5). This deletion can be explained by a

recombination and rearrangement event between dRS3 sequences in the upstream region, as downstream sequences were similar to the reference strain. These rearrangements reduced the number of dRS3 from 9 (reference strain, Z2491) to 3 in this group of isolates, thus explaining the reduction of size in PCR products from 5.6 kb to 5 kb in various *hpuAB* positive strains when compared with a strain harbouring a full length *hpuAB* system. The sequences obtained were submitted to the GenBank/EMBL/DDBJ with accession numbers JF342999-JF343005.

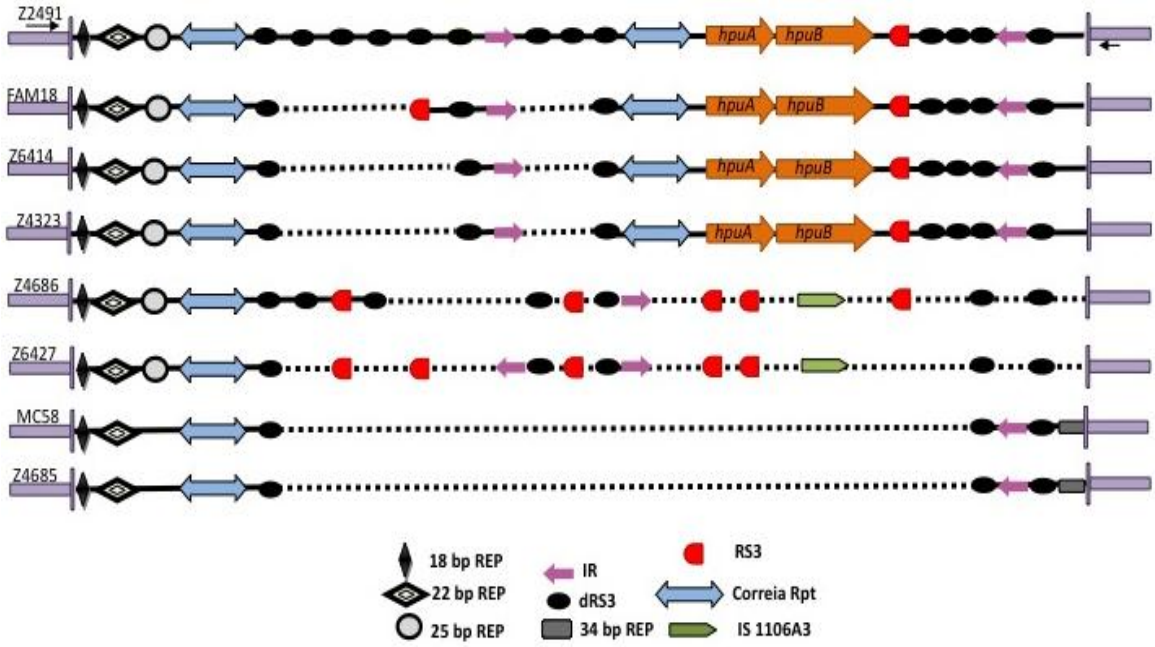

Figure 3.5: Genetic arrangement of the *hpuAB* locus in *N.meningitidis* isolates. *hpuA* and *hpuB* genes are labelled and the direction of the arrow represents the ORFs of these genes. Insertion element (IS) and repetitive sequences are represented by symbols. The sequence length of each repetitive element is also mentioned in the key. Dotted lines indicate deleted sequences in a particular clone. Note that the figure is not drawn to scale. REP, repetitive extragenic palindromic sequence; IR, invert repeat; RS3, repeat sequence 3; dRS3, duplicate repeat sequence 3 (see section 1.8.1 for details of repetitive elements). Modified from Tauseef *et al* (2011).

3.3.2 Transposition of *hpuAB* by recombination

To explain the mechanisms involved in the replacement of *hpuAB* locus, amplicons of size 2.7 or 2.3 kb were generated from strains 4686 and 6427 using flanking primers (Hpu-for1 and Hpu-Rev13), as depicted in figure 3.4. The amplicons were subsequently sequenced and new internal primers were designed from the above sequences for inward stepping to reveal the sequence. The flanking PCR products (2.7 or 2.3 kb respectively) generated in the first step were used as a template for nested or 2nd step PCR, to avoid any non-specific amplification and for chromosomal walking. The PCR was performed using primers hpuF-seq/hpuR-seq, which bind inside the flanking PCR products. Sequencing of the products generated by nested PCR was done in both directions. A blast search with the sequence from strains 4686 and 6427 revealed a high sequence identity (96%) with the published DNA sequence of IS*1106A3* (Figure 3.6).

A total of eleven IS*1106A3* sequences are present in the genome of reference strain (Z2491), in addition to a few remnants for this IS element. The alignment of sequences from both strains with those of the reference strain showed a variation in the cluster of dRS3, which probably indicate different recombination events for insertion and dRS3 rearrangement. Strain 4686 contains four RS3 (ATTCCC) elements and five dRS3 core sequences upstream to the insertion sequence. Similarly, the sequence from strain 6427 revealed different RS3 and dRS3 sequences upstream of the IS element. These RS3 elements were probably involved in the recombination and insertion of the IS*1106A3* element. The sequences obtained were submitted in GenBank with accession numbers JF342999-JF343005.

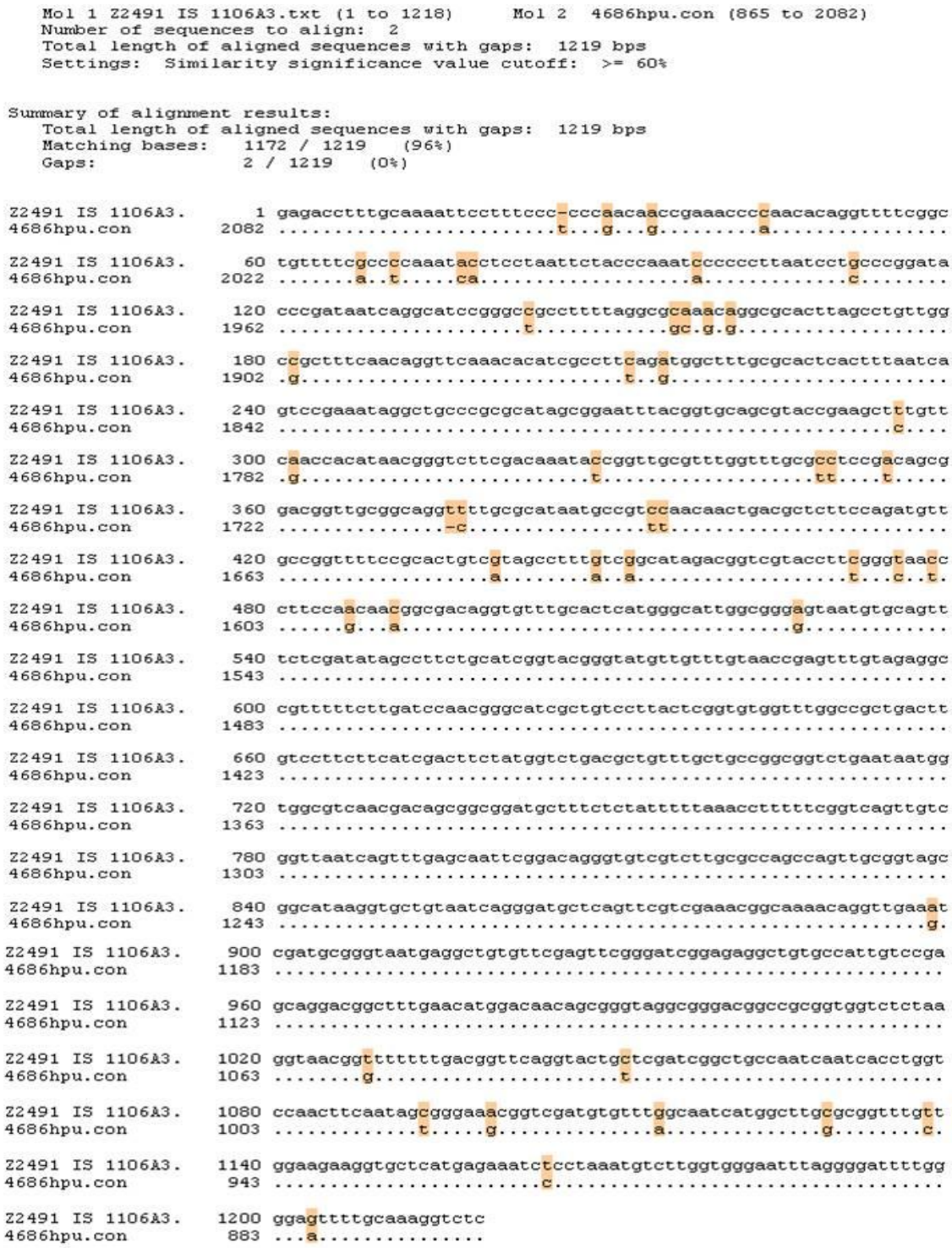

Figure 3.6: Sequence alignment of IS*1106A3* with the sequence obtained from an *hpuAB*$^-$ isolate (Z4686). The sequence of *hpu* locus from strain 4686 was obtained by sequencing the flanking region followed by inward chromosomal walking and its homology was determined by blast search. The aligned sequence replacing *hpuAB* locus in strain Z4686 showed a high homology (96%) with the published sequence of IS*1106A3* from strain Z2491 (Parkhill *et al.,* 2000).

Analysis of the sequences downstream to the IS element in both strains, 4686 and 6427, also suggests a recombination event between two dRS3 of the cluster, deleting 150 bp segment, including an inverted repeat. These rearrangements in upstream and downstream regions are collectively responsible for a decrease in size of amplicons from 5.6 kb to ~2.7 kb and ~2.3 kb in the ΔHpu3 and ΔHpu4 groups of isolates, respectively. In non- sequenced strains with ΔHpu3 and ΔHpu4 types, the presence of the insertion sequence element was confirmed by a nested PCR with Hpu-rev13, binding in one of the flanking regions, and hpuF-seq2, a primer specific for the transposase (Figure 3.7). A PCR with hpuF-seq2 and hpu-Rev13 is expected to generate a ~1 kb fragment (Lane 3 and 4) in strains with the first type of replacement (ΔHpu3) or ~0.7 kb (Lane 5 and 6) for the second type of replacement (ΔHpu4). A control PCR using a strain (MC58) with a complete deletion of this region was also used.

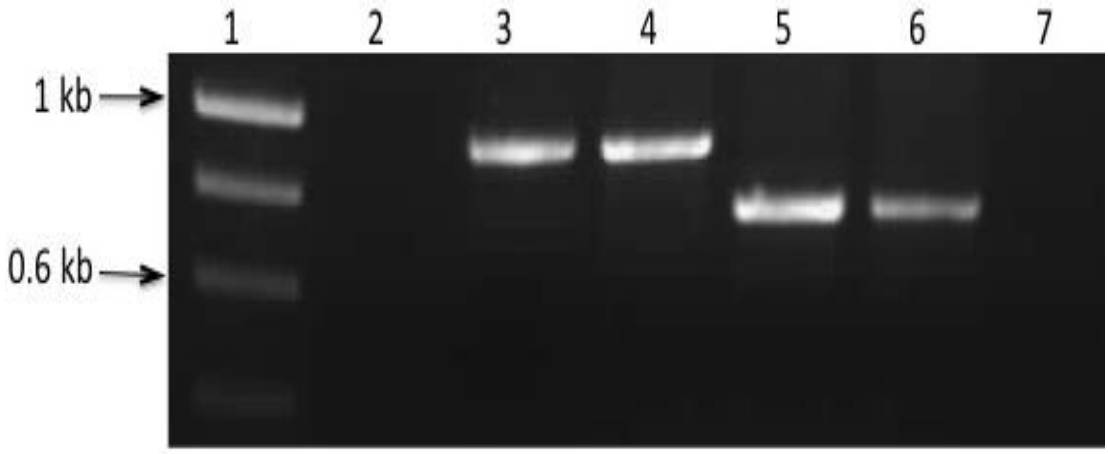

Figure 3.7: Agarose gel electrophoresis (1% agarose) loaded with the PCR products amplified with primers specific for IS*1106A3*. 5 µl from PCR reaction was loaded into the gel. Lane 1, standard molecular weight marker; Lane 2, Control PCR using *hpuAB*- strain (MC58); Lanes 3-4, DNA fragments amplified from representative isolates of ΔHpu3 (Z4692 and 3524); Lane 5-6, DNA fragments amplified from representative isolates of ΔHpu4 (Z6428 and Z4711); Lane 7, No DNA PCR negative control.

3.3.3 Complete deletion of *hpuAB*

The mechanism for complete deletion of the *hpuAB* system in MC58 and 4685 (ΔHpu5) was also elucidated. The sequence analysis (accession numbers JF342999-JF343005) revealed that the absence of the system appears to have occurred via a recombination between dRS3 sequences in the upstream and downstream regions, resulting in deletion of an ~4.5 kb fragment including the bipartite system. This event also explains the reason for production of 1 kb amplicon during PCR with flanking primers. The amplicons of all negative isolates was not sequenced, as the PCR products observed were of identical lengths within a particular group suggesting a single type of deletion event, recombination or rearrangement, in all isolates of the same group.

3.3.4 Phylogenetic association of transposition and deletion events with clonal complex

A phylogenetic analysis of all the *hpuAB* negative isolates was performed to study the distribution of deletion mechanisms within clonal complexes (Tauseef *et al.,* 2011). The analysis revealed that 72% of isolates lacking this system belonged to only 5 CCs ST-41/44, ST-18, ST-213, ST-32, and ST-269 (Table 3.4, shaded columns green and blue). Moreover, among negative isolates lacking an *hpuAB* system and containing an IS element (shaded green in table 3.4), the majority {(69/122), 57%)} of the isolates belonged to the ST41/44 complex. A minority {(12/122 isolates), 10%)} of isolates belonged to ST-18 and ST-213 {(6/122 isolates), 5%)}. Although this number seems low for both CCs, but within CCs a high prevalence of the IS replacement in ST-18 {(12/13 isolates), 92%)} and ST-213 {(6/8 isolates), 75%)} was evident. A slight bias for the type of deletions within these CCs (ΔHpu3 or ΔHpu4) was also observed. In CC ST-41/44 and ST-213, a majority (60% and 83% respectively) had the ΔHpu3 deletion

type while remaining isolates were represented with an ΔHpu4 type. Conversely, ST-18 showed a high dissemination (83%) of the ΔHpu4 deletion type within this CC. A significant minority of isolates (27%) with this type (IS element) of replacement event did not belong to any previously described clonal complex (shaded grey in table 3.4). The distribution of a ΔHpu5 deletion among clonal complexes revealed that 74% of the isolates belonged to two CCs (shaded blue in table 3.4). The majority of the isolates (45%) were from ST-32 CC while 29% were from ST-269.

In summary, transposition and replacement was principally associated with loss of *hpuAB* in the ST41-44 and ST-18 complexes, while an association of complete deletion with the ST-32 and ST-269 clonal complexes was apparent.

3.4 PHASE VARIATION STATUS OF THE *hpuAB* AND *hmbR* GENES IN DISEASE AND CARRIAGE ISOLATES

The ON or OFF status of both genes, *hmbR* and *hpuAB*, was determined by either sequencing of previously amplified products or by a GeneScan (sizing of fluorescent PCR products) method using FAM-labelled primers. Two isolate collections, MLST group (with 9 additional isolates) and isolates from 2008/2009 study, were included in this analysis. The first group represents several clinically important serogroups and disease causing clones collected from many geographical regions. The 2008/2009 carriage group was selected keeping in view that this group was formulated recently and the isolates were subject to only one *in vitro* passage, which in combination with the low rate of phase variation (1×10^{-4}) reduced the chances of a switch in the receptor status (Martin *et al.*, 2004).

For determining the PV status of *hpuAB* in both groups of strains, a combination of sequencing and GeneScan analysis were used in this study. All MLST isolates were

examined by sequencing while the GeneScan technique was also used (in combination with sequencing) for the carriage isolates. The sequenced data obtained was directly analysed to determine the length of the repeat tract and to deduce the ON-OFF status of *hpuAB*. For the carriage group, the isolates were grouped into their respective *porA* types and data was generated by GeneScan for all isolates. The GeneScan data was analysed using Peak scanner software and compared with the standard sizes to determine the size of the PCR fragments. Subsequently one or two representative isolates from each *porA* type were also sequenced for tract length correlation with that of determined by GeneScan. Two different sizes of PCR fragments were evident during GeneScan analysis i.e. 310 bp and 348 bp and the reasons for this size variation is discussed in section 3.6. A 10G repeat tract (in a predicted 310 or 348 bp amplicon) was found to represent the ON status for *hpuAB* in all strains.

For *hmbR*, the sequencing and GeneScan analysis was done with primers encompassing the poly(G) repeat tract. Again, for the MLST group, direct sequencing was employed while the carriage group was analysed by a combination of sequencing and GeneScan. In addition, the length of repeat tracts in ~25 isolates of the MLST group was derived from *hmbR* sequence alignments provided by Odile Harrison, University of Oxford. A 9G tract length represents the ON state for *hmbR* and addition or deletion of a nucleotide results in change of expression of the gene.

The ON/OFF status of both genes in disease and carriage isolates from both groups was analysed separately (Figure 3.8). The analysis revealed that majority (96%) of invasive isolates with both genes (55 isolates) had either both genes (40%) in combination or at least one of the two genes (56%) in an ON state. For carriage isolates harbouring both genes (41 Isolates), 66% of isolates had both or one gene in an ON phase while 34% of them had both OFF. In strains harbouring only *hmbR*, 97% of disease isolates had this

gene in an ON state compared with 61% of carriage isolates. The *hpuAB* only carriage strains (49 isolates) surprisingly had 82% of isolates with an ON state for this gene but only 60% in case of disease isolates. However, the latter figure is representative of a small number (10 isolates) of samples.

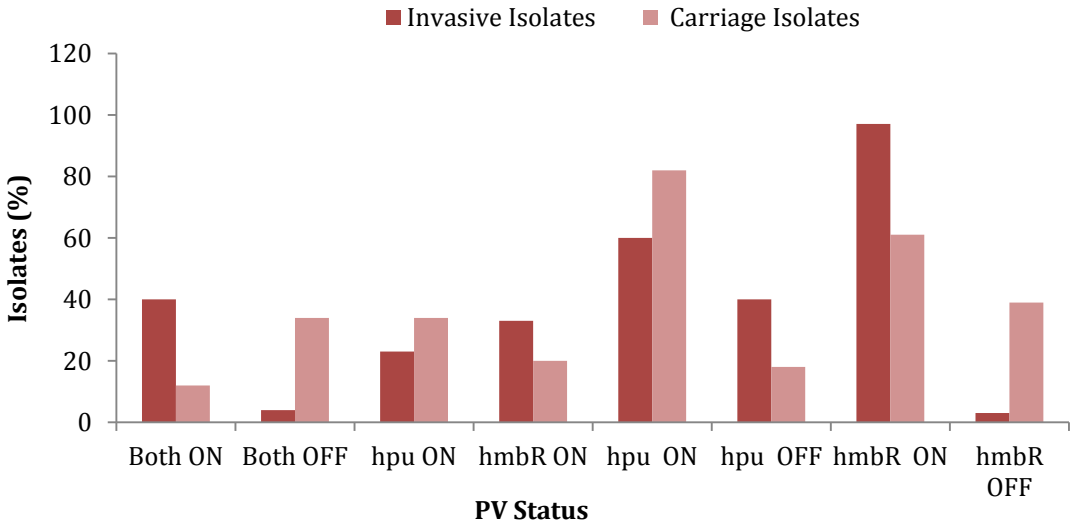

Figure 3.8: PV status of *hpuAB* and *hmbR* genes in meningococcal disease and carriage isolates. The status of both genes was determined based on the length of the repeat tract as determined by sequencing and GeneScan. The invasive isolates (88 from the MLST collection plus 9 additional isolates for a total disease of n=97) were separated from the carriage isolates to constitute the disease group. The carriage isolates from the MLST collection combined with those from carriage study 2008/2009 constituted the second group (n=105). Each group was further split into 3 subgroups based on the presence of both, *hmbR* only or *hpuAB* only phenotype. Strains harbouring both genes exhibited four PV states (ON-ON, OFF-OFF, ON-OFF or OFF-ON) while strains with a single gene will fall in to either ON or OFF genotypic groups.

Overall, more than 90% of disease (89/96) and 73% of carriage isolates (75/103) had at least one gene in an ON state, indicating a statistically significant difference between the disease and carriage PV state {p = 0.0003 (OR 4.747, 95% CI, 1.962 to 11.48)}.

3.4.1 Haemoglobin disc assay for confirmation of ON/OFF status

The phase variation status of the genes in 10 representative isolates (5 ON and 5 OFF) having one of the two systems was confirmed by a haemoglobin disc assay. This assay tests the ability of these isolates to utilize haemoglobin as the sole source of iron. The strains are grown on iron-restricted media (MHA supplemented with 40 μM desferal) and thus cannot grow without an external iron source. Growth is restricted to a zone around a filter paper disc, if the disc is impregnated with an iron source. However, the ability of an isolate to utilise a particular iron source depends upon the presence of a specific receptor for that source and its ON-OFF status. Strain N88 from the carriage study of 2008/2009 harbours only one gene, *hpuAB*, in an OFF state. When grown in the presence of Hb as sole iron source, only scattered colonies were observed around the disc containing Hb, which probably represent ON phase variants (Figure 3.9 A). On the other hand this strain exhibited confluent growth around a disc containing Tf or iron sulphate but no growth without any added iron source (Figure 3.9 B and C).

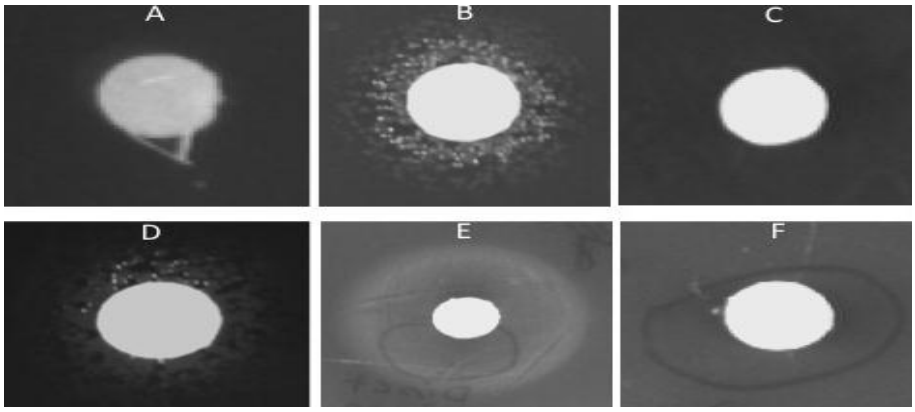

Figure 3.9: Haemoglobin disc assay for analysis of ON-OFF status of *hmbR* and *hpuAB* phase variants. An OFF variant of strain N88 (an *hpuAB* only strain) and an ON variant of MC58 (an *hmbR* only strain) were tested for Hb, Tf and/or iron acquisition as a sole iron source. Bacteria were spread on MHA plates containing 40 μM desferal. Disks containing 10 μl of either Hb (100 μM) or Tf (50 mg/ml) or without any iron source were applied to the plates, which were then incubated overnight. (A) N88, with Hb, (B) N 88, with Tf, (C) N88, no iron control, (D) MC58, with Hb, (E) MC58, with Tf, (F) MC58, no iron control.

Contrastingly, strain MC58 which harbours only *hmbR* in an ON state had confluent growth around both the Hb and Tf discs but no growth around a sterile control disc (Figure 3.10 D-F respectively). An *hmbR* mutant of the same strain was also grown as a control and exhibited no growth around Hb or a blank disc, but confluent growth around a disc containing Tf (data not shown). The phenotypic analysis of 8 other strains was also performed to correlate the expression of the receptors with the ON/OFF status determined genetically (Table 3.5). A similar confluent growth was observed around Hb discs for ON variants but no growth for OFF variants. However, confluent growth was evident around the discs impregnated with an iron source (transferrin or iron (II) sulphate heptahydrate) but no growth around sterile discs.

Table 3.5: List of the isolates tested for Hb acquisition by disc assay. The presence and status of the system is indicated along with its growth ability in the presence of haemoglobin.

Strain	Presence of Hb genes	Status of gene	Growth around Hb disc
MC58	*hmbR* only	ON	Confluent
MC58-*Δ hmbR*	Mutated-*hmbR*	-	No growth
8047 (wt)	Both	*hpuAB* ON/*hmbR* OFF	Confluent
8047-*Δ hmbR*	Mutated-*hmbR*	*hpuA*B ON	Confluent
8047-*Δ hpuAB*	Mutated-*hpuAB*	*hmbR* OFF	No growth
8047-*Δ hpuAB*:: *Δ hmbR* double knockout	Both Mutated	-	No growth
N134	Both	*hmbR* OFF/*hpuAB* ON	Confluent
N43	*hpuAB* only	ON	Confluent
Z4684	*hmbR* only	ON	Confluent
N88	*hpuAB* only	OFF	No growth
N54	*hpuAB* only	OFF	No growth
N51	*hpuAB* only	OFF	No growth
N352	*hpuAB* only	OFF	No growth
N429	*hpuAB* only	OFF	No growth

3.5 TRACT LENGTH DISTRIBUTION IN DISEASE AND CARRIAGE ISOLATES

The length of the homopolymer repeat tracts is one of the key factors in controlling the rate of phase variation and its influence on switching have been described in various studies (Bayliss *et al.,* 2004; Richardson *et al*., 2002; De Bolle *et al*., 2000). These investigations demonstrated a direct relationship between the length of repeat tracts and PV rate. Keeping this in view, the distribution and variation of homo-polymer (G) repeat tract length from the sequences of both genes, *hpuA* and *hmbR,* was determined in for the MLST and Carriage 2008/2009 group and published (Tauseef *et al*., 2011). The isolates from both groups were split into disease and carriage isolates and a direct comparison for the variation of homo-polymer repeat tracts was made for both genes.

The tracts length for *hpuA* varies between 7G and 19G in both sub-groups (Disease and carriage) with the majority (37% and 43%, respectively) of strains harbouring a 10G tract (Figure 3.10 A). This modal repeat number (10G) reflects an ON state for this gene in most of the isolates. A significant minority (20% disease and 19% carriage) of isolates from both groups was harbouring a 13G tract that also kept the reading sequence "in frame" and thus the status of gene in an ON phase.

For *hmbR*, the length of poly(G) repeat tract in disease and carriage groups ranged from 7G to 15G (Figure 3.10 B). In disease isolates, the majority (81%) was harbouring a 9G in their repeat tract, an ON number of repeats for this gene. In the case of carriage isolates, the modal repeat number observed was 9 and 10G (31% and 30% respectively), reflecting an almost equal distribution of ON and OFF states for *hmbR*. The difference in the length of repeat tracts for *hmbR* also represents the proportion of ON phase variants for this gene in both groups (disease and carriage).

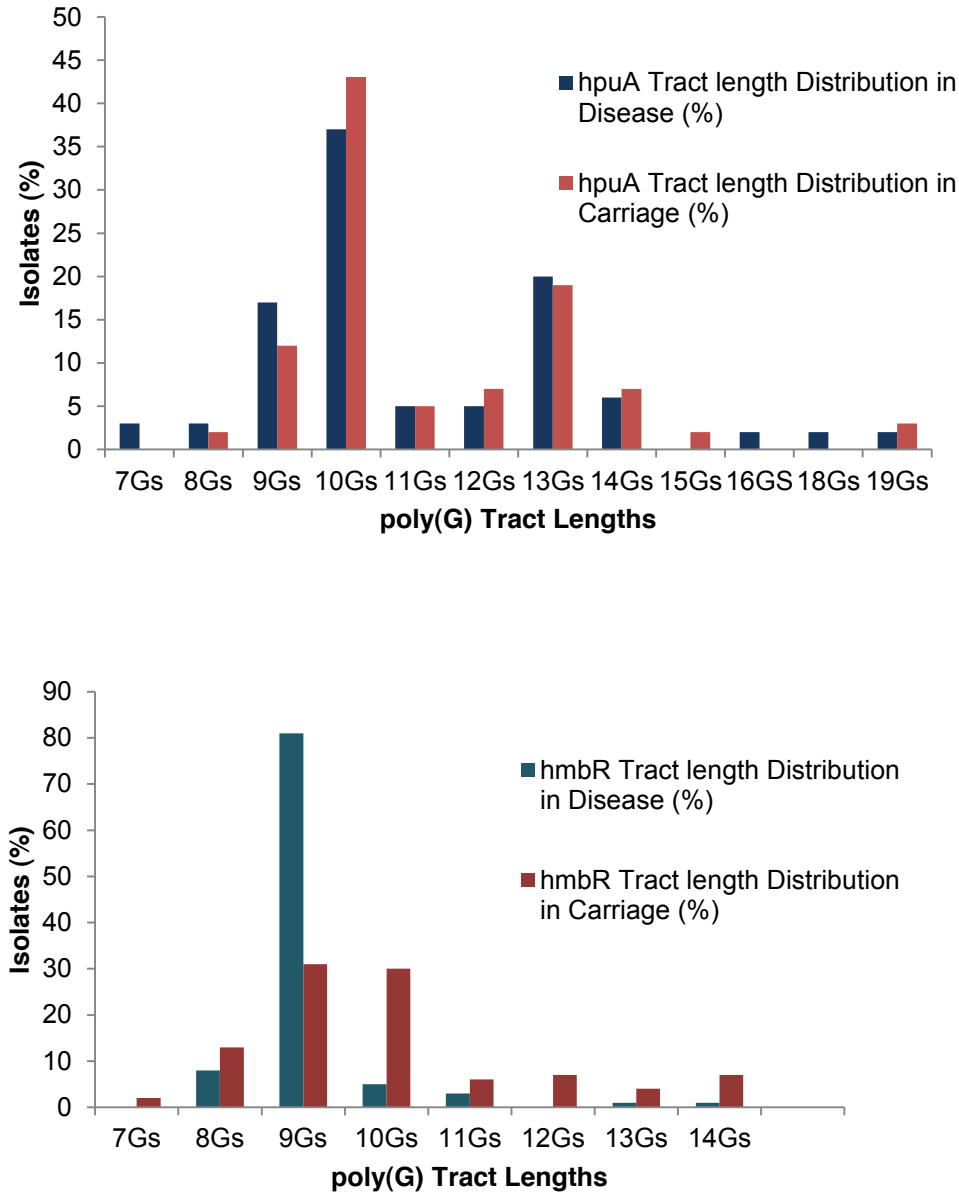

Figure 3.10: Percentage distribution of tract lengths of haemoglobin receptor encoding genes (*hpuAB* and *hmbR*) in disease and carriage meningococcal isolates. Fragment of *hpuAB* and *hmbR* genes encompassing the repeat tracts were amplified and sequenced from carriage and disease isolates. Sequenced products were analysed for the enumeration of residues in poly(G) tracts of both genes. The colours of bars represent a specific group (disease or carriage) and are described on the chart. (A) Repeat tract lengths distribution of *hpuAB* in disease and carriage groups. (B) Distribution of repeat tract lengths observed for *hmbR* gene in both groups. The tract lengths and their corresponding ON-OFF status with a gene are described in the text.

A statistical test was performed to test the frequency of longer repeat tracts as compared to shorter lengths for both genes. The tract lengths for *hpuA* and *hmbR* were split in to three groups based on the tracts length:- equal to; shorter than; and longer than the modal repeat number. For *hpuA*, we observed that 28/155 isolates had a shorter tract length than the modal number (10G) while 64/155 had tracts longer than the modal repeat number. Similarly for *hmbR*, a minority of isolates (15/140) were harbouring tracts shorter than the modal number (9G) as compared with those possessing longer tract lengths (38/140). Chi square analysis revealed a slight bias for tract lengths longer than the modal numbers and present at a significantly higher frequency when compared with those possessing shorter tract lengths ($p < 0.05$).

3.6 *hpuA* SEQUENCE VARIATION ANALYSIS AMONG ISOLATES FROM DIFFERENT CCs

Antigenic and phase variation of outer membrane proteins of *N. meningitidis* facilitates evasion of host immune selection (Alcala *et al.,* 2004). The antigenic variability and amino acid sequences variation of HpuA, a membrane associated and surface exposed component of the HpuAB bipartite system, has not been reported previously. The variability of the amino acid was analysed in the sequences generated from 10 different isolates and published (Tauseef *et al*., 2011). Complete *hpuA* nucleotide sequences from 2008/2009 carriage strains N88, N114, N117, N119, N134, N138 and N222 representing the clonal complexes CC174, CC60, CC167 and CC23 along with a serogroup B isolate (strain 8047) were obtained. In addition, partial sequences of ~200 bp length encoding the N-terminal of HpuA were also obtained from the MLST group of strains (Not shown).

The nucleotide sequences were aligned with those extracted from the Z2491 (ST-4, serogroup A) and FAM18 (ST-11, serogroup C) genome sequences (Parkhill *et al.,* 2000; Bentley *et al.,* 2007). A major variable region was observed ~100 bp downstream of the repeat tract along with other single nucleotide variations (Appendix 1). The region was characterised by a 48 bp deletion/insertion in one group of strains. This also explains the reason for two different amplicons sizes (310 bp and 348 bp) observed in GeneScan, as described in section 3.4. These nucleotide sequences were also translated to derive amino acid sequences for all isolates and a subsequent multiple sequence alignment was done using software program CLUSTALW (http://www.genome.jp/tools/clustalw/) (Figure 3.11). The predicted signal peptide sequence at the N-terminus of HpuA along with the repeat tract was removed to get only the mature protein sequence and to avoid any genetic diversity caused by slipped strand mispairing region of the homo-nucleotide repeat tract.

The size of predicted HpuA translated proteins from the above sequences varied from 326 to 342 amino acids due to an insertion/deletion of 16 amino acids in the N-terminal region. A significant level of variation (76-94% identity) between aligned sequences was also evident and the variation was attributed to four major variable regions along with >100 polymorphic sites within the aligned nucleotide sequences (Appendix 1). As the structure-function relationship of the HpuA domains, and the functional role of diversity in this surface exposed protein is largely unknown, it is hard to predict the influence of this variation on the antigenicity of the receptor and its ability to bind haemoglobin. However, the influence of amino acid sequence variations on the function of a similar meningococcal outer membrane protein TbpB has been assessed previously (Boulten *et al.*, 1998). TbpB is a component of the transferrin binding protein complex

with a high level of variability but the variability in this receptor protein did not affect binding to its substrate, transferrin (Boulten *et al.*, 1998).

