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The role of novel *Neisseria meningitidis* antigens in the pathogenesis of infection and their potential as vaccine candidates

by

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Abstract

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THE ROLE OF NOVEL NEISSERIA MENINGITIDIS ANTIGENS IN THE PATHOGENESIS OF INFECTION AND THEIR POTENTIAL AS VACCINE CANDIDATES

By Miao-Chiu Hung

Neisseria meningitidis is a major cause of bacterial meningitis and septicaemia. Although vaccines against meningococcal A, C, Y and W are available, there is no vaccine for serogroup B infection. The vaccine potential of two proteins, *Neisseria meningitidis*-macrophage infectivity potentiator (MIP, NMB1567) and *Neisseria meningitidis*-adhesin complex protein (ACP, NMB2095) were investigated.

The gene encoding each protein was cloned in the pRSETA system and propagated in *E.coli* DH5α. Recombinant MIP (rMIP) and recombinant ACP (rACP) proteins were expressed in *E. coli* BL21(DE3)pLysS and purified to high purity and yield by nickel column affinity chromatography under native and denaturing conditions, respectively. For animal immunisation, the pure proteins were refolded into i) liposomes and ii) Zwittergent 3-14 micelles, both with and without the adjuvant monophosphoryl lipid A (MPLA), iii) adsorbed onto aluminium hydroxide and iv) diluted in saline alone.

Antisera of immunised animals were subjected to immunological analyses. High antibody titres against recombinant proteins and against protein in the outer membrane (OM) were detected by ELISA and western blot. Both proteins induced serum bactericidal antibody (SBA) against homologous strain (titres from 256-1024). However, the optimal preparations for eliciting heterologous SBA were proteins in saline or liposomes alone without addition of adjuvants (titres of 256-1024). Both proteins were highly conserved amongst meningococci and showed similar expression levels amongst a collection of 13 strains surveyed.

In particular, the role of ACP in pathogenesis was investigated by generating ACP knockout mutant (MC58ΔACP) and complementation strains for *in vitro* infection assays using a variety of human cells. MC58ΔACP showed significantly decreased association with epithelial cells, endothelial cells and meningioma cells compared to MC58 in a capsulated background ($p<0.05$). Using an acapsular ACP mutant strain, the protein mediated internalisation of meningococci by epithelial cells and endothelial cells. In particular, a tropism for epithelial cell interactions mediated by ACP expression was observed.

In conclusion, both MIP and ACP are highly conserved, surface-exposed proteins capable of eliciting cross-protective SBA and they deserve to be considered as vaccine antigens, possibly in a multi-component vaccine. Notably, ACP is a new adhesin and invasin that shows specific cell tropism and antibodies against ACP might provide multi-level protection against meningococcal infection.

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Presented work

Part of this work has been presented as:

- A poster presentation at the 17th International Pathogenic *Neisseria* Conference (IPNC) in Banff, Canada, Sep 2010.
- An oral presentation at the MHLS postgraduate conference at the University of Southampton, School of Medicine in June 2011.
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In addition:

- An author's manuscript is being prepared for journal submission.

DECLARATION OF AUTHORSHIP

I, Miao-Chiu Hung,

declare that the thesis entitled

The role of novel *Neisseria meningitidis* antigens in the pathogenesis of infection and their potential as vaccine candidates

and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

- this work was done wholly or mainly while in candidature for a research degree at this University;
- where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
- where I have consulted the published work of others, this is always clearly attributed;
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Abbreviation

APS	Ammonium persulphate solution
BCA	Bicinchoninic acid
B-CSF	Blood-cerebrospinal fluid barrier
dFCS	decomplemented Foetal calf serum
ELISA	Enzyme linked immunoabsorbent assay
FACS	Fluorescence activated cell sorting
fHbp	Factor H binding protein
FITC	Fluorescein isothiocyanate
GuHCl	Guanidine hydrochloride
Hep2 cells	Human epidermoid carcinoma, larynx
HUVECs	Human umbilical vein endothelial cells
IPTG	Isopropyl-β-D-thiogalactopyranoside
LAL assay	Limulus amebocyte lysate assay
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
MPLA	Monophosphoryl lipid A
MS	Mass spectrometry
Ni-NTA	Nickel-nitrilotriacetic acid
OM	Outer membrane
OMV	Outer membrane vesicles
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulphonyl fluoride
PPIase	peptidyl-prolyl-*cis*-*trans*-isomerase
rACP	Recombinant ACP
rMIP	Recombinant MIP
SBA	Serum bactericidal antibody
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
Tfp	Type IV pilus
ZW 3-14	Zwittergent 3-14

Chapter 1 Introduction

1.1 Bacteriology

Neisseria meningitidis (the meningococcus, Kingdom Bacteria, Phylum Proteobacterium, Class β-Proteobacterium, Order *Neisseriales*, Family *Neisseriaceae*, Genus *Neisseria*, Species *meningitidis*) was first isolated from the cerebrospinal fluid of a patient with meningitis in 1887 (Weichselbaum 1887). It is an aerobic Gram-negative diplococcus and fastidious with optimal growth conditions in a moist environment at 35-37°C with 5-10% (v/v) carbon dioxide. Meningococci grow on different media, such as blood agar, supplemented chocolate agar, Mueller-Hinton agar and GC proteose-peptone agar. Variable size and shape can be observed on solid media due to its susceptibility to autolysis (Apicella 2005). Under microscopy, the non-motile diplococci with 0.6-1.0 μm in diameter are often in pairs with adjacent sides flattened (Bergey and Holt 1993). In addition, the organism metabolises glucose and maltose to acid and also have oxidase, catalase and carbonic anhydrase enzymes (Bergey and Holt 1993).

The surface of meningococci can be capsulated or non-capsulated with extending pili and a cell envelope, which contains a thin peptidoglycan layer present between the outer membrane (OM) and cytoplasmic membrane (Figure 1.1). The outer part of the OM contains proteins and lipooligosaccharide (LOS) while the inner part of the OM is composed of phospholipid. Major OM proteins include 1) the PorA and PorB proteins, which are porins that allow the selective passage of cation and anion across the cell membrane respectively (Tommassen *et al.* 1990); 2) the Rmp protein, which shares homology with the OmpA protein of *E.coli* and allows the formation of membrane pores (Klugman *et al.* 1989); and 3) opacity proteins, Opa and Opc, known to be major adhesins and invasins (Virji *et al.* 1993a). Under electron microscopy, shedding of OM blebs (Figure 1.2) is a characteristic feature of meningococcal growth both *in vivo* and *in vitro* (Stephens *et al.* 1982; Poolman *et al.* 1995).

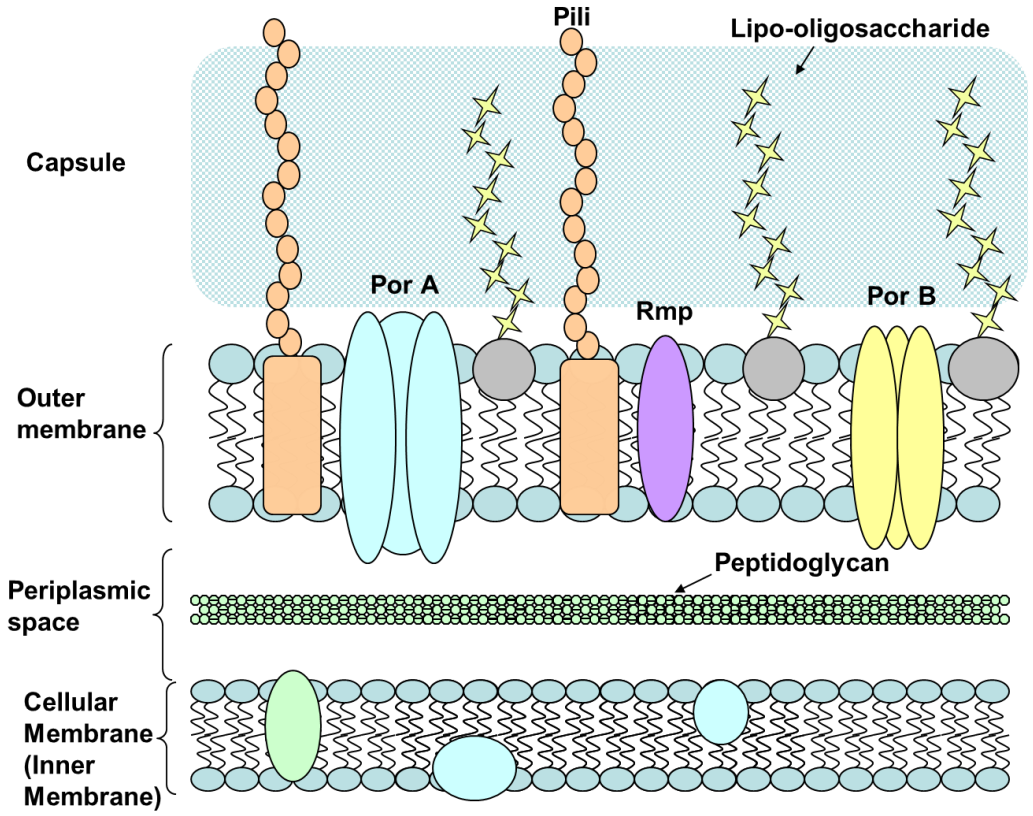

Figure 1.1 Cross-section of meningococcal cell surface.

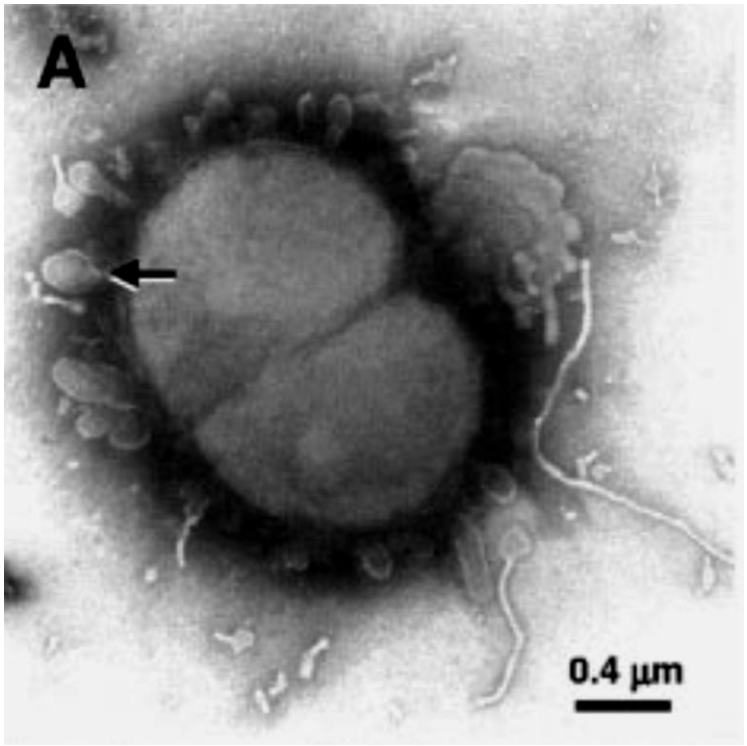

Figure 1.2 Characteristic shedding of meningococcal OM under electron microscopy (Lee *et al.* 2007). (used with permission)

Classification of meningococci depends on target antigens and 13 serogroups (A, B, C, D, 29E, H, I, K, L, W-135, X, Y and Z) are defined based on the serological reaction to capsular antigens. Among these serogroups, A, B, C, Y and W135 cause invasive disease. Furthermore, serotypes are defined by the PorB protein while sero-subtypes are dependent on the PorA protein. Moreover, 12 lipooligosaccharide (LOS) immunotypes, L1-L12, have been identified by reactivity with LOS antisera (van Deuren *et al.* 2000; Stephens *et al.* 2007).

Multilocus sequence typing (MLST) has been widely used as a tool for meningococcal genotyping (Maiden *et al.* 1998; Stephens 2007). By using the sequences of internal fragments of 7 housekeeping genes, 9227 different sequence types (ST) have been identified to date (Dec 2011) (http://pubmlst.org/neisseria/). About 450-500 base pairs of each internal fragment are sequenced by an automatic sequencer and different sequence types can be assigned even though there is only one nucleotide difference. All of these sequence types are then grouped into 50 different clonal complexes, which contain a group of different clones with close relation. The most prevalent clonal complexes are the ST-41/44 complex/lineage 3, ST-32 complex/ET-5 complex and ST-269 complex (Howitz *et al.* 2008). Significantly, different clonal complexes appear to have different pathogenic potential (Caugant 2008).

The whole genome sequence of strain MC58 (ST-32, serogroup B) has been available since 2000 (Tettelin *et al.* 2000). Subsequently, whole genome sequences of other meningococcal strains such as Z2491 (ST-4, serogroup A) (Parkhill *et al.* 2000), FAM18 (ST-11, serogroup C) and 053442 (ST-4821, serogroup C) (Rusniok *et al.* 2009) have been described. The size of the genomic chromosome is between 2.0 to 2.1 megabases with 2000 genes. There is a significant amount of repetitive DNA of variable size and location in meningococcal genomic DNA that can contribute to antigenic variability (Tettelin *et al.* 2000).

1.2 Epidemiology

According to the World Health Organisation (WHO) – Initiative for Vaccine Research, (http://www.who.int/vaccine_research/diseases/soa_bacterial/en/index1.html), *N. meningitidis* causes approximately 500,000 cases per year globally and up to 50,000 deaths. The incidence of meningococcal disease ranges from less than 0.2/100,000 to over 1,000/100,000 population/ year. The two peak attack rates occur in children less than 1 year old and in adolescents and young adults (Stephens 2007).

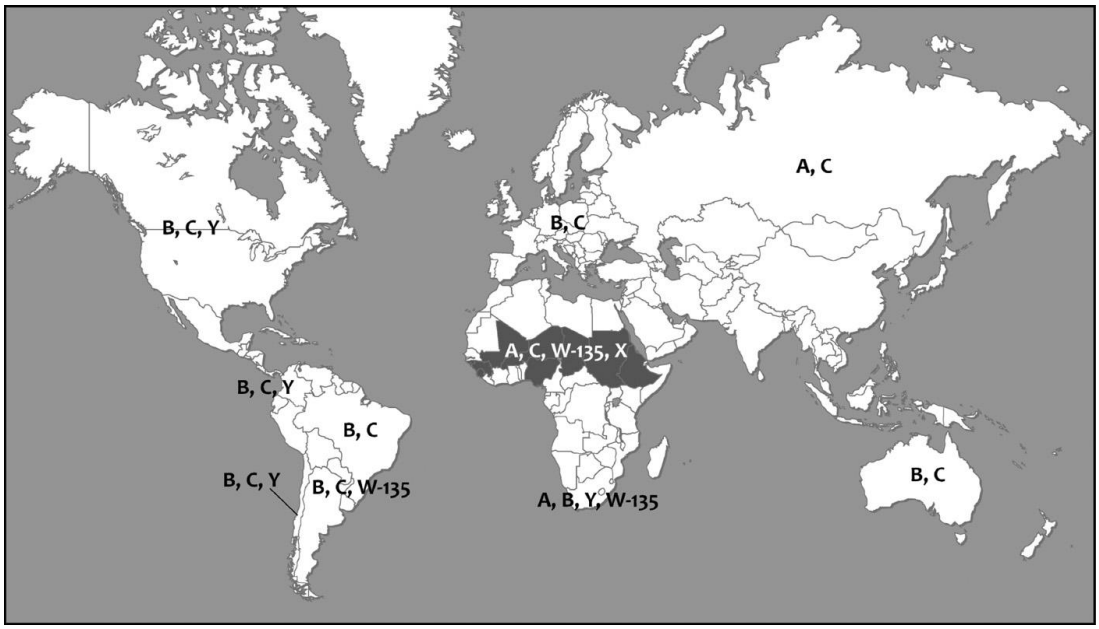

Figure 1.3 The geographical distribution of prevalent meningococcal serogroups (Harrison 2009). (used with permission)

The medically important serogroups A, B, C, Y and W135 are responsible for markedly different geographical distributions of meningococcal disease (Figure 1.3). Serogroup A bacteria are prevalent in parts of Asia and Africa and responsible for most reported meningococcal disease. In the "African meningitis belt", outbreaks are more common during the dry seasons (van Deuren *et al.* 2000; Stephens *et al.* 2007; Harrison *et al.* 2009). Serogroup B bacteria cause sporadic or endemic diseases mainly in developed countries, such as European nations, Cuba, Chile, the United States, Australia and New Zealand (Loring *et al.* 2008). Serogroup C bacteria not only cause major epidemic outbreaks in sub-Saharan Africa and South America, but also clusters of cases and local outbreaks in the US, Canada and Western Europe. Serogroup W-135 bacteria also cause outbreaks in some regions of the African belt, while serogroup X is responsible for sporadic disease in sub-Saharan African countries such as Nigeria (Boisier *et al.* 2007; Stephens *et al.* 2007). Serogroup Y meningococci have caused up to more than one- third of the cases of meningococcal disease reported in the US (Holst *et al.* 2009).

The disease burden in Asia is considered to be less well defined because of the relatively infrequent epidemics and the low level of endemic disease (Harrison *et al.* 2009). Serogroup A meningococci were responsible for up to 95% of meningococcal disease in China and recent outbreaks of serogroup A disease have also occurred in Delhi, India and in the Philippines. In Japan, the incidence is fewer than 30 cases per year and serogroup B (ST-32/ET-5) is the most prevalent strain (Horino *et al.* 2008). Although there were only a few endemic outbreaks of

meningococcal disease in Taiwan (Chiou *et al.* 2006), Thailand and Malaysia, the most common causative organism in these countries is serogroup B.

Serogroup B meningococci are responsible for about 50% of the cases of meningococcal disease worldwide with ~20,000 to 80,000 cases per annum (de Filippis 2009). In the US, 30-40% of meningococcal disease is attributable to serogroup B meningococci in comparison to up to 45-80% in Europe (Stephens *et al.* 2007; Trotter *et al.* 2007). It is also the only serogroup against which capsular polysaccharide vaccines cannot be developed, so serogroup B meningococcal infection remains a continual and potentially fatal condition.

1.3 Pathogenesis of *N. meningitidis* infection

1.3.1 Virulence factors of N. meningitidis

The key surface structures for meningococci to interact with host cells include polysaccharide capsules, lipooligosaccharide (LOS) and several OM proteins. Other virulence factors include rapid doubling time, release of OM blebs, antigenic and phase variation and molecular mimicry (Virji 2009).

I. Polysaccharide capsules and LOS

Many colonizing meningococci are non-capsulated while those causing invasive disease are almost always capsulated (Lo *et al.* 2009). The highly-hydrated capsule ensures meningococcal survival in aerosol droplets that can be transmitted between hosts.

Apart from serogroup A, the polysaccharides of invasive serogroups B, C, Y and W-135 contain sialic acids (NANA, 5-*N*-acetyl-neuramic acid), which are also commonly present on human cells. To some extent, encapsulated strains can therefore avoid immune reaction due to immune mimicry. The most well studied example is serogroup B meningococci, on which the group B capsular polysaccharide**,** α(2-8)-linked polysialic acid, is structurally identical to glycosyl residues of human neural cell adhesion molecule (NCAM) (Finne *et al.* 1983). Therefore, the capsular polysaccharides of serogroup B are particularly poor immunogens.

Capsule expression also exhibits characteristic antigenic and phase variation to evade host immune recognition. The genes encoding the biosynthesis of sialic acids for serogroups B, C, W-135 and Y are similar. Therefore, horizontal gene exchange could happen and result in capsule switching between those invasive serogroups (Swartley *et al.* 1997). Additionally, the on/off expression of capsule has an influence on interaction between meningococci and host cells (Hill *et al.* 2010).

The lipo-oligosaccharide (LOS) is composed of a lipid A, an inner and outer core oligosaccharide and is structurally distinct from lipopolysaccharide (LPS) of Gram-negative

enteric bacilli due to its lack of repeating polysaccharide O-side chain (Kahler and Stephens 1998). CD14/Toll-like receptor 4 (TLR4) has been shown to be the inflammatory signalling receptor on host cells for LOS (Zughaier *et al.* 2004). In addition, the incorporation of sialic acids into LOS in serogroups B, C, W-135 and Y makes meningococci more resistant to antibody and complement-mediated killing and phagocytosis (Vogel *et al.* 1996; Hill *et al.* 2010).

II. Major adhesins

The major adhesins of meningococci are pili, Opa and Opc proteins.

Meningococcal pili belong to the type IV pilus (Tfp) family, which is commonly found in Gram-negative bacteria (Strom and Lory 1993). Apart from imparting twitching motility by rapid extension and retraction (Merz *et al.* 2000), antigenically and structurally distinct pili also increase the transformation frequency by facilitating uptake of foreign DNA (Fussenegger *et al.* 1997). Antigenic variation of meningococcal pili has been shown both *in vitro* (Pinner *et al.* 1991) and *in vivo* (Tinsley and Heckels 1986). Even a single meningococcus is capable of producing different variants of pili. Moreover, two types of pili produced by meningococci have been identified, class I and II. Similar to gonococcal pili, Class I pili are recognised by the murine monoclonal antibody SM1 (Virji and Heckels 1983). By contrast, class II pili is not related to gonococcal pili and cannot be recognised by the SM1 antibody (Perry *et al.* 1988; Virji *et al.* 1989; Nassif *et al.* 1997).

Pili are hair-like and helically homopolymeric fibres, which mainly consist of the major pilin subunit, PilE (Nassif *et al.* 1994). Other pilus-associated proteins, including PilC, D, F, G, H, I, J, K, M, N, O, P, Q, T, V, W, X have been identified and they are related to the biosynthesis of pili. This involves 4 steps: assembly, functional maturation, emergence onto the cell surface and counter-interaction (Carbonnelle *et al.* 2005). Genes *pilM*, *pilN*, *pilO*, *pilP* and *pilQ* were known to organise as an operon. Proteins PilD, F, M, N, O and P are important in assembly and PilC, I, J, K and W are related to functional maturation (Carbonnelle *et al.* 2006). Furthermore, PilC has been demonstrated to mediate fibre retraction (Morand *et al.* 2004). The highly conserved PilG is required for pili assembly (Tonjum *et al.* 1995). PilQ is essential for the emergence of the fibres on the surface (Drake and Koomey 1995). Though low in abundance, PilX was demonstrated to mediate bacterial aggregation, which is important for bacterial adhesion (Helaine *et al.* 2005). PilW has been shown to be related to the stability and function of the fibre (Carbonnelle *et al.* 2005). In addition, antigenically variable PilE and phase variable PilC contribute to antigenic variation which enables meningococci to evade the host immune system (Carbonnelle *et al.* 2009).

Pili are one of the most efficient factors providing the initial interactions with host mucosal epithelial cells. The presence of pili enables meningococci to bind to epithelial cells and endothelial cells (Virji *et al.* 1993b), to the human meninges (Hardy *et al.* 2000) and to red blood cells (Pinner *et al.* 1991; Scheuerpflug *et al.* 1999). It has been suggested that pili facilitate adhesion by binding to a membrane-located human complement regulatory protein, CD46 (Kallstrom *et al.* 1997). The role of CD46 on endothelia of the blood brain barrier in meningococcal disease has also been investigated using a transgenic mouse model (Johansson *et al.* 2003). However, another study suggested that gonococcal pili adherence could occur via a CD46-independent process (Kirchner *et al.* 2005). Recently, meningococci have been shown to hijack the β2-adrenoceptor/ β-arrestin pathway to cross the brain microvasculature endothelium (Coureuil *et al.* 2010).

Opa protein (an 8-stranded β-barrel structure) and Opc protein (a 10-stranded β-barrel structure) are similar in size (27-31 kDa) and commonly expressed as OM proteins in meningococci. There are four *opa* genes in meningococci and their expression is known to be determined by phase variation. Opa proteins can therefore arise through a variety of genetic events and contribute to antigenic variation. In particular, certain types of Opa proteins are more predominant in invasive isolates due to their high virulence properties (Callaghan *et al.* 2006). It has been shown that Opa mainly binds to the carcinoembryonic antigen cell adhesion molecule 1 (CEACAM1, previously known as CD66a) on the epithelial surface (Virji *et al.* 1996) and induce subsequent cellular invasion (Virji *et al.* 1999). Other host cell receptors recognised by Opa include integrins, heparan sulphate proteoglycans (HSPG) and sialic acid molecules (de Vries *et al.* 1998; Moore *et al.* 2005).

By contrast, the Opc protein is encoded by a single gene, *opcA*, and is only expressed in *Neisseria meningitidis*. The expression level of Opc is phase variable resulting from transcriptional regulation (Sarkari *et al.* 1994). It has been shown that Opc mediates adhesion and invasion of epithelial and endothelial cells in acapsular strains (Virji *et al.* 1992; Virji *et al.* 1993a). Binding of Opc to human endothelial cells was shown to be mediated through a trimolecular complex that includes vitronectin or fibronectin connected to integrins in the presence of the amino acid sequence arginine-glycine-aspartic acid (RGD) (Virji *et al.* 1994). Similar findings using human brain microvascular endothelial cells were also reported (Unkmeir *et al.* 2002). In addition, the integrin receptors for vitronectin and fibronectin are αvβ3 and α5β1 respectively. Other host cell receptors recognised by Opc include heparan sulphate proteoglycans (HSPG) (de Vries *et al.* 1998) and sialic acid molecules (Virji *et al.* 1994). Recently, a study has demonstrated that sulphated tyrosines of activated vitronectin are the

targets of Opc binding for adhering to and invading human brain endothelial cells (Sa E Cunha *et al.* 2010).

III. Antigenic variation

Antigenic variation of meningococci may result from a combination of accumulating mutations, horizontal genetic exchange and phase variation. The meningococcal genome contains many repetitive and mobile sequences. Up to 1900 copies of the 9 bp DNA uptake sequence (DUS) inside the genome are available for uptake of DNA from the environment for the production of potential new variants (Lo *et al.* 2009).

1.3.2 Interaction between N. meningitidis and host cells: from the nasopharynx to the meninges

I. Adhesion to and penetration of the nasopharyngeal mucosal epithelium

The natural habitat of the meningococcus is the surface of the human nasopharyngeal mucosal membrane. Acquisition or transmission of this pathogen is mainly through close contact. Once meningococci make contact with the human nasopharyngeal epithelium, they initially adhere to non-ciliated nasopharyngeal epithelium (Pinner *et al.* 1991). There are two steps involved in meningococcal adhesion - initial adhesion mediated by pili (Nassif *et al.* 1994) followed by intimate adhesion (Deghmane *et al.* 2002).

As mentioned above, pili are the most important adhesin during initial adhesion. In addition, some minor adhesins such as App (Serruto *et al.* 2003) or NadA (Capecchi *et al.* 2005) were shown to mediate adhesion in capsulated strains and therefore considered to mediate the step of initial adhesion. At this stage, the presence of capsule can sterically hinder the surface-expressed adhesins and therefore prevent further intimate contact with the cells. To enable intimate bacterial contact with cells, capsule expression has been shown to be down-regulated through activation of the CrgA regulatory protein (Deghmane *et al.* 2002). Furthermore, after meningococci attach to the host cells, retraction of pili brings them closer to the cell surface and subsequently meningococci lose their pili (Nassif *et al.* 1999). Without the hindrance of capsules and pili, Opa and Opc then mediate the intimate contact with host epithelial cells. However, under some circumstances, such as inflammation induced by viral infection, Opa can still bind to the CEACAMs on host cells in capsulate strains. Therefore, it is likely that capsulate meningococci can penetrate epithelial cells and then spread through a hematogenous route without a need to down-regulate capsule expression (Rowe *et al.* 2007).

Resistance to mechanical flow or shear force is crucial for meningococci during colonization and adaptation in the nasophaynx. Apart from forming bacterial aggregates

associated with host cells, tfp-mediated adhesion can trigger cortical plaque formation in epithelial cells. The components of the cortical plaque include CD44v3 (a heparin sulphate proteoglycan), EGFR (a receptor tyrosine kinase), CD44/ ICAM-1 (adhesion molecules), f-actin and ezrin (a component that links the membrane components to the actin cytoskeleton) (Merz *et al.* 1999). In response to such a cross-talk with *Neisseria*, clustering of membrane protein (correlating with cytoskeletal rearrangements) on host cells has been demonstrated. Therefore, it might enable more avid binding with host cell receptors and result in greater resistance to detachment by shear stresses (Merz *et al.* 1999).

Recently, tfp-mediated adhesion was shown to contribute to a phase-variable post-translational modification, in which addition of phosphoglycerol to the pili allows detachment of the bacteria from the bacterial aggregates and further dissemination to other sites (Chamot-Rooke *et al.* 2011).

After meningococci are attached to nasopharyngeal epithelial cells, they can cross the epithelium by transcytosis, direct invasion or via phagocytosis in a 'Trojan horse' manner. If all bacteria that cross the epithelial barrier are eliminated by the human immune system, the host may become an asymptomatic carrier (Figure 1.4) (Virji 2009).

II. Survival in the blood

Following invasion of submucosal blood vessels and possible subsequent drainage to regional lymph nodes, survival of meningococci in the bloodstream is determined by their virulence factors and the host immunity. If meningococci successfully enter the blood, they could be killed by the immune system and therefore cause only a transient bacteremia in the host. However, if meningococci survive in the blood due to the absence of an appropriate immune response, they can multiply rapidly, disseminate and cause bacteremia that can then lead to septicaemia and/or meningitis.

Capsule and LOS are the most essential virulence factors for meningococci to survive in the blood (van Deuren *et al.* 2000) and are also associated with resistance to complement-mediated killing (Schneider *et al.* 2007; Kugelberg *et al.* 2008). LOS can cause coagulopathy, endothelial disruption and circulatory collapse (Devoe 1982; Brandtzaeg and van Deuren 2002). LOS can induce production of several pro-inflammatory cytokines (*e.g.* interleukin (IL)-6, tumour necrosis factor (TNF)-α and IL-1β) and some chemokines (*e.g.* IL-8, monocyte chemoattractant protein (MCP)-1 and released on activation normal T-cell expressed and secreted, RANTES). Moreover, production of excessive OM that is then shed as OM vesicle 'blebs' allows meningococci to divert antibodies and complement away from the bacterial surface.

Recently, a study has shown that the minor pilin, PilV, can trigger plasma membrane reorganization and recruit cholesterol to form filopodia in endothelial cells to enhance microcolony cohesion. These lipid rafts enable meningococci to form projections at the apical aspect of the endothelium which can protect the bacteria from shear stress (Mikaty *et al.* 2009).

III. Interaction with the meninges

By further crossing the blood-cerebrospinal fluid barrier (B-CSF), meningococci can cause meningitis (Figure 1.4). B-CSF is present in the choroid plexus inside ventricles and the arachnoid membrane. Unlike the capillaries with inter-endothelial tight junctions that form the blood-brain barrier (BBB), the B-CSF is formed by the tight junctions between cuboidal epithelial cells rather than fenestrated capillaries (Join-Lambert *et al.* 2010). Extracellular pathogens that cause meningitis do not normally penetrate intact BBB to invade brain parenchyma but cross B-CSF barrier to reach the meninges and cause an inflammatory response (Christodoulides *et al.* 2002).

Pili have been shown to maintain adherence to cerebral endothelial cells under high flow conditions *in vitro* (Mairey *et al.* 2006). The subsequent internalised meningococci might reach meninges via transcytosis or disruption of intercellular junctions (Carbonnelle *et al.* 2009). To investigate the interaction between meninges and meningococci, sections of fresh human brain and human meningioma cell culture model were established (Hardy *et al.* 2000). The cells in the latter model have essentially the same morphology and profile of cell markers as normal leptomeningeal cells. In the study of Hardy *et al*., all the three major adhesins were investigated under a capsulated background. Pili were the most important adhesin and Pil_{Ib}^{+} showed much reduced association to both fresh human brain sections and meningioma cells compared to Pil_{Ia}^{+}. Moreover, Opa protein also played a role in adherence, whereas Opc did not mediate adherence to meningoma cells. In addition, there was no evidence of invasion (Hardy *et al.* 2000) which correlates with the observation that there is little or no meningococcal invasion into the underlying brain parenchyma.

Later, it was shown that high levels of the pro-inflammatory cytokine IL-6, the CXC chemokine IL-8, the CC chemokine MCP-1 and RANTES and GM-CSF were produced by meningioma cells challenged with meningococci (Christodoulides *et al.* 2002). Apart from no secretion of the pro-inflammatory cytokines IL-1β and TNF-α, the cytokine profile is similar to that observed in CSF samples from patients with meningitis (Christodoulides *et al.* 2002).

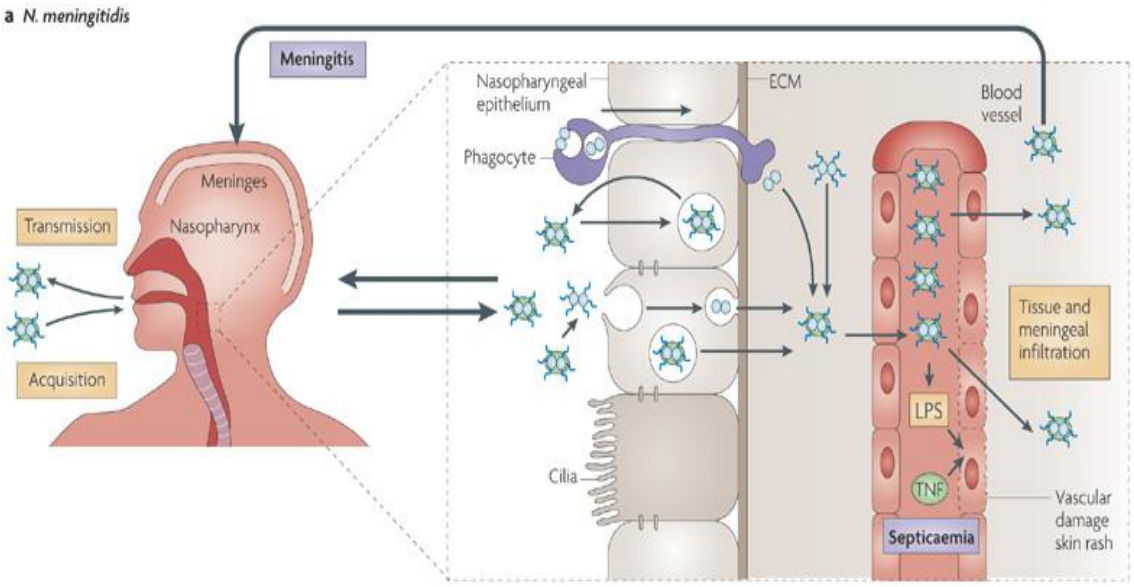

Figure 1.4 Progression of meningococci from nasopharynx and hematogenous spread to the meninges (Virji 2009). (used with permission)

1.3.3 Host immunity and susceptibility

Host defense after meningococcal invasion is determined by the innate and adaptive immune systems. Complement-mediated bacteriolysis and opsonophagocytosis play a crucial role in innate immunity against systemic meningococcal disease while adaptive immunity provides later protection by specific antibodies (van Deuren *et al.* 2000).

I. Complement-mediated killing of meningococci

In brief, there are three pathways for complement activation: (1) the classical pathway, activated by antibody-antigen interaction; (2) the alternative pathway, activated by microbial surface structures; (3) the lectin pathway (Figure 1.5) (Schneider *et al.* 2007). Without pre-existing serum antibodies, (2) and (3) are the major pathways for initial defence against meningococcal infection (Kugelberg *et al.* 2008).

To avoid complement-mediated killing, meningococci can down-regulate the complement cascade via complement regulatory proteins. The PorA protein has been shown to bind to the main inhibitor of classical pathway, component 4 binding proteins, C4bp (Jarva *et al.* 2005). The bound C4bp remained functional and enabled inhibition of C4b leading to down regulation of the classical pathway. Moreover, meningococcal factor H binding protein (fHbp) has been demonstrated to bind to the factor H on human complement, which is a main regulator of the alternative pathway, and down-regulate complement activation (Madico *et al.* 2006).

To date, serum bactericidal antibody (SBA) assay remains the gold standard for testing immunogenicity of meningococcal vaccines (Goldschneider *et al.* 1969; Vermont *et al.* 2002). SBA is based on complement-mediated killing, which starts from complement component C1q and leads to opsonization of the pathogen via the classical pathway (Figure 1.5). A fourfold or greater rise ($\geq$4) is considered as the standard to determine seroconversion after vaccination (Maslanka *et al.* 1997). However, there is difficulty regarding reproducibility and the presence of lab-to-lab variation.

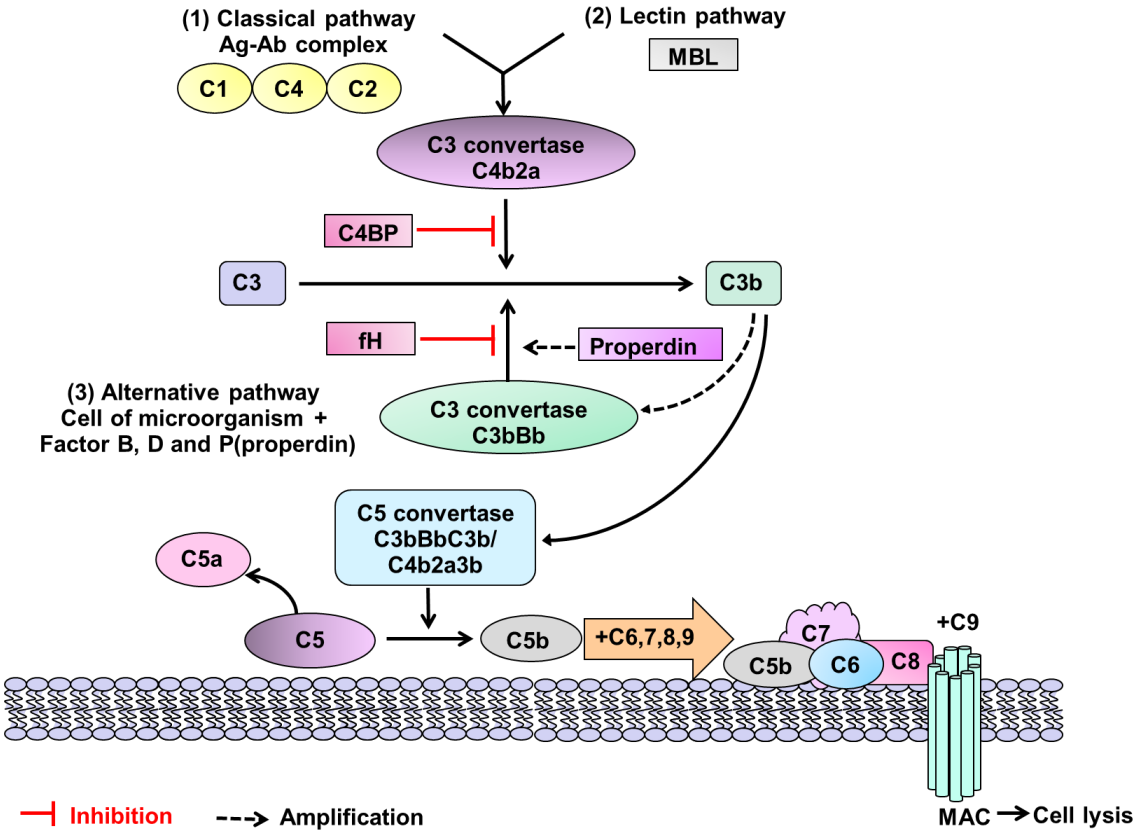

Figure 1.5 The three complement activating pathways. (adapted from Schneider *et al.* 2007*)*

II. Importance of protective antibodies

In the late 1960s, Goldschneider *et al*. provided evidence that humans are susceptible to developing meningococcal disease in the absence of protective antibodies (Goldschneider *et al.* 1969). In their study, they found that the prevalence of serum bactericidal antibody (SBA) decreases rapidly after birth and reaches the lowest point between 6 months to 24 months of age while a linear increase of SBA titre occurs from 24 months to 12 years of age. Moreover, the bactericidal activity titres of sera from adults aged 19-26 years old were slightly greater than that in the newborn infants. This is compatible with the acquisition of maternal antibodies in newborn infants followed by waning of acquired maternal antibodies after 6 months old. Furthermore, they also did a prospective survey on the recruits at the U.S. Army base, Fort Dix, N.J., where there was an outbreak of meningococcal C disease. The blood samples from new recruits were taken to determine baseline SBA. During a 4-month study period, 60 of 14,744 recruits contracted meningococcal disease. As a result, only 5.6% of the cases had baseline SBA titres $\geq$ 1:4 (and 94.4% $\leq$ 1:4) whereas 82.2% of non-cases had baseline SBA titres $\geq$ 1:4. In addition, convalescent sera from 11 cases showed high SBA titres. They therefore concluded that a lack of humoral antibodies against meningococci was related to susceptibility to systemic meningococcal disease (Goldschneider *et al.* 1969) .

To investigate the acquisition of natural immunity among the subjects without a history of meningococcal disease, blood samples from meningococcal carriers were taken to determine the SBA titres. The data showed that meningococcal carriage itself is an immunizing process with detectable protective antibodies (92% against homologous strains and 87% against heterologous strains) within 2 weeks of colonization (Goldschneider 1969). However, specific antibodies may not provide complete protection as meningococcal disease also developed in cases with presumed protective antibodies.

III. Genetic factors versus host susceptibility

Host genetic factors also affect susceptibility and outcome (Wright *et al.* 2009). Haralambous *et al.* suggested that host genetics are responsible for one-third of the total risk (Haralambous *et al.* 2003). For example, depletion of complement predisposes the host to recurrent meningococcal infections. Toll-like receptor 4 (TLR4) variants were found to be associated with invasive meningococcal disease in children less than one year old (Faber *et al.* 2006). From the same cohort, the heterozygous TLR4 Asp299Gly genotype was reported to be associated with increased mortality and need of ventilator support in paediatric patients with invasive meningococcal disease (Faber *et al.* 2009). However, contradictory results were found in another study in which the patients were analysed after stratification by age and meningococcal serogroup (Read *et al.* 2001). In terms of acquired immunity, the Fcγ receptor IIa-R/R131 allotype was believed to correlate with meningococcal disease severity (Domingo *et al.* 2002; Domingo *et al.* 2004). In addition, the genetic variability of genes encoding cytokines, *e.g.* IL-10 polymorphism (A1082G), has been shown to be associated with disease severity or outcome (Balding *et al.* 2003). Recently, a genome-wide association study suggested that single nucleotide polymorphisms (SNPs) within the region of complement factor H (CFH) and CFH-related protein 3 were related to host susceptibility to meningococcal infection (Davila *et al.* 2010).

1.4 Meningococcal disease

1.4.1 Clinical manifestations

The first description of meningococcal meningitis was made by Vieusseaux in 1805 (Vieusseux 1805), in which he reported on a 5-year-old child presenting with violent abdominal pain, vomiting and diarrhoea without headache and the presence of livid spots on the skin. The child died 12 hours later.

The distinguishing features of meningococcal disease are the fulminant clinical course and the ability to cause large-scale epidemics. If bacteria enter the bloodstream and are killed

by the host immune system, a mild transient meningococcaemia can present as a short febrile 'flu-like' episode. On the contrary, failure of the host immune system to kill the pathogen can lead to a rapid-progression of fatal disease (Brandtzaeg 1996).

Wolf and Birbara (Wolf and Birbara 1968; Apicella 2005) classified clinical meningococcal disease during an outbreak in an infantry training centre, Fort Polk in Southwest Louisiana into the following four presentations:

(1) Bacteremia without sepsis. These patients can present at hospital with febrile illness along with upper respiratory symptoms. After patients recover, the blood cultures show growth of meningococci. Normally, there are only low levels of bacteria so antibiotic treatment is often unnecessary.

(2) Meningococcemia without meningitis. These patients can present fever, a skin rash, headache, lethargy and malaise. In severe cases, circulatory collapse and multi-organ failure can occur within hours.

(3) Meningitis with or without meningococcemia. In this situation, initial presentations might be headache, vomiting, lethargy or even seizure. Signs of meningeal irritation on physical examination along with cloudy cerebrospinal fluid (CSF) can be diagnostic. The deep tendon reflexes and superficial abdominal reflexes but no pathological reflexes are present. However, it is notable that young children might present with irritability and decreased activity without classical meningeal signs.

(4) Meningoencephalitis manifestations. The patient is comatose with meningeal signs (Brudzinski sign and Kernig sign), absent or rarely hyperactive deep tendon reflexes and absent superficial abdominal reflexes. Pathological reflexes such as Babinski sign are frequently present.

The clinical presentations of meningococcal disease may vary widely and in any given patient may develop from one to another. The four potentially overlapped classifications are more useful in regards with prognosis, mortality or morbidity (Wolf and Birbara 1968). Brandtzaeg *et al.* also presented another clinical classification for research purposes, including 1) shock without meningitis; 2) shock and meningitis; 3) meningitis without shock; 4) meningococcocemia without shock or meningitis. This classification has been used in clinical studies including 862 subjects and a higher mortality rate was observed in patients with shock (Brandtzaeg and van Deuren 2012).

Meningitis is the most common presentation of invasive meningococcal disease while fulminant meningococcal septicaemia is the most devastating form of sepsis with a higher mortality rate (Stephens *et al.* 2007). Critical cases may develop disseminated intravascular coagulation (DIC) and acute adrenal haemorrhage. The Waterhouse–Friderichsen syndrome,

first reported in 1911 by Rupert Waterhouse, is characterized by petechial rash, coagulopathy, cardiovascular collapse, and bilateral adrenal haemorrhage (Waterhouse 1911). Without antibiotic treatment, all cases were fatal. Until 1938, when the sulfonamides and later penicillin became available, there were single reports of patient cases who recovered (Friderichsen 1955). The syndrome is generally associated with fulminant meningococcemia; however, other pathogens such as *Streptococcus pneumoniae* or *Haemophilus influenza* can also cause this syndrome. Early administration of antibiotics is the key factor leading to full recovery. Empirically, a third-generation cephalosporin (*e.g*., cefotaxime, ceftriaxone) should be given once the diagnosis is suspected. Penicillin G is still the drug of choice if the antibiotic susceptibility of the causative meningococci is known. Alternatively, chloramphenicol can also be effective (Varon *et al.* 1998). In addition, a comprehensive treatment guideline for bacterial meningitis and meningococcal septicaemia (NICE guideline 2010) developed by the national collaborating centre for women's and children's health can be accessed from the website: www.nice.org.uk/guidance/CG102.

The characteristic petechiae of meningococcemia are larger and deeper blue than the pinpoint petechiae caused by thrombocytopenia, vomiting or cough. Petechial lesions are observed mostly over trunk and lower limbs amongst 28-77% of patients on admission. However, haemorrhagic lesions can also appear on mucosal membranes and sclera. In cases with severe meningococcocemia, intravascular thrombosis and haemorrhagic necrosis can cause dramatic widespread purpura fulminans with potential infarction and gangrene of limbs. In addition, some patients can have transient erythematous maculopapular rashes resembling viral exanthema (Apicella 2005; Stephens *et al.* 2007).

Meningococcal pneumonia occurs in 5 to 15 percent of patients with invasive meningococcal disease. However, it can always be under-diagnosed, because growth of this organism from sputum culture does not distinguish whether it is only colonised or the causative pathogen (Rosenstein *et al.* 2001). Other syndromes associated with meningococcal disease include pneumonia, conjunctivitis, otitis media, epiglottitis, urethritis, arthritis, pericarditis, *conus medullaris* syndrome and cranial nerve dysfunction, especially of the 6^{th}, 7^{th} and 8^{th} cranial nerves. Severe pericarditis, which is likely to result from an immunological reaction or to be endotoxin-related, can complicate massive tamponade (Brasier 1987; Rosenstein 2001; Apicella 2005).

The concentration of LOS in different compartments, *e.g.* in the blood or in the subarachnoid space, has been shown to quantitatively correlate with the compartmentalised inflammatory response, which reflects the clinical presentation. For example, meningitis and septicaemia are indeed compartmentalised intracranial and intravascular inflammatory

responses (Brandtzaeg *et al.* 1992). Amongst patients with persistent septic shock, almost all the patients have a plasma LOS level higher than 10 EU/ml (Brandtzaeg and van Deuren 2002). If the plasma LOS level is higher than 100 EU/ml, the mortality rate rises up to 95% due to circulatory collapse and multiple organ failure. By contrast, the concentration of meningococci ($<10^3$/ml) and LOS (<3 EU/ml) in the blood is low but high in the CSF in patients with meningitis (Ovstebo *et al.* 2004).

1.4.2 Outcomes/Prognosis

Early recognition by family and health personnel becomes one of the most important prognostic factors. The mortality rate associated with meningococcal disease can be as high as 70-90% without treatment. Despite the availability of effective antibiotics, the mortality rate still remains between 10-15% of all cases (Girard *et al.* 2006).

Up to 25% of the survivors of meningococcal disease have lifelong sequelae, such as hearing loss, neurological disability (*e.g.* mental retardation, seizures, cognitive dysfunction), hydrocephalus, renal failure, skin scarring or loss of a limb (Rosenstein *et al.* 2001). Several studies regarding long-term outcome of children surviving from meningococcal septic shock have been carried out in Netherland and showed that one third of the cases had mild to severe neurological sequelae (Buysse *et al.* 2008), 48% of them had long-term skin scarring and 14% of the survivors had orthopaedic sequelae (Buysse *et al.* 2009). Lower self-esteem was observed amongst the survivors with scars (Vermunt *et al.* 2008). However, in a later study, there was no association observed between the long-term adverse physical and psychological outcomes (Buysse *et al.* 2010). A prospective study in the UK suggested that admission to the hospital due to meningococcal disease was related to increased psychological symptoms in a proportion of children survivors and their parents (Garralda *et al.* 2009). Amputation following meningococcal septicemia is often multiple and therefore devastating. The accompanying complications include skin necrosis, abnormal sensation, contractures, tissue breakdown, and poor stump healing or abnormal growth (Bache and Torode 2006). A cohort of multiple-amputated children were assessed and showed that they generally had good quality of life regardless the degree of amputation (Allport *et al.* 2008). Recently, a prospective study of 101 survivors of invasive meningococcal disease in adolescence has shown that 57% of cases had major physical sequelae, including seizures, amputation, hearing problems, speech problems, mobility problems and vertigo. Survivors were also reported to have poor mental health, such as depressive symptoms, fatigue and less social support, which might relate to greater reduction in quality of life and lower educational attainment (Borg *et al.* 2009).

To date, such a debilitating disease remains potentially prevalent worldwide. Development of an effective vaccine to prevent the disease would be the best strategy to eliminate fatality or poor outcome. Therefore, meningococcal vaccine research has thrived since the 1970s.

