

**Identification and characterisation of
vaccine candidate proteins in the *Neisseria*
meningitidis surface proteome**

A thesis submitted by
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Declaration

The work presented in this thesis is my own with the exceptions of complement deposition and opsonophagocytosis assays carried out at the Centre for Emergency Preparedness and Response, Health Protection Agency, under supervision of Dr S. Taylor and the immunological analysis of sera against OMV vaccines (presented in Chapter 4) which was conducted at the Division of Infectious Disease Control, Norwegian Institute of Public Health, under supervision of Dr E. Wedege. The nucleotide sequences encoding NmMIP from the panel of 106 meningococcal isolates were kindly provided by Prof M. Maiden, University of Oxford. Other people contributing materials or technical assistance to this project are appropriately referenced in the text.

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Abstract

Neisseria meningitidis is a major cause of meningitis and septicaemia in infants and children. Disease caused by serogroup A, C, W-135 and Y strains is prevented by vaccination using capsular polysaccharide preparations. For serogroup B strains for which the capsular polysaccharide is poorly immunogenic, antigenic proteins on the bacterial surface are under investigation as promising vaccine candidates. Due to the variability in sequence of antigenic proteins, vaccine formulations containing combinations of proteins and protein variants are tested for broad protection against serogroup B isolates.

In search for new members for inclusion in multi-protein vaccines, the surface proteome of *N. meningitidis* from MC58 strain was investigated using treatment of whole meningococci with proteases followed by quantitative differential in-gel electrophoresis (DIGE) of the meningococcal proteome. The majority of proteins identified included known surface antigens and other uncharacterised predicted surface proteins. Quantitative analysis also allowed the assessment of the extent of protease cleavage on the protein population and suggested proteins were either completely or partially digested. Additionally, differences in the protein content of outer membrane vesicles (OMVs) from H44/76 meningococci grown in rich or minimal culture media were assessed by DIGE. Differentially expressed proteins were related to differences in bactericidal activity detected between immune sera from mice immunised with the different OMV preparations. Of the proteins detected on the surface and OMVs, those able to induce an immune response in mice were identified by immunoproteome analysis.

Four of the previously uncharacterised proteins identified by the proteomic analyses were assessed in immunological assays for their potential as vaccine

candidates. Recombinant forms of macrophage infectivity potentiator (NmMIP), type IV pili biogenesis proteins PilP and PilO and putative adhesin complex protein NMB2095 were produced in *Escherichia coli*, purified and used to immunise mice. Immune sera against NmMIP bound strongly to the surface of MC58 whilst exhibiting opsonophagocytic activity and facilitating deposition of complement factors on the surface of various meningococcal strains. The nucleotide sequence encoding NmMIP was assessed across a panel of 106 meningococcal isolates and found to be highly conserved and under negative selective pressure.

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Table of Abbreviations

1-D	One dimensional
2-D	Two dimensional
3-D	Three dimensional
2D-PAGE	Two dimensional-polyacrylamide gel electrophoresis
ABC	ATP binding cassette
ANOVA	Analysis of variance
BHI	Brain heart infusion medium
BSA	Bovine serum albumin
BVA	Biological variation analysis
CHAPS	3-[(3-Cholamidopropyl)-dimethyl-ammonio]-1-propane sulphonate
CDA	Complement deposition assay
CID	Collision induced dissociation
Cy3	CyDye TM DIGE fluor propyl-Cy3 N-hydroxysuccinimide ester dye
Cy5	CyDye TM DIGE fluor methyl-Cy5 N-hydroxysuccinimide ester dye
DIA	Differential in-gel analysis
DIGE	Differential in-gel electrophoresis
DMF	Dimethylformamide
dOMVs	Detergent extracted outer membrane vesicles
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionisation
fHbp	Factor H-binding protein
FITC	Fluorescein isothiocyanate
FM	Frantz medium
HBPA	Heparin binding protein A
HMM	Hidden Markov model
IAA	Iodoacetamide
IC3	IC-OSu ethyl-Cy3 N-hydroxysuccinimide ester dye
IC5	IC-OSu ethyl-Cy5 N-hydroxysuccinimide ester dye
IEF	Isoelectric focusing

IgG	Immunoglobulin gamma
IPG	Immobilised pH gradient
IPTG	Isopropyl β -D-1-thiogalactopyranoside
LB	Luria-Bertani medium
Lbp	Lactoferrin binding protein
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LPS	Lipopolysaccharide
MALDI	Matrix-assisted laser desorption ionisation
MD	Multidimensional
MC.6M	Modified Catlin-6 medium
MEGA	Molecular evolutionary and genetics analysis
MH	Mueller-Hinton medium
MLST	Multilocus sequence typing
MPL	Monophosphoryl lipid A
MS	Mass Spectrometry
MS/MS	Tandem mass spectrometry
MW	Molecular weight
NadA	Neisserial adhesin A
NHS	N-hydroxysuccinimide ester
NIBSC	National Institute for Biological Standards and Control
NmMIP	<i>Neisseria meningitidis</i> macrophage infectivity potentiator
NN	Neural networks
nOMVs	Naturally released outer membrane vesicles
NspA	Neisserial surface protein A
OD	Optical density
OMP	Outer membrane protein
OMV	Outer membrane vesicle
Opa	Opacity associated protein
OPA	Opsonophagocytosis assay
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pI	Isoelectric point
PMF	Peptide mass fingerprinting

PVDF	Polyvinylidene difluoride
RmpM	Reduction modifiable protein
SBA	Serum bactericidal assay
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SILAC	Stable isotope labelling with amino acids in cell culture
SNAP	Synonymous non-synonymous analysis programme
ST	Sequence type
Tbp	Transferrin binding protein
TFA	Trifluoroacetic acid
TOF	Time of flight

Chapter 1

General Introduction

1. General Introduction

1.1 Overview

Bacterial meningitis has been recognised as a devastating disease for more than 200 years since its first description in 1804 (Stephens *et al.*, 2007). Meningococcal disease is an important cause of morbidity and mortality with approximately 500,000 reported cases and 50,000 deaths annually worldwide and presents a major threat to the health system of both industrialised and developing countries (Maiden and Caugant, 2006). The bacterium *N. meningitidis*, also known as the meningococcus, colonises the human nasopharynx and is responsible for most of the cases of meningitis and septicemia. Systemic infection occurs when the bacterium crosses the epithelial barrier and enters the bloodstream. It can then multiply rapidly and spread to sites in the brain to cause meningitis whereas uncontrollable replication leads to the more serious septicaemia (Brandtzaeg, 2006; Steven and Wood, 1995; van Deuren *et al.*, 2000).

Though antibiotic treatment is effective and can reduce mortality (van Deuren *et al.*, 2000), rapid development of the disease can lead to permanent neurological damage (Steven *et al.*, 1995) and prevention through prophylactic vaccination is the only efficient way to fight the disease (Feavers, 2001). The meningococcus is divided into serogroups based on chemical and antigenic differences on the polysaccharide capsule surrounding the bacterium (Vedros, 1987). Serogroups A, B, C, W-135 and Y account for almost all disease cases and polysaccharide-based conjugate vaccines have been developed to provide protection against one or a combination of all but disease serogroup B (Harrison, 2006). In the case of infection based on serogroup B strains vaccine development has instead focused on outer membrane proteins (OMPs) which are directly involved in interaction with host cells and are able to induce an

immune response. Outer membrane vesicle vaccines including the repertoire of OMPs have been successfully developed and used to protect against outbreaks around the world (Nokleby *et al.*, 2007; Poolman *et al.*, 2002; Thornton *et al.*, 2006). However, their specificity for the strain they are derived from has redirected studies towards the targeted identification of bacterial proteins that could elicit a cross-protective response.

Studies on vaccine discovery and understanding of the biology behind the processes involved in pathogenicity and carriage have been greatly influenced by new technologies and availability of genome sequences. Since the publication of the genome sequence of two *Neisseria* strains in 2000 (Parkhill *et al.*, 2000; Tettelin *et al.*, 2000), functional genomics has been successfully used to provide a genome-wide picture of virulence factors involved in disease (Grifantini *et al.*, 2003; Sun *et al.*, 2000) and protein antigens (Grifantini *et al.*, 2002b; Pizza *et al.*, 2000) which formed the basis for the first vaccine formulations against serogroup B-based meningococcal disease (menB) currently tested in clinical trials. Proteomics has also been used for the characterisation of the content of OMV vaccines (Wheeler *et al.*, 2007) and demonstrated the need for protein-level analysis and the potential of this technology in the identification of vaccine candidates.

1.2 *Neisseria meningitidis*

1.2.1 Microbiology

Neisseria meningitidis is an aerobic Gram-negative diplococcus which strictly colonises the human nasopharynx in an asymptomatic manner. It has the typical structure of Gram-negative bacteria with the outer and inner membranes flanking the periplasmic space and the thin peptidoglycan layer. The outer membrane can be

surrounded by a polysaccharide capsule, a typical feature of pathogenic isolates that can cross the nasopharynx and enter the circulation. Unencapsulated strains are frequently isolated from the nasopharynx of asymptomatic carriers and rarely, if ever, cause disease (Claus *et al.*, 2002; Harrison, 2006; Hoang *et al.*, 2005). The cell wall also contains lipopolysaccharide (LPS), an abundant antigenic glycolipid, and a number of variable surface exposed proteins which either traverse the outer membrane or attach to it via lipid moieties (Figure 1.1). Many members of the meningococcal cell wall are involved in interactions with the host and determine processes required for establishment of colonisation and survival in the host including adhesion to host epithelial cells and uptake of essential nutrients.

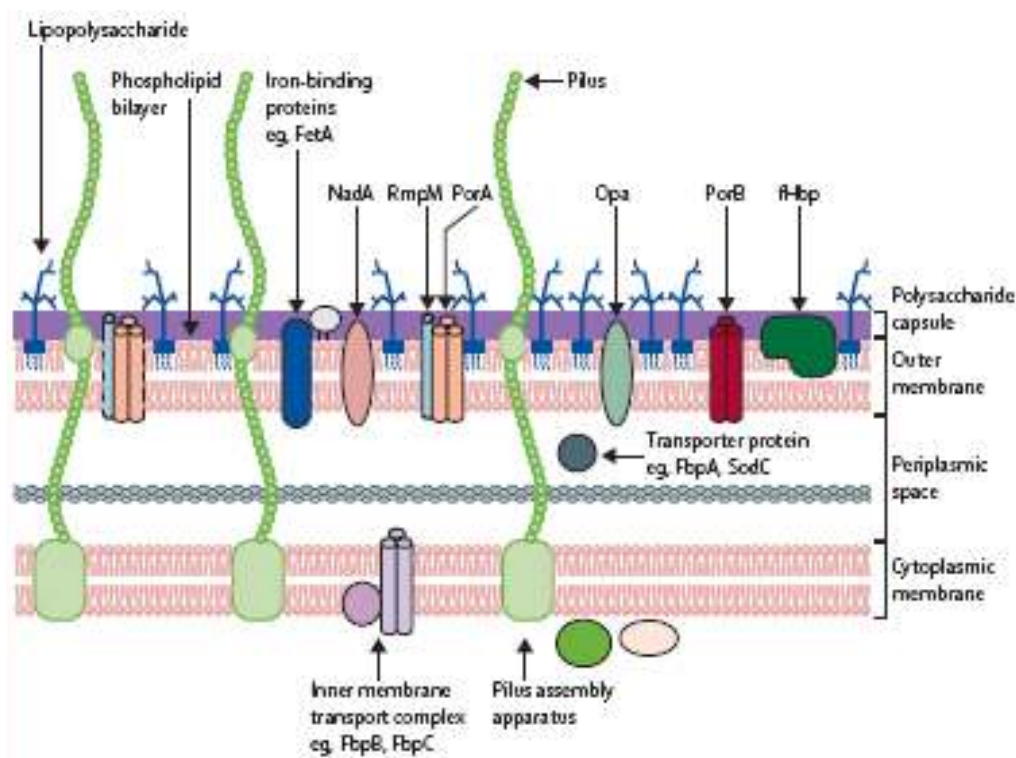


Figure 1.1 Schematic representation of meningococcal surface structures (adapted from Sadarangani and Pollard, 2010).

1.2.2 Carriage and disease

Meningococci are spread through droplets and are carried in the human nasopharynx with no symptoms (Stephens *et al.*, 2007) (Figure 1.2). Their attachment to nasopharyngeal epithelial cells is mediated by specific surface structures and their survival facilitated by acquisition of nutrients essential for bacterial growth. Carriage is a prerequisite of disease occurrence and is also considered to be required for transmission (Broome, 1986). How meningococci cross the epithelium and gain access to the circulation is still unclear. However, infection is expected to be an accidental event since host killing would prevent meningococcal spread. The magnitude and rate of bacterial growth in the bloodstream determines the severity of disease symptoms and fatality rate. The number of circulating meningococci rises gradually in patients with distinct meningitis and mild systemic infection whilst rapid multiplication occurs in the patients developing septic shock (Brandtzaeg, 2006) (Figure 1.2). Infants and children are at the greatest risk of developing meningococcal disease mainly due to immunological immaturity (Cartwright, 1995). The incidence rate is lower in adolescents and adults but can be increased by factors such as overcrowding (Baker *et al.*, 2000) and poverty which favour carriage and transmission. Individuals suffering from complement deficiencies (Fijen *et al.*, 1999; Figueroa *et al.*, 1991), respiratory infections or hyposplenism are also more vulnerable to disease progression.

1.2.3 Classification and epidemiology

Knowledge of the nature and distribution of meningococcal isolates is essential for disease management and development of preventive treatments. Various

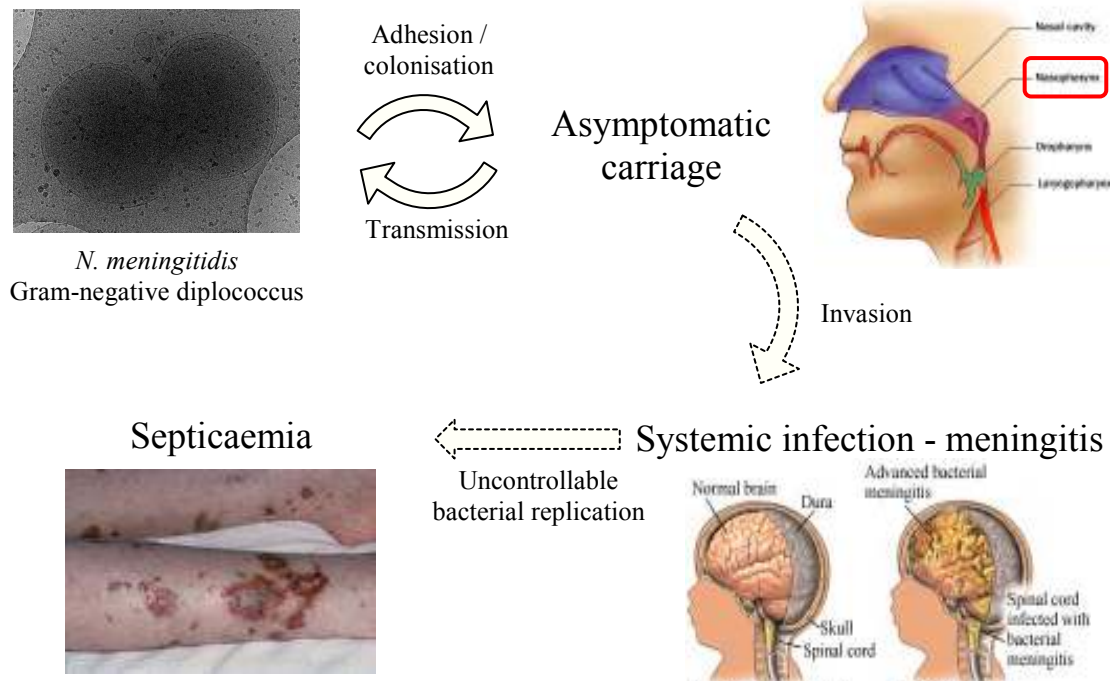


Figure 1.2 Life cycle of *N. meningitidis*. Images adapted from <http://www.meningitis.org>, <http://www.mayoclinic.com> and freely available internet content. The microscopic image of the *N. meningitidis* diplococcus was produced in this study.

classification systems have been developed and used in surveillance studies. The first such method is the serological classification of meningococcal isolates and is based on phenotypic characteristics of variable surface structures probed with specific antibodies (Kuipers *et al.*, 2001). The biochemical composition of the polysaccharide capsule defines an isolate's serogroup. *Neisseria meningitidis* strains are classified into 12 distinct serogroups, of which serogroups A, B, C, W-135, Y and occasionally X are responsible for almost all cases of invasive disease (Harrison, 2006; Vedros, 1987). Antigenic variation of the outer membrane porins PorB and PorA is used to assign the serotype and the serosubtype, respectively. The serosubtype is given by two numbers which correspond to two surface exposed loops of PorA (Frasch, 1987; Wedege *et al.*, 1990). Meningococci are further classified into 12 immunotypes based on the LPS variability (Scholten *et al.*, 1994).

Among other classification tools widely used to distinguish between closely related strains are multilocus enzyme electrophoresis (MLEE), multilocus sequence typing (MLST) and polymerase chain reaction (PCR)-based antigen sequencing. Multilocus enzyme electrophoresis uses starch gel electrophoresis of bacterial cell lysates to identify variations in the electrophoretic mobility of up to 25 metabolic enzymes which account for allelic variations of the corresponding housekeeping loci. Each electrophoretic profile is referred to as an electrophoretic type (ET) (Selander *et al.*, 1986). Meningococcal strains with similar ETs are assigned to genetically related groups or “clonal complexes” of strains. The most invasive meningococcal isolates belong to ET-37, ET-5, cluster A4 and lineage III complexes (Caugant *et al.*, 1987).

Multilocus sequence typing is the DNA-based version of MLEE. It is based on nucleotide sequencing of 6 housekeeping genes following amplification of the genes by PCR (Maiden *et al.*, 1998; Spratt, 1999). Each new sequence is assigned an allele and the combination of alleles from the 7 genes is assigned a sequence type (ST). Genetically closely related STs constitute central genotypes and can be categorized into clonal complexes with each complex including STs that match the central genotype at 4 or more loci. The ease of use, sensitivity and applicability of the method on every specimen render MLST advantageous over MLEE. Additionally, PorA and PorB PCR-based sequencing approaches have been implemented to provide finer resolution of the STs identified by MLST (Feavers *et al.*, 1992; Suker *et al.*, 1991). Sequencing is targeted to variable regions 1 and 2 and semi-variable region 3 of PorA which correspond to surface-exposed loops (Clarke *et al.*, 2003; Russell *et al.*, 2004). The number of variable loops in PorB is higher as almost all 8 surface-exposed loops show variability (Feavers *et al.*, 1992). These PCR-based typing methods find

particular use on unencapsulated strains where serogroup cannot be determined or on culture-negative cases (Newcombe *et al.*, 1997).

Meningococcal disease is both endemic and epidemic. Attack rates range from 1 to 5 cases per 100,000 population during endemics and can exceed 500 cases per 100,000 during epidemic outbreaks. Serogroup A strains are the main cause of the cyclic epidemics occurring in sub-Saharan African countries in a zone called “the Meningitis belt”. In industrialised countries in Europe and the Americas serogroup B and C strains are the most prevalent. The incidence of serogroup Y disease accounts for 30% of cases in the USA (Maiden *et al.*, 2006). The introduction of glycoconjugate vaccines against A, C, W-135 and Y has led to a rapid decrease in cases caused by these serogroups (Balmer *et al.*, 2002; Fairley *et al.*, 1996; Pace and Pollard, 2007) and has highlighted the extent and persistence of serogroup B disease. Recent serogroup B outbreaks in the Americas (Cuba, Brazil, Chile), Europe (Norway, Spain, Belgium) and New Zealand (Bjune *et al.*, 1991; Martin *et al.*, 1998; Sierra *et al.*, 1991) have also served to heighten awareness of serogroup B disease.

A small number of STs are persistent in the population whereas most STs are only observed once (Maiden *et al.*, 2006). This structuring of meningococcal populations is mainly the result of immunoselective evolutionary pressures imposed by the host and explains the high prevalence of certain invasive complexes, also called hyperinvasive lineages (Jolley *et al.*, 2005; Yazdankhah *et al.*, 2004). Sequence types 1, 4 and 5 have been associated with serogroup A pandemics whereas ST-11 (ET-37) complex was observed in B, C, W-135 and Y epidemics. Two other complexes, ST-32 (ET5) and ST-41/44 (lineage 3), are mainly associated with the recent serogroup B epidemics and triggered the development of OMV vaccines for population protection (Fredriksen *et al.*, 1991; Oster *et al.*, 2005; Sierra *et al.*, 1991).

1.3 Surface structures and their significance in carriage and disease

1.3.1 Capsule

Meningococci are surrounded by the capsule, a polysaccharide layer covalently attached to phospholipids in the cell envelope. It is found in almost all invasive strains and is considered a major virulent factor though it is highly unlikely that it is an evolution-acquired virulence determinant in an accidental pathogen like *N. meningitidis*. It has anti-bactericidal, anti-phagocytic and anti-opsonic properties which help meningococcus survive the host defense mechanisms and establish systemic disease. It also possesses anti-adherent properties allowing meningococcal transmission and spread (Stephens *et al.*, 1993). The capsule is the basis for serogrouping of meningococci due to variations in the polysaccharide structure. The polysaccharide of most of the invasive serogroups (B, C, W-135 and Y) usually consists of a 5-*N*-acetyl-neuraminic acid (sialic acid) homopolymer (Bhattacharjee *et al.*, 1975). Differentiation arises from the different linkage between sialic acid monomers, for example serogroups C and B exhibit $\alpha(2-9)$ and $\alpha(2-8)$ linked sialic acid, respectively.

1.3.2 Lipopolysaccharide (LPS)

The LPS is a glycolipid with a mass of 4.6 kDa and consists of two distinct regions, lipid A and the core oligosaccharide. Lipid A comprises a β -D-glucosaminyl-(1'→6)-D-glucosamine disaccharide backbone and is attached to the outer membrane through fatty acids. The core oligosaccharide consists of an inner and an outer core region. Structural differences on the outer core region and the oligosaccharide chains define the 12 distinct immunotypes of LPS (Scholten *et al.*, 1994). Among the genes involved in LPS biosynthesis, the *lgt* gene family encodes for glycosyl transferases

which are responsible for additions to the outer core region of the LPS and are subjected to phase variation. The LPS is known to have a significant role to the adherence functions mediated by pili and also contributes to serum resistance (Smith *et al.*, 1995; Virji *et al.*, 1996). The LPS is immunogenic and immunotypes L3, L7 and L9 have been linked to pathogenicity.

1.3.3 Outer membrane proteins (OMPs)

Outer membrane proteins are of the main components to come in direct contact with epithelial cells and possess a variety of functions to facilitate adhesion, colonization and interaction with the human immune system to escape complement mediated killing and phagocytosis (Bourdoulous and Nassif, 2006; Schneider *et al.*, 2007). As part of the mechanisms for meningococcal adaptation and immune evasion OMPs also exhibit characteristic variation, mainly phase and antigenic. Outer membrane proteins have long been considered promising targets for vaccine development and an increasing number of studies are targeted to the identification and characterisation of proteins on the meningococcal surface (Pizza *et al.*, 2000). Among well studied OMPs are the abundant porins PorA and PorB, RmpA, Opa/Opc adhesins, proteins of the type IV pili machinery and iron-regulated proteins (Derrick *et al.*, 2006).

1.3.3.1 Porins

Porins are common channels in the outer membrane of Gram-negative bacteria for passive transport of hydrophilic nutrients. *Neisseria meningitidis* possesses two porins, named PorA and PorB which together constitute approximately 60% of the outer membrane protein content. Meningococcal porins share structural similarity with *Escherichia coli* porins and consist of a high proportion of beta sheets which fold

into beta barrel structures and associate to form the native trimer (Blake and Gotschlich, 1987).

PorA, also known as class 1 protein, has a varying molecular weight of 44-47 kDa depending on the isolate and is predicted to expose 7 loops on the meningococcal surface. Sequencing of the *porA* gene from different isolates has revealed 2 hypervariable regions VR1 and VR2, located on the tip of loops 1 and 4 and one less variable region in loop 5 (VR3) (McGuinness *et al.*, 1990; van der Ley *et al.*, 1991). Interestingly, these regions have also been shown to be the antigenic epitopes targeted by strain-specific antibodies (McGuinness *et al.*, 1993).

Two distinct PorB proteins are encoded by two alleles of the *porB* locus, PorB2 (40-42 kDa, also named class 2) and PorB3 (37-39 kDa, class 3) and are mutually exclusive between different isolates (Minetti *et al.*, 1998; Tsai *et al.*, 1981). They also consist of 8 surface-exposed loops with variable regions observed in loops 1, 5, 6 and 7. Unlike PorA epitopes, PorB exhibits discontinuous epitopes formed when the molecule takes its native conformation and regions from different loops come in close proximity (Bash *et al.*, 1995). Structural characterization of PorB revealed pathways for transportation of ions and uptake of sugars (Tanabe *et al.*, 2010). PorB was shown to have a role in protection of host cells from apoptosis by translocation into the mitochondria where it binds to voltage-dependent anionic channels and blocks activation of caspases (Massari *et al.*, 2000; Massari *et al.*, 2003). PorB has also demonstrated adjuvant activity by direct binding to TLR2 receptor and activation of B cells (Massari *et al.*, 2006). Both PorA and PorB have been linked to other activities affecting immune cells including inhibition of neutrophil actin polymerisation, opsonin receptor expression and phagocytosis (Bjerknes *et al.*, 1995).

1.3.3.2 Adhesins

The first step for colonisation of the human epithelium and subsequent invasion involves adhesion of meningococci on the epithelial layer. As such, adhesion mechanisms have attracted considerable attention from many research groups. A number of surface-located meningococcal proteins are involved in the primary interactions with epithelial cells and the initiation of adhesion (Bourdoulous *et al.*, 2006). The most representative and well studied adhesins are OpcA, proteins of the Opa family and type IV pili.

At least three Opa proteins (also termed class 5 proteins) are encoded by a family of genes in *N. meningitidis* and exhibit high variability in their surface exposed domains (Aho *et al.*, 1991; Malorny *et al.*, 1998). Unlikely, OpcA is the product of a single gene sharing little homology with Opa genes and exhibits less variation (Seiler *et al.*, 1996). However, it has common physicochemical properties with Opa proteins and was also considered to be a class 5 protein. OpcA expression varies significantly and can even be absent in some meningococcal strains (Achtman *et al.*, 1988; Olyhoek *et al.*, 1991; Seiler *et al.*, 1996). Opa proteins are known to mediate adhesion via interaction with a family of receptors, known as carcinoembryonic antigen-related cell adhesion molecules, expressed on epithelial and endothelial cells (de Jonge *et al.*, 2003). Heparin and heparin sulfate proteoglycans have been shown to serve as additional ligands for both Opa and Opc protein families during adhesion (Hauck and Meyer, 2003; Merz and So, 2000).

Neisseria species produce type IV pili (tfp) essential for bacterial movement as well as interaction and colonization of the host cells. Type IV pili are filament-type structures made of pilin Pile, a small protein of about 150 aa length assembled in a helical way to form pili (Strom and Lory, 1993; Pelicic, 2008). Other minor pilins

have been identified to be part of pili structures and their sequence is well conserved across meningococcal isolates (Brown *et al.*, 2010; Cehovin *et al.*, 2010). Several proteins are required in the various steps of tfp biogenesis and function including the secretin PilQ, responsible for pili emergence on the surface (Wolfgang *et al.*, 2000) as well as PilP and PilW which help in PilQ oligomerisation and pilus stabilisation, respectively (Carbonnelle *et al.*, 2005; Drake *et al.*, 1997). Type IV pili coordinate bacterial movement, known as twitching motility, utilizing the function of PilT, a AAA ATPase. Most importantly for meningococcal disease, tfp are essential for initiation of bacterial attachment to epithelial cells and phase and antigenic variation of pilin are mechanisms used to modulate adherence (Marceau *et al.*, 1995; Nassif *et al.*, 1993). The receptor CD46 is considered to be the interacting partner for tfp in the human host cell (Källström *et al.*, 1997; Rytönen *et al.*, 2001).

1.3.3.3 Iron-regulated proteins and iron metabolism

Iron is a cofactor involved in many metabolic processes and an essential nutrient for meningococcal pathogenesis. In the human nasopharynx, iron can be a limiting factor for the pathogen's survival as it is found complexed to high affinity iron-binding proteins such as transferrin, lactoferrin and hemoglobin. Under iron-restricted conditions, meningococci produce a repertoire of outer membrane receptors to directly acquire iron from these proteins (Schryvers and Stojiljkovic, 1999). The transferrin receptor is a complex of two proteins, transferrin binding protein A (TbpA), a 80kDa, surface-exposed variable protein and TbpB, a 98kDa, highly conserved inner membrane protein (Irwin *et al.*, 1993; Legrain *et al.*, 1993). Similarly, the lactoferrin receptor also consists of two proteins, lactoferrin binding protein A (LbpA) which forms the channel for transport and LbpB (Pettersson *et al.*, 1993; Pettersson *et al.*, 1998). Finally, heme is another source of iron on the mucosal surfaces in the

human nasopharynx and meningococci recruit the hemoglobin receptor HmbR and the hemoglobin-haptoglobin utilization receptor Hpu (Lewis *et al.*, 1997; Stojiljkovic *et al.*, 1995).

Transferrin and lactoferrin binding proteins and hemoglobin receptors are transcriptionally regulated by iron through the ferric uptake regulator protein Fur which dimerises with ferrous iron and inhibits transcription of the respective genes (Escolar *et al.*, 1999). The energy required for iron transport through the cell membrane and to the cytoplasm is provided by the inner membrane protein TonB. Further to TbpA, LbpA, HpuB and HmpR, a number of other TonB-dependent receptors have also been suggested based on sequence homology and are assumed to be involved in recognition of siderophores present on the mucosal surfaces. One such example is the ferric enterobactin receptor, FetA which is predicted to expose several loops on the bacterial surface, one of which exhibits high sequence variability (Pettersson *et al.*, 1995; Thompson *et al.*, 2003).

1.3.3.4 Other OMPs

Other OMPs with important roles in virulence and bacterial survival include the reduction-modifiable protein (RmpM), Omp85, factor H binding protein (fHbp) and neisserial surface protein A (NspA). RmpM (also known as class 4 protein) participates in PorA-PorB complexes and associates with iron-regulated LbpA, TbpA and FetA (Prinz and Tommassen, 2000). RmpM is suggested to stabilise the oligomeric complexes of the outer membrane which it forms part. Omp85 is another protein involved in protein architecture in the outer membrane. Omp85 is a 85kDa protein which belongs to a family of highly conserved OMPs across bacteria. Its role in the assembly of barrel proteins makes Omp85 an essential protein for bacterial

viability (Voulhoux *et al.*, 2003). In *N. meningitidis*, Omp85 was also shown to be involved in lipid export to the outer membrane (Genevrois *et al.*, 2003).

Factor H-binding protein was first identified through genome mining as protein GNA1870 (Pizza *et al.*, 2000). It is a lipidated, surface-exposed protein which recruits factor H (fH), a negative regulator of the alternative complement pathway, to the meningococcal surface as a mechanism to evade complement-mediated killing (Madico *et al.*, 2006; Schneider *et al.*, 2006). Structural analysis of fHbp in complex with fH suggested that fHbp imitates interaction of carbohydrates with fH on human endothelial cells for recruitment of fH on the bacterial surface (Schneider *et al.*, 2009). Recently, NspA was reported as another ligand for fH (Lewis *et al.*, 2010). NspA is also surface exposed although its accessibility varies depending on the amount of polysaccharide produced by individual isolates (Moe *et al.*, 1999a).

1.4 Vaccine development

Surface structures are of great value in vaccine development as the antigenic source in vaccine preparations. Being exposed to the environment of the host, meningococcal surface components are in direct contact with the host's immune system and are most likely to evoke an immune response. Serum bactericidal activity (SBA), the accepted surrogate of protection, is used to assess whether antibodies elicited by potential vaccine candidates can confer protection against meningococcal disease by complement-mediated bacterial killing. Licensed vaccines exist against invasive serogroups A, C, W-135 and Y and are based on capsular polysaccharide preparations. Outer membrane vesicle-based vaccines protecting against specific serogroup B outbreak strains have also been used over the years, whilst protein-based

preparations aiming to protect against the majority of invasive serogroup B strains are under development.

1.4.1. Capsular polysaccharide-based vaccines

The first vaccines against meningococcal disease used capsular polysaccharide as the antigenic source and were developed 30 years ago to tackle epidemics against serogroup A and C strains among US military recruits (Gotschlich *et al.*, 1969). These vaccines demonstrated the capsule's ability to elicit bactericidal antibodies and helped identify SBA as a correlate for protection against meningococci (Goldschneider *et al.*, 1969). The reciprocal of the antibody dilution conferring killing of more than half of the meningococcal population used in the assay was defined as the protective titre. For polysaccharide vaccines, an SBA titre of ≥ 4 in the presence of human complement was found to correlate with protection.

First generation polysaccharide vaccines included bivalent (A, C), trivalent (A, C, W-135) and quadrivalent (A, C, W-135 and Y) formulations of purified, high molecular weight polysaccharides. Efficacy studies on the early monovalent preparations as well as the later multivalent polysaccharide vaccines showed an age-dependent response with infants being poorly protected (Gold *et al.*, 1979; Harrison, 2006; Law *et al.*, 1998). They also failed to induce immunological memory in people of any age.

Next generation conjugate meningococcal vaccines were developed through conjugation of polysaccharide to an immunogenic carrier protein aimed at inducing immunologic memory. The conjugation concept was first demonstrated in a vaccine against *Haemophilus influenza* type b as a strategy for inducing T-cell dependent responses. Vaccine preparation involves the use of purified or chemically synthesized

saccharide which is activated in a random or targeted way and conjugated to a carrier protein such as diphtheria toxoid, tetanus toxoid and CRM₁₉₇ (variant of diphtheria toxoid) (Ravenscroft and Feavers, 2006). The first monovalent group C conjugate vaccine was licensed in the UK in 1999. It was shown to be safe and immunogenic in all age groups and demonstrated immunological memory and higher bactericidal activity compared to the unconjugated equivalents (MacDonald *et al.*, 1998; Richmond *et al.*, 1999a; Richmond *et al.*, 1999b). Tetravalent (A, C, W-135 and Y) conjugate preparations are also available in the US and Europe for administration to adolescents and adults (Campbell *et al.*, 2002; Cooper *et al.*, 2011).

Although the introduction of conjugate vaccines has been successful, it has highlighted the lack of protection against serogroup B strains which are currently dominant in developed countries. The poor immunogenicity of the serogroup B capsule (Devi *et al.*, 1997; Zollinger *et al.*, 1997) together with the risk of producing autoimmune responses due to its structural identity with the polysialylated form of the human neural cell adhesion molecule (Finne *et al.*, 1983) has rendered the development of a conjugate group B vaccine difficult. Attempts to chemically modify the polysaccharide by replacing natural N-acetyl groups with N-propionyl groups followed by conjugation to tetanus toxoid resulted in a safe but poorly immunogenic preparation when administered to humans (Bruge *et al.*, 2004; Jennings *et al.*, 1987; Jennings *et al.*, 1986). A more recent approach involved the identification of peptide mimetics, peptides of polysaccharide epitopes that do not cross-react with human tissue (Granoff *et al.*, 1998; Lo Passo *et al.*, 2007; Moe *et al.*, 2005). One such peptide, designated 9M, was shown to elicit strong bactericidal antibodies and is considered a promising vaccine candidate (Lo Passo *et al.*, 2007).

1.4.2 Subcapsular protein-based vaccines

Proteins of the outer membrane have been recognised as alternative sources of antigens for use in vaccines. For the past two decades, the design of protein-based meningococcal vaccines has focussed on the prevention of serogroup B disease and several potential antigens have been identified on the surface of the meningococcus (reviewed in Feavers and Pizza, 2009). A common feature of surface antigens is the sequence diversity of their epitopes which are targets of protective antibodies. This diversity, which is the result of the immunoselective pressure imposed to meningococci by the host, has important implications to vaccine design. For vaccines containing variable antigens, protection will depend on the sequence variant used and be limited to only those strains expressing the same variant. To account for this, several combinations of different protein antigens or protein variants have been developed over the past years and assessed for their potential to offer cross protection against hyperinvasive serogroup B strains.

1.4.2.1 Outer membrane vesicle vaccines

Outer membrane vesicles are membrane blebs naturally released by a variety of Gram-negative bacteria during growth. They contain a significant number of membrane proteins and high levels of LPS. Meningococcal OMVs are enriched in major OMPs PorA, PorB, Omp85 and OpcA and contain a repertoire of other minor protein antigens (Vipond *et al.*, 2006). Their role in the life cycle of meningococci is still unknown though it is hypothesized that they contribute to adherence and DNA transfer. Release of OMVs might also have a role in diverting immune response during invasive disease by transporting and presenting LPS and outer membrane proteins into host cells (Alaniz *et al.*, 2007).

Outer membrane vesicle vaccines have been used for the past 20 years to control epidemics caused by serogroup B meningococcal strains. They are made of OMVs extracted from bacteria by treatment with the detergent deoxycholate to reduce LPS levels and hence toxicity of the extract (Fredriksen *et al.*, 1991). The first OMV vaccines were developed by the Finlay Institute in Cuba and the Norwegian Institute of Public Health against outbreak strains CU350 and H44/76, respectively, and were used to contain epidemics in Chile, Cuba, Brazil and Norway (Bjune *et al.*, 1991; Boslego *et al.*, 1995; de Moraes *et al.*, 1992; Sierra *et al.*, 1991). Recently, a tailor-made OMV vaccine against 98/254 strain was used to contain an outbreak in New Zealand (Oster *et al.*, 2005). The efficacy of these OMV vaccines varied between 50-80% and the protection was strain specific since bactericidal responses were mainly targeted against the highly variable PorA (Rosenqvist *et al.*, 1995; Wedege *et al.*, 2007). However, where no other vaccine against menB disease is available, OMV vaccines constitute an effective and well-established solution against disease outbreaks.

1.4.2.2 Major porins

Much of the information on the immunogenic properties of the major porins PorA and PorB has been gathered during clinical evaluation of OMVs. Additionally, independent studies on recombinant forms of PorA and PorB have been conducted. Both porins were shown to elicit a bactericidal response when incorporated into liposomes suggesting that a correct, near native conformation is vital for induction of functional antibodies (Arigita *et al.*, 2003; Wright *et al.*, 2002). With the aim of providing protection against a larger panel of isolates representative of the hypervirulent lineages, vaccine developers selected different PorA variants to be introduced simultaneously either in liposomes or OMV preparations. Tetravalent

PorA-liposome and hexa- and nonavalent PorA-OMV preparations were shown to elicit a varying, cross protective bactericidal response against homologous and heterologous strains (Humphries *et al.*, 2005; van den Dobbela *et al.*, 2007; Vermont *et al.*, 2003). In all cases, selection of variants was facilitated by epidemiological data which suggest that a limited number of variants circulating between hyperinvasive lineages would be sufficient to confer broad protection (Urwin *et al.*, 2004). These studies also highlighted the need for constant monitoring of spread of these variants especially following the use of serosubtype-specific OMV vaccines against certain epidemics.

1.4.2.3 Other antigens of known function

Other immunogenically important proteins expressed on the meningococcal surface have been identified over the years and included in tests assessing their potential as vaccine candidates. Proteins involved in iron uptake attracted attention mostly due to their vital role in the survival of the meningococcus in the host. Transferrin-binding proteins A and B were both found to protect mice when challenged with meningococci and raise bactericidal antibodies (Ala'Aldeen and Borriello, 1996). However, bactericidal response against a TbpB formulation assessed in phase I clinical trials was poor (Danve *et al.*, 1998). Lactoferrin-binding proteins were also immunogenic and elicited a bactericidal response with limited cross-reactivity as a result of sequence variation (Pettersson *et al.*, 2006). FetA, an iron-regulated OMP present in OMVs elicits antibodies which are directed against a highly variable surface-exposed loop and are thus strain-specific (Pettersson *et al.*, 1990). However, given the antigenic structuring of meningococci, a limited number of FetA variants was suggested to potentially protect against the majority of hypervirulent strains, a hypothesis that remains to be tested (Urwin *et al.*, 2004).

Proteins involved in adhesion and colonisation of epithelial cells have also been shown to be immunogenic. Major adhesins OpcA and Opa family proteins are abundant on the surface of the meningococcus and antibodies against them have been found in convalescent sera and sera from humans immunised with OMV vaccines (Mandrell and Zollinger, 1989; Poolman *et al.*, 1983; Wedege and Froholm, 1986). Although they are phase variable and antigenically diverse, Opa proteins exhibit conserved sequence combinations across hyperinvasive lineages (Callaghan *et al.*, 2006). Based on this genetic structuring, a combination of 6 Opa variants was recently shown to induce bactericidal antibodies in mice that could potentially protect against 90% of the lineages circulating in the UK (Callaghan *et al.*, 2011). Antibodies against recently characterised, surface-exposed adhesins App and NhhA were also involved in protection via serum bactericidal killing in mice (Hadi *et al.*, 2001; Pizza *et al.*, 2000). App is an autotransporter protein with autolytic properties mediating adhesion to epithelial cells (Hadi *et al.*, 2001; Serruto *et al.*, 2003). NhhA is a trimeric autotransporter adhesin with roles in protection from phagocytosis and complement mediated bactericidal killing (Scarselli *et al.*, 2006; Sjolinder *et al.*, 2008). Antibodies against App and NhhA have been detected in sera from patients and healthy colonised individuals suggesting that both are expressed *in vivo* (Litt *et al.*, 2004).

1.4.2.4 Genome-derived protein antigens

An important contribution of genomic revolution has been the ability to identify and assign a function to proteins that could not be previously revealed by conventional methods. The first *Neisseria* genomes to be sequenced were that of serogroup B MC58 and serogroup A Z2491 strains (Parkhill *et al.*, 2000; Tettelin *et al.*, 2000). Availability of genome sequences facilitated a number of genome-based approaches either for direct discovery of vaccine candidates, or for functional

characterisation of virulence factors with implications in vaccine development (Dietrich *et al.*, 2003; Exley *et al.*, 2009; Grifantini *et al.*, 2002b; Sun *et al.*, 2005). Based on the MC58 genome sequence, a novel genome-based approach, termed reverse vaccinology, was applied for the identification of novel vaccine candidates (Pizza *et al.*, 2000). In this pioneering study, the 570 of 2158 predicted open reading frames (ORFs) were chosen *in silico* as potentially encoding surface-exposed proteins (i.e. lipoproteins, outer membrane proteins). These were amplified and cloned in *E. coli* with the aim of expressing and purifying the corresponding products. Purified proteins were then used to immunise mice and immune sera were tested for bactericidal antibodies. This study identified antigens including fHbp, neisserial adhesin protein A (NadA) and heparin binding protein A (HBPA, or GNA2132).

Factor H-binding protein is expressed in the majority of meningococcal strains, however expression levels are strain-dependent. Based on sequence variation, fHbp has been classified into 3 variant families which exhibit different prevalence across strains (Maignani *et al.*, 2003). Although bactericidal activity of antibodies against fHbp is known to be variant specific, a vaccine combining variants from the different families in one formulation could in principle confer cross protection. One such formulation was developed using 2 fully lipidated fHbp variants and is currently undergoing clinical trials (Richmond *et al.*, 2008). In a different approach, the most prevalent variant 1 of fHbp was combined with NadA and HBPA in a multi-component vaccine termed 5CVMB (also known as 4CmenB) (Giuliani *et al.*, 2006). Neisserial adhesin protein A is considered to have a role in adhesion and invasion of epithelial cells and is present in 3 conserved alleles which can elicit cross reactive bactericidal antibodies in both mice and humans (Capecci *et al.*, 2005; Comanducci *et al.*, 2002). It is present in some, but not all, serogroup B isolates representing

hyperinvasive clonal complexes. Heparin binding protein A, a surface-exposed lipoprotein which binds to heparin, has also been independently shown to elicit bactericidal antibodies against some isolates in mice and confer protection against bacteremia in an infant rat model (Serruto *et al.*, 2010; Welsch *et al.*, 2003). The 5CVMB formulation also contained antigens GNA2091 and GNA1030 from the reverse vaccinology study which were fused to fHbp and HBPA, respectively (Giuliani *et al.*, 2006). In preclinical studies in mice, 5CVMB formulated with aluminum hydroxide was able to elicit cross-protective antibodies against 66 of 85 strains tested (Giuliani *et al.*, 2006). In phase II trials, 63-100% of infants immunised with 5CVMB administered together with OMVs from New Zealand strain 98/254 elicited protective titres (1:4 or greater) against strains expressing at least one of the vaccine antigens (Snape *et al.*, 2008). Regardless of the final formulation, vaccines containing fHbp might offer an additional advantage. Further to the induction of a bactericidal response, antibody binding to fHbp could inhibit the binding of fH on the meningococcal surface and hence enhance complement-mediated killing of bacteria.

Recently, the design of OMV preparations from strains engineered to overexpress different fHbp variants and NadA was reported (Zollinger *et al.*, 2010). Three strains were engineered to express increased levels of one of fHbp variant 1, fHbp variant 2 and NadA. Each strain was additionally manipulated to reduce toxicity of LPS, include a second heterologous PorA variant in addition to endogenous PorA and express consistent levels of OpcA. The resulting antigenic preparation combined the virtues of strong immunogens with the advantage of OMVs to present antigens in their native conformation. Selection of antigenic variants was also based on the rationale of providing broad protection against strains that express at least one of the vaccine antigens. Manipulations on the LPS background also allowed for native

OMVs to be safely used and detoxified LPS to serve as additional antigen. In phase I clinical trials, the vaccine was found to elicit bactericidal antibodies that were cross protective against a small panel of strains tested (Keiser *et al.*, 2011).

1.4.2.5 Hypothetical proteins

Genome-wide screening and characterization of OMVs led to the discovery of a number of protein antigens with unknown function. Proteins designated GNA992, GNA1946, GNA1162 and GNA2001 were identified during the original reverse vaccinology study conducted by Pizza *et al.* in addition to the proteins included in the 5CVMB vaccine (Pizza *et al.*, 2000). From a more recent application of the genome screening approach, four more antigens were found, namely NMB0606, NMB0873, NMB1163 and NMB0938 (Pajon *et al.*, 2009). Finally, NMB0088 and NMB0928, two proteins identified following proteomic characterization of the OMV protein content from the CU385 outbreak strain were also included in the list of potential vaccine candidates (Delgado *et al.*, 2007; Sardinas *et al.*, 2009). All of these uncharacterised proteins were shown to elicit a bactericidal response and/or confer protection in experimental animal challenge studies.

1.5 Proteomics and meningococcal disease

1.5.1 Proteomic technologies

Proteomics is a dynamic and large-scale study of the total protein content in an organism, tissue or cell. It also deals with the differences in protein expression levels between different conditions as well as networks of protein interactions (Aebersold and Mann, 2003). Proteomics exploits a wide range of separation techniques, spectroscopic methods and available genome sequences to study complex protein

samples. A typical workflow of proteome-wide analysis includes sample preparation for isolation and separation of protein mixtures, digestion of proteins with a specific protease (*e.g.* trypsin) and mass spectrometric (MS) analysis of the generated peptides for protein identification (Domon and Aebersold, 2006).

1.5.1.1 Protein isolation and separation

Protein isolation and separation methods are selected on the basis of the nature of the sample and the aim of the analysis to facilitate downstream mass spectrometric identification. Isolation methods are used to prepare samples enriched in proteins of interest (Figure 1.3). These methods involve either chemical or physical treatment to extract proteins from certain cell compartments (membrane, cytoplasm), or bearing post-translational modifications (*e.g.* glycoproteins, phosphoproteins) (Stasyk and Huber, 2004). In the former case, detergents or partitioning solutions that can create different phases upon centrifugation are commonly used for extraction of proteins from organelles and cell compartments (Abdolzade-Bavil *et al.*, 2004; Everberg *et al.*, 2008; Fialka *et al.*, 1997; Wright *et al.*, 2005). In the latter case, modified proteins are purified from whole-cell lysates by exploiting their affinity to certain chemical structures. Phosphorylated proteins are normally isolated by affinity of the phosphate group to chelating metal ions or titanium dioxide (Larsen *et al.*, 2005; Salomon *et al.*, 2003). Enrichment of glycoproteins is instead achieved by lectin affinity chromatography or by covalent capture on hydrazide moieties (Bunkenborg *et al.*, 2004; Zhang *et al.*, 2003).

Separation techniques are applied to reduce complexity of the analysed protein samples prior to MS analysis. Proteins are separated based on physicochemical parameters including molecular weight (MW), isoelectric point (pI) and hydrophobicity.

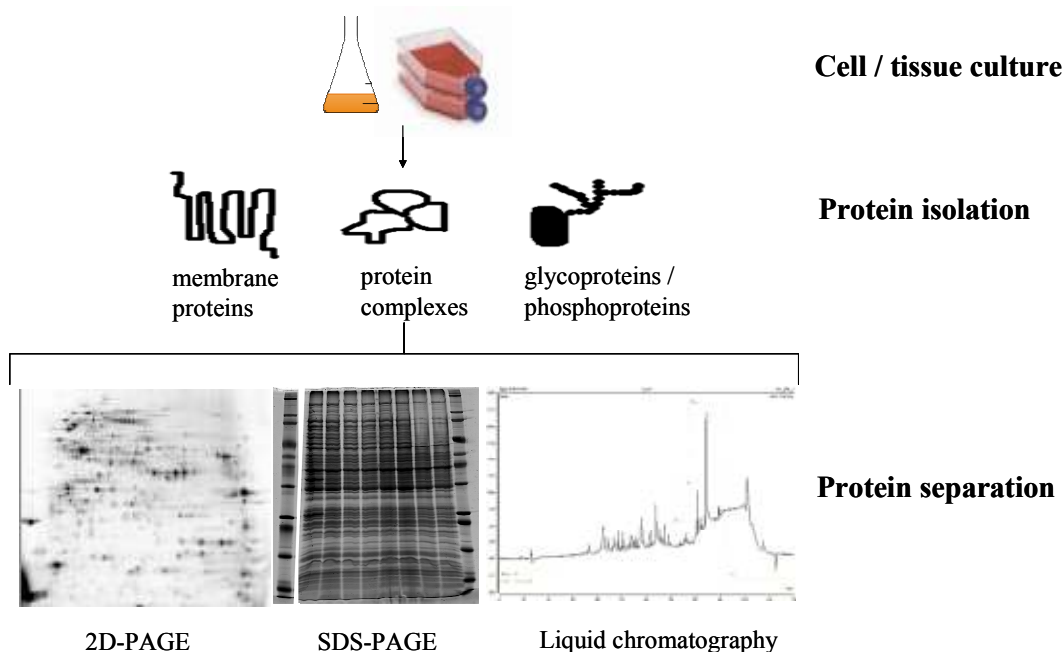


Figure 1.3 Sample preparation for MS analysis. Following cell culture, proteins of interest are isolated by chemical and/or physical methods. Proteins are subsequently separated on one or two-dimensional polyacrilamide gels (2D-PAGE or SDS-PAGE) or using liquid chromatography. Proteins from gel spots / bands or chromatographic fractions are subjected to digestion with proteases and the peptides generated are analysed by MS.

At this stage, modifications can also be introduced to allow comparative analysis of different protein fractions. The most commonly used separation technologies in proteomics include two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), one-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and multidimensional liquid chromatography (MD-LC) (Figure 1.3) (Issaq *et al.*, 2002).

In 2D-PAGE, complex protein mixtures are first separated by their pI using isoelectric focusing (IEF) and subsequently separated by MW in SDS-PAGE. Proteins in 2-D gels are then excised, digested with trypsin and extracted peptides identified by MS (Görg *et al.*, 2004). The main advantage of 2D-PAGE is the high resolving power which can be further enhanced by the use of large-format gels. Comparative analysis in 2D-PAGE can be achieved through differential in-gel electrophoresis (DIGE), a

method that involves chemical labelling of protein samples with spectrally resolvable, fluorescent dyes of similar size (Unlü *et al.*, 1997; Yan *et al.*, 2002). The most common dyes used are cyanine derivatives containing a N-hydroxysuccinimide (NHS) ester group to react and covalently attach to the epsilon amino group of lysine residues in proteins. Under minimal labelling conditions, one molecule of dye is attached to one protein molecule and fluorescence intensity is proportional to protein abundance. Protein samples to be compared are labelled with different dyes, mixed and separated in the same 2-D gel. (Yan *et al.*, 2002). Where protein patterns are identical between samples, differences in protein expression levels can be inferred from differences in fluorescence intensity between the different dyes. Alternatively, different samples can be labelled with the same dye and run in different gels together with a differently-labelled internal standard made by the pooling of all samples to be compared (Alban *et al.*, 2003; Kondo *et al.*, 2003). Following matching of the same spots between different gels, fluorescence intensities are compared and differences in protein abundance detected. This design allows for more samples to be compared simultaneously and prevents variation caused by the use of different dyes. Differential in-gel electrophoresis offers the most sensitive quantitation platform in gel-based approaches with differences of less than 20% in protein levels being detectable (Alban *et al.*, 2003).

As an alternative separation method, IEF can be omitted and samples solubilised in buffers containing SDS and separated by traditional SDS-PAGE followed by digestion of proteins in gel bands and MS analysis of peptides for protein identification (Taylor *et al.*, 2003). Each gel band usually consists of a mixture of proteins which require further chromatographic separation either directly linked to MS analysis or carried out offline. This approach, which is also known as GeLC-MS,

can be used for whole-proteome identification analysis as well as for quantitation carried out at the MS stage (Gao *et al.*, 2008). Although separation of proteins does not compare to 2D-PAGE, the use of SDS to solubilise proteins enhances the representation of more hydrophobic membrane proteins in the analysed sample.

Multidimensional liquid chromatography (MD-LC) is a gel-free strategy for analysis of complex samples at the peptide level which involves digestion of a protein mixture and subsequent separation of peptides by multiple sequential LC runs (Wolters *et al.*, 2001). The most common setup consists of a LC step using strong cation exchange (SCX) chromatography to separate proteins by charge followed by reversed-phase chromatography (RP-LC) for separation based on hydrophobicity. The RP-LC step is directly interfaced with the mass spectrometer and can also be used independently to process peptide samples generated from 2-D gel spots and 1-D gel bands, as described previously. In this so called LC-MS setup, LC offers an additional separation step for proteins already resolved by gel electrophoresis. The automation of LC together with the increased solubility of the resulting peptides has rendered MD-LC a powerful approach for fast identification of proteomes (Washburn *et al.*, 2001).

Quantitation in SDS-PAGE and MD-LC setups can be performed by comparison of peptide ion intensities in mass spectra. Samples to be compared are labelled with different isotopes to introduce a mass difference between the same proteins/peptides from different samples (Ong and Mann, 2005). Differentially-labelled samples are then pooled and separated and analysed as one sample. Relative abundance of same peptides from different samples is inferred from the relative intensities of the corresponding ion peaks in mass spectra. Stable isotopic labelling by amino acids in cell culture (SILAC) is a technology for protein tagging and relies on the *in vivo* metabolic incorporation of a 'light' and 'heavy' form of one or more

amino acids into proteins during cell culture (Ong *et al.*, 2002). When dealing with non-culturable samples, lysates can be isotopically labelled with isotope-coded affinity tags (Gygi *et al.*, 1999) and isotope-coded protein labels (Kellermann, 2008) which attach to cysteine residues and free amino groups, respectively. Chemical tags such as iTRAQ can also be introduced at the peptide level, *i.e.* after protein samples have been digested to peptides by trypsin (Ross *et al.*, 2004). Fragmentation of the tag attached to each peptide generates a low molecular mass reporter ion that is unique to the tag used to label each of the digests. Measurement of the intensity of these reporter ions enables relative quantification of the peptides in each digest and hence the proteins they originate from.

1.5.1.2 Mass spectrometry

A mass spectrometer measures the mass-to-charge ratio (m/z) of ionised analytes and consists of an ion source, a mass analyser and a detector which records the number of ions at each m/z value. Two soft ionization techniques, electrospray ionisation (ESI) and matrix-assisted laser desorption ionisation (MALDI) are commonly used for peptide analysis (Ahmed, 2008). Electrospray ionisation is based on an evaporation-ionisation process of analytes in liquid phase and can be directly coupled to LC or capillary electrophoresis for the analysis of complex peptide mixtures. In MALDI analysis, samples are applied on an inert metal surface, dried and ionised under laser with the aid of an organic material (matrix).

Two types of spectra can be acquired, a typical MS spectrum that includes the m/z of every peptide present in the sample and a tandem MS/MS spectrum which is created by fragmenting a peptide into smaller fragments and measuring the m/z of the peptide fragments (Figure 1.4) (Aebersold *et al.*, 2003). Fragmentation is achieved by collision with gas and is a process called collision-induced dissociation (CID).

Fragment ion spectra are rich in information on each peptide's amino-acid sequence and are applied in the identification and quantitation of peptides as well as the characterisation of protein post-translational modifications (Aebersold *et al.*, 2003; Witze *et al.*, 2009). Based on the types of spectra available, two bioinformatic-based methods for protein identification have been developed, namely, peptide mass fingerprinting (PMF) and MS/MS peptide sequencing (Figure 1.4). In PMF, the list of experimental peptide masses generated during acquisition of MS spectra is matched to theoretical peptide masses calculated for each entry in a protein database (www.matrixscience.com, 2011d). Since peptides from different proteins can have the same mass, PMF is particularly beneficial for simple protein samples and the identification is confirmed when a statistically significant number of peptides map to the same protein. Peptide sequencing takes advantage of the MS/MS spectra which are scanned against theoretical MS/MS spectra constructed from protein sequence databases. Cross-correlation of the predicted spectra with the measured spectra determines the best match, thus MS/MS sequence search offers more unambiguous protein identification (Steen and Mann, 2004).

The quality of the mass spectra generated determines the success of downstream analysis including identification and quantitation of proteins. Spectral quality mainly depends on the performance of the mass analyser defined by parameters including resolution, mass accuracy, sensitivity and dynamic range. Resolution describes the ability of the analyser to distinguish between peptides of similar m/z value, whereas mass accuracy accounts for the accuracy in assigning the correct mass to every peptide and measures the error in this assignment (www.matrixscience.com, 2011c). Sensitivity and dynamic range are measures of the minimal protein concentrations that can be detected in a simple or complex sample,

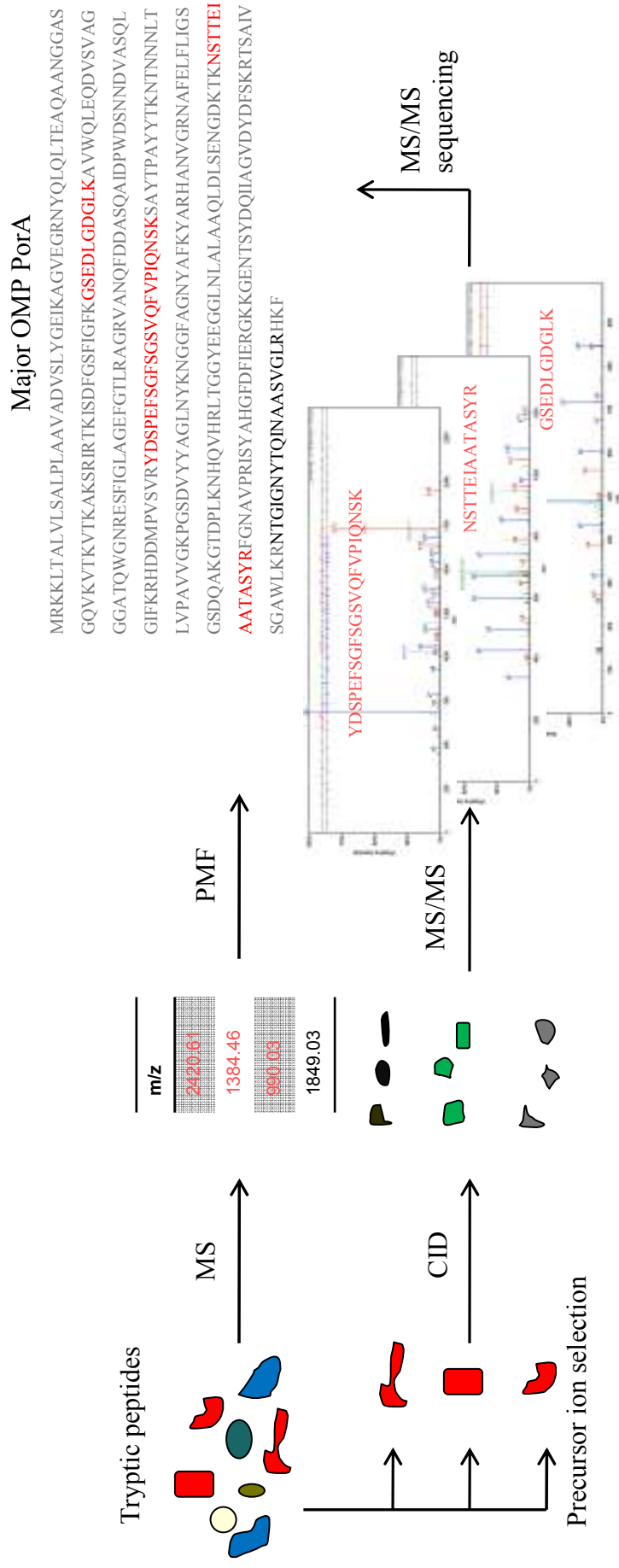


Figure 1.4 Mass spectrometry analysis for protein identification. Peptides generated following digestion of the protein sample with trypsin are subjected to MS and the list of m/z values acquired serves as a fingerprint which is compared against theoretical lists to identify the protein present in the sample (PMF). Each peptide can be further fragmented by CID and m/z values of fragment ions acquired in what is called a MS/MS spectrum. MS/MS spectra contain information about part or the complete sequence of the peptide and can be compared against theoretical spectra for identification of the protein of origin. As an example, peptides from major OMP PorA were used in this illustration.

respectively. Four main types of analysers are available, namely ion trap, time-of-flight (TOF), quadrupole and Fourier transform ion cyclotron. These can be used alone or in tandem leading to a variety of MS systems with different capabilities. Ion trap analysers can acquire MS and MS/MS spectra and fragment ions are generated in the same trapping space where peptide ions are captured. In TOF analysers, peptides of different m/z are scanned as they fly with different speed out of the analyser. The combination of TOF with quadrupole analysers has led to the development of hybrid instruments where selection of certain peptides and their fragmentation is carried out in the quadrupole part and m/z is measured in the TOF (Ahmed, 2008). More recent advances in hybrid MS systems include the use of ion traps in tandem where one trap is used for generation and selection of fragment ions while the other acquires MS spectra of high resolution. An example of such system is shown in Figure 1.5.