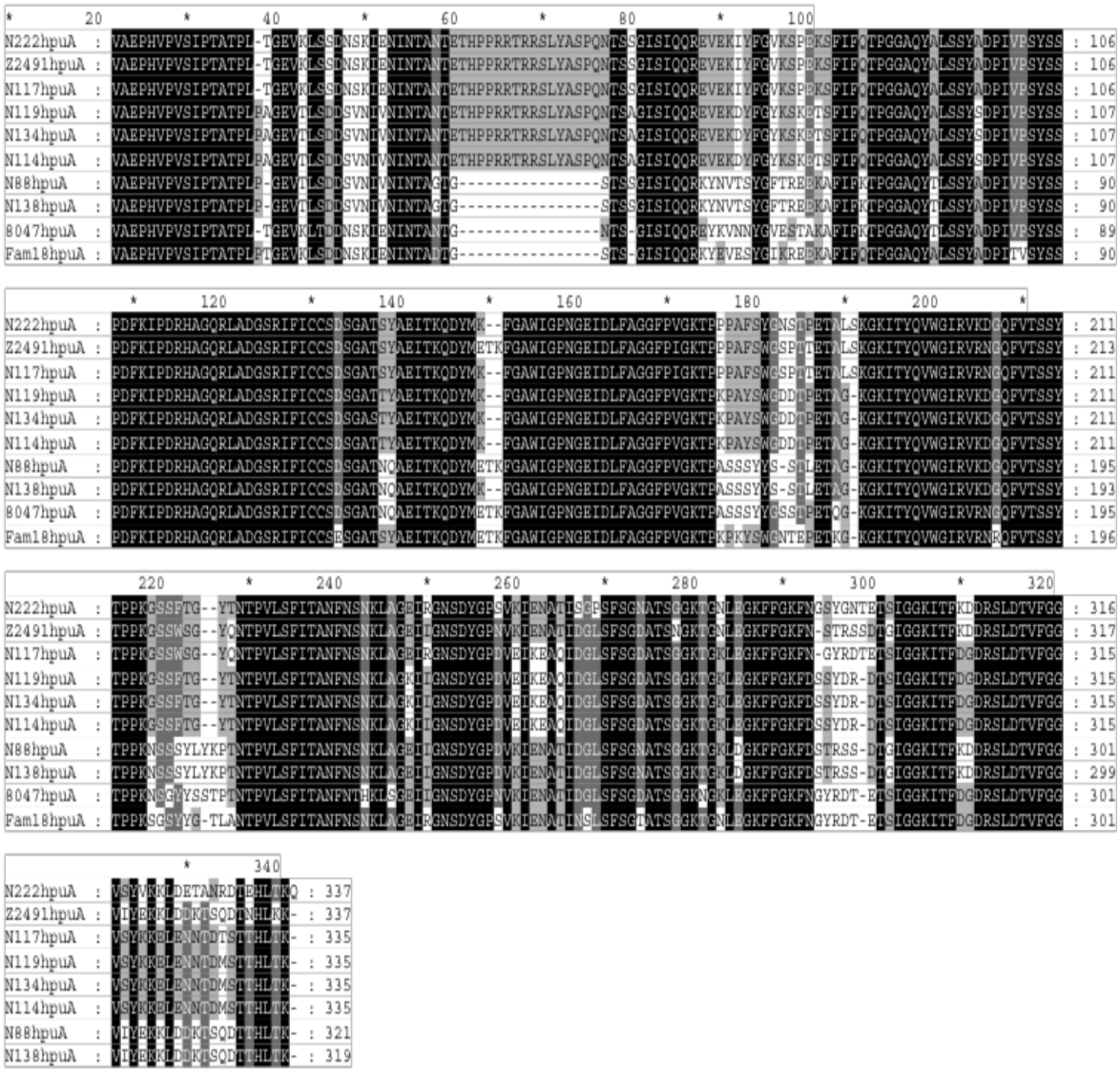

Figure 3.11: Alignment of the amino acid sequences of HpuA from carriage and disease isolates of *Neisseria meningitidis.* Full-length sequences were generated from two disease isolates (Z2491 and 8047) and six carriage isolates (N222, N117, N119, N134, N114, N88 and N138). The sequences were translated from the first codon after the repeat tract and so do not include the initiation codon, signal sequence and repeat tracts. These sequences were then aligned with HpuA sequences derived from the published genome sequences of strains Z2491 (Parkhill *et al.,* 2000) and FAM18 (Bentley *et al.,* 2007). Adapted from Tauseef *et al* (2011).

3.7 PHASE VARIATION DURING PERSISTENT CARRIAGE

Persistent carriage is associated with an increase rate of immune response against the presented antigen (Jordens *et al.,* 2004). This heightened immune response may influence the phase variation status of surface receptors. To observe the changes in *hpuAB* and *hmbR* expression due to PV during carriage, longitudinal carriage isolates obtained from healthy individuals during 2008/2009-carriage study were analysed (Bidmos *et al.,* 2011). Isolates of two different meningococcal *porA* types (P1.21,16 and P1.5,2) were chosen for this analysis (Table 3.6 and 3.7). The *porA* type P1.21,16 consisted of eight persistent carriers and all isolates were harbouring *hpuAB*-only genotype, as detected by PCR. Similarly, *porA* type P1.5,2 was represented by three persistent carriers and this strain harbours both, *hmbR* and *hpuAB*, genes. The phase variation status of each persistent carrier was determined at time of collection (1st time point), after four weeks (2nd time point), after 12 weeks (3rd time point) and after 24 weeks (4th time point) from same volunteer. The *porA* typing was performed after each time point to confirm that the same strain was isolated.

The analysis of poly(G) repeat tract of *hpuA* in the isolates belonging to P1.21,16 *porA* type revealed a variation in tract length (hence the ON-OFF status) between time points in a majority of isolates. The ON-OFF status of ~90% of isolates (seven isolates out of eight analysed for this *porA* type) was changed, when compared the ON-OFF status of *hpuA* gene determined at 3rd or 4th time point with the status at first time point (Table 3.6). Phase variation status of five isolates was in an OFF state after persistent carriage (after 12 or 24 weeks interval), which may hint at the kind of selective pressure responsible for the selection of OFF variants during carriage.

Table 3.6: Phase variation status of *hpuAB* gene in isolates of *porA* type P1. 21,16 after prolonged carriage. The PV status of each isolate was detected by analysis of poly(G) repeat tract length of *hpuAB* gene, and determined for four different time points.

		Time points[a] and PV status of *hpuAB* during persistent carriage				
porA	Isolate	1	2	3	4	Status[c]
P1. 21,16	N43	ON (13G)[b]	OFF (12G)	OFF (12G)	-	Changed
	N51	OFF (12G)	OFF (12G)	ON (13G)	ON (13G)	Changed
	N52	ON (13G)	ON (13G)	OFF (11G)	-	Changed
	N54	OFF (14G)	ON (13G)	OFF (11G)	-	Changed
	N58	ON (13G)	ON (13G)	-	OFF (12G)	Changed
	N59	ON (13G)	ON (13G)	OFF (12G)	ON (13G)	Changed
	N88	OFF (9G)	ON (10G)	ON (10G)	OFF (9G)	Changed
	N138	ON (13G)	ON (13G)	ON (13G)	-	Same

[a] Sampling time points during carriage study; 1, First sampling time; 2, sampling after four weeks from same carrier; 3, sampling after twelve weeks from same carrier; 4, sampling after twenty four weeks from same carrier.

[b] Number of consecutive G residues in the poly(G) tract and the predicted ON-OFF status of *hpuA* gene determined at a specific time point.

[c] PV status comparison between time points. Same; no switching of gene observed from 1st time point till the

The isolates of *porA* type P1.5,2 were also analysed (Table 3.7). The PV status of both genes, *hpuA* and *hmbR*, was determined for all four-time points. No obvious change in PV status was observed for both genes during carriage in the isolates of this *porA* type. The PV status of *hmbR* was in an OFF state in all of them, suggesting some selective pressure. However, due to low number of samples for this *porA* type, the affect of persistent carriage on the PV status of the isolates from this *porA* type cannot be concluded.

Table 3.7: PV status of *hpuAB* and *hmbR* genes during persistent carriage in isolates of *porA* type P1.5,2. The PV status was detected between four consecutive intervals of sampling.

		Time points[a] and PV status of *hpuA* and *hmbR* during persistent carriage				
porA type	Isolate	1	2	3	4	Status[c]
		hpuA/ hmbR	*hpuA/ hmbR*	*hpuA/ hmbR*	*hpuA/ hmbR*	
P1. 5,2	N114	OFF (9G)/OFF (10G)[b]	OFF (9G)/OFF (10G)	OFF (9G) /OFF (10G)	-	Same
	N134	ON (10G)/OFF (10G)	ON (10G) /OFF (10G)	ON (10G) /OFF (10G)	-	Same
	N185	ON (10G)/OFF (10G)	ON (10G) /OFF (10G)	-	ON (10G) /OFF (10G)	Same

[a] Sampling time points during carriage study. 1, First sampling; 2, sampling of isolates after four weeks; 3, sampling after twelve weeks; 4, sampling after twenty four weeks.

[b] Number of consecutive G residues in the poly(G) tract and the predicted ON-OFF status of *hpuA* and *hmbR* genes determined at a specific time point.

[c] PV status comparison between time points. Same; no switching of gene observed from 1st time point till the final time point. Changed; gene switched in either direction (OFF→ON or ON→OFF) atleast once between time points.

3.8 DISCUSSION

Multiple pathogenesis factors such as type IV pili and capsular polysaccharides have been identified as potential determinants for meningococcal invasion and infection. However, these factors may not be sole contributors in disease progression and a possible association of other genetic factors cannot be under estimated. This part of the study focussed on a general aim of determining the virulence association of *hpuAB* and *hmbR*. A comprehensive set of data about the distribution of these genes in disease and carriage isolates was not available at the start of this study. This type of investigation is important because there is not a good model for *in vivo* study of meningococcal virulence factors such as the receptors encoded by these genes. Epidemiological data on the presence/absence of these genes in isolates from disease or carriage backgrounds would provide an understanding of their impact on meningococcal pathogenesis and

disease progression. The results suggested a complex relationship between the distribution/phase variable expression of both haemoglobin receptors and disease or carriage states of meningococci.

In this study the presence/absence of both systems was analysed, the statistical analysis revealed a significant under-representation of *hpuAB*-only gene in clinical isolates when compared with the presence of both genes and/or an *hmbR* only genotype (Table 3.1). One explanation for this under-representation of an *hpuAB*-only genotype and over-representation of an *hmbR*-only genotype in disease isolates is, that there is greater selection against HpuAB due to higher immunogenicity of this receptor than HmbR. A comparatively strong immunogenicity may ultimately lead to stronger selection against *hpuAB*-only isolates than *hmbR*-only strains during invasive disease. However, when the HpuAB receptor is present in combination with HmbR in a strain (both receptors simultaneously), then one can expect a low level of selection against that strain due to phase variation mediated switching of HpuAB into an OFF phase and HmbR into an ON phase. This switching in pattern of expression leads to a phenotype expressing only the haemoglobin receptor (HmbR), which may enable meningococci to escape from immunity while maintaining haemoglobin acquisition.

In contrast to disease states, the selective pressure imposed by the immune system would be far less during colonization/carriage state. In this scenario, *hpuAB*-only strains can switch the gene OFF and acquire the iron from an alternate source e.g. iron-loaded lactoferrin by using lactoferrin binding receptor protein. This switching of the *hpuAB* gene into a non-expression state and also acquisition from an alternate source of iron may explain the over-representation of an *hpuAB*-only genotype in carriage as compared to disease isolates.

Another factor, which may influence the distribution of haemoglobin receptors in disease and carriage background, is the difference in the receptor's specificity and affinity for either haemoglobin or haptoglobin-haemoglobin complexes. In the nasopharynx, haemoglobin is likely to be found complexed with haptoglobin, thus Hb will be found at a low concentration. A similarly low concentration of free haemoglobin compared to haptoglobin-haemoglobin, can be expected in serum during the initial phase of invasion due to its binding with haptoglobin. The abundance of haptoglobin-haemoglobin complexes favours colonization by strains with an Hpu^+ phenotype due to its specificity for this iron source during carriage. This specificity and ability to utilize haptoglobin-haemoglobin may provide an advantage to strains with an Hpu^+ phenotype over Hpu^- phenotype, and could be the reason for over-representation of this phenotype during carriage.

However this specificity cannot be the sole explanation for this over-representation due to isolation of carriage strains harbouring an *hmbR*-only genotype, suggesting the availability of free haemoglobin during carriage. Similarly it is not clear how this substrate specificity leads to a low prevalence for an *hpu*-only genotype in clinical isolates, when the preferential substrate (haptoglobin-haemoglobin complex) may be present at comparatively higher levels in blood during initial exposure and subsequent systemic spread. It is also interesting to note that when this receptor is present in combination with *hmbR*, then a preferential switch 'for' haptoglobin-haemoglobin complex utilization and 'against' haemoglobin only was observed in a recent publication (Omer *et al.,* 2011).

An alternative explanation for the differing distributions and the published results (Harrison *et al.,* 2009) is that this may arise due to a higher affinity of the HmbR receptor for free haemoglobin compared to that of the HpuAB receptor. It is very likely

and expected that once invasion has occurred and disease has established, free haemoglobin should be available at comparatively higher levels due to tissue damage and cell lysis. FrPC, a protein containing typical RTX domain found in cytotoxins is reported in all invasive and some carriage meningococci (Osicka *et al.,* 2001), and up regulated when bacteria are grown in the presence of haemoglobin (Jordan and Saunders, 2009). Thus, expression of this protein may favour cytotoxicity and hemolysis, releasing free haemoglobin in turn. In this scenario, the presence of a heamoglobin utilizing system would be extremely beneficial and favours the distribution and accumulation of an *hmbR*$^{+}$ genotype in disease isolates. In a recent study, Jordan and Saunders (2009) have reported that virulence associated genes of meningococcal strain MC58, an *hmbR*-only strain, were induced when grown in the presence of haemoglobin. However, no such induction was observed when transferrin was supplied as a sole iron source. These findings suggest a critical role of haemoglobin utilization in disease progression and establish a direct link between its utilization and a pathogenic phenotype.

Keeping in view the above findings, haemolysis and induction of cytotoxins associated with release of free haemoglobin during disease is likely to have two impacts (i) it would favour and enhance the *hmbR*$^{+}$ strain's growth; (ii) induction of virulence–associated genes whose expression may lead to an increase in invasion of the *hmbR*$^{+}$ strain. If this is the case, then one could predict a high frequency of either an *hmbR*-only genotype or both genes (*hmbR* in combination with *hpuAB*) in clinical isolates. Our study observed similar patterns of distribution and supports the prediction for an over-representation of either *hmbR*-only phenotype or both receptors in disease isolates.

The statistical analysis revealed no significant difference between the presence of both receptors and *hmbR* only genotype. This suggests that *hmbR* only or both genes may be

important for disease. The presence of a second gene (*hpuAB*) with *hmbR* may provide an additional advantage to the isolates due to differences in haemoglobin sources, and may also provide flexibility of switching to the other system when an immune response is mounted against the ON phenotype. This observation can be supported by the fact that clonal complexes with high disease/carriage ratios (>6) harbour both receptors, as described in Table 3.4. The presence of both receptors simultaneously may increase the propensity of a strain to cause disease by immune evasion or may be connected to the rapid transmission of the strains to compensate for low carriage levels.

The distribution analysis also identified some carriage isolates {21 isolates (7% of the total carriage isolates)} lacking both systems. The strains with negative results were further tested with additional set of primers to avoid PCR false-negative results. It is unlikely that the annealing site polymorphism is present for additional set of primers as well, keeping in view that the primers are designed for regions of highest homology in published sequences. It may also unlikely that false-negative PCR only appear in carriage but not disease isolates. This observation (lack of both genes in some carriage) suggests that iron acquisition from heme of haemoglobin or haptoglobin-haemoglobin may not be essential for persistence in the human respiratory tract, as lactoferrin, transferrin or other iron sources may be available as alternative sources of iron. However, if a heme source is required for persistence then another possibility is the presence of an alternative haemoglobin system, which has yet to be identified.

Repetitive DNA within genes and non-coding regions is a “hot-spot” for recombination and rearrangement events. Neisserial genomes are replete with a variety of repetitive elements in coding and non-coding regions (Parkhill *et al.,* 2000). These elements have been associated with the deletion of genes coding for important surface determinants such as FetA and PorA (Claus *et al.,* 1997; Marsh *et al.,* 2007; van der Ende *et al.,*

1999). The deletion mechanisms in the *hpuAB* locus were identified and appear to have a similar pattern. The deletion of the locus resulted from either a replacement by an IS element or a recombination mediated deletion event(s) by repetitive extragenic palindromic sequence (dRS3) leading to rearrangements in the locus (Figure 3.5). Only three types of rearrangements/replacement events were found in *hpuAB* null locus strains. The association of specific *hpuAB* deletion mechanisms with a particular ST type was also evident (Table 3.4). Stable lineages of a particular deletion type suggest that these deletions are likely to be infrequent possibly due to a low selective advantage associated with them. High immune pressure on Hpu^+ might have driven the acquisition of *hmbR* as a replacement before the *hpu* deletion to avoid acquired immunity. However, a more detailed analysis may be required to elucidate whether the prevalence of *hpuAB*-negative isolates is driven by immune evasion.

As with many other surface exposed and virulence related proteins, the expression of the haemoglobin receptors in meningococci undergoes phase variation. Phase variation of *hpuAB* and *hmbR* is likely to contribute significantly to evasion of adaptive immune responses and may help in niche adaptation. A high rate of phase variation in these genes was reported to be associated with epidemic spread of meningococcal disease strains (Richardson *et al.,* 2002). This observation suggests that in late phase of an epidemic spread, random switching of phase variable genes facilitates the evasion of acquired immunity and spread in a population once the host population has acquired herd immunity against the circulating meningococci.

The data on the ON-OFF status of both systems indicated that the expression status of both genes is dependent on the invasive or carriage state of the examined strains. The presence of an ON state for one or both genes in a significant number of disease isolates (96%) compared to (66%) carriage reinforces the importance of Hb acquisition during

invasive disease. Furthermore, a significant number of carriage isolates with their both genes in the OFF state were also identified. This may be interpreted as a less stringent requirement for Hb acquisition during carriage and more frequent immune evasion, a hypothesis further supported by the absence of both genes in a number of carriage isolates. However, the influence of adaptive immune responses on the phase variation of Hb receptors is less clear and a detailed analysis of these specific responses would be helpful.

A comment on the quality of the phase variation data determined for both genes in this study is relevant here. One can argue that the PV status of isolates may have been switched during passage in the lab and may not represent the original *in vivo* PV status of the examined population. However, as the switching rates due to phase variation are low (1:10000) for these genes (Richardson and Stojiljkovic, 1999; Lewis *et al.,* 1999) and also the passage in lab lacks an *in vitro* selection, it can be safely said that our PV data is almost representation of *in vivo* PV status of the entire population. In addition, as the ON-OFF status was determined on the basis of DNA sequencing, the quality of interpretation of ON-OFF data was also tested by phenotypic assay of ~10 isolates (see table 3.5 and figure 3.9) which confirmed that the phase variable status determined by poly(G) enumeration was correct.

A number of factors augment the frequency of phase variation mediated by repetitive DNA. An increase or decrease in the length of repeat tract directly influences the rate of phase variation (Richardson *et al.,* 2002). Variation in repeat tract length was evident for both of the haemoglobin receptor encoding genes reflecting potential differences in phase variation rates. The analyses of tract lengths distribution of both genes demonstrated that the majority of strains have 10Gs and 9Gs tracts for *hpuAB* and *hmbR*, respectively. This indicates that a particular phase variation rate may be favoured

for adaptation. However, a skewed distribution of longer tract lengths for both genes was also observed. This may be explained by the fact that longer tandem repeats are more prone to phenotypic switching than shorter ones, resulting in an advantage in certain selective environments. However, as there are no differences in distribution of these longer tracts between invasive and carriage isolates, the significance of these longer tracts is unclear. Also, whether the change is due to selection or a molecular driver (e.g. mismatch repair system) remains to be determined.

Surface modulations mediated by antigenic variation have long been recognised in meningococci (Segal *et al.,* 1986; Stern *et al.,* 1986). Such variation is associated with bacterial escape from host immunity and has significant implications for development of an effective vaccine. Alignment of complete *hpuA* nucleotide sequences from 10 different meningococcal isolates and partial 5' end nucleotide sequences from several other isolates identified significantly variable regions interspersed with conserved sequences. A random distribution of single nucleotide polymorphisms was also detected in the aligned sequences. Such variable regions have been found in other surface exposed components and have been recognised as potential targets for human defence mechanisms (Russell *et al.,* 2004; Thompson *et al.,* 2003).

As the HpuA structure has not been resolved so far, the functional predictions based on structural analogy are inconclusive. Thus it is difficult to associate and map these polymorphisms with surface exposed loops of this lipoprotein, although it is very likely that these variations have accumulated due to selective pressures mounted by immune responses, and also provide evidence of a high level of antigenic variation in this surface exposed component of the haptoglobin-haemoglobin receptor. A thorough study focused on diversity of both *hpuA* and *hpuB* genes would be helpful to elucidate whether these modulations of the HpuAB receptor are subject to CC structuring, as

observed for other surface component encoding genes including *hmbR*, *porA* and *fetA* (Evans *et al.,* 2010; Russell *et al.,* 2004; Thompson *et al.,* 2003).

Bacteria need to constantly modulate their surface antigens during persistent carriage and a variety of mechanism including phase and antigenic variation are employed (Hill *et al.,* 2010). Longitudinal samples obtained at specific intervals provide excellent rationale for assessment of these modulations and to examine whether responses are driven by host immune systems, or required for adaptation to other changes. The phase variation status of *the hpuAB* gene of the majority of the isolates belonging to the P1.21,16 *porA* type (ST-1466, CC-174, all *hpuA*-only strains) changed during persistent carriage (Table 3.6). This change is likely to occur due to immune responses against this antigen. The tract lengths of all isolates from P1.21,16 *porA* type changed and five out of seven isolates had an OFF state at the 12th or 24th week of colonization. This OFF phase after prolonged carriage may indicate selection driven by immune responses, and may have emerged at the peak of an immune response.

In contrast, no such change of PV status was observed in the isolates belonging to P1.5,2 (ST-1383, CC-60) *porA* type, a group of strains harbouring both receptors. However the continuous OFF status of *hmbR* throughout persistence of carriage suggests a selection against this phenotype. Alternatively during carriage, free haemoglobin (the preferred source of this receptor) is present at a low level and the expression of this receptor may not be as advantageous as the expression of HpuAB counterpart. Hence, this analysis showed a change in length of repeat tract for the *hpuA* in the isolates of one of the two *porA* type analysed. The change occurred during prolonged carriage and similar changes can not be over ruled out in other phase variable genes encoding surface proteins.

In conclusion, analyses in this part of the study have implications for discovering the roles of the Hb receptors in meningococcal carriage and disease. The under-representation of the *hpuAB*-only genotype in disease isolates suggests that not all iron acquisition systems may be essential for invasion and disease progression. However, the PV status of both genes in disease strains indicated that at least one gene is in an ON state, reinforcing the need for haemoglobin acquisition during invasion. The results for distribution also suggest that the disease isolates harbour at least one gene and that invasion is facilitated by either a combination of both genes or *hmbR* alone. Finally, persistent carriage is frequently associated with changes in phase variable status of haemoglobin receptors and is likely to be driven by immune evasion.

Chapter 4

VALIDATION OF *IN VITRO* SERUM BACTERICIDAL ASSAY TO STUDY IMMUNE ESCAPE MEDIATED BY PHASE VARIATION OF PorA

Phase variation in many surface structures of meningococci has long been recognised and can potentially mediate the escape from the bactericidal activity of specific antibodies due to mutations in tandem DNA repeat tracts (Bayliss *et al.,* 2008). To investigate such type of PV mediated evasion from bactericidal antibody, Bayliss *et al.,* (2008) has published a novel assay. The assay involved the continuous incubation of a bacterial population in multiple cycles in the presence of specific antibody and human serum, mimicking an on-going and continuous immune response by adding fresh antibody after each cycle. This assay was selected to study the PV mediated immune evasion from antisera generated against HpuA in this project (see chapter 6). However, validation of this assay was required prior to use this for studying PV mediated immune evasion from the antisera. For this, immune escape mediated by PV of PorA was used to validate the assay, and also as a model to study PV mediated immune evasion from antisera raised against HpuA.

PorA antigen was selected as a model for impact of PV on immune escape due to importance of this antigen as a known target for a protective immune response and also that this component is being investigated as a potential vaccine candidate (Jodar *et al.,* 2002; Martin *et al.,* 2000; Holst *et al.,* 2009). The antibodies raised against this antigen were the most effective in preventing a meningococcal infection in the infant rat model (Saukkonen *et al.,* 1987; Saukkonen *et al.,* 1989). The production of bactericidal

antibodies specific for PorA following an infection and colonisation has also been reported and are important to protect against meningococcal infection (Mandrell *et al.,* 1989; Jordens *et al.,* 2004). The protein is predicted to contain eight surface exposed loops and structural model have suggested that variations are mainly confined to loop 1 and loop 2 (also called VR1 and VR2, respectively) regions (van der Ley *et al.,* 1991). These variable regions are known targets for bactericidal antibodies, which recognise a short peptide sequence at the apices of VR1 and/or VR2 and elicit complement-mediated bacterial killing (van den Elsen, 1997). The antigenic variation at these regions is used for serological classification (sub-typing) of meningococci.

The phase variable expression of *porA* gene is due to the presence of a poly(C) repeat tract in its core promoter (van der Ende *et al.,* 1995). The biological significance of phase variation of PorA in immune escape is not investigated before this study. Hence, the escape from bactericidal antibody, specific for 8047 PorA antigen is thoroughly investigated in this part of the project. For this analysis, MAb P1.2 was used to test the PV mediated evasion of bacterial killing by strain 8047. This antibody recognises the sequence in the VR2 of PorA in the strain 8047 and used to define its subtype (http://neisseria.org). The incubation of meningococcal population (8047) in the presence of this antibody and a complement source (human serum) demonstrated that phase variation of the PorA receptor can mediate escape from killing by MAb P1.2 due to a change in the expression of PorA via change in length of poly(C) tract, as determined by various molecular biology techniques employed. This analysis provided a background to test the biological significance of phase variation of haemoglobin receptors in evasion of adaptive immunity.

4.1 ESCAPE OF *N. meningitidis* STRAIN 8047 FROM MONOCLONAL ANTIBODY P1.2

The *porA* gene of strain 8047 contains 11C residues between the -35 and -10 region of the promoter sequence, thus a potential to phase vary which may alter the length of repeat tract. Such a change in spacing between -10 and -35 regions of a promoter may affect the confirmation of the sequence required for an optimum binding of RNA polymerase leading to an alteration in transcription efficiency (Moxon *et al.,* 2006), and a change in the expression level of the gene. The experiment was performed to assess the escape from killing of MAb P1.2, specific for PorA surface antigen.

The inoculum for the escape assay was prepared from a glycerol stock of strain 8047. The stock was previously made by streaking a single colony of strain 8047 to obtain a purified culture. The bacteria were grown on BHI plates overnight from the purified stock and used for inoculum preparation. Previous studies indicated that the *porA* switching frequency is $\sim 10^{-3}$ (Hopman *et al.,* 1994). Based on this information, inoculums of 3 different sizes {i.e. large inoculum size (5×10^6 CFU) and medium/intermediate inoculum sizes (5×10^5 CFU & 5×10^4 CFU)} were incubated in the presence of 1:4 dilutions of MAb P1.2 (obtained from NIBSC) with 5% human serum (as complement source) and the bactericidal activity of antibody was observed.

After the first passage (2 h incubation) of the continuous assay, appropriate dilutions of all inoculums were plated on BHI plates to obtain the CFU counts. We observed a strong bactericidal activity of monoclonal antibody P1.2 reducing the number of CFU by 5 folds in the case of the high inoculum (Figure 4.1). However, with the medium and

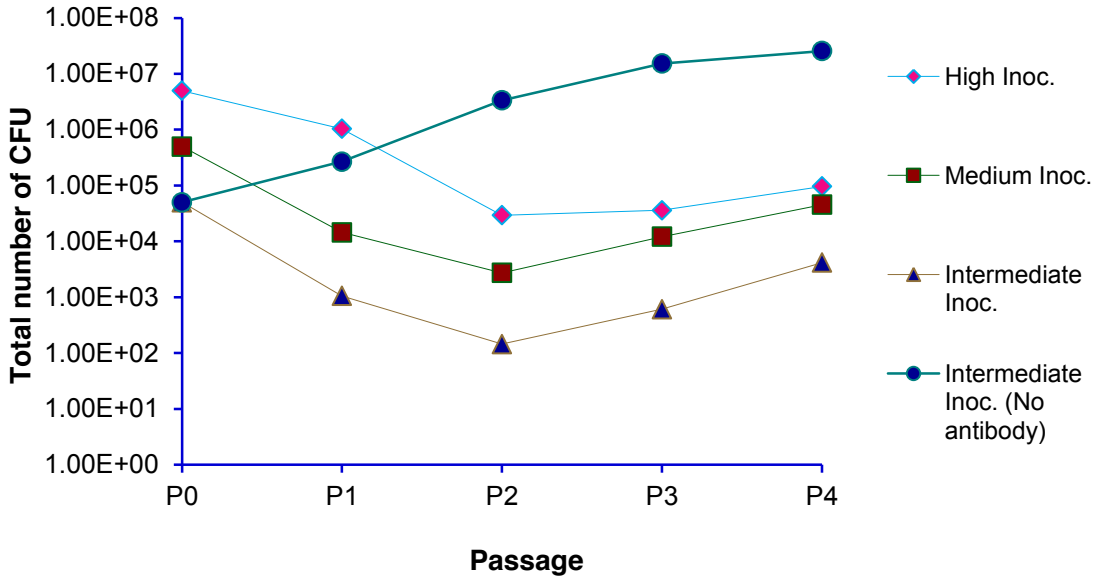

Fig 4.1: Escape from MAb P1.2 mediated serum bactericidal activity by *Neisseria meningitidis* strain 8047. The inocula of high (5×10^6 cells), medium and intermediate (5×10^5 and 5×10^4 cells respectively) sizes of strain 8047 were prepared from an overnight culture grown on BHI plates and incubated in the presence of 5% human pooled serum (diluted in a final volume of 1 ml of PBSB containing 0.1% glucose) supplemented with or without MAb P1.2. Subsequent passage (2, 3 and 4) were performed by mixing 500 µl (out of 1 ml) of the previous passage with an equal volume of PBSB containing human pooled serum (5%), and with or without antibody. The populations were passaged for four times and each passage was performed for 2 h. Serial dilutions of remaining (500 µl) passaged population were plated on BHI after each passage to obtain total viable count, which represent the number of cells (Y-axis) present in each passage (X-axis). Appropriate dilutions of inoculum were also plated and represent number of viable cells in inoculum (Y-axis). P0, sizes of population used as inoculum before incubation with/without antibody and human serum; P1, population after 2 h incubation; P2, 4 h; P3, 6 h and P4, 8 h incubation. The impact of periodic dilution is not corrected and the result relies on the difference between control population (inoculum incubated without antibody represented by filled circle) and the population of same size (filled triangle) incubated with antibody.

intermediate inoculums, the bactericidal activity was stronger reducing the total population 35 fold and 50 fold, respectively. An inoculum of ~5×10^4 CFU was also incubated without antibody but with 5% human serum, acting as a control to determine whether any change is driven by the complement source only. The CFU count obtained

for the control population was comparable with the selected population of the same size (~5×10^4 CFU) for validity (Figure 4.1).

In the second passage, after addition of fresh antibody and human serum, the reduction in population size was smaller (7 folds) for the medium and intermediate inoculum but comparatively more significant (35 folds) for the large inoculum population. This reflects that due to the large size of this population, more reactive variants survived the first passage due to antibody depletion as compared to a medium-sized population. The reduction in population size for the large inoculum even continued in the third passage but not for the medium size inoculum where no bactericidal activity was observed after the second passage. This lack of activity indicated that the populations had become resistant to MAb P1.2 mediated killing. This resistant population survived and multiplied in the subsequent passages.

The fraction of resistant population in inoculum was calculated. Firstly, the doubling time was determined for this experiment from the control population (population passaged without antibody) and was ~45 minutes. As it is clear from figure 4.1 that the population after 2nd passage (4 h incubation) was completely resistant, thus knowing the doubling time, the phase variants were counted back from passage two and calculated for inoculum. Based on these calculations, the frequency of phase variants in inoculum was ~3×10^{-4}.

4.1.1 Colony immunoblotting for analysis of passaged and un-passaged population

To determine the nature of the resistant population, the colony immunoblots were performed using MAb P1.2. Both, passaged and non-passaged populations were examined by colony immunoblotting. Similarly, the immunoblots from the inoculum were also analysed for comparison (Figure 4.2).

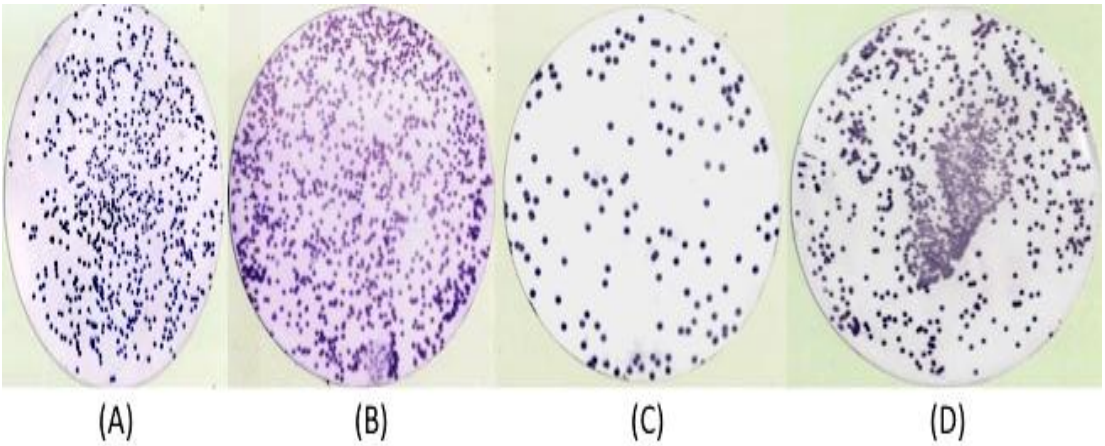

Fig 4.2: Colony immunoblots obtained from strain 8047 and probed with monoclonal antibody P1.2. Serial dilutions of inoculum and passaged populations were grown overnight on BHI plates and transferred to nitrocellulose filters. The colonies were probed with MAb P1.2 (1:2000) and its binding was detected by anti-mouse AP-conjugated secondary antibody (1:3000). The blots were developed by incubation of filters for 15 min in 1 ml of substrate. (A) Immunoblot obtained from inoculum demonstrating high reactivity to MAb P1.2; (B) Blot obtained from the population passaged in the absence of antibody, but in the presence of 5% human serum; (C) immunoblot developed from P1 of passaged population; (D) immunoblot developed from P4 of passaged population.

In the immunoblots obtained from the inoculum, all colonies reacted equally when developed with substrate solution indicating the homogeneity of population (Figure 4.2 A). This can be expected due to a smaller frequency ($\sim 3 \times 10^{-4}$) of variants in inoculum. A similar pattern was observed for blots developed from the bacterial population passaged in the presence of human serum but without monoclonal antibody (Figure 4.2 B). Surprisingly, immunoblots obtained from passaged population did not show any difference in staining intensity between the colonies (Figure 4.2 C & D). These results could not demonstrate a clear difference between the passaged and un-passaged population and hence the nature of the resistant population.

