Evaluation of a potential vaccine against hyperinvasive serogroup B *Neisseria meningitidis* by assessment of the effects of surface-expressed Opacity-associated proteins on the immune system.

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Manish Sadarangani
Linacre College, University of Oxford

Supervisor:
Professor Andrew J Pollard
University of Oxford Department of Paediatrics

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ABSTRACT

Neisseria meningitidis causes 500,000 cases of meningitis and septicaemia annually worldwide, with a mortality rate of approximately 10%. Most disease in developed countries is caused by serogroup B infection, against which there is no universal vaccine. Opa proteins are major meningococcal outer membrane proteins, and a limited number of Opa variants have been associated with hyperinvasive serogroup B meningococci, suggesting their use as a potential novel vaccine. Immunisation of mice with recombinant Opa elicited high levels of meningococcal-specific serum bactericidal antibody (SBA), demonstrating proof in principle of this approach. Opa proteins mediate bacterial adherence to host cells and modulate human cellular immunity, and there are conflicting data regarding their effects on $CD4^+$ T cells.

opa genes from *N. meningitidis* strain H44/76 were cloned into the plasmid vector pBluescript, disrupted using antibiotic resistance cassettes and transformed into H44/76 to sequentially disrupt the four *opa* genes. This produced a unique panel of 15 isogenic Opa-deficient strains, including an Opa-negative strain, which enabled investigation of the immunomodulatory role of surface-expressed Opa proteins.

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There was no consistent effect of Opa expressed on the surface of OMVs and inactivated bacteria on $CD4^+$ T cells, with significant heterogeneity of responses

between individuals. The rate of Opa phase variation was between 10^{-3} and 10^{-4}, and increased 180-fold following transformation of bacteria with unrelated DNA.

These data support further investigation of Opa as a potential meningococcal vaccine component, and highlight the importance of host and bacterial factors in the development of OMV vaccines.

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ABBREVIATIONS

7-AAD	7-aminoactinomycin D
BCIP/NBT	5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium
BHI	Brain heart infusion
BSA	Bovine serum albumin
CEACAM	Carcinoembryonic antigen cell adhesion molecule
CFSE	Carboxyfluorescein succinimidyl ester
cfu	Colony forming units
CR	Coding repeat
CSF	Cerebrospinal fluid
DIC	Disseminated intravascular coagulation
DNA	Deoxyribonucleic acid
DOC	Sodium deoxycholate
DUS	DNA uptake sequence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EryR	Erythromycin resistance cassette
ET	Electrophoretic type
fHbp	Factor H binding protein
GPI	Glycosylphosphatidylinositol
GWAS	Genome wide association study
HBSS	Hanks' Balanced Salt Solution
HCl	Hydrochloric acid
HPA	Health Protection Agency
HSPG	Heparin sulphate proteoglycan
HV	Hypervariable
Ig	Immunoglobulin
IL	Interleukin
IMD	Invasive meningococcal disease
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IS	Insertional sequence
ITIM	Immunoreceptor tyrosine based inhibition motif
KanR	Kanamycin resistance cassette
LB	Luria-Bertani
LDAO	Lauryldimethylamine-oxide
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MATS	Meningococcal antigen typing system
MLEE	Multi-locus enzyme electrophoresis
MLST	Multi-locus sequence typing
NaCl	Sodium chloride
NCS	Newborn calf serum
NHBA	Neisserial heparin binding antigen

NTA	Nanoparticle tracking analysis
NVI	Netherlands Vaccine Institute
OMP	Outer membrane protein
OMV	Outer membrane vesicle
PBA	Phosphate buffered albumin
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PHA	Phytohaemagglutinin
PPD	Purified protein derivative
PV	Phase variation
RNA	Ribonucleic acid
RT	Room temperature
SAP	Shrimp alkaline phosphatase
SBA	Serum bactericidal antibody
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEB	Staphylococcal enterotoxin B
SNP	Single nucleotide polymorphism
ST	Sequence type
SV	Semivariable
TAE	Tris-acetate-EDTA
TCR	T cell receptor
TEM	Transmission electron microscopy
TetR	Tetracycline resistance cassette
TLR	Toll-like receptor
TNF	Tumour necrosis factor
UPW	Ultrapure water
wt	Wild-type
X-Gal	5-bromo-4-chloro-3-indolyl β-D-galactopyranoside

CHAPTER 1. INTRODUCTION

1.1. Historical aspects

The first description of a disease with the characteristics of infection with *Neisseria meningitidis* (the meningococcus) was published by Vieusseux in Geneva, Switzerland during an outbreak of epidemic meningitis in March 1805 which resulted in 33 deaths[1]. The following year an outbreak was described in the United Sates at Medfield, Massachusetts[2]. Since then, invasive meningococcal disease (IMD) has become a global, endemic problem. It may be a more ancient disease, described alongside other "spotted fever syndromes", but recent analysis of the genome suggests that the organism only developed the ability to be pathogenic in the early 1800s, probably by acquisition of genes important for the capsule[3].

The organism itself was first described in 1884 by Marchiafava and Celli, who found oval micrococci in the spinal fluid of patients with meningitis[4]. Three years later Weichselbaum first isolated the organism from meningeal exudates of six out of eight cases of meningitis in Vienna, Austria[5]. He observed that it was an intracellular diplococcus, so named the organism *Diplococcus intracellularis meningitidis*. It was later renamed *Neisseria meningitidis* after Albert Neisser of Dresden, who described the gonococcus in 1879[6].

1.2. Characteristics of *Neisseria meningitidis*

1.2.1. Microbiology

N. meningitidis is a Gram-negative obligate aerobe from the family Neisseriaceae. On blood agar plates, young colonies are round, smooth, greyish and unpigmented[7]. Older cultures become more opaquely grey and sometimes cause the underlying agar to turn dark. Well-separated colonies can grow from about 1mm in diameter in 18 hours to up to 4mm after several days. Identification of the organism can be confirmed by Kovac's oxidase test, followed by carbohydrate reactions and serogroup classification. Kovac's oxidase test determines the presence of cytochrome oxidase. Tetramethyl-p-phenylenediamine hydrochloride is turned purple by organisms containing cytochrome c as part of their respiratory chain. Other Neisseria, as well as unrelated bacterial species, may also give a positive reaction so carbohydrate utilisation tests are used to further validate the identification. Neisseria produce acid from carbohydrates by oxidation, and *N. meningitidis* oxidizes glucose and maltose, but not lactose and sucrose, which is detected using phenol red. The serogroup is identified by co-agglutination using polyclonal antibodies.

1.2.2. Structure of the meningococcus

Meningococci possess the common features of Gram-negative bacteria, being enclosed by a cytoplasmic membrane, a thin peptidoglycan cell wall and an outer membrane. Virtually all pathogenic organisms are surrounded by a polysaccharide capsule, which is an essential virulence factor. The capsule provides the organism with anti-phagocytic properties and enables evasion of complement-mediated lysis during infection[8]. It is rare for non-encapsulated meningococci to cause invasive

disease, and related commensal Neisseria, such as *N. lactamica*, do not possess a capsule[3]. The outer membrane contains a number of components which are critical to transmission and virulence of the organism (figure 1.1). Many of the surface-exposed structures are immunogenic and have been investigated as vaccine candidates.

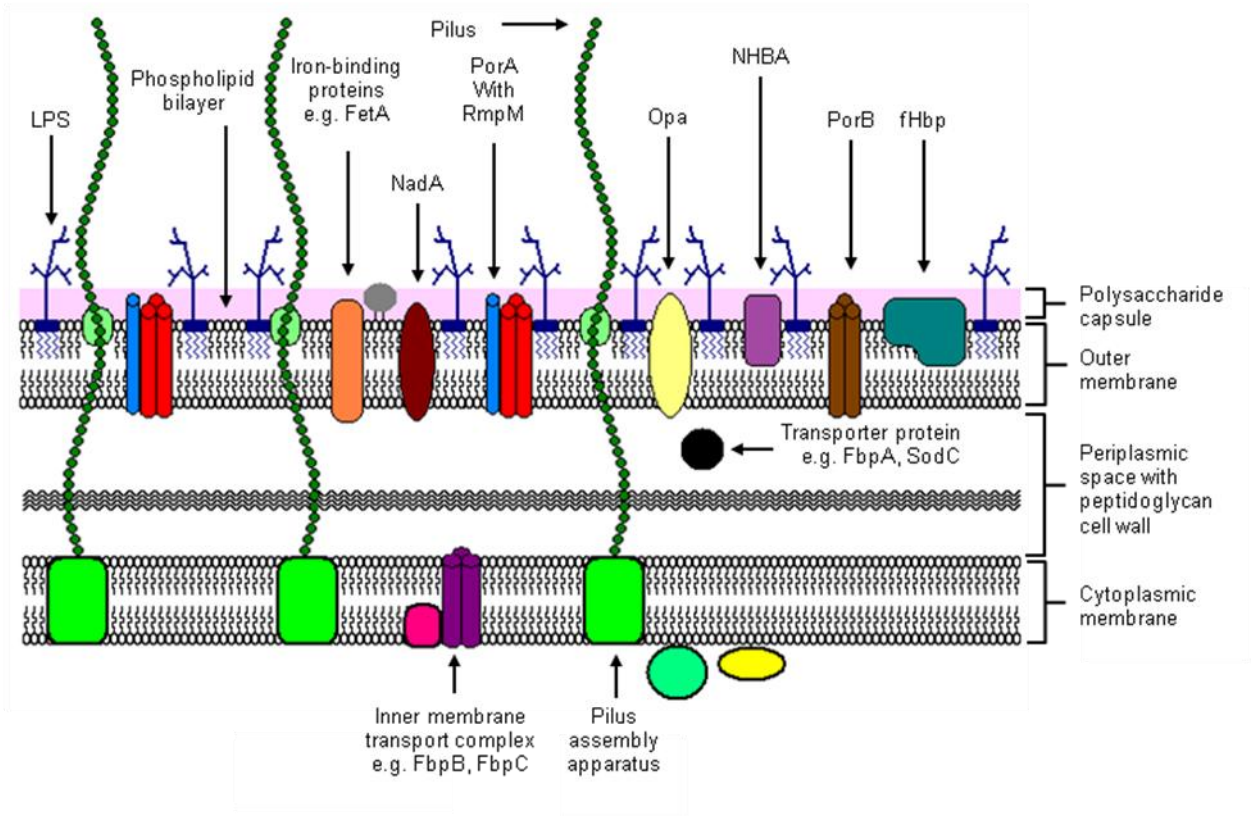

Figure 1.1 Surface structures of *Neisseria meningitidis*.

Pili and lipopolysaccharide (LPS) both extend beyond the polysaccharide capsule. Pili are important in mediating initial contact between the organism and the host, and are also involved in transformation (section 5.1) and intercellular signalling. LPS (endotoxin) is composed of a lipid A anchor and core oligosaccharide, and is the most abundant antigenic component of the outer membrane. Severity of meningococcal sepsis has been correlated with circulating levels of LPS during invasive disease[9]. The

lipid A component is responsible for much of the biological activity and toxicity of the molecule, although some isolates from cases of invasive disease possess mutated forms[9-11]. In one study, 40 strains of *N. meningitidis* from 464 different patients with IMD contained an *lpxL1* mutation and therefore produced penta-acylated rather than hexa-acylated LPS. Although patients infected with these strains had invasive disease, they were less likely to present with rash or thrombocytopenia, suggesting reduced coagulopathy and highlighting the importance of LPS in pathogenesis. LPS-deficient forms are viable *in vitro*, but the extent to which they cause invasive disease is unknown, and likely to be minimal.

The outer membrane contains a large number of proteins, but a small number of major proteins have been the focus of most investigation due to their abundance and potential as vaccine components. These were initially designated Class 1-5 proteins based on their relative mobility on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), but are now known as PorA (Class 1), PorB (Class 2/3), RmpM (Class 4), and Opa/Opc (Class 5)[12]. PorA and PorB are porins and are the predominant proteins on the bacterial surface. They form heterotrimers which function as pores, allowing small hydrophilic nutrients to diffuse into the cell[13]. PorA is the immunodominant component of outer membrane vesicle (OMV) vaccines (section 1.6.4). RmpM occurs in close association with PorA and PorB[14], as well as with the iron-regulated proteins FetA, LbpA, and TbpA[15]. This suggests that its major role may be stabilisation of protein clusters in the outer membrane. Opa and Opc are largely responsible for the close adhesion of bacteria with host epithelial cells during colonisation following initial attachment mediated by pili. Opc facilitates bacterial

binding to epithelial and endothelial cells via heparin sulphate proteoglycans (HSPGs) and the extracellular matrix (ECM) proteins vitronectin and fibronectin. Some studies suggest that Opc is important in meningococcal meningitis, assisting in breach of the endothelial blood-brain barrier[16]. Opa proteins are discussed further in section 1.7.

Iron-regulated proteins within the outer membrane are of critical importance for meningococcal survival and have also been considered as vaccine candidates. Iron is an essential co-factor in a number of important metabolic processes within the bacterial cell. In humans the majority of iron is bound to high-affinity iron binding proteins such as transferrin, lactoferrin and haemoglobin, and is therefore inaccessible to most invading organisms. During conditions of iron limitation, meningococci express a number of proteins in the outer membrane, such as FetA, LbpA and TbpA, which are responsible for iron acquisition from these sources[17]. These remove iron from their storage proteins and transport it across the outer membrane.

Other outer membrane proteins (OMPs), such as factor H binding protein (fHbp), NadA and Neisserial heparin binding antigen (NHBA), have attracted attention recently as components of vaccines currently undergoing clinical trials. fHbp is a lipoprotein responsible for recruiting factor H to the bacterial surface, resulting in dysregulation of the complement pathway and increased bacterial survival[18]. fHbp also provides protection for bacteria against killing by the anti-microbial peptide LL-37[19]. NadA aids meningococcal adhesion and invasion into epithelial cells[20], activates human monocytes/macrophages *in vitro*, and may play a role in stimulating the inflammatory cascade that occurs during IMD[21]. NHBA binds to heparin via an

arginine-rich region located between sites which can be cleaved by both bacterial and human proteases *in vitro*, and binding to heparin correlated with increased survival in human serum[22]. One of the limitations of all surface components as potential vaccines is that most exhibit significant heterogeneity between organisms and antigenic and/or phase variation (PV), providing a mechanism of immune evasion.

A number of other adhesins have been identified more recently following genome sequencing of neisserial strains, and much less is known regarding their structure, exact function and importance during infection. These include NhhA, App, MspA and HrpA[20, 21, 23-26]. To date these have only been described in *N. meningitidis*, except App, which is also present in *N. gonorrhoeae*. NhhA and App are autotransporters involved in adhesion to epithelial cells, MspA is a secreted autotransporter which binds to epithelial and endothelial cells when expressed by *E. coli*, and HrpA forms a two-partner secretion pathway to allow secretion of very large proteins, and may also be involved in bacterial adhesion to epithelial cells. How these contribute to neisserial infection and disease awaits the identification of their host cellular receptors and downstream effects of bacterial binding.

1.3. Classification of meningococci

Serological typing has historically been the most widely used to characterise meningococci[27]. The organism is designated a serogroup based on the capsular polysaccharide and further classification depends on PorB (serotype), PorA (serosubtype) and LPS (immunotype) (figure 1.1). A strain is described by its serogroup, serotype, serosubtype and immunotype, separated by a colon. Thirteen

variants of meningococcal serogroup have been described (A, B, C, D, 29E, H, I, K, L, W-135, X, Y, Z)[28] and the chemical structures of some component polysaccharides have been elucidated[29, 30]. Only 5 of these are responsible for the majority of invasive disease (A, B, C, W-135 and Y). Twelve structural variations of LPS have been identified and this variation affects the role of LPS in determining virulence of the organism. In contrast there are at least 600 *porA* alleles, and 539 *porB* alleles (http://pubmlst.org/neisseria, accessed 05/03/2011), although only a limited number have been associated with the majority of disease-causing isolates[31, 32].

Serological classification is dependent on major surface-exposed structures that are under constant immune selection pressure, rendering it unsuitable for investigating global epidemiology and population structure of the organism. Multi-locus enzyme electrophoresis (MLEE) was utilised from the 1980s and identifies naturally occurring allelic variation in chromosomal housekeeping genes, which are not under selection pressure, by indexing differential electrophoretic migration of the gene products (the enzymes) in a starch gel[33]. The allele profiles are designated as electrophoretic types (ETs) and genetically related ETs are grouped together in families known as clonal complexes. Although hundreds of distinct ETs have been identified, fewer than 10 clonal complexes have been responsible for the majority of IMD worldwide – subgroups I and III, ET-5, ET-37, cluster A4 and lineage III[34].

MLEE, however, is technically cumbersome and has poor reproducibility between laboratories. It has now been replaced by a deoxyribonucleic acid (DNA) sequence-based version, known as multi-locus sequence typing (MLST). Nucleotide sequence

data provide a fundamental measure of genetic diversity and interpretation is unambiguous, while the technique itself is entirely generic[35]. The MLST scheme for Neisseria is defined by the sequences of fragments from seven housekeeping genes. This assigns related isolates into sequence types (STs) and related STs into clonal complexes. Over 8,500 STs have been identified (http://pubmlst.org/neisseria, accessed 05/03/2011), but the majority of IMD since the 1960s has been caused by isolates from just seven clonal complexes, known as the hyperinvasive lineages (ST-1, ST-4, ST-5, ST-8, ST-11, ST-32, ST-41/44)[34, 35]. Serogroup B organisms are usually found associated with clonal complexes ST-41/44 and ST-32, and more recently ST-269[36]. Both serological and MLST type are used during surveillance and in planning vaccine development.

Methods of meningococcal classification have always progressed with the development of new technologies. The recent inclusion of FetA and fHbp as antigens in novel meningococcal vaccines in development has led to their inclusion in the Neisseria PubMLST database, in addition to PorA typing. PorB typing is no longer routinely performed. All targets, including PorA, are now assessed by sequencing of the relevant genes rather than by their reactivity with specific monoclonal antibodies (mAbs). DNA sequencing of *porA* and *fetA* are now performed routinely across Europe via ‘European Meningococcal Epidemiology in Real Time' (EMERT). The current availability of parallel sequencing technology for rapid, low-cost DNA sequencing of whole bacterial genomes has led to the development of the Bacterial Isolate Genome Sequence Database ($BIGS_{DB}$) as a freely available resource to unify all relevant databases[37]. It is likely that typing methods for *N. meningitidis* will

continue to evolve rapidly in the next few years, and it is important that the methods used in the future are able to provide data to continue to inform the development and implementation of any new vaccines, particularly against serogroup B meningococci.

1.4. Epidemiology of meningococcal disease

Humans are the only recognised reservoir for the meningococcus. In non-epidemic periods the organism is carried asymptomatically in the nasopharynx by approximately 10% of the population, with highest carriage rates in the 15-25 year age range[38, 39]. Transmission is by direct contact with nasal or oral secretions or through inhalation of large droplet nuclei[40]. Factors associated with the development of invasive disease include immunological susceptibility of the individual, time of year (peaks in winter and spring), special climatic conditions (e.g. dry season, dust storm), low socio-economic status, transmission of a virulent strain, concurrent respiratory tract infection, overcrowding and smoking[41, 42]. IMD caused by serogroup B organisms occurs sporadically in small clusters throughout the world with seasonal variations and accounts for a variable proportion of endemic bacterial meningitis. Meningococcal meningitis is the only form of bacterial meningitis which causes epidemics, and numerous epidemics have occurred throughout the world in the last 200 years, including during World War I and World War II[42]. The largest epidemics occur mainly in the semi-arid areas of sub-Saharan Africa, designated the African "meningitis belt". Serogroups B and C currently account for a large majority of cases in Europe, the Americas and Australasia. Epidemics in Africa are usually associated with serogroup A, and more recently serogroups W-135[43] and X[44, 45]. Serogroup A is usually the cause of IMD in Asia.

Approximately 500,000 cases of IMD occur globally every year, resulting in over 50,000 deaths[42]. The case fatality rate has generally remained high at approximately 10%, although some specialist centres have published improved survival with early aggressive circulatory support[46, 47]. Survivors may sustain permanent disability such as deafness, epilepsy, amputation and cognitive impairment[48]. In developed countries, where serogroup B disease is predominant, the incidence of IMD is usually up to 6 cases per 100,000 per year[42, 49]. During epidemics in these areas, attack rates can reach almost 100 per 100,000, compared to 1,000 per 100,000 during epidemics in Africa[42]. In contrast to carriage, the peak incidence of disease occurs in children between 6 months and 2 years of age, although during epidemics there may be a broader peak age range[42].

Introduction of a serogroup C conjugate vaccine in the UK from 1999, and subsequently across Europe, resulted in a 10-fold reduction in the incidence of serogroup C disease[36]. In addition to direct protection, there was a significant effect of herd immunity observed with this vaccine[50]. Since serogroup Y organisms account for a substantial proportion of cases in North America, an ACYW-135 tetravalent conjugate vaccine was introduced in the USA for adolescents in 2005[51]. Early data suggest that the overall vaccine effectiveness is 80-85% after 3-4 years[52]. A monovalent serogroup A conjugate vaccine has now been developed for use in the meningitis belt of sub-Saharan Africa[53], with the hope that this will substantially reduce the global burden of IMD. There are now vaccines available that should therefore control A, C, Y and W135 disease, but serogroup B is currently the major

cause of IMD in most temperate countries, and accounts for 85-90% of cases in the UK[36, 54-57]. Until a safe and effective serogroup B vaccine is developed, IMD will continue to cause significant morbidity and mortality in children worldwide.

1.5. Invasive meningococcal disease

1.5.1. Clinical features

IMD has an early non-specific stage, with symptoms such as fever, lethargy, irritability and poor feeding. One observational study associated leg pain, cold extremities and abnormal skin colour with the early stages of disease[58]. A more recent study suggested that confusion, leg pain, photophobia, rash and neck pain or stiffness had positive likelihood ratios for meningococcal disease compared to other infections, and did not find that cold extremities was a useful discriminator[59]. The disease usually progresses to meningitis and/or septicaemia, accompanied by a characteristic purpuric rash. Between 30 and 50% of cases have meningitis only, 7-10% suffer from septicaemia alone, and 40% exhibit both[60]. Occult bacteraemia with *N. meningitidis* has also been described[61].

1.5.2. Pathogenesis and pathophysiology

Colonisation of the nasopharynx by *N. meningitidis* is the first step in either carriage or invasive disease. Disease usually occurs 1-14 days after acquisition of the pathogen[56]. Initial contact of meningococci with host epithelial cells is mediated by type IV pili, the receptor for which may be the I-domain of integrin α chains or CD46[62]. Close adhesion is then mediated by Opa and Opc binding to carcinoembryonic antigen cell adhesion molecule (CEACAM) receptors and integrins,

respectively. Subsequent internalisation of meningococci by epithelial cells is followed by transcytosis through to the basolateral tissues and dissemination into the bloodstream. Immunoglobulin A1 (IgA1) protease secreted by invasive bacteria degrades secretory IgA on the mucosal surface, circumventing this first-line host defence mechanism[63].

Once in the bloodstream, meningococci multiply rapidly to high levels to cause septicaemia. Patients with a higher bacterial load have a more rapid clinical deterioration and longer period of hospitalisation, as well as a higher risk of death and permanent sequelae[64, 65]. Resistance to complement-mediated lysis and phagocytosis is largely mediated by the capsule and LPS. Blebs released from the surface of the organism contain LPS, OMPs, periplasmic proteins and phospholipid and play a major role in the inflammatory cascade which leads to severe disease (figure 1.2)[66].

Figure 1.2 Electron micrograph of *Neisseria meningitidis*.
The organism is seen as an encapsulated diplococcus, with several blebs being released from the surface. *(Courtesy of Meningitis UK)*

Much of the tissue damage is due to host immune mechanisms activated by meningococcal components, in particular LPS. During invasive disease LPS is bound to a circulating plasma protein, known as LPS binding protein. The host receptor complex for LPS consists of toll-like receptor 4 (TLR4), CD14 and myeloid differentiation protein 2[64]. Binding of LPS to TLR4, which is upregulated on circulating leucocytes during septicaemia, results in activation of a number of different cell types. An intense inflammatory reaction ensues due to the secretion of pro-inflammatory cytokines such as tumour necrosis factor α (TNFα), interleukin 1β (IL-1β), IL-6, IL-8, and granulocyte macrophage colony-stimulating factor, levels of which are closely associated with plasma levels of LPS. The major anti-inflammatory cytokines IL-1Ra, IL-2, IL-4 and IL-12 and transforming growth factor β are present at very low levels. Both high and low levels have been observed for IL-10 and interferon γ[64, 67].

The pathophysiological events which occur during meningococcal septicaemia are largely related to microvascular injury[67]. This leads to increased vascular permeability and the capillary leak syndrome, pathological vasoconstriction and vasodilatation, disseminated intravascular coagulation (DIC) and profound myocardial dysfunction. Increased vascular permeability can lead to dramatic fluid loss and severe hypovolaemia. Capillary leak syndrome with or without aggressive fluid resuscitation (which is essential in severe cases) leads to pulmonary oedema and respiratory failure. Initial vasoconstriction is a compensatory mechanism in response to hypovolaemia, and results in the clinical features of pallor and cold peripheries. Following resuscitation, some patients experience “warm shock” – intense vasodilatation with

bounding pulses and warm peripheries, despite persistent hypotension and metabolic acidosis. Virtually all anti-thrombotic mechanisms appear to be dysfunctional during meningococcal sepsis, leading to a procoagulant state and DIC. All of these factors contribute to depressed myocardial function, but there is also a direct negative cytokine effect on myocardial contractility, thought to be largely mediated via IL-6[68]. Hypoxia, acidosis, hypoglycaemia, hypokalaemia, hypocalcaemia and hypophosphataemia are all common in severe septicaemia and further depress cardiac function. Some patients become unresponsive to the positive inotropic effects of catecholamines, and require high levels of inotropic support during intensive care management. These processes result in impairment of microvascular blood flow throughout the body and ultimately lead to multi-organ failure, which is responsible for much of the mortality.

Following invasion of the circulation, meningococci may also penetrate the blood-brain barrier and enter the cerebrospinal fluid (CSF), facilitated by pili, and possibly Opc[16]. Once there bacteria continue to proliferate and LPS and other outer membrane products can stimulate a pro-inflammatory cascade similar to that observed in the blood[64]. This leads to upregulation of specific adhesion molecules and recruitment of leucocytes into the CSF. Central nervous system damage occurs directly by meningeal inflammation and indirectly due to circulatory collapse, and causes a high rate of neurological sequelae in affected patients. Death can occur from cerebral oedema, which leads to raised intracranial pressure and cerebral or cerebellar herniation.

1.5.3. Host genetic factors

The sibling risk ratio for meningococcal disease is similar to other diseases where susceptibility shows polygenic inheritance[69], and there are a number of host genetic factors which have now been identified which affect either susceptibility to IMD or severity of disease. The difficulties of these studies are the requirement for large numbers of cases and controls, and the need to confirm any potential associations in more than one population. Deficiencies in the complement pathways have consistently been associated with an increased risk of IMD (section 1.6.1), with specific polymorphisms in mannose binding lectin, and factor H found to be associated with disease susceptibility[70]. A genome wide association study (GWAS) of 7,522 individuals in Europe identified single nucleotide polymorphisms (SNPs) within genes encoding complement factor H (*CFH*) and CFH-related protein 3 (*CFHR3*) which were associated with host susceptibility to IMD[71]. Complement-mediated bacteriolysis is known to be extremely important in protection against IMD, giving these associations biological plausibility. In terms of disease severity, a recent meta-analysis performed to collate data from smaller studies found that SNPs in genes encoding plasminogen activator inhibitor 1 (*SERPINE1*), IL-1 receptor antagonist (*IL1RN*) and IL-1β (*IL1B*) were associated with increased mortality from IMD, which again would be predicted from the known pathophysiological changes which occur during invasive disease[72]. Given that any single specific SNP is likely to have only a small impact on disease susceptibility or severity, further large GWAS in genetically different populations are required.

1.5.4. Treatment

Prior to any specific treatment, mortality from meningococcal meningitis was up to 80%[73]. The first rational treatment was developed in 1906 when antisera raised in rabbits and horses was administered subcutaneously and then via the intrathecal route in humans[74]. In 1908 horse anti-meningococcal serum was extensively used for intrathecal treatment in the USA and Germany, reducing the mortality in treated patients by half[75]. Serum sickness and occasional cases of secondary meningitis were the major adverse effects. Sulphonamides were the first specific and highly effective therapy, forming the mainstay of treatment throughout World War II, and being used for prophylaxis as these drugs clear nasopharyngeal carriage. The spread of sulphonamide resistance in the early 1970s aroused renewed interest in disease prevention. Despite advances in intensive care and specific therapy with beta-lactam antibiotics since then, the mortality rate has remained around 10% (figure 1.3).

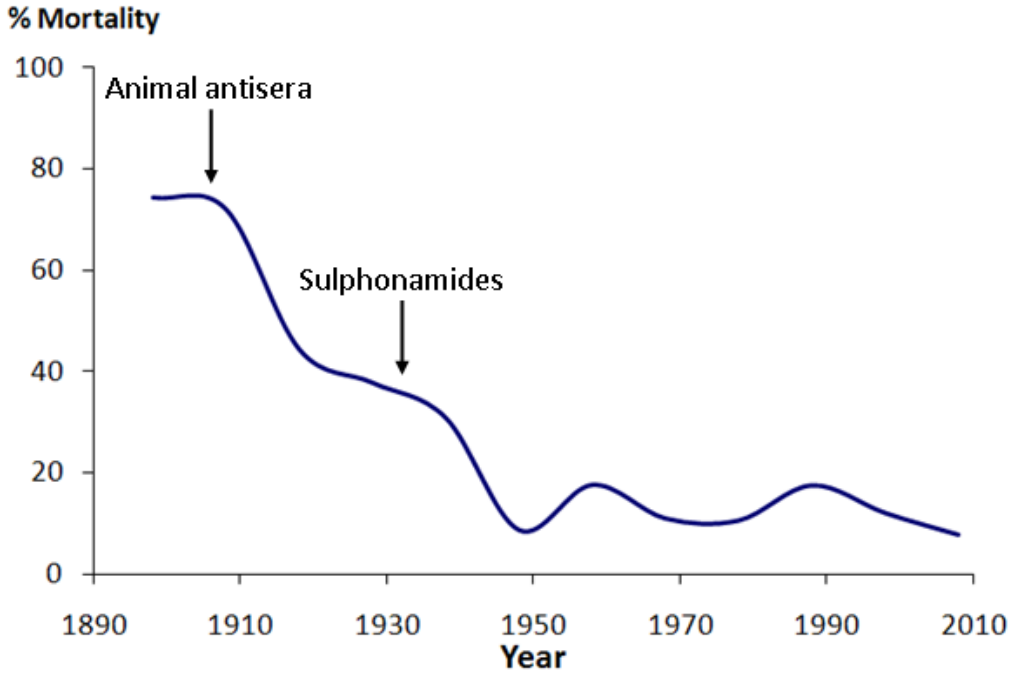

Figure 1.3 Mortality from meningococcal disease in North America and Europe.
Introduction of animal antisera and sulphonamides both had a significant impact on mortality, which has remained relatively unchanged since 1950. *(Data from www.hpa.org.uk, www.cdc.gov, Havens* et al.[76]*, Edwards* et al.[77]*, Gedde-Dahl* et al.[78]*, Andersen*[79]*, Farries* et al.[80]*, Stiehm and Damrosch*[81]*, Banks*[82]*, Glasscock*[83]*, Dunn*[84]*, and Flexner and Jobling.*[85]*)*

1.6. Serogroup B meningococcal vaccines

1.6.1. Immunity to the meningococcus and surrogate markers of protection

Serogroup B disease has a relatively low incidence in countries where it is endemic, so the large sample size required for vaccine efficacy studies prohibits such trials. It has therefore been necessary to identify a surrogate marker of protection which can be measured in trial participants. The importance of "bactericidal substances" in human blood in determining protection against IMD, and higher levels of such factors in adults compared to children, was first proposed in the early 1900s[86, 87]. In 1969, Goldschneider provided evidence that circulating antibody was the critical "substance", demonstrating an inverse correlation between the incidence of disease and the prevalence of complement-dependent serum bactericidal antibody (SBA)

(figure 1.4)[88]. This led to accepted use of the SBA assay as a surrogate marker of protection.

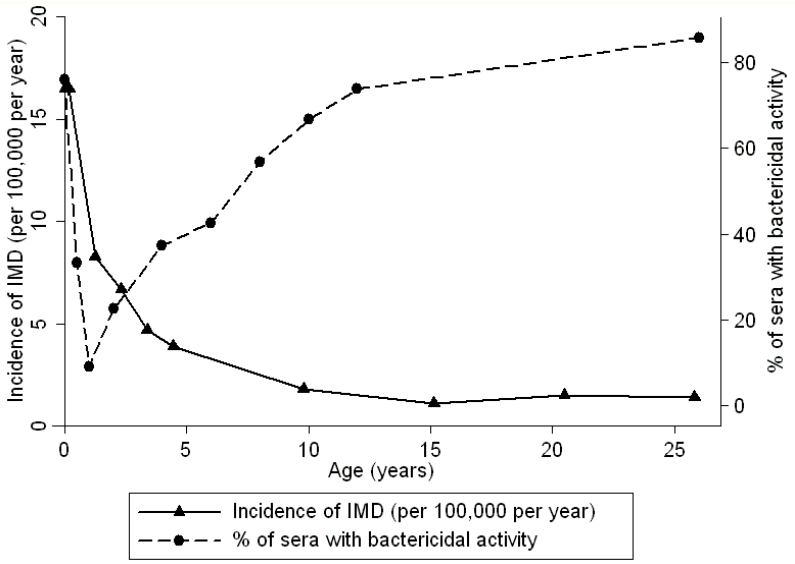

Figure 1.4 Age-related incidence of invasive meningococcal disease (IMD) and prevalence of serum bactericidal antibody against serogroup B *N. meningitidis*.
The level of bactericidal antibody is most prevalent at birth and among adults and least in children aged between 6 months and 2 years, when the highest incidence of disease occurs. A similar relationship was described for serogroups A, B and C. *(Adapted from Goldschneider* et al.[88]*)*

In the modern SBA assay, *N. meningitidis* target strains are killed in the presence of meningococcal-specific antibody (from post-vaccination serum) and exogenous complement[89]. For the meningococcal serogroup C conjugate vaccine, an SBA titre of ≥1:8 (with rabbit complement) correlated strongly with post-licensure vaccine effectiveness[89]. For serogroup B disease the data are less secure since they are based on subgroup analysis of study participants compared to the large studies for serogroup C vaccines. The proportions of serogroup B vaccine recipients with ≥4-fold rises in SBA following vaccination or SBA titres ≥1:4, using human complement, have been correlated with clinical efficacy in trials of OMV vaccines[90-92]. These cut-offs are

therefore currently considered the protective threshold in evaluation of experimental vaccines, and are required to support vaccine licensure.

There are, however, several limitations of the SBA assay. There is significant inter-laboratory variation, and there remains a lack of consensus about representative target strains. Recent data suggest that rabbit complement factor H does not bind to *N. meningitidis*, unlike human factor H. Binding of factor H to the bacterial surface downregulates complement activation, leading to higher SBA titres and overestimation of vaccine efficacy with rabbit complement[93]. Furthermore, there is increasing evidence that alternative mechanisms are important in determining protection against IMD. Firstly, the relationship between incidence of disease and prevalence of SBA described by Goldschneider was not observed in more recent studies in the UK and Canada[94, 95], where a decline in disease incidence throughout childhood was not associated with a change in the prevalence of SBA. In the UK study, the second peak of disease in teenagers coincided with a paradoxical increase in the proportion with an SBA titre ≥1:4 and adults had a low risk of disease despite a much lower prevalence of SBA activity. Secondly, in a large study in Iceland SBA titres underestimated vaccine efficacy[96]. Thirdly, disease in individuals with complement deficiency has a different age distribution, less severe clinical features, and often involves unusual serogroups[97]. In particular, complement deficiency does not appear related to an increased risk of serogroup B disease, highlighting the importance of other mechanisms of protection. Alternative surrogate markers of protection include the opsonophagocytic assay[98] and antibody avidity[99], but there are

no studies that have attempted to link these laboratory tests with vaccine efficacy or even population protection, as has been found with SBA.

The SBA assay continues to be widely utilised, but it is not known whether mechanisms of protection for novel vaccine candidates will be the same as for polysaccharide and OMV vaccines. New vaccines may elicit different mechanisms of killing and other immune responses may be important. However, SBA remains the only surrogate of protection for which there is any supportive human data, although vaccine efficacy can only be accurately evaluated during post-marketing surveillance.

1.6.2. Early vaccines

The first generation of meningococcal vaccines were crude, comprising standardised suspensions of killed meningococci. A heat-killed vaccine used during a large outbreak in England in 1915 did not protect vaccinees against disease[100], but a similar vaccine given to soldiers in a US military camp outbreak in 1917 had a short term efficacy of 87%[101]. Trials with whole cell vaccines carried out between 1915 and 1950 were unable to provide conclusive evidence of efficacy[73, 102], and resulted in unacceptable levels of adverse reactions, leading to their disuse. Following the successful use of diphtheria anti-toxin for immunisation, meningococcal exotoxin vaccines were tested in teenagers and adults during the 1930s. There was some suggestion that they induced protection against disease, but again definitive evidence was lacking[103, 104].

1.6.3. Capsular polysaccharide vaccines

The serogroup B capsule is the most obvious candidate for a universal vaccine, but failed to elicit SBA in humans[105] or animals[106] (table 1.1). Some studies of complexes of the B polysaccharide and OMPs showed antibody responses to polysaccharide and protein components[107, 108], but others have not confirmed this[109]. When polysaccharide-specific antibodies are present, they are mostly of the IgM class, not bactericidal and therefore may not be protective. The most likely explanation for this lack of immunogenicity is structural homology between the B polysaccharide and human tissue, leading to immunological tolerance. The key component of the serogroup B capsule is an α(2-8) linked sialic acid homopolymer, which contains epitopes that are cross-reactive with the polysialylated form of the neural cell adhesion molecule[110]. Any anti-capsular antibodies elicited could cross-react with host antigens and contribute to autoimmune disease.

A modified polysaccharide, in which the N-acetyl groups were replaced with N-propionyl groups, followed by conjugation with tetanus toxoid, elicited bactericidal antibodies in mice[111], but not in humans[112] (table 1.1). Although there was no evidence that it induced auto-antibodies, concerns remain about this possibility. Failure to derive a capsule-based vaccine and concerns about autoimmunity has shifted focus to subcapsular antigens.

Year	Country	Vaccine		No. of doses	n	Age group	% with SBA titre*		Vaccine efficacy (95% CI)	Ref
		Strain	Components				≥1:4	≥4-fold rise		
Capsular polysaccharide vaccines										
1972	USA	n/a	B polysaccharide	1	113	Adults	0	0		105
2004	France	n/a	N-propionylated B polysaccharide	1	17	Adult males	0	0		112
Parenteral meningococcal OMV vaccines										
1983	South Africa	M986†	OMP + B polysaccharide	1	4,400	4m-5y	41‡‡			113
1991	Cuba	Cu385/83‡	OMV + C polysaccharide + 65-95kDa envelope proteins	2	106,252	10-14y		85	83% (42 to 95)	91
1991	Norway	44/76§	OMV	2	171,800	14-16y	97	80	57% (27 to 87) 87% in first 10m	90, 114, 115
				2 + 1	311	13-14y	96	96		
				3 + 1	374	12-17y	93	90		
1992	Brazil (Sao Paulo)	Cu385/83‡	OMV + C polysaccharide + 65-95kDa envelope proteins	2	~2.7m	4-6y	52	52	74% (16 to 92)	116
						24-47m	43	45	47% (-72 to 84)	
						3-23m	13	22	-37% (-100 to 73)	
1995	Brazil (Rio de Janeiro)	Cu385/83‡	OMV + C polysaccharide + 65-95kDa envelope proteins	2	~1.6m	6m-9y			58% (31 to 74)	117
						4-9y			70% (38 to 85)	
						24-47m			42% (-47 to 77)	
						6-23m			23% (-119 to 73)	
1995	Chile	8529¶	OMP + C polysaccharide	2	40,811	1-21y			51% (-11 to 80)	118
						5-21y		35-65	69% (14 to 91)	
						1-4y		12	-23% (<-100 to 73)	
2005	New Zealand	NZ98/254‖	OMV	3 ± 1	75	Adults		96		119, 120
					608	8-12y		76		
					325	16-24m	75‡‡	75 (3 doses) 100 (4 doses)		
					294	6-8m	74‡‡	74		
					375	6-8w	54‡‡	53 (3 doses) 69 (4 doses)		
2010	Argentina	H44/76§§	OMV	3	150	Adults		5-36		121

Year	Country	Vaccine		No. of doses	n	Age group	% with SBA titre*		Vaccine efficacy (95% CI)	Ref
		Strain	Components				≥1:4	≥4-fold rise		
1999	UK	H44/76§	Hexavalent PorA OMV††	3 + 1	111	8-12w	98-100	19-81 (3 doses) 78-95 (4th dose)		122
2000	Netherlands	H44/76§	Hexavalent PorA OMV††	3	172	2-3y	28-98			123
					165	7-8y	16-100			
2007	Norway	44/76§ + NZ98/254‖	Bivalent OMV	3 + 1	91	Adults	68-87			124
2007	Spain/Belgium	Cu385/83‡ + NZ228/98‖	Bivalent OMV	3	478	12-18y		42-76		125
2010	USA	H44/76¶¶	Native OMV	3	34	Adults		54		126
2011	USA	8570‖‖	Native OMV	3	36	Adults		79		127
Other OMV vaccines										
1998	Norway	44/76§	Intranasal OMV	4 + 1	23	Adults		45-72		128
1999	USA	9162**	Intranasal OMV	3	32	Adults		75		129
2002	USA	9162**	Intranasal OMV	3 or 4	44	Adults		43		130
2009	UK	*N. lactamica*	OMV	3 ± 1	97	Adults	54-96 (3 doses) 61-94 (4 doses)	8-31 (3 doses) 6-22 (4 doses)		131
Protein vaccines										
2007	Canada	n/a	NspA	3	122	Adults	0	0		132
2008	Australia	n/a	fHbp A & B	3	103	Adults		22-100		133
2010	UK	n/a	NadA + fHbp + NHBA	3 ± 1	48	2m	78-100 (3 doses) 97-100 (4 doses)	69-97 (3 doses) 97 (4 doses)		134, 135
				3	30	6-8m	100			
2010	UK	NZ98/254‖ (OMV)	NadA + fHbp + NHBA + OMV	3 ± 1	50	2m	85-95 (3 doses) 93-100 (4 doses)	78-92 (3 doses) 84-97 (4 doses)		134, 135
				3	30	6-8m	96-100			

Table 1.1 Clinical trials of serogroup B meningococcal vaccines.

*****against vaccine strain; †M986=B:2a:P1.2; ‡Cu385/83=B:4:P1.19,15:L3,7,9; §44/76 or H44/76=B:15:P1.7,16:L3,7,9; ¶8529=B:15:P1.3:L3,7; ‖NZ98/254 and NZ228/98=B:4:P1.7-2,4; ‡‡≥1:8; §§modified H44/76: Δ*lpxL1* Δ*porA* Δ*fetA* Hsf+ TbpA+ TrL3/L7 LPS; ††contains P1.7,16; P1.5-1,2-2; P1.19,15-1; P1.5-2,10; P1.12-1,13; P1.7-2,4; ¶¶modified H44/76: Δ*synX* Δ*lpxL2* Opc+; ‖‖modified 8570=B:4:P1.19,15:L8-5 Δ*synX* Δ*lpxL1* fHbp+ Opc+ P1.22,14+; ******9162=-:15:P1.3:L3,7,9

1.6.4. Outer membrane vesicle vaccines

Bactericidal antibodies directed against subcapsular antigens develop following disease and carriage, suggesting OMPs as vaccine candidates[136, 137]. The first OMP vaccines in the 1970s were immunogenic in animals, but failed to elicit SBA in humans[138, 139], leading to the development of soluble protein vaccines in the form of OMVs. The vesicles are similar to outer membrane blebs naturally released by *N. meningitidis* during culture and infection, and contain LPS, OMPs, periplasmic proteins and phospholipid (figure 1.2)[66]. OMVs can be produced with or without the use of detergent. The majority of OMV vaccines have been based on detergent-extracted vesicles. In these preparations, most of the LPS is removed from outer membrane fragments by detergent, following which the detergent is removed and proteins in the vesicles solubilised. More recently a number of "native" OMV vaccines have been developed, and these are described in more detail below.

A number of methods exist to produce native OMVs without using detergent, including ultrafiltration or ultracentrifugation of bacterial culture supernatant, physical disruption to produce vesicles or precipitation with ammonium sulphate[107, 140, 141]. Native OMVs would have higher toxicity due to the presence of LPS, which is therefore genetically detoxified, and tend to have a lower yield[142]. However, they retain some of the proteins which may be lost during detergent extraction, and which may be important in stimulating a protective immune response[143].

