Investigation of the potential of PorA and FetA as meningococcal vaccine components

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Abstract

Investigation of the potential of PorA and FetA as meningococcal vaccine components

Holly Sanders, Hertford College - DPhil Submission, Hilary Term 2012

In the search for a vaccine providing comprehensive protection against meningococcal disease, one vaccine currently under development contains the immunogenic proteins PorA and FetA in meningococcal outer membrane vesicles (OMVs). To achieve high levels of coverage against disease-causing isolates, the antigenic variability of these proteins could be overcome using knowledge of meningococcal epidemiology and population structure. In this study, the possible implications of variable expression levels of PorA and FetA on vaccine efficacy were investigated. Producing OMVs containing consistent amounts of FetA is difficult due to iron-repressed expression; therefore, meningococcal strains were constructed which constitutively expressed FetA at increased levels for OMV vaccine production and analysis. In mice, OMVs from modified strains induced antibodies against both PorA and FetA. These antibodies acted synergistically in a serum bactericidal assay; however, antibodies against FetA were weakly bactericidal alone. The potential to increase levels of PorA- and FetA-specific bactericidal antibodies with a prime-boost strategy, using OMV and protein inoculums, was also tested. While successful for a weakly-immunogenic PorA variant, a similar strategy did not increase bactericidal activity against FetA. Although antibodies against FetA can be induced following OMV immunisation, sufficient antigen expression in target bacteria is also required for bactericidal killing; therefore, the variability and regulation of *porA* and *fetA* transcription was investigated in a range of isolates. Despite differences in regulation among clonal complexes, variable expression is unlikely to be an issue for vaccine coverage. In particular, regulation of *fetA* by iron is reduced in many isolates due to a deletion in the sequence bound by the regulatory protein, Fur. Therefore, a vaccine targeting PorA and FetA may provide high levels of protection against meningococcal disease; however, an alternative formulation or immunisation strategy is required to improve coverage against FetA.

Preface

This thesis describes work carried out in the Division of Bacteriology, National Institute for Biological Standards and Control (NIBSC). The project forms part of a collaboration with the University of Oxford and the University of Manchester, funded by a Wellcome Trust Strategic Translation Award. All work described here is my own, with the exception of the care and immunisation of animals used, which were carried out by the Biological Services Division at NIBSC. Where information or components have been obtained from other sources, these have been acknowledged in the text. No part of this work has been submitted for any other degree.

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Abbreviations

Abbreviation	Full Term
aa	Amino Acid
AdjSS	Adjusted Sum of Squares
APC	Antigen presenting cell
APS	Ammonium persulphate
BCA	Bicinchoninic Acid
bp	Base pairs
cc	Clonal complex
CC	Complement control
cDNA	Copy Deoxyribonucleic acid
cfu	Colony forming units
CIC	Complement independent control
CNS	Central nervous system
CT	Critical Threshold (of fluorescence)
CPS	Conjugate polysaccharide
DFAM	Deferoxamine mesylate salts
DNA	Deoxyribonucleic acid
dNT	Deoxynucleotide
DOC	Sodium deoxycholate
dOMV	Detergent-extracted outer membrane vesicle
ELISA	Enzyme-Linked Immunosorbent Assay
EDTA	Ethylenediaminetetraacetic acid disodium salt
fB	Factor B
fH	Factor H
FS	Frameshift
GMT	Geometric mean titre
HRP	Horseradish Peroxidase
hSBA	Serum bactericidal assay with human complement
IgG	Immunoglobulin G
IgM	Immunoglobulin G
IPTG	Isopropyl β-D-1-thiogalactopyranoside
KanR	Kanamycin resistance marker
ln	Natural logarithm
LB	Luria-Lysogeny
LPS	Lipopolysaccharide
MenA	Serogroup A *Neisseria meningitidis*
MenB	Serogroup B *Neisseria meningitidis*
MenC	Serogroup C *Neisseria meningitidis*
MH	Mueller Hinton
MHCII	Major Histocompatibility Complex II
MLST	Multi-Locus Sequence Typing
MPL	Monophosphoryl Lipid A
mRNA	Messenger ribonucleic acid

nOMV	Native outer membrane vesicle
NIBSC	National Institute for Biological Standards and Control
NUS	*Neisseria* Uptake Sequence
OMP	Outer membrane protein
OMV	Outer membrane vesicle
ORF	Open reading frame
PAGE	Poly-Acrylamide Gel Electrophoresis
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
Poly-C	Poly-cytosine
Poly-G	Poly-guanidine
PPS	Plain polysaccharide
qRT-PCR	Quantitative reverse-transcription polymerase chain reaction
RNA	Ribonucleic acid
RQ	Relative Quantity
rSBA	Serum bactericidal assay with baby rabbit complement
RT	Reverse transcription
SBA	Serum bactericidal assay
SD	Shine-Dalgarno
SDS	Sodium dodecyl sulphate
SNP	Single nucleotide polymorphism
SOC	Super Optimal broth with Catabolite repression
ST	Sequence Type
TDM	Trehalose dicorynomycolate
TEMED	Tetramethylethylenediamine
TLR	Toll-like receptor
TMB	Tetramethylbenzidine
TRIS	Trishydroxymethylaminomethane
tRNA	Transfer ribonucleic acid
TSA	Tryptone soya agar
TSB	Tryptone soya broth
VC	Viable count
VR	Variable region
X-gal	5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside

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Introduction

1.1: Background

Neisseria meningitidis (the meningococcus) is a Gram-negative, diplococcal bacterium that is naturally found exclusively in the nasopharynx of humans. Although usually a commensal organism, the meningococcus occasionally causes meningitis and septicaemia in its human hosts. There is high genetic similarity between *N. meningitidis* and other commensal and pathogenic *Neisseria* species, particularly *N. lactamica* (a commensal in the human nasopharynx) and *N. gonorrhoeae* (the gonococcus - the pathogen responsible for the sexually transmitted disease gonorrhoea). However, these bacteria are deemed as separate species due to their diverse lifestyles, ecological separation and the resulting genetic isolation between meningococci and gonococci. [1,2]

The normal lifecycle of *N. meningitidis* involves asymptomatic colonisation of the nasopharynx of humans, with transmission by direct contact or transfer of respiratory droplets. Carriage can be transient or last for several months, and multiple strains may be carried simultaneously [3]. In children less than four years of age, only about 1% carry meningococci [4]. This has been postulated to be due to competition between *N. meningitidis* and high levels of *N. lactamica*, carried in young children [5]. The carriage rate of meningococci is higher in older age groups (18-20%) [6], with the risk of carriage increasing significantly in teenage years [7].

In a process that is not well understood, some meningococci may, shortly after colonisation, pass into the bloodstream [8]. The meningococci that survive can then rapidly disseminate to various organs, causing septicaemia. In many cases the bacteria will cross the blood-brain barrier and proliferate in the central nervous system (CNS), causing inflammation of the meninges [9]. The classically recognised symptoms of meningococcal disease include neck

stiffness, photophobia or other CNS signs (associated with meningitis) and cardiovascular shock or multi-organ failure (associated with septicaemia), as well as a characteristic purpuric rash [10].

Despite a low incidence across most of the world, *N. meningitidis* is an important health concern as the case fatality rate is high even with antibiotic treatment, and up to 20% of survivors suffer from serious sequelae [11]. Permanent sequelae include deafness, seizures, amputations and mental retardation [12]. Therefore, improvement of preventative measures and the study of the spread of disease are important global priorities.

1.1.1: Classification

Meningococci are often classified into serogroups according to the chemical nature of the polysaccharide capsule. Twelve chemically and antigenically distinct capsules have been recorded, although only six types (A, B, C, X, Y and W135) are usually associated with disease [13]. Many meningococci have lost the genes necessary for capsule synthesis. These capsule-null strains are usually only found in carriage as capsule expression is an important virulence factor [14].

Although classification into serogroups through biochemical methods has been used historically to type meningococci, technological advances in high-throughput sequencing and information technology [15] mean that classification is now primarily based on genetic information. As well as sequencing the genes for capsule and protein synthesis, allowing more accurate antigenic classification, epidemiological data is collected using Multi-Locus Sequence Typing (MLST). The process of MLST involves sequencing internal fragments of about 500 base pairs (bp) of each of seven housekeeping genes. Individual, unique nucleotide sequences are then assigned an allele number in order of discovery. A new allele number is assigned whether

the sequences differ at only a single base or at many bases, as a genetic event resulting in a new allele can occur from either point mutation or by recombination. The allele numbers for each of the seven loci are then combined to give an allelic profile, which determines the sequence type (ST). STs are then assigned to a clonal complex (cc), with all isolates in a particular cc sharing at least four of the seven loci in common [15,16,17]. MLST data is now available for thousands of meningococcal isolates [17], with antigenic data also available to support the epidemiology.

1.1.2: Disease Burden

The highest incidence of meningococcal disease, as well as the highest disease:carriage ratio, is found in young children after maternal antibodies have waned [4,12]. The risk of disease, given carriage, then declines with age as repeated exposure to meningococci through carriage leads to priming of the antibody response and, consequently, humoral immunity against invasive meningococcal infection [18,19]. There is a small secondary peak in disease incidence at adolescence (15-19 years), most likely due to increased rates of carriage and transmission [20].

Historically, the majority of meningococcal disease globally has occurred in an area of Africa known as the Meningitis Belt. The Meningitis Belt is characterised by high endemic levels of meningococcal meningitis with large epidemics during the dry season every 2-10 years [21]. During epidemics, disease incidence can be as high as 1,000 per 100,000 population. The Meningitis Belt extends from Senegal in western Africa to Ethiopia in the east, although meningococcal epidemics have also been reported in Africa outside of this region [22]. Previously, meningococcal disease in this area has largely been caused by serogroup A strains belonging to the ST-1, ST-4 and ST-5 ccs [23]; however, more recently serogroups C, W135 and X have also been involved in the epidemics [24].

Meningococcal disease is generally rare in industrialised countries; the incidence in the European Union is around 1.13 cases per 100,000 population per year [25]. However, periods of high disease incidence have occurred in several developed countries over the past fifty years. For example, high rates of meningococcal disease in England and Wales were associated with a period of hyperendemic serogroup C disease, largely due to ST-11, that began in 1985 and continued through the 1990s [26]. There were also large outbreaks of serogroup C disease in USA army recruits in the 1960s [27], in Brazil in the early 1970s [28], and in Spain in the 1990s [29]. Serogroup B meningococci have caused epidemics in Brazil in the late 1980s [30], and, more recently, epidemics in New Zealand and Norway. These outbreaks are typically due to the spread of a single clonal complex; the New Zealand epidemic was predominantly due to strains from the ST-41/44 cc [31], while the Norwegian epidemic was largely due to strains from the ST-32 complex [32].

The highest rates of meningococcal disease in Europe and the Americas are associated with serogroups B and C, although serogroup Y disease has been increasing in prevalence in North America and Europe [12,33,34]. Meningococci expressing the serogroup B capsule (MenB) cause more than 3000 cases of invasive disease in Europe each year, with more than 50% of cases in children under 5 years of age (2006 data) [25]. In the USA, a third of cases of meningococcal disease are now attributed to each of the three serogroups B, C, and Y [35]. In the last ten years worldwide, there has also been a significant increase in the number of serogroup W135 isolates, particularly from the ST-11 complex [36,37]. Serogroup X meningococci have been recorded at low levels since the 1960s [38], although cases of disease caused by this serogroup are being recorded with increasing frequency in several countries in Africa and Asia [39,40,41,42].

1.1.3: Meningococcal surrogates of protection

Determining whether individuals are likely to be protected against invasive meningococcal disease is important for understanding the incidence of disease within populations, and for predicting the efficacy of meningococcal vaccines. Due to the low incidence of meningococcal disease and the unethicality of human challenge studies, true measures of protection are difficult to obtain; therefore, a surrogate of protection must be used. During an outbreak of meningococcal serogroup C (MenC) disease in USA army recruits in the 1960s, protection from disease was correlated to reactivity of sera in a serum bactericidal assay (SBA) [18]. The SBA measures the activity of complement-binding antibodies in serum. The correlation is apparent not only with respect to natural immunity, as there is a negative association between SBA titres and age-dependent susceptibility to meningococcal disease [18], but also in terms of protection induced by available vaccines, in particular against MenC [43]. Individuals with deficiencies in late-stage complement components are also highly susceptible to meningococcal disease [44], indicating that the complement cascade is a critical mechanism of bacterial clearance.

SBA is now considered the "gold standard" meningococcal surrogate of protection [45], although the results obtained vary according to the assay conditions and strains used [46,47]. The putative titre, as determined by SBA using baby rabbit complement (rSBA), indicative of short term protection against disease is generally considered to be ≥8 [48]. However, meningococci are more susceptible to lysis using rabbit complement than using human complement, and so rSBA titres may underestimate protection [49]. This is thought to be due to the absence of human factor H (fH), a negative regulator of the alternative complement pathway, which the meningococcus can bind to itself via factor H binding protein (fHbp) [50]. Therefore, the SBA titre thought to be protective in an assay with human complement (hSBA) is often listed as ≥4 [51]. Alternatively, when analysing the efficacy of a meningococcal vaccine,

a rise in SBA titres from pre- to post-vaccination sera of greater than 4-fold is considered protective [51], and can give more consistent results between laboratories [52].

Although rSBA and hSBA results generally correlate well with protection against MenC disease, there has been more difficulty correlating SBA results to protection against disease caused by other serogroups [53]. In particular, it is often difficult to correlate SBA titres with protection against MenB disease [48]. This may be in part because the sialic acid present in the MenB capsule downregulates activation of the alternative complement pathway and minimizes terminal complement activity by promoting the conversion of C3b to iC3b [54]. This would enhance the effect of fH (Figure 1.1), and therefore could result in larger changes in SBA titres due to different complement sources. Often, Immunoglobulin G (IgG) titres, as measured by Enzyme-Linked Immunosorbent Assay (ELISA), are reported alongside SBA results as a further measure of serogroup B vaccine efficacy [55]; however, as ELISAs only measure antibody binding and not functional killing, serum antibodies may not be effective at killing meningococci *in vivo*. This could be due to several factors, including low avidity, poor complement-binding, or specificity for poorly-exposed epitopes.

There is some question as to whether an SBA titre can underestimate the protective efficacy of a vaccine, as an hSBA titre of <4 does not necessarily indicate susceptibility [51]. Other assays that may indicate protection have also been investigated; for example, Welsch and Granoff (2007) found that blood from several human subjects with no detectable SBA titres were able to kill >99% of meningococci within 1 hour of incubation in a whole-blood assay [56]. This assay may be more sensitive than SBA, but also requires the incubation to be commenced within two hours of blood collection, and so is unlikely to be useful for evaluating sera from large clinical trials [55]. An opsonophagocytic assay has been found to correlate with SBA titres, but, as with SBAs, results vary according to the source of cells and complement used [55].

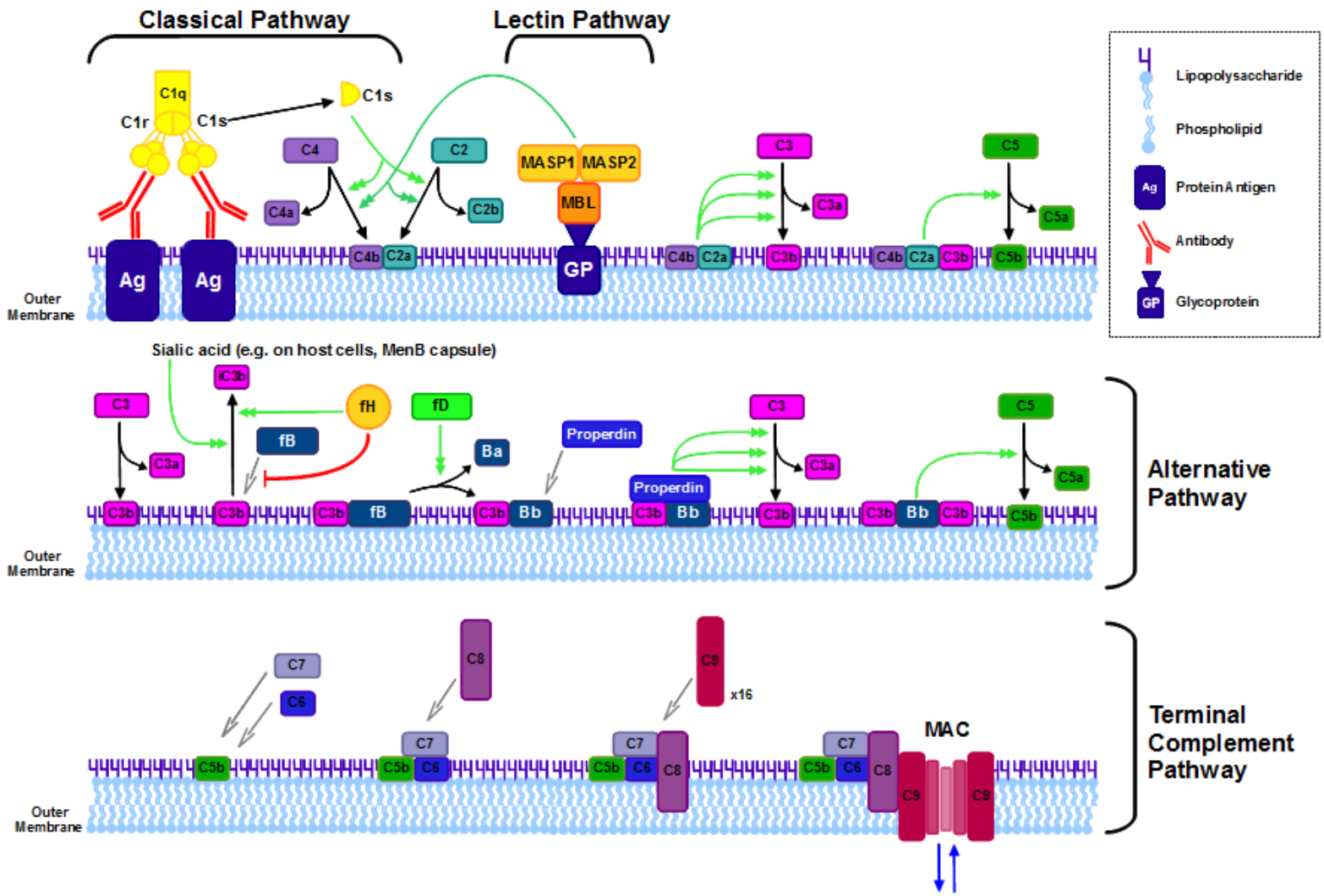


Figure 1.1: Overview of the complement pathways leading to the formation of the membrane attack complex, thought to be important in the killing of meningococci *in vivo*. The ability of antibodies to activate complement is measured by serum bactericidal assay (SBA). Factor H (fH) acts by binding to C3b and preventing the binding of Factor B (fB), by promoting the cleavage of C3b (to iC3b) by Factor I, and by promoting the dissociation of C3bBb. This promotes the inactivation of C3b to iC3b. The inactivation of C3b is also enhanced by sialic acid such as that present on host cells or in the MenB capsule [54].

There is also a lack of reliable animal models of infection, as *N. meningitidis* does not naturally infect any species other than humans. An infant rat infection model has been used widely, although using this model results in the same susceptibility issues as the use of baby rabbit complement. Rats engineered to express human fH hold the promise of a more suitable infection model [57]. Currently, for pre-clinical testing of developmental meningococcal vaccines, mice are often used for immunisations, with sera tested by SBA and ELISA. This method allows for comparisons among groups and with control groups, although does not provide an accurate prediction of whether a vaccine will be protective in humans.

In the absence of protection studies, currently-used vaccines have been licensed based on SBA results obtained during clinical development. The widespread use of these vaccines has provided further support for the correlation between SBAs and vaccine-induced protection against meningococcal disease.

1.2: Capsular polysaccharide vaccines against meningococcal disease

Vaccines based on the polysaccharide capsules of serogroups A and C meningococci have been used since the 1960s [58,59]. These plain polysaccharide (PPS) vaccines consist of purified capsular polysaccharide, and are sufficient to induce protection in adults [60]. However, as they are not peptides, polysaccharides are not presented by MHCII (Major Histocompatibility Complex 2) on antigen presenting cells (APCs), and therefore do not recruit T-cell help or result in the formation of memory B-cells [61]. The efficiency of the T-cell-independent immune response varies with age and previous exposure, and, consequently, PPS vaccines are not effective in infants [62,63].

Conjugate-polysaccharide (CPS) vaccines consist of capsular polysaccharide conjugated to a carrier protein that elicits T-cell help [64]. T-helper cells induce differentiation of naive B cells

into plasma cells and memory cells; therefore, multiple doses of the conjugate vaccine lead to immunological memory. This type of vaccine has been used effectively against disease caused by *Haemophilus influenzae* type B since 1988 [65], and is now used in several forms with several bacterial species [64,66,67], particularly *Streptococcus pneumoniae* [68].

CPS vaccines have been shown to be immunogenic in infants, children and adults [61]. Immunisation with CPS vaccines also leads to protection against carriage [69]. From 1999, two CPS vaccines against MenC disease (Meningitec®, Pfizer vaccines, Tadworth, UK; and Menjugate®, Novartis Vaccines, Sienna, Italy) were licensed in the UK, both conjugated to CRM_{197} (a non-toxic mutant of a toxin from *Corynebacterium diphtheriae*). There was a stepwise introduction of this vaccine, beginning with 15-17 year-olds, as this age group had the highest incidence and mortality due to MenC disease. The vaccine was also given at 2-4 months and 13-15 months. Other age groups were subsequently included. Coverage rates for most groups then exceeded 85% of the population [26]. Other MenC conjugate vaccines have since been licensed [70,71].

Introduction of MenC CPS vaccines in several countries has resulted in a significant drop in serogroup C cases in both vaccinated and unvaccinated individuals [69,72]. There has also been a reduction in disease in unvaccinated individuals, demonstrating that the vaccine promotes herd immunity [69,72]. Data from infant immunisations suggest that the antibody response declines significantly one year after vaccination, and therefore herd immunity is more important for community protection [71]. However, so far there are no data on how immunity and herd immunity will persist in the long term.

As well as CPS vaccines targeting serogroup C disease, a vaccine against serogroup A (MenA) disease has been rolled out in several countries in the African Meningitis Belt. This vaccine was developed as part of the Meningitis Vaccine Project, with the specific aim of providing the vaccine at an affordable price for the developing world. MenAfriVac™, manufactured by

Serum Institute of India Ltd (Pune, India), is a conjugate vaccine composed of serogroup A capsular polysaccharide conjugated to a tetanus toxoid carrier protein. The vaccine, which has been shown to induce functional antibodies [73], has now been introduced over several phases in Burkina Faso, Mali and Niger. It is currently too early to determine the impact on meningococcal epidemics, but incidence of MenA disease has dropped in these countries since widespread vaccination began [74].

There are also three tetravalent CPS vaccines available. Menactra® (Sanofi Pasteur, Lyon, France), Menveo® (Novartis Vaccines), and Nimenrix® (GlaxoSmithKline, London, UK) contain the A, C, W135 and Y polysaccharides conjugated to either a diphtheria toxoid carrier protein (Menactra®) or to CRM_{197} (Menveo® and Nimenrix®). Tetravalent vaccines are used widely in the USA; however, in most other countries cost is a significant issue surrounding whether to enter tetravalent vaccination into routine immunisation, and so such vaccines are only used for travellers or other high risk groups [75,76].

1.3: Vaccines against serogroup B meningococci

1.3.1: The serogroup B capsule

Although polysaccharide vaccines have been shown to be useful for protection against other meningococcal serogroups, no such vaccines currently exist against MenB. The MenB capsule is a linear homopolymer of α-*N*-acetylneuraminic acid, containing α-(2,8) glycosidic linkages [77]. Polysialosyl glycopeptides containing *N*-acetylneuraminic acid residues bound by α-(2,8) linkages are also found in the human foetal brain, and so there is the potential for immune cross-reactivity between MenB polysaccharide and human neural tissue, leading to a risk of autoimmune disease. This potential for cross-reactivity has been shown by inhibition studies, although it was also shown that a fairly long stretch of α-(2,8)-linked sialosyl units is required

for antibody binding, while brain gangliosides only contain α-(2,8)-linked disialosyl and trisialosyl units [78]. Furthermore, bacteria possessing polysialic acid capsules are common in the upper respiratory tracts and intestinal tracts of humans of all ages; for example, the capsule of *Escherichia coli* K1 is also a homopolymer of α-(2,8)-*N*-acetylneuraminic acid. It has been argued that, although antibodies targeting these molecules have been found to bind to foetal and adult tissues *in vitro*, there is no evidence that these antibodies would bind human cells *in vivo*, or cause autoimmune disease. There is also no significant increase in mortality or sequelae associated with autoimmune disease following MenB infection. [79]

Furthermore, the MenB polysaccharide is weakly immunogenic in humans. *E. coli* with capsular type K1 have an immunochemically identical capsule to MenB, and, similarly, does not induce a strong antibody response [80]. MenB strains resist the bactericidal activity of up to 50% normal human sera, possibly due to tolerance of the polysialic acid molecules that the capsule is composed of. There may also be decreased binding of antibodies to other surface structures as well through stearic or electrostatic hindrance [80]. The MenB capsule has also been shown to inhibit the alternative complement pathway [54]. With the limited immunogenicity of the serogroup B capsule, and potential for causing autoimmune disease, research into developing a vaccine that will induce protection against MenB disease is now predominantly focussed on subcapsular and outer membrane protein (OMP) antigens.

1.3.2: Vaccines based on sub-capsular antigens

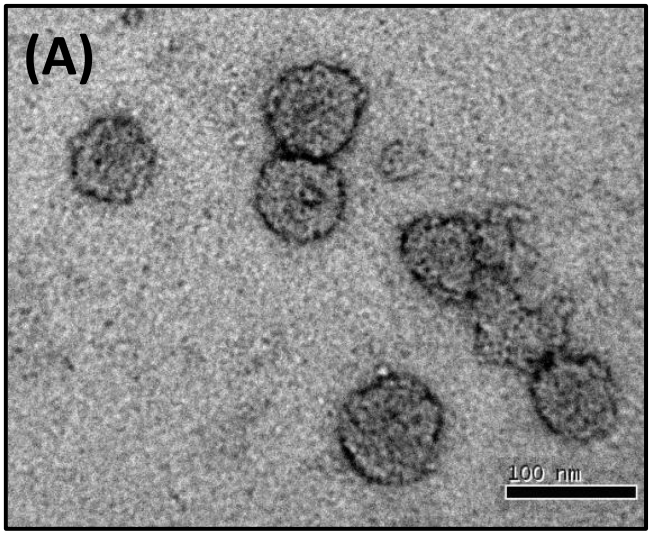
Due to immune selection, immunogenic epitopes on meningococcal sub-capsular antigens vary among isolates. Antigens with low levels of variability are often poorly immunogenic, or induce antibodies that are not functional in an SBA. In addition to epitope variability, antigens are often variably expressed, and so it is difficult to predict whether expression *in vivo* will be sufficient for specific antibodies to lead to bactericidal killing. Consequently, development of

vaccines that will provide wide coverage against meningococcal disease, including coverage against serogroup B, predominantly involves using a combination of different antigens and/or antigenic variants. These antigens are often presented in outer membrane vesicles (OMVs).

OMVs are composed of, amongst other things, lipopolysaccharide (LPS), outer membrane lipids, and OMPs (Figure 1.2). Periplasmic proteins, cytoplasmic proteins, deoxyribonucleic acid (DNA), and ribonucleic acid (RNA) can also be present [81,82]. OMVs are released constantly from the surface of live Gram-negative bacteria, both during carriage and infection [83]. As OMVs enable both pathogenic and non-pathogenic bacterial species to secrete large amounts of complex proteins and lipids into the surrounding environment, they can be potent virulence factors [84]. Pathogenic bacteria generally produce more vesicles than non-pathogenic bacteria [84], and the high levels of endotoxin released into plasma in OMVs during meningococcal infection can lead to septic shock [85]. OMVs secreted by Gram-negative bacteria may also have roles in competitive interactions with other bacteria, aggregation and biofilm formation, and transfer of material between bacteria [84]. For example, penicillin-resistance genes can be transferred between gonococcal cells in this manner [86]. OMVs can also act as immune decoys by adsorbing immune components or antibiotics. A β-lactamase enzyme in *Pseudomonas aeruginosa*, for example, can be packaged into OMVs and hydrolyse β-lactam antibiotics away from the cell, reducing the risk of damage to the bacteria [87].

Some membrane proteins are found in OMVs at higher concentrations than on the surface of the bacteria, while other proteins are less concentrated in OMVs. Deatherage *et al.,* [88] present evidence that the release of OMVs from the surface of dividing bacteria occurs at regions of the membrane where the density of specific, conserved protein associations has decreased; therefore, the protein content of OMVs is not entirely representative of the protein content of the membrane of the live organism [88]. OMVs may be an important stress-response to remove unwanted membrane proteins, for example, misfolded proteins [89], although generally very little is known about the regulation of OMV release. However, all of

the major outer membrane proteins in meningococci are represented in native OMVs, as well as several proteins associated with the periplasm or peptidoglycan [82]. Therefore, OMVs contain high concentrations of important surface antigens for immunisation.



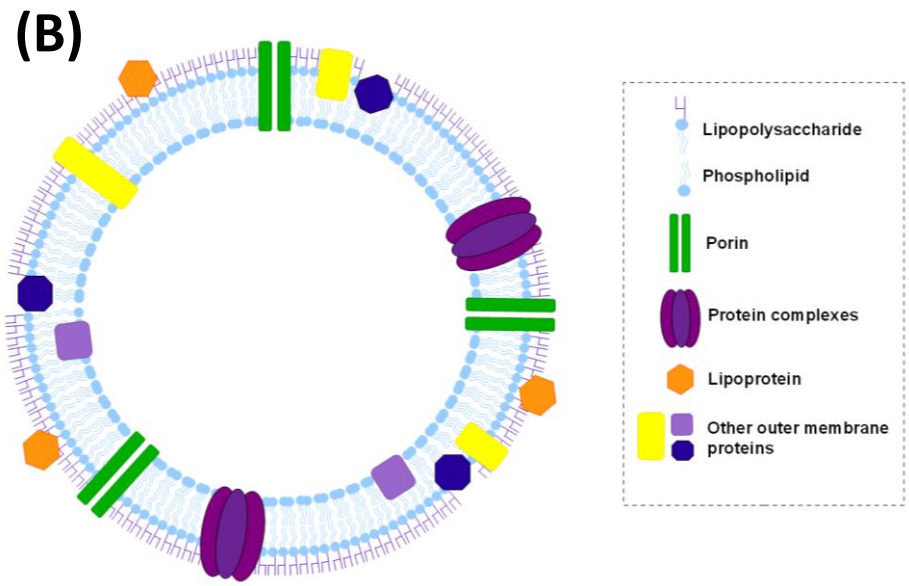


Figure 1.2: Outer membrane vesicles. **(A)** Cryo-electron microscopy image of outer membrane vesicles from *Neisseria meningitidis* H44/76 (image courtesy of Dr Anwen Bullen, University College London, London, UK). The scale bar shows 100nm. **(B)** Representation of the structure of an outer membrane vesicle.

OMV vaccines have been used for widespread immunisation in several countries to control epidemics of meningococcal disease. An OMV vaccine (MenBvac®, Norwegian Institute of Public Health, Oslo, Norway) was developed in Norway to combat an epidemic of a ST-32-related MenB disease, and contains OMVs extracted from the epidemic strain [32]. More recently, MenBvac® has been used to deal with an outbreak of meningococcal disease in Normandy [90]. MeNZB® (Norwegian Institute of Public Health) has been used in a large

immunisation campaign in New Zealand, in response to an epidemic caused by meningococci belonging to the ST-41/44 cc, and was introduced without an efficacy trial after the success of MenBvac® [91]. A vaccine that combines MenB OMVs with MenC polysaccharide has also been used in Cuba [92].

These vaccines have been found to be safe and immunogenic, although commonly associated with pain at the injection site [93]. Often, at least three doses are needed to induce long-lasting, high avidity antibodies, particularly in infants [94]. This can be a significant problem as, by the time the third dose has been administered, the child has passed the age at which MenB disease is most prevalent. Furthermore, increased doses of these OMV vaccines are associated with a higher proportion of antibodies formed against antigens that do not lead to bactericidal killing [95,96]. Consistent production of OMVs is an additional problem due to the large number of proteins and associated molecules involved [97]. The composition of OMVs may also vary significantly with changes in growth conditions [98].

Despite these issues, the OMVs listed above have been shown to be effective. However, coverage is limited due to the immunodominance of the Porin A (PorA) antigen. Most of the bactericidal antibodies induced by OMVs are only effective against strains with a homologous PorA variant; therefore, using OMVs to cover wide geographic areas where there is no single dominant meningococcal lineage (and consequently many PorA types to target) is difficult.

In order to provide high levels of coverage against meningococcal disease, including coverage against MenB isolates, multiple antigens or antigenic variants must be used. These could simply be combined as a cocktail of recombinant proteins; for example, rLP2086 (Pfizer) contains two recombinant variants of fHbp [99]. This vaccine has been shown to induce bactericidal antibodies in adults [100]. However, immune responses to proteins presented in OMVs are generally more protective than immune responses to the same antigens presented

as purified proteins [101]. Therefore, due to their inherent adjuvant properties, the majority of meningococcal protein vaccines currently in development contain OMVs.

1.3.3: Outer membrane vesicles as adjuvants

An adjuvant can be described as any compound used to induce, increase or modify the immune response to an antigen in a vaccine. These can function in a variety of ways. One particular mechanism, the recognition of pathogen-associated molecular patterns (PAMPs), is considered particularly important for the immunogenicity of OMVs. PAMPs are bound by particular receptor molecules, for example Toll-like receptors (TLRs). This recognition induces expression of co-stimulatory molecules by APCs [102].

The Lipid-A fraction of meningococcal LPS, present in OMVs, is a potent TLR4-ligand. However, the high concentration of LPS in native OMVs (25-50%) means that these OMVs are too toxic for use in immunisation. Therefore, detergent extraction of the OMVs is often used to reduce the LPS levels (to 5-8%), resulting in more acceptable toxicity levels [103]. Detergent-extracted OMVs (dOMVs) are also easier to manufacture in large quantities than native OMVs (nOMVs), and so detergent extraction is currently the method used for commercial manufacture of OMV-based vaccines. Genetic modification of the LPS to reduce the toxicity has been tested for use in nOMV vaccines [104], and although these modifications significantly reduce the toxicity, the adjuvant effect of the OMVs is also reduced [105].

Meningococcal PorA and Porin B (PorB) have also been found to act as adjuvants, binding TLR2 and activating dendritic cells [106,107], although the adjuvant effect of these proteins is likely to be minimal compared to the adjuvant effect of Lipid-A [105]. However, the presence of ligands for multiple TLRs within a vaccine may have a synergistic effect on the immune response induced [108].

The nature of OMVs to act as carrier molecules further adds to their intrinsic adjuvant properties. It has been proposed that, as carrier molecules such as liposomes or OMVs have similar dimensions to the pathogens that the immune system has evolved to target, these molecules are more likely to be internalised by APCs and translocated to the local draining lymph nodes [109]. Furthermore, it is important for antigens to be presented in a correctly folded state, as found in OMVs, to induce protective antibodies [110].

The meningococcal vaccine furthest through clinical development (Bexsero®, Novartis) utilises the adjuvant properties of OMVs by combining recombinant protein antigens with an OMV component. The vaccine includes two chimaeric proteins, containing protective epitopes for the antigens fHbp and Neisserial Heparin Binding Antigen (NHBA), alongside a third recombinant protein, Neisserial Adhesin A (NadA), and a single PorA variant contained within an OMV. The combination of the three recombinant protein antigens is predicted to give a high level of coverage against meningococcal disease [111], while the addition of the OMV contributes additional antigens, particularly PorA, to further increase coverage. The adjuvant properties of the OMV also improves efficacy of the vaccine, as bactericidal activity induced against the recombinant protein antigens is higher using formulations with the OMV than formulations without [112].

1.3.4: Genetically modified outer membrane vesicle vaccines

Other developmental meningococcal vaccines use genetic modification of strains to alter OMV properties and antigen expression, in order that a combination of OMVs can be used to provide broad coverage. Meningococci are naturally competent throughout their life-cycle and readily incorporate DNA from the environment [113]; therefore, genetic modification of meningococci is a useful tool for the development of OMV-based vaccines. Genetic alterations can be used to increase OMV production, for example using gene deletions that increase the frequency of outer-membrane blebbing [114], or to reduce OMV toxicity by modifying the LPS

structure [115,116]. Strains expressing detoxified LPS can be used to produce nOMV vaccines with reduced reactogenicity. These nOMVs also contain antigenic lipoproteins, such as fHbp, that would otherwise be removed during detergent extraction of vesicles [114]. Detoxification of LPS also allows its use as a vaccine antigen. Although LPS is antigenically variable, the number of variants is limited. Antibodies specific for LPS can be induced following immunisation or meningococcal disease, and can lead to bactericidal killing in an SBA [117]. Therefore, nOMVs including detoxified LPS, as both an adjuvant and antigenic component, are becoming more popular in meningococcal vaccine development.

A further issue with wildtype OMVs is that many meningococcal antigens have variable expression levels within and among strains [118]. As the concentration of antigen on the surface of OMVs can be important for determining the size and cross-reactivity of the antibody response [119], it is important that expression of the key antigens is consistent and at high levels.

Genetic modification can be used to increase and stabilise the expression of important vaccine antigens in OMVs while decreasing expression of unwanted vaccine components. For example, a method that has been used to improve cross-reactivity during vaccine development is the addition of multiple genes of a variable antigen, enabling a single mutant strain to express several key variants of that antigen. The strains used by the Netherland Vaccine Institute (Bilthoven, The Netherlands) to make HexaMen (two OMVs) and NonaMen (three OMVs) each contain three different PorA serosubtypes in an isogenic meningococcal background [120,121]. The PorA types included have been chosen to cover the most hyperinvasive lineages circulating in Europe [122]. As well as replacing the native PorA, additional PorA types were used to replace the genes encoding PorB and RmpM (Reduction-modifiable protein M), on the grounds that the gene products induce antibodies that do not contribute to bactericidal titres [121]. However, mutations in such major outer membrane proteins are likely to have significant effects on the meningococcus; for example, it has been shown that, following

deletion of PorB, many complexes seen in the wildtype strain are no longer present, and have been replaced by a single high-molecular weight complex consisting entirely of PorA [123]. Although the immune response to the individual PorAs were found to be unaffected [121,124], alteration of OMP complexes in this way may affect immune responses to other surface antigens.

Another OMV vaccine targets multiple antigens in order to improve coverage. This vaccine, under development by the Walter Reed Army Institute of Research (Silver Spring, Maryland, USA), contains native outer membrane vesicles produced from three genetically modified meningococcal strains. Each strain contains a different antigenic variant of LPS, detoxified by several gene deletions that also remove capsule expression. All three strains contain a different PorA variant as well as an additional, introduced variant, resulting in six variants in total. Two strains each express a different variant of fHbp, expression of which have been genetically increased, while the third strain has been modified to express NadA at increased concentrations. Furthermore, in two of the strains, expression of the antigen OpcA (Opacity protein A) has been stabilised by replacement of a poly-cytosine repeat sequence, responsible for phase variation due to slipped-strand mispairing during replication, with a sequence composed of both cytosine and guanidine bases. All OMVs were extracted following growth in media with decreased iron concentrations to unselectively de-repress iron-regulated proteins that may contribute to protection [125]. However, expression of these antigens is likely to be inconsistent among batches and so cannot be relied upon for coverage estimates.

The combination of three nOMVs has been shown to result in bactericidal antibodies, in mice, to PorA, LPS and NadA [125], and in humans against LPS, OpcA and fHbp [126]. However, as the vaccine targets a large number of diverse antigens and antigenic variants, it is difficult to separate the protective responses against individual antigens. This all-inclusive approach may also make predicting coverage difficult, as there is limited epidemiological data for several of the antigens targeted.

As with all protein-based meningococcal vaccines, variable expression of antigens is an issue for potential coverage. Sufficient concentrations of antigens on the surface of the meningococcus are critical for bactericidal killing by specific antibodies [99], as low levels of antibody-antigen complexes do not effectively activate the complement cascade [127]. While expression levels of key antigens can be stabilised during production of the vaccines, accurately predicting the levels of expression *in vivo* is impossible. With many protein antigens it is not known whether expression levels *in vivo* are sufficient, or how variation in expression is distributed at a population level. For example, although fHbp is important for serum resistance in some strains, and therefore likely to be expressed during invasive disease, expression of the *fHbp* gene is also regulated by both oxygen and iron [128,129]. Expression of OpcA is also variable among isolates and over time due to changes in a repeat sequence within the promoter region [130].

If levels of antigen are low, antibodies to multiple epitopes can act synergistically to activate complement [131,132]. This is why targeting several different proteins is more likely to be an effective vaccination strategy than targeting only a single antigen, as antibodies are more likely to result in protection regardless of expression levels *in vivo*. However, due to the association between antigen expression and complement-mediated killing, epidemiological data on antigen variation alone cannot guarantee high vaccine coverage. Additional data is also needed on the potential variation in expression to determine whether the bactericidal killing observed in an SBA is likely to extend to protection against invasive disease.

1.4: Developing an alternative vaccine candidate

This study is part of a project that aims to develop an alternative meningococcal vaccine candidate that will cover not only MenB isolates, but the majority of disease-causing isolates in Europe, regardless of serogroup. The vaccine design is based on knowledge of the biology and

detailed epidemiology of meningococcal disease. It is known that PorA, particularly when presented in OMVs, is an immunodominant protein that induces bactericidal antibodies both after vaccination and carriage, and therefore PorA is an effective vaccine antigen. This project aims to develop a vaccine (referred to here as MenPF) based on the PorA antigen, alongside another immunodominant OMP, the FetA (Ferric enterobactin transporter A) protein.

1.4.1: PorA

PorA is a porin protein with slight cationic selectivity [133], that is also known as the meningococcal Class 1 OMP. Although expression is variable due to changes in the promoter sequence of the *porA* gene, PorA can account for more than 30% of the protein in the outer membrane [134]. Changes in the length of a poly-guanine repeat between the -35 and -10 binding sites affect the strength of binding of the RNA polymerase, and consequently the rate of transcription [135]. This can lead to variable expression among strains and within strains over time.

PorA has a beta-barrel topology with eight surface-exposed loops, and is often found in trimeric complexes with the PorB protein [136]. Immunodominant variable regions (VRs), are found on the apices of loops 1 and 4 (Figure 1.3) [137]. The amino acid (aa) sequence at each VR determines the VR type, and aa sequences containing more than 80% identity are grouped into VR families. The prototype VR for each family is arbitrarily assigned based on which epitope is recognised by an existing mAb, or the first aa sequence of that family described. Successive minor variants of a family are numbered according to order of discovery [138].The two VRs collectively determine the serosubtype of the strain (documented as P1.VR1,VR2, for example P1.7,16) [139]. It is common practise to document the serosubtype of isolates alongside the sequence type, and therefore there is extensive data on the distribution of variation in this antigen. There is often more variability in VR2 (loop 4) than in VR1 (loop 1)

[138], with 10 major VR1 families and 21 major VR2 families recorded to date [38]. However, there is often an association between VR1 type and VR2 type, and between serosubtype and clonal complex [17].

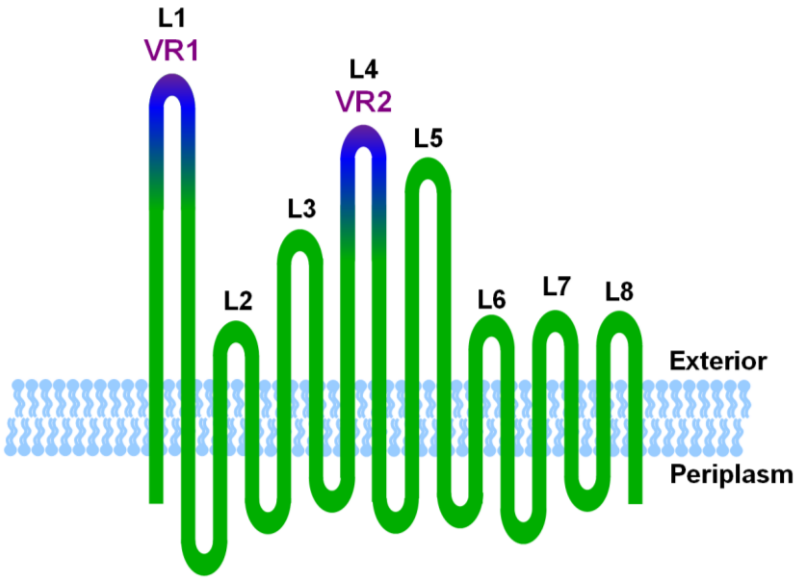


Figure 1.3: Topology of the porin protein, PorA, in *N. meningitidis*, according to van der Ley *et al*., [137]. There are 16-transmembrane regions forming a beta-barrel structure with eight surface-exposed loops (L1 – L8) [136]. Loops 1 and 4 are immunodominant and contain the variable regions VR1 and VR2, respectively [137].

Other than the VRs, most of the PorA aa sequence is conserved [137]. Loops 2 and 3 have been predicted to have a structural role, with loop 2 involved in the formation of the PorA trimers. Loop 3 lies inside the pore, possibly controlling pore function [136]. There is also some sequence variation in loops 5 and 6, suggesting that these regions may also be targets for bactericidal antibodies [140], although the majority of PorA-specific antibodies target the VRs on loops 1 and 4 [137].

PorA induces bactericidal antibodies both in OMVs and in whole cells, and is the immunodominant protein after OMV immunisation in both infants and adults [95,141]. However, these antibodies are largely serosubtype-specific, and even a single point mutation in a VR can be enough to remove the binding capacity of specific antibodies [142].

Furthermore, some serosubtypes are more immunogenic than others. For example, P1.5,2 is generally highly immunogenic, while P1.7-2,4 is often only weakly immunogenic [143,144,145].

Although bactericidal antibodies against PorA are induced against both VRs, often one VR predominates in the immune response [146]. Which VR this is seems to depend on the exact PorA serosubtype studied, as the accessibility of the VR epitopes to antibodies depends on the specific VR1-VR2 combination. For example, bactericidal antibody responses are directed predominantly against loop 1 in P1.7,16 and against loop 4 in P1.5,10 [147]. As the two VRs are in close proximity in native complexes [136], this may be because the epitopes interact in some way. Vermont *et al.,* [148] found that antibodies against P1.7-2,4 could be bactericidal against minor variants of P1.4, but not against a strain with the same VR1 (P1.7-2) and a different VR2 (other than P1.4), suggesting that only antibodies against VR2 are effective against this serosubtype [148]. Despite this, use of OMV vaccines targeting the P1.7-2,4 variant in epidemic strains have shown that this antigen is effective for inducing protection against meningococcal disease.

1.4.2: FetA

FetA, formerly designated FrpB (Ferric repressed protein B), is a 70kDa surface expressed protein that is upregulated by iron-limitation [149]. As such, FetA is predicted to have a role in iron acquisition, although the exact function is still unknown.

1.4.2.1: Predicted function of FetA

As most of the functional iron in the human body is bound to carrier molecules, *Neisseria meningitidis* has several systems that enable it to acquire iron from haemoglobin, lactoferrin, transferrin, heme and haptoglobin-hemoglobin [150]. Some of these involve direct binding of the carrier molecule to the surface of the bacteria, for example, the uptake of iron from transferrin [151]. However, many iron-uptake mechanisms in bacteria require intermediate

molecules. Siderophores are relatively low-molecular weight iron-chelating compounds secreted by bacteria to collect free or bound iron in the external environment [152]. Siderophores are too large to diffuse across the cytoplasmic membrane, so once a siderophore-iron chelate is formed it is actively taken up into the cell by specific receptors on the outer membrane. These receptors are often linked to complexes such as TonB on the inner membrane [152]. TonB functions to transduce the energy from electron transport across the membrane into conformational changes in TonB-dependent transporters located on the outer membrane [153]. These conformational changes drive transport of molecules such as iron. For example, the *E. coli* siderophore ferric enterobactin is bound by FepA (Ferric enterobactin protein A) and transported across the membrane by the FepBCDG system and TonB complex (Figure 1.4) [154].

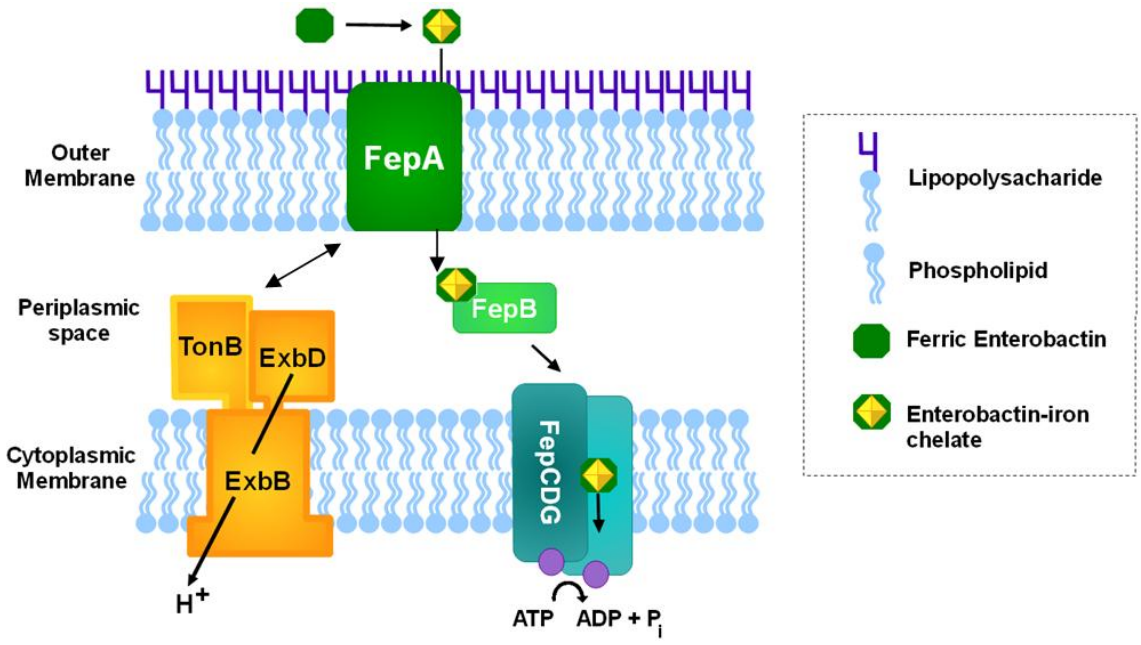


Figure 1.4: Enterobactin uptake in *E.coli* using FepA and TonB, adapted from Faraldo-Gómez and Sansom (2003). The TonB protein is charged as a result of the passage of an electron through the ExbBD complex. Charged TonB then shuttles to the outer membrane. When an iron-bound siderophore, ferric enterobactin, attaches to FepA, this induces a conformational change which, in turn, results in interaction of FepA with a charged TonB. The conformationally-stored potential energy from TonB is then transferred to FepA, enabling active transport of the enterobactin-iron chelate across the outer membrane. Transport across the inner membrane is facilitated by FepBCDG. [153,154]

FetA (which shows 91% sequence identity to meningococcal FetA [149]), is able to bind *E. coli* enterobactin and use it as an iron source for growth *in vitro* [155]. Therefore the protein, previously called FrpB (Ferric repressed protein B), was renamed FetA (Ferric enterobactin transporter A) in both gonococci and meningococci. However, the binding of enterobactin to FetA was low-affinity, and such binding has never been shown *in vivo*. Gonococci have been shown to utilise other siderophores in a FetA-dependent manner [156]. Despite this evidence, no meningococcal or gonococcal siderophores have been discovered. Gonococcal mutants that do not express FetA have also been found to have a reduced capacity to utilise iron from hemin [157], transferrin and lactoferrin [158] compared to wildtype strains. Therefore FetA may play some role in the uptake of iron from these molecules.

Genetic studies also support the role of Neisserial FetA in iron uptake. Sequence comparisons support its association with TonB [157], and the open reading frames downstream of *fetA* (shown in Figure 1.5) seem to be homologous to other siderophore transport proteins, similar to FepBCDG. It was found that a polar insertion into the downstream open reading frame removed the ability of gonococci to use ferric enterobactin as an iron source for growth; however, binding of ferric enterobactin to FetA was unaffected by this insertion [155]. This would support FetA acting as a siderophore receptor, with the downstream open reading frames comprising the transport machinery for the uptake of the siderophores into the cell. More recent evidence shows that that gonococcal *fetA* is co-transcribed with the downstream genes as an operon, although there is also a second RNA transcript produced, beginning after the 3' end of *fetA*, which codes only for the downstream genes. Furthermore, expression of the second transcript was iron-regulated to a lesser extent than transcription of *fetA* [156]. Therefore it may be that the operon produces complexes for iron-uptake that are not dependent on FetA, but utilise FetA when iron availability is very low.

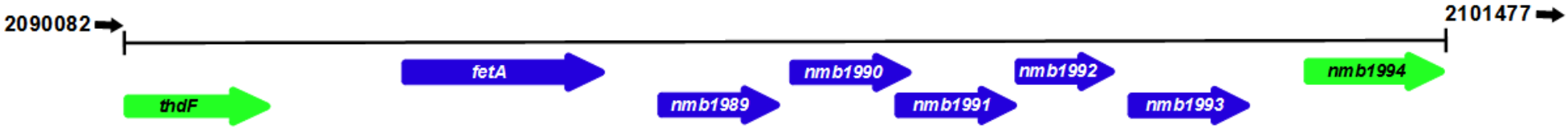


Figure 1.5: Representation of the positions and orientations of the open reading frames surrounding *fetA* in meningococcal strain MC58 [159]. Nucleotide positions in the MC58 whole genome sequence (Accession number: AE002098) are shown at either end. Open reading frames believed to be transcribed as a single operon are shown in blue, with flanking genes shown in green. Illustrated genes have the following proposed functions: *thdF* (also *trmE*) encodes a transfer-RNA modification enzyme; *fetA* encodes an iron-uptake protein; *nmb1989, nmb1990, nmb1991, nmb1992,* and *nmb1993* encode a putative iron(III) ABC transporter complex; *nmb1994* encodes NadA, an adhesin and outer membrane protein vaccine candidate. [159]

Proteins that are predicted to be involved in iron uptake, such as FetA, have been argued to be promising vaccine candidates as surface-exposed expression is likely to be required for growth *in vivo* [160]. In comparison to antigens such as NadA, where the gene is only present in certain clonal complexes [161], the vast majority of strains carry the *fetA* gene and are capable of expressing the protein. Although a small number of isolates have been found naturally with a mutation that has removed *fetA* [162,163], sensitivity of FetA-deficient strains to serum is mildly enhanced compared to wild-type strains [149] and so there may be some selection against strains not expressing the FetA protein *in vivo*.

1.4.2.2: Variable expression of FetA

FetA is known to be immunogenic *in vivo*, with FetA-specific antibodies found in sera during and after meningococcal infection [164]. Black *et al.,* [165] found Immunoglobulin M (IgM) and IgG to a 70kDa iron-regulated protein in convalescent sera. This protein is likely to be FetA, and the data therefore suggests that, as well as being immunogenic, FetA must be expressed *in vivo* [165]. However, predicting the level or consistency of expression is difficult, as several factors are known to be involved in the regulation of *fetA*.

Similar to that described for *porA*, transcription of *fetA* may be phase variable due to changes in the length of sequence between the -35 and -10 sites. In *fetA* this is due to different lengths of a poly-cytosine repeat [166], although the full effects of this and the variation at a population level have not been studied.

Expression of FetA is also known to be negatively regulated by iron-availability through the actions of the Fur (Ferric uptake regulator) protein [166]. In the presence of iron, a Fur-iron complex binds to specific sequences upstream of the *fetA* gene, blocking RNA-polymerase binding and preventing transcription. In the absence of iron, this complex dissociates and separates from the DNA, allowing expression of the gene [167]. Expression of FetA in gonococci is also regulated by an additional protein, MpeR (Mtr protein efflux Regulator),

which has been shown to bind specifically to DNA upstream of *fetA* and increase transcription when iron availability is low [156]. Expression of MpeR is negatively regulated by Fur in both gonococci and meningococci [168]. Meningococcal FetA may, therefore, also be influenced by iron availability in an indirect manner by MpeR.

Transcriptional regulation by iron is a particular problem for the study of FetA as a vaccine candidate as, under the iron replete growth conditions normally used *in vitro*, expression is repressed. This is an issue not only for the study of the activity of FetA-specific antibodies in SBAs, where target strains are grown on iron replete blood agar [46], but also for the production of OMVs containing FetA as a target antigen, where wildtype OMVs contain low and inconsistent concentrations of the protein. Although FetA-specific antibodies have been found following OMV immunisation [169], the variable expression of this antigen in wildtype production strains currently limits the use of FetA as a major antigen. Therefore, in order to produce and evaluate an OMV vaccine specifically targeting FetA, both variation in expression and variation in antigenic epitopes need to be considered.

1.4.2.3: Predicted structure of FetA

FetA had been predicted to have a topology with 11 surface-exposed loops, similar to that of other TonB-dependent siderophore receptors like FepA, with the greatest variability in loop 5. This topology has 22-membrane spanning domains forming a Beta-barrel. The N-terminal domain forms a plug that closes the barrel [170]. Loop 5 is the largest loop, and contains the highly variable immunodominant region (Figure 1.6). It has also been found that, when loop 5 is removed, antibodies are directed primarily against the second-largest loop, loop 3. Antibodies against loop 3 are only bactericidal in the absence of loop 5, suggesting that loop 5 shields other surface-exposed epitopes [170].

The variable region (VR) on loop 5 determines the FetA type that is documented alongside MLST data and PorA variant [139]. Variable regions are classified into families and minor

variants; for example, F3-6 denotes FetA containing a VR within family 3, variant 6. Monoclonal antibodies against this region are bactericidal, but are also strain specific [171]. As there is limited cross-reactivity between different VRs, it has been argued that FetA alone has restricted use as a vaccine candidate [172]. However, the extensive epidemiological data on the distribution of FetA variability, and how that variability is structured within the hyperinvasive lineages, suggest that only a few FetA types in a vaccine would cover the majority of disease-causing strains in Europe (Brehony *et al.,* unpublished).

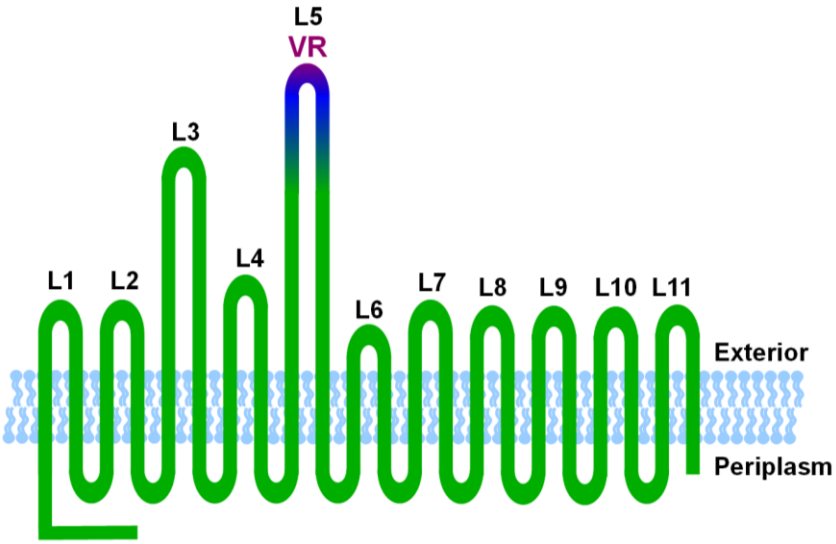


Figure 1.6: Predicted topology of FetA in *N. meningitidis*, according to Kortekaas *et al.,* [170]. The protein forms a 22-stranded Beta-barrel, with 11 surface-exposed loops (L1 – L11) and an N-terminal domain forming a plug that can block the barrel. Loop 5 is the longest of the surface-exposed regions, and contains the immunodominant variable region (VR).