4.1.2 Analysis of passaged and un-passaged populations by sequencing and GeneScan

Once the reactivity of the population resistant to MAb P1.2 killing was analysed on the immunoblots, and no clear difference was observed, the colonies were further investigated to determine whether resistance was due a change in the repeat tract of *porA*. Random colonies (*n*=24) from passaged and un-passaged populations were picked and re-streaked on fresh BHI plates for genomic DNA preparation followed by PCR amplification and analysis of the PCR products by sequencing (Table 4.1).

Table 4.1: *porA* repeat tract length analysis by sequencing of 8047 variants analysed from inoculum, passaged and un-passaged populations.

Detected variant [a]	Inoculum [b]	MAb selection (Passage) [c]				No antibody (Passage) [d]
-	-	P1	P2	P3	P4	P4
11C	3	3	0	0	0	3
10C	0	5	2	2	6	0
9C	0	0	0	0	0	0
Split of total colonies (*n*=24) analysed from inoculum, passaged and un-passaged	3	8	2	2	6	3

[a] Type of variant detected from inoculum, passaged and un-passaged population samples.

[b] Colonies analysed from an untreated population and used as inoculum in assay. A digit represents the number of that particular variant detected (down the column) from a total number of colonies analysed for this group (bottom column). Zero represents no variant of this type was detected.

[c] Colonies analysed from a population incubated in the presence of antibody. The number and type of variants in each passage are indicated (down the column). Bottom column represents total number of colonies analysed for a particular passage.

[d] Colonies analysed from a population passaged with human serum but no antibody.

The results indicated that the population incubated without antibody but with human serum only had 11C in the core promoter region of *porA*. A similar length of *porA* tract was observed for the colonies obtained from the inoculum. However, the colonies obtained from P1 of passaged populations consisted of a mixture of 11C and 10C

colony variants. In subsequent passages (P2, P3 and P4), variants with an 11C repeat tract were not detected and a population with 10C variants was observed to have accumulated. These results suggested the accumulation of 10C variants after incubation with human serum and monoclonal antibody, hence provided an evidence for the nature of resistant population.

The analysis was extended and a comparatively large number of representative colonies were analysed using GeneScan Colonies (n=39) of strain 8047 from inoculum, the population passaged with human serum only, and the population passaged in the presence of antibody were also picked and re-streaked. The analysis revealed that all variants picked and re-streaked from inoculum/ no antibody population had a product size indicative of 11C residues in the core promoter region of *porA* (Table 4.2). However in P1 of the antibody-passaged population, a mixture of variants with sizes indicative of 11C and 10C repeat tracts was detected while all isolates analysed from P4 of this population indicated a 10C tract length in their *porA* repeat tract.

These results also suggested that although 10C variants were present in P1 of passaged population, the difference between the levels of expression of 11C and 10C was perhaps not enough to differentiate on immunoblots, resulting in no visible staining difference between them (see figure 4.2 C). This was true for P4 as well, where all population tested had 10C but the staining of the colonies was not clearly different from the 11C population (see figure 4.2 D). The reason for this lack of differentiation on immunoblots was revealed later and described in sections 4.1.3-4.1.5. The analyses suggested that the difference in levels of expression between both variants was marginal, leading to a lack of clear differentiation on immunoblots. However, this difference was detected when more sensitive methods were employed (see sections 4.1.3, 4.1.4 and 4.1.5).

Table 4.2: *porA* repeat tract length analysis of 8047 variants isolated from passaged and un-passaged populations by GeneScan sizing.

Detected variant[a]	Inoculum[b]	MAb selection[c] (Passage)		No antibody[d] (Passage)	
-	-	P1	P4	P1	P4
11C	5	5	0	4	8
10C	0	9	8	0	0
9C	0	0	0	0	0
Split of total colonies (*n*=39) analysed from inoculum, passaged and un-passaged population	5	14	8	4	8

[a] Type of variant detected from inoculum, passaged and un-passaged population.

[b] Colonies analysed from an untreated population and used as inoculum in assay. A digit represents the number of that particular variant detected (down the column) from a total number of colonies analysed for this group (bottom column). Zero represents no variant of this type was detected.

[c] Colonies analysed from a population incubated in the presence of antibody. The number and type of variants in each passage are indicated (down the column). Bottom column represents total number of colonies analysed for a particular passage.

[d] Colonies analysed from a population passaged with human serum but no antibody.

The length of the repeat tract of variants was further confirmed by sequencing (Not shown) to determine whether the change in product size is due to a change in repeat tract present in the promoter region or in a poly(A) repeat tract in the 5' end of this gene. The sequenced data confirmed that the poly(A) tract remained intact in these variants while they all contained a 10C repeat tract. The sequencing and GeneScan data from all colonies streaked either from the inoculum or from the population passaged without antibody had 11C residues in the repeat tract.

These results indicated that incubation of strain 8047 in the presence of MAb P1.2 resulted in a switch of 11C phenotype to a 10C population. Interestingly, none of the phase variants tested had 9C in its repeat tract. This repeat number in the promoter sequence corresponds to a very low level of expression for this gene (see section 4.1.4 and figure 4.3). This finding indicates that a phase variable switch from an 11C

population to 10C population may lead to a change in expression level and resistance of MAb P1.2 killing.

4.1.3 Western blot analysis of the levels of PorA expression

The repeat tract analysis by sequencing and GeneScan confirmed an alteration in the repeats in the promoter of the strain 8047 *porA* gene. The impact of this change on expression level was further investigated using a combination of methods. Firstly to test whether the repeat numbers determined by sequencing or GeneScan corresponded to the levels of expression, a Western blot assay was employed. Whole cell lysates from each population were analysed by Western using MAb P 1.2 against the PorA antigen.

PorA expression was detected from three different overnight cultures (1 of 11C and 2 different variants of 10C) of 8047 containing 11C, 10C and also two different variants with a 9C repeat tract. Cell lysates from equal numbers of 8047 cells (1×10^9) of each variant were mixed with 100 µl of SDS- PAGE loading buffer and boiled. An equal volume (15 µl) from each sample was loaded onto the SDS-PAGE gel and used for the Western blot. A band of ~40 kDa was detected by MAb P 1.2 which corresponds to the expected size of the PorA protein (Figure 4.3). The lower small size bands with low intensity are attributed to degradation products. A loading control gel was also run to confirm that each sample contained equal amounts of protein (not shown).

The results of the Western blotting demonstrated that the 11C parental phenotype had the highest level of PorA expression (Lanes 2 and 3), indicating that this repeat number is optimum for expression. The variants containing 10C in their repeat tract showed a medium level of PorA expression (Lanes 4 and 5), indicating that a deletion of one nucleotide from the *porA* repeat tract results in a reduction in the expression of this

gene. The expression was at the lowest level in a phenotype containing 9C residues in the core promoter region (Lanes 6 and 7).

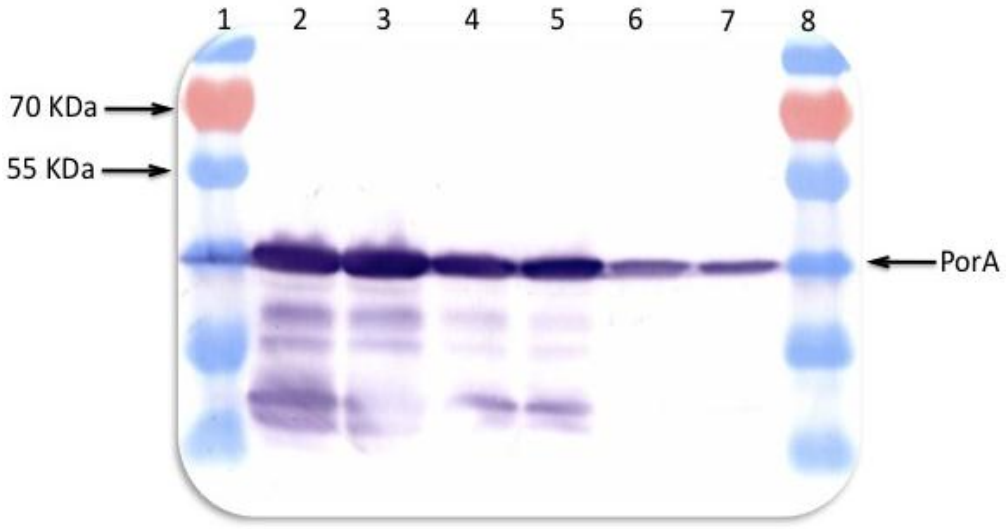

Figure 4.3: Western blot analysis of PorA expression in three different variants of strain 8047. Each variant was grown overnight in BHI and fixed in 0.05% formalin. Equal numbers (1×10^9 cells) of cells for all variants were mixed in an equal volume (100 µl) of SDS buffer and boiled. A 15 µl sample from all lysates was separated by SDS- PAGE on a 10% gel and transferred to a nitrocellulose membrane. Transferred protein was probed with the MAb P1.2 (1:2000 dilution) and its binding was detected with a secondary antibody (anti mouse AP-conjugated antibody, 1:3000) followed by the development of blot with substrate. Lane 1, protein ladder; Lane 2 -3, wild type strain 8047 variant containing an 11C repeat tract (one variant loaded in duplicate); Lane 4-5, strain 8047 variants containing 10C repeat tract (two different variants); Lane 6-7, 8047 phase variants containing 9C repeat tract (two different variants).

A quantitative analysis was performed to measure the difference in levels of expression between PorA variants. An online software ImageJ (http://rsbweb.nih.gov/ij/) was used to measure this difference. The software measures the pixels of each protein band on a gel image and generates peaks based on the intensity of each individual band. The area, based on intensity for each band probed in figure 4.3, was calculated and the mean area for each variant was obtained (Figure 4.4). The result showed a significant reduction ($p < 0.05$) in expression due to alteration of repeat tract lengths between 11C and 10C

PorA phase variants. Similarly, the significance was increased ($p < 0.01$) when the expression of 11C and 9C was compared. The difference in expression, based on the intensity of each band, from the wild type (11C variant) was calculated for 10C and 9C variants. The results suggested a 1.2 fold reduction of expression in variants with 10C tract lengths compared with 11C. Similarly the reduction in expression for 9C was 2.9 folds compared to 11C variants.

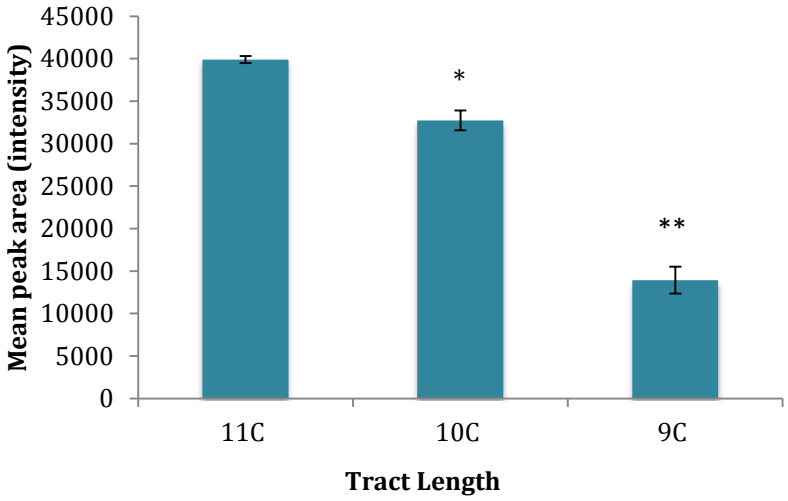

Figure 4.4. Mean peak area based on the intensity of PorA protein bands obtained from strain 8047 phase variants in figure 4.3 (11C, 10C and 9C) and measured by ImageJ. Peak area, generated based on the intensity of each band in figure 4.3, was calculated and the mean peak areas for each variant were obtained by combination of values obtained for both bands. Results are means (±SEM) of two samples for each variant and are representative of two independent experiments. Relative expression was compared between 11C & 10C and also between 11C and 9C using student unpaired t-test. *$p < 0.05$, **$p < 0.01$.

4.1.4 Whole cell ELISA assay for PorA surface expression

This method was employed for an accurate quantitative analysis in level of surface expression between the *porA* variants containing different repeat lengths. The samples were prepared by the fixing of whole cells from 8047 variants (11C, 10C and 9C) in

formalin and re-suspended in ELISA coating buffer to adjust OD550 = 0.5. A 100 µl sample of fixed cells was re-suspended in coating buffer and coated in duplicate into the well of an ELISA plate. An ELISA was performed as described in the methods sections. The binding of MAb with cells was analysed by measuring the OD405 after substrate addition and subsequent development. A wild type 8047 variant containing an 11C tract in its core promoter region showed a high level of PorA surface expression (Figure 4.4).

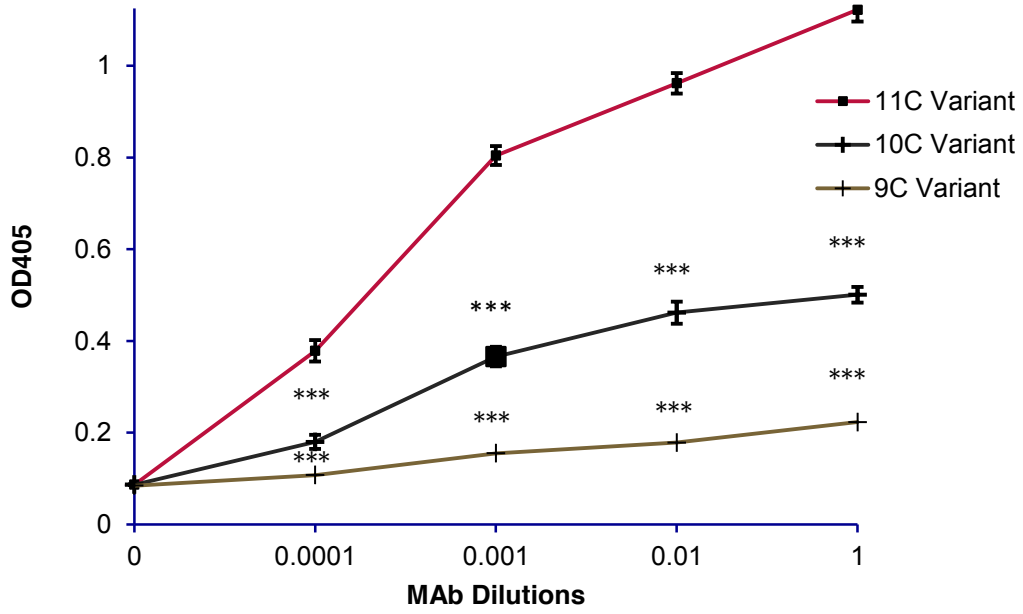

Figure 4.4: Whole cell ELISA representing the level of PorA surface expression in three phase variants of strain 8047. Whole cells were obtained from 11C, 10C and 9C *porA* variants of strain 8047. These cells were fixed in 0.05% formalin/PBS and OD550 was adjusted to 0.5 in coating buffer. A 100 µl of sample was coated onto an ELISA plate and probed with MAb P1.2. The binding of MAb to bacterial surface was assessed using an anti-mouse AP-conjugated antibody (1:10000). The OD405 was obtained (Y-axis) for a range of dilutions (X-axis) of MAb P 1.2 (ranging from 0 to 1). The results are the mean (±SEM) of duplicates and are representative of two independent experiments. ***$p < 0.0001$.

Medium and low levels of PorA expression were detected for 10C and 9C variants, respectively. These results indicated that the reduction in reactivity of MAb P1.2 observed for 11C variants versus 10C/9C variants was due to a reduction of total surface expression of PorA protein. The reduction in expression relative to wild type (11C) was significant ($p < 0.0001$). On average, the binding of MAb P 1.2 was reduced ~2 fold by a change from 11C to 10 C and another 2.5 folds between 10C and 9C variants. The assay confirmed that the change in level of expression observed by Western corresponded to the amount of PorA antigen expressed on the surface of the cell.

4.1.5 FACS (fluorescence-activated cell sorter) analysis for surface expression of PorA in phase variants

Flow cytometry, an accurate method for the analysis of surface expression, was also used to further confirm the above findings and for comparison of all data obtained from other techniques. The variants (11C, 10C and 9C) of strain 8047 were grown overnight in separate liquid BHI cultures and 200 µl of each culture was centrifuged. After primary (MAb P1.2) and secondary antibody (an anti-mouse FITC labelled conjugate) incubations as described in methods, the cells were fixed with a fixative buffer containing formalin. The relative surface expression of 11C, 10C and 9C variants was measured.

The bacteria were gated by side scatter to exclude the cell debris and aggregates, and the binding of a FITC-labelled secondary antibody was detected for 25000 bacteria. As measured by mean fluorescence of gated bacteria, the lowest level of fluorescence was detected for the control samples without adding primary antibody (P1.2), but with FITC-labelled secondary antibody (Figure 4.5). After exposing to monoclonal antibody,

the highest level of fluorescence was gained by the strain 8047 variant containing an 11C repeat tract. Similarly the results measured for 10C and 9C repeats showed significantly lower levels ($p < 0.0001$) of fluorescence than wild type (11C), respectively.

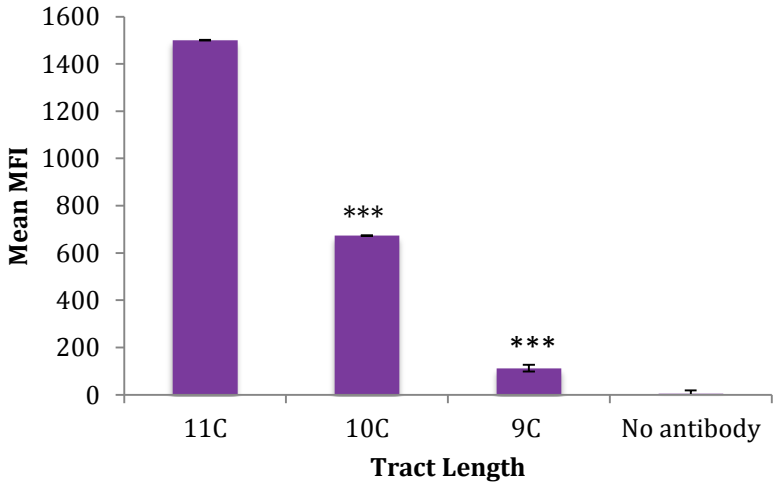

Fig: 4.5. FACS analysis (mean fluorescence intensity ±SEM) of MAb P1.2 binding with three different PorA phase variants (11C, 10C and 9C). Three different PorA phase variants (11C, 10C and 9C) of strain 8047 were grown overnight in a liquid culture and fixed with 0.05% formalin. Fixed bacteria were incubated with 1:50 dilution of MAb P1.2 followed by detection with mouse specific FITC-labelled secondary antibody (1:100 dilution). The MFI value of 25000 bacteria decorated with labelled antibody was recorded in triplicates for each sample and the mean MFI was obtained for each variant. The results are the mean (±SEM) of triplicates. ***$p < 0.0001$.

A 2 fold reduction in surface expression of a 10C variant was recorded compared with wild type with normal PorA surface expression (11C). Similarly, a 9C variant had ~14 fold lower expression as compared to 11C. An overlay of the fluorescent data obtained from the three variants was also performed for direct comparison (Figure 4.6). This demonstrated that the 11 C variant has the highest antibody binding capacity for MAb P1.2 and the binding was reduced on a shift of repeat tract lengths to 10 and 9C.

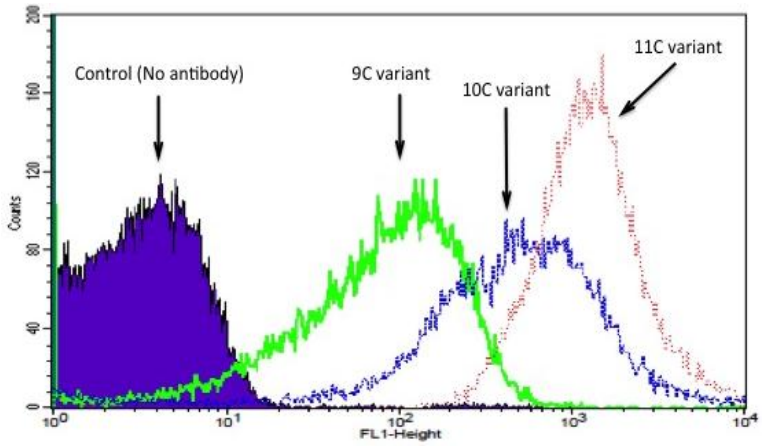

Figure 4.6: Flow cytometry histograms overlay of the strain 8047 variants (11C, 10C and 9C). A representative FACS analysis histogram overlay displaying the relative fluorescence intensity of PorA surface expression is shown for three 8047 variants. FACS analysis was carried out using Becton Dickinson FACS Calibur and the results were analysed using software supplied by the manufacturer. A shift in peaks represents the difference in surface expression determined for PorA variants. Blue fill, control sample incubated without MAb P1.2; green line, expression of 8047 variant with 9C repeat tract; blue line, 10C variant; pink line, wild type (11C). The histograms are representative of three independent readings recorded for each variant.

These results mirrored our earlier data collected by Western and ELISA techniques. All methods indicated ~1.5-2 fold decrease in expression of PorA during switching from 11C to 10C. The difference between 11C and 9C variants was ~2.5 to 6 folds, depending upon the employed method. The difference in fold reduction of expression level for variants between the three techniques was perhaps due to the sensitivity of each method.

These results can also be co-related to the reactivity of PorA antibody with strain 8047 on immunoblots and may explain the reason for unclear differentiation between 11C and 10C variants. As detected by all three methods, the reduction in expression between 11C and 10C variants is low and perhaps not enough to differentiate between both variants on immunoblots. This marginal difference in expression makes the

differentiation of 10C from 11C variants difficult in a population containing both variants.

4.2 ESCAPE FROM ANTIBODY IS DEPENDENT UPON SIZE OF INOCULUM

The escape or evasion from specific antibody response is known to be influenced by a number of factors including the size of bacterial inoculum (Bayliss *et al.,* 2008). The influence of inoculum size on escape from MAb P1.2 was also investigated. Inoculum from strain 8047 of medium (5×10^5 CFU) and small sizes (5×10^3 CFU) were passaged with monoclonal antibody (Figure 4.7). The results demonstrated that after the first passage, the inoculum containing ~5000 CFU was completely eradicated by MAb P1.2 mediated killing but not in a control without antibody. For this inoculum, the number of CFU reached an undetectable level after 2 h incubation, which indicated that this size of population was too small to contain phase variants capable of escape from an acquired antibody response. However when the inoculum size was raised up to 5×10^4 CFU as seen in the earlier experiment (Figure 4.1), escape was observed.

The colonies from the surviving population and from inoculum were analysed by GeneScan analysis (Table 4.3). The results confirmed the eradication of the 11C population and accumulation of 10C variants, as observed in previous experiments, in the passaged population. The *porA* tract length of isolates obtained from the population passaged without antibody were also determined and remained unchanged, as observed previously. This experiment demonstrated that the size of inoculum is an important factor in determining the ability of populations to evade the specific antibody mediated killing.

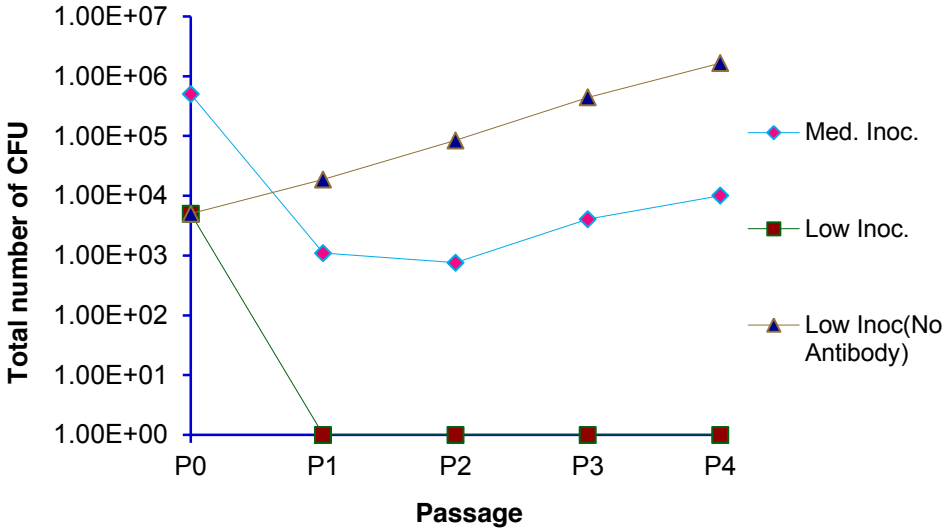

Fig 4.7: Influence of size of inoculum on escape from specific antibody by strain 8047. Medium and low inoculum sizes (5×10^5 CFU and 5×10^3 CFU, respectively) for strain 8047 were passaged in the presence or absence of MAb P1.2. The total number of CFU was derived after each passage by making dilutions. The data indicates a limit required to escape the bactericidal affects of antibody. The concentration of the antibody, human serum and the number of cycles were the same as described in the legend of figure 4.1.

4.3 INFLUENCE OF THE CONCENTRATION OF BACTERICIDAL ANTIBODY ON ESCAPE FROM KILLING

In addition to inoculum size, another important factor that affects the dynamics of escape from bactericidal clearance is the amount, affinities and functionalities of bacterial specific antibodies (Bayliss *et al.,* 2008). In contrast to earlier experiments described in section 4.1, where 10 µl of a 1:4 dilution of specific antibody was used, the affect of concentrated antibody on escape was also examined in a continuous assay using high and medium inoculum sizes (Figure 4.8). The CFU count for each passage indicates that the population structure differed from that observed for diluted antibody.

Table 4.3: GeneScan results for 8047 variants. The results are representative of variants analysed from inoculum, after passage in the absence or presence of MAb P1.2.

Detected variant[a]	Inoculum[b]	MAb selection[c] (Passage)		No antibody[d] (Passage)	
-	-	P1	P4	P1	P4
11C	4	1	0	6	8
10C	0	11	18	0	0
9C	0	0	0	0	0
Split of total colonies (n=48) analysed from inoculum, passaged and un-passaged population	4	12	18	6	8

[a] Type of variant detected from inoculum, passaged and un-passaged population.

[b] Colonies analysed from an untreated population and used as inoculum in assay. A digit represents the number of that particular variant detected (down the column) from a total number of colonies analysed for this group (bottom column). Zero represents no variant of this type was detected.

[c] Colonies analysed from a population incubated in the presence of antibody. The number and type of variants in each passage are indicated (down the column). Bottom column represents total number of colonies analysed for a particular passage.

[d] Colonies analysed from a population passaged with human serum but no antibody.

The results demonstrated that after the first passage in the presence of concentrated monoclonal antibody, the bacterial population was reduced to a minimum level for each inoculum and then recovered. This indicates that the reactive population was almost completely eradicated after 2 h incubation leaving the nonreactive phase variants. The GeneScan performed for each passage confirmed the accumulation of 10C variants in passaged populations, but not in the control population (Table 4.4).

To further clarify this phenomenon, a direct comparison was made between the CFU count obtained from the passage with neat antibody with the CFU count of a passage with diluted antibody. The CFU counts from high and intermediate inoculums of both passages were obtained and directly compared (Figure 4.9).

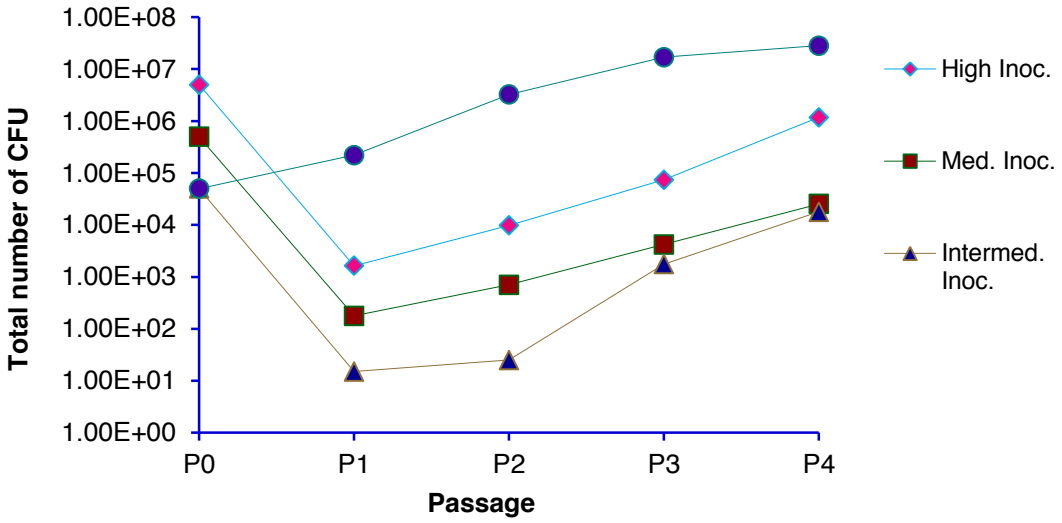

Fig 4.8: Effect of concentration of antibody on the escape by 8047 phase variants. Inoculums of high (5×10^6 CFU), medium (5×10^5 CFU) and intermediate (5×10^4 CFU) sizes were prepared and passaged in a continuous assay in the presence of concentrated antibody. Human serum concentration and the incubation cycles were kept the same as described in figure 4.1. The data represent the numbers of viable cells present in the inoculum and each passage, obtained on BHI plates from serial dilutions of appropriate sample.

Table 4.4. GeneScan analysis of 8047 variants from the passage experiment with concentrated MAb.

Detected variant[a]	Inoculum[b]	MAb selection[c] (Passage)		No antibody[d] (Passage)	
-	-	P1	P4	P1	P4
11C	4	1	0	8	4
10C	0	11	8	0	0
9C	0	0	0	0	0
Split of total colonies (n=36) analysed from inoculum, passaged and un-passaged population	4	12	8	8	4

[a] Type of variant detected from inoculum, passaged and un-passaged population.

[b] Colonies analysed from an untreated population and used as inoculum in assay. A digit represents the number of that particular variant detected (down the column) from a total number of colonies analysed for this group (bottom column). Zero represents no variant of this type was detected.

[c] Colonies analysed from a population incubated in the presence of antibody. The number and type of variants in each passage are indicated (down the column). Bottom column represents total number of colonies analysed for a particular passage.

[d] Colonies analysed from a population passaged with human serum but no antibody.

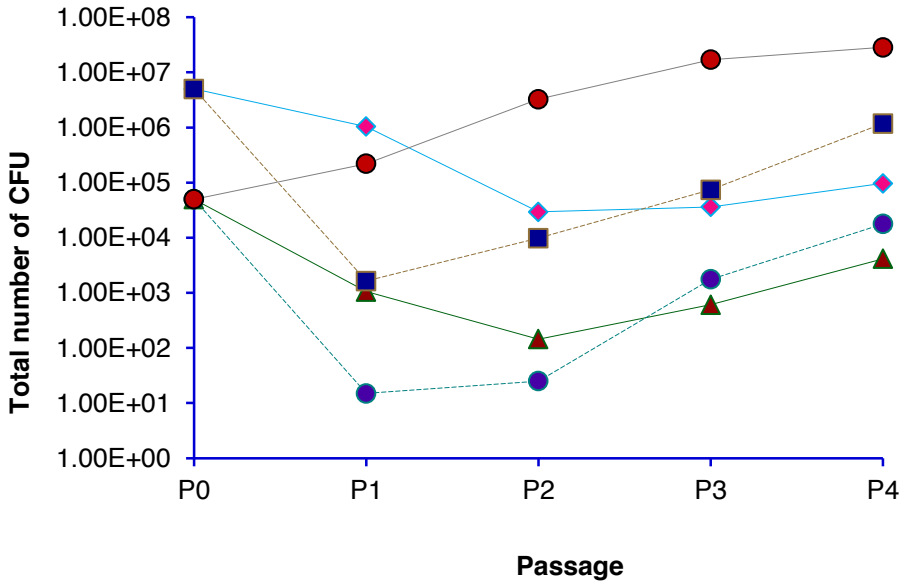

Fig 4.9: Comparison of total viable cell count after selection with high and lower concentrations of antibody. Equal sizes of inoculum incubated in the presence of concentrated (data from figure 4.8) or diluted antibody (data from figure 4.1) were compared. Solid lines represent the CFU count obtained from populations passaged with diluted antibody and dashed lines represent selection with the high concentration of antibody. Filled rectangles, high inoculum (5×10^6 CFU) of strain 8047 incubated in diluted antibody; filled triangles, intermediate inoculum (5×10^4 CFU) of strain 8047 incubated in diluted antibody; filled squares, high inoculum (5×10^6 CFU) of strain 8047 incubated in neat antibody; filled circles, intermediate inoculum (5×10^4 CFU) of strain 8047 incubated in neat antibody; open circles, intermediate inoculum (5×10^4 CFU) of strain 8047 incubated without antibody.

The results demonstrated that in the presence of a low concentration of antibody, the clearance of sensitive variants was gradual until the end of the second passage when the resistant phase variants had accumulated. In contrast, a higher concentration of bactericidal antibody for the same inoculum sizes eradicated the sensitive variants (presumably with a high level of expression 11C) within 2 h of incubation, leaving the resistant phase variants (having medium level of expression 10C). The population without antibody did not show any detectable changes in population structure and proportion of variants.

4.4 ESCAPE IS ENHANCED BY A MUTATOR PHENOTYPE, 8047 *ΔmutS*

Phase variation mediated adaptation to environmental changes by meningococci is likely to be affected by the rate of generation of phase variants. The phase variation mediated escape from specific antibody by a mutator strain, 8047 *ΔmutS*, was examined to study the affect of the rate of generation of variants. The escape was determined in a continuous assay, as described in section 4.1. The inoculums of four different sizes were used and incubated with 1:4 dilutions of bactericidal antibody to investigate the dynamics of escape. The results demonstrated a rapid accumulation of variants, resistant to monoclonal antibody killing (Figure 4.10)

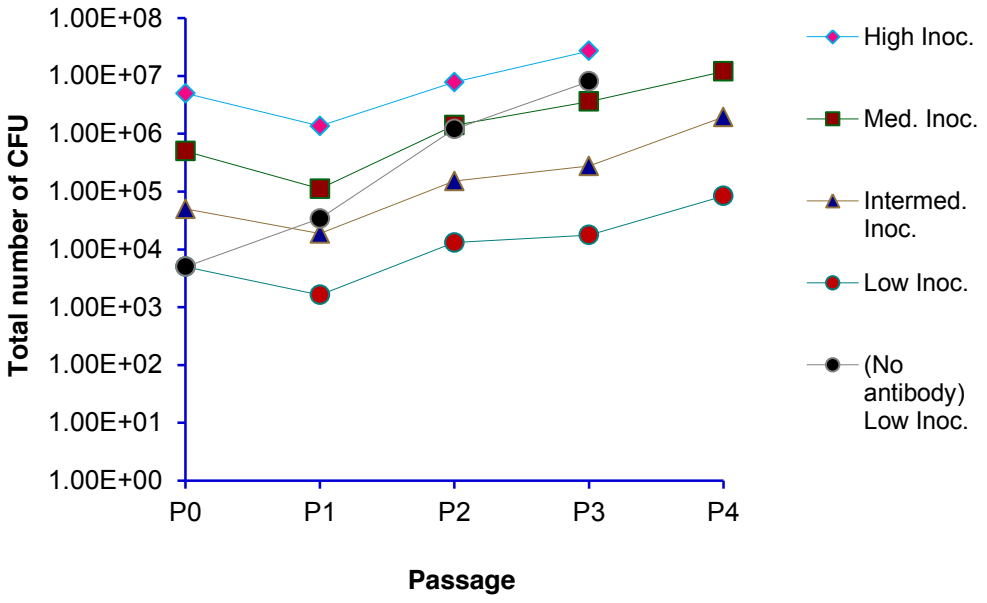

Fig 4.10: Escape by 8047 ***ΔmutS*** mutant from bactericidal activity of monoclonal antibody P1.2. The mutator phenotype (8047 *ΔmutS)* was grown overnight in BHI and inoculums of high (5×10^6 CFU), medium (5×10^5 CFU) and intermediate (5×10^4 CFU) sizes were prepared, as described in figure 4.1. The continuous assay was run by incubating the inoculum in the presence of 5% human pooled serum supplemented with or without antibody The viable cell count after each passage was obtained (Y-axis) by plating dilutions on BHI plates and plotted against respective passage (X-axis).

