The role of exopolyphosphatase in *Neisseria meningitidis* infection

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Abstract

The development of vaccines against serogroup B *Neisseria meningitidis* to reduce the morbidity and mortality of meningococcal disease is a major public health priority. We developed a novel genetic screen for immunogens present on the bacterial surface using human immune sera with bactericidal activity. We found that two mutants lacking *nmb1467* survived in high concentrations of sera from two patients, while the wild-type strain was killed. Biochemical assays using purified recombinant NMB1467 indicated that *nmb1467* encodes an exopolyphosphatase (PPX) with the ability to hydrolyse inorganic polyphosphate (poly P). In addition, we demonstrated that the Δ*ppx* mutant has at least 2-fold more poly P than the wild-type strain. Therefore, we designated NMB1467 as PPX. We showed that *N. meningitidis* mutant lacking the *ppx* had an increased resistance against normal human complement system. Substitution of the glutamic acid at residue 147 of PPX with an alanine significantly reduced the enzymatic activity *in vitro*, and contributed to increased level of poly P in *N. meningitidis* and the resistance of bacteria against the complement-mediated killing. Levels of polysaccharide capsule and lipopolysaccharide (LPS) sialylation, two important virulence factors, were not affected by the loss of *ppx* in *N. meningitidis*. Using flow cytometry, we demonstrated that binding of factor H (fH), the negative regulator of the alternative pathway of complement activation, to the bacterial surface was increased in the strain lacking PPX. By Western blot analysis, we did not detect a significant change in the expression of the fH receptor, indicting another mechanism is involved in the fH binding to the bacterial surface and resistance of bacteria against complement-mediated killing.

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Abbreviations

AP	alternative pathway
APS	ammonium persulfate
BBB	blood brain barrier
BHI	brain-heart infusion
bp	base pairs
BSA	bovine serum albumin
C	complement
CFU	colony forming unit
CMP-NANA	cytidine 5'-monophospho-N-acetyl-neuraminic acid
CP	classical pathway
CTAB	cetyl-trimethyl-ammonium bromide
CSF	cerebrospinal fluid
EGTA	ethylene glycol tetraacetic acid
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked-immunoabsorbent assay
Ery^R	erythromycin resistant
fB	factor B
fH	factor H
fI	factor I
GSI	Genetic Screening for Immunogens
Hib	*Haemophilus influenzae* type b
HRP	horse-radish peroxidase
Ig	immunoglobulin
Kan^R	kanamycin resistant
kb	kilobase pairs
kDa	kilodalton
LB	Luria Bertani
LNTn	lacto-*N*-neotetraose
LP	lectin pathway
LPS	lipopolysaccharide
mAb	monoclonal antibody
NHS	normal human serum
MAC	membrane attack complex
MASP	MBL-associated serine protease

MBL	mannose-binding lectin
MLEE	multilocus enzyme electrophoresis
MLST	multi locus sequence typing
mAb	monoclonal antibody
NHS	normal human serum
O.D.	optical density
OMP	outer membrane protein
OMV	outer membrane vesicle
ORF	open reading frame
pAb	polyclonal antibody
PBS	phosphate buffered saline
PBS-T	PBS/0.1%Tween20
PCR	polymerase chain reaction
PFA	paraformaldehyde
Por	porin
PVDF	polyvinylidene fluoride
poly P	inorganic polyphosphate
PPA	pyrophosphatase
PPK	polyphosphate kinase
PPN	endopolyphosphatase
PPX	exopolyphosphatase
p.s.i.	pound per square inch
r.p.m	revolutions per minute
SBA	bactericidal activity assay
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TAE	tris acetate EDTA
TE	tris EDTA
TEMED	N, N, N', N'-tetramethylethylenediamine
Tris	tris(hydroxymethyl)aminomethane
TSDS-PAGE	tricine sodium dodecyl sulphate polyacrylamide gel electrophoresis
UV	ultraviolet
V	volts
xg	times gravity (relative centrifugal force)

Introduction

1.1 History

Neisseria meningitidis is an aerobic and Gram-negative diplococcal bacterium that is a leading cause of bacterial meningitis. In 1805, Vieusseux documented 33 deaths from a "spotted fever" with symptoms of violent headache, fever and meningitis in Switzerland (Vieusseux, 1806). This is widely accepted as the first description of meningococcal disease. In 1887, Weichselbaum first isolated an organism from cerebrospinal fluid (CSF) from six of eight fatal cases with meningococcal disease and named the bacterium "*Diplococcus intracellularis meningitidis*" (Weichselbaum, 1887). The organism was renamed *Neisseria meningitidis* after Albert Neisser's discovery of *Neisseria gonorrhoeae* (Kampmeier, 1978). So far, *N. meningitidis* has only been isolated from humans.

In the pre-antibiotic era, the mortality rate of meningococcal disease was more than 70% (Cartwright, 1995). In the early 20th century, the German researcher Jochmamn first raised antisera in rabbits and horses, which proved to be protective in a porcine model of disease (Kolle and Wasserman, 1906). By 1908, anti-meningococcal sera were used for intrathecal treatment in Europe and the USA with a dramatic improvement in the outcome (Kolle and Jobling, 1908). However, toxicity resulting from the administration of foreign proteins and cases of secondary meningitis were often associated with serum therapy, so it was abandoned when antibiotics became available (Cartwright, 1995). In 1937, sulfanilamide was reported to be the first specific and highly effective treatment for meningococcal infection (Schwentker, 1937).

The first attempts to develop a vaccine were made by Sophian and Black in the USA using heat-killed bacteria (Sophian and Black, 1912). Until 1991, strain-specific outer membrane vesicles had been used to reduce local outbreaks of serogroup B disease (Bjune *et al.*, 1991b; Sierra *et al.*, 1991). In 1999, a protein/polysaccharide conjugate serogroup C vaccine was introduced in UK and the disease was rapidly controlled (Ramsay *et al.*, 2001). Despite the availability of antibiotics and the introduction of vaccines, the case fatality from meningococcal disease remains 5-10% and up to 25% of survivors are left with permanent sequelae such as sensorineural hearing defects and/or loss of limbs (Tzeng and Stephens, 2000).

1.2 Classification of *N. meningitidis*

1.2.1. Serological typing of *N. meningitidis*

A variety of immunological and genetic methods has been developed to categorise strains of *N. meningitidis* for epidemiological surveillance and the management of meningococcal disease. Serological typing has been traditionally used to identify surface expressed antigens including the polysaccharide capsule, lipopolysaccharide (LPS) and outer membrane proteins (OMPs).

Based on the structural and immunological differences of the polysaccharide capsule, meningococci are currently divided into 13 serogroups (A, B, C, D, X, Y, Z, E, W-135, H, I, K and L), with six serogroups (A, B, C, W-135 Y and X) commonly associated with meningococcal disease (Jodar *et al.*, 2002; Stephens *et al.*, 2007). Characterisation of the serogroup of case isolates is crucial to prevent further cases in close contacts, as most vaccines are serogroup specific (Harrison, 2006). A number of

commercial agglutination kits are available to distinguish individual serogroups directly using live bacteria recovered from the CSF or blood, or indirectly with dead bacteria. Antibodies recognising different serogroups have also been applied in enzyme linked immunosorbent assays (ELISAs) to screen large numbers of samples (Rosenqvist *et al.*, 1990). However, a proportion of carriage isolates do not express a capsule, and therefore cannot be grouped according to this typing method (Yazdankhah and Caugant, 2004).

N. meningitidis can be classified into at least 20 serotypes on the basis of the class 2/3 OMP PorB, and into at least 10 serosubtypes based on the class 1 OMP PorA, depending on the reactivity of these porins to type-specific antibodies (Hansman and Ashton, 1994). The type specific epitopes are restricted to variable regions in the surface exposed loops of these proteins (Minetti *et al.*, 1998; Russell *et al.*, 2004; Sacchi *et al.*, 1998). Serotyping and serosubtyping are usually performed with suspensions of heat-killed meningococci as antigens in whole-cell or dot-blot ELISAs (Wedege *et al.*, 1990). However, the emergence of multiple antigenic variants of PorA and PorB which are not recognised by available antibodies has limited the utility of these forms of typing (Russell *et al.*, 2004; van der Ende *et al.*, 2003).

Differences in LPS allow *N. meningitidis* to be divided into 12 immunotypes (Scholten *et al.*, 1994). Most meningococcal isolates express more than one immunotype of LPS. Immunotype L3,7,9 is the most prevalent in serogroup B and C meningococci, followed by immunotypes L2 and L1,8 (Jennings *et al.*, 1999). Immunotypes L10 and L11 are uniquely associated with and prevalent in serogroup A isolates (Scholten *et al.*, 1994). An example of this serological typing is

B:4:P1.4:L3,7,9 indicating the serogroup (B), serotype (4), serosubtype (P1.4) and immunotype (L3,7,9) (Tzeng and Stephens, 2000).

Serological typing has been important for public health decisions and vaccine development, but is less suitable for detailed epidemiological studies for the following reasons. Firstly, *N. meningitidis* is naturally competent for DNA uptake and capable of exchanging genetic information encoding surface antigens (Alexander *et al.*, 2004). For example, capsule switching from serogroup B to C, following the acquisition of the gene *siaD*, provides one mechanism by which closely related virulent meningococcal clones may not be recognised by traditional serogroup-based methods (Swartley *et al.*, 1997). In addition, surface expressed immunogenic antigens used for serological typing are likely to be under selective pressure, and therefore variants may arise rapidly in populations of *N. meningitidis* (Hammerschmidt *et al.*, 1996; Jennings *et al.*, 1999; Johansson *et al.*, 2005; Swartley *et al.*, 1997). Low level expression of capsule or other antigens used for serotyping or serosubtyping of strains recovered from carriers also limits the use of serological typing methods. Thus, since the late 1990s molecular typing techniques have been increasingly applied to understand the epidemiology and evolution of *N. meningitidis*.

1.2.2 Molecular characterisation of *N. meningitidis*

Multilocus enzyme electrophoresis (MLEE) is a method that identifies phylogenetic relationships in bacterial species by measuring allelic variation in metabolic enzymes based on the electrophoretic mobility of twenty-five housekeeping enzymes (Selander *et al.*, 1986). Each novel combination of allelic variants is referred to as an electrophoretic type (ET), and strains possessing similar ETs are grouped into clonal

complexes (Caugant *et al.*, 1987b). Investigation of global meningococcal epidemiology by MLEE reveals that the ET-5 and ET-37 complexes, which include serogroup B, C and W135 strains, have been associated with several outbreaks and most sporadic meningococcal disease in Europe and the USA (Raymond *et al.*, 1997; Swartley *et al.*, 1997; Taha *et al.*, 2000). However, as this method requires calibration with standard strains and is technically demanding, MLEE has been less often used in reference laboratories since the advent of multilocus sequence typing (MLST) (Maiden *et al.*, 1998).

MLST is a DNA sequence-based version of MLEE, recording variations in the nucleotide sequence of housekeeping genes distributed around the genome (Spratt, 1999). MLST categorises bacteria according to the nucleotide sequence of fragments of approximately 433-501 bp in length from seven housekeeping genes: *abcZ*, *adk*, *aroE*, *fumC*, *gdh*, *pdhC* and *pgm*. As these genes are dispersed across the genome, alleles are unlikely to be co-inherited through a single recombination event. In addition, the housekeeping genes are also generally not in close proximity to genes that are under strong selective pressure and therefore can be used to assess the genetic background of *N. meningitidis* (Maiden *et al.*, 1998). Every unique sequence at each locus is assigned an allele number, and every unique combination of alleles is assigned a sequence type (ST). Highly prevalent STs usually have the highest number of related STs that differ at only one or two loci, and genetically related STs surrounding a central genotype are grouped into clonal complexes (Feavers *et al.*, 1999). MLST provides unambiguous and portable results that can be compared between different laboratories on a central database (http://pubmlst.org/neisseria) via the internet (Jolley *et al.*, 2004). However, MLST might not be sufficient for outbreak

investigations, thus combinations of MLST with other approaches, such as pulsed-field gel electrophoresis, sequencing of 16S rRNA genes, multilocus variable-number tandem repeats analysis and sequencing of genes encoding outer membrane proteins, have been used (Popovic *et al.*, 2001; Sacchi *et al.*, 2002; Yazdankhah *et al.*, 2005).

Amplification and sequencing the capsule biosynthesis genes has been used to determine the serogroup of isolates, even in the absence of capsule expression (Lansac *et al.*, 2000; Porritt *et al.*, 2000). It can also be directly applied to clinical samples without culture (Abdel-Salam, 1999). Similarly, amplification of *porA* and *porB* by PCR, and querying the PorA and PorB variable region sequence database (http://neisseria.org/nm/typing/pora/ and http://neisseria.org/nm/typing/porb/) have been used to characterise strains (Jelfs *et al.*, 2000). Recently, more surface antigens have been employed as epidemiological markers. For example, FetA is found to be conserved within hyper-invasive lineages, and a database of the sequence of the variable region in *fetA* (http://neisseria.org/nm/typing/feta) is available for classifying strains (Thompson *et al.*, 2003).

1.3. Meningococcal carriage

N. meningitidis is an obligate commensal in humans, and it can colonise the nasopharynx without causing the disease, a phenomenon known as carriage (Yazdankhah and Caugant, 2004). The carriage rate ranges between 5-28% in healthy individuals (Stephens *et al.*, 2007), and very high carriage rates (> 90%) have been were reported among military recruits during serogroup A epidemics (Caugant *et al.*, 1992).

A number of factors are associated with meningococcal carriage. The carriage rate is age-dependent; it is less than 3% in children who are less than 4 years old, then increases to between 24-37% in the 15-24 year age group, and declines to about 10% in the elderly (Cartwright *et al.*, 1987; Caugant *et al.*, 1994). Carriage rates are higher in closed populations, such as military recruits, university students, and household contacts of cases (Cartwright *et al.*, 1991; Caugant *et al.*, 1992; Imrey *et al.*, 1995). Individuals with upper respiratory tract damage caused by microbial infection or smoking are at higher risk of being carriers (Artenstein *et al.*, 1967; Blackwell *et al.*, 1990; Moore *et al.*, 1990). Males have more often been reported to be carriers than females for unknown reasons (Cartwright *et al.*, 1987; Caugant *et al.*, 1994). A one year post-vaccination survey in the UK showed that introduction of meningococcal serogroup C conjugate vaccine reduced the carriage of serogroup C meningococci in young adults aged 15-17 years by 66%, but had no impact on the carriage of other serogroups strains (Maiden and Stuart, 2002).

Culturing bacteria following nasopharyngeal swabbing is the conventional method of studying *N. meningitidis* carriage. However, carriage rates obtained by this method depend on the number of swabs taken, the site swabbed and the sensitivity of nasopharyngeal culture (Broome, 1986). A recent study suggests that nasopharyngeal swabbing may underestimate the carriage rate, with carriage detected in only in 10% patients undergoing tonsillectomy, compared to carriage of 45% identified by immunohistochemistry analysis of tonsillar tissue (Sim *et al.*, 2000; Yazdankhah and Caugant, 2004).

1.4 Epidemiology

Meningococcal infection is spread by direct contact with infectious respiratory secretions and by droplet transmission. The incidence of meningococcal disease varies from 1 case per 100,000 population per year to over 1,000 cases per 100,000 population per year (Tzeng and Stephens, 2000).

Serogroup A meningococcal strains are prevalent in developing countries and are the main cause of both endemic and epidemic meningococcal disease in Africa. In a region of sub-Saharan Africa extending from Senegal to Ethiopia (referred to as the meningitis belt), sporadic infections occur in seasonal annual cycles, while large-scale epidemics happen at greater intervals with an irregular pattern (Greenwood *et al*., 1885; World Health Organisation 2005). During a major outbreak in 1996, the number of cases reached 150,000 with 20,000 deaths, and molecular and genetic analysis revealed that most the strains belonged to the ST-7 or ST-5 complexes (Tzeng and Stephens, 2000). Recently, attack rates of meningococcal disease have also increased in non-meningitis-belt countries in Africa. The emergence of the ST-7 complex was first reported in China in the early 1990s, and this ST complex then spread to Russia in 1996 (Girard *et al.*, 2006; Yang *et al.*, 2007).

Serogroup B and C meningococcal strains are associated with endemic disease in developed countries. They account for 80% of cases in certain European countries, such as the UK and Spain, and are responsible for 30-40% of cases in North America (Cartwright *et al.*, 2001). Endemic infection, with incidences ranging from 1 to 10 per 100,000 population, often occur in annual cycles with a peak incidence in the first quarter of the year (Ramsay *et al.*, 1997). Epidemics of serogroup B disease have

been reported in Cuba, Chile, Norway and New Zealand (Bovre *et al.*, 1977; Caugant *et al.*, 1987a; Oster *et al.*, 2005; Sierra *et al.*, 1991). The global incidence of serogroup B disease is about 20,000 to 80,000 cases per year, with a mortality rate of approximately 10%. Genotypic analysis suggests that most strains are ET-5/ST-32 and ET-37/ST-11 (Vogel *et al.*, 2000).

Serogroup W-135 (ET-37/ST11) has caused worldwide outbreaks associated with the Hajj pilgrimage and also disease in African meningitis belt countries such as Burkina Faso (Aguilera *et al.*, 2002; Decosas and Koama, 2002; Lingappa *et al.*, 2003). Disease resulting from serogroup Y (ET-501/ST-23) has been increasing in the USA since 1990 (Rosenstein *et al.*, 2001), and has also been reported in Israel and Sweden (Memish, 2002). Serogroup X has been identified as the cause of local outbreaks in parts of sub-Saharan Africa such as in Niger (Nathan *et al.*, 2005).

Many factors have been associated with an increased susceptibility to disease, of which the most important is the degree of host immunity. In England and Wales, the peak incidence of serogroup B and C disease occurs at 6 months of age when maternal bactericidal antibodies have largely disappeared and before active immunity has developed (Ramsay *et al.*, 2003). The relatively low case incidence compared with carriage in military recruits is related to the level of serum bactericidal antibodies induced by nasopharyngeal carriage (Almeida-Gonzalez *et al.*, 2004; Goldschneider *et al.*, 1969b; Orren *et al.*, 1987). Individuals with complement deficiencies are highly susceptible to meningococcal disease, and are likely to experience recurrent attacks (Figueroa *et al.*, 1993; Orren *et al.*, 1987; Vega and Quinby, 1994). In addition, fully functional opsonisation and phagocytosis are also

necessary for the protection of the host against meningococcal disease (Sparling, 2002).

1.5 *N. meningitidis* virulence determinants

1.5.1 Polysaccharide capsule

The polysaccharide capsule is the outermost layer of meningococcus and interacts with the external environment, mediating a variety of biological processes relevant to pathogenesis. Based on its biochemical and physical properties, the meningococcal capsule is similar to the capsule from *Escherichia coli* K1 and *Haemophilus influenzae*, and is categorised as a group II capsule (Frosch *et al.*, 1989; Frosch *et al.*, 1991). N-acetyl neuraminic acid (NANA, sialic acid) is a crucial component of the polysaccharide capsule of disease-associated serogroups B, C, W135 and Y. The serogroup B capsule is composed of α(2-8)-linked sialic acid that is also present as a modification of the mammalian neural cell adhesion molecule (NCAM), while, the serogroup C capsule is composed of α(2-9)-linked sialic acid (Kabat *et al.*, 1986; Liu *et al.*, 1971a). Serogroups Y and W135 capsules contain alternating units of sialic acid with D-glucose or D-galactose, respectively (Bhattacharjee *et al.*, 1976). As the exception, the serogroup A capsule does not contain sialic acid, and instead it is composed of α(1-6) linked N-acetyl-D-mannosamine-1-phosphate (Liu *et al.*, 1971b).

Genes involved in meningococcal capsule biosynthesis are clustered at a chromosomal locus, which is termed the capsule biosynthesis locus (*cps*). The *cps* is about 24 kb length and divided into five distinct regions: E, C, A, D and B (Frosch *et al.*, 1989). Region A encodes enzymes for the biosynthesis of the capsular

polysaccharide, while regions B and C are involved in the translocation of the polysaccharides to the cell surface. The capsule biosynthesis genes in region A (*siaA, siaB, siaC, siaD*) are transcribed co-ordinately as a single operon (Swartley *et al.*, 1996). The *siaA, siaB, siaC* genes are highly conserved in different serogroups, whereas, *siaD* gene sequence is specific for different serogroups (Claus *et al.*, 1997).

Despite the distinct chemical composition of the capsule based on genes in region A, the genes in regions B and C share a high degree of homology among all serogroups, and even in the genomes of other bacterial species expressing group II capsules (Claus *et al.*, 1997). Genes in region C (*ctrA, ctrB, ctrC, ctrD*) share a divergently transcribed promoter region with genes in region A (Swartley *et al.*, 1996). CtrABCD together with an ATP-binding cassette (ABC) transport system participate in the transport of the polysaccharide chains to the cell surface (Frosch *et al.*, 1992). Genes in region B (*lipA, lipB*), which is separate from the *sia* and *ctr* operons, are responsible for the phospholipid substitutions at the reducing end of polysaccharide chains (Frosch and Muller, 1993).

Encapsulation is a pre-requisite for meningococci to survive in the systemic circulation of host and cause disease. Epidemiologic studies of an outbreak of ET-5 in Washington revealed that expression of capsule was higher in invasive isolates recovered from the blood and CSF in comparison to strains present in the nasopharynx (Tzeng and Stephens, 2000). Similarly, the genotype and phenotype of isolates recovered from carriers were the same as the outbreak strains, with the exception that they lacked capsule, during the Stonehouse outbreak of serogroup B

meningococcal disease in England (Cartwright *et al.*, 1987; Hammerschmidt *et al.*, 1996).

The importance of the polysaccharide capsule in meningococcal pathogenesis is not only demonstrated by epidemiological and clinical studies, but also by clear experimental evidence. Encapsulation is crucial for bacteria to avoid complement-mediated bacteriolysis during systemic infection. The sialic acid capsule of serogroup B strains is indispensable for systemic infection in the infant rat and murine models (Mackinnon *et al.*, 1993; Vogel *et al.*, 1996). Interruption of *siaD* leads to loss of capsule expression, and resultant mutants are highly sensitive to complement-mediated killing compared to the parental strains (Frosch *et al.*, 1990). It is suggested that the capsule can down-regulate the alternative complement pathway and/or prevent the insertion of the Membrane Attack Complex (MAC) into the bacterial outer membrane (Jarvis and Vedros, 1987; Ram *et al.*, 1999).

In addition, the polysaccharide capsule inhibits phagocytosis of *N. meningitidis* by macrophages, with decreased rates of incorporation of the major lysosomal integral membrane protein (LAMP-1) into the phagosome (Read *et al.*, 1996). This antiphagocytic property was further confirmed by the observation that expression of a serogroup A, B, or C capsule dramatically decreased the adherence of bacteria to dendritic cells (DC) and phagocytosis (Unkmeir *et al.*, 2002). Furthermore, the capsule protects bacteria against cationic antimicrobial peptides (CAMPs), such as polymyxin B, defensins and protegrin PG-1, enabling bacteria to survive and replicate within epithelial or endothelial cells (Spinosa *et al.*, 2007). The expression of *siaD* and *lipA*, required for the capsular polysaccharide biosynthesis and translocation

respectively, are up-regulated when bacteria were recovered from eukaryotic cells in comparison with bacteria cultured in medium alone (Spinosa *et al.*, 2007).

1.5.2 Lipopolysaccharide

Lipopolysaccharide (LPS) is the most abundant antigenic component in the *N. meningitidis* cell envelope, and is a key virulence factor involved in the pathogenesis of meningococcal sepsis and meningitis (Brandtzaeg *et al.*, 2001; Verheul *et al.*, 1993).

LPS is an amphipathic glycolipid with a molecular mass of about 4.8 kDa. It consists of the hydrophobic lipid A domain embedded in the outer membrane, joined by the inner core carbohydrates to two short chains of oligosaccharides (Jennings *et al.*, 1980). Meningococcal LPS lacks the repeating O-antigen found in enteric pathogens, and only has a few sugar residues linked to the inner core, and thus is often referred to as lipooligosaccharide (LOS) (Jennings *et al.*, 1987). The lipid A backbone, also called endotoxin, is the main toxic component of LPS that induces inflammatory responses (Brandtzaeg *et al.*, 2001). The inner core region is composed of 3-deoxy-D-manno-2-octulosonic acid (KDO) and heptose (Hep) attached to lipid A. The variable side chains (α-, β-) are attached to the KDO and Hep residues (Kahler *et al.*, 1998). The carbohydrate moieties are highly diverse in the α-chain, and the biochemical and immunogenic differences in α-chain are the basis of the different immunotypes (Kurzai *et al.*, 2005).

LPS is essential for adhesion to and colonisation of host cells by *N. meningitidis*. Inactivation of *lpxA*, encoding the enzyme responsible for the first step of lipid A

biosynthesis, severely reduces bacterial adhesion to and invasion of epithelial cells (Gorter *et al.*, 2003). A *pgm* mutant, which is unable to express the α-chain of LPS, invades epithelial cells much more slowly than the wild-type strain, and has a significant defect for survival in the murine bloodstream (Plant *et al.*, 2006). The important role of LPS in early infection is further supported by the finding that a mutant without LPS is unable to persist in the lung and invade the blood in a murine model (Zarantonelli *et al.*, 2006).

LPS is important for *N. meningitidis* to escape killing by the immune system. Full length LPS is required for bacteria to avoid complement-mediated lysis, regardless of the expression of capsule (Hammerschmidt *et al.*, 1994; Kahler *et al.*, 1998). A lacto-N-neotetraose (LNnT) moiety in the α-chain of LPS is identical to a human blood group antigen, and is an excellent example of molecular mimicry used by *N. meningitidis* to subvert the immune system (Mandrell *et al.*, 1988). The LNnT epitope present in LPS can be sialylated exogenously in the presence of 5'-monophosphate-N-acetyl-neuraminic acid (CMP-NANA) or endogenously via the sialic acid biosynthesis pathway in serogroup B, C, W-135 and Y strains (Hammerschmidt *et al.*, 1994; Mandrell *et al.*, 1991). Strains with full length and sialylated L3,7,9 LPS are more frequently associated with invasive disease, whereas, L8 immunotypes predominant in carriage isolates (Jones *et al.*, 1992). Sialylation has been shown to contribute to bacteraemia in the infant rat model (Vogel *et al.*, 1996). However, the contribution of LPS sialylation to meningococcal serum resistance is controversial (Ram *et al.*, 1999; Smith *et al.*, 1995). Some but not all of Δ*lst* mutants of serogroup B *N. meningitidis* strains have increased sensitivity to complement-mediated killing in the presence of high concentrations of serum only (Vogel *et al.*, 1999). By masking

the surface molecules Opa or Opc, sialylation of the LNnT epitope renders bacteria resistant against opsonophagocytosis by human neutrophils and phagocytosis by DCs (Unkmeir *et al.*, 2002).

LPS is released from *N. meningitidis* in membrane blebs. The level of LPS in the plasma and CSF is correlated with the level of inflammatory mediators, clinical severity and the outcome from disease (Brandtzaeg *et al.*, 2001). Patients with persistent septic shock, multiple organ failure, and severe coagulopathy have extraordinarily high levels of LPS in plasma. Mortality related to shock increases 10-fold with an increase of plasma LPS from 10 to 100 endotoxin units/ml (Brandtzaeg *et al.*, 2001). Meningococcal LPS binds to the CD-14 receptor and Toll-like receptor-4 (TLR-4) with its co-factor, myeloid differentiation protein-2 (MD2), on macrophages, granulocytes and endothelial cells (Ingalls *et al.*, 2000). This triggers the activation of nuclear factor κB (NFκB) and other cytokine pathways, leading to increased production of tumour necrosis factor (Stephens *et al.*, 2007; Westendorp *et al.*, 1995).

1.5.3 Pili

Pili are filamentous, hair-like outer membrane protein organelles that extend beyond the bacterial surface (Devoe and Gilchrist, 1974). Type IV pili (Tfp) have several remarkable functions in many Gram-negative bacteria, including bacterial motility, adhesion to host cells, aggregation, biofilm formation, natural transformation and immune escape (Merz and So, 2000; Nassif *et al.*, 1997; Rudel *et al.*, 1995). The importance of pili to the pathogenesis of *N. meningitidis* has been demonstrated by

the findings that pili are ubiquitously expressed by clinical isolates from patients with meningococcal sepsis (Stephens and McGee, 1981).

Expression of Tfp facilitates *N. meningitidis* adhesion to epithelial cells during colonisation of the mucosal surface, and to endothelial cells while crossing the blood-brain barrier (Nassif *et al.*, 1997). Mutants lacking piliation adhere to both epithelial and endothelial cells several orders of magnitude less than piliated meningococci (Stephens and McGee, 1981; Stephens *et al.*, 1983; Virji *et al.*, 1991). Meningococcal pili are mainly composed of thousands of copies of the pilin protein, PilE (Potts and Saunders, 1988). *N. meningitidis* can express Class-I or Class II pili, with the former recognised by the monoclonal antibody, SM1 (Virji *et al.*, 1989). Biogenesis of Tfp includes the assembly of fibres in the periplasm, stabilisation and secretion to the bacterial surface, and requires at least 15 genes (Craig *et al.*, 2006; Tonjum and Koomey, 1997). For example, PilD, PilF and PilG are involved in fibre assembly, while PilQ contributes to the secretion and translocation of fibres to the bacterial surface (Tonjum *et al.*, 1995). In addition to proteins required for pili expression, PilT, PilU, PilC, PilX and PilW contribute to pilus function. For instance, PilX mediates bacterial aggregation but not adhesion during early interactions between *N. meningitidis* and human cells (Carbonnelle *et al.*, 2005). PilT is required for bacterial competence and twitching motility, and plays a key role in interactions with the host cells by up-regulating the expression of PilC and by mediating pilus retraction (Pujol *et al.*, 1999; Wolfgang *et al.*, 1998; Yasukawa *et al.*, 2006).

PilC alleles (PilC1 and PilC2) are required for fibre stabilisation and adhesion, as a double *pilC* knock-out mutant is non-piliated (Nassif *et al.*, 1997). Encapsulated

pilC2^{+}*/pilC1*$^{-}$ strains are piliated but not adhesive, indicating that adhesion is mediated by the expression of PilC1 but not PilC2. This could be explained by the presence of a putative cell binding domain in the PilC1 but not in PilC2 (Nassif *et al.*, 1997). The adhesiveness of bacteria is correlated with the expression of PilC1, the transcription of which is modulated by *pilA* and *pilT* (Taha *et al.*, 1998).

In addition to the expression of PilC, sequencing of *pilE* reveals that high level of adhesiveness is associated with pilin antigenic variation. For example, in the hypervariable region of PilE, an aspartic acid (Asp) residue is present at amino acid 140 in low adhesive strains, whereas a lysine (Lys) is found in highly adhesive isolates (Nassif *et al.*, 1997). The pili of highly adhesive variants mediate aggregation and the formation of large bundles, and strains grow as colonies on infected monolayers of human cells. This enhanced adhesiveness could be due to increased bacteria-bacteria interactions allowing a high density of adhesins interacting with eukaryotic receptors (Marceau *et al.*, 1995).

The proposed receptor for the Tfp is CD46, which is expressed in almost all human cells, apart from erythrocytes. Purified pili can bind to a protein of the size of CD46, and adhesion of *N. meningitidis* to cells is blocked by anti-CD46 antibodies (Kallstrom *et al.*, 1997). Furthermore, transgenic mice expressing human CD46 are susceptible to meningococcal disease (Johansson *et al.*, 2005). The initial attachment of gonococci to host epithelial cells results in phosphorylation of tyrosine at 354 amino acid on the intracellular domain of CD46 by Src-family kinase. Inhibition of these tyrosine kinases results in a significant reduction in the adhesion of the gonococcus to the epithelium (Lee *et al.*, 2002). However, the recent finding that

down-regulation of CD46 by RNA interference has no effect on the binding efficiency of piliated gonococci or purified PilC2 protein calls into questions the role of CD46 as a receptor in *N. meningitidis* infection (Kirchner *et al.*, 2005)

1.5.4 Outer membrane proteins

As a Gram-negative bacterium, *N. meningitidis* has an outer membrane (OM) containing numbers of proteins in addition to lipids and LPS. The major outer membrane proteins are categorised into five classes based on their different mobility on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Tsai *et al.*, 1981). Studies identified that class 2 and class 3 proteins are encoded by two different alleles of a single gene, *porB*, and demonstrated class 1, 4 and 5 proteins are PorA, Rmp and Opa/Opc, respectively (Aho *et al.*, 1991; Blake *et al.*, 1989; Olyhoek *et al.*, 1991; Tsai *et al.*, 1981).

The class 1 PorA and class 2/3 PorB are porins. PorA forms cation-selective pores in the OM, while, PorB forms anion-selective channels, which allow ion exchange between the inside of bacteria and the external environment (Tommassen *et al.*, 1990). The molecular mass of PorA varies between 44 and 47 kDa in different strains, whereas PorB is present as one of two mutually exclusive forms. Structural analysis predicts that PorA and PorB have eight surface exposed hydrophilic loops with variation between strains (van der Ley *et al.*, 1991). The predicted surface loops I and IV are designated as variable regions 1 and 2 (VR1 and VR2), respectively, while loops V and VI are designated as semi-variable regions (SR1 and SR2) (Sacchi *et al.*, 1998). The immunological properties of the VRs are responsible for serosubtype classification, while nucleotide sequence analysis of VRs has been be used for

epidemiological studies (Devoy *et al.*, 2005; Feavers *et al.*, 1999). As an abundant surface protein, PorA is frequently recognised by bactericidal antibodies. Monoclonal antibodies directed against PorA can activate complement-mediated killing of meningococci, and have been shown to be effective in preventing infection in an infant rat model of meningococcal infection (Saukkonen *et al.*, 1987). Serum from individuals immunised with outer membrane vesicle (OMV) vaccines revealed a correlation between protection and the presence of antibodies against PorA (Guttormsen *et al.*, 1994; Milagres *et al.*, 1994). The adjuvant property of recombinant PorA has been demonstrated by its ability to activate monocyte-derived DCs, and influence T-helper immune responses (Al-Bader *et al.*, 2004). In contrast, PorB can stimulate B lymphocyte proliferation and Ig secretion, by binding to TLR-2 (Wetzler *et al.*, 1996). PorB is also capable of inserting into the membrane of host cells and facilitating bacterial attachment by forming functional channels and interfering with Ca^{2+} signalling (Muller *et al.*, 1999). The interaction of PorB with mitochondria of host cells blocks the activation of caspase-9, caspase-3 and DNA degradation, thus preventing cell apoptosis (Massari *et al.*, 2003; Plummer *et al.*, 1993).