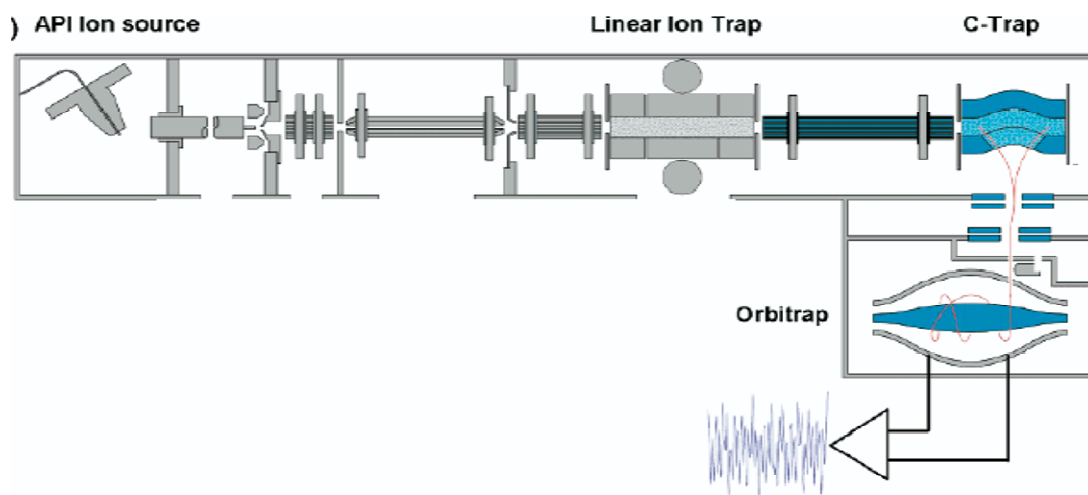


Figure 1.5 The Thermo LTQ-Orbitrap hybrid mass spectrometer (adapted from Makarov *et al.*, 2006). A typical MS method for protein identification involves the use of the Orbitrap for acquisition of high resolution MS spectra and the linear ion trap for CID fragmentation and acquisition of MS/MS spectra.

1.5.2 Proteomics of *N. meningitidis*

Sequencing of the *N. meningitidis* genome enabled the application of proteomic technology for the study of meningococcal disease at the protein level. Characterisation of the protein content of OMV preparations has dominated proteomics of *N. meningitidis*. Outer membrane vesicles used in vaccines against the Cuban CU385 and New Zealand 98/254 outbreak strain were the first to be analysed by proteomics (Uli *et al.*, 2006; Vipond *et al.*, 2006). 2D-PAGE separation of detergent extracted OMVs followed by MS identification of protein spots on gels confirmed the presence of major and minor OMPs, with PorA and PorB being the most abundant. These studies also revealed a more complex protein mixture than anticipated with many cytoplasmic proteins present in detergent-extracted preparations. Proteomic analysis of detergent extracted OMVs from other strains resulted in similar observations (Vaughan *et al.*, 2006; Williams *et al.*, 2007). Considerably fewer cytoplasmic proteins were found in naturally-released OMVs (nOMVs) prepared using a knockout strain missing a lytic transglycosylase encoded by gene *gna33* (Ferrari *et al.*, 2006). Analysis of nOMVs confirmed the contribution of detergent extraction to the presence of cytoplasmic proteins in the previous OMV vaccine preparations. It also suggested that nOMVs might be a better sample for OMV vaccine production since LPS levels were comparable to those from detergent-extracted preparations. The use of OMVs from LPS deficient mutant strains has also been suggested as an alternative way to minimize the amount of LPS without the need for detergents. The protein content of such OMVs was analysed by proteomics and found comparable to OMVs from the wild-type strain (Williams *et al.*, 2007).

Other applications of proteomics included the construction of 2-D proteome maps from strains representative of serogroups A and B (Bernardini *et al.*, 2004;

Mignogna *et al.*, 2005). Such maps aimed at providing information on protein expression under laboratory growth conditions to better understand biological processes in the meningococcus. Comparative proteomics were also used in some cases to assess the effect of certain factors on meningococcal protein expression. Proteins whose expression depends on iron availability were identified following comparison of the proteomes of bacteria grown under iron-depleted and iron-replete conditions (Basler *et al.*, 2006). There are also examples of comparative studies on the changes in protein expression induced by autoinducer AI-2, a molecule involved in quorum sensing (Schauder *et al.*, 2005) and Hfq, a conserved protein that mediates sRNA regulation (Fantappiè *et al.*, 2009). Assessment of protein content of OMV batches from different vaccine manufacturers by 2D-DIGE proteomic analysis was also documented as part of a study on the New Zealand 98/254 OMV vaccine (Vipond *et al.*, 2006).

More recent proteomic studies focussed on the identification of protein targets of immune response using antibodies raised against specific proteins or sera from patients and healthy carriers. These studies used so called immunoproteomic analysis, which involves separation of protein antigens by 2D-PAGE followed by their transfer onto membranes and blotting with antibodies (Utt *et al.*, 2002). Immunoproteomics was used in two cases to identify antigens that contribute to natural cross-protective immunity against meningococcal disease (Mendum *et al.*, 2009; Williams *et al.*, 2009). Using the same approach and specific monoclonal antibodies, NMB1468, a lipoprotein of unknown function, was identified as a novel immunogen able to confer protection in mice (Hsu *et al.*, 2008).

1.5.3 Proteomics in the analysis of bacterial surface proteomes for the identification of vaccine candidates

Recently, proteomic strategies for profiling the surface proteome of bacteria have emerged as promising tools for vaccine discovery (Cordwell, 2006). Methods involving chemical or physical treatment have been developed aiming to isolate only those proteins that are targeted by the immune system of the host. One strategy uses proteolytic enzymes to ‘shave’ the bacterial surface and release peptides originating from the surface-exposed regions of outer membrane antigens (Bledi *et al.*, 2003). Protease treatment is followed by MD-LC MS analysis of the released peptides or comparison between treated and non-treated cell lysates for the identification of the affected proteins (Rodríguez-Ortega *et al.*, 2006; Sabarth *et al.*, 2005). This method has been applied to Gram-positive bacteria including *Streptococcus* and *Bacillus* species and in some cases was specific for membrane proteins leading to the identification of potential vaccine candidates (Rodríguez-Ortega *et al.*, 2006; Rodríguez-Ortega *et al.*, 2008; Severin *et al.*, 2007; Tjalsma *et al.*, 2008).

Other strategies involve the modification of surface proteins by the application of chemical, membrane impermeable reagents to intact cells. Chemical tags consist of a reactive group for labelling lysine residues and a fluorophore or biotin molecule to directly visualise or purify the modified proteins, respectively. Modified proteins can then be separated and identified by MS analysis. Unlike protease treatment methods, proteins are permanently modified and their isolation requires additional purification steps. This approach has already been applied to several pathogenic bacteria using 2D-PAGE separation techniques (Anaya *et al.*, 2007; Ge and Rikihisa, 2007b; Ge and Rikihisa, 2007a; Mayrhofer *et al.*, 2006). In all studies using chemical modification, concerns about potential permeability of chemical tags through the bacterial

membrane were raised following the isolation and identification of numerous predicted cytoplasmic proteins.

1.6 Aim of the thesis

In the search for antigens for inclusion in meningococcal vaccines, proteins exposed on the bacterial cell surface pose as the most attractive candidates. Whilst many surface antigens have been characterised over the years, others might have been missed by the experimental methods used so far. Recent proteomic advances in the specific identification of bacterial surface proteomes offer a promising alternative and have not been fully exploited for the screening of the meningococcal surface. In this context, this study aimed to:

- refine and apply proteomic methods for the identification of proteins on the meningococcal surface.
- compare the proteome and immune responses of OMV vaccines from meningococci grown in different media for identification of protein antigens contributing to vaccine-induced immunity.
- identify protein targets of the immune system in mice through the application of immunoproteomics.
- assess the vaccine potential of proteins identified in the proteomic studies.

Chapter 2

Materials and Methods

2. Materials and Methods

2.1 Materials

All chemicals, equipment and analysis software used for protein labelling, SDS-PAGE, electro-transfer and immunoblotting were supplied by GE Healthcare (Chalfont St Giles, UK), unless otherwise stated. Enzymes, protease inhibitors, chemicals for immunological assays and solvents for liquid chromatography were provided by Sigma-Aldrich (Dorset, UK). Cell culture media were from Oxoid (Basingstock, UK). Plasmid vectors, purification kits and *E. coli* competent cells were purchased from EMD Chemicals (Gibbstown, New Jersey, USA). Liquid chromatography separation columns and equipment were from Dionex (Surrey, UK). The distributors of other materials and equipment used throughout the study are provided in the Methods section.

Monoclonal antibodies against PorA (P1.7), PorB (P3.5) and fHbp (variant 1) were produced at the National Institute for Biological Standards and Control (NIBSC). Murine immune sera against live *N. meningitidis* MC58 and OMVs from H44/76 strain were kindly provided by Dr Y. Li (CMMI, Imperial College London, London, UK) and Dr E. Wedege (Norwegian Institute of Public Health, Oslo, Norway), respectively. Nucleotide sequences of the gene coding for *N. meningitidis* macrophage infectivity potentiator (NmMIP) from 106 meningococcal isolates were kindly provided by Prof M. Maiden (University of Oxford, Oxford, UK).

2.2 Meningococcal strains and culture conditions

Neisseria meningitidis MC58 wild-type and Δ -*siaD* strains were used to generate protein extracts and as target strains in whole-cell ELISA and serum bactericidal assays and were obtained from the NIBSC strain collection. Target strains

in complement deposition and opsonophagocytosis assays were obtained from the HPA Meningococcal Reference Unit and included Norwegian and New Zealand vaccine strains H44/76 and NZ98/254, respectively, and representatives of the most prevalent UK serosubtypes (Table 2.1) (Findlow *et al.*, 2006). Strains used in phylogenetic analysis of the gene encoding NmMIP are presented in Table A1 (Appendix).

Strains were grown from frozen bead stocks in Columbia blood agar or brain heart infusion (BHI) agar supplemented with defibrinated horse blood and incubated at 37°C in a humidified atmosphere of 5% CO₂. For long term storage of strains, colonies were scraped from a blood agar plate, suspended onto beads (Technical Service Consultants Ltd, Heywood, UK) and stored at -80°C. For downstream applications requiring liquid cultures, bacteria were grown in Mueller-Hinton (MH) broth under aerobic conditions at 37°C with gentle shaking. Bacteria were collected at an optical density (O.D.) of 0.5–0.6 (late exponential phase) by centrifugation at 10,000 x g for 10 min and washed twice with cold phosphate buffered saline (PBS) until further use.

Table 2.1 Meningococcal isolates used in complement deposition and opsonophagocytosis assays.

Isolate	serogroup	sequence type	clonal complex	serosubtype	
				VR1	VR2
MC58	B	74	32	7	16-2
H44/76	B	32	32	7	16
NZ 98/254	B	44	41/44	7-2	4
M01-240101	B	1049	269	19-1	15-11
M01-240013	B	275	269	22	9
M01-240149	B	41	41/44	7-2	4
M01-240185	B	11	11	5-1	10-8
M01-240355	B	213	213	22	14

2.3 Proteinase K treatment

Bacteria from MH cultures of the MC58 Δ -*siaD* strain were used for treatment with proteinase K. Cell pellets containing approximately 12×10^9 bacteria were resuspended in 1 mL of PBS pH 7.2, containing 5 mM dithiothreitol (DTT) and proteinase K at concentrations varying from 1.25 to 80.0 $\mu\text{g/mL}$ per 10^9 cells. Cell suspensions were incubated for 45 min at 37°C in a shaking incubator. Proteinase K activity was terminated by the addition of 10 μL of protease inhibitors. Cells were subsequently washed twice in cold PBS, resuspended in 200 μL lysis buffer containing 30 mM Tris hydrochloride, pH 8.5, 7 M urea, 2 M thiourea and 2% (v/v) Triton X-100 and lysed by repeats of 30 sec vortexing followed by incubation on ice. Protein extracts (also referred to as cell extracts and protein lysates) were recovered by centrifugation at 10,000 x g for 20 min. Protein concentrations were determined using the Bradford assay (Bio-Rad, Hemel Hempstead, UK) (Bradford, 1976). Optical density measurements were performed using the Lambda 800 UV/VIS spectrophotometer (Perkin-Elmer, Waltham, MA, USA).

2.4 Preparation of OMV lysates

Outer membrane vesicles were prepared at the Division of Infectious Disease Control, Norwegian Institute of Public Health as described by Tsolakos *et al.* (available in the electronic copy of the thesis) (Tsolakos *et al.*, 2010). The meningococcal vaccine strain H44/76 (B:15:P1.7,16; ST-32) was cultured in a 2.5 L fermentor in either the semi-synthetic Frantz medium (FM) containing yeast extract and casamino acids (Fredriksen *et al.*, 1991) or the synthetic modified Catlin 6 medium (MC.6M) containing ferric citrate prepared as previously described (Frasch *et al.*, 2001). The detailed composition of the two media is presented in Table 2.2. Outer

membrane vesicles were prepared by extraction with Na-deoxycholate as previously described (Fredriksen *et al.*, 1991). Three OMV batches were prepared from bacteria grown in each of the culture media. For 2D-PAGE, the 6 samples were concentrated using Microcon YM-3 filter (Millipore, Livingston, UK) following centrifugation at 8,000 x g for 15 min and reconstituted in lysis buffer containing 30 mM Tris, 7 M urea, 2 M thiourea and 2% (v/v) Triton X-100 (Sigma-Aldrich), pH 8.5 (Vipond *et al.*, 2006). The protein concentration of the samples was determined using the Bradford assay.

Table 2.2 Composition of media used to grow the Norwegian OMV vaccine strain H44/76.

Frantz (FM)		Modified Catlin-6 (MC.6M)	
Reagent	g/L	Reagent	g/L
Na ₂ HPO ₄ · 2H ₂ O	3.5	NaCl	5.8
KCl	0.09	K ₂ HPO ₄	4.0
MgSO ₄ · 7H ₂ O	0.6	K ₂ SO ₄	1.0
Glucose	10.0	NH ₄ Cl	1.0
Casamino acids	30.0	CaCl ₂ · 2H ₂ O	0.03
D-cysteine hydrochloride	0.02	MgCl ₂ · 6H ₂ O	0.4
Yeast Extract	1.0	L-Glutamic Acid	3.9
		L-Cysteine · HCl	0.1
		L-Arginine · HCl	0.15
		L-Serine	0.5
		Glycine	0.25
		Glucose	10.0
		Fe(III) Citrate	0.04

2.5 Fluorescent labelling

Fluorescent labelling of protein samples was carried out using fluorescent cyanine dyes. Two sets of cyanine dyes were used. CyDyeTMDIGE fluor propyl-Cy3 and methyl-Cy5 (NHS) ester dyes (referred to as Cy3 and Cy5) containing 5 nmol per tube were freshly reconstituted in anhydrous dimethylformamide at 100 pmol/ μ L. IC-OSu ethyl-Cy3 and ethyl-Cy5 NHS ester cyanine dyes (referred to as IC3 and IC5) (NBS Biologicals Ltd, Huntington, UK) were dissolved in 100% methanol and individual aliquots of 5 nmol dispensed into clear 1.5 mL tubes. The dyes were immediately dried by vacuum centrifugation. The closed tubes were stored at -20°C, protected from the light. The 5 nmol IC dyes were used and prepared prior to labelling as described for CyDyeTMDIGE dyes. As part of their evaluation prior to use in subsequent studies, IC dyes were tested and found comparable to CyDyeTMDIGE dyes for labelling of protein samples. Results from this analysis were published (Tsolakos *et al.*, 2009) and are available in the electronic copy of this thesis.

A minimal labelling approach was used where 50 μ g of protein extract was mixed with 400 pmol of cyanine dye and left on ice for 30 min in the dark. The reaction was stopped with the addition of 1 μ L of 10 mM L-lysine and incubation on ice for an extra 10 min. For DIGE analysis, a two-colour experimental design was used where individual samples were labelled with IC5 or Cy5 and internal standards were made of equal quantities of all individual samples included in each experiment with IC3 or Cy3, respectively (Yan *et al.*, 2002). 50 μ g of sample and internal standard were mixed and subjected to IEF (described in section 2.6.2).

2.6 Protein separation

2.6.1 SDS-PAGE

Protein samples (25 µg) were boiled for 5 min in Laemmli sample buffer (Bio-Rad) (Laemmli, 1970) containing 62.5 mM Tris-hydrochloride, pH 6.8, 25% (v/v) glycerol, 2% (w/v) SDS and 0.01% bromophenol blue. They were then loaded on Tris-glycine-SDS polyacrylamide gels (16 x 14 x 0.15 cm) comprising a 5% stacking and a 12% resolving part. The gels were placed in a SE-600 gel tank apparatus filled with SDS running buffer made up of 25 mM Tris base, 192 mM glycine and 0.1% (w/v) SDS. Proteins were separated by the application of a constant current of 40 mA for 5 hr until the dye front migrated off the bottom of the gel.

2.6.2 Two-dimensional PAGE

Protein samples were mixed with rehydration buffer made up of 2 M thiourea, 7 M urea, 2% (v/v) Triton X-100, 1% (v/v) pharmalytes pH 3-10 and 2 mg/mL DTT and applied onto 24 cm, non-linear, immobilised pH gradient (IPG) Immobiline DryStrips, pH 3-11. Rehydration of the strips was carried out overnight at ambient temperature. The strips were then placed on an IPGphor II for IEF using six phases of stepped and gradient voltages. The first two phases were identical for all samples and included the application of 300 V for 3 hr followed by a gradient of 300-1000 V for 9 hr. The remaining phases depended on the total focusing time and maximal voltage chosen. A maximal voltage of 10,000 V for a total focusing time of 100 KVhr was used for whole-cell lysates. For OMV samples, maximal voltages of 3,500, 5,500, 8,000 and 10,000 V for a total focusing time of 45 KVhr were tested. Following IEF, the strips were either used immediately or stored at -20°C.

Prior to second dimension SDS-PAGE, each strip was equilibrated by incubation for 15 min in 5 mL of equilibration buffer containing 50 mM Tris-hydrochloride pH 8.8, 2 M thiourea, 7 M urea, 30% (v/v) glycerol, 2% (w/v) SDS and 20 mg/mL DTT. The equilibration step was repeated by replacing DTT with iodoacetamide (IAA) (48 mg/mL) in the equilibration buffer. Dithiothreitol was used to aid solubilisation of proteins separated on the strips by disrupting disulfide bonds formed by thiol groups of cysteine residues. Iodoacetamide alkylates thiol groups and prevents re-formation of disulfide bonds, thus maintaining proteins in a soluble state. In this way, transition of proteins from the strips onto the acrylamide gels is enhanced. The strips were then placed on 12% Tris-glycine-SDS polyacrylamide gels (26 x 20 x 0.1 cm) prepared in-house using an automatic gel casting system (NextGen Sciences, Huntington, UK). Proteins were separated by the application of 8 mA/1W/gel for 2 hr followed by 18 mA/1W/gel until the dye front migrated off the bottom of the gel.

2.7 Protein staining

For protein samples requiring staining for visualisation following electrophoresis, Coomassie R-350 or Sypro Ruby was used. Gels were stained overnight in a 0.1% Coomassie R 350 stain solution made by dissolving a PhastGel Blue R tablet in a 40% (v/v) methanol/10% (v/v) acetic acid solution and destained in a 40% (v/v) methanol/10% (v/v) acetic acid solution. For Sypro Ruby staining, proteins were first fixed in 50% (v/v) methanol/10% (v/v) acetic acid for 30 min, incubated in Sypro Ruby gel stain overnight and washed in 10% (v/v) methanol/7% (v/v) acetic acid for a final 30 min.

2.8 Gel Scanning

Gels containing proteins labeled with fluorescent cyanine dyes or Sypro Ruby were scanned using the Typhoon 9410 Imager (GE Healthcare). Cy3, IC3 and Sypro Ruby were excited by a green laser at a wavelength of 532 nm whilst Cy5 and IC5 by a red laser at 633 nm. Emitted light was captured by bandpass filters at 580 nm for Cy3 and IC3, 610 nm for Sypro Ruby and 670 for Cy5 and IC5. Scanning resolution was set to 100 bpi and photomultiplier tube voltages were adjusted for fluorescence intensity readings to fall within the linear dynamic range of the scanner. Images of scanned gels were visualized using the ImageQuant tools software v. 3.0.

Scanning of the gels stained with Coomassie R-350 was carried out with Bio-Rad Scanner GS-710 Imaging Densitometer (Hercules, CA, USA) using default parameters.

2.9 DIGE quantitative analysis

Images of proteins subjected to DIGE were imported into DeCyder 2-D differential analysis software v. 6.5 for spot detection and quantitative differential analysis. Pairs of Cy5/Cy3 or IC5/IC3 images corresponding to sample/internal standard pairs run on the same gel were imported simultaneously and assigned to individual samples. Co-detection of spots in the sample and internal standard images acquired from each gel was performed by the Differential In-gel Analysis (DIA) module using an estimated number of 2,500 spots. DIA also calculated and normalised fluorescence intensities (abundance) of each spot to the internal standard. In particular, normalised abundance was expressed as the ratio of abundance of the spot in the sample image against the internal standard image. Normalisation of quantitative data to compensate for variability in sample amount, total fluorescence

and laser intensity was also applied and was based on the assumption that the modal peak of spot ratios is zero (no change in protein expression between samples run in the same gel should occur) (Yan *et al.*, 2002). Images from DIA analysis were then imported to the Biological Variation Analysis module (BVA) for spot matching and comparative analysis. In BVA, samples were first grouped based on the nature of treatment. Biological replicates (*i.e.* samples subjected to the same type of treatment) were placed within the same group. With the assistance of Cy3/IC3 internal standard images and landmark spots, same protein spots were matched across all samples. Normalised abundance of same spots was compared across groups and differences were detected. Differences were expressed as the ratio of mean normalised abundance between two groups which was termed average ratio. The significance of differences observed was determined by application of Student's t-test and analysis of variance (ANOVA). Differences exhibiting a P-value of less than 0.05 were considered significant.

2.10 Protein electro-transfer and immunoblotting

Proteins from gels were transferred to low-fluorescent HyBond-LFP polyvinylidene fluoride (PVDF) membranes using a TE70 semi-dry blotter. Gels were placed in ice-cold Towbin buffer made up of 25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS, 20% (v/v) methanol, pH 8.3, for 15 min while PVDF membranes were equilibrated in ice-cold methanol for 5 min followed by 10 min in Towbin buffer. Gels and membranes were then assembled into a stack of up to 3 sandwiches consisting of 3 sheets of blotting paper, membrane, gel and 3 sheets of blotting paper with cellulose sheet in-between sandwiches. Proteins were then transferred onto the

membrane by the application of 8 mA/cm² (180 mA and 437 mA for 1- and 2-D gels, respectively) for 3 hr.

Blotting with antibodies was performed in a Roller-Blot HB-3D Hybridiser (Techne, Minneapolis, MN) at ambient temperature using the ECL Plex western blotting system for single protein detection. PBS pH 7.4 containing 0.1% (v/v) Tween-20 was used for antibody dilutions and membrane rinse steps. Membranes were blocked with 5% ECL Advance blocking agent for 1 hr and incubated for 3 hr with monoclonal or murine polyclonal antibodies in 1:200 and 1:4,000 dilutions, respectively, for 3 hrs. Antibody binding was detected by incubation with 1:2,000 dilution of a Cy5-conjugated, ECL Plex goat anti-mouse IgG (Sigma-Aldrich) for 1 hr and scanning for Cy5 fluorescence using the Typhoon 9410 imager as described in section 2.8.

2.11 Spot picking from 2-D preparative gels

Protein spots were picked from 2-D gels (termed preparative) for MS analysis and protein identification. Two-dimensional PAGE of protein samples to be used for spot picking was carried out as described in section 2.6.2 with the following modifications. A total of 200 µg of protein sample was loaded onto IPG strips including 50 µg labelled with IC5 cyanine dye. Prior to gel plate assembly for in-house gel casting, the sides of low-fluorescent gel plates in direct contact with the gel were treated with bind and repel silane, respectively. Final bind silane solution contained 18 mL water, 80 mL ethanol, 2 mL acetic acid and 100 µL bind silane. When polymerised, polyacrylamide gels were mounted on the plate treated with bind silane whilst the plate containing repel silane could be removed to allow for spot picking. Following 2D-PAGE, proteins on gels were stained with Sypro Ruby as

described in section 2.7. Two images of total protein were acquired, one of Sypro Ruby and one of IC3-labelled proteins. Images were imported into the DeCyder 2-D differential analysis software v. 6.5 and the DIA module was used for spot detection and matching between Sypro Ruby and IC3 images. Proteins were subsequently matched to their correlates in analytical gels containing information from DIGE analysis or immunoblots. Protein spots aimed for MS analysis were annotated on the Sypro Ruby image and x, y coordinates of their position in relation to reference markers placed on the gel plates were imported into the Ettan Spot Picker for automated spot picking. Gels were placed inside the picker chamber fully submerged in water. The position of the picking needle was adjusted based on the same reference markers used for annotation of spot position on the gel. The picking needle was washed twice with 200 μ L of distilled water in between applications on the gel. Gel plugs were placed into 96-well plates until further use.

2.12 In-gel protein digestion and peptide extraction

Protein spots from 2-D gels were transferred to protein LoBind tubes (Eppendorf UK Ltd, Cambridge, UK), and destained with 50 mM ammonium bicarbonate (VWR International Ltd, Lutterworth, UK), pH 8.5 in 50% (v/v) acetonitrile for 1 hr. Spots were alternatively washed with 50 mM ammonium bicarbonate, 50 mM ammonium bicarbonate in 50% (v/v) acetonitrile and 100% (v/v) acetonitrile by incubation for 20 min at ambient temperature. In-gel digestion was carried out overnight at ambient temperature using 0.1 μ g of trypsin (Promega, Southampton, UK) per spot in 50 mM ammonium bicarbonate.

Peptides were extracted sequentially by water bath sonication for 4 min, using 1% (v/v) trifluoroacetic acid (TFA) (VWR International Ltd), 50% (v/v)

acetonitrile/0.2% (v/v) TFA (twice) and 100% (v/v) acetonitrile. The pooled extraction solution was dried in the Savant SPD2010 SpeedVac concentrator (Thermo Scientific, Basingstoke, UK) and reconstituted in 0.1% (v/v) TFA.

2.13 Mass spectrometric analysis and database searching for protein identification

2.13.1 MALDI-TOF MS and LIFT-TOF MS/MS

Digests were desalted and concentrated using a ZipTip C18 (Millipore, Watford, UK) according to the manufacturer's instructions. Briefly, each tip was wetted with 3 rounds of 10 μ L of 100% (v/v) acetonitrile and equilibrated with 3 rounds of 10 μ L of 0.1% (v/v) TFA. The sample was aspirated and dispensed through the tip several times for peptide binding followed by washing with 3 rounds of 10 μ L of 0.1% (v/v) TFA. Peptides were eluted in 5 μ L of 60% (v/v) acetonitrile/0.1% (v/v) TFA. As matrix for ionisation α -cyano-hydroxycinnamic acid was prepared at a concentration of 10 mg/mL in 50% (v/v) acetonitrile/0.1% (v/v) TFA by vortexing and centrifugation for 1 min. One μ L of matrix was mixed with 1 μ L of sample and spotted onto a MALDI target.

An Autoflex II MALDI LIFT TOF with FlexControl and FlexAnalysis software was used for acquisition, calibration and display of spectra (Bruker Daltonics, Coventry, UK). A total of 700 laser shots per sample was acquired by summing sets of 50 laser shots. MS/MS spectra were acquired by application of LIFT-TOF on the most intense parent ions. A mixture of 7 reference peptides was used for external calibration of spectra. A list of peptide masses from MS and masses of fragment ions from MS/MS spectra were produced in FlexAnalysis and imported using BioTools v.2.0 (Bruker Daltonics) to a web-based Mascot search engine (Matrix

Science, Boston, MA, USA) for database searching and protein identification by PMF and MS/MS ion searching, respectively. Search parameters included the protein entries of Swiss-Prot and NCBItr for *N. meningitidis* as the protein databases used for searching, trypsin as the enzyme used for digestion allowing one missed cleavage, cysteine carbamidomethylation and methionine oxidation as fixed and variable modification, respectively, precursor and fragment mass tolerance of 100 ppm, and peptide charge of +1. Proteins with a significant (P-value < 0.05) score according to Mascot were reported.

2.13.2 Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Protein digests were analysed by nano-scale capillary LC-MS/MS using a Dionex U3000 HPLC system operated at flow rates of 500 nL/min. Peptides were trapped on a Acclaim PepMap100 C18, 5 µm, 300 µm x 5 mm µ-Precolumn for 1 min, separated on a PepMap100 C18, 3 µm, 75 µm x 5 cm analytical column and eluted with a 10 min 2-step gradient of 10-20% (0-6 min) and 20-55% (6-10 min) of 90% (v/v) acetonitrile in 0.05% (v/v) formic acid. A nano-spray source was interfaced to the analytical column to direct peptides into a LTQ Orbitrap Discovery (Thermo Scientific, Hemel Hempstead, UK) using a 10 µm coated SilicaTip emitter (New Objective, Woburn, MA). MS spectra were acquired in the Orbitrap operated on positive mode at a resolution of 30,000. The 5 most intense multiply-charged precursor ions in the m/z range of 400-2000 were selected for CID and fragment ions were analysed in data-dependent scans using the linear ion trap. The mass spectrometer was routinely tuned by direct infusion of 100 fmol of [Glu]-fibrinopeptide at a flow rate of 500 nL/min. The performance of the LC-MS/MS

method was further monitored by MS and MS/MS analysis of bovine serum albumin digests (Michrom Bioresources, Auburn, California, USA) at fmol levels.

Proteome Discoverer v.1.0 (Thermo Scientific) was used for downstream data processing and database searching using the Sequest engine and the *N. meningitidis* MC58 sequence entries (UniProtKB/SwissProt release 56.4). Search parameters included trypsin as the enzyme used for digestion with one missed cleavage allowed, methionine oxidation as a variable modification, a precursor mass tolerance of 10 ppm and fragment mass tolerance of 0.5 Da. Only proteins identified by at least three peptides of high confidence passing the single threshold filter of Xcorr (1.5, 2.0, 2.5) versus charge state (+ 1, 2, 3), respectively, were reported.

2.14 Cloning of vaccine candidate genes

2.14.1 Small scale DNA extraction and isolation

Genomic DNA was extracted from *N. meningitidis* MC58 strain using the Wizard Genomic DNA Purification kit (Promega UK). Cells from blood agar plates were resuspended in 600 µL of nuclei lysis solution and incubated for 5 min at 80°C. The suspension was cooled to room temperature and 3 µL of 4 mg/mL RNase were added followed by incubation at 37°C for 30 min. Proteins were precipitated by addition of 200 µL of protein precipitation solution and incubation on ice for 5 min. The cell-free supernatant containing genomic DNA was recovered by centrifugation at 13,000 x g for 3 min and mixed with 600 µL of isopropanol by gentle inversion until strings of DNA were visible. DNA was pelleted by centrifugation at 13,000 x g for 2 min and the isopropanol was removed carefully. The DNA pellet was washed with 600 µL of 70% (v/v) ethanol, air-dried, resuspended in sterile distilled water and stored at -20°C.

2.14.2 Primer design

Primer pairs were designed for each gene using the nucleotide sequences from the annotated genome of MC58 strain. Potential primer sequences were assessed for melting temperature and G/C content using the OligoCalc tool from Northwestern University Medical School (www.basic.northwestern.edu/biotools/oligocalc). Primer pairs are presented in Table 2.3. The annealing position of the primer pairs in the nucleotide sequences of the corresponding genes are shown in Figure A1 (Appendix).

Primer sequences were designed as close to the cleavage site of the signal peptide at the 5' end and translation termination codon at the 3' end as possible. Primer pairs were required to exhibit melting temperatures no greater than 65°C and no more than 5°C difference. A G/C content of 40-60% was also preferred where possible. Sequences were also required to terminate with a G or C whilst avoiding runs of three or more G/C at the 3' end. Sequence pairs following these properties were further assessed for complementarity and hairpin loop formation using OligoCalc tool and PrimerSelect from Lasergene software v.8 (DNASTAR, Madison, USA) and those exhibiting the lowest number of base pair matches were selected. In the case of NmMIP, the anti-sense primer included part of the gene downstream of NmMIP to achieve optimal annealing properties.

As highlighted in Table 2.3, extension sequences for ligation independent cloning were also introduced at the 5' end of each primer. Extension sequences were designed which were complementary to the overhangs present in the pET Ek/LIC plasmid vector to allow for subsequent gene insertion to the vector downstream of a six-His sequence. Anti-sense primers also included a stop codon at the 5' end after the overhang extension sequences.

Table 2.3 Primer sequences used for amplification and sequencing of *fkpA* (NMB1567), *pilP* (NMB1811), *pilO* (NMB1810) and NMB2095. The sequences corresponding to the LIC overhangs are italicized and were not included in the calculation of melting temperature and G/C content. All sequences are presented in 5' - 3' orientation.

Gene name	Protein name	Size of amplified sequence (bp)	Primer sequence	Melting temperature (°C)	G/C content (%)
<i>fkpA</i>	Macrophage infectivity potentiator (NmMIP)	763	sense: <i>GACGACGACAAGATCGCCCTGCCGCAAAAAGAAG</i>	60	55
			anti-sense: <i>GAGGAGAAAGCCCCGGTTATTGTGATGTCGACTTGAGC</i>	59	39
<i>pilP</i>	PilP	490	sense: <i>GACGACGACAAGATTTCTGAGGACCTAAACGAATG</i>	60	45
			anti-sense: <i>GAGGAGAAAGCCCCGGTTAATTTTGTCTGCCGCAGG</i>	57	43
<i>pilO</i>	PilO	529	sense: <i>GACGACGACAAGATCGGATTGTTCAAAAGCCAGATG</i>	60	45
			anti-sense: <i>GAGGAGAAAGCCCCGGTTATTTTGTCTCGGCATTTTGTG</i>	58	35
NMB2095	Putative adhesin complex protein	313	sense: <i>GACGACGACAAGATCGCTGGCAGGACAACC</i>	60	71
			anti-sense: <i>GAGGAGAAAGCCCCGGTTAACGTGGGGAACAGTCTTTG</i>	60	45

2.14.3 PCR amplification of vaccine candidate genes

PCR amplification reactions were prepared by mixing 160 μ M of each dNTP, 1 μ M of each primer (Invitrogen, Paisley, UK), 1 U of AmpliTaq DNA polymerase (Invitrogen), 10x GeneAmp PCR buffer, 1 mM $MgCl_2$ and 100 ng of genomic DNA to a final volume of 10 μ L. Reactions were carried out in a Veriti 96 well thermal cycler (Applied Biosystems, Warrington, UK) by the application of a primary denaturation step at 94°C for 2 min, 30 cycles of 60 sec denaturation, annealing and extension steps at 94°C, 63°C and 72°C, respectively, and a final extension step at 72°C for 5 min.

2.14.4 Purification of PCR products

PCR products were purified using Polyethylene Glycol 8000 (PEG₈₀₀₀) precipitation. 60 μ L of PEG₈₀₀₀ in 2.5 M NaCl were mixed with 95 μ L of PCR product and incubated at 37°C for 10 min. The mixture was centrifuged at 13,000 x g for 10 min and the supernatant discarded. The DNA pellet was washed with 500 μ L 80% ethanol, air-dried and resuspended in 25 μ L TlowE (10 mM Tris-HCl, 0.1 mM EDTA pH 8.0). DNA concentration of purified products was measured using a Nanodrop 2000 (Fisher Scientific UK Ltd, Loughborough, UK).

2.14.5 Agarose gel electrophoresis

PCR amplification products were analysed by agarose gel electrophoresis. Gels were made to 0.8% (w/v) in 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA) and 5 μ L of SafeView nucleic acid stain (NBS Biologicals Ltd) were added to the solution. The solution was poured into 40 mm gel trays and left to polymerise at room temperature. PCR products were mixed with 6x loading buffer (15% (v/v) ficoll, 15%

(v/v) glycerol, 0.1 M Tris, pH 8.0 and 0.5% bromophenol blue) and loaded into the individual wells of the gel. DNA ladders of 1,000 bp and 100 bp (New England Biolabs, Ipswich, MA, USA) were run in parallel to allow for DNA fragment size determination. Electrophoresis was carried out in 1x TAE buffer by application of 100 V for 1 hr and DNA bands were visualized using an ultraviolet transilluminator. Images of gels were photographed by the GelLogic 1500 Imaging System and analysed using Molecular Imaging software (Kodak Ltd, Hemel Hempstead, UK).

2.14.6 T4 DNA polymerase treatment and annealing of vector and PCR products

PCR products from gene amplification and pET-30 Ek/LIC plasmid vector were treated with T4 DNA polymerase for generation of compatible overhang sequences. Reactions were prepared to a final volume of 20 μ L and included 1 U of T4 DNA polymerase, 25 mM dATP, 100 mM DTT, 2 μ L of 10x reaction buffer and 0.4 pmol purified PCR product. Reactions were carried out at 22°C for 30 min and stopped by incubation at 75°C for an extra 20 min. T4 DNA polymerase treated vector and PCR products were mixed at a 1:2 ratio and annealed by incubation at 22°C for 5 min followed by addition of 1 μ L ethylenediaminetetracetic acid (EDTA) (Sigma-Aldrich) and an extra 5 min incubation step at 22°C.

2.14.7 Transformation

The construct prepared from annealing vector and PCR product was used to transform NovaBlue GigaSingles *E. coli* competent cells. 1 μ L of the annealing reaction was added to a tube containing 20 μ L of freshly thawed NovaBlue cells and incubated on ice for 5 min. The tube was then placed on a 42°C water bath for exactly 30 sec and transferred back on ice for 2 min. Cells in each tube were mixed with 250

μL of room temperature SOC medium and incubated at 37°C with shaking at 250 rpm for 60 min. Transformants were selected by plating 100 μL of the cell suspension on Luria-Bertani (LB) agar plates supplemented with 30 μg/mL kanamycin and overnight incubation at 37°C.

2.14.8 Colony-PCR

Screening for colonies carrying the vector construct was carried out by colony-PCR. The reaction mix contained 10 mM of T7 promoter and terminator primers, 10 mM dNTPs, 2.5 U AmpliTaq (Applied Biosystems), 10x GeneAmp buffer to a final volume of 50 μL. Single colonies were picked from agar plates using a sterile tip and mixed with the reaction mix. The same tips were also used to inoculate fresh LB agar plates and broths containing kanamycin for overnight growth of clones at 37°C. Thermal Cycler conditions included an initial denaturation step at 95°C for 5 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 43°C for 1 min and extension at 72°C for 2 min followed by a final extension step at 72°C for 5 min. PCR products were loaded onto 0.8% (w/v) agarose gels and the presence of a band at the size of the insert indicated a positive clone.

2.14.9 Sequencing

In-frame insertion of the cloned gene to the Ek/LIC vector was assessed by sequencing the part of the vector containing the site of insertion and the inserted gene. As a first step, the plasmid vector was purified from Novablue transformed cells using the QIAprep Spin MiniPrep kit (QIAGEN Ltd, Crawley, UK) following the manufacturer's instructions. Dye terminator sequencing was carried out using the BigDye® terminator v. 3.1 cycling kit (Applied Biosystems) and T7 promoter and

terminator primers. 10 μ L reactions included 0.5x concentrated BigDye® Ready Reaction Premix, 0.5x concentrated BigDye® Sequencing buffer, 0.5 μ M of one of T7 primers and 10-40 μ g plasmid DNA. Sequencing reactions were placed in the Veriti 96-well thermal cycler and the thermal profile conditions consisted of 25 cycles of 96°C for 30 sec, 50°C for 15 sec and 60°C for 4 min.

Sequencing reactions were purified by ethanol precipitation. 10 μ L of water, 5 μ L of 125 mM EDTA and 80 μ L of 100% (v/v) ethanol were added sequentially to each reaction and the solution was mixed by vortexing and incubated for 1 hr at room temperature. Precipitated DNA was recovered by centrifugation at 3,000 x g for 30 min and incubated with 200 μ L of 70% (v/v) ethanol for 5 min at room temperature. Following centrifugation at 1,800 x g for 15 min, the ethanol was discarded and DNA pellets were dried by centrifugation of the tubes inverted on blotting paper at 900 x g for 2 min. Samples were then resuspended in Hi-Di™ formamide buffer (Applied Biosystems) and sequencing products were detected using the ABI PRISM® 3130 Genetic Analyser (Applied Biosystems) following manufacturer's instructions.

2.15 Expression and purification of recombinant proteins

Purified plasmid vectors containing the insert of interest were used to transform the *E coli* BL21 (DE3) pLysS expression strain. Transformation, selection of positive clones and confirmation of in-frame insertion were again performed as described for Novablue cells (sections 2.14.7-9). Large-scale growth of expression cells was carried out by inoculation of 100 mL LB broth cultures containing 30 μ g/mL kanamycin and 1% (w/v) glucose and incubation at 37°C. When an O.D. of 0.6 was reached, expression cell cultures were supplemented with isopropyl β -D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM for induction of

protein expression. Cells were left to grow for an additional 2-5 hr and harvested by centrifugation at 5,500 x g for 20 min. Pellets were weighed and stored at -70°C until further use.

Proteins were extracted from cells using the BugBuster Protein Extraction Reagent following the manufacturer's instructions. Briefly, cell pellets were resuspended at room temperature by gentle vortexing with 1x concentrated BugBuster Reagent added at a ratio of 5 mL per gram of wet cell paste. To assist cell lysis, 10 µL of Lysonase Bioprocessing Reagent was added per gram of wet cell paste. Following addition of protease inhibitors at 1:100 dilution, the suspension was incubated for 20 min at room temperature with gentle shaking. Soluble proteins were recovered in the supernatant following centrifugation at 12,500 x g for 20 min at 4°C and stored at -20°C prior to purification.

Recombinant proteins were purified by metal affinity chromatography in gravity flow columns using the His-Bind Resin and His-Bind Buffer kit. Appropriate volumes of 1x concentrated buffers were prepared according to the manufacturer's instructions. Binding buffer from His-Bind Buffer kit was supplemented with 0.05% (w/v) Z3-14 and Wash and Elution Buffers with 0.2% (v/v) Triton X-100 to enhance solubility of the proteins in the respective steps. Approximately 6 mL of His-Bind Resin corresponding to 3 mL of bed volume were applied to Novagen Chromatography Columns, washed with 3 volumes of sterile deionised water, charged with 5 volumes of Charge Buffer (50 mM NiSO₄) and equilibrated with 3 volumes of Binding Buffer (0.5 M NaCl, 5 mM imidazole, 0.05% (w/v) Z3-14, 20 mM Tris-hydrochloride, pH 7.9). The protein extract was loaded on the column, mixed with resin and left for 20 min for affinity binding to occur. Unbound material was washed away by the application of 10 volumes of Binding Buffer followed by 6 volumes of

Wash Buffer (0.5 M NaCl, 60 mM imidazole, 0.2% (v/v) Triton X-100, 20 mM Tris-hydrochloride, pH 7.9). His-tag recombinant proteins were eluted with 6 volumes of Elution Buffer (0.5 M NaCl, 1 M imidazole, 0.2% (v/v) Triton X-100, 20 mM Tris-hydrochloride, pH 7.9). Eluted proteins were dialysed against a buffer containing 10 mM Tris, pH 7.9, 150 mM NaCl and 0.05% (v/v) Triton X-100 to remove imidazole using 10 kDa MW cutoff Slide-A-Lyser Dialysis cassettes (Thermo Scientific). Dialysed solutions were concentrated by application to Amicon Ultra-15 Centrifugal Filter units (Millipore Ltd, Watford, UK) and centrifugation at 4,000 x g for 20 min at room temperature. Protein concentrations were calculated using the BCA assay (Thermo Scientific).

2.16 Immunisations and immune sera

Immunisations with the recombinant meningococcal proteins were carried out by the Biological Services Department at NIBSC. Groups of 10 female BALB/c mice, 6-7 weeks old, were immunized on days 1, 21 and 28 by subcutaneous injection of 10 µg purified protein mixed with the Sigma Adjuvant system (monophosphoryl lipid A, MPL) (Sigma-Aldrich). Control mice received MPL in physiological saline. Blood was collected on day 35. Animal work was carried out under license by the Home Office, United Kingdom.

Immune sera from OMV vaccines were a gift from Dr E. Wedege following immunisations carried out as described (Tsolakos *et al.*, 2010). Briefly, one OMV preparation from each growth medium (FM or MC.6M), made by pooling similar amounts of protein from each of the 3 batches, was adsorbed to aluminium hydroxide adjuvant (Alhydrogel, Superfos Biosector, Copenhagen, Denmark). Groups of 12 female NMRI mice received 2 doses subcutaneously of 0.5 or 2.0 µg protein of each

OMV vaccine 3 weeks apart. Control mice in groups of 6 received physiological saline. Blood samples were collected from the mice 2 weeks after the second dose.

Immune sera from live *N. meningitidis* were a gift from Dr Y. Li following immunisations carried out as previously described (Li *et al.*, 2006). Briefly, groups of 10 to 15 6-week old female BALB/c mice were immunised on days 1, 21 and 28 by intraperitoneal injection of 1×10^6 colony forming units of *N. meningitidis* MC58 grown in BHI medium containing 0.5% (v/v) iron dextran (Sigma-Aldrich). Control mice were given BHI medium with iron dextran alone. Blood was collected on day 35.

2.17 Whole-cell ELISA

Whole-cell enzyme-linked immunosorbent assays (ELISA) were carried out using bacteria from MC58 wild-type and Δ -*siaD* strains. Bacteria from liquid cultures were resuspended in PBS containing 0.05% (v/v) Tween-20 and then heat-inactivated at 56°C for 30 min. 96-well microtitre plates were coated with approximately 1.5×10^7 bacteria/well by overnight incubation at 37°C. Serial dilutions of control or immune sera in PBS containing 0.5% (w/v) BSA and 0.01% (v/v) Tween-20 were added to the wells and incubated at 37°C for 1 hr. Plates were washed with 0.025% (v/v) Tween-20 before and after the addition of sera. Binding of antibodies to the surface of bacteria was detected with a horseradish peroxidase goat anti-mouse IgG diluted 1:2,000. Following a final wash, 3,3',5,5' tetramethylbenzidine (Leinco Technologies, St Louis, Missouri) was added for color development and the reaction was stopped with the addition of 1 M sulphuric acid. Optical density readings were taken at 450 nm. The assay was repeated 3 times and the mean of the reciprocal of the

dilution of serum that gave readings 3 times higher than that of negative control sera was reported (Li *et al.*, 2006).

2.18 ELISA

Antibody levels in murine immune sera against OMV vaccine were measured as U/mL in microtiter plates. Wells were coated with 100 μ L per well of a reference 44/76 OMV preparation from a FM cultivation in a 50 L fermentor (5 μ g protein/mL) and developed with alkaline phosphatase anti-mouse IgG conjugate (Sigma-Aldrich) (Rosenqvist *et al.*, 1991).

2.19 Complement deposition assay (CDA)

Complement deposition assays were performed at the Centre for Emergency Preparedness and Response, Health Protection Agency, under supervision of Dr S. Taylor as described by Pajon *et al.* (Pajon *et al.*, 2009). Deposition of complement factors C3c and C5b-9 on the surface of meningococcal strains was assessed by flow cytometry analysis performed in U-bottom 96-well microtitre plates against strains shown in Table 2.1. IgG-depleted human plasma was used as complement source. Target bacteria grown on brain heart infusion agar plates were inactivated in 0.2% (w/v) sodium azide/PMSF and made into suspension with blocking buffer (1% (w/v) BSA in PBS) to an O.D. of 0.1 (600 nm). The cell suspension (90 μ L) was mixed with 5 μ L of test serum and 5 μ L of IgG-depleted human plasma followed by incubation for 30 min with shaking at room temperature. Cells were recovered from suspension by centrifugation at 3,000 x g for 5 min, washed twice with 200 μ L of blocking buffer and incubated for 20 min at 4°C with fluorescein isothiocyanate (FITC) labeled sheep anti-human C3c and C5b-9 antibodies diluted 1:500 in blocking buffer. Following

washing with blocking buffer, cells were analysed in a Beckman Coulter FC500 flow cytometer (Beckman Coulter Ltd, High Wycombe, UK). Horizontal gates in the FITC channel were set against a no-antibody control to include approximately 10% of the population. A total of 7,500 cells were measured for each sample. A fluorescence index (FI) was calculated by multiplying the percentage of cells showing FITC fluorescence in the appropriate gate (% gated) with the mean fluorescence of the gated population. The final FI-C reported values were calculated by subtracting FI of the FITC conjugate only control from the FI of each test serum sample. The assay was performed in triplicates for each test serum and control samples.

2.20 Opsonophagocytosis assay (OPA)

Opsonophagocytosis assays were performed at the Centre for Emergency Preparedness and Response, Health Protection Agency, under supervision of Dr Steven Taylor as described by Findlow *et al.* (Findlow *et al.*, 2006). Opsonophagocytic activity of test sera was assessed by flow cytometry performed in U-bottomed 96-well microtitre plates against strains shown in Table 2.1. Bacteria from target strains were prestained internally with 10 µg of 2', 7'-bis-(2-carboxyethyl)-5-(and -6)-carboxyfluorescein (BCECF) per mL, acetoxymethyl ester (Merck Chemicals Ltd, Nottingham, UK) and fixed with 0.2% (w/v) sodium azide for 48 hrs. Bacteria were then reconstituted in OP buffer made of Hanks balanced salts solution containing 2% Marvel skimmed milk powder (Premier International Foods, UK), 1.2 mM CaCl₂ and 1 mM MgSO₄. 10 µL of 6.25 x 10⁸ cells per mL were mixed with 20 µL of test sera diluted 1:10 in OP buffer and 10 µL of IgG-depleted human complement followed by incubation for 7.5 min at 37°C with shaking. 50 µL of HL60 phagocytic cells at 7.5 x 10⁷ cells per mL in OP buffer, differentiated with 0.8% (v/v)

DMF for 5 days prior to use, were added to the mixture followed by incubation for 7.5 min at 37°C. Flow cytometry analysis was carried out as described for complement deposition assay (section 2.19) measuring 7,500 HL60 cells.

2.21 Complement-mediated serum bactericidal assay (SBA)

The serum bactericidal assay was performed in 96-well microtitre plates against serogroup B meningococcal strains H44/76 and MC58 using baby rabbit complement. Test sera were diluted to 1:4 in bactericidal buffer made by dissolving 0.5 g BSA in Geys balanced salt solution and 20 µL were heat-inactivated at 56°C for 30 min and placed in wells. Freshly grown bacteria from blood agar plates were reconstituted in bactericidal buffer to an O.D. of 0.1 and 10 µL of a 1:2,500 dilution were deposited in each well. Following addition of 10 µL of active baby rabbit complement, 96-well plates were covered and incubated at 37°C for 1 hr. As a complement-independent control, heat-inactivated complement was used in wells containing test sera, whilst killing due to complement only was assessed by the addition of active complement to wells containing bactericidal buffer in the place of test sera. Wells containing heat-inactivated complement and bactericidal buffer were also included to assess the number of viable cells irrespective of killing (viable cells control). Following incubation, 10 µL from each well were plated out using the tilt method. Plates were incubated overnight at 37°C. Colonies were counted the next day using the Protocol Colony Counter (Synbiosis, Cambridge, UK). Bactericidal titres were expressed as the reciprocal of the final dilution giving $\geq 50\%$ killing compared to the viable cells control. The assay was considered successful when the percentage of killing measured for the other two controls (killing due to complement or sera) was

less than 30. For each sample, the assay was performed in duplicates and results from two assays run on different days were included in the calculations.

2.22 Cryo-electron microscopy

Cryo-electron microscopy was performed by Dr K. McLellan at NIBSC. Gold quantifoil grids (Agar Scientific, Stansted, UK) were plasma cleaned for 20 seconds in a partially shielded custom grid holder (E.A. Fischione Instruments, Inc., Surrey, UK) in a 1020 plasma cleaner (E.A. Fischione). Bacterial suspensions from MC58 Δ -*siaD* before and after treatment with proteinase K (20 μ g/mL per 10^9 cells) were adsorbed onto the grid for 2 min, by floating the grid onto a 50 μ L drop of the suspension. The grid was transferred into a Leica EM CPC (Leica, Vienna, Austria) plunge freezing device trying to transfer the maximal amount of the sample with the grid. The grid was blotted to remove excess liquid and was plunged into liquid ethane kept at -154°C. The grid was transferred into a Gatan 924 cryo holder (Gatan, Pleasanton, U.S.A) at a temperature below -170°C and was rapidly transferred into a pre-cooled JEM 2100 TEM (JEOL, Tokyo, Japan). Images were taken with a Gatan 4K/4K ultrascan CCD camera (Gatan) at a nominal magnification of 15,000 times.

2.23 Bioinformatic analysis

2.23.1 Sequence assembly and alignments

The sequences generated from the sequencing reactions of each of the cloned genes were assembled using the SeqMan tool of the LaserGene software v.8 (DNASTAR) and the consensus sequence inferred. Sequences were assessed for in-frame insertion of the cloned gene and presence of nucleotide substitutions by

comparison to the corresponding nucleotide sequences from the MC58 annotated genome.

Sequence alignments were carried out using the MegAlign tool of DNASTAR LaserGene 8 software. Sequences were imported in FASTA format and aligned using the ClustalW method. During the report of alignments, residues differing from or matching to the consensus were highlighted with colours. Missing residues were represented by a hyphen.

2.23.2 Analysis of synonymous and non-synonymous substitutions

Synonymous and non-synonymous substitutions in NmMIP sequences across 106 meningococcal isolates (Table A1, Appendix) were detected and their rates calculated using the Synonymous Non-synonymous Analysis Program (SNAP) available at the URL site <http://www.hiv.lanl.gov/content/sequence/SNAP/SNAP.html> (Korber B, 2000). A FASTA file of data from all sequences aligned was imported into SNAP and the number of synonymous versus non-synonymous substitutions for all pairwise sequence comparisons was calculated based on a method by Nei and Gojobori (Nei and Gojobori, 1986). These numbers were subsequently compared to the number of potential substitutions to infer the proportion of observed substitutions for each pairwise comparison. Following corrections for sites with multiple substitutions, the average of synonymous (ds) and non-synonymous (dn) substitutions from all pairwise comparisons was calculated. The dn/ds ratio was then used to infer the type of immune selection imposed to NmMIP. A dn/ds value of > 1 indicated positive selection, a value of 1 no selection and a value of < 1 negative selection pressure. Using SNAP the rates of synonymous and non-synonymous substitutions per codon

across all sequences aligned were also calculated. Codon-by-codon rates were used to assess the nature and distribution of changes in the amino acid sequence of NmMIP.

2.23.3 Phylogenetic analysis

The clonal structure of the nucleotide sequences from meningococcal isolates coding for NmMIP was inferred using ClonalFrame (Didelot and Falush, 2007). ClonalFrame is developed to assess clonal relationships between isolates of the same species that have been formed from point mutations and recombination events using data from single DNA fragments to whole genomes. Version 1.2 of ClonalFrame was downloaded from the URL site <http://www.xavierdidelot.xtremhost.com/clonalframe>. Aligned NmMIP nucleotide sequences from MegAlign were transformed into FASTA format and imported into ClonalFrame. ClonalFrame calculations were performed using default parameters. The output file was imported into the Graphical User Interface module of ClonalFrame and converted into a Newick file which was then visualised in the Molecular Evolutionary and Genetics Analysis (MEGA) software using the radial tree view. Version 5.0 of MEGA was downloaded from the URL site <http://www.megasoftware.net/> (Tamura *et al.*, 2011).

2.23.4 Topology prediction analysis

The bioinformatics tools used for *in silico* prediction of sequence features related with protein subcellular location included: PsortB v. 3.0 for prediction of subcellular protein location in Gram-negative bacteria (<http://www.psort.org/>) (Yu *et al.*, 2010), Lipop v. 1.0 for prediction of lipoprotein motifs (<http://www.cbs.dtu.dk/services/LipoP/>) (Juncker *et al.*, 2003), SignalP v. 3.0 for

prediction of signal peptide sequences (<http://www.cbs.dtu.dk/services/SignalP/>) (Bendtsen *et al.*, 2004) and TOPCONS for prediction of membrane protein topology (<http://topcons.net/>) (Bernsel *et al.*, 2009). The amino acid sequence of each protein to be tested was acquired in FASTA format from the Uniprot knowledgebase (<http://www.uniprot.org/>) and imported into the relevant prediction tool. Where applicable, the setting for Gram-negative bacteria was selected. In SignalP, both analysis methods of Hidden Markov model and Neural Networks were performed.

2.23.5 Construction of 3-D structural model

The 3-D structural view of NmMIP was constructed using SWISS-MODEL, available from URL site <http://swissmodel.expasy.org>. Structural construction was carried out in the automated mode of SWISS-MODEL using the amino acid sequence of mature NmMIP from MC58 and LpMIP as template. The pdb file generated was visualized in DeepView / Swiss PdbViewer software v. 4.01 (downloaded from <http://spdbv.vital-it.ch/>).

Chapter 3

**Characterisation of the surface proteome
of the serogroup B *N. meningitidis*
MC58 strain**

3. Characterisation of the surface proteome of the serogroup B *N. meningitidis* MC58 strain

3.1 Introduction

Where polysaccharide-based vaccines have been introduced against meningococcal strains expressing serogroup A, C W-135 and Y capsular polysaccharides, serogroup B isolates remain the predominant cause of bacterial meningitis and septicaemia (Tan *et al.*, 2010). The poor immunogenicity of serogroup B polysaccharide and the possibility that it is an autoantigen mimicking human glycan structures has focused attention on subcapsular proteins as alternative vaccines (Devi *et al.*, 1997; Finne *et al.*, 1987).

As for most bacterial pathogens, the bacterial surface of meningococci constitutes the main location of immunogenic proteins. Integral outer membrane proteins (OMPs) and lipoproteins can extend beyond the surface of the bacterium. As a result of their exposure, surface proteins can be recognised as antigens by the host's immune system triggering an adaptive immune response through the production of antibody producing B cells (Rijkers *et al.*, 1998; Vos *et al.*, 2000). This acquired immunity is mimicked by vaccines containing surface proteins as their antigenic components. Any strategy that can specifically identify the surface proteome would greatly contribute towards the discovery of potential vaccine candidates.

Several proteomic strategies have attracted attention as effective ways for the identification of bacterial surface proteins (Cordwell, 2006). The aim of these strategies is to specifically isolate those proteins that are part of and/or extend beyond the outer membrane and identify them by MS analysis. They can broadly be divided into two groups: either those that chemically modify proteins on the surface to facilitate their purification from the rest of the proteome, or those that use non-

modifying reagents to extract surface proteins. Compared to genome-wide analysis (Pizza *et al.*, 2000), often described by the term reverse vaccinology, these approaches can save considerable experimental time on downstream immunological evaluation assays by narrowing down analysis to a subset of proteins with vaccine potential. They also make no pre-assumptions as to which proteins might be present on the bacterial surface.

Prior to the current study, proteomics has been used to characterise meningococcal OMVs as part of the quality assessment of OMV vaccines (Uli *et al.*, 2006; Vipond *et al.*, 2006). Outer membrane vesicles are rich in OMPs and have been considered as a suitable sample for identification of potential surface protein antigens. However, the process employed for extraction of OMVs from whole cells can affect OMV protein composition. When extraction involves the use of detergents, cytosolic proteins can contaminate the sample. Therefore, the presence of a protein in an OMV preparation is no guarantee that it is surface-expressed or immunogenic.

On the basis that cell surface antigens are accessible to proteases, treatment of bacteria with proteases provides a means of analysing the bacterial surface proteome. Whole bacteria are incubated with a specific protease (*e.g.* trypsin) and the peptides released from digestion of surface-exposed proteins are recovered in the incubation buffer (supernatant) and analysed by MS (Figure 3.1B) (Bledi *et al.*, 2003). This type of analysis has been used successfully for the identification of potentially protective antigens from streptococci (Doro *et al.*, 2009; Rodriguez-Ortega *et al.*, 2006). However, the specificity of the method largely depends on retaining the integrity of the cells and the treatment conditions (Dreisbach *et al.*, 2010; Tjalsma *et al.*, 2008). Contamination of the supernatant with cytoplasmic components is common,

especially in the case of Gram negative bacteria (Doro *et al.*, 2009; Walters and Mobley, 2009).

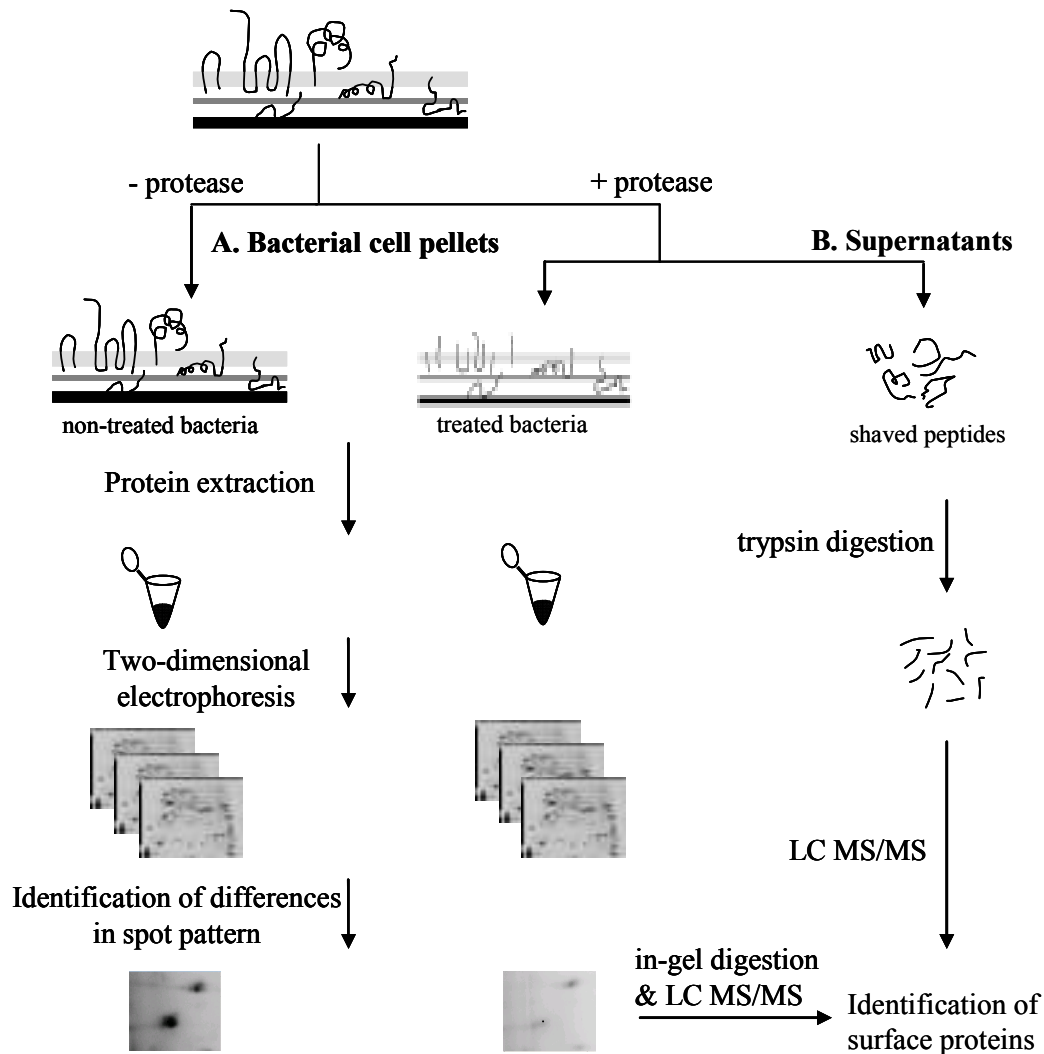


Figure 3.1 Bacterial treatment with proteases: analysis strategies for identification of surface proteins. A) Whole-cell protein lysates from bacteria incubated with or without proteinase K are collected and subjected to 2D-PAGE. Images of 2-D gels are compared to detect gel spots disappearing from treated samples as a result of cleavage of surface proteins corresponding to these spots. Proteinase K-affected protein spots are then excised from 2-D gels of untreated samples, digested with trypsin and identified by LC-MS/MS. B) Bacteria are incubated with trypsin and the peptide fragments released from digested surface proteins are recovered in the supernatant. Peptide fragments are subjected to an additional step of trypsin treatment for completion of digestion and analysed by LC-MS/MS for identification of the digested proteins.

