University of Southampton
Faculty of Medicine, Health and Biological Sciences

Studies on the *porB* gene of *Neisseria meningitidis*: Use as an epidemiological marker and as a potential vaccine candidate

by

Judith Claire Wright BSc

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ABSTRACT

FACULTY OF MEDICINE, HEALTH AND BIOLOGICAL SCIENCES

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STUDIES ON THE *PORB* GENE OF *NEISSERIA MENINGITIDIS*: USE AS AN EPIDEMIOLOGICAL MARKER AND AS A POTENTIAL VACCINE CANDIDATE

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Neisseria meningitidis is an obligate human pathogen responsible for endemic and epidemic disease world-wide. Currently available vaccine preparations are based upon group specific polysaccharides, however the structure of the polysaccharide of group B strains closely resembles that of human glycoproteins and it is non-immunogenic.

Traditional epidemiological studies of meningococcal disease have relied on detecting differences in surface antigen expression between strains by serological methods. Sequence variation in the class 1 outer membrane protein (OMP) has been shown to occur in regions not recognised by monoclonal antibodies representing a hidden source of epidemiological information. The variation of two additional OMPs, class 2/3 and Opc in a collection of strains from patients and close contacts following a disease outbreak was investigated. Comparison of the inferred amino acid sequences showed that three distinct variants within serotype 2a were present. Sequencing of the class 3 expressing strains allowed identification of two non-typeable strains. Of the 22 strain only nine were found to possess the opc gene, with four distinct variants present.

In order to evaluate the vaccine potential of a particular protein it is advantageous to prepare a source of that protein free from other meningococcal components. The porB gene, which codes for the class 3 protein was amplified by PCR and cloned into two expression systems. The highest levels of recombinant class 3 (rC13) protein expression were achieved in the Xpress™ system in E.coli JM101, which inserts the gene downstream of a N-terminal fusion encoding six histidine residues that functions as a metal binding domain, allowing a one-step purification procedure by immobilised metal affinity chromatography. Sequencing of the insert was used to ensure that no mutations had arisen as a result of the original gene amplification. Epitopes on the class 3 protein are of a conformational nature and therefore the rC13 protein was inserted into liposomes and Zwittergent 3-14 and NDSB-195 micelles in an attempt to refold the protein. The adjuvant MPLA was also included in some preparations to boost the immune response.

The various immunisation preparations all induced antibodies against the homologous class 3 protein as detected by ELISA and Western blotting. Cross-reactivity against a heterologous class 2 and class 3 protein was also seen in some sera. The ability to kill live meningococci is accepted as the correlate of protection. The highest levels of bactericidal activity were shown by sera raised against rC13 in Zwittergent 3-14 with MPLA and rC13 in NDSB-195 with MPLA. Thus, refolding of the rC13 protein had been achieved and had resulted in significant bactericidal activity.

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ABRREVIATIONS

BCA	Bicinchoninic acid
BCIP	5-bromo-4-chloro-3-indoyl phosphate
bp	Base pairs
BSA	Bovine serum albumin
cfu	Colony forming unit
CSF	cerebrospinal fluid
DMF	N,N-dimethylformamide
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DS	Dialysis sonication
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunoabsorbent assay
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
IgA/G/M	Immunoglobulin A/G/M
IMS	Industrial methylated spirit
IPTG	Isopropyl-β-D-thiogalactopyranoside
kb	Kilobase pairs
kDa	Kilodaltons
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MPLA	Monophosphoryl lipid A
NBT	Nitroblue tetrazolium
NDSB	Non-detergent sulphobetaine
Octylglucoside	octyl β-D-glucopyranoside
OMP	Outer membrane protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
rCl3	Recombinant class 3 protein

PMSF	Phenylmethylsulphonylfluoride
SEC	Size exclusion chromatography
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide electrophoresis
TBS	Tris buffered saline
TEMED	N,N,N',N'tetramethylethylenediamine
Tris	Tris (hydroxymethyl) aminomethane
TTBS	Tween-Tris buffered saline
Tween 20	Polyoxyethylenesorbitane monolaurate
UHQ	Ultra high quality water
UV	Ultra violet
v/v	Unit volume per unit volume
w/v	Unit weight per unit volume

Single and three letter amino acid codes

A	Ala	Alanine	M	Met	Methionine
C	Cys	Cysteine	N	Asn	Asparagine
D	Asp	Aspartic acid	P	Pro	Proline
E	Glu	Glutamic acid	Q	Gln	Glutamine
F	Phe	Phenylalanine	R	Arg	Arginine
G	Gly	Glycine	S	Ser	Serine
H	His	Histidine	T	Thr	Threonine
I	Ile	Isoleucine	V	Val	Valine
K	Lys	Lysine	W	Trp	Tryptophan
L	Leu	Leucine	Y	Tyr	Tyrosine

PUBLICATIONS

The work presented in this thesis have formed the basis of the following publications:

Wright, J.C., Jolley, K., Brooks, J.L., Heckels, J.E. (1997)
Sequence variation in outer membrane proteins in *Neisseria meningitidis* from patients and close household contacts
In *138th Ordinary Meeting of the Society of General Microbiology* at Southampton University in September 1997

Wright, J.C., Christodoulides, M., Heckels, J.E. (1998)
Expression and purification of meningococcal class 3 porin from *E.coli*: comparison of strategies for protein refolding on the immune response to meningococci
In *Eleventh International Pathogenic Neisseria Conference* pp 283.
Edited by X. Nassif, M.J. Quentin-Millet, M.K. Teha

Wright, J.C., Christodoulides, M., Heckels, J.E.
Immunisation with recombinant class 3 outer membrane protein from *Neisseria meningitidis*: influence of liposomes and micelles on recognition of native protein and the induction of a bactericidal immune response against meningococci
(*manuscript in preparation,* 2000)

CHAPTER 1: INTRODUCTION

1.1 Historical Background

Neisseria meningitidis is an obligate human pathogen, responsible for endemic and epidemic disease world-wide. The clinical manifestations of meningococcal disease are varied, ranging from sub-clinical nasopharangeal infection to fulminant life threatening disease (Apicella, 1990). The mortality rate in most developed countries has been reduced to 10-30% due to antibiotic therapy (Peltola, 1983). Chemotherapy must be administered early to be effective. This is hampered by the difficulty in obtaining an early diagnosis of meningococcal infection coupled with rapid onset of disease from the first presentation of symptoms.

Descriptions of meningitis have been found dating back to the sixteenth century. However cerebrospinal meningitis as a clinical entity was first identified by Vieusseaux in 1805, where it was described as malignant purpuric fever (Peltola, 1983). Subsequently the disease was reported during the nineteenth century throughout Europe and America for both its episodic and epidemic nature and its propensity for young children (Apicella, 1990). In 1887, Anton Weichselbaum isolated the etiologically agent of meningitis from cerebrospinal fluid (CSF) of a meningitis patient, which was named *Diplokokkus intracellularis meningitidis*. This was confirmed in 1898 when Councilman *et al.* isolated meningococci from both blood and CSF of meningitis patients, firmly establishing the bacterium as the causative agent of epidemic cerebrospinal meningitis (Dillon *et al*, 1983).

Neisseria meningitidis is an aerobic, encapsulated, Gram-negative diplococcus whose only natural reservoir is the human nasopharynx (Griffiss & Artenstein, 1976). Asymptomatic carriage in the nasopharynx may persist for weeks or months (Rake, 1934) but in certain individuals colonisation with a particular strain can lead to systemic infection. This infection may be limited to a mild bacteraemia or may result in a life-

threatening meningoccaemia with bacteria actively dividing in both the blood stream and CSF (Devoe, 1982).

The first effective treatment for meningococcal disease was introduced by Flexner in 1913 and known as serum therapy. Infected individuals were passively immunised with horse anti-meningococcal antiserum (Banks, 1948). In 1937, sulphonamide therapy radically altered the outcome of meningococcal infection and replaced serum therapy in treatment. Prophylaxis with sulphonamides eradicated the carrier state and provided a simple and safe method for prevention of epidemics (Apicella, 1990). Due to the widespread use of sulphonamide an increase in sulphonamide resistant meningococci was noted as early as 1938 (Feldman, 1966). These resistant strains were also present during the 1941-1943 epidemic and caused the epidemic in California in 1963. Sulphonamide resistant strains are now so widespread that this once effective drug is virtually useless in the general treatment or prophylaxis of meningococcal infection.

Fortunately by the time sulphonamide resistant meningococci had become clinically important, penicillin had been proven to be a safe and effective alternative treatment. In 1983 however, a meningococcal strain resistant to penicillin-G was isolated from the genitourinary tract. The isolate carried a β-lactamase producing plasmid, identical to that found in *N. gonorrhoeae* (Dillon *et al*, 1983). In addition to plasmid encoded resistance, several meningococcal strains possessing a chromosome-encoded resistance factor have also been isolated (Botha, 1988). These strains have yet to become clinically important.

Renewed interest in immunoprevention has occurred since the emergence of antibiotic resistant strains and the difficulty of early diagnosis of meningococcal disease. Although vaccines have been developed they do not protect against infection with all meningococcal strains and show various levels of effectiveness depending upon the age of the recipient.

1.2 Characteristics of the *Neisseria* Genus

The *Neisseria* genus takes its name from the German physician Albert Ludwig Siegmund Neisser who in 1879 described diplococci in smears of purulent secretions from acute gonorrhoea patients. The name gonorrhoea is attributed to Galen in about 130 AD. *N. meningitidis* was first described in 1887 and together with *N. gonorrhoeae* are responsible for almost all human diseases produced by the genus (Griffiss & Artenstein, 1976). The remaining members of the genus are almost invariably commensal in the upper respiratory tract of man. The *Neisseria* are organisms of the moist mucous membranes of man and other animals.

All members of the genus are Gram-negative diplococci (0.6 x 0.8μm) and exhibit a characteristic kidney shape due to the adjacent sides of the diplococcus being flattened. They are usually aerobic, although they occasionally behave as facultative anaerobes (Apicella, 1990). Both the disease causing organisms *N. meningitidis* (encapsulated) and *N. gonorrhoeae* (non-encapsulated) are considered fastidious in their growth conditions, requiring a suitable growth media containing substance such as heated blood, hemin and animal proteins and an atmosphere containing 5-10% CO_2.

All members of the genus contain cytochrome oxidase in the cell wall, allowing oxidisation of the dye tetramethylparaphenylenediamine hydrochloride (TMPD) from colourless to pink. This was thought to be specific to *Neisseria*, but subsequent studies have shown other genera exhibit TMPD-oxidase activities including *Pseudomonas*, *Azobacter*, *Aeromonas* and *Moraxella*. Individual members of the genus can be identified by the fermentation of various carbohydrates, where they produce acid but not gas.

Both morphologic and biochemical similarity between members of the genus as well as the presence of antigenically similar cell wall proteins and lipopolysaccharides suggest evolution from a common ancestor. Early DNA homology studies indicated strong sequence similarity between *N. meningitidis* and *N. gonorrhoeae* (Kingsbury, 1967). Recent publication of DNA sequences for several neisserial genes encoding for proteins

such as pilin and several outer membrane proteins confirm this (Barlow *et al*, 1989; Murakami *et al*, 1989; Diaz *et al*, 1984).

	Acid formed from:			
	Glucose	Maltose	Lactose	Sucrose or Fructose
N. gonorrhoeae	+	-	-	-
N. meningitidis	+	+	-	-
N. lactamica	+	+	+	-
N. sicca	+	+	-	+
N. subflava	+	+	-	+/-
N. mucosa	+	+	-	+
N. flavescens	-	-	-	-
N. cinerea	-	-	-	-
B. catarrhalis	-	-	-	-

Table 1.1 – Results of fermentation of carbohydrates allowing identification of members of the *Neisseria* genus

1.3 Meningococcal Disease

Currently available data shows that the meningococcus can only establish a systemic infection in a normal human host if that host lacks serum bactericidal antibodies directed against capsular or non-capsular antigens of the invading strain or if the host is deficient in certain complement components. The immunological status of the individual determines if colonisation of the nasopharynx will lead to systemic infection or asymptomatic carriage (Devoe, 1982).

The human nasopharynx is the natural reservoir of the meningococci, which are being disseminated by aerosols. The airborne organisms impinge on the mucous-covered surfaced of the pharynx, adhere and establish populations. Host antibodies of the three

major immunoglobulin classes are elicited within 7-10 days, but are unable to eliminate the homologous strain in the pharynx, which may persist for weeks or even months.

The colonisation of the nasopharynx is usually sub-clinical, although pure cultures of meningococci have been isolated from cases of tonsillitis and nasopharyngitis (Banks, 1948). The meningococci are believed to enter the systemic circulation directly from the nasopharynx although direct infection of the meninges through the Cribform plate has been suggested (Raman, 1988). There are two possible conclusions rising from meningococcaemia. In 90% of cases the meningococcal disease will progress to an inflammation of the leptomeninges (pia and arachnoid) with associated dermal lesions (petechiae or the larger purpura) in approximately half the cases. 10-20% of the patients will develop fulminant septicaemia characterised by haemorrhagic skin lesions and endotoxic shock (Devoe, 1982). However these are the two extremes and a wide spectrum of syndromes between these are seen.

Banks in 1948 attempted to classify the majority of meningococcal disease states into four clinical syndromes. This classification was reviewed and modified by Devoe, (1982).

Common Form

Patients usually present with the classic meningitis syndromes such as stiff neck, vomiting, fever and general malaise. In 20-50% of cases the papula-petechial rash is present as a result of premeningitic bacteraemia. In rare cases, coma develops within 24 hours.

Chronic Meningococcal Septicaemia

Although initially described in 1902, this disease state did not gain clinical importance until the 1940 epidemic (Banks, 1948). Intermittent fever and recurrent crops of papules with petechial centres are common and rheumatic complications such as joint swellings and muscle pain are often observed.

Fulminant Encephalitis

This state is characterised by the rapid onset of fever and vomiting which can rapidly progress to coma. The brain is oedematous and enlarged whilst the sub-arachnoid space shows a purulent meningitis. In the parenchyma of the brain, small haemorrhagic lesions characteristic of this syndrome are present. A petechial but not a purpuric rash may be present (Banks, 1948).

Fulminant Meningococcaemia

The main symptoms are the rapid onset of fever. Low blood pressure and a petechial rash, which soon becomes purpuric and can lead to skin infarction. Internally there is overwhelming septicaemia and vascular change consisting of intravascular coagulation, endothelial damage, thrombosis and necrosis. There is frequently bilateral haemorrhage of the suprarenal glands (Waterhouse-Friderichsen syndrome), although death is due to acute endotoxaemia caused by bacterial LPS not adrenal insufficiency.

Several other diseases such as arthritis, opthalmitis, pericarditis, urethritis, vaginitis and peritonitis have also been attributed to meningococci although are they are relatively rare (Peltola, 1983).

Olsen *et al,*(1991) reported that 14% of meningococcal disease survivors suffer from permanent sequelae. These can be divided into either psycho-neurological functions or tissue damage as a result of impaired circulation. The most common complication is hearing impairment, found in 1-2% of cases as opposed to 20% before the use of antibiotics (Salmi *et al*, 1976). Other neurological sequelae include myopia, microcephaly, behavioural problems and intellectual impairment (Berg *et al*, 1992).

After fulminant meningococcal disease renal dysfunction and skin infarction are the most common sequelae. In severe cases the necrosis of the purpuric lesions may lead to skin grafts or even digit or limb amputation (Hudson *et al*, 1993).

1.4 The molecular Structure of the Meningococcus

Neisseria meningitidis, like most gram negative bacteria is bounded by a cytoplasmic membrane, an intermediate layer of peptidoglycan and an outer membrane which together make up the cell envelope (Frasch & Mocca, 1978). The meningococcus may also possess a polysaccharide capsule and surface exposed filamentous pili (Blake & Gotschlich, 1987). Research has focused on the cell-surface components since this is the site of interaction with the host cell and such information is necessary if the process of infection and immunogenicity are to be understood.

1.4.1 Polysaccharide capsule

The protective, hydrophilic polysaccharide capsule is found surrounding many pathogenic bacteria. In meningococci differences in capsular structure generate immunological differences which are used as the basis of the meningococcal serogrouping system. Currently thirteen different variations have been identified, each one being chemically and immunologically distinct; A, B, C, D (Gordon & Murray, 1915) X, Y, Z (Slaterus, 1961), E29, W135 (Evans *et al*, 1968), H, I, K (Ding *et al*, 1981), and L (Ashton *et al*, 1983).

The capsules of serogroups A, B, C, X, Y, Z, 29E, W135 and L have all been isolated and characterised chemically and structurally. Groups D, H, I and K are rare groups and remain to be characterised. Each polysaccharide is a polymeric molecule constructed from several glycose units joined by a glycosidic or phosphodiester linkage. Group A polysaccharide is a homopolymer of N-acetyl-mannosamine-6-phosphate residues and serogroups B, C, Y and W135 are all polymers which contain sialic acid.

Group	Chemical Composition of Capsular Polysaccharide
A	Partially O-acetylated 2-acetamido-2-deoxy-D-mannose-6-phosphate
B	(2→8)-linked N-acetylneuraminic acid
C	O-acetylated (2→9)-linked N-acetylneuraminic acid
X	2-acetamido-2-deoxy-D-glucose-4-phosphate
Y	Partially O-acetylated alternating sequence of D-glucose and N-acetylneuraminic acid
W135	Alternating sequence of D-galactose and N-acetylneuramninc acid
29E	2-acetamido-2deoxy-galactosamine and 3-deoxy-D-manno-octulosonic acid
L	N-acetylglucosamine phosphate

Table 1.2 – Chemical composition of the capsular polysaccharide
Compiled from Griffiss & Artenstein (1976) and Apicella (1990).

The presence of a polysaccharide capsule is an important virulence determinant in invasive meningococcal infection, since virtually all disease isolates are encapsulated as opposed to only 50% of carrier isolates (Band *et al*, 1983). A capsule deficient group B strain was shown to be avirulent in a mouse model but when capsule expression was regained, so too was virulence (Masson *et al*, 1982). The group B polysialic acid capsule has been shown to confer resistance against complement-mediated killing by human sera and confers protection in an infant mouse infection model (Mackinnon *et al*, 1994). The sialic acid is also known to inhibit the alternative complement pathway by enhancing the surface binding of the regulatory protein, factor H. This down regulation is believed to protect the meningococci from both opsonisation and complement-mediated lysis *in vivo* (Estabrook *et al*, 1992).

It has been shown that the presence of capsular polysaccharide inhibits meningococcal invasion into both epithelial and endothelial cells (Virji *et al*, 1992b; Frasch & Peppler, 1982). However it is known to enhance the survival rate in the intravascular space

(Mackinnon *et al*, 1994). This has lead to the suggestion that the level of capsule production varies depending on the organism's location in the host (Virji *et al*, 1992b). This phase variation is possibly controlled via a post-translational mechanism although this is not completely understood (Frosch, 1994).

1.4.2 Major Outer Membrane Proteins

The outer membrane of *Neisseria meningitidis* contains a restricted number of major proteins. Distinct protein profiles were obtained when various strains were subjected to SDS-PAGE analysis (Frasch & Gotschlich, 1974). Tsai *et al,* (1981) showed by the techniques of radioiodination followed by peptide mapping that these major outer membrane proteins could be categorised into five structural classes according to their apparent molecular weights on SDS-PAGE. Subsequently, several of these outer membrane proteins have been shown to have immunological importance (figure 1.1).

1.4.2.1 Class 1 - PorA

The class 1 outer membrane protein is expressed by the majority of meningococcal strains and on SDS-PAGE has been shown to have an apparent molecular weight of 41-42 kDa (Tsai *et al*, 1981). The amount of protein produced varies between strains (Poolman *et al*, 1980a) and also during human nasopharyngeal carriage (Woods & Cannon, 1990). Expression is controlled at the transcriptional level via slip-strand mispairing (Farley *et al*, 1994).

Cloning and sequencing of the *porA* gene, which encodes the class 1 protein has shown strong structural similarities with other Neisserial outer membrane proteins, most noticeably the PIA and PIB gonococcal proteins (Barlow *et al*, 1989) and the class 2 and 3 meningococcal protein (Ward *et al*, 1992). By inserting the class 1 protein into a lipid bilayer, Tommassen *et al,* (1990) were able to show that it was a porin with cation selectivity similar to the OmpF protein of *E. coli* K-12. These porin proteins exist as trimers in the outer membrane where they form water filled pores which allows

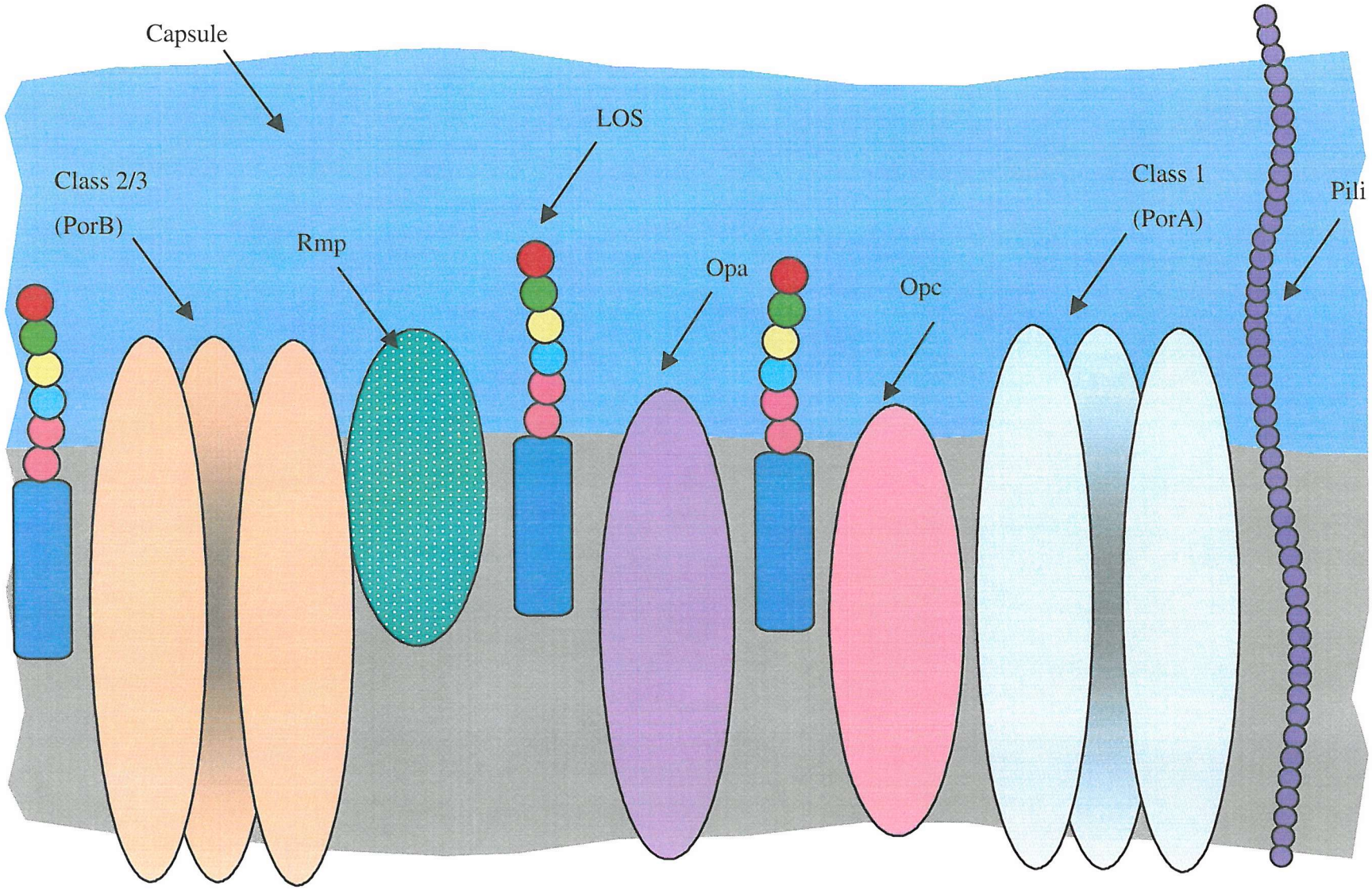


Figure 1.1 – Schematic representation of the meningococcal outer membrane (courtesy of Prof. J.E. Heckels).

transmembrane diffusion of small solutes into the bacteria (Blake & Gotschlich, 1987; Nikaido, 1992).

The predicted amino acid sequence contains alternating hydrophilic and hydrophobic regions, characteristic of an outer membrane transmembrane protein (Barlow *et al*, 1987). Comparison of a number of class 1 sequences revealed eight highly variable, discrete regions separated by stretches of highly conserved residues and from this a two-dimensional model was constructed (figure 1.2) (van der Ley *et al*, 1991). The conserved regions were proposed to be amphipathic β-strands, traversing the membrane and generating eight surface exposed loops, which correspond to the hydrophilic maxima of a hydropathy profile. This model has been extended to cover the meningococcal class 2 and class 3 proteins and the gonococcal PIA and PIB proteins. The model of the Neisserial porin structure is very similar to that of the *E. coli* porins OmpF and PhoE, where in addition the three-dimensional structure has been elucidated (Cowan *et al*, 1992). The anti-parallel β-sheet structures of these porins fold within the membrane to form a β-barrel, which then aggregates to form a trimeric complex. In the *E. coli* porins, one of the loops folds into the centre of the barrel forming a constricted region which determines the size exclusion limit for the diffusing particles. It has been suggested that the charge distribution on this internalised loop determine the ion selectivity of the porin (Cowan *et al*, 1992).

Expression of the class 1 outer membrane protein is antigenically stable within a strain. However extensive antigenic diversity is seen between strains and is the basis of sero-subtyping. Comparison of predicted amino acid sequences from class 1 proteins of different subtypes showed that within the eight surface exposed loops, most amino acid changes occurred in two variable regions termed VR1 and VR2, located at the apices of the two longest loops, 1 and 4 (McGuinness *et al*, 1990). Analysis of comparisons of a number of DNA sequences allowed a third semi-variable region to be identified VR3 or SV1, located at the apex of loop 5 (Maiden *et al*, 1991). By the reaction of monoclonal

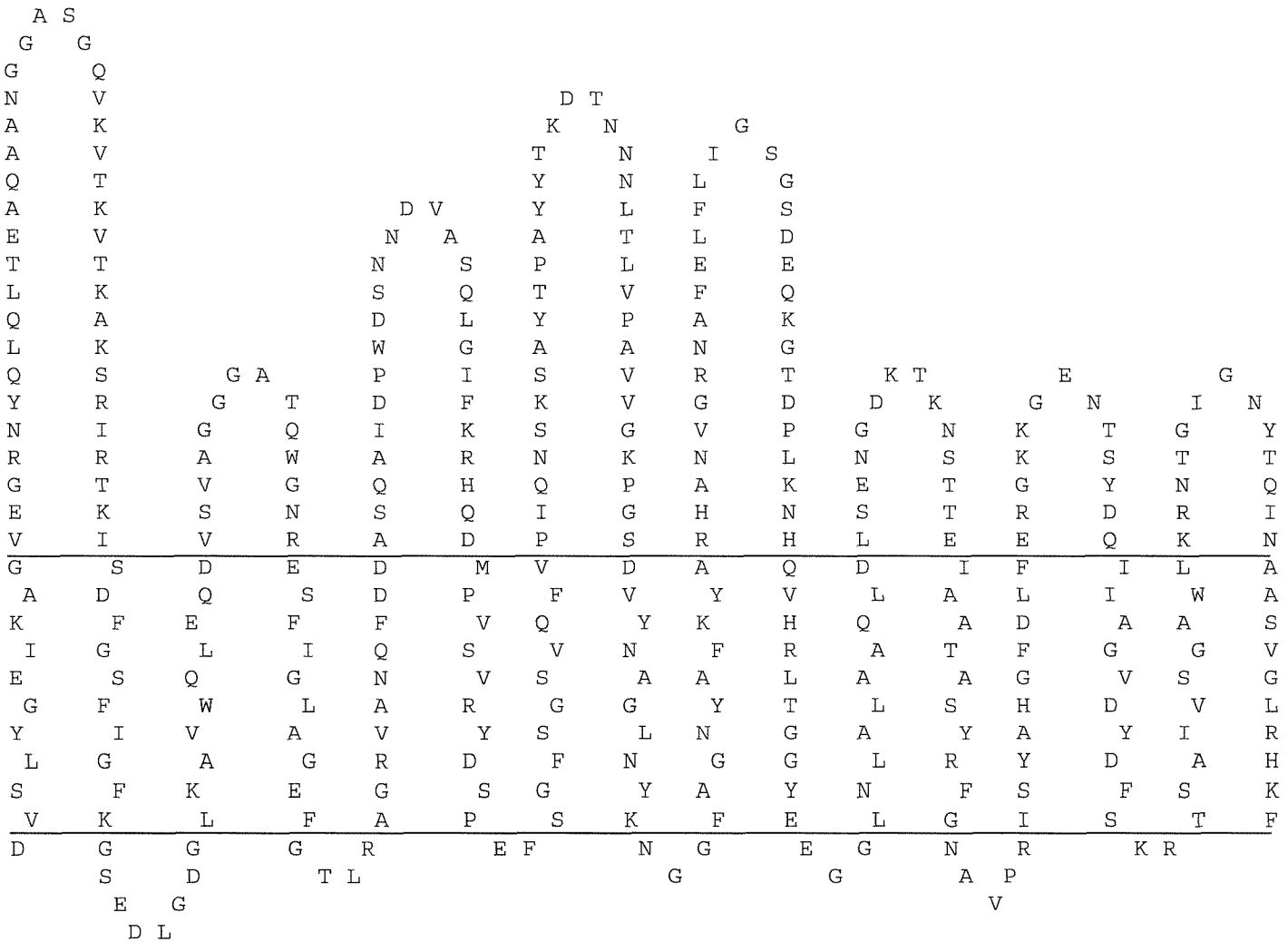


Figure 1.2 – Model of the predicted structure of the class 1 outer membrane protein within the outer membrane (van der Ley *et al*, 1991) The top part of the model shows the surface exposed regions, whereas the central part indicates the transmembrane regions. Amino acid residues are shown alternating where they can form a β strand with at least one side consisting of entirely hydrophobic residues.

antibodies with overlapping peptides the location of the subtype specific epitopes has been elucidated. Two subtype-specific epitopes; P1.7 in VR1 and P1.16 in VR2 were localised to these domains and it was further demonstrated that a single amino acid change within the subtyping epitope was sufficient to abolish subtype specificity (McGuinness *et al*, 1991). The class 1 protein shows limited heterogeneity, with seven subtypes representing more than 80% of all group B meningococcal disease world-wide (Abdillahi & Poolman, 1988).

The class 1 protein has been shown to be immunogenic following either meningococcal infection (Mandrell & Zollinger, 1989; Zollinger & Mandrell, 1983; Guttormsen *et al*, 1994) or immunisation with an experimental outer membrane complex vaccine (Wedege & Froholm, 1986). Ward *et al,* (1996) showed that a recombinant class 1 protein when inserted into liposomes induced antibodies, which not only recognised the class 1 protein on the surface of the meningococci, but also promoted complement mediated killing of homologous strains. Antibodies specific to class 1 have been shown to protect infant rats against bacterial challenge with meningococci carrying the same class 1 protein (Saukkonen *et al*, 1987). Due to antibodies being sub-type specific, current work is focusing on production of a hexavalent PorA meningococcal outer membrane vesicle vaccine (van der Voort *et al*, 1996; van der Voort *et al*, 1997). A meningococcus was engineered to contain six *porA* genes responsible for 60-80% of meningococcal disease cases in the Netherlands. Results however in human trials have shown an immunodominance skewing the immune response towards certain PorA proteins present in the vaccine (van der Voort *et al*, 1997). An alternative approach for presenting the PorA protein has been taken by Jiang *et al,*(1997) where loop 4 of the class 1 protein has been displayed on the bacteriophage T4 capsid surface and has generated an immune response in mice. It is hoped that this system can be manipulated to display more than one peptide.

1.4.2.2 Class 2/3 - PorB

All strains express either a class 2 or class 3 protein. The *porB* gene has two alleles that encode for the class 2 and class 3 proteins that are mutually exclusively expressed. Whichever PorB protein is expressed is quantitatively the major outer membrane protein. The apparent molecular weight of the class 2 and class 3 proteins are 39-41 and 35-38 kDa respectively (Tsai *et al*, 1981). The proteins exist as trimers in the outer membrane where they function as anion selective porins, allowing the passage of nutrients (Blake & Gotschlich, 1987; Tommassen *et al*, 1990). Both classes of protein exhibit antigenic variation, which is the basis of the serotyping classification scheme (Frasch *et al*, 1985). Variability is limited and currently there are approximately twenty recognised serotypes.

The class 2 allele was first cloned by Murakami *et al*, (1989) and consists of a gene of 1079 bp coding for a mature protein of 341 amino acids and a 19 amino acid leader sequence, with a molecular weight of 36 kDa. The class 3 allele has been cloned and sequenced by several workers (Swanson *et al*, 1988; Wolff & Stern, 1991) and here the gene is 939 bp long and codes for a mature protein of 313 amino acids and a 19 amino acid leader sequence, with a molecular weight of 35 kDa.

Comparisons of the class 2/3 proteins with porins from other Neisserial species and also the class 1 protein shows high levels of homology (Ward *et al*, 1992). The leader sequence is shared between the *porB* gene products, the porins of *N. sicca* and *N. lactamica* and the PIA and PIB proteins of *N. gonorrhoeae*, whilst the class 1 meningococcal protein differed in seven places. High levels of homology are seen throughout the rest of the genes. The class 2 and class 3 show a 70% homology, whilst the class 1 and class 3 exhibit a 50% homology. The greatest homology is shown between meningococcal class 3 protein and the gonococcal PIA protein at 82% (Swanson *et al*, 1988). The class 2 protein shows an 81% homology with the gonococcal PIB (Murakami *et al*, 1989). Construction of a dendrogram (figure 1.3) shows there is no clear evolutionary divergence between the proteins of the pathogenic and non-pathogenic *Neisseria* species.

Both the class 2 and class 3 proteins show large areas of highly conserved sequence interspersed with several well-defined areas of variability. The predicted amino acid sequence contains alternating hydrophobic and hydrophilic regions characteristic of an outer membrane transmembrane protein (van der Ley *et al*, 1991). There are eight discrete variable regions , which correspond to the hydrophilic maxima on a hydropathy profile suggesting surface exposure and from this a two-dimensional model has been constructed, highly similar to that of the class 1 outer membrane protein (figure 1.4) (van der Ley *et al*, 1991). The conserved regions are proposed to be membrane spanning amphipathic, hydrophobic β-strands and the variable regions are surface exposed loops. Loops designated 1 and 5 have been confirmed as surface exposed by peptide mapping techniques (van der Ley *et al*, 1991).

In contrast to the class 1 outer membrane protein, the class 2 and class 3 proteins pocess small surface exposed loops and amino acid substitutions are found in several (Feavers *et al*, 1992). Major variation is seen on loops 5 and 7 of the class 3 protein, but variation is also present at loops 1,4,6 and 8 (Ward *et al*, 1992). Each strain exhibits only a single serotype unlike the sero-subtype of the class 1 protein, where two are present. Sero-subtype specific antibodies recognise continuous epitopes whilst serotype specific antibodies largely recognise conformational epitopes (van der Ley *et al*, 1991). The greater diversity of the class 2/3 proteins could represent separate segments, which together contribute to a type specific conformational epitope (Ward *et al*, 1992).

Within a particular epidemiological setting most strains causing meningococcal disease are limited to a small number of serotypes, notably serotype 2a among class 2 protein expressing strains and serotype 15 amongst class 3 strains (Poolman *et al*, 1983). Immunisation with experimental outer membrane complex vaccines or natural infection with the meningococcus induces class 2/3 antibodies (Mandrell & Zollinger, 1989; Guttormsen *et al*, 1994), which are serotype specific. The class 2/3 antibodies are bactericidal *in vitro* and have been shown to elicit various levels of protection in the

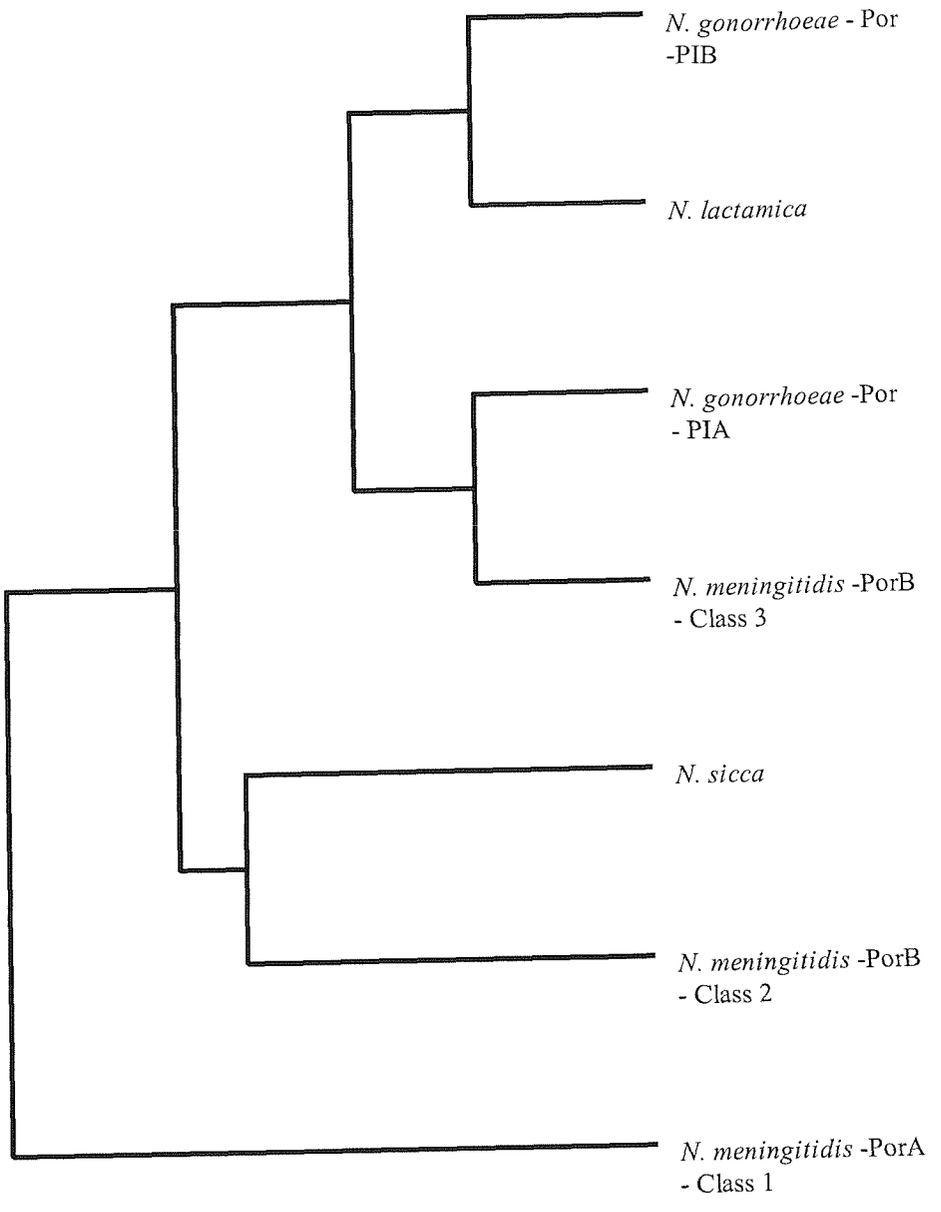
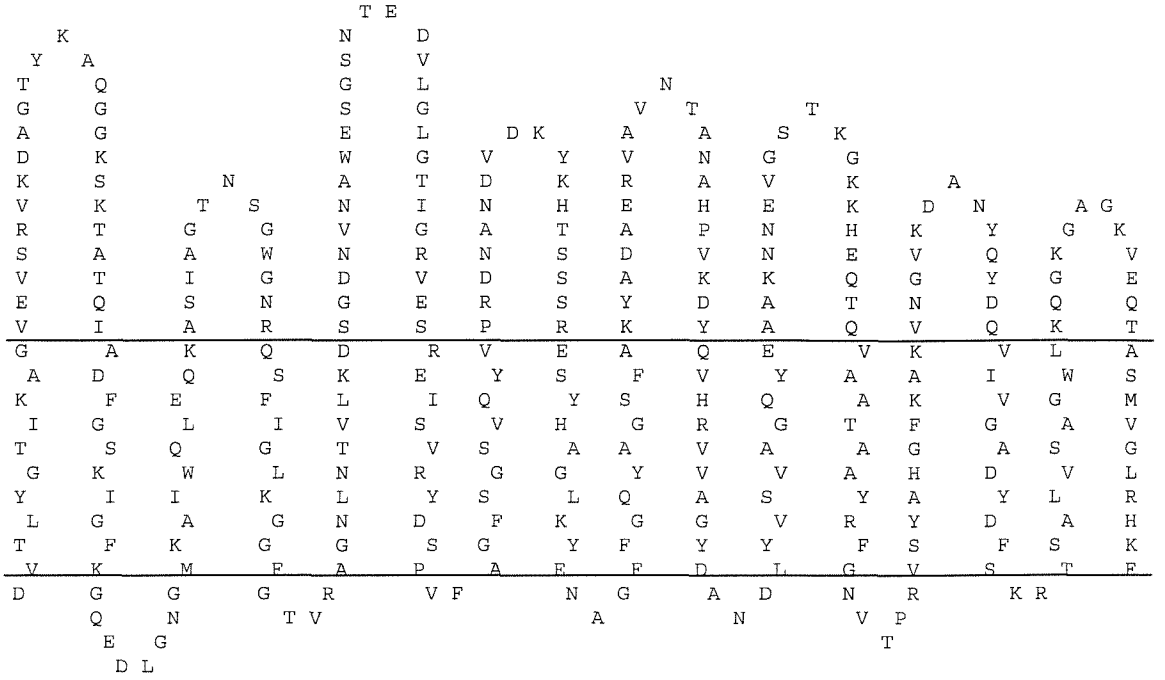
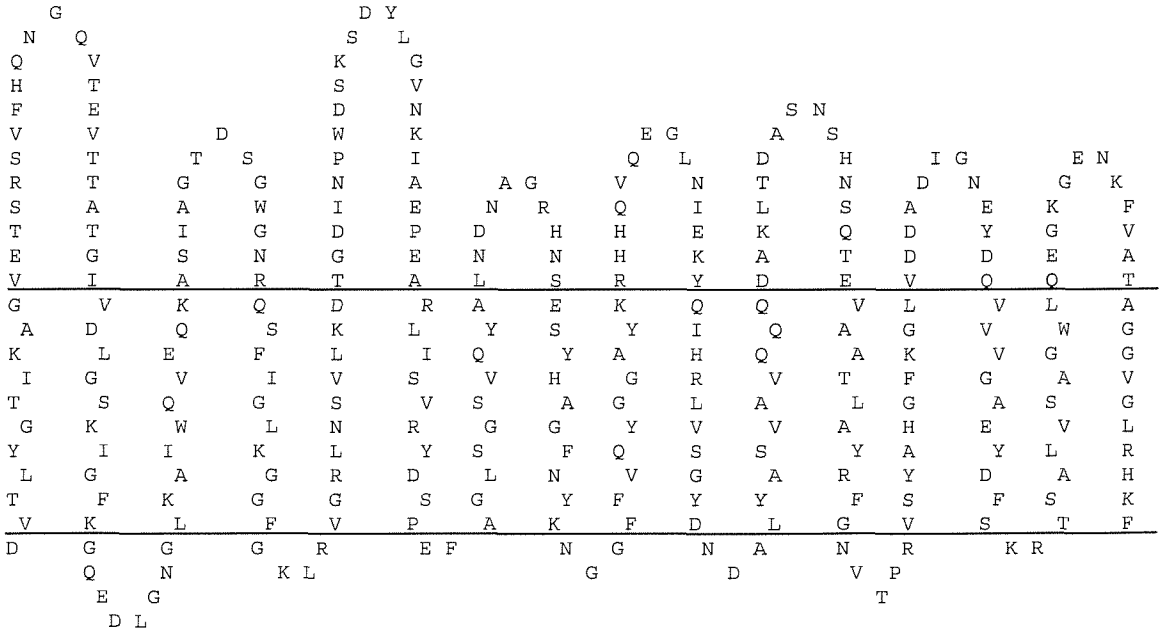


Figure 1.3 – Comparison of the homologies of the porin proteins in pathogenic and non-pathogenic Neisseria (Ward *et al*, 1992)

Figure 1.4 – Models of the predicted structures of the class 2 and 3 proteins within the outer membrane



The class 2 OMP (van der Ley *et al*, 1991)



The class 3 OMP

The top part of the models shows the surface exposed regions, whereas the central part indicates the transmembrane regions. Amino acid residues are shown alternating where they can form a β strand with at least one side consisting of entirely hydrophobic residues.

infant rat model although at lower levels than seen for the class 1 outer membrane protein (Saukkonen *et al*, 1987; Saukkonen *et al*, 1989).

As well as having a physiological role, the porins of the pathogenic *Neisseria* species may play a role in pathogenesis. These porins have been shown to transfer from the outer membrane on contact with foreign membranes (Lynch *et al*, 1984). This implies an active role for porins in interactions with cells, which is enforced by the inability of the commensal strain *N. sicca* even at high levels to transfer.

1.4.2.3 Class 5 - Opa

Meningococci express 0, 1 or more of a family of Opa (formerly class 5) proteins. These are heat modifiable proteins and range in size from 25-32 kDa (Frasch & Mocca, 1978; Poolman *et al*, 1983). The proteins show a high homology to the Opa (formerly P.II) protein in the gonococcus both at the amino acid level and in the DNA sequence where the genes are arranged in a similar manner (Stern *et al*, 1984). Four separate *opa* loci have been found in the meningococcal chromosome whilst the gonococcal chromosome has been found to have up to eleven different *opa* loci (Stern *et al*, 1984). Each Opa protein is encoded by a complete structural gene, which are from a family of genes interspersed throughout the genome (Aho *et al*, 1997). The proteins differ in their electrophoretic mobility and their procession of unique epitopes recognised by polyclonal or monoclonal antibodies (Kawula *et al*, 1988). DNA sequence analysis of several *opa* genes has shown that large regions show almost perfect homology interspersed with three regions which vary in sequence and size. The region encoding the N-terminal region of the mature protein shows moderate variability and is termed SV (semi-variable). Downstream are two hypervariable regions HV_1 and HV_2. Several different versions of these two regions have been found to be present within a single strain and different combinations of these sequences occur on different *opa* genes (Connell *et al*, 1990).

Marked heterogeneity in expression from the *opa* gene is seen between strains and within a strain. Phase variation of these proteins is seen at a rate of 10^{-3} or 10^{-4} per cell per generation (Kawula *et al*, 1988) and is controlled at the translational level. Each gene is constitutively transcribed but contains a variable number of repeats of the sequence element CTCTT positioned downstream of the initiation codon but before the codon coding for the first amino acid of the mature protein. Depending on the number of times this element is repeated puts the structural gene in or out of frame, resulting in a full sized Opa protein or a truncated protein product (Stern *et al*, 1984). Variation in the number of repeats is probably due to slipped strand mispairing of the DNA during the replication process (Belland *et al*, 1989).

Monoclonal antibodies raised against the Opa proteins of a meningococcus were found to be bactericidal only against meningococci expressing the target protein (Kawula *et al*, 1987). The epitopes recognised were found to be encoded by the HV_2 region. When the bacteria were viewed by immune electron microscopy the antibodies were found to be bound to the surface of the meningococci, implying that the HV_2 encoded region of the protein is surface exposed.

Sera from patients recovering from meningococcal disease has shown a strong, strain specific IgG antibody response to class 5 proteins (Poolman *et al*, 1983). However the results of an immunisation trial with outer membrane vesicles showed that although there was initially a strong IgG antibody response to the class 5 proteins, the levels of antibodies decreased with time after vaccination (Wedege & Froholm, 1986).

1.4.2.4 Opc

The Opc protein was initially thought to be a member of the class 5 Opa family of proteins and termed 5C or OpaC (Achtman, 1988). Subsequently, following cloning and sequencing of the gene, it was realised that this is a separate entity and was reclassified as Opc. The Opc protein is expressed by approximately 60% of meningococci belonging to different serogroups (Olyhoek *et al*, 1991). Within those strains expressing Opc,

various levels of expression are seen, with some strains expressing large amounts (Opc^{++}), others smaller amounts (Opc^{+}) and the remaining strains at undetectable levels (Opc^{-}) (Achtman, 1988). Switching between these expression levels occurs at a frequency comparable to that of phase variation of Opa proteins (Sarkari *et al*, 1994).

The gene encoding Opc has been cloned and sequenced (Olyhoek *et al*, 1991; Guillen *et al*, 1995). The predicted amino acid sequence revealed a mature protein of 250 amino acids, approximately 28 kDa, and a 19 amino acid leader peptide, typical for membrane proteins. Comparison of the predicted amino acid sequence with those from other *opa* genes showed that 10% were identical at the same positions and that a further 10% were functionally equivalent. Features shared between Opc and Opa are that they have an extremely basic pI, similar molecular weights, a trimeric structure and basic chemical properties. Consequently they have been proposed to belong to the same protein family, but to have evolved considerably since their original evolution (Olyhoek *et al*, 1991).

In a study where 108 meningococcal strains were analysed for RFLP's of the *opc* region, a surprisingly low number of variants were found (Seiler *et al*, 1996). Sequencing of a subset of 43 strains revealed 18 distinct alleles, with polymorphisms evenly distributed throughout the coding region and grouped nucleotide exchanges occurring in the 5' flanking region. Of the 18 alleles, 15 differed in the *opc* coding region leading to 13 variants at the protein level.

A further difference between Opa and Opc is seen in the regulation of expression. The pentameric repeats upstream of the *opa* start codon responsible for bringing the gene in or out of frame are completely missing in the equivalent region of the *opc* gene (Olyhoek *et al*, 1991). Regulation of expression of the Opc protein occurs at a transcriptional level as no mRNA for the gene is found in meningococci known to have the gene but not expressing it (Opc^{-}) (Sarkari *et al*, 1994). Sequencing of the upstream region of the *opc* gene, reveals a stretch of contiguous cytosine residues. Opc^{++} expression correlates with 12 or 13 C's, Opc^{+} with 11 or 14 and Opc^{-} with both a higher or lower number of cytosines. These results indicate that the length of the poly-C region

is crucial for efficient transcription and may be part of a binding site, which is important for function of the *opc* promoter (Sarkari *et al*, 1994).

Using the predicted amino acid sequence, a two-dimensional structural model has been proposed (Merker *et al*, 1997a). The model consists of the protein spanning the membrane ten times, exposing five hydrophilic loops on the cell surface with the N-terminus and four turns to the periplasm. Peptide mapping showed that the epitopes for two monoclonal antibodies to Opc were located at the apex of loop 2, confirming its surface exposure. Insertion of foreign epitopes from Semliki Forest virus in various positions along the protein reinforced this model (Merker *et al*, 1997a).

Opc^{++} bacteria are more commonly isolated from the nasopharynx than from the CSF or the bloodstream, suggesting that down regulated variants may be selected during the later stages of invasive disease (Achtman *et al*, 1991). Non-capsulated Opc^{++} meningococci have also been shown to adhere to and invade human epithelial and endothelial cells (Virji *et al*, 1992b; Virji *et al*, 1994). Although Opc is poorly immunogenic in animals it has been shown to be highly immunogenic in humans, even more so than the class 1 outer membrane protein (Wiertz *et al*, 1996). The antibodies raised however were bactericidal only against Opc^{++} strains. It has been suggested that this is part of a refined virulence mechanism (Rosenqvist *et al*, 1993), whereby a small proportion of the bacteria express a highly immunogenic protein that enables invasion of the body. Their direct descendants however express lower amounts and are therefore protected against the antibodies stimulated by the protein.

1.4.2.5 Pili

Pili are filamentous surface structures, which often play a role in bacterial attachment. Pili are found on the surfaces of the meningococcus and gonococcus where they are approximately 4-6nm in diameter and 500-6000nm in length (Stephens *et al*, 1985). Initial studies in the 1960's focused on the gonococcal pili. (Kellog *et al*, 1963) demonstrated that there was a loss of virulence seen when gonococci were subjected to

repeated subculture and that this was correlated with a morphological change of the colonies observed. Primary isolates produced small, domed, highlighted colonies (T1 and T2), whereas repeated subculture produced large flat colonies (T3 and T4). Only type T1 and T2 colonies retained their virulence; electron microscope studies revealed the presence of pili and their absence in the T3 and T4 types (Swanson *et al*, 1971). Meningococci proved to be more difficult than gonococci but a similar study in meningococci showed the association between piliation and colony phenotype to be present also (Blake *et al*, 1989).

The pilus is composed of repeated subunits of the polypeptide pilin, which has a molecular weight of between 15 and 22 kDa (Aho *et al*, 1997; Robertson *et al*, 1977). The primary sequence of pilin molecules in gonococci was determined by Schoolnik *et al,* (1984), who found by protein sequencing that the pilin subunit contained 160 amino acids with methionine at positions 7 and 92 and a disulphide loop between Cys 121 and Cys151. Cleavage at the methionine residues using cyanogen bromide resulted in three peptides; CNBr-1 (residues 1-7), CNBr-2 (residues 8-92) and CNBr-3 (residues 93-159). All gonococci react with the non-protective monoclonal antibody SM1, the epitope for which is in CNBr-2, residues 49 to 53 (Virji *et al*, 1989).