1.5 Development of meningococcal vaccines against serogroups A, C, Y and W-135

1.5.1 Polysaccharide vaccines

The development of meningococcal vaccines begins with the classical studies by Gotschlich *et al*. during the 1960s. In their study, six volunteers from their laboratory were immunized with purified polysaccharides from group A and C meningococci. Both of the polysaccharides showed excellent immunogenicity in humans with serum bactericidal antibody (SBA) lasting at least for 5 months (Gotschlich *et al.* 1969a). At that time, there was an outbreak of invasive meningococcal C disease amongst training infantry at Fort Dix in the winter and spring of 1968. In order to examine the effect of vaccination on the carrier state and potentially control the infection, 145 recruits were immunized with purified group C polysaccharide while 53 recruits were immunized with group A polysaccharide preparation. Only 2 recruits with group A polysaccharide injection failed to develop serum bactericidal activity. Amongst the unvaccinated recruits, 38, 42 and 69% of the recruits in 3 studied companies contracted meningococcal disease during a 6-week observation. By contrast, a significantly lower proportion (4.6, 24 and 31% respectively) of the individuals vaccinated with group C polysaccharide contracted group C meningococcal disease (Gotschlich *et al.* 1969b).

Following the availability of meningococcal A and C polysaccharide vaccines in the early 1970s, a quadrivalent vaccine A, C, Y, W-135 was developed in 1978 (Farquhar *et al.* 1978). These polysaccharide vaccines are safe with good efficacy (>85%) in children older than 2 years old and young adults. However, polysaccharides are T-cell-independent antigens (Coutinho and Moller 1973) and do not provide sufficient protection in children younger than 2 years old, who are particularly prone to meningococcal infection. Moreover, polysaccharide vaccines do not influence carriage status and do not induce immunological memory (Pollard *et al.* 2009). In general, the immunity to a single dose of polysaccharide vaccine lasts between 3-5 years and complications of hyper-responsiveness are not uncommon if attempts to boost immunity are made by administrating a second dose (Granoff and Pollard 2007). In addition, in terms of the immunogenicity and safety, polysaccharides are good meningococcal vaccine candidates except for serogroup B.

1.5.2 Polysaccharide-protein conjugate vaccines

In order to induce T-cell dependent immunity and protect children less than 2 years of age, new vaccine strategies have been developed based on the conjugation of capsular polysaccharide and a carrier protein, such as diphtheria and tetanus toxoids. Unconjugated capsular polysaccharides can bind to B-cell receptor (BCR) and result in B-cell differentiation and immunoglobulin production (T-cell independent). However, no new memory B cells could be produced through this process; instead, repeated immunization of polysaccharide vaccines can deplete the memory B-cell pool and therefore cause hypo-responsiveness. In contrast, when polysaccharide is conjugated to a carrier protein, the polysaccharide-specific B cells can present the protein to carrier-peptide-specific T cells. As a result, plasma cells and memory B cells are produced with the help of T cells (T-cell dependent) (Figure 1.6) (Pollard *et al.* 2009).

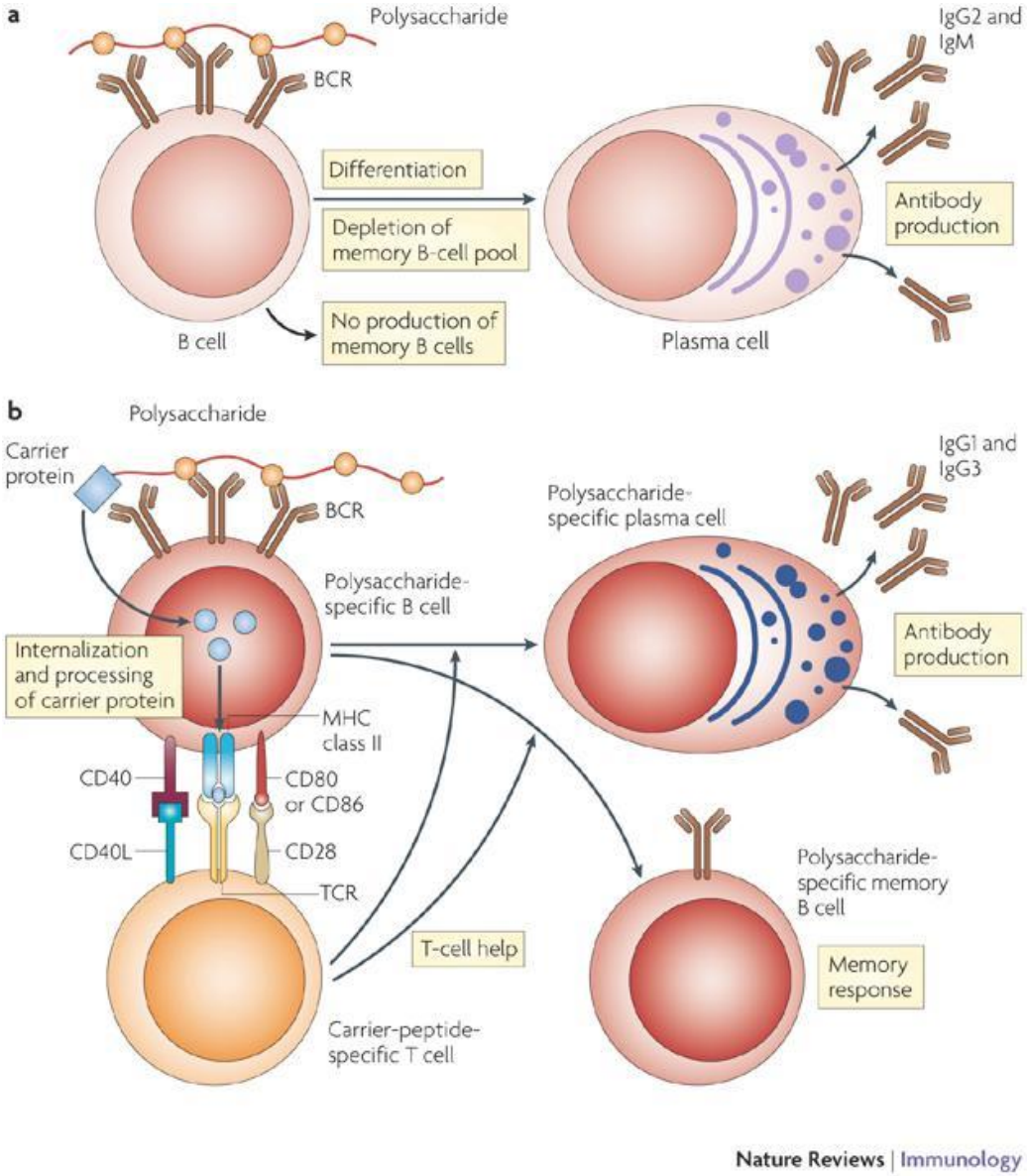

Figure 1.6 Comparison of T-cell independent and T-cell dependent immunogenicity of polysaccharide and protein-polysaccharide vaccine (Pollard *et al.* 2009). (used with permission)

The first conjugate vaccine, composed of *Haemophilus influenza* type b (Hib) capsule conjugated to diphtheria or tetanus toxoid carrier protein, was licensed in the US in 1987 for children aged 18-59 months and later recommended in children older than 2 months in 1991 (American Academy of Pediatrics Committee on Infectious Diseases 1991). The conjugate Hib vaccine was then introduced in the UK in 1992 (Howard 1992) and the incidence of invasive Hib disease dropped dramatically with a 98% reduction by 1998 (Heath and McVernon 2002). The Hib conjugate vaccines are not only immunogenic for children younger than 2 years old but also induce immunological memory. In addition, an effect on nasopharyngeal carriage leading to herd immunity has been noted (Pollard *et al.* 2009).

The conjugation strategy has been extended to serogroup C meningococci and in 1999, the MenC vaccine was introduced into the UK infant routine immunization schedule (Miller *et al.* 2001). A marked reduction of serogroup C meningococcal disease has been observed, with the vaccine showing ≥90% efficacy 3 years after administration (Ramsay *et al.* 2001). Moreover, via herd immunity, a 50-70% decrease of meningococcal disease was observed among unvaccinated individuals (Balmer *et al.* 2002; Ramsay *et al.* 2003; Maiden *et al.* 2008).

Conjugated A, C, Y, W-135 meningococcal vaccines and other combinations have also been made available since 2005. Administration of a tetravalent meningococcal glycoconjugate vaccine (MenACWY) in infants generated ≥80% levels of protective immunity (Snape *et al.* 2008). Another non-adjuvanted quadrivalent glycoconjugate vaccine (MenACWY-CRM) has shown similar levels of protection and good tolerability in infants (Perrett *et al.* 2009).

1.6 Development of vaccines against serogroup B meningococci

1.6.1 Vaccines against serogroup B capsular polysaccharide

Despite decades of effort, no successfully effective vaccine for serogroup B meningococci is commercially available at present. The formidable challenge to developing B vaccines is attributable to the molecular mimicry between the B capsule polysaccharide and human neural tissue (Finne *et al.* 1983). Therefore, a serogroup B meningococcal polysaccharide vaccine is not only likely to be poorly immunogenic but could generate auto-immune antibodies cross-reactive with human tissue (Girard *et al.* 2006; Giuliani *et al.* 2006).

An attempt was made to conjugate serogroup B meningococcal polysaccharide to tetanus toxoid carrier protein; however, this conjugate vaccine still failed to induce protective immunity (Jennings and Lugowski 1981). The result also demonstrated that a poor immunogen cannot become a good immunogen despite the use of conjugate technology. In an alternative approach, the serogroup B meningococcal polysaccharide was chemically modified by replacing the N-acetyl group of the sialic acid residues with the N-propionyl groups.

Conjugation of this N-propionylated polysaccharide with a tetanus toxoid carrier protein was shown to elicit high titres of bactericidal antibodies in mice (Jennings *et al.* 1986). Later, this N-propionylated polysaccharide was also found to mimic an epitope on group B meningococci (Jennings *et al.* 1987). However, when the chemically-modified serogroup B meningococcal conjugate vaccine was introduced in a phase I clinical trial in 17 male adults, the induced antibodies were not functional (Bruge *et al.* 2004).

1.6.2 Vaccines against outer membrane vesicles (OMV)

I. Detergent-extracted OMV vaccines

The focus of developing a group B meningococcal vaccine then shifted to outer membrane proteins (OMP) or vesicles (OMV) from 1980 onwards. OMV vaccines, which are prepared by detergent extraction of OM preparations that removes the majority of the LOS present in the membrane, have proved to be effective against certain epidemic clonal types in Chile (Boslego *et al.* 1995), Cuba (Sierra *et al.* 1991), Brazil (de Moraes *et al.* 1992), Norway (Bjune *et al.* 1991) and New Zealand (O'Hallahan *et al.* 2009). The efficacy for each vaccine was 51%, 83%, 47-74%, 57.2% and over 70% respectively. The advantage of this kind of vaccine is its wide coverage of the antigens presenting on the bacterial surface and its good physio-chemical stability (Holst *et al.* 2009). However, meningococcal OMV vaccines provide only strain-specific protection that is mostly directed towards the immunodominant protein, PorA, which is responsible for sero-subtype specificity.

II. Native (non-detergent-treated) OMV vaccines

The majority of LOS can be removed from OMV using detergent extraction; however, some minor protein antigens with potential cross protection, such as factor H binding protein (fHbp) and GNA2132, can also be removed during the process. To minimize the toxicity of LOS but preserve most potentially important protein antigens, native outer membrane vesicles can be prepared from genetically detoxified *lpxL1* knockout strains, which were first generated by the Netherlands Vaccine Institute (van der Ley *et al.* 2001). The biological activity of LOS from *lpxL1* knockout mutant has a 100-fold reduction in comparison to the wild type but retains the adjuvant function (van der Ley *et al.* 2001). Recently, a PorA knockout mutant was genetically modified to detoxify L2 and L3, 7 LOS resulting in production of non-toxic LOS-enriched OMV. This OMV preparation has been used for immunizing mice and showed a broad cross-bactericidal response (Weynants *et al.* 2009).

III. Neisseria lactamica OMV vaccines

Since the commensal *Neisseria lactamica* is believed to be involved in inducing mucosal immunity towards meningococci, it has been considered to provide potentially cross-protective immunity (Gorringe 2005). Without capsule and PorA, the immunity generated by this strain is supposed to be neither serogroup-specific nor serosubtype-specific. A previous study has shown that immunization with *N. lactamica* killed whole cells, OMV or OM protein pools could protect mice from lethal challenge of meningococcal infection; however, murine antisera raised to *N. lactamica* OMV did not elicit bactericidal antibody (Oliver *et al.* 2002; Finney *et al.* 2008). *N. lactamica* OMV vaccine was therefore assessed in a phase I human clinical study, which showed that it was safe and presented good antibody titres but only 8-32% of the volunteers had SBA titres greater than 1:4 (Gorringe *et al.* 2009).

1.6.3 Lipo-oligosaccharide (LOS)-based Vaccines

Anti-LOS antibodies have been shown to be bactericidal and linked with natural bacterial clearance in humans, and LOS is therefore considered as a potential vaccine candidate (Gidney *et al.* 2004) . LOS-based glycoconjugates were developed chemically and demonstrated the ability to elicit functional and protective antibodies against meningococcal infection (Cox *et al.* 2005). It has been shown that the glycose lacto-*N*-neotetraose on LOS could be recognised by purified human LOS- specific IgG and induces bactericidal activity for L2, L3 L4 and L7 strains (Estabrook *et al.* 2007). However, the concern of its molecular mimicry with antigens on human red blood cells might limit the development of LOS vaccine (Mandrell *et al.* 1988; Granoff 2010). Recently, the highly conserved inner core region of LOS was demonstrated to react with naturally-occurring human antisera; however, the bactericidal activity was observed only against LOS truncated mutant with a homologous LOS inner core (Jakel *et al.* 2008).

1.6.4 Vaccines based on purified meningococcal protein antigens

I. From major OM proteins to minor OM proteins

Among the outer membrane proteins present in OMV vaccines, the immunodominant antigen is the PorA porin, which has high antigenic variability. There are 2 variable regions, VR1 and VR2, with 161 and 73 unique variants respectively (Russell *et al.* 2005). The immunodominant PorA protein has been shown to induce good bactericidal activity against homologous strains, but not cross-strain. To solve this problem, a hexavalent vaccine composed of PorA and OMV has been used for phase II clinical trials and revealed immunogenicity against 6 strains in infants (Cartwright *et al.* 1999). Incorporating 20 different

PorA proteins into an OMV vaccine could lead to immunity against 80% of all circulating strains in the US (Tondella *et al.* 2000).

Without OMV, the conformation restoration of purified recombinant PorA protein is a key factor for immunogenicity. Immunization with recombinant PorA protein incorporated into liposomes or synthetic peptides containing epitopes of PorA protein have been shown to elicit bactericidal antibodies against the homologous meningococcal strain (Christodoulides *et al.* 1993; Muttilainen *et al.* 1995; Ward *et al.* 1996; Christodoulides *et al.* 1998; Humphries *et al.* 2004). To deliver cross-protection, liposome-based 4-valent PorA protein has been used to immunize mice and showed cross-strain bactericidal activity (Humphries *et al.* 2006). A fusion DNA vaccine based on a peptide epitope of PorA protein can also induce weak bactericidal activity (Zhu *et al.* 2008). However, there is currently no vaccine made with purified PorA used in clinical trials.

Other major outer membrane proteins have been studied but none of them are considered as good vaccine candidates. First of all, PorB has been incorporated into liposomes for immunizing animals but the antisera showed only serotype-specific bactericidal activity (Wright *et al.* 2002). Moreover, antisera raised against Opc showed bactericidal effect but Opc is only produced by approximately 20% of serogroup B strains (Rosenqvist *et al.* 1993). Even though purified recombinant Opc was incorporated into liposomes for immunizing animals, the variable expression level of Opc had a major impact on the magnitude of the bactericidal effect (Jolley *et al.* 2001). Recently, despite the fact that Opa is antigenically hypervariable, 14 Opa proteins from hypervirulent strains were cloned, purified and proved to be capable of eliciting bactericidal antibody in mice against at least one hypervirulent strain. It was concluded that a vaccine containing six Opa proteins can cover 90% of the disease-causing strains in the UK (Callaghan *et al.* 2011). In addition, antisera raised against antigenically conserved Rmp protein were shown to block the bactericidal effect on meningococci (Munkley *et al.* 1991). However, a later study using monoclonal antibody raised against reactive epitopes of Rmp protein showed a contradictory result (Rosenqvist *et al.* 1999). Therefore, the vaccine strategies have shifted to consider minor outer membrane proteins.

Transferrin-binding protein (Tbp), a minor OM protein, has been shown to elicit bactericidal activity in mice (Danve *et al.* 1993). When it was used for a phase I clinical trial, however, it induced only poor bactericidal activity (Danve *et al.* 1998). Another highly conserved minor OM protein, Neisserial surface protein A (NspA), was identified by generating monoclonal antibodies using different combination of the OM preparations to select highly conserved OM protein. Purified NspA was found to confer cross protection in animal studies (Martin *et al.* 1997; Martin *et al.* 2000). By contrast, another study showed that the SBA titres

of monoclonal antibodies raised against recombinant NspA did not provide broad protection (Moe *et al.* 2001). However, a phase I clinical trial of recombinant NspA with detergent-extracted outer membrane adsorbed to aluminium hydroxide revealed the safety and immunogenicity of the vaccine but not bactericidal activity (Halperin *et al.* 2007). One iron-repressible OMP, FetA, (previously known as FrpB) was found to be recognised by convalescent sera from patients with meningococcal disease; however, this hypervariable antigen has been shown to elicit mainly variant-specific protection (Ala'Aldeen *et al.* 1994; Thompson *et al.* 2003). Additionally, it has been suggested that a vaccine combining six PorA and five FetA proteins might provide broad protection (Urwin *et al.* 2004). However, that was based on analysis of genetic diversity of PorA, PorB and FetA without further immunisation study.

II. Reverse vaccinology

The availability of the MC58 whole genome sequence (Tettelin *et al.* 2000) has allowed the development of an *in silico* method commonly referred to as 'reverse vaccinology' for identifying potential vaccine antigens (Pizza *et al.* 2000). The *in silico* analysis of the whole genome showed that there were 570 open reading frames encoding surface-exposed or secreted proteins, of which 350 proteins were expressed in *E.coli*, purified and used for immunisation of mice. The resulting antisera were used in FACS analysis to determine whether the proteins were surface exposed and also tested for their bactericidal activity. However, the prediction is not always accurate (Masignani *et al.* 2002; Mora *et al.* 2003), but nevertheless this process led to the discovery of 28 novel vaccine candidates, including factor H binding protein (fHbp, GNA1870, 2086 lipoprotein or LP2086, 3 variants + sub-variants or 2 subfamilies) (Masignani *et al.* 2003; Fletcher *et al.* 2004), Neisserial adhesin A (NadA or GNA1994, 5 variants + sub-variants) (Comanducci *et al.* 2002), Neisserial heparin binding antigen (NHBA or GNA2132, 14 variants + sub-variants) (Welsch *et al.* 2003), and GNA33 (Granoff *et al.* 2001).

Recently, as whole genome sequencing of more meningococcal strains became available, genomic diversity could be analysed by comparative genomics. Therefore, a pan-genome reverse vaccinology era has evolved (Serruto *et al.* 2009). Through a systematic analysis of the neisserial genome, 20 open reading frames (ORFs) were selected, expressed and refolded for immunological assays. As a result, 5 surface-located and conserved proteins, including NMB0606, NMB0873, NMB0928, NMB0938 and NMB1163 showed positive SBA against a homologous serogroup B strain (Pajon *et al.* 2009).

III. Novartis multicomponent vaccine and the Pfizer LP2086 vaccine

Based on reverse vaccinology, the Novartis 5CVMB vaccine contains 5 recombinant protein antigens, including 2 fusion proteins, NHBA (GNA2132)-GNA1030 and fHbp-GNA2091, and NadA in tandem with an OMV from the New Zealand vaccine strain (Giuliani *et al.* 2006). Each dose of vaccine (0.5 ml) contains 50 µg of the 2 fusion proteins and NadA adsorbed to 1.5 mg of aluminium hydroxide and 25 µg of OMV. Intramuscular injection was recommended (Bai *et al.* 2011). Recently, the name of Novartis 5CVMB was changed to 4CMenB (Bexsero®). In fact, the components of the vaccine remain the same but 4 main immunogenic components in this vaccine have been used, including fHbp (variant 1.1), NHBA (variant 1.2), NadA (variant 3.1) and the NZ98/254 OMV (PorA 1.4) (Figure 1.7) (Findlow *et al.* 2010). The other 2 proteins, GNA1030 and GNA2091, were kept in the vaccine due to their importance in stabilization of fusion proteins (fHbp-GNA2091 and NHBA-GNA1030).

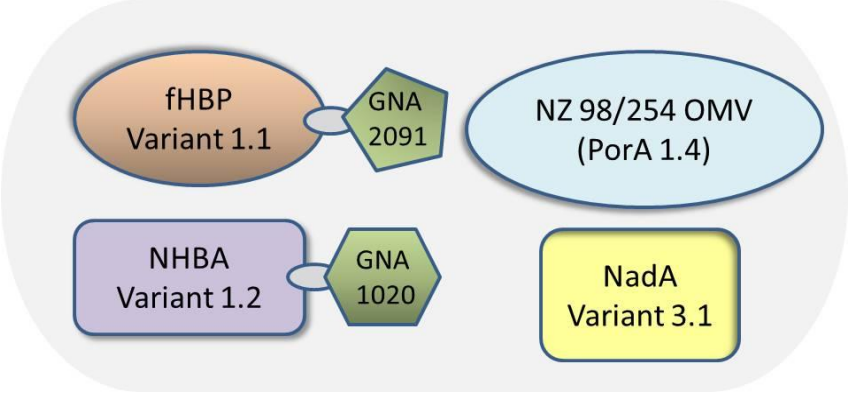

Figure 1.7 Antigens used for 4CMenB vaccines. (Adapted from Su and Snape 2011)

This vaccine was used in phase II human clinical trials following the encouraging data from a phase I trial. Two phase II clinical trials in infants comparing the effectiveness of rMenB (contains 2 fusion proteins and NadA) and rMenB + OMV were completed in the UK (Findlow *et al.* 2010; Snape *et al.* 2010). The immunogenicity of this vaccine was evaluated by conducting SBA against 7 standard strains, including 44/76-SL and M01 240101 (testing for fHbp), 5/99 and M01 240364 (testing for NadA), NZ98/254 and M00 242922 (testing for PorA 1.4; and also for other subvariants of fHbp), M01 240355 (negative control). Overall, the vaccine showed very good efficacy with ≥85% protection; however, without incorporation of the OMV component into the vaccine, only 5% of vaccinees showed SBA ≥ 1:4. Moreover, the decline of protection in infants 6 months after vaccination was observed and a booster dose at 12 month resulted in at least 93% of the participants showing SBA ≥ 1:4 (Snape *et al.* 2010; Su and Snape 2011). This vaccine has been through phase III clinical trials in the first quarter of 2008 (Rinaudo *et al.* 2009) and was submitted for regulatory approval of the European Medical

Agency in Dec 2010 (Rappuoli *et al.* 2011). Recently, the efficacy of this vaccine from phase III clinical trials was reported to be ~70% (Dull 2011) if only 4 standard strains were tested for SBA.

The safety profile was also investigated alongside the vaccine trials. The reactogenicity in infants included local reaction (*e.g*. local induration and tenderness) and systemic reaction such as fever. There was a case considered as vaccine-related reactive arthritis 1 month after Bexsero injection (Snape *et al.* 2010). In adolescents and adults, the most common reported side effect was injection site reaction such as tenderness, swelling and erythema (Bai *et al.* 2011). Overall, the vaccine appears to be safe in use.

To evaluate the efficacy of Bexsero in a more efficient way, a meningococcal antigen typing system (MATS) assay was developed that showed good correlation with the SBA assay (Donnelly *et al.* 2010). This method is basically a sandwich ELISA assay in which specific IgG is used to capture the corresponding antigen (*e.g.* fHbp, NadA and NHBA) from detergent-extracted bacteria and then the antisera from vaccinees are reacted with the specific antigens. By comparing the ELISA reactivity to strains with known expression level of specific antigens, relative potency (RP) for each antigen can be determined. The lowest RP correlated to positive bactericidal activity is defined as the positive bactericidal threshold (PBT). It has been shown that MATS RP against one antigen higher than PBT correlated with a ≥80% probability of positive bactericidal activity while the positive prediction value for MATS RP against ≥2 antigens higher than PBT was 96% (Donnelly *et al.* 2010).

The other current vaccine that is well-advanced in development is the Pfizer bivalent recombinant LP2086 vaccine, which contains both subfamily A and B (A05 and B01) of the surface-expressed LP2086 (fHbp). The subfamily A includes Novartis variant 2 and 3 while subfamily B comprises variant 1. An international collection of invasive MenB isolates has shown that more isolates (70%) are classified as subfamily B compared to subfamily A (30%) (Jiang *et al.* 2010). Sequence identity between the 2 subfamilies is 60-75% while sequence identity within either the subfamily A or B is >84%. The 2 selected components are lipidated. In a phase I study, 3 different doses of 20 μg, 60 μg and 200 μg of recombinant LP2086 were administrated to healthy children, adolescents aged 8-14 years and adults. Four fold rises in bactericidal titre against 6 meningococcal strains were reported and the highest dose generated the greatest response (Granoff 2010). Recently, phase II clinical trials included 535 adolescents aged 11-18 years with immunisation at 0, 2, and 6 months. The immunogenicity was evaluated by testing SBA against 9 serogroup B meningococcal strains with heterologous fHbp and the result suggested that the Pfizer bivalent LP2086 vaccine provides about 80% (76-100%) protection (Jansen 2011). Although safety and efficacy were considered acceptable during a phase I study in infants, they did not include infants in a phase II clinical trial with no

explanation. In addition, the SBA data were generated using a human complement source, which has been shown to have better correlation than using baby rabbit complement. This vaccine is now in a phase III trial (Jansen 2011).

IV. Proteomics and immunoproteomics

Proteomic technology provides another approach to investigate the proteins in the meningococcal outer membrane. Extracted meningococcal OM could be separated by a 2-dimensional SDS-PAGE and then subjected to mass spectrometry (MS) analysis. In combination with available genomic sequence databases, the separated OM proteins can be identified. The proteome map of outer membrane preparations from strain NZ98/254 (Vipond *et al.* 2006), wild-type MC58 and LPS-deficient MC58 (Williams *et al.* 2007) have been produced. By this method, some novel potential vaccine candidates, such as lipoprotein NMB0928 (Delgado *et al.* 2007) and NMB0088 (Sardinas *et al.* 2009b) were identified, incorporated into liposomes and were able to induce bactericidal activity in mice.

However, the proteomic analyses could not provide further information regarding whether the individual protein is immunogenic. Therefore, immunoproteomic approaches were subsequently developed by adding one more step-western blot. Sera from people colonized by meningococci or patients who recovered from meningococcal disease were reacted with the protein blot to identify potentially cross-protective *Neisseria* antigens (Mendum *et al.* 2009; Williams *et al.* 2009). In the study conducted by Mendum *et al.*, identified proteins associated with protection in convalescent sera from patients were cloned, expressed and emulsified in Freund's adjuvant for animal immunisation. However, none of the cloned proteins, induced serum bactericidal activity. It is likely that the proteins were not immunogenic themselves or they were not refolded properly in Freund's adjuvant (Mendum *et al.* 2009; Heckels and Williams 2010).

Taken together, a good vaccine candidate requires the following characteristics: 1) surface-exposure; 2) broad functional immunogenicity; 3) high conservation and 4) expression in all or most meningococcal strains. Based on different vaccine strategies, many meningococcal antigens have been shown to elicit serum bactericidal activity (Table 1.1). However, almost all of them have limitations such as antigenic variability (*e.g.* FetA), absence in hypervirulent strains (*e.g.* NadA), phase variability (*e.g.* Opc), low constitutive expression of antigen by some strains or poor expression of important conformational epitopes (*e.g.* NspA). Therefore, it is probably unlikely that a single protein antigen vaccine will provide broad protection and this has been taken into consideration. Additionally, protein refolding, carrying agents and the choice of adjuvant are important for the optimal design of a vaccine.

Table 1.1 'Minor' meningococcal proteins that have been reported to induce serum bactericidal antibody (SBA).

Protein	**SBA in animals**	**SBA in human**
Transferrin-binding protein (Tbp)	Yes (Danve *et al.* 1993; Ala'aldeen *et al.* 1994)	No (Danve *et al.* 1998)
NspA	Yes (Martin *et al.* 1997; Martin *et al.* 2000)	No (Halperin *et al.* 2007)
FetA (also known as FrpB)	Yes (Ala'Aldeen *et al.* 1994; Thompson *et al.* 2003)	-
GNA33, GNA992, GNA1162, GNA1220, GNA1946, GNA2001, GNA2132	Yes (Pizza *et al.* 2000)	-
NhhA (GNA0992)	Yes (Peak *et al.* 2000)	-
GNA33	Yes (Granoff *et al.* 2001)	-
NadA (NMB1994)	Yes (Comanducci *et al.* 2002)	Yes, all in 5CVMB (Giuliani *et al.* 2006)
GNA2132 (NHBA)	Yes (Welsch *et al.* 2003)	
fHbp (GNA1870, LP2086)	Yes (Masignani *et al.* 2003; Fletcher *et al.* 2004)	
Chimeric fHbp	Yes (Beernink and Granoff 2008)	-
App	Yes (Hadi *et al.* 2001)	-
MspA (NMB1998)	Yes (Turner *et al.* 2006)	-
NMB0543(Lctp)	Yes (Sun *et al.* 2005)	-
Lactoferrin-binding proteins (LbpA and LbpB)	Yes (Pettersson *et al.* 2006)	-
Omp85	Yes (Weynants *et al.* 2007)	-
NMB0606, NMB1163, NMB0873, NMB0938, NMB0928	Yes (Pajon *et al.* 2009)	-
NMB0088	Yes (Sardinas *et al.* 2009b)	-
ZnuD (NMB0964)	Yes (Stork *et al.* 2010)	-

'-' means no available data.

1.7 Adjuvants in vaccinology

The word 'adjuvant' derives from the latin '*adjuvo*' which means 'to help'. In the 1920s, Ramon first used the word 'adjuvant' to indicate the substances used in diphtheria or tetanus toxoid vaccines to enhance the antibody production. Interestingly, those substances included bread crumbs, agar tapioca flour, starch oil or saponin (Ramon 1926; Tagliabue and Rappuoli 2008).

A good adjuvant is able to stimulate immunity without causing undesired inflammation. Several factors affect the immunogenicity of vaccine antigens. As shown in Figure 1.8, particulate antigens or antigens with a repetitive surface organization can increase phagocytosis and the ability to activate complement and recruit other molecules of the innate humoral immune system. Binding to B cell receptor (BCR) is also facilitated by the size and repetitive organization of the antigens and also pathogen-associated molecular patterns (PAMP). Subsequently, mature antigen presenting cells, such as dendritic cells can activate T cells. T cell activation can also result from the prolonged presence of antigen through depot-forming adjuvants or perhaps vaccination regimens. As mentioned in section 1.5, activated T cells are essential for producing memory B cells, the T-cell dependent immune response (Figure 1.8) (Bachmann and Jennings 2010). Through manipulating the immune response, the immunogenicity of new generation vaccines should be considerably improved. For example, introduction of Toll-like receptor (TLR) agonists or other immunostimulants binding to pathogen recognition receptors (PRRs) can increase the activation of dendritic cells (Wack and Rappuoli 2005). To date, several adjuvants have been found to be TLR agonists or immunostimulators (Table 1.2) (Rappuoli *et al.* 2011).

In our study, aluminium hydroxide ($Al(OH)_3$) and MonoPhosphoryl Lipid A (MPLA) will be used as adjuvants in protein formulations. In 1926, Alexander T. Glenny *et al.* first reported that precipitation of diphtheria toxoid onto insoluble particles of aluminium potassium sulphate (known as 'potash alum') enhanced antibody responses (Glenny *et al.* 1926; Marrack *et al.* 2009). Later, the aluminium salts were confirmed to improve the immune response (Glenny *et al.* 1931). The immune response has been shown to act mainly through a Th2-type antibody response (Brewer 2006); however, the detailed mechanism of its adjuvancity is still not completely clear. Moreover, aluminium salts are the first licensed adjuvants and have been used in humans for more than 80 years (Lindblad 2004). Three types of aluminium salts have been used: Alum ($AlK(SO_4)_2$), Alhydrogel ($Al(OH)_3$) and Adju-Phos ($Al(PO_4)_3$). In fact, Alhydrogel and Adju-Phos have almost replaced Alum due to the manufacturing batch-to-batch variability with Alum preparations (Marrack *et al.* 2009).

MPLA is a non-toxic derivative of LPS from *Salmonella enterica* serotype Minnesota Re 595. It is a potent immunopotentiator acting on TLR4 to increase the production of functional biological antibodies (Casella and Mitchell 2008). Its immunogenicity is based on inducing a Th1-type antibody response or a combination of both Th1 and Th2. In brief, a Th1-type antibody response produces IgG1 and IgG3 along with cytotoxic $CD8^+$ T cells, so it is important for anti-viral and anti-bacterial immunity. A Th2-type antibody response plays a more important role in anti-parasitic activity or for killing extracellular pathogens. Currently, there are 2 human vaccines adjuvanted with MPLA, a human papillomavirus vaccine (Cervarix®) and a hepatitis B vaccine (Fendrix®) (Perrie *et al.* 2008). It has been shown that MPLA can enhance the adjuvant effect of liposomes (Verma *et al.* 1992). Recently, a study has shown that the combination of cationic liposomes and MPLA induced an antigen-specific CD8+ T-cell specific response and a humoral response (Nordly *et al.* 2011).

To assist protein refolding to its native form, liposomes and Zwittergent micelles will be used in our study. The term 'liposome' comes from the Greek 'lipos' (fat) and 'soma' (body) first described by Bangham *et al.* (Bangham *et al.* 1965; Henriksen-Lacey *et al.* 2011). Liposomes are composed of amphipathic phospholipids resembling cell membranes and have been used extensively for drug delivery in humans (Gregoriadis 1995). Several advantages of using liposomes as drug delivery systems have been identified: liposomes 1) protect drugs from degradation; 2) increase the solubility of some drugs; 3) shield potential toxic drugs and therefore reducing toxicity in humans; 4) change the specific targeting/distribution of the drug. For example, the nephrotoxicity of an anti-fungal drug, amphotericin B, can be reduced significantly by incorporation into liposomes (AmBisome®, Astellas Pharma, Canada). A similar situation applies to the effective chemotherapeutic drug, doxorubicin, which has notorious cardiotoxicity (Henriksen-Lacey *et al.* 2011).

Moreover, liposomes have been used for carrying vaccine antigens and shown adjuvanticity (Alving 1991). In the 1990s, incorporation of a malarial fusion protein into liposomes was shown to be safe and immunogenic in humans (Fries *et al.* 1992). Owing to its structural characteristics, a wide range of antigens, including proteins, peptides or DNA can be delivered using liposomes. Protein antigens can be encapsulated in the core, inserted inside the phospholipid bilayer or surface-adsorbed. In particular, cationic liposomes can be used for surface adsorbtion of negatively charged antigens or DNA (Heurtault *et al.* 2010). Attempts have been made to improve the immunogenicity of liposomes by introducing immunopotentiators or modifying their composition (Christensen *et al.* 2009). For example, the adjuvant, AS01 (GlaxoSmithKline, GSK) is composed of liposomes, MPL and the saponin *Quillaja saponaria* (QS) 21. Recently, combination of cationic liposomes based on dimethyl

dioctadecyl ammonium bromide (DDA) and lipid extract of *Mycobacterium bovis* BCG has been shown to be a powerful Th1 adjuvant system (Rosenkrands *et al.* 2005). Therefore, both $CD4^+$ (TH1 and/or TH2) and $CD8^+$ T cells can be stimulated, depending on the incorporation of other immunopotentiators, immunogenic lipids or bacterially derived molecules. In addition, Zwittergent micelles were shown to help partial reconstitution of the PorA protein epitope and lead to improved immunogenicity (Idanpaan-Heikkila *et al.* 1996). However, Zwittergent is a detergent, not suitable for human use unless it is removed by dialysis to produce proteosomes (Lowell *et al.* 1988; Wetzler *et al.* 1992).

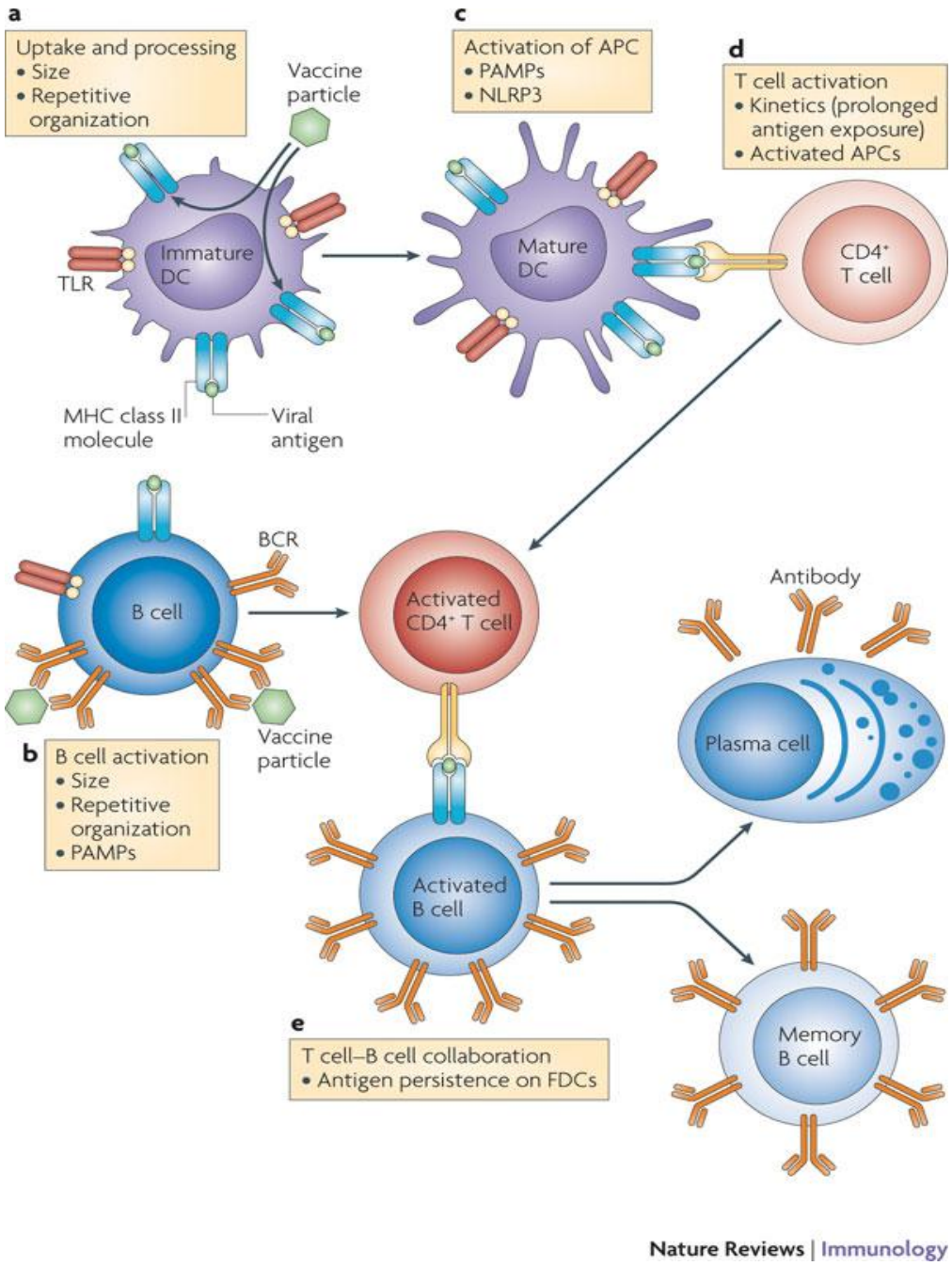

Figure 1.8 Key steps during the generation of protective immune responses (Bachmann and Jennings 2010). (used with permission)

Table 1.2 Vaccine adjuvants (adapted from *Rappuoli et al.* 2011)

Adjuvant name	**Adjuvant characteritics**	**Components, application (Company, licensed year or references)**
Licensed for human use		
Alumunium salts	Mineral salts/Depot effect	Aluminium phosphate or aluminium hydroxide ; in use since 1930s
AS04	Aluminium-absorbed TLR4 agonist	Aluminium hydroxide, MPLA ; used in hepatitis B vaccine (Fendrix) and human papilloma virus vaccine (Cervarix) (GSK; 2005);
MF59	Oil-in-water emulsion	Squalene, polysorbate 80 (Tween 80; ICI Americas), sorbitan trioleate (Span 85; Croda International) ; used in influenza vaccines (Novartis; 1997)
AS03	Oil-in-water emulsion	Squalene, Tween 80, α-tocopherol; used in influenza vaccines (GSK; 2009)
Virosomes	Liposomes	Lipids, hemagglutinin; used in seasonal influenza vaccines and also hepatitis A vaccine (Epaxal) (Berna Biotech; 2000)
Tested but not licensed for human use		
CpG 7909, CpG1018	TLR9 agonist	CpG oligonucleotides alone or combined with aluminium/emulsions
IC31	TLR9 agonist	Oligonucleotide and antimicrobial peptide $KLKL_5KLK$ (Novartis and Intercell)
Flagellin	TLR5 agonist	Bacterial flagella (Cuadros *et al.* 2004)
AS01	Liposomes	MPLA, saponin (QS21) (GSK)
AS02	Oil-in-water emulsion	MPLA, saponin (QS21) (GSK)
AF03 (adjuvant formulation 03)	Oil-in-water emulsion	Squalene, Montane 80 and Eumulgin B1 PH, used in influenza vaccines (Girard *et al.* 2011)
CAF01(cationic adjuvant formulation 01)	Liposomes	DDA (dimethyldioctadecylammonium) and TDB (trehalose dibehenate) (Christensen *et al.* 2009)
Imidazoquinolines	TLR7 and TLR8 agonists	small molecules (Hemmi *et al.* 2002)
Iscomatrix	Combination	Saponin, cholesterol and dipalmitoylphosphatidylcholine (Brown 2010)
PolyI:C(polyinosinic–polycytidylic acid)	TLR3 agonist	Double-stranded RNA analogues (Alexopoulou *et al.* 2001; Cui and Qiu 2006)
Pam3Cys (tripalmitoyl-S-glyceryl cysteine)	TLR2 agonist	Lipopeptide (Yoder *et al.* 2003)

1.8 Aims and hypotheses

In the current study, the overall aims were to investigate novel meningococcal antigens as potential vaccine candidates and their role(s) in pathogenesis. Surface exposure is believed to be a key factor for an antigen to elicit functional immunogenicity so OM proteins can therefore be potentially immunogenic. From the list of OM proteins identified in the proteomic analysis of MC58 OM (Williams *et al.* 2007), two in particular had not been investigated before and were chosen for study. The *Neisseria meningitidis*-Macrophage Infectivity Potentiator (MIP) was present in relatively high abundance in the OM and it was also predicted to be surface located using the PSORTb programme. Based on these observations, we tested the hypothesis that the MIP protein was likely to be functionally immunogenic.

The second protein, the *Neisseria meningitidis*-Adhesin Complex Protein (ACP), was chosen because bio-informatic programmes suggested that the protein was a possible adhesin. Several minor adhesins, such as NadA (Comanducci *et al.* 2002), App (Hadi *et al.* 2001), NhhA (Pizza *et al.* 2000) and MspA (Turner *et al.* 2006) have been shown to elicit bactericidal antibodies. If ACP is a meningococcal adhesin, it may also have vaccine potential. Although the predicted location of ACP using the PSORTb programme was unknown and its presence in the OM was not particularly high, we tested the hypotheses that ACP played a role during meningococcal pathogenesis and was also capable of inducing bactericidal antibodies.

1.8.1 Neisseria meningitidis-Macrophage Infectivity Potentiator (MIP)

The bacterial macrophage infectivity potentiator (MIP) proteins represent a class of bacterial pathogenicity factors. The most widely studied MIP protein to date is from *Legionella pneumophila*, the causative agent of Legionnaire's disease. The importance of the *Legionella pneumophila*-macrophage infectivity potentiator (Lp-MIP) (~24 kDa) was identified when a Lp-MIP mutant showed significantly impaired ability to infect monocytes/macrophages (Cianciotto *et al.* 1989; Wieland *et al.* 2002). Therefore, the protein was designated macrophage infectivity potentiator. Expression of this surface-located Lp-MIP (Helbig *et al.* 2001) allowed *Legionella pneumophila* to survive and multiply within monocytes and macrophages and also within protozoa (Cianciotto and Fields 1992; Wintermeyer *et al.* 1995). Moreover, sera from patients with *Legionella* infection have been shown to react with Lp-MIP protein (Bangsborg *et al.* 1991), indicating its expression during infection.

MIP homologues have also been found in other bacteria and appear to have direct relevance to the intracellular survival of important human pathogens (Hacker and Fischer 1993). For example, *Chlamydia trachomatis* and *C. psittaci* contain a protein similar to MIP (Lundemose *et al.* 1991; Rockey *et al.* 1996) that appeared to be essential for optimal intracellular infection (Lundemose *et al.* 1993). The fkpA gene of *Salmonella typhimurium*

encodes a protein similar to MIP (Horne *et al.* 1997) and the mutants showed lower levels of intracellular survival *in vitro*. A peptidyl-prolyl-*cis*-*trans*-isomerase (PPIase) homologous to MIP that was functionally involved in cell invasion has also been found in the intracellular parasite *Trypanosoma cruzi* (Moro *et al.* 1995; Pereira *et al.* 2002). The trigger factor of *Mycoplasma* also shares homology with MIP (Vogtherr *et al.* 2002) and some other intracellular pathogens including *Rickettsia*, *Ehrlichia*, *Coxiella* and *Rochalimaea* species have been shown to express homologues of MIP (Cianciotto *et al.* 1995; Mo *et al.* 1998).

MIP also shows some similarity to the immunophilin family of human FK506-binding proteins (FKBP), which are a family of conserved, widely distributed eukaryotic proteins (Schreiber 1991; Dornan *et al.* 2003). The family of FK506-binding protein has PPIase activity, which catalyses the slow *cis/trans* isomeration of prolyl peptide bonds in oligopeptides involved in the folding of proteins (Siekierka *et al.* 1990). Lp-MIP has been shown to have PPIase activity and it can be inhibited by FK506 (Fischer *et al.* 1992; Riboldi-Tunnicliffe *et al.* 2001). An intact active site of PPIase was shown to be important in enhancing the virulence of *L. pneumophila* (Helbig *et al.* 2003). However, contradictory evidence has shown that PPIase activity is not necessary for full virulence of *L. pneumophila* (Kohler *et al.* 2003).

The *Neisseria meningitidis* MIP (NMB1567, 272 aa, *Mr* ~29 kDa) has also been found in proteomic analyses of OMV vaccine prepared from the group B strain NZ98/254 (Vipond *et al.* 2006) and OMV preparations from group B meningococci (Post *et al.* 2005; Ferrari *et al.* 2006). Recently, a surface-exposed lipoprotein in *Neisseria gonorrhoeae* (272 aa, *Mr* ~28.9 kDa), Ng-MIP, was demonstrated to be highly conserved in 21 clinical gonococcal strains using western blotting (Leuzzi *et al.* 2005). To investigate if meningococcal MIP is also homologous to Ng-MIP, we did a BLAST-protein search on the NCBI website. The result (Table 1.3 and Figure 1.9) showed 5 types of MIP proteins different from MC58 but with high similarity (96-99%) and a 97-98% similarity between MIP protein of strain MC58 and 4 types of Ng-MIP. Interestingly, there is also 95% homology with MIP proteins of *Neisseria lactamica* and 42-44% homology to the Lp-MIP. Furthermore, the role of MIP located in the OM is not known, but since the Ng-MIP has been implicated to play a role in bacterial persistence within macrophages (Leuzzi *et al.* 2005), it is likely that MIP is important during interactions of meningococci with immune effector cells and potentially at other intracellular stages of infection, *e.g.* invasion of epithelial and endothelial cells.

Hypothesis 1: we will test the hypothesis that MIP, which is located in the OM of MC58 in high abundance, induces serum bactericidal antibodies and is therefore a potential vaccine candidate.

Table 1.3 BLAST protein results of MIP protein from NCBI website.