In a different strategy that could potentially overcome contamination issues following analysis of supernatants, Sabarth *et al.* compared the proteomes of whole bacteria from *Helicobacter pylori* treated with a less specific protease, such as proteinase K, with untreated bacteria by 2D-PAGE (Figure 3.1A) (Sabarth *et al.*, 2005). Spots disappearing from 2-D gels as a result of digestion of surface proteins were detected and proteins identified by MS.

This chapter describes the application of protease treatment for the identification of proteins on the surface of the serogroup B MC58 meningococcal strain. Comparison of whole-cell proteomes by 2D-PAGE was used as the main strategy for detection of the digested surface proteins. Various additions to the original method are described including the adjustment of proteinase K concentration and the use of DIGE to quantify differences in the abundance of protein spots between treated and untreated samples on 2-D gels. In addition, the peptides released in the incubation buffer upon treatment of MC58 with trypsin were identified by LC-MS/MS analysis.

3.2 Analysis of the effect of proteinase K treatment on the MC58 proteome

The main strategy for the identification of proteins on the surface of the meningococcus was to treat bacteria with proteinase K and analyse the effect on the whole-cell proteome. As a first step, the effect of treatment on the total protein content and abundance of specific proteins was assessed by SDS-PAGE and western blotting and optimal conditions for specific cleavage of surface proteins were established. Subsequent proteomic analysis included: a) quantitative comparison of the proteome of treated and untreated meningococci by DIGE for the detection of gel

spots with changes in abundance and b) mass spectrometric analysis of the gel spots for protein identification.

3.2.1 SDS-PAGE and western blotting

The capsule-deficient derivative of MC58 was incubated with proteinase K at concentrations of 1.25, 2.5, 5, 10, 20, 40 and 80 $\mu\text{g/mL}$ per 10^9 bacteria as described in section 2.3. An untreated control sample from bacteria incubated without addition of proteinase K was also prepared. Whole-cell protein extracts were separated on 12% acrylamide gels and visualised by fluorescent staining using Sypro Ruby. Similar total protein profiles were observed between the untreated sample and those treated with concentrations of up to 20 $\mu\text{g/mL}$. For concentrations of 40 $\mu\text{g/mL}$ or higher, extensive loss of proteins of high molecular weight was observed (Figure 3.2).

The effect of treatment on the abundance of specific proteins was assessed following transfer of protein extracts separated by SDS-PAGE to PVDF membranes and blotting with appropriate antibodies (Figure 3.3). The outer membrane porin PorA was used as marker for surface-exposed proteins. PorB, an outer membrane porin which is protease-resistant in live, intact cells (Minetti *et al.*, 1998), and two autofluorescent proteins present in blots (one identified as the predicted inner membrane-associated protein cytochrome c5, data presented in Table A2, Appendix) were used to assess cell integrity. Complete cleavage of PorA was detected following incubation at proteinase K concentrations as low as 5 $\mu\text{g/mL}$. In contrast, PorB and the autofluorescent proteins remained unaffected by concentrations of up to 20 $\mu\text{g/mL}$. Consistent with results from SDS-PAGE, some cleavage of the PorB and autofluorescent proteins was observed at the two highest concentrations tested. When lysed meningococci were subjected to proteinase K treatment, the same proteins were

shown to be completely digested indicating they were not resistant to proteolytic cleavage under the conditions used (Figure 3.3, right panel).

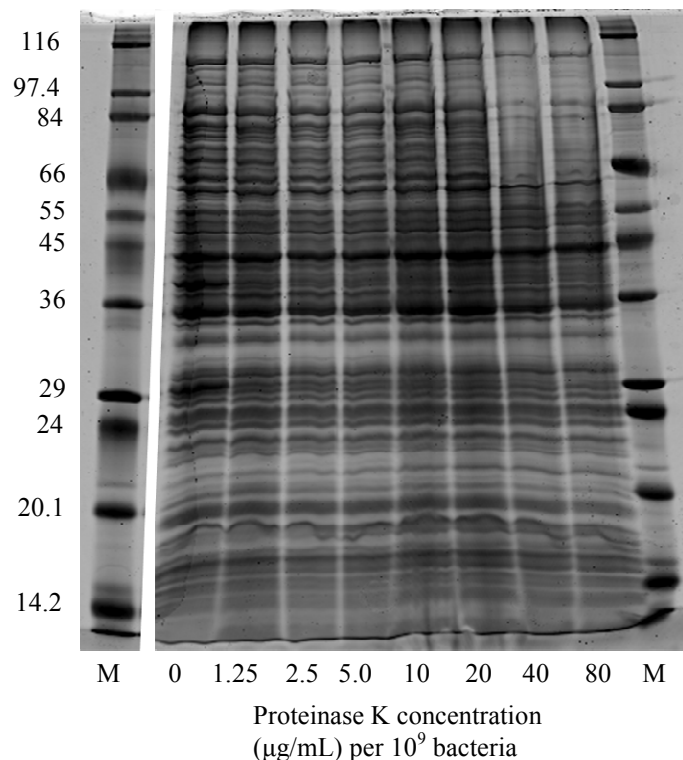


Figure 3.2 SDS-PAGE of protein extracts from meningococci treated with various concentrations of proteinase K. Protein extracts collected from bacteria were incubated with proteinase K at concentrations ranging from 0 to 80 $\mu\text{g/mL}$ (per 10^9 bacteria) and separated on a 12% polyacrylamide gel. 25 μg of protein were loaded on each lane, separated by electrophoresis for 4 hr and stained with Sypro Ruby fluorescent stain. Horizontal numbers correspond to proteinase K concentrations. M: Wide-range MW reference protein marker (SigmaMarker™, Sigma-Aldrich). The MW (in kDa) of bands of the protein marker is shown on the left.

To further assess cell integrity during protease treatment, bacteria treated with 20 $\mu\text{g/mL}$ of proteinase K were examined by cryo-electron microscopy and images were compared to those obtained from untreated bacteria. A representative image of a diplococcus before and after treatment is shown in Figure 3.4. There were no differences in the morphology and integrity of cells between treated and untreated bacteria. From images acquired from both samples, a limited number of dead cells

was detected. However, the proportion of dead cells could not be accurately calculated and compared between the two samples owing to the tendency of meningococci to clump.

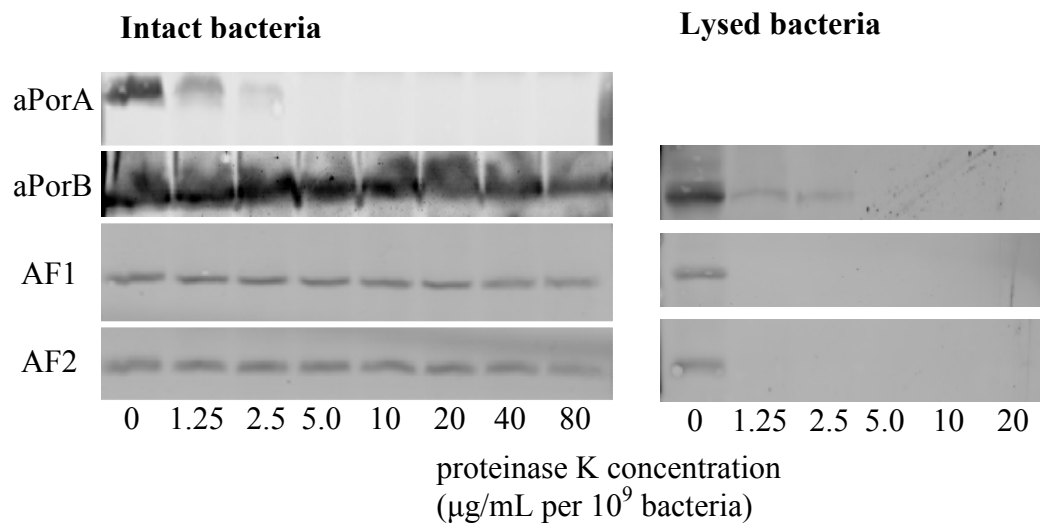


Figure 3.3 Effect of proteinase K treatment on specific proteins. Protein extracts from intact (left) or lysed (right) meningococci treated with various concentrations of proteinase K were separated on 12% polyacrylamide gels, transferred to PVDF membranes and either scanned directly for detection of autofluorescent proteins AF1 and AF2 or blotted with anti-PorA or anti-PorB monoclonal antibodies to detect PorA and PorB. A Cy5-conjugated goat anti-mouse IgG was used for detection of antibody binding. AF2 was identified as cytochrome c5 (Table A2, Appendix).

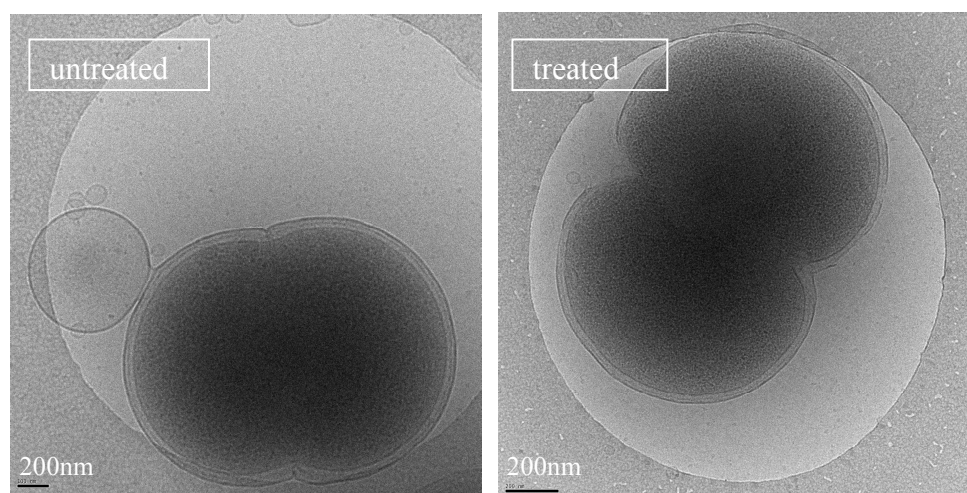


Figure 3.4 Cryo-electron microscopy of meningococci. Bacteria before (left) and after (right) digestion with 20 µg/mL proteinase K.

3.2.2 DIGE

Although used to assess total protein content from proteinase K-treated meningococci, SDS-PAGE lacks the resolving power to separate proteomes enough to allow for differences in protein abundance upon treatment to be detected. Two-dimensional DIGE was instead used which combines high resolution of protein separation with fluorescence labelling technology for the quantitative comparison of proteomes. In 2-D gels, protein spots representing the intact form of surface proteins might be expected to show a decrease in abundance or disappear from gels of treated samples. Also, new spots corresponding to protein fragments retained in the cell wall upon treatment could potentially be formed and detected by a marked increase in abundance.

Figure 3.5 summarises the DIGE analysis of proteinase K-treated meningococci. Aliquots of approximately 12×10^9 bacteria were incubated with proteinase K at concentrations ranging from 0 to 20 $\mu\text{g/mL}$. A total of 3 samples per proteinase K concentration were generated from 3 bacterial liquid cultures. Protein lysates from whole cells from each sample were labeled with IC5 fluorescent dye, mixed with IC3-labeled internal standard and separated on 2-D gels. A total of 18 2-D gels were run and generated 18 sample images, each accompanied by an internal standard image. Representative images of 2-D separation of meningococcal proteomes from treated and untreated bacteria are shown in Figure 3.6.

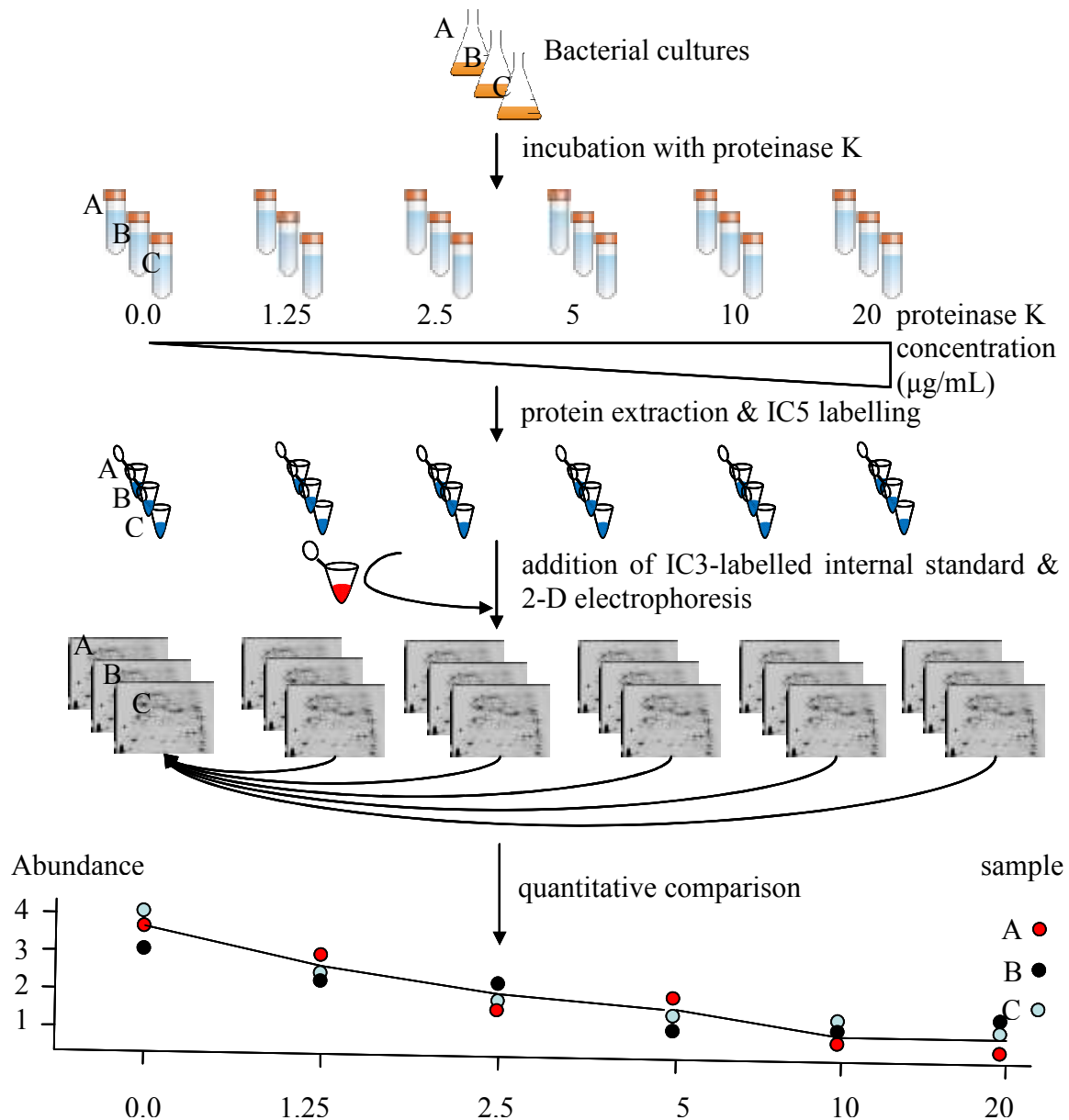


Figure 3.5 DIGE analysis of whole-cell lysates from meningococci treated with increasing concentrations of proteinase K. Aliquots from each of three bacterial cultures were incubated with proteinase K, each at a different concentration. The concentrations used were 1.25, 2.5, 5, 10 and 20 µg/mL per 10^9 bacteria. Untreated samples (0.0) were also prepared from bacterial cultures. A total of three samples per concentration were generated. Protein lysates from each sample were collected, labelled with IC5 fluorescent dye, mixed with equal amount of an IC3-labelled internal standard made up of all samples prepared and subjected to 2D-PAGE. Images from treated samples were grouped and compared to the untreated group for identification of protein spots with a significant change in abundance across all treated groups.

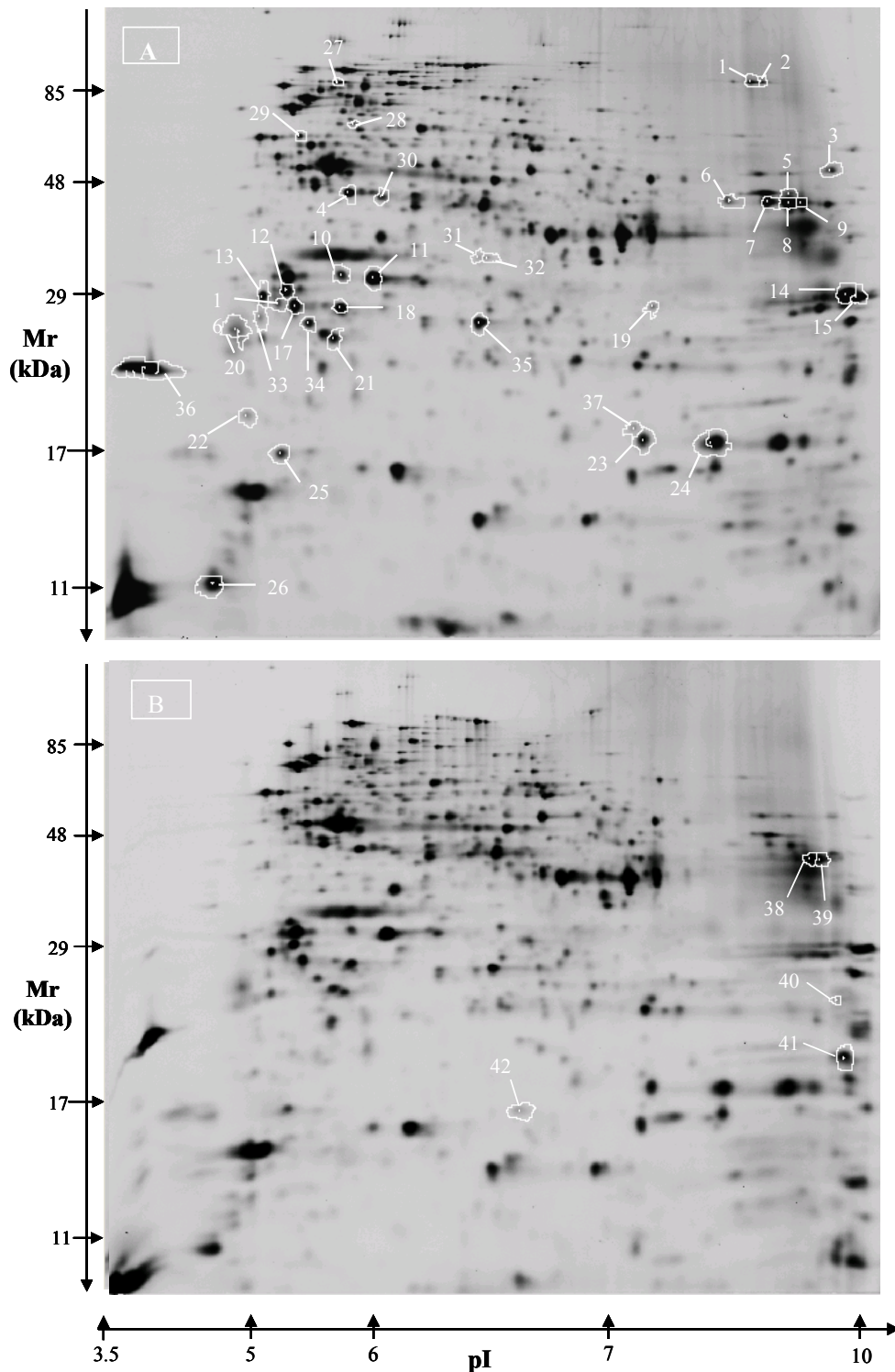


Figure 3.6 Representative images of 2D-PAGE of proteins from *N. meningitidis* MC58 Δ -siaD. Proteins from untreated meningococci (A) and meningococci treated with proteinase K (B) were separated by pI using IEF of IPG strips, pH 3-11 and subsequently by MW in 12% polyacrylamide gels. The spots exhibiting decreased abundance as a result of proteinase K treatment of meningococci are highlighted in (A) (numbers 1-37). New spots are highlighted in (B) (numbers 38-42). Quantitation was carried out using DeCyder differential analysis software and spots with consistent difference in abundance between untreated and treated samples are reported (P-value < 0.05).

Images were imported into DeCyder differential analysis software and grouped into 6 groups according to the proteinase K concentrations used during treatment. Five hundred and forty one protein spots were matched and their fluorescence intensities compared across the different sample groups. The number of spots with a significant decrease (P-value < 0.05) in each treated group compared to the untreated group are presented in Table 3.1.

Table 3.1 Total number of spots showing changes in abundance upon treatment of *N. meningitidis* Δ -*siaD* strain with proteinase K. Fluorescence intensities measured for each spot in each treated group were compared against those from the untreated group. Spots with a significant decrease or increase in abundance (P-value < 0.05) were chosen for further analysis.

	Proteinase K concentration ($\mu\text{g/mL}$ per 10^9 bacteria)				
	1.25	2.5	5	10	20
Decreased	53	56	52	45	55
Increased	13	12	14	10	8

Of the protein spots with an observed decrease in abundance in 1 or more treated groups, 37 were consistent across all treated groups, indicating the proteolytic cleavage of the corresponding intact proteins. Figure 3.6A indicates the position of these spots on the 2-D gel image. Proteinase K-affected protein spots were found in both the acidic and basic regions of the gels and varied in MW. The exact values of decrease for each protein spot across treated groups are presented in the Appendix (Table A3, spots 1-37). Values of decrease were expressed as the average ratio of the calculated standardised abundance in each treated group against that from the untreated group. Values of decrease in abundance varied from very high (30-fold) to very low (1.2-fold or 20%) across protein spots indicating different degrees of

cleavage. Spots 1, 2, 3, 5, 6, 7, 8, 9, 14 and 19 exhibited the greatest decrease (> 2.5 -fold). For the rest of the proteinase K-affected spots, the decrease was generally between 1.5 and 2-fold. Plots of the standardized abundance against proteinase K concentration were generated for each of the 37 spots. These plots are presented collectively in the Appendix (Figure A2) and as separate plots in section 3.2.4 in relation to the results from protein identification. In general, plots indicated that different proteins decreased at different proteinase K concentrations and to a different extent.

Five new spots with consistently increased abundance across treated samples were also detected corresponding to fragments of proteins digested by proteinase K. Their position on 2-D gels is indicated in Figure 3.6B and exact values of increase across sample groups are presented in the Appendix (Table A3, spots 38-42). Spots 39 and 41 exhibited the highest increase which gradually declined with increasing concentrations of proteinase K. In contrast, the increase in abundance of spot 42 followed the increase in proteinase K concentration.

3.2.3 Identification of proteinase K-affected proteins by LC MS/MS

Twenty-six of the protein spots with decreased abundance and the 5 new spots detected in gels upon proteinase K treatment were analysed by LC-MS/MS. Preparative gels from untreated samples were used to pick spots of decreased abundance. Conversely, new spots formed were picked from gels of treated samples. For each spot, 2 samples from 2 independent gels were prepared and analysed and only proteins reproducibly identified in both samples are reported. A total of 24 proteins were unambiguously identified and are presented in Table 3.2 together with information on the spots of origin and MS analysis.

Table 3.2 Proteins affected by proteinase K treatment of bacteria from *N. meningitidis* MC58 Δ -*siaD* strain. Proteins were identified following LC-MS/MS of 2-D gel spots showing consistent, significant change in abundance in treated samples compared to untreated samples. Quantitative analysis of 2-D gel images was carried out using DeCyder differential analysis software. Proteins identified by 3 or more unique peptides passing the Xcorr vs charge state threshold of Sequest search engine are reported.

Gel spot number ^a	Protein name	Accession no ^b	NMB ^c	pI / MW (kDa) ^d	No of peptides sequenced
<i>Spots of decreased abundance</i>					
1	Omp85	Q9K1H0	0182	8.37 / 86.3	18
2	Omp85	Q9K1H0	0182	8.37 / 86.3	16
3	FadL-like protein	Q9K1M2	0088	9.28 / 48.1	14
4	Spermidine/putrescine ABC transporter	Q9K0U8	0462	6.92 / 50.6	12
	Succinyl-CoA ligase [ADP-forming] subunit beta	Q9JZP4	0959	5.06 / 41.3	9
	Protein recA	P56988	1445	5.18 / 37.6	3
	PorA	Q51240	1429	8.73 / 40.1	4
6	PorA	Q51240	1429	8.73 / 40.1	9
7	PorA	Q51240	1429	8.73 / 40.1	17
	UbiH family protein	Q9K157	0323	8.34 / 41.7	4
8	PorA	Q51240	1429	8.73 / 40.1	21
9	PorA	Q51240	1429	8.73 / 40.1	17
10	Probable FKBP-type peptidyl-prolyl cis-trans isomerase (macrophage infectivity potentiator-NmMIP)	Q9JY18	1567	5.5 / 26.9	8
11	Probable FKBP-type peptidyl-prolyl cis-trans isomerase (macrophage infectivity	Q9JY18	1567	5.5 / 26.9	11

Gel spot number ^a	Protein name	Accession no ^b	NMB ^c	pI / MW (kDa) ^d	No of peptides sequenced
	potentiator-NmMIP)				
12	Outer membrane lipoprotein	Q7DD63	1946	5.01 / 29.1	13
	Amino acid ABC transporter, periplasmic amino-acid binding protein	Q9JYF0	1612	4.87 / 26.6	8
13	Thiazole synthase	Q9JXF5	2071	4.6 / 28.1	3
	Arginine biosynthesis bifunctional protein ArgJ	P63574	2005	5.67 / 42.8	3
14	OpcA	Q7DDI3	1053	9.68 / 28.1	15
15	OpcA	Q7DDI3	1053	9.68 / 28.1	18
16	Amino acid ABC transporter, periplasmic amino-acid binding protein	Q9JYF0	1612	4.87 / 26.6	8
17	Thiol-disulfide interchange protein DsbA2	Q7DDR6	0294	5.1 / 23.4	10
18	Amino acid ABC transporter, periplasmic amino-acid binding protein	Q9K035	0787	5.42 / 27.1	16
19	Factor H-binding protein	Q9JXV4	1870	8.94 / 34.3	11
20	Outer membrane protein H.8	P57026	1533	4.61 / 16.9	4
21	PilO	Q7DD78	1810	4.74 / 19.9	10
	Putative uncharacterized protein	Q7DDP8	0478	4.99 / 23.6	3
22	PilP	Q7DD77	1811	4.76 / 18.0	4
23	PilE	P05431	0018	9.21 / 15.3	3
	PilE	P05431	0018	9.21 / 15.3	3
24	30S ribosomal protein S5	P66577	0159	10.04 / 18.3	4
25	Bacterioferritin A	P0A0R1	1207	4.73 / 17.9	4
	Putative uncharacterized protein	Q9JYR2	1468	4.38 / 9.3	5
26	Outer membrane protein H.8	P57026	1533	4.61 / 16.9	3

Gel spot number ^a	Protein name	Accession no ^b	NMB ^c	pI / MW (kDa) ^d	No of peptides sequenced
<i>New spots</i>					
38	PorA	Q51240	1429	8.73 / 40.1	25
39	PorA	Q51240	1429	8.73 / 40.1	22
40	PorA	Q51240	1429	8.73 / 40.1	9
41	OpcA	Q7DDI3	1053	9.68 / 28.1	6
42	PorA	Q51240	1429	8.73 / 40.1	4

^a Spot numbers as they appear in Figure 3.6.

^b Accession numbers were derived from UniProtKB/Swiss-Prot database.

^c Gene locus numbers (NMB) are based on the annotated MC58 genome sequence.

^d Theoretical pI and molecular weight values were calculated using ProtParam tool (www.expasy.ch).

Of the identified proteins, PorA, Omp85, pilin Pile, the amino-acid ABC transporter NMB1612 and NmMIP were detected in multiple spots of similar MW and different pI. A single protein was identified in each of 20 spots. With the exception of H.8 and fHbp, the position of these spots on the gel was consistent with the predicted pI and MW of the corresponding proteins identified by MS. The H.8 protein (spot 20) migrated with a higher MW than calculated from its amino acid sequence. Conversely, the fHbp (spot 19) migrated with a lower than expected MW.

Two or more proteins were identified in each of 6 spots. Specifically, spots 7, 21, 24 and 26 contained 2 and spots 4 and 13, 3 co-migrating proteins. For these spots, differences were observed in the number of peptides matched to each protein. These differences are likely to be associated with differences in protein abundance on the spot. In general, the increased amount of a protein increases the chances of identifying more peptides. PorA, ABC transporters NMB0462 and NMB1612 and PilO were identified by the highest number of peptides in spots 7, 4, 13 and 21, respectively, and were probably the most abundant proteins in these spots. In contrast, proteins H.8 and NMB1468 identified in spot 24 and Pile and 30S ribosomal protein S5 identified in spot 26 were represented by similar amounts.

3.2.4 Detailed DIGE results of identified proteins

As mentioned in section 3.2.2, the decrease in abundance resulting from proteinase K treatment varied between proteins. How this related to the extent of proteolytic cleavage is presented in Table 3.3, which shows a 3-D view of the trace of each affected protein spot before and after treatment, in relation to the calculated fold decrease values. Spots exhibiting a decrease in abundance greater than 3.5-fold were shown to almost disappear from the gels, an indication of almost complete proteolytic

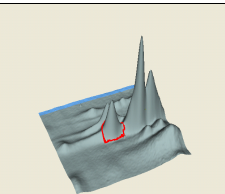
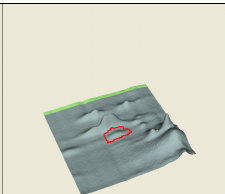
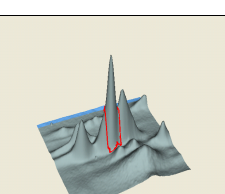
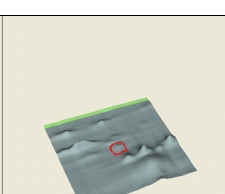
cleavage. In contrast, spots with low decrease values, falling below 2.6-fold, corresponded to partially cleaved proteins.

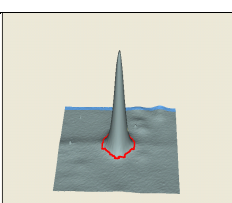
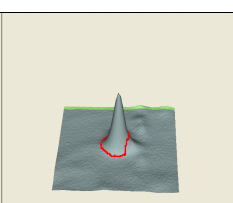
Proteins that were completely cleaved included PorA (spots 5, 6, 7, 8, and 9), OpcA (spot 14) and fHbp (spot 19). Based on the abundance profiles (Figure 3.7), the amount of proteinase K needed for the maximum cleavage to be achieved varied and could be linked to the expression level of each protein (as inferred by the size of the spot as seen in Figure 3.6). Highly abundant PorA and OpcA required proteinase K concentrations of at least 5 µg/mL to be completely digested whilst fHbp, which is expressed at lower levels was completely digested by 1.25 µg/mL.

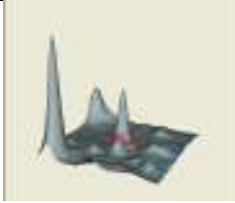
Of the proteins that were only partially cleaved, Omp85 (spots 1 and 2), fadL-like NMB0088 (spot 3) and OpcA spot 15 decreased the most. Their abundance profiles also showed gradual decrease with increasing proteinase K concentration. The remainder, including NMB1946, H.8, NmMIP, NMB1468, PilP, PilO, ABC transporters NMB0462, NMB1612 and NMB0787 and thiol-disulfide interchange protein DsbA2, decreased less and there was no correlation with their relative levels of expression. These proteins had similar abundance profiles that did not alter significantly upon increase in the concentration of proteinase K (Figure 3.7).

Total cleavage was observed for all spots identified as PorA but the values of decrease in abundance differed. Specifically, PorA spots 8, 9, 7 and 6 exhibited a decrease of up to 30.72, 13.72, 12.36 and 3.69-fold, respectively. More marked decreases might also be associated with higher expression levels as inferred by spot size (Figure 3.6). Similarly, OpcA spots 14 and 15 had markedly different fold decrease values (14.37 and 2.37, respectively), which might correspond to different degrees of cleavage since the size of the 2 spots was similar.

Table 3.3 Extent of proteolytic cleavage of surface proteins. The degree of cleavage of the proteinase K-digested surface proteins was assessed by analysis of the 3-D views of the corresponding gel spots from untreated and treated samples. Three-dimensional spot views were generated using DeCyder software. The highest fold-values of decrease measured per spot are also shown.

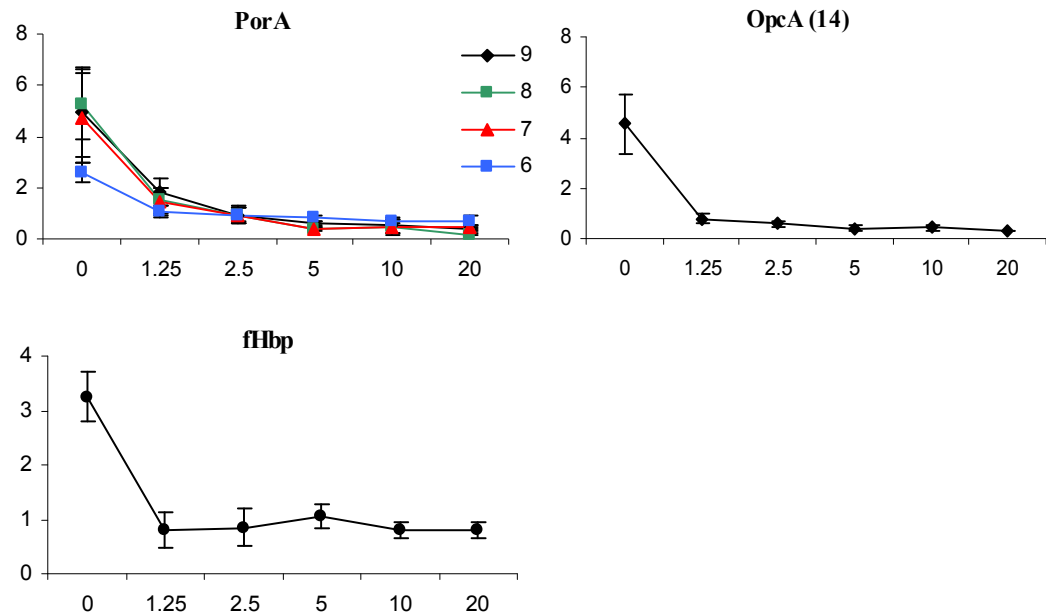
Protein name	Spot number	Fold-decrease	3-D spot views (untreated – treated)	
<i>Completely cleaved proteins</i>				
PorA	6	3.69		
	7	12.36		
	8	30.72		
	9	13.72		
OpcA	14	14.37		
fHbp	19	4.02		

Protein name	Spot number	Fold-decrease	3-D spot views (untreated – treated)	
<i>Partially cleaved proteins</i>				
Omp85	1	2.31		
	2	2.61		
OpcA	15	2.37		
fadL-like NMB0088	3	2.3		
NmMIP	11	1.91		
	10	1.41		
NMB1468	26	1.89		

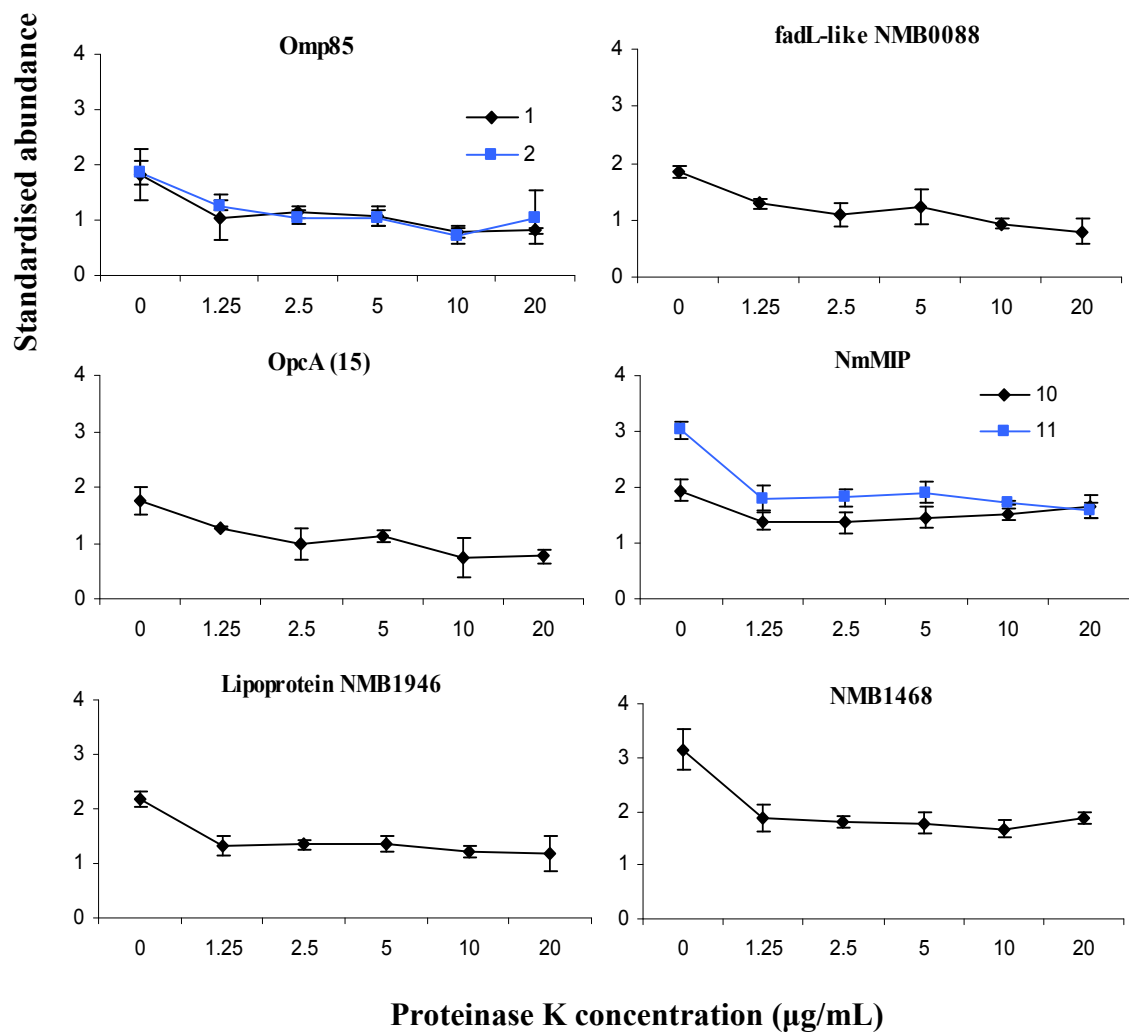
Protein name	Spot number	Fold-decrease	3-D spot views (untreated – treated)	
<i>Partially cleaved proteins</i>				
DsbA2	17	1.95		
Lipoprotein NMB1946	12	1.85		
NMB1612	13	1.67		
	16	2.17		
NMB0787	18	1.52		
H.8	20	1.59		
PilP	22	1.57		

Protein name	Spot number	Fold-decrease	3-D spot views (untreated – treated)	
<i>Partially cleaved proteins</i>				
PilO	21	1.58		
Spermidine / putrescine ABC transporter	4	1.6		
Bacterioferritin A	25	1.74		
PilE	23	1.23		
	24	1.2		

Completely cleaved proteins



Partially cleaved proteins



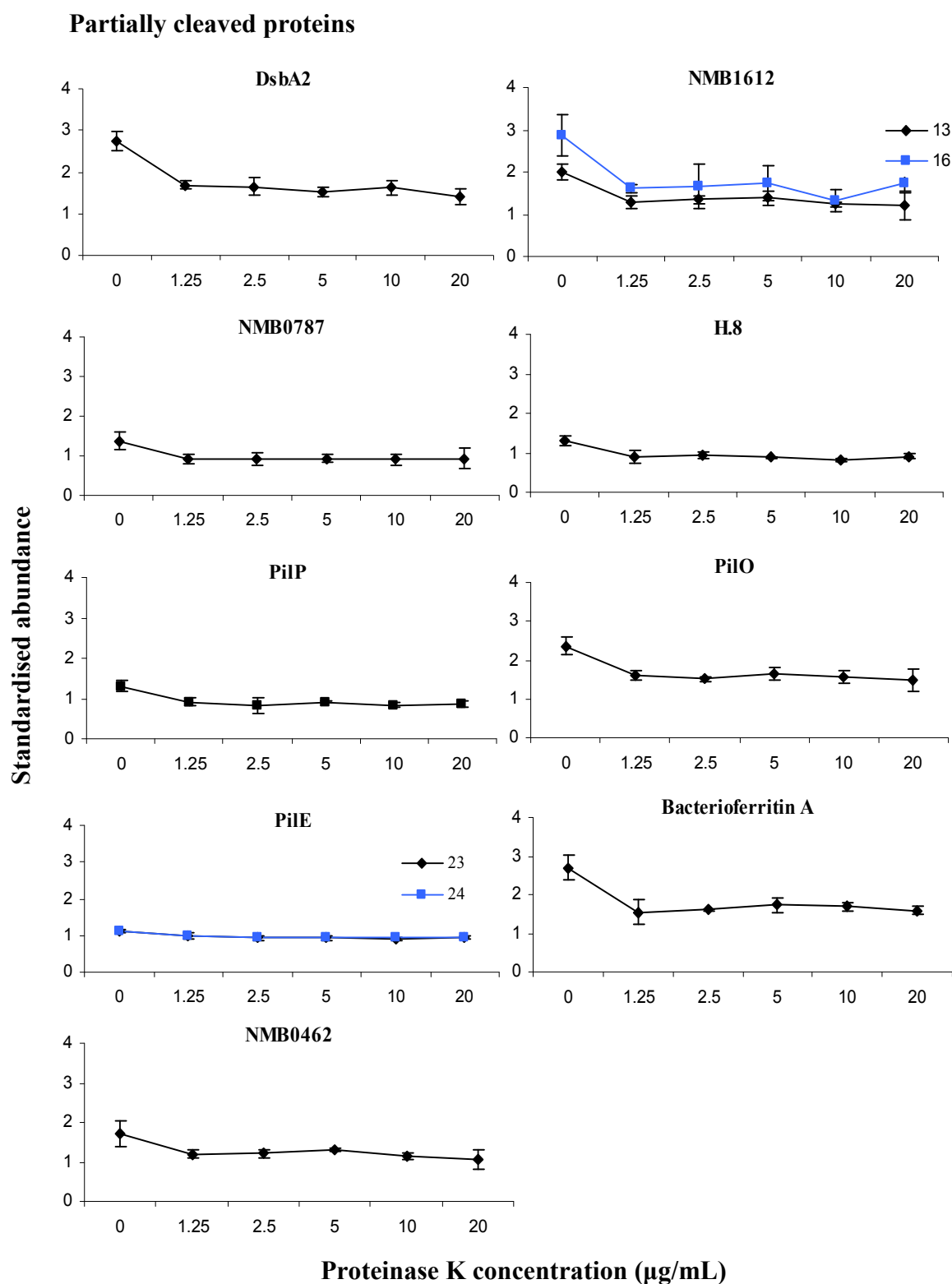


Figure 3.7 DIGE abundance profiles of identified proteins. Mean abundance values calculated for each protein spot using DeCyder software were plotted against the concentration of proteinase K used during treatment of bacteria. Error bars show the standard deviation of the values calculated from triplicates for each concentration. Spot numbers correspond to those in Figure 3.6.

3.2.5 Prediction of cellular location of proteins cleaved by proteinase K

Proteins digested by proteinase K were sorted according to their predicted location in the meningococcal cell (Table 3.4). Predictions for proteins of the outer and inner membrane, cytoplasm, periplasm and extracellular space (secreted) were based on PsortB algorithm (Yu *et al.*, 2010). LipoP, which is not part of PsortB analysis, was used to detect proteins with a lipoprotein motif recognized by signal peptidase II (grouped as lipoproteins in Table 3.4) (Juncker *et al.*, 2003).

Four outer membrane and 9 lipoprotein-predicted proteins were identified in a total of 24 proteins. Outer membrane proteins included PorA, OpcA, Omp85 and FadL-like protein NMB0088. Among predicted lipoproteins were factor H-binding protein, NmMIP, amino acid-binding ABC transporter proteins encoded by NMB0787 and NMB1612, H.8, PilP, thiol-disulfide interchange protein DsbA2, and proteins encoded by NMB1946 and NMB1468. PileE was predicted to be secreted into the extracellular space, PilO to be part of the inner membrane and the spermidine/putrescine ABC transporter protein encoded by NMB0462 to reside in the periplasmic space. Proteins RecA, bacterioferritin A, succinyl-CoA ligase [ADP-forming] subunit beta, thiazole synthase, 30S ribosomal protein S5 and arginine biosynthesis bifunctional protein ArgJ were predicted to function in the cytoplasm. Finally, no conclusion could be made for the cellular location of 2 proteins, namely UbiH family protein and the protein encoded by NMB0478. UbiH was found to contain a signal peptide sequence suggesting it is not retained in the cytoplasm. No particular features regarding the subcellular location of protein NMB0478 were predicted.

Table 3.4 Prediction of cellular location of proteins cleaved by proteinase K. Proteins were classified into outer membrane, extracellular, inner membrane, periplasmic and cytoplasmic based on PsortB algorithm. Lipoproteins were predicted using LipoP algorithm. The location of proteins for which no conclusive prediction could be made was assigned as unknown.

Cellular location	Protein name
<i>Outer membrane</i>	PorA OpcA Omp85 FadL-like protein NMB0088
<i>Extracellular</i>	PilE
<i>Lipoproteins</i>	Outer membrane lipoprotein NMB1946 Factor H binding protein Probable FKBP-type peptidyl-prolyl cis-trans isomerase (macrophage infectivity potentiator) Thiol-disulfide interchange protein DsbA2 Amino acid ABC transporter, periplasmic amino-acid binding protein NMB0787 Putative uncharacterized protein NMB1468 PilP Amino acid ABC transporter, periplasmic amino-acid binding protein NMB1612 Outer membrane protein H.8
<i>Inner membrane</i>	PilO
<i>Periplasmic</i>	Spermidine/putrescine ABC transporter NMB0462
<i>Unknown</i>	Putative uncharacterized protein NMB0478 UbiH family protein
<i>Cytoplasmic</i>	Succinyl-CoA ligase [ADP-forming] subunit beta Protein recA Bacterioferritin A 30S ribosomal protein S5 Arginine biosynthesis bifunctional protein ArgJ Thiazole synthase

3.3 Comparison of the expression levels of proteins digested by proteinase K between wild-type and Δ -siaD MC58 strains.

Protein expression levels can be critical for binding of antibodies on the bacterial surface and hence for the protective effect and vaccine potential of an antigen. Here, the proteins of the capsule-deficient Δ -siaD MC58 digested by proteinase K were investigated for their presence and relative expression levels in the wild type homologous strain. The whole-cell proteomes of Δ -siaD and wild type MC58 were compared by DIGE using the experimental design presented in Table 3.5. Triplicates of whole-cell lysates from the different strains were prepared and labelled with Cy5. An internal standard made up of the samples to be analysed was labelled with Cy3 and mixed with each of the Cy5-labelled samples prior to 2D-PAGE. A total of 6 2-D gels were prepared and the images imported in DeCyder for spot matching and relative quantitation. The exact position of protein spots digested by proteinase K in images from this DIGE experiment was confirmed by matching to the master image from the DIGE analysis of surface proteome (shown in Figure 3.6).

Table 3.5 Experimental design of DIGE comparison of proteomes from wild type and Δ -siaD MC58. Different batches were prepared from independent cell cultures of the corresponding strain. The internal standard was made from pooling equal amounts of each of the 6 samples compared.

Gel No	Cy3 label (50 μ g)	Cy5 label (50 μ g)
1	Pooled internal standard	wild type batch 1
2	Pooled internal standard	wild type batch 2
3	Pooled internal standard	wild type batch 3
4	Pooled internal standard	Δ -siaD batch 1
5	Pooled internal standard	Δ -siaD batch 2
6	Pooled internal standard	Δ -siaD batch 3

Table 3.6 lists the proteins digested by proteinase K exhibiting a significant difference in expression levels between Δ -*siaD* and wild-type MC58 strains (P-value < 0.05). Factor H-binding protein and a spot corresponding to Omp85 showed decreased expression in Δ -*siaD* MC58. In contrast, amino acid binding protein NMB0787, OpcA, one PorA and one Omp85 spot exhibited decreased expression in wild-type MC58. The remaining proteins digested by proteinase K were expressed at similar levels in the two strains.

Table 3.6 Proteins digested by proteinase K with significantly altered expression in Δ -*siaD* compared to wild-type MC58 (P-value < 0.05, n=3).

Protein name	Spot No. ^a	Fold difference	P-value
<i>Decreased expression in Δ-<i>siaD</i></i>			
Omp85	1	-1.68	0.0018
Factor H-binding protein	19	-1.75	0.0017
<i>Increased expression in Δ-<i>siaD</i></i>			
Omp85	2	4.34	2.6e-04
PorA	9	6.85	2.3e-04
OpcA	14	1.55	0.037
OpcA	15	1.5	0.044
Amino acid binding protein NMB0787	18	2.5	0.0019

^a As in Figure 3.6.

3.4 Analysis of peptides recovered in the supernatant following treatment of meningococci with trypsin

As a complementary strategy to identify the proteins digested upon treatment of whole meningococci with proteases, the peptides recovered in the incubation buffer

(cell-free supernatant) were directly analysed by LC MS/MS. Trypsin was used in place of proteinase K to facilitate generation of peptides that favour identification by MS. Same treatment conditions to those of proteinase K treatment were used and trypsin concentrations ranged from 1.25 to 20 µg/mL. Triplicate samples from 3 independent bacterial cultures were generated for each trypsin concentration used. Upon collection, supernatants were subjected to a second round of overnight trypsin digestion to ensure complete digestion of large peptide fragments. Supernatants collected before and after incubation of cells for 45 min with no protease added were used as controls (termed pre- and post-incubation control, respectively). The proteins identified in each sample by two or more peptides meeting the MS searching criteria (see Methods section 2.13.2) are shown in the Appendix (Table A4).

A total of 118 proteins were identified. The number of proteins identified per sample varied as did the number of peptides detected for each protein across different samples. Thirty-four proteins were unique to samples treated with trypsin and were termed protease-specific. For most protease-specific proteins, identification was not consistent across samples treated with different concentrations of trypsin. The remaining 84 proteins included proteins identified in both controls and treated samples and were termed non protease-specific.

Predictions on the subcellular location of the proteins identified were performed using PsortB and LipoP algorithms and results are presented in the Appendix (Table A4). The predicted cellular distribution of proteins from each category is shown in Table 3.7. The majority of both non specific and specific proteins were predicted to function in the cytoplasm.

Table 3.7 Subcellular distribution of proteins identified in the incubation buffer of trypsin-treated meningococci

Subcellular location	Non-protease specific	Protease specific
Cytoplasm	67	26
Inner membrane	3	1
Periplasm	1	-
Outer membrane	1	3
Lipoproteins	1	3
Extracellular	1	1
Unknown	10	0
Total	84	34

3.5 Discussion

This chapter presented analyses of the meningococcal proteome for identification of potentially surface-exposed proteins that hold promise as vaccine candidates. Although a considerable number of surface protein antigens have been identified based on *in silico* analysis of the meningococcal proteome (Pajon *et al.*, 2009; Pizza *et al.*, 2000), no proteomic studies specifically investigating the meningococcal surface subproteome have been reported. The method used in this study included the digestion of proteins *in situ* in whole bacteria by proteolytic enzymes followed by proteomic analysis of the resulting cell extracts and cell-free supernatants. Protease treatment provides a physical way to analyse the proteins that are exposed on the cell surface. Its specificity is mainly dependent upon the nature of the protein itself, in this case the exposure of protease cleavage sites on the bacterial surface.

Previous reports of protease treatment of bacterial proteins have highlighted the need for careful choice of treatment conditions to maximise cleavage of surface proteins without compromising cell integrity (Dreisbach *et al.*, 2010; Tjalsma *et al.*, 2008). For encapsulated bacteria like the meningococcus, the thickness of the capsular

polysaccharide on the cell surface may affect the accessibility of antigenic proteins to other proteins such as proteases and antibodies. It may, for example, explain apparent differences in the exposure of neisserial surface protein A (NspA) (Moe *et al.*, 1999a). Non-encapsulated strains offer a way of circumventing this problem and have been used in studies with streptococci (Rodriguez-Ortega *et al.*, 2006). The current study used a *siaD*, capsule-deficient, derivative of the serogroup B meningococcal strain MC58.

From analysis of total protein content and abundance of specific proteins with SDS-PAGE and western blotting, it was demonstrated that treatment can be specific to surface proteins when proteinase K is used at concentrations of up to 20 µg/mL per 10⁹ bacteria. Under these conditions, bacteria remained intact and any changes in protein abundance could be attributed to the effect of proteinase K. Limited cell lysis was also observed based on microscopic analysis of meningococci but was independent of proteinase K treatment. A limited number of dead or dying cells would be expected within a bacterial population harvested at the late exponential phase. The time lapsed between cell harvest and analysis on the microscope might have also contributed to this.

The results from DIGE suggest that combining proteinase K treatment of whole cells with comparison of proteomes between treated and untreated cells is an effective way of distinguishing potential surface antigens within the meningococcal proteome. Twenty-three of the 26 spots that were analysed (88.5%) contained proteins that could be potentially found on the meningococcal surface based on their predicted cellular location. These included 4 outer membrane proteins, 9 lipoproteins and 1 secreted protein. Experimental evidence exists for the surface exposure and antigenicity of the majority of these proteins including OMPs PorA, OpcA, Omp85

and FadL-like NMB0088, lipoproteins fHbp, NMB1946, H.8, thiol-disulfide interchange protein DsbA2, amino acid ABC transporter NMB0787 and uncharacterized protein NMB1468 and the extracellular protein Pile (summarized in Table 3.8).

The meningococcal porin PorA and the adhesin OpcA are 2 highly expressed outer membrane proteins with multiple extensive loops exposed on the bacterial surface (Tsai *et al.*, 1981). PorA and OpcA are the dominant antigens in OMV vaccines and the main targets of human immune responses as suggested by analysis of sera from human vaccinees (Wedegé *et al.*, 2007). Omp85 is another outer membrane-embedded protein forming a beta barrel-like channel for lipid transport and contributing to the assembly of other beta barrel OMPs in the meningococcus (Voulhoux *et al.*, 2003). Its susceptibility to proteolytic cleavage has been previously reported following treatment of cell envelopes with trypsin (Robert *et al.*, 2006). Omp85-specific antibodies have been identified in sera from humans vaccinated with OMVs suggesting Omp85 contributes to immune responses (Wedegé *et al.*, 2007). Its essential role in bacterial survival also suggests that Omp85 must be a conserved antigen across the meningococcal population. NMB0088 is an OMP sharing homology with FadL proteins involved in transport of hydrophobic molecules. NMB0088 has been suggested to contain 3 variable regions located in extracellular domains (Yero *et al.*, 2010). NMB0088 was able to induce bactericidal antibodies in mice (Sardinas *et al.*, 2009). Pile is the major pilin of tfp which extends beyond the meningococcal outer membrane facilitating adhesion to human epithelial cells (Pelicic, 2008). Anti-Pile antibodies have been found in acute and convalescent sera from patients (Poolman *et al.*, 1983).

Table 3.8 Known surface-exposed proteins identified following proteinase K treatment of meningococci. Information on the antigenic properties of the proteins is presented.

Protein name	Determination of surface exposure	Antigenic properties
PorA	Analysis of variable, exposed loops Flow cytometry	Induces bactericidal antibodies in mice and humans
OpcA	Structural analysis	Induces bactericidal antibodies in mice and humans
Omp85	Structural analysis	Induces bactericidal antibodies in mice and humans
fadL-like NMB0088	Flow cytometry	Induces bactericidal antibodies in mice
PilE	Functional analysis, microscopy	Induces immune response in humans
Factor H binding protein	Structural analysis, flow cytometry	Induces bactericidal antibodies in mice and humans
NMB1946	Flow cytometry	Induces bactericidal antibodies in mice
Thiol disulphide interchange proteins DsbA2	Flow cytometry	Not determined
NMB1468	Flow cytometry	Protects mice from lethal challenge with live meningococci
ABC transporter NMB0787	Flow cytometry	Does not induce bactericidal antibodies in mice
H.8	Immunogold labelling	Does not induce bactericidal antibodies in mice. Blocks bactericidal activity of antibodies against other antigens

Factor H-binding protein is a fully exposed lipoprotein found on the surface of the majority of invasive serogroup B meningococcal strains. Antibodies against fHbp are bactericidal in mice and humans and are also present in healthy carriers and convalescing patients (Litt *et al.*, 2004; Maignani *et al.*, 2003). Factor H-binding protein is a promising vaccine candidate currently undergoing clinical evaluation

alone or as part of a multi-protein vaccine preparation (Giuliani *et al.*, 2006). Using flow cytometry to assess binding of protein specific antibodies on the surface of live meningococci, exposure of predicted lipoproteins NMB1946, thiol-disulfide interchange protein DsbA2, amino acid ABC transporter NMB0787 and NMB1468 has been demonstrated (Ferrari *et al.*, 2006; Grifantini *et al.*, 2002b; Hsu *et al.*, 2008; Pizza *et al.*, 2000). NMB1946 and NMB1468 have also been shown to protect against meningococcal disease in mice (Hsu *et al.*, 2008; Pizza *et al.*, 2000). In contrast, amino acid ABC transporter NMB0787 reportedly failed to elicit bactericidal antibodies in mice (Grifantini *et al.*, 2002b). Finally, H.8 is another example of a meningococcal lipoprotein. The surface exposure of the gonococcal H.8 homologue has been studied using anti-H.8 sera in immunogold labelling experiments (Hitchcock *et al.*, 1985). Antibodies directed against H.8 were not bactericidal in mice and were recently shown to block bactericidal activity of human sera or antibodies against other meningococcal antigens (Bhattacharjee *et al.*, 1990; Ray *et al.*, 2011).

Little information exists on the cellular location and immunogenicity of the remaining proteins identified by DIGE analysis. Among proteins that warrant further investigation, the macrophage infectivity potentiator NmMIP, PilP, amino acid binding protein NMB1612 and PilO are of particular interest as judged by their predicted cellular location and function. NmMIP is a predicted lipoprotein bearing a palmitate group and belongs to the family of peptidyl-prolyl cis-trans isomerases that contribute to protein folding. Members of this family constitute virulence factors in other bacteria by initiating infection of macrophages (Cianciotto and Fields, 1992; Fischer *et al.*, 1992). The gonococcal homologue NgMIP is surface-exposed and promotes gonococcal survival in macrophages (Leuzzi *et al.*, 2005). The amino acid binding protein NMB1612 is also a predicted lipoprotein and a member of the family

of ABC transporters with a putative role in the transport of amino acids across the bacterial cell wall. NMB1612 was recently identified as a target of the human immune system as inferred by analysis of sera from colonised subjects (Williams *et al.*, 2009). PilP is a lipoprotein involved in biogenesis of type IV pili, the structures that facilitate meningococcal adherence to human epithelial cells (Drake and Koomey, 1995). PilP was also suggested to mediate processes of pilus motility through its interaction with the secretin PilQ (Balasingham *et al.*, 2007). PilO, predicted to reside in the inner membrane, is also required for tfp expression (Drake *et al.*, 1997). The gene encoding PilO was upregulated during interaction of MC58 Δ -*siaD* meningococci with epithelial HEP-2 cells (Dietrich *et al.*, 2003) suggesting a putative role in adhesion and colonisation processes.

Among the proteins identified by MS analysis were 6 proteins predicted to function in the cytoplasm. With the exception of bacterioferritin A, these proteins were found in spots containing other non-cytoplasmic proteins which were more likely targets of proteinase K. Therefore, it is suggested that the identification of these cytoplasmic proteins is more a result of their co-migration in the same spot with proteins sharing the same pI and MW and not their exposure on the surface.

As well as identifying surface proteins affected by proteinase K treatment on the meningococcal surface, results from DIGE analysis of cell extracts provided evidence for the extent of this effect on each protein. The presence of groups of completely and partially digested proteins could be inferred by the traces of proteins on 2-D gels and the values of decrease in abundance. Complete digestion of PorA, OpcA and fHbp confirmed their extensive exposure on the meningococcal surface which is also responsible for their immunodominance (Mascioni *et al.*, 2008; Prince *et al.*, 2002; van der Ley *et al.*, 1991). What caused the partial digestion of the rest of

identified proteins could be a result of partial surface exposure or weak accessibility to proteases or a combination of both. Accessibility of proteases to some surface proteins can indeed be hindered by factors including other surface structures of topological relationship (Bunikis and Barbour, 1999; Waldemarsson *et al.*, 2006). As an example, in the outer membrane of *Borrelia burgdorferi* protein P66 was masked by members of the Osp lipoproteins and could not be accessed by trypsin (Bunikis *et al.*, 1999). Uneven distribution of these structures on the bacterial surface could be accused for the non-uniform cleavage of meningococcal proteins observed. Partial exposure can be expected when proteins function in multiple locations on the cell. In this case, only part of their population would be exposed on the surface at any given point. Collectively, this information suggests a possible association between surface accessibility of proteins and their extent of cleavage by proteases. More studies on the surface exposed regions of partially digested proteins will allow for more certain conclusions to be made.