In the meningococcus, two types of pilin are found termed class I and class II. The gonococcal pilin and meningococcal class I pilin have been shown to be very similar (Diaz *et al*, 1984) and the meningococcal class I pilin will react with the monoclonal antibody SM1 raised against the gonococcal pilin. Comparison of these two amino acid sequences shows the characteristic features of the type IV family of bacterial pilins, which include pilin from various bacteria including *Moraxella bovis*, *Bacteroides nodus*, *Pseudomonas aeruginosa*, *Vibrio cholera* and enteropathogenic *E. coli*. These include a short leader sequence of 6-7 amino acids followed by methyl phenylalanine as the first residue of the mature protein, a highly conserved N-terminal domain and two conserved cysteine residues allowing a disulphide loop in the C-terminal region of the protein (Strom & Lory, 1993). Although numerous features are shared, gonococcal pilins display antigenic differences between strains and antigenic variation within

strains. DNA sequence analysis of the pilin genes, *pilE* from a number of strains has shown a gene structure composed of a highly conserved 5' region encoding the N-terminal region of the protein followed by a semi-variable region which contains five strongly conserved sequence elements designated SV1 -SV5. The remaining 3' region encodes the disulphide region and the C- terminal of the protein, which is collectively known as the hypervariable region. The greatest degree of variability is seen in the disulphide region located between two conserved sequence elements termed cys1 and cys2 and is the immunodominant region of the protein (Swanson *et al*, 1987). One or two expression loci are present in the gonococcus along with numerous truncated silent pilin genes, *pilS* that lack the promoters and 5' coding sequences of the *pilE*. Through non-reciprocal recombination events *pilS* donate genetic information to *pilE*, so that the altered *pilE* encode pilin variants that differ primarily in the hypervariable region.

The cloning and sequencing of the meningococcal class I pilin gene (Potts & Saunders, 1988) showed that there is a high level of homology with the gonococcal pilin in the predicted translation product. Numerous base substitutions are seen in the wobble position of the coding region when the DNA sequences are compared, possibly indicating a fundamental difference in codon preference between the two organisms. The meningococcal class I pilin contains the conserved N-terminal and other conserved sequence elements that are features in the gonococcal pilin and other type IV pilin. DNA sequencing of the meningococcal class II pilin (Aho *et al*, 1997) and the predicted translation product indicate that this too is a typical type IV pili. A short seven amino acid leader sequence is present and there is a conserved N-terminal region, although there are six amino acid changes out of the fifty-three amino acids making up this region when compared to class I and gonococcal pilin. The conserved Cys residues in the C-terminal region are also present along with the presence of methyl phenylalanine as the first residue of the mature protein. Other regions conserved in the gonococcal and class I pilins such as SV2 in the semi-variable region and cys2 in the hyper-variable region are also seen, and the latter is thought to play a role in facilitating recombination with the silent *pilS* loci. Several differences between the class I and class II pilin are also present. The conserved elements SV1, SV3, SV4 and SV5 all show extensive divergence as does

the cys1 element. The SM1 epitope ^{49}EYYLN^{53} is altered to ^{49}EYYSD^{53} in the class II pilin explaining its non-reactivity. The most striking difference however is the deletion of almost the entire disulphide region.

Following the observation that virulent colonial forms of gonococci differed from avirulent forms by the presence of pili, many studies have demonstrated that pili facilitate adhesion to a wide range of different cell types such as tissue culture cells, vaginal epithelial, fallopian tube epithelium and buccal epithelial cells (reviewed Heckels, 1989). Gonococcal pili play a role in the two stage attachment process; initially they are able to overcome the electrostatic barrier between the negatively charged surfaces of the gonococcus and the host cell, thus allowing the two cells to come closer together, leading to a stable adhesion process involving other components of the gonococcal surface (Heckels *et al*, 1976). In the case of the meningococcus, pili have been shown to be required in the adherence of capsulated strains to both epithelial and endothelial cells *in vitro* (Virji *et al*, 1991; Virji *et al*, 1992a). The two classes of meningococcal pili although structurally and genetically different appear to have functional similarities in their association with endothelial cells(Virji *et al*, 1991). This adherence is only seen with cells of human origin not those of bovine or porcine origin (Virji *et al*, 1993). This human specificity has also been demonstrated by the gonococcal pili (Johnson *et al*, 1977).

Among piliated strains both inter and intra strain variability exists with respect to the degree of adhesion to epithelial cells in vitro (Virji *et al*, 1992a). The sequence of the *pilE* gene from highly adhesive strains is identical but in lowly adhesive strains very different sequences were found (Nassif *et al*, 1993). Some strains producing the highly adhesive pilin have been shown to vary in their adhesiveness to human epithelial cells, suggesting other factors may play a possible role in adhesion (Nassif *et al*, 1994). In the gonococcus an additional protein termed PilC was shown to co-purify with the PilE protein (Jonsson *et al*, 1991). This protein was also found in the meningococcus where two alleles are present, *pilC1*, *pilC2* (Nassif *et al*, 1994). PilC is thought to be involved in pilus assembly, shown by the ability of a meningococcal PilC protein being able to

restore pilus assembly in a PilC deficient gonococcus (Ryll *et al*, 1997). PilC has also been demonstrated as having a central role in the adherence of gonococci to human epithelial cells (Rudel *et al*, 1992). Antisera specific to gonococcal PilC has been used in electron microscopy studies and has revealed that the PilC protein is primarily at the tip of the gonococcal pilus, suggesting a potential role in cellular attachment (Rudel *et al*, 1994).

Many organisms use strategies to escape the host defence mechanisms by varying expression of their major surface antigens. Phase variation is seen in the pilin protein of both the gonococcus and the meningococcus (reviewed Meyer & van Putten, 1989). The switching on and off of pili expression may or may not be reversible, but loss of pili may allow detachment from the initial site of colonisation and access to new locations (reviewed Heckels, 1989). Antigenic variation is also seen *in vivo* in both the meningococcus (Tinsley & Heckels, 1986) and the gonococcus (Swanson *et al,* 1987). Early studies revealed the gonococcal pili to be immunogenic in animals, and patients with gonorrhoea develop anti-pilus antibodies (Heckels, 1989). A single gonococcal strain has been shown to be able to produce at least a dozen different pilus types (Swanson and Barrera, 1983). Most pilin sequence variation occurs within six short regions termed minicassettes, which are located after the conserved, N-terminal in the central and C-terminal region of the gene (Hagblom *et al*, 1985). The disulphide loop in the hypervariable and immunodominant region is the location of the minicassette MC2. A number of non-expressed genes termed *pilS* are also present in the Neisserial chromosome. A silent locus carries one or more partial pilin gene copies, which are tandemly arranged and connected by repetitive sequences (Haas *et al*, 1992). The silent gene copies show the same arrangement as the functional *pilE* locus, but have a deleted N-terminal coding sequence. The transfer of minicassettes from the *pilS* gene to the *pilE* gene generates the high level of genetic variability seen. This recombination process is *recA* dependent (Koomey *et al*, 1987) and predominantly occurs via an intracellular pathway although transformation-mediated recombination can also be seen (Stahn *et al*, 1992).

1.5 Serological Classification of Meningococci

In 1909, Dopter showed that there were serological differences among meningococci and they could be separated into two groups (Frasch, 1979). The first classification system separated the meningococci into four groups (I-IV) by means of an agglutination test (Gordon & Murray, 1915). This classification system was subsequently modified by several workers (Branham, 1932; Branhan & Carlin, 1942) until 1950 when the Neisseriae Subcommittee of the International Association of Microbiologists unified the nomenclature, recommending division of all known strains into four groups; A, B, C, D (Branham, 1953). The bacterial component responsible for generating serogroup heterogeneity was first isolated from a group I strain (Serogroup A) in 1935 by Scherp and Rake, being identified as a high molecular weight polysaccharide (Cartwright, 1995).

Three new serogroups (X, Y, Z) were described in 1961 by Slaterus, followed in 1968 by the isolation of serogroups 29E and W135 by Evans *et al*, also in 1968. In the early 1980's, four additional groups were observed, H, I, K by Ding *et al*, in 1981 and serogroup L by Ashton *et al*, in 1983.

The interaction of group B meningococci with polymorphonuclear leukocytes and serum antibodies was studied by (Roberts, 1967), who concluded that group B meningococci organisms were heterogeneous. Independent serotyping schemes for group B and C were developed as a result of work using bacterial precipitation and antigen binding techniques (Frasch, 1979). A complex antigenic diversity within serogroup C was shown by Goldschneider *et al, (1969b)* using bactericidal assays. The serotyping scheme he proposed, was modified by Gold & Wyle, (1970) who divided the group C strains into four types (I-IV). Serotyping was also extended to group B where 10 serotypes were designated by Frasch & Chapman (1972).

Cross reactivity of several of the type specific antibodies raised by Gold & Wyle (1970) with several different serogroups demonstrated that the determinants for type specificity were expressed independently of the group specific antigen. SDS-PAGE allowed

identification of antigens responsible for serotype-heterogeneity; class 1, class 2/3 and class 5 outer membrane proteins as well as the LPS molecule (Frasch, 1979; Poolman *et al*, 1980b). A serotyping scheme proposed by Frasch utilised only the class 2/3 OMP, since this was the only molecule that was found to be antigenically stable within a strain an also exhibit diverse antigenic variability between strains. Further division within serotypes classified this way can be achieved by separation into serosubtypes on the basis of the antigenic variation occurring in the class 1 OMP.

The use of monoclonal antibodies in whole cell ELISA allowed 75% of group B and C strains to be serotyped and 80% serosubtyped (Abdillahi & Poolman, 1988). The use of synthetic peptides allowed the position of epitopes responsible for serosubtype specificity to be identified to two regions on the class 1 OMP (McGuinness *et al*, 1993), hence the dual serosubtype seen with many strains.

Differences in the LPS oligosaccharide structure have allowed even further subdivision (Verheul *et al*, 1993). 12 LPS immunotypes have currently been identified; L1-L8 are associated with group B and C meningococci (Zollinger & Mandrell, 1977) whilst L9-L11 are predominantly found in group A strains (Zollinger & Mandrell, 1980).

In summary the current serological classification system utilised is that initially described by Frasch *et al*, (1985) and is based on four groups of antigenic surface components; capsular polysaccharide, class 2/3 OMP, class 1 OMP and LPS. Therefore a meningococci isolate designated B:15:P1.7,16:L3,7 would be a group B organism of serotype 15 with subtypes 7 and 16 and an LPS immunotype of L3 and L7.

1.6 Alternative Typing Methods

Serological classification schemes were developed prior to and independently of multilocus enzyme electrophoresis (MLEE) (Frasch *et al*, 1985). Unfortunately the current array of monoclonal antibodies used in the typing procedure does not recognise every serotype and serosubtype (Achtman, 1995).

MLEE is a technique that utilises the natural variation in electrophoretic mobility of diverse cytoplasmic allozymes and in some cases outer membrane proteins as markers for different genetic alleles on the bacterial chromosome. A common electrophoretic type (ET) is assigned to bacteria indistinguishable by this method. Numerous ET's have been defined differing by at least one allozyme or OMP. Groups of related ET's (as defined by cluster analysis) have been designated subgroup in serogroup A and cluster or complex in serogroups B and C (Achtman, 1995).

It has been postulated that a cluster, subgroup, or complex consist of bacteria descended from a single ancestral cell which have undergone only minor genetic changes subsequently (Achtman, 1995).

Among endemic isolates a large number of genetic groupings are present, however most isolates from any epidemic belong to a common gouping. Only a few genetic groupings of meningococci have been associated with epidemic disease (reviewed Achtman, 1995). Since World War II, regardless of global location, most epidemics have been caused by serogroup A bacteria of subgroups I, III, IV-I and V (Wang *et al*, 1992), serogroup B of the ET-5 complex or A4 cluster (Caugant *et al*, 1987a; Caugant *et al*, 1987b) or by serogroup C of the ET-37 complex (Wang *et al*, 1993). The spread of the disease caused by some of these groupings covers countries and even continents.

The polymerase chain reaction (PCR) was used by (Maiden *et al*, 1992) for the identification of serosubtypes. This typing system amplifies the VR1 and VR2 region of the class 1 gene, which are then Southern blotted against a reference panel of pre-cloned variable regions attached to a nylon membrane. This system allowed a number of previously untypeable strains to be assigned a new serotype.

A DNA fingerprinting system utilising restriction fragment length polymorphism (RFLP) has also been used to type meningococci. Grouping of the bacteria was as a

result of the characteristic banding patterns obtained after digestion of chromosomal DNA with a defined panel of restriction endonucleases (Bjorvatn *et al*, 1984).

1.7 Epidemiology of Meningococcal Disease

The annual world-wide attack rate for meningococcal disease is approximately 1 to 3 per 100,000 population (Schwartz *et al*, 1989). However in a unique endemic area termed the meningitis belt in sub-Saharan Africa, the attack rate is much higher at 70 per 100,000 population (Peltola, 1983). This attack rate rises sharply towards the end of the dry and dusty season (the 'hamattan') and falls with the onset of the rains (Greenwood *et al*, 1985). In contrast, in temperate climates the peak incidence of disease occurs within the first quarter of the year (Jones, 1995). In developed countries the peak incidence of disease occurs in the under 5 age group, reaching a maximum between 6 and 9 months of age (Jones, 1995), a second much smaller peak of disease occurs amongst 16 to 19 year olds, with the overall adult rate of infection in the UK standing at 0.4 per 100,000 population, per annum (Jones, 1995). In comparison, children aged between 5 and 9 years old are the most susceptible to meningococcal infection in the meningitis belt.

Currently thirteen meningococcal serogroups are recognised and disease is caused only by groups; A, B, C, W-135 and Y. Groups A, B and C are responsible for the majority of epidemic and endemic disease (Peltola, 1983). However the incidence of serogroup Y disease is increasing and accounted for 26% of all disease isolates in the USA between 1992 and 1996 (Rosenstein *et al*, 1999). Serogroup A strains are usually associated with pandemic disease including the epidemic resulting after an annual Moslem pilgrimage to Mecca in 1987 (Schwartz *et al*, 1989). Group B meningococci in contrast are recognised to be the major cause of sporadic meningococcal disease in developed countries (Schwartz *et al*, 1989).

Notification of meningococcal disease since 1912 has allowed trends in meningococcal disease to be analysed. Serogroup A meningococci were responsible for large peaks in

incidence that coincided with the two world wars as well as the peak in the 1930's (Jones, 1995). Since World War II, serogroup A epidemics have been rare in developed countries (Devoe, 1982). In the UK, the incidence of meningococcal infection fell during and after the 1950's to a basic endemic level of 400-500 cases per year, which was caused mainly by serogroup B strains, with 20 – 30% of infections caused by group C strains (Jones, 1995). However in recent years, the number of cases has been increasing with the number of cases in 1995 being higher than any year since 1947 (figure 1.5) (Ramsay *et al*, 1997). Together with this increase in disease cases, the age incidence has also risen with a higher proportion of disease occurring in older children and adults.

Alternative classification systems have allowed meningococcal strains to be characterised at a molecular level using methods such as multilocus enzyme electrophoresis. This technique has been extensively used to study epidemics and has shown that several serogroup A epidemics have been caused by single meningococcal clones (Moore, 1992).

The majority of serogroup B and C isolates have been assigned to three genetically distinct groups; the A4 cluster, ET-5 complex and ET-37 complexes (Wang *et al*, 1993). The majority of serogroup C disease associated strains belong to the ET-37 complex and have frequenctly been isolated from endemic disease cases in Europe and the USA (Achtman, 1995). Serogroup B bacteria of the ET-5 complex were relatively rare prior to 1970, but since then have been responsible for elevated disease levels in Northern Europe (Caugant *et al*, 1986). Meningococci in the ET-5 complex have been associated with different serological markers, bacteria from Norway tend to be 15:P1.16, whilst bacteria from Spain and Brazil tend to be 4:P1.15 (Achtman, 1995). A B:15:P1.16 strain of the ET-5 complex has been associated both with hyper-endemic disease in Norway (Caugant *et al*, 1986) as well as localised outbreaks in the UK (Jones, 1988). In one outbreak in Gloucestershire, disease was shown to be caused by a variant strain which possessed a single amino acid change in the P1.16 sero-subtyping epitope (McGuinness *et al*, 1991).

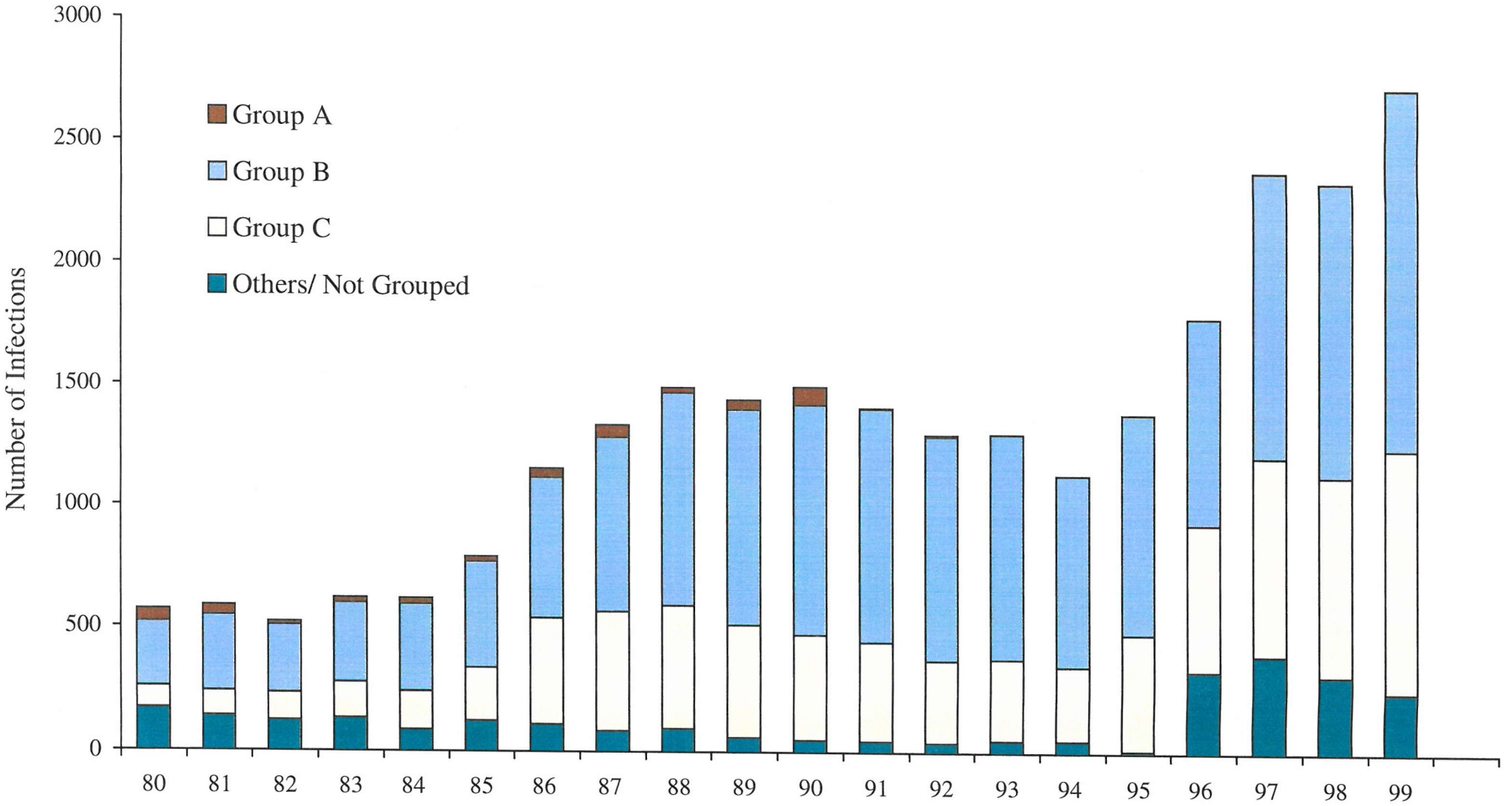


Figure 1.5 – Epidemiology of meningococcal disease in England in Wales for the period of 1980 to 1992

From 1992 onwards, groups A disease isolates were included with the others/non-grouped (CDR Review).

New strains are emerging both within and between countries over time, which appear to result from the continual evolution of strains and from the introduction of new strains from different geographical areas.

1.8 Human susceptibility to meningococcal disease

The human nasopharynx is the natural habitat of the meningococcus and asymptomatic carriage can occur for weeks or months (Rake, 1934). For some individuals however, colonisation of the nasopharynx can lead to invasive disease. In work by Heist *et al,* (1922) it was shown that the blood taken from the majority of the population studied killed meningococci, whilst approximately 5% were unable to demonstrate bactericidal activity. This latter group was considered to be the population susceptible to meningococcal infection. Heist, unfortunately confirmed his hypothesis by contracting and dying of meningococcal meningitis as a result of the lack of bactericidal antibodies in his own blood.

Goldschneider, Gotschlich and Artenstein demonstrated the importance of bactericidal antibodies in the generation of host immunity in a set of papers in the late 1960's. During a meningitis epidemic in a group of 492 military recruits, only 5% of the disease cases possessed bactericidal antibodies, whilst 82% of the healthy control group were able to promote bactericidal lysis of the homologous disease causing strain (Goldschneider *et al*, 1969b). The lack of bactericidal activity in the infected individual was shown to be due to a lack of bactericidal antibodies and not due to the absence of other factors such as complement components or the presence of inhibiting substances.

These papers also showed an inverse correlation between the age-related incidence of disease and the age-specific prevalence of serum bactericidal activity (Goldschneider *et al*, 1969a). A peak of meningococcal disease was observed between 6 and 24 months of age, which was shown to be due to the gradual loss of maternal bactericidal antibodies from the infant. From 2 to 12 years of age, there was a linear increase in bactericidal

antibody production, which correlated with a decrease in the levels of meningococcal disease.

The most compelling evidence for the importance of complement-mediated lysis in the prevention of disseminated meningococcal disease is the increased susceptibility to infection of individuals who have an acquired deficiency in one of the terminal complement components required for immune lysis (Nicholson & Lepow, 1979). The complement system plays a critical role in the host's defences against invasive meningococcal disease. Complement can be activated via the classical pathway by antigen-antibody complexes or via the alternative pathway by bacteria which express one of a number of repeating chemical structures such as LPS on their surfaces. Activated complement brings about bacterial cell death by opsonisation and lysis (Ross & Densen, 1984).

Serogroup B strains have been shown to activate the classical pathway only, whilst other serogroups can activate both pathways (Densen, 1989). Individuals deficient in the terminal complement components are associated with recurrent Neisserial infection despite possessing adequate levels of anti-meningococcal antibodies (Nicholson & Lepow, 1979). Since opsonisation cannot prevent recurrent meningococcal infection, it is believed that complement mediated lysis is the prime effector mechanism of the immune system in prevention of Neisserial infection.

1.9 Vaccination against meningococcal disease

Epidemic meningococcal meningitis causes substantial public health problems. Prevention of the disease through immunoprophylaxis has been practised for nearly 100 years. Before the advent of serum therapy, the mortality rate from meningococcal meningitis was between 60 and 80%, but this was later reduced to between 13 and 30% depending on how promptly serum therapy was initiated (reviewed Apicella, 1990).

1.9.1 Whole cell vaccines

Heat killed whole cell meningococci were first used as a vaccine by Sophian and Black in 1912. Three increasing doses of bacteria were given to eleven volunteers at weekly intervals, who all developed agglutinating and complement fixing antibodies. This vaccine preparation was then used to vaccinate 280 family case contacts and 100 nurses. Two nurses who received only two of the three doses developed meningococcal meningitis but survived. No other cases were seen in other vaccinated individuals (reviewed Frasch, 1995).

Large outbreaks of meningococcal meningitis during the Great War led to a mass immunisation trial in US army camps by Gates in 1917. His vaccine was prepared from whole cells heated to 65°C for 30 minutes and three graded doses were administered at 8 day intervals. Out of the 3700 men vaccinated, only one case of meningitis was seen in a fully vaccinated soldier and two in partially vaccinated individuals, compared to 43 cases in non-vaccinated individuals, giving an efficacy of 87% in fully vaccinated troops (reviewed Frasch, 1995).

Trials of whole cell vaccines continued in the 1920's and 1930's but the results showed uncertain efficacy combined with generally unacceptable levels of adverse reactions and so the vaccine preparations fell into disuse. Preparations using freshly isolated strains provided good evidence of protective efficacy, probably due to the presence of capsular polysaccharide. At this time strains were preserved by repeated passage which results in the loss of serogroup specific antigens (reviewed Frasch, 1995).

1.9.2 Exotoxin vaccines

Meningococci had long been known to possess cell-associated endotoxin, however in 1931 Ferry described the presence of an extracellular toxin in 4 day old glucose broth culture filtrates. When dilutions of the culture filtrates were injected intradermally an erythema was induced within 24 hours. Ferry reported that boiling for 30 minutes inactivated the toxin but Kuhns later stated autoclaving was required to inactivate the

toxin. The toxin was used to immunise 600 children aged 12 to 18. An initial skin test showed 47% had positive reactions and these children received three or four graded injections of toxin at weekly intervals. Two months later when the skin test was repeated on the immunised children, 70% had become skin test negative (Ferry, 1935). Although Ferry did not examine the relationship between skin test reactivity and protection, the equivalent skin test using a diphtheria toxin is converted to negative following vaccination. The exact nature of the toxin was unknown but may have been LPS or the more recently described iron-regulated meningococcal RTX toxin-like exoprotein (Thompson & Sparling, 1993).

1.9.3 Polysaccharide vaccines

By 1940 sulphur drugs were being routinely used to treat meningococcal meningitis and later penicillin was also used. This led to the rapid discontinuation of serum therapy and interest in meningococcal vaccines decreased. During World War II, large epidemics of serogroup A meningococcal meningitis occurred in the US and England in military recruit training camps leading to renewed interest in a preventative vaccine.

The serogroup A polysaccharide was identified in 1935 by Scherp and Rake, who also related the protective power of anti-polysaccharide meningococcal horse serum directly to the content of anti-polysaccharide antibodies. Kabat developed a polysaccharide vaccine, but this failed to induce antibodies in most recipients. Watson and Scherp using a similar method to Kabat also developed a group C polysaccharide vaccine, however this too gave disappointing results. It was later demonstrated that the polysaccharide purified, had been degraded to a low molecular size and the lack of immune response was due to this smallness in size (reviewed Frasch, 1995).

War was again to play a role in the development of a meningococcal vaccine, when in 1963 outbreaks of sulphonamide resistant serogroup B meningococcal meningitis occurred in US army camps training recruits for the Vietnam war. A new method of purifying meningococcal polysaccharide was devised by Gotschlich, which was critical

to the success of most future polysaccharide vaccines. Gotschlich was able to purify polysaccharides of a high molecular weight size, contaminated with less than 1% of nucleic acid or protein. The purified capsular polysaccharide vaccines against serogroup A and C were among the first chemically pure bacterial vaccines. Gotschlich showed this meningococcal polysaccharide vaccine to be immunogenic in humans and went on to immunise US army recruits inducing bactericidal antibodies (Gotschlich *et al*, 1969). This immunisation trial was performed during serogroup C outbreaks on several bases, with carrier rates of up to 80%. Vaccinated recruits were shown to have a significantly reduced acquisition rate of serogroup C carriage when compared with unvaccinated individuals.

A tetravalent vaccine containing in each 0.5ml dose, 50μg each of serogroup A, C, Y and W-135 polysaccharides and was licensed for use in the US in 1981. The serogroup Y and W-135 polysaccharides were added to the vaccine because up to 20% of meningococcal disease is due to these serogroups. In recent years this vaccine has been used to control outbreaks of disease in Europe and North America.

Polysaccharides are T-cell independent immunogens and are poorly immunogenic in young children, the age group as greatest risk. Duration of protection in children under 4 years of age is thought to be 2 years or less (Reingold *et al*, 1985). Long term protection can be achieved by converting serogroup A and C polysaccharides to T-cell dependent immunogens by covalent linkage to proteins. This has been done in the *Haemophilus influenzae* type B polysaccharide-protein conjugate vaccine which has been shown to be highly effective in children as young as 2 months (Black *et al*, 1985). A vaccination programme against meningococcal serogroup C disease using a conjugated polysaccharide vaccine has been launched in the UK in the last six months (Bradbury, 1999). The vaccine consists of depolymerised capsular polysaccharide conjugated to the protein carrier CRM_{197}, a non-toxic mutant diphtheria toxin (Lieberman *et al*, 1996). Trials in toddlers using this vaccine resulted in an increase in antibodies against serogroup C polysaccharide and after three doses resulted in bactericidal activity against a serogroup C strain (MacDonald *et al*, 1998). The results of this study showed that the

meningococcal C conjugate vaccine is highly immunogenic in toddlers and induces immunogenic memory to meningococcal polysaccharide that persists for at least 12 months.

1.9.4 Group B Vaccines

Serogroup B strains have been responsible for most meningococcal disease in developed countries since the late 1940's, yet an effective vaccine is still elusive. Purified high molecular weight serogroup B polysaccharide fails to induce measurable increase in antibodies. Possible explanations for this poor immunogenicity include sensitivity to neuraminidases and the polysaccharide's similarity to sialic acid moieties on human tissues inducing immunotolerance.

As attempts to use the group B polysaccharide as a vaccine have largely been unsuccessful, interest in other possible immunogens has arisen. The outer membrane proteins show considerable diversity with approximately 20 different serotypes based in variation in the PorB protein and additional antigenic diversity is also seen in the PorA protein, the basis of the sero-subtyping scheme. The use of outer membrane proteins in a vaccine is supported by the observation that during serogroup B infections, bactericidal antibodies are directed against non-capsular surface antigens (Frasch & Chapman, 1973). Meningococci release large amounts of essentially pure outer membranes in culture broth as blebs or vesicles during normal growth. These membranes can be purified and used as the starting material for a vaccine (Frasch & Peppler, 1982).

Various vaccine trails have been performed using outer membrane vesicles in Norway, Cuba, Chile and Brazil. In the Norwegian trial (Bjune *et al*, 1991) the outer membrane vesicle vaccine from the strain H44/76, was administered as two injections, 6 weeks apart. After 30 months observation an efficacy of 57%was achieved. No cases of meningococcal disease were observed in the first seven months following the second dose in vaccinated individuals demonstrating that antibodies induced to non-capsular surface antigens could protect against meningococcal disease.

In all these trials a single serotype and sero-subtype has been used, in conditions where most infection was from a single strain. In non-epidemic conditions several strains of meningococci are responsible for disease and so a non-subtype specific vaccine is required. To avoid the problem of subtype specificity an outer membrane vesicle vaccine has been constructed from two meningococcal strains that have been manipulated to each express three class 1 outer membrane proteins and therefore contains six sero-subtypes (van der Voort *et al*, 1996; van der Voort *et al*, 1997). The six sero-subtypes present cover 90% of meningococcal disease in the Netherlands. In preliminary trials in mice, sero-subtype specific bactericidal antibodies were induced to five of the subtypes present, with some epitopes seeming to be more immunogenic that others (Claassen *et al*, 1996). The results of a trial in UK infants using this vaccine have shown that serum bactericidal antibodies were induced. The immune response after a fourth dose of the vaccine was consistent with priming of the immune system and generation of immunological memory (Cartwright *et al*, 1999). However at present there is no licensed vaccine against meningococcal serogroup B infections.

1.10 Thesis Aims

Traditional epidemiological studies of meningococcal infection have largely relied upon detecting differences in surface antigen expression between strains by serological methods. Even with the availability of monoclonal antibodies against the class 1 and class 2/3 outer membrane proteins, the information obtained is still restricted. Sequencing studies allow the identification of individual base changes generating amino acid substitutions within epitopes, which may or may not modify recognition by the relevant antibody and represents a hidden source of epidemiological information. The aim in the work described in the first part of the thesis was to use a collection of meningococcal strains from patients and close household contacts and to use sequence analysis of three outer membrane proteins to see if any differences between the case and carrier strains could be identified.

In the second part of the thesis, the class 3 outer membrane protein was targeted as a potential vaccine candidate. The class 1 outer membrane protein had previously been demonstrated to induce bactericidal antibodies (Ward *et al*, 1996), but these were subtype specific. The class protein 3 is less variable than the class 1 protein and like the class 1 protein, although to a lesser extent is immunogenic during meningococcal infection. Therefore the aim of the work described was to clone the *porB* allele coding for the class 3 protein into *E. coli* and generate a source of class 3 protein free from other meningococcal components. This purified protein could then be used to evaluate the vaccine potential of the class 3 outer membrane protein.

CHAPTER 2: MATERIALS AND METHODS

2.1 Bacterial growth media

2.1.1 Sterilisation of solutions and media

All growth media was routinely sterilised by autoclaving at 15lb in^{-2} for 20 min. Antibiotics, supplements and isopropyl-β-D-thiogalactopyranoside (IPTG) were sterilised by filtration through 0.45μm sterile membrane filters (Millipore).

2.1.2 Luria-Bertani broth (LB)

The following components were dissolved in 1 litre of distilled water and the pH was adjusted to 7.5 with 5M NaOH before autoclaving (Sambrook *et al*, 1989):

Materials:	**Amount per litre**
Bacto-tryptone (Difco)	10g
Yeast extract (Difco)	5g
NaCl	10g

2.1.3 LB agar

Bacto-agar (Difco) was added to LB broth at a final concentration of 1.5% (w/v) before autoclaving. The resulting medium was cooled to 55°C and then poured into sterile petri dishes. The plates were dried in a laminar downflow cabinet before being stored at 4°C.

2.1.3.1 LB-ampicillin agar (LB-amp)

Ampicillin (Sigma) was prepared as a 25mg ml^{-1} stock in UHQ water, filter sterilised and stored in aliquots at -20°C. LB agar was prepared and cooled to 55°C. Ampicillin

was added to the media at a final concentration of 50μg ml^{-1} before pouring. All plates were dried in a laminar downflow cabinet before being stored at 4°C.

2.1.4 Minimal E agar

A Minimal salt solution (Vogel & Bonner, 1956) was prepared as a 50x stock concentrate as described below, and was diluted 1:50 in distilled water containing 0.2% (w/v) glucose and 1.5% (w/v) Bacto agar. After autoclaving the media was cooled and sterile thiamine chloride was added at a final concentration of 1μg ml^{-1}. The agar was then poured into sterile petri dishes, dried and stored at 4°C.

Materials:	**Amount per litre**
$MgSO_4.7H_2O$	10g
K_2HPO_4	500g
NH_4NaHPO_4	175g
Citric acid	100g

2.1.5 Proteose Peptone agar

Proteose peptone media was prepared by the method of (Zak *et al*, 1984) from the components listed below. Both A and B supplements were added to the autoclaved media after cooling to 55°C.

Materials:	**Amount per litre**
Proteose peptone (Difco)	10g
Bacto agar (no. 1 Oxoid)	10g
Starch	1g
$K_2HPO_4.3H_2O$	5.24g
$K_2H_2PO_4$	1g
NaCl	5g
Supplement A	8ml
Supplement B	2ml

Supplement A	Amount per 800ml
Glucose	100g
L-glutamine	10g
para-amino-benzoic acid (Sigma)	13mg
β-nicotinamide adenine dinucleotide (Sigma)	250mg
Thiamine hydrochloride (Sigma)	3mg
Co-carboxlase (Sigma)	100mg
Cyanocobalamin (Sigma)	10mg
Ferric nitrate	20mg

The above were dissolved in distilled water, filter sterilised and stored in 5ml aliquots at -20°C.

Supplement B:	Amount per 200ml
L-cysteine hydrochloride	26g
Adenine	1g
Guanine hydrochloride	30mg
Uracil	800mg
Hypoxanthine	320mg

All of the above bases with the exception of cysteine were dissolved in 100ml of boiling 0.1M hydrochloric acid. After cooling to room temperature, the cysteine was added and the final volume was made up to 200ml with distilled water. The solution was filter sterilised and stored in 1ml aliquots a -20°C.

2.1.6 SOB medium

The following components were dissolved in 950ml distilled water and the pH was adjusted to 7.0 with 5M NaOH. The volume was adjusted to 1l prior to autoclaving. After autoclaving 10ml sterile 1M $MgCl_2$ was added.

Materials:	Amount per litre
Bacto-tryptone (Difco)	20g
Yeast extract (Difco)	5g
NaCl	0.5g
KCl	186mg

2.1.7 2YT media

The following components were dissolved in 1l distilled water and autoclaved:

Materials:	Amount per litre
Bacto-tryptone (Difco)	16g
Yeast extract (Difco)	10g
NaCl	5g

2.1.8 Superbroth media

The following components were dissolved in 1l distilled water and autoclaved:

Materials:	Amount per litre
Bacto-tryptone (Difco)	25g
Yeast extract (Difco)	15g
NaCl	5g

2.1.9 Soft agar overlay

Bacto-agar (no.1 Oxoid) was added to LB broth at a concentration of 0.65% (w/v). After autoclaving, the media was stored as 3ml aliquots. Prior to use, the soft agar was melted in a microwave oven.

2.2 Bacterial strains and growth conditions

2.2.1 Growth and storage of Neisseria

The *N. meningitidis* strains used were stored as thick suspensions in protease peptone media (Difco) containing 10% (v/v) glycerol, and stored in liquid nitrogen. For bacterial growth the frozen stocks were used to inoculate protease peptone agar plates which were subsequently incubated at 37°C, in an atmosphere of 5% CO_2 for 16-18 hours.

2.2.2 Growth and storage of E.coli

All of the *Escherichia coli* (*E.coli*) strains used are listed in table 2.1. Working stocks were kept at 4°C on minimal E agar plates supplemented with thiamine hydrochloride (1ug ml^{-1}) and sub-cultured every 3 weeks. For long term storage, suspensions were kept at -70°C in SOB medium (15% glycerol).

Strain	Genotype	Reference
JM101	*sup*E, *thi*, Δ(*lac-pro*AB), [F', *tra*D36, *pro*AB, *lac*I^qZΔM15]	(Yanisch-Perron *et al*, 1985)
JM109	*end*A1, *rec*A1, *gyr*A96, *thi*, *hsd*R17, (rk-mk+), *rel*A1, *sup*E44, Δ(*lac-pro*AB), [F', *tra*D36, *pro*AB, *lac*I^qZΔM15]	(Yanisch-Perron *et al*, 1985)
JM109(DE3)	*end*A1, *rec*A1, *gyr*A96, *thi*, *hsd*R17, (rk-mk+), *rel*A1, *sup*E44, Δ(*lac-pro*AB), [F', *tra*D36, *pro*AB, *lac*I^qZΔM15],λ(DE3)	(Yanisch-Perron *et al*, 1985)
BL21	F-, *ompT*, *hsdSB* (r_B^- m_B^-), *gal*, *dcm*, pLysS (Cam^R)	

Table 2.1 – *E. coli* strains used

2.3 Isolation of Plasmid DNA

Two methods of preparation of plasmid DNA were routinely employed.

2.3.1 MINIPREP plasmid isolation system

The Wizard™ Miniprep DNA purification system (Promega) was routinely used for the isolation of plasmid DNA. The procedure followed was as described in the manufacturer's instructions.

2.3.2 QIA-Prep MiniPrep Plasmid Preparation

The QIA-Prep MiniPrep plasmid preparation (Qiagen) was used as an alternative to the Wizard ™ Miniprep system. It was also used routinely in the preparation of plasmid template for use in sequencing reactions. The procedure followed was as described by the manufacturer.

2.4 DNA manipulation

2.4.1 Quantification of DNA

The quantity of DNA present in aqueous solutions was determined by measurement of the absorbance at 260nm (A_{260}) using a CE599 automatic scanning spectrophotometer (Cecil Instruments). DNA-containing samples were diluted 1 in 200 with UHQ water and A_{260} measured against an UHQ water blank. An A_{260} of 1.0 was taken to be equivalent to a DNA concentration of 50μg ml^{-1} double stranded DNA, 40μg ml^{-1} single stranded DNA or 33μg ml^{-1} oligonucleotide (Sambrook *et al*, 1989).

An alternative method for estimating the concentration of DNA involved analysing a sample of the DNA by agarose gel electrophoresis. The intensity of the ethidium bromide staining of the DNA sample was compared against 1μg of a 1kb DNA ladder and the sample concentration then estimated. This method allowed only an approximate value of DNA concentration to be obtained.

2.4.2 *GenecleanII™ purification of DNA*

The GenecleanII™ kit (Stratech) contains a specially formulated silica matrix called glassmilk. This matrix allows DNA to be purified from both aqueous solutions and agarose gels of size 500bp upwards. All of the solutions were supplied with the kit and were used according to the manufacturer's instructions.

2.4.3 *Restriction enzyme digestion of DNA*

Restriction enzymes and their appropriate buffers (10x concentrates) were stored at -20°C as recommended by the manufacturers (Promega and New England Biolabs). Digests typically contained 1-2 μg of DNA and 5-10 units of enzyme in a total reaction volume of 10-50μl and were routinely incubated at 37°C for 1-3 hours. Bovine serum albumin was included in the reaction mix, as suggested by the manufacturers, at a final concentration of 1μg μl^{-1}. Depending on the source of DNA and its purity, RNAse at a concentration of 1174U μl^{-1} was included in the reaction mix when appropriate. Enzyme digestion was stopped by storing the sample at -20°C, by the addition of 6x gel loading buffer prior to agarose gel electrophoresis or by DNA purification using the Wizard DNA Miniprep kit. (section 2.3.1)

2.4.4 *Ligation of DNA*

The ligation of DNA molecules utilised the activity of bacteriophage T4 DNA ligase (Promega). The following ligation components were dissolved in sterile 10x ligase buffer:

10x ligation buffer	700mM Tris-HCl	pH7.5
	700mM $MgCl_2$	

ligation mix:	BSA (1mg ml^{-1})	1µl	
	DTT (15.4mg ml^{-1})	1µl	10mM final concentration
	ATP (6.1mg ml^{-1})	1µl	1mM final concentration
	ligase	1µl	2 units
	vector	1-5µl	10ng
	insert	1-5µl	30ng

Vector and insert were added in the ratio 3:1. The mixture was incubated at 14°C overnight.

2.5 Agarose gel electrophoresis

The method used was that described by Sambrook *et al.* (1989) and was routinely used to separate and characterise DNA on a size basis.

Materials:

1) **50x Tris-acetate running buffer (TAE):** 40mM Tris, 2mM Na_2EDTA adjusted to pH8.0 with glacial acetic acid.

2) **6x Gel loading buffer (GLB):** 18% (w/v) Ficoll 400, 0.18% (w/v) Bromophenol blue, 0.18% (w/v) Xylene cyanol FF, 0.18% (w/v) Orange G, 600mM EDTA (pH8.0)

A gel matrix consisting of either 1 or 2% (w/v) agarose (type II: medium, Sigma) was routinely used to resolve DNA in the 200bp - 10kbp range. Depending on the number of DNA samples to be analysed, the agarose gels were cast in a BRL Horizon 58 (GIBCO BRL), a Wide Mini-Sub™ Cell (BioRad) or a DNA Sub Cell™ (BioRad), the dimensions and gel volumes used are listed in table 2.2.

	Gel dimension	Bed volume	Gel thickness
Horizon 58	5x8cm	30ml	7.5mm
Wide Mini-Sub™ Cell	15x10cm	80ml	5.3mm
DNA Sub Cell™	15x25cm	120ml	3.2mm

Table 2.2 – Dimensions of gel casting apparatus

When using the DNA Sub Cell™ a 2% agarose gel was used to reduce the fragility of the gel due to its size. A 1% gel was used in the two other tanks.

Agarose gels were constructed by melting the required amount of agarose in 1x TAE buffer in a microwave oven, which was then poured into the desired gel former with the well-forming comb in position. After approximately 30min the set agarose was submerged in 1x TAE buffer and the comb removed.

The DNA samples to be analysed were mixed with 1/5th the volume of 6x gel loading buffer and then loaded into the pre-formed wells. Electrophoresis was carried out at room temperature at 90V until the Orange G dye front reached the end of the gel.

2.5.1 Visualisation of DNA

After electrophoresis, the DNA gels were soaked with a 1μg ml^{-1} solution of ethidium bromide (Sigma) for 30min in the dark, at room temperature. Excess stain was removed by rinsing with water. The DNA was visualised by Ultra-violet illumination using a transilluminator (UVP) and a permanent photographic image obtained using Polaroid 665 film.

2.5.2 Estimation of DNA size

The sizes of the visualised DNA fragments were estimated by comparing their relative mobility through the gel matrix against fragments of a known size. The standard markers used (1kb ladder, GIBCO BRL) ranged from 75bp to 12kb in size (table 2.3).

Size (bp)	Size (bp) cont.	Size (bp) cont.
12,216	5,090	394
11,198	4,072	344
10,180	3,054	298
9,162	2,036	220
8,144	1,635	154
7,126	1,018	142
6,108	516	75

Table 2.3 – Standard 1kb DNA ladder sizes

2.6 Oligonucleotide synthesis and manipulation

Oligonucleotides were synthesised in a 3' to 5' direction on a model 318A automated synthesiser (Applied Biosystems Inc.) employing β-cyanoethyl phosphoramidite (CEP) chemistry. Oligonucleotides were synthesised either onto a column or a filter.

2.6.1 Removal from column

To remove the oligonucleotide from the column, a 1ml syringe was attached to one end of the column and a second syringe containing 0.8ml of ammonia (0.88 specific gravity) attached to the other. The ammonia was passed through the column in 0.2ml aliquots, with each aliquot being allowed to incubate with the column for 20min at room temperature. After the final incubation step, the ammonia was passed back and forth through the column several times, transferred to a clean Reacti-vial (Pierce) which was fitted with a gas tight cap and then incubated at 55°C for 16hr.

2.6.2 Removal from filter

To remove the oligonucleotides from the filter, the filter was removed from the plastic mount and transferred to a clean React-vial (Pierce) fitted with a gas tight cap. Ammonia (0.8ml, 0.88 specific gravity) was added to the vial, which was then incubated at 55°C for 16hr.

2.6.3 Deprotection of oligonucleotides

After incubation the tube was allowed to cool to room temperature and then divided into four aliquots. The oligonucleotide was precipitated by the addition of 1/10th volume 3M sodium acetate (pH5.0) and 3 volumes of absolute ethanol, followed by incubation at –20°C for 2 hours. The oligonucleotide was harvested by centrifugation (10,000g, 10min). The pellets were washed with 1ml of ice cold 80% ethanol, recovered by centrifugation (10,000g, 5min) then dried under vacuum for 15min. One pellet was resuspended in UHQ water (50µl) and the concentration determined by measuring the A_{260}. The concentrated oligonucleotide stock and remaining pellets were all stored at -20°C.

2.7 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is an *in vitro* method of producing large amounts of a specific DNA fragment from small amounts of DNA template. The reaction is based on the cyclic annealing and extension of two oligonucleotide primers, which flank the desired DNA target sequence.

2.7.1 Preparation of template DNA

The strain of *Neisseria meningitidis* required for DNA amplification was grown overnight on protease peptone agar. A single colony was picked and resuspended in UHQ water (10µl) in a sterile microcentrifuge tube. The bacteria were lysed by the

addition of potassium hydroxide (10µl, sterile 0.25M) and then denatured by boiling at 100°C for 5 min and the tubes briefly centrifuged to remove moisture from lids. Hydrochloric acid (10µl, sterile 0.25M) was added to neutralise the mixture and Tris-HCl, (10µl sterile 0.5M, pH7.5) was added to ensure the correct pH for PCR. The stock preparation of bacterial DNA was stored at -20°C.

2.7.2 Primer design

Oligonucleotide sequences used as primers for the amplification of DNA by PCR were selected with the following criteria taken into consideration, and were synthesised as described (section 2.6).

1) Primers should not contain any palindromic sequences.
2) The primer pair should not be able to form 3`-5` concatemers.
3) The primers should ideally be between 17 and 40 bases in length.
4) The termini of the primer pairs should not be complementary.
5) The PCR product should ideally be between 100bp and 2kb in length.
6) Primers should if possible be in a GC rich region, which results in better binding to template DNA.
7) The primers should ideally have similar Tm values, which were calculated as follows:
If the primer was 24 nucleotides or less:
Tm °C = 4(G+C) + 2(A+T) (Sambrook *et al.* 1989).
If the primer was larger than 24 nucleotides:
Tm °C = 81.5 –16.6($\log_{10}[Na^+]$) + 0.4(G+C%) – (600/n)
Where $[Na^+]$ is the salt concentration, which is usually taken as 50mM and n is the length of the primer.

2.7.3 Standard PCR reactions

The mixture was prepared for a standard PCR reaction as follows:

5µl 10x Taq buffer containing 15mM $MgCl_2$ (Promega)
5µl dNTP mix (2mM, Boehringer Mannheim)
5µl forward primer (50ng μl^{-1})
5µl reverse primer (50ng μl^{-1})
25µl UHQ water
5µl template DNA
0.5µl Taq polymerase (5U μl^{-1}, Promega)

To avoid possible sources of contamination, filter tips were used throughout this process. To remove any sources of double stranded DNA, the reaction mixture was irradiated for 10min with short wave UV (256nm) in an Amplirad UV illuminator (Genetic Research Instrumentation Ltd.) before the addition of the template DNA and polymerase. These procedures were used routinely on all reaction mixtures.

2.7.4 Thermal cycling conditions

Amplification of template DNA was achieved in cycles comprising of three steps:

1) DNA Denaturation:
This was routinely carried out at 94°C for 15sec in all reactions.

2) Annealing of primers:
The annealing temperature of each set of primers was initially tested at 10°C below the lowest Tm of the primer pair. In a series of experiments this was gradually increased towards the Tm to maximise the annealing to the template DNA and therefore the

amplification. The reaction mix was held at the optimised annealing temperature for 30sec.

3) Extension of DNA chains:

The temperature at which the Taq polymerase was allowed to polymerise the extension of the new DNA fragment was always 72°C. The time allowed for this extension was 1 min since the desired PCR products were between 500bp and 1kb in length.

For the generation of PCR products from cell lysates the standard number of cycles used was 30, and was performed in a GeneAmp PCR System 9600 thermal cycler (Perkin Elmer Cetus). The reactions were given a hot start (70°C) to prevent premature annealing and samples were held at 4°C until removed from the thermal cycler.

2.8 DNA sequencing reactions

2.8.1 Preparation of PCR Product Template

When sequencing PCR products, the enzyme Bio-X-Act (Bioline) which has improved proof reading capabilities was used in order to attempt to minimise possible mutations being introduced by the PCR procedure.

1) Reaction mix:

5µl 10x buffer (Bioline)

5µl dNTPs (2mM, Boehringer Mannheim)

5µl forward primer (50ng μl^{-1})

5µl reverse primer (50ng μl^{-1})

2µl $MgCl_2$ (50mM, Bioline)

23µl UHQ water

5µl template DNA

0.5µl Bio-X-Act (4U μl^{-1}, Bioline)

The same precautions against possible contamination sources were taken as before.

2) Thermal cycling conditions:

These were essentially the same as before (section 2.7.4) except that 35 cycles were performed.

2.8.2 Sequencing Reactions

DNA sequencing was performed using the PRISM™ ready reaction dyedeoxy terminator cycle sequencing kit (Applied Biosystems Inc.) as described by the manufacturers.

1) PCR Product Sequencing:

The PCR products obtained from amplification using the Bio-X-Act polymerase were purified by the GeneClean II™ procedure (section 2.4.2).

The sequencing mixture contained:

50ng template DNA

25ng primer (12.5ng μl^{-1})

8µl ready reaction mix (PE Applied Biosystems)

Total volume adjusted to 20µl by addition of appropriate volume of UHQ water.

2) Plasmid Sequencing:

The plasmid template was prepared by extraction of the plasmid using the QIAprep procedure (section 2.3.2).

The sequencing mixture contained:

500ng template DNA

12.5ng primer (12.5ng μl^{-1})

8µl ready reaction mix (PE Applied Biosystems)

Total volume adjusted to 20µl by addition of appropriate volume of UHQ water.

As with the PCR protocols, every care was taken to avoid contamination, which involved the use of filtered tips and irradiation of the empty reaction tubes for 10min.

3) Cycling conditions:

1. Denaturation - 94°C, 40sec
2. Annealing - optimised temperature, 40sec
3. Extension - 60°C, 4min.

For generation of sequencing products the standard number of cycles used was 30, and was performed in a Perkin Elmer Cetus 9600 Thermal Cycler, using a hot start (60°C).

2.8.3 Removal of excess dyedeoxy terminators from completed sequencing reactions

At the end of the sequencing reactions, high concentrations of unincorporated terminators are present which need to be removed. This was achieved by precipitating the sequencing products with 2M sodium acetate, pH4.5 (2µl) and 95% ethanol (50µl) at -20°C for a minimum of 4 hours. The sequencing products were recovered by centrifugation at 12,000g, for 30 min and washed with 70% ethanol (200µl, room temperature, 10min). The ethanol was removed and the pellet stored at -20°C.

2.8.4 Preparation of DNA sequencing gels

Sequencing gels were poured between two horizontal, low fluorescence glass plates separated by 0.4mm thick spacers (Applied Biosystems Inc.). Before assembly, the plates were scoured with a detergent powder (Alconox Inc.) and then rinsed with hot running water. The plates were then hot washed with methanol (HPLC grade, BDH), before being left to air dry. Any smears were removed with methanol and lint free tissue.