The class 4 protein (33-34 kDa) was designated reduction-modifiable protein M (RmpM), as its electrophoretic mobility is influenced by the presence of reducing reagents. RmpM of meningococci shares 94.2% homology with protein III of *N. gonorrhoeae* and limited homology with the C-terminus of *E. coli* OmpA (Klugman *et al.*, 1989). Rmp is found in close association both with porins, PorA and PorB, and iron-regulated outer membrane proteins, LbpA, TbpA and FrpB (Jansen *et al.*, 2000; Prinz and Tommassen, 2000). Antibodies against RmpM fail to promote

complement-mediated bactericidal killing of meningococci, and some anti-protein III mAbs have been shown to inhibits the killing of meningococci by blocking the bactericidal antibodies against proteins and LPS (Plummer *et al.*, 1993; Virji and Heckels, 1988). However, later studies showed that purified anti-RmpM antibodies have no blocking effect on the bactericidal activity induced by PorA (Rosenqvist *et al.*, 1999).

The Class 5 proteins, Opa and Opc, are opacity-associated (Opa) proteins. Opa is a highly variable protein and encoded by up to four genes (Aho *et al.*, 1991), whereas Opc is relatively conserved and encoded by a single gene (Olyhoek *et al.*, 1991). Opa proteins are subject to antigenic and phase variation, the latter is mediated by multiple CTCTT repeats upstream of the *opa* open reading frame (ORF) (Belland *et al.*, 1997). Opa and Opc bind to the human CD66 and heparin sulphate proteoglycan receptors, respectively, and are important for intimate adhesion of *N. meningitidis* to host cells following initial attachment mediated by pili (de Vries *et al.*, 1998; Virji *et al.*, 1993; Virji *et al.*, 1996b). However, the interaction between bacterial Opa and Opc with host cells is often masked by the polysaccharide capsule and sialylated LPS (Unkmeir *et al.*, 2002). The Opa proteins may also act as immunomodulators, interacting with macrophages, neutrophils, and $CD4^+$ T cells, by binding to members of the carcinoembryonic antigen cell adhesion molecule (CEACAM) family of proteins (Boulton and Gray-Owen, 2002; Gray-Owen *et al.*, 1997).

1.6 Phase variation

Pathogenic bacteria have evolved sophisticated strategies to adapt to the highly selective environments they encounter within their hosts. Phase variation enables *N. meningitidis* to produce multiple combinations of phenotypes, some of which may have increased fitness in specific niches, enhancing virulence during systemic infection (Henderson *et al.*, 1999; Moxon *et al.*, 1994)

Morphologic changes of *N. gonorrhoeae* colonies in their colour, opacity and size were first reported by Kellogg (Kellogg *et al.*, 1963), and similar findings for *N. meningitidis* were observed by Blake (Blake *et al.*, 1989). These phenotypic variations are linked to changes in the expression of surface phase variable structures, such as the polysaccharide capsule (Hammerschmidt *et al.*, 1996), pili (Seifert, 1996), LPS (de Vries *et al.*, 1996), PorA (van der Ende *et al.*, 2003) and Opa proteins (van der Ende *et al.*, 2003).

Phase variation is the reversible on-off switching of gene expression in cells of a clonal population (van der Woude and Baumler, 2004). The variation is heritable between generations and can affect more than 60 *N. meningitidis* genes (Alexander *et al.*, 2004; Snyder *et al.*, 2001). The genome of *N. meningitidis* is relatively small (~ 2 Mb) and has a limited number of classic regulatory mechanisms compared to *E. coli*, such as sigma factors and two-component systems (Jolley *et al.*, 2005). However, it has a number of mechanisms to promote gene variation through mutation (Jolley *et al.*, 2005).

Repetitive DNA sequences are highly prevalent in the genome of *N. meningitidis*. Most phase variation events are based on the loss or gain of repetitive DNA sequences during DNA replication or repair (Stern and Meyer, 1987). Typically, loss or gain of repeat sequences in an ORF can result in frameshift mutants and or translation of non-functional proteins (Chen *et al.*, 1998). Changes in repetitive sequence located in promoters can alter the transcriptional activity of genes. For example, the presence of TAAA repeats within the promoter of *nadA* mediates phase variation by altering interactions with the transcription factor IHF and its DNA binding site (Martin *et al.*, 2003). In addition to repetitive DNA, insertion elements and atypical recombination are also responsible for phase variation of *N. meningitidis*. For example, insertion of IS*1301* within *siaA* mediates phase variation of this gene and abolishes capsule expression (Hammerschmidt *et al.*, 1996). Homologous recombination between *pilE* encoding subunits of pili and copies of the silent gene leads to the on/off expression of pili (Virji *et al.*, 1992; Virji *et al.*, 1993)

Several environmental factors affect the frequency of phase variation, such as the level of available iron (Schryvers and Stojiljkovic, 1999). Iron starvation is associated with increased DNA recombination and repair, and elevated rates of phase variation of pili in *N. gonorrhoeae* (Sechman *et al.*, 2005; Serkin and Seifert, 2000). Transformation of *N. meningitidis* with donor DNA from heterologous strains can also increase the rate of phase variation up to 73-fold (Alexander *et al.*, 2004). Adherence to host cells has similar effect on the up-regulation of the rate of phase variation (Morelle *et al.*, 2005). The length or number of repetitive sequences can also affect the phase variation rate. For instance, increased numbers of G residues in the

poly G tract of the *hmbR* gene results in an increase in the rate of phase variation (Tapsall *et al.*, 2001).

Antigenic variation refers to the expression of functionally conserved moieties within a clonal population that are antigenically distinct (Davidsen and Tonjum, 2006; van der Woude and Baumler, 2004). The genetic information for producing a family of antigenic variants can be available within the bacterium, with only one variant expressed at a given time (van der Woude and Baumler, 2004). In *N. meningitidis*, type IV pili and Opa proteins are typical structures that vary through intragenomic recombination (Nassif *et al.*, 1993). Alternatively, antigenic variation can arise by spontaneous mutation or by transformation (Koomey *et al.*, 1987; Seifert *et al.*, 1988).

1.7 Competence of *N. meningitidis*

Transformation is the one of the major routes for bacteria to obtain novel genetic information. Transformation consists of binding of exogenous DNA, transport through the membranes surrounding the bacteria, and incorporation of the DNA into the chromosome (Chen *et al.*, 1998). *Neisseria* spp. are naturally competent for transformation throughout their whole life cycle (Lorenz and Wackernagel, 1994).

Experiments performed on the closely related species *N. gonorrhoeae* indicated that a 10-bp DNA uptake sequence (5'-GCCGTCTGAA-3', DUS) in the incoming DNA enhances the frequency of transformation (Graves *et al.*, 1982). DUS is the most frequent repetitive sequence element in the meningococcal genome and is present in approximately 1,900 copies (Alexander *et al.*, 2004). The requirement of DUS for

Neisseria transformation was first described in 1988 when DNA segments from *N. gonorrhoeae* were used for transformation (Goodman and Scocca, 1988). Further experiments showed that *N. gonorrhoeae* exhibited preferential uptake of DNA containing DUS when incubated with homologous and foreign DNA (Hamilton and Dillard, 2006). In addition to the presence of PilE on the bacterial surface, PilQ and PilP, which form a channel for the transport of incoming DNA (Collins *et al.*, 2005), and PilT, which mediates pilus retraction, are essential for DNA uptake (Wolfgang *et al.*, 1998). Once the incoming DNA passes through the outer membrane, ComE in the periplasm is required for DNA uptake by an unknown mechanism (Chen *et al.*, 1998). ComA, an inner membrane protein, is crucial for DNA transport into the cytosol (Rudel *et al.*, 1995). Integration of the homologous incoming DNA into the chromosome is RecA-dependent, and also partially requires RecBCD, RecN, RecX (Mehr and Seifert, 1998; Stohl *et al.*, 2002).

1.8 Complement system

1.8.1 Complement pathway

Complement was first identified as a heat-labile activity in serum that "complemented" antibodies in the killing of bacteria (Bordet, 1895). The complement system consists of more than 30 fluid phase proteins circulating in the plasma, and several membrane-associated complement-receptor proteins and regulators. Activation of the complement system can be achieved through three different pathways, the classical (CP), alternative (AP) and lectin (LP) pathways (Ikeda *et al.*, 1987; Ingwer *et al.*, 1978) (Figure 1.1). The importance of complement in the immune system has been well established (Walport, 2001). First, it defends the host against micro-organisms

through opsonisation, lysis of bacteria and chemotaxis and activation of leukocytes. Secondly, it can bridge innate immunity with adaptive immunity, by inducing antibody responses and immunologic memory, or attracting leukocytes to the site of infection (Nielsen and Leslie, 2002). For example, C5a, the cleavage product of C5 can recruit phagocytic cells, and C3a can regulate the inflammatory response by triggering the release of lysosomal enzymes, histamine secretion from mast cells (Fretland *et al.*, 1991). Thirdly, complement is involved in the elimination of immune complexes and the clearance of apoptotic cells (Walport, 2001).

The CP is mainly initiated by binding of antibodies to antigens. Binding of C1q of the C1 complex (which consists of C1q, C1s and C1r) to the heavy chain of IgM or IgG leads to the activation of C1s (Loos, 1982; Ziccardi, 1983). Activated C1s first cleaves C4 into C4a and C4b, and then separates C2 into C2a and C2b. Binding of C4b to C2a forms C4b2a, which is the C3 convertase of the CP. This C3 convertase cleaves C3 into active C3b, which then binds to an activator surface (Walport, 2001).
Initiation of AP occurs by spontaneous low-rate hydrolysis of the thioester bond in C3 and formation of C3(H_2O) in plasma (Pangburn, 1983). C3(H_2O) binds to factor B (fB), and factor D (fD) can cleave fB in the complex, resulting the C3(H_2O)Bb. C3(H_2O)Bb is the fluid-phase C3 convertase and can cleave C3 into C3a and C3b. C3b on the bacterial surface can then bind to fB and form C3bBb in the presence of fD (Pangburn *et al.*, 1983). Binding to properdin stabilises the activity of C3bBb, which is C3 convertase of the AP. C3bBb can cleave further C3 molecules, and this amplification loop is a key feature of the AP (Fishelson *et al.*, 1983). C3b is also able to bind to C3bBb to form C3bBb3b, a C5 convertase capable of cleaving C5 into C5a and C5b (DiScipio *et al.*, 1983).

The LP is activated when mannose-binding lectin (MBL) or ficolins bind to carbohydrates or other ligands on a micro-organism in a calcium-dependent manner (Fujita *et al.*, 2004). MBL is associated with three MBL-associated serine proteases (MASPs), MASP-1, MASP-2 and MASP-3. The MBL-MASP-2 complex is able to cleave C4 and C2, forming C4b2a, the C3 convertase that splits C3 into C3a and C3b (Sim and Laich, 2000). In addition to activating complement, binding of MBL to some carbohydrate structures on the microbial surface can enhance phagocytosis via complement receptor 1 (CR1/CD35) (Ghiran *et al.*, 2000).

C3 plays a pivotal role in complement activation as the three complement pathways converge at this complement component (Daha *et al.*, 1976). Binding of C3b to C3 convertase (C4b2a or C3bBb) forms the C5 convertase that cleaves C5 into C5a and C5b (DiScipio *et al.*, 1983). C5a is an inflammatory mediator that activates a variety of cells through G protein-linked receptors (Pellas and Wennogle, 1999). C5b binds to C6, and the resulting complex binds to C7. The C5b67 complex is hydrophobic and preferentially inserts into membrane lipid bilayers initiating the formation of the membrane attack complex (MAC). C8 binds to C5b67 at C5b and forms small transmembrane channels, and acts as the receptor for the polymerisation of C9, which completes the MAC transmembrane channel, leading to osmotic lysis of cells (Podack and Tschopp, 1982).

In addition to complement-mediated lysis, C3 has an important role in phagocytosis (Griffin and Mullinax, 1985). C3b functions as an opsonin, mediating uptake of coated particles by phagocytic cells through complement receptor 3 (CR3)

(Ehlenberger and Nussenzweig, 1977). A breakdown product of C3b, iC3b, can itself function as an opsonin leading to complement receptor 3- and 4-mediated uptake (Winkelstein *et al.*, 1975).

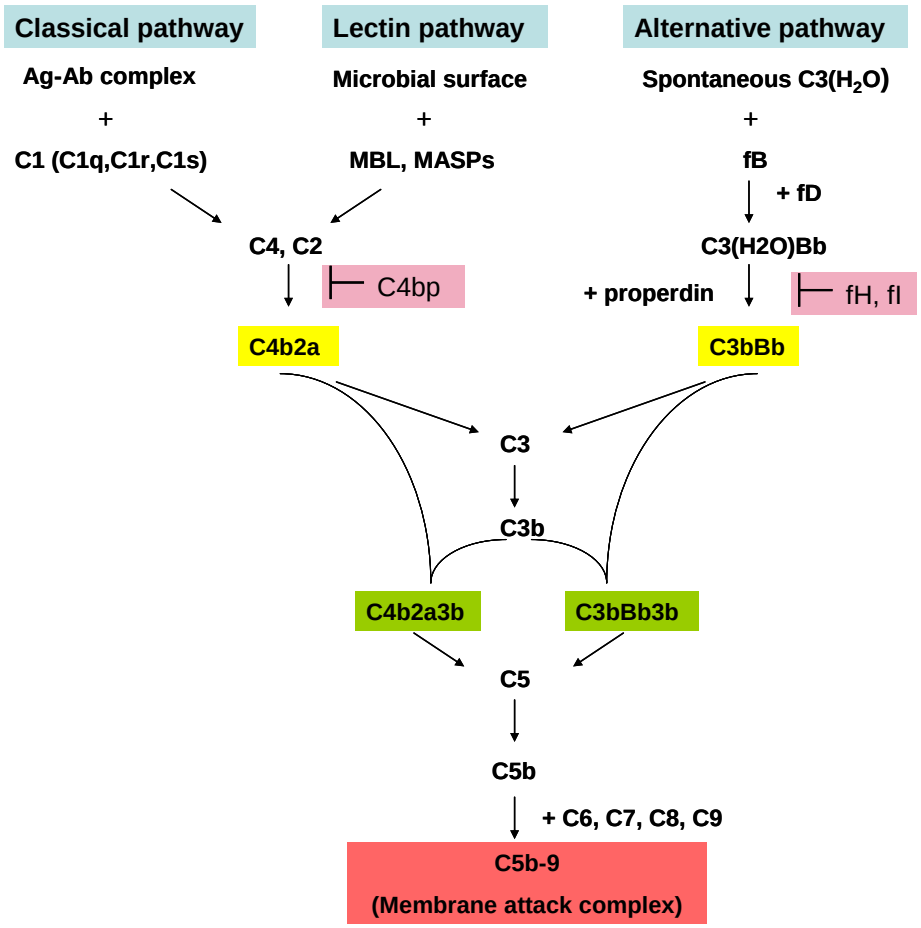


Figure 1.1 **The three complement activation pathways.** The classical pathway (CP) and the lectin pathway (LP) use C2 and C4 to generate C4b2a as a C3 convertase, while the alternative pathway forms C3bBb as a C3 convertase. Cleavage of C3 by a C3 convertase (C4b2a or C3bBb, in yellow) results in C3b, and then forms C5 convertase (C4b2a3b or C3bBbC3b, in green). The C5 convertase splits C5 into C5a and C5b, and C5b binds to C6, C7, C8 and C9 to form the membrane attack complex (MAC). The MAC inserts into the membrane of bacteria and causes cell lysis. A few of negative regulators of complement pathways are indicated in pink.

1.8.2 Regulation of the complement cascade

Inappropriate and uncontrolled activation of complement could potentially damage host cells, and therefore the complement system is tightly regulated, with activation confined to foreign particles and damaged tissue (Haupt and Fischer, 1962; Humphrey, 1971). Regulation of the complement system occurs at different stages of the cascade, from the initial recognition of activator molecules to the formation of the MAC. The soluble protein C1-inhibitor inhibits the activation of the C1 complex in the initial stages of the CP (Yonemasu, 1988). C4bp is a co-factor in the factor I-mediated cleavage of C4b and participates in the dissociation of C4b2a, the C3 convertase of the CP and the LP (Nagasawa and Stroud, 1977; Ruddy and Austen, 1971). Binding of C4bp to the surface of *N. meningitidis* confers protection of the bacterium against the CP activation (Jarva *et al.*, 2005). The analogous regulator of the AP is factor H, a co-factor in the factor I-mediated cleavage and inactivation of C3b. fH also contributes to the dissociation of C3bBb, and thus has decay-accelerating activity for the AP (Zipfel *et al.*, 1999). Recruitment of fH to the surface of *N. meningitidis* contributes to avoidance of complement-mediated killing (Schneider *et al.*, 2006). It has been found that S-protein can block the formation of MAC (C5b-9) by preventing C9 polymerisation, and blocking the attachment of the C5b67 to the cell surface (Hetland *et al.*, 1987). In addition to these soluble proteins, several membrane-associated proteins, such as complement receptors (CRs) and homologous restriction factor (CD55), take part in complement regulation. For example, CD59 can inhibit the formation of the MAC by binding to C8 and/ or C9, and contribute the survival of the *Helicobacter pylori* on the gastric mucosa (Rautemaa *et al.*, 2001).

1.8.3 Complement deficiencies and meningococcal infection

The importance of the complement system for host defence against meningococcal infection is illustrated by the fact that individuals with complement deficiencies are highly susceptible to meningococcal disease (Ellison *et al*., 1983). Individuals with deficiencies of the terminal complement components (C5-C9) have a 5-10,000 fold greater risk of developing meningococcal disease than the normal population, and half of them experience recurrent attacks (Pollard and Scheifele, 2001). In addition, infection with W-135, Y and other rare serogroups are found more often in people with terminal complement deficiencies (Sjoholm *et al.*, 2006).

Meningococcal infections have also been associated with defects in the AP and the LP. Individuals with either properdin or factor D deficiency have a 250-fold increased susceptibility to meningococcal infection especially with serogroup Y and W-135 strains (Figueroa and Densen, 1991), and have higher mortality rates than normal individuals (Sjoholm *et al.*, 2006). Polymorphisms of major complement regulatory proteins of the AP have also been associated with meningococcal infection. For example, a genetic susceptibility study showed that individuals possessing fH (C-496T C/C) genotype have a higher risk of *N. meningitidis* infection, due to increased serum fH level and reduced bactericidal activity of the serum (Haralambous *et al.*, 2006). A large study in children with meningococcal disease revealed that low concentration or decreased biological activity of mannose-binding lectin is also associated with higher incidence of bacterial infections during childhood (Bathum *et al.*, 2006).

1.9 Pathogenesis of meningococcal infection

The development of meningococcal disease requires exposure of a susceptible host to pathogenic meningococci, colonisation and penetration of bacteria of the nasopharyngeal mucosa and survival of the bacterium in the bloodstream.

Meningococci are transferred in droplets from a carrier to a new host and the transmission rate of virulent strains is higher than for non-pathogenic isolates (van Deuren *et al.*, 2000). Acquisition of a virulent isolate can lead to meningococcal disease within one week, but most individuals carry the bacterium asymptomatically. For instance, a study in Norway suggested that in endemic situations, only 1% of people harbouring ET-5 and ET-37 isolates become ill (Caugant *et al.*, 1994).

N. meningitidis colonises selectively the microvilli of non-ciliated epithelial cells in the mucosa of the human nasopharynx (Stephens *et al.*, 1983). Electron microscopic studies using human nasopharyngeal tissue as a model distinguished two steps in the adhesion of *N. meningitidis*. The initial attachment is characterised by dispersed microcolonies of 10-100 meningococci on the surface of epithelial cells, which is mediated primarily by Tfp (Nassif *et al.*, 1997; Stephens *et al.*, 1983). Binding of Tfp to its putative receptor CD46 can trigger an increase in cytosolic calcium and induce signal transduction pathways in host cells (Kallstrom *et al.*, 1998). After initial attachment, the microvilli of these nonciliated cells elongate and surround the organisms. Eight to sixteen hours post-infection, bacteria disperse from the microcolonies, which is mediated by PilT and down-regulation of pilus expression (Pujol *et al.*, 1999; Yasukawa *et al.*, 2006). Individual diplococci become intimately attached to the cell surface by binding of Opa/Opc proteins to their receptors (Virji *et*

al., 1993; Virji *et al.*, 1996a). This process is promoted by down-regulation of capsule, which is mediated by a negative transcription regulator CrgA (Deghmane *et al.*, 2002).

The nasopharyngeal epithelium has barrier functions, mediated by tight junctions between cells (Pujol *et al.*, 1997). Traversal of the epithelial layer by meningococci is not well understood. Encapsulated meningococci are transported through the cell in large membrane-bound phagocytic vacuoles, whereas non-capsulated strains traverse the cytoplasm without vacuoles (Stephens *et al.*, 1993). Six to twelve hours after infection, endocytic vacuoles containing meningococci can be observed in the apical portion of some non-ciliated columnar cells (Stephens *et al.*, 1983). Expression of polysaccharide capsule hinders invasion and is down-regulated during this stage (de Vries *et al.*, 1996). Expression of the surface antigen NadA promotes bacterial adhesion and invasion in a capsule-negative background (Capecchi *et al.*, 2005). During invasion, translocation of PorB into target cells membranes affects the maturation of nascent phagolysosomes (Muller *et al.*, 1999). IgA1 protease, produced by *N. meningitidis* but not by non-pathogenic bacteria such as *N. lactamica*, can inactivate specific IgA1 (Mulks and Plaut, 1978; Vitovski *et al.*, 1999). Accelerated degradation of LAMP-1, resulting from infection with meningococci, can promote the survival of *N. meningitidis* in epithelial cells (Ayala *et al.*, 1998). Within twenty-four hours, meningococci are observed in the submucosa in close proximity to local immune cells and blood vessels (Stephens *et al.*, 1993).

After mucosal penetration, bacteria gain access to the circulation. Expression of the polysaccharide capsule, which protects bacteria against complement-mediated killing and phagocytosis, is an essential bacterial virulence factor for survival in the

bloodstream (Mackinnon *et al.*, 1993; Vogel *et al.*, 1996). PorA can inhibit the phagocytic capacity of human neutrophils by down-regulation of the Fcγ receptor, and C1 and C3 receptors (Bjerknes *et al.*, 1995). In addition to the properties of the meningococcus, the host immune system is important for bacteria to survive in bloodstream. As the production of antibodies takes at least one week after acquisition of the bacterium, the innate immune response is indispensable. For example, X-linked properdin deficiency involved in the AP may lead to overwhelming invasive disease (Fijen *et al.*, 1995). Once viable meningococci reach the bloodstream, different disease manifestations can develop.

Following dissemination in the vascular system, circulating *N. meningitidis* is capable of crossing the blood-brain barrier (BBB) to cause meningitis. The BBB is made of two different structures. One structure consists of the endothelium of brain capillaries with tight junctions limiting paracellular flux. The second structure is the choroid plexus, which is the major site of CSF synthesis and is located in the ventricles (Rubin *et al.*, 1991). Meningococci adhere to the endothelial cells of both the choroid plexus and the meninges. Expression of PilC was much higher in bacteria isolated from the CSF in comparison to expression from isolates obtained from the systemic circulation (Pron *et al.*, 1997). The mechanism by which meningococci invade the meninges and cross the BBB is not fully understood. Once in the subarachnoid space, where the principal humoral and cellular host defence mechanisms are absent, *N. meningitidis* proliferate in an uncontrolled fashion (Brandtzaeg *et al.*, 1989; Simberkoff *et al.*, 1980; van Deuren *et al.*, 2000).

1.10 Clinical manifestations

The initial clinical manifestations of meningococcal disease are similar to a viral infection, which makes early diagnosis difficult. The number of bacteria and the level of circulating LPS are associated with different clinical presentations and outcomes (Stephens *et al.*, 2007).

Bacterial meningitis, resulting from hematogenous dissemination, is the most common clinical manifestation of meningococcal disease, and observed in about 50% of patients (Manchanda *et al.*, 2006; van Deuren *et al.*, 2000). Most patients experience a rapid onset of high fever, headache and neck stiffness. Some patients also have nausea, vomiting and mental confusion. In infants, headache and nuchal rigidity are not typical, while a swollen fontanelle, irritability and poor feeding are more typical (Welch and Nadel, 2003). The application of quantitative real-time PCR reveals that patients with meningitis usually have low concentrations of meningococci ($<10^3$ CFU/ml) and endotoxin (<3 EU/ml) in plasma, but high concentrations in the CSF (Ovstebo *et al.*, 2004)

Meningococcal septicaemia (meningococcaemia), with rapid proliferation of *N. meningitidis* in the circulation, occurs in approximately 5-15% of cases of meningococcal disease and has a high mortality (van Deuren *et al.*, 2000). It is characterised by the abrupt onset of fever, prostration and often a petechial rash, and may progress to purpura and/or fulminant septicaemia (van Deuren *et a*l., 2000). Patients who develop septicaemia are hypotensive, and may have acute adrenal hemorrhage (Waterhouse-Friderichsen syndrome) with multiple organ failure

(Stephens *et al.*, 2007). The concentration of bacteria is about 10^5–10^8 CFU/mL with 10^1–10^3 EU/ml of endotoxin in plasma (Brandtzaeg *et al.*, 1989; Hackett *et al.*, 2002).

Chronic meningococcaemia is a rare form of meningococcal disease. Patients normally have intermittent fever, lasting from a few days to weeks, and a non-specific maculopapular rash (Hansen *et al.*, 2003). Pneumonia has been found in 5-15% of patients with this syndrome; other manifestations of *N. meningitidis* infection include septic arthritis, urethritis and pericarditis (Stephens *et al*., 2007).

1.11 Diagnosis

The gold standard for the diagnosis of meningococcal disease is the isolation of *N. meningitidis* from a normally sterile body site (Rosenstein *et al.*, 2001). As the meningococcus is a fastidious organism, samples should be cultured as soon as they are collected, ideally within one hour (Manchanda *et a*l., 2006). Even so, the sensitivity of diagnosis by bacterial culture is not very high, especially when prior antibiotic treatment has been administered. Gram staining of CSF is another important method for the rapid detection of *N. meningitidis*. However, the definitive diagnosis of meningococcal disease relies on bacteriologic culture (Dunbar *et al.*, 1998).

Molecular methods, which do not require recovery of viable bacteria, are less compromised by antibiotic treatment. These methods reduce the time for identification and increase the sensitivity of the diagnosis (Abdel-Salam, 1999; Fontanals *et al.*, 1996; Newcombe *et al.*, 1996). For instance, PCR is used to amplify

either conserved genes encoding 16S rRNA or species-specific targets such as *porA* or *ctrA* (Atobe *et al.*, 2000; Jordens and Heckels, 2005; Whiley *et al.*, 2003). The sensitivity of PCR is highest within the first 24 hours after antibiotic treatment. In the UK, more than half of cases are now diagnosed by PCR without culture (Atobe *et al.*, 2000; Gray *et al.*, 2006).

Additionally, molecular methods are essential for the identification of the serogroup of *N. meningitidis* and bacterial susceptibility to antibiotics. Serogroups B, C, Y and W135 can be detected by amplification of their different alleles of *siaD* (Borrow *et al.*, 1997; Borrow *et al.*, 1998). Serogroup A strains can be identified by amplifying the *mynB*/*sacC* gene (Taha *et al.*, 2005). Agglutination is widely used to detect capsular antigen of serogroups A, B, C, Y and W-135 in clinical samples (Martynov Iu *et al.*, 1990). False negatives are more common in cases of serogroup B disease and in non-encapsulated strains. Antibiotic resistance of *N. meningitidis* has been increasingly observed in recent years. Modifications of the *penA* gene, encoding penicillin binding protein 2 (PBP2), is responsible for most reduced susceptibility to penicillin. PCR can be used to characterise this alteration and is increasingly used in reference laboratories (Taha *et al.*, 2006).

1.12 Treatment

As meningococcal disease can be fatal within a few hours of onset, admission to hospital or a health centre is necessary. Early recognition of disease prompts initial antibiotic treatment and intensive monitoring, which reduces the rate of meningococcal sepsis and fatality (Gardner, 2006). Effective antibiotic treatment

rapidly halts the proliferation of bacteria in the CSF and vascular system. Within 3-4 hours of the administration of antibiotics, all meningococci in the CSF are killed and the concentration of endotoxin in plasma falls by approximately 50% (Brandtzaeg *et al.*, 1989; Kanegaye *et al.*, 2001; Ovstebo *et al.*, 2004). The concentration of key cytokines and chemokines falls in parallel (Moller *et al.*, 2005; van Deuren *et al.*, 1995). In general, antibiotics are given by the parenteral route. In developed countries, benzylpenicillin alone or a third generation cephalosporin remains the treatment of choice (Stephens *et al*., 2007). For patients who are allergic to penicillin, intravenous chloramphenicol can be given (Feldman and Zweighaft, 1979). The duration of treatment varies. A recent study of patients with serogroup B disease in New Zealand showed that a three-day treatment course with intravenous benzylpenicillin was sufficient and not associated with relapse (Ellis-Pegler *et al.*, 2003; Latorre *et al.*, 2000). The resistance of meningococci against penicillin has been increasing worldwide since the late 1980s (Latorre et al., 2000). However, there is no evidence of any association between a poor clinical outcome and reduced susceptibility to penicillin. In developing countries, a single dose of ceftriaxone or chloramphenicol can be sufficient for the treatment of patients with meningococcal meningitis (Nathan *et al.*, 2005).

Treatments to increase the circulating blood volume, decrease blood coagulation or haemorrhage are given to patient to reduce mortality (Welch and Nadel, 2003). Attempts to inactivate meningococcal LPS and thereby block the inflammatory cascade have not been shown to significantly improve survival (Derkx *et al.*, 1999; Levin *et al.*, 2000).

1.13 Vaccines against serogroup A, C, Y and W135 meningococcal disease

Over the past 80 years there have been many attempts to develop effective vaccines against the meningococcus, including the use of purified capsular polysaccharide, protein polysaccharide conjugates, outer membrane proteins, and reverse vaccinology (Girard *et al.*, 2006; Harrison, 2006). Immunological correlates of protection, which can consistently predict immunity, are essential for evaluating the efficacy of vaccines. In studies conducted in US military recruits in 1969, Goldschneider showed that the presence or absence of serum bactericidal antibodies (SBA) against serogroup C *N. meningitidis* predicted the risk of subsequent disease in individuals, which established the SBA titre as a surrogate measure of immunity (Goldschneider *et al.*, 1969b). Subsequent studies revealed that the percentage of individuals with an SBA titre of ≥ 4 had an inverse relationship with the incidence of serogroup C disease (Goldschneider *et al.*, 1969a). Commercially available baby rabbit complement is now recommended for standard bactericidal assays (SBAs) in evaluating the efficacy of vaccines. A SBA titre of ≥ 8 is accepted as a correlation of protection against meningococcal serogroup C strains (Borrow *et al.*, 2001) .

1.13.1 Polysaccharide vaccines

The bactericidal activity of serum from healthy volunteers was abolished when antibodies against the capsular polysaccharide were removed by adsorbing serum with purified capsule. This result suggested that immune responses against the polysaccharide capsule were responsible for protection (Gotschlich *et al.*, 1969). Thus, high molecular weight capsular polysaccharide was purified and used as a vaccine.

A serogroup C capsular polysaccharide vaccine proved effective in preventing serogroup C disease in military recruits in the USA, and in the 2-29 year age group during an outbreak in Texas (Artenstein *et al.*, 1970; Rosenstein *et al.*, 1998). Similarly, the efficacy of a serogroup A capsular polysaccharide vaccine in school-aged children was about 89% during a serogroup A outbreak in Europe in 1970s (Wahdan *et al.*, 1977). In the following decades, bivalent (A, C), and tetravalent (A, C, W-135, Y) combinations of purified capsular polysaccharides have been widely used to control outbreaks of meningococcal disease (Vodopija *et al.*, 1983). More recently, a trivalent (A, C, W-135) vaccine was licensed against the emergent W-135 serogroup of *N. meningitidis*, which causes epidemics in the countries of the meningitis belt (Sacchi *et al.*, 2002).

However, there are several limitations of polysaccharide vaccines. Firstly, capsular vaccines are poorly immunogenic in children less than 2 years old, who are most at risk of meningococcal disease. Secondly, waning levels of antibody are age-dependent, with lower persistence and protective immunity in the younger age group. In addition, polysaccharide vaccines induce T-cell independent immune responses, and therefore fail to induce immunological memory. Finally, polysaccharide vaccines have limited effects on carriage rates (Girard *et al.*, 2006).

1.13.2 Polysaccharide/Protein conjugate vaccines

The shortcomings of capsular polysaccharide vaccines are largely attributable to a lack of T cell-dependent responses, and can be overcome by incorporating a T cell-dependent antigen into the polysaccharide. The introduction of the *H. influenzae* type b glycoconjugate vaccine successfully eradicated this disease in developed countries

(Robbins *et al.*, 1996), and led to the application of the same strategy to meningococcal capsular polysaccharides. The carrier proteins used for meningococcal conjugate vaccines include tetanus toxoid proteins, diphtheria toxoid, and diphtheria cross-reactive material (CRM_{197}) (Girard *et al.*, 2006).