From analysis of the 2-D gel spots, the formation of spot trails of same protein origin was also demonstrated. Proteins identified in multiple spots included OMPs PorA and Omp85, pilin Pile, the amino-acid ABC transporter NMB1612 and NmMIP. Spot trails is a common phenomenon in 2-D gels and is usually attributed to post-translational modifications, most likely phosphorylation, which results in a shift in the protein's pI (Schaefer *et al.*, 2006). From the proteins identified in multiple spots in this study, Pile has been previously shown to undergo glycosylation (Power *et al.*, 2000). NmMIP and NMB1612 are also predicted lipoproteins with NmMIP predicted to undergo palmitoylation. For NmMIP, which has a potential peptidyl-prolyl cis-trans isomerase activity, multiple spots could also represent different enzymatic isoforms (isozymes). For PorA and Omp85, similar spot trails have been

previously reported following 2D-PAGE of meningococcal OMVs (Vipond *et al.*, 2006). Although their post-translational modification cannot be excluded, it has been suggested that PorA and Omp85 multiple spots are more likely the result of different conformational equilibria formed during protein denaturation under the conditions used for 2-D PAGE. In 2-D gels of *Porphyromonas gingivalis* and *Methylococcus capsulatus*, a single spot from a 2-D separated spot trail could give rise to the same spot trail when re-subjected to 2-D analysis highlighting a conformational response of the protein to the experimental conditions (Berven *et al.*, 2003; Veith *et al.*, 2001). The presence of multiple transmembrane domains in PorA and Omp85 sequence which form highly hydrophobic beta barrels in the outer membrane could explain the model of multiple conformational isoforms.

Mass spectrometric analysis of the peptides recovered in the incubation buffer following treatment of meningococci with trypsin was alternatively used for identification of surface-exposed proteins. This type of analysis suffered from under-representation of proteins known or predicted to reside on the meningococcal surface and over-representation of cytoplasmic predicted proteins. Of the 34 proteins identified in the incubation buffer of trypsin-treated samples only 6 were predicted lipoproteins or OMPs. These included proteins NMB1468, PilE, NmMIP and amino acid-binding protein NMB0787 previously identified by DIGE to be digested by proteinase K, PilQ, an OMP involved in tfp biogenesis and TspA, a predicted OMP involved in cell wall degradation. None of PorA, OpcA or fHbp that were demonstrated to be completely cleaved by proteinase K were among the proteins identified in the incubation buffer of trypsin-treated meningococci. This is rather surprising considering these proteins are extensively exposed on the meningococcal surface and therefore expected to release multiple peptides in the incubation buffer

upon trypsin treatment. It is possible that the size of the exposed peptides released from these proteins might have not been ideal for MS analysis. Other proteins, shown to be partially cleaved by proteinase K in DIGE, also failed to be identified by analysis of the incubation buffer. The absence of enough accessible trypsin cleavage sites in these proteins might have possibly hindered the release in the incubation buffer of enough peptides to allow their identification.

The high proportion of cytoplasmic predicted proteins identified in the trypsin-treated samples is in agreement with studies from other bacteria (Doro *et al.*, 2009; Dreisbach *et al.*, 2010; Tjalsma *et al.*, 2008; Walters *et al.*, 2009). Contamination with cytoplasmic components was observed in Gram-positive bacteria such as *Bacillus subtilis* and *Staphylococcus aureus* which persisted even after extensive optimisation of treatment conditions (Dreisbach *et al.*, 2010; Tjalsma *et al.*, 2008). The leakage of proteins from the cytoplasm could be the result of a possible destabilisation of the bacterial cell wall upon proteolytic cleavage of surface structures, which would otherwise provide mechanical support to the cell. In Gram-negative bacteria, this leakage could be further enhanced by the presence of large porin complexes transversing the outer membrane which pose as potential escape sites for proteins through passive diffusion (Tan *et al.*, 2008). This explains higher levels of contamination observed in this study and in a study of protease treatment of *E. coli* (Walters *et al.*, 2009).

Extensive representation of cytoplasmic proteins was also observed in control samples. The most likely explanation for this observation is the lysis of cells as a result of osmotic stress during incubation in the buffer selected. Cell lysis was found to be limited in cell images from cryo-TEM. Although not enough to alter total protein abundance levels, few dead cells can seemingly provide the incubation buffer

with proteins from various cell compartments and at sufficient concentrations to be detected by a highly sensitive mass spectrometer. Some of the proteins identified could also represent proteins loosely attached to the meningococcal surface which were physically detached upon resuspension in the buffer (Tjalsma *et al.*, 2008). Of the proteins identified in controls, elongation factor Tu, chaperone DnaK, enolase, peroxiredoxin 2 family protein and macrophage infectivity potentiator-related protein have been previously detected on the surface of meningococci by flow cytometry (Ferrari *et al.*, 2006; Grifantini *et al.*, 2002b; Knaust *et al.*, 2007). Similarly, elongation factors Ts and G and the 30S ribosomal protein S1 have been found on the surface of other bacteria (Lei *et al.*, 2000; Sanchez-Campillo *et al.*, 1999). It is possible that the attachment of these proteins on the bacterial surface occurred following their release from dead cells during cell culture (Tjalsma *et al.*, 2008). Upon release, these proteins could have been attracted to the surface of live bacteria through affinity to cell wall structures.

3.6 Concluding remarks

In this part of the study, treatment of meningococci with proteases coupled with proteomic analysis was used for the identification of the meningococcal surface proteome. The success of this strategy was dependent upon the cell fraction analysed following protease treatment. The analysis of whole-cell proteomes provided more specific and conclusive results compared with the analysis of the peptides released in the incubation buffer. The surface proteome consisted of known antigens as well as other potentially surface-exposed proteins that warrant further investigation as possible vaccine candidates.

Chapter 4

Characterization of meningococcal serogroup B OMV vaccines from strain H44/76 after growth in rich and minimal media

4. Characterization of meningococcal serogroup B OMV vaccines from strain H44/76 after growth in rich and minimal media

4.1 Introduction

The meningococcus is highly adaptive to changes in its environment and has evolved mechanisms for survival under the conditions posed by its host. One strategy involves regulation of its gene expression for selective production of proteins involved in acquisition of nutrients essential for its growth during colonisation and infection. Such proteins are normally located on the bacterial surface to facilitate direct interaction with nutrient sources. The significance of these proteins in meningococcal survival and infection can also render them key targets of the immune system of the host. Typical examples include OMPs involved in iron acquisition under iron-limiting conditions such as proteins involved in binding of lactoferrin and transferrin (Lbps and Tbps) (Legrain *et al.*, 1993; Pettersson *et al.*, 1993; Pettersson *et al.*, 1998). Expression of Lbps and Tbps is regulated by the ferric uptake regulatory protein (Fur) which, in the presence of iron, binds to a consensus sequence next to the promoter region of the iron-regulated gene and blocks transcription by RNA polymerase (Escobar *et al.*, 1999). The ability of Lbp and Tbp to protect against meningococcal disease has also been confirmed in experimental animal models (Ala'Aldeen *et al.*, 1996; Pettersson *et al.*, 2006).

In this chapter, the effect of growth medium on the expression of meningococcal proteins was investigated. Frantz medium (FM) containing yeast extract and casamino acids was chosen as a rich medium for bacterial growth whilst the synthetic modified Catlin-6 medium (MC.6M) was used for growth under minimal nutrient supply. Growth conditions under these media have been previously established during production of OMV vaccines against outbreak strains CU385,

H44/76 and NZ98/254 used in protection trials in Cuba, Norway and New Zealand, respectively (Fredriksen *et al.*, 1991; Oster *et al.*, 2005).

Meningococci from H44/76, a strain of similar genetic background to MC58, were grown in each of the 2 media and OMV preparations were assessed for changes in expression levels of proteins present in the outer membrane. Analysis was carried out using both traditional SDS-PAGE and DIGE. SDS-PAGE has been used for analysis of the presence and abundance of major antigens as part of the evaluation of OMV vaccines (Frasch *et al.*, 2001; Fredriksen *et al.*, 1991; Vipond *et al.*, 2005). DIGE has also been used to investigate the consistency in the manufacturing of OMV vaccines by comparing expression levels of both key and minor antigens across different OMV batches (Vipond *et al.*, 2006). In general, 2D-PAGE coupled to mass spectrometry has facilitated characterisation of the OMV proteome of the strains responsible for outbreaks of serogroup B disease in Cuba and New Zealand (Uli *et al.*, 2006; Vipond *et al.*, 2006). Together with DIGE comparative analysis, similar characterisation of the protein content of OMVs from H44/76 strain was attempted and is presented in this chapter.

In addition, in collaboration with the Norwegian Centre of Public Health, OMV vaccine formulations from meningococci grown in the different media were prepared and their efficacy compared in ELISA and serum bactericidal assays (SBA). In this way, possible relationships between the detected changes in protein levels and vaccine-induced immune responses could be investigated and novel protein targets for vaccine development suggested. Collectively, results from analysis of both antigen composition and immunogenicity would offer additional insights to such an important variable as the growth medium during OMV vaccine manufacturing and its effect on protein-based vaccines quality and efficacy.

4.2 SDS-PAGE

Batches of OMVs, produced from meningococci grown in FM or MC.6M were first compared by SDS-PAGE (Figure 4.1). Relative expression levels for the most abundant proteins present on the gels were measured by scanning densitometry of the corresponding protein bands. The identity of protein bands was confirmed by immunoblotting using protein-specific antibodies.

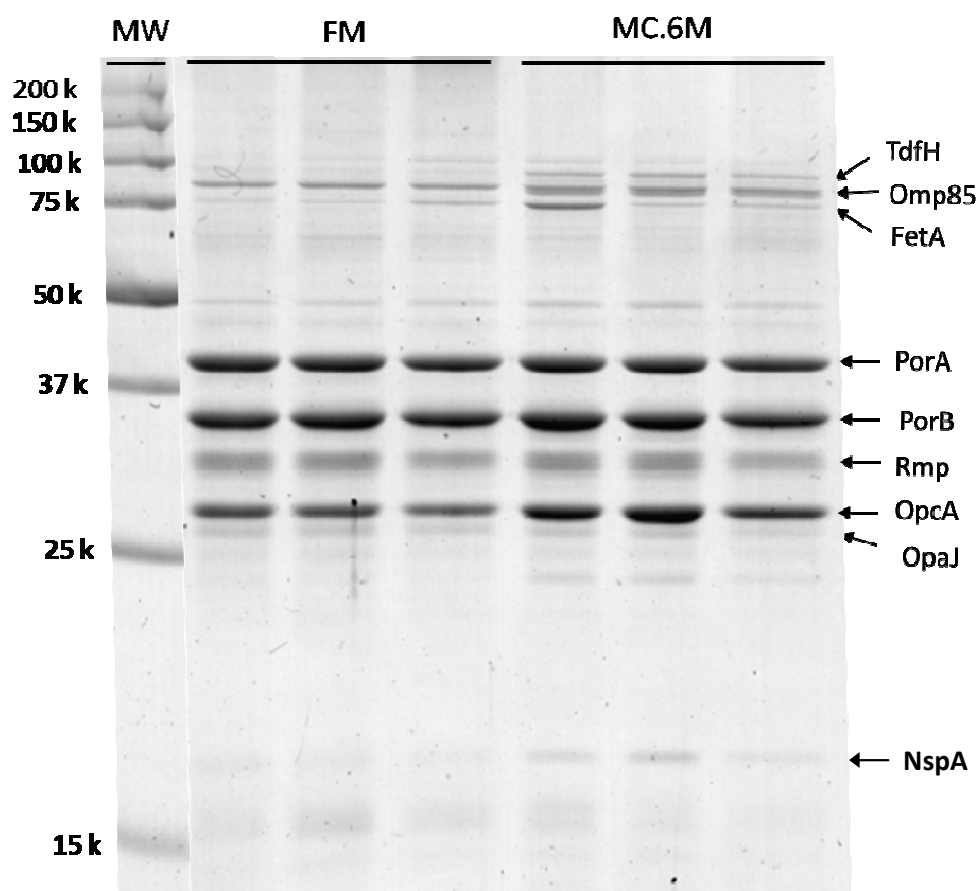


Figure 4.1 SDS-PAGE of three batches of OMVs from meningococcal strain 44/76 grown in either FM or MC.6M. Two μg of OMV protein was applied in each lane. The MW reference is shown to the left, and the main outer membrane protein bands are identified to the right. The gel was stained with Coomassie R-350 and photographed with Bio-Rad Scanner GS-710 Imaging Densitometer.

No significant differences in the expression levels of the major OMPs PorA (P1.7,16), PorB3 (serotype 15) and RmpM were detected. The ranges of the staining

intensities of these protein bands in percentage of total band intensity were 18-24%, 25-33% and 15-20%, respectively. The level of Omp85 (range 4-6%) was also similar among the different preparations. Two high MW proteins, a 100 kDa species identified as TonB-dependent receptor TdfH and a 80 kDa species just below Omp85 were more abundant in MC.6M OMVs (band intensity levels not determined), as was OpcA with an intensity range of 21-25% compared with 16-19% in FM OMVs (P-value = 0.008). Relative to the intensity of the PorA band, there was 1.6-fold more OpcA in the MC.6M OMVs than in those from FM (P-value = 0.021). NspA also exhibited somewhat higher levels in OMVs produced in MC.6M (band intensity levels not determined). Batch-to-batch variations in both media were observed with respect to the level of expression of the iron-regulated protein FetA (range 1-8%).

Scanning of the L3 and L8 LPS bands in silver-stained gels after loading equivalent amounts of OMV protein from the 6 batches showed higher levels of both bands in MC.6M OMVs (P-value < 0.005) compared with the FM OMVs. From the sum of L3 and L8 bands in reference LPS samples, applied in the same gel, the MC.6M OMVs contained 0.13 μg LPS/ μg protein (range 0.12-0.16 μg LPS/ μg protein) and the FM OMVs 0.09 μg LPS/ μg protein (range 0.08-0.10 μg LPS/ μg protein).

4.3 2D-PAGE

SDS-PAGE has relatively low resolution efficiency during protein separation meaning protein bands do not necessarily represent single protein species. This, in turn, prevents its use as a fully quantitative method since differences in expression levels cannot be directly linked to a single protein. The superior separation performance of 2D-PAGE over traditional SDS-PAGE was therefore utilised in this

study and coupled to DIGE technology for comparative analysis of OMVs from meningococci grown in the different media. Prior to DIGE, tests on 2D-PAGE conditions were carried out to ensure optimal quality of separation of proteins present in OMVs.

4.3.1 Establishing optimal 2D-PAGE conditions for separation of H44/76 OMVs

Outer membrane vesicles, rich in highly hydrophobic OMPs, are difficult to solubilise in the buffers used during IEF, the first dimension of 2D-PAGE. Additionally, meningococcal proteins are dominated by major OMPs like PorA and PorB whose efficient separation in 2-D gels can be challenging. A previous study on meningococcal OMVs reported the use of the surfactant Triton X-100 in the solubilisation buffer for effective protein solubility and a total focusing time of 45 kVh (Vipond *et al.*, 2006). Both conditions were incorporated in the protocol used for the 2D-PAGE analysis presented in this chapter. Optimisation of additional parameters that can impact on protein solubilisation and separation during IEF were attempted and presented here.

As a first step, two surfactants, Triton X-100 and CHAPS, were tested for their effect on protein solubilisation when added in the buffer used to rehydrate the IPG strips prior to IEF. Two-dimensional gels were produced from H44/76 OMV samples applied to IPG strips and rehydrated overnight with the different buffers. All other parameters were kept identical throughout 2D-PAGE. The results are presented in Figure 4.2. Considerable under-representation of proteins on the gel from the sample rehydrated with CHAPS was observed judging by the number and pattern of 2-D spots compared to that rehydrated with Triton X-100. Many spots present on the gel from the Triton X-100 sample were missing on that from CHAPS sample, especially

in the low MW region (indicated by arrows in Figure 4.2). Therefore, a rehydration buffer containing Triton X-100 was chosen for all subsequent optimisation and investigative work.

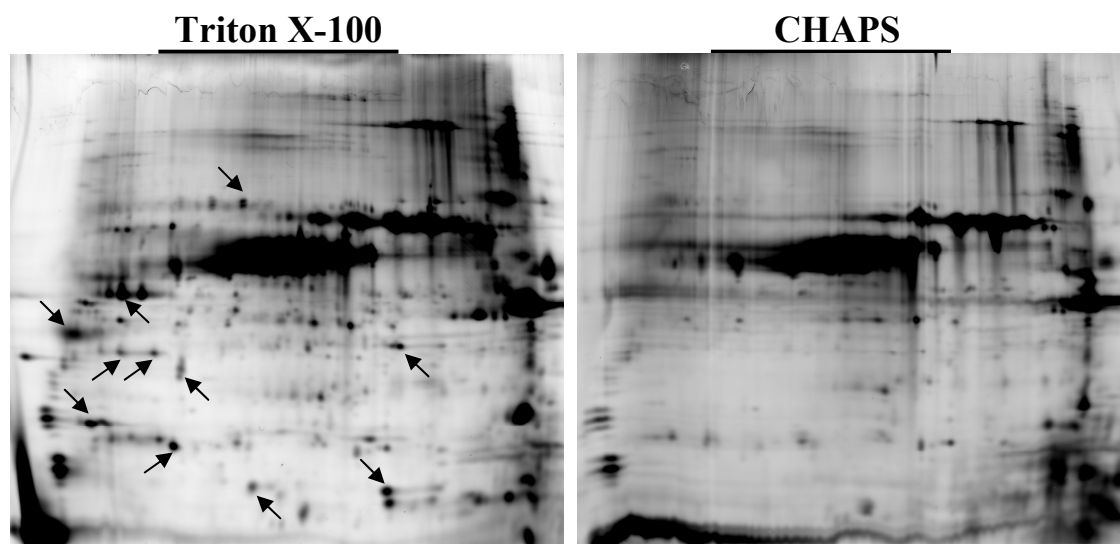


Figure 4.2 Effect of IPG strip rehydration buffer on 2D-PAGE of H44/76 OMVs. Outer membrane vesicles (100 μ g) from meningococci grown in FM were labeled with Cy5 dye and applied to IPG strips (3-11, non-linear). IPG strips were rehydrated overnight with buffers containing either Triton X-100 or CHAPS and subjected to isoelectric focusing for 45 KVhr using a maximal voltage of 3,500 V. Second-dimension SDS-PAGE was carried out in 12% polyacrylamide gels. Gel images were acquired by fluorescence scanning at the Cy5 channel. Arrows in the 'Triton X-100' image highlight protein spots missing in the 'CHAPS' image.

Two more parameters of the 2D-PAGE protocol were assessed, the maximal voltage used for IEF and the duration of strip equilibration prior to second dimension SDS-PAGE. Maximal voltages tested were 3,500, 5,500, 8,000 and 10,000 V. Strips focused under these voltages were then incubated sequentially with DTT- and iodoacetamide-containing equilibration buffer at durations of 10 and 5 min, respectively, or 15 min for both incubations. Images of the gels produced from each combination of conditions tested are presented in Figure 4.3. No major differences in protein separation and representation were observed across images. Spot patterns were reproducible regardless of IEF voltage or equilibration time.

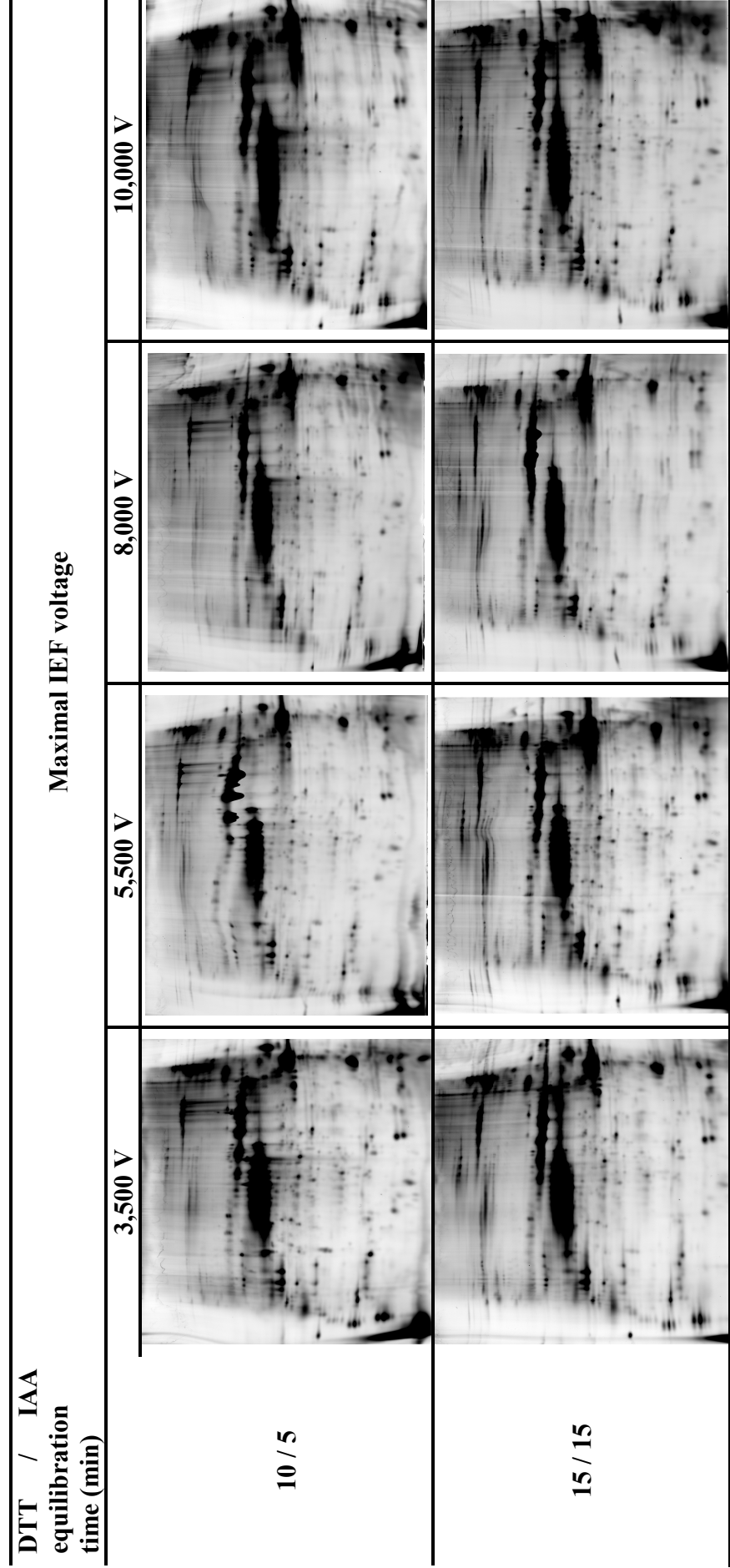


Figure 4.3 Evaluation of IEF maximal voltages and duration of equilibration steps during 2D-PAGE of H44/76 OMVs. Outer membrane vesicles (100 µg) from meningococci grown in FM were labeled with Cy5 dye, applied to IPG strips (3-11, non-linear) and subjected to IEF for 45kVh using maximal voltages of 3,500, 5,500, 8,000 or 10,000. Equilibration steps with buffers containing DTT and IAA were carried out, equilibration times stated on the left. Second-dimension SDS-PAGE was carried out using 12% polyacrylamide gels and images acquired by fluorescence scanning.

The highest voltage (10,000 V) was chosen for subsequent analysis to reduce IEF experimental times. Fifteen-minute equilibration steps were also chosen for consistent and easy to handle experimental times.

4.3.2 DIGE

DIGE was used to compare protein expression levels between the two OMV preparations made from H44/76 meningococci grown in FM or MC.6M media. Three individual OMV batches were prepared for each growth medium. A 2-colour DIGE experimental design was used where individual batches were labeled with Cy5 dye and an internal standard sample generated from pooling all individual batches was labeled with Cy3 dye (Table 4.1A). Each batch was run on a single gel together with the internal standard resulting in a total of 6 gels. Images acquired from fluorescence scanning of gels are presented in Figure 4.4. Acquired gel images were imported on DeCyder software v.6.5 and grouped on the basis of culture medium (3 gels per group) for subsequent statistical analysis. Fluorescence intensities were calculated for each protein spot across all images and compared between the 2 groups.

Results from quantitative analysis are summarised in Table 4.1B. A total of 2,005 spots were matched across the 6 gel images. The level of expression of 1941 spots (97%) was less than 10% different between the two OMV preparations (Table 4.1B, fold change < 1.1). Sixty four spots exhibited a greater than 10% difference in the amount of protein (P-value = 0.00023 – 0.049).

Table 4.1 DIGE analysis of OMVs from meningococcal strain H44/76 grown in FM or MC.6M. A). Detailed two-colour DIGE experimental design; B). Summary of quantitative data analysis.**A).**

Gel No	Cy3 label (50 µg)	Cy5 label (50 µg)
1	Pooled internal standard ^a	FM batch 1
2	Pooled internal standard	FM batch 2
3	Pooled internal standard	FM batch 3
4	Pooled internal standard	MC.6M batch 1
5	Pooled internal standard	MC.6M batch 2
6	Pooled internal standard	MC.6M batch 3

^a made from equal quantity of all batches**B).**

Fold change	Total no. of spots showing differential expression ^b	Protein expression in MC.6M compared to FM (no. of spots) ^b	
		Increased	Decreased
≥ 2	41	24	17
< 2 and ≥ 1.1	23	17	6
< 1.1	1941	-	-
Total	2005	41	23

^b results in number of spots from the application of a P-value < 0.05 are displayed (n=3).

The majority of these spots exhibited greater than 2-fold change (41 out of 64, 64%). Forty-one proteins were more abundant in OMVs produced in MC.6M, whereas 23 were more abundant in OMVs produced in FM. The position of spots with differential expression between the two OMV preparations is highlighted in Figure 4.5. Most spots were found in the basic region and included a range of molecular masses. High abundance spots, identified as the major OMPs, *i.e.* PorA, PorB and OpcA, were excluded from accurate quantitative comparison due to their saturation in fluorescence intensity which exceeded the linear range of scanning conditions. Details about the extent of change in expression levels are provided in Table 4.2 for those protein spots for which MS identification was carried out.

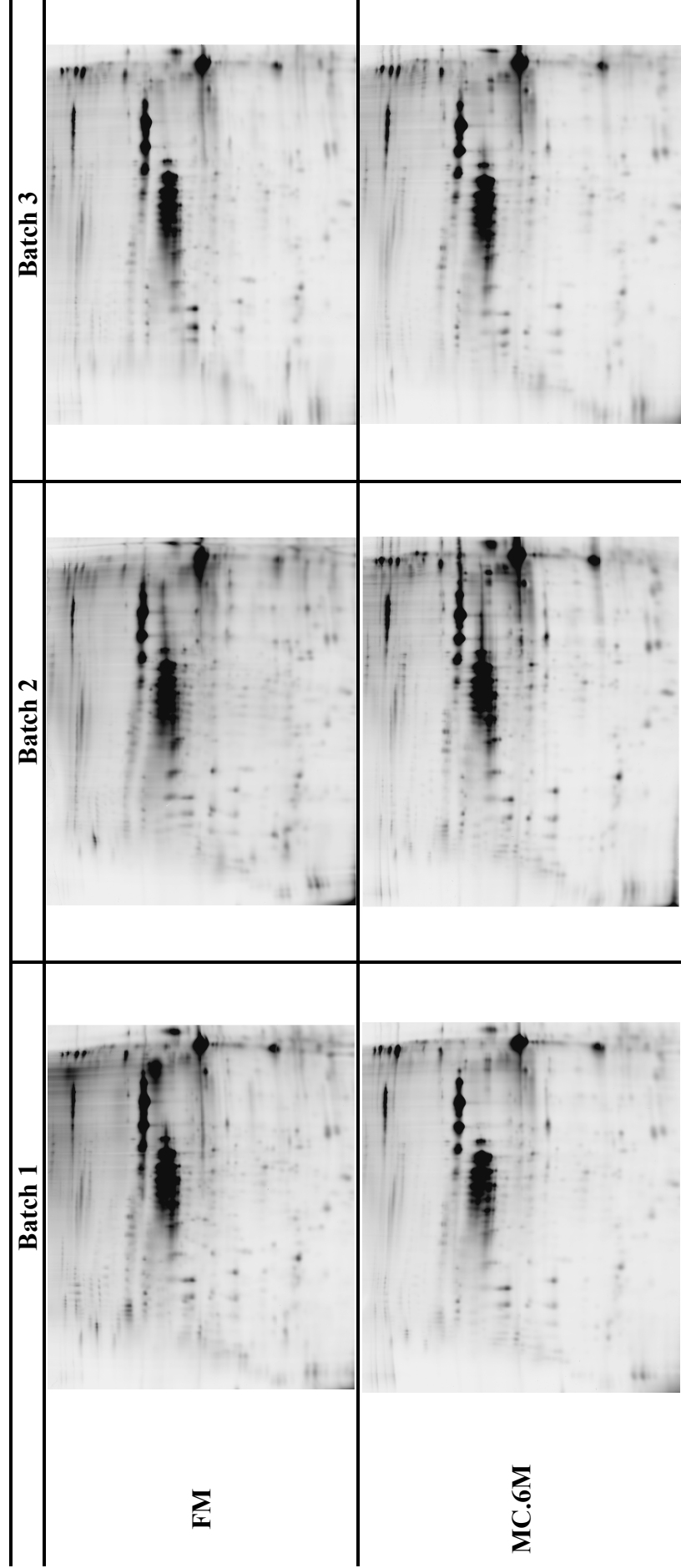


Figure 4.4 2D-PAGE of OMVs from H44/76 meningococci grown in FM and MC.6M. Three meningococcal batches were prepared from each medium and OMVs were extracted using deoxycholate. Outer membrane vesicles were labeled for DIGE analysis (see Table 4.1A), applied to IPG strips (3-11, non-linear) and subjected to IEF for 45 kVhr using 10,000 V. SDS-PAGE was carried out in 12% polyacrylamide gels and images acquired by fluorescence scanning. Cy5 images corresponding to the different batches made in each medium are presented.

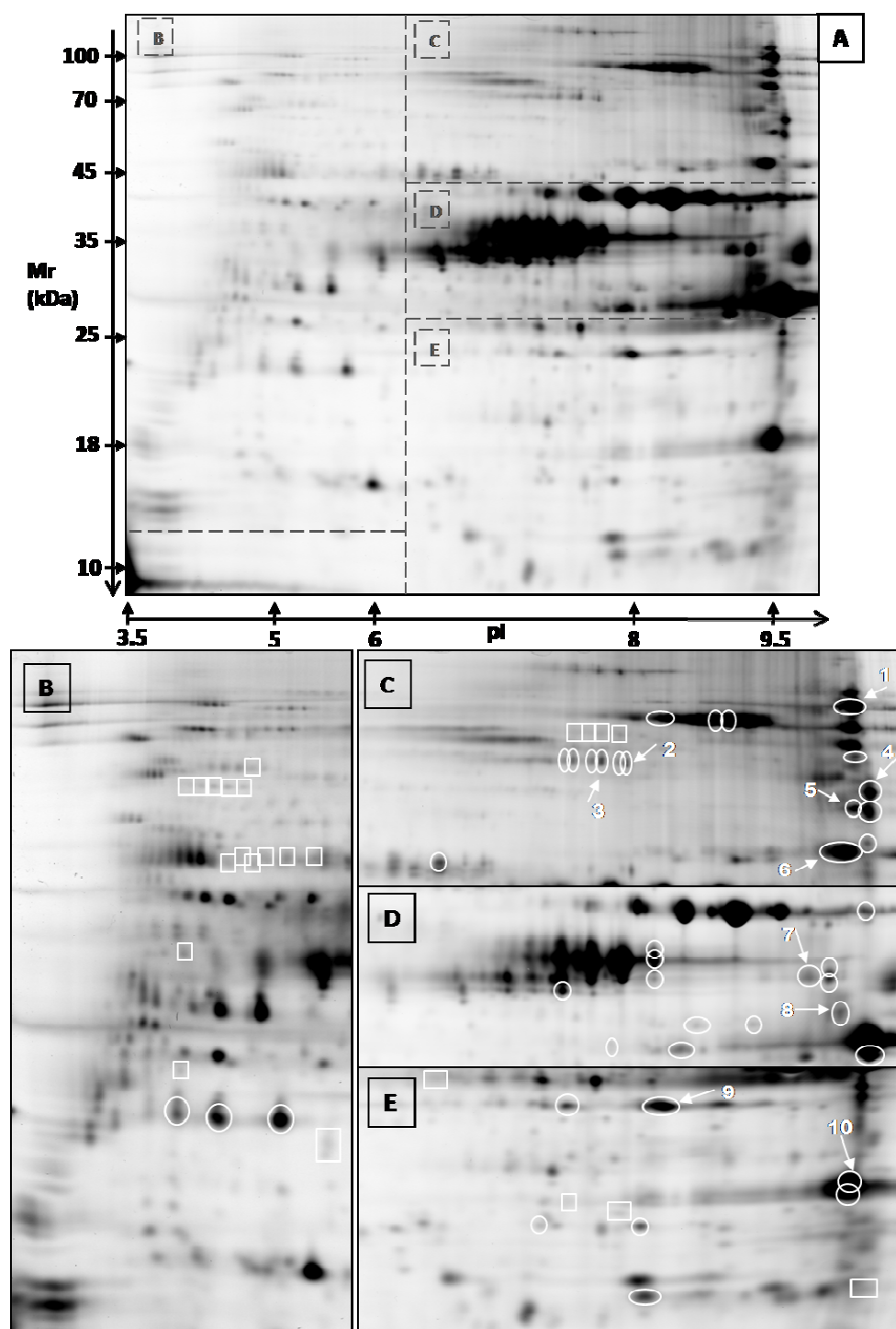


Figure 4.5 Position on 2-D gel of proteins with differential expression in OMVs from meningococci grown in FM and MC.6M. A) Complete gel image from 2D-PAGE of H44/76 OMVs. B) – E). Enlarged sections of A) highlighting the protein spots with increased (circles) and decreased (squares) expression in MC.6M OMVs (fold change > 1.1, P-value = 0.00023 – 0.049). Arrows indicate the spots identified by MS (shown in Table 4.2).

4.4 MS identification of proteins expressed at different levels in FM and MC.6M OMVs

Spots showing different expression levels between OMVs prepared in FM and MC.6M were picked from a set of preparative gels. Following in-gel digestion of spots by trypsin, proteins were identified using MALDI PMF as the main mass spectrometric method. MALDI measures the masses of peptides present in the sample which are then correlated to theoretically predicted masses from proteins present in the database used during searching. Combined with the resolving power of 2D-PAGE, MALDI PMF can accurately identify proteins from 2-D spots. For some of the spots, additional peptide fragmentation (MS/MS) was performed to increase confidence of identification through correlation to peptides sequences.

Seven proteins were identified from MS analysis of 10 spots and are presented in Table 4.2. TonB-dependent receptor TdfH, OpcA and protein encoded by NMB2134 were each identified in 2 spots whilst FadL-like NMB0088, adhesin MafA, lipoprotein NMB1126/1164 and NspA were each identified in a single spot. Proteins identified belonged to spots with increased levels in OMVs from MC.6M medium (Table 4.2). Two-fold or greater increase was observed for all identified proteins. TdfH exhibited the highest increase in expression in MC.6M OMVs among the identified proteins. Differences were observed in the fold-increase values calculated for the same protein appearing in different spots, *i.e.* TdfH (spots 1 and 5), OpcA (spots 4 and 8) and NMB2134 (spots 2 and 3) and can be mostly attributed to differences in the amount of protein present in each spot.

Taking together the results from SDS-PAGE and DIGE-MS, TdfH, OpcA and NspA were confirmed by both methods to be produced at higher levels in MC.6M OMVs. Additionally, FadL-like NMB0088, MafA, NMB2134 and NMB1126 were

identified by DIGE MS to also be part of the proteins over-expressed in MC.6M OMVs. The 80 kDa protein below Omp85 that was shown by SDS-PAGE to be more abundant in MC.6M OMVs was not identified by DIGE-MS. Spots of higher expression in MC.6M OMVs were detected in the area of Omp85 migration on 2-D gels (Figure 4.5) which might belong to the 80 kDa protein of interest. However, the high abundance of Omp85 and low spot resolution in the area prevented confident detection of the differentially expressed protein.

Table 4.2 Summary of DIGE and MS results from comparison of FM and MC.6M OMVs. Quantitative comparison between OMVs grown in either FM or MC.6M was carried out using DeCyder 2-D differential analysis software v. 6.5. Differentially-expressed proteins were subjected to MALDI PMF and/or MS/MS for identification. Spot numbers correspond to the numbers in Figure 4.5.

Protein name	Spot No.	Fold increase in MC.6M OMVs	P value	MS method	Protein coverage / No. of unique peptides sequenced or matched
TdfH (TonB-dependent receptor NMB1497)	1	8.27	0.000023	MALDI PMF	26% / 19
	5	6.72	0.0013	MALDI PMF	32% / 26
Hypothetical protein NMB2134	2	2.90	0.023	MALDI PMF	13% / 7
	3	3.22	0.038	MALDI PMF	25% / 13
Class 5 OMP OpcA	4	3.14	0.026	LC MS/MS	28% / 5
	8	1.75	0.037	MALDI PMF	45% / 9
OMP NMB0088				MALDI PMF	39% / 14
	6	2.36	0.034	MALDI MS/MS	7% / 2
Adhesin MafA	7	5.46	0.011	MALDI PMF	55% / 14
Lipoprotein NMB1126/NMB1164	9	4.0	0.016	MALDI PMF	45% / 7
				MALDI PMF	30% / 6
NspA	10	2.29	0.033	MALDI MS/MS	10% / 1

4.5 Antibody levels in immunised mice

OMVs from meningococci grown in either FM or MC.6M were adsorbed to aluminium hydroxide and used to immunise groups of mice. Doses of 2.0 μg for both OMV vaccine formulations were given. A lower 0.5 μg dose of MC.6M OMV was also administered in one group. Antibodies present in the generated immune sera were first tested for total levels in ELISA. Mice immunised with 2.0 μg of MC.6M OMVs had significantly higher serum IgG levels (P-value = 0.0002) than those receiving the 0.5 μg dose (Figure 4.6A). There was no significant difference between the IgG levels induced by the 2.0 μg dose of the MC.6M and FM OMV vaccines. No antibodies were detected in the negative control sera from mice immunised with physiological saline.

Serum bactericidal activity induced by OMV vaccines has been shown to correlate with protection against meningococcal disease in humans (Holst *et al.*, 2003). Here, murine immune sera from the different OMV vaccine preparations were tested and compared for bactericidal activity against the H44/76-SL strain in the presence of human complement source (hSBA). hSBA titers of ≥ 8 (3 when expressed in $\log_2$ scale) were measured and results are presented in Figure 4.6B. The titers obtained with the 2.0 μg dose of both vaccines were significantly higher (P-value < 0.001) than those of the saline control group, whereas no significant differences were observed between the saline control and 0.5 μg of the MC.6M OMV vaccine. However, the 2.0 μg dose of MC.6M OMV vaccine induced significantly higher titers (P-value = 0.032) than the same dose of the FM OMV vaccine. MC.6M OMV vaccine also elicited a significant dose response in bactericidal titers (P-value $\leq$ 0.0003), as observed by comparing results from the two MC.6M OMV doses used.

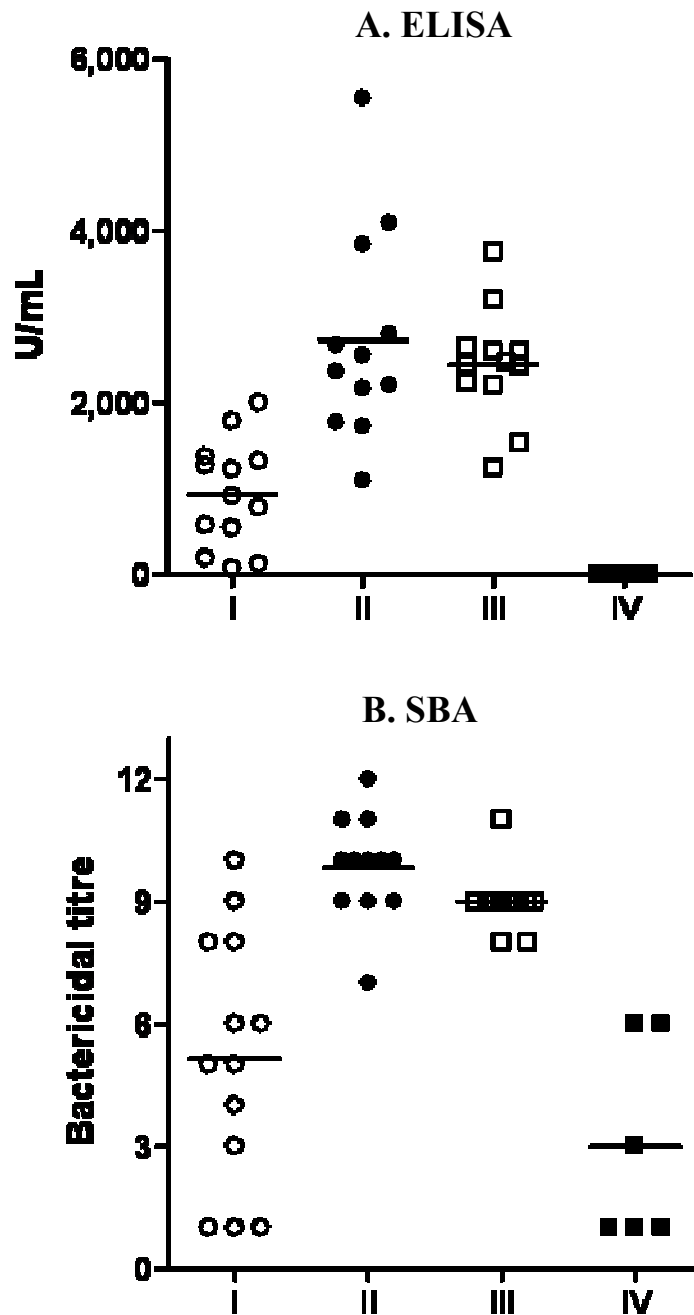


Figure 4.6 Antibody levels in mice immunised with FM or MC.6M OMV vaccines. Groups I and II received 0.5 μ g and 2.0 μ g of the MC.6M OMV vaccine, respectively; group III 2.0 μ g of the FM OMV vaccine and group IV physiological saline. A). IgG antibody levels (U/mL) measured in ELISA. B). Bactericidal antibodies measured in SBA. The titres are given as log₂ of the highest reciprocal serum dilution showing > 50% killing of the 44/76-SL target strain. The starting dilution was 1:8 (3 in log₂ scale) and lower titres were assigned a value of 1. The SBA was performed blinded with respect to the groups of mice.

Specific antibody levels in immunized mice to the major OMPs were measured on immunoblots using the MC.6M OMV as antigen. The 2.0 µg dose of both vaccines induced similar Ig levels to Omp85, PorA, PorB, RmpM, OpcA, and OpaJ129 which were the main immunogenic bands on the blots. Significantly lower levels (P-value = 0.001 – 0.046) to these antigens were induced by 0.5 µg of the MC.6M vaccine. Antibodies to PorA contributed markedly to the bactericidal activity of the murine sera as there was a significant correlation between the Ig binding intensity to PorA on the blots and the bactericidal titres with both doses of each OMV vaccine (range of Pearson product moment correlation or Spearman rank order correlation coefficients 0.580–0.856; P-value = 0.0004–0.048).

4.6 Characterisation of the protein content of H44/76 OMVs

Further to OMV proteins with altered expression levels due to bacterial growth conditions, other proteins present in H44/76 OMVs were identified by analysis of additional 2-D spots picked from preparative gels. In total, 94 spots picked from gels of OMVs prepared in both media were assigned a protein identity following LC-MS/MS. For 26 spots MALDI analysis (PMF and/or MS/MS) was carried out in parallel. A list of the identified proteins is presented in Table 4.3. In total, 74 proteins were identified as part of H44/76 OMVs.

Table 4.3 Characterisation of the protein composition of OMVs from the Norwegian meningococcal vaccine strain 44/76. Three OMV batches were made from meningococci grown in FM or MC.6M. One mg of pooled OMV protein lysate from all batches was separated on 2D-PAGE and the gel was post-stained with Coomassie R-250. Spots with high intensity were manually excised and proteins digested and analysed by nano-scale capillary LC-MS/MS as described in section 2.13.2 with the following modifications. Liquid chromatography was carried out in a nanoAcquity UPLC (Waters, Elstree, UK) delivering a flow of approximately 300 nL/min. A C18 Symmetry 5 μ m, 180 μ m x 20 mm μ -Precolumn (Waters, Elstree, UK), trapped the peptides prior to separation on a C18 BEH130 1.7 μ m, 75 μ m x 100 mm analytical UPLC column (Waters). Tandem mass spectra were searched against Uniprot KB13.6 entries using MASCOT. Scaffold (version 2.1.03, Proteome Software Inc., Portland, OR) was used to validate MS/MS based protein identifications. Protein identifications were accepted when probability was greater than 95% and at least 2 peptides were identified.

Gene locus ^a	Protein name ^a	Protein coverage / No. of unique peptides ^b	Subcellular location ^c
NMB2134	Putative uncharacterised protein	21% / 13	Outer membrane
NMB2130	Putative uncharacterised protein	38% / 2	Unknown
NMB2101	30S ribosomal protein S2	24% / 4	Unknown
NMB2095	Putative adhesion complex protein	25% / 5	Outer membrane
NMB2039	Major outer membrane protein PorB	53% / 10	Outer membrane
NMB1998	Serine-type peptidase	1.7% / 2	Outer membrane
NMB1988	Iron-regulated outer membrane protein FrpB (FetA)	53% / 32	Outer membrane
NMB1985	Adhesion and penetration protein App	4.4% / 5	Outer membrane
NMB1972	60kDa chaperonin	57% / 27	Cytoplasm (surface-exposed) (Ferrari <i>et al.</i> , 2006)
NMB1969	Putative serotype-1-specific antigen	6.7% / 6	Outer membrane
NMB1934	ATP synthase subunit beta	11% / 3	Inner membrane
NMB1829	TonB-dependent receptor	7.5% / 3	Outer membrane
NMB1812	Type IV pilus biogenesis and competence protein PilQ	50% / 27	Outer membrane
NMB1762	Putative hemolysin activation protein HecB	5.9% / 3	Outer membrane
NMB1737	Putative secretion protein	16% / 6	Outer membrane
NMB1714	Multidrug efflux pump channel protein	14% / 4	Outer membrane
NMB1710	Glutamate dehydrogenase, NADP-specific	7.2% / 2	Cytoplasm (surface-exposed) (Gharbia and Shah, 1995)
NMB1668	Hemoglobin receptor	14% / 8	Outer membrane
NMB1623	Copper-containing nitrite reductase	6.2% / 2	Outer membrane
NMB1580	Putative uncharacterised protein	22% / 5	Cytoplasm
NMB1567	Macrophage infectivity potentiator (NmMIP)	25% / 6	Outer membrane
NMB1559	Glutathione synthetase	19% / 5	Cytoplasm
NMB1540	Lactoferrin-binding protein A	25% / 17	Outer membrane
NMB1498	Aspartokinase	11% / 3	Cytoplasm

Gene locus ^a	Protein name ^a	Protein coverage / No. of unique peptides ^b	Subcellular location ^c
NMB1497	Probable TonB-dependent receptor	30% / 21	Outer membrane
NMB1483	Putative lipoprotein NlpD	6.7% / 2	Non-cytoplasmic
NMB1429	Major outer membrane protein PorA	42% / 12	Outer membrane
NMB1313	Trigger factor	11% / 3	Cytoplasm (surface-exposed) (Ferrari <i>et al.</i> , 2006)
NMB1190	Sulfite reductase [NADPH] flavoprotein alpha-component	16% / 5	Unknown
NMB1053	Class 5 outer membrane protein OpcA	59% / 14	Outer membrane
NMB0964	Probable TonB-dependent receptor	30% / 16	Outer membrane
NMB0956	2-oxoglutarate dehydrogenase, E2 component, dihydrolipoamide succinyltransferase	16% / 3	Cytoplasm / Inner membrane
NMB0928	Uncharacterised protein NMB0928 (NlpB)	13% / 3	Periplasm
NMB0758	Polyribonucleotide nucleotidyltransferase	25% / 13	Cytoplasm
NMB0707	Putative rare lipoprotein B	42% / 6	Unknown
NMB0703	Competence lipoprotein ComL	41% / 9	Non-cytoplasmic
NMB0700	IgA-specific serine endopeptidase	11% / 15	Outer membrane
NMB0663	Outer membrane protein NspA	20% / 2	Outer membrane
NMB0634	Major ferric iron-binding protein	15% / 4	Periplasm
NMB0631	Putative phosphate acetyltransferase Pta	30% / 10	Non-cytoplasmic
NMB0589	50S ribosomal protein L19	17% / 2	Cytoplasm
NMB0554	Chaperone protein DnaK	45% / 20	Cytoplasm (surface-exposed) (Montigiani <i>et al.</i> , 2002)
NMB0496	Hemolysin activator-related protein	6.4% / 3	Outer membrane
NMB0462	Spermidine/putrescine ABC transporter, periplasmic spermidine/putrescine-binding protein	10% / 3	Periplasm
NMB0461	Transferrin-binding protein 1	21% / 15	Outer membrane
NMB0426	Cell division protein FtsA	23% / 7	Cytoplasm
NMB0387	ABC transporter, ATP-binding protein	19% / 7	Inner membrane
NMB0382	Outer membrane protein class 4 RmpM	38% / 7	Outer membrane
NMB0375/ NMB0652	Adhesin MafA	54% / 12	Outer membrane
NMB0359	Glutamine synthetase	5.5% / 2	Cytoplasm
NMB0325	50S ribosomal protein L21	19% / 2	Unknown
NMB0313	TPR repeat-containing protein NMB0313	31% / 12	Non-cytoplasmic
NMB0281	Peptidyl-prolyl cis-trans isomerase	18% / 5	Periplasm
NMB0280	LPS-assembly protein	24% / 13	Outer membrane
NMB0219	3-oxoacyl-(Acyl-carrier-protein)	23% / 5	Inner membrane

Gene locus ^a	Protein name ^a	Protein coverage / No. of unique peptides ^b	Subcellular location ^c
	synthase II		
NMB0182	Outer membrane protein Omp85	67% / 40	Outer membrane
NMB0167	30S ribosomal protein S4	21% / 3	Cytoplasm
NMB0166	30S ribosomal protein S11	18% / 3	Unknown
NMB0161	50S ribosomal protein L15	14% / 2	Unknown
NMB0158	50S ribosomal protein L18	22% / 2	Cytoplasm
NMB0157	50S ribosomal protein L6	28% / 5	Unknown
NMB0154	50S ribosomal protein L5	21% / 3	Cytoplasm
NMB0144	50S ribosomal protein L23	35% / 2	Unknown
NMB0143	50S ribosomal protein L4	41% / 6	Unknown
NMB0142	50S ribosomal protein L3	14% / 2	Cytoplasm
NMB0140	30S ribosomal protein S10	30% / 5	Cytoplasm
NMB0138			Cytoplasm (surface-exposed) (Ferrari <i>et al.</i> , 2006)
	Elongation factor G	20% / 10	Unknown
NMB0128	50S ribosomal protein L1	62% / 12	Cytoplasm (surface-exposed) (Ferrari <i>et al.</i> , 2006)
NMB0124/ NMB0139	Elongation factor Tu	40% / 12	Outer membrane / non-cytoplasmic
NMB0109	Putative uncharacterised protein	6.2% / 2	Outer membrane
NMB0088	Outer membrane protein NMB0088	32% / 10	Outer membrane
NMB0010	Phosphoglycerate kinase	12% / 4	Cytoplasm
Not available	Opacity protein OpaB128	40% / 10	Outer membrane
Not available	Opacity protein OpaJ129	6.8% / 3	Outer membrane

^a The name and corresponding gene locus of the identified proteins were derived from the annotated MC58 genome (<http://www.tigr.org>).

^b For proteins identified to be present in more than one spot the highest protein coverage detected is reported.

^c Prediction of protein localisation was carried out using the PSORTb v.3.0 subcellular localisation prediction tool.

Based on the protein localisation prediction algorithm PsortB v. 3.0 (Yu *et al.*, 2010), 32 out of the 74 identified proteins (43%) were predicted OMPs (Table 4.3). Four proteins were predicted to be located in the inner membrane and four in the periplasm. For proteins NMB0313, NMB1126/NMB1164, putative lipoprotein NlpD, putative phosphate acetyltransferase Pta and competence lipoprotein ComL, a signal peptide sequence was detected, but no further prediction as to their exact position on the cell could be deduced. Of the remaining proteins, 19 were predicted cytoplasmic

proteins 6 of which have also been detected on the meningococcal surface (Ferrari *et al.*, 2006; Gharbia *et al.*, 1995; Montigiani *et al.*, 2002). There were also examples of proteins bearing no sequence features to be associated with a specific cell compartment (labeled “unknown” in Table 4.3).

PsortB-predicted distribution in the bacterial cell of identified proteins from H44/76 OMVs was compared to that from previously published OMV protein datasets (Table 4.4). Specifically, results from proteomic studies on detergent-extracted OMVs from the New Zealand outbreak strain NZ98/254 (Ferrari *et al.*, 2006; Vipond *et al.*, 2005; Vipond *et al.*, 2006), the Cuban outbreak strain CU385 (Gil *et al.*, 2009; Ramos *et al.*, 2008; Uli *et al.*, 2006), and MC58 (Williams *et al.*, 2007) were used for comparison. Presence of each of the PsortB-predicted outer membrane and membrane-associated periplasmic proteins from H44/76 OMVs in OMVs derived from the above mentioned meningococcal strains was also assessed (Table 4.5). Similar numbers of OMPs were present in all detergent-extracted OMVs whilst the number of proteins of the cytoplasm and other cell compartments varied, as did the total number of identified proteins in each study. Of the 35 outer membrane and membrane-associated proteins found in H44/76 OMVs, 13 were also present in OMVs from all other strains compared and 32 were present in OMVs from at least one of the strains compared. Putative lipoprotein NlpD, hemolysin activator-related protein and copper-containing nitrite reductase AniA were identified in H44/76 OMVs alone. Additionally, 21 of the 35 OMPs from H44/76 OMVs were also found in naturally-released OMVs from *gna33* mutant strain (Table 4.5) (Ferrari *et al.*, 2006).

Table 4.4 Number and distribution on the bacterial cell of proteins identified in detergent-extracted OMVs. Results from this study on H44/76 OMVs were compared to datasets from proteomic studies on OMVs derived from other meningococcal strains. The strains compared and corresponding studies are highlighted in the top row.

Cellular location	H44/76 (this study)	NZ98/254 (Ferrari <i>et al.</i> , 2006)	NZ98/254 (Vipond <i>et al.</i> , 2005; Vipond <i>et al.</i> , 2006)	CU385 (Gil <i>et al.</i> , 2009)	CU385 (Ramos <i>et al.</i> , 2008)	MC58 (Williams <i>et al.</i> , 2007)
outer membrane	32	34	29	31	28	23
cytoplasm	19	75	25	23	37	45
other^a	23	39	9	52	32	43
total	74	148	63	106	97	111

^a Includes proteins located in the periplasm, inner membrane and proteins with unknown or multiple locations in the bacterial cell.

Table 4.5 Presence of OMPs from H44/76 OMVs in OMVs from other strains. Presence / absence is indicated by ✓ / -, respectively. dOMVs, detergent-extracted OMVs; mOMVs, OMVs derived from *gna33* mutant strain.

Protein name	Gene Locus	MW	Function (known or putative)	dOMVs				mOMVs
				NZ98/254 (Ferrari <i>et al.</i> , 2006; Vipond <i>et al.</i> , 2005; Vipond <i>et al.</i> , 2006)	dOMVs CU385 (Gil <i>et al.</i> , 2009; Ramos <i>et al.</i> , 2008)	dOMVs MC58 (Williams <i>et al.</i> , 2007)		
Outer membrane protein OMP85	NMB0182	88	lipid export	✓	✓	✓		✓
Fe-regulated protein B (FetA)	NMB1988	80	Iron transport and metabolism	✓	✓	–		✓
Type IV pilus biogenesis and competence protein pilQ	NMB1812	82	intracellular trafficking and secretion	✓	✓	✓		✓
Outer membrane protein OpcA	NMB1053	28	adhesion	–	✓	–		–
Putative TonB-dependent outer membrane receptor	NMB1497	104	Inorganic ion transport and metabolism	✓	✓	✓		✓
OMP PorA protein	NMB1429	42	transport	✓	✓	✓		✓
Major outer membrane protein PorB	NMB2039	36	transport	✓	✓	✓		✓
Outer membrane protein class 4 (RmpM)	NMB0382	26	cell wall/membrane biogenesis	✓	✓	✓		✓
IgA-specific serine endopeptidase	NMB0700	173	protein fate: degradation of proteins, (glyco)peptides	–	✓	–		✓
LPS-assembly protein	NMB0280	89	transport of LPS	✓	✓	✓		–
Outer membrane protein P1	NMB0088	46	lipid transport and metabolism	✓	✓	✓		✓

Protein name	Gene Locus	MW	Function (known or putative)	dOMVs			mOMVs (Ferrari <i>et al.</i> , 2006)
				NZ98/254 (Ferrari <i>et al.</i> , 2006; Vipond <i>et al.</i> , 2005; Vipond <i>et al.</i> , 2006)	dOMVs CU385 (Gil <i>et al.</i> , 2009; Ramos <i>et al.</i> , 2008)	dOMVs MC58 (Williams <i>et al.</i> , 2007)	
Hemoglobin receptor	NMB1668	89	Iron transport and metabolism	✓	✓	–	–
Transferrin-binding protein 1	NMB0461	102	Iron transport and metabolism	✓	✓	✓	✓
MafA protein	NMB0375/ NMB0652	34	adhesion	✓	✓	–	–
	NMB1540	105	Iron transport and metabolism	–	✓	✓	–
Competence lipoprotein comL	NMB0703	31	establishment of competence for transformation	✓	✓	–	✓
Opa protein	O30756_NEI ME	27	adhesion	✓	✓	✓	✓
Putative uncharacterized protein	NMB0313	56	unknown	✓	✓	–	✓
IgA-specific serine endopeptidase	NMB1985	160	App autotransporter	–	✓	✓	–
Putative uncharacterized protein	NMB2134	70	shares homology with bacterial protective antigen 15	✓	✓	✓	–
Macrophage infectivity potentiator (NmMIP)	NMB1567	29	posttranslation modification, protein turnover	✓	–	✓	✓
Serine-type peptidase (AusI)	NMB1998	165	AusI, phase-variable autotransporter serine protease	–	✓	–	–
Putative serotype-1-specific antigen	NMB1969	114	NalP autotransporter	✓	–	–	–
TonB-dependent receptor	NMB1829	77	Inorganic ion transport and	–	✓	–	–

Protein name	Gene Locus	MW	Function (known or putative)	dOMV's			mOMV's
				NZ98/254 (Ferrari <i>et al.</i> , 2006; Vipond <i>et al.</i> , 2005; Vipond <i>et al.</i> , 2006)	dOMV's CU385 (Gil <i>et al.</i> , 2009; Ramos <i>et al.</i> , 2008)	dOMV's MC58 (Williams <i>et al.</i> , 2007)	
metabolism							
Neisserial surface protein NspA	NMB0663	18	adhesion	✓	✓	✓	✓
Putative adhesin complex protein	NMB2095	20	unknown	–	✓	✓	✓
Opa protein	O30752_NEI ME	26	adhesion	✓	✓	✓	✓
Putative secretion protein	NMB1737	52	unknown	–	✓	–	–
Multidrug efflux pump channel protein	NMB1714	50	cell wall/membrane biogenesis	✓	✓	✓	✓
Putative hemolysin activation protein HecB	NMB1762	67	hemolysin transport	✓	✓	–	–
Putative lipoprotein NlpD	NMB1483	43	cell wall metabolism	–	–	–	✓
Putative uncharacterized protein	NMB0928	37	unknown	✓	✓	✓	✓
Hemolysin activator-related protein	NMB0496	66	hemolysin transport	–	–	–	–
Copper-containing nitrite reductase AniA	NMB1623	40	nitrogen metabolism	–	–	–	–
Putative uncharacterised protein	NMB1126/ NMB1164	24	unknown	✓	–	✓	✓

4.7 Discussion

As part of its adaptation to the environments it encounters during survival and infection of the human host, the meningococcus has established ways to differentiate its protein repertoire. Being the result of phase-variable genes, sequence substitutions or regulation of gene expression, the protein content of the organism can vary substantially under different conditions posed by the host. In this chapter, the differences in antigen composition and immunogenicity of OMV vaccines in response to meningococcal growth in media of different nutrient composition were assessed. Growth media were selected on the grounds of their previous use in OMV vaccine preparations and included the rich complex FM and the synthetic minimal MC.6M medium.

Overall, the results showed that the OMVs produced using the two culture media had a similar protein composition. The major porins, PorA and PorB, were expressed at similar levels, as were Omp85 and RmpM, which are involved in outer membrane synthesis and stability, respectively (Jansen *et al.*, 2000; Voulhoux *et al.*, 2003). About 3% (64/2,005) of the proteins were differentially expressed. The majority (41/64, 64%) of the differentially expressed proteins were present in higher amounts in OMVs produced in MC.6M. They included the proteins OpcA, MafA, NspA, TdfH, OMP NMB0088, lipoprotein NMB1126/1164 and the uncharacterized OMP NMB2134, which constitute confirmed or predicted proteins of the cell surface. As MC.6M is less complex than FM, it was not surprising to find that in adapting to the synthetic medium the meningococcus increased the expression of specific cell surface proteins.