The sequencing gel was prepared as follows:

32ml Sequagel-6 (National Diagnostics)

5.28ml 10x TBE containing 89mM Tris

89mM Boric acid

2mM disodium EDTA

Once dissolved in distilled water, the solution was filtered through a sintered glass filter.

2.72ml UHQ water

20µl TEMED

200µl Ammonium persulphate (100mg ml^{-1})

The gel mixture was poured between the horizontal plates, taking care to avoid air bubbles. The top spacer was inserted, secured using bulldog clips and the gel was left to polymerise for a minimum of 4 hours.

2.8.5 Preparation and loading of samples

Running buffer: 224ml 10x TBE

1476ml distilled water

The dried sequencing reaction pellets were resuspended in 4µl of a solution composed of deionised formamide (Applied Biosystems Inc.) and 50mM EDTA (pH8.0) mixed at a 5:1 (v/v) ratio, containing a trace of dextran blue dye. Before loading the gel, the samples were heated at 90°C for 2min. Electrophoresis was carried out in an Applied Biosystems Inc. 373A automated DNA Sequencer for 12 hours at 30W. The subsequent data was analysed using the LASERGENE software package supplied by DNASTAR.

2.9 Transformation of *E.coli*

2.9.1 Preparation of competent cells

The desired *E.coli* strain was grown statically for 16-18hr in 10ml LB at 37°C. This culture (1ml) was used to inoculate 25ml of LB and the culture was incubated at 37°C with shaking until early log phase (A_{550} 0.4-0.5), then transferred to ice cold sterile tubes. All solutions, apparatus and tips used from this point had to be ice-cold to ensure maximum competence. After incubating on ice for 20min, the cells were harvested (1000g, 10min, 4°C), resuspended in ice cold $MgCl_2$ (10ml, sterile 0.1M) and immediately re-harvested (1000g, 10min, 4°C). The cells were finally resuspended in ice cold $CaCl_2$ (1ml, sterile 0.1M) and incubated on ice for a minimum of 1hr. The cells were then competent for the uptake of DNA and could be stored at 4°C without significant loss of competence for 24 hours.

2.9.2 Transformation of competent cells

Competent *E.coli* cells were transferred to sterile microcentrifuge tubes in 100µl aliquots and kept on ice. The quantity of DNA used in a typical transformation reaction ranged between 0.1ng-10ng per 100µl of cells and was usually contained within a 10µl volume. The DNA was added to the cells, which were then incubated on ice for 30min with intermittent mixing. To promote the uptake of DNA, the cells were subjected to heat shock in a 42°C water bath for 2min and then immediately transferred back to ice for a further 30min incubation period. LB (100µl) at room temperature was added to the transformed cells and the bacteria were incubated at 37°C for 1hr before plating out on selective media and incubating overnight at 37°C.

2.9.3 Screening for constructs

STETS buffer:

8%	Sucrose
5%	Triton-X-100
50mM	EDTA
50mM	Tris-HCl, pH8.0

Transformed bacteria were screened for the presence of the construct by an adapted method of Holmes and Quigley (1981). An LB-amp agar plate was divided into 8 sections and individual colonies from the transformation plate picked off and streaked on the sectioned plate which was incubated at 37°C overnight.

From each colony picked and streaked from the transformation plate a small loopful of cells was taken and resuspended in 50µl STETS buffer. A freshly prepared solution of lysosyme (Sigma, 8µl,10mg ml^{-1}) was used to lyse the cells, which were incubated at room temperature for 5min. The cells were boiled for 5min then were subjected to centrifugation at 8,500g for 10min to remove cell debris. The plasmid DNA contained within the supernatant was precipitated at -20°C for 1hr with an equal volume of isopropanol. The construct plasmid was harvested by centrifugation at 8,500g for 15min and the pellet was vacuum dried, before resuspending in UHQ water (15µl). The resultant plasmid DNA was then used in restriction enzyme digests to ascertain the presence or absence of the insert.

2.10 Expression Trials of the fusion protein in *E.coli*

Those *E.coli* strains shown to contain the recombinant plasmid were grown overnight in 10ml LB-amp at 37°C, with shaking at 200rpm. A volume of this culture (30µl) was used to inoculate 5ml LB-amp. The culture was incubated at 37°C with shaking to mid-log phase (A_{600} 0.3). At this point, a 1ml time zero sample was removed and kept. Expression of the plasmid encoded protein was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1mM. When using the *E.coli*

strain JM101 an additional induction step had to be used. One hour after the addition of IPTG, M13/T7 phage were added at a concentration of 10pfu ml^{-1}. After further incubation at 37°C, with shaking at 200rpm for 5 hours, the final A_{600} was noted and a 1ml final sample taken. Cells from the two samples were harvested by centrifugation at 13,000rpm for 15min. The cells were resuspended in 3x SDS-PAGE loading buffer in a volume depending on their cell density as estimated from A_{600} readings. A sample with an A_{600} reading of 1.0 was resuspended in 100µl buffer, whilst a sample reading of 1.2 was resuspended in 120µl buffer. The levels of protein expression were then assessed by running the samples on an SDS-PAGE gel and Western blotting with an appropriate antibody.

2.10.1 M13/T7 Phage Production

The *E.coli* strain JM109 was grown overnight in 10ml LB at 37°C, with shaking at 200rpm. A volume of this culture (0.5ml) was used to inoculate 25ml LB which was incubated with 50µl of M13/T7 phage at 37°C, 180rpm and grown overnight. Cellular debris was removed by centrifugation of the culture at 1,000g for 15min. The supernatant solution was removed and stored at 4°C until required.

To ascertain the titre of the phage stock, serial dilutions were made down to 10^{-12}. Dilutions of phage (100µl) were mixed with an overnight culture of *E.coli* strain JM101 (200µl) which in turn was mixed with 3ml molten soft agar overlay, which was then poured onto minimal media plates and incubated at 37°C overnight.

Phage titre was calculated as follows:

pfu/ml = no. plaques x dil^{n} x (vol. plated ml^{-1})$^{-1}$

2.11 Measurement of protein concentration by Micro bicinchoninic acid (BCA) protein assay

The micro BCA protein assay, initially described by Smith *et al.* (1985) was performed according to the manufacturer's instructions (Pierce) and was routinely used to determine protein concentrations.

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

Materials:

1) **Acrylamide monomer solution:** 50% (w/v) acrylamide and 1.3% (w/v) N,N'-methylene bisacrylamide (BDH). The mixture was warmed under a hot running tap to facilitate the endothermic solubilisation of the acrylamide. The solution was stored at 4°C.

2) **SDS solution:** 2% (w/v) SDS , stored at room temperature.

3) **Ammonium persulphate solution:** (APS)1% (w/v) ammonium persulphate, freshly prepared.

4) **Glycerol**

5) **N,N,N',N'-tetramethylethylenediamine:** (TEMED) (BDH), stored at 4°C.

6) **Separating gel buffer:** 1.2M Tris-HCl, pH8.8, stored at 4°C.

7) **Stacking gel buffer:** 0.25M Tris-HCl, pH6.8, containing 0.08% (v/v) TEMED, stored at 4°C.

8) **Running buffer:** 25mM Tris, 192mM glycine, 0.1% (w/v) SDS, pH8.3, freshly prepared.

9) **Dissociating buffer:** 125mM Tris-HCl, pH6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, 0.002% (w/v) bromophenol blue.

2.12.1 Construction of gradient gel

SDS-PAGE was performed using a 10-25% linear acrylamide gradient as described by Heckels (1981), with the discontinuous buffer system of Laemli (1970).

The gel casting plates were cleaned with IMS (80%), separated by 1mm thick plastic spacers (BDH) and sealed with 1% (w/v) molten agarose (1 x 150 x 200mm). Two acrylamide solutions of 10% and 25% (table 2.4) were prepared and deaerated to remove any dissolved oxygen that may have prevented complete acrylamide polymerisation. TEMED and APS were added and the separating gel was cast by pumping the stock acrylamide mixes through a triple channel peristaltic pump (Pharmacia P-3). In order to exclude air, isobutanol was layered onto the surface of the acrylamide and the gel was left to polymerise at room temperature for a minimum of 1 hour. After polymerisation, the isobutanol was poured off and the surface of the gel washed with distilled water. The stacking gel (1 x 30 x 200mm) was then cast on top of the separating gel and the desired comb was inserted. After polymerisation of the stacking gel (minimum 30min), the comb was removed and the wells were washed with water applied using a needle and syringe.

2.12.2 Construction of linear gel

The preparation of the plates was identical to above. A 10% acrylamide solution was prepared using double the volumes specified in the table 2.4. The separating gel was cast by pipetting the gel mixture between the two glass plates and layering with isobutanol. The stacking gel was prepared as above.

Separating Gel:

Acrylamide concentration	**25%**	**10%**
Acrylamide monomer solution	6.25ml	2.5ml
Separating gel buffer	3.1ml	3.1ml
SDS solution	0.62ml	0.62ml
Glycerol	1.25ml	-
TEMED*	6.2µl	6.2µl
UHQ water	1.1ml	6ml
APS*	0.125ml	0.25ml

Stacking Gel:

Acrylamide concentration	4%
Acrylamide monomer solution	1.0ml
Stacking gel buffer	6.25ml
SDS solution	0.62ml
UHQ water	3.37ml
APS*	1.25ml

* - added to gel mixture after degassing and just before casting.

Table 2.4 - Composition of solutions used in the casting of polyacrylamide gels

2.12.3 Sample preparation

For each gel track between 1-20µg of total protein was loaded. The sample volume was adjusted to 20µl and mixed with an equal volume of dissociation buffer. All samples were heated in a boiling water bath for 10 min and 40µl of sample was run per well.

2.12.4 Preparation of cell lysates for SDS-PAGE

Bacterial strains were grown overnight on solid media and scraped into 1ml UHQ water containing 0.05% sodium azide and 0.01mM PMSF, then centrifuged at 700g to remove large cell clumps. The supernatant was transferred to a clean microcentrifuge tube and stored at -20°C. The protein concentration was determined by diluting the cell lysate solution 1 in 10, in 1% SDS and 0.01% NaOH and measuring A_{260}, using a CE599 automatic scanning spectrophotometer (Cecil Instruments). Absorbance at 260nm measures the DNA concentration and this has previously been correlated to protein concentration. An A_{260} of 12 was taken to be a 1mg ml^{-1} protein concentration.

2.12.5 Gel running conditions

Wells containing sample were overlaid with running buffer prior to filling both gel tank reservoirs with buffer (GIBCO BRL V15.17 gel tanks). Gradient gels were run at 200V for 18-20hr at 4°C, whilst linear gels were run at 200V for 3hr at room temperature or 40V for 18hr.

2.12.6 Standards used for the determination of the relative molecular masses of the proteins

A standard curve was produced by plotting the $\log_{10}$ (distance migrated) against the molecular mass of the protein standards. The apparent molecular masses (M_r) of desired sample proteins were calculated by extrapolating the distances migrated by these proteins on a gel to the standard curve. A seven standards system was used (Sigma - Dalton Mark VII-L, table 2.5).

Proteins	Approx. kDa
Albumin (bovine)	66
Albumin (egg)	45
Glyceraldehyde-3-P-dehydrogenase	36
Carbonic anhydrase (bovine)	29
Trypsinogen (bovine pancreas)	24
Trypsin inhibitor (soybean)	20
α-lactalbumin (bovine milk)	14.2

Table 2.5 - Molecular weight standards (kDa)

2.12.7 Page blue stain

After electrophoresis, the stacking gel was removed and the proteins stained by a modification of the method of Fairbanks *et al.* (1971). Gels were fixed and stained in a solution of 10% (v/v) glacial acetic acid and 20% (v/v) isopropanol, containing 0.5mgml^{-1} PAGE Blue 83 (BDH) for 1hr. The gels were destained in 10% (v/v) isopropanol and 10% (v/v) glacial acetic acid until the background staining was minimal. Gels were either photographed or dried for storage.

2.12.8 Long term storage of acrylamide gels

Two methods were employed for long term storage of gels. In the first, individual gels were shaken in 200ml of solution containing 25% (v/v) methanol and 5% (v/v) glycerol for 30min, twice. The soaked gel was then sandwiched between 2 sheets of gel drying film (Promega), clamped into a drying frame and allowed to dry overnight. The second method of drying required the gel to be shaken in destain buffer containing 1% glycerol overnight, followed by washing of the gel with distilled water 4 times. The gel was transferred onto a piece of chromatography paper (17 Chr, Whatman) by placing the gel upside down on a glass plate, laying the filter paper on top of the gel and then sliding

the glass plate free. The gel was covered with a piece of Saran wrap and then placed in a gel dryer (BioRad) and vacuum dried at 80°C for 1hr 15min.

2.13 Western blotting of polyacrylamide gels and detection of protein

2.13.1 Electrophoretic transfer of proteins to nitro-cellulose sheets

This procedure was adapted from that of Towbin *et al,* (1979).

Materials:

1) **Blotting buffer:** 20mM Tris, 150mM glycine, 20% (v/v) methanol, 0.1% SDS (w/v), pH 8.8

2) **Nitrocellulose:** Hybond™C, pore size 0.45μm (Amersham)

The gel was equilibriated in blotting buffer by gentle shaking at room temperature for 30min. Four pieces of chromatography paper (17 Chr, Whatman) and a sheet of nitrocellulose were cut to the same size as the gel and were soaked in blotting buffer. On the anode plate of a BioRad Trans blot semi-dry transfer cell the following sandwich was constructed in order: 2 sheets of filter paper, sheet of nitrocellulose, polyacrylamide gel, 2 sheets of filter paper. All air bubbles were expelled from each layer and a constant current was applied at 0.8mA cm^{-2} for 1hr.

2.13.2 Immune detection

Materials:

1) **Tris buffered saline (TBS):** 20mM Tris-HCl, 500mM NaCl, pH 7.5

2) **Tween Tris buffered saline (TTBS):** TBS plus 0.05% Tween 20

3) **Blocking buffer:** 5% (w/v) non-fat powdered milk (Marvel™) in TTBS

4) **Antibody dilution buffer:** 1% (w/v) gelatine in TTBS

5) **Substrate buffer:** 100mM Tris, 100mM NaCl, 2mM $MgCl_2$, pH9.5

After the electrophoretic transfer of protein was completed, the nitrocellulose was washed twice for 5min in TTBS to remove traces of SDS and then incubated in blocking buffer at room temperature for 1hr to prevent any non-specific binding. After washing

twice for 5min in TTBS, the nitrocellulose sheet was shaken for 1hr at room temperature in dilution buffer, containing monoclonal antibody at the desired dilution (between 1:100 and 1:10,000). Unbound antibody was washed from the nitrocellulose sheet using three washes of 5min each in TTBS. The blot was then shaken for 1hr in blocking buffer containing 1:1000 dilution of the appropriate anti-species Ig alkaline phosphatase conjugated antibody (BioRad) at room temperature. The blot was then washed three times in TTBS for 5min, followed by three times in TBS for 5min. Bound antibody was detected by addition of the following substrates:

Substrate components:

Substrate buffer - 100mM Tris, 100mM NaCl, 2mM $MgCl_2$, pH9.5

NBT solution - 30mg/ml in 70% DMF (Sigma)

BCIP solution - 15mg/ml in 100% DMF (Sigma)

The colour reagent was prepared by mixing 1ml of NBT solution and 1ml BCIP solution with 100ml substrate buffer. Nitrocellulose sheets were incubated with the colour substrate until the desired band intensity was achieved then terminated by washing in distilled water. The blots were either photographed or stored dry in the dark.

2.14 Low Molecular Weight Gels

Low molecular weight polyacrylamide gels were used for the detection of lipopolysaccharide. Polyacrylamide gels were constructed according to the method of Schagger & von Jagow (1987) and used a discontinuous buffer system. This system enhanced the resolution of low molecular weight proteins (2kDA to 17kDa) when compared to SDS-PAGE gel electrophoresis and was therefore routinely used for the visualisation of lipopolysaccharide.

Materials:

1) **Acrylamide monomer solution:** 48% (w/v) acrylamide and 1.5% (w/v) N-N'-methylene bisacrylamide (BDH). The mixture was warmed under a hot running tap to aid the solubilisation of the acrylamide. The solution was stored at 4°C.
2) **SDS solution:** 20% (w/v) SDS, stored at room temperature.
3) **Ammonium persulphate solution:** (APS) 10% (w/v) ammonium persulphate solution, freshly prepared.
4) **N,N,N',N'-tetramethylethylenediamine:** (TEMED) (BDH), stored at 4°C.
5) **Gel buffer:** 3M Tris-HCl, pH8.5, containing 0.3% (w/v) SDS, stored at 4°C.
6) **Anode buffer:** 0.2M Tris-HCl, pH8.9, freshly prepared.
7) **Cathode buffer:** 0.1M tricine, 0.1% (w/v) SDS, 0.1M Tris-HCl, pH8.2, freshly prepared.
8) **Sample buffer:** 4% (w/v) SDS, 12% (v/v) glycerol, 2% (v/v) 2-mercaptoethanol, 0.02% (w/v) brilliant blue-G, 0.05M Tris-HCl, pH6.8, stored at room temperature.

2.14.1 Construction of Gel

The gel casting plates were cleaned with 80% IMS, separated by 1mm thick spacers (BDH) and sealed with 1% (w/v) molten agarose (1 x 150 x 200mm). Both the separating and spacer gel solutions were prepared according to table 2.6 and degassed to remove any dissolved oxygen that may have prevented complete acrylamide polymerisation. The appropriate quantities of TEMED and APS were added to these solutions and gently mixed. The separating gel mixture was poured between the sealed plates to a height of 10cm and then carefully overlaid with 3cm of spacer gel mixture which was in turn overlaid with water. The gels were left to polymerise for 1 hour at room temperature. After polymerisation the water was removed, the stacking gel mixture containing APS and TEMED was cast on top of the spacer gel and the desired comb was inserted.

Solution	Separating Gel	Spacer Gel	Stacking Gel
Acrylamide monomer sol.	6.7ml	2.0ml	1.0ml
Gel buffer sol.	6.7ml	3.33ml	3.1ml
Glycerol	2.1ml	-	-
Water	4.5ml	4.63ml	8.4ml
TEMED*	6.7µl	3.3µl	10µl
APS*	67µl	33µl	100µl

* add after degassing.

Table 2.6 – Composition of solutions used in casting low molecular weight gels

2.14.2 Sample preparation

Samples were made up to a total volume of 20µl with sample buffer, heated at 40°C for 30 minutes and loaded into a single well.

2.14.3 Gel running conditions

Gels were placed into GIBCO BRL V15.17 gel tanks containing the respective anode and cathode buffers. The samples were then stacked by running the gel at 30V for 1 hour and then separated by increasing the voltage to 90V for 16 hours.

2.14.4 Protein Staining

Materials:

1) **Fixing solution:** 50% (v/v) methanol, 10% (v/v) glacial acetic acid.
2) **Staining solution:** 0.025% brilliant blue-G in 10% glacial acetic acid. This was stirred to aid solubilisation of the dye for 30 minutes and then undissolved dye was removed by filtration.
3) **Destain solution:** 10% glacial acetic acid.

After electrophoresis, the stacking gel was removed and the gel was stained for protein. Gels were placed in fixing solution for 30 minutes and gently shaken. The proteins were visualised by covering the gel with a staining solution and gently shaking for 2 hours. The gels were then placed in destining solution which was changed every 30 minutes until the background staining was minimal. Gels were either photographed or dried for storage.

2.15 Silver Staining for LPS

The silver staining method is that described by Hitchcock & Brown (1983). All the gel staining procedures were performed at 37°C in a clean glass dish using pre-warmed solutions.

Materials:

1) **Fixing solution:** 25% (v/v) isopropanol, 7% (v/v) glacial acetic acid, freshly prepared
2) **Oxidising solution:** 0.7% (w/v) periodic acid, 2.67% (v/v) fixing solution, freshly prepared
3) **Silver staining solution:** 0.56ml of a 35% (w/v) ammonia solution was slowly added to 18.8ml of 0.1M NaOH with constant stirring. 3.4ml of 20% (w/v) silver nitrate solution was added dropwise to this constantly stirring solution until saturation point was reached. This solution was finally diluted to 100ml with water.
4) **Developing solution:** formalin (37% formaldehyde) was diluted to 0.05% (v/v) with water and citric acid added to a final concentration of 0.05% (w/v).

The gel was placed in fixing solution for 30 minutes and then shaken in oxidising solution for 5 minutes. After washing eight times with distilled water for 4 minutes, the gel was incubated with silver staining solution for 10 minutes. Excess stain was removed with four water washes of 10 minutes and the LPS was visualised by incubation of the gel with developing solution in the dark. When the desired band intensity was reached, the reaction was stopped by incubation with 0.1% (v/v) glacial

acetic acid for 1 hour and the gel was washed with water. A permanent image of the gel was obtained by photography.

2.16 Purification of Protein on Ni-NTA Resin

Purification of $(His)_6$ tagged proteins was performed using Ni-NTA resin (Qiagen). Ni-NTA resin was supplied pre-swollen and charged with Ni^{2+} as a 50% aqueous suspension containing 30% ethanol as a preservative. The resin contains 8 – 12μmol Ni^{2+} ml^{-1} gel and has a binding capacity of ~ 5-10mg ml^{-1} resin for a $(His)_6$ tagged protein.

Materials:

1) **Buffer B:** 8M urea, 0.1M sodium dihydrogen orthophosphate mono-hydrate, 0.01M Tris, pH8.0. Filter sterilised and stored at 4°C.
2) **Buffer C:** 8M urea, 0.1M sodium dihydrogen orthophosphate mono-hydrate, 0.01M Tris, pH6.3. Filter sterilised and stored at 4°C.
3) **Buffer D:** 8M urea, 0.1M sodium dihydrogen orthophosphate mono-hydrate, 0.01M Tris, pH5.9. Filter sterilised and stored at 4°C.
4) **Buffer E:** 8M urea, 0.1M sodium dihydrogen orthophosphate mono-hydrate, 0.01M Tris, pH4.5. Filter sterilised and stored at 4°C.
5) **Buffer F:** 6M guanidium hydrochloride, in 0.2M acetic acid
6) **SDS solution:** 2% SDS
7) **EDTA solution:** 100mM EDTA, pH8.0
8) **Nickel sulphate solution:** 100mM nickel sulphate

2.16.1 Preparation and equilibration of column

The Ni-NTA resin was resuspended in water and the desired volume was transferred to a sealed column and allowed to settle, avoiding air bubbles. Excess liquid was drained off and the column was equilibriated by washing with 5 volumes of UHQ water, followed by 10 volumes of buffer B.

2.16.2 Preparation of bound protein

Pelleted cells were resuspended in buffer B at 0.2g ml^{-1} and lysed by sets of sonication at 10 – 12 microns in 1 minute bursts, followed by resting on ice, until a clear cell lysate was achieved.

The cleared cell lysate was carefully loaded onto the column avoiding disturbance of the packed resin and was drawn through the column by gravity. The bound protein was washed with 5 volumes buffer B and 5 volumes buffer C. The initial flow through and both washes were collected for later analysis.

2.16.3 Elution of bound protein

Elution of the bound protein was achieved using two methods.

2.16.3.1 Elution by pH

Reduction of pH results in protonation of the histidine residues causing dissociation from the Ni-NTA resin. Therefore the protein was eluted from the column with 4 volumes buffer D (pH5.9) and 4 volumes buffer E (pH4.5) and fractions were collected.

2.16.3.2 Elution by imidazole

The molecule imidazole is structurally similar to histidine and so can compete with histidine for binding on the Ni-NTA resin and at high concentrations is able to displace the bound protein. Following the two wash stages, elution was achieved by the addition of 4 volumes buffer C containing 250mM imidazole and fractions collected.

2.16.4 Recharging the Ni-NTA resin

The re-use of the Ni-NTA resin was recommended only for purification of identical recombinant proteins and a maximum 3-5 runs per column can be performed.

Regeneration of the column was as follows:

1) Wash the column with 2 volumes buffer F
2) Wash the column with 2 volumes water
3) Wash the column with 3 volumes SDS solution
4) Wash the column with 1 volume 25% ethanol
5) Wash the column with 1 volume 50% ethanol
6) Wash the column with 1 volume 75% ethanol
7) Wash the column with 5 volumes 100% ethanol
8) Wash the column with 1 volume 75% ethanol
9) Wash the column with 1 volume 50% ethanol
10) Wash the column with 1 volume 25% ethanol
11) Wash the column with 1 volume water
12) Wash the column with 5 volumes EDTA solution
13) Wash the column with 2 volumes water
14) Recharge the column with 2 volumes nickel sulphate solution
15) Wash the column with 2 volumes water
16) Wash the column with 2 volumes buffer F
17) Equilibrate column

The ethanol gradient steps are designed to prevent precipitation of the protein on the Ni-NTA column and are essential.

2.17 Protein precipitation

The eluted protein was solubilised in an 8M urea buffer and in order to obtain pure protein pellets, the eluted protein fractions were precipitated with 4 volumes of ethanol to give a final concentration of 80% ethanol. These were incubated overnight at –20°C for 16 hours. The precipitated protein was harvested by centrifugation at 12,000g for 15 minutes. Ethanol will also precipitate out salts and urea, but these were removed by the additional step of washing the pellet in UHQ water, followed by centrifugation at 12,000g for 10 minutes. The resulting protein pellet was stored at –20°C. Immediately

prior to use, to ensure the pellet was absolutely pure, the pellet was washed with 70% ethanol and centrifuged at 1,000g for 10 minutes. All traces of ethanol were removed by washing the pellet with UHQ water.

2.18 Enzyme linked immunoabsorbent assay (ELISA)

Materials:

1) **Coating buffer:** 0.1M sodium hydrogen carbonate, 0.05M sodium carbonate pH9.6
2) **Wash:** 0.15M sodium chloride, 0.05% (v/v) Tween 20.
3) **Antibody diluent:** 50mM Tris, 0.15M sodium chloride, 0.05% (v/v) Tween 20, containing 1% (w/v) BSA, adjusted to pH 7.4 with acetic acid.
4) **Colour substrate:** immediately before use 3.6mg 3,3,5,5-tetramethyl benzadine was dissolved in 1ml dimethyl sulphoxide and added to 100ml of 0.1M sodium acetate (adjusted to pH 6.0, with 1M citric acid) and to this 10µl of hydrogen peroxide added.

The wells of a 96-well, flat bottomed microtitre plate (Sterilin) were coated with the desired antigen by diluting the antigen to 1µg ml^{-1} in coating buffer and incubating 100µl of this solution in each well. The plate was covered and incubated in a moist chamber at 37°C for 16 hours. The plate was washed three times with ELISA wash, with care being taken to ensure that no residual wash solution remained in the wells. To prevent non-specific binding, the plate was blocked with 100µl diluent solution per well and incubated at 37°C for 1 hour. The diluent was removed and a four–fold dilution series in ELISA diluent was made in the wells with the serum under investigation, so that each well contained a final volume of 100µl. The plate was again incubated at 37°C for 1 hour. The plate was washed three times with ELISA wash and then incubated with 100µl per well of the appropriate anti-species Ig horseradish peroxidase conjugated antibody (Zymed) diluted 1:2000 in diluent. After incubation for 1 hour at 37°C the wells were washed five times in ELISA wash. To develop the colour reaction, 100µl of the colour substrate was added to each well and light was excluded from the plate. The reaction was left to proceed for 10 minutes before being stopped by the addition of 50µl

1M sulphuric acid. The A_{450} of each well was measured on a plate reader (Anthos, HTLL). The absorbencies were corrected to 1 hour reaction time and the titre was taken as the final dilution that would have produced an increase in A_{450} of 0.1 in one hour.

In order to determine the antibody subclasses present in sera, isotyping ELISA's were performed using anti-species Ig subclass conjugates.

2.19 Immunofluorescence

This assay was used to test whether antibodies were able to recognise whole meningococci. The desired strain of meningococci was grown overnight and harvested into a 1ml PBS solution and then further diluted 1/100 in PBS. The bacterial suspension (30µl) was added to a pre-cleaned microscope slide and left to air dry. The meningococci were fixed by the addition of 70% ethanol for 15 minutes and then allowed to air dry. To prevent non-specific binding the fixed meningococci were incubated with 50µl of a 1% BSA solution diluted in PBS and incubated at room temperature for 30min. Excess solution was tapped off and 50µl of the serum dilution being tested was added to the slide, which was incubated for 1 hour at room temperature with gentle shaking. The slide was washed three times in PBS before the addition of an anti-species Ig fluorescein isothiocyanate (FITC) conjugate (Dako) diluted 1 in 100 in PBS. The slide was incubated at room temperature for 1 hour in the dark with gentle shaking then washed five times in PBS before counter staining with propidium iodide diluted 1 in 400 in PBS. The slide was incubated for 5 min in the dark at room temperature with gentle shaking before washing five times in PBS and allowing to air dry. Coverslips were added to the slides using an anti-fade mountant (Dako). The slides were viewed either under a fluorescent or a confocal microscope.

2.20 Bactericidal assays

Materials:

PBSB: 0.05% (v/v) Dulbecco B salts in PBS

PBSB/FCS: 2% foetal calf serum in PBSB

The desired strain of meningococci was grown overnight for single colony growth and all areas of the plate except the initial inoculation region scraped into 2ml of PBSB. Cellular aggregates were sedimented by centrifugation at 450g for 1min. A 100µl fraction of the supernatant was added to 0.1M sodium hydroxide containing 1% (w/v) SDS and the A_{260} of the lysed cells measured. The volume of the bacterial suspension required to give approximately 200-300 cfu per plate was calculated as $y\mu l = 200/A_{260}$. This volume was transferred to 5ml PBSB, mixed and then 30µl were transferred to 5ml PBSB/FCS. Using a 96 well, flat bottomed plate, 25µl of the bacterial suspension was added to each well. Serial dilutions of the sera under investigation were made in PBSB and 10µl of each dilution added to a well. The sera and foetal calf serum had been previously heat treated at 56°C for 30 min to inactivate complement. Each antibody dilution was set up in duplicate and to one well 5µl of freshly thawed baby rabbit complement was added and to the other 5µl of heat inactivated baby rabbit complement was added. Each well was made up to a total volume of 100µl with PBSB with a final antibody dilution of 1 in 8 to 1 in 1024. The plate was briefly agitated and 15µl was removed from each well containing the inactivated complement and plated out on protease peptone agar plates. The microtitre plate was incubated at 37°C for 30 min before each well containing active complement was sampled three times. Sampling consisted of removing 15µl and plating out on protease peptone plates. The plates were incubated overnight and the number of surviving colonies were counted. A positive control antibody was included which promoted high level bactericidal activity against the meningococci strain being tested, whilst the negative control used PBSB instead of antibody.

The bactericidal activities of the various dilutions of antisera were expressed as the percentage increase in killing observed between the pre and post – immune serum.

$$\% \text{ killed} = 100 - \left(\frac{\text{(mean no. of CFU in the wells containing antisera and complement)}}{\text{(mean no of CFU in the wells containing no antisera and inactive complement)}} \right) \times 100$$

2.21 Preparation of Outer Membrane Vesicles

Outer membrane vesicles were prepared from whole meningococci for use in ELISA's as the antigen. Two protease peptone plates were inoculated for semi-confluent growth and incubated overnight. The following day, after checking for contamination, the overnight plates were used to inoculate a further 12 plates which were again incubated overnight. The bacterial growth from these twelve plates was scraped into 4ml protease peptone broth. Five drops of this bacterial suspension were used to inoculate one 15cm diameter, protease peptone plate for confluent growth. In total 40 large plates were prepared in this manner and incubated overnight.

Bacteria from the 40 large plates were scraped into 40ml 0.2M lithium acetate, pH5.8 in a screw capped glass bottle. Approximately 15ml of glass beads and a magnetic follower were added to the bottle which was then sealed in a bag containing neat hycolin in case of spillage. The bottle was clamped in position in a water bath at 45°C and vigorously stirred for 2 hours. The glass beads and bacterial suspension were transferred to plastic bottles and subjected to centrifugation at 8000g, for 10 min, at 4°C in a Beckman Ultracentrifuge, using rotor 55.2. The resulting pellet contained viable bacteria and the glass beads, whilst the outer membrane vesicles remained in the supernatant solution, which was re-centrifuged as before to remove any remaining bacteria. The supernatant solution containing the outer membrane vesicles was then centrifuged at 112,000g for 2 hours at 4°C. The outer membrane vesicles were present as a clearish pellet with an orange tinge due to the presence of cytochromes. The pellet was resuspended in ~1.5ml UHQ water where they had an opalescent appearance. A BCA assay was performed to determine the protein concentration. Typically the protein concentration was found to be 5–10mg ml^{-1}.

2.22 Electron Microscopy

All of the transmission electron microscopy was undertaken on a Hitachi S-800 transmission electron microscope.

2.22.1 Negative staining

Carbon sprayed, formvar coated copper grids were used as the support matrix for the samples. A copper grid was clamped between a pair of reverse action tweezers and 2µl of liposome solution allowed to adsorb to the surface of the grid for 1 min. Excess solution was carefully blotted off with filter paper and 5µl of 1.5 % phosphotungstic acid, pH7.2 allowed to adsorb to the grid for 10sec. Excess solution was removed with filter paper and the grid was allowed to air dry before viewing.

2.22.2 Immunogold labelling of liposomes

Materials:

1) **PBSA:** 0.1% BSA in PBS

2) **PBSAG:** 1% glycine in PBSA buffer

A sample of liposome solution (5µl) was placed on the surface of a carbon sprayed formvar coated copper grid for 5 min and excess blotted off with filter paper. The grid was washed by placing a drop of PBSA (5µl) onto a square of Parafilm (America Can Company) and inverting the grid on the surface of the drop for 5min. Any non-specific binding was similarly blocked by inverting the grid on the surface of a drop of PBSAG (5µl) for 5 min. The grid was re-washed in PBSA and inverted on a drop of antibody (5µl) diluted 1 in 10 in PBSA and left at room temperature for 1 hour in a moist chamber. The grid was washed three times with PBSA before inverting on a drop of PBSA containing a 1 in 50 dilution of gold-labelled goat anti-mouse IgM and IgG conjugate (15nM gold, Biocell). The grids were incubated at room temperature for 1 hour in a moist chamber before blotting off excess solution with filter paper. The grids were washed three times in PBS and then fixed with 2.5% glutaraldehyde in PBS by inverting on a droplet for 30 sec. The grids were washed three times and then negatively stained as above before viewing.

CHAPTER 3: SEQUENCE VARIATION IN OUTER MEMBRANE PROTEINS IN *N. MENINGITIDIS* FROM PATIENTS AND CLOSE HOUSEHOLD CONTACTS

3.1 Introduction

Generally, traditional epidemiological studies of meningococcal infection have largely relied upon detecting differences in surface antigen expression between strains by serological methods. The availability of monoclonal antibodies against the class 1 and class 2/3 outer membrane proteins that are responsible for the serosubtype and serotype respectively has greatly aided the analysis of these two variables (Abdillahi & Poolman, 1988), however the information obtained is restricted. Sequencing studies have identified base changes, generating amino acid substitutions within subtype epitopes, that may or may not modify recognition by the relevant antibody (McGuinness *et al*, 1991; Suker *et al*, 1994; Brooks *et al*, 1995). In these studies it has been the sequence of the class 1 outer membrane protein that has been analysed. In this chapter, the sequence of the class 2/3 outer membrane protein has been analysed to see if additional information could be obtained since any 'silent' microheterogeneity represents a potential hidden source of epidemiological information.

3.2 Sequencing of an outbreak of meningococcal disease

During a recent outbreak of meningococcal disease in Glasgow in 1991-1992, cultures of meningococci were obtained from blood or cerebrospinal fluid of ten index cases with meningococci infection and from the nasopharynx of close contacts living in the same household. All meningococci were forwarded from the Scottish Meningococcus Pneumococcus Reference Laboratory, where they had been identified, serogrouped and serotyped by whole cell ELISA (table 3.1). In a previous study by Brooks *et al*, (1995) the VR1, VR2 and SV1 regions of the *porA* gene of these isolates had been sequenced. Base changes were seen in all three regions, resulting in altered amino acid sequence and so identifying new variants within an already designated subtype. In this study the

entire genes for the outer membrane proteins PorB and Opc were sequenced in addition to see if further microheterogeneity could be seen.

Family Group	Case/Carrier relationship (age)	Serological profile	Lab No. assigned
1	Case (4yr)	C:4,21:P1.15	MC139
1	Sister (6yr)	C:4,21:P1.15	MC140
2	Case (2yr)	B:4:P1.15	MC130
2	Mother (28yr)	B:4:P1.15	MC131
2	Father (28yr)	B:4:P1.15	MC132
3	Case (17yr)	B:NT:P1.15	MC121
3	Brother (n.a.)	B:NT:P1.15	MC122
4	Case (1yr)	C:2a:P1.2	MC126
4	Father (19yr)	C:2a:P1.2	MC125
5	Case (16yr)	C:2b:P1.2	MC133
5	Brother (13yr)	C:2b:P1.2	MC134
6	Case (2m)	B:2b:P1.2	MC135
6	Father (24yr)	B:2b:P1.2	MC136
7	Case (62yr)	Y:14:P1.2	MC120
7	Son (n.a.)	Y:14:P1.2	MC119
8	Case (1yr)	B:2b:P1.10	MC117
8	Brother (3yr)	B:2b:P1.10	MC118
9	Case (5yr)	B:2b:P1.10	MC128
9	Sister(7yr)	B:2b:P1.10	MC129
9	Mother (38yr)	B:2b:P1.10	MC127
10	Case (2m)	B:2b:P1.10	MC137
10	Grandfather (n.a.)	B:2b:P1.10	MC163

Table 3.1 – Serological profile of meningococcal isolates from an outbreak of meningococcal disease in Glasgow in 1991/2

n.a. – not available

Primer name		Sequence	Annealing temperature used in sequencing reactions	Source
		porB - Class 2		
PB2f	5'	TAT GAA AAG GAA TAC AGC	40°C	this study
PB2b	5'	TCA GAT TAG AAT TTG TGA	40°C	this study
B1f	5'	CGG TAC AAT TAA AGC AGG	45°C	this study
B6b	5'	CGT AGC GTA CGG AGA TTT	45°C	this study
B6f	5'	GTG AAA TCT CCG TAC GCT	45°C	this study
B11b	5'	ATA CTG ACC GGC AAC AGA	45°C	this study
B11f	5'	CTG TAC GTT TCT GTT GCC	40°C	this study
B16b	5'	TTG TGA CGC AGA CCA AC	45°C	this study
		porB - Class 3		
B21	5'	CCA AAA AAG GAA TAC AGC		Dr.M.J.Ward
CW1	5'	GCA GAT TAG AAT TTG TGG		Dr.M.J.Ward
3B1f	5'	GTA CGG CAC CAT CAA AGC	50°C	this study
3B6b	5'	GAG AAT CGT AGC GTA CGG	50°C	this study
3B6f	5'	CGC CTC ATT TCC GTA CGC	50°C	this study
3B11b	5'	TGT ACG GCT ACG GAA GCG	50°C	this study
3B11fa	5'	CTT CCG TAG CCG TAC AGC	50°C	this study
3B16b	5'	TTT GTG ACG CAG ACC GAC	50°C	this study
		opc		
opc-1	5'	GCA CAA GAG CTT CAA ACC	50°C	Dr.J.L. Brooks
opc-2	5'	TTA TGC CGA CGC GCA AG	50°C	Dr.J.L. Brooks
opc-3	5'	ATT TCC GTG TGG GTG CCG	50°C	Dr.J.L. Brooks
opc-4	5'	CGG CAC CCA CAG GGA AAT	50°C	Dr.J.L. Brooks
opc-5	5'	CCC ACC GGC GTT CCT GAA	50°C	Dr.J.L. Brooks
opc-6	5'	CAA AGC CCC AGC CGT TG		Dr.J.L. Brooks

Table 3.2 – Sequence of primers used in the amplification and sequencing of the *porB* alleles and the *opc* gene

The *porB* gene has two alleles that code for the class 2 and class 3 outer membrane proteins that are mutually exclusively expressed (Tsai *et al*, 1981). The topological model for both the class 2 and class 3 proteins suggests 16 conserved transmembrane regions and eight surface exposed loops (van der Ley *et al*, 1991; Bash *et al*, 1995). Two sets of primers, each consisting of a forward and reverse primer for each allele of the gene were used to amplify the whole gene by PCR. As the class 2 gene is ~ 1200 bp and the class 3 gene ~990 bp a further six primers were designed within the conserved regions of each gene to enable sequencing of both of the DNA strands. Sequencing of the *opc* gene utilised two external primers and a set of four internal primers corresponding to conserved regions of the gene to enable sequencing of both strands. The sequence of the primers and the annealing temperatures used are listed in table 3.2, the exact binding positions of each primer to the gene are shown in Appendix 1.

DNA was extracted as described (Section 2.7.1) and the genes were amplified by PCR using the proof-reading polymerase Bio-X-act (Bioline). The PCR products were purified using the GeneClean II™ kit (section 2.4.2). The annealing temperatures used in the PCR amplification procedure and the expected product sizes are listed in table 3.3.

Target gene	Forwards primer	Reverse primer	Annealing temperature for PCR	Expected product size
Class 2, *porB*	PB2f	PB2b	45°C	1106 bp
Class 3, *porB*	B21	CW1	49°C	1012 bp
opc	Opc-1	Opc-2	54°C	713 bp

Table 3.3 – Annealing temperatures and expected product sizes in the PCR amplification of the *porB* alleles and the *opc* gene.

Sequencing reactions were performed as described (section 2.8.2), using 50ng template DNA and 25ng primer. The DNA of the *porB* and *opc* gene and the complimentary strand from all 22 isolates were sequenced and the translated amino acid sequence

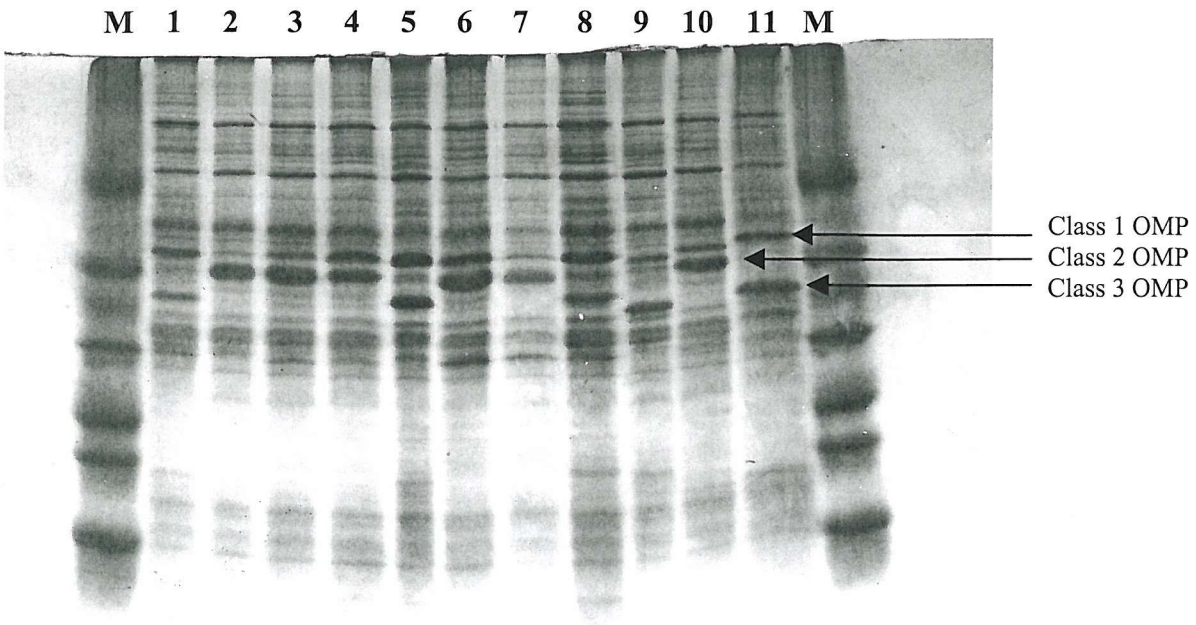
determined. These results were then aligned and dendrograms constructed using either the Pileup program from the GCG suite of programs or ClustalX from NCBI and DrawTree from Phylip. Pileup allows comparison of the homologies of the sequences to be compared, but the length of the branches is not proportional to the relationships between the sequences. ClustalX uses a pairwise alignment algorithm to align the sequences. This was then used to construct a non-rooted tree by the Neighbour Joining method with bootstrapping.

3.2.1 Results of sequencing of the porB gene

The *porB* gene exists as two alleles, coding for the class 2 or 3 outer membrane proteins. Analysis by SDS-PAGE showed that of the 22 isolates obtained, 13 were class 2 strains and 9 were class 3 strains. (figure 3.1). Serological typing of the class 2 strains showed just two recognised serotypes to be present; 2a and 2b. Comparison of the translated sequences of the *porB* gene was performed using the Pileup program from the GCG suite of programs (figure 3.2) and showed that in a single family grouping the PorB sequences were indistinguishable between case and contacts. However between the five family groupings which were typed as 2b, there were three distinct variants of the 2b type present. Family groups 6, 8 and 9 possessed identical type 2b sequences, whilst family groups 5 and 10 had sequences that each differed by only a few amino acids. Family 10 had one amino acid change in transmembrane region 15. Family group 5 possessed 5 amino acid differences in comparison with family groups 6, 8 and 9. One amino acid change was present in transmembrane region 5 and there were four amino acid substitutions at the apex of the surface exposed loop 4.

Each family grouping possessing a class 3 allele was identified as a different serotype by serological methods, except for family group 3, which was designated as non-typable. Sequencing revealed that for family groups 1, 2 and 7 the case and carrier isolates within each group could not be distinguished on the basis of the PorB sequence (figure 3.3). Family group 3, proved to be very interesting. The two isolates from this group could not be serologically typed, but *porB* sequencing showed the two isolates to

Figure 3.1 – SDS-PAGE analysis of the strains obtained from an outbreak on meningococcal disease in Glasgow in 1991/2.



Whole cell lysates were prepared from case isolates from an outbreak of meningococcal disease and subjected to SDS-PAGE. The proteins were visualised with PAGE blue-83.

The loading order is as follows:

M - Molecular weight standards
1 - H44/76
2 - MC117
3 - MC119
4 - MC121
5 - MC125
6 - MC127
7 - MC130
8 - MC133
9 - MC135
10 - MC137
11 - MC139
M - Molecular weight standards

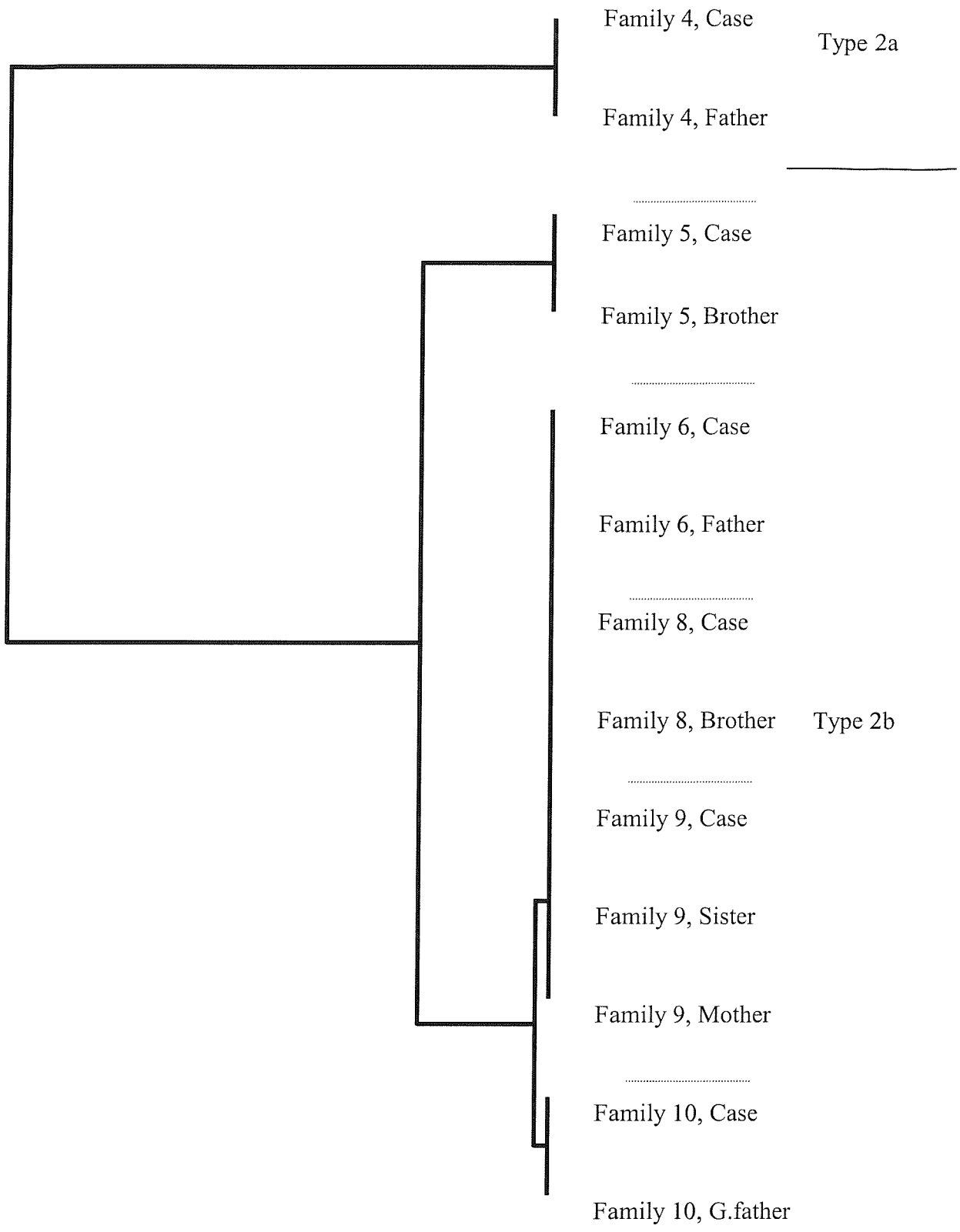


Figure 3.2 – Comparison of the inferred amino acid sequence of isolates from an outbreak of meningococcal disease expressing the class2 PorB protein

The sequences were aligned using the Pileup program from the GCG suite of programs. The branch length does not indicate the relationship between sequences

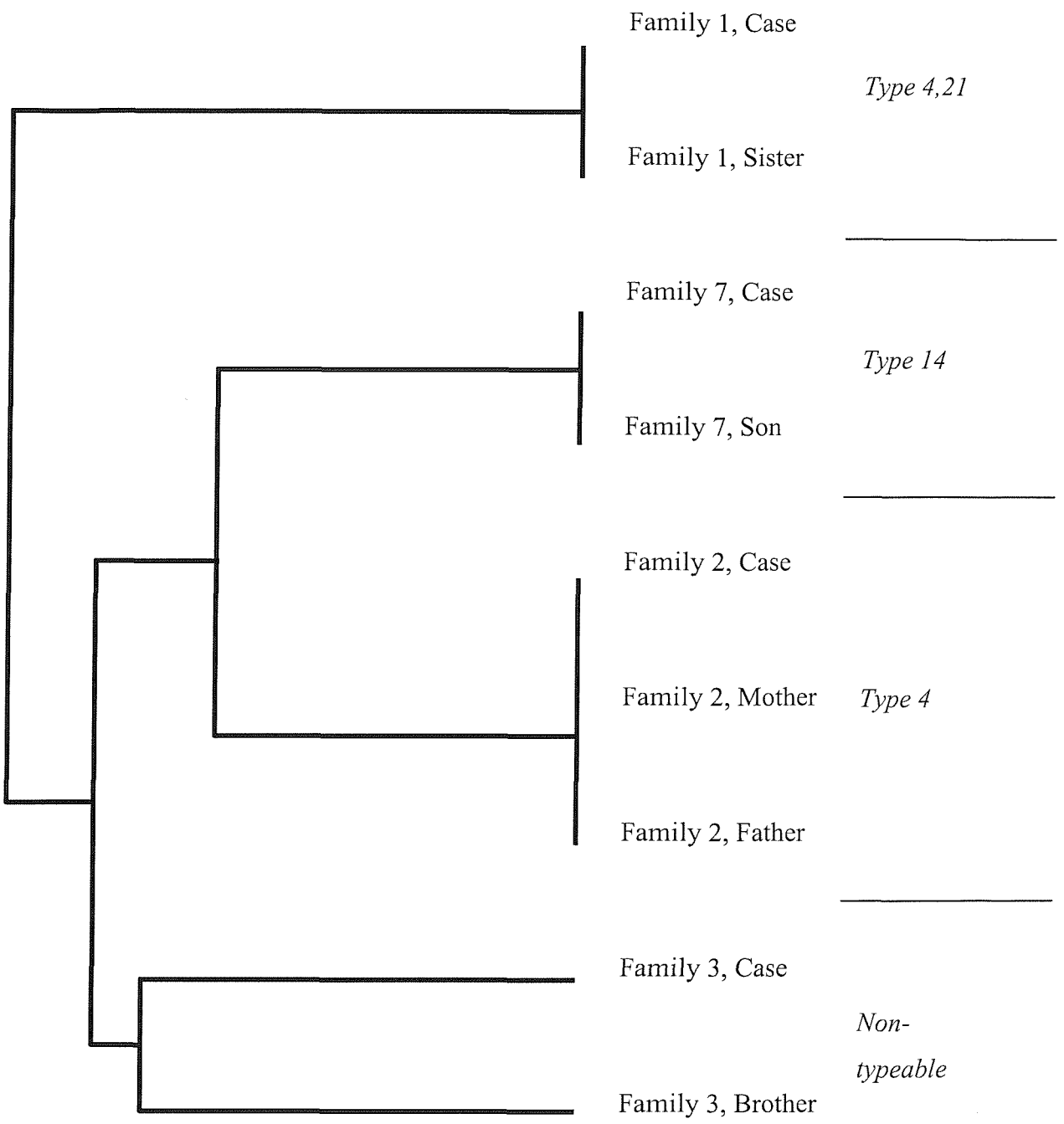


Figure 3.3 - Comparison of the inferred amino acid sequence of isolates from an outbreak of meningococcal disease expressing the class3 PorB protein

The sequences were aligned using the Pileup program from the GCG suite of programs. The branch length does not indicate the relationship between sequences

vary enormously, with in total 13 amino acid differences between the two isolates. Two single amino acid substitutes were present in transmembrane regions 7 and 11 whilst most variation was seen in loops 5 and 6 and resulted in substitutions and 3 insertions.