Commercial meningococcal serogroup C conjugate vaccines, consisting of fractionated capsular polysaccharide linked to tetanus toxoid or CRM_{197}, were introduced into the UK routine immunisation programme in 1999. These vaccines proved highly effective against serogroup C disease, with a 90% decrease in incidence across all age groups, and a 66% decrease in carriage (Balmer *et al.*, 2002; Ramsay *et al.*, 2003). In addition to the protection reported in the vaccinated population, the conjugate vaccine also reduces the incidence of disease in non-vaccinated people by 67% through herd immunity (Ramsay *et al.*, 2003). In recent years, serogroup C conjugate vaccines were licensed in Australia, Canada and in other European countries such as Spain (Welte *et al.*, 2005).

A tetravalent conjugate vaccine (MCV4) incorporating the polysaccharide capsule from serogroups A, C, Y and W-135 linked to diphtheria toxoid was licensed in the USA for people between 11-55 years of age in 2005 (Bilukha and Rosenstein, 2005). However, it is not recommended to routinely immunise children aged from 2-10 years of age unless the child is at increased risk for disease (2008).

Currently, the World Health Organisation (WHO) and Meningitis Vaccine Project (MVP) are working to develop cheaper and cost-effective serogroup A conjugate vaccines to prevent epidemic disease in the African meningitis belt (Jodar *et al.*,

2003). They are aiming to license the monovalent A conjugate vaccine for use in single-dose mass vaccination campaigns in approximately 400-500 million people aged 1 to 29, hopefully starting in 2008-2009 (Girard *et al.*, 2006).

1.14 Serogroup B vaccines

There is no effective polysaccharide vaccine against serogroup B strains, as the serogroup B capsule is poorly immunogenic and has structural similarity to a polysialylated form of a neural cell adhesion molecule, NCAM-1 (Finne *et al.*, 1983; Finne *et al.*, 1987). Attempts have been made to replace the N-acetyl sialic acid residues of the serogroup B capsular polysaccharide with N-propionylated (NPR) groups in a conjugate vaccine, but no serum bactericidal activity was induced in human volunteers (Bruge *et al.*, 2004; Pon *et al.*, 1997). Consequently, vaccine research against serogroup B meningococci has focused on OMVs and OMPs.

1.14.1 Outer membrane vesicles and proteins

OMVs have been used to control prolonged serogroup B outbreaks, which tend to be caused by a single clone. The efficacy of OMV vaccines was first evaluated in the late 1970s using aggregated OMPs from OMVs, from which LPS had been depleted by treatment with detergents. Despite promising results in animal studies, this vaccine failed to induce bactericidal antibodies in either adults or children, which was probably due to the altered conformation of OMPs (Zollinger *et al.*, 1978). Thus, a soluble OMV vaccine, in which the native conformation of OMPs was retained, was prepared by purifying OMVs from bacteria during growth and selectively removing LPS. Modest increases in SBA titres were found after immunisation of adults with

this vaccine (Frasch and Peppler, 1982). Significantly increased immunogenicity was found by incorporating serogroup B polysaccharide into soluble OMVs (Yang *et al.*, 2007). In addition, a further increase in bactericidal titres resulted from the addition of aluminium hydroxide as an adjuvant (Frasch *et al.*, 1988).

A number of efficacy trials with serogroup B OMV vaccines have been conducted, with the two most extensively studies developed in Cuba and Norway. The B+C meningococcal vaccine licensed in Cuba in 1989 (commercially marketed as VA-MENGOC-BC® by the Finlay Institute and GSK) consists of LPS-depleted OMVs from a B:4:P1.19,15 strain with serogroup C capsular polysaccharide in a high molecular weight complex, all adsorbed onto aluminium hydroxide. The vaccine was administered in two doses, and approximately 83% efficacy was observed. However, the efficacy of the vaccine is dependent on age, with very low protection in children less than 4 years old (de Moraes *et al.*, 1992). A Norwegian serogroup B vaccine containing LPS-depleted OMVs from a serogroup B:15:P1.7,16 strain, adsorbed onto aluminium hydroxide, showed an efficacy of 57% in an trial conduced in secondary school children in Norway (Bjune *et al.*, 1991a). In New Zealand, an OMV vaccine containing PorA, PorB and LPS from the New Zealand serogroup B strain (B:4:P1.7b, 4) was licensed in 2004 for use in a national campaign immunisation (O'Hallahan *et al.*, 2004). This vaccine successfully reduced the incidence of disease and raised bactericidal antibodies in 70% of children from 6 month of age in 90% of teenagers (Oster *et al.*, 2005).

PorA is the most abundant OMP in OMVs and sufficient to induce bactericidal antibodies following nasopharyngeal carriage, invasive meningococcal infection, and

immunisation with serogroup B OMVs (Idanpaan-Heikkila *et al.*, 1995; Jones *et al.*, 1998; Rosenqvist *et al.*, 1995). However, anti-PorA bactericidal antibodies are directed against the two longest external variable loops of the protein, and the protective immune response is serosubtype-specific (Idanpaan-Heikkila *et al.*, 1995; van der Ley *et al.*, 1991). Thus, single-strain based PorA vaccines are only able to prevent clonal disease outbreaks and not sporadic disease. To overcome this problem, an OMV vaccine containing PorA from six different prevalent serogroup B isolates was developed by the Rijksinstituut voor Volksgezondheid en Milieu (RIVM) in the Netherlands to cover 80% of disease-causing strains in the UK (Cartwright *et al.*, 1999). This hexavalent PorA-OMV vaccine is able to increase SBA titres by at least 4-fold after a fourth doses of immunisation and elicit memory responses in 78-95% of children (Longworth *et al.*, 2002). However, the effectiveness of a PorA-based vaccine is likely to be limited by the rapid evolution of antigenic variation of PorA in the meningococcal population under selective pressure and by the emergence of PorA-deficient meningococcal strains (Cartwright *et al.*, 1999; van der Ende *et al.*, 2003).

In addition to porins, a number of other OMPs are under investigation for their vaccine potential, such as class 5 proteins, Neisserial surface protein A (NspA), the iron regulated protein (FrpB) and transferrin binding proteins B (TbpB) (Ala'Aldeen *et al.*, 1994; Martin *et al.*, 1997; Rokbi *et al.*, 1997). Immunisation with recombinant NspA was able to elicit bactericidal antibodies against serogroup B *N. meningitidis*, but it only killed about 50% of genetically diverse strains (Moe *et al.*, 2001). Anti-TbpB bactericidal antibodies have been found in sera from carriers and patients with meningococcal disease, but not from healthy individuals (Filatova and Gamzulina,

2000). Immunisation with affinity-purified Tbps from *N. meningitidis* was able to elicit bactericidal antibodies and to induce protection against challenge in mice (Ala'Aldeen and Borriello, 1996; Danve *et al.*, 1993). However, a recent Phase I study showed that adults sera raised by recombinant TbpB purified from *E. coli* were not active in killing serogroup B strains (Danve *et al*., 1998). Furthermore, an OMP-based vaccine with six PorA and five FrpB sequence variants could induce a protective immune response against most circulating *Neisseria* isolates (Urwin *et al.*, 2004).

1.14.2 *N. lactamica*

The cross-reactivity of immune responses between non-pathogenic *Neisseria* spp. and *N. meningitidis* provides another approach for vaccine development (Cann and Rogers, 1989; Kim *et al.*, 1989; Troncoso *et al.*, 2000). The carriage of *N. lactamica* often occurs in infants and young children, and the carriage rate declines with increasing age, which is in contrast to the age-specific carriage rate of *N. meningitidis* (Gold *et al.*, 1978). Thus, carriage of *N. lactamica* is often thought to be important for the development of natural immunity (Troncoso *et al.*, 2002). Immunisation with live *N. lactamica* elicits high titres of bactericidal antibodies and protects mice against challenge with live *N. meningitidis* (Li *et al.*, 2006). Differences were found between different *N. lactamica* strains in their ability to induce protective immune responses, indicating the need to identify specific surface antigens responsible for cross-reactivity.

1.14.3 Reverse vaccinology

With the availability of the complete genome sequence, all proteins can be tested as vaccines without any prior selection based on their in *vivo* expression or role in virulence (Tettelin *et al.*, 2000). This method of identification of vaccine candidates based on whole genome sequence has been termed "reverse vaccinology" (Pizza *et al.*, 2000; Rappuoli, 2001). There are approximately 2,158 ORFs in *N. meningitidis* serogroup B strain MC58 predicted by algorithms and whole-genome homology searches. Based on bioinformatic analysis, 570 ORFs were predicted to encode surface-exposed or secreted proteins, which are potential immunological targets because of their accessibility to antibodies. Taking advantage of recombinant DNA technology, genes encoding 334 candidates were successfully cloned in *E. coli*, expressed and purified as soluble recombinant proteins, and used to immunise mice (Pizza *et al.*, 2000). Using assays including immunoblot, ELISA and fluorescence-activated cell sorting (FACS), 91 novel surface-expressed proteins were identified. All sera were analysed for complement-mediated bactericidal activity, and 28 recombinant proteins were identified as able to induce a protective immune response. The most promising antigens which elicited the highest bactericidal titres were evaluated for their sequence conservation in a large number of *N. meningitidis* strains of genetic and geographic diversity (Tettelin *et al.*, 2000).

One of these novel surface antigens, referred to as Genome-derived Neisserial Antigen (GNA), is lipoprotein GNA1870. GNA1870 has a predicted molecular mass of 27 kDa, and three variants have been identified based on the sequence of the gene in 71 strains (Masignani *et al.*, 2003). Conservation within each variant ranges between 91.6% and 100%, while conservation between the variants can be as low as

62.8% (Jacobsson *et al.*, 2006). The bactericidal activity against GNA1870 is variant-specific, as serum raised against a recombinant form of one variant of the protein is effective in killing strains carrying the same variant but not strains carrying other variants (Welsch *et al.*, 2004). A recent study showed that using OMVs prepared from a *N. meningitidis* strain over-expressing GNA1870 could elicit greater bactericidal antibodies than that induced by either GNA1870 or OMV alone (Hou *et al.*, 2005). Recombinant lipoprotein rLP2086, a homologue of GNA1870 from a serogroup A strain Z2491, has similar effect to the rGNA1870 in its capacity to induce bactericidal antibodies against many serogroup B isolates (Fletcher *et al.*, 2004).

Several other genome-derived antigens have been under investigation for their vaccine potential such as GNA2132 and GNA33. Immunisation of mice with recombinant GNA2132 failed to elicit bactericidal antibodies, but was able to protect sinfant rats against meningococcal bacteraemia after challenge with serogroup strain NZ394/98 (Welsch *et al.*, 2003). Immunisation with GNA33 was able to elicit bactericidal antibodies against serotype P1.2 serogroup B strains, as a result of cross-reaction with an epitope on loop IV of PorA, indicating that molecular mimetics have potential as meningococcal vaccine candidates (Granoff *et al.*, 2001)

1.14.4 Genetic Screening for Immunogens

Based on the availability of the whole genome sequence of serogroup B *N. meningitidis* MC58 and *in vitro* mutagenesis technology, our group developed a new approach (Genetic Screening for Immunogens, GSI) to identify genes that encode surface expressed antigens that could be potential used as vaccine candidate (more

details are provided in the Chapter 3). Two independent mutants were identified with Tn*5* transposon in ORF of *nnmb1467*. According to bioinformatic analysis, NMB1467 is a homologue to *Escherichia coli* exopolyphosphatase, an enzyme involved in cleavage single phosphate (Pi) from Inorganic polyphosphate (poly P).

1.15 Inorganic polyphosphate

Inorganic polyphosphate (poly P) is a linear chain composed of tens or many hundreds of orthophosphate (Pi) residues linked by high-energy phosphor-anhydride bonds (Figure 1.2) (Kornberg, 1999). Poly P was first isolated from yeast and subsequently found in all living cells including bacteria, fungi, protozoans, plants and animals (Wood and Clark, 1988). It has been found on the surface of cells, in the cytoplasm, and in the periplasm of microbes (Glonek *et al.*, 1971). Various biological functions are associated with the presence of poly P, depending on its location and the bacterial species. For instance, poly P can act as a reservoir of energy and phosphate, as a chelator of metal ions (e.g., Mn^{2+} and Ca^{2+}), as a buffer against alkali, as a regulator of gene expression and can promote competence for bacterial DNA uptake (Kornberg, 1999).

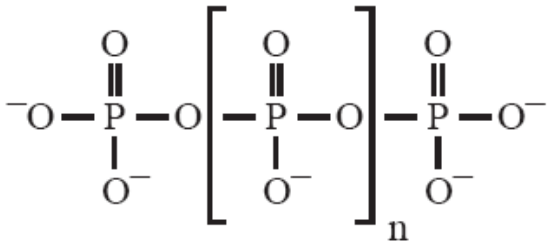


Figure 1.2 Inorganic polyphosphate (Kornberg, 1999)

Poly P was first observed using light microscopy as metachromatic granules, when it stained pink by basic blue dyes (Kornberg, 1999). Poly P appears highly refractive and quickly vanishes under an electron beam (Ogawa and Amano, 1987). Nuclear magnetic resonance (NMR) analysis is also able to detect highly concentrated poly P, but not in aggregated forms or in metal complexes (Deslauriers *et al.*, 1980).

As a polyanion, poly P is able to bind to basic dyes, such as toluidine blue, and shift its absorption to a shorter wavelength (Kornberg, 1999). Similar to nucleic acids which are also polyanions, poly P can nonspecifically bind to basic proteins, such as histones and basic domains of proteins (Kornberg, 1999; Stros *et al.*, 1984). Recently, it has been reported that poly P can specifically bind to the ATP-dependent protease Lon, which promotes the degradation of most ribosomal proteins and competence with DNA binding (Kuroda *et al.*, 2001; Nomura *et al.*, 2004).

Polyphosphate kinase (PPK) was first enzyme purified to homogeneity from *E. coli* that catalyses the reversible conversion of terminal(γ) phosphate of ATP to poly P (Poly P_n + ATP ↔ poly P_{n+1} + ADP) (Kornberg *et al.*, 1956; Ahn and Kornberg, 1990). This membrane protein is mainly responsible for the synthesis of longer chains of poly P, but in an excess of ADP, this enzyme can convert poly P to ATP (Ahn and Kornberg, 1990). PPK is highly conserved in most bacterial species including pathogens, but no PPK activity has been found in yeast or mammalian cells (Ault-Riche *et al.*, 1998). Recently, an actin-related protein complex was identified in mammalian cells, which synthesise poly P from GTP or ATP, and is called polyphosphate kinase 2 (PPK2) (Ishige *et al.*, 2002; Zhang *et al.*, 2002).

Aside from PPK, there are several enzymes involved in the utilisation of poly P (list in Table 1.1). For example, exopolyphosphatase (PPX) splits the terminal phosphate until only pyrophosphate (PP_i) is left (poly P_n+ (n-2) $H_2O \rightarrow$ (n-2) P_i + PP_i) (Akiyama *et al.*, 1993). Endopolyphosphatase (PPN) cleaves poly P internally to generate shorter chains of poly P and tripolyP (Kowalczyk and Phillips, 1993). Pyrophosphatase (PPA), which is traditionally considered to be responsible for the hydrolysis of pyrophosphate (PP_i), has been recently shown to be able to catalyse the breakdown of poly P in *Trypanosoma brucei* (Lemercier *et al.*, 2004).

The importance of poly P in bacterial pathogens has been illustrated mainly in experiments with *ppk* mutants, which have decreased levels of cellular poly P. In *E. coli*, a *ppk* mutant has a defect in long term survival during stationary phase and is more sensitive to oxidative, osmotic and heat stresses (Rao and Kornberg, 1996). All the defects appear to be related to a decreased expression of *rpoS*, which encodes a sigma factor that controls the expression of more than 50 genes responsible for adaptation to stress and the survival in the stationary phase (Shiba *et al.*, 1997). Poly P is also required for motility of bacterial pathogens such as *Salmonella* spp., *Pseudomonas aeruginosa*, *Vibrio cholerae* (Rashid *et al.*, 2000). In addition, *P. aeruginosa*, poly P is necessary for swarming motility, twitching motility, quorum sensing and the development of biofilms (Rashid and Kornberg, 2000).

Table 1.1 Enzymes involved in inorganic polyphosphate metabolism

Enzymes	Functions
Polyphosphate kinase (PPK)	Poly P_n + ATP ↔ poly P_{n+1} + ADP
	Poly P_n + GDP → poly P_{n-1} + GTP
	Poly P_n + GDP → poly P_{n-2} + ppppG
Polyphosphate kinase 2 (PPK2)	Poly P_n + ATP ↔ poly P_{n+1} + ADP
	Poly P_n +GTP ↔ poly P_{n+1} + GDP
AMP-phosphotransferase	Poly P_n + AMP → poly P_{n-1} + ADP
Glucokinase	Poly P_n + glucose → poly P_{n-1} + glucose-6-P
Exopolyphosphtase (PPX)	Poly P_n + (n-2) H_2O → poly P_{n-2} + PP_i
Endopolyphosphtase (PPN)	Poly P_{750} + H_2O → n poly P_{60} + 3n P_3
Pyrophosphatase (PPA)	PP_i→P_i + P_i
	Poly P_n + H_2O → poly P_{n-x} + P_x

Due to its co-transcription with *ppk*, a mutant lacking only *ppx* has not been constructed in *E. coli* (Rao and Kornberg, 1996). In *Bacillus cereus*, a *ppx* mutant, which accumulates 1000-fold more of poly P than the wild-type, has defects in motility, biofilm formation and sporulation (Shi *et al.*, 2004). However, in *Saccharomyces cerevisiae*, a mutant harbouring an inactivation of the gene encoding a major exopolyphosphatase (PPX1) has similar levels poly P as the wild-type strain (Lichko *et al.*, 2002).

The membrane localization of PPK in bacteria suggests that poly P might be made and localized at that site (Kornberg, 1999). In *S. cerevisiae*, poly P is found in nucleus, mitochondria, vacuole (lysosome), and cytosol (Lichko *et al.*, 2006). In *N. gonorrhoeae*, more than 10% of cellular phosphate is present in the form of poly P and at least half of poly P is loosely associated with the cell surface and can be easily

washed off (Noegel and Gotschlich, 1983). The presence of cell surface poly P in addition to its polyanionic character suggests that it may form a protective layer around the cell, such as observed around *N. gonorrhoeae* as a pseudo-capsule (James and Swanson, 1977; Noegel and Gotschlich, 1983).

According to the whole genome sequence of *N. meningitidis* serogroup B MC58 and protein blast, *nmb0641 nmb1467* and *nmb1900* encode PPA, PPX and PPK, respectively, which are enzymes involved in the metabolism of poly P (Tettelin *et al.*, 2000). A *ppk*-mutant of serogroup B *N. meningitidis* strain M1080, with reduced total poly P, was more sensitive to complement-mediated killing through unknown mechanisms (Tinsley *et al.*, 1993; Tinsley and Gotschlich, 1995), suggesting a capsule-like function of poly P in *N. meningitidis*.

Aim of the thesis

The function of *nmb1467*, loss of which renders serogroup B *N. meningitidis* MC58 resistant against complement-mediated killing, has not been reported before. The aims of this study were to:

1) identify meningococcal factors involved in interactions with the complement system by GSI,
2) assess the vaccine potential role of NMB1467,
3) investigate the biochemical function of this protein,
4) understand the mechanisms of serum resistance of the Δ*nmb1467* mutant.

Chapter 2 Materials and Methods

2.1 Bacterial strains and growth conditions

All *N. meningitidis* and *E. coli* strains used in this study are listed in Table 2.1. For overnight culture, *N. meningitidis* was grown on the Brain Heart Infusion (BHI) agar plates with Levanthal's supplement in the presence of 5% CO_2 at 37°C. To prepare Levanthal's supplement base, 37 g of BHI broth (Oxoid) and 500 ml of horse blood (Oxoid) were dissolved in 1 litre H_2O and autoclaved, and the supernatant was collected after centrifugation at 6000 *xg* at 4°C for 30 minutes. Aliquots were stored at -20°C. To make BHI agar plates, 37 g of BHI broth (Oxoid), 1 g of soluble starch and 15 g of bacteriological agar (1.5% wt/vol final concentration, Oxoid) were dissolved in 1 litre of H_2O, autoclaved, and 50 ml of Levanthal's base was added.

E. coli was either grown in LB (20 g of Lennox L Broth base in 1 litre H_2O, Invitrogen) liquid medium with shaking at 200 r.p.m or on LB agar plates (1.5% agar wt/vol) without shaking at 37°C. To make frozen stocks, bacteria were suspended in liquid medium used for growth with 15% glycerol, and stored at -80°C.

Table 2.1 Bacterial strains and plasmids used in this study

Strains/Plasmid	Genotype/Description	Reference
N. meningitidis		
MC58	Wild-type serogroup B	(Tettelin *et al.*, 2000)
Δ*nmb1467* (Δ*ppx*)	Insertional inactivation of *nmb1467*; kanamycin resistant	This study
Δ*siaD*	Insertional inactivation of *siaD*; erythromycin resistant	(Exley *et al.*, 2005)

Δ*siaD:ppx*	Insertional inactivation of *siaD* and *ppx*	This study
Δ*lst*	Insertional inactivation of *lst*; tetracycline resistant	(Exley *et al.*, 2005)
Δ*lst:ppx*	Insertional inactivation of *lst* and *ppx*	This study
Δ*nmb1467*^{com} (Δ*ppx*^{com})	Complemented Δ*nmb1467* with *ppx*	This study
Δ*ppx*^{E147A}	Complemented Δ*ppx* with *nmb1467*^{E147A}	This study
Δ*ppk*	Insertional inactivation of *ppk*; tetracycline resistant	This study
Δ*gna1870*	Insertional inactivation of *gna1870*; tetracycline resistant	This study
Δ*gna1870:ppx*	Insertional inactivation of *lst* and *nmb1467*	This study
E. coli		
TOP10	F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mc*rBC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*araleu*) 7697 *gal*U *gal*K rpsL (Str^R) *end*A1 *nup*G	Invitrogen
BL21(DE3)pLysE	F$^-$ *ompT hsdSB* ($r_B^-m_B^-$) *gal dcm* (DE3) pLysE (Cam^R)	Invitrogen
TT24554	*E. coli* Top10/pHIS-*ppk*	(Price-Carter *et al.*, 2005)
Plasmids		
pCR®2.1-TOPO®	Cloning vector	Invitrogen
pET-15b	Vector for protein expression	Novagen
pYHS25	Vector for complementation	(Winzer *et al.*, 2002)
p*nmb1467*-pET-15b	pET-15b containing *nmb1467*	This study
p*nmb1467*^{E147A}-pET-15b	pET-15b containing *nmb1467* with modification at 147 residue	This study
p*nmb1467*-pYSH25	pYSH25 containing *nmb1467*	This study

2.2 Antibiotics

Antibiotics were used at following concentrations: for *E. coli* strains, 50 µg/ml of kanamycin, 50 µg/ml of ampicillin, 200 µg/ml of erythromycin and 15 µg/ml of tetracycline; for *Neisseria* strains, 100 µg/ml of kanamycin, 2.5 µg/ml of tetracycline and 2.5 µg/ml of erythromycin. To make stocks of higher concentrations, all the antibiotics were dissolved in H_2O, with the exceptions of tetracycline that was dissolved in 70% ethanol, and erythromycin which was dissolved in 100% ethanol, filter sterilised, and aliquots kept at -80°C.

2.3 Isolation of genomic DNA from *N. meningitidis*

Between one quarter to one half loop (10 µl loop) of bacteria was harvested after overnight growth on solid media, and re-suspended in 567 µl of TE buffer (10 mM Tris, pH 7.6, 1 mM EDTA), 30 µl of 10% SDS and 3 µl of proteinase K (> 600 mAU/ml, Qiagen). After a 1 hour incubation at 37°C, 100 µl of 5 M NaCl and 80 µl of cetyl-trimethyl-ammonium bromide (CTAB)/NaCl (10% CTAB, 0.7 M NaCl) were added to the mixture, and incubated at 65°C for 10 minutes. An equal volume of phenol was added to the mixture, which was mixed by inverting the tube several times, and centrifuged at 16,000 *xg* for 10 minutes. The aqueous layer was transferred to a clean tube, mixed with an equal volume of chloroform by inverting the tube, and centrifuged at 16,000 *xg* for 10 minutes. The top layer containing DNA was transferred to another clean tube, and the DNA was precipitated by adding 0.7 volumes of isopropanol and centrifuged at 16,000 *xg* for 10 minutes. The DNA pellet was washed with 70% ethanol, and dried using DNA speed Vacuum DNA110 (Savant, ThermoQuest). The DNA was re-suspended in distilled H_2O. For Southern

blot analysis, the DNA was further treated with 2 mg/ml RNAse A (100 mg/ml, Qiagen).

To isolate plasmid DNA, the QIAprep Spin Miniprep Kit (Qiagen) was used following the protocol provided by the manufacturer.

2.4 Restriction endonuclease digestion of DNA

Restriction enzymes and buffers were either purchased from New England Biolabs or Invitrogen. Digestion of DNA was completed by mixing 1x restriction endonuclease buffer, 0.5 U/µl of restriction endonuclease and target DNA, and incubating the reaction mixture at the recommended temperature (in general, 37°C) for at least 1 hour. For certain enzymes from New England Biolabs, 100 µg/ml of bovine serum albumin (BSA) was added to the reaction.

2.5 Gel electrophoresis and purification of DNA from agarose gels

Purified DNA was normally analysed by agarose gel electrophoresis. DNA samples were mixed with a 1/6 volume of 6x loading buffer (0.25% bromophenol blue, 30% glycerol, 0.25% xylene cyanol), and separated on 0.8% agarose gels in 1x Tris-Acetate-EDTA (TAE) buffer (40 mM Tris, 20 mM acetic acid and 2 mM Na_2-EDTA). The DNA was either stained with 0.1% ethidium bromide or 0.1% SYBR Green I (Invitrogen). The stained DNA was visualised under UV illumination and images were taken using the Gene Flash Bio Imaging System (Syngene, UK).

To recover DNA samples from agarose gels, the QIAEX II Gel Extraction Kit (Qiagen) was used following the protocol provided by the supplier. This kit was also used to clean DNA fragments from enzymatic reactions.

2.6 Ligation of DNA

Ligation of DNA fragments was performed in two different ways depending on the nature of the inserts and vectors. PCR products synthesised by *Taq* polymerase with a single 3'-A overhang were ligated to the TA cloning vector pCR®2.1-TOPO® (Invitrogen) directly according to manufacturer's protocol. For other ligations, T_4 DNA ligase was required. Purified DNA fragments (1-10 µg/ml in total) with ratio of insert to vector ranging from 2:1 to 6:1 were incubated with a 1/20 volume of T_4 DNA ligase and ligase buffer. Using T_4 ligase (5 U/µl, Invitrogen), the incubation was performed at 16°C from 1 hour to overnight, whereas, using Quick-Stick Ligase (10 U/µl, Bioline), the ligation was incubated for 5 minutes at room temperature.

2.7 Transformation

Chemically competent cells One Shot® Top10 and One Shot® BL21 (DE3) pLysE *E. coli* strains were purchased from Invitrogen for transformations. Ligation products or plasmids (5-10 µl) were added to competent cells, and the mixture incubated on ice for 15-30 minutes. After a brief heat-shock for 45 second at 42°C, the mixture was placed on ice immediately for 2 minutes. The cells were then incubated with 250 µl of rich medium SOC (Invitrogen) at 37°C with shaking at 200 r.p.m for 60 minutes.

Bacteria were plated on appropriate selective solid medium and incubated at 37°C overnight without shaking.

To transform *N. meningitidis*, strains were grown on BHI agar plates overnight, and re-suspended in phosphate buffered saline (PBS). A small portion of the bacterial suspension (10 µl) was spotted on a BHI agar plate, and after 5-10 minutes, 5 µl of DNA was added and allowed to dry. Plates were next incubated at 37°C in the presence of 5% CO_2. After 4-5 hours incubation, bacteria were re-streaked on BHI plates containing appropriate antibiotics, and incubated overnight at 37°C in the presence of 5% CO_2.

2.8 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) was used to amplify target genes from either genomic or plasmid DNA. PCRs containing 0.2 µM of each primer (listed in Table 2.2), 200 µM dNTPs, a 0.1 volume of 10x PCR buffer, 2-50 ng of template DNA and 2 U of *Taq* DNA polymerase (Sigma) were performed in a Perkin Elmer GeneAmp® System 9700 thermal cycler (Applied Biosystems, UK). Expand High Fidelity PCR System (Roche) was used to amplify DNA fragments for complementation or protein expression, and a long template PCR system (Roche) was used for products with size larger than 3 kb. Colony PCR was used for rapid screening of bacterial colonies. *E. coli* colonies were picked with sterile toothpicks, dabbed into the PCR mixture and directly used as the DNA template. *N. meningitidis* colonies were boiled in 50 µl of distilled H_2O for 10 minutes, and the supernatant was used as the DNA template. Typical PCR conditions started with an initial step of denaturation at 95°C for 5 minutes and followed by 30 cycles each of 30 seconds of denaturation at 95°C, 30

seconds of annealing at 55°C and 2 minutes of extension at 72°C (for fragments larger than 3 kb, 1 minute per 1 kb incubation at 68°C was used). The reaction ended with 7 minutes of extension at 72°C (or 68°C for if PCR product was larger than 3 kb). PCR products were stored at 4°C overnight, or at -20°C for longer periods.

Tables 2.2 Primers used in this study

Name	Sequence
NG551	GCGCTAGCTGCCGTCGGCAACATCCGTGG
	(with a *Nhe*I site at the 5' end)
NG552	GCGGATCCTCAGACGGCCTCGACTTTGAACG
	(with a *Bam*HI site at the 5' end)
NG559	GCCATATGACCACCACCCCCGCAAAC
	(with a *Nde*I site at the 5' end)
NG591	GCGGATCCTCAGCCTCCGAGCAGTGT
	(with a *Bam*HI site at the 5' end)
NG607	GGCGGTTCGACAGCATTTGTCATCGGCTCGACG
NG608	CGTCGAGCCGATGACAAATGCTGTCGAACCGCC
NG663	GCCATATGCCGTCTGAAACCGGCGCA
	(with a *Nde*I site at the 5' end)
NG1153	GCGGATCCATGCCGTCTGAAACC
	(with a *Bam*HI site at the 5' end)
NG1157	GCAAGCTTTATTTCGAGGGTAACAT
	(with a *Hin*dIII site at the 5' end)
NG1347	GGATCCATGCCGTCTGAACC
	(with a *Bam*HI site at the 5' end)
NG1348	GAATTCGAACTCTCCACTCTCCAA
	(with a *Eco*RI site at the 5' end)
NG1349	GAATTCCAAGTATACAAACAAAGCC
	(with a *Eco*RI site at the 5' end)
NG1350	TTCGAATTATTGCTTGGCGGCAAG
	(with a *Bst*BI site at the 5' end)

2.9 Southern analysis

RNA-free genomic DNA was digested with *Cla*I and *Dra*I separately at 37°C. The digested DNA was separated overnight by agarose gel electrophoresis at 15 V with DNA ladders on both sides of the digested samples. The gel was washed in H_2O and equilibrated in depurination buffer (0.25 M HCl) by slow shaking for 12 minutes. After rinsing in H_2O briefly, the gel was immersed in denaturing buffer (0.5 M NaOH, 1.5 M NaCl) for 15-18 minutes, then equilibrated in neutralising solution (1 M Tris, 3 M NaCl) for 15-18 minutes. A nitrocellulose membrane (10 cm x 15 cm, Hybond N+, Amersham) was pre-wetted in H_2O for 30 seconds, soaked into 20x SSC (3 M NaCl, 0.3 M $C_6H_8NaO_7$, pH 7.0). The DNA gel was covered with the membrane without any air bubbles. Three pieces of Whatman paper (Fisher) pre-soaked in 20x SSC were placed on top of the membranes followed by three pieces of dry Whatman paper. Transfer of DNA from the gel to the membrane was completed in 4 hours, and the DNA was fixed by exposing membranes to UV light for 30 seconds. The membrane was soaked in 2x SSC buffer for a few seconds and washed in a hybridisation tube with solution containing 0.5% SDS and 0.1x SSC for 20 minutes at 65ºC. Alkphos Direct Labelling (Amersham) was used to label DNA probes following the instructions provided by the manufacturer. DNA probes (10 ng/µl) were denatured by heating at 100°C for 5 minutes, and then placed on ice immediately. The probes were mixed gently with 10 µl of reaction buffer, 2 µl of labelling reagent, and 10 µl of cross linker (diluted 1:5 in H_2O), spun briefly, and incubated at 37°C for 30 minutes. The labelled DNA probes could be used immediately or kept on ice for up to 2 hours. When the probe was ready, the membrane was pre-incubated with hybridisation buffer (0.5 M NaCl, 4% blocking reagent, Amersham) at 55°C for at least 15 minutes, then hybridised with labelled probes (5-10 ng/ml) at 55°C overnight in a

hybridisation oven. The membrane was carefully transferred to another clean hybridisation bottle and washed with pre-heated buffer (0.2 M urea, 0.1% SDS, 0.05 M sodium phosphate pH 7.0, 1 mM $MgCl_2$, 0.2% blocking reagent) at 55°C for 10 minutes. The membrane was washed twice with buffer (1 M Tris-HCl, pH 10, 2 M NaCl) at room temperature, and then placed in an X-ray film cassette. Signals on Southern blots were visualised on Hyperfilm film by autoradiography (Amersham).

2.10 DNA sequencing

DNA samples were mixed with 3.2 pmol of primer and diluted with H_2O to a final volume of 10 µl and sent to sequencing service centre at Imperial College London.

2.11 Real time-PCR

Bacteria were grown in 10 ml of liquid BHI with shaking at 150 r.p.m for 4 hours to mid-logarithmic phase, and were harvested by centrifugation at 15,000 *xg* for 5 minutes. Total RNA was extracted using the RNeasy Mini® Kit (Qiagen), and cDNA was synthesised from RNA using QuantiTec Rev Transcription Kit (Qiagen). Transcription levels were measured by qrtRT-PCR using QuantiTect SYBR Green PCR Kit (Qiagen) on a thermal cycler (Rotor-Gene 3000; Corbett Research). Data were analysed using the comparative quantitation method by Rotor-Gene software (version 6.0; Corbett Research). Controls included reactions with no template and samples of RNA that had not been treated with RT. qrtRT-PCRs were performed in triplicate on cDNA samples derived from three independent cultures.