Expression of the FetA protein, which belongs to the family of TonB-dependent receptors, is normally repressed in iron-rich media (Carson *et al.*, 1999). Its

inconsistent expression in both FM and MC.6M suggested that batches of both media varied in the amount of readily available iron for meningococcal growth. However, variations in iron availability alone were unlikely to account for all observed changes. There was no evidence of increased expression of other iron-repressed surface proteins, such as transferrin-binding protein or haem receptors, in the OMV preparations from bacteria grown in MC.6M. Like iron-regulated proteins, TdfH also belongs to the family of TonB-dependent receptors. It shares homology with haem receptors but does not appear to be involved in iron uptake (Turner *et al.*, 2001). Unlike FetA, it was found to be expressed consistently and at increased levels by different batches of meningococci grown in MC.6M, suggesting that the induction of TdfH was not dependent upon fluctuations in iron levels. Other metal ions present solely or in higher amounts in MC.6M might be responsible for the observed regulation of the gene coding for TdfH. Zinc was recently suggested to regulate expression of another member of the TonB-dependent receptor family encoded by the NMB0964 gene locus (Stork *et al.*, 2008). Similarly to TdfH, NspA, a surface antigen encoded by an iron-activated gene had consistently elevated levels in MC.6M (Shaik *et al.*, 2007). Increased production of NspA in MC.6M suggests that the expression of the respective *nspA* gene is affected by additional factors that have yet to be identified.

Consistent with the similar expression levels observed for major antigens PorA, PorB, RmpM and Opc85, the two OMV vaccines induced the same levels of specific antibodies to these proteins in mice. A high correlation between the titres in SBA of the mice sera and the levels of PorA-specific antibodies was observed in support of previous findings that PorA is the primary target for bactericidal antibodies in mice (Saukkonen *et al.*, 1989; Weynants *et al.*, 2007). However, mice vaccinated

with 2.0 µg of the MC.6M OMV vaccine elicited significantly higher bactericidal titres than those vaccinated with the same dose of the FM OMV vaccine. The SBA result was probably attributable to the increased expression of the small number of surface proteins, LPS or a combination of the two. Of the surface proteins with increased expression in MC.6M, OpcA, MafA, NspA and NMB0088 have all previously been shown to induce bactericidal antibodies in humans or mice (Grifantini *et al.*, 2002b; Poolman *et al.*, 1983; Sardinas *et al.*, 2009). Proteins encoded by NMB1126 and NMB2134 have been included in a study for discovery of novel vaccine candidates with no reports for their vaccine potential (Pajon *et al.*, 2009) whilst TdfH remains to be tested. LPS also induces bacterial antibodies in mice (Saukkonen *et al.*, 1989) but can also act as an adjuvant through the induction of a TLR4-dependent response (Zughaier *et al.*, 2004). The expression of meningococcal LPS has previously been shown to be affected by growth conditions (Zhu *et al.*, 2001). The relative contribution of antibodies to OpcA may have been underestimated in this study, as the target strain used in the SBA only expressed low levels of the protein (Jolley *et al.*, 2001; Rosenqvist *et al.*, 1995; Rosenqvist *et al.*, 1993). In addition, combination of antibodies to less abundant upregulated OMPs may also have contributed synergistically to increase the bactericidal titres obtained with the vaccine prepared from cells grown in MC.6M (Weynants *et al.*, 2007).

In contrast to SBA results, total IgG levels were similar between the 2 OMV vaccines. This finding may be explained by the use of a reference FM OMV as the common antigen in ELISA; however, it is more likely that the relatively few antigens with increased expression in MC.6M OMVs contributed only marginally to the total antibody levels.

The protein composition of OMVs extracted from H44/76 strain by deoxycholate treatment was similar to that reported for OMVs from outbreak strains CU385 and NZ98/254 and laboratory strain MC58 prepared in the same way (Ferrari *et al.*, 2006; Gil *et al.*, 2009; Ramos *et al.*, 2008; Uli *et al.*, 2006; Vipond *et al.*, 2005; Vipond *et al.*, 2006; Williams *et al.*, 2007). Varying numbers of cytoplasmic proteins were found in all OMV preparations as a result of the detergent extraction process. Since the studies differed in the method and conditions used during protein separation and MS analysis, some differences in the number and identity of identified proteins were expected. Inter-strain variations in the expression of specific proteins on the meningococcal outer membrane are also possible. The majority of identified OM proteins are involved in transport systems, adhesion to host cells, cell wall biogenesis and post-translation protein modification and folding. Whilst including many characterised potential vaccine candidates, the list of identified proteins contained additional interesting members of the bacterial cell surface not previously evaluated for their vaccine potential. Examples included the macrophage infectivity potentiator NmMIP, putative adhesin complex protein (NMB2095), putative secretion protein (NMB1737) and proteins potentially involved in hemolysin activation (NMB1762, NMB0496).

4.8 Concluding remarks

This study demonstrated that changes in the composition of the growth medium used for the production of OMV vaccines affected the expression of both protein and LPS antigens. Differences in antigen expression levels were associated with the ability of the vaccines to elicit a functional antibody response. Known protective protein antigens were identified which could have contributed to the

observed increase in vaccine-induced SBA in the minimal MC.6M medium. Antibodies against other differentially expressed proteins or OM proteins present in H44/76 OMVs with a yet unknown protective character might also have had a synergistic effect on results. Evaluation of such proteins in immunological assays would indicate their potential as new vaccine candidates.

Chapter 5

Immunoproteomics of serogroup B *N. meningitidis*

5. Immunoproteomics of serogroup B *N. meningitidis*

5.1 Introduction

The proteomic approaches presented in Chapters 3 and 4 helped to identify proteins on the surface of the meningococcus. The next step was to address which of these proteins can elicit an immune response and thus constitute potential antigens. Western blots using immune sera from various sources have often been used to assess response against meningococcal OMPs. Conventional 1-D gel blots allow quick detection of immunoreactive proteins but often fail to pinpoint the identities of these proteins as a result of protein co-migration onto the same band (Utt *et al.*, 2002). With the development of 2D-PAGE these limitations could be minimised and the workflow of immunoproteomics followed. In immunoproteomics, proteins are separated on 2-D gels and transferred to membranes which are then blotted with immune sera for detection of those proteins eliciting an IgG response (Kornilovs'ka *et al.*, 2002; Utt *et al.*, 2002). Immunoreactive proteins can be subsequently identified by MS analysis of the respective gel plugs.

Two-dimensional immunoproteomics has been used for analysis of immunogenic proteins from a range of bacterial pathogens including different species of *Helicobacter* (Kornilovs'ka *et al.*, 2002; Utt *et al.*, 2002) and *Streptococcus* (Lei *et al.*, 2000; Ling *et al.*, 2004), *Staphylococcus epidermidis* (Sellman *et al.*, 2005) *Clostridium difficile* (Wright *et al.*, 2008) and *Haemophilus parasuis* (Zhou *et al.*, 2009). Two reports on the meningococcal immunoproteome have also been recently published (Mendum *et al.*, 2009; Williams *et al.*, 2009). Using sera from colonised or infected individuals, both studies aimed at identifying protein targets of natural cross-protective immunity. In the study by Mendum *et al.* (Mendum *et al.*, 2009), pairs of acute and convalescent sera from patients with mild or severe septicaemia were

assessed on immunoblots whilst Williams *et al.* (Williams *et al.*, 2009) used sera from humans collected before and after their colonisation by serogroup B meningococcal strains. A varying set of immunoreactive proteins depending on the sera tested was identified by these studies some of which might be involved in protection against meningococcal disease.

In this chapter, the meningococcal proteins with ability to evoke an immune response in mice were identified by immunoproteomics. Although not a natural host for meningococci, mice are widely used for generation of immune sera to assess vaccine-induced responses during evaluation of vaccine preparations. Immune sera were raised against live meningococci and OMVs from MC58 and H44/76 strains, respectively, both formulated with aluminium hydroxide for administration in mice. Protein lysates from MC58 whole cells and H44/76 OMVs separated by 2D-PAGE were used as antigens in immunoblots. Immunoreactive protein spots detected in 2-D gels were subsequently identified by MS and also by matching to the information on protein identity of these samples presented in Chapters 3 and 4.

5.2 Immunoproteome of MC58 whole cells

5.2.1 Protein separation and immunoblotting

Total protein extracts from the *N. meningitidis* MC58 Δ -*siaD* strain were separated on 2-D gels using the same experimental conditions as those employed during analysis of the surface proteome (Chapter 3). A small amount of the protein sample labelled with the IC3 fluorescent dye was used as ‘spike’ to generate an image of total protein profile. This allowed the tracking of the efficiency of 2-D electrophoresis and transfer and facilitated subsequent matching of immunoblots to the surface proteome map generated in Chapter 3. Total protein profiles on-gel and

on-membrane following 2D-PAGE and electro-transfer are shown in Figure 5.1A and B, respectively. Comparing gel and membrane images, an almost identical spot pattern was revealed indicating the uniform and efficient transfer of proteins from gels to membranes. Membranes were incubated with serum from mice immunised with live *N. meningitidis* MC58 in the presence of iron dextran. The immunoreactive proteins detected are presented in Figure 5.2A (shown in pseudo-red) superimposed on the total protein content (shown in pseudo-green). As negative control, serum from mice given medium with iron dextran alone was used (Figure 5.2B). Immunoblots revealed a number of immunoreactive proteins the position of which matched to proteins present on the total-protein image. Most fluorescence signals of bound antibody were detected in the region of medium to low MW with varying intensities. Low fluorescence signals were detected on negative control blots which did not match to any of the proteins reacting to immune sera. These are the autofluorescent proteins also observed in SDS-PAGE gels at similar molecular masses (Table A2, Appendix).

5.2.2 Identification of immunoreactive proteins from MC58 whole cells

Immunoreactive proteins were identified by comparison with the surface proteome from which protein identities were available (Chapter 3). Spot matching between immuno- and surface 2-D proteomes was carried out using the IC3 images. In this way, proteins sharing both characteristics of immunoreactivity and surface exposure were detected. Results from immuno- and surface proteome comparison are presented in Figure 5.3, together with information on protein identity. In total, 18 out of the 26 spots of the surface proteome identified by MS also evoked an immune response in mice.

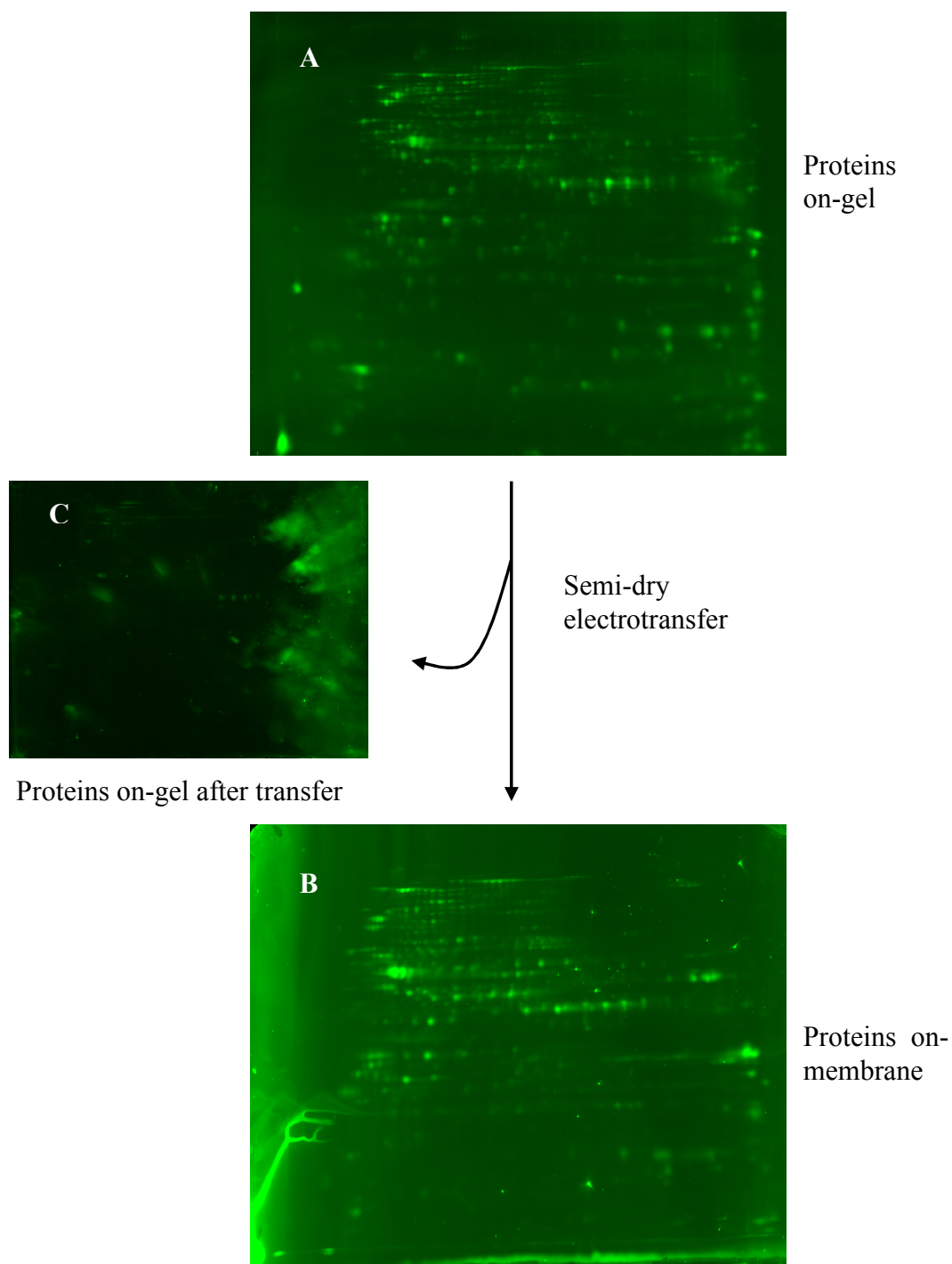


Figure 5.1 Electrotransfer of proteins from *N. meningitidis* MC58 Δ -*siaD*. Proteins labeled with IC3 dye (green) were separated on 2-D gels (pI 3-11, 12% polyacrylamide gels) (A). Proteins were subsequently transferred to PVDF membranes by application of a 437 mA current for 3.5 hr on a semi-dry device (B). Images of gels post-transfer were also acquired to check efficiency of transfer (C).

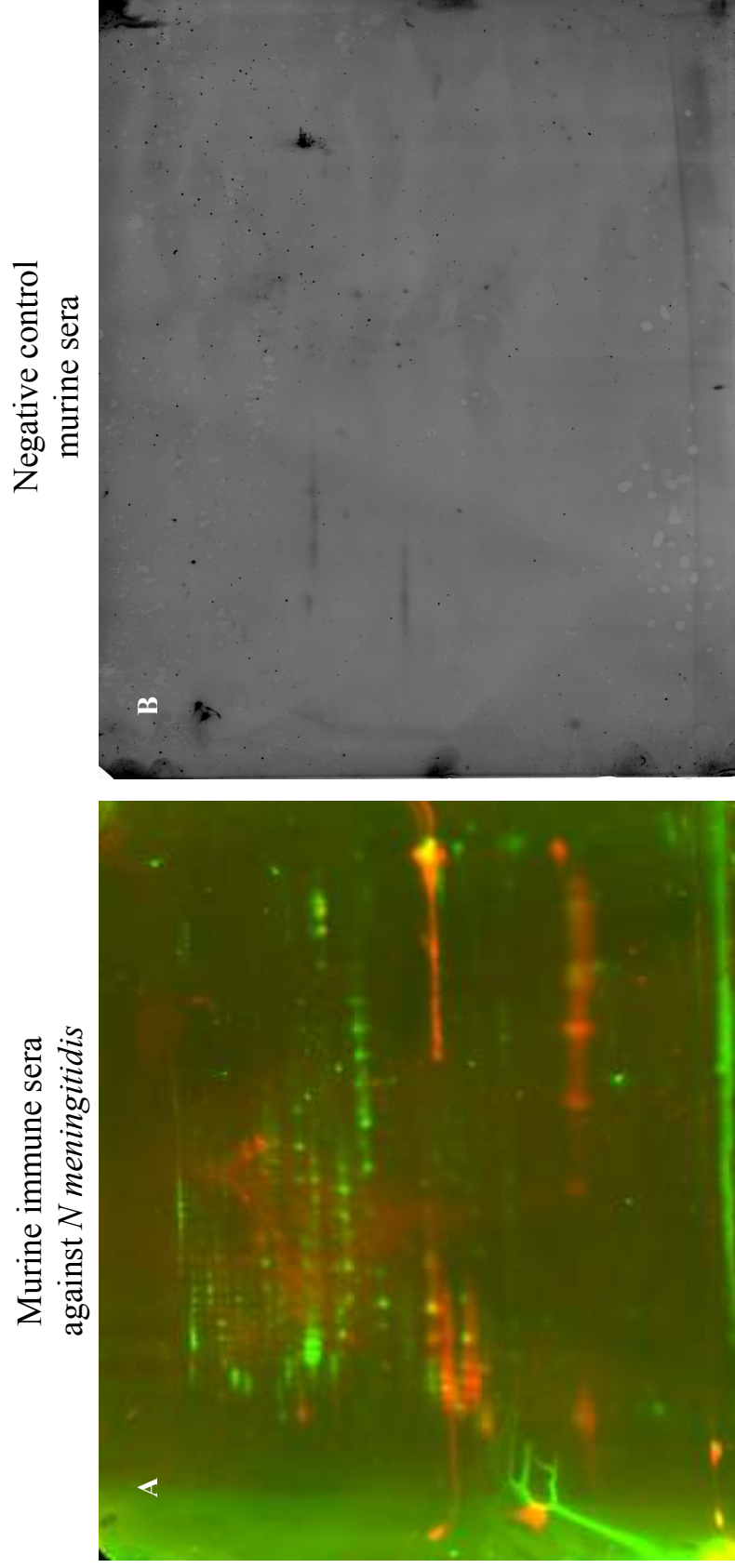


Figure 5.2 Immunoblotting of proteins from *N meningitidis* MC58 Δ -siaD. (A) Proteins transferred to PVDF membranes (green) were blotted with immune sera from mice immunised with live *N meningitidis* bacteria from MC58 strain. Immunoreactive proteins were detected using a Cy5-conjugated goat anti-mouse IgG (red/yellow). The overlaid image of total and immunoreactive proteins was created using ImageQuant tools v3.0. (B) As negative control, sera from mice immunised with culture medium alone were used for blotting and results from the Cy5 channel are shown in grey scale.

Immunoreactive spots belonged to 12 distinct proteins including PorA, OpcA, fHbp, lipoproteins NMB1946 and H.8, uncharacterized protein NMB1468, NmMIP, Pile, PilP, amino acid ABC transporters NMB1612 and NMB0787 and thiol-disulfide interchange protein DsbA2. The presence of different protein isoforms identified in previous analysis of 2-D gels was also observed in immunoblots. Trains of spots from antibodies binding to isoforms of PorA, NmMIP and Pile were detected. Also, lipoprotein NMB1946 and DsbA2 possibly generated spot trails although MS analysis for all isoform spots was not carried out. Antibody spot trails suggest different isoforms of the same protein could bind to specific antibodies present in immune serum. Smears on the trains also indicate their higher immunoreactivities compared to the other antigens.

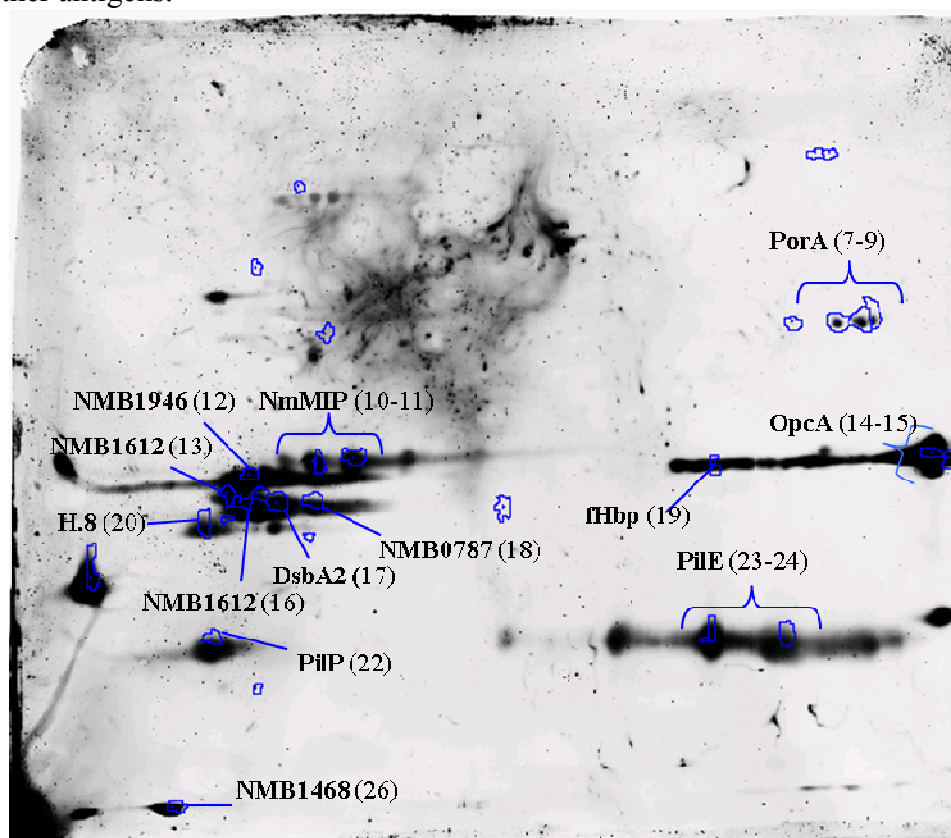


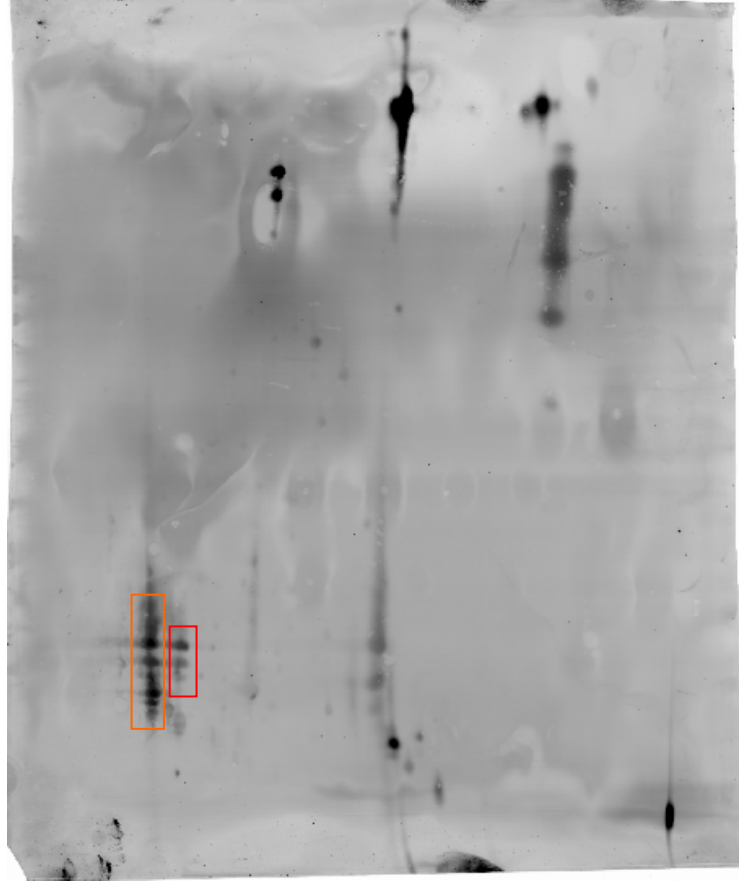
Figure 5.3 Identity of proteins reacting to sera from mice immunised with live bacteria from *N. meningitidis* MC58 strain. Images from 2-D immunoblots were matched to those from DIGE analysis of proteins from proteinase K-treated meningococci. Immunoreactive spots were matched to protein spots affected by proteinase K (highlighted by blue areas) and identities assigned based on MS results presented in Table 3.2. Matched spots are numbered according to Figure 3.6.

5.2.3 Comparison of immunoblots using MC58 wild-type and Δ -*siaD* proteins as antigens

Immunoblots were also prepared using the protein extract from wild-type MC58 strain as antigen. In this way, differences in the presence of immunogenic proteins between wild-type and Δ -*siaD* strains could be investigated and correlated with differences in protein expression levels observed by DIGE (Chapter 3, section 3.3). Immunoreactive proteins from wild type MC58 is shown in Figure 5.4, in comparison to those from Δ -*siaD*.

Differences in the antibody binding profiles between blots from the two strains were observed. Differences were mainly associated with immunoreactive spots being present solely or exhibiting more intense fluorescent signals of bound antibody in one of the blots. Antibody binding against the amino acid ABC transporter NMB0787, PilP and a third protein of an approximate molecular weight of 45 kDa was detected in the Δ -*siaD* blot only. Likewise, proteins forming a train of spots at about 70 kDa reacted with sera only in wild-type blots. The intensity in the fluorescence signal was somewhat higher for thiol-disulfide interchange protein DsbA2 in Δ -*siaD* blots whilst the trains of spots formed by DsbA2 and lipoprotein NMB1946 in Δ -*siaD* were not obvious in wild-type blots. Also in blots from wild-type MC58, an increase in signal and number of spots binding to antibody was observed for proteins forming a train of spots at an approximate molecular weight of 80 kDa. Although fluorescence signals of bound antibodies against other proteins could also be regarded as differing considerably, the significance of these variations in relative levels of protein-specific antibody binding was not assessed.

wild-type



Δ -siaD

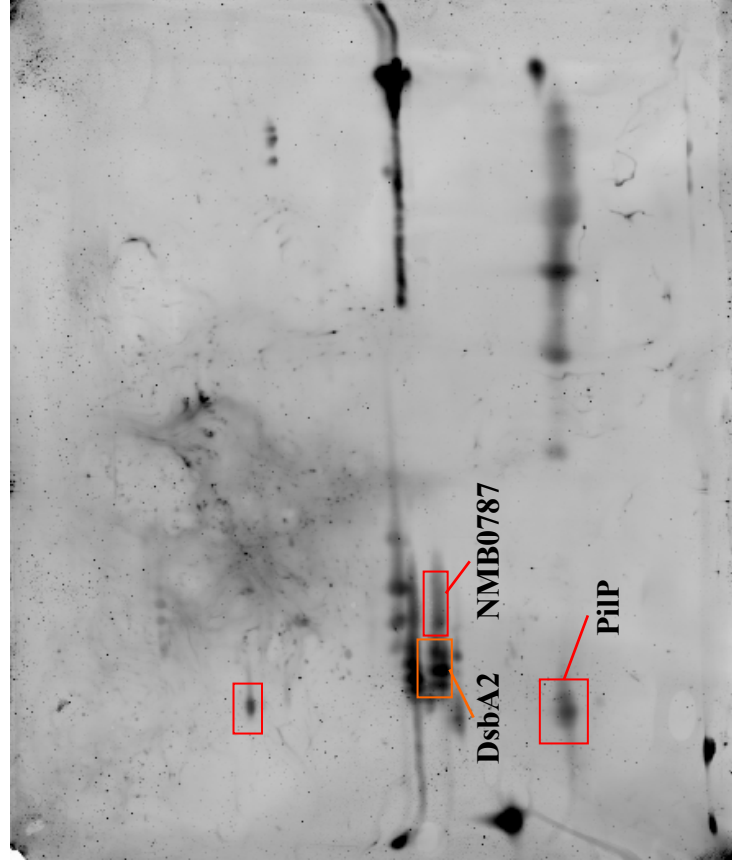


Figure 5.4 Comparison of 2-D immunoblots from wild-type and Δ -siaD MC58 strains. 200 μ g of protein from either strain were separated by 2D-PAGE and transferred onto PVDF membranes by semi-dry electro-transfer. Membranes were blotted with immune sera from mice immunised with live meningococci from wild-type MC58 strain. Bound antibodies were detected using a Cy5-conjugated goat anti-mouse IgG. Differences in antibody binding between blots from different strains are highlighted by coloured boxes, red indicating binding specific to one strain and orange indicating increased fluorescence signals for certain immunogenic proteins in one of the blots.

5.3 Immunoproteome of H44/76 OMVs

5.3.1 Protein separation and immunoblotting

Images of gels and PVDF membranes following 2D-PAGE and electro-transfer of OMV proteins from H44/76 strain, respectively, are shown in Figure 5.5. Similar protein patterns were detected from gels and membranes indicating efficient protein transfer. Membranes were blotted with serum from mice immunised with MC.6M OMV vaccine. Immunoreactive proteins in pseudo-red are shown in Figure 5.6A superimposed onto the total OMV protein (shown in pseudo-green). Response was mainly directed against the major OMPs as judged by the overlaid image. A fluorescence signal of similar magnitude to major OMPs was also detected against a minor protein of acidic pI and an approximate molecular mass of 30 kDa. Detectable fluorescence signals of antibodies binding to other minor proteins were also observed in gel areas around major OMPs. Vertical streaking caused by major OMPs during second dimension SDS-PAGE was apparent in images from both gels and blotted membranes.

5.3.2 Identification of immunoreactive proteins from H44/76 OMVs

Identification of OMV proteins reacting to immune sera was carried out by MS analysis of gel plugs from preparative 2-D gels. Preparative gels of OMVs were made under identical 2D-PAGE conditions used for immunoblots. Following matching of protein patterns from gels and blots, those protein spots from gels corresponding to the immunoreactive spots on blots were excised and subjected to trypsin digestion. Protein digests were subsequently analysed by LC MS/MS for identification of immunoreactive proteins.

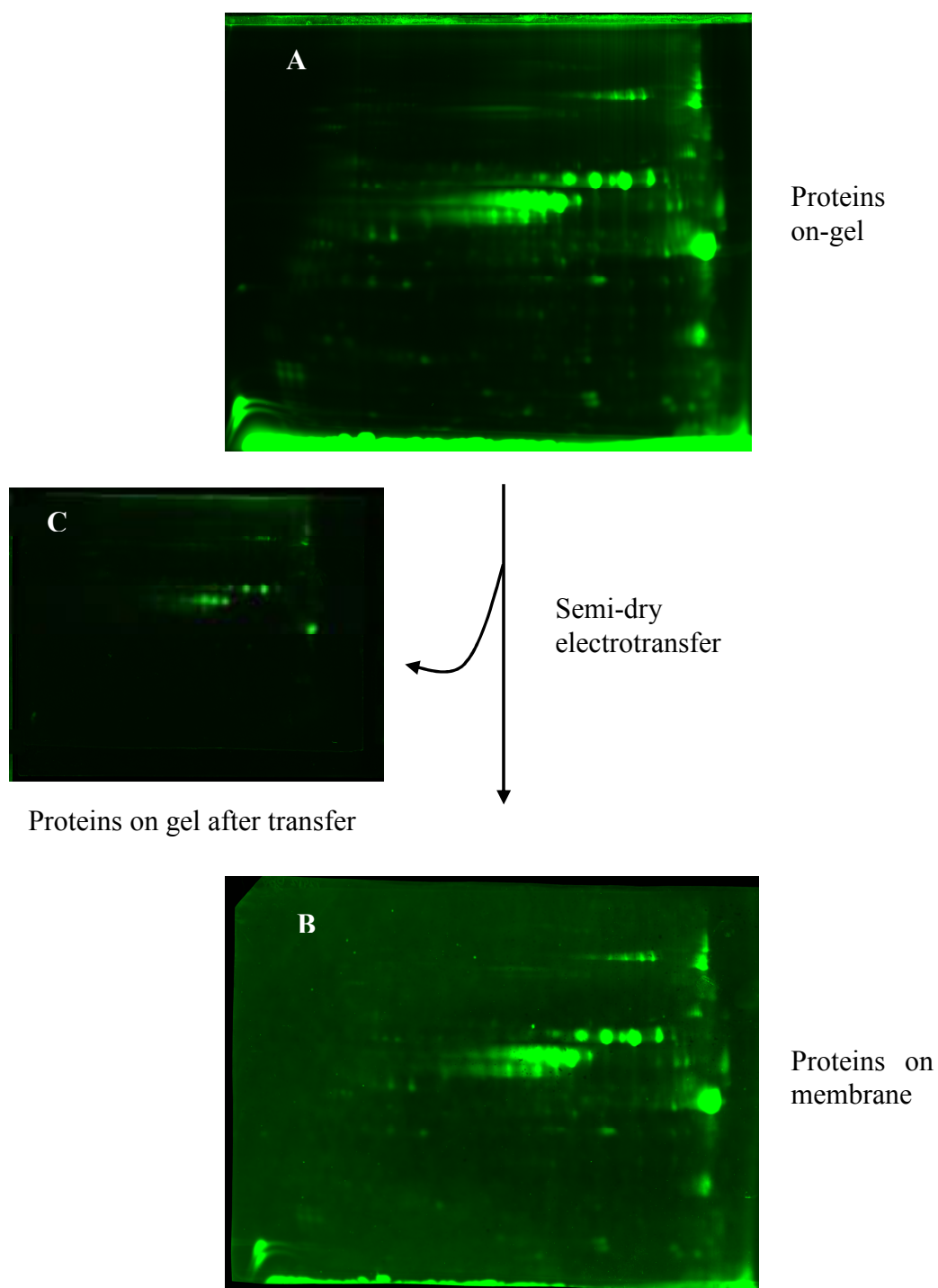
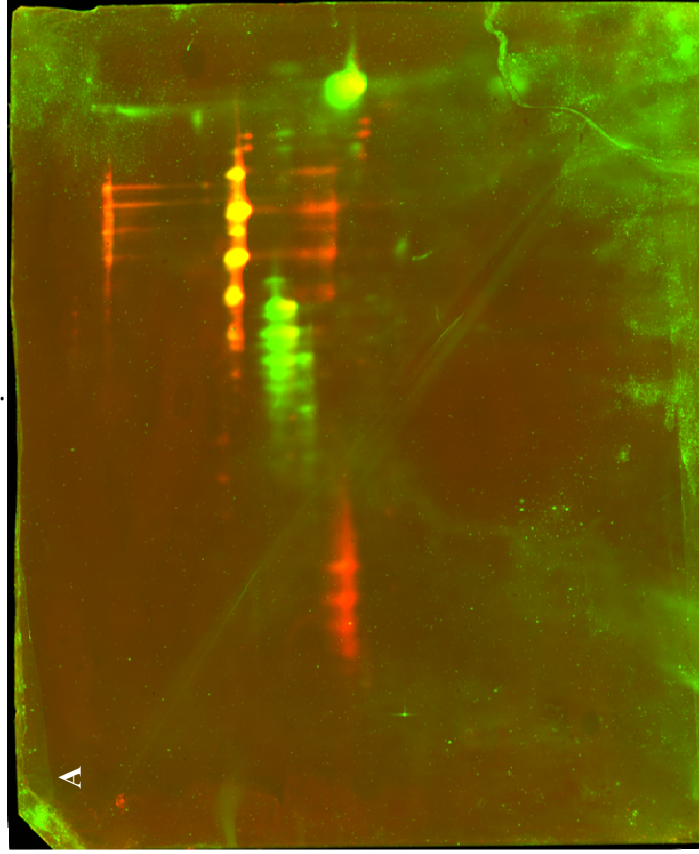


Figure 5.5 Electrotransfer of OMV proteins from *N. meningitidis* H44/76 strain. Proteins labeled with IC3 dye (green) were separated on 2-D gels (pI 3-11, 12% polyacrylamide gels) (A). Proteins were subsequently transferred to PVDF membranes by application of a 437 mA current for 3.5 hr on a semi-dry device (B). Images of gels post-transfer were also acquired to check efficiency of transfer (C).

Murine immune sera
against H44/76 OMV



Negative control
murine sera



Figure 5.6 Immunoblotting of OMV proteins from *N. meningitidis* H44/76. (A) Proteins transferred to PVDF membranes (green) were blotted with immune sera from mice immunised with MC.6M OMV vaccine. Immunoreactive proteins were detected using a Cy5-conjugated goat anti-mouse IgG (red/yellow). The overlaid image of total and immunoreactive proteins was created using ImageQuant tools v3.0. (B) As negative control, sera from mice immunised with aluminium hydroxide alone were used for blotting and results from the Cy5 channel are shown in grey scale.

In total, 24 protein spots were analysed and 8 distinct proteins identified. The position and name of identified immunoreactive OMV proteins is shown in Figure 5.7. Additional information on identified proteins and MS analysis is presented in Table 5.1. Among the proteins with strong reaction to sera were major OMPs PorA, PorB, Omp85 and OpcA. The 30 kDa minor protein of acidic pI reacting with sera at levels similar to the major OMPs was identified as the NmMIP. Other proteins binding to antibodies present in OMV sera were major OMP RmpM, a component of a multidrug efflux pump channel (MtrE) and FadL-like protein encoded by NMB0088.

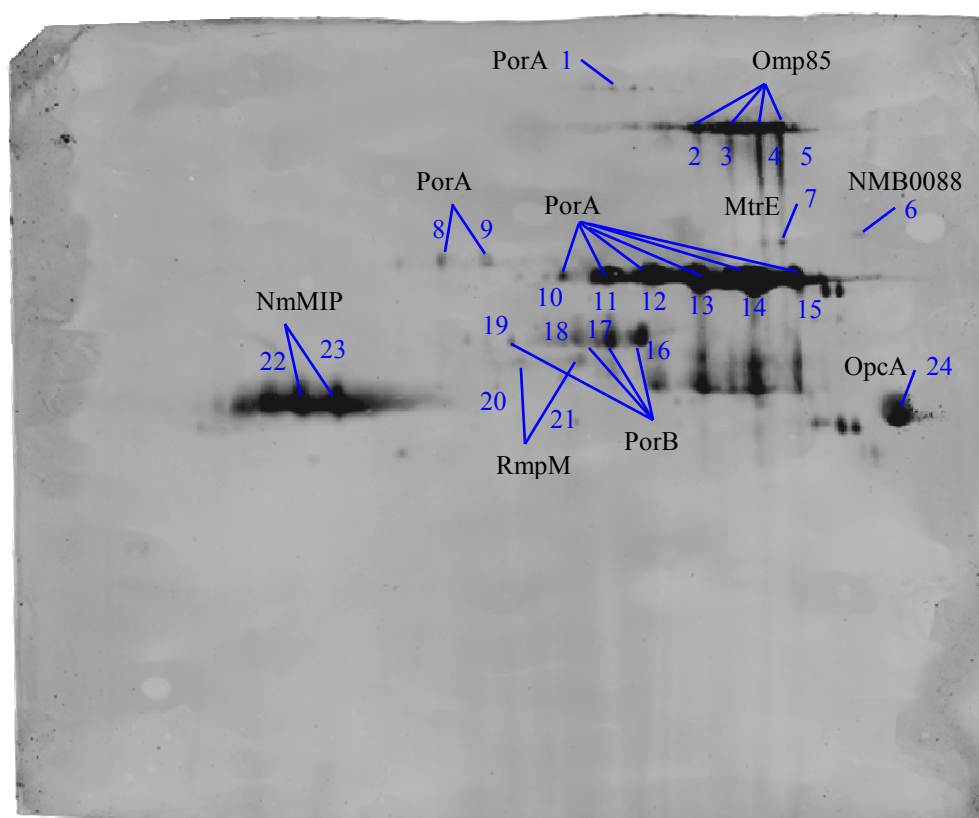


Figure 5.7 Identity of proteins reacting to sera from mice immunised with H44/76 OMV vaccine. Two-dimensional immunoblots were matched to images from preparative gels of OMV proteins using DeCyder software v.6.5. Protein spots from preparative gels matching to immunoreactive proteins on blots were excised and subjected to digestion with trypsin followed by LC-MS/MS analysis. Immunoreactive spots identified by LC-MS/MS are highlighted and numbered. Information on MS analysis is presented in Table 5.1.

Table 5.1 Mass spectrometric identification of proteins reacting to sera from mice immunised with H44/76 OMV vaccine. Gel plugs corresponding to the position on 2-D gels of proteins binding to antibodies present in sera were excised and digested by trypsin. Digests were analysed by LC-MS/MS on an Orbitrap Discovery. Proteins identified by three or more unique peptides passing the Xcorr vs charge state threshold of Sequest search engine are reported.

Gel spot number ^a	Protein name	Accession No ^b	NMB ^c	pI / MW (kDa)	No of peptides sequenced
1	PorA	Q51240	1429	8.73 / 40.1	5
2	Omp85	Q9K1H0	0182	8.37 / 86.3	34
3	Omp85	Q9K1H0	0182	8.37 / 86.3	32
4	Omp85	Q9K1H0	0182	8.37 / 86.3	28
5	Omp85	Q9K1H0	0182	8.37 / 86.3	10
6	FadL-like protein NMB0088	Q9K1M2	0088	9.28 / 48.1	16
5	PorA	Q51240	1429	8.73 / 40.1	4
6	PorA	Q51240	1429	8.73 / 40.1	9
7	Multidrug efflux pump channel protein (MtrE)	Q9JY68	1714	8.34 / 50.5	17
8	PorA	Q51240	1429	8.73 / 40.1	6
9	PorA	Q51240	1429	8.73 / 40.1	6
10	PorA	Q51240	1429	8.73 / 40.1	11
11	PorA	Q51240	1429	8.73 / 40.1	15
12	PorA	Q51240	1429	8.73 / 40.1	21
13	PorA	Q51240	1429	8.73 / 40.1	20
14	PorA	Q51240	1429	8.73 / 40.1	23
15	PorA	Q51240	1429	8.73 / 40.1	18
16	PorB	P30690	2039	6.54 / 33.8	29
17	PorB	P30690	2039	6.54 / 33.8	23
18	PorB	P30690	2039	6.54 / 33.8	22
19	PorB	P30690	2039	6.54 / 33.8	17
20	RmpM	P0A0V3	0382	6.26 / 24.0	6
	PorB	P30690	2039	6.54 / 33.8	3

Gel spot number ^a	Protein name	Accession No ^b	NMB ^c	pI / MW (kDa)	No of peptides sequenced
21	RmpM	P0A0V3	0382	6.26 / 24.0	5
	PorB	P30690	2039	6.54 / 33.8	5
22	Macrophage infectivity potentiator (NmMIP)	Q9JYI8	1567	5.5 / 26.9	12
23	Macrophage infectivity potentiator (NmMIP)	Q9JYI8	1567	5.5 / 26.9	9
24	OpcA	Q7DDI3	1053	9.68 / 28.1	19

^a Spot numbers as they appear in Figure 5.7.

^b Accession numbers were derived from UniProtKB/Swiss-Prot database.

^c Gene locus numbers (NMB) are based on the annotated MC58 genome sequence.

5.4 Discussion

The use of immunoproteomics in combination with fluorescence technology offered an accurate tool for detection of immunoreactive meningococcal proteins. Overlaid images of gels and blots from both whole-cell and OMV protein preparations helped matching individual immunoreactive proteins to their position on 2-D gels. Correct assignment of the position of proteins on gel, in turn, allowed identification of proteins by correlation to previously existing protein identities or MS analysis of the corresponding gel plugs from preparative gels.

Among the identified immunoreactive proteins were major OMPs and known immunogens PorA, OpcA, PorB, RmpM and Omp85. Response against these proteins has been reported in studies assessing immunity induced by OMV vaccines as well as analysis of sera from naturally infected humans (Delvig *et al.*, 1996; Litt *et al.*, 2004; Mandrell *et al.*, 1989; Wedege *et al.*, 2007; Wedege *et al.*, 1998). Antibodies to major OMPs PorB, RmpM and Omp85 were detected in 2-D immunoblots using sera from mice immunised with OMV vaccines but not the whole organism. A different topology leading to weaker exposure of these proteins to immune system mechanisms on the surface of live meningococci as opposed to OMVs could explain this

observation. Weak binding of PorB antibodies on the surface of live meningococci has been previously documented in flow cytometry experiments (Michaelson *et al.*, 2001).

Twelve minor proteins were shown to react to murine sera raised against whole meningococci or OMV vaccine. Of these, 9 (fHbp, PilE, H.8, NMB1946, NMB1612, NMB1468, DsbA2, NMB0787 and PilP) were recognized by sera against whole meningococci and 2 (MtrE and FadL-like NMB0088) by sera against OMV vaccine. NmMIP was the only minor protein that reacted to both sera tested. The 9 proteins that were not recognised by OMV sera had not been identified during characterisation of H44/76 OMVs (section 4.6) and might indeed not be present in OMV preparations. These proteins could either be located away from the outer membrane or be stripped off the membrane upon detergent extraction of OMVs. On the other hand, absence of MtrE and FadL-like specific antibodies in sera from whole meningococci might reflect weak exposure of these proteins on the surface of live bacteria. The possible enrichment of these proteins in OMV preparations as opposed to whole bacteria might have also contributed to their ability to evoke an immune response in mice immunized with OMV vaccine formulations.

Minor immunoreactive proteins included previously reported immunogens fHbp, pilin PilE, lipoproteins H.8 and NMB1946, the amino acid ABC transporter encoded by NMB1612, FadL-like protein and protein encoded by NMB1468. Antibodies against fHbp and H.8 have previously been detected in human sera from convalescing patients (Black *et al.*, 1985; Litt *et al.*, 2004). Pilin PilE and amino acid ABC transporter NMB1612 have also exhibited reactivity to sera from humans colonised by serogroup B meningococcal strains (Williams *et al.*, 2009) whilst antibodies specific for NMB1468 have been detected in sera from mice immunised

with a menB strain different to the one used in this study (Hsu *et al.*, 2008). The presence of antibodies against lipoprotein NMB1946 or FadL-like protein in murine sera generated against whole meningococci has not been reported elsewhere, however, specific antibodies generated against these proteins in mice have been assessed in SBA and confirmed to be bactericidal (Pizza *et al.*, 2000; Sardinas *et al.*, 2009).

For minor proteins NmMIP, PilP, DsbA2 and amino acid ABC transporter encoded by NMB0787 this study was the first to report their immunogenic properties. NMB0787 has been previously assessed for its potential as vaccine candidate but failed to produce bactericidal antibodies in a study by Grifantini *et al.* (Grifantini *et al.*, 2002b), the rest remain to be tested. Low expression of NMB0787 was detected in wild-type MC58 by DIGE (section 3.3) which was also confirmed by immunoblots from this strain where no antibody binding to NMB0787 was observed. Low expression levels could explain failure of NMB0787 specific antibodies to kill meningococci in SBA. Conversely, the decreased antibody binding to DsbA2 observed in blots from wild-type MC58 compared to Δ -*siaD* was not the result of lower expression levels of DsbA2 in the wild-type strain. Similarly, antibody binding to PilP was absent from immunoblots against wild-type MC58 which could not be explained by differences in relative expression levels between the two strains. On the contrary, a strong response against NmMIP was detected regardless of sera or meningococcal strain type used in blots suggesting NmMIP could be a promising candidate to be prioritised in immunological assays.

The immunoproteomes presented in this study bear little similarity to the two published immunoproteomes assessing reactivity to human sera from colonised individuals and convalescing patients (Mendum *et al.*, 2009; Williams *et al.*, 2009).

PorA was the only protein present on immunoblots from all studies whilst H.8, Pile and amino acid ABC transporter NMB1612 were recognised by both post-colonisation sera and murine sera against whole meningococci. The remaining proteins reacting to murine sera were not recognised by human sera in the published immunoproteomes. On the other hand, there were proteins recognised by human sera, which were absent in blots with murine sera presented in this study. These results highlight a highly variable response depending on the source of sera which can be explained when considering human is the only host for meningococci as opposed to mice which do not contract meningitis. Part of the discrepancies in the results presented here and in published studies could also be attributed to technical variation during 2-D immunoblotting. Such variations can be caused during the choice of meningococcal strain, protein sample used as antigen and 2D-PAGE conditions. In fact, this and published immunoproteomic studies varied in all these parameters. This could also explain why OpcA and fHbp with known ability to elicit an immune response in humans were identified in this study but not in the published immunoproteomes. Another explanation to the absence of known immunogenic proteins from blots might be the fact that electrophoretic protein separation is carried out under denaturing conditions. In this way, proteins recognised by antibodies through conformational epitopes would be missed.

Chapter 6

Evaluation of meningococcal surface proteins as potential vaccine candidates

6. Evaluation of meningococcal surface proteins as potential vaccine candidates

6.1 Introduction

Surface exposure is not the only prerequisite for a protein to be considered as a vaccine candidate. The protein's ability to elicit an immune response, *i.e.* functional antibodies that offer the potential of protecting against meningococcal infection should be demonstrated. Factors that can influence this ability include the sequence diversity and abundance of the protein on the bacterial surface. High protein expression levels can result in high antibody density on the surface of meningococci facilitating recruitment of complement and initiation of bacterial killing. Sequence variability as a result of positive immune selection is also considered an indication of a strong immunogen. Addressing these qualities is therefore an important element of the evaluation of a protein's vaccine potential.

This chapter presents information towards the evaluation of the vaccine candidacy of 4 meningococcal proteins, namely NmMIP, PilP, PilO and the putative adhesion complex protein NMB2095. These proteins were identified through proteomic analyses described in chapters 3, 4 and 5 as potentially surface-exposed, and in some cases, immunoreactive. During the design and performance of this study, no report existed regarding the characterisation of any of these proteins as protein vaccine candidates.

Macrophage infectivity potentiator, NmMIP, is a predicted lipoprotein and a member of the family of FKBP-type peptidyl-prolyl cis-trans isomerases involved in protein folding. Macrophage infectivity potentiators are virulence factors in intracellular bacterial pathogens contributing to persistence or survival in macrophages (Cianciotto *et al.*, 1992). In the current study, NmMIP was found to be

part of both the surface and OMV proteomes. NmMIP peptides were also detected in the supernatants of trypsin-treated meningococci. A strong reaction of NmMIP to antibodies present in sera from mice immunised with live *N. meningitidis* or meningococcal OMV preparations was also observed in 2-D immunoblots.

PilP and PilO are 2 of the proteins required for tfp expression (Drake *et al.*, 1997). Lipoprotein PilP is also involved in tfp assembly through interactions with the secretin PilQ (Balasingham *et al.*, 2007). Both PilP and PilO were identified in the study of the surface proteome where they were partially cleaved by proteinase K. PilP was additionally shown to react with sera from mice immunised with live bacteria from *N. meningitidis* MC58 strain. In a previous study, the gene encoding PilO was upregulated during interaction of meningococci with human epithelial cells (Dietrich *et al.*, 2003).

The putative adhesin complex protein NMB2095 shares homology with adhesion components from other neisserial and bacterial species and bears a signal peptide sequence. Based on its detection in supernatants prior to incubation of meningococci with proteases, this protein was suggested to be loosely attached to the surface of meningococci. NMB2095 was also found to be part of the OMV preparations analysed in this and other studies (Ferrari *et al.*, 2006; Williams *et al.*, 2007).

As part of their evaluation, the genes corresponding to these proteins were cloned in plasmid expression vectors and expressed in *E. coli*. The resulting recombinant proteins were extracted, purified and used to immunise groups of mice for production of protein-specific polyclonal antibodies. Immune sera were subsequently used in protease-treatment assays and whole-cell ELISA for assessing the surface exposure of proteins. Immune sera were also evaluated in immunological

assays for detection of antibody-dependent complement deposition, opsonophagocytosis and complement-mediated killing of meningococci.

NmMIP was further assessed for sequence variation using sequence data from a panel of 106 meningococcal strains encompassing isolates from various serogroups and clonal complexes. Sequence data were also used to detect regions of selective pressure and draw phylogenetic relationships between sequence variations and genotypic background of strains. Information on a possible topological and structural model of NmMIP is also presented.

6.2 Bioinformatic analysis of potential vaccine candidates

To facilitate subsequent primer design and cloning into plasmid vectors, the potential candidates were assessed for their prevalence and sequence conservation across the annotated *N. meningitidis* genomes as well as for presence of predicted signal peptide sequences.

6.2.1 Sequence characteristics and alignments

At the time of analysis of sequence data, the annotated genome sequences of 10 *N. meningitidis* strains were freely available from Genbank (www.ncbi.nlm.nih.gov/genbank). These included invasive strains MC58 (ST-32, serogroup B), Z2491 (ST-4, serogroup A), FAM18 (ST-11, serogroup C), 8013 (ST-18, serogroup C), 053442 (ST-4821, serogroup C), WUE2594 (ST-5, serogroup A) and carriage strains alpha14 (ST-53, unencapsulated), alpha153 (ST-60, serogroup 29E), alpha275 (ST-22, serogroup W-135) and alpha710 (ST41/44, serogroup B). The presence of ORFs encoding NmMIP, PilP, PilO and adhesin complex protein in these genomes is presented in Table 6.1. Open reading frames corresponding to NmMIP

and adhesin complex protein were detected in all annotated genomes. In contrast, PilP and PilO were detected in some strains and their presence was irrespective of serogroup or sequence type. No homologues of PilP were present in carrier strains. However, these results are only based on the 10 publically available annotated genomes and this limited dataset may not be representative of the entire meningococcal population.

Table 6.1 Presence of potential vaccine candidates in the annotated *N. meningitidis* genomes. Genome sequences for the strains presented are available from Genbank. The + / - symbols correspond to presence / absence of a homologous gene sequence in the genome of strains following BLAST with the corresponding nucleotide sequence of each candidate from MC58 genome.

<i>N. meningitidis</i> strain	Protein name			
	NmMIP	PilP	PilO	Putative adhesin complex protein
MC58	+	+	+	+
Z2491	+	+	-	+
FAM18	+	+	+	+
8013	+	-	-	+
053442	+	+	+	+
WUE2594	+	-	+	+
alpha14	+	-	-	+
alpha153	+	-	-	+
alpha275	+	-	+	+
alpha710	+	-	-	+

The amino acid sequences deduced for each potential candidate were aligned using the MegAlign tool from DNASTAR Lasergene software v.8. Alignments are presented in Appendix (Figure A3). For NmMIP sequences, a 30-amino acid amino-terminal (N-terminal) extension was observed in some strains while missing from others. A 4-amino acid deletion was also present in some strains. The remaining sequence was 96.5% identical across all ten strains. PilP and PilO sequences exhibited 99.5% and 99% identity, respectively. Finally, adhesion complex protein appeared to have a 62-amino acid-extended N-terminal domain in 4 out of 10 strains. From the 124 amino acid sequence that was common across all strains, 123 amino acids were identical (99.2%).

6.2.2 Signal peptide sequences

Signal peptides are cleavable sequences at the N-terminal of the protein which are responsible for activating the translocation machinery for protein secretion across the cytoplasmic inner membrane and into the periplasm. Here, the chosen proteins were examined for the presence and exact cleavage site of signal peptides to determine the sequence of the mature protein. The nucleotide sequence corresponding to the mature protein sequence subsequently formed the template for primer design and cloning into plasmid vectors.

The presence and location of signal peptide cleavage sites in the amino acid sequences from the annotated MC58 genome was investigated using SignalP 3.0 (Bendtsen *et al.*, 2004). SignalP predicts signal peptidase I cleavage sites based on a combination of neural network (NN) and hidden Markov model (HMM) algorithms. The HMM calculates the probability of the presence of a signal peptide to differentiate between secreted and non-secreted proteins whilst the NN detects the site

of cleavage by assigning different scores to amino acids indicating if they are part of the signal sequence and if they are next to a cleavage site.

The amino acid sequence corresponding to NmMIP was shown to contain multiple possible cleavage sites with similar scores based on the NN algorithm. Two positions between amino acids 31-32 and 22-23 were suggested as the most likely cleavage sites. However, a low S-score was calculated for the sequence between amino acids 23 and 31 suggesting that this region has a low probability to form part of the signal peptide. Further confirmation that cleavage would more likely occur between amino acids 22 and 23 was provided by the HMM algorithm, which calculated a high cleavage probability for this site.

PilP amino acid sequence also exhibited multiple cleavage sites based on results from NN with the position between amino acids 23-24 being the most probable cleavage site and position 19-20 exhibiting the second best score. The HMM algorithm suggested position 19-20 as the most probable cleavage site with high probability score. According to von Heijne (Von Heijne, 1983), the most common amino acids expected at position -1 of the cleavage site are alanine, glycine, serine, cysteine, glutamine and threonine whilst the least expected amino acids at position -3 are lysine, arginine, asparagine, glutamine, tyrosine, aspartic acid, glutamic acid, tryptophan, phenylalanine and histidine. Taking this into account, the site between amino acids 19-20 which has glycine and serine at positions -1 and -3, respectively, was the most likely cleavage site as opposed to the site between amino acids 23-24 with leucine and glutamic acid at positions -1 and -3, respectively.

The amino acid sequence of PilO contained a possible cleavage site between amino acids 32 and 33 according to the NN algorithm. Although the HMM also detected a cleavage site at the same position, it calculated a higher score for the

position between amino acids 40 and 41. However, the HMM is mainly designed to predict the presence of the signal sequence and is not as accurate in predicting the cleavage site as the NN algorithm. Additionally, the average length of signal peptides from Gram-negative bacteria is expected to lie within 20 to 30 amino acids (Bendtsen *et al.*, 2004); therefore, cleavage after the 40th amino acid for PilO would be highly unlikely.

Finally, for the putative adhesin complex protein, both the NN and HMM were in agreement suggesting that signal peptide cleavage site was between amino acids 21 and 22.

6.3 PCR amplification

Conditions for PCR amplification were optimised using the primer pair designed for NmMIP. Parameters assessed included the annealing temperature, the starting amount of genomic DNA and concentration of MgCl₂. Annealing temperatures were tested in the range of 54°C to 69°C and MgCl₂ at concentrations between 0 and 2 mM. Figure 6.1 shows the PCR products yielded for each combination of conditions tested. PCR reactions at 63°C in the presence of 1 mM MgCl₂ gave the most specific product. The different amounts of genomic DNA tested had little effect on results and the lowest amount was chosen for subsequent analysis. Under these conditions, PCR products for all 4 genes were obtained (Figure 6.2). From agarose gel electrophoresis, the size of PCR fragments was shown to correspond to the expected size of each amplified gene.

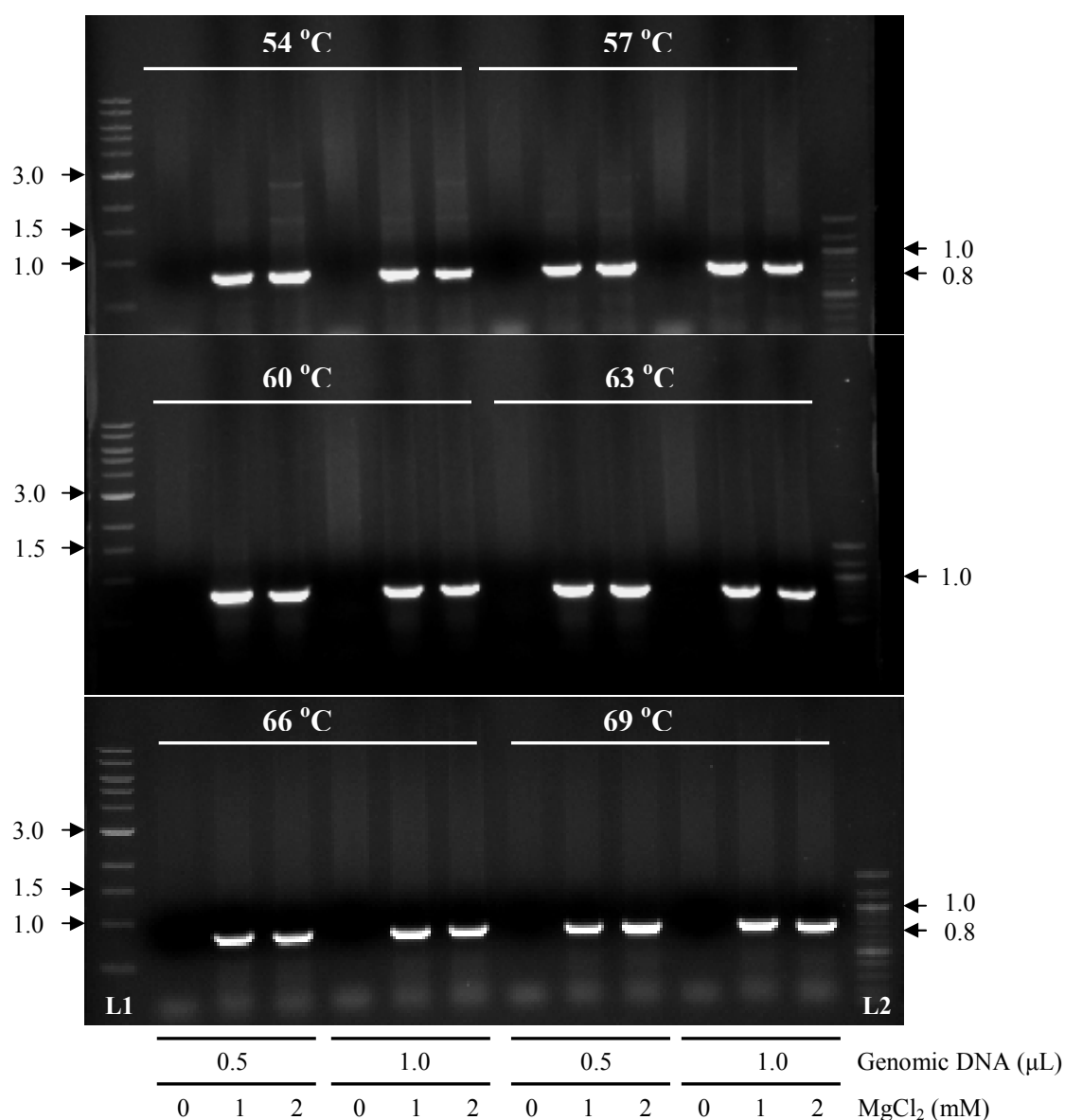


Figure 6.1 Optimisation of PCR amplification conditions for *fkpA* (NmMIP). Reaction mixtures containing 0.5 or 1 μ L of genomic DNA extracted from MC58 *N meningitidis* were prepared and supplemented with MgCl₂ at concentrations of 1 or 2 mM. Mixtures with no added MgCl₂ were also prepared (0 mM). Aliquots were subjected to PCR at different annealing temperatures at a range of 54 to 69 °C. PCR products were run on a 0.8% TAE agarose gel at 40 mA for 2 hr. DNA was stained with SafeView nucleic acid stain and visualized using the KODAK Gel Logic 1500 imaging system. DNA ladder sizes are shown in kbp on the left and right edges. L1: 1 kbp DNA ladder, L2: 100 bp DNA ladder.