Sequencing of the *porB* gene and comparisons of the translated amino acid sequences differentiated between three variants of serotype 2b which were indistinguishable by serological methods. Monoclonal antibodies are unable to identify a significant number of meningococci and as a result these are classed as non-typable. Sequencing of the *porB* gene demonstrated that within one family group, two isolates that had been classed as non-typable, whilst both were expressing class 3 proteins, the actual amino acid sequences were different and hence that the case and carrier were not epidemiologically linked.

3.2.1.1 Comparison of PorB *sequencing with representatives types from the sequence databases*

The *porB* gene has been sequenced in many laboratories world-wide and consequently over 150 sequences are available through Genbank. Thirty different types have been assigned to these sequences, although some of these type differences are not seen at the predicted amino acid level. Representatives of all these different types were obtained and the predicted amino acid residues aligned against the results of the Glasgow outbreak sequencing using the pairwise alignment of the ClustalX program from NCBI. The strains used are listed in Appendix 2. For ease of clarity, only the case sequence from each group has been included on the resulting dendrograms except for family group 3, where the PorB sequences diverged. The unrooted trees were drawn using the Neighbour Joining method with Bootstrapping using the DrawTree program from Phylip.

Far fewer sequences are available for class 2 strains and indeed there have only been 7 serotypes assigned to class 2 strains. The Glasgow outbreak isolates were all either type 2a or 2b and as can be seen on the dendrogram (figure 3.4), this is where they fit when

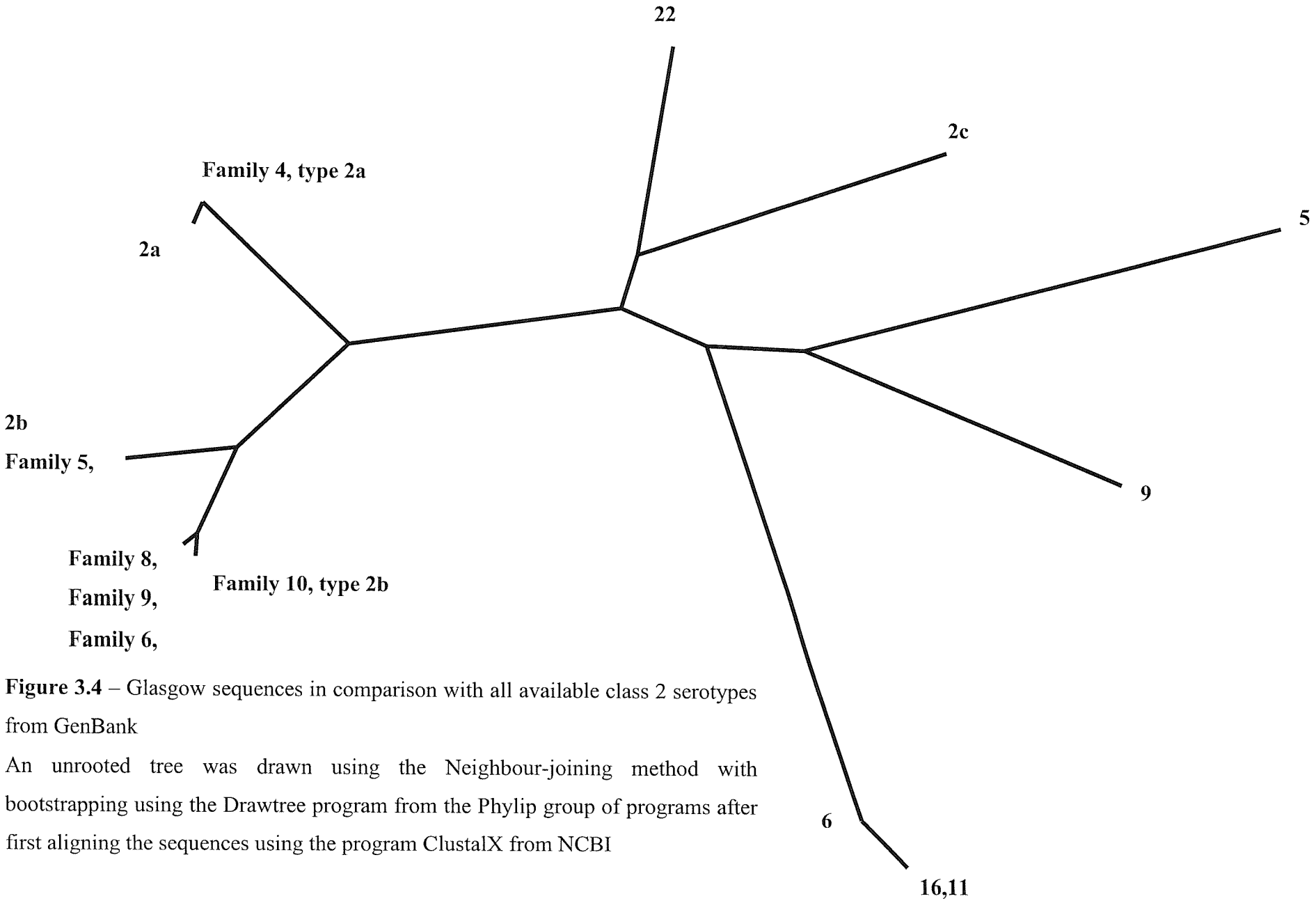


Figure 3.4 – Glasgow sequences in comparison with all available class 2 serotypes from GenBank

An unrooted tree was drawn using the Neighbour-joining method with bootstrapping using the Drawtree program from the Phylip group of programs after first aligning the sequences using the program ClustalX from NCBI

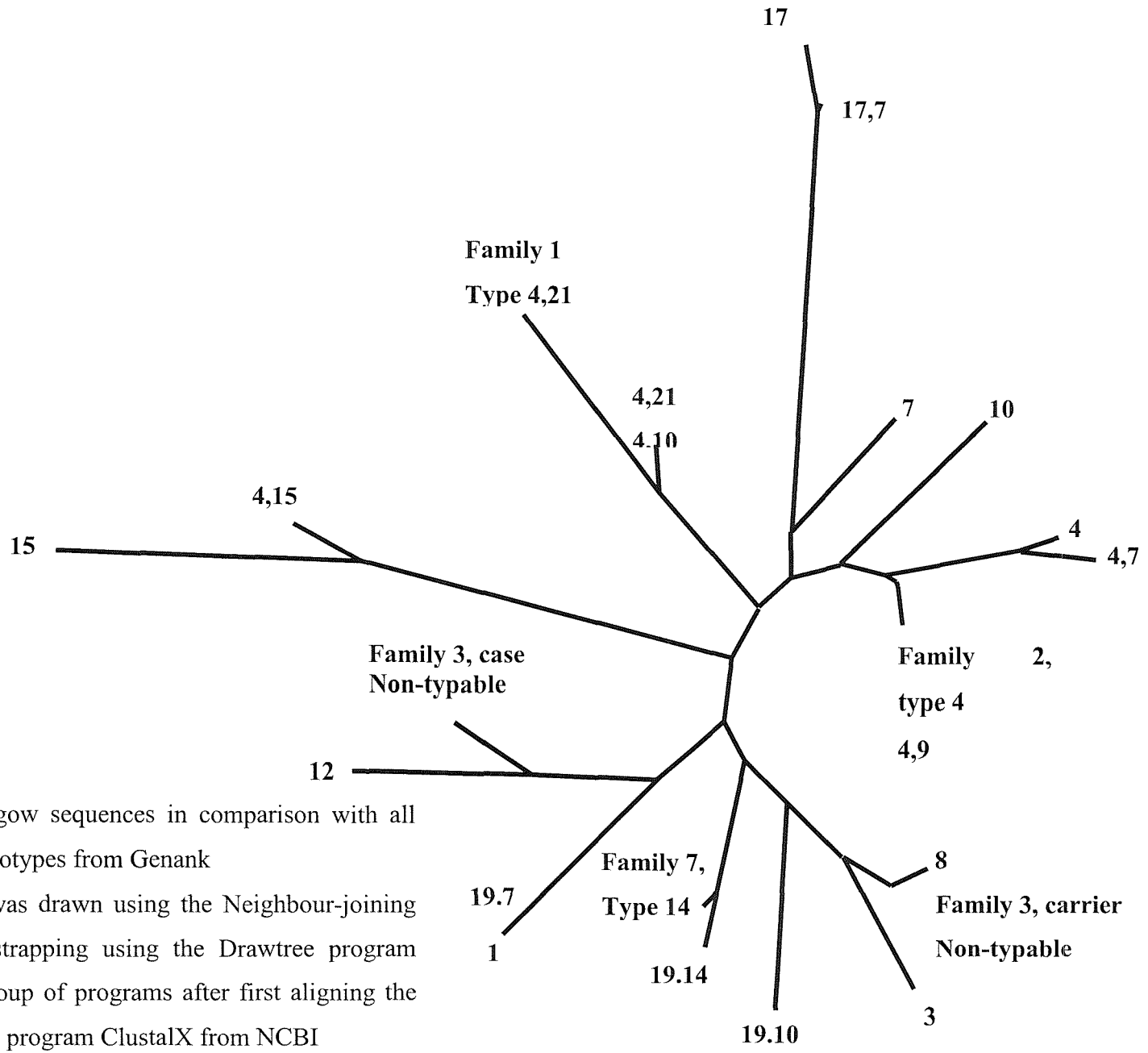


Figure 3.5 – Glasgow sequences in comparison with all available class 3 serotypes from Genank

An unrooted tree was drawn using the Neighbour-joining method with bootstrapping using the Drawtree program from the Phylip group of programs after first aligning the sequences using the program ClustalX from NCBI

compared to the other class 2 strains. The five family groups serologically typed as 2b, but identified by sequence analysis as belonging to three distinct variants are responsible for branching as the end of the type 2b spur, although family 5 has an identical sequence to a type 2b sequence in the database.

There are 15 different class 3 serotypes assigned. Those isolates from Glasgow defined as specific types by serological methods, all fit in at the appropriate places when compared with the representative class 3 serotypes on the dendrogram (figure 3.5). Using serological methods alone, isolates from family group 3 were unable to be assigned a serotype. Comparison of the sequences obtained however, identifies the carrier isolate to be a type 8 strain possessing an identical sequence to a type 8 strain present in the database. The case isolate is more like a type 12 strain.

3.2.2 Results of sequencing of the opc gene

In order to determine whether further discrimination between these strains was possible sequencing was also performed on the *opc* gene, which codes for the Opc outer membrane protein. The *opc* gene is possessed by approximately 60% of all meningococci and within this group, varying levels of expression are seen (Olyhoek *et al*, 1991). The *opc* sequences reported show this protein to be more conserved with limited variability in comparison to the highly variable PorA and PorB proteins (Seiler *et al*, 1996). Of the isolates from the Glasgow outbreak, nine isolates were identified as possessing the *opc* gene by PCR using specifically designed primers, all belonging to four family groupings. Dr. JL Brooks and Dr. K Jolley aided sequencing of these nine isolates.

Isolates from family groups 1, 2 and 7 could not be distinguished within families on the basis of the predicted amino acid sequences of the *opc* gene (figure 3.6). Similar sequences had been expected but four variants of the *opc* gene were clearly distinguishable, with differences present at 7 different amino acid sites. The Opc protein has been predicted to consist of 10 transmembrane regions and 5 surface exposed loops

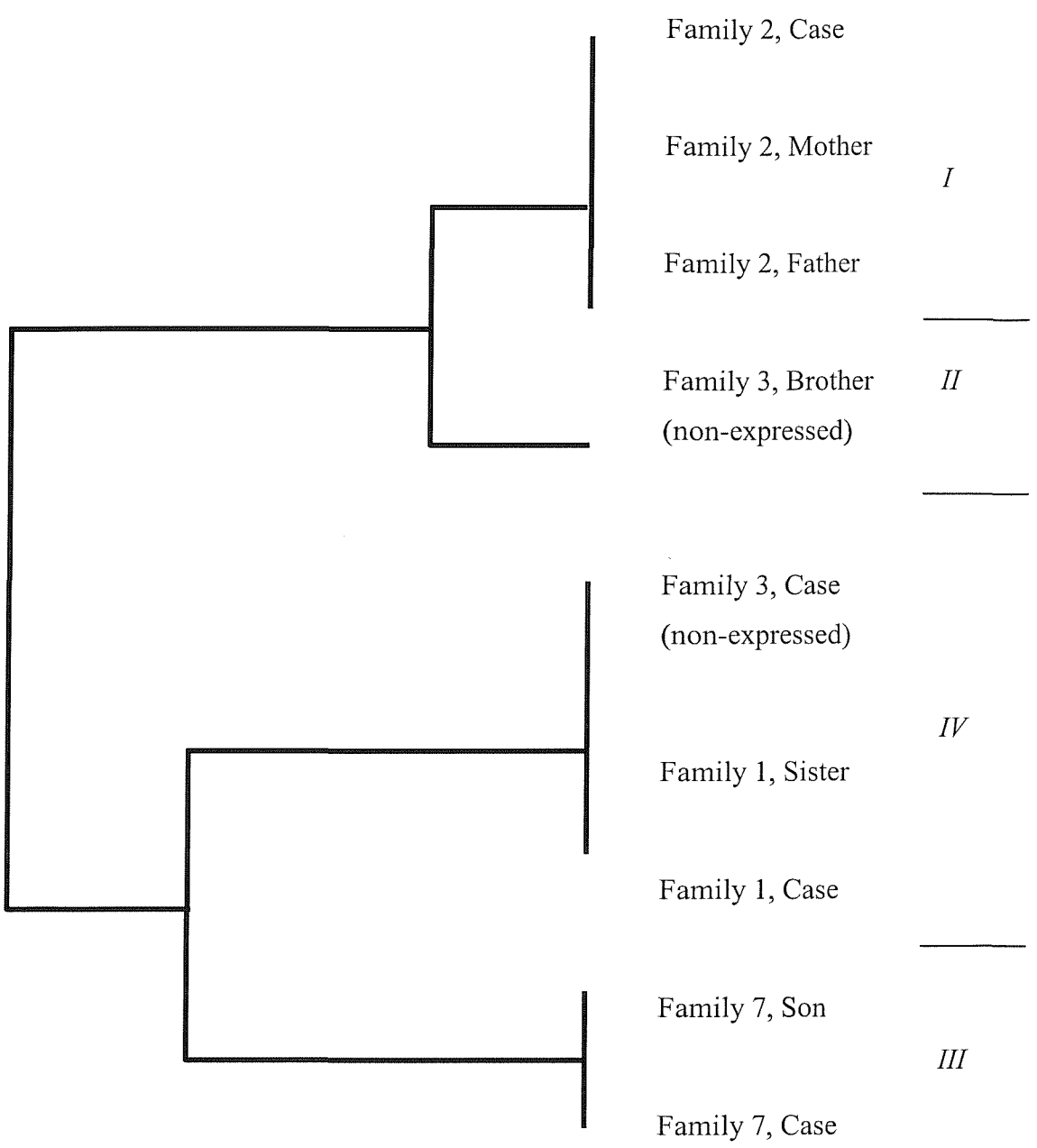


Figure 3.6 - Alignment of isolates from an outbreak of meningococcal disease possessing the *opc* gene

The sequences were aligned using the Pileup program from the GCG suite of programs. The branch length does not indicate the relationship between sequences

(Merker *et al*, 1997b). Three of the amino acid site differences occurred within a transmembrane region, on the internal surface or at the base of a loop and so would not be expected to result in altered epitopes. The remaining four amino acid differences were near the apexes of loops 4 and 5. Sequencing of family group 3, enabled the case and carrier isolates to be easily separated, with four amino acid differences present between the two isolates. The case isolate from family group 3, when aligned with other isolates was seen to posses an identical *opc* gene to that of family group 1, whereas the carrier isolate formed a new variant of the *opc* gene, although similar to that of family group 2.

Western blotting had been previously performed on cell lysates of all 22 isolates in this study by Dr JL Brooks. The monoclonal antibody B306, an anti-Opc antibody was used to identify isolates expressing the Opc protein. A positive result was obtained for all members of family groups 1, 2 and 7 as expected, but not family group 3. Expression of the *opc* gene is phase variable and is controlled at a transcriptional level by an upstream poly-cytosine tract, bringing the mRNA in and out of frame for translation (Sarkari *et al*, 1994).

3.2.3 Conclusions of sequencing results

When information from sequencing of the *porA*, *porB* and *opc* gene is compared (table 3.4), then it can be seen that sequencing of the different genes allows identification of differences between case and carrier isolates for three family groupings.

Sequencing is a much more precise method than using monoclonal antibodies for identifying different variants present. Sequencing of the *porA* gene showed that within the subtypes assigned by monoclonal antibodies variation was present amongst strains serosubtyped as P1.2 or P1.10 (Brooks *et al*, 1995). Sequencing of this gene also showed differences between the case isolates and the carrier isolates of two groups, families 6 and 9 to be present.

Family Group	Case/carrier relationship (age)	Serological Profile	*porA* VR1	*porA* VR2	*porB*	*opc*
1	Case (4yr)	C:4,21:P1.15	19	15b	4,21	IV
1	Sister (6yr)	C:4,21:P1.15	19	15b	4,21	IV
2	Case (2yr)	B:4:P1.15	19	15b	4	I
2	Mother (28yr)	B:4:P1.15	19	15b	4	I
2	Father (28yr)	B:4:P1.15	19	15b	4	I
3	Case (17yr)	B:NT:P1.15	19	15b	NT 1	IV
3	Brother (n.a.)	B:NT:P1.15	19	15b	NT 2	II
4	Case (1yr)	C:2a:P1.2	29	2d	2a	
4	Father (19yr)	C:2a:P1.2	29	2d	2a	
5	Case (16yr)	C:2b:P1.2	29	2d	2b i	
5	Brother (13yr)	C:2b:P1.2	29	2d	2b i	
6	Case (2m)	B:2b:P1.2	29	2	2b ii	
6	Father (24yr)	B:2b:P1.2	5	2	2b ii	
7	Case (62yr)	Y:14:P1.2	19c	2c	14	III
7	Son (n.a.)	Y:14:P1.2	19c	2c	14	III
8	Case (1yr)	B:2b:P1.10	5b	10h	2b ii	
8	Brother (3yr)	B:2b:P1.10	5b	10h	2b ii	
9	Case (5yr)	B:2b:P1.10	29	10i	2b ii	
9	Sister (7yr)	B:2b:P1.10	29	10i	2b ii	
9	Mother (38yr)	B:2b:P1.10	5b	10	2b ii	
10	Case (2m)	B:2b:P1.10	5b	10j	2b iii	
10	Grandfather (n.a.)	B:2b:P1.10	5b	10j	2b iii	

Table 3.4 – Compilation of results from sequencing the *porA, porB* and *opc* genes.

Isolates within family group 3 were unable to be assigned a serotype by conventional serological methods. Analysis by SDS-PAGE showed these two isolates to be expressing the class 3 protein. Sequencing of the isolates showed there to be 13 differences in the predicted amino acid sequence. Comparison with sequences in the databases showed that the carrier isolate was in fact a type 8 strain, whereas the case was similar to a type 12 strain. Current monoclonal antibodies identified 11 isolates as all being serotype 2b. Sequencing of the *porB* gene enabled further differentiation between these isolates into three variants of type 2b.

Western blotting of the isolates for expression of the outer membrane protein Opc identified seven isolates. Expression of the *opc* gene is phase variable and by using PCR to amplify the *opc* gene a further two isolates; both members of family group 3, were identified as possessing the gene. Sequencing of this gene in family group 3, revealed four differences in the predicted amino acid sequences between the case and the carrier isolate. Sequencing of the *opc* gene from the other strains identified four gene variants.

Sequencing of the *porB* and the *opc* genes, whilst being less discriminating than the sequencing of the *porA* gene did allow identification of non-expressing isolates and identification of gene variants within previously assigned groupings. In the case of family group 3, the *porB* and *opc* sequencing allowed clear identification of the case and carrier isolates that was not possible by *porA* sequencing alone.

3.3 Distribution of genes

On completion of sequencing the isolates from the Glasgow outbreak, an interesting observation was noted; all the class 3 expressing isolates possessed the *opc* gene, although not all expressed the protein, whilst none of the class 2 expressing isolates did. From this small collection of strains, it appeared that expression of the class 3 protein might be linked to possession of the *opc* gene.

Another meningococcal surface protein subject to allelic variation is pilin. The pilus is composed of repeated subunits of the polypeptide pilin. In the meningococcus, two types of pilin are found, termed class I and class II coded for by two alleles of the *pilE* gene. The class I pilin has been shown to be have a very similar structure to the gonococcal pilin subunit and is able to react with the monoclonal antibody SM1 raised against the gonococcal pilin (Diaz *et al*, 1984). A minority of meningococci possess the class II pilin, which is smaller in size (15-17kDa) than the class I pilin (17-22kDa). The class II pilin is unable to react with the SM1 antibody. As expression of the class of pilin is mutually exclusive then there is the possibility that this too could be linked to expression of the class 2 and class 3 outer membrane proteins.

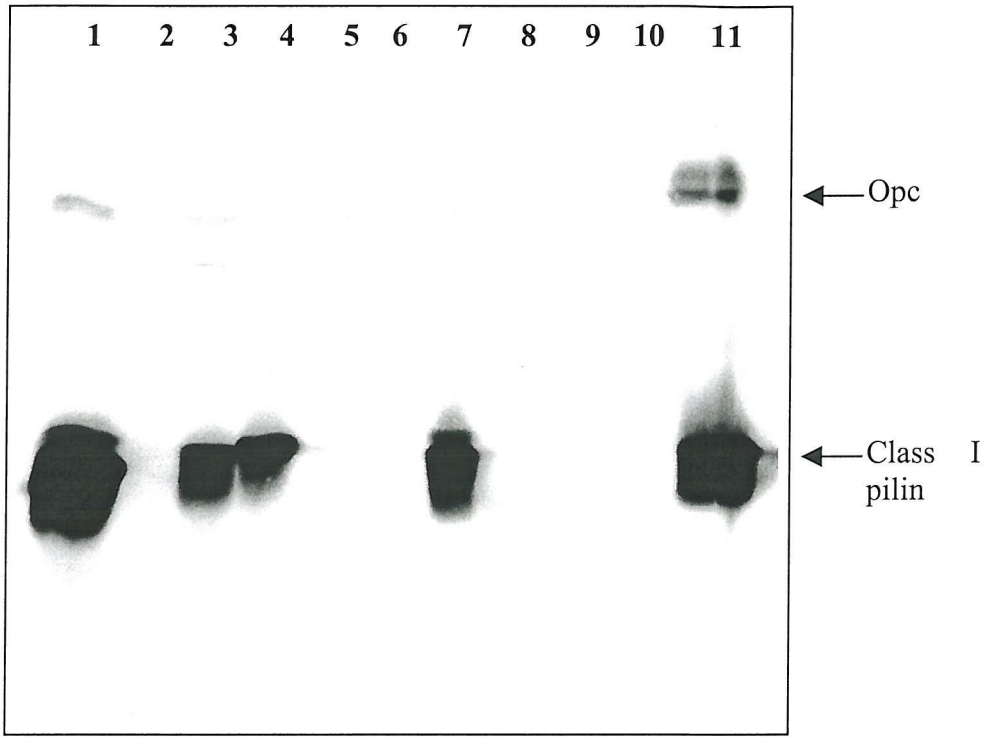
A Western blot was performed using all the case isolates from the Glasgow outbreak. Following transfer to nitrocellulose, the blot was probed with two monoclonal antibodies; B306 an anti-Opc antibody (diluted 1 in 5000) and the anti-class I pilin antibody, SM1 (diluted 1 in 25,000) (figure 3.7). All isolates expressing the Opc protein expressed class I pili. The two isolates that had been shown by PCR to possess the *opc* gene, but in a switched off state also expressed class I pili.

From this small subset of isolates, a pattern of the distribution of genes seemed to be emerging. The class 3 porin was being associated with possession of the *opc* gene and with expression of class I pili. In order to establish whether this observation was universal a second study was undertaken using a larger number of strains obtained from laboratory stocks. These strains were chosen as belonging to a variety of serogroups, serotypes and serosubtypes and are listed in appendix 3.

3.3.1 Primer design

Because the expression of the Opc protein and both classes of pili are phase variable, PCR was the method employed to determine the presence of the gene, rather than relying on detection of expression from the gene by Western blotting.

Figure 3.7 –Western blotting for the detection of class I pilin and the outer membrane protein Opc



Whole cell lysates were prepared from the case isolates of an outbreak of meningococcal disease and subjected to SDS-PAGE. The proteins were transferred to nitrocellulose and probed with the monoclonal antibody SM1 for the presence of class I pilin and B306 for the presence of Opc protein. Lanes 3 and 7 contain low Opc expressing strains.

The lane order is as follows:

1 - H44/76
2 - MC117
3 - MC119
4 - MC121
5 - MC125
6 - MC127
7 - MC130
8 - MC133
9 - MC135
10 - MC137
11 - MC139

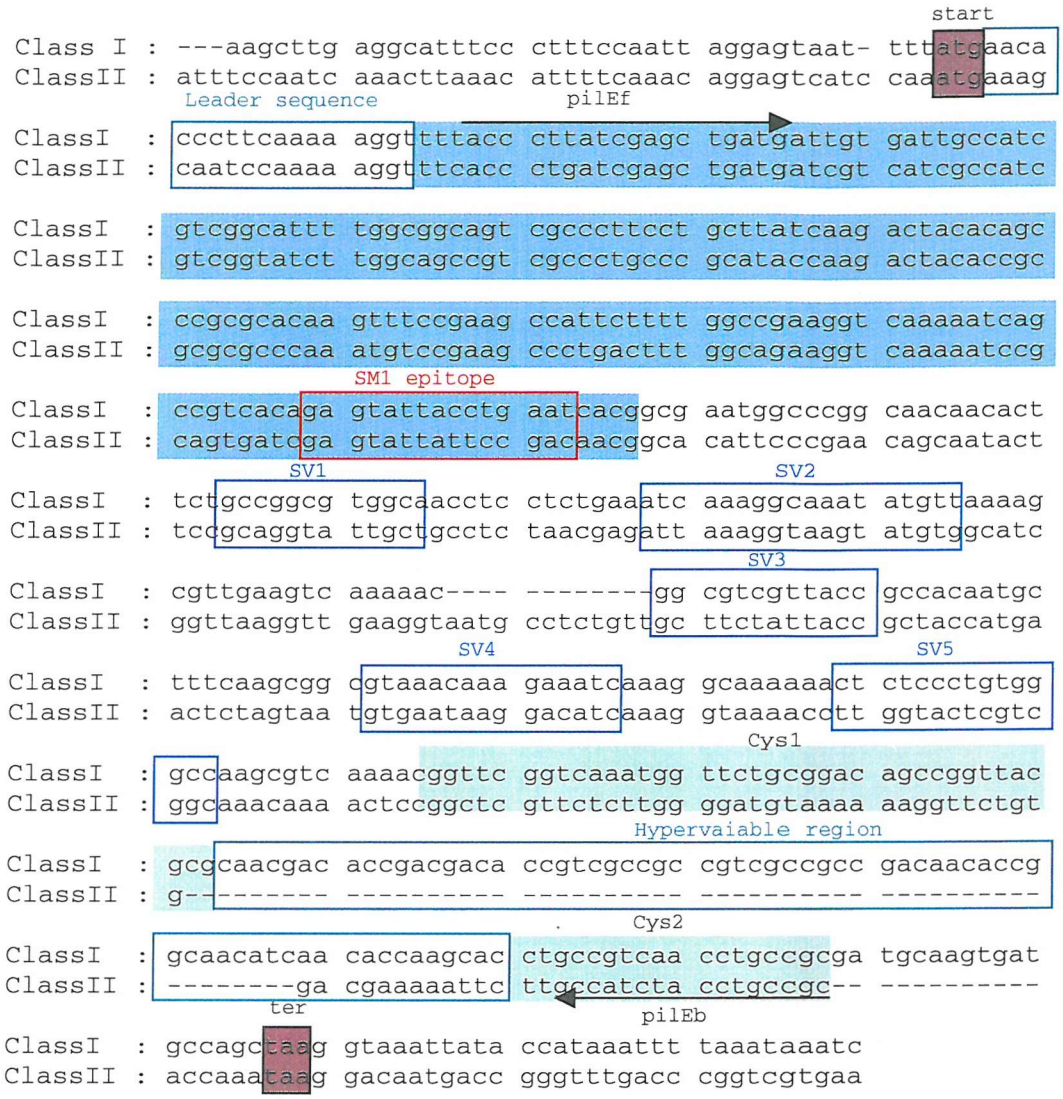
Primers were designed for the *pilE* gene using the sequences obtained from the sequence database (Genbank), for the two alleles (Potts & Saunders, 1988; Aho *et al*, 1997). Alignment of these two sequences (figure 3.8) shows that there are regions of homology, including the first 32 amino acids at the N-terminus. The class II sequence shows an increased diversity following amino acid residue 55, through the central and C-terminus, regions, although there are several small stretches of perfect identity. A feature of the class I sequence are the two regions towards the C-terminus, termed Cys1 and Cys2, containing two Cys residues, so allowing formation of a disulphide bond. The region in between the two Cys regions is termed the hypervariable region, which as the name suggest does vary between different class I strains. In the class II pilin gene, the sequence within Cys1 is altered and the majority of the hypervariable region is deleted, but the Cys2 region is conserved. Primers were therefore designed to bind within the region coding for the conserved 32 amino acid region at the N-terminus and at the region coding for the conserved Cys2 region at the C-terminus. These sites are slightly internal to the gene, but following amplification both the class I and class II gene can be distinguished between on the basis of the product size; 458 bp for the class I allele and 413bp for the class II allele. The sequence of the primers used to amplify the *pilE* alleles are listed in table 3.5.

Primer name		Sequence	
PilEf	5'	ACC CTG ATC CAG CTG ATG	3'
PilEb	5'	GCG GCA GGT $^{A}/_{T}$GA $^{T}/_{C}$GG C	3'

Table 3.5 – Primers used in the amplification of the *pilE* alleles

The sense primer binds at all bases in the class II allele, and at seventeen of the eighteen bases in the class I allele. Four-fold degeneracy was included in the anti-sense primer due to this divergence in sequence at these two positions.

The primers were tested for the ability to amplify and allow identification on the basis of the size of the product, using two strains; C311 P+, which was known to possess a class I allele and C114 P+, which was known to possess a class II allele (Potts &



Conserved region, the first 55 amino acids are homologous between gonococcal pilin and meningococcal class I pilin, and this region includes the SM1 epitope. The meningococcal class II pilin shares only the first 32 amino acid residues

SV – semi-variable regions

Figure 3.8 – Alignment of the class I and class II alleles of the *pilE* gene showing binding sites of primers used in amplification of gene by PCR

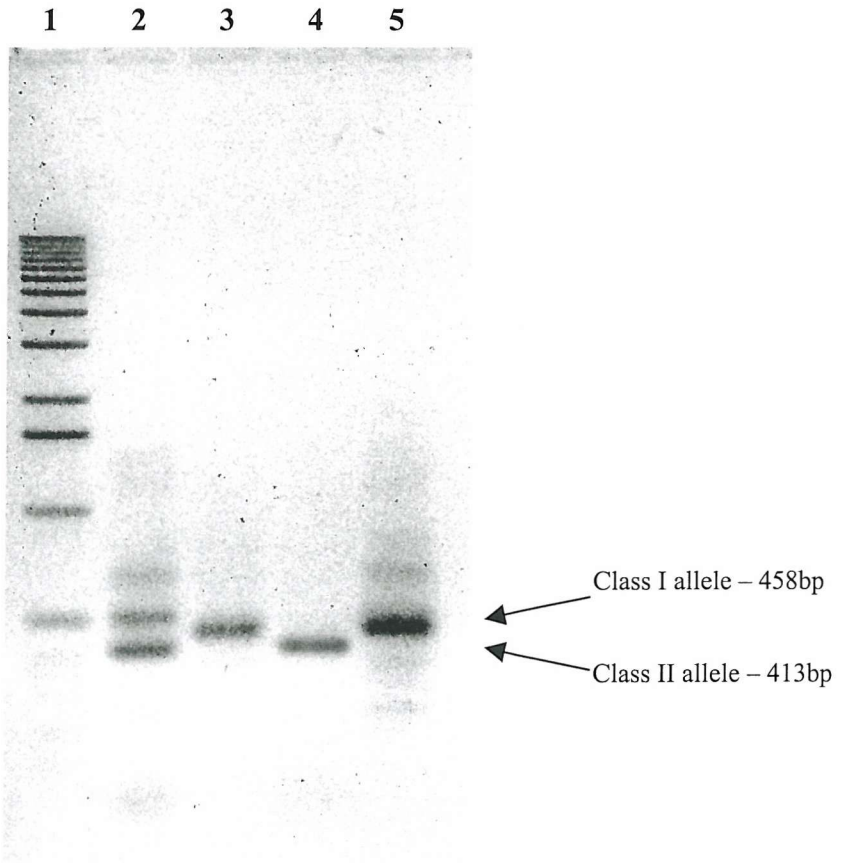
Saunders, 1988). Also included was the strain H44/76 which reacted with the anti-class I antibody, SM1 and the strain MC117 which did not react (figure 3.9). Following amplification a single bright band can be seen of the one of the expected sizes for all strains and the size difference does allow identification of the two alleles. When non-piliated variants of the class I and class II strains (C311 P- and C114 P-) were used as template DNA, then no band of the expected size was observed confirming the absence of an intact *pilE* gene. Antigenic variation is achieved at the *pilE* locus by homologous recombination with copies of the pilin gene which are not expressed (*pilS*). A silent locus carries one or more partial pilin gene copies, which are tandemly arranged and connected by repetitive sequences (Haas & Meyer, 1986) and the larger sized bands generated by the *pilE* primers are probably due to amplification of this.

3.3.2 Amplification by PCR of three different genes in a random collection of strains

The two primers; PB2f and PB2b previously used (section 3.2) were designed for amplification of the class 2 allele of the *porB* gene. Binding of these primers is in the identical position to the primers designed for the class 3 allele. The two alleles differ at the binding site of the sense primer by four bases and at the 5' end by one base at the 5' end of the reverse primer, therefore lowering the annealing temperature should permit amplification of the class 3 allele also. The two PCR products of the two alleles could then be differentiated between on the basis of the size of the product.

DNA was extracted from the 24 new strains and each was subjected to PCR to amplify the three genes of interest. The primers and annealing temperatures used in the PCR amplification procedure and the expected product sizes are listed in table 3.6. The PCR products from a selected group of strains are shown in figure 3.10.

Figure 3.9 - Amplification of the *pilE* gene



The primers; pilEf and pilEb, had been designed to amplify both alleles of the *pilE* gene. Strains of known pilin classification were used to test whether the *pilE* gene could be amplified and the two alleles identified when the products were separated by agarose gel electrophoresis.

The lanes are as follows:

1 - 1 Kb ladder

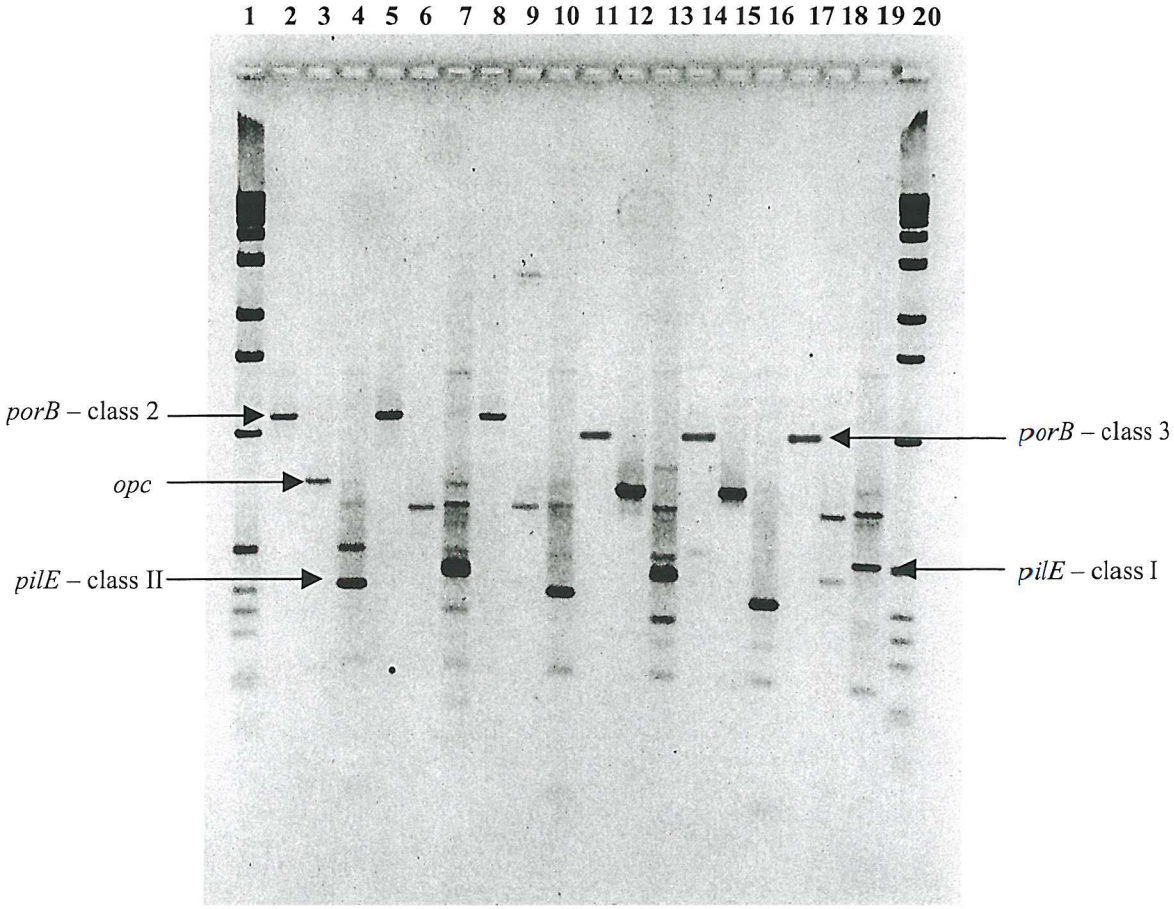
2 - C114 P+, a known class II strain

3 - C311 P+, a known class I strain

4 - MC117, a strain that does not react with the anti-classI antibody, SM1

5 - H44/76, a strain that does react with the anti-classI antibody, SM1

Fig. 3.10 – Amplification by PCR of the *porB*, *opc* and *pilE* genes



A collection of strains was analysed by PCR for the presence of the *opc* gene and the identification of the allele present for the *porB* and *pilE* genes. These genes were amplified using primers designed to amplify each gene and identify the allele present when the products were subjected to agarose gel electrophoresis.

The lane order is as follows:

1 -	1 kb ladder			11 -	H44/76	*porB*	Class 3
2 -	MC81	*porB*	Class 2	12 -		*opc*	Present
3 -		*opc*	Present	13 -		*pilE*	Class I
4 -		*pilE*	Class II	14 -	MC86	*porB*	Class 3
5 -	MC50	*porB*	Class2	15 -		*opc*	Present
6 -		*opc*	Not present	16 -		*pilE*	Class II
7 -		*pilE*	Class I	17 -	MC80	*porB*	Class 3
8 -	MC117	*porB*	Class 2	18 -		*opc*	Not present
9 -		*opc*	Not present	19 -		*pilE*	Class I
10 -		*pilE*	Class II	20 -	1 kb ladder		

Primers	Annealing temperature	Gene	Expected product sizes
PB2f / PB2b	45°C	*porB*	Class2 : 1106 bp Class 3: 1012bp
opc-1 / opc –2	54°C	*opc*	713 bp
pilEf / pilEb	54°C	pilE	Class I: 458 bp Class II: 413 bp

Table 3.6 – Primers, annealing temperatures and expected product sizes for amplification of the *porB*, *opc* and *pilE* genes.

Cell lysates were also prepared from each strain and the proteins were separated by SDS-PAGE and subjected to Western blotting using the anti-Opc monoclonal antibody, B306 (1 in 5000 dilution) and the anti-class I pili monoclonal antibody, SM1 (1 in 25,000 dilution), to test for expression of these proteins.

3.3.3 Results of PCR amplification and Western blotting

The results from these three separate PCR protocols and two Western blots were compiled in table 3.6. Of the further 12 class 3 expressing strains examined, ten were found to fit the hypothesis and also possess the *opc* and the class I *pilE* allele. However when the results of the further 12 class 2 expressing strains were inspected, an even distribution of the *opc* gene and *pilE* alleles was seen. Overall, in the 34 strains tested, 13 strains did not fit with the hypothesis, so disproving the possible linkage between the class 3 allele, *opc* gene and the class I *pilE* allele.

The results in this chapter show that the PorB protein is less variable than the PorA protein, but this minor variation can still be used to obtain additional epidemiological information. Approximately 65% of the strains tested possessed the *opc* gene, but only

Strains	Profile			PCR				Western	Blott
	Group	Type	Subtype	*porB* Class 2	*porB* Class 3	*opc*	*pilE* Class I	*pilE* Class II	Opc	PilE - Cl I
MC125	C :	2a :	P1.2	+				+		
MC82	B :	2b :	P1.2	+				+		
MC135	B :	2b :	P1.2	+				+		
MC133	C :	2b :	P1.2	+				+		
MC117	B :	2b :	P1.10	+				+		
MC127	B :	2b :	P1.10	+				+		
MC137	B :	2b :	P1.10	+				+		
MC90	B :	9 :	P1.9	+			+			+
PM	B :	16 :	P1.10	+			+			+
AN	B :	16 :	P1.16	+			+			+
MC50	C :	NT :	P1.16a	+			+			+
MC116	C :	2a :	nt	+		+	+			+
MC87	B :	5 :	P1.7	+		+	+			
SC	29E :	16 :	P1.2	+		+	+		+	+
283V	Z/29E :	nt :	nt	+		+	+			
MC81	B :	2a :	P1.2	+		+		+		
C114 P+	C :	15,16 :		+		+		+		
292U	W135 :	nt :	nt	+		+		+	+	
MC80	B :	1 :	P1.11.7		+		+			
MC130	B :	4 :	P1.15		+	+	+		+	+
MC139	C :	4,21 :	P1.1		+	+	+		+	+
MC93	B :	14 :	P1.6		+	+	+		+	+
MC119	Y :	14 :	P1.2		+	+	+		+	+
MC58	B :	15 :	P1.17,16b		+	+	+		+	+
H44/76	B :	15 :	P1.7,16		+	+	+		+	+
MC59	B :	15 :	P1.7,16		+	+	+			+
MC160	B :	nt :	P1.2,5		+	+	+		+	+
MC121	B :	nt :	P1.15		+	+	+			+
MC157	B :	nt :	P1.15		+	+	+			+
C311 P+	B :	nt :			+	+	+		+	+
312F	29E :	nt :	P1.9		+	+	+		+	+
428E	Y :	nt :	P1.15		+	+	+			+
137Q	Y :	nt :	P1.5		+	+	+		+	+
MC86	A :	4 :	nt		+	+		+	+	

Table 3.7 – Compilation of PCR and Western blotting results for the genes; *porB*, *opc*, *pilE* and the proteins expressed in a collection of strains.

half, demonstrated expression of the protein. Sequencing of this gene showed low levels of variation but this too was useful epidemiologically.

An allele of the *porB* gene is always expressed in meningococci and is quantitatively the major outer membrane protein (Tsai *et al*, 1981). This information, coupled with the lower levels of variation in comparison to the PorA protein, make the PorB protein an attractive vaccine candidate. In the remainder of the thesis, the class 3 protein is isolated and tested as a vaccine antigen.

CHAPTER 4: CLONING, EXPRESSION AND PURIFICATION OF THE PORB OUTER MEMBRANE PROTEIN

4.1 Introduction

Currently available vaccine preparations are based on group specific capsular polysaccharides. However, due to the close structural resemblance of the polysaccharide of group B strains to human glycoproteins it is non-immunogenic. As a result, alternative sub-capsular antigens are being investigated as potential vaccine candidates. Expression of the class 2 or class 3 outer membrane protein is mutually exclusive and whichever is expressed is quantitatively the major outer membrane protein (Tsai *et al*, 1981), therefore these proteins appear attractive vaccine candidates. The proteins are coded for by the two alleles of the *porB* gene which have both been cloned and sequenced (Murakami *et al*, 1989; Wolff & Stern, 1991). .

In this chapter the *porB* gene from strain H44/76 was cloned into two expression systems; Xpress™ (Invitrogen) and IMPACT™ (New England Biolabs) and protein expression levels compared.

4.2 Cloning and Expression in the Xpress™ System

The Xpress System ™ (Invitrogen) was chosen in which to clone and express the class 3 protein, following the successful expression of the meningococcal class 1 outer membrane protein (Christodoulides *et al*, 1998). The pRSET vectors of this system are pUC-derived expression vectors designed for high level protein expression in *E.coli*. Inserting a gene into the multiple cloning site positions the DNA downstream and in frame with a sequence that encodes an N-terminal fusion peptide. This sequence includes an ATG initiation codon and a series of six histidine residues that function as a metal binding domain in the translated protein allowing a one-step protein purification procedure by immobilised metal affinity chromatography. Expression of the

recombinant protein is driven by a T7 promoter and T7 RNA polymerase is introduced to the system via an M13 phage carrying the T7 RNA polymerase gene fused to the *E.coli lac* promoter. Expression of the gene can then be induced by the addition of IPTG.

4.2.1 Construction of pRSETB Recombinant Plasmid

DNA was obtained from the laboratory strain H44/76 (B:15:P1.7,16) using the method described (section 2.7.1). Primers were designed to amplify by PCR the mature class 3 protein whilst omitting the 19 amino acid leader sequence, and to include appropriate restriction enzyme sites for cloning into the multiple cloning site (figure 4.1).

Binding of the sense primer H44f1 to *porB* gene:

```
 M   K   K   S   L   I   A   L   T   L   A   A   L   P   V
atg aaa aaa tcc ctg att gcc ctg act ttg gca gcc ctt cct gtt
```

initiation codon

```
 A   A   M   A | D   V   T   L   Y   G
gca gca atg gct|gac gtt acc ctg tac ggc tac ggc acc atc aaa
              || ||| ||| ||| ||| ||| ||| ||| ||| ||
      g|gat cct gac gtt acc ctg tac ctg tac ggc ac
```

mature protein

*BamH*I

Primer – H44f1

Binding of the antisense primer H44b1 to *porB* gene:

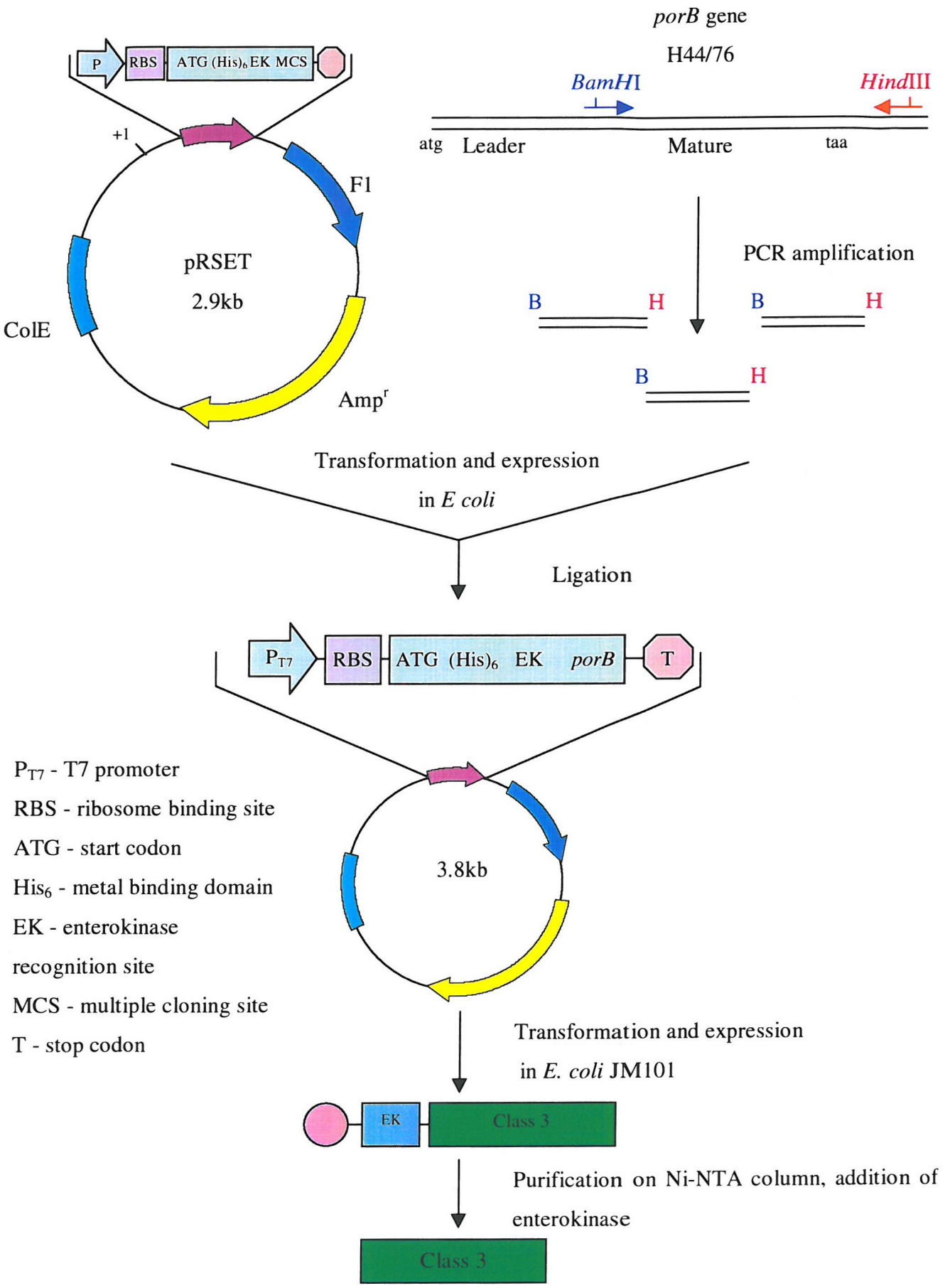
```
 A   A   S   V   G   L   R   H   K   F   #
gcc gcc tcc gtc ggt ttg cgc cac aaa ttc taa
                ||| ||| ||| ||| ||| ||| |||
                cca aac gcg gtg ttt aag att agt tcg aac gtg ac
```

stop codon *Hind*III

Primer – H44b1

Figure 4.1 – Binding of primers for amplification of the *porB* gene into the Xpress™ system.

Figure 4.2 – Cloning and expression of the *porB* gene from strain H44/76 into the Xpress™ system



The sequence of the primers used are listed in table 4.1. The primers were used in PCR to amplify the gene using the Bio-X-act enzyme, with an annealing temperature of 55°C and 30 cycles (section 2.7.3). These conditions produced a product of the expected size, 942 bp. The amplified DNA was purified using the GenecleanII™ purification procedure (section 2.4.2).