1.4.3: Potential coverage of a vaccine targeting PorA and FetA

Despite their antigenic variability, PorA and FetA VRs are relatively stable, surface exposed peptides that are easily defined. There is extensive epidemiological data on the distribution of PorA and FetA types [38], and how the distribution of the variants is structured among clonal complexes [17]. Certain PorA and FetA types are associated with particular genetic lineages in

non-overlapping antigen combinations (Figure 1.7). These associations are generally stable geographically and temporally, and cannot be explained by simple clonal expansion [173].

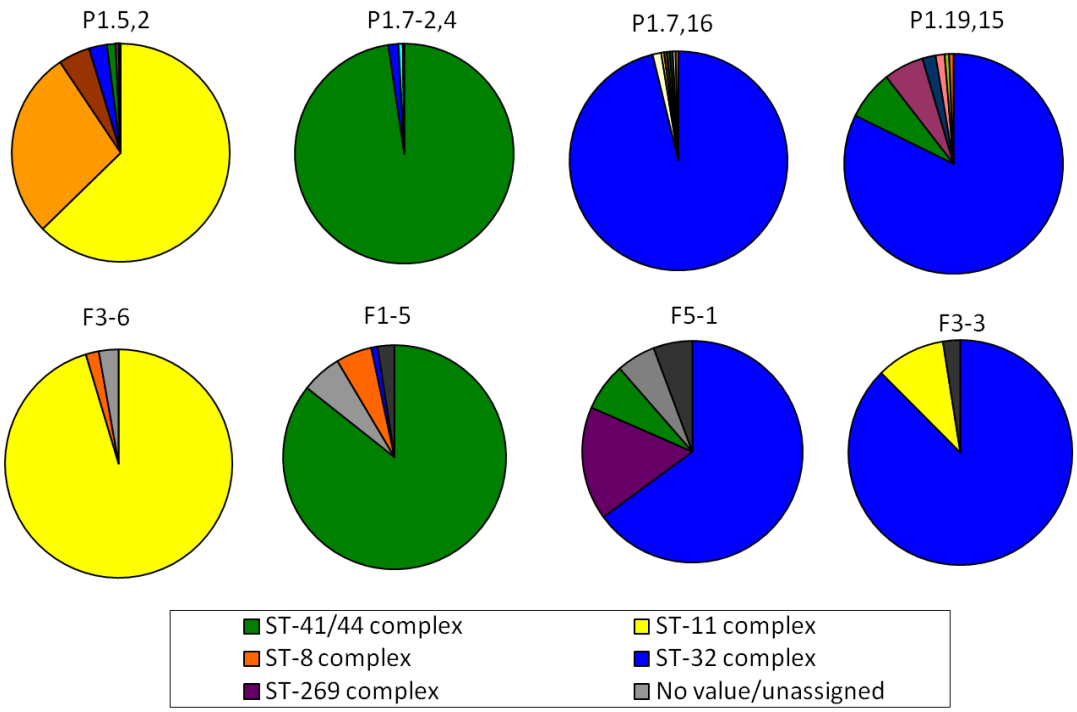


Figure 1.7: Association between PorA and FetA variants and clonal complex. The predominant four variants of PorA and FetA in the EU-MenNet dataset are shown [17,174]. Pie-charts are divided according to the clonal complexes that are present which contain those variants. Image courtesy of Dr Carina Brehony (University of Oxford, Oxford, UK).

This population structuring fits a model driven and maintained by host immunity, and by competition among lineages for those hosts. Lineages with random assortments of variants of immunodominant antigens are more likely to encounter hosts that have previously developed some immunity to meningococci expressing a similar variant of at least one of those antigens. In this way, lineages in which the combination of antigenic variants changes often over time are at a selective disadvantage due to development of host immunity against certain variants. Over time, this selection results in dominant lineages having non-overlapping combinations of antigens, as hosts that have not previously encountered a particular lineage will be completely immunologically naive to that antigen combination [175]. As the distributions of both PorA and FetA are structured in this way, this provides further evidence that both antigens are

immunogenic *in vivo,* and that antibodies to these proteins are likely to be capable of bacterial clearance both during disease and carriage.

The strong structuring of immunogenic variants of the two antigens also implies that a combination of PorA and FetA in a vaccine could be particularly effective. For example, in a survey of 78 isolates belonging to the major disease associated lineages reported over the last 60 years, a combination of 6 PorA types and 5 FetA types would potentially provide homologous protection against all 78 isolates [173].

By using knowledge of the association of different clonal complexes with disease [25], a vaccine combining certain PorA and FetA variants has been designed that should cover most of the disease-causing meningococcal isolates in Europe. By combining PorA and FetA, each circulating strain contains three possible target epitopes (two PorA VRs and one FetA VR). It is predicted that a combination of four OMVs (each expressing a different PorA and FetA variant) should cover 78.8% of disease-causing isolates (as represented by meningococci characterised by the EU-MenNet project [174]) with at least one antigenic epitope (Brehony *et al.,* unpublished, Table 1.1). The four clonal complexes specifically represented in the vaccine recipe will each be targeted by antibodies against all three epitopes, which may result in greater levels of protection against these STs due to synergistic effects between antibodies.

Clonal Complex	PorA VR1	PorA VR2	FetA	Coverage (Total)	Coverage with 1 epitope	Coverage with 2 epitopes	Coverage with 3 epitopes
ST-11	5	2	F3-6				
ST-41/44	7-2	4	F1-5				
ST-32/ST-269	19	15	F5-1				
ST-32	7	16	F3-3				
Number of isolates:				**2216/2813 (78.8%)**	**697**	**664**	**855**

Table 1.1: Vaccine recipe and potential coverage (based on isolates characterised by the EU-MenNet project) (Brehony *et al.,* unpublished). [174]

1.5: Aims of this DPhil

1) The iron-regulated expression of FetA has so far hindered its investigation and potential use as a vaccine antigen. Under most *in vitro* growth conditions FetA expression in many strains is undetectable. This is a problem as, in order to produce OMVs that can induce bactericidal antibodies against FetA or to study the FetA-specific antibodies in *in vitro* assays, FetA must be expressed at sufficient levels. The first aim of this study was to genetically modify and characterise meningococcal strains with which an OMV vaccine targeting both FetA and PorA could be produced and the bactericidal response to both antigens investigated.

2) The second aim of this study was to determine whether the modified strains developed could be used to:

(a) produce OMVs capable of inducing PorA- and FetA-specific bactericidal antibodies in a murine model; and

(b) study the individual and combined effects of antibodies specific for PorA and FetA in a serum bactericidal assay.

3) Presenting antigens in OMVs has several immunological advantages over presentation of the same antigens as recombinant proteins. However, due to the complex nature of OMVs, it is difficult to target the antibody response towards antigens that will lead to protection. The third aim of this study was to determine whether a two-dose heterologous prime boost strategy, involving subsequent doses of OMV and protein, could be used to increase the bactericidal antibody responses specifically against the PorA and FetA antigens compared to the responses induced by OMV vaccination alone.

4) Although PorA is often a highly abundant protein in the outer membrane, and current evidence suggests that FetA is expressed *in vivo*, very little is known about the

variation in expression of these antigens at a population level. The fourth aim of this study was to use sequence data and gene expression assays to compare transcription of *porA* and *fetA* in wildtype meningococcal isolates. This data would improve understanding of the transcriptional regulation of both genes and of the implications that the variable expression of the antigens may have on the efficacy and coverage of the vaccine MenPF.

Chapter 2:

Materials and Methods

Unless otherwise stated, chemicals and antibiotics were obtained from Sigma Aldrich (St. Louis, Missouri, USA) or BDH AnalR grade (Surrey, UK); enzymes from New England Biolabs (Ipswich, UK); bacterial growth media from Oxoid (Cambridge, UK). Water used was purified with a Milli-Q purification system (Millepore, Billerica, Massachusetts, USA).

2.1: Bacterial Strains and culture conditions

Escherichia coli used for cloning are listed in Table 2.1.

Strain	Genotype	Source
OneShot® TOP10 Chemically Competent *E. coli*	*F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu) 7697 galU galK rpsL (StrR) endA1 nupG λ-*	Invitrogen, Paisley, UK
5-alpha High Efficiency competent *E. coli*	*fhuA2Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	New England Biolabs, Ipswich, UK
5-alpha F´I^q High Efficiency Competent *E. coli*	F´ *proA*$^+$*B*$^+$ *lacI*^{q} *Δ(lacZ)*M15 *zzf::Tn10* (Tet^R) / *fhuA2Δ(argF-lacZ)U169 phoA glnV44 Φ80Δ(lacZ)*M15 *gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	New England Biolabs, Ipswich, UK

Table 2.1: *Escherichia coli* strains used.

E. coli were grown in Luria-Lysogeny (LB) Broth (1% (w/v) tryptone, 0.8% (w/v) sodium chloride, 0.5% (w/v) yeast extract, pH 7.5) or on 25ml Luria-Lysogeny agar plates (LB broth containing 1.5% (w/v) Agar Bacteriological) with or without antibiotic selection. Broth cultures of *E. coli* were incubated at 37°C with rotational shaking at 150rpm. Plate cultures were incubated at 37°C. Antibiotics were used in the concentrations shown in Table 2.2.

For blue/white selection of *E. coli,* 10µl of 40µg/ml 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal), dissolved in *N,N*-Dimethylformamide (USB Corporation, California, USA) was spread onto the surface of plates before use, and allowed to dry at 37°C. When using 5-alpha F´I^q High Efficiency Competent *E. coli*, 5µl of 100µg/ml Isopropyl β-D-1-thiogalactopyranoside (IPTG) was also added to plates before use.

Antibiotic	**Final Concentration for *E. coli* (µg/ml)**	**Final Concentration for *N. meningitidis* (µg/ml)**
Ampicillin (Ampicillin sodium salt)	50	-
Kanamycin (Kanamycin disulfate salt from *Streptomyces kanamyceticus*)	50	100
Erythromycin	300	50

Table 2.2: Antibiotics and concentrations used for selection during cloning of *E. coli* and *Neisseria meningitidis*.

Neisseria meningitidis strains were obtained from The National Institute of Biological Standards and Control (NIBSC, Potters Bar, UK) -80°C stocks. For a full list of meningococcal strains used, see Appendix 1 (page 218).

Meningococci were grown from frozen stocks onto 25ml Colombia Blood agar plates with 5% Defibrinated Horse Blood, with subsequent growth in Mueller Hinton (MH) broth [176] or on 25ml MH plates with 1% (w/v) Agar Bacteriological. Alternatively, meningococci were grown in Tryptone Soya Broth [177] (TSB) or on Tryptone Soya Agar (TSA) or in Frantz medium [178] (provided by the Norwegian Institute of Public Health, Oslo, Norway). Antibiotics for selection of meningococci were used at the concentrations shown in Table 2.2. For growth conditions with reduced availability of iron, MH broth was incubated overnight at room temperature with 50µM Deferoxamine mesylate salts (DFAM) before use. Broth cultures of meningococci were incubated at 37°C with rotational shaking at 150rpm. Plate cultures were incubated at 37°C with 5% CO_2.

2.2: Antibiotic Susceptibility Testing

Adapted from Jorgensen *et al.,* [179]. Antibiotic diffusion discs were prepared using 10mm sterile filter paper discs (Sigma Aldrich) soaked for 3 minutes in the appropriate antibiotic. Antibiotics were used at the concentrations shown in Table 2.3.

Antibiotic	Solvent	Final Concentration (µg/ml)
Polymyxin B sulphate	Deionised water	1250
Vancomycin	Deionised water	2500
Rifampicin	Methanol*	50
Erythromycin	70% (v/v) Ethanol*	50

* VWR, Lutterworth, UK.

Table 2.3: Antibiotics and concentrations used for antibiotic susceptibility testing of *Neisseria meningitidis*.

Discs were allowed to air dry for 30 minutes before use. After overnight growth on blood agar, meningococci were resuspended in sterile saline solution (145mM sodium chloride) to an OD_{600nm} of 0.15. Using a sterile swab, bacterial suspension was inoculated onto the surface on MH agar plates containing 5% Defibrinated Horse Blood, ensuring that the bacteria covered the entire surface of the plate. Antibiotic discs were then placed on the surface of the plates. After overnight incubation, plates were imaged using an Allied Vision Firewire Camera (Kaiser Fototechnik, Buchen, Germany). Images were analysed using the ProtoCOL software (Synbiosis, Cambridge, UK) using the manual measure mode. Calibration was set according to the diameter of the plate.

2.3: Plasmid Production

2.3.1: Polymerase Chain Reaction

DNA was amplified from genomic DNA by Polymerase Chain Reaction (PCR) using Amplitaq® DNA Polymerase (Applied Biosystems, California, USA), with custom primers (Thermo Fisher, Massachusetts, USA) as listed in Table 2.4. For site-directed mutagenesis, Phusion DNA Polymerase (New England Biolabs) was used as described in Appendix 3 (page 228).

Reagent	Final Concentration
10x Buffer I*	1x
GeneAmp® dNTPs (A, G, C, T)**	40nM each
Forward and Reverse Primers	1µM each
DNA Template	10ng/µl
AmpliTaq® DNA Polymerase*	0.025 units/µl

* Reagents from Amplitaq® DNA Polymerase kit (Applied Biosystems).
** Also Applied Biosystems.

Table 2.4: Reagent concentrations used for PCR with Amplitaq® DNA Polymerase.

DNA was quantified using a NanoDrop micro-volume Ultra-Violet Spectrophotometer (Thermo Fisher), with measurements taken at 260nm. For colony PCRs, a sterile pipette tip was touched to the surface of a single colony on an agar plate before mixing cells into a PCR mix containing no template DNA. Thermocycling conditions used were as listed in Table 2.5. Annealing temperatures and extension times varied according to the primers used, and are listed in Table 2.6. Annealing temperatures were calculated using Primer Express 2.0 software (Applied Biosystems).

Step	Temperature (°C)	Time	Cycles
Activation	95	10 min	1
Melting	95	30s	34
Annealing	Various	30s	
Extension	72	60s per kb product	
Final Extension	72	300s	1

Table 2.5: Thermocycling conditions used for PCR with Amplitaq® DNA Polymerase.

Forward Primer	Forward Primer Sequence	Reverse Primer	Reverse Primer Sequence	Annealing Temperature (°C)	Extension Time
M13F	5′-GTAAAACGACGGCCAG-3′	M13R	5′-CAGGAAACAGCTATGAC-3′	50	2 min
FetApartialF	5′-CATGAAGACGTATCGGTTTGGATTTACTTCCC-3′	FetApartialR	5′-TTATCCAAGCTTTGAGCAGGTCTTGGGC-3′	56	1 min
ThdFpartialF	5′-TCATGAATGCTGGTCGAAGCGA-3′	ThdFtotalR	5′-TTCGAACGATCCGTTTATTTTCCGAT-3′	60	1 min
KanRev	5′-TGCGTGCAATCCATCTTGT-3′	S4	5′-GCGGTTTGATTTCCTGATGG-3′	62.5	2 min
A2	5′-TGGCAATTCCGGTTCGCTTGCT-3′	SDM3	5′-GGCAGGTTTTGCCCATGCGCAGAA-3′	65	30 sec
		SDM5	5′-TGCTCTCGCTTACCCTGGGGCAGGTTTTG-3′		
		SDM7	5′-CCATGCGGCAGAAAATAATGCAAGGTCGTACT-3′		
		SDM9	5′-GGCAGAAAATAATGCCAAGGCGTACTGGATACCGTTAC-3′		
InsRev	5′-TCATTGTTGCGGTTGTAAGAG-3′	SDM4	5′-CTTGGCATTATTTTCTGCGCATGGGCAAAA-3′	65	30 sec
		SDM6	5′-GCATGGGCAAAACCTGCCCCAGGGT-3′		
		SDM8	5′-CGGTAACGGTATCCAGTACGACCTTGCATTATTTTCT-3′		
		SDM10	5′-CCTTTTACGGTAACGGTATCCAGTACGCCTTGGCAT-3′		

Table 2.6: Sequences, annealing times and extension temperatures for all primer pairs used for genetic modification. All primers were obtained from Thermo Fisher.

2.3.2: *Escherichia coli* transformations

PCR products were cloned into pCR2.1TOPO vector (Invitrogen), according to manufacturer's instructions. The complete vector was subsequently transformed into either TOP10 *E. coli* (Invitrogen), 5-alpha F´I^q High Efficiency Competent *E. coli* (New England Biolabs) or 5-alpha High Efficiency competent cells (New England Biolabs) following the regular chemical transformation protocols provided by the manufacturer. Briefly:

- Vials of competent cells were thawed from -80°C on ice for 10 minutes.
- Between 5µl and 10µl DNA was added to the cells, and the vials were incubated on ice for 30 minutes.
- The cells were then heat shocked at 42°C for 30 seconds before further incubation on ice for 5 minutes.
- SOC (Super Optimal broth with Catabolite repression) media provided with the cells was added (250µl to vials of TOP10 *E. coli*, or 950µl to vials of 5-alpha F´I^q or 5-alpha *E. coli*) and the samples were incubated at 37°C with shaking for one hour.
- Vials were centrifuged at 9600x g for 1 minute and the supernatant was removed. The cell pellets were resuspended in 50µl SOC media and the entire cell suspension was plated selective plates.
- When cloning into a pCR2.1TOPO vector, blue/white screening was used as described above (see 2.1). Colonies were screened by colony PCR using M13F and M13R.

2.3.3: DNA extraction

After amplification in *E. coli*, plasmids were purified using a GenElute™ Plasmid Purification kit (Sigma Aldrich), or a Plasmid Plus Midi kit (Qiagen, Hilden, Germany).

Genomic DNA was isolated from meningococcal strains using a Wizard Genomic DNA Purification Kit (Promega, Wisconsin, USA), following manufacturer's instructions.

2.3.4: Restriction digest and ligation of plasmid DNA

Restriction digest of plasmids was performed using the reagent concentrations listed in Table 2.7.

Reagent	Final Concentration
10x Buffer*	1x
Restriction Enzyme(s)*	2 units/µl (total)
100x Bovine Serum Albumin*	0.1mg/ml
Plasmid DNA	50µg

* All from New England Biolabs.

Table 2.7: Reagent concentrations used for digestion of plasmid DNA with restriction enzymes.

Buffers used were those supplied with individual restriction enzymes or, if more than one enzyme was used in a single reaction, reaction conditions were as advised by New England Biolabs [180]. Restriction digest reactions were incubated for 3 hours, unless otherwise stated. Incubation temperatures used were as advised by New England Biolabs for each enzyme.

Restriction digest fragments were separated by agarose gel electrophoresis (described in 2.4.1). Appropriate fragments were purified from agarose gel slices using a High Pure PCR Product Purification Kit (Roche Applied Sciences, Burgess UK). Fragments were ligated using T4 DNA ligase at 16°C for 15 hours before transformation into *E. coli*.

2.4: Analysis of DNA

2.4.1: Agarose Gel Electrophoresis of DNA Samples

Electrophoresis of DNA products was carried out under the following conditions:

- Electrophoresis Buffer used was 1x TRIS-Acetate-EDTA:
  - 40mM Tris(hydroxymethyl)aminomethane (TRIS),

- 1mM Ethylenediaminetetraacetic acid disodium salt (EDTA),
- 0.1% (v/v) Glacial acetic acid.

- Agarose gels: 0.8% (w/v) Agarose (NBS Biologicals, Huntingdon, UK) dissolved in 1x TRIS-Acetate-EDTA, containing 0.005% (v/v) Safeview Nucleic Acid stain (NBS Biologicals).
- Loading Buffer: Blue/Orange Loading Dye, 6x (Promega).
- DNA Ladder: 1kb Plus DNA ladder (Invitrogen).
- Electrophoresis: 100V, 1 hour.
- Imaged using a Kodak Gel Logic 1500 Imaging System (Kodak, Hemel Hempstead, UK).
- Image processing using Kodak Molecular Imaging Software (v.4.0.5).

2.4.2: Acrylamide Gel Electrophoresis of DNA Samples

For small or low-concentration DNA products, electrophoresis was carried out under the following conditions:

- Electrophoresis Buffer used was 1x TRIS-Borate-EDTA:
  - 0.22M TRIS pH 8.3,
  - 180mM Borate,
  - 5mM EDTA.
- Acrylamide TRIS-Borate-EDTA Gel:
  - 13% (v/v) Bis-Acrylamide (29:1) (BioRad Laboratories, California, USA),
  - 0.02M TRIS pH 8.3,
  - 18mM Borate,
  - 0.5mM EDTA,

- 0.0016% (w/v) Ammonium persulphate (APS),
- 0.0008% Tetramethylethylenediamine (TEMED, BioRad Laboratories).

- Loading Buffer: Blue/Orange Loading Dye, 6x (Promega)
- DNA Ladder: 100bp DNA ladder (Invitrogen)
- Electrophoresis: 100V, 1 hour.

After running, gels were incubated for 20 minutes in TRIS-Borate-EDTA containing 0.005% v/v Safeview Nucleic Acid stain (NBS Biologicals) at room temperature in the dark. Gels were then imaged under the same conditions as agarose gels.

2.4.3: Sequencing

The deoxynucleotide (dNT) sequence of the DNA was determined using custom primers (Thermo Fisher) and BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). Reagent concentrations are listed in Table 2.8.

Reagent	Final Concentration
BigDye® 2.5x Ready Reaction Premix*	1x
BigDye® 5x Sequencing Buffer*	1x
Primer	0.5µM
Template DNA	5ng/µl

* Reagents from BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems).

Table 2.8: Reagent concentrations used for dNT sequencing.

Thermocycling involved 30 cycles of: 95°C for 15 seconds, 50°C for 15 seconds, 72°C for 4 minutes. Following thermocycling, PCR products (10µl each) were prepared for sequencing as follows:

- A combination of 5µl 125mM EDTA, 10µl dH_2O and 80µl 96% Ethanol (VWR) was added and the sample was incubated at room temperature for 15 minutes.

- Following centrifugation at 2567x g for 30 minutes, supernatants were drained and 200µl 70% Ethanol (VWR) was added to wash the sample.
- The sample was incubated at room temperature for 5 minutes, and then centrifuged at 2567x g for 10 minutes.
- The supernatant was drained and pellets were dried in a centrifuge at 576x g for one minute.
- Samples were rehydrated in 10µl each HiDi™ Formamide (Applied Biosystems).

Samples were sequenced using an ABI-Prism 3130*xl* Genetic Analyser (Applied Biosystems). Sequence viewing, alignments and vector diagrams were completed using the Lasergene Software Suite (v. 8.0.2, DNASTAR®, Madison, USA), or VectorNTI Advance™ v.10 (Invitrogen).

2.5: Transformation of *Neisseria meningitidis*

The transformation method was adapted from that used by van der Ley *et al.,* [120].

Meningococci were resuspended from overnight growth to an OD_{600nm} of 0.2-0.3 in MH broth containing 8mM magnesium chloride. 1µg undigested plasmid DNA was added to 1ml of meningococcal suspension and incubated at 37°C with 150rpm rotational shaking for 4 hours. The culture was then centrifuged at 9600x g for 1 minute to pellet the cells. The supernatant was removed and the cell pellet was resuspended in 50µl MH broth. The resuspended pellet was then grown on selective MH plates (with 8mM magnesium chloride) for 36 hours at 37°C plus 5% CO_2. Initial screening of transformants was carried out by colony PCR.

Following transformation, meningococcal strains were frozen at -80°C in MH broth with 50% (v/v) glycerol, or in Protect Beads (Technical Services Consultants Ltd, Heywood, UK).

2.6: Production of protein samples

2.6.1: Preparation of Outer Membrane Proteins by Spheroplast Lysis

Outer membrane protein samples were prepared from growth on agar plates or growth in broth cultures.

- Cells were suspended in 200µl 100mM TRIS/1mM EDTA. 200µl of 4°C 200mM TRIS/1M sucrose/1mM EDTA and 24µl of 1mg/ml Lysozyme were then added.
- The cell suspension was diluted with 400µl deionised water and incubated at room temperature with gentle shaking for 30 minutes.
- Spheroplasts were sedimented at 43140x g for 20 minutes, and resuspended in 800µl deionised water.
- The final outer membrane protein preparation was then sedimented at 43140x g for 20 minutes and resuspended in deionised water.

2.6.2: Outer Membrane Vesicle Preparation

OMVs were prepared using the following method, adapted from a protocol prepared by Gunnstein Norheim (Oxford Vaccines Group, Oxford, UK):

- Growth from overnight plate cultures (from frozen stocks) was subcultured onto blood agar plates and incubated for 7 hours. Cells from this plate were then resuspended in broth to an OD_{600nm} of 0.15-0.16, and incubated overnight at 37°C with shaking.
- The culture was then centrifuged in pre-weighed centrifuge tubes at 5468x g for 30 minutes at 4°C. The supernatant was removed and the wet weight of the harvested cells determined.
- Harvested cells were resuspended in 5ml of 0.1M TRIS (pH 8.6)/10mM EDTA/0.01% (w/v) thimerosal per gram of cells.

- Per ml of the previous buffer, 0.05ml 10% (w/v) sodium deoxycholate (DOC)/0.1M TRIS (pH 8.6)/10mM EDTA/0.01% (w/v) thimerosal was added to the resuspension. The mixture was stirred for 30 minutes at room temperature to extract the OMVs.
- The cell mass was then harvested by centrifugation at 5468x g for 30 minutes at 4°C.
- The supernatant containing the OMVs was transferred to clean centrifuge tubes and centrifuged again at 5858x g for 30 min at 4°C.
- The supernatant was then ultracentrifuged at 227000x g for 90 minutes at 4°C. The pellet was washed briefly with 0.05M TRIS (pH 8.9)/2mM EDTA/2.5% DOC/0.01% (w/v) thimerosal before being resuspended in 6ml of the same buffer.
- A total of 12ml 0.05M TRIS (pH 8.9)/2mM EDTA/0.5% DOC/30% (w/v) sucrose/0.01% (w/v) thimerosal was then added and the solution as homogenized in an ultrasound bath for 1 minute.
- The sample was ultracentrifuged at 227000x g for 60 minutes at 4°C.
- The supernatant was removed and the pellet was washed with 3% (w/v) sucrose solution/0.01% (w/v) thimerosal, before being resuspended in a pre-calculated volume of the same solution (resulting in a solution with approximately 1mg/ml protein).

The protein concentration of outer membrane protein preparations and OMV preparations were determined using a Pierce Bicinchoninic Acid (BCA) Protein Assay kit (Thermo-Fisher). After incubation, the plate was read in a Labsystems Multiskan MS spectrophotometer (Labsystems, Helsinki, Finland) at 570nm. A standard curve was plotted and the protein concentrations calculated from the curve.

2.7: Analysis of protein samples

2.7.1: 1-Dimensional protein electrophoresis

Samples were mixed in a 1:1 ratio with 2x Sample Buffer (10% (w/v) sucrose/5% (v/v) 2-Mercaptoethanol/0.02% (w/v) Sodium dodecyl sulphate (SDS)/0.001% (w/v) APS/0.75M TRIS (pH 7.2)/0.001% Bromophenol blue) and heated to 95°C for five minutes. Denatured proteins were then run on 25cm x 20.5cm x 1.5mm gels of the composition shown in Table 2.9.

Reagent	Resolving Gel	Stacking Gel
Bis-Acrylamide (29:1) (BioRad Laboratories)	12%, 10% or 8% (v/v)	5% (v/v)
TRIS	0.375M (pH 8.8)	0.2M (pH 6.8)
SDS	0.001% (w/v)	
APS	0.001% (w/v)	
TEMED (BioRad Laboratories)	0.0004% (v/v)	

Table 2.9: Reagent concentrations used for SDS-PAGE gels.

Molecular weight markers used were Prestained SDS-PAGE Standards, Broad Range (BioRad).

SDS-PAGE (Poly-Acrylamide Gel Electrophoresis) gels were run at 10mA in 0.4M Glycine/0.1M TRIS/0.2% (w/v) SDS. Gels were then stained with Coomassie strain (50% (v/v) Methanol/0.1% (v/v) Glacial acetic acid/0.005% (w/v) Brilliant Blue R-250) overnight, and destained in several changes of destain (50% (v/v) Methanol/0.1% (v/v) Glacial acetic acid) until bands were visible. Alternatively, gels were stained using a PlusOne Silver Staining kit (GE Healthcare, Connecticut, USA) according to manufacturer's instructions.

For Silver-stained gels, 10µg total protein was loaded per lane. For Coomassie-stained or western-blotted gels, 50µg total protein was loaded per lane.

2.7.2: Immunoblotting of protein samples

Proteins were transferred from SDS-PAGE gels onto Amersham Hybond-C nitrocellulose membrane (GE Healthcare) using a BioRad TransBlot® electrophoretic system. Blotting apparatus, membrane and gel were assembled according to manufacturer's instructions. Protein was transferred at 0.1A overnight in 0.025M TRIS/0.2M Glycine/20% (v/v) Methanol.

Immunoblotting of the membrane was carried out according to the following method:

- The nitrocellulose membrane was blocked for 1 hour at room temperature. Blocking buffer consisted of TRIS-Buffered Saline (0.025M TRIS (pH 7.6)/137mM sodium chloride) containing 5% (w/v) Marvel milk powder (Premier International foods, Lincolnshire, UK).
- The membrane was then incubated at room temperature in sera (1:200 dilution) or monoclonal antibody (NIBSC, 1:1000 dilution) in TRIS-Buffered Saline/5% (w/v) milk powder for 2 hours.
- The membrane was washed in 3 x 10 minute changes of TRIS-Buffered Saline, and then incubated at room temperature with secondary antibody (1:1000 dilution) in TRIS-Buffered Saline/5% milk powder for 1 hour.

The membrane was washed in 3 further 10 minutes changes of TRIS-Buffered Saline, then stained with SIGMAFAST™ 3,3′-Diaminobenzidine tablets (Sigma Aldrich) dissolved in deionised water or with SuperSignal West Pico Chemiluminescent substrate (Thermo Fisher). Blots were imaged using a Kodak Gel Logic 1500 Imaging System. For imaging of blots stained with chemiluminescent substrate, three times five minutes exposures for luminescence were used, with all exposures combined to give the final image.

2.8: Murine immunisations for antibody production

OMV samples were prepared as described above and filter sterilised using a 200nm filter before protein quantification by BCA assay. Recombinant protein samples used were prepared by Hema Patel (NIBSC). The following strains of mice were used for immunisations (all from Harlan Laboratories, Blackthorn, UK):

- NIH/OlaHsd mice (female),
- Balb/cOlaHsd (female),
- C3H/HeNHsd (female),
- C3H/HeJOlaHsd-*Tlr4*$^{LPS\text{-}d}$ (male).

All mice were 18-22g at the start of immunisation. Mice were immunised subcutaneously and bled by staff in the Biological Services Division at NIBSC.

2.8.1: Preparation of Inoculums with Aluminium Hydroxide Adjuvant

Samples were diluted to the final required concentration with $Al(OH)_3$ Diluent Buffer: 10mM TRIS (pH 7.9)/0.15M sodium chloride/0.05% (v/v) Triton X-100/0.25mg/ml $Al(OH)_3$ (Alhydrogel).

For OMV immunisations, samples were mixed continuously using a magnetic stirrer at 4°C overnight before use. Recombinant protein inoculums were mixed by vortexing briefly before use.

2.8.2: Preparation of Inoculums with Monophosphoryl Lipid A Adjuvant

OMV and protein samples were diluted to the final required concentration with sterile 145mM sodium chloride solution. Vials of Sigma Adjuvant System (Monophosphoryl Lipid A, MPL, + Trehalose dicorynomycolate, TDM oil-in-water emulsion, Sigma Aldrich) were warmed to 42°C before use. 2.4ml diluted sample was added to each vial of adjuvant. Vials were then mixed vigorously by vortexing for 3 minutes.

2.8.3: Production of FetA-specific Polyclonal Mouse Sera

A group of 10 female NIH mice (Harlan Laboratories, Indiana, USA), 18-22g, were immunised with 10µg purified FetA F3-3 protein (purified by Hema Patel, NIBSC) in Sigma Adjuvant System (Sigma Aldrich). Inoculums were prepared as described above. Mice were immunised on days 0 and 21, with terminal bleeds collected on day 42. Sera from all mice was then pooled for use in downstream applications.

2.9: Enzyme-Link Immunosorbent Assays

2.9.2: Preparation of Whole Cells as a Coating Antigen

- *N. meningitidis* were grown in liquid culture to an OD_{600nm} of 0.6. Cells from 3ml of culture were pelleted by centrifugation at 9600x g for 10 minutes.
- Supernatant was removed and the pellet was resuspended in 1ml Phosphate-Buffered Saline (PBS: 137mM Sodium chloride/2.7mM Potassium chloride/10mM Disodium hydrogen phosphate/1.8mM Potassium dihydrogen phosphate/pH adjusted to 7.4 with Hydrochloric acid) containing 0.05% (v/v) Tween-20 (Polyethylene glycol sorbitan monolaurate)**.**
- Cells were heat killed at 56°C for 30 minutes.

2.9.2: Enzyme-Link Immunosorbent Assay procedure

- For whole-cell ELISA, cell samples were diluted 1:10 in PBS and 100µl was added to each well of a NUNC flat-bottomed 96-well microtitre plate (Thermo Fisher). Plates were allowed to dry overnight at 37°C.
- For protein ELISAs, recombinant protein samples were diluted to 0.5µg/ml in PBS. 100µl was added to each well of a NUNC flat-bottomed 96-well microtitre plate. Plates were incubated overnight at 4˚C.

- The following morning, plates were washed three times with ELISA Wash Buffer (0.025% (v/v) Tween-20 in dH_20) using a Skatron SkanWasher 400 (Skatron Instruments, Lierskogen, Norway).
- 100µl per well of sample or standard was added, diluted in ELISA Dilution Buffer (PBS/0.5% (w/v) bovine serum albumin/0.01% (v/v) Tween-20). All monoclonal antibody standards were obtained from NIBSC. Samples were added in columns 1 to 10, with a standard added in duplicate in columns 11 and 12. Serial dilutions of factor 2 were made of all samples from row A to row H.
- Plates were incubated at 37°C for 1 hour, then washed three times before addition of 100µl per well of secondary antibody Horseradish Peroxidase (HRP) conjugate at an appropriate dilution in ELISA Dilution Buffer. Secondary antibodies used were: Goat Anti-Mouse IgG (Sigma Aldrich); Goat Anti-Mouse IgG1 (Caltag Laboratories, California, USA); Goat Anti-Mouse IgG2a (Caltag Laboratories); Goat Anti-Mouse IgG2b (Caltag Laboratories); and Goat Anti-Mouse IgG2 (Autogen Bioclear, Calne, UK).
- Plates were incubated at 37°C for 1 hour, then washed three times before addition of 100µl per well of Tetramethylbenzidine (TMB) ELISA substrate (Universal Biologicals, Cambridge, UK).
- Plates were incubated at room temperature for ten minutes before addition of 100µl per well of 1M sulphuric acid.
- Absorbance of each well was recorded at 450nm with a Multiskan MS plate reader (Labsystems). Concentrations of antibody, relative to the standard, were calculated using CDC ELISA software (CDC, Atlanta, USA).

2.10: Serum Bactericidal Assays

Adapted from Maslanka *et al.,* [46]. All dilutions of sera and bacteria were made in Bactericidal Buffer (0.5% (w/v) bovine serum albumin in Gey's Balanced Salt Solution).

- Following overnight growth of the required strain on Columbia blood agar, ~20 colony forming units (cfu) were spread over the entire surface of another Columbia blood agar plate and incubated at 37°C with 5% CO_2 for 5 hours.
- Sera was heat inactivated at 56°C for 30 minutes, and 20µl was added to each well of a sterile round-bottomed 96-well plate (Sterilin, Caerphilly, UK) in serial two-fold dilutions from columns 1 to 9. Sera were added in duplicate on each plate. 20µl of the starting dilution was also added to column 12 (Complement Independent Control, CIC), and 20µl bactericidal buffer was added to columns 10 (Complement Control, CC) and 11 (Viable Count, VC).
- Bacteria were resuspended in bactericidal buffer to an OD_{600nm} of 0.1. The cell suspension was diluted 1:10, and this was further diluted 1:250 before addition of 10µl to every well of the SBA 96-well plate.
- 10µl Baby rabbit complement (Pel-Freez Biologicals, Arkansas, USA) containing 0.007% (w/v) Colominic acid was added to each well in columns 1 to 10. Complement added to columns 11 and 12 was first heat inactivated at 56°C for 30 minutes.
- The plate was incubated at 37°C for 1 hour.
- Cfu for each well was calculated by plating out 10µl from each well onto a 100mm x 100mm x 15mm square Columbia blood agar plate (square plates from Cole Palmer, Illinois, USA). Each column was inoculated onto a single agar plate using a multichannel pipette, tilting the plate and allowing the cell suspensions to flow down the plate in 8 lanes. After incubation overnight, the number of colonies in each lane was counted using an Allied Vision Firewire Camera (Kaiser Fototechnik) and ProtoCOL computer software (Synbiosis).
- The serum bactericidal activity (SBA titre) was calculated as the reciprocal of the serum dilution that gave 50% killing compared to the VC. The average of the two replicates of

each serum sample was recorded. Killing in the CIC and CC lanes had to be below 30% for the assay to be valid.

2.11: Gene expression analysis

2.11.1: RNA Extraction

- Following overnight growth from frozen stocks, meningococcal strains were resuspended in 5ml MH to an OD_{600nm} of 0.2. Cultures were then incubated at 37°C with 150rpm shaking for 5 hours, to an OD_{600nm} of 0.6-0.7, during which the cultures achieved logarithmic growth.
- 500µl culture was added to 1ml of Bacteria Protect Reagent (Qiagen) and mixed by vortexing for 5 seconds. The mixture was incubated at room temperature for 5 minutes before pelleting cells by centrifugation at 9600x g for 10 minutes.
- Total RNA was then extracted from the cell pellets using an RNeasy Mini Kit (Qiagen) with an additional RNase-free DNAse step (Qiagen) to remove DNA contamination.
- Total RNA was eluted in 50µl RNase free water and stored at -80°C.

2.11.2: Quantitative Reverse-Transcription PCR Assay

Custom primers (Thermo Fisher) and probes (TIBMolbiol, Berlin, Germany) used for gene expression assays are listed in Table 2.10. Primer and probe sequences were determined using Primer Express 2.0 software (Applied Biosystems). Reverse-transcription PCR assays (RT-PCR) were completed in MicroAmp® Fast Optical 96-Well Reaction Plates (Applied Biosystems). Reactions were set up as listed in Table 2.11, with *gdh* used as an endogenous control in each well. A negative RNA-free control and a standard positive control sample of H44/76 total RNA following growth in MH were run on every plate. All reactions were run in triplicate. For reactions containing SYBR®-Green dye (SYBR®-Green RNA-to-CT™ 1-Step Kit, Applied Biosystems), *gdh* amplification was carried out separately, and probes were omitted.

Gene	Forward Primer	Reverse Primer	Probe
fetA (SYBR-Green)	FetA663F 5’-CTGCTCAAAGGCTTGGATAAAAA-3’	FetA734R 5’-GCTTACGCCTTCGTTGCTG-3’	-
nmb1989	NMB1989F 5’-GCCCCACAAGCCAAAGAA-3’	NMB1989R 5’-CATACCCAAATCGTAAACGGC-3’	-
nmb1993	NMB1993F 5’-CGCCAACGCGCCAT-3’	NMB1993R 5’-TGTTCAGCGGTTCGTCCA-3’	-
gdh	gdh350F 5’-TCGCCATTAAAGCCGAAATC-3’	gdh417R 5’-CTTGCCGGTACGCAGGTAGA-3’	gdh374T 5’-6JOE-ACGAACGCTGGAAGGGCGTTC-BBQ-3’
fetA	FetA377F 5’-TGCTCAAAGGCTTGGATAAAAAC-3’	FetA452R 5’-CCGTAGCTTACGCCTTCGTT-3’	FetA409T 5’-6FAM-CGCCTCAACAGCGGCTTTGCC-BBQ-3’
porA	PorA984F 5’-ACCGATCCCTTGAAAAACCAT-3’	PorA1050R 5’-ATTCAAGCCGCCTTCCTCAT-3’	PorA1006T 5’-Cy5-AGGTACACCGCCTGACGGGCG-BBQ-3’

Table 2.10: Primer and probe sequences used for quantitative RT-PCR assays. Primers were obtained from Thermo Fisher, and labelled probes from TIBMolBiol.

Reagent	Volume (µl)	Final Concentration
RT Enzyme Mix (40x)*	0.625	1x
RT-PCR Mix (2x)*	12.5	1x
Target Forward Primer	*fetA* = 0.75 *porA* = 0.50	*fetA* = 300nM *porA* = 200nM
Target Reverse Primer	*fetA* = 0.75 *porA* = 0.50	*fetA* = 300nM *porA* = 200nM
Control Forward Primer**	0.75	300nM
Control Reverse Primer**	0.75	300nM
Target Probe**	2.0	200nM
Control Probe**	2.0	200nM
Total RNA sample	2.0	
RNase-free water	To 25µl.	

*Reagents in the TaqMan® or SYBR®-Green RNA-to-CT™ 1-Step Kits (Applied Biosystems)

** Omitted from reactions with SYBR®-Green.

Table 2.11: Reagent concentrations used for quantitative RT-PCR assays. Primers and probes specific for the *gdh* gene were used as an endogenous control in each reaction.

Plates were sealed with a MicroAmp® Optical Adhesive Film (Applied Biosystems) and run on an Applied Biosystems 7500 Fast RT-PCR machine according to the thermocycling conditions listed in Table 2.12.

Step	Temperature (°C)	Duration	Cycles
Reverse Transcription	48	15 min	1
Enzyme Activation	95	10 min	1
Denture	95	15 sec	40
Anneal/Extend	60	1 min	

Table 2.12: Thermocycling conditions used for quantitative RT-PCR reactions.

Fluorescence was recorded at the end of each extension step according to the probes present in the reaction, at wavelengths determined by the Applied Biosystems 7500 Fast System Sequence Detection Software.

Relative Quantity (RQ) values were calculated by the Applied Biosystems 7500 Fast System Sequence Detection Software using the $2^{-\Delta\Delta CT}$ method. The *gdh* reaction was used as an endogenous control and all samples were calibrated to the positive control sample.

RQ values were compared to gene and promoter sequences from total genome sequence data present in the Maiden Group (University of Oxford, Oxford, UK) BIGSdb [181]. Gene sequences were found using the BLAST function and then aligned using MegAlign (part of the DNASTAR® Lasergene Software Suite).

2.12: Statistical Analysis

All data was analysed by student's t-test or General Linear Model for analysis of covariance using Minitab® 15 software (Minitab Inc., Pennsylvania, USA).

Chapter 3:

Strain Construction and Characterisation

3.1: Introduction

Neisseria meningitidis isolates are often genetically modified for research and vaccine-development. For example, genetic modification can be used increase the expression of important vaccine antigens in OMVs [182,183]. In this study, the natural competence of meningococci was utilised to construct strains required for the production and investigation of an OMV vaccine intended to be broadly protective against meningococcal disease. This vaccine, MenPF, will be based on the PorA and FetA outer membrane protein antigens.

PorA is one of the most abundant proteins in the outer membrane [184]. Furthermore, the protection induced by PorA in OMVs is well documented following the use of the OMV vaccines MenBvac and MeNZB [95,185]. Although antibodies to FetA have been found following meningococcal infection [164,165], relatively little is known about the FetA protein, due largely to the variability of its expression. As discussed previously (Chapter 1), *fetA* transcription is down-regulated by available iron via the binding of the regulatory protein, Fur [166]. Therefore, under many *in vitro* growth conditions, FetA levels are very low. As FetA-specific antibodies are present following disseminated infection, it is assumed that FetA is expressed by meningococci during replication in the bloodstream [164,165]; however, the limited expression *in vitro* is a fundamental problem facing the use of FetA as an antigen in an OMV vaccine. Under the conditions in which the OMVs are produced, FetA expression is unpredictable at best and undetectable at worst. Studying FetA-specific functional antibodies is also a problem, as the FetA protein is not expressed under the iron replete growth conditions used for *in vitro* serological assays.

In order to produce and study an OMV vaccine containing FetA as a major antigen, the first aim of this project was to produce meningococcal strains that constitutively expressed FetA at detectable levels. This was achieved by replacing the promoter sequence upstream of the *fetA* gene. In wildtype meningococcal isolates, the *fetA* promoter contains a poly-cytosine (poly-C) tract that is responsible for phase variation by slipped-strand mispairing (Figure 3.1). There are also Fur-binding regions overlapping the promoter region [166], resulting in iron-regulation of protein expression.

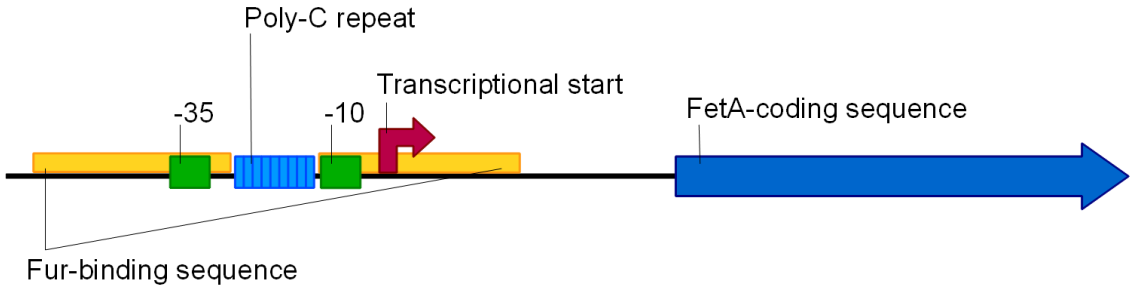


Figure 3.1: Transcriptional control of *fetA* expression. The promoter upstream of the *fetA* gene (dark blue) is shown: the -10 and -35 RNA-polymerase binding sites (green); the transcriptional start site (red); the poly-C repeat leading to phase variation of *fetA* (light blue); the sites bound by the Fur protein responsible for iron-regulation (yellow) [166].

As well as removing the poly-C tract upstream of *fetA*, the Fur-binding regions were removed to eliminate negative regulation by this protein. The *fetA* promoter was replaced with a sequence modified from the *porA* promoter region. The modified promoter was chosen to give a higher level of expression that is more likely to induce protective antibodies. Expression from the native *porA* promoter is variable due to slipped-strand mispairing altering the length of the poly-guanidine (poly-G) tract between the -10 and -35 regions [186]. Therefore, to prevent phase variation by this mechanism, this region in the modified promoter was taken from the

porB gene. The -10 to -35 sequence in the *porB* promoter does not readily undergo slipped-strand mispairing, but maintains optimal spacing for high expression.

An OMV vaccine produced from the modified strain requires immunogenicity testing in a murine model prior to clinical trials in humans. As there is currently no effective animal model that can be used to estimate protection against meningococcal disease *in vivo*, the efficacy of meningococcal vaccines is determined by an *in vitro* SBA. This assay measures the ability of complement-binding antibodies present in serum samples to kill live meningococci. It has been established that, for human serum samples post-vaccination, SBA titres against the PorA antigen or meningococcal capsule correlate with protection against invasive meningococcal disease [45]. Following immunisation with OMVs, antibodies will be produced to a range of antigens. Therefore, a genetically modified panel of strains was developed to investigate the immune response induced by the vaccine against PorA and FetA. These strains were constructed so that *porA* and *fetA* genes were either switched on or off in various combinations. This panel of strains will facilitate SBA analysis that can separate the effects of antibodies against each of the vaccine antigens and against other antigens present in the OMVs.

Specific modification of vaccine antigen expression may have further effects on meningococcal survival or immunogenicity. Any resulting changes in growth or expression of other genes could also be used to provide insight into the role of FetA in meningococcal survival. Therefore, modified strains were characterised to ensure that any changes introduced would not be detrimental to the use of the strains for OMV vaccine production or serological investigation.

3.2: Results

3.2.1: *Neisseria meningitidis* strains used

Meningococcal strains used and produced in this chapter are listed in Table 3.1. Following the first introduction of each strain, strains will be referred to as listed below.

Strain	Parent Strain	Source	Genotype	Modifications from wildtype	Referred to in text as
H44/76	-	NIBSC collection.	*fetA+ porA+*	None.	Wildtype
3043	H44/76	NIBSC collection. Produced in [187].	*fetA::kan*	*fetA* gene interrupted by a Kanamycin-resistance cassette insertion [187]. Native *fetA* promoter present. PorA expression as wildtype.	FetA-off
3200	H44/76	This study.	$fetAp_{17bp}$ $fetA_{\Delta 65C}$	*fetA* promoter replaced with the modified *porA* promoter. -1 Frame-shift introduced at the start of the *fetA* gene, resulting in low FetA expression. PorA expression as wildtype.	FetA-on (Frame shift)
3207	H44/76	This study.	$fetAp_{17bp}$	*fetA* promoter replaced with the modified *porA* promoter, resulting in high expression. PorA expression as wildtype.	FetA-on
3311	3043	This study.	*fetA::kan* *porA::ermC*	*fetA* gene interrupted by a Kanamycin-resistance cassette insertion [187]. *porA* gene interrupted by an Erythromycin-resistance cassette insertion.	FetA-off/PorA-off
3312	3207	This study.	$fetAp_{17bp}$ *porA::ermC*	*fetA* promoter replaced with the modified *porA* promoter. *porA* gene interrupted by an Erythromycin-resistance cassette insertion.	FetA-on/PorA-off
3313	H44/76	This study.	$fetAp_{17bp}$ $fetA_{\Delta 66G}$	*fetA* promoter replaced with the modified *porA* promoter. -1 Frame-shift introduced at various positions at the start of the *fetA* gene. PorA expression as wildtype.	3313
3314			$fetAp_{17bp}$ $fetA_{\Delta 47C}$		3314
3315			$fetAp_{17bp}$ $fetA_{\Delta 81C}$		3315
3316			$fetAp_{17bp}$ $fetA_{\Delta 86T}$		3316

Table 3.1: *Neisseria meningitidis* strains used and produced in this chapter.

3.2.2: Constitutive expression of *fetA*

As FetA is regulated by both Fur-binding sequences and variation in the -10 to -35 region [166], it was necessary to replace both the Fur-binding regions and the promoter region for constitutive expression. The region upstream of *fetA* was replaced with a *porA*-derived promoter to give high levels of expression. A promoter shown to give high expression, containing a 17bp spacer between the -10 and -35 regions, was used [186]. To prevent phase variation via slipped-strand mispairing, the spacer region in this promoter contained an alternative sequence from the *porB* -10 to -35 region (Figure 3.2). A plasmid containing this promoter sequence in a pUC19 background (pUC19PorApro) was provided by Dr Rory Care (NIBSC).

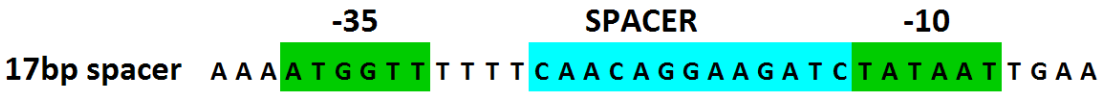


Figure 3.2: Sequence of the modified *porA* promoter region used to replace the native *fetA* promoter. Bases constituting the -10 and -35 regions are highlighted in green. Bases taken from the *porB* promoter are shaded in blue.

The strain H44/76 (see Appendix 1, page 218), was chosen as the parent strain. The growth characteristics are well characterised, as are the content, immunogenicity and safety of OMVs produced from this strain. The strain was first isolated in 1976 in Norway, from a patient with invasive meningococcal disease. As a representative of the meningococcal lineage responsible for the Norwegian epidemic, H44/76 is the isolate used to produce MenBvac [32]. H44/76 is also readily transformable, and is the parent strain used to create the HexaMen and NonaMen vaccine strains [121]. A wide variety of other H44/76 mutants have been produced previously [115,123].

In order to replace the promoter of the *fetA* gene, plasmid pUC19FetApartialKanThdF was constructed (Figure 3.3). For a detailed description of the cloning method used, see Appendix 2

(page 223). Briefly, the plasmid was constructed to contain the modified PorA promoter upstream of the 5' end of the *fetA* open reading frame. This 514bp region of *fetA* sequence (FetApartial) provided homology at the 3' end of the promoter. The plasmid also contained a Kanamycin resistance marker for selection of positive clones. Homology with the 3' end of the upstream gene, *thdF*, was included to allow recombination both upstream and downstream of the altered promoter. As the *Neisseria* Uptake Sequence (NUS, 5'-GCCGTCTGAA-3'), is required for efficient transformation of meningococci [188], inverted repeats of the NUS were included at the 3' end of the incorporated *fetA* sequence.

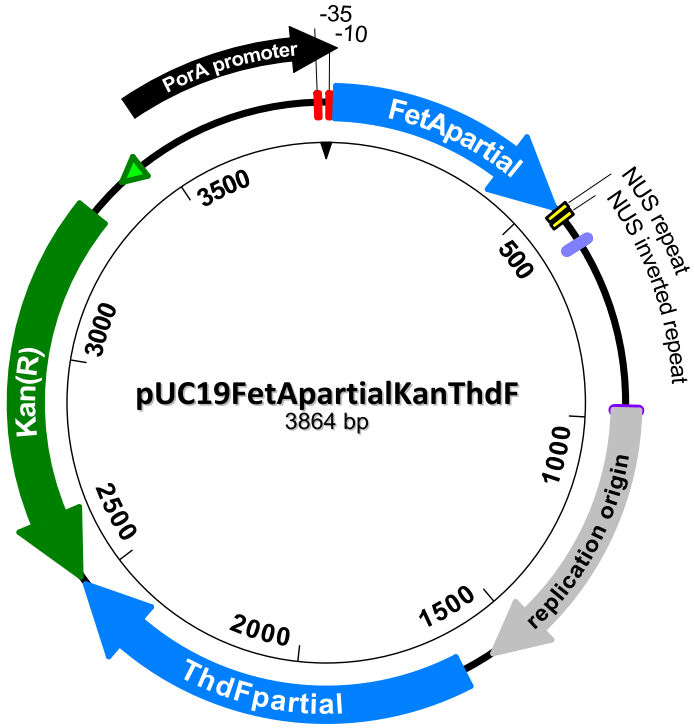


Figure 3.3: Plasmid pUC19FetApartialKanThdF, showing the positions of the Kanamycin resistance selective marker (KanR); the *porA*-derived promoter; the 5' end of *fetA* (FetApartial); the 3' end of the upstream gene *thdF* (ThdFpartial) and the uptake sequence (NUS).

Plasmid pUC19FetApartialKanThdF was used directly to transform wildtype H44/76. Clones in which the Kanamycin resistance marker (KanR) from the plasmid had been incorporated were selected for on Kanamycin-containing media. Clones in which KanR had been inserted upstream of the *fetA* gene along with the *porA*-derived promoter were then screened for using

colony PCR. Primers used for screening were KanRev, binding within the Kanamycin resistance marker, and S4, binding within the *fetA* gene outside of the sequence present in the plasmid (see Table 2.6, page 37). A PCR product of the size 1747bp indicated successful recombination (Figure 3.4). Sequencing across the *fetA* promoter confirmed correct integration.

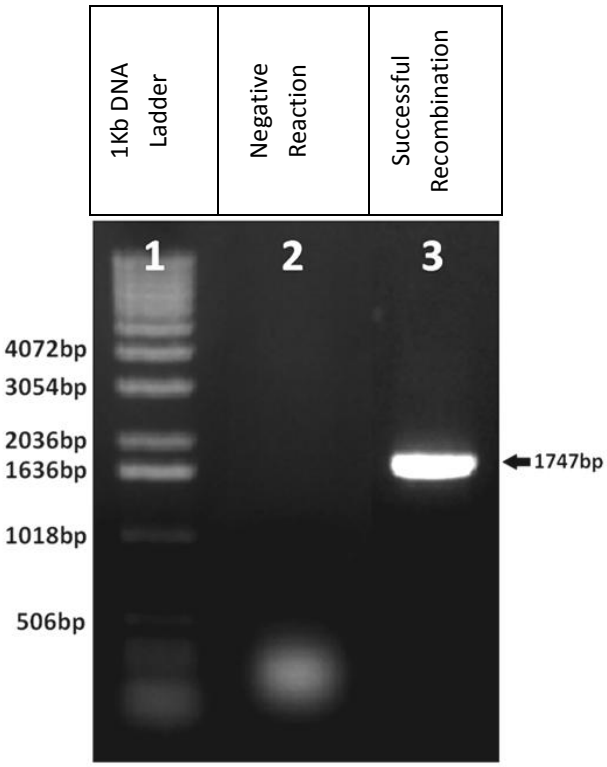


Figure 3.4: Agarose gel electrophoresis of PCR products following colony PCR of *N. meningitidis* H44/76 transformation with plasmid pUC19F33KanThdF. Lanes: 1) 1kb DNA ladder, 2) Negative reaction, 3) PCR from a colony containing the correct genomic integration. Sizes of the DNA standards are indicated on the left.

Homologous recombination was expected to occur across both *thdF* and *fetA*. However, in all clones investigated recombination of the plasmid with genomic DNA was found to have occurred only across the *fetA* gene. This single recombination event resulted in incorporation of the whole plasmid. The native *fetA* promoter region and 5' end of the genomic *fetA* gene were present in all clones, but displaced from the rest of the *fetA* gene by the incorporated plasmid DNA.

3.2.3: FetA expression levels in strains containing the modified *porA* promoter

SDS-PAGE gels of outer membrane protein preparations were used to confirm increased expression of FetA in clones containing the *porA*-based promoter upstream of the *fetA* gene.

Resolution of FetA was optimal on 8% or 10% Acrylamide gels visualised using silver-stain, although in many cases probing a western blot with FetA-antisera was required to confirm FetA expression.

Clones were compared following growth in MH broth or MH supplemented with the iron-chelator DFAM. The addition of DFAM was used to chelate the available iron in the growth media, therefore changing conditions from high iron availability to a lower iron availability. The wildtype strains showed increased FetA expression in the presence of DFAM, with low FetA expression levels in MH without DFAM. A strain in which *fetA* has been interrupted by an antibiotic resistance cassette (FetA-off strain 3043) showed no detectable FetA expression under either condition tested. Comparisons of protein preparations from transformed clones grown in MH+DFAM versus MH alone suggested that one clone (strain 3200) constitutively expressed FetA regardless of iron availability in the growth media. However, the level of FetA expression was low compared to the expression of FetA in wildtype H44/76 in MH+DFAM (Figure 3.5).