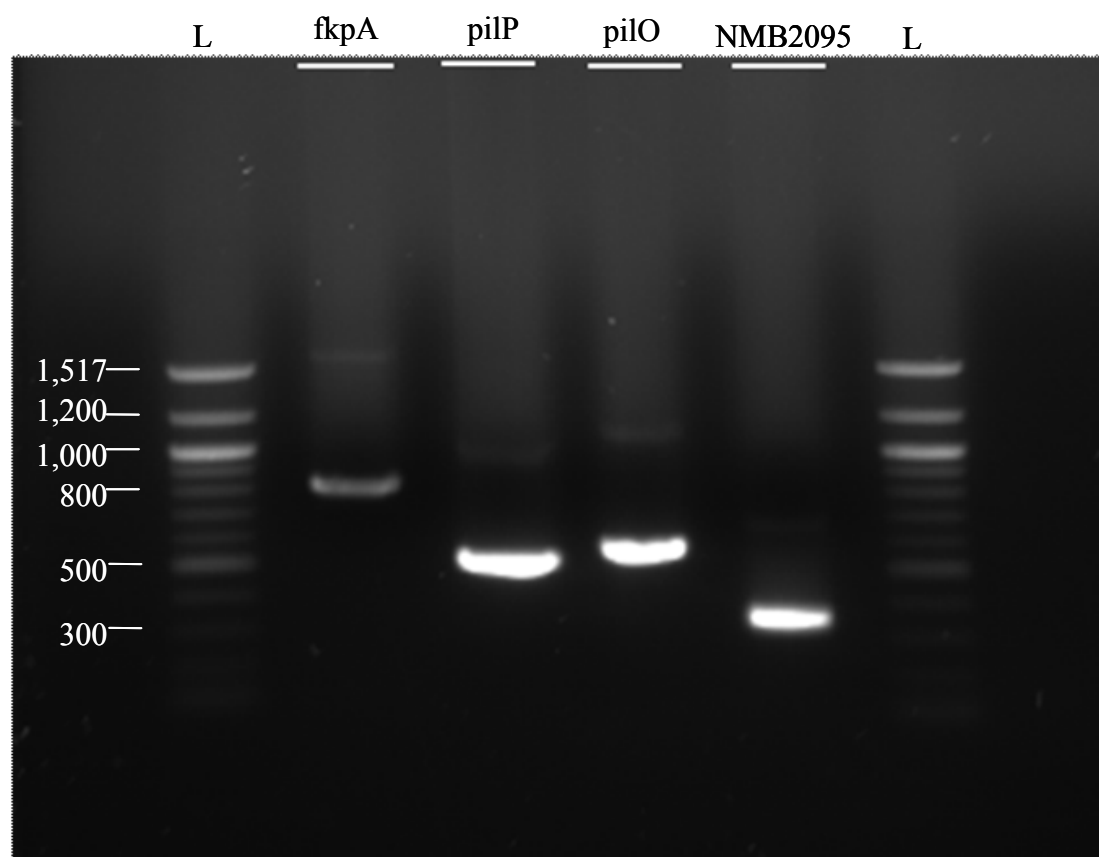


Figure 6.2 Agarose gel electrophoresis of PCR products. Genes *fkpA* (NMB1567), *pilP* (NMB1811), *pilO* (NMB1810) and NMB2095 were amplified from *N. meningitidis* MC58 genomic DNA. Primers were designed to introduce sequences for ligation-independent cloning (LIC) of the genes to the pET30 Ek/LIC vector system (listed in Table 2.3). The size of bands from the DNA ladder is presented on the left in bp. L: 100 bp DNA ladder.

6.4 Production and purification of recombinant meningococcal proteins

PCR products were cloned into pET Ek/LIC plasmid vectors in frame and downstream of a sequence coding for a 6-Histidine tag to facilitate subsequent protein purification by affinity chromatography. Plasmids containing the gene of interest were then used to transform Novablue *E. coli* competent cells. Successful in-frame insertion of all 4 genes into plasmid vectors was achieved as assessed by a) colony-PCR of *E. coli* transformants and b) sequencing of plasmids purified from *E. coli* cultures. No nucleotide substitutions were detected when sequencing data were compared to the sequences from the annotated MC58 genome.

Escherichia coli BL21 pLysS strain was used for expression of protein candidates following transformation with the purified plasmids containing the corresponding gene. BL21 pLysS cells express the T7 RNA polymerase required for transcription of the gene of interest, which is under the control of T7 promoter in the pET plasmid vector. Protein expression is achieved through IPTG induction of the *lac* UV5 promoter of T7 RNA polymerase. Additionally, the T7 lysozyme encoded by the pLysS gene ensures low background expression levels of the target protein in the absence of IPTG. Protein production for up to 5 hr upon IPTG induction was assessed by SDS-PAGE of crude extracts from *E. coli* cell cultures and presented in Figure 6.3. Crude extracts were prepared from resuspension of *E. coli* cell pellets into Laemmli sample buffer and were directly applied onto polyacrylamide gels. SDS-PAGE of extracts from non-induced cultures is also shown in comparison. All 4 proteins were shown to be produced in relatively high levels in the IPTG-induced samples. The amount of NmMIP produced reached a maximum after 3 hours of IPTG induction. Similarly, the highest yield for PilP, PilO and adhesin complex protein was achieved at 5, 4 and 2 hours following addition of IPTG, respectively. Bands corresponding to over-expressed proteins were not present in non-induced samples. Total *E. coli* protein levels were shown to be reduced in IPTG-induced samples probably as a compensation for the high rate of protein synthesis required for expression of recombinant meningococcal proteins.

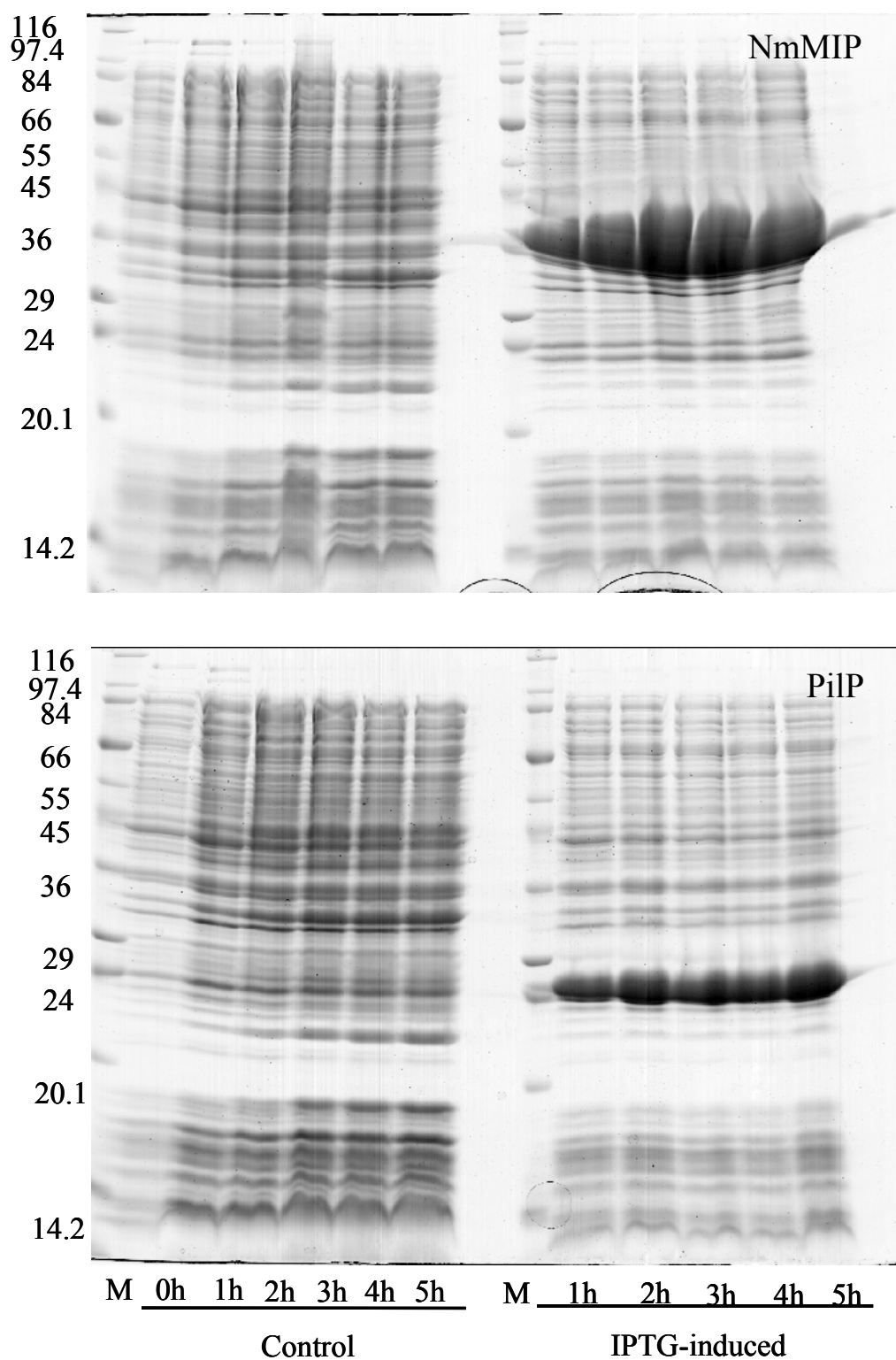


Figure 6.3 SDS-PAGE of crude extracts from cultures of *E. coli* cells transformed with plasmid expression vectors (continues in the next page).

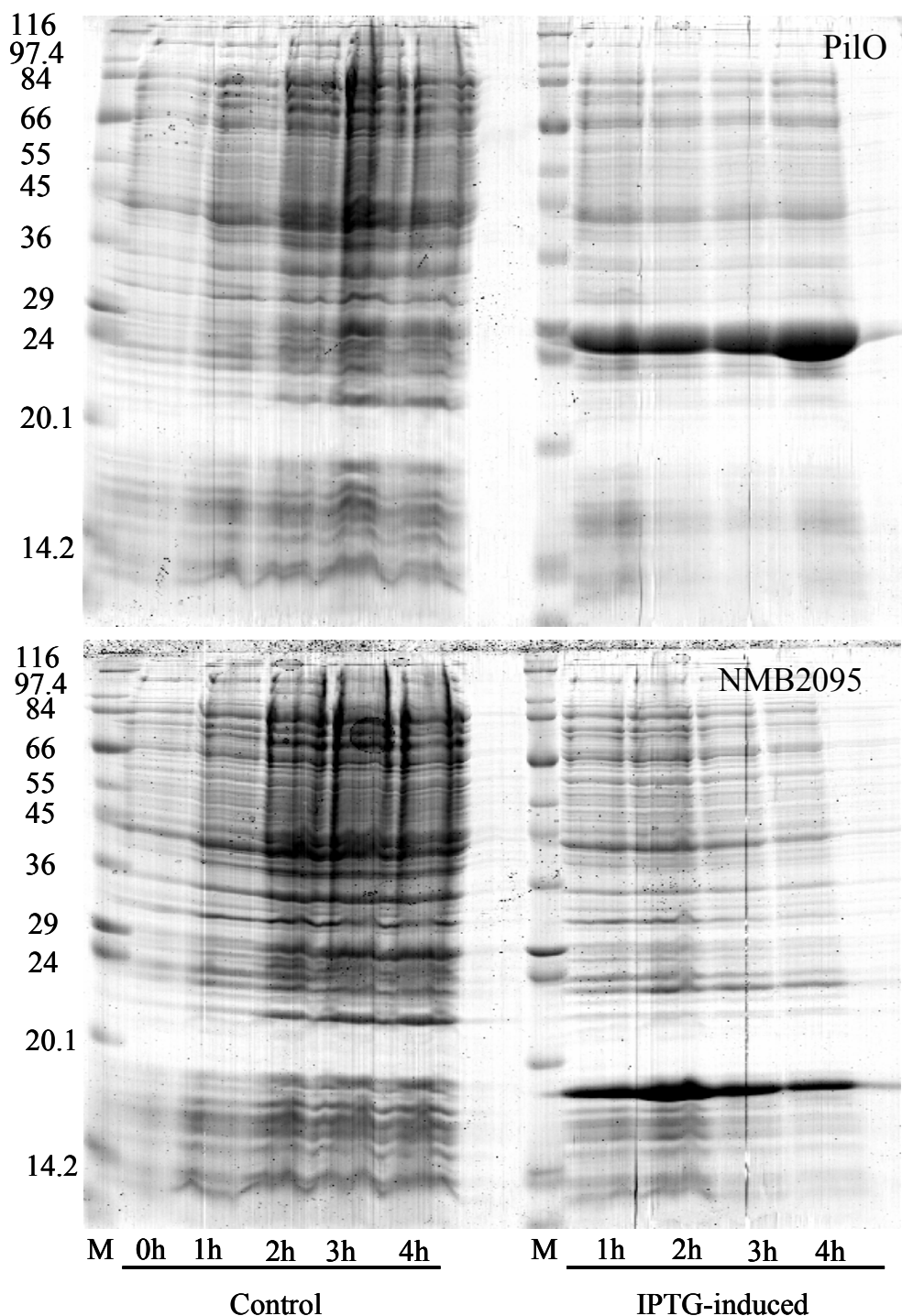


Figure 6.3 SDS-PAGE of crude extracts from cultures of *E. coli* cells transformed with plasmid expression vectors (continues from previous page). Equal volumes of samples were collected from cell cultures over a period of 4-5 hr following induction with IPTG, reconstituted in Laemmli sample buffer and run on a 12% polyacrylamide gel. Samples from non-induced cultures were also collected and run alongside as a negative control. The MW (in kDa) of bands of the protein marker is shown on the left. M: SigmaMarkerTM wide-range MW protein marker.

Following the lysis of *E. coli* cells using the BugBuster extraction reagent, both the soluble supernatant and insoluble cell debris fractions were collected and assessed for the presence of the recombinant proteins in SDS-PAGE. NmMIP, PilP and adhesin complex protein were found in the soluble fraction whilst PilO was in the insoluble fraction (Figure 6.4). Proteins in the soluble fractions were directly processed for affinity purification. The insoluble cell pellet containing PilO was instead processed for purification of inclusion bodies by urea treatment. During this process, the majority of PilO was recovered in the soluble fractions prior to urea treatment, which were then used for affinity purification (Figure 6.4, PilO lane 3).

The soluble fractions containing the recombinant proteins were applied to nickel-charged resins and retained by affinity binding of their His-tag sequence to nickel ions. Selective elution of recombinant proteins was achieved by increasing concentrations of imidazole. After *E. coli* proteins were washed away at low imidazole concentrations, His-tagged proteins were released at high imidazole concentrations through the competition of imidazole for nickel ions. Elution fractions containing the purified recombinant proteins were analysed by SDS-PAGE and are presented in Figure 6.5. High purity was achieved for NmMIP, PilO and adhesin complex protein. The bands corresponding to these proteins exhibited approximate MW of 36, 26 and 18 kDa, respectively. In the PilP fraction, two main bands of similar high abundance and MW of 25 and 12 kDa were detected. The higher MW band probably corresponded to intact PilP whilst the 12 kDa band was more likely a truncated version of PilP. Traces of other proteins with potential non-specific affinity to nickel ions were also observed in the PilP fraction. The experimental MW of the intact form of all 4 proteins was higher by a factor of 6 kDa compared to the theoretical values for the mature proteins. This 6 kDa increase corresponds to the

plasmid vector sequence upstream of the corresponding cloned gene which contains the His-tag sequence.

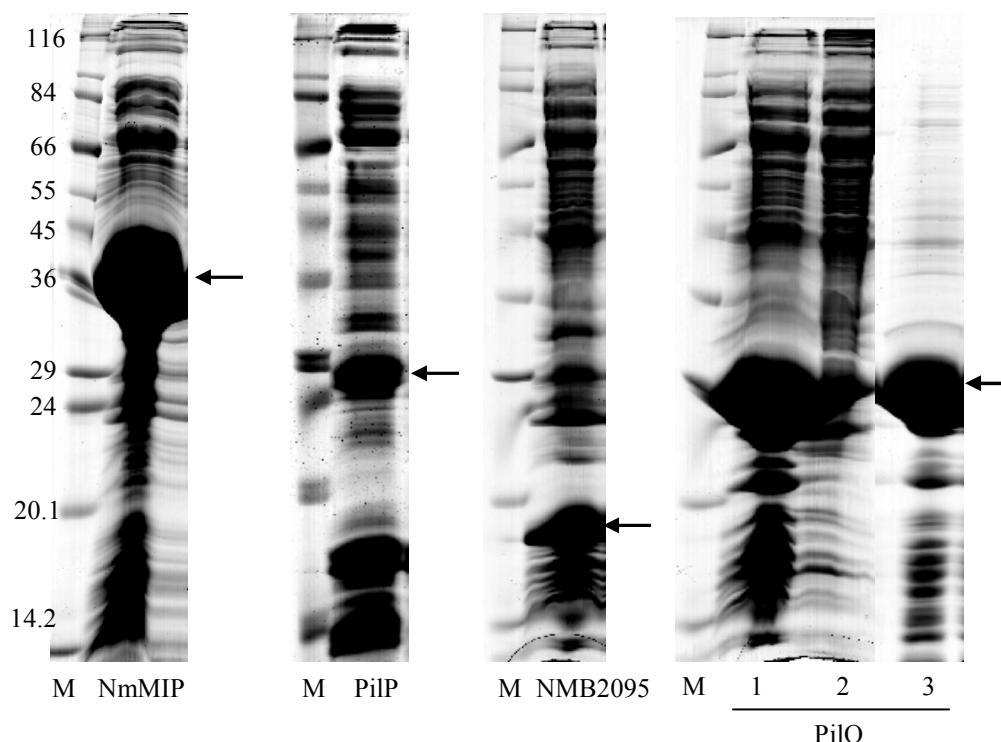


Figure 6.4 SDS-PAGE of fractions used for affinity purification of recombinant proteins. The soluble supernatant fractions following BugBuster protein extraction are shown for NmMIP, PilP and NMB2095. For PilO, the crude fraction containing soluble and insoluble material (lane 1), the soluble supernatant (lane 2) and the soluble fraction with recovered PilO during inclusion body purification (lane 3) are shown. 25 μ g of each fraction were loaded on a 12% polyacrylamide gel and a 40mA current was applied for 4 hr. The position of the expressed recombinant proteins is highlighted with arrows. The MW (in kDa) of bands of the protein marker is shown on the left. M: SigmaMarkerTM wide-range MW protein marker.

6.5 Production of protein-specific polyclonal antibodies

Immune sera from mice immunised with purified fractions of each of the recombinant proteins were assessed in western blots for generation of protein-specific antibodies. Whole-cell extracts from *N. meningitidis* MC58 strain separated on SDS-PAGE were used as antigen. Antibody binding was detected in the Cy5 channel by a Cy5-conjugated goat anti-mouse IgG. Immune serum against each of the recombinant

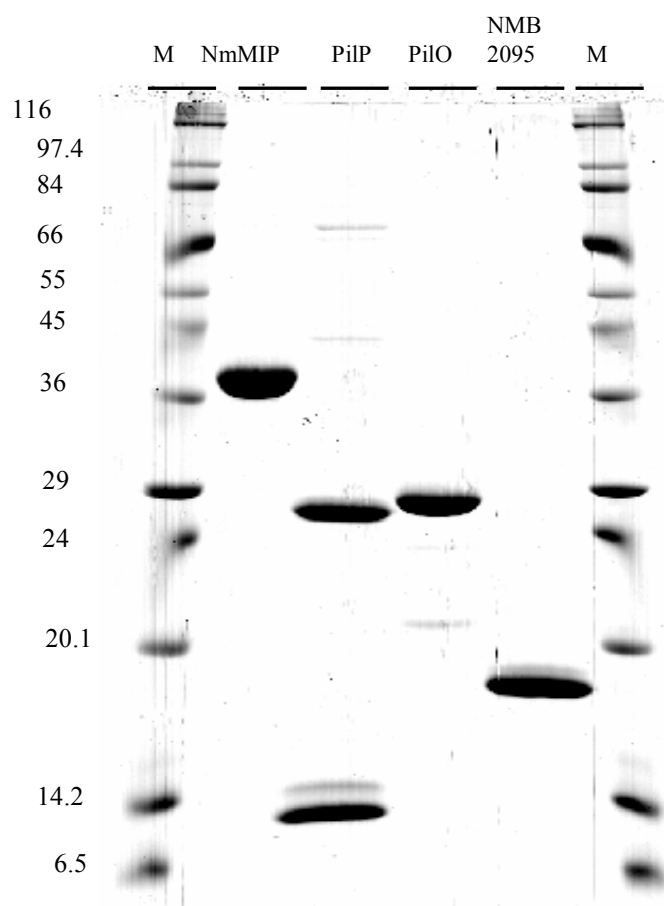


Figure 6.5 SDS-PAGE of His-tag purified recombinant proteins. Proteins NmMIP, PilP, PilO and NMB2095 were produced in *E. coli* BL21 (DE3) pLysS cells and purified by gravity-flow affinity chromatography using a NiSO₄-charged resin. 10 µg of each protein were loaded on a 12% polyacrylamide gel and a 40 mA current was applied for 4 hr. The MW (in kDa) of bands of the protein marker is shown on the left. M: SigmaMarker™ wide-range MW protein marker.

proteins reacted strongly with a single band (Figure 6.6, pseudo-red). Reacting bands were detected at positions corresponding to the theoretical MW of the mature form of the meningococcal proteins. In PilP western blots, weak binding to a high MW protein was also observed. The position of the band correlated with a protein, which co-purified with PilP during affinity purification, and was present in the fraction used to immunise mice (shown in Figure 6.5). Other bands were also detected in the green channel across all blots (Figure 6.6, pseudo-green) and were attributed to protein autofluorescence signals. As shown in Table A2 (Appendix), intrinsic fluorescence of

certain meningococcal proteins can be detected during fluorescence scanning. Although more prevalent in the green channel, these protein bands were also present in the red channel suggesting wide emission spectra, a common property of intrinsically fluorescent proteins.

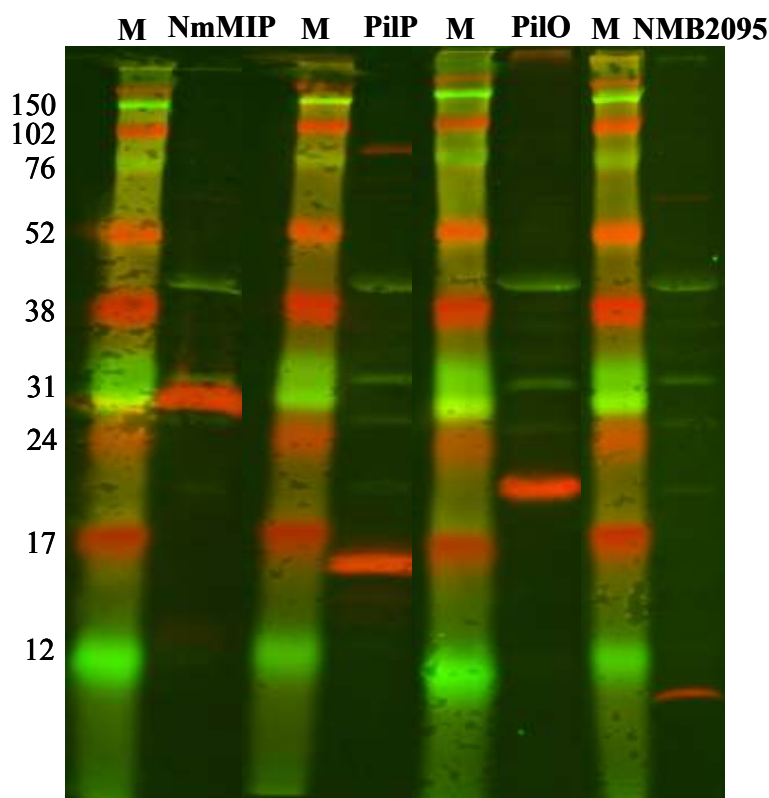


Figure 6.6 Western blots of cell extracts from *N. meningitidis* MC58 strain with murine immune sera against NmMIP, PilP, PilO and adhesin complex protein NMB2095. Cell extracts were separated on 12% polyacrylamide gels and transferred to PVDF membranes. Serum from mice immunised with each protein was used as the primary antibody (1:2,000 dilution for NmMIP and 1:1,000 for PilP, PilO and NMB2095). Cy5-conjugated goat anti-mouse IgG was used to detect antibody binding (red bands). Green bands correspond to autofluorescent proteins. The MW (in kDa) of bands of the protein marker is shown on the left. M: Pre-stained wide range MW protein marker (GE Healthcare).

6.6 Investigation of surface exposure of meningococcal vaccine candidate proteins

The immune serum generated against each of the selected meningococcal proteins was used to investigate their potential exposure on the bacterial surface. Immune sera were assessed for binding to the surface of meningococci in whole-cell ELISA and for susceptibility of proteins to protease cleavage carried out *in situ* in live meningococci.

6.6.1 Whole-cell ELISA

Wild-type and Δ -*siaD* MC58 strains were used to assess any differences in binding due to the presence of the capsule. Figure 6.7 shows the O.D. measured across the series of serum dilutions. Starting dilutions as low as 1:50 were used for immune sera against PilP, PilO and adhesin complex protein. Due to signal saturation, the starting dilution for NmMIP immune sera and positive controls was 1:1,000. A cutoff line corresponding to 3 times the optical density measured from the negative control serum was generated to determine antibody binding. Readings falling above this line were considered as a result of specific binding of antibodies to the meningococcal surface (Li *et al.*, 2006). Table 6.2 presents the antibody binding titers expressed as the reciprocal of the lowest dilution at which specific binding was observed. NmMIP, PilP and adhesin complex protein specific antibodies were shown to bind to the surface of both strains tested. NmMIP sera exhibited the highest titers in both strains. Differences in optical density and titers measured between the 2 strains were also observed but were not significant for any sera tested.

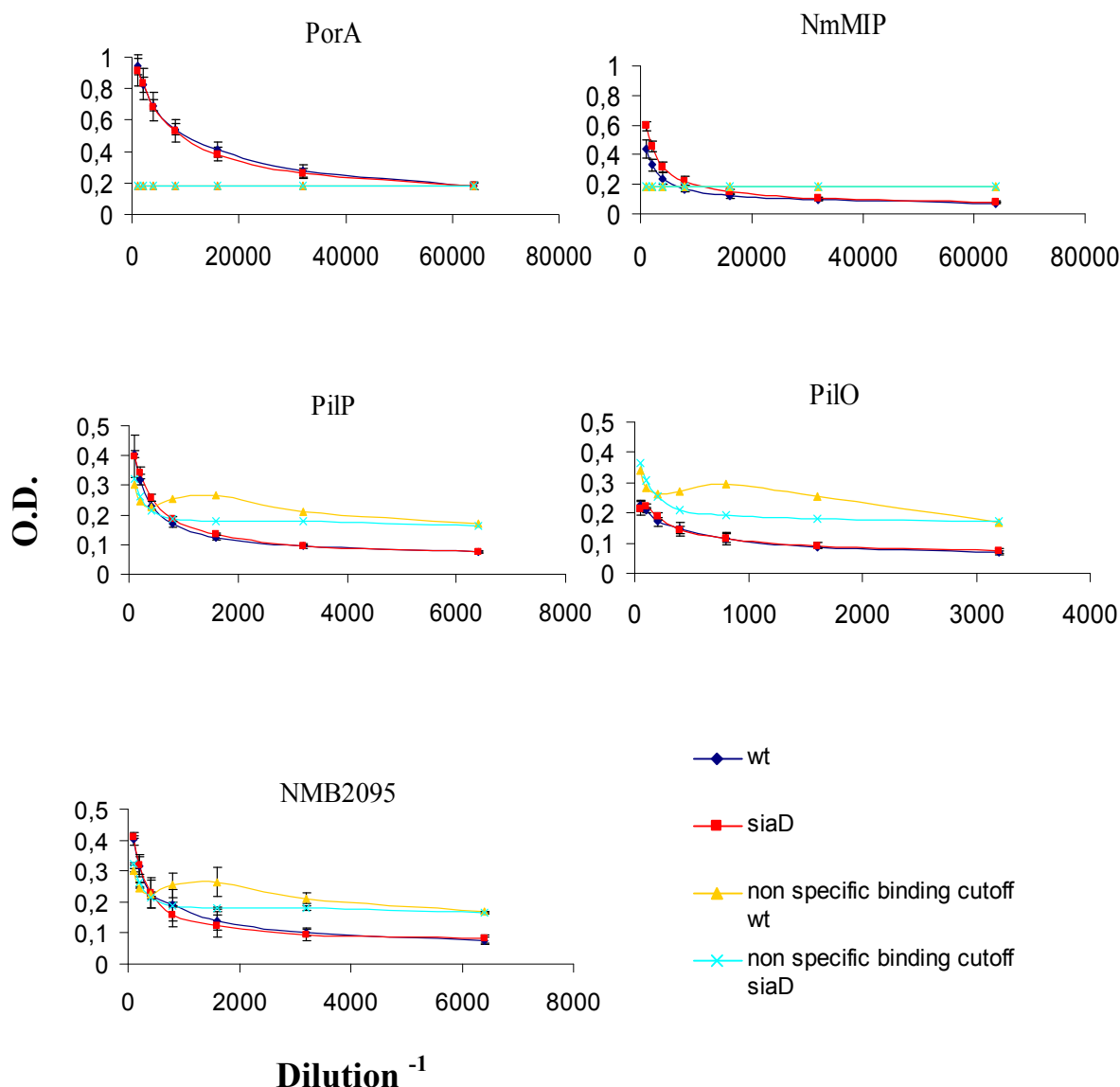


Figure 6.7 Optical density measurements from binding of immune sera on the surface of *N. meningitidis* MC58 wild-type and Δ -*siaD* meningococci in whole-cell ELISA. Immune sera were collected following immunisation of mice with purified fractions of NmMIP, PilP, PilO or adhesion complex protein NMB2095 mixed with MPL adjuvant. As positive control, binding of the anti-PorA monoclonal antibody P1.7 was tested. Cutoff thresholds corresponded to 3 times the optical density measured with immune sera collected from mice immunised with adjuvant alone. Error bars show the standard deviation of the mean values calculated from 3 readings.

Table 6.2 Whole-cell ELISA titers showing binding of protein-specific immune sera to the surface of MC58 wild-type and Δ -siaD strains^a.

Protein	Antibody	wild-type	Δ -siaD	P-value ^b
NmMIP	a-NmMIP pooled sera	7,751	12,393	0.054
PilP	a-PilP pooled sera	689	844	0.17
PilO	a-PilO pooled sera	-	-	-
NMB2095	a-NMB2095 pooled sera	890	778	0.64
PorA	P1.7 monoclonal	84,045	81,180	0.89

^a Whole-cell ELISA was performed by coating plates with the target strain and probing with pooled immune sera from groups of 10 mice immunised with the equivalent protein. Results are given as the reciprocal of the dilution of serum that gave positive readings 3 times greater than the negative control serum (from mice immunised with adjuvant alone). Mean values from triplicate analysis is reported. The hyphen corresponds to negative readings.

^b P-values from one-way ANOVA of titers from wild-type and Δ -siaD strains for each protein are given.

6.6.2. Digestion of proteins by proteinase K in whole meningococci

In Chapter 3, NmMIP and PilP were shown to be partially cleaved upon treatment of whole meningococci from MC58 Δ -siaD strain with proteinase K. Analysis was based on quantitative comparison of the amount of protein on 2-D gels before and after treatment. Here, immune sera were used to assess the effect of proteinase K treatment on the amount of each protein on 1-D western blots. Treatment of both wild-type and Δ -siaD meningococci was carried out using the same range of proteinase K concentrations as described in Chapter 3. The results are shown in Figure 6.8. Monoclonal antibodies against PorA, which was identified to be completely cleaved by proteinase K on the surface of meningococci, were used as positive control. Binding of NmMIP sera was observed in treated and non-treated fractions from both strains. A reduction in binding in the treated samples from Δ -siaD was observed. No obvious reduction in antibody binding was observed for PilP between treated and non-treated samples in both strains. However, bands of lower

MW appeared in the treated samples of PilP blots which might correspond to proteolytic fragments. PilP fragments were more abundant in Δ -*siaD* blots but were also present in blots from wild-type. Complete cleavage of PorA was confirmed in blots from both strains.

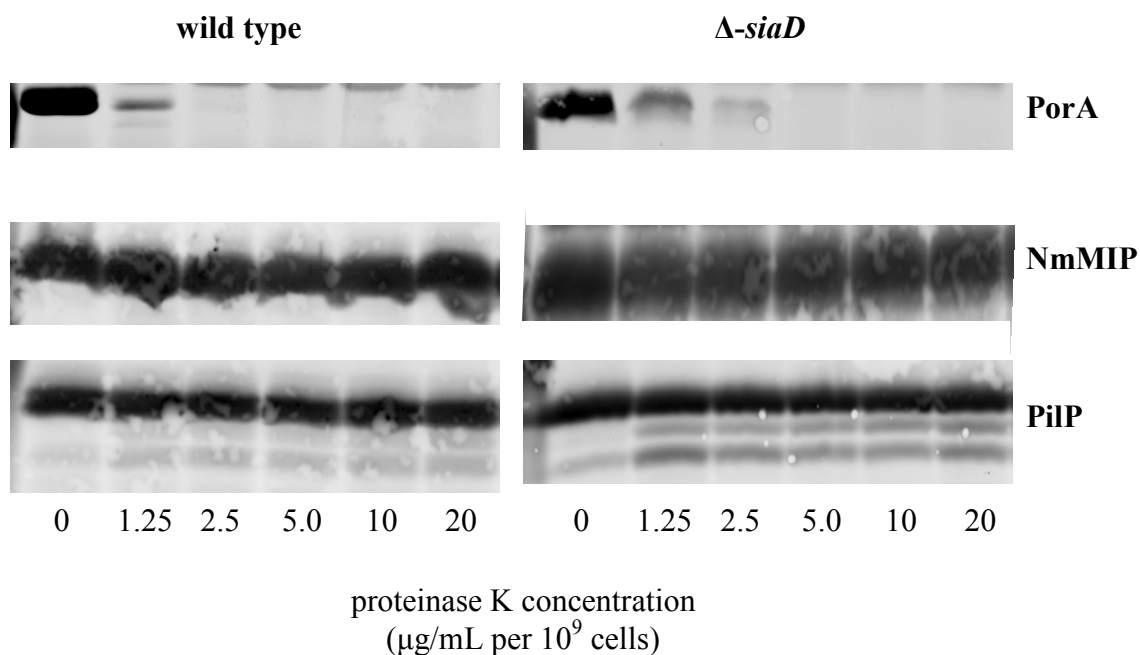


Figure 6.8 Western blots of proteins digested with proteinase K in whole meningococci. Whole-cell protein extracts from MC58 strains treated with different concentrations of proteinase K were separated on 12% polyacrylamide gels and transferred to PVDF membranes. Membranes were incubated with immune sera from mice immunised against NmMIP or PilP. Monoclonal antibodies against the P1.7 variable region of PorA were used as positive control. Cy5-conjugated goat anti-mouse IgG was used for detection of antibody binding.

6.7 Immunologic evaluation of potential vaccine candidate proteins

Complement deposition, opsonophagocytosis and complement-mediated meningococcal killing were used to assess whether protein-specific antibodies generated in mice are functional.

6.7.1 Complement deposition assay (CDA)

C3c and C5b-9 are two factors formed in the late stages of both classical and alternative complement pathways and their binding on the surface of meningococci is essential for initiation of cell lysis and bacterial clearance (Densen, 1989). Binding of C3c and C5b-9 on the meningococcal surface in the presence of immune sera against the chosen vaccine candidate proteins and human complement source was assessed by flow cytometry (Figure 6.9). Binding to the surface of serogroup B strains from various clonal complexes was tested. Significant increases in the deposition of both C3c and C5b-9 were observed with NmMIP sera compared to negative control sera in all strains tested except H44/76-SL. Binding of C3c in the presence of PilP, PilO and NMB2095 sera increased significantly only on the homologous MC58 strain. No significant increase in C5b-9 deposition was observed in the presence of sera against these 3 proteins in all strains tested. The highest increase in C3c deposition on the MC58 strain was observed with NmMIP sera.

C3c deposition

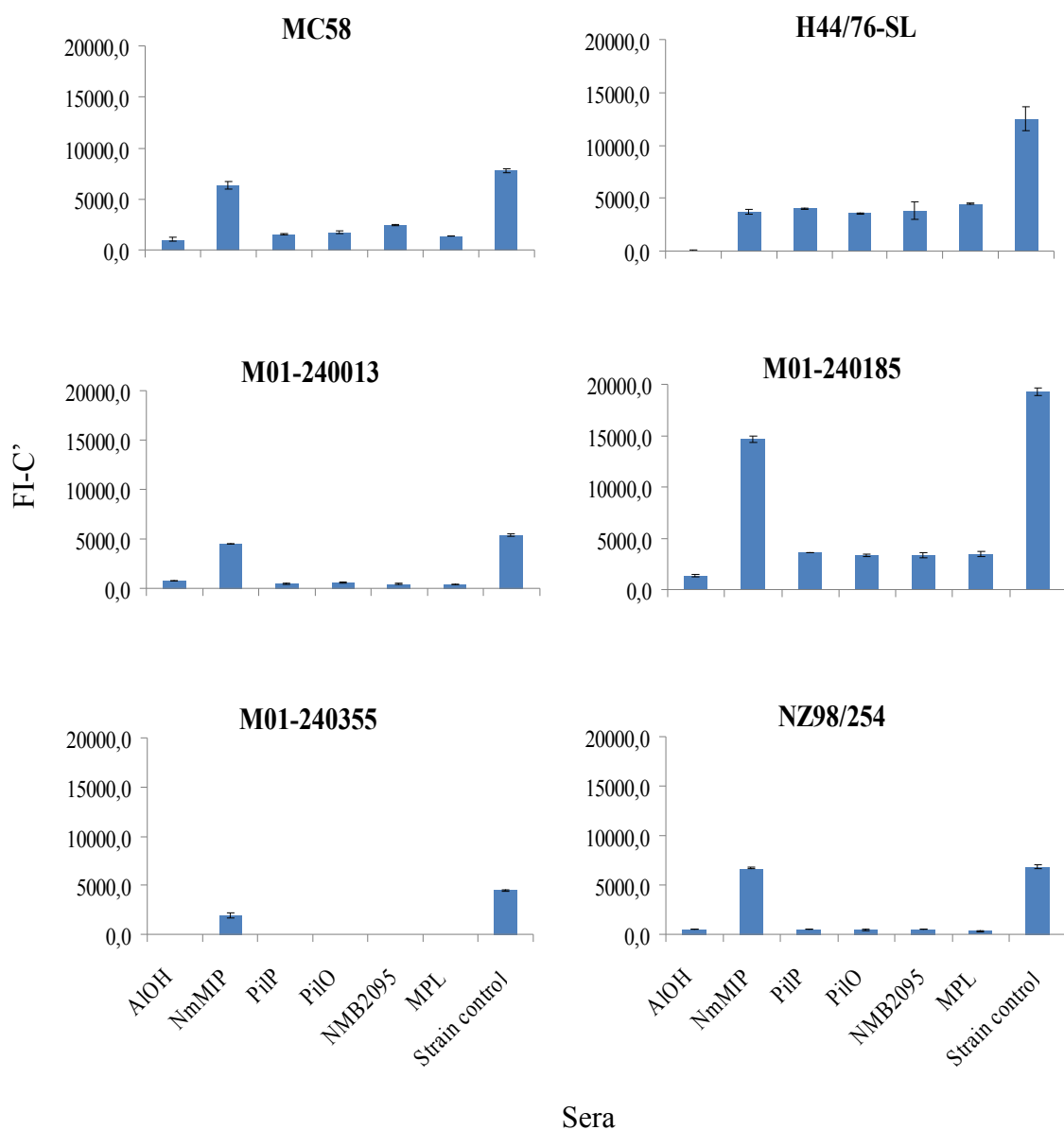


Figure 6.9 Deposition of complement factors on the surface of meningococci in the presence of protein-specific immune sera (continues in the next page).

C5b-9 deposition

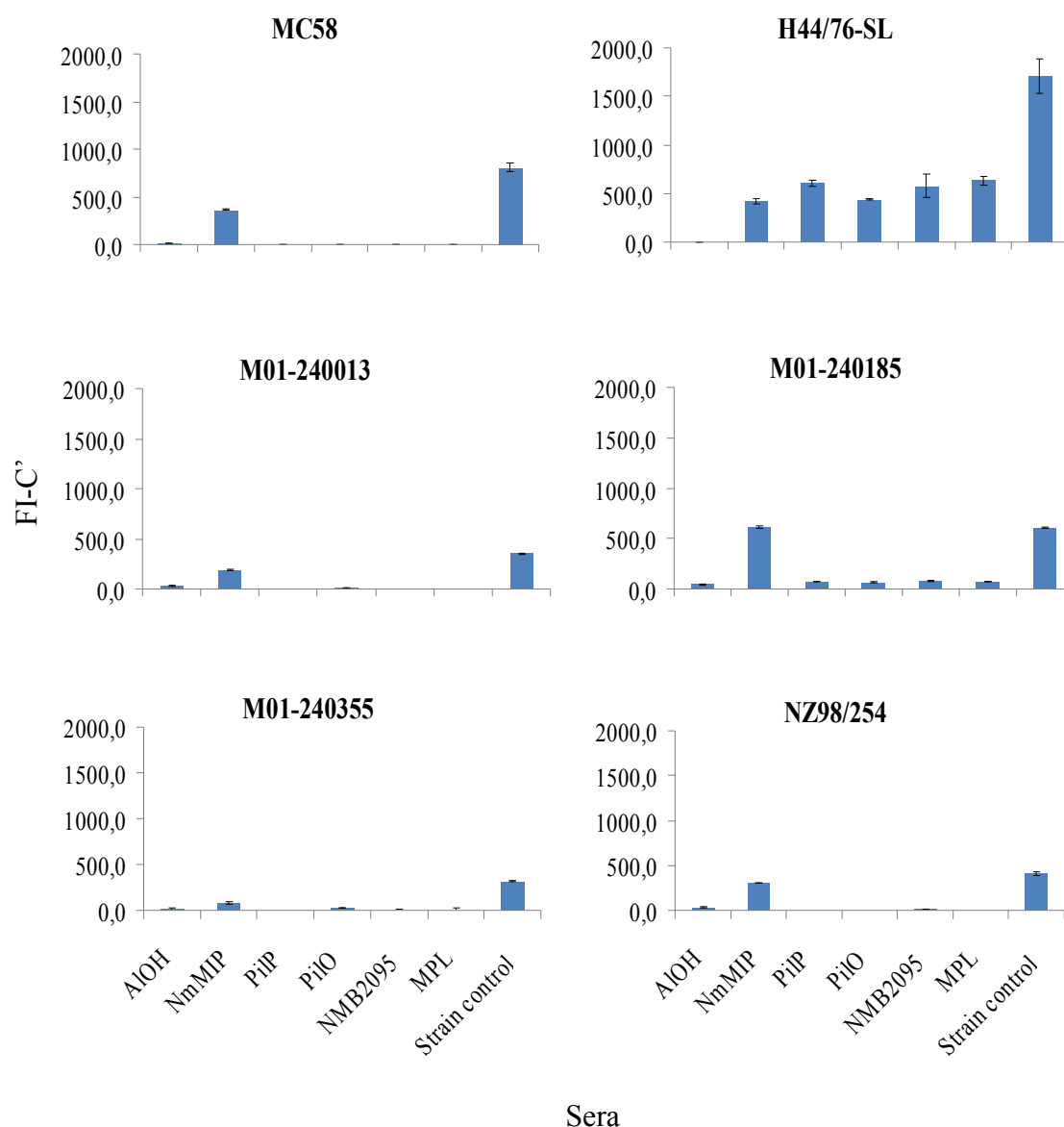


Figure 6.9 Deposition of complement factors on the surface of meningococci in the presence of protein-specific immune sera (continues from previous page). Meningococci from each of the tested strains were incubated with IgG-depleted human plasma and murine immune sera against NmMIP, PilP, PilO and adhesin complex protein (NMB2095). Immune sera from mice immunised against adjuvant alone (aluminium hydroxide-AIOH or MPL) or against OMV preparations from each of the tested strains were used as negative and positive controls, respectively. Binding of C3c and C5b-9 complement factors was detected using FITC-labelled sheep anti-human C3c and C5b-9 antibodies and analysis of FITC fluorescence on a flow cytometer. FI-C' values correspond to FITC fluorescence measured for each test serum corrected for fluorescence from a FITC-labelled conjugate only control. Information on the strains used can be found in Table 2.1. Error bars show the standard deviation of the mean values calculated from 3 readings.

6.7.2 Opsonophagocytic assay (OPA)

The ability of immune sera to induce phagocytosis of meningococci in the presence of HL60 phagocytic cells and human complement source was investigated. Internalisation of stained bacteria by HL60 cells was detected by flow cytometry (Figure 6.10). Opsonophagocytic (OP) activity was tested against the homologous MC58 and other serogroup B strains from various clonal complexes. NmMIP specific antibodies demonstrated OP activity against 7 out of the 8 strains tested. The only exception was the NZ98/254 strain. Immune sera against PilP, PilO and adhesion complex proteins failed to show OP activity against any of the strains tested.

6.7.3 Serum bactericidal assay (SBA)

The serum bactericidal assay was used to test the potential of protein-specific antibodies to kill bacteria in the presence of complement source. Results from SBA of human sera correlate with protection against meningococcal disease in the presence of human complement source (Borrow *et al.*, 2005). Given the scarcity of human complement and the use of sera generated in mice, rabbit complement was used in SBAs in this study. Bactericidal titers were expressed as the reciprocal of antibody dilution where 50% killing of meningococci is observed. Sera generated against each of the chosen candidates were tested at dilutions of 8 or greater. This titer has been previously used during preclinical evaluation of murine antibodies against other potential protein antigens in SBAs in the presence of rabbit complement. When immune sera generated against the chosen candidate proteins were tested in SBAs against H44/76 strain, no killing of $\geq 50\%$ of meningococci was observed for dilutions $\geq 1:8$ (Figure 6.11). In contrast, monoclonal antibodies against the PorA type expressed by H44/76 strain exhibited a titer of $\geq 16,000$. Immune sera from mice

against OMVs derived from H44/76 were also bactericidal against the homologous strain at a titre of 4,000. Bactericidal activity of NmMIP serum was also tested against MC58 strain and similarly no killing was detected. A bactericidal titer of 500 was measured for PorA monoclonal antibodies against MC58 strain.

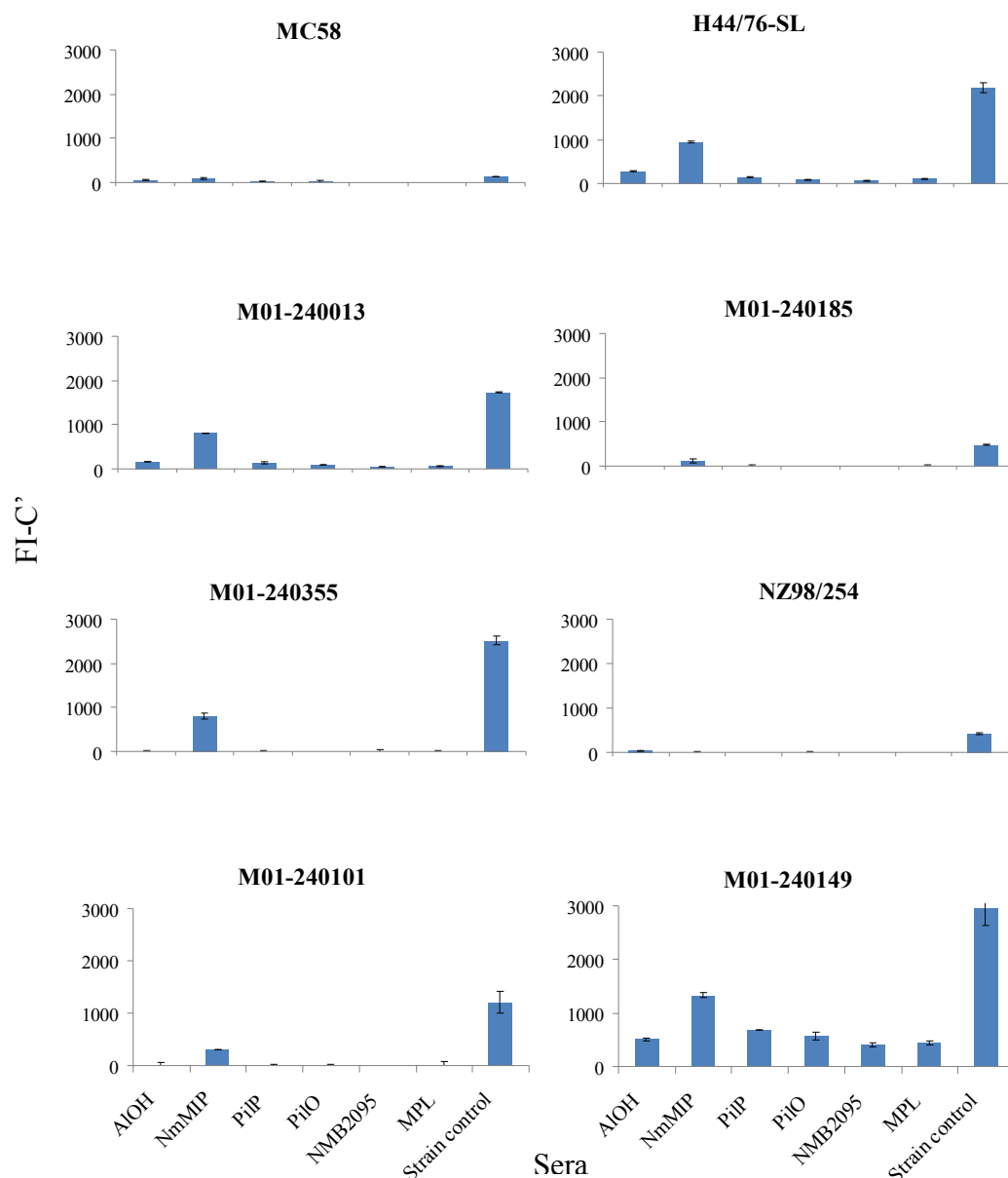


Figure 6.10 Opsonophagocytosis of meningococci in the presence of protein-specific immune sera. BCECF-stained meningococci from each of the tested strains were incubated with human complement and immune sera against one of NmMIP, PilP, PilO and adhesin complex protein NMB2095 followed by incubation with HL60 phagocytic cells. Internalisation of stained bacteria by HL60 cells was assessed by detection of BCECF fluorescence (fluorescein equivalent) by flow cytometry. Positive and negative controls were used and FI-C' values calculated as described in Figure 6.9 and section 2.19. Error bars show the standard deviation of the mean values calculated from 3 readings.

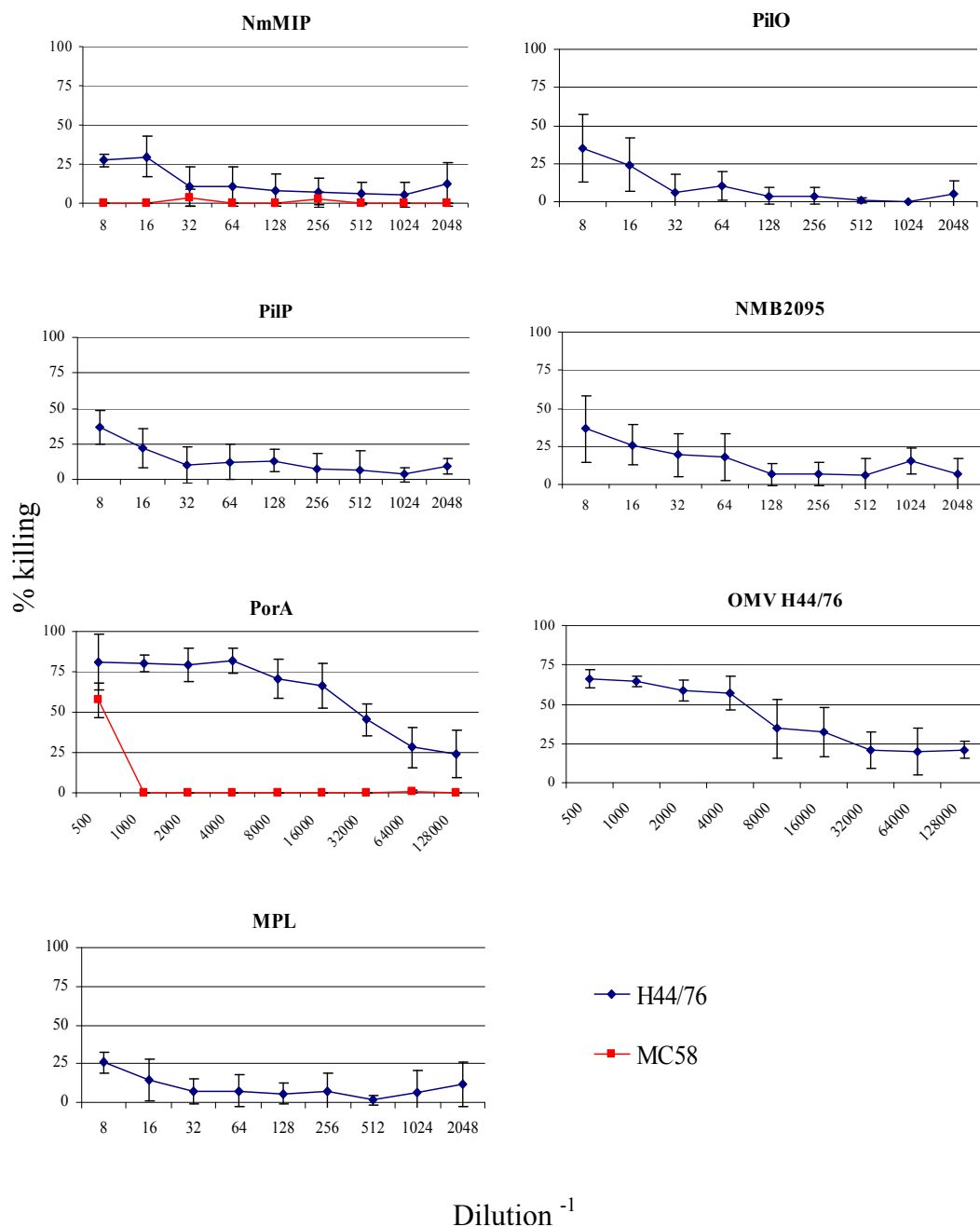


Figure 6.11 Complement-mediated killing of meningococci in the presence of protein-specific immune sera. Meningococci were incubated with serial dilutions of immune sera against NmMIP, PilP, PilO and adhesin complex protein NMB2095 in the presence of rabbit complement source. The percentage of killing was calculated by comparison of colony counts between test samples and control samples containing heat-inactivated complement. Additional controls testing bacterial killing by active complement alone were included. Anti-PorA monoclonal antibodies P1.7 and P1.16 were used for MC58 and H44/76, respectively, as positive SBA control. Serum against MPL adjuvant alone was used as negative SBA control. Error bars show the standard deviation of the mean values calculated from 4 readings.

6.8 Investigation of the meningococcal macrophage infectivity potentiator (NmMIP)

From immunologic analysis of the 4 potential candidate proteins, NmMIP was the only one to provide evidence of potential to protect against meningococci. This prompted further investigation into other properties of NmMIP including sequence variability and structural predictions inferred from comparison with NmMIP orthologues in other bacteria.

6.8.1 Sequence analyses

6.8.1.1 Sequence characteristics and phylogenetic analysis

Nucleotide and amino acid sequences from 106 meningococcal isolates encoding for NmMIP were extracted from BIGS database (Jolley and Maiden, 2010), access to which was kindly provided by Prof M. Maiden group, University of Oxford. The panel of meningococcal isolates corresponds to the MLST dataset and encompasses strains from various hypervirulent serogroup B lineages as well as invasive and carrier strains from other serogroups (Table A1, Appendix). Sequences were aligned using the MegAlign tool from DNASTAR Lasergene software v.8 and alignments are presented in the electronic copy provided. Results on the number of substitutions and the sequence types are shown in Table 6.3. Thirty polymorphic nucleotide sites and 13 polymorphic amino acid sites were detected resulting in 18 unique nucleotide sequences (allele types) and 10 unique amino acid sequences (sequence types), respectively. The 18 unique allele types are presented aligned in Appendix (Figure A4). In total, 777 out of 807 (96.3%) base pairs and 255 out of 268 (95.2%) amino acids were identical across all aligned nucleotide and protein sequences, respectively. An in-frame deletion of 12 bp was also observed in 28

isolates. As presented in Table A1, isolates carrying the deletion belonged to the lineages ST-5, ST-11 and ST-37.

Table 6.3 Polymorphism and allele statistics for NmMIP based on sequences from 106 meningococcal isolates. Allele types and amino acid sequence types correspond to unique nucleotide and amino acid sequences. The panel of isolates used in analysis is presented in Table A1 (Appendix).

Alignment (bp)	819
Number of allele types	18
Number of polymorphic nucleotide sites	30
Number of amino acid sequence types	10
Number of polymorphic amino acid sites	13

To assess a possible connection between clonal complexes and MIP allele type phylogenetic trees were constructed by ClonalFrame using the aligned nucleotide sequences from the 106 isolates. ClonalFrame assesses the clonal relationships of isolates by accounting for both point mutations and homologous recombination. Results from ClonalFrame were visualized using MEGA software v.5 and presented as radial trees (Figure 6.12). In these phylogenetic views, each leaf represents a meningococcal isolate and each internal node corresponds to the most recent common ancestor of the descendant isolates. Based on NmMIP sequences, isolates were divided into 2 groups / clades linked to a single common ancestor. One group contained the isolates carrying the 12 bp deletion. The only exceptions were isolates numbered 657 and 658 which do not carry the deletion and formed a distinct clade within this group. Isolates of the same clonal complex, namely ST-5, ST-11 and ST-37 were shown to cluster together. The other group contained all remaining isolates which further clustered by clonal complex into four subclades. ST-4, ST-8 and ST-32

formed distinct subclades whilst ST-1, ST-18 and ST41/44 grouped together in the same subclade. Notably, 2 isolates from ST-5 lacking the deletion (numbered 239 and 299) clustered together with ST-4 isolates. Overall, there was an evident relationship between clonal complex and changes in the NmMIP gene and a low rate of sequence reassortment between isolates of different lineages.

ClonalFrame was also used to calculate the ratio of changes resulting from recombination to those occurring through point mutations (r/m). Recombination versus mutation values less than 1 indicate low, 1-2 intermediate, 2-10 high and greater than 10 very high recombination rates. The r/m value calculated for NmMIP gene was 1.54 suggesting intermediate levels of recombination between meningococcal isolates affecting NmMIP gene.

6.8.1.2 Synonymous and non-synonymous substitutions

Base substitutions are the most common form of mutation which can either alter (non-synonymous) or have no effect (synonymous) on the amino acid sequence due to redundancy of the genetic code. The ratio of non-synonymous (dn) against synonymous (ds) substitutions is used as an indicator of whether or not sequences are under selective pressure. Positive selective pressure works towards sequence variability and favours non-synonymous substitutions. A protein under positive selective pressure would therefore be expected to exhibit a dn/ds ratio ≥ 1 (Nei and Kumar, 2000). This is a common observation for immunogenic proteins where alterations occur in the epitopic regions and help meningococci to evade host immunity (Derrick *et al.*, 1999). To assess possible selective pressures imposed to NmMIP by the immune system, the dn/ds ratio was calculated using SNAP programme which is based on a method developed by Nei and Gojobori (Nei and Gojobori, 1986).

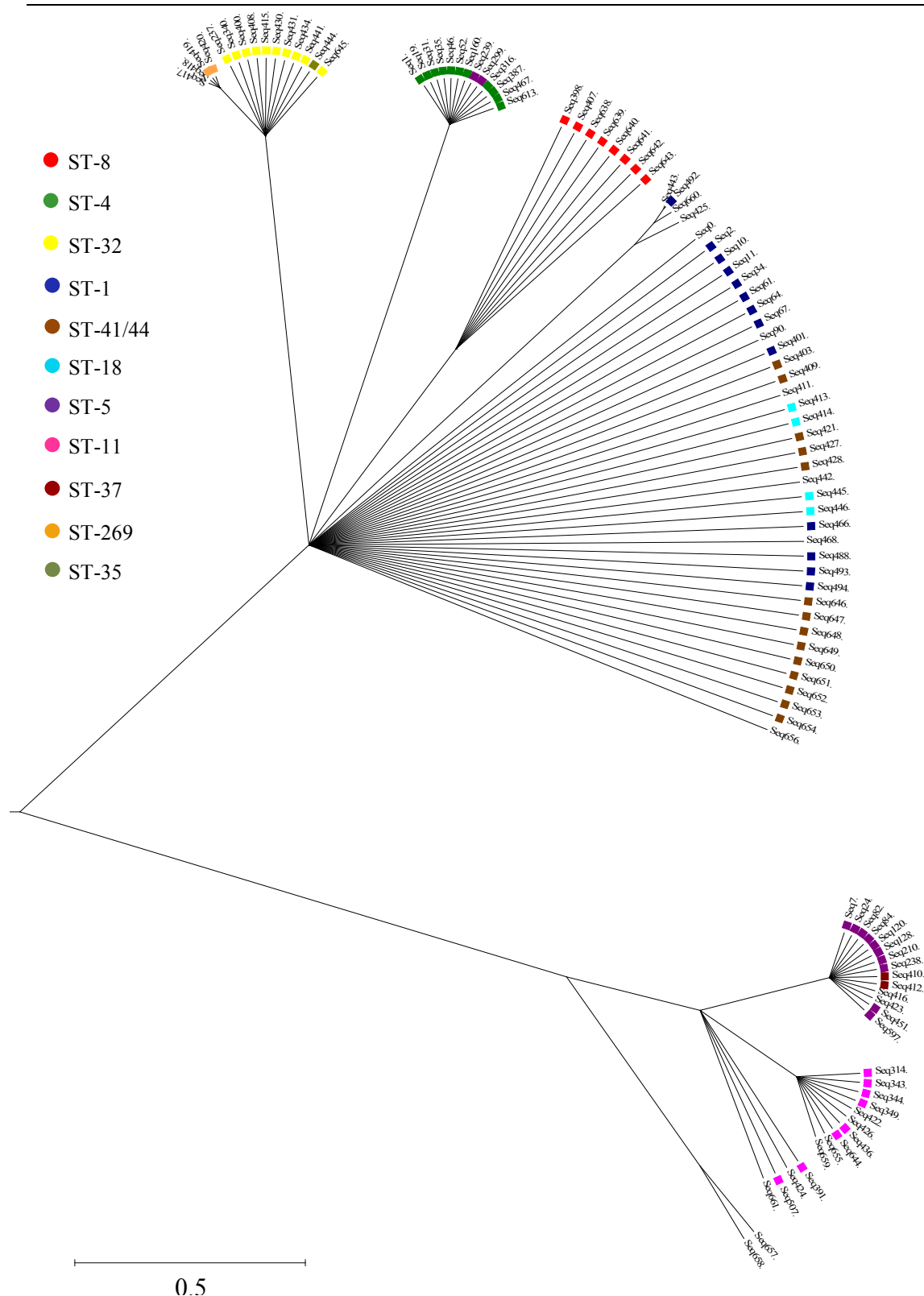


Figure 6.12 Clonal relationships of meningococcal isolates based on NmMIP nucleotide sequence. Aligned NmMIP sequences from 106 meningococcal isolates were analysed by ClonalFrame and results visualised using MEGA v.5. Evolutionary distance is presented using the radial tree view. Isolates are numbered according to Table A1 (Appendix) and color-coded by sequence type (ST).