The colony blots from P1 antibody-selected populations indicated a mixture of reactive and non-reactive variants, and this combination of both variants was maintained at the end of the 4th passage, as found on blot obtained at P4 (Figure 4.11). Blots obtained from non-selected populations and inoculums were uniform and consisted of all reactive population (data not shown).

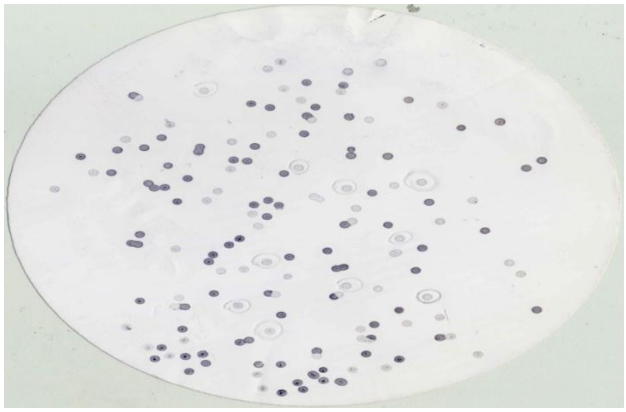

Figure 4.11: Colony immunoblots from 8047 ***ΔmutS*** after passage with antibody and probed with monoclonal antibody P1.2. Serial dilutions of passaged population (P4) were grown overnight on BHI plates in the presence of CO_2. The colonies were transferred to nitrocellulose filters and were blocked in the presence of 5% skimmed milk in PBS. The colonies were probed with a 1:2000 dilution of MAb P1.2 and the binding was detected with an anti-mouse AP-conjugated (1:1000) secondary antibody). Immunoblots were developed in the presence of 1 ml of substrate for 15 min and consisted of a mixture of reactive (dark circles) and non-reactive (light coloured) colonies.

The tract lengths analysis from the PCR products of variants picked from inoculum, and also from passaged populations (with or without antibody) was performed by gene sizing (Table 4.5). The tract length of inoculum and non-selected population variants contained an 11C tract in their repeat tract. In the case of MAb passaged population, the reactive variants on blots contained repeat tracts of 10C while non-reactive variants had 9C residues in their repeat tract. In P1, these reactive variants (10C) were present in

comparatively higher numbers than 9C (~10:1). After subsequent cycles and at the end of the 4th passage, the nonreactive population consisting of 9C residues appeared to be in an equal proportion to that of 10C variants (Figure 4.11). This phenomenon is described in details in section 4.6.

Table 4.5. GeneScan analysis of 8047 *ΔmutS* variants. The results are representative of variants analysed from inoculum, population passaged in the presence or absence of MAb P1.2.

Detected variant[a]	Inoculum[b]	MAb selection[c] (Passage)		No antibody[d] (Passage)	
-	-	P1	P4	P1	P4
11C	4	0	0	4	4
10C	0	6	9	0	0
9C	0	7	12	0	0
Split of total colonies (*n*=46) analysed for inoculum, passaged and un-passaged population	4	13	21	4	4

[a] Type of variant detected from inoculum, passaged and un-passaged population.

[b] Colonies analysed from an untreated population and used as inoculum in assay. A digit represents the number of that particular variant detected (down the column) from a total number of colonies analysed for this group (bottom column). Zero represents no variant of this type was detected.

[c] Colonies analysed from a population incubated in the presence of antibody. The number and type of variants in each passage are indicated (down the column). Bottom column represents total number of colonies analysed for a particular passage.

[d] Colonies analysed from a population passaged with human serum but no antibody.

Interestingly, no 11C variants were detected on the immunoblots of selected populations. However, as the difference in colonies on immunoblots of 11C and 10C is marginal, it is likely that 11C variants may be present but not picked and analysed by GeneScan. The population incubated without antibody but with human serum, did not show any noticeable change in the proportion of nonreactive variants.

Keeping in view the above data and to study the difference in escape between parental wild type and 8047 *ΔmutS* mutant strain, a direct comparison was made by incubating

similar sizes (medium and low inoculum) of populations from both strains in the presence of mAb (Figure 4.12). As expected from the previous data, in the presence of antibody, a decrease in total population size was observed after the first passage at an almost similar size for both wild type and mutator phenotype. However in subsequent passages, a decrease in population was observed for wild type, but the population increased for the *mutS* mutant. The results also demonstrated that even a small sized inoculum of *mutS* mutant, contained enough variants to resist the bactericidal killing, but these inoculum were too small for the parental type, which was eradicated.

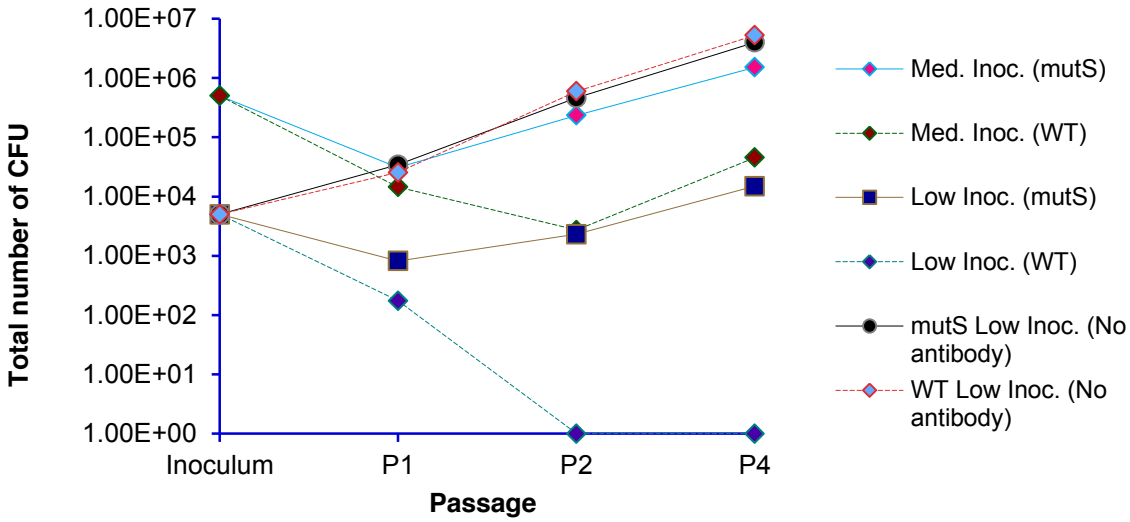

Fig: 4.12: A direct comparison of CFU count obtained after incubation of wild type 8047 and a *mutS* mutant of 8047 in the presence of monoclonal antibody P1.2. Similar sizes of inoculums from wild type 8047 and 8047 *ΔmutS* mutant were incubated in the presence of diluted antibody and passaged in the continuous assay. Serum concentration and the cycles of incubation are described in figure 4.1. Dotted lines represent viable cell counts obtained for wild type inoculum and solid lines represent the mutator phenotype. Medium inoculum, 5×10^5 CFU; low inoculum, 5×10^3 CFU.

These results indicated that 8047 *ΔmutS* population escapes from specific immune responses more rapidly than the wild type due to a higher number of pre-existing phase variants in the inoculum, resulting in a MAb P1.2 resistance due to a medium or low

level of PorA expression. The CFU count obtained from the inoculum of both parental and mutated types incubated without antibody but human serum only, demonstrated no significant difference in population structure and repeat numbers.

For lower (5×10^3 CFU) and medium (5×10^5 CFU) sizes of wild type inoculum, a difference of ~ 4-10 folds was observed in bactericidal activity, compared with results obtained in section 4.2, at the same MAb concentration. A strong bactericidal activity was observed for these inoculum sizes in those earlier experiments. This difference in activity compared to results depicted in figure 4.7, where a small sized population was totally eradicated after the first passage (Section 4.2), is either due to use of a different aliquot of antibody in this later experiment or loss of MAb activity due to storage of the aliquots at 4°C.

4.5 RESISTANCE OF 10C VARIANTS TO THE BACTERICIDAL ACTIVITY OF MAb P1.2

Earlier experiments described in section 4.1, and performed on 8047 wild type strains containing an 11C repeat tract in *porA* demonstrated that in the presence of the selective pressure of MAb P1.2, this reactive population is eradicated by the bactericidal activity of the antibody resulting in an accumulation of a comparatively less reactive but more resistant population having a medium level of PorA expression. Interestingly, after four passages, none of the phase variants isolated had 9C, which corresponded to the lowest expression level for PorA.

To determine the effect on escape from monoclonal antibody mediated killing by continuous incubation of phase variants having a medium level (10C) of PorA expression, two different inoculum sizes were analysed in the presence of concentrated and dilutions of antibody.

4.5.1 Escape of 10C phase variants from serum bactericidal activity at low antibody concentration

This assay determined the dynamics of escape of 10C phase variants in the presence of a 1:4 dilution of antibody. The continuous assay was performed on medium and low size inoculums prepared from re-growth of 10C mutants, collected in previous experiments. The CFU counts obtained for each population size are described in figure 4.13. Although not as prominent as observed for 8047 wild type (11C), a slight difference was observed in the total size of population in P4 for populations incubated with and without antibody, which may suggest a weak bactericidal activity at this altered level of expression. However the size of population remained high as compared to the inocula, which suggests resistance to bactericidal activity by these variants.

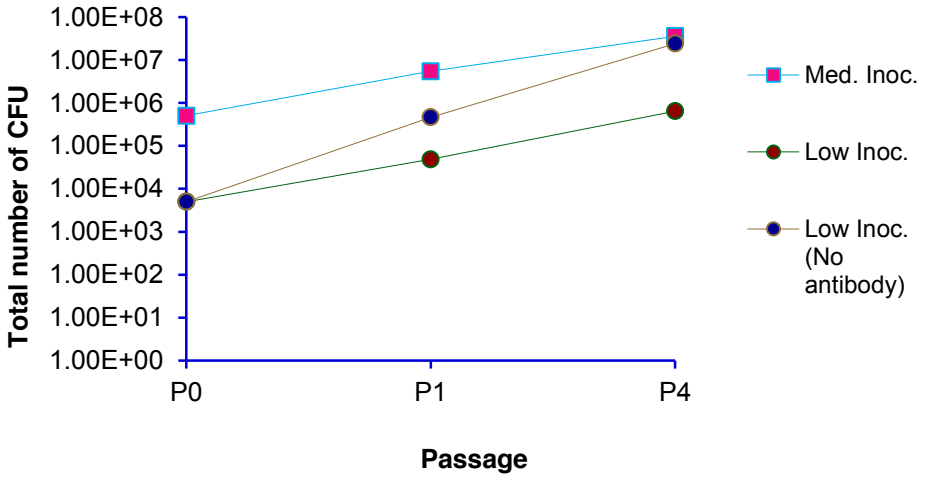

Fig: 4.13: Escape from bactericidal activity of MAb P.12 by 8047 variant having a medium level of expression (10C). The inoculum was prepared from overnight growth of a 10C variant of strain 8047 on BHI plates and incubated in the presence of 1:4 dilution of antibody. The serum concentration and duration of each incubation cycle is described in figure 4.1.The viable cell counts were obtained for inoculum and each passage and plotted on the Y-axis. Medium inoculum, 5×10^5 CFU; low inoculum, 5×10^3 CFU.

Subsequent analysis by immunoblotting and GeneScan analysis of the passaged variants showed that the entire population was comprised of medium reactive phase variants and no nonreactive variants were detected after the 4th passage (Table 4.6). These results indicated that under low antibody concentrations, the medium level of PorA expression results in escape from the bactericidal activity, which allows the growth of these 10C variants without switching to a low reactive phase (9C) of expression.

Table 4.6. GeneScan analysis of 8047 10C passaged assay. The results are representative of variants analysed from inoculum, population passaged in the presence or absence of MAb P1.2.

Detected variant[a]	Inoculum[b]	MAb selection[c] (Passage)		No antibody[d] (Passage)	
-	-	P1	P4	P1	P4
11C	0	0	0	0	0
10C	4	12	6	4	4
9C	0	0	0	0	0
Split of total colonies (n=30) analysed for inoculum, passaged and un-passaged population	4	12	6	4	4

[a] Type of variant detected from inoculum, passaged and un-passaged population.

[b] Colonies analysed from an untreated population and used as inoculum in assay. A digit represents the number of that particular variant detected (down the column) from a total number of colonies analysed for this group (bottom column). Zero represents no variant of this type was detected.

[c] Colonies analysed from a population incubated in the presence of antibody. The number and type of variants in each passage are indicated (down the column). Bottom column represents total number of colonies analysed for a particular passage.

[d] Colonies analysed from a population passaged with human serum but no antibody.

4.5.2 Escape of 10C phase variants at higher antibody concentrations

The experiment performed with a low concentration of antibody on 10C variants demonstrated a slight reduction in total population size of passaged variants compared to those without antibody. This effect was further elaborated by incubating similar sized populations (section 4.5.1) in the presence of concentrated monoclonal antibody.

The CFU count demonstrated a comparatively stronger bactericidal activity in the first passage but the population had recovered by the end of the 4th passage (Figure 4.14).

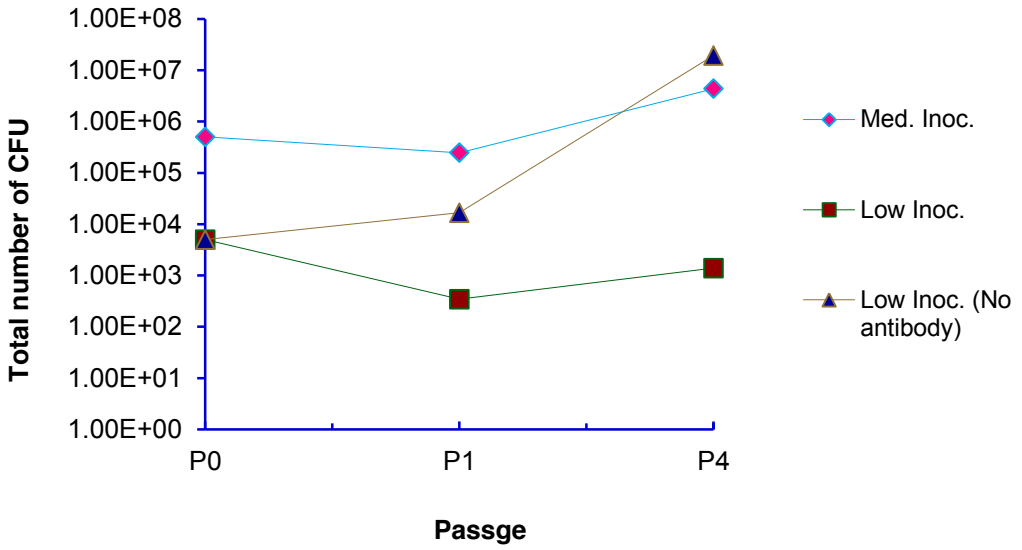

Fig 4.14: Escape of strain 8047 variants containing 10C repeat tracts, from bactericidal activity at a high antibody concentration. The inoculum was prepared from overnight growth of a 10C variant on BHI plates and incubated in the presence of concentrated antibody. The serum concentration and duration of each incubation cycle is described in figure 4.1. The legends describe the size of population tested. Viable CFU count from inoculum and passaged population are indicated by the Y-axis. Medium inoculum, 5×10^5 CFU; low inoculum, 5×10^3 CFU.

Interestingly, the immunoblots and GeneScan data (not shown) obtained after the first passage showed that the resistant population was still entirely comprised of 10C variants. However, after the 4th passage, phase variants containing 9C repeats and with the lowest PorA expression began to emerge, but at a low frequency (~1:2000). The data indicates that, under higher antibody concentrations, a medium level of expression of PorA is low enough to enable escape of killing by the bactericidal antibody but not enough to permit rapid growth. Thus the balance of growth is maintained in such a way that it resembles a bacteriostatic effect, however continuous incubation of this medium

reactive variant in the presence of specific antibody results in the accumulation of variants with expression at the lowest level.

4.6 VARIANTS WITH THE LOWEST EXPRESSION (9C) OUT COMPETE THE VARIANT WITH MEDIUM EXPRESSION (10C)

The results obtained and described in section 4.4 indicated that 10C phase variants were in a significantly higher number than 9C variants in the first passage, but eventually at the end of the 4th passage, the number of 9C variants increased significantly suggesting a selective advantage of this variant over a 10C variant. To further explore this phenomenon, a competition assay with a mixture of variants containing two different concentrations of variants was subjected to a selection with concentrated (neat) P1.2 monoclonal antibody. Medium sized inoculums (5×10^5 CFU) containing either an ~1:1 population of both 10C and 9C variants or a mixture containing an excess of 10C (9:1) variants were tested in four passages using the continuous assay. The CFU counts were obtained for the first and 4th passage of the population, which showed no apparent killing of population after incubation in the presence of antibody (not shown).

Immunoblots were performed for detection of 10C and 9C variants and used to calculate the ratio after initial and final passages. In the presence of MAb P1.2 and using a 1:1 mixture of variants, the ratio of 10C to 9C variants was reduced to ~0.082 after the first passage, and it reached a final ratio of ~0.02 after the 4th passage, indicating that 9C phase variants had outcompeted the 10C population (Figure 4.15). This competition was also obvious in the inoculum where an excess of 10C variants (9:1) was used, in this case the final ratios was decreased to as low as 0.25 by the end of eight hour continuous incubation. In both cases, a completely MAb P1.2 nonreactive population dominated by the end of the assay.

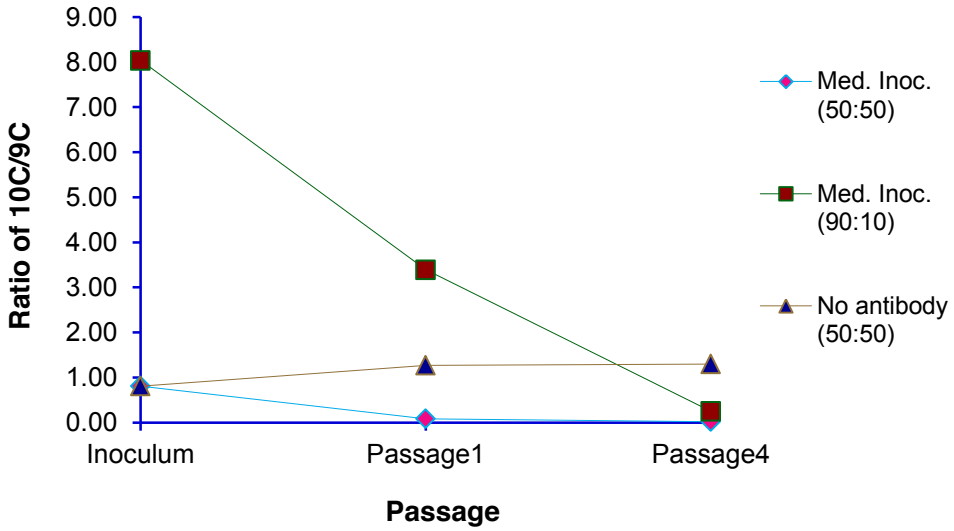

Fig 4.15: Competition assay between two different phase variants of strain 8047. Variants with medium (10C) and low (9C) expression levels were grown overnight and used for inoculum preparation. The inoculums were tested by continuous assay and escape from bactericidal activity was monitored. The inoculum contained two different mixtures of both variants and ratios are indicated in the legend. The ratios of variants for each passage were calculated from immunoblots.

The blots obtained from the population incubated without antibody maintained the initial ~1:1 ratio at the end of the 4th passage. This indicates that 9C variants did not provide a growth advantage in the absence of antibody but outcompetes the 10C when incubated in the presence of antibody. Blots were also obtained for the inoculum containing mixture as well as for both variants separately. Variants from the inoculum and from passaged populations were analysed for repeat tract lengths, and all of them corresponded with the level of expression exhibited on immunoblots. This experiment indicated that a 9C variant with the lowest level of PorA expression provides a competitive advantage over 10C, probably due to the lower reactivity of MAb P1.2 conferred by a ~2.5 fold reduction in expression of this antigen, as measured by ELISA and described in section 4.1.

4.7 DISCUSSION

Spontaneous mutations occur at very low rates reducing their usefulness for adaptation. An increase of the mutation rate (hypermutations) above the levels of normal mutations can increase the genetic diversity of a population and gears up the population for adaptation, especially when multiple mutations are needed to neutralize a number of stresses applied simultaneously i.e. host defence mechanism, antibiotic administration, co-infection etc (Jayaraman, 2011).

The role of such hypermutation of *porA* was analysed in the second part of the study and aimed at assessing the PV mediated immune evasion from a bactericidal antibody specific for this surface antigen. Such analysis is particular interest and relevant due to importance of PorA as vaccine candidate. A modified serum bactericidal/immune evasion assay was used for this purpose. Using this assay, escape from MAb B5-dependent killing due to phase variation of *lgtG* has already been established (Bayliss *et al.,* 2008). It was observed that the accumulation of pre-existing non-reactive phase variants was due to an alteration in an in-frame number of mononucleotide repeat of this gene.

The role of phase variation in immune evasion from MAb P1.2 specific for PorA has not been robustly documented, and also the validation of the modified assay for this type of escape due to differential levels of expression was required. The meningococcal population of strain 8047 was tested for validation of the immune evasion assay. This was used as a background to test the capacity for PV mediated immune escape from polyclonal antisera generated against HpuA.

In these *in vitro* experiments, we observed an accumulation of phase variants resistant to MAb P1.2 killing within ~4 h of incubation (Figure 4.1). The subsequent sequencing

and GeneScan analyses identified a resistant population of cells with an altered repeat tract lengths (10C) compared with the parental type (11C). To elucidate the mechanism for development of this resistant population with a comparatively low reactivity immunotype, parental phenotypes and a number of variants (after selection) to MAb P1.2 were examined for their repeat tract lengths. The initial population (inoculum) consisted of 11C while phase variants contained either 10C or 9C in the *porA* promoter sequences. The levels of expression for these mutants were determined by Western, whole cell ELISA and FACS. All analyses confirmed a significant level ($p < 0.0001$ as determined by ELISA and FACS or $p < 0.05$ to $p < 0.01$ determined by Western) for 10C/9C variants, compared with normal wild type (11C). This reduction in phase variable expression of PorA level compared to parental phenotype resulted in accumulation of a resistant population and was due to lower availability of MAb P1.2 target epitopes in phase variants.

These experimental findings revealed the ability of meningococcal populations to escape from antibody mediated killing due to the repeat mediated phase variation in *porA* promoter sequences, and a prolonged incubation in the presence of antibody selected for a medium expression level of PorA. The results also demonstrated how rapidly phase variants can be accumulated when a specific selection pressure is applied. These results also indicated that prolonged incubation of meningococcal strain 8047 in the presence of bactericidal antibody selects for phase variants with the lowest PorA expression level, which can mediate escape from killing by antibody.

Previous studies have identified that persistent carriage results in dramatic fluctuations in antibody titers specific for a particular surface antigen or against the bacteria (Jordens *et al.,* 2004; Robinson *et al.,* 2002). The impact of this varying level of antibody on the proportion and rate of accumulation of phase variants was investigated *in vitro* by

Bayliss and co-workers who demonstrated that the rate was dependent upon the concentration of antibody and the size of the population being tested (Bayliss *et al.,* 2008).

Inside the host, bacteria face a change in the levels of antibody from a low level following initial colonisation leading to a gradual rise in the titer as the bacterial population persists (up to 6 months). This varying level of antibody may gradually select for a population non-reactive to antibody, if the antibody is raised against a phase-variable antigen. This type of selection may drive a sensitive population to become a resistant phase-variable type and may have important implications for bacteria in their natural environment. The results herein for accumulation of non-reactive phase variants by imposing selection confirms the previous findings shown by Bayliss *et al* (2008). The results suggest that a low titer of antibody leads to a slow accumulation of *porA* phase variants in a meningococcal population with reduced expression of this antigen. The phase variants may be low in number at initial exposure but a continuous presence of antibody leads to a high number of non-reactive phase variants in the resulting population than the initial non-exposed population. These surviving phase variants, which are now found in comparatively higher numbers than the pre-exposed population, facilitate the recovery of the population during subsequent increases in antibody titer.

Another important factor influencing the escape of a population from specific immune responses is the population size being investigated. Transmission of meningococci from one host to another by respiratory droplets is likely to involve a small number of bacteria i.e. < 10,000 cells (Bayliss *et al.,* 2008). In a such scenario, due to the low basal mutation rate, it is unlikely that bacterial inocula would consist of resistant variants for a specific immune response, so inocula size would restrict the transmission of

meningococci in an already immune population. Our results in this study show that in the case of wild type a small population size of 5×10^4 CFU is sufficient to contain the phase variants required for evasion from bactericidal killing (Figure 4.1). These results suggest that phase variable antigens facilitate the survival of a small population in the presence of specific immune responses by rapidly generating resistant variants, this phenomenon has implications in the transmission of meningococci between immune individuals especially during the late phase of an epidemic outbreak.

Differing PV rates have profound impact on the adaptation and fitness of bacteria (Bayliss, 2009). The rate of PV is influenced by various factors such as mismatch repair deficiency. Mismatch repair deficient mutants with an elevated mutation rate are frequently found in natural population (Richardson *et al.,* 2002). Presence of hypermutable strains with a relatively higher frequency and phase variation rates led the authors to suggest that higher mutation rate may provide an advantage to meningococci during selection and mutators evolved in subsequent epidemic spread. These findings were interpreted as evidence for efficient transmission or fitness being associated with these increased PV rates.

We also found that a meningococcal mutator phenotype (8047 *ΔmutS*) increases the escape from specific immunity compared to wild type in our *in vitro* studies (Figure 4.11). As for many other surface antigens (Martin *et al.,* 2004; Richardson *et al.,* 2002), this mutator phenotype (8047 *ΔmutS*) elevates the PV rate of *porA* whose optimum expression is required for full scale binding of MAb P1.2. This elevated rate is likely to increase production of phase variants having a reduced expression level of PorA, thus fewer reactive epitopes available for binding leading to an escape from killing.

It has been proposed that mutator phenotypes reported for serogroup A strain epidemics (Richardson *et al.,* 2002), may accumulate due to selection imposed by specific immune responses. Thus, once a large proportion of a host population in an epidemic area develops adaptive immunity against the antigenic determinants of circulating meningococci, it would be less advantageous for bacteria to express these surface structural determinants or to have expression of these determinants at the maximum level in the presence of such a selective pressure. Absence or lower levels of expression would be favoured and would lead to a selection for these strains. If this selection continues then clones with higher or elevated mutation rates would have an advantage over their competitors. This would impose a secondary selection for a mutator clone i.e. (*mutS* mutant) which will further elevate this mutation rate and the genetic diversity of the population leading to rapid adaptation. In such a case, a mutator phenotype would be more prevalent than wild type due to a higher capacity for immune evasion. The results of this study suggest that the reason for a higher prevalence of mutator phenotypes during epidemic spread is because of elevated PV rates which helps in rapid adaptation to high levels of antigen specific antibodies.

The *in vitro* PorA experiments demonstrated that exposure of 11C variants to MAb led to the rapid accumulation of 10C variants. This mimics an *in vivo* exposure to a low concentration of antibody and may occur during the early phase of colonization. Our results demonstrate that lowering of the expression level from high to a medium level (a less reactive population to MAb P1.2) is enough to resist antibody mediated killing during prolonged exposure at low antibody concentrations (Figure 4.13). Interestingly, the population did not exhibit a shift for a further lowering of PorA expression (9C variants) indicating that at this level of selection pressure, a population with medium

expression is able to survive. This indicates that a meningococcal population can maintain a balance between the expression of this antigen and immune evasion.

In contrast, an increase in antibody concentration, mimicking the late phase of an infection does not permit the rapid growth of medium reactive (10C) variants (Figure 4.14), but rather favours the gradual appearance of variants with the lowest PorA expression (9C). Indeed, once these variants (9C) appeared, they outcompete the 10C variants at even lower ratios (1:10) when incubated together at higher antibody concentration levels. Thus, in the late phase of infection or in the epidemic spread when the population is immune, a mixed population with medium and low levels of reactivity to the phase variable antigen would accumulate. This mixed population circulating in immune individuals would increase transmissibility and adaptation to host with differing immune statuses. A clear advantage of normal PorA expression is not identified yet. However, keeping in view the function of PorA as an adhesin, a medium level of expression (10C) could maintain the function of PorA on one hand and immune evasion on other hand.

In conclusion, our results in this part of the study support the previous findings observed by Bayliss and co-workers that localized hypermutation has a significant impact on escape of selection imposed by specific immune responses. Phase variation mediated changes in expression confer evasion from adaptive immunity, which has implications for transmission, fitness and the development of vaccines targeting the PorA protein.

Chapter 5

MUTAGENESIS OF HAEMOGLOBIN RECPTOR ENCODING GENES, *hpuAB* & *hmbR,* AND SERUM GROWTH ASSAYS

Mutagenesis of both haemoglobin receptors was performed in strain 8047 and MC58 in order to use these mutants as a control for phenotypic growth assays, ELISAs and in immunoblotting for the identification of phase variants by antisera generated against haemoglobin receptor proteins. The growth assay was performed in order to evaluate the utilisation of iron sources by meningococcal strain MC58 (Wt and *hmbR* mutant) when grown in human serum treated with desferal to chelate the free iron.

5.1 MUTAGENESIS OF *hpuAB* GENE IN STRAIN 8047

Strain 8047 is an *hpuAB* and *hmbR* positive strain and sequencing of the repeat tracts of both genes suggested that the strain grown in our lab has *hpuAB* in an ON phase and *hmbR* in an OFF state (13G repeat tract for *hpuA* and 8G for *hmbR*). The *hpuAB* gene was mutated by the deletion of ~120 bp of 3'- end *hpuA* sequence and ~1.5 kb of *hpuB* 5' end sequences.

The deletion of the internal sequences was performed by an inverse PCR using the primers HA-Inv 938 and HB-Inv1 from a previously cloned full-length *hpuAB* gene in pGEMT vector system. This PCR generated a 4.8 kb fragment (Figure 5.1, Lane 2), containing both *hpuA* and *hpuB* fragments and the whole plasmid sequence. These 0.9 kb fragments of both 5' and 3'-termini sequences of *hpuA* and *hpuB*, respectively, were

kept to allow recombination and integration of the construct into the genome of strain 8047 in subsequent steps.

Both primers contained an EcoRV restriction site, which allowed the digestion and re-ligation of PCR products. The re-ligated construct was used to transform *E. coli* and minipreps were prepared.

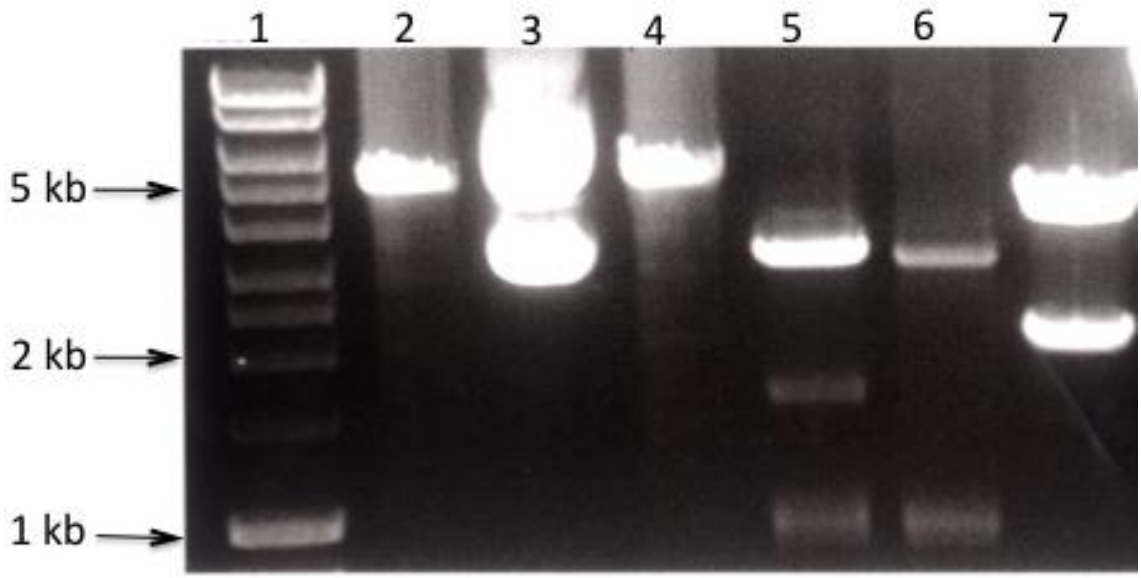

Figure 5.1: Inverse PCR product of the *hpuAB* gene and restriction digests of the p*hpuAB*-Kan constructs. PCR products, 5 µl from a 10 µl reaction, and restriction digests, 5 µl of plasmid construct was digested with the respective enzyme (s) in a total volume of 10 µl, were loaded onto a 1% agarose gel and analysed under UV. Lane 1, standard molecular weight marker; Lanes 2, inverse PCR of *hpuAB* cloned in pGEMT; Lane 3, miniprep of a re-ligated and re-transformed inverse PCR product; Lane 4, p*hpuAB*-GemT EcoRV digest; Lane 5, p*hpuAB*-GemT EcoRI and EcoRV double digest; Lane 6, inverse PCR product digested with EcoRI; Lane 7, p*hpuAB*-Kan XmnI and SpeI double digest.

Recombinant plasmid (lane 3) was digested with EcoRV to allow the insertion of a kanamycin cassette and to confirm that the restriction site had been inserted (lane 4). The presence of both *hpuA* and *hpuB* fragments was confirmed by restriction analysis of phpuAB with a double digest by EcoRV and EcoRI (lane 5). The restriction digest

released two fragments of 0.9 kb and a 3 kb fragment for the vector. An additional band of 1.8 kb was also obtained which is perhaps the fragment released from the plasmid by complete EcoRI but only partial EcoRV digestion. The size of the fragment (1.8 kb) also suggests that both fragments are intact. However, the presence of 0.9 kb fragments also confirms the presence of an EcoRV site within this fragment. The Inverse PCR product was also digested with EcoRI alone (lane 6), which released both fragments.