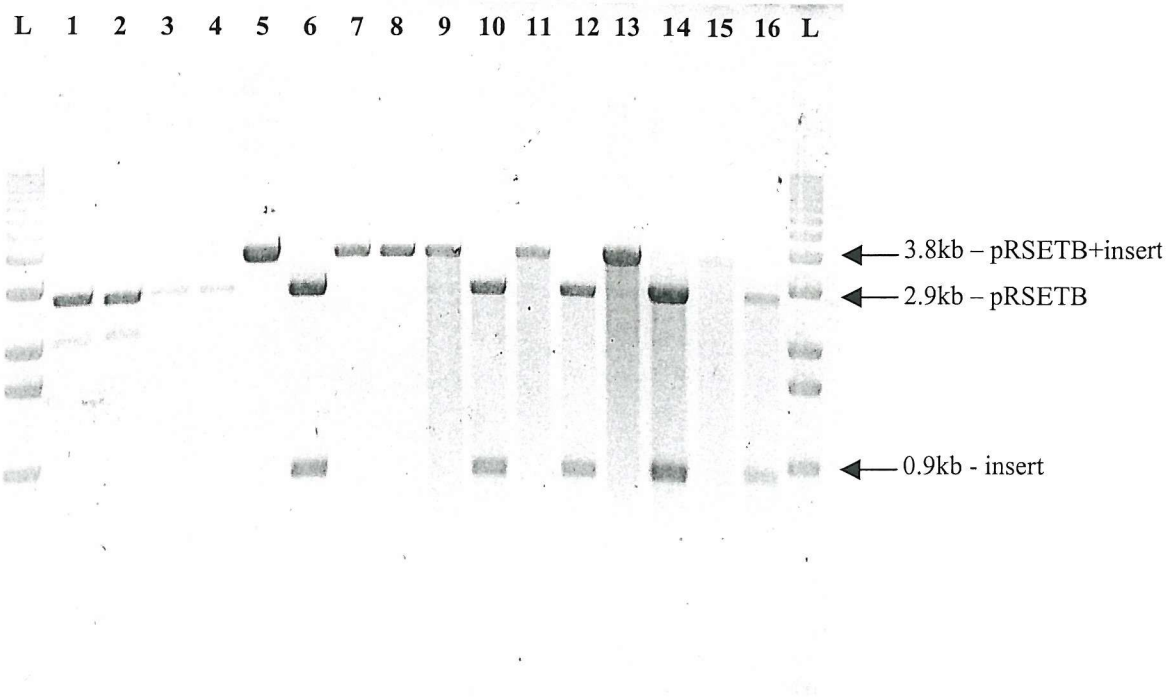
Primer name		Sequence	
H44f1	5′	gcg att gga tcc tga cgt tac cct gta cgg cac	3′
H44b1	5′	cag tgc aag ctt gat tag aat ttg tgg cgc aaa cc	3′

Table 4.1 – Primers used in the amplification of the *porB* gene for cloning into the Xpress™ system

The vector, pRSETB (figure 4.2) was obtained as a frozen glycerol stock in *E.coli* JM109 and plasmid DNA was prepared using the Promega Wizard Mini-Prep kit (section 2.3.1). The vector was prepared for ligation by digestion with the restriction enzymes BamH*I* and Hind*III* (Promega) and the cleaved vector was purified using the Wizard DNA Miniprep kit (Promega). The insert DNA was similarly treated to produce complimentary 'sticky ends'. Vector DNA (10ng) and insert DNA (30ng) were ligated overnight at 14°C then transformed into competent JM101 cells. This resulted in a large number of colonies. Plasmid DNA was isolated from 16 of these which were screened using the plasmid preparation procedure as described (section 2.9.3).

In order to determine the incorporation and correct orientation of the inserted DNA, the recombinant plasmids were isolated and used in two separate digestions. To establish if the construct contained the insert, a double digestion was performed using *BamHI* and *Hind*III to excise the inserted DNA. To check that a single copy of the insert was present the recombinant plasmid was linearised by digestion with *Sca*I. A unique site for *Sca*I is present on the vector DNA and analysis of the *porB* gene showed no site to be present. Of the recombinant plasmids screened nine were found to contain a single copy of the insert and were used in further work (figure 4.3).

Figure 4.3 – Screening of the isolated pRSETB constructs for the presence of a single copy of the *porB* gene



Plasmid DNA was prepared from a number of constructs resulting from the ligation of the *porB* gene into the pRSETB vector. The plasmid DNA's were digested with the restriction enzymes; *Sca*I to linearise the plasmid, and with *BamH*I and *Hind*III to remove the insert. The digested products were subjected to agarose gel electrophoresis. The lane order is as follows:

L	-	1Kb ladder		
1	-	pRSETB (1)	cut with	*Sca*I
2	-			*BamH*I + *Hind*III
3	-	pRSETB (2)	cut with	*Sca*I
4	-			*BamH*I + *Hind*III
5	-	Construct A1	cut with	*Sca*I
6	-			*BamH*I + *Hind*III
7	-	Construct A2	cut with	*Sca*I
8	-			*BamH*I + *Hind*III
9	-	Construct A4	cut with	*Sca*I
10	-			*BamH*I + *Hind*III
11	-	Construct B1	cut with	*Sca*I
12	-			*BamH*I + *Hind*III
13	-	Construct B3	cut with	*Sca*I
14	-			*BamH*I + *Hind*III
15	-	Construct B8	cut with	*Sca*I
16	-			*BamH*I + *Hind*III
L	-	1kb ladder		

4.2.2 Expression Trials in E.coli JM101

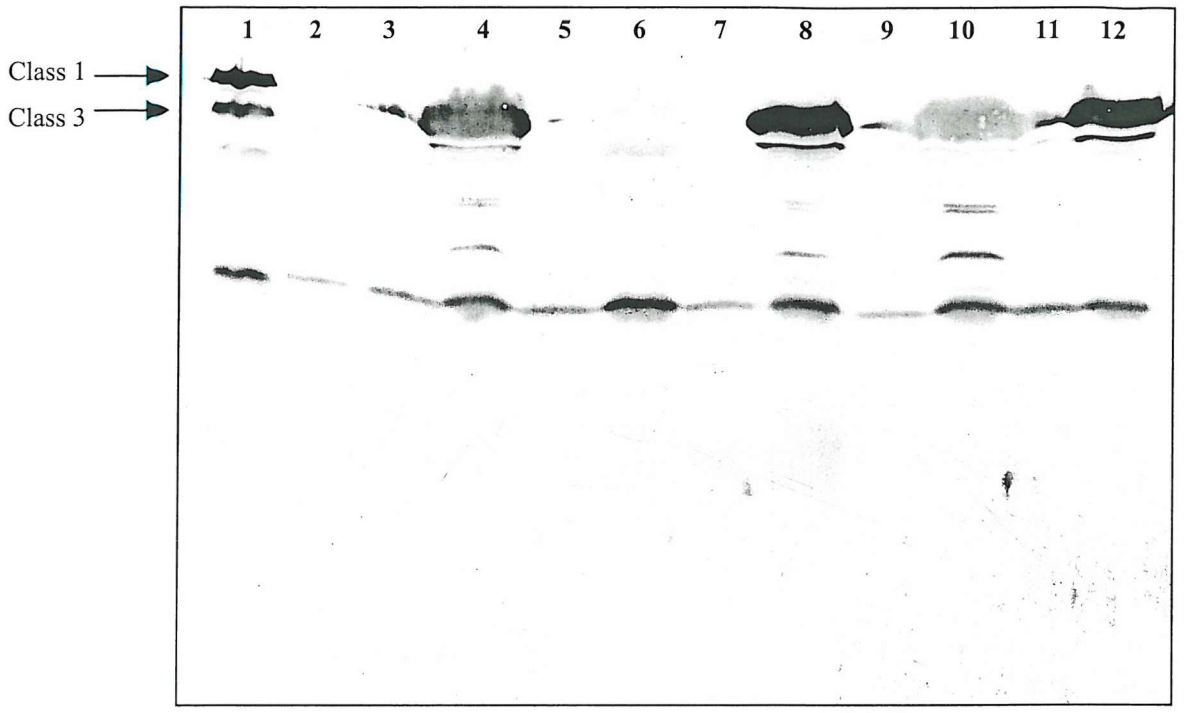
In order to establish if class 3 protein could be expressed from any of these constructs, expression trials in 5ml LB-amp were performed (section 2.10). Five hours after induction with IPTG (1mM) and M13/T7 phage (10pfu ml^{-1}) the cells were harvested and protein expression was assessed by SDS-PAGE and Western blotting with the general anti-porin antibody R141 (1/1000 dilution). Good expression was seen in six constructs (figure 4.4). A strong band of ~32kDa was present following induction of expression.

4.3 Transformation and expression in *E.coli* JM109(DE3)

The *E.coli* strain JM109 (DE3) has been constructed to contain the T7 RNA polymerase gene fused to the *E.coli lac* promoter, so eliminating the phage induction step during expression of the protein. Recombinant plasmid DNA from five different constructs was transformed into competent *E.coli* JM109 (DE3) cells and a selection of the resulting colonies were screened for recombinant plasmids as before.

Expression in JM109 (DE3) allows the omission of the phage induction step and therefore simplifies and reduces the total time for this procedure. Expression trials in 5ml LB-amp were performed using the five recombinant plasmids transformed into JM109 (DE3). Protein expression was induced using IPTG (1mM) and 5 hours after induction, cells were harvested and analysed by SDS-PAGE and Western blotting with the antibody R141 (1/1000 dilution). Only one construct produced a band of ~32kDa that reacted with the antibody R141 confirming expression of the class 3 protein, B1 (figure 4.5).

Figure 4.4 - Expression trials of pRSETB constructs in *E.coli* JM101

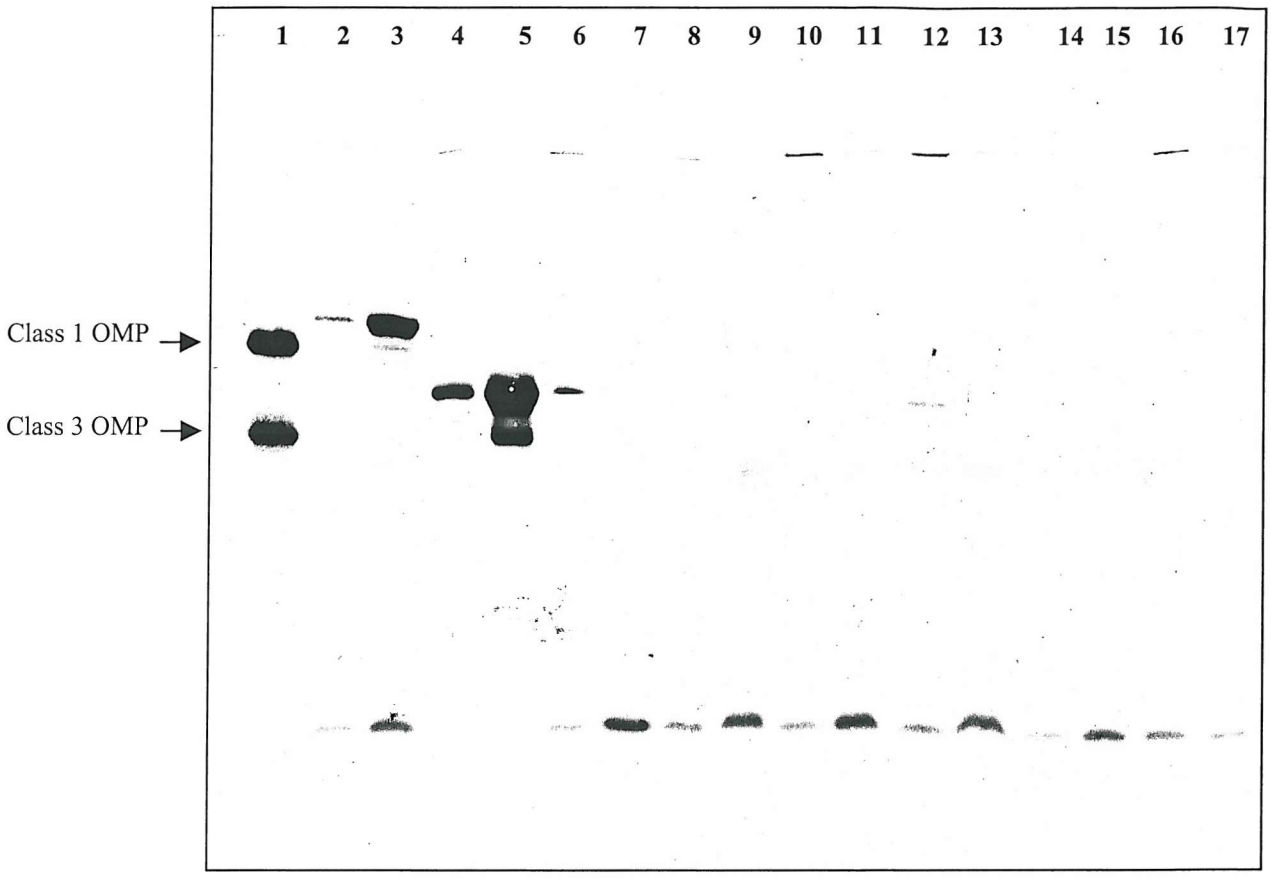


Various constructs in *E.coli* JM101, that had been shown to possess the *porB* gene were analysed for protein expression. Five hours after induction of protein expression, cell lysates were prepared and subjected to SDS-PAGE. The separated proteins were transferred to nitrocellulose and probed with the anti-porin antibody, R141.

The lane order is as follows:

1	-	H44/76	
2	-	PRSETB in JM101	
3	-	Construct B1 in JM101	Start
4	-		End
5	-	Construct B2 in JM101	Start
6	-		End
7	-	Construct B3 in JM101	Start
8	-		End
9	-	Construct B7 in JM101	Start
10	-		End
11	-	Construct B8 in JM101	Start
12	-		End

Figure 4.5 – Expression trials of pRSETB constructs in *E.coli* JM109(DE3)



Constructs that had been shown to contain the *porB* gene were transformed into *E.coli* JM109 (DE3) and levels of protein expression analysed. Five hours after induction of protein expression, cell lysates were prepared and subjected to SDS-PAGE. The separated proteins were transferred to nitrocellulose and probed with the anti-porin antibody, R141.

The lanes are as follows:

1	-	H44/76	
2	-	+ve control: *porA* in pRSET in JM109 (DE3)	Start
3	-		End
4	-	Construct B1 in JM109(DE3)	Start
5	-		End
6	-	Construct B2 in JM109(DE3)	Start
7	-		End
8	-	Construct B3 in JM109(DE3)	Start
9	-		End
10	-	Construct B4 in JM109(DE3)	Start
11	-		End
12	-	Construct B5 in JM109(DE3)	Start
13	-		End
14	-	Construct B6 in JM109(DE3)	Start
15	-		End
16	-	pRSETB in JM109(DE3) – negative control	Start
17	-		End

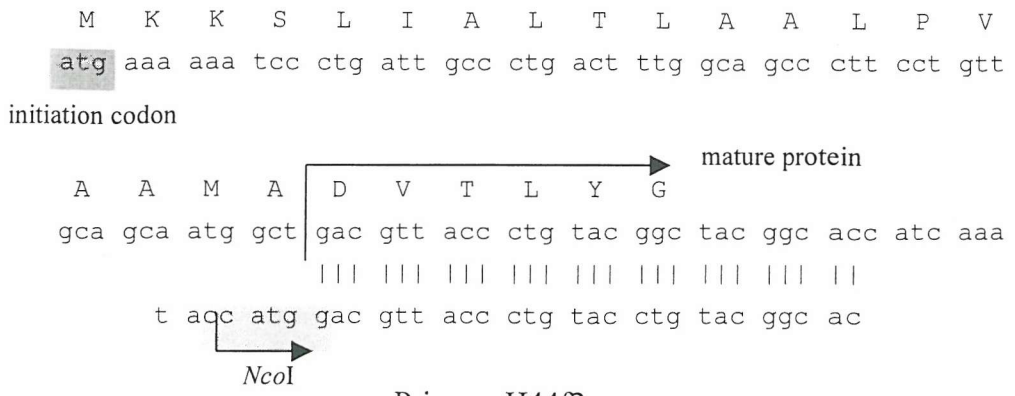
4.4 Cloning and Expression in the IMPACT™ System

Previous studies with the class 1 outer membrane protein (Christodoulides *et al*, 1998) had utilised the Xpress system. As an alternative the IMPACT™ system (New England Biolabs) was tried here. IMPACT™ (Intein Mediated Purification with an Affinity Chitin-binding Tag) system is a novel and simple protein purification system that has evolved from studies of protein splicing mechanisms. The IMPACT T7 system utilises a protein splicing element, an intein from *Saccharomyces cerevisiae*, that has been modified such that at low temperatures and in the presence of thiols, self-cleavage occurs at the N-terminus. The gene of interest is cloned into a multiple cloning site to create an in frame fusion between the C-terminus of the gene and the N-terminus of the intein encoding gene. At the C-terminus of the intein encoding gene, DNA encoding a 5kDa chitin binding domain (CBD) from *Bacillus circulas* has been added. This allows a one-step purification procedure whereby crude cell extract is passed over a chitin column, allowing the fusion protein to bind. Intein mediated self-cleavage of the fusion protein occurs by the addition of a thiol such as β-mercaptoethanol and the target protein is released leaving the intein–CBD bound to the column.

4.4.1 Construction of pCYB3 Recombinant Plasmid

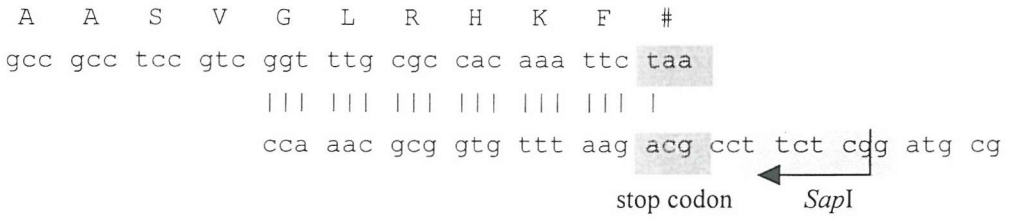
As for the cloning into the Xpress™ system, DNA was obtained from the laboratory strain H44/76 (B:15:P1.7,16) (section 2.7.1). Primers for PCR were designed to amplify the mature protein and omit the 19 amino acid leader sequence. Appropriate restriction enzyme sites were included for cloning into the vector (figure 4.6).

Binding of the sense primer H44f2 to *porB* gene:



Primer –H44f2

Binding of the antisense primer H44b2a to *porB* gene:



Primer – H44b2a

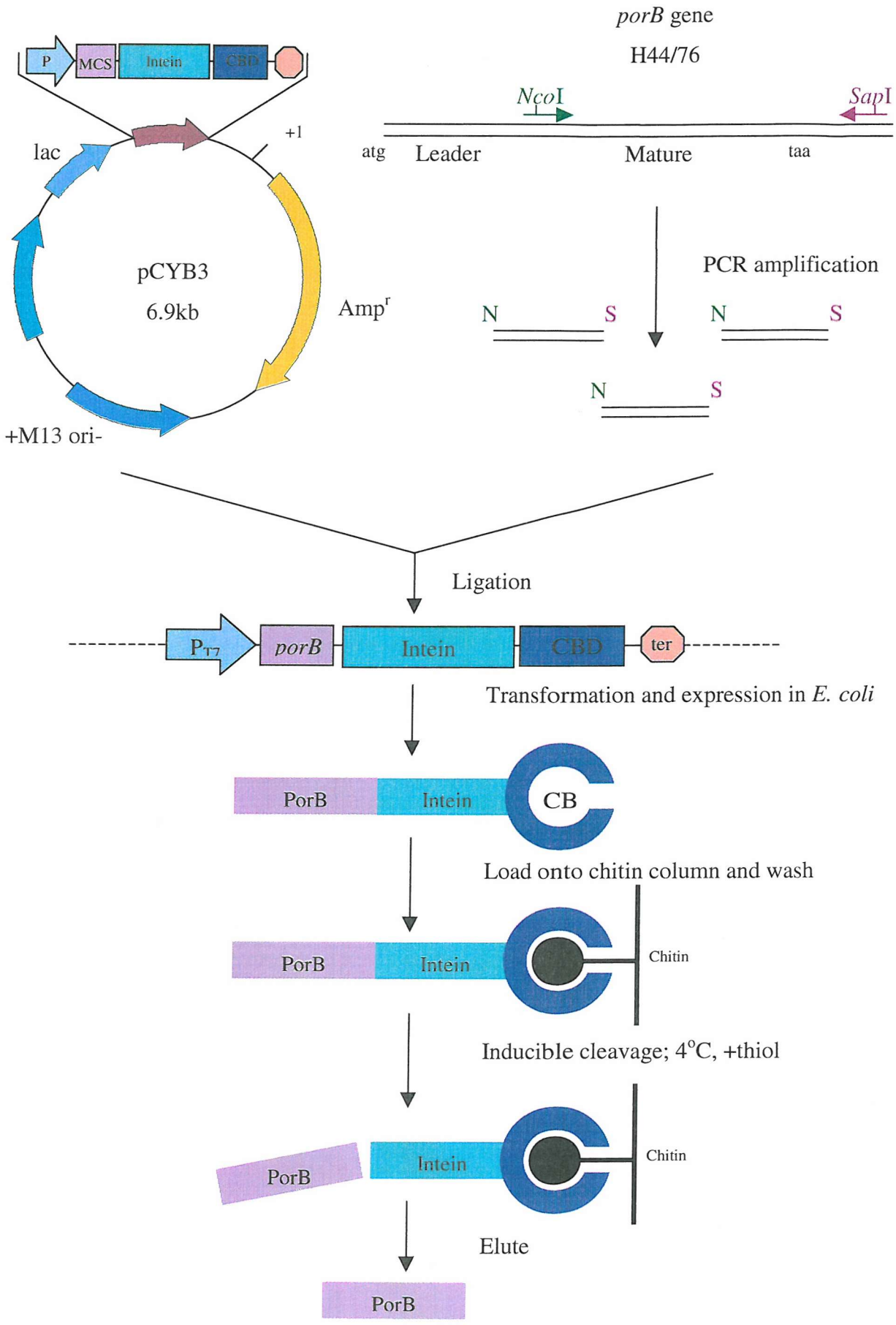
Figure 4.6 – Binding of primers for amplification of the *porB* gene into the IMPACT™ system.

The sequence of the primers used are listed in table 4.2. The primers were used in PCR to amplify the gene (section 2.7.3) using the proof-reading enzyme Bio-X-act, with an annealing temperature of 60°C and 30 cycles, resulting in a 944bp product. The amplified DNA was purified using the GeneCleanII™ purification procedure.

Primer name		Sequence	
H44f2	5′	gcg tac cat gga cgt tac cct gta cgg c	3′
H44b2a	5′	gcg tag gct ctt ccg cag aat ttg tgg cgc aaa cc	3′

Table 4.2 – Primers used in the amplification of the *porB* gene for cloning into the IMPACT™ system

Figure 4.7 – Cloning and expression of the *porB* gene from strain H44/76 in the IMPACT™ system



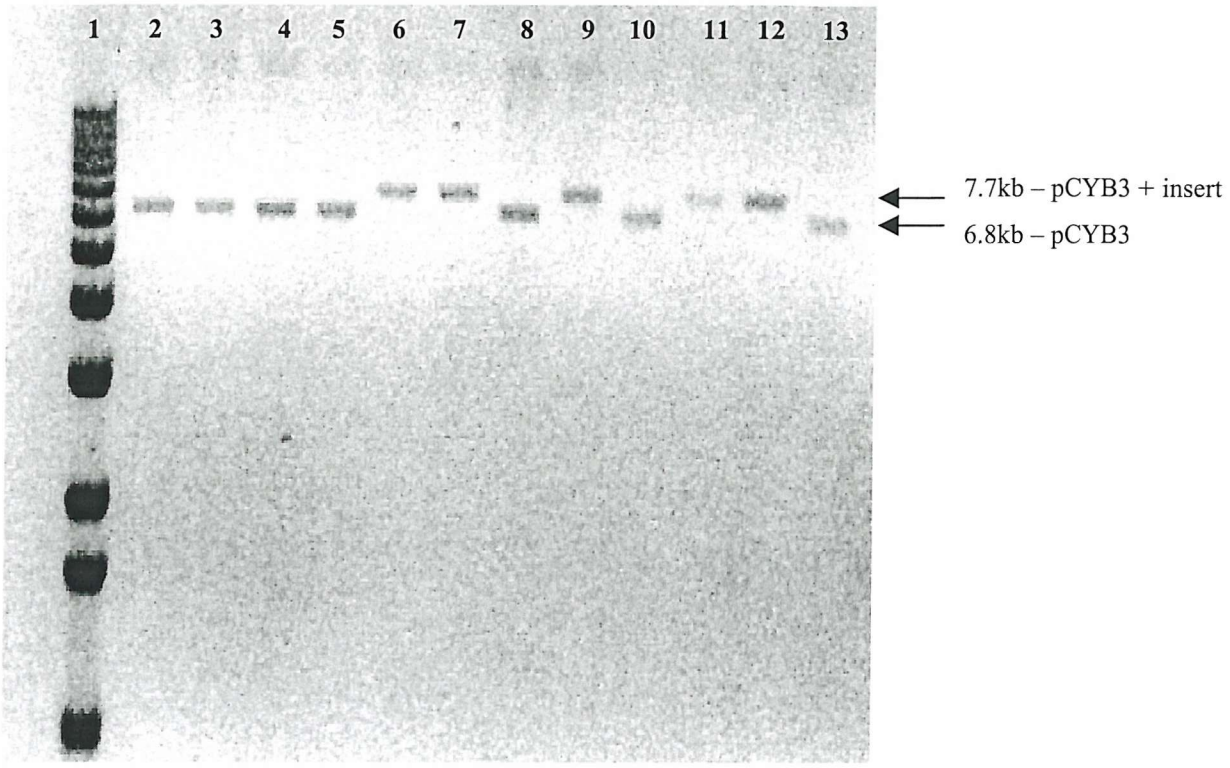
The vector, pCYB3 (figure 4.7) was obtained as a frozen glycerol stock in *E.coli* JM109 (DE3) and plasmid DNA was prepared using the Promega Wizard Miniprep kit (section 2.3.1). The vector DNA was prepared for ligation by digestion with the restriction enzymes *Nco*I and *Sap*I (New England Biolabs) and the cleaved vector was purified using the Wizard DNA Miniprep Kit (Promega). The insert DNA was similarly treated to produce complimentary 'sticky ends'. Vector DNA (10ng) and insert DNA (30ng) were ligated overnight at 14°C then transformed into competent JM101 cells. This resulted in a large number or colonies. Individual colonies were picked, streaked out onto selective media and plasmid DNA extracted (section 2.9.3).

In order to determine if the insert had been successfully ligated into the vector, restriction enzyme digests were performed. The insert could not be excised using the two enzymes used in the cloning procedure as the *Sap*I site is not regenerated following ligation. The enzyme *Hind*III (Promega) was used to linearise the plasmid. This enzyme was known to have no recognition sites within the insert and a unique site in the vector, so that recombinant plasmids could be identified by a size increase of ~1kb and therefore should be clearly visible by agarose gel electrophoresis. Of the twenty-four recombinant plasmids screened, sixteen were found to contain a single copy of the insert (figure 4.8).

4.4.2 Expression Trials in E.coli JM101

To determine whether the class 3 protein could be expressed from any of the recombinant plasmid constructs, expression trials in 5ml LB-amp were performed (section 2.10). Five hours after inducing expression with IPTG (1mM) the cells were harvested and protein expression was assessed by SDS-PAGE. Due to the nature of the system, whereby thiols will cause the intein to undergo self-cleavage, the loading buffer used in the SDS-PAGE gel contained no β-mercaptoethanol. As a consequence the samples ran only a small distance. The separated proteins were transferred to nitrocellulose and subjected to Western blotting with the general anti-porin antibody

Figure 4.8 – Screening of the isolated pCYB3 constructs for the presence of a single copy of the *porB* gene

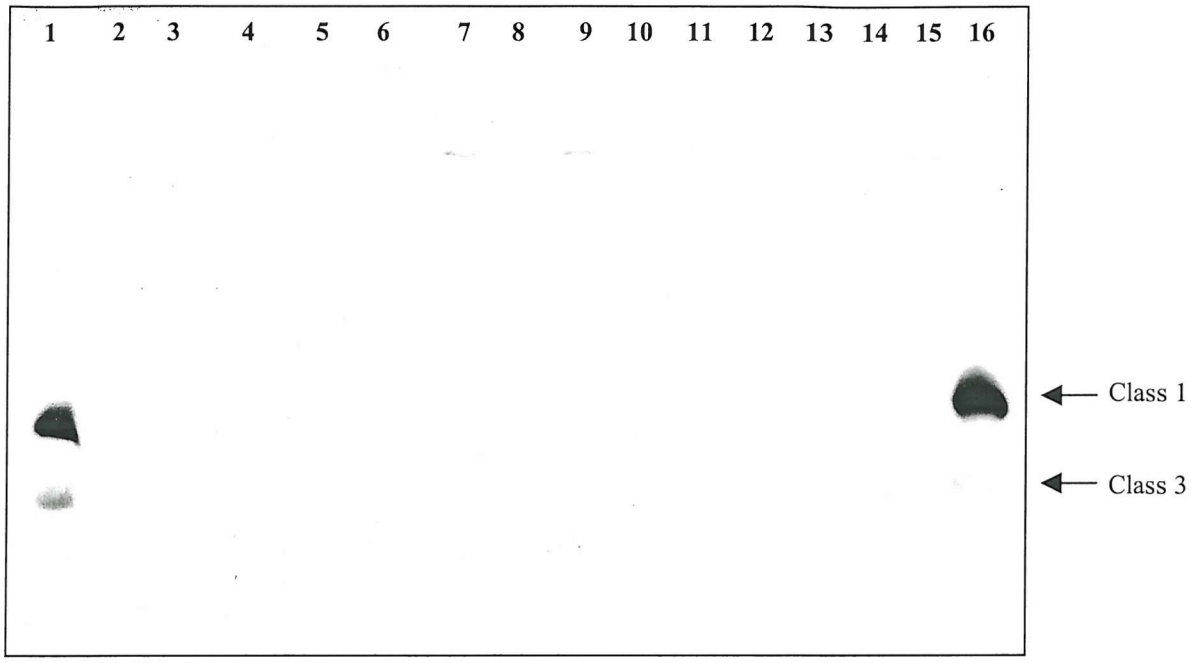


Plasmid DNA was prepared from a number of constructs resulting from the ligation of the *porB* gene into the pCYB3 vector. The plasmid DNA's were digested with the restriction enzyme *Hind*III to linearise the plasmid, and the digested products were subjected to agarose gel electrophoresis.

The lane order is as follows:

1 - 1Kb ladder
2 - pCYB3 (1) cut with *Hind*III
3 - pCYB3 (2) cut with *Hind*III
4 - pCYB3 (3) cut with *Hind*III
5 - pCYB3 (4) cut with *Hind*III
6 - Construct Y5 cut with *Hind*III
7 - Construct Y6 cut with *Hind*III
8 - Construct Y7 cut with *Hind*III
9 - Construct Y8 cut with *Hind*III
10 - Construct Y9 cut with *Hind*III
11 - Construct Y10 cut with *Hind*III
12 - Construct Y11 cut with *Hind*III
13 - Construct Y12 cut with *Hind*III

Figure 4.9 – Expression trials of pCYB3 constructs in *E.coli* JM101



Constructs that had been shown to contain the *porB* gene were transformed into *E.coli* JM101 and levels of protein expression analysed. Five hours after induction of protein expression, cell lysates were prepared and subjected to SDS-PAGE. The separated proteins were transferred to nitrocellulose and probed with the anti-porin antibody, R141.

The lanes are as follows:

1 - H44/76 in loading buffer containing β-mercaptoethanol
2 - Construct Y9 in JM101 Start
3 - End
4 - Construct Y8 in JM101 Start
5 - End
6 - Construct Y7 in JM101 Start
7 - End
8 - Construct Y6 in JM101 Start
9 - End
10 - Construct Y5 in JM101 Start
11 - End
12 - Construct Y4 in JM101 Start
13 - End
14 - Construct Y3 in JM101 Start
15 - End
16 - H44/76 in loading buffer containing β-mercaptoethanol

R141 (1/1000 dilution). Bands from eleven constructs reacted with the anti-porin antibody confirming expression of the class 3 protein (figure 4.9). Levels of expression were greatly reduced when compared to that in the Xpress™ system.

4.4.3 Transformation and Expression in E.coli strains JM109 (DE3) and BL21

In order to attempt to increase the levels of protein expression, seven constructs that had shown some protein expression in JM101 cells were transformed into two more *E.coli* strains; JM109(DE3) and BL21. The *E.coli* strain BL21 lacks two proteases; *ion* and *ompT,* which might be degrading the fusion protein as it is being produced. Recombinant plasmids were isolated and then transformed into competent JM109 (DE3) and BL21 cells. Large numbers of colonies resulted from the transformation and individual colonies were picked and screened for the presence of the recombinant plasmid. The integrity of the plasmid was also checked.

Expression trials were performed in 5ml LB-amp and five hour after induction with IPTG (1mM) the cells were harvested and expression of the class 3 protein analysed on an SDS-PAGE gel, again omitting β-mercaptoethanol from the loading buffer. The samples were run in duplicate and upon completion transferred to nitrocellulose. The two membranes were probed with the anti-porin antibody R141 and an anti-intein antibody (New England Biolabs). Protein bands that reacted with the anti-porin antibody did not react with anti-intein antibody, indicating that no fusion protein had been expressed and that the anti-porin antibody had probably cross-reacted with an *E.coli* protein.

4.5 Choice of Expression System

The *porB* gene had been successfully cloned into two expression systems that utilised different mechanisms for purifying the recombinant protein. However, the IMPACT™ system consistently gave very low levels of protein or none at all in the three *E.coli* backgrounds tested, whereas the Xpress™ system gave high levels of protein expression

in both *E.coli* strains used. The Xpress™ system was therefore used for further experiments.

4.6 Sequencing of Inserts

Although protein expression in *E.coli* strains JM101 and JM109 (DE3) had been established, possible mutations arising as a result of the original PCR amplification had not been eliminated. Therefore the five constructs known to give good expression of class 3 protein were chosen for sequencing. Primers for sequencing the class 3 gene had been designed previously (section 3.2) and were sited in the conserved, transmembrane regions of the mature protein.

Construct plasmid DNA was isolated using the QIAprep procedure (section 2.3.2) and sequencing reactions set up (section 2.8) using the primers and annealing temperatures listed in table 4.3.

Sequencing reaction products were separated by electrophoresis on an Applied Biosystems Inc 373A automated DNA Sequencer for 12 hours at 30W and the data generated was analysed using the LASERGENE software package supplied by DNASTAR.

Primer	Sequence	Annealing Temperature
3B1f	5' gta cgg cac cat caa agc	50°C
3B6b	5' gag aat cgt agc gta cgg	50°C
3B6f	5' cgc ctc att tcc gta cgc	50°C
3B11b	5' tgt acg gct acg gaa gcg	50°C
3B11fa	5' ctt ccg tag ccg tac agc	50°C
3B16b	5' ttt gtg acg cag acc gac	50°C

Table 4.3 – Primers and annealing temperatures used in sequencing the pRSETB constructs

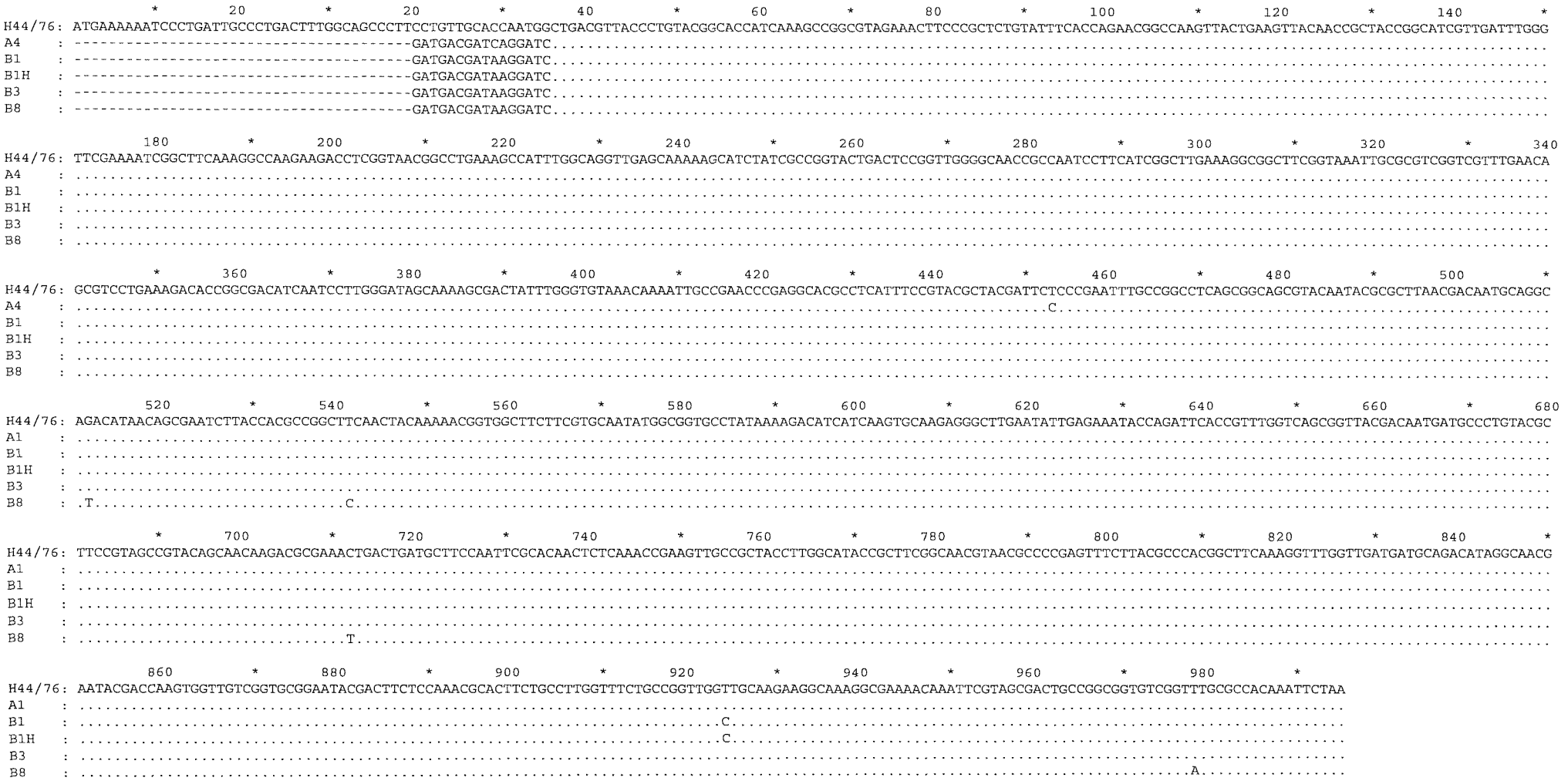


Figure 4.10 – Alignment of sequenced *porB* constructs against the gene from the parental strain H44/76.
- indicates missing residues
. indicates homology

The DNA sequence of each construct was aligned against each other and also the sequence of the host strain H44/76 which had previously been sequenced (figure 4.10). Of the five constructs sequenced, A4, B1 and B1h showed one mutation at the DNA level, whilst B8 contained three base changes. Construct B3 contained no mutations and was renamed pPORB3 and used in all further work.

4.7 Media Trials

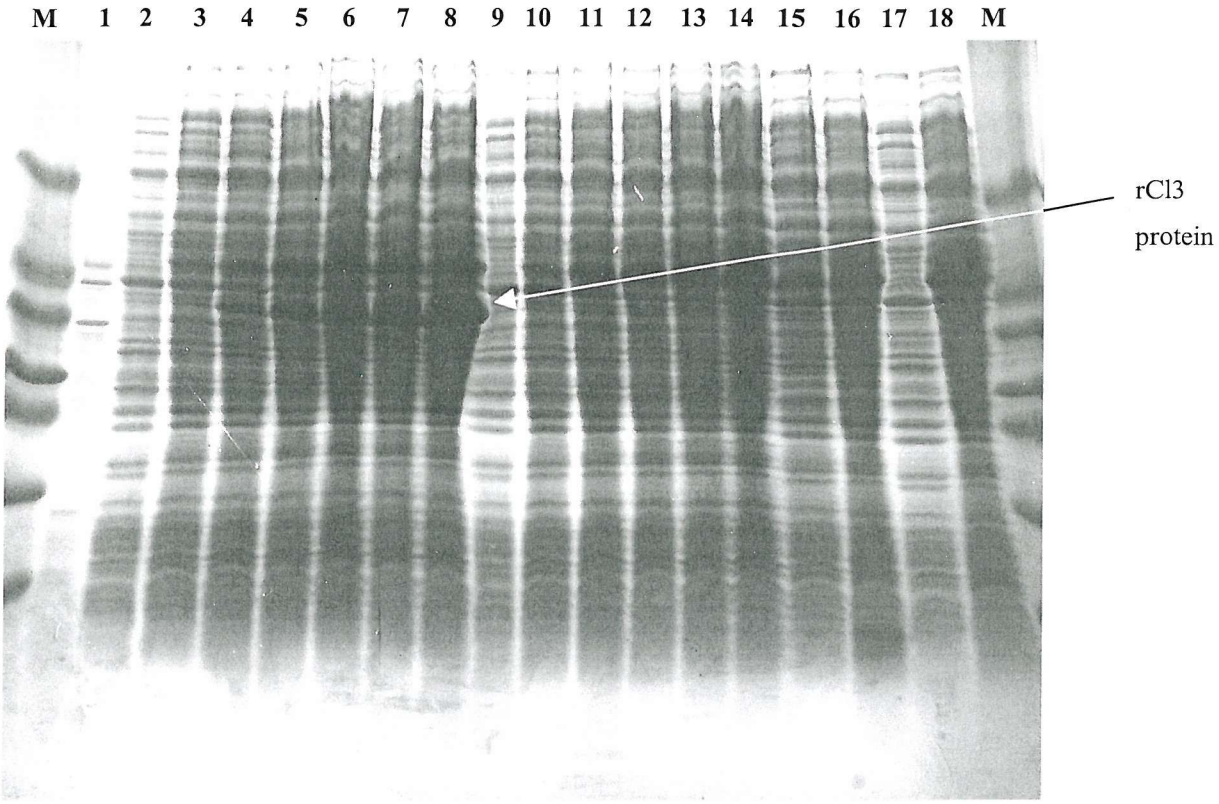
Having established that pPORB3 contained no mutations, expression trials using different growth media were performed. The construct pPORB3 had been previously transformed into both JM101 and JM109 (DE3) and both these strains were used in these trials. An overnight culture (200µl) was used to inoculate 25ml of the different media tested; LB, SOB, 2YT and superbroth. Protein expression was induced as appropriate to the host cell type and a 1ml sample removed every hour for five hours post-induction. These samples were subjected to SDS-PAGE.

The results of these media trials showed that in all media little protein was produced by the construct in the host cell JM109 (DE3). Large amounts of protein were produced in JM101, where expression was induced by the addition of T7 phage and IPTG, in all media types, with highest levels of recombinant class 3 protein being produced in the superbroth medium (figure 4.11).

4.8 Location of the recombinant class 3 protein

The location of the recombinant protein within the *E.coli* cells needed to be determined. Pelleted cells that had been induced for protein expression were resuspended at a concentration of 0.2g ml^{-1} in TE buffer. The resuspended cells were lysed by repeated sets of sonication; 30 sec bursts, 10-12 microns, with resting on ice in between. Lysed cells were centrifuged at 7,250g for 15 minutes to separate the soluble and insoluble fractions. At each stage samples were taken for analysis by SDS-PAGE. The recombinant class 3 protein was found to be present only in the insoluble cellular fraction.

Figure 4.11 – Expression trials of pPORB3 in *E.coli* JM101 and *E.coli* JM109 (DE3) in superbroth medium



The pPORB3 construct was transformed into *E.coli* JM101 and *E.coli* JM109 (DE3) and levels of protein expression compared in superbroth medium. Cell lysates were prepared from samples taken prior to induction and hourly afterwards. These samples were subjected to SDS-PAGE and the proteins visualised by PAGE-blue 83.

The lanes are as follows:

M	–	molecular weight standards	
1	–	H44/76 – parental strain	
2	–	pPORB3 in JM101	prior to induction with IPTG
3	–		prior to addition of phage
4	–		+ 1 hour
5	–		+ 2 hour
6	–		+ 3 hour
7	–		+ 4 hour
8	–		+ 5 hour
9	–	pPORB3 in JM109 (DE3)	prior to induction with IPTG
10	–		+ 1 hour
11	–		+ 2 hour
12	–		+ 3 hour
13	–		+ 4 hour
14	–		+ 5 hour
15	–	pRSETB in JM101	prior to induction
16	–		+ 5 hour
17	–	pRSETB in JM109(DE3)	prior to induction
18	–		+ 5 hour
M	–	molecular weight standards	

4.9 Purification Trials

The recombinant class 3 protein has an N-terminal fusion with a short leader peptide. Included in this leader peptide is a $(His)_6$ tag that functions as a metal binding domain. NTA (Qiagen) resin has been developed to have four chelating sites that can interact with metal ions. Thus NTA occupies four of the six ligand binding sites in the co-ordination of the Ni^{2+} ion, leaving two sites free to interact with the $(His)_6$ tag at an extremely high affinity, allowing any proteins bound non-specifically to be easily washed away under relatively stringent conditions without disrupting the $(His)_6$ tag bound protein (Invitrogen manual). The presence of detergents or high salt concentrations (0.1-1M) also has no effect on the protein binding. Elution of the tagged protein can be achieved by two methods. Reducing the pH results in the histidine residues becoming protonated and dissociating from the Ni-NTA resin. Imidazole is a molecule that is structurally similar to histidine and so competes with the histidine for binding on the Ni-NTA resin, displacing the tagged protein. Purification of the recombinant protein was performed using a 1.5ml Ni-NTA resin column. The column and cell lysate were prepared (section 2.17). Elution of the protein was achieved using four methods:

1) Reduction of pH: the column was eluted with 4 volumes buffer D (pH 5.9), followed by 4 volumes buffer E (pH4.5). The eluted protein was collected in 0.5ml fractions.
2) Reduction of pH, following selective washing: the solutions used to wash the bound protein contained 20mM imidazole to selectively elute weakly bound contaminants. Elution of the protein was achieved as above by lowering the pH with buffers D and E and collecting 0.5ml fractions.
3) Competition with imidazole: the protein was eluted with 4 volumes buffer C containing 250mM imidazole and fractions of 0.5ml were collected.
4) Competition with imidazole, following selective washing: the solutions used to wash the bound protein contained 20mM imidazole to selectively elute weakly

bound contaminants. Elution of the protein was achieved as above by including 250mM imidazole in buffer C and collecting 0.5ml fractions.

To determine in which fraction the recombinant class 3 protein had been eluted, samples from each of the washes and eluted fraction were analysed on an SDS-PAGE gel. A BCA protein assay was performed on each of the washes and fractions to determine the amount of purified recombinant class 3 that had been recovered in each trial (figure 4.12).

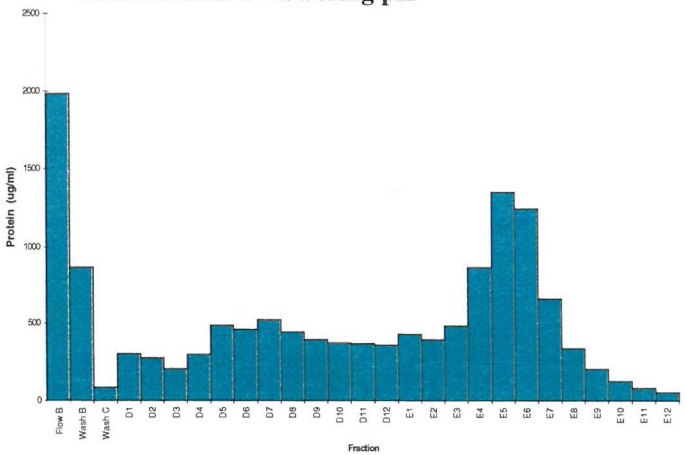
The fractions were analysed by SDS-PAGE and were shown to have a major band at 40kDA corresponding to the recombinant class 3 protein, followed by a series of bands of a smaller molecular weight. A Western blot was performed on these fractions using the anti-porin antibody R141 at 1/100 dilution (figure 4.13). The class 3 protein band and all the smaller bands reacted with the antibody and these were assumed to be breakdown products of the class 3 protein. Elution by competition with imidazole following removal of weakly bound contaminants by including a low concentration of imidazole in the wash buffers gave the highest protein concentration in fewer fractions (figure 4.14). This method was chosen as the method of elution for all further work.

4.10 Solubility Trials

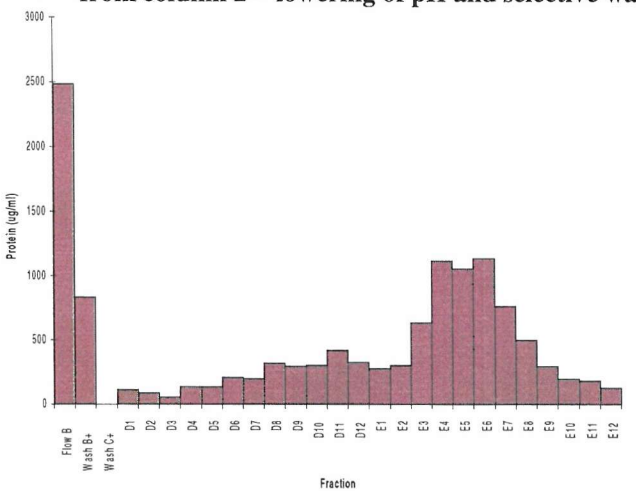
For future work, the solubility of the protein in different solutions needed to be established. A purified protein pellet was obtained by precipitating 300µl of the purification trial fractions containing the recombinant class 3 protein in 80% (section 2.17). The protein was solubilised in a variety of solutions by briefly vortexing and then incubating at 37°C for 1 hour. Any non-solubilised protein was removed by centrifugation at 12,250g for 10 minutes. The soluble and insoluble fractions were subjected to SDS-PAGE for analysis.

Figure 4.12 - Analysis of protein content of all fractions from purification trials

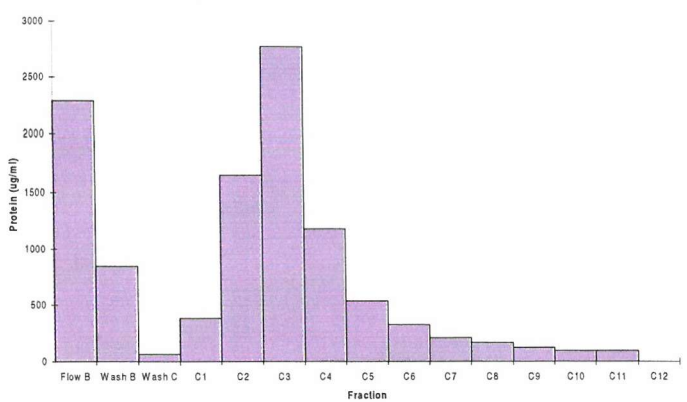
A) Protein conc^{n} from all washes and elute fractions from column 1 - lowering pH



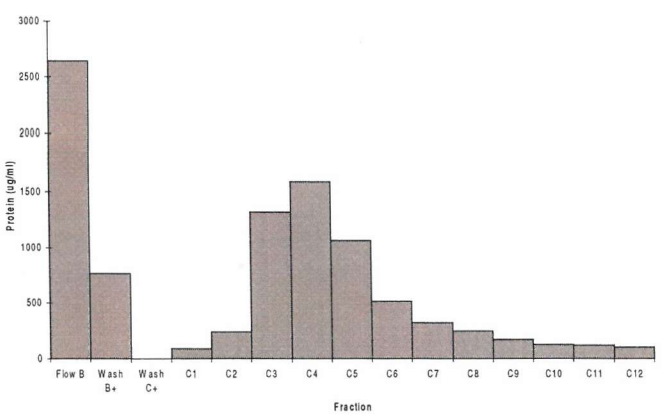
B) Protein conc^{n} from all washes and elute fractions from column 2 - lowering of pH and selective washing



C) Protein conc^{n} from all washes and elute fractions from column 3 - competition with imidazole

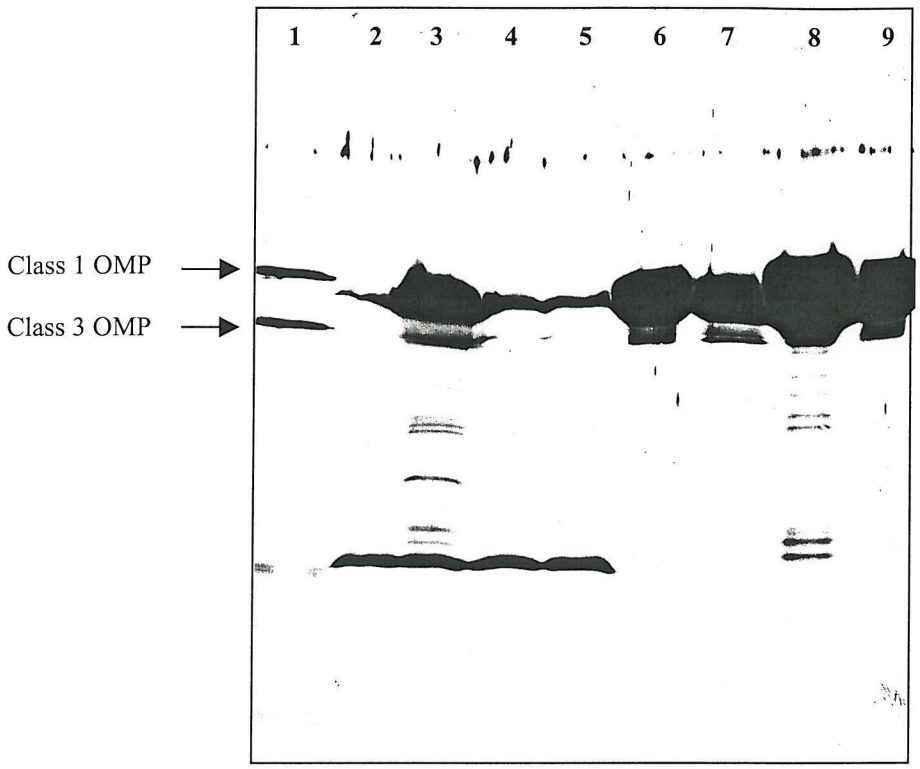


D) Protein conc^{n} from all washes and elute fractions from column 4 - competition with imidazoleand selective washing



Following productions of rCl3 protein in *E.coli* JM101, the protein was purified by elution from an Ni-NTA column. Elution of the protein was achieved by lowering of the pH or by the addition of imidazole. Both these methods were tested with and without the additional step of washing the bound column with low levels of imidazole to remove weakly bound contaminants. Equal volume samples from the washes and elution fractions were analysed for protein content by performing a BCA assay.