Studies of early OMV vaccines during the late 1970s and early 1980s demonstrated ≥4-fold rise in SBA in 70-85% of adults following a single dose, but children under 6

years had lower responses[107, 144, 145]. Immunogenicities were enhanced with the addition of capsular polysaccharide[145, 146] or aluminium hydroxide[109], and the latter was included as an adjuvant in most subsequent OMV vaccines. A number of serogroup B OMV vaccines have since undergone efficacy trials (table 1.1).

The Cuban OMV vaccine, which had an efficacy of 83%, was the first serogroup B vaccine to demonstrate high efficacy[91]. Two subsequent case-control studies of this vaccine in Brazil demonstrated an efficacy of 70-74% in children 4-9 years of age, but the vaccine was ineffective in younger children[116, 117]. Similar results were found with an OMP vaccine in Chile[118]. These studies also indicated that responses to OMV vaccines were largely restricted to the vaccine strain. In Norway, an OMV vaccine based on an epidemic strain had an efficacy of 57% after 2 doses[114]. There was significant waning of immunity over time, with an efficacy of 87% in the first 10 months following vaccination, compared to only 30% after 21-29 months[90]. Subsequent studies demonstrated boosting of SBA titres with additional doses, which also resulted in SBA activity against strains of a different serosubtype[90, 115].

The second serogroup B meningococcal vaccine to be licensed following the Cuban vaccine was utilised recently during an epidemic New Zealand[147]. After 3 doses of an OMV vaccine based on the outbreak strain, ≥4-fold rise in SBA titre was seen in 53% of young infants[120], 74-76% of older children and 96% of adults[119]. Again there was significant waning of immunity over time. The proportion of seroresponders increased to 100% of toddlers and 69% of young infants following a fourth dose[120]. The vaccine was rolled out nationally in 2004, contributing to a decrease in number of cases,

although the disease burden was already waning. The vaccination programme was discontinued in 2008, and ongoing surveillance will determine the longer term impact of the vaccine campaign and its cessation and provide important information for strategies to control future outbreaks.

The SBA response induced by OMV vaccines is largely serosubtype-specific, being directed predominantly against PorA[148-150]. In order to increase vaccine coverage, a hexavalent PorA OMV vaccine was developed in The Netherlands, consisting of two OMVs each expressing 3 different PorA proteins. In phase II trials, the proportion of children aged between 8 weeks and 8 years with significant SBA responses was 16-100%, dependent on the PorA variant[122, 123]. A nonavalent PorA OMV vaccine has been developed, adding a third OMV containing 3 further PorA proteins, and is in clinical trials[151].

An alternative approach to increase breadth of protection is by combining OMVs. Two such vaccines have been investigated, one included the Norwegian and New Zealand OMVs[124] and the other was based on the Cuban and New Zealand OMV vaccine strains[125]. In teenagers and adults, 42-87% of vaccine recipients achieved SBA responses against homologous or PorA-related strains, with similar results for both vaccines. Interestingly, 28-62% of vaccinees had a response to PorA heterologous strains after immunisation with the Cuba/New Zealand vaccine, suggesting this could provide broader protection, not restricted by serosubtype in this age group.

OMV vaccines from strains which have had multiple genetic manipulations to improve immunogenicity and potential breadth of coverage have also been tested in phase I studies. One of the problems with the conventional method of detergent extraction for removal of potentially toxic LPS from OMV vaccines is that this may also remove other desirable surface antigens, including lipoproteins such as fHbp, as well as the LPS[143]. The production of native OMVs does not require detergent, but the LPS has to be genetically detoxified since it is present at much higher levels than in detergent-extracted OMVs. Native OMV vaccines often contain modifications in addition to those in the LPS biosynthesis genes to improve immunogenicity, usually by upregulation of more conserved minor OMPs, with or without disruption of *porA*. This is a further strategy to try and avoid the serosubtype-specific response usually seen with OMV vaccines.

Two similar native OMV vaccines have undergone phase I trials in the USA (table 1.1). They both contain stable expression of Opc and modified LPS via a disruption of either *lpxL1* or *lpxL2*[126, 127]. One has been further manipulated to express fHbp and a second PorA in addition to the PorA which is normally present[127]. These vaccines produced a ≥4-fold rise in SBA titre against the respective vaccine strains in 54% or 79% of adults, with the better response seen with the vaccine with fHbp and a second PorA. A vaccine including this OMV and 2 additional OMVs from different genetically modified strains, each expressing 2 PorA variants and different detoxified LPS immunotypes, with overexpression of the OMPs fHbp, NadA or Opc, elicited SBA in mice and rabbits[152]. All three sets of antigens were involved in the bactericidal

response. These data suggest that such a formulation may be able to provide broad protection in humans.

OMVs with no PorA and overexpression of the conserved proteins TbpA, Hsf, NspA or Omp85 only induced an SBA response in mice when combined[153]. This suggested that a critical density of bactericidal antibodies is required on the bacterial surface to mediate bacteriolysis, which can be achieved either by a single major protein, such as PorA, or multiple minor OMPs. However, a similar OMV with a modified LPS, manufactured using low concentrations of detergent to increase LPS content, only elicited an SBA response in 5-36% of adults (depending on the target strain) in a phase I study in Argentina[121]. Although these approaches are appealing and have shown some promise, further work is required to identify the key modifications which will enhance the immunogenicity of native OMV vaccines.

OMV vaccines remain a useful tool in the control of serogroup B outbreaks, but a number of hurdles remain. The problem of serosubtype-restricted responses has yet to be overcome to produce a vaccine with a wide breadth of coverage. Lack of persistence of immune responses suggests that multiple doses throughout childhood would be required to maintain protection[90, 117, 118, 150, 154]. This is already necessary with vaccines against tetanus and diphtheria, which have been acceptable for decades worldwide. The lower vaccine efficacy in younger children remains unresolved[116-118].

1.6.5. Other vaccines in clinical trials

Several vaccines based on other subcapsular antigens are currently in development (table 1.1). One promising candidate, 4CMenB (also known as 5CVMB), is based on 3 proteins – NadA, fHbp (also known as GNA1870 or LP2086) and NHBA (also known as GNA2132) – combined with the New Zealand OMV[155]. Including multiple antigens reduces the risk of escape variants, which might readily occur with single antigen vaccines given the high rate of PV, recombination and mutation in *N. meningitidis*. This vaccine was developed by "reverse vaccinology" – 570 novel putative surface-exposed or secreted proteins were identified from the *N. meningitidis* genome sequence[156], and 350 of the encoded proteins were used to immunise mice[157]. Twenty-nine induced bactericidal antibodies and the most immunogenic were included in 4CMenB. Sera from mice immunised with 4CMenB (excluding the OMV) killed 78% of 85 representative global isolates[155], suggesting this vaccine has the potential to provide broad coverage against serogroup B disease.

In phase II studies, SBA titres ≥1:4 were achieved in 93-100% of young infants after 4 doses of 4CMenB, and 96-100% of 6-8 month old children after 3 doses, against homologous strains[158, 159]. A phase II study in infants across Europe has been completed, and this vaccine was submitted for licensure in December 2010 (www.novartisvaccines.com, accessed 05/03/2011). In the phase II studies, sera from vaccinated individuals were tested in the SBA assay against a panel of target strains. Strains were chosen such that they expressed different vaccine antigens to independently assess the response to each protein. Additional strains not expressing any vaccine antigens, or containing different subvariants of these proteins were also

tested, to establish potential vaccine coverage. Responses against strains not expressing the vaccine antigens or following fewer doses were much lower, suggesting that the problems of breadth of coverage and persistence of bactericidal antibody seen with OMV vaccines may also be an issue with protein-based vaccines.

fHbp has also been studied as an independent vaccine candidate. Different classification systems have divided fHbp into either 3 variants (1, 2 and 3)[160] or 2 subfamilies (A and B)[161]and a unified nomenclature has also been proposed whereby unique fHbp sequences are arbitrarily assigned an allele number depending on the order in which they are described[162]. A vaccine containing proteins of subfamilies A and B, which therefore has the potential for broad coverage, elicited SBA responses in 22-100% of adults in a phase I trial in Australia, dependent on dose and target strain[133]. Data from completed phase I trials in toddlers and teenagers are awaited.

The genes encoding fHbp and GNA2132 appear to be present in all meningococci, whereas only 38-50% contain *nadA*[163-166]. NadA, fHbp and NHBA have now been sub-divided into 14, 3 and 5 variants, respectively, with a number of additional sub-variants, and it is unclear how broad the protection is against strains containing more genetically distant variants. Variant 1.1 (subfamily B) of fHbp is present in 4CMenB and is the most commonly occurring. Sera from individuals vaccinated with 4CMenB had lower SBA titres against strains expressing fHbp sub-variants of variant 1 other than 1.1, and this was more pronounced in infants compared to adults[167], confirming results of animal studies[160].

A potential problem with these OMPs as vaccine components is variation in expression level between strains. For example, studies using rabbit complement demonstrated killing of bacteria expressing low fHbp levels by antisera from immunised mice[160]. Studies using human complement, however, found that mAbs recognising different epitopes which were not bactericidal individually achieved bacteriolysis when combined[168, 169]. This was thought to occur because the overall antibody density on the bacterial surface was sufficient to allow complement deposition and activation of the classical pathway. Similar synergy has been found with antibodies targeting fHbp and NHBA in human sera following immunisation with 4CMenB[170]. Whether this occurs with antibodies generated *in vivo* requires investigation. A meningococcal antigen typing system (MATS) has been developed in an attempt to predict bactericidal activity against strains with different expression levels of the antigens in 4CMenB, with the aim to screen large panels of strains in order to predict vaccine coverage[171]. The limited volume of sera obtained in clinical trials following infant immunisation means that testing against large panels of target strains in the SBA assay is extremely difficult. The MATS does appear to correlate with SBA, but its ability to predict vaccine efficacy would probably only be clear from post-marketing surveillance.

An additional potential problem with these protein vaccines is the absence of immunodominant surface structures such as PorA, which is exposed to the immune system *in vivo,* is under immune selection pressure and therefore shows sequence variability. The relative conservation of NadA, fHbp and NHBA may indicate that

they are not naturally immunologically exposed (during colonising infection), and as such may not be as immunogenic as more variable OMPs.

Several other vaccines have been tested in clinical trials (table 1.1). NspA is a highly conserved protein present in most *N. meningitidis* strains, but did not induce bactericidal antibodies in humans after promising murine studies and is not being pursued[132, 172]. Transferrin-binding proteins purified from *N. meningitidis* elicited SBA in mice and rabbits[173, 174], but not in humans[175]. Intranasal OMV vaccines have been tested to mimic the natural immunising effect of colonisation. Nasal IgA levels increased in the majority of vaccinees in phase I trials, but only 43-75% had significant SBA responses[128-130]. Further data are required, in particular to determine whether mucosal IgA plays a role in protection against colonisation with virulent meningococci. *N. lactamica* is a commensal carried in the nasopharynx of young children and has been implicated in the acquisition of natural immunity to the meningococcus. An *N. lactamica* OMV vaccine was developed on the basis that it would avoid PorA-dependent strain restriction as it does not express PorA. No bactericidal responses were elicited following immunisation in mice or rabbits[176], and only 6-31% of adults (dependent on the target strain) showed ≥4-fold rise in SBA after 3 or 4 doses during a phase I trial[131]. Antisera from immunised rabbits, however, did mediate opsonophagocytosis, suggesting this as a mechanism for the protection against meningococcal bacteraemia observed in mice. With the paucity of data currently available for alternative assays to the SBA, it is uncertain whether vaccines that do not induce SBA could be licensed[177].

1.6.6. Vaccines in pre-clinical studies

A number of additional approaches have provided promising data in animal models (table 1.2). Iron-regulated proteins (e.g. FetA, LbpA, LbpB) are upregulated under iron-limited conditions, so the amount present in OMV vaccines is dependent on growth conditions during manufacture. FetA induced SBA in rabbits[178] and OMVs with overexpression of LbpA and purified LbpB[179] have induced bactericidal antibodies in mice, as have liposomal and recombinant Opa proteins[180, 181] (section 1.8). Although these vaccines are immunogenic, antibodies are usually directed against variable regions of the proteins and exhibit limited cross-reactivity. The additional problem of variable expression levels on target bacteria must also be overcome if these vaccines are to be developed further. In addition to these antigens, recent analysis of meningococcal genome sequences, similar to the methods used during the development of 4CMenB, has revealed five novel putative surface proteins (NMB0606, NMB0873, NMB0928, NMB0938 and NMB1163) which have elicited bactericidal antibodies following immunisation of mice and which require further assessment[182].

LPS has been implicated in the immune response to natural infection[183], but must be detoxified before inclusion in a vaccine. Meningococcal strains which possess a disrupted *lpxL1* gene produce penta-acylated instead of the usual hexa-acylated LPS, resulting in significantly lower toxicity. Such strains have been used in the preparation of native or partially detergent-extracted OMVs, including the OMVs with over-expressed fHbp described above and a vaccine based on OMVs enriched with different immunotypes of genetically detoxified LPS. Both of these vaccines elicited

bactericidal antibodies in mice, and avoid loss of antigens caused by the detergent used in conventional OMV production[184]. Different approaches using LPS have demonstrated that oligosaccharides conjugated to tetanus toxoid induced immunotype-specific SBA in rabbits[185] and the relatively conserved LPS inner core elicited SBA in mice[186]. An alternative function of LPS could be as an adjuvant. OMV vaccines without LPS resulted in lower immune responses, whereas an *lpxL1* mutant LPS retained adjuvant activity similar to wild-type with reduced toxicity in mice[187], although more recent *in vitro* studies using human dendritic cells suggested this adjuvant property of the mutant LPS may not occur in humans[188].

Vaccine	SBA in mice	SBA in rabbits	Passive protection in rats/mice	Ref
OMV vaccines				
Nonavalent PorA OMV	✓			151
OMV with overexpression of fHbp	✓			143
OMV with overexpression of TbpA, Hsf, NspA, Omp85	✓			153
Hexavalent PorA, trivalent LPS OMV with overexpression of fHbp, NadA, Opc	✓	✓		189
OMV with overexpression of LbpA + LbpB	✓			179
OMV with genetically detoxified LPS	✓			184
N. lactamica OMV	×	×	✓	176
OMP preparations				
Chimeric fHbp	✓			190
Transferrin-binding proteins	✓	✓	✓	173, 174
FetA (FrpB)		✓		178
Opa	✓			180, 181
NMB0606	✓			182
NMB0928	✓			182
NMB0873	✓			182
NMB1163	✓			182
NMB0938	✓			131
Other vaccine candidates				
Capsule mimetics	✓		✓	191, 192
Liposomal PorA + PorB	✓			193
LPS		✓		185
LPS inner core	✓		✓	186
Live attenuated *N. meningitidis*	✓			194, 195
DNA vaccines	✓			196

Table 1.2 Preclinical studies of serogroup B meningococcal vaccines.

1.6.7. The current state of serogroup B meningococcal vaccines

Lack of immunogenicity of the serogroup B polysaccharide was demonstrated in 1972 and yet a universal vaccine remains elusive. During the past 40 years there has been some success, with OMV vaccines contributing to disease control in Cuba and New Zealand. These vaccines, however, elicit only limited protection against PorA heterologous strains, are poorly immunogenic in young children and require multiple doses to provide long-term immunity. Current vaccine candidates are based on subcapsular antigens and, if successful, could have an impact on other serogroups. Recent clinical trials have produced encouraging initial results, but the persistence and breadth of protection they induce remain unknown. A single vaccine is unlikely to protect against all serogroup B disease and a more realistic goal is a vaccine which protects against a significant proportion of strains, hence reducing the burden of disease.

1.7. Opa

The Opacity-associated adhesin (Opa) proteins are one of the major proteins found in the outer membrane of pathogenic Neisseria (figure 1.1). Meningococci and gonococci can express up to four or eleven Opa proteins, respectively, encoded at loci dispersed throughout the genome[156, 197, 198]. Their name is derived from the fact that their expression often correlates with increased opacity of agar-grown bacterial colonies when viewed by oblique sub-stage lighting[198, 199]. Opa proteins play a critical role in meningococcal pathogenesis by mediating bacterial adherence to the nasopharynx and modulating human cellular immunity via interactions with T cells and neutrophils[200, 201].

1.7.1. Expression of opa *genes*

opa genes are constitutively transcribed, but Opa protein expression undergoes PV at the translational level due to the presence of the pentameric coding repeat (CR) sequence 5'-CTCTT-3', which encodes a portion of the amino-terminal leader peptide[202]. Modulation of the number of repeats occurs by slipped-strand mispairing during DNA replication, resulting in regular frameshifting (figure 1.5). The frequency of PV in gonococci is estimated to be approximately 10^{-3} per cell per generation, and occurs independent of homologous recombination[203]. In addition, promoter strength influences PV of gonococcal *opa* genes, as well as regulating Opa expression levels[204]. As a result, any given bacterial population can include bacteria expressing none, one or multiple Opa proteins at varying levels of expression. The ability of bacteria to exist in the absence of Opa proteins is presumably a key mechanism of immune evasion, as Opa proteins are known to induce antibodies following meningococcal infection and after immunisation with vaccines containing outer membrane complexes or vesicles[148, 205, 206]. Moreover, transparent Opa$^-$ variants of *N. gonorrhoeae* predominate during menstruation but represent a minority of bacteria at other times[207].

Figure 1.5 Control of Opa protein phase variation.
Nucleotide and protein sequences from *opaA* of *N. meningitidis* strain H44/76 from the ATG start codon until the start of the mature protein (arrow). Differences in the number of coding repeat (CR) sequences determine whether the protein is successfully expressed. If the number of CR sequences is a multiple of three the protein sequence is in frame.

1.7.2. Structure of Opa proteins

Opa proteins are predicted to contain eight anti-parallel beta strands forming a barrel structure in the bacterial outer membrane, linked by four extracellular loops (figure 1.6)[208]. Loops 1, 2 and 3 contain variable regions[209], and sequence diversity in these regions confers specificities for host receptors. Sequence variation is observed predominantly within loops 2 and 3, which have been termed hypervariable (HV1 and HV2, respectively). Loop 1 contains some structural similarities between Opa proteins and is termed semivariable (SV).

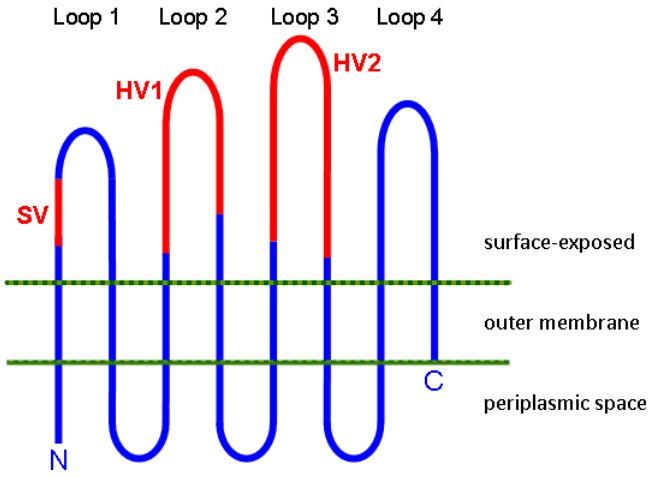

Figure 1.6 Predicted two-dimensional structure of an Opa protein.
The semivariable (SV) region is located in loop 1. The hypervariable regions HV1 and HV2 are within loops 2 and 3, respectively.

Receptor tropism of Opa proteins can be broadly divided into two categories. The first, which is represented by a relatively small number of Opa variants, bind to HSPGs[210-212] and ECM proteins, such as vitronectin and fibronectin[213, 214]. Most Opa variants instead bind to the human CEACAM family of receptors expressed by a variety of cell types, including epithelial cells, neutrophils, and T and B cells. CEACAM1, CEACAM3, CEA and CEACAM6 have all been shown to bind to

neisserial Opa proteins, whereas CEACAM4, CEACAM7 and CEACAM8 do not[212, 215-220]. Haplotypic diversity in CEACAM3 and CEACAM6 has been associated with susceptibility to meningococcal disease, suggesting that binding of these molecules is involved in modulation of the immune response[221].

1.7.3. Molecular interactions between Opa and CEACAM

CEACAM family members are differentially recognised by Opa protein variants, so the distribution of each CEACAM has the potential to influence the cellular tropism of Neisseria expressing different Opa proteins *in vivo*[212, 214, 216, 218, 219, 222]. Furthermore each CEACAM mediates different cellular processes[223-225], suggesting that the cellular response to neisserial binding will depend upon the specific combination of CEACAMs engaged. Opa-CEACAM interactions occur most effectively with acapsulate organisms[212, 222], although Opa-mediated adhesion of apparently fully encapsulated bacteria to transfected cells expressing high levels of CEACAM1 has been demonstrated[226-228].

Binding of CEACAM by Opa proteins requires a conformational interaction between both HV regions of Opa, whereas the SV region appears dispensable[229]. Deletion of the conserved fourth loop resulted in a severe decrease in Opa expression, so any specific effect it may have on CEACAM binding has not been tested. Antibodies directed against the HV2 region of a gonococcal Opa protein inhibited interactions with neutrophils[230] and two meningococcal Opa variants differing only in their HV1 regions varied in their interactions with CEACAM3 and CEACAM6[212], consistent with the importance of both HV1 and HV2 regions. Chimeric Opa variants,

containing combinations of HV regions derived from different CEACAM-binding Opa proteins, lost most of their receptor binding activity, however, suggesting a specific interaction between HV1 and HV2 is necessary for CEACAM binding[229].

The high degree of sequence variation in the HV regions of Opa proteins raises the question of how the binding sites for CEACAM are conserved. *N. meningitidis* strain H44/76 possesses four different Opa proteins, of which OpaA and OpaJ bind to CEACAM1, while OpaB and OpaD bind to CEACAM1 and CEA. A sequence motif involved in binding to CEACAM1 was identified by alanine scanning mutagenesis of conserved amino acid residues within the HV regions of all four Opa proteins. Hybrid Opa variants with different combinations of HV1 and HV2 showed reduced binding to CEACAM1 and CEA, and strongly suggested the existence of a conserved binding site for CEACAM by the interaction of HV1 and HV2 regions. This included a relatively conserved binding motif in HV2, consisting of Gly_{172}, Ile_{174} and Gln_{176}[231]. Similar motifs can be found in almost all CEACAM-binding Opa variants, suggesting that the receptor binding contacts are conserved in spite of the high degree of Opa sequence variation or that these residues are integral to the functional conformation of all Opa variants.

Opa-expressing isolates of meningococci and gonococci bind to the CEACAM N-domain, which is largely conserved between members of the CEACAM family, and binding occurs to a region within the N-terminal 108 amino acids[217, 220, 222, 226]. Recognition is mediated by the CEACAM protein backbone and not by glycosylated moieties, so differences in glycosylation should not influence Opa-CEACAM

mediated responses[226, 232]. Using chimeric constructs of different N-domains, distinct binding regions have been identified for specific groups of Opa variants, which are therefore likely to dictate differential recognition of CEACAM by Opa. The binding surface of the CEACAM1 N-domain is the non-glycosylated face composed of the beta strands C'', C', C, F and G, and similar structures exist on the other Opa-binding receptors of the CEACAM family. This is the region of highest sequence divergence between human and animal CEACAMs[233], which may partly explain why Neisseria are obligate human pathogens, and confirms the difficulty of examining these interactions in animal models. Opa proteins bind to exposed residues of the CC'FG face, and binding appears to require Tyr_{34} and Ile_{91}[212]. Further efficient interaction of distinct Opa proteins depends on different non-adjacent amino acids which spatially lie in close proximity to Tyr_{34} and Ile_{91}, making continuous conformational binding domains. Variation of an amino acid triplet sequence (residues 27-29) between different CEACAMs is responsible for the differential binding of Opa proteins to CEACAM1, CEA and CEACAM6[220] .

CEACAM1 and CEACAM3 have different transmembrane and cytoplasmic domains, and CEA and CEACAM6 are glycosylphosphatidylinositol (GPI)-anchored, so it is likely that distinct processes mediate neisserial uptake following Opa protein binding to each of these receptors. CEACAM-dependent bacterial engulfment has been compared using a panel of transfected HeLa epithelial cell lines, each expressing a different CEACAM[225]. CEACAM1 and CEACAM3 each contain proteinaceous transmembrane and cytoplasmic domains, but gonococcal uptake mediated by these receptors differed with respect to their susceptibility to tyrosine kinase inhibitors and

the actin microfilament-disrupting agent cytochalasin D, indicating the stimulation of different cell signalling pathways. Bacterial uptake mediated by CEA was reduced by cleavage of the GPI anchor by phosphatidylinositol-specific phospholipase C after bacterial binding, consistent with a single zipper-like mechanism[225]. Regardless of the CEACAM expressed by the transfected HeLa cells, internalised gonococci were effectively killed by a microtubule-dependent process that required acidification of the bacterium-containing phagosome. A more recent study utilised a CEACAM-deficient cell line which was transfected with different CEACAMs. Bacterial internalisation via CEACAM1 or CEA, which are present within cholesterol-rich membrane microdomains on the epithelial cell surface, was inhibited by cholesterol-chelating agents, unlike CEACAM3-mediated internalisation[224]. Given the phase variable nature of Opa proteins, these results indicate that the mechanism of bacterial engulfment and the cellular response to infection depend on the receptor specificities of the Opa variants expressed and the spectrum of CEACAM present on target cells.

1.7.4. Effects of Opa-CEACAM interactions during meningococcal infection

Opa proteins mediate bacterial attachment to and invasion of nasopharyngeal epithelial cells via binding to HSPG and CEACAM[210, 211, 234]. Initial attachment between meningococci and epithelial cells is mediated by pili. Pilus retraction then allows tight secondary binding via an interaction between Opa proteins and HSPG. Upregulation of CEACAM, which can occur in response to pro-inflammatory cytokines, could subsequently lead to translocation of bacteria across the mucosal surface and into the bloodstream to cause a rapid onset of disseminated disease. This suggests a rationale for observations that individuals with prior mucosal

inflammation, such as respiratory tract infections, carry an increased risk of IMD. The pro-inflammatory cytokine TNFα also stimulates CEACAM1 expression on human umbilical vein endothelial cells, leading to an increase in Opa-dependent bacterial binding and invasion[215]. Gonococcal Opa proteins and binding of LPS to TLR4 elicit secretion of pro-inflammatory cytokines, providing mechanisms for meningococci to potentiate invasive disease[235, 236].

The response of T cells, and particularly $CD4^+$ T cells, is important during infection with pathogenic Neisseria as these cells are involved in directing the magnitude and quality of humoral immune response. There is good evidence that antibody directed against surface structures of *N. meningitidis* are important in immunity but gonococci do not induce a strong, protective antibody response following infection[237]. T cells are also important in generation of immunological memory and possibly cell-mediated immunity, so these responses are also important to meningococcal vaccine design. There are conflicting data regarding the effect of meningococcal Opa proteins on $CD4^+$ T cells, with both stimulatory and inhibitory effects described.

Studies comparing the meningococcal proteins PorA, PorB, Opa and Opc showed that Opa induced the strongest T cell proliferative responses and was more immunogenic for T cells than the other OMPs[238, 239]. Recently live meningococci, heat-killed bacteria and meningococcal OMVs have been shown to stimulate $CD4^+$ T cell proliferation, independent of their Opa phenotype[240]. Moreover, several OMV vaccines, containing variable amounts of Opa, have been proven to be immunogenic in humans[91, 114, 116, 117, 119]. In other studies, when $CD4^+$ T cells were exposed to

OMVs from Opa-expressing meningococci, their activation and proliferation in response to a variety of stimuli were effectively halted via Opa binding to CEACAM1[241]. This effect was specific to CEACAM1-binding Opa variants, and was not observed with non-CEACAM1 binding Opa, or Opa-negative preparations, including OMVs from Opa^- variants of *N. lactamica*. To further support this, gonococcal Opa proteins on the surface of whole bacteria were able to suppress activation and proliferation of $CD4^+$ T cells via binding to CEACAM1[242]. Infection of $CD4^+$ T cells with gonococci expressing the HSPG-specific Opa_{50} resulted in increased expression of the activation marker CD69, which did not occur during infection with bacteria containing the CEACAM1-binding Opa variants. This down-regulating effect was accompanied by a reduced proliferative response of $CD4^+$ T cells to activation stimuli such as IL-2 and/or T cell receptor (TCR) ligation. These effects resulted from a specific arrest in cell division, rather than from infection-induced cytotoxicity[242].

A suggested mechanism by which Opa proteins could inhibit T cells is attributed to the co-inhibitory function of CEACAM1. Activation of the TCR results in stimulation of Src kinase, which phosphorylates the cytoplasmic tyrosine residues of cell surface-expressed CEACAM1 that is bound by Opa[243]. The phosphorylated immunoreceptor tyrosine based inhibition motif (ITIM) recruits activated tyrosine phosphatases SHP-1 and SHP-2, which effectively oppose the activation response[243-245] by increasing the activation threshold of the T cells. Overwhelming stimulus can, therefore, overcome the co-inhibitory effect of CEACAM1[240, 242]. As always, it is the relative levels of activation and inhibitory signals that determines the cellular response (figure 1.7). It is

pertinent to note that the phospho-ITIM dependent recruitment of SHP-1 upon Opa binding to CEACAM1 also explains the bacteria's ability to suppress TLR-2 dependent innate responses in epithelial cells[246], suggesting that both adaptive and innate immune mechanisms may be depressed.

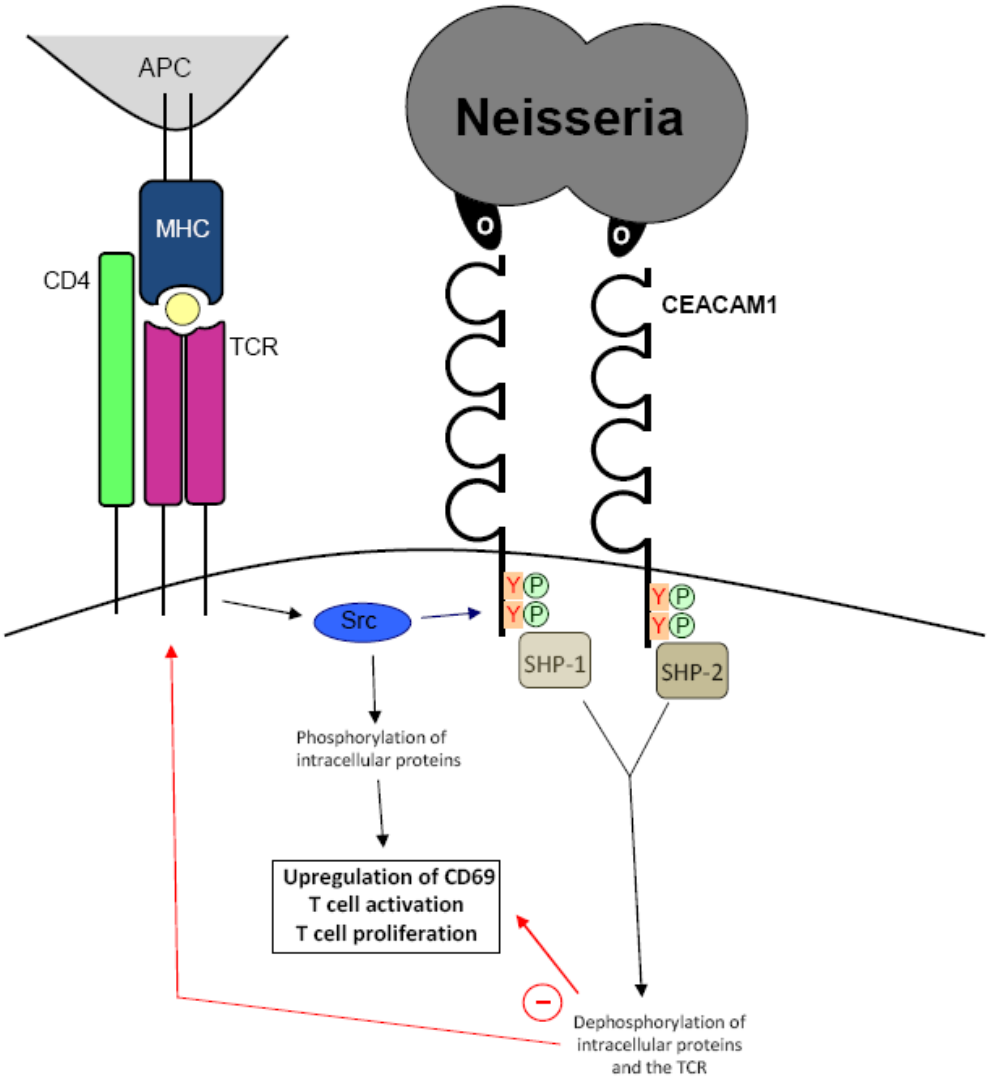

Figure 1.7 Possible mechanism of Opa-mediated inhibition of $CD4^+$ T cells via binding to CEACAM1.
Activation of the T cell receptor (TCR) by binding to an antigen presented with MHC class II by an antigen presenting cell (APC) results in stimulation of Src kinase. This leads to tyrosine phosphorylation of CEACAM1 as well as activation of downstream signalling pathways, resulting in T cell activation. Binding of CEACAM1 by Opa (O) allows recruitment of tyrosine phosphatases SHP-1 and SHP-2 to the ITIM, which dephosphorylate CEACAM1, the TCR and other intracellular proteins, inhibiting T cell activation.

There may be a number of reasons to explain the contrasting published data. Methods differed with regards to the antigens (proteins, OMVs and bacteria) and human cells (peripheral blood mononuclear cells (PBMCs) or purified $CD4^+$ T cells) used. Furthermore, different studies included different Opa variants and different strains of *N. meningitidis*. It is not clear which of the *in vitro* conditions used reflects the situation that occurs *in vivo* in infected patients or immunised individuals, although it is known that recognition of antigens by T cells requires accessory cells (such as monocytes) and that the activation of many T cell functions and responses depends on their interaction with antigen presenting cells. It is possible that purified or recombinant protein may have a different effect to Opa expressed on the bacterial surface, and differential expression of other surface components due to PV may also have influenced T cell responses. If Opa does indeed down-regulate immune responses *in vivo*, the deletion of Opa from potential vaccine preparations could improve immunogenicity of OMVs. Previous clinical trials of OMV vaccines, which contained variable amounts of Opa, have already demonstrated that these vaccines are immunogenic, and that most of the antibody response is directed against PorA[91, 114, 119, 120, 148-150]. There is significant variation in anti-Opa antibody responses, both between studies and among individuals within studies[205, 247, 248]. Given the heterogeneous nature of OMV vaccines, it is difficult to verify the key determinants of this response, and therefore the significance of Opa proteins contained in the vaccines. Any proposed OMV or protein-based meningococcal vaccine would need to demonstrate its ability to elicit bactericidal antibodies in clinical trials to be considered for licensure. Further investigation of this interaction is necessary, and an ideal *in vitro* test should aim to mimic *in vivo* conditions as closely as possible.

Opa has been demonstrated to inhibit antibody production from human B cells which have been induced to express CEACAM1 by IL-2 and then stimulated[249]. The interaction of *N. gonorrhoeae* and CEACAM1 on human peripheral B cells also resulted in induction of cell death. The same findings were observed in the DT40 B cell line. This CEACAM1-promoted cell death pathway did not involve the inhibitory signals or SHP-1 and SHP-2, but were dependent on Bruton's tyrosine kinase. This observation led the authors to suggest that Opa-CEACAM1 interactions could suppress immunoglobulin expression by killing B cells[249], however it is important to consider that CEACAM1 binding is not generally considered to deliver apoptotic signals in all contexts[250]. It is, therefore, equally plausible that Opa-dependent association of these bacteria with B cells may contribute to activation-induced cell death rather than death being a specific consequence of CEACAM1-dependent signals. Opa expression has also been found to enhance resistance against complement-mediated killing, although the exact mechanism of this is unclear[251]. It may be that Opa protein expression reduces antibody production or blocks deposition on the bacterial cell surface, or it could be a direct inhibitory effect on one or more components of the complement cascade.

The majority of Opa-expressing Neisseria interact with neutrophils, hence their original name of leucocyte association proteins[252]. Opa mediates intimate attachment of meningococci to neutrophils, as well as subsequent non-opsonic phagocytosis and stimulation of the bactericidal oxidative burst response[216, 217, 230, 253-259]. The primary Opa receptor on neutrophils appears to be CEACAM3[260], although CEACAM1 is also

important[216]. Phagocytosis by and stimulation of neutrophils would seem to confer a selective disadvantage to Opa-expressing bacteria, which would be killed by the bactericidal response. Phagocytosis of large numbers of bacteria, however, may facilitate bacterial dissemination. Alternatively, binding of outer membrane blebs which are naturally released during neisserial infection may result in activation of neutrophils and diversion of the immune response. This lends credence to the proposal that neutrophils use CEACAM3 as a decoy to capture bacteria expressing Opa adhesins intended to allow colonisation of other cell types[261, 262].

1.7.5. Population diversity of Opa

There is a high level of diversity of Opa proteins, generated by mutation within and genetic recombination between *opa* loci, both among loci in the same chromosome and different organisms (figure 1.8)[199, 263]. Neisseria are naturally highly transformable bacteria, having the ability to take up extracellular DNA efficiently, and then incorporate it into the chromosome by homologous recombination[264]. This mechanism may have evolved to aid in the repair of damaged chromosomes, as bacteria are most likely to encounter DNA from nearby cells of the same species, but transformation is also a powerful mechanism for generating genetic diversity by horizontal gene exchange. In addition, the presence of multiple *opa* genes allows genetic recombination between loci in the same chromosome, as well as between different organisms. These recombination events can involve exchange of entire or partial *opa* genes. Finally, mutations can occur within *opa* loci during bacterial growth as a further source of diversity. In the case of *opa* genes, intra- and inter-genomic recombination events are much more likely to occur than mutations in both

N. meningitidis and *N. gonorrhoeae*[199, 265, 266]. This was recently quantified in a study of gonococci, which estimated that 77% of diversity was due to recombination between genes of the same isolate, a further 16% due to import of genes from other isolates, and only 7% due to *de novo* mutation[266].

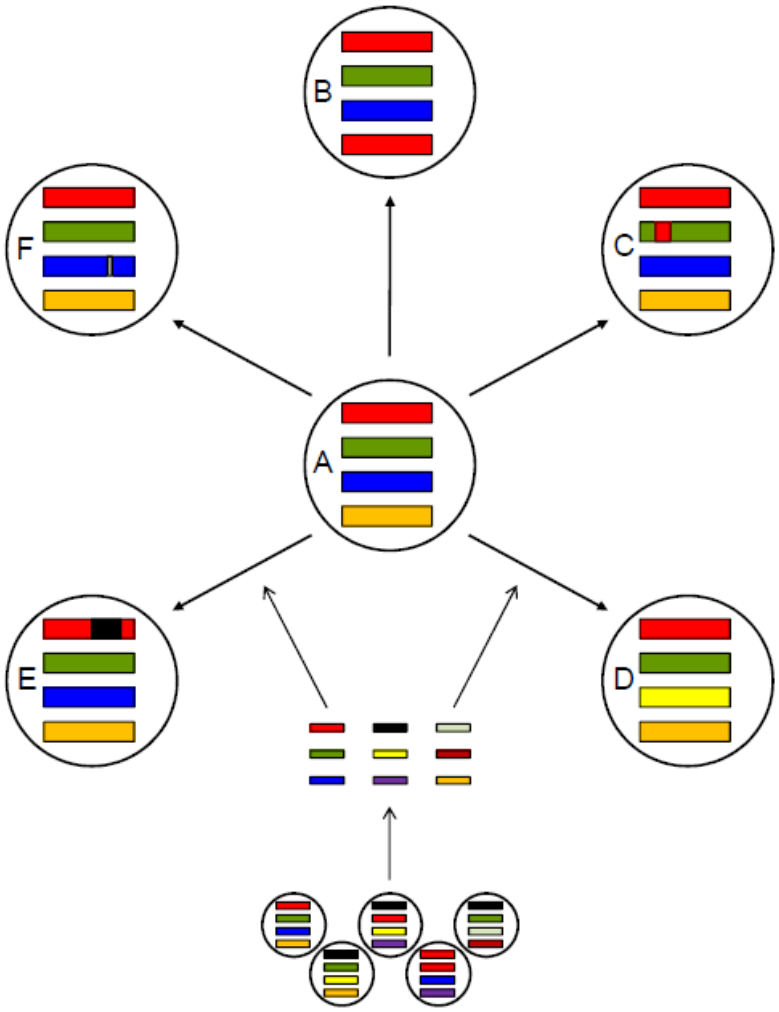

Figure 1.8 Mechanisms of genetic variation of neisserial *opa* genes.
Population diversity of *opa* genes can be generated by recombination or, less frequently, mutation. In this illustration, the initial strain (A) contains four different *opa* genes depicted in different colours. Intra-genomic recombination can result in duplication and replacement of an entire *opa* gene (B), or part of a gene (C). Alternatively, inter-genic recombination can occur when the bacteria imports exogenous DNA from death or damage of bacteria in the local environment. This can also result in total (D) or partial (E) replacement of one of the *opa* genes. Homologous recombination is more likely at the same locus due to the presence of similar upstream and downstream sequences, but can also occur at a different locus. Finally, new variants can also occur by *de novo* mutation (F). Although the figure shows *N. meningitidis*, with four *opa* genes, the same principles also apply for *N. gonorrhoeae*, which typically contain 11 *opa* genes.

One of the difficulties in quantifying *opa* diversity has been the lack of a standardised nomenclature for describing alleles[32]. The Opa sequence database sited at the University of Oxford was developed in part to standardise Opa classification. There are currently 338 distinct *opa* alleles in the database (www.neisseria.org, accessed 10/03/2011), and this variability had resulted in Opa proteins being dismissed as potential vaccine candidates. These include 26 different SV regions, 96 HV1 variants and 127 HV2 sequences. If all possible combinations of these variable regions existed there would be over 300,000 *opa* alleles, suggesting restriction in permissible combinations which may be due to stability of the resulting protein structures or a survival advantage of certain variants.

The majority of IMD has been caused by isolates from only a small number of clonal complexes, known as the hyperinvasive lineages (section 1.3). Analysis of the distribution of *opa* alleles within these clonal complexes revealed significant structure to the diversity, such that a limited number of Opa variants were associated with hyperinvasive meningococci globally over the last 60 years (table 1.3)[32, 267]. The association of particular Opa variants with individual clonal complexes over decades of global spread could be maintained if specific combinations of Opa proteins confer fitness advantage for transmission between hosts.

Isolate	Serogroup	Year	Country	ST	*opaA*	*opaB*	*opaD*	*opaJ*
H44/76	B	1976	Norway	32	96	288	147	218
NG 80	B	1981	Norway	32	96	288	71	218
NG144/82	B	1982	Norway	32	96	313	147	218
BZ 83	B	1984	The Netherlands	34	96	335	147	218
BZ 169	B	1985	The Netherlands	32	96	185	147	218
EG 329	B	1985	East Germany	32	96	185	147	218
NG PB24	B	1985	Norway	32	96	185	185	218
196/87	C	1987	Norway	32	96	185	147	218
8680	B	1987	Chile	32	96	185	147	218
204/92	B	1992	Cuba	32	261	185	147	322

Table 1.3 *opa* gene repertoire of 10 hyperinvasive meningococci from the ST-32 clonal complex. Numbers represent the allele number for each *opa* gene according to the *Neisseria meningitidis* Opa sequence database at http://neisseria.org/nm/typing/opa. Similar patterns are found for other hyperinvasive lineages. *(Adapted from Callaghan* et al.[32]*)*

Opa diversity may reflect different functions of different Opa variants, or could be the result of immune selection pressure, as has been found for other meningococcal outer membrane proteins, such as PorA[268]. Analysis of 216 meningococcal carriage isolates from the Czech Republic revealed the presence of discrete, non-overlapping *opa* repertoires with low within-repertoire diversity, consistent with a model of strong immune selection where identical alleles can occupy different loci[267]. This also supports a theory that *opa* diversity is greater in gonococci because there is less immune pressure due to the lower prevalence of the organism in the population. However, there are no similar studies of gonococcal *opa* diversity and, conversely, it is also possible that *opa* diversity in gonococci is greater because of greater immune pressure relating to differences in transmission of the two organisms. *N. meningitidis* can infect most young children and therefore has a continuously replenishing population of naïve hosts. Sexually transmitted infections, such as gonorrhoea, however, persist by repeated infections in a relatively small 'core group' of sexually active or risky individuals (e.g. commercial sex workers). It is therefore critical that they can continue to evade the immune response to survive in the population, requiring increased *opa* diversity. Alternatively, functionally important Opa proteins

may be conserved to optimise meningococcal adhesion to host cells and interaction with the immune system. Further information on *opa* diversity and immune responses against both Neisseria species, in particular *N. gonorrhoeae*, would be required to test these hypotheses.

1.8. Opa proteins as a serogroup B meningococcal vaccine candidate

Anti-Opa IgG antibodies, including bactericidal antibodies, have been demonstrated in patients following meningococcal infection and in recipients of serogroup B OMV vaccines, suggesting that Opa proteins are immunogenic in humans. Nasal immunisation of mice with liposomes containing Opa resulted in production of anti-Opa IgA antibodies in nasal lavages, and in some cases anti-Opa IgA and IgG in lung lavages[269]. Significant SBA titres were obtained with one of three Opa proteins investigated. More recently, subcutaneous immunisation of mice with a panel of 14 different Opa proteins elicited high levels of meningococcal-specific bactericidal antibodies, demonstrating proof in principle of this approach[181]. Bactericidal activity against Opa is thought to be mediated by epitopes within the HV1 and HV2 regions[209].