The EcoRV digested plasmid (lane 4) was ligated with a kanamycin cassette recovered from a HincII digestion of puc4-Kan plasmid. This fragment has blunt ends. The transformants were selected by double antibiotic selection (kanamycin and ampicillin) and recombinant plasmid DNA was isolated.

The insertion of the antibiotic cassette was confirmed by digesting the construct using XmnI and SpeI enzymes (lane 7). XmnI digests inside the plasmid and in the presence of SpeI should release an ~2 kb band of plasmid DNA and a 4 kb band containing the remaining plasmid DNA, the insert DNA (*hpuA* and *hpuB* fragments) and kanamycin resistance gene cassette. These restriction results confirmed the insertion of the antibiotic cassette in the plasmid construct.

The p*hpuAB*-Kan construct was linearized using SpeI and used to transform meningococcal strain 8047 using a standard protocol. The transformants selected in the presence of kanamycin were used for obtaining DNA preps and the mutation was confirmed by a couple of PCR amplification reactions.

In the first PCR, the *hpuAB* mutated locus was amplified using HpuA-NTERM and HpuB-CTERM primers (Figure 5.2). The amplification generated an ~3 kb sized fragments in 8047 *hpuA* mutants (lanes 5 & 6). A sample obtained from wild type 8047 was also run as a control (lane 2), which produced an ~3.4 kb fragment, a size

corresponding to an un-deleted *hpuAB* gene. The p*hpuAB*-Kan construct was also amplified (lane 3) and produced a fragment of similar size to the mutant.

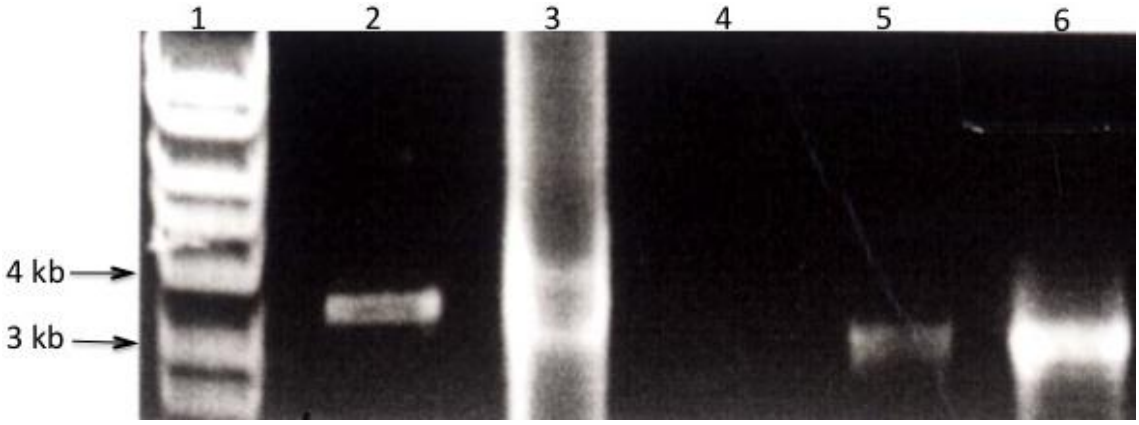

Figure 5.2: Analysis by agarose gel electrophoresis of the amplified PCR products for the *hpuAB* gene from 8047 wild type and 8047 *ΔhpuAB* mutant. PCR reaction products (5 µl) amplified from strain 8047 wild type and 8047 *ΔhpuAB* mutant using HpuA-NTERM/CTERM primers were loaded into a 1% agarose gel for electrophoresis and visualized under UV. Lane 1, standard molecular weight marker; Lane 2, wild type strain 8047; Lane 3, phpuAB-plasmid construct; Lane 4, No DNA PCR negative control; Lanes 5 & 6, two different 8047-*ΔhpuAB* mutants.

A second PCR was also performed using either HpuB-1659F/ HpuB-2356R or HpuA-end-For/HpuB-2356R primers. The first pair of primers binds inside *hpuB* and generates a 0.8 kb fragment in the wild-type 8047 strain (Figure 5.3, lane 2). However, in the mutant, no amplification is expected due to a deletion inside of *hpuB* containing the binding site for both primers. The PCR results confirmed this fragment was absent in the *hpuAB* knock out strains (figure 5.3, lane 4 & 5). The p*hpuAB*-Kan construct was also added as a control (lane 3).

The second set of primers (HpuA-end-For/HpuB-2356R) is designed in such a way that one of the primers (HpuA-end-For) binds within the *hpuA* un-deleted region while HpuB-2356R binds in the deleted region of *hpuB*. A negative PCR was observed for the

p*hpuAB*-Kan construct and mutants (Figure 5.3, lanes 7, 8 & 9, respectively), but not for wild type generating a 1.6 kb fragment (lane 6), hence confirming the mutation of *hpuAB* in strain 8047.

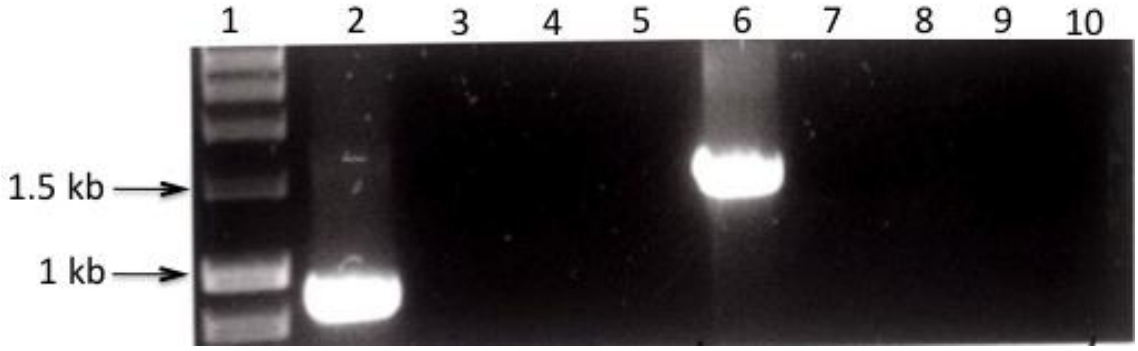

Figure 5.3: Analysis by agarose gel electrophoresis (1% agarose) loaded with of the amplified PCR products for the *hpuAB* gene from strain 8047 wild type and 8047 ***ΔhpuAB*** mutant. PCR amplification of *hpuAB* gene from wild type and mutant was performed with two different set of primers to confirm the mutation of *hpuAB* gene. 5 µl of sample from PCR reaction was loaded onto a agarose gel and visualised under UV. Lane 1, standard molecular weight marker; Lanes 2, wild type strain 8047; Lane 3, phpuAB-plasmid construct; Lanes 4 & 5, two different 8047-*ΔhpuAB* mutants; Lane 6, wild type strain 8047; Lane 7, p*hpuAB*-plasmid construct; Lane 8 & 9, two different 8047-*ΔhpuAB* mutants; Lane 10, No DNA PCR negative control. Lanes 2-5, PCR products obtained with HpuB 1659-F/ HpuB 2356-R primers. Lanes 6-10, PCR products obtained with hpuA-end-for/hpuB-2356-R primers.

5.2 MUTAGENESIS OF THE *hmbR* GENE IN STRAINS 8047 AND MC58

In contrast to *hpuAB*, the mutagenesis of *hmbR* was done by insertional inactivation of the gene. Previously, the *hmbR* gene was cloned into pGem-Teasy vector and a unique EcoRV restriction site was identified in the middle of the cloned gene. This restriction site allowed insertion of either a blunt ended kanamycin cassette or an erythromycin cassette, recovered by HincII or HaeIII digestion from puc4Kan or peR2 plasmids, respectively.

After ligation, subsequent transformation of *E. coli* and miniprep preparations from the transformants obtained after their respective antibiotic selection, the recombinant plasmid was digested with EcoRI to confirm the insertion of cassette (Figure 5.4). The digestion released a fragment of ~3.5 kb separating it from the plasmid (~3 kb).

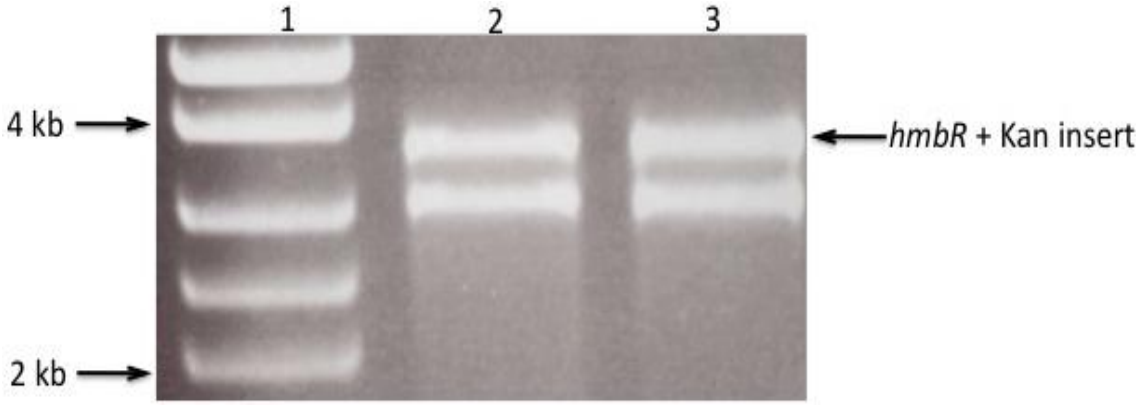

Figure 5.4: Digestion of *hmbR*-Kan construct with EcoRI restriction enzyme. A miniprep of the p*hmbR*-kan construct was purified and digested with EcoRI in a 10 µl reaction mixture. The digested products were separated on a 1% agarose gel and visualised under UV. Lane 1, standard molecular weight marker; Lane 2 & 3, EcoR1 digests of two p*hmbR*-Kan constructs.

The recombinant plasmid was linearized with SpeI and the reaction products were used to transform either MC58 or 8047 using standard protocols. The transformants were selected in the presence of the appropriate antibiotic and re-streaked to obtain the genomic DNA. A series of PCR amplification reactions were used for the confirmation of *hmbR* mutagenesis. The first PCR was performed using HmbR-NTERM and HmbR-CTERM primers, (figure 5.5). The PCR generated a 3.5 kb fragment for the p*hmbR*-Kan construct and mutant strain (lane 3 & 4 respectively). Wild type strain 8047 was also used as a control generating a 2.4 kb fragment which corresponded to the un-mutated *hmbR* gene. The difference in sizes between wild type and mutant PCR products arise due to insertion of the antibiotic cassette in mutants. A number of other

combinations of primers i.e HmbR-NTERM /hmbR-RF4, and HmbR-CTERM/hmbR-RF3 were also tested on mutants for the confirmation of *hmbR* mutagenesis (not shown). Two additional sets of PCR primers i.e. HpuA350-Rev/HpuAC and HpuAC/p26.85 were used for confirmation that all isolates were meningococci (data not shown), and to check Hpu ON-OFF status which was observed to be in an ON phase (13G repeat tract).

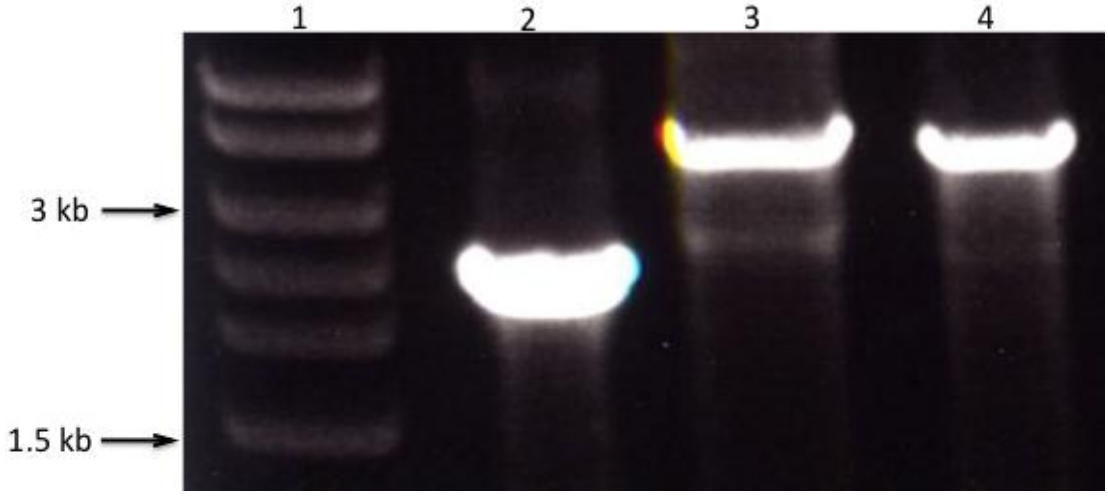

Figure 5.5: PCR amplification of wild type and 8047 *ΔhmbR* mutant strains using HmbR N- and C-terminal primers. PCR amplification of *hmbR* gene from wild type and mutant strain were amplified. A 5 µl sample of amplified product was analysed on a 1% agarose gel by electrophoresis and visualised under UV. Lane 1, standard molecular weight marker; Lane 2, 8047 WT; Lane 3, 8047 hmbR-Kan construct; Lane 4, 8047*ΔhmbR* mutant.

5.3 CONSTRUCTION OF DOUBLE KNOCKOUT STRAIN 8047 MUTANT

A double knockout mutant of strain 8047 was also constructed. For this, the genomic DNA of an 8047 *hmbR*-Ery knockout mutant was extracted and used to transform the 8047 *hpuA*-Kan mutant strains. The transformants were selected in the presence of both antibiotics simultaneously i.e. kanamycin and erythromycin. The confirmatory PCRs

described above for individual mutagenesis of *hpuAB* and *hmbR* were also performed on these transformants (data not shown).

5.4 WESTERN BLOTTING OF 8047, MC58 WT AND MUTANT STRAINS USING POLYCLONAL ANTISERA

Western blotting using polyclonal antisera recognising both proteins was used to further confirm the mutations in the haemoglobin receptor proteins. Both strains were tested separately. All mutants generated above for both strains were grown in BHI broth to mid-log phase (OD600 ~0.5) and then whole cell lysates were prepared. An anti-HpuA antiserum raised against purified recombinant HpuA from strain 8047 during this project was used to probe the expression of HpuA while an anti-HmbR antiserum (a gift from Ian Feavers) was used to probe for HmbR in strain MC58. Wild type strains were also grown and used as controls.

The HpuA antiserum clearly identified that *hpuAB* was mutated successfully, as no corresponding protein band was detected in whole cell lysates of mutants (8047 Δ*hpuA* mutant and 8047 Δ*hpuA::*Δ*hmbR* double knockout) but appeared in the wild type (Figure 5.6). Purified HpuA protein was used as a positive control to compare and locate the HpuA protein. This analysis confirmed that the *hpuAB* mutagenesis had abrogated expression of the genes and supported the restriction results and PCR confirmatory test performed during the mutant's construction.

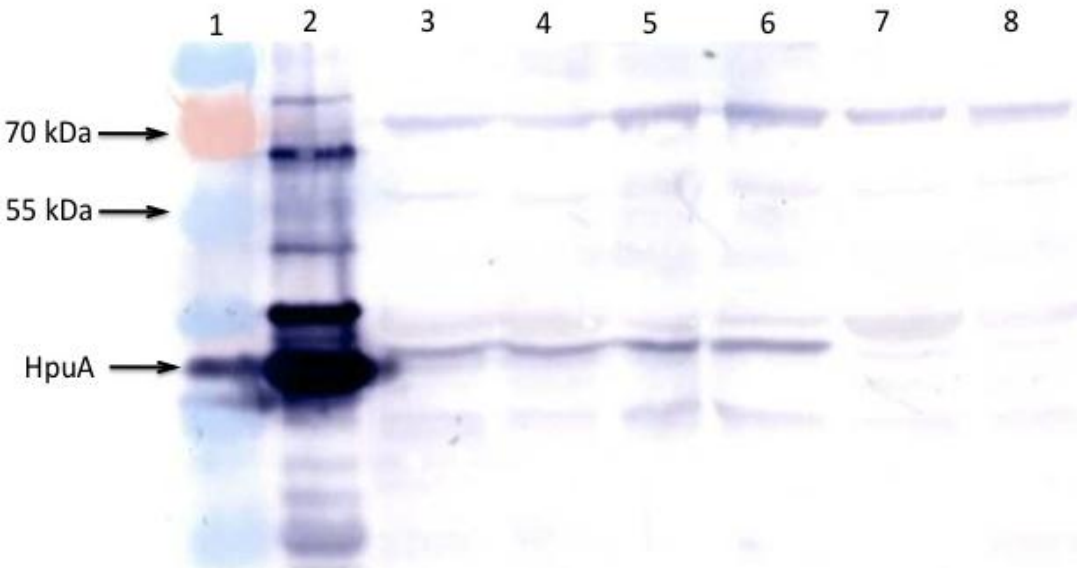

Figure 5.6: Western blot analysis of 8047 wild type and 8047 *ΔhpuA* mutants with polyclonal HpuA antibodies. 5 µl of purified protein (300 µg/ml) and 15 µl of whole cell lysate from wild type and knockouts was electrophoresed on 10% SDS-PAGE gels, transferred to a membrane and probed with a 1:500 dilution of HpuA antisera. The binding of primary antibody was detected with anti-mouse AP-conjugated secondary antibody (1:3000) and the blot was developed. Lane 1, protein ladder; Lane 2, Purified HpuA protein; Lane 3 to 6, Cell lysates obtained from wild type strain 8047; Lanes 7 & 8, cell lysates from two different 8047 *ΔhpuA* mutants. Lysates in lane 4 and 5 were obtained from bacteria grown in the presence of desferal (15 µM and 20 µM, respectively).

Similarly, whole cell lysates from (wild type and *hmbR* knockout mutants) of strain MC58 were probed against anti-HmbR antiserum (Figure 5.7). A band of ~90 kDa was found to be absent in mutants (lanes 9 to 11) but detected in wild type (lanes 2 to 8). This confirmed the successful disruption of the *hmbR* gene by insertion of antibiotic cassette in strain MC58. These mutants (8047Δ*hpuA* and 8047Δ*hpuA*::*ΔhmbR* double knockout) were also tested by the disc method to check their ability for Hb utilization (described in chapter 3). All tests confirmed the mutation of these genes when compared with wild type.

In conclusion this part of the project demonstrated a successful mutagenesis of both haemoglobin genes in two different meningococcal strains, resulting in the loss of the ability to utilize human haemoglobin when provided as the sole iron source.

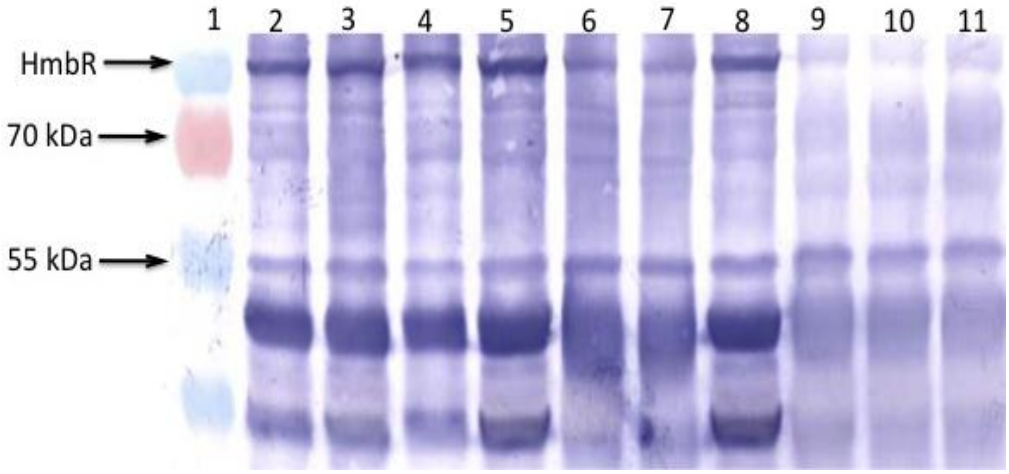

Figure 5.7: Western blot analysis of MC58 wild type and MC58 *ΔhmbR* mutants with rabbit polyclonal HmbR antisera. Whole cell lysate (15 µl) from wild type and knockout mutants was electrophoresed on 10% SDS-PAGE gels, transferred to membrane and probed with 1:100 dilutions of HmbR antisera. Anti-rabbit AP-conjugated secondary antibody (1:3000) was used to detect the binding of polyclonal antisera following addition of substrate. Lane 1, protein ladder; Lane 2-8, Cell lysates obtained from wild type strain MC58; Lanes 9 -11, cell lysates from three different MC58 *ΔhmbR* mutants.

5.5 SERUM ASSAY TO TEST THE IRON SOURCE UTILISED BY STRAIN MC58

This experiment was performed to analyse the utilization of iron source when meningococci are grown in human serum *in vitro*. This assay was a qualitative test to check the ability of wild type and haemoglobin knockouts to grow in the presence of human serum. Approximately 1×10^8 cells of meningococcal strain MC58 were used to inoculate 10 ml of PBS with a 15% serum concentration and also containing 50 µM desferal to sequester free iron. Two different control cultures were also setup. The first

control included the PBS without serum but with desferal and was inoculated with MC58 bacteria. The second control consisted of PBS, human serum (15%) and desferal (50 μM) but without bacterial inoculation. After overnight incubation, 10 μl and 100 μl samples from the cultures were plated on BHI agar and growth was analysed (Table 5.1).

Table 5.1: Growth of MC58 wild type and MC58 *ΔhmbR* mutant in the presence of human serum.

	Growth Conditions	Strain	Growth
1	PBS + serum (15%) + desferal (50 μM)	MC58 WT	Confluent
2	PBS + desferal (50 μM)	MC58 WT	No growth
3	PBS + serum (15%) + desferal (50 μM)	No Bacteria	No growth
4	PBS + serum (15%) + desferal (50 μM)	MC58 *ΔhmbR*	Confluent

The results indicated that despite the presence of desferal, strain MC58 was able to grow in the human serum indicating the utilisation of an alternate source of iron. To check a possible utilisation of haemoglobin by the strain, the experiment was repeated using an *hmbR* mutant of MC58 strain. A confluent growth was observed for the mutant, indicating that the mutant also utilised an iron source other than haemoglobin. As transferrin is the major iron bound complex in serum, it could be expected that the bacteria utilise transferrin when grown in serum. Construction of a transferrin mutant of this strain and a further experiment is required to investigate this possibility.

5.6 DISCUSSION

In this chapter, mutant strains (MC58 and 8047) lacking haemoglobin utilization genes were constructed. These mutants were required for comparison while examining the ability of their respective wild type strains to utilise Hb (see chapter 3, Hb disc assay). The mutants were also required as controls for subsequent Western, ELISA and immunoblot assays while testing the recognition of native HpuA proteins by newly generated polyclonal sera. The analysis of the mutant strains using PCR and Western blotting confirmed the successful inactivation of *hmbR* and *hpuAB* in strains 8047 and MC58. In later sections of this chapter, strain MC58 (WT) and MC58 *ΔhmbR* were investigated in order to check which iron source is utilized during growth in human serum. The results suggested that this strain can grow in the absence of free iron and Hb in serum, probably using Tf which is the most abundant source of iron found in serum. The assay also suggested that the absence of any iron source severely restricts bacterial growth, as seen by the growth assay in PBS only, and this effect looked like bactericidal activity due to the fact that no colonies were recovered after overnight incubation despite using a very high inoculum size (1×10^8 CFU).

Chapter 6

CLONING, EXPRESSION AND GENERATION OF HpuA POLYCLONAL ANTISERA

Meningococci possess two different haem-acquisition systems, HpuAB and HmbR, for extraction of iron from haemoglobin to support its growth. Iron acquisition by meningococci using these systems is important for prolonged septicaemia, as the availability of Tf (Major iron source in serum) is restricted by hypoferremic responses during late phase of infection (Holbein *et al.,* 1981; Wyngaarden and Smith, 1985). The results of this study have also provided epidemiological evidence of association of haemoglobin acquiring genes with virulence of meningococci (see chapter 3). Thus, keeping in view the importance of these genes, and importance of PV mediated immune evasion in bacterial fitness; an analysis of impact of phase variation of these genes in immune escape is relevant. This part of the project mainly focussed on HpuA (a component of HpuAB system) for such analysis.

HpuAB is a two-component system, which binds and extracts the iron not only from haemoglobin, but also its haptoglobin complexes (Lewis *et al.,* 1995; Lewis *et al.,* 1997). HpuA component of this system is a surface exposed lipoprotein and is important for ligand binding (Lewis *et al.,* 1997). The DNA sequence of *hpuA* gene contains a poly(G) region susceptible to translational phase variation (Lewis *et al.,* 1999). As *hpuB* lacks its own promoter sequence and co-transcribed with *hpuA*, a lack of expression of *hpuA* gene abolished the *hpuB* expression.

Previous studies by Bayliss *et al* (2008) have demonstrated the role of PV in immune evasion. Subsequently, the successful validation of PV mediated immune escape of

MAb P1.2 in strain 8047 was performed in this study (see details in chapter 4). This analysis was used as a rationale to generate an HpuA polyclonal bactericidal antisera and to investigate HpuA-associated PV mediated immune evasion. Cloning and expression of HpuA and subsequent polyclonal antisera generation was the first step towards this analysis. The polyclonal sera developed against HpuA were tested using modified SBA for analysis of immune evasion mediated by phase variation of HpuA in strain 8047. In addition, taking advantage of previously generated HmbR polyclonal antisera, a similar assay (immune evasion assay) was performed on strain MC58 to analyse the role of PV of HmbR.

6.1 AMPLIFICATION AND CLONING OF *hpuA* FROM STRAIN 8047

Neisserial HpuA is encoded by an open reading frame of ~1 kb coding a protein of ~37 kDa in strain 8047. For the expression of HpuA protein and subsequent production of polyclonal antibodies, the *hpuA* gene was cloned into an expression plasmid (see appendix 2 for plasmid map and the cloning site) and fused to a six His-tag to facilitate purification. A pair of primers (HpuA-NTERM and p26.85) was designed from the published genome sequences. The forward primer (HpuA-NTERM) was designed in such a way that it binds just downstream of the predicted signal peptide sequence and the poly(G) repeat tract to avoid any frame shift mutations due to changes in the repeat tract during cloning. This primer also included a four nucleotide sequence "CACC" on the 5' end for directional cloning of blunt ended PCR product. Using these primers, the *hpuA* gene was successfully amplified generating an ~1.1 kb fragment (Figure 6.1).

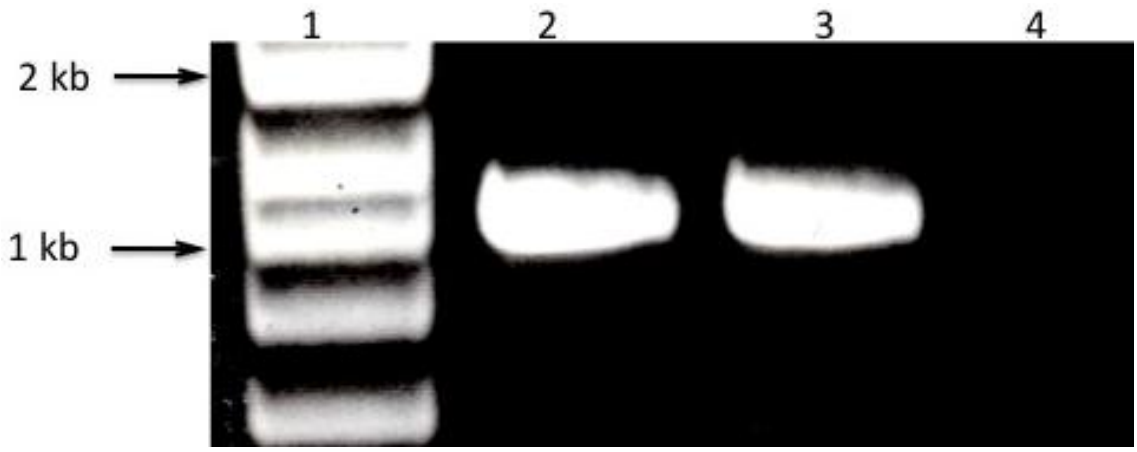

Figure 6.1: Agarose gel electrophoresis of *hpuA* gene amplified from strain 8047. Complete *hpuA* locus was amplified using 5' and 3' termini specific primers for cloning into an expression vector. A 5 µl sample from PCR reaction was loaded into the agarose gel for electrophoresis and the amplified product was detected under UV. Lane 1, standard molecular weight marker; Lanes 2-3, DNA fragments of the *hpuA* gene loaded in duplicate; Lane 4, No DNA PCR negative control.

The PCR product was purified and visualized on a 1% agarose gel to confirm the recovery of product and finally cloned into a pET-200 Directional TOPO expression vector (see figure 6.2 for p*hpuA*-pET200 expression construct). The ligation reaction mix was used to transform *E. coli* DH5-alpha and the cells were plated on LA plates containing kanamycin. Five colonies were grown in liquid media containing antibiotic and minipreps were prepared. The plasmids were digested with EcoRV restriction enzyme and the DNA bands were separated on an agarose gel to confirm the successful insertion of an ~1 kb fragment in to the vector (not shown). The p*hpuA*-pET200 construct was also sequenced to confirm that insert was in frame and no PCR mediated mutations had been introduced.

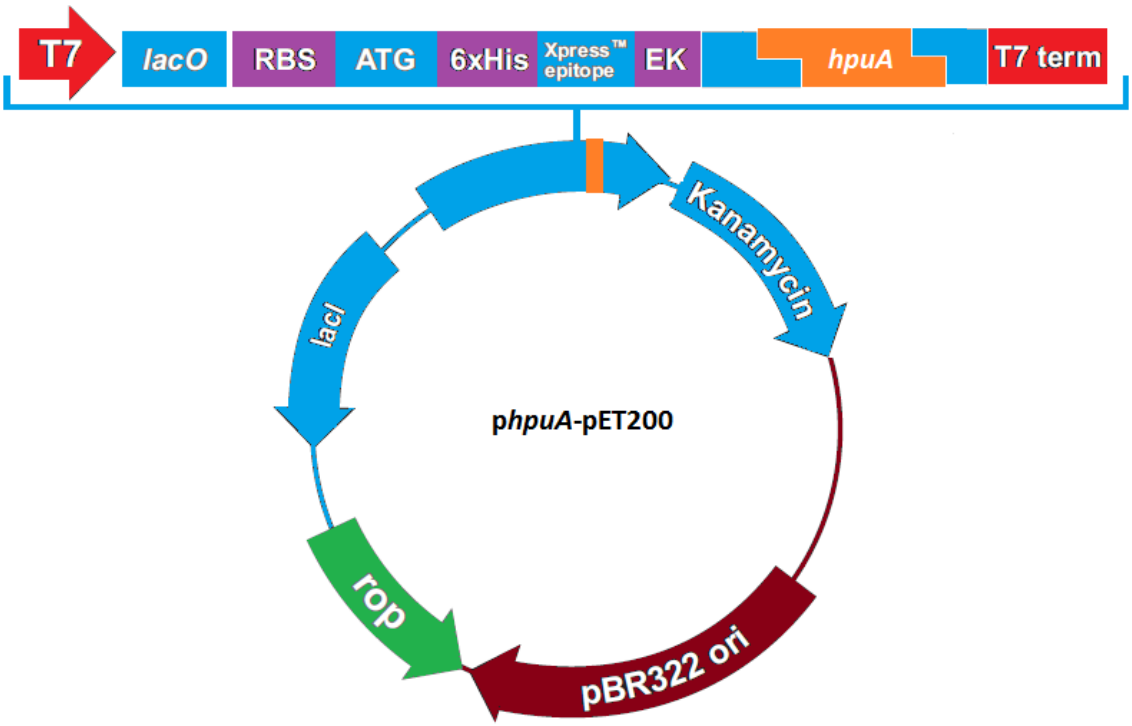

Figure 6.2: Diagrammatic representation of p*hpuA*-pET200 recombinant construct. The position of start codon, sequence encoding His-tag and enterokinase restriction site relative to cloned *hpuA* gene are indicated. (Modified map of pET200/D-TOPO, see appendix 2 for complete features of plasmid).

6.2 EXPRESSION OF HpuA PROTEIN

Expression of recombinant proteins decorated with a His-tag at either N- or C-termini is a valuable tool in studying protein structure and functions. It not only facilitates the detection and purification of recombinant protein by using immobilized Nickel affinity chromatography (IMAC), but also a His-tag adds only a few amino acids (6xHis-tag in this case) to the expressed protein which are poorly immunogenic and does not interfere with folding. This allows the recombinant protein to be used for production of antibodies with or without prior removal of tag.

6.2.1 Pilot HpuA protein expression

In a preliminary experiment, HpuA protein was expressed using *E. coli* BL21 (DE3) strain system grown in LB medium. The bacteria were transformed with pET200/D-TOPO recombinant plasmid and cultures were set up for expression. At OD600 of ~0.6, 1 ml of culture was taken and then 1 mM IPTG was added. Samples of 1ml induced and un-induced cultures were taken after every hour. From these samples collected at different time points, pellets were obtained by centrifugation and were mixed with 80 µl of 2× SDS-PAGE buffer. An equal volume of each whole cell lysate taken from boiled samples was analysed on SDS-PAGE. A protein band of the expected size (~40 kDa), which showed increased expression from 0 h induction to the end of 5 h was identified (Figure 6.3).

A control was set up containing only BL21 *E. coli* that had not been transformed with recombinant construct (Lane 2). At zero h of induction, the expression of recombinant HpuA fragment was detectable in both samples, with and without 1mM IPTG, but no visible difference between induced and un-induced was identified (Lane 3 & 4). However, after 1 h of IPTG induction (Lane 5), the recombinant protein was expressed at higher levels making it easily visible in comparison to zero hour samples. A band of comparable size was absent in the control (Lane 2) and only found in transformed cells suggesting that the induced protein is not from the host genome. The increase in amount of expressed protein is correlated with the time lapse after addition of IPTG.

The expression reached maximum level after 4 h of induction (Lane 6 to 8) as determined by the thickness of staining intensity of the bands on Coomassie stained SDS-Page gel. The staining intensity of the protein remained unchanged after 5 h (Lane 9). This suggested the optimum level of recombinant protein was obtained after 4 h. An

un-induced sample collected after 5 h (Lane 10) was also loaded to compare with those induced previously (Lanes 4 to 9). The results indicated a successful induction and expression of recombinant protein, which was absent in an untransformed or non-induced host system.