Figure 4.13 – Analysis by Western blotting of recombinant class 3 protein resulting from four purification trials

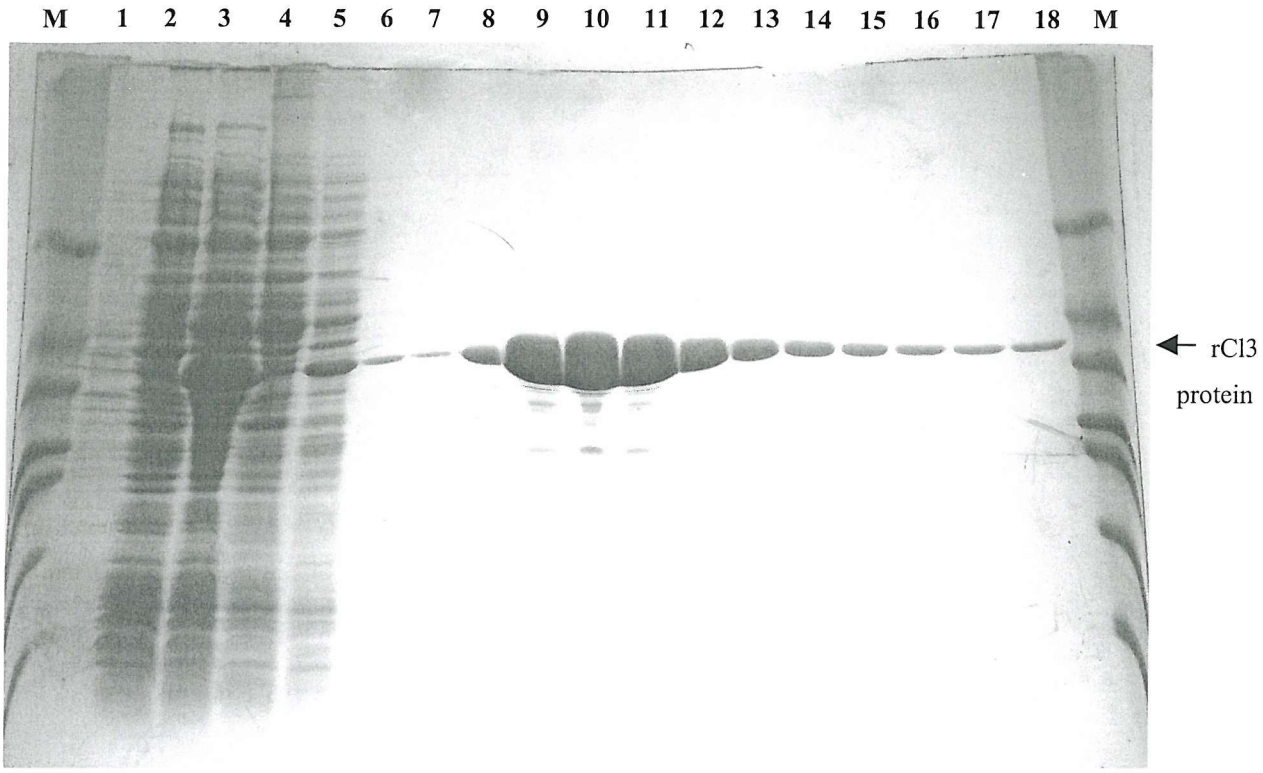


Protein that had been eluted from the Ni-NTA column in the four purification trials was analysed for protein purity by Western blotting. The eluted proteins were subjected to SDS-PAGE and the separated proteins transferred to nitrocellulose and probed with the anti-porin antibody, R141.

The lanes are as follows:

1 – H44/76 – parental strain
2 – pPORB3 in JM101, prior to induction
3 – pPORB3 in JM101, 5 hours after induction
4 – negative control: pRSETB in JM101, prior to induction
5 – negative control: pRSETB in JM101, 5 hours after induction
6 – purified protein from elution by lowering pH
7 – purified protein from elution with imidazole
8 – purified protein from elution by lowering pH, plus low conc^{n} imidazole washing
9 – purified protein from elution with imidazole, plus low conc^{n} imidazole washing

Figure 4.14 – Purification of the recombinant class 3 protein



Lysed cells that had previously been induced for expression of the recombinant class 3 protein were loaded onto a Ni-NTA column. The bound protein was washed with low levels of imidazole and then eluted by competition with imidazole. Samples from the washes and eluted fractions were subjected to SDS-PAGE and visualised by PAGE-blue 83.

The lane order is as follows:

M	–	molecular weight standards
1	–	H44/76 – parental strains
2	–	pPORB3 in JM101, prior to expression induction
3	-	pPORB3 in JM101, 5 hours after induction
4	–	cell lysate flow through
5		wash B, containing 20mM imidazole
6	–	wash C, containing 20mM imidazole
7	–	elute fractions: C1
8	–	C2
9	–	C3
10	–	C4
11	–	C5
12	–	C6
13	–	C7
14	–	C8
15	–	C9
16	–	C10
17	–	C11
18	–	C12
M	–	molecular weight standards

1) Solubility in urea:

Individual pellets of the recombinant class 3 protein were solubilised in 10mM Tris, pH7.4 (200µl) containing increasing concentrations of urea; 0.25M, 0.5M, 1M and 2M.

2) Reduction of the urea concentration by dilution:

The recombinant class 3 protein had readily solubilised in the buffer used in the purification procedure which contained 8M urea therefore a protein pellet was solubilised in 10mM Tris, pH8.0 (400µl) containing 8M urea and then diluted with 10mM Tris, pH8.0 to 1M urea.

3) Solubility in Octyl glucoside and SDS:

Individual pellets of the recombinant class 3 protein were solubilised in 10mM Tris, pH7.4 containing 10% octyl glucoside and varying concentrations of SDS; 0.01%, 0.05% and 0.2%.

4) Reduction of SDS concentration by dilution:

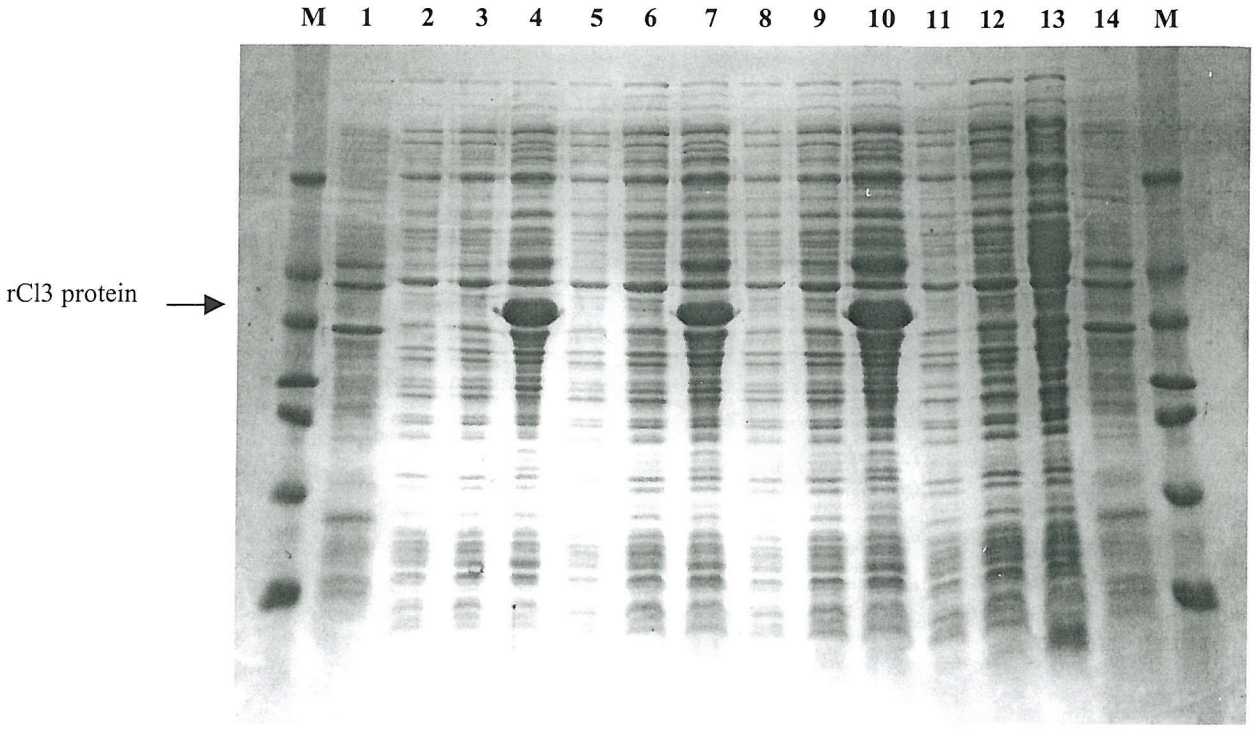
A pellet of recombinant class 3 protein was solubilised in 10mM Tris, pH8.0 containing 2% SDS. The concentration of SDS was then diluted from 2% to 0.004%. Due to the volume of the samples containing 0.0625% to 0.004% SDS, these were placed under vacuum to reduce the volume by approximately half.

The recombinant class 3 protein remained soluble only when diluted from a higher concentration solution, i.e. from 8M urea or 2% SDS. The protein was insoluble in octyl glucoside and in low concentrations of urea. A BCA assay performed on the diluted urea samples, found that the protein level remained constant in each of the dilutions. The level of protein in the diluted SDS samples remained constant from 2% to 0.0625%, however each further dilution approximately halved the protein content.

4.11 Large scale production and purification of recombinant class 3 protein

The protein expression procedure was scaled up to produce large amounts of the recombinant class 3 protein. Flasks containing 750ml superbroth-amp were inoculated with 15ml of an overnight culture and grown until an OD_{600} 0.3 was reached. Protein

Figure 4.15 – Large scale growth of recombinant class 3 protein

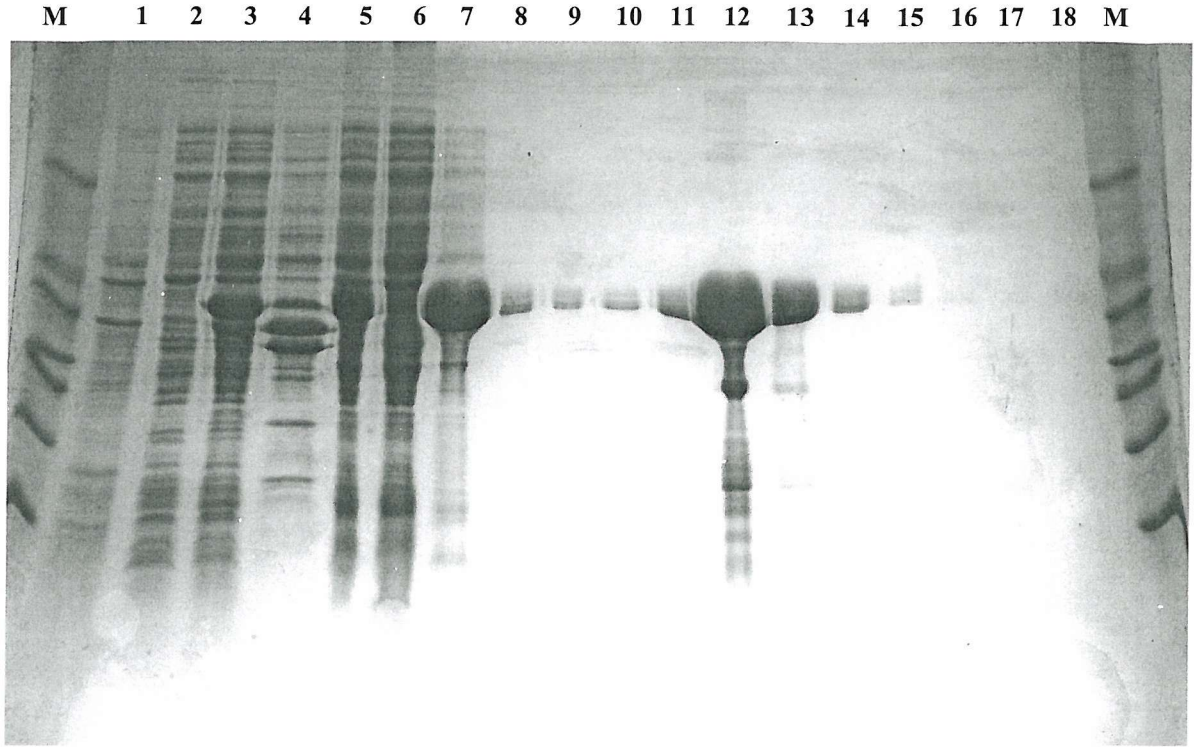


The pPORB3 construct in *E.coli* JM101 was induced to produce large amounts of recombinant class 3 protein. Samples were taken during the growth period, cell lysates prepared and subjected to SDS-PAGE. The separated proteins were visualised using PAGE-Blue 83.

The lane order is as follows:

M	–	molecular weight markers	
1	–	H44/76 – parental strain	
2	–	pPORB3 in JM101 (1)	t = -1 , prior to induction with IPTG
3	–		t = 0, addition of phage
4	–		t = +5 hours
5	–	pPORB3 in JM101 (2)	t = -1 , prior to induction with IPTG
6	–		t = 0, addition of phage
7	–		t = +5 hours
8	–	pPORB3 in JM101 (3)	t = -1 , prior to induction with IPTG
9	–		t = 0, addition of phage
10	–		t = +5 hours
11	–	pRSETB in JM101 (-ve control)	t = -1 , prior to induction with IPTG
12	–		t = 0, addition of phage
13	–		t = +5 hours
14	–	H44/76 – parental strain	
M	–	molecular weight standards	

Figure 4.16– Large scale purification of recombinant class 3 protein



Following production of large amounts of recombinant class 3 protein, the protein was purified by elution from an Ni-NTA column by competition with imidazole following washing of the column with low levels of imidazole to remove weakly bound contaminants. Samples from each of the washes and eluted fractions were analysed by SDS-PAGE and the proteins visualised by PAGE-blue 83.

The order of the lanes is as follows:

M	–	molecular weight markers
1	–	H44/76 – parental strain
2	–	pPORB3 in JM101, prior to induction
3	–	pPORB3 in JM101, 5 hours after induction
4	–	pellet obtained from centrifugation following sonication to lyse the cells
5	–	cell lysate loaded onto column
6	–	cell lysate flow through
7	–	wash B, containing 20mM imidazole
8	–	wash C, containing 20mM imidazole
9	–	elute fractions: C1
10	–	C2
11	–	C3
12	–	C4
13	–	C5
14	–	C6
15	–	C7
16	–	C8
17	–	C9
18	–	C10
M	–	molecular weight standards

expression was induced by the addition of IPTG (1mM) for 1 hour followed by the addition of M13/T7 phage (10pfu ml^{-1}) and incubation at 37°C with shaking. Cells were harvested by centrifugation at 6000g for 30 minutes at 10°C (figure 4.15).

Purification of the protein was performed as in the purification trials, but on a larger scale using a 5ml Ni-NTA column. The bound protein was washed with low levels of imidazole to remove weakly bound contaminants and then eluted with 250mM imidazole in buffer C (figure 4.16).

The eluted factions were analysed by SDS-PAGE and a BCA protein assay was performed to determine the protein levels in each. The fractions containing the recombinant class 3 protein were pooled. In total 75mg of recombinant class 3 protein was obtained. The recombinant class 3 protein was separated from the purification buffers by precipitation with 80% ethanol (section 2.18) and the purified protein pellets were stored at -20°C.

4.12 Removal of $(His)_6$ tag

The vector plasmid pRSETB contains a short leader sequence before the multiple cloning site. This contains the codons for the $(His)_6$ tag and also for an $(Asp)_4$-Lys element immediately preceding the multiple cloning site. $(Asp)_4$-Lys is the cleavage recognition site for enterokinase allowing removal of the leader sequence.

Approximately 1mg, purified, precipitated protein was solubilised in 10mM Tris, pH8.0 (200µl) containing 2% SDS then diluted to 0.032% SDS. The same amount of protein was also solubilised in 2M urea (200µl) and diluted to 1M. Reactions were performedusing recombinant enterokinase (rEK) from Novagen. Reactions were performed as suggested by the manufacturer containing 2µg protein, 1x cleavage buffer (supplied) and 0.2U μl^{-1} rEK enzyme in a final volume of 50µl made up with UHQ water. Samples were mixed and incubated at room temperature for 16 hours. The

reactions were terminated by lyophylisation. The samples were resuspended in 20µl SDS-PAGE loading buffer and analysed by SDS-PAGE (figure 4.17).

The gel showed that when the protein sample was solubilised in urea at both 2M and 1M concentrations, the rEK enzyme was inhibited and no cleavage seen. At 2% SDS the enzyme was again inhibited resulting in no cleavage. At SDS concentrations of 1%, 0.5% and 0.25%, non-specific cleavage of the protein occurred, possibly due to the SDS fully denaturing the protein and cleavage occurring non-specifically. At SDS concentrations of 0.125%, 0.063% and 0.032% some specific cleavage was present, although smaller sized bands were also present suggesting that following removal of the $(His)_6$ tag, non-specific cleavage had taken place, resulting in loss of most of the protein. The $(His)_6$ tag is very small (<4kDa) and has been shown to be non- immunogenic or at most poorly immunogenic in all species except monkeys (Invitrogen manual). Therefore on the results of this trial and the small size of the tag, removal of the $(His)_6$ tag was deemed unnecessary.

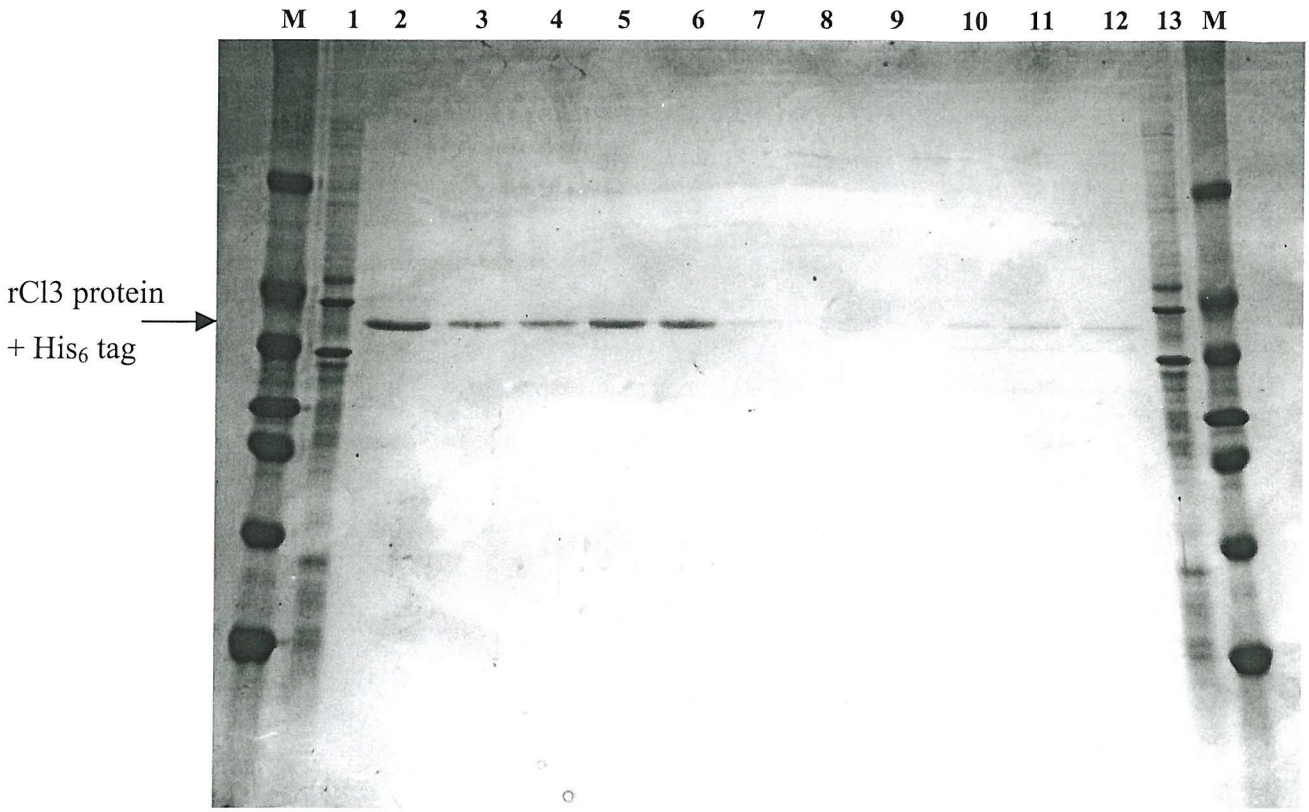
4.13 Protein Purity

Following purification of the recombinant class 3 from crude cell lysate, samples were analysed by SDS-PAGE and Western blotted with the antibody R141 (1/1000 dilution). As in the earlier trials, weak bands were visible below the recombinant class 3 protein band at 40kDa on the SDS-PAGE gel. However as these all reacted with the antibody R141 these were assumed to be break down products (figure 4.18).

To ensure no contaminating LPS was present a low-molecular weight gel was run and silver stained for the presence of LPS. LPS was shown to be present in crude cell lysate samples before purification but absent in the purified protein samples (figure 4.19).

This purified, recombinant class 3 protein was used in further studies to look at the immune response when used in immunisations.

Figure 4.17 – Digestion of purified recombinant class 3 protein with enterokinase

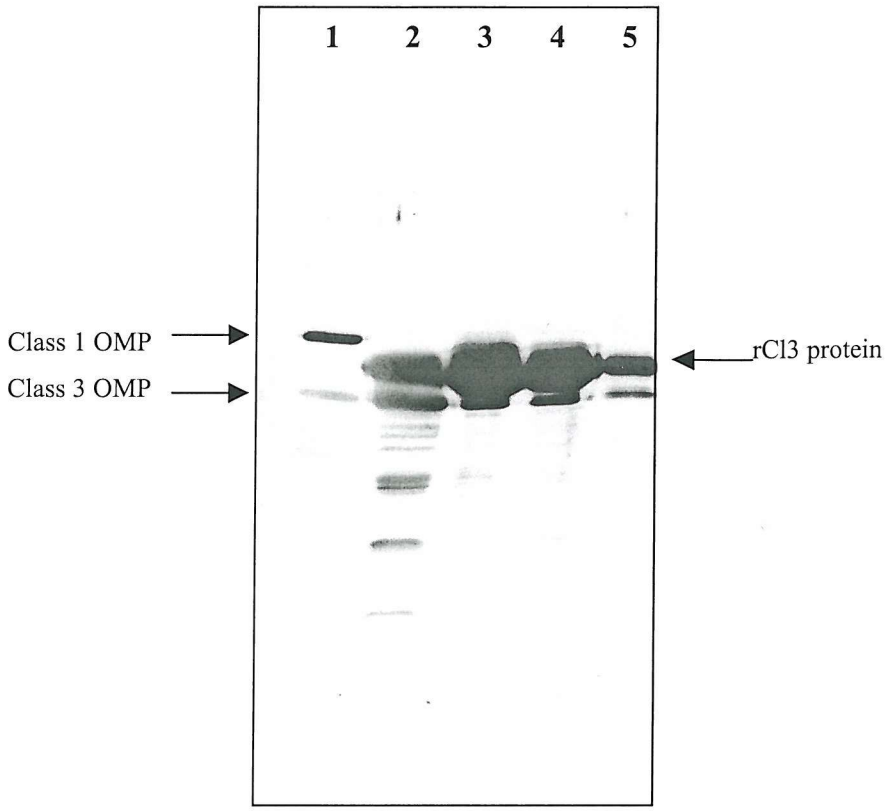


Recombinant class 3 protein was solubilised in urea or SDS and digested with enterokinase to remove the His_6 tag. The digested proteins were subjected to SDS-PAGE and the separated proteins visualised by PAGE-blue 83.

The lane order is as follows:

M	-	Molecular weight markers		
1	-	H44/76		
2	-	rCl3 in purification elution buffers		
3	-	rCl3 solubilised in	2M urea	digested with rEK
4	-		1M urea	digeste with rEK
5	-	rCl3 in purification elution buffers		
6	-	rCl3 solubilised in	2% SDS	digested with rEK
7	-		1% SDS	digested with rEK
8	-		0.5% SDS	digested with rEK
9	-		0.25%SDS	digested with rEK
10	-		0.125% SDS	digested with rEK
11	-		0.063% SDS	digested with rEK
12	-		0.032% SDS	digested with rEK
13	-	H44/76		
M	-	Molecular weight markers		

Figure 4.18 – Assessment of protein purity of the purifed recombinant class 3 protein by Western blotting

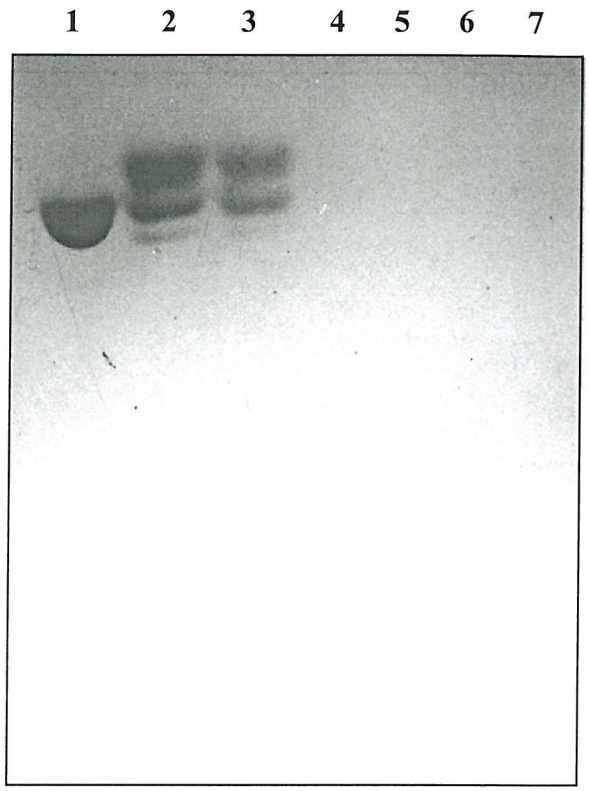


To determine the purity of the purified recombinant class 3 protein, varying amounts of protein were subjected to SDS-PAGE and the separated proteins transferred to nitrocellulose and probed with the anti-porin antibody, R141.

The lane order is as follows:

1 – H44/76 – parental strain
2 – pPORB in JM101, 5 hours after induction of expression
3 – 10μg purified, recombinant class 3 protein
4 – 5μg purified, recombinant class 3 protein
5 – 1μg purified, recombinant class 3 protein

Figure 4.19 – Assessment of protein purity of the purified recombinant class 3 protein by silver staining for LPS contamination



To determine the purity of the purified recombinant class 3 protein, varying amounts of protein solubilised in different solutions were separated on a low molecular weight gel, which was silver stained to detect for the presence of LPS. As controls, the parental meningococcal strain and non-purified protein in *E.coli* JM101 were included.
The lane order is as follows:

1 – H44/76 – parental strain
2 – pPORB3 in JM101, prior to induction
3 – pPORB3 in JM101, five hours after induction
4 – 5μg purified protein in elution buffer
5 – 1μg purified protein in elution buffer
6 – 1μg purified protein solubilised in 2M urea
7 – 1μg purified protein solubilised in 2% SDS

CHAPTER 5: IMMUNISATION WITH RECOMBINANT CLASS 3 PROTEIN

5.1 Introduction

In the previous chapter successful production and purification of the recombinant class 3 (rCl3) protein was demonstrated. The class 3 protein is proposed to exist as a transmembrane protein, threading through the membrane sixteen times with limited regions being presented to the immune system as eight surface exposed loops (van der Ley *et al*, 1991). Previous studies within the department had shown that immunisations with recombinant purified class 1 protein, which has an analogous structure to the class 3 protein, generated antiserum that reacted with the class 1 protein on SDS-PAGE and with meningococcal outer membranes in ELISA (White *et al*, 1990). The antibodies however also cross-reacted with both the class 2 and class 3 proteins on SDS-PAGE (White *et al*, 1990). Epitope mapping showed that the antibodies raised recognised sequences that are conserved amongst all meningococcal porin proteins and are non-surface exposed regions of the protein. Most importantly, the antibodies were unable to promote complement-mediated killing of meningococci *in vitro*. Therefore it was concluded that the purified class 1 protein did not resemble its native conformation and as a result was unable to direct the immune response towards the surface exposed, protective epitopes.

In an attempt to refold the protein to resemble the native conformation, Ward *et al*, (1996) inserted recombinant class 1 protein into the amphipathic bilayers of liposomes. Immune electron microscopy showed that the epitopes present on the surface of the liposomes reacted in a similar manner to protein in its native conformation. Following immunisations with this preparation, the antibodies raised recognised the class 1 protein on the surface of meningococci in a subtype specific manner and promoted complement mediated killing of the homologous strain.

In an alternative strategy Wetzler *et al,* (1992) attempted to overcome the refolding of the analogous gonococcal PI porin protein in a slightly different way. Amphipathic bilayers of detergent micelles were used to encourage refolding. When used in immunisations the antibodies raised recognised the PI protein in its natural conformation and it was concluded that the PI protein had correctly refolded and that the immune response had been directed against surface exposed loops, the antibodies raised however, proved to be non-bactericidal.

In the current study, in an attempt to deliver the rC13 protein in a state resembling its native conformation, so that the surface exposed loops would be presented to the immune system, two alternative approaches were undertaken. The rC13 was inserted into liposomes and incorporated into micelles.

5.2 Production of Liposomes

Two alternative methods of liposome production were employed; size-exclusion chromatography (Muttilainen *et al*, 1995a) and dialysis-sonication (Ward *et al*, 1996).

5.2.1 Production of liposomes by size-exclusion chromatography (SEC)

A lipid film was achieved by dissolving phosphatidylcholine (PC, 20mg, Sigma) and octyl-β-D-glucoside (100mg, Sigma) in 3ml chloroform : methanol (2:1) in an acid cleaned round bottom glass flask. Solvent was removed under vacuum with rotation of the flask in a 25°C water bath using a Büchi rotary evaporator to produce an even lipid film. The dried lipid film was solubilised in 5ml of 100mM Tris, pH8.0 buffer, containing 100mM KCl and 2% (w/v) octyl glucoside.

The 5ml liposome solution was loaded onto the top reservoir of a pre-equilibriated 50ml Sephadex G-50 column, carefully avoiding disturbance of the Sephadex. Once loaded on the column, liposomes were drawn through by gravity and the column was eluted with PBS. Twenty fractions (4ml) were collected. The fractions 4, 5 and 6 appeared

opalescent and the presence of liposomes in these fractions was confirmed by electron microscopy.

5.2.1.1 Incorporation of protein into liposomes

A pellet of purified rCl3 protein was solubilised in 100mM Tris buffer, pH8.0 containing 2% (w/v) SDS with sonication in a water bath (37°C; five sets of 30sec each). The protein concentration was determined by a BCA protein assay and the solubilised protein was diluted to a concentration of 1mg ml^{-1}. To ensure complete denaturation of the protein, a 1ml sample was boiled at 100°C for 5 minutes, diluted by addition to 4ml 100mM Tris buffer, pH8.0, containing 100mM KCl and 2% (w/v) octyl glucoside and left at room temperature for a minimum of 1 hour before use. This protein solution was used to solubilise the lipid film made as described above. Following vesicle formation by gel filtration as described, a sample of each eluted fraction was analysed by SDS-PAGE to determine the location of the recombinant class 3 protein. The protein was present in the same fractions that appeared opalescent confirming that the protein and liposomes were present in the same fractions.

5.2.2 Liposomes produced by dialysis sonication (D-S)

Liposomes were prepared using PC and cholesterol combined at a 7:2 molar ratio. A total of 20mg PC and cholesterol was dissolved in 3ml of chloroform in a glass round bottom flask. The solvent was removed under vacuum with rotation of the flask in a 25°C water bath using a rotary evaporator to produce an even lipid film. The dried lipid film was solubilised in 5ml 10mM HEPES buffer, pH7.2 containing 2% (w/v) octyl glucoside.

The solubilised liposome solution was transferred to a Colloidion sac (12,000 MW cut off, Sartorius), which had been soaked in water for one hour prior to use. The solution was extensively dialysed against PBS (pH7.2) for 72 hours at 4°C, to remove the detergent and induce multilamellar vesicle formation. On completion of dialysis, the resulting milky solution was sonicated for 30 x 1 minute at 10–12 microns amplitude,

with cooling on ice between each sonication. Sonication cleared the solution and promoted the formation of small, unilamellar membrane vesicles, which were observed by electron microscopy. Any insoluble material was removed by centrifugation at 1000g for 10 minutes.

5.2.2.1 Incorporation of protein into liposomes

A pellet of purified rCl3 protein was solubilised in 10mM HEPES buffer, pH7.2, 2% (w/v) SDS with sonication in a water bath (37°C; five sets of 30 sec each). The protein concentration was determined by performing a BCA protein assay and the solubilised protein was diluted to a concentration of 1mg ml^{-1}. To ensure complete denaturation of the protein a 1ml sample was boiled at 100°C for 5 minutes, then diluted by addition to 4ml 100mM HEPES buffer, pH7.2, containing 2% (w/v) octyl glucoside and left at room temperature for a minimum of 1 hour prior to use. This solution was used to solubilise a lipid film prepared as before and formation of liposomes achieved by extensive dialysis and sonication.

5.2.2.2 Incorporation of MPLA into liposomes

Although liposomes are able to act as adjuvants, addition of monophosphoryl lipid A (MPLA) to liposomes containing the class 1 protein has been shown to boost the immune response to protein antigens (Ward *et al*, 1996; Christodoulides *et al*, 1998). MPLA is a synthetic analogue of the LPS molecule of *Salmonella minesota* which is approximately 40-fold less pyrogenic than that of the intact lipid A moiety of LPS (Richards *et al*, 1989). Although MPLA has a reduce pyrogenicity, it is still a potent stimulator of the immune system (Verma *et al*, 1992). This adjuvant was incorporated in the lipid film at a ratio of 20mg lipid : 1mg MPLA, by inclusion in the initial preparation of the lipid film of 500µl of a 2mg ml^{-1} MPLA solution. Formation of liposomes was achieved by extensive dialysis and sonication.

5.3 Incorporation of solubilised recombinant class 3 protein in micelles

Detergent monomers are amphipathic molecules that self-aggregate in aqueous solution above a characteristic concentration called the critical micellar concentration (CMC) to form micelles. The micelle structurally resembles that of a phospholipid membrane and can therefore be used to re-nature proteins. The detergent Zwittergent 3-14 was chosen and the non-detergent amphipathic molecule NDSB-195.

5.3.1 Zwittergent 3-14

Zwittergent 3-14 is a synthetic zwitterionic detergent and belongs to the sulfobetaine group of detergents. Unlike most other amphipathic surfactants, Zwittergent retains its zwitterionic character over a wide pH range. A pellet of purified rCl3 protein was solubilised in 50mM Tris buffer, pH8.0, containing 100mM NaCl and 2% (w/v) SDS. A BCA protein assay was performed to determine the protein concentration (~4mg ml^{-1}). A 1ml solution was made containing 1mg of the solubilised protein, 8mg Zwittergent 3-14 and 2mg SDS in 50mM Tris, pH8.0, containing 100mM NaCl. This was solubilised by incubation at room temperature for 16 hours.

5.3.2 NDSB-195

NDSB-195 is a member of a class of non-detergent sulfobetaines. NDSB's are non-denaturing at high concentrations and have been used to improve the efficiency of solubilisation of membrane proteins. NDSB's also prevent protein aggregation and have been shown to facilitate the renaturation of chemically and thermally denatured proteins (Goldberg *et al*, 1996). A pellet of purified recombinant class 3 protein was solubilised in 50mM Tris, pH8.0, 100mM NaCl and 2% (w/v) SDS. A BCA protein assay was performed to determine the protein concentration (~4mg ml^{-1}). A 1ml solution was made containing 1mg of the solubilised protein 8mg NDSB-195 and 2mg SDS in 50mM Tris, pH8.0, 100mM NaCl. This was solubilised by incubation at room temperature for 16 hours.

5.4 Preparation of Immunogens

A series of immunogens containing the rC13 protein were prepared for immunisation of groups of mice.

5.4.1 Liposomes preparations

Liposome preparations containing recombinant class 3 protein were diluted with saline to give a concentration of 100μg ml^{-1}. Those liposomes containing both recombinant class 3 protein and the adjuvant MPLA were diluted in saline to give a protein and MPLA concentration of 100μg ml^{-1}. Liposomes containing MPLA only and liposomes containing class 3 protein only were each diluted with saline to a concentration of 200μg ml^{-1} and then mixed in equal proportions to give a final concentration of each of 100μg ml^{-1}.

As controls, equivalent volumes of liposomes without protein were diluted with the appropriate volume of saline. Liposomes containing MPLA only were diluted with saline to give a concentration of 100μg ml^{-1} MPLA.

5.4.2 Micelle preparations

The micelles prepared with Zwittergent 3-14 and NSBD-195 were each diluted with saline to give a protein concentration of 100μg ml^{-1}. A second preparation of each was made containing the adjuvant MPLA. A sample of the protein solution was diluted with saline and mixed with MPLA (1mg ml^{-1}) to give a final concentration of each of 100μg ml^{-1}.

5.4.3 Aluminium hydroxide ($Al(OH)_3$)

As a control, the recombinant class 3 protein was adsorbed to Alum. Alum is a 2% aluminium hydroxide gel adjuvant and together with aluminium phosphate are currently the only adjuvants licensed for human use. A pellet of purified rC13 protein was

solubilised in 50mM Tris buffer, pH8.0, containing 100mM NaCl, 0.2% (w/v) SDS and a BCA assay performed to determine the protein concentration (~4mg ml^{-1}). A sample was diluted with saline to give a concentration of 200μg ml^{-1} in a 700μl volume. This was then mixed with an equal volume of the adjuvant alum and mixed for 16 hours on a rotary mixer at 4°C.

5.5 Immunisation Protocol

The immunogen preparations prepared in section 5.4 were used for immunisation of mice. Seven week old, Balb/C (H-2^d) haplotype female mice were bled 24 hours prior to the first immunisation. Mice in groups of five were injected inter-peritoneally (ip) with each of the antigens described in table 5.1, on days 0, 14, 28 and 42. For each immunisation, mice received 20μg of protein or equivalent control in a 200μl volume. Two weeks after the last immunisation the mice were terminally bled by cardiac puncture. Serum was collected and was stored at -20°C.

Liposome preparations:
rC13 - Liposomes (SEC)
rC13 - Liposomes (D-S)
rC13 + MPLA - Liposomes (D-S)
[rC13 - Liposomes (D-S)] + [MPLA - Liposomes (D-S)]
Empty liposomes (SEC) – control
Empty liposomes (D-S) – control
MPLA - Liposomes (D-S) – control
Other immunogens:
rC13 + alum
rC13 + Zwittergent 3-14
rC13 + Zwittergent 3-14 + MPLA
rC13 + NDSB-195
rC13 + NDSB-195 + MPLA

Table 5.1 – Immunisation preparations

Seven mice housed with those receiving the immunogen preparations did not receive any injections and were bled at the same time points as normal controls.

5.6 Analysis of the immunogenicity of the sera raised against the various rCl3 immunisations

In order to test whether an immune response had been generated by any of the various immunisation preparations, antisera raised was tested by a number of methods against the recombinant protein and three meningococcal strains (table 5.2).

Strain	Serological profile
H44/76 – homologous, parental strain	B:15:P1.7,16
MC139 – heterologous, class 3 protein expressing strain	C:4,21:P1.1
MC114 – heterologous, class 2 protein expressing strain	B:2a:P1.2

Table 5.2 – Meningococcal strains used to test raised sera against

The antisera were tested against the above strains for the ability to react with rCl3 and outer membrane preparations in ELISA, against denatured cell lysates in Western blotting, against whole fixed meningococci and for the ability to promote complement mediated killing of live meningococci. The proportion of different antibody subclasses was also measured.

5.6.1 Immune response to immunisation antigens

a) ELISA against rCl3 protein

The antisera raised were first tested for their ability to recognise the immunogen, solubilised, denatured rCl3 protein in ELISA. Each mouse serum was tested individually and the mean antibody titre per group of mice was calculated for each immunisation preparation. All the terminal bleed antisera, raised by immunisation with

preparations containing the rCl3 protein reacted strongly against the homologous protein, whereas sera raised with the control preparations which contained no rCl3 protein did not react. Mouse sera from the pre-bleeds were pooled and showed no reactivity against the rCl3 protein. A group of control mice, which were housed together with those receiving immunisations and bled at the same time, also showed no reactivity to rCl3 protein (figures 5.1 and 5.2). Therefore each immunisation preparation used had induced antibodies against the rCl3 protein.

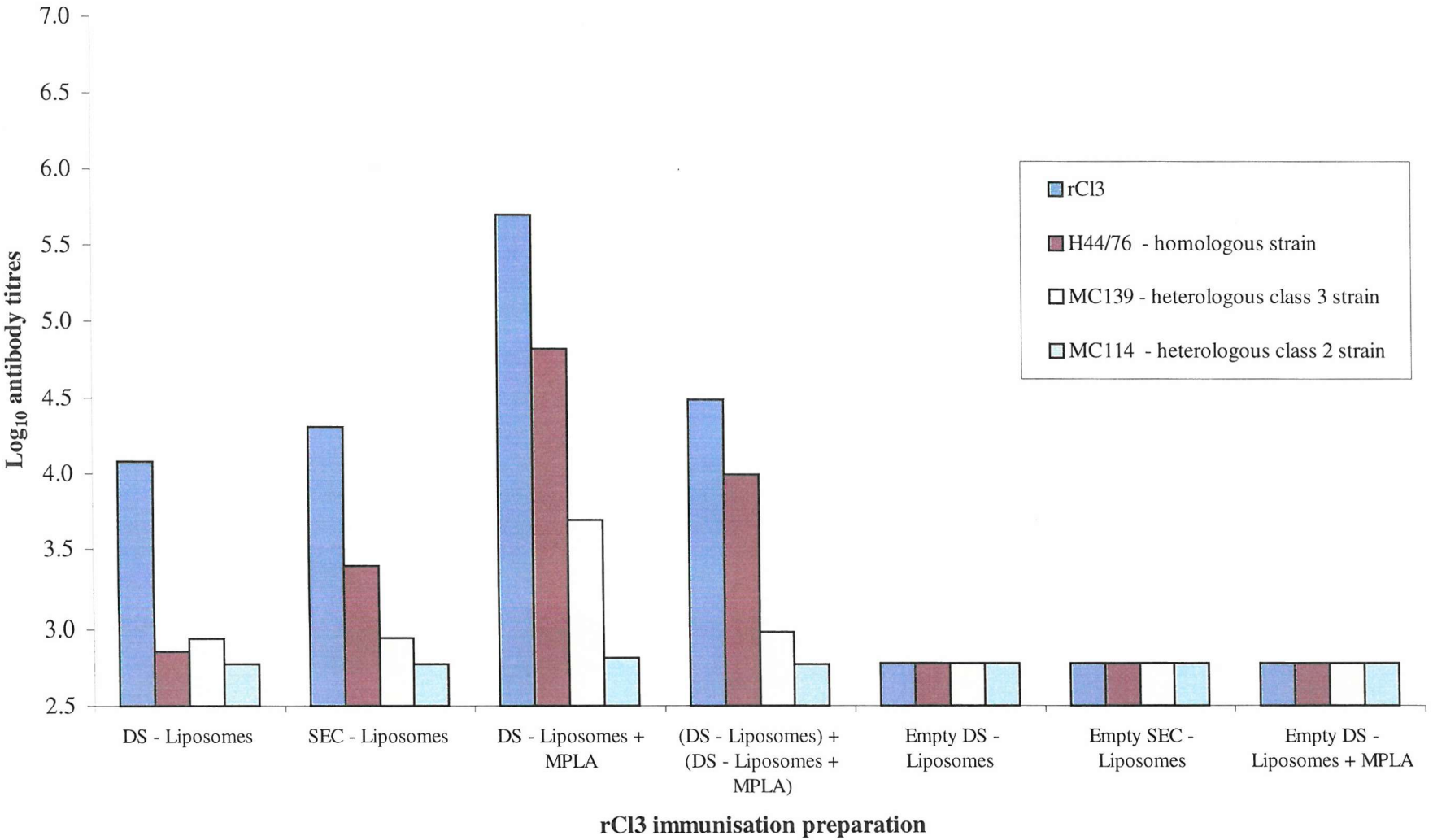
b) ELISA against outer membrane preparations

The class 3 protein is present in a more native conformation in outer membrane preparations. Therefore the antisera raised were also tested in ELISA against outer membranes from three strains: H44/76, the homologous class 3 strain; MC139, a heterologous class 3 strain and MC114, a heterologous class 2 strain. Each serum was tested individually and the mean antibody titre calculated (figures 5.1 and 5.2). The control immunisation preparations which contained no rCl3 protein failed to recognise any of the antigens used and showed the same antibody titres as sera from non-immunised mice.

The immunisation preparation consisting of rCl3 adsorbed to Alum resulted in antibody titres of 984 x 10^3 against denatured rCl3 protein, but showed only a low response against outer membranes of the homologous strain H44/76. No attempt at refolding the rCl3 protein was made in this preparation and so the protein remained in a denatured state. Therefore it is unsurprising that antibodies to a more native form of the protein were not detected.

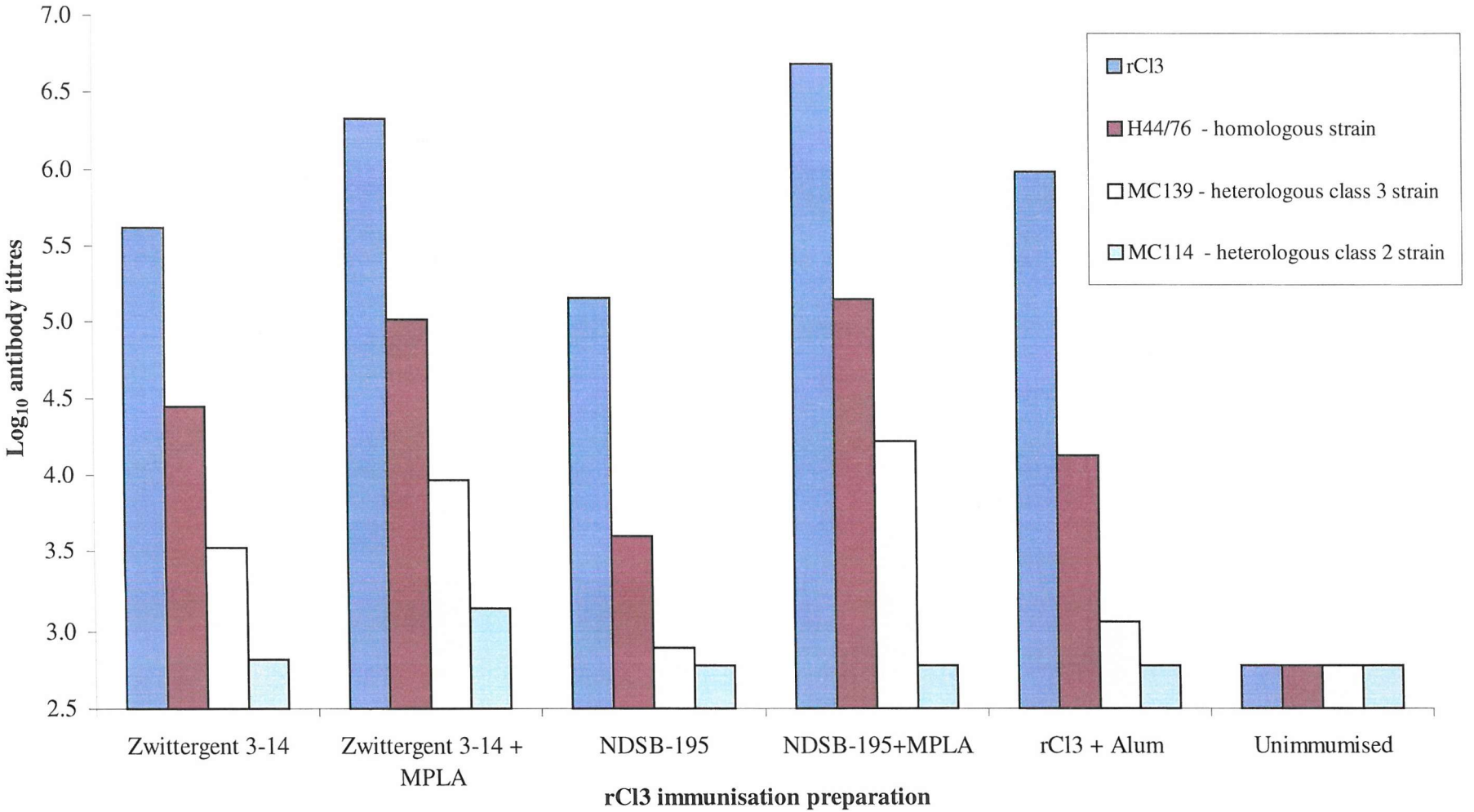
The various liposome preparations containing the rCl3 protein were all able to induce antibodies that were able to recognise the denaturecd rCl3 protein with titres ranging from 11.9 x 10^3 to 506 x 10^3. The liposomes made by the two different methods; dialysis-sonication and size exclusion chromatography, induced similar levels of

Figure 5.1 – ELISA reactivity of antisera obtained following immunisation with rCl3 liposome preparations



Sera resulting from immunisation with the liposome preparations were tested against denatured rCl3 protein and outer membranes from the homologous class 3 strain, the heterologous class 3 strain and the heterologous class 2 strain in ELISA.

Figure 5.2 – ELISA reactivity of antisera obtained following immunisation with rCl3 micelle preparations



Sera resulting from immunisation with the micelle preparations were tested against denatured rCl3 protein and outer membranes from the homologous class 3 strain, the heterologous class 3 strain and the heterologous class 2 strain in ELISA.

antibodies against the rC13 protein. The SEC liposomes induced antibodies that recognised outer membranes of the homologous class 3 strain, but not from a heterologous class 3 strain. When the adjuvant MPLA was included with the rC13 protein in the DS liposomes then the immune response was markedly increased and antibody titres of 506 x 10^3 were seen against the rC13 protein. High antibody titres were also observed using this immunisation preparation against outer membranes of the homologous class 3 strain and against a outer membranes from a heterologous class 3 strain. When a mixed population of liposomes containing either rC13 protein or the adjuvant MPLA was used for immunisation, then the antibody response was increased when compared to the levels observed when no adjuvant was included.

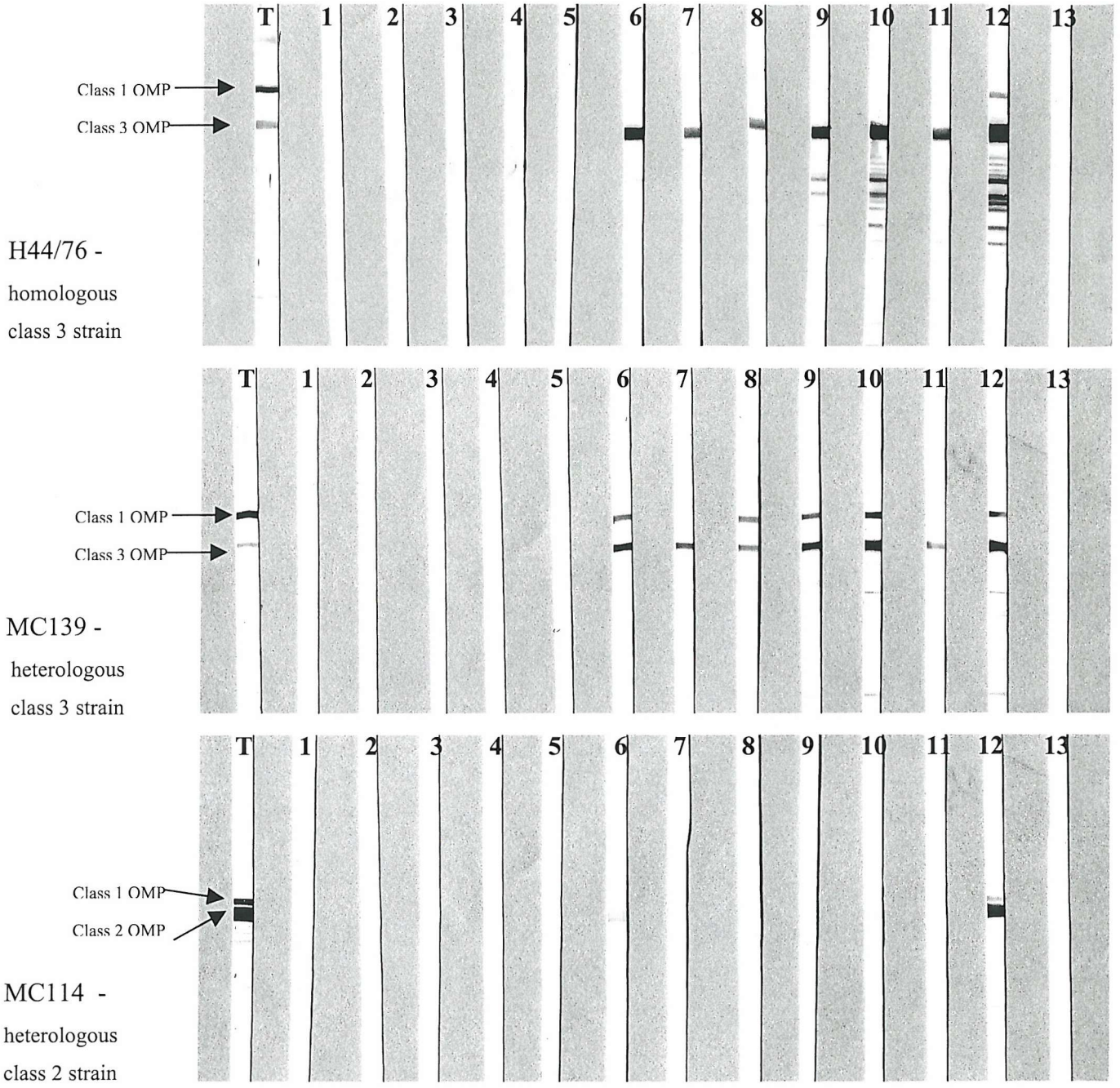
All the micelle preparations produced high antibody titres against the rC13 protein, ranging from 145 x 10^3 to 4915 x 10^3. The induced antibodies from the micelle preparations also reacted against outer membranes of the homologous class 3 strain and to a lesser extent outer membranes from a heterologous class 3 strain. When the adjuvant MPLA was included in the micelle preparations then the immune response to each of the antigens tested was boosted. When the adjuvant MPLA was included in the Zwittergent micelles then in addition to high levels of antibody against the two class 3 strains tested, a low level titre of antibodies against a heterologous class 2 strain were also detected (1.4 x 10^3).