	Protein length	Identities	% similarity	Other strains with 100% similarity
Neisseria meningitidis				
MC58	272	272/272	100	Nm ATCC13091, alpha153 (aa 31-302)
Nm 053442	302	271/272	99	
Nm Z2491	272	271/272	99	
Nm α14	272	269/272	99	Nm 961-5945, G2136
Nm FAM18	268	262/272	96	Nm 8013, M6190, ES14902
Nm WUE 2594	268	262/272	96	
Nm alpha275	298	262/272	96	the same as FAM18 (aa 31-298)
Neisseria gonorrhoeae				
Ng FA1090	272	266/272	98	Ng DGI18, PID24-1, SK-93-1035, F62
Ng FA6140	272	265/272	97	Ng PID18, PID1, PID332, SK-92-679, DGI2
Ng FA19	272	263/272	97	
Ng NCCP11945	272	264/272	97	Ng 1291, 35/02, MS11
Neisseria lactamica				
N. lactamica 020-06	273	259/273	95	Nl ST-640
N. lactamica ATCC 23970	273	258/273	95	
N. lactamica Y92-1009	273	259/273	95	
Other *Neisseria*				
N. polysaccharea ATCC 43768	272	261/272	96	
N. cinerea ATCC 14685	273	253/273	93	
N. flavescens NRL30031/H210	271	213/271	79	
N. mucosa C102	272	212/272	78	
N. subflava NJ9703	271	212/271	78	
N. flavescens SK114	271	209/271	77	

```
MC58        1   MNTIFKISALTLSAALALSACGKKEAAPASASEPAAASSAQGDTSSIGSTMQQASYAMGV   60
Nm 053442  31   MNTIFKISALTLSAALALSACGKKEAAPASASEPAAASSAQDDTSSIGSTMQQASYAMGV   90
Nm Z2491    1   MNTIFKISALTLSAALALSACGKKEAAPASASEPAAASSAQGDTSSIGSTMQQASYAMGV   60
Nm α14      1   MNTIFKISALTLSAALALSACGKKEAAPASASEPAAASSAQGDTSSIGSTMQQASYAMGV   60
FAM18       1   MNTIFKISALTLSAALALSACGKKEAASEPAA----ASAAQGDTSSIGSTMQQASYAMGV   56
WUE2594     1   MNTIFKISALTLSAALALSACGKKEAASEPAA----ASAAQGDTSSIGSTMQQASYAMGV   56
Ng FA1090   1   MNTIFKISALTLSAALALSACGKKEAAPASASEPAAASAAQGDTSSIGSTMQQASYAMGV   60
Ng FA6140   1   MNTIFKISALTLSAALALSACGKKEAAPASASEPAAASAAQGDTSSIGGTMQQASYAMGV   60
Ng FA19     1   MNTIFKISALTLSAALALSACGKKEAAPAPASEPAAASAAQGDTFSIGGTMQQASYAMGV   60

MC58       61   DIGRSLKQMKEQGAEIDLKVFTEAMQAVYDGKEIKMTEEQAQEVMMKFLQEQQAKAVEKH  120
Nm 053442  91   DIGRSLKQMKEQGAEIDLKVFTEAMQAVYDGKEIKMTEEQAQEVMMKFLQEQQAKAVEKH  150
Nm Z2491   61   DIGRSLKQMKEQGAEIDLKVFTEAMQAVYDGKEIKMTEEQAQEVMMKFLQEQQAKAVEKH  120
Nm α14     61   DIGRSLKQMKEQGAEIDLKVFTEAMQAVYDGKEIKMTEEQAQEVMMKFLQEQQAKAVEKH  120
FAM18      57   DIGRSLKQMKEQGAEIDLKVFTEAMQAVYDGKEIKMTEEQAQEVMMKFLQEQQAKAVEKH  116
WUE2594    57   DIGRSLKQMKEQGAEIDLKVFTEAMQAVYDGKEIKMTEEQAQEVMMKFLQEQQAKAVEKH  116
Ng FA1090  61   DIGRSLKQMKEQGAEIDLKVFTDAMQAVYDGKEIKMTEEQAQEVMMKFLQEQQAKAVEKH  120
Ng FA6140  61   DIGRSLKQMKEQGAEIDLKVFTDAMQAVYDGKEIKMTEEQAQEVMMKFLQEQQAKAVEKH  120
Ng FA19    61   DIGRSLKQMKEQGAEIDLKVFTDAMQAVYDGKEIKMTEEQAQEVMMKFLQEQQAKAVEKH  120

MC58      121   KADAKANKEKGEAFLKENAAKDGVKTTASGLQYKITKQGEGKQPTKDDIVTVEYEGRLID  180
Nm 053442 151   KADAKANKEKGEAFLKENAAKDGVKTTASGLQYKITKQGEGKQPTKDDIVTVEYEGRLID  210
Nm Z2491  121   KADAKANKEKGEAFLKENAAKDGVKTTASGLQYKITKQGEGKQPTKDDIVTVEYEGRLID  180
Nm α14    121   KADAKANKEKGEAFLKENAGKESVKTTASGLQYKITKQGEGKQPTKDDIVTVEYEGRLID  180
FAM18     117   KADAKANKEKGEAFLKENAAKDGVKTTASGLQYKITKQGEGKQPSKDDIVTVEYEGRLID  176
WUE2594   117   KAEAKANKEKGEAFLKENAAKDGVKTTASGLQYKITKQGEGKQPTKDDIVTVEYEGRLID  176
Ng FA1090 121   KADAKANKEKGEAFLKENAAKDGVKTTASGLQYKITKQGEGKQPTKDDIVTVEYEGRLID  180
Ng FA6140 121   KADAKANKEKGEAFLKENAAKDGVKTTASGLQYKITKQGEGKQPTKDDIVTVEYEGRLID  180
Ng FA19   121   KADAKANKEKGEAFLKENAAKDGVKTTASGLQYKITKQGEGKQPTKDDIVTVEYEGRLID  180

MC58      181   GTVFDSSKANGGPVTFPLSQVIPGWTEGVQLLKEGGEATFYIPSNLAYREQGAGDKIGPN  240
Nm 053442 211   GTVFDSSKANGGPVTFPLSQVIPGWTEGVQLLKEGGEATFYIPSNLAYREQGAGDKIGPN  270
Nm Z2491  181   GTVFDSSKANGGPVTFPLSQVILGWTEGVQLLKEGGEATFYIPSNLAYREQGAGDKIGPN  240
Nm α14    181   GTVFDSSKANGGPVTFPLSQVIPGWTEGVQLLKEGGEATFYIPSNLAYREQGAGDKIGPN  240
FAM18     177   GTVFDSSKANGGPVTFPLSQVIPGWTEGVQLLKEGGEATFYIPSNLAYREQGAGDKIGPN  236
WUE2594   177   GTVFDSSKANGGPVTFPLSQVIPGWTEGVQLLKEGGEATFYIPSNLAYREQGAGDKIGPN  236
Ng FA1090 181   GTVFDSSKANGGPATFPLSQVIPGWTEGVRLLKEGGEATFYIPSNLAYREQGAGEKIGPN  240
Ng FA6140 181   GTVFDSSKANGGPATFPLSQVIPGWTEGVRLLKEGGEATFYIPSNLAYREQGAGEKIGPN  240
Ng FA19   181   GTVFDSSKANGGPATFPLSQVIPGWTEGVRLLKEGGEATFYIPSNLAYREQGAGEKIGPN  240

MC58      241   ATLVFDVKLVKIGAPENAPAKQPAQVDIKKVN  272
Nm 053442 271   ATLVFDVKLVKIGAPENAPAKQPAQVDIKKVN  302
Nm Z2491  241   ATLVFDVKLVKIGAPENAPAKQPAQVDIKKVN  272
Nm α14    241   ATLVFDVKLVKIGAPENAPAKQPAQVDIKKVN  272
FAM18     237   ATLVFDVKLVKIGAPENAPAKQPAQVDIKKVN  268
WUE2594   237   ATLVFDVKLVKIGAPENAPAKQPAQVDIKKVN  268
Ng FA1090 241   ATLVFDVKLVKIGAPENAPAKQPDQVDIKKVN  272
Ng FA6140 241   ATLVFDVKLVKIGAPENAPAKQPDQVDIKKVN  272
Ng FA19   241   ATLVFDVKLVKIGAPENAPAKQPDQVDIKKVN  272
```

Figure 1.9 BLAST result- MIP amino acid sequence alignment of MC58, 5 *N. meningitidis* (Nm) (96-99% similar to MC58) and 3 *N. gonorrhoeae* (Ng) (97-98% similar to MC58) strains. The colours and grey shadings denote the amino acid differences.

1.8.2 Neisseria meningitidis-Adhesin Complex Protein (ACP)

The *Neisseria meningitidis* -Adhesin Complex Protein (ACP), NMB2095 (124 amino acids, 13.3 kDa) has been found in other proteomic studies of OMV (Post *et al.* 2005; Ferrari *et al.* 2006; Gil *et al.* 2009) though not found in OMV of vaccines prepared from the New Zealand MenB strain (Vipond *et al.* 2006). Microarray analysis showed that under iron-depleted conditions, the *acp* gene was reported to be up-regulated (Grifantini *et al.* 2003). This protein has been found as one of the 5 most abundant proteins in MC58ΔGNA33 mutant although it was not so dominant in the MC58 wild type lysate (du-Bobie *et al.* 2004). A similar finding was noted in NMB0573 knockout mutant (Ren *et al.* 2007). The up-regulation of *acp* gene either in certain mutants or under nutrient limiting conditions suggests the importance of this protein for cellular survival.

There is no information regarding the role of ACP in meningococcal pathogenesis or whether it can be considered as a candidate vaccine antigen. The BLAST-protein result from the NCBI website showed that this protein is highly conserved amongst not only *Neisseria meningitidis* strains but also in *Neisseria lactamica* (Figure 1.10). In order to investigate the functions of this protein we will test the following hypothesis:

Hypothesis 2: we will test the hypothesis that ACP, possibly located in the OM of MC58 in high abundance, induces serum bactericidal antibodies and is therefore a potential vaccine candidate.

In order to test *Hypothesis 1 and 2*, we will clone the genes encoding the MIP protein and ACP, express each protein in a heterologous host, and purify the recombinant proteins to high purity and yield. We will prepare the recombinant proteins in different immunization formulae, including a variety of delivery vehicles, such as liposomes or ZW 3-14 micelles and adjuvants, including aluminium hydroxide ($Al(OH)_3$) and MPLA. Finally, the immunogenicity of the recombinant proteins will be assessed using a series of assays for biological and functional activity.

Hypothesis 3: we will test the hypothesis that ACP is an adhesin that plays a role in meningococcal attachment to human cells and subsequent invasion.

To test this hypothesis, we will generate an ACP knockout mutant from the wild-type MC58 strain. Furthermore, an ACP mutant will be prepared in an acapsulate MC58 strain without expression of the major adhesins Opa, Opc and pilin. The ability of wild-type and mutant bacteria to adhere to human cells and subsequently invade will be investigated using *in vitro* infection assays.

```
MC58          1 MKLLTTAILSSAIALSSMAAAAGTDNPTVAKKTVSYVCQQGKKVKVTYGFNKQGLTTYAS  60
Nm8013        1 MKLLTTAILSSAIALSSMAAAAGTNNPTVAKKTVSYVCQQGKKVKVTYGFNKQGLTTYAS  60
Ng FA1090    63 MKLLTTAILSSAIALSSMAAAAGTNNPTVAKKTVSYVCQQGKKVKVTYGFNKQGLTTYAS  122
Ng FA19       1 MKLLTTAILSSAIALSSMAAA-GTDNPTVAKKTVSYVCQQGKKVKVTYGFNKQGLTTYAS  59
Nc14685       1 MKLLTTVILSSALALSGMAAAAGTSNPTVAKKTVSYVCQQGKKVKVTYGFNKQGLTTYAS  60

MC58         61 AVINGKRVQMPVNLDKSDNVETFYGKEGGYVLGTGVMDGKSYRKQPIMITAPDNQIVFKD  120
Nm8013       61 AVINGKRVQMPVNLDKSDNVETFYGKEGGYVLGTGVMDGKSYRKQPIMITAPDNQIVFKD  120
Ng FA1090   123 AVINGKRVQMPINLDKSDNMDTFYGKEGGYVLSTGAMDSKSYRKQPIMITAPDNQIVFKD  182
Ng FA19      60 AVINGKRVQMPINLDKSDNMDTFYGKEGGYVLSTGAMDSKSYRKQPIMITAPDNQIVFKD  119
Nc 14685     61 AAIKGKQVQMPINLDKSDNMDTFYGKEGGYVLSTGAMDSKSYRKQPIMITAPDNQIVFKD  120

MC58        121 CSPR  124
Nm8013      121 CSPR  124
Ng FA1090   183 CSPR  186
Ng FA19     120 CSPR  123
Nc 14685    121 CSPR  124
```

Figure 1.10 BLAST result- ACP amino acid sequence alignment with MC58. MC58 shares the same amino acid sequence type with *N. meningitidis* H44/76, CU385, 053442 (serogroup C), *N. lactamica* ATCC 23970, Y92-1009, ST-640, 020-06 and *N. polysaccharea* ATCC 43768. *N.meningitidis* 8013 (Nm 8013) shares the same aa sequence type with *N. meningitidis* ATCC 13091, alpha153, alpha275, alpha710, WUE 2594, N1568, OX99.30304, M6190, M13399, M0579, ES14902, 961-5945, M01-240013, G2136, M01-240149, M01-240355, M04-240196 and NZ-05/33 with only 1 amino acid difference (99% similarity). The same amino acid sequence is also present in *N. meningitidis* FAM18, alpha14 and Z2491 but from amino acid number 63 to 186 instead of 1 to 124. *N. gonorrhoeae* FA 1090 (Ng FA19) has 95% (117/124) similarity with MC58. *N. gonorrhoeae* FA19 shares the same amino acid sequence with (117/124, 95% similarity) *N. gonorrhoeae* 35/02, DGI18, FA6140, MS11, PID18, PID1, PID24-1, PID332, SK-92-679, SK-93-1035, DGI2. Nc 14685 stands for *N. cinerea* ATCC 14685 (111/124, 90% similarity). The colours and grey shadings denote the amino acid differences.

Chapter 2 Materials and Methods

2.1 Bacteria, plasmids and growth conditions

Neisseria meningitidis strain MC58 (B:15:P1.7,16b: $Cap^+Opa^+Opc^+$ $PorA^+PorB^+Pil^+$[type IV; Class I] Rmp^+LPS^+) was isolated from an outbreak of meningococcal infections that occurred in Stroud, Gloucestershire in the mid-1980's (McGuinness *et al.* 1991). Meningococci were grown on supplemented proteose-peptone agar (GC-agar; Appendix I) incubated at 37°C in an atmosphere containing 5% (v/v) CO_2. Twelve other strains with different phenotypic characteristics, including different serogroups (polysaccharide), serotypes (PorB) and serosubtypes (PorA), isolated either from patients or carriers, were selected for investigating whether MIP and ACP are highly conserved in meningococci (Table 2.1). Meningococci were stored in proteose peptone liquid medium containing 30% (v/v) glycerol (Fisher, UK) in liquid nitrogen.

Table 2.1 Characteristics of meningococcal strains used in this study.

Sequenced strains	Phenotypic characteristics (serogroup:serotype:serosubtype)	Reference
serogroup **C**		
MENC11	C:16,P17a,1	(Williams *et al.* 2003)
MC173	C:2b:P1.5,2	(Jordens *et al.* 2004)
MC161	C:2-37,P1.5-1,10-4	(Humphries *et al.* 2006)
MC162	C:2-36:P1.5,2	(Williams *et al.* 2003)
Serogroup **B**		
MC168	B:4:P1.5,2	(Jordens *et al.* 2004)
MC172	B:1:P1.22,14	(Jordens *et al.* 2004)
MC179	B:1:P1.19-2,13-1	(Williams *et al.* 2009)
MC180	B:1:P1.22,14	(Williams *et al.* 2009)
MC54	B:2b:P1.18-1,3	(Humphries *et al.* 2006)
MC90	B:9:P1.5,2	(Humphries *et al.* 2006)
L2470	B:4:P1.7-2,4	(Humphries *et al.* 2006)
Serogroup **29E**		
MC174	29E:4:P1.5-1,10-8	(Jordens *et al.* 2004)

The mutant strain MC58¢18 was kindly provided by Professor Mumtaz Virji (School of Cellular and Molecular Medicine, University of Bristol, Medical Sciences Building, Bristol, UK). This derivative of MC58 was initially selected using single colony isolation (McNeil *et al.* 1997).

This phase variant was pilE$^-$, Opa$^-$, Opc$^-$, PilC$^+$ and SM82 non-reactive (L8 LOS). An erythromycin antibiotic cassette was inserted for mutagenesis of sialyl transferase and therefore the mutant was also acapsulate. In addition, the Cap$^-$ mutant was selected using erythromycin (5 µg/ml).

E. coli strain DH5α and BL21(DE3)pLysS (Invitrogen, UK) were stored in 30% (v/v) glycerol at -80°C. The pRSETA plasmid (Invitrogen, UK), maintained in *E. coli* DH5α, was used for cloning genes encoding meningococcal proteins. *E. coli* BL21(DE3)pLysS was transformed by recombinant pRSETA for protein expression. Both Luria-Bertani (LB) broth and agar were used for growing *E. coli* (Appendix II). For protein expression, transformed BL21(DE3)pLysS bacteria were cultured on SOB medium (Appendix III) supplemented with the antibiotics ampicillin (50 µg/ml; Sigma-Aldrich, UK) and chloramphenicol (30 µg/ml; Sigma-Aldrich, UK). Both antibiotic stocks were sterilized through a 0.22 µm filter (Millipore, Ireland) and stored at -20°C.

2.2 Competent *E.coli* DH5α and BL21(DE3)pLysS bacteria

LB broth (10 ml) was inoculated with a single colony of *E.coli* grown on LB agar overnight and incubated for 18 h at 37°C with shaking (200 rpm; Gallenkamp, UK). From this culture, 1 ml was added to 25 ml of fresh LB broth and this was cultured at 37°C with shaking until an optical density (OD) at λ 550 nm 0.4-0.5 was reached. Optical density was measured in a HITACHE U-1100 Spectrophotometer. The culture was chilled on ice for 20 min and a 20 ml volume was centrifuged (1500*g*) for 10 min at 4°C. The pellet was then suspended in 10 ml of ice cold 0.1 M $MgCl_2$ (Fisher, UK) and centrifugation repeated. The washed pellet was then suspended in 1 ml of ice cold 0.1 M $CaCl_2$ (Fisher, UK) and kept on ice for at least 1 h. The competent cells were then stored at -80°C in 0.1 M $CaCl_2$ containing 30% (v/v) glycerol.

2.3 Transformation of pRSETA plasmid into competent *E. coli* cells

The pRSETA plasmid (10 ng) was added to 100 µl of competent *E. coli* DH5α cells and left for 30 min on ice. The bacteria were then heat-shocked by placing at 42°C in a water bath for 45 seconds and left once more on ice for 5 min. Next, LB broth (500 µl) was added and the suspension incubated for 1 h at 37°C in a water bath to allow the ampicillin-resistance cassette to express its resistance marker. The suspension was then centrifuged for 1 min (2,500*g*) and 500 µl of the supernantant solution was removed and the pellet was suspended in the remaining volume. This was then brought to room temperature and a 100 µl volume plated onto a single ampicillin-LB agar selective medium plate that was later incubated overnight at 37°C.

2.4 Cloning of *mip* and *acp* genes

The gene sequences encoding both MIP (NMB1567) and ACP (NMB2095) proteins were accessed from the NCBI website (http://www.ncbi.nlm.nih.gov/). The Seqbuilder (Lasergene, DNASTAR) program was used to identify restriction enzyme sites absent from each gene sequence, leading to the choice of *XhoI* and *HindIII* enzymes for cloning experiments.

2.4.1 Preparation of insert

I. Extraction of genomic DNA from strain MC58 as PCR template

Genomic DNA of strain MC58 was extracted by alkaline lysis as described previously (Christodoulides *et al.* 2001). Briefly, a single colony of MC58 was suspended in 10 µl of ultra-high quality (UHQ) water (Triple Red, UK). Next, 10 µl of 0.25 M potassium hydroxide solution (BDH, UK) was added and the sample boiled for 5 min. To neutralize the preparation prior to polymerase chain reaction (PCR), 10 µl of 0.5 M Tris-HCl buffer (pH 7.5) (Fisher, UK) was added. The final volume was brought to 130 µl with the addition of UHQ water. Extracted genomic DNA was stored at -20°C until used.

II. Primer design

The primers used for cloning both genes are shown in Table 2.2. The 5' terminal sequence *ggctat* in each primer is the overhang and sequences **ctcgag** and **aagctt** represent the restriction sites for *XhoI* and *HindIII* enzymes respectively. For each forward primer, the 20 nucleotide bases following the enzyme restriction site represents the first 20 bases of the target DNA sequence and in the reverse primers the sequences represent the reverse complementary of the last 20 bases. These primers were synthesized commercially by Eurogentec (UK).

Table 2.2 Primers used for cloning *mip* and *acp* genes.

***mip* gene**	
Forward primer (NMB1567F)	5'-*ggctat***ctcgag**atgaacaccattttcaaaat-3'
Reverse primer (NMB1567R)	5'-*ggctat***aagctt**ctattaatttacttttttgatgt-3'
***acp* gene**	
Forward primer (NMB2095F)	5'-*ggctat***ctcgag**atgaaacttctgaccaccgc-3'
Reverse primer (NMB2095R)	5'-*ggctat***aagctt**ctattaacgtggggaacagtctt-3'

III. Amplification of target DNA sequences from MC58 genomic DNA

The 2X Phusion™ PCR master mix (Finnzymes, UK), which contains a proof-reading enzyme (Phusion DNA polymerase), was used to amplify target DNA sequences from MC58 genomic DNA (Table 2.3). The PCR conditions (Table 2.4) were carried out in a Biometra T3 thermocycler (Germany).

Table 2.3 PCR reactions (50 µl) for amplifying insert DNA sequences.

	Volume (µl)	**Final concentration**
2X Phusion™ PCR master mix	25	1X
Forward primer (10 µM)	2.5	0.5 µM
Reverse primer (10 µM)	2.5	0.5 µM
UHQ water	19	-
Template DNA	1	-

Table 2.4 PCR conditions for amplification of *mip* and *acp* genes.

	Initial denaturation	**Denaturation**	**Annealing**	**Extension**	**Final extension**	**Cycles**
mip	98°C, 30 s	98°C, 10 s	60°C, 30 s	72°C, 25 s	72°C, 5 min	30
acp	98°C, 30 s	98°C, 10 s	65°C, 30 s	72°C, 10 s	72°C, 5 min	30

IV. Agarose gel electrophoresis

Materials:

i) **Tris acetate EDTA (TAE) buffer**: 1X TAE buffer contains 40 mM Tris (Fisher, UK) and 1 mM EDTA (Fisher, UK). Glacial acetic acid (Fisher, UK) was used for adjusting pH to 8.0.

ii) **Agarose** (Fisher, UK)

iii) **Hyperladder I** (Bioline, UK)

iv) **5X DNA loading buffer** (Bioline, UK)

The concentration of agarose used in gels ranged from 1-1.5% (w/v). The voltage of power supply was set at 70 V for a small gel (size: 83 mm x 60 mm). After ~1 h, the gel was stained with 0.5 µg/ml of ethidium bromide (EtBr, Fisher, UK) in 1X TAE buffer for 15 min. Next, the gel was photographed using a transilluminator (BioDoc-It™ imaging system, Upland, USA).

V. Gel Purification of PCR product

The PCR product was mixed with 5X DNA loading buffer and Sybr Green (10:1; v/v) and subjected to agarose gel electrophoresis. After ~1 h, the visible apple-green bands were cut

carefully by a clean scalpel on a long wave-length UV (360 nm) light box. Next, Wizard® SV gel and PCR clean-up system (Promega, UK) was used to purify the PCR product. In addition, EtBr was later replaced by Sybr Safe (Invitrogen, UK). The DNA bands could be visualised under UV light by addition of Sybr Safe in agarose-TAE solution (1:20,000 dilution) during agarose gel preparation.

VI. Wizard® SV gel and PCR clean-up system

Materials: buffers provided in the kit include

i) **Membrane binding solution**: 4.5 M guanidine isothiocyanate and 0.5 M potassium acetate (pH 5.0)

ii) **Membrane wash solution**: 10 mM potassium acetate (pH 5.0), 80% (v/v) ethanol and 16.7 µM EDTA (pH 8.0).

Wizard® SV gel and PCR clean-up systems (Promega, UK) are based on the affinity between DNA and the silica membrane of the mini-column in the presence of chaotropic salts. The procedure was performed as described by the manufacturer's instructions.

Every 10 µg of excised gel (Section 2.4.1 V) was dissolved in 10 µl of membrane binding solution heated in a water bath at 56-65°C. When this kit was used for a PCR product, an equal amount of membrane binding solution was added. The mixture was vortex-mixed every 2-3 minutes until the gel pieces were dissolved thoroughly. The mixture was then transferred to a mini-column inserted into a collecting tube and left at room temperature for 1 min and then centrifuged at 16,000*g* (Eppendorf Centrifuge 5417C, UK) for 1 min. Next, the fluid in the collecting tube was discarded. Membrane wash solution (700 µl) was added into the mini-column which was re-inserted into the collecting tube and the centrifugation repeated. Another 500 µl of membrane wash solution was used to wash the mini-column followed by centrifugation at the same speed for another 5 min. The collecting tube was discarded carefully to avoid carrying over any residual ethanol left on the mini-column. Next, the mini-column was inserted into a clean 1.5 ml eppendorf tube, 50 µl of nuclease-free water was added into the mini-column and the sample was left at room temperature for 1 min. Finally, centrifugation at 16,000*g* was repeated for 1 min to elute the DNA bound to the mini-column. The eluted DNA was stored at -20°C until used and 5 µl of sample was applied to an agarose gel to check both the concentration and size of the purified DNA.

VII. Digestion of the PCR product by restriction enzymes

The remaining 45 µl of clean-up PCR product was mixed with 1.5 µl (10 U/µl) of *XhoI* enzyme (Promega, UK), 1.5 µl (10 U/µl) of *HindIII* enzyme (Promega, UK), 6 µl of 10X bovine

serum albumin (BSA) (Promega, UK) and 6 µl of 10X buffer B (Promega, UK) and incubated at 37°C for 3 h. The PCR clean-up system was carried out before the concentration of DNA was quantified using agarose gel electrophoresis.

VIII. Determination of DNA concentration

The DNA concentration was determined either by comparing with a standard DNA ladder, such as Hyperladder I (Bioline, UK) on agarose gel electrophoresis or more accurately, by reading optical density (O.D.) at a UV wavelength of 260 nm. The O.D. λ 260nm =1 is equivalent to 50 ng/µl of double-stranded DNA or 40 ng/µl of single-stranded DNA.

2.4.2 Preparation of vector

The key features of pRSETA vector system include the T7 promoter gene (base 20-39), an ATG translation initiation codon, a polyhistidine tag (base 112-219), the multiple cloning sites (MCS, bases 202-248), the enterokinase cleavage recognition sequence and the ampicillin resistance gene (base 1042-1902) (Figure 2.1).

Lambda lysogen of *E.coli* BL21(DE3)pLysS contains T7 RNA polymerase gene, whose expression can be induced by the addition of isopropyl β-D-thiogalactopyranoside (IPTG). T7 RNA polymerase then binds with the ribosome binding site of recombinant pRSETA and hence induces gene expression downstream until the stop codon following the insert gene.

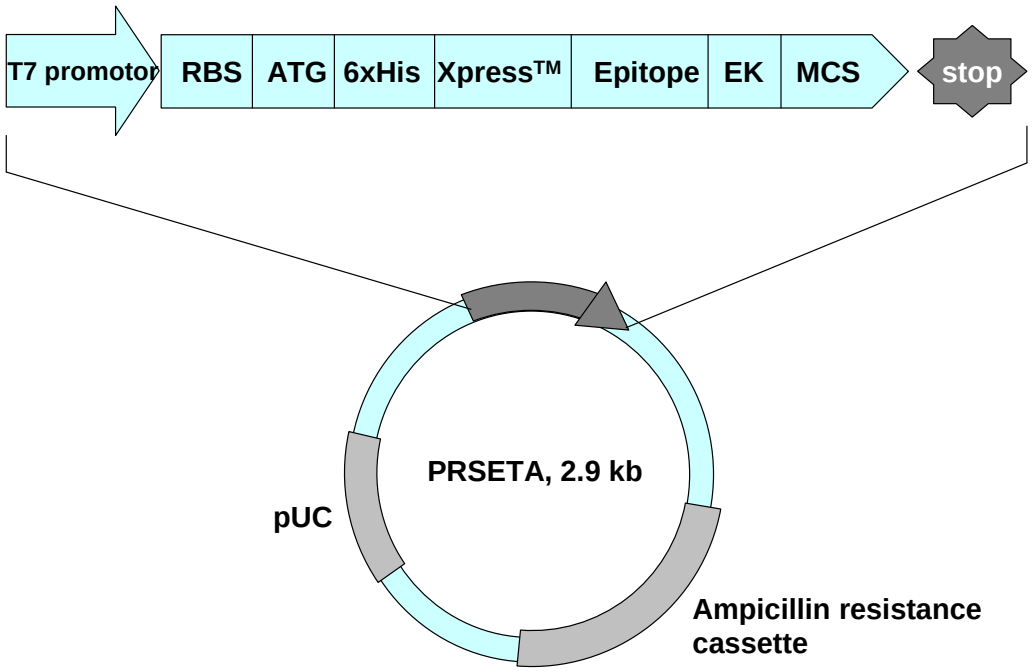

Figure 2.1 The features of pRSETA plasmid.

I. Purification of pRSETA from E. coli

To purify the pRSETA from overnight cultures of *E.coli* DH5α-pPRSETA, the Wizard® Plus SV Minipreps DNA purification system (Promega, UK) was used as described by the manufacturer.

Materials: buffers provided in the kit include:

i) **Cell resuspension solution**: 50 mM Tris-HCl (pH 7.5); 10 mM EDTA; 100 µg/ml RNase A.

ii) **Cell lysis solution**: 0.1 M NaOH; 1% (w/v) SDS

iii) **Alkaline protease**

iv) **Neutralization solution** (Final pH is approximately 4.2): 4.09 M guanidine hydrochloride; 0.759 M potassium acetate and 2.12 M glacial acetic acid.

v) **Column wash solution**: 60% (v/v) ethanol, 60 mM potassium acetate; 8.3 mM Tris-HCl (pH 7.5) and 0.04 mM EDTA (pH 8.0).

A 1-5 ml overnight culture of DH5α-pRSETA was centrifuged at 2,200*g* for 5 min. The pellet was suspended in cell resuspension solution (250 µl), followed by addition of cell lysis solution (250 µl) and alkaline protease (10 µl). Next, the mixture was inverted 6 times and incubated at room temperature for 5 min. Neutralization solution (350 µl) was added and the mixture inverted 4 times. Next, the mixture was centrifuged at 20,000*g* for 10 min and the bacterial lysate decanted into a spin column, which was inserted into a collection tube. After centrifugation at the same speed for 1 min, the flow-through was discarded and the spin column re-inserted into the collection tube. Next, wash buffer (750 µl) was added, the tube was centrifuged at 20,000*g* for 1 min and the flow-through discarded. The wash step was repeated with the addition of 250 µl of wash buffer and centrifugation at 20,000*g* for 10 min. The spin column was then inserted into a clean 1.5 ml microcentrifuge tube. Nuclease-free water (50-100 µl) was added into the column, incubated for 1 min and centrifuged at 20,000*g* for another 1 min. A 2-5 µl sample of DNA elution was used for 1% (w/v) agarose gel electrophoresis.

II. Digestion and dephosphorylation

The digestion reaction contained pRSETA (1.5 µg), 10X buffer B (5 µl), 10X bovine serum albumin (BSA, 5 µl), 1 µl (10 U) of both *XhoI* and *HindIII* enzymes along with UHQ water to make up a final volume of 50 µl. The digestion enzymes were added last. The reaction was carried out by incubating in a 37°C water bath for 3 h.

To prevent recircularization and religation of linearized cloning vehicle DNA, calf intestinal alkaline phosphatase (CIAP) (Promega, UK) was used to remove phosphate groups

from both 5'-ends. Calf intestinal alkaline phosphatase (8 µl, 1 U/µl), 10X CIAP buffer (8 µl), 10X BSA (8 µl) and UHQ water (6 µl) were added into the aforementioned 50 µl reaction. The mixture was incubated at 37°C in a water bath for 30 min and then 56°C for 15 min.

Next, the Wizard® SV gel and PCR clean-up system was used and the concentration of vector was determined by agarose gel electrophoresis and spectrophotometer (section 2.4.1 VI and VII).

2.4.3 Ligation of insert and vector

For 10 ng of vector DNA, the desired amount of insert for the ligation reaction was calculated using the following formula. The molar ratio of insert/vector used was 3/1.

$$\frac{\text{10 ng of vector x Size of insert (kb)}}{\text{Size of vector (kb)}} \text{ x3 = ng of insert}$$

A 10 µl ligation reaction contained 10 ng of vector, the desired amount of insert, 1 µl (=3 U) of T4 DNA ligase (Promega, UK) and 5 µl of 2X rapid ligase buffer (Promega, UK). The mixture was incubated at room temperature for 15 min and ready for transformation later.

2.4.4 Transformation of recombinant plasmids into DH5α competent cells.

The whole ligation reaction was used for transformation into DH5α competent cells (section 2.3). One LB-ampicillin plate containing the same ligation reaction without insert (10 ng vector without insert) was included as a negative control. Those colonies which uptake either recombinant plasmids or recirculized pRSETA would grow on the selective plates. Colonies with recombinant plasmids were screened by PCR.

2.4.5 Screening of colonies with recombinant plasmids by PCR

Six colonies were randomly selected from the transformed plate and grown separately on an ampicillin-containing LB agar plate overnight. To prepare the DNA templates for PCR screening, a colony of the bacteria was added into 0.25 M KOH (10 µl) along with UHQ water (10 µl) and then boiled for 5 min. Next, 0.25 M HCl (10 µl) and UHQ water (70 µl) were added. Table 2.5 and 2.6 show the PCR reactions and PCR conditions respectively.

2.4.6 Sequencing of inserts

Plasmids from PCR-confirmed transformants were purified using Miniprep columns and the sequencing was carried out commercially (Geneservice, Oxford, UK) using their stock primers, T7 forward primer (T7F) and T7 reverse primer (T7R). For *mip* insert gene, T7F and T7R were used whereas only T7R was used for sequencing the *acp* insert gene. The results were analysed by Seqman (Lasergene, DNASTAR).

Table 2.5 PCR reactions (25 µl) for screening colonies with recombinant plasmids.

	Volume (µl)	Final concentration
GoTaq® Green Master Mix (Promega, UK)	12.5	1X
Forward primer (10 µM)	2.5	1 µM
Reverse primer (10 µM)	2.5	1 µM
UHQ	6.5	-
Template DNA	1	-

Table 2.6 PCR conditions for screening colonies with recombinant plasmids.

	Initial denaturation	Denaturation	Annealing	Extension	Final extension	Cycles
mip	95°C, 2 min	95°C, 30 s	52°C, 30 s	72°C, 60 s	72°C, 5 min	30
acp	95°C, 2 min	95°C, 30 s	53°C, 30 s	72°C, 30 s	72°C, 5 min	30

2.5 Expression of recombinant proteins

The recombinant plasmids identified from the sequencing reactions with the correct insert sequence were then transformed into competent *E.coli* BL21(DE3)pLysS (Section 2.3). A colony of transformed bacteria was inoculated in 2-3 ml of SOB-ampicillin (50 µg/ml) – chloramphenicol (30 µg/ml) liquid medium and grown overnight at 37°C with shaking (200 rpm). On the next day, the overnight culture (1 ml) was inoculated into fresh SOB medium (25 ml) without antibiotics and the bacteria were grown until the O.D. λ 600 nm reached 0.4-0.6. Protein expression was induced by the addition of IPTG (Fisher, UK) to the final concentration of 1 mM. At every hour after addition of IPTG for up to 6 h, samples of the culture (1 ml) were removed and centrifuged at 15,000*g* for 1 min and the pellets were suspended in 100 µl of phosphate buffered saline (PBS) containing 1 mM phenylmethylsulphonyl fluoride (PMSF; Sigma-Aldrich, UK). As a negative control, the same strain was grown and sampled without IPTG induction. Subsequently, all collected samples were frozen and thawed 3 times to obtain

whole bacterial lysate. The optimal expression time, defined as the time point at which maximal protein expression was induced, was determined by SDS-PAGE analysis (Section 2.9).

In order to determine if the expressed protein was present as an insoluble protein in the cellular debris or soluble in the supernatant fluid of the lysate, the supernatant fluid and a suspension of the cellular debris were analysed by SDS-PAGE.

2.6 Purification of recombinant proteins

The purification procedure followed the protocol described in the QIAexpressionist system manual (Qiagen, UK). The components of the lysis, wash and elution buffers are shown in Table 2.7.

2.6.1 Pilot growth and protein induction

E.coli BL21(DE3)pLysS containing the recombinant plasmid was grown overnight in 10 ml of SOB medium containing ampicillin (50 μg/ml) and chloramphenicol (30 μg/ml) at 37°C with shaking (200 rpm). From this overnight culture, 5 ml were removed and inoculated into 100 ml of SOB without antibiotics and this culture grown until the O.D. λ 600 nm reached ~0.6. A 1 ml sample was removed immediately before addition of IPTG, centrifuged at 15,000*g* for 1 min and the pellet was stored at -80°C as the un-induced control (time =0 h). IPTG was added to a final concentration of 1 mM and the bacteria were cultured until the optimal expression time, at which point another 1 ml sample was removed and processed in the same way as the un-induced control. The total bacterial culture was centrifuged at 2,500*g* (Boeco centrifuge U-32R, Germany) at 4°C for 20 min and the pellet was stored at -80°C before use.

2.6.2 Pilot purification under native conditions

I. Preparation of the lysate under native conditons

The pellet obtained in section 2.6.1 was thawed on ice for 10-15 min before being suspended in native lysis buffer (4-5 ml per g pellet) (Table 2.7). The suspension was frozen and thawed three times and the resulting slurry was sonicated (MSE Soniprep 150 sonicator) on ice, using 15 µm for 10-20 bursts of 10-15 s each. The sonicated sample was then centrifuged (10,000*g*; Biofuge 13, Heraeus Sepatech) for 30 min at 4°C. Next, the supernatant fluid, which contained the majority of the recombinant protein, was removed for further purification and a sample (labelled SN) was kept for SDS-PAGE. The cellular debris was suspended in an equal volume of lysis buffer (labelled CD).

II. Protein purification under native conditions

Protein purification was done with metal-affinity chromatography using nickel-nitrilotriacetic acid (Ni-NTA) to bind the 6xHis-tagged protein. In a pilot experiment, 4 ml of the supernatant and 1 ml of 50% (w/v) Ni-NTA resin (Qiagen, UK) were mixed on an angled rotary mixer at 4°C for 1 h. The mixture was loaded into a small column (3.4 mm in diameter and 55 mm in height) and the flow-through was collected (labelled FT). The column was washed with wash buffer and 2 fractions of 4 ml each (labelled W1 and W2) were collected. Next, the 6xHis-tagged protein was eluted by elution buffer and 4 fractions of 0.5 ml each were collected (labelled E1-E4).

2.6.3 Pilot purification under denaturing conditions

To prepare the bacterial lysate under denaturing condition, the pellet obtained in section 2.6.1 was thawed for 10-15 min on ice followed by suspending the pellet in buffer A or B (4-5 ml per g pellet) (Table 2.7) at room temperature until it looked translucent. Next, the lysate was centrifuged at 10,000*g* for 30 min at room temperature. The supernatant fluid was used for protein purification and the cellular debris was suspended in an equal volume of buffer A or buffer B.

The procedure for protein purification under denaturing conditions was similar as that described in section 2.6.2.II. but the supernatant was mixed with Ni-NTA resin at room temperature. In addition, in a pilot purification, 2 elution buffers, buffer D (pH 5.9) and buffer E (pH 4.5), were used.

For chromatography under native and denaturing conditions, samples were removed from each collected fraction for SDS-PAGE.

2.6.4 Scale-up growth and protein purification

The conditions for optimising bacterial expression of recombinant proteins and their subsequent purification described in the pilot experiments were subsequently used for bulk production. In scale-up growth, the ratio of overnight bacterial culture to SOB was 1:50. For induction, the final concentration of IPTG remained 1 mM. A 1 ml sample of un-induced culture (H0) and another 1 ml sample of culture obtained at optimal expression time were also processed for SDS-PAGE analysis. To prepare the lysate for purification, the relative volumes used for suspending bacterial pellets were unchanged. In scale-up purification, the amount of Ni-NTA resin used was dependent on the binding capacity of the resin for recombinant protein, normally 5-10 mg protein per ml (Qiagen, UK) and the volumes of wash and elution buffers adjusted accordingly. Generally, the volume of wash buffer used was 10 times the nickel resin volume and collected in 4 aliquots (labelled W1-W4). The elution volume was 4-5 times the nickel resin volume and collected in 1-ml aliquot (labelled E1-E10 and so on). Samples from each collected fraction were analysed by SDS-PAGE.

Figure 2.2 shows a summary from gene cloning to protein purification. In brief, the ligation reaction of pRSETA vector and *mip* or *acp* gene insert was transformed into *E.coli*, BL21(DE3)pLysS and addition of IPTG induced gene expression. The protein was subsequently purified through nickel column chromatography.

Table 2.7 Buffers for protein purification by Ni-NTA column chromatography under native and denaturing conditions.

	Native conditions buffer base: 50 mM NaH_2PO_4, **pH 8.0** containing 300 mM NaCl **and**	**Denaturing conditions buffer base:** 100 mM NaH_2PO_4 and 10 mM Tris containing either **6 M GuHCl** or **8 M urea**
Lysis buffer	**10 mM** imidazole	Buffer **A**: containing 6 M GuHCl, **pH 8.0**
		Buffer **B**: containing 8 M urea, **pH 8.0**
Wash buffer	**20 mM** imidazole	Buffer **C**: **pH 6.3**
Elution buffer	**250 mM** imidazole	Buffer **D**: **pH 5.9**
		Buffer **E**: **pH 4.5**

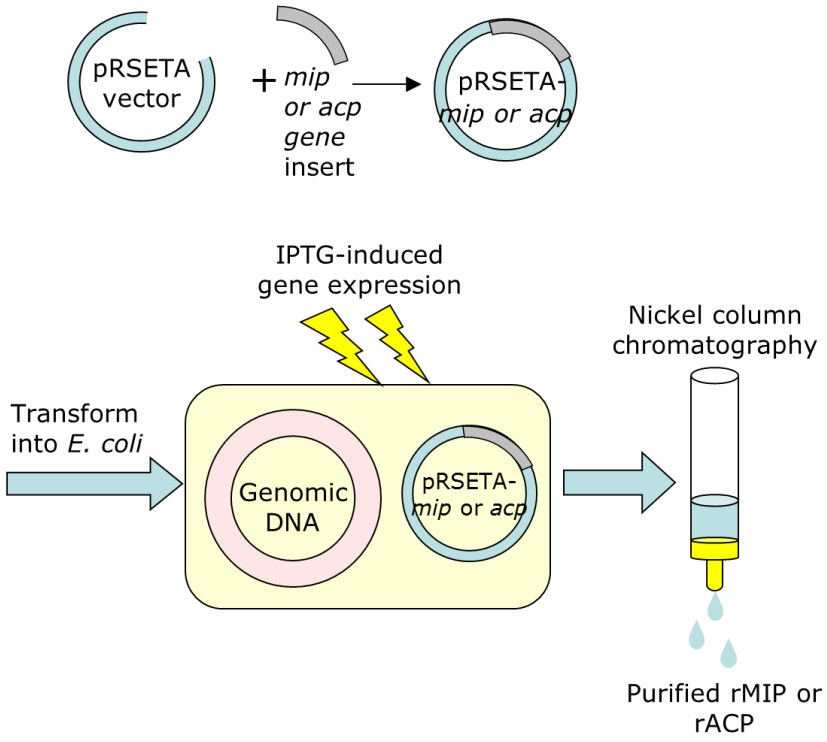

Figure 2.2 Summary of the gene cloning, gene expression and purification procedures.

2.7 Dialysis

Dialysis against PBS was used to remove imidazole (250 mM) from the eluted fractions purified under native conditions. Pooled fractions were dialysed against 2 L volumes of PBS containing 0.0001% (w/v) thimerosal (Sigma-Aldrich, UK) at 4°C, changed 3 times within 24 hours with minimal interval duration of 3-4 hours. After dialysis, SDS-PAGE was used to confirm the presence of recombinant proteins and the protein concentration was determined by the BCA assay (Pierce, UK) (section 2.10).

2.8 Precipitation of denatured recombinant proteins

To precipitate the denatured recombinant proteins from the eluate containing GuHCl (BDH, UK), each sample was diluted with a 6-fold excess volume of UHQ water and an equal volume of 10% (v/v) trichloroacetic acid (TCA; BDH, UK) was added. Next, the samples were left on ice for 20 min and then centrifuged at 10,000*g* for 15 min. The resulting pellet was washed with 100% ice-cold ethanol, dried in air and stored at -20°C. For SDS-PAGE, the pellet was suspended in dissociation buffer (Section 2.9).

Protein samples containing urea were precipitated by addition of ethanol to a final concentration of 80% (vol/vol) at -20°C for 18 h followed by centrifugation at 10,000*g* for 15 min. The resulting pellet was washed with water and stored at -20 °C until used.

2.9 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE with a linear gradient of 10-25% (w/v) was done as described previously (Heckels 1981). The discontinuous buffer system of Laemmli (Laemmli 1970) was used.

Materials:

i) **50% (w/v) Acrylamide/Bis-acrylamide** 37:1 (Sigma-Aldrich, UK), stored at 4°C.

ii) **2% (w/v) sodium dodecyl sulphate (SDS) Solution**, stored at room temperature.

iii) **Glycerol** (Fisher, UK).

iv) **TEMED** (Bio-Rad)**: N,N,N',N'-tetramethylethylenediamine,** stored at 4°C.

v) **Ammonium persulphate solution** (**APS**; Fisher, UK)**,** 1% (w/v), prepared immediately before use.

vi) **Separating gel buffer**: 1.2 M Tris-HCl (pH 8.8), stored at 4°C.

vii) **Stacking gel buffer:** 0.25 M Tris-HCl (pH 6.8) containing 0.08% (v/v) TEMED, stored at 4°C.

viii) **Running buffer**: 25 mM Tris-HCl (pH 8.3) containing 192 mM glycine and 0.1 % (w/v) SDS.

ix) **Dissociation buffer or 2X SDS sample buffers**: 125 mM Tris-HCl (pH 6.8), 4% (w/v) SDS, 20% (v/v) Glycerol, 10% (v/v) beta-mercaptoethanol and 0.002% (w/v) bromophenol blue, stored at 4°C.

x) **Staining solution:** 10% (v/v) acetic acid (Fisher, UK), 20% (v/v) isopropanol (Fisher, UK) and 0.5 mg/ml PAGE-blue-83, stored at room temperature.

xi) **Destaining solution:** 10% (v/v) acetic acid and 10% (v/v) isopropanol.

xii) **Gel drying solution:** 5% (v/v) glycerol and 20% (v/v) methanol.

2.9.1 Preparation of separating gel slabs

A separating gel slab was prepared in the space between 2 glass plates. The dimensions were 175 mm x 120 mm x 1.2 mm. Glass plates were cleaned using Whatman® filter paper and 70% (v/v) ethanol. A U-shaped rubber spacer was placed between 2 plates and fixed by clips to avoid leakage. Next, 10% (w/v) and 25% (w/v) acrylamide solutions were prepared (Table 2.8) and degassed immediately before addition of APS. Then, the 2 solutions were gradually mixed using a triple channel peristaltic pump (Pharmacia P-3) to form a gradient from 25% to 10% upwards. A thin layer of butanol (BDH, UK) was overlaid on top of the separating gel and the gel was left to polymerize at room temperature for 1 h.

2.9.2 Stacking gels

Stacking gels contained 4% (w/v) acrylamide (Table 2.9). After the separating gel was set, the butanol was poured off and the remaining solvent and any unpolymerized residual acrylamide were removed by capillary action onto thin filter paper. Stacking gel was introduced using a plastic liquid transfer pipette. A comb was placed on top of the stacking gel to form the wells and the gel was left to set at room temperature for 1 h.

2.9.3 Preparing and loading samples

The rubber spacer was removed and glass plates were mounted onto the gel tank (Bio-metra, Germany). The comb and unpolymerized stacking gel solution in the wells were removed carefully. Running buffer was poured into the lower and upper tank. In addition, bubbles below the gels and inside the wells were removed using a syringe and bent needle.

Each protein sample was mixed with an equal volume of dissociation buffer and boiled for 10 min. After brief centrifugation at 800*g* for 1 min, the samples were applied to individual wells at 4°C. Either 5-10 µl of PageRuler (Fermentas, UK) or 5-10 µl of Precision plus protein standards (Bio-rad, UK) were loaded as protein ladders. The power supply was set at 200 V and electrophoresis lasted for 14-16 h.

Table 2.8 Components of the 25% and 10% acrylamide solutions.

Acrylamide conc	25 %	10 %	Comments
50% (w/v) Acrylamide/Bis-acrylamide	6.25 ml	2.5 ml	-
Separating gel buffer	3.1 ml	3.1 ml	0.3 M Tris
2% (w/v) SDS solution	620 µl	620 µl	0.1% (w/v)
Glycerol	1.25 ml	-	10% (v/v)
UHQ water	1.1 ml	6.0 ml	-
TEMED	6.2 µl	6.2 µl	0.05% (v/v)
APS*	125 µl	250 µl	0.01% / 0.02% (w/v)
Total volume	12.5 ml	12.5 ml	25 ml

* APS was added after the mixture was degassed.

Table 2.9 Components of the stacking gels.

	Volume	Final concentration
50% (w/v) Acrylamide/ Bis-acrylamide	2.5 ml	4 % (w/v)
Stacking gel buffer	15.6 ml	0.125 M Tris
2% (w/v) SDS solution	1.55 ml	0.1% (w/v)
UHQ water	8.4 ml	-
APS*	3.1 ml	0.1 % (w/v)

* APS was added after the mixture was degassed.

2.9.4 Coomassie blue stain

The separating gel slab was immersed in staining solution for 1 h. After the buffer was drained off, the gel was immersed in destaining buffer for 30 min to 1 h. The destaining buffer was changed 2-3 times.

2.9.5 Drying of the gels

The gels were immersed in the gel drying solution and left overnight. Two gel drying films (Promega, UK) were used to dry a single gel according to the manufacturer's protocol.

2.10 BCA assay

The BCA assay (Pierce®, Thermo-Scientific, UK) was used to determine the concentration of proteins. In this assay, the presence of proteins can reduce Cu^{+2} to Cu^{+1} in an alkaline solution. Next, chelation of 2 molecules of bicinchoninic acid (BCA) with one Cu^{+1} forms the purple-coloured reaction product. The protein quantity can be determined by colorimetric detection.

Materials provided in the Pierce® BCA protein assay kit:

i) **BCA reagent A**: sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide

ii) **BCA reagent B**: containing 4% (w/v) cupric sulfate

iii) **Albumin standard ampoules**: 2.0 mg/ml of bovine serum albumin (BSA) in 0.9% (w/v) saline and 0.05% (w/v) sodium azide

A 200 µl aliquot of reagent (ratio of reagent A to reagent B of 50:1) was mixed with 25 µl of albumin standard (Appendix IV) or diluted protein samples in a 96-well flat-bottomed polystyrene microtitre plate (Nunc, UK). After incubation at 37°C for 30 min, the absorbance at λ 570 nm was read in a microplate reader (iMark™, Bio-rad, UK). The absorbance value of the albumin standards was plotted into a standard curve and the protein concentration of the samples was obtained by comparing the absorbance value with the standard curve. In addition, some substances in protein samples can interfere with the BCA results. The compatible concentrations of some interfering substances are listed in Appendix V.

2.11 Tricine-SDS-PAGE and silver stain

The method was described by Schagger and Von Jagow (1987) and used for analysing low molecular weight proteins or LPS using a discontinuous buffer system with tricine as the trailing ion (Schagger 2006).

2.11.1 Preparation of gels

Materials:

i) **48% (w/v) Acrylamide/Bis-acrylamide solution** (**1:29)** (Sigma-Aldrich, UK)

ii) **Gel buffer** (**3X** buffer): 3 M Tris-HCl, pH 8.5. SDS 0.3% (w/v) was added after the pH was adjusted.

iii) **10% (w/v) Ammonium persulphate** (APS, Fisher, UK)

iv) **TEMED** (Bio-Rad)

v) **Anode buffer**: 0.2 M Tris-HCl, pH 8.9.

vi) **Cathode buffer**: 0.1 M tricine (Merck-BDH); 0.1% (w/v) SDS; 0.1 M Tris, pH 8.2-8.3.

vii) **Sample buffer**: 0.05 M Tris-HCl, pH 6.8, containing 4% (w/v) SDS, 12% (v/v) glycerol, 2% (v/v) 2-mercaptoethanol and 0.02% (w/v) Brilliant blue G.