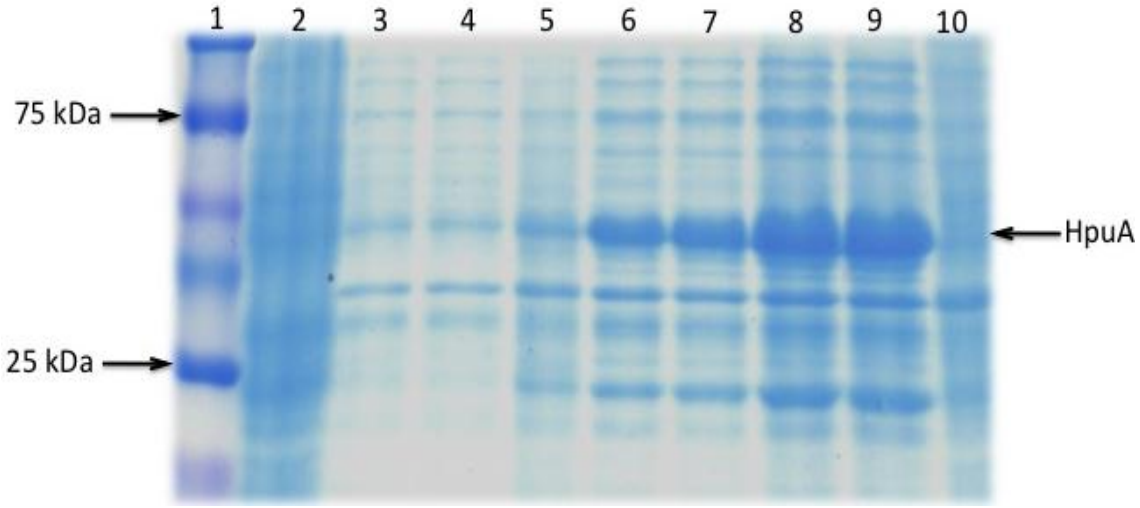

Figure 6.3: Expression of HpuA recombinant protein in the presence and/or absence of 1mM IPTG. The recombinant protein was expressed in BL21 cells from *hpuA*-pET200/D-TOPO recombinant plasmid and the cells were recovered at 0, 1, 2, 3, 4 and 5 h from induced and un-induced cultures. The cells were mixed with 100 µl of SDS loading buffer and boiled. Samples (15 µl) from cell lysates were electrophoresed on a 10% SDS-PAGE gel and analysed for the expression of HpuA recombinant protein. The recombinant protein is indicated with an arrow. Lane 1, protein ladder (Prosieve, Lonza); Lane 2, bacterial lysate taken from untransformed *E. coli* BL21; Lane 3, un-induced at 0 hr; Lane 4, induced at 0 hr; Lane 5-9, induced after 1 to 5 h of IPTG induction; Lane 10, un-induced sample after 5 h of incubation.

6.2.2 Western blot analysis of the over-expressed protein

As described earlier, the pET Topo 200 expression system adds a His-tag on the N-terminus of the recombinant protein. This stretch of engineered amino acids can be used as a target to identify the recombinant proteins, by using a monoclonal antibody, specific for this histidine tag. An equal amount of samples taken at different time

intervals after induction (1 to 5 h) were loaded onto a gel and subsequently probed by Western blotting using anti-His tag antibody (Figure 6.4).

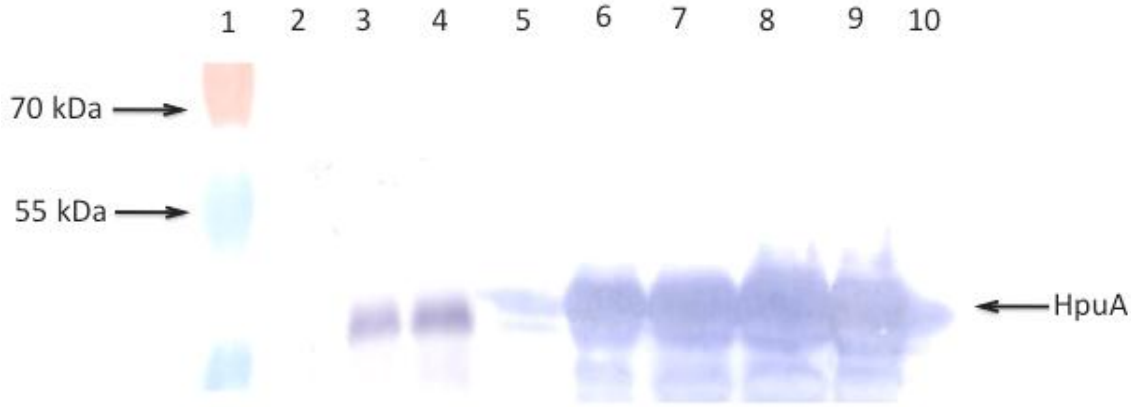

Figure 6.4: Western blot analysis of HpuA recombinant protein expressed in BL21 (DE3) strain and identified by anti His-tag antibodies. The samples and loading sequence on this gel is the same as described in figure 6.3. 15 µl of the cell lysates from indicated samples were loaded and electrophoresed on a 10% SDS-PAGE gel. The Protein was transferred onto a nitrocellulose membrane and probed with anti His-tag monoclonal antibody (1:2500). A second antibody (anti mouse AP-conjugated) was used (1:3000) to detect the binding of primary antibody and the blot was developed. Lane 1, protein ladder (pageRuler, Fermentas); Lane 2, bacterial lysate taken from untransformed *E. coli* BL21; Lane 3, un-induced at 0 hr; Lane 4, induced at 0hr; Lane 5-9, induced after 1 to 5 h of IPTG induction; Lane 10, un-induced sample after 5 h of incubation.

Cell lysates obtained from untransformed *E. coli* (Lane 2) were used as a control to confirm that the induced protein identified by PAGE did not come from the host expression system. An antibody reactive band of ~40 kDa was identified at zero h in both induced and un-induced samples with the same intensity (Lane 3 and 4). However, the intensity and strength of the signals increased after addition of IPTG indicating the induction of a His-tagged recombinant protein (Lanes 5 to 9). This intensity remained unaltered in un-induced samples collected after 5th h (Lane 10) of incubation. Protein bands of lower molecular weight can be attributed to the degradation of recombinant protein. These results demonstrated that a His-tagged recombinant protein was

expressed successfully in *E. coli* BL21 expression system and that the band identified by PAGE (Figure 6.3) was highly likely to be the recombinant His-tagged HpuA protein.

6.3 LARGE-SCALE PRODUCTION AND PURIFICATION OF RECOMBINANT HpuA PROTEIN

Once the optimal expression conditions and identity of the protein was confirmed, large-scale production of the protein was performed. The protein was expressed in a larger volume (500 ml) of media and induced by adding IPTG, as described in the pilot expression experiments.

Purification of recombinant His-tagged HpuA protein from cell lysates was performed. IMAC-Ni Sepfast columns were used for the purification of recombinant protein from the mixture of host proteins. The sample flow through, and column wash with varying concentrations of imidazole were collected and run on a 10% SDS-PAGE gel followed by Coomassie staining under reducing conditions to determine the optimum concentration of imidazole required for elution and to confirm the recovery of protein (Figure 6.5).

Analysis of each fraction demonstrated that the recombinant protein has a high affinity for the Ni column and did not elute at low imidazole concentrations (20 mM and 50 mM, Lane 3 and 4 respectively), though unbound impurities were eluted. The recombinant protein only began to elute at 100 mM imidazole concentration (Lane 5). This wash also removed some impurities that persisted after low imidazole washes. The recovery of protein remained low from 120mM to 200 mM imidazole elution fractions (Lanes 6 & 7). Maximum protein was recovered at 250 mM imidazole concentration (Lane 8), although the recovery with a doubled concentration (500 mM) was also

prominent (Lane 9). Besides the high concentration of recovered protein at high imidazole concentrations, a low concentration of impurities were also observed which were removed by an additional purification step described in a later section 6.5 (His-tag removal). The fractions obtained at 200 mM, 250 mM and 500 mM (lanes 7, 8 and 9) were pooled together and subjected to buffer exchange (20 mM phosphate buffer, pH 7.4) using centrifugal columns (Millipore, 30 kDa cut out size).

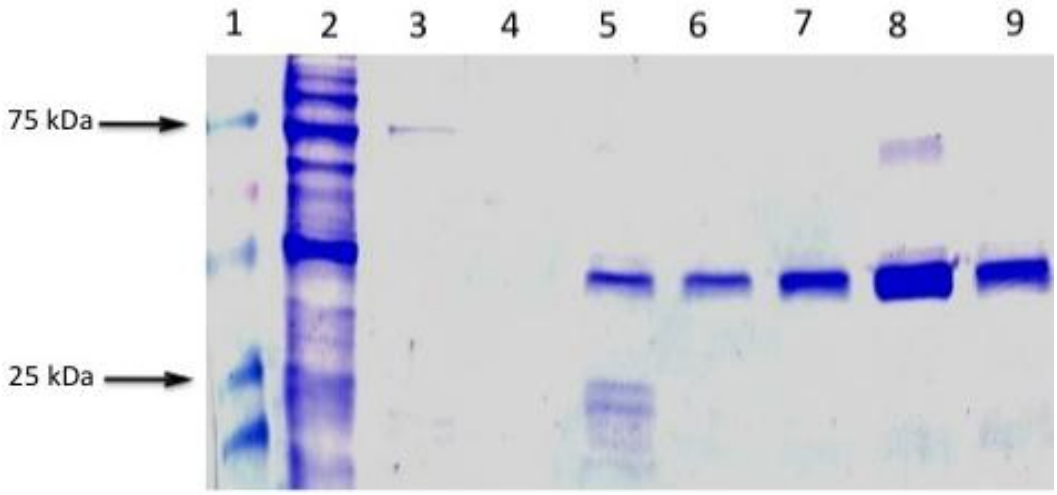

Figure 6.5: Purification of recombinant HpuA protein using IMAC Ni column. Induced *E. coli* BL21 cells were disrupted by a combination of methods to release the recombinant protein. Clarified lysates were loaded into a Ni column for separation of recombinant protein from impurities. The Ni bound recombinant HpuA protein was eluted using different concentration gradients of imidazole. A 15 µl sample from each fraction was run on a SDS-PAGE gel (10%) and stained with Coomassie stain. Lane 1, Protein Ladder (Prosieve); Lane 2, flow through; Lane 3, 20 mM imidazole wash; Lane 4, 50mM wash; Lane 5-9, elution fractions taken with 100 mM, 120 mM, 200 mM, 250 mM and 500 mM, respectively.

A low concentration of recombinant protein in the flow through (Lane 2) was also detectable and can be explained by overloading the column and resulted in the elution of protein which had not bound to the column. The protein in the flow through was recovered in a separate experiment by re-running it on Ni column and eluted again using 250 mM imidazole in a phosphate buffer.

6.4 WESTERN BLOT TO CONFIRM THE PROTEIN PURIFICATION AND MATRIX-ASSISTED LASER DESORPTION/IONIZATION (MALDI)

The identity of the purified 40 kDa recombinant protein was further confirmed by Western blot analysis using mouse anti His-tag monoclonal antibodies, as described in section 6.2.2. The samples were loaded in the same sequence as described in figure 6.5, except that lysate from untransformed *E. coli* was also loaded (Figure 6.6, Lane 1). High levels of reactivity were observed for the recombinant protein in the 100 mM to 500 mM eluted fractions (Lanes 5 to 9) and in the flow through (Lane 2) confirming the results obtained and described in figure 6.5.

A closer look at the results obtained from a Western blot of the eluants demonstrated a combination of two bands of similar sizes (Lanes 5 to 9), rather than one unique band expected for the recombinant protein. In addition, comparatively low intensity bands of higher molecular weights were also detected in Western blots of the samples collected at higher imidazole concentrations (Lane 7 to 9). On re-examining the Coomassie gel (Figure 6.5, visible in lane 8 and 9), a band of slightly low intensity found to be fused with the high intensity band indicating that both are of similar sizes. Similarly, a high molecular weight band was also observed on the Coomassie gel (Figure 6.5, most prominent in lane 8).

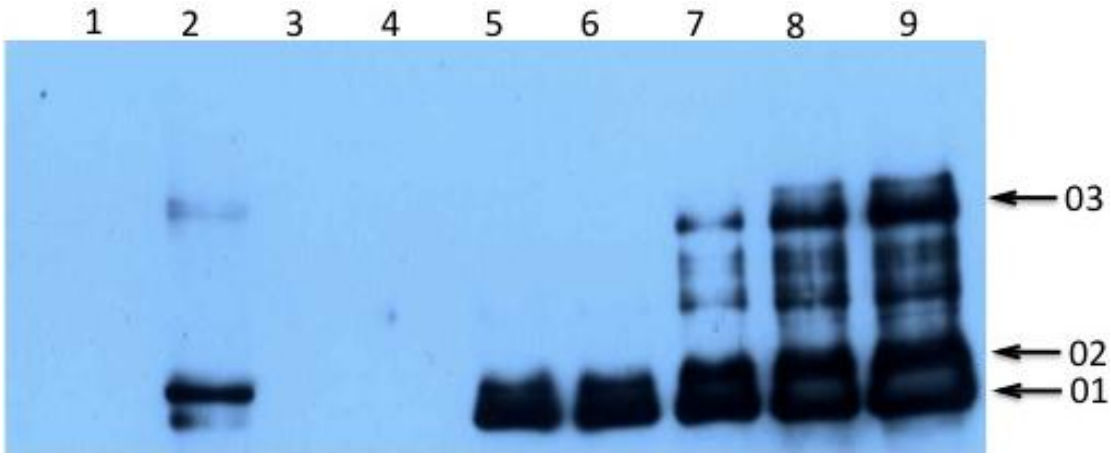

Figure 6.6: Western blot analysis of purified fractions using anti His-tag monoclonal antibody (1:10,000 dilution). A sample of 10 µl purified protein from each fraction, depicted in figure 6.4, were separated on a 10% SDS-PAGE gel and transferred to nitrocellulose membrane. The transferred protein was probed with anti His-tag monoclonal antibody (1:10,000) conjugated with HRP. The blot was treated with appropriate dilution of ECL solution and the signals were recorded on an X-ray film. Lane 1, Untransformed *E. coli* BL21; Lane 2, Flow through; Lane 3, 20mM imidazole column wash; Lane 4, 50mM wash; Lane 5-9, Elution fractions taken with 100mM, 120mM, 200mM, 250mM and 500mM, respectively.

The identity of these bands was examined by matrix-assisted laser desorption/ionization (MALDI-TOF) mass spectrometry, to confirm the differences or similarities of bands with each other. A total of three bands were analysed, as mentioned by their respective numbers in figure 6.6. The protein bands were digested with trypsin and peptide mass fingerprint was recorded for each band by the PNACL staff, University of Leicester using Voyager DE STR (Applied Biosystems). The peptide mass tolerant was kept at 50 ppm and maximum one missed cleavage was allowed. The database search was done against the translated HpuA sequence of strain 8047 HpuA in MASCOT and a total of 11 mass values for band 01 and 26 values for band 02 were searched. Both bands, 01 and 02, were identified as the strain 8047 HpuA protein, as the spectra of both samples and MASCOT search results confirmed that both have peptides that match with each

other and with the peptides predicted using the translated sequence of HpuA from strain 8047 (Figure 6.7).

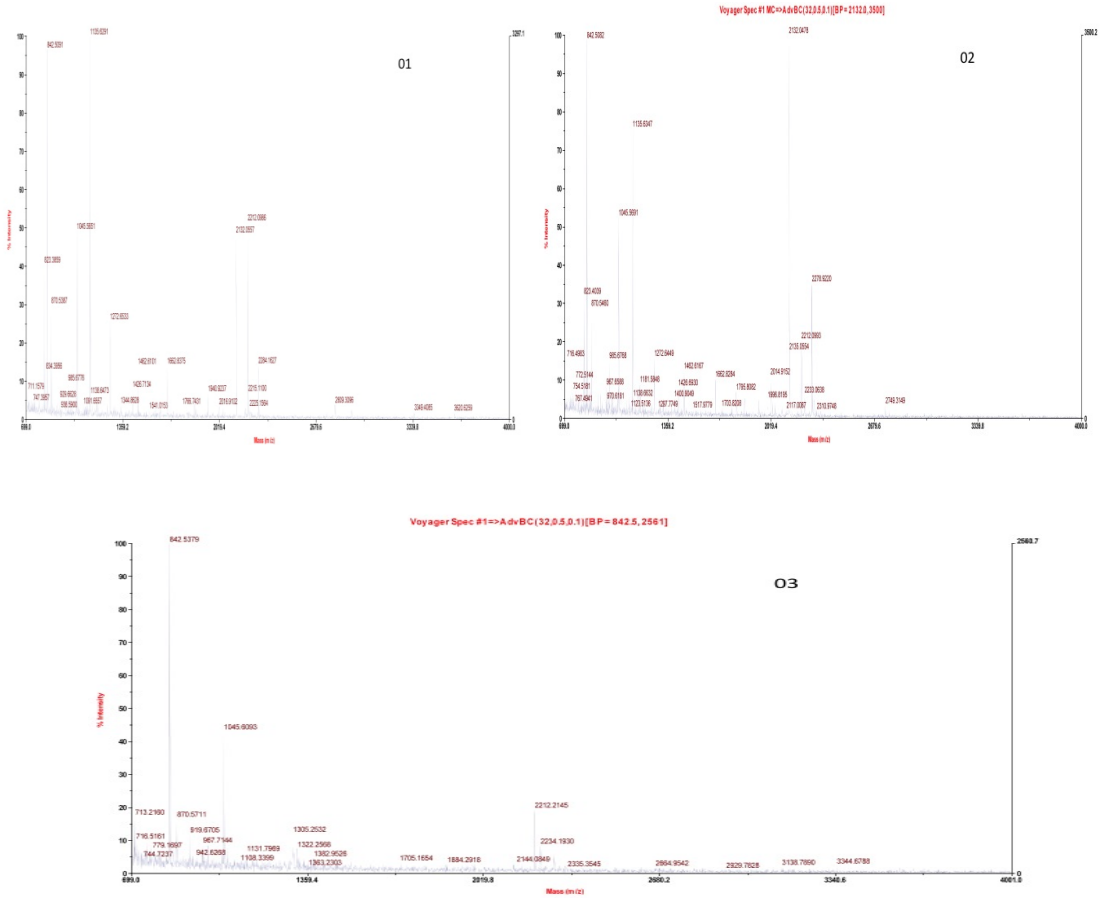

Figure 6.7. MALDI-TOF mass spectrometry of the HpuA protein samples. The recombinant protein was purified using Ni-Sep fast BG resin in a gravity flow column and 10 µl of sample was run on a 10% SDS-PAGE gel. Three protein samples indicated as 01, 02 and 03 on figure 6.5 were taken from the Coomassie stained gel, digested with trypsin followed by peptide mass fingerprint to acquire mass to charge (m/z) data for each band. The individual peaks on 01 and 02 spectra correspond to the HpuA peptides matched with the 8047 HpuA amino acid sequence ($p < 0.05$). The peaks on 03 spectra matched with the trypsin peptide sequence only, hence no HpuA peptide detected. X-axis mass (m/Z), Y-axis intensity (%).

A high score (>100) was recorded (protein score greater than 50 are significant, $p < 0.05$) and the fragments matched throughout the protein covering 56% of the total

sequence. Band 03 was not identified on the spectra and the peaks present correspond to trypsin peaks, which could mean that there was not enough protein or the protein (fragment) was small and the peptides were not detected. However, the binding of band 03 and other fragments of larger and/ or lower molecular weights with anti-His-tag antibody leads to speculation that these products are likely to be the aggregates or split products of the HpuA protein. In this scenario, one can expect that they have the same affinity for the Ni column and so are eluted at the concentration of imidazole optimum for recombinant protein.

6.5 HIS–tag REMOVAL TO ATTAIN HIGHER PURITY OF THE HpuA PROTEIN

Considering the small levels of impurities as aggregates or split products of recombinant protein, and also keeping in view that antibodies will be mainly raised against the major antigen, the recombinant protein was pure enough to immunize animals for the production of polyclonal antibodies. In addition small impurities can also be neglected due to the fact that if these are from *E. coli*, then in theory, antibodies to the impurities should not react with neisserial proteins.

However, to reduce the risk of contamination and subsequent cross reactivity by polyclonal antisera, the samples were subjected to a re-run on a Ni column after His-tag removal to attain the highest possible levels of purity. This experiment was based on the observation that the impurities have a similar affinity for the Ni column as that of the recombinant protein and only elute at high imidazole concentrations. As the binding affinity of recombinant protein is due to the His-tag on its N-terminus, removal of the tag should theoretically allow the recombinant protein to pass through the column

without binding whilst the impurities due to their affinity for Ni should be retained on the column. This step should yield a comparatively high purity protein.

The His-tag of the recombinant protein was removed by treatment with enterokinase, which cleaves the tag added by the pET-Topo expression system (see figure 6.2 for EK site relative to cloned gene). The reaction mixture was re-run on a Ni column and the flow through was collected. The protein was further purified using FPLC to remove any remaining contaminant. The samples were analysed on a SDS-PAGE gel for visual comparison with that of the His-tagged recombinant protein (Figure 6.8, lane 2 & 3).

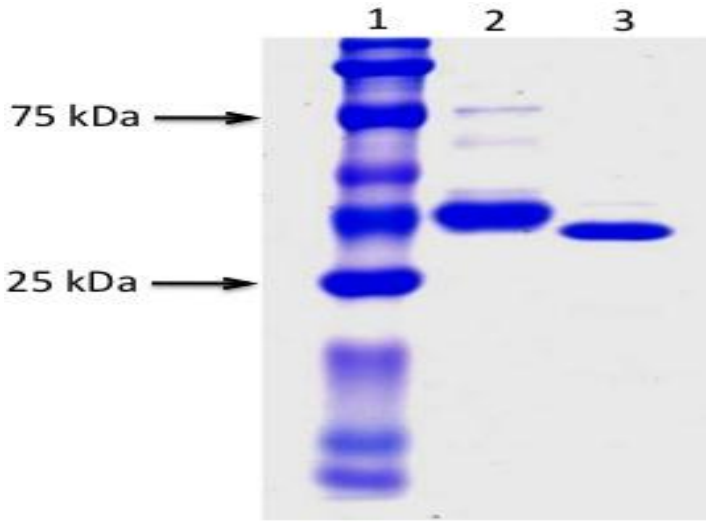

Figure 6.8: Purified His-tagged and His-removed HpuA protein electrophoresed on 10% SDS gel and stained with Coomassie stain. Recombinant HpuA protein was digested with enterokinase (0.00016 µg per 25 µg of recombinant protein) overnight. The digested product was re-run through Ni column to obtain high purity HpuA fractions. A 5 µl of sample from His-tagged (~700 µg/ml stock) and His-removed (~330 µg/ml stock) purified HpuA protein was loaded for comparison and run on a 10% SDS-PAGE gel. The difference in sizes between both samples is due to removal of His-tag, leaving the protein 3 kDa smaller than the His-tagged protein. Lane 1, protein ladder; Lane 2, His-tagged HpuA protein; Lane 3, His-removed HpuA protein.

This step demonstrated that most of the impurities were removed from the recovered protein. The degree of purification was likely to be sufficient to immunize animals for

production of antisera against the HpuA protein. A difference in size of the protein was due to the removal of the His-tag which left the remaining protein 3 kDa smaller than the His-tagged recombinant protein.

6.6 GENERATION OF POLYCLONAL ANTIBODIES AND PRELIMINARY ELISA TESTS AGAINST PURIFIED HpuA PROTEIN

For the production of polyclonal antibodies against HpuA, three mice designated as M1, M2 and M3 were immunized with purified HpuA protein (300 µl from a 1 mg/ml stock). Animals were boosted and after the 3rd week of immunization, serum samples were collected to test the reactivity of antisera against HpuA using ELISA. Two animals (M1 & M3) showed a high and equal titer against HpuA antigen while M2 showed a slightly lower level of reactivity (Figure 6.9).

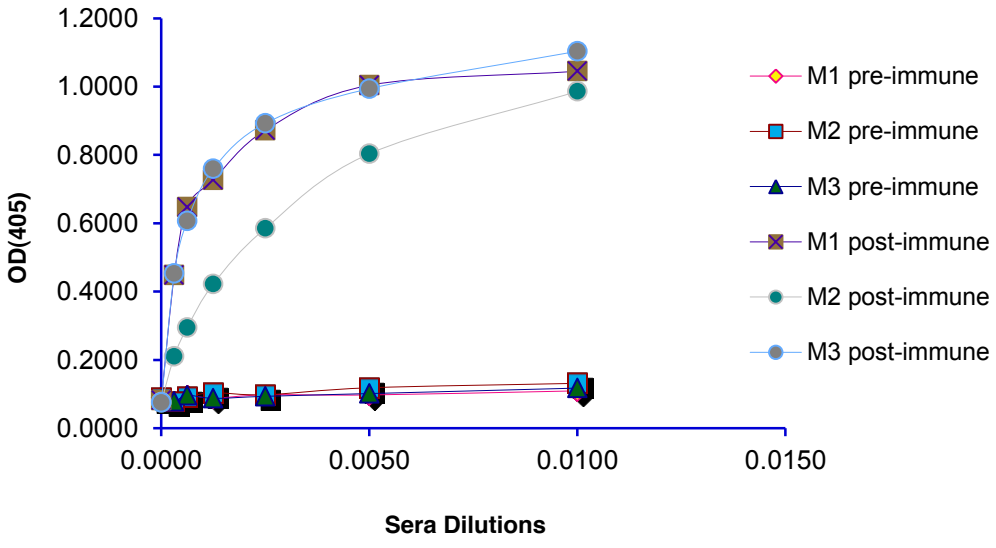

Figure 6.9: ELISA with polyclonal sera from mice (M1, M2 & M3) immunized with HpuA recombinant protein. The ELISA plates were coated with100 µl of HpuA recombinant protein (5 µg/100 ul) and probed with HpuA polyclonal antisera. A 1:2000 dilution of secondary antibody (anti-mouse AP conjugated) was used to detect the binding of polyclonal antisera. The ELISA assays were performed in duplicate for each dilution and the results are an average of these readings.

Pre-immune sera collected previously from each mouse were used as a control. The antisera reacted with purified protein at a concentration as low as 1/3200 with an OD405 = 0.45 measured after one minute of substrate addition, confirming that the antisera recognised the recombinant protein with a high titre. Two animals with high titer were boosted again as the third mouse did not survive. The antisera from the boosted animals was collected and re-screened by ELISA (Not shown) against purified HpuA protein and stored for further experiments.

6.6.1 Western blot analysis to test reactivity of polyclonal sera against purified protein

The recognition of purified HpuA protein by polyclonal antisera was confirmed using Western blots. The purified HpuA protein (5 µl from a 300 µg/ml solution) was mixed with SDS loading buffer and run on an SDS-PAGE (10%) gel, followed by probing of blots by Western with mouse polyclonal antisera (Figure 6.10). The antisera reacted to purified protein with high specificity at a 1:1000 dilutions (Lanes 3 & 4). Whole cell lysate (10 µl) obtained from untransformed *E. coli* BL21 strain was also loaded as a control and to check the cross reactivity of polyclonal antibodies. The antisera did not react with the *E. coli* proteins (Lane 2) demonstrating that it was specific for the HpuA antigen. Only the M1 mouse antiserum was tested by Western, while other antisera were tested by ELISA.

6.6.2 Western blot on iron-induced and un-induced lysates of *N. meningitis* strain 8047 with HpuA polyclonal antisera

The experiments described above demonstrated recognition by the polyclonal antisera of the purified HpuA protein, which was used to immunize the mice. Recognition by these antisera of native 8047 HpuA protein was tested in this experiment. This assay

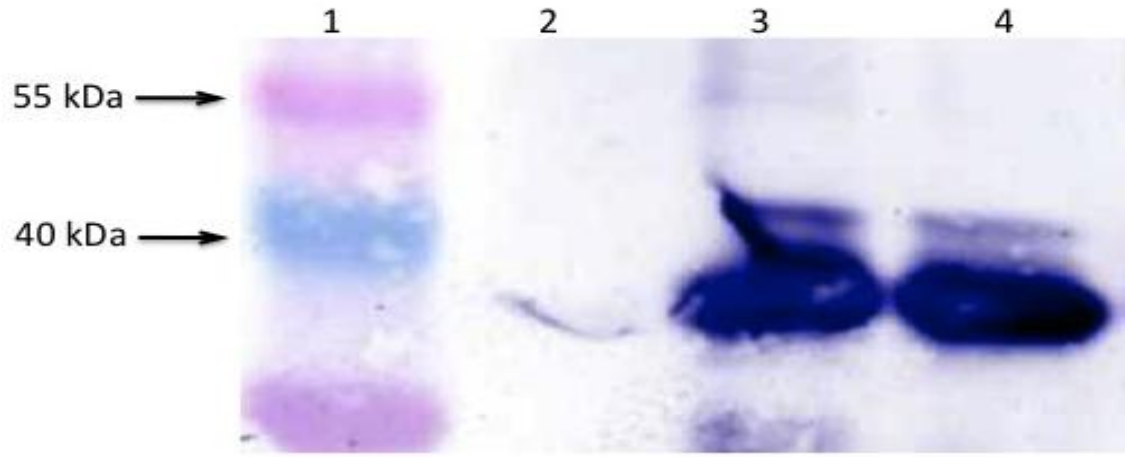

Figure 6.10: Western blot analysis using purified recombinant protein (His-tag removed) with polyclonal HpuA antibodies. Purified HpuA protein (5 µl) and whole cell lysate (15 µl) from untransformed *E. coli* strain BL21 were electrophoresed on a 10% SDS-PAGE gel. The protein was transferred to a nitrocellulose membrane and probed with 1:1000 dilution of polyclonal antisera collected from mouse (M1). The binding of polyclonal antisera was detected with anti mouse AP-conjugated secondary antibody (1:3000). Lane 1, protein ladder; Lane 2, untransformed *E. coli* BL21; Lane 3-4, purified HpuA protein loaded in duplicate.

provided two important types of information. (i) Do anti-HpuA polyclonal antibodies recognize the HpuA receptor protein produced by strain 8047 in a mixture of other neisserial proteins? (ii) If so, does growth of strain 8047 in an iron restricted environment induce the expression of HpuA?

Wild type strain 8047 was grown in either MHB which contains iron, at a low concentration but enough for bacterial growth, or MHB containing desferal with a final concentration of 15 µM, 20 µM and 30 µM to chelate the free iron. Human transferrin loaded with iron (Sigma) was added to the tube containing 30 µM desferal. An *hpuA* knockout mutant was also grown in the presence of desferal and Tf as a control. Equal numbers of cells (1×10^9 cells) from all tubes were centrifuged and mixed with 100 µl SDS loading buffer followed by boiling. An equal amount (20 µl) of each sample was loaded on to the gel and a Western blot was performed using the polyclonal HpuA

antisera (Figure 6.11). Purified HpuA protein was used as a positive control and to identify the HpuA protein in whole cell lysates (Lane 2).

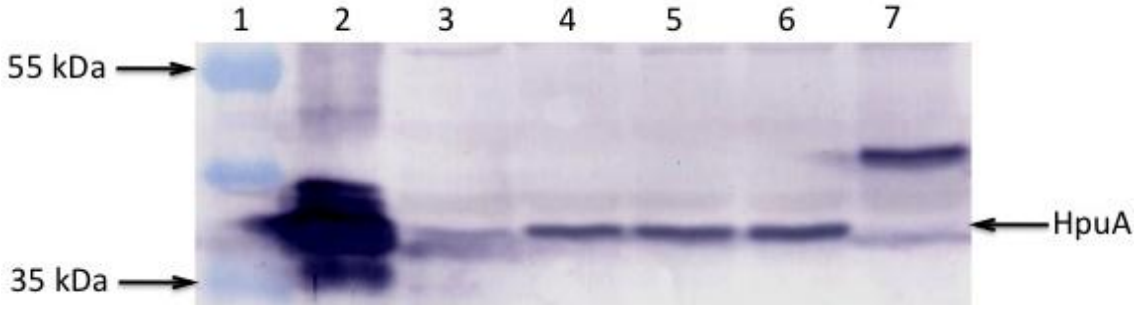

Figure 6.11: Expression and induction of HpuA in wild type and mutant meningococcal strain 8047. Whole cell lysates from cultures induced in the presence of either 15 µM and 20 µM desferal only or 30 µM desferal with human transferrin were separated by SDS- PAGE on a 10% gel, transferred to a nitrocellulose membrane and probed with the HpuA polyclonal antisera (1:1000 dilution). Cell lysates from un-induced strain 8047 and mutant 8047 (induced) strain were run for comparison. Purified HpuA protein was also loaded for confirmation of size of the protein. An arrow indicates the band corresponding to HpuA. Lane 1, protein ladder; Lane 2, purified protein; Lane 3, un-induced strain 8047; Lane 4, induced 8047 (15 µM desferal); Lane 5, induced 8047 (20 µM desferal); Lane 6, induced 8047 (30 µM desferal + Tf); Lane 7, induced 8047 *hpuA* mutant (30 µM desferal + Tf).

The results demonstrated that in the absence of induction, HpuA was detected at very low levels (lane 3). However, when iron restricted conditions were imposed, HpuA was up-regulated, and was detected at high levels (Lanes 4, 5 and 6 respectively). A band of identical size was absent in *hpuA* knockout mutants (Lane 7). However, an additional band in the mutant lane was also observed. The identity of this band is un-clear and may have resulted from fusion of *hpuA* and *hpuB* fragments in mutants due to shedding of resistance gene cassette, as mutants were not grown in the presence of antibiotic. Cross reactivity against other 8047 proteins was also evident, an issue often associated with the use of polyclonal antibodies. The results confirmed that polyclonal antibodies

recognize the HpuA antigen and also that the HpuA is induced in strain 8047 under iron limited conditions.

6.6.3 Whole cell ELISA against induced and un-induced strain 8047 with HpuA polyclonal antibodies

After confirmation by Western blot that the polyclonal antisera recognized the native HpuA protein in strain 8047, an ELISA assay was performed to correlate the whole cell protein to that of surface expressed protein in both induced and uninduced cells. The cells were grown either in MHB without induction and/or in the presence of varying concentrations of desferal to induce the expression of this surface receptor. An *hpuA* knockout 8047 mutant was also grown under induced conditions as a control (30 μM desf. + Tf). After overnight growth the cells were fixed with 0.05% formalin and then the OD550 of all samples was adjusted to 0.5 and used to coat the wells of an ELISA plate. The ELISA was performed using a range of HpuA antisera dilutions, then probed with an anti mouse AP-conjugated secondary antibody and developed with the substrate to obtain the OD405 (see figure 6.12).

The results indicated that a low level of surface expression of HpuA was detected in uninduced cells, but when cells were grown in the presence of 15-20 μM desferal, the binding of polyclonal antibody was increased about two fold suggesting induction of surface expression of HpuA. The surface expression level was also 2.5 fold higher when uninduced cells were compared to cells grown at a higher level of desferal (30 μM) and with added transferrin (100 μg/ml). However, a slight binding of antisera with mutants was also detected which showed similar OD as that of uninduced wild type cells. This may arise due to a difference in growth conditions (mutants are grown under iron limited conditions while un-induced wild type in excess of iron). The difference in

levels of expression (2 fold) between induced wild type and mutants grown under same conditions (30 µM desf. + Tf) also suggested that the reactivity in mutants is non-specific and that the fusion protein observed in figure 6.11 is not surface expressed. Otherwise the difference (2 fold) between two should be far less, or may expect higher for mutant keeping in view the strength of band in Western blot. This observation (that fusion protein is not surface expressed) is also supported by later experiments (see figure 6.12).