Opa proteins provide a number of advantages as a potential novel vaccine candidate:

(i) It is one of the major meningococcal outer membrane proteins;

(ii) Diversity of the *opa* repertoire is highly structured, suggesting that a relatively small number of Opa protein variants could provide broad protection against serogroup B meningococcal disease. Examination of the HV regions from a collection of 227 isolates from IMD in the UK between 1996 & 2001[221] gives

a theoretical coverage of 90% with a vaccine containing the six most common Opa variants[181];

(iii) There is the potential to elicit adhesion-blocking antibodies which could lead to herd immunity and significantly enhance vaccine effectiveness at the population level;

(iv) The inclusion of multiple antigens in the form of multiple Opa proteins reduces the risk of vaccine failure by the generation of escape variants;

(v) Different Opa proteins on the meningococcal surface may be targeted by different antibodies following vaccination, which may act synergistically to increase complement activation and hence bacteriolysis;

(vi) Use of a sub-capsular antigen provides an opportunity for protection from disease caused by other serogroups. It also prevents escape from vaccine-induced immunity by capsule switching, which has been described for meningococci[270];

(vii) Unambiguous characterisation of protein vaccines is possible, unlike OMV vaccines;

(viii) Recombinant protein vaccines can easily be adjusted to overcome changes in epidemiology, which can be detected by ongoing surveillance.

The interaction between meningococcal Opa proteins and host cell receptors, specifically CEACAM receptors need to be more fully understood before Opa proteins can be included as a component in a future vaccine. Determining the effects on the adaptive immune response are particularly important as many previous studies

have involved *N. gonorrhoeae* rather than *N. meningitidis*, and there are conflicting data regarding the effect on of Opa proteins on T cells.

1.9. Aims and objectives

The overall aim of this study was to provide data to evaluate the potential of a serogroup B meningococcal vaccine based on Opa proteins. The immune response to Opa proteins on the meningococcal surface were investigated, considering implications for vaccine design and pathogenesis. Specific objectives were:

(i) to construct a set of isogenic mutant meningococci which expressed different Opa proteins, and an Opa-negative mutant;

(iii) to assess the role of surface-expressed Opa proteins on particular aspects of human cellular immunity *in vitro* as an immunomodulator of importance in meningococcal pathogenesis, to assist vaccine formulation;

(iv) to investigate PV of meningococcal Opa proteins using the isogenic mutant strains, to provide further insights into the role of Opa proteins during infection with *N. meningitidis*.

CHAPTER 2. MATERIALS AND METHODS

2.1. Growth and storage of bacteria

2.1.1. Bacterial growth media and supplements

Escherichia coli was grown in Luria-Bertani (LB) media containing 10g/l tryptone (Fluka), 5g/l yeast extract (Fluka) and 4g/l sodium chloride (NaCl) (VWR). *N. meningitidis* was grown on brain heart infusion (BHI) media containing 37g/l BHI (Merck). Gol freezing broth for long-term storage of *N. meningitidis* contained BHI (1% w/v) and glycerol (10% w/v) (Sigma-Aldrich). For LB agar or BHI agar, the relevant broth was supplemented with agar (1.5% w/v) (Bioconnections). All media was sterilised by autoclaving and stored at room temperature (RT), or at 4^{o}C after addition of antibiotics when appropriate. Agar was melted and cooled to 50^{o}C before any required antibiotics or supplements were added prior to pouring into petri dishes. Agar plates were allowed to dry and stored at 4^{o}C for a maximum of one month.

All BHI agar was supplemented with 1ml Levinthal's base per 10ml media. Levinthal's base was prepared by adding 500ml of defibrinated horse blood (TCS Biosciences) to 1 litre of BHI broth and heating in a water bath at 95^{o}C for 40 minutes with occasional stirring. This was cooled to RT and subjected to centrifugation at 1,900 x *g* for 25 minutes at 4^{o}C. The supernatant was decanted and stored at -20^{o}C.

Antibiotics, and supplements (Sigma-Aldrich) were prepared and used at the concentrations given in table 2.1. They were all sterilised by filtration through a 0.2μm polyethersulfone membrane and stored at -20^{o}C.

Antibiotic/ supplement	Stock concentration	Final concentration in media	
		LB (*E. coli*)	BHI (*N. meningitidis*)
Ampicillin	100mg/ml‡	100µg/ml	100µg/ml
Kanamycin	50mg/ml‡	100µg/ml	100µg/ml
Erythromycin	50mg/ml§	300µg/ml	5µg/ml
Tetracycline	12mg/ml§	12µg/ml	2µg/ml
X-gal*	10mg/ml¶	20µg/ml	–
IPTG†	2% w/v‡	0.004% w/v	–

Table 2.1 Antibiotics and supplements used during growth of bacteria.
*X-gal = 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside;
†IPTG = isopropyl β-D-1-thiogalactopyranoside; ‡prepared in ultrapure water (Milli-Q); §prepared in 50% ethanol; ¶prepared in N, N-dimethylformamide (DMF)

2.1.2. Growth and storage of Escherichia coli

E. coli strains were stored at -80^{o}C as a thick suspension in LB broth containing glycerol (16% v/v). Bacteria were revived from storage by either inoculating into 5ml of LB broth or plating onto LB agar, with or without antibiotic and supplements as appropriate. Broth cultures were incubated at 37^{o}C, 220rpm for 16-18 hours and agar plates were incubated at 37^{o}C for 16-18 hours. If required, grown *E. coli* cultures (in broth or agar) were stored at 4^{o}C for up to one month before stock cultures were prepared from re-growth.

2.1.3. Growth and storage of Neisseria meningitidis

N. meningitidis strains were stored at -80^{o}C as a thick suspension in Gol freezing broth. Bacteria were revived from storage by plating onto BHI agar, with or without antibiotic and other supplements as appropriate, and incubating at 37^{o}C, 5% CO_2 for 16-18 hours.

2.1.4. Estimating concentration of Neisseria meningitidis *in suspension*

The concentration of bacteria was estimated by measuring the concentration of bacterial DNA. Cells were lysed by diluting 1:100 with a solution containing sodium dodecyl sulphate (SDS) (1%) and sodium hydroxide (0.1M). The DNA concentration was determined by measuring the optical density at 260nm, and using the following equation:

$$C = \frac{A260 \times 10^{11}}{t}$$

where C = bacterial concentration (colony forming units/ml), A260 = optical density at 260nm and t = 1.8 where bacteria have been grown for 16-18 hours and t = 2.2 where bacteria have been grown for 4-8 hours.

2.1.5. Inactivation of Neisseria meningitidis

N. meningitidis was inactivated using either heat or ethanol prior to use in immunological assays (section 2.11). For both methods, bacteria were first grown on BHI agar at 37^{o}C, 5% CO_2 for 16-18 hours. Cells from the entire plate were scraped and resuspended in 2ml of phosphate buffered saline (PBS), and the concentration of bacteria in the suspension was estimated by measuring the concentration of bacterial DNA in the sample (section 2.1.4). To inactivate bacteria using heat, the suspension was incubated at 56^{o}C for 1 hour. When ethanol was used, 4.7ml of 100% ethanol was added to the suspension to obtain a final concentration of 70% ethanol. To validate inactivation following either method, 50µl of the suspension was plated onto BHI agar and incubated at 37^{o}C, 5% CO_2 for 16-18 hours. Inactivated bacteria were stored at 4^{o}C for upto 12 months.

2.2. Transformation of *Escherichia coli*

2.2.1. Preparation of competent Escherichia coli *cells*

Chemically competent cells of *E. coli* strain DH5α were prepared using calcium chloride, based on the method of Cohen *et al.*[271, 272]. *E. coli* cells were grown in 5ml LB broth by incubating at 37°C, 220rpm for 16-18 hours, and 125µl or 250µl was inoculated into 25ml LB broth. These cultures were incubated at 37°C, 220rpm until the optical density at 650nm had reached a value between 0.2 and 0.5. The cultures were then chilled on ice for 10 minutes. Cells were recovered by centrifugation at 900 x *g* for 10 minutes at 4°C, resuspended in 10ml ice-cold magnesium chloride (0.1M) and incubated on ice for 10 minutes. Cells were again recovered by centrifugation at 900 x *g* for 10 minutes at 4°C, resuspended in 5ml ice-cold calcium chloride (0.1M) and incubated on ice for a further 10 minutes. Cells were finally recovered by centrifugation at 900 x *g* for 10 minutes at 4°C and resuspended in 2ml ice-cold calcium chloride (0.1M). Cell suspensions from different cultures were pooled and incubated on ice at 4°C for 1-24 hours to further increase competency. Competent cells were stored at -80°C in glycerol (16% v/v). For some transformation reactions, different *E. coli strains* were used (Invitrogen) (table 2.2).

E. coli strain	Genotype
DH5α	F⁻ Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (r_K^-, m_K^+) *pho*A *sup*E44 λ⁻ *thi*-1 *gyr*A96 *rel*A1
DH10B	F⁻ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80d*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L *nup*G λ⁻
Mach1-T1	F⁻ Φ80*lac*ZΔM15 Δ*lac*X74 *hsd*R(r_K^-, m_K^+) Δ*rec*A1398 *end*A1 *ton*A
OmniMAX 2-T1	F´ {*pro*AB+ *lac*I^q *lac*ZΔM15 Tn10(Tet^R) Δ(*ccd*AB)} *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *end*A1 *rec*A1 *sup*E44 *thi*-1 *gyr*A96 *rel*A1 *ton*A *pan*D
Stbl2	F⁻ *mcr*A Δ(*mcr*BC-*hsd*RMS-*mrr*) *rec*A1 *end*A1 *lon gyr*A96 *thi sup*E44 *rel*A1 λ⁻ Δ(*lac-pro*AB)
Stbl3	F⁻ *mcr*B *mrr hsd*S20 (r_B^-, m_B^-) *rec*A13 *sup*E44 *ara*-14 *gal*K2 *lac*Y1 *pro*A2 *rps*L20 (Str^R) *xyl*-5 λ⁻ *leu mtl*-1
TOP10	F⁻ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara leu*) 7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G

Table 2.2 Genotypes of *E. coli* strains (Invitrogen) used for transformation reactions.

2.2.2. Transformation of chemically competent Escherichia coli

A standard method for transformation of *E. coli* was used, and is based on the observation that bacteria treated with ice-cold calcium chloride and then briefly heated could be transfected with bacteriophage λ DNA[273]. DNA to be transformed was chilled on ice in a sterile 2ml screw-cap tube. Competent *E. coli* cells were thawed from -80^{o}C on ice. For each transformation, 200µl *E. coli* was added to the DNA and mixed gently by swirling with a pipette tip. Cells were incubated on ice for a minimum of 45 minutes before being subjected to heat shock in a water bath at 42^{o}C for 2 minutes and incubation on ice for a further 2 minutes. Following the addition of 500µl 2x LB broth, pre-warmed to 37^{o}C, the cells were incubated at 37^{o}C, 220rpm for 1 hour. Cells were recovered by centrifugation at 16,100 x *g* for 5 minutes and resuspended in 50-100µl LB broth. Between 20µl and 50µl cells was plated onto selective LB agar and incubated at 37^{o}C for 16-18 hours, or 36-40 hours when selecting for erythromycin resistance.

2.2.3. Screening of transformed Escherichia coli

Following transformation, single *E. coli* colonies were picked and plated onto one section of a selective LB agar plate divided into up to 100 sections. Plates were incubated at 37^{o}C for 16-18 hours. Plasmid DNA was purified from at least 6 colonies per transformation (section 2.7.1) for further screening by restriction enzyme digestion (section 2.9.2) to confirm successful transformants. If transformation resulted in fewer than 6 colonies, all were screened. Once the required plasmid had been identified, stocks were made from the corresponding *E. coli* colony (section 2.1.2). If more than one such plasmid was identified then stocks from two different colonies were stored.

2.3. Transformation of *Neisseria meningitidis*

2.3.1. Transformation reactions

N. meningitidis was transformed using the spot transformation technique which can be used for transformation of both *N. meningitidis* and *N. gonorrhoeae*[274]. Bacteria were grown on BHI agar at 37^{o}C, 5% CO_2 for 16-18 hours. A few individual colonies were picked and resuspended in 100µl of PBS. Transformation reactions typically consisted of 10µl of bacterial suspension and approximately 1µg of plasmid DNA, either linearised by prior restriction enzyme digestion or supercoiled, or 5-10µl of chromosomal DNA, plated over a 1-2cm diameter region on BHI agar. Reactions were incubated at 37^{o}C, 5% CO_2 for 4-8 hours and bacteria were resuspended in 100-200µl PBS prior to screening.

In some cases transformations were carried out using fewer bacterial cells. The concentration of bacteria in the original suspension was estimated by measuring the concentration of bacterial DNA in the sample (section 2.1.4), and the cells were diluted in PBS to achieve a concentration of 2 x 10^8 colony forming units (cfu)/ml. This was serially diluted in PBS, and 10^4-, 10^5- and 10^6-fold dilutions were used for transformation. Reactions were performed as above and incubated at 37^{o}C, 5% CO_2 for 16-18 hours before bacteria were resuspended in 100-200µl PBS prior to screening.

2.3.2. Screening transformed cells for introduction of antibiotic resistance

Bacteria were plated in 50-100µl aliquots onto selective BHI agar containing an appropriate antibiotic. Plates were incubated for 16-18 hours (36-40 hours for the introduction of erythromycin resistance) at 37^{o}C, 5% CO_2. Up to 96 individual *N. meningitidis* colonies were picked, plated onto selective media (8 colonies per plate) and incubated at 37^{o}C, 5% CO_2 for 16-18 hours. For each sample, a 1µl loopful of bacteria was resuspended in Gol freezing broth and stored at -80^{o}C and a further 1µl loopful of bacteria was used for isolation of chromosomal DNA using potassium hydroxide (section 2.7.2). DNA was used to screen samples by polymerase chain reaction (PCR) and further bacterial stocks were made for any desired transformants (section 2.1.3). Following successful transformation, stocks from two different colonies were stored when possible.

2.3.3. Screening transformed cells for removal of antibiotic resistance

The concentration of bacteria in the suspension post-transformation was estimated by measuring the concentration of bacterial DNA (section 2.1.4). Cells were serially diluted in PBS and bacteria were plated onto non-selective BHI agar to achieve 500-1,000 cfu per plate. Plates were incubated at 37^{o}C, 5% CO_2 for 16-18 hours, following which a nitrocellulose membrane (pore size 0.45μm) was used to transfer colonies to selective BHI agar plates containing the appropriate antibiotic. Both sets of plates were incubated at 37^{o}C, 5% CO_2 for 16-18 hours. Paired plates were visually screened to identify colonies which were present on non-selective media only. These colonies were picked, re-plated onto selective and non-selective media and incubated at 37^{o}C, 5% CO_2 for 16-18 hours. Those which continued to grow only on non-selective media were chosen for DNA isolation and further screening, as in section 2.3.2.

2.4. Production and characterisation of meningococcal outer membrane vesicles

2.4.1. Production of outer membrane vesicles

OMVs were produced from *N. meningitidis* using a scaled-down version of the method used to produce OMV vaccines (section 1.6.4)[275]. Bacteria were grown on BHI agar at 37^{o}C, 5% CO_2 for 16-18 hours. A few individual colonies were picked and resuspended in 200-1000μl of PBS, and 25-50μl of this suspension was plated onto BHI agar plates. Following incubation at 37^{o}C, 5% CO_2 for 16-18 hours, bacteria were resuspended in a Tris-EDTA buffer (10mM EDTA, 0.1M Tris-HCl, pH 8.6) to achieve a final ratio of 5ml buffer per 1g bacteria. Sodium deoxycholate (DOC) was added to achieve a final concentration of 0.5% (w/v) and the suspension stirred for 30 minutes at RT to inactivate the bacteria and allow recovery of OMVs released during

normal bacterial growth in addition to those produced as a result of the detergent treatment. Bacterial cell debris were removed by centrifugation at 5,000 x *g* for 30 minutes and the supernatant was transferred to a new tube. To validate the inactivation step, 50µl of this solution was plated onto BHI agar and incubated at 37^{o}C, 5% CO_2 for 16-18 hours; during this time the supernatant was stored at 4^{o}C. If no viable bacteria were detected, further cell debris were removed by centrifugation at 12,000 x *g* for 30 minutes at 4^{o}C. The supernatant was transferred to a new tube, and the OMVs were purified by centrifugation at 125,000 x *g* for 90 minutes at 4^{o}C. After the supernatant was discarded the pellet was washed with 1ml of a buffer containing 2.5% DOC (2.5% DOC, 2mM EDTA, 50mM Tris-HCl, pH 8.9). The pellet was then resuspended in 5ml of the same buffer before 10ml of a DOC-sucrose buffer (0.5% DOC, 30% sucrose, 2mM EDTA, 50mM Tris-HCl, pH 8.9) was added, to achieve a final concentration of 1.2% DOC and 20% sucrose. The solution was transferred to a Dounce tissue grinder and homogenised using 20-30 strokes of the 'tight' pestle. OMVs were finally isolated by centrifugation at 125,000 x *g* for 60 minutes at 4^{o}C. The supernatant was discarded and the OMV pellet washed with 1ml of 3% sucrose before being resuspended in 3% sucrose, using 1ml per 1g of bacteria, aiming for a final total protein concentration of approximately 1mg/ml. A further homogenisation step was performed using a Dounce tissue grinder as above, and the OMVs were stored at 4^{o}C for up to 12 months.

2.4.2. Determination of protein content

The total protein concentration of OMVs was estimated using the total protein micro Lowry kit with Peterson's modification (Sigma-Aldrich), which utilises SDS to

facilitate the dissolution of relatively insoluble lipoproteins[276]. The micro Lowry kit was used according to manufacturer's instructions, using sample volumes of 100µl in a flat-bottomed 96-well plate. OMVs were diluted two-fold between 1:5 and 1:80 in ultrapure water (UPW) (Milli-Q) and compared to a protein standard solution at concentrations up to 400µg/ml. Following the biuret reaction and reduction of the Folin & Ciocalteu's phenol reagent, the absorbance of each sample was read at 620nm. All samples were analysed in duplicate. The concentration of OMVs was estimated by comparison to the calibration curve of the protein standard solution, using values that were within the linear section of the curve.

2.4.3. Sodium dodecyl sulphate polyacrylamide gel electrophoresis

The protein profiles of the OMVs were assessed using SDS-PAGE. The required amount of OMV (measured by total protein content) was mixed 1:1 with Laemmli sample buffer (Bio-rad) and the proteins denatured by incubating at 95^{o}C for 15 minutes in a dry block heater. Samples were loaded into wells of a pre-cast Criterion XT 12% Bis-Tris gel (Bio-rad) and submerged in NuPAGE MES SDS running buffer (Invitrogen). Protein molecular weight was determined by comparison to SDS-PAGE low-range molecular weight standards (Bio-rad) (table 2.3). Standards were diluted 1:10 in UPW, and then mixed 1:1 in Laemmli sample buffer before heating at 95^{o}C for 15 minutes in a dry block heater. Where required, 10µl of this mixture containing approximately 1µg of each protein was loaded into a well adjacent to the sample wells. A voltage of 150V was applied across the gel until the tracking dye was approximately 0.3cm from the bottom of the gel. To visualise the protein bands, the

gel was detached and stained with InstantBlue Coomassie stain (Novexin) on an orbital shaker until a suitable intensity of protein bands was achieved.

Band size (daltons)	Protein
97,400	Phosphorylase b
66,200	Serum albumin
45,000	Ovalbumin
31,000	Carbonic anhydrase
21,500	Trypsin inhibitor
14,400	Lysozyme

Table 2.3 Molecular weights of proteins in SDS-PAGE low-range molecular weight standards (Bio-rad).

2.4.4. Transmission electron microscopy

A negative staining technique similar to that described previously was used[277, 278]. OMVs were diluted to a concentration of 400-800μg/ml, and 6-10μl droplets were added to a carbon mesh grid. Samples were negatively stained with 0.5% phosphotungstic acid (pH 6.8) for one minute. The stain was removed and the samples air-dried prior to examination by transmission electron microscopy (TEM). TEM was carried out by Professor David Ferguson at the Nuffield Department of Clinical Laboratory Sciences, University of Oxford.

2.4.5. Nanoparticle tracking analysis

OMVs were diluted in PBS to a concentration of 2 - 10 x 10^8 particles/ml for nanoparticle tracking analysis (NTA), which was carried out using the Nanosight NS500 system (NanoSight) by Dr Chris Gardiner at the Nuffield Department of Obstetrics and Gynaecology, University of Oxford. The system focuses a laser beam through a suspension of the particles of interest. These are visualised by light scattering, using a conventional optical microscope aligned normally to the beam axis

which collects light scattered from every particle in the field of view. A 60 second video records all events for further analysis by NTA software. The Brownian motion of each particle is tracked between frames, thus allowing calculation of the size through the application of the Stokes–Einstein equation (http://www.nanosight.com/applications/exosomes/exosomes, accessed 16/04/2010)[279].

2.5. Preparation of recombinant Opa proteins

Recombinant Opa proteins were prepared by Dr Claire Jones in the Department of Paediatrics, University of Oxford based on a method which has been previously described[181, 280]. In summary, plasmids containing the relevant *opa* gene cloned into the vector pET22b(+) (Novagen) were first transformed into competent *E. coli* TOP10 (table 2.2). *E. coli* containing the pET22b/opa plasmid was grown in DYT broth supplemented with glucose (20mM) and ampicillin (50μg/ml) and Opa expression induced by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) (1mM). Opa proteins were expressed as inclusion bodies, which were harvested by centrifugation at 3,400 x *g*, and stored in Tris-HCl (50mM, pH 9.0) at -20^{o}C. Inclusion bodies were sonicated and proteins then solubilised in Tris-HCl (50mM, pH 9.0) containing lauryldimethylamine-oxide (LDAO) (5% v/v) before being refolded by adding dropwise into Tris-HCl (50mM, pH 9.0) containing NaCl (250mM) and LDAO (5% v/v). This was dialysed overnight at 4^{o}C against Tris-HCl (50mM, pH 9.0) containing LDAO (0.1%).

Opa proteins were purified by a 2-step chromatography process. Heparin affinity chromatography was performed using a HiTrap Heparin column (GE Healthcare) followed by ion exchange chromatography with a Resource S column (GE Healthcare) equilibrated in sodium acetate (50mM, pH 4.6) containing LDAO (0.1%). Opa protein was eluted by application of a gradient from 0 to 1M NaCl. Fractions of 1ml were collected and analysed by SDS-PAGE (section 2.4.3). Fractions containing Opa proteins were pooled, dialysed into PBS containing LDAO (0.1%) and stored at -20^{o}C.

2.6. Protein blotting

Blotting with anti-Opa mAbs was performed to identify expression of specific Opa proteins on the surface of live or inactivated bacteria, or OMVs. Colony blotting was used to examine Opa expression within colonies of *N. meningitidis*, dot blotting was done with inactivated suspensions of *N. meningitidis* (section 2.1.5), and immunoblotting was performed following SDS-PAGE (section 2.4.3) for analysis of Opa expression in OMVs.

2.6.1. Colony blotting

Bacteria were grown on BHI agar at 37^{o}C, 5% CO_2 for 16-18 hours. A few individual colonies were picked and resuspended in 1ml of PBS. The concentration of bacteria was estimated by measuring the concentration of bacterial DNA in the sample (section 2.1.4). Cells were diluted in PBS to achieve a concentration of 1 x 10^5 cfu/ml, and 15µl or 30µl of this suspension was plated onto BHI agar to achieve approximately 1,500 or 3,000 colonies per plate. Plates were incubated at 37^{o}C, 5%

CO_2 for 16-18 hours, following which a nitrocellulose membrane (pore size 0.45µm) was used to adsorb the colonies. The nitrocellulose membrane was air-dried and then blocked for 1 hour with 5% skimmed milk in PBST (PBS containing 0.1% Tween 20 and 7.7mM sodium azide). Excess buffer was discarded and the membrane washed several times with wash buffer (154mM NaCl, 0.05% Tween 20). All following incubation steps were performed on an orbital shaker at RT in a sterile petri dish using sufficient liquid to completely submerge the membrane. The mAb was added after being diluted as recommended in PBST containing 1% bovine serum albumin (BSA). Following incubation for 1-2 hours, the membrane was washed several times with wash buffer. The membrane was incubated with anti-mouse IgG (Fab specific)-alkaline phosphatase antibody produced in goat (Sigma-Aldrich), diluted 1:10,000 in PBST (without sodium azide) containing 1% BSA, for 1-2 hours. Following further wash steps with wash buffer, alkaline phosphatase activity was detected by the addition of 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) (Sigma-Aldrich) (0.15mg/ml BCIP, 0.30 mg/ml NBT, 100mM Tris, 5mM MgCl2, pH 9.5). The membrane was incubated for 10 minutes and the reaction stopped by rinsing with UPW.

2.6.2. Dot blotting

Dot blotting of inactivated meningococci was performed using a method similar to that described previously[281]. For each suspension of inactivated *N. meningitidis*, 3µl (for heat-inactivated bacteria) or 10µl (for ethanol-inactivated bacteria) was dotted onto a nitrocellulose membrane (pore size 0.2um) and air dried for 15 minutes. All incubation steps were performed on an orbital shaker at RT in a sterile petri dish using

sufficient liquid to completely submerge the membrane. The membrane was initially blocked for 60 minutes with PBS containing 3% BSA. Excess liquid was discarded and the mAb was added, after being diluted as recommended in PBS containing 3% BSA. Following incubation for 1-2 hours, the membrane was washed several times with PBS. The membrane was incubated with anti-mouse IgG (Fab specific)-alkaline phosphatase antibody produced in goat (Sigma-Aldrich), diluted 1:10,000 in PBS containing 3% BSA, for 1-2 hours. Following a further wash steps with PBS, alkaline phosphatase activity was detected by the addition of BCIP/NBT (Sigma-Aldrich). The membrane was incubated for 10 minutes and the reaction stopped by rinsing with UPW.

2.6.3. Immunoblotting

Immunoblotting of OMVs was based on a method similar to that described previously[282]. Proteins contained in OMVs were first separated on an SDS-PAGE gel before being transferred to a nitrocellulose membrane (pore size 0.2um). Between 5μg and 10μg of protein (estimated by the micro Lowry assay, section 2.4.2) was used for SDS-PAGE (section 2.4.3). The gel transfer sandwich was equilibriated for 15-20 minutes prior to blotting in transfer buffer (25mM Tris, 192mM glycine, 20% v/v methanol) which had previously been chilled at 4^{o}C for 16-18 hours. When SDS-PAGE was complete, the gel was detached and cut to the appropriate size before being placed into the transfer sandwich. The transfer sandwich was submerged in transfer buffer and a voltage of 100V was applied across it for 60 minutes in a mini trans-blot electrophoretic transfer cell containing a frozen cooling unit (Bio-rad) and placed on ice. The buffer was continuously stirred throughout the transfer process.

Following transfer, the gel was stained with InstantBlue Coomassie stain (section 2.4.3). The nitrocellulose membrane was air-dried for 15 minutes before immunoblotting was performed. The membrane was initially blocked for 30 minutes with PBS containing 3% BSA, before being cut into sections to enable blotting with different antibodies. Blotting of large sections of membrane were performed in a sterile petri dish and when the membrane was cut into strips, these were placed into an 8-well incubation tray. All incubation steps were performed on an orbital shaker at RT using sufficient liquid to completely submerge the membrane. The remaining steps used the same method as dot blotting (section 2.6.2).

2.7. Purification of DNA

2.7.1. Purification of plasmid DNA from Escherichia coli

Selected transformants were inoculated into 5ml LB broth and incubated at 37^{o}C, 220 rpm for 16-18 hours. Plasmid DNA was purified by one of two methods. Alkaline lysis with SDS was used if screening of a large number of samples was required, based on the methods of Birnhoim and Doly and Ish-Horowicz and Burke[272, 283, 284]. The QIAprep Miniprep kit (Qiagen), based on the same principle, was used if fewer samples were being screened, and for preparation of plasmid DNA for further use where higher purity of DNA was required. The kit was used according to manufacturer's instructions and DNA was eluted by adding 50µl Buffer EB (10mM Tris-HCl, pH 8.5). All plasmid DNA was visualised by agarose gel electrophoresis (section 2.8.1) and stored at 4^{o}C (short-term) or -20^{o}C (long-term).

For purification of plasmid DNA by alkaline lysis with SDS, bacterial cells were first harvested by centrifugation of the broth culture at 1,900 x *g* for 10 minutes. The supernatant was discarded and the pellet resuspended in 150µl GTE solution (50mM glucose, 10mM ethylenediaminetetraacetic acid (EDTA), 25mM Tris-HCl, pH 8.0) and incubated at RT for 5 minutes. The suspension was transferred to a 1.5ml microcentrifuge tube and 200µl of a freshly prepared solution containing SDS (1%) and sodium hydroxide (0.2M) was added before mixing by inversion and incubating on ice for at least 5 minutes. When the solution appeared clear, indicating cell lysis, 150µl ice-cold potassium acetate (3M potassium, 5M acetate) was added. This was mixed by inversion and incubated on ice for a further 5 minutes. Cell debris were removed by centrifugation at 16,100 x *g* for 5 minutes and the supernatant was transferred to a new tube. To precipitate DNA, 2 volumes of ethanol (100%) was added and the sample incubated at -20^{o}C for 1-24 hours. Precipitated DNA was separated by centrifugation at 16,100 x *g* for 10 minutes and the supernatant discarded. The DNA pellet was washed by adding 500µl ethanol (70%) and recovered by centrifugation at 16,100 x *g* for 15 minutes. The supernatant was carefully aspirated and the open tube stored at RT until the residual ethanol had evaporated. DNA was resuspended in 50µl TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) supplemented with RNase A (50µg/ml) (Sigma-Aldrich) and incubated at 37^{o}C for 1 hour.

2.7.2. Purification of chromosomal DNA from **Neisseria meningitidis**

Meningococcal chromosomal DNA was isolated either by using the Wizard Genomic DNA Purification Kit (Promega), by specific binding to a silica gel membrane with

the QIAamp DNA Mini Kit (Qiagen), or with potassium hydroxide. The Wizard Genomic DNA Purification Kit was used according to manufacturer's instructions. A 10µl loopful of bacteria was scraped from an agar plate following growth for 16-18 hours and resuspended in 1ml of Hanks' Balanced Salt Solution (HBSS) (Sigma-Aldrich), following which DNA was purified. DNA was resuspended in 100µl of DNA Rehydration Solution and incubated at 65^{o}C for 1 hour before being stored at -20^{o}C. The QIAamp DNA Mini Kit (Qiagen) was used according to manufacturer's instructions. A 10µl loopful of bacteria was scraped from an agar plate following growth for 16-18 hours and resuspended in 180µl of Buffer ATL, following which DNA was purified. DNA was eluted from the column using 200µl of UPW and stored at -20^{o}C.

A crude preparation of meningococcal chromosomal DNA was isolated using potassium hydroxide, for rapid screening following transformation. Potassium hydroxide specifically lyses Gram-negative bacteria, and this method is used to distinguish Gram-negative from Gram-positive bacteria[285]. A 1µl loopful of bacteria was scraped from an agar plate following growth for 16-18 hours and resuspended in 50µl of UPW. Bacterial cells were lysed by adding 50µl of potassium hydroxide (0.25M) and incubating at 95^{o}C for 5 minutes. Potassium hydroxide was neutralised by adding 50µl of hydrochloric acid (HCl) (0.25M). This was followed by the addition of 50µl of Tris-HCl (0.5M, pH 7.6) and 250µl of UPW before the DNA was stored at -20^{o}C.

2.8. Agarose gel electrophoresis

2.8.1. Visualisation of DNA

The electrophoresis buffer was tris-acetate-EDTA (TAE). A stock solution of 50x TAE buffer was prepared consisting of 242.2g Tris base (Sigma-Aldrich) and 37.2g EDTA disodium salt (VWR), titrated to pH 8.0 with glacial acetic acid (VWR) and made up to 1 litre with UPW. The ends of a gel tray (Bio-Rad) were sealed with masking tape and one or more combs inserted as required. Agarose solution (1% w/v) (Severn Biotech) in 1x TAE buffer was heated in a microwave oven. The gel tray sizes and gel volumes are shown in table 2.4. Ethidium bromide (Sigma-Aldrich) was added to the molten gel at a final concentration of 0.5μg/ml before pouring into a gel tray. The gel was allowed to solidify at RT and combs removed before it was submerged in 1x TAE buffer to cover it to a depth of approximately 1mm.

Gel tray dimensions	Gel volume	Gel thickness
7 x 10 cm	45 ml	0.75 cm
15 x 10 cm	90 ml	0.75 cm
15 x 25 cm	270 ml	0.75 cm

Table 2.4 Characteristics of agarose gels.

DNA loading buffer was prepared as a 6x stock solution containing ficoll 400 (18% w/v), bromophenol blue (0.18% w/v), xylene cyanol FF (0.18% w/v) and orange G (0.18% w/v) in EDTA (60mM, pH 8.0). DNA was mixed with DNA loading buffer in a 5:1 ratio before loading into the wells of the submerged gel. DNA size and concentration was determined by comparison to DNA Hyperladder I (Bioline), 3μl of which was loaded into adjacent slots (table 2.5). A voltage of 5V/cm was applied across the gel until DNA fragments had separated sufficiently. DNA was visualised

by examination under ultraviolet light at a wavelength of 302nm using a transilluminator.

Band size (bp)	Concentration of DNA (ng/µl)
10,000	20
8,000	16
6,000	12
5,000	10
4,000	8
3,000	6
2,500	5
2,000	4
1,500	3
1,000	20
800	16
600	12
400	8
200	4

Table 2.5 Concentration of DNA in bands of DNA Hyperladder I (Bioline).

2.8.2. DNA extraction from agarose gels

The QIAquick gel extraction kit (Qiagen) was used to extract plasmid DNA from agarose gels by binding to a silica membrane, according to manufacturer's instructions. DNA was eluted with 30µl Buffer EB (10mM Tris-HCl, pH 8.5) and stored at 4^{o}C (short-term) or -20^{o}C (long-term).

2.9. Manipulation of DNA

2.9.1. Polymerase chain reaction

Purified desalted PCR primers (Sigma-Aldrich) were used at a final concentration of 0.2µM per reaction. Each reaction contained 1.25 units Taq DNA Polymerase (Qiagen) and 200µM each of dATP, dCTP, dGTP and dTTP, supplemented with 1x PCR Buffer and 1x Q-Solution, according to manufacturer's instructions, providing a final Mg^{2+} concentration of 1.5mM. Each reaction was made up to 49µl with UPW

before the addition of 1µl DNA. PCR was carried out in a MJ Research PTC-200 thermal cycler. PCR conditions were typically as follows: initial denaturation at 95^{o}C for 5 minutes; followed by 30 cycles of denaturation at 95^{o}C for 30 seconds, annealing at 55^{o}C for 30 seconds, extension at 68^{o}C for 3-8 minutes; final elongation was at 68^{o}C for 10 minutes. PCR products were visualised by agarose gel electrophoresis (section 2.8.1), and stored at -20^{o}C.

2.9.2. Restriction enzyme digestion of plasmid DNA

Restriction enzymes (Promega, New England Biolabs, Fermentas), buffers (10x concentration) and acetylated BSA (10µg/µl) were stored at -20^{o}C. Analytical restriction enzyme digestions, for screening plasmid DNA samples, contained approximately 50ng DNA and 4 units of each enzyme. To prepare DNA fragments for use in subsequent ligations, reactions contained approximately 2.5µg DNA and 20 units of each enzyme. For preparation of plasmids to be transformed into *N. meningitidis*, reactions contained approximately 1µg DNA and 10 units of each enzyme. Reactions were supplemented with acetylated BSA at a final concentration of 0.1mg/ml if recommended by the manufacturer. Mixtures were made up to a total volume of 10µl (analytical scale), 40µl (preparative scale) or 15µl (for transformation) with UPW and incubated at the recommended temperature (typically 37^{o}C) for 1-16 hours. Reactions were terminated either by heating to 65^{o}C for 15-20 minutes, storing at -20^{o}C or adding DNA loading buffer. DNA fragments were visualised by agarose gel electrophoresis (section 2.8.1).

2.9.3. TOPO TA cloning

PCR products were cloned into the plasmid vector pCR2.1-TOPO (Invitrogen), according to manufacturer's instructions. Each cloning reaction contained 2-4µl from the PCR and 1µl (10ng) pCR2.1-TOPO vector, supplemented with salt solution containing NaCl (200mM) and magnesium chloride (10mM), made up to a total volume of 6µl with UPW. Reactions were incubated at RT for 30 minutes and then transferred to ice, for transformation into *E. coli* (section 2.2.2).

2.9.4. Dephosphorylation of plasmid DNA

Plasmid vector DNA was dephosphorylated prior to use in some ligation reactions to prevent self-ligation. This was performed either immediately after restriction enzyme digestion or immediately prior to the ligation reaction. Shrimp alkaline phosphatase (SAP) (Sigma-Aldrich) was stored at -20^{o}C and dephosphorylation buffer (10x concentration) was stored at RT. When performed after restriction enzyme digestion the restriction enzyme was first heat-inactivated. Five units of SAP and dephosphorylation buffer (5mM magnesium chloride, 0.05M Tris-HCl, pH 8.5) were added to the reaction mixture to increase the total volume from 40µl to 50µl. This was incubated at 37^{o}C for 10 minutes for 5'-overhang or 5'-recessive ends, and for 60 minutes for blunt ends. SAP was inactivated by incubating at 65^{o}C for 15 minutes. DNA fragments were then visualised by agarose gel electrophoresis before gel extraction (section 2.8).

For dephosphorylation of plasmid vector DNA prior to ligation, vector DNA was incubated with 1 unit of SAP and dephosphorylation buffer, using UPW to maintain

the appropriate buffer concentration. This was incubated at 37^{o}C for 10 minutes for 5'-overhang or 5'-recessive ends, and for 60 minutes for blunt ends. SAP was inactivated by incubating at 65^{o}C for 15 minutes. Remaining components of the ligation reaction were added and the mixture processed as in section 2.9.5.

2.9.5. DNA ligation

T4 DNA ligase (Novagen, New England Biolabs), buffers (10x concentration) and supplements were stored at -20^{o}C. Vector and insert DNA fragments were digested with the appropriate restriction enzymes (section 2.9.2) and extracted from agarose gels prior to ligation (section 2.8). Ligation reactions contained 2-136ng of vector DNA and 0.6-408ng of insert DNA. The ratio of vector : insert DNA was typically 1:3, although this varied between 1:0.1 and 1:6. Reactions contained 0.2-0.3 Weiss units of T4 DNA ligase for ligations involving 2-4 base overhangs and 2-3 Weiss Units for ligation of blunt ends (Novagen), or 1,000 cohesive end units (New England Biolabs). Reactions were supplemented with dithiothreitol (5mM) and adenosine triphosphate (1mM) if recommended by the manufacturer, made up to a total volume of 10µl with UPW and incubated at 16^{o}C for 13-24 hours. The entire ligation mixture was used directly for transformation of *E. coli* (section 2.2.2).

2.10. DNA sequencing

Dye-terminator DNA sequencing was used to obtain the DNA sequence of *opa* genes after first amplifying the region of interest by PCR (section 2.9.1). PCR products were purified by precipitation with polyethylene glycol (PEG) 8000 and NaCl to remove the PCR primers, template DNA and unincorporated dNTPs. Following PCR, 60µl of

PEG 8000 (20% w/v)/NaCl (2.5M) was added to each sample and incubated at RT for 30 minutes. Precipitated DNA was separated by centrifugation at 2,750 x *g* for 1 hour at 4^{o}C and the supernatant discarded by centrifugation at 500 x *g* for 1 minute at 4^{o}C. The DNA pellet was washed twice by the addition of 150µl of ice-cold ethanol (70%) followed by centrifugation at 2,750 x *g* for 5 minutes at 4^{o}C and removal of the supernatant as above. If DNA sequencing reactions were not performed immediately, DNA was stored for a maximum of 24 hours at -20^{o}C.

The DNA pellet was resuspended in 75µl of UPW immediately prior to performing the sequencing reactions. For sequencing reactions, oligonucleotide primers purified by high performance liquid chromatography were used (Sigma-Aldrich). Each reaction contained 1.3pmol oligonucelotide primer, 0.5µl BigDye Terminator Ready Reaction Mix (2.5x) (Applied Biosystems), 1.75µl BigDye sequencing buffer (5x) and 5µl DNA template in a total of 10µl. Reactions were carried out in a MJ Research PTC-200 thermal cycler with the following conditions: 30 cycles of 95^{o}C for 10 seconds, 50^{o}C for 6 seconds and 60^{o}C for 2 minutes. Extension products were purified by precipitation with ethanol/sodium acetate. After the sequencing reaction 10µl of UPW was added to each sample, followed by 52µl of ethanol (100%)/sodium acetate (115mM, pH 4.6). This was incubated at RT for 45 minutes, and the precipitated DNA separated by centrifugation at 2,750 x *g* for 1 hour at 4^{o}C. The supernatant was discarded as above and the DNA pellet washed by adding 150µl of ice-cold ethanol (70%) and centrifugation at 2,750 x *g* for 10 minutes. After the supernatant was discarded, DNA was stored at -20^{o}C prior to electrophoresis.

Labelled extension products were separated by capillary electrophoresis on the 3730xl DNA Analyzer (Applied Biosystems) at the Department of Zoology Sequencing Facility, University of Oxford. Sequence trace chromatograms were assembled into consensus sequences using the Staden sequence analysis package[286], using Gap4 shotgun assembly. Consensus sequences were visualised and manually checked to confirm quality of the individual chromatograms and appropriate alignment of overlapping sequences. Consensus sequences were only assigned if there was exact alignment of at least two satisfactory chromatograms, with at least one for each strand of DNA.

2.11. T cell proliferation assays

2.11.1. Study subjects

Blood was obtained from 46 healthy adult volunteers aged over 18 years after informed consent was obtained. Anyone with a history of previous IMD, a known immunodeficiency, or who was enrolled in another study which may affect their immune responses was excluded. The study was approved by the Oxfordshire C Research Ethics Committe (OxRec Number: 07/H0606/84; Study Code Number: 2007/3).

2.11.2. Separation of peripheral blood mononuclear cells

A maximum of 20ml of blood was collected from each study participant into a centrifuge tube containing 350µl of heparin (1000 units/ml). All subsequent procedures were performed under sterile conditions in a class 2 microbiological safety cabinet. Blood was diluted in an equivalent volume of buffer R0 (RPMI-1640

medium, HEPES modification, 25mM HEPES, 50 units/ml penicillin, 50µg/ml streptomycin, 2mM L-glutamine [Sigma-Aldrich]) and gradually layered over Lymphoprep (Axis-Shield), using a volume of Lymphoprep equivalent to the original blood sample. PBMCs were separated by density gradient centrifugation at 1,000 x *g* for 30 minutes with the brake minimised. The buffy coat, containing PBMCs, was transferred into a new centrifuge tube and the cells washed twice by addition of buffer R0 to a total volume of 30ml, followed by centrifugation at 850 x *g* for 20 minutes and aspiration of the supernatant. The number of PBMCs was established by resuspending the cell pellet in 5ml of buffer R10 (buffer R0 supplemented with 10% heat-inactivated newborn calf serum (NCS) [Sigma-Aldrich, lot numbers 016K8404 and 057K8416]), mixing 50µl PBMCs with 50µl PBS and 50µl trypan blue (0.4%), and adding 10µl of this mixture to an improved Neubauer haemocytomer. The number of cells in an area of 0.4mm^2 (equivalent to 40nl) was counted to determine the total number of viable PBMCs in the original suspension. Cells were subsequently either labelled with carboxyfluorescein succinimidyl ester (CFSE) (section 2.11.4) prior to stimulation with antigen (section 2.11.5), or used for purification of CD4$^+$ T cells (section 2.11.3).

2.11.3. Purification of CD4$^+$ T cells

CD4$^+$ T cells were purified from PBMCs by magnetic bead separation using the AutoMACS cell separator and the CD4$^+$ T cell isolation kit II (Miltenyi). The number of PBMCs was determined using a haemocytometer (section 2.11.2) prior to purification. CD4$^+$ T cells were purified by negative selection, according to manufacturer's instructions. Non-CD4$^+$ PBMCs were labelled with a mixture of

biotin-conjugated mAbs (against CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCRγ/δ and glycophorin A) as the primary labelling reagent and anti-biotin antibodies conjugated to MicroBeads as the secondary labelling reagent. The magnetically labelled non-$CD4^+$ cells were depleted by retaining them on a magnetic activated cell sorting (MACS) column in the magnetic field of the cell separator, while the unlabelled $CD4^+$ T cells were collected as they passed through the column. The number of $CD4^+$ lymphocytes was determined using an improved Neubauer haemocytometer (section 2.11.2). Following separation, cells were labelled with CFSE prior to stimulation with antigen (section 2.11.4).