2.12 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Polyacrylamide gels were prepared using a Mini-Protean II gel apparatus (Bio-Rad) and were run in SDS-PAGE buffer (200 mM glycine, 248 mM Tris, 34 mM SDS) at 120 V for 1-2 hours. Reagents used to make a 12% polyacrylamide gel are listed in the following table.

Table 2.3 Reagents for 12% polyacrylamide gel

	Separating buffer (5ml)	Stacking buffer (1.5ml)
30% Acrylamide (Bio-Rad)	2 ml	225 µl
1.5 M Tris-HCl	1.25 ml, pH 8.8	125 µl, pH 6.8
10% SDS	375 µl	11.25 µl
dH_2O	1.75 ml	1.125 ml
10% APS	16 µl	15 µl
TEMED	3.5 µl	1.5 µl

2.13 Staining and de-staining of polyacrylamide gel

Polyacrylamide gels were immersed in Coomassie blue staining buffer (0.2% Coomassie blue R-250, 40% ethanol, 10% glacial acetic acid) with slow shaking at room temperature for at least 10 minutes. De-staining of gels was completed by incubation in de-staining buffer I (40% methanol, 10% glacial acetic acid) for 10 minutes and with de-stain buffer II (10% glacial acetic acid, 4% glycerol) overnight.

2.14 Preparation of whole cell lysates of *N. meningitidis*

N. meningitidis strains were harvested after overnight growth on BHI agar plates and re-suspended in PBS. The concentration of the bacterial suspension was determined

by measuring the optical density (O.D.) of a mixture containing 20 µl of the bacterial suspension and 980 µl of lysis buffer (1 M NaOH, 1% SDS) at 260 nm using a UV-VIS specphotometer (Shimadzu UK). The bacterial suspension was adjusted to 10 x 10^9 CFU/ml, mixed with an equal volume of 2x SDS-PAGE loading buffer (100 mM Tris-HCl pH 6.8, 20 µM β-mercaptoethanol, 4% SDS, 0.2% bromophenol blue, 20% glycerol), and boiled for 10 minutes. The samples were centrifuged at 16,000 *xg* for 20 seconds, and 10 or 20µl of samples was analysed by SDS-PAGE.

2.15 Western blot analysis

When proteins were separated on 12% polyacrylamide gel, one piece of 7cm x 9 cm Immobilon Polyvinylidene Fluoride (PVDF) membrane (Millipore) pre-wetted with methanol, and two fibre pads (Bio-Rad) and two pieces of Whatman paper (VWR) were soaked in cold wet blot transfer buffer (48 mM Tris, 39 mM glycine, pH 8.3) before use. To transfer proteins from gels to PVDF membranes, one pad and one piece of wet Whatman paper were put on the dark side of the gel transfer holder cassette. Then the polyacrylamide gel and PVDF membrane, wet Whatman paper and a fibre pad were placed on top of each other in that sequence. Transfer of proteins was performed at 70 V for 1 hour using a Mini Trans-Blot Cell (Bio-Rad). The PVDF membrane was blocked in PBS with 5% milk at room temperature for 1 hour or at 4°C overnight. Next, the membrane was rinsed with PBS and incubated with the primary antibody diluted in PBS containing 1% milk with slow shaking. The concentration of and incubation time of primary antibodies were optimised for each antibody. After incubation with the primary antibody, membranes were washed three times in PBS containing 0.1% Tween-20 (PBS-T) for 10 minutes at room temperature.

Membranes were incubated with 1:1,000 diluted anti-mouse, anti-goat, or anti-human horseradish peroxidase conjugated immunoglobulin G (DakoCytomation, UK) for 1 hour at room temperature with shaking. After another three washes with PBS-T, membranes were incubated with ECL Western blot detection reagent (Amersham) for 1 minute at room temperature. After removing excess detection reagent, membranes were wrapped in SaranWarp, placed in an X-ray film cassette, and exposed to an autoradiography Hyperfilm film (Amersham).

2.16 Flow cytometry analysis (FACS)

N. meningitidis was harvested after overnight growth on BHI plates, and 2 x 10^7 CFU bacteria were fixed with 3% paraformaldehyde (PFA) at room temperature for 20 minutes. The fixed bacteria were washed three times with PBS-T and incubated with 50 µl PBS containing the primary antibody at 37°C for 30 minutes. After three washes with PBS-T, bacteria were incubated with a secondary, FITC-conjugated antibody that was 1:100 diluted in PBS at 4°C for 30 minutes in the dark. After three washes, bacteria were re-suspended in 500 µl of PBS. Binding of the secondary antibody to meningococci was detected by flow cytometry using FACSCalibur machine (Becton Dickinson).

2.17 Whole cell enzyme linked immunosorbent assay (ELISA)

N. meningitidis was harvested after overnight growth on solid medium, re-suspended in PBS, and killed by heating at 56°C for 1 hour. Bacteria were re-suspended in ELISA coating buffer (Sigma), and 100 µl of bacterial suspension (10^8 CFU) were

coated onto each well of ELISA plates (Maxisorb, NuncTM) at 4°C overnight. The wells were washed three times with 200 µl of PBS-T, and incubated with 100 µl of PBS containing different concentrations of the primary antibody at 37°C for 1 hour. The unbound primary antibody was removed by three washes with PBS-T, and the wells were incubated with the HRP-conjugated, secondary antibody diluted 1:100 in PBS at 37°C for 1 hour. After three washes, 100 µl of substrate (0.4 mg/ml o-phenylenediamine dihydrochloride, 0.4 mg/ml urea hydrogen peroxide and 0.05 M phosphate-citrate, pH 5.0) was added to each well, and incubated at room temperature for 10-15 minutes. Reactions were stopped by the addition of 100 µl of 3 M HCl, and absorbance was read at 492 nm.

2.18 Over-expression of recombinant NMB1467 in *E. coli*

For protein expression, *E. coli* BL21 cells carrying the p*nmb1467*-pET-15b were grown overnight in the LB liquid medium with 50 µg/ml ampicillin at 37°C with shaking. Bacteria were sub-cultured into fresh LB culture to give an initial O.D. A_{600} of 0.1. After 1.5-2 hours incubation at 37°C with shaking, the O.D. A_{600} of cultures reached approximately 0.6, and isopropyl-D-thiogalactoside (IPTG) was added to a final concentration of 1 mM. Protein samples from bacteria were separated by SDS-PAGE, and the over-expression of recombinant protein was analysed by Coomassie blue staining and Western blot analysis using a HRP-conjugated anti-His monoclonal antibody (Qiagen).

2.19 Antibodies

A list of antibodies used in this work is shown in Table 2.4.

Table 2.4 Antibodies used in this work

Antibody	Isotype	Reference
Anti-penta His HRP conjugate	Mouse, monoclonal	Qiagen
Anti-meningococcal serogroup B	Mouse, monoclonal	NIBSC
Anti-meningococcal immunotype L3,7,9	Mouse, monoclonal	NIBSC
3F11	Mouse, monoclonal	Gift (M. Apicella)
FITC-conjugated goat anti-C3	polyclonal	EMD
Anti-human C5b-9	Rabbit, polyclonal	Calbiochem
Anti-human factor H	Goat, polyclonal	Calibochem
Anti-RecA	Rabbit, polyclonal	Gift (D. Holden)
Anti-rNMB1467	Mouse, serum	This study
Anti-rGNA1870	Mouse, serum	Gift (YW. Li)

2.20 Purification of recombinant NMB1467

After 3-4 hours incubation with 1 mM IPTG, bacteria over-expressing recombinant protein were harvested by centrifugation at 4,000 *xg* at 4°C for 30 minutes. The bacterial pellet was either directly used for protein purification or stored at -80°C until use. Bacteria were re-suspended in 10 ml of washing/equilibration buffer (500 mM sodium phosphate, pH 7.0, 300 mM NaCl), and lysed in a French press (three times at 1,500 p.s.i). Intact cells and the insoluble fraction were removed by centrifugation at 17,000 *xg* at 4°C for 1 hour. The supernatant was filtered using a 0.4 µm pore and applied to a 1 ml volume of Nickel-Sepharose High performance pre-packed HisTrap™ column (GE healthcare), pre-equilibrated with washing/equilibration buffer. The column was washed with 15 ml of washing/equilibration buffer to remove non-specifically bound proteins. The over-expressed rNMB1467 with His-tag was eluted with washing/equilibration containing

200 mM imidazole. Samples collected from different steps were analysed by SDS-PAGE followed by Coomassie blue staining and Western blot analysis using anti-penta His HRP conjugated mAb.

2.21 Estimation of protein concentration

Protein concentrations were determined using the Bradford reagent (Sigma) following the manufacturer's protocol. For purified recombinant, BSA (New England Biolab) diluted in washing/equilibration buffer with 200 mM imidazole was used as a standard.

2.22 Construction of *nmb1467*^{E147A}

The point mutation in *nmb1467* was constructed using Quick Change Site-Direct Mutagenesis Kit (Stratagene). The gene encoding NMB1467^{E147A} was amplified by PCR using p*nmb1467*-pET-15b as DNA template, *PfuTurbo* DNA polymerase and primers NG607 and NG608. The PCR product was digested with 1 U/µl of *Dpn*I at 37°C overnight, and transformed into TOP10 cells. The mutated gene was verified by DNA sequencing, and then the corresponding plasmids were transformed in to BL21 cell for protein over-expression and purification as described above.

To construct complemented Δ*nmb1467*^{E147A} strain, plasmid p*nmb1467*^{E147A}-pET-15b was digested *Sma*I and *Bam*HI sequentially. The DNA fragment containing *nmb1467*^{E147A} point mutation was recovered from an agarose gel, and ligated into p*nmb1467*-YHS25 digested with the same enzymes. The ligation product was

transformed into TOP10 competent cells, and plasmids were analysed. The mutation was verified by DNA sequencing, and p*nmb146*^{E147A}-pYSH25 was used for transformation of *N. meningitidis*.

2.23 Generation of polyclonal anti-serum against recombinant NMB1467

The immunisation of mice to generate polyclonal anti-serum against rNMB1467 was performed by Dr. YW Li. Six- to eight-week-old BALB/c mice (Harlan, UK) were immunised by subcutaneous inject with 20 µg of rNMB1467 in 100 µl of PBS mixed with 100 µl of Freund's incomplete adjuvant. Booster doses were given on days 21 and 28 post-immunisation. On day 35, blood was obtained by cardiac puncture under terminal anaesthesia, and sera were collected by centrifugation at 2,000 *xg* at 4°C for 20 minutes. Sera containing antibody were aliquoted and kept at -80°C.

2.24 Extraction of LPS from *N. meningitidis*

N. meningitidis strains were harvested after overnight growth on BHI agar plates and re-suspended in PBS. Bacterial suspensions were adjusted to 6 x 10^9 CFU/ml and mixed with an equal volume of 2x LPS loading buffer (100 mM Tris-HCl, pH 8.0, 6% β-mercaptoethanol, 6% SDS, 0.2% bromophenol blue, 46% glycerol, 10 mM dithiothreitol), and boiled for 10 minutes. Proteins in the mixture were digested with 0.1 mg/ml proteinase K at 37°C overnight. Prior to loading, the samples were boiled for 10 minutes and treated with 0.1 mg/ml proteinase K at 56°C for another 3 hours.

2.25 Electrophoresis and staining of LPS

Tricine sodium dodecyl sulphate-polyacrylamide gel electrophoresis (TSDS-PAGE) was used to obtain high resolution separation of LPS. The reagents used to make the 16% of separating gel and the stacking gel are listed in Table 2.5. Electrophoresis of LPS was the same as SDS-PAGE except for the running buffers and voltage. The anode buffer (0.2M Tris-HCl, pH 8.9) was filled carefully into the inner chamber of a Mini-PROTEAN 3 cell (Bio-Rad) and the cathode buffer (0.1 M Tris-HCl, pH 8.25, 0.1 M Tricine, 0.1% SDS) was added to the rest of the tank. Samples were run in the stacking gel at 15 V, then separated in the separating gel at 90-150 V. LPS were visualised using a Silver Staining Kit (GE Healthcare).

Table 2.5 Reagents for 12% Tricine-SDS polyacrylamide gel

	Separating buffer (5ml)	Stacking buffer (1.5ml)
30% Acrylamide (Bio-Rad)	2 .67ml	225 µl
1.5 M Tris-HCl	1.25 ml, pH 8.8	125 µl, pH 6.8
10% SDS	375 µl	11.25 µl
Urea	1.2 g	-
dH_2O	To make up to 5 ml	1.125 ml
10% APS	16 µl	15 µl
TEMED	3.5 µl	1.5 µl

2.26 Bacterial growth curves

N. meningitidis strains were harvested after overnight growth on BHI agar plates and re-suspended in PBS. The suspension was centrifuged at 1,000 *xg* for 2 minutes and the supernatant was transferred to a new tube. The concentration of bacteria was determined by measuring the O.D. at 600 nm. The suspension was diluted to a O.D. A_{600} of 0.1 in 10 ml of BHI liquid, and the culture was incubated at 37ºC with 150

r.p.m. Bacterial samples were taken every hour to measure the O.D. at 600 nm, and the number of CFU was determined by plating the aliquots of the culture to solid media and counting the number of colonies after overnight incubation.

2.27 Serum bactericidal activity assay (SBA)

N. meningitidis strains were harvested after overnight growth on BHI agar plates and re-suspended in PBS. The suspension was centrifuged at 1,000 *xg* for 2 minutes, and the supernatant transferred to a new tube. The concentration of bacteria was determined by measuring the O.D. at 260 nm of the mixture of the bacterial suspension in lysis buffer as above. The bacterial suspension was diluted in SBA buffer (PBS with 0.1% glucose) to a final concentration of 2 x 10^4 CFU/ml and incubated with an equal volume (12.5 µl per well in a 96-well plates) of baby rabbit complement (Pelfreeze, US) diluted 1:4 in SBA buffer, and 25 µl of serial dilutions of sera in SBA buffer at 37°C for 1 hour in the absence of CO_2. The number of surviving bacteria was determined by plating 10 µl of each well onto solid medium and counting the colonies after overnight incubation. The bactericidal activity was expressed as the reciprocal of the dilution of sera required to kill more than 50% of bacteria.

2.28 Human serum sensitivity assay

Bacterial strains were grown on BHI agar plates overnight and re-suspended in PBS. After centrifugation at 1,000 *xg* for 2 minutes, the bacterial suspension was transferred to a new tube. Bacteria were diluted to a final concentration of 1 x 10^5

CFU/ml in DMEM-glutaMAX™ medium (Invitrogen), and incubated with different concentrations of normal human sera (NHS) at 37°C in the presence of CO_2 for 1 hour. Serum was heated at 56°C for 30 minutes to inactivate complement as required. The survival of bacteria in the presence of sera was determined by plating aliquots onto BHI plates and counting the number of colonies after overnight incubation.

2.29 Quantification of poly P

The concentration of poly P was quantified by two different methods based on the metachromatic reaction with toluidine blue (Mullan *et al.*, 2002) and the activity of PPK (Ault-Riche *et al.*, 1998).

Method 1: Samples (10 µl) were added to 990 µl of 6 mg/L toluidine blue in 40 mM acetic acid. The O.D. of the mixture was read at 530 and 630 nm.

Method 2: Samples were converted to ATP by incubation with 50 mM Tris-HCl, pH 7.4, 40 mM ammonium sulphate, 4 mM $MgCl_2$, 5 µM ADP and 24,000 U of PPK in a 100 µl ATP reaction at 37°C for 40 minutes, and then at 90°C for 2 minutes. The concentration of resulting ATP was quantified by mixing an equal volume of the ATP reaction with CellTiter-Glo® Reagent (Promega), and measuring the luminescence using a luminometer (Turner Designs). The concentration of poly P is given in terms of P_i residues.

2.30 Exopolyphosphatase activity assay

The activity of purified recombinant NMB1467 was measured by the loss of standard poly P_{75} by the toluidine blue assay. A 100 µl reaction containing 50 mM Tricine/KOH (pH 8.0), 175 mM KCl, 15 mM poly P_{75}, 1 mM $MgCl_2$ and recombinant rNMB1467 was incubated at 37°C for 30 minutes. The concentration of poly P_{75} was determined using the toluidine blue assay. To determine the kinetics of rNMB1467 activity, different concentrations of protein were added to the reaction buffer.

2.31 Polyphosphate kinase activity

Purified protein was assayed in a 250 µl reaction volume consisting of 50 mM Tris–HCl buffer, pH 7.0, 40 mM glycylglycine-KCl, 10 mM potassium phosphate (pH 7.0), 10 mM $MgCl_2$, 15% (v/v) glycerol, 2.5 mM ATP and 3 mM poly P_{75}. To avoid inhibition of PPK by ADP formed during the reaction, 6 mM of phosphoenolpyruvate and 10 U of pyruvate kinase were also added to the assay mixture to permit the regeneration of ATP. The increased poly P concentration was subsequently determined using the toluidine blue assay.

2.32 Extraction of poly P from *N. meningitidis*

Poly P was extracted from *N. meningitidis* by two different methods according to reports by Tinsley (Tinsley *et al.*, 1993) and Ault-Riché (Ault-Riche *et al.*, 1998).

Method 1: *N. meningitidis* strains were harvested in PBS from BHI plates after overnight incubation. Bacteria were centrifuged at 16,000 *xg* for 5 minutes, and the pellet was re-suspended in 5.4% sodium hypochloride solution (7.5 ml/g of cells) with stirring at room temperature for 45 minutes. The cell debris containing poly P was collected after centrifugation at 16,000 *xg* for 30 minutes, and washed twice with ice-cold 1.5 M NaCl with 1 mM EDTA. Poly P was extracted twice with cold water with an equal volume sodium hypochlorite, poly P then precipitated by the addition of a 1/50 volume of 5 M sodium chloride, washed with 70% ethanol, and finally re-suspended in 100 µl of 10 mM Tris-HCl, pH 7.5.

Method 2: ***N.*** *meningitidis* strains were harvested into PBS from BHI plates after an overnight incubation. Bacteria (2×10^9 CFU) were re-suspended in 0.5 ml of GITC lysis buffer (4 M guanidine isothiocyanate, 50 mM Tris-HCl, pH 7.0, pre-warmed to 95°C), vortexed briefly and incubated in a heat block at 95°C for 5 minutes. The bacterial suspension was sonicated for 30 seconds at 12 W (Bandelin Sonoplus), and added with 30 µl of 10% SDS, 0.5 ml of 95% ethanol and 5 µl of Glassmilk (Sigma). The mixture was vortexed briefly and centrifuged at 16,000 *xg* for 2 minutes. The pellet was washed twice with 0.5 ml of cold New Wash buffer (5 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM EDTA, 50% ethanol) by sonication. The washed pellet was then re-suspended in 100 µl of 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 20 µg/ml DNase and RNase, and incubated at 37°C for 10 minutes. After centrifugation, the pellet was washed with 150 µl of 4 M GITC lysis buffer and 150 µl of 95% ethanol once and New Wash buffer twice. Poly P was eluted from the pellet with 50 µl of 50 mM Tris-HCl, pH 8.0, at 95°C for 2 minutes; recovery of poly P was completed by two additional elutions.

2.33 Crystallisation of native rNMB1467

Following purification, rNMB1467 was dialysed against buffer containing 20 mM Tri-HCl, pH 8.0, 200 mM NaCl, 5 mM DTT and 5 mM pentasodium triphosphate using a Slide-A-Lyzer 10 MWCO Dialysis Cassettes (Pierce) at 4°C overnight. The dialysed protein was concentrated to 10 mg/ml using an Amicon® Ultra10K Centrifugal Filter devices (Millipore) at 4°C, and used for a broad search of crystallisation conditions.

The crystallisation of all PPX structures was carried out using the sitting drop vapour diffusion methods (Chayen, 1998). The initial crystallisation screen was performed using commercial available plates (Table 2.6).

After optimisation, the diffraction of PPX crystals were tested on the Rigaku RU-H3RHB model X-ray generator in Imperial College, a rotating anode technique based generator, prior to data collection at the SRS synchrotron in Daresbury, UK.

Table 2.6 Plates with buffers used for the initial crystallisation screen

Plate ID	Screen	Company
ICL1	Crystal Screen 1 & 2	Hampton Research
ICL2	Wizard Screen 1 & 2	Emerald BioSystems
ICL3	PEG/Ion & Natrix	Hampton Research
ICL4	Index	Hampton Research
ICL5	SaltRX	Hampton Research
ICL6	Mem-Start & Mem-Sys	Molecular Dimensions Ltd
ICL7	PACT	Molecular Dimensions Ltd
ICL8	JCSG+	Molecular Dimensions Ltd
ICL9	MemGold	Molecular Dimensions Ltd

2.34 Crystal mounting and freezing techniques

To reduce radiation damage, X-ray data collection was carried out at cryogenic temperature, which allowed data to be collected from a singe crystal. This effect is due to the fact that by lowering the temperature to about 100K, diffusional processes and therefore propagation of highly reactive agents within crystals are slowed down or virtually eliminated.

Nylon loops (0.2-0.4 mm, Hampton Research) were attached to CrystalCap Copper Magnetic bases (Hampton Research) using adhesive. A single crystal was fished out from the well using loops, briefly dipped into a drop appropriate cryo-protectant (mother liquor containing 15% glycerol) on a small cover slip. The CrystalCap was then transferred onto the goniometer, which is mounted onto the X-ray generator, and crystal is rapidly frozen in a gaseous stream of nitrogen (100K).

2.35 Statistics

Data were compared by the Student's t test. Differences with p values of < 0.05 were considered significant.

Chapter 3 Identification and evaluation of rNMB1467 as a vaccine candidate

3.1 Strategy of GSI

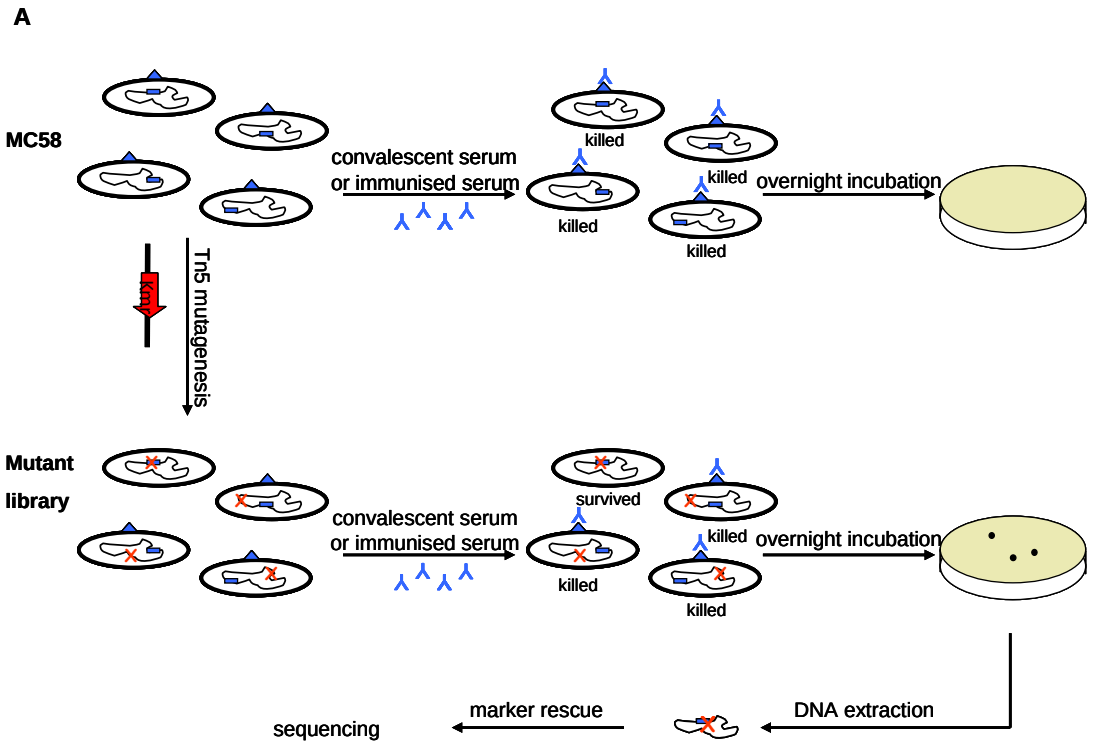
Meningococcal disease remains a leading cause of childhood mortality and morbidity (Rosenstein *et al.*, 2001; Stephens *et al.*, 2007). In addition to antibiotic treatment, vaccination would provide an alternative and effective approach to combat meningococcal infection (Jodar *et al.*, 2002). Unlike the polysaccharide capsules of serogroup A, C, W-135 and Y *N. meningitidis*, which have been successfully included in conjugate vaccines to reduce the global disease burden, the capsule of serogroup B shares antigenic similarities with neural tissue and is a poor immunogen in humans (Balmer *et al.*, 2002; Bilukha and Rosenstein, 2005; Finne *et al.*, 1987). The use of vaccines based on the PorA is effective in diminishing disease caused by strains with the corresponding serosubtypes, but is hindered by the genetic diversity of PorA (Idanpaan-Heikkila *et al.*, 1995). Reverse vaccinology is based on bacterial genome sequencing and computer algorithms but is totally reliant on murine immune responses (Pizza *et al.*, 2000; Rappuoli, 2001).

Therefore, the production of a universal and safe vaccine against serogroup B *N. meningitidis* remains a challenge. An alternative strategy named GSI was developed by our group to identify the targets of bactericidal antibodies, which can be used as vaccine candidates, and to further understand the interaction between the meningococcus and the complement system.

In GSI, a library of insertional mutants of *N. meningitidis* was constructed and then screened for strains which are less susceptible to killing by bactericidal antibodies in human immune serum. It was anticipated that insertional inactivation of genes encoding important antigens recognised by antibodies in immune sera would at a selective advantage compared with the wild-type parental strain in the presence of bactericidal antibodies. Therefore, this approach should identify bacterial factors which modulate resistance against complement and/or might isolate important antigens on the bacterial surface (Figure 3.1A). Due to the availability of its complete genome sequence, MC58, a serogroup B ET-5 isolate of *N. meningitidis*, was used as the parental strain in these experiments (Tettelin *et al.*, 2000). A library containing 40,000 mutants was generated by *in vitro* mutagenesis with Tn*5* (Sun *et al.*, 2000). The diversity of transposon insertions in mutants was confirmed by performing Southern analysis on 30 mutants selected at random from the library. Genomic DNA was prepared from the mutants and digested with *Cla*I or *Dra*I. The DNA fragments were transferred to membranes that were then probed with the kanamycin resistance cassette. As there is *Cla*I recognition site within the kanamycin cassette, two bands are recognised in DNA digested with this enzyme. The 30 mutants all had distinct fragments recognised by Southern analysis, demonstrating that *in vitro* mutagenesis with Tn*5* generates a wide diversity of insertions in *N. meningitidis* (Figure 3.1B).

To avoid PorA-mediated immune responses, acute and convalescent sera were obtained from patients who had been infected with *N. meningitidis* isolates expressing different serosubtypes from MC58 (B:15:P1.7,16b) and vaccinees given outer membrane vesicles prepared from *N. meningitidis* isolate H44/76 (B:15:P1.7,16) (Moe *et al.*, 2002; Sorhouet Pereira *et al.*, 2008). All the selected sera were tested

against MC58, and proved to have bactericidal antibodies with SBAs of > 1:64 when using a 1:4 dilution of baby rabbit complement (data not shown).



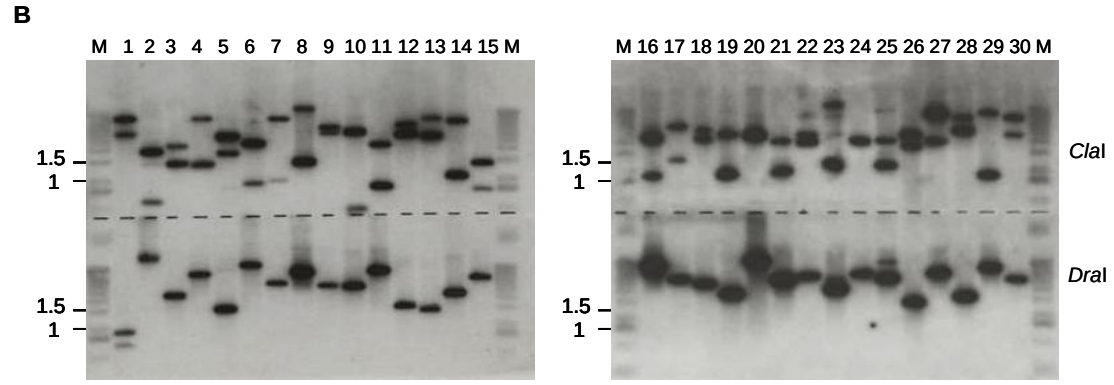


Figure 3.1 Strategy of GSI

(A) GSI. Bacteria express targets (blue triangles) of bactericidal antibodies (blue inverted Y) on their surface. In a library of mutants, the transposon (red cross) may insert in the gene (blue box) encoding an important surface antigen. Loss of this target may enable bacteria to escape recognition of corresponding bactericidal antibodies and subsequent bacterial lysis.

(B) Southern blot analysis of the diversity of mutants generated by Tn*5* mutagenesis. Genomic DNA was extracted from 30 mutants selected at random from library, digested with *Cla*I or *Dra*I and probed with the kanamycin resistance cassette. The sizes of DNA makers are shown in kb.

3.2 Identification of Δ*nmb1467* mutants

Preliminary experiments were performed to establish conditions in which mutants that are resistant against complement-mediated killing would be selected from the library of mutants. As the library contained around 40,000 mutants, sufficient numbers of bacteria had to be included in assays. Furthermore, there was only limited amount of human immune serum. Initial assays were performed with wild-type MC58, with dilutions of heat-treated sera from patients and baby rabbit complement. Different total volumes (100 µl to 2 ml), number of bacteria (10^4 to 10^7 CFU), concentrations of human sera (1:64 to 1:1,024) and complement (1:2 or 1:4) were used to determine the maximum number of CFU that could be included in assays with the lowest concentrations of serum that gave killing of all wild-type bacteria.

To screen for mutants with enhanced survival, the mutant library (10^5 CFU) was incubated with baby rabbit complement (1:4) and with serial dilutions of human immune serum in a 2 ml volume for 1 hour. Bacteria were recovered at the end of this time by plating to solid medium. The wild-type isolate MC58 was screened in parallel with the mutant library on each occasion. Using sera from patient H, there were no colonies recovered when MC58 was inoculated in assays in the presence of a 1:1,024 dilution of serum. In contrast, a few colonies (5-10) were recovered when the mutant library was screened under identical conditions. To confirm the enhanced resistance of these colonies was due to the transposon insertion, the mutations were re-introduced into the parental strain from the mutants. The newly constructed mutants were then tested individually for resistance against complement-mediated killing in standard SBAs. Only mutants exhibiting consistently increased serum resistance were analysed for further detail.

To determine the transposon insertion sites, genomic DNA was extracted from the mutants, and digested with *Eco*RV and *Dra*I, which do not recognise sites within the transposon. After self-ligation, the DNA was transformed into *E. coli*. Only cells containing the transposon, which harbours a functional origin of replication in *E. coli*, were recovered. Plasmids were extracted from these colonies, and the sequences flanking the transposon were determined by DNA sequencing using primers that are complementary to each end of the transposon. Interestingly, mutants with Tn*5* insertions were found in the gene *nmb1467* in independent screens with sera from different individuals. In the mutant HT1024 L14, Tn*5* is located 165 bp downstream of the first ATG of the predicted ORF of *nmb1467*, and in another mutant, T1024 L4, the insertion is 187 bp downstream of the first ATG (Figure 3.2A).

As these two mutants exhibited very similar enhanced resistance against serum killing in preliminary experiments (data not shown), one of the mutants was used in subsequent analysis and was designated MC58Δ*nmb1467*. To confirm the increased resistance resulted from mutation of *nmb1467*, the recovery of the Δ*nmb1467* mutant and MC58 was examined in standard SBAs using convalescent serum from patient H and serum from mice immunised with the attenuated serogroup B *N. meningitidis* strain 23A10 (Li *et al.*, 2004). The SBA titre of patient H' serum against the Δ*nmb1467* mutant was 1024, which was two-fold less than that against MC58 (Figure 3.2B). The killing of both MC58 and Δ*nmb1467* by the α-23A10 serum was more efficient than the serum from patient H. However, the survival of the Δ*nmb1467* mutant was increased compared with MC58 in the presence of 1:8,192 or 1:16,384 diluted α-23A10 serum (Figure 3.2C).

The protein product of *nmb1467* had not been characterised previously. Insertion in genes encoding structural components of the cell wall or regulators could affect expression of a number of surface antigens. Therefore, to determine whether mutation of *nmb1467* led to pleiotropic effects, whole cell extracts were obtained from the Δ*nmb1467* mutant and MC58, and subjected to Western blot analysis using the serum from mice immunised with the attenuated *N. meningitidis* strain 23A10 (Li *et al.*, 2004). There was no significant difference in the binding of antibodies to proteins from the mutant compared with MC58, suggesting that loss of NMB1467 does not have multiple effects on the cell surface (data not shown).