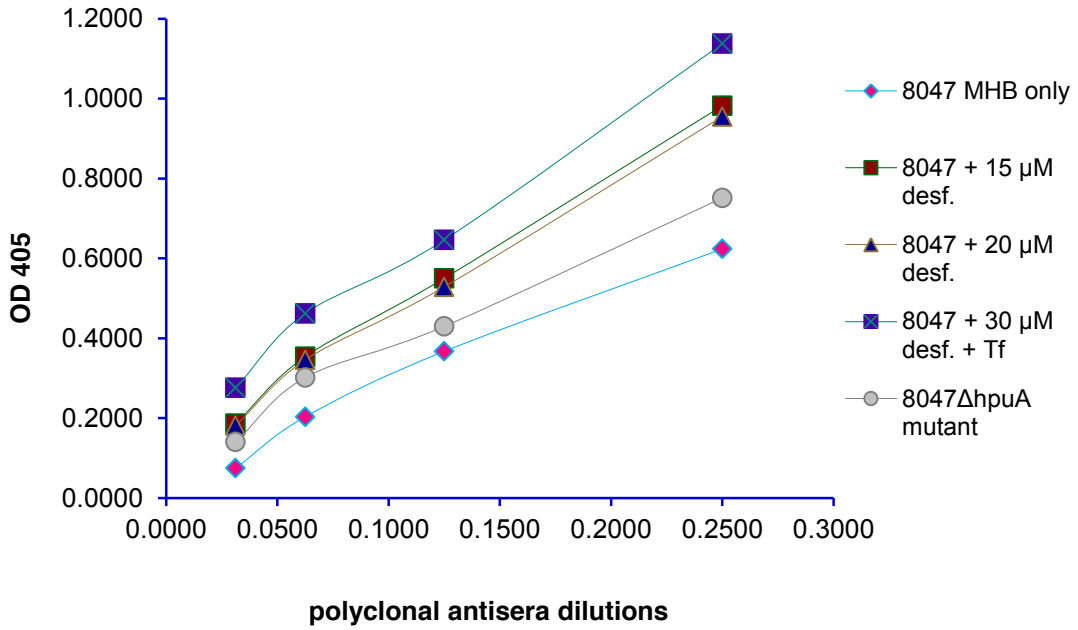

Figure 6.12: Surface expression of HpuA analysed by whole cell ELISA for meningococcal strain 8047 (WT and mutant) grown in the presence or absence of iron. Whole cells were obtained from the indicated growth conditions, fixed in coating buffer containing formalin (0.05%) and OD550 of cells was adjusted to 0.5 in ELISA coating buffer. The ELISA plate was coated with 100 µl/well of the samples. Anti-HpuA polyclonal antibody was used (1:100) for the detection of surface expression and induction of HpuA. The binding of antisera was detected with secondary antibody (1:2000) and developed by substrate. The results are the mean OD of samples run in duplicate.

Another explanation is that during preparations of cells for ELISA, the extensive washing and mixing by aggressive pipetting, may break a few cells and fusion protein may leak out which slightly lifts the OD compared to the uninduced cells. Another potential reason is that as the polyclonal antisera is cross reactive to many proteins, if it weakly reacts with any surface expressed inducible protein which may result in a slight difference in OD compared to un-induced wild type cells. These results correspond to those observed in the Western blot (Figure 6.11), and indicate that the surface expression of HpuA is induced in iron-limited conditions and is detectable with polyclonal antibodies by ELISA.

6.6.4 Colony immunoblotting of wild type strain 8047 and/or 8047 *hpuA* knockout mutant under low iron conditions

The reactivity of HpuA polyclonal antisera against strain 8047 was also tested by colony immunoblotting. Both wild type and *hpuA* knockout 8047 mutants were either streaked side by side or appropriate dilutions of both strains were spread on the same MHA plate to obtain single colonies (Figure 6.13 A & B respectively). The expression of the HpuA receptor was induced by growing the bacteria in iron-limited conditions (15 μM desferal). The results demonstrated that the polyclonal antibodies reacted with wild type strain 8047 but not the isogenic strain lacking the *hpuA* gene, indicating that the sera were specific for surface expressed HpuA protein. The result also demonstrate a lack of surface expression of HpuA protein in the mutant.

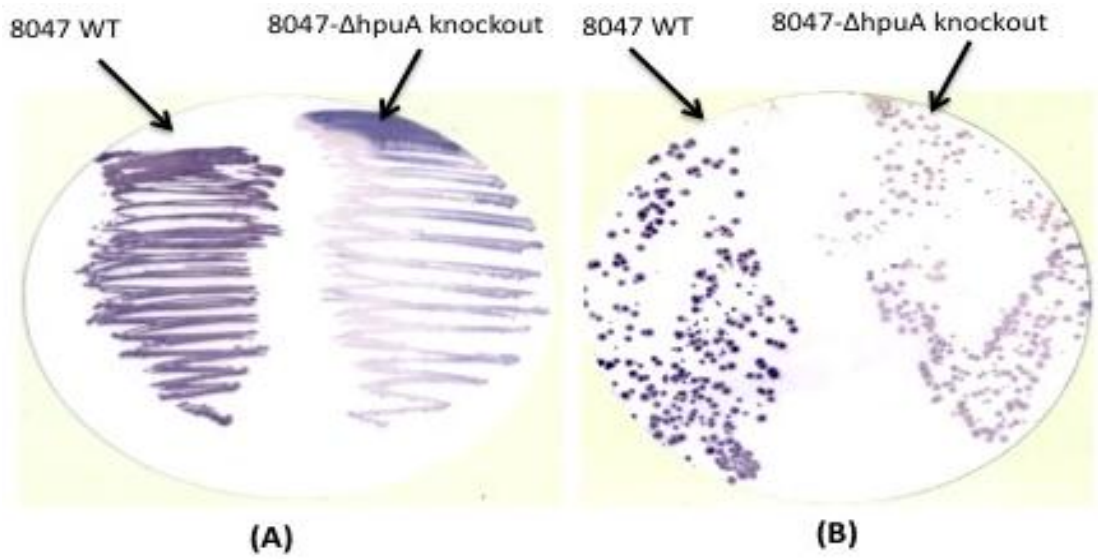

Figure 6.13: Colony immunoblotting of induced wild type strain 8047 compared to *hpuA* knockout strain 8047. Wild type and 8047 *ΔhpuA* mutant were either streaked or spread on MHA plates with restricted iron supply. Bacteria were transferred to blots and probed with a 1:100 dilutions of polyclonal antisera. A 1:2000 dilution of anti-mouse AP-conjugated secondary antibody was used followed by the development of blots. (A) Wild type and knockout mutant streaked side by side on an MHA plate containing 15 μM desferal (B) Dilutions of wild type and mutant spread on an MHA plate containing 15 μM desferal.

6.7 MODIFIED SERUM BACTERICIDAL ASSAY WITH HpuA POLYCLONAL ANTISERA ON UNINDUCED AND INDUCED MENINGOCOCCAL STRAIN 8047

Once all the experiments described above (section 6.6) confirmed that the antisera were recognising the HpuA protein, a preliminary assay was performed to test whether polyclonal antisera against HpuA surface antigen mediate bactericidal activity or not. In the first part of this trial, strain 8047 was used without induction, as binding of polyclonal antisera was detected by Western (Figure 6.11) and ELISA (Figure 6.12) in un-induced 8047 cells, though at lower levels.

An inoculum of medium (5×10^5 CFU) or low size (5×10^3 CFU) were prepared from overnight growth of bacteria on plain MHA plates and incubated in the presence of 5%

human serum in PBSB, with 10 µl (in a total volume of 1 ml resulting in a 1:100 final dilution of the antisera) of HpuA polyclonal antisera, as described in chapter 4. This dilution of antibody was based on the results obtained from Western and ELISA, which showed detectable reactivity at this dilution.

The bacterial population was incubated for two cycles of 2 h each in a continuous assay, as a reduction in population size can be detected after 4 h if the antisera elicit bactericidal activity. This pattern was observed in previous experiments by bactericidal PorA of MAb P1.2 antibodies as described in section 4.1 and the study conducted by Bayliss and co-workers (Bayliss *et al.,* 2008). Growth from appropriate dilutions of passaged populations was obtained on BHIA plates. A control containing only human serum but without polyclonal antibodies was also set up. The CFU count obtained for each population demonstrated no significant killing activity by the HpuA antisera (Figure 6.14), as observed previously for PorA monoclonal antibody in section 4.1. The CFU count remained equal in the populations incubated with and without antibody. This indicated that either the surface expression of HpuA was too low to mediate a bactericidal response or the polyclonal antibody did not elicit bacterial killing.

The bactericidal activity of HpuA polyclonal antisera was further investigated on induced strain 8047 cells. As observed in Western and ELISA described earlier (Figures 6.11 & 6.12), the expression of HpuA in strain 8047 was induced in the presence of desferal and used for the preparation of inoculums. Bacteria were grown overnight in MHB containing 30 µM desferal and 100 µg/ml iron loaded human transferrin. Inoculums of medium (5×10^5 CFU) and low sizes (5×10^3 CFU) from induced population were used for this experiment. The bacterial population was incubated in the

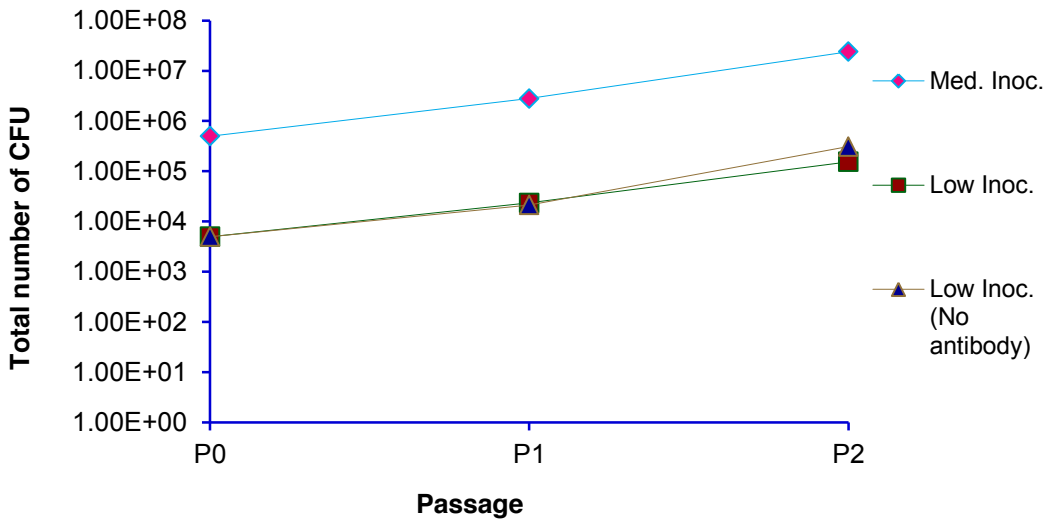

Figure 6.14: Analysis of the Serum Bactericidal activity of HpuA polyclonal antisera against un-induced *Neisseria meningitidis* strain 8047.In a continuous assay, strain 8047 was incubated in the presence of 5% human serum diluted in a final volume of 1 ml of PBSB containing 0.1% glucose, and 10 µl of polyclonal antibody (without antibody in control population). The inocula of the indicated sizes for the first passage were prepared from an overnight culture grown on MHA plates. Bacteria were passaged 2 times and each passage was performed for 2 h. The second passage was performed by mixing 500 µl passaged population with equal volume of PBSB containing 5% human serum and polyclonal antibody. The Y-axis data is the representative of viable count obtained from plating of appropriate dilutions of inoculum, and each passage. X-axis represents the number of passage performed.

presence of 5% human serum and 10 µl of polyclonal antibody and a continuous bactericidal assay was performed. Fresh antibody and serum was added after each cycle, as described for the PorA bactericidal assay in section 4.1. A CFU count for each population was obtained from the appropriate dilutions after the 1st and 4th passage (Figure 6.15).

The data obtained did not demonstrate any difference to that obtained from uninduced populations and again no bactericidal activity was observed. The CFU count was similar for the populations passaged with and without antibody. Colony immunoblots were probed with the HpuA anti sera for the inoculum, and after passage 4 of the

incubation. The analysis confirmed the homogeneity of the population in passaged and non-passaged populations (Figure 6.16).

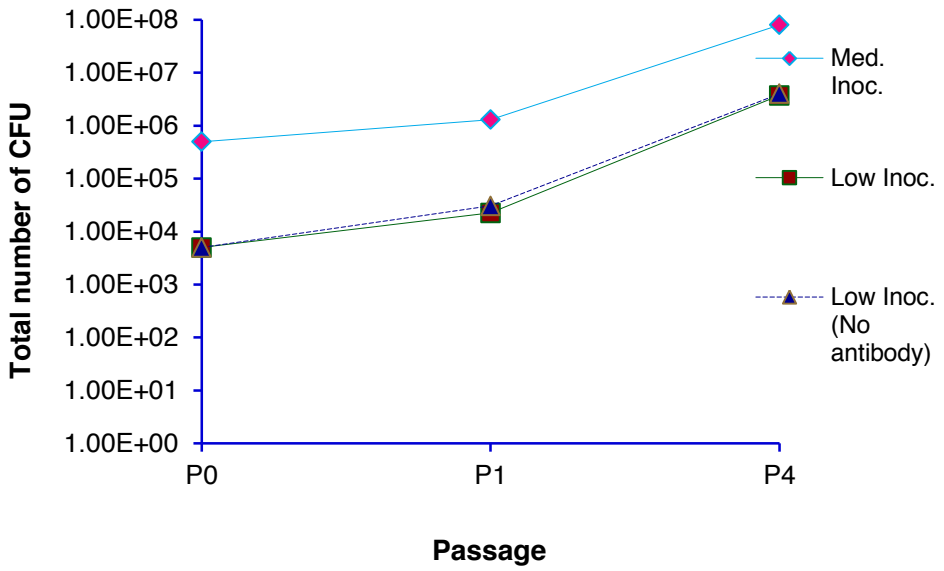

Figure 6.15: Serum Bactericidal activity analysis of HpuA polyclonal antisera against induced *Neisseria meningitidis* strain 8047. Inocula of strain 8047 were prepared from an overnight culture grown in MHB containing desferal and human transferrin, and were incubated as described in figure 6.13. The bacteria were passaged four times and each passage was performed for 2 h. Total CFU counts were obtained by plating serial dilutions of inoculum and each passage. The Y-axis data is the representative of viable count obtained from plating of appropriate dilutions of inoculum, and each passage. X-axis represents the number of passage performed. Filled rectangles, 5×10^5 CFU of strain 8047 incubated with antibody; filled square, 5×10^3 CFU of strain 8047 incubated with antibody; triangles, 5×10^3 CFU of strain 8047 incubated without antibody.

Neither of the blots showed any negative variants. Five colonies from both inoculum and the passaged population (4^{th} passage) were re-streaked and sequenced. The analysis did not show any change in repeat tract of the *hpuA* gene after passaging the population in the presence of antisera. These results demonstrated that although polyclonal

antibodies were raised against the correct antigen, they do not elicit a bactericidal response when the bacterial population is incubated with human serum.

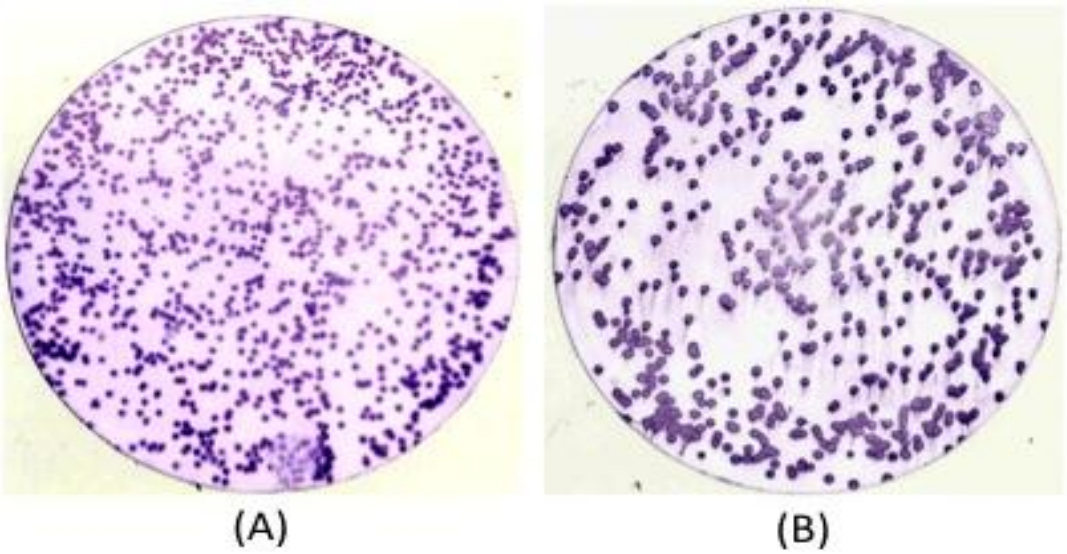

Figure 6.16: Colony immunoblots obtained from strain 8047 and developed against HpuA polyclonal antisera. Serial dilutions of inoculum and passaged populations were grown overnight on MHA agar plates, transferred to nitrocellulose filters and probed with 1:100 dilution of HpuA polyclonal antisera. The secondary antibody (anti-mouse HRP conjugated) was used at a concentration of 1:2000 (A) an immunoblot developed from inoculum (B) immunoblot obtained from a population incubated in the presence of polyclonal antibody (after 4^{th} passage).

6.8 PRELIMINARY SERUM BACTERICIDAL ASSAY BY ANTI HmbR POLYCLONAL ANTIBODY ON INDUCED STRAIN MC58

Polyclonal antibodies against the HmbR protein of strain MC58 were raised in rabbits, a gift by Ian Feavers, National Institute for Biological Standards, UK. A preliminary bactericidal assay was performed using medium (5×10^5 CFU) and low (5×10^3 CFU) inoculum sizes. The inoculums were prepared from an overnight culture induced in the presence of 30 μM desferal in MHB containing 100 μg/ml iron loaded human

transferrin. The bactericidal activity of HmbR polyclonal antisera was tested by continuous assay, as described in earlier sections. CFU counts were obtained after the 1st and 4th passage by spreading the appropriate dilutions on BHIA plates followed by an overnight incubation at 37°C (Figure 6.17). The data revealed no obvious difference of CFU count between the populations incubated in the presence of polyclonal antibodies to that incubated with out antibody. This suggested that like HpuA polyclonal antisera, the HmbR rabbit polyclonal antibody could not induce a bactericidal activity in the presence of human serum.

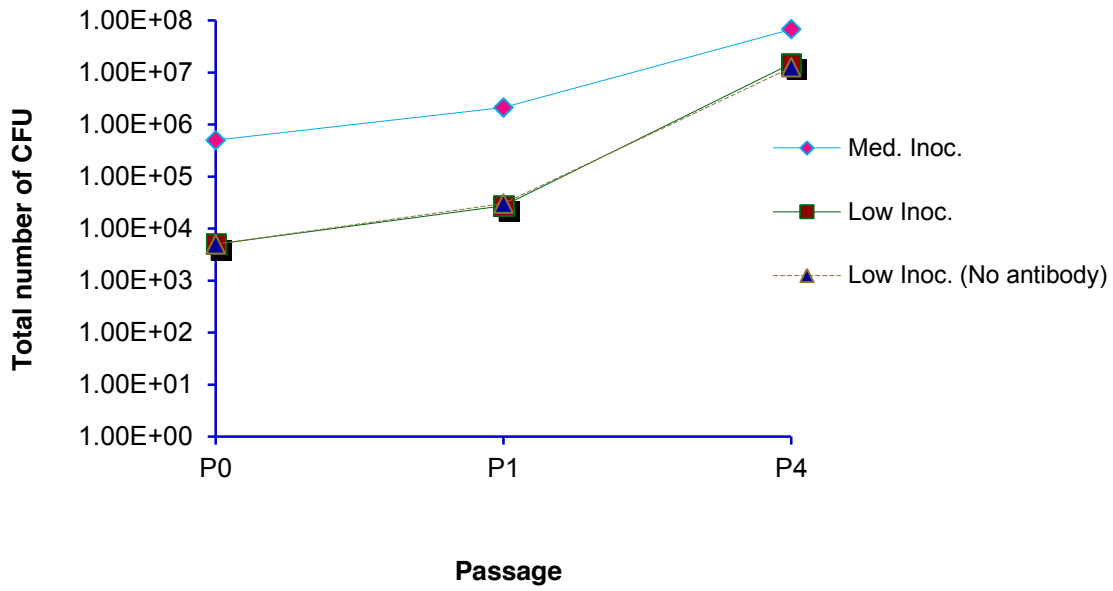

Figure 6.17: Serum Bactericidal activity analysis of HmbR polyclonal antisera against induced *Neisseria meningitidis* strain MC58. Strain MC58 was subjected to four cycles of selection in the modified serum bactericidal assay as described in figure 6.14 except that 10 µl of an anti-HmbR antiserum was used for each passage. The inocula of the indicated sizes were prepared from an overnight culture grown in MHB containing desferal and human transferrin. CFU counts were obtained by plating dilutions on MHA plates. The Y-axis data is the viable count obtained from plating of appropriate dilutions of inoculum, and each passage. X-axis represents the passages at which samples were collected. Filled rectangles, 5×10^5 CFU of strain MC58 incubated with antibody; filled square, 5×10^3 CFU of strain MC58 incubated with antibody; triangles, 5×10^3 CFU of strain MC58 incubated without antibody.

6.9 DETECTION OF HpuA SPECIFIC ANTIBODIES IN HUMAN SERUM

The *in vitro* assays performed in sections 6.6 suggested the expression and induction of HpuA surface antigen after detection with polyclonal antisera raised against this receptor. However, the *in vivo* expression of HpuA is not well documented. A preliminary study using Bioplex xMAP assay was performed to analyse *in vivo* expression of HpuA by detecting the specific antibodies against this antigen in human serum. The human sera selected for this assay included a serum obtained from a recently recovered patient (convalescent serum) and the sera collected from a healthy carrier designated as V88 in the carriage study (Bidmos *et al.*, 2011). Sera analysed were collected from this volunteer at the start of the study (week 0) and then at an interval of four and 24 weeks. The presence of *hpuA* was first detected by PCR to confirm that the gene was present in the strains from this volunteer and the strain isolated from the convalescent patient. Both PCRs confirmed the presence of the *hpuA* locus in these strains (data not shown).

Purified HpuA protein from strain 8047 was coated on magnetic fluorescently labelled beads and incubated with appropriate dilutions of human serum, as described in the methods section. The binding of HpuA specific antibody, present in human serum, with that of purified HpuA protein was detected by a secondary antibody conjugated with phycoerythrin and specific for human antibodies. The mean flourescent index value (MFI) for each sample was obtained and the values were plotted against the respective sample (Figure 6.18). Polyclonal antibodies, raised against HpuA in mice, were used as a positive control. Serum collected from a non-carrier volunteer was also included as a negative control. The carrier and non-carrier were defined on the basis of the presence

or absence growth on selective plates following the application of nasopharyngeal swabs. A carrier was a person whose swab gave growth on GC agar selective plates and growth was identified as meningococci. Swabs from a non-carrier gave either no growth or growth not identified as meningococci.

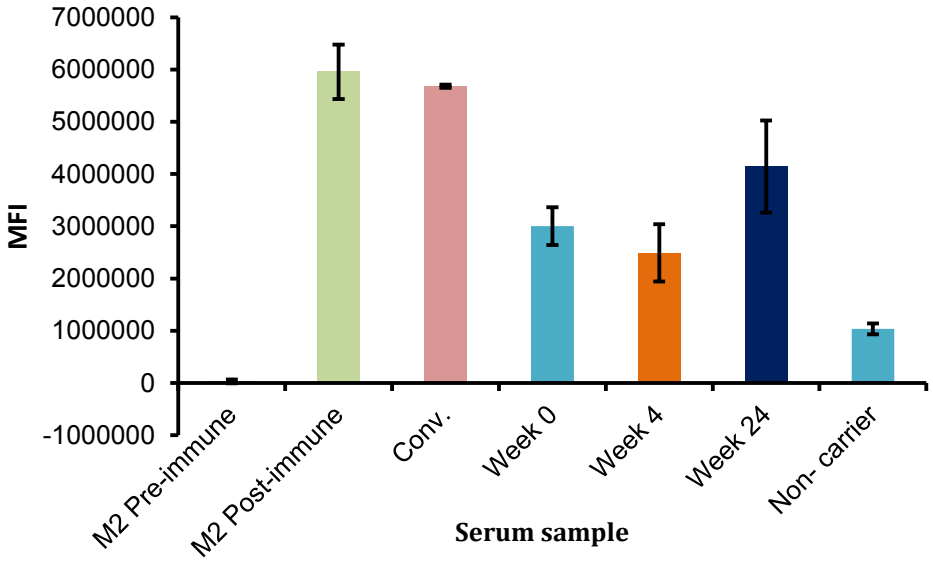

Figure 6.18: Detection of HpuA specific antibodies in human serum samples using a Bioplex assay. Purified HpuA protein coated on magnetic beads was mixed with appropriate dilution of human serum (1:100, 1:500, 1:2000 and 1:5000 dilutions) and/or mouse serum (1:100, 1:1000, 1:5000, 1:10000). Presence and binding of HpuA specific antibodies with coated HpuA protein was detected by human specific (mouse specific in case of control) phycoerythrin (PE) conjugated secondary antibody. The MFI values were recorded for each sample and plotted (Y-axis). The results are the mean (±SEM) of duplicates and are representative of two independent experiments. M2 Pre immune; sera from mouse M2 before immunization with recombinant HpuA protein, M2 Post; post-immune sera from mouse M2 inoculated with recombinant HpuA, Conv.; serum collected from a recovered patient with known meningococcal infection, Week 0: serum collected from a carrier (V88) at the first time point of sampling, Week 4: serum collected from same persistent carrier (V88) after four weeks of first sampling, Week 24: serum collected from same persistent carrier (V88) after 24 weeks of first sampling, Non-carrier; serum obtained from a volunteer (V57) without meningococcal carriage during sampling.

The results based on the reactivity suggested an *in vivo* expression of HpuA in all serum samples obtained from either a recovered patient or from a healthy carrier, as antibodies were detected in these samples specific for the purified HpuA protein (Figure 6.18). A high MFI value was obtained for convalescent serum and was approximately equal to that obtained for the positive control. The immune response was low in week zero of carriage (Figure, data series week 0) and this level persisted for 4 weeks (data series week 4). However, the immune response in this volunteer reached a peak after 24 weeks of carriage (data series week 24). The reactivity was lowest in the serum obtained from a non-carrier suggesting the absence of carriage in this volunteer.

These results indicated HpuA is expressed *in vivo* and that antibodies specific for HpuA are generated which can be detected in human serum. The level of antibody is dependent on the length of carriage. The experiment was also repeated on 11 non-carriers to observe the serum binding with HpuA protein. However, 5 non-carrier showed a medium or low cross reactivity with HpuA protein. Thus, although the assay worked for serum obtained from carrier, the cross reactivity in few other non-carrier samples suggest that more optimisation and considerations are required. However, as data about the history of past colonization in these non-carriers is not available, it is likely that these non-carriers (with medium or low cross reactivity) may acquired meningococci transiently before sampling and then cleared it.

6.10 DISCUSSION

The third aim of my project was to clone and express a recombinant HpuA protein in order to generate a polyclonal antibody specific for this antigen. Production of bactericidal antibodies against HpuA was required in order to search for a biological role for phase variation of this surface exposed protein. The pET Topo200 expression

vector was used to clone and express the recombinant protein in *E. coli* BL21(DE3) strain. This expression system permitted a high level of expression of recombinant HpuA protein and a high yield this protein was recovered in a single step purification.

Surprisingly, we observed two recombinant protein bands instead of a single unique band. Both bands reacted with anti His-tag antibody and mass spectrometry analysis confirmed the identity of both bands as HpuA (Figures 6.6 and 6.7). One obvious explanation for these two bands in recombinant protein preparations is that they represent modifications of the protein which is likely as HpuA is a lipo-protein whose lipid moiety is anchored to the bacterial outer membrane. It is likely that during expression, some molecules are lipidated while the others are not. This difference in lipidation leads to a slight difference in their physical properties and obviously in their electrophoresis pattern. Biochemical analysis of a counterpart protein, TbpB, has confirmed the presence of lipidation on a cysteine residue which is adjacent to the amino acid at the N-terminal tag and exposed for lipidation after the removal of the signal sequence (Lissolo, *et al.,* 1994). This modification of meningococcal TbpB not only known to occur naturally but also found at lower level when TbpB is expressed in *E. coli* B expression system (Legrain *et al.,* 1995). As expression system used in this study (BL21) is a derivative of *E. coli* B expression system carrying a λ prophage with T7 RNA polymerase gene (Studier and Moffatt, 1986), it is likely that a small amount of protein is lipidated during expression and affects the mobility of recombinant protein during electrophoresis.

A second purification step helped to significantly reduce impurities and enabled us to obtain a high purification of HpuA protein for immunization of mice. The subsequent part of the work demonstrated a successful generation of polyclonal antisera against HpuA by immunizing mice. Screening by ELISA and Western blotting identified and

confirmed the specificity of antibody against purified HpuA protein (Figures 6.9 and 6.10 respectively).

The polyclonal antisera were expected to recognize the purified protein used for immunization. Recognition of the native protein following induction with or without iron was also examined (Figure 6.11). Despite high cross reactivity for other meningococcal proteins, the induction and recognition of native HpuA protein by the polyclonal antisera was confirmed. A couple of unusual and unexpected bands with high reactivity for the antisera appeared in the *hpuA* mutant. These bands were absent in un-induced mutants but present in induced cells. One explanation of these bands is that they are fusion proteins which arose due to fusion of HpuA N-terminal and HpuB C-terminal fragments after shedding the cassette in some cells of the population. The shedding of antibiotic resistance cassette may have occurred due to growth of mutants for this assay in a medium without antibiotic. However, it remains unclear, as the identity of the bands has not been determined.

The whole cell ELISA results confirmed a two-fold induction in surface expression of HpuA under iron limited conditions when compared with uninduced 8047 cells (Figure 6.12). The difference in reactivity was also 2 fold between induced wild type cells and the mutants grown under the same conditions. This result confirmed that the additional proteins observed in Western blotting of mutants (Figure 6.11) are not likely to be surface expressed, otherwise the difference in induction would have been far less between induced wild type cells and mutants.

Other evidence for surface expression and induction came from immunoblotting. As the cells are grown under the same conditions and on the same plate, the comparison for induction, surface expression and specificity of antisera for the wild type and knockouts

should be more conclusive. This comparison confirmed the expression and induction of HpuA in wild type but not in mutants (Figure 6.13). These results support those observed in ELISA for the surface expression of HpuA and the specificity of polyclonal antisera.

The antiserum was tested for immune evasion analysis by using a modified serum bactericidal assay. This analysis was performed to analyse the bactericidal activity and immune evasion due to phase variation of HpuA. The results obtained from both uninduced and induced cultures tested for bactericidal activity did not demonstrate any significant killing in the presence of human complement (Figures 6.14 and 6.15). Subsequent immunoblotting confirmed that the selective pressure was not enough to eradicate the reactive population and also we were unable to identify any negative variant on immunoblots. Similar results were obtained for the analyses performed using an antisera raised against the HmbR receptor (Figure 6.17).

The evaluation of bactericidal activity of an antibody is not straightforward because of the complexity of the immune responses required for bactericidal activity, and also each strain may be killed by antibodies to multiple antigens (Giuliani *et al.,* 2010). Our results demonstrate that antisera raised against both haemoglobin receptors were not able to elicit demonstrable serum cidal activity, so the establishment of this assay for haemoglobin receptors remained inconclusive. One possible reason for the lack of bactericidal activity may be the level of surface expression of these receptors. A high expression level of antigen is required for optimum binding of the antibody, hence lower expression may reduce the bactericidal activity (Beernink *et al.,* 2008; Koeberling *et al.,* 2011).

As we have observed in PorA phase variable expression in chapter 4, a minor shift in level of expression leads to almost no bactericidal activity. Compared to PorA, which is expressed at high levels constitutively, the expression of these receptors is inducible. Though we have repeated the assay on induced cells, but it is likely that we may not be able to attain a sufficient level of expression *in vitro* to elicit bactericidal activity and immune evasion responses. One solution to address the reduced expression level of is antigen *in vitro* is the construction of over-expression mutants. An analysis of immune evasion in such a mutant would be useful to test this hypothesis. Another explanation is that we may require a combination of antibodies, i.e. anti-HpuB and anti-HmbR, to elicit these responses and to compensate for low expression levels (Beernink *et al.,* 2008).

Another reason for the lack of bactericidal activity in our assay is likely to be the lower levels of antibody used in the assay. As in this assay, we have used a larger volume (1 ml) and number of bacteria (atleast 5000 CFU), it is likely that polyclonal antibody (10 µl) may not be enough to elicit complement mediated killing. Compared to this, standard serum bactericidal assay requires less number (3000 CFU) of bacteria and performed in smaller volumes (~200 µl). So, to compensate the size of population and dilution caused by larger volumes of our assay, a standard SBA may be helpful to test the serum cidal activity and to determine the suitable concentration of antibody required for the assay used in this study.

A lack of bactericidal activity by sera is also likely to be associated with concentration of each immunoglobulin isotype in the sera (Gomez *et al.,* 1998). Each isotype has a different effect on activation of the complement pathway. Isotypes IgG2a, IgG2b, IgG3 and IgM are reported to be an effective activator while IgA and IgG1 have less or even no affect on complement activation (Christodoulides *et al.,* 1994). IgG1 may even

inhibit the other effective antibodies, as cited by authors. A higher concentration of this isotype also reduces the bactericidal effects elicited by a bactericidal antibody. A potential solution for this is the production of monoclonal antibodies specific for these antigens and would be another valuable test to consider.

Studies have reported that the presence and type of adjuvant used during immunization may also influence the bactericidal activity (Gomez *et al.,* 1998; Hung *et al.,* 2011). Although Gomez *et al* (1998) reported that FCA (an adjuvant also used in our study) were effective to promote the lysis of bacteria, a trial with a different adjuvant might be helpful for generating an effective bactericidal response. Thus, due to a lack of such data, these results remain inconclusive and further investigation is required.

The immunological data describing the *in vivo* expression of a protein by the detection of antibodies present in the convalescent serum or the serum of a carrier host is a potential support to determine the candidacy of the target for a future vaccine (Thompson *et al.,* 2003). The analysis of *in vivo* expression of HpuA was performed using sera obtained from healthy carriers. The correlation of the level of antibody with that of the phase variable status was analysed for a limited number of samples. Our results from this experiment suggest an *in vivo* expression of HpuA and generation of antibodies against the expressed antigen. This indicated that antibodies specific for HpuA are induced at the gain of carriage due to *in vivo* HpuA expression, and persistence is associated with an increased amount of specific HpuA antibodies.