2.11.4. Cell labelling with carboxyfluorescein succinimidyl ester

Separated PBMCs or purified $CD4^+$ T cells were labelled with CFSE prior to stimulation with antigen to assess proliferation[287]. All procedures involving CFSE, or cells that had been previously labelled with CFSE, were done with protection from light. Cells were isolated by centrifugation at 850 x *g* for 20 minutes and resuspended in 5ml of CFSE buffer (PBS containing 2mM EDTA and 0.5% NCS). The number of cells required was diluted to a concentration of 1 x 10^6/ml in CFSE buffer, to which CFSE was added to a final concentration of 2μM. Cells were incubated at 37^{o}C, 5% CO_2 for 10 minutes to allow labelling with CFSE and then made up to a total volume of 50ml with buffer R10 before centrifugation at 700 x *g* for 10 minutes. The supernatant was aspirated and the cell pellet resuspended in 20ml of buffer R10 and washed by centrifugation at 700 x *g* for 10 minutes. Cells were resuspended in buffer R10, with or without IL-2, to obtain the appropriate concentration of cells for culture, which was 2 x 10^6 cells/ml for PBMCs and 1 x 10^6 cells/ml for purified $CD4^+$ T cells.

When assays involved pre-incubation of cells with IL-2, this was added at a final concentration of 1,000 units/ml (Roche).

2.11.5. Cell culture

All steps involving CFSE-labelled cells were performed with protection from light. Assays using PBMCs contained 2 x 10^5 cells per well and purified $CD4^+$ T cells were used at 1 x 10^5 cells per well, in a total volume of 100µl per well. After being labelled with CFSE and diluted in R10 with or without IL-2 as needed (section 2.11.4), cells were placed into flat-bottomed 96-well tissue culture plates (Thermo Scientific Nunc). For *ex vivo* studies, antigens were diluted to the required concentration in R10 and 100µl of antigen was added per well (table 2.6). Cultures were then incubated for 120 hours. When pre-incubation with IL-2 was required, cells diluted in R10 with IL-2 were incubated for 48 hours prior to addition of antigens. Antigens were diluted to the required concentration in R10 containing IL-2 (1,000 units/ml), with or without anti-CD3 and anti-CD28 antibodies. Antigen solutions contained 2µg/ml of each antibody when required, to achieve a final concentration of 1µg/ml of each antibody in the assay. Cultures were then incubated for a further 96 hours. For all assays, $CD4^+$ T cell proliferation was assessed by flow cytometric analysis of CFSE dilution (section 2.11.6). Each antigen was set up in triplicate, and for each 96-well plate two sets of controls (also in triplicate) were set up, containing media only with or without CFSE-labelled cells.

Antigen	Stock concentration	Final concentration in assay
PHA* (Sigma-Aldrich)	5mg/ml	5µg/ml
Tuberculin PPD† (Statens Serum Institut)	1mg/ml	10µg/ml
Anti-CD3 + anti-CD28 antibodies (Biolegend)	1mg/ml‡	1µg/ml‡
Isotype control antibody (DAKO)	15mg/ml	25µg/ml
Polyclonal anti-CEACAM antibody (DAKO)	2mg/ml	25µg/ml
OMVs	Variable	50µg/ml
Inactivated bacteria§	Variable	ratio of 500 bacteria : 1 cell¶

Table 2.6 Antigens used for T cell proliferation assays.
All antigens were diluted in R10 to the required concentration.
*PHA = phytohaemagglutinin; †PPD = purified protein derivative; ‡concentration per antibody; §Bacteria were washed 3 times in R10 by centrifugation at 16,100 x *g* for 1 minute and finally resuspended in R10; ¶1 x 10^8 cfu per well for assays with PBMCs and 5 x 10^7 cfu per well for assays with purified $CD4^+$ T cells.

2.11.6. Analysis of cells using flow cytometry

$CD4^+$ T cell proliferation was assessed using flow cytometric measurement of CFSE dye dilution[287]. $CD4^+$ T cells were positively identified by detection of CD4 expression, with exclusion of dead cells. All steps were performed with protection from light. Cells were first transferred from the flat-bottomed 96-well plates to 96-well plates with a V-bottom. Cells were initially resuspended by pipetting up and down a few times, and harvested by centrifugation at 500 x *g* for 5 minutes. The supernatants were transferred to new 96-well plates, which were sealed and stored at -30°C, for any future work on cytokine secretion. Cells were washed twice by addition of 180µl of phosphate buffered albumin (PBA) (PBS containing 0.1% BSA and 10mM sodium azide), centrifugation at 500 x *g* for 2 minutes and aspiration of the supernatant. Cells were incubated for 20 minutes at 4°C with 50µl of PBA containing 2.4% (v/v) anti-CD4 antibody conjugated to allophycocyanin (BD Pharmingen), which was followed by 2 wash steps with PBA as above. If cells were to be stored for longer than 1-2 hours prior to flow cytometric analysis, they were fixed by adding 100µl of Cellfix (Becton Dickinson), incubating at 4°C for 10 minutes and washing twice with PBA as above. To identify apoptotic cells, cells were stained with 50µl of

PBA containing 1μg/ml of 7-aminoactinomycin D (7-AAD) (Sigma-Aldrich). Following incubation at for 20 minutes at RT in the dark, cells were washed twice with PBA as above. Cells were finally resuspended in 150μl of PBA and transferred to 1.2ml tubes.

Flow cytometry was performed using the FACSCalibur (Becton Dickinson). Cells were kept on ice and protected from light throughout flow cytometric measurement. Data from 10,000 $CD4^{high}$ $7\text{-}AAD^{low}$ events were collected for each sample. CFSE labelling was detected using channel FL1 at a wavelength of 530nm. CD4 expression was detected using channel FL4 at a wavelength of 661nm, and 7-AAD staining was detected through channel FL3 at a wavelength of 670nm. Data were collected and stored using CellQuestPro for Macintosh (Becton Dickinson). The percentage $CD4^+$ T cell proliferation was calculated as the percentage of cells with a CFSE concentration lower than the end of the initial peak.

2.11.7. Statistical analysis

For all culture conditions, cells from all participants were incubated with each antigen in triplicate. $CD4^+$ T cell proliferation was calculated as the mean of the triplicate wells, and the mean proliferation of the wells containing media only was then subtracted. For all antigens in all culture conditions, a Shapiro-Wilk analysis was performed to assess if the values were normally distributed ($p > 0.05$). For normally distributed variables, statistical significance was calculated using a paired two-tailed Student's t-test. For non-normally distributed variables, statistical significance was calculated using a Wilcoxon signed-rank test. For an individual sample and a single

culture condition, values of the triplicate wells for each antigen were compared to values of the media only wells to assess if each antigen was causing stimulation or inhibition. Statistical significance was calculated using a paired two-tailed Student's t-test. For all analyses, a *p*-value of <0.05 was considered statistically significant.

2.12.

CHAPTER 3. CONSTRUCTION OF LOCUS-SPECIFIC *OPA* PLASMIDS

3.1. Introduction

The first step to disrupting the *opa* genes of *N. meningitidis* strain H44/76 was to clone and disrupt each *opa* gene in a plasmid vector and amplify the plasmids in *E. coli*, before using the disrupted genes for transformation of *N. meningitidis*. This would ultimately enable construction of isogenic Opa-deficient strains by sequential *opa* disruption. To target a specific *opa* gene during transformation, plasmids which included locus-specific sequences upstream and downstream of each *opa* gene were required (figure 5.1). Plasmids containing a modified *opa* gene, either with or without an antibiotic resistance cassette, could subsequently be used to sequentially disrupt all four *opa* genes of *N. meningitidis* (section 5.2). The conventional approach to disrupting a neisserial chromosomal gene is to clone the gene into a plasmid vector, disrupt it with a selectable marker, such as an antibiotic resistance cassette, and use the resulting plasmid for transformation of the wild-type strain. Bacteria which have successfully incorporated the disrupted gene can be identified by growth on selective media. This approach is more difficult when applied to the construction of Opa-deficient meningococci due to the presence of four *opa* genes in the genome.

N. meningitidis strain H44/76 from the ST-32 clonal complex was chosen as the parent strain from which Opa-deficient isogenic mutant strains would be constructed. This strain was chosen for a number of reasons. Firstly, meningococci of the ST-32 clonal complex have caused IMD throughout Europe, South America and Asia[289-294]. Organisms from this clonal complex tend to be associated with the serogroup B

capsule[36], against which there is no vaccine. Secondly, *opa* genes are highly conserved within the ST-32 clonal complex[32], suggesting the possibility of broad coverage of these organisms with a vaccine containing a small number of Opa protein variants (table 1.3). Thirdly, H44/76 has been used as the basis of OMV vaccines manufactured in Norway, The Netherlands and the USA, and as such has been very well characterised[114, 126, 127, 295]. These vaccines have been studied in clinical trials in several European countries, the USA, Chile and New Zealand (table 1.1). Finally, H44/76 is closely related to strain MC58, also from the ST-32 clonal complex, and for which the entire genome sequence had been published when the study started[156]. The genome sequence of H44/76 has now also been published (GenBank accession numbers AEQZ00000000 and CP002420)[296, 297]. Three of the four *opa* genes within H44/76 are identical to those found in MC58.

This chapter describes a series of experiments in which an attempt was made to construct eight plasmids. Each plasmid contained one of the four *opa* genes from *N. meningitidis* strain H44/76, which had been modified to include a 185 bp deletion. Four plasmids also contained an antibiotic resistance cassette inserted within the *opa* gene.

3.2. Methods

The methods used in this chapter were transformation of *E. coli* (section 2.2), purification of DNA (section 2.7), agarose gel electrophoresis (section 2.8) and manipulation of DNA (section 2.9).

3.3. Amplification of *opa* genes from *Neisseria meningitidis* by PCR

Nucleotide sequences of the four *opa* genes from H44/76 have been published and are summarised in table 3.1[32]. The closely related strain MC58 (GenBank accession number AE002098) was used to predict the sequences of adjacent upstream and downstream segments[156]. For each *opa* locus, two primers within the *opa* gene and two primers in neighbouring genes were used to amplify the 5' and 3' ends of each *opa* gene separately (figure 3.1). PCR primers were based on those that have previously been published wherever possible. Additional primers were designed with Primer3Plus[298], using the following criteria:

- Primer length: 15-30 bp;
- Primer melting temperature: 55-68^{o}C;
- Primer GC content: 40-70%;
- Maximum length of any homopolymeric tract: 5 bp;
- CG clamp at 3' end: 2 bp.

All primers were adapted to introduce *Sal*I, *Sac*II or *Bam*HI sites, one site at each end of every PCR product. Primer sequences are shown in table 3.2. PCR was performed as in section 2.9.1, using an extension time of 3 minutes. The 5' and 3' ends of all four *opa* genes were successfully amplified.

opa gene	Allele number	SV	HV1	HV2
opaA	96	2-1	19-10	14-3
opaB	288	5-1	1A-2	8-1
opaD	147	2-2	1A-2	8-1
opaJ	218	2-1	19-10	14-5

Table 3.1 *opa* gene repertoire of *N. meningitidis* strain H44/76.
Numbers in SV, HV1, and HV2 columns are given according to the following convention: amino acid sequence family-amino acid sequence variant. Classification is according to the *Neisseria meningitidis* Opa sequence database at http://neisseria.org/nm/typing/opa. *(Adapted from Callaghan* et al.[32]*)*

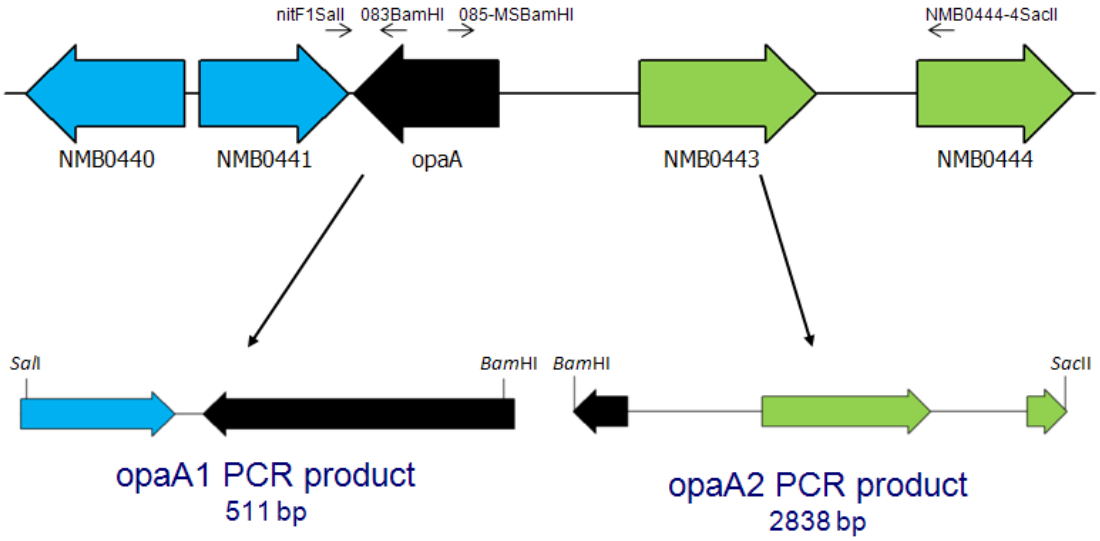

Figure 3.1 Amplification of 5' and 3' ends of *opaA* by PCR.
The 5' end was amplified along with locus specific sequences using primers 085-MSBamHI and NMB0444-4SacII, introducing *Bam*HI and *Sac*II restriction sites into the PCR product. The 3' end was similarly amplified using primers nitF1SalI and 083BamHI to introduce *Sal*I and *Bam*HI sites. A similar process was used for all *opa* genes.

opa gene	Adjacent gene	PCR product	Forward primer	Reverse primer	Product size (bp)
opaA	*nmb0441*	opaA1	nitF1SalI§ (5'-GCACGTGTCGACACAGCATGATTGTCGATCC-3')	083BamHI¶ (5'-CTATATGGATCCGCGCGTCGCCTACGGAC-3')	511
	*nmb0444**	opaA2	085-MSBamHI¶ (5'-TTTTCTGGATCCGGCATAATCTGCCGCTATTC-3')	NMB0444-4SacII§ (5'-TGAAGCCCGCGGGTCAGCACATAGTTGACG-3')	2838
opaB	*nmb1634*†	opaB1	NMB1634-4SalI§ (5'-GCCGTAGTCGACTTCTTCCGATCCCAACC-3')	085BamHI¶ (5'-TTTTCTGGATCCGGCATAATCTGCCGCTATCC-3')	2556
	nmb 1637	opaB2	083BamHI¶ (5'-CTATATGGATCCGCGCGTCGCCTACGGAC-3')	0464 opaBrevSacII‖ (5'-TTACCGCCGCGGAAGGCGAGGTAGGATTGC-3')	941
opaD	*nmb 1464*	opaD1	NMB1464-3SalI§ (5'-CAAAAGGTCGACTGCCAAAGCCTGAGATTGC-3')	083BamHI¶ (5'-CTATATGGATCCGCGCGTCGCCTACGGAC-3')	1559
	ppx‡	opaD2	085BamHI¶ (5'-TTTTCTGGATCCGGCATAATCTGCCGCTATCC-3')	opaDrevdSacII§ (5'-TTTCGACCGCGGAGGCGGAATGCTTGTGATAG-3')	2671
opaJ	*nmb 0925*	opaJ1	acthR2SalI§ (5'-GCGACGGTCGACAGGAGCAGTTCGCCTTGAG-3')	083BamHI¶ (5'-CTATATGGATCCGCGCGTCGCCTACGGAC-3')	1968
	pip	opaJ2	085-MSBamHI¶ (5'-TTTTCTGGATCCGGCATAATCTGCCGCTATTC-3')	pipSEQRSacII** (5'-CCGGTTCCGCGGATTTTCAGCAATCGGCGCG-3')	2360

Table 3.2 PCR primers used for amplification of *opa* genes.
Restriction sites within primer sequences are underlined. ******nmb0443* is adjacent to *opaA* but no unique primer site could be identified within *nmb0443* so *nmb0444* was used; **†***nmb1635* is adjacent to *opaB* but the putative coding sequence is only 222 bp so *nmb1634* was used; **‡***nmb1466* is adjacent to *opaD* but primers within *ppx* have been published following successful use; **§**designed using Primer3Plus[298]; **¶**from Hobbs *et al.*[263]; **‖** from Morelli *et al.*[299]; ******from Maiden *et al.*[35]

3.4. TA cloning of *opa* PCR products

The TOPO TA vector pCR2.1 was used for initial cloning of all PCR products. The plasmid pCR2.1 is a linearised vector with 3'-T overhangs and topoisomerase I-activated to directly accept PCR products with 3'-A overhangs within the *lacZα* fragment. In addition, pCR2.1 contains ampicillin and kanamycin resistance genes. These features enabled selection of transformed *E. coli* by growth on selective media and blue/white screening of recombinant colonies. *E. coli* strain DH5α was used for transformation reactions. This strain supports blue/white screening due to the presence of *lacZ*ΔM15. In addition, *rec*A1 and *end*A1 mutations increase insert stability and improve the quality of subsequently purified plasmid DNA. The full genotype of DH5α is given in table 2.2.

PCR products containing the 5' and 3' ends of each *opa* gene were cloned separately into pCR2.1 (section 2.9.3; figure 3.2). DNA from TA cloning reactions was used to transform *E. coli* DH5α (section 2.2.2), which was grown on LB agar containing ampicillin, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) and IPTG. Plasmid DNA was purified from selected colonies and screened by restriction enzyme digestion using *Sal*I and *Bam*HI or *Bam*HI and *Sac*II, to demonstrate successful introduction of these restriction sites (table 3.3).

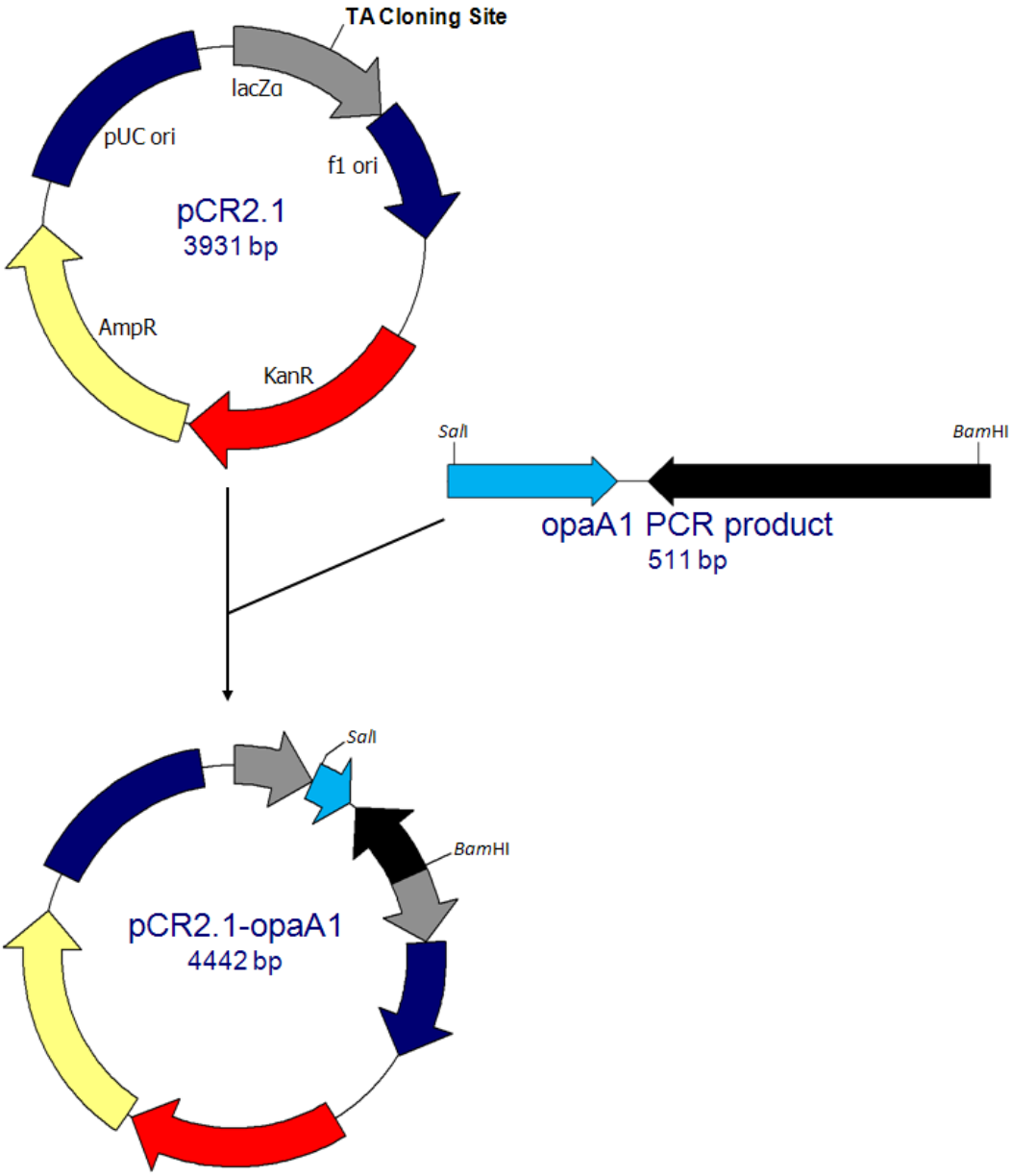

Figure 3.2 TA cloning of opaA1 PCR product into pCR2.1.
Other PCR products were similarly cloned.

Plasmid	Size (bp)	Restriction enzyme fragment sizes (bp)	
		***Sal*I + *Bam*HI**	***Bam*HI + *Sac*II**
pCR2.1-opaA1	4442	49, 493, 3900	–
pCR2.1-opaA2	6769	–	49, 2823, 3897
pCR2.1-opaB1	6487	49, 2538, 3900	–
pCR2.1-opaB2	4872	–	49, 926, 3897
pCR2.1-opaD1	5490	49, 1541, 3900	–
pCR2.1-opaD2	6602	–	49, 2656, 3897
pCR2.1-opaJ1	5899	49, 1950, 3900	–
pCR2.1-opaJ2	6291	–	49, 2345, 3897

Table 3.3 Sizes of plasmids and restriction enzyme fragments following cloning of *opa* PCR products into pCR2.1.

3.5. Cloning of ends of *opa* genes into pBluescript

The phagemid vector pBluescript II (SK-) was used for further cloning steps. This is a commonly used cloning vector which contains an ampicillin resistance gene and the N-terminal portion of a *lacZ* gene fragment[300]. Within the *lacZ* sequence is an extensive polylinker flanked by T7 and T3 ribonucleic acid (RNA) polymerase promoters. Inserts within the polylinker result in disruption of *lacZ.* Following transformation of *E. coli* DH5α, recombinant colonies can therefore be identified by growth on selective media and blue/white screening.

The aim of the next two steps was to sequentially introduce the 5' and 3' ends of each *opa* gene adjacent to each other, creating a modified *opa* gene unable to encode functional protein (figure 3.3). Vector and insert DNA were prepared by double digestion with *Sal*I and *Bam*HI or *Bam*HI and *Sac*II, sites for which had been introduced during PCR. Digests were carried out for 16 hours and the desired fragments were extracted from agarose gels. Initial ligation reactions contained 6ng of vector DNA and 18ng of insert DNA, estimated by comparison to DNA Hyperladdder I (Bioline) during agarose gel electrophoresis. The ligation reactions are summarised in table 3.4.

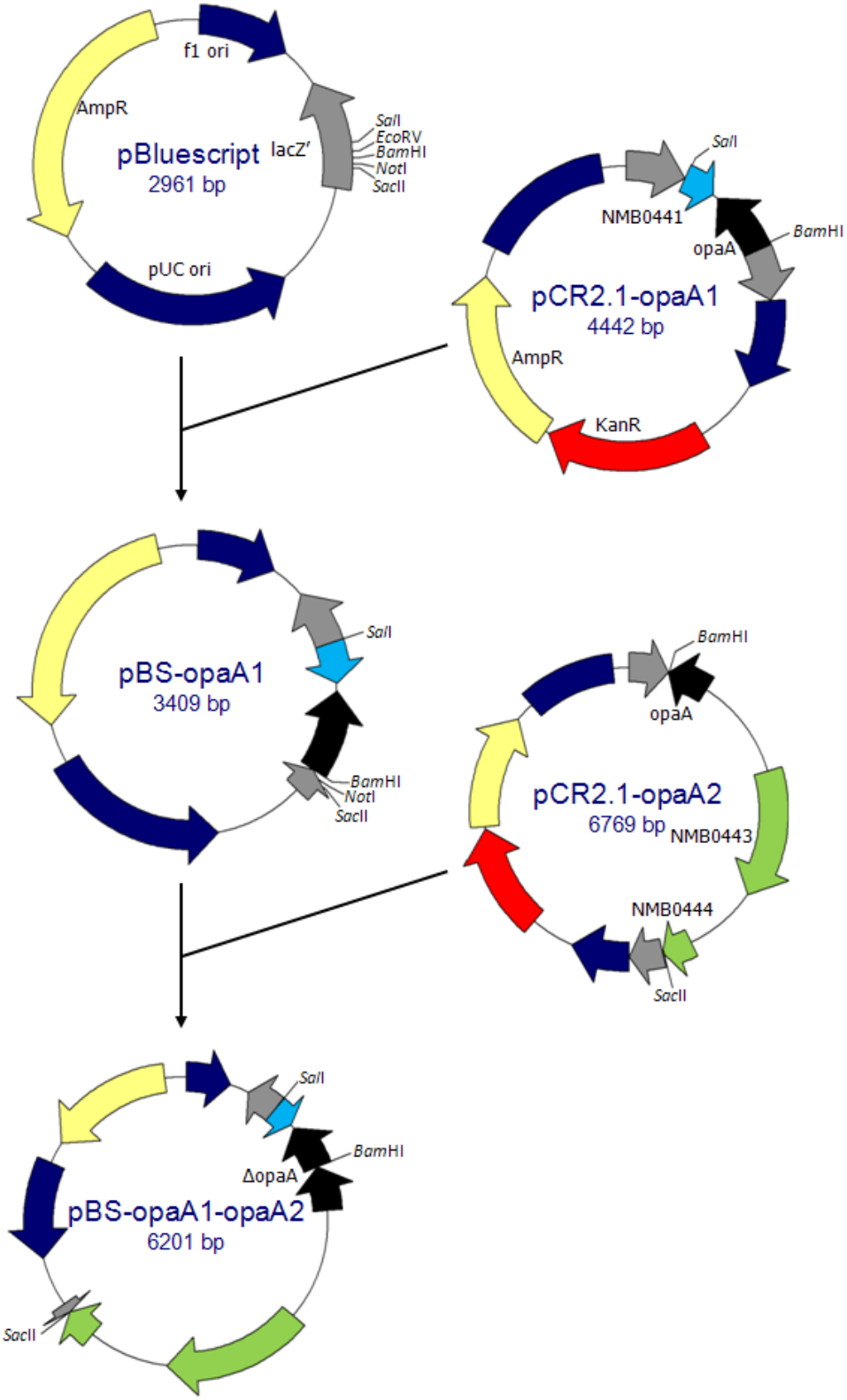

Figure 3.3 Sequential cloning of the 5' and 3' ends of *opaA* into pBluescript.
The first step involved cloning of a 493 bp *Sal*I-*Bam*HI fragment from pCR2.1-opaA1 containing the 3' end of *opaA* into pBluescript, producing pBS-opaA1. In the second step a 2,823 bp *Bam*HI-*Sac*II fragment from pCR2.1-opaA2, containing the 5' end of *opaA*, was cloned into pBS-opaA1 to construct pBS-opaA1-opaA2. This plasmid therefore produced a modified *opaA* gene, Δ*opaA*, which contained a 185 bp deletion. The same process can be used for all *opa* genes, and the same final plasmid can be obtained by performing step two before step one.

Plasmid	Plasmid size (bp)	Vector	Vector size (bp)	Insert*	Insert size (bp)	Number of ligations	Number of *E. coli* colonies screened	Number of successful clones
pBS-opaA1	3409	pBluescript	2916	opaA1	493	1	6	4
pBS-opaA1-opaA2	6201	pBS-opaA1	3378	opaA2	2823	8	223	2
pBS-opaB1	5454	pBluescript	2916	opaB1	2538	11	105	0
pBS-opaB2	3856	pBluescript	2930	opaB2	926	6	57	0
pBS-opaD1	4457	pBluescript	2916	opaD1	1541	1	22	2
pBS-opaD1-opaD2	7082	pBS-opaD1	4426	opaD2	2656	3	311	2
pBS-opaJ1	4866	pBluescript	2916	opaJ1	1950	5	59	2
pBS-opaJ1-opaJ2	7180	pBS-opaJ1	4835	opaJ2	2345	1	7	1

Table 3.4 Summary of cloning of ends of *opa* genes into pBluescript.
*****Inserts were derived from pCR2.1-based plasmids into which the PCR products listed had been cloned (figure 3.2).

Plasmid	Size (bp)	Restriction enzyme fragment sizes (bp)				
		***Ava*I**	***Ava*II**	***Bss*HII**	***Pvu*II**	***Sca*I**
pBS-opaA1	3409	56, 3353	–	621, 2788	–	3409
pBS-opaA1-opaA2	6201	56, 499, 1654, 3992	–	2788, 3413	–	6201
pBS-opaB1	5454	2488, 2966	–	–	2513, 2941	5454
pBS-opaB2	3856	–	222, 1392, 2242	–	1343, 2513	3856
pBS-opaD1	4457	–	222, 1582, 2653	1669, 2788	–	4457
pBS-opaD1-opaD2	7082	143, 166, 199, 463, 632, 706, 1127, 3646	–	–	2513, 4569	7082
pBS-opaJ1	4866	–	222, 1567, 3077	2078, 2788	–	4866
pBS-opaJ1-opaJ2	7180	171, 297, 423, 437, 684, 5168	–	2788, 4392	–	7180

Table 3.5 Sizes of plasmids and restriction enzyme fragments following cloning of ends of *opa* genes into pBluescript.

DNA from ligation reactions was used to transform *E. coli* DH5α (section 2.2.2), which was grown on LB agar containing ampicillin, with X-gal and IPTG added for the first cloning step. Following the second cloning step blue/white screening was not possible due to disruption of *lacZ* in the first step. Plasmid DNA was purified from selected colonies and screened by restriction enzyme digestion using at least three enzymes (table 3.5). These included:

(i) an enzyme to excise the insert, confirming its presence;

(ii) an enzyme to cut asymmetrically within the insert, confirming its orientation;

(iii) an enzyme to linearise the plasmid, confirming a single copy of the insert was present.

The results of these experiments are described separately for each of the four *opa* genes below.

3.5.1. opaA

The first cloning step was successfully achieved, resulting in the plasmid pBS-opaA1. The second step was initially unsuccessful despite the growth of transformed *E. coli* on selective media. Analysis of plasmid DNA purified from these colonies demonstrated the presence of pBS-opaA1, rather than pBS-opaA1-opaA2. This suggested pBS-opaA1 was present in a circular form following the second ligation reaction since circular plasmid DNA has a much higher transformation efficiency than linear plasmid DNA. Prior to the second reaction, pBS-opaA1 was prepared by double digestion with *Bam*HI and *Sac*II. Any plasmid DNA digested by only one enzyme would co-purify with double-digested DNA during gel extraction due to its similar

size. This could potentially self-ligate during the ligation reaction and undergo successful transformation. The ligation reaction products were therefore digested with *Not*I prior to transformation of *E. coli*. The *Not*I restriction site is present in pBS-opaA1 and not in pBS-opaA1-opaA2 (figure 3.3), resulting in selective linearisation of pBS-opaA1 and a much lower transformation efficiency of the unwanted plasmid. The second cloning step was successful using this strategy, resulting in the plasmid pBS-opaA1-opaA2.

3.5.2. opaB

It was not possible to complete the first cloning step for either the 5' end or 3' end of *opaB*. Modifications were made in order to improve ligation and transformation efficiency. The ligation reaction products were digested with either *Eco*RV (during cloning of opaB1) or *Not*I (during cloning of opaB2) prior to transformation of *E. coli*. Both sites are present in pBluescript, but not in pBS-opaB1 and pBS-opaB2, respectively. This strategy was successfully employed during cloning of *opaA*, and the rationale is described in section 3.5.1. A further modification was alteration of the vector : insert DNA ratio in the ligation reaction. In general, an optimum yield of monomeric circular recombinant plasmids is achieved with a slight excess of insert DNA relative to vector DNA, although the exact ratio is variable. Equimolar amounts of vector and insert DNA have also been described as optimal for successful ligation[301, 302]. Using a vector : insert DNA ratio of 1:1 did not result in successful cloning of either opaB1 or opaB2 into pBluescript. No further attempts were made to persist with cloning *opaB* into pBluescript in order to make progress with cloning of the other *opa* genes.

3.5.3. opaD

The first cloning step was achieved, resulting in the plasmid pBS-opaD1. The second step also appeared successful, although the desired plasmid pBS-opaD1-opaD2 co-purified with a second, smaller plasmid in two different colonies (figure 3.4). Restriction enzyme digestion of plasmid DNA from these colonies revealed the presence of all desired fragments as well as some additional fragments (figure 3.5), confirming the presence of two different plasmids. This may have been due to two colonies being inadvertently inoculated into a single culture during screening, or to the presence of two plasmids within one *E. coli* colony following transformation.

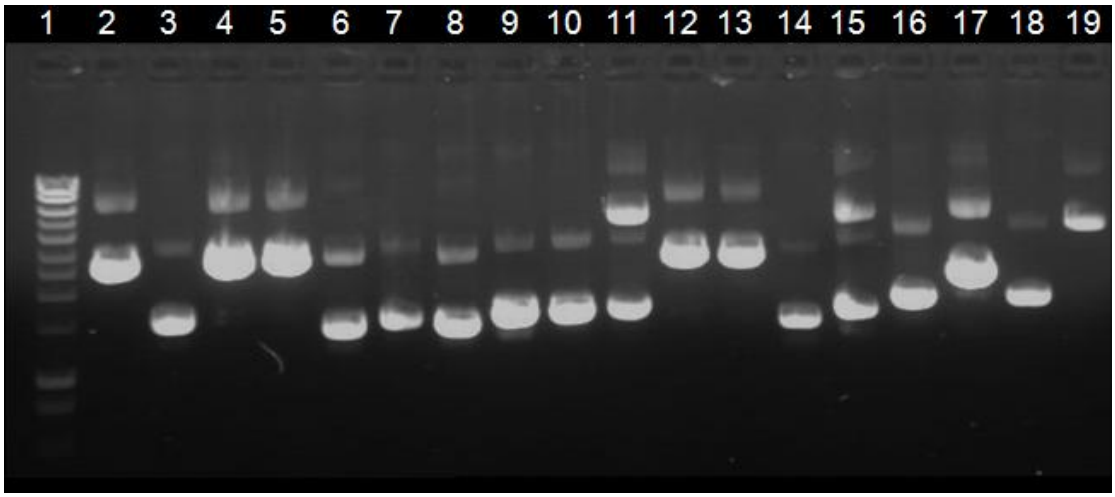

Figure 3.4 Agarose gel showing pBS-opaD1-opaD2 plasmid DNA from different *E. coli* colonies. Samples in lanes 11 and 15 contained two distinct plasmids. Lane 1= DNA ladder

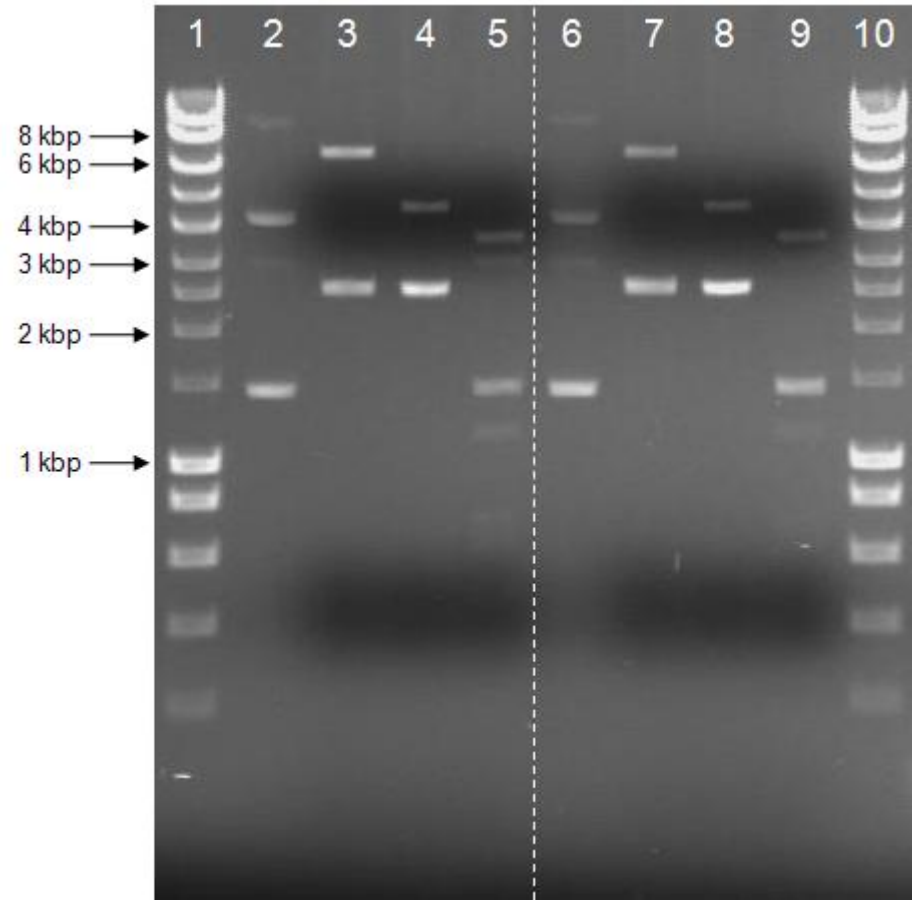

Figure 3.5 Agarose gel of fragments of pBS-opaD1-opaD2 following restriction enzyme digestion. Lanes 2-5 contained plasmid DNA from the sample in lane 11 of figure 3.4 and lanes 6-9 contained DNA from the sample in lane 15 of figure 3.4. Lanes 2 and 6 = undigested DNA; lanes 3 and 7 = *Sca*I digest; lanes 4 and 8 = *Pvu*II digest; lanes 5 and 9 = *Ava*I digest. Lanes 1 and 10 = DNA ladder

A number of procedures were performed to obtain a pure sample of pBS-opaD1-opaD2. Firstly, further purification of DNA from the original *E. coli* colonies resulted in the same plasmid mixture being obtained. Secondly, the plasmid mixture was used to transform *E. coli*, and colonies screened to identify those that had been transformed with pBS-opaD1-opaD2 only. Thirdly, the two plasmids were separated by agarose gel electrophoresis, pBS-opaD1-opaD2 was gel extracted in its supercoiled form and used to transform *E. coli*. Finally, the two plasmids were separated by agarose gel electrophoresis after linearisation with *Sap*I, pBS-opaD1-opaD2 was gel extracted and used to transform *E. coli*, both in its re-circularised form (following self-ligation with

T4 DNA ligase) and its linear form. The last three methods resulted in either no colonies following transformation, or when colonies did grow the plasmid DNA obtained was not pBS-opaD1-opaD2.

Since it did not appear possible to purify pBS-opaD1-opaD2, the part of the plasmid containing Δ*opaD* was amplified by PCR using primers M13 forward (5'-GTAAAACGACGGCCAG-3') and M13 reverse (5'-CAGGAAACAGCTATGAC-3') to obtain a 4,350 bp product. PCR was performed as in section 2.9.1, using an extension time of 5 minutes, and the product was cloned into the TOPO TA vector pCR2.1 resulting in pCR2.1-opaD1-opaD2.

3.5.4. opaJ

Both ends of *opaJ* were successfully cloned into pBluescript using standard methods.

3.6. Insertion of antibiotic resistance cassettes into *opa* genes

3.6.1. Purification of antibiotic resistance cassettes

Cassettes conferring kanamycin (KanR) and erythromycin (EryR) resistance were used for insertion into the *opa* genes using the plasmids constructed in section 3.5 (figure 3.6). For KanR, it has been demonstrated that the orientation must be the same as the target gene to obtain the resistance phenotype in *N. meningitidis*[303]. For EryR, insertion could be in either orientation. The KanR cassette was obtained from pUC4-kan (GenBank accession number X06404) and EryR from pER2. The plasmid pUC4-kan contains KanR from the transposon Tn903 cloned in pUC7, which is derived from pUC19[304]. The transposon Tn903 confers resistance to kanamycin by encoding

aminoglycoside 3'-phosphotransferase[305]. The plasmid pER2 contains the *ermC* gene from pIM13, cloned in pBluescript[306]. *ermC* is constitutively transcribed and encodes an rRNA methylase which confers resistance to the macrolide-lincosamide-streptogramin B antibiotics.

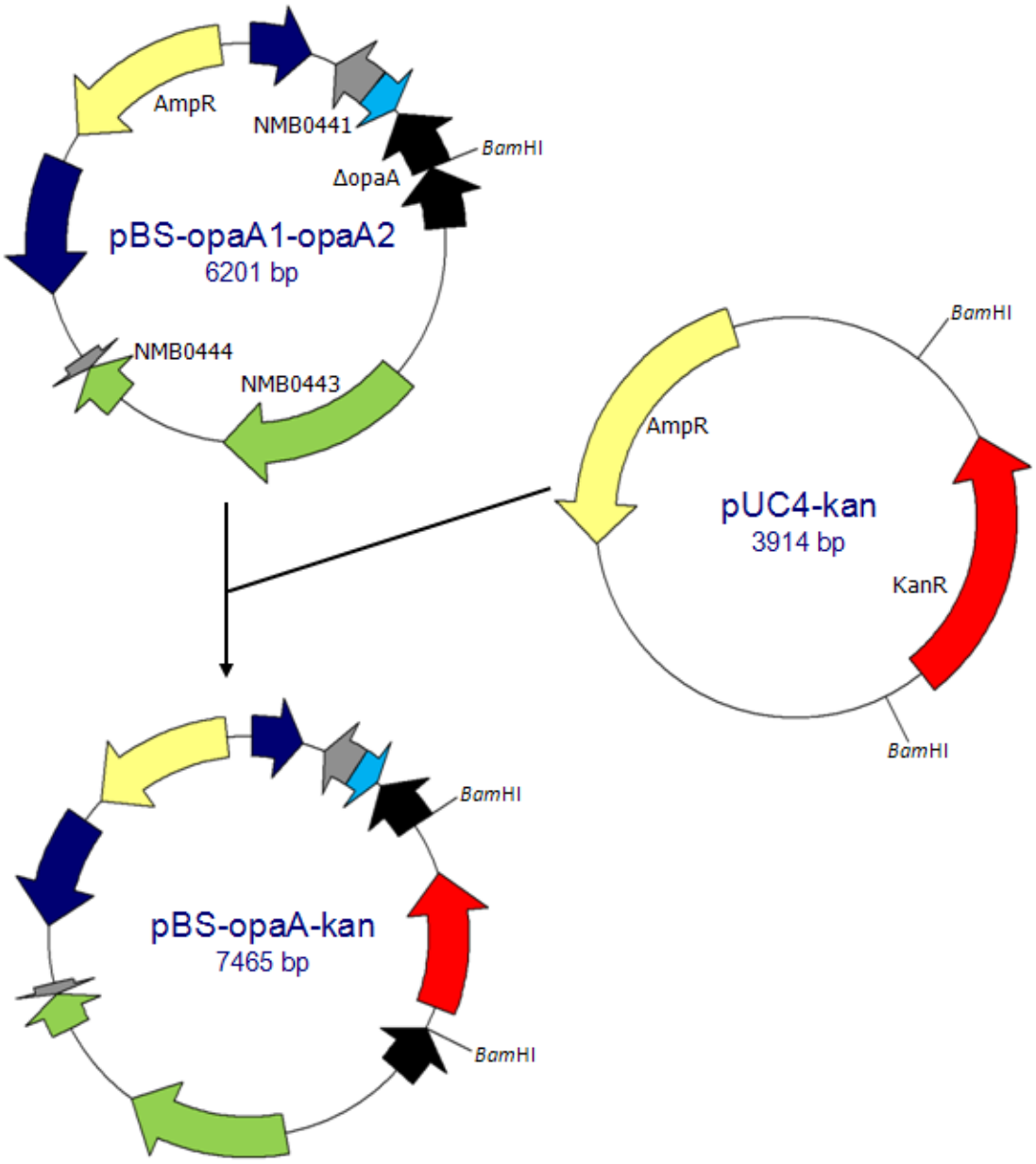

Figure 3.6 Insertion of KanR into pBS-opaA1-opaA2.
A 1,264 bp *Bam*HI fragment of pUC4-kan carrying KanR is cloned into pBS-opaA1-opaA2 to produce pBS-opaA-kan. Similar constructs can be obtained based on other *opa* genes by the same method.