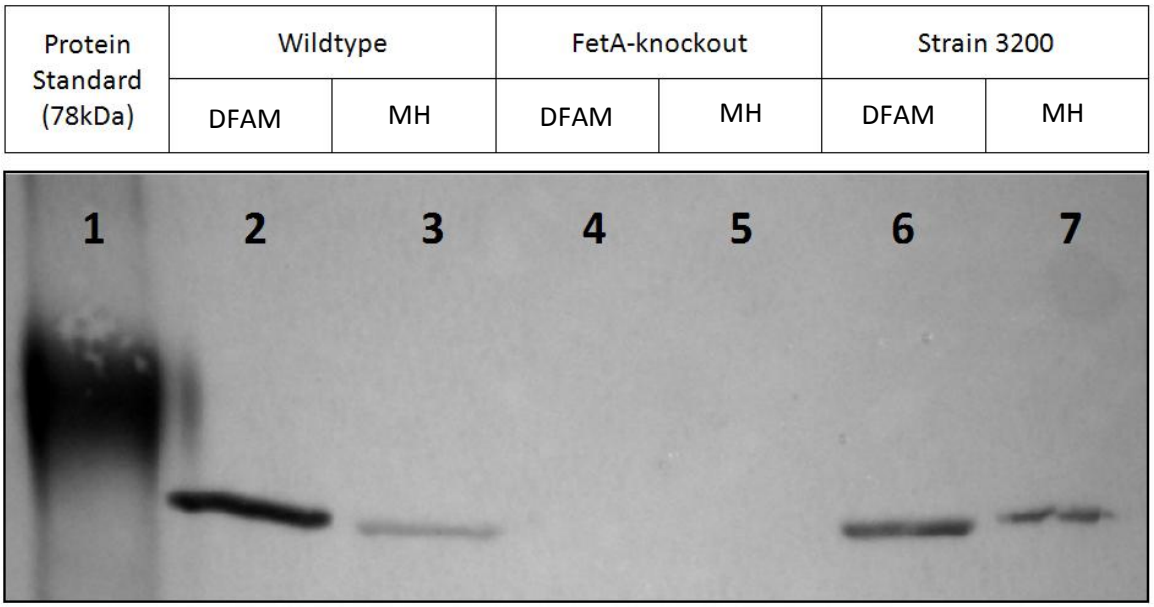


Figure 3.5: Western Blot of OMP preparations, probed with polyclonal FetA-specific mouse sera. Strains were grown in MH media (high iron) or MH supplemented with DFAM to reduce levels of available iron. Lanes: 1) Protein Standard (78kDa marker shown), 2) Wildtype Fe-, 3) Wildtype Fe+, 4) FetA-off (3043) Fe-, 5) FetA-off (3043) Fe+, 6) 3200 Fe-, 7) 3200 Fe+.

Another transformed clone (FetA-on strain 3207) showed constitutive expression of FetA at higher levels (Figure 3.6).

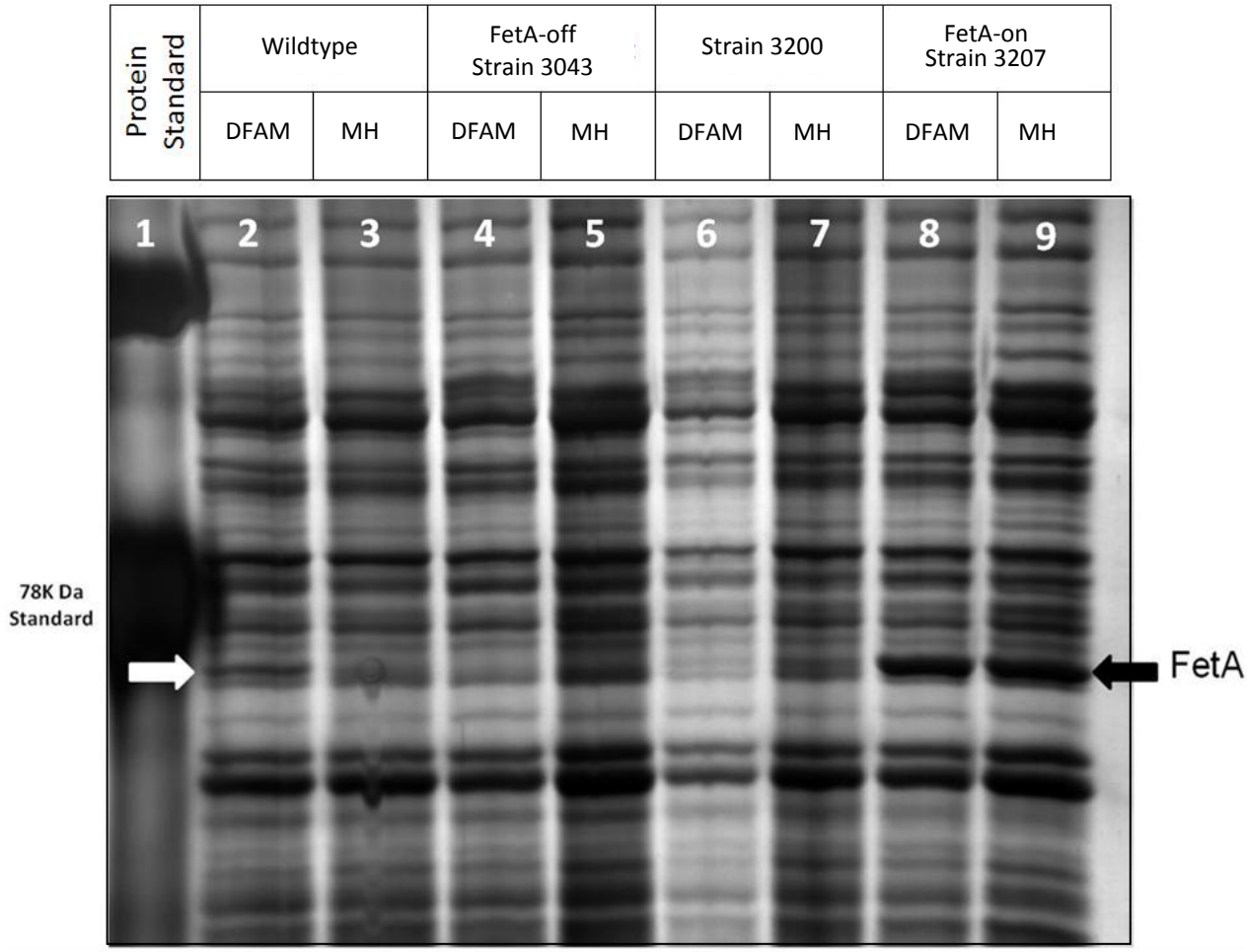


Figure 3.6: Silver-stained 10% SDS-PAGE gel of OMP preparations. Strains were grown in MH broth or MH supplemented with DFAM to chelate available iron.

Lanes: 1) Protein Standard, 2) Wildtype Fe-, 3) Wildtype Fe+, 4) FetA-off (3043) Fe-, 5) FetA-off (3043) Fe+, 6) 3200 Fe-, 7) 3200 Fe+, 8) 3207 Fe-, 9) 3207 Fe+.

The composition of growth media can affect expression levels of many meningococcal proteins [98]. Many of these effects will be due to the availability of nutrients other than iron. Therefore, it was necessary to investigate whether the FetA-on strain 3207 also expressed FetA at high levels in alternative growth media that may be used for OMV production. Protein expression was compared in the wildtype and FetA-on strains following growth in TSB and Frantz medium. Strains were inoculated from overnight growth on blood agar into the

different growth media. After six hours of growth, cells were harvested and outer membrane proteins were purified. Proteins from the wildtype and FetA-on strains were subjected to SDS-PAGE electrophoresis and compared (Figure 3.7). As growth conditions in both media were iron replete, FetA expression in the wildtype strain was repressed. Similar to results seen following growth in MH (Figure 3.6), over-expression of FetA compared to wildtype was seen in the FetA-on strain following growth in both TSB and Frantz media. No other changes were visible between that strain and the wildtype strain.

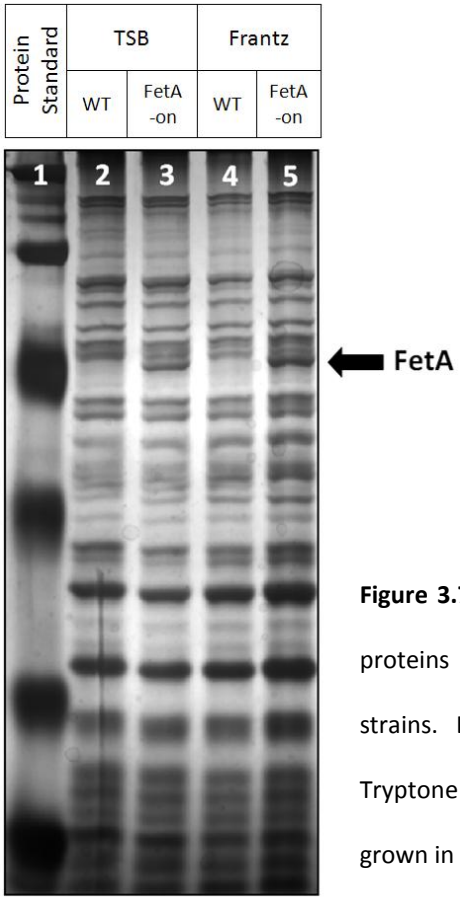


Figure 3.7: Silver-stained 10% SDS-PAGE gel of outer membrane proteins preparations from the wildtype (WT) and FetA-on strains. Lanes: 1) Protein Standards, 2) Wildtype grown in Tryptone Soya Broth (TSB), 3) FetA-on grown in TSB, 4) Wildtype grown in Frantz Medium, 5) FetA-on grown in Frantz medium.

The aim of this project was to construct a strain for production of an OMV vaccine. As OMVs contain a number of key outer membrane proteins, the protein profile of OMVs from the FetA-on strain were compared to wild-type OMVs. Proteins from outer membrane vesicles were separated by SDS-PAGE electrophoresis (Figure 3.8(A)), and densitometric analysis was subsequently completed for six key outer membrane proteins identified by comparison with [184]. Protein expression was quantified by comparing the size and intensity of each protein

band. Protein expression was recorded as a percentage of the total protein content (Figure 3.8(B)). FetA was excluded from the calculation of total protein content as expression of this OMP has been modified.

(A)

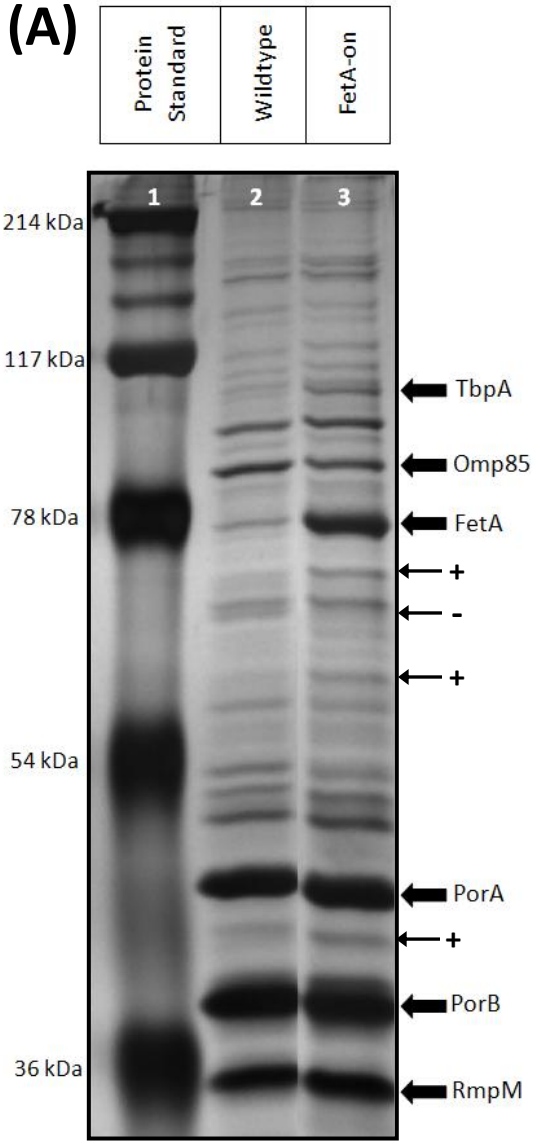


Figure 3.8: Comparison of outer membrane vesicles from the wildtype and FetA-on strains. OMVs were extracted following growth in MH broth. **(A)** Silver-stained 10% SDS-PAGE gel. Lanes: 1) Protein Standards, 2) Wildtype, 3) FetA-on. 10µg total protein was loaded per lane. Molecular weights of the protein standards are shown to the left of the image. Proteins that could be identified by comparison with [184] are labelled, and were subsequently analysed by densitometry. Other proteins that show increased (+) or decreased (-) expression are indicated with small arrows. **(B)** Results of densitometric analysis of the identifiable (labelled) protein bands seen on the SDS-PAGE gel shown in (A). Proteins from the Wildtype and FetA-on strains were quantified as a percentage of the total protein content based on band size and density. FetA was excluded from calculation of the total protein value.

(B)

	Strain	% Total Protein
TbpA	Wildtype	2.55
	FetA-on	3.91
Omp85	Wildtype	3.03
	FetA-on	3.02
FetA	Wildtype	2.52
	FetA-on	7.91
PorA	Wildtype	11.90
	FetA-on	12.16
PorB	Wildtype	17.09
	FetA-on	12.97
RmpM	Wildtype	10.79
	FetA-on	11.91

As well as an increase in the concentration of FetA within OMVs, several other minor OMPs were also present at increased or decreased concentrations compared to wildtype OMVs. When FetA expression was quantified by densitometry, it was seen that FetA levels in the FetA-on strain were three times that seen in the wildtype (7.9% compared to 2.5%). Expression of several other key OMPs (Omp85, PorA and RmpM) was found to be similar to wildtype. An increase was observed in expression of TbpA relative to wildtype (3.9% compared to 2.6%). A decrease in PorB expression was also observed in the FetA-on strain relative to wildtype (13% compared to 17%). Differences such as these are often found between batches of OMVs [184] and so may be unrelated to the genetic modification in strain 3207. Alternatively, the differences may be artefacts of the experimental technique. As protein loading was determined by measurements taken of the total protein content of OMV samples, and concentrations of many antigens change, consistent loading of multiple samples is difficult. This is a particular problem when comparing samples in which the expression of particular antigens has been up- or down-regulated, as these will markedly alter the total protein content of the OMVs.

3.2.4: Investigation of the frame-shift mutation introduced in *fetA* during plasmid amplification in *E. coli*

Following transformation of H44/76 with the plasmid pUC19FetApartialKanThdF, a clone (strain 3200) was isolated that expressed FetA constitutively, although at low levels compared the FetA-on strain 3207. The presence of a -1 frame-shift (FS) mutation at the start of the *fetA* gene from strain 3200 was revealed using end terminal sequencing (Figure 3.9), and provides an explanation for the low levels of FetA expression observed in this strain. The mutation appeared during amplification of the plasmid in *E. coli*, regardless of the strain of *E.coli* used. The single base deletion arose in all plasmids in which *fetA* was present downstream of the

porA-based promoter, but was not found when *fetA* was amplified without a promoter sequence present. However, the appearance of the mutation was not dependent on the presence of a fully functional *fetA* coding region.

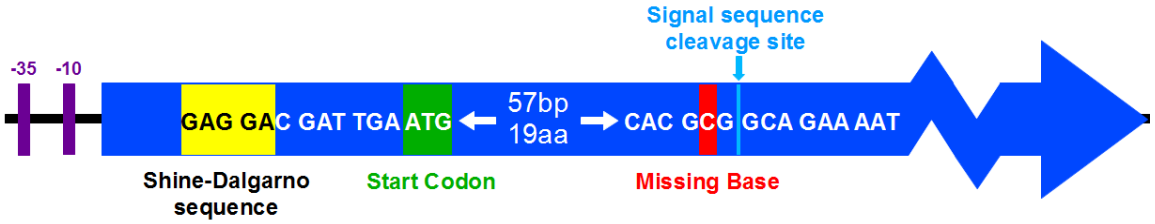


Figure 3.9: Relative position of the nucleotide base that is deleted during amplification of *fetA* in *E. coli*. The positions of the ATG start codon, and signal-sequence cleavage site are shown [149].

Sequence analysis suggests that the single base deletion would result in a 35-aa peptide. Expression of FetA in *E. coli* carrying the pUC19FetApartialKanThdF plasmid, containing the FS mutation, was tested by immunoblot of whole-cell lysates with FetA polyclonal sera. No FetA expression was detected (data not shown).

When the FS was introduced into the meningococcal *fetA* gene with the *porA*-based promoter, the levels of expression were low. However, as in strain 3207, when homologous recombination occurred upstream of this point and the FS mutation was not introduced, expression was both constitutive and at high levels. Sequence analysis showed that there were no compensatory mutations downstream in 3200 that would put translation back into the correct frame. Therefore, the FS in H44/76 reduced expression levels but did not, as would be expected, result in a truncated protein. One possible explanation is that there can be a movement between frames occurring during translation, and that this movement has a reduced efficiency compared to normal translation. This would result in reduced expression levels of the complete protein. If the mechanism that allows this movement between frames could be better understood, this may enable replacement of the mutation seen in 3200 with an alternative mutation that would still be stable in *E. coli* while allowing a higher efficiency of shifted translation (corresponding to higher levels of FetA expression) in meningococci. This

alternative FS mutation could then be used for further strain-construction required to produce other strains for the vaccine MenPF.

Site-directed mutagenesis, as described by Ho *et al.,* [189], was used to introduce similar FS mutations at other nucleotide positions at the start of the *fetA* gene. The positions of these mutations are shown in Figure 3.10. The method used is described in Appendix 3 (page 228). Introduced mutations were found to be stable during plasmid amplification in *E. coli*, replacing the original FS mutation but not resulting in expression of the full-length FetA protein (data not shown).

Codon:	Thr	Leu	Ala	Ala	Gly	Phe	Ala	His	Ala	Ala	Glu	Asn	Asn	Ala	Lys	Val	Val
3200:	ACA	CTT	GCG	GCA	GGT	TTT	GCC	CAC	GCG	GCA	GAA	AAT	AAT	GCC	AAG	GTC	GTA
3313:	ACA	CTT	GCG	GCA	GGT	TTT	GCC	CAC	GCG	GCA	GAA	AAT	AAT	GCC	AAG	GTC	GTA
3314:	ACA	CTT	GCG	GCA	GGT	TTT	GCC	CAC	GCG	GCA	GAA	AAT	AAT	GCC	AAG	GTC	GTA
3315:	ACA	CTT	GCG	GCA	GGT	TTT	GCC	CAC	GCG	GCA	GAA	AAT	AAT	GCC	AAG	GTC	GTA
3316:	ACA	CTT	GCG	GCA	GGT	TTT	GCC	CAC	GCG	GCA	GAA	AAT	AAT	GCC	AAG	GTC	GTA

Figure 3.10: Positions of the frame-shift mutations introduced into the start of the *fetA* gene by site-directed mutagenesis, and the codons affected (underlined). Deleted bases are shown in red. The position of the original frame-shift found in 3200 is shown for comparison. Strains are listed according to the strain number of the final *N. meningitidis* transformant strains.

Following plasmid transformation into *N. meningitidis*, whole-cell lysates were probed with FetA F3-3 polyclonal sera to detect the presence of the FetA protein (Figure 3.11). The F3-3 polyclonal sera was seen to cross react with other proteins present in all *N. meningitidis* whole-cell lysates, both of higher and lower molecular weights than the FetA protein.

A band corresponding to FetA was present in the lysate of the wildtype strain, although this band was faint due to repressed expression following growth of the strains in iron replete media. There were no bands of the correct size found in lysates from the negative control strain 3043 (FetA-off). Bands corresponding to the complete FetA protein were also present in lysates from strains 3200 (FetA-on, FS) and 3207 (FetA-on), with the expected increases in levels of FetA present in 3200 and 3207 compared to wildtype.

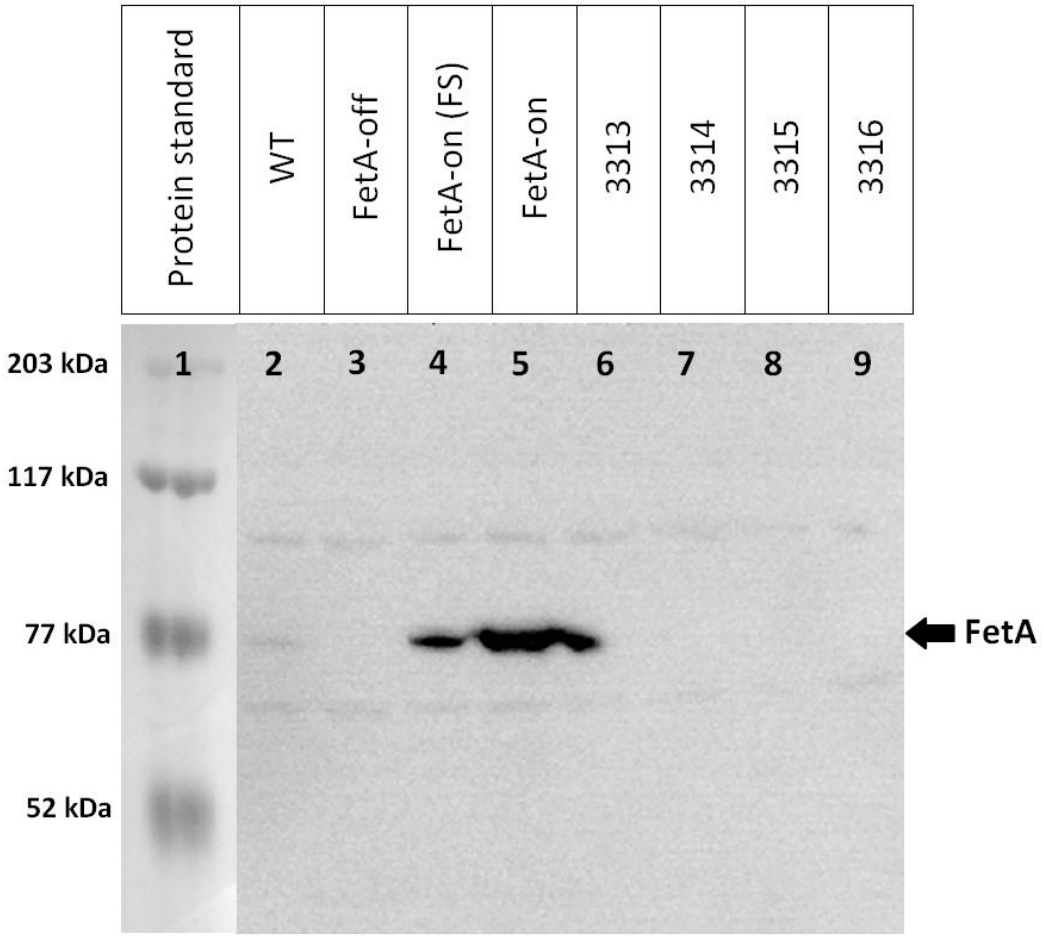


Figure 3.11: Western blot of whole-cell lysates (50µg total protein per lane) probed with F3-3 polyclonal mouse sera and imaged with chemiluminescent substrate. Lanes: 1) Protein Standards (visualised with white light), 2) Wildtype, 3) FetA-off, 4) FetA-on + FS (3200), 5) FetA-on, 6) 3313, 7) 3314, 8) 3315, 9) 3316.

In strain 3200, movement between frames of translation, resulting in expression of the complete FetA protein, could occur in several ways. Mutations in genes encoding transfer-RNAs (tRNAs) can lead to the anticodon loop reading two bases [190] or four bases [191] rather than the usual three bases; however, these mutant tRNAs are often less efficient than wildtype RNAs, resulting in reduced levels of alternate-frame translation [192]. As the mutation in 3200 resulted in sequence GG GCA (rather than GCG GCA), movement into the correct frame for full protein expression could occur through doublet-decoding of GG by

$tRNA^{Gly}$ [190]. If this was the case, removal of the third base in the codon (strain 3313), resulting in sequence GC GCA, would be expected to abolish this, while the mutation introduced into strain 3314 (resulting in an identical sequence to that found in 3200, GG GCA, positioned upstream of the original mutation) would still result in full protein expression. However, neither strain 3313 nor 3314 were found to express the complete FetA protein.

Wildtype tRNAs may also occasionally slide from one frame into the next during translation in the ribosome, particularly if there is low specificity in the third base position of the codon [193]. As the missing base occured in the first of two sequential alanine codons, movement back into the zero frame (for full protein translation) could occur by slippage of the $tRNA^{Ala}$. Therefore, the mutation introduced into strain 3313 would be expected to result in expression of a full-length protein, as this mutation maintains two sequential alanine codons, $\overrightarrow{\underrightarrow{GCGCA}}$, allowing slippage from the first (GCG, underlined) to the second (GCA, overlined). Following the same hypothesis, the mutation introduced into strain 3315, a base removed in a single alanine codon rather than a sequential pair of identical codons, would not result in full protein expression as $tRNA^{Ala}$ would not be able to slide between that codon and the adjacent lysine codon. To investigate whether the movement between frames was restricted to alanine codons, a similar base-deletion was also introduced into a pair of valine codons, resulting in strain 3316. As shown in Figure 3.11, none of the strains 3313, 3315 or 3316 were found to express the complete FetA protein.

These results suggest that the translational shift in 3200 that allows expression of a full-length FetA protein is dependent on the precise context of the missing base. This context-dependent movement may be due to tRNAs for adjacent codons affecting the bases available for binding [194]. Alternatively, it may be that frame-shifting does occur in other mutational contexts (for example in strain 3314), but because of the context of the mutation shifting is constrained to occur towards the -1 frame, which would not allow full FetA expression. In 3200, the surrounding codons could enable movement in towards the +1 frame in some peptides

(movement into the correct frame for full protein expression), while some frameshifting will still occur in the opposite direction and some translation will continue without additional movement between frames. This would result in the expression of full length FetA, albeit at a lower level of expression than that seen in 3207 when no FS mutation is present.

As the alternative mutations did not result in FetA expression in strains 3313, 3314, 3315 or 3316, further investigation of the mechanism allowing FS suppression is unlikely to provide information that will aid further strain construction.

3.2.5: Construction of strains required for investigation of FetA as a vaccine candidate

Due to the iron-regulation of FetA in wildtype strains, investigating FetA-specific antibodies employing *in vitro* assays is difficult. Growth conditions used for *in vitro* serological assays are often iron replete, resulting in a culture of meningococcal cells which do not express FetA. The important antigenic components of the vaccine MenPF are PorA and FetA, although, as the antigens are presented within OMVs, other outer membrane proteins will also elicit an antibody response. To determine the relative contribution of each antigen to the immune response, meningococcal strains were constructed which expressed either FetA or PorA exclusively. A strain was also constructed that expressed neither PorA nor FetA, to differentiate antibodies to other surface proteins. The FetA-knockout strain 3043 (*fetA::kan*) was already available [187].

To complete the panel the following additional strains were constructed:

- A double FetA/PorA-knockout was constructed by transformation of strain 3043 (FetA-off) with plasmid PorAEryF (provided by Dr Gunnstein Norheim and Ojas Mehta, CCVTM, Oxford), containing the *porA* gene interrupted by an erythromycin resistance

marker to remove expression of the full-length PorA protein. This resulted in strain 3311 (FetA-off/PorA-off, *fetA::kan porA::ermC*).

- Strain 3207 (FetA-on, $fetAp_{17bp}$) was transformed with plasmid PorAEryF, resulting in strain 3312 (FetA-on/PorA-off, $fetAp_{17bp}$ *porA::ermC*), in which FetA was expressed constitutively at high levels while PorA expression was interrupted.

Following transformation with plasmid DNA and selection of clones containing the appropriate antibiotic resistance markers, correct genomic configurations were confirmed by sequencing. Outer membrane proteins of these strains were harvested and separated by SDS-PAGE electrophoresis (Figure 3.12).

	H44/76	3207	3043	3311	3312
FetA	WT	+	-	-	+
PorA		+	+	-	-

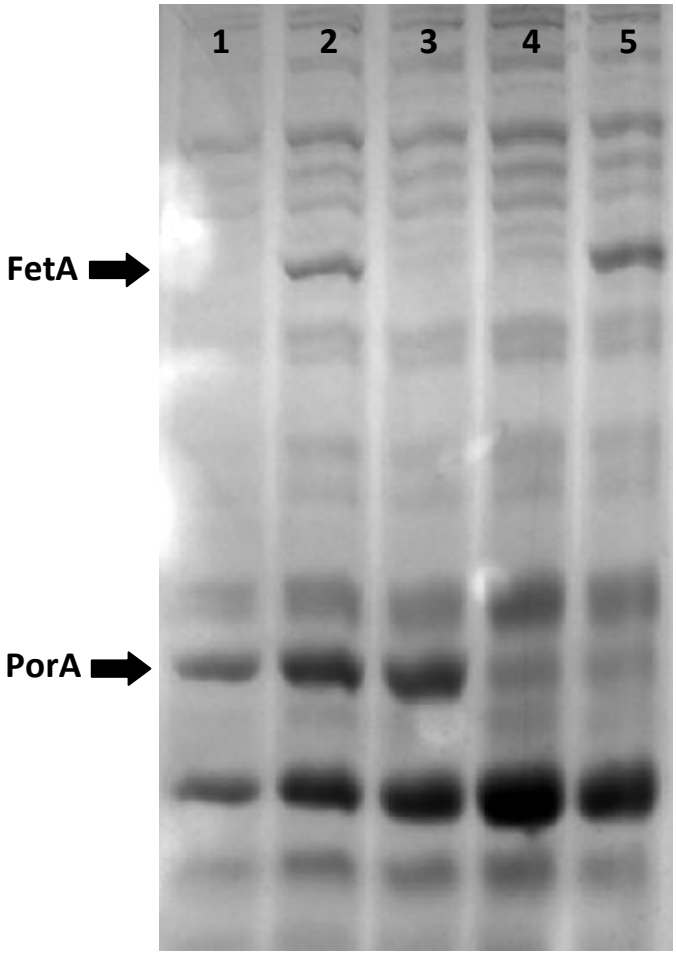


Figure 3.12: Coomassie-stained SDS-PAGE gel of outer membrane protein preparations from constructed strains. 50µg total protein was loaded per lane. Lanes: 1) H44/76 wildtype, 2) 3207 (FetA-on), 3) 3043 (FetA-off), 4) 3311 (FetA-off, PorA-off), 5) 3312 (FetA-on, PorA-off).

Strain 3311 (FetA-off/PorA-off) showed no expression of PorA or FetA, while 3312 (FetA-on/PorA-off) showed no expression of PorA, with FetA expression similar to that in the FetA-on strain (strain 3207). Both the FetA-off/PorA-off and FetA-on/PorA-off strains also showed increased expression of PorB compared to the strains expressing the PorA protein. However, this may be an artefact of gel loading based on total protein concentration.

3.2.6: Characterisation of Strains

3.2.6.1: Effect of *fetA*-modifications on transcription of downstream genes

It has been suggested that *fetA* may form the first open reading frame (ORF) in an operon containing genes for iron transport machinery [155]. A quantitative reverse transcription-PCR (qRT-PCR) was used to investigate whether transcription of the downstream open reading frames is altered by the upregulation of *fetA*. Strain 3043 (FetA-off), in which full transcription of the *fetA* open reading frame has been interrupted by insertion of a Kanamycin resistance marker [187], was also compared to the wildtype and FetA-on strains to indicate whether these genes are co-transcribed. The ORFs compared were *nmb1989*, the ORF immediately downstream of *fetA*; and *nmb1993*, potentially the last ORF in the operon (Figure 1.5, page 25). Total RNA was isolated from the wildtype, FetA-off and FetA-on strains following growth in MH and in MH supplemented with DFAM to chelate available iron. Total RNA was extracted from each strain on three separate days. Short (500-150bp) regions of *fetA, nmb1989* and *nmb1993* were amplified from copy-DNA (cDNA) using a one-step RNA-to-CT PCR reaction containing SYBR®-Green dye. cDNA was first synthesised from total messenger RNA (mRNA) before amplification of the cDNA of interest with specific primers. Primers used were: FetA663F, FetA734R; NMB1989F, NMB1989R; NMB1993F, NMB1993R. A region of the housekeeping gene *gdh* was amplified as a positive control from each RNA sample using primers gdh350F and gdh417R. Reactions were subsequently run on an acrylamide gel to verify

that the correct PCR product was present (Figure 3.13). As the incorporated SYBR-green dye releases fluorescence in the presence of double-stranded DNA, the amplification of the desired products was followed in real-time. The cycle number at which the quantity of amplified DNA resulted in a critical threshold of released fluorescence (the CT value) was determined. Relative Quantity (RQ) values for each gene were calculated from CT values relative to *gdh* and normalised to *fetA* RQ from one sample of wildtype grown in MH (Figure 3.14).

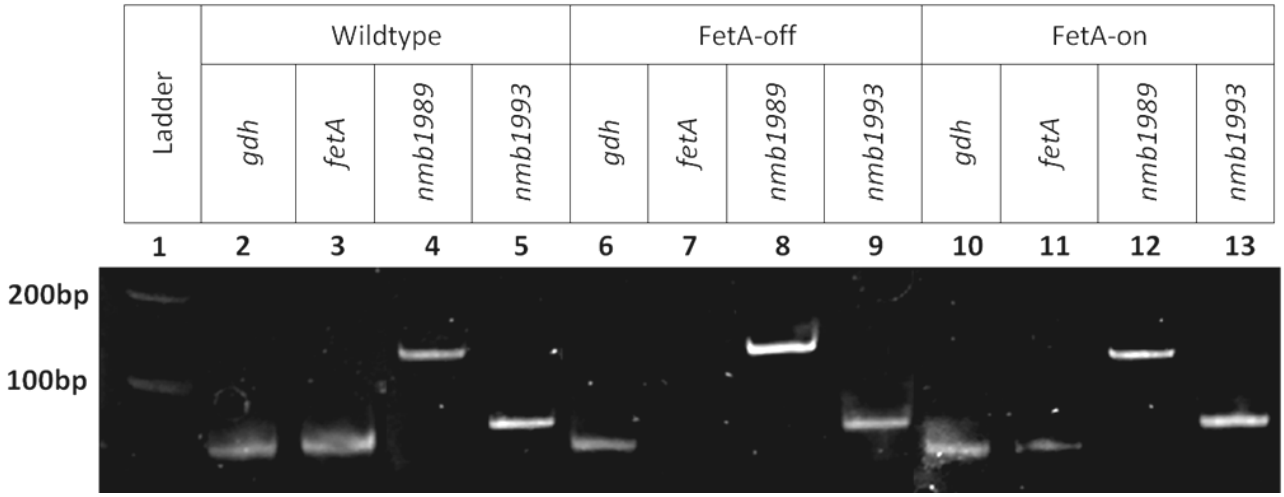


Figure 3.13: Acrylamide-gel electrophoresis for verification of PCR products following amplification of regions of *gdh* (69bp), *fetA* (72bp), *nmb1989* (132bp), *nmb1993* (73bp). Lanes: 1) DNA ladder, 2) Wildtype *gdh,* 3) Wildtype *fetA*, 4) Wildtype *nmb1989*, 5) Wildtype *nmb1993*, 6) FetA-off *gdh,* 7) FetA-off *fetA*, 8) FetA-off *nmb1989*, 9) FetA-off *nmb1993*, 10) FetA-on *gdh,* 11) FetA-on *fetA*, 12) FetA-on *nmb1989*, 13) FetA-on *nmb1993*.

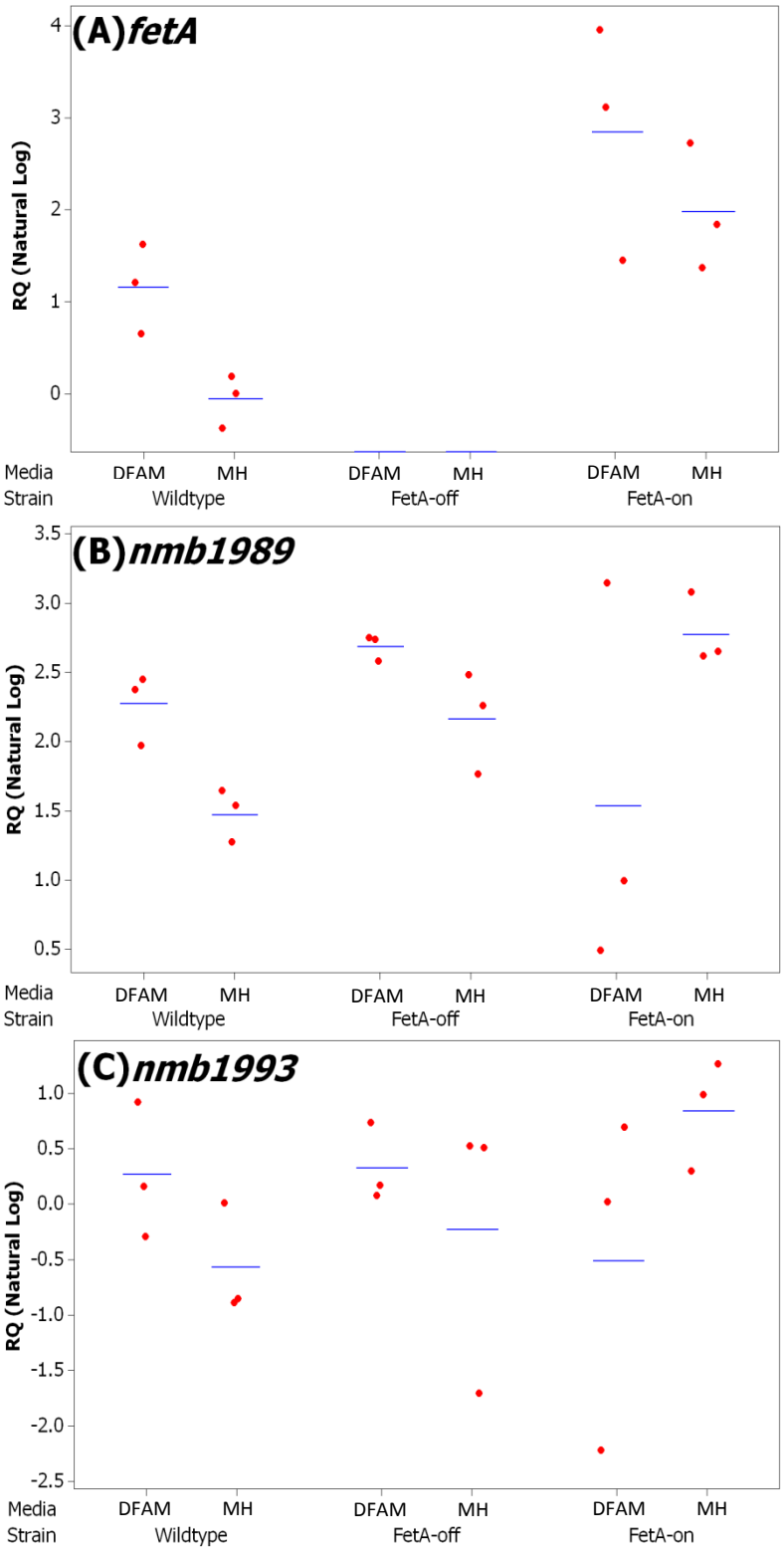


Figure 3.14: Relative Quantity (RQ) of RNA of three genes relative to *gdh* RNA in total RNA extracted following growth in MH or MH supplemented with DFAM. **(A)** *fetA,* **(B)** *nmb1989,* **(C)** *nmb1993*. Strains investigated were H44/76 (Wildtype); 3043 (FetA-off); and 3207 (FetA-on). Individual readings are shown in red, with mean values shown in blue.

In the wildtype strain, transcription of both *fetA* and *nmb1989* were repressed by the presence of iron (P = 0.033 and P = 0.024, respectively). The use of the DFAM in the growth media had no effect on transcription of *nmb1993* in any of the three strains (P = 0.978).

Transcription of *fetA* was not detected in the FetA-off strain. The primers used for amplifying the *fetA* cDNA are specific for a region at the 3' end of the gene, and so amplify cDNA downstream of the Kanamycin resistance cassette insertion. The lack of *fetA* expression in this strain is supported by electrophoresis of PCR products following qRT-PCR, as seen in Figure 3.12, where no band is present for the *fetA* PCR from 3043 cDNA. Transcription of *nmb1989* in the FetA-off strain was detected, but, with the low statistical power of this experiment, was not found to be significantly altered by iron availability (P = 0.141).

In the FetA-on strain, the native *fetA* promoter and Fur-binding regions have been removed and replaced with an alternative promoter. In this strain, transcript levels of *fetA* were both higher than in the wildtype grown in the presence of DFAM (P = 0.049), and were not significantly affected by the availability of iron (P = 0.379). Similarly, transcription of *nmb1989* was not significantly affected by iron availability in the FetA-on strain (P = 0.273), although variability between repeats was high. Transcript levels of *nmb1989* were not significantly higher in FetA-on compared to wildtype (P = 0.091).

This data does not support the theory the *fetA* is transcribed as part of an operon, as the high-strength promoter introduced upstream of *fetA* in the FetA-on strain would be expected to result in increased levels of mRNA coding for downstream co-transcribed genes as well as mRNA for the *fetA* gene itself. Similarly, in the FetA-off strain, the transcriptional terminator present in the insertion should prevent transcription of the targeted region of *fetA* and of any co-transcribed genes. Although no transcription of *fetA* was detected in this strain, transcription of the downstream genes *nmb1989* and *nmb1993* suggests that these genes are transcribed independently of *fetA*.

Conversely, the lack of iron-regulation of *nmb1989* seen in the FetA-on and FetA-off strains suggests that the iron regulated transcription of *nmb1989* in the wildtype strain may be at least partially controlled by the *fetA* promoter. This is consistent with data that the genes downstream of *fetA* in gonococci are expressed in two transcripts: one with *fetA* and one without [156]. The results seen in the FetA-on and FetA-off strains suggest that the majority of transcription is independent of *fetA,* but that iron-regulated expression is dominated by the *fetA* promoter.

3.2.6.2: Growth of strains with modified *fetA* expression

In order to determine whether modification of FetA expression had any effect on the growth of the meningococcus, the strains H44/76 (wildtype), 3043 (FetA-off) and 3207 (FetA-on) were resuspended from overnight growth on Columbia blood agar into 10mls MH or MH with DFAM. Strains were resuspended to an OD_{600nm} of 0.15-0.20, and the OD_{600nm} was then recorded after 3 hours growth and every subsequent hour for a further 8 hours (11 hours total growth). Each growth curve was run in triplicate on each day, and repeated on three separate days. The growth curves of the three strains are shown in Figure 3.15.

The OD_{600nm} reached after 3 hours was compared to indicate any differences in the length of the lag phase of growth. The growth rate during log phase (Time = 4 hours to Time = 8 hours), and the OD_{600nm} reached at stationary phase (at 11 hours growth) were also compared.

For all criteria tested there were found to be no significant differences between any strains following growth in MH or MH with DFAM ($P > 0.05$). It may be that mutant strains are capable of compensating for deficiencies in FetA expression. Alternatively, FetA may not be required for growth under the conditions used *in vitro*, although the protein may be required for growth under conditions *in vivo*.

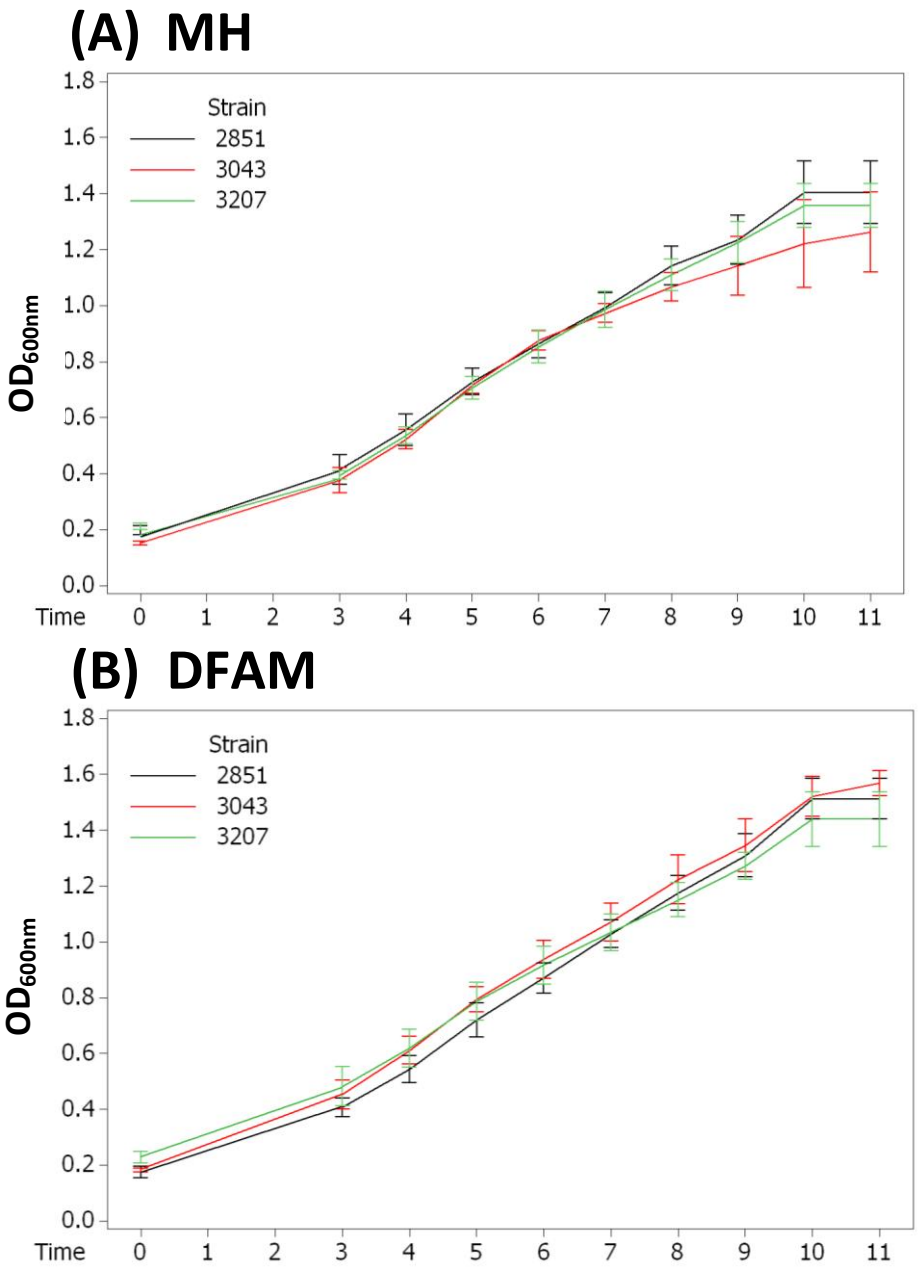


Figure 3.15: Comparison of growth of the wildtype (H44/76), FetA-off (3043) and FetA-on (3207) strains in **(A)** MH, and **(B)** iron MH supplemented with DFAM, over time (hours). Curves show the average OD_{600nm} at each time point for each strain. Error bars show 95% confidence intervals of the mean.

3.2.6.3: Effects of *fetA*- and *porA*-modifications on outer membrane protein complexes

As FetA is known to be involved in complexes with other outer membrane proteins such as PorA, the effect of altered FetA expression levels of these complexes was investigated using SDS-PAGE. Outer membrane protein samples were prepared from the strains H44/76 (wildtype), 3043 (FetA-off), 3207 (FetA-on), 3311 (FetA-off/PorA-off) and 3312 (FetA-on/PorA-

off) following growth in MH without DFAM. In strains 3043 and 3207, expression of *porA* has not been directly altered from the wildtype strain. Samples were then either denatured in sample buffer containing β-mercaptoethanol at 95°C for 5 minutes, or incubated in sample buffer without β-mercaptoethanol at 37°C for 20 minutes. The latter, non-denaturing treatment was used to maintain the proteins in the complexes present in the outer membrane. Samples were then run on an 8% Acrylamide SDS-PAGE gel. Results, shown in Figure 3.16, show that the wildtype sample subjected to the denaturing treatment does not contain many of the high-molecular weight protein bands seen in the samples that underwent the non-denaturing treatment. Therefore, these high molecular weight bands are likely to correspond to protein complexes.

PorA-deficient strains (3311 and 3312) show several differences in protein profile from strains expressing PorA at wildtype levels (H44/76, 3043 and 3207). Several bands (for example, bands labelled A, B, E and H), can be seen to be at a higher intensity in PorA-deficient strains, while other bands (bands labelled D and G) that are present in the wildtype strains are absent in 3311 and 3312. Bands at position C are also of a higher molecular weight than those seen in the wildtype. This is consistent with previous evidence that PorA is a major component of many OMP complexes [123,195]. When comparing the strains 3043 or 3207 with wildtype, there are fewer differences in protein profile. There are no visible differences between the protein profiles of 3043 (FetA-off) and 3207 strains (FetA-on). There are also no visible differences when comparing strain 3311 with 3312. As these strains have different phenotypes with respect to FetA, this suggests that modification of FetA expression has limited effects on OMP complexes.

Bands at position F in the wildtype are absent from all four modified strains. However, these bands are present in the wildtype denatured sample, and so may represent monomeric proteins rather than complexes.

Treatment	95°C	Non-denaturing				
Strain	H44/76	H44/76	3043	3207	3311	3312
FetA	WT	WT	-	+	-	+
PorA			+	+	-	-

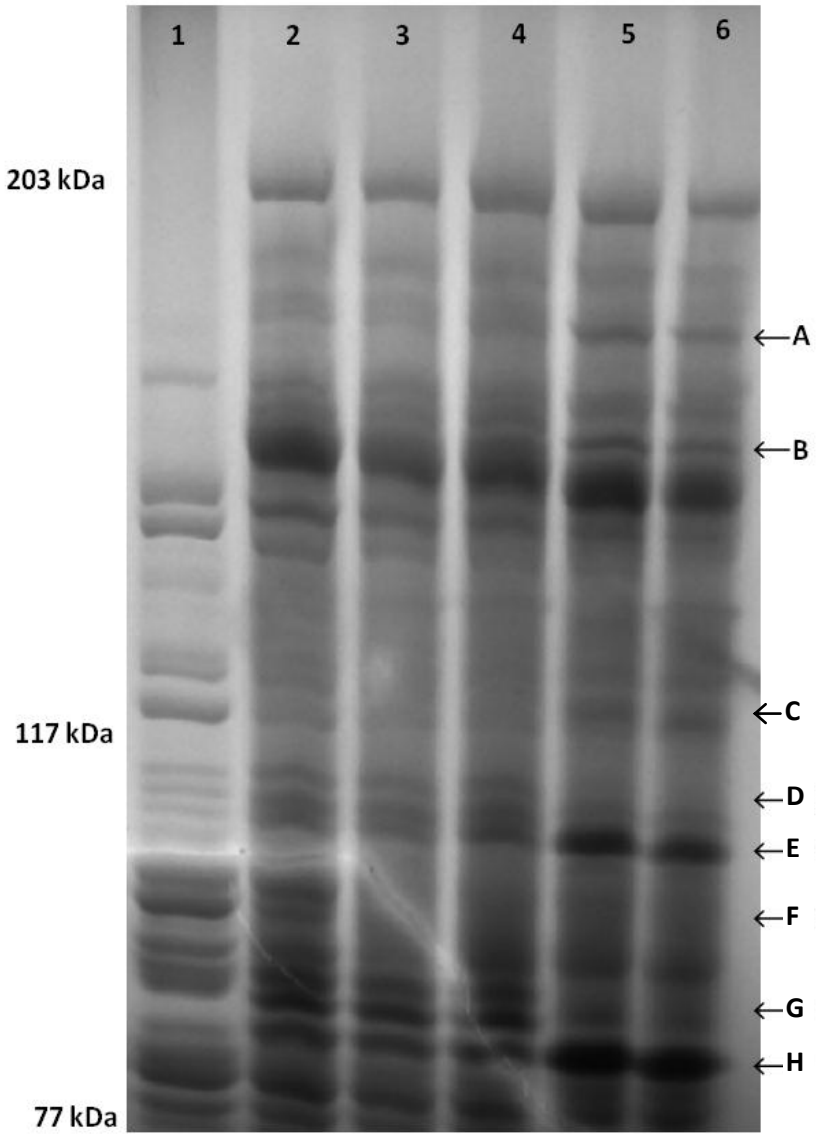


Figure 3.16: Comparison of protein profiles of modified strains on an 8% Acrylamide SDS-PAGE gel. Outer membrane protein samples were either denatured by boiling (95°C) with β-mercaptoethanol for five minutes, or incubated without β-mercaptoethanol at 37°C for 20 minutes (non-denaturing treatment). Lanes: 1) Wildtype (WT) following denaturing treatment, 2) Wildtype following non-denaturing treatment, 3) 3043 following non-denaturing treatment, 4) 3207 following non-denaturing treatment, 5) 3311 following non-denaturing treatment, 6) 3312 following non-denaturing treatment. Molecular weights of protein standards run concomitantly are shown at the left. Some bands of interest are indicated on the right.

3.2.6.4: Antibiotic susceptibility of modified strains

Expression of gonococcal FetA in *E. coli* has been found to lead to an increased susceptibility to hydrophobic antibiotics [157]. Therefore, in order to investigate whether increased expression of FetA in meningococci has any effects on cellular integrity or permeability, the susceptibility of H44/76 (wildtype), 3043 (FetA-off) and 3207 (FetA-on) to four antibiotics was compared.

The antibiotics chosen for the susceptibility tests are those that evidence suggests have a role in altering membrane permeability in bacteria, or those that require particular levels of membrane permeability for cellular uptake. If the modification of *fetA* regulation had any effect on membrane integrity, for example by increasing the number of outer membrane pores, modified strains would be expected to have an increased or decreased susceptibility to these antibiotics. The following antibiotics were compared:

- Erythromycin,
- Rifampicin,
- Vancomycin,
- Polymyxin B.

Polymyxin B interacts with the anionic LPS in bacterial membranes, displacing the ions that link the LPS and destabilising the membrane. Polymyxin B itself can then be inserted into the membrane, increasing permeability and creating “cracks” through which a variety of molecules can pass, eventually leading to the death of the cell [196].

Vancomycin inhibits the latter stages of peptidoglycan cell wall synthesis [197]. Although Gram-positive bacteria are susceptible to vancomycin, most Gram-negative bacteria are resistant, as the high molecular weight, hydrophilic molecules cannot pass through the pores in the Gram-negative outer membrane [198]. Mutations that results in larger or unstable pores in the membrane would, therefore, be expected to increase susceptibility of meningococci to this antibiotic.

Rifampicin and Erythromycin are both hydrophobic antibiotics. Rifampicin acts by binding to and inhibiting RNA polymerase [199], while Erythromycin promotes dissociation of peptidyl tRNA from the ribosome [200]. As both are lipophilic molecules, uptake occurs largely via diffusion through the lipid bilayer of the outer membrane, rather than through pores. Therefore, changes in membrane permeability, for example through changes in lipid composition, can increase or decrease sensitivity to these antibiotics [198].

Results, as seen in Figure 3.17, show the diameters measured for the zone of clearance around each antibiotic disc. The mean diameters for the different strains are similar for each antibiotic. There was no significant difference between the susceptibility of each strain to the four antibiotics tested (P = 0.569). These results suggest that removal or upregulation of FetA expression has no effect on membrane stability or pore size.

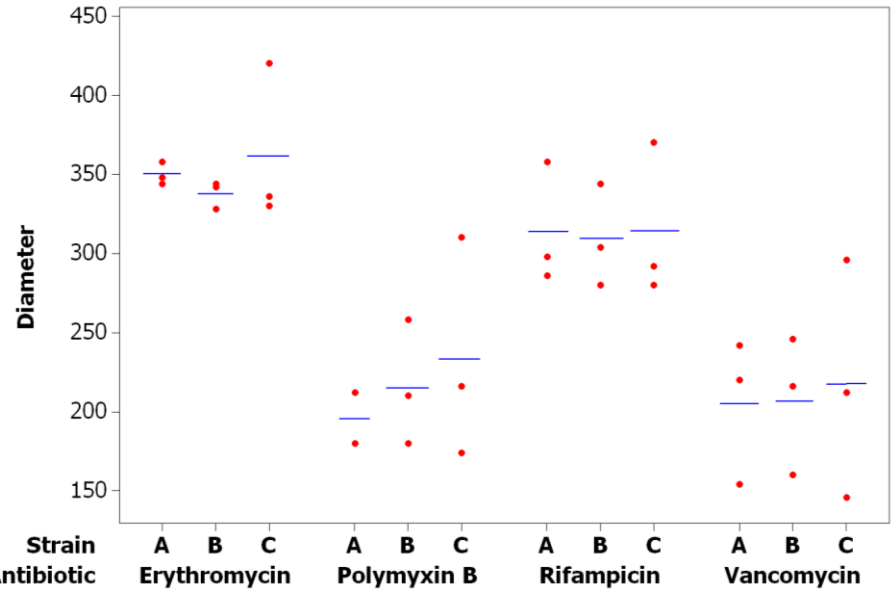


Figure 3.17: Comparison of susceptibility of strains to four antibiotics. Susceptibility was measured on three separate days. Individual measurements of the diameter of the zone of clearance surrounding the antibiotic discs are plotted in red. Average values for each strain are plotted in blue. Strains: A) Wildtype, B) FetA-off (3043), C) FetA-on (3207).

3.3: Discussion

To overcome the regulation of FetA by iron, in order to produce an OMV vaccine containing sufficient levels of this antigen, meningococcal strains were modified to constitutively express the FetA protein. As the vaccine MenPF will be based on both the FetA and PorA antigens, and PorA is known to be immunodominant in the meningococcal outer membrane [201], other strains were also constructed that could be used to differentiate the immune response to these individual antigens, as well as to other meningococcal antigens. This completed a panel of isogenic strains, containing PorA and FetA switched off and on in various combinations, which would enable serological evaluation of the separate contributions of antibodies against PorA and FetA to the bactericidal response.

Replacement of the promoter region upstream of *fetA,* and removal of the Fur-binding sequences, resulted in expression of FetA at high levels compared to those seen in the wildtype strain. This expression was independent of iron availability and growth media. Furthermore, alteration of *fetA* expression was found to have no effect on growth of the meningococcus and to be stable over ten passages without antibiotic selection (data not shown). These data suggest that the modification has no detrimental effect on the meningococcus *in vitro*. This is important for strains intended for production of OMV vaccines, as the strains needs to grow well under manufacturing conditions. Therefore, for production of OMVs containing consistent levels of FetA, it is likely that the constructed strain can be used with existing manufacturing protocols and specifications for production of MenBvac from the wildtype H44/76 strain.

Protection afforded by OMVs is likely to involve antibodies to major surface antigens such as PorA and FetA, as well as antibodies to more conserved minor antigens. These antibodies can be difficult to detect individually, and are unlikely to be bactericidal alone, but may act synergistically with antibodies to major proteins to improve overall protection [131].

Therefore, modifications that also affect expression or surface exposure of a large number of other surface proteins may be detrimental to the overall cross-reactivity of the final vaccine. This effect could be positive or negative; however, as OMVs from wildtype strains have been used successfully for vaccination against epidemic meningococcal disease, mutant strains intended for use in OMV vaccine production should ideally remain consistent with wildtype strains in all respects other than the intentionally-modified vaccine antigens. Apart from the upregulation of FetA, there were minimal other differences between the OMV protein composition of the FetA-on strain and the wildtype. There were also minimal differences in transcription of the genes downstream of *fetA*, as transcription of these genes may be largely independent of *fetA* [156]. Therefore, the effect of the modification in the FetA-on strain on the open reading frames downstream of *fetA* should not affect the immune response induced against OMVs from that strain.