5.6.2 Reactivity of antisera raised against various antigens by Western blotting

The different immunisation preparations containing the rC13 protein had all induced antibodies that recognised the rC13 protein and also recognised outer membranes of the homologous strain, H44/76 as detected by ELISA.

The sera were now tested in Western blotting experiments to assess the specificity of the sera. Whole cell lysates were prepared from three strains; H44/76, the homologous class 3 strain; MC139, the heterologous class 3 strain and MC114 the heterologous class 2 strain. To identify the PorA and PorB proteins, a strip from each strain was reacted with

Figure 5.3 – Reactivity of the antisera against denatured whole cell lysates as detected by Western blotting



Proteins present in whole cell lysates from the three test strains were separated by SDS-PAGE and transferred to nitrocellulose which was probed using neat sera which had been raised against the various immunisation preparations. The anti-porin antibody, R141 was also used to identify the class 1, 2 and 3 proteins.

The strip order is as follows:

T – test strip, blotted with R141, anti-porin antibody
1 – Empty liposomes (SEC)
2 – Empty liposomes (DS)
3 – MPLA + Empty liposomes (DS)
4 – rCl3 + liposomes (SEC)
5 – rCl3 + liposomes (DS)
6 – rCl3 + MPLA + liposomes (DS)
7 – [rCl3 + liposomes (DS)] + [MPLA + liposomes (DS)]
8 – rCl3 + Alum
9 – rCl3 + Zwittergent 3-14
10 – rCl3 + Zwittergent 3-14 + MPLA
11 – rCl3 + NDSB-195
12 – rCl3 + NDSB-195 + MPLA
13 – normal mouse sera

the anti-porin antibody R141 at 1/1000 dilution. The raised sera from individual mice in each group was pooled and used to probe nitrocellulose membrane strips (figure 5.3). To aid comparison the band intensity was scored relative to each other and a table compiled (table 5.3).

Antisera raised against immunisation preparation	Homologous Strain (H44/76)	Heterologous class 3 strain (MC139)	Heterologous class 2 strain (MC114)
Liposome Preparations:			
Empty liposomes (SEC)	-	-	-
rC13 + Liposomes (SEC)	2	1	-
Empty liposomes (D-S)	-	-	-
MPLA + Liposomes (D-S)	-	-	-
rC13 + Liposomes (D-S)	2	1	-
rC13 + MPLA + Liposomes (D-S)	4	3	3
[rC13 + liposomes (D-S)] + [MPLA + liposomes (D-S)]	3	2	1
Micelle preparations:			
rC13 + Zwittergent 3-14	4	3	-
rC13 + Zwittergent 3-14 + MPLA	5	4	2
rC13 + NDSB-195	3	2	3
rC13 + NDSB-195 + MPLA	6	4	4
rC13 + Alum	3	3	2
Non-immunised:	-	-	-

Table 5.3 - Results of immunoblotting using sera raised against the various immunisation preparations.

Sera were diluted 1 in 500 and the immune detection colour reaction allowed to develop for 10 min. before quenching. The band intensity was blind scored relative to each other for ease of comparison with 1 representing a weak reaction and 6 a very strong reaction, the symbol - indicates no reactivity.

Normal mouse sera and sera resulting from immunisation with the control preparations, i.e. Those not containing the rC13 protein showed no reactivity with any of the strains tested.

Liposomes containing rC13 protein alone, made by either of the two methods, resulted in sera that showed the same patterns of reactivity. Both sets of sera showed weak reactivity against the homologous strain H44/76 and barely detectable reactivity against the heterologous class 3 strain, MC139. When MPLA was included in the liposomes the reactivity of the resulting antisera was markedly increased against the homologous strain H44/76. It also showed significant reactivity against the heterologous class 3 protein and weak reactivity against the heterologous class 2 protein. When a mixed population of liposomes containing rC13 protein and MPLA were used for immunisation, the antisera raised did not react as strongly as when MPLA was included in the same liposomes as the rC13 protein. Cross-reactivity with the heterologous class 3 protein was also decreased. No cross-reactivity with the higher molecular weight, class 1 protein was seen for any sera raised against any of the liposome preparations.

The antisera to micelle preparations, in general produced higher levels of reactivity than the sera raised against the liposome preparations. The sera raised against the rC13 protein adsorbed to Alum showed equivalent levels of reactivity with the homologous heterologous class 3 proteins. Sera raised against rC13-Zwittergent 3-14 micelles showed good reactivity against the homologous and heterologous class 3 proteins but none against the heterologous class 2 protein. When MPLA was included in this preparation then the levels of reactivity were increased and cross-reactivity with the heterologous class 2 protein was seen. Sera raised against rC13-NDSB-195 micelles showed good reactivity with all three strains tested and this reactivity was markedly increased by the inclusion of MPLA in the preparation.

The antisera raised against the micelle preparations also showed reactivity against a band of higher molecular weight ~42kDa, this was most apparent when using the

heterologous class 3 strain, MC139. The class 1, class 2 and class 3 porins share large regions of homology, which are mostly confined to the transmembrane regions. When the general anti-porin antibody R141 was used the higher band was confirmed as the class 1 porin.

5.6.3 Reactivity of antisera raised against whole meningococci

The antisera raised against the various immunisation preparations had been shown in the immunoblotting experiments to be directed against a two proteins present in denatured cell lysates. The antisera were now tested for the ability to recognise the class 3 protein on the surface of whole meningococci.

Fixed meningococcal cell suspensions from the three test strains were incubated with pooled antisera, raised against the different immunisation preparations. Sera from individual mice in each group was pooled and used at a 1 in 10 dilution and the assay was performed as in section 2.20. The slides were viewed by confocal microscopy and images stored (figure 5.4). For ease of comparison the reactivity results were scored against each other (table 5.4).

Sera raised against the control preparations lacking the rC13 protein did not react with whole meningococci; similarly, normal mouse serum also failed to react. Antisera raised against liposomes containing rC13 protein alone, made by either of the two methods reacted very weakly against the homologous strain and were unable to recognise either heterologous strain. When MPLA was included in the liposomes then reactivity was greatly enhanced. Sera raised against the mixture of liposomes containing either rC13 protein or MPLA showed slightly lower levels of reactivity.

Sera raised against rC13 adsorbed to Alum were unable to recognise any strain of meningococci. The antisera to rC13-NDSB-195 micelles recognised meningococci of the homologous strain only weakly but this reactivity was increased by the inclusion of MPLA in the immunisation preparation. The antisera to rC13-Zwittergent 3-14 micelles

Figure 5.4 - Reactivity of sera obtained following immunisation with rC13 preparations against whole meningococci

To determine whether the sera raised against the rC13 protein could recognise whole meningococci of the homologous strain, immunofluorescence was used. The sera was added to fixed meningococci and then labelled with anti-mouse FITC conjugate (green). The slides were then counter stained with propidium iodide (red) to stain the meningococci.

The sera tested in the panels opposite are as follows:

A – empty liposmes (SEC)

B – rC13 in liposomes (D-S)

C – rC13 in Zwittergent 3-14

D – rC13 in Zwittergent 3-14 with MPLA

E – rC13 in NDSB-195

F – rC13 + alum

G – normal mouse sera

The bar represents 10μm

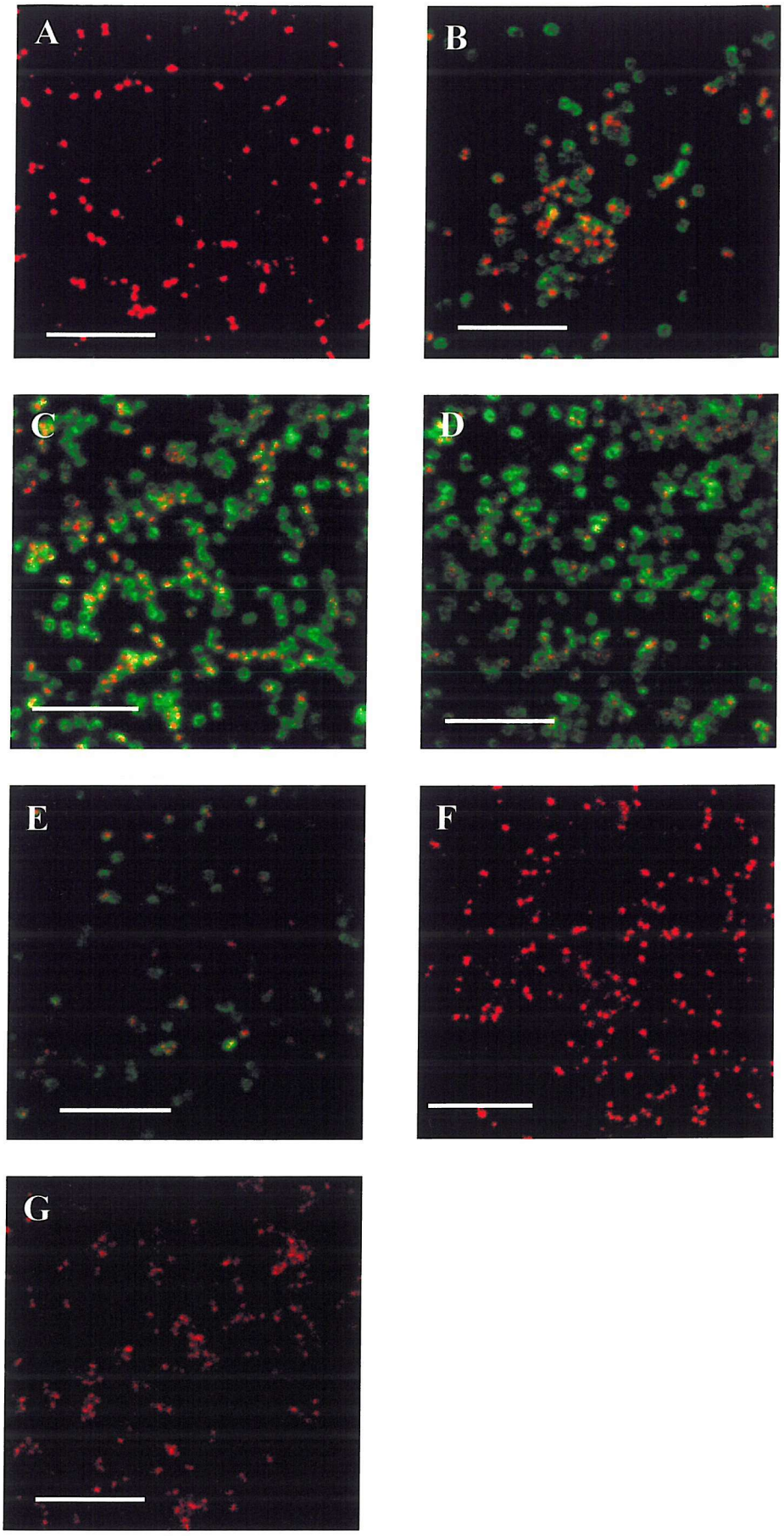


Figure 5.4 - Reactivity of sera obtained following immunisation with rCl3 preparations against whole meningococci

reacted strongly to the homologous strain and this reactivity was increased further by the addition of MPLA. The latter was the only one to recognise the heterologous class 3 strain albeit very weakly.

Antisera raised against immunisation preparation	Homologous strain (H44/76)	Heterologous class 3 strain (MC139)	Heterologous class 2 strain (MC114)
Liposome Preparations:			
Empty liposomes (SEC)	-	-	-
Liposomes + rCl3 (SEC)	+	-	-
Empty liposomes (D-S)	-	-	-
MPLA + Liposomes (D-S)	-	-	-
rCl3 + Liposomes (D-S)	+/-	-	-
rCl3 + MPLA + Liposomes (D-S)	+++	-	-
[rCl3 + liposomes (D-S] + [MPLA +liposomes (D-S)]	++	-	-
Micelle preparations:			
rCl3 + Alum	-	-	-
rCl3 + Zwittergent 3-14	+++	-	-
rCl3 + Zwittergent 3-14 + MPLA	++++	+/-	-
rCl3 + NDSB-195	+	-	-
rCl3 + NDSB-195 + MPLA	++	-	-
Non-immunised:	-	-	-

Table 5.4 - Reactivity of antisera raised against the various immunisation preparations against whole fixed meningococci

The results were blind scored against each other for ease of comparison.

These results show that antibodies capable of recognising the rC13 protein on the surface of whole meningococci had been elicited from all but one of the immunisation preparation containing rC13 protein, with the rC13 in Zwittergent 3-14 micelles with the addition of the adjuvant MPLA showing the strongest reactivity.

5.6.4 Determination of antibody subclasses present in antisera raised

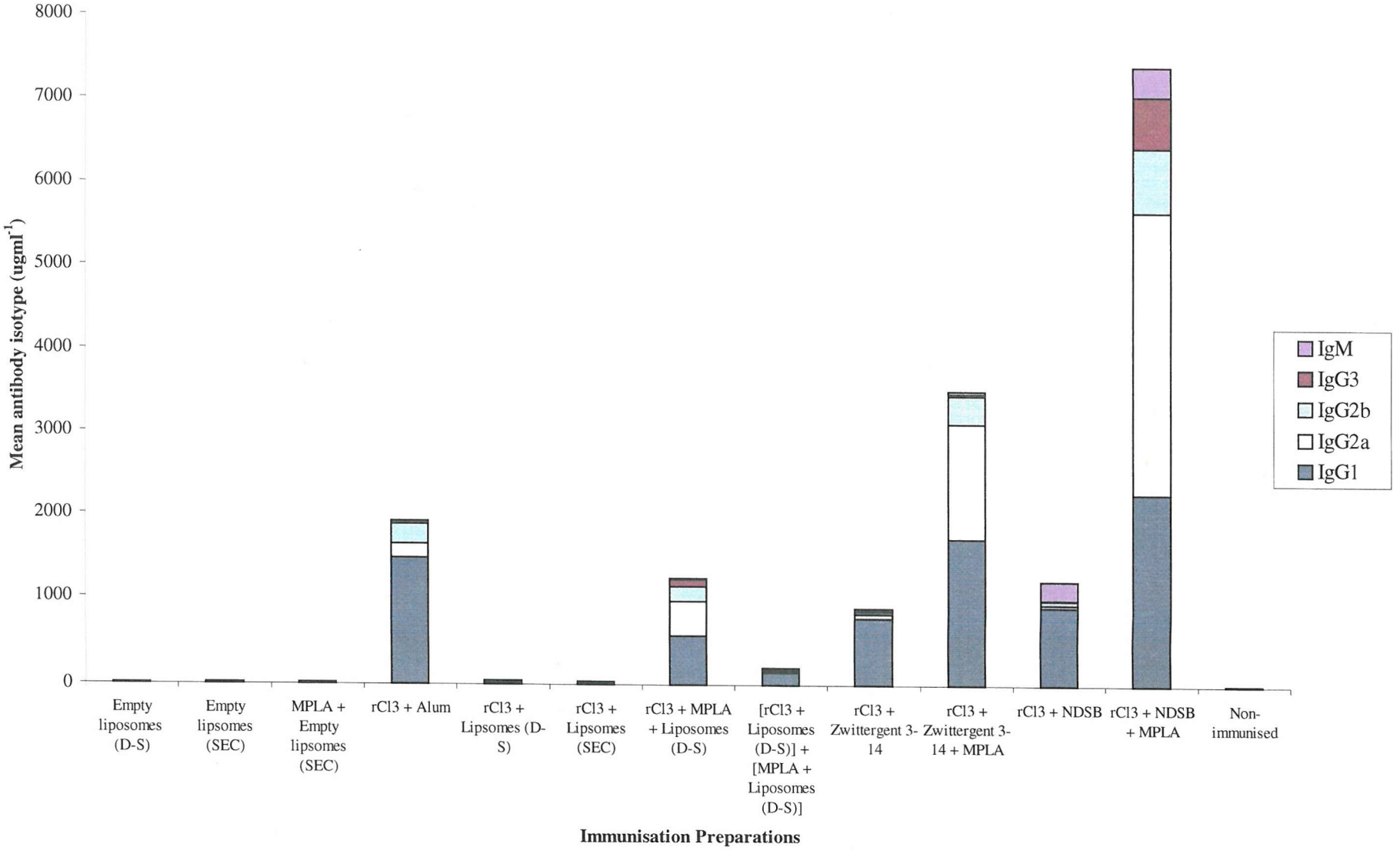
In order to determine the distribution of antibody subclasses present in the antisera the technique of isotyping ELISA was used (section 2.18) To quantify the levels of antibody in murine antisera, standard curves had been constructed using ^{125}I – labelled pure Ig sub-classes of defined specific activity (Christodoulides *et al*, 1998). The raised sera was reacted against the rC13 protein and outer membranes from the homologous class 3 strain (figures 5.5 and 5.6). The results show that a range of antibody subclasses were present in those sera which demonstrated high levels of reactivity against the rC13 protein or the homologous class 3 strain. The sera raised against the rC13 – NDSB-195 + MPLA showed the broadest range of subclass antibodies to be present.

5.6.5 Evaluation of bactericidal activity of antisera raised

The rC13 protein had been shown on immunisation to elicit a range of antibodies that were able to recognise whole meningococci. However as a potential vaccine candidate a vital attribute of the raised antibodies is whether they are able to promote *in vitro* complement-mediated killing of meningococci. The pooled antisera were tested initially for their ability to kill the homologous strain, H44/76. Bactericidal activity was assessed as described in section 2.21, and killing was quantified as the lowest dilution of sera able to kill 50% of meningococci present. The antisera raised against the different immunisation preparations showed a range of bactericidal activities (figure 5.7).

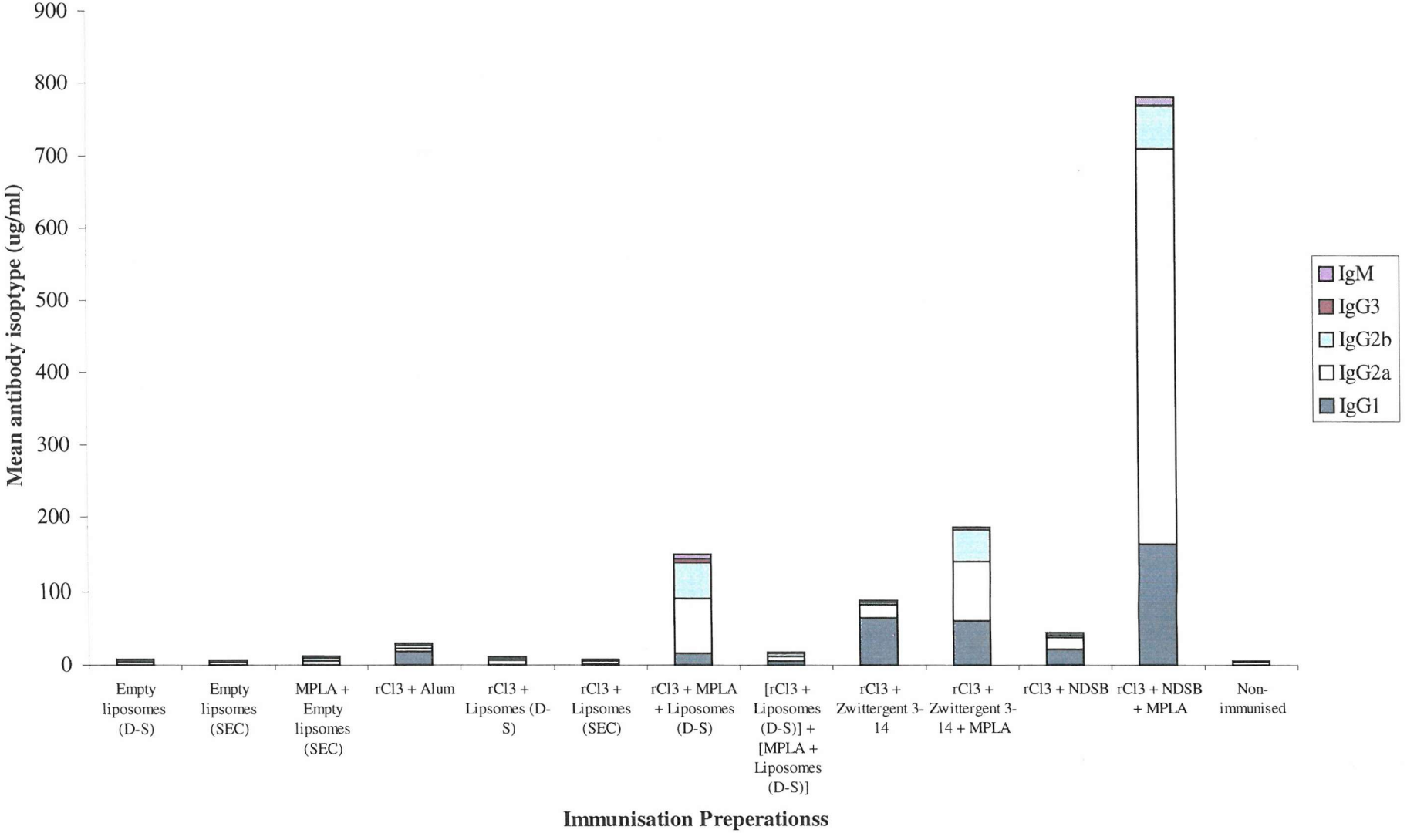
The antisera to rC13 protein adsorbed to Alum were unable to promote killing. Antisera raised to the liposomes containing rC13 alone had bactericidal titres of 1:8. When the adjuvant MPLA was included in the preparations, either in the same liposomes or in

Figure 5.5 – Analysis of isotypes reacting against rCl3 protein present in sera obtained following immunisation with rCl3 preparations



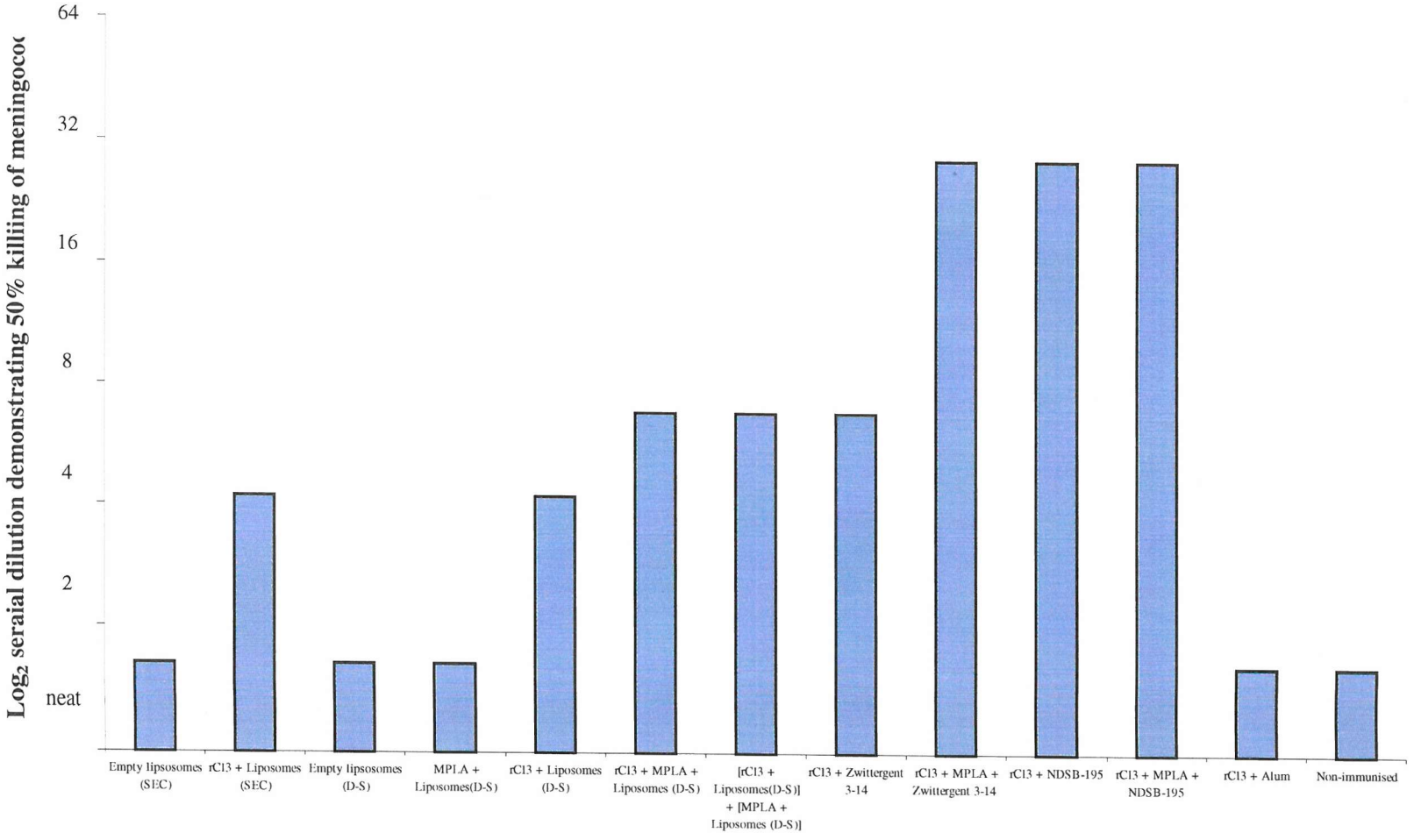
Concentrations of antibody subclasses in murine antisera were determined by ELSIA using enzyme conjugates of antibodies specific for mouse Ig subclasses. The ELISA was performed using denatured rCl3 protein.

Figure 5.6 – Analysis of isotypes reacting against proteins present in the outer membrane of H44/76 with sera obtained following immunisation with rCl3 preparations



Concentrations of antibody subclasses in murine antisera were determined by ELSIA using enzyme conjugates of antibodies specific for mouse Ig subclasses. The ELISA was performed using outer membranes for the homologous class 3 strain, H44/76.

Figure 5.7 – Bactericidal activity of sera obtained following immunisation with rCl3 preparations



Following immunisation with the various preparations, the antisera raised against the rCl3 protein were tested for ther ability to promote complement mediated killing of the homologous class 3 strain, H44/76. The bars represents the highest serum dilution at which 50% killing of live meningococci was achieved.

separate liposomes, this titre was increased to 1:16. The control liposomes were unable to promote killing.

Antisera raised against rC13-Zwittergent 3-14 micelles had a titre of 1 in 16, which was increased to 1 in 128 by the inclusion of MPLA. A titre of 1 in 128 was also demonstrated by rC13-NDSB-195 micelles, with and without the inclusion of MPLA. Antisera from these immunisation preparations was also tested for the ability to kill the heterologous strain MC139, but no significant killing of meningococci was achieved, suggesting that the ability to promote killing was serotype specific.

The rC13 protein had been successfully used in immunisations to produce antibodies able to recognise whole meningococci and had been able to promote complement mediated killing of live meningococci of the homologous strain. The rC13 had been presented in a variety of preparations with varied levels and types of antibodies being produced. In general, the micelle preparations produced much higher immune responses, with the rC13-NDSB-195 + MPLA preparation showing high antibody titres; cross-reactivity with the heterologous class 3 protein and also the class 1 and class 2 proteins; presence of a broad range of antibody subclasses and significant levels of bactericidal activity.

CHAPTER 6: DISCUSSION

Neisseria meningitidis is an obligate human pathogen, responsible for endemic and epidemic disease world-wide. Since 1980, the reported number of meningococcal disease cases in England and Wales has quadrupled (Ramsay *et al*, 1997). Meningococcal disease is largely due to serogroups A, B, C, W-135 and Y. For a number of years a tetravalent vaccine containing polysaccharide from serogroups A, C, W-135 and Y has been available, although it is poorly immunogenic in young children. In the last six months, an immunisation programme has been introduced in the UK, using the new Group C conjugate vaccine (Bradbury, 1999). This vaccine consists of depolymerised capsular polysaccharide from serogroup C, conjugated to the protein carrier CRM_{197}, a non-toxic mutant diphtheria toxin (Lieberman *et al*, 1996). Trials in toddlers using this vaccine resulted in an increase in antibodies against serogroup C polysaccharide and after three doses resulted in bactericidal activity against a serogroup C strain. (MacDonald *et al*, 1998). The results in the study show that the meningococcal C conjugate vaccine is highly immunogenic in toddlers and induces immunogenic memory to meningococcal polysaccharide that persists for at least 12 months.

Group B disease was responsible for about 60% of meningococcal disease in England and Wales in 1999 and with the arrival of a vaccine against group C disease that is effective in young children, a corresponding vaccine against serogroup B disease is ever more vital. Attempts to use the group B polysaccharide as a vaccine fails to induce measurable increases in antibodies. Possible explanations for this poor immunogenicity include sensitivity to neuraminidases and the polysaccharide's similarity to sialic acid moieties on human tissues which induces immunotolerance.

In an alternative approach the sub-capsular antigens in the outer membrane from group B strains have been viewed as possible immunogens. The outer membrane proteins show considerable diversity with approximately 20 different serotypes, based on the PorB protein and additional, more extensive antigenic diversity seen in the PorA

protein, the basis of the sero-subtyping scheme. Analysis of disease isolates has shown that a minority of serotypes predominates. In England and Wales in 1999, out of 541 typeable serogroup B strains, 355 were serotype 4, whilst 390 out of 492 typeable serogroup C strains were serotype 2a (Ramsay *et al*, 1997). The use of outer membrane proteins in a vaccine is supported by the observation that during group B infections, the bactericidal antibodies produced, are directed against non-capsular surface antigens (Frasch, 1979). Meningococci release large amounts of essentially pure outer membranes in culture broth as blebs or vesicles during normal growth. These have been used as the starting material for OMV vaccines in which the toxic LPS is removed by detergent extraction. Vaccine trials in Norway (Bjune *et al*, 1991) resulted in an efficacy of 57%, and importantly showed that antibodies induced to non-capsular surface antigens could protect against meningococcal disease. The outer membrane contains a number of proteins, including PorA, PorB, Opa, and Opc. In the OMV trials all these proteins are able to act as immunogens, but in order to fully evaluate the vaccine potential of a particular protein, it would be an advantage to have a source of protein that is free from other meningococcal components. Extensive work on the vaccine potential of the class 1 protein has been performed (Muttilainen *et al*, 1995a; Ward *et al*, 1996; Christodoulides *et al*, 1998). In each of these studies, the *porA* gene was cloned into an expression vector and expressed in *Bacillus subtilis* or *E. coli*. Antibodies resulting from immunisation with the purified recombinant class 1 protein whilst being bactericidal were sero-subtype specific.

All strains express either a class 2 or class 3 protein, which are encoded for by the two alleles of the *porB* gene. Whichever is expressed is quantitatively the major outer membrane protein (Tsai *et al*, 1981). Following the course of meningococcal disease the amount of antibodies specifically recognising the class 3 protein are significantly increased (Guttormsen *et al*, 1993). In this study the majority of the specific anti-class 3 IgG antibodies induced in patients infected with class 2 or non-typeable strains were shown to recognise surface exposed epitopes of the class 3 protein on whole bacteria, indicating the cross reactivity of the PorB and other outer membrane proteins. In a separate study by Lehmann *et al*, (1999), higher anti-PorB opsonic activity was

observed following meningococcal disease than anti-PorA opsonic activity. These studies coupled with the lesser variability of the PorB protein make it a good candidate for a potential vaccine.

Epidemiology of PorB

In the first section of work in this thesis, the epidemiology of the outer membrane protein, PorB was studied. Traditionally, epidemiological studies have relied on serological methods to differentiate between strains, with banks of monoclonal antibodies being available to identify the most common serotypes and sero-subtypes of the meningococci. The PorA protein shows most variability in loops 1 and 4 with a third semi-variable region at the apex of loop 5. The PorB protein has smaller surface exposed loops with major variation seen in loops 5 and 7 and to a lesser extent in loops 1, 4, 6 and 8. The epitopes that have been identified on the PorA protein are mostly linear, whereas with the exception of a linear epitope on the first loop of the class 3 protein (Delvig *et al*, 1995; Delvig *et al*, 1997), the PorB epitopes are conformational (Frasch, 1979). Sequencing studies of the *porA* gene have identified base changes, generating amino acid substitutions within subtype epitopes, that may or may not modify recognition by the relevant antibody (McGuinness *et al*, 1991; Suker *et al*, 1994; Brooks *et al*, 1995).

Since sequencing studies have revealed epidemiological information on the class 1 protein, work in this thesis was undertaken to investigate whether the class 2/3 protein could be used as an additional epidemiological marker. A collection of 22 strains from patients and close household contacts from an outbreak of meningococcal disease in 1992 allowed the opportunity to analyse the actual amino acid sequences of three of the outer membrane proteins as inferred from their DNA sequence. The sequencing of the *porA* gene had been previously been undertaken by Brooks *et al,* (1995). Brooks showed that although isolates may be serologically identical, a number of amino acid changes were present in VR1, VR2 and SV1 representing a hidden epidemiology. The *porB* gene of these isolates together with the *opc* gene was sequenced and the inferred

amino acid sequences compared in this thesis. Three groups had been shown by serotyping to be class 3 expressing isolates with each group being assigned a separate serotype. Within these family groupings, no distinction could be made between the case and the contact isolates on the basis of the PorB sequence. Six groups had been previously identified as class 2 expressing strains. Of the five families identified serologically as serotype 2b, three distinct variants were identified within this type that could not be detected serologically. In all the groups assigned a serotype no differences could be detected between the case and the contacts within a family grouping. One group of two isolates had been designated non-typeable by serological methods. Analysis by SDS-PAGE showed the isolates to express the class 3 protein. Sequencing of the *porB* gene revealed 13 amino acid differences between these two isolates. When the amino acid sequences were compared with representative class 3 serotypes from GenBank on a dendrogram, then the case was identified as being most like a serotype 12 stain and the carrier isolate was identical to a serotype 8 sequence in the database.

A third outer membrane protein from these isolates was also sequenced. The Opc protein is expressed by approximately 60% of meningococci (Olyhoek *et al*, 1991). In a study by Seiler *et al,* (1996) of 152 strains, eighteen distinct alleles were identified with polymorphisms evenly distributed throughout the coding region. Currently, the only available monoclonal antibody against Opc simply recognises its presence or absence. The *opc* gene is phase variable, with regulation of expression occurring at the transcriptional level (Sarkari *et al*, 1994). Of the 22 isolates, nine were identified as possessing the *opc* gene by PCR using specifically designed primers. These nine isolates belonged to four family groups and analysis of the inferred amino acid sequences showed four distinct Opc sequences. Three groups showed no differences between case and carrier isolates. In the fourth group the case isolate proved to have an identical *opc* gene to that of another family, whilst the carrier was a distinct variant. Western blotting experiments showed that the case and carrier isolate that were non-identical, were the only two isolates that possessed an *opc* gene, but did not express the Opc protein.

Comparison of the results of the *porA*, *porB* and *opc* sequencing showed that for one family group, the case and contact could not previously be distinguished between on the basis of *porA* sequencing, but were clearly different following *porB* and *opc* sequencing representing further hidden epidemiological information, that was non-detectable by serological methods.

Possible linkage of the *porB* gene

Whilst analysing these 22 isolates an apparent pattern regarding the distribution of the genes emerged. All the class 3 expressing strains possessed the *opc* gene, although not all expressed the protein, whilst none of the class 2 expressing strains possessed the *opc* gene. In this small collection of strains, it appeared that expression of the class 3 protein might be linked to possession of the *opc* gene. Therefore the possibility of linkage was investigated. Another meningococcal surface protein, which exhibits allelic variation, is pilin. The pilus is composed of repeated subunits of the polypeptide pilin. In the meningococcus, two types of pilin are found, termed class I and class II, coded for by two alleles of the *pilE* gene. The majority of meningococci express class I pilin which is similar in structure to the gonococcal pilin subunit and is able to react with the monoclonal antibody SM1, raised against the gonococcal pilin. As expression of the class of pilin is mutually exclusive, then there was the possibility that this too might be linked to expression of class 2 and class 3 outer membrane proteins. Western blotting of all the case isolates revealed that all the class 3 expressing strains expressed class I pilin. From this small subset of isolates a pattern of genes appeared to be emerging. The class 3 porin appeared to be associated with possession of the *opc* gene and expression of class I pili. In order to establish whether this observation was universal, a second study was undertaken using a larger number of strains obtained from the laboratory collection. These strains were chosen as belonging to a variety of serogroups, serotypes and sero-subtypes. Distribution of the genes was analysed by using primers in PCR that allowed amplification of the gene and allowed identification of alleles based upon size. Analysis of the PCR products showed that the genes were randomly distributed and no association was present.

Sequencing of the *porB* gene and comparisons with those from GenBank had shown that PorB demonstrates less variability than PorA and therefore work now focussed on using PorB as a vaccine candidate.

Vaccine studies using the PorB protein

The strain H44/76 was chosen as the parental strain from which the *porB* gene would be used. H44/76 (B:15:P1.7,16) was first associated with an increased rate of infection in Norway in the 1970's and was subsequently found to be responsible for increased infection rates in many parts of England and Wales and in Europe (Poolman *et al*, 1986). In the Norwegian vaccine trial, outer membrane vesicles were prepared from H44/76 and used to immunise 171,800 students (Bjune *et al*, 1991). The protection rate against group B diseases was calculated as being 57%. Bactericidal antibodies against Opc and Class 1 outer membrane proteins were found after three doses of the vaccine and along with antibodies recognising the class 3 and class 5 outer membrane proteins (Rosenqvist *et al*, 1995). These results suggest that the outer membrane proteins have the ability to provide immunity, but alone are not good enough. A number of studies have been undertaken looking at the potential of the class 1 protein as a vaccine candidate, therefore in this thesis work was focussed on the class 3 protein.

The PorB protein is functionally and immunologically equivalent to the gonococcal protein I, which in early studies was found to be lethal if expressed in *E. coli* (Carbonetti, 1987). However, removal of the 19 amino acid leader sequence, results in large amounts of protein being expressed as inclusion bodies (Qi *et al*, 1994). The PorA protein is highly homologous to the PorB protein and this has been successfully cloned and expressed on a plasmid vector in *Bacillus subtilis* (Muttilainen *et al*, 1995b). The PorA protein has also been expressed in the Xpress™ system in *E.coli* (Christodoulides *et al*, 1998) and this system was initially investigated as a method of preparing class 3 protein free from other meningococcal cell components.

The *porB* gene was amplified by PCR and cloned into two expression systems. The Xpress™ System (Invitrogen) uses pUC-derived expression vectors designed for high-level protein expression in *E.coli*. The gene is inserted in a multiple cloning site downstream and in frame with a sequence that encodes an N-terminal fusion peptide. This sequence includes an ATG initiation codon and a series of six histidine residues that function as a metal binding domain in the translated protein, allowing a one-step purification procedure by immobilised metal affinity chromatography. A T7 promoter drives expression of the recombinant protein and a T7 RNA polymerase is introduced to the system via an M13 phage carrying the T7 RNA polymerase gene fused to the *E.coli lac* promoter. Expression of the gene can then be induced by the addition of IPTG (Invitrogen manual).

As an alternative the *porB* gene was also cloned into the IMPACT™ System (New England Biolabs). IMPACT™ (Intein Mediated Purification with an Affinity Chitin binding Tag) has evolved from studies of protein splicing mechanisms. The IMPACT™ system utilises a protein splicing element, an intein from *Saccharomyces cerevisiae* that has been modified so that at low temperatures and in the presence of thiols, self-cleavage occurs at the N-terminus. The gene of interest is cloned into a multiple cloning site to create an in frame fusion between the C-terminus of the gene and the N-terminus of the intein coding gene. At the C-terminus of the intein gene, DNA encoding a 5kDa chitin binding domain from *Bacillus circulas* has been added. One step purification is achieved by passing crude cell extract over a chitin column, allowing the fusion protein to bind. Addition of a thiol, such as β-mercaptoethanol causes self-cleavage of the fusion protein, leaving the intein-chitin binding domain bound to the column. In trials of this system a number of proteins were successfully purified (Chong *et al*, 1997)

In order to achieve maximum protein expression in these systems a number of constructs were made and tested in a number of cell types. The Xpress™ System gave significantly more rC13 protein as detected by SDS-PAGE and Western blotting than did the IMPACT™ System, and was therefore used in all further work. Five constructs, which consistently gave high expression levels, were sequenced to ensure that there

were no possible mutations that might have arisen from the original PCR amplification of the gene. A construct with an identical sequence to that of the original gene in H44/76 was selected. To further maximise levels of protein expression, the construct was transformed into two different cell type; JM101 and JM109 (DE3). Expression in JM101 requires the addition of M13 phage, whereas *E.coli* strain JM109 (DE3) has been constructed to contain the T7 RNA polymerase gene fused to the *E.coli lac* promoter, so eliminating the phage step (Yanisch-Perron *et al*, 1985). Expression in these two cell types was tested in a variety of media and maximum expression was achieved using the *E. coli* JM101 grown in superbroth medium. Analysis of the *E.coli* cells following induction of expression, showed that the rCl3 protein was not secreted and remained in the insoluble cellular fraction, following lysis by sonication.

Having expressed large quantities of rCl3 protein in *E.coli*, the $(His)_6$ tag was utilised to allow purification from the *E.coli* components. The crude cell lysates were run through a Ni-NTA column allowing the rCl3 protein to bind to the column and extraneous material to be washed away. The bound protein was eluted by the addition of imidazole, which competes with the histidine for binding on the Ni-NTA resin and displaces the tagged protein. The eluted protein was shown by SDS-PAGE analysis, Western blotting and silver staining for the presence of LPS to be of a highly pure nature.

To remove the $(His)_6$ tag a sequence encoding an $(Asp)_4$-Lys element was included immediately preceding the multiple cloning site. $(Asp)_4$-Lys is the cleavage recognition site for enterokinase, allowing removal of the leader sequence. A small amount of the purified rCl3 protein was digested with enterokinase and the results analysed by SDS-PAGE. The class 3 protein does not contain the $(Asp)_4$-Lys element, however non-specific cleavage was observed, resulting in loss of most of the protein. The $(His)_6$ tag is very small (<4kDa) and in studies by the manufacturer (Invitrogen), the tag has never been found to interfere with the structure or function of the purified protein. Invitrogen also state that the $(His)_6$ tag is non-immunogenic or at most very poorly immunogenic in all species except some monkeys. In the study by Christodoulides *et al,* (1998), the class 1 protein was expressed in the Xpress™ System, the $(His)_6$ tag was not removed and

following immunisation, the antibodies induced were able to recognise the homologous protein. Therefore, the non-removal of the $(His)_6$ tag appeared unlikely to be detrimental and was therefore left attached to the rC13 protein.

The class 3 protein like other meningococcal porin proteins is proposed to exist as a transmembrane protein, threading through the membrane 16 times, with only limited regions being presented to the immune system as eight surface exposed loops (van der Ley *et al*, 1991). In a study by White *et al,* (1990), sera raised following immunisation with purified recombinant class 1 protein with a large leader sequence was analysed. Epitope mapping showed that the antibodies raised recognised sequences that are conserved amongst all meningococcal porin proteins, particularly in non-surface exposed regions of the protein. These antibodies were unable to promote complement-mediated killing of meningococci *in vitro*, the most important attribute of a potential vaccine candidate. In an attempt to refold the protein to resemble the native conformation, Ward *et al,*(1996) inserted the class 1 protein into the amphipathic bilayers of liposomes. Immune electron microscopy showed that the epitopes present on the surface of the liposomes reacted in a similar manner to protein in its native conformation. Following immunisation with this preparation, the antibodies raised were able to recognise class 1 on the surface of meningococci in a subtype specific manner and were also able to promote complement mediated killing of the homologous strain.

In an alternative approach, Wetzler *et al,* (1992) attempted to overcome the refolding of the analogous gonococcal P1 porin protein in a slightly different way. Amphipathic bilayers of detergent micelles were used to encourage refolding. The antibodies raised against this preparation were able to recognise native PI protein, but did not promote complement mediated killing, however it was concluded that the protein had correctly refolded.

The PorB proteins are porins, which exist as trimers, with each monomer forming an ion-conducting channel (Lynch *et al*, 1984). The electrophysiological properties of the class 3 protein have been investigated by Song *et al,* (1998) and Minetti *et al,* (1997). In

both these studies, the class 3 protein was over-expressed in *E. coli* as inclusion bodies. In the study by Song, the class 3 protein was refolded by insertion into phospholipid membranes and in the study by Minetti, by solubilisation in Zwittergent 3-14. As a comparison, the class 3 protein was isolated from meningococcal strains lacking the class 1 and class 4 protein and reconstituted in a similar manner to the recombinant protein. These studies showed the need for the isolated proteins to be re-assembled in order to function normally.

Unlike the class 1 protein, epitopes on the class 3 protein are conformational (Frasch, 1979). Only one linear epitope has so far been identified in the class 3 protein, which is in the surface exposed loop 1 region (Delvig *et al*, 1995). Therefore in order to induce antibodies that can recognise native class 3 expressed in meningococci, it was vital refolding of the rC13 protein was attempted.

The rC13 protein was incorporated into liposomes in an attempt at making the protein resemble its native conformation. Two alternative methods of producing the liposomes were used; size exclusion chromatography (Muttilainen *et al*, 1995a) and dialysis-sonication (Ward *et al*, 1996). As an alternative to liposomes, two amphipathic molecules were used; Zwittergent 3-14 and NDSB-195. Zwittergent had been shown by Mandrell and Zollinger (1984) to restore antibody binding to denatured meningococcal outer membrane proteins. Ward, (1995) also used Zwittergent 3-14 in an attempt to refold the class 1 protein. Following immunisation, the antibodies raised were able to direct the immune response against exposed regions of the native protein. NDSB-195, is a member of a new class of mild solubilisation agents (Vuillard *et al*, 1995a). NDSB-195 has been successfully used to solubilise proteins (Vuillard *et al*, 1995b) and has also been shown to facilitate the renaturation of chemically and thermally denatured proteins (Goldberg *et al*, 1996). NDSB-195 has also been used to renature the separate subunits of human DNA helicase II/Ku autoantigen and restore functional activity (Ochem *et al*, 1997). Therefore the rC13 protein was incorporated into both the Zwittergent 3-14 and NDSB-195 micelles.

In order to boost immune response, the adjuvant MPLA was included in a liposome preparation and in two micelle preparations. MPLA is a synthetic analogue of the LPS molecule of *Salmonella minesota* which is approximately 40-fold less pyrogenic in humans than that of the intact lipid A moiety of LPS (Richards *et al*, 1989). Although MPLA has a reduce pyrogenicity, it is still a potent stimulator of the immune system (Verma *et al*, 1992).

In order to evaluate the immunogenicity of the refolded rC13 protein, all the above preparations were used to immunise mice. As a control, the rC13 protein was adsorbed to Alum, with no attempt at refolding. Alum is a 2% aluminium hydroxide gel adjuvant and together with aluminium phosphate are the only adjuvants currently licensed for human use. The sera raised were tested against the homologous strain, a heterologous class 3 strain and a heterologous class 2 strain.

ELISA with rC13 protein showed that each of the preparations had induced antibodies, which reacted with denatured protein. Meningococcal outer membranes were used as a source of PorB in a more native conformation. All the immunisation preparations induced antibodies that demonstrated some reactivity with the native homologous protein. Sera raised against rC13 in liposomes with MPLA, rC13 in Zwittergent 3-14 with MPLA and rC13 in NDSB-195 with MPLA showed the highest antibody titres. Sera resulting from these immunisation preparations also showed cross-reactivity with outer membranes containing the heterologous class 3 protein. Only the sera raised against rC13 in Zwittergent 3-14 with MPLA reacted with the heterologous class 2 strain.

To define the proteins recognised by the antibodies raised, Western blotting was performed using whole cell lysates. The sera raised from all the immunisation preparations reacted with the homologous class 3 protein. Levels of reactivity were higher for the sera resulting from the micelle preparation than sera resulting from the liposome preparations. Some of the sera were also shown to react with the class 1 protein. The class 1 protein is closely related to the class 3 protein and shares large

regions of homology, particularly in the transmembrane regions (Ward *et al*, 1996). This suggests that the antibodies induced by the rC13 protein were directed against transmembrane regions as well as surface exposed loops. The three sera that showed the highest antibody titres in ELISA were also seen to react the strongest in Western blotting with the homologous class 3 protein and also showed the most cross-reactivity with the heterologous class 2 and 3 proteins.

For PorB to be an effective vaccine the antibodies raised must be at least able to recognise whole meningococci and this was demonstrated by immuno-fluorescence detection of antibody binding to fixed meningococci. The sera raised against the rC13 in liposomes with MPLA, rC13 in Zwittergent 3-14 with MPLA and rC13 in NDSB-195 with MPLA showed the highest levels of reactivity. Only the sera raised against rC13 in Zwittergent 3-14 with MPLA showed any reactivity with the strain expressing the heterologous class 3 protein and this was very low. These results suggest that the rC13 in these three immunisation preparations had achieved a more native like conformation than in the other preparations. The lack of cross-reactivity suggests that the cross-reactivity seen in the ELISA and Western blotting experiments was due to the presence of antibodies directed against non-exposed or non-conformational epitopes.

The ability of an antigen to induce a broad range of isotypes with different immune effector functions is likely to be required for protection against infection. ELISA, using mouse Ig-isotype specific conjugates determined the subclass-specific response to the rC13 protein and native class 3 in outer membranes. The sera raised against the rC13 in NDSB-195 with MPLA quantitatively produced the highest antibody titres, with a broad range of isotypes present. The rC13 in liposomes with MPLA and the rC13 in Zwittergent 3-14 with MPLA also induced a broad range of isotypes.

All the above analysis of the sera raised against the rC13 protein suggests that refolding of the protein had been achieved to some degree in several of the immunisation preparations. The key test for a potential vaccine candidate is that the antibodies raised must not only be able to recognise a meningococcus, but also be able to kill the bacteria.

This can be measured *in vitro* by bactericidal assays where the sera is tested for the ability to induce complement - mediated killing of live meningococci which is accepted as the correlate of protection. The highest dilution of the sera at which 50% of the meningococci were killed was 1 in 32, which was demonstrated by sera raised against three preparations; rC13 in Zwittergent 3-14 with MPLA, rC13 in NDSB-195, and rC13 in NDSB-195 with MPLA. In contrast the sera raised against rC13 in liposome with MPLA showed levels of 50% killing at a 1 in 8 dilution. None of the sera raised promoted killing of either of the heterolgous strains. When the class 1 protein was tested as a vaccine candidate (Christodoulides *et al*, 1998), the sera raised was sero-subtype specific. Likewise protection against the class 3 protein has proved to be serotype specific. The exposed regions of the class 3 protein are loops which vary in sequence between serotypes, however the epitopes in the class 3 protein are conformational, not linear, so antibodies able to recognise these epitopes must have been induced by similar conformational moieties, indicating some correct refolding of the protein.

The levels of bactericidal activity observed were lower than when the class 1 protein was incorporated into liposomes in a comparable assay by Christodoulides *et al,* (1998). Sera dilutions of 1 in 512 were able to promote killing of 50% of meningococci of the homologous strain, which is comparable to the titre of 1024 obtained when an outer membrane vesicle was used for immunisation. The results seen in the bactericidal activity with the rC13 protein may be related to the cross-reactivity seen in the ELISA, Western blotting and immuno-fluorescence experiments, suggesting that the rC13 protein had not completely refolded and therefore that a significant proportion of the antibodies raised were directed against non-exposed or non-conformational epitopes. These results also indicate the superiority of using live meningococci in comparison to fixed bacteria or outer membranes as a source of native protein. (Aase *et al*, 1998), suggested that most class 3 epitopes were more accessible on dead meningococci, than on live bacteria.