The same gel tank system was used as described in section 2.9. The separating gel was poured to a height of 10 cm with a spacer gel (3 cm) on top of the separating gel (Table 2.10). A thin layer of UHQ water was overlaid and the gel was left to polymerize at room temperature for 1 h. After the water was removed, the stacking gel was introduced on top of the spacer gel with a desired comb and allowed to set for 30 min.

Table 2.10 Components of gels for tricine-SDS-PAGE.

	Separating gel (16%)	Spacer gel	Stacking gel (4%)
48 % (w/v) Acrylamide solution	10 ml	2 ml	1.0 ml
Gel buffer (3X)	10 ml	3.3 ml	3.1 ml
Glycerol	3 ml	-	-
UHQ water	6.7 ml	4.7 ml	8.4 ml
*TEMED	10 µl	3.3 µl	10 µl
*10% (w/v) APS	100 µl	33 µl	100 µl
Total volume	30 ml	10 ml	12.6 ml

*TEMED and APS should be added last.

2.11.2 Sample preparation and voltage setting

Each sample containing 10 µg of protein was mixed with sample buffer to a volume of 20 µl, boiled at 100^{o}C for 10 min and loaded into a single well on a 20 well gel. The plates were mounted onto the apparatus (Biometra, Germany) and the top tank reservoir was filled with cathode buffer and the bottom reservoir was filled with anode buffer. The voltage was set at 30 volts for 1 h and increased to 90 volts for a further 16 h.

2.11.3 Silver staining of LPS

The silver staining method of Hitchcock and Brown (Hitchcock and Brown 1983) was used.

Materials:

i) **Fixation solution:** 25% (v/v) isopropanol, 7% (v/v) glacial acetic acid.

ii) **Oxidation solution:** 0.7% (w/v) periodic acid (Sigma-Aldrich, UK), 2.6% (v/v) fixation solution

iii) **Silver staining solution**: 0.56% (v/v) of 35% (w/v) ammonia hydroxide solution (Fisher, UK), 18.6% (v/v) of 0.1 N sodium hydroxide and 3.3% (v/v) of 20% (w/v) silver nitrate (Fisher, UK).

iv) **Developing solution**: 0.005% (w/v) citric acid (Merck-BDH), 0.05% (v/v) of 37% (v/v) formaldehyde solution (BDH, UK)

v) **Stop solution:** 0.1% (v/v) acetic acid.

The staining was carried out at 30-35°C in clean glassware and all the solutions were prepared immediately before use. The gel was taken carefully from the plates and immersed in fixation solution for 30 min, followed by freshly prepared oxidation solution for 5 min. After 8 washes in distilled water (4 min /wash), the gel was stained in the silver staining solution for 10 min. Next, the gel was washed another 4 times (10 min /wash) in distilled water and then immersed in developing solution until the LPS bands had reached the desired intensity. To stop the reaction, the gel was left in stop solution for 1 h. The gel was scanned to keep the image.

2.12 Limulus Amebocyte Lysate (LAL) assay

The reaction depends on catalyzing the activation of a proenzyme in the limulus amebocyte lysate by Gram-negative bacterial endotoxin. A Gram-negative bacterial infection was first found to cause fatal intravascular coagulation in *Limulus polyphemus,* the horseshoe crab, in 1956 (Bang 1956). Later, it was proved to be a reaction between the bacterial endotoxin and a protein in the circulating amebocytes of *Limulus* (Levin and Bang 1964). The lysate from washed amebocytes was prepared and became an excellent reagent for detecting the presence of endotoxin (Levin and Bang 1968). By comparing the absorbance values of the endotoxin standard using the linear correlation of OD value and endotoxin concentration between 0.1 EU/ml-1 EU/ml, the endotoxin level of the protein samples can be quantified. Every 1 endotoxin unit (EU) of LPS is equivalent to ~0.5 μg of LPS (Humphries *et al.* 2005). The experiments were carried out according to the manufacturer's instruction (Lonza, UK).

Materials:

i) **Limulus Amebocyte lysate (LAL), *E. coli* endotoxin, Chromogenic substrate** and **LAL reagent water** were provided in the kit.

v) **Stop reagent:** 10% (w/v) sodium dodecyl sulfate (SDS) in UHQ water.

A sterile 96-well microplate was pre-warmed in a 37°C incubator and it was kept at 37°C during incubation throughout the whole experiment. The *E.coli* endotoxin was mixed thoroughly on a shaker for 15 minutes before it was diluted into 1, 0.5, 0.25 and 0.1 EU/ml. LAL water (50 µl) was used as blank. Protein samples were diluted to 0.001 µg to 10 µg per 50 µl (per reaction). Endotoxin, LAL water and protein samples were prepared in duplicate. At time =0 min, 50 µl of LAL was added into each well. The order and speed of adding reagents was the same and at time =10 min, chromogenic reagent (100 µl) was added. After 6 min incubation at 37°C, the stop reagent (100 µl) was added and the absorbance at λ 415 nm was obtained using the microplate reader (iMark™, Bio-rad, UK).

2.13 Immunization preparations

2.13.1 Liposome preparations with or without Monophosphoryl Lipid A (MPLA)

I. Preparation of protein samples with octyl-β-D-glucopyranoside

The concentration of soluble rMIP protein used for preparing samples for immunization was 1.75 mg/ml. To make liposome preparations, 10 mM HEPES buffer containing 0.5% (w/v) SDS was used to dissolve the rACP pellet and the final concentration was ~1 mg/ml.

Recombinant protein (1 mg) and octyl β-D-glucopyranoside (100 mg) (Sigma-Aldrich, UK) were mixed and dissolved in 5 ml of 10 mM HEPES (Sigma-Aldrich, UK; pH 7.2) buffer. The mixture was incubated at room temperature for 3 hours before use. A liposome control was made under the same conditions without protein.

II. Making the lipid shell

The method of preparing liposomes was based on the procedure originally described by Ward *et al.* (1996). All glassware used for liposome manufacture was cleaned overnight by soaking with 70% (v/v) nitric acid, followed by extensive washing with distilled water and drying at 160°C. The lipid shell was composed of L-α-phosphatidylcholine (PC) (molecular weight: 768) (Sigma-Aldrich, UK) and cholesterol (Sigma-Aldrich, UK) (molecular weight: 386.66) with a molecular ratio of 7:2 and 20 mg in total. The mixture was dissolved in chloroform (BDH, UK) to give a final volume of 3 ml. Next, a rotary evaporator (Buchi Rotavapor, Switzerland) was used to evaporate chloroform under vacuum and coat the lipid mixture evenly onto the inner wall of a round-bottom flask.

For the liposome with MPLA preparations, 500 µg of MPLA (Sigma-Aldrich, UK) dissolved in 500 µl of chloroform was added to the 10 mg mixture of cholesterol and L-α-phosphaditylcholine before the lipid film was made.

III. Incorporation of the protein into liposomes

The prepared sample with detergent was carefully introduced into the lipid-coated flask and vortex-mixed gently to dissolve the lipid coating in the inner glass wall. The mixture was then left at room temperature for 1 h.

IV. Dialysis and sonication

The purpose of dialysis is to remove any detergents and SDS; and once removed from the mixture, the appearance of the milky lipid would become visible. The mixture was introduced into dialysis tubing (0.63 cm in diameter), which had been previously boiled for 5 min at 100°C, and dialyzed against phosphate buffer saline (PBS) containing 0.0001% (w/v) thimerosal at 4°C. Two litres were used for each dialysis and the buffer was changed twice per day. The total duration of dialysis was 72 hours.

To generate small unilamellar liposomes, sonication was done on ice using 15 µm of amplitude, with 1 min for each burst (MSE Soniprep 150 sonicator) and repeated 15-30 times until the sample looked translucent. The sample was chilled on ice for 1 min between each burst. Liposome preparations were stored in aliquots at -20°C until used. The injection dose was 20 µg of protein per mouse.

2.13.2 Micelle preparations with and without MPLA

The concentration of the soluble rMIP stock used was 1.75 mg/ml. The rACP pellet was dissolved in PBS containing 0.5% (w/v) SDS to a final concentration of 2 mg/ml.

Zwittergent 3-14 (ZW 3-14) (Calbiochem, UK) detergent (8 mg), protein (0.5 mg) with or without MPLA (0.5 mg dissolved in 500 µl of PBS) were mixed in a Bijoux tube, brought to a final volume of 1 ml using PBS and left at room temperature overnight. A ZW 3-14 control and a ZW 3-14 micelles + MPLA control were prepared without protein. Next, aliquots of 280 µl from each preparation were diluted in 420 µl of 0.9% (w/v) NaCl before use. Otherwise, the preparations were stored at -20°C. The injection dose for each mouse was 20 µg of protein.

2.13.3 Aluminium hydroxide preparations

Aluminium hydroxide gel adjuvant (2.0%) (Superfos Biosector, Denmark) was used to adsorb either recombinant protein or OM preparation from MC58 (supplied by Dr. M. Christodoulides). A 1 ml preparation was composed of aluminium hydroxide gel (500 µl), 200 µg of recombinant protein or OM and 0.9% (w/v) NaCl. Next, the preparation was mixed on an angled rotary mixer at 4°C overnight. This preparation was made fresh one day before immunization and the injection dose for each animal was 100 µl containing 20 µg of protein.

2.13.4 Saline preparations

Recombinant protein (200 µg) was diluted with saline to a final volume of 1 ml. This preparation was made immediately before use and the injection dose for each animal was 100 µl, equivalent to 20 µg of protein.

2.13.5 Freund's adjuvant

Complete Freund's adjuvant (Sigma-Aldrich, UK) contains dried and inactivated *Mycobacterium tuberculosis* and was used for the first injection dose. Incomplete Freund's adjuvant (Sigma-Aldrich, UK) is a water-in-oil emulsion and was used for all subsequent injection. To make a 1.5 ml preparation, 60 µg of each protein was mixed with 750 µl of either complete or incomplete Freund's adjuvant. After all the components were mixed in a Bijoux tube, emulsification was achieved by drawing the emulsion up-and-down repeatedly with a 2 ml syringe (Terumo, Belgium) fitted with a 19 gauge needle (Terumo, Belgium). The preparations were made and used immediately. The immunization dose for each rabbit was 500 µl, equivalent to 20 µg of protein.

2.14 Animal immunization and processing of animal antisera

BALB/C mice (H-2^d haplotype) and New Zealand white rabbits were housed under standard conditions of temperature and humidity with a 12 h lighting cycle and with food and water available *ad libitum.* For each recombinant protein, 14 groups of 5 mice, of approximate equal size and weight, were immunized intra-peritoneally with different preparations (Table 2.11) using a 1 ml syringe (Terumo) and 25G x 5/8" needle (Terumo). The immunization schedule was 3 doses on day 0, 14 and 28 followed by terminal bleeding by cardiac puncture under anaesthesia on day 42.

Vaccine formulations containing the Freund's adjuvants were also used for immunizing rabbits. Initially, a pre-immune bleed was taken from each rabbit from the middle-ear vein. A pair of rabbits was each immunized with rMIP and rACP, with Freund's Complete Adjuvant used for the primary injection and the subsequent 3 injections using Freund's Incomplete Adjuvant. Injections were administered subcutaneously over various sites using a 1 ml syringe with 19G x 11/2" needle (Terumo) at 14 day intervals. Each dose contained 20 µg of protein. The rabbits were terminally bled from the middle ear vein and by cardiac puncture under anaesthesia 2 weeks after the last dose.

All animal experiments were carried out following Home Office regulations on project licence PPL30/2563 Prevention and Diagnosis of Infectious Diseases (Dr. Myron Christodoulides).

Table 2.11 Immunization groups for mice**.**

A.	**Without recombinant protein** (control groups)
1.	$Al(OH)_3$
2.	Liposome
3.	Liposome + MPLA
4.	Saline
5.	Zwittergent 3-14 micelles
6.	Zwittergent 3-14 micelles + MPLA
B.	**With recombinant protein**
1.	$Al(OH)_3$ + rMIP or rACP
2.	Liposome + rMIP or rACP
3.	Liposome + MPLA + rMIP or rACP
4.	Saline + rMIP or rACP
5.	Zwittergent 3-14 micelles + rMIP or rACP
6.	Zwittergent 3-14 micelles + MPLA + rMIP or rACP
C.	**MC58 outer membrane preparation on alum**
D.	**Normal mouse (for normal serum)**

Whole blood from mice and rabbits was allowed to clot at 37°C for 1 h and then left at 4°C overnight to express serum. Samples were centrifuged at 6,000*g* for 6 min and the antisera were removed to new sterile microtubes and stored at -20°C until analysed.

2.15 Enzyme linked immunoabsorbent assay (ELISA)

Materials:

i) **Coating buffer**: 35 mM sodium hydrogen carbonate ($NaHCO_3$) and 15 mM sodium carbonate (Na_2CO_3).

ii) **Wash buffer**: 0.15 M sodium chloride, 0.05% (v/v) Tween 20 (Sigma-Aldrich, UK)

iii) **Antibody diluent**: 50 mM Tris, 0.15 M sodium chloride, 0.05% (v/v) Tween 20 (Sigma-Aldrich, UK), containing 1% (w/v) BSA (Sigma-Aldrich, UK) adjusted to pH 7.4 using acetic acid.

iv) **Anti-species horseradish peroxidase conjugated** (Bio-Rad)

v) **Colour substrate**: TMB solution (1 ml), hydrogen peroxide (10 µl) and 0.1 M sodium acetate buffer (100 ml) were mixed immediately before use.

a) **3,3',5,5'-tetramethyl benzidine (TMB) solution:** 3.6 mg of TMB (Sigma-Aldrich, UK) was dissolved in 1 ml dimethyl sulphoxide (DMSO) (BDH,UK)

b) **30% (w/v) Hydrogen peroxide** (BDH, UK)

c) **0.1 M sodium acetate** (BDH, UK) **buffer**, which was adjusted to pH 6.0 with 1 M citric acid (BDH, UK).

Recombinant proteins were diluted to 1 µg/ml in coating buffer and 100 µl was loaded into each well of a 96-well, flat-bottomed polystyrene microtitre plate (Nunc, UK). The plate was incubated in a moist environment at 37°C overnight. Next, all the wells were washed 3 times before antibody diluent (100 µl) was loaded into each well and incubated at 37°C for 1 h. The diluent was then removed without wash. Serially diluted antisera (100 µl) was loaded into each well and incubated at 37°C for 1 h. After 4 washes, anti-species horseradish peroxidase conjugated antibody (100 µl, 1:2000) was added and incubated at 37°C for 1 h. Following 4 washes, the colour substrate (100 µl /well) was added and the plate was incubated at room temperature in the dark for 10 min. Finally, 1 M sulphuric acid (Fisher, UK) (50 µl) was added to stop the reaction. The absorbance at λ 450 nm was measured with a microplate reader (iMark^{TM}, Bio-rad, UK). The geometric mean of endpoint titres with 95% confidence intervals for each immunization group was calculated and student t-Test was used to compare the mean values between groups with $p<0.05$ considered significant.

2.16 Western blot

Materials:

i) **Blotting buffer**: 20 mM Tris-HCl (pH 8.3), 153.6 mM glycine, 0.08% (w/v) SDS and 20% (v/v) methanol.

ii) **Tris buffered saline (TBS)** (pH 7.5): 20 mM Tris and 0.5 M NaCl.

iii) **Tween Tris buffered saline (TTBS)**: TBS with 0.05% (v/v) Tween 20.

iv) **Blocking buffer**: TTBS with 5% (w/v) non-fat milk powder.

v) **Goat Anti-species IgG - alkaline phosphatase conjugate** (Bio-Rad): 1:3000 diluted in blocking buffer.

vi) **Alkaline phosphatase substrate**: substrate buffer with 1% (v/v) NBT solution and 1% (v/v) BCIP solution.

a) **Substrate buffer (pH 9.5)**: 0.1 M Tris, 0.1 M NaCl and 2 mM $MgCl_2$

b) **Nitro Blue Tetrazolium** (**NBT**) **solution**: 30 mg of NBT (Sigma-Aldrich, UK) was dissolved in 1 ml of 70% (v/v) dimethylformaldehyde (DMF).

c) **5-Bromo-4-Chloro-3-Indolyl Phosphate (BCIP)** solution: 15 mg of BCIP (Sigma-Aldrich, UK) was dissolved in 1 ml of 100% dimethylformaldehyde (DMF).

SDS-PAGE gels containing separated protein samples were immersed in blotting buffer for 30 minutes on a gentle shaker. Next, 8 layers of thin filter papers (3M, Whatman, UK) and a piece of nitrocellulose in the same size as the gel were cut and rinsed with the blotting buffer. The blotting sandwich was made in the order of 4 layers of thin filter papers, a piece of nitrocellulose, a gel slab and another 4 layers of thin filter papers from the bottom to the top. They were then placed into a semi-transfer blotter (Bio-Rad, UK) and the applied amperes limit was calculated using the following formula: length (cm) x width (cm) x 0.8 mA. The voltage limit was set at 25 V. After 1 h, the gel slab was subjected to Coomassie blue stain and the nitrocellulose was oriented and then either cut into strips or the whole piece kept for the following steps or the whole blot stored dry in the dark.

The blot was washed using TTBS for 5 min twice and then immersed in blocking buffer for 1 h. Next, the blot was washed using TTBS for 5 min twice and left in primary antibodies (1:100 to 1:500 dilution in TTBS) on a gentle shaker at 4°C overnight or at room temperature for 1 h. Following 3 washes with TTBS, goat anti-species IgG - alkaline phosphatase conjugate was added and left at room temperature for 1 h. Next, the TTBS wash step was repeated 3 times followed by 3 times with TBS. The alkaline phosphatase substrate was made right before use and added onto the blot. The reaction was stopped after the blue-purple colour started to appear and images were later obtained by a scanner (Epson Perfection V300 scanner).

As shown in Table 2.12, some monoclonal antibodies were used to determine the presence of OM proteins.

Table 2.12 Monoclonal antibodies for testing Opa, Opc and PilE protein expression.

OM protein	Monoclonal Ab (mAb)	Working concentration	Source
Opa	4B12C11	1/5000	A gift from Dr M. Blake, NAV Inc., Beltsville, MD, USA
	B33	1/5000	A gift from Dr. G.F.Brooks, University of California, USA
Opc	B306	1/5000	A gift from Dr M. Achtman; (Achtman *et al.* 1992)
PilE	SM1	1/25,000	(Virji and Heckels 1983)

2.17 Serum bactericidal antibody (SBA) assay

Materials:

i) Phosphate buffered saline-B salts (PBSB): PBS with 9 mM $CaCl_2$ and 5 mM $MgCl_2$.

ii) Phosphate buffered saline-B salts-decomplemented foetal calf serum (PBSB-dFCS): PBSB with 2 % (v/v) heat-decomplemented foetal calf serum (Lonza, UK)

iii) Baby rabbit complement (AbD Serotec, UK). Aliquots were made after the dry powder was reconstituted in sterile distilled water and stored at -80°C until used.

A lawn of meningococci from 16-18 hours culture grown on supplement GC culture plates was suspended in 2 ml of PBSB. After brief centrifugation (200*g*, 1 min), the supernatant was transferred into a new Bijoux tube and labelled as the master suspension. To obtain the final bacterial colony count on each agar plate of between 200-250 CFU, the working bacterial suspension was estimated to be 6.6 x 10^4 CFU/ml. Therefore, 100 µl of the master suspension was mixed with 900 µl of 1% (w/v) SDS/ 0.1% (w/v) NaOH solution and UV λ 260 nm measured. The O.D. λ 260 nm of 1 is equivalent to ~4 x 10^8 CFU/ml of MC58 in the master suspension, as previously determined by viable counting. To make a 2 ml working bacterial suspension, the O.D. λ 260 nm value was applied in the following formula for calculation the x ml of a 1/1000 dilution from the master suspension needed in working bacterial suspension.

$$X\ (ml) = 0.6 \times 2\ (ml) / 4 \times O.D.\ \lambda\ 260\ nm$$

The antisera were decomplemented by heating at 56°C in a water bath for 30 minutes. A sterile 96-well flat-bottom polystyrene plate was used. The components were added in the following order: 55 µl (with antisera) or 65 µl (without antisera, negative control) of PBSB, bacterial suspension (25 µl), decomplemented antisera (10 µl) and then baby rabbit complement (5 µl or 10 µl). An aliquot of baby rabbit complement was thawed immediately

before use. After a short agitation, the plate was incubated at 37°C in a humidified incubator with 5% (v/v) CO_2 for 30 minutes and then 15 µl was plated out onto each GC agar plate. On the next day, viable count was obtained by a colony counter (PotoCol model 60000, Synoptics Ltd, Cambridge, UK) and the mean CFU was subjected to the following formula.

% killing = 100 x (CFU of serum-negative control – mean CFU of a serum sample)/ CFU of serum-negative control

The complement-mediated serum bactericidal titre was recorded as the reciprocal of the highest serum dilution yielding more than 50% bacterial killing compared to the number of CFU in the absence of serum.

2.18 Immunofluorescence

The reactivity of antisera with either MIP or ACP on the surface of meningococcal cells was investigated by immunofluorescence (IF). An overnight culture of MC58 from a lawn was suspended in PBS and diluted to 1/100. Each well on 10-well pattern microscope slides (Polysciences, USA) was loaded with 30 µl of the suspension, and allowed to air dry. Next, the samples were fixed in 70% (v/v) methanol for 10 min at room temperature and then blocked by the addition of PBS containing 1% (w/v) bovine serum albumin for 30 min. Pooled murine antisera (1/100 dilution) were reacted with the fixed organisms for 1 h at room temperature or at 4°C overnight. After 4 washes with PBS, binding of antibodies was detected by reactivity of anti-mouse IgG-FITC (fluorescein isothiocyanate) conjugate (Dako, UK), at a 1/100 dilution in PBS containing 1 % (w/v) BSA, for 1 h in the dark. The cells were examined using a confocal microscope (Leica TCS SP2, Germany) and IF reactivity is indicated as follows: non-reactivity (-), weak (±), medium (+) and strong (++) determined as previously described (Jolley *et al.* 2001).

2.19 Fluorescence Activated Cell Sorting (FACS)

An overnight (16-18 h) culture of bacteria was collected using centrifugation. Cold 70% (v/v) ethanol (2-3 ml) was added onto the pellet at -20°C for 1 h to permeabilize the capsule. Bacteria were washed twice with sterile PBS containing 1% (w/v) BSA and suspended to 2-4 x 10^8 CFU/ml. Next, bacterial pellet from the 1 ml suspension was centrifuged at 2,200*g* for 3 min, suspended in 200 µl neat rabbit antiserum and incubated at 37°C for 30 min. After 2 washes, bacteria were incubated with 100 µl of 1/50 FITC-conjugated goat anti-rabbit IgG (Dako, UK) at room temperature for 30 min. Bacteria were washed twice and then fixed with 0.4% (w/v) para-formaldehyde (BDH, UK) solution at room temperature for 30 min. Samples were stored at 4°C before analysis. Fluorescence was measured with a FACSAria Flow

Cytometer (BD Biosciences, USA)recording 100,000 events and the data compared to samples containing either PBS-1% (w/v) BSA, PBS alone or bacteria reacted with pre-immunization sera (negative control).

2.20 Sequencing *mip* gene and *acp* gene of 12 meningococcal strains

Twelve meningococcal strains with markedly different serogroups, serotypes and serosubtypes were selected for sequencing (Table 2.1). The genomic DNA was extracted according to the manufacturer's instructions (QIAamp DNA Mini and Blood Mini Handbook, Qiagen, UK). Next, the target genes were amplified by PCR with primers SF-1567 (5'-gaaacattcaaactcggcta-3') and SR-1567 (5'-gtttcagacggcatttgccg-3'). The PCR mixture (Table 2.3) and PCR reaction were the same as for cloning the *mip* gene (Table 2.4). The PCR product was gel purified (Section 2.4.1.V) and cleaned (Section 2.4.1 VI) and sequencing was done commercially (Geneservice, Oxford, UK) using the SF-1567 primer.

To sequence the *acp* gene of the target strains, primers KO2095F (5'-cgggctgaaccagatagact-3') and KO2095R (5'-gctccagtttggtacggaga-3') were used to amplify a 1.3 kb DNA segment including *acp* gene. The primer for sequencing was Seq2095 (5'-cgggatacgccgacattaga-3').

2.21 Constructing an ACP knockout mutant

2.21.1 DNA manipulation

Constructing an ACP knockout mutant was achieved by double cross-over of a segment of gene containing a mini-transposon. The genomic DNA of ACP knockout mutant of strain 8013 (serogroup C) was kindly provided by Dr. Vladimir Pelicic (Imperial College, London, UK) (Rusniok *et al.* 2009). The library which has more than 4000 mutant strains was generated by random insertion of a mini-transposon (1.6 kb) which contains meningococcal DNA uptake sequences (DUS) for facilitating DNA uptake and kanamycin-resistance cassette for mutant selection. In strain 8013, the gene encoding ACP (NMV_2302) starts at 2,219,241 and finishes at 2,219,615 of the genomic DNA. In the mutant 35/11, the mini-transposon is inserted after 2,219,561 (Figure 2.3).

Primers KO2095F (5'-cgggctgaaccagatagact-3') and KO2095R (5'-gctccagtttggtacggaga-3') were used to amplify the specific segment of gene (Figure 2.2). The amplified gene size was 1,300 bp in wild type or 2,900 bp in mutant strain. The 2.9 kb PCR product was gel-purified,

cleaned and used for transformation of MC58. In addition, this DNA segment has 98.38% identity with MC58 genomic DNA.

cgggctgaaccagatagacttctggggcaaggtcggcgttacccagtctggaggctcgcgctacgaaac
cggccgcaaaatgcccaaacccgtacgcgaactgctccgcctcgtccatatcgaatgcatcgatttggc
gaaagtcaacaaaaaagatatggaaatcgccgccctgttgaaaaaacaccatcccgacctgtatgccga
attgtccaaacagaccaagtccgaaagaaaaaaacaaagttaaaccgcaacctccggatgcccgaaggt
ttttcatttccgaaaaacgcaaacaatgccgtctgaaacaccggacaggccgccgtatcccgcctgccg
cccctgcctcaaaccgccgaaccgcccgaacccgccttttttacaaactttatccaatttcctgtttatt
tcaggatacgccgacattagaatgtcaaacagctcgaaacgggcaaactccacatccatccaaaggaat
aaaaATGAAACTTCTGACCACCGCAATCCTGTCTTCCGCAATCGCGCTCAGCAGTATGGCTGCTGCTGC
CGGCACGAACAACCCCACCGTTGCCAAAAAAACCGTCAGCTACGTCTGCCAGCAAGGTAAAAAAGTCAA
AGTAACCTACGGCTTCAACAAACAGGGCCTGACCACATACGCTTCCGCCGTCATCAACGGCAAACGTGT
GCAAATGCCTGTCAATTTGGACAAATCCGACAATGTGGAAACATTCTACGGCAAAGAAGGCGGTTATGT
TTTGGGTACCGGCGTGATGGATGGCAAATCCTATCGCAAACAGCCTA Mini-transposon 1.6 kb
TTATGATTACCGCACCTGACAACCAAATCGTCTTCAAAGACTGTTCCCCACGTTAAcaagccaacaaaa
aacagcgttttcagaaatgaaaacgctgtttttttgaccgttccattattcgcaaaagggaaaaaacga
ttacctgccccgtgtatcaaaacctgccctgccggatgaagggcataaccggcagggacggcgtcaaca
ccatatgggggtacggcttttcttgaaagattcggcttaaatatccaatactttcgcggtataggcgat
aatttcatccgccctttcagggttttcgttcaacttgatgccgtaacccggtaccagctctttcagacg
gtcttcccaagacggggcgcgctcggggaagcattggtgcatcagccggatcatcagcggcacagcggt
cgatgcgcccggcgacgcgcccagcaatgcggcgagtgagccgtcggcgtgggcgacaatctccgtacc
aaactggagc

Figure 2.3 The 2.9 kb PCR product used for transformation. The region of NMV_2302 is in upper case and grey shading. The blue box indicates where the mini-transposon was inserted. Primers KO2095F and KO2095R are underlined.

In addition, we also cloned the 2.9 kb DNA construct into another plasmid system-pGEM®-T easy vector (Promega, UK) to increase the concentration of DNA for the following transformation.

2.21.2 Transformation of meningococci

An overnight (16-18 h) culture of MC58 was prepared to a concentration of ~1 x 10^8 CFU/ml in GC liquid broth (Appendix IV) containing 5 mM $MgCl_2$. Bacterial suspension (200 µl) was added into each well of a 24-well tissue culture plate. PCR product or homologous genomic DNA was added to a saturated concentration of 1 µg/ml. The plate was incubated with shaking at 37°C with 5% (v/v) CO_2 for 30 min. Next, 1.8 ml of pre-warmed GC liquid broth was added to the wells and the plate was incubated for another 3 h without shaking under the same condition. The bacteria (200 -300 µl) was plated onto selective GC agar plates (with kanamycin 100 µg/ml) and grown at 37°C with 5% (v/v) CO_2 overnight and viable counts were determined on the next day as described in section 2.17. The transformants were screened by PCR (section 2.4.5). A 2.9 kb band indicated successful replacement of the 1.3 kb DNA segment within MC58 genome with the exogenous 2.9 kb DNA segment (containing the mini-transposon and the similar DNA sequence from genomic DNA of strain 8013) via double cross-over. Therefore, the colony was identified as an ACP knockout mutant (MC58ΔACP).

Another transformation protocol of Bogdan *et a.l* was adapted for transforming the non-piliated strain MC58¢18 (Bogdan *et al.* 2002; van Dam and Bos 2012) due to the low transformation rate for non-piliated strains. Overnight culture of a meningococcal lawn was suspended in GC broth. The bacterial suspension was diluted to 1x 10^7 CFU/ml (section 2.17) in a Falcon tube (50 ml, Greiner Bio-one, Germany) and grown for 2 h at 37°C in an orbital shaker (120 rpm; Gallenkamp, UK). Next, the bacteria were centrifuged (10 min, 3000*g*) and the pellet was suspended in 0.5 ml of ice cold transformation solution (TS), which contained 10% (w/v) of PolyEthylene Glycol (PEG) 8000 (Sigma, UK), 5% (v/v) DMSO (BDH, UK) and 50 mM of $MgCl_2$. A 190 µl sample of the suspension was transferred into a new ice-cold Falcon tube, 12 µl of DNA was added and left for 15 min on ice. Subsequently, 1.5 ml of GC broth was added and the bacteria were grown for 2 h at 37°C with shaking (180 rpm) followed by centrifugation at 7200*g* for 4 min. The pellet was suspended in 200 µl of GC broth and plated onto GC-agar plates supplemented with the appropriate antibiotics (kanamycin).

2.22 Constructing complemented strains

2.22.1 Cloning the acp gene into pGCC4 plasmids

The pGCC4 plasmids (Figure 2.4) for complementation were kindly provided by Dr. Hank Steven Seifert (Department of Microbiology-Immunology, North Western University Medical School, Chicago, IL, USA). The cloning method was similar to that described in section 2.4, with minor modification, as follows. Based on the multiple cloning sites of this plasmid and the restriction enzyme absent sites of the *acp* gene, the decided restriction enzymes were *PacI* (New England Biolab, UK) and *PmeI* (New England Biolab, UK). Therefore, the primers used were Com2095F (5'-GGCTAT**TTAATTAA**atgaaacttctgaccaccgc-3') and Com2095R (5'-ttaacgtggggaacagtctt-3'). The sequence **TTAATTAA** represents the restriction site for *PacI*. Next, for vector preparation, the pGCC4 vector was doubly digested by both enzymes at 37°C for 2 h while the insert preparation from the amplified PCR product containing the *acp* gene was digested by *PacI* using the same conditions to create 5' sticky end and 3' blunt end. To increase the ligation efficiency, the insert and the vector were not dephosphorylated and the ligation reaction was incubated for 30 min instead of 15 min. In addition, to sequence the insert, an upstream primer- LacP (5'-CGGTTCTGGCAAATATTCTG-3') was used.

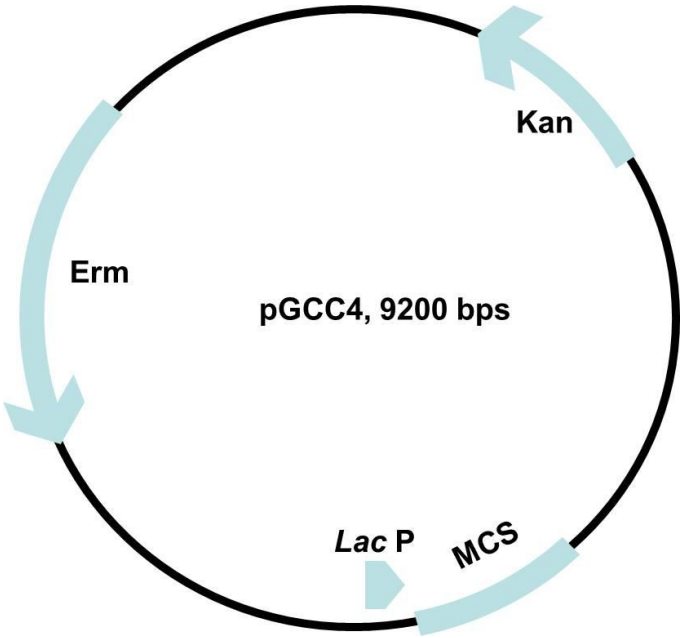

Figure 2.4 Features of pGCC4. Erm, erythromycin cassette; Kan, kanamycin cassette; MCS, multiple cloning sites (4350 NheI, FseI, PmeI, NsiI, ScaI, PacI 4375).

2.22.2 Transformation

The transformation method of Stohl and Seifert was used (Stohl and Seifert 2001). A suspension (~1 x 10^8 CFU/ml) from 18 h culture of MC58ΔACP was made using GC liquid broth (Appendix IV) containing 5 mM $MgCl_2$ plus the appropriate concentration of supplement A and supplement B. In a 24-well tissue culture plate, 200 µl of bacterial suspension was added into each well. Sequenced-proved recombinant rpGCC4-*acp* was added (~1 µg/ml). The plate was incubated without shaking at 37°C with 5% (v/v) CO_2 for 20 min. Next, 1.8 ml of pre-warmed GC liquid broth was added to the wells and the plate was incubated without shaking under the same condition for another 4 h. The transformation reaction (200 µl- 300 µl) was plated out on double selective GC agar plates (with kanamycin 100 µg/ml and erythromycin 1 µg/ml) and grown at 37°C with 5% (v/v) CO_2 overnight. Viable count was determined as described previously (section 2.17). The transformants were screened by PCR (section 2.4.5) and a ~0.375 kb band indicated successful complementation of MC58ΔACP.

2.23 Cell culture

2.23.1 Human meningioma cells

Materials:

i) **Growth medium**: Dulbecco's modified Eagles medium with Glutamax-1 and sodium pyruvate (DMEM) (Lonza, UK) supplemented with 10 % (v/v) decomplemented foetal calf serum (dFCS). Foetal calf serum was decomplemented using a 56°C water bath for 1 h. Antibiotics with penicillin (100 IU/ml), streptomycin (10 µg/ml) and gentamicin (10 µg/ml) were added for a newly resuscitated vial of cells.

ii) **Storage medium**: growth medium with 10% (v/v) dimethyl sulfoxide (DMSO).

iii) **Trypsin-EDTA**: Hank's Balanced Salt solution containing 0.5 g/l of trypsin and 0.2 g/l EDTA (Lonza, UK).

iv) **Collagen coating solution**: 50 µg/ ml of collagen, type I from rat-tail (BD Biosciences) in 0.02 M acetic acid (Fisher, UK).

Meningioma cell lines were obtained from surgically removed tumours as described previously (Hardy *et al.* 2000). Both meningothelial (M61) and transitional (M53) histological subtypes were shown previously to express the characteristic markers of desmosomal desmoplakin, epithelial membrane antigen, vimentin and cytokeratin.

To grow meningioma cells, all tissue culture flasks and plates were coated with collagen at room temperate for 1 h and then the excessive collagen was discarded and washed twice using PBS. To resuscitate a vial of meningioma cells from liquid nitrogen storage, the cells were thawed, transferred to growth medium (10 ml) with antibiotics and spun down at 1,000*g* for 3-5 min. The cells between passage 5-9 were suspended in growth medium and grown on flasks or plates coated with collagen. Cells were maintained at 37°C with 5% (v/v) CO_2. In addition, the growth medium was changed every 2-3 days until the cells reached confluence.

For long-term preservation, cells were added to DMEM containing 10 % (v/v) dFCS and 10% (v/v) dimethylsulphoxide (DMSO; BDH, Poole, England) in sterile cryogenic vials (Nunc, Roskilde, Denmark) and were frozen stepwise from -20°C to -80°C to liquid nitrogen.

2.23.2 Chang conjunctival epithelium

Chang conjunctival epithelial cells were obtained originally from the European Type Culture Collection (ETCC, Proton Down, UK). Culture and storage of Chang cells were the same as described in section 2.23.1 except that 5% (v/v) dFCS was used in DMEM for maintaining the culture.

2.23.3 Human epidermoid carcinoma, larynx (Hep2 cells)

Hep2 cells were originally obtained from the European Type Culture Collection (ETCC, Proton Down, UK). The procedure and media used for Hep2 cell culture were the same as for Chang cells.

2.23.4 Human umbilical vein endothelial cells (HUVECs)

HUVECs were obtained both from PromoCell (Heidelberg, Germany) and also from Dr. Timothy Millar (Clinical and Experimental Sciences, University of Southampton Medical School). The growth medium was DMEM supplemented with 20% (v/v) dFCS, endothelial cell growth supplement (ECGS; bovine hypothalamic extract; PromoCell, Germany; 2 ml/500 ml medium) and antibiotics. In addition, the culture flasks or plates were treated with sterile 0.2% (v/v) gelatin and incubated at room temperature for 30 minutes before the cells were seeded.

2.24 Infection experiments

2.24.1 Total bacterial association

The method followed the procedure described by Virji *et al.* (1991). After cells reached confluence in a 24-well tissue culture plate, the culture medium was changed to DMEM containing 1% (v/v) dFCS and no antibiotics. A bacterial suspension from 16-18 h culture was adjusted to 5 x 10^7 CFU/ml in DMEM containing 1% (v/v) dFCS. Next, the culture media was removed and 1 ml of bacterial suspension was added into each well in triplicate. After 3 h incubation at 37°C with 5% (v/v) CO_2, the monolayers were washed 4 times with PBS followed by addition of 250 µl lysis solution containing 1% (w/v) saponin (Sigma-Aldrich, UK) and 1% (v/v) dFCS. After incubation at 37°C with 5% (v/v) CO_2 for 15 min, triplicate cell lysates were pooled and 15 µl from each pooled sample was plated out onto GC agar plates in triplicate. Viable counts were obtained 18-20 hours later and data were analysed by student t-Test, $p<0.05$ considered significant.

In addition, to calculate the multiplicity of infection (M.O.I. = challenged bacteria number/cell) quantification of cells per monolayer was carried out using the improved Neubauer cell counting chamber (Weber, England) and trypan blue stain. Following addition of 250 µl of trypsin into an intact monolayer in a 24-well tissue culture plate, the plate was incubated at 37°C with 5% (v/v) CO_2 for 2 min. Next, 10 µl of the trypsinised cells was mixed gently with 10 µl of trypan blue on a small sheet of parafilm. After the cells with trypan blue

were loaded into the counting chamber, the cell count in the 4 squares was obtained and the following formula used to calculate cell number:

cell count/ml= (total cell count in the 4 squares/2) x 10^4 x 0.25 (ml)

2.24.2 Gentamicin assay

Similar to section 2.24.1, cell monolayers in 24-well tissue culture plates were challenged with bacteria for 3 h. After 4 washes, 1 ml of gentamicin (200 μg/ml) in DMEM containing 1% (v/v) dFCS was added per well and incubated for 90 min to eliminate the attached but not internalised bacteria. The monolayers were washed 4 times and then lysed to release the internalised bacteria by addition of 250 μl 1% (w/v) saponin lysis solution. The following procedure was described in section 2.24.1. To validate the gentamicin assay, a triplicate of monolayers was pre-treated with 2 μg/ml of Cytochalasin D (CD, Sigma-Aldrich, UK) for 30 minutes before addition of bacterial suspension. The cells were incubated at the concentration of 1 μg/ml CD during bacterial challenge. CD has been shown to disrupt polymerisation of actin and therefore inhibit cytoskeleton activity or microfilament function of the cells. As a result, the number of internalised bacteria decreased remarkably. In a validated gentamicin assay with pre-treatment of CD, the attached bacteria should be killed by gentamicin so only very few internalised bacteria can be harvested by viable counts (Virji *et al.* 1991). A parallel total association experiment was also carried out and the data were shown as percentage internalisation using the following formula:

$$\frac{\text{the number of internalised bacteria}}{\text{the number of associated bacteria}} \times 100\%$$

Chapter 3 Immunogenicity of recombinant macrophage infectivity potentiator (rMIP) protein

3.1 Cloning and expression of rMIP protein (NMB1567)

The ligation reaction of *mip* gene insert and pRSETA vector was transformed into *E.coli* DH5α competent cells and transformants grown on ampicillin-LB agar plates were screened by PCR. Recombinant plasmids from 6 PCR-positive transformants were purified and sequenced. Next, the recombinant plasmids with correct sequence data (Figure 3.1) were transformed into *E.coli* BL21(DE3)pLysS for protein expression.

TAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**CGGGGTTCTCATCATCATCATCATCATGGTATG
GCTAGCATGACTGGTGGACAGCAAATGGGTCGGGATCTGTACGACGATGACGATAAGGATCGATGGG
GATCCGAGCTCGAGatgaacaccattttcaaaatcagcgcactgacccttccgccgctttggcactttccgcctgcggcaaaaaa
gaagccgccccgcatctgcatccgaacctgccgccgcttcttccgcgcagggcgacacctcttcgatcggcagcacgatgcagcaggc
aagctatgcgatgggcgtggacatcggacgctccctgaagcaaatgaaggaacagggcgcggaaatcgatttgaaagtctttaccgaa
gccatgcaggcagtgtatgacggcaaagaaatcaaaatgaccgaagagcaggctcaggaagtcatgatgaaattccttcaggaacaa
caggctaaagccgtagaaaaacacaaggcggacgcgaaggccaataaagaaaaaggcgaagcctttctgaaagaaaatgccgcca
aagacggcgtgaagaccactgcttccggcctgcaatacaaaatcaccaaacagggcgaaggcaaacagccgaccaaagacgacatc
gttaccgtggaatacgaaggccgcctgattgacggtacggtattcgacagcagcaaagccaacggcggcccggtcaccttccctttgag
ccaagtgattccgggttggaccgaaggcgtacagcttctgaaagaaggcggcgaagccacgttctacatcccgtccaaccttgcctacc
gcgaacagggtgcgggcgacaaaatcggtccgaacgccactttggtatttgatgtgaaactggtcaaaatcggcgcacccgaaaacgc
gcccgccaagcagccggctcaagtcgacatcaaaaaagtaaattaaTAGAAGCTTGATCCGGCTGCTAACAAAGCCCG
AA

Figure 3.1 Sequencing data of *mip* gene insert. The bold and underlined 'ATG' is the start codon of the recombinant protein. The box denotes the DNA sequence for 6x HisTag and the grey shadow shows the correct insert of *mip* gene.

In pilot expression experiments, *E.coli* BL21(DE3)pLysS- recombinant plasmid (pRSETA-*mip*) was grown overnight in SOB-ampicillin (50 μg/ml)- chloramphenicol (30 μg/ml) liquid broth (2-3 ml) and was added into fresh medium without antibiotics (1 ml into 25 ml). Protein expression was induced by the addition of 1 mM IPTG and at every hour thereafter culture samples were removed and analysed by SDS-PAGE (Figure 3.2). Expression of recombinant MIP (rMIP) protein was evident as early as 1 h after induction rising to maximal levels by 5 h. As a

negative control, the same transformant was grown and sampled without IPTG induction and as expected showed no expression (Figure 3.2).

Native MIP protein contains 272 amino acids with a predicted molecular weight of 28.9 kDa. The recombinant protein expressed in the pRSETA system, which adds 39 amino acids at the N-terminus, including the 6xHisTag, produces a protein of 33.4 kDa (Figure 3.2).

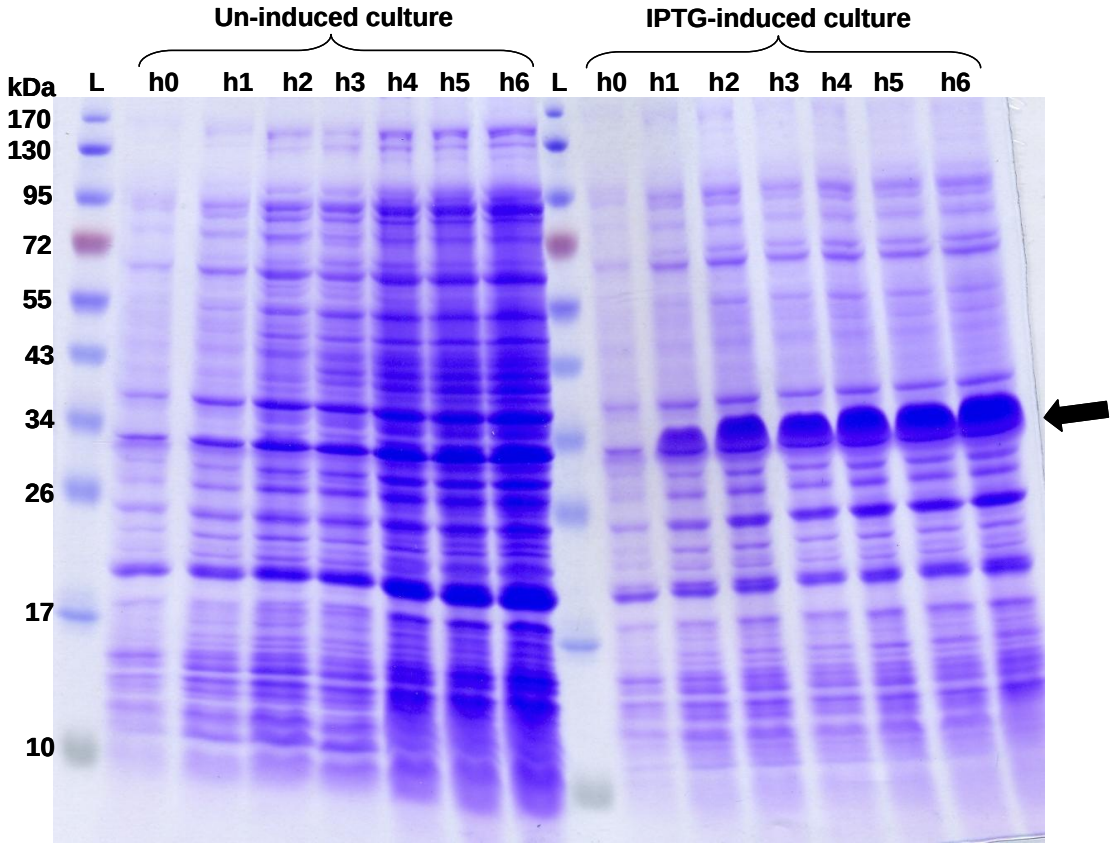

Figure 3.2 SDS-PAGE analysis of hourly samples from pilot expression of rMIP protein. L contains protein ladder (kDa). Samples (20 µl) taken from hourly lysate (h0-h6) of both un-induced and IPTG-induced culture of BL21(DE3)pLysS-pRSETA-*mip* were analysed in order. The black arrow indicates the presence of ~33.4 kDa bands. Maximal expression of rMIP was present 5 hours after induced expression.

In order to determine the solubility of rMIP, both un-induced and IPTG-induced culture of *E.coli* BL21(DE3)pLysS-pRSETA-*mip* were lysed by 3 freeze-thaw cycles. A 20 µl sample of the whole lysate was taken before centrifugation to separate the supernatant sample and the pellet. The whole lysate (L), the supernatant sample (SN) and the pellet (P) were analysed using SDS-PAGE (Figure 3.3). Recombinant MIP protein was present in the supernatant fraction of the IPTG-induced culture, demonstrating that the protein was soluble and could be purified, therefore, under native conditions.

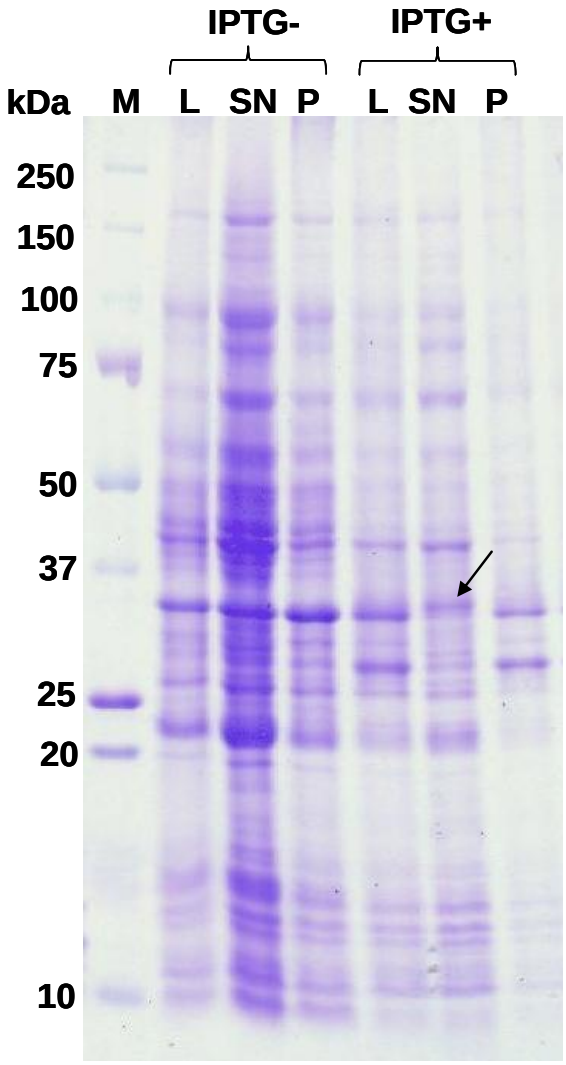

Figure 3.3 SDS-PAGE analysis of samples from solubility experiment of rMIP.
Lane M contains protein ladders. The whole lysate (L), the supernatant sample (SN) and the pellet (P) from un-induced (IPTG-) culture of *E.coli* BL21(DE3)pLysS-pRSETA-*mip* were analysed as negative controls. In the same order, the whole lysate (L), the supernatant sample (SN) and the pellet (P) from IPTG-induced (IPTG+) culture were compared. Each lane contained 20 µg of proteins. The black arrow indicates a ~33.4 kDa band present in the supernatant sample of IPTG-induced culture.