The KanR cassette was excised from pUC4-kan by digestion with *Bam*HI. The EryR cassette was developed by initial amplification of EryR from pER2 by PCR. The primers were designed to introduce the same restriction sites, either *Bam*HI or *Sgr*AI, at both ends of the PCR product. To obtain *Bam*HI sites (underlined), primers ery-bamf (5'-GATCCCGGATCCTGCAGGAATTCGATATCAAGC-3') and ery-bamr (5'-CCGGGCGGATCCTCGAGGTCGACGGTATCG-3') were used to amplify a 1,210 bp product. To introduce *Sgr*AI sites (underlined), primers ery-sgrf (5'-TGGATCCACCGGTGTGCAGGAATTCGATATCAAGC-3') and ery-sgrr (5'-TACCGGCACCGGCGTCGAGGTCGACGGTATCG-3') were used to obtain a 1,214 bp product. PCR was performed as in section 2.9.1, using an extension time of 3 minutes. Each PCR product was cloned into the TOPO TA vector pCR2.1 (section 2.9.3) and DNA from the TA cloning reactions used to transform *E. coli* DH5α (section 2.2.2), which was grown on LB agar containing erythromycin. Plasmid DNA was purified from selected colonies and screened by restriction enzyme digestion using *Bam*HI or *Sgr*AI, as appropriate (table 3.6). The EryR cassette was then be excised from these plasmids using *Bam*HI or *Sgr*AI, as appropriate. When EryR was required for blunt-ended ligation reactions it was excised directly from pER2 by digestion with *Pvu*II.

Plasmid	**Size (bp)**	**Restriction enzyme fragment sizes (bp)**	
		***Bam*HI**	***Sgr*AI**
pCR2.1-ery-bam	5141	49, 1192, 3900	–
pCR2.1-ery-sgr	6769	–	1194, 3951

Table 3.6 Sizes of plasmids and restriction enzyme fragments following cloning of EryR into pCR2.1.

3.6.2. Insertion of KanR and EryR into **opa** *genes*

Following the experiments described in section 3.5, the following plasmids were available for insertion of an antibiotic resistance cassette: pBS-opaA1-opaA2; pCR2.1-opaD1-opaD2; pBS-opaJ1-opaJ2. EryR was suitable for all plasmids, whereas KanR was not suitable for pCR2.1-opaD1-opaD2 due to the presence of a kanamycin resistance gene in pCR2.1.

Vector and insert DNA were prepared by digestion with either *Bam*HI (for insertion into pBS-opaA1-opaA2 or pBS-opaJ1-opaJ2) or *Sgr*AI (for insertion into pCR2.1-opaD1-opaD2). Digests were carried out for 16 hours and the desired fragments were extracted from agarose gels. Initial ligation reactions contained 6ng of vector DNA and 18ng of insert DNA, estimated by comparison to DNA Hyperladdder I (Bioline) during agarose gel electrophoresis. The ligation reactions are summarised in table 3.7.

DNA from the ligation reactions was used to transform *E. coli* DH5α (section 2.2.2), which was grown on LB agar containing kanamycin or erythromycin, as appropriate. Plasmid DNA was purified from selected colonies and screened by restriction enzyme digestion using at least three enzymes, chosen as described in section 3.5 (table 3.8).

Plasmid	Plasmid size (bp)	Vector	Vector size (bp)	Insert	Insert size (bp)	Number of ligations	Number of *E. coli* colonies screened	Number of successful clones†
pBS-opaA-kan	7465	pBS-opaA1-opaA2	6201	KanR	1264	28	88	0 (74)
pBS-opaA-ery	7393	pBS-opaA1-opaA2	6201	EryR	1192	19	41	0
pBS-opaA-eryB*	5557	pBS-opaA1-opaA2	3966	EryR	1631	2	2	0
pCR2.1-opaD-ery	9475	pCR2.1-opaD1-opaD2	8281	EryR	1194	23	29	0
pCR2.1-opaD-eryB*	8695	pCR2.1-opaD1-opaD2	7104	EryR	1631	1	1	0
pBS-opaJ-kan	8444	pBS-opaJ1-opaJ2	7180	KanR	1264	38	21	0 (4)
pBS-opaJ-ery	8372	pBS-opaJ1-opaJ2	7180	EryR	1192	14	35	1

Table 3.7 Summary of cloning of antibiotic resistance cassettes into locus-specific *opa* plasmids.
*****Blunt-ended ligation; † numbers in parentheses indicate clones in which KanR inserted in the opposite direction to the *opa* gene.

Plasmid	Size (bp)	Restriction enzyme fragment sizes (bp)						
		***Ava*I**	***Ava*II**	***Bss*HII**	***Eco*ICRI**	***Nsi*I**	***Pvu*II**	***Sca*I**
pBS-opaA-kan	7465	56, 224, 274, 1265, 1654, 3992	–	2788, 4677	–	–	–	7465
pBS-opaA-ery	7393	56, 448, 1243, 1654, 3992	–	2788, 4605	–	7393	–	–
pBS-opaA-eryB	5557	–	–	–	364, 646, 4547	–	2513, 3044	5557
pCR2.1-opaD-ery	9475	–	–	–	–	4087, 5388	360, 717, 2433, 5965	9475
pCR2.1-opaD-eryB	8695	–	–	–	364, 1661, 2714, 3956	–	360, 717, 2433, 5185	8695
pBS-opaJ-kan	8444	171, 224, 274, 297, 423, 437, 1450, 5168	222, 244, 3077, 4901	–	–	–	2513, 5931	8444
pBS-opaJ-ery	8372	171, 297, 423, 437, 633, 1243, 5168	–	–	–	8372	2513, 5859	–

Table 3.8 Sizes of plasmids and restriction enzyme fragments following cloning of antibiotic resistance cassettes into locus-specific *opa* plasmids.

Cloning of KanR into pBS-opaA1-opaA2 and pBS-opaJ1-opaJ2 did not result in any plasmids where the orientation of KanR and *opa* were the same, although KanR did insert in the opposite direction. Cloning of EryR into pBS-opaA1-opaA2 and pCR2.1-opaD1-opaD2 was unsuccessful. EryR was successfully cloned into pBS-opaJ1-opaJ2 to create pBS-opaJ-ery after some modifications were made to the original method, which are described below.

In order to systematically investigate the lack of success of these cloning steps, a series of experiments was performed using pBS-opaA1-opaA2 and EryR (table 3.9). This confirmed that failure of the ligation reaction was likely to be the primary problem, although the transformation process could not be excluded as an additional cause.

Experiment number	Ligation reaction			Plasmid transformed	Number of colonies following transformation (25µl per plate)		Media used*
	Vector	Insert	T4 DNA ligase		18 hours	42 hours	
1	pBS-opaA1-opaA2	EryR	yes	pBS-opaA-ery	0	0	LB + ery / LB + amp
2	pBS-opaA1-opaA2	–	no	pBS-opaA1-opaA2 (linear)	0	0	LB + amp
3	pBS-opaA1-opaA2	–	yes	pBS-opaA1-opaA2	1	1	LB + amp
4	pBS-opaA1-opaA2	EryR	yes	pBS-opaA-ery	PCR analysis following ligation – no products seen†		
5‡	pBluescript	opaA1	yes	pBS-opaA1	3	3	LB + amp + X-gal + IPTG
6	purified plasmid used, no ligation done			pBS-opaA1	confluent growth	–	LB + amp + X-gal + IPTG
7	purified plasmid used, no ligation done			pER2	585	1,060	LB + ery
					confluent growth	–	LB + amp
8	No DNA transformed, bacteria only				confluent growth	–	LB
					0	0	LB + ery / LB + amp

Table 3.9 Results of ligations and transformations used to investigate failure of cloning.
*amp = ampicillin; ery = erythromycin. †PCR was performed using primer nitF1SalI (table 3.2) and either ery-bamf or ery-bamr (section 3.6.1) as described in section 2.9.1, using an extension time of 5 minutes. ‡See table 3.4 for results from previous cloning. Experiment 1 confirmed lack of successful recombinant clones. Experiment 2 suggested that no circular vector plasmids were remaining after restriction enzyme digestion prior to ligation. Low number of transformed colonies using self-ligated vector (experiment 3), absence of PCR products following ligation (4) and lower yield of colonies compared to previous cloning experiments (5) suggested failure of ligation and possibly transformation. Transformation of whole plasmids remained very efficient (6 and 7) so ligation was likely to be the limiting step. There was no natural development of resistance to ampicillin or erythromycin by *E. coli* (8).

A number of modifications were therefore made to improve the ligation and transformation process:

(i) The vector : insert ratio in the ligation reaction was varied between 1:0.2 and 1:5 to optimise the reaction, for reasons described in section 3.5.2;

(ii) The total amount of DNA in the ligation reactions was increased. Self-ligation of the vector plasmids was more likely during these reactions compared to those described in section 3.5 because the vectors had complementary ends. An increase in total DNA favoured intermolecular rather than intramolecular reactions, improving the likelihood of successful ligation;

(iii) SAP was used to dephosphorylate vector DNA prior to ligation. This removed terminal 5'-phosphate groups from the vector, minimising self-ligation because DNA ligase catalyses the formation of a phosphodiester bond between adjacent nucleotides only if one carries a 5'-phosphate residue and the other a 3'-hydroxyl terminus. The insert DNA with intact 5'-terminal phosphate residues can then be ligated more efficiently into the vector;

(iv) Blunt-ended ligation reactions were performed. Vector and insert DNA were prepared by utilising *Fsp*AI, *Ale*I and *Pvu*II sites which were already present, rather than the *Bam*HI sites (figure 3.7). Insertion of the antibiotic resistance genes at the *Bam*HI site may have resulted in unstable plasmids. Insertion at different sites, as well as excision of a larger fragment of the vector could improve plasmid stability;

(v) T4 DNA ligase was purchased from a different manufacturer;

(vi) Different strains of *E. coli* (table 2.2) were used for transformation. If the ligation reactions were successful, the resulting plasmid may have been

unstable or toxic in *E.coli* DH5α, but possibly not with other strains. All competent cells were used according to manufacturer instructions, which were essentially as described in section 2.2.2. The only differences were slight variation of the incubation periods: all reactions were initially incubated for 30 minutes on ice; heat shock was 45 seconds for DH5α, DH10B and Stbl3, 30 seconds for Mach1-T1, OmniMAX 2-T1 and TOP10 and 25 seconds for Stbl2; recovery period was 1 hour for all strains except Stbl2, which was 1.5 hours.

(vii) *E. coli* cells were transformed with lower volumes of the ligation reaction to avoid any possible effect of the ligation reaction components on the transformation process;

(viii) The length of recovery of *E. coli* cells in the transformation process after heat shock was increased from 1 hour to 2-4 hours (section 2.2.2). This allowed increased time for plasmid replication and potentially increased expression of the antibiotic resistance gene.

Implementation of (ii), (iii) and (v) led to the construction of pBS-opaJ-ery, but none of the modifications resulted in successful insertion of KanR or EryR into pBS-opaA1-opaA2 or EryR into pCR2.1-opaD1-opaD2.

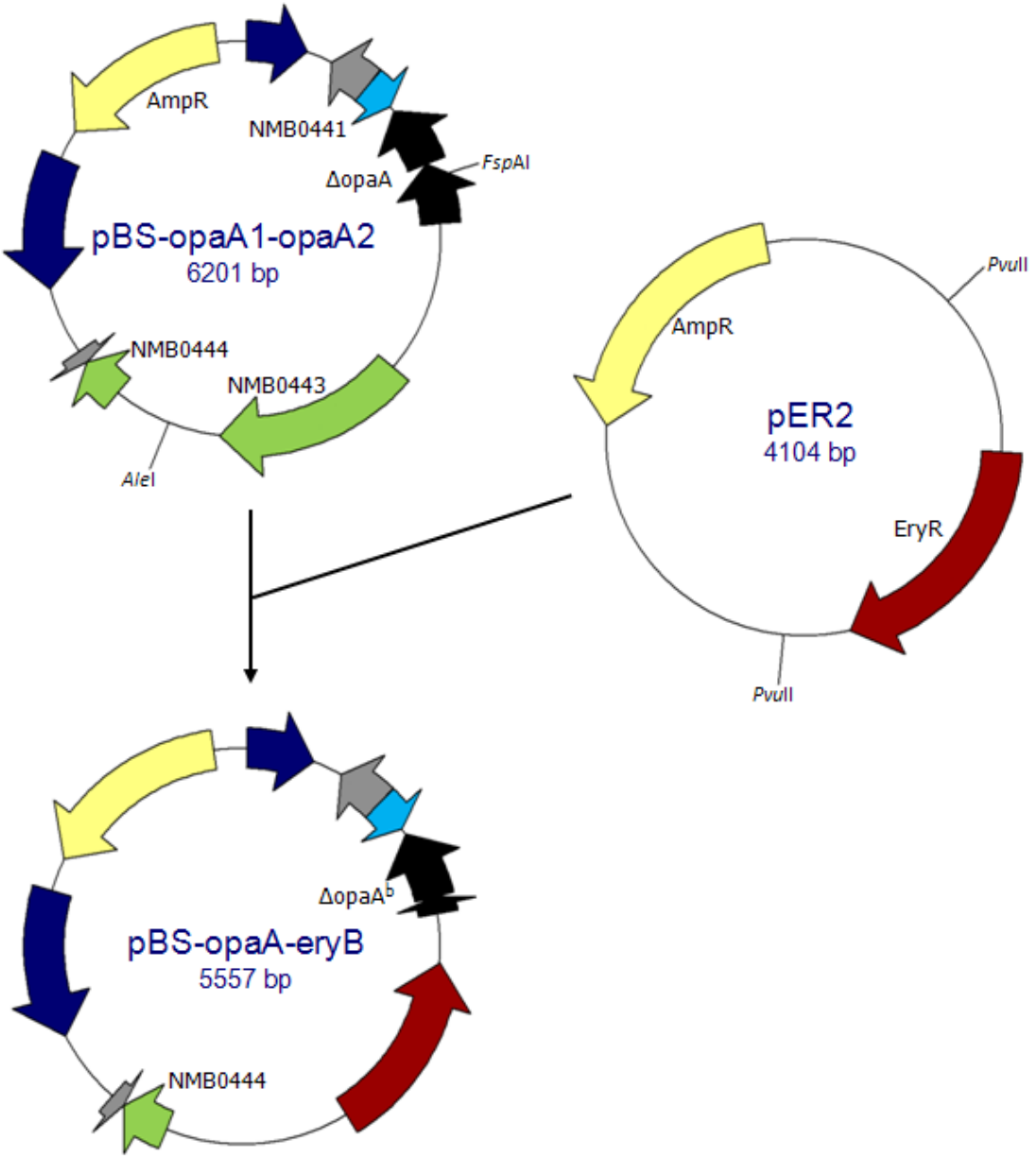

Figure 3.7 Insertion of EryR into pBS-opaA1-opaA2 by blunt ended ligation.
A 1,591 bp *Pvu*II fragment of pER2 carrying EryR is cloned into pBS-opaA1-opaA2 following *Fsp*AI-*Ale*I digestion. This deletes further *opaA* sequence, including the CR tract. The resulting plasmid pBS-opaA-eryB contains EryR in an intergenic region.

3.7. Discussion

The aim of the experiments described in this chapter was to construct plasmids to enable sequential, locus-specific disruption of the four *opa* genes of *N. meningitidis* strain H44/76. Two plasmids for each *opa* gene would be required, and contain the 5' and 3' ends of the gene ligated together with or without the insertion of an antibiotic

resistance cassette. Both plasmids were obtained for *opaJ*, but only those without an antibiotic resistance cassette were constructed for *opaA* and *opaD*. For the *opaB* gene, neither plasmid was successfully created.

The construction of only four of the desired plasmids raises a number of questions regarding the reasons for failure, given that the same approach was used for all four *opa* genes and that the *opa* sequences are highly homologous. The major difference between the four *opa* gene fragments used in these experiments was the length and sequence of the associated locus-specific regions amplified in the initial PCR. In order to create locus-specific plasmids, one PCR primer from each pair was targeted to an adjacent gene, resulting in PCR products varying between 511 bp and 2,838 bp in length. In addition to the length, the sequence of adjacent DNA also varied between PCR products. The series of experiments summarised in table 3.9 suggested that failure of the ligation reaction was the likely reason for the lack of success. A number of modifications were made to the ligation and transformation reactions, resulting in some success, but the construction of four plasmids remained elusive.

One possible explanation is the relatively large size of some of the PCR products. However, the phagemid pBluescript is a common vector into which large DNA fragments have previously been inserted[307, 308] and some of the larger PCR products were successfully cloned, suggesting that other factors were important. A second possible reason is differences in DNA secondary structure between different plasmids, with some being inherently unstable. The observation that KanR inserted in the incorrect orientation into both *opaA* and *opaJ*, despite the fact that 50% of successful

recombinant clones should have contained KanR in the correct orientation, provides some evidence to support this. A third possibility is that the nucleotide sequences of the different *N. meningitidis* fragments were influencing the process. The *opa* CR tract may have interfered with transcription or translation of the antibiotic resistance gene. Successful cloning of EryR into *opaJ* suggests that this may not have been a major factor, although the tracts are of different lengths in the four *opa* genes[156]. Sequence analysis was not undertaken at the time as this would not have provided definitive evidence of an effect of the CR tract. Subsequent DNA sequencing of the CR tracts (section 5.5) did not provide any evidence that differences in CR tract lengths were a reason for failure of cloning of some *opa* genes. Alternatively, the presence of repetitive elements within the intergenic DNA, such as Correia repeats, may have caused instability of some plasmids. Correia repeats are found frequently throughout the *N. meningitidis* genome, and these elements are present adjacent to all four *opa* genes[156, 309, 310]. Fourthly, some of the foreign gene products may have been toxic to *E. coli*, resulting in failure of transformation. Finally, it is possible that ligation and transformation for these plasmids was very inefficient and success would only be achieved by continued replication of these experiments. However, these procedures were already carried out on a large number of occasions (table 3.7) making this an unreasonable long term strategy.

A number of approaches were attempted to achieve construction of the locus-specific *opa* plasmids, with varying success for each of the four *opa* genes. The success rate of ligation reactions was improved by increasing the total amount of DNA, dephosphorylation of vector DNA and use of T4 DNA ligase from a different

manufacturer. However, alteration of the vector : insert ratio during ligation, blunt-ended ligation, or alteration of *E. coli* transformation conditions were not helpful. It is ultimately not possible to unambiguously state why some plasmids were successfully constructed and others were not. The principal factors may have been different for each plasmid. Although the plasmids obtained would have enabled disruption of *opaA*, *opaD* and *opaJ*, only the latter would have utilised an antibiotic resistance gene, making the process inefficient, time-consuming and possibly unsuccessful. An alternative approach for constructing plasmids which would be more suitable to create Opa-deficient meningococci is described in chapter 4.

CHAPTER 4. CONSTRUCTION OF GENERIC *OPA* PLASMIDS

4.1. Introduction

It was not possible to disrupt all four *opa* genes of *N. meningitidis* using the locus-specific plasmids constructed in chapter 3. An alternative strategy was therefore required to obtain suitable plasmids to achieve this objective. Review of the DNA sequence of the four *opa* genes of strain H44/76 revealed 96% similarity of the 253 bp at the 5' end and 93% similarity of the 228 bp at the 3' end to each other. More specifically the similarity between *opaA* and *opaJ* was 99%, and between *opaB* and *opaD* was 97% within these regions. This suggested that it may be possible to disrupt all four *opa* genes using two groups of plasmids – one group based on *opaA* or *opaJ* and the other based on *opaB* or *opaD*. Each group would contain the 5' and 3' ends of an *opa* gene without any locus-specific sequences, flanking different antibiotic resistance cassettes.

Generic *opa* plasmids could be derived from the locus-specific plasmids described in chapter 3 by specifically amplifying the *opa* sequences using generic PCR primers and cloning these products (figure 4.1). The resulting plasmids would be smaller than the locus-specific plasmids, would not contain DNA from the intergenic regions or from adjacent genes, and would exclude the *opa* CR tract. This would therefore avoid some of the proposed causes of failure described in chapter 3. In addition the plasmid pUC4NmnDUS, containing a tetracycline resistance cassette, was acquired and could be utilised as an additional selectable marker.

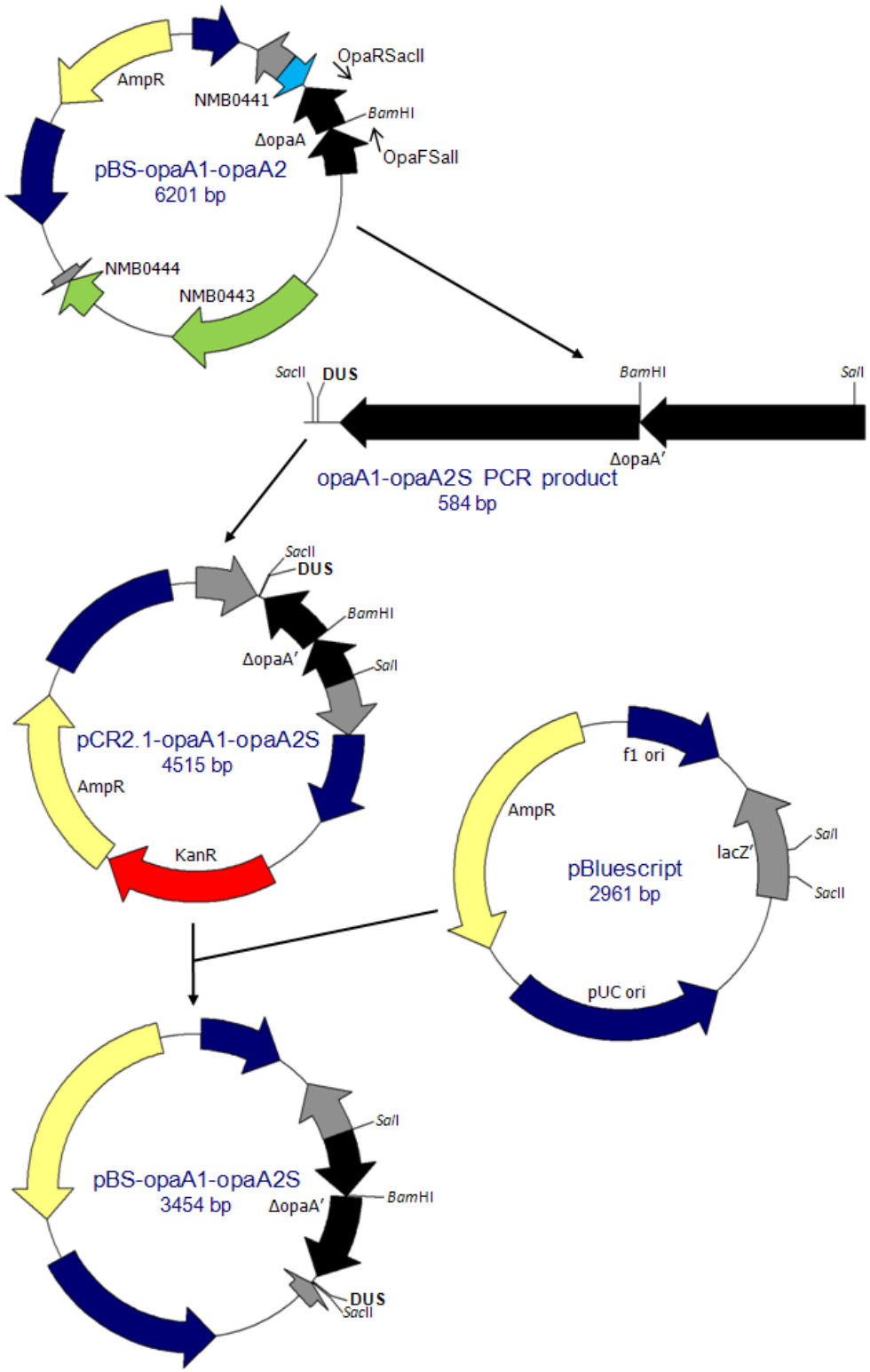

Figure 4.1 Construction of generic *opaA* plasmid from locus-specific *opaA* plasmid.
Firstly, Δ*opaA* was amplified from pBS-opaA1-opaA2 using generic *opa* primers OpaFSalI and OpaRSacII. The resulting product opaA1-opaA2S was a further modified version of *opaA*, Δ*opaA*', which also contained *Sal*I and *Sac*II sites, and included the neisserial DNA uptake sequence (DUS) which is present downstream of *opaA*. In the second step, opaA1-opaA2S was cloned into the TOPO TA vector pCR2.1 to obtain pCR2.1-opaA1-opaA2S. Finally, a 563 bp *Sac*II-*Sal*I fragment containing Δ*opaA*' from pCR2.1-opaA1-opaA2S was cloned into pBluescript to construct pBS-opaA1-opaA2S. Similar plasmids based on other *opa* genes were produced in the same way.

This chapter describes a series of experiments which were performed to construct generic *opa* plasmids based on *opaA*, *opaD* and *opaJ*. These plasmids contained the 5' and 3' ends of each *opa* gene ligated together with and without (for *opaA* and *opaD*) an antibiotic resistance cassette.

4.2. Methods

The methods used in this chapter were transformation of *E. coli* (section 2.2), purification of DNA (section 2.7), agarose gel electrophoresis (section 2.8) and manipulation of DNA (section 2.9).

4.3. Construction of plasmids without antibiotic resistance cassettes

The 5' and 3' ends of *opaA* and *opaD* had previously been ligated together to construct plasmids pBS-opaA1-opaA2 and pCR2.1-opaD1-opaD2 (section 3.5). These plasmids were used as the basis for constructing generic *opa* plasmids. The *opa* sequences from pBS-opaA1-opaA2 and pCR2.1-opaD1-opaD2 were amplified by PCR using the generic *opa* primers OpaFSalI (5'-TTCCGCGTCGACGGCGGCAAGTGAAGACG-3') and OpaRSacII (5'-ATGCCGCCGCGGGGTTCAGACGGCATCG-3'). Primers were designed to amplify as much of the *opa* genes and adjacent homologous regions as possible, including a downstream neisserial DNA uptake sequence (DUS) (section 5.1) and without the CR tract, and to introduce *Sal*I and *Sac*II sites (underlined) at the ends of the PCR products. PCR was performed as in section 2.9.1, using an extension time of 3 minutes to produce products of 584 bp. Agarose gel electrophoresis of the PCR

products revealed multiple bands, so the desired bands were gel extracted prior to cloning.

The PCR products opaA1-opaA2S and opaD1-opaD2S were cloned separately into the TOPO TA vector pCR2.1 (section 2.9.3). DNA from the TA cloning reactions was used to transform *E. coli* DH5α (section 2.2.2), which was grown on LB agar containing ampicillin, X-gal and IPTG. Plasmid DNA was purified from selected colonies and screened by restriction enzyme digestion using *Sal*I and *Sac*II to produce fragments of 563 bp and 3,952 bp, demonstrating successful introduction of these restriction sites.

The modified *opaA* and *opaD* genes were subsequently cloned into pBluescript. Vector and insert DNA were prepared by double digestion with *Sal*I and *Sac*II for 16 hours and the vector pBluescript was additionally dephosphorylated before the desired fragments were extracted from agarose gels. Ligation reactions (table 4.1) contained 13-20ng of vector DNA and 39-60ng of insert DNA, estimated by comparison to DNA Hyperladdder I (Bioline) during agarose gel electrophoresis, and the vector : insert ratio was 1:3. The ligation reaction products were digested with *Not*I prior to transformation because a site for this restriction enzyme is present in pBluescript and not in pBS-opaA1-opaA2S or pBS-opaD1-opaD2S, as described in section 3.5.1. The dephosphorylation step, use of increased amounts of DNA and restriction enzyme digestion post-ligation were introduced based on their previous successful use (section 3.6.2).

Plasmid	Plasmid size (bp)	Vector	Vector size (bp)	Insert	Insert size (bp)	Number of ligations	Number of *E. coli* colonies screened	Number of successful clones
pBS-opaA1-opaA2S	3454	pBluescript	2885	opaA1-opaA2S	563	1	5	5
pBS-opaA-kanS	4718	pBS-opaA1-opaA2S	3454	KanR	1264	1	5	5
pBS-opaA-tetS	5501	pBS-opaA1-opaA2S	3454	TetR	2047	2	6	1
pBS-opaD1-opaD2S	3454	pBluescript	2885	opaD1-opaD2S	563	1	5	5
pBS-opaD-kanS	4718	pBS-opaD1-opaD2S	3454	KanR	1264	1	5	5
pBS-opaD-tetS	5501	pBS-opaD1-opaD2S	3454	TetR	2047	3	11	2
pCR2.1-opaD-eryS	5709	pCR2.1-opaD1-opaD2S	4515	EryR	1194	1	1	1

Table 4.1 Summary of cloning of generic *opa* plasmids.

Plasmid	Size (bp)	Restriction enzyme fragment sizes (bp)						
		***Ava*I**	***Ava*II**	***Bam*HI**	***Eco*ICRI**	***Pvu*II**	***Sac*II**	***Sca*I**
pBS-opaA1-opaA2S	3454	173, 3281	222, 3232	–	–	941, 2513	–	3454
pBS-opaA-kanS	4718	173, 224, 274, 4047	222, 1921, 2575	–	–	2205, 2513	–	4718
pBS-opaA-tetS	5501	–	222, 5279	–	–	2513, 2988	5501	2550, 2951
pCR2.1-opaD1-opaD2S	4515	218, 4297	222, 591, 1682, 2020	–	–	–	–	4515
pBS-opaD1-opaD2S	3454	173, 3281	222, 1570, 1662	–	–	941, 2513	–	3454
pBS-opaD-kanS	4718	173, 224, 274, 4047	222, 259, 1662, 2575	–	–	2205, 2513	–	4718
pBS-opaD-tetS	5501	–	222, 1662, 3617	–	–	2513, 2988	5501	2550, 2951
pCR2.1-opaD-eryS	5709	–	–	1586, 4123	1086, 4623	–	–	5709
pCR2.1-opaJ-eryS	5707	–	–	–	1269, 4438	360, 717, 2197, 2433	–	5707

Table 4.2 Sizes of plasmids and restriction enzyme fragments following cloning of generic *opa* plasmids.

DNA from the ligation reactions was used to transform *E. coli* DH5α (section 2.2.2), which was grown on LB agar containing ampicillin, X-gal and IPTG. Plasmid DNA was purified from selected colonies and screened by restriction enzyme digestion using at least three enzymes, chosen as described in section 3.5 (table 4.2). These cloning steps resulted in the construction of the generic *opa* plasmids pBS-opaA1-opaA2S, pCR2.1-opaD1-opaD2S and pBS-opaD1-opaD2S.

4.4. Construction of plasmids with antibiotic resistance cassettes

The tetracycline resistance cassette, which was not available during the experiments described in chapter 3, was obtained from pUC4NmnDUS (gift from M Deadman). The plasmid pUC4NmnDUS is based on pUC4-kan, in which KanR has been excised and replaced by a derivative of the transposon Tn10. In addition, neisserial DNA uptake sequences have been cloned at both ends of the inserted transposon (figure 4.2). The transposon Tn10 encodes resistance to tetracycline and contains two genes, *tetA* and *tetR*, which are transcribed from divergent tetracycline-inducible promoters. *tetA* encodes a tetracycline efflux protein and *tetR* encodes a regulatory protein which prevents transcription from the *tetA* and *tetR* promoters in the absence of tetracycline. Tetracycline interacts with the TetR protein to relieve repression and induce transcription of both *tetR* and *tetA*. The entire tetracycline resistance cassette will henceforth be referred to as TetR. The TetR cassette was excised from pUC4NmnDUS by digestion with *Bam*HI. The KanR and EryR cassettes were prepared as described in section 3.6.1.

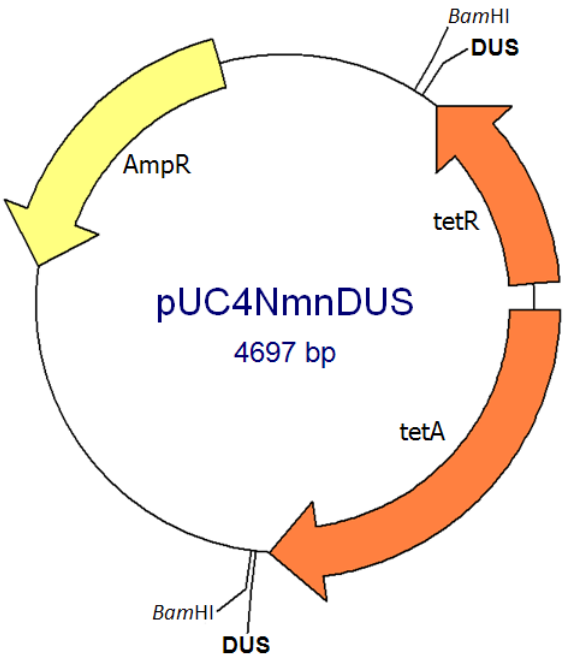

Figure 4.2 Plasmid pUC4NmnDUS.

In order to target all four *opa* genes of *N. meningitidis* using each of the three antibiotic resistance cassettes for selection, six plasmids containing KanR, EryR or TetR were required. For each antibiotic resistance cassette, one plasmid was based on *opaA* or *opaJ*, and one plasmid was based on *opaD* (table 4.1). To produce pBS-opaA-kanS, pBS-opaA-tetS, pBS-opaD-kanS and pBS-opaD-tetS, KanR or TetR were cloned into the *Bam*HI site within the *opa* genes (figure 4.3). To obtain pCR2.1-opaD-eryS, EryR was cloned into the *Sgr*AI site within *opaD*. The plasmid pCR2.1-opaJ-eryS was constructed from the locus-specific plasmid pBS-opaJ-ery (section 3.6.2). This was achieved by amplification of the *opa* sequences along with EryR by PCR using primers opaFSalI and opaRSacII, gel extraction of the desired fragment and TA cloning into pCR2.1, as described in section 4.2.

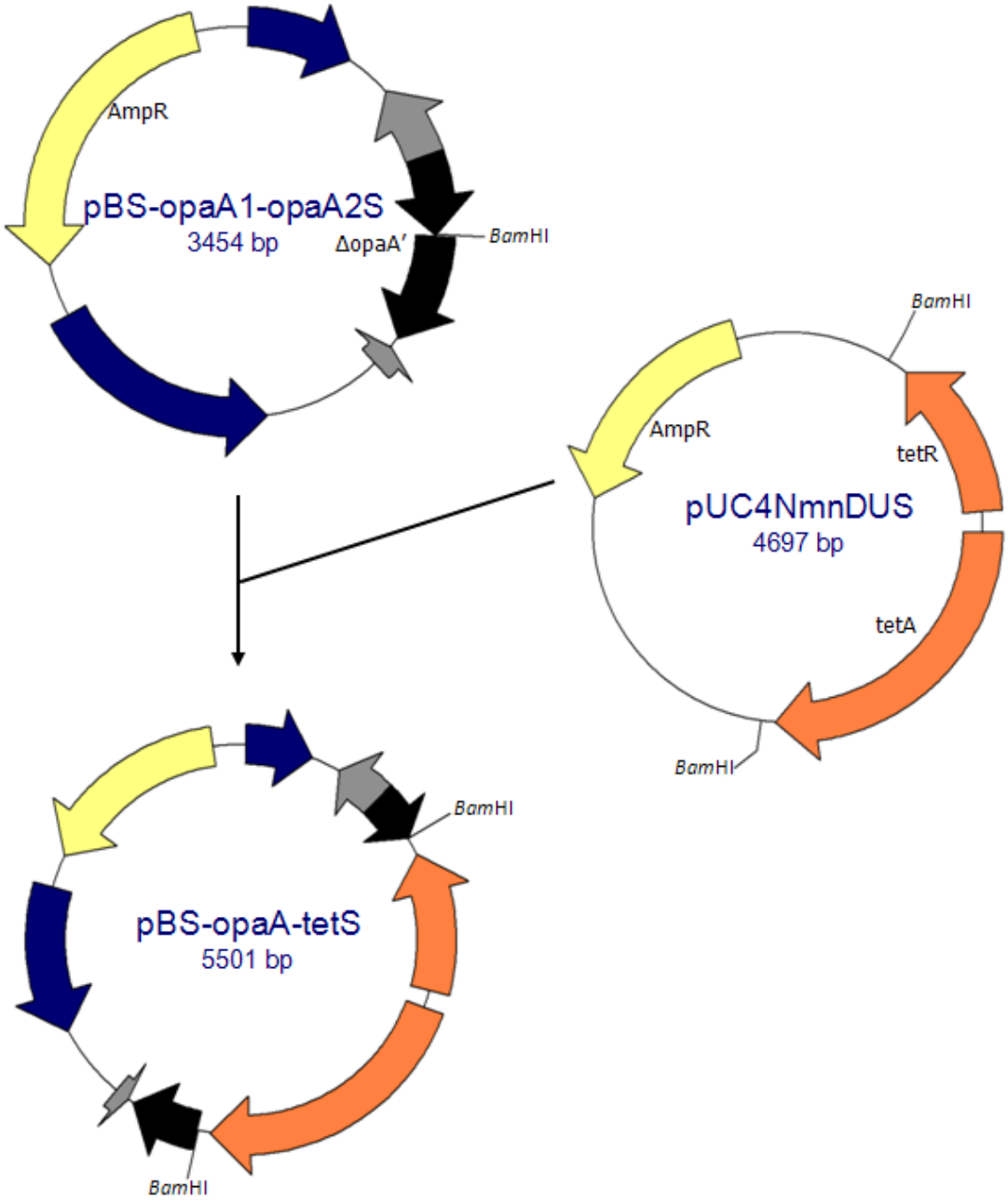

Figure 4.3 Insertion of TetR into pBS-opaA1-opaA2S.
A 2,047 bp *Bam*HI fragment of pUC4NmnDUS containing the TetR cassette, which includes the *tetA* and *tetR* genes, was cloned into pBS-opaA1-opaA2S. Similar plasmids based on other *opa* genes and different antibiotic resistance cassettes were produced in the same way.

For ligation reactions, vector and insert DNA were prepared by digestion with either *Bam*HI (for insertion into pBS-opaA1-opaA2S or pBS-opaD1-opaD2S) or *Sgr*AI (for insertion into pCR2.1-opaD1-opaD2S) for 16 hours, and the vectors were additionally dephosphorylated before the desired fragments were extracted from agarose gels.

Ligation reactions (table 4.1) contained 32-108ng of vector DNA and 97-324ng of insert DNA, estimated by comparison to DNA Hyperladdder I (Bioline) during agarose gel electrophoresis, and the vector : insert ratio was 1:3. DNA from the ligation reactions was used to transform *E. coli* DH5α (section 2.2.2), which was grown on LB agar containing kanamycin, erythromycin or tetracycline, as appropriate. Plasmid DNA was purified from selected colonies and screened by restriction enzyme digestion using at least three enzymes, chosen as described in section 3.5. (table 4.2). The following plasmids were constructed: pBS-opaA-kanS; pBS-opaA-tetS; pBS-opaD-kanS; pBS-opaD-tetS; pCR2.1-opaD-eryS and pCR2.1-opaJ-eryS. All three antibiotic resistance cassettes had been successfully introduced into *opaA* or *opaJ*, and *opaD*, as required.

4.5. Discussion

This chapter describes the successful construction of generic *opa* plasmids containing the antibiotic resistance cassettes KanR, EryR and TetR. There is a high degree of similarity between *opaA* and *opaJ* and between *opaB* and *opaD* for strain H44/76, so for each antibiotic resistance cassette one plasmid was produced based on *opaA* or *opaJ* and a second based on *opaD*. These plasmids would therefore be suitable to use for sequential disruption of all four *opa* genes of *N. meningitidis*.

Following only partial success in the construction of locus-specific plasmids, described in chapter 3, a number of modifications were made to the ligation reactions used in the experiments described in this chapter. A higher total concentration of DNA was used, all vectors were dephosphorylated with SAP prior to gel extraction

and T4 DNA ligase was purchased from a different manufacturer. Where possible the ligation reaction products were digested with a restriction enzyme prior to transformation of *E. coli*, to linearise any persisting self-ligated vector DNA. The rationale for these is given in section 3.5.1. These adaptations were implemented because they appeared to improve the success rate of some experiments described in chapter 3.

There are a number of possible reasons for the increased success observed during the construction of generic *opa* plasmids compared to the locus-specific plasmids. It is difficult to know whether the improved success of cloning was due to modifications made to the ligation reactions, or the fact that the generic plasmids were much smaller than locus-specific plasmids. In particular the generic plasmids do not contain any repetitive elements from the intergenic regions, or the *opa* CR tract. These plasmids would now theoretically allow the construction of Opa-deficient strains of *N. meningitidis* by sequential transformation, described in chapter 5.

CHAPTER 5. CONSTRUCTION AND CHARACTERISATION OF OPA-DEFICIENT STRAINS

5.1. Introduction

N. meningitidis is one of the naturally transformable bacterial species, having the ability to take up extracellular DNA efficiently, and then incorporate it into the chromosome by homologous recombination[311]. This mechanism may have evolved to aid in the repair of damaged chromosomes, as bacteria are most likely to encounter DNA from nearby cells of the same species. Meningococci have harnessed transformation as a powerful mechanism for generating genetic diversity, spreading advantageous allelic variants and mediating some forms of antigenic variation.

Efficient transformation of Neisseria is dependent upon the presence of a DUS in the exogenous DNA[312], surface expression of type IV pilus[313, 314] and homologous recombination mediated by RecA. The 10 bp DUS sequence 5'-GCCGTCTGAA-3' is by far the most frequent repeat sequence element in the meningococcal genome, with 1910 occurrences in strain MC58[156]. Genome scanning of different Neisseria has revealed that 76% of these sequences have two additional semi-conserved base pairs extending from the 5' end of the DUS to constitute the 12 bp sequence 5'-ATGCCGTCTGAA-3'[315]. Plasmids containing the 12 bp sequence were more efficient at transformation than those with the 10 bp DUS. The 12 bp DUS is present 16 bp downstream of the 3' end of all *opa* genes of strain H44/76.

Transformation of DNA by piliated meningococci and gonococci generally occurs at a frequency of 10^{-1} to 10^{-3} per cell. Type IV pili are long, thin appendages present on

the meningococcal surface (figure 1.1), and are important in bacterial cell-to-cell interactions and adhesion to host cells. DUS-specific binding of DNA to a bacterial cell depends on the presence of the major pilin (PilE) and can be modulated by the expression of different minor pilins, although it is unclear whether pili themselves are directly involved in transformation[316]. Only one strand of the DNA molecule is effectively transported into the cytoplasm, while the other strand is degraded. Once single-stranded DNA is within the cytoplasm, it can be integrated into the meningococcal chromosome via a RecA-dependent process which requires sequence homology between the exogenous and chromosomal DNA[317].

The ability of *N. meningitidis* to naturally incorporate exogenous DNA into its chromosome can be manipulated *in vitro* to disrupt specific genes. Exogenous DNA, such as antibiotic resistance cassettes, flanked by two regions of homology can be integrated stably into the chromosome via a double-crossover event (figure 5.1). The plasmids described in chapter 3 and chapter 4 were all suitable for transformation of *N. meningitidis* because they contained the neisserial DUS. The generic *opa* plasmids were constructed such that the DUS downstream of each *opa* gene was retained (figure 4.1) and TetR contained additional uptake sequences (figure 4.2). The locus-specific *opa* plasmids contained two homologous regions, up to 2,826 bp in length, which were identical to the targeted *opa* gene. In contrast, the generic plasmids included 253 bp of homology at the 5' end and 228 bp of homology at the 3' end of the *opa* gene where homologous recombination could occur. Furthermore, the generic plasmids would only be identical to the gene on which they were based, but only 97-99% similar in these homologous regions to the second *opa* gene which was being

targeted. It was therefore likely that transformation would be more efficient using locus-specific plasmids than generic plasmids, and for the generic plasmids recombination at the identical locus would be more frequent than at the second locus being targeted. Generic plasmids based on *opaA* and *opaJ* were used to target both of these genes, and plasmids based on *opaD* were used to target *opaD* and *opaB*.

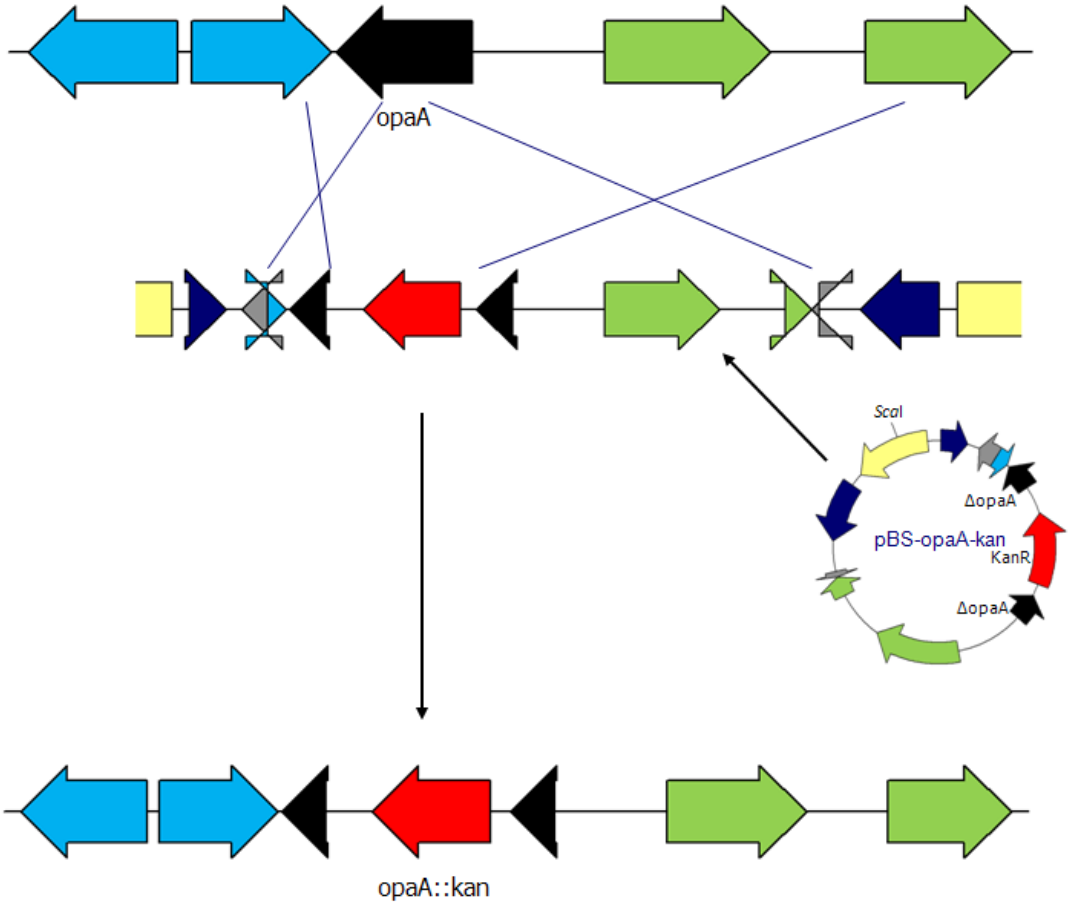

Figure 5.1 Homologous recombination in *N. meningitidis*.
The plasmid pBS-opaA-kan contains sequences identical to the 5' and 3' ends of *opaA* and adjacent locus-specific sequences flanking KanR. Crossover events between chromosomal and plasmid DNA are illustrated using linearised plasmid. A double crossover event between homologous regions on the plasmid and chromosome allow KanR to be stably inserted into the chromosome at the *opaA* locus.