Expression of gonococcal FetA in *E. coli* has been found to lead to an increased susceptibility to hydrophobic antibiotics, such as erythromycin and rifampicin. This was hypothesised to be because FetA expression in *E. coli* may either be involved in forming pores or in some other way affecting the integrity of the outer membrane [157]. However, neither upregulation nor removal of FetA expression had a significant effect on membrane stability or permeability in meningococci, including intrinsic permeability and permeability due to the presence or size of outer membrane pores. Although FetA has been identified in high molecular weight complexes with other outer membrane proteins including PorA and PorB [195], previous analysis of outer membrane complexes in a *fetA* knockout strain by blue-native PAGE showed no differences from the wildtype strain [123]. This is in contrast to the modifications seen in the HexaMen and NonaMen-production strains where expression of PorB and RmpM have been removed [121]. Removal of either of these proteins has been shown to have considerable effects on OMP complexes [123], and so may influence immune responses to minor protein antigens.

When OMP complexes of the modified strains in this study were compared, removal or increase of FetA expression had limited effect on visible complexes. This is similar to the results reported following blue-native PAGE electrophoresis of meningococcal protein complexes [123]. In contrast, removal of PorA expression had a large effect on OMP complexes. Removal of PorA expression has been shown previously to lead to increased expression of PorB and significant changes in many high-molecular weight complexes [123]. Analysis of the OMP profiles of the PorA-off strains also show increased expression of PorB compared to wildtype. Therefore, although the strain panel produced for serological evaluation may be useful for separating the effects of antibodies against PorA and FetA, the deletion of PorA may indirectly affect the SBA results obtained when 3311 and 3312 are used as target strains through altering the surface exposure of other antigens. This could be overcome using further strain production to include a PorA variant lacking both variable regions, while retaining the rest of the protein, as this will remove most of the effects of PorA antibodies without the same impact on the OMP complexes.

The proposed vaccine MenPF will also require additional strains to be constructed which express alternative FetA variants at high levels. However, during the cloning strategy used in this study, replication of the *fetA* gene preceded by the modified promoter in *E. coli* resulted in selection for a frame-shift mutation at the 5' end of *fetA*. Previous attempts to express meningococcal or gonococcal FetA in *E. coli* have also had difficulties [149,155], and it was found that the protein could only be expressed under an inducible promoter in inclusion bodies [202], or in a low-copy number plasmid [157]. Kortekaas *et al.,* [170] tried a similar method to constitutively express meningococcal FetA. This method first involved inserting the coding region of the *fetA* gene into a plasmid in *E. coli*, preceded by a *porA* signal peptide and promoter sequence. The authors did not report any difficulties when amplifying this plasmid in *E. coli*, unlike those that have been found with a similar construct in this study. This construct was then successfully transformed into H44/76, resulting in strain CE1528, which showed

constitutive expression of FetA. However, the SDS-PAGE gel analysis shown comparing OMVs from the wildtype strain and CE1528 suggests that levels of FetA in the modified strain are less than the levels seen in the wildtype grown in the presence of an iron chelator, as seen in strain 3200 from this study. In order to give high levels of FetA expression, the authors used a Neisserial replicative plasmid, containing a region encoding the *fetA* gene controlled by an IPTG-inducible *lac* promoter. [170]

It may be that Kortekaas *et al.,* [170] have encountered similar problems during their cloning, although sequence data was not reported. Another possibility is that the *porA* promoter used had undergone slipped-strand mispairing to a less optimal promoter. This may have reduced expression to levels tolerated by *E. coli*, but also led to reduced expression levels in meningococci. As higher expression levels were only obtained using an IPTG-inducible promoter, this method would not be ideal for construction of the strains required by this study. Consequently, development of the additional strains required for MenPF will need to involve several recombination events with multiple plasmids that do not contain the complete *fetA* gene and the constitutive promoter within the same plasmid.

For the purposes of this study, a meningococcal strain, 3207, has been constructed that expresses the FetA protein at increased levels regardless of iron availability or growth medium. With the exception of FetA expression, growth and protein profiles of the modified strain are highly similar to the wildtype. Characterisation of 3207 using various methods provides no evidence that the modification used will have any detrimental effect on the potential use of this strain to produce a meningococcal OMV vaccine.

Chapter 4:

Investigation of a prime-boost strategy to improve *in vivo* bactericidal responses against PorA

4.1: Introduction

In the developmental meningococcal vaccine MenPF, the antigens PorA and FetA will be presented in OMVs. The aim of this study was to investigate whether the ability of OMVs to induce an effective, targeted immune response to PorA could be improved using a prime-boost strategy involving both OMVs and recombinant protein inoculums. Such a strategy may reduce both the level of antibodies produced to ineffective antigens, and the number of doses of OMV required for protection against meningococcal disease.

The efficacy of a meningococcal vaccine is determined by measuring the serum bactericidal activity of post-vaccination sera. As described previously (see 1.1.3, page 5), SBA is currently considered the most effective correlate of protection for meningococcal disease [45]. This assay measures the ability of antibodies in sera to elicit complement-mediated killing of meningococci. As such, there are several factors that are important in determining whether an antibody response will be bactericidal. For example, inducing the production of sufficient quantities of antibody is a key factor in vaccine efficacy [203]. The complement-binding ability of the various IgG subclasses also varies [204]; therefore a vaccine must induce the correct IgG profile for bactericidal killing (IgG2 in mice [205], IgG1 and IgG3 in humans [206]). However, many antigens are not associated with bactericidal killing. Consequently, antibodies must also be specific for appropriate antigens, the binding of which results in effective formation of membrane attack complexes for meningococcal killing.

OMV vaccines used against epidemic strains of *Neisseria meningitidis* serogroup B in Norway, Cuba and New Zealand have been shown to be protective [32,207,208], inducing bactericidal

antibodies against PorA. This is, in part, due to the inherent adjuvant properties of OMVs (described in 1.3.3, page 15), resulting in the appropriate IgG subclasses for complement binding [101]. Furthermore, presentation of proteins in OMVs retains those proteins in their native conformation, exposing only the surface epitopes of each antigen; therefore, antibodies induced are capable of binding live meningococci. However, three or four doses of OMV are required to induce a bactericidal antibody response [94]. Furthermore, as OMVs are complex molecules containing many different antigens, antibodies are often formed to protein antigens that do not induce killing in SBA, such as the RmpM protein [209]. Antibodies to non-bactericidal epitopes in OMVs can dominate the immune response and reduce the potential efficacy [96], resulting in the need for more doses to induce protection. This increases the cost of vaccination, and, when used in infants, means that by the time the last dose has been administered the child has passed that age at which susceptibility to disease is highest.

In comparison to OMVs, recombinant protein vaccines induce antibodies that are specific for the antigen used but are poorly bactericidal unless additional adjuvants or alternative formulations are used. Additional adjuvants are required to induce switching to complement-binding subclasses, which are required for bactericidal activity [101]. Immunisation with recombinant proteins also induces antibodies against hidden epitopes. These antibodies are unlikely to be bactericidal as they do not bind the surface of the bacteria [210].

For meningococcal vaccines, the use of a prime-boost strategy may improve immune responses to targeted antigens within an OMV. A prime-boost strategy can be defined as one involving sequential administration of vaccines that use different antigen delivery systems, the effects of which can be additive or synergistic [211]. Heterologous doses can be used to induce the level and type of immune response most appropriate for the targeted pathogen. For example, heterologous prime-boost strategies have been investigated for use against pathogens such as Human Immunodeficiency Virus [212] and Malaria [213], where both cellular and humoral immunity are required for effective protection.

For the meningococcal PorA antigen, a recombinant protein dose could effectively target the immune response to the desired antigen while the adjuvant effects of OMVs could improve antibody concentrations to surface epitopes and induce the correct IgG profile required for bactericidal killing. The aim of this study was to determine, using OMV and recombinant protein inoculums, whether a prime-boost schedule involving only two antigenic doses could elicit a greater bactericidal response against PorA than the use of OMV alone.

4.2: Results

4.2.1: Immunisation Strategies

Groups of ten female NIH mice were immunised on days 0 and 21 with either OMV (1µg total protein) or recombinant PorA P1.7-2,4 protein (10µg). OMVs used were produced from strain NZ98/254 expressing P1.7-2,4 (Appendix 1, page 218). OMVs expressing this PorA variant were used as P1.7-2,4 has been found to be relatively weak at inducing bactericidal antibodies in OMVs compared to other serosubtypes [122]. Groups 1 to 10 were immunised with the schedules shown in Table 4.1. Terminal bleeds were taken on day 21 or on day 35.

The strategies were tested with aluminium hydroxide ($Al(OH)_3$), an adjuvant commonly used for OMV vaccines. As much of the difference in protection induced by OMVs compared to recombinant protein antigen presentation forms has been attributed to the presence of the TLR4 ligand Lipid-A in the meningococcal outer membrane [214], an adjuvant containing the Lipid-A mimetic Monophosphoryl Lipid A (MPL) was also tested as a method to improve the immunogenicity of the antigen when presented as a recombinant protein.

Group	Day 0	Day 21	Day 35
1	Adjuvant	OMV	Terminal Bleed
2	OMV	Terminal Bleed	
3	OMV	Adjuvant	Terminal Bleed
4	OMV	OMV	Terminal Bleed
5	OMV	Protein	Terminal Bleed
6	Adjuvant	Protein	Terminal Bleed
7	Protein	Terminal Bleed	
8	Protein	Adjuvant	Terminal Bleed
9	Protein	OMV	Terminal Bleed
10	Protein	Protein	Terminal Bleed

Table 4.1: Immunisation schedules used. Groups of 10 mice were immunised on the days shown with the following: outer membrane vesicle (OMV), recombinant PorA protein (Protein) or adjuvant alone. Inoculums were formulated with either $Al(OH)_3$ or MPL adjuvant.

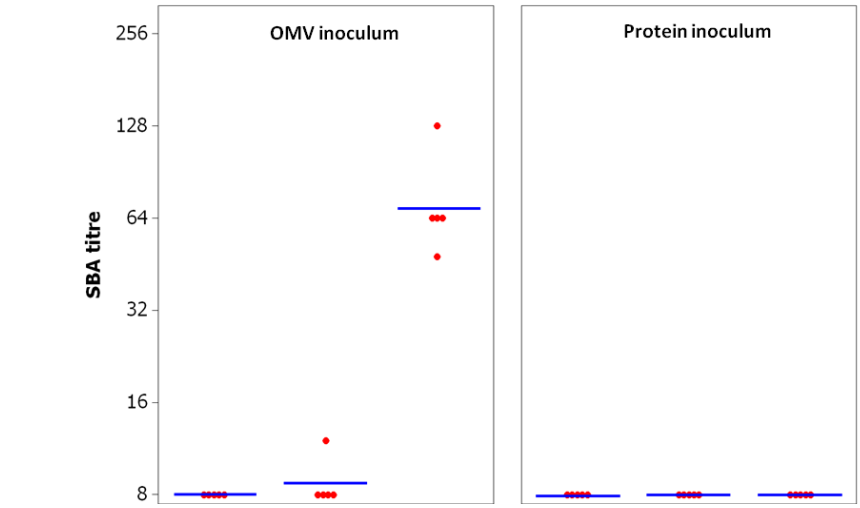
4.2.2: Comparison of serum bactericidal titres

To determine whether the immunisation strategies used induced functional antibodies capable of killing live meningococci, pools of paired mouse sera from each group were used in serum bactericidal assays (SBAs) with baby rabbit complement (Pel-Freez Biologicals, Batch 00528). The target strain for all groups was NIBSC strain 91/40, a serogroup B strain belonging to the same clonal complex as the OMV-parent strain (NZ98/254, ST-41/44 complex) and expressing the homologous PorA variant. This strain was used as it was found to be compatible with the baby rabbit complement available. All sera were used at a starting dilution of 1:8.

4.2.2.1: $Al(OH)_3$ adjuvant:

The first groups compared were those given only a single dose of antigen, either as OMV or recombinant protein (groups 1-3 and 6-8 as listed in Table 4.1). These groups only differ in the presentation of the antigen (OMV or recombinant protein) and the number of days between immunisation and serum collection (14, 21 or 35 days).

Sera from mice given protein inoculum (groups 6, 7 and 8) showed no detectable bactericidal activity (SBA titres < 8, Figure 4.1). When mice were given OMV inoculum, there was a general increase in SBA titres over time. Sera from mice given OMV inoculum 14 days before bleeding (group 1) showed no detectable bactericidal activity. Sera from mice given a single dose of OMV inoculum 21 days before bleeding (group 2) also showed limited bactericidal activity, with a single paired serum pool showing SBA titres >8. However, sera from mice given a primary dose of OMV 35 days prior to bleeding (group 3) gave a mean bactericidal activity of 64. This was significantly higher than SBA titres from groups 1 and 2 ($P < 0.001$). These results indicate that, using OMV inoculum with $Al(OH)_3$ adjuvant, more than 21 days are required for a bactericidal response to develop.

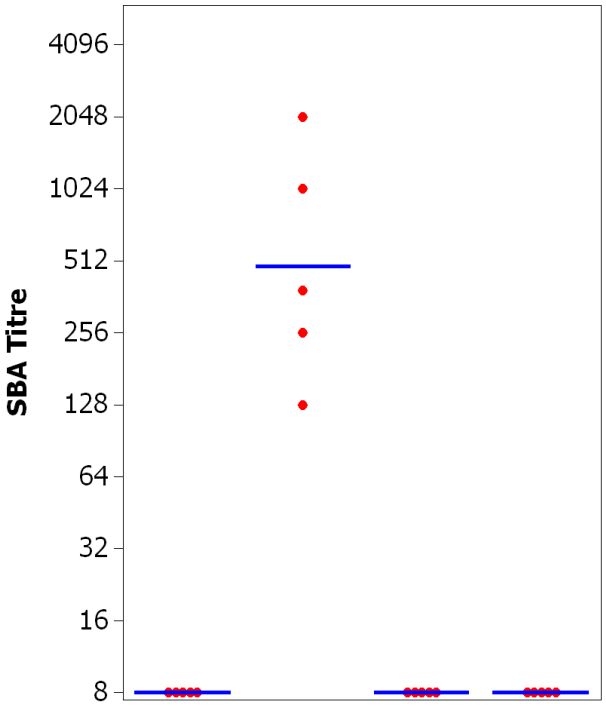


Group:	1	2	3	6	7	8
Prime (Day 0):	Adjuvant	OMV	OMV	Adjuvant	Protein	Protein
Boost (Day 21):	OMV	[Bleed]	Adjuvant	Protein	[Bleed]	Adjuvant
Time between antigen dose and bleed (days):	14	21	35	14	21	35

Figure 4.1: SBA titres obtained for sera from mice given a single dose of antigen formulated with $Al(OH)_3$ adjuvant. The antigen (PorA P1.7-2,4) was presented either as outer membrane vesicles (OMV) or as recombinant protein. Values for pools of paired mouse sera are shown in red, with mean values for each group shown in blue.

Sera from mice given two doses of antigen were then compared (groups 4, 5, 9 and 10). Serum bactericidal activity was only detected in serum from mice given a primary dose of OMV followed by a boosting dose of recombinant protein (group 5, Figure 4.2, Geometric mean SBA titre = 512). SBA titres in this group were significantly higher than those in mice given only a prime of OMV (group 3), indicating that the protein secondary dose improved the bactericidal activity. On the other hand, sera from mice given two doses of OMV (group 4) did not result in detectable SBA titres, suggesting that a secondary dose of OMV inoculum was detrimental to the bactericidal response.

Sera from mice give a primary dose of antigen as recombinant protein (groups 9 and 10) did not show any detectable bactericidal activity.



Group:	4	5	9	10
Prime (Day 0):	OMV	OMV	Protein	Protein
Boost (Day 21):	OMV	Protein	OMV	Protein

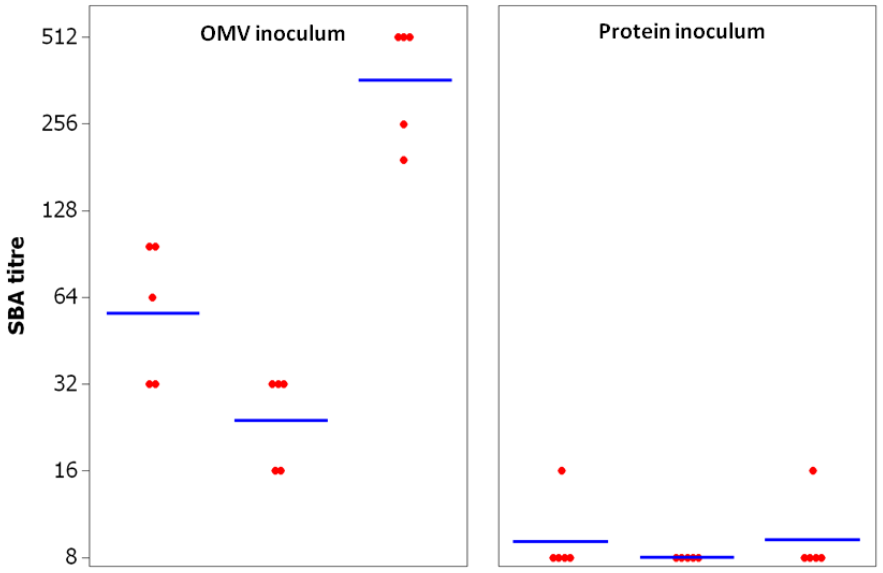
Figure 4.2: SBA titres obtained for sera from mice given two doses of antigen formulated with $Al(OH)_3$ adjuvant. Antigen was presented either as outer membrane vesicles (OMV) or as recombinant PorA protein. Values for pools of paired mouse sera are shown in red, with mean values for each group shown in blue.

4.2.2.2: MPL adjuvant

As with $Al(OH)_3$ adjuvant, with MPL adjuvant immunisation of mice with a single dose of protein (groups 6, 7 and 8) resulted in very low levels of bactericidal activity (Figure 4.3). A

single pool from two of these groups (groups 6 and 8) were found to have SBA titres >8; however there was no significant difference between the three schedules (P = 0.619).

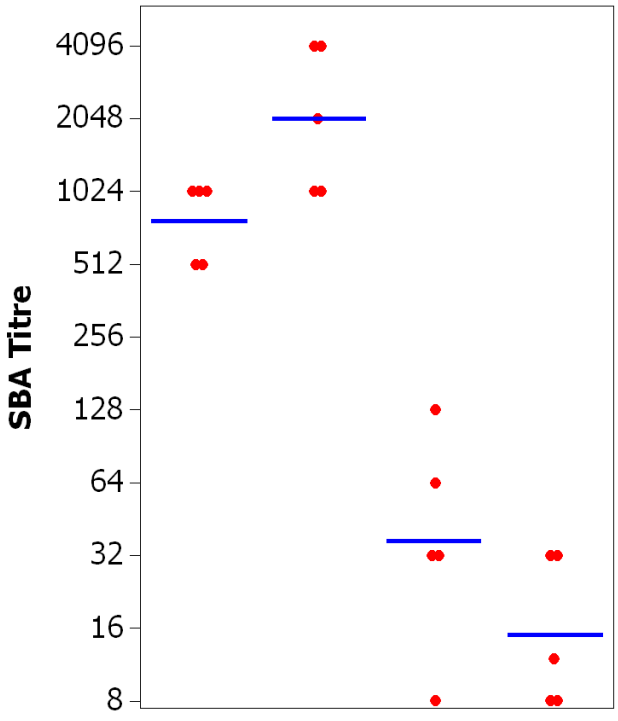
A single dose of OMV inoculum with MPL adjuvant resulted in high SBA titres, although, unlike with $Al(OH)_3$ adjuvant, this response did not increase steadily over time. A single dose of OMV 35 days prior to bleeding (group 3) showed significantly higher bactericidal activity than doses 14 or 21 days prior to bleeding (groups 1 and 2, P < 0.001). However, SBA titres seen following a single dose of OMV 14 days prior to bleeding (group 1) were significantly higher than SBA titres obtained when OMVs were given 21 days prior to bleeding (group 2) (Geometric mean SBA titre = 64 vs. 25, P = 0.025). These results suggest that, with MPL adjuvant, there is an initial bactericidal response, not seen with $Al(OH)_3$ adjuvant, that wanes after 14 days post-vaccination but develops further after 21 days post-vaccination.



Group:	1	2	3	6	7	8
Prime (Day 0):	Adjuvant	OMV	OMV	Adjuvant	Protein	Protein
Boost (Day 21):	OMV	[Bleed]	Adjuvant	Protein	[Bleed]	Adjuvant
Time between antigen dose and bleed (days):	14	21	35	14	21	35

Figure 4.3: SBA titres obtained for sera from mice given a single dose of antigen formulated with MPL adjuvant. The antigen (PorA P1.7-2,4) was presented either as outer membrane vesicles (OMV) or as recombinant protein. Values for pools of paired mouse sera are shown in red, with mean values for each group shown in blue.

When serum from groups of mice given two doses of antigen formulated with MPL adjuvant were compared (groups 4, 5, 9 and 10, Figure 4.4), all schedules were found to result in detectable bactericidal activity. The use of a primary dose of OMV (groups 4 and 5) was found to result in significantly higher SBA titres than the use of a recombinant protein primary dose (groups 9 and 10, $P < 0.001$) indicating that the primary response initiated by an OMV inoculum contributes to high bactericidal activity. Bactericidal activity was higher following a secondary dose of protein (group 5) after an OMV prime compared to a secondary dose of OMV (group 4, Geometric mean SBA titre = 2048 vs. 768, $P = 0.033$), while both groups showed significantly higher SBA titres than mice given only a primary dose of OMV (group 3, $P < 0.05$). These results show that the secondary dose increases the bactericidal antibody response, and that the use of a secondary dose of protein increases SBA titres further than a secondary dose of OMV.



Group:	4	5	9	10
Prime (Day 0):	OMV	OMV	Protein	Protein
Boost (Day 21):	OMV	Protein	OMV	Protein

Figure 4.4: SBA titres obtained for sera from mice given two doses of antigen with MPL adjuvant. Antigen was presented either as outer membrane vesicles (OMV) or as recombinant PorA protein. Values for pools of paired mouse sera are shown in red, with mean values for each group shown in blue.

Although the use of an OMV secondary dose following a protein prime (group 9) resulted in higher mean SBA titres than the use of two doses of protein (group 10, Geometric mean SBA titre = 32 vs. 16), this difference was not significant (P = 0.155).

When comparing the two adjuvants tested, the use of MPL adjuvant resulted in significantly higher SBA titres in all groups than the use of $Al(OH)_3$ adjuvant (P < 0.001).

4.2.3: Comparison of total IgG in sera by whole-cell enzyme-linked immunosorbent assay

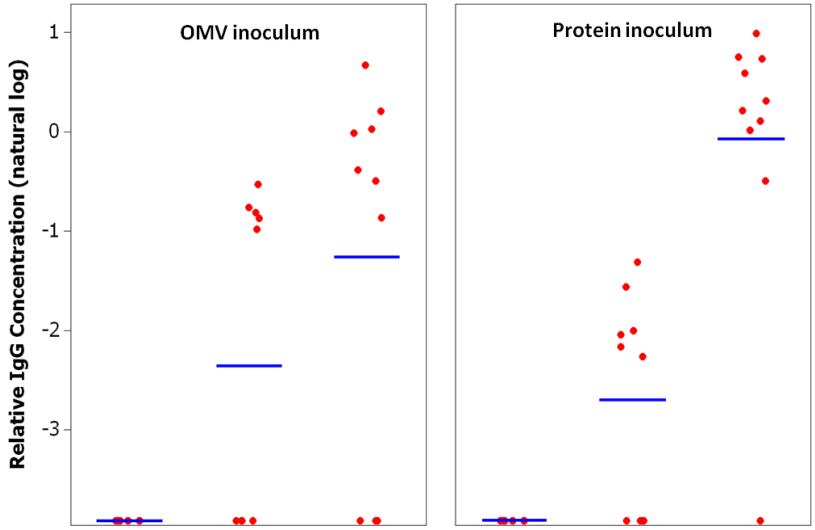
Bactericidal killing in SBAs is dependent on the presence of sufficient concentrations of antibodies capable of binding live bacteria [203]. Therefore, in order to determine why SBA titres varied between groups, the relative concentrations of IgG in the mouse sera capable of binding meningococcal whole cells was measured by ELISA. Total IgG was quantified against whole cells of the OMV-parent strain, NZ98/254. Titres were calculated relative to a PorA P1.4 monoclonal antibody standard.

4.2.3.1: $Al(OH)_3$ adjuvant

Of the groups given only a single dose of OMV (groups 1-3), sera showed increasing IgG titres over time (Figure 4.5). Sera from mice given OMV 14 days prior to bleeding showed no detectable IgG, while sera from mice given OMV 35 days prior to bleeding gave the highest IgG titres of the three groups. This is similar to the trend seen with SBA titres, which also increased over time since inoculation.

Groups given a single dose of protein (groups 6-8) also showed an increase in IgG over time. Sera from mice given a dose of protein 35 days prior to bleeding (groups 8) contained significantly higher IgG titres than sera from mice given a dose of protein 21 days prior to bleeding (group 7, P < 0.001). A booster dose should, ideally, be administered after the primary serum IgG response has waned [215]. Therefore, as the antibody response to all antigens was

still increasing at day 21 when the second dose was given, this would not have resulted in a true booster response.



Group:	1	2	3	6	7	8
Prime (Day 0):	Adjuvant	OMV	OMV	Adjuvant	Protein	Protein
Boost (Day 21):	OMV	[Bleed]	Adjuvant	Protein	[Bleed]	Adjuvant
Time between antigen dose and bleed (days):	14	21	35	14	21	35
Mean SBA titre:	<8	9	69	<8	<8	<8

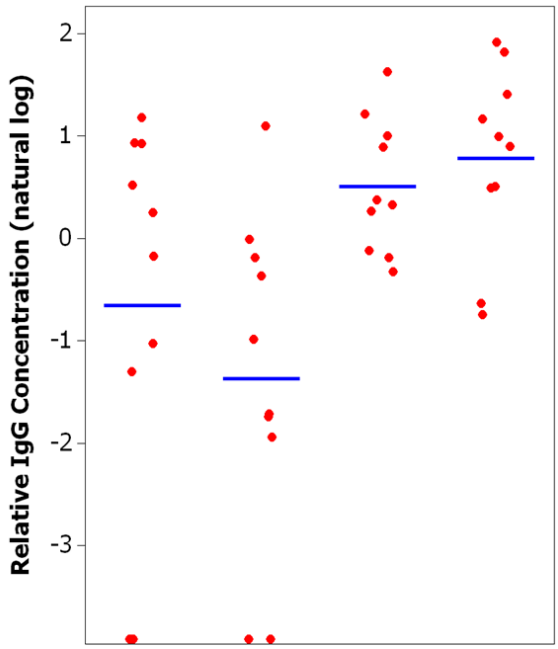
Figure 4.5: Relative IgG titres obtained for sera from one dose of antigen given with $Al(OH)_3$ adjuvant. Antigen was presented either as outer membrane vesicles (OMV) or as recombinant PorA protein. Values for sera from individual mice are shown in red, with mean values for each group shown in blue.

Although IgG responses were detected 21 and 35 days after protein immunisation (groups 7 and 8), no bactericidal activity was detected in any groups given a primary dose of protein. Therefore, the antibodies induced by protein with $Al(OH)_3$ adjuvant are not capable of complement-mediated killing.

Groups give a primary dose of protein followed by either OMV or protein (groups 9 and 10) showed high IgG titres (Figure 4.6) and no detectable SBA titres, consistent with the

observation that antibodies induced by a primary dose of protein do not contribute to bactericidal killing.

Following an OMV primary dose, IgG titres were similar between groups given a secondary dose of OMV or protein (groups 4 and 5). There was also no significant difference in IgG titres between these groups and those given only a primary dose of OMV (group 3, $P > 0.05$). Therefore, the use of a secondary dose following a primary dose of OMV does not affect the magnitude of the IgG response. This contradicts SBA data, where the use of a secondary dose of OMV following an OMV prime had a large, detrimental effect on bactericidal activity.



Group:	4	5	9	10
Prime (Day 0):	OMV	OMV	Protein	Protein
Boost (Day 21):	OMV	Protein	OMV	Protein
Mean SBA titre:	9	484	<8	<8

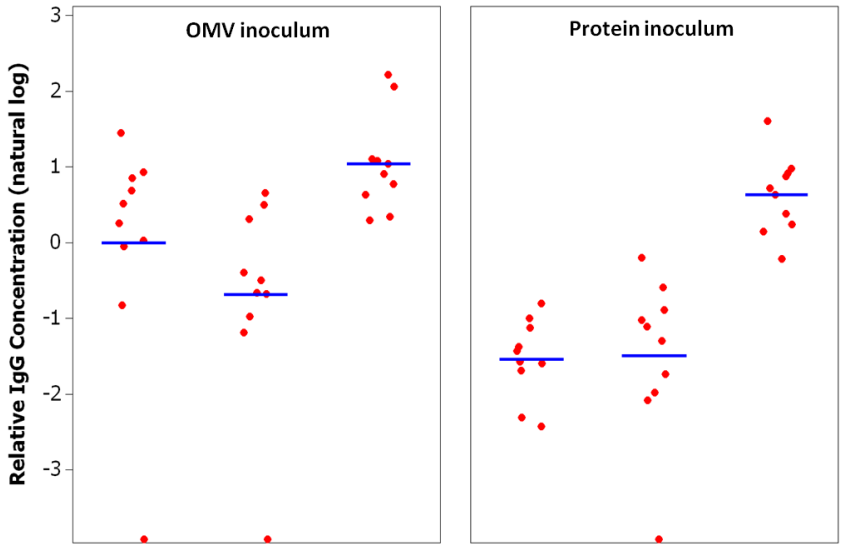
Figure 4.6: Relative IgG titres obtained for sera from two doses of antigen given with $Al(OH)_3$ adjuvant. Antigen was presented either as outer membrane vesicles (OMV) or as recombinant PorA protein. Values for sera from individual mice are shown in red, with mean values for each group shown in blue.

4.2.3.2: MPL adjuvant

When MPL adjuvant was used, IgG was detected in all groups. When a single dose of OMV inoculum was given, serum IgG titres were highest when the dose was administered 35 days prior to bleeding (group 3, Figure 4.7) and lowest when the dose was administered 21 days

prior to bleeding (group 2). Therefore, as seen with the SBA titres in these groups, there is a primary IgG response that wanes after 14 days and then increases to peak after 21 days.

When a single dose of protein inoculum was used, IgG titres increased over time. Titres were highest 35 days after the antigenic dose (group 8), while IgG titres were similar at 14 and 21 days (groups 6 and 7). However, SBA titres for these groups were very low, indicating that the antibodies measured are not capable of bactericidal activity.

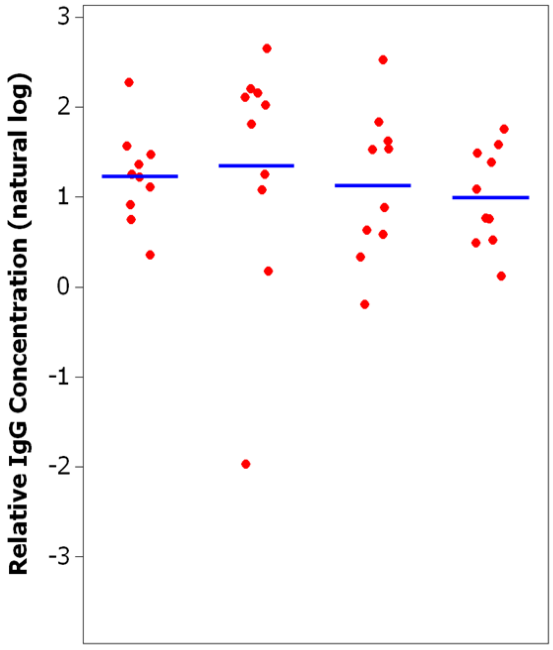


Group:	1	2	3	6	7	8
Prime (Day 0):	Adjuvant	OMV	OMV	Adjuvant	Protein	Protein
Boost (Day 21):	OMV	[Bleed]	Adjuvant	Protein	[Bleed]	Adjuvant
Time between antigen dose and bleed (days):	14	21	35	14	21	35
Mean SBA titre:	57	24	366	9	<8	9

Figure 4.7: Relative IgG titres obtained for sera from one dose of antigen given with MPL adjuvant. Antigen was presented either as outer membrane vesicles (OMV) or as recombinant PorA protein. Values for sera from individual mice are shown in red, with mean values for each group shown in blue.

When two doses of antigen were given with MPL adjuvant, IgG titres were similar across all groups regardless of the strategy used (Figure 4.8). There were no significant differences among the four groups given two doses of antigen (groups 4, 5, 9, and 10, $P > 0.05$). IgG titres is these groups were also not significantly different from titres obtained from a single dose of either OMV or protein given 35 days prior to bleeding (groups 3 and 8, $P > 0.05$). However, SBA

titres were very different among these groups, suggesting that the nature of the second dose influences bactericidal activity in a way that is not dependent on the magnitude of the total antibody response.



Group:	4	5	9	10
Prime (Day 0):	OMV	OMV	Protein	Protein
Boost (Day 21):	OMV	Protein	OMV	Protein
Mean SBA titre:	776	2048	37	15

Figure 4.8: Relative IgG titres obtained for sera from two doses of antigen given with MPL adjuvant. Antigen was presented either as outer membrane vesicles (OMV) or as recombinant PorA protein. Values for sera from individual mice are shown in red, with mean values for each group shown in blue.

4.2.4: Comparison of IgG subclass distribution in sera by whole-cell enzyme-linked immunosorbent assay

Many groups that contained high IgG levels did not show any detectable SBA activity, showing that high levels of total antibody are not sufficient for serum bactericidal killing. Furthermore, the immunisation strategy found to give the highest SBA titres, an OMV prime followed by a protein boost, did not show the highest antibody levels. Therefore, the success of this strategy is not due to increasing total IgG.

As different IgG subclasses have variable abilities to bind complement [204, 216], differences in the IgG subclass ratio may account for variation in bactericidal activity among the strategies

tested. The relative quantities of the subclasses IgG1, IgG2a and IgG2b in sera were determined. In mice, T-helper cells can be classified as Th1 cells or Th2 cells. Th1 cells mediate cell-mediated immunity and switching to IgG2a, IgG2b and IgG3. Th2 cells promote switching to IgG1, IgA and IgE. Efficient killing of meningococci in SBAs is associated with effective complement binding of IgG2a and IgG2b [205], and consequently with a Th1 response.

Total strain-specific IgG1, IgG2a and IgG2b were quantified by ELISA against NZ98/254 whole cells and geometric mean titres (GMT) were calculated relative to a total pool of group 10 with $Al(OH)_3$ (IgG1), PorA P1.7 (IgG2a) or P1.16 (IgG2b) monoclonal antibody standards. No IgG3 was detected in any group (data not shown).

When inoculums were administered with $Al(OH)_3$ adjuvant, groups 1, 2, 6, and 7 (a single dose of either inoculum) gave very low or undetectable levels of all subclasses, as expected from low total IgG GMTs (see 4.2.2).

	Group	Prime	Boost	$Al(OH)_3$			
		(Day 0)	(Day 21)	SBA	IgG1	IgG2a	IgG2b
OMV	1	Adjuvant	OMV	<8	-	-	0.04
	2	OMV	(Bleed)	9	0.02	-	0.27
	3	OMV	Adjuvant	69	0.09	0.08	0.58
	4	OMV	OMV	9	0.03	0.31	0.82
	5	OMV	Protein	484	0.03	0.15	0.83
Protein	6	Adjuvant	Protein	<8	-	-	-
	7	Protein	(Bleed)	<8	-	-	-
	8	Protein	Adjuvant	<8	0.57	-	0.03
	9	Protein	OMV	<8	0.7	0.02	0.06
	10	Protein	Protein	<8	0.91	-	0.04

Table 4.2: Relative GMT for total IgG1, IgG2a, and IgG2b specific for NZ98/254 whole cells. Antigen was presented either as outer membrane vesicles (OMV) or as recombinant PorA protein with $Al(OH)_3$ adjuvant.

Protein inoculums (groups 8-10) gave high levels of IgG1 and very low levels of IgG2a and IgG2b (Table 4.2), indicative of a Th2 response. As sera from these groups did not contain any

detectable bactericidal activity, the IgG1 subclass does not contribute to complement-mediated killing. This is consistent with the poor complement-binding ability of murine IgG1 [217].

OMV inoculums with $Al(OH)_3$ adjuvant gave low levels of IgG1 and higher levels of IgG2a and IgG2b, consistent with a Th1 response. However, the use of OMV as a secondary dose, following a primary dose of protein, resulted in a predominantly Th2 response, indicating that it is the primary dose that is important in determining the IgG subclass profile.

The highest SBA titres were seen in groups containing high levels of IgG2b, suggesting that this subclass elicits bactericidal activity. This is supported by previous observations that killing of meningococci in SBAs is associated with a Th1 response in mice [205]. However, sera from mice given two doses of OMV did not fit this trend, showing high levels of IgG2b but very low SBA titres. Sera from groups 4 and 5 contained very similar levels of all three IgG subclasses, but showed very different SBA titres; therefore, other factors are also affecting the ability of the antibodies to induce complement-mediated killing.

The use of MPL adjuvant induced higher levels of all subclasses than $Al(OH)_3$ adjuvant, particularly in groups where only one dose of antigen had been given (groups 1, 2, 3, 6, 7, and 8, Table 4.3). When two doses of antigen were given with protein as the priming dose (groups 9 and 10), levels of IgG1 measured were higher with $Al(OH)_3$ adjuvant than with MPL, resulting in an overall decrease in the IgG1:IgG2 ratio with MPL adjuvant compared with $Al(OH)_3$ adjuvant. This indicates that MPL promotes a mixed Th1/Th2 response while $Al(OH)_3$ adjuvant promotes a Th2 response in mice. However, although the use of protein with MPL resulted in the presence of Th1 IgG subclasses, bactericidal activity in these sera were low, suggesting that the majority of the antibodies detected are not contributing to bactericidal killing.

	Group	Prime	Boost	MPL			
		(Day 0)	(Day 21)	SBA	IgG1	IgG2a	IgG2b
OMV	1	Adjuvant	OMV	57	0.05	0.11	1.97
	2	OMV	(Bleed)	24	0.03	0.79	1.45
	3	OMV	Adjuvant	366	0.19	2.99	4.36
	4	OMV	OMV	776	0.09	3.24	3.61
	5	OMV	Protein	2048	-	1.66	0.21
Protein	6	Adjuvant	Protein	9	0.04	0.04	0.21
	7	Protein	(Bleed)	<8	0.03	0.06	0.03
	8	Protein	Adjuvant	9	0.52	1.53	1.02
	9	Protein	OMV	37	0.57	1.44	1.19
	10	Protein	Protein	15	0.79	2.44	1.57

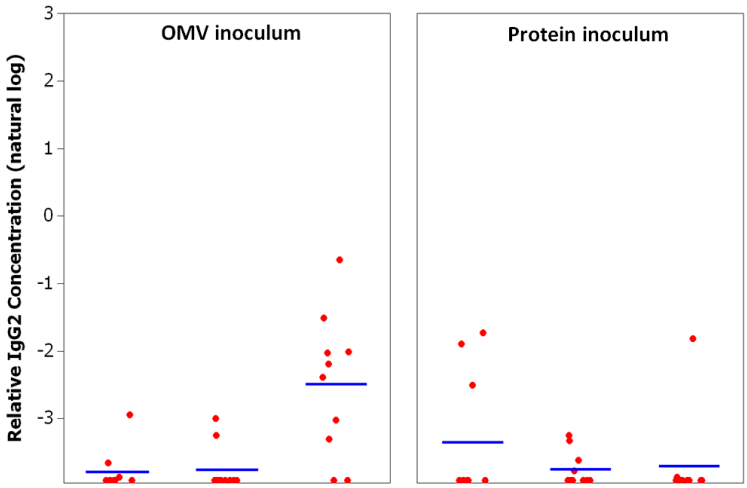
Table 4.3: Relative geometric mean titres (GMT) for total IgG1, IgG2a, and IgG2b specific for NZ98/254 whole cells. Antigen was presented either as outer membrane vesicles (OMV) or as recombinant PorA protein with MPL adjuvant.

As with $Al(OH)_3$ adjuvant, administration of OMVs with MPL adjuvant resulted in higher levels of IgG2a and IgG2b than administration of protein with the same adjuvant, providing further evidence that OMVs promote switching to a Th1 response. The highest levels of IgG2a and IgG2b were seen in groups 3 and 4 with MPL adjuvant (a priming dose of OMV followed by a dose of either OMV or adjuvant alone). These groups also showed high bactericidal activity, consistent with the role of IgG2b in complement-mediated killing. Sera from these groups also contained high levels of IgG2a, which is also likely to be contributing to bactericidal activity [205]. However, sera from group 5 (OMV prime followed by a protein boost), despite containing the highest bactericidal activity, showed low levels of IgG2b and reduced levels of IgG2a compared to sera from mice given one or two doses of OMV (groups 3 and 4). Therefore the secondary dose of protein is altering the antibody response to improve complement-mediated killing. This improvement is not dependent on the total concentration of Th1 IgG subclasses.

4.2.5: Comparison of PorA-specific IgG2 in sera by recombinant-protein enzyme-linked immunosorbent assay

Bactericidal activity among the immunisation strategies tested was found to be linked to the presence of IgG2a and IgG2b subclasses. However, although IgG2 seemed to be necessary for bactericidal activity, some groups had high IgG2 concentrations and low bactericidal activity. Therefore, high levels of surface-binding IgG2 are not sufficient for complement-mediated killing. Although PorA is usually the immunodominant protein in the meningococcal outer membrane, and antibodies to this protein are known to be bactericidal, immunisation with OMVs results in the production of antibodies to a variety of antigens. Antibodies targeting many of these other antigens, for example PorB, do not normally induce killing in SBAs [95], but will be included in the IgG measured by whole-cell ELISA. As the aim of this study was to compare the ability of prime-boost immunisation strategies to induce bactericidal antibodies specific for the PorA protein, PorA-specific IgG2 in the sera was quantified by ELISA using recombinant PorA P1.7-2,4 as the coating antigen. Sera from groups 1, 2, 6, and 7 (groups give a single dose of inoculum <35 days prior to bleeding), which showed very low levels of IgG against whole cells, were not tested.

When used with $Al(OH)_3$ adjuvant, a primary dose of protein inoculum resulted in PorA-specific IgG2 in some mice (groups 8, 9, and 10, Figure 4.9). However, sera from these mice did not contain IgG2 that bound in a whole-cell ELISA (Table 4.2), indicating that these antibodies are to non-surface-exposed epitopes and therefore unable to attach to live meningococci in an SBA.



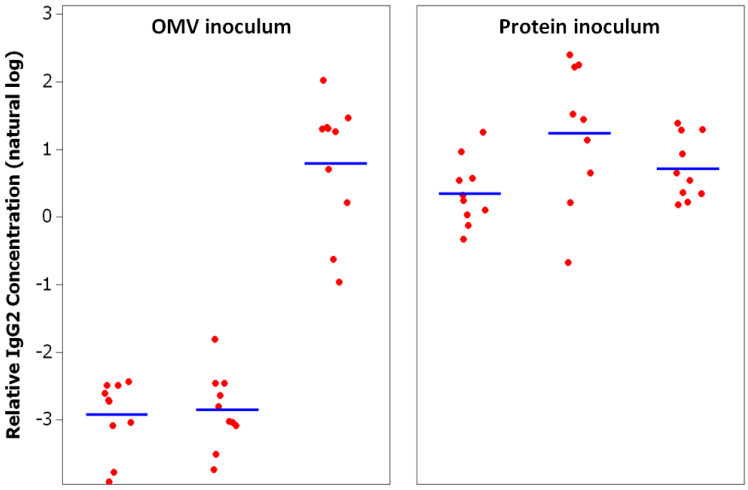
Group:	3	4	5	8	9	10
Prime (Day 0):	OMV	OMV	OMV	Protein	Protein	Protein
Boost (Day 21):	Adjuvant	OMV	Protein	Adjuvant	OMV	Protein
IgG2a + IgG2b GMT:	0.66	1.13	0.98	0.03	0.08	0.04
Mean SBA titre:	69	9	484	<8	<8	<8

Figure 4.9: Relative PorA P1.7-2,4-specific IgG2 titres obtained for sera from immunisations with $Al(OH)_3$ adjuvant. Antigen was presented either as outer membrane vesicles (OMV) or as recombinant PorA protein. Values for sera from individual mice are shown in red, with mean values for each group shown in blue. Mean IgG2 titres against whole cells and SBA titres are listed in the table.

When a primary dose of OMV was used, the use of a secondary dose of protein inoculum (group 5) resulted in the highest levels of PorA-specific IgG2. Levels of IgG2 were significantly higher in group 5 than in all other groups with this adjuvant (P = 0.006), supporting the high bactericidal activity detected in these sera. Levels of IgG2 specific for PorA were low in groups given only OMV inoculum (groups 3 and 4). As levels of IgG2 measured by whole-cell ELISA were high in these groups, it is likely that the antibodies induced by the OMVs are to antigens other than PorA. However, while a single dose of OMV (group 3) resulted in a mean SBA titre of 69, two doses of OMV resulted in very low bactericidal killing (group 4, Geometric mean SBA titre = 9). Therefore, while the non-PorA antigens targeted following a single dose of OMV are capable of leading to bactericidal killing, the antigens targeted following two doses of OMV are not. It can be concluded that, following an OMV primary dose with $Al(OH)_3$ adjuvant, the

presence and nature of a second dose influences which antigens are immunodominant in the antibody response.

Immunisation with MPL adjuvant gave significantly higher levels of IgG2 than immunisation with $Al(OH)_3$ adjuvant ($P < 0.001$). With MPL adjuvant, sera from groups given at least one dose of protein inoculum (groups 5, 8, 9, and 10) showed high levels of PorA-specific IgG2 (Figure 4.10).



Group:	3	4	5	8	9	10
Prime (Day 0):	OMV	OMV	OMV	Protein	Protein	Protein
Boost (Day 21):	Adjuvant	OMV	Protein	Adjuvant	OMV	Protein
IgG2a + IgG2b GMT:	7.35	6.85	1.87	2.55	2.63	4.01
Mean SBA titre:	366	776	2048	9	37	15

Figure 4.10: Relative PorA P1.7-2,4-specific IgG2 titres obtained for sera from immunisations with MPL adjuvant. Antigen was presented either as outer membrane vesicles (OMV) or as recombinant PorA protein. Values for sera from individual mice are shown in red, with mean values for each group shown in blue. Mean IgG2 titres against whole cells and SBA titres are listed in the table.

Titres were similar across groups 5, 8, 9, and 10. However, while sera from group 5 (OMV prime followed by protein boost) showed high bactericidal activity, other groups gave low SBA titres. This difference is unlikely to be due to antibodies in groups 8, 9, and 10 targeting non-surface epitopes, as these groups also contained high levels of IgG2 as measured by whole-cell ELISA (see 4.2.4). It could be that the high levels of non-complement-binding IgG1 in these sera

are inhibiting the binding of the complement-binding IgG2 subclasses. Alternatively, there may be other differences in the nature of these antibodies that result in poor SBA activity. The low bactericidal activity of these antibodies could not be attributed to antibody affinity (data not shown).

Sera from mice given only OMV inoculum (groups 3 and 4), showed significantly lower levels of PorA-specific IgG than groups given protein inoculum (groups 5, 8, 9, and 10, $P < 0.001$). These groups showed high levels of IgG2 specific for whole meningococcal cells, indicating that the majority of antibodies are to other surface proteins. Mean PorA-specific IgG2 in these groups were similar to those seen following an OMV prime/protein boost schedule with $Al(OH)_3$ adjuvant (group 5), which also showed similar SBA titres. Therefore, it may be that these low levels of PorA-specific IgG2 are sufficient for bactericidal activity, or are contributing to the bactericidal activity of antibodies to a range of outer membrane proteins.

Overall, while IgG2 specific for surface-exposed epitopes on PorA are generally linked to bactericidal activity, antibodies specific for certain other outer membrane proteins are also capable of bactericidal activity. Furthermore, while the secondary dose and adjuvant used affect the antigens primarily targeted by the antibody response, a primary dose of OMV is important for ensuring that antibodies formed are specific for surface-exposed epitopes and, consequently, capable of bactericidal activity.

4.2.6: Analysis of immunoreactive outer membrane proteins

Combined results from whole-cell and recombinant PorA ELISAs suggest that, while the use of a prime-boost strategy targets the immune response towards the PorA protein, use of OMV inoculum alone results in the production of antibodies to a range of other outer membrane

proteins. Some of these proteins seem to be capable of inducing a bactericidal antibody response, while other proteins induce antibodies that are not functional in an SBA.

In order to determine which proteins were immunoreactive among the different strategies, and to confirm the targeting of PorA using a prime-boost strategy, outer membrane protein preparations from NZ98/254 (the OMV parent strain) were run on a 1-dimensional 12% acrylamide SDS-PAGE gel. Proteins were then transferred to a nitrocellulose membrane by western blot and subsequently incubated with sera from groups 3, 4, 5, 8, 9, and 10 (pools of all ten mice in each group) before detecting bound IgG. As sera from groups 1, 2, 6 and 7 showed very low levels of IgG against whole cells, sera from these groups were not tested. Sera from mice immunised with both $Al(OH)_3$- and MPL-adjuvanted formulations were analysed. Total IgG was imaged using chemiluminescence.

Groups that received a primary dose of protein inoculum (groups 8, 9, and 10) showed similar immunoreactive protein profiles regardless of adjuvant (Figure 4.11). The majority of antibodies in these sera reacted with the PorA protein, although, as evidenced from whole-cell ELISAs, many of these react with non-surface epitopes or do not effectively recruit complement. Sera from group 10 (two doses of protein) also reacted with protein at two low molecular weight bands that may be PorA-degradation products. Alternatively, these may represent antibodies to non-surface epitopes that are conserved across several proteins in the outer membrane rather than being confined to PorA. For group 8, 9, and 10, PorA-specific bands were more intense when MPL adjuvant was used, which is consistent with previous ELISA results that showed higher concentrations of IgG with this adjuvant compared to $Al(OH)_3$.

	$Al(OH)_3$						MPL					
	OMV			Protein			OMV			Protein		
Group	3	4	5	8	9	10	3	4	5	8	9	10
Prime	OMV	OMV	OMV	Protein	Protein	Protein	OMV	OMV	OMV	Protein	Protein	Protein
Boost	$Al(OH)_3$	OMV	Protein	$Al(OH)_3$	OMV	Protein	MPL	OMV	Protein	MPL	OMV	Protein
GMT SBA	69	9	484	<8	<8	<8	366	776	2048	9	37	15

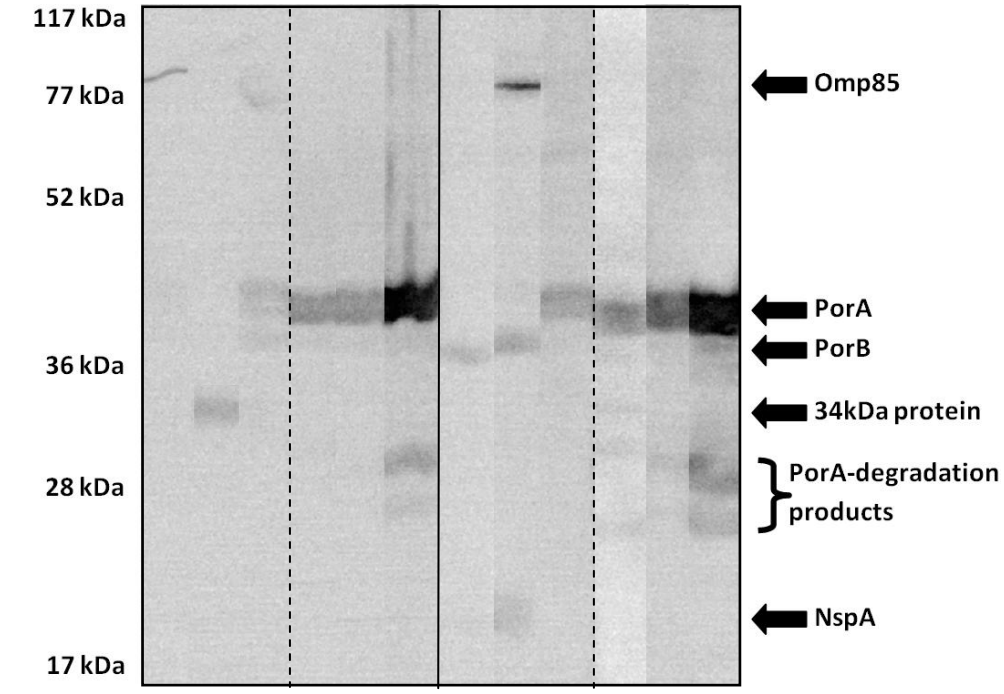


Figure 4.11: Outer membrane proteins of NZ98/254 that react with total IgG from group pools of mouse sera (1:200 dilution). Sera from mice immunised with both $Al(OH)_3$-adjuvanted and MPL-adjuvanted formulations were compared. Sizes of molecular weight markers run concomitantly are shown to the left of the image. Immunoreactive proteins (determined by by comparison with [184]) are putatively labelled to the right of the image. Geometric mean SBA titres are listed in the table.

The immunoreactive proteins detected with sera from groups given only OMV inoculum (groups 3 and 4) were very different between the two adjuvants. Two doses of OMV administered with $Al(OH)_3$ adjuvant resulted in antibodies solely reactive with a 34kDa protein,

confirming ELISA results that there was no PorA-specific IgG in those sera. The lack of SBA activity in sera from this group suggests that antibodies to this protein do not result in bactericidal killing.

The 34kDa protein was also found to be present at consistent levels in H44/76 mutants devoid of RmpM or OpcA expression and in a range of strains from other clonal complexes (data not shown). This consistent expression and epitope conservation also suggest that this protein is unlikely to be one of the Opa proteins, and is, therefore, likely to be a minor outer membrane protein.

When administered with $Al(OH)_3$ adjuvant, a single dose of OMV (group 3) resulted in antibodies to an ~80kDa protein consistent with the position of Omp85 [184]. Antibodies to this protein are therefore likely to have contributed to the bactericidal activity of sera from this group. When administered with MPL adjuvant, the immunodominant antigens within the OMVs were altered. A single dose of OMV resulted in antibodies specific for PorB, while two doses of OMV resulted in antibodies specific for three proteins: PorB, an ~80kDa protein (putatively Omp85) and a ~21kDa protein with a similar mass to NspA [184]. Both Omp85 and NspA are known to be immunoreactive following OMV immunisation [95].

Both groups 3 and 4 with MPL adjuvant (one or two doses of OMV) displayed serum bactericidal activity. Therefore, it may be that, as well as antibodies specific for Omp85 showing bactericidal activity, the antibodies specific for PorB are also contributing to complement-mediated killing. This has been found in previous studies [218,219], although antibodies against PorB are often found to be non-functional in SBAs [220]. Alternatively, it may be that the bactericidal activity can be attributed to antibodies directed against multiple minor antigens that could not be detected individually using the methods in this study.

The band patterns produced by sera from group 5 (OMV prime followed by protein boost) were similar when $Al(OH)_3$ and MPL adjuvant were compared. As with groups given a primary

dose of protein, the majority of antibodies were specific for the PorA protein. However, use of this strategy with $Al(OH)_3$ adjuvant resulted in a lower intensity band at the molecular weight for PorA when compared to MPL and also showed reactivity with a protein consistent with the size of PorB [184].

The presence of PorA-specific antibodies following an OMV prime and protein boost, with either adjuvant, suggests that this strategy is effectively targeting the immune system to the PorA antigen and overcoming the immunodominance of other antigens seen following immunisation with one or two doses of OMV alone (groups 3 and 4). Other evidence presented here also shows that the use of this strategy resulted in antibodies that are both specific for surface-exposed epitopes and of the appropriate IgG subclasses to induce complement-mediated killing in an SBA.

4.2.7: Use of alternative mouse strains to test the efficacy of the prime-boost strategy

It is generally accepted that different strains of mice respond differently to immunisation with outer membrane vesicles. In NIH mice, the use of two doses of OMV adjuvanted with $Al(OH)_3$ resulted primarily in antibodies specific for non-bactericidal antigens, while an OMV primary dose followed by a secondary dose of recombinant PorA protein resulted in an effective, PorA-specific bactericidal antibody response. To determine whether this response was an artefact of the strain of mice used, the two strategies were also compared in three additional inbred strains of mice: Balb/c, C3H, and C3H $Tlr4^{LPS\text{-}d}$. Ten individuals from each mouse strain were tested with the two strategies using $Al(OH)_3$ adjuvant.

Balb/c and C3H mice were chosen as common strains used for pre-clinical studies, which are known to be phenotypically biased towards Th2 and Th1 responses, respectively [221]. These strains may therefore respond differently to the combination of OMV and protein inoculum;

for example, different IgG subclass distributions could be induced, leading to varying bactericidal activities. C3H *Tlr4*$^{LPS\text{-}d}$ mice, in contrast to other mouse strains used, express no TLR4 and do not respond to LPS. These mice were tested as much of the difference in protection induced by OMVs compared to recombinant protein antigen presentation forms has been attributed to the presence of the TLR4 ligand Lipid-A in the meningococcal outer membrane [214]. The response induced in the C3H *Tlr4*$^{LPS\text{-}d}$ mice, compared to the wildtype C3H mice, would therefore indicate whether the prime-boost strategy used would be successful without the primary adjuvant effect of the OMVs.

Relative PorA-specific IgG titres in sera from all four strains of mice were measured by recombinant protein ELISA using P1.7-2,4 as a coating antigen. When two doses of OMV were used, all four strains of mice showed low levels of PorA-specific IgG. Of the four strains of mice, C3H mice showed significantly higher IgG titres than the other strains tested ($P < 0.001$, Figure 4.12). Although Balb/c mice also showed higher PorA-specific IgG than NIH mice or C3H *Tlr4*$^{LPS\text{-}d}$ mice, this difference did not achieve significance ($P > 0.05$). Therefore, the murine antibody response is poorly targeted towards PorA using OMV inoculum alone. This response is similar regardless of the strain of mice used.

When the prime boost strategy was used (OMV prime followed by protein boost), all strains of mice showed significantly higher PorA-specific IgG titres than when two doses of OMV were used ($P < 0.001$). Sera from C3H *Tlr4*$^{LPS\text{-}d}$ mice showed the lowest IgG titres with the prime-boost strategy and, although not significantly lower than titres achieved in NIH or Balb/c mice, the titres measured in C3H *Tlr4*$^{LPS\text{-}d}$ mice were significantly lower than titres measured in wildtype C3H mice ($P = 0.002$).