3.2 Purification of recombinant MIP under native conditions

3.2.1 Pilot purification of rMIP

In pilot purification experiments, samples (20 µl) from each collected fraction were analysed by SDS-PAGE (Figure 3.4). By comparison to uninduced culture (H0), the lysate obtained at optimal expression time (H5) showed the presence of a ~33.4 kD protein. Recombinant MIP protein was present in the supernatant (SN) rather than cellular debris (CD). Some rMIP was found in the flow through (FT), suggesting over-loading of the Ni-NTA resin. A single protein band of ~33.4 kDa in size was observed in each 0.5 ml eluate fraction (E1-E4) and the maximal level of protein was found in eluate samples E1 and E2.

3.2.2 Scale-up purification of rMIP protein

In order to produce sufficient rMIP for immunisation studies, overexpression and purification were scaled up to a volume of 2 L of SOB medium. The culture was then processed under native conditions and resulted in ~100 ml of the supernatant which contained soluble rMIP protein. Figure 3.5 shows the SDS-PAGE analysis of a representative large scale experiment for rMIP purification. In this experiment, a volume of 25 ml of the supernatant prepared under native conditions was purified on 5 ml of Ni-NTA resin. In comparison to the lysate before induction (H0), the whole lysate 5 hours after induction (H5) showed a strong band of rMIP. Although there was some protein present in the cellular debris (CD), the amount was relatively low compared to that present in the supernatant (SN). The rMIP was also present in the flow-though (FT) indicating that the rMIP binding exceeded the binding capacity of the Ni-NTA resin. Little protein was present in wash fractions W1 and W2 and maximal elution of rMIP was observed from fraction E2-E4/5.

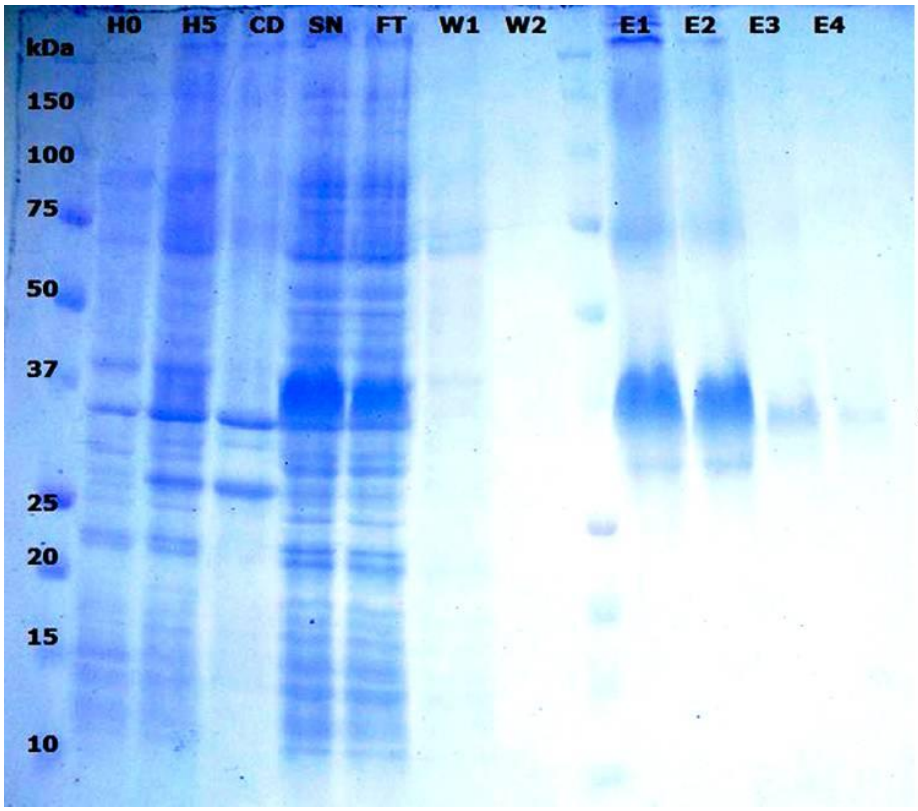

Figure 3.4 SDS-PAGE analysis of samples from pilot purification of rMIP.

In order, the samples (20 µl each) were whole lysate of *E.coli* before IPTG induction (H0), whole lysate at hour 5 after IPTG induction (H5), cellular debris (CD), the supernatant sample (SN), flow through (FT), 2 wash fractions (W1 and W2) and 4 fractions of eluate (E1-E4). The arrow indicates the presence of ~33.4 kDa bands.

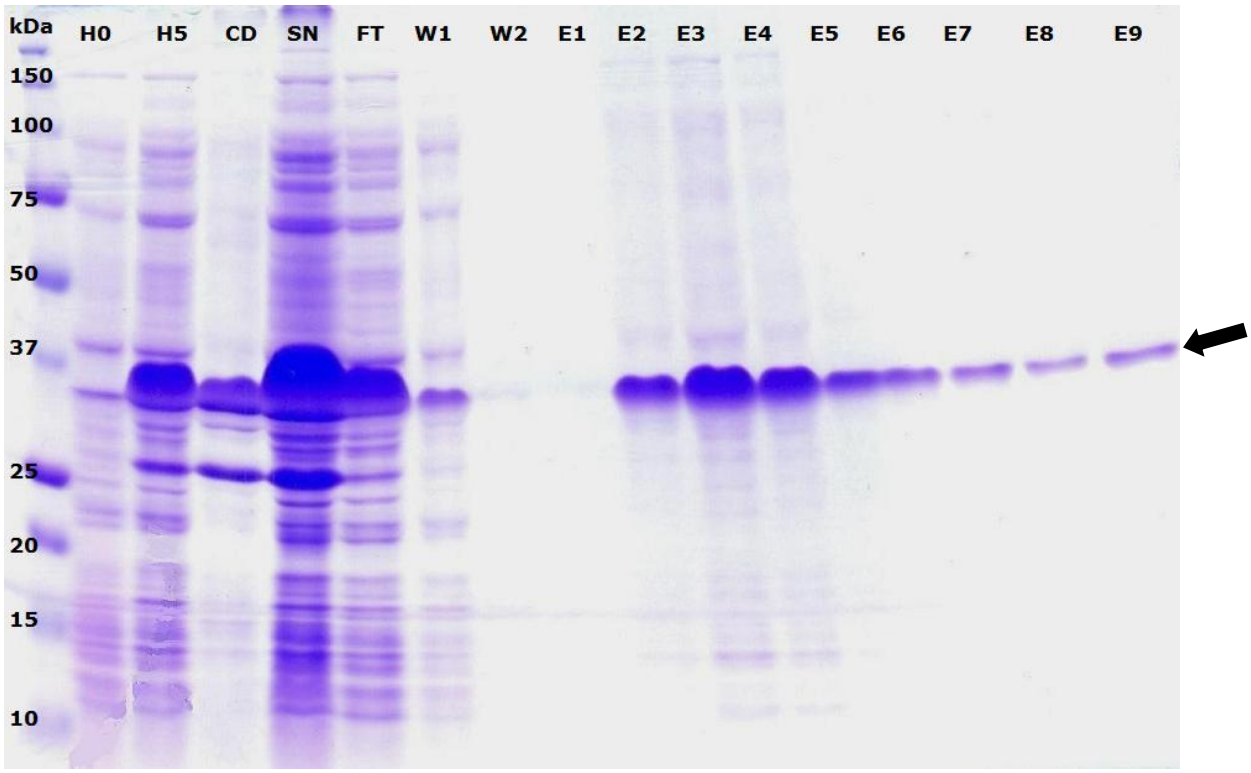

Figure 3.5 SDS-PAGE analysis of samples from scale-up purification of rMIP.
Samples (20 µl) taken from a scale-up purification were analysed by SDS-PAGE. In order, they were whole lysate before IPTG induction (H0), whole lysate at hour 5 after IPTG induction (H5), cellular debris (CD), the supernatant sample (SN), flow through (FT), 2 wash fractions (W1 and W2) and 9 fractions of eluate (E1-E9). The arrow indicates the presence of ~33.4 kDa bands.

Although several purification runs with higher ratios of the supernatant to Ni-NTA resin were done, rMIP was still found in the flow through suggesting that higher amounts of Ni-NTA resin were needed to bind total protein expressed. To estimate the yield rate of rMIP per litre of bacterial culture, 0.5 ml of the supernatant sample from the same lysate preparation was purified on 10 ml of Ni-NTA resin. Samples from collected fractions were analysed by SDS-PAGE (Figure 3.6). There was no visible band in the flow through (FT) or wash fractions (W1 and W2). Therefore, the rMIP present in the sample did not exceed the binding capacity of the Ni-NTA resin. In addition, recombinant MIP was present from the third to seventh eluate fraction (E3-E7).

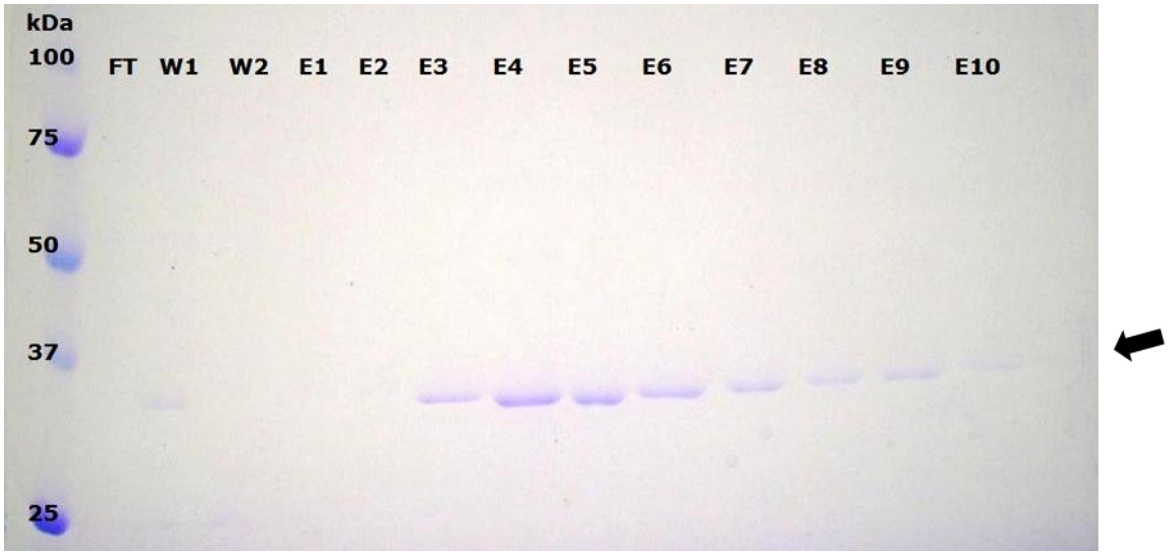

Figure 3.6 SDS-PAGE analysis of scale-up purification of rMIP with a lowest protein to Ni-NTA resin ratio. In order, samples (20 µl) were flow through (FT), 2 wash fractions (W1 and W2) and fractions of eluate (E1-E9). The arrow indicates the presence of ~33.4 kDa bands.

The fractions from the purification without excessive rMIP appearing in the flow-through were pooled and protein amount was determined by BCA assay. The yield of rMIP was estimated to be approximately 74.2 mg per litre of culture.

In order to remove imidazole, fractions containing the relatively high level of rMIP protein were pooled and dialysed against PBS containing 0.0001% (w/v) thimerosal. SDS-PAGE analysis of rMIP protein (10 µg) before and after dialysis showed that dialysis did not result in protein loss (Figure 3.7). After dialysis, rMIP was stored at -20°C before use.

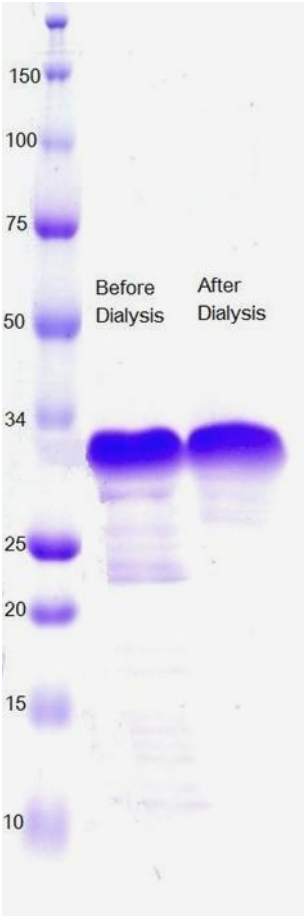

Figure 3.7 SDS-PAGE analysis of rMIP protein (10 µg), before and after dialysis.

3.3 Detection of LPS in protein samples

The presence of any contaminating LPS in rMIP preparations was analysed by low molecular weight SDS-PAGE/silver staining and by Limulus Ameboycte Lysate (LAL) assay. As a result, silver stain for LPS showed that no contaminating LPS was visible in the rMIP preparation (Figure 3.8). In addition, LAL assay confirmed the presence of a very low level of LPS (≤0.7% wt/wt) in the rMIP protein samples.

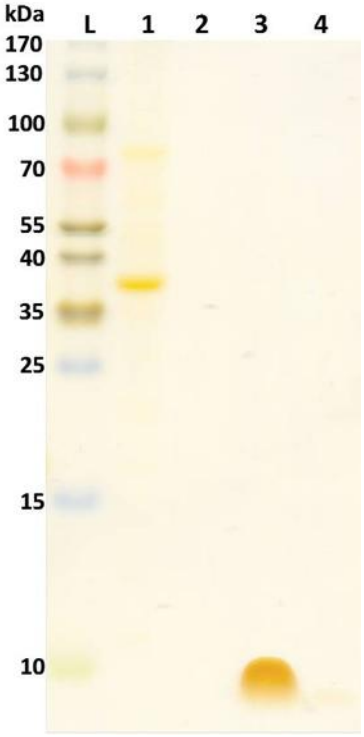

Figure 3.8 Silver stain for LPS. Lane 1 contains protein ladder. Lane 2, rMIP (10 μg); lane 3, loading buffer only; lane 4 and 5, *E.coli* LPS (10 μg and 1 μg respectively).

3.4 Humoral immune response to rMIP protein

The antigenicity and immunogenicity of rMIP was investigated by immunizing mice and rabbits with recombinant proteins in a variety of different adjuvant formulae (Section 2.13). The resulting antisera were tested for biological functions in a variety of *in vitro* assays.

3.4.1 ELISA of antisera raised to rMIP

Murine antisera raised against different formulations containing rMIP showed high levels of antibodies that reacted with rMIP (Figure 3.9 A) and also showed cross reactivity with native MIP in the OM of MC58 (Figure 3.9 B). Moreover, rMIP in ZW 3-14 micelles (mean titre of 1,151,000) or adsorbed to $Al(OH)_3$ (mean titre of 1,550,000) induced statistically higher titres than protein incorporated in liposomes (mean titre of 212,000) or liposomes + MPLA (mean titre of 214,000) ($p<0.05$). The addition of MPLA into micelles (mean titre of 1,435,000) or liposomes increased the production of antibodies though it did not reach statistical significance compared to antibody titres induced by liposomes or micelles preparations alone

(p>0.05). In particular, antibodies to rMIP could be induced by immunisation with rMIP in saline (mean titre of 822,000). Immunisation with MC58 OM on alum also induced antibodies (mean titre of 83,000) that reacted with the rMIP protein (Figure 3.9 A). Animals that were sham immunised did not produce antibodies that reacted with rMIP in ELISA (Figure 3.9 A).

Murine antisera raised against rMIP were also tested against OM from the homologous strain MC58 (Figure 3.9 B). Significant reactivity to MIP present in OM was observed with antisera raised to all the different rMIP formulations (mean titres between 1,200 to 18,000) though there were no statistically significant differences between the mean antibody levels observed for any of the groups (p>0.05). As expected, significantly higher (p<0.05) anti-OM antibodies were induced by immunisation with OM (mean titre of 226,000) compared with rMIP protein formulae. No significant reactivity against OM was observed with antisera from sham immunised animals (Figure 3.9 B).

Rabbit antisera raised against rMIP protein in Freund's adjuvant also showed high levels of antibody. The reciprocal ELISA endpoint titres of 2 rabbit antisera were 1,023,000 and 1,072,000 against rMIP protein, but lower at 1,000 and 2,500 against MC58 OM. Pre-immune rabbit sera showed little reactivity (<100) either against rMIP or MC58 OM.

3.4.2 Antibodies recognised native MIP in lysate and OM of MC58

The specificity of the immune response against rMIP was also investigated by western blotting using whole-cell lysate and OM preparations of the homologous strain MC58 (Figure 3.10). All murine antisera raised to rMIP tested at 1/100 dilution showed strong reactivity with a band of *Mr* ~29 kDa and sera from the corresponding sham immunised animals were non-reactive (Figure 3.10). Similarly, both rabbit antisera raised to rMIP tested at 1/500 dilution (*Mr* ~29 kDa) showed specific reactivity with both lysate and OM of MC58 (Figure 3.11).

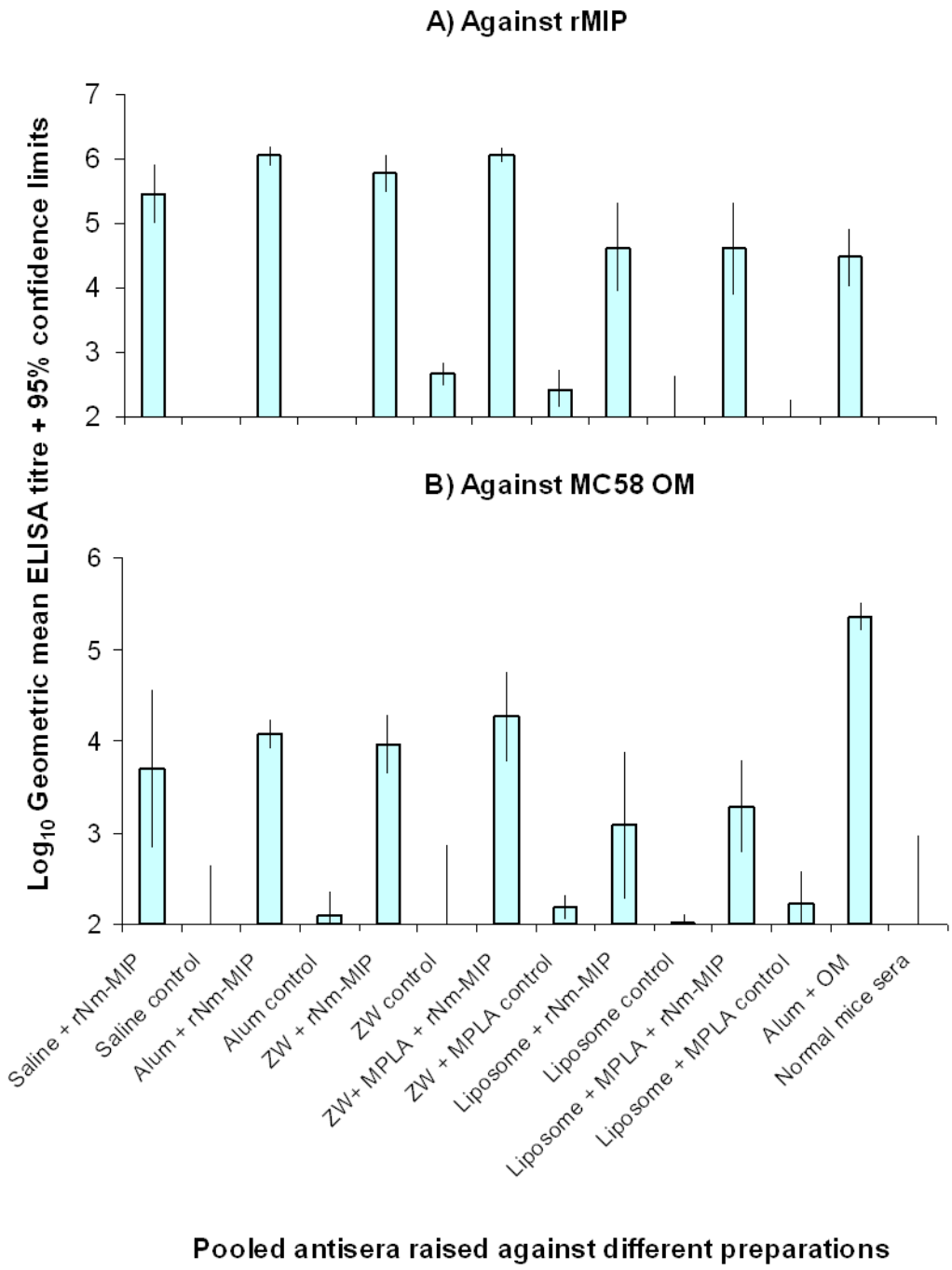

Figure 3.9 ELISA reactivity of murine antisera raised against different rMIP formulations. Murine antisera were reacted with purified rMIP (A) and OM of MC58 (B). The columns represent the geometric mean of reciprocal ELISA titres (*n*=5 animals per group) and the error bars the 95% confidence intervals.

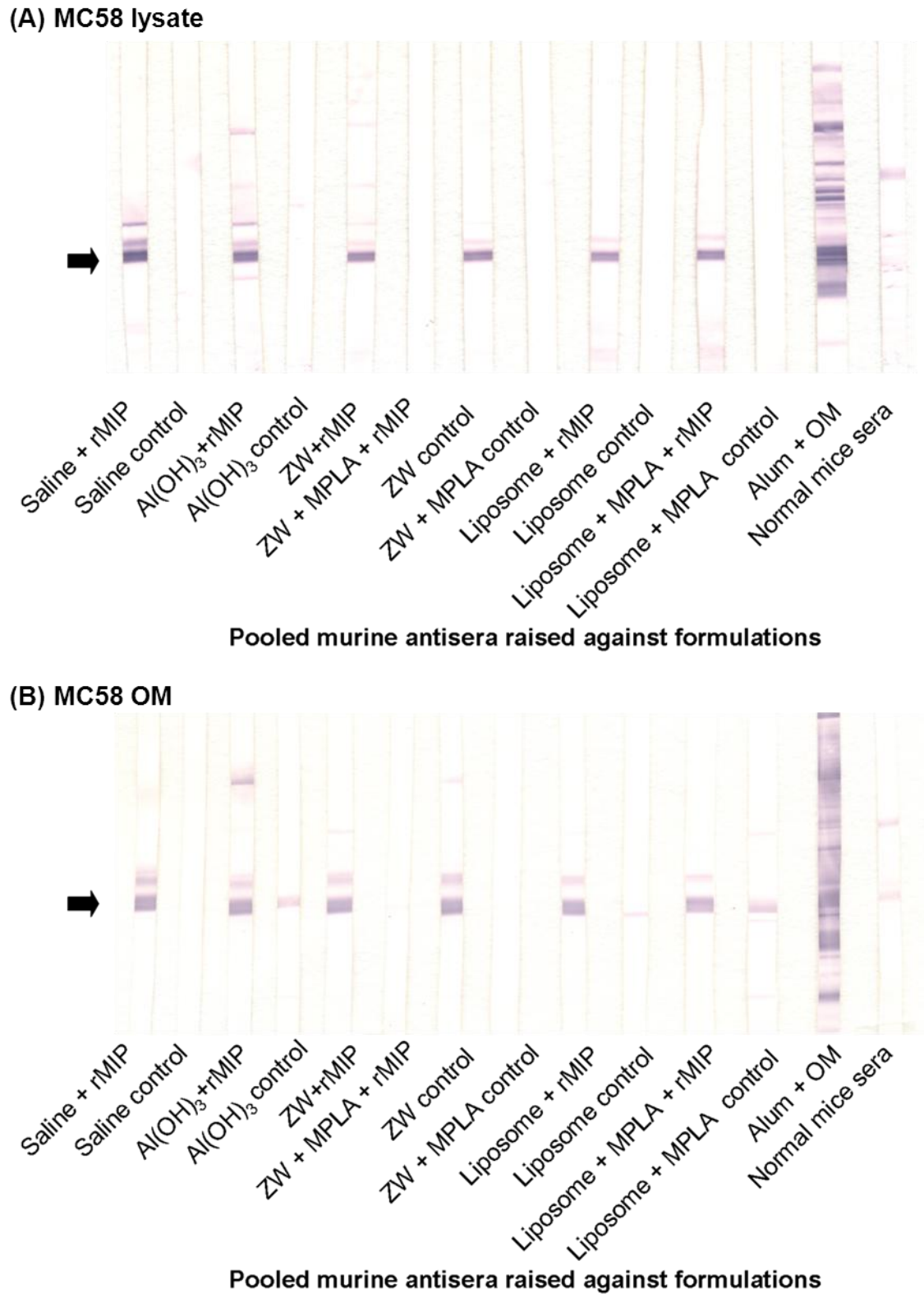

Figure 3.10 Western Blots of whole cell lysate (A) and OM (B) of MC58 were reacted with pooled murine antisera raised against different rMIP-formulations. The black arrows indicate the presence of native MIP with *Mr* of ~29 kDa.

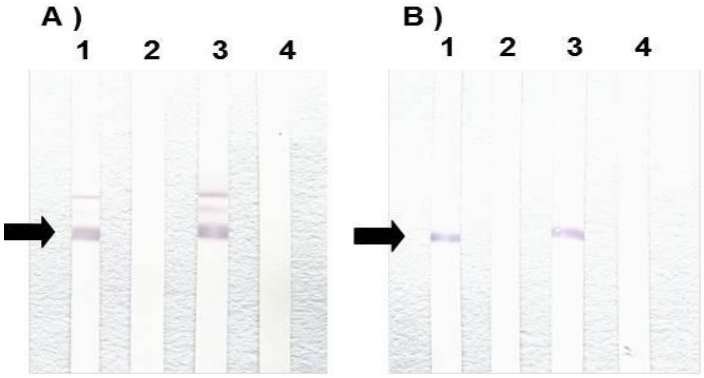

Figure 3.11 Western blot using rabbit anti-rMIP sera (1/500 dilution) reacted with A) whole cell lysate and B) OM of MC58. Blot 1 and 2 were reacted with post-immunization and pre-immune sera of rabbit 1. Blot 3 and 4 were reacted with post-immunization and pre-immune antiserum from rabbit 2. The black arrows indicate the presence of native MIP with *Mr* ~29 kDa.

3.5 Antibody binding to MIP on the surface of MC58

Pooled antisera of each group of animals immunized with the different rMIP formulations, was reacted (1/100 dilution) with fixed whole meningococcal cells and antibody binding detected with fluorescence conjugate (Figure 3.12). Antisera raised to rMIP in saline, ZW 3-14 micelles + MPLA, liposomes + MPLA and adsorbed to aluminium hydroxide, preparations showed strong reactivity with MC58 compared to those raised to ZW 3-14 micelles and liposomes. Sera from the corresponding sham immunised animals were non-reactive (Table 3.1).

Table 3.1 Reactivity of antisera raised against rMIP on whole MC58 meningococcal cells determined by IF.

	IF reactivity	
Formulation	**+ rMIP**	**-rMIP**
Saline	++	-
$Al(OH)_3$	++	-
ZW 3-14 micelles	±	-
ZW 3-14 micelles + MPLA	++	-
Liposome	±	-
Liposome + MPLA	++	-

IF reactivity is indicated as follows: non-reactivity (-), weak (±), medium (+) and strong (++) determined as previously described (Jolley *et al.* 2001). For comparison, antiserum raised to OM showed very strong (+++) reactivity. Data are representative of 2 independent experiments.

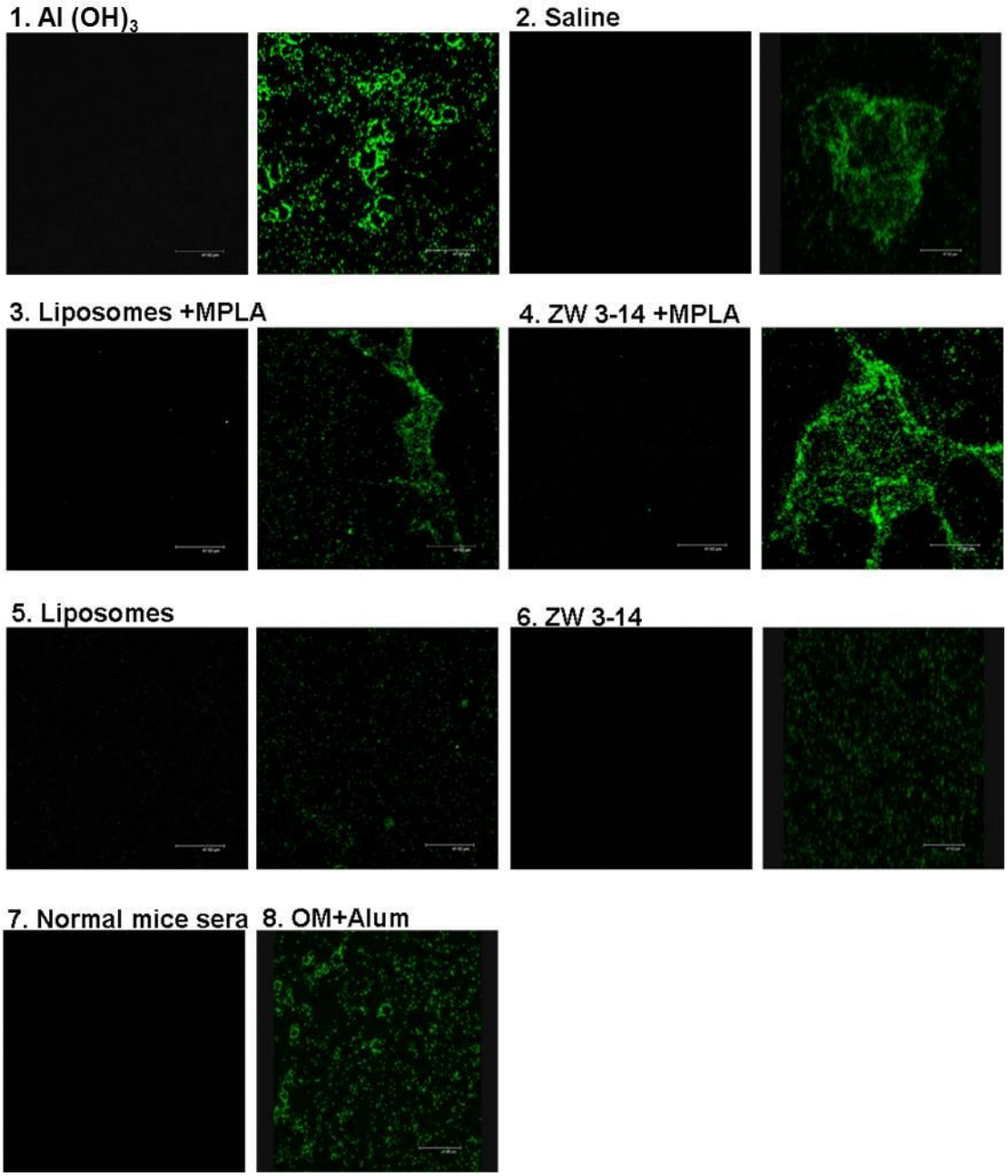

Figure 3.12 Binding of antibodies with MIP protein on the bacterial surface of MC58 detected by immunofluorescence (IF). Pooled murine antisera (1/100 dilution) were reacted with fixed MC58. For each pair of figures, the left figure shows no antibody binding with sera from sham immunization while the right figure shows reaction of MC58 with anti-rMIP sera raised against different formulae (1. $Al(OH)_3$; 2. Saline; 3. Liposomes + MPLA; 4. ZW 3-14 micelles + MPLA; 5. Liposomes and 6. ZW 3-14 micelles). Normal mouse serum was not reactive whereas and strong reactivity with sera raised to OM + Alum was observed.

3.6 Recombinant MIP can elicit bactericidal antibodies against MC58

Pooled murine antisera raised to different rMIP preparations were tested for their bactericidal activity against the homologous strain MC58. As shown in Table 3.2, the highest bactericidal antibody titres were produced by murine antisera raised against rMIP either in ZW 3-14 + MPLA or incorporated to liposomes + MPLA (1024). Recombinant MIP in saline or liposomes elicited similar bactericidal antibody titres (128). By contrast, adsorption of rMIP onto aluminium hydroxide or incorporation of rMIP in to ZW 3-14 micelles did not generate bactericidal activity.

Table 3.2 Bactericidal activity of pooled antisera raised against rMIP protein for the homologous strain MC58.

	Serum bactericidal titre[a] against MC58	
Formulation	**+ rMIP**	**-rMIP**
Saline	128	<8
$Al(OH)_3$	<4	<4
ZW 3-14 micelles	<4	8
ZW 3-14 micelles + MPLA	1024	<4
Liposome	128	<4
Liposome + MPLA	1024 (512, 2048)	<4

[a]Titres are expressed as the reciprocal of the highest dilution at which 50% killing was observed. Normal mouse serum and sera from mice immunised with MC58 OM showed titres <8 and 20,000 respectively. Data are the median values, with the range of values in parenthesis, for SBA from three or more independent measurements of bactericidal activity of all pooled serum samples. Single values denote that the SBA titres were identical from the independent experiments. Data are representative of 3 or more independent measurements of bactericidal activity of all pooled serum samples.

3.7 Conservation of MIP protein amongst surveyed meningococci

3.7.1 The mip gene was highly conserved

The DNA sequencing result of *mip* gene from each strain was translated into amino acid sequence and aligned using MegAlign programme in Lasergene 8 (Figure 3.13). The chosen strains were very different in serogroup, serotype and serosubtype. MIP protein showed high conservation amongst the surveyed strains with very few amino acid differences (98-99% similarity). Five strains, including MC168, L2470, MC172, MC174 and MC180 shared the same MIP protein sequence type as MC58 (type I). As shown in Figure 3.13, another 5 strains (MC90,

MC161, MC162, MC179 and MENC11) were of sequence type II MIP, which is devoid of 4 amino acids (aa 27-30 and has 2 amino acid differences (Ser^{39} →Ala^{39} and Thr^{165} →Ser^{165}) compared to type I MIP. The other 2 strains (MC54 and MC173) were of type III MIP protein, which has 3 amino acid changes very close to each other (Ala^{140} →Gly^{140}, Asp^{142} →Glu^{142} and Gly^{143} →Ser^{143}).

```
I   (MC58)     1  MNTIFKISALTLSAALALSACGKKEAAPASASEPAAASSAQGDTSSIGSTMQQASYAMGV  60
II  (MC90)     1  MNTIFKISALTLSAALALSACGKKEA----ASEPAAASAAQGDTSSIGSTMQQASYAMGV  60
III(MC54)      1  MNTIFKISALTLSAALALSACGKKEAAPASASEPAAASSAQGDTSSIGSTMQQASYAMGV  60

I   (MC58)     61 DIGRSLKQMKEQGAEIDLKVFTEAMQAVYDGKEIKMTEEQAQEVMMKFLQEQQAKAVEKH  120
II  (MC90)     61 DIGRSLKQMKEQGAEIDLKVFTEAMQAVYDGKEIKMTEEQAQEVMMKFLQEQQAKAVEKH  120
III(MC54)      61 DIGRSLKQMKEQGAEIDLKVFTEAMQAVYDGKEIKMTEEQAQEVMMKFLQEQQAKAVEKH  120

I   (MC58)    121 KADAKANKEKGEAFLKENAAKDGVKTTASGLQYKITKQGEGKQPTKDDIVTVEYEGRLID  180
II  (MC90)    121 KADAKANKEKGEAFLKENAAKDGVKTTASGLQYKITKQGEGKQPSKDDIVTVEYEGRLID  180
III(MC54)     121 KADAKANKEKGEAFLKENAGKESVKTTASGLQYKITKQGEGKQPTKDDIVTVEYEGRLID  180

I   (MC58)    181 GTVFDSSKANGGPVTFPLSQVIPGWTEGVQLLKEGGEATFYIPSNLAYREQGAGDKIGPN  240
II  (MC90)    181 GTVFDSSKANGGPVTFPLSQVIPGWTEGVQLLKEGGEATFYIPSNLAYREQGAGDKIGPN  240
III(MC54)     181 GTVFDSSKANGGPVTFPLSQVIPGWTEGVQLLKEGGEATFYIPSNLAYREQGAGDKIGPN  240

I   (MC58)    241 ATLVFDVKLVKIGAPENAPAKQPAQVDIKKVN  272
II  (MC90)    241 ATLVFDVKLVKIGAPENAPAKQPAQVDIKKVN  272
III(MC54)     241 ATLVFDVKLVKIGAPENAPAKQPAQVDIKKVN  272
```

Figure 3.13 Amino acid sequence alignment of MIP from surveyed strains with MIP of MC58. Amino acid sequences of MC168, MC172, MC174, MC180 and L2470 were identical to MC58 (I); MC90, MC161, MC162 and MC179 and MENC11were the same (II) and MC54 and MC173 (III) showed a third amino acid sequence type. Red and green shading denotes amino acid deletions or changes for type II and type III MIP respectively.

3.7.2 Similar expression level of MIP protein in each strain

Whole cell lysate from each strain was subjected to SDS-PAGE analysis and then transferred onto nitrocellulose. Rabbit antisera (1/500) were then reacted with the blot and similar expression levels of MIP protein were found across the strains (Figure 3.14). The lower molecular weight of type II MIP protein was due to the absence of the 4 amino acids. In addition, the presence of double bands indirectly suggested that the protein can be present both in lipidated and non-lipidated forms.

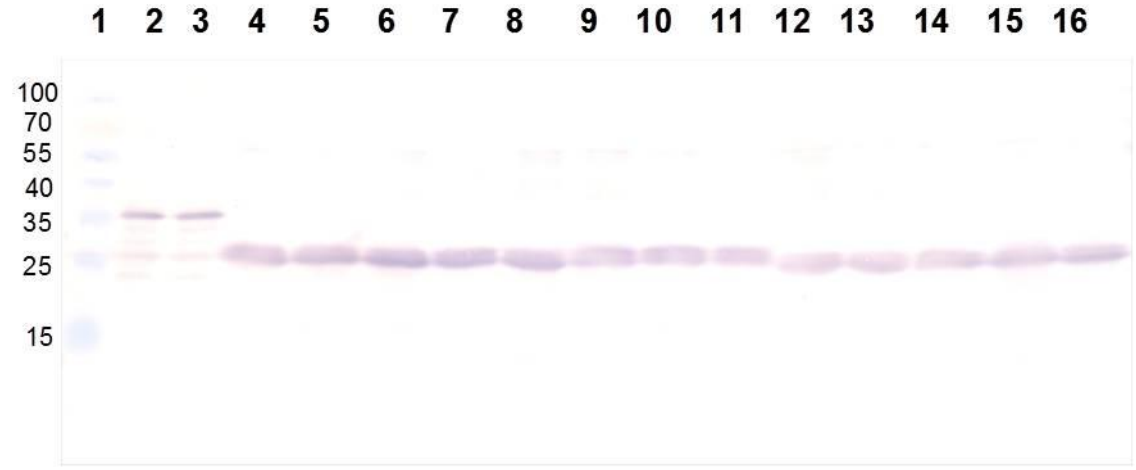

Figure 3.14 Western blot showed reactivity of rabbit anti-rMIP sera (1/500) with lysates (15 µg) of sequenced meningococcal strains. Lane 1 contains protein ladder. Lane 2 and 3 contains rMIP (0.1 µg). Lanes 4-11 in order are MC58, L2470, MC168, MC172, MC174, MC180, MC173 and MC54 (type I and III strains). Lanes 12-16 in order are MC90, MC161, MC162 MC179 and MENC11 (type II).

3.8 Antibody binding to MIP on other meningococcal strains

To test if the antibody raised to type I MIP could recognize different types of MIP expressed by other meningococcal strains, type I murine antisera, which showed heterologous killing (Section 3.9) were reacted against MC90 (expressing type II MIP) and MC54 (expressing type III MIP). Immunofluorescence experiments showed similar reactivity of antibody binding with different types of MIP proteins (Table 3.3).

Table 3.3 IF reactivity of antisera raised against type I rMIP with meningococcal strains expressing type II and III MIP proteins.

Sequence type/ Representative strains	Liposome	Saline
I /MC58	±	+
II /MC90	±	+
III /MC54	±	+

(See previous Table 3.1 legend for IF reactivity interpretation.)

3.9 Heterologous bactericidal activity of anti-rMIP (MC58) sera

Antisera that were bactericidal for homologous strain MC58 were tested for their ability to induce complement-mediated killing of other strains. As shown in Table 3.4, rMIP in saline or liposomes elicited similar bactericidal antibody titres (128-256) for strains with all the 3

different types of MIP. Although antisera raised to rMIP in liposomes + MPLA killed MC90, there was no bactericidal activity towards strain MC54 (type III MIP). Similarly, antisera to rMIP in ZW 3-14 micelles + MPLA killed both MC90 and MC54 but the bactericidal titre for the later was much lower (64).

In addition, rabbit antisera raised against rMIP in Freund's adjuvant also showed cross-strain bactericidal activity. It has been shown that different complement sources had an influence on SBA titres (Skarnes 1978a; Skarnes 1978b). To test a complement source different from the antisera (rabbit), guinea pig complement was chosen, since this has been used with success previously (Christodoulides *et al.* 1993; Christodoulides and Heckels 1994) and the data were compared with those using baby rabbit (BR) complement. The killing titres for MC58 was 1/512 using baby rabbit (BR) complement and 1/64 using guinea pig complement; for MC179 (expressing type II), the titre was 1/64 using guinea pig complement; the killing titres for MC54 was 1/64 using BR complement and 1/32 using guinea pig complement. Overall, the bactericidal titres were lower using guinea pig complement.

Table 3.4 Bactericidal activity of pooled murine anti-rMIP protein for heterologous strains.

	Serum bactericidal titre[a] of antisera raised to rMIP in:			
Sequence type/representative strain	**Saline**	**Liposomes**	**Liposomes + MPLA**	**ZW 3-14 micelles + MPLA**
I/MC58	128	128	1024 (512, 2048)	1024
II/MC90	256 (256, 512)	256 (128, 256)	512 (512, 1024)	2048 (1024, 4096)
III/MC54	256	256	<4	64

[a]Pooled antisera raised to rMIP from MC58 were tested against heterologous strains with variations in MIP sequence. The titres are expressed as the reciprocal of the highest dilution at which 50% killing was observed. Titres for normal mouse sera and sera from mice immunised with controls without rMIP were <4. Data are the median values, with the range of values in parenthesis, for SBA from three or more independent measurements of bactericidal activity of all pooled serum samples. Single values denote that the SBA titres were identical from the independent experiments. Data are representative of 3 independent measurements of bactericidal activity of all pooled serum samples.

3.10 Effect of MPLA on the immunogenicity of rMIP

Based on the data above, the addition of MPLA to either liposomes or ZW 3-14 micelles seemed to induce higher bactericidal titres for the homologous strain. Therefore, we asked the question 'Whether addition of MPLA to rMIP alone without liposomes or ZW 3-14 micelles could elicit bactericidal antibodies?' To answer this question, another 5 groups of mice were immunized and antisera were analysed. The experimental design included rMIP in saline as a positive control and also as a repeat experiment. Apart from normal mouse sera, the other 3 immunization preparations included rMIP with MPLA (in saline), saline control and MPLA control (in saline).

3.10.1 Humoral immune response to rMIP protein preparations

Murine antisera raised against both formulations containing rMIP showed reactivity with rMIP and also showed cross reactivity with native MIP in the OM of MC58. As shown in Figure 3.15 A, rMIP with MPLA induced comparably high antibody titres (mean titre of 1,213,000) against rMIP compared to rMIP in saline (762,000) ($p>0.05$). Moreover, rMIP with MPLA preparation (mean titre of 2,000) induced significantly higher antibody titres against OM compared to rMIP in saline (mean titre of 900) ($p<0.05$) (Figure 3.15 B). Animals that were sham immunised did not produce antibodies that reacted with either rMIP or MC58 OM in ELISA (Figure 3.15 A and B).

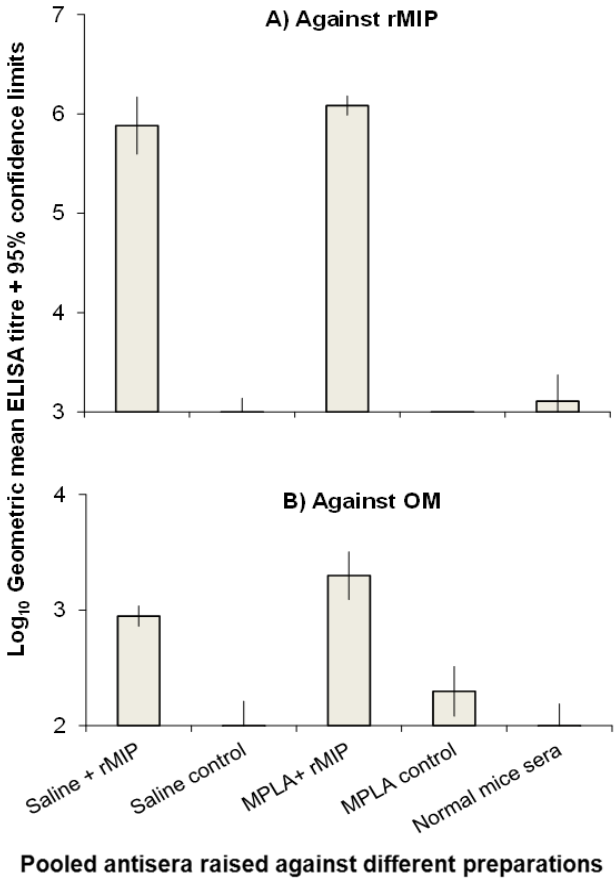

Figure 3.15 ELISA reactivity of murine antisera raised against different rMIP formulations. Pooled antisera were reacted with purified rMIP protein (A) or OM of MC58 (B). The columns represent the geometric mean of reciprocal ELISA titres (*n*=5 animal per group) and the error bars the 95% confidence intervals.

In addition, western blot also showed that antisera raised against rMIP in saline and rMIP with MPLA recognized a *Mr* ~29 kDa band either in whole cell lysate of MC58 or MC58 OM (Figure 3.16).

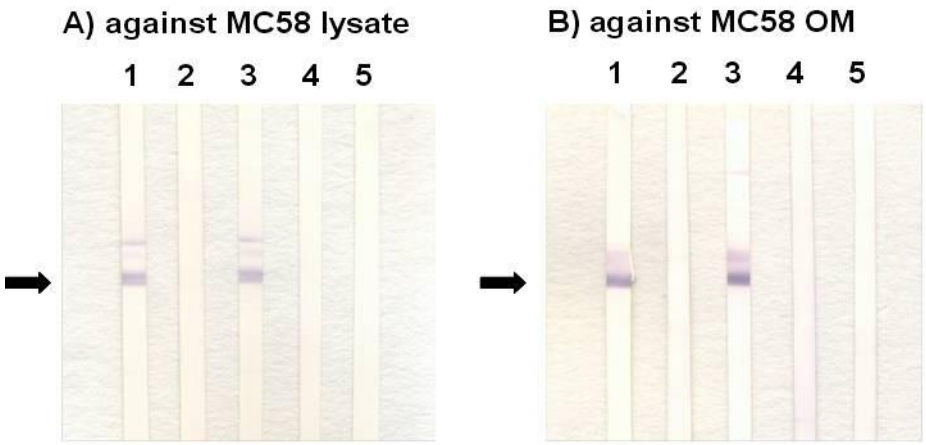

Figure 3.16 Western blots of whole cell lysate (A) and OM (B) of MC58 were reacted with pooled murine antisera raised against different formulations (1/100). Lanes 1-5, in order, were reacted with anti-sera raised against rMIP + saline, saline control, rMIP + MPLA, MPLA control and normal mouse sera. The black arrows indicate the presence of native MIP with *Mr* ~29 kDa.

3.10.2 Antibody binding on meningococcal cells

Immunofluorescence showed positive reactivity (+) for both antisera raised against rMIP + saline and rMIP + MPLA. By contrast, the reactivity was negative (-) using the pooled antisera raised against saline control and MPLA control.

3.10.3 Addition of MPLA to rMIP does not induce bactericidal antibodies.

However, antisera raised to MPLA + rMIP showed no bactericidal activity (<1/4) while antisera raised to saline + rMIP showed a reproducible result with a killing titre (1/256) comparable with that of the first batch of antisera raised to rMIP in saline (1/128) (Table 3.2).

Chapter 4 Immunogenicity of the recombinant Adhesin Complex Protein (rACP) and the role of ACP in pathogenesis

4.1 Cloning and expression of *acp* gene

The ligation reaction of *acp* gene insert and the pRSETA vector was transformed into *E.coli* DH5α competent cells and the transformants grown on LB-ampicillin plates were screened by PCR. Plasmids from 6 PCR-positive transformants were obtained and sequenced. Next, the recombinant plasmids with correct sequence data (Figure 4.1) were transformed into *E.coli* BL21(DE3)pLysS for protein expression.

In pilot expression experiments, *E.coli* BL21(DE3)pLysS – recombinant plasmid (pPRSETA-*acp*) were grown overnight in SOB-ampicillin (50 μg/ml)- chloramphenicol (30 μg/ml) liquid medium (2-3 ml) and then used to inoculate fresh medium (1 ml into 25 ml). Protein expression was induced by the addition of 1 mM IPTG and at every hour thereafter samples were removed and analysed by SDS-PAGE (Figure 4.2). Expression of recombinant ACP (rACP) protein was evident as early as 1 h after induction rising to maximal levels by 4 h. As a negative control, the same transformant was grown and sampled without IPTG induction and as expected showed no expression (Figure 4.2).

Native ACP protein contains 124 amino acids with a predicted molecular weight of 13.3 kDa. The recombinant protein produced in the pRSETA system produces a protein of 17.8 kDa (Figure 4.2).

ATAATTTTGTTTAACTTTAAGAAGGAGATATACAT**ATG**CGGGGTTCTCATCATCATCATCATCATGGTATGGCTAG
CATGACTGGTGGACAGCAAATGGGTCGGGATCTGTACGACGATGACGATAAGGATCGATGGGGATCCGAGCTCGAG
atgaaacttctgaccaccgcaatcctgtcttccgcaatcgcgctcagcagtatggctgccgccgctggcacggaca
accccactgttgcaaaaaaaaccgtcagctacgtctgccagcaaggtaaaaaagtcaaagtaacctacggcttcaa
caaacagggtctgaccacatacgcttccgccgtcatcaacggcaaacgcgtgcaaatgcctgtcaatttggacaaa
tccgacaatgtggaaacattctacggcaaagaaggcggttatgttttgggtaccggcgtgatggatggcaaatcct
accgcaaacagcccattatgattaccgcacctgacaaccaaatcgtcttcaaagactgttccccacgttaaTAGAA
GCTTGATCCGGCTGCTAACAAAGCCCGA

Figure 4.1 Sequencing data of *acp* gene insert. The bold and underlined 'ATG' shows the start codon of the recombinant protein. The box denotes the DNA sequence for 6xHisTag and the grey shadow shows the correct insert of *acp* gene.