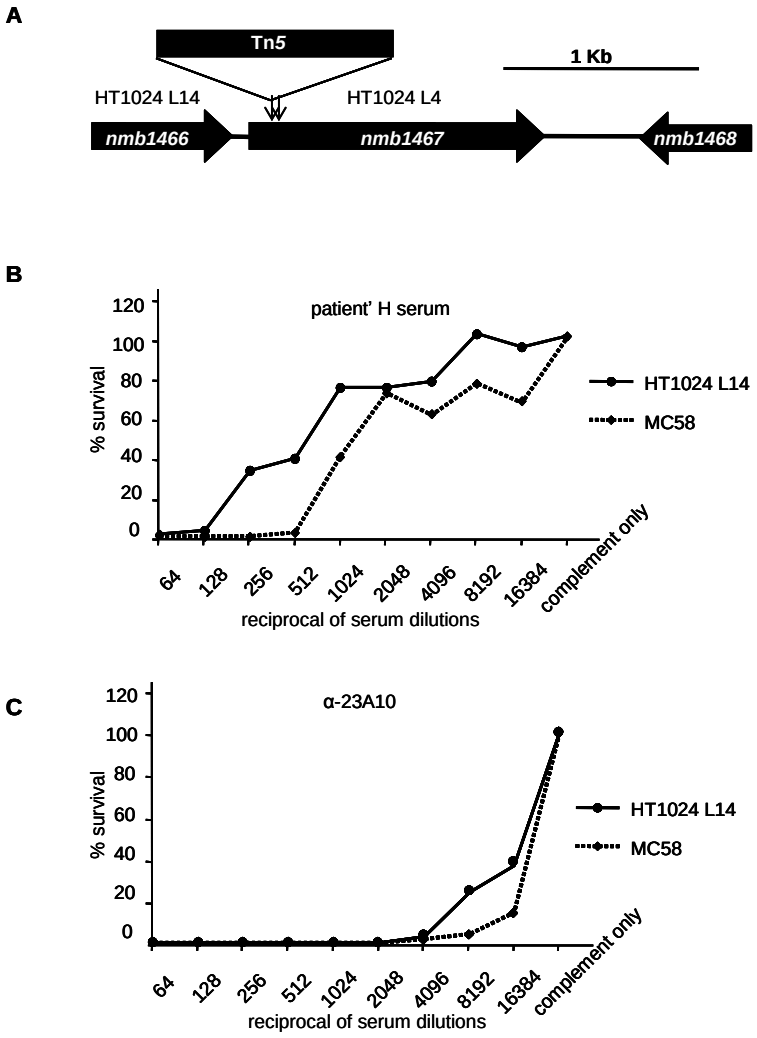


Figure 3.2 Δ*nmb1467* mutants are relatively resistant to killing mediated by bactericidal antibodies

(A) Schematic representation of open reading frames around *nmb1467* and the locations of Tn*5* insertions in mutants, HT1204 L14 and HT1024 L4. The size of 1 kb is indicated. NMB1466 and NMB1468 encode proteins of unknown function.

(B) and **(C)** Increased resistance of the Δ*nmb1467* mutant in patient's and murine immune serum, respectively. SBAs were performed using sera from patient H **(B)** and mice immunised with *N. meningitidis* 23A10 **(C)**.

3.3 Enhanced resistance of the Δ*nmb1467* mutant in normal human serum

After the relatively enhanced resistance of the Δ*nmb1467* mutant was confirmed in the patient H's serum and α-23A10 serum with baby rabbit complement, the survival of the Δ*nmb1467* was examined in the presence of human complement using normal human serum (NHS). Analysis of the orientation of ORFs around *nmb1467* demonstrated that the transposon insertion is unlikely to have polar effects on downstream genes (Figure 3.3A). However, to exclude this possibility the Δ*nmb1467* mutant was complemented by introducing a single wild-type copy of the gene at an ectopic chromosomal location. To construct the complemented strain Δ*nmb1467*^{com}, *nmb1467* and its own promoter were amplified from MC58 by PCR using primers NG551 and NG552, and inserted into the complementation vector pYHS25 in *E. coli* (Winzer *et al.*, 2002). After transformation of *N. meningitidis*, integration of the vector by double crossover leads to a single chromosomal copy of the complementing gene in the intergenic region between *nmb102* and *nmb103*, which are ORFs orientated in a tail-to-tail fashion (Figure 3.3A). The complemented strain MC58Δ*nmb1467*^{com} was verified by PCR and Southern blot analysis (data not shown).

To examine the contribution of *nmb1467* to bacterial survival in the presence of human complement, the recovery of MC58, the Δ*nmb1467* mutant and the complemented strain Δ*nmb1467*^{com} was determined after a 1 hour incubation in NHS. In the presence of a 1:8 dilution of NHS, the number of colonies recovered from the Δ*nmb1467* mutant was about twice that of the wide-type strain; the relatively increased resistance of the mutant was abolished in the complemented strain, Δ*nmb1467*^{com} (Figure 3.3B). In contrast, when bacteria were incubated with serum which had been incubated at 55°C for 30 minutes to inactivate the complement

system, there was no significant difference in the survival of the strains (Figure 3.3C). Taken together, the results demonstrate that insertion of Tn*5* in *nmb1467* results in relatively increased resistance against complement-mediated killing, and inactivation of *nmb1467* is solely responsible for this enhanced resistance.

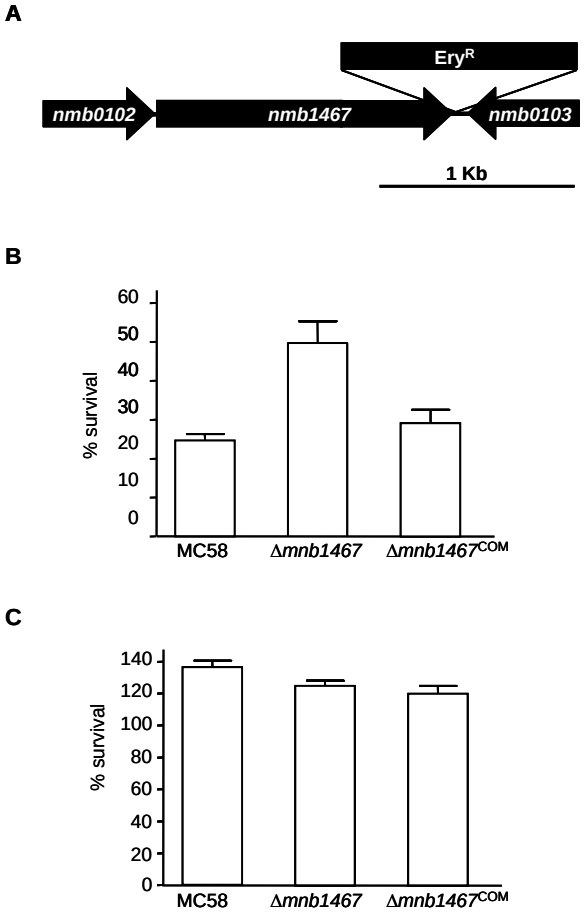


Figure 3.3 Loss of NMB1467 leads to increased survival in normal human serum

(A) Schematic representation of the construction of the complemented strain, Δ*nmb1467*^{com}. *nmb1467* was inserted with an erythromycin resistance gene into the intergenic region between *nmb102* and *nmb103,* as a single copy in the genome.

(B) Increased resistance of MC58Δ*nmb1467* against NHS. The survival of the Δ*nmb1467* mutant was significantly greater than the wild-type strain after incubation with 1:8 diluted NHS for 1 hour ($p < 0.05$). The increased resistance was abolished in the complemented strain Δ*nmb1467*^{com}. The figure shows representative results of three independent experiments performed in triplicate, and error bars show the SEM of assays performed in triplicate**.**

(C) Similar survival of MC58, the Δ*nmb1467* mutant and MC58Δ*nmb1467*^{com} in heat-inactivated serum. Data ***represent*** the average of ***three*** independent ***experiments performed in triplicate,*** and error bars represent the SEM.

3.4 Over-expression and purification of recombinant NMB1467 in *E. coli*.

To allow assessment of NMB1467 as a vaccine candidate, the protein was over-expressed and purified in *E. coli* as a recombinant protein. The whole ORF of *nmb1467* was amplified from MC58 by PCR using primes NG552 and NG559, and initially cloned into the expression vector pET15-b. The resulting plasmid was transformed into TOP 10 competent cells, which do not express T7 RNA polymerase. Once the sequence of *nmb1467* in pET15-b was verified, the plasmid p*nmb1467*-pET15-b was transferred into the expression host BL21, which contains T7 RNA polymerase under the control of *lac* operator. To investigate the effect of over-expression of rNMB1467 on cell growth and to determine the optimal induction strategy for expression of NMB1467, the protein profile of small scale cultures (20 ml) was examined. Bacteria were grown overnight in liquid culture, and sub-cultured to fresh LB to an O.D. A_{600} of 0.1. Strains were grown to an O.D. A_{600} of 0.6, with IPTG added to a final concentration of 1 mM. The growth of bacteria in the presence and absence of IPTG was monitored by measuring O.D. A_{600} every 30 minutes, and aliquots of cultures were taken every hour to analyse protein over-expression. The O.D. A_{600} of cultures with 1 mM IPTG measured at every time point was similar to that of cultures without IPTG (Figure 3.4A), indicating that induction with 1 mM IPTG was not toxic to the host strain carrying p*nmb1467*-pET-15b. Whole cell lysates were separated by SDS-PAGE, and proteins were stained with Coomassie blue. Over-expression of a protein with a molecular mass of approximately 56 kDa appeared in samples after incubation with 1 mM IPTG for 1 hour, and the amount of the over-expressed protein increased over time (Figure 3.4B). Thus, over-expression of rNMB1467 was successfully induced with 1 mM IPTG at 37°C for 1 hour, and induction for 4 hours was optimal for large scale protein purification.

To purify rNMB1467, bacteria were harvested from large scale cultures (500 ml) 4 hours after induction, then lysed using a French press. The soluble and insoluble fractions of the lysates were separated by centrifugation; the over-expressed protein appeared in the soluble fraction in the supernatant (Figure 3.4C). Next, the soluble fraction was loaded onto a His-Trap column, washed with equilibration buffer and the protein was eluted in the presence of 200 mM imidazole (Figure 3.4C). Using Western blot analysis, the purified his-tagged rNMB1467 was recognised by an HRP-conjugated anti-His mAb as a protein with a molecular weight around 56 kDa (Figure 3.4D). Precipitation was observed when the N-terminal His-tag of rNMB1467 was cleaved by thrombin (data not shown), so the His-tag was not removed for all subsequent analyses.

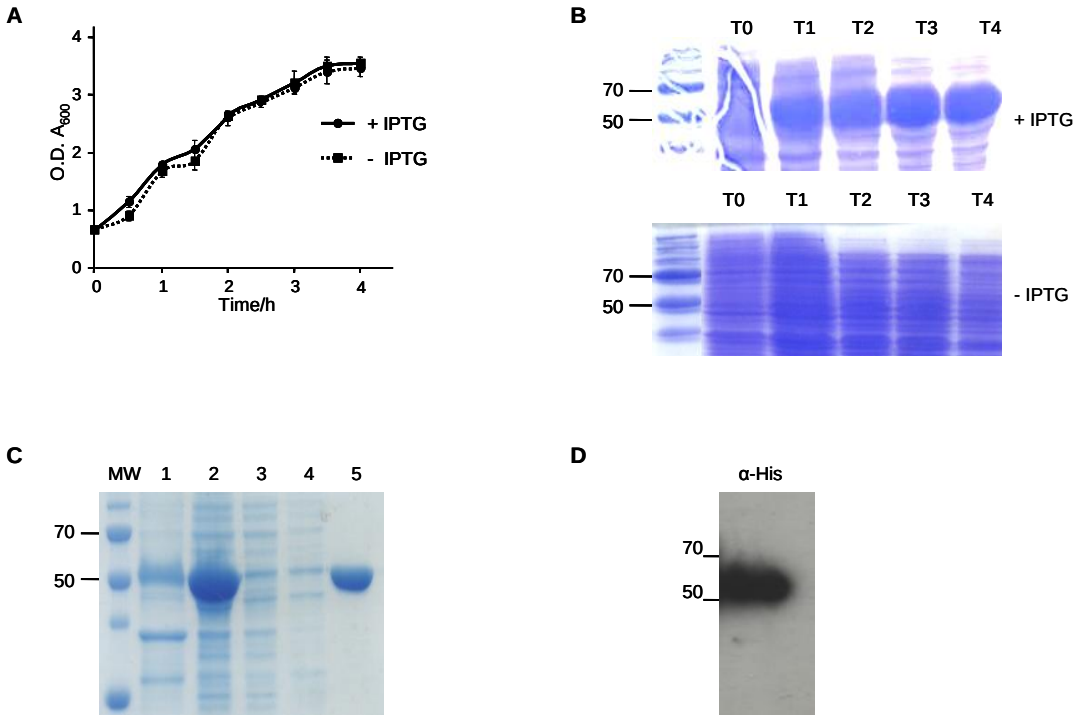


Figure 3.4 Over-expression and purification of rNMB1467 in *E. coli*

(A) Growth of *E. coli* BL21 cells harbouring p*nmb1467*-pET15-b in the presence (solid line with circles) or absence (dashed line with squares) of 1 mM IPTG from early exponential phase.

(B) Analysis of proteins expressed by *E. coli* BL21 containing p*nmb1467*-pET15-b. Whole cell lysates were prepared from bacteria grown with or without IPTG for 0, 1, 2, 3, 4 hours (T0, T1, T2, T3, T4, respectively), and analysed by SDS-PAGE. Proteins were stained with Coomassie blue, and the sizes of molecular weight markers (in kDa) are shown.

(C) Analysis of the purification of rNMB1467 by SDS-PAGE and Coomassie blue staining. Bacteria over-expressing rNMB1467 were lysed with a French press. The soluble fraction (Lane 1) of whole cell lysates was separated from insoluble pellet (Lane 2) by centrifugation and loaded onto a His-trap column. There was no detectable protein with a molecular mass of 56 kDa in the flow-through (Lane 3) and wash solution (Lane 4), but a protein of this mass was eluted from the column in the presence of 200 mM imidazole (Lane 5).

(D) Western blot analysis of purified rNMB1467 using an HRP-conjugated α-poly-His antibody.

3.5 Recognition of rNMB1467 by antibodies in convalescent serum

Purified recombinant NMB1467 (25 µg) was used to generate anti-sera by immunising six-eight week old female BALB/c mice on three occasions by the subcutaneous route. To confirm the specificity of the immune serum, pre-immune and immune (α-rNMB1467) sera were tested by Western blot analysis. First, it was confirmed that the immune but not pre-immune serum recognised purified recombinant rNMB1467 as a single band of about 56 kDa (Figure 3.5A). Next, whole cell extracts from the MC58 and the Δ*nmb1467* mutant were tested with the α-rNMB1467 serum. As expected, the 56 kDa band was present in lysates from MC58 but not in samples of the Δ*nmb1467* mutant (Figure 3.5B).

To test the hypothesis that the increased resistance of the Δ*nmb1467* mutant was due to the loss of NMB1467 as a target of bactericidal antibodies, the presence of α-rNMB1467 antibodies in patient H's serum was examined. By Western blot analysis, purified rNMB1467 (detected as a 56 kDa band) was recognised by antibodies in this serum at a 1:500 dilution (Figure 3.5C). To confirm this result, ELISAs were performed using higher concentrations of patient H's serum. Recombinant rNMB1467 (100 ng) was coated onto the wells of ELISA plates, and dilutions of patient's serum, pre-immune and immune α-rNMB1467 murine serum were added to the wells. ELISA readings detecting rNMB1467 with serum from patient H were lower than that with α-rNMB1467 serum. These results are consistent with findings by Western blot that demonstrated weaker binding was detected when rNMB1467 was incubated with patient H's serum compared with α-rNMB1467 immune serum. In contrast, results with patient's H serum were higher than those obtained with pre-immune murine serum (Figure 3.5D). Taken together, ELISA and Western blot

analysis show that α-rNMB1467 antibodies were present in convalescent serum from patient H, which was used to select Δ*nmb1467* mutants by GSI.

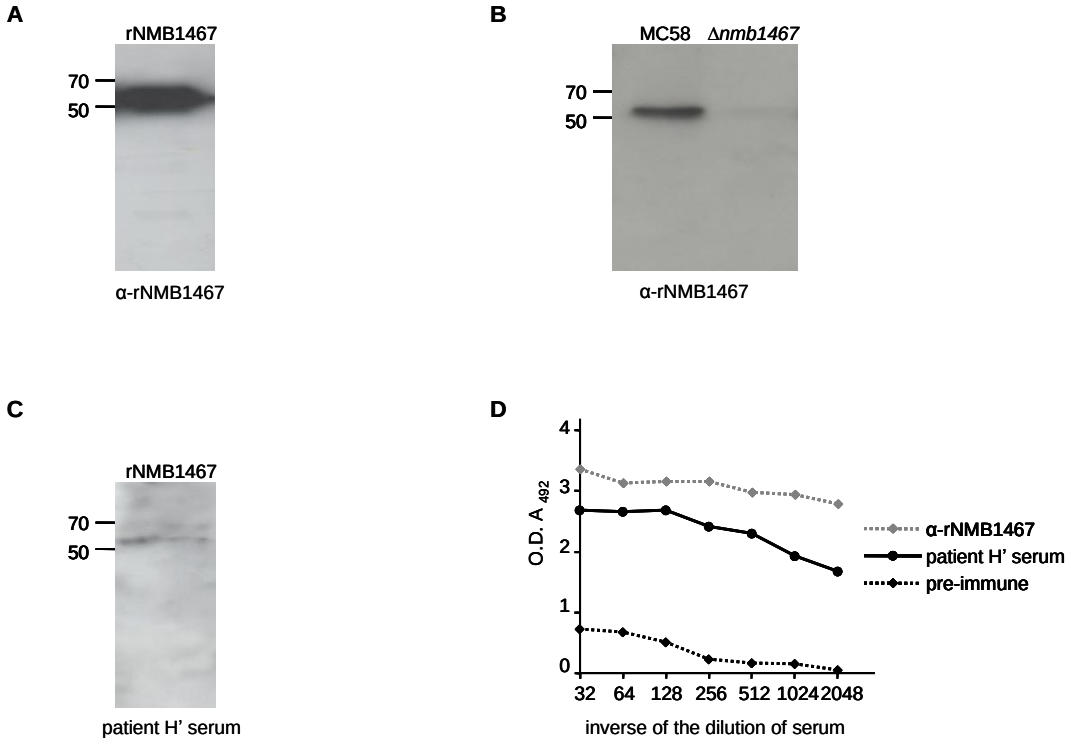


Figure 3.5 Recognition of rNMB1467 by antibodies in serum from patient H

(A) Western blot analysis of binding of anti-rNMB1467 serum (1:5,000 dilution) to the recombinant protein rNMB1467. The sizes of a molecular weight marker are indicated in kDa.

(B) Western blot analysis of the presence of NMB1467 in MC58 but not in the Δ*nmb1467* mutant using a 1:2,000 dilution of anti-rNMB1467serum.

(C) Western blot analysis of binding to rNMB1467 by patient H' serum (1:500 diluted).

(D) ELISA analysis of binding to rNMB1467 of total immunoglobulins in the pre-immune serum (dark dashed line with squares), anti-rNMB1467 (grey dashed line with squares) and patient H's serum (dark solid line with circles).

3.6 Assessment of the vaccine potential of rNMB1467

As an effective vaccine, a selected antigen should be present in all *N. meningitidis* serogroup B strains and be able to elicit bactericidal antibodies that are cross-reactive with a range of strains. To examine whether *nmb1467* is conserved in serogroup B strains, genomic DNA was extracted from 21 clinical isolates (Appendix 1) and used as template in PCRs to detect the presence of *nmb1467*. Using a pair of primers flanking *nmb1467*, a 1.5 kb PCR product was amplified from all these 21 strains as well as MC58, and 10 of 20 PCR products are shown in Figure 3.6A. The presence of homologues of *nmb1467* in the serogroup A and C strains was confirmed by PCR and BLAST of DNA sequence (data not shown).

To examine whether α-rNMB1467 antibodies have bactericidal activity, standard SBAs were performed. In the first assay, the SBA titre of the α-rNMB1467 serum against MC58 was 512, whereas, the SBA titre against the Δ*nmb1467* mutant was < 64. These results were consistent with the hypothesis that α-rNMB1467 serum has bactericidal antibodies which binds to MC58. Similar killing of BZ167 and H44/76, which belong to ET-5, by the α-rNMB1467 serum was also found (Figure 3.6B). However, these SBA results were not reproducible in the subsequent experiments using sera from second and third immunisation experiments (Figure 3.6B).

To establish whether rNMB1467 can confer protective immunity, groups of mice (15 animals per group) received 25 μg of rNMB1467 by subcutaneous administration on days 0, 21, and 28. Control groups were given either medium alone or a previously characterized live attenuated *N. meningitidis* strain, 23A10 (Li *et al.*, 2004). On day 43, animals were challenged with 5 x 10^6 CFU or 5 x 10^7 CFU of live MC58. As described previously, animals immunised with the attenuated *N. meningitidis* strain

survived much better than animals that only received medium following challenge with the homologous strain (Li *et al.*, 2004). However, vaccination with rNMB1467 did not provide significant protection to animals against challenged with MC58 (Figure 3.6D and 3.6E).

To further analyse the vaccine potential of rNMB1467, the localisation of NMB1467 in MC58 was analysed by FACS. No significant shift to the right was observed when fixed bacteria were incubated with α-rNMB1467 (1:10 dilution) compared with negative control in which cells were incubated in PBS alone. This result indicates that NMB1467 is unlikely to be present on the bacterial surface. Therefore, these series of results indicate that rNMB1467 is not a good vaccine candidate against serogroup B *N .meningitidis*.

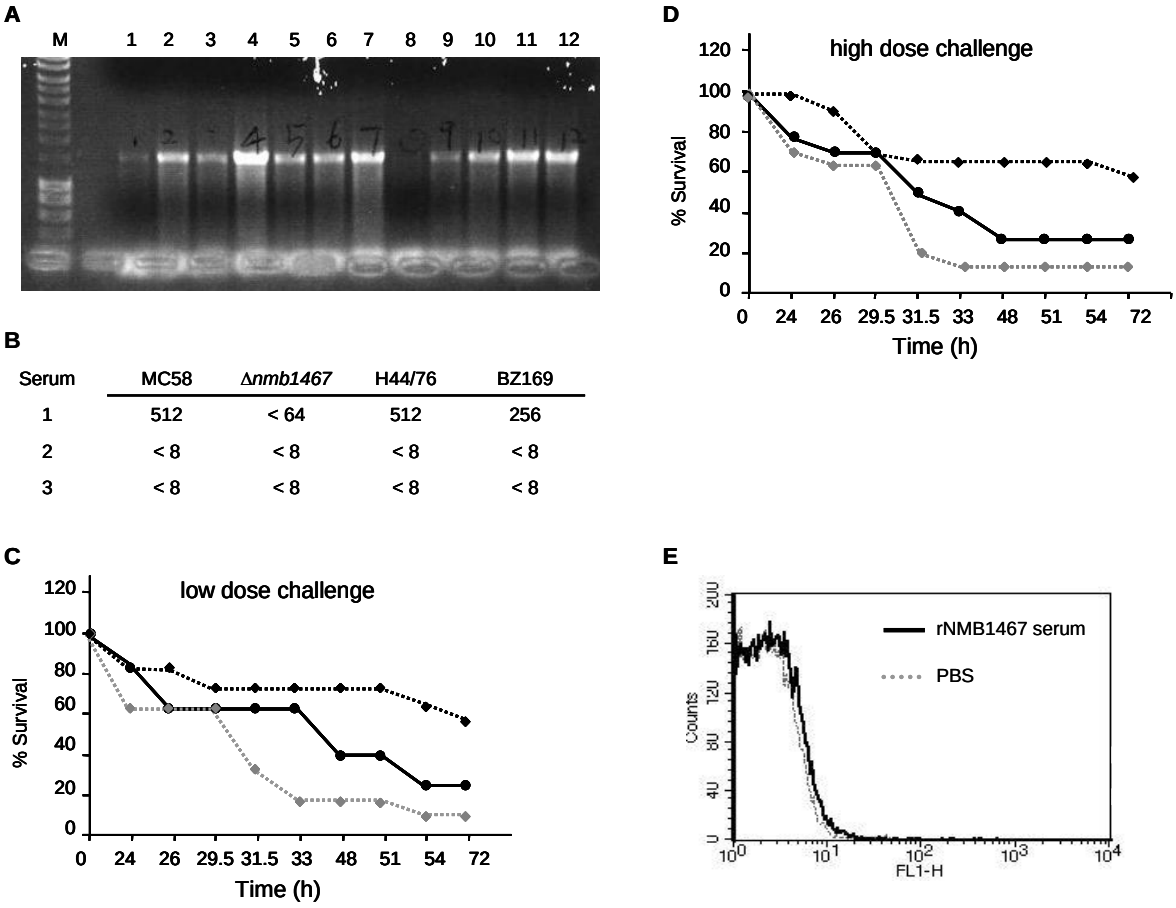


Figure 3.6 Assessment of the vaccine potential of rNMB1467

(A) Detection of *nmb1467* gene by PCR in a range of clinical isolates, MC58 (Lane 1), H44/76 (Lane 2), BZ169 (Lane 3), BZ83 (Lane 4), SWZ107 (Lane 5), NGF26 (Lane 6), NG6/88 (Lane 7), 860800 (Lane 9), 2996 (Lane 10), A22 (Lane 11) and 2059001 (Lane 12). H_2O instead of genomic DNA was used as negative control (Lane 8).

(B) SBA titres against MC58, MC58Δ*nmb1467*, H44/76 and BZ169 using anti-rNMB1467 sera raised on three independent immunisations.

(C) and **(D)** Protection by immunisation of rNMB1467 against live meningococcal challenge. Group of 15 mice were vaccinated with 25 µg of rNMB1467 on days 0, 21, and 28 (dark solid line with circles). Control animals (15 per group) were immunised with either *N. meningitidis* 23A10 (dark dash line with squares) or PBS alone (grey dash line with squares). On day 43, animals were challenged with low dose 5 x 10^6 CFU of MC58 **(C)** or 10^7 CFU MC58 **(D)**, and survival was recorded over the following 72 hours.

(E) FACS analysis of binding of anti-rNMB1467 to MC58. There was no significant binding of anti-rNMB1467 serum to MC58 compared to negative control PBS.

Chapter 4 Biochemical characterisation of NMB1467

4.1 Homology of NMB1467 to PPX from *E. coli*

The ORF of *nmb1467* is predicted to encode a protein consisting of 502 amino acids, with a predicted molecular weight of 55,921.07 Da and pI of 7.68. BLASTP searches showed that NMB1467 belongs to the PPX/GppA Super Family, and shares 35% amino acid identity with the biochemically characterised PPX of *E. coli* (Figure 4.1). Examination of the whole genome sequence of *N. meningitidis* MC58 demonstrated that *nmb1467* is the only ORF with identity to *E. coli ppx* (cut off 3.00E-14). A BLASTP with *E. coli* PPX revealed the presence of PPX in many but not all bacterial species (Table 4.1).

It has been reported that over-expression of *S. cerevisiae* PPX in *E. coli* reduces the cellular level of poly P and decreases resistance against heat and oxidative stress (Shiba *et al.*, 1997). Therefore, the effect of over-expressing rNMB1467 on the resistance of *E. coli* against heat shock and oxidative stress was examined. Over-expression of rNMB1467 was performed by inducing exponential phase bacteria with 1 mM IPTG at 37°C with shaking at 200 r.p.m for 4 hours. For the oxidative stress assay, bacteria (10^9 CFU) were spread over the surface of agar plates and filter disks soaked in 8 mM H_2O_2 were placed in the centre of plates. Zones of growth inhibition were observed after an overnight incubation. The zone of inhibition by H_2O_2 was significantly larger on the plates inoculated with a strain over-expressing rNMB1467 compared with *E. coli* containing the vector alone, indicating that over-expression of rNMB1467 enhances the susceptibility to the oxidative stress (Figure 4.2 A). To examine sensitivity to heat, bacteria were incubated at 55°C for 5 minutes and

survival was determined by plating aliquots to solid LB media. After an overnight incubation, the strain over-expressing rNMB1467 only exhibited about 10% survival compared to the strain containing the vector alone. Taken together, these data demonstrate that over-expression of rNMB1467 in *E coli* has similar effects to over-expression of yeast PPX on resistance against heat and oxidative stress, suggesting that *nmb1467* may encode a functional exopolyphosphatase.

```
NMB1467         ----MTTTPANVLASVDLGSNSFRLQICENNNGQLKVIDSFKQMVRFAAGLDEQKNLSAA 56
PPX             MPIHDKSPRPQEFAAVDLGSNSFHMVIARVVDGAMQIIGRLKQRVHLADGLGPDNMLSEE 60
                     .:. .: :*:********:: *..  :* :::*. :** *::* **. :: **

NMB1467         SQEQALDCLAKFGERLRGFRPEQVRAVATNTFRVAKNIADFLPKAEAALGFPIEIIAGRE 116
PPX             AMTRGLNCLSLFAERLQGFSPASVCIVGTHTLRQALNATDFLKRAEKVIPYPIEIISGNE 120
                :  :.*:**: *.***:** * .*  *.*:*:* * * :*** :** .: :*****:*.*

NMB1467         EARLIYTGVIHTLPPGGGKMLVIDIGGGSTEFVIGSTLNPDITESLPLGCVTYSLRFFQN 176
PPX             EARLIFMGVEHTQPEKG-RKLVIDIGGGSTELVIGENFEPILVESRRMGCVSFAQLYFPG 179
                *****: ** ** *  * : **********:***..::* :.**  :***:::  :* .

NMB1467         -KITAKDFQSAISAARNEIQRISKNMRREGWDFAVGTSGSAKSIRDVLAAEMPQEADITY 235
PPX             GVINKENFQRARMAAAQKLETLTWQFRIQGWNVAMGASGTIKAAHEVLMEMGEKDGIITP 239
                  *. ::** *  ** :::: :: ::* :**:.*:*:**: *: ::**     ::. **

NMB1467         KGMRALAERIIEAGSVKKAKFENLKPERIEVFAGGLAVMMAAFEEMKLDRMTVTEAALRD 295
PPX             ERLEKLVKEVLRHRNFASLSLPGLSEERKTVFVPGLAILCGVFDALAIRELRLSDGALRE 299
                : :. *.:.::.  .. . .: .*. **  **. ***:: ..*: : : .: :::.***:

NMB1467         GVFYDLIGRGLNEDMRGQTVAEFQHRYHVSLNQAKRTAETAQTFMDSLCHAKNVTVQELA 355
PPX             GVLYEMEGRFRHQDVRSRTASSLANQYHIDSEQARRVLDTTMQMYEQWREQQ--PKLAHP 357
                **:*:: **  ::*:*.:*.:.: ::**:. :**:*. :*:  : :.  . :  .    .

NMB1467         LWQQYLGRAAALHEIGLDIAHTGYHKHSAYILENADMPGFSRKEQTILAQLVIGHRGDMK 415
PPX             QLEALLRWAAMLHEVGLNINHSGLHRHSAYILQNSDLPGFNQEQQLMMATLVRYHRKAIK 417
                  :  *  ** ***:**:* *:* *:******:*:*:***.:::* ::* **  **  :*

NMB1467         KMSG---IIGTNEMLWYAVLSLRLAALFCRSRQDLSFPKNMQLRTDTESCGFILRIDREW 472
PPX             LDDLPRFTLFKKKQFLPLIQLLRLGVLLNNQRQATTTPPTLTLITDDS--HWTLRFPHDW 475
                  .     : .:: :   :  ***..*: ..**  : * .: * ** .   : **: ::*

NMB1467         LERHPLIADALEYESVQWQKIN-MPFKVEAV------- 502
PPX             FSQNALVLLDLEKEQEYWEGVAGWRLKIEEESTPEIAA 513
                 :.::.*:   ** *.  *: :    :*:*
```

Figure 4.1 Amino acid alignment of NMB1467 with *E. coli* PPX

Identical residues are shown with an asterisk, conserved substitutions are shown with two dots and semi-conserved substitutions are shown with one dot. The residues shown in grey are highly conserved in 20 micro-organisms (Kristensen *et al.*, 2004).

Table 4.1 Presence or absence of predicted PPX in microbial genomes

Presence	Absence
Acinetobacter calcoaceticus	*Archaeoglobus fulgidus*
Aquifex aeolicus	*Bacillus subtilis*
Bacillus cereus	*Campylobacter coli*
Bordetella pertussis	*Dictyostelium discoideum*
Clostridium acetobutylicum	*Enterococcus faecalis*
Deinococcus radiodurans	*Mycobacterium leprae*
Escherichia coli	*Mycoplasma pneumoniae*
Haemophilus influenzae	*Mycobacterium tuberculosis*
Helicobacter pylori	*Myxococcus xanthus*
Klebsiella pneumonia	*Propionibacterium shermanii*
Neisseria gonorrhoeae	*Streptomyces coelicolor*
Neisseria meningitidis	*Thermotoga maritima*
Plasmodium falciparum	
Propionibacterium shermanii	
Pseudomonas aeruginosa	
Saccharomyces cerevisiae	
Salmonella typhi	
Shigella flexneri	
Synechocystis spp.	
Vibrio cholerae	
Yersinia pestis	

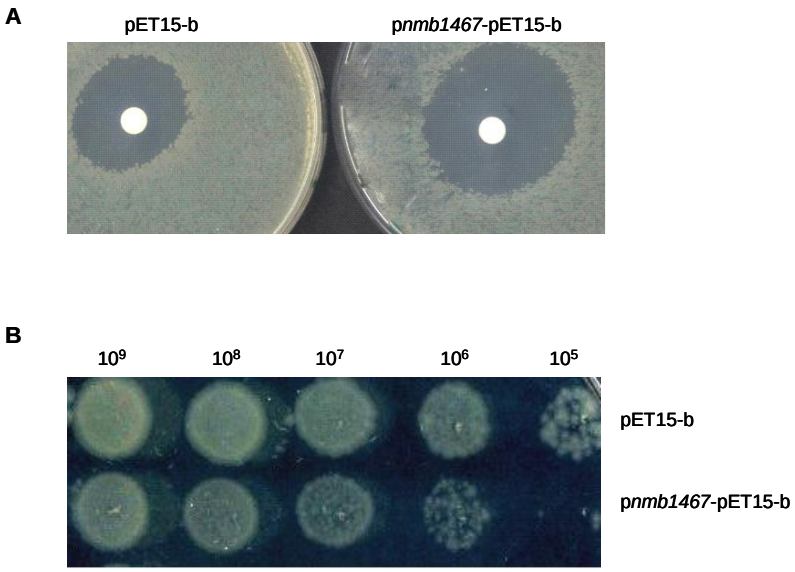


Figure 4.2 Effects of over-expression of rNMB1467 on *E. coli*

(A) The effect of over-expression of rNMB1467 on sensitivity to H_2O_2. Protein over-expression in *E. coli* strain BL21 carrying p*nmb1467*-pET15-b or an empty plasmid (pET15-b) was induced with 1 mM IPTG for 4 hours, then 10^9 CFU of each strain spread onto LB plates. Filter disks containing 10 µl of 8.8 M H_2O_2 were added and the plates were incubated overnight at 37°C.