Thus refolding of the rC13 protein had been partially achieved and had resulted in significant bactericidal activity. However better refolding of the protein would be

expected to improve the specificity of the antibodies raised allowing better recognition of native class 3 protein. For better analysis of the class 3 protein as a potential vaccine candidate, further work involving refined refolding of the rCl3 protein needs to be performed.

The advantage of the class 3 protein however is there are only approximately 15 different class 3 serotypes as opposed to the many different class 1 sero-subtypes. In 1999, of the serogroup B typeable disease isolates, 66% were serotype 4, 12% serotype 15 and 11% serotype, all of which are class 3 proteins. In comparison, the majority of serogroup C disease isolates were class 2 expressing strains (Ramsay *et al*, 1997). Therefore for an effective vaccine using the class 3 protein, a vaccine constructed of serotypes 1, 4 and 15 proteins could potentially protect against 89% of serogroup B meningococci currently causing disease in England and Wales.

CHAPTER 7: BIBLIOGRAPHY

Aase, A., Hoiby, E.A., Kolberg, J., Rosenqvist, E. & Michaelsen, T.E. (1998) Most antibodies to PorB do not bind to viable meningococci, but bind strongly to ethanol-killed bacteria. *Eleventh International Pathogenic Neisseria Conference* (ed. by X. Nassif, M.J. Quentin-Millet and M.K. Taha), p.284.

Abdillahi, H. & Poolman, J.T. (1988) Definition of meningococcal class 1 outer membrane protein subtyping antigens by monoclonal antibodies. *FEMS Microbiology Immunology*, **47**, 139

Achtman, M. (1995) Global epidemiology of meningococcal disease. *Meningococcal Disease* (ed. by K.A.V. Cartwright), p.159. John Wiley and sons,

Achtman, M., Wall, R.A., Bopp, M., Kusecek, B., Morelli, G., Saken, E. & Hassanking, M. (1991) Variation in class 5 protein expression by serogroup A meningococci during a meningitis epidemic. *Journal of Infectious Diseases*, **164**, 375-382.

Achtman.M. (1988) Purification and characterisation of eight class 5 outer membrane protein variants from a clone of *Neisseria meningitidis* serogroup A. *Journal of Experimental Medicine*, **168**, 507-525.

Aho, E.L., Botten, J.W., Hall, R.J., Larson, M.K. & Ness, J.K. (1997) Characterization of a class II pilin expression locus from *Neisseria meningitidis*: Evidence for increased diversity among pilin genes in pathogenic *Neisseria* species. *Infection and Immunity*, **65**, 2613-2620.

Apicella, M.A. (1990) *Neisseria meningitidis*. Principles and Practise of Infectious Diseases (ed. by Mandrell, Douglas and Bennett), p.1600. Wiley and sons,

Ashton, F.E., Ryan, A., Benito, D. & Jennings, H.J. (1983) A new serogroup (L) of *Neissseria meningitidis*. *Journal of Clinical Microbiology*, **17**, 722-727.

Band, J.D., Chamberland, M.E., Platt, T., Weaver, R.E., Thornsberry, C. & Fraser, D.W. (1983) Trends in meningococcal disease in the United states, 1975-1980. *Journal of Infectious Diseases*, **4**, 754-758.

Banks, H.S. (1948) Meningococcosis: a protean disease. *Lancet*, **ii**, 635-640.

Barlow, A.K., Heckels, J.E. & Clarke, I.N. (1989) The class 1 outer membrane protein of *Neisseria meningitidis*: gene sequence and structural and immunological similarities to gonococcal porins. *Molecular Microbiology*, **3**, 131-139.

Barlow, D.J., Edwards, M.S. & Thornton, J.M. (1987) Continuous and discontiuous protein antigenic determinants. *Nature*, **322**, 747-748.

Bash, M. C., Lesiak, K. B., Banks, S. D., and Frasch C.E. (1995) Analysis of *Neisseria meningitidis* class 3 outer membrane protein gene variable regions and type identification using genetic techniques. *Infection and Immunity*, **63**, 1484-1490.

Belland, R.J., Morrison, S.G., van der Ley, P. & Swanson, J. (1989) Expression and phase variation of gonococcal PII genes in *Escherichia coli* involves ribosomal frameshifting and slipped- strand mispairing. *Molecular Microbiology*, **3**, 777-786.

Berg, S., Trollfors, B., Alestig, K. & Jodal, U. (1992) Incidence, serogroups and case-fatality rates of invasive meningococcal infections in a swedish region 1975-1989. *Scandanavian Journal of Infectious Diseases*, **24**, 333-338.

Bjorvatn, B., Lund, V., Kristiansen, B.E., Korsnes, L., Spanne, O. & Lindqvist, B. (1984) Applications of restriction endonuclease fingerprinting of chromosomal DNA of *Neisseria meningitidis*. *Journal of Clinical Microbiology*, **19**, 763-765.

Bjune, G., Gronnesby, J.K.H., Closs, O. & Nokleby, H. (1991) Results of an efficacy trial with an outer membrane vesicle vaccine against systemic serogroup b meningococcal disease in Norway. *N.I.P.H.Annals*, **14(2)**, 125-132.

Black, J.R., Black, W.J. & Cannon, J.G. (1985) Neisserial antigen H.8 is immunogenic in patients with disseminated gonococcal and meningococcal infections. *Journal of Infectious Diseases*, **151**, 650-657.

Blake, M.S. & Gotschlich, E.C. (1987) Functional and immunologic properties of pathogenic Neisseria surface proteins. *In Bacterial Outer Membranes as Model Systems.* (ed. Inouye,M) J.Wiley and sons.*pp377-400*, chp 14, 377-399.

Blake, M.S., MacDonald, C.M. & Klugman, K.P. (1989) Colony morphology of piliated *Neisseria meningitidis*. *Journal of Experimental Medicine*, **170**, 1727-1736.

Botha, P. (1988) Penicillin-resistant *Neisseria meningitidis* in Southern Africa. *Lancet*, **i**, 54

Bradbury, J. (1999) New meningitis C vaccine to be used in the UK. *Lancet* **354**, 310.

Branham, S.E. (1932) Serological diversity among meningococci. *Journal of Immunology*, **23**, 49-49.

Branham, S.E. (1953) Serological relationships among meningococci. *Bacteriological Reviews*, **17**, 175-188.

Branhan, S.E. & Carlin, S.A. (1942) Comments on a newly recognised group of the meningococcus. *Proceedings of the Society for Experimental Biology and Medicine*, **49**, 141-144.

Brooks, J.L., Fallon, R.J. & Heckels, J.E. (1995) Sequence variation in class 1 outer membrane protein in *Neisseria meningitidis* isolated from patients with meningococcal infection and close household contacts. *FEMS Microbiology Letters*, **128**, 145-150.

Butcher, S., Sarvas, M. & Runeberg-Nyman, K. (1991) Class-3 porin of *Neisseria meningitidis*: cloning and structure of the gene. *Gene*, **105**, 125-128.

Cartwright, K.A., Morris, R., Rumke, H.C., Fox, A., Borrow, R., Begg, N., Richmond, P. & Poolman, J.T. (1999) Immunogenicity and reactogenicity in UK infants of a novel meningococcal vesicle vaccine containing multiple class 1 (PorA) outer membrane proteins. *Vaccine*, **17**, 2612-2619.

Cartwright, K.A.V. (1995) Introduction and historical aspects. *Meningococcal disease* (ed. by K.A.V. Cartwright), p.1. Wiley and Sons,

Caugant, D.A., Froholm, L.O., Bovre, K., Holten, E., Frasch, C.E., Mocca, L.F., Zollinger, W.D. & Selander, R.K. (1986) Intercontinental spread of a genetically distinctive complex of clones of *Neisseria meningitidis* causing epidemic disease. *Proceedings of the National Academy of Sciences of the United States of America*, **83**, 4927-4931.

Caugant, D.A., Mocca, L.F., Frasch, C.E., Froholm, L.O., Zollinger, W.D. & Selander, R.K. (1987a) Genetic-structure of *Neisseria meningitidis* populations in relation to serogroup, serotype, and outer-membrane protein pattern. *Journal of Bacteriology*, **169**, 2781-2792.

Caugant, D.A., Zollinger, W.D., Mocca, L.F., Frasch, C.E., Whittam, T.S., Froholm, L.O. & Selander, R.K. (1987b) Genetic-relationships and clonal population-structure of serotype-2 strains of *Neisseria meningitidis*. *Infection and Immunity*, **55**, 1503-1513.

Chong, S., Mersha, F. B, Comb, D. G., Scott, M. E., Landry, D., Vence, L. M., Perler, F. B., Benner, J., Kucera, R. B., Hirvonen, C. A., Pelletier, J. J., Paulus, H., and Xu, M. Q. (1997) Single column purification of free recombinant proteins using a self-cleavable affinity tag derived from a protein splicing element. *Gene* **192**, 277-281.

Christodoulides, M., Brooks, J.L., Rattue, E. & Heckels, J.E. (1998) Immunisation with recombinant class 1 outer-membrane protein from *Neisseria meningitidis*: influence of liposomes and adjuvants on antibody avidity, recognition of native protein and the induction of a bactericidal immune response against meningococci. *Microbiology*, **144**, 3027-3037.

Claassen, I., Meylis, J., van der Ley, P., Peeters, C., Brons, H., Robert, J., Borsboom, D., van der Ark, A., van Stratten, I., Roholl, P., Kuipers, B. & Poolman, J. (1996) Production, characterization and control of a *Neisseria meningitidis* hexavalent class 1 outer membrane protein containing vesicle vaccine. *Vaccine*, **14**, 1001-1008.

Connell, T.D., Shaffer, D. & Cannon, J.G. (1990) Characterization of the repertoire of hypervariable regions in the protein. *Molecular Microbiology*, **4**, 439-449.

Cowan, S.W., Schirmer, T., Rummel, G., Steiert, M., Ghosh, R., Pauptit, R.A., Jansonius, J.N. & Rosenbusch, J.P. (1992) Crystal structures explain functional properties of two *E.coli* porins. *Nature*, **358**, 727-733.

Delvig, A.A., Rosenqvist, E., Oftung, F. & Robinson, J.H. (1997) T-Cell epitope mapping the PorB protein of serogroup B *Neisseria meningitidis* in B10 congenic strains of mice. *Clinical Immunology and Immunopathology*, **85**, 134-142.

Delvig, A.A., Wedege, E., Caugant, D.A., Dalseg, R., Kolberg, J., Achtman, M. & Rosenqvist, E. (1995) A linear B-cell epitope on the class 3 outer-membrane protein of *Neisseria meningitidis* recognised after vaccination with the Norwegian group B outer-membrane vesicle vaccine. *Microbiology*, **141**, 1593-1600.

Densen, P. (1989) Interaction of complement with *Neisseria meningitidis* and *Neisseria gonorrhoeae*. *Clinical Microbiology Reviews*, **2**, S11-S17

Devoe, I.W. (1982) The meningococcus and mechanisms of pathogenicity. *Microbiological Reviews*, **46** , 162-190.

Diaz, J.L., Virji, M. & Heckels, J.E. (1984) Structural and antigenic differences between 2 types of meningococcal pili. *FEMS Microbiology Letters*, **21**, 181-184.

Dillon, J.R., Pauze, M. & Yeung, K.H. (1983) Spread of penicillinase-producing and transfer plasmids from the gonococcus to *Neisseria meningitidis*. *Lancet*, **ii**, 779-781.

Ding, S., Ye, R.-B. & Zhang, H.-C. (1981) Three new serogroups of *Neisseria meningitidis*. *Journal of Biological Standardisation*, **9**, 307-315.

Estabrook, M.M., Christopher, N.C., Griffiss, J.M., Baker, C.J. & Mandrell, R.E. (1992) Sialylation and human neutrophil killing of group C *Neisseria meningitidis*. *Journal of Infectious Diseases*, **166**, 1079-1088.

Evans, J.R., Artenstein, M.S. & Hunter, D.H. (1968) Prevalence of meningococcal serogroups and description of three new groups. *American Journal Of Epidemiology*, **87**, 643-646.

Fairbanks, G., Steck, T.L. & Wallach, D.F.H. (1971) Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry*, **10**, 2606-2616.

Farley, J., Masi, A., Novitsky, B. & Hazelo, R. (1994) Transcriptional regulation of the class 1 outer membrane protein in group B *Neisseria meningitidis*. *The Proceedings of the Ninth International Pathogenic Neisseria Conference* (ed. by J.S. Evans, S.E. Yost, M.C.J. Maiden and I.M. Feavers), p.92. NIBSC, London.

Feavers, I.M., Suker, J., Mckenna, A.J., Heath, A.B. & Maiden, M.C. (1992) Molecular analysis of the serotyping antigens of *Neisseria meningitidis*. *Infection and Immunity*, **60**, 3620-3629.

Feldman, H.A. (1966) Meningococcal disease, 1965. *Journal of the American Medical Association*, **196**, 391-393.

Frasch, C.E. (1979) Noncapsular surface antigens of *Neisseria meningitidis*. in *Seminars in Infectious Disease, Volume* 2, 304-337 (eds .L.Weinstein & B.Feilds) Stratton Intercontinental Medical Book Corporation,New York.

Frasch, C.E. (1995) Meningococcal vaccines: Past, present and future. *Meningococcal disease* (ed. by K.A.V. Cartwright), p.245. John Wiley & Sons, Chichester, UK.

Frasch, C.E. & Chapman, S.S. (1972) Classification of *Neisseria meningitidis* group B into distinct serotypes II. extraction of type-specific antigens for serotyping by precipitin techniques. *Infection and Immunity*, **6**, 127-133.

Frasch, C.E. & Chapman, S.S. (1973) Classification of *Neisseria meningitidis* group B into distinct serotypes.III. application of a new bactericidal inhibition technique to distribution of serotypes among cases and carriers. *Journal of Infectious Diseases*, **127**, 149-154.

Frasch, C.E. & Gotschlich, E.C. (1974) An outer membrane protein of *Neisseria meningitidis* group B responsible for serotype specificity. *Journal of Experimental Medicine*, **140**, 87-104.

Frasch, C.E. & Mocca, L.F. (1978) Heat-modifiable outer membrane proteins of *Neisseria meningitidis* and their organisation within the membrane. *Journal of Bacteriology*, **136**, 1127-1134.

Frasch, C.E. & Peppler, M.S. (1982) Protection against group B *Neisseria meningitidis* disease: preparation of soluble protein and protein-polysaccharide immunogens. *Infection and Immunity*, **37**, 271-280.

Frasch, C.E., Zollinger, W.D. & Poolman, J.T. (1985) Serotype antigens of *Neisseria meningitidis* and a proposed scheme for designation of serotypes. *Reviews of Infectious Diseases*, **7**, 504-510.

Frosch, M. (1994) Molecular basis and biological significance of encapsulation and capsule modification in meningococci. *Proceedings of the Ninth International Pathogenic Neisseria Conference.* (ed. by J.S. Evans, S.E. Yost, M.C.J. Maiden and I.M. Feavers), p.5. NIBSC, London.

Gold, R. & Wyle, F.A. (1970) New classification of *Neisseria meningitidis* by means of bactericidal reactions. *Infection and Immunity*, **1**, 479-484.

Goldberg, M. E., Bezancon, N. E., Vuillard, L., and Rabilloud, T. (1996) Non-detergent sulphobetaines: a new class of molecules that facilitate in vitro protein renaturation. *Folding and Design* **1**, 21-27. 1996.

Goldschneider, I., Gotschlich, E.C. & Artenstein, M.S. (1969b) Human immunity to the meningococcus. I. The role of humoral antibodies. *Journal of Experimental Medicine*, **129**, 1307-1326.

Goldschneider, I., Gotschlich, E.C. & Artenstein, M.S. (1969a) Human immunity to the meningococcus. II. Development of natural immunity. *Journal of Experimental Medicine*, **129**, 1327-1348.

Gordon, M.H. & Murray, E.G. (1915) Identification of the meningococcus. *Journal of the Royal Army Medical Corps*, **25**, 411-423.

Gotschlich, E.C., Goldschneider, I. & Artenstein, M.S. (1969) Human immunity to the meningococcus V, the effect of immunisation with meningococcal group C polysaccharide on the carrier state. *Journal of Experimental Medicine*, **129**, 1385-1394.

Greenwood, B.M., Bradley, B.M. & Wall, R.A. (1985) Meningococcal disease and season in sub-Saharan Africa. *Lancet*, **ii**, 829-830.

Griffiss, J.M. & Artenstein, M.S. (1976) The ecology of the genus *Neisseria*. *The Mount Sinai Journal of Medicine*, **43**, 746-761.

Guillen, G., Leal, M. J., Alvarez, P. A., elgado, M., Silva, R., and Herrera, L. (1995) Cloning and expression of the *Neisseria meningitidis* 5C outer membrane protein. *Acta Biotechnology* **15**, 97-106.

Guttormsen, H.K., Wetzler, L.M. & Naess, A. (1993) Humoral immune response to the class 3 outer membrane protein during the course of meningococcal disease. *Infection and Immunity*, **61**, 4734-4742.

Guttormsen, H.K., Wetzler, L.M. & Solberg, C.O. (1994) Humoral immune response to class 1 outer membrane protein during the course of meningococcal disease. *Infection and Immunity*, **62**, 1437-1443.

Haas, R. & Meyer, T.F. (1986) The repertoire of silent pilus genes in *Neisseria gonorrhoeae* - evidence for gene conversion. *Cell*, **44**, 107-115.

Haas, R., Veit, S. & Meyer, T.F. (1992) Silent pilin genes of *Neisseria gonorrhoeae* ms11 and the occurrence of related hypervariant sequences among other gonococcal isolates. *Molecular Microbiology*, **6**, 197-208.

Hagblom, P., Segal, E., Billyard, E. & So, M. (1985) Intragenic recombination leads to pilus antigenic variation in *Neisseria gonorrhoeae*. *Nature*, **315**, 156-158.

Heckels, J.E. (1981) Structural comparison of *Neisseria gonorrhoeae* outer membrane proteins. *Journal of Bacteriology*, **145**, 736-742.

Heckels, J.E. (1989) Structure and function of pili of pathogenic *Neisseria* species. *Clinical Microbiology Reviews*, **2**, S66-S73

Heckels, J.E., Blackett, B., Everson, J.S. & Ward, M.E. (1976) The influence of surface charge on the attachment of *Neisseria gonorrhoeae* to human cells. *Journal of General Microbiology*, **96**, 359-364.

Heist, G.D., Solis-Cohen, S. & Solis-Cohen, M. (1922) A study of the virulence for man and of human susceptibility to meningococcal infection. *Journal of Immunology*, **7**, 1-33.

Hitchcock, P.J. & Brown, T.M. (1983) Morphological heterogeneity among salmonella lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. *Journal of Bacteriology*, **154**, 269-277.

Holmes, D.S. & Quigley, M. (1981) A rapid boiling method for the preparation of bacterial plasmids. *Analytical Biochemistry*, **114**, 193-197

Hudson, D.A., Goddard, E.A. & Millar, K.N. (1993) The management of skin infarction after meningococcal septicaemia in children. *British Journal of Plastic Surgery*, **46**, 243-246.

Jiang, J., Abu Shilbayeh, L. & Rao, V.B. (1997) Display of a PorA peptide from *Neisseria meningitidis* on the bacteriophage T4 capsid surface. *Infection and Immunity*, **65**, 4770-4777.

Johnson, A.P., D, Taylor-Robinson, Z.A. & McGee. (1977) Species specificity of attachment and damage to oviduct mucosa by *Neisseria gonorrhoeae*. *Infection and Immunity*, **18**, 833-839.

Jones, D.M. (1988) Epidemiology of meningococcal infection in England and Wales. *Journal of Medical Microbiology*, **26**, 165-168.

Jones, D.M. (1995) Epidemiology of meningococcal disease in Europe and the USA. *Meningococcal disease* (ed. by K.A.V. Cartwright), p.147. John Wiley & Sons, Chichester, UK.

Jonsson, A.B., Nyberg, G. & Normark, S. (1991) Phase variation of gonococcal pili by frameshift mutation in *pilC*, a novel gene for pilus assembly. *Embo Journal*, **10**, 477-488.

Kawula, T.H., Aho, E.L., Barritt, D.S., Klapper, D.G. & Cannon, J.G. (1988) Reversible phase variation of expression of *Neisseria meningitidis* class 5 outer membrane proteins and their relationship to gonococcal proteins II. *Infection and Immunity*, **56**, 380-386.

Kawula, T.H., Spinola, S.M., Klapper, D.G. & Cannon, J.G. (1987) Localization of a conserved epitope and an azurin-like domain in the H.8 protein of pathogenic *Neisseria*. *Molecular Microbiology*, **1**, 179-185.

Kellog, D.S., Peacock, W.L., Deacon, W.E., Brown, L. & Pirkle, C.I. (1963) *Neisseria gonorrhoeae*.I.virulence genetically linked to clonal variation. *Journal of Bacteriology*, **85**, 1274-1279.

Kingsbury, D.T. (1967) Deoxyribonucleic acid homologies among species of the genus *Neisseria*. *Journal of Bacteriology*, **94**, 870-874.

Koomey, M., Gotschlich, E.C., Robbins, K., Bergstrom, S. & Swanson, J. (1987) Effects of *recA* mutations on pilus antigenic variation and phase- transitions in *Neisseria gonorrhoeae*. *Genetics*, **117**, 391-398.

Laemli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage t4. *Nature*, **227**, 680-685.

Lehmann, A. K., Halstensen, A., Aaberge, I. S., Holst, J., Michaelsen, T. E., Sornes, S., Wetzler, L. M., and Guttormsen, H. K. (1999) Human opsonins induced during meningococcal disease recognise outer membrane proteins PorA and PorB. *Infection and Immunity* **67**, 2552-2560.

Lieberman, J. M., Gheesling, L. L, Carlone, G. M., and Ward, J. I. (1996) Safety and immunogenicity of serogroups A/C *Neisseria meningictidis* oligosaccharide protein conjugate vaccine in young children. *Journal of the American Medical Association* **275**, 1499-1503.

Lynch, E.C., Blake, M.S., Gotschlich, E.C. & Mauro, A. (1984) Studies of porins spontaneously transferred from whole cells and reconstituted from purified proteins of *Neisseria gonorrhoeae* and *Neisseria meningitidis*. *Biophysical Journal*, **45**, 104-107.

MacDonald, N. E., Halperin, S. A, Law, B. J., Forrest, B., Danzig, L. E., and Granoff, D. H. (1998) Induction of immunologic memory by conjugated vs. plain meningococcal C polysaccharide vaccine in toddlers. *Journal of the American Medical Association* **280**, 1685-1689.

Mackinnon, F.G., Borrow, R., Fox, A.J., Robinson, A. & Jones, D.M. (1994) Effects of capsule and lipooligosaccharide sialylation on the serum resistance *of Neisseria meningitidis. Proceedings of the ninth international pathogenic Neisseria conference* (ed. by J.S. Evans, S.E. Yost, M.C.J. Maiden and I.M. Feavers), p.38. NIBSC, London.

Maiden, M.C.J., Bygraves, J.A., McCarvil, J. & Feavers, I.M. (1992) Identification of meningococcal serosubtypes by polymerase chain reaction. *Journal of Clinical Microbiology*, **30**, 2835-2841.

Maiden, M.C.J., Suker, J., Mckenna, A.J., Bygraves, J.A. & Feavers, I.M. (1991) Comparison of the class 1 outer membrane proteins of 8 serological reference strains of *Neisseria meningitidis. Molecular Microbiology*, **5**, 727-736.

Mandrell, R.E. & Zollinger, W.D. (1984) Use of a zwitterionic detergent for the restoration of the antibody-binding capacity of electroblotted outer membrane proteins. *J Immunol Methods*, **67**, 1-11.

Mandrell, R.E. & Zollinger, W.D. (1989) Human immune response to meningococcal outer membrane protein epitopes after natural infection or vaccination. *Infection and Immunity*, **57** , 1590-1598.

Masson, L., Holbein, B.E. & Ashton, F.E. (1982) Virulence linked to polysaccharide production in serogoup B *Neisseria meningitidis. FEMS Microbiology Letters*, **13**, 187-190.

McGuinness, B.T., Barlow, A.K., Clarke, I.N., Farley, J.E., Anilionis, A., Poolman, J.T. & Heckels, J.E. (1990) Deduced amino acid sequences of class 1 protein (*porA*) from 3 strains of *Neisseria meningitidis* - synthetic peptides define the epitopes responsible for serosubtype specificity. *Journal of Experimental Medicine*, **171**, 1871-1882.

McGuinness, B.T., Clarke, I.N., Lambden, P.R., Barlow, A.K., Poolman, J.T., Jones, D.M. & Heckels, J.E. (1991) Point mutation in meningococcal *porA* gene associated with increased endemic disease. *Lancet*, **337**, 514-517.

McGuinness, B.T., Lambden, P.R. & Heckels, J.E. (1993) Class 1 outer membrane protein of *Neisseria meningitidis*: epitope analysis of the antigenic diversity between strains, implications for subtype definition and molecular epidemiology. *Molecular Microbiology*, **7**, 505-514.

Merker, P., Tommassen, J., Kusecek, B., Virji, M., Sesardic, D. & Achtman, M. (1997a) Two-dimensional structure of the Opc invasin from *Neisseria meningitidis*. *Molecular Microbiology*, **23**, 281-293.

Merker, P., Tommassen, J. & Kusecek, B. (1997b) Two-dimensional structure of the Opc invasin from *Neisseria meningitidis*. *Molecular Microbiology*, **23**, 281-293.

Meyer, T.F. & van Putten, J.P.M. (1989) Genetic mechanisms and biological implications of phase variation in pathogenic *Neisseriae*. *Clinical Microbiology Reviews: Supplement*, **2**, S139-S145

Minetti, C.A., Tai, J.Y., Blake, M.S., Pullen, J.K., Liang, S.M. & Remeta, D.P. (1997) Structural and functional characterization of a recombinant PorB class 2 protein from *Neisseria meningitidis* - Conformational stability and porin activity. *Journal of Biological Chemistry*, **272**, 10710-10720.

Moore, P.S. (1992) Meningococcal meningitis in sub-saharan Africa - a model for the epidemic process. *Clinical Infectious Diseases*, **14**, 515-525.

Murakami, K., Gotschlich, E.C. & Seiff, M.E. (1989) Cloning and characterization of the structural gene for the class 2 protein of *Neisseria meningitidis*. *Infection and Immunity*, **57**, 2318-2323.

Muttilainen, S., Idanpaan-Heikkila, I., Wahlstrom, E., Nurminen, M., Makela, P.H. & Sarvas, M. (1995a) The *Neisseria meningitidis* outer membrane protein P1 produced in *Bacillus subtilis* and reconstituted into phospholipid vesicles elicits antibodies to native P1 epitopes. *Microbial Pathogenesis*, **18**, 423-436.

Muttilainen, S., Butcher, S.J., Runeberg, K., Nurminen, M., Idanpaan-Heikkila, I., Wahlstrom, E. & Sarvas, M. (1995b) Heterologous production of the P1 porin of *Neisseria meningitidis* in *Bacillus subtilis*: The effect of an N-terminal extension on the presentation of native-like epitopes. *Microbial Pathogenesis*, **18**, 365-371.

Nassif, X., Beretti, J.L., Lowy, J., Stenberg, P., Ogaora, P., Pfeifer, J., Normark, S. & So, M. (1994) Roles of pilin and Pilc in adhesion of *Neisseria meningitidis* to human epithelial and endothelial-cells. *Proceedings of the National Academy of Sciences of the United States of America*, **91**, 3769-3773.

Nassif, X., Lowy, J., Stenberg, P., Ogaora, P., Ganji, A. & So, M. (1993) Antigenic variation of pilin regulates adhesion of *Neisseria meningitidis* to human epithelial cells. *Molecular Microbiology*, **8**, 719-725.

Nicholson, A. & Lepow, I.H. (1979) Host defence against *Neisseria meningitidis* requires a complement-dependent bactericidal activity. *Science*, **205**, 298-299.

Nikaido, H. (1992) Porins and specific channels of bacterial outer membranes. *Molecular Microbiology*, **6**, 435-442.

Ochem, A.E., Skopac, D., Costa, M., Rabilloud, T., Vuillard, L., Simoncsits, A., Giacca, M. & Falascni, A. (1997) Functional properties of the separate subunits of human DNA helicase II/Ku autoantigen. *Journal of Biological Chemistry*, **272**, 29919-29926.

Olsen, S.F., Djurhuus, B., Rasmussen, K., Joensen, H.D., Larsen, S.O., Zoffman, H. & Lind, I. (1991) Pharyngeal carriage of *Neisseria meningitidis* and *Neisseria lactamica* in households with infants within areas with high and low incidences of meningococcal disease. *Epidemiology And Infection*, **106**, 445-457.

Olyhoek, A.J.M., Sarkari, J., Bopp, M., Morelli, G. & Achtman, M. (1991) Cloning and expression in Escherichia coli of opC, the gene for an unusual class 5 outer membrane protein from *Neisseria meningitidis* (meningococci surface-antigen). *Microbial Pathogenesis*, **11**, 249-257.

Peltola, H. (1983) Meningococcal disease: still with us. *Reviews of Infectious Diseases*, **5**, 71-91.

Poolman, J.T., Hopman, C.T.P. & Zanen, H.C. (1980b) Immunochemical characterisation of *Neisseria meningitidis* serotype antigens by immunodiffusion and SDS-PAGE immunoperoxidase techniques and the distribution of serotypes among cases and carriers. *Journal of General Microbiology*, **116**, 465-473.

Poolman, J.T., Hopman, C.T.P. & Zanen, H.C. (1983) Immunogenicity of meningococcal antigens as detected in patient sera. *Infection and Immunity*, **40**, 398-406.

Poolman, J.T., Lind, I., Jonsdottir, K. & et al (1986) Meningococcal serotypes and serogroup disease in North West Europe. *Lancet*, **iii**, 555-557.

Poolman, J.T., Marie, S. & de Zanen, H.C. (1980a) Variability of low molecular weight, heat-modifiable outer membrane proteins of *Neisseria meningitidis*. *Infection and Immunity*, **30**, 642-664.

Potts, W.J. & Saunders, J.R. (1988) Nucleotide sequence of the structural gene for class 1 pilin from *Neisseria meningitidis*- homologies with the *pilE* locus of *Neisseria gonorrhoeae*. *Molecular Microbiology*, **2**, 647-653.

Qi, H.L., Tai, J.Y. & Blake, M.S. (1994) Expression of large amounts of Neisserial porin proteins in *Escherichia coli* and refolding of the proteins into native trimers. *Infection and Immunity*, **62**, 2432-2439.

Rake, G. (1934) Studies on meningococcus infection.VI. The carrier problem. *Journal of Experimental Medicine*, **59**, 553-576.

Raman, G.V. (1988) Meningococcal septicaemia and meningitis: a rising tide. *British Medical Journal*, **296**, 1141-1142.

Ramsay, M., Kaczmarski, E., Rush, M., Mallard, R., Farrington, P., and White, D.(1997) Changing patterns of case ascertainment and trends in meningococcal disease in England and Wales. *CDR Review,* 7, R49-R54. 1997.

Reingold, A.L., Broome, C.V., Hightower, A.W., Ajelo, G.W., Bolan, G.A., Adamsbaum, C., Jones, E.E., Philips, C., Tiendrebeogo, H. & Yada, A. (1985) Age-specific differences in duration of clinical protection after vaccination with meningococcal polysaccharide A. *Lancet*, **2**, 114-118.

Richards, R.L., Swartz, G.M., Schultz, C., Hayre, M.D., Ward, G.S., Ballou, W.R., Chulay, J.D., Hockmeyer, W.T., Berman, S.L. & Alving, C.R. (1989) Immunogenicity of liposomal malaria sporozoite antigen in monkeys: adjuvant effects of aluminium hydroxide and non-pyrogenic liposomal lipid A. *Vaccine*, **7**, 506-512.

Roberts, R.B. (1967) The interaction in vivo between group B meningococci and rabbit polymorphonuclear lymphocytes. *Journal of Experimental Medicine*, **126**, 795-818.

Robertson, J.N., Vincent, P. & Ward, M.E. (1977) The preparation and properties of gonococcal pili. *Journal of General Microbiology*, **102**, 169-177.

Rosenqvist, E., Hoiby, E.A., Wedege, E., Bryn, K., Kolberg, J., Klem, A., Ronnild, E., Bjune, G. & Nokleby, H. (1995) Human antibody responses to meningococcal outer membrane antigens after three doses of the Norwegian group B meningococcal vaccine. *Infection and Immunity*, **63**, 4642-4652.

Rosenqvist, E., Hoiby, E.A., Wedege, E., Kusecek, B. & Achtman, M. (1993) The 5c protein of *Neisseria meningitidis* is highly immunogenic in humans and induces bactericidal antibodies. *Journal of Infectious Diseases*, **167**, 1065-1073.

Rosenstein, N., Perkins, B. A., Stephens, D. S., Lefkowitz, L., Cartter, M. L., Danila, R., Crieslak, P., Shult, K. A., Popovic, T., Schuchat, A., Harrison, L. H, and Reingold, A. L. (1999) The changing epidemiology of meningococcal disease in the US 1992-1996. *Journal of Infectious Diseases* **180**, 1894-1901.

Ross, S.C. & Densen, P. (1984) Complement deficiency states and infection: Epidemiology, pathogenesis and consequences of Neisserial and other infections in an immune deficiency. *Medicine*, **63**, 243-273.

Rudel, T., Scheuerpflug, I., Kahrs, A.F., Maier, J. & Meyer, T.F. (1994) Function of PilC as the pilus adhesin and the interaction of PilC with host cells and other Neisserial factors. *Proceedings of the Ninth International Pathogenic Neisseria Conference* (ed. by J.S. Evans, S.E. Yost, M.C.J. Maiden and I.M. Feavers), p.63. NIBSC, London.

Rudel, T., Vanputten, J.P.M., Gibbs, C.P., Haas, R. & Meyer, T.F. (1992) Interaction of 2 variable proteins (PilE and PilC) required for pilus-mediated adherence of *Neisseria gonorrhoeae* to human epithelial cells. *Molecular Microbiology*, **6**, 3439-3450.

Ryll, R.R., Rudel, T., Scheuerpflug, I., Barten, R. & Meyer, T.F. (1997) Pilc of *Neisseria meningitidis* is involved in class II pilus formation and restores pilus assembly, natural transformation competence and adherence to epithelial cells in Pilc-deficient gonococci. *Molecular Microbiology*, **23**, 879-892.

Salmi, I., Pettay, O., Simula, I., Kallio, A.-K. & Waltimo, O. (1976) An epidemic due to sulfonamide-resistant group A meningococci in the Helsinki area (Finland): epidemiological and clinical observations. *Scandanavian Journal of Infectious Diseases*, **8**, 249-254.

Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989) *Molecular cloning- a laboratory manual*, Cold Spring Harbour Press, Cold Spring Harbour, NY, USA.

Sarkari, J., Pandit, N., Moxon, E.R. & Achtman, M. (1994) Variable expression of the Opc outer membrane protein in *Neisseria meningitidis* is caused by size variation of a promoter containing poly-cytidine. *Molecular Microbiology*, **13**, 207-217.

Saukkonen, K., Abdillahi, H., Poolman, J.T. & Leinonen, M. (1987) Protective efficacy of monoclonal antibodies to class 1 and class 3 outer membrane proteins of *Neisseria meningitidis* B:15:P1.16 in infant rat infection model - new prospects for vaccine development. *Microbial Pathogenesis*, **3**, 261-267.

Saukkonen, K., Leinonen, M., Abdillahi, H. & Poolman, J.T. (1989) Comparative evaluation of potential components for group B meningococcal vaccine by passive protection in the infant rat and *in vitro* bactericidal assay. *Vaccine*, **7**, 325-328.

Schagger, H. & von Jagow, G. (1987) Tricine-sodium dodecyl sulphate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Analytical Biochemistry*, **166**, 368-379.

Schoolnik, G., Fernandez, R. & Rothbard, J. (1984) Synthetic peptide analogs of gonococcal pill - common and type- specific epitopes. *Clinical Research*, **32**,

Schwartz, B., Moore, P.S. & Broome, C.V. (1989) Global epidemiology of meningococcal disease. *Clinical Microbiology Reviews* , **2**, S118-S124

Seiler, A., Reinhardt, R., Sarkari, J., Caugant, D.A. & Achtman, M. (1996) Allelic polymorphism and site-specific recombination in the *opc* locus of *Neisseria meningitidis*. *Molecular Microbiology*, **19**, 841-856.

Slaterus, K.W. (1961) Serological typing of meningococci by means of micro-precipitation. *Antonie van Leeuwenhoek Journal of Microbiology and Serology*, **29**, 265-271.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. & Klenk, D.C. (1985). Measurement of protein using bicinchoninic acid. *Analytical Biochemistry* **150**, 76-85.

Song, J.M., Minetti, C.A., Blake, M.S. & Colombini, M. (1998) Successful recovery of the normal electrophysiological properties of PorB (class 3) porin from *Neisseria meningitidis* after expression in *Escherichia coli* and renaturation. *Biochimica Et Biophysica Acta-Biomembranes*, **1370**, 289-298.

Stephens, D.S., Whitney, A.M., Rothbard, J. & Schoolnik, G.K. (1985) Pili of *Neisseria meningitidis* - analysis of structure and investigation of structural and antigenic relationships to gonococcal pili. *Journal of Experimental Medicine*, **161**, 1539-1553.

Stern, A., Nickel, P., Meyer, T.F. & So, M. (1984) Opacity determinants of *Neisseria gonorrhoeae* - gene-expression and chromosomal linkage to the gonococcal pilus gene. *Cell*, **37** , 447-456.

Strom, M.S. & Lory, S. (1993) Structure-function and biogenesis of the type IV pili. *Annual Reviews in Microbiology*, **47**, 565-596.

Suker, J., Feavers, I.M., Achtman, M., Morelli, G., Wang, J.F. & Maiden, M.C.J. (1994) The *porA* gene in serogroup A meningococci: evolutionary stability and mechanism of genetic variation. *Molecular Microbiology*, **12**, 253-265.

Swanson, J. & Barrera, O. (1983) Gonococcal pilus subunit size heterogeneity correlates with transitions in colony piliation phenotype, not with changes in colony opacity. *Journal of Experimental Medicine*, **158**, 1459-1472.

Swanson J, R.K.B., Corwin, D., Boslego, J., Ciak, , J, , Blake, M. & Koomey, M.J. (1987) Gonococcal pilin variants in experimental gonorrhoea. *Journal of Experimental Medicine*, **165**, 1344-1357.

Swanson, J., Barrera, O., Sola, J. & Boslego, J. (1988) Expression of outer-membrane protein-ii by gonococci in experimental gonorrhea. *Journal of Experimental Medicine*, **168**, 2121-2129.

Swanson, J., Kraus, S.J. & Gotschlich, E.C. (1971) Studies on gonococcus infection.I. pili and zones of adhesion: their relation to gonococcal growth patterns. *Journal of Experimental Medicine*, **134**, 886-906.

Swanson, J., Robbins, K., Barrera, O., Corwin, D., Boslego, J., Ciak, J., Blake, M.S. & Koomey, J.M. (1987) Gonococcal pilin variants in experimental gonorrhoea. *Journal of Experimental Medicine*, **165**, 1344-1357.

Thompson, S.A. & Sparling, P.F. (1993) The rtx cytotoxin-related frpa protein of *Neisseria meningitidis* is secreted extracellularly by meningococci and by hlybd+ *Escherichia coli*. *Infection and Immunity*, **61**, 2906-2911.

Tinsley, C.R. & Heckels, J.E. (1986) Variation in the expression of pili and outer membrane protein by *Neisseria meningitidis* during the course of meningococcal infection. *Journal of General Microbiology*, **132**, 2483-2490.

Tommassen, J., Vermeij, P., Struyve, M., Benz, R. & Poolman, J.T. (1990) Isolation of *Neisseria meningitidis* mutants deficient in class 1 (PorA) and class 3 (PorB) outer membrane proteins. *Infection and Immunity*, **58**, 1355-1359.

Towbin, H., Stachelin, T. & Gordon, J. (1979) Electrophoretic transfer of proteins from acrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences of the United States of America*, **76**, 4350-4354.

Tsai, C.M., Frasch, C.E. & Mocca, L.F. (1981) Five structural classes of outer membrane proteins of *Neisseria meningitidis*. *Journal of Bacteriology*, **146**, 69-78.

van der Ley, P., Heckels, J.E., Virji, M., Hoogerhout, P. & Poolman, J.T. (1991) Topology of outer membrane porins in pathogenic *Neisseria* spp. *Infection and Immunity*, **59**, 2963-2971.

van der Voort, E.R., Kuipers, B., Brugghe, H.F., Van Unen, L.M.A., Timmermans, H.A.M., Hoogerhout, P. & Poolman, J.T. (1997) Epitope specificity of murine and human bactericidal antibodies against PorA P1.7,16 induced with experimental meningococcal group B vaccines. *FEMS Immunology and Medical Microbiology*.**17**, 139-148.

van der Voort, E.R., van der Ley, P., van der Biezen, J., George, S., Tunnela, O., van Dijken, H., Kuipers, B. & Poolman, J. (1996) Specificity of human bactericidal antibodies against PorA P1.7, 16 induced with a hexavalent meningococcal outer membrane vesicle vaccine. *Infection and Immunity*, **64**, 2745-2751.

Verheul, A.F.M., Snippe, H. & Poolman, J.T. (1993) Meningococcal lipopolysaccharides - virulence factor and potential vaccine component. *Microbiological Reviews*, **57**, 34-49.

Verma, J.N., Rao, M., Amselem, S., Krzych, U., Alving, C.R., Green, S.J. & Wassef, N.M. (1992) Adjuvant effects of liposomes containing Lipid A - enhancement of liposomal antigen presentation and recruitment of macrophages. *Infection and Immunity*, **60**, 2438-2444.

Virji, M., Alexandrescu, C., Ferguson, D.J.P., Saunders, J.R. & Moxon, E.R. (1992a) Variations in the expression of pili - the effect on adherence of *Neisseria meningitidis* to human epithelial and endothelial- cells. *Molecular Microbiology*, **6**, 1271-1279.

Virji, M., Heckels, J.E., Potts, W.J., Hart, C.A. & Saunders, J.R. (1989) Identification of epitopes recognised by monoclonal antibodies SM1 and SM2 which react with all pili of *Neisseria gonorrhoeae* but which differentiate between 2 structural classes of pili expressed by *Neisseria meningitidis* and the distribution of their encoding sequences in the genomes of Neisseria spp. *Journal of General Microbiology*, **135**, 3239-3251.

Virji, M., Kayhty, H., Ferguson, D.J.P., Alexandrescu, C., Heckels, J.E. & Moxon, E.R. (1991) The role of pili in the interactions of pathogenic *Neisseria* with cultured human endothelial cells. *Molecular Microbiology*, **5**, 1831-1841.

Virji, M., Makepeace, K., Ferguson, D.J.P., Achtman, M., Sarkari, J. & Moxon, E.R. (1992b) Expression of the Opc protein correlates with invasion of epithelial and endothelial cells by *Neisseria meningitidis*. *Molecular Microbiology*, **6**, 2785-2795.

Virji, M., Makepeace, K. & Moxon, R. (1994) Distinct mechanisms of interactions of Opc-expressing meningococci at apical and basolateral surfaces of human endothelial cells; the role of integrins in apical interactions. *Molecular Microbiology*, **14**, 173-184.

Virji, M., Saunders, J.R., Sims, G., Makepeace, K., Maskell, D. & Ferguson, D.J.P. (1993) Pilus-facilitated adherence of *Neisseria meningitidis* to human epithelial and endothelial cells: modulation of adherence phenotype occurs concurrently with changes in primary amino acid sequence and the glycosylation status of pilin. *Molecular Microbiology*, **10**, 1013-1028.

Vogel, H.J. & Bonner, D.M. (1956) Acetylornithinease of Escherichia coli: partial purification and some properties. *Journal of Biological Chemistry*, **218**, 97-106.

Vuillard, L., Braun-Breton, C. & Rabilloud, T. (1995a) Non-detergent sulphobetaines: a new class of mild solubilisation agents for protein purification. *Analytical Biochemistry*, **230**, 290-294.

Vuillard, L., Madern, D., Franzetti, B. & Rabilloud, T. (1995b) Halophilic protein stabilisation by the mild solubilising agents non-detergent sulphobetaines. *Biochemical Journal*, **305**, 337-343.

Wang, J., Caugant, D.A., Li, X., Hu, X., Poolman, J.T., Crowe, B.A., & Achtman, M. (1992). Clonal and antigenic analysis of serogroup A *Neisseria meningitidis* with particular reference to epidemiological features of epidemic meningitis in the Peoples Republic of China. *Infection and Immunity* **60**, 5267-5282

Wang, J.F., Caugant, D.A., Morelli, G., Koumare, B. & Achtman, M. (1993) Antigenic and epidemiologic properties of the ET-37 complex of *Neisseria meningitidis*. *Journal of Infectious Diseases*, **167**, 1320-1329.

Ward, M.J., Lambden, P.R. & Heckels, J.E. (1992) Sequence analysis and relationships between meningococcal class 3 serotype proteins and other porins from pathogenic and non-pathogenic *Neisseria* species. *FEMS Microbiology Letters*, **95**, 187-194.

Ward, S. J. Characterisation and immunogenicity of recombinant class 1 outer membrane protein of *Neisseria meningitidis*. 1995. (PhD thesis)

Ward, S.J., Scopes, D.A., Christodoulides, M., Clarke, I.N. & Heckels, J.E. (1996) Expression of *Neisseria meningitidis* class 1 porin as a fusion protein in *Escherichia coli*: the influence of liposomes and adjuvants on the production of a bactericidal immune response. *Microbial Pathogenesis*, **21**, 499-512.

Wedege, E. & Froholm, L.O. (1986) Human antibody response to a group B serotype 2a meningococcal vaccine determined by immunoblotting. *Infection and Immunity*, **51**, 571-578.

Wetzler, L.M., Blake, M.S., Barry, K. & Gotschlich, E.C. (1992) Gonococcal porin vaccine evaluation - comparison of por proteosomes, liposomes, and blebs isolated from rmp deletion mutants. *Journal of Infectious Diseases*, **166**, 551-555.

White, D.A., Barlow, A.K., Clarke, I.N. & Heckels, J.E. (1990) Stable expression of meningococcal class 1 protein in an antigenically reactive form in outer membranes of *Escherichia coli*. *Molecular Microbiology*, **4**, 769-776.

Wiertz, E.J.H.J., Delvig, A., Donders, E.M.L.M., Brugghe, H.F., Vanunen, L.M.A., Timmermans, H.A.M., Achtman, M., Hoogerhout, P. & Poolman, J.T. (1996) T-cell responses to outer-membrane proteins of *Neisseria meningitidis* - comparative-study of the Opa, Opc, and PorA proteins. *Infection and Immunity*, **64**, 298-304.

Wolff, K. & Stern, A. (1991) The class-3 outer-membrane protein (Porb) of *Neisseria meningitidis* - gene sequence and homology to the gonococcal porin pia. *FEMS Microbiology Letters*, **83**, 179-185.

Woods, J. P. and Cannon, J. G. (1990) Variation in expression of class 1 and class 3 outer membrane proteins during nasophayngeal caariage of *Neisseria meningitidis*. *Infect Immun.* **58**, 569-572. 1990.

Yanisch-Perron, C., Vieira, J. & Messing, J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and PUC19 vectors. *Gene*, **33**, 103-119.

Zak, K., Diaz, J.L., Jackson, D. & Heckels, J.E. (1984) Antigenic variation during infection with *Neisseria gonorrhoeae*: detection of antibodies to surface proteins in sera of patients with gonorrhea. *Journal of Infectious Diseases*, **149**, 166-173.

Zollinger, W.D. & Mandrell, R.E. (1977) Outer membrane protein and lipopolysaccharide serotyping of *Neisseria meningitidis* by inhibition of a solid phase radioimmunoassay. *Infection and Immunity*, **10**, 975-984.

Zollinger, W.D. & Mandrell, R.E. (1980) Type-specific antigens of group A *Neisseria meningitidis*: lipopolysaccharide and heat modifiable outer membrane proteins. *Infection and Immunity*, **28**, 451-458.

Zollinger, W.D. & Mandrell, R.E. (1983) Studies of the human antibody response to specific meningococcal outer membrane proteins of serotypes 2 and 15. *Medicine Tropicale*, **43**, 143-147.

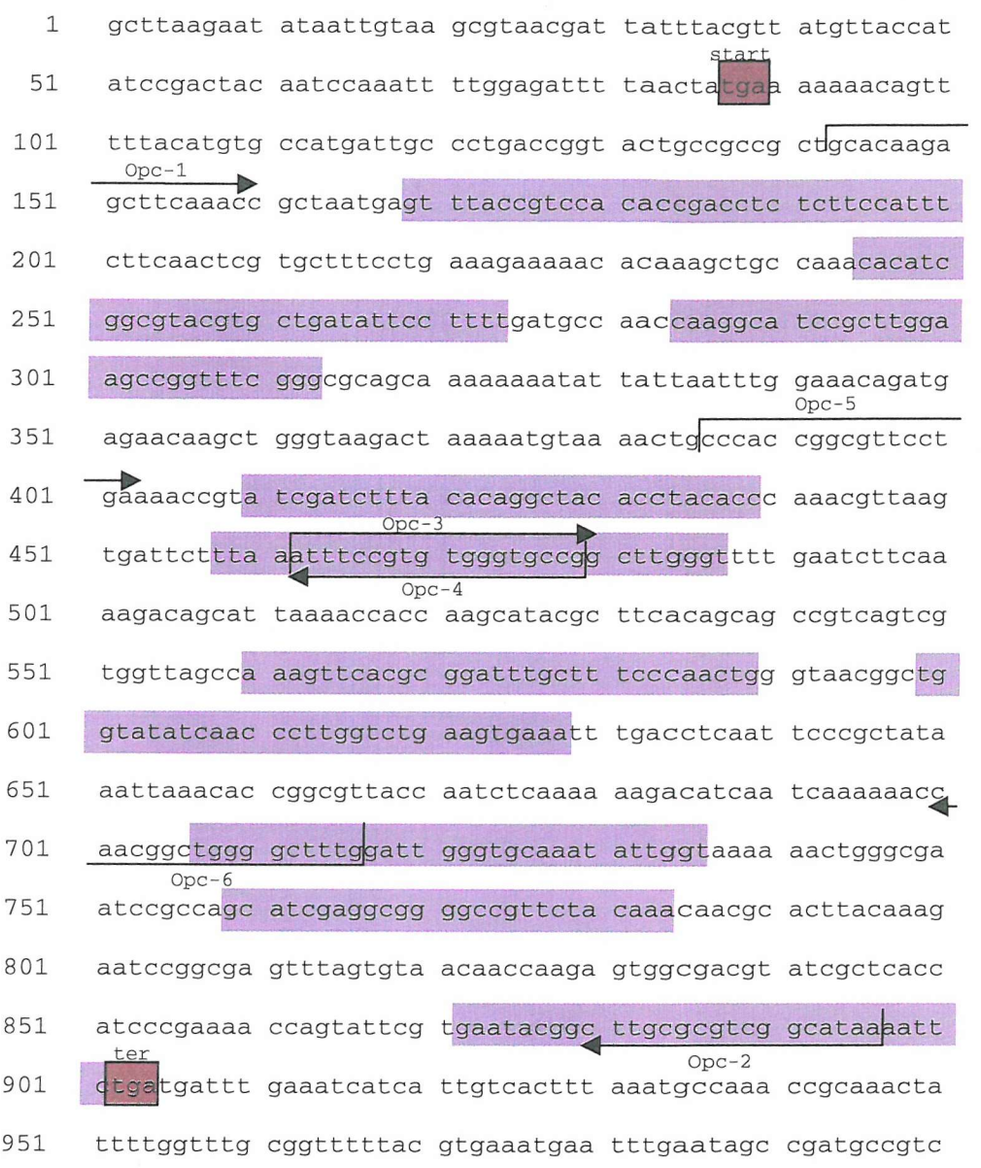
Appendix 1 – Binding positions of primers used in PCR amplification and sequencing

indicates conserved transmembrane regions of the protein

Binding of primers to the class 2 allele of the *porB* gene

indicates conserved transmembrane regions of the protein

Binding of primers to the class 3 allele of the *porB* gene



indicates conserved transmembrane regions of the protein

Binding of primers to the *opc* gene

Appendix 2

List of *porB* sequences obtained from GenBank, including the accession numbers and the author

Type	Genbank Accession no.	Author
1	nmporb1g	Ward
2a	nmporbi	Maiden
2b	nmporbk	Maiden
2c	nmu92911	Sacchi
3	nmu07191	Bash
4	nmporbg3	Ward
4.10	nmu59869	Sacchi
4.15	nmaf002250	Sacchi
4.21	nmporbf	Maiden
4.7	nmu59866	Sacchi
5	nmu92909	Sacchi
6	nmporbh	Maiden
7	nmu92901	Sacchi
8	nmu07189	Bash
9	nmporbj	Maiden
10	nmu92904	Sacchi
12	nmporb12g	Ward
15	nmporbgen	Butcher
16.11	nmu95366	Sacchi
17	nmu07190	Basch
17.7	nmu92900	Sacchi
18	nmu07193	Basch
19	nmu11030	Basch
19.10	nmu34194	Sacchi
19.14	nmaf001318	Sacchi
19.7	nmu94961	Sacchi
22	nmu92906	Sacchi