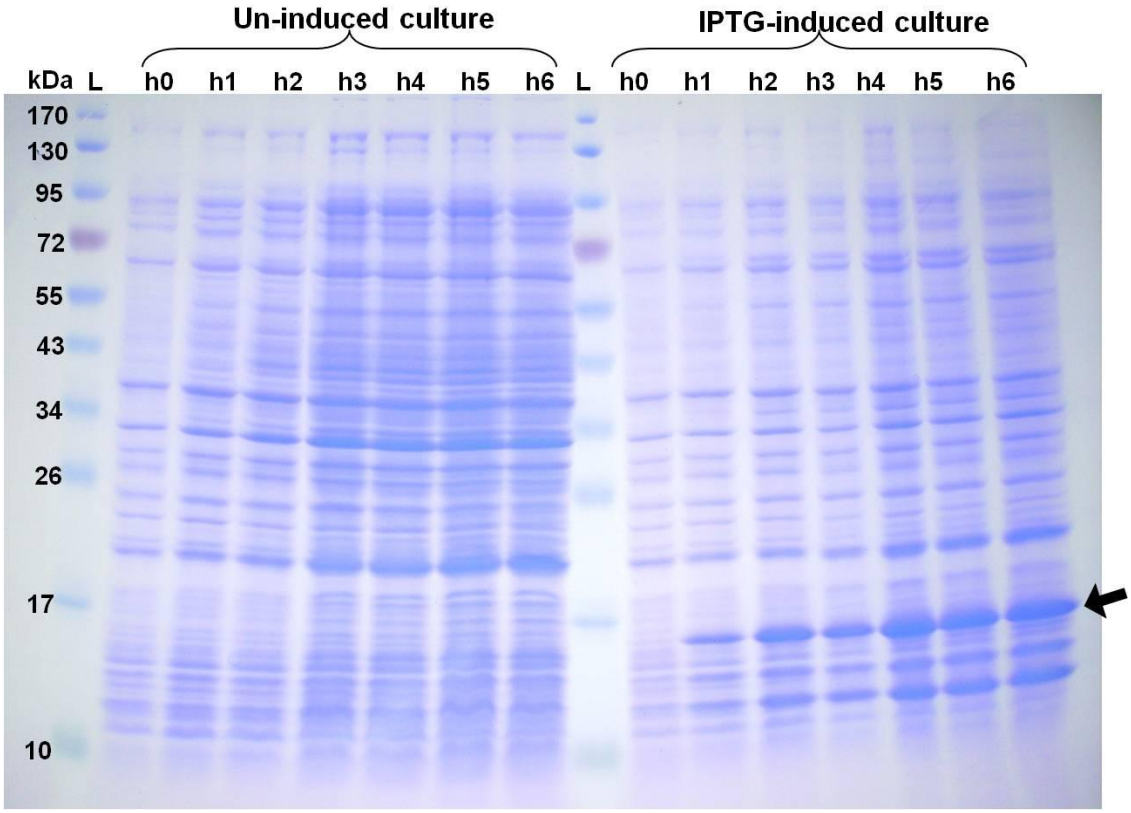

Figure 4.2 SDS-PAGE analysis of hourly samples from pilot expression of rACP. L contains protein ladder (kDa). Samples (20 µl) taken from hourly lysate (h0-h6) of both un-induced and IPTG-induced culture of BL21(DE3)pLysS-pRSETA-*acp* were analysed in order. The black arrow indicates the presence of ~17 kDa bands. Maximal expression of rACP was present 4 hours after induced expression.

To determine the solubility of rACP, IPTG-induced cultures of *E.coli* BL21(DE3)pLysS-pRSETA-*acp* were lysed by 3 freeze-thaw cycles and the resulting supernatants and pellets produced by centrifugation were analysed by SDS-PAGE (Figure 4.3). By contrast to rMIP, the rACP protein appeared to be in the cell pellet (P) predominantly, rather than in the supernatant (SN), demonstrating that the protein was insoluble and should be purified therefore under denaturing conditions.

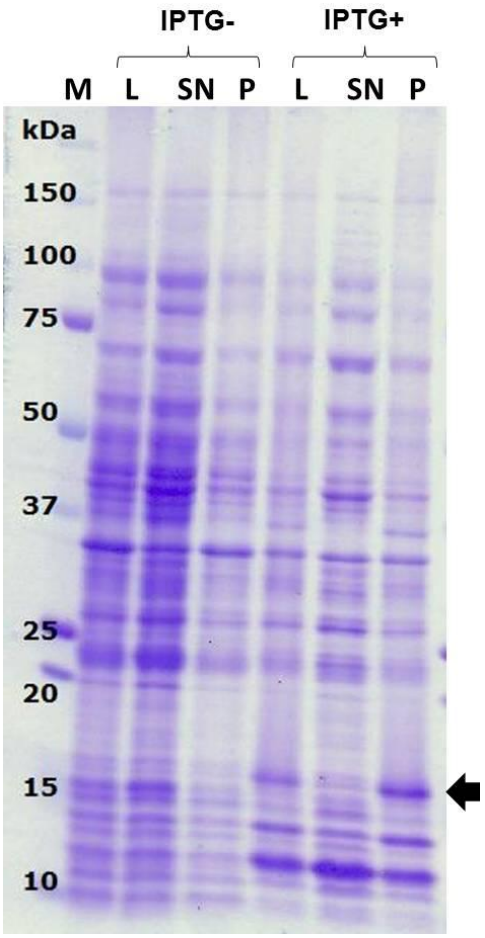

Figure 4.3 SDS-PAGE analysis of samples from solubility experiment of rACP. Lane M contains protein ladders. The whole lysate (L), the supernatant sample (SN) and the pellet (P) from un-induced (IPTG-) culture of E.coli BL21(DE3)pLysS-pRSETA-*acp* were analysed as negative controls. The samples from IPTG-induced (IPTG+) culture were analysed in the same order. Each lane contained 20 µg of proteins. The black arrow indicates a ~17 kDa band present in the pellet sample of IPTG-induced culture.

4.2 Purification of rACP under denaturing conditions

4.2.1 Pilot purification

In pilot purification experiments under denaturing conditions in the presence of 8M urea, samples from each collected fraction were analysed by SDS-PAGE (Figure 4.4). In comparison to the lysate of un-induced culture (H0), the lysate obtained at the optimal expression (H4) showed a doublet of protein bands. Recombinant ACP appeared predominately in the supernatant (SN) rather than in cellular debris (CD). Only a little rACP was present in the flow-through (FT) and wash fractions (W1-W4). A small amount of rACP was eluted by buffer D (DE1-DE4) and rACP was eluted mostly with buffer E (EE1-EE4). In addition to some degraded proteins appearing at lower molecular weight, dimers and trimers of the protein were observed at approximately 2 and 3 times the molecular weight of monomer. From the pilot experiment, a doublet of protein bands of ~17 kDa in size was observed in each 0.5 ml eluate fraction (EE1-EE4).

4.2.2 Mass spectrometry of rACP double bands

Due to the presence of double bands coincident with rACP, both bands were excised from the gel for mass spectrometric analysis. By comparing the amino acid sequence with the annotation of MC58 genome, both protein bands were determined to contain the same protein, rACP.

4.2.3 Scale-up purification of rACP

The volume of bacterial culture was increased to 1 L. After the lysate was prepared under denaturing conditions, 20 ml of supernatant was mixed with 5 ml of 50% (w/v) Ni-NTA resin and purification carried out. Based on the pilot purification experiments, only buffer E (pH 4.5) was used for elution. Figure 4.5 shows the SDS-PAGE analysis of samples from each collected fraction. In comparison to un-induced culture (H0), IPTG-induced culture at 4 hour (H4) contained an increased amount of rACP. The cellular debris (CD) contained negligible rACP protein while the majority of rACP was present in the supernatant. The flow-through (FT) and wash fractions (W1 and W2) all showed minimal rACP. The doublet of bands was observed again on elution and most rACP was present in fractions 3-6 (E3-E6).

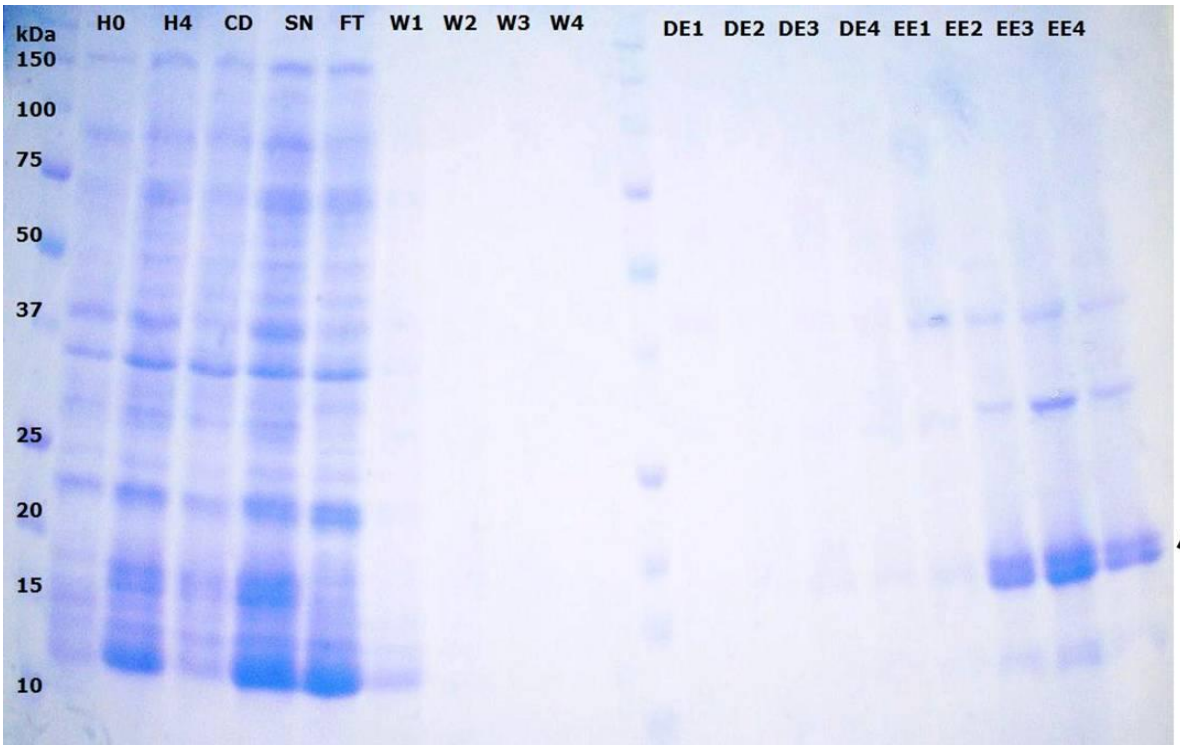

Figure 4.4 SDS-PAGE analysis of samples from pilot purification of rACP.

In order, the samples (20 µl each) were whole lysate of *E.coli* before IPTG induction (H0), whole lysate at hour 4 after IPTG induction (H4), cellular debris (CD), the supernatant sample (SN), flow through (FT), 4 wash fractions (W1 - W4), 4 fractions eluted with buffer D (pH 5.9) (DE1-DE4) and 4 fractions eluted with buffer E (pH 4.5) (EE1-EE4). The arrow indicates the presence of double bands of *Mr* ~17 kDa.

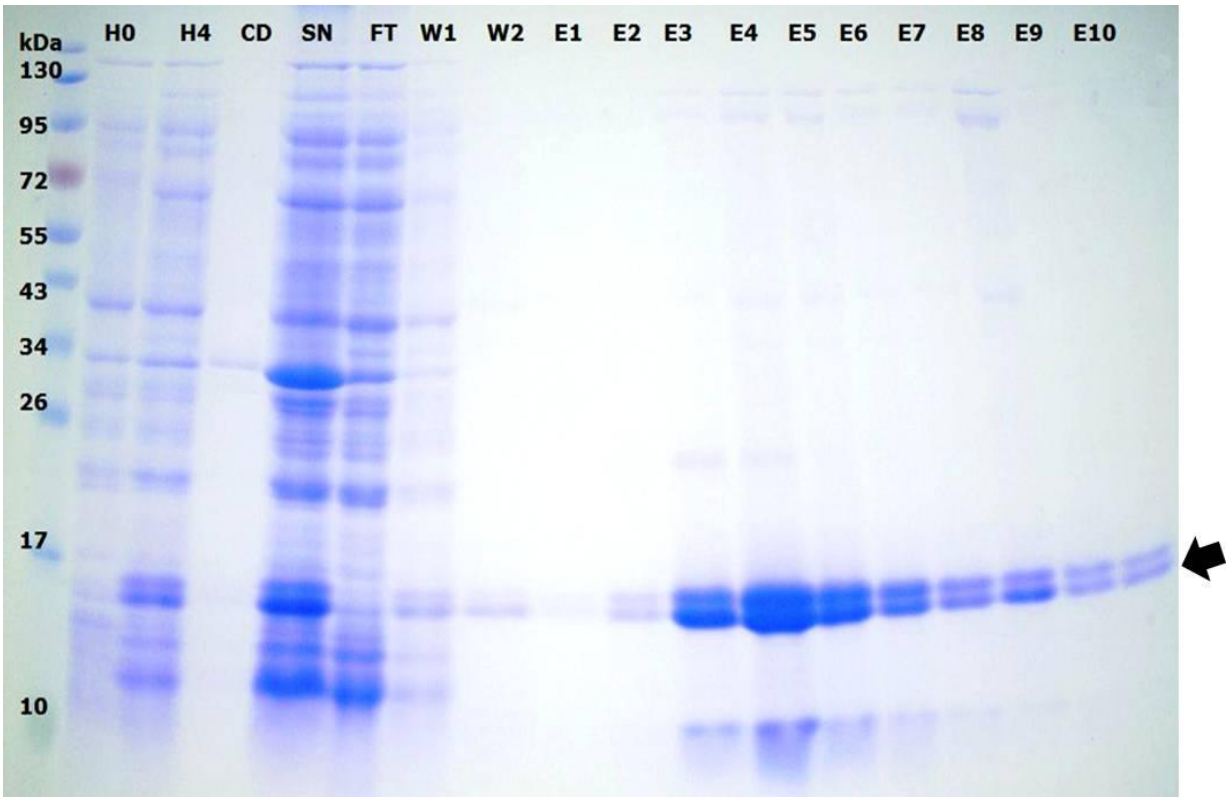

Figure 4.5 SDS-PAGE analysis of samples from scale-up purification of rACP.

Samples (20 µl) taken from a scale-up purification were analysed by SDS-PAGE. In order, they were whole lysate before IPTG induction (H0), whole lysate at hour 4 after IPTG induction (H4), cellular debris (CD), the supernatant sample (SN), flow through (FT), 2 wash fractions (W1 and W2) and 10 fractions of eluate with buffer E(E1-E10). The arrow indicates the presence of ~17 kDa bands.

4.3 Presence of urea leads to the rACP doublet

Since the doublet of protein bands appeared only in purified rACP, we reviewed the SDS-PAGE analysis for rACP solubility (Figure 4.3) and pilot expression of rACP (Figure 4.2). The result showed that double bands of rACP were not present in both experiments in which PBS was used to prepare the samples. However, to purify the protein under denaturing conditions, 8 M urea was added in every buffer (Buffer B-E) used.

Based on these observations, we tested the hypothesis that the presence of 8M urea was responsible for modifying the protein. To test this hypothesis, a small scale IPTG induction of bacterial growth until hour 4 was carried out and then 1 ml of culture was centrifuged to obtain the pellet. The pellet was suspended in 100 µl of i) PBS, ii) lysis buffer with 8 M urea (Buffer B) and iii) lysis buffer with 6 M guanidine hydrochloride (GuHCl) (Buffer A). The bacterial suspension was sonicated to obtain a lysate and a 10 µl sample from each lysate was used for SDS-PAGE analysis. As shown in Figure 4.6, rACP appeared as a single band in both PBS and Buffer A (6 M GuHCl buffer). However, the characteristic double bands appeared only when 8M urea was present in the protein solution.

Therefore, we decided to replace 8 M urea with 6 M GuHCl throughout the whole purification procedure. Samples (20 µl) from each collected fraction were analysed by SDS-PAGE (Figure 4.7). In comparison to whole lysate obtained before IPTG induction (H0), the sample from hour 4 after IPTG induction (H4) showed a strong band of rACP. Little or no rACP was present in flow-through (FT) or wash fractions (W1 and W2). A single band of rACP eluted mainly between fractions 2-6 (E2-E6). Minor protein degradation was observed with lower molecular weight bands visible. Interestingly, the doublet of rACP was no longer present. All eluted fractions were pooled before protein precipitation and the protein concentration was determined by BCA assay. The estimated purification rate of rACP was ~34 mg per litre of culture. Suspension of the precipitated protein was analysed by SDS-PAGE and showed a single band with high purity (Figure 4.8 A).

4.4 LPS is not present in rACP samples

A 10 µg rACP sample and *E.coli* LPS (1 µg and 10 µg) were analysed on a low molecular weight gel. Silver stain for LPS showed no visible LPS bands in the rACP sample (Figure 4.8 B). LAL assay showed that the absorbance of the rACP sample (20 µg) was lower than LAL water, which was assumed to be blank. Therefore, there was no detectable LPS in rACP samples.

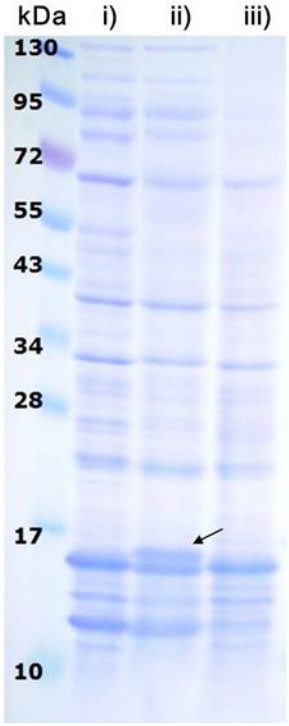

Figure 4.6 SDS-PAGE analysis for comparing rACP prepared in different buffers. The whole lysate of IPTG-induced culture from BL21(DE3)plysS-pRSETA-*acp* was prepared in

i) PBS;

ii) lysis buffer containing 8 M urea (Buffer B);

iii) lysis buffer containing 6 M GuHCl (Buffer A).

The arrow indicates the rACP doublet protein present only in 8M urea buffer.

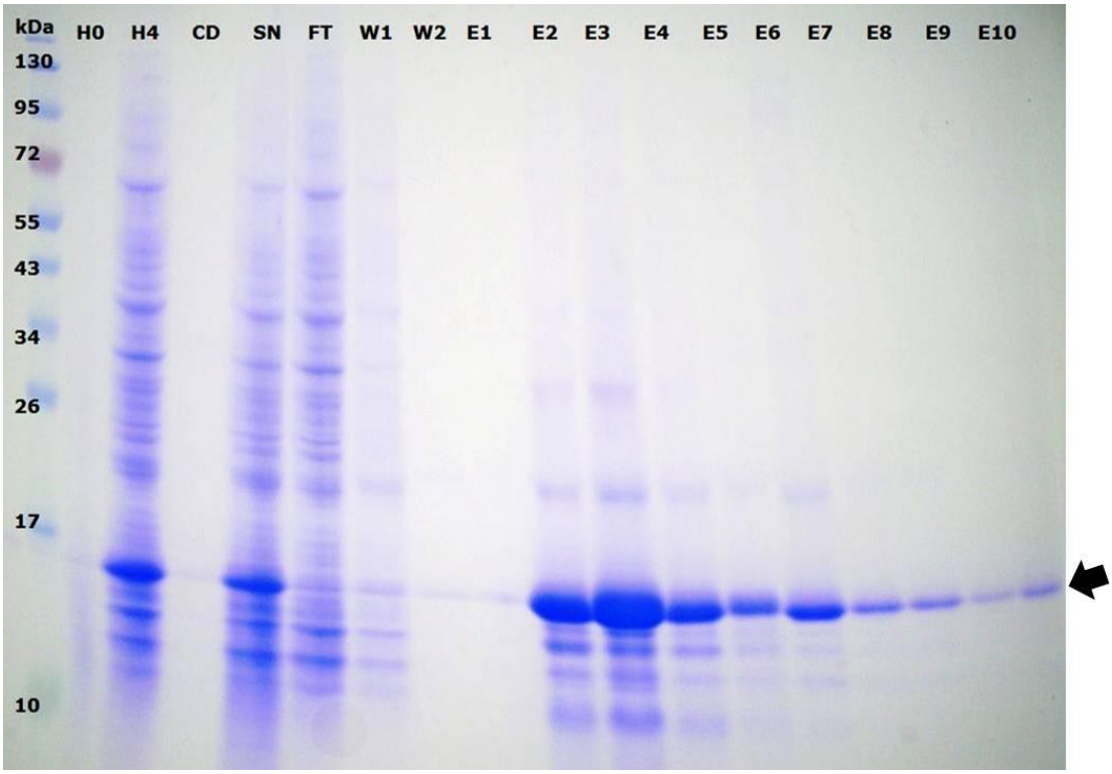

Figure 4.7 SDS-PAGE analysis of samples from scale-up purification of rACP with buffers containing 6 M GuHCL under denaturing conditions. In order, they were whole lysate before IPTG induction (H0), whole lysate at hour 4 after IPTG induction (H4), cellular debris (CD), the supernatant sample (SN), flow through (FT), 2 wash fractions (W1-W2) and 10 fractions of eluate with buffer E (E1-E10). The arrow indicates the presence of ~17 kDa bands.

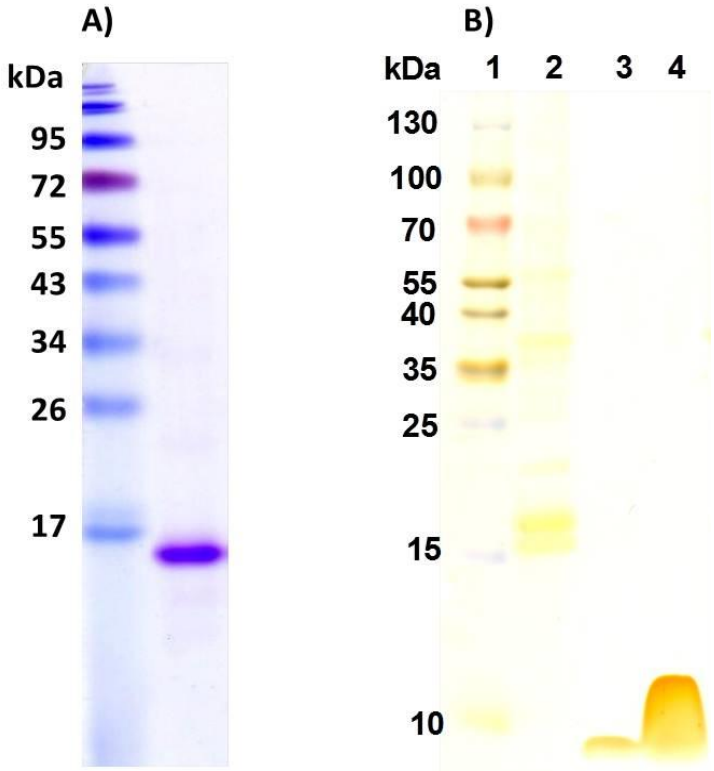

Figure 4.8 A) SDS-PAGE analysis of purified rACP (10 μg); B) Silver stain for LPS. Lane 1 contains molecular weight markers. Lane 2-4 in order contain rACP (10 μg) and *E.coli* LPS (1 μg and 10 μg respectively). No LPS is visible in the rACP sample.

4.5 Humoral immune response to rACP protein

The antigenicity and immunogenicity of rACP was investigated by immunizing mice and rabbits with recombinant proteins in a variety of different adjuvant formulae (Section 2.13). The resulting antisera were tested for biological functions in a variety of *in vitro* assays as follows.

4.5.1 ELISA of antisera raised to rACP

Murine antisera raised against rACP in different formulations showed high levels of anti-rACP antibody (Figure 4.9 A) and also showed cross reactivity with native ACP in the OM preparation of MC58 (Figure 4.9 B). Moreover, rACP adsorbed to $Al(OH)_3$ (mean titre of 133,000) induced statistically higher titres than rACP in saline (mean titre of 22,000), ZW 3-14 micelles (mean titre of 10,000) or incorporated into liposomes (mean titre of 8,600) ($p<0.05$). The addition of MPLA in micelles (mean titre of 49,000) or liposomes (mean titre of 41,000) increased antibody titres though it did not reach statistical significance ($p>0.05$). In particular, rACP in saline preparation induced high titre antibodies, which were significantly higher than that induced by rACP in liposomes ($p<0.05$). In addition, immunisation with MC58 OM on alum also induced antibodies that reacted with the rACP (Figure 4.9 A). Animals that were sham immunised did not produce antibodies that reacted with rACP in ELISA (Figure 4.9 A).

Murine antisera raised against rACP were also tested against OM from the homologous strain MC58 (Figure 4.9 B) and showed significant reactivity; however, the overall antibody titres were not as high as those for rMIP, suggesting that ACP levels may be lower in the OM compared to rMIP. Incorporation of rACP into liposomes with MPLA (mean titre of 2,400) induced statistically significant higher antibody titres than rACP in saline (mean titre of 300) or in liposomes (mean titre of 400) ($p<0.05$). Similarly, addition of MPLA to rACP in ZW 3-14 preparation (mean titre of 6,500) had an effect on increasing antibody production though there was no statistically significant difference ($p>0.05$). As expected, significantly higher ($p<0.05$) anti-OM antibodies were induced by immunisation with OM (mean titre of 161,000) compared with rACP formulae. No significant reactivity against OM was observed with antisera from sham immunised animals (Figure 4.9 B).

Rabbit antisera raised against rACP in Freund's adjuvant also showed high endpoint titres of antibodies. The reciprocal ELISA endpoint titres from those 2 rabbit antisera were 141,000 and 155,000 against rACP and both 3,000 against MC58 OM. Pre-immune rabbit sera showed little reactivity (<100) with rACP and MC58 OM.

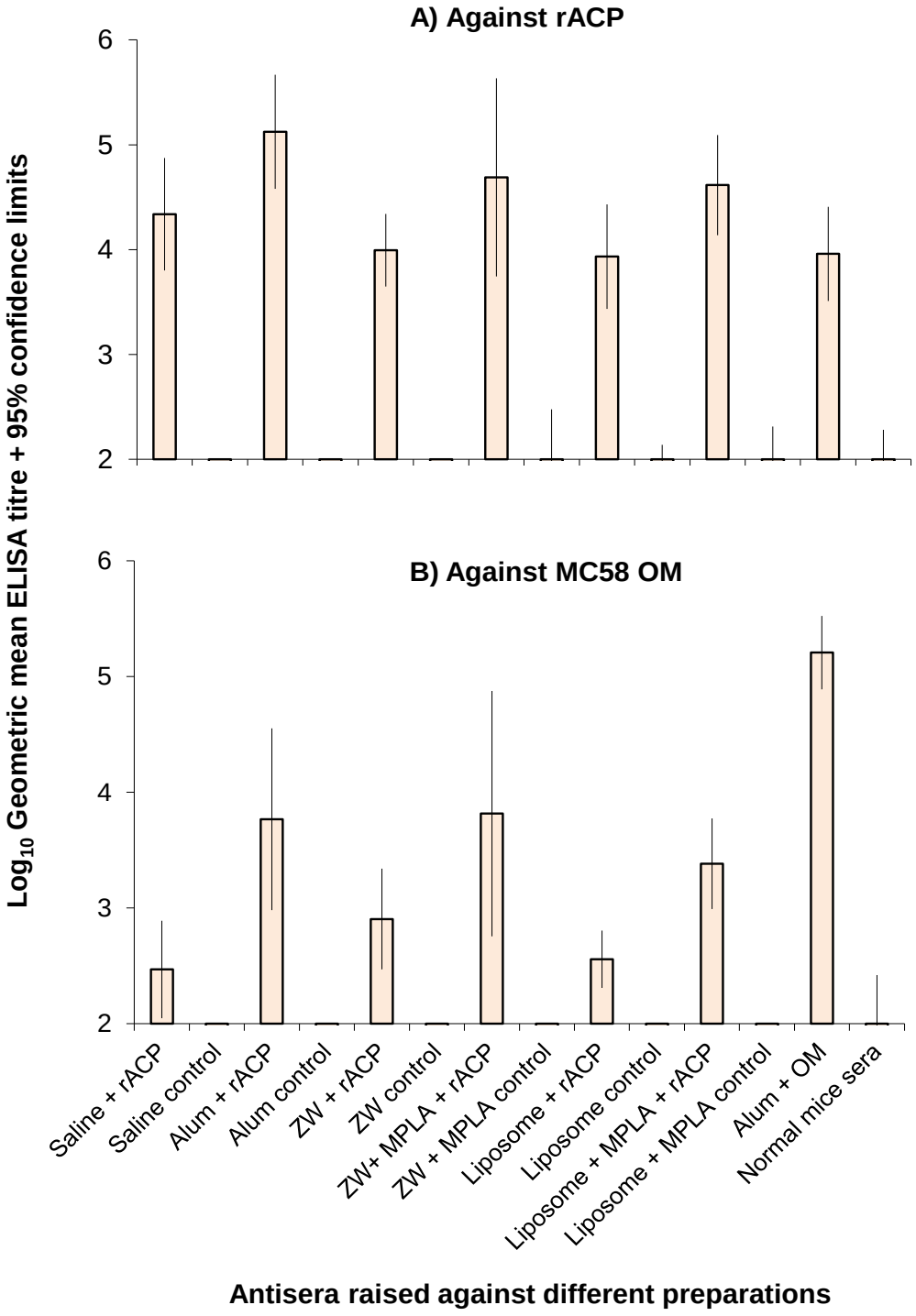

Figure 4.9 ELISA reactivity of murine antisera, raised against different preparations with or without rACP. Murine antisera were reacted with purified rACP (A) and OM of MC58 (B). The columns represent the geometric mean of reciprocal ELISA titres (*n*=5 animals per group), and the error bars represent the 95% confidence limits.

4.5.2 Recognition of native ACP in lysate and OM of MC58 by antibody

Murine antisera raised against the same preparations were then pooled and reacted with blot strips of the bacterial cell lysate and OM of MC58. As a result, antisera, raised against the preparations containing rACP (1/200 dilution), reacted with both the lysate and OM of MC58 and showed bands with molecular weight ~13 kDa (Figure 4.10).

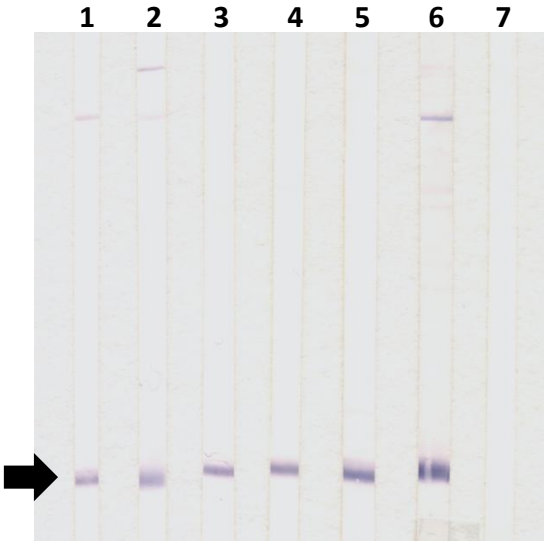

Figure 4.10 Western blot of MC58 OM reacted with pooled murine antisera raised against different formulations (1/200 dilution), including 1) rACP in saline; 2) rACP on Aluminium hydroxide; 3) rACP in micelles; 4) rACP in ZW 3-14 micelles + MPLA; 5) rACP in liposomes; 6) rACP in liposomes + MPLA; 7) negative control (micelles alone). The black arrow indicates the presence of native ACP with molecular weight ~13 kDa. Similar results were obtained with the MC58 cell lysate (data not shown).

4.6 FACS analysis detected the presence of ACP on the surface of MC58

Initially, immunofluorescence (IF) was used to detect the binding of anti-rACP sera to the meningococcal cell surface. Pooled murine antisera (1/100 dilution) were reacted with methanol-fixed MC58 for 1 h at room temperature. Following wash steps and incubation with anti-mouse IgG FITC-conjugate antibody, the samples were checked using confocal microscopy. However, meningococci reacted with anti-rACP sera showed similar FITC binding in comparison to negative controls (meningococci reacted with sera from sham-immunized animals). To increase the binding of anti-rACP antibody, the incubation period of primary antibody was increased to overnight at 4°C, but similar negative IF results were obtained.

FACS analysis was therefore carried out for more sensitive detection. Pre-immune rabbit sera were used as a negative control and also the baseline. The recorded events were defined as 1% compared to the gated area (% parent). Post-immune rabbit anti-rACP sera showed increased FITC

fluorescence recorded events (15.9%) with a right-shift (Figure 4.11 A). As another negative control, post-immune sera were reacted with MC58ΔACP and showed ~1% of parent recorded events (Figure 4.11 B).

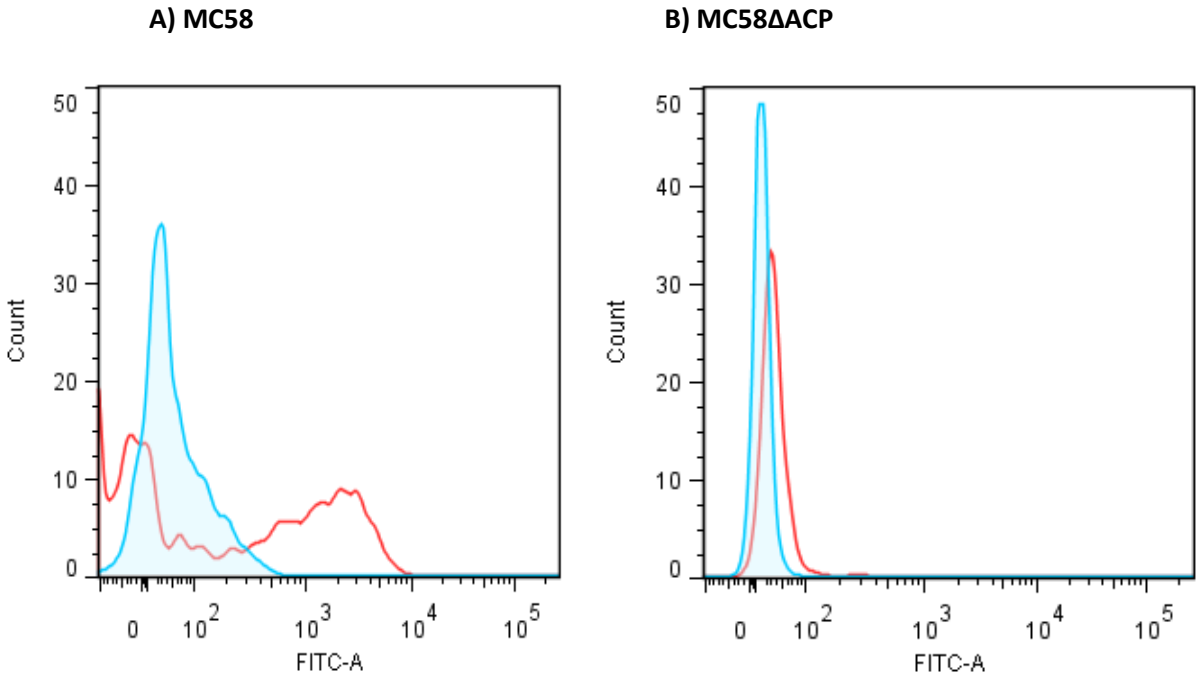

Figure 4.11 FACS analysis showing reactivity of neat rabbit polyclonal anti-rACP sera to A) MC58 and B) MC58ΔACP. Blue tinted profiles show reactivity of pre-immune sera and red profiles show reactivity of post-immunized antisera. Both figures were generated using the FlowJo programme.

4.7 Recombinant ACP induced bactericidal antibody against MC58

Pooled murine antisera raised to different rACP preparations were tested for their bactericidal activity against the homologous strain MC58. As shown in Table 4.1, the highest bactericidal antibody titres (512) were elicited by the formulations containing rACP in liposomes, in ZW 3-14 micelles and in saline alone. Noticeably, rACP adsorbed to $Al(OH)_3$ also induced bactericidal activity (128). By contrast, addition of MPLA to rACP in liposomes and micelles resulted in a complete loss of bactericidal activity.

Table 4.1 Bactericidal activity of pooled antisera, raised against rACP formulations, for the homologous strain MC58.

	Serum bactericidal titre[a] against MC58	
Formulation	**+ rACP**	**-rACP**
Saline	512	<4
Al(OH)3	128 (128, 512)	<4
ZW 3-14 micelles	512	<4
ZW 3-14 micelles + MPLA	<4	<4
Liposome	512 (128,1024)	<4
Liposome + MPLA	<4	<4

[a] The titres are expressed as the reciprocal of the highest dilution at which 50% killing was observed. Titres for normal mouse serum and sera from mice immunised with MC58 OM were <8 and 20,000 respectively. Data are the median values, with the range of values in parenthesis, for SBA from three or more independent measurements of bactericidal activity of all pooled serum samples. Single values denote that the SBA titres were identical from the independent experiments.

4.8 Conservation of ACP protein amongst surveyed meningococci

4.8.1 The acp genes are highly conserved

The DNA sequencing results of the *acp* gene in all the strains in our collection were translated to amino acid sequences and aligned (Figure 4.12). There were 3 types of ACP found: type I (MC58 and MC168), type II (MC54, L2470, MC161, MC162, MC172, MC173, MC174, MC179, MC180 and MENC11) and type III (MC90). Type II ACP has only one amino acid difference compared to MC58 (type I ACP) ($Asp^{25} \rightarrow Asn^{25}$). With 1 more amino acid substitution ($Ala^{12} \rightarrow Ser^{12}$) to type II ACP, type III ACP also shares high similarity (98%) with type I ACP.

As mentioned in section 1.8, the BLAST-protein results from the NCBI website showed that there are 2 types of ACP in meningococcal strains. These 2 types are actually the type I and type II proteins that we identified in our strain collection. We also accessed the BIGS database, which includes the whole genome sequencing data of 205 *Neisseria* strains (31 DNA alleles in total) amongst which 173 were *Neisseria meningitidis*. In addition, the most predominant DNA alleles were allele 13 (73 strains), allele 4 (45 strains) and allele 3 (28 strains).

The DNA sequences from the 31 meningococcal alleles and our 13 strains were aligned using the ClustalW tool on the EMBL-EBI website (European Bioinformatics Institute) (http://www.ebi.ac.uk/Tools/services/web_clustalw2) and a phylogenetic tree was generated to

compare the similarity of the DNA sequences amongst all the strains. As a result, the corresponding allele number in the BIGS database can be found for all the strains in our collection except for the strain MC90, which is a new allele (Table 4.2).

Next, the 31 alleles along with the new allele of the strain MC90 were translated into amino acid sequences using 'Transeg' tool on the EMBL-EBI website and then the phylogenetic trees were generated based on calculating the average distance of % identity using the Jalview programme. The 31 alleles were shown to form 12 clusters based on amino acid sequences (Figure 4.13 A). Next, the phylogenetic tree was re-organised based on the 12 clusters of proteins (Figure 4.13 B). Interestingly, *N. meningitidis* (Nm) only expresses type I (encoded by representative allele 1; including 15 strains in the database and 2 of our strains); type II (encoded by representative allele 2; including 158 strains in the database and 10 of our strains); and type III (encoded by the new allele of MC90, only 1 strain) ACP proteins. Additionally, gonococci had only 2 clusters of proteins derived from allele 6 and allele 10.

Since we know that only MC90 expresses the type III ACP, we wondered if type I and II ACP protein expression was also limited to meningococcal strains. As shown in Figure 4.14, a DNA dendrogram including the 18 alleles encoding type I and type II ACP was generated. All the type II ACP were exclusively expressed by meningococci while type I ACP was also found in a variety of *Neisseria* strains, including *N. sicca* (Ns), *N. polysaccharea* (Np) and *N. lactamica* (Nl). Notably, both types of proteins were not expressed in *N. gonorrhoeae*.

We also raised another question 'What is the DNA allele profile for all meningococcal strains?' As shown in Figure 4.15, a DNA dendrogram including all the DNA alleles of meningococci was generated. All the alleles encoding type II ACP were present in meningococci while only 2 alleles encoding type I ACP appeared in meningococci. In particular, DNA allele 1 was also present in one strain of *N. polysaccharea* (Np). The new allele MC90 (type III ACP) was more similar to alleles encoding type II ACP, especially allele 13. In addition, 6 different alleles (allele 1, 3, 4, 13, 17 and 18) and the new allele were present in the serogroup B meningococcal strains (including 84 strains in the database and 7 of our strains). Therefore, serogroup B could express type I, type II and type III ACP.

```
MC58(I)     1 MKLLTTAILSSAIALSSMAAAAGTDNPTVAKKTVSYVCQQGKKVKVTYGFNKQGLTTYAS 60
MC54(II)    1 MKLLTTAILSSAIALSSMAAAAGTNNPTVAKKTVSYVCQQGKKVKVTYGFNKQGLTTYAS 60
MC90(III)   1 MKLLTTAILSSSIALSSMAAAAGTNNPTVAKKTVSYVCQQGKKVKVTYGFNKQGLTTYAS 60

MC58(I)    61 AVINGKRVQMPVNLDKSDNVETFYGKEGGYVLGTGVMDGKSYRKQPIMITAPDNQIVFKD 120
MC54(II)   61 AVINGKRVQMPVNLDKSDNVETFYGKEGGYVLGTGVMDGKSYRKQPIMITAPDNQIVFKD 120
MC90(III)  61 AVINGKRVQMPVNLDKSDNVETFYGKEGGYVLGTGVMDGKSYRKQPIMITAPDNQIVFKD 120

MC58(I)   121 CSPR 124
MC54(II)  121 CSPR 124
MC90(III) 121 CSPR 124
```

Figure 4.12 Alignment of amino acid sequences between MC58 and the surveyed strains in our collection. The red colour and green colour denote the differences of protein type II (MC54) and type III (MC90) ACP in comparison to MC58.

Table 4.2 Corresponding allele number in the BIGS database of our 13 strains.

Names of surveyed strains	**allele number in the BIGS database**
MC54, MC161, MC162, MC173, MENC11, **MC174**	4
L2470, MC172 and MC180	13
MC179	18
MC58, MC168	1
MC90	A new allele, unassigned

The grey shadings denote serogroup C strains and the bold (MC174) indicates serogroup 29E. All the rest of the strains belong to serogroup B.

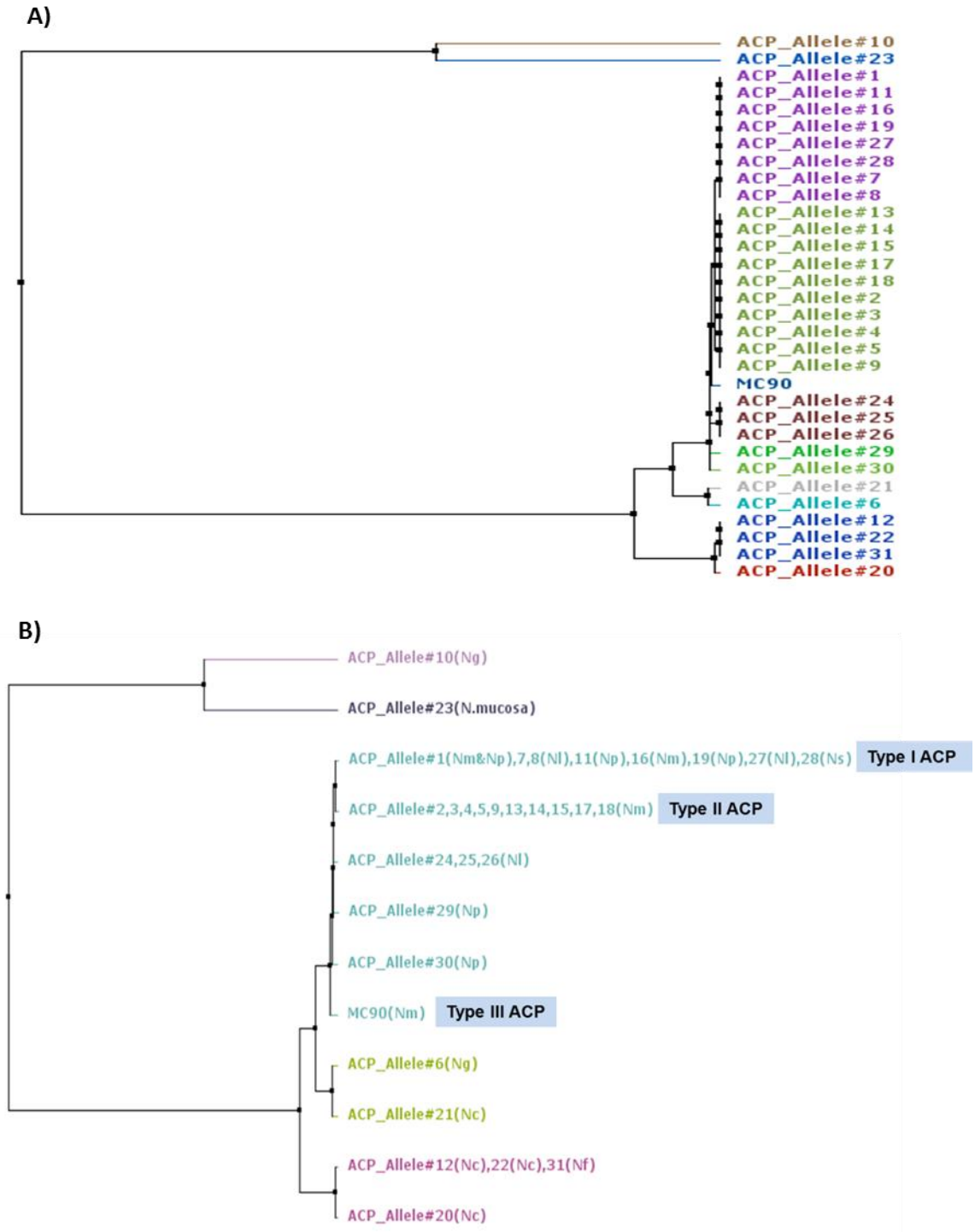

Figure 4.13 A) Phylogenetic tree of amino acid sequence translated from the 31 alleles in the BIGS database and the new allele of MC90. The strain clustering was based on calculating the average distance of % identity using Jalview programme. There were 12 clusters of proteins identified. Different colours show different clusters of proteins; B) a phylogenetic tree of all the 12 clusterings of proteins. The brackets denote *Neisseria species* expressing the allele (s), including *N. cinerea* (Nc), *N. sicca* (Ns), *N. polysaccharea* (Np), *N. lactamica* (Nl), *N. gonorrhoeae* (Ng), *N. flavescens* (Nf) and *N. mucosa*.

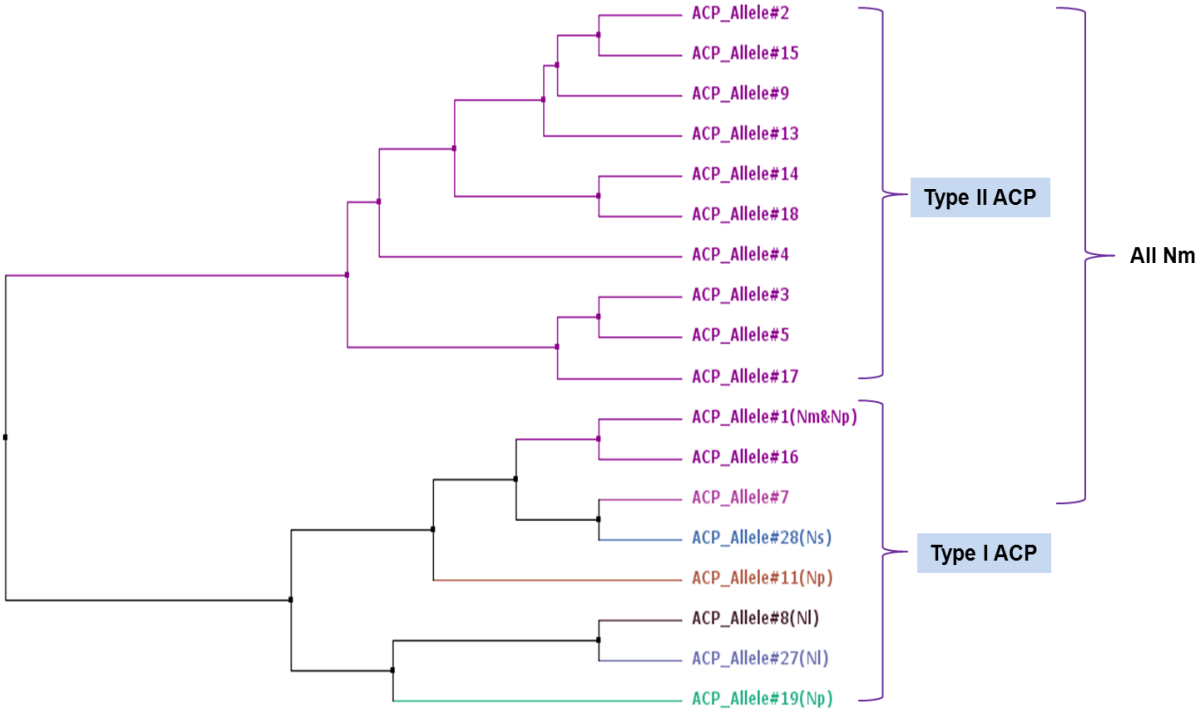

Figure 4.14 The DNA dendrogram of all alleles encoding type I and type II ACP protein. The result was based on calculating the average distance of % identity using Jalview programme. Purple colours denote clustering of *N. meningitidis*, except that one strain of *N. polysaccharea* also has allele 1 in the BIGS database. Type I ACP protein could be expressed by a variety of *Neisseria* strains, including *N. sicca* (Ns), *N. polysaccharea* (Np) and *N. lactamica* (Nl).