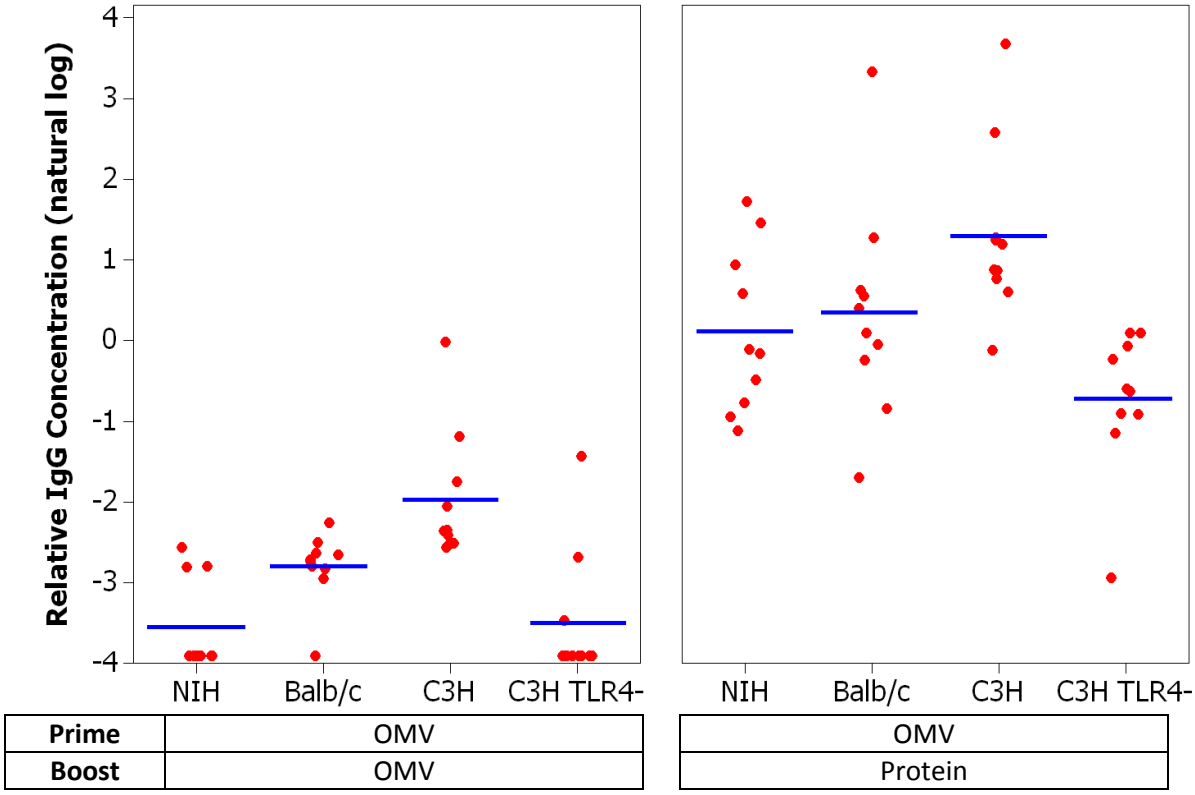


Figure 4.12: Relative PorA-specific IgG titres obtained for sera from immunisations with $Al(OH)_3$ adjuvant in four strains of mice. Antigen was presented either as outer membrane vesicles (OMV) or as recombinant PorA protein. Values for sera from individual mice are shown in red, with mean values for each group shown in blue.

The reduced PorA-specific IgG seen in C3H *Tlr4*$^{LPS\text{-}d}$ mice compared with wildtype C3H mice, following both a two-dose OMV strategy and an OMV/protein heterologous prime boost strategy, suggests that the presence of TLR4 ligand in the OMVs is important for inducing antibody production. However, all PorA-specific antibodies from all four mouse strains were found to be of the IgG2 subclass (data not shown), indicating that the presence of TLR4 ligands in OMVs is not necessary for switching to a Th1 response.

To determine whether the antibodies measured in the four mouse strains were capable of bactericidal killing, SBAs were completed as described previously. There was no significant differences among the mouse strains using either immunisation strategy ($P > 0.05$, Figure 4.13), although following two doses of OMV SBA titres were highest in C3H mice.

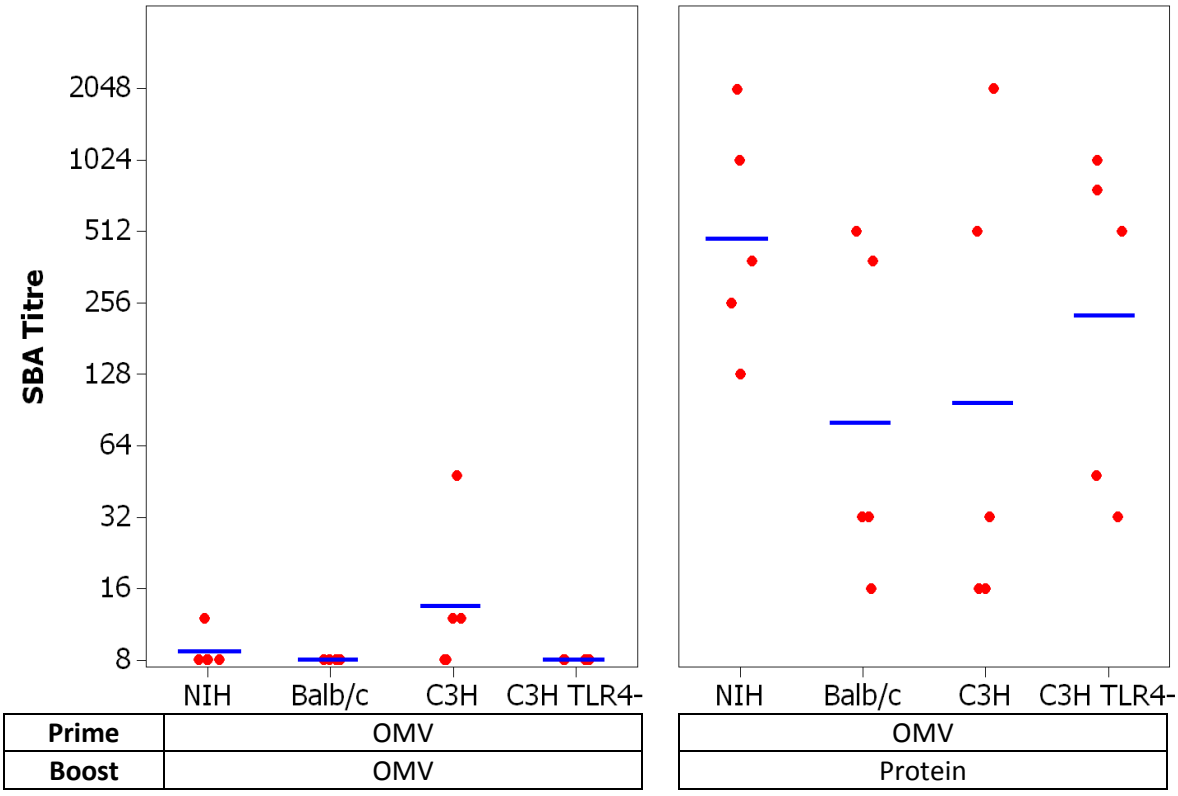


Figure 4.13: SBA titres obtained for sera from immunisations with $Al(OH)_3$ adjuvant in four strains of mice. Antigen was presented either as outer membrane vesicles (OMV) or as recombinant PorA protein. Values for pools of paired mouse sera are shown in red, with mean values for each group shown in blue.

For NIH, Balb/c and C3H *Tlr4*$^{LPS\text{-}d}$ mice, immunisation with the prime boost strategy resulted in significantly higher SBA titres than immunisation with OMV alone ($P < 0.001$). Although the same trend could be seen in wildtype C3H mice, the difference did not achieve significance in that mouse strain ($P = 0.133$). This may be due to the high levels of PorA-specific IgG seen in sera from C3H mice following two doses of OMV (Figure 4.12) resulting in higher SBA titres in that strain compared to other mice.

Overall, the use of an OMV primary dose followed by a recombinant protein boost was found to be more successful at inducing PorA-specific bactericidal antibodies than use of OMV inoculum alone in all mouse strains tested.

4.3: Discussion

In the developmental vaccine MenPF, the PorA and FetA antigens will be presented in *Neisseria meningitidis* OMVs. In this study, the potential to increase bactericidal antibody responses to the PorA antigen using a two-dose, prime-boost strategy involving both OMVs and recombinant protein inoculums was tested in mice. Bactericidal activity is determined by several factors, including: the magnitude of the antibody response, the quality of that antibody response (the IgG subclasses induced), and the antigens and epitopes targeted by those antibodies. Therefore, the effect of a prime-boost strategy on these determinants, and the consequent effect on SBA activity, was investigated.

The prime boost strategies tested involved two doses of the different inoculums, although schedules involving only a single antigenic dose were also tested for comparison. These single dose groups showed that the antibody response generally increased over time, although a small primary peak in antibody levels was observed with MPL adjuvant in the first two weeks after dosing. As total antibody levels were still increasing after three weeks, the second antigenic dose given at that time was unlikely to be a true booster dose. However, the nature of the primary and secondary doses was important in determining the efficacy of the antibody response. In this study, the strategy that resulted in the highest levels of bactericidal antibodies against the PorA antigen, with either adjuvant, was the use of an OMV prime followed by a booster dose of recombinant protein.

As SBAs measure the ability of antibodies in sera to kill meningococci through complement-mediated lysis, antibodies present in sera must be capable of activating the complement cascade in order to be effective. Antibodies capable of complement-mediated killing are associated with a Th1 response in mice [205]. This type of response was effectively induced by a primary dose of meningococcal OMVs, while the use of a primary dose of recombinant protein alone induced an ineffective Th2 response with $Al(OH)_3$ as adjuvant. The use of MPL

adjuvant also improved switching to a Th1 response. The ability of MPL to stimulate a mixed Th1/Th2 response in mice, compared to the predominant Th2 response induced by $Al(OH)_3$, has been reported previously [101]. The presence of LPS in OMVs has also been shown to be important for inducing antibody class-switching from IgG1 to IgG2 [222], supporting the data obtained in this study.

For both MPL and LPS, the induction of a Th1 response has been linked to signalling pathways induced by TLR-binding [223,224], particularly TLR4. However, when the OMVs were used to immunise mutant mice that do not express TLR4, although the magnitude of the IgG response was reduced compared to wildtype mice, serum bactericidal titres were not affected. Therefore, the adjuvant effects of OMVs, and their ability to induce class-switching to complement-binding IgG2, are not confined to their TLR4 ligands. Other intrinsic adjuvant properties of OMVs, including their particle-like nature and ability to bind TLR1 and TLR2 via the porin proteins, have been discussed previously (see 1.3.3, page 15).

The particle-like nature of OMVs was also important, during the primary dose, for targeting antibodies to bactericidal epitopes on the surface of meningococcus. When recombinant proteins were used as the primary dose, the majority of antibodies induced were specific for non-surface epitopes, and were therefore unable to bind live meningococci in an SBA. When MPL was used, although antibodies were to surface-epitopes, bactericidal activity was limited. This may be because non-complement binding IgG1 in the serum was competing for antigen-binding.

Alternatively, it may be that the recombinant PorA protein used was not correctly folded. The protein was produced by expression in inclusion bodies in *E.coli* and denatured with urea prior to purification; the protein was then refolded by dialysis to remove the urea (Patel *et al.,* unpublished). Although denatured PorA has been shown to be capable of inducing production of PorA-specific antibodies, these antibodies are not capable of killing meningococci in an SBA

[110]. Therefore, if the recombinant PorA was not correctly folded, or contained a mixture of folded and unfolded protein, this could have lead to the low bactericidal killing measured in sera from mice given recombinant protein with MPL, despite the presence of antibodies specific for surface-exposed epitopes. Incorrect protein folding may also have contributed to the lack of bactericidal antibodies in sera from mice given recombinant protein with $Al(OH)_3$ adjuvant. Correct folding of the protein could be investigated using circular dichroism, for example, to study the secondary structure of the protein. Comparison of the protein used with a sample of the same protein that had been denatured would indicate whether the protein was likely to be folded [110].

PorA is often listed as the immunodominant antigen in meningococcal OMVs. However, there is much published literature showing that antibodies to other antigens are also induced following OMV vaccination, and that antibodies to many of these antigens are not functional in an SBA [95,169]. This was also found to be the case in this study. High levels of PorA-specific antibodies were only seen in mice that had received at least one dose of recombinant PorA protein. The use of OMV alone was not effective at inducing antibodies specific for PorA, but, in some cases, antibodies were elicited to other protein antigens, such as Omp85, which seemed to contribute to bactericidal killing.

The immunodominant antigen in the OMVs varied according to whether one or two doses of OMV were given. Although the use of MPL adjuvant increased the number of antigens targeted by the antibody response, this adjuvant did not improve responses to the PorA protein. High levels of PorA-specific bactericidal antibodies were only induced following a primary dose of OMV and a secondary dose of recombinant PorA. This strategy was more effective than OMV alone in all four strains of mice tested, and using both adjuvants. Therefore, a prime-boost strategy such as this may allow improved immune responses to poorly immunogenic PorA serosubtypes such as P1.7-2,4. This is in contrast to previous studies that suggest a specific prime, rather than a specific boost as used in this case, is more effective

at inducing effective antibodies against a particular antigen [225]. In this study, the use of a protein prime followed by an OMV boost was ineffective, as the protein prime induced a predominantly Th2 response, with the majority of antibodies directed to non-surface epitopes on the PorA protein. Therefore, the antibodies present were unable to bind live meningococci or recruit complement in an SBA. The use of OMVs as a secondary dose, in this case, was insufficient for inducing antibody class switching or for enhancing antibody production to surface epitopes.

Using a two-dose schedule with a specific boost, the adjuvant properties of the OMV primary dose resulted in a Th1 response suitable for complement-mediated bactericidal killing, with antibodies specific for surface epitopes. Antibody responses were then effectively targeted to the PorA antigen, rather than to other OMP antigens, by the recombinant protein booster dose. For inducing a bactericidal antibody response against the PorA protein, therefore, the use of an OMV primary dose followed by a protein secondary dose was the most effective strategy tested here.

Although the use of MPL generally increased bactericidal antibody titres, this effect was not specific to PorA. Furthermore, the MPL-adjuvant formulation used in this study is not currently licensed for use in humans. This is in contrast to $Al(OH)_3$ adjuvant, which is widely used in human vaccines. We have shown that a prime-boost strategy can be used to increase PorA-specific bactericidal activity using currently-licensed adjuvants. This strategy may also allow for a reduced number of doses compared to the three or four doses required when OMV is used alone [94], decreasing the cost of vaccination and increasing protection at a younger age. Therefore, heterologous prime-boost strategies, using sequential administration of OMV followed by recombinant protein inoculums, may be a more effective way to induce protection against specific meningococcal antigens than the use of OMV inoculums alone.

Chapter 5:

Investigation of a prime-boost strategy to improve *in vivo* bactericidal responses against FetA

5.1: Introduction

In the developmental vaccine MenPF, it is hoped that a high level of coverage will be achieved against meningococcal disease using multiple variants of the antigens PorA and FetA in OMVs. Due to the intrinsic adjuvant properties of OMVs (discussed in 1.3.3, page 15), antibodies induced by OMVs are often more effective at bacterial clearance than antibodies induced by purified protein inoculums [101]. For *N. meningitidis*, protective efficacy is determined by SBA. This assay measures the ability of antibodies in serum to kill live meningococci in the presence of complement, and is considered to be the best correlate of protection against meningococcal disease [45]. For an antibody response to induce killing in an SBA, the antibodies need to: be of sufficient quantity, be of the appropriate IgG subclasses for complement-binding, and target surface-exposed epitopes of appropriate antigens. However, OMVs induce antibodies against a range of outer membrane proteins, and many of these antibodies target antigens that are not protective [95].

The PorA protein is often immunodominant within OMVs [169]. While other developmental vaccines target multiple variants of PorA [121], the inclusion of FetA as a major antigen in MenPF is predicted to improve protection against specific hyperinvasive meningococcal clonal complexes (expressing homologous PorA and FetA variants) as well as providing broader coverage against lineages expressing heterologous PorA variants (Brehony *et al*., unpublished). Consequently, in order for this coverage to be achieved, the vaccine must induce reliable bactericidal antibody responses against FetA as well as PorA.

Although antibodies to FetA are known to be induced during meningococcal infection [164], there are often variable levels of this antigen in OMVs due to negative regulation by iron [166,184]. As consistent concentrations of FetA would be required to reliably induce production of specific-antibodies, this issue was addressed using genetic modification of meningococcal strain H44/76, as described in Chapter 3, to express FetA constitutively at increased levels compared to wildtype. Additional strains were also constructed, in which expression of PorA and FetA was switched either on or off in various combinations, allowing the relative contributions of antibodies targeting PorA, FetA, and other proteins to be analysed using *in vitro* immunological assays.

The PorA variant in the modified strains, P1.7,16, is known to be highly immunogenic [145]. The antibody responses induced by the OMVs are, therefore, likely to predominantly target PorA. Other, non-protective antigens are also known to be targeted following OMV immunisation [95]. It was previously shown (Chapter 4) that a strategy involving a primary dose of OMV followed by a dose of recombinant protein effectively targeted a bactericidal antibody response to a weakly-immunogenic PorA variant in mice. By using this strategy, both the IgG subclasses induced and the specificity of the antibodies could be altered to improve the efficacy of the bactericidal response against PorA. Immunisation with subsequent doses of OMV and protein inoculum, using OMVs derived from modified strains, may also be an effective strategy to increase vaccine efficacy and vaccine coverage against the FetA antigen.

As the vaccine MenPF aims to target both PorA and FetA in order to provide high levels of coverage against disease, it is important to determine that antibodies to both of these antigens are contributing to bactericidal activity. The first aim of this study was to determine whether use of OMVs from a modified strain, expressing increased levels of FetA, was sufficient to induce bactericidal antibodies against both antigens. As the immune response may be dominated by antibodies against PorA, or other outer membrane antigens, this study also aimed to investigate whether a heterologous prime-boost strategy using a combination of

OMV and recombinant FetA protein could increase bactericidal antibody responses specific for FetA, thereby increasing the potential coverage of a FetA-based vaccine.

5.2: Results

5.2.1: *Neisseria meningitidis* strains used

This chapter made use of strains described in Chapter 3 (see 3.2.5, page 71). All strains were derived from a wildtype H44/76 parent strain and contained mutations that either constitutively increase FetA expression (FetA-on), or disrupt FetA expression (FetA-off). Expression of PorA was either disrupted (PorA-off), or not modified from the high level of expression found naturally in the wildtype (PorA-wt). Strains used are listed below:

- 3043 (*fetA::kan*, FetA-off/PorA-wt),
- 3207 ($fetAp_{17bp,}$ FetA-on/PorA-wt),
- 3311 (*fetA::kan porA::ermC,* FetA-off/PorA-off),
- 3312 ($fetAp_{17bp}$ *porA::ermC,* FetA-on/PorA-off).

5.2.2: Immunisation Strategies

Groups of ten female NIH mice were immunised on days 0 and 21 with either OMV (1µg total protein) or recombinant FetA F3-3 protein (10µg) formulated with $Al(OH)_3$. Serum samples were taken on day 35. OMVs used were produced from strain 3207 (FetA-on/PorA-wt), expressing P1.7,16 and increased levels of F3-3 compared to wildtype H44/76. Groups 1 to 6 were immunised with the schedules shown in Table 5.1.

Group	Day 0	Day 21	Day 35
1	OMV	Adjuvant	Terminal Bleed
2	OMV	OMV	Terminal Bleed
3	OMV	Protein	Terminal Bleed
4	Protein	Adjuvant	Terminal Bleed
5	Protein	OMV	Terminal Bleed
6	Protein	Protein	Terminal Bleed

Table 5.1: Immunisation schedules used. Groups of 10 mice were immunised on the days shown with the following: outer membrane vesicle (OMV), recombinant FetA F3-3 protein (Protein) or adjuvant alone. Inoculums were formulated with $Al(OH)_3$ adjuvant.

5.2.3: Comparison of sera by Enzyme-Linked Immunosorbent Assay

5.2.3.1: Quantification of antibodies reactive with recombinant FetA protein

In order to determine the immunisation strategy that resulted in the highest levels of FetA-specific antibodies, IgG in sera specific for this antigen was quantified by ELISA using recombinant homologous FetA (F3-3) as the coating antigen. IgG1 and IgG2 were quantified separately (both relative to FetA polyclonal sera), as these subclasses have differing abilities to induce complement-mediated killing in an SBA; IgG1 is associated with poor complement binding while IgG2 can effectively activate the complement pathway [205].

High levels of IgG1 were found in sera from groups of mice given a primary dose of protein inoculum (groups 4, 5, and 6, Table 5.2). In mice that received only protein inoculum (groups 4 and 6), while levels of IgG1 were high, levels of FetA specific IgG2 were low. These results support previous evidence that recombinant protein alone with $Al(OH)_3$ adjuvant induces a Th2 response in mice, resulting in IgG1 production (see 4.2.4, page 99).

Group	Prime	Boost	IgG1	IgG2
1	OMV	Adjuvant	-	0.004
2	OMV	OMV	-	4.870
3	OMV	Protein	0.002	1.703
4	Protein	Adjuvant	0.205	0.003
5	Protein	OMV	0.117	0.921
6	Protein	Protein	0.698	0.006

Table 5.2: Relative geometric mean titres (GMT) for total IgG1 and IgG2 specific for recombinant FetA F3-3. Each groups contained 10 mice. Antigen was presented either as outer membrane vesicles (OMV) or as recombinant FetA protein.

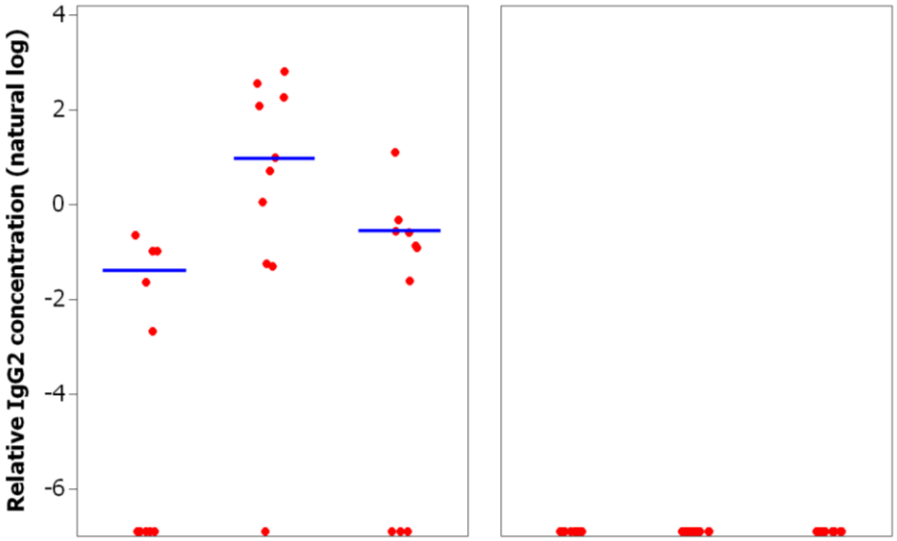
Levels of FetA-specific IgG2 were also low in mice that received only a single dose of OMV inoculum (group 1), but high in mice that received two doses of OMV (group 2) or alternating doses of OMV and recombinant protein (groups 3 and 5). These data support previous observations that use of OMV inoculums induced IgG class-switching to IgG2 (see 4.2.4, page 99), but suggest that a single dose of OMV is not sufficient for inducing FetA-specific antibodies. Of the groups in which FetA-specific IgG2 was detected, titres were highest in mice that received two doses of OMV. When alternating doses of OMV and protein were given, FetA-specific IgG2 was higher in mice given a primary dose of OMV than in mice given a primary dose of protein. Mice given a primary dose of protein showed a mixed IgG1/IgG2 antibody response.

5.2.3.2: Quantification of antibodies reactive with meningococcal whole cells

The FetA-specific IgG2 measured using recombinant protein ELISA will include antibodies against non-surface epitopes that do not induce bactericidal killing of meningococci. Therefore, whole-cell ELISAs were used to compare the total concentration of all antibodies in sera that could bind the OMV parent strain (3207), expressing the homologous FetA variant,

F3-3. As the IgG2 subclasses are associated with complement-mediated killing, total IgG2 in sera was quantified (relative to an LPS 3,7,9 monoclonal antibody).

Sera from mice given a primary dose of recombinant protein (groups 4, 5, and 6) did not contain any IgG2 that bound 3207 whole cells (Figure 5.1). This is in contrast to data obtained from ELISAs targeting recombinant FetA, where group 5 was found to contain high levels of specific IgG2. Therefore, FetA-specific IgG2 present in these sera may bind to epitopes that are not surface-exposed. Alternatively, the sensitivity of the assay used may be too low to measure the binding of antibodies in these sera.



Group:	1	2	3	4	5	6
Prime (Day 0):	OMV	OMV	OMV	Protein	Protein	Protein
Boost (Day 21):	Adjuvant	OMV	Protein	Adjuvant	OMV	Protein

Figure 5.1: Relative IgG2 in sera specific for 3207 (FetA-on/PorA-on) whole cells (relative to an LPS 3,7,9 monoclonal antibody). Mice were immunised with either outer membrane vesicles (OMV) or FetA (F3-3) recombinant protein with $Al(OH)_3$ adjuvant. Values for pools of paired mouse sera are shown in red, with mean values for each group shown in blue. Mice for which no IgG2 was detected were excluded from the calculation of the mean.

IgG2 capable of binding 3207 whole cells was present in all groups given a primary dose of OMV. However, sera from several mice in each group (five in group 1, one in group 2, and three in group 3), did not contain any IgG2 that bound the meningococcal cells. Consequently,

although none of the strategies tested induced IgG2 in all mice, the use of two doses of OMV gave the most consistent antibody titres.

Of the mice that showed a detectable response in this assay (five from group 1, nine from group 2, and seven from group 3), antibody titres were highest in mice that had been given two doses of OMV (group 2, GMT = 2.69), and lowest in mice given only a single dose of OMV (group 1, GMT = 0.25). Geometric mean titres were significantly higher following two doses of OMV (group 2) than one dose (group 1, $P = 0.003$), although neither group was significantly different from the group 3 (OMV followed by protein, GMT = 0.58, $P > 0.05$).

5.2.4: Determination of immunoreactive proteins by immunoblot

In order to investigate which antigens are being targeted following the different immunisation strategies, outer membrane protein preparations from the OMV parent strain, 3207 (FetA-on/PorA-on) were separated by 1-dimensional SDS-PAGE in a single well and, subsequently, transferred to a nitrocellulose membrane by western blot. The membrane was incubated in pooled sera from all ten mice in each group. Total bound IgG was imaged using chemiluminescence (Figure 5.2).

As expected, sera from groups given only protein inoculum (groups 4 and 6) showed reactivity only with the FetA protein. Furthermore, using this method, FetA-specific IgG was detected only in groups given at least one dose of recombinant protein inoculum (groups 3, 4, 5, and 6).

The use of OMV resulted in reactivity with a wide range of proteins, including PorA, PorB, Omp85 and the 34kDa protein identified in the PorA-prime-boost immunisations (see 4.2.6, page 106). In particular, all groups given at least one dose of OMV inoculum (groups 1, 2, 3, and 5) contained antibodies that reacted with Omp85, while sera from all groups given a primary dose of OMV (groups 1, 2, and 3) showed reactivity with the PorA antigen.

No FetA-specific IgG was detected in groups given only OMV inoculum (groups 1 and 2). For mice given only a single dose of OMV, this supports data from ELISAs, which showed no FetA-specific IgG1 or IgG2 was present. For this group, analysis by immunoblot showed that the antibodies that reacted with meningococcal whole cells were specific for the PorA and Omp85 proteins.

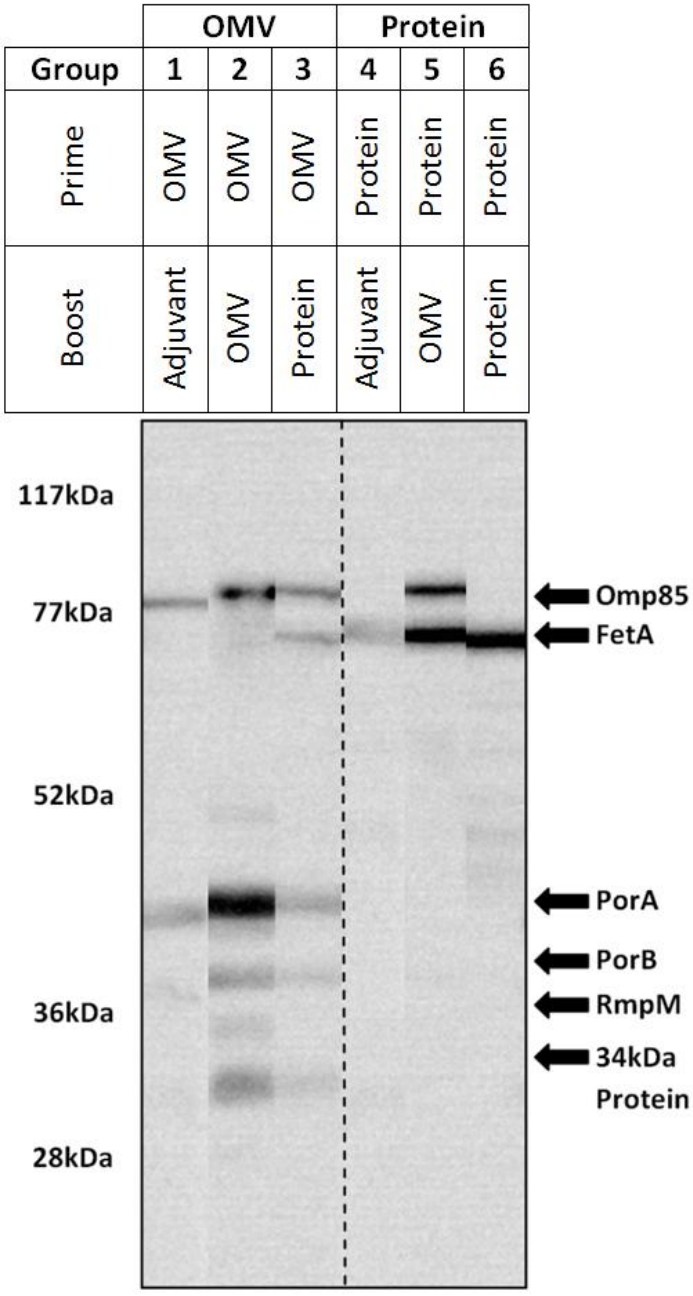


Figure 5.2: Western Blot of 3207 outer membrane proteins probed with total group pools of sera and imaged for total IgG with chemiluminescence. Mice were immunised with either OMV or recombinant FetA protein. Sizes of molecular weight markers run concomitantly are shown to the left of the image. Proteins identified by comparison with [184] are indicated to the right of the image.

Sera from mice given two doses of OMV (group 2) also showed no reactivity with the FetA protein during the immunoblot. This is in contrast to the results obtained from ELISAs, which showed the presence of high levels of FetA-specific IgG2 in these sera. As FetA presented in

OMVs is likely to induce antibodies against only the surface-exposed epitopes, these results indicate that antibodies targeting the surface epitopes of FetA are dependent on the conformation of the protein. Antibodies to conformational epitopes were not able to bind the denatured protein on the nitrocellulose membrane. Conversely, sera from mice given doses of recombinant protein (groups 3, 4, 5, and 6) react with non-surface epitopes that are still present on the denatured protein. Consequently, these antibodies may not be capable of serum bactericidal activity, as antibodies must target surface-exposed epitopes to be functional in an SBA.

5.2.5: Comparison of serum bactericidal assay titres

5.2.5.1: Serum bactericidal activity against FetA-on/PorA-wt strain 3207

To determine whether the immunisation strategies and inoculums used had induced antibodies capable of complement-mediated killing of meningococci, pools of paired mouse sera from each group were used in SBAs with baby rabbit complement (Pel-Freez Biologicals, Batch 04332EL). Bactericidal activity was first measured against the FetA-on/PorA-wt OMV-parent strain, 3207, to determine the total killing by antibodies specific for the homologous variants of all antigens in the OMV.

Sera from mice that received only protein inoculum (groups 4 and 6) showed very low or no bactericidal activity (Figure 5.3). Higher levels of bactericidal activity were observed in sera from all groups that received OMV inoculum (groups 1, 2, 3, and 5). This is consistent with ELISA data that the use of recombinant protein with $Al(OH)_3$ adjuvant results in antibodies predominantly of the IgG1 subclass, which do not effectively recruit complement, while the adjuvant properties of OMVs induce the IgG2 subclasses required for complement-mediated killing [205].

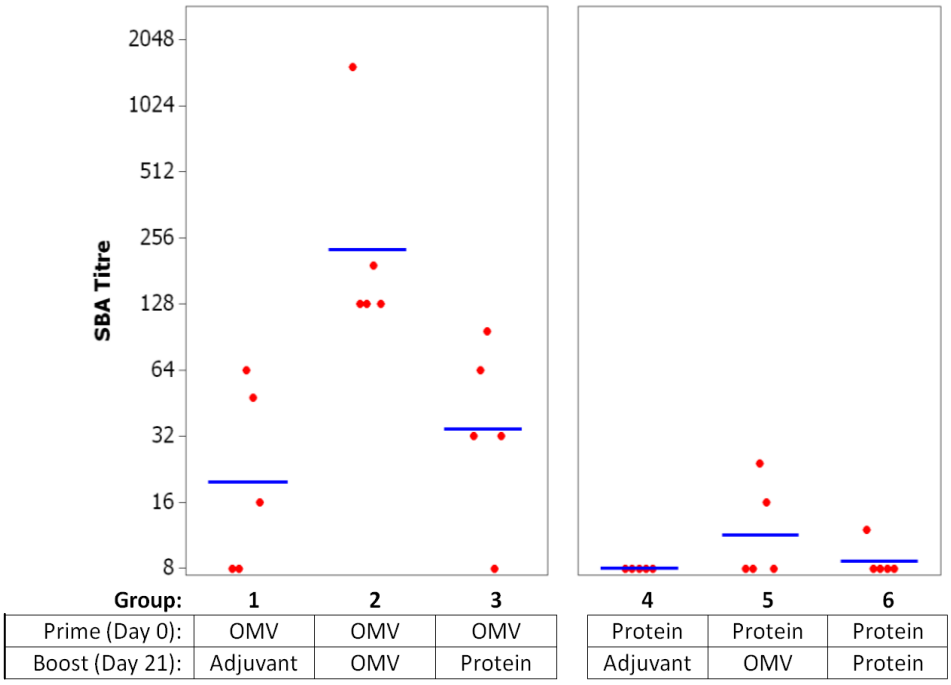


Figure 5.3: SBA titres obtained against the FetA-on/PorA-wt strain, 3207. Mice were immunised with either OMV or FetA F3-3 recombinant protein. Values for pools of paired mouse sera are shown in red, with mean values for each group shown in blue.

Within groups given only a single dose of OMV (groups 1, 3, and 5), at least one pool in each group showed no bactericidal activity. The number of pools that did not show any activity in the SBA was highest in mice given a primary dose of protein followed by a dose of OMV (group 5, three pools). Groups given OMV as a primary dose (groups 1 and 3) contained fewer pools with no detectable bactericidal activity (two and one pools, respectively). This suggests that the use of OMV as a primary dose is more effective at inducing bactericidal antibodies than when used as a secondary dose. Data from ELISA and immunoblot suggests that this may be because use of a primary dose of OMV is more effective at inducing switching to IgG2 subclasses compared to a secondary dose of OMV (Table 5.2), and because a primary dose of OMV also induces antibodies against the PorA protein while a primary dose of protein dose not (Figure 5.2).

Of the groups given a primary dose of OMV, SBA titres were similar between mice given only a single dose of OMV (group 1) and mice given a dose of OMV followed by a dose of protein (group 3). Therefore, the secondary dose of protein does not increase overall bactericidal

activity. As the main difference between these groups, as determined by previous experiments, was the presence of FetA-specific IgG2 in sera from group 3, these data would also suggest that the antibodies measured against FetA are not contributing to bactericidal activity. This may be due to antibody specificity for non-surface epitopes, or to low epitope density.

Following two doses of OMV inoculum (group 2), bactericidal titres were significantly higher than all other groups (P = 0.004), indicating that a second dose of OMV increases the magnitude or quality of the antibody response. This increased bactericidal activity could be due to the increased levels of FetA-specific IgG2 measured by ELISA, or the increased levels of antibody against a range of other proteins detected by immunoblot. As the vaccine aims to target both PorA and FetA, in order to provide high levels of coverage against disease, it is important to determine that antibodies to both of these antigens are contributing to bactericidal activity.

5.2.5.2: Comparison of bactericidal activity against mutant strains

Antibodies induced by immunisation with OMVs will be specific for a variety of antigens present in the meningococcal outer membrane. In particular, PorA is considered to be the immunodominant antigen in OMVs [169]. In order to determine whether bactericidal killing measured against the OMV-parent strain was directed against PorA or FetA individually or in combination, or against other antigens, pools of paired sera were subsequently analysed further by SBA against strains in which FetA or PorA expression had been removed. Comparison of SBA titres against strain 3043 (FetA-off/PorA-wt) to those measured against the OMV parent strain, 3207 (FetA-on/PorA-wt), was used to determine the contribution of FetA-specific antibodies to bactericidal killing; FetA-specific antibodies that contribute to bactericidal activity against the FetA-on strain will not be functional in an SBA against the FetA-off strain. Similarly, as antibodies against PorA would be active against a PorA-wt strain but not

against a PorA-off strain, comparison of SBA titres against strain 3312 (FetA-on/PorA-off) to those against strain 3207 (FetA-on/PorA-wt) indicated the contribution of PorA-specific antibodies to bactericidal killing.

Sera from groups that did not contain detectable bactericidal activity against the OMV parent strain (groups 4 and 6) did not result in bactericidal killing of either mutant strain (Table 5.3). In the control group, given only a single dose of OMV (group 1), mean SBA titres were higher against strain 3043 (FetA-off/PorA-wt) than 3207 (FetA-on/PorA-wt). Titres obtained for this group were not significantly different between these two strains (P = 0.119), suggesting that FetA-specific antibodies are not contributing to bactericidal killing. Sera from this group showed no detectable bactericidal activity against strain 3312 (FetA-on/PorA-off). Previous analysis of these sera has also shown a lack of FetA-specific IgG2 and the presence of PorA-specific antibodies. Collectively, these data support a conclusion that, following a single dose of OMV inoculum, bactericidal antibodies were predominantly targeted against the PorA antigen.

Group	Prime	Boost	SBA Titres			IgG2 specific for	
			3207	3043	3312		
			FetA-on	FetA-off	FetA-on		
			PorA-wt	PorA-wt	PorA-off	FetA	3207 cells
1	OMV	Adjuvant	20	64	8	0.004	0.022
2	OMV	OMV	228	21	11	4.870	1.308
3	OMV	Protein	35	32	22	1.703	0.106
4	Protein	Adjuvant	8	8	8	0.003	-
5	Protein	OMV	11	8	25	0.921	-
6	Protein	Protein	9	8	8	0.006	-

Table 5.3: Geometric mean SBA titres against three meningococcal target strains following murine immunisation with either OMV or recombinant FetA protein with $Al(OH)_3$ adjuvant. Target strains used were: the OMV parent strain (3207, FetA-on/PorA-wt); a mutant with no FetA expression (3043, FetA-off/PorA-wt); and a mutant with no PorA expression (3312, FetA-on/PorA-off). Geometric mean titres of IgG2 measured by ELISA against FetA F3-3 and against 3207 whole cells are also listed.

Following two dose of OMV (group 2), mean SBA titres were higher against the OMV parent strain, 3207, than against either strain 3043 (FetA-off/PorA-wt) or 3312 (FetA-on/PorA-off). As both FetA- and PorA-specific antibodies were detected during previous analysis, these data indicate that antibodies against both proteins are contributing to bactericidal activity in this group. Compared to data from mice given only a single dose of OMV (group 1), these results suggest that a second dose of OMV is sufficient to induce bactericidal antibodies against FetA. Although these antibodies were detected by ELISA, they were not seen in an immunoblot (Figure 5.2), possibly due to the conformation nature of the epitope.

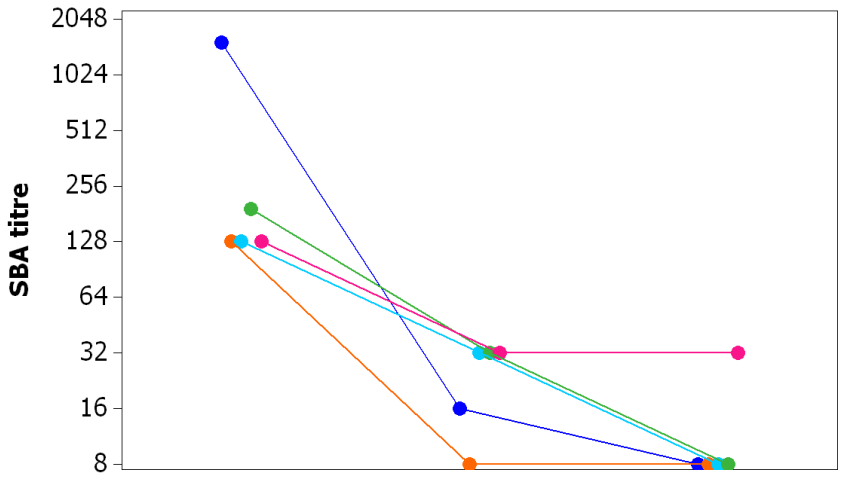
When alternating doses of OMV and protein inoculum were administered (groups 3 and 5), mean SBA titres varied depending on the order at which the inoculums were given. When the recombinant protein dose was given first (group 5), SBA titres were low against both FetA-on strains (3207 and 3312). No bactericidal activity was detected against strain 3043, which did not express FetA. Therefore, as FetA-specific IgG was detected by ELISA, the limited bactericidal activity seen against the OMV parent strain was likely to be targeting FetA. Although whole-cell ELISA data suggested that antibodies in these sera were specific for non-surface epitopes, the presence of some complement-mediated killing indicates that IgG was capable of binding the meningococcal cell surface. Antibodies were also found to be present to the outer membrane antigen Omp85, which could only have been induced by surface-exposure on the OMVs. It may be that antibodies in these sera, specific for both FetA and Omp85, could not be detected by whole-cell ELISA due to low epitope density.

Conversely, when the recombinant protein dose was given after a primary dose of OMV, mean SBA titres were similar across all three target strains. As the target strains differ in expression of both FetA and PorA, these results suggest that bactericidal activity in these sera may target antigens other than PorA and FetA, despite the presence of antibodies to both proteins. However, individual mice respond differently to immunisation. It may be that the serum pools

tested have varying responses against the target strains that cannot be seen by investigating the group means.

As the aim of this chapter was to determine the optimum two-dose strategy for inducing FetA-specific bactericidal antibodies, SBA titres of individual pools of groups 2, 3 and 5 were compared to investigate the consistency of the response to each strategy. Groups 1, 4 and 6 were not investigated further as these groups either showed no bactericidal activity (groups 4 and 6) or had a response dominated by PorA (group 1).

All pools from group 2 (two doses of OMV) showed lower SBA titres against strain 3043 (FetA-off/PorA-wt) than against strain 3207 (FetA-on/PorA-wt, Figure 5.4), indicating that, in all pools, FetA-specific antibodies are contributing to bactericidal activity. However, in four of the five pools, bactericidal activity was still present against the FetA-off strain, indicating that the FetA-specific antibodies are not necessary for complement-mediated killing. In these pools, antibodies to PorA or other antigens are also responsible for bactericidal activity.



Target Strain:	3207	3043	3312
	FetA-on	FetA-off	FetA-on
	PorA-wt	PorA-wt	PorA-off

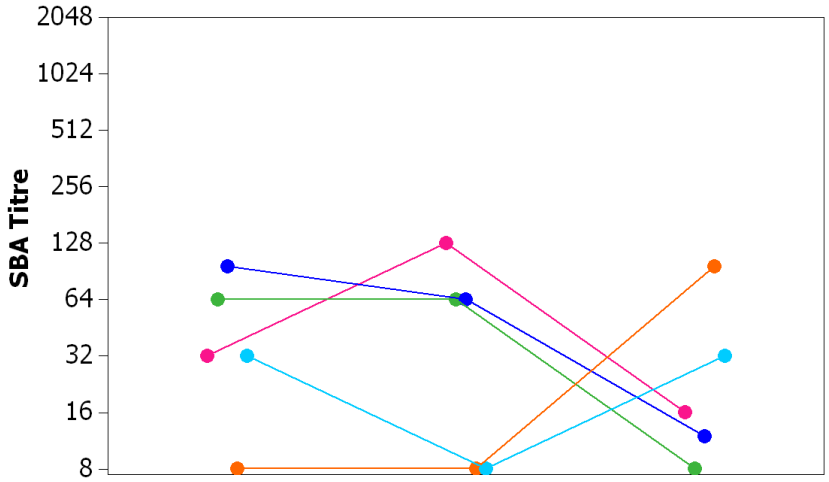
Figure 5.4: SBA titres obtained against three target strains following immunisation of mice with two doses of OMV inoculum. Symbols represent pools of paired mouse sera, each shown in a different colour. Five pools were tested in total against three target strains; 3207 (FetA-on/PorA-wt), 3043 (FetA-off/PorA-wt), and 3312 (FetA-of/PorA-off).

Three pools showed positive SBA titre against 3043 (FetA-off/PorA-wt) but no bactericidal activity against strain 3312 (FetA-on/PorA-off), suggesting that PorA-specific antibodies were necessary for bactericidal activity in these pools. This would indicate that, although FetA-specific antibodies in these sera may have contributed to bactericidal killing, the majority of bactericidal activity in these sera was due to PorA-specific antibodies; antibodies targeting the FetA protein were incapable of inducing complement-mediated killing alone. Bactericidal activity mediated by antibodies against FetA was only detected when enhanced by a synergistic effect with PorA-specific antibodies.

In one pool, no bactericidal activity was detected against either mutant strain, suggesting that antibodies to both proteins were necessary for killing in an SBA. The fifth pool showed equal SBA titres against both mutant strains; in this pool antibodies to PorA and FetA seemed to act equally, and antibodies to only one of the antigens was sufficient for bactericidal activity. Therefore, following two doses of OMV, SBA results suggest that all pools contain FetA-specific bactericidal activity; however, the strength of this response varied relative to the PorA-specific antibodies in different pools.

When mice given a dose of OMV followed by a dose of protein were compared (group 3), SBA titres varied among pools (Figure 5.5). Three pools showed similar titres against the strain 3207 and 3043 (expressing PorA at wildtype levels), and very low bactericidal activity against strain 3312 (expressing no PorA). In these pools, data suggest that the bactericidal response was targeted predominantly against PorA.

One pool showed similar SBA titres against 3207 and 3312 (both expressing FetA) and no bactericidal activity against 3043 (which did not express FetA), suggesting that, in this pool antibodies to FetA were responsible for complement-mediated killing.



Target Strain:	3207	3043	3312
	FetA-on	FetA-off	FetA-on
	PorA-wt	PorA-wt	PorA-off

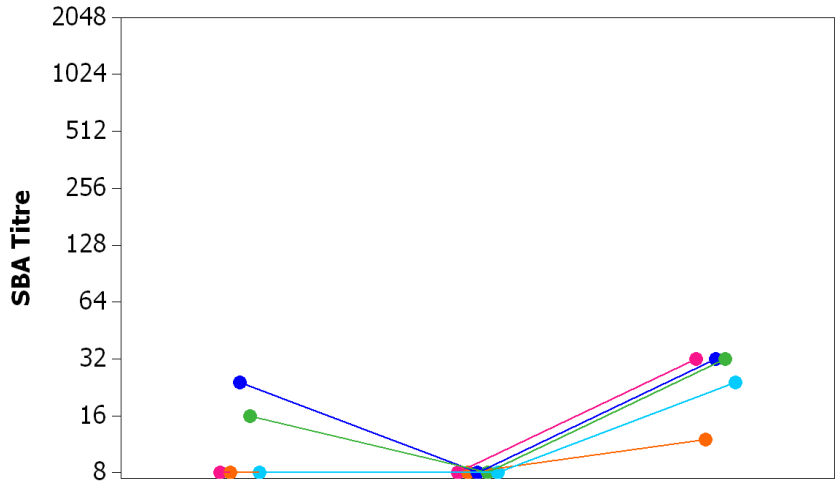
Figure 5.5: SBA titres obtained against three target strains following immunisation of mice with a dose of OMV inoculum followed by a dose of recombinant FetA. Symbols represent pools of paired mouse sera, each shown in a different colour. Five pools were tested in total against three target strains; 3207 (FetA-on/PorA-wt), 3043 (FetA-off/PorA-wt), and 3312 (FetA-of/PorA-off).

The fifth pool showed no bactericidal activity against 3207 or 3043 (FetA-on/PorA-wt and FetA-off/PorA-wt), but showed high bactericidal activity against 3312 (FetA-on/PorA-off), likely due to antibodies against antigens other than PorA and FetA. As antibodies capable of bactericidal activity against the PorA-off strain would also be expected to kill the PorA-wt strain, 3207, these data suggest that the removal of PorA expression in 3312 may make this target strain more susceptible to complement-mediated killing. This could be due to a general increase in complement-sensitivity in 3312, or to upregulation of other antigens for which antibodies are present. Alternatively, the increased killing against 3312 compared to 3207 may be due to changes in outer membrane protein complexes seen in Chapter 3 (Figure 3.16, page 80). Consequently, use of this strain in SBAs may overestimate the potential coverage against antigens other than PorA.

When mice were given a dose of recombinant protein followed by a dose of OMV (group 5, Figure 5.6), two serum pools showed similar SBA titres against strain 3312 (FetA-on/PorA-off)

compared to strain 3207 (FetA-on/PorA-wt), but had no detectable bactericidal activity against strain 3043 (FetA-off/PorA-wt). As bactericidal activity was only detected in strains that expressed FetA, in these pools the majority of bactericidal activity was due to FetA-specific antibodies.

Three pools in group 5 showed no detectable SBA activity against PorA-wt strains 3207 and 3043, but were capable of complement mediated killing against PorA-off strain 3312, proving further evidence that strain 3312 may overestimate potential coverage. However, as all SBA titres seen in this group were low (≤32), it is difficult to determine whether the differences seen between target strains are due to antibody specificity or to variability within the assay. Therefore, although the use of a protein prime followed by an OMV boost seemed to show FetA-specific bactericidal responses for two pools, bactericidal activity in other pools could not, using these target strains, be linked to the presence of FetA.



Target Strain:	3207	3043	3312
	FetA-on	FetA-off	FetA-on
	PorA-wt	PorA-wt	PorA-off

Figure 5.6: SBA titres obtained against three target strains following immunisation of mice with a dose of recombinant FetA inoculum followed by a dose of OMV. Symbols represent pools of paired mouse sera, each shown in a different colour. Five pools were tested in total against three target strains; 3207 (FetA-on/PorA-wt), 3043 (FetA-off/PorA-wt), and 3312 (FetA-of/PorA-off).

5.3: Discussion

The developmental vaccine MenPF aims to provide high levels of coverage against meningococcal disease by targeting both the PorA and FetA outer membrane protein antigens. These antigens will be presented in meningococcal outer membrane vesicles. Although immunisation with OMVs has been shown to induce protective antibodies against PorA, low concentrations of FetA in the vesicles restrict production of antibodies specific for that protein. The first aim of this study was to determine whether OMVs produced from a strain modified to express high levels of FetA (see Chapter 3) were capable of inducing bactericidal antibodies against both the PorA and FetA antigens in a murine model.

Although FetA-specific antibodies were not induced following a single dose of OMV, two doses resulted in high levels of antibodies specific for both target antigens. Using previously constructed strains, which have expression of PorA and FetA switched on or off in various combinations, it was shown that, following two doses of OMV, antibodies targeting PorA and FetA contribute to complement-mediated killing in a synergistic manner, resulting in high levels of bactericidal activity.

The potential for antibodies to act synergistically during complement-mediated killing was described by Weynants *et al.,* [131] using several minor outer membrane protein antigens. Similar results were reported by Vu *et al.,* [226] for antibodies against the vaccine candidates Factor H Binding protein (fHbp) and Neisserial surface protein A (NspA). Both papers present evidence that, while antibodies to sparsely distributed antigens may have limited bactericidal activity when only a single antigen is targeted, mixtures of antibodies targeting multiple minor outer membrane proteins can result in bactericidal killing. This may be due to increased antibody density on the surface of the bacterium promoting high levels of complement deposition [131,226]. In this study, although antibodies specific for PorA dominated the bactericidal response, the additional binding of FetA-specific antibodies enhanced this

bactericidal activity. However, FetA specific-antibodies induced by OMV immunisation were generally not capable of complement-mediated killing alone in the absence of antibodies to PorA.

The second aim of this study was to determine whether the use of a heterologous prime-boost strategy, involving subsequent doses of OMV or recombinant protein inoculum, could be used to effectively target and increase the bactericidal response towards the FetA antigen. Such a strategy was previously shown to work for the weakly immunogenic PorA P1.7-2,4 (Chapter 4). As seen with PorA, the use of OMV inoculums effectively induced switching to a Th1 response, associated with effective recruitment of complement [205], while use of recombinant protein inoculum resulted in a predominantly Th2 response. As the IgG subclasses present in a Th2 response are ineffective at complement-binding, these do not result in bactericidal killing of meningococci in an SBA [205], and are therefore not associated with protection. Consequently, bactericidal antibody responses were only induced when at least one dose of OMV inoculum was used.

Unlike results seen with OMVs containing P1.7-2,4, where PorA-specific responses were weak, OMVs used in this chapter showed a high level of immunodominance of the PorA and Omp85 antigens. The improved immunogenicity of PorA P1.7,16 (present in the FetA-on strain 3207) compared to P1.7-2,4 has been documented previously [145]. Wedege *et al.,* [169] also reported higher Omp85-specific IgG titres following three doses of MenBvac (H44/76-derived OMVs) than following the same number of doses of MeNZB (NZ98/254-derived OMVs) [169]. This suggests that there may be additional properties of the P1.7-2,4 OMVs derived from strain NZ98/254 in Chapter 4 that result in low immunogenicity compared to OMVs harvested from the H44/76-derived FetA-on strain in this chapter.

Although the use of a heterologous prime-boost strategy improved the concentration of bactericidal antibodies to PorA P1.7-2,4 compared to two doses of OMV, similar strategies did

not improve the concentration of antibodies to FetA here. Levels of FetA-specific antibodies measured by ELISA were lower following immunisation with the prime-boost strategies than using two doses of OMV. Furthermore, it is likely that many of the FetA-specific antibodies induced by the protein dose were directed towards non-surface exposed epitopes and were not responsible for bactericidal killing. Of the strategies tested, the use of two doses of OMV was the most effective at inducing bactericidal antibodies specific for FetA. While some mice immunised with the two heterologous prime-boost strategies showed bactericidal killing against FetA, the levels of bactericidal activity achieved were low and inconsistent; many mice immunised with these strategies did not show any FetA-specific activity in an SBA. As responses in these groups were variable, the potential coverage that could be achieved using these strategies is likely to be unpredictable. Consequently, a two-dose heterologous prime-boost strategy may not be ideal for inducing FetA-specific protection against meningococcal disease.

Overall, antibodies specific for FetA were poorly bactericidal alone. This may be due to low epitope density on the surface of the bacteria, rather than limiting concentrations of antibodies or absolute antigen concentration, as serum induced by immunisation of mice with recombinant FetA in MPL adjuvant was capable of bactericidal activity (data not shown). Results suggest that FetA-specific antibodies induced by a primary dose of OMV immunisation are specific for conformational epitopes. If these antibodies all target a single site, likely to be the variable region [170], then antibody density on the bacterial surface will be limited, resulting in low complement-deposition [206]. In contrast, the use of a recombinant FetA dose may induce antibodies to other surface-exposed FetA epitopes, increasing the antibody density, and consequently increasing complement deposition enough for bactericidal killing without requiring higher antigen density or antibodies to other OMPs. The OMV dose could then induce switching to a Th2 response, required for bactericidal activity, and promote antibody production to FetA-surface epitopes. This may explain the limited FetA-specific

bactericidal activity seen using the prime-boost strategies. The use of more doses, for example two doses of OMV followed by a dose of recombinant protein, may provide higher levels of bactericidal activity against FetA.

A potential issue with the results in this study is that, in the strains used, FetA-expression is increased compared to wildtype strains. As antigen expression levels have been found to be an important determinant of SBA titres [99], and SBA titres following human immunisations are used to predict the protection provided [45], bactericidal activity of FetA-specific antibodies against these FetA-on target strains may not correlate with effective protection against naturally occurring strains *in vivo*. Use of a strain such as 3200 (see 3.2.3, page 61), expressing FetA constitutively at levels similar to wildtype, may be a more reliable estimate of protection. On the other hand, it is difficult to predict whether wildtype expression levels *in vitro* correlate with expression *in vivo*. This is true not only for FetA, but for all antigens with variable expression. However, the presence of FetA-specific antibodies in convalescent sera [164] suggests that FetA is expressed *in vivo*.

We have shown, in mice, that an OMV vaccine containing upregulated FetA F3-3 is capable of inducing antibodies to both the PorA and FetA antigens, and that these antibodies are bactericidal *in vitro*. It is likely that FetA-specific antibodies induced by these OMVs could contribute to protection against meningococcal disease, in particular against strains expressing a homologous PorA type. However, due to low epitope density, an improved formulation or immunisation strategy would be required to increase protective coverage specifically against FetA.

Chapter 6:

Investigation of sequence variation affecting transcription of the *porA* gene

6.1: Introduction

As with many meningococcal proteins, the vaccine antigens PorA and FetA show variation in expression levels [118,166,186]. In developmental meningococcal vaccines, where protein antigens are presented in OMVs, low expression of antigens can result in poor production of specific IgG [227]. Therefore, genetic modification can be used to increase and stabilise expression of antigens in the meningococcal outer membrane for OMV vaccine production [182,227]. This genetic modification, as used previously for the vaccine antigen FetA (Chapter 3), focuses on altering the transcriptional regulation of the gene of interest by replacing the promoter region upstream of the gene. Regulation of *fetA* was altered both by removal of negative regulation by iron and increase of transcriptional initiation using a promoter based on that of the highly-expressed *porA* and *porB* genes. This strategy was optimised using existing knowledge of the genetic factors affecting PorA expression in wildtype meningococci [135]. The aim of the work described in this chapter was to extend that knowledge by investigating additional sequence variation in the promoter region of the *porA* gene that could be associated with differences in transcription among meningococcal isolates.

Sequence variation in a gene or within its control region can alter both the rate of initiation of transcription and the stability of the RNA. Such variation can ultimately change the amount of protein expressed from the RNA transcript without affecting expression levels of other proteins. Although protein expression can also be affected by factors acting post-transcription [228], many of these factors act across multiple genes simultaneously and cannot be targeted to a single gene or operon. Therefore, to control expression levels of specific antigens,

knowledge of factors directly affecting transcription could be used during genetic modification of meningococcal strains for OMV vaccine production. This genetic modification enables optimisation of expression of both vaccine antigens and unwanted proteins to ensure that the appropriate antigens are correctly targeted by the immune system.

Variable expression of the antigens targeted by protein based vaccines in meningococci can alter the ability of specific antibodies to facilitate killing. If antigen levels are low, the reduced density of antibody binding corresponds to reduced levels of complement deposition. At very low antigen densities, complement deposition and formation of membrane attack complexes can be below the threshold at which bactericidal killing occurs [99]. Therefore, this study also aimed to investigate the distribution of transcriptional variation among isolates. As the meningococcal vaccine MenPF (discussed in Chapter 1) aims to target the main disease-causing ccs in Europe, patterns of expression in different meningococcal lineages could affect vaccine efficacy against the targeted complexes.

As a major vaccine antigen, the regulation of PorA expression has been well characterised [135]. Variable expression of the PorA protein has been linked to the presence of a poly-G repeat region between the -10 and -35 sites of the *porA* promoter [135,186]. As the RNA-polymerase binds to both the -10 and -35 sites during transcriptional initiation, changes in the length of this repeat and the twist of the DNA affect the relative positions of these binding sites. Particular orientations are optimal for RNA-polymerase binding, while other orientations lead to weak binding and, consequently, to reduced rates of transcriptional initiation [229].