The aim of sequential disruption of *opa* genes was to construct a panel of Opa-deficient strains with different patterns of Opa expression so the immunological

effects of different Opa variants could be compared. One of the major difficulties in achieving this is the issue of PV. Although *opa* genes are constitutively transcribed, Opa protein expression undergoes PV at the translational level due to the presence of the pentameric CR sequence 5'-CTCTT-3' within the sequence encoding the amino-terminal leader peptide[202] (section 1.7.1). Therefore, a strain in which an *opa* gene has been disrupted is unable to express that particular Opa protein, but may or may not express the other proteins. The ideal method to circumvent this problem would be to construct strains that constitutively express different Opa proteins.

Construction of strains which constitutively express Opa has been described for an *opa* gene of *N. gonorrhoeae*[318], but has a number of inherent difficulties. Firstly, the N-terminal sequence would need to be modified such that the pentameric repeat sequence 5'-CTCTT-3' no longer occurs, but the resulting amino acid sequence LFSSL remains unchanged. This may be possible using a series of PCRs with mutagenic primers, due to codon redundancy, but would require the entire resulting modified CR sequence to be a multiple of 15 base pairs to ensure the protein is expressed (figure 1.5), and to be relatively short in order to technically achieve this. The length of the repeat tract varies between 30 and 285 nucleotides[319], and the effect of lengthening or shortening the tract on Opa protein expression is unknown. Secondly, an additional marker would be required to enable selection of a transformant with an *opa* gene with a modified CR sequence into the parent strain, and as described in chapter 4 only 3 marker genes were available. Thirdly, the cloning steps required would have to be performed in *E. coli*, as with the other cloning steps performed (chapter 3 and chapter 4) and high expression of Opa in *E. coli* has

previously proven toxic to the host cells[318]. Finally, it would be necessary to confirm that the entire *opa* sequence had not been significantly modified (i.e. a nucleotide point mutation resulting in an amino acid substitution) throughout the process. Standard DNA polymerases used in PCR lack 3' → 5' exonuclease activity to remove incorrectly incorporated bases, and high fidelity enzymes produce PCR products that usually have a lower cloning efficiency, so it is likely that a number of products at each step would have to be screened to obtain a single, successful transformant.

This chapter describes the sequential disruption of the four *opa* genes of *N. meningitidis* strain H44/76 to construct a set of Opa-deficient isogenic strains, using the plasmids described in chapter 3 and chapter 4. These plasmids each contain two regions of homology with the chromosomal DNA, including the 5' and 3' ends of the *opa* genes, flanking an antibiotic resistance cassette which would allow for selection of bacteria which had incorporated plasmid DNA. All mutant strains were characterised in terms of their *opa* CR DNA sequence and Opa phenotype. Specific strains which were identified as exhibiting different patterns of Opa expression on the bacterial cell surface were identified and assessed in greater detail prior to their further use.

5.2. Methods

The methods used in this chapter were transformation of *N. meningitidis* (section 2.3), production and characterisation of OMVs (section 2.4), protein blotting (section 2.6), purification of DNA (section 2.7.2), agarose gel electrophoresis (section 2.8), PCR (section 2.9.1) and DNA sequencing (section 2.10).

5.3. Strategies to construct Opa-deficient strains of *Neisseria meningitidis*

Two strategies were designed to enable the construction of Opa-deficient meningococci. The first involved using locus-specific plasmids and only one or two antibiotic resistance cassettes. Following the relative lack of success in construction of these plasmids (chapter 3) a second strategy was developed based on the generic plasmids (chapter 4), with the additional benefit that a third antibiotic resistance cassette was available. Ideally four different, selectable markers would be available to disrupt the four *opa* genes, but the strategies described below were designed so that the same marker could be utilised to disrupt more than one gene.

The original strategy devised to construct Opa-deficient meningococci was sequential, specific disruption of *opa* genes using locus-specific plasmids (figure 5.2). Disruption of each *opa* gene would occur in two steps. In the first step, a plasmid containing the 5' and 3' ends of an *opa* gene flanking an antibiotic resistance cassette would be used to specifically disrupt the same *opa* gene. Successfully transformed bacteria could be identified by growth on selective media and confirmed by PCR analysis. During the second step, a similar locus-specific plasmid without an antibiotic resistance cassette would be used to target the same *opa* gene, resulting in a modified *opa* gene with reversion to antibiotic sensitivity. In this case, successfully transformed bacteria could be identified by growth on non-selective media only after replica plating on selective and non-selective media, and confirmed by PCR analysis. The same antibiotic resistance cassette could then be used to disrupt each *opa* gene in turn and the resulting strains would ultimately contain no antibiotic resistance genes.

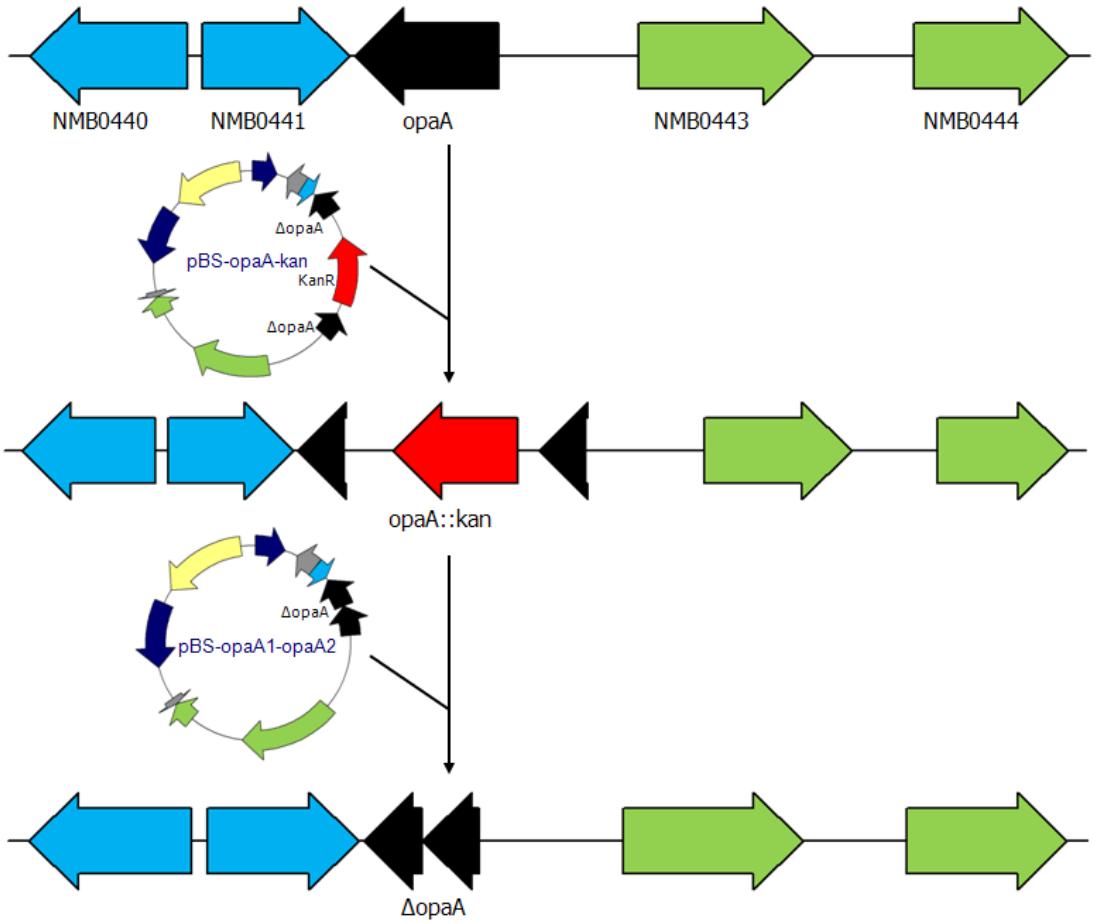

Figure 5.2 Transformation of *N. meningitidis* using locus-specific plasmids to disrupt *opa* genes. In the first transformation, pBS-opaA-kan is used to introduce KanR at the *opaA* locus and confer kanamycin resistance to successfully transformed bacteria. A second transformation with pBS-opaA1-opaA2 then replaces *opaA::kan* with Δ*opaA*, which contains a 185 bp deletion. The *opaA* gene is successfully disrupted and the KanR cassette can be used in a similar fashion to sequentially disrupt all *opa* genes.

The second strategy, utilising generic plasmids, included some modifications from the first because generic plasmids could not be used efficiently in the same manner as locus-specific plasmids (figure 5.3). The first step would result in disruption of different *opa* genes in different bacterial cells, since all *opa* genes would be potential targets for homologous recombination. This is acceptable when introducing antibiotic resistance because the aim was to ultimately disrupt all four *opa* genes. However, it becomes problematic when trying to specifically remove the antibiotic resistance gene during the second step. A generic plasmid would have a lower transformation

efficiency than a locus-specific plasmid (section 5.1). Moreover, screening for loss of antibiotic resistance is more difficult than screening for its introduction. For these reasons using a generic *opa* plasmid for removal of antibiotic resistance would be an extremely inefficient process. The availability of the TetR cassette during the construction of generic *opa* plasmids provided a third selective marker, in addition to EryR and KanR, allowing three *opa* genes to be disrupted without removal of the antibiotic resistance cassette at each step. Removal of the antibiotic resistance cassette on a single occasion, using a locus-specific plasmid, would then be sufficient to create all the desired strains. In contrast to using locus-specific plasmids, this strategy would result in all strains retaining their antibiotic resistance genes.

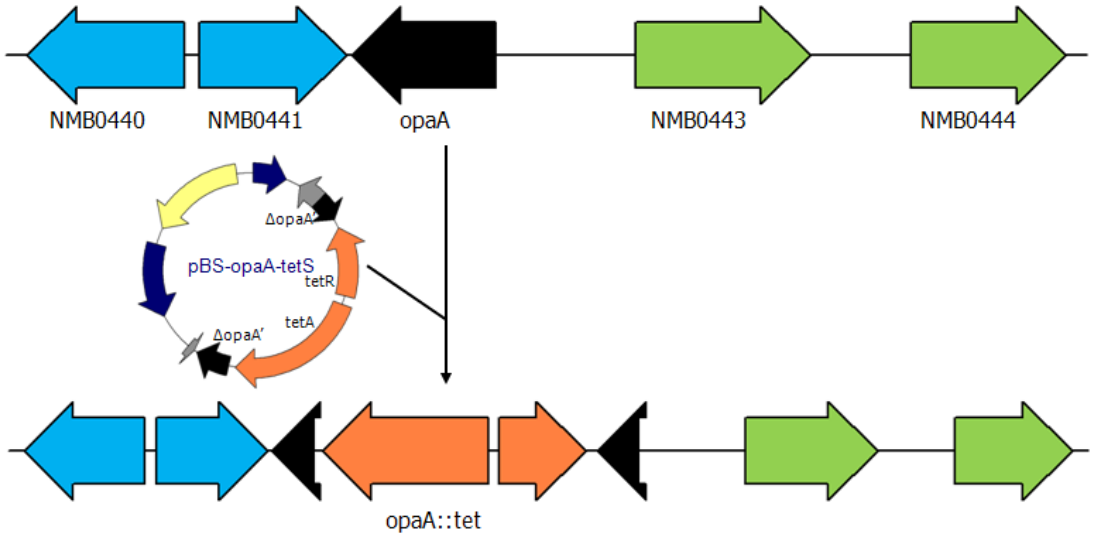

Figure 5.3 Transformation of *N. meningitidis* using generic plasmids to disrupt *opa* genes.
The TetR cassette is introduced into the *opaA* locus by transformation with pBS-opaA-tetS, conferring tetracycline resistance to successfully transformed bacteria. This generic plasmid could also be used to disrupt *opaJ*. The TetR cassette is not removed, so sequential *opa* disruption is achieved by similarly introducing different antibiotic resistance cassettes into different *opa* loci.

5.4. Construction of Opa-deficient meningococci

The sequence of transformations used to construct all Opa-deficient meningococci is summarised in figure 5.4 and table 5.1. Following transformation, chromosomal DNA was purified from selected colonies and screened by PCR as described in section 2.9.1, using an extension time of 3-8 minutes (table 5.2). Firstly, presence of the antibiotic resistance cassettes was confirmed using primers specific to each cassette. Secondly, locus-specific amplification of each *opa* gene using one primer in the neighbouring 5' gene and a second primer in the 3' neighbouring gene confirmed *opa* disruption. Finally, PCR using one primer in a neighbouring gene and a second primer within the antibiotic resistance cassette established the specific location of each antibiotic resistance cassette. Plasmids containing EryR were used to obtain strains with a single *opa* gene disrupted. The locus-specific plasmid pBS-opaJ-ery was used to create an OpaJ-negative strain, whereas the other single Opa-deficient strains were constructed using generic *opa* plasmids based on *opaD* and *opaJ*. Six double Opa-deficient strains were constructed using two different generic plasmids based on *opaA* and *opaD*, and containing KanR. As predicted, generic *opa* plasmids based on *opaA* or *opaJ* resulted in recombination at both loci, and those based on *opaD* could also be used to target the *opaB* locus. In all cases recombination at the identical *opa* locus occurred with a higher frequency than at the other targeted locus.

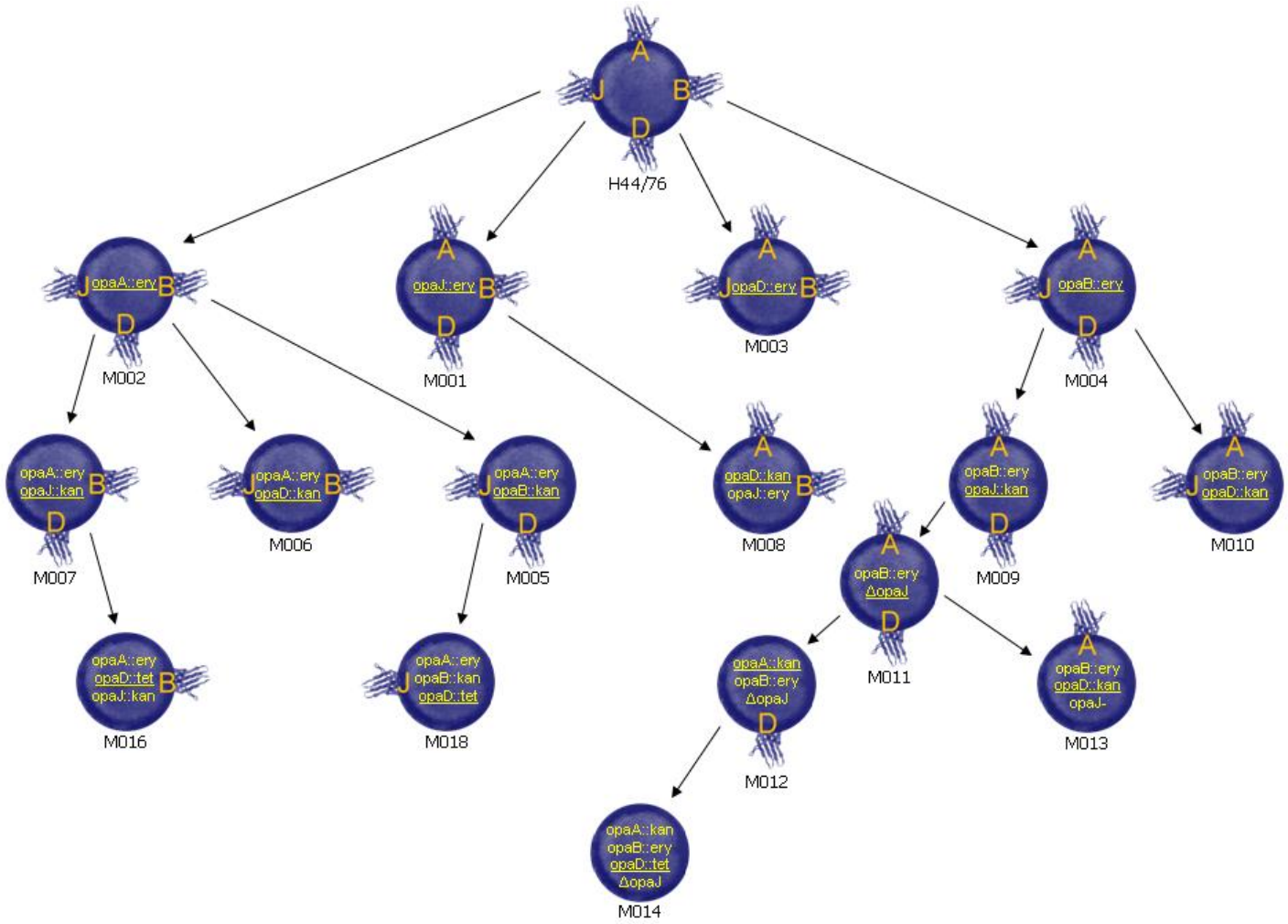

Figure 5.4 Summary of Opa-deficient isogenic mutant meningococci constructed from parent strain H44/76.
The *opa* genotype of each strain is shown, with the new mutation at each step underlined.

Parent strain	Transforming DNA	Adaptations to transformation	Colonies screened	Resulting strain	
				Name	Genotype
H44/76	pBS-opaJ-ery	None	All	M001	*opaJ::ery*
M001	pBS-opaJ1-opaJ2	None	21,860		No new strain
H44/76	pCR2.1-opaJ-eryS	None	All	M002	*opaA::ery*
H44/76	pCR2.1-opaD-eryS	None	All	M003	*opaD::ery*
				M004	*opaB::ery*
M002	pBS-opaD-kanS	None	All	M005	*opaA::ery opaB::kan*
				M006	*opaA::ery opaD::kan*
M002	pBS-opaA-kanS	None	All	M007	*opaA::ery opaJ::kan*
M001	pBS-opaD-kanS	None	All	M008	*opaD::kan opaJ::ery*
M004	pBS-opaA-kanS	None	All	M009	*opaB::ery opaJ::kan*
M003	pBS-opaD-kanS	None	All		No new strain
M004	pBD-opaD-kanS	None	All	M010	*opaB::ery opaD::kan*
M010	pCR2.1-opaD1-opaD2	None	10,000		No new strain
M009	pBS-opaJ1-opaJ2	None	10,000	M011	*opaB::ery ΔopaJ*
M011	pBS-opaA-kanS	None	All	M012	*opaA::kan opaB::ery ΔopaJ*
M011	pBS-opaD-kanS	None	All	M013	*opaB::ery opaD::kan ΔopaJ*
M006	pCR2.1-opaD1-opaD2	None	30,000		No new strain
M006	pCR2.1-opaD1-opaD2	Decreased bacteria	30,000		No new strain
M013	pCR2.1-opaD1-opaD2	None	30,000		No new strain
M012	pBS-opaD-tetS	None	All	M014	*opaA::kan opaB::ery opaD::tet ΔopaJ*
M013	pBS-opaA-tetS	None	All	M015	*opaA::tet opaB::ery opaD::kan ΔopaJ*
M007	pBS-opaD-tetS	None	All	M016	*opaA::ery opaD::tet opaJ::kan*
M008	pBS-opaA-tetS	None	All	M017	*opaA::tet opaD::kan opaJ::ery*
M005	pBS-opaD-tetS	None	All		No new strain
M005	pBS-opaD-tetS	Decreased bacteria	All		No new strain
M010	pBS-opaA-tetS	None	All		No new strain
M010	pBS-opaA-tetS	Decreased bacteria	All		No new strain
M005	M014 chromosomal DNA (*opaD::tet*)	None	All	M018	*opaA::ery opaB::kan opaD::tet*
M010	M015 chromosomal DNA (*opaA::tet*)	None	All	M019	*opaA::tet opaB::ery opaD::kan*

Table 5.1 Summary of transformation reactions to construct Opa-deficient isogenic mutant meningococci from parent strain H44/76.

Target of PCR	Forward primer	Reverse primer	Extension time in PCR (minutes)	Product size (bp)				
				***opa* (wt*)**	***opa::ery***	***opa::kan***	***opa::tet***	**Δ*opa***
EryR	ery-bamf	ery-bamr	3	–	1210	–	–	–
KanR	kan-if	kan-ir	3	–	–	963	–	–
TetR	tetF	tetR	3	–	–	–	1603	–
opaA	nitF1SalI	NMB0444-4SacII	8	3510	4523	4595	5378	–
opaB	NMB1634-4SalI	0464 opaBrevSacII	8	3676	4673	4743	–	–
opaD	NMB1464-7SalI	NMB1466-0SacII	8	2703	3700	3770	4553	–
opaJ	acthR2SalI	pipSEQRSacII	8	4489	5502	5574	–	4310
opaA::ery	nitF1SalI	ery-bamf	5	–	1703	–	–	–
opaA::kan	nitF1SalI	kan-5-out	5	–	–	887	–	–
opaA::tet	nitF1SalI	tetF	5	–	–	–	2302	–
opaB::ery	ery-bamf	0464 opaBrevSacII	5	–	1950	–	–	–
opaB::kan	NMB1634-4SalI	kan-3-out	5	–	–	2881	–	–
opaD::ery	ery-bamr	NMB1466-0SacII	5	–	2459	–	–	–
opaD::kan	kan-3-out	NMB1466-0SacII	5	–	–	1407	–	–
opaD::tet	tetR	NMB1466-0SacII	5	–	–	–	2923	–
opaJ::ery	ery-bamr	pipSEQRSacII	5	–	3552	–	–	–
opaJ::kan	kan-3-out	pipSEQRSacII	5	–	–	2685	–	–
Δ*opaJ*	acthR2SalI	opaFSalI	5	2370	–	–	–	2191

Table 5.2 PCR primers used for screening of *opa* genes following transformation.

*****wt = wild-type. Primer sequences were: kan-if, 5'-AGCCATATTCAACGGGAAAC-3'; kan-ir, 5'-TTTGCTTTGCCACGGAAC-3'; tetF, 5'-TTGATGCTCTTGATCTTCC-3'; tetR, 5'-TAACAGCAAACAGTAATGG-3'; NMB1464-7SalI, 5'-TGCAGAGTCGACGGCATCAACACCCATGC-3'; NMB1466-0SacII, 5'-CCGCCTCCGCGGTTATGTTGTGCGACCAGTCC-3'; kan-5-out, TCAAAAATATGGTATTGATAATCC-3'; kan-3-out, 5'-TGTAACATCATTGGCAACGC-3'. Other primer sequences are in section 3.6.1 (EryR primers), table 3.2 (*opa* locus-specific primers) and section 4.2 (opaFSalI).

In order to produce a strain with all four *opa* genes disrupted, replacement of an *opa* gene containing an antibiotic resistance cassette with a modified, non-functional *opa* gene without a selective marker was required. In order to target a specific *opa* gene, locus-specific plasmids were used to transform *N. meningitidis* and the resulting colonies were grown in replicate on non-selective and selective media to identify those that had developed antibiotic sensitivity, summarised in table 5.1. Strain M009 (*opaB::ery opaJ::kan*) was successfully transformed with pBS-opaJ1-opaJ2 to remove KanR from the *opaJ* locus. Generic *opa* plasmids based on *opaA* or *opaD* and including KanR were then used to produce triple Opa-deficient strains retaining only *opaA* or *opaD*. The TetR cassette was used to obtain triple Opa-deficient strains in which only *opaB* or *opaJ* remained, as well as a strain with all four *opa* genes disrupted. To construct the strain in which all *opa* genes were disrupted except *opaJ*, chromosomal DNA containing the TetR cassette within the *opaA* locus was required for transformation because the generic plasmids did not result in any transformant colonies.

The locus-specific and generic plasmids described in chapter 3 and chapter 4 were used to successfully construct a set of isogenic Opa-deficient meningococci from *N. meningitidis* strain H44/76. Four single Opa-deficient strains, six double Opa-deficient strains, four triple Opa-deficient strains and an Opa-negative strain were obtained.

5.5. DNA sequence analysis of *opa* genes

The ideal method to determine Opa expression of the different strains, and therefore identify suitable strains for further studies (chapter 7 and chapter 8), would be to use specific mAbs, able to recognise only one of the four Opa proteins expressed in *N. meningitidis* strain H44/76. However, no such antibodies were available when the mutant strains were initially constructed. In order to predict Opa expression of the Opa-deficient mutant strains, the CR sequence of each of the 4 *opa* genes in each mutant strain was therefore determined by dye-terminator DNA sequencing (section 2.10). The N-terminal sequence of the *opa* gene is such that if the number of pentameric CR sequences is a multiple of 3 (e.g. 3, 6, 9) then the mature protein should be 'in-frame' and would be expected to be expressed on the bacterial surface (figure 1.5). If however, the number of CR sequences is not a multiple of 3, then transcription of the DNA sequence will result in a premature stop codon, and the full-length protein would not be expressed.

The N-terminal sequence of each *opa* gene from every mutant strain was therefore determined, in order to identify appropriate strains for use in future work. For almost all meningococcal transformations, stocks from two different colonies were stored (denoted by the suffix /01 or /02) (section 2.3) and both were subjected to DNA sequencing. The *opa* genes were first amplified by PCR, as described in section 2.9.1, using locus-specific primers (table 5.3) and an extension time of 6 minutes.

Target of PCR	Forward primer	Reverse primer	Product size (bp)
opaA	nitF1SalI	NMB0444-4SacII	3510
opaA::ery	ery-bamr	NMB0444-4SacII	4030
opaA::kan	kan-3-out	NMB0444-4SacII	3163
opaA::tet	tetR	NMB0444-4SacII	4679
opaB	NMB1634-4SalI	0464 opaBrevSacII	3676
opaB::ery	NMB1634-4SalI	ery-bamr	3933
opaB::kan	NMB1634-4SalI	kan-3-out	2881
opaD	NMB1464-7SalI	NMB1466-0SacII	2703
opaD::ery	ery-bamr	NMB1466-0SacII	2459
opaD::kan	kan-3-out	NMB1466-0SacII	1407
opaD::tet	tetR	NMB1466-0SacII	2923
opaJ	acthR2SalI	pipSEQRSacII	4489
Δ*opaJ*	acthR2SalI	pipSEQRSacII	4310
opaJ::ery	ery-bamr	pipSEQRSacII	3552
opaJ::kan	kan-3-out	pipSEQRSacII	2685

Table 5.3 PCR primers used for amplification of *opa* loci prior to DNA sequencing.
Primer sequences are in table 5.2.

Following purification of the PCR products, DNA sequencing reactions were performed as described in section 2.10 using oligonucleotides O85 (5'-GGCATAATCTGCCGCTATCC-3')[265] and OpaFwdII (5'-TATATTGTGTTGAAACATCG-3'), which was based on the PCR primer O3510[32]. Where it was not possible to assign a sequence due to the poor quality of the chromatograms, the PCR and sequencing reactions for that *opa* locus were repeated. Table 5.4 summarises the sequencing results.

Strain	*opaA*	*opaB*	*opaD*	*opaJ*	Parent strain
H44/76 wt	10	8	14	11	n/a
M001/01	9*	8	15*	Δ	H44/76 wt
M001/02	10	8	14	Δ	H44/76 wt
M002/01	Δ	8	15*	11	H44/76 wt
M002/02	Δ	8	14	11	H44/76 wt
M003/01	10	8	Δ	11	H44/76 wt
M003/02	10	8	Δ	11	H44/76 wt
M004/01	10	Δ	14	11	H44/76 wt
M004/02	10	Δ	14	11	H44/76 wt
M005/01	Δ	Δ	15	11	M002/01
M005/02	Δ	Δ	15	11	M002/01
M006/01	Δ	8	Δ	11	M002/01
M006/02	Δ	8	Δ	11	M002/01
M007/01	Δ	8	15	Δ	M002/01
M008/01	10	8	Δ	Δ	M001/02
M008/02	10	8	Δ	Δ	M001/02
M009/01	10	Δ	14	Δ	M004/02
M009/02	10	Δ	14	Δ	M004/02
M010/01	10	Δ	Δ	11	M004/02
M010/02	10	Δ	Δ	11	M004/02
M011/01	10	Δ	14	Δ	M009/01
M012/01	Δ	Δ	14	Δ	M011/01
M012/02	Δ	Δ	14	Δ	M011/01
M013/01	10	Δ	Δ	Δ	M011/01
M013/02	10	Δ	Δ	Δ	M011/01
M014/01	Δ	Δ	Δ	Δ	M012/02
M014/02	Δ	Δ	Δ	Δ	M012/02
M015/01	Δ	Δ	Δ	Δ	M013/01
M015/02	Δ	Δ	Δ	Δ	M013/01
M016/01	Δ	8	Δ	Δ	M007/01
M017/01	Δ	8	Δ	Δ	M008/01
M018/01	Δ	Δ	Δ	11	M005/01
M018/02	Δ	Δ	Δ	11	M005/01
M019/01	Δ	Δ	Δ	11	M010/01
M019/02	Δ	Δ	Δ	11	M010/01

Table 5.4 Number of CR sequences in the N-terminal region of each *opa* gene in the wild-type and isogenic Opa-deficient mutant strains.
Genes where Opa protein expression would be expected based on the nucleotide sequence are shown in green, and where Opa protein expression would not be expected are shown in red.
Δ: *opa* gene disrupted, so protein expression not possible
*****Change in number of CRs from parent strain.

It was intriguing to discover that the wild-type strain used in this study would not be expected to express any Opa proteins on the bacterial surface. The first transformation, in which the *opaJ* gene was disrupted, resulted in two phenotypically different strains being constructed – one which was now predicted to express both OpaA and OpaD (M001/01) and another which should express neither (M001/02). In

addition, one of the two colonies stored from the second transformation performed should express OpaD only, having had *opaA* disrupted (M002/01). The altered number of CR sequences in this strain compared to the wild-type strain was maintained during subsequent transformations (M005 and M007). All other strains constructed would not be expected to express any Opa proteins, based on the nucleotide sequence, although they would have the capacity for PV at those *opa* loci which had not been disrupted.

On the basis of these results, strains H44/76, M001/01, M002/01 and M014/01 were chosen for further development. The finding that PV had occurred at 5% of the *opa* loci assessed (3/58) following transformations, suggesting a PV rate of between 10^{-2} and 10^{-3} (figure 6.1) also initiated further analysis of PV, which is described in chapter 6.

5.6. Characterisation of strains for immunological studies

Four strains from the panel constructed were chosen to enable any differences between Opa proteins to be tested:

(i) H44/76 wild-type – no Opa proteins expressed, but capacity to express all;

(ii) M014/01 – All *opa* genes disrupted, so no Opa proteins could be expressed.

(iii) M001/01 – OpaA and OpaD expressed, plus capacity to express OpaB;

(iv) M002/01 – OpaD expressed, and able to express OpaB and OpaJ;

In strain H44/76, OpaA and OpaJ are very similar, as are OpaB and OpaD, so being able to compare OpaA and OpaD was useful. In addition, the combination in strain

M001/01 would enable some assessment of whether there may be a synergistic or additive effect when more than one Opa protein is expressed on the bacterial surface.

Bacteria and OMVs were characterised by a number of methods using anti-Opa mAbs (gift from P van der Ley)[320], which were acquired once the strains had been constructed. There was some cross-reactivity of the mAbs between the different Opa variants found in H44/76. The minimal epitope for mAb 15-1-P5.5 is GGPIIQ, which is found in the HV2 region of OpaA and OpaJ, and mAb MN20E12.70 targets the epitope GIWQELK, which is present within the HV1 region of OpaB and OpaD.

Bacteria from the four chosen strains were inactivated using two different methods – heat and ethanol (section 2.1.5) – to identify which method maintained optimal Opa expression. Dot blotting performed with the anti-Opa mAbs (section 2.6.2) revealed that the ethanol-fixed bacteria preserved the expected Opa phenotype better than the heat-killed bacteria (figure 5.5). With ethanol-fixed bacteria, mAb MN20E12.70 bound to strains M001/01 and M002/01, and mAb 15-1-P5.5 bound to strain M001/01 only. When heat-killed bacteria were used, mAb 15-1-P5.5 also bound to strain M002/01 and both mAbs bound to the wild-type strain, which would not be expected based on their CR sequence (table 5.4). Ethanol-fixed bacteria were therefore used in the T cell assays (chapter 7).

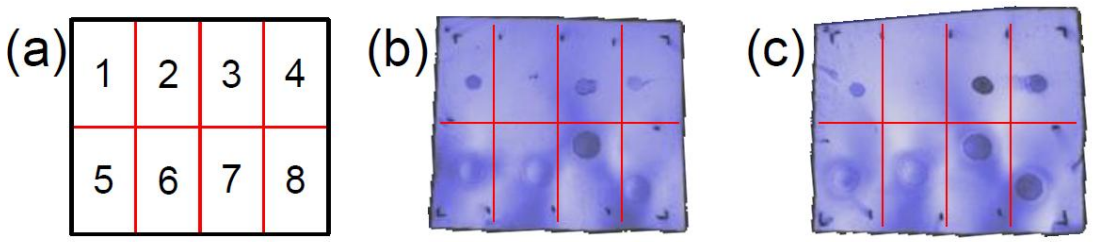

Figure 5.5 Dot blots of killed bacteria from strains chosen for further studies.
Dot blot layout is shown in (a) and blotting using mAbs 15-1-P5.5 (anti-OpaA) and MN20E12.70 (anti-OpaD) in (b) and (c) respectively.
1 and 5 = H44/76 (wild-type); 2 and 6 = M014/01 (Opa-negative); 3 and 7 = M001/01 (OpaA+ OpaD+); 4 and 8 = M002/01 (OpaD+); 1-4 = ethanol-fixed bacteria; 5-8 = heat-killed bacteria

The text originally presented here cannot currently be made freely available via ORA.

Two batches of OMVs were required to complete the experiments. The total protein content was determined using a modified micro Lowry assay (section 2.4.2; table 5.5).

Strain	Estimated total protein concentration (µg/ml)	
	Batch 1	Batch 2
H44/76 wt	2020	1018
M014/01	978	1619
M001/01	1598	606
M002/01	1083	754

Table 5.5 Total protein concentrations of OMVs determined by the modified micro Lowry assay.

Once the protein concentration had been estimated, denatured OMVs were visualised using SDS-PAGE (section 2.4.3), and the Opa phenotype was assessed by immunoblotting with mAbs 15-1-P5.5 and MN20E12.70 (section 2.6.3; figure 5.6). On the SDS-PAGE gel multiple major bands were visualised, and from their positions the major bands would be likely to be PorA, PorB, RmpM and Opa/OpcA, as expected[321]. Strains M001/01 and M002/01 did contain a major band where Opa would be expected, whereas H44/76 and M014/01 did not. This suggested that Opa

was only present in M001/01 and M002/01, which was predicted from the DNA sequence analysis (table 5.4) and dot blotting of inactivated bacteria from the same strains. Immunoblotting confirmed expression of OpaA and OpaD in the relevant strains, as well as low level expression of Opa in the wild-type strain, and no Opa expression in the Opa-negative strain.

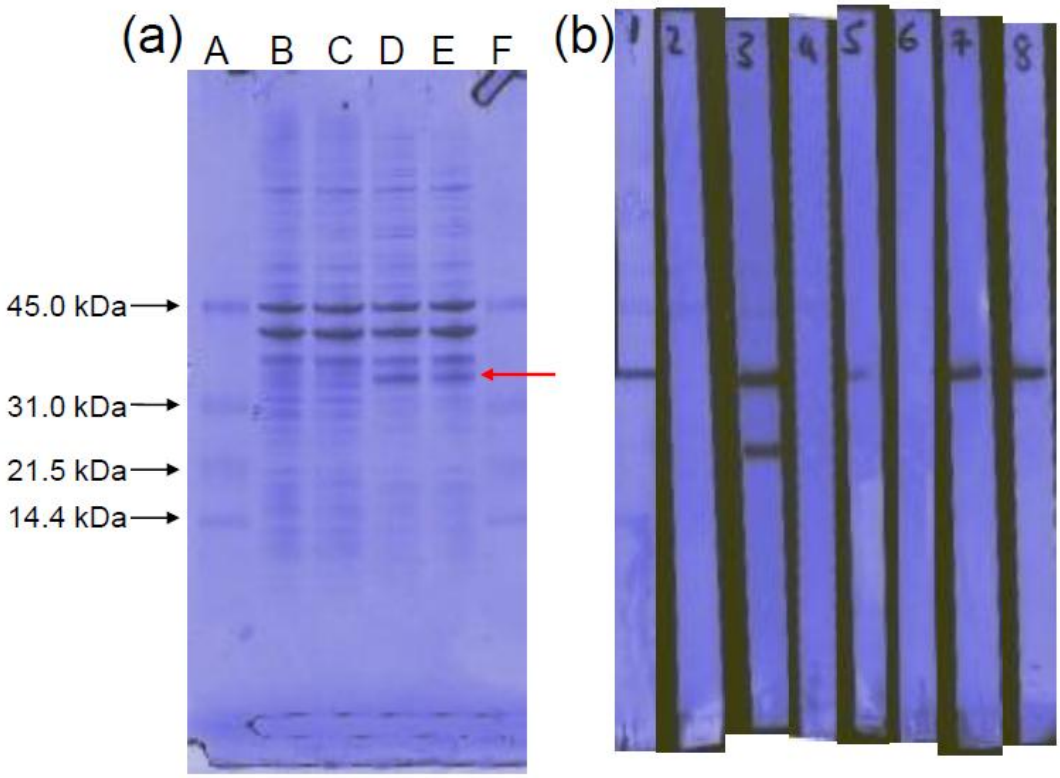

Figure 5.6 (a) SDS-PAGE & (b) immunoblotting of OMVs.
(a) Lanes A and F = low-range molecular weight standards; lane B = H44/76 (wild-type); lane C = M014/01 (Opa-negative); lane D = M001/01 (OpaA+ OpaD+); lane E = M002/01 (OpaD+). The red arrow highlights an additional band present in strains M001/01 and M002/01 only, which represents Opa.
(b) Lanes 1-4 = blotting with mAb 15-1-P5.5 (anti-OpaA); lanes 5-8 = blotting with mAb MN20E12.70 (anti-OpaD). Lanes 1 and 5 = H44/76 (wild-type); lanes 2 and 6 = M014/01 (Opa-negative); lanes 3 and 7 = M001/01 (OpaA+ OpaD+); lanes 4 and 8 = M002/01 (OpaD+)

The integrity of the OMVs was inspected using TEM (section 2.4.4; figure 5.7), and particle size distribution was assessed using NTA (section 2.4.5; figure 5.8). These

confirmed that samples contained mostly intact vesicles with some cell debris, and the size of the particles was largely in the expected range of 50-200nm[275].

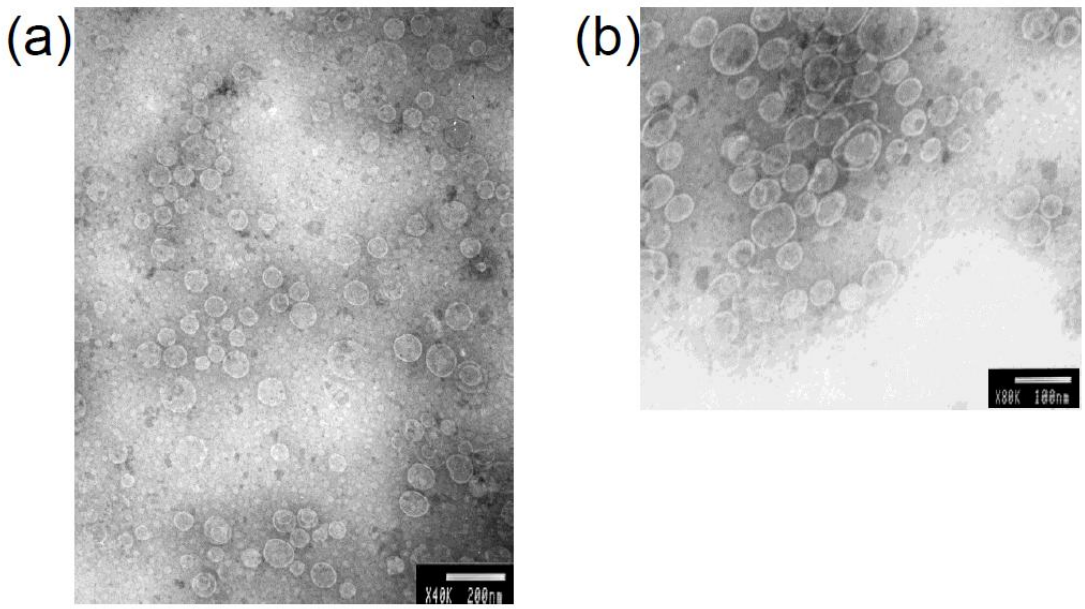

Figure 5.7 Transmission electron microscopy (TEM) of Opa-negative OMVs at (a) x40,000 and (b) x80,000 magnification.
TEM of other OMVs revealed similar findings.

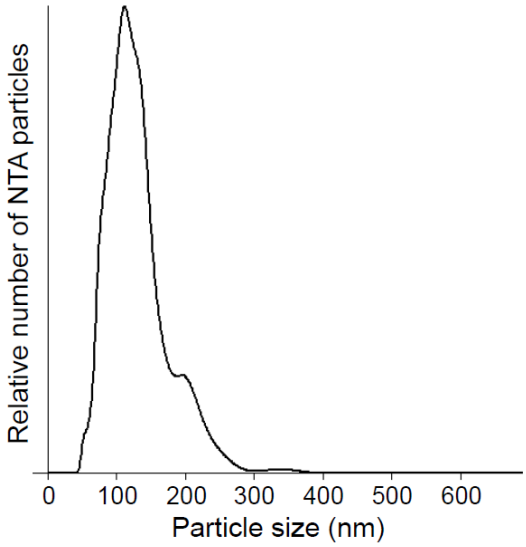

Figure 5.8 Nanoparticle tracking analysis (NTA) of Opa-negative OMVs.
NTA of other OMVs revealed similar findings.

5.7. Discussion

The first aim of the experiments described in this chapter was to construct isogenic strains with different patterns of Opa expression derived from H44/76, including an Opa-negative strain. This was achieved through a succession of transformations which introduced erythromycin, kanamycin or tetracycline resistance cassettes into the *opa* genes. Most attempted transformations with the locus-specific and generic plasmids were successful, although two transformations required chromosomal DNA (table 5.1). Chromosomal DNA has a significantly greater transformation efficiency compared to plasmid DNA due to the presence of a high number of DNA uptake sequences and the increased length of DNA where homologous recombination can occur. A key breakthrough was the removal of KanR from strain M009, which ultimately enabled construction of the Opa-negative strain. There has been no previously published description of such a collection of Opa-deficient meningococci, making them a valuable new tool for the investigation of the effects of Opa proteins.

Strains in which *opa* genes were disrupted would not be able to express that particular Opa protein. Where wild-type genes were still present, Opa proteins may or may not be expressed due to PV. Nucleotide sequence analysis of *opa* genes in all strains was performed and 4 strains with different patterns of Opa expression were identified for further development. The *opa* DNA sequence was later confirmed to predict the Opa phenotype, such that *opa* genes where the number of CR sequences was a multiple of 3 did express the protein and others did not. There has been no previous direct correlation of Opa expression with DNA sequence. These data suggest that *opa* sequencing would be a useful tool in future studies of Opa proteins, given that specific

mAbs are not available for the large number of Opa variants. It would be interesting to identify any variants which had a tendency to be switched ‘on’ or ‘off’. This would be particularly helpful to predict potential coverage during vaccine development and may also be important for investigation of the pathogenesis of meningococcal infection.

A number of methods are available for characterisation of inactivated meningococci and derived OMVs. The methods used for this study were chosen primarily to confirm the Opa phenotype of the different strains and to establish that OMV preparations were composed mainly of vesicles rather than membrane fragments, and there was no significant aggregation of vesicles.