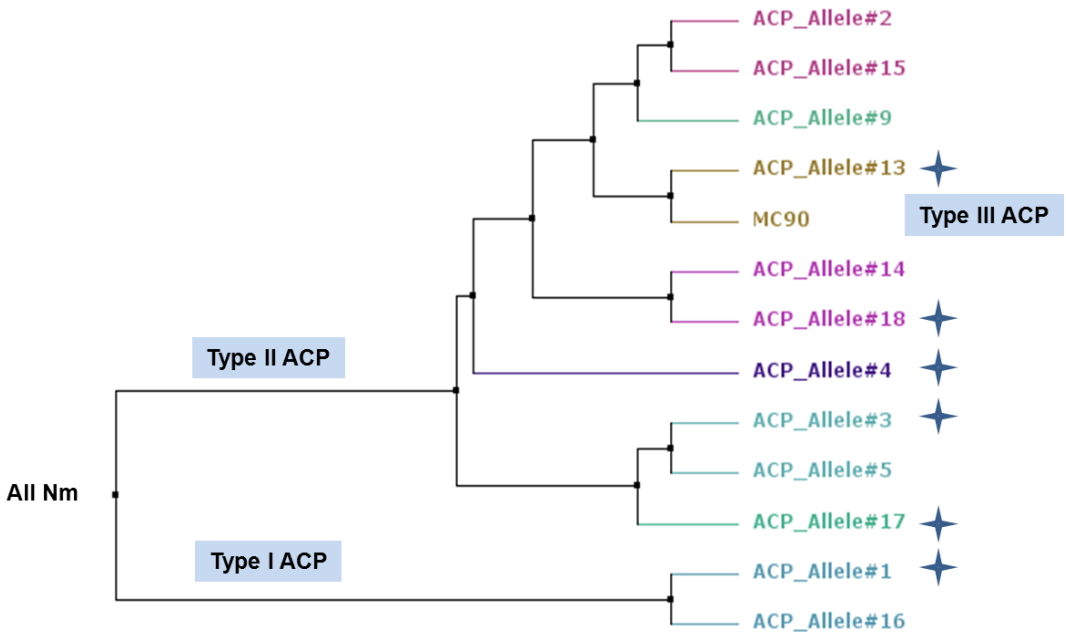

Figure 4.15 The DNA dendrogram of alleles present in all meningococcal (Nm) strains. The result was based on calculating the average distance of % identity using Jalview programme. The new allele of MC90 (encoding type III) is closer to type II ACP, especially allele 13. All the alleles encoding type II ACP belong to meningococci while only 2 alleles encoding type I ACP are present in meningococci. In addition, the stars denote the alleles present in all the serogroup B meningococcal strains.

4.8.2 Expression levels of ACP amongst surveyed strains

All the sequenced strains were grown for ~18 h and lawns of meningococci were prepared as lysates for western blot. A ~13 kDa band was observed in the lysates of all the surveyed meningococcal strains using rabbit anti-rACP sera (Figure 4.16). In addition, the lysate of MC58ΔACP was used as a negative control and as expected, showed no reactivity with the antisera.

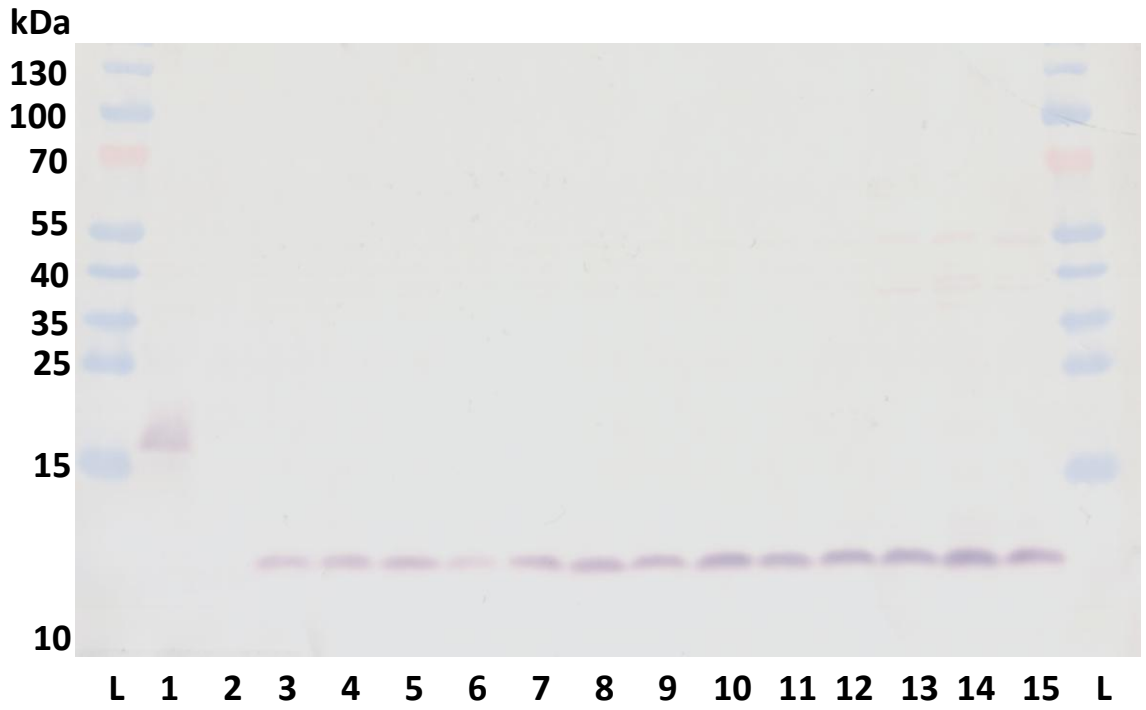

Figure 4.16 Western blot reactivity of antisera, raised to rACP, against lysates of meningococcal strains from our collection. Rabbit antisera (1/400) to rACP from strain MC58 was reacted with lysates of meningococcal strains. Lane M contains protein markers. Lane 1 contains rACP (0.1 µg) and lane 2 contains lysate of MC58ΔACP (20 µg). In order, lane 3-15 are lysates (20 µg) of MC58, MC168, L2470, MC54, MC161, MC162, MC172, MC173, MC174, MC179, MC180, MENC11 (type I and II strains) and MC90 (type III strain).

4.9 Cross-strain protection

Pooled murine antisera with bactericidal antibodies to type I ACP were tested for their bactericidal activity on strains with type II and type III ACP. Antisera raised against rACP (type I ACP) in saline and liposomes showed a similar killing effect on MC179 (type II ACP) and MC90 (type III ACP). Significantly, this was also observed with antisera raised to rACP adsorbed to $Al(OH)_3$ (Table 4.3).

Table 4.3 Bactericidal activity of pooled murine anti-rACP sera for the heterologous strains.

Sequence type/representative strain	Serum bactericidal titre[a] elicited by rACP in		
	Saline	**Liposomes**	**$Al(OH)_3$**
I/MC58	512	512	128
		(128, 1024)	(128, 512)
II/MC179	512	512	64
	(128, 4096)	(128, 2048)	(64,128)
III/MC90	512	256	64
	(128, 4096)	(128, 2048)	(64,128)

[a]The titres are expressed as the reciprocal of the highest dilution at which 50% killing was observed. Data are the median values, with the range of values in parenthesis, for SBA from three or more independent measurements of bactericidal activity of all pooled serum samples. Single values denote that the SBA titres were identical from the independent experiments.

4.10 Generating ACP knockout mutants

4.10.1 Confirmation of the MC58∆ACP and MC58¢18∆ACP mutants by PCR

Using the 2.9 kb DNA segment amplified from mutant 35/11 of strain 8013 in transforming wild-type MC58, the transformation rate was low. PCR screening of the transformant showed a 2.9 kb band, which indicated successful mutagenesis by interrupting the *acp* gene in MC58. Therefore, the colony was identified as an ACP knockout mutant (MC58ΔACP). As shown in Figure 4.17, the MC58ΔACP mutant showed a 2.9 kb DNA band while the positive control (MC58) showed a 1.3 kb DNA band. The mutant was subsequently stored in proteose peptone liquid medium containing 30% (v/v) glycerol in liquid nitrogen. Importantly, sequencing of the 2.9 kb DNA segment amplified from the MC58ΔACP was done commercially (Geneservice, Oxford, UK) and showed that the adjacent proteins, NMB2094 and NMB2096, were not affected by the mutagenesis procedure.

Later, we also used pGEM®-T vector (Promega, UK) to carry this 2.9 kb DNA segment and extracted genomic DNA from this MC58ΔACP mutant to repeat transformation of MC58. As a result, the transformation rate increased significantly. However, when the same method was used to transform the non-capsulated and non-piliated mutant MC58¢18 ($Cap^-Opa^-Opc^-Pil^-$), the transformation rate was low. Similarly, the mutant was also confirmed by PCR and showed a 2.9 kb band of expected PCR product.

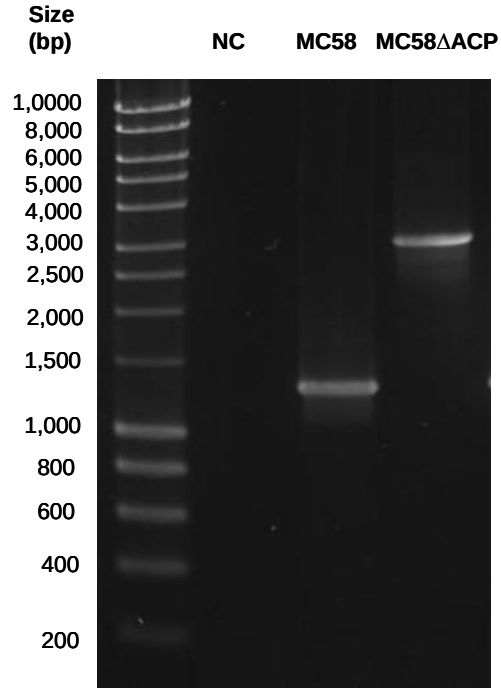

Figure 4.17 Confirmation of MC58ΔACP by PCR. Lane 1 contains Hyperladder I. NC contains negative control sample. PCR product from MC58 genomic DNA template shows a ~1,300 bp band in comparison to a ~2,900 bp band of MC58ΔACP.

4.10.2 Confirmation of MC58∆ACP and MC58¢18∆ACP by western blot

The whole lysate of MC58¢18 ($Cap^-Opa^-Opc^-Pil^-$) was subjected to SDS-PAGE and then it was transferred onto nitrocellulose by western blotting. The blot was then reacted with monoclonal antibody generated against known adhesins, including 4B12C11 (anti-Opa; 1:5,000), B306 (anti-Opc; 1:5,000) and SM1 (anti-PilE; 1:25,000). The blot result showed that the wild type MC58 expressed Opa (*Mr* 26-31 kDa), Opc (*Mr* 26-31 kDa) and pilin (*Mr* ~18 kDa) while the lysate of MC58¢18 and MC58¢18ΔACP did not show reactivity with these monoclonal antibodies (Figure 4.18).

The lysate of the 4 strains were also blotted and reacted with rabbit anti-rACP sera (1/400). As a result, neither MC58ΔACP nor MC58 ¢18ΔACP showed reactivity (Figure 4.19).

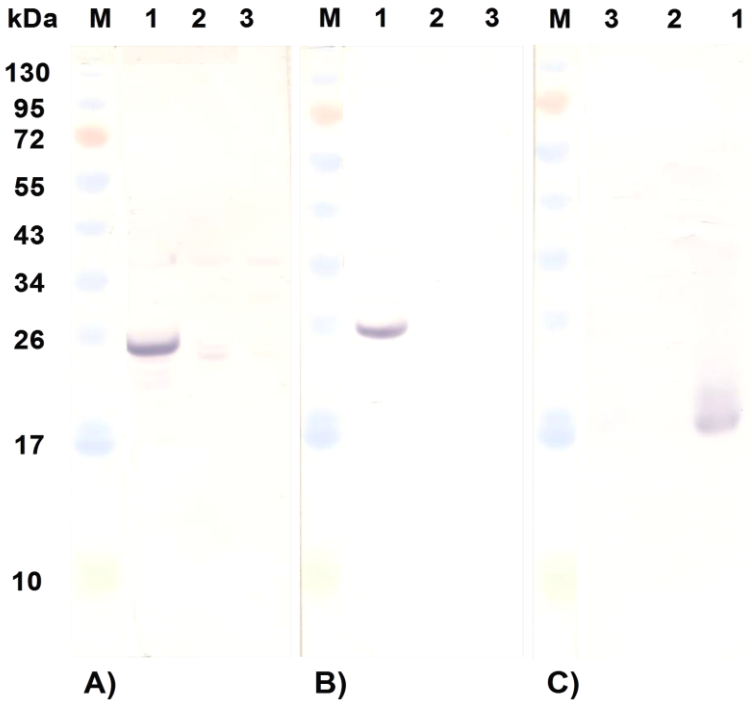

Figure 4.18 No expression of Opa, Opc and PilE in MC58¢18 and MC58¢18ΔACP.
M: protein ladder; Lane 1, 2 and 3 contain lysate of MC58 (20 µg), MC58¢18 (20 µg) and MC58¢18 ΔACP (20 µg). The blot was reacted with monoclonal antibody raised against A) Opa (4B12C11), B) Opc (B306) and C) PilE (SM1).

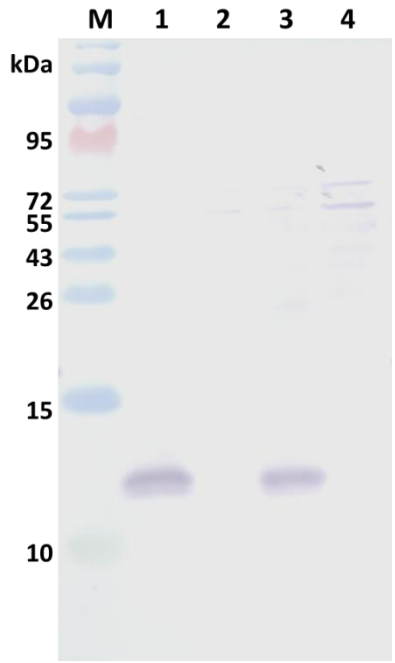

Figure 4.19 Confirmation of ACP mutants by western blot. The whole lysates (20 µg) of MC58 (1), MC58ΔACP (2), MC58¢18 (3) and MC58¢18ΔACP (4) were reacted with rabbit anti-rACP sera (1/400). M contains protein marker. The reactive bands show *Mr* ~13 kDa.

4.10.3 Confirmation of MC58⊿ACP complemented strain by PCR

To prove that the depletion of biological function in MC58ΔACP was attributed to the lack of ACP expression, a functional copy of *acp* gene was cloned into pGCC4 plasmid and propagated in *E. coli*. Next, extracted pGCC4-*acp* recombinant plasmids from 3 transformed *E.coli* colonies were sequenced commercially (Geneservice, Oxford, UK) and all showed the correct *acp* gene sequences. The pGCC4-*acp* recombinant plasmids were then used for transforming MC58ΔACP and 12 out of several hundreds of transformants were screened by PCR. As shown in Figure 4.20, 7 screened transformants were the complemented strains, showing a ~375 bp band.

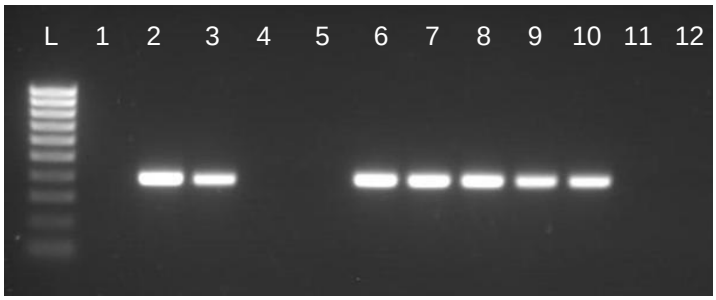

Figure 4.20 PCR screening of 12 MC58ΔACP complemented transformants. L contains Hyperladder IV. Lanes 1-12 contain PCR products from each transformed colony. The complemented strains showed ~375 bp bands.

4.11 Comparison of the growth curve of wild type (ACP^+) and mutant (ACP^-)

Bacterial suspensions of MC58, MC58ΔACP, MC58¢18 and MC58¢18ΔACP were prepared from 16-18 h cultures and adjusted to ~10^8 CFU/ml in 1 ml of DMEM containing 1% (v/v) decomplemented foetal calf sera. Viable counts were obtained by plating out onto 3 GC agar plates at 0 h, 1 h and 3 h. As shown in Figure 4.21, the growth curves of MC58ΔACP and MC58 are similar. Additionally, MC58¢18 and MC58¢18ΔACP showed similar growth rates (Figure 4.21).

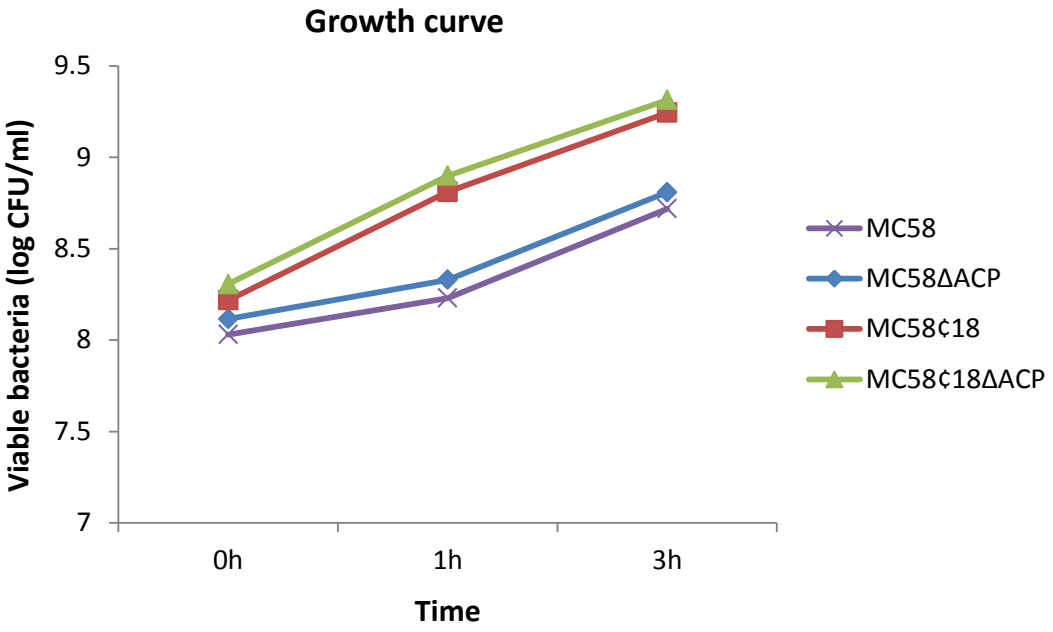

Figure 4.21 Growth curves of MC58, MC58ΔACP, MC58¢18 and MC58¢18ΔACP (n=2).

4.12 Role of ACP in bacterial association of meningococci with epithelial cells, endothelial cells and meningioma cells

To investigate the potential role of ACP in pathogenesis, such as adhesion to host cells and subsequent invasion, we challenged different human cells, including epithelial cells, endothelial cells and meningioma cells with MC58 and MC58ΔACP and compared the differences of associated bacterial numbers. We tested the hypothesis that a reduction in total associated bacterial numbers of the ACP^- mutant indicates that ACP plays a role in adhesion to and possibly subsequent invasion into host cells.

Experiments with different cell lines and bacteria were done repeatedly and the overall trend was similar between experiments, although quantitative variations between experiments were observed. Therefore, following the method of presentation used by Virji *et al*. and others, data from representative experiments are shown below with the number of experiments done cited.

4.12.1 Total association with epithelial cells

I. Chang conjunctival epithelial cells

Each monolayer of Chang cells in 24-well tissue culture plates was challenged with ~2 x 10^7- 5 x 10^7 CFU/ml of both MC58 and MC58ΔACP. The cell count per monolayer of Chang cells in 24-well tissue culture plates was ~2.43 x 10^5 ± 8.42 x 10^4 cells/monolayer (n=12 monolayers counted). After 3 h incubation at 37°C with 5% (v/v) CO_2, the monolayer were lysed by addition of 250 µl of 1% (w/v) saponin in PBS with 1% (v/v) decomplemented foetal calf sera and 15 µl of the lysate was taken,

diluted and plated onto GC agar plates. Viable counts were obtained on the next day. The mean value and standard deviation of the total associated bacterial numbers from triplicate wells were calculated. As shown in Figure 4.22, MC58ΔACP showed a ~4 fold reduction of associated bacterial number in comparison to MC58 in a typical experiment. The MC58ΔACP complemented strain showed a restoration in adherence to the mutant, to levels similar to wild-type strain ($P>0.05$). The lower levels of associated bacterial numbers of MC58ΔACP compared to those of MC58 reached statistical significance (independent t-test, $p<0.001$). Although there was variation between 6-8 experiments, the significant reduction of associated bacterial number of the ACP deficit mutant was consistent and similar.

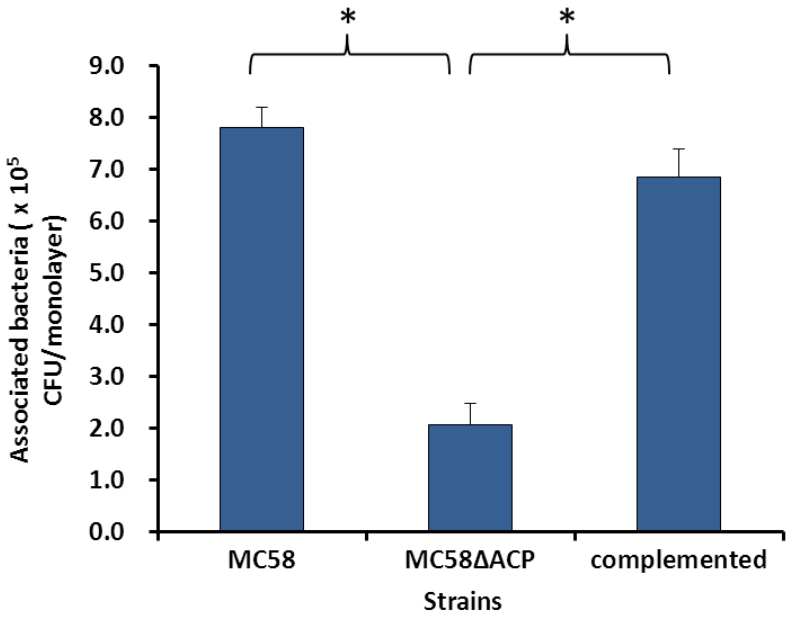

Figure 4.22 Associated bacterial numbers per monolayer of Chang cells challenged with MC58, MC58ΔACP and the MC58ΔACP complemented strain; The data are from one representative experiment (n=6 experiments) using an inoculum of 2 x 10^7 CFU/monolayer (M.O.I. =~100). The columns denote the mean associated bacterial numbers and the error bars the standard deviations from triplicate wells.'*' denotes the comparison between 2 groups of data showed statistical significance ($p<0.001$).

II Human epidermoid carcinoma, larynx (Hep2 cells) cells

Each monolayer of Hep2 cells in 24-well tissue culture plates was challenged with ~2 x 10^7- 5 x 10^7 CFU/ml of both MC58 and MC58ΔACP. The cell count per monolayer of Hep2 cell in 24-well tissue culture plates was 3.52 x 10^5 ± 1.04 x 10^5 cells/monolayer (n=5). In a typical experiment and similar to the observations with Chang cells, MC58ΔACP showed a ~4 fold reduction of total association with another epithelial cell line, Hep2, in comparison to MC58 (Figure 4.23). The MC58ΔACP complemented strain also showed a restoration of adhesion to the mutant. The associated bacterial numbers of MC58ΔACP compared to those of MC58 reached statistical significance (independent t-test, $p<0.001$). Although there was variation between n=6 experiments, the significant reduction of associated bacterial number of the ACP deficit mutant was consistent and similar.

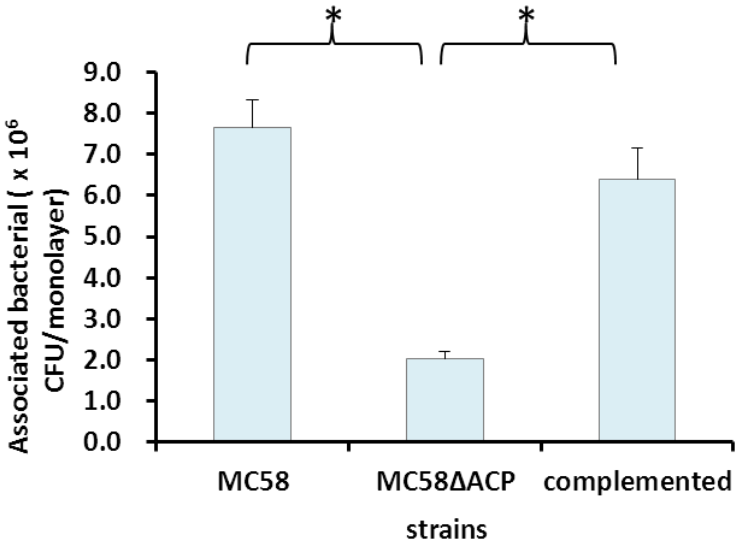

Figure 4.23 Associated bacterial numbers per monolayer of Hep2 cells challenged with MC58, MC58ΔACP and MC58ΔACP complemented strain. The data show one representative experiment (n=6) using an inoculum of 3 x 10^7 CFU/monolayer (M.O.I. =100). The columns represent the mean associated bacterial numbers and the error bars the standard deviations from triplicate wells. '*' reports that a comparison between 2 groups of data showed statistical significance ($p<0.001$).

III. Human primary nasal epithelial cells (HpNECs)

HpNECs is another epithelial cell line chosen for surveying the role of ACP in adhesion. In addition, the cell count per monolayer of HpNECs in 24-well tissue culture plates was 1.44 x 10^5± 3.65 x 10^4 cells/monolayer (n=5). From one typical experiment, there was ~50% reduction of associated MC58ΔACP bacteria compared to the wild-type strain (Figure 4.24). In addition, the MC58ΔACP complemented strain also showed a restoration of adhesion to the mutant. The lower levels of associated bacterial numbers of MC58ΔACP compared to that of MC58 reached statistical significance (t-test, p=0.034). However, these particular primary cells tended to overgrow monolayers and exhibit different morphologies. Consequently, there were higher levels of inter-assay variation in the 4 independent experiments (showing a range of 13-71% reduction of associated mutant bacteria compared to wild-type).

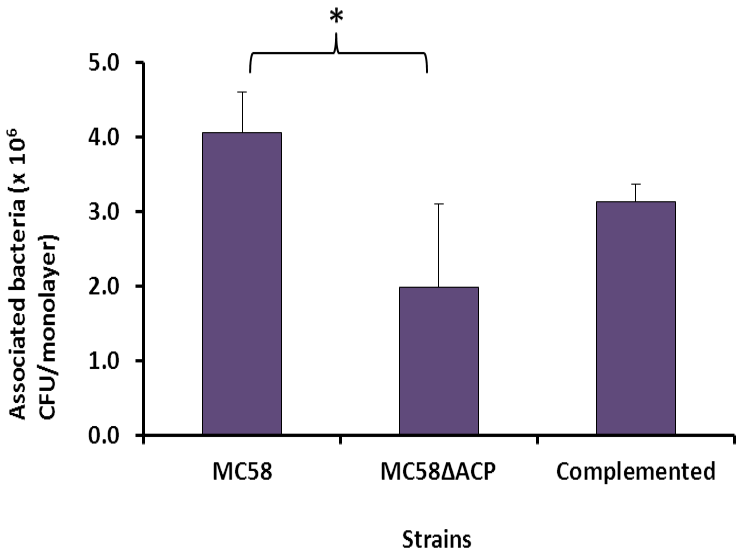

Figure 4.24 Associated bacterial numbers per monolayer of HpNECs challenged with MC58, MC58ΔACP and MC58ΔACP complemented strain. The data show one representative experiment (n=6) using an inoculum of 2 x 10^8CFU/monolayer (M.O.I. =1000). The columns represent the mean associated bacterial numbers and the error bars the standard deviations from triplicate wells. '*' reports that a comparison between 2 groups of data showed statistical significance (p=0.034).

4.12.2 Human umbilical vein endothelial cells (HUVECs)

To investigate if ACP also plays a role in interacting with endothelial cells, similar association experiments were done with human umbilical vein endothelial cells (HUVECs). The cell count of a HUVECs monolayer in a 96-well tissue culture plate was $9.46 \times 10^3 \pm 1.92 \times 10^3$ cells (n=3) and a monolayer of HUVECs in 24-well tissue culture plates had $4.0 \times 10^4 \pm 1.43 \times 10^4$ cells (n=11). From a typical experiment, MC58ΔACP showed ~40% reduction of total association with HUVECs in comparison to MC58 (Figure 4.25). The MC58ΔACP complemented strain also showed a restoration of adhesion to the mutant. The lower levels of associated bacterial numbers of MC58ΔACP compared to those of MC58 reached statistical significance (independent t-test, p=0.034). Although there was variation of between n=11 independent experiments, the reduction of associated bacterial number of the ACP mutant strain was consistent and reproducible.

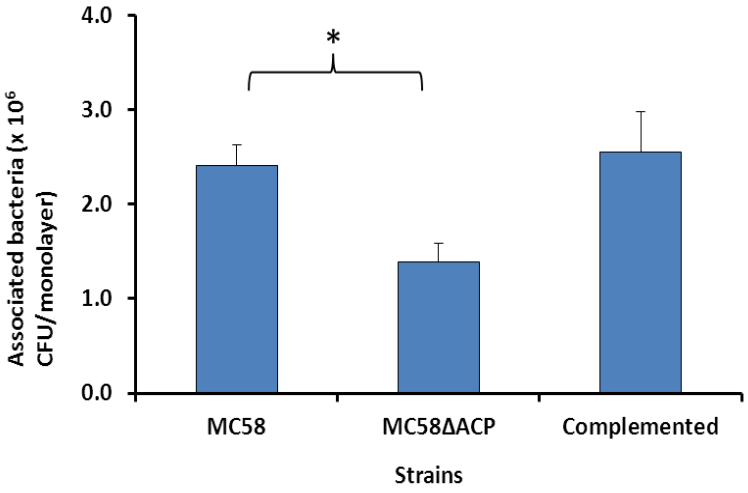

Figure 4.25 Associated bacterial numbers per monolayer of HUVECs challenged with MC58, MC58ΔACP and MC58ΔACP complemented strain. The data show one representative experiment (n=6) using an inoculum 2×10^8 CFU/monolayer (M.O.I. =5000). The columns represent the mean associated bacterial numbers and the error bars the standard deviations from triplicate wells. '*' denotes that a comparison between 2 groups of data showed statistical significance (p=0.034).

4.12.3 Meningioma cells (lines M53 and M61)

Since the ACP was shown to mediate the adhesion to both epithelial cells and endothelial cells, we hypothesized that it might also play a role in adhesion to the meninges. To test our hypothesis, we used the meningioma cell culture model (Hardy *et al.* 2000) and initially using a meningioma transitional histological subtype cell line (M53). The cell count per monolayer of meningioma cells (M61) in 24-well tissue culture plates was $1.21 \times 10^5 \pm 2.66 \times 10^4$ cells/monolayer (n=5). In a typical experiment, MC58ΔACP showed an ~20% reduction of total association with M53 cells compared to MC58 (M.O.I. =250) (Figure 4.26 A). The MC58ΔACP complemented strain showed a restoration in adherence to the mutant, to levels similar to wild-type strain ($p>0.05$). The lower levels of associated bacterial numbers of MC58ΔACP compared to those of MC58 reached statistical significance (independent t-test, $p<0.019$). Although there was variation between 5 experiments, the significant reduction of associated bacterial number of the ACP deficit mutant was consistent and similar. In order to demonstrate this result was not influenced by the cell line used, we repeated the association experiments using another meningioma cell line of different histological subtype, *i.e.* meningothelial (M61) cells. With this cell line, similar levels of reduction (~35% for M.O.I. =800) in associated bacterial numbers of MC58ΔACP compared to MC58 ($p<0.001$) were observed in a typical experiment (n=6) (Figure 4.26 B). Similarly, the MC58ΔACP complemented strain also showed a restoration of adhesion to the mutant to M61 cells (Figure 4.26 B).

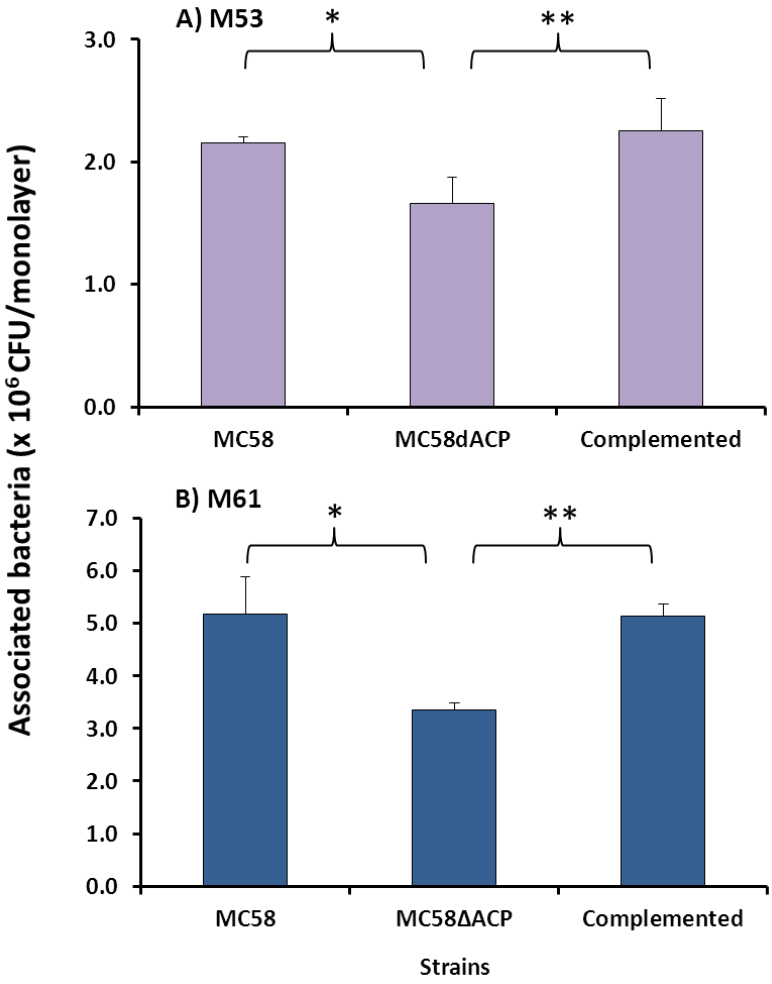

Figure 4.26 Associated bacterial numbers per monolayer of meningioma cells M53 (A) and M61 (B) challenged with MC58, MC58ΔACP and MC58ΔACP complemented strain. (A) The data shows one representative experiment (n=5) using an inoculum 3 x 10^7 CFU/monolayer (M.O.I. =250) and (B) one representative experiment in M61 using inoculum 1 x 10^8 CFU/monolayer (M.O.I. =800). The columns represent the mean associated bacterial numbers and the error bars the standard deviations from triplicate wells. '*' and '**'denotes that a comparison between 2 groups of data showed statistical significance (* p=0.019; ** p<0.001 for A and * p=0.018; ** p<0.001 for B).

4.13 Anti-rACP antibody inhibits the association of wild-type MC58 with Chang cells

To provide further evidence that the reduction of associated bacterial number of MC58ΔACP with Chang cells was indeed related to ACP expression, anti-rACP antibody was added to the monolayers at the time of infection with meningococci. As shown in Figure 4.27, addition of 10% (v/v) decomplemented rabbit anti-rACP sera decreased the total associated bacterial numbers by ~85% ($p < 0.001$). Moreover, the use of only 1% (v/v) sera also showed ~75% reduction of association. From a pilot experiment using different doses (10^4-10^8 CFU/monolayer) of wild-type MC58 in the presence of 10% (v/v) anti-rACP antisera, the binding of antibody to ACP seemed to reach saturation when the inoculum was higher than 10^7 CFU/monolayer. Therefore, the experiments were carried out using an inoculum 10^4-10^5 CFU/monolayer.

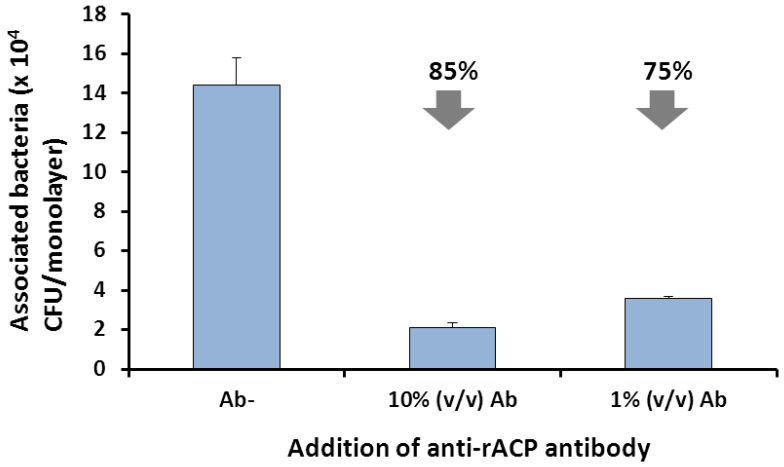

Figure 4.27 Addition of decomplemented anti-rACP antibody inhibited association of wild-type MC58 with Chang cells. The data are from a representative experiment (n=3) using an inoculum 5 x 10^4 CFU/monolayer. The columns represent the mean associated bacterial numbers and the error bars the standard deviations from triplicate wells.

4.14 ACP plays a role in invasion in acapsular strains

Since ACP played a role in association of meningococci with various kinds of human cell lines, we next investigated if it also played a role in cellular invasion. The standard gentamicin assay was used to investigate invasion of human cells. Furthermore, to investigate whether internalisation of bacteria was dependent on the microfilament function of the cells, monolayers were also treated with cytochalasin D for validation.

In these experiments, the data were presented as the percentage internalisation (=% internalised bacteria/associated bacteria) and the differences were compared using the Mann-

Whitney U test. Similar to measurements of total association (Section 4.12), there was inter-experiment variation, but the overall trend was similar between experiments. Therefore, one representative experiment was used to present the data.

4.14.1 Role of ACP in Invasion of capsule-expressing bacteria

First of all, we compared the percentage of internalisation between MC58 and its ACP mutants. Notably, with capsulated meningococci, the internalised bacterial counts were low. There were no statistically significant differences between the percentage internalisation of MC58 and MC58ΔACP into A) Chang cells, B) Hep2 cells, C) HpNECs and D) HUVECs (Figure 4.28).

4.14.2 Role of ACP in invasion of acapsular meningococci into epithelial cells and HUVECs

I. Chang cells and Hep2 cells

Since capsulated MC58 showed low numbers of internalised bacteria and ACP was not involved in invasion, we investigated whether ACP was important for the invasion of meningococci lacking capsule. For these experiments, we used the acapsular $Opa^{-}Opc^{-}PilE^{-}$ MC58¢18 and its ACP mutant, in order to determine the role, if any, of ACP for invasion without the hindrance of capsules or the contribution of other major adhesins/invasins. As shown in Figure 4.29, the percentage internalisation of the ACP mutant was significantly lower compared to its parent strain in both A) Chang cells and B) Hep2 cells. As expected, the cytochalasin (CD)-treated cells showed even low numbers of internalised bacteria, which indicated that active cell-related uptake of bacteria was dependent on cytoskeletal function.

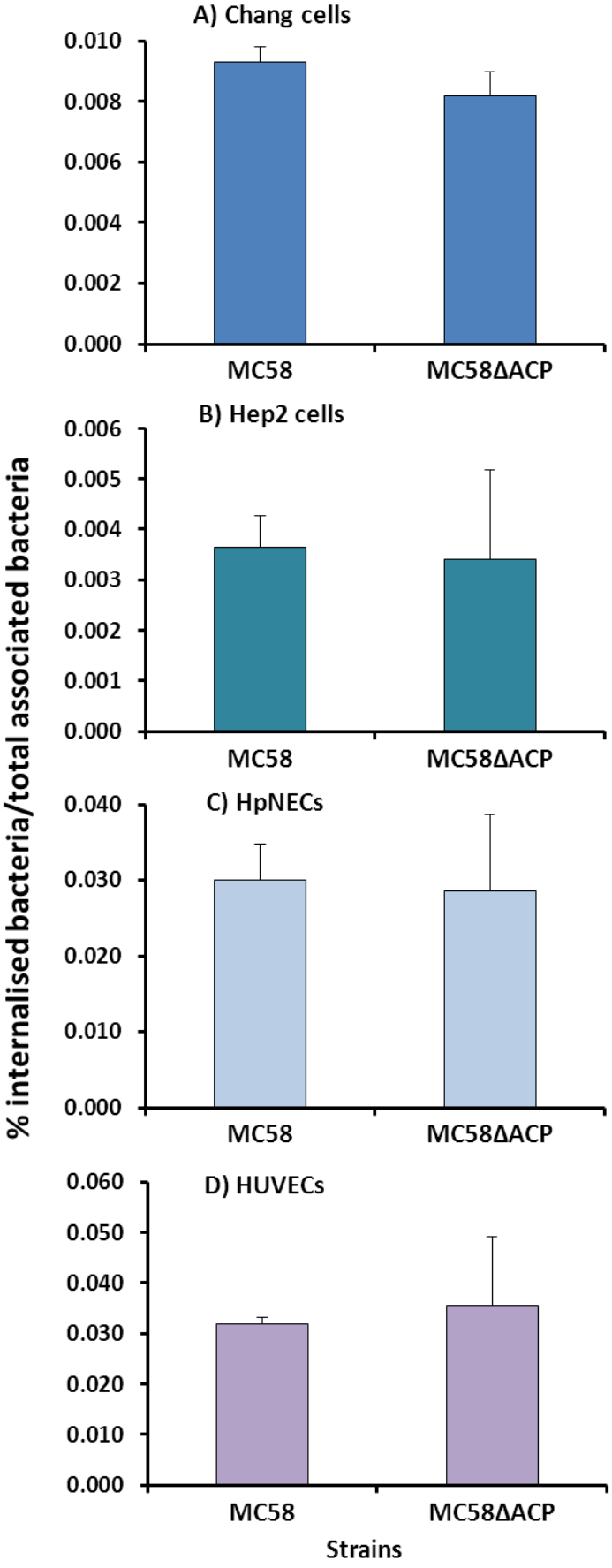

Figure 4.28 Gentamicin assay- comparison of percentage internalisation between MC58 and MC58ΔACP in different cell lines. The data show one representative experiment for

A) **Chang cells**, M.O.I. =100, n=5 experiments

B) **Hep 2 cells**, M.O.I. =33, n=5 experiments;

C) **HpNEC**s, M.O.I. =1000, n=3 experiments;

D) **HUVECs**, M.O.I. =5000, n=3 experiments;

Even with different M.O.I. between independent experiments, the trend was identical for each cell line. The columns represent the mean associated bacterial numbers and the error bars the standard deviations from triplicate wells. Comparison between 2 groups of data showed no statistical significance ($p>0.05$).

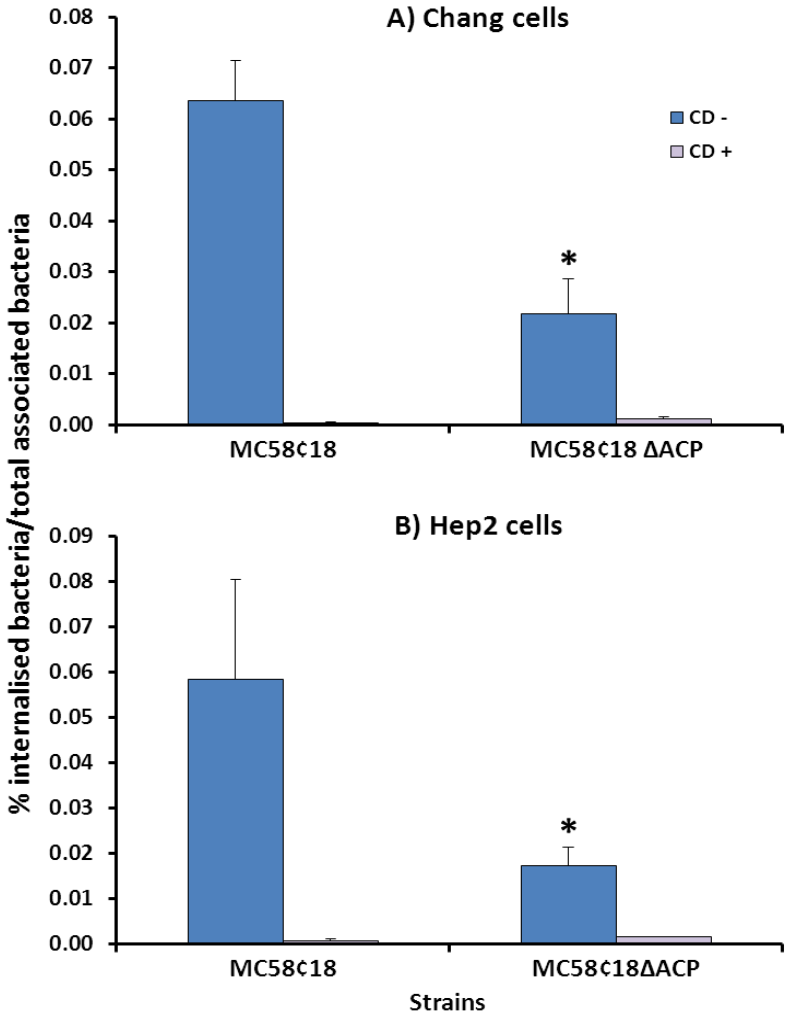

Figure 4.29 Gentamicin assay- percentage internalisation per monolayer of A) Chang cells and B) Hep2 cells challenged with MC58¢18 and MC58¢18ΔACP. The data show one representative experiment for A) Chang cells, M.O.I =1000, n=5 experiments and B) Hep2 cells, M.O.I. =750, n=3 experiments. CD- and CD+ represent without and with pre-treatment of cytochalacin D. The columns represent the mean associated bacterial numbers and the error bars the standard deviations from triplicate wells. '*' reports that a comparison between 2 groups of data showed statistical significance (p=0.043).

II. HUVECs

ACP clearly plays an active role in meningococcal invasion of epithelial cells. We tested the hypothesis that it also plays a role in invasion of endothelial cells. The standard gentamicin assay was done with HUVECs infected with strain MC58¢18 and its ACP mutant. By contrast to epithelial cells, the HUVECs-internalised bacterial numbers were low (<100 CFU/monolayer) in all experiments (n=4); however, the trend of reduced internalisation of the ACP mutant compared to MC58¢18 was consistent. One representative experiment is shown in Figure 4.30, the difference between this 2 percentage internalisation reached statistical significance (p=0.043). In addition, the percentage internalisation of MC58¢18ΔACP in HUVECs without and with CD treatment were identical (p>0.05).

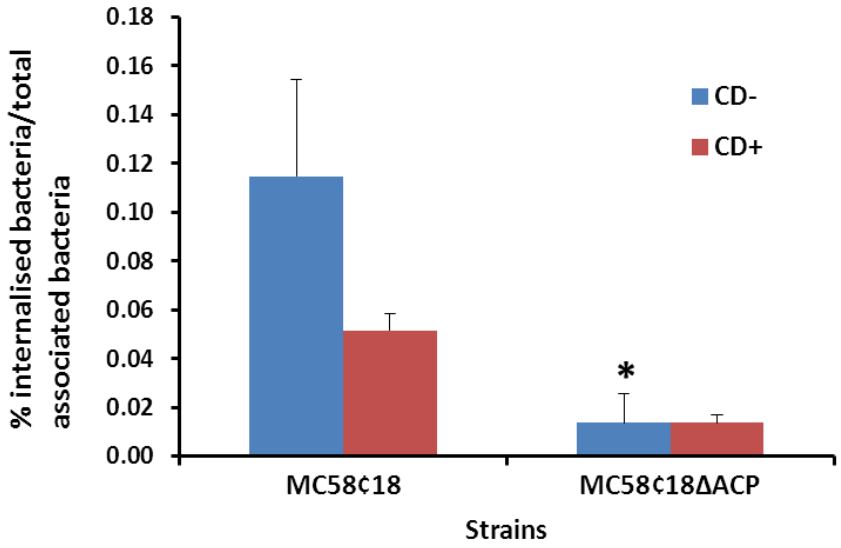

Figure 4.30 Gentamicin assay- percentage internalisation per monolayer of HUVECs challenged with MC58¢18 and MC58¢18ΔACP. The data show one representative experiment (n=4) using a M.O.I. =1000. CD- and CD+ represent without and with cytochalasin D treatment. The columns represent the mean associated bacterial numbers and the error bars the standard deviations from triplicate wells. '*' reports that a comparison between 2 groups of data showed statistical significance (p=0.043).

Chapter 5 Discussion

Currently, the most promising meningococcal B vaccine is the Novartis 4CMenB (Bexsero®), which has reported a ~70% efficacy in a phase III clinical trial (Dull 2011). However, without incorporating the OMV of the New Zealand vaccine strain (NZ98/254), only 5% of the vaccinees showed SBA titres ≧ 4 against the NZ98/254 strain (Su and Snape 2011). The contribution of the individual purified proteins to the overall immunogenicity is questionable, as there are many antigen components present in the OMV. Moreover, the efficacy evaluation was based on testing SBA against 4 standard strains representative for each protein (Dull 2011). The validated protein-specific bactericidal antibody toward one standard strain showed low or no activity to kill other strains bearing the same variant of protein. Hence, it is very likely that the efficacy would be less than 70% if more standard strains are tested or the manner in which the standard strain is chosen and defined is changed.

In fact, there were some noticeable findings from the clinical trials (Findlow *et al.* 2010; Snape *et al.* 2010). First of all, Bexsero contains only one variant of fHbp (variant 1.1) and the elicited antibody response of the participants did not even show cross-strain bactericidal activity towards other subvariants (*e.g.* fHbp variant 1.14 in the NZ98/254 strain). The protection coverage might decrease significantly than expected initially. Secondly, although the NadA (variant 3.1) in the vaccine was shown to provide cross-variant protection to variants 1, 2 and 3, a recent epidemiological study of 87 strains in England and Wales showed that only 20 strains expressed NadA and 5 strains were variant 1, 2 or 3 (Lucidarme *et al.* 2010). The protection rate contributed from NadA is therefore a concern. Thirdly, the antisera raised against the 3 recombinant proteins without OMV did not kill the strains with homologous NHBA variant 1.2 (including the NZ98/254 strain and the M00242922 strain). It was therefore suggested that the immunogenicity of NHBA needs further evaluation (Snape *et al.* 2010).