(B) Effect of over-production rNMB1467 on heat sensitivity of *E. coli*. Bacteria over-expressing rNMB1467 were incubated at 56°C for 5 minutes. The viability of bacteria was determined by plating serial dilutions onto LB agar plates and incubation overnight at 37°C. The expected number of bacteria in aliquots (CFU) are indicated above each spot.

4.2 NMB1467 is an exopolyphosphatase

Several approaches have been used to quantify the level of poly P in cells, among which the toluidine blue assay is an easy, fast and inexpensive method (Ault-Riche *et al.*, 1998; Kornberg, 1999; Mullan *et al.*, 2002). Toluidine blue is a metachromatic dye, and the addition of negatively charged poly P to toluidine blue changes the colour of the dye from blue to pink, shifting the absorption of the dye from approximately 630 nm to 530 nm.

To examine the sensitivity of the toluidine blue assay for quantification of poly P, different concentrations of poly P_{75} were diluted in 10 µl of 10 mM Tris-HCl and added to 990 µl of dye solution (6 g/L in 40 mM acetic acid), and the absorbance of the solution was measured at 530 and 630 nm. The ratio of O.D. A_{630} /O.D. A_{530} was approximately 0.15 when 10 µl of 10 mM Tris-HCl alone was used as control. The ratio increased with increasing concentrations of poly P_{75} from 1 mM to 15 mM in 10 µl of samples, but the relationship was linear only over a narrow range of concentrations (approximately 6-12 mM), and did not increase over 15 mM (Figure 4.3A).

To determine the biochemical function of rNMB1467, different concentrations of the protein were incubated with 10 mM of poly P_{75} in 100 µl of exopolyphosphatase reaction buffer, and the concentration of poly P_{75} was determined at 0, 1, 15, and 30 minutes using the toluidine blue assay. In the presence of rNMB1467 with a final concentration of 50 µg/ml, the level of poly P_{75} declined to almost 0 mM within 15 minutes (Figure 4.2B); there was no change in the poly P_{75} level using the same concentration of BSA (data not shown). Lower concentrations of rNMB1467 (5 µg/ml and 10 µg/ml) decreased the level of poly P_{75} within 30 minutes at slower rates

compared to 50 µg/ml (Figure 4.3B). These results demonstrate that rNMB1467 has exopolyphosphatase activity, and the kinetic activity of rNMB1467 is related to the concentration of protein. Thus, NMB1467 was designated PPX.

Divalent cations are required for the activity of many phosphatases. It has been reported that removal of divalent cations reduces the activity of *E. coli* PPX to 0.3% of the maximum level, and that activity using Ca^{2+} only reaches 12% of that obtained with Mg^{2+} (Bolesch and Keasling, 2000). Thus, the effect of divalent cations on the activity of rPPX was examined using 1 mM $CaCl_2$. Different final concentrations of rPPX (5 µg/ml, 10 µg/ml and 50 µg/ml) were used in reactions with 1 mM $CaCl_2$ instead of 1 mM $MgCl_2$. rPPX activity was not detected when 5 µg/ml of protein was used in the reaction buffer with 1 mM Ca^{2+} (Figure 4.3C). In the presence of 50 µg/ml of rPPX, the level of poly P_{75} reduced at a lower rate than obtained with 10 µg/ml of rPPX and 1 mM Mg^{2+} (Figure 4.3C). These results indicate that Ca^{2+} is able to sustain the enzymatic activity of rPPX of *N. meningitidis*, but it reaches less than 20% of the activity obtained with Mg^{2+}.

The effect of the temperature on rPPX activity was examined by incubating reactions containing 5 µg/ml of rPPX and 1mM Mg^{2+} at different temperatures. The level of poly P_{75} decreased much slower when the reaction was incubated at 20°C or 56°C than at 37°C over 30 minutes (figure 4.3D). These results indicate that rPPX is most active at 37°C.

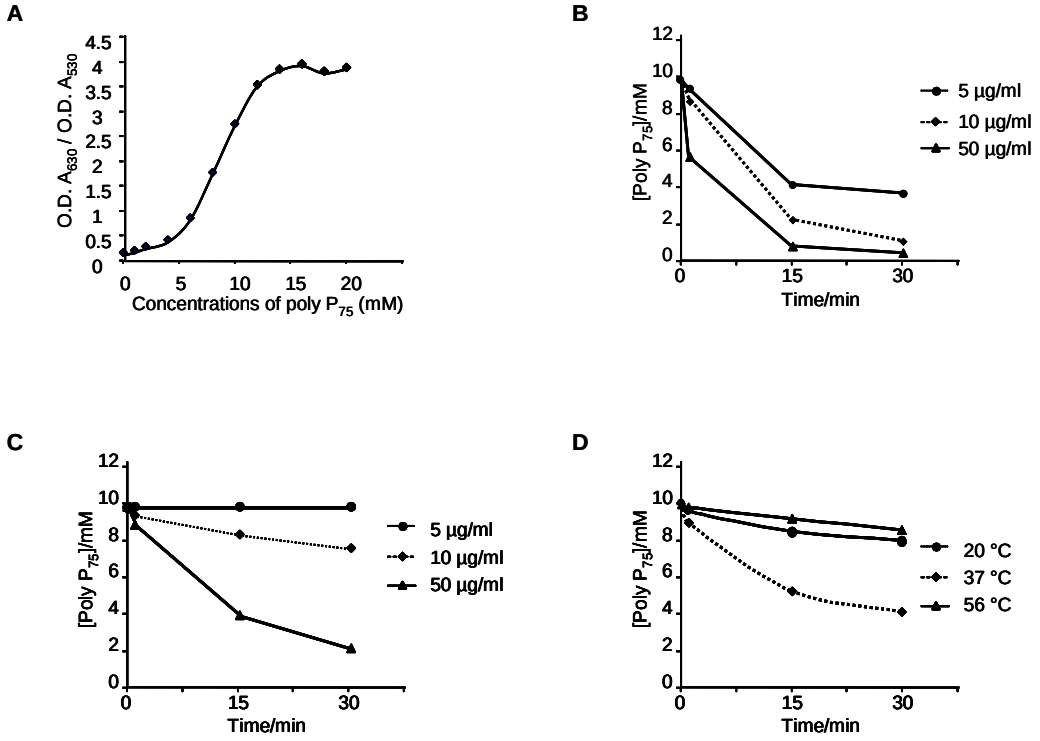


Figure 4.3 Biochemical characterisation of rNMB1467

(A) Quantification of poly P_{75} using the toluidine blue assay. Different concentrations of poly P_{75} in 10 µl of Tris-HCl were added to 990 µl of dye solution, and the absorbance of the solution was measured at 530 and 630 nm.

(B) Biochemical characterisation of rPPX. Different concentrations of purified rNMB1467 were added to 100 µl of exopolyphosphatase reaction buffer and incubated at 37°C. Concentrations of poly P_{75} were measured at 1, 15 and 30 minutes using the toluidine blue assay.

(C) Activity of rPPX in the presence of Ca^{2+}. Reactions containing 1 mM $CaCl_2$ instead of 1 mM $MgCl_2$ with indicated concentrations of rPPX were incubated at 37°C. The resulting levels of poly P_{75} were measured at 1, 15 and 30 minutes using the toluidine blue assay.

(D) Effect of temperature on rPPX activity. PPX assay reactions containing 1 mM $MgCl_2$, 50 mM Tricine/KOH (pH 8.0), 175 mM KCl, and 5 µg/ml rNMB1467 were incubated at 20°C, 37°C and 56°C, respectively. Levels of poly P_{75} were determined at 1 min, 15 min and 30 min using the toluidine blue assay.

4.3 Requirement of Glu^{147} for the full catalytic activity of rPPX

The crystal structure of the *A. aeolicus* PPX suggests that the Glu^{148} residue is involved in binding to a divalent cation (Figure 4.4A) (Kristensen *et al.*, 2004). This residue is conserved in most PPX sequences, and corresponds to Glu^{147} in PPX of *N. meningitidis* MC58 (data not shown). To examine the role of the Glu^{147} in the activity of rPPX, this residue was replaced with alanine (A) by site-directed mutagenesis. $rPPX^{E147A}$ was over-expressed and purified from *E. coli* using the same method as used for rPPX. This protein had the same mobility as the wild-type protein when analysed by SDS-PAGE (Figure 4.4B), and was recognised by the anti-rPPX serum using Western blot analysis (data not shown). The exopolyphosphatase activity of $rPPX^{E147A}$ was measured under identical conditions to those used to measure the activity of rPPX. However, in the presence of 5 µg/ml, 10 µg/ml and 50 µg/ml of $rPPX^{E147A}$, the level of poly P_{75} remained constant over 30 minutes. When the concentration of $rPPX^{E147A}$ was increased to 500 µg/ml, a slow decrease in the level of poly P_{75} was detected (Figure 4.4C). The level of poly P_{75} after incubation with 500 µg/ml $rPPX^{E147A}$ for 30 minutes was about 7 mM, which was close the level (about 6 mM) obtained after incubation with 5 µg/ml of rPPX over the same time. These results indicate that substitution of Glu with Ala at residue 147 dramatically reduces the activity of rPPX to about 1% of the wild-type enzyme, and that Glu^{147} is required for the full enzymatic activity of the protein.

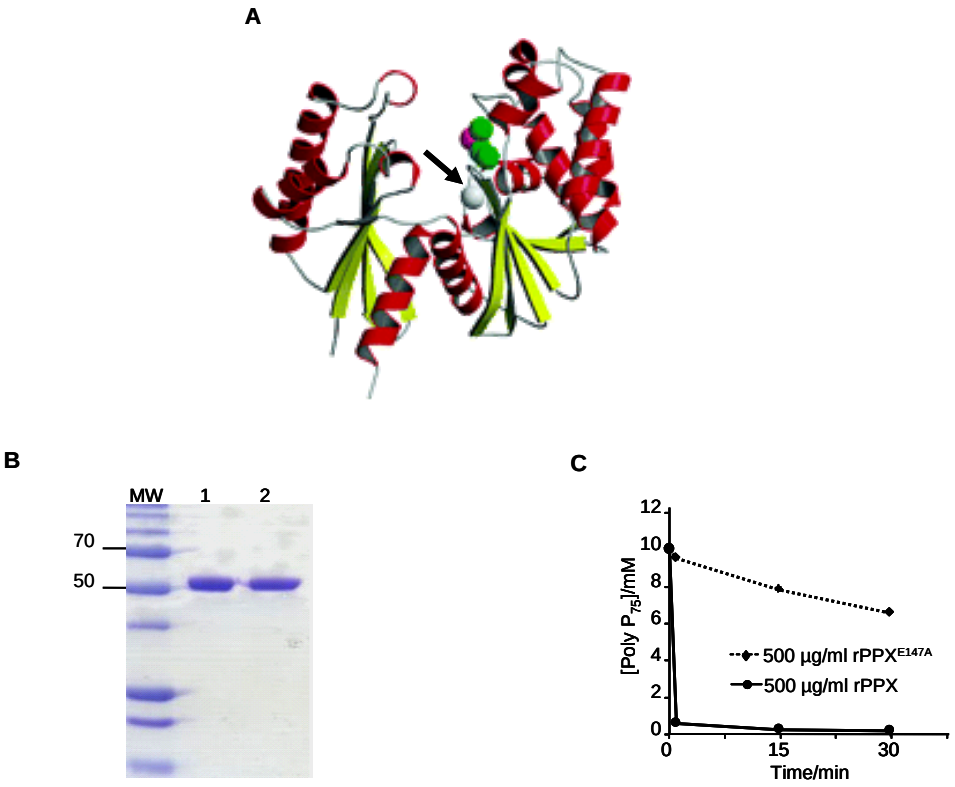


Figure 4.4 Effect of the rPPX^{E147A} substitution on PPX activity

(A) Structure of *Aquifex aeolicus* PPX/GppA phosphatase. Three chloride ions (green spheres) and a calcium ion (grey sphere, indicated with an arrow) indicate the position of the putative active site (Kristensen *et al.*, 2004).

(B) Over-expressed rPPXE^{147A} was purified from *E. coli* and analysed by SDS-PAGE. rPPX^{E147A} (Lane 1) and wild-type rPPX (Lane 2) were visualised by Coomassie staining, and the sizes of molecular weight markers (in kDa) are indicated.

(C) rPPX^{E147A} has significantly reduced PPX activity. PPX assays containing 10 mM poly P_{75}, 1 mM $MgCl_2$, 50 mM Tricine/KOH (pH 8.0), 175 mM KCl, and 500 µg/ml of recombinant protein were incubated 37°C, and concentrations of poly P_{75} were measured at 1, 15, 30 minutes by the toluidine blue assay.

4.4 Mutation of *ppx* leads to increased cellular poly P levels in *N. meningitidis*

To compare the level of poly P in MC58 and the Δ*ppx* mutant, a Δ*ppk* mutant was generated as a control. Interruption the ORF of *ppk* was initially performed using *in vitro* Tn*5* transposition (data not shown). A linearised plasmid containing *ppk*::Tn*5* was introduced into MC58 by transformation. However, there was significant heterogeneity in the sizes of Δ*ppk* mutant colonies. To examine whether this phenotype was caused by the insertion of the Tn*5* transposon, another Δ*ppk* mutant was then constructed by insertion of a cassette containing a tetracycline resistance gene at bp 1701 of the ORF, at the location of an *Eco*RI recognition site (data not shown). The resulting Δ*ppk* mutants were confirmed by PCR and DNA sequencing, but size variation was still observed (data not shown). Thus, large and small single colonies were kept separately for the following assays.

Levels of poly P in MC58, MC58Δ*ppx*, MC58Δ*ppk* with large and small colonies were initially analysed based on the methods reported by Tinsley (Tinsley *et al.*, 1993). Poly P was extracted from bacteria (10×10^9 CFU) using NaClO, precipitated and dissolved in 100 µl of 10 mM Tris-HCl. The level of poly P was determined using the toluidine blue assay as described above. However, poly P was not detected in any of these strains. Several attempts were made to improve the sensitivity of the assay, by increasing the amount of bacteria and the concentration of toluidine blue solution, and by varying the ratio of the poly P sample and toluidine blue dye. However, no difference was observed in poly P levels between MC58, MC58Δ*ppx* and MC58Δ*ppk* (data not shown). This result could have been due to contamination with other polyanions (such as capsular polysaccharide) during poly P extraction, which also could lead to a shift in the wavelength of toluidine blue, and mask any differences in poly P levels between the mutants and the parental strain.

Thus, a more sensitive and specific method was used to quantify the poly P levels in *N. meningitidis* strains based on the activity of PPK. Poly P was extracted from cells with GITC lysis buffer and bound to Glassmilk, while binding of ATP or protein to glass was prevented by adding ethanol and SDS (Ault-Riche *et al.*, 1998; Kornberg, 1999). Poly P was eluted from the Glassmilk with hot low-ionic buffer, and quantified by its conversion to ATP by PPK in the presence of a 10-fold excess of ADP. The generated ATP was then determined using a luciferase assay (Ault-Riche *et al.*, 1998).

To purify rPPK of *N. meningitidis* from *E. coli*, the ORF of *nmb1900* was amplfied by PCR using primers NG591 and NG663, and then cloned into pET-15b. The over-expression of rPPK of *N. meningitidis* in *E. coli* BL21 was induced at 37°C overnight to increase the protein level. However, no polyphosphate kinase activity was found with purified rPPK. This was probably due to the presence of a poly-His-tag at the N-terminus instead of the C-terminus (Zhu *et al.*, 2003). Thus, a plasmid with a His-tag on the C-terminus of *E. coli* PPK (generous gift provided by Professor J. Roth) was used to produce recombinant protein. The polyphosphate kinase activity of the *E. coli* protein was confirmed by detecting increased levels of poly P_{75} when incubated with rPPK and ATP (Figure 4.5A). In addition, the ability of PPK to generate ATP from poly P was examined by incubating poly P_{75} with rPPK and ADP, and measuring the resulting ATP based on luminescence (data not shown). This method is able to detect concentrations of poly P_{75} as low as 0.5 µM, which is 2,000 times more sensitive than the toluidine blue assay (Figure 4.5B).

Poly P was extracted from different *N. meningitidis* strains after overnight growth, and converted to ATP. The level of poly P in MC58Δ*ppx* was about 750 pmol/10^9 CFU, which was about twice as much as the poly P level in MC58. The level of poly P extracted from large colonies of MC58Δ*ppk* was similar to that from small colonies, and was only approximately 10% of MC58 ($p < 0.05$, Figure 4.5D), consistent with previous reports (Tinsley and Gotschlich, 1995).

To examine the role of the activity of PPX on the levels of cellular poly P, a complemented strain MC58Δ*ppx*^{E147A} was constructed by introducing the *ppx*^{E147A} allele as a single chromosomal copy in the intergenic region between *nmb102* and *nmb103* in the Δ*ppx* mutant. Western blot analysis confirmed the expression of a 56 kDa protein by MC58Δ*ppx*^{E147A} recognised by the α-rPPX serum (Figure 4.5C). The level of poly P in the Δ*ppx*^{E147A} mutant was similar to levels in the Δ*ppx* mutant which dose not express PPX, and was approximately twice as much as that in strain complemented with the wild-type *ppx* allele ($p < 0.05$, Figure 4.5D). These results demonstrate that modification of Glu^{147} in PPX not only reduces the enzymatic activity of rPPX to less than 1% *in vitro*, but is also necessary for the maintenance of the level of poly P in *N. meningitidis*.

To examine the effect of modification of PPX activity on bacterial resistance against complement mediated-killing, different *N. meningitidis* strains were incubated with NHS at a 1:8 dilution for 1 hour. The survival of the Δ*ppx*^{E147A} mutant was 100% more than that of the MC58 and MC58Δ*ppx*^{com} ($p < 0.05$, Figure 4.5E), indicating that full enzymatic activity of PPX is required for the relatively enhanced resistance against complement-mediated killing.

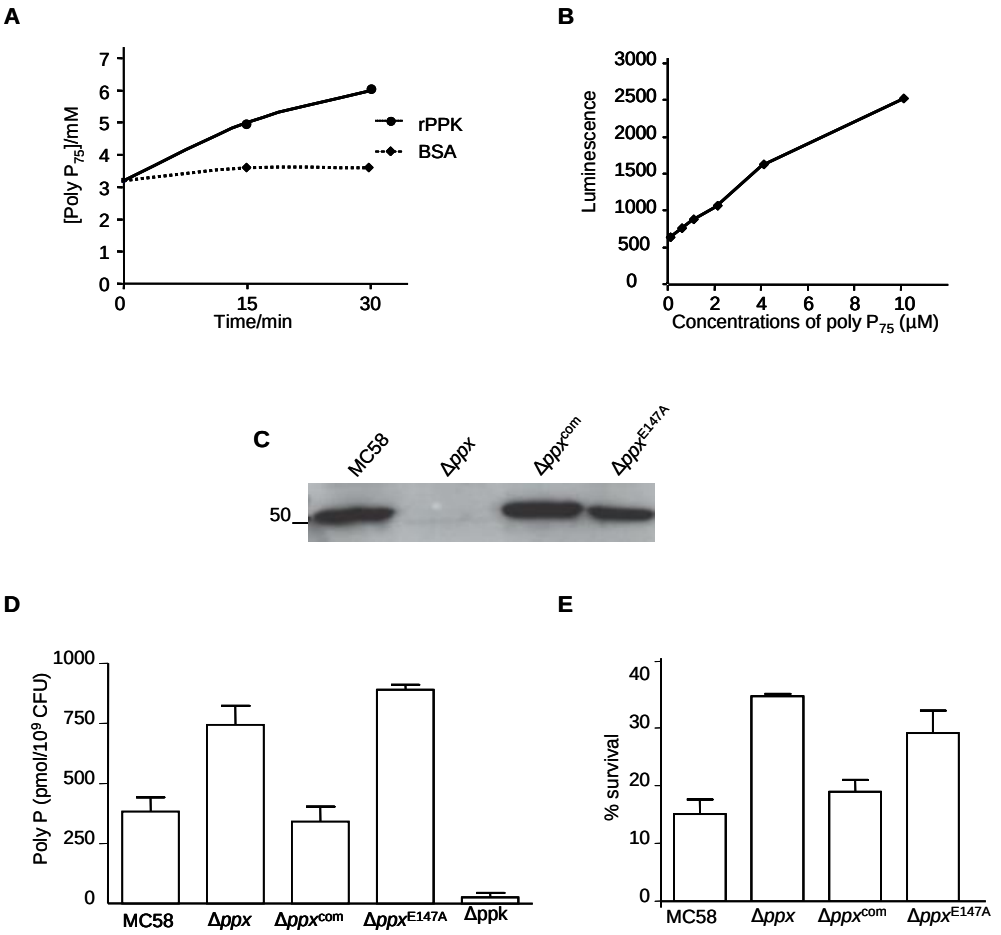


Figure 4.5 Quantification of poly P levels in *N. meningitidis* using rPPK

(A) Polyphosphate kinase activity of rPPK. Purified rPPK (0.1 mg/ml) was incubated with poly P_{75} at 37°C over 30 minutes, and levels of poly P_{75} were determined by the toluidine blue assay. The same concentration of BSA was used as control.

(B) Quantification of the concentration of poly P_{75} by luminescence reaction. Poly P_{75} was converted to ATP in the presence of rPPK and an excess of ADP; the level of resulting ATP was measured by luminescence.

(C) Western blot analysis of the presence of PPX in *N. meningitidis* strains (indicated above each lane) using the α-rPPX serum. The size of a molecular weight maker is shown in kDa.

(D) Quantification of poly P levels in *N. meningitidis*. Poly P was extracted from 2 x 10^9 CFU after overnight growth using Glassmilk and converted to ATP, which was measured by luminescence. The figure is a representative of three independent experiments performed in triplicate, and the error bars show the SEM.

(E) Increased survival of the Δ*ppx*^{E147A} mutant. *N. meningitidis* strains were harvested after overnight growth, and exposed to NHS at a 1:4 dilution. The survival of bacteria was determined after 1 hour. The results are a representative of three independent experiments performed in triplicate, and the error bars show the SEM.

4.5 Levels of poly P at different stages of meningococcal growth

To measure cellular levels of poly P in *N. meningitidis* at different growth stages, bacteria were grown overnight then inoculated into fresh liquid BHI medium and incubated at 37°C with shaking at 150 r.p.m. Samples were taken every three hours, and poly P was extracted by Glassmilk. Levels of poly P both in MC58 and the Δ*ppx* mutant increased during bacterial growth and reached the maximum at stationary phase (Figure 4.6A). The difference in poly P levels between MC58 and the Δ*ppx* mutant became more marked after 12 hours growth ($p < 0.05$, Figure 4.6A).

It has been reported that the Δ*ppx* mutant of *B. cereus* has approximately 10 fold more PPK and 1,000 fold more poly P than the wild-type strain (Shi *et al.*, 2004), so the increased level poly P in the Δ*ppx* mutant of *B. cereus* could be due to increased activity of PPK. Western blot analysis of PPX expression of *N .meningitidis* MC58 showed that the level of PPX does not significantly change during growth (Figure 4.6B). This result indicates that the increased level of poly P in MC58 during growth is not due to reduced expression of PPX. Thus, it is possible that the increased level of poly P in the Δ*ppx* mutant partly resulted from the increased level of PPK. To test this hypothesis, the levels of PPK transcript were analysed at mid-log phase by quantitative real-time PCR (qrtRT-PCR). The results were analysed by comparison with a control housekeeping gene, *gdh*. Surprisingly, there was no significant difference in PPK transcript level between MC58 and the Δ*ppx* mutant (Figure 4.6C). Taken together, these results demonstrate that the increased level of poly P in the Δ*ppx* mutant is caused by loss of PPX and not changes in PPK expression.

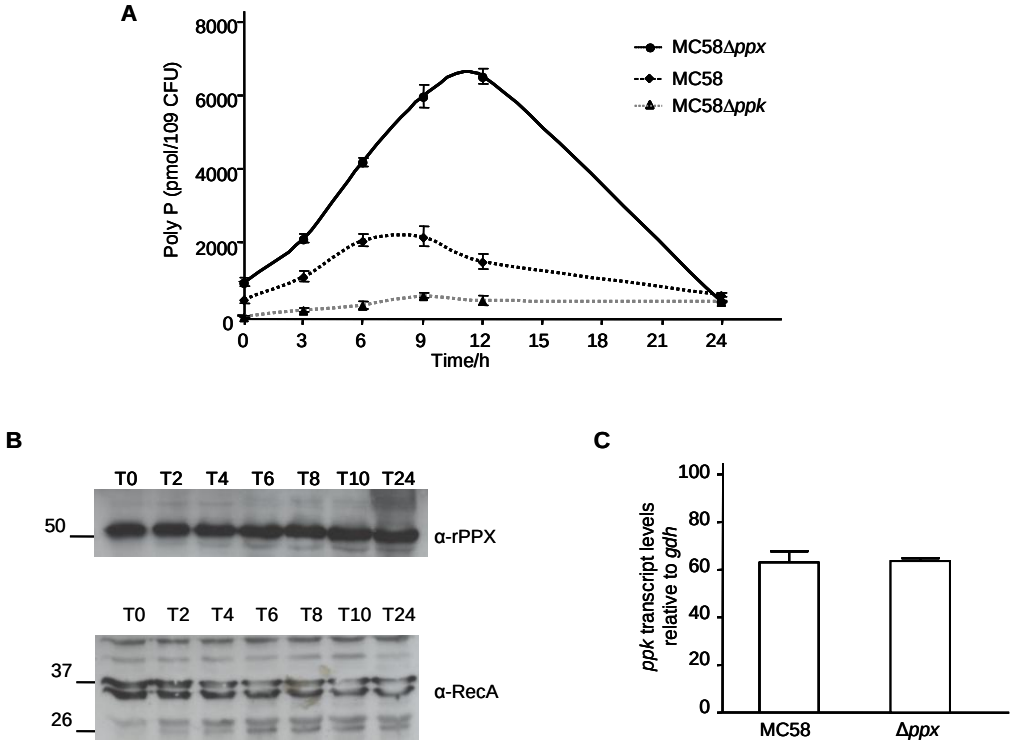


Figure 4.6 Quantification of cellular poly P of *N. meningitidis* at different growth stages

(A) Quantification of poly P produced by *N. meningitidis* strains during different growth stages. Bacteria were grown overnight on BHI plates, and sub-cultured to liquid BHI medium at an initial O.D. A_{600} of 0.1. Bacterial cultures were incubated at 37°C with shaking at 150 r.p.m and samples were taken at 0, 3, 6, 9, 12, 24 hours and assayed for poly P. The figure shows representative results of two independent experiments performed in triplicate, and error bars show the SEM.

(B) Quantification of PPX expression by Western blot analysis using the α-rPPX serum. Bacteria were grown in liquid medium under identical conditions as described above, and samples were taken 0, 2, 4, 6, 8, 10, 24 hours post-subculture

(C) Quantification of *ppk* transcription by qrtRT-PCR. Bacteria were grown in liquid BHI at 37°C with shaking at 150 r.p.m for 4 hours. Total RNA was extracted from bacteria, and the transcription of *ppk* and *gdh* measured by qrtRT-PCR. The results are representative of three independent experiments, and error bars show the SEM of assays performed in triplicate.

4.6 Crystallisation of *N. meningitidis* rPPX

Crystallisation is a phase transition phenomenon. Crystals grow from an aqueous protein solution when the solution is brought into supersaturation (Chayen, 1998). The most popular and successful techniques to establish crystallisation conditions rapidly and efficiently are based on vapour diffusion, including the hanging drop, sitting drop and sandwich drop methods. These techniques utilise the difference in precipitant concentration between the protein drop and the mother liquor to drive volatile components to evaporate from the drop until the concentration of the precipitant in drop equals that of the well solution to achieve the supersaturation state of protein macromolecules (Chayen, 1998).

Purified rPPX was stable in buffer consisting of 20 mM Tris-HCl, pH 8.0, 200 mM NaCl, 5 mM DTT and 5 mM pentasodium triphosphate at 4°C or -20 °C for about one month. The initial screen for PPX crystals was performed using nine commercially available 96-well plate screens kits and the Mosquito Nanodrop crystallisation robot. Each well of the 96 well plates contains different crystallisation solutions, with a chamber of the mother liquor (100 µl) and one drop (200 nl) sitting on a bridge which sits over the reservoir. Using the Mosquito Nanodrop crystallisation robot, 200 nl of 10 mg/ml of PPX was added to each drop in the wells, then, the plates were sealed with tape and stored at 20°C. As the crystallisation solution in the drop was mixed with an equal volume of protein solution, the solution in the mother liquor had double concentration of that in the drop. This allows the water to evaporate from the drop gradually. During the first week each drop was viewed by microscopy every day, and in the second week the samples were checked every other day. Most crystals appeared after 24 hours incubation and positive hits were found in 78 conditions. Different

shapes of rPPX crystal were observed under the microscope, and 5 samples of conditions are listed in Figure 4.7.

Once the crystals were found, the conditions were optimised in 24-well plates using 1 ml of mother liquor and 1 µl of protein. Optimising the crystal conditions was achieved by varying all crystallisation parameters, including precipitant, salt, protein and pH.

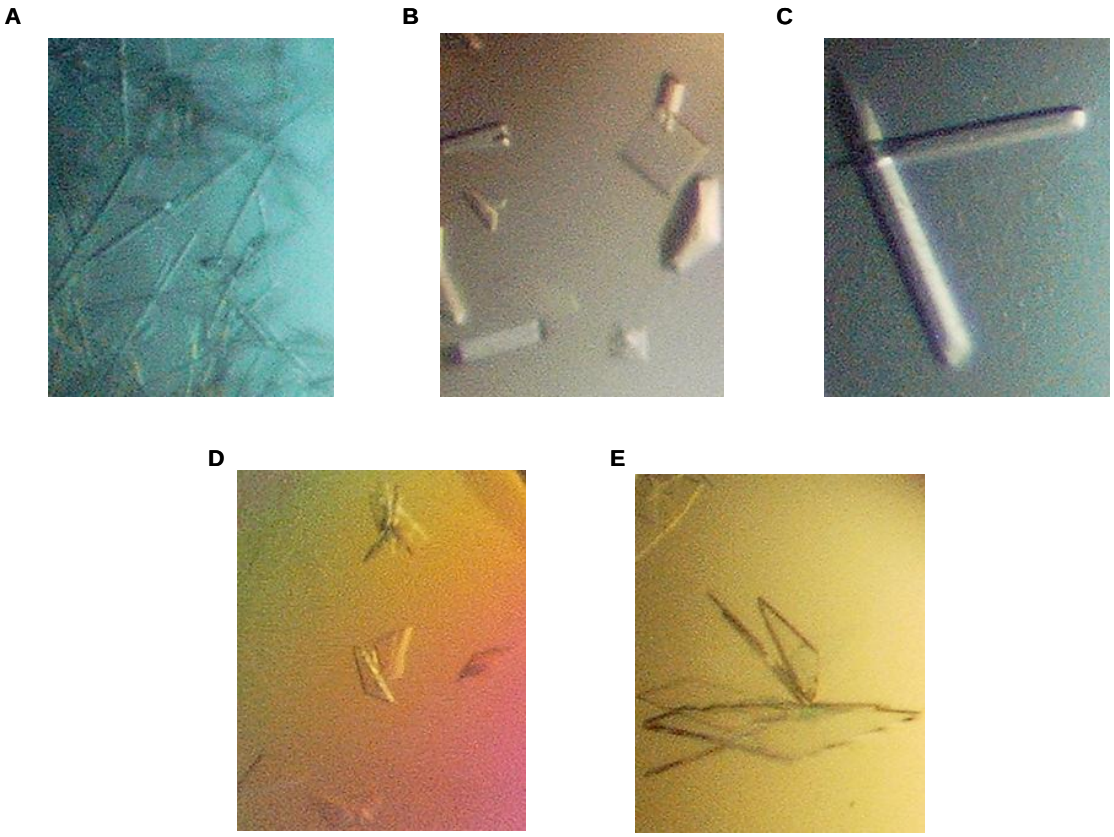


Figure 4.7 Typical shapes of rPPX crystals under different conditions

(A) Clustered needle shapes of rPPX were obtained in the conditions of 0.2 M NaCl, 0.1 M CHES pH, 9.0, and 12% PEG 8,000.

(B) Small cubic crystals of rPPX were obtained in conditions of 0.07 M NaCl, 0.01 M 0.05 M sodium citrate and 22% PEG 400.

(C) Long rod shapes of rPPX crystals were obtained in conditions of 0.225 M MES/Bis-Tris pH 6.6, and 6,6% PEG6K.

(D) Small plate shapes of rPPX crystals were obtained in conditions of 0.02 M $CaCl_2$, 0.02 M MES pH 6.5, and 7.7% PEG 1,500.

(E) Big plate shapes of rPPX crystals were obtained in conditions of 0.1 M Li_2SO_4, 0.1 M Na citrate pH 5.5, and 12% PEG 4,000.