Differences were observed between the protein concentrations of the 2 OMV batches for all strains. This would be expected since the final OMV pellet was resuspended in a volume of 3% sucrose based on the weight of bacteria initially harvested, aiming for a final concentration of 1000μg/ml. This was a small scale of OMV production, compared to the large scale required for manufacturing, which would result in increased variation of protein concentration. Furthermore, the OMVs were further diluted before being used, so OMVs from both batches were used at the same final concentration in all experiments.

Visualisation of the protein profiles of the different OMVs by SDS-PAGE demonstrated the presence of all major proteins, as previously described. Proteins that are thought to be the most important in the immunogenicity of OMVs are Omp85,

FetA, PorA, PorB, RmpM, Opa, OpcA and NspA[321]. The majority of these appeared to be present in the OMVs produced, although the only way to confirm the identity of the proteins would be using specific mAbs or by mass spectrometry. These OMVs were to be used to evaluate differences between Opa proteins. The presence or absence of Opa was confirmed by anti-Opa mAbs and protein profiles appeared visually similar in other regards, so further evaluation of other proteins was not performed. Immunoblotting with the anti-OpaA mAb 15-1-P5.5 against the OMV from strain M001/01 did highlight a second smaller band in addition to the main Opa band, and this was presumed to be Opa degradation products. Such products have been described during OMV production[321] and would also need mass spectrometry to be performed for confirmation. The presence of some Opa proteins in the wild-type strain suggested that some PV had occurred during bacterial growth for OMV production, and may result in differences between H44/76 and M014/01 (which is Opa-negative)

This finding suggests that PV during large scale OMV production may contribute to the batch-to-batch variation which occurs during industrial-scale manufacturing of OMV vaccines[322], which may have significant implications for vaccine immunogenicity or reactogenicity.

TEM and NTA confirmed the physical integrity of the OMVs. The use of 3% sucrose stabilises OMV membranes and reduces any aggregation that may otherwise occur[275]. Membrane fragments are thought to be less immunogenic than intact vesicles since proteins may not be presented to the immune system in their normal conformation[278], so it was important to assess that the majority of each sample contained intact

vesicles. NTA is a relatively new technology that is able to confirm particle size for particles between 10nm and 1,000nm. It has not previously been described for the assessment of meningococcal OMVs, and may be an additional method of characterisation that could be adopted for routine use in the future.

Although a number of OMV vaccines have now been produced, and licensed, for human use there are no standard global guidelines for quality control of these products. A number of features need to be characterised, including those that have been described above. In addition other aspects, such as quantitation of LPS, polysaccharide, DNA and detergent levels, endotoxin activity and sterility, are usually required for products manufactured for human use. These were not assessed for these batches of OMVs since the main aim of further experiments was to evaluate differences mediated by variation in Opa proteins. If, however, any of these OMVs would be used in future as a vaccine strain then it would be important to perform additional characterisation. In addition, it would also be vital to confirm that the mutant strains were similar to the wild-type strain in terms of bacterial growth.

In this chapter, construction and characterisation of Opa-deficient isogenic mutant strains of *N. meningitidis* strain H44/76 have been described. A unique panel of strains has been produced, which can be used to evaluate Opa as a vaccine component and investigate the potential effects of different Opa variants during meningococcal infection. Four strains with different patterns of Opa expression were identified, with the different Opa expression of these strains being confirmed both genotypically and

phenotypically. Inactivated bacteria and OMVs were produced from these four strains, and their use in further experiments is described in chapter 7 and chapter 8.

CHAPTER 6. PHASE VARIATION OF MENINGOCOCCAL OPA PROTEINS

6.1. Introduction

PV describes the phenomenon whereby pathogens can genetically switch 'on' and 'off' the expression of specific components. This is distinct from antigenic variation, where there is variation in the molecular structure of a component, such that different variants of the same component can be expressed at different times. For *N. meningitidis* in particular, PV is a major mechanism of variability. This is highlighted by genome sequence analysis of *N. meningitidis* strains, which revealed the presence of over 100 putative phase-variable genes[323]. Several meningococcal surface structures are subject to PV, including the capsule[324], LPS[325, 326], pili[327, 328] and outer membrane proteins including PorA[329, 330], NadA[331], Opc[332] and Opa[202]. PV of these components is important to *N. meningitidis* for a number of reasons. The meningococcus occupies different niches during infection (i.e. nasopharynx, blood, meninges) and PV enables adaptation to each environment. PV is vital for the organism to evade the host immune response by regular alteration of its surface, which is especially important for *N. meningitidis* since it is frequently carried asymptomatically in the nasopharynx. It is therefore important to understand PV to enable appropriate development of vaccines based on subcapsular antigens, such as OMVs.

A number of different mechanisms exist to enable PV in *N. meningitidis*. The most common is the presence of repetitive DNA sequences, which can be homopolymeric tracts or tandem repeats with different numbers of nucleotides, and can be within

coding sequences, promoter regions or other regulatory areas. Homopolymeric tracts of guanosine or cytosine are the most common. One example of this is *porA*, where variation in the polyG tract between the -10 and -35 residues in the promoter region alters transcriptional activity and therefore protein expression levels[329]. A similar mechanism exists for PV of NadA[333], and is thought to be due to the 3D structure of the DNA and consequent alterations in the binding site of RNA polymerase in relation to the transcription start site. Tandem repeats, such as the pentanucleotide 5'-CTCTT-3' sequence present within the *opa* open reading frame, are thought to result in PV as a result of slipped-strand mispairing during DNA replication, resulting in frameshifting and translation of non-functional, truncated proteins, as described in section 1.7.1.

Additional methods of PV in the meningococcus include homologous recombination and insertional sequences (IS). Pili expression is an example of PV mediated by homologous recombination. Pili are composed of subunits encoded by the *pilE* gene. Up to 8 additional silent pili genes also exist, known as *pilS*. Homologous recombination between *pilE* and *pilS* can result in PV of pilus expression (although it more commonly results in antigenic variation), which can affect transformability, immunogenicity and adherence of bacteria[334]. One mechanism of capsule PV is insertion and exact excision of the naturally occurring insertional sequence element IS1301 into *siaA*[335] or *siaD*[336]. This mechanism has also been described for other genes, including *porA*[337] and *nadA*[338].

PV of meningococcal Opa proteins is of particular interest for a number of reasons. Opa PV may be a key mechanism of immune evasion by the meningococcus since Opa proteins induce antibodies following meningococcal infection and after immunisation with vaccines containing outer membrane complexes or vesicles[148, 205, 206]. Differences in Opa PV rates may also result in distinct functions of Opa variants during colonisation and invasive disease. Knowledge of PV rates of different Opa proteins will aid design of any future Opa-containing meningococcal vaccines.

PV of Opa proteins may occur due to slipped-strand mispairing of the CR sequence, or site-specific recombination, which can be either intra- or inter-genomic (sections 1.7.1 and 1.7.5). Homologous recombination can occur between *opa* alleles in the same chromosome, or following DNA uptake of whole or fragments of *opa* genes from the environment (figure 1.8). For gonococci, promoter strength is known to additionally regulate Opa expression levels[204]. The frequency of Opa PV in gonococci is estimated to be approximately 10^{-3} per cell per generation[203], but this has not been studied in detail for the meningococcus.

This chapter describes analysis of Opa PV in *N. meningitidis* strain H44/76 by analysis of *opa* nucleotide sequence and Opa phenotype. The effect of transformation of *N. meningitidis* with unrelated plasmid DNA on Opa PV was also explored.

6.2. Methods

The methods used in this chapter were transformation of *N. meningitidis* (section 2.3) colony blotting (section 2.6.1), purification of DNA (section 2.7), agarose gel electrophoresis (section 2.8), PCR (section 2.9.1) and DNA sequencing (section 2.10).

6.3. Estimation of phase variation rate

There are a number of published methods used for calculating PV rate[339]. The most accurate of these incorporates a number of factors, and is defined by the equation:

$$\alpha = \frac{2}{1+x}\left[1-\sqrt[n]{1-(1+x)p_n}\right],$$

where α represents the PV rate per cell per generation, the back-variation rate is x times the forward rate, n is the number of generations of bacterial cell division and p_n is the proportion of mutants at n generations[339].

This also assumes that there is no fitness advantage between the 2 phase-variable states, which it is reasonable to assume in this study given that this was an *in vitro* experiment without any specific selection pressure. In this study, there was no reason to expect the back-variation rate to be different from the forward rate, i.e. $x = 1$. To estimate the number of generations of cell division, picked colonies were plated out at serial 10-fold dilutions between 10^{-2} and 10^{-4}. This revealed that each colony contained approximately 1.40×10^6 cfu, which is approximately equivalent to 20 generations, since $2^{20} = 1.05 \times 10^6$. Based on these factors, the equation above can be simplified to:

$$\alpha = 1-\sqrt[20]{1-2p},$$

where p is the proportion of mutants after overnight growth.

This results in the curve depicted in figure 6.1, which reveals that the alteration in repeat tract length in 5% of *opa* alleles during construction of mutant strains (described in section 5.5) should reflect a PV rate in the order of 10^{-2} to 10^{-3}, which therefore warranted further investigation.

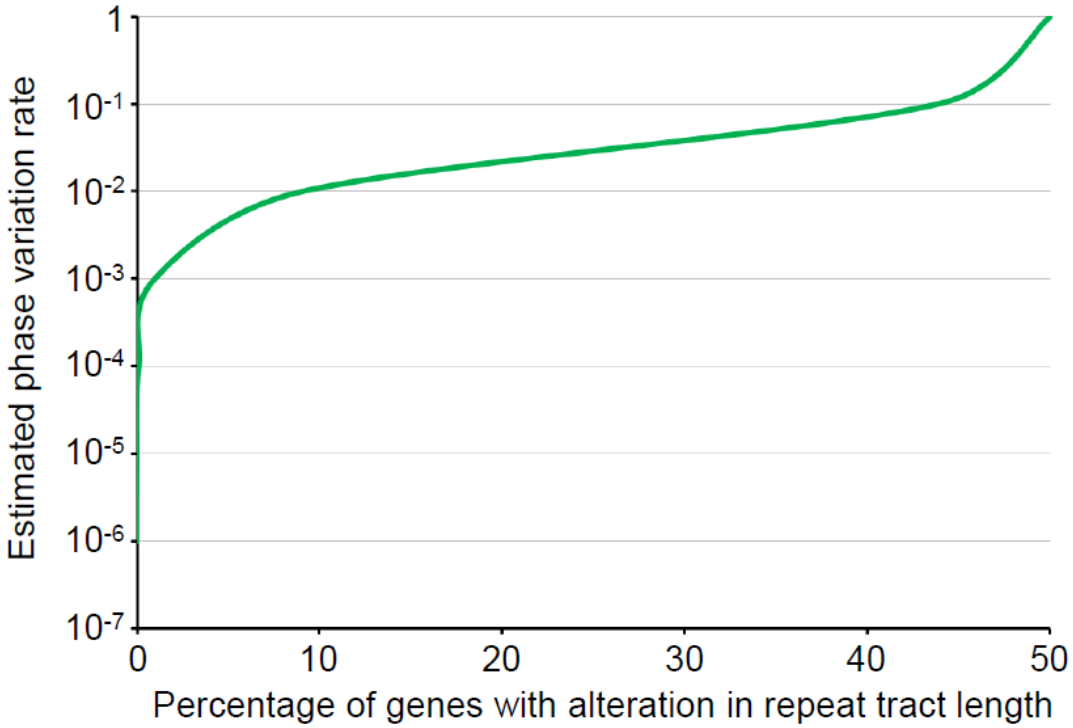

Figure 6.1 Relationship between the estimated phase variation rate and the percentage of genes with an alteration in the repeat tract length.
Graph based on the method described in Saunders *et al.*[339].

6.4. Assessment of Opa phase variation by *opa* sequence analysis

The PV rates of the 4 Opa variants in *N. meningitidis* strain H44/76 were first examined by analysis of DNA sequence of the *opa* CR tract during serial passage. Bacteria were initially revived from storage by plating onto BHI agar to obtain single colonies. Genomic DNA was extracted from 18 colonies (section 2.7.2), and one of these colonies was also used for further passage in a similar fashion. This was

repeated a further 4 times, with 19 colonies picked following the final passage. DNA was therefore extracted from a total of 91 colonies for DNA sequencing. For each DNA sample, all 4 *opa* genes were amplified by PCR, followed by sequencing of the CR regions as previously described (section 5.5). For some samples, satisfactory consensus sequences could not be obtained, despite multiple attempts.

The first 18 colonies reflected variation of the CR sequences of the *opa* genes in the bacterial stocks, rather than aid analysis of PV. There was some heterogeneity of the CR sequences of *opaA* and *opaD*, but not *opaB* and *opaJ* (table 6.1), suggesting that the highest PV rates would be observed for OpaA and OpaD. Analysis of the CR sequences from the following 73 colonies screened appeared to confirm this, with only *opaA* and *opaD* exhibiting changes in the number of repeats, suggesting PV of protein expression (table 6.2). Rates were calculated as described above. These data suggested that the rates of PV for OpaB and OpaD of H44/76 were both $<1.39 \times 10^{-3}$ per cell per generation, which was the minimum rate that could be detected by this screening method.

Gene	Number of colonies screened	Number (%) of 5'-CTCTT-3' repeats							
		8	**9**	**10**	**11**	**12**	**13**	**14**	**15**
opaA	16	0	3 (19)	12 (75)	1 (6)	0	0	0	0
opaB	16	16 (100)	0	0	0	0	0	0	0
opaD	18	0	0	0	0	0	1 (6)	10 (56)	7 (39)
opaJ	12	0	0	0	12 (100)	0	0	0	0

Table 6.1 Number of pentameric CR sequences for each *opa* gene among 18 colonies screened following direct plating of *N. meningitidis* strain H44/76 from frozen stocks.

Gene	Passage	Number of colonies screened	Number of colonies with altered number of CR sequences	PV rate (per cell per generation)	Average PV rate (per cell per generation)
opaA	1	18	0	0	1.47×10^{-3}
	2	18	1	5.87×10^{-3}	
	3	18	0	0	
	4	19	0	0	
opaD	1	18	0	0	1.39×10^{-3}
	2	18	0	0	
	3	18	0	0	
	4	19	1	5.55×10^{-3}	

Table 6.2 Estimation of PV rate of the four *opa* genes of *N. meningitidis* strain H44/76 by DNA sequencing of 73 colonies during serial passage.

6.5. Assessment of Opa phase variation by colony blotting

Assessing PV by sequence analysis is very labour-intensive, and allows only a small number of colonies to be screened. Lower rates of PV therefore cannot be detected. To improve sensitivity, further analysis was performed using anti-Opa mAbs (gift from P van der Ley)[320]. There was some cross-reactivity of the mAbs between the different Opa variants found in H44/76. The minimal epitope for mAb 15-1-P5.5 was GGPIIQ, which is found in the HV2 region of OpaA and OpaJ, whilst mAb MN20E12.70 targets the epitope GIWQELK, which is present within the HV1 region of OpaB and OpaD. In order to analyse PV rates of each Opa protein separately, the Opa-deficient mutant strains M001/02, M002/02, M003/01 and M004/01 were used (figure 6.2). None of these strains would be predicted to express any Opa proteins,

based on their DNA sequence (section 5.5), and all had a single *opa* gene disrupted. For each strain, only one protein was therefore targeted by the mAb (figure 6.2).

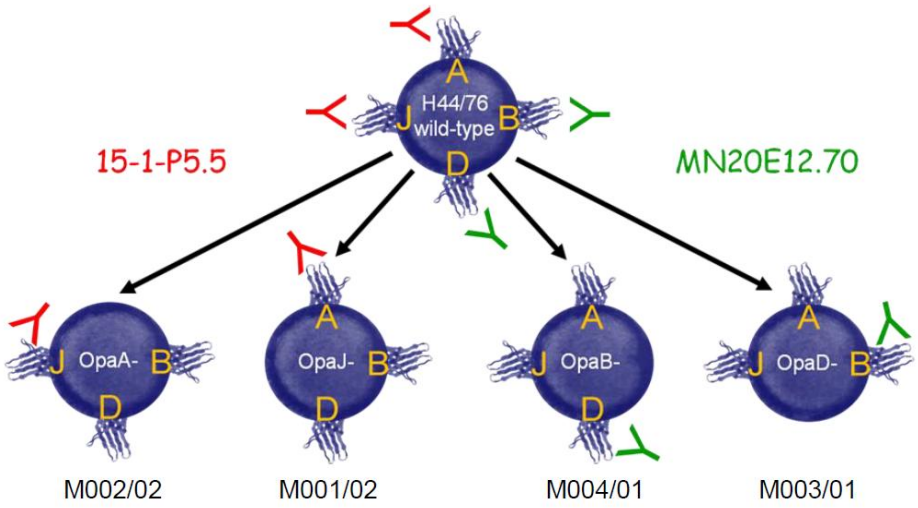

Figure 6.2 Colony blotting using mAbs 15-1-P5.5 (anti-OpaA and anti-OpaJ) and MN20E12.70 (anti-OpaB and anti-OpaD).
Strains M002/02 and M001/02 were blotted against mAb 15-1-P5.5 and strains M004/01 and M003/01 were blotted against mAb MN20E12.70 to separately estimate PV rates of the four Opa proteins.

Opa PV rates were therefore determined by colony blotting of these 4 mutant strains using the 2 mAbs, as described in section 2.6.1. The strain M014/01 was used as a negative control, and M001/01 as a positive control (table 6.3). PV rates were calculated as described above with the switching rate at the DNA level assumed to be twice the rate calculated using the mAbs, since two-thirds of CR tract lengths would not result in protein expression. This allowed comparison between the two methods. For example, OpaA contained 10 CR sequences at the start, which should not result in protein expression. A decrease in CRs to 9 would result in expression of OpaA on the cell surface, whereas an increase to 11 CRs would not. Both changes would be detected with DNA sequencing, but only if the protein was expressed would it be detected using the mAb.

Gene	Number of colonies screened	Number of colonies with Opa protein expression	Phenotypic PV rate (per cell per generation)	Genotypic PV rate (per cell per generation)
opaA	2617	25	9.64×10^{-4}	1.93×10^{-3}
opaB	2830	9	3.19×10^{-4}	6.38×10^{-4}
opaD	2690	90	3.46×10^{-3}	6.91×10^{-3}
opaJ	2279	9	3.96×10^{-4}	7.93×10^{-4}

Table 6.3 Estimation of PV rate of the four *opa* genes of *N. meningitidis* strain H44/76 by colony blotting.

The results of the study of Opa phenotype using the mAbs were in close agreement with those found from DNA sequencing. OpaD in strain H44/76 appeared to have the highest PV rate, followed by OpaA, which in turn was higher than OpaB and OpaJ, which were similar to each other.

6.6. The effect of transformation on Opa phase variation

A final experiment evaluating Opa PV was performed to test the hypothesis that transformation of *N. meningitidis* with plasmid DNA increases the PV rate of unrelated genes, which has been demonstrated in a previous study of *N. meningitidis*[340]. This phenomenon is increasingly important in the current era of genetically-modified strains being used as the basis for development of meningococcal OMV vaccines (section 1.6.4; table 6.4). Transformation of bacteria with plasmids containing meningococcal genes which have been modified to be constitutively expressed, over-expressed or disrupted is the standard method of creating these vaccine strains. There are a large number of phase variable genes in the meningococcal genome, and some of these are important determinants in the immunogenicity of the resulting OMVs, but detailed studies of any changes in the expression of molecules other than the vaccine antigens under consideration are rarely undertaken.

OMV vaccine	Parent strain	Studies done	Refs
Hexavalent PorA	H44/76	Phase II clinical	122, 123
Nonavalent PorA	H44/76	Phase I clinical	151
Overexpresed fHbp	H44/76	Pre-clinical	143
Overexpressed TbpA, Hsf, NspA, Omp85	H44/76	Pre-clinical	153
Overexpressed LbpA + LbpB	H44/76	Pre-clinical	179
Genetically detoxified LPS	multiple	Phase I clinical Pre-clinical	121, 126, 127 176, 184, 189

Table 6.4 OMV vaccines based on genetically modified strains of *N. meningitidis*.

In order to test this hypothesis, OpaA expression in strain M001/02 was assessed using mAb 15-1-P5.5 following transformation with the plasmid pT7-E2F2-kan, which contains the LPS biosynthesis gene *lpt-3* disrupted with a kanamycin resistance cassette[341]. M001/02 was transformed with pT7-E2F2-kan (section 2.3), following which bacteria were plated out onto both selective and non-selective media. Following overnight incubation, OpaA expression was determined by colony blotting (section 2.6.1). Strains M001/01 and M007/01 were used as the positive and negative controls, respectively. When the total number of bacteria were considered (i.e. following growth on non-selective media), 14/5041 colonies expressed OpaA, giving a PV rate of 2.78×10^{-4} per cell per generation. In the population of successfully transformed bacteria (i.e. following growth on selective media), 63/196 colonies expressed OpaA, which was a PV rate of 5.02×10^{-2} per cell per generation and was 180-fold higher than the rate observed overall. This was also consistent with the previous finding of an alteration in the CR tract length in 5% of opa alleles during construction of the Opa-deficient strains (section 5.5), which suggested a PV rate in that context of between 10^{-2} and 10^{-3} per cell per generation.

6.7. Discussion

This chapter describes a series of experiments which examined meningococcal Opa PV in greater detail than previously described in published literature. Opa PV can occur via an alteration in the number of CR sequences, or homologous recombination between *opa* alleles, but in these relatively short *in vitro* studies any effects of recombination were likely to be minimal.

A number of factors are known to modulate the PV rates of different meningococcal proteins, although the different methods used to calculate PV rates in different studies make it difficult to compare them directly. For example, Opa PV rates have previously been estimated based on restriction enzyme digestion and Southern blotting[342], analysis of mRNA, binding to anti-Opa mAbs, construction of fusion proteins[204] and assessment of colony opacity by stereomicroscopy[203]. The length of the repeat tract is thought to be a key determinant, and an increased repeat tract length has been associated with higher PV rates[343]. The type of repeat sequence also influences PV rate, with poly-G/C tracts being less stable than those containing poly-A/T[344]. The location of a gene within the chromosome is important, since the sequence of nearby DNA affects PV, in particular additional repetitive DNA in close proximity, as does transcription levels of that specific region of the chromosome. Mutator strains have been detected in bacterial populations, and have PV rates that are 10- to 1000-fold higher than basal rates. Mutations in the mismatch repair genes *mutS* and *mutL*, for example, result in 1000-fold higher PV rates of some genes[345, 346]. Of importance to this study, transformation of Neisseria with exogenous DNA has been reported to increase the rate of PV of unrelated genes between 24- and 73-fold[340]. This

increase was due to inhibition of the activity MutS and MutL by single-stranded DNA molecules, which are imported into the cytoplasm during transformation.

In this study, OpaD of strain H44/76 had the highest PV rate, consistent with the theory that genes with longer repeat tract lengths have higher PV rates. This has been shown in *Haemophilus influenzae* and is thought to be due to increased instability of the region of DNA[343]. However, if this was the main determinant then the PV rate of OpaJ should have been higher than it was, as it has a similar number of CR sequences to OpaA. This suggested that other factors were also important in determining PV rates. Whether there was also an interaction between different Opa proteins to regulate PV rates is unknown, and this may be a factor for Opa since there are multiple homologous genes present within the genome.

Future studies of meningococcal Opa PV would ideally include examination of a number of diverse strains, including those from invasive disease as well as carriage, with different lengths of CR sequences. *In vitro* studies are unable to evaluate the effects of the complex environmental pressures found *in vivo*, but there may be effects of different growth conditions (e.g. different media, liquid vs. solid media) that could be examined further. In addition, the effect of non-bactericidal polyclonal anti-Opa sera (e.g. from animal immunisations) could be used as a model of what might occur *in vivo* following immunisation with Opa. Assessment of a large number of strains may allow estimation of the influence of interaction between different Opa proteins in the same chromosome in determining PV rates. Further knowledge of meningococcal

Opa PV rates may provide useful information if Opa proteins are to be developed further as a vaccine candidate in humans.

The finding that transformation with unrelated plasmid DNA resulted in a transient 180-fold increase in PV rate is similar to previous observations, but has potential significance for vaccine development. A number of meningococcal OMV vaccines in pre-clinical or clinical studies have been based on strains which have been genetically modified via transformation (table 6.4). Characterisation of the modified bacteria and resulting OMVs focuses on those antigens of interest for the vaccine developer, and assessment of other antigens is usually not undertaken, other than visual inspection of an SDS-PAGE gel. It is highly likely, however, that these modifications result in changes in expression of other phase variable meningococcal proteins. These changes may not have a significant impact on vaccine development. Alternatively, it is possible that modification of just one or two proteins may significantly alter the immunogenicity or reactogenicity of the resulting OMV, or have an effect on bacterial growth and therefore influence vaccine production. Further study of the major phase variable proteins during development of OMV vaccines may provide additional insights into the determinants of immunogenicity of these vaccines, and therefore allow development of more refined products with better immunogenicity and reactogenicity profiles, which may also be more easily produced.

CHAPTER 7. EFFECT OF OPA PROTEINS ON CD4$^+$ T CELL PROLIFERATION

7.1. Introduction

N. meningitidis can persist in the human nasopharynx without causing symptoms for several months, and *N. gonorrhoeae* can cause prolonged mucosal infection of the genito-urinary tract. This ability to persist relies on their adaptability to the host and their capacity to evade the immune system. Opa protein binding to CEACAMs on the surface of host cells confers the ability to associate with human epithelial, endothelial and leucocytic cells encountered during neisserial infection, indicating a direct effect on the immune response. As a result, Opa proteins have the capacity to influence bacterial attachment, engulfment and immunological responses. As well as being important for prolonged colonisation of humans, these interactions may also be critical during IMD, and may have implications for the development of Opa-containing vaccines.

CEACAM family members are differentially recognised by Opa protein variants, so the distribution of each CEACAM has the potential to influence the cellular tropism of Neisseria expressing different Opa proteins *in vivo*[212, 214, 216, 218, 219, 222]. Furthermore each CEACAM mediates different cellular processes[223-225], suggesting that the cellular response to neisserial binding will depend upon the specific combination of CEACAMs engaged. Although Opa proteins are able to bind to a number of different CEACAMs, CEACAM1 is the only member of the family present on the surface of T cells.

The response of T cells, and particularly $CD4^+$ T cells, is important during infection with pathogenic Neisseria as these cells are involved in directing the magnitude and quality of humoral immune response. There is good evidence that antibody directed against surface structures of *N. meningitidis* are important in immunity but gonococci do not induce a strong, protective antibody response following infection[237]. T cells are also important in the generation of immunological memory and possibly cell-mediated immunity, which is therefore relevant to vaccine development. The interaction between meningococci and human T cells and the particular role of Opa proteins in this interaction has therefore been the subject of intense, and conflicting, study in the last few years.

A number of studies suggest Opa proteins are good stimulators of T cell proliferation. Studies comparing the meningococcal proteins PorA, PorB, Opa and Opc showed that Opa induced the strongest T cell proliferative responses[238, 239] and whole bacteria and OMVs have been shown to stimulate $CD4^+$ T cell proliferation, independent of their Opa phenotype[240]. Several Opa-containing OMV vaccines are immunogenic in humans[91, 114, 116, 117, 119]. In other studies, when $CD4^+$ T cells were exposed to OMVs from Opa-expressing meningococci or to gonococci expressing CEACAM-binding Opa variants, their activation and proliferation in response to a variety of stimuli were effectively halted[241].

There may be a number of reasons to explain the contrasting published data. Purified, denatured Opa proteins induced strong proliferation when incubated with freshly isolated PBMCs[238], which support cytokine-dependent interactions and receptor

signalling between cells. However, studies utilising whole bacteria or OMVs, which contain Opa in its native conformation but also other potentially important neisserial antigens, have yielded conflicting data[240-242]. It is possible that the different observations simply relate to variation in behaviour of different Opa proteins or variation of their expression levels relative to other outer membrane proteins in the various studies. The experiments with inhibitory effects were performed using purified $CD4^+$ T cells to specifically investigate Opa-CEACAM effects, and cells were incubated with IL-2 and anti-CD3 and anti-CD28 mAbs, rather than a standard antigen proliferation assay[241, 242]. The combination of IL-2, anti-CD3 and anti-CD28 results in potent non-specific, non-physiological stimulation of T cells which will inevitably identify inhibitory effects. The IL-2 induced up-regulation of CEACAM1 surface expression, in combination with cross-linked CD3- and CD28-specific antibodies, may have amplified any Opa-mediated effects. Various other methodological differences in the studies may also have influenced the results.

It is possible that purified or recombinant Opa may have a different effect to Opa expressed on the bacterial surface with other proteins. Furthermore, isolated T cells will behave differently to PBMCs *in vitro*. Considering that Neisseria have the capacity to randomly and reversibly switch many surface antigens 'on' and 'off' by a process of PV, it is also possible that the differential expression of uncharacterised factors other than Opa may influence T cell responses during infection. It is not clear which of the *in vitro* conditions used reflects the situation that occurs *in vivo* in infected patients or immunised individuals, and further investigation of this interaction is necessary to determine the utility of Opa as a vaccine candidate.

This chapter describes a series of experiments in which the isogenic mutant bacteria constructed in chapter 5, and the OMVs derived from these strains, were used to investigate the effects of surface-expressed Opa proteins on the interaction between meningococci and human $CD4^+$ T cells.

7.2. Methods

T cell proliferation assays were performed as described in section 2.11.

7.3. Study subjects

A maximum of 20ml of blood was taken from a total of 46 healthy adults after informed consent was obtained. All subjects were 18 years or over, and those with a history of previous IMD or a known immunodeficiency were excluded.

7.4. Antigens used for proliferation assays

Antigens used to stimulate PBMCs and $CD4^+$ T cells were OMVs and killed bacteria from the strains constructed in this study, which have been described in detail in chapter 5. These were the wild-type parent strain H44/76 (which expressed a small amount of Opa protein), an Opa-negative strain (M014/01), a strain which expressed OpaD only (M002/01), and one which expressed both OpaA and OpaD (M001/01). In addition, OMVs used in previously published studies of T cell proliferation in response to Opa were acquired as gifts from other groups and included in the assays. This was to enable further comparison with previous studies. OMVs from Opa-negative and OpaJ-positive strains constructed from H44/76 were provided by the

Netherlands Vaccine Institute (NVI) (gift from P van der Ley). In previous studies, these OMVs stimulated $CD4^+$ T cell proliferation when incubated with purified $CD4^+$ T cells pre-stimulated with IL-2, anti-CD3 and anti-CD28, but there was no difference between the two preparations[240]. OMVs from a wild-type H44/76 strain and an Opa-negative *N. lactamica* strain were provided by the Health Protection Agency (HPA) (gift from A Gorringe). The *N. lactamica* OMVs have also stimulated $CD4^+$ T cell proliferation when Opa-positive strains did not[241]. The OMVs obtained from NVI and the HPA were only available after recruitment to the study had started, so a smaller number of samples were tested with these reagents.

A number of control antigens were included in the assays. Positive controls included phytohaemagglutinin (PHA) and purified protein derivative (PPD) from *Mycobacterium tuberculosis*. The plant lectin PHA is a T cell mitogen, which results in polyclonal T cell stimulation after binding to cell membrane glycoproteins, including the TCR-CD3 complex[347, 348], and would be expected to stimulate T cells in almost all individuals. PPD is also a potent stimulator of T cell responses, and sensitisation to PPD is common in the UK (although not universal) since the majority of the population has been vaccinated with the BCG vaccine[349]. Other protein antigen controls used in some assays were tetanus toxoid, diphtheria toxoid and Staphylococcal enterotoxin B (SEB). When cells were not being treated with anti-CD3 and anti-CD28, these were used as additional positive controls for proliferation. A rabbit polyclonal anti-CEACAM antibody (DAKO) was used as an inhibitory control since this antibody is known to bind to CEACAM1 on the T cell surface and

result in inhibition of T cell responses when cells have been pre-stimulated[242]. An isotype antibody was used as a control for comparison of the anti-CEACAM response.

7.5. Cell culture conditions

In order to comprehensively investigate the effects of surface-expressed Opa proteins on $CD4^+$ T cell proliferation, different Opa-positive and Opa-deficient antigens were tested using different T cell culture conditions. The following culture conditions were tested:

(i) PBMCs cultured for 5 days with antigen *ex vivo*;

(ii) PBMCs cultured for 4 days with antigen and IL-2 following 48 hours pre-incubation with IL-2;

(iii) PBMCs cultured for 4 days with antigen, IL-2, anti-CD3 and anti-CD28, following 48 hours pre-incubation with IL-2;

(iv) Purified $CD4^+$ T cells cultured for 4 days with antigen and IL-2 following 48 hours pre-incubation with IL-2;

(v) Purified $CD4^+$ T cells cultured for 4 days with antigen, IL-2, anti-CD3 and anti-CD28, following 48 hours pre-incubation with IL-2.

A standard method of assessing *in vitro* T cell responses to vaccine candidates utilises freshly isolated PBMCs in an *ex vivo* culture to assess antigen-specific proliferation[350]. There are no recent data exploring the role of Opa in T cell proliferation using these conditions. In order to compare data with previous published studies, assays using pre-stimulated PBMCs and purified $CD4^+$ T cells were also performed. In this case cells were pre-incubated for 48 hours with IL-2, prior to

antigen addition with or without anti-CD3 and anti-CD28. Culture conditions including anti-CD3 and anti-CD28 were performed as a priority over those with IL-2 alone when the number of cells recovered from blood was insufficient for all assays. IL-2 is a growth factor for T cells and is known to increase the expression of CEACAM1 on the T cell surface[242]. Any stimulation or inhibition of proliferation mediated via CEACAM1, such as that proposed for Opa proteins, may therefore be enhanced. Anti-CD3 antibodies provide an initial activation signal for T cells, and binding of CD28 by specific antibodies is able to provide a co-stimulatory signal which results in receptor cross-linking and extensive proliferation[242]. Anti-CD3 and anti-CD28 also result in increased expression of CEACAM1 on the T cell surface[242]. Incubation of cells with IL-2, anti-CD3 and anti-CD28 therefore results in non-specific T cell stimulation, which will therefore identify inhibitory rather than stimulatory effects. Conversely, *ex vivo* culture, which is more conventional, can be used to assess T cell stimulation. Priority was therefore given to assays involving *ex vivo* PBMCs, as the culture condition of most interest to vaccine development.

Typical examples of the flow cytometry analysis are shown in figure 7.1. PBMCs *ex vivo* stimulated with PHA resulted in strong $CD4^+$ T cell proliferation compared to media alone. PBMCs stimulated with IL-2 only proliferated slightly, with the addition of anti-CD3 and anti-CD28 after 48 hours resulting in proliferation of a similar magnitude to PHA. This could be inhibited by the anti-CEACAM antibody, but not the isotype control antibody. Addition of bacteria to pre-stimulated cells resulted in further proliferation in many cases.

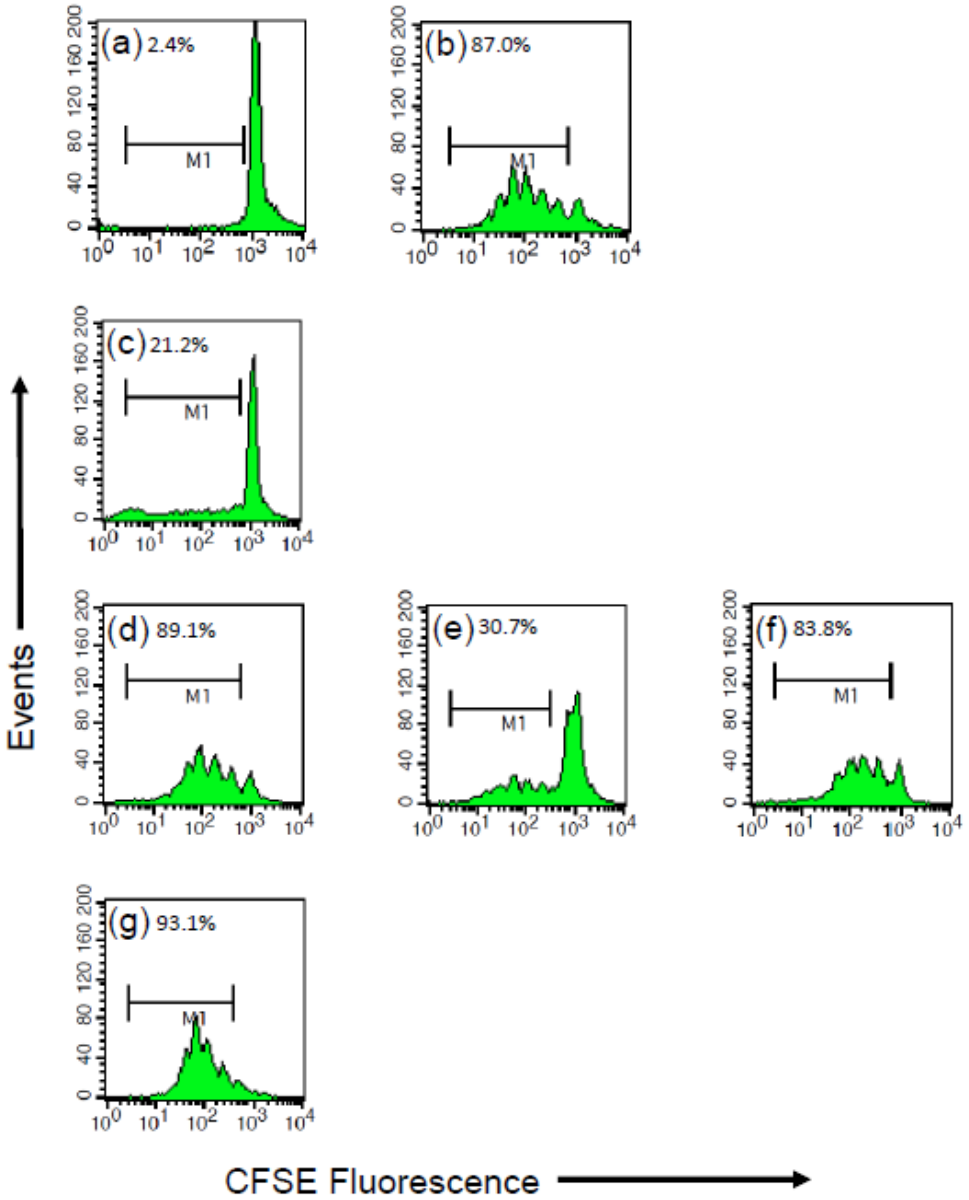

Figure 7.1 Examples of flow cytometry analysis of $CD4^+$ T cell proliferation.
CFSE fluorescence decreases as cells proliferate. Numbers represent the percentage $CD4^+$ T cell proliferation in each plot, which is indicated by the marker M1. PBMCs *ex vivo* from most subjects showed strong proliferation with PHA compared to media alone. When IL-2 was added some proliferation was observed. Proliferation following pre-incubation with IL-2 + anti-CD3 + anti-CD28 was similar to that seen with PHA, and could be inhibited by the anti-CEACAM antibody. Inactivated bacteria also stimulated strong proliferation following pre-incubation with IL-2 + anti-CD3 + anti-CD28.
(a) - (b) PBMCs *ex vivo* with (a) media alone, (b) PHA; (c) PBMCs + IL-2;
(d) - (g) PBMCs + IL-2 + anti-CD3 + anti-CD28 with (d) media alone, (e) anti-CEACAM antibody, (f) isotype antibody, (g) OpaA+ OpaD+ bacteria.

7.6. Assessment of $CD4^+$ T cell proliferation

7.6.1. PBMCs ex vivo

Priority was given to assays involving *ex vivo* PBMCs, as the culture condition of most interest to vaccine development, and 34 samples were used in these assays (table 7.1; figure 7.2).

Antigen	Mean % $CD4^+$ T cell proliferation					
	n	vs. media	*p*-value	n	vs. Opa-	*p*-value
Control antigens						
PHA	**34**	**+61.32**	**<0.0001 (w)**		n/a	
Anti-CD3 + anti-CD28	**12**	**+65.78**	**0.0022 (w)**		n/a	
PPD	**34**	**+4.22**	**<0.0001 (w)**		n/a	
Tetanus toxoid	10	+0.14	0.9609 (t)		n/a	
Diphtheria toxoid	10	-1.83	0.1394 (w)		n/a	
SEB	12	+0.06	0.4802 (w)		n/a	
Isotype antibody	34	-0.79	0.2485 (w)		n/a	
Anti-CEACAM antibody	34	-1.17	0.4417 (w)		n/a	
OMVs						
OMV buffer (3% sucrose)	34	-0.85	0.1997 (w)		n/a	
Opa- OMV	34	-0.80	0.4995 (w)		REF	
wt OMV	34	-0.70	0.7453 (w)	34	+0.10	0.7523 (w)
OpaD+ OMV	34	-0.96	0.6321 (w)	34	-0.17	0.1461 (w)
OpaA+ OpaD+ OMV	34	-0.77	0.4727 (w)	34	+0.02	0.5104 (w)
NVI OMV buffer	14	+0.37	0.5173 (t)		n/a	
NVI Opa- OMV	**14**	**+2.11**	**0.0003 (t)**		REF	
NVI OpaJ+ OMV	**14**	**+1.86**	**0.0076 (w)**	14	-0.25	0.1771 (w)
HPA OMV buffer	14	+0.35	0.4673 (t)		n/a	
HPA *N. lactamica* OMV	**14**	**+2.88**	**0.0013 (t)**		REF	
HPA wt H44/76 OMV	14	-0.07	0.8555 (t)	**14**	**-2.95**	**<0.0001 (t)**
Inactivated bacteria						
Opa-	34	-0.90	0.3604 (w)		REF	
wt H44/76	34	-1.36	0.3256 (w)	34	-0.46	0.3648 (w)
OpaD+	**31**	**+0.32**	**0.0289 (w)**	**31**	**+1.27**	**0.0002 (w)**
OpaA+ OpaD+	34	-0.36	0.0889 (w)	**34**	**+0.55**	**0.0076 (w)**

Table 7.1 $CD4^+$ T cell proliferation following incubation of PBMCs *ex vivo* with OMVs, inactivated bacteria and control antigens.
Values in bold represent antigens where the % $CD4^+$ T cell proliferation was statistically significant compared to media alone or compared to the relevant Opa-negative antigen (REF), with a *p*-value <0.05 using the Student's t-test (t) or Wilcoxon signed-rank test (w) as appropriate.
Comparisons were done using the Student's t-test unless the values were not normally distributed according to the Shapiro-Wilk analysis, in which case significance testing was done using the Wilcoxon signed-rank test.

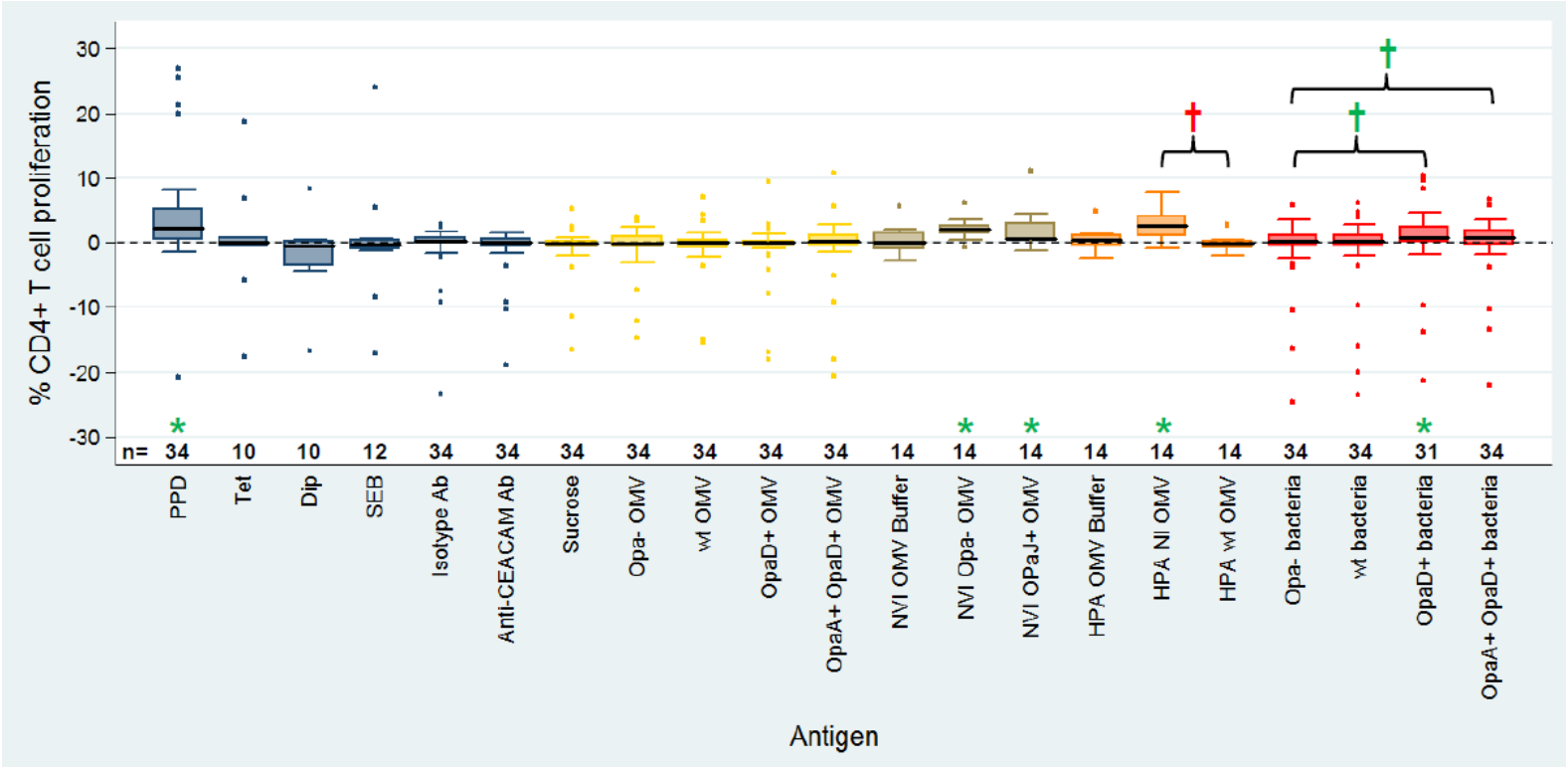

Figure 7.2 CD4$^+$ T cell proliferation following incubation of PBMCs *ex vivo* with OMVs, inactivated bacteria and control antigens.
Incubation with PPD, some OMVs and OpaD+ bacteria resulted in statistically significant stimulation of T cells compared to media alone, although the percentage increase in proliferation was <5% in all cases. There were some statistically significant differences between Opa+ and Opa- preparations, but no consistent Opa-dependent effect.
For each antigen, percentage CD4$^+$ T cell proliferation was plotted after subtracting the proliferation seen in wells containing media alone.
Control antigens are in blue, OMVs constructed in this study are yellow, NVI OMVs in brown, HPA OMVs in orange and inactivated bacteria in red.
Antigens where proliferation was statistically significant ($p < 0.05$) compared to media alone are indicated by ***** (green if increased proliferation and red if decreased proliferation). Antigens where proliferation was statistically significant ($p < 0.05$) compared to the relevant Opa-negative antigen are indicated by † (green if increased proliferation and red if decreased proliferation).