In terms of a 'universal vaccine' against meningococcal B infection, it remains uncertain whether this 4CMenB will succeed. Therefore, there is still a continuing need for a MenB vaccine which can offer more comprehensive protection. In the current thesis, the vaccine potential of 2 novel OM proteins, MIP and ACP was tested. This was achieved by cloning the *mip* and *acp* genes into the pRSETA system, expressing the N-terminal 6xHisTag recombinant proteins and purifying the proteins using nickel column chromatography. Purified rMIP and rACP were used for animal immunization. The antisera were then tested for their bactericidal activity, which remains the 'gold-standard' and immune surrogate of protection against meningococcal disease. In addition, we also investigated the role of ACP during pathogenesis of meningococcal infection, as effective antibody elicited against different virulence factors could contribute to different levels of protection.

In our study, the genes encoding MIP and ACP proteins from strain MC58 were successfully cloned using the pRSETA system. Both recombinant proteins were expressed and purified by nickel column chromatography with high yield. Since both the proteins can induce serum bactericidal activity, it is worth considering using this manufacturing method to produce meningococcal vaccine antigens. However, the recombinant proteins contained a N-terminal leader peptide (39 amino acids, including 6xHisTag). The presence of the leader sequence may affect refolding of recombinant proteins and hence reduce their immunogenicity. The leader peptide can be removed by protease treatment, but use of protease enzymes can be either inefficient, leading to low protein yield, or non-specific, leading to degradation of the protein (Ward *et al.* 1996). The leader peptide of both rMIP and rACP was not removed in our study; moreover, their ability to elicit bactericidal antibodies indicated that the proteins were likely to retain their functional conformation. This finding is consistent with a previous study showing that the leader sequence is not likely to interfere with protein folding to native conformation (Ward *et al.* 1996). However, if these antigens are going to be introduced into humans, it may be necessary to check if the sequences themselves are immunogenic or even induce autoimmune responses.

Alternatively, there are options of using other plasmid systems that allow cleavage and removal of leader peptides. For example, the leader peptide of an intein-tagged recombinant PorA protein prepared using the IMPACT-TWIN system could be cleaved via binding with chitin on-column (Humphries *et al.* 2004). Furthermore, the 6xHisTag recombinant proteins in the 4CMenB were cloned into pET24b+ system and purified through nickel column chromatography (Giuliani *et al.* 2006). There is no information in the published papers on 4CMenB regarding how many extra amino acids were added to the recombinant proteins; however, it does contain 6xHisTag in the final vaccine product (Bai *et al.* 2011).

In the following sections, the vaccine potentials of these 2 novel antigens (Section 5.1 and 5.2) and the role of ACP as a new adhesion/invasin (Section 5.3) will be discussed.

5.1 The *Neisseria meningitidis* rMIP protein induces cross-strain serum bactericidal activity and is a potential serogroup B vaccine candidate

This is the first report demonstrating the significant vaccine potential of a recombinant *Neisseria meningitidis* macrophage infectivity potentiator (rMIP) protein, which can induce cross-strain bactericidal activity. As judged by IF, the native MIP protein is surface-located. Bactericidal activity elicited by rMIP also provided supporting evidence of its surface-exposure. Moreover, the MIP protein is highly conserved and expressed to similar levels amongst 13 surveyed strains. There were only 3 types of MIP identified from the clinical strains or colonised strains of different serogroups, serotypes and serosubtypes. The MIP protein of MC58 was defined as type I. Type II MIP showed 98% similarity to type I MIP, with the absence of 4 amino acid (aa 27-30) and 2 amino acids changes (aa 39 and 165). Notably, type II MIP has not been found in the NCBI database. The type III MIP has 3 amino acid changes within 4 aa space (aa 140, 142 and 143) compared to type I MIP (99% similarity). In addition, the type III MIP can be found in other meningococcal strains, including α14, 961-5945 and G2136 base on the NCBI database. The limited amino acid variations are likely to have a minimal effect on epitope presentation and this has been proved indirectly by the reactivity of anti-type I MIP sera with the different types of MIP and the positive cross-strain bactericidal activity.

The key findings from our study were that rMIP elicited murine antibodies that induced complement-mediated killing of meningococci. Antisera raised against rMIP in liposomes induced bactericidal activity against the homologous strain (1/256). Incorporation of other meningococcal outer membrane proteins, such as PorA protein (Humphries *et al.* 2006), NM0938 (Sardinas *et al.* 2009a), NMB0928 (Delgado *et al.* 2007) and NMB0088 (Sardinas *et al.* 2009b) into liposomes has been shown previously to elicit serum bactericidal activity. However, antisera raised against rMIP incorporated into ZW 3-14 micelles + MPLA and liposome + MPLA showed the highest SBA titres against the homologous strain, MC58 (1/1024). Addition of MPLA not only increased the quantity of antibody production but also the SBA titres killing homologous strain. Similarly, previous studies have shown that incorporation of PorB (Wright *et al.* 2002) or Opc protein (Jolley *et al.* 2001) into liposomes or ZW 3-14 micelles containing MPLA produced the most effective formulations to induce bactericidal antibody.

A significant finding from our study was that rMIP protein in saline alone, without any exogenous adjuvant, induced bactericidal antibody titre comparable to rMIP in liposomes. Since the rMIP protein was purified under native conditions, it appears that the protein can be present in its native confirmation without the need to refold or to use other delivery vehicles. It is therefore possible that the rMIP itself acts as a pathogen-associated molecular pattern

(PAMP), which can activate antigen presenting cells (*e.g.* dendritic cells) or B cells and trigger adaptive immune responses.

By contrast, the rMIP protein adsorbed onto aluminium hydroxide did not elicit bactericidal activity, despite the fact that high titres of antibody that bound to the surface of meningococci were induced. It is possible that adsorption of rMIP onto aluminium hydroxide interferes with the structure of its immunogenic epitope(s). A previous study has demonstrated that the adsorption of lysozyme and ovalbumin to aluminium-based adjuvants could alter their tertiary structure and thermal stability (Jones *et al.* 2005).

Notably, the SBA titres elicited by rMIP in micelles + MPLA and liposomes + MPLA (1/1024) were comparable to those elicited by recombinant PorA protein in liposomes (1/512-1/2048) (Christodoulides *et al.* 1998; Humphries *et al.* 2006). Therefore, the bactericidal activity of antibodies to rMIP in these 2 formulations was equivalent to the activity observed for antibodies induced against the most immuno-dominant OM protein of meningococci.

An important attribute of any potential meningococcal vaccine candidate is the ability to induce antibodies that show bactericidal activity against other, heterologous, meningococcal strains. In our study, anti-rMIP sera also showed bactericidal activity for heterologous strains expressing different types of MIP. Antisera raised against rMIP in liposomes and rMIP in saline showed similar SBA titres in killing heterologous strains. However, addition of MPLA showed deleterious effect on killing of strain MC54. This deleterious effect has also been observed in a previous study in which the liposomal PorA elicited SBA (1/512), while addition of MPLA resulted in a loss of bactericidal activity (Christodoulides *et al.* 1998). By contrast, in the study of Arigita *et al*., liposomal PorA with or without addition of MPLA showed no differences in eliciting homologous SBA; however, the dose of MPLA used in this study might be too low (protein/adjuvant (w/w) =2) to enhance immune responses or cause a deleterious effect on immunogenicity (Arigita *et al.* 2005).

It is possible that addition of MPLA has an effect on the epitope structure and/or recognition during antigen presentation. The mechanism remains unknown. MPLA may associate with OM proteins in liposomes, in a similar manner to LPS-protein interactions in OM, and subtly alter epitope conformation. Interestingly, MPLA appeared to affect the immunogenicity of type III MIP rather than type II MIP, leading to overall lower titres of bactericidal antibody. This raises an important caveat in the selection of adjuvants for meningococcal proteins.

A concern of SBA assay that needed to be addressed was whether it was possible that a MIP^- mutant could be selected during or after SBA experiment. Theoretically, such a selection force would not normally happen so quickly. But in order to investigate this, the SBA assay

survivors were immunoblotted and reacted with anti-rMIP sera. As expected, the SBA assay survivors all expressed MIP proteins, demonstrating that there was no selection of MIP non-expressing bacteria.

In addition, it has been shown that complement source can also affect the result of the SBA assay. Human complement tends to give lower titres compared to baby rabbit complement (Maslanka *et al.* 1997). That was mainly attributed to the presence of factor H on human complement but not on baby rabbit complement. Meningococcal factor H binding protein (fHbp) has been shown to bind to human factor H protein, subsequently down-regulate alternative complement pathway and allow meningococci to evade complement-mediated killing (Madico *et al.* 2006). Therefore, it is likely that the bactericidal titre might be 'falsely' high using baby rabbit complement. Nevertheless, sources of human complement with inherently low bactericidal activity against meningococci are extremely difficult to find. If time permitted and a reliable source of human complement could be found, SBA assay could be repeated in the current study using 25% (v/v) human complement and the data then compared with data generated using 5% (v/v) baby rabbit complement.

In summary, rMIP is an excellent vaccine candidate as it fulfils several important criteria: rMIP is 1) surface-located, 2) highly conserved, 3) expressed to similar levels in all strains sequenced thus far, and 4) able to induce antibodies that show cross-strain bactericidal activity. Recently, in an *ex vivo* human whole-blood model of wild type MC58 infection, *mip* gene expression was shown to be up-regulated (Echenique-Rivera *et al.* 2011). Although there is no study, yet, regarding the role of MIP in pathogenesis during meningococcal infection, based on a study of the similar gonococcal MIP, meningococcal MIP protein may play a role in persistence in macrophage infection (Leuzzi *et al.* 2005). In the future, large-scale gene screening will be necessary to discover if the *mip* gene is highly conserved and expressed in meningococci. Recently, interrogation of the ~200 sequenced strains in the BIGS database has suggested that type I, II and III MIP proteins are found in ~90% of meningococci and that inclusion of a type IV MIP protein would provide ≥95% strain coverage (personal communication, M. Christodoulides). In addition, the investigation of other novel adjuvants or combination of different types of MIP protein incorporated into liposomes may improve functional immunogenicity.

5.2 The *Neisseria meningitidis* ACP protein induces cross-strain serum bactericidal activity and is a potential serogroup B vaccine candidate

In this study, recombinant ACP was prepared by cloning into the pRESTA system, followed by gene expression and purification of protein by nickel column affinity chromatography. Recombinant ACP protein was insoluble and was purified initially under denaturing conditions in the presence of urea but this process, however, yielded two bands on SDS-PAGE. The doublet was present as long as urea was used in the purification buffers. Since there were no internal translation starts or premature termination sites and the doublet was not present before purification was carried out, we hypothesized that the presence of urea may be responsible for the doublet formation. In order to confirm that both protein bands were rACP, the individual bands were excised from the gel and subjected to mass spectrometry (MS) following protein digestion by trypsin, and the amino acid sequences of fragments were compared against the MC58 proteome. This demonstrated that both bands were indeed composed of identical proteins.

Urea is commonly used for denaturing proteins, but can lead to carbamylation (Figure 5.1), which has been widely reported as a problem in 2-D SDS-PAGE and associated with sample preparation steps (McCarthy *et al.* 2003; Righetti 2006). Temperatures higher than 37°C and pH values >8.5-9.5 favour the occurrence of this post-translational modification on lysine, arginine and cysteine residues. However, our experiments were carried out at room temperature (25°C) and at a pH $\leq$8.0. We therefore replaced urea with GuHCl in all buffers used for the purification of rACP and this resulted in a single band on SDS-PAGE. In addition, although the molecular basis for denaturation by both urea and GuHCl are unclear, GuHCl was shown to be more efficient at denaturing globular proteins (Del Vecchio *et al.* 2002; Camilloni *et al.* 2008).

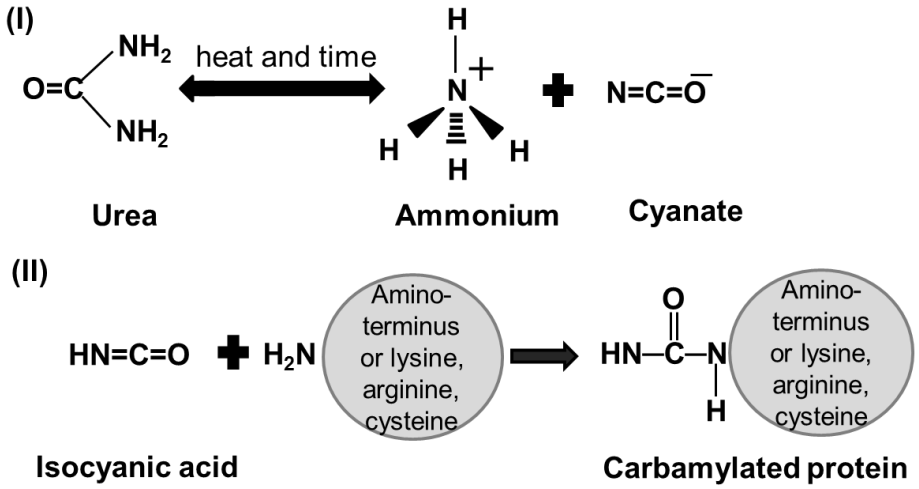

Figure 5.1 Mechanism of carbamylation. (Adapted from McCarthy, 2003)

The purified rACP was used for preparing different formulae to immunize animals and the animal experiments, bio-informatics analyses and protein studies showed that ACP fulfils several criteria for an ideal meningococcal vaccine antigen. First of all, ACP is present in all surveyed strains and it is highly conserved. In our 13 collected strains of different serogroups, serotypes and serosubtypes, which were isolated either from patients or carriers, only 3 types of ACP were identified. Notably, there are only 1-2 amino acid differences amongst the 3 types of ACP. Type II ACP has only one amino acid difference compared to type I ACP ($Asp^{25} \rightarrow Asn^{25}$). With 1 more amino acid substitution ($Ala^{12} \rightarrow Ser^{12}$) to type II ACP, type III ACP also shares high similarity (98%) with type I ACP. Next, similar to the BLAST-protein results from the NCBI website, interrogation of the BIGS database showed that only type I (15 strains) and type II ACP (158 strains) proteins are present in all meningococcal strains (173). Since only strain MC90 expresses type III ACP, we repeated sequencing of this strain using genomic DNA prepared from different batches of bacteria and the result was reproducible.

Interestingly, the type I ACP protein is not only present in meningococci but also in other colonizing *Neisseria* strains, including *N. sicca*, *N. polysaccharea* and *N. lactamica*. It suggests that the protein might be important in colonization of the nasopharyngeal mucosal epithelium. This hypothesis could be indirectly supported by the fact that this cluster of type I protein does not include gonococcal ACP. Different ACP proteins for colonizing the nasopharynx and urogenital tract mucosae might suggest that *Neisseria* ACP is produced in different conformations in order to show a tropism for interactions with respiratory tract cells and urogenital tract cells.

Regarding the high conservation of ACP amongst *Neisseria* spp., it is possible that a vaccine eliciting effective anti-ACP antibody can wipe out all carriage of *Neisseria spp.*. The non-pathogenic *Neisseria*, such as *N. lactamica*, have been suggested to provide protection from meningococcal infection by niche occupation or generating cross-protective bactericidal antibodies (Gold *et al.* 1978). Nevertheless, the relative role of non-pathogenic *Neisseria* in the upper respiratory tract and how they provide protection against meningococcal infection are not clear. Theoretically, if a vaccine can decolonise all the *Neisseria spp.* in the upper respiratory tract, the protection from the colonised strains might be no longer needed. However, whether decolonisation of all *Neisseria* will expose host niche to other colonising bacteria with pathogenic potential needs further investigation.

Notably, the majority of *N. meningitidis* strains express type II ACP, which is present exclusively in *N. meningitidis*. There is no information on whether meningococci with type II ACP are more virulent or more important for colonization. However, if we check the types of proteins expressed by serogroup B meningococci (91 strains in total), type II ACP is present in the majority (80 strains).

Secondly, the rACP induced complement-mediated serum bactericidal activity, which is indeed one of the most important criteria for a meningococcal vaccine antigen. The rACP protein was purified under denaturing conditions using GuHCl and then precipitated by 10% (v/v) trichloroacetic acid. Notably, the resulting rACP in saline alone induced bactericidal antibodies similar to immunisation with rACP incorporated into liposomes (1/512). Therefore, it is possible that the rACP protein can refold naturally after precipitation or the purification procedure does not affect the conformation of the immunogenic epitope(s). Furthermore, rACP adsorbed to aluminium hydroxide also induced bactericidal antibodies (1/128). By contrast, antisera raised against rACP formulations containing MPLA showed bactericidal titres <1/4 although MPLA did increase the ELISA end point titres. In some way similar to the observations with rMIP, MPLA might also profoundly change the conformation of the rACP protein as presented to immune cells in mice. Possibly, if rACP is also a Toll-like receptor 4 agonist, MPLA and rACP might compete for the receptors and therefore affect functional immunogenicity of rACP in the presence of MPLA.

Antisera raised against preparations which induced homologous bactericidal activity were also tested against heterologous strains with different types of ACP, *i.e.* type II and type III. As a result, the heterologous strain SBA data were similar to homologous SBA results. Therefore, rACP can induce high and comparable SBA titres towards heterologous strains to provide broad protection.

Surface location of ACP was confirmed by FACS analysis and indirectly also by the SBA results. When immunofluorescence showed negative results, the incubation of murine antibody was prolonged from 1 hour under room temperature to overnight at 4°C. However, no increased fluorescence signal was observed. This is likely to be due to the low expression level of ACP in the OM, compounded by low sensitivity of the IF assay; or, ACP may not be expressed on the outer surface of the OM, but perhaps with an orientation towards the inner membrane. Therefore, we used FACS analysis which provides a more sensitive way of detecting the surface location of proteins. Although this might not be the most accurate representation of surface location, it has been widely used to determine the surface exposure of other meningococcal antigens (Pizza *et al.* 2000; Masignani *et al.* 2003; Fletcher *et al.* 2004). A significant shift of the immunofluorescence intensity demonstrated that ACP was surface located.

Taken together, the *acp* gene is present in all meningococci (186 strains in total) and expressed to similar levels in our 13 surveyed strains. Importantly, recombinant type I ACP in liposomes and saline alone can elicit high SBA titres (1-256-1/512) towards heterologous strains expressing type II and III ACP. Adsorption of rACP to aluminium hydroxide also elicited cross-strain SBA although the titres were lower (1/64-1/128). Moreover, ACP appears to be surface-exposed. Therefore, it deserves to be considered as a vaccine candidate either as a single protein vaccine or in a multiple component vaccine.

5.3 The ACP protein plays a role in mediating meningococcal interactions with human cells

5.3.1 ACP is a minor adhesion showing cell tropism

The key findings in our infection experiments are that the MC58ΔACP showed a decreased binding to different host cells compared to wild type MC58. MC58ΔACP mutant showed approximately 4-fold reduction (75%) of total associated bacterial numbers with Chang epithelial cells and Hep2 epithelial cells compared to wild type MC58. Moreover, MC58ΔACP showed approximately a 50% reduction of total association with HUVECs. However, only 20-30 % of reduction in adhesion was observed for meningioma cells (either transitional cell type, M53 or meningothelial type, M61), but this did reach statistical significance. Thus, the variation of difference between different cell lines showed a decreased trend from epithelial cells, endothelial cells to meningioma cells. It is likely that ACP protein interacts with as yet uncharacterised host cell receptors and the distribution of these receptors is higher on epithelial cells compared to endothelial cells through to meningioma cells. This kind of cell tropism has also been observed with other major adhesins. For example, Opc is the most

effective protein in increasing bacterial interaction with HUVECs (Virji *et al.* 1993a). In addition, an OpaB-expressing strain showed higher association with Chang cells compared to HUVECs (Virji *et al.* 1993a). Interaction with host cells using different profiles of adhesins/invasins at different stages of infection can be a well-designed strategy for meningococci to successfully evade host immune selection pressure.

To date, there is little information regarding the role of minor adhesins used by meningococci for interacting with meningeal cells. In one study, a T-cell stimulating protein A (TspA) mutant of MC58 has been reported to decrease association with meningioma cells compared to wild-type MC58, but the data were not shown (Oldfield *et al.* 2007). In a previous study by Hardy *et al*, meningioma cells were first used to study the interaction with meningococci expressing different combinations of adhesins (Hardy *et al.* 2000). In this study, the most important adhesin for meningioma cells (of both meningothelial and transitional types) is the pilus; moreover, the type of pilus affects meningococcal association. In addition, without the presence of pili, expression of the Opc protein did not appear to have an effect on total association in the capsulate background. By contrast, with the expression of class Ia pili and Opc protein in capsulate strains, the absence of the Opa protein resulted in a 13-26% reduction of associated bacteria (Hardy *et al.* 2000). Interestingly, the importance of the Opa protein interacting with meningioma cells is similar to the observations in our current study of 20-30% less associated MC58ΔACP bacteria compared to wild type MC58.

Our study has also suggested that ACP plays a role in adhesion under capsulate background. It has been shown that the major adhesins, including Opa and Opc, effectively mediate adhesion of meningococci to epithelial or endothelial cells only in acapsular strains. Therefore, capsule-deficient and non-piliated variants were chosen for Opa and Opc study (Virji *et al.* 1993a). Some minor adhesins, including App (Serruto *et al.* 2003), NadA (Capecchi *et al.* 2005) and NhhA (Scarselli *et al.* 2006), have also been demonstrated to contribute to adhesion in a capsulated background. Therefore, it is possible that ACP plays a role in initial adhesion leading to colonisation of the nasopharyngeal mucosa. However, whether this occurs in concert or independently of other major or minor adhesins needs to be determined.

To confirm that the absence of ACP expression is the cause of decreased association with host cells, the MC58ΔACP mutant was complemented by inserting a copy of the *acp* gene. As a result, the complemented strain restored the function of adhesion, demonstrating similar associated bacterial numbers compared to wild type MC58. Moreover, addition of decomplemented rabbit anti-rACP sera during infection of epithelial cells also inhibited meningococcal adherence. Therefore, it appears that the reduction in associated bacterial number of the MC58ΔACP strain is directly due to the absence of ACP.

5.3.2 ACP is a new invasin that facilitates internalisation into human epithelial and endothelial cells

To investigate the role of ACP in invasion with minimal effect of other adhesins/invasins, we generated an ACP$^-$ mutant from a Cap$^-$, Pil$^-$, Opa$^-$, Opc$^-$ strain, MC58¢18 (McNeil and Virji 1997). *Neisseria* are naturally competent for transformation; however, without expression of pili, the transformation competency is significantly low (Seifert *et al.* 1990). Therefore, we finally used an adapted protocol from *E.coli* transformation (Bogdan *et al.* 2002; van Dam and Bos 2012), which increased the transformation efficiency greatly. Notably, since the specific non-piliated strain was selected by phase variation (McNeil and Virji 1997), we found that reversion to pilus expression occurred with a high percentage (10 out of 12 screened colonies) after transformation. Therefore, it was important to demonstrate that the bacteria harvested from the monolayers during infection experiments retained the original phenotype of the infecting strain.

Our major findings were that ACP is a new invasin, which is important for meningococci to enter human epithelial and endothelial cells. By comparison to MC58¢18, there was an approximately 3-fold reduction (66%) of percentage internalisation of MC58¢18ΔACP into Chang and Hep2 cells. A 5-fold reduction (80%) of percentage internalisation of MC58¢18ΔACP was observed in HUVECs. Notably, a much smaller number of internalised bacteria were quantified in invasion experiments using HUVECs. This is consistent with a previous study showing that the Opc protein is the most important invasin that enables meningococci to invade HUVECs (Virji *et al.* 1992; Virji *et al.* 1993a). Although the number of internalised bacteria was low, the reduction of internalised MC58¢18ΔACP compared to its parent strain was consistent. In addition, since it has been shown that meningococci do not invade meningioma cells with any certainty (Hardy *et al.* 2000), invasion experiments with these cells were unnecessary.

5.3.3 Proposed Model of ACP in pathogenesis

Following entry through epithelia, if meningococci can survive in the blood, they can cause septicaemia and/or penetrate the blood-cerebrospinal fluid barrier to reach the meninges and cause meningitis. In capsular strains, type IV pili have been shown to mediate the initial adhesion of meningococci to nasopharyngeal epithelial cells, endothelial cells and meningioma cells and different classes of pili also affect the process of adhesion (Virji *et al.* 1993b; Hardy *et al.* 2000). Based on our study, ACP in Cap$^+$Pil$^+$ strains also contributes to the initial process of adhesion of meningococci to human mucosal epithelial cells, endothelial cells

and meningioma cells although it appears to be more important in interacting with epithelial cells. As shown in Table 5.1, other minor adhesins, including App, NadA and NhhA have also been shown to contribute to the initial step of adhesion of $Cap^{+}Pil^{+}$ strains to epithelial cells. MspA was demonstrated to mediate adhesion to human bronchial epithelial cells and human brain microvascular endothelial cells, but this study was done using *E.coli* expressing MspA rather than meningococci (Turner *et al.* 2006). Therefore, to determine if MspA is important for adhesion of meningococci to host cells, further study comparing associated bacterial number of wild type MC58 and MC58ΔMspA would be needed.

After initial attachment, pili retract to bring the meningococci closer to the host cells (Nassif *et al.* 1999) and then disappear subsequently. At this stage, the synthesis of the polysaccharide capsule is down-regulated (Deghmane *et al.* 2002). During the second step of adhesion, a different repertoire of adhesins, including Opc and Opa, is involved that enable intimate association between the bacterial cell membrane and the host cell plasmalemma (Virji *et al.* 1993a). These two major adhesins are also important invasins. Furthermore, it has been shown that a low expression level of Opc can allow more Opa-dependent invasion of primary nasopharyngeal cells (de Vries *et al.* 1996). However, the mechanism of how these proteins interact with each other remains unclear.

During this stage, another invasin, NadA, has been shown to mediate invasion into Chang cells (Capecchi *et al.* 2005). Our study showed that ACP also plays a role in invasion to both epithelial cells and endothelial cells. We chose an acapsular mutant without expression of Opa, Opc and pilin based on the hypothesis that the absence of these major OM antigens would demonstrate the role of ACP in invasion, if this protein was only a minor invasin whose function could be possibly masked by the dominant invasins. However, since the particular strain does not express these major adhesins/invasins, we could not observe the interaction between ACP and other major adhesins/invasins. It might be interesting to investigate whether the interaction of ACP with other proteins by generating ACP mutants in an acapsular background with differing expression of Opa, Opc and other important components.

In summary, redundancy of adhesins/invasins is possibly one of the most important survival tactics of *Neisseria* (Stephens 2009). ACP is not only a new adhesin, but also a new invasin which is important for meningococcal interactions with both epithelial and endothelial cells. We can postulate that an effective antibody response to ACP could interfere not only with attachment of meningococci to nasopharyngeal mucosa but also with invasion into the blood and therefore provide effective protection.

Table 5.1 Minor adhesins expressed by *Neisseria meningitidis*.

Protein, *Mr*	**Homology/conservation**	**Biological function**	**Interactions with host cells**	**SBA**
NadA, Neisseria adhesin A, NMB1994, 38 kDa	➢ Similar to a novel class of adhesins (Oca family, including YadA and UspA2); ➢ present in 52 out of 53 hypervirulent strains (Comanducci *et al.* 2002)	A good immunogen with self-adjuvanting properties (Mazzon *et al.* 2007)	**I.** Capecchi *et al.* reported that 1) NadA *E. coli* mediated bacterial adhesion to **Chang cells** but not to HUVECs; 2) Cap^{+}MC58ΔNadA mutant showed 3-fold reduction adhering to Chang cells and 3) NadA was proved to be an **invasin** for Chang cells in Cap^{-}MC58 (Capecchi *et al.* 2005). **II.** NadA targets human monocytes /macrophages (Franzoso *et al.* 2008). **III.** Receptors: human **β1 integrins** (Nagele *et al.* 2011).	**Yes** (Comanducci *et al.* 2002)
App , Adhesion and penetration protein, 160 kDa	➢ Highly homologous to the *Haemophilus* adhesion and penetration protein (Hap); ➢ present in all genomes that have been sequenced.	Autotransoprter; autocatalytic serine protease activity (Serruto *et al.* 2003);	**I.** Serruto *et al.* reported that 1) App *E. coli* mediated bacterial adhesion to **Chang cells** but not to HUVECs; 2) Cap^{+}MC58ΔApp mutant showed 3 to 10-fold reduced adhering to **Chang cells**.; 3) no App-mediated association with Hep2 and HUVECs (Serruto *et al.* 2003). **II.** No invasion study. **III.** Receptors: unknown.	**Yes** (Hadi *et al.* 2001)

NhhA, *Neisseria* hia/hsf homologue, GNA0992, 57 kDa	➢ Similar to Hsf and Hia adhesins of *H. influenzae*; ➢ expressed in the majority of disease-associated strains (Peak *et al.* 2000)	A multifunctional trimeric autotransoprter (Scarselli *et al.* 2006)	**I.** Scarselli *et al* reported that 1) NhhA *E. coli* mediated bacterial adhesion to **epithelial cells**, including Chang cells and Hec-1-B; 2) Cap^{+}MC58ΔNhhA adhered to Chang cells 10-fold less than the wild-type strain; 3) A NhhA complemented strain was also investigated to augment the evidence (Scarselli *et al.* 2006). **II.** No invasion study and also no other cells lines studied. **III.** Receptors: **laminin** and **heparin sulphate** (Scarselli *et al.* 2006)**.**	**Yes** (Pizza *et al.* 2000)
MspA, Meningococcal serine protease A, NMB1998, 157 kDa	➢ Homology to IgA1 protease and App of meningoccci; ➢ not present in all meningococcal strains	An autotransporter (Turner *et al.* 2006)	**I.** MspA *E. coli* mediated adhesion to **human bronchial epithelial cells** and **human brain microvascular endothelial cells** (HBME) (Turner *et al.* 2006). No further study in meningococcal strains. **II.** No study of its role in invasion. **III.** Receptors: unknown.	**Yes** (Turner *et al.* 2006)
HrpA, NMB1779 180 kDa; **HrpB**, NMB1780	➢ Homology to *Bordetella pertussis* FHA (filamentous hemagglutinin) ➢ Expressed by all strains	A functional two-partner secretion (TPS) system; HrpB as a transporter for HrpA	**I.** Decreased percentage association of MC58ΔhrpA and MC58ΔhprB with **Hep2 cells** and **FaDu epithelial cells** compared to Cap^{+}MC58 (Schmitt *et al.* 2007). **II.** Essential for the intercellular survival of meningococci (Tala *et al.* 2008) **III.** Receptors: unknown.	NA

Fructose-1,6-bisphosphate aldolase (**FBA)** , NMB1869, 38 kDa	➢ Potential virulence factors in a variety of organisms; ➢ Expressed in all meningococci	fructose bisphosphate aldolase activity	**I.** FBA was shown to mediate association with **Hep-2** cells and human brain microvascular endothelia (**HBME**) in Cap^{+}MC58. **II.** No role in invasion. (Tunio *et al.* 2010b). **III.** Receptors: unknown.	NA
Glyceraldehyde 3-phosphate dehydrogenase (**GapA-1**), NMB0207	➢ Based on upregulation during adhesion; ➢ Expressed in all meningococci	a glycolytic enzyme	**I.** GapA-1 was shown to mediate association with **Hep-2** cells and human brain microvascular endothelia (**HBME**) both in Cap^{+}MC58 and Cap^{-}MC58. (Tunio *et al.* 2010a) **II.** Receptors: unknown.	NA
T-cell stimulating protein A (**TspA**)	➢ Sequence similarity to FimV (related to pili twitching) of *Pseudomonas aeruginosa;* ➢ Expressed in all meningococcal strains	An immunogenic , T and B cell stimulating proteins	**I.** Required for optimal adhesion to **Hep2 cells** and **meningioma cells** (data not shown) in Cap^{+}MC58 (Oldfield *et al.* 2007). **II.** No study of its role in invasion. **III.** Receptors: unknown.	NA

5.4 Conclusion and Prospective studies

In comparison to the antigens contained in the two vaccines currently in advanced clinical trials and pre-licensure, the MIP and ACP proteins are more highly conserved and capable of inducing significant cross-strain bactericidal activity. Therefore, they both deserve consideration as novel vaccine candidates for inclusion in current or future multicomponent MenB vaccines. To optimise the design of the two vaccine antigens, additional studies can be proposed. These studies include:

1. Generation of monoclonal antibodies for epitope mapping. If the epitope(s) that induce SBA are identified, then vaccines can be made as synthetic polypeptides (Christodoulides *et al.* 1999) or peptide epitope-based DNA vaccines. As long as the DNA sequence encoding the target antigen is known, it can be inserted into to a carrying plasmid and induced expression controlled by a strong viral promoter. This kind of vaccine can trigger both antibody production and also T-cell immunity. In addition, the double-strand DNA itself can be a good adjuvant (Rice and Christodoulides 2012). Liposomes can be also considered as a vehicle and adjuvant for DNA or synthetic peptides vaccines (Henriksen-Lacey *et al.* 2011).
2. Testing different adjuvants in order to enhance immunogenicity. If a MenB vaccine is going to contain these two proteins, our studies show that aluminium hydroxide and MPLA are not suitable adjuvants due to lack of SBA elicited by rMIP on aluminium hydroxide and rACP with any MPLA present. Although these proteins demonstrate inherent immunogenicity in the absence of exogenous adjuvant, the inclusion of appropriate adjuvants will enhance immunogenicity and therefore potentially increase vaccine efficacy.
3. Crystallization could be done to provide more information on the structure of the proteins and their protective epitopes (Saleem *et al.* 2012). A combination of protein structure and immunology is likely to elaborate vaccine development.
4. Are the purified recombinant proteins lipidated?

   Both native MIP and ACP are predicted to be lipoproteins in *Neisseria* using an online software program (http://www.cbs.dtu.dk/services/LipoP/). In MIP, there is a lipobox motif ($L_{18}XXC_{21}$) where the lipid can attach to the cysteine followed by the cleavage of the signal peptide (the first 20 amino acids). This cleavage is predicted to occur through the action of a lipoprotein signal peptidase II (SPase II). By contrast, without the similar lipobox, the ACP is also predicted to be a lipoprotein and cleaved by signal peptidase I (SPaseI) between aa 21 and 22 (Juncker *et al.* 2003).

The importance of the signal peptide in lipidation has been shown in gonococcal MIP, which also has a lipobox motif ($L_{18}XXC_{21}$) The gonococcal *mip* gene with or without the signal peptide (the first 20 aa) was cloned and expressed in *E.coli*. Using radioactive [9-10-^{3}H]-palmitic acid labelling, the gonococcal MIP was demonstrated to be lipidated in *E.coli* only with the signal peptide present (Leuzzi *et al.* 2005). In fact, up to the first 38 amino acids in meningococcal MIP are the same as gonococcal MIP and it is therefore very likely that meningococcal MIP is also a lipoprotein with the same signal peptide.

Notably, rMIP and rACP proteins are immunogenic without exogenous adjuvants. It is possible that the proteins themselves are good immunogens because they were purified as lipoproteins, which can be self-adjuvants (Moyle and Toth 2008). In contrast to the gonococcal MIP with C-terminal 6xHisTag (Leuzzi *et al.* 2005), our rMIP protein had a N-terminal leader peptide containing 6xHisTag (Figure 5.2). Since the rMIP protein was purified through nickel column chromatography, it means that the purified protein retained the signal peptide of the MIP protein sequence following the N-terminal leader peptide. It would be interesting to investigate whether rMIP with N-terminal leader peptide can also be lipidated. In that case, lipidation should happen without subsequent cleavage of the signal peptide. In addition, to demonstrate the contribution of lipidation to functional immunogenicity, a rMIP protein without the leader peptide or a full-length rMIP with C-terminal 6xHisTag can be produced for immunisation studies. Similar studies can be suggested for the rACP.

5. Although both proteins were not detected in immunoproteomic studies, it did not exclude the possibility that the proteins can be recognised by the convalescent sera from patients or sera from the carriers. Thus, if possible, we can use the immune human sera and non-immune human sera to react with the purified rMIP or rACP. If the proteins can be recognised only by the immune sera rather than non-immune sera, that will provide further evidence of the potential immunogenicity of rMIP or rACP in humans.

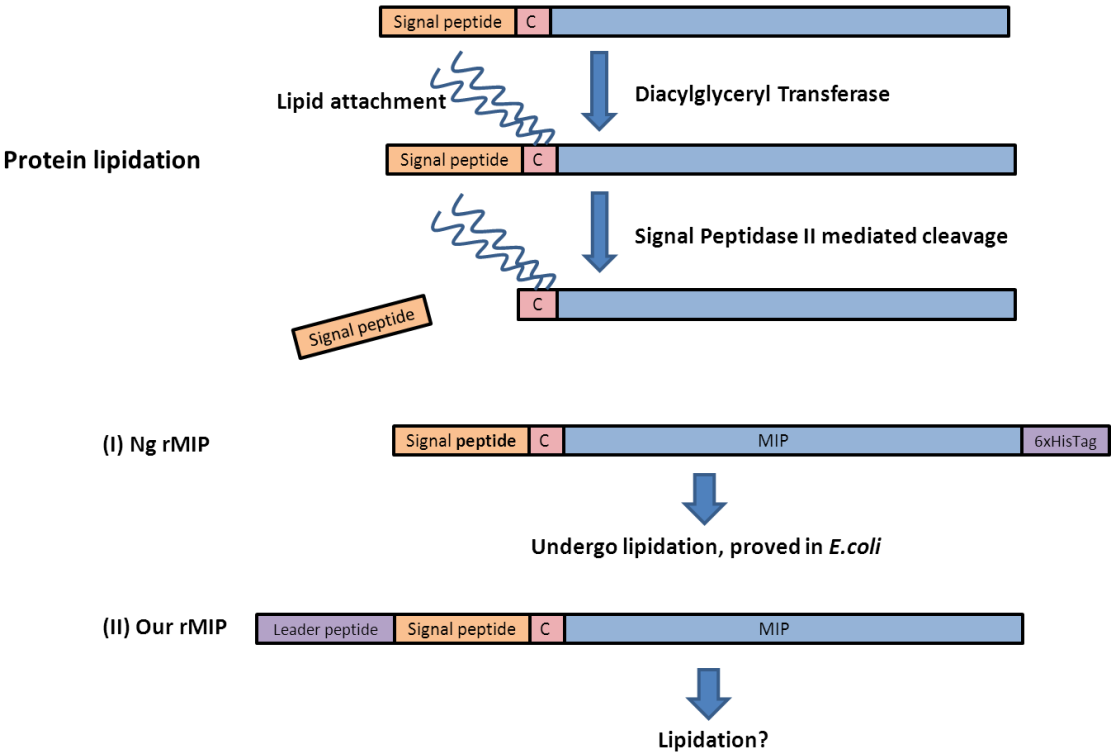

Figure 5.2 Protein lipidation followed by cleavage of the signal peptide. The Ng-MIP (Leuzzi *et al.* 2005) was cloned with a C-terminal 6xHisTag while our rMIP was cloned with a N-terminal leader peptide (39 aa including 6xHisTag for nickel column chromatography). Whether lipid can attach to the cysteine in this aa sequence without cleavage needs further investigation.

6. Since these 2 proteins are highly conserved amongst the two pathogenic *Neisseria*, we would test the hypothesis that the antibody elicited against the meningococci is also bactericidal towards gonococci. It might not be cost-effective to develop a vaccine against gonococci but if a meningococcal vaccine can provide cross protection against gonococci, this might be a multi-functional vaccine.

I. Future work for MIP protein

1. The BLAST result showed that the meningococcal MIP protein has a 30-40% similarity with human FK506 binding protein at the C-terminal end (Figure 5.3). However, the similarity is not in continuous stretches but rather discrete so the MIP protective epitope might not be found in human proteins. It is still possible that the vaccine containing the whole rMIP protein would induce no antibody production in humans or even an autoimmune reaction. Truncation of the protein and production of N-terminal fragment for immunisation, to avoid the similar sequence with human protein(s) can be considered to obviate this possibility. Thus, this increases the importance of identifying the protective epitope within MIP.

```
MIP                150 GLQYKITKQGEG-KQPTKDDIVTVEYEGRLIDGT-VFDS-------SKANGGPVTFPL-- 198
I.  FKBP506-BP6     27 GVLKDVIREGAG-DLVAPDASVLVKYSGYLEHMDRPFDS-------NYFRKTPRLMKLGE 78
II. FKBP52          32 GVLKVIKREGTGTEMPMIGDRVFVHYTGWLLDGT-KFDS-------SLDRKDKFSFDLGK 83
III.FKBP52          57 -----IKREGTGTEMPMIGDRVFVHYTGWLLDGT-KFDS-------SLDRKDKFSFDLGK 102
IV. FKBP51          35 ---KIVKRVGHGEETPMIGDRVYVHYNGKLANGK-KFDS-------SHDRNEPFVFSIGK 83
V.  FKBP51          39 ---KIVKRVGNGEETPMIGDKVYVHYKGKLSNGK-KFDS-------SHDRNEPFVFSLGK 87
VI. FKBP25          17 ---------------PKKGDVVHCWYTGTLQDGT-VFDTNIQTSAKKKKNAKPLSFKVGV 60

MIP                199 SQVIPGWTEGVQLLKEGGEATFYIPSNLAYREQGAGD-KIGPNATLVFDVKLVKI 252
I.  FKBP506-BP6     79 DITLWGMELGLLSMRRGELARFLFKPNYAYGTLGCPP-LIPPNTTVLFEIELLDF 132
II. FKBP52          84 GEVIKAWDIAIATMKVGEVCHITCKPEYAYGSAGSPP-KIPPNATLVFEVELFEF 137
III.FKBP52         103 GEVIKAWDIAIATMKVGEVCHITCKPEYAYGSAGSPP-KIPPNATLVFEVELFEF 157
IV. FKBP51          84 GQVIKAWDIGVATMKKGEICHLLCKPEYAYGATGSLP-KIPSNATLFFEVELL-- 135
V.  FKBP51          88 GQVIKAWDIGVATMKKGEICHLLCKPEYAYGSAGSLP-KIPSNATLFFEIELL-- 250
VI. FKBP25          61 GKVIRGWDEALLTMSKGEKARLEIEPEWAYGKKGQPDAKIPPNAKLTFEVELVDI 115
```

Human proteins	Similarity (%)
I. Chain A, crystal structure of human Fk506-binding protein 6, 134 aa	32/106 (30%)
II. Chain A and B, crystal structure of the N-terminal domain of human Fkbp52, 140 aa	39/106 (37%)
III. Chain A, crystal structure of N(1-260) of human Fkbp52, 280 aa	38/101 (38%)
IV. Chain A, structure of the large Fkbp-Like protein, Fkbp51, involved in steroid receptor complexes, 457 aa	39/101 (39%)
V. Chain A and B, crystal structure of A fragment of Fkbp51 comprising the Fk1 and Fk2 domains, 254 aa	39/101 (39%)
VI. Chain A, homologous domain of human Fkbp25, 116 aa	40/99 (40%)

Figure 5.3 Alignment of the MIP protein with human FK binding proteins using ClustalW2 programme (http://www.ebi.ac.uk/Tools/services/web_clustalw2/). The identical amino acids (in red colour) between MIP with human Fkbp proteins are discontinuous. The full name of the human proteins and % similarity are listed.

2. Gonococcal MIP (Ng-MIP) has been shown to be involved in persistence in macrophages and to have a peptidyl-prolyl cis/trans isomerase (PPIase) activity (Leuzzi *et al.* 2005). According to the homology of Ng-MIP and meningococcal MIP protein, it is very likely that MIP protein also has similar biological functions. In the future, a MIP deficient mutant could be generated from wild-type MC58. Further infection experiments using different cells lines, such as macrophages, epithelial cells, endothelial cells and meningeal cells could be carried out to investigate the role of MIP protein in different stage of infection, such as adhesion, invasion or intracellular survival. We can also investigate whether the presence of Ng-MIP could promote conversion of the *cis* form to *trans* form more quickly (Figure 5.4) and whether this enzymatic activity could be inhibited by rapamycin.

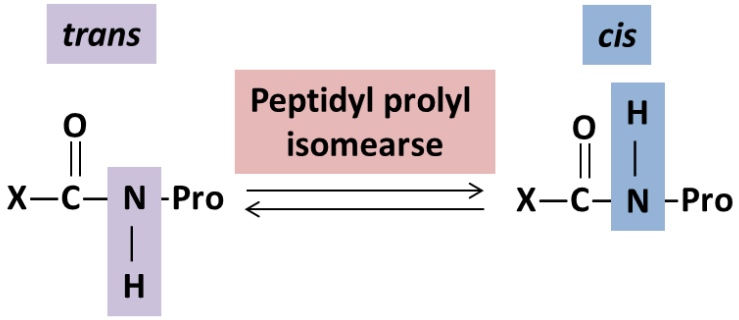

Figure 5.4 The action of peptidyl prolyl isomerase (PPIase).

3. There is no information on the receptors on host cells that interact or recognise MIP, Ng-MIP or Lp-MIP. Recently, the receptor on human cells for *Chlamydia trachomatis* MIP have been studied and shown to be a complex of TLR2/TLR1/TLR6 and CD14 (Bas *et al.* 2008). Although *Chlamydial* MIP and *Neisseria* MIP proteins shared low homology, they both belong to immunophilin family. Therefore, it would be interesting, initially, to investigate if *Neisseria* MIP protein also signals through the same receptors on host cells, or uses alternative mechanisms.

II. Future work for ACP protein

1. What are the cell receptors recognised by ACP?

Since ACP is shown to be a new adhesin and a new invasin, we would test the hypothesis that specific receptors on host cells are the targets of ACP. The cell tropism of ACP suggests that the putative host cell receptors for ACP are in greater abundance on epithelial cells, and lower on endothelial cells and meningeal cells. In comparison to Opa, the cell tropism of ACP appears to show a similar pattern. It has been shown that either $OpaB^+$ or $OpaD^+$ variants associated considerably more with Chang epithelial cells than with HUVECs (Virji *et al.* 1993a). Additionally, a similar pattern of Opa^- mediated and ACP^- mediated association was observed on meningioma cells (Hardy *et al.* 2000). Therefore, it is possible that ACP binds to similar receptors that are recognised by Opa protein, including CEACAM (1,3,5 and 6), heparin sulphate proteoglycan (HSPG), extracellular matrix (ECM) and integrins (Virji 2009).

Recently, it was reported that *N. meiningitidis* can interact with CEACAM1 using another binding pathway which is independent of Opa, Opc and NadA (Kuespert *et al.* 2011). This study suggested the presence of a second CEACAM1-binding invasin that associates with the protein backbone rather than the carbohydrate structure of CEACAM1. Our study

has shown that ACP is a new invasin, so it might be interesting to investigate if ACP is this second invasin that could also bind to CEACAM1.

2. A previous study showed that human monocytes/macrophages are a target of the minor adhesin, NadA protein (Franzoso *et al.* 2008). In our study, we surveyed the role of ACP in pathogenesis using human epithelial cells, endothelial cells and also meningioma cells. Whether ACP also targets human monocytes/macrophages also deserves further study.
3. Currently, there is no study regarding gonococcal ACP. The BLAST-protein result on the NCBI website and our interrogation of the BIGS database showed that gonococcal ACP shows a high degree of similarity (95% similarity) to meningococcal ACP. The limited differences between meningococcal ACP and gonococcal ACP imply that they may be produced in different conformations to interact with human nasopharyngeal and urogenital epithelial cells respectively. Since meningococcal ACP has been proved to be an adhesin and invasin, it is likely that gonococcal ACP is also important in adhering to urogenital epithelial cells and maybe related to subsequent invasion. To test this hypothesis, an ACP mutant could be generated from a gonococcal strain. Next, both strains could be used to challenge urogenital epithelial cells and differences of associated bacterial number can be compared.

In summary, the meningococcal MIP and ACP proteins show considerable promise as potential vaccine candidates and they are also likely to be important in the pathogenesis of meningococcal infection. Both proteins could be considered as components of current and newer multi-component MenB vaccines and are thus worthy of further investigation.

Appendix

I. **GC agar plates** (For 1 L)

Proteose peptone #3 (BD Bacto™)	10 g
Bacto-agar	12 g
Starch (BDH, UK)	1 g
$K_2HPO_4 \cdot 3H_2O$ (Fisher, UK)	5.24 g
KH_2PO_4 (BDH, UK)	1 g
NaCl (BDH, UK)	5 g

Make up with distilled water to 1 L. After autoclaving with a pressure cooker with pressure 2.68 Kg/cm^2 at 121°C for 15 min, supplement A (8 ml) and supplement B (2 ml) were added to agar cooled to 45-50°C

Supplement A: (per 800 ml)

Glucose	100 g
L-glutamine	10 g
Para-amino-benzoic acid	13 mg
β-nicotinamide adenine dinucleotide	250 mg
Thiamine hydrochloride	3 mg
Co-carboxylase	100 mg
Cyanocobalamin	10 mg
Ferric nitrate	20 mg

The above compounds were dissolved in distilled water, filtered-sterilized and stored at -20°C.

Supplement B: (per 200 ml)

L-cysteine hydrochloride	26 g
Adenine	1 g
Guanidine hydrochloride	30 mg
Uracil	800 mg
Hypoxanthine	320 mg

All the components were dissolved in 100 ml boiling 0.1 M hydrochloric acid. Cysteine was added after the solution was cooled down to 25°C and the final volume was brought to 200 ml by distilled water. Supplement B was stored at -20°C.

II. Luria-Bertani (LB) broth or agar plates (For 1 L)

	Liquid broth	Agar plates
Proteose peptone #3	10 g	10 g
Yeast extract (BD Bacto™)	5 g	5 g
Sodium chloride	5 g	5 g
Bacto-agar (Oxfoid, UK)	-	10 g

The components were dissolved in distilled water with final volume of 1 L and then autoclaved. Filtered sterilized antibiotics were added after the agar medium was cooled to ~45-50°C.

III. SOB medium (For 1 L)

Proteose peptone #3	20 g
Yeast extract	5 g
Sodium chloride	0.5 g
Potassium chloride	186 mg

All compounds were dissolved in 950 ml distilled water and the pH was adjusted to 7.0 by addition of 10 M NaOH. After autoclaving, 10 ml of 1 M $MgCl_2$ was added immediately before use.

IV. Serial dilutions of albumin standard for the BCA assay. (working concentration: 20 - 2,000 µg/ml)

Label	Volume of diluent	volume and source of BSA	Final concentration of BSA (µg/ml)
A	0	300 µl from Stock	2,000
B	125 µl	375 µl from Stock	1,500
C	325 µl	325 µl from Stock	1,000
D	175 µl	175 µl from B	750
E	325 µl	325 µl from C	500
F	325 µl	325 µl from E	250
G	325 µl	325 µl from F	125
H	400 µl	100 µl from G	25
I	400 µl	0	0 µg/ml = Blank

V. Interfering substances used and their compatible concentrations with the BCA kit.

	Maximum concentration used in BCA reaction	**Compatible concentration**
Urea	1.6 M	< 3 M
Guanidine HCl	1.5 M	< 4 M
Tris	2 mM	< 100 mM
Imidazole	50 mM	< 50 mM

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