For *porA*, a spacer length of 17bp or 18bp between the -10 and -35 sites leads to the highest level of protein expression [135]. However, studies in other organisms have shown that additional mechanisms exist to alter protein expression through transcriptional regulation. For example, in *E. coli*, promoter sequence variation can affect binding of transcriptional factors that can enhance or inhibit transcription [230]. Nucleotide changes within the RNA can also

alter its stability, varying final protein concentrations by affecting the length of time that the RNA is available as a template for translation [231]. Similar mechanisms may be employed in the meningococcus to regulate *porA* expression in addition to the previously described regulation mediated by the length of the poly-G repeat.

Here, genetic sequences upstream of the *porA* gene were analysed alongside quantitative RT-PCR (qRT-PCR) measurements of *porA* RNA. As this assay measures the total quantity of RNA present, the results were affected by both the production and degradation rates of the RNA. The aim of collecting this data was not only to corroborate previous evidence on the effect of spacer length with information from an additional panel of isolates, but also to extend understanding of the sequence variation that could affect *porA* expression levels by other mechanisms.

6.2: Results

6.2.1: Diversity in the spacer length between the *porA* -35 and -10 sites

As variation between the -35 and -10 sites has been shown to affect PorA expression levels [135], sequences upstream of the *porA* gene were extracted from whole genome sequence data (Bratcher *et al.,* unpublished) available for 107 meningococcal reference isolates [232] (listed in Appendix 1, page 218). For one isolate (DK 24), no *porA* sequence data was available as both the *porA* gene and the upstream promoter region had been deleted.

For all remaining isolates, the sequences upstream of *porA* were aligned according to the ClustalW algorithm using MegAlign Lasergene Software (DNASTAR) and a consensus sequence was established (Figure 6.1). The -35 and -10 regions, as described previously [186], are separated by a spacer region containing a poly-G repeat. This repeat contained between 9 and 15 bases. The length of this repeat region could not be determined for 8 isolates due to

termination of the sequencing read within the poly-G repeat (Appendix 4, page 231). All variation in the length of the spacer region was due to varying numbers of bases in the poly-G repeat. Other variation within the spacer region will be described separately (6.2.3).

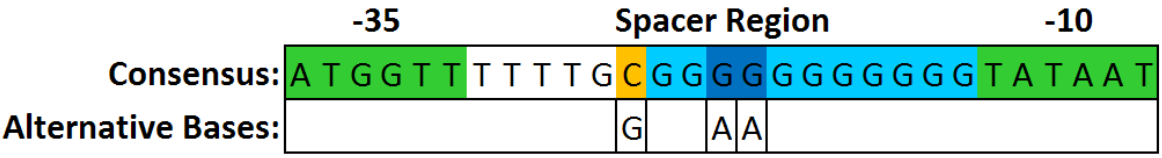
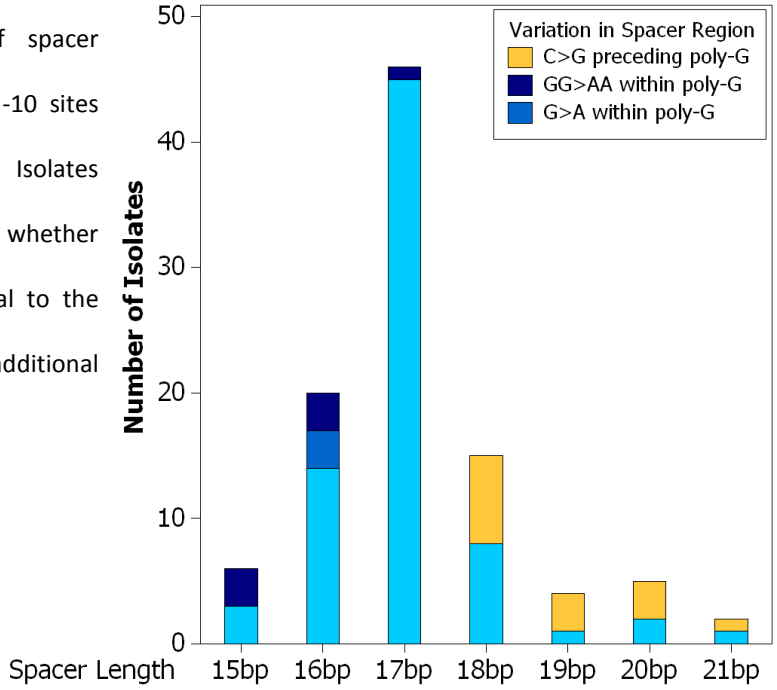


Figure 6.1: Consensus sequence for the promoter upstream of the *porA* gene in the meningococcal reference strains. The -10 and -35 sequences are highlighted in green. The poly-G repeat in the spacer region is shown in light blue. Bases at which interruptions occur in the repeat region are highlighted in dark blue. A C>G polymorphism is highlighted in orange.

Of the 98 isolates for which the length of the spacer region in the *porA* promoter could be determined, almost half (47 isolates) had a total spacer length of 17bp (Figure 6.2). Spacer lengths of 16bp and 18bp were also common, present in 20 and 15 isolates, respectively. Other spacer lengths were less common, collectively only found in 17 isolates (Figure 6.2).

Figure 6.2: Distribution of spacer lengths between the -35 and -10 sites upstream of the *porA* gene. Isolates were characterised according to whether the spacer region was identical to the consensus or contained additional variation, shown in Figure 6.1.



6.2.2: Variation in *porA* transcript levels due to spacer length

Quantitative reverse-transcription PCR (qRT-PCR) was used as a high-throughput method of comparing transcription of *porA* in the 107 meningococcal reference isolates set. The housekeeping gene *gdh* was chosen as a constitutively-expressed internal control, as it has been used for meningococcal transcription studies previously [233]. Relative Quantity (RQ) values of *porA* RNA were calculated relative to levels of *gdh* RNA in each sample.

Of the 107 reference isolates, 9 were no longer present in the NIBSC collection (Appendix 1, page 218). Total RNA was isolated from the remaining 98 isolates following growth in MH broth. Total RNA from H44/76 was also extracted for use as a positive control, as expression of both PorA and FetA in this isolate had already been characterised (Chapter 3). RQ values for each total RNA sample were normalised to expression levels in this positive control sample. All normalised RQ values were subsequently transformed using the natural logarithms (ln) transformation prior to further analysis. Unless otherwise stated, all statistical analysis was completed using General Linear Models for analysis of variance. This analysis allowed the variance in measured ln(RQ) values to be partitioned according to multiple discrete sequence differences.

Complete *porA* promoter sequences and total RNA were available for 94 isolates (Appendix 4, page 231). Levels of *porA* transcription were linked to the length of the spacer region between the -10 and -35 sequences (Figure 6.3, $P < 0.001$). Levels of *porA* RNA values were highest in isolates containing the 18bp or 19bp spacer regions (Mean ln(RQ) = 0.33 and 0.40, respectively), although only 4 isolates with the 19bp spacer region were available for analysis. With the removal of bases from the 18bp or 19bp maximum, RNA levels decreased in a stepwise manner. Similarly, isolates containing longer spacer lengths than the 19bp maximum showed comparatively reduced levels of *porA* RNA. However, isolates containing the 21bp spacer showed a higher mean RNA quantities than isolates containing the 20bp spacer region

(Mean ln(RQ) = -0.36 versus -0.55), although only two and five data points were present, respectively.

Quantities of *porA* RNA were found to be significantly higher in isolates containing a promoter with a 17bp or 18bp spacer than in isolates containing the 16bp spacer region ($P < 0.001$, mean ln(RQ) = -0.03 versus -0.84). However, RQ values were not significantly different between isolates containing the 17bp and 18bp spacer regions ($P = 0.109$). There were too few data points for isolates containing other spacer lengths for statistical comparison.

Overall, there was a high level of residual variability of expression that could not be explained by spacer length. Differences in spacer length alone accounted for only a small proportion of the total variation in ln(RQ) values among isolates (Spacer Length Adjusted Sum of Squares (AdjSS) = 22.68, Residual AdjSS = 58.68, Adjusted R^2 = 22.90%).

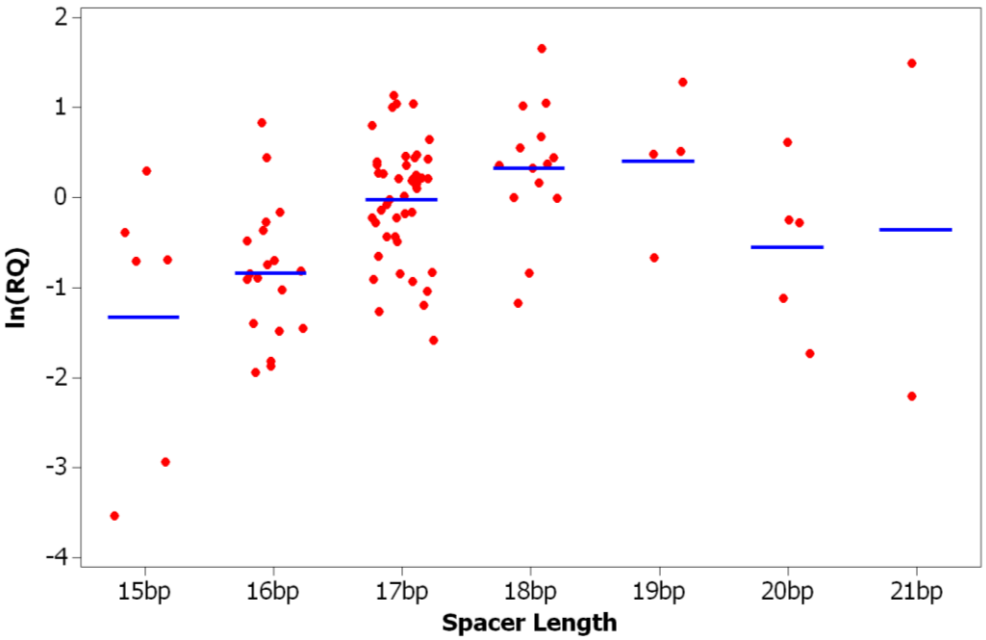


Figure 6.3: ln(RQ) values obtained for *porA* according to the length of the spacer region between the -35 and -10 sequences in the *porA* promoter. The ln(RQ) values for individual isolates are plotted in red, with mean values for each spacer length shown as blue lines.

6.2.3: Other diversity between the *porA* -35 and -10 sites

As isolates containing the same spacer lengths showed very diverse *porA* transcript levels, the contribution of other sequence differences was investigated. As well as spacer length, nucleotide replacements between the -35 and -10 sites have also been found to affect PorA expression, with previous evidence suggesting that substitutions within the poly-G repeat lead to increased [135] or decreased PorA expression levels [234].

Within the reference isolates, the poly-G repeats in three isolates (312 901, 94/155, and 38VI) were interrupted by a single adenine base. The poly-G repeat regions of seven isolates (500, NG P20, MA-5756, M597, D1, 90/18311, and F1576) were interrupted by two sequential alanine bases (Figure 6.1). These interruptions were only found in isolates containing the shorter spacer lengths (15 - 17bp, Figure 6.2), and predominantly within the ST-11 complex. It may be that the presence of the substitutions is associated with a higher error rate in ST-11. Alternatively, the substitutions may reduce the frequency of slipped-strand mispairing and, consequently, isolates containing these substitutions are more likely to replicate with the same spacer length rather than undergoing phase variation to longer lengths.

Whether there was an association between these interruptions in the poly-G repeat and the levels of *porA* RNA measured was investigated statistically for those isolates containing the 16bp spacer length (for which 5/20 contained G>A or GG>AA interruptions). For these isolates, using a two-sample t-test, there was no significant difference between isolates containing interruptions within the poly-G and those without ($P = 0.517$, Figure 6.4).

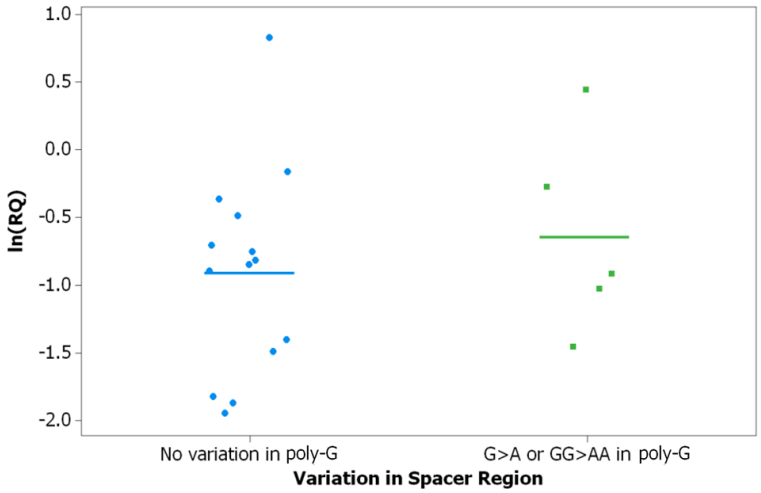


Figure 6.4: ln(RQ) values obtained for *porA* for isolates containing the 16bp spacer length. Isolates are grouped according to whether the poly-G repeat sequence contains G>A substitutions (shown in green) or whether the poly-G repeat is uninterrupted (shown in blue). The ln(RQ) values for individual isolates are plotted, with mean values for each group shown as horizontal lines.

In 14 isolates (44/76, 71/94, 50/94, 400, 91/40, L93/4286, 26, 139M, EG 327, 79128, 690, 255, 254, and NG144/82), there was a substitution of C>G preceding the poly-G repeat (Figure 6.1), lengthening the poly-G repeat sequence while retaining the same spacer length. This C>G polymorphism was only found in isolates containing the longer length spacer regions (18 - 21bp, Figure 6.2) and was not associated with any particular clonal complex (data not shown). This suggests that this substitution has arisen independently in multiple isolates and that the probability of this substitution occurring may be increased by longer poly-G repeat sequences. This is consistent with an observation by Richardson *et al.,* [235] that longer lengths of repeat sequence within the meningococcal *hmbR* gene are associated with increased chances of error during DNA replication [235].

The effect of the C>G polymorphism on *porA* RNA levels was tested using a two-sample t-test for those isolates containing the 18bp spacer length, as 5/13 isolates containing this spacer

length also had the C>G substitution (Figure 6.5). In these isolates there was no significant association between this polymorphism and ln(RQ) values (P = 0.323). Therefore, besides differences in spacer length, sequence variation between the -35 and -10 sites did not significantly influence variation in *porA* RNA expression within this data set.

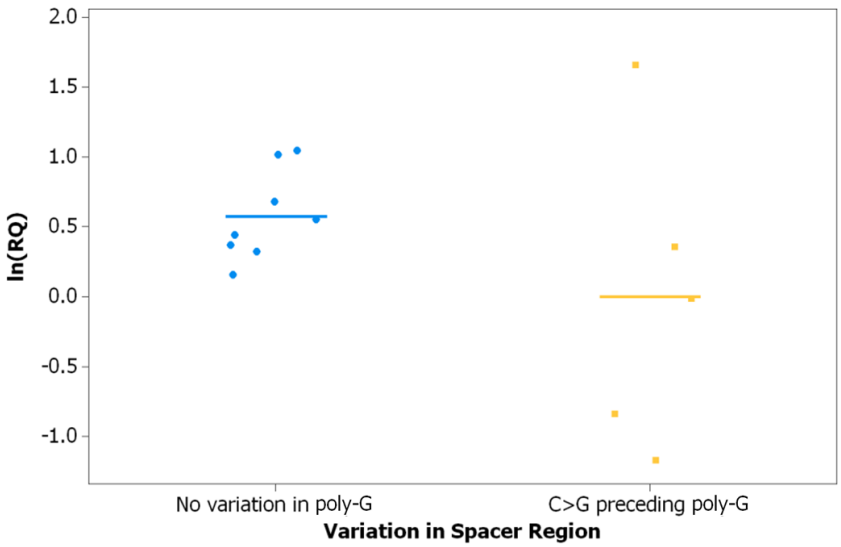


Figure 6.5: ln(RQ) values obtained for *porA* for isolates containing the 18bp spacer length. Isolates are grouped according to whether or not there is a C>G substitution preceding the poly-G repeat sequence (isolates with the substitution are shown in yellow, while those without are shown in blue). The ln(RQ) values for individual isolates are plotted, with mean values for each group shown as horizontal lines.

6.2.4: Variation in *porA* transcription due to sequence variation surrounding the -35 and -10 sites

As sequence variation within the spacer region between the -35 and -10 sites could only account for a small proportion of the total variance in levels of *porA* RNA measured among the 107 isolates, sequence variation outside of this region was also compared. Nucleotide polymorphisms surrounding the -35 and -10 sites can affect transcriptional initiation or mRNA stability [230,231]. Therefore, the sequence between the ATG start codon and position -85 from the transcriptional start site was investigated (Figure 6.6). Overall, the *porA* promoter

was found to be highly conserved, with an average sequence identity of 97.4%. Nine points of sequence variation within this region were observed, although single nucleotide polymorphisms (SNPs) found in fewer than 5 isolates were not documented as no statistical analysis could be completed to investigate associations between those base changes and *porA* transcript levels within this set of isolates.

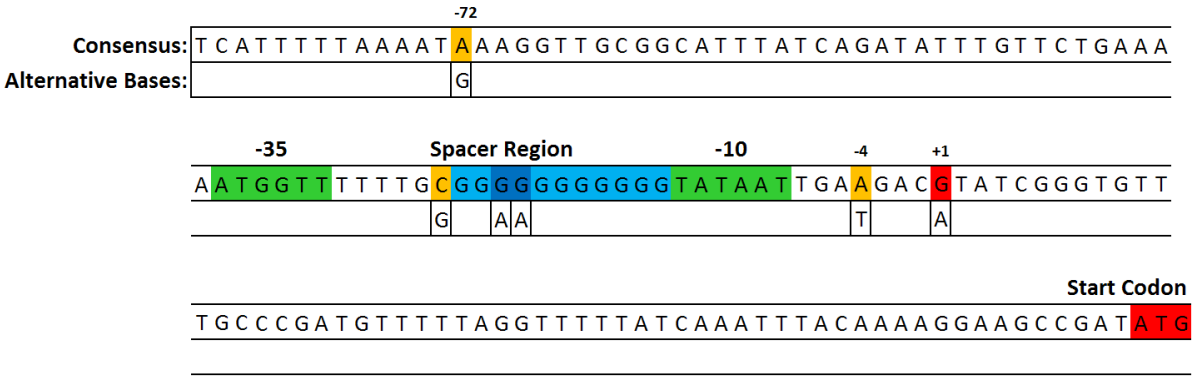


Figure 6.6: Consensus sequence for the promoter upstream of the *porA* gene in the 107 meningococcal reference isolates. The sequence investigated was between the ATG start codon and position -85 from the transcriptional start site. The transcriptional start site (+1) and translational start codon are highlighted in red. The -10 and -35 sequences are highlighted in green. The poly-G repeat is highlighted in light blue, and bases at which interruptions occur in the repeat region are shown in dark blue. Positions of other SNPs are highlighted in orange.

In twelve isolates (F6124, 204/92, Z2491, 26, 243, D1, NG E30, 690, 38VI, 255, S3131, and 10) a substitution of A>G at position -72 from the transcriptional start site (Figure 6.6) was present. There was also variation in the -4 to +1 sequence, with three alternative sequences present: AGACA (type 1), AGACG (type 2), TGACA (type 3).

Whether such sequence variation to could be linked to *porA* RNA levels was assessed by analysis of variance to determine whether the polymorphisms are associated with transcription or mRNA stability. The effect of the A>G polymorphism at position -72 on RNA levels was tested using isolates containing the 18bp spacer length (of which 3/13 contained

the A>G substitution at position -72). However, a two-sample t-test showed no significant difference between isolates with an A or a G base at this position (Figure 6.7, P = 0.649).

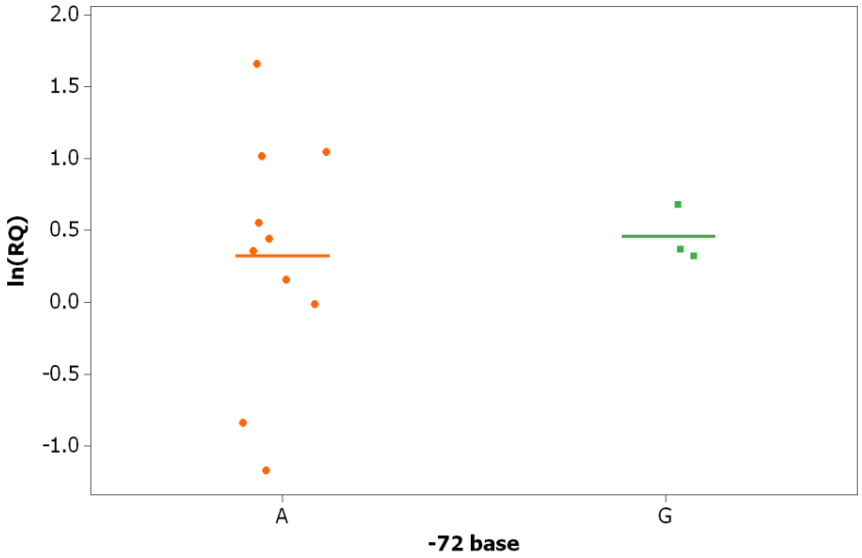


Figure 6.7: ln(RQ) values obtained for *porA* for isolates containing the 18bp spacer length. Isolates are grouped according to whether they contain an A base (shown in orange) or a G base (shown in green) at position -72 with respect to the transcriptional start site. The ln(RQ) values for individual isolates are plotted, with mean values for each group shown as horizontal lines.

There was a significant correlation between the -4 to +1 sequence and the residual ln(RQ) values obtained after variance attributed to spacer length had been removed (Figure 6.8). Only values for isolates containing the 16bp, 17bp or 18bp spacers were analysed, as these spacer lengths have sufficient data points for confidence in the mean. RNA from isolates containing -4 to +1 sequence type 1 (AGACA) showed significantly higher residual ln(RQ) values than RNA from isolates containing type 2 (AGACG, P = 0.021) sequences. Although the mean value obtained for isolates containing the type 1 sequence was also higher than the mean value from isolates containing the type 3 sequence, this difference did not achieve significance (P = 0.052). There was no significant difference between isolates containing type 2 and type 3 sequences (P = 0.752). However, the spacer length and the sequence present at the -4 to +1 sites

collectively could only account for a small proportion of the total variance in the *porA* RNA concentrations measured. After variance due to spacer length (AdjSS = 9.38) and -4 to +1 sequence (AdjSS = 1.92) had been partitioned, the unexplained variance was still high (Error AdjSS = 32.92, Adjusted R^2 = 26.4%).

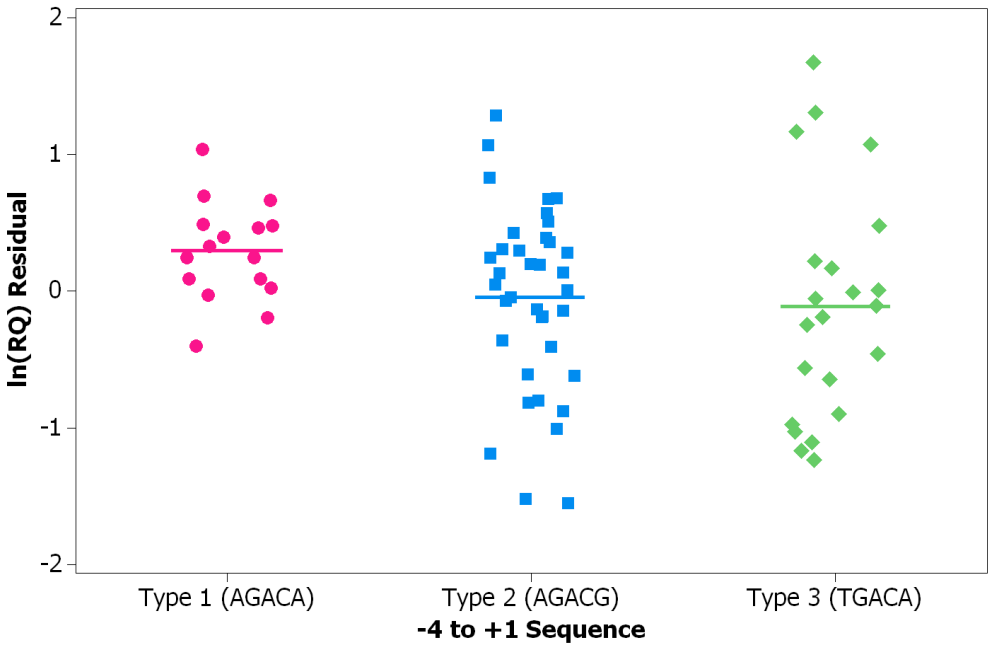


Figure 6.8: Residual ln(RQ) values obtained for *porA* after variance due to spacer length had been partitioned. Values are grouped according to the -4 to +1 sequence upstream of the *porA* gene: Type 1 (AGACA, shown in pink); Type 2 (AGACG, shown in blue); Type 3 (TGACA, shown in green). Only values for isolates containing the 16bp, 17bp or 18bp spacer lengths were analysed. The residual values for individual isolates are plotted, with mean values for each sequence shown as horizontal lines.

6.2.5: Comparison of *porA* transcript levels among clonal complexes

Variation within the *porA* promoter region was catalogued according to cc, as isolates within the same cc are closely related [232] and therefore more likely to share similar genetic polymorphisms throughout the genome than isolates from different ccs. If variation in genetic

factors affecting *porA* transcription is phylogenetically structured in this way, the resulting patterns of expression may affect the efficacy of a PorA-based vaccine against particular ccs.

The panel of 107 isolates contained meningococci from 50 different STs across 23 ccs, with 9 isolates not characterised into a cc (Appendix 1, page 218). The most common ccs were the ST-41/44 complex (14 isolates); ST-5 complex (12 isolates); ST-4 complex (11 isolates); ST-11 complex and ST-32 complex (10 isolates each); ST-8 complex (8 isolates). Eleven ccs were present only once.

Possible associations between *porA* RNA levels and cc were investigated for all ccs with greater than two isolates within the meningococcal reference set (Figure 6.9). Clonal complexes containing ≤2 isolates, or isolates not assigned to a cc, were not analysed, as these groups contained insufficient data points for statistical comparison, although values for these isolates were included in the calculation of the sample mean.

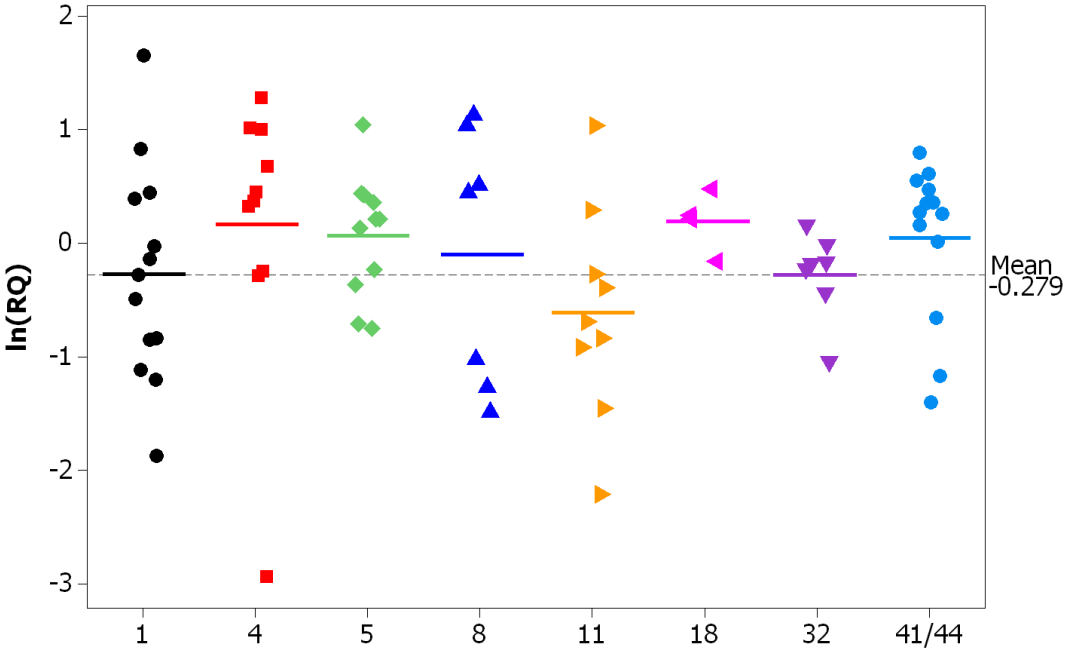


Figure 6.9: Distribution of *porA* ln(RQ) values among clonal complexes. Symbols show ln(RQ) values for individual isolates. Means for each group are plotted as lines, with the mean for the total sample (all available data points) shown as a dashed lined. Clonal complexes for which >2 isolates are available are shown. Isolates from all other clonal complexes are not shown, but were included in the calculation of the mean.

From these data, several trends were apparent. Levels of *porA* RNA were highest in isolates belonging to the ST-4 complex. However, one isolate in the ST-4 complex, A4/M1027, showed very low *porA* transcription compared to all other isolates. A4/M1027 was the only isolate in that clonal complex to contain the type 3 sequence (TGACA) at the -4 to +1 region rather than the type 1 sequence (AGACA), and also the only isolate in that cc to contain a 15bp spacer length rather than a longer spacer length. These combined factors may explain the low transcript levels.

Mean *porA* transcription was also high in isolates belonging to the ST-5, ST-18 and ST-41/44 complexes. Isolates belonging to the ST-11 complex showed low *porA* RNA concentrations compared to the sample mean. Mean transcription from other clonal complexes were similar to the sample mean.

Variability in *porA* transcription was highest in ST-1, ST-8 and ST-11 complexes (ln(RQ) Standard deviations = 0.95, 1.13 and 0.94, respectively), while transcription from the ST-5, ST-18 and ST-32 complexes showed less variability (ln(RQ) Standard deviations = 0.54, 0.27 and 0.39, respectively). Overall, no significant link was found between clonal complex allocation and *porA* RNA levels by analysis of variance (P = 0.119).

6.2.6: Distribution of variation among clonal complexes

Some of the variation in *porA* transcription among isolates has already been attributed to sequence variation within the *porA* promoter region, in particular with the spacer length between the -35 and -10 sites. To determine whether this sequence variation could account for the differences in *porA* transcription among clonal complexes, the distribution of the various spacer lengths among the clonal complexes was investigated.

The different spacer lengths were found to be evenly distributed among ccs (Figure 6.10). Overall, the *porA* promoter was variable within ccs; most ccs contained at least 3 different spacer lengths. The ST-32 complex showed less variability, exclusively containing the 17bp or 18bp spacer lengths.

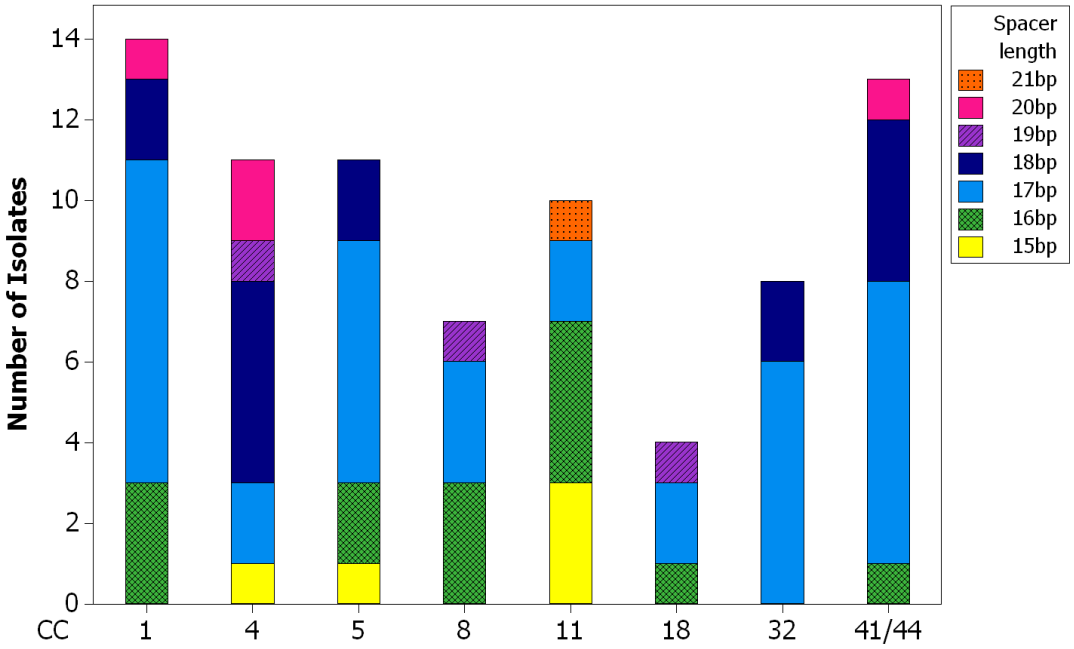


Figure 6.10: Distribution of spacer lengths between the -35 and -10 regions upstream of the *porA* gene across the ccs represented by >2 isolates.

Isolates in the ST-32 complex may have more effective mismatch-repair systems that reduce the overall rate of phase variation by this mechanism [236]. Similarly, isolates belonging to the ST-4 and ST-11 complexes, containing a wide variety of spacer lengths, may have polymorphisms in genes encoding mismatch-repair proteins, which make nucleotide substitutions in repeat regions more likely. A high proportion of meningococci expressing the serogroup A capsule (associated with the ST-1, ST-4 and ST-5 complexes), have been found to show elevated mutability due to defective mismatch-repair systems [235]. Alternatively, the distributions found here may be an artefact of the sample, and a larger sample size may reveal a normal distribution among all ccs.

The variation in transcription among ccs may be due to differences in the frequencies of the spacer lengths. For example, as the 17bp and 18bp spacer lengths have been linked to the highest transcript levels, the presence of only these spacer lengths in ST-32 isolates could be expected to result in high levels of *porA* RNA in this cc. In order to test this association, variance in ln(RQ) values was first partitioned by spacer length. Only values for isolates containing the 16bp, 17bp or 18bp spacers were analysed, as these spacer lengths have sufficient data points for confidence in the mean. Following partition of variance according to spacer length, the residual sums of squares (residuals) were analysed by cc (Figure 6.11).

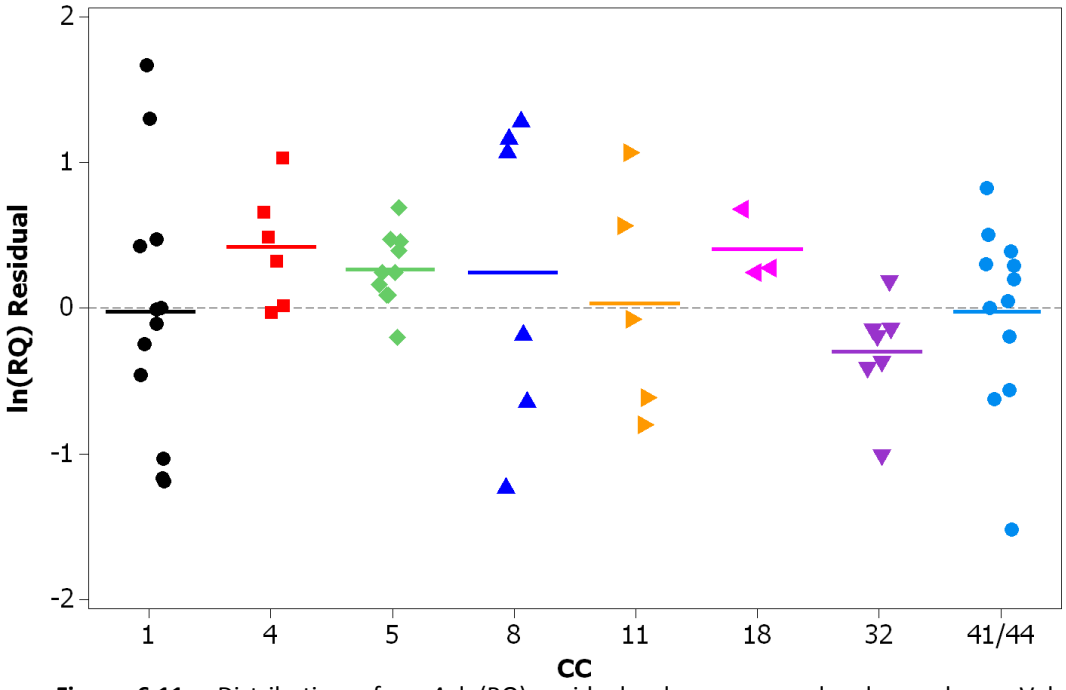


Figure 6.11: Distribution of *porA* ln(RQ) residual values among clonal complexes. Values plotted are residual sums of squares following partitioning of variance by spacer length. Only values for isolates containing the 16bp, 17bp or 18bp spacer lengths were analysed. Symbols show residual values for individual isolates. Means for each group are plotted as lines, with the expected residual (a value of 0) shown as a dashed line. Clonal complexes for which >2 isolates are available are shown. Isolates from all other clonal complexes are not shown, but were included in the calculation of the mean values for each spacer length (from which the residuals were calculated).

There was no significant association between the residual values and ccs when tested by analysis of variance (P = 0.534). However, average residual values for ST-4, ST-5 and ST-18 complexes were still higher than expected (residual values > 0). Mean residual values were lower than expected for the majority of isolates belonging to the ST-32 complex (residual values < 0). Isolates within the ST-8 complex could be divided into two distinct groups: 3 isolates with high residual values and 3 isolates with low residual values.

As the sequence present at the -4 to +1 sites was significantly associated with varying transcript levels, the distribution of this sequence variation among ccs was investigated to determine if this could account for the remaining variation in *porA* RNA levels among ccs (Figure 6.12).

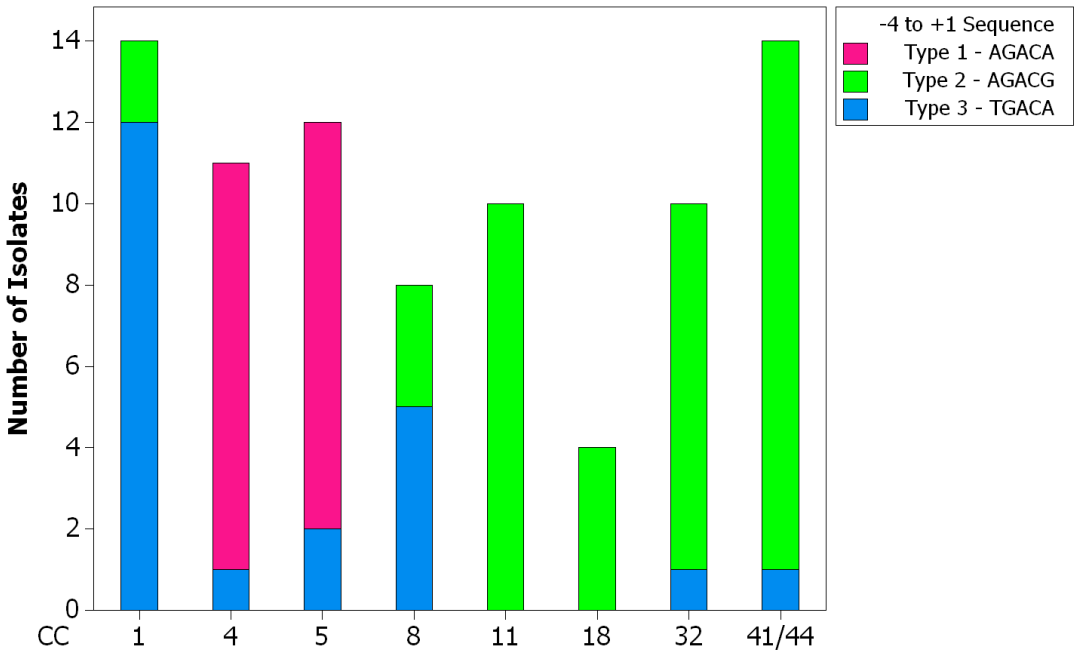


Figure 6.12: Distribution of the variation in the -4 to +1 sequence upstream of the *porA* gene across the ccs represented by >2 isolates.

The variation at the -4 to +1 sequence was unevenly distributed among clonal complexes. The type 1 sequence (AGACA), associated with high transcript levels, was predominantly present in the ST-4 and ST-5 complexes. Only 1/11 and 2/12 isolates from ST-4 and ST-5, respectively, contained alternative sequence at these positions (type 3 sequence, TGACA). The type 1

sequence was not restricted to these complexes, however, as 1/2 isolates belonging to the ST-269 complex also contained AGACA bases at the -4 to +1 sites (data not shown).

Of isolates belonging to the ST-1 complex, 12/14 contained the type 3 sequence (TGACA, associated with low expression). The two isolates within the ST-1 complex that contained the type 2 sequence rather than the type 3 sequence belonged to a distinct sequence type (ST-3) within that cc.

All isolates belonging to ST-11 and ST-18 complexes exclusively contained the type 2 sequence (AGACG). Similarly, the majority of isolates belonging to the ST-32 and ST-41/44 complexes contained the type 2 sequence. The variation within these complexes was segregated according to sequence type (data not shown). Isolates belonging to the ST-8 complex contained either the type 2 or type 3 sequence, although variation in this cc could not be assigned to distinct STs.

As the type 1 sequence, associated with the highest transcript levels, is predominant within ST-4 and ST-5 complexes and has limited presence in other ccs, it was not possible to complete an intra- or inter-cc comparison of the effect of this sequence. It may be that the association between the type 1 sequence and high transcript levels is not due to a direct effect but rather to an indirect link to other genetic factors associated with the ST-4 and ST-5 complexes.

However, comparisons between clonal complexes containing the same -4 to +1 sequence showed differences in *porA* RNA levels (Figure 6.11). Isolates belonging to the ST-11, ST-18, ST-32 and ST-41/44 complexes predominantly contain the type 2 sequence at the -4 to +1 sites. After variation due to spacer length has been accounted for, residual variation in RNA levels among these isolates differs among the ccs. Residual values for the ST-11 and ST-41/44 complexes have means close to the expect residual (zero). The mean residual value for the ST-18 complex is higher than expected, while the mean residual value for the ST-32 complex is lower than expected. These discrepancies in *porA* RNA levels cannot be attributed to any

sequence variation documented in this study and is likely to be due to other factors not investigated here.

Within the ST-8 complex, three isolates showed higher transcript levels than expected and three isolates showed lower levels than expected. This high variation, both within the cc and within STs belonging to this complex, could not be attributed to sequence variation studied here. None of spacer length, poly-G interruptions or the -4 to +1 sequence present could be used to divide the isolates in the ST-8 complex into the high- and low-expression groups (Table 6.1).

Relative Expression	Isolate	ST	Spacer Length	Interruption in poly-G	-4 to +1 type	ln(RQ)	Residual
Low	G2136	8	18	-	3	-1.27	-1.24
	B6116/77	10	17	-	3	-1.49	-0.65
	94/155	66	17	A	2	-1.02	-0.18
High	AK22	153	18	-	3	1.04	1.07
	SB25	8	18	-	3	1.13	1.16
	312 901	8	17	A	2	0.44	1.28

Table 6.1: Documented sequence variation upstream of the *porA* gene in the isolates belonging to the ST-8 complex. Isolates are grouped into those with high relative *porA* transcription and those with low *porA* transcription. The residual value is the residual sum-of-squares after variance due to spacer length has been partitioned.

6.3: Discussion

For the vaccine antigen PorA, levels of protein expression have been linked to the length of the spacer region between the -35 and -10 sites upstream of the gene [135,186]. In this chapter, the diversity of sequences upstream of the *porA* gene was investigated alongside variation in *porA* transcript levels for 107 meningococcal reference isolates to determine whether differences in promoter sequences other than those already described may affect gene expression.

Within the 107 isolates studied, the length of the spacer region between the -35 and -10 sites varied between 15bp and 21bp. Variation in the length of the spacer was found to correlate with transcript levels; spacer lengths from 17bp to 19bp resulted in the highest levels of *porA* RNA. The association between the length of the poly-G repeat and *porA* transcription described here shows a similar pattern to that described for PorA protein expression by van der Ende *et al.,* [135,186].

Earlier studies have also shown that other sequence variation between the -35 and -10 sites can also affect PorA expression. Sawaya *et al.,* [234] showed that introducing a mutation in the poly-G tract reduced PorA expression, possibly by affecting the orientation of the -35 and -10 binding sites relative to each other. Conversely, data presented by van der Ende *et al.,* [135] suggest that substitutions in the poly-G tract lead to increased levels of PorA expression. Here, there was found to be no significant association between sequence variation within the spacer region and transcript levels. This may suggest a low sensitivity in this assay, or that these associations are not being detected due to high levels of transcriptional variation among isolates due to other factors.

However, in an observation unique to this study, the sequence between the -4 to +1 positions was associated with *porA* RNA quantities. The sequence associated with highest transcription was present in isolates belonging to the ST-4 and ST-5 complexes. Deviation at either the -4 position or at the +1 position was linked with reduced transcription. It may be that additional transcription factors binds across these positions to stabilise the RNA-polymerase association with the DNA [230] and that nucleotide polymorphisms at these positions can improve or reduce binding of those transcription factors. Alternatively, the association between this polymorphism and high *porA* transcription may be due to an indirect link with cc rather than a direct link between sequence and transcript levels. This highlights the difficulty of investigating the causes of transcriptional differences among ccs, as, within each cc, the whole genome is expected to be closely-related. Therefore there may appear to be an association between a

particular genotype and phenotype, while the two are only indirectly linked by shared ancestry. The effect of this sequence variation at the -4 and +1 positions upstream of *porA* could, in future, be confirmed by genetic modification and analysis of mutant strains. If successful, a single nucleotide change at either of these positions could be used in genetic modification to increase expression of antigens in meningococci for use in OMV vaccine production.

There was a high level of variation in *porA* transcription that could not be statistically associated with any of the sequence variation documented here. It is difficult to determine how much of the residual variability among isolates is due to additional factors acting to control transcription, or whether this variability is inherent within the assay used, or as a result of sample collection. For example, Baart *et al.,* [134] showed that the growth rate at which meningococcal OMVs are harvested affects the concentration of the PorA protein within those OMVs. Similar effects may act in different ways among isolates, or it may be that, although RNA was sampled at the same time point for all isolates, the RNA was not extracted at the same growth phase for all isolates. In this case the observation made by Baart *et al.,* [134] may account for the high variation in the transcript levels measured.

There was a high level of variation in *porA* transcription that could not be statistically associated with any of the sequence variation documented here. It is difficult to determine how much of the residual variability among isolates is due to additional factors acting to control transcription, or whether this variability is inherent within the assay used, or as a result of sample collection. For example, Baart *et al.,* [134] showed that the growth rate at which meningococcal OMVs are harvested affects the concentration of the PorA protein within those OMVs. Similar effects may act in different ways among isolates, or it may be that, although RNA was sampled at the same time point for all isolates, the RNA was not extracted at the same growth phase for all isolates. In this case the observation made by Baart *et al.,* [134] may account for the high variation in the transcript levels measured. There is also likely to be a high

level of variation between RNA samples taken from the same isolate on multiple days, due to the competition and interactions of the meningococcal cells in the culture during growth, and the changes that are selected for as a result of that. These changes will vary among cultures of the same isolate, and, as transcription of each gene is affected by competition with other genes for RNA polymerase and other transcription factors, small changes in the transcription rates of unconnected genes will indirectly affect levels of *porA* and *gdh* transcript. Much of the variation seen in relative *porA* transcript levels measured here could, therefore, be a result of sampling.

Although the level of variation in *porA* transcription was high among isolates, none of the ccs investigated showed low enough *porA* transcript levels to cause concern for vaccine coverage. Almost most half of all isolates investigated contained the 17bp spacer, associated with high expression levels, between the -35 and -10 sites upstream of *porA*. This suggests that PorA expression is likely to be high in the majority of meningococci. However, there was variation in spacer lengths among clonal complexes that lead to differences in transcription. For example, a high proportion of isolates in the ST-11 complex contained sub-optimal 15bp and 16bp spacer lengths and showed correspondingly low levels of *porA* transcription. Isolates within the ST-41/44 complex, on the other hand, contained a high proportion of the optimal 17bp and 18bp spacer lengths and showed comparatively high levels of transcription.

While isolates here contained spacer lengths between 15bp and 21bp in length, van der Ende *et al.,* [135] found lengths to range from 14bp to 24bp. Within the strain panel, meningococci were also found to contain varying numbers of thymine bases within the spacer region, or substitutions in various places within the poly-G repeat [135]. The same was not found in the meningococci studied here. Thymine bases within the spacer regions were identical for all isolates, and substitutions (all G>A) within the poly-G repeat were only found in two positions. Therefore, the variation described in the 107 isolates used here, and in panels described previously, is not representative of total meningococcal diversity upstream of the *porA* gene.

Isolates may also have differences in the rates of translation of the PorA protein that are not measured by the assay used here but could affect vaccine coverage. van der Ende *et al.,* [135] report that some meningococci developed mutations within the coding region that stopped expression of the full protein. These frame-shift mutations were due to slipped strand mispairing in a poly-adenine repeat region downstream of the ATG start codon, adding or removing bases from the 7bp repeat that allows in-frame translation. Such difference in translation would not be detected using the qRT-PCR employed in this study, although all but one isolate in the 107 meningococcal reference set used here were found to contain a 7bp poly-adenine repeat (data not shown). The single isolate (120M) found to deviate from the 7bp repeat was not available for transcriptional analysis.

Investigation of meningococcal diversity is highly dependent on the strain panel used. Although chosen to be representative of meningococcal diversity, the panel used here is dominated by isolates from invasive disease and from the main disease-causing ccs at the time the set was established [232]. Due to the population structure of meningococci, genetic diversity is known to be greatest in carriage isolates. Furthermore, ccs such as the ST-23 and ST-269 complexes have become common both in carriage and disease in recent years [237], but are poorly represented in the 107 isolates here. Although the sequence and transcriptional variation described here may not be exhaustive of meningococcal diversity, the information collected adds to that presented by van der Ende *et al,.* [135,186], and also suggests other points of sequence variation that may contribute to differences in *porA* transcription among ccs. This sequence variation can be investigated further using additional isolates or genetic modification and, if shown to be effective, can be used to optimise expression of antigens for the development of OMV vaccines.

Chapter 7:

Investigation of promoter sequence variation affecting transcription of the *fetA* gene

7.1: Introduction

The meningococcal outer membrane protein FetA has previously been considered a poor vaccine antigen due to its variable expression [172]. In particular, the negative regulation of FetA expression under iron replete conditions is considered a problem for the use of this protein as a vaccine component. To ensure a good response against FetA is induced following vaccination with an OMV vaccine the antigen must be present in sufficiently high concentrations. This was achieved, as discussed in detail in Chapter 3, by modifying the vaccine strain to constitutively express increased quantities of FetA. However, sufficient concentrations of the protein in the outer membrane of meningococci *in vivo* are essential for killing by specific antibodies [99]. Therefore, coverage offered by a vaccine containing FetA could be influenced by variation in expression across diverse strains. In this study, differences in *fetA* transcription, which are likely to result in differences in antigen expression, were measured. In addition, the promoter sequence, assumed to play a role in the control of transcription, was analysed across a large sample of meningococcal isolates to evaluate what effect transcriptional regulation may have on the potential coverage of a FetA-based vaccine.

Variation in the length of the poly-cytosine (poly-C) repeat region between the -35 and -10 sites upstream of *fetA* alters the level of gene expression. Delany *et al.,* [166] found that a mutant containing a run of 10 bases in the poly-C repeat transcribed higher levels of *fetA* RNA than a strain modified to contain a repeat of 11bp. In the case of the meningococcal protein PorA, the length of the repeat sequence between the -35 and -10 hexamers is part of the control mechanism determining levels of protein expression [135]. This is attributed to the

distance between the -35 and -10 sites affecting the strength of the association between the DNA and the RNA-polymerase (Figure 7.1 and [229]). A similar mechanism may be used to control *fetA* transcription.

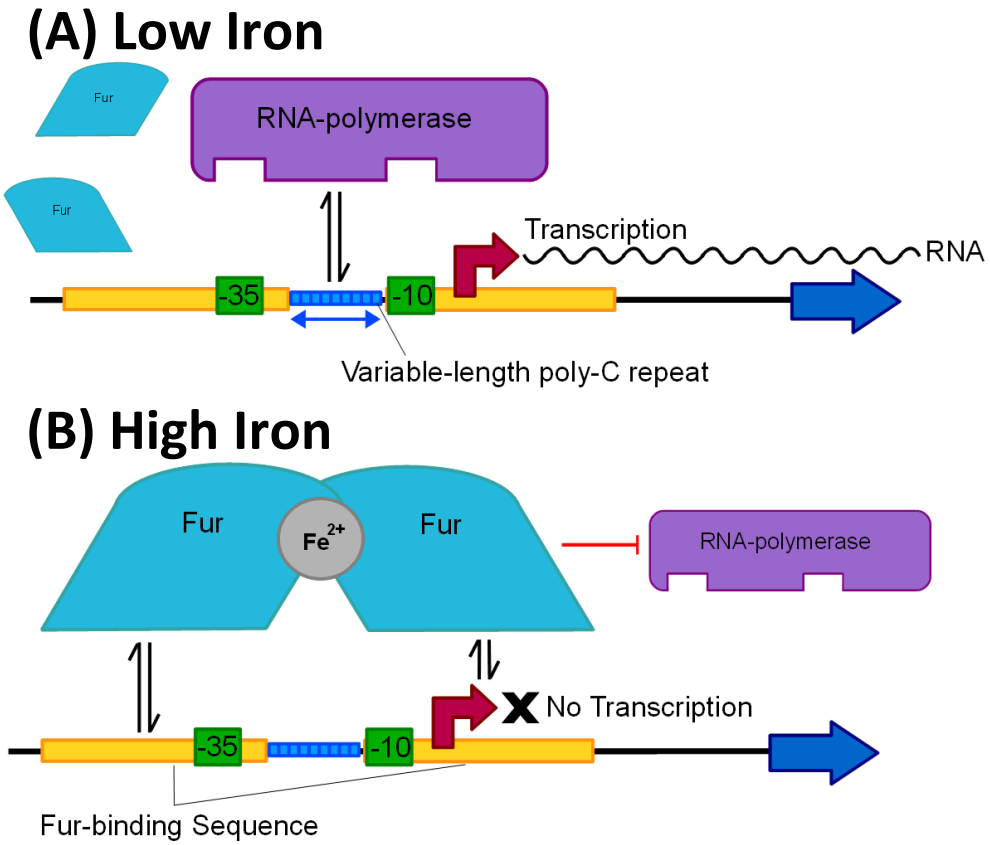


Figure 7.1: Transcriptional control of *fetA* expression as understood from previous data. The promoter upstream of the *fetA* gene (dark blue) is shown: the -10 and -35 RNA-polymerase binding sites (green); the transcriptional start site (red); the poly-C repeat leading to phase variation of *fetA* (light blue); the sites bound by the Fur protein responsible for iron-regulation (yellow) [166]. **(A)** When iron availability is low, Fur is not active. The RNA-polymerase binds across the promoter and initiates transcription. The strength of this interaction is affected by the distance between the -35 and -10 binding sites, which is altered by a variable-length poly-C repeat. **(B)** Under conditions of high iron availability, the Fur protein forms a dimer that binds across the promoter, preventing RNA-polymerase binding and stopping transcription. The strength of this interaction may also be affected by the variable-length poly-C repeat.

Delany *et al.,* [166] present further evidence that Fur binds with high affinity to two sites upstream of the *fetA* coding region. One of these sites overlaps the transcriptional start site, while the other binding site overlaps the -35 region [166]. In the presence of iron, the Fur protein forms a dimer and binds across the *fetA* promoter. When bound, the Fur dimer blocks association of the RNA-polymerase with the promoter (Figure 7.1(B)), thereby preventing transcription [167].

Investigation of transcriptional variation at a population level by quantitative RT-PCR (qRT-PCR) was used in the previous chapter alongside whole-genome sequence data to investigate the association between sequence variation upstream of the *porA* gene and expression of that gene (Chapter 6). Here, the same method was used to identify the sequence variation that may be associated with transcriptional regulation of the *fetA* gene, and how that regulation differs among ccs that may be targeted by a meningococcal vaccine.

The first aim of this study was to investigate the variation in the promoter sequence of *fetA,* both between the -35 and -10 regions and within the *fetA* Fur-binding regions, for the 107 meningococcal isolates for which whole genome sequences are available (Appendix 1, page 218). Transcription of *fetA* was also measured following growth under conditions of high iron-availability (MH), or lower iron-availability (MH with DFAM), in order to determine whether any of the documented sequence variation might be associated with overall changes in *fetA* transcription or with changes in iron-regulation.

The aim of this investigation was both to corroborate and to expand upon information presented by Delany *et al.,* [166]. Knowledge of such sequence variation would also allow improved strategies for increasing expression of the FetA protein by genetic modification (as described in Chapter 3). Furthermore, as the FetA protein is a major antigenic component of the developmental vaccine MenPF, transcription was compared by cc to determine whether

differences in transcription or regulation of *fetA* among ccs could affect the potential coverage of a vaccine containing FetA as a major antigenic component.

7.2: Results

7.2.1: Diversity in the spacer length between the *fetA* -35 and -10 sites

The sequences upstream of the *fetA* gene were extracted from whole-genome sequence data (Bratcher *et al.,* unpublished) for 107 meningococcal isolates from diverse geographical and temporal locations (Appendix 1, page 218). The isolates were those belonging to the reference set described by Maiden *et al.,* [232]. Sequences were aligned by the ClustalW algorithm using MegAlign Lasergene Software (DNAStar). Meningococci were characterised as having one of nine different sequences between the putative -10 and -35 sites described by Pettersson *et al.,* [149]. The consensus promoter sequences, listed as promoter types A - I, are shown in Figure 7.2. The different promoter sequences result in different spacer lengths from 16bp to 21bp.

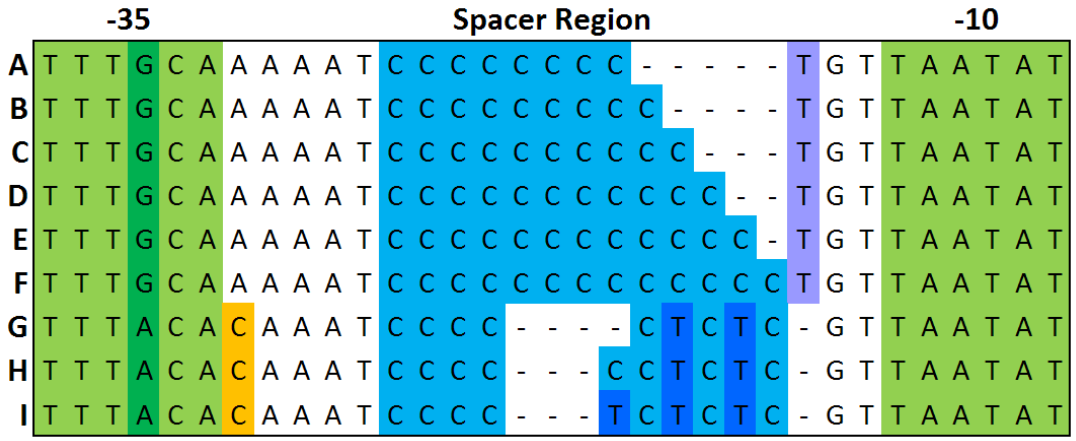


Figure 7.2: Consensus sequences derived for sequence between -35 and -10 sites (according to Pettersson *et al.*, [149], shown in green) upstream of the *fetA* gene. Nine different consensus sequences (promoter types A to I) were found within the 107 reference isolates investigated. These contain varying lengths of repeats (shown in blue). Regions of non-homology are highlighted: substitutions within the repeat are shown in dark blue; substitutions within the -35 sequence are shown in dark green; other substitutions are shown in orange; insert sites are highlighted in purple.