There was statistically significant stimulation of T cell proliferation by both of the NVI OMVs, although no difference between the Opa- and OpaJ+ vesicles. Stimulation was also observed with the *N. lactamica* OMV, which was statistically significantly greater than the HPA H44/76 OMV, although the magnitude of this difference was not large. Most bacterial strains did not demonstrate significant stimulation or inhibition, although the Opa-positive strains stimulated more (OpaD+) or inhibited less (OpaA+ OpaD+) than the Opa-negative strain. In summary, there was no consistent effect of surface-expressed Opa on T cell proliferation observed with *ex vivo* PBMCs.

7.6.2. PBMCs and purified CD4$^+$ T cells with IL-2, anti-CD3 and anti-CD28

A total of 22 samples were processed to examine the effects of the various antigens on PBMCs pre-stimulated with IL-2, anti-CD3 and anti-CD28, with a smaller number involving OMVs from NVI and the HPA (table 7.2; figure 7.3). Under these conditions, T cells are highly stimulated, so any inhibitory effects would expect to be seen. Most of the in-house OMVs (i.e. those constructed in this study) did not exert a significant effect on CD4$^+$ T cells in these conditions, although the wild-type OMV did cause some inhibition compared to the Opa-negative strain. All of the bacteria were able to additionally stimulate CD4$^+$ T cells, with the Opa-negative strain providing more stimulation than the wild-type or OpaD+ strains. There was no suggestion from these studies that Opa induced inhibition of CD4$^+$ T cells, and the differences between the Opa-negative and wild-type strains are likely to be Opa-independent since neither of these strains contained high levels of Opa proteins.

Antigen	Mean % $CD4^+$ T cell proliferation					
	n	vs. media	*p*-value	n	vs. Opa-	*p*-value
Control antigens						
PHA	**22**	**+10.03**	**0.0002 (w)**		n/a	
PPD	**22**	**+3.19**	**0.0048 (t)**		n/a	
SEB	**4**	**+5.26**	**0.0308 (t)**		n/a	
Isotype antibody	**22**	**-2.88**	**0.0057 (t)**		n/a	
Anti-CEACAM antibody	**22**	**-24.51**	**<0.0001 (t)**		n/a	
OMVs						
OMV buffer (3% sucrose)	21	+1.52	0.0537 (w)		n/a	
Opa- OMV	**22**	**+1.87**	**0.0293 (t)**		REF	
wt OMV	22	-2.23	0.0949 (t)	**22**	**-4.11**	**0.0099 (w)**
OpaD+ OMV	22	+0.54	0.6049 (t)	22	-1.33	0.1311 (w)
OpaA+ OpaD+ OMV	22	-0.68	0.5382 (t)	22	-2.56	0.1311 (w)
NVI OMV buffer	8	+1.98	0.4632 (t)		n/a	
NVI Opa- OMV	8	+4.21	0.3621 (w)		REF	
NVI OpaJ+ OMV	8	+3.06	0.1170 (t)	8	-1.15	0.8886 (w)
HPA OMV buffer	8	+3.70	0.1741 (t)		n/a	
HPA *N. lactamica* OMV	8	+8.35	0.1341 (t)		REF	
HPA wt H44/76 OMV	**8**	**+7.21**	**0.0365 (t)**	8	-1.13	0.1614 (w)
Inactivated bacteria						
Opa-	**21**	**+9.26**	**0.0001 (w)**		REF	
wt H44/76	**21**	**+7.15**	**0.0003 (w)**	**21**	**-2.11**	**0.0008 (w)**
OpaD+	**18**	**+7.63**	**0.0006 (w)**	**18**	**-2.79**	**0.0263 (w)**
OpaA+ OpaD+	**21**	**+8.89**	**0.0001 (w)**	21	-0.36	0.4761 (w)

Table 7.2 $CD4^+$ T cell proliferation following incubation of PBMCs + IL-2 + anti-CD3 + anti-CD28 with OMVs, inactivated bacteria and control antigens.

Values in bold represent antigens where the % $CD4^+$ T cell proliferation was statistically significant compared to media alone or compared to the relevant Opa-negative antigen (REF), with a *p*-value <0.05 using the Student's t-test (t) or Wilcoxon signed-rank test (w) as appropriate.

Comparisons were done using the Student's t-test unless the values were not normally distributed according to the Shapiro-Wilk analysis, in which case significance testing was done using the Wilcoxon signed-rank test.

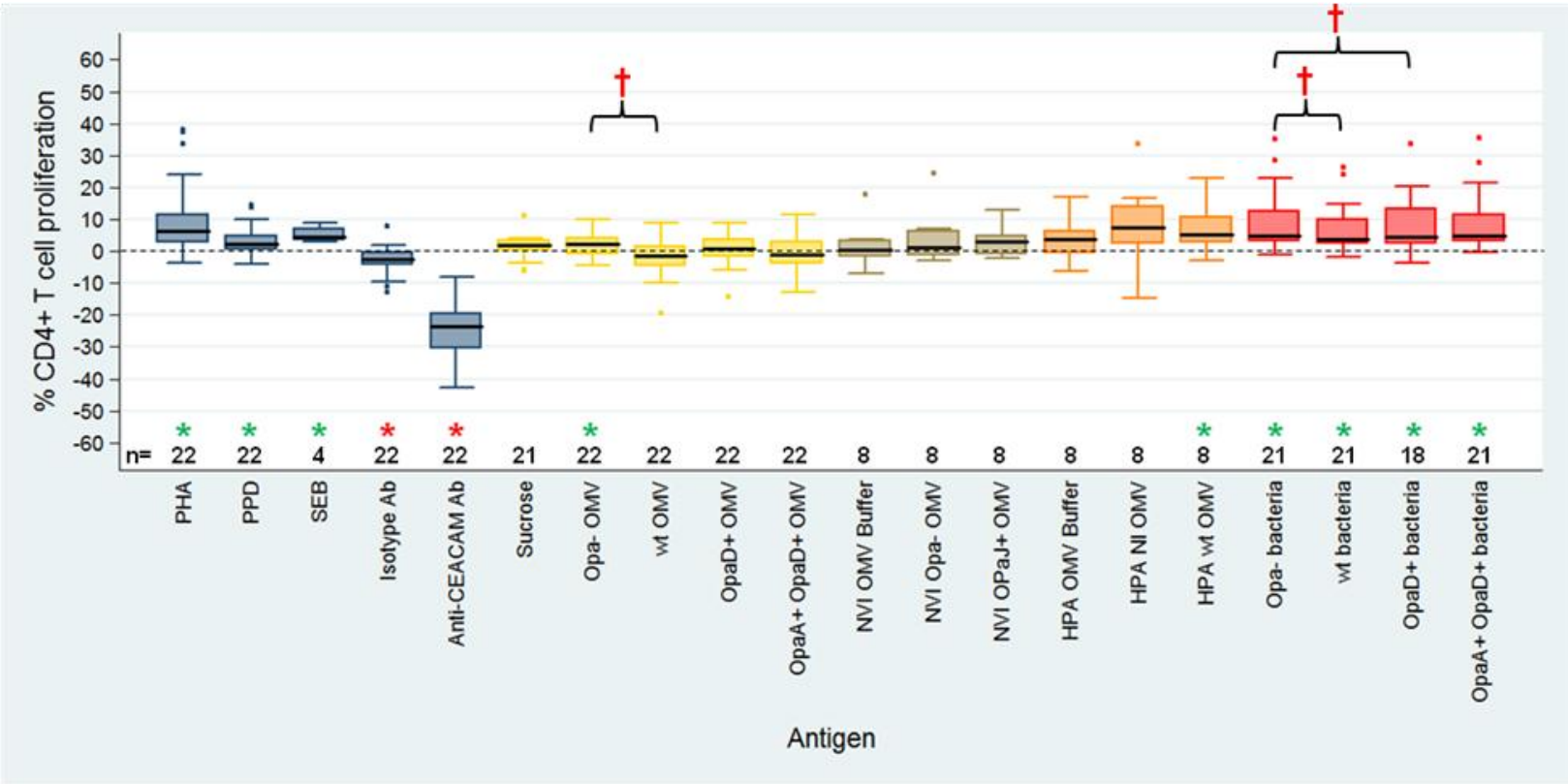

Figure 7.3 CD4$^+$ T cell proliferation following incubation of PBMCs + IL-2 + anti-CD3 + anti-CD28 with OMVs, inactivated bacteria and control antigens.
Incubation with some OMVs and all bacteria resulted in statistically significant stimulation of T cells compared to media alone. There were some statistically significant differences between Opa+ and Opa- preparations, with Opa+ preparations occasionally resulting in less stimulation but this was not seen for all preparations.
For each antigen, percentage CD4$^+$ T cell proliferation was plotted after subtracting the proliferation seen in wells containing media alone.
Control antigens are in blue, OMVs constructed in this study are yellow, NVI OMVs in brown, HPA OMVs in orange and inactivated bacteria in red.
Antigens where proliferation was statistically significant ($p < 0.05$) compared to media alone are indicated by ***** (green if increased proliferation and red if decreased proliferation). Antigens where proliferation was statistically significant ($p < 0.05$) compared to the relevant Opa-negative antigen are indicated by † (green if increased proliferation and red if decreased proliferation).

A smaller number of samples (n=10) were used to purify $CD4^+$ T cells, which were then pre-stimulated with IL-2, anti-CD3 and anti-CD28 before addition of the different antigens (table 7.3; figure 7.4). Under these conditions, there was inhibition of $CD4^+$ T cell proliferation by Opa-negative OMVs, which was similar to that observed following incubation with anti-CEACAM antibody. All of the other OMVs were significantly less inhibitory than the Opa-negative OMVs. All of the bacteria were able to provide additional stimulation to $CD4^+$ T cells in these conditions, similar to the results observed with PBMCs in similar conditions. There were no significant differences between the different bacterial strains. These data suggested that Opa proteins in OMVs reduced inhibition of $CD4^+$ T cells in these *in vitro* conditions.

Antigen	**Mean % $CD4^+$ T cell proliferation**					
	n	**vs. media**	***p*-value**	**n**	**vs. Opa-**	***p*-value**
Control antigens						
PHA	**10**	**+26.63**	**0.0002 (t)**		n/a	
PPD	4	+1.32	0.6787 (t)		n/a	
Isotype antibody	10	-1.67	0.3894 (t)		n/a	
Anti-CEACAM antibody	**10**	**-17.86**	**0.0006 (t)**		n/a	
OMVs						
OMV buffer (3% sucrose)	9	-0.15	0.9294 (t)		n/a	
Opa- OMV	**10**	**-13.94**	**0.0008 (t)**		REF	
wt OMV	8	-3.70	0.0979 (t)	**8**	**+11.00**	**0.0046 (t)**
OpaD+ OMV	**10**	**-8.43**	**0.0067 (t)**	**10**	**+5.51**	**0.0120 (t)**
OpaA+ OpaD+ OMV	**9**	**-6.52**	**0.0497 (t)**	**9**	**+8.08**	**0.0087 (t)**
Inactivated bacteria						
Opa-	**7**	**+15.33**	**0.0005 (t)**		REF	
wt H44/76	**5**	**+15.30**	**0.0138 (t)**	5	-1.44	0.2902 (t)
OpaD+	**7**	**+16.30**	**0.0085 (t)**	7	+0.97	0.7696 (t)
OpaA+ OpaD+	**7**	**+14.40**	**0.0029 (t)**	7	-0.93	0.7352 (t)

Table 7.3 $CD4^+$ T cell proliferation following incubation of purified $CD4^+$ T cells + IL-2 + anti-CD3 + anti-CD28 with OMVs, inactivated bacteria and control antigens.
Values in bold represent antigens where the % $CD4^+$ T cell proliferation was statistically significant compared to media alone or compared to the relevant Opa-negative antigen (REF), with a *p*-value <0.05 using the Student's t-test (t) or Wilcoxon signed-rank test (w) as appropriate.
Comparisons were done using the Student's t-test unless the values were not normally distributed according to the Shapiro-Wilk analysis, in which case significance testing was done using the Wilcoxon signed-rank test.

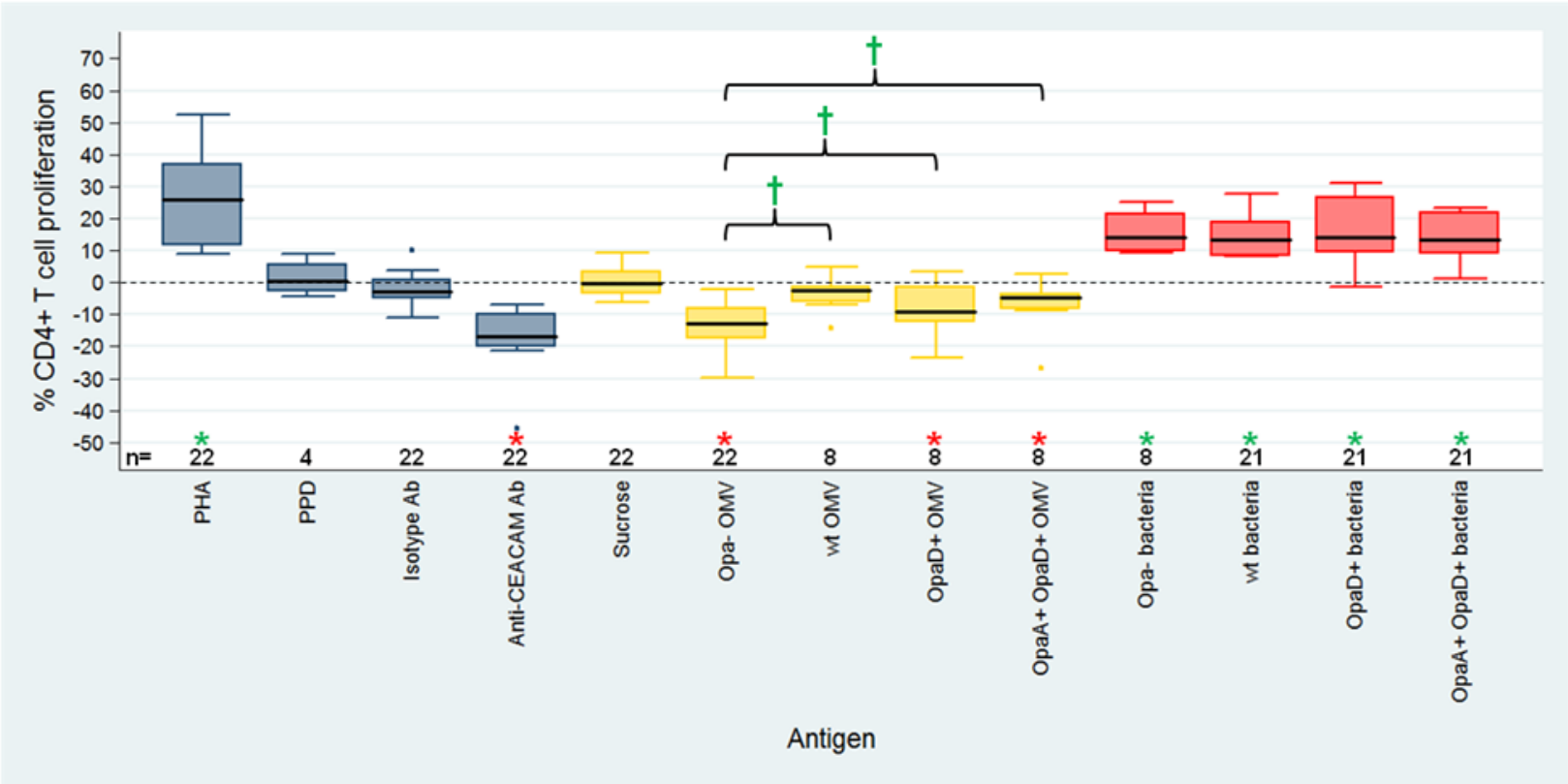

Figure 7.4 CD4$^+$ T cell proliferation following incubation of purified CD4$^+$ T cells + IL-2 + anti-CD3 + anti-CD28 with OMVs, inactivated bacteria and control antigens.

Incubation with the anti-CEACAM antibody and most of the OMV preparations resulted in statistically significant inhibition of T cells compared to media alone. All bacteria caused statistically significant stimulation of T cells compared to media alone. Opa+ OMVs all resulted in less inhibition than Opa- OMVs.

For each antigen, percentage CD4$^+$ T cell proliferation was plotted after subtracting the proliferation seen in wells containing media alone.

Control antigens are in blue, OMVs constructed in this study are yellow and inactivated bacteria in red.

Antigens where proliferation was statistically significant ($p < 0.05$) compared to media alone are indicated by ***** (green if increased proliferation and red if decreased proliferation). Antigens where proliferation was statistically significant ($p < 0.05$) compared to the relevant Opa-negative antigen are indicated by † (green if increased proliferation and red if decreased proliferation).

7.6.3. PBMCs and purified CD4$^+$ T cells with IL-2 only

A small number of samples contained sufficient blood to test the effects of OMVs and bacteria on PBMCs and CD4$^+$ lymphocytes which had been stimulated with IL-2 alone (table 7.4; table 7.5). Although such cells are not as strongly stimulated as those with anti-CD3 and anti-CD28, the increased CEACAM1 expression induced may be apparent, via differences in T cell proliferation between Opa-positive and Opa-negative antigens.

Antigen	Mean % CD4$^+$ T cell proliferation					
	n	**vs. media**	***p*-value**	**n**	**vs. Opa-**	***p*-value**
Control antigens						
PHA	**6**	**+53.55**	**0.0277 (w)**		n/a	
PPD	6	+1.55	.08035 (t)		n/a	
Isotype antibody	6	+4.14	0.1738 (t)		n/a	
Anti-CEACAM antibody	**6**	**-12.86**	**0.0264 (t)**		n/a	
OMVs						
OMV buffer (3% sucrose)	6	-1.30	0.6373 (t)		n/a	
Opa- OMV	6	-2.72	0.4071 (t)		REF	
wt OMV	6	-0.79	0.7568 (t)	6	+1.93	0.1177 (t)
OpaD+ OMV	6	+0.99	0.7754 (t)	**6**	**+3.70**	**0.0040 (t)**
OpaA+ OpaD+ OMV	6	+2.87	0.4154 (t)	**6**	**+5.59**	**0.0030 (t)**
Inactivated bacteria						
Opa-	5	+4.30	0.4179 (t)		REF	
wt H44/76	5	+1.97	0.6701 (t)	5	-2.33	0.2782 (t)
OpaD+	2	+9.06	0.1766 (t)	2	-5.10	0.3946 (t)
OpaA+ OpaD+	5	+1.33	0.7872 (t)	**5**	**-2.97**	**0.0349 (t)**

Table 7.4 CD4$^+$ T cell proliferation following incubation of PBMCs + IL-2 with OMVs, inactivated bacteria and control antigens.

Values in bold represent antigens where the % CD4$^+$ T cell proliferation was statistically significant compared to media alone or compared to the relevant Opa-negative antigen (REF), with a *p*-value <0.05 using the Student's t-test (t) or Wilcoxon signed-rank test (w) as appropriate.

Comparisons were done using the Student's t-test unless the values were not normally distributed according to the Shapiro-Wilk analysis, in which case significance testing was done using the Wilcoxon signed-rank test.

Antigen	Mean % $CD4^+$ T cell proliferation					
	n	vs. media	*p*-value	n	vs. Opa-	*p*-value
Control antigens						
PHA	**4**	**+69.59**	**0.0001 (t)**		n/a	
Anti-CD3 + anti-CD28	**4**	**+54.94**	**0.0010 (t)**		n/a	
Isotype antibody	2	-0.05	0.7952 (t)		n/a	
Anti-CEACAM antibody	2	-0.78	0.0857 (t)		n/a	
OMVs						
OMV buffer (3% sucrose)	4	-0.57	0.0679 (w)		n/a	
Opa- OMV	4	-0.55	0.1089 (t)		REF	
wt OMV	4	-0.56	0.1549 (t)	4	-0.01	0.9511 (t)
OpaD+ OMV	**4**	**-0.43**	**0.0287 (t)**	4	+0.12	0.4518 (t)
OpaA+ OpaD+ OMV	4	-0.36	0.4652 (w)	4	+0.20	0.4338 (t)
Inactivated bacteria						
Opa-	3	+0.19	0.7049 (t)		REF	
wt H44/76	2	+0.55	0.4637 (t)	2	-0.04	0.8855 (t)
OpaD+	3	+0.20	0.8183 (t)	3	+0.01	0.9796 (t)
OpaA+ OpaD+	2	+1.64	0.3757 (t)	2	+1.04	0.4250 (t)

Table 7.5 $CD4^+$ T cell proliferation following incubation of purified $CD4^+$ T cells + IL-2 with OMVs, inactivated bacteria and control antigens

Values in bold represent antigens where the % $CD4^+$ T cell proliferation was statistically significant compared to media alone or compared to the relevant Opa-negative antigen (REF), with a *p*-value <0.05 using the Student's t-test (t) or Wilcoxon signed-rank test (w) as appropriate.

Comparisons were done using the Student's t-test unless the values were not normally distributed according to the Shapiro-Wilk analysis, in which case significance testing was done using the Wilcoxon signed-rank test.

Opa-positive OMVs did stimulate $CD4^+$ proliferation in the PBMC culture significantly more than the Opa-negative OMVs, although the converse was observed following incubation with bacteria where the OpaA+ OpaD+ bacteria were less stimulatory than the Opa-negative bacteria. There were no significant differences between the Opa-positive and Opa-negative antigens in the purified $CD4^+$ T cell culture. These assays included only a small number of participants, making it difficult to draw any firm conclusions.

7.6.4. Individual variation in $CD4^+$ T cell proliferative responses

Further examination of the results revealed interesting heterogeneity of the responses between different individuals (table 7.6). In all culture conditions, most OMVs and

bacteria did not cause significant stimulation or inhibition of either PBMCs or $CD4^+$ T cells in the majority of individuals. When stimulation or inhibition was observed, the in-house OMVs appeared equally likely to stimulate or inhibit, with the exception of culture with pre-stimulated purified $CD4^+$ T cells, when none of the OMVs stimulated proliferation in any individuals. OMVs from NVI and the HPA were more likely to stimulate than to inhibit, suggesting that differences between OMVs were more likely to be Opa-independent. Bacteria were much more likely to stimulate than inhibit in all conditions, with no differences observed between the Opa-positive and Opa-negative strains.

Antigen	PBMCs *ex vivo* (*n = 34*)			PBMCs + IL-2 (*n = 6*)			PBMCs + IL-2 + anti-CD3/CD28 (*n = 22*)			$CD4^+$ T cells + IL-2 (*n = 4*)			$CD4^+$ T cells + IL-2 + anti-CD3/CD28 (*n = 10*)		
	S	**I**	**NS**	**S**	**I**	**NS**	**S**	**I**	**NS**	**S**	**I**	**NS**	**S**	**I**	**NS**
Control antigens															
PHA	30	0	4	5	0	1	12	0	10	4	0	0	9	0	1
Anti-CD3 + anti-CD28	10	0	2	–	–	–	–	–	–	4	0	0	–	–	–
PPD	16	0	18	2	0	4	3	1	18	–	–	–	0	0	4
Tetanus toxoid	0	0	10	–	–	–	–	–	–	–	–	–	–	–	–
Diphtheria toxoid	0	1	9	–	–	–	–	–	–	–	–	–	–	–	–
SEB	0	3	9	–	–	–	3	0	1	–	–	–	–	–	–
Isotype antibody	7	2	25	2	0	4	0	5	17	0	0	2	1	2	7
Anti-CEACAM antibody	5	2	27	0	3	3	0	20	2	0	0	2	0	7	3
OMVs															
OMV buffer (3% sucrose)	1	2	31	0	0	6	4	0	17	0	0	4	0	1	8
Opa- OMV	5	3	26	0	0	6	5	1	16	0	0	4	0	7	3
wt OMV	4	3	27	0	0	6	3	3	16	0	0	4	0	2	6
OpaD+ OMV	5	4	25	1	0	5	2	1	19	0	0	4	0	5	5
OpaA+ OpaD+ OMV	6	6	22	0	0	6	1	4	17	0	0	4	0	2	7
NVI OMV buffer	2	2	10	–	–	–	2	0	6	–	–	–	–	–	–
NVI Opa- OMV	9	0	5	–	–	–	1	0	7	–	–	–	–	–	–
NVI OpaJ+ OMV	6	0	8	–	–	–	0	0	8	–	–	–	–	–	–
HPA OMV buffer	3	2	9	–	–	–	1	0	7	–	–	–	–	–	–
HPA *N. lactamica* OMV	8	0	6	–	–	–	5	1	2	–	–	–	–	–	–
HPA wt H44/76 OMV	1	2	11	–	–	–	2	1	5	–	–	–	–	–	–
Inactivated bacteria															
Opa-	7	5	22	3	0	2	10	0	11	0	1	2	4	0	3
wt H44/76	9	3	22	2	0	3	9	0	12	0	0	2	2	0	3
OpaD+	7	1	23	2	0	0	8	0	10	0	0	3	5	0	2
OpaA+ OpaD+	8	3	23	2	1	2	12	0	9	2	0	0	5	0	2

Table 7.6 Individual responses to OMVs, inactivated bacteria and control antigens in all culture conditions.

Numbers represent the number of individuals where there was statistically significant (p-value <0.05) stimulation (S) or inhibition (I) of $CD4^+$ T cell proliferation compared to media alone, and the number of individuals where the response to each antigen was not statistically significant (NS).

7.7. Discussion

This chapter described the effect of surface-expressed Opa proteins on $CD4^+$ T cells. The conflicting published data regarding the effects of meningococcal Opa proteins on $CD4^+$ T cell proliferation have created a potentially significant hurdle in the future development of any vaccines containing Opa proteins. Whereas some studies have suggested that Opa proteins stimulate T cell proliferation, others have demonstrated

inhibition and yet others have shown no significant differences between Opa-positive and Opa-negative bacteria and OMVs[238, 240-242]. In terms of vaccine development, it is difficult to use many of these *in vitro* assays to predict what happens *in vivo*, either during infection or following immunisation. In an attempt to clarify the effects of surface-expressed Opa proteins on $CD4^+$ T cells, a number of assays were undertaken using different cell culture conditions, and a variety of Opa-positive and Opa-negative antigens.

Overall, there was no consistent Opa-dependent effect of meningococcal OMVs or inactivated bacteria on $CD4^+$ T cells across the conditions examined. Most antigens did not result in significant stimulation of $CD4^+$ T cells in culture of PBMCs *ex vivo*, and even when stimulation did occur, the magnitude was much lower than that observed for control antigens. In a culture of pre-stimulated purified $CD4^+$ lymphocytes, Opa-negative OMVs actually caused more inhibition than Opa-positive strains, and inactivated bacteria from all strains resulted in a similar degree of stimulation. These effects were not seen when $CD4^+$ lymphocytes were examined following culture of antigens with pre-stimulated PBMCs. Unfortunately it was not possible to examine the NVI and HPA OMVs with purified $CD4^+$ T cells, to be able to directly compare this assay with published data.

The most striking feature of the results from these healthy adults was the heterogeneity of results between different individuals. Whereas cells from some individuals clearly did undergo significant stimulation or inhibition with some antigens in some conditions, the majority of antigens did not cause a significant

response in the majority of individuals. The reasons for these differences have not been assessed in this study, and may be a result of differences in previous exposure to *N. meningitidis*, or variation in host genetics and specifically polymorphism in the major histocompatibility complex (MHC) molecule.

The results from these experiments do not support a proposed hypothesis that surface-expressed Opa proteins inhibit $CD4^+$ T cell proliferation. However, this was a study of only two Opa variants, and it may be that different Opa proteins have different effects. The proposed mechanism of inhibition is mediated via an interaction between Opa and CEACAM1 on the T cell surface (figure 1.7), and both the OpaA and OpaD protein from H44/76 bind to CEACAM1[231]. Previous studies have suggested that all CEACAM1-binding Opa variants would mediate the same effect on T cells, so any inhibitory effect should therefore have been apparent. Initial data using recombinant Opa proteins as antigens in similar T cell assays in a small number of individuals suggested there may be some Opa-dependent inhibition of T cell proliferation, and more robust data in a larger number of subjects are awaited[351].

Previous studies have demonstrated both stimulatory and inhibitory effects of Opa proteins, and more recent studies have suggested that the interaction between *N. meningitidis* or meningococcal OMVs and $CD4^+$ T cells is not Opa-dependent (table 7.7). There may be a number of reasons to explain the differences in these data. Different studies have used different Opa variants. Opa variants from H44/76 have not previously been shown to have an inhibitory effect. Although all Opa proteins encoded by H44/76 are CEACAM1-binding, there may be other differences between

CEACAM-binding Opa variants that mediate distinct downstream effects. A major difference between studies is the culture conditions and method of assessment of proliferation. PBMCs and purified $CD4^+$ T cells have been used, with most studies including assays with IL-2 pre-incubation and/or addition of anti-CD3 and anti-CD28. Even when IL-2 pre-incubation is done, there are differences in duration, which may affect CEACAM1 expression on the T cell surface. These conditions may be useful in exploring specific interactions, but are unlikely to represent what occurs *in vivo*, and should be interpreted with caution when predicting the potential effects of Opa proteins during meningococcal infection or following immunisation in humans. Immune responses to previous OMV vaccines have included the induction of anti-Opa antibodies, which strongly suggests that Opa does not have a significant immunosuppressive effect.

Subjects	Antigens	Culture conditions	Method of assessment	Results	Ref
18 healthy adults (29-55y)	Purified NM Opa proteins (3 variants)	PBMCs *ex vivo*	[^{3}H]thymidine incorporation	Opa induced proliferation in most donors, greater than Opc or PorA	238
≥4 individuals per experiment, no further details	Live NG expressing different Opa variants (strain MS11)	Purified CD4$^+$ T cells***** +/- IL-2 (48 hours pre-incubation) +/- anti-CD3 +/- anti-CD28	Lymphocyte density, assessed by direct counting	HSPG-binding Opa$^+$ bacteria induced greater proliferation than CEACAM-binding Opa$^+$ bacteria	242
Not stated	OMVs from Opa$^+$ NM (strain K454) and Opa$^-$ NL (strain Y92 1009) OMVs from NG expressing different Opa variants (strain MS11)	Purified CD4$^+$ T cells + IL-2 (48 hours pre-incubation) + anti-CD3 + anti-CD28 Jurkat CD4$^+$ T cells† +/- IL-2 (48h) and/or anti-CD3	Cell culture density, assessed by direct counting	NL Opa$^-$ OMVs induced greater proliferation than NM Opa$^+$ OMVs Opa$^-$ or HSPG-binding Opa$^+$ OMVs induced greater proliferation than CEACAM-binding Opa$^+$ OMVs	241
Healthy adults and buffy coats, number not stated	Live Opa$^+$ or Opa$^-$ NM (strain C751 or H44/76)	Purified CD4$^+$ T cells‡ +/- IL-2 (48 hours pre-incubation) +/- anti-CD3	[^{3}H]thymidine incorporation	No difference in proliferation between isogenic Opa$^+$ and Opa$^-$ strains	240
	Live Opa$^+$ or Opa$^-$ NM (strain C751) **or** heat-killed Opa$^+$ or Opa$^-$ NM (strain H44/76) **or** Opa$^+$ or Opa$^-$ NM OMVs (strain H44/76)	Purified CD4$^+$ T cells + IL-2 (96 hours pre-incubation) +/- anti-CD3 and anti-CD28	Flow cytometric analysis of CFSE dilution	No difference in proliferation between isogenic Opa$^+$ and Opa$^-$ strains	
46 healthy adults (≥18 years)	Opa$^+$ or Opa$^-$ NM OMVs **or** ethanol-fixed Opa$^+$ or Opa$^-$ NM (strain H44/76)	PBMCs +/- IL-2 (48 hours) +/- anti-CD3 and anti-CD28 **or** purified CD4$^+$ T cells + IL-2 (48 hours) +/- anti-CD3 and anti-CD28	Flow cytometric analysis of CFSE dilution	No consistent difference in proliferation between isogenic Opa$^+$ and Opa$^-$ strains	this study

Table 7.7 Studies of the effects of Opa on CD4$^+$ T cell proliferation.
NM = *N. meningitidis*, NG = *N. gonorrhoeae*, NL = *N. lactamica*; *****conditions included CD4$^+$ T cells alone, with IL-2 or anti-CD3 only, with IL-2 and anti-CD3, with anti-CD3 and anti-CD28, and with IL-2, anti-CD3 and anti-CD28; †conditions included CD4$^+$ T cells alone, with IL-2 or anti-CD3 only, and with IL-2 and anti-CD3; ‡conditions included CD4$^+$ T cells alone, with anti-CD3 only, and with IL-2 and anti-CD3.

Methods of measuring proliferation vary and include the use of [^{3}H]thymidine, CFSE dilution and direct counting of lymphocyte density. Dead cells can be easily excluded from the analysis when CFSE dilution is evaluated by flow cytometry, as was done in this study, but this is more difficult when using the other methods. One study did assess proliferation using both CFSE dilution and [^{3}H]thymidine incorporation with the same antigens, but these were done with slightly different culture conditions, so there is uncertainty whether these different methods provide comparable data.

One major advantage of this study was the inclusion of a large number of healthy adult subjects, which highlighted the variation between individuals (table 7.6). Most previous studies have not stated sample size and the use of buffy coats from the National Blood Service in one study[240] would make it impossible to have specific inclusion and exclusion criteria. Comparison of Opa-positive and Opa-negative strains to determine Opa-dependent effects is only valid when comparing otherwise isogenic strains. The isogenic strains constructed in this study (chapter 5) were therefore critical for these experiments. Inclusion of a strain expressing 2 different Opa proteins allowed additional assessment of the potential immunomodulatory effects of Opa, since previous studies included Opa-positive strains which usually expressed a single Opa protein. Although most studies used isogenic strains, one study compared Opa-positive OMVs from *N. meningitidis* with Opa-negative OMVs from *N. lactamica*. It would be expected that these OMVs would differ significantly with regards to expression of other OMPs, so it is difficult to conclude that any differences are due to Opa. Even when isogenic strains are used, there may be alterations in the expression of other phase variable proteins, as discussed in chapter 6, which may result in

differences between strains. The large number of proteins in addition to Opa on the surface of OMVs and bacteria provide an additional difficulty in interpreting results, and any Opa-dependent phenomenon would have to be sufficiently dominant to be detectable using these antigens.

This is the first study of the effects of Opa proteins on $CD4^+$ T cells to include a range of culture conditions, including the use of PBMCs and purified $CD4^+$ T cells, using the same set of antigens with the same measure of proliferation. Moreover, a number of different forms of surface-expressed Opa have been included in order to try and detect a consistent effect of Opa on $CD4^+$ T cells. Finally, OMVs used in previous studies have been included to allow some comparison between this study and published data, although continuing these experiments with a greater number of individuals would be required to enable firm conclusions to be made.

Results from this study are consistent with findings from recent studies of meningococcal OMVs, including some used in this study, that there is no significant Opa-dependent stimulation or inhibition of $CD4^+$ T cells[240]. Other studies which have demonstrated inhibition have utilised different Opa variants, which were not available for this study. The significance of any *in vitro* findings to *in vivo* mechanisms is unknown, and this is particularly the case when cells are being artificially stimulated *in vitro*. In studies of OMVs or bacteria, it is likely that a number of other antigens will have a significant effect on determining T cell proliferation, and it may be that differences between studies can be explained by changes in the bacterial strains used and differences in methodology, rather than being an inhibitory effect of Opa proteins.

CHAPTER 8.

8.1.

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CHAPTER 9. CONCLUSIONS

9.1. Introduction

The aim of this study was to provide data to aid evaluation of a strategy of using meningococcal Opa proteins in a future vaccine, and to examine the immunomodulatory role of surface-expressed Opa proteins. There are conflicting published data on the effects of Opa proteins on $CD4^+$ T cell proliferation so the potential of using Opa-negative OMVs as the basis for a future vaccine was assessed. There was also exploration of PV of meningococcal Opa proteins, and the implications of this for the design of future OMV vaccines.

9.2. Construction of Opa-deficient meningococci

The original aim of this study was to ideally construct a set of isogenic mutant meningococci each able to constitutively express a single different Opa protein, and an Opa-negative strain. The original strategy required the construction of locus-specific *opa* plasmids with and without antibiotic resistance cassettes to specifically and sequentially disrupt each *opa* gene using the same selectable marker by transformation of *N. meningitidis* strain H44/76. Construction of some of these plasmids was unsuccessful, so an alternative strategy involving generic *opa* plasmids was devised. Each generic plasmid targeted two of the four *opa* genes during transformation, and the genotype of transformants could be identified by PCR analysis.

A panel of 15 genotypically different Opa-deficient meningococci was obtained using these plasmids, including an Opa-negative strain. Although this unique panel of

isogenic mutant strains was different to the original objective, it enabled the additional study of Opa PV using the mAbs which were available, and allowed assessment of OMVs and bacteria expressing one or two different Opa proteins, which would not have been possible with the four different mutant strains originally planned. This panel of strains remain a potentially valuable tool in the assessment of Opa proteins as a vaccine candidate and in further work on their role in the pathogenesis of meningococcal infection. Future work should aim at developing these strains further, and to construct strains which do constitutively express Opa. Ideally, this would involve a marker which is not an antibiotic cassette, and which ideally could easily be inserted and removed, such as *lacZ*, where a colour change would be readily detectable when picking transformant colonies.

Transformation of *N. meningitidis* is a tool which is commonly used to create genetically modified strains. However, few published methods are available to provide guidance on the optimal methods of DNA cloning related to creating mutant meningococcal strains. One of the challenges of creating the Opa-deficient strains was the requirement to disrupt four homologous genes, which therefore relied on using sequences adjacent to the *opa* genes for homologous recombination to occur. The failures and successes experienced during cloning of *opa* genes suggests that the following factors would increase the success of genetic manipulation of meningococcal genes:

(i) smaller lengths of DNA fragments, in particular avoiding regions of repetitive DNA;

(ii) high concentrations of DNA in ligation reactions;

(iii) dephosphorylation of vector DNA in ligation reactions;

(iv) selective restriction enzyme digestion of self-ligated vector DNA post-ligation;

9.3.

9.4. The effect of Opa proteins on $CD4^+$ T cell proliferation

There are conflicting data regarding the effect of Opa proteins on $CD4^+$ T cell proliferation, and this is a potential major obstacle to the inclusion of Opa proteins in future meningococcal vaccines. To investigate this, the effects of Opa-positive and Opa-negative OMVs and inactivated bacteria on $CD4^+$ T cells were assessed in different culture conditions. There was no evidence that Opa proteins resulted in inhibition of $CD4^+$ T cells in any conditions. Culture of PBMCs *ex vivo* revealed little stimulation or inhibition by any of the antigens tested. When PBMCs or purified $CD4^+$ T cells were pre-stimulated, all bacteria provided additional stimulation. Differences between OMVs were more apparent when comparing OMVs from different sources rather than being dependent on their Opa phenotype. Finally, Opa-negative OMVs caused the greatest degree of inhibition of pre-stimulated purified $CD4^+$ T cells, compared to Opa-positive OMVs.

These data do not provide any evidence that Opa proteins should be excluded from future vaccines, or that consideration of Opa as a vaccine candidate should cease. Indeed, many OMVs that have been used in clinical trials, including those that have become licensed, contain Opa[205, 247, 248]. To complete these experiments, data are required on the effect of recombinant Opa proteins on $CD4^+$ T cells using the same conditions. A study of a small number of individuals suggested that recombinant Opa proteins inhibited $CD4^+$ T cell proliferation in culture of PBMCs and purified $CD4^+$ T cells[351], although data in a larger number of individuals is required. In the same study, no effect was seen during stimulation of *ex vivo* PBMCs. The significance of the results from these *in vitro* assays on the *in vivo* situation remains unclear. The effect

of Opa proteins on other immune cells also requires further evaluation. In particular, Opa proteins are known to interact with CEACAM receptors on neutrophils, and possibly B cells. The effect on B cells is of particular interest, as any effect on $CD4^+$ T cells would also influence B cell responses, and ultimately production of antibody, which is the key determinant of protection against IMD. Finally, a recent study suggested that other meningococcal proteins interact with CEACAM[356] and further work is required to identify those proteins and the influence they might have.

The individual variation observed in all culture conditions and with all antigens highlights the importance of the host in determining the immune response. Unfortunately, participant DNA was not collected as a part of this study, so it was not possible to explore the host genetic determinants of the differences in response to the different antigens tested. Host determinants of responses to OMV vaccines are not known, and would be an important area for future study given the number of OMV vaccines in development, not only for meningococcal disease but also for other infections[357-360]. In the future it may be possible to identify groups of individuals who will respond poorly to certain OMVs, and target and tailor vaccines specifically to defined groups.

9.5. Phase variation of meningococcal Opa proteins

The PV rates of meningococcal Opa proteins from H44/76 were similar to rates previously described for *N. gonorrhoeae*[203]. Opa PV may be important for immune evasion in pathogenic Neisseria, and differences in PV rates may also suggest variations in function of different Opa proteins. Following this exploratory analysis, a

number of questions remain. It is clearly important to establish if the patterns seen for H44/76 Opa proteins are also present with other strains, and in different growth conditions. Of most interest is perhaps the effect of PV during infection with *N. meningitidis*, and whether or not it has an influence on prolonging or shortening the duration of carriage, or stimulating invasion of a particular organism. This would require a large number of isolates from both disease and carriage, and ideally isolates from a longitudinal study in which bacteria have not been serially passaged, therefore reducing the likelihood of PV occurring *in vitro*.

Opa PV rates were also increased following transformation of an unrelated plasmid, which has been described previously[340]. This has potentially significant implications for vaccine design, since several meningococcal OMV vaccines in pre-clinical development and early clinical trials are based on strains that have been genetically modified by transformation with exogenous DNA. Alteration in a small number of phase-variable proteins may result in changes in immunogenicity and reactogenicity of the resulting OMVs. Although OMVs have been thought of as relatively "crude" vaccines they are effective in eliciting a bactericidal immune response. However, a number of problems remain since the immune responses are serosubtype-specific, multiple doses are required and efficacy is limited in young children. In the future these vaccines could be improved if the key determinants of immunogenicity and reactogenicity could be identified, enabling further manipulation of these vaccines. The large number of phase-variable proteins in *N. meningitidis* means that increased PV rates during transformation may have a significant effect on OMVs, which could be manipulated to optimise future vaccines.

9.6. Summary

In this study, a panel of Opa-deficient meningococci were constructed to provide a tool to assess the potential of Opa proteins as a future meningococcal vaccine.

There was no consistent evidence of $CD4^+$ T cell inhibition or stimulation by any surface-expressed Opa proteins, suggesting that development of Opa as a vaccine candidate should continue, including further immunological studies on the immunomodulatory effects of Opa proteins, and good epidemiological data are required to identify which Opa variants should be included in any developing vaccines. The finding of significant individual variation in responses to OMVs and bacteria, and the increased rates of Opa PV following transformation of *N. meningitidis* highlights the importance of future work in refining the bacterial and host factors involved in producing a good quality immune response following immunisation with OMV vaccines, which in the case of meningococcal vaccines equates to high levels of bactericidal antibodies. Future vaccines can then be optimised by genetic manipulation of vaccine strains, and targeting of different vaccines to specific population groups to produce vaccines that provide comprehensive protection against meningococcal disease in all potentially susceptible individuals.

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