The average dn and ds numbers calculated from all pairwise sequence comparisons were 0.0037 and 0.0241, respectively, resulting in a dn/ds value of 0.15, indicative of negative selective pressure favoring sequence conservation. SNAP was also used to calculate the rate of synonymous and non-synonymous substitutions for every single codon. Figure 6.13 shows the rate, type and distribution of substitutions across the amino acid sequence. No region of high variability was identified. Instead, the few substitutions were shown to be evenly distributed along the amino acid sequence.

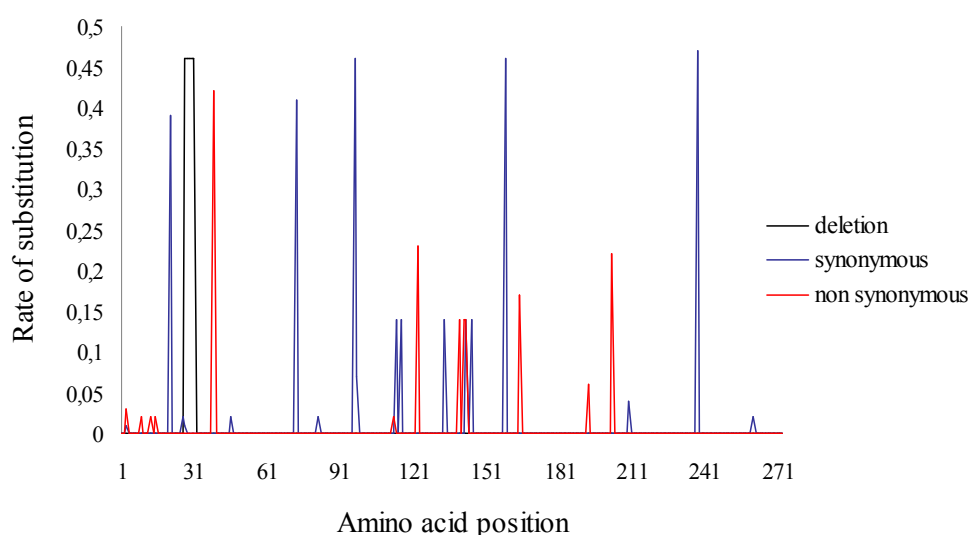


Figure 6.13 Distribution of synonymous and non-synonymous substitutions in the NmMIP amino acid sequence. Rates of substitutions were calculated using the SNAP algorithm (<http://www.hiv.lanl.gov>) following alignment of NmMIP amino acid sequences from 106 strains (Table A1, Appendix).

6.8.2 Topological and structural characteristics of NmMIP protein

6.8.2.1 Topology predictions

Based on PsortB and LipopP analysis described in Chapter 3, NmMIP is a predicted lipoprotein associated with the outer membrane via a palmitoyl group. In Gram-negative bacteria like *N meningitidis*, lipoproteins possess a signal peptide

sequence which targets them to the inner (cytoplasmic) membrane and a lipobox sequence, usually L-(S/A)-(G/A)-C. In NmMIP, the first 22 amino acids serve as the signal peptide sequence (see section 6.2.2) and amino acids 18-21, a sequence of L-S-A-C, constitute the lipobox. Following cleavage of the signal peptide by peptidases and lipid modification of the cysteine residue of the lipobox, the protein can be either retained on the inner membrane or released for association to the outer membrane (Tokuda and Matsuyama, 2004). This is determined by sequence signals recognised by the Lol system, a group of inner membrane, periplasmic and outer membrane proteins found in *E. coli* and other Gram-negative bacteria. Homologues of the Lol system in MC58 were identified through BLAST searches with *E. coli* amino acid sequences for proteins LolA-E. Open reading frames coding for Lol proteins in MC58 included NMB0622 (LolA), NMB0873 (LolB), NMB1235 (LolC/E) and NMB1234 (LolD). A known lipoprotein-sorting signal recognized by Lol proteins is the first residue carboxy-terminal to the modified cysteine (position 2). Any amino acid other than aspartic acid in this position allows for the release of the protein from the inner membrane and its recognition by proteins LolA and LolB for subsequent attachment to the outer membrane. In NmMIP, a glycine residue is found in position 2 suggesting NmMIP is associated to the outer membrane. What determines the orientation of an outer membrane-attached lipoprotein is still poorly understood and no general sequence patterns have been proposed so far.

The membrane topology prediction server TOPCONS was used to identify other potential sequence features of NmMIP in an attempt to conclude to a more accurate model of NmMIP topology on the cell envelope. TOPCONS brings together different methods for prediction of transmembrane domains based on both sequence statistics (PRO-TMHMM, PRODIV-TMHMM) and physicochemical principles

(SCAMPI) (Bernsel *et al.*, 2009). One transmembrane domain was identified in the region between amino acids 3 and 22 which exhibited the lowest ΔG value (Figure 6.14). This is the region corresponding to the signal peptide sequence which is cleaved upon targeting of the lipoprotein to the inner membrane and does not form part of the mature protein. No additional transmembrane helices were identified.

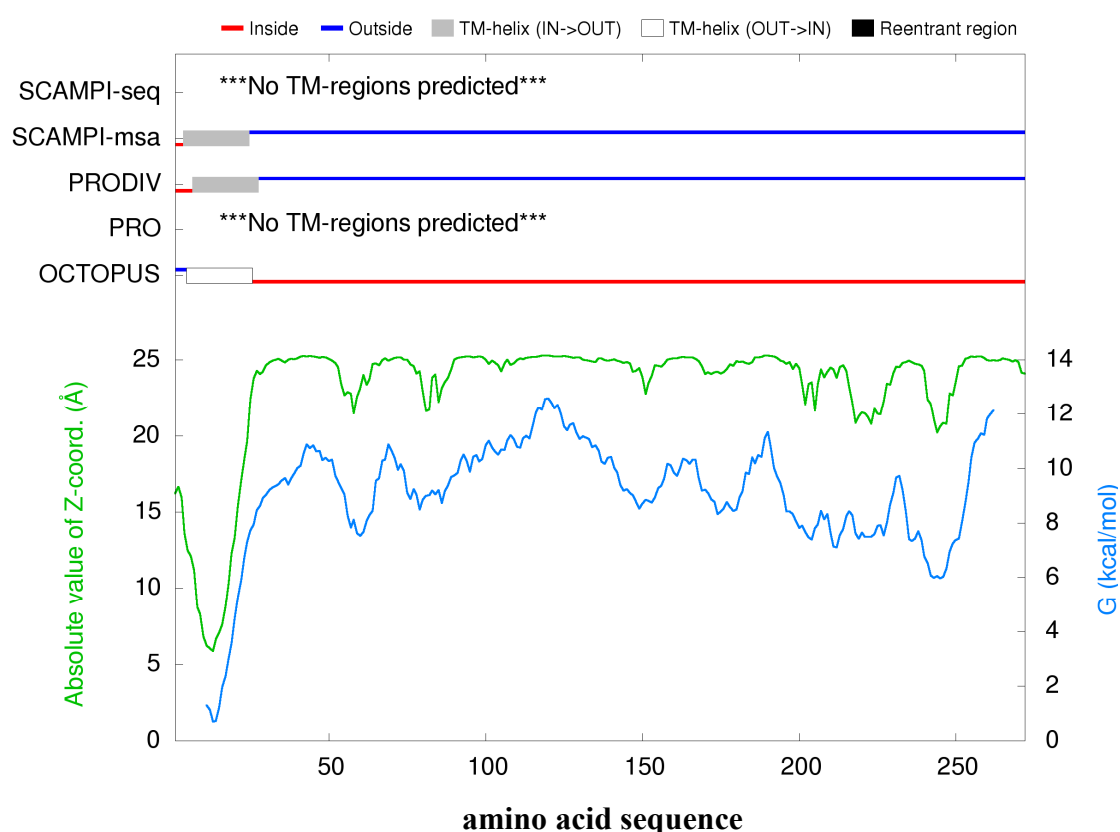


Figure 6.14 TOPCONS membrane topology prediction for NmMIP protein. The proposed topological model from the individual methods is presented (top). Graphs indicate predicted Z-coordinates and free energy (ΔG) values across the sequence.

6.8.2.2 Structural model

NmMIP belongs to a group of proteins predominantly present in intracellular bacterial pathogens which assist to the survival and multiplication of the pathogen within macrophages (Cianciotto *et al.*, 1992). Bacterial MIPs also belong to the wider family of FK506-binding proteins (FKBP) whose functional role is the catalysis of the

cis-trans isomerisation of peptidyl-prolyl bonds during protein folding (Galat and Metcalfe, 1995). FKBP-type proteins are also found in humans. *Legionella pneumophila* MIP (LpMIP) is the most studied bacterial MIP and its structure has been resolved (Riboldi-Tunncliffe *et al.*, 2001). LpMIP consists of two distinct domains. The N-terminal domain is composed of alpha helices, which are involved in protein dimerisation and exhibit high sequence diversity across different species. The C-terminal domain forms 6 beta-strands with a fold typical of FKBP-type proteins. The C-terminal domain exhibits isomerase activity and is well conserved across FKBP. LpMIP has been confirmed to reside on the cell surface of *L. pneumophila* (Helbig *et al.*, 2001).

The structural characteristics of NmMIP were investigated by a) alignment of NmMIP amino acid sequence from MC58 with MIP orthologues from other bacteria and a member of the FKBP family from human (Figure 6.15) and b) reconstruction of a 3-D model based on LpMIP published structure (Figure 6.16). Sequence sites involved in dimerisation and interactions with FK506 were inferred from LpMIP structural elucidation and are marked in the alignment. A good degree of homology was observed across bacterial MIP sequences where approximately 40% of amino acid residues were identical. The human FKBP2 sequence included in the alignment exhibited similarity with the C-terminus of bacterial MIPs. Most identical residues across all aligned sequences were found in the C-terminal domain which carries the enzymatic centre of FKBP and is therefore expected to be more conserved. Two methionines in helix $\alpha 2$ of LpMIP are thought to be involved in interactions between the monomers for formation of the dimeric structure. One of these methionines is also present in NmMIP (position 85) with the other residue being a phenylalanine (position 81). Residues involved in interaction with FK506 have also been identified in LpMIP.

FK506 is an immunosuppressant macrolide antibiotic which inhibits the prolyl isomerase activity of FKBP. All residues are found in the C-terminal domain and were conserved across bacterial MIPs and the human FKBP2 sequence. This provides further evidence for the conserved character of the enzymatic centre of FKBP.

The predicted structure of NmMIP protein is presented as a 3-D model in Figure 6.16. The generated structure belongs to the region between amino acids 49 and 255 of the 272 amino acid-long NmMIP sequence which aligned with LpMIP protein sequence. Two antiparallel α -helices are formed in the N-terminus by amino acids 50-67 (α_1) and 79-90 (α_2). A long, exposed helix composed of amino acids 98-139 links the N and C-terminal domains. Like in LpMIP, the C-terminal domain consists of 6 β -strands, which form an antiparallel β -sheet and a α -helix (aa 203-211) lying across the sheet. The amino acids involved in dimerisation in N-terminal helices and in interaction with FK506 are highlighted. An example of the structure of the NmMIP dimer is shown in Figure 6.16B.

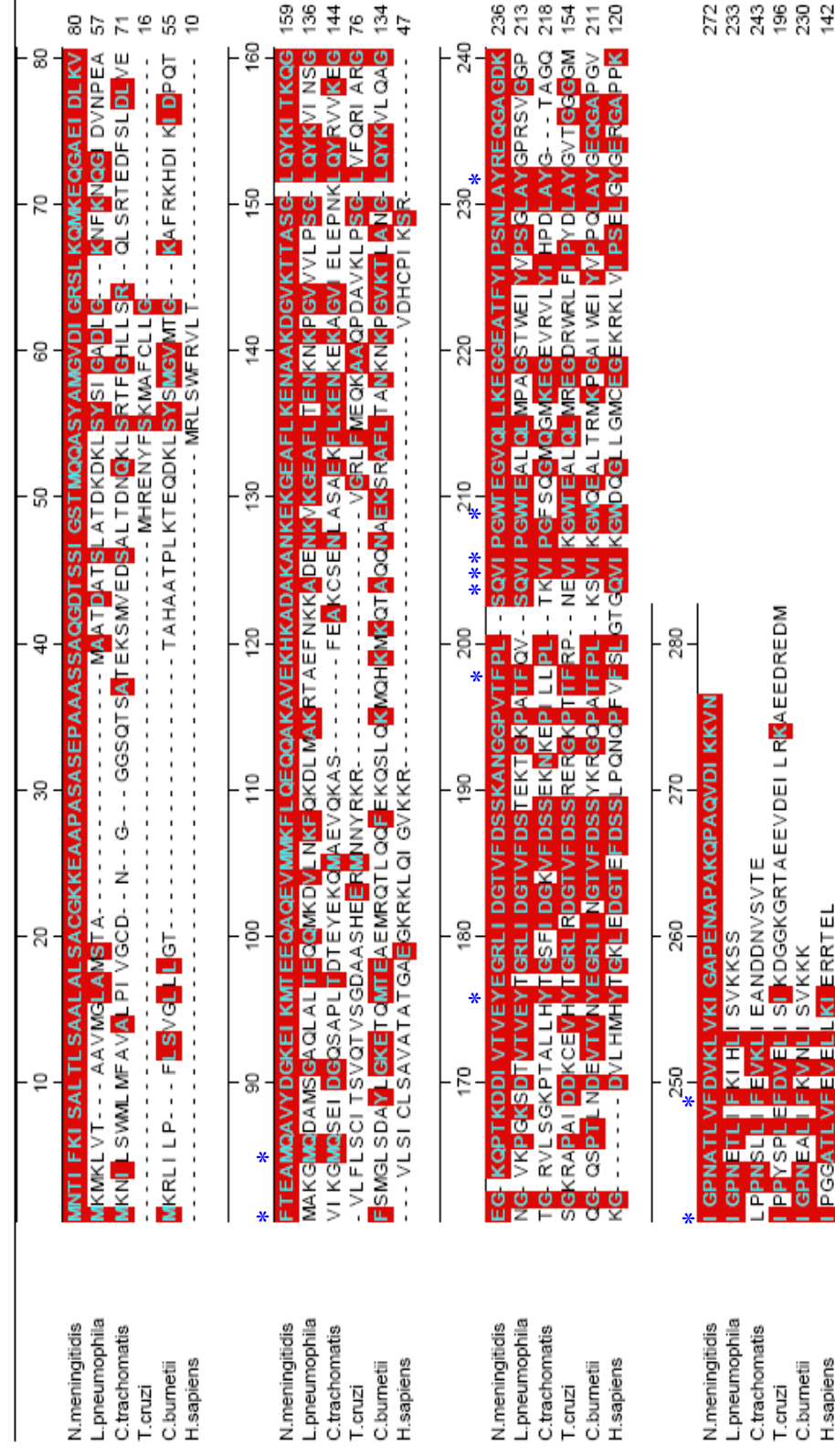


Figure 6.15 Alignment of homologous NmMIP amino acid sequences from bacterial species and human. Swiss-Prot accession numbers of proteins from each organism are as follows: *Neisseria meningitidis*: Q9JYI8, *Legionella pneumophila*: Q5ZXE0, *Chlamydia trachomatis*: P26623, *Trypanosoma cruzi*: Q4D932, *Coxiella burnetii*: P51752, *Homo sapiens*: P26885 (FKBP2). Residues matching those of NmMIP sequence are coloured in deep red. Residues involved in dimerisation and interaction with FK506 are highlighted with blue asterisks.

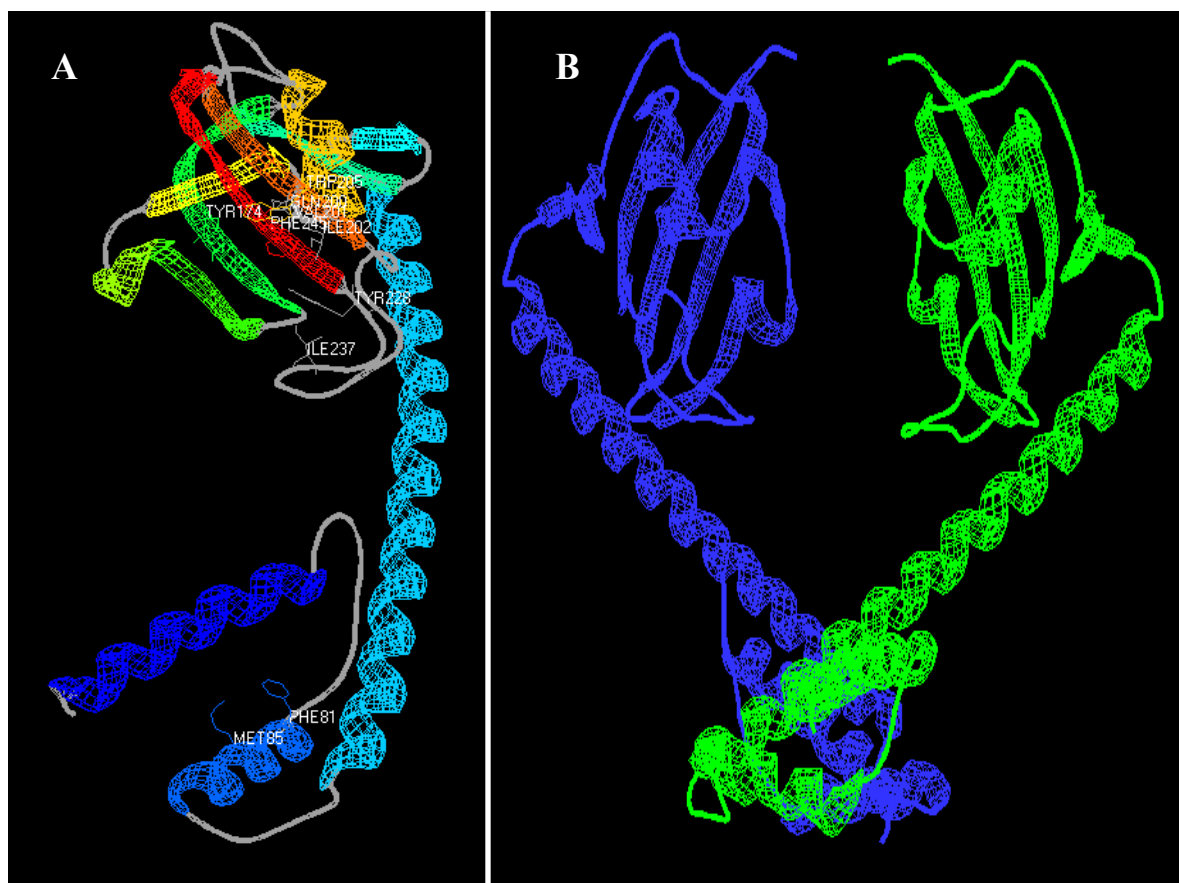


Figure 6.16 3-D model of the predicted structure of NmMIP protein from MC58. NmMIP monomer (A) and dimer representations (B) are shown. Secondary structures in A are coloured in sequence succession from dark blue to red. The monomeric forms of the dimer are coloured green and blue. The position of important residues for NmMIP structure and function are highlighted in A.

6.9 Discussion

The vaccine potential of 4 proteins identified from proteomic analysis of the meningococcal surface and OMV proteome was assessed in immunological assays using protein-specific antibodies raised in mice. Meningococcal macrophage infectivity potentiator (NmMIP), tfp biogenesis assembly proteins PilP and PilO and putative adhesin complex protein encoded by NMB2095 had not previously been characterised as potential vaccine candidates despite their putative functional roles in meningococcal pathogenesis.

Recombinant forms of all four proteins were purified from soluble fractions of *E. coli* protein lysates and there was no evidence of formation of inclusion bodies during protein production. Inclusion bodies constitute aggregates of partially folded or misfolded proteins which in the case of recombinant protein production are considered to be the result of the bacterial stress response mechanisms to cope with the large amounts of accumulated protein product (Villaverde and Carrió, 2003). The solubility of the meningococcal proteins accumulated in the *E. coli* cytoplasm suggests the proteins were in their folded state although their exact conformation might be different to the one obtained in their natural location. It also suggests proteins did not undergo any degradation often observed in inclusion body-forming proteins. Only exception was PilP which exhibited a second truncated version appearing at a lower MW in SDS-PAGE of *E. coli* crude extracts (Figures 6.5 and 6.6). However, this PilP fragment must be the result of treatment with BugBuster since it was only present in SDS-PAGE of BugBuster-lysed cells.

The murine polyclonal antibodies were shown to specifically recognise the protein they were raised against and enabled evaluation of individual proteins in immunological assays. The assays performed assessed 2 different mechanisms of protection involved in immunity against meningococcal disease. Complement deposition and serum bactericidal assays test the attachment of certain components of the complement pathway and the complement-dependent killing of meningococci, respectively. Opsonophagocytosis assays assess ingestion of meningococci by phagocytic cell lines in the presence of complement source. Serum bactericidal assays have been shown to correlate with efficacy of serogroup B OMV vaccines and a titer of ≥ 4 (or ≥ 4 -fold rise from pre to post immunisation) has been proposed as a suitable correlate of protection (Holst *et al.*, 2003). Opsonophagocytosis has also been

suggested to contribute independently to the observed protection especially in cases where SBA activity is absent (Plested and Granoff, 2008).

NmMIP was the only protein tested whose antibodies provided positive results in one or more assays. This can, in part, be explained by the differences in surface exposure observed for the 4 proteins in whole-cell ELISA. The extent of a protein's exposure can affect the accessibility of antibodies on the bacterial surface and the subsequent attraction of complement factors for formation of the attack complex. NmMIP was shown to be sufficiently exposed on the meningococcal surface to bind to NmMIP immune sera in whole-cell ELISA. In contrast, only weak binding was observed for PilP and NMB2095 sera and no binding for PilO sera.

Antibodies against NmMIP induced deposition of complement factors on the bacterial surface but failed to kill meningococci in a complement-mediated manner. Differences in bacterial treatment between the 2 assays might have contributed to this apparent paradox. Bacteria were inactivated by treatment with azide/PMSF prior to complement deposition assay whilst live untreated bacteria were used in SBAs. Inactivation of bacteria by heat or ethanol has previously been accused for modulation of the bacterial outer membrane facilitating the accessibility of antibodies to protein epitopes that were otherwise buried in untreated bacteria (Kolberg *et al.*, 2008). A possible exposure of additional NmMIP epitopes on azide-treated meningococci used in CDA could have enhanced antibody binding to the bacterial surface leading to the observed attraction of complement factors. In contrast, absence of antibody binding in live bacteria would not support attachment of complement and hence induction of bactericidal killing in SBA.

Another parameter that could have potentially affected the SBA result is relevant to the folding properties of purified protein injected into mice. Acquisition of

a conformation different to that of the native protein can impact on the formation of the epitopic regions and hence, the generation of antibodies which can efficiently bind to the protein on the meningococcal surface and activate the complement cascade. During writing of this thesis, Hung *et al.* showed that incorporation of a similar recombinant NmMIP preparation into liposomes or Zwittergent 3-14 micelles followed by injection into mice in the presence of MPL adjuvant produced bactericidal antibodies with a titer of 1,024 (Hung *et al.*, 2011). Liposomes and micelles form a lipid bilayer mimicking the bacterial cell wall thus allowing the protein to obtain a near native conformation. Liposomes have been previously used for administration of meningococcal protein vaccine candidates in mice for subsequent evaluation of resulting antibodies in SBAs (Delgado *et al.*, 2007; Wright *et al.*, 2002).

The opsonic activity of NmMIP antisera suggests that NmMIP antibodies might offer protection against meningococcal disease by an alternative mechanism to complement-mediated bacterial lysis. Vaccine-induced opsonophagocytic activity of antibodies present in human sera against subcapsular antigens has been observed following immunisation with OMVs derived from H44/76 strain (Findlow *et al.*, 2006). Whether OPA can provide immunity in humans independently of SBA activity has not been experimentally demonstrated. Yet, subcapsular antigens may involve a more direct role for cell-mediated immunity compared to the capsular polysaccharide which is the main effector of vaccine-induced protection in menC (Gomez-Lus *et al.*, 2003).

An important observation was the limited sequence variation of NmMIP. Antigenic variation is the result of positive immunoselective pressure and provides a method for meningococcal immune evasion. Sequence variation is common across

meningococcal immunogenic proteins including immunodominant PorA as well as minor antigens fHbp and FadL-like protein coded by NMB0088 (Brehony *et al.*, 2009; Derrick *et al.*, 1999; Yero *et al.*, 2010). Interestingly, this variation occurs in the exposed epitopic regions of meningococcal antigens, which are the targets for functional antibodies. Unlike these immunogens, NmMIP exhibited a highly conserved sequence with only a small number of substitutions scattered across the sequence precluding the presence of regions under strong positive selective pressure. Although following a structuring similar to other meningococcal antigens (Brehony *et al.*, 2009; Urwin *et al.*, 2004), the limited sequence diversity could suggest that NmMIP might have evolved to evade immunity and thus represent a weak antigen. In a different scenario, immune selection might have recently been introduced in the evolution of NmMIP sequence hence the limited variation observed. The limited reassortment observed between different allele types as a result of recombination events might provide support to this argument.

Sequence similarities between NmMIP and orthologues from other bacteria supports NmMIP functional role as a prolyl isomerase. The structural model presented in this study confirmed the different conformation observed for N- and C- terminal domains with N-terminal helices being fully exposed and C-terminal strands containing the enzymatic centre (Riboldi-Tunnicliffe *et al.*, 2001). However, structural and topological data did not conclude on the exact orientation of NmMIP on the meningococcal outer membrane. Results from whole-cell ELISA support a possible surface exposure which however could also be attributed to the heat inactivation of bacteria used in the assay. Additional methods would be needed to assess surface exposure of NmMIP under physiological conditions including flow cytometry and immunogold labelling using anti-NmMIP immune sera.

A study on the MIP orthologue from the related species *N. gonorrhoeae* by Leuzzi *et al.* supported a role for gonorrhoeal MIP in bacterial persistence in macrophages (Leuzzi *et al.*, 2005). This is in line with observations from intracellular bacterial pathogens which exploit MIP proteins to initiate infection of host macrophages (Cianciotto *et al.*, 1992). In a study of *Legionella pneumophila* MIP, the N- and C-terminal domains were assessed for contribution to virulence and both were found to be necessary for infection in animal models whilst only the dimer was essential for virulence against monocellular systems (Köhler *et al.*, 2003). Respective studies on *N. meningitidis* would greatly improve our understanding into the role of NmMIP in meningococcal pathogenesis.

6.10 Concluding remarks

Of the 4 proteins evaluated in immunological assays, NmMIP provided evidence for its potential to induce functional antibodies. The ability of anti-NmMIP antibodies to attract complement components on the meningococcal surface in combination with their opsonic and bactericidal activity in mice observed in this and other studies supports a role in vaccine-induced protective immune response against meningococcal disease. On the other hand, the absence of variable regions in the NmMIP sequence as a result of negative immune selection might have implications in the induction of a similar response in humans and hence the suitability of NmMIP as a vaccine candidate.

Chapter 7

General Discussion

7. General Discussion

7.1 Bacterial surface proteomics for the discovery of protein vaccine candidates

7.1.1 Bacterial treatment with proteases

Characterisation of the repertoire of protein antigens on the surface of bacteria for identification of potential vaccine candidates has been the objective of various proteomic strategies. A common feature of these strategies is the direct chemical treatment of whole bacteria to facilitate downstream isolation and analysis of surface-exposed proteins. Certain methods involve chemical agents to introduce a modification, such as biotin or fluorophores, on accessible amino acid residues of surface-exposed proteins (Ge *et al.*, 2007a; Ge *et al.*, 2007b; Mayrhofer *et al.*, 2006). Modified surface proteins can be separated from the total proteome by detection of fluorescence or purification using affinity interactions to pull down chemically-tagged proteins. However, non-uniform modification of proteins, cell-permeable chemicals and downstream purification steps might introduce variability and reduce specificity of these methods. In this study, treatment of whole bacteria with proteases was used for detection and characterisation of protein antigens on the surface of meningococci. This method provides a physical way to identify surface antigens through direct digestion of their exposed, epitopic domains (Bledi *et al.*, 2003; Rodriguez-Ortega *et al.*, 2006). Unlike other methods, the specificity of protease treatment is more dependent upon the extent of protein exposure on the bacterial surface, and less upon the success of chemical modification and purification steps.

The potential of protein treatment with proteases for discovery of putative antigens has been demonstrated in studies with Gram-positive bacteria, such as streptococci (Doro *et al.*, 2009; Rodriguez-Ortega *et al.*, 2006). These studies also highlighted certain limitations in the specificity of the method related with the direct

MS analysis of supernatant samples containing the protease-digested surface-exposed peptides. Contamination of supernatant samples with non-surface proteins was observed in most studies which varied depending on the organism and conditions of treatment (Dreisbach *et al.*, 2010; Tjalsma *et al.*, 2008; Walters *et al.*, 2009). Analysis of meningococcal supernatants following treatment with trypsin identified proteins, the majority of which were not unequivocally shown to be expressed at the bacterial surface. Gram-negative bacteria could be suggested to be more vulnerable to contamination phenomena as a result of the absence of physical barriers to protect the outer membrane. On the contrary, the membrane of Gram-positive organisms is surrounded by a thick peptidoglycan layer that could potentially offer better resistance to proteases. Comparative studies on bacteria of different Gram stain will be required to establish possible differences in their cell wall resistance to protease treatment.

An additional limitation to the analysis of supernatants is the need for more specific proteases like trypsin to facilitate downstream MS analysis. Specific proteases hydrolyse peptide bonds after certain residues. The number of protease cleavage sites across the exposed domains of surface proteins determines the number of peptides released in the supernatant. For a protein requiring 2 peptides to be identified by MS, 3 cleavage sites are needed. This, in turn, suggests that proteins containing less than 3 cleavage sites digested by the respective specific protease will fail to be identified. Direct MS analysis also produces inconsistencies in the number of peptides matched to each protein which can be the result of differences in the proteins abundance. Such inconsistencies further confuse interpretation of data and correct assignment of surface proteins. Based on these limitations, it is arguable whether the analysis of peptides released in the supernatant can reliably characterise the bacterial surface.

Comparative analysis of the total protein content before and after treatment with proteases poses as an attractive alternative strategy to identify the proteins digested by proteases in whole bacteria. This type of analysis avoids contamination phenomena related to the analysis of supernatants and therefore does not require exhaustive optimisation of treatment conditions. By uncoupling treatment from direct MS analysis, it also allows the use of less specific proteases, such as proteinase K, to enhance digestion efficiency. Before this report, this strategy had only been used for identification of *Helicobacter pylori* surface proteins (Sabarth *et al.*, 2005). When applied to meningococci, it provided a more realistic picture of antigens on the meningococcal surface compared to the analysis of supernatants. Major antigens PorA, OpcA, Omp85 and fHbp, potential antigens fadL-like NMB0088, NMB1468 and NMB1946 as well as other known and novel immunogens were part of the proteins identified. The specificity of analysis was also improved and only a few cytoplasmic proteins were identified mainly due to their co-detection in the same spot with proteins of the surface. Although not yet applied to Gram-positive bacteria, this approach could potentially find application across bacterial species regardless of their cell wall composition.

Quantitative DIGE analysis applied in this study also provided a way to obtain additional information on the individual proteins found on the bacterial surface. Quantitative results on the decrease in abundance of a protein spot upon treatment with protease are indicators of the extent of protein digestion which, in turn, can be associated with the protein's exposure and accessibility on the bacterial surface. In other words, completely digested proteins would be expected to be more accessible on the bacterial surface as opposed to partially digested proteins. In the context of vaccine development, this information could prove useful when choosing candidates

to be prioritised in immunological evaluation studies. Completely digested, highly accessible surface proteins are more likely strong immunogens with variable exposed epitopes due to positive immune selection. On the other hand, partially digested proteins would be expected to be more conserved and exhibit modest antigenic properties influenced by their weak accessibility to antibodies on the surface of individual isolates. For bacterial species with little previous knowledge of the antigenic protein content, this analysis can highlight and narrow down subsequent studies to proteins with the greatest potential as protective immunogens. For organisms like meningococci where major antigens are well studied, this approach can identify additional minor candidates which, proven protective, could complement vaccine formulations.

The nature of protease treatment and 2D-PAGE analysis can impose potential limitations to the application of this approach to certain protein groups. One such group includes proteins with inherent resistance to proteases. By resisting digestion these proteins will fail to be identified even when present on the surface. Outer membrane protein PorB is an example of a meningococcal protein with resistance to various proteases under native conditions (Minetti *et al.*, 1998). PorB was not found among the proteins affected by proteinase K although it is known to expose several loops on the meningococcal surface.

Another protein category that could be missed from this type of analysis includes highly hydrophobic members of the outer membrane. These proteins are difficult to solubilise in the mild detergents used during 2D-PAGE and might be under-represented in 2-D gels. Considering their association with the bacterial membrane, absence of these proteins from 2-D analysis could suggest a considerable loss of many potentially surface-exposed proteins. Identification of several OMPs on

the meningococcal 2-D gels prepared in this study suggests that the representation of membrane proteins on 2D gels can be enhanced by the use of certain conditions. Other strategies might also be implemented to overcome the issues related to 2D-PAGE. An attractive alternative would be the use of conventional SDS-PAGE coupled to chemical isotopic labelling for comparative analysis of protease treated and untreated bacteria (Soufi *et al.*, 2010). Proteins from different samples are first modified to contain different isotopic tags (*e.g.* SILAC, ICPL). They are subsequently solubilised in SDS-containing buffers, mixed and separated by SDS-PAGE. The use of SDS during sample preparation improves solubilisation and representation of membrane proteins. Several gel bands are then excised and proteins digested into peptides and analysed by MS. Differential analysis occurs in MS by comparing the peak intensity of each peptide in the different samples. The difference in mass introduced by isotopic labelling allows for differentiation between peptides from different samples. Considering a gel band contains tens of proteins, this type of analysis has the capacity to screen the entire proteome for potential surface proteins.

7.1.2 Immunoproteomics

Immunoproteomics is another proteome-wide analysis that can assist in the search for protein candidates to be qualified for further immunological evaluation of their vaccine potential. Blotting with immune sera from animals or humans is a common strategy to detect proteins that evoke an immune response. Conventional SDS-PAGE immunoblots have been used during the evaluation of OMV vaccines to assess consistency in IgG responses against major OMPs across different batches (Frasch *et al.*, 2001; Fredriksen *et al.*, 1991). However, the low resolution of proteins in SDS-PAGE does not allow for accurate identification of other minor immunogenic

proteins. Through combination of 2D-PAGE and MS analysis, immunoproteomics offers a strategy to not only detect but also identify the proteins involved in immune responses. Using this approach, a 2-D map was constructed containing the proteins identified to react to immune sera from mice immunised with whole meningococci or OMV vaccine preparations. Identification of immunogens not previously reported in the literature demonstrated the potential of immunoproteomics to support the discovery of novel potential antigens.

Similar to the surface proteome analysis, there are limitations to the information that can be inferred from immunoblots. Reactivity of a protein to antibodies present in immune sera does not necessarily mean that these antibodies are functional and able to offer protection. Bactericidal killing and opsonophagocytosis assays are necessary for the assessment of the functional activity of protein specific antibodies and for immunoreactive proteins to be considered as potential antigens. Additionally, antibody binding on immunoblots does not represent the true conditions of antibody recognition on the bacterial surface. The denaturing conditions used during protein separation on gels alter the conformation acquired by protein epitopes when presented to the immune system. Immunogenic proteins for which interaction with antibodies is based on conformational epitopes are therefore missed by immunoproteomic analysis. Similarly, denaturation may reveal otherwise hidden epitopes and give a positive result on immunoblots when the native protein would not normally react.

The use of animals as a source of immune sera is another possible cause of unrealistic results. Especially in the case of meningococci where the human is the only host, immune responses raised in mice and other animals can vary greatly and should be treated with some scepticism. This also highlights the importance of

studying sequence variation as a more relevant way to assess human immune responses. From all of the above, immunoblots are essentially not expected to be the protagonists but instead play a supportive role in the screening of bacterial proteomes for identification of potential antigens.

7.1.3 Integrated 2-D proteome maps

Proteomic methods often generate a great amount of data that require further organisation and processing if comprehensive conclusions are to be drawn. The methods described for the analysis of bacterial surface antigens potentially provides a variety of datasets that need to be compared and integrated into a single data file. The use of 2D-PAGE and the generation of 2-D proteome maps provide the starting platform where integration of results from different experiments can be achieved. A schematic example of an integrated proteome map is presented in Figure 7.1. The map incorporates results from the two proteomic experiments performed in this study to provide a picture of the meningococcal antigenic proteome. Surface proteins digested by proteases are highlighted using dark and light green circles corresponding to complete and partial digestion, respectively. Proteins reacting with sera from immunised mice are demonstrated by red dots. The resulting map can be supplemented with information from comparative studies on protein expression levels between different isolates, like the one presented in Chapter 3 between wild-type and Δ -*siaD* MC58 strains. Additionally, the size of each spot on 2-D gels can be used to provide information on the relative expression levels of the corresponding proteins in the bacterial proteome.

Integrated maps can form the basis for the generation of more comprehensive, publicly available “antigen-ome” databases, *i.e.* databases that incorporate results

from application of the described methods to more isolates and more bacterial species and link them to information from antigenic variation and immunological studies. Construction of such databases are feasible using packages like the Make2D-DB II (Mostaguir *et al.*, 2003) which have been used for the design of existing 2-D databases such as SWISS-2DPAGE and World-2DPAGE Repository. The contribution of “antigen-ome” databases to vaccine discovery will be marked considering that the researcher will have simultaneous access to information on the coverage, level of expression, surface exposure and immunological responses of protein vaccine candidates, prior to any clinical testing.

7.2 The future of proteomics in meningococcal disease

The advances seen in the field of whole genome sequencing have enabled the generation of genome-wide sequence data from an increasing number of meningococcal isolates which can be used in comparative genomics and transcriptomics studies. There are various reports from studies comparing the genomes of invasive and carrier isolates in an attempt to identify virulent genes associated with the invasive meningococcal phenotype (Hotopp *et al.*, 2006; Joseph *et al.*, 2010; Schoen *et al.*, 2008). Available genome sequences have also facilitated construction of microarrays which have been used to screen for differences in the gene content of isolates from various serogroups and of different disease phenotype (Stabler *et al.*, 2005). Microarray technology was also used in transcriptomics studies assessing changes in gene expression upon interaction with human epithelial cells or exposure to human serum for identification of genes involved in meningococcal pathogenesis (Dietrich *et al.*, 2003; Grifantini *et al.*, 2002a; Kurz *et al.*, 2003).

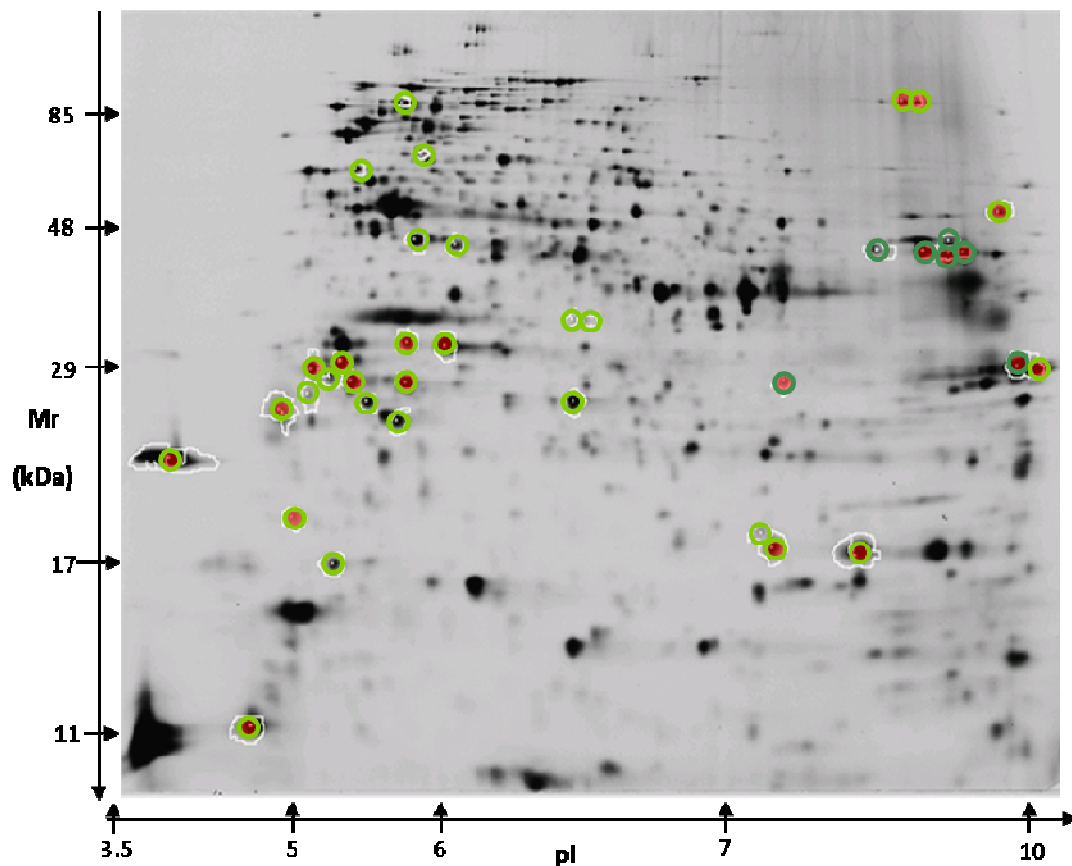


Figure 7.1 Integrated 2-D map of the antigenic proteome of *N. meningitidis* MC58. Proteins on the meningococcal surface digested by proteinase K are indicated by green rings, dark and light colours corresponding to complete and partial digestion, respectively. Rings filled with red colour highlight those proteins binding to antibodies from murine immune sera in immunoproteomic analysis.

So far, meningococcal genomes have been sequenced using Sanger technology, which is based on electrophoretic separation of dNTPs with single-base resolution. Next generation sequencing-by-synthesis methods, such as pyrosequencing and single molecule sequencing, with a yield of gigabases per run are able to sequence megabase-long microbial genomes in just a few days (Shendure *et al.*, 2004). The first example of application of high-throughput genome sequence analysis in meningococcal research was recently demonstrated by whole-genome analysis of 105 meningococcal reference isolates (Bratcher *et al.*, 2011). Information on gene

presence and sequence diversity that would once require years to collect is now immediately available for hundreds of proteins across a wide panel of isolates.

Understanding the impact of differences in genome sequence and expression profiles on the protein level is essential to elucidate the processes underlying meningococcal pathogenesis. Proteome-wide analysis has been used in cases to characterise sub-proteomes or compare pairs of samples subjected to different treatment conditions (Marzoa *et al.*, 2009; Mignogna *et al.*, 2005; Schauder *et al.*, 2005; Vipond *et al.*, 2006). Similar to the methods reported here, these studies used 2D-PAGE and DIGE for protein separation and differential analysis, respectively. Given its robust but labour-intensive character, 2-D gel-based analysis is more likely to continue its application to small scale projects with a restricted number of samples. Where there is need for high throughput proteome analysis, gel-free MS-based methods will come into play.

Following advances in LC for protein separation and instrumentation for mass spectrometry, MS-based proteomics promise large-scale analysis of several proteomes in relatively short experimental times. It is now possible to separate whole proteomes using 2 steps of high performance liquid chromatography in an automated way (Wolters *et al.*, 2001). Each fraction collected from the first separation step is directly applied to the second chromatographic column, which is interfaced to the mass spectrometer for immediate analysis. The use of 2 chromatographic steps offers the advantage of increasing peak capacity and hence improving resolution of separated peptides. The automation in the whole process is an added benefit when processing large numbers of samples. The evolution of hybrid mass spectrometers has also enabled faster and more accurate acquisition of mass spectra. By combining 2 or more analysers into hybrid systems, scan times have decreased substantially meaning more

mass spectra can be acquired in less time (Frese *et al.*, 2011). Following a typical shotgun proteomics workflow where proteins are subjected to trypsin digestion followed by LC separation and MS analysis of peptides, a complete MS analysis of a bacterial proteome would require a day to complete.

Given the availability of sequencing data and hence protein databases, characterisation of the total protein content from a large panel of isolates would be one possible application of MS-based analysis. Relative protein expression levels could also be inferred based on comparison of ion intensities across spectra from the different isolates (Neilson *et al.*, 2011). Peptide ion intensities are indicators of the relative abundance of proteins in the samples. This method of comparative proteomics, also known as label-free quantitation, has no limitations to the number of samples to be compared. This is a considerable advantage over isotope labelling methods for comparative analysis where the number of compared samples is restricted by the number of available isotope tags. Label-free quantitation could be useful in many hypothesis-driven studies to identify which proteins are solely or over-expressed in invasive meningococcal strains as opposed to carriers. It could also be used to assess the changes in meningococcal proteome upon interaction with host epithelial cells or cells of the host's innate immune system. Results from such studies can highlight single virulent factors or whole protein networks involved in meningococcal colonisation and survival in humans. Assessment of the relative expression levels of protein vaccine candidates across a wide panel of hypervirulent strains will also be possible with label-free approaches. Combined with sequencing data on the antigenic diversity of proteins across the same meningococcal population this information will provide strong evidence on the potential coverage and efficacy of protein candidates and guide plans for their consideration in clinical trials.

Further to their application in whole-proteome comparative studies, MS-based methods can offer additional opportunities in the manufacturing evaluation of novel menB vaccines. Given the presence of different proteins or variants of the same protein in some vaccine preparations, an analytical method to accurately quantify the different components in the same batch or across different batches will be essential. Protein variants comprise different amino acid sequences, which upon fragmentation inside the mass spectrometer will generate unique MS/MS spectra. Based on the knowledge of protein variant sequences the MS/MS spectra and hence the peptides that belong to each variant will be inferred. Assessing the consistency in protein levels across batches can then be possible by comparing peptide ion intensities in a label-free manner. Alternatively, the abundance of each variant in the same sample can be quantified in absolute numbers using absolute quantitation (AQUA) technology (Gerber *et al.*, 2003). In AQUA, a known amount of a synthetic peptide incorporating a stable isotope-labelled amino acid is spiked in the sample to be analysed. The spiked peptide is selected to have the exact same sequence as a tryptic peptide unique to the protein variant to be quantified. During MS analysis, the synthetic peptide ion will generate a peak in MS spectrum with a mass difference from that of the native peptide owing to the isotope-labeled amino acid. The quantity of the native protein is subsequently calculated by comparing the ion intensity ratio between the 2 peptides.

7.3 MIP as a potential vaccine candidate

Screening of the meningococcal proteome identified NmMIP as a novel immunoreactive protein expressed at relatively high levels on the bacterial surface. Subsequent evaluation of the antigenic and sequence properties of NmMIP offered contradicting results as to the potential of NmMIP as a target antigen for use in

vaccines. On one hand, results from opsonophagocytosis and complement deposition assays in combination with the bactericidal activity observed by Hung *et al.* (Hung *et al.*, 2011) suggest the potential of anti-NmMIP antibodies to confer protection against menB strains. In contrast, the absence of positive selection over NmMIP sequence raises concerns over the potential involvement of NmMIP in acquired immunity and hence its ability to induce a strong response in humans.

During the writing of this thesis, Echenique-Rivera *et al.* reported up-regulation of the gene *fkpA* coding for NmMIP in a model of human blood infection (Echenique-Rivera *et al.*, 2011). Expression of NmMIP was also shown to be required for meningococcal survival in human blood. It is not yet clear whether this is associated with the putative role of NmMIP in facilitating persistence in macrophages. However, these observations suggest that NmMIP is an important virulent factor expressed in the host during meningococcal infection. Testing of human blood from infected patients for presence of anti-NmMIP antibodies would provide further evidence on this expression as well as the potential contribution of NmMIP to protective immune responses in humans. Following experiments should also include analysis of surface exposure and expression levels of NmMIP from a wide panel of isolates. The information gathered from such analysis combined with the results reported here should form the basis of possible clinical evaluation of NmMIP.

7.4 Prospects for meningococcal vaccines

The idea of a meningococcal vaccine able to protect against all major invasive serogroups remains elusive primarily due to the difficulties encountered in the development of a vaccine protecting against menB disease. Instead, various serogroup-specific vaccine formulations have been developed over the years against

the other invasive serogroups. Development of serogroup-specific vaccines has to some extent been supported by the different distribution of strains and hence the different needs for vaccine formulations in every continent.

Currently, conjugate polysaccharide vaccines against serogroups A, C, W-135 and Y are licensed and distributed across the globe. The first conjugate vaccine was a monovalent group C formulation introduced into schedules of routine infant immunisations in the UK in 1999 (Richmond *et al.*, 1999b). The vaccine achieved considerable decline in menC disease owing to the induction of herd immunity (Maiden *et al.*, 2008). Following the success of menC vaccine, an initiative has been introduced for massive immunisations across countries of the African Meningitis belt where the major disease burden resides. The introduced vaccine, termed MenAfriVac, is a monovalent formulation against serogroup A, the most prevalent serogroup in that area (Bishai *et al.*, 2011). Although the circulation of serogroup W-135 strains in Africa remains a concern, this initiative is the first encouraging step towards availability of vaccines to those suffering the most. The emergence of serogroup Y disease in the United States and European countries is also being treated with licensure of tetravalent A, C, W-135 and Y vaccines. The most recent formulation, MenVeo from Novartis Vaccines, was recently approved for administration to all ages from 2 to 55 years old whilst promising protection to infants under the age of 2 (www.novartis.com, 2011b; Cooper *et al.*, 2011). Long-term epidemiological studies following their use will be essential to assess whether tetravalent vaccines can be as successful as their monovalent counterparts. The eventual establishment of tetravalent conjugate vaccines as the sole polysaccharide-based formulation might also be considered on the basis that their global and cost effective distribution can be guaranteed. Any future alterations to the existing immunisation schedules including

replacements, additions or withdrawals of vaccines should also follow epidemiological data and surveillance studies.

With OMV vaccines able to protect against only a limited group of serogroup B isolates of the same serosubtype, the development of a broadly protective menB vaccine has been the main focus of meningococcal vaccine research for the past decade. The slow progress seen in the development of such vaccine is mainly attributable to the nature of meningococcal candidate antigens. On one side, highly immunogenic proteins exhibit high sequence variability which confines the breadth of protection to variant specific isolates. On the other side, proteins with more conserved epitopes are not immunodominant and often do not guarantee good levels of protection.

To overcome the limitations imposed by either group of protein antigens, 2 strategies have been suggested. One strategy involves the combination of the most common variants of an immunodominant protein in a single formulation. For antigens PorA, FetA, Opa and fHbp, the number of variants required for broad protection has been calculated and found to be conveniently small owing to the antigenic structuring of these proteins. A vaccine containing two fHbp variants developed by Pfizer (formerly Wyeth) was shown to elicit protective bactericidal titres in a high proportion of adults and adolescents in phase II clinical trials (Richmond *et al.*, 2010). Alternatively, vaccines combining variable and conserved proteins in the same formulation have been developed and tested. The Novartis multicomponent 4CMenB is an example of such vaccine preparation which has now completed clinical trials and awaits regulatory review from European authorities under the brand name Bexsero (www.novartis.com, 2010). 4CmenB comprises recombinant forms of variant 1 of fHbp which is found in approximately 70% of circulating menB isolates, the more

conserved NadA and HBPA and OMVs from the New Zealand 98/254 strain (www.novartis.com, 2011a). Based on results from clinical trials in infants following a three-dose immunisation schedule, 4CmenB claims coverage of 79% or greater against the strains tested (www.novartis.com, 2011a).

While the licensure of the first menB vaccine seems to be a matter of time, menB vaccine research should remain active in various directions. Judging by the first reports on the efficacy of the protein-based vaccines under trials, there is still room for improvement in the current combinations of meningococcal proteins and protein variants. It is thus important that the screening of different combinations of existing potential antigens for improved vaccine coverage continues. Formulations based on multiple variants from immunodominant proteins like PorA, Opa and FetA, carefully selected to represent the hypervirulent strains, could possibly offer the desired breadth of coverage without compromising efficacy. The effectiveness of such formulations in clinical trials remains to be tested together with the ease and cost effectiveness of their manufacturing. Alternatively, conserved minor antigens could be combined and act synergistically to confer broad protection. In one such example, 4 proteins of relatively low expression in meningococci, namely TbpA, NspA, Omp85 and Hsf, were over-expressed in a strain where expression of PorA was abolished. Outer membrane vesicles derived from this strain were shown to induce bactericidal killing of unrelated strains, which was the result of an additive effect of the antigens expressed (Weynants *et al.*, 2007). This example also highlights the importance of studies for the discovery of novel potential antigens like NmMIP to feed such protein combinations.

Investigation of vaccine preparations based on OMVs and/or non-protein meningococcal surface structures could offer an attractive alternative and should also

be pursued. The use of naturally-released OMVs containing detoxified lipooligosaccharide presents a promising approach for production of a cross-protective vaccine. By combining the antigenic properties of LOS and OMPs from native OMVs, these formulations were recently shown to induce good bactericidal response against homologous and heterologous strains in mice (Weynants *et al.*, 2009). Reproducing this response in humans has so far been difficult and additional trials will be required. Other strategies could involve the use of peptide mimics of the serogroup B capsular polysaccharide (Moe *et al.*, 1999b) or live bacteria from strains with an avirulent, attenuated phenotype (Li *et al.*, 2004; Newcombe *et al.*, 2004; Tang *et al.*, 1999).

Upon administration of the developed menB vaccines, constant monitoring of their impact would also be essential. One particular concern involves the possible emergence of protein variants not covered by the introduced vaccines as a result of meningococcal immune evasion. Constant surveillance will be required to assess this possibility and modify the formulations used accordingly. Attention should also be focussed on the efficacy of the vaccines and the generation of immunity especially in infants which constitute the most vulnerable group. Parameters including formulation and adjuvant used could potentially affect vaccines efficacy and should be carefully chosen (Sanders and Feavers, 2011).

Protein-based vaccines will also need to be assessed for induction of herd immunity. Herd immunity can occur as a result of the disruption of transmission of invasive strains following vaccination. Herd immunity has been considered to be responsible for the long-term protection of infants in the absence of protective antibody levels following introduction of the serogroup C conjugate vaccine in the UK (Borrow and Miller, 2006). So far, the same herd effect has not been reported for

OMV vaccines. If herd immunity would be induced by protein-based vaccines, sufficient levels of protection could be achieved using fewer vaccine doses in infants or even by directing routine immunisations to teenagers and young adults, who exhibit the highest rates of carriage and are mostly responsible for transmission.

Finally, the cost of multicomponent vaccines needs to be addressed. The more complex the vaccine, the more complicated and expensive its manufacturing becomes. Based on preliminary probabilistic studies, the introduction of the current multicomponent vaccines into routine infant immunisations schedules in the UK may not prove to be cost effective (Christensen *et al.*, 2010). The efficacy and breadth of coverage of the final formulations will also influence the financial viability of the vaccine by determining the immunisation schedule (*i.e.* number of doses, age groups to be immunised). Eventually, a single meningococcal vaccine protecting against the major invasive serogroups might pose as the most likely formula to sustain affordable routine immunisations without compromising population protection.

7.5 Conclusion

This study presented a refined version of the analysis of the bacterial surface proteome following cell treatment with proteases. Two-dimensional DIGE of the proteomes from protease-treated and untreated bacteria was shown to specifically detect surface proteins with antigenic properties. Given the implication of these proteins in immune responses, this approach can aid the identification of protein antigens from various other bacterial pathogens. Quantitative data on the extent of protease cleavage can also be exploited during selection of proteins for preclinical assessment. Combination of this information with genomic studies of antigenic diversity could eventually lead to a more integrated strategy for the selection of

candidates for clinical evaluation. The *N. meningitidis* macrophage infectivity potentiator, which was identified from proteomic analysis of the meningococcal surface proteome, was suggested to be a novel immunogen under negative selective pressure. Providing it is sufficiently accessible on the surface of invasive strains to induce bactericidal antibodies in humans, NmMIP could pose as a promising vaccine candidate. While the discovery of novel antigens is vital for improving current formulations, the assessment of the quality, efficacy and cost effectiveness of forthcoming vaccines should be the future aim of meningococcal vaccine research.

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Appendix

Table A1. Meningococcal isolates used in phylogenetic analysis of the gene coding for NmMIP. Information on the isolates was derived from the Neisseria MLST database (<https://pubmlst.org/neisseria/>) (Jolley *et al.*, 2010)

id	isolate	sero-group	sequence type	clonal complex	serosubtype		NmMIP deletion ^a
					VR1	VR2	
1	A4/M1027	A	4	ST-4 complex/subgroup IV	5-2	10	n
2	120M	A	1	ST-1 complex/subgroup I/II	5-2	10	n
7	7891	A	5	ST-5 complex/subgroup III	20	9	d
10	6748	A	1	ST-1 complex/subgroup I/II	18-1	3	n
11	129E	A	1	ST-1 complex/subgroup I/II	5-2	10	n
13	139M	A	1	ST-1 complex/subgroup I/II			
19	S3131	A	4	ST-4 complex/subgroup IV	7	13-1	n
24	S4355	A	5	ST-5 complex/subgroup III	5-1	9	d
31	10	A	4	ST-4 complex/subgroup IV	7	13-1	n
34	20	A	1	ST-1 complex/subgroup I/II	5-2	10	n
35	26	A	4	ST-4 complex/subgroup IV	7	13	n
46	255	A	4	ST-4 complex/subgroup IV	7-2	13-1	n
52	243	A	4	ST-4 complex/subgroup IV	7	13	n
61	393	A	1	ST-1 complex/subgroup I/II	5-2	10	n
64	254	A	1	ST-1 complex/subgroup I/II	5-2	10	n
67	S5611	A	1	ST-1 complex/subgroup I/II	5-2	10	n
82	11-004	A	5	ST-5 complex/subgroup III	20	9	d
84	IAL2229	A	5	ST-5 complex/subgroup III	20	9	d
90	CN100	A	21		5-2	10	n
120	F4698	A	5	ST-5 complex/subgroup III	20	9	d
128	F6124	A	5	ST-5 complex/subgroup III	20	9	d
160	1014	A	4	ST-4 complex/subgroup IV	7	13-1	n
210	H1964	A	5	ST-5 complex/subgroup III	20	9	d
237	44/76	B	32	ST-32 complex/ET-5 complex	7	16	n
238	153	A	5	ST-5 complex/subgroup III	20	9	d
239	154	A	6	ST-5 complex/subgroup III	20	9	n
299	80049	A	5	ST-5 complex/subgroup III	5-2	10	n
314	D1	C	11	ST-11 complex/ET-37 complex	5	2-1	d

id	isolate	sero- group	sequence type	clonal complex	serosubtype		NmMIP deletion ^a
					VR1	VR2	
316	D8	A	4	ST-4 complex/subgroup IV	7	13-1	n
340	196/87	C	32	ST-32 complex/ET-5 complex	7-2	16-12	n
343	500	C	11	ST-11 complex/ET-37 complex	5	2	d
344	F1576	C	11	ST-11 complex/ET-37 complex	5	2	d
349	38VI	B	11	ST-11 complex/ET-37 complex	5	2	d
369	M597	C	11	ST-11 complex/ET-37 complex	5	2-1	
387	2059001	A	4	ST-4 complex/subgroup IV	7	13	n
391	90/18311	C	11	ST-11 complex/ET-37 complex	5	2-1	d
398	BZ 10	B	8	ST-8 complex/Cluster A4	5-1	2-2	n
400	BZ 83	B	34	ST-32 complex/ET-5 complex	5-2	10	n
401	BZ 133	B	1	ST-1 complex/subgroup I/II	7	16	n
403	BZ 147	B	48	ST-41/44 complex/Lineage 3	18-2	1-2	n
407	BZ 163	B	9	ST-8 complex/Cluster A4	21	16	n
408	BZ 169	B	32	ST-32 complex/ET-5 complex	5-2	16	n
409	BZ198	B	41	ST-41/44 complex/Lineage 3	7-2	4	n
410	BZ 232	B	38	ST-37 complex	5-2	2-2	d
411	DK 24	B	16		delete d	delete d	n
412	DK 353	B	37	ST-37 complex	5-2	2-2	d
413	EG 328	B	18	ST-18 complex	22	14	n
414	EG 327	B	19	ST-18 complex	7	14-1	n
415	EG 329	B	32	ST-32 complex/ET-5 complex	7-1	16	n
416	EG 011	B	36		18-1	3	d
417	NG 3/88	B	12		7-1	1	n
418	NG 4/88	B	30		18	25-7	n

id	isolate	sero- group	sequence type	clonal complex	serosubtype		NmMIP deletion ^a
					VR1	VR2	
419	NG 6/88	B	13	ST-269 complex	7-1	1	n
420	NG F26	B	13	ST-269 complex	31	16	n
421	NG H15	B	43	ST-41/44 complex/Lineage 3	19	15-2	n
422	NG H41	B	27		5-2	10	d
423	NG H38	B	36		18-1	3	d
424	NG E31	B	15	ST-364 complex	12-1	13-1	d
425	NG G40	B	25		19	15-1	n
426	NG E28	B	26		22-1	14-1	d
427	NG E30	B	44	ST-41/44 complex/Lineage 3	21	16	n
428	NG H36	B	47	ST-41/44 complex/Lineage 3	5-1	2-2	n
430	NG 080	B	32	ST-32 complex/ET-5 complex	7	16	n
431	NG144/82	B	32	ST-32 complex/ET-5 complex	7	16	n
434	NG PB24	B	32	ST-32 complex/ET-5 complex	7-2	16-7	n
436	NG P20	B	11	ST-11 complex/ET-37 complex	5	2	d
441	8680	B	32	ST-32 complex/ET-5 complex	7-2	3	n
442	297-0	B			19	15-1	n
443	3906	B	17		12-1	16-8	n
444	SWZ107	B	35	ST-35 complex	22-1	14-1	n
445	528	B	18	ST-18 complex	22	14	n
446	1000	B	20	ST-18 complex	5-1	10-4	n
451	14/1455	A	5	ST-5 complex/subgroup III	20	9	d
466	371	A	1	ST-1 complex/subgroup I/II	5-2	10	n
467	690	A	4	ST-4 complex/subgroup IV	7	13-1	n
468	BRAZ10	C	11	ST-11 complex/ET-37 complex	5-1	10-1	n
488	106	A	1	ST-1 complex/subgroup I/II	5-2	10	n
492	79128	A	3	ST-1 complex/subgroup I/II	7-1	10	n
493	322/85	A	2	ST-1 complex/subgroup I/II	5-2	10	n

id	isolate	sero- group	sequence type	clonal complex	serosubtype		NmMIP deletion ^a
					VR1	VR2	
494	79126	A	3	ST-1 complex/subgroup I/II	7-3	10-5	n
507	MA-5756	C	11	ST-11 complex/ET-37 complex	5	2-1	d
597	92001	A	7	ST-5 complex/subgroup III	20	9	d
613	Z2491	A	4	ST-4 complex/subgroup IV	7	13-1	n
638	G2136	B	8	ST-8 complex/Cluster A4	5-2	10-1	n
639	B6116/77	B	10	ST-8 complex/Cluster A4	5-1	2-2	n
640	SB25	C	8	ST-8 complex/Cluster A4	18-1	3	n
641	94/155	C	66	ST-8 complex/Cluster A4	5	2	n
642	312 901	C	8	ST-8 complex/Cluster A4	5	2	n
643	AK22	B	153	ST-8 complex/Cluster A4	5-2	10	n
644	L93/4286	C	11	ST-11 complex/ET-37 complex	5-1	10-4	d
645	204/92	B	33	ST-32 complex/ET-5 complex	19	15	n
646	400	B	40	ST-41/44 complex/Lineage 3	7-2	13-2	n
647	AK50	B	41	ST-41/44 complex/Lineage 3	7-2	4	n
648	M-101/93	B	41	ST-41/44 complex/Lineage 3	7-2	4	n
649	50/94	B	45	ST-41/44 complex/Lineage 3	7-2	4	n
650	M40/94	B	41	ST-41/44 complex/Lineage 3	7-2	4	n
651	931905	B	41	ST-41/44 complex/Lineage 3	7-2	4	n
652	N45/96	B	41	ST-41/44 complex/Lineage 3	7-2	4	n
653	91/40	B	42	ST-41/44 complex/Lineage 3	7-2	4	n
654	88/03415	B	46	ST-41/44 complex/Lineage 3	7-2	4	n
655	E32	Z	31	ST-334 complex	5-2	10-9	d
656	E26	X	39	ST-198 complex	18	25-1	n
657	860060	X	24	ST-750 complex	12-1	13-5	n

id	isolate	sero- group	sequence type	clonal complex	serosubtype		NmMIP deletion ^a
					VR1	VR2	
658	890326	Z	28	ST-103 complex	5-4	2-2	n
659	A22	W-135	22	ST-22 complex	18-1	3	d
660	71/94	Y	23	ST-23 complex/Cluster A3	5-1	2-2	n
661	860800	Y	29	ST-167 complex	5-1	10-4	d

^a It refers to the four amino acid deletion and its prevalence across strains. n: strains without deletion, d: strains with deletion.