Chapter 5 Investigation of the contribution of PPX to avoidance of complement-mediated killing

5.1 The *in vitro* growth of the Δ*ppx* mutant

The increased survival of the Δ*ppx* mutant in the presence of NHS could be due to a growth advantage of the mutant compared with the wild-type strain. To examine this possibility, the growth of both strains was analysed *in vitro*. The growth of the strains in BHI liquid medium was monitored at hourly intervals from the lag phase to stationary phase by measuring the O.D. at 600 nm. Given the same starting O.D. A_{600} of 0.1, the growth of the Δ*ppx* mutant at each time point was slightly lower than that of the wild-type on three independent experiments (Figure 5.1A), indicating a slower replication rate of the Δ*ppx* mutant. Reducing the agitation of cultures from 200 r.p.m to 150 r.p.m during incubation significantly reduced the growth rate of both strains. When the bacteria were propagated at 150 r.p.m, the lower growth rate of the Δ*ppx* mutant compared with MC58 was still observed, with the difference between two strains becoming more marked (data not shown). Similar results were found when bacteria were grown in DMEM that was used to dilute bacteria in the normal human serum assays (data not shown).

In addition to the measurement of O.D., the growth of the Δ*ppx* mutant and the parental strain was compared by measuring cell viability expressed as colony forming units (CFU). Aliquots of cultures grown at 200 r.p.m were collected at each time point, plated to solid media, incubated overnight, and colonies were counted. The numbers of the Δ*ppx* mutant recovered were similar to MC58 over the whole growth phase (Figure 5.1B). Therefore, taken together, these results indicate that the Δ*ppx*

mutant does not grow faster than MC58, and therefore the resistance of the Δ*ppx* mutant cannot be explained simply by an increased replication rate of the mutant.

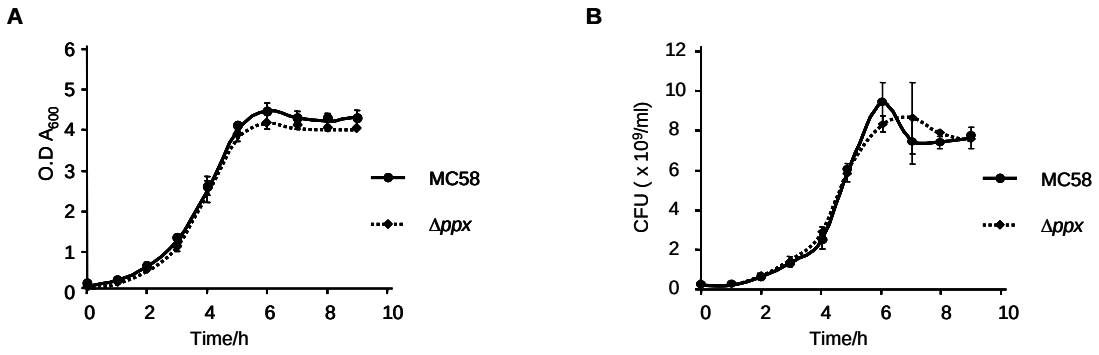


Figure 5.1 Growth of MC58 and MC58Δ*ppx* *in vitro*

(A) Analysis of bacterial growth by measuring the O.D. at 600 nm. Bacteria were inoculated into BHI liquid medium at a starting O.D. A_{600} of 0.1, then incubated at 37°C with shaking at 200 r.p.m. Growth was monitored by measuring the O.D. A_{600}. The result shows the mean from three independent experiments performed in triplicate and error bars represent the SEM. **(B)** Analysis of the viability of MC58 and MC58Δ*ppx* during the growth. The initial concentration of bacteria was 10^8 CFU/ml. The number of viable bacteria was determined by plating aliquots to solid media and counting the number of colonies after an overnight incubation. Results represent the mean of three independent experiments performed in triplicate, and the error bars show the SEM.

5.2 The resistance of the Δ*ppx* mutant is independent of the polysaccharide capsule

Expression of a polysaccharide capsule by *N. meningitidis* is critical for bacterial virulence (Vogel et al., 1996). Loss of *siaD*, which encodes polysialyltransferase, leads to loss of capsule, and marked sensitivity to complement-mediated killing compared with the parental strain, MC58 (Edwards *et al.*, 1994; Kahler *et al.*, 1998). As opposed to *N. meningitidis*, no capsule has been identified in *N. gonorrhoeae*. However, half of total cellular poly P was found to be loosely attached to the gonococcal surface. Thus it has been proposed that poly P on the surface of the gonococcus has a role of a pseudocapsule (Kornberg, 1999; Noegel and Gotschlich, 1983; Tinsley *et al.*, 1993; Tinsley and Gotschlich, 1995).

To examine whether the resistance of the Δ*ppx* mutant was due to changes in the capsule, the levels of capsule expression of MC58 and the Δ*ppx* mutant were determined initially by whole cell ELISA. Strains were coated onto the wells of ELISA plates and incubated with serial dilutions of an anti-serogroup B capsule mAb. Absorbance readings taken at 492 nm were dependent on the dilutions of the α-serogroup B capsule mAb used for detection. The O.D. A_{492} from MC58 incubated with a 1:10 dilution of the mAb was higher than that obtained with the Δ*ppx* mutant, but the cut off (O.D. $A_{492} < 0.4$) dilution for both strains was the same (Figure 5.2A). To determine whether there was a significant difference in the level of capsule expression between the Δ*ppx* mutant and MC58, capsule expression was quantified using a more sensitive method, flow cytometry. Bacteria were fixed with 3% PFA and incubated with the α-serogroup B capsule mAb at a 1:10 dilution in PBS. An increase in fluorescence intensity and thereby capsule expression was detected as a shift to the right of the FACS tracing. The amount of capsule was calculated as the

mean fluorescence intensity (MFI, calculated as the geometric mean multiplied by the percentage of positive cells), and MC58Δ*siaD* was used as a negative control. The value obtained from the Δ*siaD* mutant was less than 1% that of MC58, confirming that *siaD* is essential for capsule production and validating the assay. The MFI of the Δ*ppx* mutant was not significantly different from that of MC58 (Figure 5.2B), indicating that loss of *ppx* does not affect capsule expression.

To investigate the role of capsule in the enhanced resistance of the Δ*ppx* mutant against complement-mediated killing, the impact of PPX on survival was examined in a capsule-negative background. Due to the inherent susceptibility of capsule-minus strains to complement-mediated killing, lower concentrations of NHS were used in the serum assays. After a 1 hour incubation with serum at 1:16 dilution in DMEM, loss of PPX in MC58Δ*ppx:siaD*, still resulted in significantly enhanced survival, with an approximately 100% increased survival compared with the strain expressing PPX, MC58Δ*siaD* ($p < 0.05$, Figure 5.2C). This result indicates that the relative serum resistance of bacteria caused by loss of PPX does not require the expression of capsule. Overall, these findings indicate that the relative resistance of the Δ*ppx* mutant against complement–mediated killing is capsule independent.

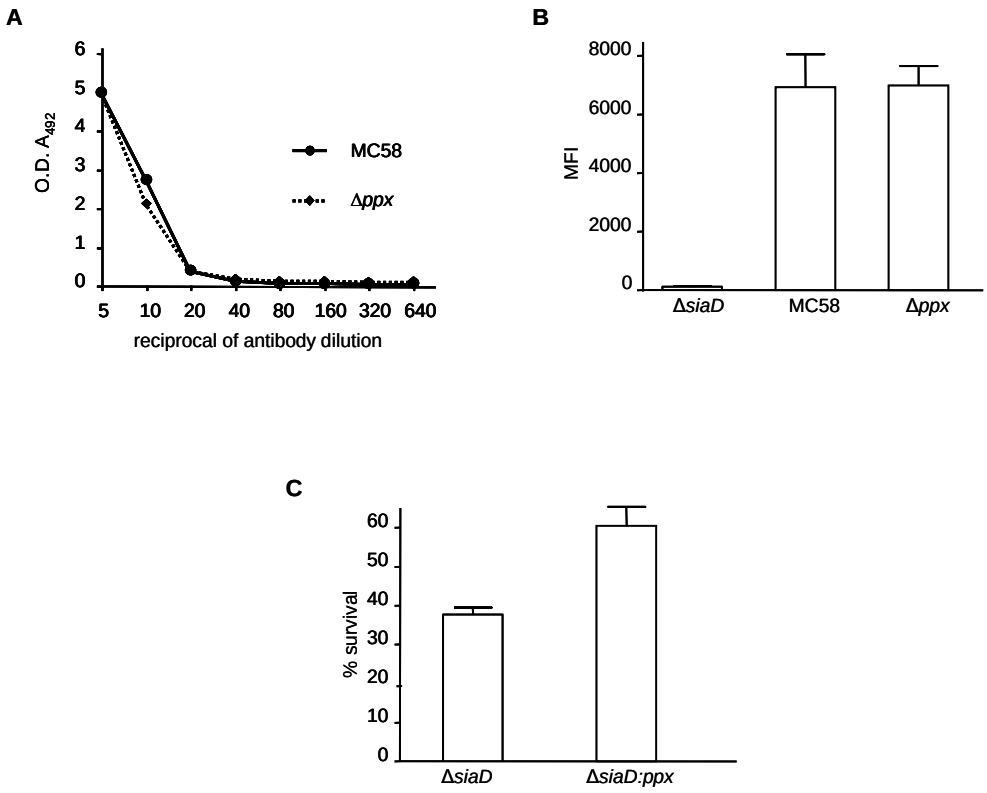


Figure 5.2 The increased resistance of the Δ*ppx* mutant is independent of capsule expression

(A) Detection of capsule expression by ELISA. Heat-inactivated bacteria (10^8 CFU) were coated onto the wells of microtitre plates and capsule expression was detected by incubation with serial dilutions of an α-serogroup B capsule mAb.

(B) Quantification of capsule expression by FACS using an α-serogroup B capsule mAb. Fixed bacteria (2 x 10^7 CFU) were incubated with the α-serogroup B capsule mAb. Capsule expression is shown as the mean fluorescence index (MFI, calculated as the geometric mean multiplied by the percentage of positive cells). The figure is a representative example of three independent experiments with similar results performed in the triplicate, and the error bars show the SEM.

(C) Serum resistance of MC58Δ*siaD* and MC58Δ*siaD:ppx*. The *ppx* mutation was introduced into MC58Δ*siaD*. The survival of bacteria was determined after a 1 hour incubation in a 1:16 dilution of NHS. There was a significant increase in the survival of MC58Δ*siaD:ppx* compared with MC58Δ*siaD* ($p < 0.05$). The figure shows the representative results of three independent experiments performed in the triplicate, and the error bars show the SEM.

5.3 The resistance of the Δ*ppx* mutant is independent of LPS sialylation

In *N. gonorrhoeae,* sialylated LPS and PorB have been proposed to bind to fH, which enhances cleavage and inactivation of C3 by factor I, leading to serum resistance of bacteria (Ram *et al.*, 1998). In contrast, the role of LPS sialylation in serum resistance of *N. meningitidis* remains controversial (Ram *et al.*, 1999). To investigate the impact of inactivation of *ppx* on LPS sialylation, the extent of sialylation of LPS in MC58 and the Δ*ppx* mutant was analysed. First, total LPS of MC58 and the Δ*ppx* mutant were extracted, analysed on Tricine-SDS gels, and visualised by silver staining. The migration of the LPS of the Δ*ppx* mutant was indistinguishable from the wild-type strain (Figure 5.3A), indicating it is unlikely that there is a significant difference in LPS sialylation of the Δ*ppx* mutant.

To quantify the levels of LPS sialylation in the different strains, total LPS and unsialylated LPS were measured by flow cytometry. Initially, FACS analysis was performed with the immunotyping antibody against L3,7,9 LPS. The results showed that the total amount of L3,7,9 immunotype LPS produced by MC58 and the Δ*ppx* mutant were similar (Figure 5.3B). Next, the extent of LPS sialylation was assessed using mAb 3F11, which recognises the terminal Gal1-4GlcNAc epitope on the α-chain of meningococcal LPS, and therefore detects unsialylated LPS (Mandrell *et al.*, 1991). An Δ*lst* mutant, which is unable to sialylate LPS, was used as a control (Exley *et al.*, 2005). The level of MFI showed that binding of 3F11 to the Δ*ppx* mutant was similar to that of the wild-type strain; the result for both strains was significantly lower than MC58Δ*lst* (Figure 5.3C). The results of FACS indicate that the unsialylated LPS expressed by MC58Δ*ppx* is similar to that expressed by MC58.

Taken together, the results demonstrate that loss of *ppx* does not change the level of LPS sialylation.

To exclude the role of LPS sialylation in the serum resistance of the Δ*ppx* mutant, the effect of PPX was evaluated in an *lst*-negative background. Normal human serum assays were performed with MC58Δ*lst:ppx* and MC58Δ*lst*. Increased survival of MC58Δ*lst:ppx* was observed compared with MC58Δ*lst* ($p < 0.05$) in the presence of NHS diluted in DMEM. Therefore, sialylation of LPS was not required for the increased resistance of the Δ*ppx* mutant against complement-mediated killing.

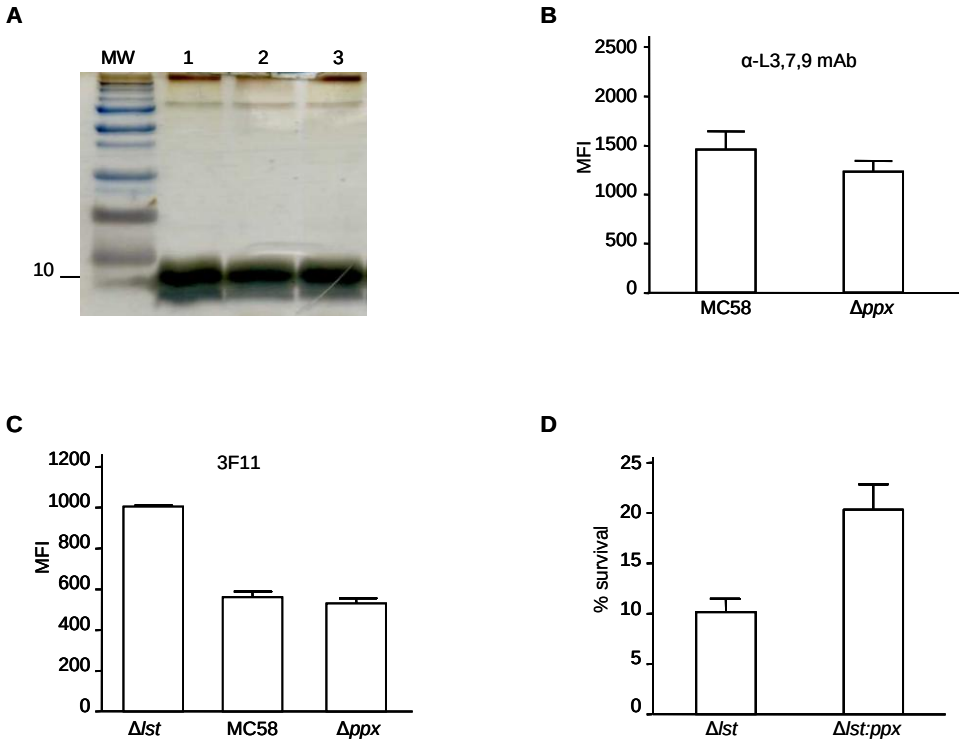


Figure 5.3 The relative resistance of the Δ*ppx* mutant is independent LPS sialylation

(A) Silver staining of the LPS from MC58 (Lane 1), the Δ*ppx* mutant (Lane 2) and the complemented strain Δ*ppx*^{com} (Lane 3), separated by Tricine-SDS-PAGE. The size of molecular weight maker (in kDa) is shown.

(B) Quantification of LPS expression by FACS using an α-L,3,7,9 mAb. The results show the MFI from one representative experiment performed on three independent occasions in triplicate, and error bars represent the SEM.

(C) Quantification of the expression of unsialylated LPS by FACS using mAb 3F11, which recognises unsialylated LPS. Binding of 3F11 to the Δ*lst* mutant was significantly higher than to parental strain MC58 ($p < 0.05$), but there was no significant difference in binding of 3F11 to MC58 and MC58Δ*ppx*. The figure shows representative results from three independent experiments performed in triplicate, and error bars represent the SEM.

(D) Increased resistance of the Δ*lst:ppx* mutant. The survival of Δ*lst:ppx* in the presence of 1:4 diluted NHS for 1 hour was significantly increased compared with the Δ*lst* mutant ($p < 0.05$). The figure shows one representative experiment performed on three independent occasions in triplicate, and error bars represent the SEM.

5.4 Examination of the deposition of complement factors on *N. meningitidis*

To further understand the basis of the enhanced resistance against complement-mediated killing of strains lacking PPX, the deposition of different complement components on bacteria after incubation with NHS was analysed. The formation of the MAC, a high molecular weight protein complex which inserts into cell membranes, is required for complement-mediated lysis of bacteria (Walport, 2001). Increased survival of bacteria is likely to be associated with less deposition of MAC on the resistant strains (Uria *et al.*, 2008). To test this hypothesis, binding of the MAC to the bacterial surface was determined by flow cytometry using a pAb recognising the C5b-9 complex. As expected, there was significantly less MAC deposition on the Δ*ppx* mutant compared with MC58 after incubation in NHS ($p < 0.05$, Figure 5.4A).

The complement factor C3, the site of convergence of the CP, the AP and the LP, is one of most important components of the complement cascade. Deposition of C3 on bacterial surfaces has been studied in several pathogens, including *Staphylococcus aureus* and *N. meningitidis* (Vogel *et al.*, 1997). C3 activation is essential for the assembly of the MAC and bacteriolysis, demonstrated by the finding that animals lacking C3 have no complement-mediated bactericidal activity (Densen *et al.*, 1988). Therefore, the level of C3 deposition on the bacterial surface after incubation in NHS was compared by flow cytometry. There was a significant decrease in the amount of C3 binding to the strain lacking PPX compared with MC58, with approximately 60% more C3 present on the Δ*ppx* mutant than MC58 ($p < 0.05$, Figure 5.4B).

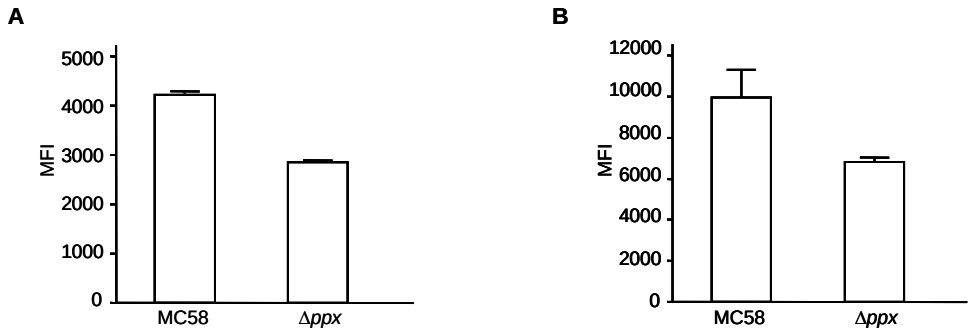


Figure 5.4 Loss of *ppx* affects C3 and MAC deposition on the bacterial surface

(A) Quantification of MAC deposition on the bacterial surface by FACS analysis. Fixed bacteria (2 x 10^7 CFU) were incubated with NHS at 37°C for 30 minutes and MAC deposition detected by an α-human C5-9 pAb. The deposition of MAC to the Δ*ppx* mutant surface was significantly less than to wild-type MC58 ($p < 0.05$). The figure shows representative results from three independent experiments performed in triplicate, and error bars give the SEM.

(B) Quantification of C3 deposition on the bacterial surface by FACS after incubation with NHS. Binding of C3 to bacteria was detected using a FITC-conjugated, α-human C3 mAb and shown as the MFI. There was significantly less binding of C3 to the Δ*ppx* mutant than to MC58 ($p < 0.05$). The figure shows representative results from three independent experiments performed in triplicate, and error bars represent the SEM.

5.5 Reduced activity of the AP contributes to the relative resistance of MC58Δ*ppx*

Antibody recognition and binding to antigens are the initial steps in the activation of the CP. As mentioned above, Western blot analysis using the α-23A10 showed there is no clear change in surface antigen expression upon loss of PPX. It is unlikely that reduction of C3 on bacterial surface and increased survival of bacteria in NHS were due to loss of PPX as an antigen from the surface of bacteria. However, to exclude this possibility, the presence of α-PPX antibodies in the NHS was examined. By Western blot analysis, no antibody binding to purified rPPX could be detected in NHS using a 1:500 dilution of serum. Subsequently the concentration of NHS was increased during incubation with the immunoblots, but this led to extensive background staining. To overcome this problem and to exclude the possibility that the undetectable signal was due to inadequate levels of NHS, ELISAs were performed using higher concentrations of NHS; pre-immune serum from mice was used as a control. The level of fluorescence detected at A_{492}, representing binding antibodies in NHS to rPPX, was similar to results obtained when bacteria were incubated with pre-immune serum, even when the concentration of NHS was as high as 1:16. As a control, binding of rPPX recognised by α-PPX serum led to a much higher O.D. A_{492} (Figure 5.5A). These data suggest that α-PPX antibodies are undetectable in NHS, and indicate that there is no difference in the initial antibody-antigen binding to the Δ*ppx* mutant compared with MC58.

The AP serves to amplify the amount of complement activation on pathogen surfaces (Walport, 2001). To address the role of the AP in the increased resistance of the Δ*ppx* mutant, the CP and the LP were blocked by the addition of 10 mM EGTA-$MgCl_2$

when bacteria were incubated with NHS during killing assays. Both MC58 and the Δ*ppx* mutant were recovered at higher levels in serum without the activation of the CP and the LP compared with NHS. Despite this, there was at least 50% more survival of the Δ*ppx* mutant compared with MC58 after a 1 hour incubation. This result indicates that the AP contributes to the relatively increased resistance of the Δ*ppx* mutant against complement-mediated killing (Figure 5.5B).

To examine the difference in the extent of the AP activity, C3 and MAC levels on the bacterial surface were determined after incubation with NHS without the CP and the LP. As expected, inhibition of the CP and the LP resulted in significantly reduced total levels of C3 and MAC present on bacterial surface. However, amounts of C3 and MAC on the Δ*ppx* mutant were about 60% lower than of those on MC58, demonstrating that loss of PPX resulted in reduced activity of the AP on their surface.

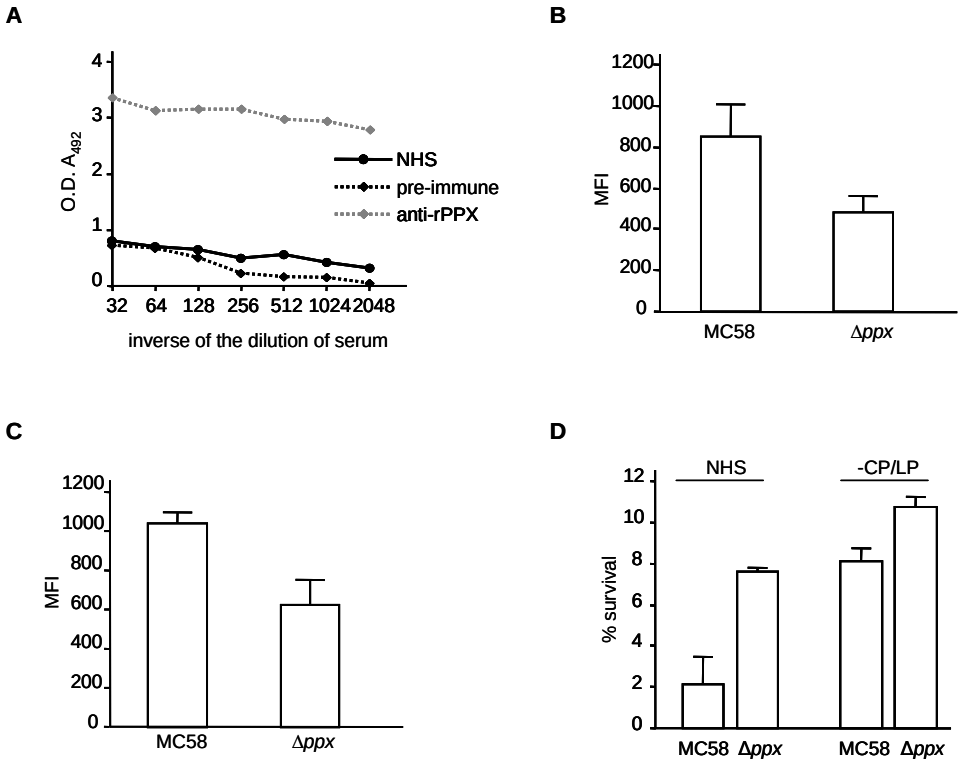


Figure 5.5 Reduced activity of the AP on the Δ*ppx* mutant

(A) Quantification of binding of antibodies in different sera to rPPX ELISA. Binding of rPPX (100 ng) to NHS was similar to that detected with the pre-immune serum from mice. Both were significantly less than binding of murine α-rPPX serum.

(B) and **(C)** FACS analysis of binding of MAC and C3 to bacteria in the absence of the CP and the LP. Fixed bacteria were incubated in NHS with 10 mM EGTA-$MgCl_2$ to block the CP and the LP (-CP/LP). Binding of MAC **(B)** and C3 **(C)** to the bacterial surface was quantified by FACS using an α-C3 mAb and an α-human C5b-9 pAb, respectively. There was significantly less activity of the AP on bacterial surface of the Δ*ppx* mutant compared with the wild-type MC58 ($p < 0.05$). Both figures are representative for at least three independents experiments that gave similar results, and the error bars show the SEM.

(D) Increased survival of the Δ*ppx* mutant in the absence of the CP and the LP. MC58 and MC58Δ*ppx* were incubated with NHS with 10 mM EGTA-$MgCl_2$ for 1 hour, and more survival of the Δ*ppx* mutant was found compared with MC58 ($p < 0.05$). NHS was used as a control. The figure shows representative results of three independent experiments performed in the triplicate, and error bars show the SEM.

5.6 Increased binding of fH to the Δ*ppx* mutant

fH is the major negative regulator of the AP and is the second most abundant complement factor present in serum (Walport, 2001). fH is recruited by many bacteria pathogens to evade killing by the complement system. It has been demonstrated by our group that fH binds to *N. meningitidis* MC58, and recruitment of fH to the bacterial surface protects the meningococcus against complement-mediated killing (Schneider *et al.*, 2006). The reduced activity of the AP on the Δ*ppx* mutant could be due to increased fH deposition. To test this hypothesis, the effect of loss of *ppx* on fH binding to the bacterial surface was initially examined by flow cytometry. Fixed bacteria were incubated in a 1:4 dilution of NHS and deposition of fH to the bacterial surface was detected using a α-human fH pAb. Incubation of bacteria with PBS was used as a control, and the MFI representing the level of fH was determined by subtracting the MFI obtained when bacteria were incubated with PBS from the MFI using NHS. Deposition of fH was detected on both strains, but the level of fH binding to the Δ*ppx* mutant was about 100% more than that to the parental strain MC58 ($p < 0.05$, Figure 5.6A). Similar results were observed in a capsule–minus background, with significantly increased binding of fH to the Δ*siaD:ppx* mutant compared with the Δ*siaD* mutant ($p < 0.05$, Figure 5.6B). Taken together, these results indicate that inactivation of *ppx* leads to increased binding of fH to the bacterial surface, irrespective of capsule expression.

To exclude the possibility that the increased binding of fH to the Δ*ppx* mutant was due to changes as a result of fixation, live *N. meningitidis* stains were incubated with serum from C7-deficient patient, which is unable to form terminal MACs to lyse bacteria. The level of fH deposition was significantly increased on the surface of the live Δ*ppx* mutant compared with MC58 ($p < 0.05$, Figure 5.6C). Consistent results,

with increased binding of fH to the Δ*ppx* mutant, were observed when bacteria were incubated with purified 440 μg/ml fH instead of NHS as the source of fH ($p < 0.05$, Figure 5.6D).

To examine whether the increased fH binding was physiologically relevant, the survival of strains was examined in fH-depleted plasma (kind gift from Professor R. B. Sim, University of Oxford). Although depletion of fH leads to a decreased level of C3, fH depleted plasma still retains about 50% of its bactericidal activity (Schneider *et al.*, 2006). The relatively increased resistance of the Δ*ppx* mutant was abolished in the serum without fH, indicating the requirement of fH for the increased resistance of the Δ*ppx* mutant against complement-mediated killing (Figure 5.7).

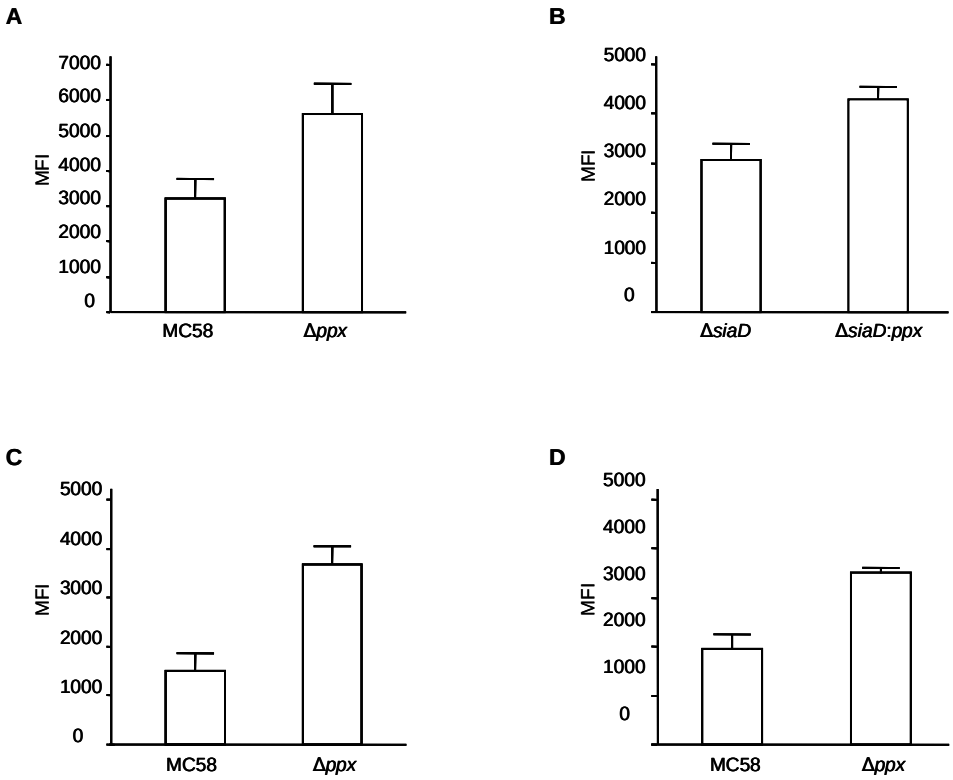


Figure 5.6 Increased binding of fH to the Δ*ppx* mutant using different sources of fH.
(A) and **(B)** FACS analysis of binding of fixed bacteria to fH in NHS. Bacteria (2 x 10^9 CFU) were incubated with NHS at 37°C for 30 minutes, and the deposition of fH to bacterial surface was detected by FACS using an α-human fH pAb, and shown as the MFI. Binding of fH to the surface of the Δ*ppx* mutant and Δ*siaD:ppx* was significantly increased compared with the parental strain MC58 **(A)** and Δ*siaD* **(B)**, respectively ($p < 0.05$). Figures show representative results of three independent experiments performed in the triplicate, and error bars show the SEM.
(C) and **(D)** Quantification of binding of live bacteria to different sources of fH by FACS. Live bacteria (2 x 10^9 CFU) were incubated with C7-deficient serum **(C)** or with 440 μg/ml purified fH **(D)**, and deposition of fH to bacterial surface was quantified by FACS. There was significantly increased binding of fH to the live Δ*ppx* mutant compared with MC58 ($p < 0.05$). Figures are representative results of at least three independents experiment performed in triplicate, and error bars show the SEM.

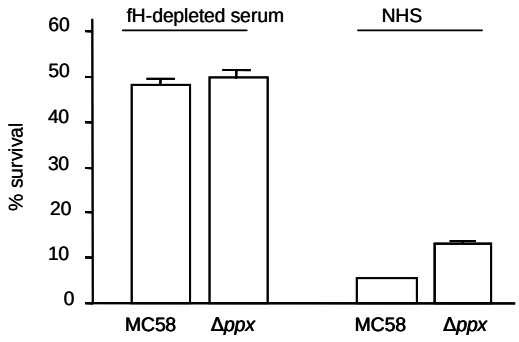


Figure 5.7 Reduction of the relative resistance of the Δ*ppx* mutant in the fH-depleted serum. MC58 and MC58Δ*ppx* were incubated with fH-depleted serum or NHS, and the survival of each strain was determined after a 1 hour incubation. The Δ*ppx* mutant had similar recovery number as MC58 in the presence of fH-depleted serum. As control, there was significantly increased survival of the Δ*ppx* mutant compared with MC58 in the presence of NHS ($p < 0.05$). The figure shows representative results of three independent experiments performed in the triplicate, and error bars show the SEM.

5.7 Increased binding of fH to the Δ*ppx* mutant is independent of the fH receptor NMB1870

Lipoprotein GNA1870 has been identified as a fH receptor in *N. meningitidis* serogroup B strain H44/76 (Madico *et al.*, 2006). The sequence of GNA1870 in H44/76 is identical to NMB1870 in *N. meningitidis* MC58 (data not shown). Previous work in our group has demonstrated that an isogenic mutant of MC58 with a kan^R cassette in *nmb1870* has significantly less fH deposition on its surface compared with the parental strain, MC58. To examine whether the increased binding of fH to the Δ*ppx* mutant was due to alterations in NMB1870, the expression of NMB1870 was examined by Western blot. Equal amounts of whole cell lysates of MC58 and MC58Δ*ppx* were separated by SDS-PAGE and verified by immunobloting with an α-RecA mAb. A single band of around of 33 kDa with similar intensity was recognised by α-rNMB1870 serum (kind gift from Dr. YW.Li) in both strains, indicating mutation of *ppx* does not affect NMB1870 expression levels (Figure 5.8A).