Of the nine consensus sequences found within the 107 isolates (Figure 7.2), only promoter types A to F contained an uninterrupted poly-C repeat, varying in length from 8bp to 13bp and resulting in spacer lengths from 16bp to 21bp. Promoter types G to I contained 2 or 3 thymine interruptions within the poly-C repeat region, and were also associated with additional sequence variation within the -35 region, immediately downstream of the -35 hexamer, and following the poly-C repeat region.

Almost half of all isolates investigated (50 isolates) were found to contain promoter type H (17bp spacer length, Figure 7.3, and Appendix 5, page 234). Other promoter types were less frequent, with promoter types B (17bp), C (18bp), D (19bp) and I (17bp) present in between 12 and 15 isolates each. Promoter types A (16bp), E (20bp), F (21bp) and G (16bp) were rare, present in 6 isolates in total. Overall, the 17bp spacer length (promoter types B, H, and I) was most prevalent, being found in 76/107 isolates. The 18bp and 19bp spacer lengths (types C and D, respectively) were less common, present in 12 and 13 isolates, respectively. The 16bp (types A and G), 20bp (type E) and 21bp (type F) spacer lengths were rare, present in 6 isolates in total.

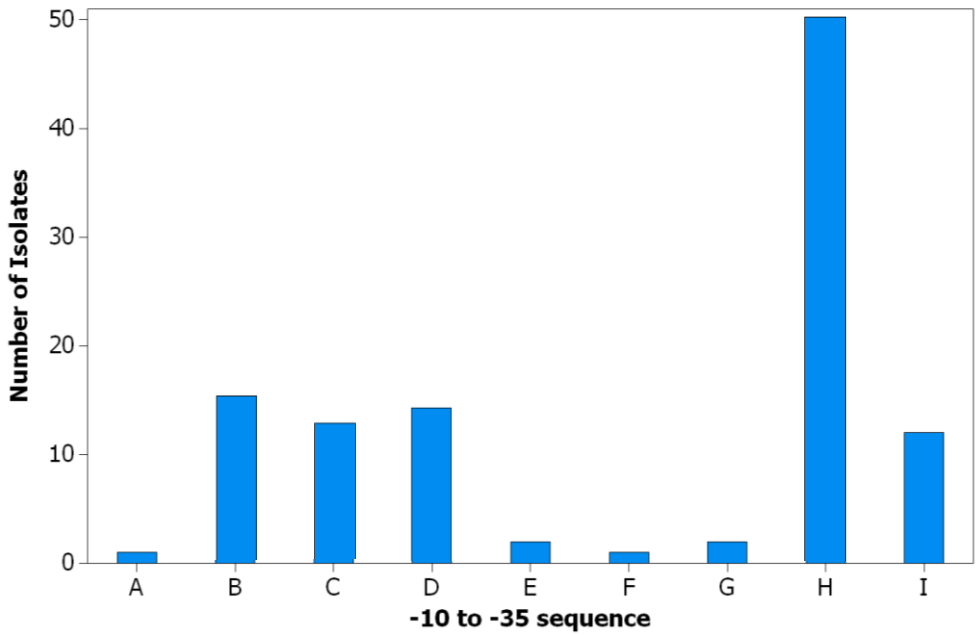


Figure 7.3: Distribution of the consensus promoter sequences upstream of the *fetA* gene in the 107 reference isolates.

7.2.2: Comparison of *fetA* transcription in the presence of different spacer length

A qRT-PCR assay, using primers and fluorescently-tagged oligonucleotide probes specific for the *fetA* gene and the control gene *gdh*, was used to measure levels of *fetA* transcription. Initially, associations between sequence variation and transcription rates of the *fetA* gene were investigated within meningococcal cultures grown in MH broth supplemented with the iron-chelator DFAM prior to RNA extraction. Under these conditions, the concentration of iron available to bind to Fur is reduced, thus reducing transcriptional repression of *fetA*, and other meningococcal genes, by the Fur-iron complex.

RQ values were calculated using the $2^{-\Delta\Delta Ct}$ method [238] relative to transcript levels of *gdh* and normalised to a positive control RNA sample (extracted from isolate H44/76 grown under iron replete conditions). All normalised RQ values were subsequently transformed using the natural logarithms (ln) transformation prior to further analysis. Unless otherwise stated, all statistical analysis was completed using General Linear Models for analysis of variance, allowing the variance in measured ln(RQ) values to be partitioned according to multiple discrete sequence differences.

Transcription of *fetA* was highest in isolates that contained an 18bp spacer length between the -10 and -35 regions (Figure 7.4). However, there was no significant differences in *fetA* expression levels between the 17bp, 18bp and 19bp spacer lengths (P = 0.325). Limited data points for spacer lengths 16bp, 20bp and 21bp prevented reliable statistical analysis of these groups.

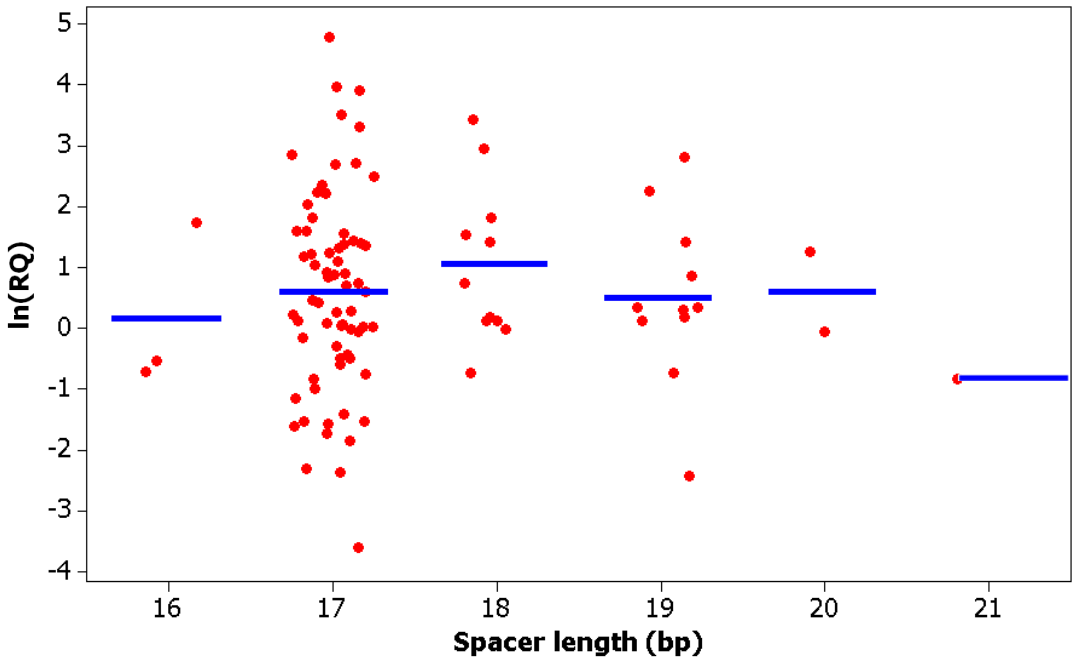


Figure 7.4: Relationship between ln(RQ) values obtained for *fetA* (following growth in MH supplemented with DFAM) and the length of the spacer region between the -35 and -10 sequences in the *fetA* promoter. The ln(RQ) values for individual isolates are plotted in red, with mean values for each spacer length shown in blue.

As promoter types G, H and I were linked to additional sequence differences not present in types A-F, in order to investigate the effect of varying spacer length separately from those that may be due to other nucleotide polymorphisms promoter types A to F were compared alone (Figure 7.5). In this subset, all containing a continuous poly-C repeat, a trend could be seen between *fetA* transcript levels and spacer length. Isolates containing an 18bp spacer length gave, on average, higher transcript levels than those containing either 17bp or 19bp spacer lengths. Isolates containing 16bp or 21bp spacer lengths showed the lowest transcript levels, although only a single isolate of each was present for comparison.

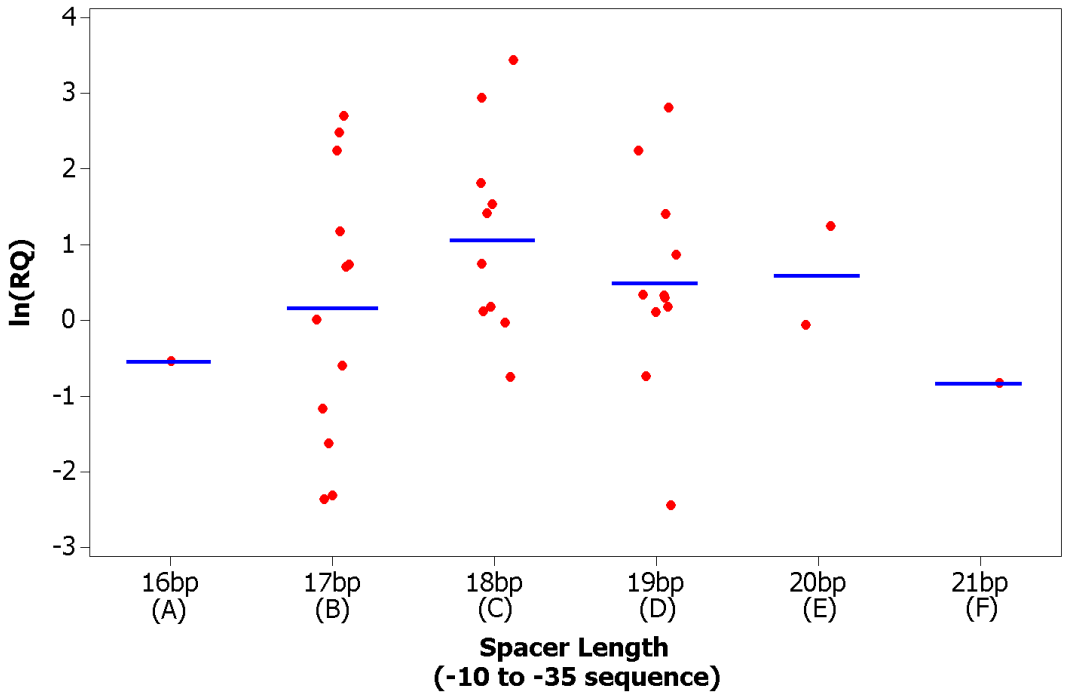


Figure 7.5: Relationship between ln(RQ) values obtained for *fetA* (following growth in MH supplemented with DFAM) and the length of the spacer between the -10 and -35 sites, for promoter types containing a continuous poly-C repeat (A – F). The ln(RQ) values for individual isolates are plotted in red. Mean values for each sequence are shown in blue.

This trend was similar to that seen for transcription of *porA*, for which a spacer length of 18bp between the -35 and -10 sites results in optimal binding for high transcript levels, while longer or shorter spacer lengths result in reduced transcription (Chapter 6 and [135]). Despite this trend being consistent across both genes, there were no significant differences in transcription of *fetA* among the spacer lengths ($P > 0.05$).

The high variation in *fetA* transcription seen among isolates containing the same spacer length suggests that other factors are likely to be important in regulating expression. The standard deviations were similar for the 17bp, 18bp and 19bp spacer lengths (-10 to -35 sequence types B, C and D, standard deviations 1.69, 1.51 and 1.61, respectively), suggesting that these other factors have an equal effect across the different spacer lengths.

7.2.3: Comparison of *fetA* transcription in response to substitutions within the -10 to -35 sequence

As spacer length alone could not be significantly linked to *fetA* transcript levels, sequence variation between the -35 and -10 sites was investigated further to determine whether the different consensus sequences could be associated with *fetA* RNA levels.

Three of the consensus promoter sequence types (B, H, and I) contained a total spacer length of 17bp (Figure 7.1). Type B contained a continuous poly-C repeat, while type H contained 2 C>T substitutions in the poly-C repeat and type I contained 3 C>T substitutions. Types H and I also contained additional bases changes in the -35 hexamer and within the spacer when compared to type B.

When isolates containing promoter sequence types B, H or I were compared, mean transcript levels were highest in isolates containing promoter type I (mean ln(RQ) for B, H, I = 0.16, 0.57, 1.20, respectively, Figure 7.6). As all isolates in the subset compared contained the same spacer lengths, the differences in transcription between these promoter types may be due to the different patterns of C>T substitutions within the poly-C repeat. Such substitutions could affect the twist of the DNA and, consequently, alter the relative positions of the -35 and -10 binding sites without changing the length of the spacer DNA [234]. Alternatively, the nucleotide substitution within the -35 hexamer may directly affect the strength of RNA-polymerase binding. For example, the -35 sequence present in promoter types H and I (TTTACA), is more similar to the *E. coli* consensus -35 binding site (TTGACA) [239] than the sequence present in promoter type B (TTTGCA). Therefore RNA-polymerase binding may be stronger in isolates containing promoter types H and I than type B, explaining the higher mean transcript levels seen in those isolates. However, the differences in *fetA* RNA levels among the three promoter types were not significant (P = 0.322). This may be due to other factors

affecting transcription of the *fetA* gene and increasing variability among isolates containing the same -10 to -35 sequence.

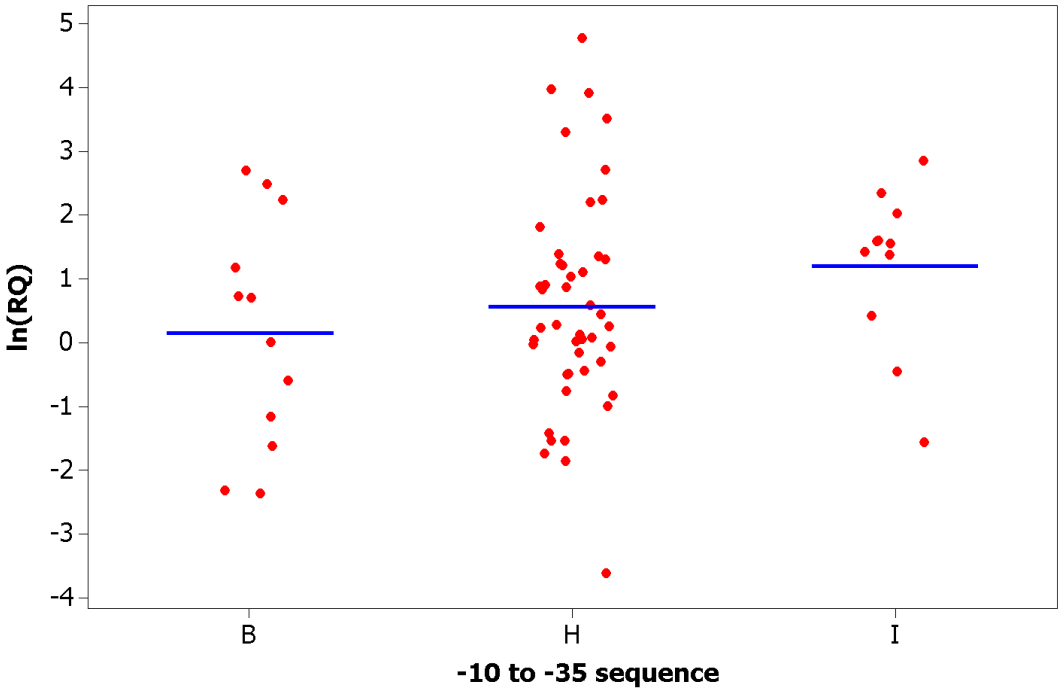


Figure 7.6: Relationship between ln(RQ) values obtained for *fetA* (following growth in MH supplemented with DFAM) and the promoter type present between the -10 and -35 sites, for isolates containing a 17bp spacer length. The ln(RQ) values for individual isolates are plotted in red. Mean values for each sequence are shown in blue.

7.2.4: Investigation of *fetA* transcription associated with sequence variation surrounding the -10 to -35 sites

As sequence differences between the -35 and -10 sites could not explain much of the variability in *fetA* transcript levels, all sequence variation between the ATG start codon and position -144 from the transcriptional start site was investigated (Figure 7.7). This span of sequence was chosen to encompass both sequence within the mRNA that may affect RNA stability and also all sequence within the Fur-binding regions described by Delany *et al.,* [166]. Across all isolates the region investigated (including the -10 to -35 spacer) showed 96.8% sequence identity, although polymorphisms were found at 16 positions outside of the -35 and

-10 sites. Nucleotide polymorphisms found in fewer than 5 isolates were not investigated further as the statistical power would be limited.

Between positions -144 and +24 relative to the transcriptional start site, all sequence variation present in ≥5 isolates was associated with the nine different promoter consensus sequences described above (7.2.1, Figure 7.7). When compared to promoter types A – F, which contained a continuous poly-C repeat, promoter types G, H and I (containing substitutions within the poly-C repeat) were found to contain additional variation: a T>C substitution at position -1 from the transcriptional start site, and a 4bp deletion of ATTA at the +15 to +18 positions. The link between interruptions within the poly-C and these additional sequence polymorphisms suggests that the two groups of promoters may have each arisen from a common ancestor and that spontaneous mutations within the poly-C repeat are rare.

Sequence variation associated with the different promoter types had no significant effect on *fetA* transcription under the growth conditions compared (MH with DFAM, see 7.2.3).

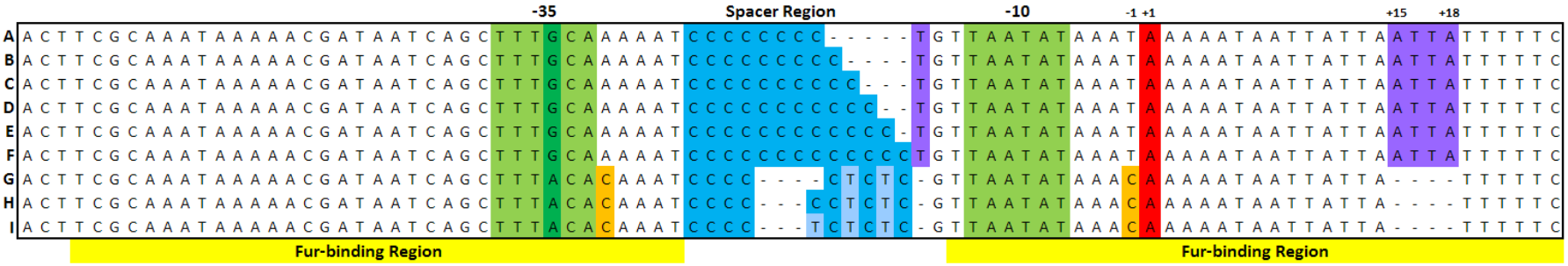


Figure 7.7: Consensus sequences derived for the promoter upstream of the *fetA* gene. Nine different consensus sequences (promoter types A to I) could be derived for the 107 reference strains investigated. These contain varying lengths of repeats (shown in blue) within the spacer region between the -35 and -10 sequences (according to Pettersson *et al.*, [149], shown in green). Positions where variation occurs in >5 strains are coloured: nucleotide substitutions are highlighted in orange; sites of insert/deletions are highlighted in purple. The transcriptional start site (+1) and fur-binding regions determined by Delany *et al.,* (2006) [166] are shown in red and yellow, respectively.

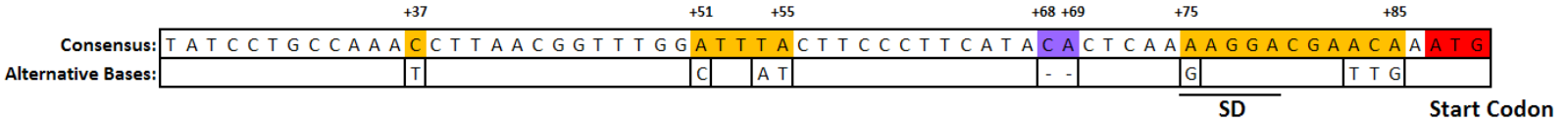


Figure 7.8: Consensus sequence derived for the promoter upstream of the *fetA* gene between the ATG start codon (shown in red) and position +25 from the transcriptional start site [149]. Positions where variation occurs in >5 strains are coloured: nucleotide substitutions are highlighted in orange; sites of insert/deletions are highlighted in purple.

Between the ATG start codon and positions +25 from the transcriptional start site, there were several sites of sequence variation (Figure 7.8). This variation was not linked to the promoter sequence found between the -10 and -35 sites.

Within the signal sequence of 28 isolates (see Appendix 5, page 234), there was a C>T polymorphism at position +37 with respect to the transcriptional start site. Polymorphisms were also found at the +51 to +55 positions (ATTTA>CTTAT, present in 14 isolates: 1000, BZ198, M40/94, M-101/93, 890326, 88/03415, N45/96, 50/94, 400, 931905, 91/40, NG 6/88, BRAZ10, NG H15), and at the +68 to +69 positions (a CA deletion found in 8 isolates: 8680, BZ198, 44/76, NG PB24, 204/92, NG 080, NG144/82, BZ 83). These polymorphisms did not significantly affect *fetA* transcription ($P > 0.05$, data not shown).

Sequence variation was also found within the Shine-Dalgarno (SD) sequence. An A>G polymorphism at the first base of the SD site, present in 31 isolates (see Appendix 5, page 234) was also associated with an ACA>TTG polymorphism in the four bases immediately upstream of the ATG start codon. Improved ribosome-binding as a result of an altered SD sequence could increase mRNA stability by protecting the RNA from endoribonucleases [231], leading to higher RNA levels.

To investigate any association between this polymorphism and transcript levels, isolates containing the type B promoter sequence were compared. This subset was chosen as the largest group of isolates containing a comparable number of both types of SD sequence (6 isolates of each). Isolates containing the A>G substitution showed higher *fetA* transcript levels than those isolates with the consensus adenine residue at this position (Figure 7.9). Although this association approached significance ($P = 0.056$), there was not enough evidence within this subset to reject the null hypothesis (of no effect of SD sequence on transcript levels).

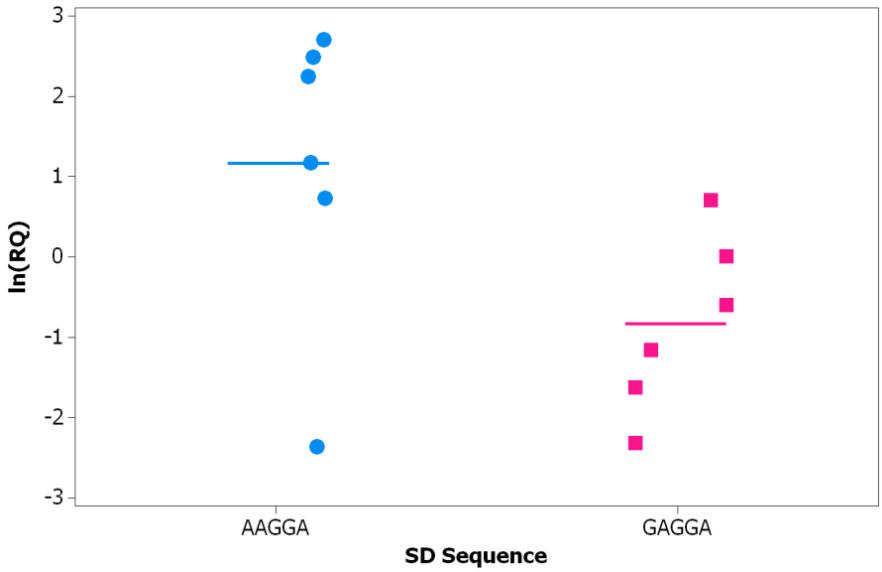


Figure 7.9: ln(RQ) values obtained for *fetA* for isolates containing the type B promoter sequence (Figure 7.7). Isolates are grouped according to the Shine-Dalgarno (SD) sequence present. The ln(RQ) values for individual isolates are plotted, with mean values for each group shown as horizontal lines.

Changes in the SD sequence could also affect the efficiency of translation from the RNA by changing the strength of ribosome-binding [240], although these differences would not be measured by the methods used here. Genetic modification could be used to confirm the effect of SD sequence variation on transcription or translation of *fetA*. If successful, changing the SD sequence could be used to improve FetA expression in modified strains for vaccine production or evaluation.

7.2.5: Comparison of *fetA* transcription in response to iron availability

FetA expression is regulated by iron availability; therefore, transcription of *fetA* was also quantified in total RNA extracted from isolates following growth under iron replete growth conditions. As seen previously for FetA protein expression (Figure 3.6, page 63), transcription of the *fetA* gene was significantly higher following growth in MH with DFAM than in MH

(Figure 7.10, P < 0.001). Mean *fetA* RNA levels following growth in MH with DFAM were 6.8 times higher than in MH (Mean RQ (untransformed) = 6.26 versus 0.92).

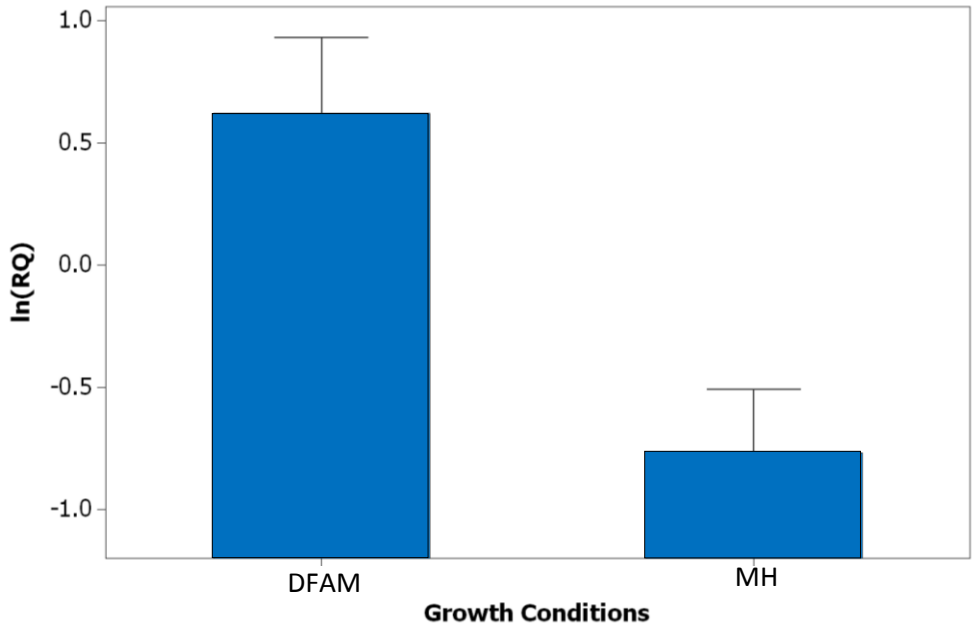


Figure 7.10: Mean ln(RQ) values obtained for *fetA* for isolates investigated following growth in MH or in MH supplemented with DFAM. Error bars show 95% confidence intervals of the mean.

As the different promoter sequences found upstream of *fetA* contain several polymorphisms and deletions within the regions bound by the Fur-protein, the effect of these differences on iron-regulation of *fetA* transcription was investigated.

Promoter types were divided into two groups:

1) Promoter types A – F, which contained a continuous poly-C repeat;

2) Promoter types G, H and I, which contained nucleotide substitutions both within and surrounding the poly-C repeat and a 4bp deletion between the +15 and +18 bases.

When grown in MH with DFAM, there was no significant differences in *fetA* transcription between these two promoter groups (for all isolates containing a 17bp spacer length, see

7.2.3). When the same isolates were grown in MH without DFAM, isolates in promoter group 2 had significantly higher *fetA* RNA levels than those in promoter group 1 (Figure 7.11, P = 0.009). Following growth under iron replete conditions, RNA levels measured for isolates containing this deletion were twofold higher than RNA levels obtained for isolates without the deletion (Mean RQ (untransformed) = 1.16 versus 0.54).

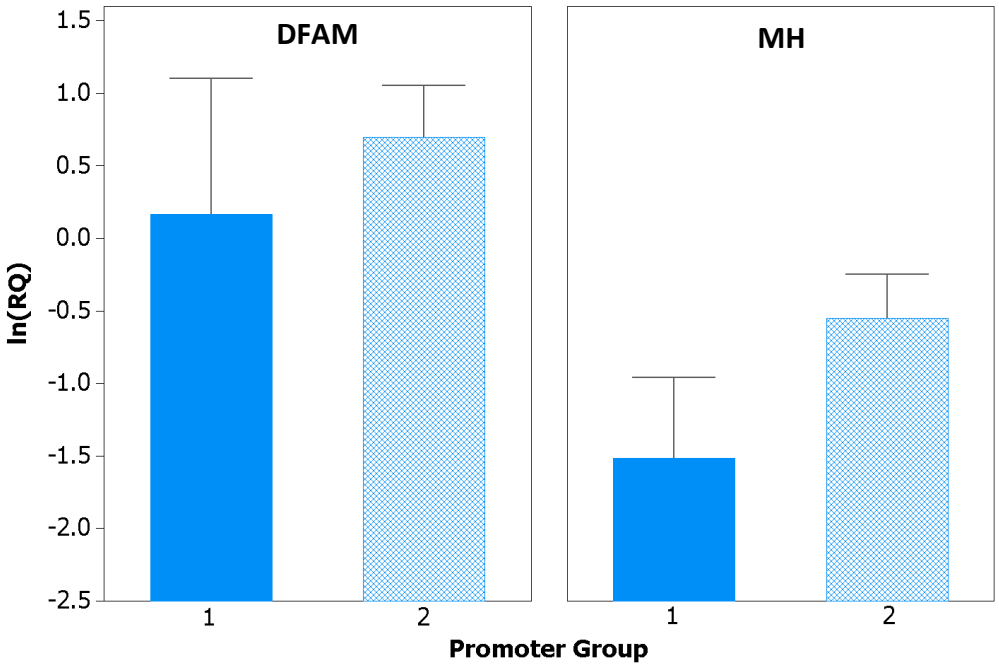


Figure 7.11: Mean ln(RQ) values obtained for *fetA* following growth in MH or MH supplemented with DFAM, for isolates containing the 17bp spacer length. Isolates were categorised into two promoter groups: 1) Promoter types A-F, and 2) Promoter type G-I. Error bars show 95% confidence intervals of the mean.

The difference in transcription between the two promoter groups is likely to be because several of the sequence polymorphisms are found within the sites that are bound by the regulatory Fur protein (Figure 7.7). The deletion at the +15 to +18 positions would be expected to reduce the efficiency of Fur-binding in this region, consequently increasing the chances of the RNA-polymerase binding to the promoter and initiating transcription. The difference between isolates with and without the deletion is only seen following growth in MH as, when

iron is chelated using DFAM, the Fur protein is not present in an active form to bind to the *fetA* promoter.

7.2.6: Comparison of *fetA* transcript levels among clonal complexes

As isolates belonging to the same cc are genetically closely related [232], they are more likely to share genetic polymorphisms affecting transcription of the *fetA* gene. Shared genetic polymorphisms will include those outside of the sequence directly investigated above, but could potentially affect *fetA* transcription. Therefore, differences in *fetA* transcription among ccs were investigated to determine whether such variation could affect the efficacy of a FetA-based vaccine against particular meningococcal lineages.

Possible associations between *fetA* RNA levels and cc were investigated for all ccs with greater than 2 isolates within the meningococcal reference set (Figure 7.12). Although values for all ccs were included in the calculation of the sample mean, ccs containing ≤2 isolates or isolates not assigned to a cc were not analysed further as these groups contained insufficient data points for statistical comparison.

When grown in MH with DFAM, mean RNA levels for all ccs investigated were close to the sample mean (0.619, Figure 7.12(A)). When grown under iron replete conditions, *fetA* RNA levels for isolates belonging to the ST-4 complex were significantly higher than the sample mean (Figure 7.12(B), P= 0.040). Under iron replete conditions mean *fetA* RNA levels were low compared to the sample mean for isolates belonging to the ST-32 complex, although this difference did not achieve significance (P = 0.204). *fetA* RNA levels for other ccs were similar to the sample mean (-0.769).

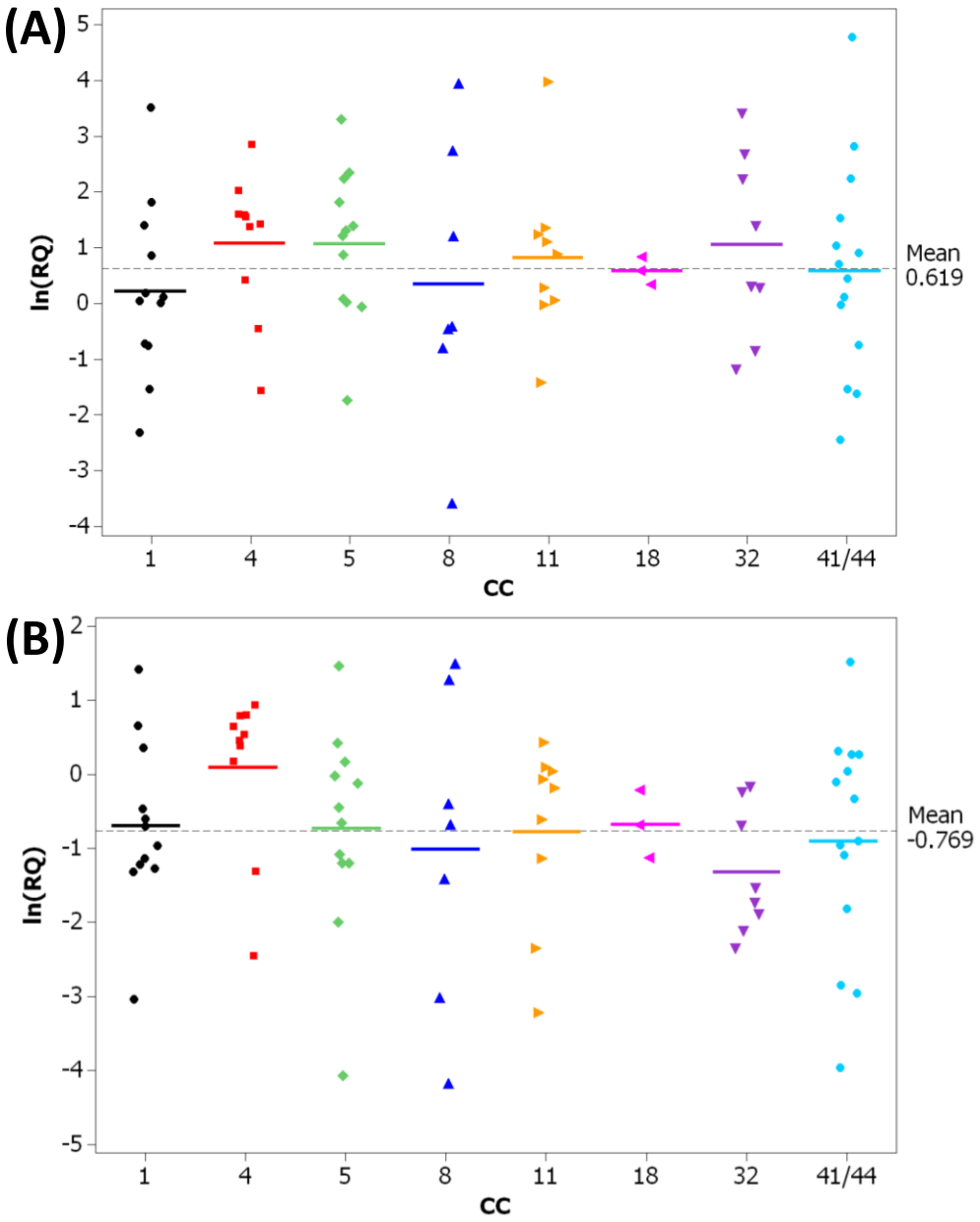


Figure 7.12: Distribution of *fetA* ln(RQ) values among clonal complexes (cc) following growth of isolates in **(A)** MH with DFAM, or **(B)** MH media. Symbols show ln(RQ) values for individual isolates. Means for each group are plotted as lines, with the mean for the total sample (all available data points) shown as a dashed lined. Clonal complexes for which >2 isolates are available are shown. Isolates from all other clonal complexes are not shown, but were included in the calculation of the mean.

Under both growth conditions variation within ccs was high, although the level of intra-complex variation was similar among most ccs (Figure 7.13). Exceptions were isolates belonging to the ST-18 complex and the ST-8 complex.

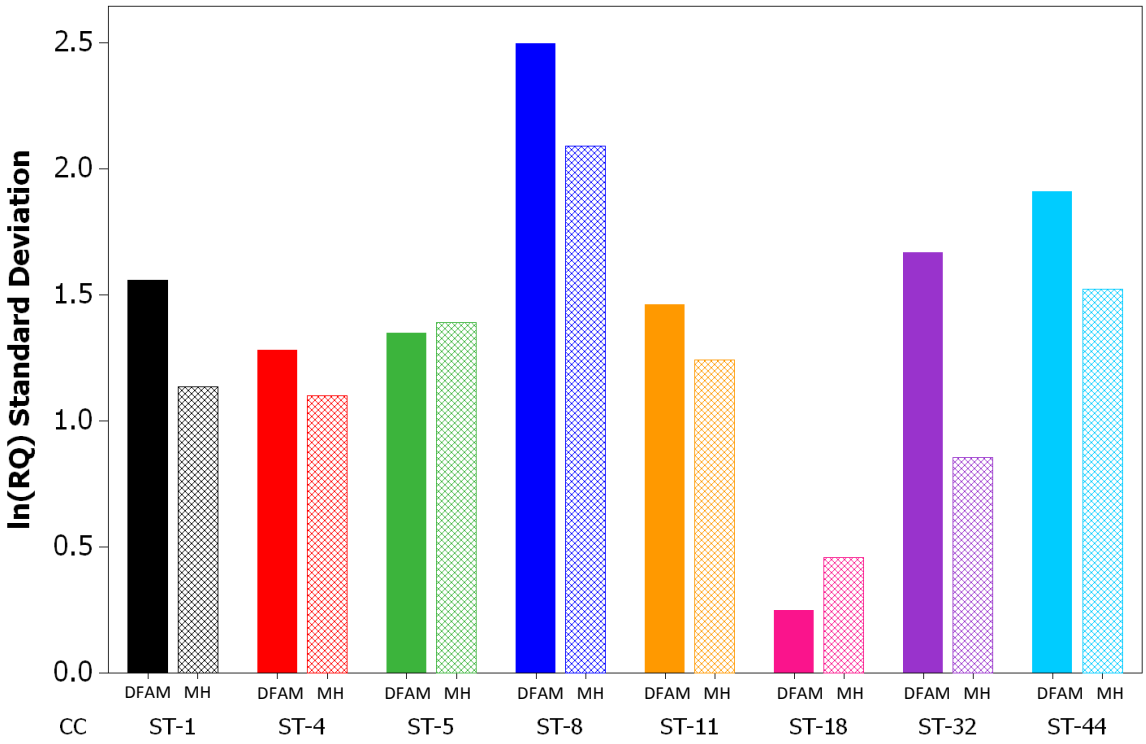


Figure 7.13: Standard deviations for *fetA* ln(RQ) values. Bars shown the standard deviation of ln(RQ) values within ccs for which >2 isolates are available. Standard deviations following growth in MH are shown in crosshatch. Standard deviations following growth in MH with DFAM are shown in block colour.

The particularly high variation in transcript levels within the ST-8 complex, under both growth conditions, suggests additional levels of transcriptional regulation may act within that cc. Some of this variation with the ST-8 complex may be due to genetic differences among sequence types (STs), as isolates belonging to ST-66 and ST-10 show relatively low transcript levels compared to the ST-8 isolates within that complex (data not shown).

Under both growth conditions standard deviation was lowest in isolates belonging to the ST-18 complex, for which all measured RNA levels were close to the sample mean. However, all

isolates within the cc belonged to different STs (ST-18, ST-19 and ST-20). Therefore regulation of *fetA* transcription is not variable among STs within the ST-18 complex. It is likely that the high variation seen in the ST-8 cc and low variation seen in ST-18 cc are a result of isolate sampling, as only 7 and 3 isolates from each complex were analysed, respectively. A larger sample of isolates from each complex may give a distribution in variation similar to that seen for other complexes.

Alone, cc could account for little of the total variance in ln(RQ) values (Adjusted R^2 = 0.00%). Overall, there was no significant association between cc and *fetA* transcript levels under either growth conditions ($P > 0.05$).

7.2.7: Investigation of the distribution of promoter sequences among clonal complexes

Although no significant association was found between *fetA* transcript levels and the promoter sequence, trends were seen that could link spacer length and *fetA* RNA levels. Certain ccs were associated with particular *fetA* promoter sequences (Figure 7.14).

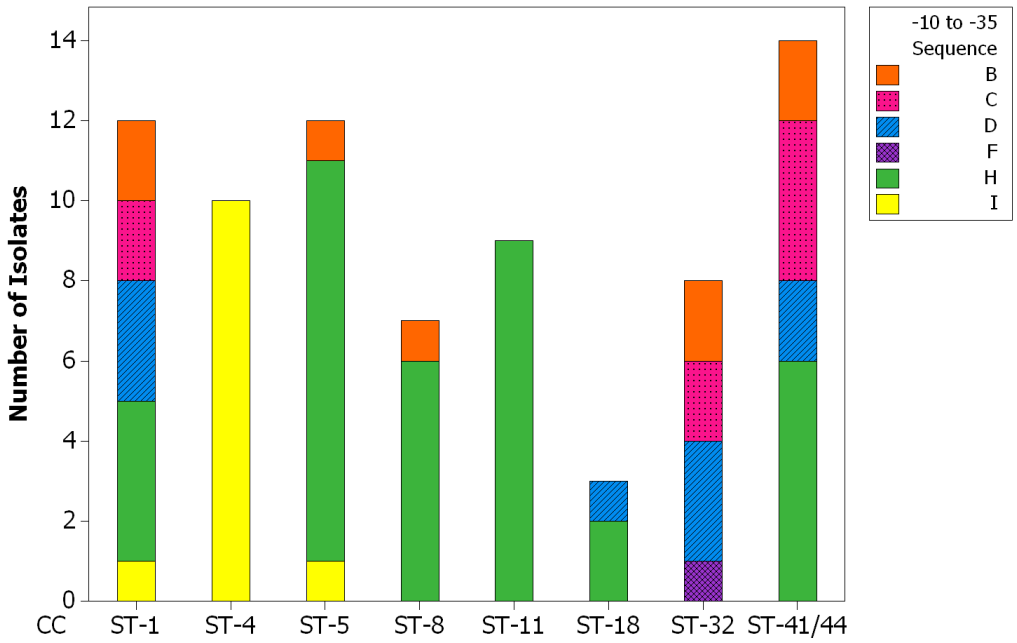


Figure 7.14: Distribution of the consensus promoter sequences upstream of the *fetA* gene across the ccs represented by the reference isolates.

Isolates belonging to the ST-4 complex exclusively contained promoter type I, while isolates belonging to the ST-11 complex contained only promoter type H. The promoter sequences of isolates belonging to the ST-1, ST-32 and ST-41/44 complexes were diverse; strains from each complex contained at least three of the nine different promoter sequences.

The high transcript levels seen in the ST-4 complexes in both MH and MH with DFAM may be due to all isolates within that cc containing promoter type I. This promoter type was found to be associated with high transcript levels compared to other promoter types, although the difference did not achieve significance. However, as promoter type I is present in only 2 isolates from other ccs, it would be necessary to study additional isolates to determine whether the high transcript levels are a result of this promoter type or of other factors associated with the ST-4 complex. Furthermore, despite the ST-4 and ST-11 complexes containing only a single promoter type upstream of the *fetA* gene, the standard deviation of transcript levels was similar to other ccs that contained multiple promoter types. This suggests that the promoter sequence is not an important factor determining variation in *fetA* transcription.

To investigate the contribution of promoter type to variance in *fetA* transcript levels, ln(RQ) values were partitioned by promoter type. Values for isolates containing promoter types A, E, F, and G were not analysed as there were insufficient data points for confidence in the mean. Following partition of variance according to promoter type, the residual sums of squares (residuals) were analysed by cc. The standard deviations of the residual values were very similar to the standard deviations of the ln(RQ) levels for each cc (data not shown), confirming that promoter sequence type contributes little to the variation seen in *fetA* transcription.

7.2.8: Comparison of the effect of iron regulation among clonal complexes

The standard deviations of transcript levels within the ST-1, ST-8, ST-32 and ST-41/44 complexes were lower in MH than in MH with DFAM (Figure 7.13). Some of the variation in *fetA* transcription in MH has been attributed to multiple sequence polymorphisms within the Fur-binding regions of the promoter, which differentiate the promoter types into two distinct promoter groups. The polymorphisms within promoter types G, H and I (promoter group 2) resulted in high levels of *fetA* RNA under iron replete conditions, possibly by reducing the binding efficiency of the Fur protein in the presence of iron.

Isolates belonging to the ST-4, ST-5, ST-8 and ST-11 complexes almost exclusively contained these polymorphisms (promoter type 2, Figure 7.15). The ST-1, ST-32 and ST-41/44 complexes contained a high proportion of isolates with promoter type 1 (associated with low transcript levels under iron replete conditions).

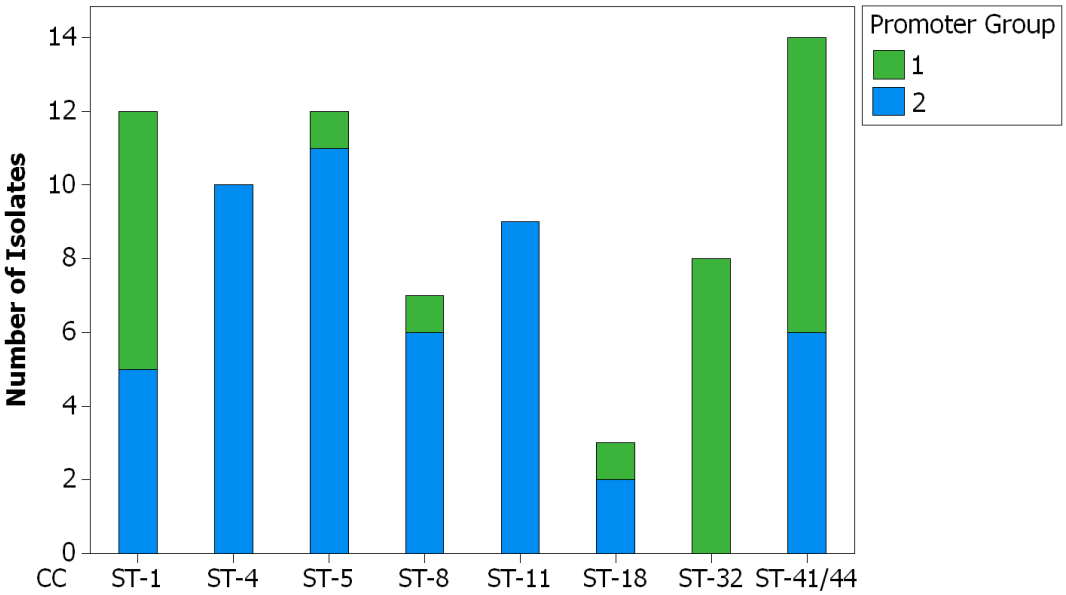


Figure 7.15: Distribution of the two *fetA* promoter groups across the ccs represented by the reference isolates.

The presence of promoter group 2, associated with high transcript levels under iron replete conditions, in all isolates belonging to the ST-4 complex may explain why isolates in the cc have

higher *fetA* RNA levels than other ccs in the presence of iron (Figure 7.12(B)). Similarly, as all isolates in the ST-32 complex contain promoter group 1, this could be the reason for the low transcript levels in this cc in the presence of iron. However, average transcript levels for all other ccs are similar to the population mean, regardless of which promoter group dominates within each cc.

On the other hand, for the ST-1, ST-32 and ST-41/44 complexes, the differences in standard deviation between transcript levels in MH and MH with DFAM conditions (Figure 7.13) suggest that repression by iron in these ccs can override other causes of transcriptional variation. For isolates in the ST-4, ST-5 and ST-11 complexes, similar standard deviations under both growth conditions show that, in these ccs, other causes of variance act equally regardless of the effect of iron-repression.

7.3: Discussion

The variable expression of FetA, regulated primarily by iron availability [172], is a potential issue for its use as a vaccine antigen. For antibodies induced by a vaccine to be capable of bactericidal killing, sufficient quantities of the vaccine antigens must be expressed on the surface of bacteria [99]. As FetA expression is repressed in the presence of iron, and growth conditions *in vivo* cannot be determined, the potential efficacy of a FetA-specific vaccine is difficult to predict. Iron-regulated expression of FetA in meningococci is due to the binding of the transcription factor Fur to a specific sequence in the *fetA* promoter. The aim of this study was to identify sequence variation within the *fetA* promoter that could affect the binding of Fur, or other transcription factors, thereby affecting transcriptional regulation at a population level. Knowledge of sequence variation associated with regulation of expression could be used to improve efficacy estimates for a vaccine targeting the FetA antigen.

Sequence variation upstream of the *fetA* gene was compared to the quantity of *fetA* RNA present in wildtype meningococcal isolates following growth in both MH and MH with DFAM. The quantity of *fetA* RNA is likely to correlate with the level of translation of the FetA protein, and is influenced by the relative rates of initiation of RNA transcription and RNA degradation. Both transcriptional initiation and RNA stability can be affected by sequence variation within the promoter.

Promoter sequences upstream of the *fetA* gene were found to contain different lengths of poly-C repeats between the -10 and -35 sites, resulting in total spacer lengths from 16bp to 21bp. Isolates containing the 18bp spacer length showed the highest transcript levels. This is similar to the trend seen previously for transcription of the *porA* gene (Figure 6.3, page 144). However, neither spacer length nor interruptions within the poly-C repeat could account for much of the variation in *fetA* transcript levels among isolates. This was true both within and among ccs, indicating that variation in spacer length is not an important mechanism by which the meningococcus alters expression of *fetA*. This is further supported by the observation that the majority of isolates shared an identical sequence between the -10 and -35 sites. Many isolates contain promoters that have several C>T substitutions within the poly-C repeat. These substitutions may stabilise the repeat sequence and reduce the frequency of slipped-strand mispairing. Alternatively, the distribution of promoter sequences seen here may be as a result of isolate sampling; the majority of meningococci investigated here were isolated from invasive disease, while the diversity found in *fetA* promoter sequences may be higher in carriage isolates. Further study with a larger number of carriage isolates would be required to confirm this.

The reduced impact of changes in spacer length on expression levels may also be a result of uncertainty in the position of the -35 RNA-polymerase binding site. There were two distinct groups of promoters present in the isolates investigated. Among other differences both within and surrounding the spacer, the two promoter types contain different -35 sequences. In the

first promoter type the sequence present is a single base deviation from the consensus -35 hexamer in *E. coli*, while other promoters contain two base pair changes from this consensus. RNA-polymerase binding, and therefore the rate of transcriptional initiation, is expected to be higher when the -35 binding site present is similar to the consensus, which has been shown to be optimal in *E. coli* [239]. While this may explain the small increase in transcript levels in the first promoter type compared to the second, it is difficult to predict exactly where the -35 binding site lies. The -35 site listed here was that putatively assigned by Pettersson *et al.,* [149]. Conversely, Delany *et al.,* [166] predict the -35 site to lie 1bp towards the transcriptional start. In the second promoter type, this position would increase the sequence conservation with the *E. coli* -35 consensus. Therefore, it is likely that multiple -35 sites may be used within and among isolates. As the -10 site is more fixed, this would similarly result in multiple spacer lengths being used (Figure 7.16). As different isolates may show a biased towards alternative -35 sites, this would increase the variation in transcript levels seen among isolates containing the same promoter sequence.

The two promoter groups also contained several polymorphisms within the sequence shown previously to be bound by the Fur protein during iron-repression [166]. The most noticeable of these differences was a 4bp deletion in one group (promoter types G, H and I). Isolates containing this deletion showed higher *fetA* RNA levels in when grown under iron replete conditions but not in the presence of an iron chelator. These results suggest that the polymorphisms reduce the efficiency of Fur-binding to the *fetA* promoter, consequently increasing the chances of the RNA-polymerase binding and initiating transcription. The differences seen in *fetA* transcription among the different promoter types may therefore be a combination of the effects of the poly-C repeat, differences in the -35 sequence and other polymorphisms in the Fur-binding regions. As these polymorphisms are all connected, however, in order to separate the effects of these sequence differences from each other, genetic modification would need to be used to introduce individual factors into an isogenic

background. Transcriptional analysis of these isogenic strains would indicate the relative effects of each polymorphism while removing the effects of other, unknown differences that may act among isolates.

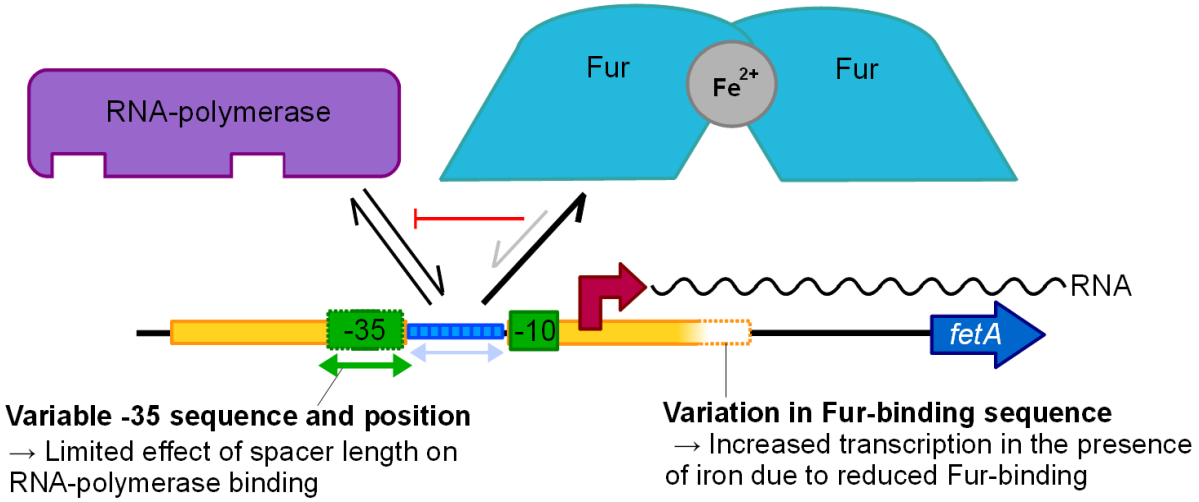


Figure 7.16: Transcriptional control of *fetA* expression, including conclusions drawn from data presented in this chapter. The promoter upstream of the *fetA* gene (dark blue) is shown: the -10 and -35 RNA-polymerase binding sites (green); the transcriptional start site (red); the poly-C repeat leading to phase variation of *fetA* (light blue); the sites bound by the Fur protein responsible for iron-regulation (yellow) [166]. When Fur is not active, the RNA-polymerase binds across the promoter and initiates transcription. The strength of this interaction is not strongly affected by the distance between the -35 and -10 binding sites, possibly due to the presence of multiple potential -35 binding sites. Under iron replete conditions, binding of the Fur protein is reduced in many isolates due to sequence variation in the Fur-binding sequences. Consequently, *fetA* transcription is increased in the presence of iron. Under both conditions, additional factors are likely to affect transcription.

Much of the variation in *fetA* transcript levels among isolates could not be explained by sequence differences or by cc. This could be due to other levels of regulation, for example, in *Neisseria gonorrhoeae*, the promoter upstream of the *fetA* gene is bound by the MpeR (Mtr protein efflux regulator) protein [156]. MpeR is negatively regulated by Fur [241], and, in the

absence of iron, the protein is expressed and enhances transcription of *fetA* [156]. MpeR in meningococci has also been shown to be iron regulated via Fur [166,168] and, as such, could similarly be involved in transcriptional regulation of meningococcal *fetA*. However, the MpeR protein is not expressed in ST-32 isolates due to the presence of a mutation within the gene [168], and transcription of *fetA* in the ST-32 strain MC58 is negatively iron-regulated in a meningococcal mutant not expressing the Fur protein [166]. Although the effect of iron on *fetA* transcription was reduced compared to the wildtype strain, this suggests that there are additional mechanisms of regulation acting in the meningococcus that are not dependent on Fur or MpeR.

As well as transcriptional regulation, additional levels of regulation may also act on FetA protein expression. When the *fetA* promoter is replaced with one derived from the *porA* promoter (Chapter 3), FetA expression remains lower than PorA expression with a similar promoter (data not shown). This difference may be due to inherent properties in the *fetA* RNA compared to *porA* RNA, for example codon usage bias leading to reduced rates of translation, or to increased protein degradation. Such differences would not be measured using the methods employed here.

Much of the unexplained variation in *fetA* transcription in this study could be introduced during RNA extraction. As expression of FetA is highly dependent on nutrient availability, there are likely to be differences in the transcription of the *fetA* gene between different samples from the isolate or between samples taken at different growth phases. Studies with multiple repeats of a small number of isolates would be required to determine the extent of this sample variability and how it may affect the results obtained in this study.

Despite this unexplained variation, there is no evidence that the variable expression of FetA will lead to reduced vaccine efficacy. Iron availability during growth in the blood stream is expected to be low [242], resulting in increased levels of FetA expression. The presence of

FetA-specific antibodies in convalescent sera is further evidence that the FetA protein is expressed during meningococcal disease [165]. Many of the isolates studied here contain a 17bp spacer length, resulting in high levels of transcription. Several of the ccs studied also contain a large proportion of isolates with polymorphisms within the Fur-binding regions, making them less susceptible to negative *fetA* regulation under iron replete conditions. Therefore, clearance of meningococci by FetA-specific antibodies, particularly those targeting ST-4 and ST-11 isolates, is likely to be higher, even under iron replete conditions.

None of the ccs investigated showed very low overall levels of *fetA* transcription. As such isolates would be likely to escape killing by FetA-specific antibodies, this suggests that a FetA-specific vaccine could be effective against all ccs expressing the targeted variable regions. Furthermore, it was noted that *fetA* transcription in ccs associated with epidemic meningococcal disease (the ST-1, ST-4, ST-5, ST-8, ST-11, ST-32 and ST-41/44 complexes) was higher than transcription in isolates belonging to other ccs (data not shown). Therefore FetA may play a role in the ability of meningococci to cause epidemic disease, and vaccine efficacy against these ccs is likely to be high.

The majority of work on the expression of FetA, or the use of FetA as a vaccine candidate, has been done using isolates belonging to the ST-32 complex. Of the ccs investigate here, the ST-32 complex showed the highest variability in spacer lengths and greatest transcriptional response to iron availability. Across other ccs there was limited variability in the spacer length as the promoters in many isolates were stabilised by interruptions within the poly-C repeat. Compared to ST-32, the majority of isolates showed increased expression compared to ST-32 under both iron-repressed and -replete conditions. These differences are likely to be due to polymorphisms within the -35 hexamer and the Fur-binding regions. Therefore, although a vaccine targeting FetA may show reduced efficacy against the ST-32 cc, efficacy against other ccs is likely to be higher and more consistent than predicted from previous studies regardless of iron availability *in vivo*.

Chapter 8:

General Discussion

8.1: Discussion

8.1.1: Background

Due to the similarity of the *Neisseria meningitidis* serogroup B polysaccharide capsule with human antigens, the development of vaccines that will provide comprehensive coverage against meningococcal disease has focussed on sub-capsular antigens. As immunogenic meningococcal proteins have evolved to be antigenically variable or transiently expressed, it is generally accepted that an effective vaccine will need to contain a cocktail of antigens. One vaccine currently under pre-clinical development is MenPF, based on multiple variants of two outer membrane proteins, PorA and FetA. Antigenic variation of these proteins is structured among meningococcal lineages; therefore, a combination of variants has been chosen that should provide effective coverage against meningococci belonging to the most prevalent disease-causing clonal complexes in Europe (Brehony *et al.*, unpublished). The use of both PorA and FetA provides three immunodominant epitopes (two on PorA and one on FetA), which should provide broad coverage against meningococci expressing a homologous variants of any epitope, and high protection against those clonal complexes expressing homologous variants of all three epitopes.