The results also suggest that fluctuations in level of antibody may select for an ON or OFF state. Higher antibody levels were detected at week 24 in the volunteer and the meningococcal strain isolated at this time point contained an OFF variant of HpuA, while lower antibody levels were obtained for sera from carriers of ON variants (week

4). A time course of 20 weeks was observed for HpuA titer to reach at a level required for the selection of HpuA OFF state. However these results are representative of only one experiment and a detailed investigation including more samples would be beneficial. In addition, further optimization may be required for this assay due to non-specific binding in few non-carrier samples. This may be a genuine HpuA titer, as we do not have data about past or transient colonization between sampling points in these non-carriers. An analysis of antibody titer against immunodominant PorA would be useful, as it is known that the PorA titer is found once colonization occur (Jordens *et al.,* 2004). This will help to correlate the results of HpuA titer with PorA and to determine any past or transient colonization.

In conclusion, in this part of the study we were able to successfully clone and express the HpuA component of the HpuAB receptor complex from strain 8047. The subsequent immunization of animals with this antigen also successfully generated the polyclonal antisera specific not only for the purified antigen, but it also recognised the native protein on bacterial surface. However, the antiserum was not able to elicit bactericidal activity in immune evasion assays. Hence, the development of assay to determine the possible role of phase variation of this haemoglobin receptor (HpuA) in immune evasion remains to be concluded.

SUMMARY

Neisseria meningitidis (the meningococcus) is an important (asymptomatic) human commensal colonizing about ~10% of the general population at a given time (Yazdankhah and Caugant, 2004), but with the potential to colonize up to 50% of a specific population (Bidmos *et al.,* 2011). However, disease incidence is not always dependent on a high carriage rate rather intrinsic virulence, the acquisition rates of the strains associated with high levels of disease (i.e. the hypervirulent strains) and whether these strains are circulating in a population are more important.

Although meningococcal vaccines based on capsular polysaccharides are available and effectively protect against specific serogroup (A, C, Y and W-135) isolates, the absence of vaccine based protective immunity against serogroup B isolates, and also the isolation of non-encapsulated isolates with invasive potential (Johswich *et al.,* 2012), prompts the replacement of current capsule based vaccines with a universal protein based vaccine covering all clinically important serogroups. In this scenario, a continuous epidemiological surveillance of meningococci for novel virulence factors, with a potential use as a future vaccine candidate, may be of great value.

This project investigated the virulence association of haemoglobin genes in meningococci. The study of the distribution of haemoglobin encoding genes revealed a significant association between disease and the presence of both genes (in combination) or *hmbR*-only. This detailed analysis of the distribution of both genes, in depth analysis of phase variation status and extent of structural diversity has implications for understanding the host–pathogen interaction during infection. The fact that both haemoglobin-binding systems are surface exposed and widely distributed (99%) in meningococci, suggests that further investigation of their structures and immune-

associated status would be useful in understanding infection. Further investigation of these receptors may also be important for development of an effective vaccine covering all clinically important serogroups.

The project was also useful to understand how phase variation impacts on infection and adaptation to the surrounding environment. The demonstration of escape mediated by phase variation of *porA* in a meningococcal strain, 8047, have further strengthened the previous findings that the rate of switching is important for adaptation (Bayliss *et al.,* 2008). This study is the first to demonstrate a functional role of *porA* phase variation in immune evasion, and also provide a perfect demonstration of how PV influences bacterial fitness by evading adaptive immunity. A significant point to note, and an important implication of this work, is that virtually all meningococcal isolates have the potential of *porA* phase variation (van der Ende, 2000). Hence, all meningococcal isolates can potentially generate phase variants, by high frequency switching, with reduced PorA expression. A specific immune response can then be a driving force to accumulate and select for phase variants capable of escaping immunity. These factors in combination, with other variation generators, may indicate that the efficacy of a PorA based vaccine would be limited. Another importance of this work is that if PV of PorA mediates escape from antibody-mediated killing, then in a general perspective, many other meningococcal surface proteins are also regulated by this type of mechanism and so may also escape due to PV. Thus meningococcal population could generate a repertoire of antigenic variants which is difficult, if not impossible, to eliminate.

This successful demonstration of PV mediated immune evasion of *porA* in strain 8047 laid the foundation for a similar study of *hpuA*. Following the expression of HpuA, a polyclonal antibody recognising the surface antigen was generated. However despite the

recognition of native protein by polyclonal antisera, we could not obtain evidence of bactericidal activity and immune evasion. One reason could be that a lack of activity is due to an adjuvant (FCA) mediated alteration in the confirmation of the immunizing protein. However, such a change in protein confirmation usually results in a lack of surface recognition of antigen (Hung *et al.,* 2011). As in our case, the surface expression is recognised, the lack of SBA is likely due to either the expression of HpuA was not enough to reach the critical density required for a bactericidal response (Koeberling *et al.,* 2011) or the concentration (may be isotype) of antibody generated is not able to elicit a complement mediated bactericidal response as seen in other studies (Christodoulides *et al.,* 1994). However, whatever the reason, this and other published data (Hung *et al.,* 2011) indicate that surface accessibility and recognition of a native protein are not enough to predict the SBA, as a number of complicated factors influence the antibody-mediated killing.

In conclusion, the study has thoroughly investigated the distribution of haemoglobin encoding genes in a comprehensive set of meningococcal isolates collected from diverse geographical backgrounds. This analysis successfully achieved the first aim of the study and proposed a clear link between haemoglobin utilisation and meningococcal virulence by providing epidemiological evidence for the association of both genes or *hmbR*-only with virulence. This finding confirmed that a hypothesis of an association of haemoglobin utilisation with virulence holds true.

The demonstration of immune evasion by PV of *porA* helped to successfully achieve the second aim. This analysis not only validated the assay but also provided a strong background to test the capacity of immune evasion by phase variation of *hpuA.* However, despite the successful cloning, expression and generation of native protein recognising polyclonal antisera, a lack of bactericidal activity restricted the conclusion

of the final aim of this study. Overall, the study has significantly contributed to our understanding of host-pathogen interactions during infection, disease progression and immune evasion.

Future Work

A detailed investigation of bactericidal and immune evasion assays using a single antibody or a combination of antibodies against the Hb receptors is required. Once the immune evasion assay is established for antibodies against these receptors, the growth and reverse selection of an OFF to ON phase variant in human blood would be useful to demonstrate the phase variable adaptation by Hb receptors. Similarly, the resolution of HpuA structure, its crystallization and mapping of variable regions in different variants could be of great value for a future vaccine consideration.

Appendix 1

hpuA sequence alignment

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CLUSTAL 2.0.10 multiple sequence alignment


Z2491hpuA   GTAGCCGAACCGCACGTCCCCGTGTCCATCCCCACCGCCACGCCGCTG---ACCGGTGAG
117hpuA     GTAGCCGAACCGCACGTCCCCGTGTCCATCCCCACCGCCACGCCGCTG---ACCGGTGAG
222hpuA     GTAGCCGAACCGCACGTCCCCGTGTCCATCCCCACCGCCACGCCGCTG---ACCGGTGAG
114hpuA     GTAGCCGAACCGCACGTCCCCGTGTCCATCCCCACCGCCACCCCGCTGCCCGCCGGCGAG
134hpuA     GTAGCCGAACCGCACGTCCCCGTGTCCATCCCCACCGCCACCCCGCTGCCCGCCGGCGAG
119hpuA     GTAGCCGAACCGCACGTCCCCGTGTCCATCCCCACCGCCACCCCGCTGCCCGCCGGCGAG
88hpu       GTAGCCGAACCGCACGTCCCCGTGTCCATCCCCACCGCCACGCCGCTACCC---GGTGAG
138hpuA     GTAGCCGAACCGCACGTCCCCGTGTCCATCCCCACCGCCACGCCGCTACCC---GGTGAG
8047hpuA    GTAGCCGAACCGCACGTCCCCGTGTCCATCCCCACCGCCACGCCGCTGACC---GGTGAG
Fam18hpuA   GTAGCCGAACCGCACGTCCCCGTGTCCATCCCCACCGCCACCCCGCTGCCCACCGGCGAG
            ........................................ .....       .. ...


Z2491hpuA   GTAAAGTTATCAAGCGATAACAGCAAAATCGAAAACATCAACACCGCCAACACTGAAACA
117hpuA     GTAAAGTTATCAAGCGATAACAGCAAAATCGAAAACATCAACACCGCCAACACTGAAACA
222hpuA     GTAAAGTTATCAAGCGATAACAGCAAAATCGAAAACATCAACACCGCCAACACTGAAACA
114hpuA     GTAACGTTATCAGACGATAGCGTCAATATCGTAAACATCAACACCGCCAACACTGAAACA
134hpuA     GTAACGTTATCAGACGATAGCGTCAATATCGTAAACATCAACACCGCCAACACTGAAACA
119hpuA     GTAACGTTATCAGACGATAGCGTCAATATCGTAAACATCAACACCGCCAACACTGAAACA
88hpu       GTAACGTTATCAGACGATAGCGTCAATATCGTAAACATCAACACCGCCGGCACAGGAA--
138hpuA     GTAACGTTATCAGACGATAGCGTCAATATCGTAAACATCAACACCGCCGGCACAGGAA--
8047hpuA    GTAAAGTTAACAGACGACAATAGCAAAATCGAAAACATCAACACCGCCAACACTGGAA--
FAM18hpuA   GTAAAGTTATCAGACGACAATAGCAAAATCGAAAACATCAACACCGCCGACACCGGAA--
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Z2491hpuA  CATCCGCCGCGCCGCACCAGACGCTCCCTCTATGCCTCCCCGCAAAACACATCCTCCGGT
117hpuA    CATCCGCCGCGCCGCACCAGACGCTCCCTCTATGCCTCCCCGCAAAACACATCCTCCGGT
222hpuA    CATCCGCCGCGCCGCACCAGACGCTCCCTCTATGCCTCCCCGCAAAACACATCCTCCGGT
114hpuA    CATCCGCCGCGCCGCACCAGACGCTCCCTCTATGCCTCCCCGCAAAACACATCCGCCGGT
134hpuA    CATCCGCCGCGCCGCACCAGACGCTCCCTCTATGCCTCCCCGCAAAACACATCCGCCGGT
119hpuA    CATCCGCCGCGCCGCACCAGACGCTCCCTCTATGCCTCCCCGCAAAACACATCCGCCGGT
88hpu      ----------------------------------------------GCACATCCTCCGGC
138hpuA    ----------------------------------------------GCACATCCTCCGGC
8047hpuA   ----------------------------------------------ACACATCC---GGC
Fam18hpuA  ----------------------------------------------GCACATCC---GGC
                                                           .......   ..

Z2491hpuA  ATTTCCATTCAGCAGCGGGAAGTAGAAAAAATCTATTTCGGTGTTAAATCACCAGAAAAA
117hpuA    ATTTCCATTCAGCAGCGGGAAGTAGAAAAAATCTATTTCGGTGTTAAATCACCAGAAAAA
222hpuA    ATTTCCATTCAGCAGCGGGAAGTAGAAAAAATCTATTTCGGTGTTAAATCACCAGAAAAA
114hpuA    ATTTCCATTCAGCAGCGGGAAGTAGAAAAAGACTATTTCGGCTATAAATCAAAAGAAACA
134hpuA    ATTTCCATTCAGCAGCGGGAAGTAGAAAAAGACTATTTCGGCTATAAATCAAAAGAAACA
119hpuA    ATTTCCATTCAGCAGCGGGAAGTAGAAAAAGACTATTTCGGCTATAAATCAAAAGAAACA
88hpuA     ATTTCTATTCAACAGCGAAAATATAACGTAACAAGTTACGGCTTCACAAGAGAAGAAAAA
138hpuA    ATTTCTATTCAACAGCGAAAATATAACGTAACAAGTTACGGCTTCACAAGAGAAGAAAAA
8047hpuA   ATTTCCATTCAGCAGCGGGAATATAAAGTAAACAATTACGGCGTCGAATCAACAGCAAAA
Fam18hpuA  ATTTCCATTCAGCAGCGGAAATATGAAGTAGAAAGTTACGGCATCAAAAGAGAAGAAAAA
           ..... ..... .....  ..    .   .     .. ...      .  .  .. .. .

Z2491hpuA  TCATTCATCTTCCAAACACCCGGCGGCGCGCAATACGCCCTTTCATCCTACGCCGACCCC
117hpuA    TCATTCATCTTCCAAACACCCGGCGGCGCGCAATACGCCCTTTCATCCTACGCCGACCCC
222hpuA    TCATTCATCTTCCAAACACCCGGCGGCGCGCAATACGCCCTTTCATCCTACGCCGACCCC
114hpuA    TCTTTCATCTTCCAAACACCCGGCGGCGCGCAATACGCCCTTTCATCTTATTCCGACCCC
134hpuA    TCTTTCATCTTCCAAACACCCGGCGGCGCGCAATACGCCCTTTCATCTTATTCCGACCCC
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119hpuA     TCTTTCATCTTCCAAACACCCGGCGGCGCGCAATACGCCCTTTCATCTTATTCCGACCCC
88hpu       GCATTCATCTTCAAAACACCTGGCGGCGCGCAATACACCCTTTCATCCTACGCCGACCCC
138hpuA     GCATTCATCTTCAAAACACCTGGCGGCGCGCAATACACCCTTTCATCCTACGCCGACCCC
8047hpuA    GCATTCATCTTCAAAACACCTGGCGGCGCGCAATACACCCTTTCATCCTACGCCGACCCC
Fam18hpuA   GCATTCATCTTCCAAACACCCGGCGGCGCGCAATACGCCCTTTCATCCTACGCCGACCCC
             . ......... ....... ............... .......... ..  ........

Z2491hpuA   ATCGTCCCCAGCTACTCCTCCCCCGACTTCAAAATTCCCGACCGCCACGCAGGGCAGCGG
117hpuA     ATCGTCCCCAGCTACTCCTCCCCCGACTTCAAAATTCCCGACCGCCACGCAGGGCAGCGG
222hpuA     ATCGTCCCCAGCTACTCCTCCCCCGATTTCAAAATTCCCGACCGCCACGCAGGGCAGCGG
114hpuA     ATCGTCCCCAGCTATTCCTCCCCCGATTTCAAAATTCCCGACCGCCACGCCGGGCAGCGG
134hpuA     ATCGTCCCCAGCTATTCCTCCCCCGATTTCAAAATTCCCGACCGCCACGCCGGGCAGCGG
119hpuA     ATCGTCCCCAGCTATTCCTCCCCCGATTTCAAAATTCCCGACCGCCACGCCGGGCAGCGG
88hpu       ATCGTCCCCAGCTACTCCTCCCCCGATTTCAAAATTCCCGACCGCCACGCAGGGCAGCGG
138hpuA     ATCGTCCCCAGCTACTCCTCCCCCGATTTCAAAATTCCCGACCGCCACGCAGGGCAGCGG
8047hpuA    ATCGTCCCCAGCTACTCCTCCCCCGATTTCAAAATTCCCGACCGCCACGCAGGGCAGCGG
Fam18hpuA   ATAACCGTCAGCTACTCCTCCCCCGATTTCAAAATTCCCGACCGCCACGCAGGGCAGCGG
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Z2491hpuA   CTTGCCGACGGCAGCCGCATCTTTATCTGCTGCAGCGACTCCGGGGCTACTTCGTATGCG
117hpuA     CTTGCCGACGGCAGCCGCATCTTTATCTGCTGCAGCGACTCCGGGGCTACTTCGTATGCG
222hpuA     CTTGCCGACGGCAGCCGCATCTTTATCTGCTGCAGCGACTCCGGGGCTACTTCGTATGCG
114hpuA     CTTGCCGACGGCAGCCGCATCTTTATCTGCTGCAGCGACTCCGGGGCTACTACGTATGCG
134hpuA     CTTGCCGACGGCAGCCGCATCTTTATCTGCTGCAGCGACTCCGGGGCTTCTACGTATGCG
119hpuA     CTTGCCGACGGCAGCCGCATCTTTATCTGCTGCAGCGACTCCGGGGCTACTACGTATGCG
88hpu       CTTGCCGACGGCAGCCGCATCTTTATCTGCTGCAGCGACTCCGGGGCAACCAATCAAGCG
138hpuA     CTTGCCGACGGCAGCCGCATCTTTATCTGCTGCAGCGACTCCGGGGCAACCAATCAAGCG
8047hpuA    CTTGCCGACGGCAGCCGCATCTTTATCTGCTGCAGCGACTCCGGGGCAACCAATCAAGCG
Fam18hpuA   CTTGCCGACGGCAGCCGCATCTTTATCTGTTGCAGCGAATCCGGGGCTACTTCGTATGCG
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Z2491hpuA  GAAATTACCAAACAAGATTATATGAAATTCGGGGCGTGGATAGGCCCCAACGGCGAAATC
117hpuA    GAAATTACCAAACAAGATTATATGAAATTCGGGGCGTGGATAGGCCCCAACGGCGAAATC
222hpuA    GAAATTACCAAACAAGATTATATGAAATTCGGGGCGTGGATAGGTCCCAACGGCGAAATC
114hpuA    GAAATTACCAAACAAGATTATATGAAATTCGGGGCGTGGATAGGCCCCAACGGCGAAATC
134hpuA    GAAATTACCAAACAAGATTATATGAAATTCGGGGCGTGGATAGGCCCCAACGGCGAAATC
119hpuA    GAAATTACCAAACAAGATTATATGAAATTCGGGGCGTGGATAGGCCCCAACGGCGAAATC
88hpu      GAAATTACCAAACAAGATTATATGAAATTCGGGGCGTGGATAGGTCCCAACGGCGAAATC
138hpuA    GAAATTACCAAACAAGATTATATGAAATTCGGGGCGTGGATAGGTCCCAACGGCGAAATC
8047hpuA   GAAATTACCAAACAAGATTATATGAAATTCGGGGCGTGGATAGGTCCCAACGGCGAAATC
Fam18hpuA  GAAATCACCAAACAAGATTATATGAAATTCGGGGCGTGGATAGGTCCCAACGGCGAAATC
           ..... ..................................... ..............

Z2491hpuA  GACCTCTTCGCCGGCGGCTTCCCCATCGGCAAAACGCCGCCTCCTGCGTTCTCCTGGGGC
117hpuA    GACCTCTTCGCCGGCGGCTTCCCCATCGGCAAAACGCCGCCTCCTGCGTTCTCCTGGGGC
222hpuA    GACCTCTTCGCCGGCGGCTTCCCCGTCGGCAAAACGCCACCTCCTGCGTTCTCCTACGGC
114hpuA    GACCTCTTCGCCGGCGGCTTCCCCGTCGGCAAAACGCCGAAACCGGCATACTCCTGGGGC
134hpuA    GACCTCTTCGCCGGCGGCTTCCCCGTCGGCAAAACGCCGAAACCGGCATACTCCTGGGGC
119hpuA    GACCTCTTCGCCGGCGGCTTCCCCGTCGGCAAAACGCCGAAACCGGCATACTCCTGGGGC
88hpu      GACCTCTTCGCCGGCGGCTTCCCCGTCGGCAAAACACCGGCATCAAGCAGTTATTAC---
138hpuA    GACCTCTTCGCCGGCGGCTTCCCCGTCGGCAAAACACCGGCATCAAGCAGTTATTAC---
8047hpuA   GACCTCTTCGCCGGCGGCTTCCCCGTCGGCAAAACACCGGCATCAAGCAGTTATTACGGC
Fam18hpuA  GACCTCTTCGCCGGCGGCTTCCCCGTCGGCAAAACGCCGAAGCCTAAATACTCTTGGGGC
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Z2491hpuA  AGCCCCACGACCGAAACCGCATTGTCAAAAGGCAAAATCACTTATCAGGTTTGGGGCATC
117hpuA    AGCCCCACGACCGAAACCGCATTGTCAAAAGGCAAAATCACTTATCAGGTTTGGGGCATC
222hpuA    AACAGCACGCCCGAAACCGCATTGTCGAAGGGCAAAATCACTTATCAGGTTTGGGGCATC
114hpuA    GACGACACGCCCGAAACCGC--CGGC-AAGGGCAAAATCACTTATCAGGTTTGGGGCATC
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134hpuA     GACGACACGCCCGAAACCGC--CGGC-AAGGGCAAAATCACTTATCAGGTTTGGGGCATC
119hpuA     GACGACACGCCCGAAACCGC--CGGC-AAGGGCAAAATCACTTATCAGGTTTGGGGCATC
88hpu       AGCAGCACGCTCGAAACCGC--CGGC-AAGGGCAAAATCACTTATCAGGTTTGGGGCATC
138hpuA     AGCAGCACGCTCGAAACCGC--CGGC-AAGGGCAAAATCACTTATCAGGTTTGGGGCATC
8047hpuA    AGCAGCACGCCCGAAACCCA--AGGC-AAGGGCAAAATCACTTATCAGGTTTGGGGCATC
Fam18hpuA   AACACCGAACCCGAAACCAA--AGGC-AAGGGCAAAATCACTTATCAGGTTTGGGGCATC
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Z2491hpuA   CGCGTCAGAAACGGGCAATTTGTTACTTCCTCTTATACGCCGCCCAAAGGCAGCTCTT-G
117hpuA     CGCGTCAGAAACGGGCAATTTGTTACTTCCTCTTATACGCCGCCCAAAGGCAGCTCTT-G
222hpuA     CGCGTCAAAGACGGGCAATTTGTTACCTCCTCTTATACGCCGCCCAAAGGCAGCTCTT-T
114hpuA     CGCGTCAAAGACGGGCAATTTGTTACCTCCTCTTATACGCCGCCCAAAGGCAGCTCTT-T
134hpuA     CGCGTCAAAGACGGGCAATTTGTTACCTCCTCTTATACGCCGCCCAAAGGCAGCTCTT-T
119hpuA     CGCGTCAAAGACGGGCAATTTGTTACCTCCTCTTATACGCCGCCCAAAGGCAGCTCTT-T
88hpu       CGCGTCAAAGACGGGCAATTTGTTACCTCCTCTTATACGCCGCCGAAAAACAGTAGTTCT
138hpuA     CGCGTCAAAGACGGGCAATTTGTTACCTCCTCTTATACGCCGCCGAAAAACAGTAGTTCT
8047hpuA    CGCGTCAGAAACGGGCAATTTGTTACTTCCTCTTATACGCCGCCGAAAAACAGTGGTTAT
Fam18hpuA   CGCGTCAGAAACAGGCAATTTGTTACTTCCTCTTATACGCCGCCGAAAAGCGGTTCTTAT
            ....... . .. ............. ................. ...  . .   ..

Z2491hpuA   GAGTGGT-----TATCAAAACACCCCCGTCCTTTCCTTTATCACCGCCAATTTCAACAGC
117hpuA     GAGTGGT-----TATCAAAACACCCCCGTCCTTTCCTTTATCACCGCCAATTTCAACAGC
222hpuA     TACCGGC-----TACACCAACACCCCCGTCCTTTCCTTTATCACCGCCAATTTCAACAGC
114hpuA     TACCGGC-----TACACCAACACCCCCGTCCTTTCCTTTATCACCGCCAATTTCAACAGT
134hpuA     TACCGGC-----TACACCAACACCCCCGTCCTTTCCTTTATCACCGCCAATTTCAACAGT
119hpuA     TACCGGC-----TACACCAACACCCCCGTCCTTTCCTTTATCACCGCCAATTTCAACAGT
88hpu       TATTTGTACAAACCTACCAACACCCCCGTCCTTTCCTTTATCACCGCCAATTTCAACAGC
138hpuA     TATTTGTACAAACCTACCAACACCCCCGTCCTTTCCTTTATCACCGCCAATTTCAACAGC
8047hpuA    TATTCGTCCACACCTACCAACACCCCCGTCCTTTCCTTTATCACCGCCAATTTCAACACC
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Fam18hpuA  TATGGG---ACACTTGCCAACACCCCCGTCCTTTCCTTTATCACTGCCAATTTCAACAGC

            .   .            ......................... ............


Z2491hpuA  AACAAACTGGCAGGCGAAATCATCGGCAACAGCGATTACGGGCCAAATGTGAAAATTGAA
117hpuA    AACAAACTGGCAGGCGAAATCCGCGGCAACAGCGATTACGGGCCGGATGTGGAAATTAAA
222hpuA    AACAAACTGGCAGGCGAAATCCGCGGCAACAGCGATTACGGACCGAGTGTGAAAATTGAA
114hpuA    AACAAACTGGCAGGCAAAATCATCGGCAACAGCGATTACGGGCCGGATGTGGAAATTAAA
134hpuA    AACAAACTGGCAGGCAAAATCATCGGCAACAGCGATTACGGGCCGGATGTGGAAATTAAA
119hpuA    AACAAACTGGCAGGCAAAATCATCGGCAACAGCGATTACGGGCCGGATGTGGAAATTAAA
88hpu      AACAAACTGGCAGGCGAAATCATCGGCAACAGCGATTACGGGCCGGATGTGAAAATTGAA
138hpuA    AACAAACTGGCAGGCGAAATCATCGGCAACAGCGATTACGGGCCGGATGTGAAAATTGAA
8047hpuA   CATAAACTGTCAGGCGAAATCATCGGCAACAGCGATTACGGGCCAAATGTGAAAATTGAA
Fam18hpuA  AACAAACTGGCAGGCGAAATCCGCGGCAACAGCGATTACGGACCGAGTGTGAAAATTGAA

            . ...... ..... .....  .................. ..   .... ..... ..


Z2491hpuA  AACGCAACGATTGACGGACTATCCTTTTCCGGCGATGCCACCTCCAACGGCAAAACCGGC
117hpuA    GAAGCACAGATTGACGGATTATCCTTTTCCGGCGATGCCACCTCCGGCGGCAAAACCGGC
222hpuA    AACGCAACGATCAGCGGTCCGAGCTTTTCCGGCAATGCCACCTCCGGCGGCAAAACCGGC
114hpuA    GAAGCACAGATTGACGGATTATCCTTTTCCGGCGATGCCACCTCCGGCGGCAAAACCGGC
134hpuA    GAAGCACAGATTGACGGATTATCCTTTTCCGGCGATGCCACCTCCGGCGGCAAAACCGGC
119hpuA    GAAGCACAGATTGACGGATTATCCTTTTCCGGCGATGCCACCTCCGGCGGCAAAACCGGC
88hpu      AACGCAACGATTGACGGACTATCCTTTTCCGGCAATGCCACCTCCGGCGGCAAAACCGGC
138hpuA    AACGCAACGATTGACGGACTATCCTTTTCCGGCAATGCCACCTCCGGCGGCAAAACCGGC
8047hpuA   AACGCAACGATTGACGGACTATCCTTTTCCGGCGATGCCACGTCCGGCGGCAAAAACGGC
Fam18hpuA  AACGCAACGATTAACAGTTTATCCTTTTCCGGCACTGCCACGTCCGGCGGGAAAACCGGC

            . ...  ...   . .      ..........  ...... ...  ... .... ....


Z2491hpuA  AACTTGGAAGGCAAGTTTTTTGGCAAGTTCAACAGCACCC---GCAGCAGCGACACCGGC
117hpuA    AAATTGGAAGGCAAGTTTTTTGGAAAATTCAACGGCTACC---GGGACACTGAAACCAGC
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222hpuA     AACTTGGAAGGCAAGTTTTTTGGAAAATTCAACGGCAGCTACGGCAACACTGAAACCAGC
114hpuA     AAGTTGGAAGGCAAGTTTTTTGGCAAATTCGACAGCAGCT---ATGACAGAGACACCAGC
134hpuA     AAGTTGGAAGGCAAGTTTTTTGGCAAATTCGACAGCAGCT---ATGACAGAGACACCAGC
119hpuA     AAGTTGGAAGGCAAGTTTTTTGGCAAATTCGACAGCAGCT---ATGACAGAGACACCAGC
88hpu       AAATTGGACGGCAAGTTTTTTGGCAAGTTCGACAGCACCC---GCAGCAGCGACACCGGC
138hpuA     AAATTGGACGGCAAGTTTTTTGGCAAGTTCGACAGCACCC---GCAGCAGCGACACCGGC
8047hpuA    AAATTGGAAGGCAAGTTTTTTGGAAAATTCAACGGCTACC---GGGACACTGAAACCAGC
Fam18hpuA   AATTTGGAAGGCAAGTTTTTTGGAAAATTCAACGGCTACC---GGGACACTGAAACCAGC
            .. ..... .............. .. ... .. ..  .        ..  .. ... ..

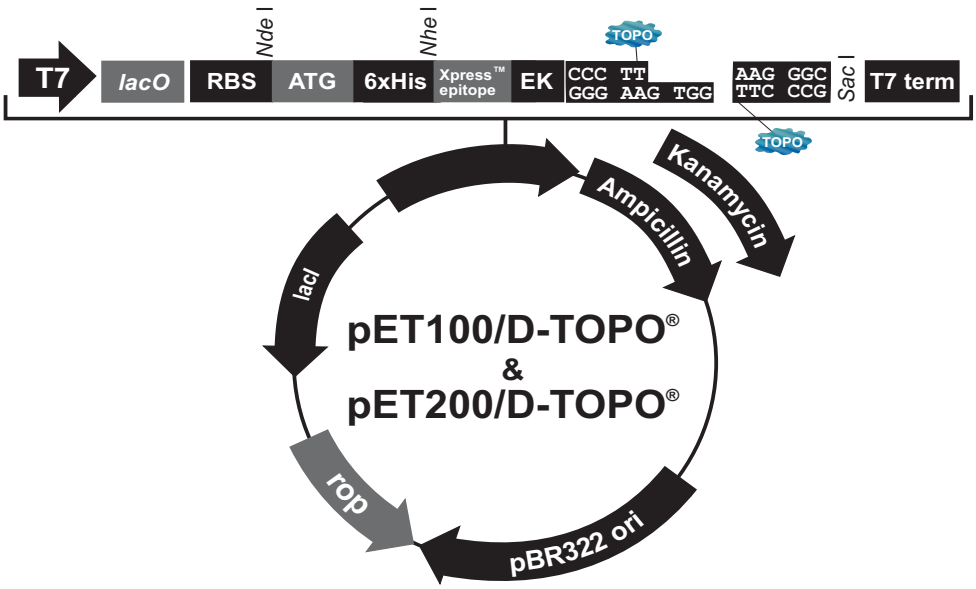
Z2491hpuA   ATAGGCGGCAAAATCACTTTTAAAGACGACCGCTCCCTCGATACCGTATTCGGCGGCGTG
117hpuA     ATAGGCGGCAAAATCACCTTTGACGGCGACCGCTCCCTCGATACCGTATTCGGCGGTGTG
222hpuA     ATAGGCGGCAAAATCACTTTTAAAGACGACCGCTCCCTCGATACCGTATTCGGCGGTGTG
114hpuA     ATAGGCGGCAAAATCACCTTTGACGGCGACCGCTCCCTCGATACCGTATTCGGCGGTGTG
134hpuA     ATAGGCGGCAAAATCACCTTTGACGGCGACCGCTCCCTCGATACCGTATTCGGCGGTGTG
119hpuA     ATAGGCGGCAAAATCACCTTTGACGGCGACCGCTCCCTCGATACCGTATTCGGCGGTGTG
88hpu       ATAGGCGGCAAAATCACTTTTAAAGACGACCGCTCCCTCGATACCGTATTCGGCGGCGTG
138hpuA     ATAGGCGGCAAAATCACTTTTAAAGACGACCGCTCCCTCGATACCGTATTCGGCGGCGTG
8047hpuA    ATAGGCGGCAAAATCACCTTTGACGGCGACCGCTCCCTCGATACCGTATTCGGCGGTGTG
Fam18hpuA   ATAGGCGGCAAAATCACCTTTGACGGCGACCGCTCCCTCGATACCGTATTCGGCGGTGTG
            ................. ... . . ............................. ...

Z2491hpuA   ATTTATGAGAAAAAACTTGATGACAAAACCAGTCAAGATACCAACCATCTCAAAAAATAA
117hpuA     AGTTATAAAAAAGAACTTGAAAACAACACTGATACAAGTACCACCCATCTCACAAAATAA
222hpuA     AGTTATGTAAAAAAACTTGATGAAACCGCCAATCGAGATACCGAACACCTTACTAAACAA
114hpuA     AGTTATAAAAAAGAACTTGAAAACAACACTGATATGAGTACCACCCATCTCACAAAATAA
134hpuA     AGTTATAAAAAAGAACTTGAAAACAACACTGATATGAGTACCACCCATCTCACAAAATAA
119hpuA     AGTTATAAAAAAGAACTTGAAAACAACACTGATATGAGTACCACCCATCTCACAAAATAA
```

```
88hpu       ATTTATGAGAAAAAACTTGATGACAAAACCAGTCAAGATACCACCCATCTCACAAAATAA
138hpuA     ATTTATGAGAAAAAACTTGATGACAAAACCAGTCAAGATACCACCCATCTCACAAAATAA
8047hpuA    AGTTATAAAAAAGAACTTGAAAACAACACTGATACAAGTACCACCCATCTCACAAAATAA
Fam18hpuA   AGTTATAAAAAAGAACTTGAAAACAACACTGATACAAGTACCACCCATCTCACAAAATAA
            . ....   ... .......  . .   .   .     ....   .. .. .  ... ..
```

Appendix 2

Map and Features of pET200/D-TOPO® (Invitrogen)



Features of pET200/D-TOPO®

Feature	Benefit
T7 promoter	Permits high-level, IPTG-inducible expression of your recombinant protein in *E. coli* strains expressing the T7 RNA polymerase.
T7 forward priming site	Allows sequencing in the sense orientation.
lac operator (lacO)	Binding site for lac repressor that serves to reduce basal expression of your recombinant protein.
Ribosome binding site	Optimally spaced from the TOPO® Cloning site for efficient translation of PCR product.
N-terminal 6xHis tag	Permits purification of recombinant fusion protein on metal-chelating resin (*i.e.* ProBond™ or Ni-NTA). In addition, allows detection of recombinant protein with the Anti-HisG Antibodies.
Xpress™ epitope (Asp-Leu-Tyr-Asp-Asp-Asp-Asp-Lys)	Allows detection of the fusion protein by the Anti-Xpress™ Antibodies.
Enterokinase (EK) recognition site (Asp-Asp-Asp-Asp-Lys)	Allows removal of the N-terminal tag from your recombinant protein using an enterokinase such as EKMax™.
TOPO® Cloning site (directional)	Permits rapid cloning of your PCR product for expression in *E. coli*.
T7 Reverse priming site	Allows sequencing of the insert.
T7 transcription termination region	Sequence from bacteriophage T7 which permits efficient transcription termination.
bla promoter	Allows expression of the ampicillin resistance gene.
Ampicillin resistance gene (β-lactamase) (pET100/D-TOPO® only)	Allows selection of the plasmid in *E. coli*.
Kanamycin resistance gene (pET200/D-TOPO® only)	Allows selection of the plasmid in *E. coli*.
pBR322 origin of replication (*ori*)	Permits replication and maintenance in *E. coli*.
ROP ORF	Interacts with the pBR322 origin to facilitate low-copy replication in *E. coli*.
lacI ORF	Encodes lac repressor which binds to the T7*lac* promoter to block basal transcription of the gene of interest and to the *lacUV5* promoter in the host chromosome to repress transcription of T7 RNA polymerase.

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