To confirm that the increased binding of fH to the Δ*ppx* mutant is independent of expression NMB1870, the effect of loss of PPX on fH deposition was compared in strains lacking *nmb1870*. As there was already a kan^R cassette in *ppx*, a new Δ*nmb1870* mutant was constructed by inserting a tetracycline resistance gene into *nmb1870* to allow construction of the double mutant, MC58Δ*ppx:nmb1870*. The upstream and downstream regions of *nmb1870* were amplified by PCR using primers NG1347 and NG1348, and NG1349 and NG1350, respectively. PCR products were ligated to a tetracycline resistance gene; the resulting construction was used to transform MC58 (Figure 5.6A). The successful construction of Δ*nmb1870* mutants was verified by PCR with primers flanking *nmb1870*, which generated products of 2.3 kb from mutants compared with a product of 0.9 kb from MC58 (Figure 5.6B).

The loss of NMB1870 in the Δ*nmb1870* mutants was also confirmed by Western blot analysis using the α-rNMB1870 serum; no band of around 33 kDa was detected in the mutants (Figure 5.8C). In addition, significantly reduced binding of fH to the Δ*nmb1870* mutant was confirmed by flow cytometry using the α-human fH pAb (data not shown). Genomic DNA from the Δ*nmb1870* mutants was extracted and used to transform the Δ*ppx* mutant to generate an isogenic Δ*nmb1870:ppx* mutant. The resulting transformants were verified by PCR and Western blot analysis (Figure 5.8B and 5.8D).

Fixed Δ*nmb1870* and Δ*nmb1870:ppx* mutants were incubated with NHS then with α-human fH pAbs to detect fH binding. About 100% more binding of fH to Δ*nmb1870:ppx* compared with the parental strain Δ*nmb1870,* indicating that *nmb1870* is dispensable for the increased binding of fH to the mutant lacking *ppx* (Figure 5.9C and 5.9D). Serum assays showed significantly increased survival of Δ*nmb1870:ppx* compared with Δ*nmb1870* (Figure 5.9B), demonstrating *nmb1870* is not involved in increased binding of fH, which contributes to the decreased activity of the AP and serum resistance of the Δ*ppx* mutant.

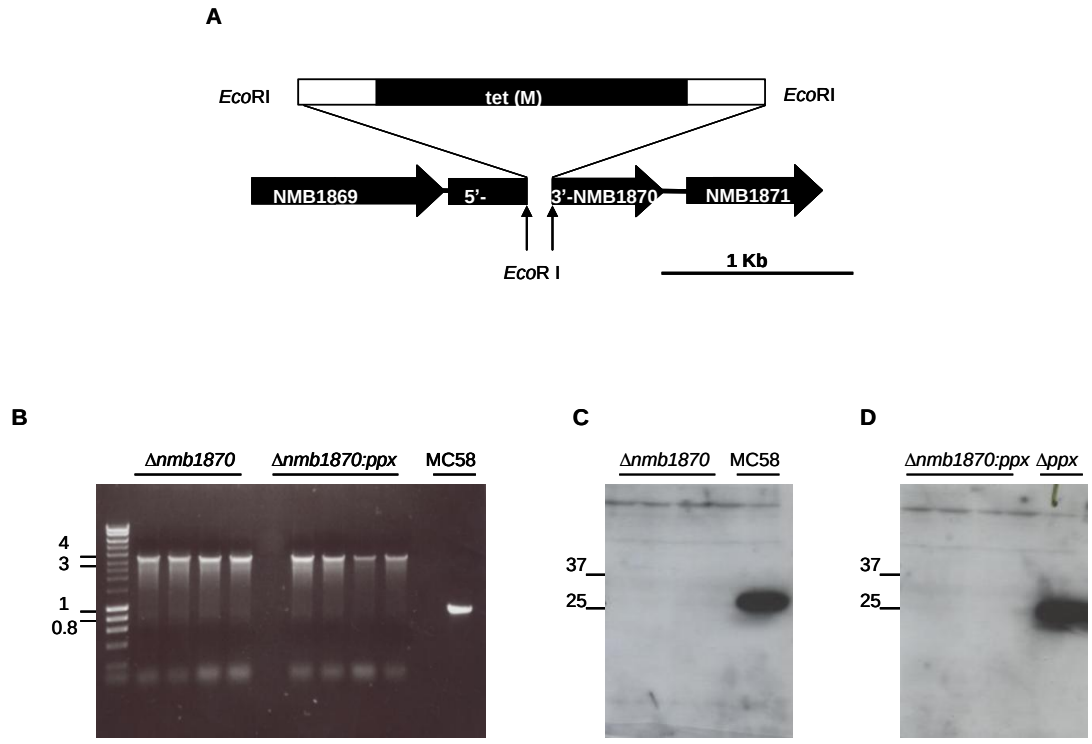


Figure 5.8 Construction of MC58Δ*nmb1870*

(A) Strategy to generate the Δ*nmb1870* mutant. The upstream (5') and downstream (3') region of *nmb*1870 with additional *Eco*RI sites were amplified and cloned to pTrcHis vector. The resulting plasmid was digested with *Eco*RI and ligated to tetracycline resistance gene flanked by *Eco*RI.

(B) Verification of the presence of the tetracycline resistance gene in Δ*nmb1870* by PCR using primers flanking *nmb1870*. The size of PCR products amplified from genomic DNA of four Δ*nmb1870* mutants and four Δ*nmb1870:ppx* mutants was about 2.4 kb bigger than that from MC58.

(C) Western blot analysis of Δ*nmb1870* mutants using an α-rNMB1870 serum. A band of about 33 kDa was recognised by the α-rNMB1870 serum (1:1,000 dilution in PBS) in the whole cell lysates of MC58 but not in the Δ*nmb1870* mutants.

(D) Western blot analysis of Δ*nmb1870:ppx* mutants using an α-rNMB1870 serum. A band of about 33 kDa recognised by the α-rNMB1870 serum (1:1,000 diluted in PBS) was present in the whole cell lysates of the Δ*ppx* mutant but not in the Δ*nmb1870:ppx* mutants.

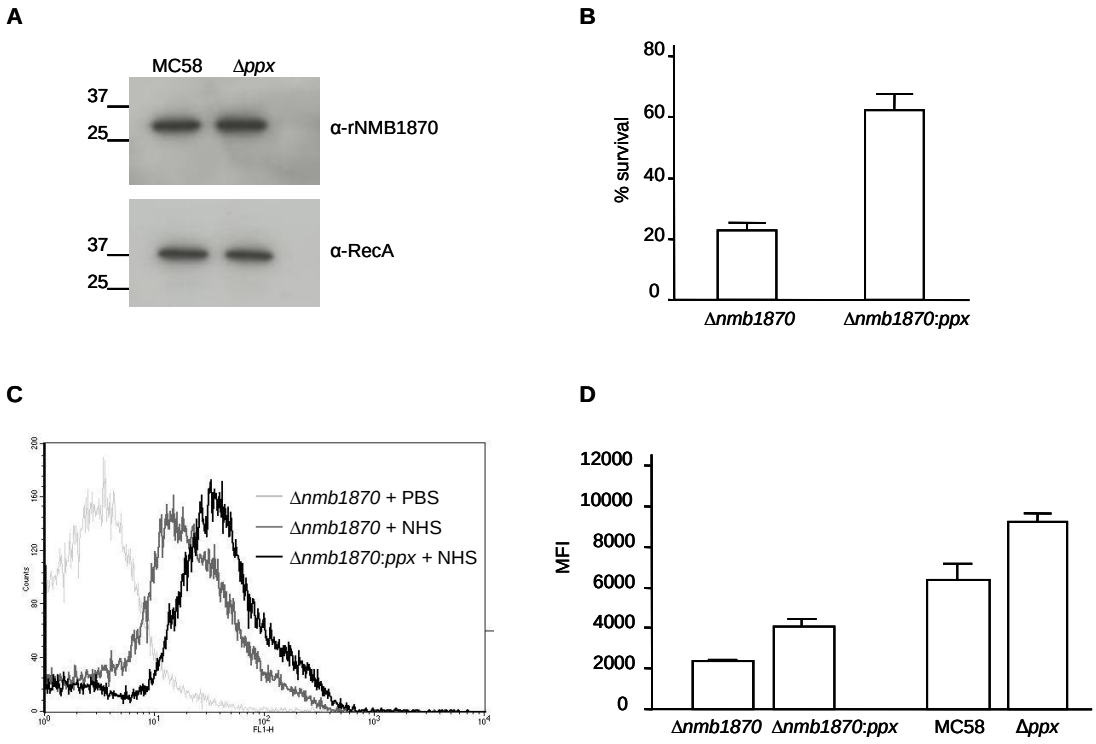


Figure 5.9 Increased fH binding to the Δ*ppx* mutant is independent of the fH receptor NMB1870

(A) Western blot analysis of NMB1870 expression in whole cell lysates. The strains are indicated above each lane, and the protein detected with the α-rNMB1870 serum was about 33 kDa. An α-RecA serum was used as the loading control. There was no significant difference in expression of NMB1870 between MC58 and the Δ*ppx* mutant.

(B) The percent survival of Δ*nmb1870:ppx* and Δ*nmb1870* after a 1 hour incubation with NHS. There was increased survival of the Δ*nmb1870:ppx* mutant compared with the Δ*nmb1870* mutant ($p < 0.05$). The results are representative of three independent experiments performed in triplicate, and error bars show the SEM.

(C) FACS analysis of binding of fH to the Δ*nmb1870:ppx* mutant and the Δ*nmb1870* mutant. Fixed bacteria were incubated in NHS, and fH deposition was detected using an α-human fH pAb. A shift to the right was seen with the Δ*nmb1870:ppx* mutant compared with the Δ*nmb1870* mutant.

(D) Deposition of fH on bacterial surface was quantified by FACS analysis and expressed as MFI. The increased fH binding on the surface of Δ*nmb1870:ppx* compared with Δ*nmb1870* was significant ($p < 0.05$), and the increased fH binding on the surface of MC58Δ*ppx* compared to the MC58 was used as control.

Chapter 6 Discussion

This thesis describes the isolation of a mutant with a transposon insertion in the gene *nmb1467* (referred to as *ppx* from now on), the characterisation of the gene product as an exopolyphosphatase (designated PPX), and investigation into the role of this enzyme in interactions between *N. meningitidis* and the complement system.

The complement system is essential for immunity against meningococcal infection. This is demonstrated by the exquisite susceptibility to meningococcal disease of individuals lacking components of the MAC (C5-C9 inclusive), who are at 1,000-10,000 increased lifetime risk of disease compared to immunocompetent people (Figueroa *et al.*, 1993). It also highlights the importance of bacteriolytic activity of complement as the MAC is required for this effector function. The *ppx* mutant was originally identified through an approach called Genetic Screening for Immunogens (GSI). This method attempted to take advantage of the power of bacterial genetics to identify vaccine candidates, by performing an unbiased screen for transposon mutants that have increased resistance to complement-mediated killing in the presence of bactericidal antibodies. The rationale was that a mutant that had lost an important surface antigen, would have a survival advantage under immune selection, and could be identified by positive selection.

The major advantage of GSI was the ability to use human sera to isolate potential immunogens for inclusion in vaccines. Most strategies to identify vaccine candidates rely on analysing immune responses generated in animals such as mice or rabbits (Rappuoli, 2001). This has proved to be a significant stumbling block as several

promising meningococcal surface molecules, including NspA and N-propionylated polysaccharide capsule of serogroup B *N. meningitidis,* have provided encouraging results in murine models, but have failed to elicit bactericidal antibodies in humans (Bruge *et al.*, 2004; Moe *et al.*, 2001; Pon *et al.*, 1997). In contrast, GSI was designed to identify vaccine candidates through the use of bactericidal antibodies in sera from convalescent patients. In our screening, we included baby rabbit complement in the assays because of the lack of availability of a reliable source of human complement which lacked bactericidal activity in large supply. In the future, it would be possible to repeat the screening with a human complement source.

The library of mutants was constructed by *in vitro* mutagenesis. This approach has been described previously with mariner and Tn*10*; both transposons have generated a wide range of insertions in *N. meningitidis* (Pelicic *et al.*, 2000; Sun *et al.*, 2000). Here, a modified version of Tn*5* was employed which contained a pACYC origin of replication that is functional in *E. coli* (but not *N. meningitidis*) to facilitate the identification of transposon insertion sites. The transposase included in the *in vitro* reactions was commercially available (Epicenter), and has been shown to have less target site specificity than wild-type Tn*5* transposase (Goryshin *et al.*, 1998; Muller *et al.*, 1999; Naumann and Reznikoff, 2002). Southern analysis demonstrated that *in vitro* Tn*5* mutagenesis successfully provided a diverse series of mutants in the library, and could be used for GSI.

The original GSI screen required considerable initial optimisation. The ideal situation was to be able to screen the entire library of over 40,000 individual mutants in a single assay in which there was no survival of the wild-type strain, while a few

resistant mutants were recovered when the library was screened. The main constraints were the amounts of available human immune serum (which was in limited supply), and the complement source. The use of a human complement source would have been preferable, but there were only small amounts of serum from a C7 deficient individual available, so this was not possible. Instead, baby rabbit complement was included in the screens. This source of complement was considered to be a suitable alternative as it is routinely used in SBAs as recommended by the Centers for Disease Control (CDC).

Several other parameters had to be adjusted before screening was undertaken. These included the total volume of assays, the number of bacteria, the duration of incubation, and the concentration of the complement source. Additionally, it would have been possible to adjust the growth conditions of bacteria as these are known to affect gene expression. In particular, it would have been interesting to propagate the bacterium in iron-restricted conditions prior to screening as this is relevant to the environment *in vivo* and affects the profile of surface antigens (Brener *et al.*, 1981).

Independent *ppx* mutants (with distinct transposon insertion sites) were isolated from separate screens performed with different immune sera, highlighting the role of this gene for survival in the presence of an active complement system. Both insertions were in the initial portion of the open reading frame and would be expected to result in a null phenotype. However, a recurrent issue with performing genetic screens is the issue of polar effects on downstream genes (resulting from the insertion of the transposon) and of second site mutations. The latter is a particular issue with *N. meningitidis*, in which there are multiple phase variable genes which can give rise to

phenotypic variants at high frequency (Richardson and Stojiljkovic, 2001). Phase variation is a particular feature of surface expressed molecules (e.g. capsule, LPS, PorA), and variants are likely to emerge under immune selection (Hammerschmidt *et al.*, 1996; Jennings *et al.*, 1999; van der Ende *et al.*, 2000). Therefore to demonstrate that loss of PPX was responsible for the observed relative resistance to complement, the mutation was reconstructed in the wild-type background, and was complemented by introducing a single copy of the gene at an ectopic chromosomal site. The results from these experiments validated the isolation of *ppx* mutants as the resistant phenotype was seen in the newly-constructed mutant and was lost upon complementation. To exclude the possibility that the effect of PPX was restricted to interactions between the meningococcus and rabbit complement, the sensitivity of the strains was examined in human serum assays.

Initial experiments on PPX were designed to examine its candidacy as an antigen in vaccines against meningococcal disease. The criteria for an attractive immunogen include: i) it should be expressed by a wide-range of strains, ii) it should expressed on the surface of the bacterium and be accessible to antibodies, iii) immunisation should elicit protection against live challenge, and iv) preferably the antigen should elicit bactericidal antibody responses. Furthermore it would be predicted that the sera that was used to select the *ppx* mutants would contain antibodies against the recombinant protein.

Therefore, recombinant PPX was expressed in *E. coli* and purified to >95% homogeneity. This was achieved using available vectors with standard *E. coli* strains and expression conditions. The protein was produced with an N terminal poly-His tag

to allow purification using a nickel column. Interestingly, removal of the tag by digestion with thrombin led to precipitation of the protein. Therefore all subsequent experiments were performed with the recombinant protein containing the His tag. This was not thought to affect the results of the immunogenicity, function or crystallisation of the protein, but if this were the case, an alternative tag or placing the tag at another site could have been attempted. Western blot analysis demonstrated the presence of antibodies in sera that recognised recombinant PPX, and this was also confirmed by ELISA. Furthermore PCR demonstrated that the *ppx* gene was present in all 22 meningococcal strains examined. However this did not address the issue of whether the strains expressed the protein. Western analysis could have been performed with the anti-PPX sera to confirm the expression of the protein by the strains.

Whole cell ELISA has been used to determine the surface localisation of antigens. However, this method can be misleading and provide false-positive results if there is lysis of bacteria before proteins are coated onto the wells of ELISA plates. Instead, FACS analysis was performed using specific anti-sera raised in mice, although there was no detectable surface PPX with this method. There are several potential explanations for this negative result. It could have been due to the lack of PPX expression under the growth conditions used. However this is unlikely as the bacteria were grown in the identical way for serum assays in which a clear phenotype of the Δ*ppx* mutant was observed. Alternatively, fixation of cells destroyed the epitopes recognised by the anti-PPX sera. Again, this explanation is unlikely as the experiments were also performed in which the anti-sera was incubated with live bacteria before fixation (not shown). Electron microscopy with immunogold labelling

of anti-PPX sera could have been used, but in the light of other negative results, this was not attempted.

The ability of an antigen to elicit bactericidal antibodies is highly desirable for any candidate vaccine against *N. meningitidis*. The presence of SBA is a proven correlate of protection against serogroup C disease (Goldschneider *et al.*, 1969a), and has been used as a surrogate marker of immunity in studies on polysaccharide and protein: polysaccharide conjugate vaccines (Borrow *et al.*, 2001). However, the validity of SBA for non-capsular antigens and for protection against serogroup B meningococcal disease remains unproven. The advantage of measuring SBA is that is can be done with stored serum samples, and is technically straightforward, although there are concerns about reproducibility of the assay between reference laboratories. We used the CDC recommended protocol to measure the SBA which includes baby rabbit complement. On a single occasion, immunisation with recombinant PPX was found to elicit bactericidal antibodies that killed the wild-type strain but not the mutant in dilutions of serum of up to 1 in 512. Unfortunately, this result was not reproducible when new batches of sera were raised by immunisation of further groups of mice. Overall, it was concluded that PPX failed to elicit SBA with Freund's incomplete adjuvant. It would have been possible to use alternative adjuvants to elicit immune responses, or to combine PPX with other antigens. However, given the lack of evidence of surface expression and lack of protection in animal challenge models, these experiments were not performed, and were not considered to be worthwhile.

It has been suggested that antibodies that mediate opsonophagocytosis can also be protective against meningococcal disease. Again given the lack of other supportive

data, opsonophagocytosis assays were not performed, and it was concluded that PPX would not be an effective vaccine candidate.

Therefore, GSI not only identifies gene products that are the targets of bactericidal antibodies, but also strains that have inherent resistance against complement-mediated lysis, such as the Δ*ppx* mutants, To further understand the interaction between the meningococcus and complement, experiments were undertaken to define the function of PPX at the biochemical level. During the initial analysis, advantage was taken of the available *E. coli* strains that had been constructed to express recombinant PPX as a heterologous protein. These strains were examined for their sensitivity to heat and for resistance against oxidative stress, as *E. coli* expressing a biochemically characterised PPX from yeast had increased resistance against these stresses (Shiba *et al.*, 1997). The results obtained with *E. coli* expressing *N. meningitidis* PPX were entirely consistent with those for the yeast enzyme.

Over the past 10 years, only a few laboratories have been consistently working on enzymes involved in the metabolism of poly P. Therefore assays for determining the function of PPX and measuring cellular poly P levels had to be developed. The standard assay to measure the activity of exopolyphosphatases includes toluidine blue. This cheap metachromatic dye can be simply included in reactions with recombinant protein along with a defined source of poly P to measure decreases in a poly P over time. However this assay has several disadvantages. Toluidine blue is not specific for poly P but recognises polyanions, so is not very useful for analysing total cellular poly P. For the meningococcus, the capsule and LPS are abundant surface polyanions that could be recognised by toluidine blue. Furthermore, the assay is less sensitive

than radioactive methods. Indeed, the assay was unable to detect poly P at concentrations of less than 1 mM; the concentration of poly P in cells has been estimated as 0.1~50 mM and 120 mM in prokaryotic and eukaryotic cells respectively (Kornberg, 1999).

In spite of these shortcomings, the toluidine assay proved sufficiently robust for demonstrating the biochemical activity of PPX and its dependence on divalent cations. To date, no active sites have been determine for prokaryotic exopolyphosphatases, although point mutations of yeast PPX have been shown to lead to a loss of activity. Active site residues in the meningococcal PPX were predicted through sequence alignments and the available crystal structures of the *E. coli* and *A. aeolicus* enzymes. Glu147 is a residue in the highly conserved putative ATP binding region of PPX, and is expected to bind divalent cations (Kristensen *et al.*, 2004; Rangarajan *et al.*, 2006). In the *A aeolicus* structure, a calcium ion is in close proximity with this residue (Kristensen *et al.*, 2004). This amino acid was replaced with alanine, and the resulting enzyme was found to have dramatically reduced, but not entirely absent activity. Attempts were made to construct other point mutants in PPX but these proved to be unsuccessful.

The modification of a single amino acid is frequently used to identify critical residues that are necessary for enzymatic function. However, it is entirely possible that alteration of a single amino acid, especially if the change is not conservative, could disrupt the overall structure of a protein, with loss of function not necessarily indicating that the change is restricted to the active site. Therefore PPX^{E147A} change may affect the structure of the protein. Studies are underway to determine the

structure of this modified enzyme to exclude this possibility. The modified *ppx* allele did prove valuable in experiments as it was included as an enzymatically inactive control that could be used for complementation of the *N. meningitidis ppx* mutant. The results demonstrated that the enzymatic activity of PPX is required for interactions with the complement system, providing further evidence that PPX is not an important surface antigen.

Having demonstrated that PPX is indeed a functional exopolyphosphatase, I next sought to determine the effect of this enzyme on cellular poly P levels in the meningococcus. No other exopolyphosphatases were identified in the MC58 genome using bioinformatic analysis by performing BLASTP searches with the sequences of the characterised polyphosphates from *E. coli* and *S. cerevisiae*. However there can be compensatory changes in the expression of PPK in response to loss of PPX that maintain wild-type levels of poly P. Therefore a *ppk* mutant was constructed as a strain that was predicted to have decreased or undetectable levels of cellular poly P. Interestingly two heritable colony phenotypes were observed in *ppk* mutants constructed both with the kanamycin and the tetracycline resistance cassettes. Therefore, these morphotypes are likely to result from compensatory second site mutations. Both large and small colonies were sensitive to complement-mediated lysis as described previously (Tinsley and Gotschlich, 1995), and gave identical results for quantification of cellular poly P, so size of colonies did not the utility of the *ppk* mutants in these experiments.

Initial studies to quantify total cellular poly P were performed using the toluidine blue assay. However despite modifying this assay, no significant differences were detected

in poly P levels in the wild-type strain and the *ppx* or *ppk* mutants. Therefore it is likely that the dye was detecting molecules other than poly P in the bacteria, and/or the assay was insufficiently sensitive. It would have been possible to examine the effect of these mutations in strains specifically lacking polyanions such as capsule and LPS. However this was not undertaken it would still not have excluded the possibility that further molecules were being detected by the toluidine blue assay. Furthermore, loss of LPS or capsule could inadvertently affect poly P metabolism. Instead, a more sensitive and specific method was performed based on the reversible activity of PPK to generate ATP from poly P in an excess of ADP. First poly P was extracted from cells and bound to Glassmilk beads. This approach has proven effective for purifying poly P from organisms such as *E. coli*, *P. aeruginosa*, *B. cereus* and *S. enterica* (Ault-Riche *et al.*, 1998; Price-Carter *et al.*, 2005; Rashid and Kornberg, 2000; Shi *et al.*, 2004). In early experiment recombinant *N. meningitidis ppk* was cloned, expressed and purified but was found to be enzymatically inactive, possibly due to the N terminal poly-His tag include to facilitate purification; the sequence of the plasmid used for expressed confirmed the wild-type allele had been successfully ligated into expression vector. Instead, purified *E coli* PPK was obtained and a method developed that detected poly P down to a concentration of 500 nM; this is over three orders of magnitude more sensitive than achieved with the toluidine blue assay. The PPK assay demonstrated significant differences in the level of poly P between the strains, with the *ppx* mutant having approximately twice the level of total poly P than the wild-type strain. Of interest, this was not associated with any change in expression of *ppk* as determined by qrtRT-PCR; therefore there was no evidence for a compensatory change in expression of this gene in response to loss of PPX unlike in *B. cereus* (Shi *et al.*, 2004). As expected complementation with a fully

functional PPX enzyme, but not with the Glu^{E147A} allele, restored poly P levels back to wild-type levels in the *ppx* mutant.

It has been reported that expression of PPX and poly P levels vary during different growth stages in bacteria (Shi *et al.*, 2004). However I was unable to detect any major alteration in PPX in *N meningitidis* between early log to stationary phase cultures by Western blot analysis, even though there was a ten-fold increase in poly P levels between these phases. qrtRT-PCR could be performed to confirm that there was no changes in expression of *ppx* or *ppk* during growth phase.

The determination of the atomic level structure of proteins can provide key insights into their function. Despite the availability of two structures for prokaryotic PPXs, it was decided to attempt to crystallise the *N. meningitidis* PPX during the course of this thesis as it might have provided essential information about the role of the enzyme in this organism that could not have been predicted from sequence alignments alone. Furthermore recombinant PPX was already available and there are close links between the CMMI and the Centre for Structural Biology at Imperial College. The crystallisation studies were performed in collaboration with Professor Paul Freemont's group. I performed all the trials of crystallisation, and screened the trays for potential crystals. The diffraction obtained with crystals was undertaken at the Synchrotron by Dr. Duo Lu. After several attempts, several distinct conditions supported the growth of PPX crystals which diffracted adequately to provide an overall structure of PPX at a 1.8 A resolution. Unfortunately, there were excessive differences in the structure at the active site, so this portion of the structure could not be solved during the course of my thesis. This variation in structure may have been

caused by examining crystals both with and without a divalent cation bound at the active site. Therefore further attempts are ongoing to crystallise the Glu^{E147A} variant of PPX, and to try and stabilise the active site by crystallising wild-type PPX with its substrate, poly P. The latter could be particularly revealing as it should demonstrate not only the active site residues but also show how the enzyme binds its substrate.

In the final chapter, experiments were directed at understanding the underlying basis of the change in interactions with the complement system mediated by PPX and poly P. There were two simple explanations for the enhanced recovery of the *ppx* mutant in the presence of human complement. First the mutant may have an increased growth rate compared with the wild-type strain. This was a distinct possibility as *ppx* mutants have pleiotropic effects in other organisms. Several conditions of growth were examined, but under no circumstances was there increased survival of the mutant. The conditions included growth in rich medium (BHI) and survival in heat treated serum (Figure 5.1 and Figure 3.3). Therefore, this trivial explanation could be discounted. Secondly, extracellular poly P has been proposed to have a capsule like function in the gonococcus. However, extensive attempts to identify extracellular poly P from the meningococcus were unsuccessful. Overnight grown bacteria were re-suspended in the 10 mM Tris-HCl, pH 7.4 by pipetting 20 times. The supernatant collected after centrifuge was filtered with 0.22 μm pore, and used to detect the presence of poly P in the *N. meningitidis* surface. Using the toluidine blue assay poly P was detected in the supernatant from 4 $x10^{10}$ CFU/ml of *N. gonorrhoeae* but not in the supernatant from 4 $x10^{10}$ CFU/ml or 1 $x10^{11}$ CFU/ml of *N. meningitidis*. Using the more sensitive poly P assay based on the rPPK, the luminance generated from the supernatant in the presence of rPPK was lower than that from the supernatant in the

absence of PPK, from all *N. meningitidis* strains we tested. There are a few explanations for the failure of detecting extracellular poly P. Firstly, poly P might attach more tightly to the *N. meningitidis* surface compared to the *N. gonorrhoeae*, it could not be collected by washing with Tris-HCl. Secondly, the chain of extracellular poly P on *N. meningitidis* might composed of about 3-20 residues, which can not be detected by the enzymatic poly P based on the rPPK (Kornberg, 1999). Future work can be carried on using i) prior ^{32}P labelling and polyacrylamide gel analysis, ii) High-performance liquid chromatography, and iii) electronic microscopy and direct labelling of polyphosphate at the ultrastructural level by using the affinity of the polyphosphate binding domain of PPX (Kornberg, 1999; Saito *et al.*, 2005).

The major structures on the surface of *N. meningitidis* that mediate resistance against complement killing are the polysaccharide capsule and LPS. Therefore, experiments were performed to determine whether the change in resistance of the *ppx* mutant was caused by alterations in these molecules. However no change in the level of capsule or LPS sialylation could be detected following loss of PPX, using FACS or ELISA analysis. Furthermore the impact of *ppx* on serum resistance was still evident in mutants lacking either a capsule or which were unable to sialylate their LPS, which can affect interactions with the complement system.

To gain further insights into the basis of the enhance complement resistance of the *ppx* mutant, the deposition of complement factors on fixed bacterial strains was examined after incubation in immune sera. Strains were fixed in initial experiments as live bacteria are lysed in the presence of human immune sera. The deposition of the MAC was reduced on the *ppx* mutant compared with the wild-type strain, as would be

predicted for a strain that is less sensitive to complement-mediated lysis. However the amount of C3 on the surface of the *ppx* mutant was also reduced. This could not be accounted for by reduced activity of the classical pathway through the loss of PPX as a surface antigen, and thence reduced antibody binding. Experiments in preceding chapters provided no evidence that PPX is expressed on the surface of *N. meningitidis*, and there were no detectable anti-PPX antibodies in the serum in which bacteria were incubated to estimate the binding of complement factors. Therefore, we examined complement deposition in the absence of the CP and the LP, which were inhibited by the addition of Mg and EGTA (Uria *et al.*, 2008). There were still significant differences in the level of C3 binding with only the AP active, consistent with the difference in resistance of strains lacking PPX being caused by changes in this pathway of complement activation. This was supported by the finding of increased survival of the *ppx* mutant in the presence of human immune sera which only retained activity of the AP. Clearly these results do not entirely exclude the possibility that changes in other complement pathways are involved. Furthermore, it would be preferable to measure the deposition of C3b rather than total C3 on bacteria. C3b is the cleavage product of complement that covalently binds to pathogen surfaces, through the formation of thioester bonds, and which initiates the formation of the MAC. However there are few antibodies available that can differentiate between C3b and C3.

As fH is the major regulator of the AP and is recruited by the meningococcus (Schneider *et al.*, 2006), the deposition of this complement factor was examined. Strikingly, the binding of fH to the *ppx* mutant was significantly increased regardless of whether fH was provided as a purified protein or in heat-inactivated human serum.

These assays were initially performed with fixed bacteria, however identical results were obtained using live bacteria incubated with either purified fH or with serum from a patient with C7 deficient (which lacks bactericidal activity).

fH binds to the surface lipoprotein and lead vaccine candidate variously named GNA1870, rLP2086, LP2086 or more recently factor H binding protein (fhbp) (Fletcher *et al.*, 2004); (Madico *et al.*, 2006). Of note loss of PPX did not affect the expression of GNA1870 by FACS or Western blot analysis (FACS analysis, not shown). Instead, PPX apparently affects the expression of other targets of factor H on the surface of *N. meningitidis*, as introduction of a *ppx* mutation into strains lacking GNA1870 still led to an increased of factor H binding. The targets of fH binding to structures on the meningococcus other than GNA1870 have not been defined as yet. It is possible that fH binds to poly P directly on the exterior of the bacterium; fH binds polyanions such as heparin and glucosaminoglycans (Schneider *et al.*, 2006). The latter charged carbohydrates are the cognate receptor for fH on the endothelial cells along with sialic acid. However against this hypothesis was the inability to detect poly P on the surface of *N. meningitidis*. Furthermore, there was no concomitant reduction in fH binding to the *ppk* mutant (not shown) which has diminished total cellular poly P levels. Other groups have identified putative fH receptors on the meningococcus (M. Pizza, unpublished data), so these are candidates for being altered in response to PPX.

Changes in poly P levels can have pleiotropic effects in bacteria. In *E. coli*, many changes resulting from changes in poly P are mediated by the alternative sigma factor, RpoS (Shiba *et al.*, 1997). Interestingly we observed relatively few differences in the

phenotype of the Δ*ppx* mutant aside from survival in the presence of complement. For example, growth, and expression of capsule, LPS sialylation, and GNA1870 were not affected by the presence/absence of PPX Therefore it might be that poly P affects much more limited repertoire of phenotypes in the meningococcus than found in other bacteria. The reason for this could be that the meningococcus does not have RpoS. To establish whether this is indeed the case, microarray analysis could be undertaken of the *ppx* mutant and compared with the wild-type to determine changes in patterns of genes expression. This might also provide clues to the basis of the increased fH deposition on the surface of the *ppx* mutant that would form the basis of future work.

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Appendix

Table A.1 Strains analysed by PCR for the presence of NMB1467 homologue

No.	Strain	Serogroup	Clonal complex	Disease
1	Z2491	A	ST-4/subgroup IV	Invasive
2	2059001	A	ST-4/subgroup IV	Invasive
3	FAM18	C	ST-11/ET-37	Invasive
4	BZ133	C	ST-1/subgroup I/II	Invasive
5	A22	W-135	ST-22	Carrier
6	860800	Y	Unassigned	Invasive
7	MC58	B	ST-32/ET-5	Invasive
8	BZ169	B	ET-5	Invasive
9	BZ83	B	ST-32/ET-5	Invasive
10	SWZ107	B	ST-35	Invasive
11	NGF26	B	ST-269	Invasive
12	2996	B	Unassigned	Unspecified
13	NG6/88	B	ST-269 complex	Invasive
14	2996	B	Unassigned	Carrier
15	NG3/88	B	Unassigned	Invasive
16	BZ147	B	ST-41/44	Invasive
17	1000	B	ST-18	Invasive
18	BZ198	B	ST-41/44 lineage3	Invasve
19	NGH36	B	ST-41/44 lineage3	Invasive
20	NGP165	B	ST-11/ET-37	Unspecified
21	C311	B	Unassigned	Invasive
22	NGH38	B	Unassigned	Carrier