In this study, the potential of PorA and FetA as effective vaccine components was investigated. Efficacy of meningococcal vaccines is determined by quantifying the bactericidal activity of serum after vaccination. Although it has been argued that SBAs can underestimate the protection induced by a vaccine [56], SBAs have been shown to correlate with protection induced by conjugate polysaccharide vaccines and vaccines targeting PorA, and is currently considered the best available surrogate of protection for meningococcal disease [45]. Whether

a particular antigen can effectively provide protection against meningococcal disease, as determined by SBA, is dependent on a combination of properties of that antigen. The antigen must, firstly, be capable of inducing the production of sufficient quantities of complement-binding antibodies which are specific for accessible epitopes on the surface of live meningococci. Secondly, in a large proportion of the target meningococcal population, the antigen must be present with a sufficiently high epitope density for complement-activation and bactericidal killing by specific-antibodies.

8.1.2: Construction of a strain for OMV vaccine production

The quantity and quality of antibodies produced can be largely influenced by the formulation used. In MenPF, the PorA and FetA antigens will be presented in OMVs. Presentation of antigens in OMVs not only ensures that antigens are correctly folded and displayed, but also results in production of appropriate IgG subclasses (IgG2 in mice [205], IgG1 and IgG3 in humans [206]) for activation of the complement cascade. However, OMVs are highly complex, and the concentration of particular antigens can vary over time or under different growth conditions. This is a particular problem with FetA, as the phase-variable and iron-repressed expression of this antigen can result in low and inconsistent levels of the protein in OMVs. Consequently, using wildtype meningococci, it would be difficult to produce an OMV vaccine specifically targeting FetA, as batches containing low levels of that antigen would be unlikely to induce high levels of FetA-specific antibodies [119,182].

In this study, a genetically-modified meningococcal strain has been produced that expresses a single variant of FetA constitutively at increased levels regardless of iron-availability or growth medium. This FetA-on strain is genetically stable and, in many other respects, identical to the wildtype parent strain. Therefore, methods for the large-scale production of vesicles from this strain can easily be developed using existing protocols and specifications for OMV vaccine

production. Furthermore, as the meningococcal parent strain chosen for this study is the same as that used for production of the Norwegian OMV vaccine [93], the reactogenicity and immunogenicity of OMVs from the FetA-on strain are likely to be similar to that seen during the widespread use of MenBvac, with the exception of the response to FetA.

8.1.3: Use of OMVs to induce a bactericidal response against PorA and FetA

Using a two-dose schedule in mice, OMVs from the modified strain were capable of inducing high levels of complement-binding IgG specific for that antigen, as well as to PorA and other outer membrane proteins such as Omp85. As these antigens are known to be immunogenic following immunisation with MenBvac [243], the responses induced in mice suggests that the vaccine could also induce antibodies against PorA and FetA in humans. Therefore, further strain construction, to produce a combination of strains expressing all the required PorA and FetA variants, may be an effective strategy for inducing protection based on these two antigens in an OMV vaccine. However, it is difficult to predict how immunogenic the different variants would be when combined.

With the multivalent vaccines Hexamen and Nonamen (Netherlands Vaccine Institute), containing two or three OMVs each expressing three PorA types, the different variants are not equally targeted by the antibody response in either mice [145] or humans [143]. While certain PorA variants are highly immunogenic, such as P1.5,2, others, such as P1.7-2,4, are weakly immunogenic [143,144,145]. This was supported by evidence presented in this study, where two doses of OMV containing the P1.7-2,4 variant from the NZ98/254 strain failed to induce PorA-specific IgG in mice, while only one dose of OMV from the H44/76-derived FetA-on strain was required to induce a response against P1.7,16. The differences in immunogenicity may, in part, be due to other characteristics of the OMVs as the antibody responses to other antigens also varied between the OMVs [169]. This would support the use of H44/76 as a parent strain

for the production of all vaccine strains, rather than using multiple strain backgrounds that may increase the variability in the antibody responses targeted to each OMV.

8.1.4: Use of a prime-boost strategy to increase bactericidal antibodies against PorA and FetA

It is likely that, as with PorA, different variants of FetA will also have varying abilities to induce bactericidal antibodies. It will be important, when developing a multicomponent vaccine, to use an immunisation strategy that can enhance the antibody response against poorly immunogenic variants as well as reducing antibodies against proteins that do not result in bactericidal killing. In this study, we have shown the potential of using a heterologous prime boost strategy, involving a primary dose of OMV followed by a secondary dose of recombinant protein, to increase the bactericidal antibody response against the weakly-immunogenic P1.7-2,4 variant. The success of this strategy highlighted the benefits of using OMVs as carrier molecules: induction of switching to complement-binding IgG subclasses; and exposure of bactericidal, surface-exposed epitopes [244]. The recombinant protein dose served to target the antibody response towards the appropriate antigen rather than to other proteins within the OMV.

While the heterologous prime-boost strategy was more effective at inducing bactericidal antibodies against the PorA protein than the use of OMV alone, the same was not true for FetA, as the majority of FetA-specific antibodies induced by the recombinant protein seemed to be specific for non-surface epitopes. Although antibodies were also induced to non-surface epitopes of PorA (data not shown), there were sufficient antibodies targeting the extracellular loops for complement-mediated killing. However, the responses seen here in mice may not be representative of those that would be seen in humans using a similar strategy.

The difference in the efficacy of the strategy between the two antigens, seen in mice, may be due to the reduced quantity of FetA in the OMVs compared to PorA and to the immunodominance of the P1.7,16 antigen over F3-3. When immunised with OMVs, B-lymphocytes are activated that are specific to a range of antigens present in the OMVs. Some B-cells will undergo clonal expansion and avidity maturation in germinal centres before differentiating into memory B-cells and long-lived plasma cells, which secrete the antibody measured in sera collected from the mice. During this process, developing B-cells will compete for resources and to interact with follicular dendritic cells and T-cells. B-cells that are unsuccessful undergo apoptosis [245]. It may be that, due to the reduced level of FetA in the OMVs compared to PorA, many of the B-cells specific for FetA are outcompeted and undergo apoptosis. Those that survive may not be effectively boosted by a second dose of recombinant protein without additional adjuvant. The efficacy of the prime-boost strategy may, therefore, be improved by further increasing the level of FetA in the OMVs, to encourage the activation of more FetA-specific B-cells following OMV immunisation.

There may also be differences in the responses to PorA and FetA when given as a dose of recombinant protein rather than presented in OMVs. When presented as recombinant proteins, antibodies will be induced against a range of epitopes that are not exposed on the surface of the bacteria. The surface-epitopes of FetA may not be presented effectively by T-cells, resulting in a large proportion of activated B-cells specific for non-surface epitopes compared to surface epitopes. Consequently, the majority of the induced antibody response will not be functional. In contrast, the surface epitopes of PorA may be more effectively presented by T-cells following a dose of recombinant protein, leading to a higher proportion of antibodies that are functional in an SBA.

Incorporation of FetA into carrier molecular such as liposomes may be a more effective strategy for increasing bactericidal antibodies against this antigen, as this formulation will not only aid correct folding of the protein but also predominantly expose only surface epitopes.

Furthermore, using liposomes, multiple variants of PorA and FetA could easily be combined at equal concentrations, potentially balancing the antibody responses against the two antigens.

8.1.5: Variable expression of PorA and FetA

Although use of an OMV vaccine, or an alternative formulation, could induce high quantities of complement-binding antibodies against FetA, these antibodies may not be sufficient for protecting humans against meningococcal disease. Whether antibodies against PorA and FetA are likely to lead to bacterial clearance is dependent on antigen expression by the meningococcal clones circulating in the human population, as sufficient concentrations of antigen are required for specific antibodies to be bactericidal [99]. Although there is evidence that both PorA and FetA are expressed *in vivo* [165,246], this expression is variable among isolates [135,166]. For PorA, much of the variation in transcription seen in this study was due to differences in the spacer length between the -35 and -10 binding sites, consistent with phase-variability described previously [135,186]. This consistency demonstrates the potential for using quantitative RT-PCR alongside whole-genome sequence data for investigating gene regulation at a population level. Furthermore, the distribution of promoter sequence variation seen among clonal complexes, for both *porA* and *fetA*, highlights the importance of investigating gene expression across diverse isolates rather than relying on mutants of a single strain [244].

Unlike regulation of *porA*, variable spacer lengths in the *fetA* promoter had little effect on transcript levels measured here. However, for both *porA* and *fetA*, the majority of isolates investigated contained promoter sequences with optimal spacing for high expression. In *fetA*, these are likely to be stable over time due to interruptions within the repeat region, which are likely to reduce the probability of slipped-strand mispairing during replication. Therefore,

phase variability due to promoter sequence variation is unlikely to be an issue for vaccine coverage against either antigen.

Expression of FetA is also variable as a result of iron-regulation [172], although data presented here suggests that the strength of *fetA*-regulation by iron is weaker than that predicted from studies with ST-32 isolates [166]. This is due to variation in the sequence bound by the regulatory Fur protein. It is generally accepted that conditions *in vivo* are iron-limiting [242], but are likely to vary in different parts of the human body. This study provides evidence that, in many clonal complexes, *fetA* expression *in vivo* is likely to be subject to only low levels of repression both during carriage and invasive disease, regardless of iron availability in the bloodstream or the nasopharynx. Higher expression was seen in isolates from ccs associated with epidemic or hyperendemic disease, when compared to ccs associated with low levels of disease; as these ccs are predominantly those that are specifically targeted by developmental meningococcal vaccines, coverage against these lineages is likely to be high. Further study of the relationship between pathogenicity and FetA-expression, using a wider variety of ccs and more carriage isolates, could provide additional support for this observation. Furthermore, data on the correlation between transcription and protein concentration, and the threshold required for killing by specific antibodies, would be required for more reliable estimates of coverage. However, data presented here suggests that the variable expression of FetA in wildtype meningococci is unlikely to be an issue for vaccine coverage.

8.1.6: Potential issues identified for the use of FetA as an OMV vaccine component

The key issue identified in this study regarding the use of FetA as a vaccine candidate was that antigen concentration alone is not sufficient for complement-mediated killing. Constructed meningococcal strains, containing PorA and FetA switched on and off in various combinations, were effectively used to separate and analyse the bactericidal responses targeting each

antigen individually or other outer membrane proteins. Results obtained using these strains show that, although the FetA-on OMVs induced high levels of bactericidal antibodies against both PorA and FetA, the antibodies targeting FetA were not capable of complement-mediated killing alone, even against a strain with increased expression. The same was not true of FetA-polyclonal sera (following immunisation with recombinant FetA with MPL adjuvant), which were bactericidal against the wildtype strain (data not shown). This suggests that it is not the absolute concentration of FetA that is an issue, but rather that a sufficient density and distribution of targeted epitopes is required.

Previous studies by Kortekaas *et al.,* [170] showed that, following OMV immunisation, antibodies are predominantly induced against the variable region present on loop 5 of FetA (Figure 1.6, page 28), and that this loop shields the activity of antibodies against another immunogenic loop, loop 3 [170]. In this study, although bactericidal antibodies against FetA were detected after OMV immunisation, these were incapable of binding denatured protein. In contrast, antibodies induced against other proteins by OMV immunisation, including PorA, PorB, and Omp85, were capable of binding denatured protein. Alongside results presented by Kortekaas *et al.*, [170], these data suggest that FetA-specific antibodies induced by OMVs here were specific for a single epitope, likely to be the VR, and that this epitope is conformational.

Following OMV immunisation, antibodies binding FetA may be widely dispersed in the meningococcal outer membrane, leading to low antibody density on the cell surface, which would not result in complement-mediated killing [127]. If FetA is present in multimers in the membrane, the distance or orientation of the targeted epitopes on different molecules may not be suitable for the antibodies to cooperatively recruit complement. In comparison to OMV immunisation, immunisation with recombinant FetA with MPL adjuvant is likely to have induced antibodies that bind multiple surface-exposed epitopes rather than just the variable region or the hidden epitope on loop 3. Such antibodies, in combination with VR-specific binding, would be capable of bactericidal killing without requiring the presence of antibodies

specific for other proteins. This is consistent with previous evidence of the bactericidal activity of monoclonal antibodies against fHbp: in strains that expressed low concentrations of the antigen, bactericidal killing was only achieved in the presence of antibodies to multiple epitopes that were within a particular distance of each other on the antigen [247]. The specificity of antibodies for non-VR epitopes of FetA could be investigated using a modified FetA that does not contain the VR, as described by Kortekaas *et al.,* [170].

Due to the dominance of the VR, unless SBAs underestimate FetA-specific bactericidal activity, antibodies induced against FetA by an OMV vaccine may only be effective when combined with antibodies specific for another antigen, such as PorA. Antibodies to FetA and PorA were seen to act synergistically to enhance meningococcal killing. Therefore, the multivalent MenPF vaccine should provide a high level of protection against lineages expressing a homologous combination of both PorA and FetA, while protection may be limited against meningococcal clones expressing heterologous PorA types not included in the vaccine. A vaccine formulation that induces antibodies against multiple FetA surface epitopes rather than simply the VR would increase cross-reactivity with other FetA variants and meningococcal lineages. Further immunisations with different vaccine formulations, such as liposomes containing FetA, would indicate whether improved coverage against FetA could be achieved in this manner.

8.2: Conclusions

In this study, we have shown that it is possible to genetically modify a meningococcal strain to express FetA constitutively at increased concentrations for OMV vaccine production. OMVs from this strain are capable of inducing bactericidal antibodies against both PorA and FetA; however, an alternative vaccine formulation or immunisation strategy is likely to improve protection and cross-reactivity of antibodies against both antigens. A heterologous prime-boost strategy can be used to increase the quantity of bactericidal antibodies against weakly-

immunogenic PorA variants, while a formulation that induces antibodies against multiple surface-epitopes may be required to increase bactericidal activity against FetA.

If a strategy is used that removes the immunodominance of the FetA VR epitope, variable gene regulation of *porA* and *fetA* are unlikely to be an issue for vaccine coverage. In particular, the strength of iron-repression on *fetA* in many clonal complexes is reduced due to sequence variation. This provides further evidence that, in many disease-associated clonal complexes, FetA is likely to be expressed *in vivo* regardless of the conditions. Along with verification that antibodies against PorA and FetA can work synergistically to enhance complement-mediated killing, these data suggest that, provided an appropriate vaccine formulation and schedule is developed, a vaccine targeting the PorA and FetA antigens can offer high levels of protection against meningococcal disease.

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Appendix 1:

Neisseria meningitidis strains

A1.1: Strains used for cloning and immunology

Strain	Designation	Genotype	Source
H44/76	B: P1.7,16: F3-3: ST-32 (ST-32 complex)	Wildtype	Manchester Public Health Laboratory Services, Manchester, UK
NZ98/254	B: P1.7-2,4: F1-5: ST-42 (ST-41/44 complex)	Wildtype	Norwegian Institute of Public Health, Oslo, Norway
91/40	B: P1.7-2,4: F1-5: ST-42 (ST-41/44 complex)	Wildtype	Max Planck Institute of Molecular Genetics, Berlin, Germany
3043	B: P1.7,16: F-: ST-32 (ST-32 complex)	*fetA::kan*	Produced in [187]

A1.2: Strains produced in this study

Strain	Parent Strain	Genotype	Modifications from wildtype
3200	H44/76	$fetAp_{17bp}$ $fetA_{\Delta 65C}$	*fetA* promoter replaced with the modified *porA* promoter. -1 Frame-shift introduced at the start of the *fetA* gene, resulting in low FetA expression. PorA expression as wildtype.
3207	H44/76	$fetAp_{17bp}$	*fetA* promoter replaced with the modified *porA* promoter, resulting in high expression. PorA expression as wildtype.
3311	3043	*fetA::kan* *porA::ermC*	*fetA* gene interrupted by a Kanamycin-resistance cassette insertion [187]. *porA* gene interrupted by an Erythromycin-resistance cassette insertion.
3312	3207	$fetAp_{17bp}$ *porA::ermC*	*fetA* promoter replaced with the modified *porA* promoter. *porA* gene interrupted by an Erythromycin-resistance cassette insertion.
3313	H44/76	$fetAp_{17bp}$ $fetA_{\Delta 66G}$	*fetA* promoter replaced with the modified *porA* promoter. -1 Frame-shift introduced at various positions at the start of the *fetA* gene. PorA expression as wildtype.
3314		$fetAp_{17bp}$ $fetA_{\Delta 47C}$	
3315		$fetAp_{17bp}$ $fetA_{\Delta 81C}$	
3316		$fetAp_{17bp}$ $fetA_{\Delta 86T}$	

A1.3: Strains used for gene expression analysis

All isolates sourced from the Max Planck Institute of Molecular Genetics (Berlin, Germany). Isolates not available for RNA extraction are indicated by *.

Isolate	Country	Year	Disease Status	Serogroup	Clonal Complex	Sequence Type	PorA	FetA
500	Italy	1984	Unknown	C	ST-11 complex	11	P1.5,2	F1-1
1000	USSR	1988	Invasive	B	ST-18 complex	20	P1.5-1,10-4	F1-9
8680*	Chile	1987	Invasive	B	ST-32 complex	32	P1.7-2,3	F3-1
BZ198	Netherlands	1986	Invasive	B	ST-41/44 complex	41	P1.7-2,4	F1-5
44/76*	Norway	1976	Invasive	B	ST-32 complex	32	P1.7,16	F3-3
BZ 10	Netherlands	1967	Invasive	B	ST-8 complex	8	P1.5-1,2-2	F3-9
BZ 169	Netherlands	1985	Invasive	B	ST-32 complex	32	P1.5-2,16	F3-3
NG PB24	Norway	1985	Invasive	B	ST-32 complex	32	P1.7-2,16-7	F3-3
NG P20	Norway	1969	Invasive	B	ST-11 complex	11	P1.5,2	F1-1
3906	China	1977	Invasive	B		17	P1.12-1,16-8	F5-9
F6124	Chad	1988	Invasive	A	ST-5 complex	5	P1.20,9	F3-1
F4698	Saudi	1987	Carriage	A	ST-5 complex	5	P1.20,9	F3-1
14/1455	USSR	1970	Unknown	A	ST-5 complex	5	P1.20,9	F3-1
153	China	1966	Invasive	A	ST-5 complex	5	P1.20,9	F3-1
297-0	Chile	1987	Carriage	B	ST-254 complex	8523	P1.19,15-1	F3-6
AK50	Greece	1992	Invasive	B	ST-41/44 complex	41	P1.7-2,4	F1-5
860060	Netherlands	1986	Invasive	X	ST-750 complex	24	P1.12-1,13-5	F5-5
B6116/77	Iceland	1977	Invasive	B	ST-8 complex	10	P1.5-1,2-2	F1-4
M40/94	Chile	1994	Invasive	B	ST-41/44 complex	41	P1.7-2,4	F1-5
M-101/93	Iceland	1993	Invasive	B	ST-41/44 complex	41	P1.7-2,4	F1-5
312 901	England	1996	Invasive	C	ST-8 complex	8	P1.5,2	F1-7
890326	Netherlands	1989	Invasive	Z	ST-103 complex	28	P1.5-4,2-2	F3-9
88/03415	Scotland	1988	Invasive	B	ST-41/44 complex	46	P1.7-2,4	F1-5
G2136	England	1986	Invasive	B	ST-8 complex	8	P1.5-2,10-1	F3-6
SB25	South Africa	1990	Invasive	C	ST-8 complex	8	P1.18-1,3	F3-9
2059001	Mali	1990	Invasive	A	ST-4 complex	4	P1.7,13	F1-5
A22	Norway	1986	Carriage	W-135	ST-22 complex	22	P1.18-1,3	F4-1

Isolate ID	Country	Year	Disease Status	Serogroup	Clonal Complex	Sequence Type	PorA	FetA
E26	Norway	1988	Carriage	X	ST-198 complex	39	P1.18,25-1	F5-5
N45/96	Norway	1996	Invasive	B	ST-41/44 complex	41	P1.7-2,4	F1-5
860800	Netherlands	1986	Invasive	Y	ST-167 complex	29	P1.5-1,10-4	F4-1
E32	Norway	1988	Carriage	Z	ST-334 complex	31	P1.5-2,10-9	F1-5
71/94	Norway	1994	Invasive	Y	ST-23 complex	23	P1.5-1,2-2	F5-8
204/92	Cuba	1992	Invasive	B	ST-32 complex	33	P1.19,15	F5-1
94/155	New Zealand	1994	Invasive	C	ST-8 complex	66	P1.5,2	F3-9
50/94	Norway	1994	Invasive	B	ST-41/44 complex	45	P1.7-2,4	F1-5
400	Austria	1991	Invasive	B	ST-41/44 complex	40	P1.7-2,13-2	F1-5
931905	Netherlands	1993	Invasive	B	ST-41/44 complex	41	P1.7-2,4	F1-5
91/40	New Zealand	1991	Invasive	B	ST-41/44 complex	42	P1.7-2,4	F1-5
AK22	Greece	1992	Invasive	B	ST-8 complex	153	P1.5-2,10	F3-9
L93/4286	England	1993	Invasive	C	ST-11 complex	11	P1.5-1,10-4	F3-6
7891	Finland	1975	Invasive	A	ST-5 complex	5	P1.20,9	F3-1
NG 3/88	Norway	1988	Invasive	B		12	P1.7-1,1	F5-8
D8	Mali	1990	Invasive	A	ST-4 complex	4	P1.7,13-1	F1-5
Z2491	Gambia	1983	Invasive	A	ST-4 complex	4	P1.7,13-1	F1-5
26*	Niger	1963	Invasive	A	ST-4 complex	4	P1.7,13	F1-5
S5611	Australia	1977	Invasive	A	ST-1 complex	1	P1.5-2,10	F5-1
154	China	1966	Invasive	A	ST-5 complex	6	P1.20,9	F3-1
20	Niger	1963	Invasive	A	ST-1 complex	1	P1.5-2,10	F1-7
CN100	England	1941	Invasive	A		21	P1.5-2,10	F3-9
371*	India	1980	Invasive	A	ST-1 complex	1	P1.5-2,10	F5-1
DK 353	Denmark	1962	Invasive	B	ST-37 complex	37	P1.5-2,2-2	F5-7
NG 6/88	Norway	1988	Invasive	B	ST-269 complex	13	P1.7-1,1	F5-6
EG 328	East Germany	1985	Invasive	B	ST-18 complex	18	P1.22,14	F5-5
NG G40	Norway	1988	Carriage	B		25	P1.19,15-1	F5-2
BZ 147	Netherlands	1963	Invasive	B	ST-41/44 complex	48	P1.18-2,1-2	F3-9
528*	USSR	1989	Invasive	B	ST-18 complex	18	P1.22,14	F1-5

Isolate ID	Country	Year	Disease Status	Serogroup	Clonal Complex	Sequence Type	PorA	FetA
EG 011	East Germany	1986	Invasive	B		36	P1.18-1,3	F4-1
79126	China	1979	Invasive	A	ST-1 complex	3	P1.7-3,10-5	F5-5
NG 080	Norway	1981	Invasive	B	ST-32 complex	32	P1.7,16	F3-3
243	Cameroon	1966	Unknown	A	ST-4 complex	4	P1.7,13	F1-5
MA-5756	Spain	1985	Unknown	C	ST-11 complex	11	P1.5,2-1	F5-5
M597*	Israel	1988	Invasive	C	ST-11 complex	11	P1.5,2-1	F5-5
BRAZ10	Brazil	1976	Unknown	C	ST-11 complex	11	P1.5-1,10-1	F1-10
92001	China	1992	Invasive	A	ST-5 complex	7	P1.20,9	F3-1
6748	Canada	1971	Invasive	A	ST-1 complex	1	P1.18-1,3	F5-1
D1	Mali	1989	Carriage	C	ST-11 complex	11	P1.5,2-1	F5-4
90/18311	Scotland	1990	Unknown	C	ST-11 complex	11	P1.5,2-1	F5-5
BZ 163*	Netherlands	1979	Invasive	B	ST-8 complex	9	P1.21,16	F1-7
NG H41	Norway	1988	Carriage	B		27	P1.5-2,10	F3-6
NG F26	Norway	1988	Carriage	B	ST-269 complex	13	P1.31,16	F3-9
NG E28	Norway	1988	Carriage	B		26	P1.22-1,14-1	F5-2
80049	China	1963	Carriage	A	ST-5 complex	5	P1.5-2,10	F1-5
NG H36	Norway	1988	Carriage	B	ST-41/44 complex	47	P1.5-1,2-2	F1-7
139M*	Philippines	1968	Unknown	A	ST-1 complex	1	P1.5-2,10	F5-1
H1964	UK	1987	Invasive	A	ST-5 complex	5	P1.20,9	F3-1
SWZ107	Switzerland	1986	Invasive	B	ST-35 complex	35	P1.22-1,14-1	F4-1
EG 327	East Germany	1985	Invasive	B	ST-18 complex	19	P1.7,14-1	F5-2
NG H15	Norway	1988	Carriage	B	ST-41/44 complex	43	P1.19,15-2	F1-5
79128	China	1979	Invasive	A	ST-1 complex	3	P1.7-1,10	F5-5
BZ 232	Netherlands	1964	Invasive	B	ST-37 complex	38	P1.5-2,2-2	F5-2
DK 24	Denmark	1940	Invasive	B	ST-4240	16		F1-8
A4/M1027	USA	1937	Invasive	A	ST-4 complex	4	P1.5-2,10	F1-5
NG E30	Norway	1988	Carriage	B	ST-41/44 complex	44	P1.21,16	F1-7
690	India	1980	Invasive	A	ST-4 complex	4	P1.7,13-1	F1-5
S4355	Denmark	1974	Invasive	A	ST-5 complex	5	P1.5-1,9	F3-1
38VI	USA	1964	Unknown	B	ST-11 complex	11	P1.5,2	F1-1

Isolate ID	Country	Year	Disease Status	Serogroup	Clonal Complex	Sequence Type	PorA	FetA
A4/M1027	USA	1937	Invasive	A	ST-4 complex	4	P1.5-2,10	F1-5
NG E30	Norway	1988	Carriage	B	ST-41/44 complex	44	P1.21,16	F1-7
690	India	1980	Invasive	A	ST-4 complex	4	P1.7,13-1	F1-5
S4355	Denmark	1974	Invasive	A	ST-5 complex	5	P1.5-1,9	F3-1
38VI	USA	1964	Unknown	B	ST-11 complex	11	P1.5,2	F1-1
106	Morocco	1967	Invasive	A	ST-1 complex	1	P1.5-2,10	F5-1
196/87	Norway	1987	Unknown	C	ST-32 complex	32	P1.7-2,16-12	F3-3
322/85	East Germany	1985	Invasive	A	ST-1 complex	2	P1.5-2,10	F5-2
255	Burkina Faso	1966	Invasive	A	ST-4 complex	4	P1.7-2,13-1	F1-5
254	Djibouti	1966	Invasive	A	ST-1 complex	1	P1.5-2,10	F1-7
S3131	Ghana	1973	Invasive	A	ST-4 complex	4	P1.7,13-1	F1-5
NG E31	Norway	1988	Carriage	B	ST-364 complex	15	P1.12-1,13-1	F1-6
NG 4/88	Norway	1988	Invasive	B		30	P1.18,25-7	F1-5
IAL2229	Brazil	1976	Unknown	A	ST-5 complex	5	P1.20,9	F2-1
NG H38	Norway	1988	Carriage	B		36	P1.18-1,3	F4-1
EG 329	East Germany	1985	Invasive	B	ST-32 complex	32	P1.7-1,16	F1-2
NG144/82	Norway	1982	Invasive	B	ST-32 complex	32	P1.7,16	F3-3
BZ 83	Netherlands	1984	Invasive	B	ST-32 complex	34	P1.5-2,10	F5-1
1014	Sudan	1985	Invasive	A	ST-4 complex	4	P1.7,13-1	F1-5
120M	Pakistan	1967	Invasive	A	ST-1 complex	1	P1.5-2,10	F5-1
393	Greece	1968	Carriage	A	ST-1 complex	1	P1.5-2,10	F5-1
BZ 133	Netherlands	1977	Invasive	B	ST-1 complex	1	P1.7,16	F5-1
10	Burkina Faso	1963	Invasive	A	ST-4 complex	4	P1.7,13-1	F1-5
11-004	China	1984	Invasive	A	ST-5 complex	5	P1.20,9	F3-8
F1576	Ghana	1984	Unknown	C	ST-11 complex	11	P1.5,2	F1-1
129E	West Germany	1964	Invasive	A	ST-1 complex	1	P1.5-2,10	F3-6

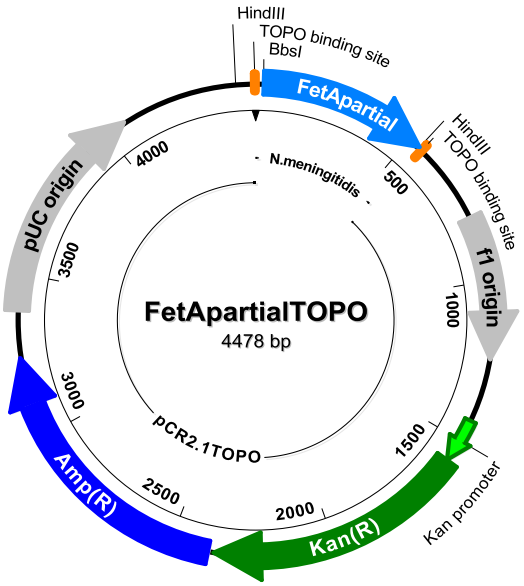
Appendix 2:

Production of plasmid pUC19FetApartialKanThdF

Plasmid pUC19PorApro was provided by Dr Rory care (NIBSC). Plasmids pUC19Kan(NoAmp) and pUC19PorAproNUS were provided by Dr Hannah Chan (NIBSC).

Plasmid pUC19FetApartialKanThdF was constructed as follows:

1) FetApartial was amplified from H44/76 gDNA using primers FetApartialF and FetApartialR (PCR annealing temperature 65°C, extension time 1 minute), introducing a *Bbs*I site preceding the gene and *Hind*III site following the gene. The 514bp PCR product was then cloned into a pCR2.1TOPO vector to give FetApartialTOPO.

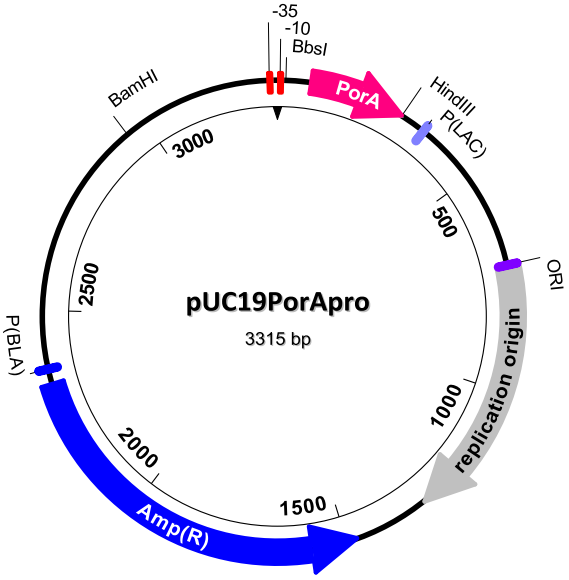


2) FetApartialTOPO was digested with *Bbs*I and *Hind*III to give the following fragments:

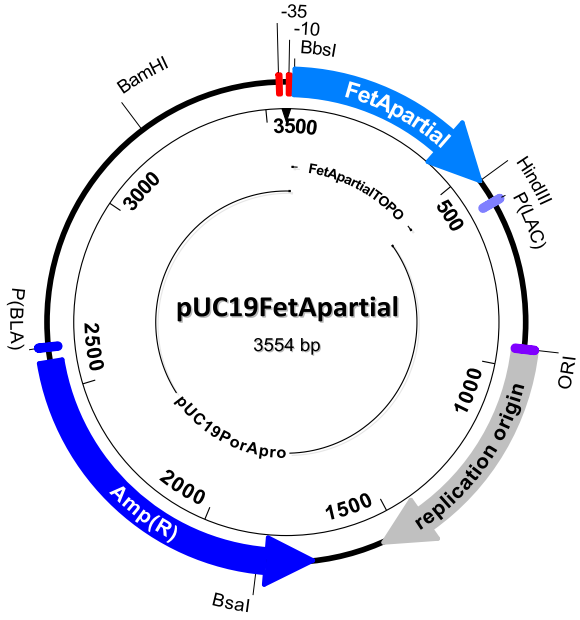
   - 3880bp (TOPO vector),
   - 514bp (FetApartial) – **FRAGMENT A,**
   - 84bp (TOPO binding site).

3) pUC19PorApro was digested with *Bbs*I and *Hind*III to give the following fragments:

- 3040bp (pUC19PorApro vector without partial *porA* gene) – **FRAGMENT B**,
- 275bp (partial *porA* gene).



4) The FetApartial fragment (Fragment A) was ligated into the pUC19PorApro vector fragment (Fragment B) to give pUC19FetApartial.

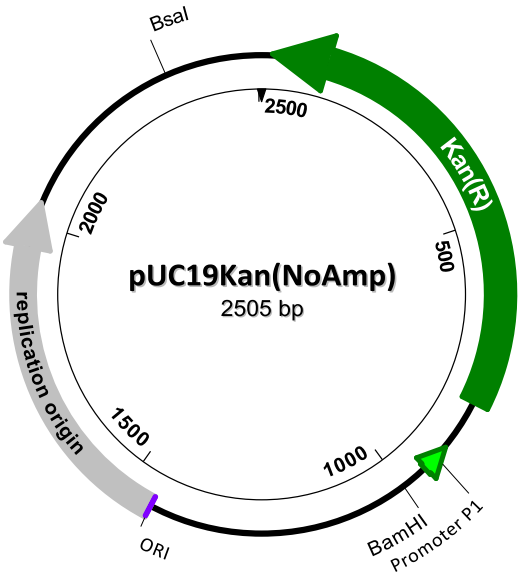


5) pUCFetApartial was digested with *Bsa*I and *Bam*HI to give the following fragments:

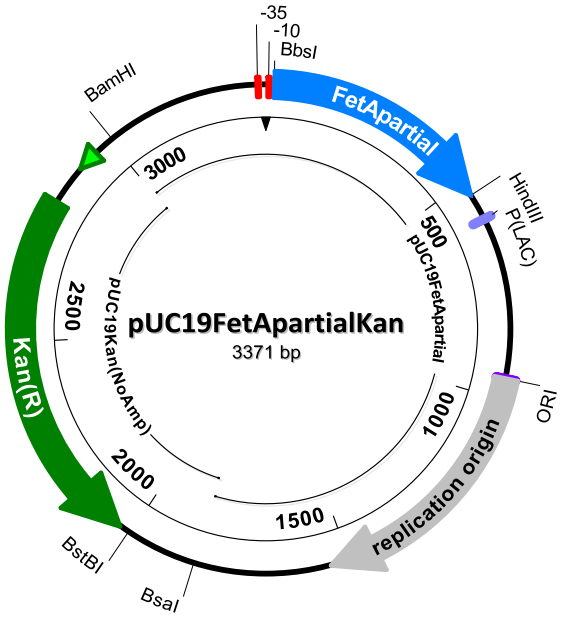
- 2211bp (Vector - PorApromoter, FetApartial and replication origin) – **FRAGMENT C,**
- 1343bp (AmpR).

6) pUC19Kan(noAmp) was digested with *Bsa*I and *Bam*HI to give the following fragments:

- 1343bp (Replication origin),
- 1162bp (KanR plus promoter) – **FRAGMENT D.**



7) The KanR fragment (Fragment D) was ligated into the pUC19FetApartial vector fragment (Fragment C) to give pUC19FetApartialKan.

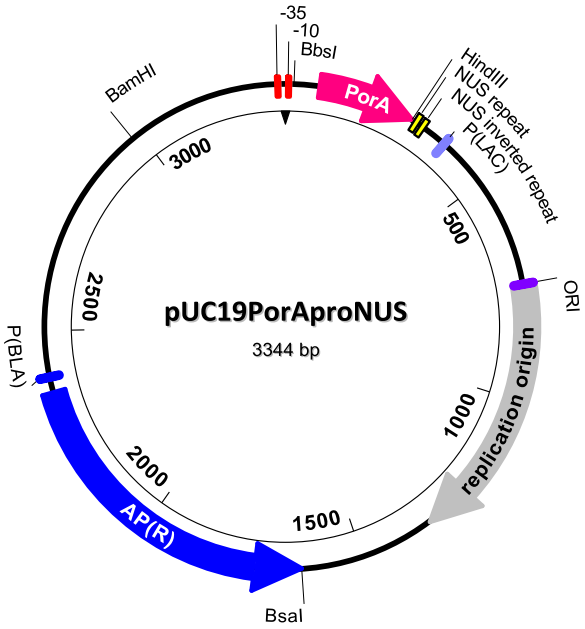


8) pUC19FetApartialKan was digested with *Bsa*I and *Hind*III to give the following fragments:

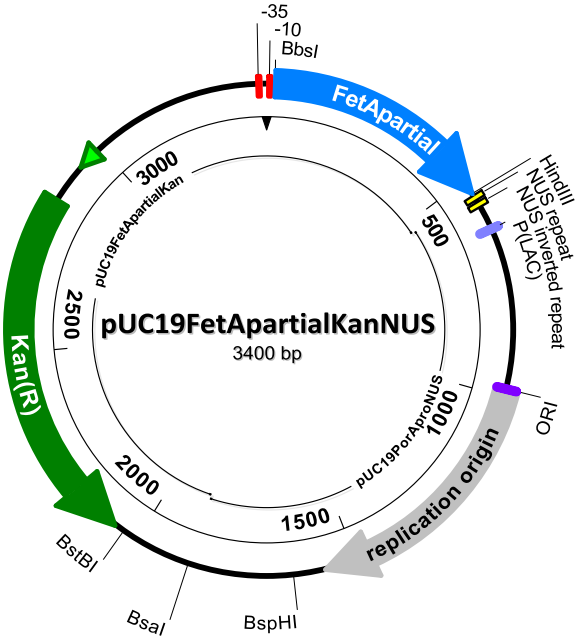
- 2060bp (KanR, FetApartial) – **FRAGMENT E,**
- 1313bp (Replication origin).

9) pUC19PorAproNUS was digested with *Bsa*I and *Hind*III to give the following fragments:

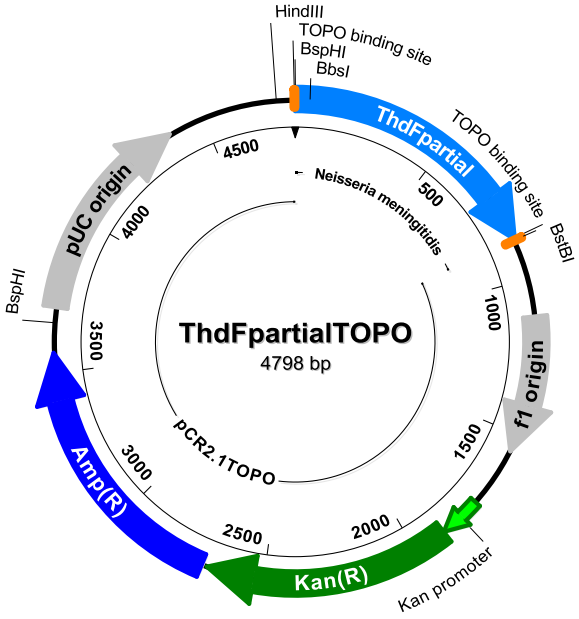
- 2002bp (AmpR, PorApro),
- 1342bp (Replication origin plus NUS) – **FRAGMENT F.**



10) The replication origin plus NUS fragment (Fragment F) was ligated into pUC19FetApartialKan (Fragment E, replacing the existing replication origin) to give pUC19FetApartialKanNUS.



11) ThdFpartial was amplified from H44/76 gDNA with primers ThdFBspHI and ThdFtotalR (PCR annealing temperature 65°C, extension time 1 minute), which introduced a *Bsp*HI and *Bst*BI site at the start and end of the gene, respectively. ThdFpartial was cloned into a pCR2.1TOPO vector to give ThdFpartialTOPO.



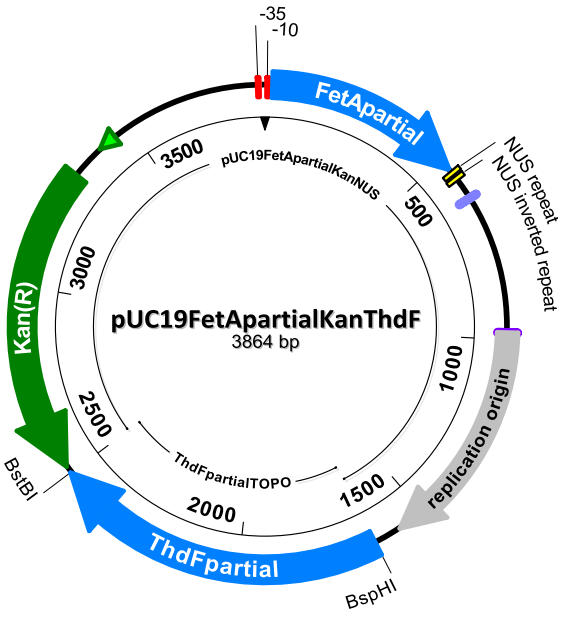
12) pUC19F33KanNUS was digested with *Bsp*HI and *Bst*BI to give the following fragments:

- 3007bp (pUC19FetApartialKanNUS vector) – **FRAGMENT G,**
- 861bp (fragment downstream of KanR).

13) ThdFendTOPO was digested with *Bsp*HI and *Bst*BI to give the following fragments:

- 2797bp (TOPO vector),
- 1140bp (pUC origin),
- 861bp (ThdFpartial) – **FRAGMENT H.**

14) ThdFpartial (Fragment H) was ligated into the pUC19FetApartialKanNUS vector (Fragment G) to give pUC19FetApartialKanThdF.

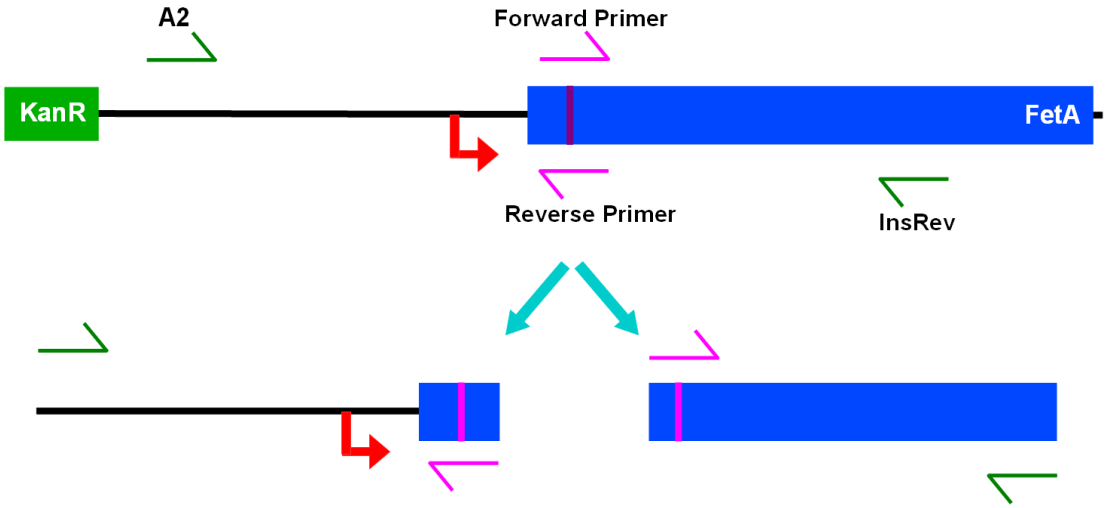


Appendix 3:

Site-directed mutagenesis method

Two sets of primers were used to amplify two overlapping fragments from 3207 genomic DNA. The internal primers contained the desired base change. For primer sequences, see Table 2.6 (page 37). External primers were A2 and InsRev. Internal primer pairs were as follows:

- Forward Primer: SDM3, Reverse Primer: SDM4 → Plasmid SDM3;
- Forward Primer: SDM5, Reverse Primer: SDM6 → Plasmid SDM5;
- Forward Primer: SDM7, Reverse Primer: SDM8 → Plasmid SDM7;
- Forward Primer: SDM9, Reverse Primer: SDM10 →Plasmid SDM9.



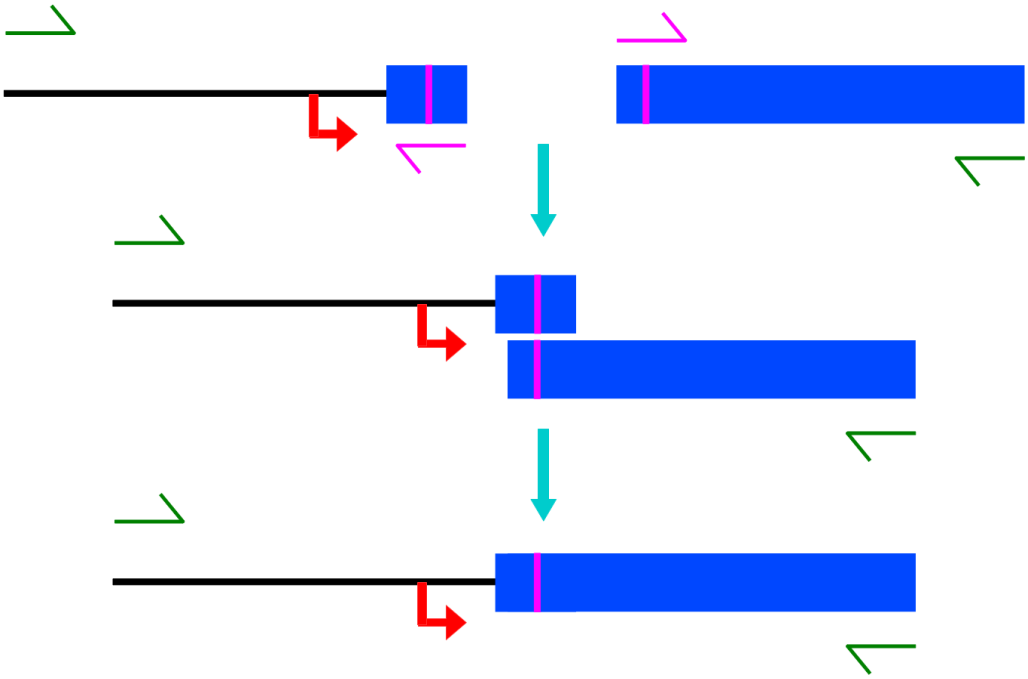
The fragments were amplified using Phusion DNA polymerase (New England Biolabs) with the internal primer at one tenth dilution (1µM) of the external primer (10µM). Thermocycling was run using the following conditions:

Reagent	Final Concentration
Phusion® 2x Master Mix	1x
Internal Primer	1µM
External Primer	10µM
DNA Template	10ng/µl

Thermocycling conditions:

Step	Temperature (°C)	Duration	Cycles
Activation	98	30s	1
Melting	98	5s	34
Annealing	65	10s	
Extension	72	30s	
Final Extension	72	300s	1

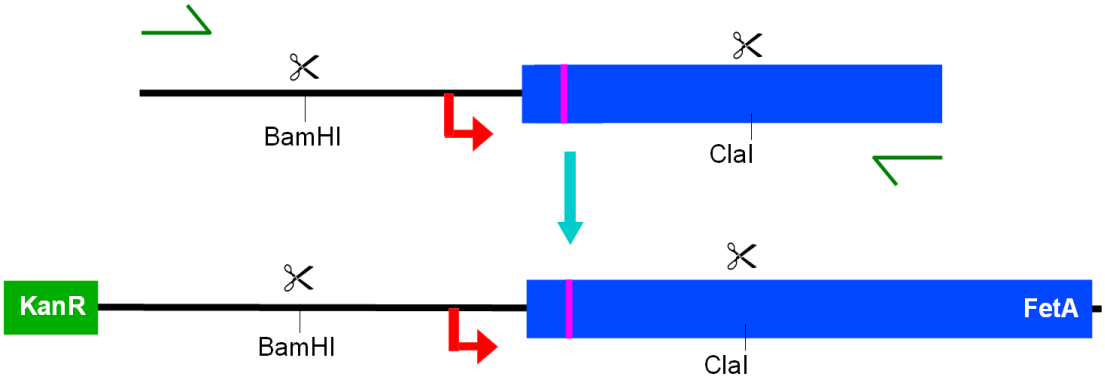
Following amplification from 3207 genomic DNA, the reactions containing the two fragments to be annealed were combined in equal volumes. The thermocycling reaction was then repeated. The same cycling conditions were used, with the exception of the annealing time, which was increased to 15 seconds.



The annealed fragments were then cloned into a pCR2.1TOPO vector, producing plasmids SDM3TOPO, SDM5TOPO, SDM7TOPO and SDM9TOPO, and sequenced to verify the correct mutation.

Plasmids containing the annealed fragments were then digested using *Bam*HI and *Cla*I, resulting in a fragment of size 646bp. Plasmid pUC19FetApartialKanThdF was digested with the same enzymes, allowing the 647bp fragment containing the start of FetApartial to be removed. The fragment containing the new mutation was

then ligated into the remaining 3218bp fragment of pUC19FetApartialKanThdF, producing plasmids SDM3, SDM5, SDM7 and SDM9.



Plasmids were used to transform H44/76 as described in Chapter 2 (see 2.5, page 42).

Appendix 4:

Sequence variation affecting transcription of the *porA* gene

Isolate	Spacer Length (bp)*	-4 to +1 Sequence**	*porA* ln(RQ)†
500	15	2	-0.392
1000	17	2	0.247
8680	NK	2	-
BZ198	17	2	0.262
44/76	18	2	-
BZ 10	19	3	0.513
BZ 169	17	2	-0.168
NG PB24	17	2	0.157
NG P20	15	2	0.293
3906	17	3	-0.934
F6124	16	1	-0.751
F4698	17	1	0.212
14/1455	18	1	0.440
153	17	1	0.212
297-0	17	2	0.197
AK50	18	2	0.549
860060	NK	2	-1.013
B6116/77	16	3	-1.487
M40/94	17	2	0.017
M-101/93	17	2	0.273
312 901	16	2	0.444
890326	16	2	-0.816
88/03415	17	2	0.356
G2136	17	3	-1.269
SB25	17	3	1.130
2059001	18	1	1.014
A22	16	3	-1.945
E26	17	2	0.641
N45/96	17	2	0.473
860800	NK	2	0.348
E32	15	3	-3.540
71/94	19	3	-0.671
204/92	NK	2	-0.711
94/155	16	2	-1.024
50/94	18	2	0.356
400	20	2	0.613
931905	17	2	-0.654
91/40	18	2	-1.171
AK22	17	3	1.037
L93/4286	21	2	-2.207
7891	15	1	-0.709

* NK = Not Known.

** Type 1 = AGACA, Type 2 = AGACG, Type 3 = TGACA.

† Where no value is given RQ could not be determined.

Isolate	Spacer Length (bp)*	-4 to +1 Sequence**	*porA* ln(RQ)†
NG 3/88	17	2	-0.914
D8	20	1	-0.282
Z2491	18	1	0.370
26	18	1	-
S5611	16	3	0.831
154	17	1	-0.229
20	16	3	-1.871
CN100	17	3	0.184
371	17	3	-
DK 353	20	2	-1.732
NG 6/88	17	2	-1.585
EG 328	17	2	0.213
NG G40	17	2	0.099
BZ 147	NK	2	-0.699
528	16	2	-0.161
EG 011	16	3	-1.820
79126	17	2	0.394
NG 080	17	2	-1.041
243	18	1	0.678
MA-5756	16	2	-0.270
M597	16	2	-
BRAZ10	17	2	1.036
92001	16	1	-0.365
6748	17	3	-1.201
D1	17	2	-0.835
90/18311	16	2	-0.914
BZ 163	NK	2	-
NG H41	16	3	-0.896
NG F26	17	1	-0.435
NG E28	17	2	-0.851
80049	17	3	0.131
NG H36	16	3	-1.402
139M	18	3	1.655
H1964	18	1	1.045
SWZ107	16	2	-0.485
EG 327	19	2	0.481
NG H15	18	2	0.158
79128	18	2	-0.837
BZ 232	16	2	-0.703
DK 24‡	-	-	-
A4/M1027	15	3	-2.937
NG E30	17	2	0.796
690	19	1	1.280
S4355	NK	3	-1.008
38VI	16	2	-1.452

* NK = Not Known.

** Type 1 = TGACA, Type 2 = AGACA, Type 3 = AGACG.

† Where no value is given RQ could not be determined.

‡ Deletion of *porA* gene and promoter

Isolate	Spacer Length (bp)*	-4 to +1 Sequence**	*porA* ln(RQ)†
106	17	3	0.443
196/87	17	2	-0.177
322/85	17	3	-0.027
255	20	1	-0.247
254	20	3	-1.118
S3131	18	1	0.323
NG E31	21	2	1.487
NG 4/88	17	2	-0.081
IAL2229	17	1	0.427
NG H38	NK	3	-0.103
EG 329	17	2	-0.440
NG144/82	18	2	-0.012
BZ 83	17	3	-0.223
1014	17	1	1.004
120M	17	3	-0.140
393	16	3	-0.849
BZ 133	17	3	-0.493
10	17	1	0.455
11-004	17	1	0.362
F1576	15	2	-0.691
129E	17	3	-0.278

* NK = Not Known.

** Type 1 = TGACA, Type 2 = AGACA, Type 3 = AGACG.

† Where no value is given RQ could not be determined.

Appendix 5:

Sequence variation affecting transcription of the *fetA* gene

Isolate	Promoter Sequence	Spacer Length	SD Sequence*	*fetA* ln(RQ)†	
				Fe-	Fe+
500	H	17	A	1.238	-2.354
1000	D	19	A	0.342	-1.130
8680	H	17	G	-	-
BZ198	B	17	G	0.705	0.312
44/76	B	17	G	-	-
BZ 10	B	17	A	1.177	-0.703
BZ 169	B	17	A	2.702	-2.096
NG PB24	D	19	G	0.302	-1.871
NG P20	H	17	A	3.971	0.096
3906	H	17	A	-0.501	-2.847
F6124	H	17	A	1.818	-0.020
F4698	H	17	A	1.389	-1.197
14/1455	H	17	A	1.209	-2.002
153	H	17	A	0.081	-0.448
297-0	H	17	G	1.171	-0.870
AK50	H	17	A	1.041	-0.103
860060	H	17	A	-0.155	-2.087
B6116/77	H	17	A	-0.491	-1.435
M40/94	C	18	G	-0.747	-0.904
M-101/93	C	18	G	0.118	-1.094
312 901	H	17	A	2.715	1.252
890326	H	17	G	-0.293	-0.711
88/03415	C	18	G	1.535	-0.957
G2136	H	17	A	-0.440	-0.425
SB25	H	17	A	3.912	1.475
2059001	I	17	A	1.430	0.939
A22	H	17	G	0.127	-0.648
E26	B	17	G	-0.598	-1.402
N45/96	H	17	A	2.243	1.514
860800	G	16	A	1.730	0.116
E32	A	16	A	-0.541	-1.008
71/94	E	20	A	-0.060	-0.931
204/92	B	17	G	-1.162	-2.333
94/155	H	17	A	-3.612	-4.200
50/94	D	19	G	-2.442	-3.963

* A = AAGGA, G = GAGGA

† Where no value is given RQ could not be determined.

Fe- = MH with DFAM, Fe+ = MH

Isolate	Promoter Sequence	Spacer Length	SD Sequence*	*fetA* ln(RQ)†	
				Fe-	Fe+
400	C	18	G	-0.027	-2.847
931905	B	17	G	-1.625	-2.957
91/40	H	17	G	-1.537	-1.814
AK22	H	17	A	-0.828	-3.037
L93/4286	H	17	A	-1.419	-3.219
7891	H	17	A	-1.737	-4.075
NG 3/88	D	19	G	-0.732	-3.079
D8	I	17	A	-0.445	-2.453
Z2491	I	17	A	-1.565	-1.306
26	H	17	A	-	-
S5611	B	17	G	-2.313	-3.037
154	H	17	A	1.313	-0.654
20	C	18	A	1.819	-0.705
CN100	C	18	A	0.176	-1.298
371	B	17	G	-	-
DK 353	E	20	A	1.247	0.189
NG 6/88	C	18	A	2.944	1.418
EG 328	H	17	A	0.839	-0.212
NG G40	H	17	A	0.257	0.035
BZ 147	H	17	A	4.782	0.269
528	C	18	A	-	-
EG 011	H	17	A	2.206	0.457
79126	D	19	A	0.181	-1.221
NG 080	D	19	G	0.329	-0.152
243	I	17	A	1.374	0.536
MA-5756	H	17	A	0.057	0.036
M597	H	17	A	-	-
BRAZ10	H	17	G	1.360	-0.069
92001	H	17	A	-0.057	-0.123
6748	H	17	G	-0.753	-0.464
D1	H	17	A	0.888	-0.184
90/18311	H	17	A	-0.029	-0.611
BZ 163	D	19	A	-	-
NG H41	H	17	A	-1.852	-1.415
NG F26	H	17	A	-0.997	-2.189
NG E28	D	19	A	0.114	-1.650
80049	I	17	A	2.344	0.166
NG H36	D	19	A	2.814	-0.329
139M	B	17	G	-	-

* A = AAGGA, G = GAGGA

† Where no value is given RQ could not be determined.

Fe- = MH with DFAM, Fe+ = MH

Isolate	Promoter Sequence	Spacer Length	SD Sequence*	*fetA* ln(RQ)†	
				Fe-	Fe+
H1964	H	17	A	3.307	0.424
SWZ107	B	17	A	0.734	-1.666
EG 327	H	17	A	0.592	-0.681
NG H15	H	17	G	0.449	0.036
79128	G	16	A	-0.722	1.414
BZ 232	B	17	A	-2.364	-2.087
DK 24	D	19	A	-	-
A4/M1027	I	17	A	1.595	0.453
NG E30	H	17	A	0.911	0.266
690	I	17	A	1.562	0.789
S4355	H	17	A	0.869	1.463
38VI	H	17	A	1.107	0.434
106	C	18	G	0.119	-0.962
196/87	F	21	G	-0.830	-1.715
322/85	H	17	A	-1.542	0.356
255	I	17	A	1.603	0.644
254	D	19	G	1.407	0.653
S3131	I	17	A	2.025	0.176
NG E31	H	17	A	0.229	1.469
NG 4/88	C	18	G	0.748	-0.041
IAL2229	B	17	A	2.244	-1.082
NG H38	B	17	A	2.483	0.140
EG 329	C	18	G	1.419	-1.514
NG144/82	C	18	G	3.438	-0.673
BZ 83	D	19	G	2.249	-0.226
1014	I	17	A	0.427	0.387
120M	D	19	G	0.865	-0.607
393	B	17	G	0.011	-1.277
BZ 133	H	17	G	0.043	-1.321
10	I	17	G	2.851	0.802
11-004	H	17	A	0.021	-1.204
F1576	H	17	A	0.286	-1.136
129E	H	17	A	3.517	-1.133

* A = AAGGA, G = GAGGA

† Where no value is given RQ could not be determined.

Fe- = MH with DFAM, Fe+ = MH