NMB1567 (NmMIP)

ATGAACACCATTTTCAAAATCAGCGCACTGACCCTTTCGCCGCTTTGGCACTTTC  CGCCTGCGGCAAA
AAAGAAGCCGCCCCGCATCTGCATCCGAACCTGCCGCCGCTTCTTCCGCGCAGGGCGACACCTCTTCG
ATCGGCAGCACGATGCAGCAGGCAAGCTATGCGATGGGCGTGGACATCGGACGCTCCCTGAAGCAAATG
AAGGAACAGGGCGCGGAAATCGATTTGAAAGTCTTTACCGAAGCCATGCAGGCAGTGTATGACGGCAAA
GAAATCAAAATGACCGAAGAGCAGGCTCAGGAAGTCATGATGAAATTCCTTCAGGAACAACAGGCTAAA
GCCGTAGAAAAACACAAGGCGGACGCGAAGGCCAATAAGAAAAAGGCGAAGCCTTTCTGAAAGAAAAT
GCCGCCAAGACGGCGTGAAGACCACTGCTTCCGGCCTGCAATACAAAATCACCAAACAGGGCGAAGGC
AAACAGCCGACCAAAGACGACATCGTTACCGTGGAATACGAAGGCCGCTGATTGACGGTACGGTATTC
GACAGCAGCAAAGCCAACGGCGGCCCCGGTCACCTTCCCTTTGAGCCAAGTGATTCCGGGTGGACCGAA
GGCGTACAGCTTCTGAAAGAAGGCGGCGAAGCCACGTTCTACATCCCGTCCAACCTTGCCTACCGCGAA
CAGGGTGCGGGCGACAAAATCGGTCCGAACGCCACTTTGGTATTTGATGTGAAACTGGTCAAAATCGGC
GCACCCGAAAACGCGCCCGCCAAGCAGCCGGCTCAAGTCGACATCAAAAAAGTAAATTAA  GTCCGAATC
CATGCCCGAAACAGGTTTTTCGGGCATTTTTACGGCA

NMB1811 (PiIP)

ATGAAACACTATGCCTTACTCATCAGCTTTCTGGCTCTCTCCGCGTGTCCCAAGGT  TCTGAGGACCTA
AACGAATGGATGGCACAAACGCGACGCGAAGCCAAAGCAGAAATCATACCTTTCCAAGCACCTACCCTG
CCGTTGCGCCGGTATACAGCCCCCGCAGCTTACAGGGCCGAACGCATTGCACTTCCGCCGCATGGAA
ACCGACAAAAAAGGGGAAAATGCCCCGACACCAAGCGTATTAAAGAAACGCTGGAAAAATTGAGTTTG
GAAATATGCGTTATGTCGGCATTTTGAAGTCCGGACAGAAAGTCTCCGGCTTCATCGAGGCTGAAGGT
TATGTCTACACTGTGCGGTGTCGGCAACTATTTGGGACAAAACCTACGGTAGAATCGAAAGCATTACCGAC
GACAGCATCGTCCTGAACGAGCTAATAGAAGACAGCACGGGCAACTGGGTTTCCCGTAAAGCAGAACTG
CTGTTGAATTCTTCCGACAAAAACACCGAACAAGCGGCAGCACCTGCCGCAGAACAAAATTAA 

Figure A1. Amplified nucleotide sequence and primer annealing position
(continues in the next page).

NMB1810 (PilO)

ATGGCTTCTAAATCATCTAAAACCAACTTGGATCTCAACAACCTTCACCTGCTCAACCTTCCTGCCAGG
CTTTTTATCGCCCTGCTGGCCGTTGCCGCCGTGCTGGGGCTCGGTTATGC   CGGATTGTTCAAAAGCCAG
 ATGGAATCCCTTGAGGAATACGAAGCAAAAGAAACCGAACTGAAAAACACCTACAAACAGAAAAGTATC
GACGCGGCCAGCCTGAACAACCTGAGGGACGAACTTGCCTCAATCCGCTCTGCCTTCGATATCATGTTG
AAACAGCTGCCGACAGATGCAGAAATTCCAATCTGGTTCAAGAGCTTCATCAGGCAGGTTTCGAGCAAC
GGTCTGCGCTTGACAGCGTTATGCCCCAACCTCCCGTAGATGACGGCCCCATCAAAAGATTACCCTAT
TCCATTTCCATTACCGGAAATTACGAACAGATCAGCCAATTTACCCGCGATGTCGGCAGCCTCTCCCGA
ATCATTACCCTTGAGTCGCTGAAAAATCGCCCAATCTCCGGAAAAACGGCGGCAATCCTGACGGCAAGAGC
AGCATCCTGAACCTCAGCGCCATTGCCACCACCTACCAAGCAAAATCCGTAGAAGAGCTTGCCGCAGAA
GCGGCACAAAATGCCGAGCAAAAATAA 

NMB2095 (adhesion complex protein)

ATGAAACTTCTGACCACCGCAATCCTGTCTTCCGCAATCGCGCTCAGCAGTATGGCTGCCGC   CGCTGGC
 ACGGACAACCCCACTGTTGCAAAAAAACCGTCAGCTACGTCTGCCAGCAAGGTAAAAAAGTCAAAGTA
ACCTACGGCTTCAACAAACAGGGTCTGACCACATACGCTTCCGCCGTATCAACGGCAAACGCGTGCAA
ATGCCTGTCAATTTGGACAAATCCGACAATGTGGAAACATTCTACGGCAAAGAAGGCGTTATGTTTTG
GGTACCGGCGTGATGGATGGCAAATCCTACCGCAAACAGCCCATTATGATTACCGCACCTGACAACCAA
ATCGTCTTCAAAGACTGTTCCCCACGTAA 

Figure A1. Amplified nucleotide sequence and primer annealing position. The length of primer and position of annealing to the nucleotide sequence is presented by thick horizontal arrows. Short vertical arrows represent the cleavage site of the signal peptide. The sequence to be amplified by the pair of primers is highlighted in blue. The red-coloured sequence corresponds to the ORF downstream of NMB1567.

Table A2. Analysis of meningococcal proteins with intrinsic fluorescent properties. Protein lysates were extracted from MC58 Δ -*siaD* strain and subjected to SDS-PAGE and 2D-PAGE following the methods described in sections 2.6. No pre-labelling with fluorescent dyes was performed. Gels were directly scanned in the Cy3 and Cy5 channel of the Typhoon 4100. Images from 2-D gels were imported into DeCyder v.6.5 and matched to 2-D images of total-protein profiles of the same sample from previous experiments. The position of the gel spot corresponding to autofluorescent protein 2 (AF2) was identified in preparative gels which were subsequently used for spot picking. The AF2 protein spot picked was digested with trypsin and identified by LC MS/MS as cytochrome c5 (UniProtKB accession number Q9JYA2 – NMB1677) (7 peptides matched, sequence coverage of 38%). The meningococcal cytochrome c5 is a predicted inner membrane-associated protein involved in electron transfer during nitrite respiration (Deeudom *et al.*, 2008). The sizes (in kDa) of protein marker bands are shown on the left. M: Pre-stained wide range MW protein marker.

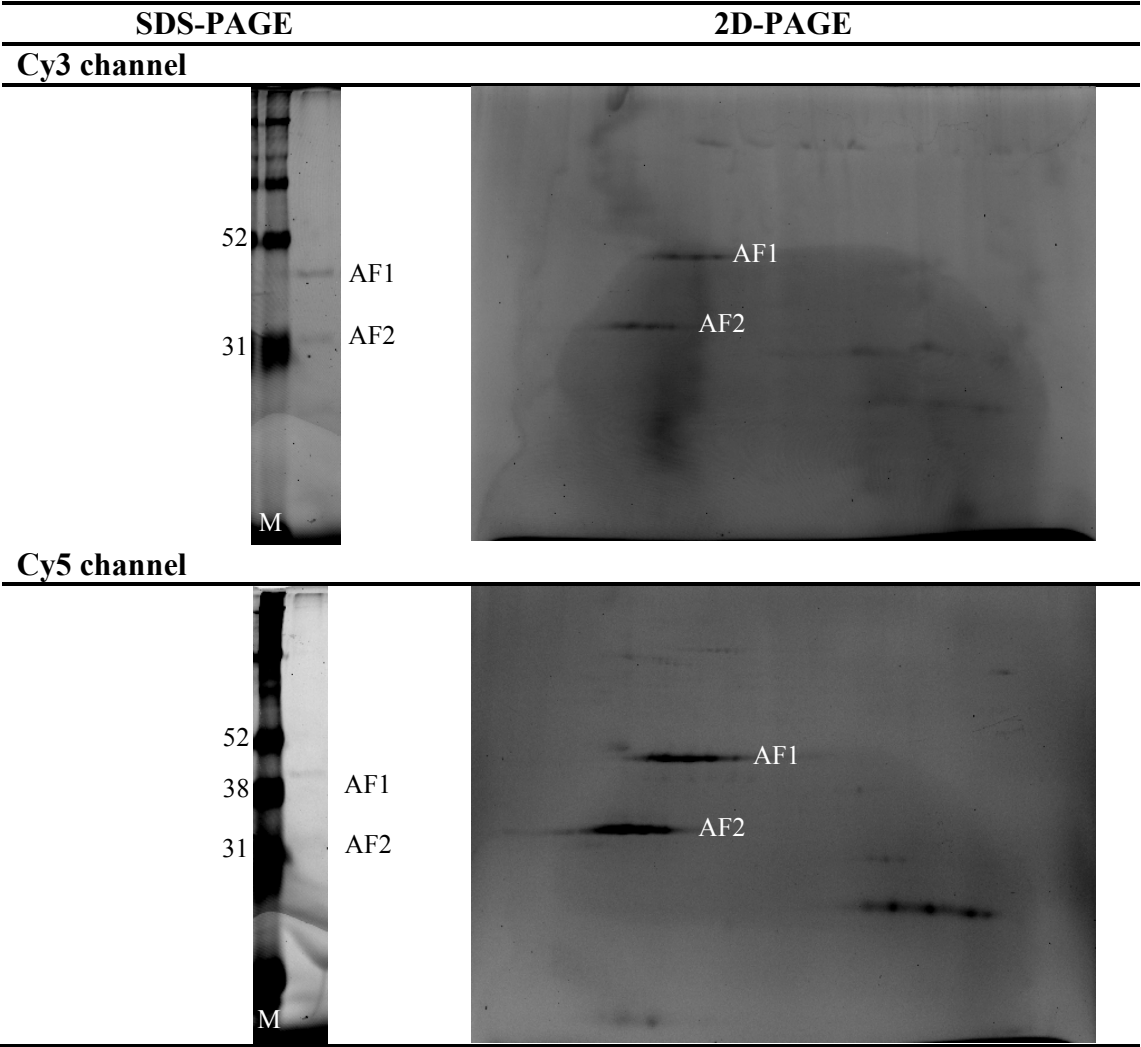


Table A3. Changes in abundance of proteinase K-digested proteins. Spots exhibiting consistently different fluorescence intensity levels of > 10% in all treated groups compared to the untreated control group are presented (P-value < 0.05, n=3).

Gel spot number ^a	Proteinase K concentration (µg/mL per 10 ⁹ bacteria)				
	1.25	2.5	5	10	20
Fold change (P-value ^b)					
Spots with decreased abundance					
1	1.73 (ns) ^c	1.6 (0.043)	1.71 (0.4)	2.31 (0.0074)	2.25 (0.026)
2	1.47 (0.0073)	1.77 (0.0037)	1.8 (0.0052)	2.61 (0.0022)	1.78 (ns)
3	1.42 (0.0018)	1.68 (0.013)	1.48 (0.04)	1.96 (2.1e-04)	2.3 (0.0059)
4	1.44 (0.038)	1.42 (0.044)	1.32 (ns)	1.49 (0.027)	1.6 (0.049)
5	1.63 (ns)	2.09 (0.024)	2.32 (0.02)	2.23 (0.048)	2.54 (0.05)
6	2.45 (0.0037)	2.72 (0.0088)	3.16 (1.6e-04)	3.67 (4.5e-04)	3.69 (0.0021)
7	3.22 (0.023)	5.28 (0.0056)	12.36 (6.3e-04)	10.25 (0.0036)	10.89 (5.7e-04)
8	3.52 (0.0096)	5.53 (0.003)	12.53 (1.6e-04)	11.63 (0.0014)	30.72 (7.5e-05)
9	2.76 (0.011)	5.52 (0.0018)	9.88 (6.2e-04)	9.74 (6.6e-04)	13.72 (0.0014)
10	1.38 (0.025)	1.41 (0.025)	1.32 (0.04)	1.28 (0.026)	1.18 (ns)

	Proteinase K concentration (µg/mL per 10 ⁹ bacteria)				
	1.25	2.5	5	10	20
Gel spot number ^a	Fold change (P-value ^b)				
11	1.56 (0.017)	1.67 (0.001)	1.59 (0.0019)	1.75 (8.4e-05)	1.91 (5.6e-04)
12	1.65 (0.0036)	1.32 (7.1e-04)	1.59 (0.0027)	1.77 (7.4e-04)	1.85 (0.018)
13	1.57 (0.0057)	1.49 (0.0036)	1.44 (0.013)	1.62 (9.5e-04)	1.67 (0.041)
14	5.87 (0.0011)	7.92 (7.3e-04)	11.29 (4.2e-04)	10.35 (4.5e-04)	14.37 (9.7e-05)
15	1.37 (0.022)	1.77 (0.042)	1.57 (0.012)	2.37 (ns)	2.3 (0.0032)
16	1.79 (0.025)	1.72 (ns)	1.65 (0.049)	2.17 (0.02)	1.64 (0.015)
17	1.62 (0.0012)	1.66 (0.0045)	1.8 (6.3e-04)	1.69 (0.0025)	1.95 (0.0017)
18	1.49 (0.026)	1.49 (0.043)	1.47 (0.025)	1.52 (0.027)	1.48 (ns)
19	4.09 (0.0061)	3.81 (0.0041)	3.05 (0.0054)	3.98 (0.0024)	4.02 (4.9e-04)
20	1.47 (0.026)	1.4 (0.0092)	1.48 (0.0014)	1.59 (0.001)	1.46 (0.0037)
21	1.47 (0.0051)	1.56 (0.0077)	1.44 (0.008)	1.49 (0.007)	1.58 (0.017)
22	1.42 (0.014)	1.57 (0.048)	1.43 (0.0054)	1.56 (0.0042)	1.54 (0.0075)
23	1.14	1.2	1.2	1.23	1.19

Proteinase K concentration (µg/mL per 10 ⁹ bacteria)					
	1.25	2.5	5	10	20
Gel spot number ^a	Fold change (P-value ^b)				
	(ns)	(0.031)	(0.014)	(0.0042)	(0.015)
24	1.14 (ns)	1.19 (ns)	1.19 (0.045)	1.2 (0.035)	1.19 (0.019)
25	1.74 (0.016)	1.68 (0.0016)	1.56 (0.0085)	1.6 (0.0039)	1.7 (0.0024)
26	1.68 (0.0076)	1.74 (0.0021)	1.77 (0.0039)	1.89 (0.0022)	1.67 (0.003)
27	1.51 (0.038)	1.48 (0.015)	1.23 (ns)	1.57 (ns)	1.7 (0.045)
28	1.38 (0.035)	1.26 (0.018)	1.2 (ns)	1.34 (0.0032)	1.54 (0.016)
29	1.5 (0.021)	1.43 (0.0039)	1.76 (0.012)	1.46 (0.012)	1.56 (0.0048)
30	1.25 (0.01)	1.28 (4.7e-04)	1.29 (0.0021)	1.38 (0.0017)	1.39 (0.006)
31	2.42 (0.0033)	2.47 (0.0047)	2.49 (0.0079)	1.88 (0.0077)	2.08 (0.0021)
32	2.21 (0.0021)	2.23 (0.0043)	2.71 (0.0024)	2.22 (8.7e-04)	1.95 (0.0038)
33	1.9 (0.021)	1.62 (0.0052)	1.54 (0.012)	1.95 (0.0089)	1.67 (0.026)
34	1.71 (0.0017)	1.72 (0.0017)	1.75 (0.0034)	1.65 (0.0021)	1.89 (1.4e-05)
35	1.44 (0.014)	1.56 (0.019)	1.42 (0.011)	1.6 (0.0028)	1.56 (0.041)

Gel spot number ^a	Proteinase K concentration (µg/mL per 10 ⁹ bacteria)				
	1.25	2.5	5	10	20
Fold change (P-value ^b)					
36	1.56 (0.017)	1.66 (0.018)	1.56 (0.011)	1.83 (0.03)	1.75 (0.0085)
37	1.36 (0.01)	1.31 (0.016)	1.21 (0.019)	1.38 (0.0014)	1.32 (0.033)
Spots with increased abundance					
38	2.30 (0.023)	3.01 (0.0064)	2.71 (0.0077)	2.04 (0.045)	1.45 (ns)
39	9.91 (3.1e-06)	10.36 (2.7e-06)	9.78 (1.7e-05)	6.36 (0.0011)	2.35 (0.002)
40	2.29 (0.022)	2.3 (ns)	2.73 (0.003)	1.89 (ns)	1.61 (0.0011)
41	9.09 (0.0071)	8.24 (0.011)	5.32 (0.027)	1.62 (ns)	1.35 (ns)
42	1.88 (ns)	2.63 (0.025)	4.15 (0.0022)	5.05 (9.3e-04)	5.93 (7.5e-05)

^a Spot numbers as they appear in Figure 3.6.

^b Fold-change values were calculated using DeCyder v.6.5 software and are expressed as the reciprocal of the average ratio of the mean standard abundance of each protein spot between the compared sample groups. Student's t-test was used to calculate the significance of change in abundance (n=3).

^c ns: not significant (P-value > 0.05)

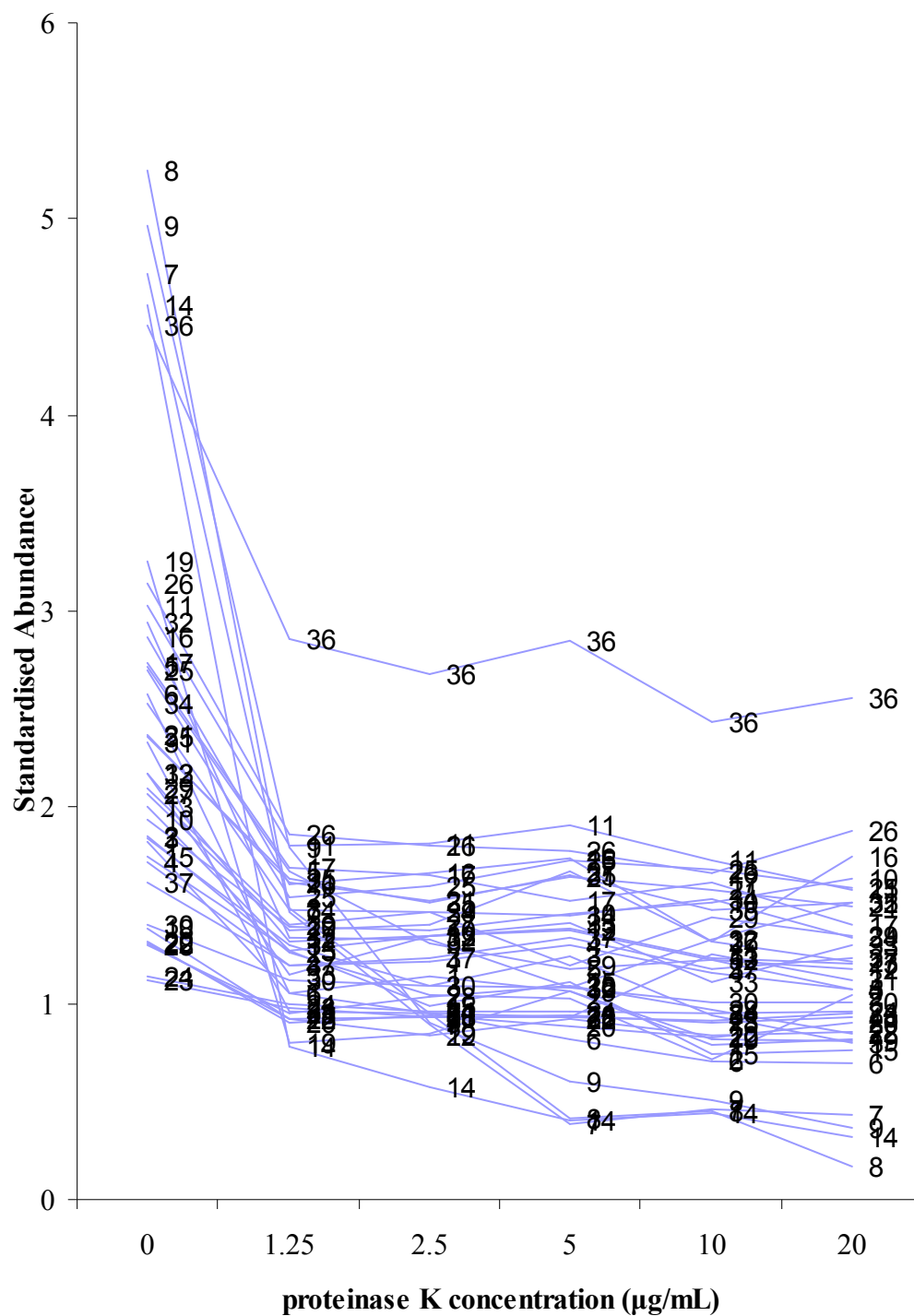


Figure A2 Abundance profiles of proteinase K-affected protein spots. The mean standardised abundance for each spot was calculated using DeCyder differential analysis software and plotted against the proteinase K concentration used for treatment of meningococci.

Table A4 Proteins identified by LC-MS/MS analysis of the peptides recovered in the incubation buffer following treatment of meningococci with trypsin. Bacteria from MC58 Δ -*siaD* strain were treated with trypsin at concentrations ranging from 1.25 to 20 μ g/mL per 10^9 bacteria. The incubation buffer used during trypsin treatment was directly applied to LC-MS/MS for identification of meningococcal proteins. Three biological replicates were used per sample and the number of peptides detected for each protein across the different samples analysed is displayed. The hyphen indicates that less than 2 peptides were detected. The predicted cellular location of proteins identified was inferred using PsortB and LipoP algorithms.

Accession number	Protein name	Cellular location	0 μ g (pre-incubation control)	0 μ g (post-incubation control)	1.25 μ g	2.5 μ g	5 μ g	10 μ g	20 μ g
P64027	Elongation factor Tu	cytoplasmic	12	21	18	13	13	11	14
Q7DDK4	Peroxisome 2 family protein/glutaredoxin	unknown	9	14	9	7	8	5	5
Q9K0P0	Alcohol dehydrogenase, propanol-preferring	cytoplasmic	5	10	12	12	9	9	7
P64389	DNA-binding protein HU-beta	cytoplasmic	4	4	3	3	3	3	3
P64051	Elongation factor Ts	cytoplasmic	3	8	7	9	8	8	6
P66088	50S ribosomal protein L1	cytoplasmic	3	4	5	8	4	4	4
Q7DDL5	Cysteine synthase	cytoplasmic	3	-	-	2	4	6	2
P66614	30S ribosomal protein S7	cytoplasmic	3	5	4	3	3	3	4
P61057	50S ribosomal protein L4	cytoplasmic	2	-	-	-	-	-	-
Q9K0H1	Putative phosphate acetyltransferase Pta	unknown	2	5	3	4	5	4	4
Q7DDR0	Putative cell-binding factor	lipoprotein	2	4	4	4	3	-	-
Q9JZL8	Macrophage infectivity potentiator-related protein	unknown	2	-	-	-	-	-	-
Q9JXD8	Putative adhesin complex protein	unknown	2	6	4	3	4	4	3
P65235	Ribose-phosphate pyrophosphokinase	cytoplasmic	2	3	3	2	2	4	2
Q9K1I1	50S ribosomal protein L17	cytoplasmic	2	-	-	-	-	-	3
Q9K1I5	50S ribosomal protein L2	cytoplasmic	2	3	2	4	-	-	6
P66628	30S ribosomal protein S8	cytoplasmic	2	-	-	-	-	-	2
Q9K1K7	Putative uncharacterized protein (NMB0109)	unknown	2	4	5	3	3	2	3
Q9K0K5	50S ribosomal protein L19	cytoplasmic	-	4	4	4	2	2	3
Q9JY16	Protein-export protein secB	cytoplasmic	-	2	2	2	-	-	-
Q7DDL3	Peptidyl-prolyl cis-trans isomerase	cytoplasmic	-	5	4	3	4	3	3
P60444	50S ribosomal protein L3	cytoplasmic	-	2	4	3	3	2	4

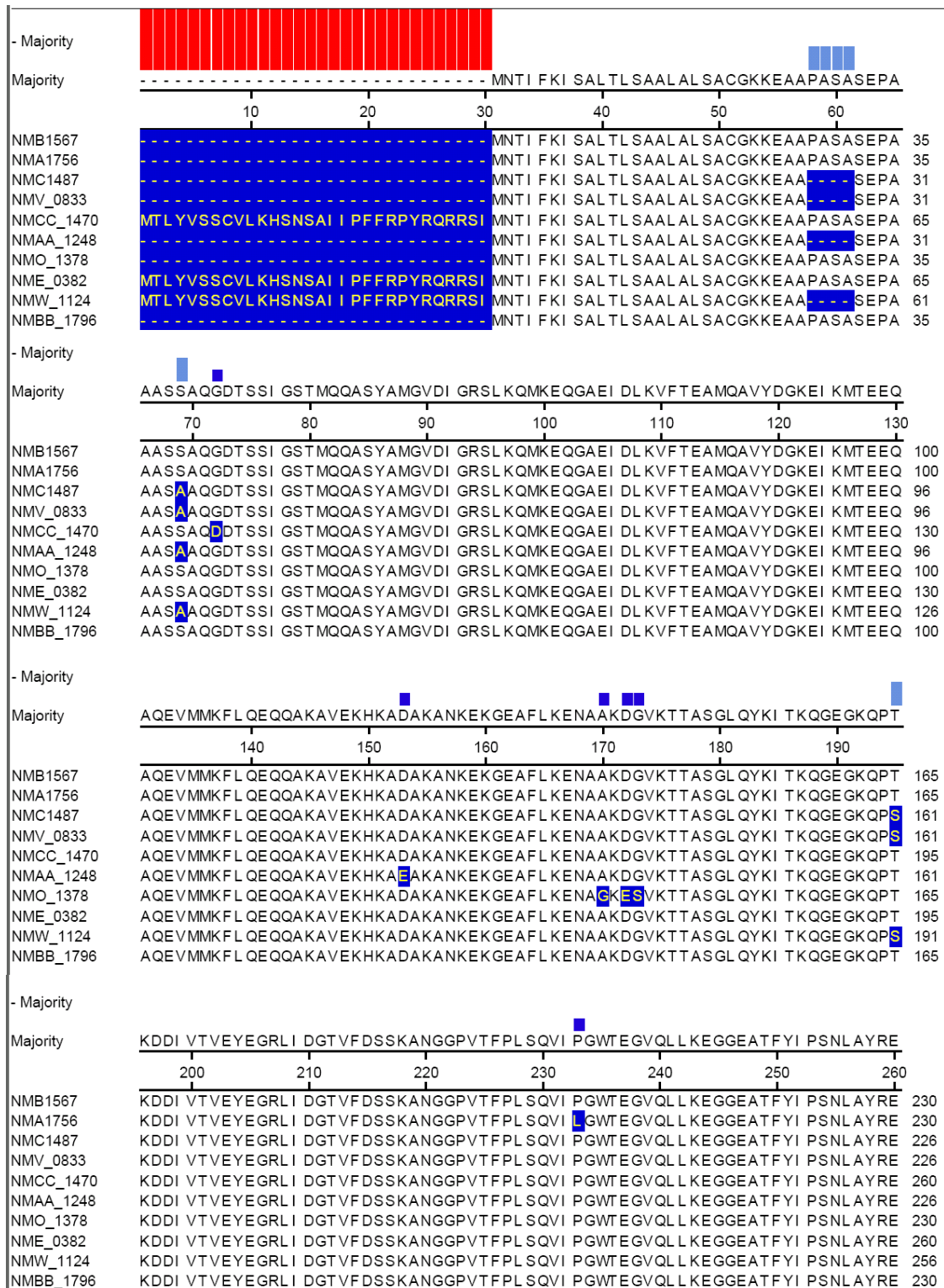
Accession number	Protein name	Cellular location	0 µg (pre-incubation control)	0 µg (post-incubation control)	1.25 µg	2.5 µg	5 µg	10 µg	20 µg
Q9K1I4	50S ribosomal protein L5	cytoplasmic	-	4	3	4	3	2	
Q9JZ31	50S ribosomal protein L9	cytoplasmic	-	2	2	-	-	2	2
P66333	30S ribosomal protein S10	cytoplasmic	-	3	3	4	4	3	4
Q9JYN4	Putative uncharacterized protein (NMB1500)	unknown	-	3	2	2	2	-	3
Q9JZB7	GTP-binding protein TypA	Inner membrane	-	4	2	3	3	-	
Q9K1I8	Elongation factor G	cytoplasmic	-	9	11	11	12	14	13
Q9K0N4	Chaperone protein dnaK	cytoplasmic	-	8	3	6	5	3	3
P0A0S6	Cell division protein ftsZ	cytoplasmic	-	8	7	8	7	6	2
Q9JX99	Electron transfer flavoprotein, beta subunit	periplasmic	-	8	4	4	4	4	3
Q9JZ44	30S ribosomal protein S1	cytoplasmic	-	8	11	12	13	7	6
Q9JZ37	Trigger factor	cytoplasmic	-	7	6	4	4	4	4
P0A0X1	50S ribosomal protein L7/L12	unknown	-	6	5	5	3	3	3
Q9JXV5	Fructose-bisphosphate aldolase	cytoplasmic	-	6	5	2	3	3	2
Q9JY14	Aconitate hydratase 2	cytoplasmic	-	6	5	5	4	4	5
Q9JZP3	Succinyl-CoA ligase [ADP-forming] subunit alpha	cytoplasmic	-	5	4	6	3	3	4
Q9JZ89	ABC transporter, ATP-binding protein	cytoplasmic	-	5	5	5	6	3	4
Q9JXM4	10 kDa chaperonin	cytoplasmic	-	4	4	4	2	2	3
Q9JZ53	Enolase	cytoplasmic	-	4	3	4	3	2	3
Q9JZ12	Pyruvate dehydrogenase, E1 component	cytoplasmic	-	4	2	3	2	-	-
P66642	30S ribosomal protein S9	cytoplasmic	-	4	2	2	2	2	4
Q9JS61	Dihydroxy-acid dehydratase	cytoplasmic	-	3	3	2	2	2	2
Q9JZ19	3-isopropylmalate dehydrogenase	cytoplasmic	-	3	2	2	2	-	-
Q7DD59	50S ribosomal protein L30	cytoplasmic	-	3	2	2	-	-	-
P66540	30S ribosomal protein S2	cytoplasmic	-	3	5	5	5	6	4
Q9JZ80	Phosphoribosylformylglycinamide cyclo-ligase	cytoplasmic	-	3	-	-	2	-	-
Q9JZ29	30S ribosomal protein S6	cytoplasmic	-	3	3	3	2	-	-
Q9JXR1	3-oxoacyl-(Acyl-carrier-protein) reductase	unknown	-	3	3	-	2	-	-
Q9JYM1	Acetate kinase 1	cytoplasmic	-	3	2	2	2	2	2
Q9JXW8	Carbamoyl-phosphate synthase large chain	unknown	-	3	2	4	3	2	-

Accession number	Protein name	Cellular location	0 µg (pre-incubation control)	0 µg (post-incubation control)	1.25 µg	2.5 µg	5 µg	10 µg	20 µg
P65533	Nucleoside diphosphate kinase	extracellular	-	3	2	2	-	-	-
P65592	Transcription antitermination protein nusG	cytoplasmic	-	3	2	4	3	3	2
Q9JXK5	Phosphoribosylformylglycinamide synthase	cytoplasmic	-	3	3	2	-	-	-
Q59622	DNA-directed RNA polymerase subunit beta	cytoplasmic	-	3	3	-	-	-	-
Q7DD69	Acetyl-CoA carboxylase, biotin carboxyl carrier protein	cytoplasmic	-	2	2	2	-	-	-
P0A0T5	Glutathione peroxidase homolog	cytoplasmic	-	2	-	-	-	-	-
Q9K1J3	50S ribosomal protein L11	cytoplasmic	-	2	-	-	-	-	-
P66849	Single-stranded DNA-binding protein	cytoplasmic	-	2	-	2	-	-	-
Q9JYH6	3-hydroxyacid dehydrogenase	cytoplasmic	-	2	2	3	-	-	-
Q9JYQ8	Chaperone protein clpB	cytoplasmic	-	2	2	2	4	2	-
P0A0V3	RmpM	outer membrane	-	2	2	3	3	2	5
Q9K0I2	Phosphoenolpyruvate synthase	cytoplasmic	-	2	2	5	4	2	-
P65932	Uridylate kinase	cytoplasmic	-	2	-	-	-	-	-
Q9JZW3	50S ribosomal protein L25	cytoplasmic	-	2	3	2	2	2	-
P66130	50S ribosomal protein L27	cytoplasmic	-	2	2	-	-	-	-
Q9JXW3	Acetyl-CoA carboxylase, biotin carboxylase	cytoplasmic	-	2	-	-	-	-	-
Q9JYJ5	Putative uncharacterized protein (NMB1557)	unknown	-	2	-	-	-	-	-
Q9K1D8	3-oxoacyl-(Acyl-carrier-protein) synthase II	inner membrane	-	2	-	-	-	-	-
Q9K0G7	UTP--glucose-1-phosphate uridylyltransferase	cytoplasmic	-	2	-	-	-	-	-
P63442	Acyl carrier protein	cytoplasmic	-	2	2	2	2	2	2
Q9JXQ0	ATP synthase subunit alpha	inner membrane	-	2	-	2	2	2	2
Q9JYI0	Cysteine desulfurase	cytoplasmic	-	2	3	-	-	-	-
P56988	Protein recA	cytoplasmic	-	2	2	-	2	-	-
P60733	50S ribosomal protein L24	cytoplasmic	-	2	2	3	4	2	5
P66386	30S ribosomal protein S13	cytoplasmic	-	2	2	3	4	2	3
Q9JZP4	Succinyl-CoA ligase [ADP-forming] subunit beta	cytoplasmic	-	2	-	-	-	2	2
Q9K1R6	Glutamyl-tRNA synthetase	cytoplasmic	-	2	2	2	-	-	2
Q9K1H7	Valyl-tRNA synthetase	cytoplasmic	-	2	-	2	2	2	2

Accession number	Protein name	Cellular location	0 µg (pre-incubation control)	0 µg (post-incubation control)	1.25 µg	2.5 µg	5 µg	10 µg	20 µg
Q9JYC7	UPF0082 protein NMB1648	cytoplasmic	-	2	-	-	2	-	-
Q9JYD3	N utilization substance protein A	cytoplasmic	-	2	2	3	2	2	-
Q9JZH7	Threonine synthase	cytoplasmic	-	2	2	3	3	2	-
Q9JYI2	Ketol-acid reductoisomerase	cytoplasmic	-	2	-	3	3	-	-
P66169	50S ribosomal protein L29	cytoplasmic	-	2	2	3	2	2	2
Q9JYR2	Putative uncharacterized protein (NMB1468)	lipoprotein	-	-	5	2	3	4	4
Q9K035	Amino acid ABC transporter, periplasmic amino-acid binding protein	lipoprotein	-	-	4	4	6	2	3
Q9K112	ABC transporter, ATP-binding protein (NMB0387)	cytoplasmic	-	-	4	-	3	3	-
P66551	30S ribosomal protein S3	cytoplasmic	-	-	3	3	3	2	3
Q9K1F8	Ribonuclease E	cytoplasmic	-	-	3	-	-	-	-
P66704	DNA-directed RNA polymerase subunit alpha	cytoplasmic	-	-	2	3	3	3	3
P05431	Fimbrial protein	outer membrane	-	-	2	3	3	2	2
Q9JZQ0	Putative 2-oxoglutarate dehydrogenase, E3 component, lipoamide dehydrogenase	cytoplasmic	-	-	2	-	-	-	-
Q9JXE1	Putative hemolysin	extracellular	-	-	2	-	-	-	-
Q9JXQ2	ATP synthase subunit beta	inner membrane	-	-	2	-	-	-	-
Q70M91	Type IV pilus biogenesis and competence protein	outer membrane	-	-	2	-	-	2	2
Q9K1J1	DNA-directed RNA polymerase subunit beta'	Cytoplasmic	-	-	2	2	-	3	3
Q9JY10	Acetolactate synthase	Cytoplasmic	-	-	2	-	-	-	-
P66561	30S ribosomal protein S4	Cytoplasmic	-	-	2	3	-	-	-
Q7DDM6	DNA-binding response regulator	Cytoplasmic	-	-	5	2	2	-	-
Q9JXA0	Electron transfer flavoprotein, alpha subunit	Cytoplasmic	-	-	-	2	-	2	2
Q9JZ28	Thioredoxin reductase	Cytoplasmic	-	-	-	-	-	2	2
Q9K0H3	1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino)methylideneamido]carboxamide isomerase	Cytoplasmic	-	-	2	-	-	-	-
Q9JZM7	Bifunctional purine biosynthesis protein purH	Cytoplasmic	-	-	3	2	-	2	2

Accession number	Protein name	Cellular location	0 µg (pre-incubation control)	0 µg (post-incubation control)	1.25 µg	2.5 µg	5 µg	10 µg	20 µg
Q9K1Q0	Methionyl-tRNA synthetase	Cytoplasmic	-	-	-	2	3	-	-
Q9JYX9	NifU protein	cytoplasmic	-	-	2	-	-	-	-
Q9JY18	Probable FKBP-type peptidyl-prolyl cis-trans isomerase	lipoprotein	-	-	-	-	-	-	2
Q9K062	Polyribonucleotide nucleotidyltransferase	cytoplasmic	-	-	-	2	-	-	-
Q9JXB3	Peptide chain release factor 2	cytoplasmic	-	-	-	-	-	-	2
Q9JYZ9	Glutamyl-tRNA(Gln) amidotransferase subunit A	cytoplasmic	-	-	-	2	2	2	-
Q9K147	TspA protein	outer membrane	-	-	-	2	-	-	-
Q9K012	Adenylosuccinate synthetase	cytoplasmic	-	-	-	3	3	2	2
P66487	30S ribosomal protein S19	cytoplasmic	-	-	-	2	-	-	-
Q9K048	Uracil phosphoribosyltransferase	cytoplasmic	-	-	-	2	-	-	-
Q7DDK0	Citrate synthase	cytoplasmic	-	-	-	-	2	2	2
Q7DDT5	50S ribosomal protein L22	cytoplasmic	-	-	-	-	-	-	2
Q9K113	50S ribosomal protein L6	cytoplasmic	-	-	-	-	-	-	2
Q7DDT2	50S ribosomal protein L14	cytoplasmic	-	-	-	-	-	-	2
Q7DDT0	50S ribosomal protein L18	cytoplasmic	-	-	-	-	-	-	2

NmMIP amino acid sequence alignment



- Majority

Majority	QGAGDKI GPNATLVFDVKLVKI GAPENAPAKQPAQVDI KKVN	
	270 280 290 300	
NMB1567	QGAGDKI GPNATLVFDVKLVKI GAPENAPAKQPAQVDI KKVN	272
NMA1756	QGAGDKI GPNATLVFDVKLVKI GAPENAPAKQPAQVDI KKVN	272
NMC1487	QGAGDKI GPNATLVFDVKLVKI GAPENAPAKQPAQVDI KKVN	268
NMV_0833	QGAGDKI GPNATLVFDVKLVKI GAPENAPAKQPAQVDI KKVN	268
NMCC_1470	QGAGDKI GPNATLVFDVKLVKI GAPENAPAKQPAQVDI KKVN	302
NMAA_1248	QGAGDKI GPNATLVFDVKLVKI GAPENAPAKQPAQVDI KKVN	268
NMO_1378	QGAGDKI GPNATLVFDVKLVKI GAPENAPAKQPAQVDI KKVN	272
NME_0382	QGAGDKI GPNATLVFDVKLVKI GAPENAPAKQPAQVDI KKVN	302
NMW_1124	QGAGDKI GPNATLVFDVKLVKI GAPENAPAKQPAQVDI KKVN	298
NMBB_1796	QGAGDKI GPNATLVFDVKLVKI GAPENAPAKQPAQVDI KKVN	272

PilP amino acid sequence alignment

- Majority

Majority	MKHYALLI SFLAL SACSQGS EDL NEWMAQTRREAKAEI I PFQAPTL PVAPVYSPQL TGPNAFDF	
	10 20 30 40 50 60	
NMB1811	MKHYALLI SFLAL SACSQGS EDL NEWMAQTRREAKAEI I PFQAPTL PVAPVYSPQL TGPNAFDF	65
NMA0651	MKHYALLI SFLAL SACSQGS EDL NEWMAQTRREAKAEI I PFQAPTL PVAPVYSPQL TGPNAFDF	65
NMC0409	MKHYALLI SFLAL SACSQGS EDL NEWMAQTRREAKAEI I PFQAPTL PVAPVYSPQL TGPNAFDF	65
NMCC_0417	MKHYALLI SFLAL SACSQGS EDL NEWMAQTRREAKAEI L PFQAPTL PVAPVYSPQL TGPNAFDF	65

- Majority

Majority	RRMETDKKGENAPDTKRI KETLEKFSLENMRYVGI LKSGQKVS GFI EAEGYVYTVGVGN YL GQNY	
	70 80 90 100 110 120 130	
NMB1811	RRMETDKKGENAPDTKRI KETLEKFSLENMRYVGI LKSGQKVS GFI EAEGYVYTVGVGN YL GQNY	130
NMA0651	RRMETDKKGENAPDTKRI KETLEKFSLENMRYVGI LKSGQKVS GFI EAEGYVYTVGVGN YL GQNY	130
NMC0409	RRMETDKKGENAPDTKRI KETLEKFSLENMRYVGI LKSGQKVS GFI EAEGYVYTVGVGN YL GQNY	130
NMCC_0417	RRMETDKKGENAPDTKRI KETLEKFSLENMRYVGI LKSGQKVS GFI EAEGYVYTVGVGN YL GQNY	130

- Majority

Majority	GRI ESI TDDSI VLNELI EDSTGNWWSRKAELLLNSSDKNTEQAAAPAAEQN	
	140 150 160 170 180	
NMB1811	GRI ESI TDDSI VLNELI EDSTGNWWSRKAELLLNSSDKNTEQAAAPAAEQN	181
NMA0651	GRI ESI TDDSI VLNELI EDSTGNWWSRKAELLLNSSDKNTEQAAAPAAEQN	181
NMC0409	GRI ESI TDDSI VLNELI EDSTGNWWSRKAELLLNSSDKNTEQAAAPAAEQN	181
NMCC_0417	GRI ESI TDDSI VLNELI EDSTGNWWSRKAELLLNSSDKNTEQAAAPAAEQN	181

PilO amino acid sequence alignment

- Majority		
Majority	MASKSSKTNL DLNNL HLLNL PARLFI ALLAVA AVLGLGYAGL FKSQMESLEEYEAKETELKNTYK	
	10 20 30 40 50 60	
NMB1810	MASKSSKTNL DLNNL HLLNL PARLFI ALLAVA AVLGLGYAGL FKSQMESLEEYEAKETELKNTYK	65
NMC0410	MASKSSKTNL DLNNL HLLNL PARLFI ALLAVA AVLGLGYAGL FKSQMESLEEYEAKETELKNTYK	65
NMCC_0418	MASKSSKTNL DLNNL HLLNL PARLFI ALLAVA AVLGLGYAGL FKSQMESLEEYEAKETELKNTYK	65
NMAA_0333	MASKSSKTNL DLNNL HLLNL PARLFI ALLAVA AVLGLGYAGL FKSQMESLEEYEAKETELKNTYK	65
NMW_1806	MASKSSKTNL DLNNL HLLNL PARLFI ALLAVA AVLGLGYAGL FKSQMESLEEYEAKETELKNTYK	65
- Majority		
Majority	QKSI DAASLNNLRDELASI RSAFDI MLKQLPTDAEI PNLVQELHQAGSSNGLRLDSVMPQPPVDD	
	70 80 90 100 110 120 130	
NMB1810	QKSI DAASLNNLRDELASI RSAFDI MLKQLPTDAEI PNLVQELHQAGSSNGLRLDSVMPQPPVDD	130
NMC0410	QKSI DAASLNNLRDELASI RSAFDI MLKQLPTDAEI PNLVQELHQAGSSNGLRLDSVMPQPPVDD	130
NMCC_0418	QKSI DAASLNNLRDELASI RSAFDI MLKQLPTDAEI PNLVQELHQAGSSNGLRLDSVMPQPPVDD	130
NMAA_0333	QKSI DAASLNNLRDELASI RSAFDI MLKQLPTDAEI PNLVQELHQAGSSNGLRLDSVMPQPPVDD	130
NMW_1806	QKSI DAASLNNLRDELASI RSAFDI MLKQLPTDAEI PNLVQELHQAGSSNGLRLDSVMPQPPVDD	130
- Majority		
Majority	GPI KRLPYSI SI TGNYEQI SQFTRDVGSL SRI I TLESLKI AQSPENGGNPDGKSSI LNL SAI ATT	
	140 150 160 170 180 190	
NMB1810	GPI KRLPYSI SI TGNYEQI SQFTRDVGSL SRI I TLESLKI AQSPENGGNPDGKSSI LNL SAI ATT	195
NMC0410	GPI KRLPYSI SI TGNYEQI SQFTRDVGSL SRI I TLESLKI AQSPENGGNPDGKSSI LNL SAI ATT	195
NMCC_0418	GPI KRLPYSI SI TGNYEQI SQFTRDVGSL SRI I TLESLKI AQSPENGGNPDGKSSI LNL SAI ATT	195
NMAA_0333	GPI KRLPYSI SI TGNYEQI SQFTRDVGSL SRI I TLESLKI AQSPENGGNPDGKSSI LNL SAI ATT	195
NMW_1806	GPI KRLPYSI SI TGNYEQI SQFTRDVGSL SRI I TLESLKI AQSPENGGNPDGKSSI LNL SAI ATT	195
- Majority		
Majority	YQAKSVEELAAEAAQNAEQK - - -	
	200 210	
NMB1810	YQAKSVEELAAEAAQNAEQK	215
NMC0410	YQAKSVEELAAEAAQNAEQK	215
NMCC_0418	YQAKSVEELAAEAAQNAEQK	215
NMAA_0333	YQAKSVEELAAEAAQNAEQK MAS	218
NMW_1806	YQAKSVEELAAEAAQNAEQK	215

Adhesin complex protein amino acid sequence alignment

- Majority		
Majority	-----MKL	
	10 20 30 40 50 60	
NMB2095	-----MKL	3
NMA0335	MPSETPDRPPYPACRPCLKPPNRPNPPFYKLYPI SCLFQDTPTLECQTVRNGQTPHPSKGI	65
NMC2075	MPSETPDRSPYPACRPCLKPPNRPNPPFYKLYPI SCLFQDTPTLECQTARNGQTPHPSKGI	65
NMV_2302	-----MKL	3
NMCC_2060	MPSETPDRPPYPACRPCLKPPNRPNPPFYKLYPI SCLFQDTPTLECQTARNGQTPHPSKGI	65
NMAA_0064	-----MKL	3
NMO_0072	MPSETPDRSPYPACRPCLKPPNRPNPPFYKLYPI SCLFQDTPTLECQTARNGQTPHPSKGI	65
NME_1590	-----MKL	3
NMW_1683	-----MKL	3
NMNB_2409	-----MKL	3

- Majority		
Majority	L T T A I L S S A I A L S S M A A A A G T N N P T V A K K T V S Y V C Q Q G K K V K V T Y G F N K Q G L T T Y A S A V I N G K R V	
	70 80 90 100 110 120 130	
NMB2095	L T T A I L S S A I A L S S M A A A A G T N N P T V A K K T V S Y V C Q Q G K K V K V T Y G F N K Q G L T T Y A S A V I N G K R V	68
NMA0335	L T T A I L S S A I A L S S M A A A A G T N N P T V A K K T V S Y V C Q Q G K K V K V T Y G F N K Q G L T T Y A S A V I N G K R V	130
NMC2075	L T T A I L S S A I A L S S M A A A A G T N N P T V A K K T V S Y V C Q Q G K K V K V T Y G F N K Q G L T T Y A S A V I N G K R V	130
NMV_2302	L T T A I L S S A I A L S S M A A A A G T N N P T V A K K T V S Y V C Q Q G K K V K V T Y G F N K Q G L T T Y A S A V I N G K R V	68
NMCC_2060	L T T A I L S S A I A L S S M A A A A G T N N P T V A K K T V S Y V C Q Q G K K V K V T Y G F N K Q G L T T Y A S A V I N G K R V	130
NMAA_0064	L T T A I L S S A I A L S S M A A A A G T N N P T V A K K T V S Y V C Q Q G K K V K V T Y G F N K Q G L T T Y A S A V I N G K R V	68
NMO_0072	L T T A I L S S A I A L S S M A A A A G T N N P T V A K K T V S Y V C Q Q G K K V K V T Y G F N K Q G L T T Y A S A V I N G K R V	130
NME_1590	L T T A I L S S A I A L S S M A A A A G T N N P T V A K K T V S Y V C Q Q G K K V K V T Y G F N K Q G L T T Y A S A V I N G K R V	68
NMW_1683	L T T A I L S S A I A L S S M A A A A G T N N P T V A K K T V S Y V C Q Q G K K V K V T Y G F N K Q G L T T Y A S A V I N G K R V	68
NMBB_2409	L T T A I L S S A I A L S S M A A A A G T N N P T V A K K T V S Y V C Q Q G K K V K V T Y G F N K Q G L T T Y A S A V I N G K R V	68
- Majority		
Majority	Q M P V N L D K S D N V E T F Y G K E G G Y V L G T G V M D G K S Y R K Q P I M I T A P D N Q I V F K D C S P R	
	140 150 160 170 180	
NMB2095	Q M P V N L D K S D N V E T F Y G K E G G Y V L G T G V M D G K S Y R K Q P I M I T A P D N Q I V F K D C S P R	124
NMA0335	Q M P V N L D K S D N V E T F Y G K E G G Y V L G T G V M D G K S Y R K Q P I M I T A P D N Q I V F K D C S P R	186
NMC2075	Q M P V N L D K S D N V E T F Y G K E G G Y V L G T G V M D G K S Y R K Q P I M I T A P D N Q I V F K D C S P R	186
NMV_2302	Q M P V N L D K S D N V E T F Y G K E G G Y V L G T G V M D G K S Y R K Q P I M I T A P D N Q I V F K D C S P R	124
NMCC_2060	Q M P V N L D K S D N V E T F Y G K E G G Y V L G T G V M D G K S Y R K Q P I M I T A P D N Q I V F K D C S P R	186
NMAA_0064	Q M P V N L D K S D N V E T F Y G K E G G Y V L G T G V M D G K S Y R K Q P I M I T A P D N Q I V F K D C S P R	124
NMO_0072	Q M P V N L D K S D N V E T F Y G K E G G Y V L G T G V M D G K S Y R K Q P I M I T A P D N Q I V F K D C S P R	186
NME_1590	Q M P V N L D K S D N V E T F Y G K E G G Y V L G T G V M D G K S Y R K Q P I M I T A P D N Q I V F K D C S P R	124
NMW_1683	Q M P V N L D K S D N V E T F Y G K E G G Y V L G T G V M D G K S Y R K Q P I M I T A P D N Q I V F K D C S P R	124
NMBB_2409	Q M P V N L D K S D N V E T F Y G K E G G Y V L G T G V M D G K S Y R K Q P I M I T A P D N Q I V F K D C S P R	124

Figure A3. Amino acid sequence alignments for NmMIP, PilP, PilO and adhesin complex protein across strains with annotated genomes. Proteins are represented by the locus number of the corresponding gene from each meningococcal strain. The gene tag used for each strain is as follows: NMB for MC58, NMA for Z2491, NMC for FAM18, NMV for 8013, NMCC for 053442, NMAA for WUE2594, NMO for alpha14, NME for alpha153, NMW for alpha275 and NMBB for alpha710. The sequence marked “majority” corresponds to the consensus. Residues that differ from the consensus are highlighted with dark blue. Colour marking on top of the consensus sequence describes the extent of disagreement across the aligned sequences for dissimilar residues, with increasing degree of disagreement from red to dark blue.

	10	20	30	40	50	60	70	80
2.seq								
237.seq								
425.seq								
403.seq								
660.seq								
419.seq								
19.seq								
1.seq								
443.seq								
507.seq							T.	
314.seq							T.	
7.seq							T.	
659.seq							T.	
398.seq								
411.seq	AA.		A.		G.	A.		T.
641.seq								
657.seq								
658.seq								
	90	100	110	120	130	140	150	160
2.seq								
237.seq								
425.seq								
403.seq								
660.seq								
419.seq								
19.seq								
1.seq								
443.seq								
507.seq				G.				
314.seq				G.				
7.seq				G.				
659.seq				G.				
398.seq								
411.seq	T.			G.		A.		
641.seq								
657.seq				G.				
658.seq				G.				
	170	180	190	200	210	220	230	240
2.seq								
237.seq								
425.seq								
403.seq								
660.seq								
419.seq								
19.seq								
1.seq								
443.seq								
507.seq							T.	
314.seq							T.	
7.seq							T.	
659.seq							T.	
398.seq								
411.seq								
641.seq								
657.seq							T.	
658.seq							T.	
	250	260	270	280	290	300	310	320
2.seq								
237.seq								
425.seq								
403.seq								
660.seq								
419.seq						G.		
19.seq								
1.seq								
443.seq								
507.seq							T.	
314.seq							T.	
7.seq							T.	
659.seq							T.	
398.seq							T.	
411.seq								
641.seq							T.	
657.seq							T.	
658.seq	T.					T.		

	330	340	350	360	370	380	390	400
2.seq								
237.seq								
425.seq								
403.seq								
660.seq								
419.seq								
19.seq								
1.seq		G.						
443.seq								
507.seq								
314.seq								
7.seq					A.			
659.seq								
398.seq		A.	T.					
411.seq								
641.seq		A.	T.					
657.seq								
658.seq								
	410	420	430	440	450	460	470	480
2.seq								
237.seq								
425.seq								T.
403.seq								T.
660.seq								T.
419.seq								
19.seq								
1.seq								
443.seq								T.
507.seq								
314.seq								T.
7.seq								
659.seq								T.
398.seq	C.	G.	AA. T.	A.				T.
411.seq								
641.seq	C.	G.	AA. T.	A.				T.
657.seq								
658.seq								
	490	500	510	520	530	540	550	560
2.seq								
237.seq								
425.seq								
403.seq								
660.seq								
419.seq								
19.seq								
1.seq								
443.seq								
507.seq								
314.seq		T.						
7.seq								
659.seq		T.						
398.seq								
411.seq								
641.seq								
657.seq								
658.seq								
	570	580	590	600	610	620	630	640
2.seq								
237.seq								
425.seq								
403.seq								
660.seq		A.						
419.seq								
19.seq					T.			
1.seq					T.			
443.seq		A.					A.	
507.seq								
314.seq								
7.seq								
659.seq								
398.seq								
411.seq								
641.seq								
657.seq								
658.seq								

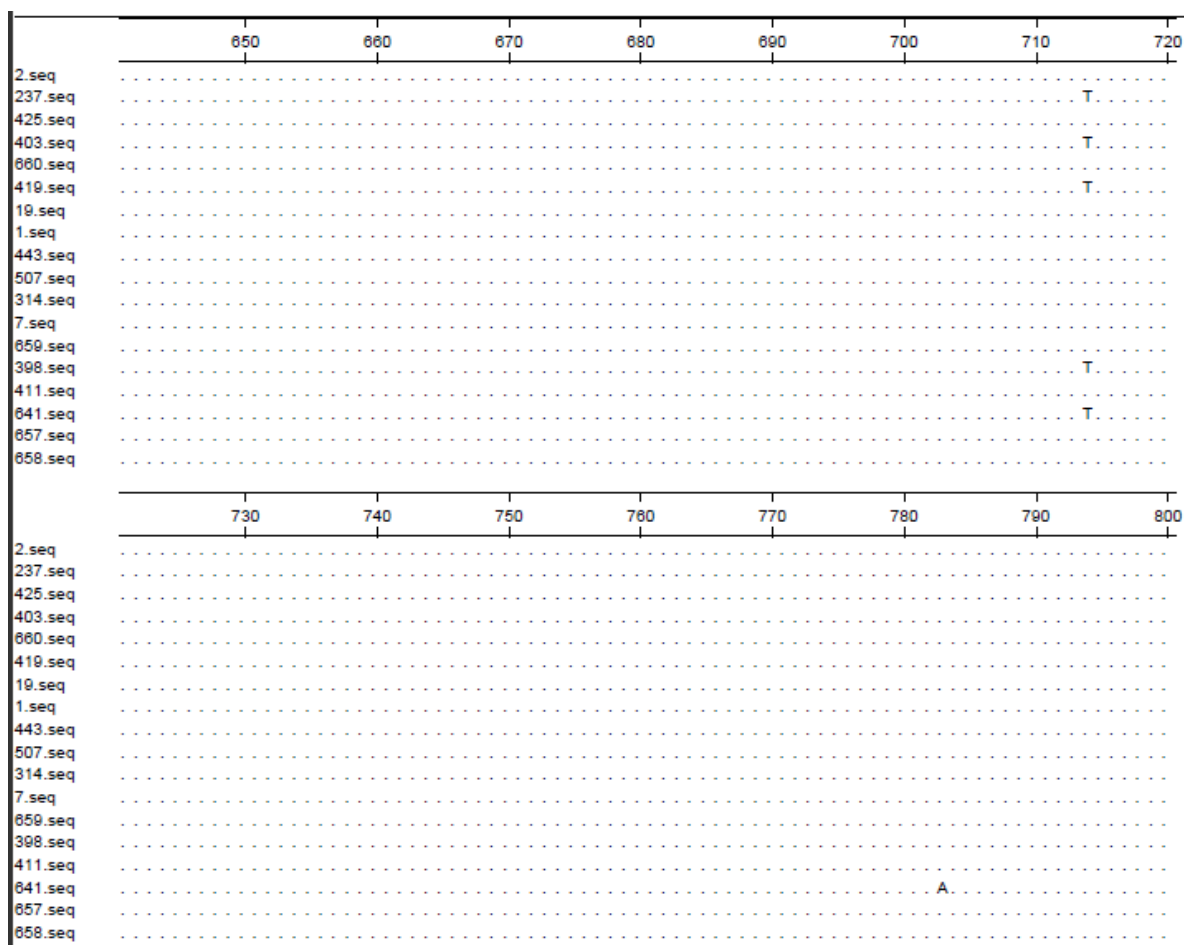


Figure A4 Distribution of sequence polymorphisms in the different allele types of the gene coding for NmMIP. The NmMIP nucleotide sequences of a representative isolate from each allele type were aligned using the MegAlign tool of the DNASTAR Lasergene software v.8. Isolates are presented on the left and numbered according to Table A1 (Appendix). Identity to nucleotide sequence of isolate 2 is represented by a dot and base pair deletion by a hyphen.

Supplementary Material

Supplementary information is provided in the electronic copy that accompanies this thesis and includes:

- A copy of the publications related to this thesis (Tsolakos *et al.*, 2009; Tsolakos *et al.*, 2010).
- The nucleotide and amino acid sequences of NmMIP from the 106 meningococcal isolates of the MLST database aligned using DNASTAR Lasergene software v.8.